CONTROL OF TRANSCRIPTION IN EMBRYONAL CARCINOMA CELLS

a thesis presented by

Panayotis Tassios

for the degree of Doctor of Philosophy
at University College,
University of London

under the supervision of
N.B. La Thangue (MRC National Institute for Medical Research, Mill Hill, London)

and
N.A.Mitchison and P.C.L.Beverley (University College, London)

October 1992

ProQuest Number: 10105662

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

ProQuest 10105662

Published by ProQuest LLC(2016). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code.
Microform Edition © ProQuest LLC.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

To my parents

ABSTRACT

The aim of this thesis was to examine the mechanisms that regulate transcription during early murine embryogenesis. F9 embryonal carcinoma (EC) cells were used as a model system, since their *in vitro* differentiated derivatives, the parietal endoderm-like (PE) cells, are similar, by a number of criteria, to cells of the early mouse embryo.

Members of the ATF transcription factor family bind DNA through a domain rich in basic amino acid residues, adjacent to a leucine zip dimerization domain. They are required for unstimulated activity of ATF site-containing promoters but can also modulate transcription in response to diverse stimuli, such as viral *trans*-activators, intracellular cAMP, and Ca^{+2} ions.

In this study, six ATF site-binding activities were defined in F9 EC and PE cells. They could be distinguished by their electrophoretic mobilities, their regulation during differentiation, and the effect that point mutations in or flanking the ATF site had on their ability to bind DNA. Two promoters containing ATF sites, that of the adenovirus *E4* and that of the human vasoactive intestinal polypeptide (VIP) genes, bound different subsets of these activities. This correlated with these promoters' distinct transcriptional activities *in vivo*, since *E4* was active in F9 EC cells and was further activated during differentiation, while *VIP* was inactive in both cell types. In addition, the regulation of transcription from the *E4* promoter *in vivo* was reflected in a differentiation-specific change in the pattern of ATF site-binding proteins that recognized this promoter *in vitro.*

Five human cDNAs derived from different members of the *ATF* family were used to measure *ATF* RNA levels in F9 EC and PE cells. *ATF2, ATF4* and *ATF6* RNA levels remained constant during F9 EC cell differentiation, whereas *ATF3* RNA levels were higher in PE cells and *ATF1* transcripts were undetectable in both cell types. This regulation of *ATF* genes contrasted with that of members of the related AP1 transcription factor family : *junD* RNA levels were constant during differentiation while c*jun* and c*fos* were only expressed in PE cells.

It is known that members of both the ATF and AP1 transcription factor families bind DNA as homodimers or heterodimers with members of their own or the related family. Here it was shown that heterodimerization can not only alter but also broaden DNA binding site specificity.

Finally, after an ATF2-specific anti-peptide rabbit serum was raised, ATF2 protein levels were also shown to remain constant during F9 EC cell differentiation, and a similar group of nuclear polypeptides, with apparent molecular weights of 41-45 kD, was found to be present in a number of different cell types.

This study has therefore shown that the regulation of ATF-dependent transcription during differentiation of EC cells can potentially occur at several levels, including *ATF* gene expression, as well as differential heterodimerization, binding site preference and promoter specificity of ATF proteins. The significance of these results with respect to transcriptional control during development is discussed.

ACKNOWLEDGEMENTS

It would be an understatement simply to thank my supervisor, Nick La Thangue, whose readiness to offer, at any time, help and advice, both theoretical and practical, I hope I have not abused. He has been an invaluable and constant source of enthusiasm, encouragement, information and intellectual stimulation.

I am grateful to the Director of the MRC National Institute for Medical Research, John Skehel, and the Head of the Eukaryotic Molecular Genetics Division, Peter Rigby, for making my time at the Institute much more easy and rewarding than it would otherwise have been, and to my academic supervisors at University College, London, Av Mitchison and Peter Beverley, for interesting discussions and valuable assistance with administrative matters.

I would also like to thank Nick La Thangue for gifts of purified DRTF-1, F9 EC, F9 PE, HeLa, C138 and 293 Manley whole cell extracts, and C138 and 293 Dignam extracts. Special thanks to Janet Partridge for gifts of F9 EC and PE Manley extracts, and to Mahmud Shivji for gifts of purified DRTF-1. I wish to thank Julian Gannon (ICRF, Clare Hall) for supervising the DRTF-1 immunisations, producing the hybridomas, gifts of purified SV40 T antigen and anti-T monoclonal antibody 419, as well as for his invaluable advice, Rowena Girling for advice and assistance during the screening of the hybridomas, for gifts of F9 EC nuclear and cytoplasmic extracts, and monoclonal antibody 6A7, and, together with Maryam Zamanian and Lan Bandara, for useful comments and suggestions. I thank Kevin Lee for plasmids E4Δ240, E4Δ138 and VIPcat4, M.R.Green for plasmids ATF1S, ATF2S, ATF2L, ATF3S, ATF3L, pGEM3-ATF3, pGEM3-ATF4, pGEM3-ATF5 and pGEM3-ATF6, M.Yaniv for plasmids AH119, RSVjunB and RSVjunD, S.Ishii for plasmid pUCCRE-BP1Eco3.2, Rolf Müller for plasmid pTZfosB and NIH 3T3 cells, T.Curran for plasmid pSP65fra-1, D.Bentley for plasmid pPX490, Elaine Dzierzak for plasmid pSP64γactin, David Stott for plasmid pCMVβgal, D.Solter for α-SSEA1 MC480 antibody, Stephen Jackson for anti-Sp1 antibodies, Siu-Pok Yee for C2 myotube extracts, M.Bignami for the SV40 origin oligonucleotide, Ted Murray for advice on transfections and Joe Chan for cellular RNA preparation and riboprobe preparation protocols. Last but not least, I would like to thank the staff of the Oligonucleotide Synthesis, Peptide Synthesis and Animal Facilities for synthesis of oligonucleotides, peptides and for rabbit immunisations, respectively, and the staff of the Photography and of the Graphics & Illustrations Divisions at N.I.M.R. for their expert assistance in the preparation of the figures.

During the first two years of this work I was supported by a Biotechnology Training Grant from the Commission of the European Communities.

TABLE OF CONTENTS

ABSTRACT 3

ACKNOWLEDGEMENTS 5

TABLE OF CONTENTS 7

LIST OF FIGURES AND TABLES 13

LIST OF ABBREVIATIONS 18

CHAPTER 1 : GENERAL INTRODUCTION 25

1.1 Regulation of eukaryotic RNA polymerase II-directed transcription 26

1.1.1 *Cis*-acting elements 26

1.1.2 The basal *trans*-acting factors 28

1.1.3. The regulatory *trans*-acting factors 33

1.1.4 Mechanisms of *trans*-activation 35

1.1.5 The effects of transcription factors on nucleosome DNA templates 38

1.1.6 Co-activators 39

1.1.7 Repression of transcription 42

1.2 Early differentiation events during murine embryogenesis 43

1.3 F9 embryonal carcinoma cells 45

1.3.1 F9 embryonal carcinoma cell differentiation as a model for early differentiation events in the mouse embryo 45

1.3.2 Regulation of viral transcription in F9 EC cells 45

1.4 The adenovirus transcriptional regulator E1A 47

1.5 Transcriptional regulation through the ATF site 50
1.6 A family of ATF site-binding proteins and *ATF* genes 51
    1.6.1 ATF protein domains 52
    1.6.2 Diverse properties and responses of ATF family members 54
    1.6.3 ATF "homologues" in non-mammalian organisms 55
1.7 Aim of the project 55

CHAPTER 2 : MATERIALS AND METHODS 63
Cell culture 64
Plasmids for transfections, gel retardation probes and oligonucleotides 64
Transfection and CAT assays 65
Protein extracts 66
Gel retardation assay 66
"Super-shifts" with anti-peptide sera 67
Construction of BS-ATG vectors 67
In vitro transcription and translation 68
Isolation of total cellular RNA with guanidine isothiocyanate 69
Templates for synthesis of anti-sense RNA probes 69
Synthesis of radiolabelled anti-sense RNA probes 70
Ribonuclease protection assay 70
Computer-assisted sequence comparisons, manipulations and predictions 71
Production of anti-peptide sera 71
Anti-peptide enzyme-linked immuno-sorbent assay (ELISA) 72
Anti-ATF/AP1 Immunoblots 72
Immunoprecipitations 73
Purification of DRTF-1 74
Production of hybridomas 74

Anti-DRTF-1 ELISA 75

Anti-DRTF-1 Immunoblots 75

CHAPTER 3 : REGULATION AND NUCLEOTIDE SPECIFICITY OF ATF SITE -BINDING ACTIVITIES IN F9 EC AND PE CELLS 77

3.1 Sequence specificity and regulation during differentiation of F9 ATF site -binding activities 78

3.2 Discussion 81

CHAPTER 4 : TRANSCRIPTIONAL REGULATION OF ATF SITE-CONTAINING PROMOTERS IN F9 EC AND PE CELLS 88

4.1 Introduction 89

4.2 Regulation of ATF site-containing promoters in F9 EC and PE cells 89

4.3 dbcAMP treatment of F9 EC cells does not affect *in vivo* transcription . . . 91

4.4 Discussion 92

CHAPTER 5 : ATF SITE-DEPENDENT PROTEIN BINDING TO THE *E4* AND *VIP* PROMOTERS 99

5.1 Introduction 100

5.2 *E4* promoter ATF site-binding activities are regulated during differentiation 100

5.3 dbcAMP treatment of F9 EC cells does not affect the ECRE activities . . . 102

5.4 Promoter-specific and common ATF site-dependent complexes 102

5.5 Discussion 103

CHAPTER 6 : *ATF* RNA LEVELS IN F9 EC AND PE CELLS 112

6.1 Introduction 113

6.2 Results 114
6.3 Discussion 117

CHAPTER 7 : DNA BINDING SPECIFICITY OF *IN VITRO* TRANSLATED ATF AND AP1 FAMILY PROTEINS 125
7.1 Introduction 126
7.2 Construction of *in vitro* expression vectors 126
7.3 DNA binding specificity of *in vitro* translated ATF dimers 127
7.4 ATF/AP1 heterodimerization causes changes in DNA binding specificity 128
7.5 Discussion 129

CHAPTER 8 : GENERATION AND CHARACTERISATION OF ANTI-PEPTIDE POLYCLONAL ANTIBODIES AGAINST MEMBERS OF THE ATF AND AP1 TRANSCRIPTION FACTOR FAMILIES 141
8.1 Introduction 142
8.2 Choice of peptide immunogens 143
8.3 Immunisation 144
8.4 ELISAs with immobilised peptides 144
8.5 Immunoblotting of *in vitro* translated polypeptides 144
8.6 Immunoprecipitation of ^{35}S-labelled *in vitro* translated polypeptides 146
8.7 Effects of antisera on antigen/DNA complexes 148
8.8 Discussion 150

CHAPTER 9 : IMMUNOCHEMICAL STUDY OF ATF AND AP1 PROTEINS IN F9 EC AND PE CELLS 167
9.1 Introduction 168
9.2 ATF2 protein levels remain constant during differentiation 168

9.3 ATF2 is expressed in a variety of cell types 169
9.4 Reaction of anti-ATF3 sera with F9 EC and PE polypeptides 170
9.5 Reaction of anti-P10 sera with cellular proteins 172
9.6 Reactivity of anti-Jun sera with polypeptides in whole cell extracts 173
9.7 Effects of anti-ATF and anti-AP1 sera on ECRE/DNA complexes 174
9.8 Discussion 176

CHAPTER 10 : PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST THE DIFFERENTIATION-REGULATED TRANSCRIPTION FACTOR DRTF-1 190
10.1 Introduction 191
10.2 Immunisation 191
10.3 ELISA 192
10.4 Gel retardation assays 192
10.5 Immunoblots 194
10.6 Production of hybridomas 194
10.7 Discussion 196

CHAPTER 11 : GENERAL DISCUSSION 206
11.1 Summary and perspectives 207
11.2 The versatility of transcription factor families 210
    11.2.1 Alternative splicing 211
    11.2.2 Antagonists of transcriptional activators 212
    11.2.3 Different members of transcription factor families display distinct expression patterns and responses to stimuli 214
    11.2.4 Transcription factor dimerization 216

11.2.5. Distinct DNA binding specificities of transcription factor family members 217

11.3 Transcription factors as instruments of development 219

11.3.1 Transcription factors as developmental "switches" 219

11.3.2 Post-translational regulation of transcription factor activity 221

11.4 Conclusions 223

REFERENCES 226

LIST OF FIGURES AND TABLES

FIG.1.1 : *Assembly of the initiation complex* 58

FIG.1.2 : *Protein-DNA and protein-protein interactions on the promoter of a pol II -transcribed gene* 59

FIG.1.3 : *Early development of the mouse embryo* 60

FIG.1.4 : *Comparison of F9 EC cell differentiation with differentiation events in the early mouse embryo* 61

FIG.1.5 : *Schematic representation of a bzip dimer binding to the DNA* 62

TABLE 3.1 : *Sequences of ATF sites and variant oligonucleotides used in this study* . . 83

FIG. 3.1 : *Distinct but ATF site-specific protein complexes are formed on different variants of the ATF site and on a TRE* 84

FIG. 3.1 (cont'd) : *Distinct but ATF site-specific protein complexes are formed on different variants of the ATF site and on a TRE* 85

FIG. 3.2 : *Specificity of probe DA for ECRE activities* 86

TABLE 3.2 : *Regulation and binding site specificity of F9 ATF site-binding activities* 87

FIG. 4.1 : *Schematic representation of transcription regulatory regions contained in the CAT reporter constructs used for transfection in F9 EC and PE cells* 94

FIG. 4.2 : *Transcriptional activity of ATF site-containing promoters in F9 EC and PE cells* 95

FIG. 4.3 : *dbcAMP treatment of F9 EC cells has no effect on transcriptional activity* 96

FIG. 4.4 : *Summary of results* 97

FIG. 4.5 : *Immunochemical staining of F9 EC cells (a, b), F9 EC cells treated with dbcAMP (c, d) and PE cells (e, f) for stage-specific embryonic antigen 1* . . . 98

FIG. 5.1 : *Complexes formed on the full length* E4 *promoter in F9 EC and PE whole cell extracts* 106
FIG. 5.2 : *Specificity of protein complexes formed on the* E4Δ *and* VIP *promoters in F9 EC and PE cell extracts* 107
FIG. 5.2 (cont'd.) : *Specificity of protein complexes formed on the* E4Δ *and* VIP *promoters in F9 EC and PE cell extracts* 108
FIG. 5.3 : *dbcAMP treatment of F9 EC cells has no effect on protein/DNA complex formation* 109
FIG. 5.4 : *Schematic representation of a possible model for promoter-specific binding of ECRE activities* 110
TABLE 5.1 : *Promoter and oligonucleotide binding specificity of the regulated ATF site-binding activities in F9 EC and PE cells* 111

FIG. 6.1 : *Schematic representation of sequences contained in the anti-sense RNA probes* 119
FIG. 6.2 : *Specificity of anti-sense RNA probes* 120
FIG. 6.3 : *γ-actin and* ATF *RNA levels in F9 EC and PE cells* 121
FIG. 6.4 : *RNA levels of* AP1 *family members and c-*myc *in F9 EC and PE cells* 122
FIG. 6.5 : ATF1 *is expressed in HeLa but not in F9 EC and PE cells* 123
TABLE 6.1 : *Regulation of* ATF *and* AP1 *family members' RNA levels during differentiation* 124

FIG. 7.1 : *Design of the BS-ATG vectors* 132
FIG. 7.2 : *Properties of the BS-ATG vectors* 133
FIG. 7.3 : In vitro *translated and co-translated* ^{35}*S-Met-labelled polypeptides* 134
FIG. 7.4 : *Binding of* in vitro *translated ATF proteins to probes P, Pm1 and E2A-3'* 135
FIG. 7.5 : *No ATF polypeptides bind to the E2A-3' probe* 136

FIG. 7.6 : *Binding of* in vitro *translated ATF homodimers and ATF2/Jun heterodimers to probes P and Pm2* 137

FIG. 7.7 : *Dimerization controls DNA binding specificity* 138

FIG. 7.8 : *Regulation of possible ATF3 dimers during F9 EC cell differentiation* 139

TABLE 7.1 : *Summary of DNA binding specificities of* in vitro *translated polypeptide complexes* 140

FIG. 8.1 : *Hydropathy and hydrophilicity plots of regions within ATF2 and ATF3* . . . 151

FIG. 8.2 : *The ATF2D and ATF3C sequences are unique* 152

FIG. 8.3 : *Alignment of cJun PEP1 and PEP2 sequences with other members of the ATF/AP1 families* 153

FIG. 8.3 (cont'd.): *Alignment of cJun PEP1 and PEP2 sequences with other members of the ATF/AP1 families* 154

FIG. 8.4 : *Alignment of cFos P10 sequence with other members of the ATF/AP1 families* 155

FIG. 8.5 : *Position of the peptide epitopes in the polypeptides* 156

FIG. 8.6 : *Specificity of the antisera tested by ELISA* 157

FIG. 8.7 : *Specificity of the antisera tested by immunoblotting against* in vitro *translated polypeptides* 158

FIG. 8.8 : *Immunoprecipitation of ^{35}S-labelled* in vitro *translated polypeptides by anti-ATF2D and anti-ATF3C sera* 159

FIG. 8.9 : *Immunoprecipitation of ^{35}S-labelled* in vitro *translated polypeptides by anti-ATF2D and anti-ATF3C sera* 160

FIG. 8.10 : *Immunoprecipitation of* in vitro *translated polypeptides by anti-P10, anti-PEP1 and anti-PEP2 sera* 161

FIG. 8.11 : *Effects of anti-ATF3C and anti-P10 sera on ATF3/DNA complexes* 162

FIG. 8.12 : *Effects of anti-ATF2D sera on ATF2/DNA complexes* 163

FIG. 8.13 : *Effects of anti-P10, anti-PEP1 and anti-PEP2 sera on* in vitro *co-translated cJun-cFos/DNA complexes and 3T3 AP1/DNA complexes* 164

FIG. 8.14 : *Differential effects of sera on DNA binding dimers* 165

TABLE 8.1 : *Summary of effects of antisera on* in vitro *translated polypeptides* 166

FIG. 9.1 : *Immunoblotting of F9 EC and PE whole cell extracts with anti-ATF2D sera* 178

FIG. 9.2 : *Immunoprecipitation with anti-ATF2D sera from ^{35}S-Met-labelled F9 EC and PE lysates* 179

FIG. 9.3 : *Detection of ATF2 polypeptides and ATF site-binding activity in various cell types* 180

FIG. 9.4 : *Detection of ATF2 in HeLa cells* 181

FIG. 9.5 : *Immunoblotting and immunoprecipitation experiments with anti-ATF3C sera in F9 EC and PE whole cell extracts* 182

FIG. 9.6 : *Lack of anti-ATF3C reactivity in HeLa cell extracts* 183

FIG. 9.7 : *Immunoprecipitation from ^{35}S-Met-labelled cell lysates with anti-cFos sera* 184

FIG. 9.8 : *Immunoblotting with anti-cFos and anti-cJun sera against F9 EC and PE whole cell extracts* 185

FIG. 9.9 : *Immunoprecipitation from 3T3, F9 EC and PE ^{35}S-Met-labelled cell lysates with anti-cJun sera* 186

FIG. 9.10 : *Effects of the anti-peptide sera on ECRE complexes* 187

FIG. 9.11 : *Anti-PEP2 serum cross-reacts with JunD but not ATF2 or ATF3* 188

FIG. 9.12 : *cJun amino acid sequence : amino acid similarities of other ATF/AP1 family members to peptide PEP2* 189

FIG. 10.1 : *ELISAs with test sera from mouse 2* 199

FIG. 10.2 : *Control "super-shift" experiment with α-Sp1 polyclonal antibody* 200

FIG. 10.3 : *a) Titration of test sera in a gel retardation assay*

b) Specificity of "super-shifts" produced by test sera 201

FIG. 10.4 : *Immunoblots with test sera* 202

FIG. 10.6 : *Immunoblot with the first supernatants from 24-well plates* 204

FIG. 10.7 : *Immunoblot with 24-well plate second supernatants* 205

LIST OF ABBREVIATIONS

A : adenine ; alanine
Ad5 : adenovirus serotype 5
ADA2 : adaptor gene 2
AFP : α-foetoprotein
αGH : glycoprotein hormone α subunit
AP1 : activating protein 1
APαM : alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulins
APαR : alkaline phosphatase-conjugated swine anti-rabbit immunoglobulins
ATF : activating transcription factor
ATP : adenosine 5'-triphosphate

BBF-2 : box B binding factor 2
bHLH : basic domain/helix-loop-helix
bp : base pairs
BSA : bovine serum albumin
BS-KS : Bluescript-KS
bzip : basic domain/leucine zip

C : cytosine ; cysteine
°C : degrees Caelsius
CaM : calcium-calmodulin dependent
cAMP : adenosine 3':5'-cyclic monophosphate
CAP : catabolite activator protein
CAT : chloramphenicol acetyl transferase
cDNA : complementary DNA
C/EBP : cellular enhancer binding protein

Cf1-a : C element binding factor 1a
CKII : casein kinase II
CMV : cytomegalovirus
CR : conserved region
CRE : cAMP-responsive element
CREB : CRE-binding protein
CREBP-1 : CRE-binding protein 1
CREM : CREB modulator
CTD : carboxy-terminal domain of RPB1
CTF : CCAAT box-binding transcription factor
CTP : cytidine 5'-triphosphate

D : aspartic acid
dbcAMP : N^6,2'-O-dibutyryl cAMP
dCREB-A : *Drosophila* CREB A
DEPC : diethyl pyrocarbonate
DIF : de-ionised formamide
DMEM : Dulbecco's modified Eagle's medium
DNA : deoxyribonucleic acid
DNase I : deoxyribonuclease I
dNTP : deoxynucleotide 5'-triphosphate
dpc : days *post coitum*
Dr1 : TFIID repressor 1
DRTF-1 : differentiation-regulated transcription factor 1
DTT : dithitothreitol

E : glutamic acid
E1A : adenovirus early gene 1A

E2A : adenovirus early gene 2
E2F : E2A promoter binding factor
E4 : adenovirus early gene 4
E4BP4 : E4 promoter binding protein 4
E4F : E4 promoter binding factor
EivF : E4 promoter binding factor
EC : embryonal carcinoma
ECRE : EC CRE binding protein
EDTA : ethylene diamine tetra-acetate
ELISA : enzyme-linked immuno-sorbent assay

F : phenylalanine
FCS : foetal calf serum

G : guanine ; glycine
g : gramme
GAL4 : galactose catabolism gene activator 4
GATA-1 : GATA motif binding factor 1
GCN4 : general control of nitrogen metabolism gene 4
GHF-1 : growth hormone promoter binding factor 1
GTP : guanosine 5'-triphosphate

H : histidine
h : hours
HAP4 : haeme activator protein 4
HBV : hepatitis B virus
HNF : hepatic nuclear factor
Hox : murine homoeobox gene

HRPαM : horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins
HRPαR : horseradish peroxidase-conjugated swine anti-rabbit immunoglobulins
HS : heparin Sepharose
HSF : heat shock factor
HSV : herpes simplex virus
HTLV-I : human T cell leukaemia virus I

I : isoleucine
ICM : inner cell mass
Id : inhibitor of differentiation
IE : immediate early gene
I-POU : inhibitor of POU
ITR : inverted terminal repeat

K : lysine
kD : kiloDaltons
KLH : keyhole limpet haemocyanin

L : leucine
LCR : locus control region

M : methionine ; molar
Met : methionine
mg : milligramme
μg : microgramme
min : minutes
ml : milliliter
μl : microliter

mM : millimolar
μM : micromolar
μm : micrometre
M_r : relative molecular weight

N : asparagine
NC1 : negative component 1
NC2 : negative component 2
NFκB : nuclear factor binding to the B element of the immmunoglobulin κ light chain enhancer
ng : nanogramme
nM : nanomolar
nmol : nanomoles
NP40 : Nonidet P40

Oct : octamer motif binding protein
ORF : open reading frame

P : proline
PBS : phosphate-buffered saline
PCRE : PE CRE binding protein
PE : parietal endoderm-like
PEPCK : phospho*enol*pyruvate carboxykinase
Pit-1 : pituitary-specific transcription factor 1
PKA : protein kinase A
PKC : protein kinase C
pmol : picomoles
POU : Pit-1, Oct and Unc86 DNA binding domain

pRb : retinoblastoma gene product

PrIE : pseudorabies virus immediate early protein

PVC : polyvinylchloride

Q : glutamine

R : arginine

RA : (all)*trans*-retinoic acid

RAP30 : RNA polymerase II-associated protein of 30 kD

RAR : retinoic acid receptor

RNA : ribonucleic acid

RNase : ribonuclease

RPB : RNA polymerase B (II) subunit

rpm : revolutions per minute

r.t. : room temperature

RXR : retinoid X receptor

S : serine

SDS : sodium dodecyl sulphate

SDS-PAGE : SDS polyacrylamide gel electrophoresis

SNF : sucrose non-fermenting

Sp1 : Sph motif binding protein 1

SRF : serum response factor

SSEA-1 : stage-specific embryonic antigen 1

SV40 : simian virus 40

SWI : switch

T : thymine ; threonine

TAF : TBP-associated factor
TAT : tyrosine aminotransferase
Tax : HTLV-I *trans*-activator protein
TBP : TATA box-binding protein
TFII : RNA polymerase II transcription factor
TFE3 : immunoglobulin enhancer μE3 motif-binding transcription factor
tk : thymidine kinase
TRE : 12-*O*-tetradecanoylphorbol-13-acetate responsive element
TREB : Tax-responsive element binding protein
tRNA : transfer RNA

Y : pyrimidine (C or T) ; tyrosine
YY1 : Ying Yang 1

USF : upstream stimulating factor
UTP : uridine 5’-triphosphate

V : valine ; Volts
VIP : vasoactive intestinal polypeptide
VP16 : HSV virion protein 16

W : tryptophan ; Watts

CHAPTER 1

GENERAL INTRODUCTION

Development of the mouse, as of all higher eukaryotic organisms, relies on the temporally and spatially controlled expression of genes, the products of which determine cellular phenotype and dictate cellular interactions. A major control point of gene expression is the initiation of transcription and, indeed, a number of genes first identified through their importance for normal development of the *Drosophila* embryo, as well as their mouse homologues, are now known to encode proteins that regulate RNA synthesis by binding to the transcriptional control regions of genes in a sequence-specific fashion (reviewed by Biggin and Tjian, 1989 ; Kessel and Gruss, 1990).

This chapter reviews some aspects of current knowledge about eukaryotic RNA polymerase II-directed transcription and its regulation by transcription factors, through protein/DNA and protein/protein interactions. It also describes the reasons for using the F9 murine embryonal carcinoma cell system as a model for differentiation during early murine embryogenesis, and the relevance of studying the control of adenovirus transcription in this system. Finally, it introduces the ATF (activating transcription factor) transcription factor family, with which the main body of this thesis is concerned ; properties of ATF family members are presented in further detail in the General Discussion (Chapter 11), where the experimental results of this thesis are discussed in the context of transcription factor families in general, and their role during murine development.

1.1 Regulation of eukaryotic RNA polymerase II-directed transcription

1.1.1 *Cis*-acting elements

Transcription of protein-encoding genes by eukaryotic RNA polymerase II usually depends on DNA regions that flank the coding sequences of the gene : these ensure correct initiation of the RNA and regulate its levels. Historically, these DNA sequences have been classified into promoter or enhancer elements. A promoter was originally defined as the proximal upstream region of the gene that incorporated the transcriptional start site and was necessary for synthesis of uninduced levels of correctly initiated RNA molecules (reviewed by Wasylyk, 1988). An enhancer, on the other hand, could be positioned up to several hundred nucleotides away from the start site in either direction, and regulate the levels of transcription in a tissue-specific or signal-inducible manner (reviewed by Maniatis *et al.*, 1987). However, these elements were soon discovered to be built of smaller nucleotide sequences which were often encountered, though in different combinations, in both promoters and enhancers (reviewed by Dynan, 1989). Excluding the region around the transcriptional start site which is only present in promoters, there therefore do not appear to be any major differences between their composition and that of enhancers ; thus, a more valid distinction would be between the basal transcriptional elements and the regulatory elements.

The former are only found in promoters and are reponsible for the basal level of correctly initiated transcripts. Two types of such mammalian *cis*-acting elements have been characterised in protein-encoding genes : the TATA box (nucleotide sequence consensus : TATA(A/T)A), located at approximately nucleotide -30 relative to the transcriptional start site, and the initiator (consensus : YAYTCYYY, where Y may be C or T), centered around nucleotide +1 (reviewed by Roeder, 1991). Genes may contain one or the other, or both.

Regulatory elements, on the other hand, of which there is a greater variety, occur further upstream or downstream from the basal elements. They do not function independently of the basal elements but modulate their efficiency in initiating transcription, and thus regulate the levels of RNA transcripts synthesized (reviewed by Hatzopoulos *et al.*, 1988). As discussed in the following sections, the distinct roles of these two classes of transcriptional control elements can be explained by the nature of proteins which bind to their DNA sequences and thereby direct RNA synthesis.

1.1.2 The basal *trans*-acting factors

Protein-encoding genes are transcribed by RNA polymerase II (pol II), a high molecular weight, multi-subunit enzyme. Genes for all ten subunits, RPB1 to RPB10 (RNA polymerase B subunit), have been cloned from *Saccharomyces cerevisiae* : they are single copy genes and are conserved to 30-50%, at the predicted amino acid level, with respect to their homologues from other eukaryotes (reviewed by Woychik and Young, 1990). The two largest subunits, RPB1 and RPB2, also bear regions of amino acid similarity to the β' and β subunits, respectively, of the *Escherichia coli* RNA polymerase. The carboxy-terminal domain (CTD) of RPB1, which lies outside these regions of similarity, contains a large number of repeats of the PTSPSTS heptapeptide consensus sequence. The CTD, which, due to its hydrophilicity is predicted to be exposed at the surface of the RPB1 subunit, is essential for *S.cerevisiae* viability ; its role in the initiation of transcription is discussed below. Interestingly, the three eukaryotic polymerases, I, II and III, share common subunits : for example, pol III contains RPB5, 6, 8 and 10 in common with pol II (reviewed by Gabrielsen and Sentenac, 1991). As will be discussed later, transcription by the three polymerases also requires at least one other common polypeptide, indicating that polymerase specificity relies on combinations of unique and shared components, rather than on three sets of distinct factors.

Pol II alone is not sufficient for correct initiation of transcription ; for this, it also requires the general or basal pol II transcription factors (TFIIs) (reviewed by Roeder, 1991). *In vitro* reconstitution studies, with TFII fractions chromatographically purified from cell extracts, have shown that these assemble, in an orderly fashion, around the TATA box (Buratowski *et al.*, 1989 ; reviewed by Peterson and Tjian, 1992) (Fig.1.1). Binding of TFIID fraction to this DNA sequence is the first step in the formation of the initiation complex. TBP (TATA binding protein), the DNA binding component of TFIID, is the only basal transcription factor to bind DNA directly. TBP cDNAs have been cloned from *S.cerevisiae* (Horikoshi *et al.*, 1989), *Arabidopsis thaliana* (Gasch *et al.*, 1990), *Drosophila melanogaster*

(Hoey *et al.*, 1990) and human cells (Peterson *et al.*, 1990). All these organisms have a single *TBP* gene (with the exception of *A.thaliana* which has two closely related genes) that is predicted to encode a 22-39 kD polypeptide, depending on the species. There is 80-90% similarity in the 180 carboxy-terminal amino acids of the various TBPs (Hoffmann *et al.*, 1990). This domain contains two amino acid repeats flanking a basic region ; the second repeat overlaps with a region of homology to the *E.coli* σ^{70} initiation factor, mapping to the σ^{70} domain required for recognition of the bacterial TATA box (Horikoshi *et al.*, 1989). TBP binds to the TATA box as a monomer *via* its two carboxy-terminal repeats (Horikoshi *et al.*, 1990 ; Reddy and Hahn, 1991) ; specific amino acid positions in the second repeat determine its specificity for variants of the TATA consensus sequence (Strubin and Struhl, 1992). However, the amino-terminal region of TBP is species-specific in its composition and length ; for example, human TBP contains 155 amino acids, whereas yeast only 61 (Hoffmann *et al.*, 1990). Not surprisingly, the domains needed for TATA box binding and transcriptional initiation from TATA box-containing promoters co-localise, by deletion analysis, to the carboxy-terminus, emphasizing the importance of direct contact of TBP with the DNA (Horikoshi *et al.*, 1990). Despite the conservation of the carboxy-terminal domain though, the human TBP carboxy-terminus fused to the yeast amino-terminus cannot support the growth of yeast cells that lack the endogenous molecule (Cormack *et al.*, 1991 ; Gill and Tjian, 1991). This is most likely due to subtle differences between the two proteins, especially since their *in vitro* TATA box binding specificities are very similar but not identical (Wobbe and Struhl, 1990). Nevertheless, human TBP is able to activate transcription of at least one tested gene in yeast, and therefore its failure to support growth may be partly explained by the stringency of the viability assay (Strubin and Struhl, 1992).

TBP is also required for transcription by pol I and pol III (reviewed by Sharp, 1992). This has been shown for yeast *in vivo* and *in vitro*, using strains carrying temperature-sensitive mutant TBP alleles and assaying transcription of genes by pol I, II and III (Cormack and Struhl, 1992 ; Schultz *et al.*, 1992). Furthermore, two different single amino acid substitution mutations in or next to the first DNA binding repeat display distinct polymerase specificities : both of them support pol I but not pol II transcription, consistent with the fact that they do not bind to the TATA box, but only one functions in conjunction with pol III (Schultz *et al.*, 1992). This implies that, though TBP is required by all three polymerases, there are differences in the way it interacts with them to promote transcriptional initiation. That TBP protein is important for pol III-directed RNA synthesis *in vitro* has also been demonstrated by White *et al.* (1992), while Comai *et al.* (1992) have shown that TBP is present in the pol I selectivity factor (SL1). How does TBP bind to pol I- and pol III-driven TATA box-less promoters ? A clue to this question is provided by the fact that only SL1 but not TBP alone can restore activity of an extract immunodepleted by anti-TBP antibodies. This is because, in the SL1 fraction, TBP is complexed with three other polypeptides or TBP-associated factors (TAFs) : only when these are added to TBP, is SL1 activity reconstituted (Comai *et al.*, 1992). Similarly, TBP recognizes a TATA box-less pol II promoter in association with six TAFs (Pugh and Tjian, 1991). TBP's TATA box-binding ability is therefore not its main activity during the initiation process, but simply one of the mechanisms for tethering it to a promoter. When a TATA box is lacking, binding of TBP near the transcriptional start site presumably occurs indirectly, *via* its association with TAFs.

The next step in the assembly of the initiation complex is entry of TFIIA, which, though not absolutely necessary for initiation, stabilises TBP binding (Buratowski *et al.*, 1989 ; Maldonado *et al.*, 1990 ; Sumimoto *et al.*, 1990). TFIIA is followed by TFIIB, whose presence is required before pol II can join the complex (Buratowski *et al.*, 1989 ; Lu *et al.*, 1991). Yeast TFIIB is essential for correct *in vivo* transcriptional initiation of at least two tested genes, since *TFIIB*$^-$ mutant strains produce transcripts initiated at sites downstream from those used in wild type strains (Pinto *et al.*, 1992). TFIIB also contains a region of amino acid similarity to *E.coli* σ^{70}, namely to the domain that is involved in contacting the bacterial TATA box (Ha *et al.*, 1991). However, since TFIIB has no specific double-stranded DNA binding activity and, furthermore, requires the presence of sequences downstream of +1 in order to complex with TFIID, TFIIA and DNA *in vitro* (Maldonado *et al.*, 1990), the biological significance of this similarity is unclear.

When pol II binds to the pre-assembled factors, it is already complexed to TFIIF, *via* the latter's small subunit, termed RAP30 (RNA polymerase II-associated polypeptide of 30 kD) in yeast (reviewed by Greenblatt, 1991). The similarities of the eukaryotic pol II transcriptional machinery with the prokaryotic one, already observed in the two largest pol II subunits, TFIID and TFIIB, are again apparent here, as RAP30 contains sequences with amino acid similarity to σ^{70} regions responsible for binding to the polymerase. This sequence similarity reflects a functional similarity, since TFIIF can bind to both the prokaryotic and eukaryotic polymerases. The homology to σ^{70} extends further : as σ^{70} directs the bacterial RNA polymerase to the promoter by increasing its affinity for specific promoter sequences, so TFIIF prevents binding of pol II to non-specific sites. In addition, the TFIIF-containing initiation complex is also able to "melt" the double-stranded DNA around the start site (Greenblatt, 1991).

Two populations of pol II exist in cells, distinguished by whether or not they are phosphorylated at the CTD (Woychik and Young, 1990). The unphosphorylated form, IIA, preferentially joins the initiation complex ; however, it is mostly its phosphorylated derivative, IIO, that is found associated with nascent RNA, suggesting that IIA complexes with the basal transcription factors and is converted to the IIO form before it starts synthesizing the transcript (Lu *et al.*, 1991). In fact, an unphosphorylated CTD, but not its phosphorylated derivative, can bind directly to TBP (Usheva *et al.*, 1992). A number of protein kinases, including $p34^{cdc2}$, which is involved in cell cycle regulation, are capable of phosphorylating the CTD *in vitro* (reviewed by Corden, 1990). However, the fact that CTD phosphorylation can occur even in the presence of purified basal factors suggested that an appropriate kinase was present in those fractions (reviewed by Peterson and Tjian, 1992). Indeed, TFIIH, which associates with the initiation complex after the addition of pol II and TFIIF, was shown to contain such an activity, which was enhanced in the presence of the TFIIE fraction (Lu *et al.*, 1992). Phosphorylation of the CTD by TFIIH would therefore lead to release of pol II from the initiation complex after dissociation from the TBP (Usheva *et al.*, 1992), and allow elongation of the transcript. Another component, TFIIJ, is also required for initiation, but its precise function and polypeptide composition have not been defined (Flores *et al.*, 1992).

The sequence of events leading to initiation from promoters containing an initiator rather than a TATA box have not been fully characterised yet, though two distinct proteins, YY1 (Ying Yang 1) and TFII-I, have been described that can bind to the initiator and direct transcription from it (Shi *et al.*, 1991 ; Seto *et al.*, 1991 ; Roy *et al.*, 1991). TFII-I can function in conjunction with purified TFIID, B, E and F (Roy *et al.*, 1991), and therefore may represent a TFIIA-equivalent for initiator-containing promoters, though its direct and specific DNA binding activity constitutes a distinct difference from TFIIA.

This relatively simple picture of the regular assembly of an initiation complex on a basal element leading to transcription, needs to be qualified in two ways. Firstly, though basal, the TFII factors do not seem to be general. For example, while *in vitro* transcription from the adenovirus major late promoter required TFIIE, this was dispensable for immunoglobulin heavy chain promoter activity (Parvin *et al.*, 1992). In addition, TFIIG, while chromatographically distinct from TFIIA, could functionally substitute for it during initiation complex assembly (Sumimoto *et al.*, 1990). Secondly, a number of factors are able to repress basal transcription. NC1 (negative component 1), for example, presumably exerts this effect by binding to TFIID, thus blocking its association with TFIIA, when the three factors are incubated together *in vitro* (Meisterernst *et al.*, 1991), while NC2 can repress transcriptional initiation by actually displacing TFIIA from a pre-formed TFIID/TFIIA complex (Meisterernst and Roeder, 1991). By contrast, Dr1 (TFIID repressor 1) is able to prevent both TFIIA and TFIIB from complexing with TFIID, by binding to the latter, and thus repress basal transcription in a phosphorylation-dependent manner (Inostroza *et al.*, 1992).

Basal transcription, therefore, occurs as a result of the physical association of pol II with the basal transcription factors and the DNA. Its level can be repressed by proteins that prevent formation of the initiation complex, but also activated by transcription factors which bind to the DNA upstream or downstream from the initiation complex, at a distance of a few nucleotides to several hundred nucleotides away from it, as discussed in the following section.

1.1.3. The regulatory *trans*-acting factors

The importance of the regulatory *cis*-acting elements in transcriptional control lies in their ability to bind *trans*-acting factors which increase or decrease the activity of the promoter (reviewed by La Thangue and Rigby, 1988b ; Levine and Manley, 1989 ; Renkawitz, 1990). The molecular mechanisms by which they achieve this are beginning to be understood, thanks to the fact that, in many cases, the protein regions that are required for transcriptional regulation, and especially activation, have been characterised, as have the regions necesssary for DNA binding (reviewed by Mitchell and Tjian, 1989 ; Johnson and McKnight, 1989). It has become apparent that these two properties map to distinct and functionally independent parts of the polypeptides. Furthermore, such regions are often similar among different transcription factors, which can therefore be grouped according to the type of their DNA binding and activation domains. Inevitably, similarities in the DNA binding region usually reflect similarities in the DNA sequence recognized. This means that, at least *in vitro*, closely related DNA binding domains may be able to bind the same DNA motif, as do, for example, members of the ATF family (for example, Hai *et al.*, 1989). The converse is also true : a single transcription factor may bind to more than one related DNA sequences (for example, Cowell *et al.*, 1992). It is possible that this flexibility is exploited *in vivo* to enhance the repertory of responses mediated by a single *cis*-acting element, and increase the number of target genes that a single *trans*-acting factor can regulate.

Eukaryotic protein motifs that bind DNA include the three α-helices encoded by homoeoboxes (found, for example, in octamer binding proteins and transcription factor Pit-1 (pituitary-specific transcription factor 1)) (reviewed by Levine and Hoey, 1988), zinc ion-coordinated polypeptide structures (present, for example, in the glucocorticoid and oestrogen nuclear receptors, and transcription factor Sp1 (Sph motif binding protein 1)) (reviewed by

Vallée *et al.*, 1991 ; Luisi, 1992), and the α-helix formed by basic domains (as in AP1 (activator protein 1) and ATF transcription factors) (reviewed by Busch and Sassone-Corsi, 1990). It should be stressed that, though the DNA binding domains of transcription factors within the same family may have structural and amino acid similarities and recognize similar DNA elements, they still retain some specificity. This is dictated both by the non-conserved amino acids in the protein and by DNA determinants such as precise nucleotide sequence (for example, Blackwell and Weintraub, 1990), and spacing of the recognition motifs (Näär *et al.*, 1991 ; Luisi *et al.*, 1991 ; reviewed by Rhodes and Schwabe, 1991).

In order to bind efficiently to their sites, some transcription factors require, apart from the domains described above that directly recognize the DNA, additional protein sequences, such as ligand binding and dimerization domains. Nuclear hormone receptors' recognition of their sites, for example, is enhanced by hormone binding which induces a conformational change leading to dimerization through the same ligand-binding region of the receptor (reviewed by Green and Chambon, 1988). CREB (adenosine 3':5'-cyclic monophosphate (cAMP) responsive element binding protein), on the other hand, an activator which contacts the DNA *via* a basic domain, relies on an adjacent dimerization domain of the "leucine zip" type (see section 1.6) for DNA binding (Dwarki *et al.*, 1990). Furthermore, formation of a CREB dimer is promoted by phosphorylation (Yamamoto *et al.*, 1988) ; post-translational modifications are therefore also important for transcription factor activity (discussed in detail in Chapter 11).

In addition, transcription factors can be grouped according to the amino acid content of their activation domains. Thus, acidic (eg. in the yeast factor GAL4) (galactose catabolism gene activator 4), glutamine-rich (eg. in Sp1) and proline-rich (eg. in CTF) (CCAAT box binding transcription factor) polypeptide sequences have been shown to activate basal transcription (Mitchell and Tjian, 1989). The significance of amino acid content probably lies in imposing a local structure capable of interacting with the initiation complex. It has been suggested, for example, that the activating function of acidic domains depends on the formation of an amphipathic α-helix, where charged and hydrophobic residues are exposed on opposite sides, and that net negative charge *per se* is not important (reviewed by Ptashne, 1988).

This modular nature of transcriptional regulators has been repeatedly demonstrated by the construction of hybrids which combine the DNA binding specificity of one transcription factor with the activation domain of another (reviewed by Frankel and Kim, 1991 ; Ptashne, 1988), and by the existence of *trans*-activators, such as viral early gene products (reviewed by Nevins, 1991), which do not bind DNA but contain activation domains and exert their effect by interacting with both regulatory and basal factors (see section 1.1.4 and 1.4).

1.1.4 Mechanisms of *trans*-activation

A number of observations led to the conclusion that regulatory *trans*-acting factors modulate transcription levels by physically interacting with components of the initiation complex. For example, it was shown that mammalian ATF, when incubated with TFIID, was able to increase the latter's area of contact with the DNA, and promote the assembly of an initiation complex by stabilising its first step (Horikoshi *et al.*, 1988a ; Hai *et al.*, 1988a). GAL4 also modified the interaction of TFIID with the DNA and, furthermore, this effect depended on the presence of a functional activation domain (Horikoshi *et al.*, 1988b).

In other experiments, GAL4, though an activator under physiological conditions, could in fact inhibit the transcription of genes that lacked GAL4 binding sites, when over-expressed in cells (Gill and Ptashne, 1988). This effect was greater with wild type GAL4, and decreased with decreasing *trans*-activating potential of GAL4 mutants, indicating that both DNA-independent inhibition and DNA-dependent activation of transcription were mediated by the activation domain. Similar results were obtained with the non-DNA binding activator of herpes simplex virus type 1 (HSV), VP16 (virion protein 16) (Triezenberg *et al.*, 1988). This effect, called "squelching", was attributed to the ability of activation domains to interact with basal factors even in the absence of DNA, thus sequestering them away from a promoter and inhibiting the formation of an initiation complex.

Another characteristic of transcription factor activity that led to the same idea of physical association with basal factors is synergy, that is activation from multiple binding sites to levels higher than those predicted by the addition of single binding site-directed transcription levels. Steroid receptors, for example, bind DNA cooperatively to multiple hormone-responsive sites arranged *in tandem*, resulting in super-additive levels of activation (Tsai *et al.*, 1989). However, synergistic *trans*-activation from multiple binding sites, for the same or two different factors, can also occur when the levels of DNA binding proteins are saturating (Lin *et al.*, 1988 ; Lin *et al.*, 1990 ; Carey *et al.*, 1990), suggesting that, in this case, it is the activating functions of the factors, rather than cooperation at the DNA binding step, that are responsible for the effect. The proposed interpretation was that the proteins bound to the DNA could all simultaneously contact the initiation complex. Furthermore, a combination of "squelching" and synergy experiments suggested that different classes of activation domains interacted with distinct, though sometimes overlapping, basal components (Martin *et al.*, 1990 ; Tasset *et al.*, 1990).

Other experiments directly addressed the ability of *trans*-activators to bind basal transcription factors. Thus, both VP16 and the adenovirus *trans*-activator protein E1A (early gene 1A) can bind to bacterially expressed yeast TFIID (Stringer *et al.*, 1990 ; Horikoshi *et al.*, 1991). In addition, VP16 can bind to bacterially expressed human TFIIB, in an activation domain-dependent fashion (Lin *et al.*, 1991). Given that both these non-DNA binding proteins can also interact with *trans*-regulatory factors, VP16 with Oct-1 (octamer binding protein 1) (Stern *et al.*, 1989), and E1A with ATF2 (Liu and Green, 1990), for example, it was proposed that such viral activators could enhance transcription by contacting both classes of cellular factors. Association with DNA-bound regulatory proteins would bring them in the proximity of the transcriptional start site, to which they would recruit basal factors bound to their activation domains, thus promoting the formation of the initiation complex (Lillie and Green, 1989). DNA binding activators, like GAL4, on the other hand, capable of directly binding to the promoter, would assist the assembly of the initiation complex by interacting with basal factors through their own activation domains.

The initially defined orientation-independent activity of enhancers can now be explained by the various modes of protein/protein interaction described above, from cooperative DNA binding of regulatory factors to interactions of activators with the initiation complex. Indeed, electron microscopy experiments have demonstrated that long sequences of DNA can be looped out between two transcription factors which are bound at positions hundreds of nucleotides apart but directly contact each other (Théveny *et al.*, 1987 ; Su *et al.*, 1991 ; Li *et al.*, 1991). The DNA looping model had been proposed to explain the action of enhancers, in opposition to the sliding model, where transcription factors, once bound to the enhancer, would slide along the DNA and finally contact the initiation complex, and a third model which postulated DNA conformation changes being transmitted from the enhancer to the promoter (reviewed by Ptashne, 1986). The sliding model seemed unlikely to apply to eukaryotic DNA which is rarely totally naked of associated histones. The third model was shown to be inappropriate by the demonstration that an enhancer can function even when attached to a promoter *via* an avidin-biotin link rather than a phosphodiester bond (Müller *et al.*, 1989). Therefore, proteins bound at a great distance from the initiation site could interact with proteins bound near it by looping out the intervening DNA ; in that case, the precise orientation, 5' or 3', of the distal proteins would be irrelevant. A prediction of this model would be that the precise position and orientation of proximally bound proteins would be much more important, since there would be higher thermodynamic costs in introducing sharp bends in small stretches of DNA. Protein/protein interactions of proximally bound factors would be limited by protein and DNA flexibility, hence the importance of orientation for "promoter" *cis*-acting elements. The fixed orientation of such elements, together with the asymmetric nature of the initiation complex and TBP itself, would then determine the direction of transcription.

Regulatory factors, therefore, can activate transcription by directly associating with basal factors and recruiting them to the promoter. Protein/protein interactions between transcription factors are also important both for cooperative DNA binding and for bringing a number of activation domains together near the initiation complex.

1.1.5 The effects of transcription factors on nucleosome DNA templates

The observations and conclusions described in the previous section were mostly derived from transcription experiments with DNA that was free of histones and other chromatin proteins. However, in the nucleus, DNA is tightly packed with histones into nucleosomes which are themselves compacted further into higher order structures. Transcriptional initiation *in vitro* is inhibited by nucleosomes ; conversely, in yeast cells where histone synthesis has been inhibited, a number of previously inactive genes become expressed (reviewed by Felsenfeld, 1992 ; Kornberg and Lorch, 1991). DNA binding proteins, therefore, must access their sites in direct competition with histones, before they can exert their effects on transcription. Two non-exclusive models can be envisaged for how such proteins activate transcription in the presence of histones. They could actively compete with, and displace histones in order to contact their binding sites (dynamic competition). Alternatively, binding of transcription factors, for example when the DNA becomes exposed during replication, would preclude histone binding (pre-emptive competition). Both models are supported by experimental data.

GAL4 can bind to its site when this is pre-assembled in a nucleosome, arguing for the possibility of dynamic competition. In contrast, the heat shock transcription factor (HSF) cannot, unless TBP is already bound to the TATA box in the pre-assembled nucleosome (Taylor *et al.*, 1991). Such cooperative protein/protein interactions are a recurring feature of transcription factor associations with nucleosomal templates, as seen in the following cases which provide examples illustrating how pre-emptive competition by one factor could promote dynamic competition by a second one.

For instance, if DNA is incubated with a TFIID fraction before nucleosome assembly, transcription can initiate upon subsequent addition of the other basal factors (Workman and

Roeder, 1987). However, addition of TFIID during nucleosome assembly can only lead to initiation if GAL4 is also allowed to bind to the DNA template during the assembly reaction, or if the pseudorabies virus immediate early activator protein (PrIE) is included in the reaction (Workman *et al.*, 1991 ; Workman *et al.*, 1988). Furthermore, this effect relies on a functional PrIE or a GAL4 derivative containing an activation domain, indicating that contact of the activator with the initiation complex is responsible. Interestingly, *in vitro* activation by GAL4 derivatives, Sp1 and VP16 is higher in the presence of histones than in their absence ; this correlates with the suppressed basal levels of transcription from nucleosome templates, and may partly explain the fact that *trans*-activation effects observed *in vivo* are generally higher than those obtained *in vitro* (Laybourn and Kadonaga, 1991 ; Workman *et al.*, 1991).

DNA regions that are hypersensitive to deoxyribonuclease I (DNase I) *in vivo*, and therefore presumably not packed into chromatin, do exist, like, for example, the β globin locus control region (LCR) (reviewed by Orkin, 1990 ; Felsenfeld, 1992). The LCR, positioned more than 20 kilobases upstream from the globin genes, contains binding sites for many transcription factors and, in transgenic mice, confers high level expression of the *trans*-gene independently of its position of integration. Presumably, proteins recognizing the LCR cause cooperative binding of other factors further downstream, leading to transcriptional activation. The LCR does not function as an "enhancer" in transient transfections ; its influence is only observed after stable integration in the genome, arguing that it has a chromatin-mediated effect.

One of the mechanisms, therefore, by which *trans*-activators can enhance transcription, is by promoting the binding of basal factors on nucleosomal DNA templates.

1.1.6 Co-activators

As mentioned in section 1.1.4, some transcriptional activators have been shown to contact the initiation complex directly in direct binding assays. However, *trans*-activation experiments using the products of cDNAs encoding basal and regulatory factors subsequently indicated that other proteins, in addition to the TFIIs, may in fact be required to mediate the effects of the regulatory proteins (reviewed by Lewin, 1990).

In *in vitro* transcription studies, it was observed that recombinant yeast TBP, fractionated yeast TFIID, recombinant *Drosophila* TBP or recombinant human TBP, did not support binding site-specific *trans*-activation by purified human Sp1 (Hoey *et al.*, 1990 ; Peterson *et al.*, 1990 ; Pugh and Tjian, 1990). These were perfectly adequate for basal levels of transcription, but only human and *Drosophila* TFIID fractions were able to also mediate stimulation by Sp1. In addition, when a heat-treated nuclear extract, which lacks TFIID activity but contains all other necessary factors, was used, only a full length human TBP, but not a deletion mutant that lacked the species-specific amino-terminus, restored the response to Sp1. Two conclusions can be drawn from these and similar experiments with other transcription factors, including VP16 (Kelleher *et al.*, 1990 ; Berger *et al.*, 1990), USF (upstream stimulatory factor) (Hoffmann *et al.*, 1990) and CTF (Pugh and Tjian, 1990). Firstly, proteins, termed co-activators, mediators or adaptors, distinct from the previously characterised basal and regulatory factors, are required for stimulation of transcription above basal levels. Secondly, the interactions of co-activators with the initiation complex, and specificially with TBP, can be species-specific, and mediated by the TBP amino-terminus. It was clear, however, that whereas the co-activator(s) defined by the glutamine-rich activator Sp1 were associated with endogenous TBP (Peterson *et al.*, 1990), those required by acidic activators in yeast were not (Kelleher *et al.*, 1990 ; Flanagan *et al.*, 1991).

The discrepancy between the large chromatographic size of native TFIID fractions (Reinberg *et al.*, 1987) and the relatively small molecular weight of TBP (see section 1.1.2) had already suggested that native TBP was complexed with other cellular proteins. Indeed, two distinct cellular fractions of 300 and >700 kD molecular weight, contained TBP protein (Timmers and Sharp, 1991). However, though they could both reconstitute basal transcription, the 300 kD fraction responded extremely poorly to *trans*-activation by Sp1, USF or an acidic activator. A number of TAFs have now been characterised from human (Pugh and Tjian,

1991) and *Drosophila* cells (Dynlacht *et al.*, 1991) and shown to be required for stimulated but not basal transcription. TAFs, therefore, have multiple roles, including bringing TBP in the proximity of the initiation site (see Section 1.1.2 above), and mediating activation by regulatory factors. Consistent with the observation that co-activators for acidic domain transcription factors were not present in a TFIID fraction (see above), the isolated human TAFs could mediate *trans*-activation by the proline-rich CTF and the glutamine-rich Sp1 but not the acidic VP16 (Tanese *et al.*, 1991). Instead, a genetic screen in yeast yielded a protein, ADA2 (adaptor 2), necessary for transcriptional response to VP16 and the also acidic GCN4 (general control of nitrogen metabolism gene 4) both *in vivo* and *in vitro* (Berger *et al.*, 1992); interestingly, ADA2 was dispensable for activation by HAP4 (haeme activator protein 4), which also possesses an acidic activation domain. Finally, an activity that potentiated *trans*-activation co-fractionated with a separable activity that inhibited basal transcription, suggesting that negative control of unstimulated transcription may be a prerequisite for positive regulation (Meisterernst *et al.*, 1991).

A number of *bona fide* co-activator genes, such as *SWI1* (switch 1), *SWI2/SNF2* (sucrose non-fermenting 2), *SWI3*, *SNF5* and *SNF6*, that do not encode DNA binding proteins but are necessary for the transcription of many different genes, have been characterised in yeast (Peterson and Herskowitz, 1992; Laurent and Carlson, 1992). However, there seems to be some specificity in this effect since, for example, *SWI1* is required for activity of a promoter containing a GAL4 but not a SWI4/SWI6 binding site (Peterson and Herskowitz, 1992). Furthermore, some of the encoded proteins act cooperatively, suggesting that they could be present in the same functional complex (Laurent and Carlson, 1992).

The requirement for protein/protein interactions for transcriptional regulation therefore seems to also involve molecules that act as a "bridge" between the DNA-bound basal and regulatory factors.

1.1.7 Repression of transcription

The term "repression" covers two distinct phaenomena : repression of basal transcription (see section 1.1.2) and inhibition of *trans*-activation. The latter can in turn be due to a number of different mechanisms. For example, proteins that share a common binding site with an activator can compete with it for binding to the DNA and thus prevent *trans*-activation, as is the case for some *Drosophila* homoeodomain proteins (Jaynes and O'Farrell, 1988 ; Han *et al.*, 1989). Alternatively, an activator, e.g. the thyroid hormone receptor, may bind to its own site but not activate transcription because it is in a non-active conformation, e.g. in the absence of thyroid hormone (reviewed by Levine and Manley, 1989). Finally, a DNA binding protein may simply be unable to interact productively with the initiation complex. This effect may be concentration-dependent, as for the *Drosophila* Krüppel protein, which activates at low and represses at high concentrations (Sauer and Jäckle, 1991), or position-dependent, as in the case of YY1, which is an activator when bound to the initiator but represses when bound upstream of a TATA box in a synthetic promoter (Shi *et al.*, 1991 ; Seto *et al.*, 1991). Protein domains specific for repression have not been as extensively characterised as activation domains, though an alanine-rich region in Krüppel has been implicated in its repressor function (Licht *et al.*, 1990).

Finally, *trans*-activation can be prevented by sequestering activators into inactive complexes. Three such mechanisms have been described so far and are discussed in greater detail in Chapter 11. Briefly, IκB, the inhibitor of NFκB, a transcription factor initially described as binding to the B element of the immunoglobulin κ light chain enhancer, binds NFκB in a cytoplasmic complex and prevents it from reaching the nucleus and activating transcription (Bauerle and Baltimore, 1988). CREM (CREB modulator) proteins, on the other hand, bind to the ATF site as homodimers or heterodimers with CREB ; some CREM isoforms prevent *trans*-activation presumably because they lack an activation domain (Foulkes *et al.*, 1991). Finally, Id (inhibitor of differentiation) heterodimers with basic domain/helix-loop-helix (bHLH) *trans*-activators are unable to bind DNA since Id lacks a basic domain (Benezra *et al.*, 1990).

Thus, although some proteins with an inherent ability to repress transcription have been described, inhibition of *trans*-activation can clearly also occur by DNA-dependent and -independent antagonists of activating transcription factors.

Three classes of proteins are therefore necessary for regulated transcription : the general factors, including pol II, which assemble near the initiation site, activators or repressors that may or may not bind to the DNA at varying distances from the initiation complex, and co-activators which "mediate" between the two previous classes (reviewed by Lewin, 1990) (Fig.1.2). Transcriptional control relies not only on the interactions of these proteins with the DNA but also on positive and negative interactions among them.

1.2 Early differentiation events during murine embryogenesis

The first two cell divisions after fertilisation of the mouse oocyte give rise to cleavage cells that remain loosely associated. These blastomeres are equipotent and totipotent : any one of them can give rise to a whole embryo. However, after the third cleavage, the eight blastomeres compact, resulting in a structure, the morula, which has a clear external surface and an internal core. In the morula, intercellular junctions are established and cells on the outside layer acquire distinct polar and basal membranes, accompanied by cytoskeletal and organelle re-organisation. This polarisation and the subsequent exposure of cells to different microenvironments, depending on their position, underlie the first differentiation event which occurs after the fourth cleavage. Cells on the inside become the inner cell mass (ICM), while cells on the outside form an epithelial layer, the trophoblast, surrounding the ICM and a fluid-filled cavity, the blastocoel (Fig.1.3). These two new groups of cells have distinct developmental fates : the trophoblast will give rise to exclusively extra-embryonic, supporting structures while the ICM will contribute to both embryonic and extra-embryonic lineages.

After the 4.5 days *post coitum* (dpc) blastocyst has hatched out of the zona pellucida, and before it implants into the uterus, both ICM and trophoblast undergo further differentiation. Depending on their position, trophoblast cells become polar or mural trophectoderm, in contact with the ICM or the blastocoel, respectively. ICM cells exposed to the blastocoel are then organised into a layer of primitive endoderm, restricted to the extra-embryonic lineage, surrounding the rest of the ICM cells which differentiate into primitive ectoderm or epiblast cells, destined to form the embryo proper (Fig.1.3). After implantation, cell movement and differentiation intensify. Primitive endoderm cells proliferate to coat the expanding primitive ectoderm and become visceral endoderm, or line the mural trophectoderm and become parietal endoderm (Fig.1.3) (reviewed by Hogan *et al.*, 1986 ; Jackson, 1989). Parietal and visceral endoderm have distinct functions and, consequently, morphological and biochemical properties (reviewed by Hogan *et al.*, 1986). Visceral endoderm cells form an epithelial layer around the embryonic ectoderm, displaying microvilli on their apical surfaces, and other specialised morphological characteristics. Their main functions are to absorb substances from the maternal circulation and to secrete substances required by the foetus, such as α-foetoprotein (AFP). Parietal endoderm cells, on the other hand, are not in close contact with each other, are motile, and display a number of morphologies, from stellate to round (Hogan *et al.*, 1983). Their most important function is to secrete Reichert's membrane which serves as a barrier between the foetal and the maternal environments. For this, they synthesize large amounts of basement membrane proteins, such as laminin and type IV procollagen.

1.3 F9 embryonal carcinoma cells

1.3.1 F9 embryonal carcinoma cell differentiation as a model for early differentiation events in the mouse embryo

F9 embryonal carcinoma (EC) cells are ultimately derived from a 6 dpc male embryo which had been transplanted into the testis to give rise to the transplantable tumour OTT6050 (Bernstine *et al.*, 1973). They have a very low rate of spontaneous differentiation. However, when treated *in vitro* with (all)*trans*-retinoic acid (RA) in monolayers or as aggregates, they differentiate into primitive endoderm-like or visceral endoderm-like cells, respectively (Fig.1.4). If compounds that elevate intracellular cAMP levels, such as N^6,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate (dbcAMP), are also added to F9 EC cell monolayers being treated with RA, these differentiate further into parietal-endoderm-like (PE) cells. Though it is not clear whether undifferentiated F9 EC cells are ICM-like, the similarities between F9 PE cells and parietal endoderm cells found *in vivo* have been well established. Like them, they are flat cells, often with long processes, which do not contact each other. They secrete tissue plasminogen activator, type IV procollagen and laminin, have low levels of alkaline phosphatase and lactate dehydrogenase activity, and do not synthesize AFP (Strickland and Mahdavi, 1978 ; Strickland *et al.*, 1980 ; Hogan *et al.*, 1981). F9 EC cells, therefore, provide a good model system for studying the molecular mechanisms of early differentiation events in the mouse embryo.

1.3.2 Regulation of viral transcription in F9 EC cells

F9 EC cells are particularly suitable for the study of transcriptional regulation since the activities of a number of viral transcriptional control sequences are regulated during their differentiation. The advantage of using viral, instead of cellular, control elements to study transcription is that they are small and well defined. Since viruses use their hosts' transcriptional apparatus, proteins important for viral transcription will also be responsible for controlling the expression of host genes (reviewed by Jones *et al.*, 1989). Thus, viral transcriptional control regions whose activity is altered during the differentiation of EC cells can be used as probes for cellular transcription factors which are regulated during differentiation, and which will also affect the expression of cellular genes. For example, the simian virus 40 (SV40) and the polyoma virus A enhancers are inactive in undifferentiated EC cells but enhance transcription in their differentiated derivatives. This has been attributed both to the presence of enhancer-binding repressors of transcription in, and the absence of enhancer-binding transcriptional activators from, EC cells (Herbomel *et al.*, 1984 ; Gorman *et al.*, 1985 ; Sleigh and Lockett, 1985 ; Kryszke *et al.*, 1987 ; La Thangue and Rigby, 1987 ; Sleigh *et al.*, 1987 ; Wasylyk *et al.*, 1988). Similarly, transcription of murine leukaemia and sarcoma viruses occurs only after differentiation of EC cells, and its repression in undifferentiated cells has been correlated with the binding of EC cell-specific proteins to its transcriptional control sequences (Linney *et al.*, 1984 ; Gorman *et al.*, 1985 ; Loh *et al.*, 1988 ; Loh *et al.*, 1990 ; Akgün *et al.*, 1991). On the other hand, transcription from the adenovirus type 5 (Ad5) *E2A* (early gene 2A) promoter occurs in F9 EC cells (Imperiale *et al.*, 1984), in mouse oocytes and in pre-implantation embryos (Suemori *et al.*, 1988 ; Dooley *et al*, 1989) in the absence of viral E1A, the *trans*-activator of adenovirus early genes, which is normally required in other cell types (Jones and Shenk, 1979). This led to the suggestion that a cellular "E1A-like" activity exists in these experimental systems ; when assayed by Ad5 *E2A* gene expression, it is seen to be reduced after the differentiation of F9 EC cells or implantation of the mouse embryo (Imperiale *et al.*, 1984 ; Suemori *et al.*, 1988 ; Dooley *et al*, 1989). The analogy between transcriptional regulation in F9 EC cells and cells expressing adenovirus E1A extends further. In F9 EC cells, the *hsp70* gene, which is activated by E1A, is constitutively expressed (Imperiale *et al.*, 1984). In contrast, and again similarly to the situation in F9 EC cells, E1A represses the SV40 and polyoma enhancers but not their promoters (Velcich and Ziff, 1985 ; Velcich *et al.*, 1986). However, it is unlikely that F9 EC cells contain an E1A "look-alike" molecule, since expression of E1A in these cells leads to their differentiation, rather than inhibits it (Montano and Lane, 1987). It is more probable, therefore, that the "E1A-like" activity is the result of a number of biochemical activities specific to the undifferentiated state.

1.4 The adenovirus transcriptional regulator E1A

Trans-activation by E1A has been extensively studied (reviewed by Berk, 1986). The protein domain responsible for *trans*-activation was originally mapped to conserved region 3 (CR3), a region of homology among all adenovirus serotypes, and unique to the product of the largest, 13S, *E1A* RNA (reviewed by Moran and Mathews, 1987). CR3 has been shown to be necessary and sufficient for transcriptional stimulation by E1A, *in vivo* and *in vitro*, and in the absence of protein synthesis (Lillie *et al.*, 1987 ; Green *et al.*, 1988). Since E1A does not bind DNA in a sequence-specific fashion (Ferguson *et al.*, 1985 ; Chatterjee *et al.*, 1988), these results suggested that it regulates transcription by modifying the binding and/or activation properties of existing cellular factors. During the attempt to identify those *cis*-acting sequences in E1A-inducible promoters which were responsive to E1A, it emerged that a large number of DNA motifs, including some TATA boxes, could mediate this activation (Pei and Berk, 1989 ; Taylor and Kingston, 1990, and references therein). Furthermore, the E1A-responsive elements were also required for unstimulated transcription (Williams *et al.*, 1989 ; Murthy *et al.*, 1985), and the extent of *trans*-activation by E1A was inversely proportional to the strength of the uninduced promoter (Taylor and Kingston, 1990). These two observations suggested that either E1A was acting at a single general step in the transcription process, or by speeding up different rate-limiting steps through different mechanisms, depending on the promoter.

That E1A may in fact affect transcription by various pathways, seems to be supported by the variety of its reported direct or indirect interactions. Studies by Lillie and Green (1989) and Martin *et al.* (1990) have defined two sub-regions in the CR3 activation domain (amino acids 139-189) and demonstrated the importance of the C-C type zinc finger that is found in this region. The amino-terminal end, including the zinc finger (amino acids 140-178) is required for activation proper and is thought to interact with the basal transcriptional machinery. The carboxy-terminal amino acids (179-189), on the other hand, serve to bring E1A to the proximity of an E1A-inducible promoter, presumably by protein-protein associations with DNA-bound transcription factors, such as ATF2 (Liu and Green, 1990). A complex between E1A and TBP over-expressed *in vivo* or *in vitro*, involving the basic domain of TBP which lies between the two DNA binding repeats, and the activation domain of E1A, has been defined (Lee, W.S. *et al.*, 1991). Not all E1A molecules which are mutated in the amino-terminal activation sub-region affect formation of this complex, however, implying that the E1A/TBP interaction is necessary but not sufficient for *trans*-activation.

In addition, "squelching" experiments suggested that E1A interacted with a component of the transcriptional machinery not required by the VP16 acidic activator, perhaps a co-activator (Martin *et al.*, 1990). The idea that E1A might be stabilising the interactions of transcription factors with co-activators, or act as a co-activator itself, was supported by studies of the interactions between E1A and the octamer motif binding protein, Oct-4, or the retinoic acid receptor $\beta 2$ (RAR$\beta 2$). The octamer motif activates transcription efficiently in F9 EC cells, which contain high levels of Oct-4, and in 293 cells, but not HeLa cells, transfected with an Oct-4 expression vector. 293 cells constitutively express E1A and F9 EC cells contain an "E1A-like" activity ; similarly, HeLa cells responded to Oct-4 when co-transfected with an E1A expression vector. However, only specific combinations of Oct-4 and E1A concentrations optimally activated transcription, suggesting an equilibrium between "squelching" and productive interaction with the initiation complex. The Oct-4/E1A interaction and *trans*-activation required the activation domains of both molecules and could be reproduced *in vitro* (Schöler *et al.*, 1991). In a similar experiment, expression of TBP and RAR$\beta 2$ in embryonal carcinoma cells led to cooperative *trans*-activation, but simultaneous expression of E1A was needed in COS cells (Berkenstam *et al.*, 1992). Thus, one mechanism of transcriptional stimulation by E1A seems to consist of providing a "link" between the basal and regulatory factors.

Two *cis*-acting elements and their cognate binding proteins have received particular attention with respect to their ability to mediate activation by E1A. One is the E2F (*E2A* promoter binding factor) binding site (consensus TTTCGCGC ; Mudryj *et al.*, 1990), which was initially characterised as an E1A-inducible motif in the adenovirus *E2A* promoter recognized by the transcription factor E2F in adenovirus-infected HeLa cells (Kovesdi *et al.*, 1986) and by DRTF-1 (differentiation-regulated transcription factor 1) in F9 EC cells (La Thangue *et al.*, 1990). In the absence of E1A, E2F/DRTF-1 is bound to the retinoblastoma gene product (pRb) and cell cycle regulators, such as cyclin A, in a transcriptionally inactive complex (Bandara and La Thangue, 1991 ; Bandara *et al.*, 1991). E1A, however, is able to dissociate this complex by binding to pRb, and release active E2F/DRTF-1 (Zamanian and La Thangue, 1992) ; E1A conserved regions 1 and 2 rather than CR3 are required for this (Bandara and La Thangue, 1991). After dissociation from pRb, DNA binding by E2F can be stabilised by complexing with the 19 kD product of the ORF6/7 transcript of the adenovirus *E4* gene (Huang and Hearing, 1989 ; Bagchi *et al.*, 1990).

Phosphorylation has been claimed to be the E1A-induced mechanism that activates a different transcription factor, E4F, binding to a site (consensus (A/T)CGTCA ; Lee, K.A.W *et al.*, 1987), known as the cAMP-responsive element (CRE) or the ATF site (Raychaudhuri *et al.*, 1989). E4F is one of the many characterised proteins recognizing this site, and its binding is ehanced after adenovirus infection of HeLa cells (Raychaudhuri *et al.*, 1987). Another ATF site-binding protein, ATF2, mediates E1A *trans*-activation apparently by interacting with E1A ; for this, the E1A CR3 region and the ATF2 amino-terminus are required (Liu and Green, 1990).

Thus, the mechanisms by which E1A activates transcription seem to include direct protein/protein interactions, resulting in the formation of active complexes, as in the Oct-4/E1A case, or in the dissociation of transcriptionally inert ones, as with E2F/pRb, as well as induction of phosphorylation.

1.5 Transcriptional regulation through the ATF site

The ATF site, or CRE, is present in many cellular and viral promoters (reviewed by Roesler *et al.*, 1988 ; Lee, K.A.W *et al.*, 1987), and is required for both basal and induced transcription. For simplicity, it will be henceforward referred to solely as the ATF site, irrespective of its functional role.

Its importance for uninduced transcription has been demonstrated both *in vivo*, for the somatostatin and Ad5 *E2A* genes (Andrisani *et al.*, 1987 ; Murthy *et al.*, 1985), and *in vitro*, for the phosphoenolpyruvate carboxykinase (PEPCK) gene (Klemm *et al.*, 1990). In addition, the ATF site is responsible for the activation of, for example, the somatostatin, the vasoactive intestinal polypeptide (VIP), and the glycoprotein hormone α subunit (αGH) genes by cAMP, and can act as a cAMP-responsive enhancer of a heterologous promoter (Montminy *et al.*, 1986 ; Silver *et al.*, 1987 ; Deutsch *et al.*, 1987 ; Fink *et al.*, 1988). A functional cAMP-dependent protein kinase A (PKA) is required for this response (Montminy *et al.*, 1986). The same sequence in the context of the adenovirus *E2A* and *E4* promoters contributes to their E1A-inducibility (Murthy *et al.*, 1985 ; Lee and Green, 1987), while it is a Ca^{+2}-responsive element in the enkephalin and c-*fos* promoters (Sheng *et al.*, 1988 ; Van Nguyen *et al.*, 1990). Finally, the two closely spaced αGH ATF sites are part of a species- and tissue-specific enhancer which allows expression of the human αGH gene in the placenta as well as the pituitary ; the bovine αGH gene, which lacks these ATF sites, is only expressed in the pituitary, both in the animal and in transgenic mice (Delegeane *et al.*, 1987 ; Bokar *et al.*, 1989).

Despite their sequence similarity, however, ATF sites are not functionally interchangeable. For example, an E1A-inducible *E4* ATF site cannot respond to cAMP, nor is the cAMP-inducible *VIP* ATF site E1A-responsive (Lee *et al.*, 1989). Furthermore, the protein binding properties of sites which are all cAMP-responsive can still differ. For example, protein binding to the tyrosine aminotransferase (TAT) ATF site is enhanced *in vivo* by forskolin treatment, which increases intracellular cAMP levels, and *in vitro* by incubation with PKA, while binding to the somatostatin and PEPCK ATF sites remains unaltered ; similarly, one of the TAT ATF site complexes is reduced when protein synthesis is blocked, while no such effect is seen on the somatostatin ATF site (Weih *et al.*, 1990 ; Nichols *et al.*, 1992). Given that not all ATF sites form the same protein/DNA complexes *in vitro* or are equally potent in their response to cAMP, functional differences are likely to be due to the binding of different proteins which would all recognize the ATF site but would be influenced in their precise specificities by flanking DNA sequences (Andrisani *et al.*, 1988 ; Deutsch *et al.*, 1988).

1.6 A family of ATF site-binding proteins and *ATF* genes

Polypeptide fractions with molecular weights ranging from 30 to 80 kD were able to form ATF site-specific protein/DNA complexes (Cortes *et al.*, 1988), and indeed, a number of ATF site-binding proteins with distinct properties were soon purified. These included CREB, which has a molecular weight of 43 kD (Montminy and Bilezikjian, 1987), ATF (43 and 47 kD) (Hai *et al.*, 1988b), E4F (50 kD) (Raychaudhuri *et al.*, 1989), and EivF (65-72 kD) (Cortes *et al.*, 1988). Apart from their relative molecular weights, other differences, such as precise binding specificities, also became apparent. For example, E4F bound exclusively to an adenovirus *E4* ATF site, and with distinct nucleotide requirements from those of purified ATF (Raychaudhuri *et al.*, 1987 ; Rooney *et al.*, 1990). EivF, on the other hand, bound only to two out of three *E4* ATF sites, though, contrary to E4F, it was also able to bind to ATF sites from cellular promoters (Cortes *et al.*, 1988). Similarly, though the methylation interference patterns of ATF1 and ATF2 were indistinguishable, binding by ATF3 required an additional nucleotide outside the core ATF site (Hai *et al.*, 1989).

The variety in functions of the ATF site and binding characteristics of purified ATF proteins was eventually reflected in the cloning of at least ten distinct mammalian cDNAs encoding ATF site-binding proteins : CREB (Hoeffler *et al.*, 1988 ; Gonzalez *et al.*, 1989), CREBP1/ATF2 (Maekawa *et al.*, 1989 ; Hai *et al.*, 1989), ATF1, ATF3, ATF4 and ATF6 (Hai *et al.*, 1989), ATFa (Gaire *et al.*, 1990), TREB5 (Yoshimura *et al.*, 1990), CREM (Foulkes *et al.*, 1991) and E4BP4 (Cowell *et al.*, 1992). Although these cDNAs were different from each other, they shared a region of homology, encoding a DNA binding domain rich in basic amino acids, adjacent to a "leucine zip" dimerization domain (Landschulz *et al.*, 1988b). The *ATF* gene family was therefore shown to be a relative of the *C/EBP* (cellular enhancer binding protein) and the *AP1* basic domain-"leucine zip" (bzip) transcription factor gene families, whose members bind DNA as homo- or heterodimers (reviewed by Busch and Sassone-Corsi, 1990 ; Habener, 1990).

1.6.1 ATF protein domains

Studies on the *Jun*, *Fos* and *C/EBP* bzip families had already shown that both the basic domain and the "leucine zip" were required for DNA binding, and led to the hypothesis that, in a dimer, the leucine heptad repeats are aligned parallel to each other, with the leucine side chains interdigitating and the basic domains contacting the DNA (Landschulz *et al.*, 1989 ; Turner and Tjian, 1989 ; Gentz *et al.*, 1989). Crystallographic experiments confirmed that the "leucine zip" has a coiled coil conformation, where the α-helical leucine repeat regions of the two monomers coil around each other, in parallel fashion, held together by electrostatic and hydrophobic interactions (Fig.1.5) (O'Shea *et al.*, 1991). Modelling and physicochemical studies of the basic domain, on the other hand, have indicated that it undergoes a structural change upon binding to the DNA : the two basic regions of the dimer adopt an α-helical conformation and contact, in opposite orientations, the major groove of the DNA (Fig.1.5) (Talanian *et al.*, 1990 ; Shuman *et al.*, 1990 ; O'Neill *et al.*, 1990).

Domains of ATF transcription factors that are important for functions other than DNA binding have been most extensively studied for CREB, in which a number of regions important for activation have been defined. Its amino-terminus (amino acids 1-87) has a 20% glutamine content ; its deletion reduces basal CREB activity to 15% of the wild type molecule but has no effect on its response to cAMP (Gonzalez *et al.*, 1991). Interestingly, glutamine-rich regions are absent in the α and β CREM isoforms, which inhibit CREB function, but present in the τ isoform which is an activator (Foulkes *et al.*, 1991 ; Foulkes *et al.*, 1992). CREB amino acids 87-151 are critical for both basal and cAMP-induced *trans*-activation (Lee *et al.*, 1990 ; Gonzalez *et al.*, 1991). They include peptide α, which is predicted to form an amphipathic α-helix (Yamamoto *et al.*, 1990) and is one of the alternatively spliced CREB domains (Ruppert *et al.*, 1992), and serine 133, which is phosphorylated by PKA, an event required for cAMP-dependent stimulation of transcription (Gonzalez and Montminy, 1989 ; Lee *et al.*, 1990). However, serine 133 is necessary but not sufficient : residues lying amino-terminally to it are also required for CREB activity, presumably due to their ability for phosphorylation *in vivo*, as are carboxy-terminal sequences (Lee *et al.*, 1990). Interestingly, the latter contain a tetrapeptide, DLSS, that is conserved in ATF1, which is also cAMP-responsive ; mutation of the aspartic acid residue to asparagine resulted in a five-fold decrease in both basal and cAMP-induced activity (Gonzalez *et al.*, 1991). Since these carboxy-terminal sequences do not contribute to phosphorylation (Lee *et al.*, 1990), their role may be structural, especially as introduction of a triglycine spacer between the DLSS tetrapeptide and serine 133 totally abolishes CREB activity (Gonzalez *et al.*, 1991).

Two regions that are important for the function of other ATF proteins have also been described. One is the ATF2 amino-terminus (amino acids 1-109), through which it mediates E1A *trans*-activation ; it contains a putative zinc-binding region whose precise molecular involvement in the response to E1A, however, has not been studied (Liu and Green, 1990). The carboxy-terminus of E4BP4 is responsible for ATF site-dependent repression ; this region does not bear any similarity to characterised repression domains but encodes a putative second "leucine zipper", though, again, the importance of this structure has not been assessed (Cowell *et al.*, 1992).

1.6.2 Diverse properties and responses of ATF family members

The complexity of the *ATF* gene family is not restricted to the large number of its members ; it is discussed briefly here, and at greater length in Chapter 11, in the light of results presented in this thesis and in the context of other transcription factor families.

Heterodimerization increases the number of potential ATF site-binding protein complexes. Dimerization, however, is not promiscuous : ATF2, for example, can complex with ATF3, but ATF1 cannot (Hai *et al.*, 1989). Nevertheless, members of the ATF family can also dimerize with proteins belonging to the related bzip AP1 family, and the choice of dimerization partner influences DNA binding specificity (Ivashkiv *et al.*, 1990 ; Benbrook and Jones, 1990 ; Macgregor *et al.*, 1990 ; Hai and Curran, 1991). Alternative splicing of transcripts from the same gene adds another level of complexity and regulation, as has been reported for ATFa (Gaire *et al.*, 1990), CREM (Foulkes *et al.*, 1991) and CREB (Ruppert *et al.*, 1992).

Functional differences between the different ATF proteins also exist. ATF2, for example, responds to E1A *trans*-activation (Liu and Green, 1990), while ATF1 and CREB activate transcription in response to cAMP (Gonzalez *et al.*, 1989 ; Liu and Green, 1990 ; Rehfuss *et al.*, 1991).

The ATF family, therefore, contains transcriptional activators, like CREB (Yamamoto *et al.*, 1990), repressors, like E4BP4 (Cowell *et al.*, 1992), and antagonists, like CREM, which lack an activation domain and prevent the activators from stimulating transcription by forming inactive heterodimers with them (Foulkes *et al.*, 1991). With different members of this family responding to distinct stimuli, a variety of regulatory functions can be fulfilled by a single *cis*-acting element and through a conserved DNA binding domain.

1.6.3 ATF "homologues" in non-mammalian organisms

ATF proteins are not restricted to mammalian organisms ; a 66 kD ATF site-binding protein has been purified from yeast (Lin and Green, 1989a), and two cDNAs each from tobacco and wheat encoding bzip proteins have been isolated that bind to similar DNA sequences (Katagiri *et al.*, 1989 ; Tabata *et al.*, 1991). The versatility of "ATF" site-binding proteins was again apparent, since expression of the two tobacco cDNAs varies among different plant tissues and in its response to light (Katagiri *et al.*, 1989). A *Drosophila* bzip *trans*-activator cDNA has also been cloned that bears 66% similarity to mammalian CREB in the basic region (Abel *et al.*, 1992 ; Smolik *et al.*, 1992). *BBF-2* (box B binding factor 2)/d*CREB-A* (*Drosophila* CREB A) is expressed throughout *Drosophila* development (Abel *et al.*, 1992), and the RNA is detected specifically in regions of the brain and the reproductive organs of both sexes in adult animals (Smolik *et al.*, 1992). In addition, the protein binds to and activates fat body-specific enhancers (Abel *et al.*, 1992) ; it has therefore been postulated that it may play a role in tissue-specific gene expression. Surprisingly, since the mechanisms of induction by cAMP are different in bacteria and higher eukaryotes, there are nucleotide similarities between some *E.coli* catabolite activator protein (CAP) binding sites and the ATF site, and the *crp* gene CAP binding site can function as a cAMP-inducible transcriptional control element in eukaryotic cells (Lin and Green, 1989b). These results suggest that there has been both structural and, in some respects, functional conservation of *ATF* genes in evolution.

1.7 Aim of the project

The main body of this thesis focuses on the regulation of ATF proteins during the differentiation of F9 EC cells. The adenovirus *E4* promoter contains at least three consensus ATF sites which have been shown to be its most important *cis*-acting elements *in vivo* and *in vitro*, both for basal expression and for E1A-mediated *trans*-activation (Handa and Sharp, 1984 ; Gilardi and Perricaudet, 1984 ; Gilardi and Perricaudet, 1986 ; Hanaka *et al.*, 1987). The *E4* gene is the second viral gene to be expressed after *E1A* during the early stages of adenovirus infection. It contributes to the activation of the *E2A* gene, then its transcription is rapidly repressed by the 72 kD E2A product (Nevins *et al.*, 1979 ; Nevins and Jensen-Winkler, 1980 ; Goding *et al.*, 1985). There were two reasons for studying its transcription and the ability of its promoter to bind transcriptional regulator proteins in F9 EC and PE cells. The first was that *E4* expression, as mentioned above, activates *E2A* transcription (Goding *et al.*, 1985). It was therefore possible that in the original experiments where *E2A* expression, from mutant viruses which were only defective in the *E1A* gene, was interpreted as an indication of a cellular "E1A-like" activity in F9 EC cells (Imperiale *et al.*, 1984), *E4* expression was also activating *E2A*, and thereby contributing to the so-called "E1A-like" activity. If this were true, it would be reasonable to postulate the existence of EC cell-specific transcription factors that activate *E4* transcription in the absence of E1A. The second reason was that ATF site-binding activities, most likely to control *E4* transcription through its ATF sites, had not been studied in the F9 EC cell system. Given that F9 EC cells can be differentiated, they offered an ideal system for studying the possible regulation of ATF-site binding proteins.

A different transcription factor, DRTF-1, contributes to the high transcriptional activity of the Ad5 *E2A* gene in F9 EC cells and is down-regulated during their differentiation, as assayed by gel retardation (La Thangue *et al.*, 1990 ; Shivji and La Thangue, 1991 ; Zamanian and La Thangue, 1992). DRTF-1 binds to the previously characterised binding site of E2F, a transcription factor originally defined in adenovirus-infected HeLa cells (Kovesdi *et al.*, 1986 ; Yee *et al.*, 1989). The relationship of F9 DRTF-1 to HeLa E2F is, at the moment, unclear, though it is evident that there exist more than one cDNA products with E2F/DRTF1 properties (Helin *et al.*, 1992 ; Kaelin *et al.*, 1992 ; R.Girling *et al.*, submitted). Chapter 10 describes an attempt to generate monoclonal antibodies against DNA binding site affinity-purified DRTF-1, the results of which were inconclusive.

The diversity of ATF transcription factors in other biological systems, and of responses mediated by the ATF site, prompted the present study of the number and regulation of ATF site-binding proteins during the differentiation of embryonal carcinoma cells. The results described here suggest that the regulation of ATF-dependent transcription during differentiation of embryonal carcinoma cells can potentially occur at several levels, including *ATF* gene expression, as well as differential heterodimerization, binding site preference, and promoter specificity of ATF proteins.

FIG.1.1 : *Assembly of the initiation complex (adapted from Roeder, 1991)*

a) Order of assembly of basal transcription factors ; it is not clear whether TFIIA is required for assembly of all initiation complexes or whether it remains in the complex throughout the process (Buratowski *et al.*, 1989 ; Maldonado *et al.*, 1990 ; Sumimoto *et al.*, 1990). A fraction called TFIIJ has also been purified, but is not included in this diagram, since its polypeptide composition has not been characterised (Flores *et al.*, 1992).

b) Schematic representation of the initiation complex.

a

$$\text{Promoter} \xrightarrow{D} \xrightarrow{A} \xrightarrow{B} \xrightarrow{\text{Pol II/F}} \xrightarrow{E+H} \text{Initiation Complex}$$

b

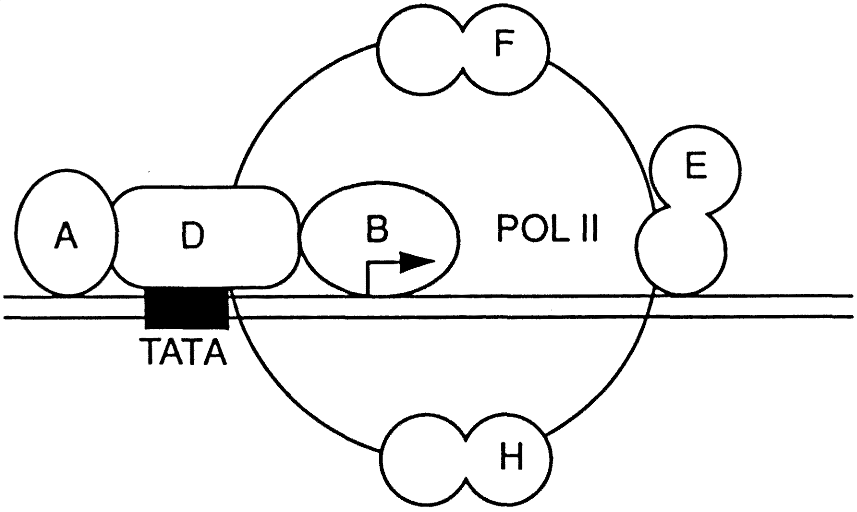

F
E
A
D
B
POL II
TATA
H

FIG.1.2 : *Protein-DNA and protein-protein interactions on the promoter of a pol II -transcribed gene*

The DNA is depicted as a single line and the transcriptional start site is indicated by an arrow. TF : DNA binding transcription factor ; CO-ACT : co-activator ; TA : non-DNA binding *trans*-activator ; IC : initiation complex.

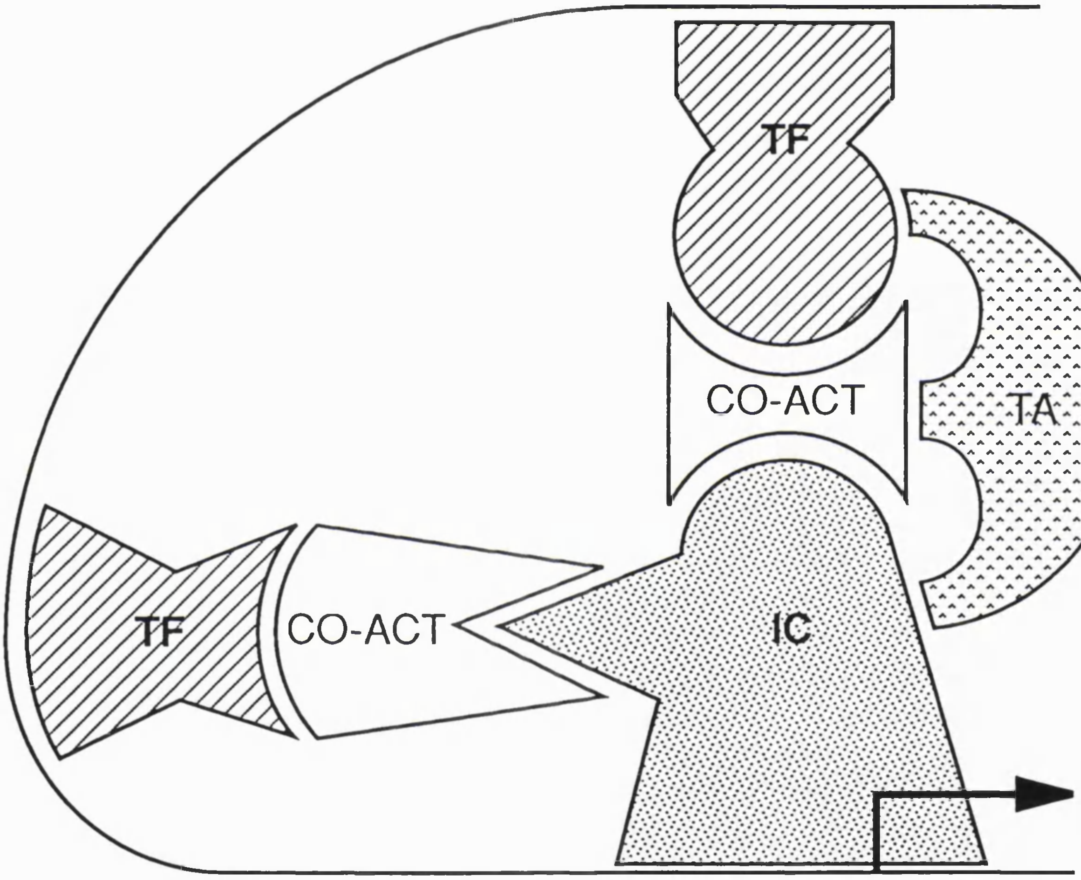
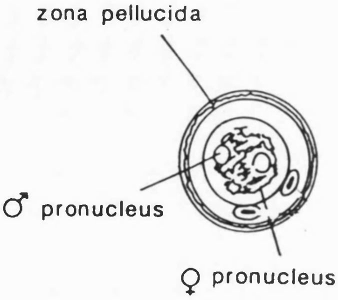
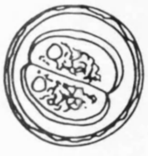

TF
CO-ACT
TA
TF
CO-ACT
IC

FIG.1.3 : *Early development of the mouse embryo (adapted from Jackson, 1989)*

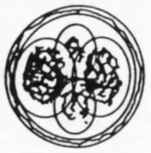

zona pellucida
♂ pronucleus
♀ pronucleus

~8 hours p.c.



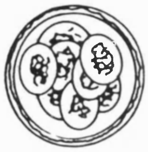
early cleavage :

~24 hours p.c.



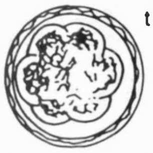
cells loosely associated

~40 hours p.c.

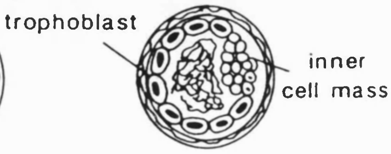


8 cell embryo

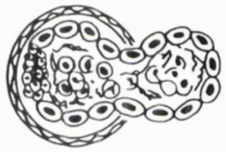
~48 hours p.c.



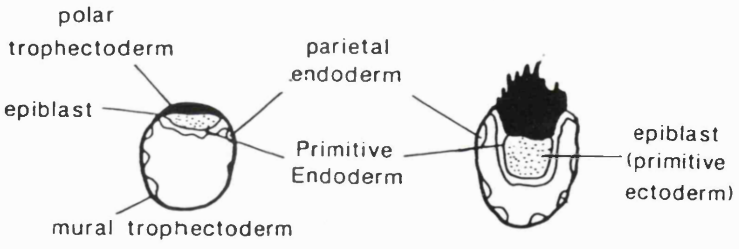
compacted morula
tight junction formed
~54 hours p.c.


trophoblast
inner cell mass

early blastocyst
64 cells
~96hours p.c.

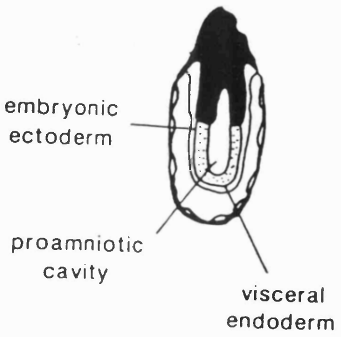


~108 hours p.c.
hatching blastocyst


polar trophectoderm
epiblast
parietal endoderm
Primitive Endoderm
mural trophectoderm
epiblast (primitive ectoderm)

4.5 dpc

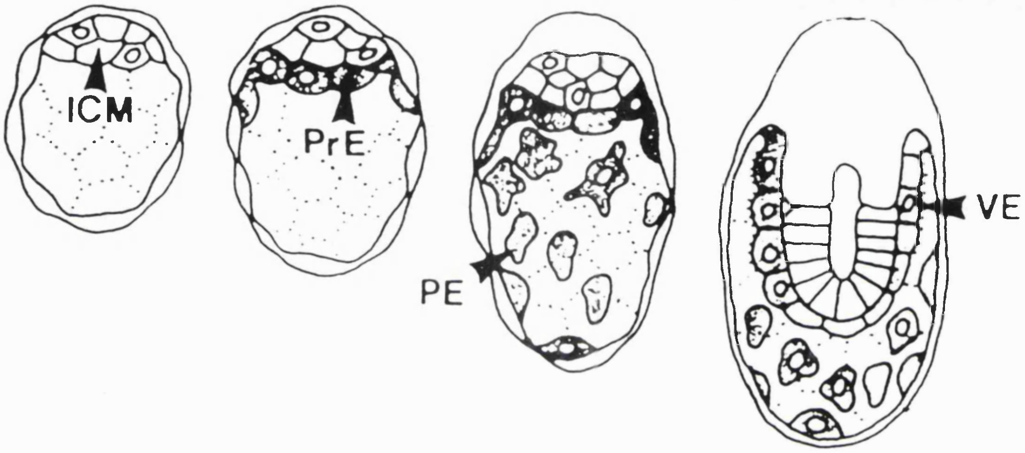
. 5.5 dpc


embryonic ectoderm
proamniotic cavity
visceral endoderm

6 dpc (egg cylinder)

FIG.1.4 : *Comparison of F9 EC cell differentiation with differentiation events in the early mouse embryo (adapted from Kurkinen* et al.*, 1983)*

ICM : inner cell mass, PrE : primitive endoderm, PE : parietal endoderm, VE : visceral endoderm, RA : retinoic acid, Agg. : aggregation.


ICM
PrE
PE
VE

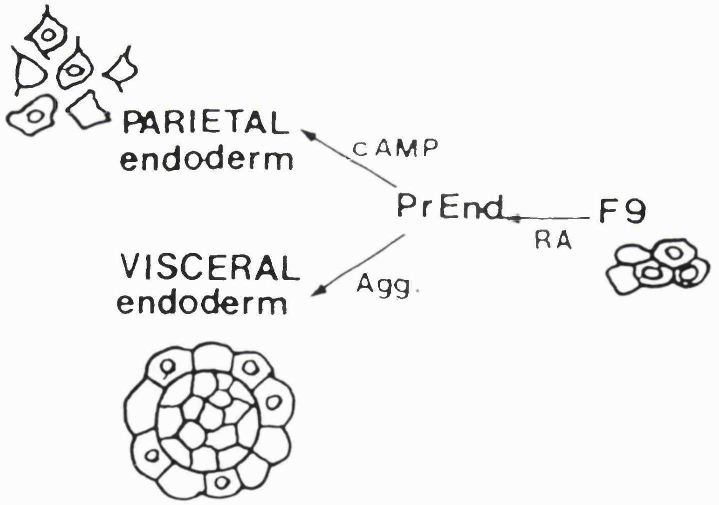
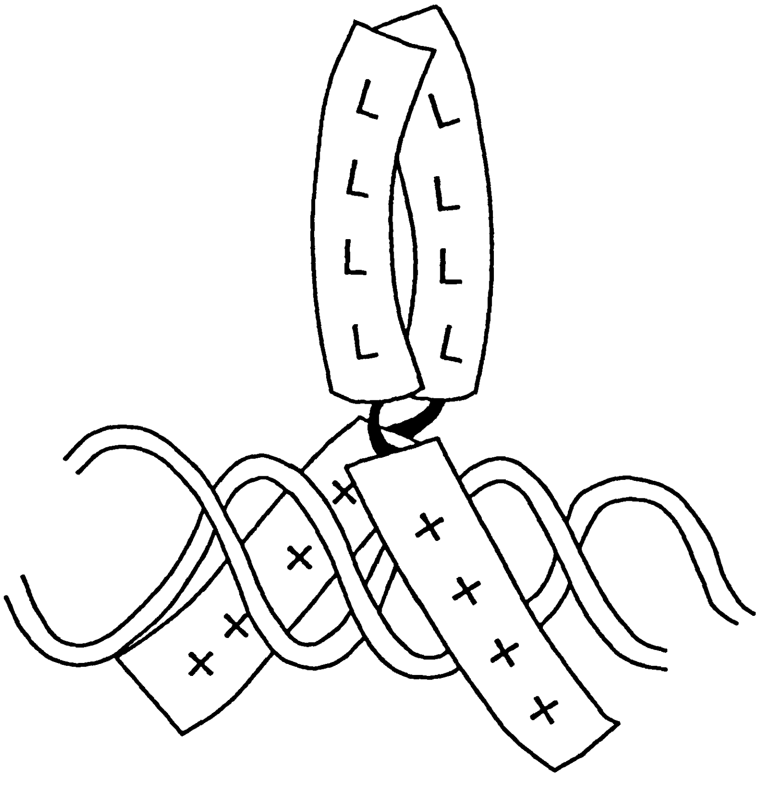

PARIETAL endoderm
cAMP
PrEnd
F9
RA
VISCERAL endoderm
Agg.

FIG.1.5 : *Schematic representation of a bzip dimer binding to the DNA*

"L"s indicate the "leucine zip" helices, "+"s indicate the basic domains. The DNA is represented as a double helix, with the basic domains of the dimer contacting its major groove.


L
L
L
L
L
L
L
L
+
+
+
+
+
+
+
+
+
+

CHAPTER 2

MATERIALS AND METHODS

DNA preparation and manipulations were performed as described in Sambrook *et al.* (1989). Any deviations from the procedures described here, are indicated in the figure legends.

Cell culture

F9 EC cells were cultured in Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum (Imperial Laboratories (Europe)), 2-4 mM L-glutamine (GIBCO), 100 units/ml penicillin (GIBCO) and 10 mg/ml streptomycin (GIBCO) (= EC medium), at 37 °C in a 5% CO_2 atmosphere ; they were re-plated at a density of approximately 4.5×10^4 cells/ml every 2-3 days. Differentiation to F9 PE cells was obtained by plating F9 EC cells at a density of 10^5/ml in EC medium supplemented with 5×10^{-8} M (all)*trans*-retinoic acid (RA) (Sigma), 10^{-3} M N^6,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate (dbcAMP) (Sigma) and 10^{-4} M isobutylmethylxanthine (Sigma) for 5 days. The undifferentiated F9 EC cell phenotype was assessed by immunoperoxidase staining with an α-SSEA1 monoclonal antibody (MC480) (Solter and Knowles, 1978), according to the procedure described by Harlow and Lane (1988), after fixing the cells in 4% formaldehyde for 10 min and treating with 0.2% Triton X-100 in PBS for 10 min. F9 EC cells and F9 EC cells treated with dbcAMP (see "Transfection and CAT assays") were morphologically indistinguishable and both stained for SSEA1 ; F9 PE cells had a distinct morphology and had lost the SSEA1 surface marker (see Fig.4.5). Growth conditions for all other cells was as for F9 EC cells, except for NIH 3T3 cells which were grown in EC medium containing 1% FCS and transferred to 10% FCS for 17 h prior to harvesting.

Plasmids for transfections, gel retardation probes and oligonucleotides

pBLcat2 contains nucleotides -105/+51 of the HSV thymidine kinase (*tk*) gene, and pBLcat3 is a promoterless vector (Luckow and Schütz, 1987). pE2A-E-cat (designated pE2A) contains the adenovirus type 5 *E2A* promoter and has been previously described (Murthy *et al.*, 1985).

pIEP1cat (designated pCMV) contains nucleotides -301/+72 as well as the polyadenylation signal of the major immediate early gene of human cytomegalovirus (CMV) and has been previously described (La Thangue and Rigby, 1988a). VIPcat4 (designated pVIP) contains nucleotides -94/+146 of the human vasoactive intestinal polypeptide (*VIP*) gene (Tsukada *et al.*, 1987). Plasmids E4Δ240 and E4Δ138 contain nucleotides -240/+35 and -138/+35, respectively, of the adenovirus 5 *E4* gene (Lee and Green, 1987). Plasmids pE4 and pE4Δ were constructed by ligating BglII linkers to the EcoRI/TaqI *E4* promoter fragments from E4Δ240 and E4Δ138, respectively, and cloning into the BglII site of the pBLcat3 polylinker. Plasmid pCMV-*β*gal (constructed by D.Stott) contains nucleotides -301/+72 of the human CMV immediate early gene cloned upstream of the *E.coli lacZ* gene followed by the simian virus 40 (SV40) small t intron and polyadenylation signal. Gel retardation probes *E4* and *E4*Δ were the BglII inserts from pE4 and pE4Δ, respectively, and probe *VIP* was a HindIII/NcoI fragment from VIPcat4, containing nucleotides -94/+146 of the VIP gene. The HSV *tk* promoter probe was a BamHI/BglII fragment from pBLcat2, containing nucleotides -105/+51 of the HSV *tk* gene. The SV40 origin of replication probe was a 60 base pairs (bp) long synthetic oligonucleotide, containing nucleotides 5175 to 37 of the SV40 genome (Tooze, 1981). Synthetic single stranded oligonucleotides were annealled in ligase buffer (Sambrook *et al.*, 1989).

Transfection and CAT assays

Differentiation of F9 EC cells to F9 PE cells was obtained by plating F9 EC cells at a density of 10^5/ml in EC medium supplemented with 5×10^{-8} M RA, 10^{-3} M dbcAMP and 10^{-4} M isobutylmethylxanthine for 3 days before transfection. By this time, the cells were morphologically indistiguishable from F9 PE cells after 5 days of treatment. Both F9 EC and PE cells were re-plated at 10^5/ml approximately (in EC medium without any additives, or containing RA and dbcAMP, or dbcAMP alone) 4 h before transfection with a DNA (20 μg of construct)/calcium phosphate co-precipitate, as previously described (Gorman *et al.*, 1982). Transfections were performed in parallel : two identical precipitates were pooled, divided in two equal parts and transfected into F9 EC and F9 PE cells, or untreated F9 EC and F9 EC cells treated with dbcAMP. The cells were washed with DMEM approximately 18 h after transfection, re-fed with EC medium containing the appropriate additives, and harvested after a further 24 h. By that time, F9 PE cells had been treated with differentiation agents for 5 days. Chloramphenicol acetyl transferase activity was assayed as previously described (Gorman *et al.*, 1982) ; approximately 50 μg of extract were incubated with substrate at 37 °C for 1-1.5 h. The results were quantitated by cutting out converted substrate from the silica gel thin layer chromatography plates (Merck) and counting in a scintillation counter. Cells transfected with pCMV-βgal, were fixed and stained for β-galactosidase activity at the time of harvest, essentially as previously described (Dannenberg and Suga, 1981).

Protein extracts

Whole cell Manley extracts were prepared as previously described (Manley *et al.*, 1980). dbcAMP-treated F9 EC cells were harvested after 42 h of treatment, F9 PE cells after 5 days of treatment with differentiation agents. Whole cell micro-extracts were prepared from approximately 10^7 cells, as described by Schöler *et al.* (1989) except that sonication was replaced by freeze-thawing three times. Protein concentration was measured according to the Bradford method, using the Bio-Rad protein assay. Dignam nuclear and cytoplasmic extracts were prepared as previously described (Dignam *et al.*, 1983).

Gel retardation assay

The binding reaction was performed in buffer containing 50 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 40 mM $(NH_4)_2SO_4$, 0.2 mM ethylene diamine tetra-acetate (EDTA), 1 mM dithiothreitol (DTT) and 15% glycerol. Each reaction (20 μl) contained 0.5 ng of a promoter fragment or 1.3 ng of a synthetic oligonucleotide probe end-labelled with the appropriate α-^{32}P-labelled deoxynucleotide triphosphate (α-^{32}P-dNTP) (Amersham), 5 μg protein extract or 2 μl of an *in vitro* translation reaction, and 2 μg denatured salmon sperm DNA as non-specific competitor. All reaction components, including oligonucleotide competitors where applicable, were incubated at 30 °C for 10 min in the absence of probe ; this was followed by probe addition and a further incubation at 30 °C for 10 min. Where anti-DRTF-1 serum or hybridoma tissue culture supernatant was included in the assay, the antigen was first incubated with the antibody for a minimum of 2 h at 4 °C, then denatured salmon sperm DNA was added and the reaction continued as above. The reaction was then loaded onto a non-denaturing 4% polyacrylamide gel (acrylamide : *bis*-acrylamide = 20 : 1) and run in Tris-Acetate-EDTA buffer (Sambrook *et al.*, 1989), at +4° C and with buffer recirculation, for 3 h (*VIP* promoter fragment), 2.5 h (*E4*, *E2A* and HSV *tk* promoter fragments, and SV40 origin oligonucleotide) or 1.5 h (other oligonucleotides) at 150 Volts (V) after an initial pulse of 500 V for 2 min. The gels were vacuum-dried and exposed to X-ray film (Fuji) at -70 °C.

"Super-shifts" with anti-peptide sera

2 μl of serum were pre-incubated with all gel retardation components except for the probe, for 1 h at +4°C, in the presence or absence of 400 μM peptide in phosphate-buffered saline (PBS). The reaction was then continued as above.

Construction of BS-ATG vectors

Bluescript-KS (Stratagene) was modified according to the scheme presented in Chapter 8.

In vitro transcription and translation

ATF1S (Hai *et al.*, 1989) was linearized with NcoI and transcribed by T7 RNA polymerase (Stratagene). ATF2S (Hai *et al.*, 1989) was linearized with BglII and transcribed by SP6 RNA polymerase (Stratagene). ATF2L (Hai *et al.*, 1989) was linearized with XhoI and transcribed by T7 RNA polymerase. BA1-CREBP1 was constructed by digesting pUCCRE-BP1Eco3.2 (Maekawa *et al.*, 1989) with EcoRI and cloning the *CREBP1* cDNA fragment into BS-ATG1. It was linearized with BamHI and transcribed by T3 RNA polymerase (Stratagene). ATF3S (Hai *et al.*, 1989) was linearized with NcoI and transcribed by T7 RNA polymerase. ATF3L (Hai *et al.*, 1989) was linearized with NcoI and transcribed by T7 RNA polymerase. BA0-ATF4 was constructed by digesting pGEM3-ATF4 (Hai *et al.*, 1989) with EcoRI and cloning the ATF4 cDNA into BS-ATG0. It was linearized with BamHI and transcribed with T3 RNA polymerase. BA1-ATF6 was constructed by digesting pGEM3-ATF6 (Hai *et al.*, 1989) with EcoRI and cloning the ATF6 cDNA into BS-ATG1. It was linearized with BamHI and transcribed with T3 RNA polymerase. BS-cjun was constructed by digesting AH119 (Ryseck *et al.*, 1988) with EcoRI and PstI and cloning the c*jun* cDNA into Bluescript-KS (Stratagene). It was linearized with PstI and transcribed with T3 RNA polymerase. BS-JunB was constructed by digesting RSVJunB (Ryder *et al.*, 1988) with EcoRI and PstI and cloning the c*jun* cDNA into Bluescript-KS (Stratagene). It was linearized with PstI and transcribed with T3 RNA polymerase. BS-JunD was constructed by digesting RSVJunD (Hirai *et al.*, 1989) with EcoRI and PstI and cloning the c*jun* cDNA into Bluescript-KS (Stratagene). It was linearized with PstI and transcribed with T3 RNA polymerase. BA1-cfos was constructed by digesting pGEM3-ATF5 (Hai *et al.*, 1989) with EcoRI and cloning the ATF5 (= cFos ; M.Green, pers. comm.) cDNA into BS-ATG0. It was linearized with BamHI and transcribed by T3 RNA polymerase. *In vitro* transcription in the presence of $m^7G(5')ppp(5')G$ (Pharmacia) and *in vitro* translation in rabbit reticulocyte lysate (Promega) were performed according to the manufacturers' instructions. A standard *in vitro* translation reaction was in a volume of 20 μl.

Isolation of total cellular RNA with guanidine isothiocyanate

(adapted from Sambrook *et al.*, 1989)

Approximately 10^8 tissue culture cells were lysed by vortexing with 8 ml homogenising solution (4.25 M guanidine isothiocyanate, 0.5% sodium lauroylsarcosine, 5mM sodium citrate pH7, 0.1 M β-mercaptoethanol) and overlaid on 3.5 ml CsCl solution (5.7 M CsCl, 5 mM sodium citrate pH7, 50 mM EDTA) in a centrifugation tube. They were centrifuged in an SW40-Ti (Beckman) rotor at 30,000 rpm at 22 °C for 18-20 hr. The RNA pellet was resuspended in water treated with diethyl pyrocarbonate (DEPC), extracted and precipitated. It was finally resuspended at 20 mg/ml in DEPC-treated water and stored at -70 °C.

Templates for synthesis of anti-sense RNA probes

pSP64γ-actin (Enoch *et al.*, 1986) was linearized with HindIII and transcribed by SP6 RNA polymerase. pGEM3-ATF1 (Hai *et al.*, 1989) was linearized with XhoI, which cuts in the middle of the basic domain, and transcribed by T7 RNA polymerase. ATF2L was linearized with XhoII, which cuts at the N-terminus of the DNA binding domain, and transcribed by SP6 RNA polymerase. pGEM3-ATF3/X was constructed by digesting pGEM3-ATF3 (Hai *et al.*, 1989) with XbaI and religating. It was linearized with PvuII, which cuts in the middle of the basic domain, and transcribed by SP6 RNA polymerase. BA0-ATF4 was linearized with XhoI, which cuts in the plasmid polylinker, and transcribed by T7 RNA polymerase. BA1-ATF6 was linearized with ClaI, which cuts in the plasmid polylinker, and transcribed by T7 RNA polymerase. BS-cjun was linearized with NarI, which cuts after the end of the coding sequences, and transcribed by T7 RNA polymerase. BS-junD/HI was constructed by digesting BS-junD with BamHI and religating. It was linearized with PvuII, which cuts 3' to the DNA binding domain, and transcribed by T7 RNA polymerase. BA1-cfos/S was constructed by digesting BA1-cfos with SmaI and StuI and religating. It was linearized with

EcoRI, which cuts in the plasmid polylinker, and transcribed by T7 RNA polymerase. αfra1 was constructed by inverting the orientation of the EcoRI *fra1* cDNA insert of pSP65fra1 (Cohen and Curran, 1988) in the same vector. It was linearized with XbaI, which cuts after the end of the coding sequences, and transcribed by SP6 RNA polymerase. αfosB was constructed by cloning the EcoRI/BamHI *fosB* cDNA fragment from pTZfosB (Zerial *et al.*, 1989) into Bluescript-KS (Stratagene) digested with EcoRI and BamHI. It was linearized with NcoI, which cuts 5' to the DNA binding domain, and transcribed by T7 RNA polymerase. pPX490, containing a mouse c-*myc* PvuII/XhoI exon I fragment (Roberts *et al.*, 1992), was linearized with EcoRI and transcribed with T3 RNA polymerase.

Synthesis of radiolabelled anti-sense RNA probes

(adapted from Sambrook *et al.*, 1989)

2 μl template plasmid (approximately 0.2 pmol) were mixed with 0.5 μl ribonucleotide mix (ATP, CTP, GTP at 10 mM, UTP at 0.5 mM) (all from Stratagene), 0.5 μl DTT 750 mM, 0.2 μl "RNA guard" (Pharmacia), 2 μl 5x transcription buffer (Stratagene), and 5 μl α-^{32}P-UTP (800 Ci/mmol) (NEN Du Pont). The mixture was incubated with 12 units of the appropriate phage RNA polymerase for 30 min at 37 °C. 1 μl ribonuclease-free DNase (1 mg/ml) (Stratagene) was added and the incubation continued for 15 min at 37 °C. The probe was extracted and ethanol-precipitated with 20 μg yeast transfer RNA (tRNA) as carrier. It was pelletted, dried and resuspended in 25 μl DEPC-treated water.

Ribonuclease protection assay

(adapted from Williams *et al.*, 1988)

1 μl total cellular RNA (20 mg/ml, unless otherwise indicated in the figure legends) was mixed with 1 μl anti-sense RNA probe, 3 μl 7x hybridization buffer (1x final concentration : 40 mM piperazine-N,N'-bis[2-ethane-sulfonic acid], 1 mM EDTA, 400 mM NaCl) and 16.8

μl de-ionised formamide (DIF) in a 1.5 ml Eppendorf tube. The tubes were heated at 90 °C for 5 min and quickly transferred to the hybridization bath set at 55 °C where they were incubated overnight. The tubes were then chilled on ice and 300 μl of ice-cold ribonuclease (RNase) mix (8 μg/ml ribonuclease A (Boehringer), 40 units/ml ribonuclease T1 (Boehringer), 300 mM NaCl, 50 mM Tris, 25 mM EDTA) were added to each tube. The tubes were incubated for 1 h at 37 °C, then each received 20 μl 10% sodium dodecylsulphate (SDS) and 5 μl proteinase K (BCL) (10 mg/ml) and were further incubated for 15 min at 37 °C. The contents were extracted once with phenol:chloroform and precipitated with 750 μl ethanol and 20 μg yeast tRNA for 20 min on dry ice. The precipitates were pelletted, dried, resuspended in 5 μl DIF loading buffer (80% DIF, 0.05% bromophenol blue (BDH), 0.15% xylene cyanol FF (BDH), 200 μM EDTA, 10% glycerol), denatured for 5 min at 90 °C, quenched on ice for 3 min, and loaded on a 4% or 6% polyacrylamide/Tris-borate-EDTA/urea gel run at 40 Watts (W) constant power. The gels were dried and exposed to Kodak XR film, with intensifying screens.

Computer-assisted sequence comparisons, manipulations and predictions

Hydrophilicity and hydropathy predictions, and prediction of amino acid sequences from cDNA sequences were performed using the MGS package, written by P.Gillett at N.I.M.R.. Sequence comparisons and predictions of modification sites were performed using the GCG Wisconsin package (Devereux *et al.*, 1984).

Production of anti-peptide sera

All procedures were according to Harlow and Lane (1988). Peptides ATF2D, ATF3C, PEP1 and PEP2 were conjugated to keyhole limpet haemocyanin (KLH) (Sigma) with glutaraldehyde, P10 with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Sigma). Sandylop rabbits were immunised subcutaneously with 0.5 ml of a peptide-KLH conjugate emulsion, containing approximately 180-1000 μg (approximately 100-250 nmol) of peptide, assuming 100% coupling efficiency, in Freund's complete adjuvant (GIBCO) for the first injection, and the same amount of peptide-KLH conjugate in Freund's incomplete adjuvant (GIBCO) for subsequent injections, spaced by approximately 6 week periods. Test bleeds were collected and treated by standard procedures.

Anti-peptide enzyme-linked immuno-sorbent assay (ELISA)

Flat-bottomed 96-well polyvinylchloride (PVC) plates (Falcon) were coated with 50 μl of peptide (400 pmol/ml) in 0.1 M sodium carbonate buffer pH 9.6, at 4 °C overnight ; all subsequent manipulations were performed at room temperature (r.t.). The plates were washed twice for 2 min in PBS. They were blocked in 10% bovine serum albumin (BSA ; Sigma) for 70 min, washed twice for 2 min with 0.1% polyoxyethylene-sorbitan monolaurate (Tween-20 ; Sigma) in PBS (= PBS/T), and incubated with sera diluted 1:100 in blocking buffer for 80 min. They were then washed three times for 2 min in PBS/T, and incubated with horseradish peroxidase-conjugated swine anti-rabbit immunoglobulins (HRPαR ; DAKOPATTS) diluted 1/2500 in blocking buffer, for 90 min, washed 3x2 min in PBS/T, and incubated with a 67 mg/l solution of 1,2-phenylenediamine dihydrochloride (DAKOPATTS) in 0.1 M citrate-phosphate buffer pH 5.0, for 5 min. The reaction was stopped with an equal volume of 1M sulphuric acid.

Anti-ATF/AP1 Immunoblots

20 μg of protein extract or 5 μl of an *in vitro* translation reaction were loaded in each lane of a 7.5% or 10% SDS-polyacrylamide mini-gels (Bio-Rad Mini Protean II) alongside "Rainbow" protein molecular weight standards (Amersham). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the manufacturers' instructions. The polypeptides were transferred onto 0.45 μm pore nitrocellulose membrane (Schleicher &

Schüll) using a Bio-Rad Mini Protean Trans-Blotter, according to the manufacturers' instructions. The polypeptides were visualised by staining the membrane with a Ponceau-S (Sigma) solution. After blotting, each lane was divided in two halves, to be incubated with pre-immune and immune sera, or in the presence or absence of peptide competitor (20 μM). The membrane was rinsed in PBS, blocked in blocking buffer (10% BSA in PBS) for 1 h, and incubated with sera diluted 1/200 in blocking buffer for 3 h. It was then washed twice for 10 min in PBS/0.1% Nonidet P40 (BDH) (PBS/NP40), incubated with alkaline phosphatase conjugated swine anti-rabbit immunoglobulins (APαR) (Promega) (diluted 1/5000 in blocking buffer) for 2 h at r.t., washed twice for 10 min in PBS/NP40 and incubated with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma), according to the manufacturers' instructions. The reaction was stopped by immersing the membrane in 1% acetic acid.

Immunoprecipitations

Cells which had been labelled with 20 μCi/ml of ^{35}S-methionine for 17 h were lysed in buffer A (50 mM Tris pH 7.9, 125 mM NaCl, 0.2 mM EDTA, 1mM DTT, 1% NP40) by rotation for 1 h at +4°C. 50 μg of cell lysate or 3 μl of an *in vitro* translation reaction were incubated with 5 μl of serum, in the presence or absence of peptide competitor (200 μM) made up to a total volume of 40 μl with buffer A or C (= A + 1% sodium deoxycholate, 0.1% SDS), rotating for 1 h at +4°C. After addition of 10 μl of protein A-Sepharose beads (Sigma) : buffer A (1 : 5 vol/vol), the reaction was incubated for 1 h, with rotation, at +4°C. The pellet was washed with 180 μl of the appropriate buffer, three times for cell lysates, four for *in vitro* translations, washed once with the appropriate buffer without NP40, denatured and used for SDS-PAGE alongside ^{14}C-labelled protein molecular weight standards (Amersham). The gels were dried and exposed to Kodak XR film, with intensifying screens.

Purification of DRTF-1

DNA binding site affinity-purified DRTF-1 was kindly provided by M.K.K.Shivji and N.B.La Thangue ; its purification has been described elsewhere (Shivji and La Thangue, 1991).

Production of hybridomas

All immunological and immunochemical procedures were as described (Harlow and Lane, 1988), unless otherwise indicated. Four BALB/c female mice (referred to as mouse 1, 2, 3 and 4) were injected with six doses of affinity purified DRTF-1 (mice 1 and 2 : 50 μl/injection ; mice 3 and 4 : 25 μl affinity fraction mixed with 25 μl of affinity matrix), as described, at a rate of approximately one injection every 2-3 weeks. Tail bleeds were taken one week after the 3rd, 5th and 6th injections and the serum was extracted. A booster injection was given to mouse 2 on the 35th week after the first injection. Five days later, the mouse was sacrificed, its spleen cells were fused, by stirring, to SP2 myeloma cells, and the fusion products were plated out in sixteen 96-well plates (Falcon). They were grown in DMEM supplemented with 20% FCS (Imperial), 5.8 μM azaserine (Sigma), 100 μM hypoxanthine (Sigma), and 1ng/ml recombinant interleukin-6 (British Biotechnology), at 37 °C in 8% CO_2. Medium supernatants (140 μl) were collected at 9, 11 and 14 days after the fusion and the wells replenished with an equal volume of fresh medium. The contents of wells that were positive in the ELISA assay were transferred into 24-well plates (Falcon) and diluted 1:5 into a total volume of 0.5-1 ml. Medium supernatants were collected when the cells reached confluence. All positive wells were eventually frozen down, after 1-3 passages, and stored in liquid nitrogen.

Anti-DRTF-1 ELISA

Flat-bottomed 96-well PVC plates (Falcon) were coated with 35 μl of a heparin Sepharose DRTF-1 fraction (17 μg/ml), unless otherwise stated, at 4 °C overnight ; all subsequent manipulations were performed at r.t.. The plates were washed 2x2 min in PBS. They were blocked in 10% BSA, unless otherwise stated (= blocking buffer) for 1 h, washed 2x2 min with PBS/T, and incubated with sera diluted in blocking buffer or neat hybridoma supernatants for 1-3 h. They were then washed 3x2 min in PBS/T, and incubated with horseradish peroxidase-conjugated rabbit α-mouse immunoglobulins (HRPαM ; DAKOPATTS) diluted 1/2500 in blocking buffer, for 1-3 h, washed 3x2 min in PBS/T, and incubated with a 67 mg/l solution of 1,2-phenylenediamine dihydrochloride (DAKOPATTS) in 0.1 M citrate-phosphate buffer pH 5.0, for 45 min. The reaction was stopped with an equal volume of 1M sulphuric acid.

Anti-DRTF-1 Immunoblots

Protein samples were loaded alongside protein molecular weight markers (Sigma) onto 7.5% or 10% SDS-polyacrylamide mini-gels (Bio-Rad Mini Protean II) prepared and run according to the manufacturers' instructions. The polypeptides were transferred onto 0.45 μm pore nitrocellulose membrane using a Bio-Rad Mini Protean Trans-Blotter, according to the manufacturers' instructions. The polypeptides were visualised by staining the membrane with a Ponceau-S solution. The membrane was rinsed in PBS, blocked in blocking buffer (10% BSA in PBS, unless otherwise indicated) for 1 h, washed 2x10 min in PBS/T, and incubated with sera diluted in blocking buffer or neat hybridoma supernatants for 1-3 h. It was then washed 2x10 min in PBS/T, incubated with either HRPαM (diluted 1/500 in blocking buffer) or alkaline phosphatase-conjugated rabbit α-mouse immunoglobulins (APαM ; DAKOPATTS), diluted 1/1000 in blocking buffer, as indicated in the figure legends, washed

2x10 min in PBS/T and incubated with the appropriate substrate : 3,3' diaminobenzidine tetrahydrochloride (Sigma) for HRPαM or nitro blue tetrazolium dimethylformamide and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma) for APαM, according to the manufacturers' instructions. The HRP reaction was stopped by rinsing the membrane with distilled water ; the AP reaction was stopped by immersing the membrane in 1% acetic acid.

CHAPTER 3

REGULATION AND NUCLEOTIDE SPECIFICITY OF ATF SITE-BINDING ACTIVITIES IN F9 EC AND PE CELLS

3.1 Sequence specificity and regulation during differentiation of F9 ATF site -binding activities

Given that a multitude of different ATF site-binding proteins had been described in other cell types, it was decided to investigate the number and regulation of ATF site-binding activities in F9 EC and PE cells. For this purpose, the panel of oligonucleotides shown in Table 3.1 was designed. The wild type ATF site (P) and a double point mutation already shown to abolish ATF binding (Pm1) (Hurst and Jones, 1987), together with eight other oligonucleotides were used to assess the specificity of F9 EC and PE cell ATF site-binding activities. Four of these oligonucleotides were designed to distinguish between ATF and E4F, the latter being a cellular factor whose binding site overlaps that of ATF but also extends over neighbouring nucleotides (Raychaudhuri *et al.*, 1987). Oligonucleotides PATFm and Pm2 are mutated in the core ATF site, and oligonucleotides PE4Fm and Pm3 are mutated within the additional nucleotides required by E4F ; these oligonucleotides were therefore used in order to distinguish between ATF and E4F complexes. A TRE (12-*O*-tetradecanoylphorbol-13-acetate responsive element) was also included, since its cognate binding activity, AP1 (Lee, W. *et al.*, 1987), has been reported to bind to the ATF site (Hai *et al.*, 1988). Similarly, another site whose cognate binding protein, C/EBP, is a member of a distantly related bzip family (Landschulz *et al.*, 1988a), was included in the panel, as the complexes it forms in liver nuclear extracts have been shown to be competed by an *E4* ATF site (Bakker and Parker, 1991). Finally, two sites, one that is located 3' to the *E2A* transcriptional initiation site, E2A-3', bears some homology to an ATF site, and is protected from DNase I cleavage in F9 EC cell extracts (N.B. La Thangue, unpublished observation), and one that is a distal ATF site, DA, from the Ad5 inverted terminal repeat (ITR) situated immediately upstream of the *E4* promoter, were also included in the panel of variant ATF site oligonucleotides.

The above oligonucleotides were used as binding sites in a gel retardation assay with F9 EC and PE whole cell extracts. As expected, no ATF site-specific complexes formed on mutant Pm1 either in F9 EC or PE extracts (Fig.3.1a : lanes 1-2). Oligonucleotide PATFm produced one complex, ECRE-3 (for EC CRE binding activity), the abundance of which was down-regulated during differentiation (Fig.3.1a : compare lane 10 to lane 9). Probe E2A-3' produced the same complex, though more efficiently (Fig.3.1a : compare lane 11 to lane 9). Mutant Pm2 produced two complexes in F9 EC extracts : ECRE-1, of slower mobility , and ECRE-4, a diffuse complex of faster mobility than ECRE-3, and complex PCRE-1 in F9 PE extracts (Fig.3.1a : lanes 13-14 ; ECRE-4 is resolved better in the experiment shown in Fig.3.2B : lane 6, where the two distinct complexes are labelled ECRE-4a and ECRE-4b). The distinction between ECRE-4 and PCRE-1 was made on the basis of a difference in mobility as well as the number of retarded bands in each complex, which are clearer when ECRE-4 and PCRE-1 are resolved on *E4*Δ, a fragment of the Ad5 *E4* promoter containing one ATF site (see Chapter 5). There was no apparent qualitative difference between the protein binding patterns of P, PE4Fm and Pm3 (Fig 3.1a : lanes 3-8) since they all bound an activity, ECRE-2, equally abundant in F9 EC and PE cell extracts and more abundant than any of the other ECRE activities, as well as ECRE-1, ECRE-3, ECRE-4 and PCRE-1 (see Fig.3.1d and e, and Chapter 5). There was, however, a quantitative difference in the efficiency of binding to these oligonucleotides : P bound ECRE-2 more efficiently than PE4Fm and Pm3. That there was no qualitative difference between the complexes bound by P, PE4Fm and Pm3 was confirmed when these oligonucleotides were used to compete for complexes formed on *E4*Δ, since they competed for the binding of all ATF site-specific activities (Chapter 5). This suggested that there was no detectable E4F-like activity in F9 EC whole cell extracts, given that PE4Fm and Pm3 were designed so that they would not bind E4F. Rooney *et al.* (1990) have indeed shown that Pm3 (called E4.pm4 by them) does not bind E4F purified from HeLa cells. The intensity of the ECRE-2 complex formed on P presumably masked the other ATF-site specific complexes, ECRE-3, ECRE-4 and PCRE-1 that migrated to a similar position. The ECRE-2 activity also served as a control for extract integrity since it was consistently of equal abundance in all pairs of F9 EC and PE extracts tested.

The TRE formed one complex common to F9 EC and PE extracts and one slower migrating complex which was induced during differentiation (Fig.3.1a : lanes 15 and 16). The DNA binding properties of this slower migrating complex were those of AP1 which is undetectable in F9 EC cells but is induced during differentiation (Kryszke *et al.*, 1987), and its mobility as well as its specificity were distinct from those of ECRE-1, -2, -3, -4 and PCRE-1 (Fig.3.1d and e).

One possibility that needed to be eliminated was that the different ECRE complexes were created by sequences in the mutated binding sites unrelated to the ATF site. This was eliminated by showing that they could be competed only by a wild type site (P) but not by one where the central CG nucleotides had been mutated (Pm1) (Fig.3.1b and c), indicating that all these activities bound to the wild type ATF site, P. The AP1 complex was also competed by P (Fig.3.1 b and c : lane 14) and therefore must have also been capable of binding when P was used as a probe, but was then presumably masked by ECRE-2.

The precise DNA binding specificities of the different activities were then confirmed by assessing how the mutated oligonucleotides competed for each other's complexes (Fig.3.1 d and e). These studies demonstrated that while all the defined activities bound to the wild type ATF site, they did so with distinct nucleotide requirements. For example, complexes ECRE-1, ECRE-4 (Fig.3.1d : lane 6) and PCRE-1 (Fig.3.1e : lane 6) bound to Pm2 but not E2A-3' (Fig.3.1 d and e : compare lane 8 to lane 9), while both Pm2 and E2A-3' were unable to bind ECRE-2 (Fig.3.1 d and e : lanes 3 and 4 ; note that Pm2 did compete for ECRE-1), confirming that ECRE-2 was not just composed of the other comigrating ECRE complexes, but was indeed a complex distinct from them. Significantly, neither the TRE (Fig.3.1d and e : compare lanes 5 to 1, 10 to 6, 15 to 11, 20 to 16) nor the C/EBP site (Fig.3.2B : compare lanes 5 to 1, 10 to 6, 15 to 11) bound any of the ECRE activities, emphasizing that, although they have distinct requirements for nucleotides within the ATF site, the primary specificity of the ECRE activities is for the ATF site, rather than the related TRE and C/EBP sites.

When the same set of experiments was conducted with oligonucleotide DA, this probe bound ECRE-1 (Fig.3.2A : lane 1), albeit more weakly than either Pm2 (Fig.3.2B : compare lane 4 to lane 3 ; Fig.3.2A : compare lane 1 to lane 7) or P (Fig.3.2B : compare lane 4 to lane 2 ; Fig.3.2A : compare lane 1 to lane 5). DA also formed a faster-migrating complex in both EC and PE extracts (indicated by brackets in Fig.3.2A : compare lanes 9 and 10) which was also ATF site-specific (Fig.3.2A : compare lane 3 to lane 2). The composition of this latter complex was elucidated by using DA to compete for the previously defined ECRE complexes (Fig.3.2B). When DA was used as a competitor against complexes formed on probe Pm2, it bound ECRE-1 and the slower migrating of the two ECRE-4 complexes, ECRE4a, but not the faster one, ECRE-4b (Fig.3.2B : compare lane 9 to lane 6). When E2A-3' was used as the probe, DA competed for ECRE-3 as efficiently as either P or E2A-3' (Fig.3.2B : compare lane 14 to lanes 12 and 13). Finally, when P was used as the probe, DA competed for ECRE-1, albeit weakly, ECRE-3, and ECRE-4a, but also for ECRE-2, so that only ECRE-4b and some residual ECRE-1 remained bound to P (Fig.3.2B : compare lane 4 to lane 1). Therefore, although DA did not define any new ECRE activities, it did have binding characteristics that were distinct from those of P, Pm2 or E2A-3' (summarized in Table 3.2), and it distinguished between ECRE-4a and ECRE-4b.

3.2 Discussion

In summary, these oligonucleotides, which contained wild type and variant ATF sites, defined six ATF site-binding activities in F9 EC and PE cell extracts which could be distinguished from each other by their electrophoretic mobility, their precise DNA sequence specificity and their regulation during F9 EC cell differentiation (summarized in Table 3.2). For example, the abundance of ECRE-2 was not altered during differentiation of F9 EC cells, whereas ECRE-1 and ECRE-3 were down-regulated during differentiation.

Because oligonucleotides that bound ECRE-2 all had an intact core ATF site, ECRE-2 would appear to have the DNA binding specificity of purified ATF as defined by others (Hurst and Jones, 1987 ; Lee *et al.*, 1987). Their "ATF-43" and "ATF-47" were eventually identified as the products of the *ATF1* and *CREB* genes, respectively (Hurst *et al.*, 1991 ;

Hurst *et al.*, 1990). This would, at first sight, imply that ECRE-2 was encoded by *CREB* or *ATF1*. However, there is no detectable *ATF1* RNA in F9 EC and PE cells (Chapter 7) and therefore *ATF1* is unlikely to be encoding ECRE-2. On the other hand, both ATF2 and ATF3, both products of genes which are expressed in F9 EC cells, bind to the wild type ATF site, P, (Chapter 6) and would therefore also be potential candidates for ECRE-2.

Failure to detect E4F, which was reported to be present in EC cells (Raychaudhuri *et al.*, 1989), may have been due to differences in experimental conditions. E4F was detected in fractionated extracts whereas whole cell extracts were used in this study. Although C/EBP has been shown to bind to the ATF site (Bakker and Parker, 1991), the converse was not true in the experiments presented here, although it should be mentioned that the C/EBP site used was not identical to the one used by Bakker and Parker (1991).

The failure of an AP1 site to compete for ECRE complexes does not contradict the results presented by Hai *et al.* (1988). Before anything was known about heterodimerization between ATF and AP1 proteins, these workers had shown that some of the proteins purified by ATF binding site affinity purification cross-reacted with anti-cJun antibodies, and some of the proteins purified by AP1 binding site affinity purification cross-reacted with anti-ATF antibodies. At the time, when none of the *ATF* genes had been cloned, it was thought that "ATF" was a single and homogeneous DNA binding activity, and those results were interpreted as indicating that "ATF" and "AP1" were immunologically related and could bind to each other's site. Today, of course, we know that, as illustrated in Chapter 7, members of the ATF and AP1 families can heterodimerize to produce DNA binding activities specific either for the ATF site, or the AP1 site, or both. It is therefore possible that Hai *et al.* (1988) were detecting ATF/Jun heterodimers in their affinity-pure ATF and AP1 preparations.

In conclusion, the data presented here constitute a novel demonstration of a family of DNA binding proteins with distinct nucleotide specificities and modes of regulation during F9 EC cell differentiation (summarized in Table 3.2) (Tassios and La Thangue, 1990).

TABLE 3.1 : *Sequences of ATF sites and variant oligonucleotides used in this study*

Complete sequences of the oligonucleotides used in this study, including GATC or other 5' overhangs. The position of these sequences relative to the transcriptional start sites of the indicated genes are shown where appropriate. Deviations from the ACGTCA ATF site consensus are double-underlined and in bold. Sequences from : *E4* (Steenbergh and Sussenbach, 1979), *E2A* (Murthy *et al.*, 1985), c*fos* TRE (Kryszke *et al.*, 1987), C/EBP (Vinson *et al.*, 1988).

Sequences of ATF sites and variant oligonucleotides

			ATF site	
		-58		-39
E4	**P**	GATCTAACCGTT ATTGGCAA	ACGTCA TGCAGT	TTTTTT AAAAAACTAG
	Pm1	GATCTAACCGTT ATTGGCAA	A**AA**TCA TTTAGT	TTTTTT AAAAAACTAG
	PATFm	GATCTAACCGTT ATTGGCAA	ACGTC**C** TGCAGG	TTTTTT AAAAAACTAG
	PE4Fm	GATCTAACC**T**TT ATTGGAAA	ACGTCA TGCAGT	TTTTTT AAAAAACTAG
	Pm2	GATCTAACCGTT ATTGGCAA	AC**A**TCA TGTAGT	TTTTTT AAAAAACTAG
	Pm3	GATCTAACCG**A**T ATTGGCTA	ACGTCA TGCAGT	TTTTTT AAAAAACTAG
		-274		-255
(Ad5 ITR)	**DA**	GATCCCGCGCC GGCGCGG	ACGTCA TGCAGT	CAAACTC GTTTGAGCTAG
		+10		+26
E2A	**E2A-3'**	GATCCC GG	**G**CGTC**T** CGCAGA	TCCAGCGTG AGGTCGCACCTAG
			AP1 site	
		-307		-291
c-fos	**TRE**	GATCGCTG CGAC	AC-TCA TG-AGT	GATGTCCT CTACAGGACTAG
	C/EBP	AATTCAATTGGG GTTAACCC	CAATCA GTTAGT	GG CCTTAA

FIG. 3.1 : *Distinct but ATF site-specific protein complexes are formed on different variants of the ATF site and on a TRE*

a) Complexes formed on the indicated oligonucleotide probes in F9 EC and PE whole cell extracts are indicated by arrows.

b) Competition of the F9 EC complexes formed on the indicated oligonucleotide probes with 50-fold molar excess of wild type (P) or mutant (Pm1) ATF site oligonucleotide. The fastest migrating complex is non-specific.

c) Competition of the F9 PE complexes formed on the indicated oligonucleotide probes with 50-fold molar excess of wild type (P) or mutant (Pm1) ATF site oligonucleotide.

(continued opposite next page)

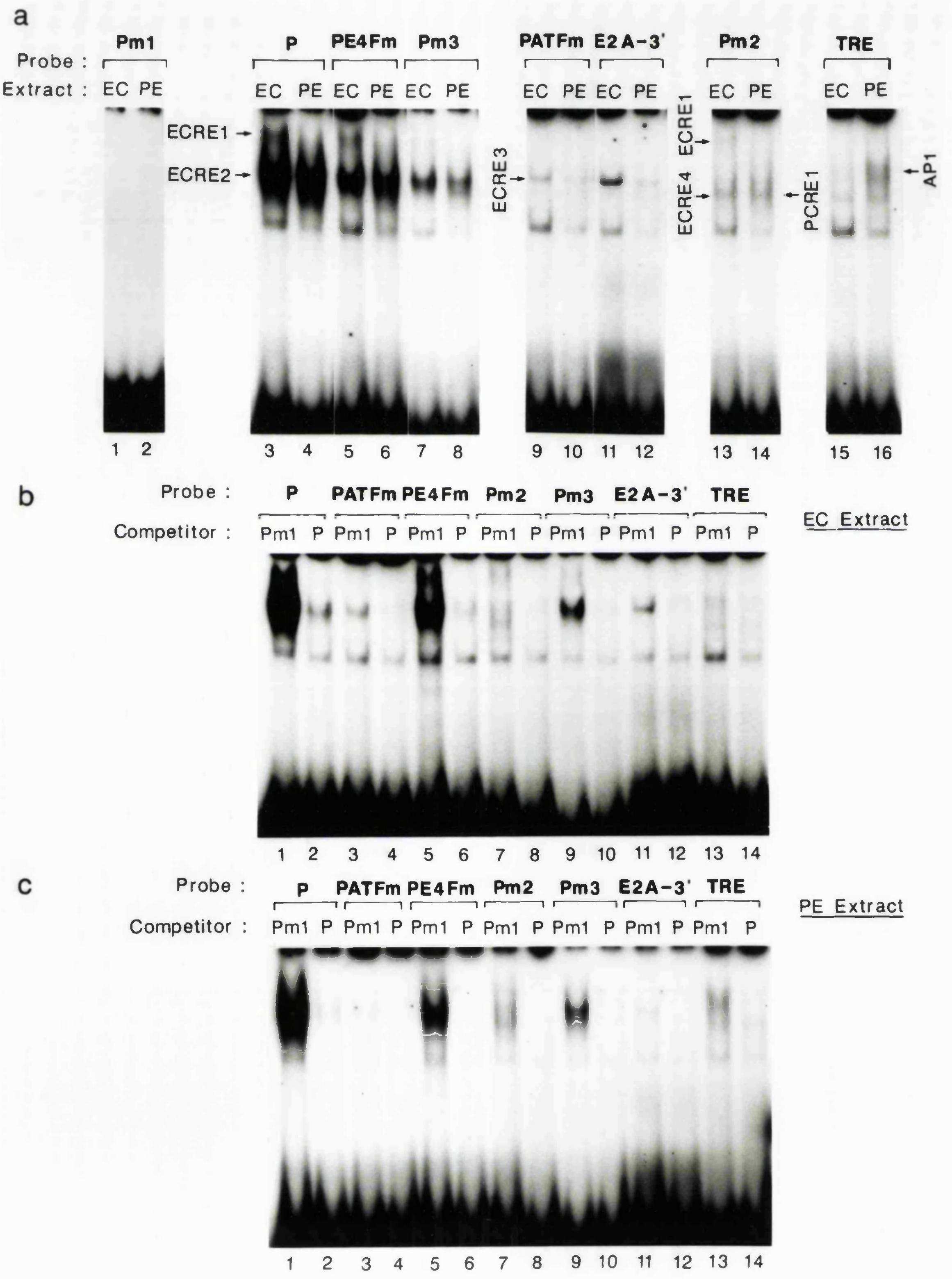
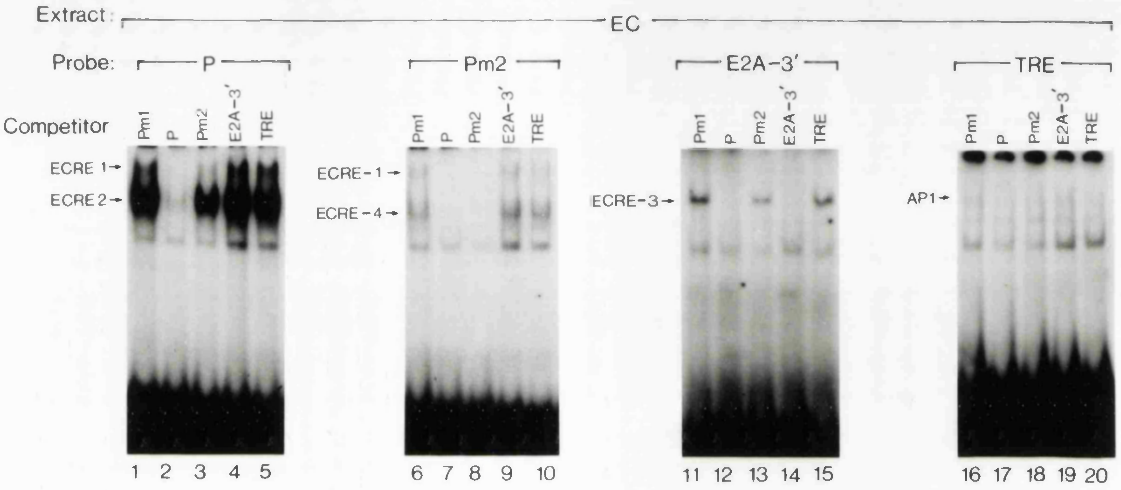

a
Probe :
Extract :
Pm1
EC PE
P
PE4Fm
Pm3
EC PE EC PE EC PE
PATFm
E2A-3'
EC PE EC PE
Pm2
EC PE
TRE
EC PE
ECRE1
ECRE2
ECRE3
ECRE1
ECRE4
PCRE1
AP1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
b
Probe :
Competitor :
P
PATFm
PE4Fm
Pm2
Pm3
E2A-3'
TRE
Pm1 P Pm1 P Pm1 P Pm1 P Pm1 P Pm1 P Pm1 P
EC Extract
1 2 3 4 5 6 7 8 9 10 11 12 13 14
c
Probe :
Competitor :
P
PATFm
PE4Fm
Pm2
Pm3
E2A-3'
TRE
Pm1 P Pm1 P Pm1 P Pm1 P Pm1 P Pm1 P Pm1 P
PE Extract
1 2 3 4 5 6 7 8 9 10 11 12 13 14

FIG. 3.1 (cont'd) : *Distinct but ATF site-specific protein complexes are formed on different variants of the ATF site and on a TRE*

d) Competition for the F9 EC complexes formed on the indicated oligonucleotide probes with 50-fold molar excess of the indicated ATF site variant and TRE oligonucleotides. The fastest migrating complex is non-specific.

e) Competition for the F9 PE complexes formed on the indicated oligonucleotide probes with 50-fold molar excess of the indicated ATF site variant and TRE oligonucleotides.

d.


Extract:
EC
Probe:
P
Pm2
E2A-3′
TRE
Competitor
Pm1
P
Pm2
E2A-3′
TRE
ECRE 1
ECRE 2
ECRE-1
ECRE-4
ECRE-3
AP1
1 2 3 4 5
6 7 8 9 10
11 12 13 14 15
16 17 18 19 20

e.

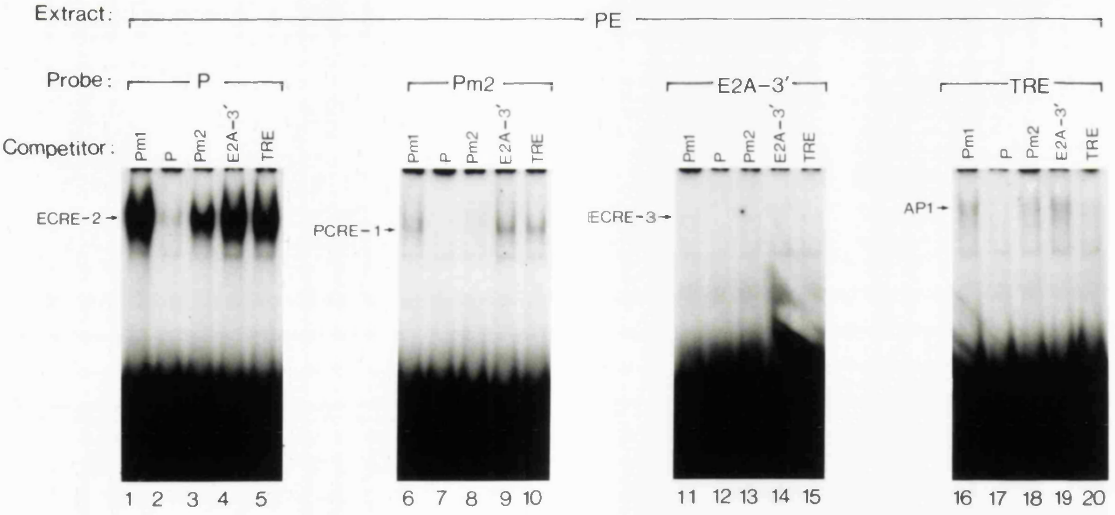

Extract:
PE
Probe:
P
Pm2
E2A-3′
TRE
Competitor:
Pm1
P
Pm2
E2A-3′
TRE
ECRE-2
PCRE-1
ECRE-3
AP1
1 2 3 4 5
6 7 8 9 10
11 12 13 14 15
16 17 18 19 20

FIG. 3.2 : *Specificity of probe DA for ECRE activities*

A) Lanes 1-4 : Competition for the F9 EC complexes formed on probe DA with 50-fold molar excess of the indicated ATF site variant oligonucleotides. Lanes 5-10 : Comparison of protein complexes formed on probes P, Pm2 and DA in F9 EC and PE whole cell extracts. All lanes are from the same experiment. The fastest migrating complex is non-specific.

B) Competition for the F9 EC complexes formed on the indicated oligonucleotide probes with 50-fold molar excess of the indicated ATF site variant oligonucleotides. Lanes containing the same probe are from the same experiment.

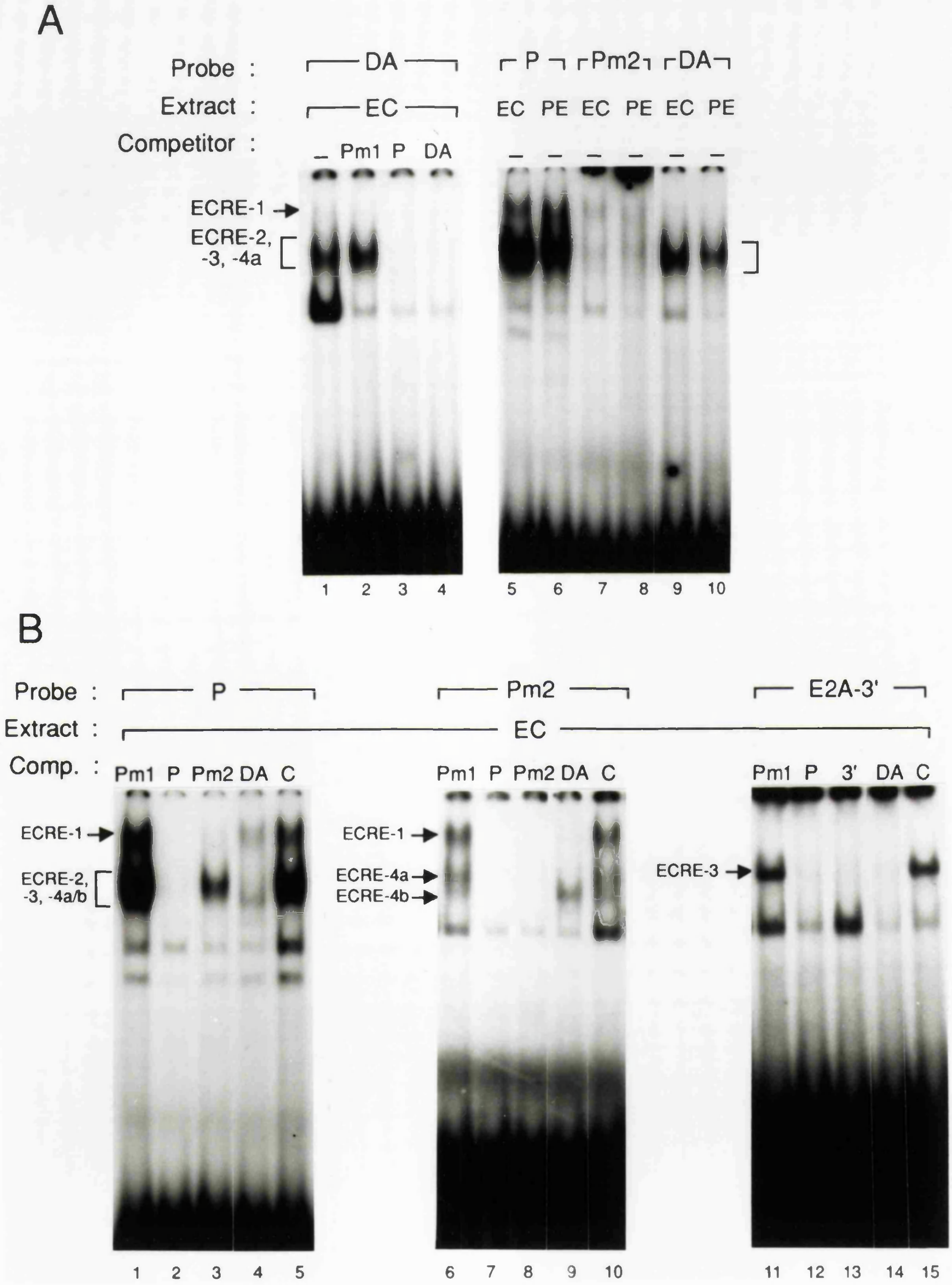
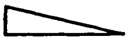
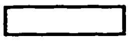
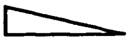
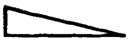
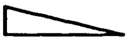
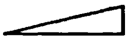

A
Probe :
DA
P
Pm2
DA
Extract :
EC
EC
PE
EC
PE
EC
PE
Competitor :
–
Pm1
P
DA
–
–
–
–
–
–
ECRE-1
ECRE-2,
-3, -4a
1
2
3
4
5
6
7
8
9
10
B
Probe :
P
Pm2
E2A-3'
Extract :
EC
Comp. :
Pm1
P
Pm2
DA
C
Pm1
P
Pm2
DA
C
Pm1
P
3'
DA
C
ECRE-1
ECRE-2,
-3, -4a/b
ECRE-1
ECRE-4a
ECRE-4b
ECRE-3
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

TABLE 3.2 : *Regulation and binding site specificity of F9 ATF site-binding activities*

The regulation of ECRE activities during differentiation of F9 EC cells to PE cells and their relative binding to the various oligonucleotides are shown. The TRE and C/EBP sites, like Pm1, also did not bind any of the ECRE activities. NT = not tested.

F9 ATF site Binding Activities

	Regulation	Oligonucleotide Specificity							
	EC PE	Pm1	P	PE4Fm	Pm3	Pm2	DA	E2A-3	PATFm
ECRE-1		-	+++	+++	++	++	+	-	-
ECRE-2		-	+++	++	+	-	++	-	-
ECRE-3		-	+++	++	++	-	+++	+++	++
ECRE-4a		-	+++	++	++	++	+++	-	-
ECRE-4b		-	+++	++	++	++	-	-	-
PCRE-1		-	+++	++	++	++	NT	-	-

CHAPTER 4

TRANSCRIPTIONAL REGULATION OF ATF SITE-CONTAINING PROMOTERS IN F9 EC AND PE CELLS

4.1 Introduction

The ATF site-binding activities which are regulated during differentiation of F9 EC cells are presumably responsible for regulating transcription from ATF site-containing promoters. It was therefore of interest to study the transcription of such promoters in F9 EC cells, and its regulation during differentiation. The promoters of the E1A-inducible Ad5 *E4* and *E2A* genes, and that of the cAMP-inducible human *VIP* gene were chosen for this study, since it is known that their ATF sites are important *cis*-acting elements both for their basal transcription and their induction by E1A and cAMP (Lee and Green, 1987 ; Murthy *et al.*, 1985 ; Tsukada *et al.*, 1987).

4.2 Regulation of ATF site-containing promoters in F9 EC and PE cells

Transcription was assayed by transfecting promoter-driven chloramphenicol acetyl transferase (CAT) reporter constructs (Fig.4.1) in F9 EC and PE cells and measuring the CAT activity of protein extracts from transfected cells, as previously described (Gorman *et al.*, 1982). Construct pE4 contained nucleotides -240 to +35 (four ATF sites), and pE4Δ, nucleotides -138 to +35 (one ATF site) of the Ad5 *E4* gene (Lee and Green, 1987). Sequences up to -240 had already been shown to be sufficient for wild type expression *in vivo* and *in vitro* (Lee and Green, 1987). The transcriptional activity of the *E2A* promoter (nucleotides -96 to +68), containing one ATF site (Murthy *et al.*, 1985 ; La Thangue *et al.*, 1990), and the *VIP* promoter (-94 to +146), containing two ATF sites (Tsukada *et al.*, 1987), were also tested in these cells ; the *VIP* promoter has previously been shown to be activated by cAMP *via* its ATF sites (Fink *et al.*, 1988).

Several control constructs were used to determine transfection efficiency. Construct pCMV, containing nucleotides -301 to +72 of the immediate early (IE) human cytomegalovirus (CMV) gene, was used as a positive control for transcription as these transcriptional control sequences are exceptionally active in the CV1 cell line (Boshart *et* *al.*, 1985). The promoterless vector pBLcat3 and the vector pBLcat2, which contains the the HSV *tk* promoter (-105/+51), were chosen in order to assess basal transcription. Transfection efficiency was determined by transfecting cells with an expression vector containing nucleotides -301 to +72 of the CMV IE gene cloned upstream of the *E.coli lacZ* reporter gene : cells that take up the plasmid should express the enzyme, allowing the number of cells transfected in cultures of F9 EC and PE cells to be determined by assaying for β-galactosidase activity *in situ.* The proportion of β-galactosidase-positive cells in each transfected culture ranged from 0.5% to 6%. Although pBLcat3 has no recognizable promoter elements, extracts prepared from either F9 EC or PE cells transfected with it did have low CAT activity, presumably resulting from initiation of transcription at sites in the vector sequence. However, the ratio of pBLcat3 activity in F9 EC and PE cells generally reflected the ratio of transfection efficiency in the two cell types, suggesting that quantitative differences in transcriptional activity of pBLcat3 resulted from expression in different numbers of cells, rather than differences in the rate of transcriptional initiation in the two cell types. For this reason, the activity of the test promoters was normalised with respect to pBLcat3 activity, thus eliminating differences that arose from the different efficiencies of DNA uptake by these two cell types (Rickles *et al.*, 1989).

For any of the constructs studied, a degree of variation in absolute CAT activity values was observed, although the trend for each construct was clear (Fig.4.2). As discussed above, pBLcat3 activity reflected the difference in transfection efficiencies between the two cell types (Fig.4.2 : lanes 11 and 12) and was therefore set to an arbitrary value of 1 in both cell types. The positive control, pCMV, was active in F9 EC cells but was induced further during differentiation (Fig.4.2 : compare lanes 13 and 14). pBLcat2, which had low activity, was also activated during differentiation (Fig.4.2 : compare lanes 9 and 10). Of the four test promoters, *E4* was the most active in F9 EC cells (Fig.4.2 : compare lane 1 to lanes 3, 5 and 7), whereas a deletion of 102 bp (nucleotides -240/-139) reduced its activity 36-fold (Fig.4.2 : compare lane 3 to 1). This was in agreement with the results of Lee and Green (1987) who demonstrated that these sequences are required for full promoter activity in 293 cells and

HeLa cell extracts ; both *E4* promoter constructs were nevertheless activated further during differentiation (Fig.4.2 : compare lane 2 to lane 1, 4 to 3). In contrast, and in agreement with previous *in vitro* results (La Thangue *et al*., 1990), pE2A activity was reduced during differentiation (Fig.4.2 : compare the average CAT activity of lane 6 to that of lane 5). Surprisingly, the *VIP* promoter was inactive in both cell types (Fig.4.2 : compare lanes 7 and 8), although this construct was transcriptionally active as well as forskolin- and cAMP-inducible in PC12 cells (Tsukada *et al*., 1987), indicating that its lack of activity in F9 cells was cell-specific and not due to a generally transcriptionally inert construct.

In summary, the -240 *E4* promoter deletion (containing four ATF sites) was active in F9 EC and PE cells, whereas the -138 deletion (containing one ATF site) was less active in both cell types, and both promoters were induced during differentiation. In contrast, Ad5 *E2A* promoter activity was down-regulated (see summary in Fig.4.4). The *VIP* promoter, on the other hand, which contains two ATF sites, was inactive in both cell types. These results demonstrate that different ATF site-containing promoters have distinct transcriptional activities in F9 EC cells.

4.3 dbcAMP treatment of F9 EC cells does not affect *in vivo* transcription

Since one of the agents used to differentiate F9 EC cells was dbcAMP, it was important to determine whether the increase in *E4* promoter activity during differentiation was due to a cAMP-dependent stimulation *via* its ATF sites rather than a response to differentiation *per se*. However, when F9 EC cells were treated with dbcAMP alone, no increase in transcription was apparent with either *E4*, *E4*Δ or the cAMP-inducible promoter *VIP* (Fig.4.3), suggesting that activation was not caused by dbcAMP treatment alone, but rather that it was dependent upon differentiation (summarized in Fig.4.4). In addition, dbcAMP treatment of F9 EC cells did not alter their morphology or reactivity with α-SSEA-1 (Solter and Knowles, 1978), which recognizes an early embryonic antigen that is present in EC cells but disappears after differentiation (Fig.4.5 ; see also Strickland *et al*., 1981), nor did it alter the pattern of proteins binding to the *E4*Δ promoter assayable by gel retardation (see Chapter 5). This result is consistent with those of Rickles *et al.* (1989) who demonstrated that cAMP-dependent *trans*-activation of the tissue plasminogen activator gene cannot occur in F9 EC cells, Gonzalez and Montminy (1989), who reported very low activity of the somatostatin ATF site in F9 EC cells, and Imperiale *et al.* (1984) who found that *E2A* expression was not altered during treatment of F9 EC cells with cAMP.

4.4 Discussion

The first conclusion to be drawn from these experiments is that different ATF site-containing promoters have distinct transcriptional activities in F9 EC and PE cells. Down-regulation of transcription from the *E2A* promoter is explained by the fact that DRTF-1, a transcription factor that binds to a *cis*-acting element adjacent to the ATF site and is transcriptionally active in F9 EC cells (La Thangue *et al.*, 1990 ; Shivji and La Thangue, 1991), is also down-regulated during F9 EC cell differentiation (La Thangue and Rigby, 1987). The DRTF-1 site has been shown to be important for *E2A* transcription *in vivo* and *in vitro* (Murthy *et al.*, 1985 ; La Thangue *et al.*, 1990 ; Murray *et al.*, 1991 ; Zamanian and La Thangue, 1992). However, in the *E4* and *VIP* promoters, the ATF site is the most important *cis*-acting element for *in vivo* and *in vitro* transcription (Gilardi and Perricaudet, 1984 ; Gilardi and Perricaudet, 1986 ; Lee and Green, 1987 ; Tsukada *et al.*, 1987 ; Fink *et al.*, 1988). The fact, therefore, that one ATF site-containing promoter, *VIP*, is inactive while another, *E4*, is active in F9 EC cells suggests a difference in the activity of their ATF sites, which may result from them being recognized by different ATF site-binding proteins. Functional differences between the *E4* and *VIP* ATF site have also been demonstrated by Lee *et al.* (1989), who showed that a *VIP* ATF site is cAMP-, but not E1A-inducible in HeLa cells, while the converse is true for an *E4* ATF site. Another possible explanation would be that a *VIP*-specific repressor inhibits *VIP* transcription in these cells. Alternatively, other transcription factors could be important for *E4* promoter activity in F9 EC cells and its increase in PE cells. With respect to that last possibility, it is worth noting that no similarity to the defined retinoic acid responsive elements (Vasios *et al.*, 1989 ; de Thé *et al.*, 1990 ; Lucas *et al.*, 1991), which might be expected to be induced during differentiation of F9 EC cells with retinoic acid and dbcAMP, was revealed by visual inspection of the *E4* promoter sequences. These two possibilities are addressed in the next chapter, where *in vitro* protein binding to the *E4* and *VIP* promoters is investigated.

The second conclusion, that transcription in F9 EC cells is insensitive to dbcAMP treatment, is in agreement with the findings of Rickles *et al.* (1989) as well as Gonzalez and Montminy (1990). The latter attributed this to a lack of functional cAMP-dependent protein kinase A and transcription factor CREB, since the somatostatin ATF site was transcriptionally active only on the presence of exogenous PKA catalytic subunit and CREB. However, recent reports from Masson *et al.* (1992) and Ruppert *et al.*(1992) indicate that both CREB RNA and protein are present in F9 EC cells even though the somatostatin and *VIP* promoters are inactive. In addition, there is measurable cAMP-stimulated kinase activity in F9 EC cells which furthermore increases during differentiation (Plet *et al.*, 1982). Lack of cAMP inducibility, therefore, may be due to insufficiently high endogenous PKA and CREB levels, or factors that inhibit their productive interaction and subsequent ATF site-dependent *trans*-activation.

Finally, this study demonstrated that differentiation affects the level of transcription from the ATF site-containing promoter of the adenovirus *E4* gene. Given that ATF site-binding activities are themselves regulated during differentiation of F9 EC cells, one could speculate that this transcriptional regulation results from different or modified ECRE activities regulating the *E4* promoter in the two cell types ; this possibility is examined in the next chapter.

FIG. 4.1 : *Schematic representation of transcription regulatory regions contained in the CAT reporter constructs used for transfection in F9 EC and PE cells*

Some of the known transcription factor binding elements are indicated. Sequences and nucleotide numbering from : HSV *tk* (McKnight and Kingsbury, 1982), *E4* and *E4Δ* (Steenbergh and Sussenbach, 1979), *E2A* (Murthy *et al.*, 1985), *VIP* (Tsukada *et al.*, 1985), CMV (Akrigg *et al.*, 1985).

Transcriptional Control Regions used for Transfection

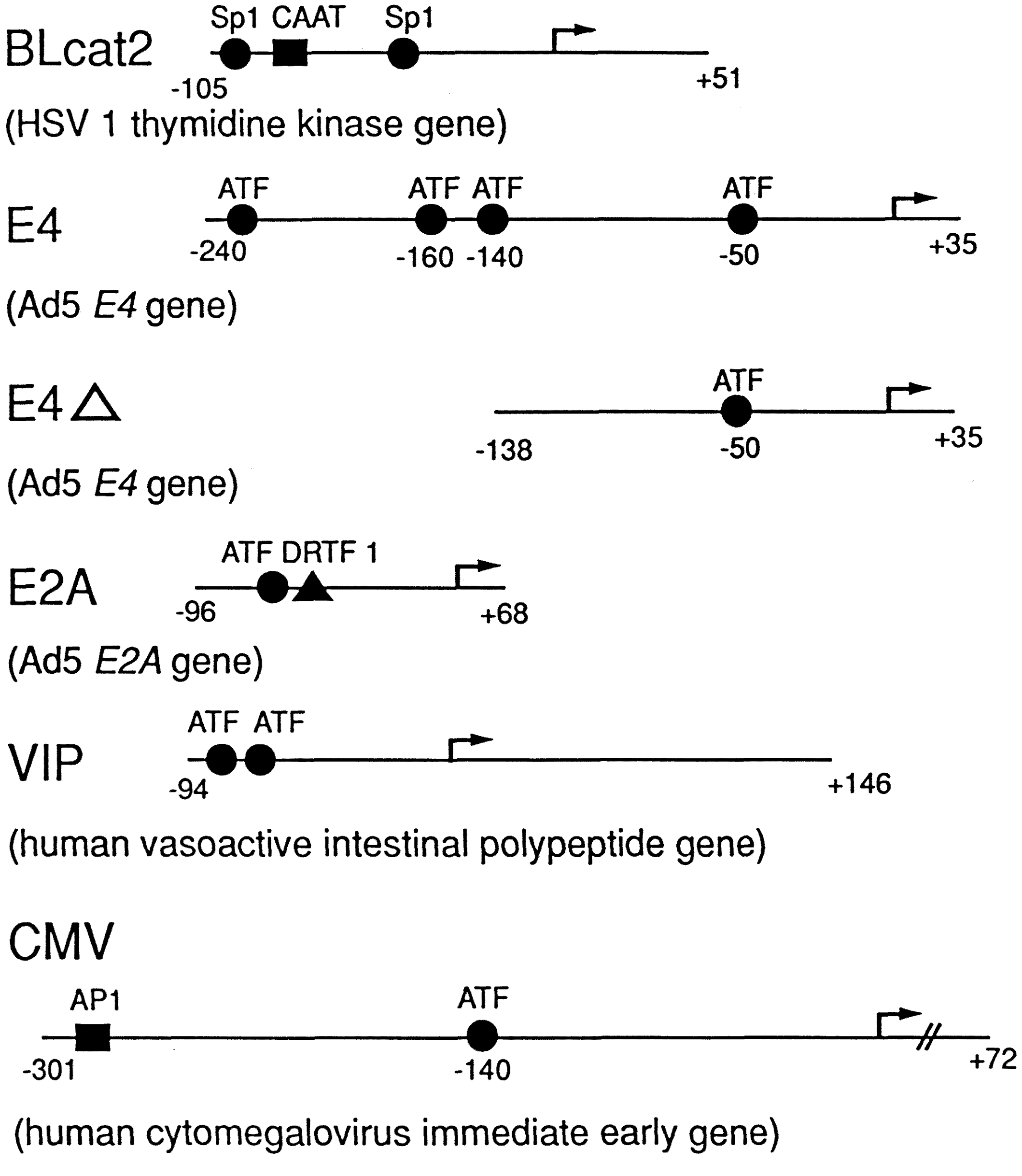

BLcat2
Sp1
CAAT
Sp1
-105
+51
(HSV 1 thymidine kinase gene)
E4
ATF
ATF
ATF
ATF
-240
-160
-140
-50
+35
(Ad5 *E4* gene)
E4△
ATF
-138
-50
+35
(Ad5 *E4* gene)
E2A
ATF
DRTF 1
-96
+68
(Ad5 *E2A* gene)
VIP
ATF
ATF
-94
+146
(human vasoactive intestinal polypeptide gene)
CMV
AP1
ATF
-301
-140
+72
(human cytomegalovirus immediate early gene)

FIG. 4.2 : *Transcriptional activity of ATF site-containing promoters in F9 EC and PE cells*

Activities of CAT constructs transfected into F9 EC and PE cells. * "Ave. CAT Act." represents the average CAT activity for each construct from the number of experiments indicated below, after normalisation with respect to pBLcat3 activity (lanes 11 and 12) in order to take transfection efficiency differences into account (see text). Construct : number of different plasmid preparations used for transfection / number of experiments performed / number of experiments quantitated and averaged = pE4 : 3/5/2, pE4Δ : 3/5/5, pE2A : 3/5/5, pVIP : 2/3/3, pBLcat2 : 2/3/3, pBLcat3 : 3/5/5, pCMV : 1/4/3 ; not all experiments were quantitated since in some cases the substrate became limiting. Two contaminants of the chloramphenicol preparation can be seen, one migrating in front of, and one behind the 1-acetate chloramphenicol product (the slowest of the two mono-acetylated forms), most clearly in lane 10, for example.

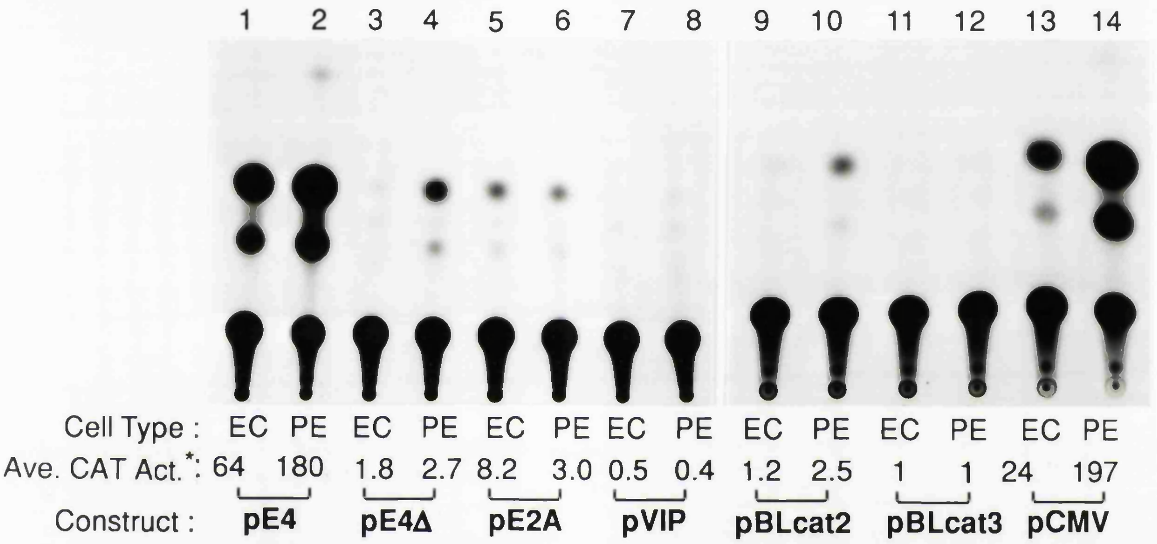

1 2 3 4 5 6 7 8 9 10 11 12 13 14
Cell Type : EC PE EC PE EC PE EC PE EC PE EC PE EC PE
Ave. CAT Act.*: 64 180 1.8 2.7 8.2 3.0 0.5 0.4 1.2 2.5 1 1 24 197
Construct : pE4 pE4Δ pE2A pVIP pBLcat2 pBLcat3 pCMV

FIG. 4.3 : *dbcAMP treatment of F9 EC cells has no effect on transcriptional activity*

Activities of CAT constructs transfected into F9 EC cells and F9 EC cells treated with 10^{-3} M dbcAMP (cA). * "Ave. CAT Act." represents the average CAT activity for each construct from the number of experiments indicated below, after normalisation with respect to pBLcat3 activity, in order to take into account transfection efficiency differences between the two cell types (see text). Construct : number of different plasmid preparations used for transfection / number of experiments performed / number of experiments quantitated and averaged = pE4 : 2/3/3, pE4Δ : 2/2/2, pVIP : 2/2/2, pBLcat3 : 2/2/2. A contaminant of the chloramphenicol preparation can be seen migrating between the two acetylated products.

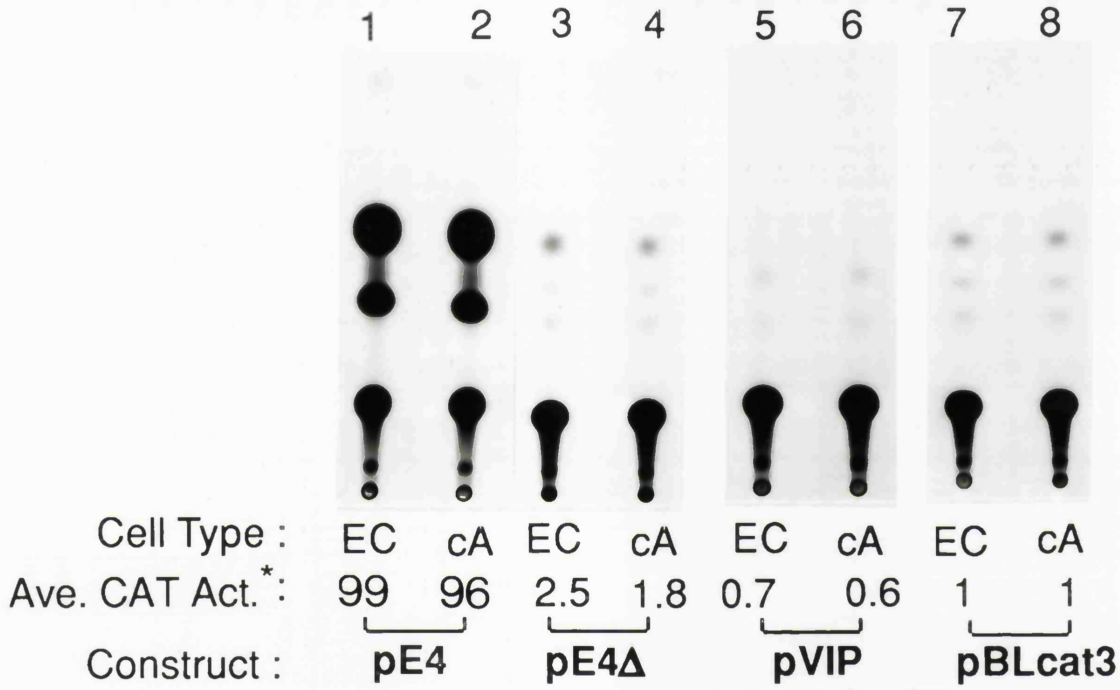
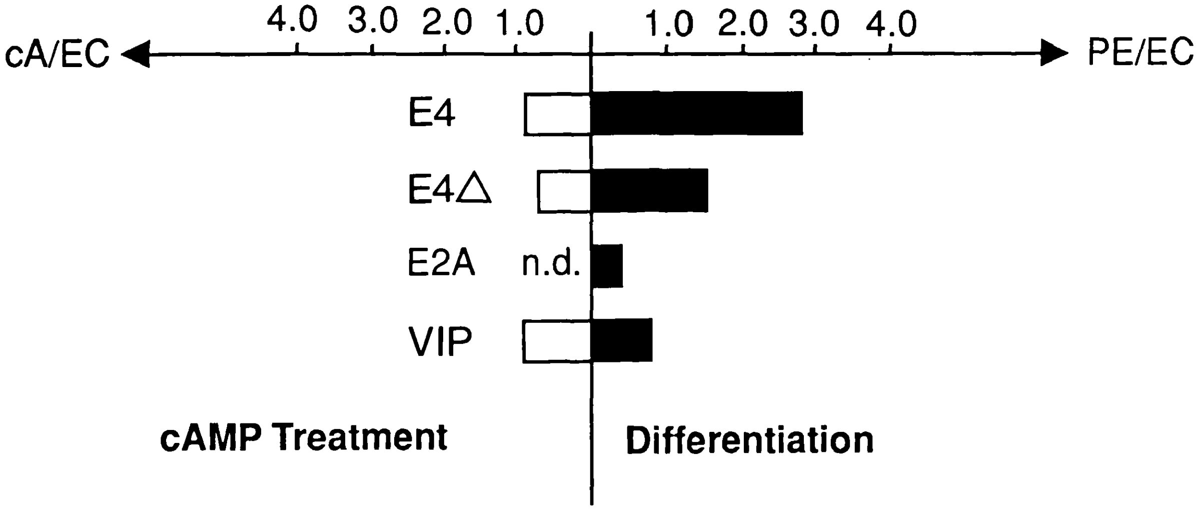

1
2
3
4
5
6
7
8
Cell Type :
EC
cA
EC
cA
EC
cA
EC
cA
Ave. CAT Act.*:
99
96
2.5
1.8
0.7
0.6
1
1
Construct :
pE4
pE4Δ
pVIP
pBLcat3

FIG. 4.4 : *Summary of results*

Ratio of CAT activities of the indicated constructs : in F9 PE relative to EC cells (PE/EC), and in dbcAMP-treated (cA) relative to untreated F9 EC cells (cA/EC) ; n.d. : dbcAMP-treated EC cells were not transfected with pE2A.

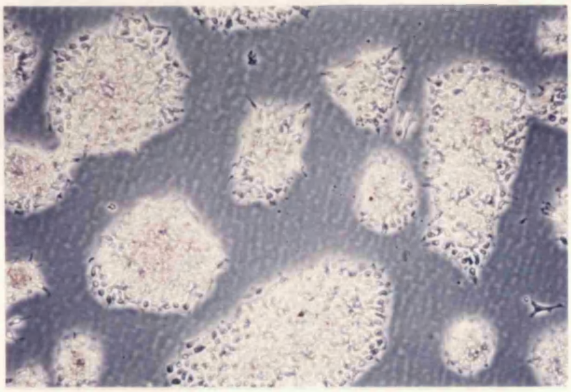


cA/EC
4.0
3.0
2.0
1.0
1.0
2.0
3.0
4.0
PE/EC
E4
E4△
E2A
n.d.
VIP
cAMP Treatment
Differentiation

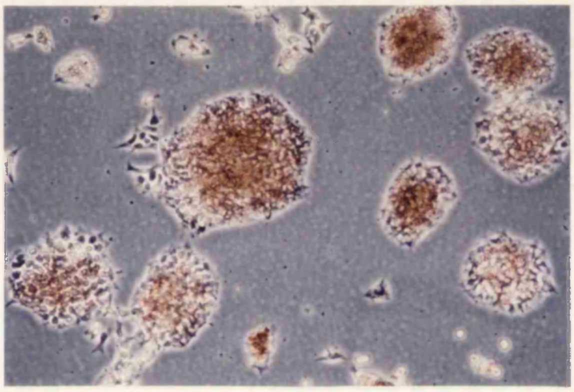
FIG. 4.5 : *Immunochemical staining of F9 EC cells (a, b), F9 EC cells treated with dbcAMP (c, d) and PE cells (e, f) for stage-specific embryonic antigen 1*

a), c) and **e)** no primary antibody ; secondary antibody : horseradish peroxidase-conjugated rabbit α-mouse immunoglobulins.

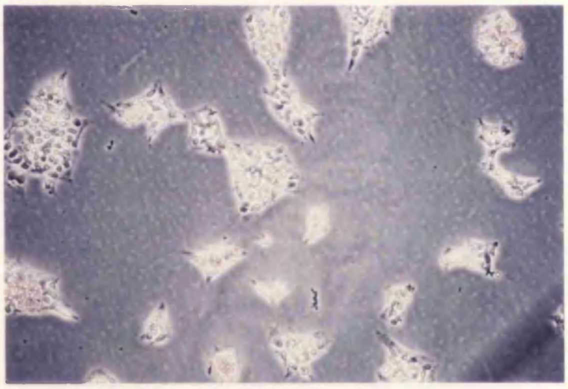
b), d) and **f)** primary antibody : α-SSEA1 MC480 ; secondary antibody : horseradish peroxidase-conjugated rabbit α-mouse immunoglobulins.



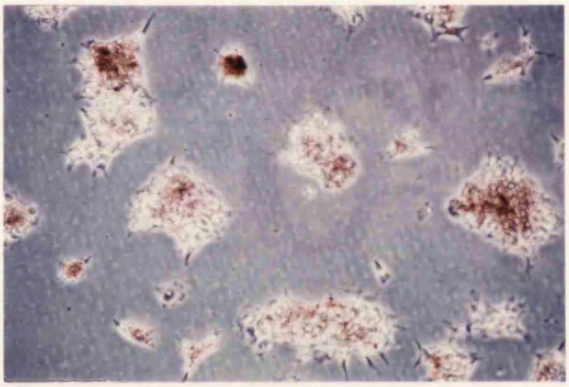
a



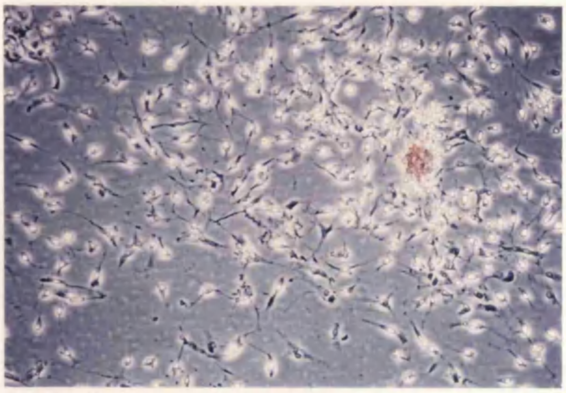
b



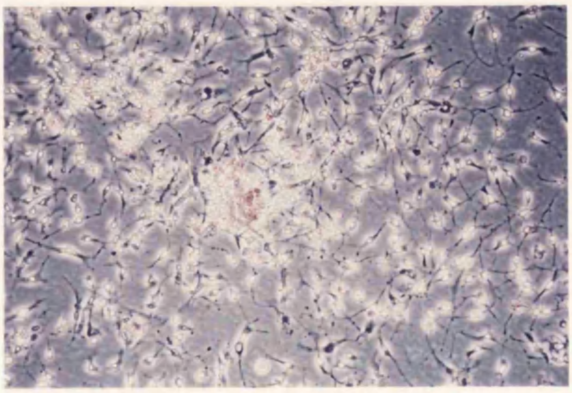
c



d



e



f

CHAPTER 5

ATF SITE-DEPENDENT PROTEIN BINDING TO THE *E4* AND *VIP* PROMOTERS

5.1 Introduction

This chapter describes the results from experiments that investigated the DNA binding activities which recognize the *E4* and *VIP* promoters *in vitro*. If the difference in their levels of transcription *in vivo* were indeed due to differences in the activity of their ATF sites, two models could be envisaged to explain it. In the first model, each promoter would bind different ATF site-binding activities : those binding to *E4* would activate transcription while those binding to *VIP* would inhibit it. In the second model, the two promoters would bind common ECRE activities but these would not activate transcription from the *VIP* promoter by failing, for example, to interact productively with the initiation complex. By a similar reasoning, the increase in *E4* transcriptional activity during differentiation would be caused by ECRE species of higher *trans*-activation potential binding to this promoter in F9 PE than in EC cells.

5.2 *E4* promoter ATF site-binding activities are regulated during differentiation

To test whether the regulation of *E4* transcription during differentiation correlated with regulated binding of ATF site-binding activities to this promoter, gel retardation experiments were performed, in which the oligonucleotides used previously to define the ECRE activities (Chapter 3) were used to compete for complexes formed on probe *E4*Δ. This allowed the assignment of the *E4*Δ complexes to the most appropriate ECRE activities. The *E4*Δ rather than the *E4* promoter was used because it afforded better resolution, though the full length *E4* promoter, as expected, also bound ATF site-specific activities in both EC (Fig.5.1 : compare lane 3 to lane 4) and PE (Fig.5.1 : compare lane 6 to lane 7) cells.

Three ATF site-specific complexes, ECRE-1, ECRE-3 and ECRE-4, formed in F9 EC cell extracts on the *E4*Δ promoter (Fig.5.2a : lane 2) and two, ECRE-3, much reduced with respect to its F9 EC cell levels, and PCRE-1, in F9 PE cell extracts (Fig.5.2b : lane 2).

As expected, they were all competed by the wild type ATF site (P) but not by the mutant site Pm1 (Fig.5.2 a and b : compare lanes 5 and 6 to lanes 3 and 4) indicating that they were all ATF site-specific. However, each single point mutation, in or around the ATF site, affected the formation of each complex differently. Mutant Pm2 competed preferentially for complexes ECRE-1 (composed of a single band) and ECRE-4 (composed of at least two closely spaced bands) in F9 EC cells, and PCRE-1 (composed of a single band) in F9 PE cells (Fig.5.2 a and b : lanes 15 and 16). The mobility difference between the ECRE-1 and ECRE-4 complexes on *E4*Δ was similar to the mobility difference of the two complexes formed in the same extract on the Pm2 oligonucleotide ; this correlation, combined with their sequence specificity and regulation, established their identity as ECRE-1 and ECRE-4. Similarly, oligonucleotides PATFm and E2A-3' competed for a complex which was unaffected by competition with Pm2 (Fig.5.2a : compare lanes 11-14 to 15 and 16), and down-regulated during F9 EC cell differentiation (compare lane 2 in Fig.5.2a to lane 2 in Fig.5.2b), suggesting it was caused by ECRE-3. Again, competition for promoter-bound ECRE-3 reflected the already observed binding activity of the oligonucleotides, since E2A-3' showed higher affinity than PATFm (Fig.5.2a : compare lane 13 to 11). When a similar competition was performed in F9 PE cell extracts, PATFm and E2A-3' competed for the ECRE-3 complex, but this was less apparent because ECRE-3 was down-regulated during differentiation of F9 EC to PE cells (Fig.5.2b : lanes 11-14) ; they did not, however, affect the PCRE-1 complex. On the other hand, mutant sites PE4Fm and Pm3, though initially designed to bind ATF in preference to E4F, competed equally for all complexes in both cell types, albeit to a weaker extent than did P (Fig.5.2 a and b : lanes 5-10), suggesting that E4F could not be detected as binding to the *E4*Δ promoter in F9 EC whole cell extracts.

These experiments allowed the relationship of all the complexes formed on the *E4*Δ promoter with those formed on the isolated variant ATF binding sites to be established. From the activities defined, ECRE-1, -3 and -4 bound to the *E4*Δ promoter in F9 EC cell extracts, whereas PCRE-1 and reduced levels of ECRE-3 bound in F9 PE extracts. The regulation of ECRE-1 and ECRE-3 promoter complexes reflected their regulation as defined with the oligonucleotide probes. In contrast, the most abundant binding activity, ECRE-2, defined with the isolated ATF site in both F9 EC and PE extracts, did not bind to the *E4*Δ promoter. This was somewhat surprising and suggests that the P oligonucleotide, which contains *E4* promoter sequences from -58 to -39, has different binding characteristics in the context of the promoter, indicating that other parameters, apart from sequence specificity, must influence binding efficiency.

There are two conclusions to be made from these experiments. First, the *in vivo* differentiation-regulated activity of the *E4*Δ promoter is reflected in a differentiation-regulated change in the ATF site-binding activities binding to it. Second, the promoter binds a subset of the total available ECRE activities.

5.3 dbcAMP treatment of F9 EC cells does not affect the ECRE activities

Consistent with its lack of effect on transcriptional activity (Chapter 4), dbcAMP treatment of F9 EC cells did not affect the pattern of ECRE activities binding to the *E4*Δ promoter (Fig.5.3 : compare lane 2 to lane 1). This suggests that, as for its transcriptional regulation, the regulation of protein binding to the *E4*Δ promoter was due to differentiation *per se* and not simply to an increase of intracellular cAMP. It also reinforces the correlation between *in vivo* transcriptional activity of, and *in vitro* protein binding to promoters : a change in *E4*Δ promoter activity was accompanied by a change of ECRE complexes formed on this piece of DNA during differentiation, while identical rates of transcription in dbcAMP-treated and untreated F9 EC cells were reflected in the unaltered pattern of ECRE activities binding to *E4*Δ.

5.4 Promoter-specific and common ATF site-dependent complexes

The complexes formed on the *VIP* promoter which, in contrast to *E4*Δ, was inactive during differentiation, were investigated next. Three ATF site-specific complexes were formed on this promoter in F9 EC extracts and two in PE extracts (Fig.5.2 c and d : lane 2). As for the *E4*Δ promoter, their identity was established by competing with the panel of variant ATF sites. In F9 EC extracts, two complexes (jointly labelled as ECRE-2) were only competed by P, PE4Fm and Pm3 (Fig.5.2c : lanes 4, 6 and 9) indicating an ECRE-2 specificity, whereas the slower migrating complex (labelled ECRE-3), which migrated just above a non ATF site-specific complex, was competed by PATFm and E2A-3' (Fig.5.2c : lanes 5 and 10). Pm2 did not compete efficiently for any of the complexes in F9 EC or PE extracts, in contrast to its effect on complexes formed on the *E4*Δ promoter (Fig.5.2 c and d : lanes 7 and 8), indicating that ECRE-1 and ECRE-4 did not bind to the *VIP* promoter. In F9 PE extracts, the two ATF site-specific complexes were, again, only competed by P, PE4Fm and Pm3 (Fig.5.2d : lanes 4, 6 and 9) arguing that the *VIP* promoter binds only one activity, ECRE-3, in common with the *E4*Δ promoter, and another activity, of ECRE-2 specificity, present in both extracts and not bound by *E4*Δ. The lack of regulation of the *VIP* ECRE2 complex was consistent with the constitutive presence of ECRE2 during differentiation (see Chapter 3).

5.5 Discussion

The data presented here could be accommodated within a combination of the two models described in the Introduction (5.1), which proposed either that distinct ATF activities bound to *E4* and *VIP*, or that common ATF site-binding activities had different functions in the context of each promoter. The *E4*Δ and *VIP* promoters, which had different *in vivo* transcriptional activities, bind distinct but overlapping subsets of the total available ATF site-binding activities in F9 cells. They both bind ECRE-3, but ECRE-1, ECRE-4 and PCRE-1 bind exclusively to *E4*Δ, whilst ECRE-2 binds exclusively to *VIP* (summarized in Table 5.1). While it cannot be excluded that non-ATF-specific complexes binding to the *VIP* promoter, such as can be seen in Fig.5.2 c and d, inhibited its transcription, it is likely that ECRE-2 and ECRE-3 are inactive in the context of this promoter. The hypothesis that

ECRE-2 is not an ATF site-binding *trans*-activator is supported by the fact that it does not bind to the transcriptionally active *E4*Δ promoter. On the other hand, because ECRE-3 binds to both the transcriptionally active *E4*Δ and the transcriptionally inactive *VIP*, its *trans*-activating potential cannot be safely surmised.

The *E4*Δ promoter experiments demonstrated that a correlation exists between *in vivo* transcription and *in vitro* protein binding. Its transcriptional induction during differentiation is reflected in a change in its ATF site-dependent complex formation between F9 EC and PE cells : binding of ECRE-1, ECRE-3 and ECRE-4 in EC cells was replaced by binding of PCRE-1 in PE cells. PCRE-1 may be related to ECRE-4, since they both have the same specificity, and may result from a PE cell-specific post-translational modification of ECRE-4. This would explain its reduced electrophoretic mobility compared to ECRE-4 (Fig.5.3 : compare lane 3 to lane 1). Therefore, ECRE-1, ECRE-4 and possibly ECRE-3 in F9 EC cells, and PCRE-1 in PE cells, are probably responsible for *E4*Δ transcription and its activation during differentiation.

The correlation between *in vivo* transcription and *in vitro* protein binding is further supported by the observation that the same ECRE activities bind to the *E4*Δ promoter in EC cells and dbcAMP-treated EC cells reflecting the fact that, as was shown in Chapter 4, dbcAMP treatment of F9 EC cells does not affect the transcriptional activity of this promoter.

Another interesting point arising from these experiments is that ECRE-2 did not bind to *E4*Δ although it bound P, whose sequence is taken from within the *E4*Δ sequence. Two possible models may explain this. According to the first, ECRE-2 would be excluded from its binding site by other DNA binding proteins ; according to the second, ECRE-2 would be unable to bind to its site when this is present in the context of this promoter. The first model seems unlikely since, in the binding reaction, the probe was in excess over the protein extract. However, the possibility cannot be excluded that the promoter-specific binding of ECRE-1, ECRE-2 and ECRE-4 is determined by "auxiliary" DNA binding proteins, which do not form discrete complexes on their own but selectively promote the binding of specific ECRE activities ; this is presented, in cartoon form, in Fig.5.4. According to this model, "auxiliary" proteins would promote binding of ECRE-1 and ECRE-4, but not ECRE-2 to *E4*Δ. Different "auxiliary" proteins would promote binding of ECRE-2 to *VIP*. Alternatively, and according to the second model, in the context of the larger DNA fragment, *E4*Δ, the P DNA sequence would adopt a conformation that precludes binding of ECRE-2. This second model could also incorporate a modification of the first, since the *E4*Δ-induced conformation of P could exclude ECRE-2 indirectly by increasing the affinity of the P sequence for other ECRE activities. The same arguments can be applied to AP1, whose complex with the TRE in F9 PE cells could be competed by P but which did not bind to the *E4*Δ promoter.

In conclusion, ATF site-containing promoters that have different transcriptional activities bind distinct ATF site-binding proteins in F9 EC and PE cells (summarized in Table 5.1). In addition, a change in a promoter's *in vivo* transcriptional activity during differentiation is reflected by a change in the pattern of ECRE proteins that this promoter binds *in vitro*. These results imply that ECRE activities differ, not only in their precise nucleotide specificity and their regulation during differentiation, but also in their affinities for different promoters and, presumably, their ability to activate transcription.

FIG. 5.1 : *Complexes formed on the full length* E4 *promoter in F9 EC and PE whole cell extracts*

Gel retardation of the full length *E4* promoter with F9 EC and PE whole cell extracts in the presence or absence of 770-fold molar excess wild type (wt)(ACGTCA) or mutant (mut) (AAATCA) ATF oligonucleotides. The ATF site-specific complexes are indicated by arrows. All lanes are from the same experiment.

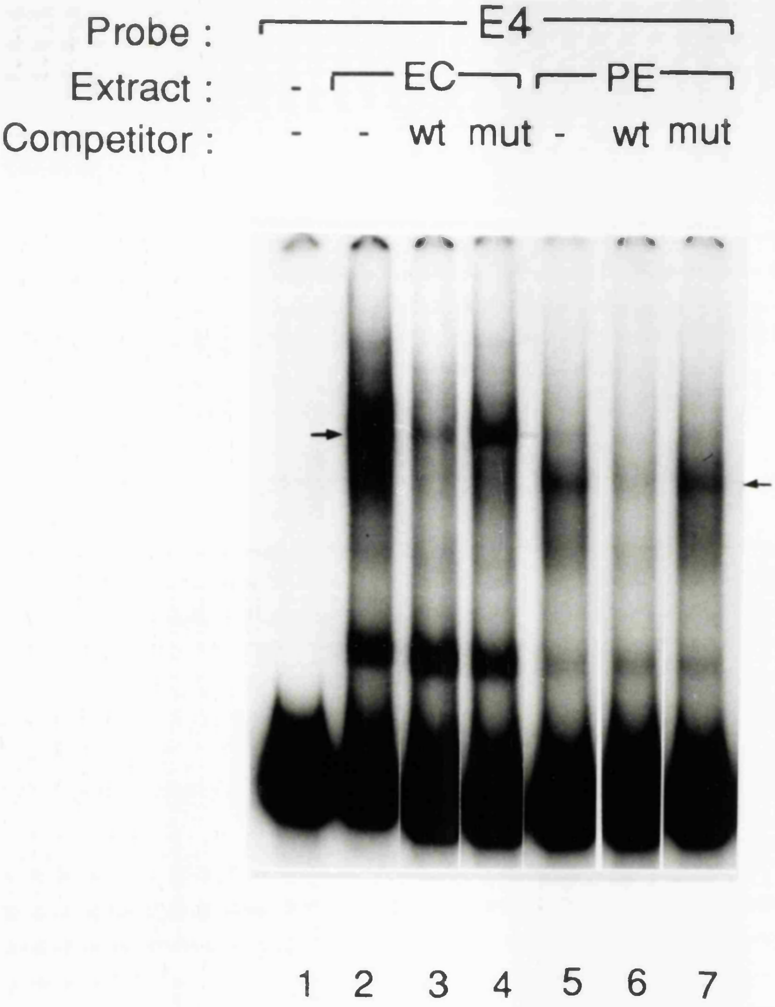
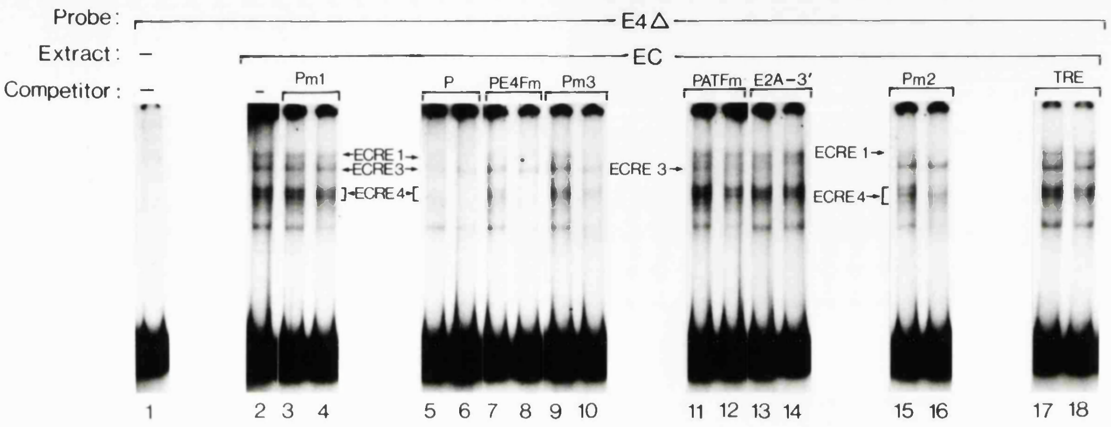

Probe :
E4
Extract :
-
EC
PE
Competitor :
-
-
wt
mut
-
wt
mut
1
2
3
4
5
6
7

FIG. 5.2 : *Specificity of protein complexes formed on the* E4Δ *and* VIP *promoters in F9 EC and PE cell extracts*

Gel retardation with probe *E4*Δ in F9 EC **(a)** and PE **(b)** whole cell extracts, in the presence of the indicated oligonucleotide competitors. Lanes 3, 5, 7, 9, 11, 13, 15, and 17 : 100-fold molar excess competitor ; lanes 4, 6, 8, 10, 12, 14, 16 and 18 : 1000-fold molar excess competitor. The fastest migrating complex, which is competed both by P (lane 4) and Pm1 (lane 6), is non-specific.

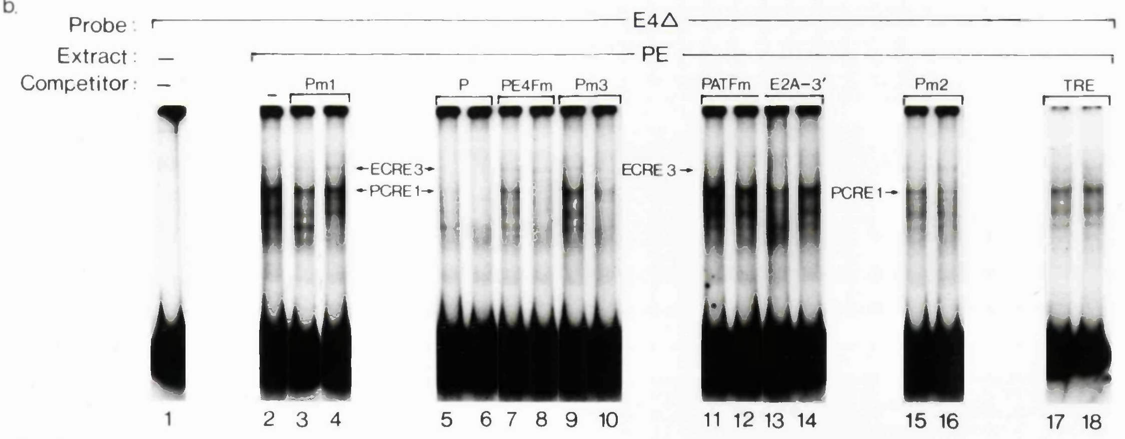
(continued opposite next page)

a.



Probe:
E4Δ
Extract:
–
EC
Competitor:
–
–
Pm1
P
PE4Fm
Pm3
PATFm
E2A-3′
Pm2
TRE
ECRE 1
ECRE 3
ECRE 4
ECRE 3
ECRE 1
ECRE 4
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18

b.

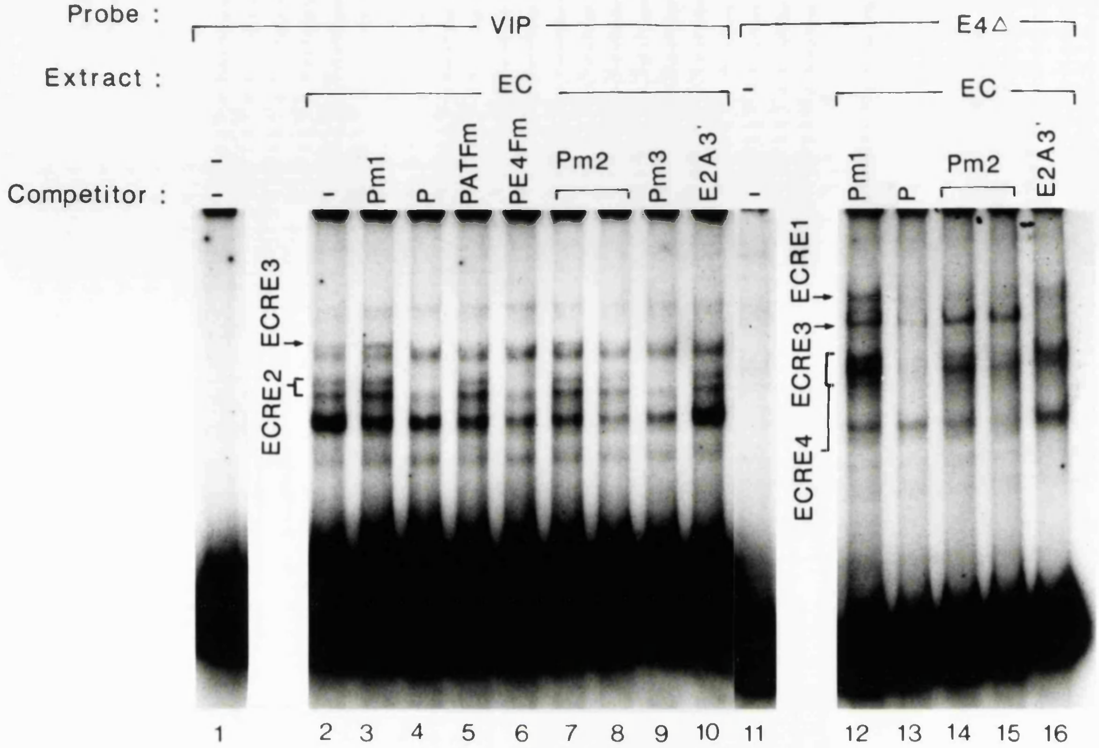


Probe:
E4Δ
Extract:
–
PE
Competitor:
–
–
Pm1
P
PE4Fm
Pm3
PATFm
E2A-3′
Pm2
TRE
ECRE 3
PCRE 1
ECRE 3
PCRE 1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18

FIG. 5.2 (cont'd.) : *Specificity of protein complexes formed on the* E4Δ *and* VIP *promoters in F9 EC and PE cell extracts*

Gel retardation with probes *VIP* and *E4*Δ in F9 EC (c) and F9 PE (d) whole cell extracts in the presence of the indicated oligonucleotide competitors. All lanes contained 1000-fold molar excess competitor, except lanes 8 and 18 which contained 2000-fold excess. All lanes are from gels run simultaneously.

c.


Probe :
VIP
E4△
Extract :
EC
-
EC
Competitor :
-
-
-
Pm1
P
PATFm
PE4Fm
Pm2
Pm3
E2A3'
-
Pm1
P
Pm2
E2A3'
ECRE3
ECRE2
ECRE1
ECRE3
ECRE4
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16

d.

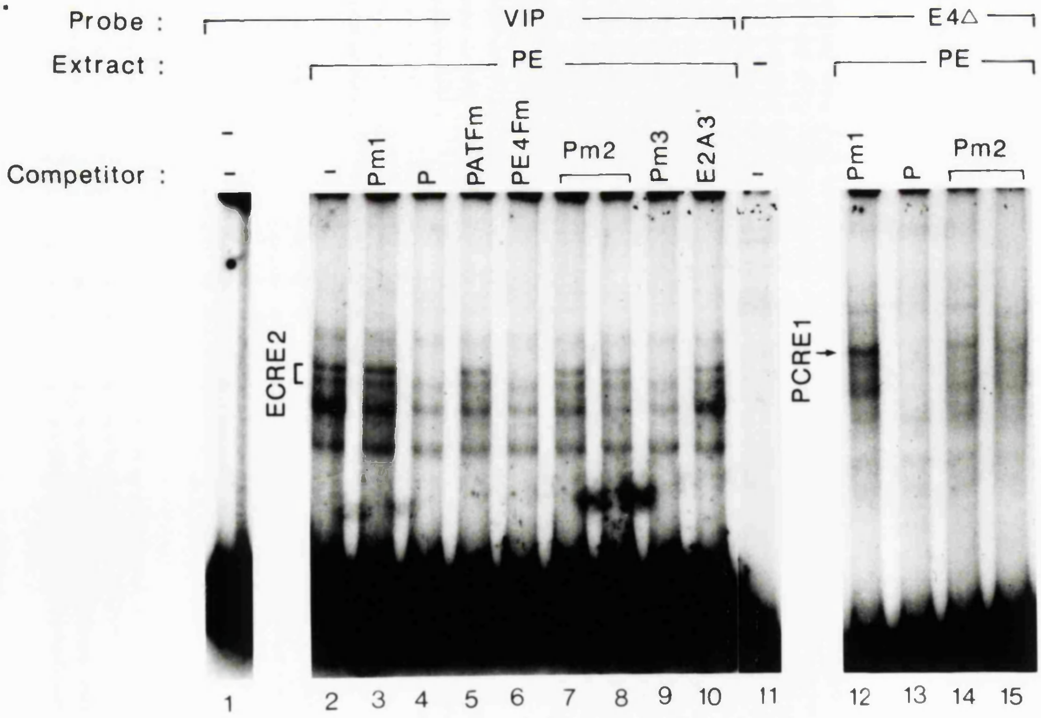

Probe :
VIP
E4△
Extract :
PE
-
PE
Competitor :
-
-
-
Pm1
P
PATFm
PE4Fm
Pm2
Pm3
E2A3'
-
Pm1
P
Pm2
ECRE2
PCRE1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

FIG. 5.3 : *dbcAMP treatment of F9 EC cells has no effect on protein/DNA complex formation*

Gel retardation with probe *E4Δ* in F9 EC cells (EC), F9 EC cells treated with dbcAMP (cA), or F9 PE (PE) whole cell extracts.

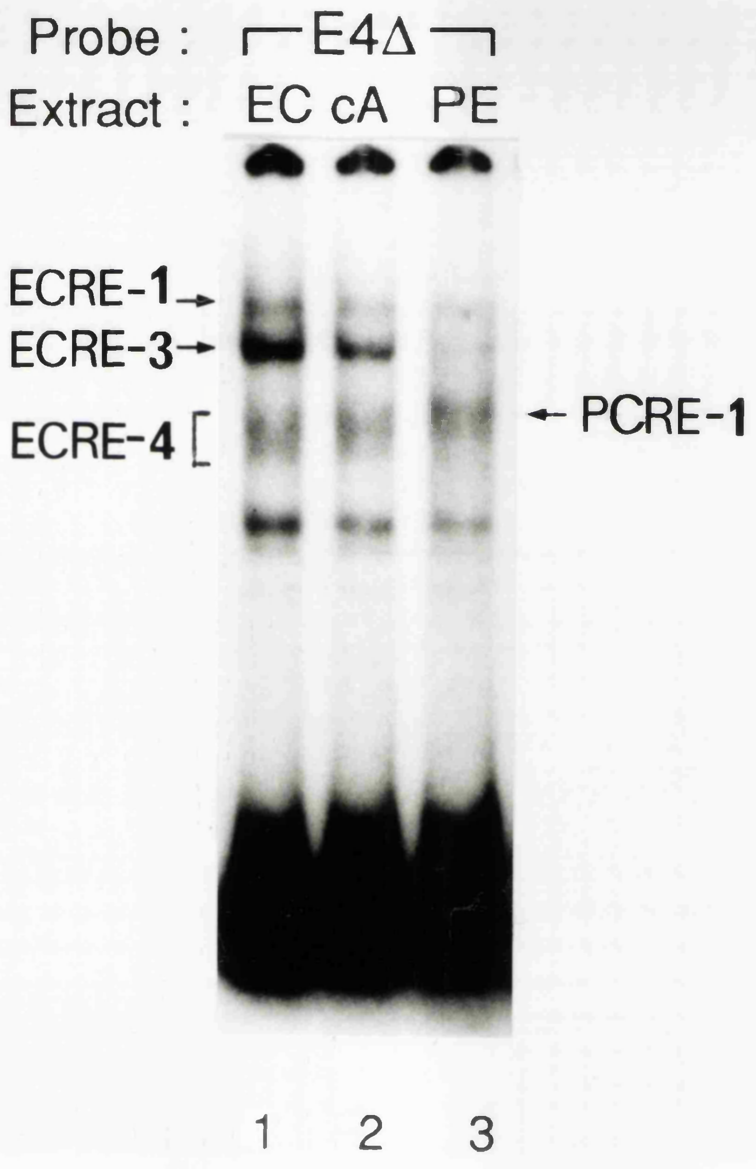

Probe : E4Δ
Extract : EC cA PE
ECRE-1
ECRE-3
ECRE-4
PCRE-1
1
2
3

FIG. 5.4 : *Schematic representation of a possible model for promoter-specific binding of ECRE activities*

Two different "auxiliary" proteins, which do not form discrete DNA complexes on their own, direct binding of ECRE-2 only to the *VIP* promoter and binding of ECRE-4 only to *E4* ; ECRE-3, on the other hand, can bind to both promoters without the aid of an "auxiliary" protein.

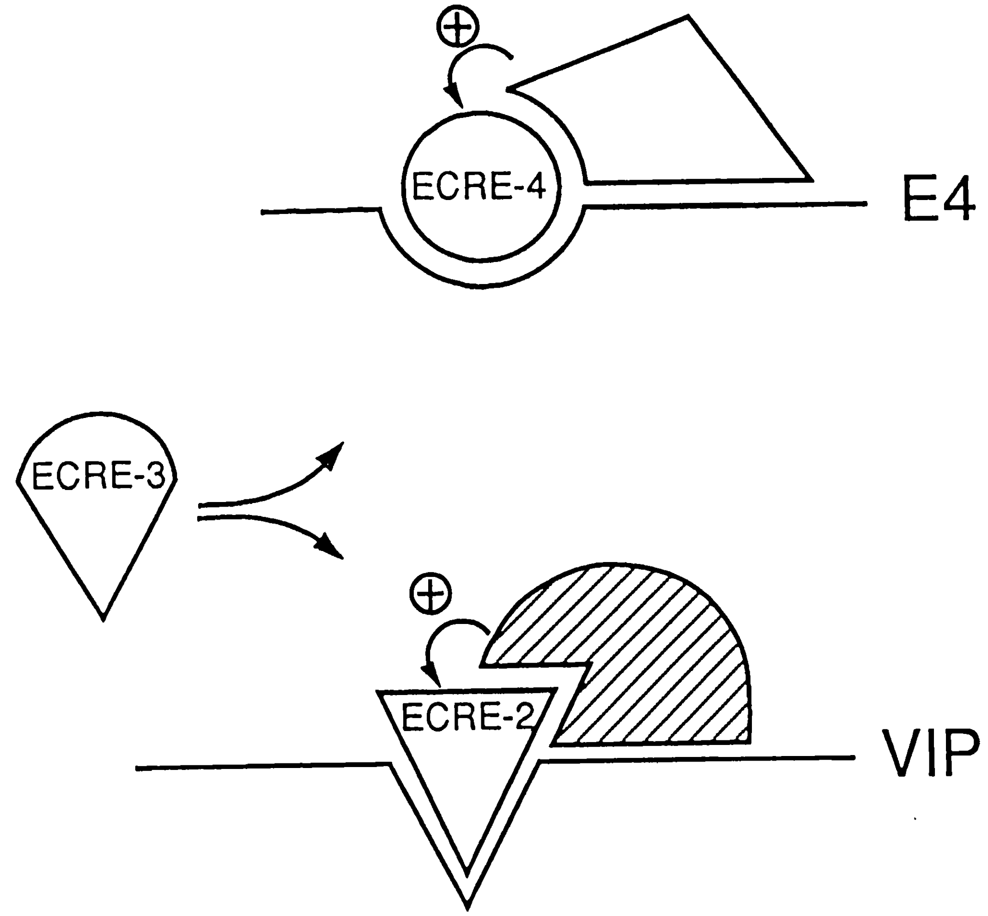

⊕
ECRE-4
E4
ECRE-3
⊕
ECRE-2
VIP

TABLE 5.1 : *Promoter and oligonucleotide binding specificity of the regulated ATF site binding activities in F9 EC and PE cells*

The regulation of F9 ATF site-binding activities during differentiation of F9 EC cells to PE cells, and their relative ability to bind to the listed oligonucleotides and promoters are shown.

F9 ATF site Binding Activities

	Regulation	Oligonucleotide Specificity								Promoter Specificity	
	EC PE	Pm1	P	PE4Fm	Pm3	Pm2	DA	E2A-3	PATFm	E4	VIP
ECRE-1	◺	-	+++	+++	++	++	+	-	-	✓	-
ECRE-2	▭	-	+++	++	+	-	++	-	-	-	✓
ECRE-3	◺	-	+++	++	++	-	+++	+++	++	✓	✓
ECRE-4a	◺	-	+++	++	++	++	+++	-	-	✓	-
ECRE-4b	◺	-	+++	++	++	++	-	-	-	✓	-
PCRE-1	◿	-	+++	++	++	++	NT	-	-	✓	-

CHAPTER 6

ATF RNA LEVELS IN F9 EC AND PE CELLS

6.1 Introduction

Having identified a number of ATF site-binding proteins in F9 EC and PE cells that had distinct sequence and promoter preferences and were regulated during differentiation, it was of interest to address the mechanisms of their regulation and sequence selectivity, as well as to determine their different effects on transcription. To achieve this, it would be necessary to study each activity in a pure state, preferably as the product of a cDNA. Two approaches were available. One would involve purification of each activity in turn by DNA binding site affinity purification, and cloning of the corresponding cDNA(s) on the basis of the protein sequence ; the other approach would be to assess whether any of the ECRE activities were encoded by any of a growing number of cDNAs that were being isolated by other laboratories. The first approach had two main disadvantages : DNA affinity purification would not be entirely practical since some activities, for example ECRE-1 and ECRE-4a, shared the same DNA specificity, and such an effort would be extremely time-consuming. The second approach, on the other hand, offered the hope of a relatively rapid identification, by comparing the regulation and specificity of known cDNA products to those of the ECRE activities. This approach was therefore chosen, especially since, even in the case that its primary aim were to fail, it would still yield pertinent information on the regulation of the ATF transcription factor gene family during cellular differentiation.

During the course of the identification of ATF site-binding activities in F9 cells, a number of laboratories had isolated cDNAs encoding distinct ATF site-binding proteins (Hoeffler *et al.*, 1988 ; Gonzalez *et al.*, 1989 ; Maekawa *et al.*, 1989 ; Hai *et al.*, 1989), using a variety of cloning techniques including screening an expression library with an oligomerized *E4* ATF site (Hai *et al.*, 1989). The predicted polypeptides were highly homologous across a region rich in basic amino acids followed by a "leucine zip" region, similar to the DNA binding domains of the Jun, Fos and C/EBP families of transcription factors (reviewed in Busch and Sassone-Corsi, 1990).

Since the proteins that bind to the ATF site were encoded by a gene family, an investigation of their RNA levels in F9 EC and PE cells was carried out. This would determine whether there were any instances where *ATF* RNA regulation reflected the regulation of ECRE activities and, therefore, if there were any potential candidates among the *ATF* genes for encoding ECRE activities.

6.2 Results

The available *ATF* cDNAs were all derived from human cells. Therefore, two prerequisites regarding the specificity of mouse F9 EC and PE cell RNA detection needed to be satisfied : that experimental conditions allow only the closest mouse homologue of each human cDNA to be detected, and that cross-hybridization across the highly homologous DNA binding domain be avoided. These two prerequisites seemed most efficiently met by excluding, wherever possible, the DNA binding domain from probe sequences and by using the ribonuclease (RNase) protection assay. RNase protection affords maximum specificity of detection by eliminating weak cross-hybridization at the RNase digestion step. This is because very few consecutive base-pairing mismatches are tolerated in each protected band, since RNase A detects and cleaves approximately 50% of possible base pair mismatches (Sambrook *et al.*, 1989). With the exception of *ATF2*, *ATF4*, *ATF6*, c*fos* and *fosB*, no probe contained sequences coding for a complete DNA binding domain (see Fig.6.1 and Chapter 2). Furthermore, when a selection of the probes used in this study were hybridized to *in vitro* transcribed RNA from the same cDNAs, under the same conditions which were used subsequently to assay F9 cellular RNA, no cross-hybridization was observed between, for example, human *ATF1* and human *ATF2* sequences (Fig. 6.2 : compare lanes : 4 to 3, 6 to 7), human *ATF4* and human *ATF6* (Fig.6.2 : compare lanes : 10 to 9, 12 to 13), or mouse c*jun* and mouse *junD* (Fig.6.2 : compare lane 16 to 15). The lack of a distinct protected fragment in this control experiment was due to the presence of RNA molecules that were smaller than full length, due to premature termination by RNA polymerase *in vitro*, and gave rise to a continuous series of protected probe fragments appearing as a smear. This lack of cross-hybridization confirmed that, though different ATF and AP1 family members share some amino acid similarity in their DNA binding domains, they lack significant overall nucleotide similarity.

As a control for RNA amounts, a probe for the human γ-actin gene was used because levels of γ-actin RNA are constant in different human cell lines and mouse tissues (Enoch *et al.*, 1986 ; Dzierzak *et al.*, 1988).

This study also addressed the RNA induction of members of the *AP1* (*jun* and *fos*) families during F9 EC cell differentiation to PE cells with dbcAMP and retinoic acid, as this had previously been studied only during retinoic acid treatment of EC cells (Yang-Yen *et al.*, 1990), which does not give rise to parietal endoderm-like cells (Strickland *et al.*, 1980).

A second control probe was derived from the proliferation-regulated c-*myc* mouse cellular oncogene (reviewed in Marcu, 1987) whose expression was known to be down-regulated as the proliferation rate decreases during F9 EC cell differentiation (Dony *et al.*, 1985).

The γ-actin signals from F9 EC and PE total RNA were indeed equal in equal amounts of total cellular RNA (Fig.6.3 : compare lanes 3 and 4), and hybridizations with this probe were always included in every experiment.

No specific protected probe fragments were detected with the *ATF1* probe (Fig.6.3 : compare lanes 7 and 8 to lane 6) in F9 EC or PE cell RNA, although HeLa RNA did give rise to a protected probe fragment (Fig.6.5 : lane 11), as expected since the *ATF1* cDNA was cloned from HeLa cells (Hai *et al.*, 1989). On the other hand, multiple protected probe fragments, indicated by arrows and brackets, whose intensity did not alter during differentiation, were observed with the *ATF2*, *ATF4* and *ATF6* probes (Fig.6.3 : compare lane 12 to lane 11, 20 to 19, 24 to 23). In contrast, the intensity of the probe fragments protected with the ATF3 probe was higher in PE cells (Fig.6.3 : compare lane 16 to lane 15). The multiplicity of mouse RNA-specific bands was presumably due to species-specific nucleotide differences giving rise to imperfect base pair matching and subsequent RNase cleavage, and possibly also to alternative splicing, which has been documented for *ATF2* (Georgopoulos *et al.*, 1992) and two other members of the *ATF* family, *CREB* (Berkowitz and Gilman, 1990 ; Ruppert *et al.*, 1992) and *CREM* (Foulkes *et al.*, 1991 ; Foulkes *et al.*, 1992).

In agreement with results published by others, *junD* was constitutively transcribed (Fig.6.4 : compare lanes 7 and 8), while *jun* and c-*fos* transcripts were detectable only in PE cells (Fig.6.4 : compare lane 4 to lane 3, 12 to 11) (Yang-Yen *et al.*, 1990). Yang-Yen *et al.* (1990) also found that, similarly to c*jun*, *junB* expression is transiently induced by RA treatment of F9 EC cells. *FosB* transcripts were undetectable in both cell types (Fig.6.4 : compare lanes 19 and 20 to lane 18) (Mumberg *et al.*, 1991), and *fra-1* transcripts were similarly not detected in either cell type (Fig.6.4 : compare lanes 15 and 16 to lane 14), though in this case, because the probe only contained 3' untranslated RNA, the possibility cannot be excluded that lack of hybridization was due to lack of conservation between rat and mouse 3' untranslated sequences.

Finally, transcripts initiating at both c-*myc* promoters (reviewed by Fahrlander and Marcu, 1986) were only present in the rapidly proliferating EC cells and not in the terminally differentiated PE cells (Fig.6.4 : compare lane 23 to lane 24), consistent with previous results which have shown that the decrease in c-*myc* RNA during differentiation is due to a post-transcriptional mechanism (Dony *et al.*, 1985).

The occasionally high non-specific hybridization background was due to the fact that all probes were hybridized to the RNA synchronously and conditions were not optimized for any one specific probe.

In conclusion, most *ATF* genes were expressed to the same levels in F9 EC and PE cells, with the exception of *ATF1*, whose RNA was undetectable, and *ATF3*, whose RNA levels increased during differentiation (Table 6.1).

6.3 Discussion

Two important conclusions can be drawn from this study of *ATF* RNA levels in F9 EC and PE cells. First, it has shown that the *ATF* gene family is not coordinately regulated during differentiation. *ATF2*, *ATF4* and *ATF6* are constitutively expressed while *ATF3* RNA levels increase during differentiation, and *ATF1* transcripts are not detected in either cell type. These distinct modes of regulation during differentiation provide one more example of the diverse responses and functions of ATF proteins, as discussed in Chapters 1 and 11.

Second, the RNA regulation of some *ATF* genes was consistent with their protein products contributing to an ECRE activity. For example, ATF2, ATF4 or ATF6 could be involved in ECRE-2 or ECRE-4/PCRE-1, since their levels of expression did not change during differentiation. ATF3, whose RNA levels increased during differentiation, could nevertheless also be a potential candidate, since RNA and protein levels need not necessarily reflect DNA binding activity. This can be modulated by post-translational modifications (reviewed by Berk, 1989), and by the choice of dimerization partner. This latter possibility will be illustrated in the next chapter, but it is worth noting here that, in a theoretical equilibrium of homodimers and heterodimers displaying distinct DNA binding properties, it would suffice for the levels of one monomer to be altered in order to produce a new set of dimers with different properties. In this light, it is interesting to note that expression of *jun/fos* family members was also non-coordinately regulated during F9 EC cell differentiation. This could allow, for example, the substitution of an ATF3/JunD dimer in EC cells by an ATF3/cJun dimer in PE cells. *ATF1*, on the other hand, was ruled out as a possible ECRE candidate sice its transcripts were not detected in F9 EC or PE cells.

As will be seen in the next chapter, the list of ECRE gene candidates was narrowed down by assaying the DNA binding activity of *in vitro* translated polypeptides. However, no *ATF* RNA was down-regulated as sharply as ECRE-1 or ECRE-3 during differentiation, and therefore the possible identity of the genes encoding these activities remained elusive.

Finally, this study guided the choice of two *ATF* members for study at the protein level : *ATF2*, whose expression remains constant during F9 EC cell differentiation, and *ATF3*, whose RNA levels increase during differentiation.

FIG. 6.1 : *Schematic representation of sequences contained in the anti-sense RNA probes*

Arrows indicate the sequences contained in the anti-sense RNA probes synthesized from the indicated cDNAs. Boxes indicate coding cDNA regions (open-sided boxes indicate that the cDNA was partial), lines indicate non-coding cDNA regions. B = basic domain, LZ = "leucine zipper".

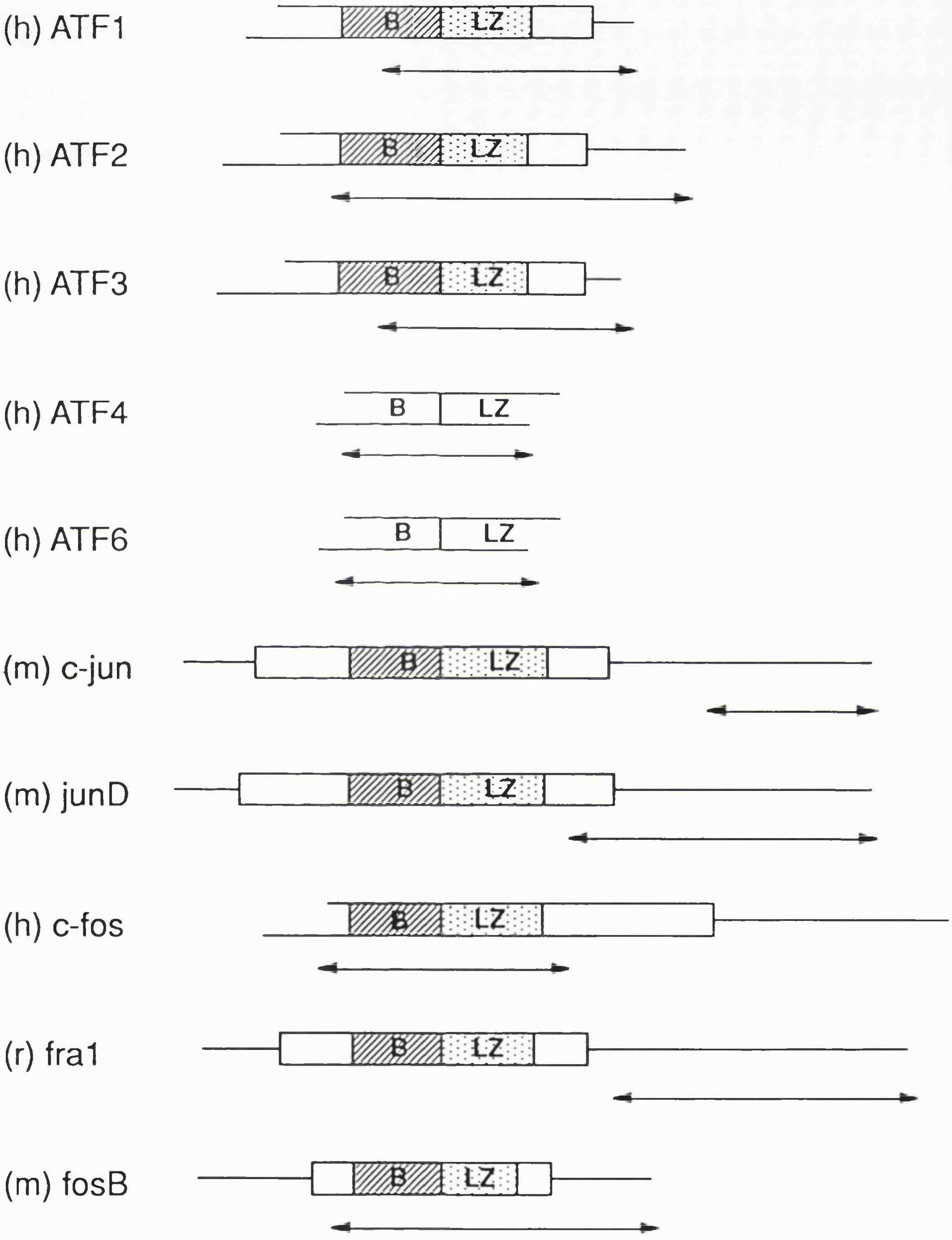

(h) ATF1
B
LZ
(h) ATF2
B
LZ
(h) ATF3
B
LZ
(h) ATF4
B
LZ
(h) ATF6
B
LZ
(m) c-jun
B
LZ
(m) junD
B
LZ
(h) c-fos
B
LZ
(r) fra1
B
LZ
(m) fosB
B
LZ

FIG. 6.2 : *Specificity of anti-sense RNA probes*

RNase protections of the indicated probes by the indicated *in vitro* translated RNAs (1 = *ATF1*, 2 = *ATF2*, 4 = *ATF4*, 6 = *ATF6*, jD = *junD*, cj = c*jun*). The lack of distinct protected fragments was due to the presence of prematurely terminated RNA molecules, a common occurrence during *in vitro* transcription, which gave rise to a continuous series of protected probe fragments appearing as a smear.

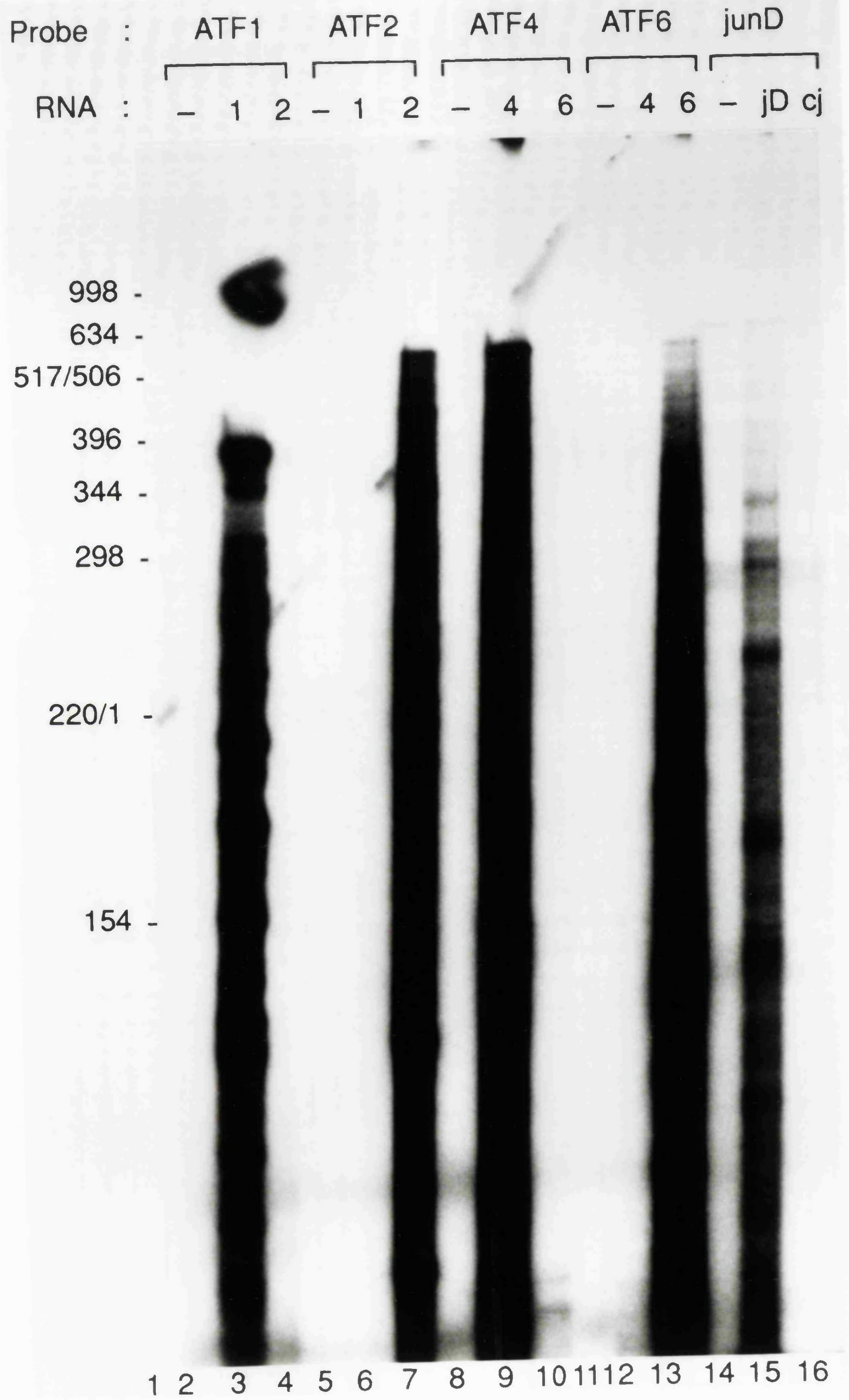

Probe : ATF1 ATF2 ATF4 ATF6 junD
RNA : – 1 2 – 1 2 – 4 6 – 4 6 – jD cj
998 -
634 -
517/506 -
396 -
344 -
298 -
220/1 -
154 -
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

FIG. 6.3 : *γ-actin and* ATF *RNA levels in F9 EC and PE cells*

RNase protections of the indicated probes (h = human) by the indicated RNAs (20 μg, except for lanes 1-4 : 2 μg) (t = yeast tRNA). Specifically protected fragments are indicated by arrows and brackets. Lanes 1, 5, 9, 13, 17 and 21 contained 1 μl of a 1:1000 dilution of the indicated undigested probe. Positions and sizes, in nucleotides, of denatured DNA size standards are shown to the left of each panel. The following groups of lanes are from the same experiment (autoradiography times indicated in brackets) : 1-4 (overnight) and 21-24 (5 days) ; 5-12 (4 days) ; 13-16 (5 days) and 17-20 (overnight).

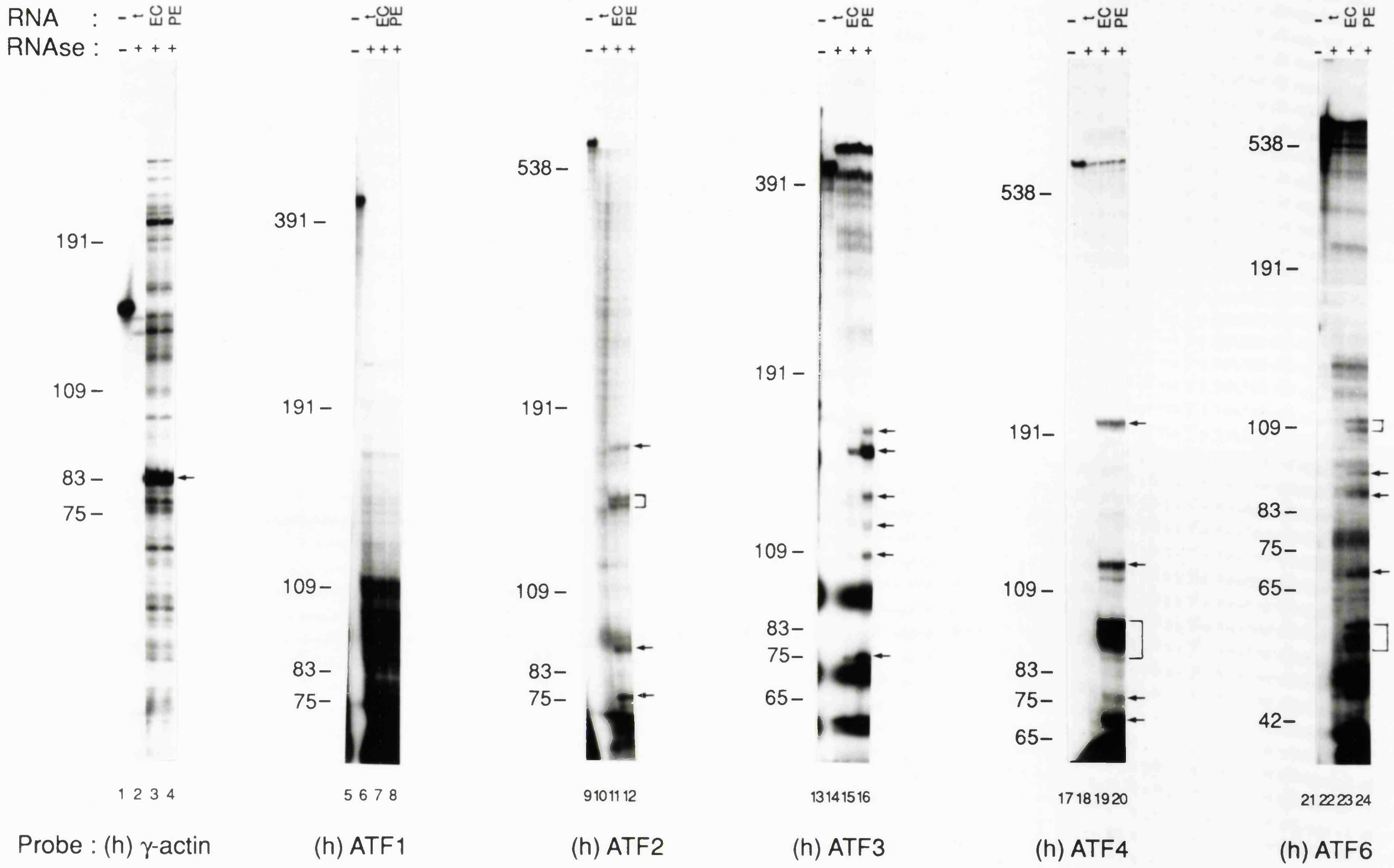

RNA : - t EC PE
RNAse : - + + +
191
109
83
75
1 2 3 4
Probe : (h) γ-actin
391
191
109
83
75
5 6 7 8
(h) ATF1
538
191
109
83
75
9 10 11 12
(h) ATF2
391
191
109
83
75
65
13 14 15 16
(h) ATF3
538
191
109
83
75
65
17 18 19 20
(h) ATF4
538
191
109
83
75
65
42
21 22 23 24
(h) ATF6

FIG. 6.4 : *RNA levels of* AP1 *family members and c-*myc *in F9 EC and PE cells*

RNase protections of the indicated probes (m = mouse, h = human, r = rat) by the indicated RNAs (20 μg) (t = yeast tRNA). Specifically protected fragments are indicated by arrows and brackets. Lanes 1, 5, 9, 13, 17 and 21 contained 1 μl of a 1:1000 dilution of the indicated undigested probe. Positions and sizes, in nucleotides, of denatured DNA size standards are shown to the left of each panel. The following groups of lanes are from the same experiment (autoradiography times indicated in brackets) : 1-4 (overnight) ; 5-8 (overnight) and lanes 5-12 of Fig.6.3 ; 9-20 (10 days) ; 21-24 (2 days).

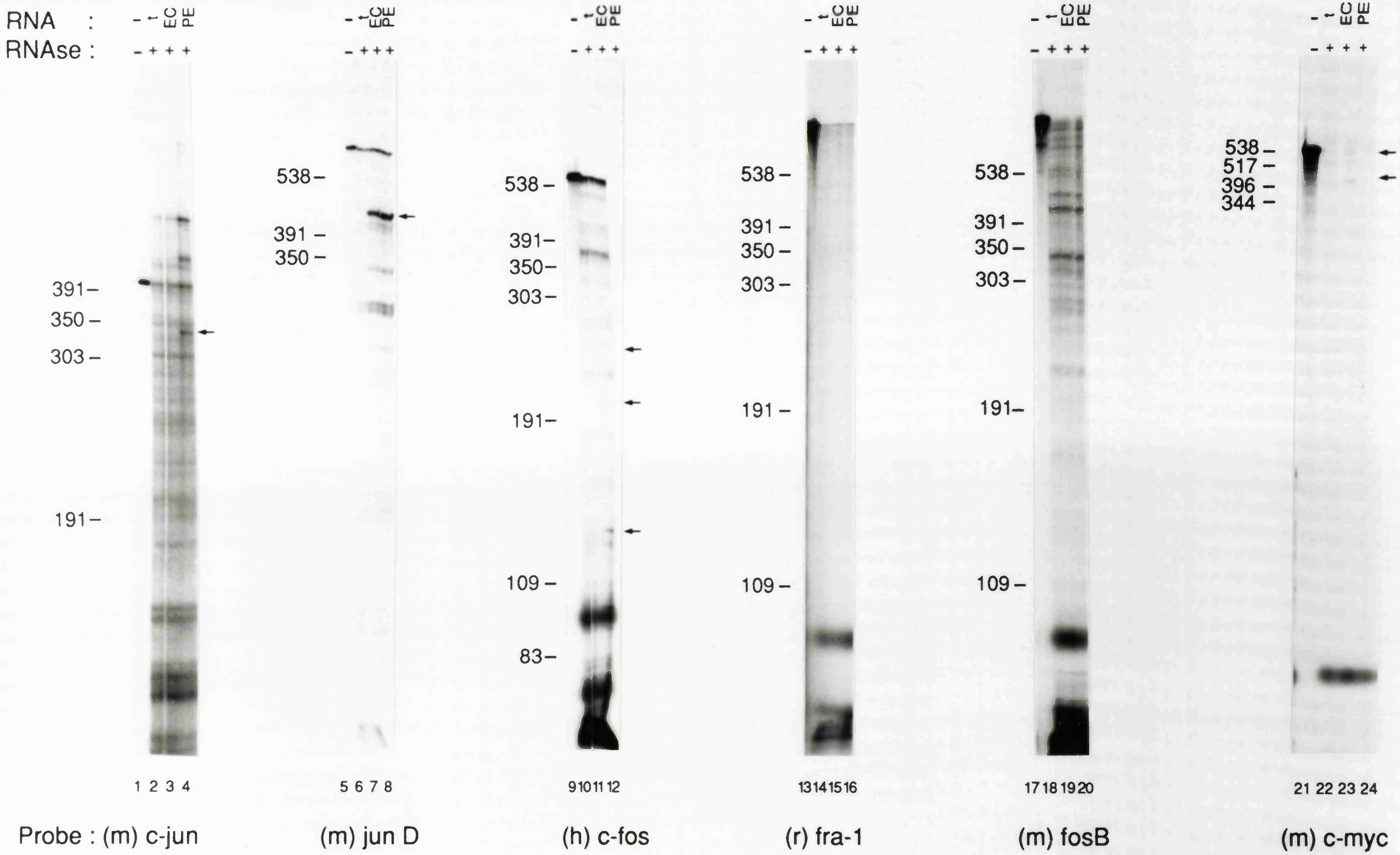

RNA :
RNAse :
- t EC PE
- + + +
391–
350–
303–
191–
538–
391–
350–
538–
391–
350–
303–
191–
109–
83–
538–
391–
350–
303–
191–
109–
538–
391–
350–
303–
191–
109–
538–
517–
396–
344–
1 2 3 4
5 6 7 8
9 10 11 12
13 14 15 16
17 18 19 20
21 22 23 24
Probe : (m) c-jun
(m) jun D
(h) c-fos
(r) fra-1
(m) fosB
(m) c-myc

FIG. 6.5 : ATF1 *is expressed in HeLa but not in F9 EC and PE cells*

RNase protections of the indicated probes (h = human) by the indicated RNAs (20 μg) (t = yeast tRNA). Specifically protected fragments are indicated by arrows. Lanes 2 and 7 contained 1 μl of a 1:1000 dilution of the indicated undigested probe. Lane 1 : denatured DNA size standards whose sizes, in nucleotides, are indicated to the left. Autoradiography time : 2 days.

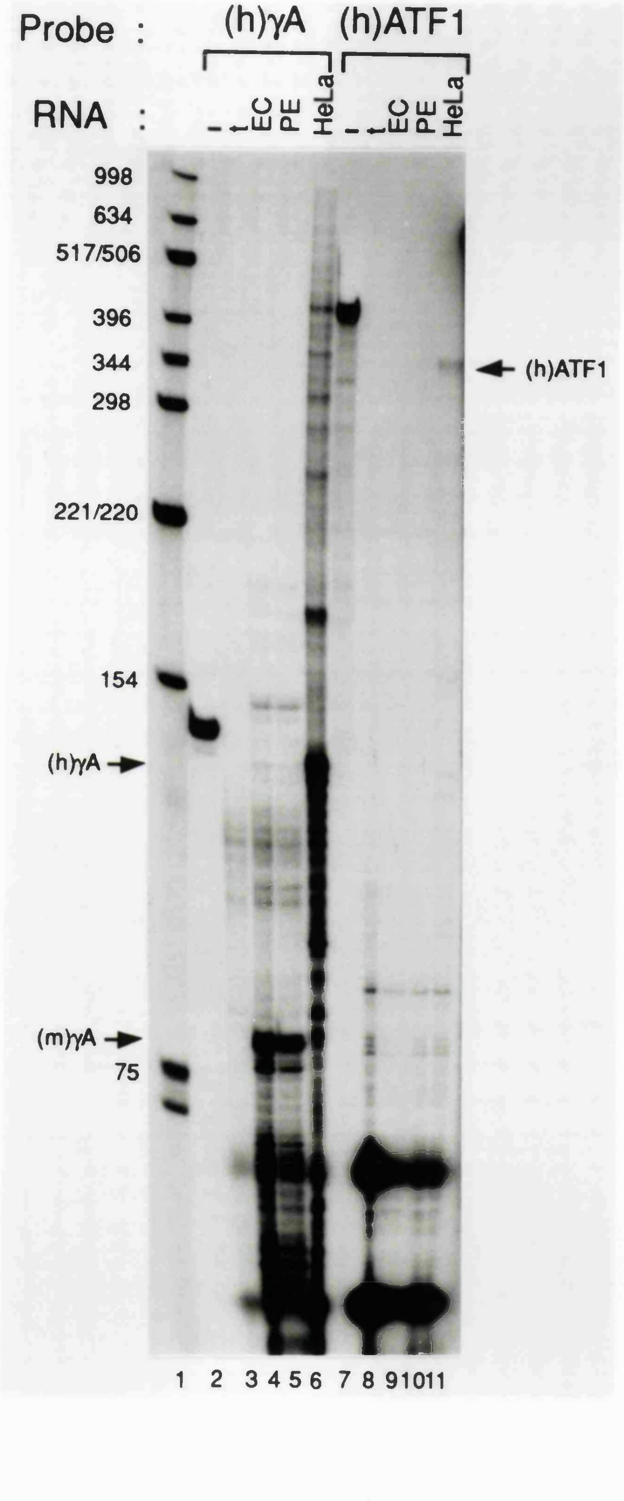

Probe :
(h)γA
(h)ATF1
RNA :
-
+
EC
PE
HeLa
-
+
EC
PE
HeLa
998
634
517/506
396
344
298
221/220
154
(h)γA
(m)γA
75
(h)ATF1
1 2 3 4 5 6 7 8 9 10 11

TABLE 6.1 : *Regulation of* ATF *and* AP1 *family members'RNA levels during differentiation*

NT : not tested

Regulation of RNA levels

	EC	PE	HeLa
γ - actin	+	+	+
ATF1	-	-	+
ATF2	+	+	NT
ATF3	+	++	NT
ATF4	+	+	NT
ATF6	+	+	NT
c-jun	-	+	NT
jun D	+	+	NT
c-fos	-	+	NT
fra-1	-	-	NT
fosB	-	-	NT
c-myc	+	-	NT

CHAPTER 7

DNA BINDING SPECIFICITY OF *IN VITRO* TRANSLATED ATF AND AP1 FAMILY PROTEINS

7.1 Introduction

As discussed in the previous chapter, the study of *ATF* transcript levels suggested some candidate genes that could, by virtue of their regulation during differentiation, encode ECRE activities. ATF2, ATF4 and ATF6 could contribute to ECRE-2 and/or ECRE-4/PCRE-1. So could ATF3, because its induction, at the RNA level, during differentiation may not directly reflect an increase in DNA binding activity. No *ATF* gene, on the other hand, was down-regulated at the RNA level during differentiation, which made it difficult to speculate on the relation between ATF proteins and ECRE-1 or ECRE-3.

Since ECRE activities were distinguishable in their ability to bind variant ATF sites (Table 3.2), another way of relating them to known ATF proteins was to assess the binding of *in vitro* translated ATF polypeptides to the panel of ATF probes.

7.2 Construction of *in vitro* expression vectors

Such a study required *in vitro* transcription and translation of the ATF cDNAs, some of which did not contain an translational initiation codon, while the complete sequence of others was not available. For this reason, a set of three vectors was constructed which would allow *in vitro* transcription and translation of an inserted cDNA in any of the three possible translational frames. The Stratagene vector Bluescript-KS (BS-KS) was modified by the insertion, 3' to the T3 promoter, of an oligonucleotide containing the Kozak consensus for vertebrate initiation of translation (Kozak, 1987) followed by an ATG initiation codon and an EcoRI cloning site. The three vectors differed in the number of nucleotides inserted after the ATG codon, thus allowing translation in all three translational frames (Fig.7.1). They also differed from each other and from the parent vector, BS-KS, in their restriction maps (Fig.7.1 and Fig.7.2). These vectors did indeed fulfill their purpose since, when the *ATF6* cDNA, whose reading frame was unknown because of partial sequence information, was cloned in all three, only one construct produced a polypeptide after *in vitro* transcription by phage T3 RNA polymerase and translation in a rabbit reticulocyte lysate, and this polypeptide had the correct molecular weight as predicted from the size of the cDNA (Fig.7.2B : compare lane 2 to lanes 1 and 3 ; see Fig.7.3B for predicted molecular weight of ATF6).

7.3 DNA binding specificity of *in vitro* translated ATF dimers

Having engineered constructs that could be used for *in vitro* expression of the ATF cDNAs which are expressed in F9 EC and PE cells, the apparent relative molecular weight (M_r) of the resulting polypeptides was determined by ^{35}S-methionine labelling and SDS-PAGE (Fig.7.3). In all cases, the predominant translated product matched the expected M_r within an acceptable margin.

The DNA binding specificity of the polypeptides was then tested by gel retardation experiments using the oligonucleotide binding sites that define the ECRE activities. This study was restricted to the ATF and AP1 family members which are expressed in F9 EC and PE cells and were available in cDNA form. For each experiment, synthesis of the polypeptides was checked by performing parallel translations with either unlabelled L-methionine or ^{35}S-labelled methionine and examining the products of the latter after SDS-PAGE and autoradiography (see, for example, Fig.7.3A). The rabbit reticulocyte lysate itself did contain weak P-binding activity (Fig.7.4A : lane 1) but this did not mask the binding of ATF2 (Fig.7.4A : lanes 2-4)) or ATF3 (Fig.7.4A : lanes 5 and 6) polypeptides to a wild type ATF site. Neither ATF2 nor ATF3 bound to the Pm1 mutant (Fig.7.4A : compare lanes 8, 9, 10, 11 to lanes 2, 3, 5, 6, respectively), or to the E2A-3' site (Fig.7.4A : compare lanes 13, 14, 15, 16 to lanes 2, 3, 5, 6, respectively), though they did bind to the Pm2 mutant, albeit less efficiently than to P (Fig.7.6B : compare lanes 9, 10, 11, 12, 13, 14 to lanes 1, 2, 3, 4, 5, 6, respectively). However, as shown in Fig.7.4B, contrary to ATF3 (lane 1) and ATF2 (lane 6), ATF4 (lane 2) and ATF6 (lane 8) showed only negligible binding, indistinguishable from cFos (lane 3) or the endogenous activity in reticulocyte lysate (lane 5). In order to investigate the possibility of ATF4 and ATF6 binding only as heterodimers, they were co-translated with either ATF2 or ATF3. The complexes formed by these co-translations on the DNA were, however, indistinguishable from those seen with ATF2 or ATF3 alone (Fig.7.4B : compare lanes 9 and 10 to 6, lane 11 to 7). Similarly, ATF4 and ATF6 did not bind to the E2A-3' site, either alone or in a co-translation (Fig.7.4B : lanes 12-14 ; Fig.7.5 : lanes 8-11).

These results confirmed the RNA studies by indicating that none of the *ATF* cDNA products tested is likely to participate in ECRE-3, defined by the E2A-3' sequence. ATF2 and ATF3 could, however, contribute to ECRE-1 and ECRE-4, since they bound both to the Pm2 mutant and the wild type site P. Finally, the inability of ATF4 and ATF6 to bind to the wild type P probe seems to preclude them from being related to the ECRE activities.

7.4 ATF/AP1 heterodimerization causes changes in DNA binding specificity

While this study was in progress several reports were published showing heterodimerization between members of the ATF and AP1 transcription factor families (Benbrook and Jones, 1990 ; Hai and Curran , 1990 ; Ivashkiv *et al.*., 1990 ; Macgregor *et al.*, 1990). Co-translation of *ATF* and *AP1* family RNAs can yield DNA binding heterodimers so that, for example, ATF2 can heterodimerize with cJun or JunD to form DNA-binding complexes which migrate to a position between the ATF2 and Jun homodimers (Fig.7.6A : compare lane 2 to lanes 1 and 3 ; Fig.7.6B : compare lane 7 to lanes 6 and 8).

Importantly, the choice of heterodimerization partner affects DNA binding specificity. The cJun/cFos heterodimer, for example, bound to the TRE and not to the ATF site (Fig.7.7A : compare lane 7 to lane 1), while the cJun/ATF2 heterodimer bound exclusively to the ATF site (Fig.7.7A : compare lane 9 to lane 3). ATF3, on the other hand, as a homodimer, bound only to the ATF site (Fig.7.7B : compare lane 2 to lane 8) ; as a heterodimer with cJun or JunD, however, it bound both to the ATF site and to the TRE, albeit more efficiently to the former (Fig.7.7B : compare lane 1 to lane 7 ; Fig.7.7A : compare lane 2 to lane 8). ATF2/cJun, ATF3/cJun and ATF3/JunD also bound to Pm2 (Fig.7.7A :

lanes 5 and 6 ; Fig.7.7B : lane 4). Again, no ATF2/Jun or ATF3/Jun heterodimer bound to the E2A-3' probe (Fig. 7.5 : compare lanes 4-7 to the negative control in lane 3, and the positive control in lane 2).

Therefore, heterodimerization can occur between members of the ATF and AP1 families as well as within each family (Hai *et al.*, 1989 ; Nakabeppu *et al.*, 1988). Furthermore, the resulting heterodimers can display a specificity for either an ATF site (ATF2/cJun), or an AP1 site (cJun/cFos), or both (ATF3/JunD).

In conclusion, this part of the study increased the possible contributors to ECRE activities to include JunD in F9 EC and PE cells and cJun in PE cells, since they too bound to the ATF site, as heterodimers with ATF2. ATF3/Jun dimers, on the other hand, would seem unlikely to correspond to an ECRE activity, since they also bound to the TRE, in contrast to all ECRE activities. These results are summarized in Table 7.1.

7.5 Discussion

The DNA binding specificity of *in vitro* translated ATF proteins and their RNA levels during F9 EC cell differentiation suggested that ATF2, ATF3, JunD and cJun could be components of the ECRE activities (summarized in Table 7.1). ATF2, whose RNA levels remained constant during differentiation, bound to both P and Pm2 but not E2A-3'. ATF3, whose RNA levels are higher in PE cells also bound both P and Pm2 but not E2A-3'. Finally, JunD and cJun also bound to both P and Pm2 as heterodimers with ATF2 and ATF3. ATF4 and ATF6, on the other hand, which were constitutively expressed at the RNA level during differentiation, did not bind to the ATF site in solution ; this is in agreement with Hai and Curran (1990), who showed that ATF4 binds to the ATF site only as a heterodimer with Fos proteins. It cannot be excluded, however, that the inability of ATF4 and ATF6 to bind to the ATF site was due to partial cDNA sequences. Nonetheless, these were obviously able to bind to a multimerized ATF site when expressed as fusion proteins in bacteria and immobilized on a membrane, since this is how they were isolated (Hai *et al.*, 1989). The DNA binding domain alone, however, may not allow stable binding to a monomeric site in solution, although it has been shown to be sufficient for DNA binding of Jun and Fos (Abate *et al.*, 1990). None of the ATF proteins examined bound to the E2A-3' site ; this was consistent with the lack of any *ATF* gene that would reflect the regulation of ECRE-3, by being transcribed in EC cells and not in PE cells. This is not too surprising since the E2A-3' site, despite its similarity, is quite unlike, in terms of nucleotide sequence and position relative to the transcriptional start site, any other ATF site. Significantly, a detailed analysis of this site by point mutation revealed that ECRE-3 binding requires not only the CG nucleotides of the ATF core site but also the CG nucleotides further downstream, at positions +23 and +24 relative to the *E2A* transcriptional start site (M.Zamanian, unpublished observations).

Although it was clear, therefore, that neither ATF2 nor ATF3 could be involved in ECRE-3, it was not possible to assign them or their heterodimers with Jun proteins to specific ECRE activities. They all bound to both P and Pm2 and could therefore equally contribute to ECRE-1 and ECRE-4/PCRE-1. However, the common binding properties of these different ATF and Jun complexes could be due to a relaxed specificity of *in vitro* synthesized polypeptides, since they were often translated from incomplete cDNAs. It is therefore possible, for example, that one of these complexes contributes to ECRE-2, which, when produced in the cell, only binds to P, but under the conditions used in these experiments also binds more weakly to the Pm2 site.

Furthermore, although the sequence specificity of ATF2 and ATF3 is consistent with either or both being present in an ECRE-1 complex, the down-regulation of ECRE-1 during differentiation is in contrast to the unaltered RNA levels of *ATF2* and *ATF3*. RNA levels, however, need not always reflect levels of protein (Mueller *et al.*, 1990 ; Cusella-De Angelis *et al.*, 1992) and even the presence of protein does not always guarantee DNA binding activity, since association with other proteins may sequester it away from the DNA (Bauerle and Baltimore, 1988). Furthermore, even if, for example, ATF3 protein levels increased during differentiation, and an ATF3 homodimer were binding to the ATF site in

EC cells, heterodimerization with a PE cell-specific bzip protein could change its specificity and prevent it from binding to the ATF site in F9 PE cells.

In conclusion, therefore, although no clear assignations of ATF proteins to ECRE activities could be made, these DNA binding experiments have suggested three ECRE activities that may be related to the examined ATF polypeptides : ECRE-1, ECRE-2 and ECRE-4/PCRE-1 may be encoded, solely or jointly, by *ATF2*, *ATF3*, c*jun* and *junD*.

The combinations of possible ATF/AP1 dimers that might bind DNA are numerous, and even if this study were exhaustive, it could not hope to be conclusive, given the limitations described above. It has, nevertheless, served as a pointer to potential candidates, but it has also illustrated the importance of differential dimerization for determining DNA binding specificity, a mechanism that has important implications for the regulation of transcription during differentiation. Taking ATF3 as an example, let us assume that, in EC cells, several dimers exist in equilibrium, say ATF3/ATF3, ATF3/ATF2, ATF3/JunD. In PE cells, two kinds of changes known to occur will upset this equilibrium : the increase in ATF3 levels and the appearance of cJun and cFos. Not only is there now more ATF3 to complex with JunD, but cJun is also available for dimerization ; on the other hand, both cJun and JunD have an increased choice in partners with the appearance of cFos (presented in cartoon form in Fig.7.8). Thus, both the levels of existing dimers can change and new dimers can be formed after differentiation. Given that different dimers have an ATF site and/or a TRE specificity, the ability of the cell to regulate transcription *via* these sites will be modified as the levels of *trans*-regulating dimers of the appropriate specificity change. Therefore, changes in transcription during differentiation could be partly caused by changes in the levels of heterodimers that exhibit a given DNA binding specificity. Of course, not only would this mechanism be combined with others, such as differential modification of activation domains of transcription factors, but also influenced by them, since dimerization itself can be controlled by post-translational modifications (discussed in Chapter 11).

FIG. 7.1 : *Design of the BS-ATG vectors*

Construction of BS-ATG Vectors

1. Digest BS-KS with HindIII and EcoRI
2. Insert oligonucleotides containing Kozak sequence - ATG - EcoRI cloning site, as follows :

BS - ATG0 → EcoRV site deleted from BS-KS

```
AGCTTGCCACCATG
    ACGGTGGTACTTAA
```

BS - ATG1 → EcoRV site deleted from BS-KS
HindIII site destroyed after re-ligation

```
AGCTCGCCACCATGG
    GCGGTGGTACCTTAA
```

BS - ATG2 → EcoRV site deleted from BS-KS
HindIII site destroyed after re-ligation
NruI site created

```
      NruI
AGCTCG|CGAGCCACCATGGG
    GC↓GCTCGGTGGTACCCTTAA
```

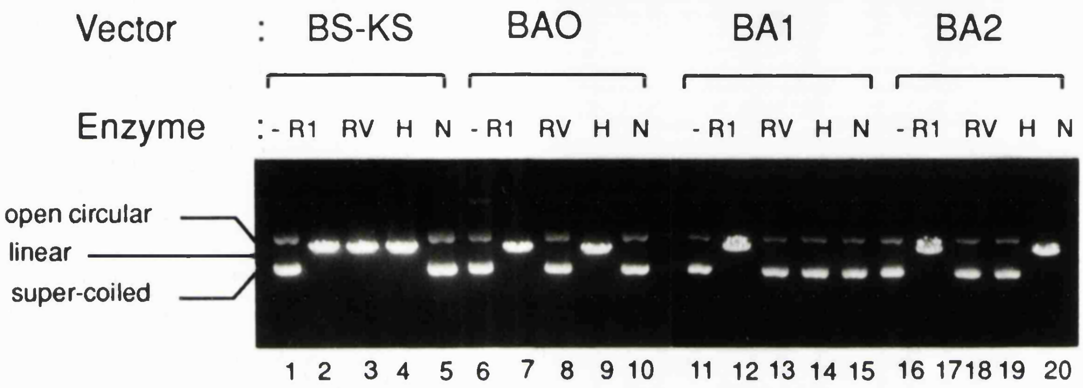
Vector	Expect linearisation by :			
	EcoRI	HindIII	EcoRV	NruI
BS-KS	✓	✓	✓	–
BS-ATG0	✓	✓	–	–
BS-ATG1	✓	–	–	–
BS-ATG2	✓	–	–	✓

FIG. 7.2 : *Properties of the BS-ATG vectors*

A) Diagnostic restriction digests of the three BS-ATG vectors and the parent vector BS-KS (refer to Fig.7.1 for predicted patterns). BS-KS = Bluescript-KS, BA0 = BS-ATG0, BA1 = BS-ATG1, BA2= BS-ATG2. The conformations of plasmid DNA are indicated to the left of lane 1.

B) The *ATF6* cDNA was cloned into, and transcribed from, all three vectors but was only translated from the one which provided the correct translational frame. The positions and sizes, in kD, of protein molecular weight standards (M_r) are shown to the left of lane 1.

A


Vector
:
BS-KS
BAO
BA1
BA2
Enzyme
:
- R1 RV H N
- R1 RV H N
- R1 RV H N
- R1 RV H N
open circular
linear
super-coiled
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

B

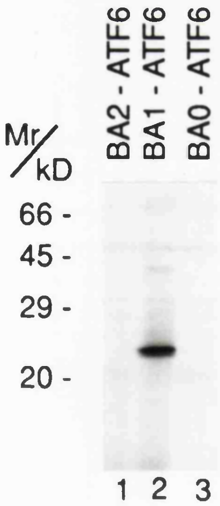
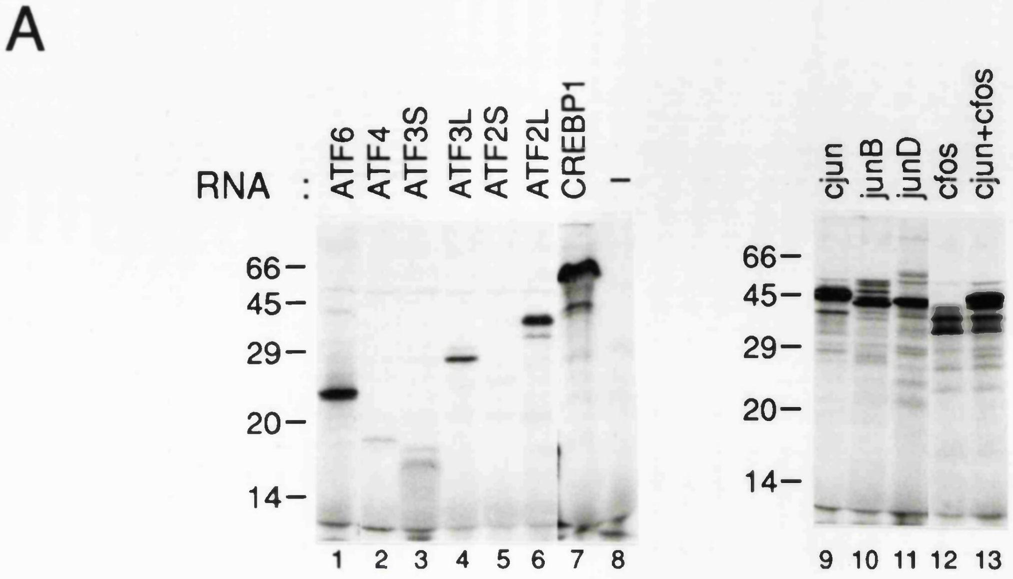

BA2 - ATF6
BA1 - ATF6
BA0 - ATF6
Mr/kD
66 -
45 -
29 -
20 -
1 2 3

FIG. 7.3 : In vitro *translated and co-translated ^{35}S-Met-labelled polypeptides*

A) SDS-PAGE of the indicated ^{35}S-Met-labelled *in vitro* translations (2 μl) ; lane 8 : the reticulocyte lysate was incubated with ^{35}S-metionine in the absence of any RNA. The positions and sizes, in kD, of protein molecular weight standards are shown to the left of each panel.

B) Table of vectors used to obtain the polypeptides shown in (A), observed and expected relative molecular weights (M_r), in kD. Vectors ATF2S, ATF2L, ATF3S and ATF3L were constructed by T.Hai. The following expected molecular weights were taken from the references listed in brackets : CREBP1 (Maekawa *et al.*, 1989), cJun (Ryseck *et al.*, 1988), ATF2L, ATF2S, ATF3L, ATF3S (Hai *et al.*, 1989) ; the others were calculated by multiplying the number of codons in each cDNA by the weighted average molecular weight of an amino acid, 0.126 kD (calculated from Harlow and Lane, 1988).

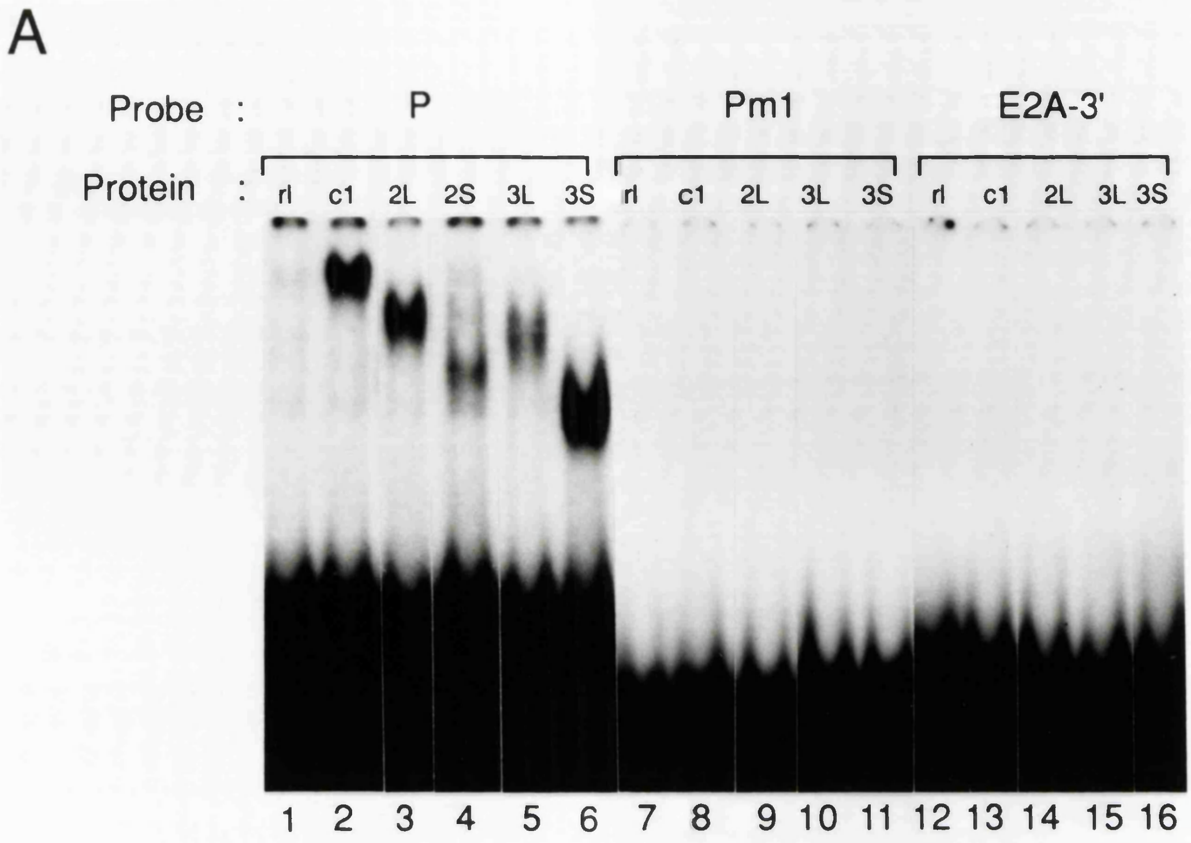

A
RNA :
ATF6
ATF4
ATF3S
ATF3L
ATF2S
ATF2L
CREBP1
–
66–
45–
29–
20–
14–
1 2 3 4 5 6 7 8
cjun
junB
junD
cfos
cjun+cfos
66–
45–
29–
20–
14–
9 10 11 12 13

B

cDNA Expression Vector	Approx. Observed Mr/kD	Expected Mr/kD
BA1-CREBP1	66	51.4
ATF2L	36	31
ATF2S	24	18
ATF3L	29	26
ATF3S	17	13
BA0-ATF4	19	19
BA1-ATF6	22	22
BS-cjun	44	35.9
BS-junB	40	43
BS-junD	42.6	42
BA1-cfos	32-37	37

FIG. 7.4 : *Binding of* in vitro *translated ATF proteins to probes P, Pm1 and E2A-3'*

Gel retardation of the indicated probes with the indicated *in vitro* translated polypeptides (rl = reticulocyte lysate unprogrammed with RNA, or programmed with RNA for : c1 = CREBP1, 2L = ATF2L, 2S = ATF2S, 3L = ATF3L, 3S = ATF3S, 4 = ATF4, 6 = ATF6 ; CREBP1 is a longer cDNA for ATF2, isolated from a human brain, rather than a HeLa cell, cDNA library). This figure is a composite of five experiments from which lanes that were not relevant have been omitted ; the following groups of lanes are from the same gels : A) 1-6, 7-16 : the two gels were run simultaneously but lanes 7-16 were exposed for three times as long as lanes 1-6, B) 1-3, 4-8, 9-14.


A
Probe :
P
Pm1
E2A-3'
Protein :
rl c1 2L 2S 3L 3S
rl c1 2L 3L 3S
rl c1 2L 3L 3S
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

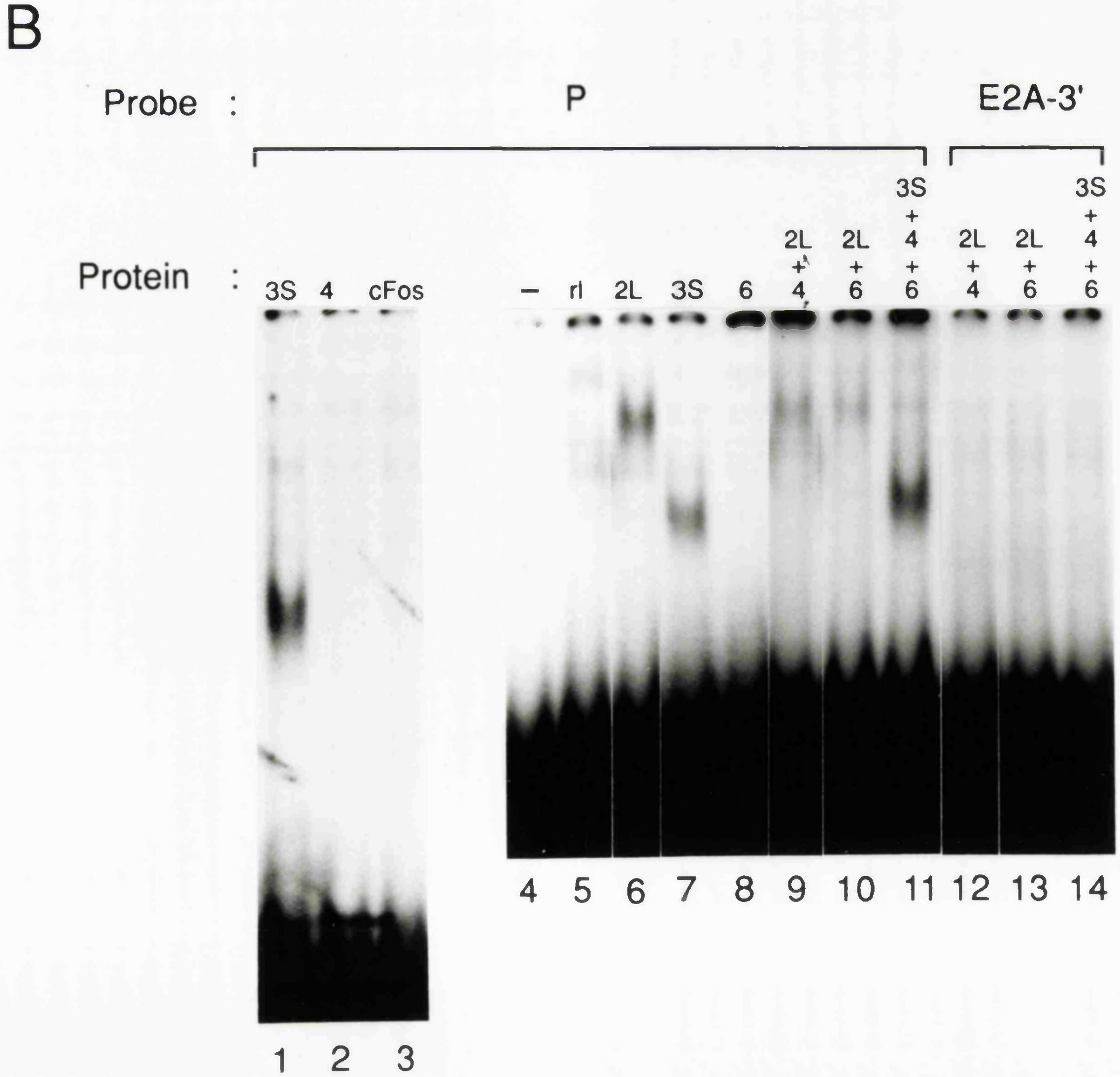

B
Probe :
P
E2A-3'
Protein :
3S 4 cFos
– rl 2L 3S 6 2L + 4 2L + 6 3S + 4 + 6
2L + 4 2L + 6 3S + 4 + 6
1 2 3
4 5 6 7 8 9 10 11 12 13 14

FIG. 7.5 : *No ATF polypeptides bind to the E2A-3' probe*

Gel retardation of the E2A-3' probe with the indicated *in vitro* co-translated polypeptides. Lane 2 contained F9 EC whole cell extract as a positive control for binding ; lane 3 contained retliculocyte lysate incubated in the absence of RNA, as a negative control.

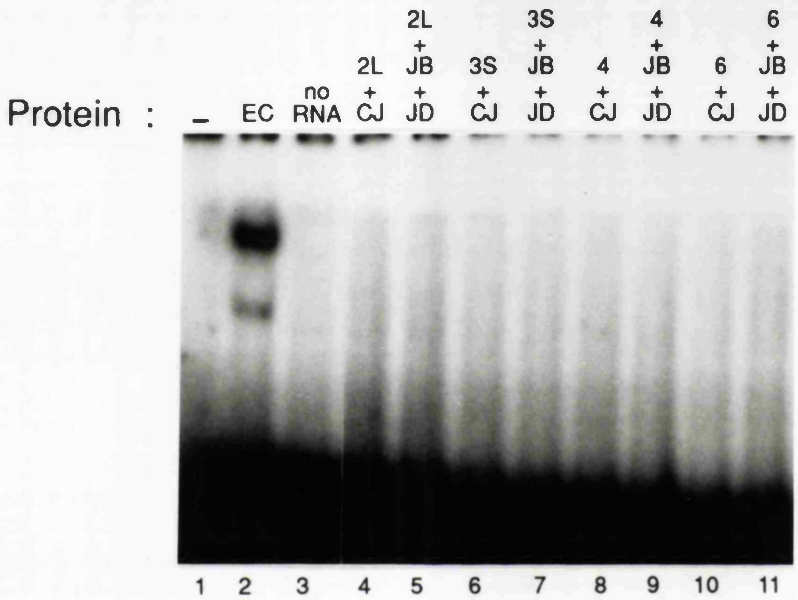

Protein :
–
EC
no RNA
2L + CJ
2L + JB + JD
3S + CJ
3S + JB + JD
4 + CJ
4 + JB + JD
6 + CJ
6 + JB + JD
1
2
3
4
5
6
7
8
9
10
11

FIG. 7.6 : *Binding of* in vitro *translated ATF homodimers and ATF2/Jun heterodimers to probes P and Pm2*

A) Gel retardation of probe P with *in vitro* translated cJun (cJ) and CREBP1 (C1) homodimers and the intermediate mobility co-translated CREBP1/cJun (C1+cJ) heterodimer.

B) Gel retardation of the indicated probes with the indicated *in vitro* translated polypeptides (3S = ATF3S, C1 = CREBP1, 2S = ATF2S, 2S+JD = ATF2S and JunD co-translated) (lanes 1, 3, 5, 7, 8, 9, 11, 13 : 4 μl ; lanes 2, 4, 6, 10, 12, 14 : 2 μl). The arrows indicate the JunD and ATF2S homodimers (JD/JD, 2S/2S) and the intermediate mobility ATF2S/JunD (2S/JD) heterodimer. All lanes are from the same experiment.

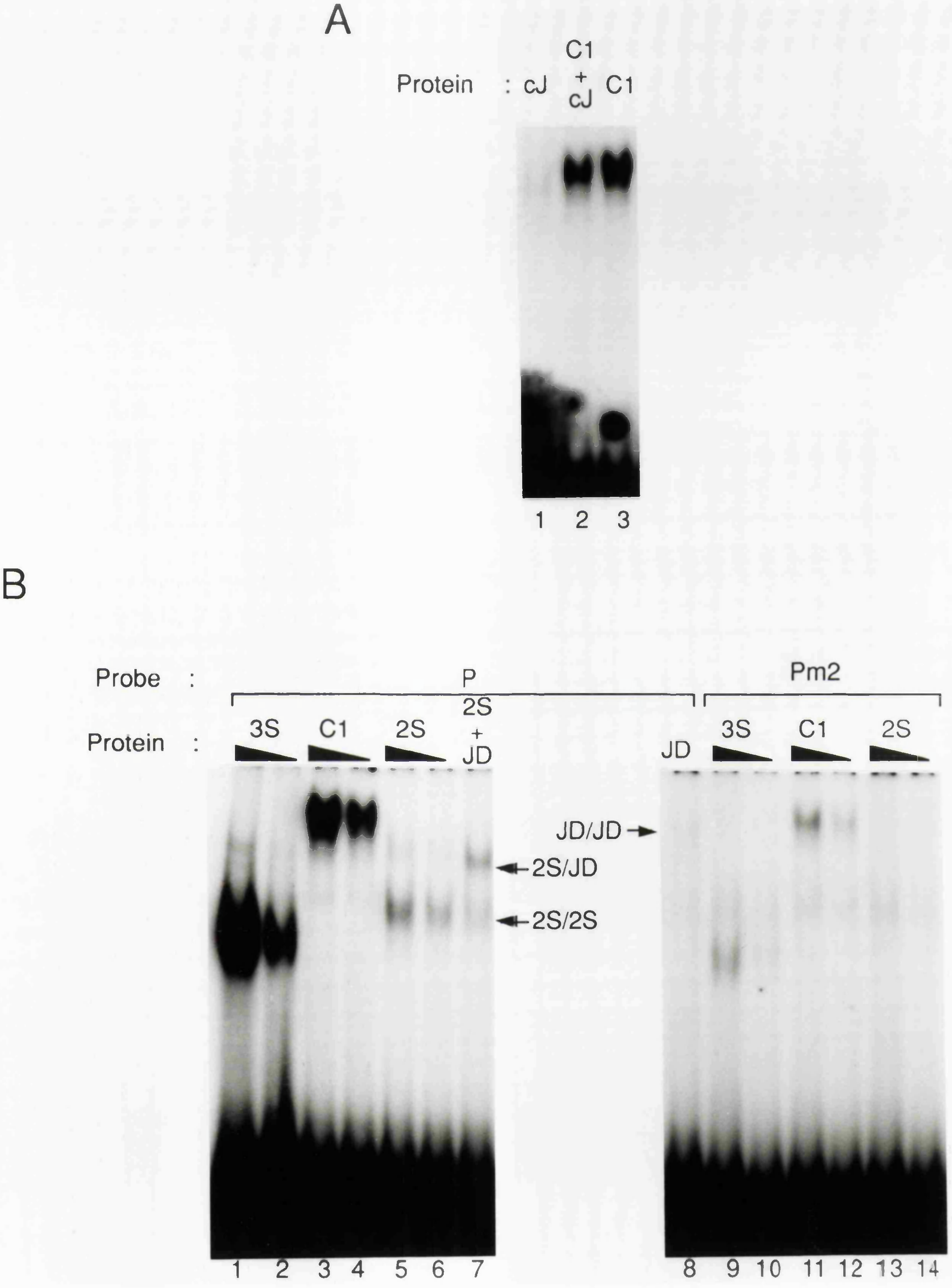

A
Protein : cJ C1 + cJ C1
1 2 3
B
Probe :
P
Pm2
Protein :
3S
C1
2S
2S + JD
JD
3S
C1
2S
JD/JD
2S/JD
2S/2S
1 2 3 4 5 6 7
8 9 10 11 12 13 14

FIG. 7.7 : *Dimerization controls DNA binding specificity*

DNA binding of the indicated *in vitro* co-translated dimers to the indicated probes. All lanes in (A) are from the same experiment, as are all lanes in (B).

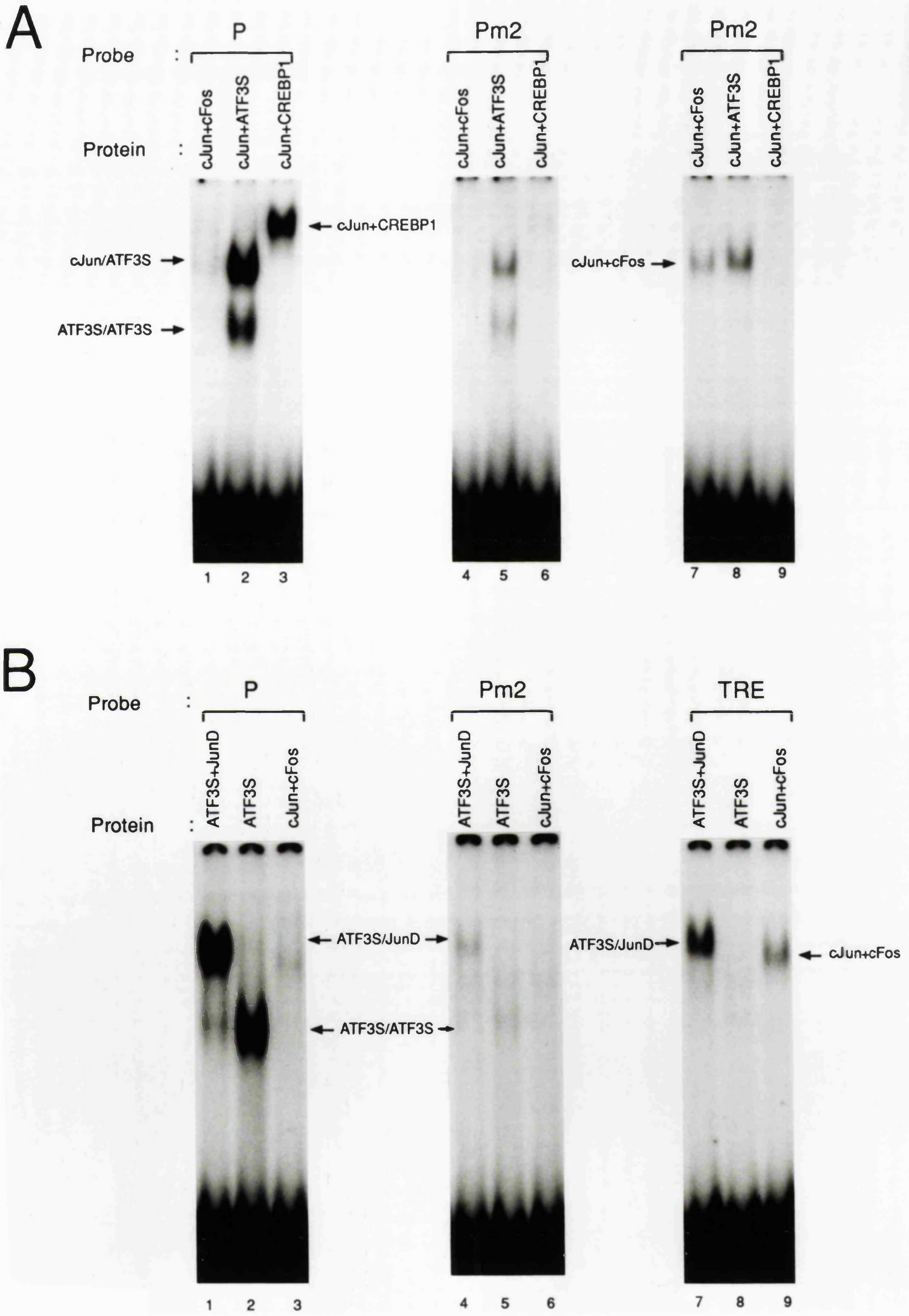

A
Probe :
P
Pm2
Pm2
Protein :
cJun+cFos
cJun+ATF3S
cJun+CREBP1
cJun+cFos
cJun+ATF3S
cJun+CREBP1
cJun+cFos
cJun+ATF3S
cJun+CREBP1
cJun+CREBP1
cJun/ATF3S
ATF3S/ATF3S
cJun+cFos
1
2
3
4
5
6
7
8
9
B
Probe :
P
Pm2
TRE
Protein :
ATF3S+JunD
ATF3S
cJun+cFos
ATF3S+JunD
ATF3S
cJun+cFos
ATF3S+JunD
ATF3S
cJun+cFos
ATF3S/JunD
ATF3S/ATF3S
ATF3S/JunD
cJun+cFos
1
2
3
4
5
6
7
8
9

FIG. 7.8 : *Regulation of possible ATF3 dimers during F9 EC cell differentiation*

This cartoon illustrates the levels of some ATF and AP1 family proteins in F9 EC and PE cells, possible homo- and hetero-dimers and their DNA binding specificity.

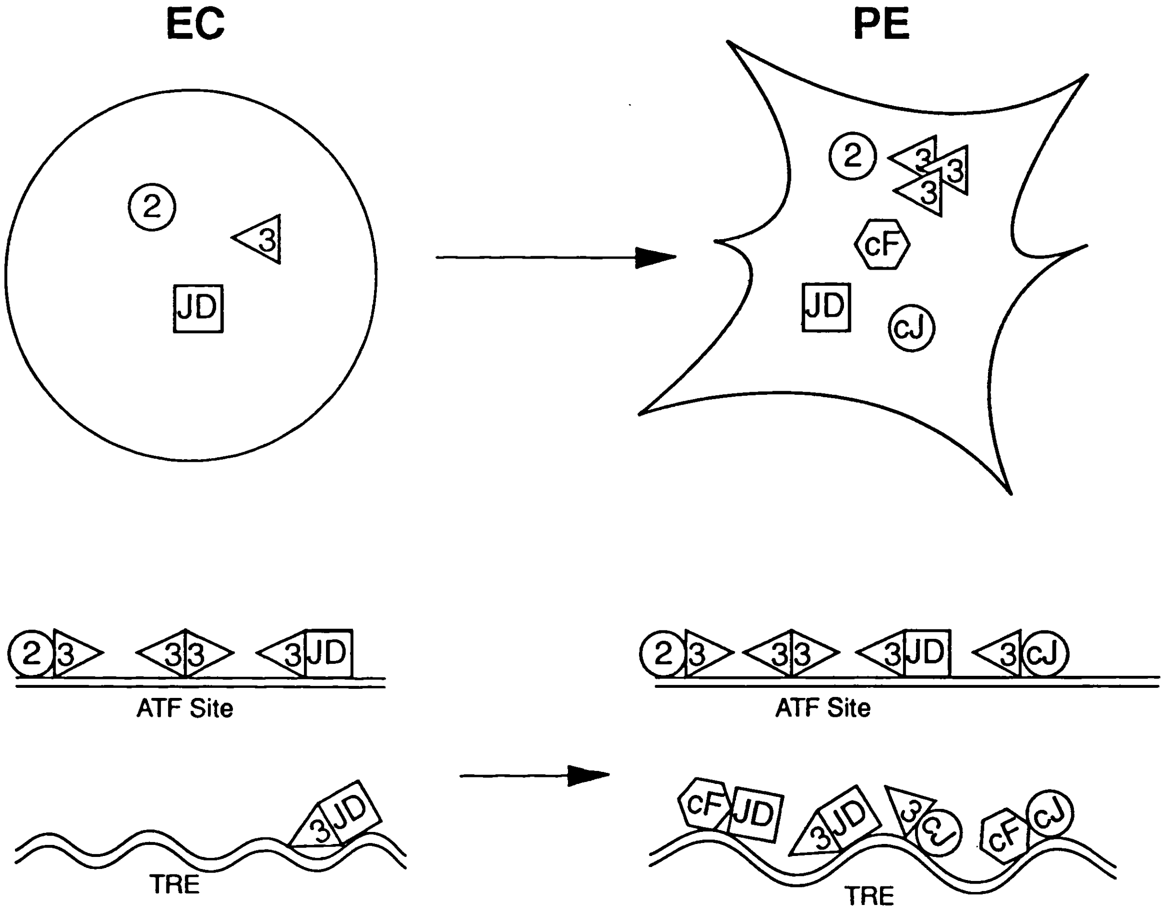
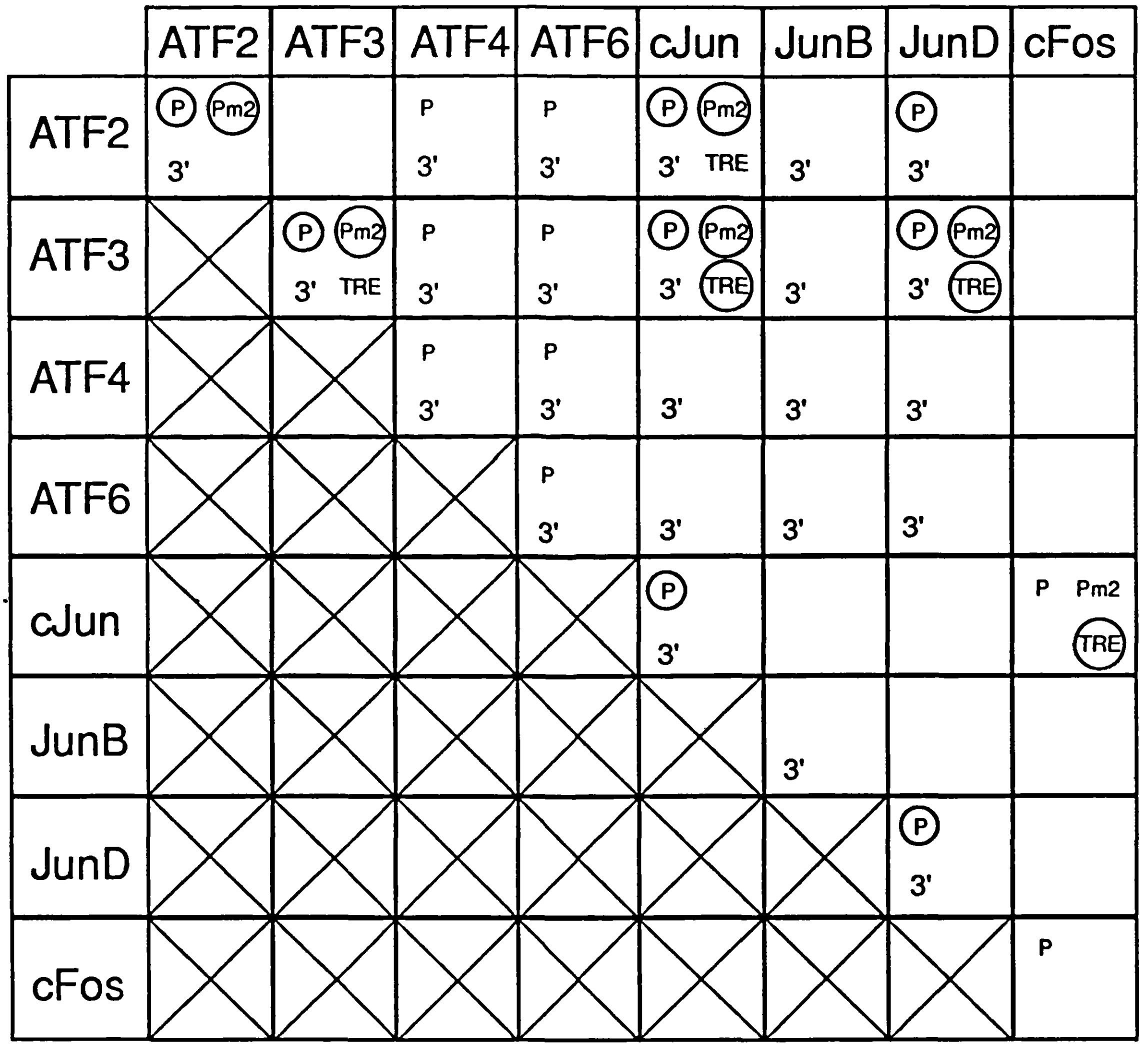

EC
PE
2
3
JD
2
3
3
3
cF
JD
cJ
2 3 33 3JD
ATF Site
2 3 33 3JD 3cJ
ATF Site
3JD
TRE
cF JD 3JD 3cJ cF cJ
TRE

TABLE 7.1 : *Summary of DNA binding specificities of* in vitro *translated polypeptide complexes*

Probe names listed in boxes indicate that the corresponding translated or co-translated polypeptides were tested for binding to these probes ; probe names in circles indicate that they bound the relevant dimer.

DNA Binding of In Vitro Translated Polypeptides



	ATF2	ATF3	ATF4	ATF6	cJun	JunB	JunD	cFos
ATF2	(P) (Pm2) 3'		P 3'	P 3'	(P) (Pm2) 3' TRE	3'	(P) 3'	
ATF3	☒	(P) (Pm2) 3' TRE	P 3'	P 3'	(P) (Pm2) 3' (TRE)	3'	(P) (Pm2) 3' (TRE)	
ATF4	☒	☒	P 3'	P 3'	3'	3'	3'	
ATF6	☒	☒	☒	P 3'	3'	3'	3'	
cJun	☒	☒	☒	☒	(P) 3'			P Pm2 (TRE)
JunB	☒	☒	☒	☒	☒	3'		
JunD	☒	☒	☒	☒	☒	☒	(P) 3'	
cFos	☒	☒	☒	☒	☒	☒	☒	P

CHAPTER 8

GENERATION AND CHARACTERISATION OF ANTI-PEPTIDE POLYCLONAL ANTIBODIES AGAINST MEMBERS OF THE ATF AND AP1 TRANSCRIPTION FACTOR FAMILIES

8.1 Introduction

The next stage in this study involved preparing antibodies against particular ATF proteins : these would be useful reagents for three purposes. Firstly, they would allow a direct study of the regulation of ATF protein levels during F9 EC cell differentiation. Secondly, they should, in principle, provide a means of relating the ECRE activities to specific ATF proteins through a study of the effects of anti-ATF antibodies on the ECRE/DNA complexes. As shown elsewhere in this study (Chapter 10) and by others (Hay *et al.*, 1989 ; Huang and Hearing, 1989), antibodies that react with a given DNA binding protein will often inhibit DNA binding or further retard the protein/DNA complex in a gel retardation assay. Thirdly, they could be used to study *in vivo* dimerization. The analysis of *ATF* RNA levels during the differentiation of F9 EC cells (Chapter 6) combined with the study of the DNA binding properties of *in vitro* translated ATF polypeptides (Chapter 7) suggested that two ATF species should be studied further at the protein level : namely, ATF2, whose RNA levels remain constant during differentiation, and ATF3, whose RNA levels are higher in PE cells. ATF2 and ATF3 were the only members of the family that were both expressed in F9 cells and bound efficiently to the ATF site *in vitro*. Finally, both polypeptides were also of intrinsic interest because of their heterodimerization properties. ATF2/ATF3 dimers bind to the ATF site (Hai *et al.*, 1989) but heterodimerization with members of the AP1 transcription factor family alters or broadens the DNA binding specificity of the constituent proteins (Chapter 7). Furthermore, cJun and JunD, which both can dimerize with ATF2 and ATF3 *in vitro*, differ in their RNA regulation during F9 EC cell differentiation : c*jun* RNA is detectable only in PE cells, while the levels of *jun*D are identical in both EC and PE cells. Thus, antibodies against ATF2, ATF3 and Jun proteins should allow a study of *in vivo* heterodimerization and its regulation during F9 EC cell differentiation.

8.2 Choice of peptide immunogens

It was therefore decided to raise antibodies against ATF2 and ATF3. However, in order to avoid cross-reactivity to the similar DNA binding domains of other ATF proteins, it was considered advisable to choose potential epitopes outside this region.

A computer-assisted prediction of hydropathic (Kyte and Doolittle, 1982) and hydrophilic (Hopp and Wood, 1983) regions in ATF2 and ATF3 was performed, since it is thought that hydrophilic regions are immunogenic, based on the general belief that they are exposed to water and therefore also at the surface of the protein. Not surprisingly, the most hydrophilic region in both molecules was in the conserved highly charged basic domain (Fig.8.1). Two other regions were therefore chosen, from which the peptides ATF2D and ATF3C were derived, based on their relatively high hydrophilicity value (Fig.8.1) and their position, flanking the DNA binding domain (Fig.8.2A). It was speculated that this might make them accessible to antibodies since they would be adjacent to the two α-helices of the basic region and the "leucine zip" (Talanian *et al.*, 1990 ; Shuman *et al.*, 1990 ; Patel *et al.*, 1990), and therefore relatively near the surface of the protein that was exposed and available for DNA binding. The lack of regions of significant amino acid similarity among other members of the ATF and AP1 families was checked by a computer-assisted comparison, which showed that no more than three consecutive amino acids of the peptide were identical in any of these related proteins (Fig.8.2B and C).

For a comparative study of AP1 family proteins, peptides from the basic domain (PEP1) and a region near the middle of the amino acid sequence (PEP2) of cJun, as well as a peptide from the DNA binding domain of cFos (P10), were also used for immunisation. Previously, PEP1 and PEP2 had been successfully used by Bohmann *et al.* (1987) to obtain rabbit antisera that recognized affinity purified human AP1, as well as *in vitro* translated and bacterially expressed vJun. Anti-PEP1 sera were expected to cross-react with JunB and JunD, due to the similar amino acid sequences of their DNA binding domains, while anti-PEP2 sera were expected to be cJun-specific, as the PEP2 region lies outside the DNA binding domain (Fig.8.3). P10 had also been successfully used by Adamkiewicz *et al.* (1990) to obtain rabbit antisera that recognized native *in vitro* translated Fos and inhibited DNA binding of HeLa cell AP1. Anti-P10 serum might also be expected to cross-react with ATF3, given that the ATF3 DNA binding domain is the most similar to that of cFos among all members of the ATF/AP1 families (Fig.8.4). The positions of the chosen peptide epitopes are schematically summarized in Fig.8.5.

8.3 Immunisation

All peptides were coupled to keyhole limpet haemocyanin, and used to immunise rabbits subcutaneously, as described in Chapter 2. Pre-immune sera are referred to by the rabbit identification number (see Table 8.1) preceded by "R" (for example, the pre-immune serum from rabbit 42 is referred to as serum R42). Sera from test bleeds were numbered with the rabbit identification number to which a last digit was added, indicating the serial number of the test bleed (for example, serum from the first bleed of rabbit 42 is identified as serum 421).

8.4 ELISAs with immobilised peptides

The specificity of the rabbits' immune response was first assayed by testing the sera against immobilised peptide in an ELISA. As shown in Fig.8.6, each immune serum recognized only the peptide against which it had been raised, and none of the pre-immune sera showed any anti-peptide reactivity.

8.5 Immunoblotting of *in vitro* translated polypeptides

Each serum was then tested for reactivity against the denatured *in vitro* translated polypeptide which contained the peptide used as immunogen. As a control for antigen specificity, reactivity with *in vitro* translated ATF1S was used in all cases. As can be seen in Fig.8.7, sera from rabbits immunised with cFos-derived P10 reacted specifically only with a polypeptide (indicated by arrows) that was only present in a cFos *in vitro* translation and not in an ATF1S *in vitro* translation (compare lanes 2 to 1 and 5 to 4, and lanes 3 to 2, 6 to 5). Sera from rabbits immunised with cJun-derived PEP2, on the other hand, recognized *in vitro* translated cJun (indicated by arrows) but not ATF1S (Fig.8.7 : compare lanes : 26 to 25 and 29 to 28, 27 to 26 and 30 to 29). The high background of cross-reacting bands in the reticulocyte lysate is presumably due to the high amounts of lysate (200-300 mg per lane) and serum (1/20 dilution) that were used, the long incubation time (overnight) and the fact that the sera were not purified. It is clear, however, that the above sera recognized their cognate antigens above this high background, since the bands indicated by arrows were present only in reticulocyte lysate that had been programmed with the appropriate *in vitro* transcribed RNA rather than *ATF1*S RNA. Unfortunately, none of the sera raised against ATF2D, ATF3C or PEP1 were able to specifically recognize denatured *in vitro* translated ATF2, ATF3 or cJun, respectively (Fig.8.7 : compare lanes : 9 to 8 and 12 to 11, 15 to 14 and 18 to 17, 21 to 20 and 24 to 23), despite the fact that they did specifically react with their cognate peptides (see Fig.8.6). Later test sera likewise did not show any reactivity either (not shown). This lack of reactivity could be due either to the sensitivity of the antibodies, since only small amounts of antigen were present in the *in vitro* translation reactions, or to denaturation-sensitive epitopes. However, when the *in vitro* translated polypeptides were labelled by ^{35}S-methionine incorporation, thus enhancing detection, and used in the native state, they were all recognized by the appropriate sera (see Section 8.6).

In conclusion, only anti-P10 and anti-PEP2 sera could react, in an immunoblotting experiment, with the denatured *in vitro* translated polypeptides which contained the P10 and PEP2 sequences, respectively.

8.6 Immunoprecipitation of ^{35}S-labelled *in vitro* translated polypeptides

Detection of *in vitro* translated polypeptides was improved when these were labelled by ^{35}S-methionine incorporation and used in an immunoprecipitation assay. Indeed, ATF2, either as ATF2L or as the longer *ATF2* cDNA product CREBP1, could be immunoprecipitated specifically by an anti-ATF2D serum but not by the corresponding pre-immune serum (Fig.8.8A : compare lanes 6 to 5 and 12 to 11 ; Fig.8.9A : compare lanes 6 to 5) ; an unrelated immune serum had the same effect as the pre-immune (Fig.8.8A : compare lanes 7, 8 and 5 to lane 6 ; Fig.8.9A : compare lanes 11-14 and 5 to lane 6). The immunoprecipitation was specific for ATF2 since when ATF3 was used as the antigen in the same experiment, only background bands were observed (Fig.8.8 : compare lane 9 in panel A to 15 in panel B). It was clear, nevertheless, that the efficiency of immunoprecipitation was low (Fig.8.8A : the band in lane 6 results from immunoprecipitation of three times the amount of neat antigen loaded in lane 2) when compared with that of anti-ATF3C sera (Fig.8.8B : the band in lane 16 results from immunoprecipitation of three times the amount of neat antigen loaded in lane 10), and that not all animals immunised with ATF2D were capable of immunoprecipitating *in vitro* translated ATF2 (Fig.8.9A : compare lanes 4, 8 and 10 to lane 6 ; the first test serum from animal 1034 also seemed to be reacting weakly with ATF2L - subsequent test sera from this animal were not tested and therefore it could not be confirmed that this was a specific response). All anti-ATF2D sera were, however, capable of detecting with equal efficiency a cluster of ATF2 proteins in cell extracts, both by immunoblotting and by immunoprecipitation (see Chapter 9). This "inconsistency" may be due to post-translational modifications of the peptide region occurring in the reticulocyte lysate but not in the cell, or association with endogenous reticulocyte lysate proteins that prevented the antibody from recognizing its epitope. Conversely, the epitope may adopt a non-reactive conformation in the *in vitro* translated polypeptide but may be exposed in the cellular protein polypeptide *via* a post-translationally induced conformational change. Potential phosphorylation sites for protein kinase C (PKC) and casein kinase II (CKII) in or around the immunogen peptide sequences in ATF2 (PKC : S^{464} ; CKII : S^{437}, S^{466}) and ATF3 (PKC : S^{109} ; CKII : S^{101}, T^{107}, T^{118}) were in fact identified by a computerized search for modification sites.

All anti-ATF3C sera, on the other hand, specifically immunoprecipitated ATF3 as compared to the corresponding pre-immune sera (Fig.8.9B : compare lanes 4 to 3, 6 to 5, 8 to 7, and 10 to 9) or an unrelated immune serum (Fig.8.9B : compare lane 11 to lane 10). The immunoprecipitating efficiency of the antiserum increased with successive booster immunisations (Fig.8.9B : compare lane 7 (third test bleed) to lane 6 (second test bleed)). Immunoprecipitation by anti-ATF3C sera was also antigen-specific since they failed to immunoprecipitate ATF2 (Fig.8.8B : lanes 4 and 8). An anti-P10 serum, directed against the cFos DNA binding domain, immunoprecipitated ATF3 (Fig.8.8B : compare lane 14 to lane 13) as well as cFos (Fig.8.8B : compare lane 14 to lane 12) and with the same efficiency as an anti-ATF3C serum (Fig.8.8B : compare lane 14 to lane 16). This was expected, since the ATF3 DNA binding domain is very similar to that of cFos (see Fig.8.4). Anti-ATF3C serum, on the other hand, did not cross-react with cFos (Fig.8.8B : lane 17) confirming the lack of cFos/ATF3 similarity outside the DNA binding domain.

Both anti-P10 sera immunoprecipitated cFos specifically when compared to the corresponding pre-immune sera (Fig.8.10 : compare lanes 4 to 3, 6 to 5) or a serum raised against a similar region of cJun (Fig.8.10 : compare lanes 4 and 6 to lane 7). This reaction was antigen-specific, since neither ATF2 nor cJun were recognized by an anti-P10 serum (Fig.8.10 : lanes 8 and 18).

Both anti-PEP2 sera immunoprecipitated cJun specifically when compared to the corresponding pre-immune sera (Fig.8.10 : compare lanes 11 to 10, 13 to 12) or a serum raised against the DNA binding domain of cFos (Fig.8.10 : compare lanes 11 and 13 to lane 18). Both anti-PEP2 sera cross-reacted with JunD (Fig.8.10 : compare lanes 31 to 30, 33 to 32), though not with JunB (Fig.8.10 : compare lanes 24 to 23, 26 to 25). This was unexpected, since PEP2 is not in the conserved DNA binding domain. However, when a computer-assisted comparison was performed, JunD, but not JunB, was seen to contain a sequence showing extensive similarity (9/11 identical amino acids, compared with 4/11 for JunB) to PEP2 (see Fig.8.3), which may explain its cross-reactivity with anti-PEP2.

Only one of the anti-PEP1 sera, 333, immunoprecipitated cJun specifically (Fig.8.10 : compare lane 15 to 14), though they both reacted against immobilised PEP1 peptide in an ELISA assay (Fig.8.6). 333 also immunoprecipitated JunD (Fig.8.10 : compare lane 29 to lane 28), but not JunB (Fig.8.10 : compare lane 22 to lane 21), or the unrelated ATF3 (Fig.8.10 : lane 19 ; the background levels of ATF3L present in this lane are not higher than those in lane 5 of Fig.8.9, where a pre-immune serum was used). Cross-reactivity with JunD but not JunB is presumably due to the longer uninterrupted sequences of amino acid identity between JunD and PEP1 than between JunB and PEP1 (Fig.8.3).

In conclusion, in an immunoprecipitation assay, at least one of the sera raised against each peptide specifically recognized the polypeptide from which the peptide sequence was derived. All of the observed cross-reactions could be explained by the presence of similar amino acid sequences in the cross-reacting polypeptides.

8.7 Effects of antisera on antigen/DNA complexes

The antisera that were capable of immunoprecipitating should also be able to react with the native antigen in a gel retardation assay. Depending on the epitope they recognize, they should either inhibit the formation of the antigen/DNA complex, by interfering with the antigen's ability to bind DNA, or retard that complex even further, by forming an antibody-antigen/DNA complex .

Indeed, both anti-ATF3C sera were able to "shift the shift" when incubated with ATF3 and its DNA recognition site, the P oligonucleotide, as compared to the corresponding pre-immune sera (Fig.8.11A : compare lanes 3 to 2, 5 to 4) or an anti-ATF2D serum (Fig.8.11A : compare lanes 3 and 5 to 6). This "super-shift" was abolished when ATF3 was incubated with antibody in the presence of ATF3C, but not ATF2D, peptide (Fig.8.11A : compare lanes 12 and 13 to lane 11). Consistent with the immunoprecipitation experiments, anti-P10 could also react with ATF3 : in fact, and as expected, anti-P10, which recognizes the DNA binding domain, inhibited the ATF3/DNA complex (Fig.8.11A : compare lane 9 to lane 8). In contrast, anti-ATF3C, which reacts with a region outside the DNA binding domain, did not inhibit DNA binding but rather, by reacting with ATF3, caused a larger antibody-ATF3/DNA complex which migrated more slowly than the ATF3/DNA complex. This reaction was antigen-specific, since anti-ATF3C had no effect on an ATF2/DNA complex (Fig.8.11B : compare lane 9 to lane 5).

Under the same conditions though, no effect was seen with anti-ATF2D on an ATF2/DNA complex (Fig.8.11B : compare lanes 7 to 6, 12 to 11) ; this lack of effect did not respond to titration (not shown). When, however, the binding reaction was carried out in immunoprecipitation buffer, rather than "Manley C" (Manley *et al.*, 1980) buffer used in all previous gel retardation experiments, the anti-ATF2D sera responded as predicted from the immunoprecipitation experiments. Namely, anti-ATF2D serum 423 "shifted" the ATF2/DNA complex to a slower mobility complex (Fig.8.12 : compare lanes 8 to 7, 13 to 12). This effect was specific since it could be inhibited by excess ATF2D, but not ATF3C, peptide (Fig.8.12 : compare lane 8 to lanes 9 and 10). The efficiency of this "super-shifting" was lower than that of anti-ATF3C sera 433 or 443 on the ATF3/DNA complex (see Fig.8.11), reflecting the relative efficiencies of immunoprecipitation previously observed. Similarly, consistent with the inability of anti-ATF2D serum 314 to immunoprecipitate ATF2, its small inhibitory effect on the ATF2/DNA complex was non-specific since it was not affected by the presence of ATF2D peptide (Fig.8.12 : compare lanes 5 and 6 to lane 4). The same was true of an unrelated anti-PEP2 serum (Fig.8.12 : compare lanes 16 and 17 to lane 15).

Anti-P10, anti-PEP1 and anti-PEP2 sera also interfered with the binding of a cJun/cFos complex to DNA whether the proteins were *in vitro* translated (Fig.8.13 : compare lanes 4 to 3, 6 to 5, 8 to 7) or present in the AP1 complex from NIH 3T3 cells (Fig.8.13 : compare lanes 11 to 10, 13 to 12, 15 to 14).

Finally, it was also apparent that sera which recognize their antigen in a given dimer may not neccessarily do so when the dimerization partner is altered. For example, anti-P10 inhibited ATF3 binding to DNA (Fig.8.14 : compare lane 14 to 13) but had no such effect on co-translated ATF3/JunD (Fig.8.14 : compare lane 11 to 10) or co-translated ATF3/CREBP1 heterodimers (Fig.8.14 : compare lane 8 to 7). Both anti-ATF2 and anti-ATF3 sera, on the other hand, "super-shifted" an ATF2/ATF3 heterodimer (Fig.8.14 : compare lanes : 4 to 3, 6 to 5) as they did ATF2 or ATF3 homodimers (see above). This difference may have arisen from the unequal stabilities of different dimers. The ATF3/CREBP1 and ATF3/JunD heterodimers, for example, were presumably more stable than the ATF3/ATF3 homodimer and prevented the anti-P10 antibodies from reaching their epitope which is in the basic domain, adjacent to the dimerization domain. Anti-ATF2D and anti-ATF3C, on the other hand, react with epitopes outside the DNA binding domain and for this reason did not disrupt the protein/DNA complexes but rather "super-shifted" them.

In conclusion, all antisera capable of immunoprecipitating were also able to interact with their antigens in a gel retardation assay, causing either an inhibition of antigen/DNA complexing or a "super-shift" of the antigen/DNA "shift".

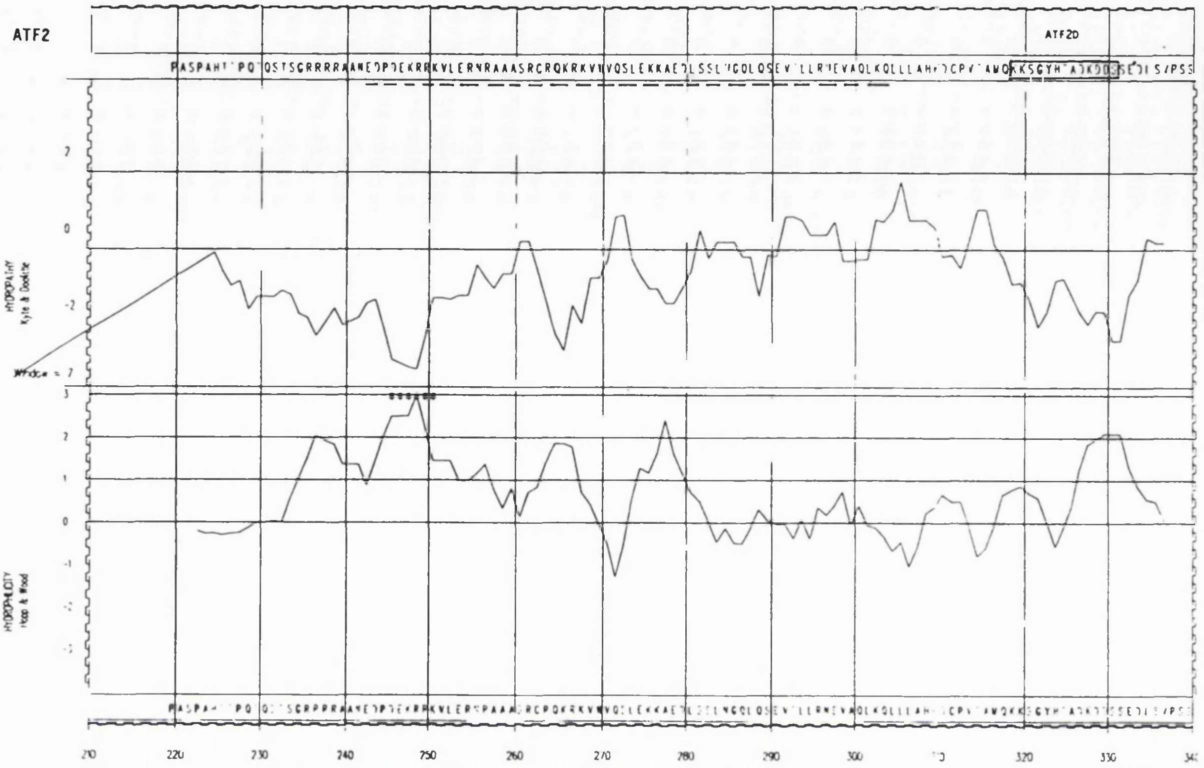
8.8 Discussion

The peptide immunisation strategy was successful in producing antisera that recognized the polypeptides from which the immunogen peptide sequences were derived, in at least two different assays, as summarized in Table 8.1. It was also successful in avoiding extensive cross-reactivity with *in vitro* translated polypeptides other than the parent one, unless such cross-reactivity could be explained by extensive amino acid similarity. Finally, that the antisera reacted with the native polypeptides should allow them to be used for the study of protein levels during differentiation of F9 EC cells and the investigation of the relation of ECRE activities to ATF proteins.

FIG. 8.1 : *Hydropathy and hydrophilicity plots of regions within ATF2 and ATF3*

Computer-generated hydropathy and hydrophilicity plots of ATF2 and ATF3 regions. The DNA binding domains are underlined ; the peptide antigen sequences are boxed.

ATF2


ATF2D
PASPAH PQ QSTSGRRRRAANEDPDEKRRKVLERNRAAASRCRQKRKVWVQSLEKKAEDLSSLNGQLQSEVTLLRNEVAQLKQLLLAHKDCPVTAMQKKSGYHTADKDDSSEDISVPSS
HYDROPATHY
Kyte & Doolittle
Window = 7
2
0
-2
HYDROPHILICITY
Hopp & Wood
3
2
1
0
-1
-2
-3
210
220
230
240
250
260
270
280
290
300
310
320
330
340

ATF3

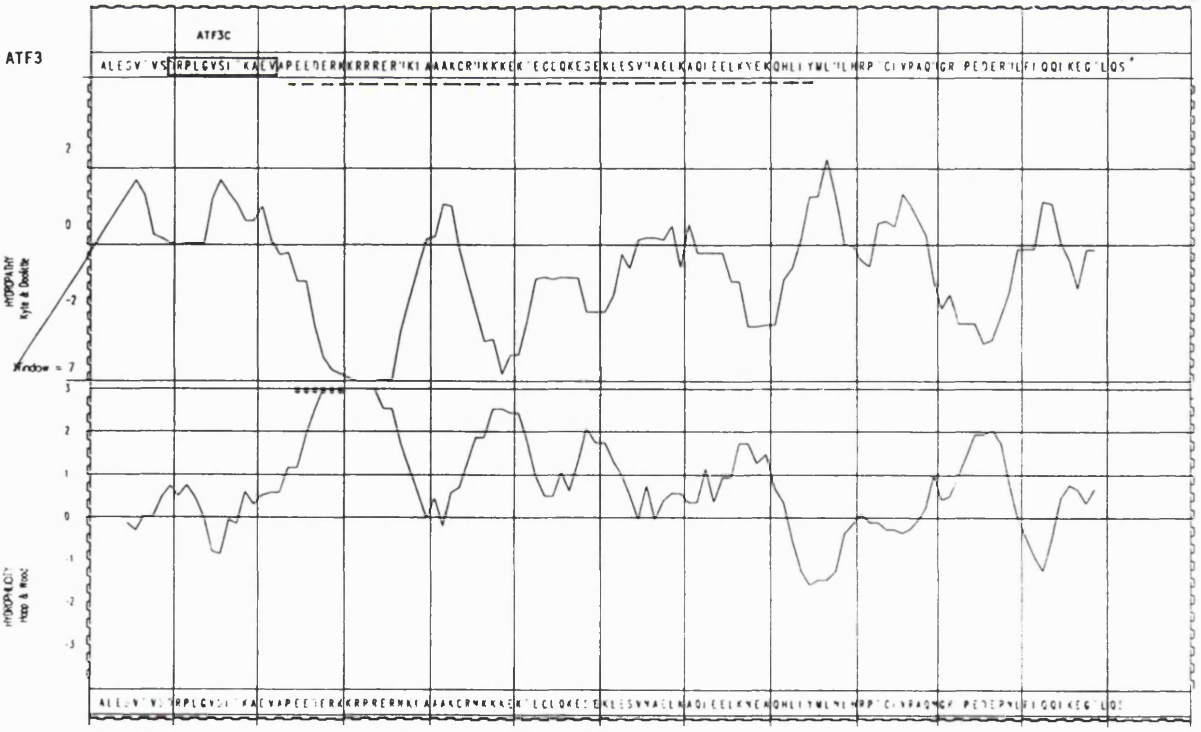

ATF3C
ALESVTVSIRPLGVSITKAEVAPEEDERKKRRRERNKIAAAKCRNKKKEKTECLQKESEKLESVNAELKAQIEELKNEKQHLIYMLNLHRPTCIVRAQNGRTPEDERNLFIQQIKEGTLQS*
HYDROPATHY
Kyte & Doolittle
Window = 7
2
0
-2
HYDROPHILICITY
Hopp & Wood
3
2
1
0
-1
-2
-3
100
110
120
130
140
150
160
170
180
190
200
210
220
230

FIG. 8.2 : *The ATF2D and ATF3C sequences are unique*

A) Alignment of ATF2 and ATF3 amino acid sequences (incomplete, due to partial cDNAs). Similarity between amino acids is indicated, in decreasing order, by a vertical line, two dots, one dot. Gaps have been introduced in order to maximize alignment of similar sequences. The broken line indicates the DNA binding domain. The antigen peptide sequences are underlined and in bold.

B) Alignment of ATF2D peptide with other ATF/AP1 family members. Identical amino acids are indicated by a single dot. (h) = human, (m) = mouse.

C) Alignment of ATF3C peptide with other ATF/AP1 family members. Identical amino acids are indicated by a single dot. (h) = human, (m) = mouse.

(Sequences from : CREB : Berkowitz and Gilman, 1990 ; ATF2, ATF3, ATF4, ATF6 : Hai *et al.*, 1989 ; cJun : Ryseck *et al.*, 1988 ; JunB : Ryder *et al.*, 1988 ; JunD : Ryder *et al.*, 1989 ; cFos : Van Beveren *et al.*, 1983, and Meijlink *et al.*, 1985).

A) Alignment of ATF2 and ATF3 amino acid sequences

```
ATF2 101 PIVPVPGPFPLLLHLPSGQTMPVAIPASITSSNVHVPAAVPLVRPVTMVP 150
                    :   ||:.. :|. ..| .: .  .|.:|| :     .|
ATF3   1 ..........EFRAHPSARAPAVSPRCSPAGQTNSPPDPVPTL....AAP 36
                  .         .         .         .         .

     151 SVPGIPGPSSPQPVQSEAKMRLKAALTQQHPPVTNGDTVKGHGSGLVRTQ 200
         |  :  ::|.|.|. : :    ...:.:.. ||. |. | :. ..|..
      37 SELEQNDASTPRPGLCLG....SECFCHRPLPVPPGSLVFEDFANLTPFV 82
                  .         .         .         .         .
                                                   --------
     201 SEESRPQSLQQPATSTTETPASPAHTTPQTQSTSGRRRRAANEDPDEKRR 250
         .|| |  .:|..  :   ..| .. |..: . . :  : ....:.||:::
      83 KEELRF.AIQNKHLCHRMSSALESVTVSDRPLGVSITKAEVAPEEDERKK 131

                                     [   ATF3C   ]


                           DNA binding domain
         --------------------------------------------------
     251 KVLERNRAAASRCRQKRKVWVQSLEKKAEDLSSLNGQLQSEVTLLRNEVA 300
         :  |||: ||.:||.|:| ..::|:|..|.|.|:|::|..::. |:|| .
     132 RRRERNKIAAAKCRNKKKEKTECLQKESEKLESVNAELKAQIEELKNEKQ 181

                           [   ATF2D   ]
         ----
     301 QLKQLLLAHKDCPVTAMQKKSGYHTADKDDSSEDISVPSSPHTEAIQHSS 350
         :|  :|  |:...:.          |:.: ..||      .:.  ||: .
     182 HLIYMLNLHRPTCIV.........RAQNGRTPED......ERNLFIQQIK 216

     351 VSTSNGVSS 359
          :| .:
     217 EGTLQS... 222
```

B) Alignment of ATF2D peptide with other ATF/AP1 family members

```
(h)ATF2  319 KKSGYHTADKDDS 331
                    .   .
(h)CREB  237 SNQVVVQAASGDV 249
             . .
(h)ATF3  157 KESEKLESVNAEL 169
             ..     .
(h)ATF4   25 KKRAEQEALTGEC 37
               .      .
(h)ATF6   16 RESACQSRKKKKE 28
                    ..   .
(m)cJun   46 QSMTLNLADPVGS 58
               . . ..
(m)JunB  201 TGSSYPTATISYL 213
             . .    .    .
(m)JunD  149 KQSQLGAATAATS 161
                .   .    .
(m)cFos  223 LTGGLPEASTPES 235
```

C) Alignment of ATF3C peptide with other ATF/AP1 family members

```
(h)ATF3  110 DRPLGVSITKAEV 122
               .      . ..
(h)CREB  276 TQPAEEAARKREV 288
                   ...   .
(h)ATF2  122 PVAIPASITSSNV 134
                .     . .
(h)ATF4    2 GEKLDKKLKKMEQ 14
              .     .   .
(h)ATF6    6 LRRQQRMIKNRES 18
               .  .     .
(m)cJun  230 EEPQTVPEMPGET 242
             .          .
(m)JunB   75 DTGASLKLASTEL 87
                .  .
(m)JunD   52 KDALTLSLAEQGA 64
             .  .  .   .
(m)cFos  291 DVDLSGSFYAADW 303
```

FIG. 8.3 : *Alignment of cJun PEP1 and PEP2 sequences with other members of the ATF/AP1 families*

The complete amino acid sequence of mouse cJun is shown, with the antigen peptide sequences in bold and indicated by a broken line. Identical amino acids are indicated by a single dot. (h) : human, (m) : mouse.

(continued opposite next page)

cJun amino acid sequence : Homologies of other ATF/AP1 family members to peptides PEP1 and PEP2

```
  1  MTAKMETTFY DDALNASFLQ SESGAYGYSN PKILKQSMTL NLADPVGSLK

                                                       PEP2
                                                 ----------
 51  PHLRAKNSDL LTSPDVGLLK LASPELERLI IQSSNGHITT TPTPTQFLCP
                                                 ....
                                     (m)JunB 102 TPTPPGQYFY
                                                 ... .... .
                                     (m)JunD 117 TPTSTQFLYP
                                                 ..... ..
                                   (h)CREBP1  69 TPTPTRFLKN
                                                 ..     .
                                      (h)ATF3 203 TPEDERNLFI

     -
101  KNVTDEQEGF AEGFVRALAE LHSQNTLPSV TSAAQPVSGA GMVAPAVASV

     P 112
     .
     K 127

     C 79

     Q 217

151  AGAGGGGGYS ASLHSEPPVY ANLSNFNPGA LSCGGGAPSY GAAGLAFPSQ
```

FIG. 8.3 (cont'd.): *Alignment of cJun PEP1 and PEP2 sequences with other members of the ATF/AP1 families*

The complete amino acid sequence of mouse cJun is shown, with the antigen peptide sequences in bold and indicated by a broken line. Identical amino acids are indicated by a single dot. (h) : human, (m) : mouse.

cJun amino acid sequence : Homologies of other ATF/AP1 family members to peptides PEP1 and PEP2 (cont'd.)

```
                                                       ----
201  PQQQQQPPQP PHHLPQQIPV QHPRLQALKE EPQTVPEMPG ETPPLSPIDM

                                               (h)ATF2 RRAA

                                               (h)ATF3 AEVH

                                               (h)ATF4

                                               (h)ATF6
                                                       .. .
                                               (m)JunB PINM
                                                       ....
                                               (m)JunD PIDM

                                               (m)cFos VEQL


        PEP1
     --------------
251  ESQERIKAER KRMRNRIAAS KCRKRKLERI ARLEEKVKTL KAQNSELAST
           .
     NEDPDEKRRK VLE
        .     .  .
     APEEDERKKR RRE
           .    .
     KGEKLDKKLK KME

     SDIAVLRRQQ RMI
     . ..... .. ..
     EDQERIKVER KRL
       ........ ..
     DTQERIKAER KRL
        .  .     .
     SPEEEEKRRI RRE

301  ANMLREQVAQ LKQKVMNHVN SGCQLMLTQQ LQTF
```

FIG. 8.4 : *Alignment of cFos P10 sequence with other members of the ATF/AP1 families*

The complete amino acid sequence of mouse cFos is shown, with the antigen peptide sequence in bold and indicated by a broken line. Identical amino acids are indicated by a single dot. (h) : human, (m) : mouse.

cFos amino acid sequence : homologies of ATF/AP1 family members to peptide P10

```
  1  MMFSGFNADY EASSSRCSSA SPAGDSLSYY HSPADSFSSM GSPVNTQDFC

 51  ADLSVSSANF IPTVTAISTS PDLQWLVQPT LVSSVAPSQT RAPHPYGLPT

                                                    P10
                                          -----------------
101  QSAGAYARAG MVKTVSGGRA QSIGRRGKVE QLSPEEEEKR RIRRERNKMA
                                             ... .   ...  .
                                  (h)ATF2 DPDEKR RKVLERNRAA
                                          ..       ...... .
                                  (h)ATF3 EEDREK KRRRERNKIA
                                          .   .      . .. .
                                  (h)ATF4 EKLDKK LKKMEQNKRA
                                               .   .   .
                                  (h)ATF6 IAVLRR QQRMIKNRES
                                           .  .     . ..  .
                                  (m)cJun QERIKA ERKRMRNRIA
                                           .  .     . ..  .
                                  (m)JunB QERIKV ERKRLRNRLA
                                           .  .     . ..  .
                                  (m)JunD QERIKA ERKRLRNRIA

     -------------
151  AAKCRNRRRE LTDTLQAETD QLEDEKSALQ TEIANLLKEK EKLEFILAAH
     .  ..  .
     ASRCRQKRKV WV
     ......   .  .
     AAKCRNKKKE KT
     .   .   .
     ATRYRQKKRA EQ
     .   .    .
     ACQSRKKKKE YM
     . ... .  .
     ATKCRKRKLE RI
     . ... .  .
     ASKCRKRKLE RI
     . ... .  .
     ASKCRKRKLE RI

201  RPACKIPDDL GFPEEMSVAS LDLTGGLPEA STPESEEAFT LPLLNDPEPK

251  PSLEPVKSIS NVELKAEPFD DFLFPASSRP SGSETSRSVP DVDLSGSFYA

301  ADWEPLHSNS LGMGPMVTEL EPLCTPVVTC TPGCTTYTSS FVFTYPEADS

351  FPSCAAAHRK GSSSNEPSSD SLSSPTLLAL
```

FIG. 8.5 : *Position of the peptide epitopes in the polypeptides*

Schematic representation of the peptides' position within the proteins. Stipled boxes : basic domain, dotted boxes : "leucine zipper", black boxes : peptide epitopes ; numbers indicated underneath refer to the amino acid boundaries of the peptides.

Location of Epitopes

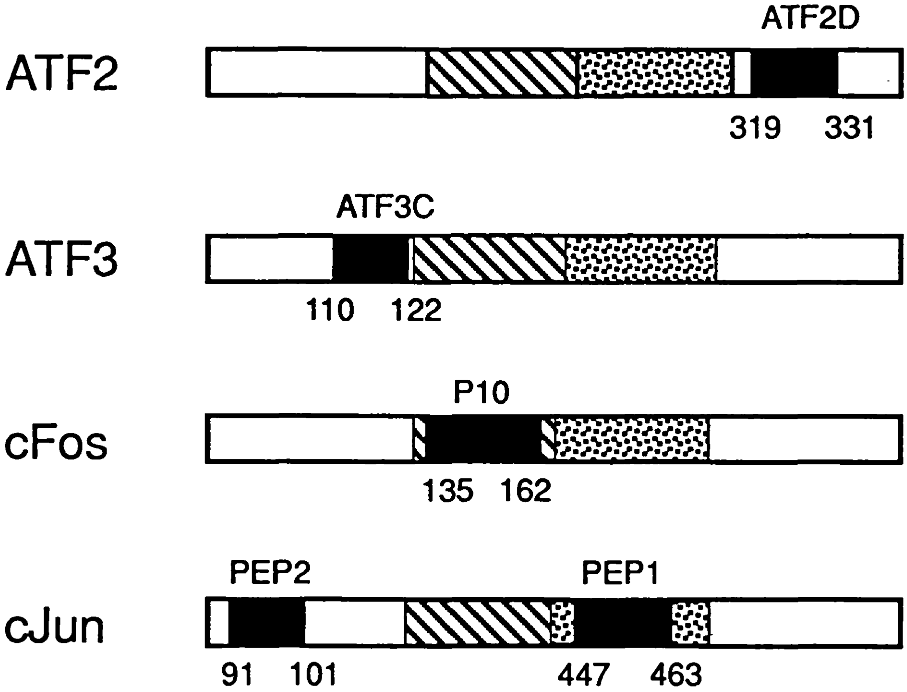

ATF2
ATF2D
319
331
ATF3
ATF3C
110
122
cFos
P10
135
162
cJun
PEP2
PEP1
91
101
447
463

FIG. 8.6 : *Specificity of the antisera tested by ELISA*

ELISA with the indicated immobilised peptides and the indicated sera.

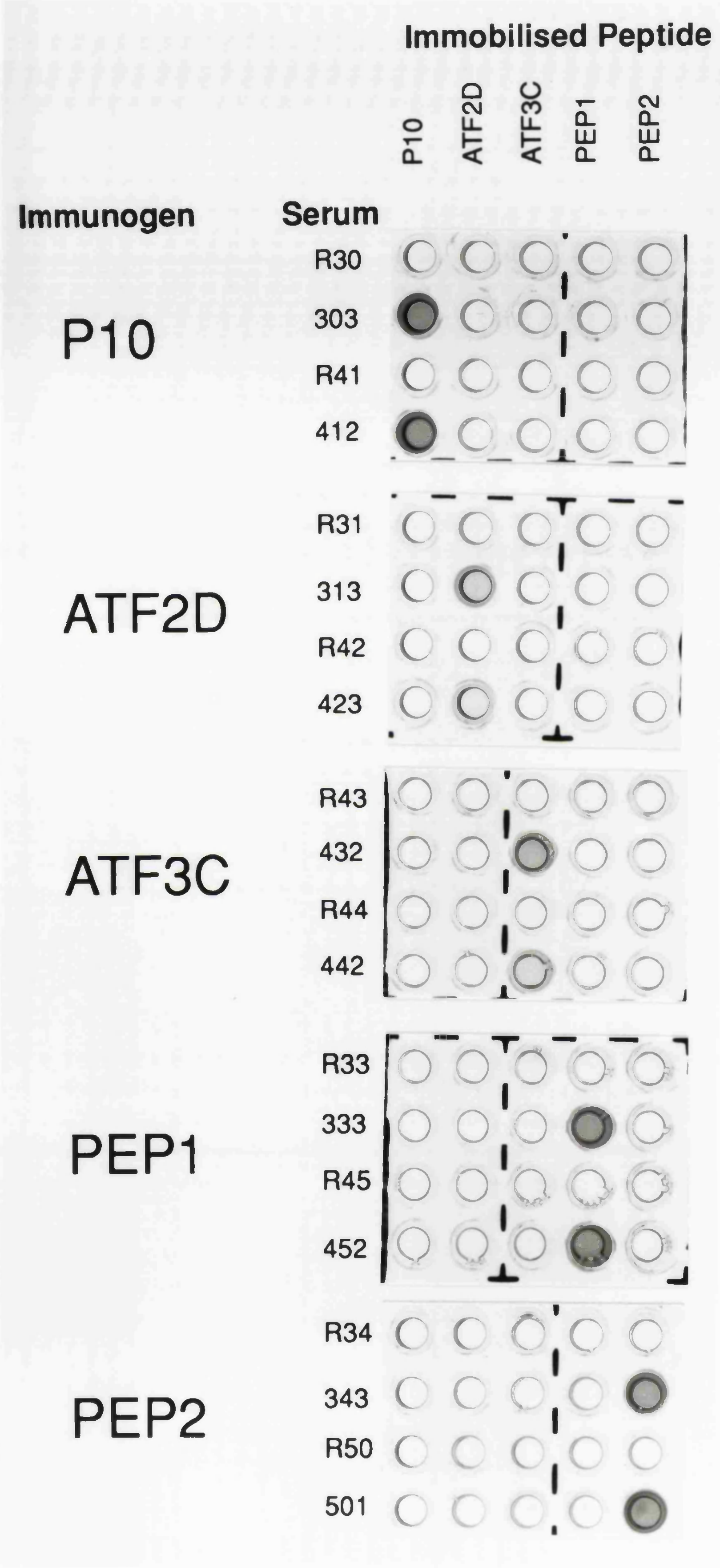
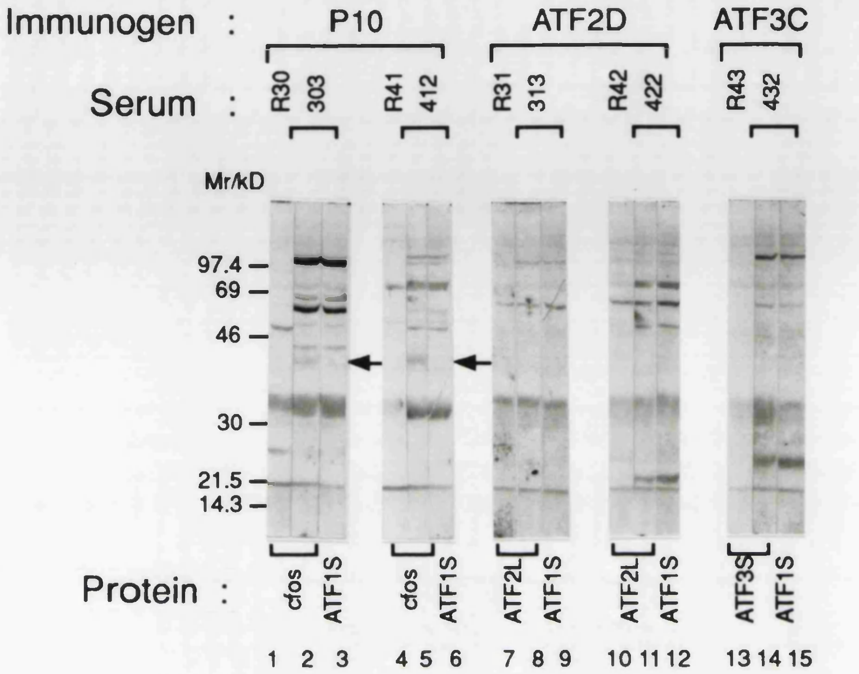
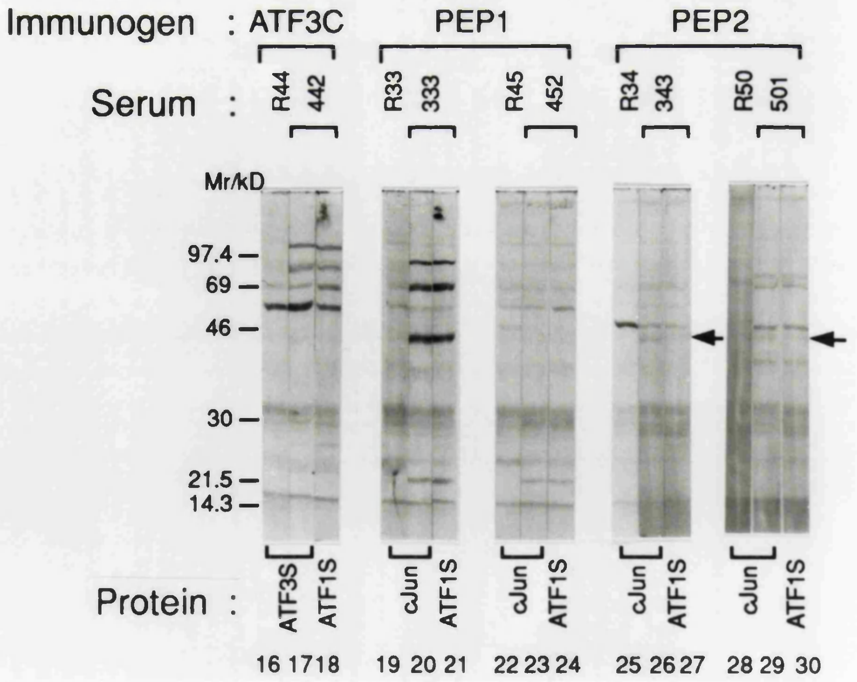

Immobilised Peptide
P10
ATF2D
ATF3C
PEP1
PEP2
Immunogen
Serum
P10
R30
303
R41
412
ATF2D
R31
313
R42
423
ATF3C
R43
432
R44
442
PEP1
R33
333
R45
452
PEP2
R34
343
R50
501

FIG. 8.7 : *Specificity of the antisera tested by immunoblotting against* in vitro *translated polypeptides*

Immunoblotting experiment of the indicated *in vitro* translations (5 μl) by the indicated sera (diluted 1:20 in 10% BSA). Incubation with the antisera was overnight at +4°C. Positions and sizes, in kD, of protein molecular weight standards are indicated to the left of lanes 1 and 16. Arrows indicate the specifically recognized bands that were only present in the appropriate *in vitro* translation.



Immunogen :
P10
ATF2D
ATF3C
Serum :
R30
303
R41
412
R31
313
R42
422
R43
432
Mr/kD
97.4
69
46
30
21.5
14.3
Protein :
cfos
ATF1S
cfos
ATF1S
ATF2L
ATF1S
ATF2L
ATF1S
ATF3S
ATF1S
1 2 3
4 5 6
7 8 9
10 11 12
13 14 15

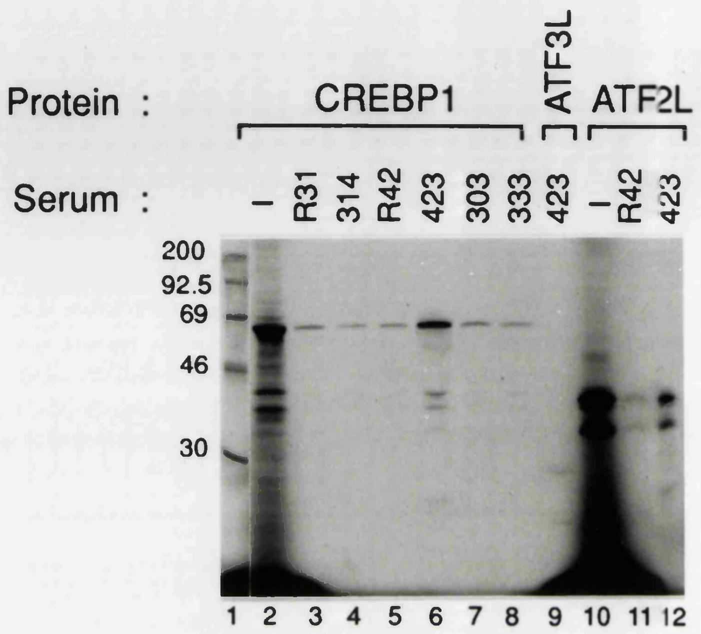


Immunogen :
ATF3C
PEP1
PEP2
Serum :
R44
442
R33
333
R45
452
R34
343
R50
501
Mr/kD
97.4
69
46
30
21.5
14.3
Protein :
ATF3S
ATF1S
cJun
ATF1S
cJun
ATF1S
cJun
ATF1S
cJun
ATF1S
16 17 18
19 20 21
22 23 24
25 26 27
28 29 30

FIG. 8.8 : *Immunoprecipitation of ^{35}S-labelled* in vitro *translated polypeptides by anti-ATF2D and anti-ATF3C sera*

Immunoprecipitation of the indicated ^{35}S-labelled *in vitro* translated polypeptides (3 μl) by the indicated sera. Lanes A2, A10, B10 and B11 were loaded with 1 μl of the indicated *in vitro* translated polypeptide. The sizes, in kD, of ^{14}C-labelled protein molecular weight standards loaded on lanes A1, B1 and B9, are indicated to the left of these lanes.

A


Protein :
CREBP1
ATF3L
ATF2L
Serum :
–
R31
314
R42
423
303
333
423
–
R42
423
200
92.5
69
46
30
1 2 3 4 5 6 7 8 9 10 11 12

B

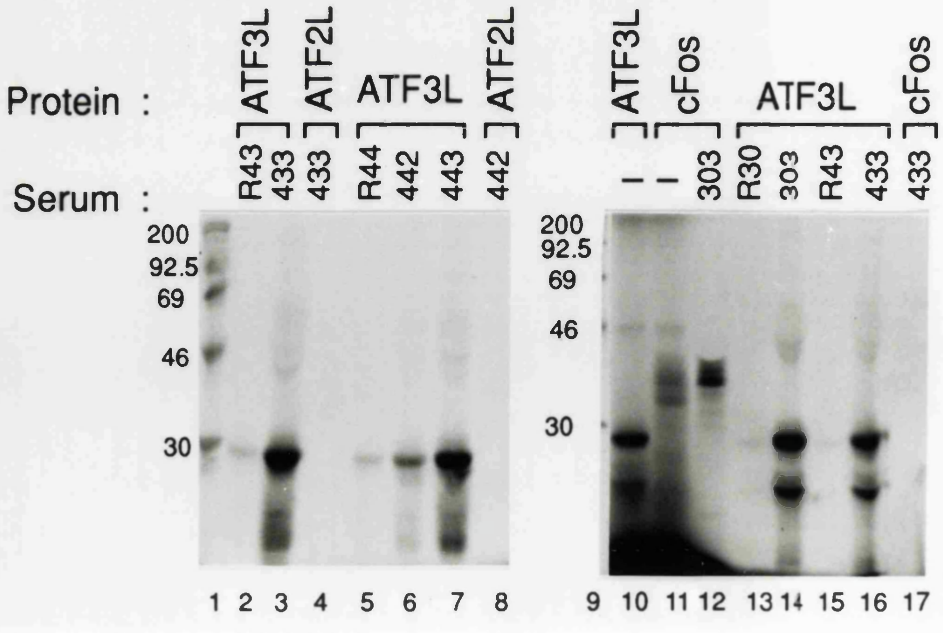
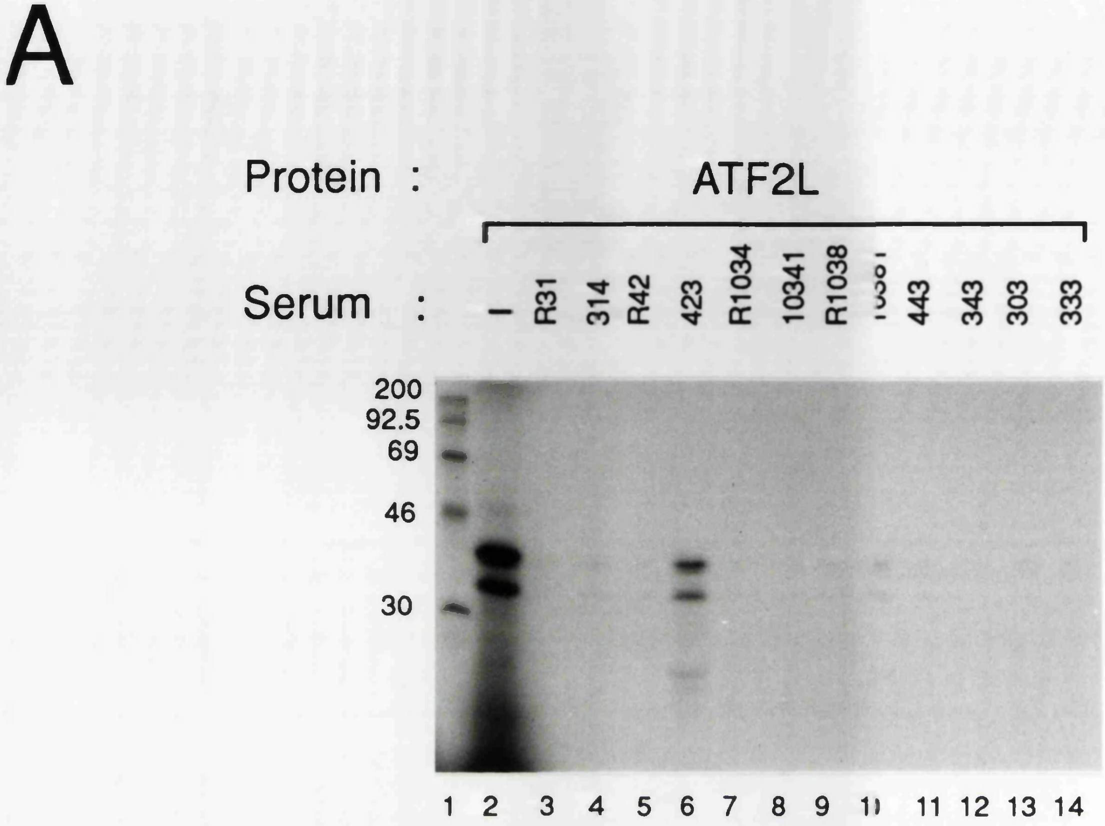

Protein :
ATF3L
ATF2L
ATF3L
ATF2L
ATF3L
cFos
ATF3L
cFos
Serum :
R43
433
433
R44
442
443
442
–
–
303
R30
303
R43
433
433
200
92.5
69
46
30
200
92.5
69
46
30
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16 17

FIG. 8.9 : *Immunoprecipitation of ^{35}S-labelled* in vitro *translated polypeptides by anti-ATF2D and anti-ATF3C sera*

Immunoprecipitation of the indicated ^{35}S-labelled *in vitro* translated polypeptides (3 μl) by the indicated sera. Lanes A2 and B2 contain 1 μl of the indicated *in vitro* translated polypeptide. The sizes, in kD, of ^{14}C-labelled protein molecular weight standards loaded on lanes A1 and B1 are indicated to the left of these lanes.


A
Protein :
ATF2L
Serum :
–
R31
314
R42
423
R1034
10341
R1038
443
343
303
333
200
92.5
69
46
30
1
2
3
4
5
6
7
8
9
10
11
12
13
14

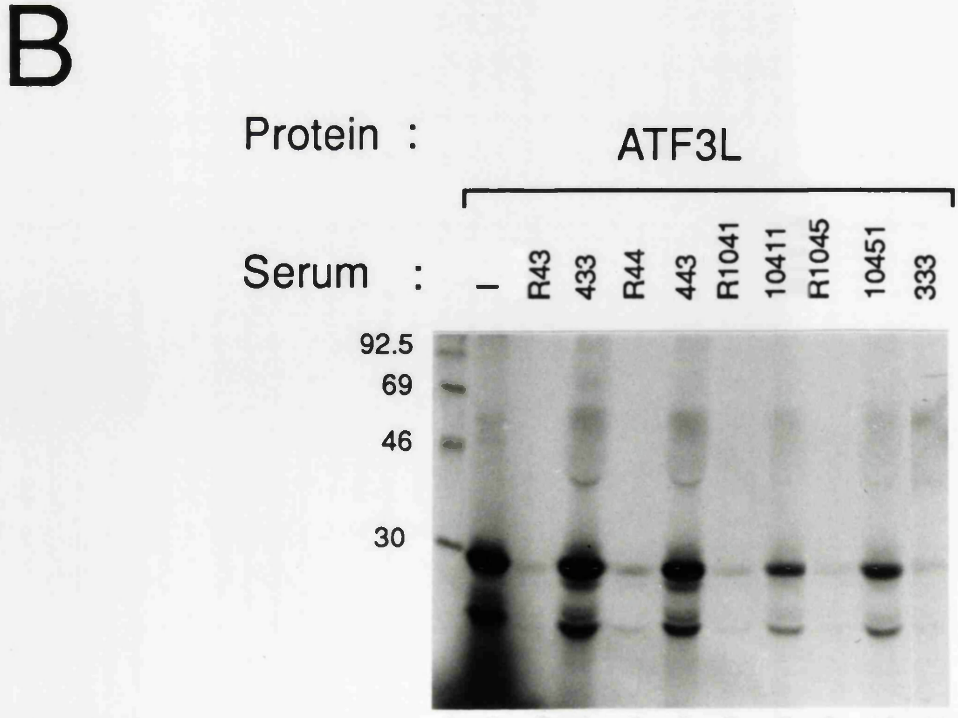
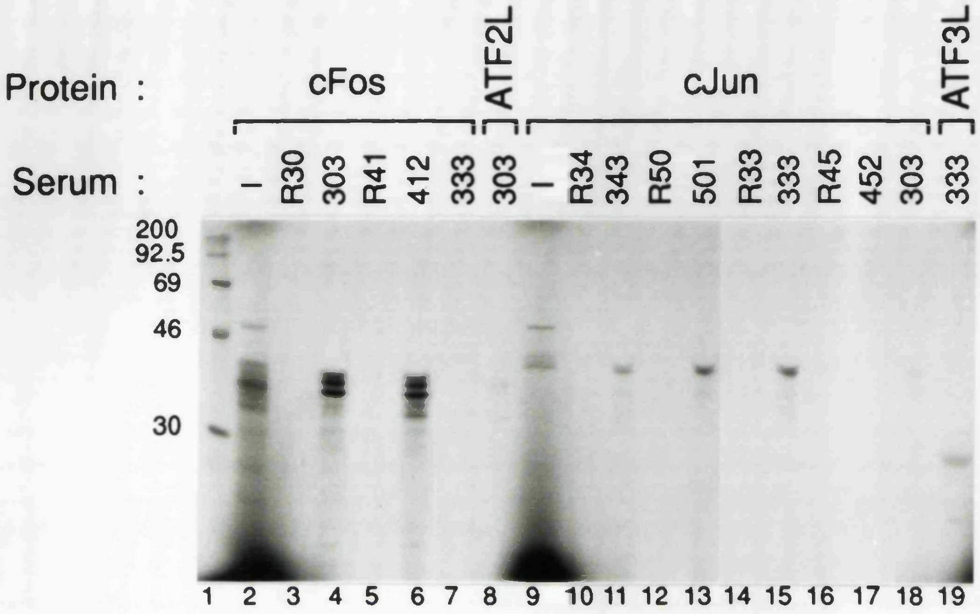

B
Protein :
ATF3L
Serum :
–
R43
433
R44
443
R1041
10411
R1045
10451
333
92.5
69
46
30
1
2
3
4
5
6
7
8
9
10
11

FIG. 8.10 : *Immunoprecipitation of* in vitro *translated polypeptides by anti-P10, anti-PEP1 and anti-PEP2 sera*

Immunoprecipitation of the indicated ^{35}S-labelled *in vitro* translated polypeptides (3 μl) by the indicated sera. Lanes 2, 9, 20 and 27 contain 1 μl of the indicated *in vitro* translated polypeptide. The sizes, in kD, of protein molecular weight standards loaded on lane 1 are indicated to its left. The following lanes come from a single experiment : 1-19 ; 20-33.


Protein :
cFos
ATF2L
cJun
ATF3L
Serum :
–
R30
303
R41
412
333
303
–
R34
343
R50
501
R33
333
R45
452
303
333
200
92.5
69
46
30
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19

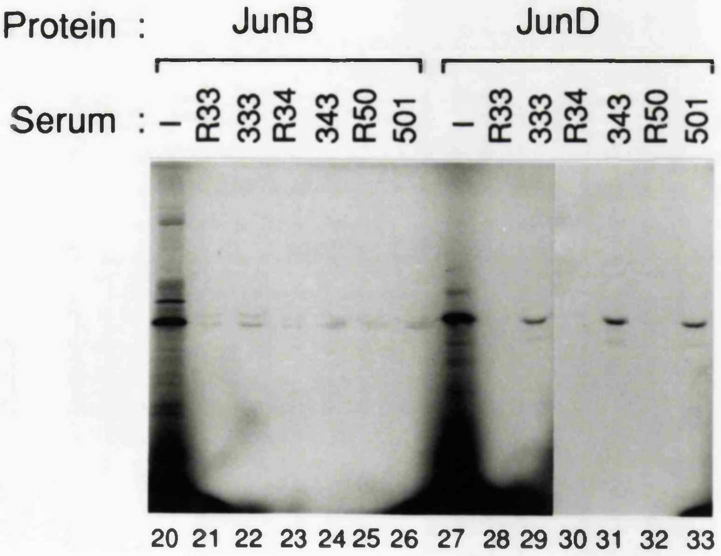
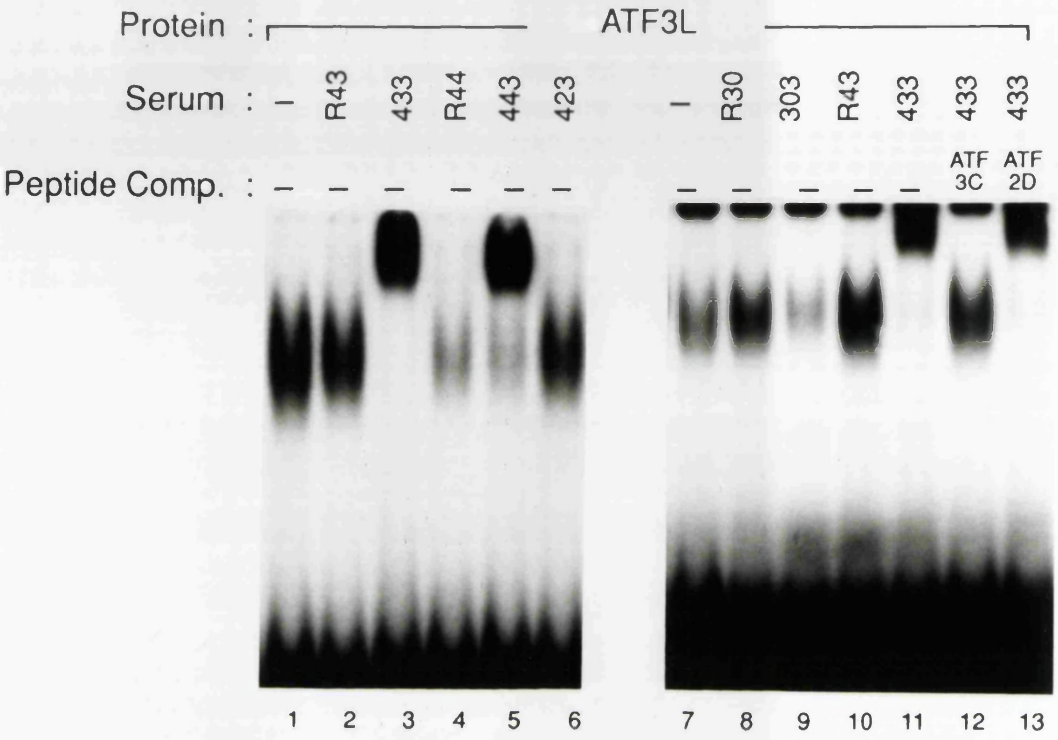

Protein :
JunB
JunD
Serum :
–
R33
333
R34
343
R50
501
–
R33
333
R34
343
R50
501
20
21
22
23
24
25
26
27
28
29
30
31
32
33

FIG. 8.11 : *Effects of anti-ATF3C and anti-P10 sera on ATF3/DNA complexes*

A) Gel retardation of oligonucleotide P with the indicated *in vitro* translated proteins in the presence or absence of the indicated sera and peptide competitors.

B) Anti-ATF2D sera do not recognize *in vitro* translated ATF2D in "Manley C" buffer (Manley *et al.*, 1980). The asterisk indicates a DNA binding activity present in rabbit serum.

A


Protein :
ATF3L
Serum :
–
R43
433
R44
443
423
–
R30
303
R43
433
433
433
Peptide Comp. :
–
–
–
–
–
–
–
–
–
–
–
ATF 3C
ATF 2D
1
2
3
4
5
6
7
8
9
10
11
12
13

B

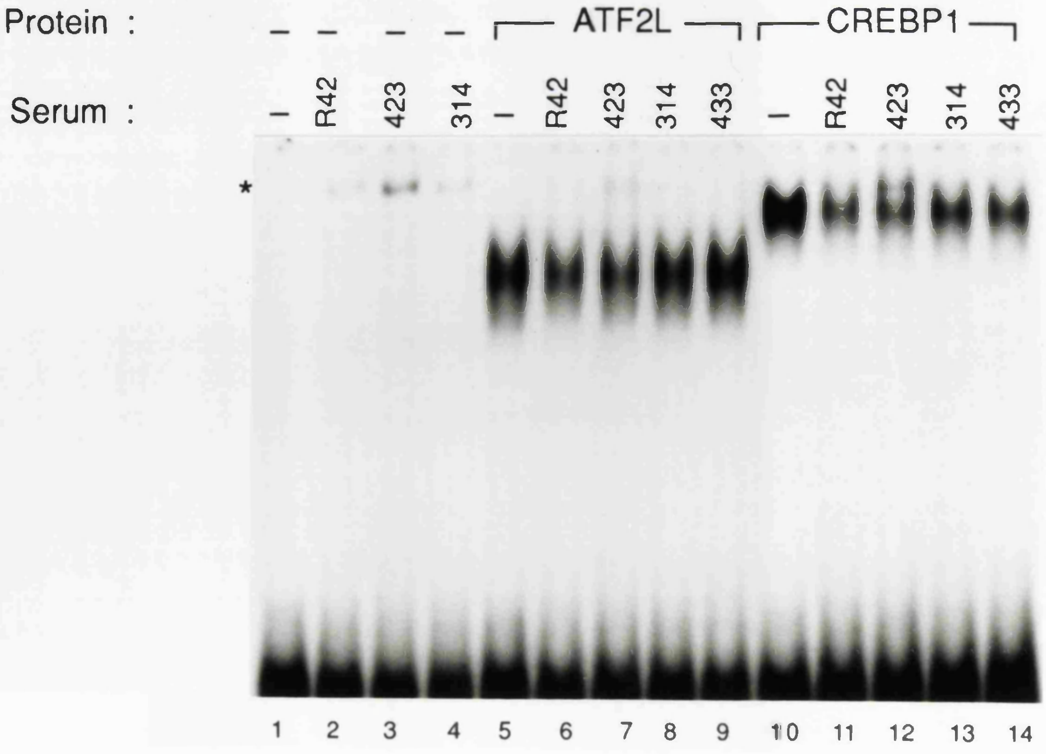

Protein :
–
–
–
–
ATF2L
CREBP1
Serum :
–
R42
423
314
–
R42
423
314
433
–
R42
423
314
433
*
1
2
3
4
5
6
7
8
9
10
11
12
13
14

FIG. 8.12 : *Effects of anti-ATF2D sera on ATF2/DNA complexes*

Gel retardation of oligonucleotide P with the indicated *in vitro* translated proteins in the presence or absence of the indicated sera and peptide competitors. CREBP1 is a longer *ATF2* cDNA product than ATF2L.

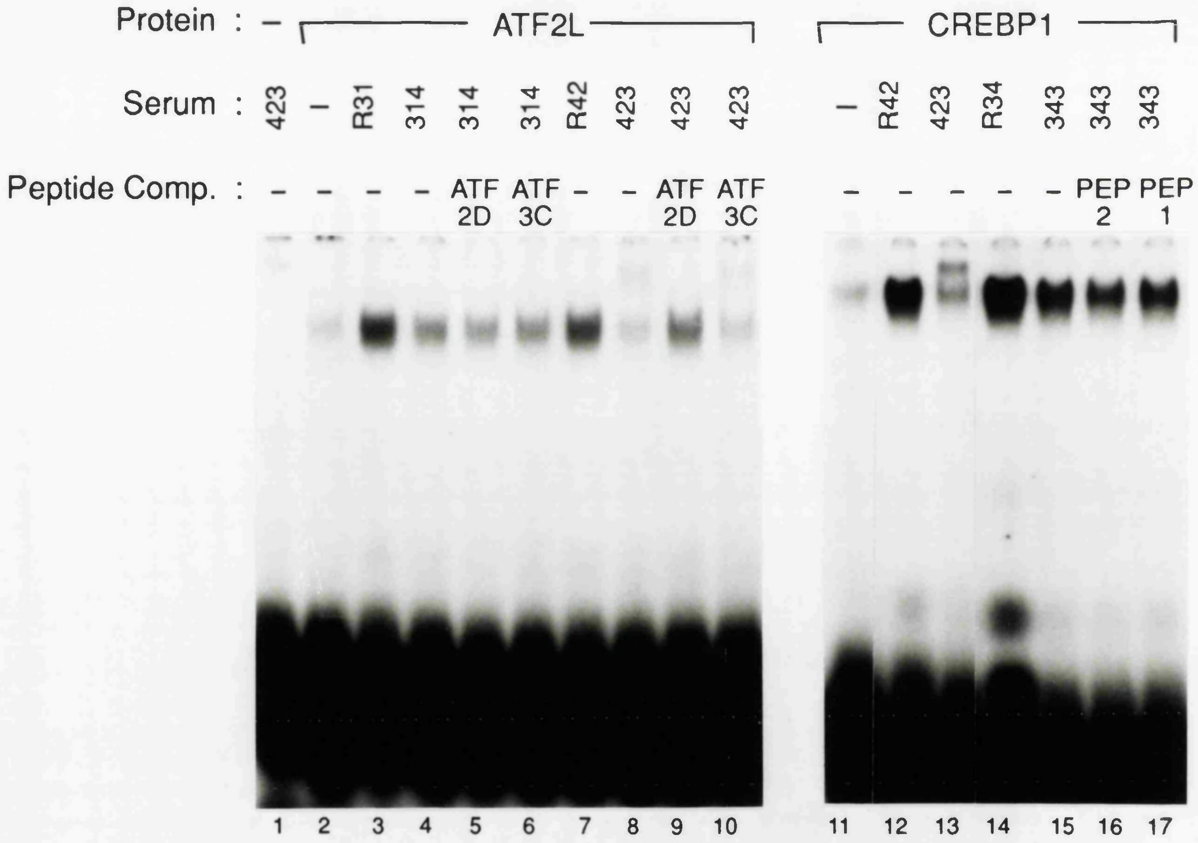

Protein : –
ATF2L
CREBP1
Serum : 423 – R31 314 314 314 R42 423 423 423
– R42 423 R34 343 343 343
Peptide Comp. : – – – – ATF 2D ATF 3C – – ATF 2D ATF 3C
– – – – – PEP 2 PEP 1
1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17

FIG. 8.13 : *Effects of anti-P10, anti-PEP1 and anti-PEP2 sera on* in vitro *co-translated cJun-cFos/DNA complexes and 3T3 AP1/DNA complexes*

Gel retardation of oligonucleotide TRE with *in vitro* co-translated cJun/cFos or 3T3 AP1 in the presence or absence of the indicated sera. The arrow indicates a DNA binding activity present in rabbit serum.

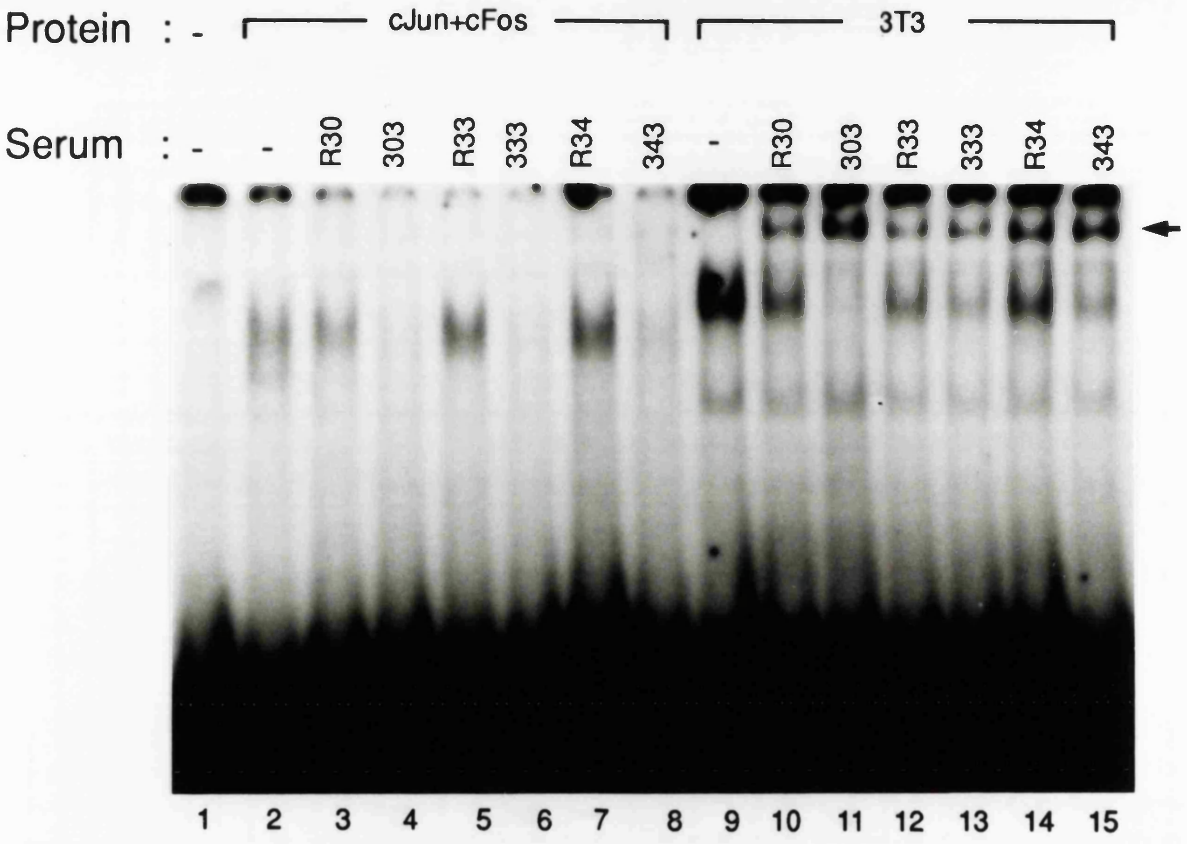

Protein : -
cJun+cFos
3T3
Serum : -
-
R30
303
R33
333
R34
343
-
R30
303
R33
333
R34
343
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

FIG. 8.14 : *Differential effects of sera on DNA binding dimers*

Gel retardation of oligonucleotide P with the indicated *in vitro* translated or co-translated polypeptides in the presence or absence of the indicated sera. Notice that the mobility of the CREBP1/ATF3L dimer (lane 2) is intermediate between that of the CREBP1 homodimer (lane 1) and the ATF3L homodimer (lane 12), and that of the ATF3L/JunD (lane 9) dimer slower than that of the ATF3L homodimer (lane 12). Pairs of lanes are labelled with a rabbit number : the left lane contains pre-immune serum, the right lane contains serum from the third test bleed. All lanes are from the same experiment ; lane 1 and lanes 2-14 are taken from two gels run simultaneously for the same length of time.

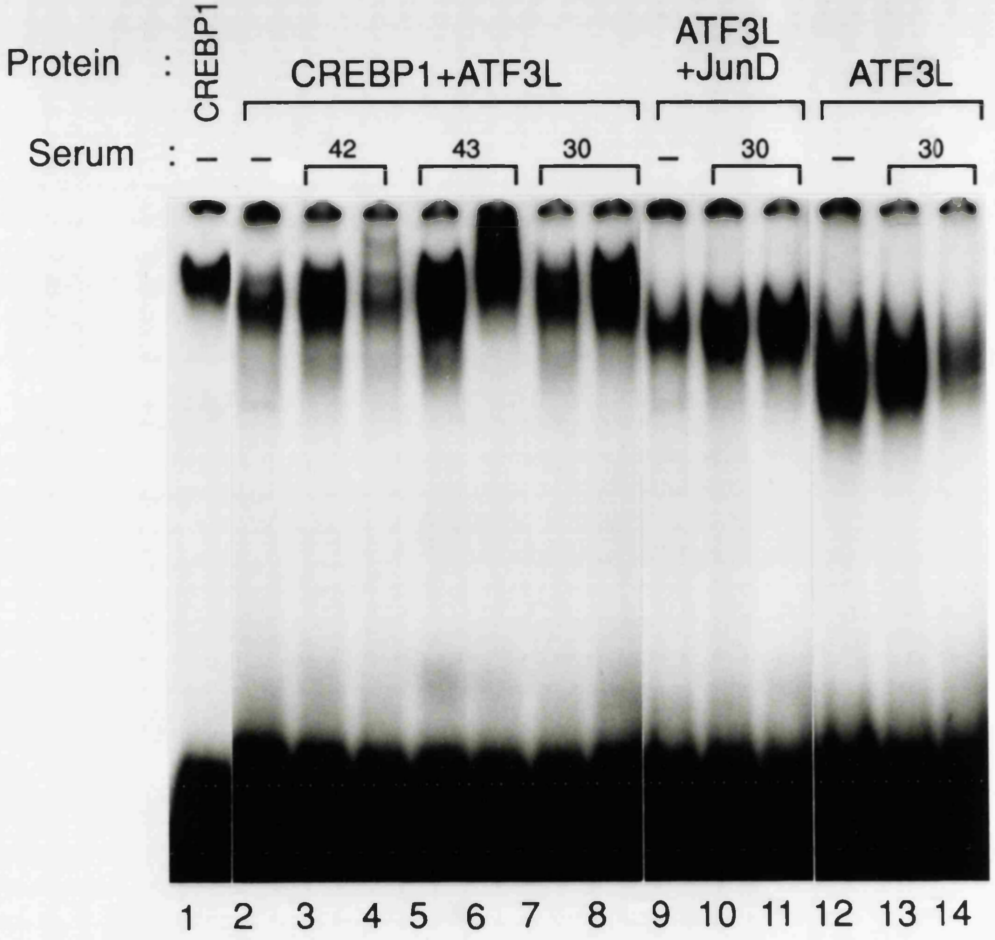

Protein :
CREBP1
CREBP1+ATF3L
ATF3L +JunD
ATF3L
Serum :
–
–
42
43
30
–
30
–
30
1
2
3
4
5
6
7
8
9
10
11
12
13
14

TABLE 8.1 : *Summary of effects of antisera on* in vitro *translated polypeptides*

IP : immunoprecipitation ; NT : not tested.

Summary of Effects of Antisera on *in vitro* Translated Polypeptides

Immunogen (antigen)	Rabbit No.	ELISA	Immuno blotting	IP	Gel Retardation
ATF2D (ATF2)	31	+	-	-	-
	42	+	-	+	super-shift
	1034	NT	NT	+?	NT
	1038	NT	NT	-	NT
ATF3C (ATF3)	43	+	-	+	super-shift
	44	+	-	+	super-shift
	1041	NT	NT	+	NT
	1045	NT	NT	+	NT
P10 (cFos)	30	+	+	+	inhibit
	41	+	+	+	NT
PEP1 (cJun)	33	+	-	+	inhibit
	45	+	-	–	NT
PEP2 (cJun)	34	+	+	+	inhibit
	50	+	+	+	NT

CHAPTER 9

IMMUNOCHEMICAL STUDY OF ATF AND AP1 PROTEINS IN F9 EC AND PE CELLS

9.1 Introduction

Since the anti-ATF and -AP1 sera reacted with the appropriate *in vitro* translated antigens, it was possible to use them for the purposes for which they were originally raised, namely, to study the regulation during differentiation of ATF2 and ATF3 at the protein level, and to assess whether the ECRE activities were immunologically related to known ATF family members.

The antisera were again tested in immunoblotting and immunoprecipitation assays with F9 EC and PE cell extracts, and for their ability to affect ECRE/DNA complexes in a gel retardation assay in crude cell extracts.

9.2 ATF2 protein levels remain constant during differentiation

All anti-ATF2D sera specifically immunoblotted the same cluster of four polypeptides with approximate relative molecular weights ranging from 41 to 45 kD in EC whole cell extracts (Fig.9.1A : compare lane 2 to lane 1, 8 to 7, 10 to 9, 12 to 11). The same polypeptides, qualitatively and quantitatively, were specifically recognized in PE whole cell extracts (Fig.9.1A : compare lane 5 to lane 4 and to lane 2). Furthermore, these bands were present exclusively in nuclear and not in cytoplasmic extracts (Fig.9.1B : compare lane 1 to lane 3). The exclusive presence of ATF2 in the nucleus was consistent with nuclear, rather than cytoplasmic, proteins binding to an ATF site (Fig.9.1C : compare lane 2 to lane 1) and with the function of ATF2 as a transcription factor. Recognition by the antisera was blocked by ATF2D peptide but not by ATF3C (Fig.9.1A : compare lane 3 to lane 2, 6 to 5 ; Fig.9.1B : 2 to 1). The same cluster of bands was immunoprecipitated from ^{35}S-methionine-labelled EC and PE cell extracts, only by an anti-ATF2D immune serum (Fig.9.2A : compare lane 3 to lane 2, 6 to 5) and only in the absence of ATF2D peptide (Fig.9.2A : compare lane 3 to lane 4, 6 to 7). The ATF2 cluster migrated faster in these experiments due to the distortion caused in the 45 kD region by the presence of large amounts of immunoglobulins in the immunoprecipitate ; this was confirmed when anti-ATF2D immunoprecipitates were immnunoblotted with anti-ATF2D after electrophoresis (not shown). In addition to the 41-45 kD bands, however, anti-ATF2D immunoprecipitates contained two polypeptides of molecular weights 48 and 52 kD (corrected for distortion) from both EC and PE cell extracts (indicated by an asterisk in Fig.9.2A : compare lane 3 to lane 2, 6 to 5) which were not recognized in the immunoblotting assays (see Fig.9.1). Their precipitation was also specific, since it was competed by ATF2D peptide (Fig.9.2A : compare lane 3 to lane 4, 6 to 7), but, contrary to the 41-45 kD cluster, they were not recognized by anti-ATF2D under harsher conditions of immunoprecipitation (Fig.9.2B : compare ATF2 and polypeptides indicated by an asterisk in lanes 5 and 3). These 48 and 52 kD polypeptides could therefore be ATF2-related, cross-reacting with anti-ATF2D only in the native state and under mild conditions. Alternatively, they could be immunologically unrelated proteins that co-immunoprecipitated with ATF2, and whose complexing to ATF2 was disrupted by stringent immunoprecipitation conditions.

ATF2, therefore, appeared as a population of nuclear polypeptides, of approximate molecular weights 41-45 kD, that were present at the same levels in F9 EC and PE cells. Two other constitutively expressed polypeptides, of approximate molecular weights 48 and 52 kD, were either related to ATF2 or complexed with it.

9.3 ATF2 is expressed in a variety of cell types

When an anti-ATF2D serum was used to immunoblot whole cell extracts from a number of different cell types, similar clusters of polypeptides were specifically recognized (Fig.9.3A : compare the odd-numbered to their adjacent even-numbered lanes ; Fig.9.4A : compare lane 6 to lanes 5 and 7 ; ATF2 polypeptides indicated by brackets). The similar abundance of these polypeptides in the different cell lines tested correlated with similar levels of ATF site binding activity (Fig.9.3B and Fig.9.4C). In fact, those extracts in which immunoblotting detected polypeptides that were migrating faster than the 41-45 kD cluster, and which were presumably ATF2 degradation products, also showed a pattern of partly degraded, faster migrating ATF site complexes in the gel retardation experiment (compare, for example, lane 12 in Fig.9.3A with lane 4 in Fig.9.3B ; the presumed ATF2 degradation products and the corresponding DNA complexes are indicated by asterisks). It was also noticeable that there were fewer ATF2 species in, for example, HeLa cells than in F9 EC cells (Fig.9.4A : compare lane 6 to lane 3). To test the possibility that different populations of ATF2 molecules were phosphorylated to different extents and consequently not migrating as a single band, extracts were incubated with alkaline phosphatase prior to SDS-PAGE and immunoblotting : the number of bands was not, however, reduced by this treatment (Fig.9.4B : compare lane 2 to lane 1, 4 to 3, 6 to 5), suggesting that phosphorylation was unlikely to be responsible for the multiplicity of ATF2 polypeptides observed.

ATF2 polypeptides were therefore present in various cell types, as initially suggested by ATF2 RNA distribution in different animal tissues (Maekawa *et al.*, 1989 ; Kara *et al.*, 1990), and in all cases migrated as a cluster with approximate molecular weights 41-45 kD.

9.4 Reaction of anti-ATF3 sera with F9 EC and PE polypeptides

When anti-ATF3C sera were used to immunoblot F9 EC and PE whole cell extracts, only one polypeptide, of 35 kD, was detected that was specific for the immune serum and the peptide epitope (indicated by an arrow in Fig.9.5A : compare lane 2 to lanes 1 and 3), and which was down-regulated during differentiation (Fig.9.5A : compare lane 5 to lane 2). All four anti-ATF3C sera raised shared this reactivity (indicated by an arrow in Fig.9.5A : compare lanes 8, 10, 12, 14). Contrary to ATF2 (Fig.9.1B), the 35 kD polypeptide recognized by anti-ATF3C was cytoplasmic (Fig.9.5A : compare lane 17 to lane 15). Given that the ATF3 cDNA was isolated from a HeLa cell cDNA library (Hai *et al.*, 1989), immunoblotting against extracts from HeLa cells and another human cell line, JM, was performed : neither the 35 kD polypeptide (indicated by an arrow) nor any other specific polypeptides were detected in either (Fig.9.6 : compare lane 2 to lane 1, 6 to 5), while ATF2 could be detected in the same extracts during the same experiment (Fig.9.6 : compare lane 4 to lane 3, 8 to 7). Three pieces of evidence, therefore, suggested that the 35 kD polypeptide detected in F9 EC cells was not ATF3 : its absence in HeLa cells, which are known to express the *ATF3* gene, its down-regulation during differentiation of F9 EC cells, which contradicted the increased *ATF3* RNA levels found in F9 PE cells (Chapter 6), and its absence from a nuclear protein fraction, which was inconsistent with it being a DNA binding protein. The possibility that F9 PE cells contained the 35 kD "ATF3" protein but that a PE cell-specific modification was blocking antibody recognition was addressed by pre-incubating equal amounts of F9 EC with PE extracts prior to SDS-PAGE and immunoblotting. The 35 kD polypeptide was still detected in the mixed extract as it was in the F9 EC extract (Fig.9.6 : compare lane 14 to lane 10), suggesting that no PE cell-specific modification that could be reproduced *in vitro* under those coditions could be responsible for the lack of the 35 kD polypeptide in F9 PE cells.

It was possible that cellular ATF3, like *in vitro* translated ATF3, was not being detected by immunoblotting because the ATF3C epitope was denaturation-sensitive. However, contrary to its strong reactivity with native *in vitro* translated ATF3, anti-ATF3C did not reproducibly immunoprecipitate any specific polypeptides from ^{35}S-methionine labelled F9 EC and PE cell extracts (Fig.9.5B : compare lane 4 to lanes 5 and 2, 8 to 9 and 6), suggesting perhaps that, although ATF3 transcripts were present in both cell types, the protein levels were very low.

Anti-ATF3C sera, therefore, failed to detect ATF3 by immunoblotting in F9 EC, PE or HeLa cells, and by immunoprecipitation from F9 EC and PE cell extracts, despite the fact that they recognized *in vitro* translated ATF3. This could be due to a denaturation-sensitive epitope and low levels of ATF3 protein.

9.5 Reaction of anti-P10 sera with cellular proteins

Anti-P10 sera were first tested for their ability to recognize cFos in ^{35}S-methionine-labelled extracts from serum-stimulated NIH 3T3 cells which are known to express *cfos* (Almendral *et al.*, 1988). The anti-P10 serum 303 immunoprecipitated specifically a polypeptide of approximate molecular weight 55 kD, which would be compatible with it being cFos (indicated by an arrow in Fig.9.7A : compare lane 3 to lane 2) (Curran *et al.*, 1984). This reaction was competitively inhibited by excess P10 peptide but not by the related cJun basic domain, PEP1, or the unrelated ATF2D (Fig.9.7A : compare lane 6 to lanes 5 and 4). However, no similar polypeptide was recognized in *cfos*-expressing F9 PE cells, though a number of polypeptides were specifically immunoprecipitated and were competed by excess P10 in both EC and PE cells (indicated by arrows in Fig.9.7B : lane 8), presumably due to cross-reactivity with anti-P10. The 412 serum, on the other hand did not immunoprecipitate a 3T3 polypeptide of a molecular weight compatible with that of cFos, though it did specifically recognize a 46 kD polypeptide (indicated by an arrow in Fig.9.7A : lane 8), and was therefore not tested in F9 PE cells. Neither 303 nor 412 immunoblotted a PE-specific polypeptide of the appropriate molecular weight for cFos in whole cell extracts, though, again, a number of polypeptides specifically cross-reacted in both EC and PE extracts (indicated by arrows in Fig.9.8 : compare lanes : 3 to 2, 6 to 5, 9 to 8, 12 to 11).

Anti-P10 sera, therefore, though capable of immunoblotting and immunoprecipitating *in vitro* translated cFos, were not capable of recognizing cFos in a crude F9 PE whole cell extract. This may have been due to low levels of cFos protein rather than weak antibody affinity, especially since a protein of the appropriate molecular weight was recognized by immunoprecipitation from 3T3 cells.

9.6 Reactivity of anti-Jun sera with polypeptides in whole cell extracts

Anti-PEP1 and anti-PEP2 sera presented a similar picture as the anti-P10 sera : neither immunoprecipitated a 3T3- or PE-specific polypeptide of a size consistent with that of cJun, which varies from 39-47 kD, depending on cell type (Curran *et al.*, 1985 ; Bohmann *et al.*, 1987 ; Angel *et al.*, 1988) though they did specifically immunoprepitate polypeptides (indicated by arrows) common to EC and PE extracts (anti-PEP2 : Fig.9.9A : compare lane 5 to lane 4, Fig.9.9B : compare lane 7 to 3, 16 to 12 ; anti-PEP1 : Fig.9.9A : compare lanes 3, 8, 10). Immunoblotting of F9 EC or PE cell extracts did not yield any PE-specific bands either (Fig.9.8 : compare lane 20 to lane 14, 23 to 17).

Neither anti-PEP1 nor anti-PEP2, therefore, recognized any polypeptides whose presence in PE but not EC cells would be consistent with them being cJun, either by immunoblotting or immunoprecipitation. As shown in Chapter 8, anti-PEP1 did not immunoblot *in vitro* translated cJun but anti-PEP2 did, and both immunoprecipitated *in vitro* translated cJun and JunD. Therefore, the specifically immunoprecipitated polypeptides that were common to EC and PE cells and migrated just above (Fig.9.9A : lanes 8 and 10 ; indicated by arrows) or below (Fig.9.9B : lanes 3 and 7) the 46 kD marker could conceivably correspond to JunD, which is expressed, at the RNA level, in both cell types (Chapter 6). The size of *in vitro* translated JunD is 42.6 kD (Chapter 7) ; however, post-translational modifications in the cell may increase its apparent molecular weight, much as, although the predicted molecular weight of human cJun is 37 kD, the cellular protein migrates as a 44-45 kD polypeptide (Angel *et al.*, 1988). The failure of these sera, however, to recognize cellular cJun may reflect its low levels in crude whole cell extracts as well as the fact that unpurified sera, rather than purified immunoglobulins, were used.

9.7 Effects of anti-ATF and anti-AP1 sera on ECRE/DNA complexes

Given that all antisera raised were capable of affecting *in vitro* translated protein/DNA complexes, either by inhibiting DNA binding (anti-P10, anti-PEP1, anti-PEP2) or by further retarding the complexes (anti-ATF2D, anti-ATF3C), with varying efficiencies, they should also, in principle, have the same effects on the corresponding cellular DNA binding proteins. It was therefore interesting to test whether they inhibited or "super-shifted" any of the ECRE/DNA complexes. Such effects would argue for an immunological, at least, relationship between ECRE activities and ATF/AP1 proteins.

The wild type ATF site, P, was used as probe in these experiments, since it bound all ECRE activities very strongly. No inhibition of DNA binding or "super-shifting" was seen with anti-ATF2D (Fig.9.10B : compare lane 9 to lane 8), anti-ATF3C (Fig.9.10B : compare lane 11 to lane 10), anti-P10 (Fig.9.10A : compare lane 14 to lane 13), or anti-PEP1 (Fig.9.10A : compare lane 12 to lane 11). Surprisingly, anti-PEP2, which is directed against a sequence in cJun that lies N-terminal to the DNA binding domain, was the only antiserum that had a detectable effect in this assay. Both 343 and 501 "super-shifted" ECRE-1 (indicated by an open arrow in Fig.9.10A : compare lane 6 to lane 5, 10 to 9). It was confirmed that the slower migrating complex that was generated contained both ECRE-1, since it was specific for the ATF site and could be competed by wild type P but not by mutant Pm1 (Fig.9.10A : compare lane 7 to lane 8), and anti-PEP2 antibody, since its formation was inhibited by PEP2 peptide but not by PEP1 or ATF2D (Fig.9.10B : compare lane 5 to lanes 6 and 7). As can be seen in Fig.9.12, there is considerable similarity between PEP2 and an amino acid sequence in JunD, and, as shown in Chapter 8 (Fig.8.10), anti-PEP2 immunoprecipitated *in vitro* translated JunD but not JunB, which has a less extended region of similarity. 343 also specifically immunoprecipitated a a polypeptide from F9 EC cells, of a size compatible with it being JunD (Fig.9.9B). This reaction of anti-PEP2 with ECRE-1 therefore suggested that JunD, which is expressed in F9 EC cells (Chapter 6 : Fig.6.4), or an unidentified protein cross-reacting with anti-PEP2, was present in the ECRE-1 complex. A comparison of the PEP2 sequence with other ATF/AP1 family members also yielded a region of similarity at the N-terminus of ATF2 (Fig.9.12). To test whether this similarity was immunologically significant, an immunoprecipitation experiment was performed where *in vitro* translated ATF2, ATF3 and JunD were incubated with their cognate antisera or with anti-PEP2. Neither the longest ATF2 cDNA, CREBP1, which contained the region of amino acid similarity, nor the truncated polypeptide ATF2L which did not, were immunoprecipitated by anti-PEP2 (Fig.9.11A : compare lane 3 to lane 5, 8 to 10). ATF3L was also not immunoprecipitated by anti-PEP2, as expected from the lack of extensive amino acid similarity (Fig.9.11A : compare lane 13 to 15). JunD, therefore, was the only polypeptide with amino acid similarity to PEP2 that could be immunoprecipitated by anti-PEP2 (Fig.9.11A : lane 18), making it the only known member of the ATF/AP1 families that could be involved in ECRE-1. Since JunD binding to the ATF site was almost undetectable, while ATF2/JunD or ATF3/JunD heterodimers bound very efficiently (Chapter 7 : Fig.7.6B), it was possible that ECRE-1 contained such a heterodimer. This possibility was tested by incubating *in vitro* translated or co-translated polypeptides with anti-PEP2 in the presence of the ATF site, P, and assaying for a "super-shift". Anti-PEP2, however, had no specific effect on either CREBP1/JunD (Fig.9.11B : compare lanes 8-10 to lane 7), or ATF3/JunD (Fig.9.11B : compare lanes 13-15 to lane 12), under conditions that "super-shifted" ECRE-1 ; as expected from the lack of immunoprecipitation, anti-PEP2 did not have any effect on a CREBP1 homodimer (Fig.9.11B : compare lanes 3-5 to lane 2) or a CREBP1/ATF3 heterodimer either (Fig.9.11B : compare lanes 18-20 to lane 17). Presumably therefore, although anti-PEP2 did recognize JunD in isolation, in an immunoprecipitation assay, *in vitro* heterodimerization with ATF2 or ATF3 in the presence of DNA blocked the anti-PEP2 cross-reacting epitope. Unless *in vivo*, rather than *in vitro*, heterodimerization of JunD with full length, rather than truncated, ATF2 or ATF3 did not block the PEP2-like epitope, these results would suggest that it is unlikely that ECRE-1 is a JunD/ATF2 or /ATF3 heterodimer. Nevertheless, of the known ATF/AP1 family members, JunD is the only one that may be involved in ECRE-1, possibly complexed with other, unidentified proteins ; alternatively, ECRE-1 may contain an unrelated, unknown protein that cross-reacts with anti-PEP2 serum.

9.8 Discussion

The study of ATF and AP1 protein levels proved most fruitful in the case of ATF2. As with its RNA levels, ATF2 protein levels remained constant during differentiation : a cluster of four polypeptides, with molecular weights ranging from 41 to 45 kD, was recognized by anti-ATF2D serum in both F9 EC and PE cells. ATF2 was found exclusively in the nuclear fraction of F9 EC cells, an observation consistent with its function as a DNA binding protein. Similar clusters of polypeptides reacted with anti-ATF2D in a number of other cell types, indicating that ATF2 is widely expressed. The different ATF2 polypeptides within each cluster could be products of alternatively spliced ATF2 RNA, as reported by Georgopoulos *et al.* (1992), or differentially modified products of the same transcript. Although a computerized search for modification sites in ATF2 revealed a number of potential protein kinase A, protein kinase C and casein kinase II phosphorylation sites, it is unlikely that the ATF2 polypeptides are differentially phosphorylated products of the same transcript, since treatment of the extract with alkaline phosphatase did not reduce the number of polypeptides recognized by the antiserum. The same search, nevertheless, also yielded two potential N-glycosylation sites near the amino terminus ; whether they are used *in vivo*, however, and whether glycosylation at these sites would affect electrophoretic mobility is unclear.

None of the anti-ATF3C, anti-Fos and anti-Jun sera reacted specifically with F9 EC and PE polypeptides that were consistent with being their correct protein targets, and therefore no information about the protein levels of ATF3, cFos and Jun proteins in these cells could be obtained. This lack of detection could be due to a number of reasons, including low serum affinity, low amounts of the cognate polypeptides in whole cell extracts, and denaturation-sensitive epitopes.

Unfortunately, anti-ATF2D, which did recognize native F9 EC and PE ATF2 polypeptides in an immunoprecipitation assay, and was able to "super-shift" *in vitro* translated ATF/DNA complexes (Chapter 8), did not affect the ECRE complexes formed on the P probe in a gel retardation assay. This would suggest either that ATF2 is not involved in those ECRE activities or that binding of cellular, as opposed to *in vitro* translated, ATF2 to DNA prevented antibody recognition. The anti-ATF3C sera also did not affect the ECRE complexes formed on P ; this was consistent with their inability to react with native F9 EC cell proteins in an immunoprecipitation assay.

However, the anti-PEP2 sera, one of which, 343, also immunoprecipitated a polypeptide of approximately 46 kD molecular weight, in F9 EC and PE cell extracts, did specifically "super-shift" the ECRE-1/DNA complex in a gel retardation assay. A comparison of the cJun PEP2 amino acid sequence with those of other ATF and AP1 family members, and immunoprecipitation assays with anti-PEP2 against various *in vitro* translated ATF and AP1 family members, revealed that only JunD cross-reacted with anti-PEP2. This would suggest that JunD may be involved in ECRE-1, which would be compatible with the presence of *JunD* transcripts in F9 EC cells. However, the possibility that an unknown DNA binding protein may be cross-reacting with anti-PEP2 cannot, at present, be excluded.

FIG. 9.1 : *Immunoblotting of F9 EC and PE whole cell extracts with anti-ATF2D sera*

A) Immunoblots of the indicated whole cell extracts with the indicated sera, in the presence or absence of the indicated peptide competitors. The specific ATF2 polypeptides are indicated by brackets on the right of lanes 6 and 12. Positions and sizes, in kD, of protein molecular weight standards are shown to the left of lane 1.

B) Immunoblots of F9 EC nuclear (NUC.) or cytoplasmic (CYT.) extracts with the 314 anti-ATF2D serum, in the presence of the indicated peptide competitors. The specific ATF2 polypeptides are indicated by brackets on the right of lane 4. Positions and sizes, in kD, of protein molecular weight standards are shown to the left of lane 1.

C) Gel retardation of probe P by F9 EC nuclear (NUC.) or cytoplasmic (CYT.) extracts. The positions of the ECRE complexes are indicated.

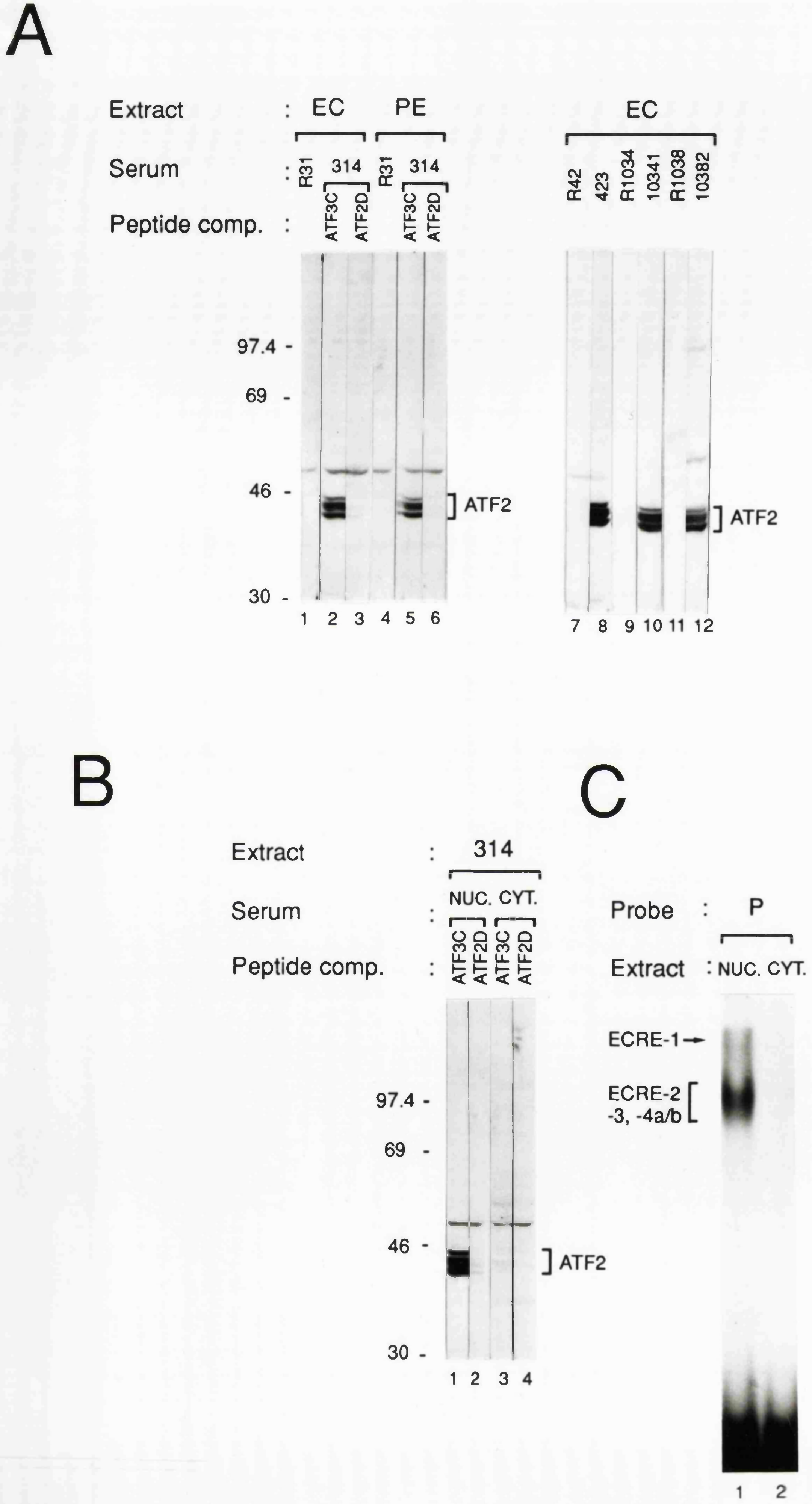
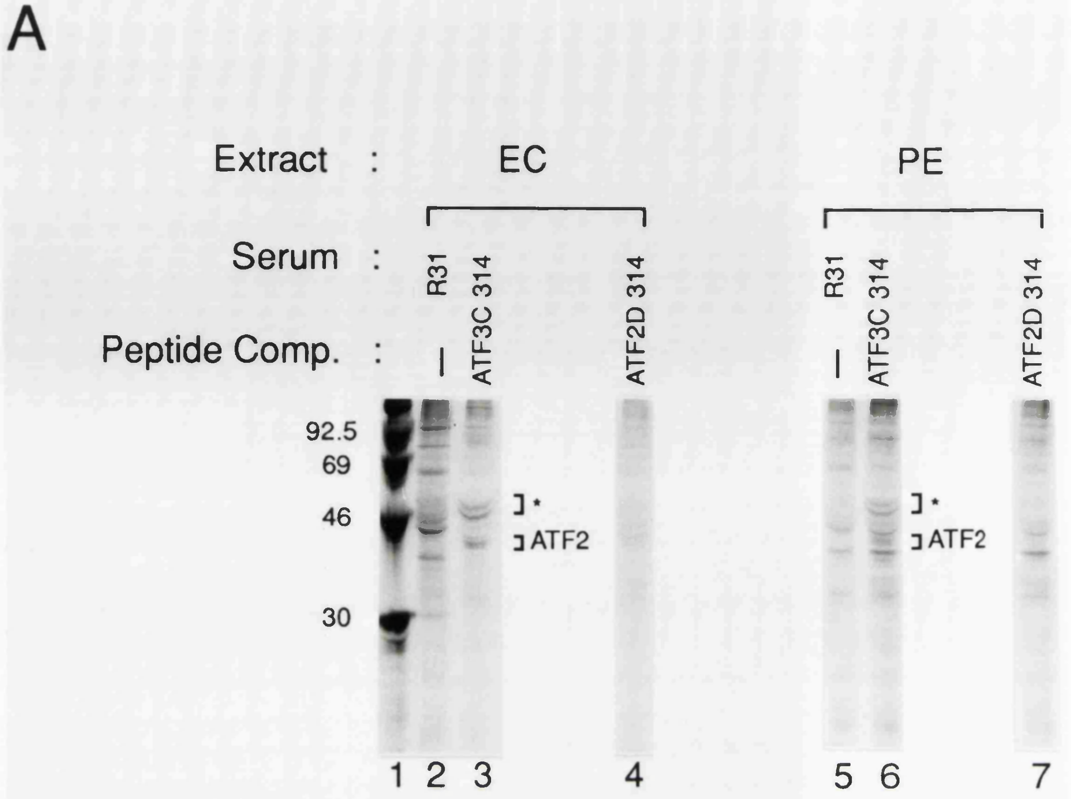

A
Extract : EC PE EC
Serum : R31 314 R31 314
R42 423 R1034 10341 R1038 10382
Peptide comp. : ATF3C ATF2D ATF3C ATF2D
97.4 -
69 -
46 -
30 -
ATF2
1 2 3 4 5 6
7 8 9 10 11 12
ATF2
B
Extract : 314
Serum : NUC. CYT.
Peptide comp. : ATF3C ATF2D ATF3C ATF2D
97.4 -
69 -
46 -
30 -
ATF2
1 2 3 4
C
Probe : P
Extract : NUC. CYT.
ECRE-1
ECRE-2 -3, -4a/b
1 2

FIG. 9.2 : *Immunoprecipitation with anti-ATF2D sera from ^{35}S-Met-labelled F9 EC and PE lysates*

A) Immunoprecipitations with the indicated extracts and sera in the presence or absence of the indicated peptide competitors. The specific ATF2 polypeptides are indicated by brackets ; the asterisk denotes the co-precipitating polypeptides that were not recognized by immunoblotting with anti-ATF2D. All lanes are from the same experiment. Lane 1 : ^{14}C-labelled protein molecular weight standards whose sizes, in kD, are shown to the left of the lane. Background bands in the pre-immune serum lanes were largely due to non-specific binding of proteins to the protein A beads, as can be seen in (B) : compare lane 2 to lane 1.

B) Immunoprecipitations from F9 EC whole cell extracts with the indicated sera in buffer A or C (= A + 1% DOC, 0.1% SDS). The specific ATF2 polypeptides are indicated by brackets ; the asterisk indicates the polypeptides that are only precipitated in the milder buffer, A. Positions and sizes, in kD, of protein molecular weight standards are shown to the left of lane 1. All lanes are from the same experiment.


A
Extract :
EC
PE
Serum :
R31
ATF3C 314
ATF2D 314
R31
ATF3C 314
ATF2D 314
Peptide Comp. :
—
—
92.5
69
46
30
]*
]ATF2
]*
]ATF2
1
2
3
4
5
6
7

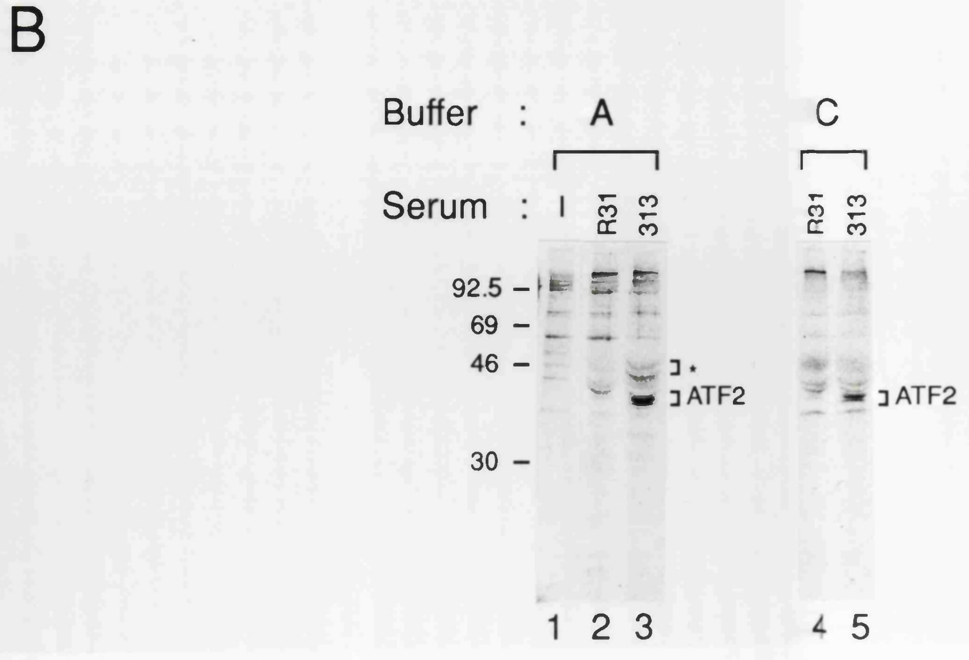
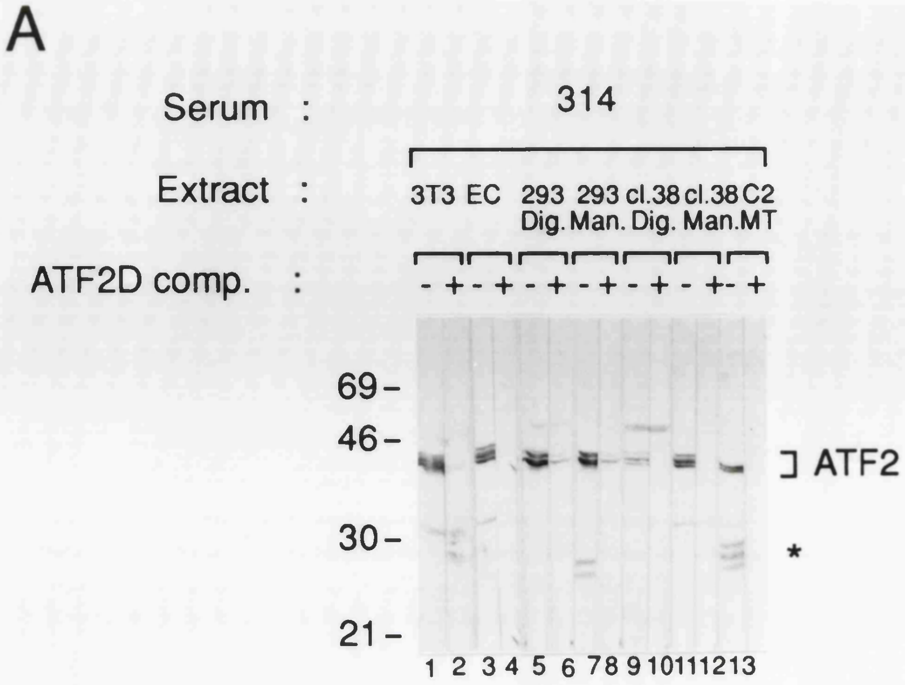

B
Buffer :
A
C
Serum :
—
R31
313
R31
313
92.5 –
69 –
46 –
30 –
]*
]ATF2
]ATF2
1
2
3
4
5

FIG. 9.3 : *Detection of ATF2 polypeptides and ATF site-binding activity in various cell types*

A) Detection of ATF2 (brackets) by immunoblotting against whole cell extracts (3T3, F9 EC, 293 Man., cl.38 Man., C2MT = C2 myotubes) or nuclear extracts (293 Dig., cl.38 Dig.) in the presence of ATF2D (+) or ATF3C (-) peptide competitor ; the asterisk indicates presumed ATF2 degradation products. Positions and sizes, in kD, of protein molecular weight standards are shown to the left of lane 1.

B) Gel retardation of probe P with the indicated extracts (see A). The position of ECRE complexes is shown to the left of lane 1. Asterisks indicate diffuse complexes presumably due to protein degradation products. All lanes are from the same experiment.


A
Serum :
314
Extract :
3T3
EC
293 Dig.
293 Man.
cl.38 Dig.
cl.38 Man.
C2 MT
ATF2D comp. :
- + - + - + - + - + - + - +
69 -
46 -
30 -
21 -
ATF2
*
1 2 3 4 5 6 7 8 9 10 11 12 13

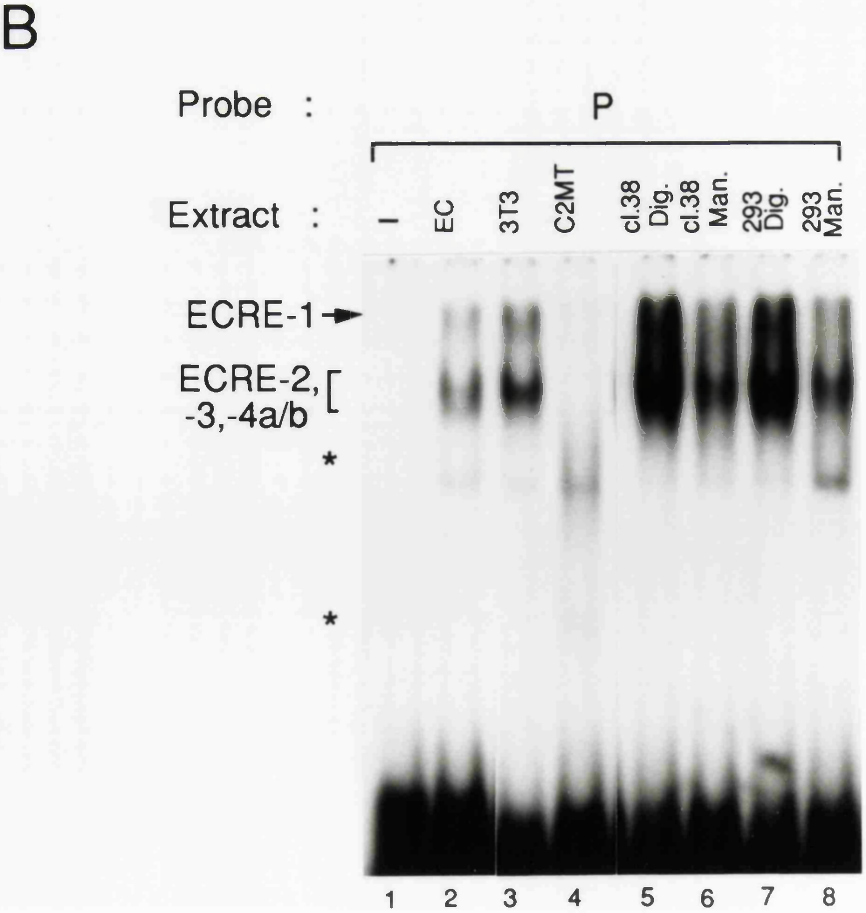

B
Probe :
P
Extract :
—
EC
3T3
C2MT
cl.38 Dig.
cl.38 Man.
293 Dig.
293 Man.
ECRE-1
ECRE-2, -3, -4a/b
*
*
1 2 3 4 5 6 7 8

FIG. 9.4 : *Detection of ATF2 in HeLa cells*

A) Immunoblots of F9 EC and HeLa whole cell extracts with the indicated sera and peptide competitors. The ATF2 polypeptides are indicated by brackets to the right of lane 7. Positions and sizes, in kD, of protein molecular weight standards in lane 1 are shown to the left of the lane.

B) Immunoblots of F9 EC, PE and HeLa whole cell extracts with the anti-ATF2D serum, 314. Where indicated (+), the extracts were pre-treated with 1 unit of calf intestinal alkaline phosphatase (Stratagene) for 30 min at 37°C. The ATF2 polypeptides are indicated by brackets to the right of lane 6. Positions and sizes, in kD, of protein molecular weight standards are shown to the left of lane 1.

C) Gel retardation of probe P with F9 EC or HeLa whole cell extracts.

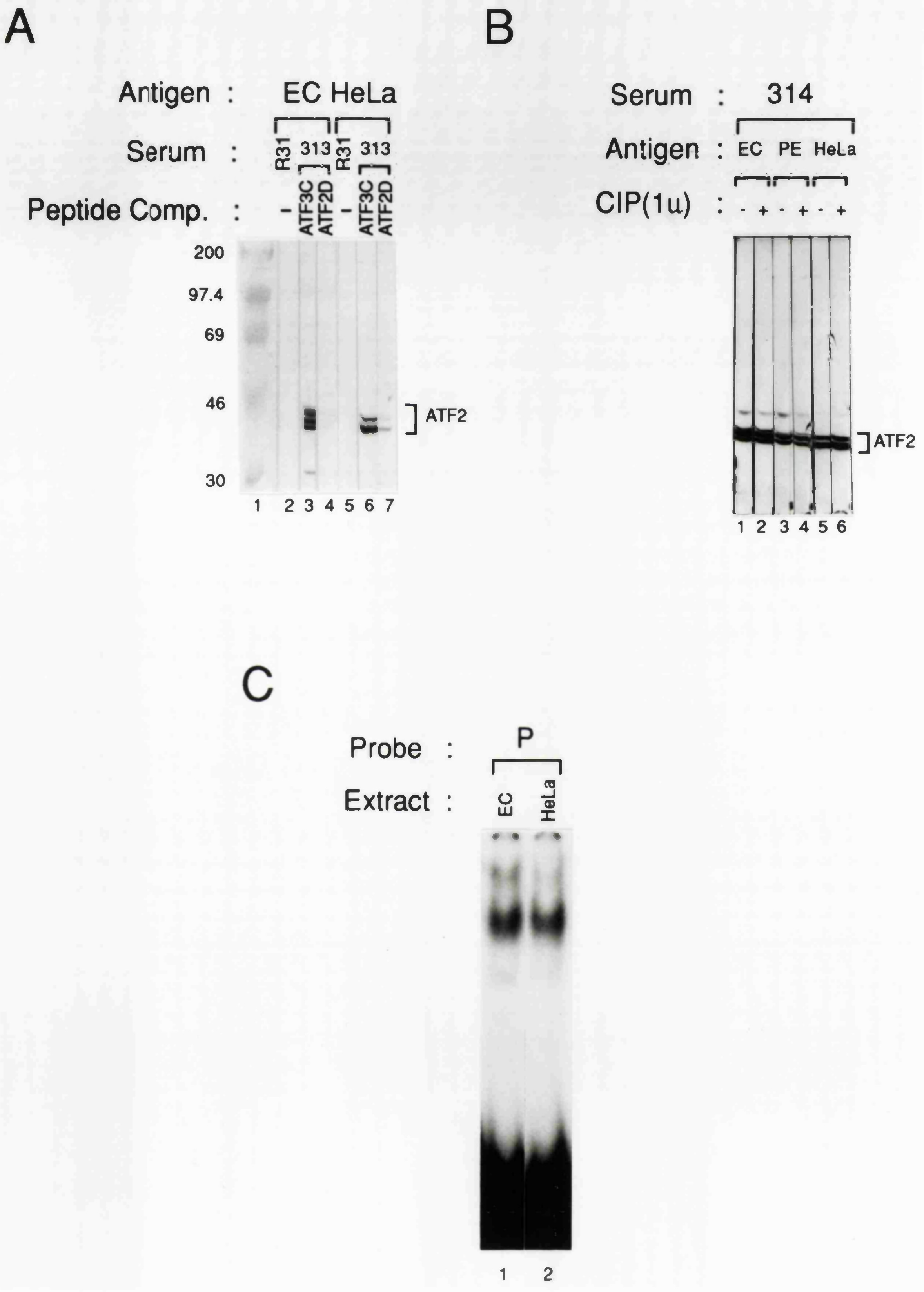

A
Antigen : EC HeLa
Serum : R31 313 R31 313
Peptide Comp. : - ATF3C ATF2D - ATF3C ATF2D
200
97.4
69
46
30
ATF2
1 2 3 4 5 6 7
B
Serum : 314
Antigen : EC PE HeLa
CIP(1u) : - + - + - +
ATF2
1 2 3 4 5 6
C
Probe : P
Extract : EC HeLa
1 2

FIG. 9.5 : *Immunoblotting and immunoprecipitation experiments with anti-ATF3C sera in F9 EC and PE whole cell extracts*

A) Immunoblots of the indicated extracts with the indicated sera in the presence or absence of the indicated peptide competitors. (nuc.) : F9 EC cell nuclear protein extract, (cyt.) : F9 EC cell cytoplasmic protein extract. The arrows indicate the specifically recognized 35 kD polypeptide. Positions and sizes, in kD, of protein molecular weight standards are shown to the left of lanes 1, 7 and 15. Lanes 1-6 are from the same experiment.

B) Immunoprecipitations with the indicated sera in the presence or absence of the indicated peptide competitors from the indicated ^{35}S-methionine-labelled cell lysates. The sizes, in kD, of the ^{14}C-labelled protein molecular weight standards (lane 1), are shown to the left of that lane.

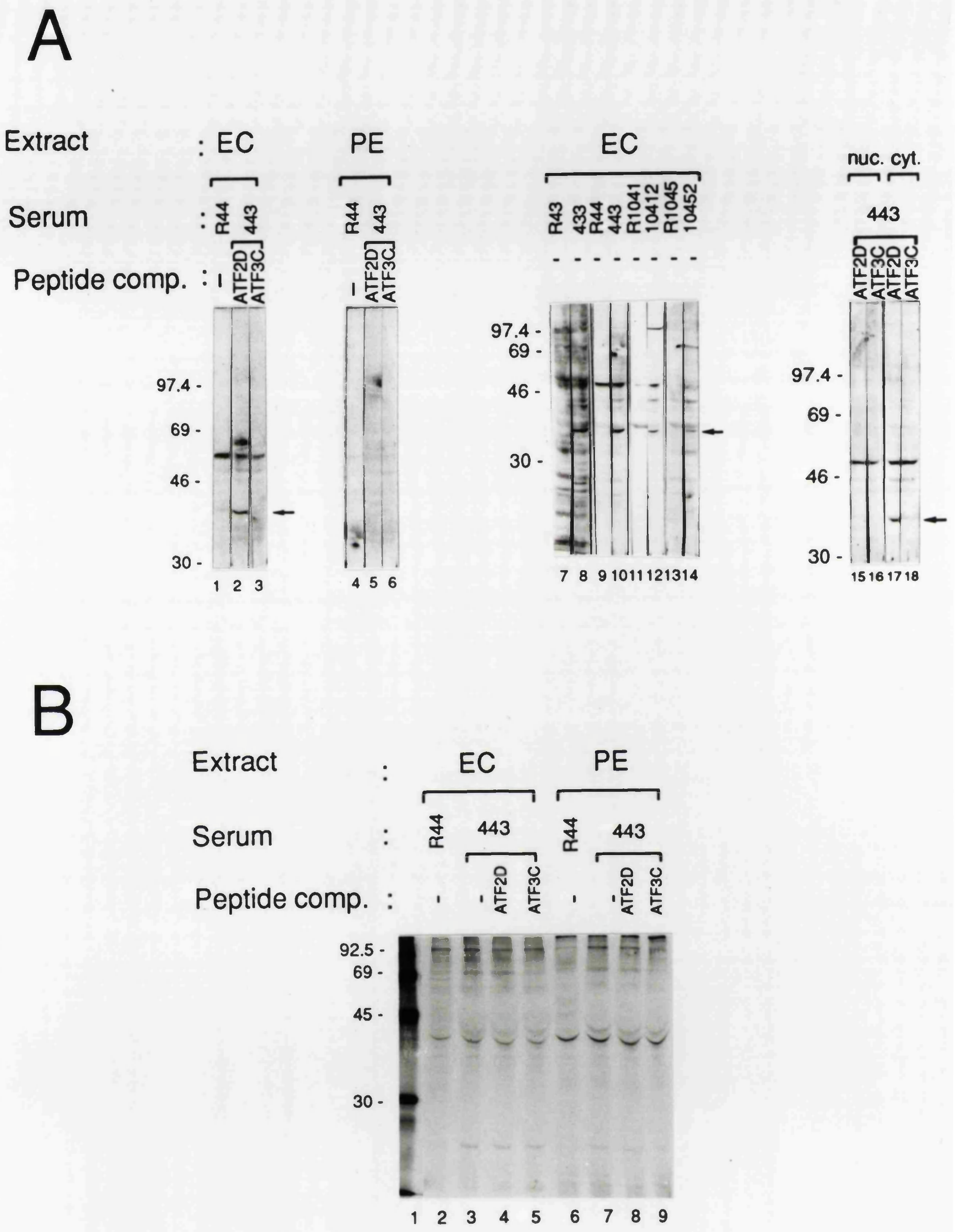

A
Extract
: EC
PE
EC
nuc. cyt.
Serum
:
R44
443
R44
443
R43
433
R44
443
R1041
10412
R1045
10452
443
Peptide comp.
:
-
ATF2D
ATF3C
-
ATF2D
ATF3C
- - - - - - - -
ATF2D
ATF3C
ATF2D
ATF3C
97.4 -
69 -
46 -
30 -
1 2 3
4 5 6
97.4 -
69 -
46 -
30 -
7 8 9 10 11 12 13 14
97.4 -
69 -
46 -
30 -
15 16 17 18
B
Extract
:
EC
PE
Serum
:
R44
443
R44
443
Peptide comp.
:
-
-
ATF2D
ATF3C
-
-
ATF2D
ATF3C
92.5 -
69 -
45 -
30 -
1 2 3 4 5 6 7 8 9

FIG. 9.6 : *Lack of anti-ATF3C reactivity in HeLa cell extracts*

Immunoblots of the indicated whole cell extracts with the indicated sera. The extracts electrophoresed in lanes 9-14 were incubated for 30 min at 30°C prior to SDS-PAGE. Positions and sizes, in kD, of protein molecular weight standards are shown to the left of lane 1.

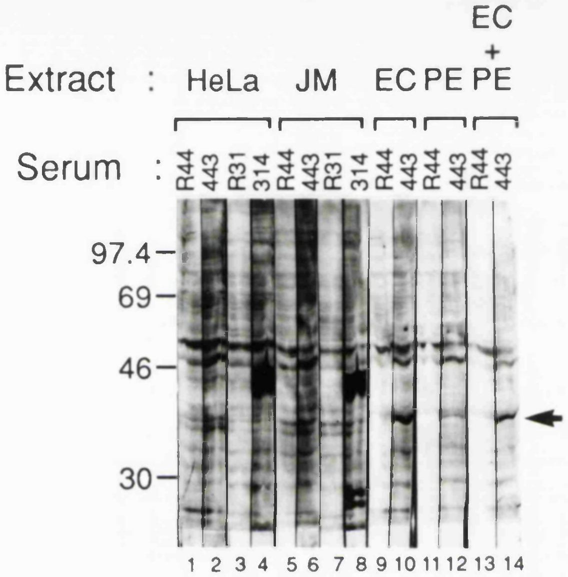
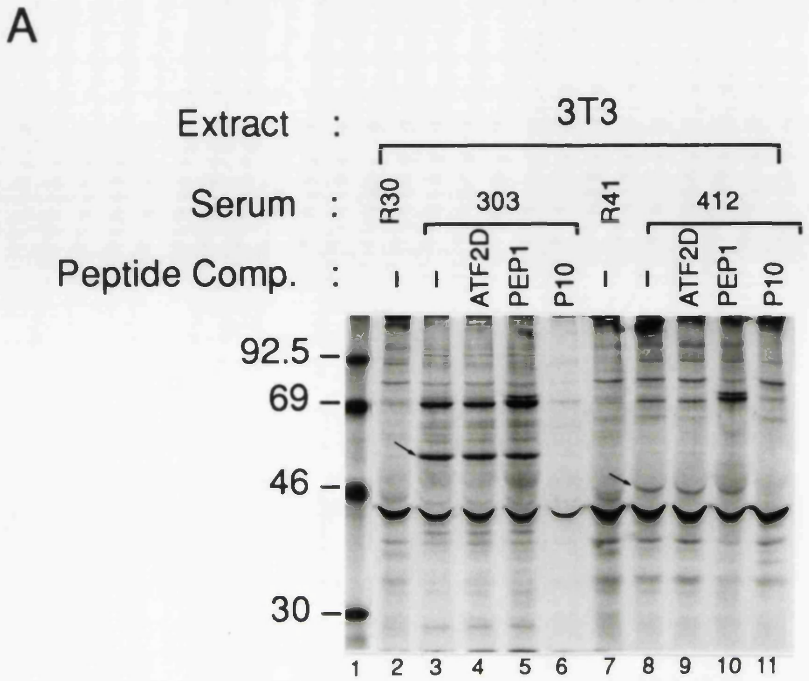

Extract :
HeLa
JM
EC
PE
EC + PE
Serum :
R44
443
R31
314
R44
443
R31
314
R44
443
R44
443
R44
443
97.4
69
46
30
1 2 3 4 5 6 7 8 9 10 11 12 13 14

FIG. 9.7 : *Immunoprecipitation from ^{35}S-Met-labelled cell lysates with anti-cFos sera*

Immunoprecipitations from the indicated ^{35}S-Met-labelled cell lysates with the indicated sera in the presence or absence of the indicated peptide competitors. Lane 1 : ^{14}C-labelled protein molecular weight standards ; their sizes, in kD, are indicated to the left.


A
Extract :
3T3
Serum :
R30
303
R41
412
Peptide Comp. :
–
–
ATF2D
PEP1
P10
–
–
ATF2D
PEP1
P10
92.5 –
69 –
46 –
30 –
1 2 3 4 5 6 7 8 9 10 11

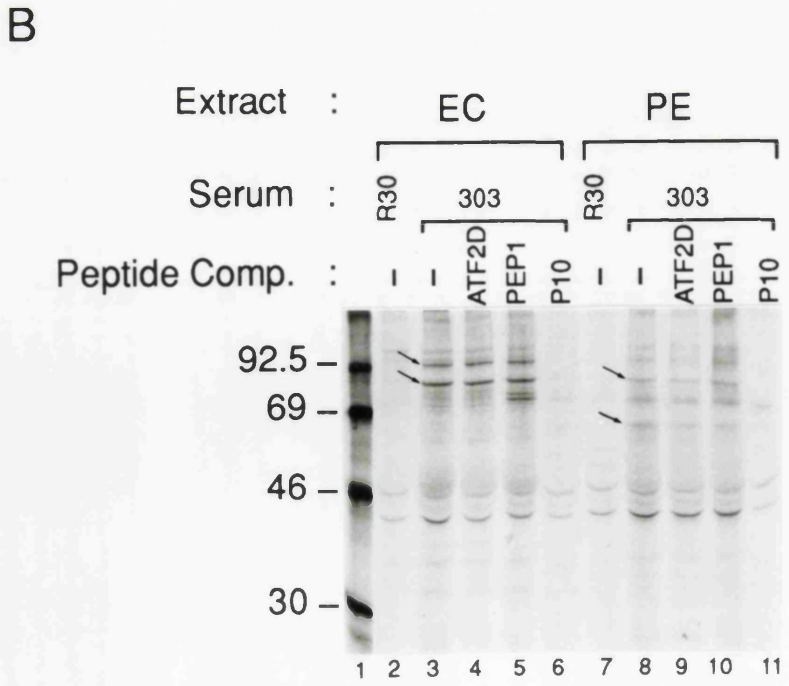

B
Extract :
EC
PE
Serum :
R30
303
R30
303
Peptide Comp. :
–
–
ATF2D
PEP1
P10
–
–
ATF2D
PEP1
P10
92.5 –
69 –
46 –
30 –
1 2 3 4 5 6 7 8 9 10 11

FIG. 9.8 : *Immunoblotting with anti-cFos and anti-cJun sera against F9 EC and PE whole cell extracts*

Immunoblotting with the indicated sera against the indicated extracts in the presence or absence of the indicated peptide competitors. Positions and sizes, in kD, of protein molecular weight standards are shown to the left of lane 1.

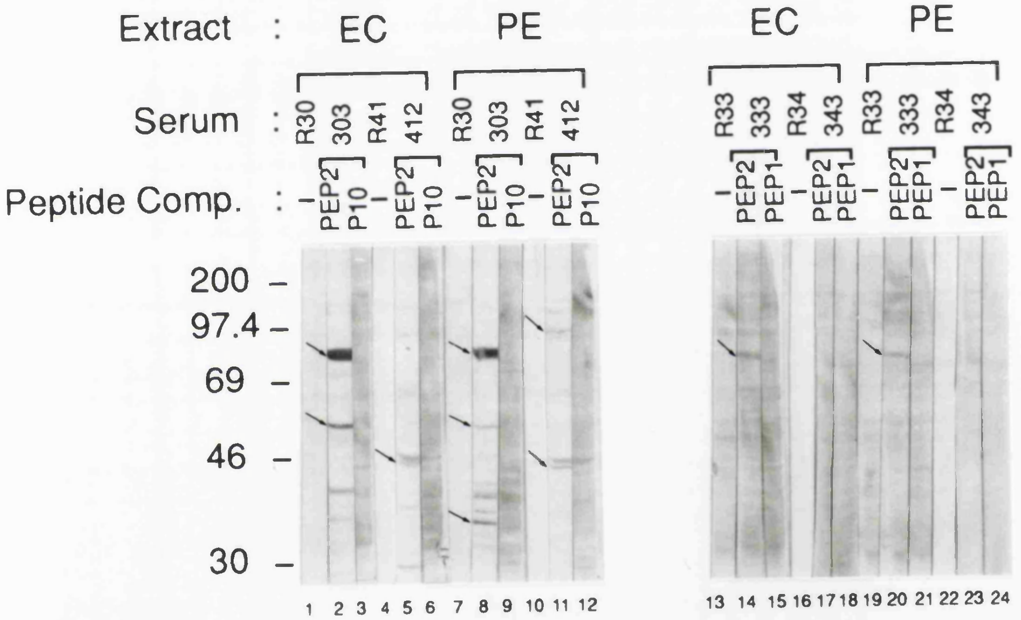

Extract :
EC
PE
EC
PE
Serum :
R30
303
R41
412
R30
303
R41
412
R33
333
R34
343
R33
333
R34
343
Peptide Comp. :
–
PEP2
P10
–
PEP2
P10
–
PEP2
P10
–
PEP2
P10
–
PEP2
PEP1
–
PEP2
PEP1
–
PEP2
PEP1
–
PEP2
PEP1
200 –
97.4 –
69 –
46 –
30 –
1 2 3 4 5 6 7 8 9 10 11 12
13 14 15 16 17 18 19 20 21 22 23 24

FIG. 9.9 : *Immunoprecipitation from 3T3, F9 EC and PE ^{35}S-Met-labelled cell lysates with anti-cJun sera*

A) Immunoprecipitations from ^{35}S-Met-labelled 3T3, F9 EC and PE cell lysates with the indicated sera in the presence or absence of the indicated peptide competitors. Lanes 1 and 6 : ^{14}C-labelled protein molecular weight standards ; their sizes, in kD, are shown to the left of the lanes.

B) Immunoprecipitations from ^{35}S-Met-labelled F9 EC and PE cell lysates with the indicated sera in the presence or absence of the indicated peptide competitors. Lanes 1 and 10 : ^{14}C-labelled protein molecular weight standards ; their sizes, in kD, are shown to the left of the lanes.

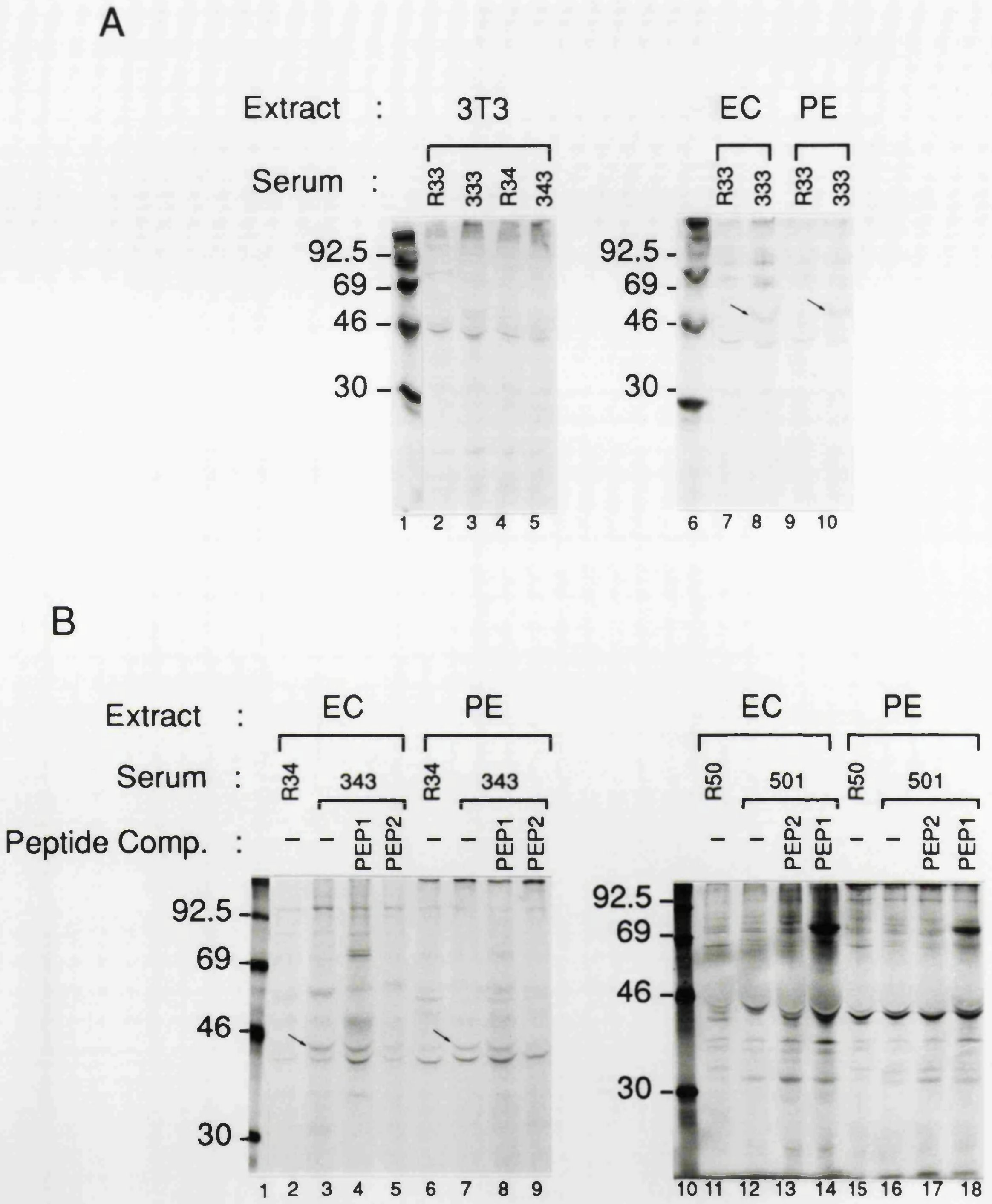

A
Extract :
3T3
EC
PE
Serum :
R33
333
R34
343
R33
333
R33
333
92.5
69
46
30
1 2 3 4 5
6 7 8 9 10
B
Extract :
EC
PE
Serum :
R34
343
R34
343
Peptide Comp. :
-
-
PEP1
PEP2
-
-
PEP1
PEP2
92.5
69
46
30
1 2 3 4 5 6 7 8 9
EC
PE
R50
501
R50
501
-
-
PEP2
PEP1
-
-
PEP2
PEP1
92.5
69
46
30
10 11 12 13 14 15 16 17 18

FIG. 9.10 : *Effects of the anti-peptide sera on ECRE complexes*

A) Gel retardation of probe P with F9 EC whole cell extract in the presence of the indicated sera and oligonucleotide competitors. Where only a rabbit number is given over a pair of lanes, the left lane contains pre-immune serum, the right one serum from the third test bleed - except for lane 10 which contains serum from the first test bleed. The position of the ECRE-1 complex is indicated ; the open arrow indicates the position of the "super-shifted" ECRE-1 complex. All lanes are from the same experiment.

B) Gel retardation of probe P with F9 EC whole cell extract in the presence of the indicated sera and peptide competitors. Where only a rabbit number is given over a pair of lanes, the left lane contains pre-immune serum, the right one serum from the third test bleed.

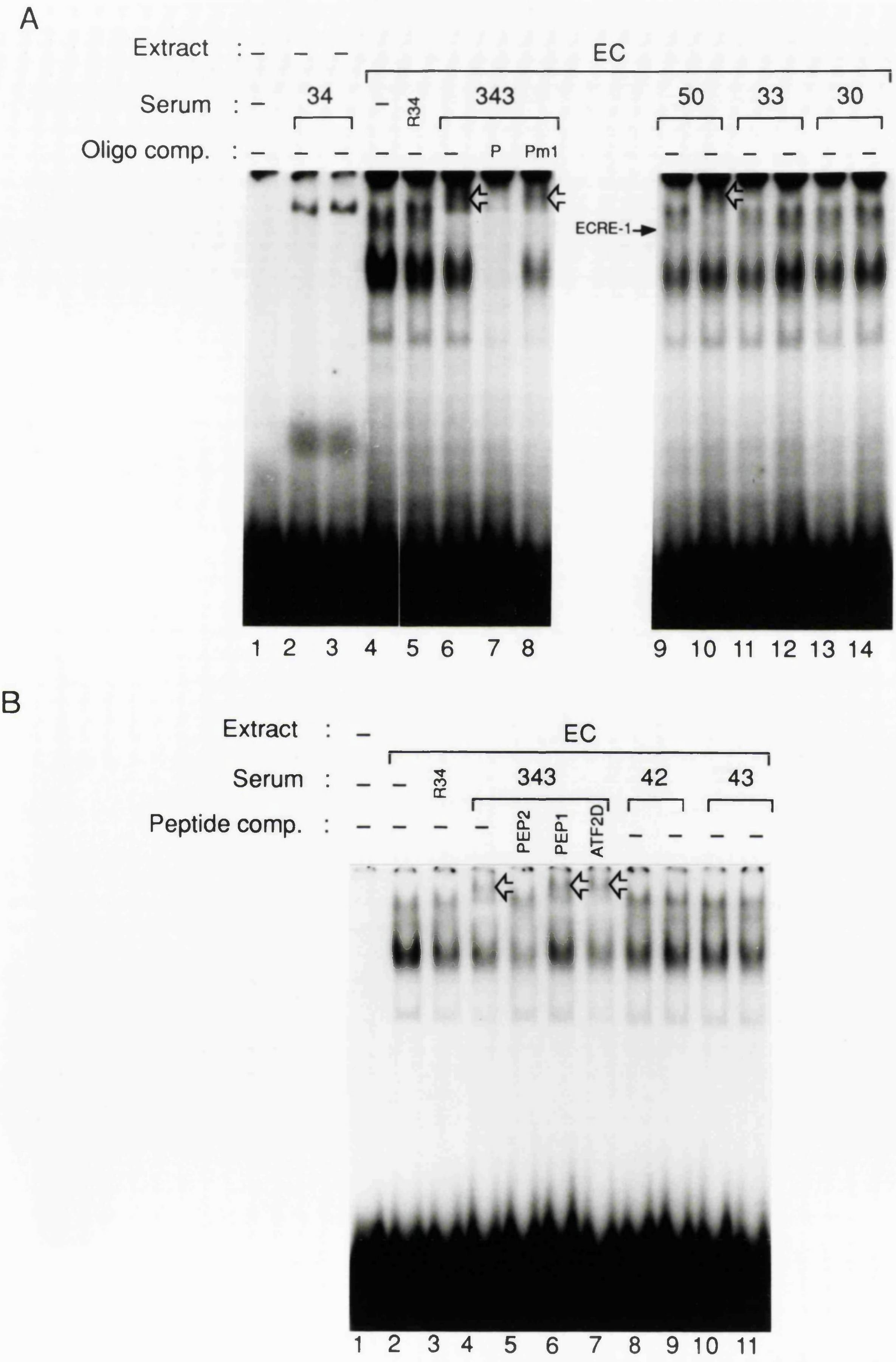

A
Extract : – – – EC
Serum : – 34 – R34 343 50 33 30
Oligo comp. : – – – – – – P Pm1 – – – – – –
ECRE-1
1 2 3 4 5 6 7 8 9 10 11 12 13 14
B
Extract : – EC
Serum : – – R34 343 42 43
Peptide comp. : – – – – PEP2 PEP1 ATF2D – – – –
1 2 3 4 5 6 7 8 9 10 11

FIG. 9.11 : *Anti-PEP2 serum cross-reacts with JunD but not ATF2 or ATF3*

A) Immunoprecipitations of the indicated ^{35}S-Met-labelled *in vitro* translated polypeptides with the indicated sera. For each pair of bands labelled with a rabbit number, the left lane contains pre-immune serum, the right one contains serum from the third test bleed. Lane 19 : ^{14}C-labelled protein molecular weight standards ; their sizes, in kD, are shown to the right of the lane. All lanes are from the same experiment.

B) Gel retardation of probe P with the indicated *in vitro* translated and co-translated polypeptides, in the presence or absence of the indicated sera and peptide competitors. This experiment was performed simultaneously with the experiment in Fig.8.14, where super-shifts were observed as predicted.

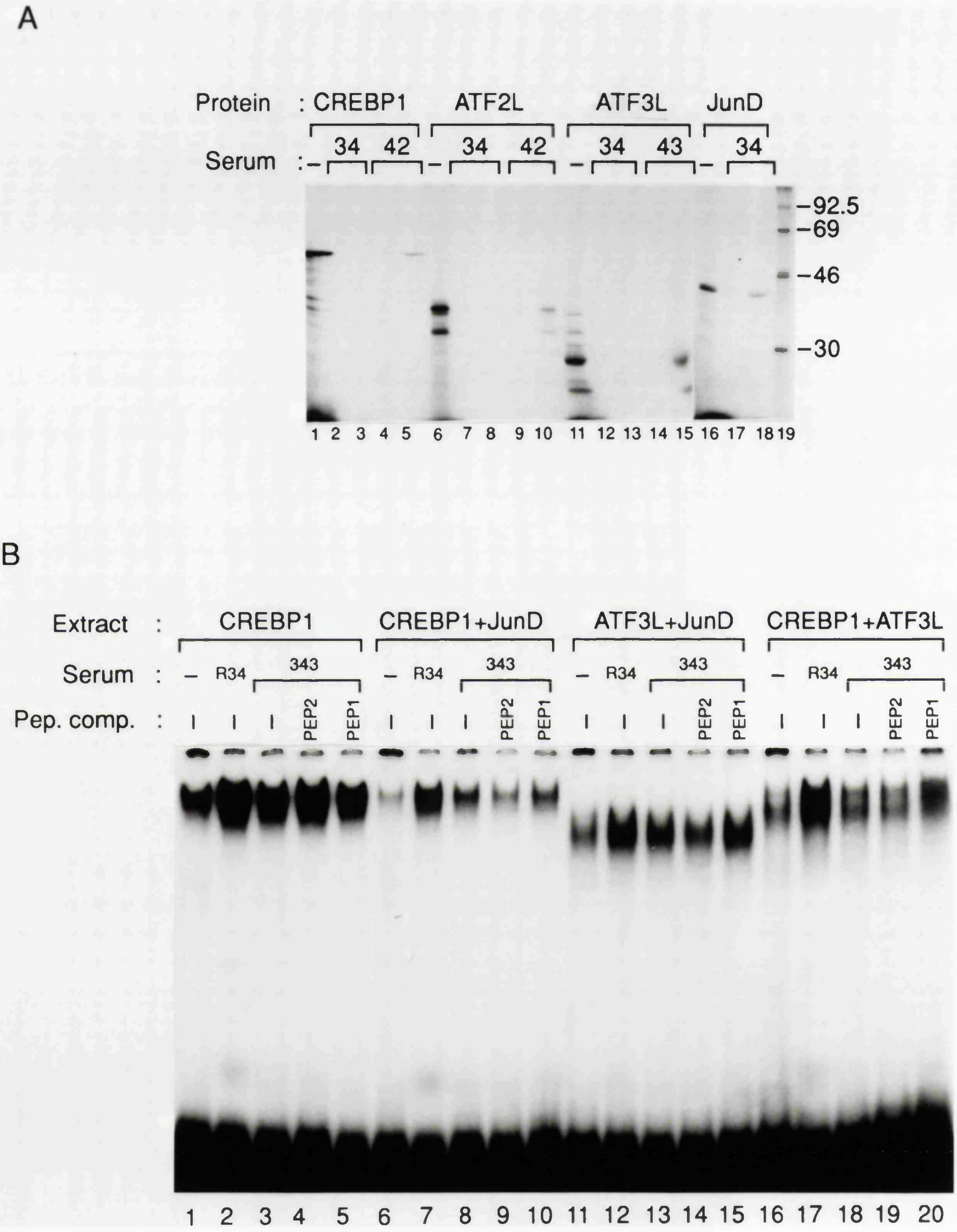

A
Protein : CREBP1 ATF2L ATF3L JunD
Serum : – 34 42 – 34 42 – 34 43 – 34
–92.5
–69
–46
–30
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
B
Extract : CREBP1 CREBP1+JunD ATF3L+JunD CREBP1+ATF3L
Serum : – R34 343 – R34 343 – R34 343 – R34 343
Pep. comp. : – – – PEP2 PEP1 – – – PEP2 PEP1 – – – PEP2 PEP1 – – – PEP2 PEP1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

FIG. 9.12 : *cJun amino acid sequence : amino acid similarities of other ATF/AP1 family members to peptide PEP2*

Identical amino acids are indicated by a single dot. (h) = human, (m) = mouse.

cJun amino acid sequence : Homologies of other ATF/AP1 family members to peptide PEP2

```
  1  MTAKMETTFY DDALNASFLQ SESGAYGYSN PKILKQSMTL NLADPVGSLK

                                                       PEP2
                                                 ----------
 51  PHLRAKNSDL LTSPDVGLLK LASPELERLI IQSSNGHITT TPTPTQFLCP
                                                 ....
                                     (m)JunB 102 TPTPPGQYFY
                                                 ... .... .
                                     (m)JunD 117 TPTSTQFLYP
                                                 ..... ..
                                   (h)CREBP1  69 TPTPTRFLKN
                                                 ..     .
                                     (h)ATF3 203 TPEDERNLFI

     -
101  KNVTDEQEGF AEGFVRALAE LHSQNTLPSV TSAAQPVSGA GMVAPAVASV

     P 112
     .
     K 127

     C 79

     Q 217

151  AGAGGGGGYS ASLHSEPPVY ANLSNFNPGA LSCGGGAPSY GAAGLAFPSQ
```

CHAPTER 10

PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST THE DIFFERENTIATION-REGULATED TRANSCRIPTION FACTOR DRTF-1

10.1 Introduction

As discussed in Chapter 1, the Ad5 *E2A* gene is transcriptionally regulated during differentiation of F9 EC cells (La Thangue *et al.*, 1990 ; Murray *et al.*, 1991). In F9 EC cells, its promoter is *trans*-activated by transcription factor DRTF-1, which had been purified in the laboratory by DNA binding site affinity chromatography (Shivji and La Thangue, 1990). It was decided to raise monoclonal antibodies against the purified factor in order to screen an expression library and isolate a cDNA encoding DRTF-1.

10.2 Immunisation

Four BALB/c female mice (referred to as mouse 1, 2, 3, and 4) were immunised, according to standard procedures, with six doses of antigen. The antigen consisted of an affinity-purified fraction containing DRTF-1 activity (purified by Mahmud K.K.Shivji and Nicholas B. La Thangue) ; when this fraction was analysed by SDS-PAGE, two polypeptides were prominent after silver staining : one in the region of 50 kD, the other, less intense, in the region of 30 kD (Shivji and La Thangue, 1990). Two of the mice were immunised with the affinity-pure fraction alone, while the other two were immunised with aliquots of the same fraction which had been mixed with the oligonucleotide-conjugated resin, since the resin beads might serve as an adjuvant to enhance the immune response. Three tail bleeds were taken from each mouse after the third, fifth and sixth immunisations (referred to as test sera TX/1, TX/2 and TX/3, respectively, where X is the number assigned to each mouse) and compared with the pre-immune sera (referred to as PX, where X is the number assigned to each mouse) from the same mice.

Three assays were used to monitor the immune response of the mice to the injected antigen : 1) an ELISA, 2) the gel retardation assay , and 3) immunoblotting.

10.3 ELISA

96-well PVC plates were coated with F9 EC whole cell extract and the rabbit anti-mouse immunoglobulins were conjugated to horseradish peroxidase. When the first immune sera were compared to the pre-immune sera, there was a clear difference in the intensity of the colour reaction produced by the enzyme. However, the absolute value of the signal was low ; a photograph of a representative plate is shown in the top half of Fig.10.1. Subsequent test sera were compared to the immediately preceding ones. When the second test serum was compared to the first, a low signal appeared to persist at lower antigen and serum concentrations ; this was interpreted as indicating the appearance of high affinity but low titre DRTF-1-specific antibodies. Such antibodies, being of low titre, would not cause a large signal but, being of high affinity, they would be able to recognize low amounts of antigen efficiently. A similar modest increase in conversion of the chromogenic substrate was apparent when the third test serum was compared to the second (bottom half of Fig.10.1 : compare, for example, column 8 to column 3).

Thus, it appeared that the mice were responding weakly to the injected antigen. The next assay was designed to assess the specificity of this response.

10.4 Gel retardation assays

The use of this assay to detect an antigen/antibody complex was based on the rationale explained in Chapter 8.

Given the limited amounts of only partially pure DRTF-1 available, it was clear that the most sensitive and specific method available for assaying for an interaction of an antibody with DRTF-1 was the gel retardation assay. This is because this assay not only allows detection of very small amounts of antigen but also the specificity of any complex can be assessed by competition with the appropriate DNA sequence, in the form of an oligonucleotide, as described in Chapter 3.

That an antibody/DNA binding protein interaction could be detected under the normal conditions of this assay, was demonstrated by incubating an F9 EC whole cell extract with a polyclonal antibody raised against transcription factor Sp1 (Dynan and Tjian, 1983), expressed as a β-galactosidase fusion protein in *E.coli*, or a non-immune serum, before adding a DNA probe which contained two Sp1 binding sites. A "super-shift" formed with as little as 0.5 μl of α-Sp1 serum but not with the same amount of non-immune serum (Fig.10.2 : compare lanes 5, 7, 9 to lanes 4, 6, 8, respectively).

The effects of the third test serum from the mice that had been injected with affinity-purified DRTF-1, and the non-immune serum from the same mice on the complex formed between affinity purified DRTF-1 and the *E2A* promoter were then compared. An increasing "super-shifting" of the complex was apparent with increasing amounts of T2/3 but not P2 (Fig.10.3a : compare lanes 5 and 7 to 3 and 4, respectively). Although the "super-shift" was not as clearly resolved as that with the α-Sp1 serum (Fig.10.2), and the non-immune serum seemed to enhance the intensity as well as slightly affect the mobility of the complex (Fig.10.3a : compare lane 3 to 2), possibly through non-specific interactions of the proteins in the complex with serum proteins which were in large excess, the difference between the effect of the test serum and the non-immune serum on the mobility of the DRTF-1/*E2A* complex was clear. Test sera from all four mice gave similar results (Fig.10.3b : compare lanes 4 to 3, 8 to 7, 12 to 11, 16 to 15). That this "super-shift" involved DRTF-1 was demonstrated by including oligonucleotide competitors in the reaction. The "super-shift" was competed by the wild type DRTF-1 site (19/20 ; see legend to Fig.10.3 for sequence) but not by a point mutated site (63* ; see legend to Fig.10.3 for sequence) that fails to bind DRTF-1 (La Thangue *et al.*, 1990) (Fig.10.3b : compare lanes 5 to 6, 9 to 10, 13 to 14, 17 to 18).

In conclusion, there was a specific "super-shifting" of the DRTF-1 complex with the third test sera, compared to the non-immune sera, although it was poorly resolved. This indicated that antibodies recognizing DRTF-1 were present in the test sera, but were presumably of low titre and/or affinity.

10.5 Immunoblots

The evidence from the ELISAs and the gel retardation assays suggested that the mice were producing specific antibodies against DRTF-1. An immunoblot would indicate whether this immune response was directed against the 50 kD and 30 kD DRTF-1 polypeptides.

Because of the limited availability and low protein concentration of affinity purified DRTF-1, it was decided to use heparin Sepharose (HS) fractions which contained DRTF-1, for immunoblotting. When the third test sera were compared to the non-immune sera, a specific quantitative increase in reaction against a 50 kD protein was observed with T1/3 (Fig.10.4a : compare lanes 4 to 3, 5 to 6) and a similar increase in recognition of a 25 kD protein was observed with T2/3 (Fig.10.4b : compare lane 2 to 1). That the response appeared quantitative rather than qualitative may have been due to inefficient blocking. In addition, more than one protein is likely to migrate to the region of 50 kD ; different but co-migrating proteins could therefore be recognized by the pre-immune and the test sera. Test sera from mice 3 and 4 did not show any difference in the pattern of bands they recognized, compared with the corresponding non-immune sera (Fig.10.4a : compare lane 2 to 1 ; Fig.10.4b : compare lane 4 to 3).

Therefore, the combined results from the three types of assay (ELISA, gel retardation and immunoblotting) suggested that mice 1 and 2 had mounted the most readily detectable immune response against DRTF-1.

10.6 Production of hybridomas

During the interval between the sixth injection and the fusion, mouse 1 died. After a booster injection of antigen, mouse 2 was sacrificed and its spleen cells were fused, by stirring, to Sp2 myeloma cells (Köhler and Milstein, 1976). Tissue culture medium supernatants from the resultant hybridomas were tested by ELISA, in duplicate, at 9, 11 and 14 days after the fusion. The ELISA plates were coated with a HS fraction enriched in

DRTF-1 activity ; the same fraction was used in gel retardation assays and for immunoblots. Cells from duplicated positive wells were transferred to 24-well plates and supernatants from these were also subsequently tested when the cells reached confluence. From the sixteen 96-well plates screened, approximately 1.5% of the wells were identified as positives (data not shown) ; due to fungal contamination, cells from only 20 positive wells survived until the conclusion of the experiments.

The initial 96-well supernatants, as well as the 24-well supernatants, were also tested for their ability to affect the complex formed between a HS DRTF-1 fraction and the *E2A* promoter in a gel retardation assay. None, however, "super-shifted" or abolished the DRTF-1 "shift" (an example is shown in Fig.10.5 : compare lanes 7,8 (positive supernatants) to 9, 10 (negative supernatants)), though a control experiment using the SV40 origin of DNA replication, purified SV40 large T antigen and a supernatant from a hybridoma clone producing α-T monoclonal antibody, did produce a "super-shift" under identical conditions (Fig.10.5 : compare lane 13 to 12). Using an affinity purified DRTF-1 fraction instead of a HS fraction did not alter the result (data not shown). This indicated that either the α-DRTF-1 antibodies produced were unable to bind to DRTF-1 under gel retardation conditions, or that the antibodies reacting in the ELISA assay recognized antigens present in an affinity purified DRTF-1 fraction but different from DRTF-1 itself.

Immunoblots performed with supernatants from the 24-well plates gave a variety of results (Fig.10.6b) : some ELISA-positive supernatants did not recognize any protein under these conditions (lanes 4, 6, 8, 12, 14-17), several reacted strongly with a group of proteins in the region of 140 kD (lanes 5, 7, 9-11, 13), and strongly (lane 7) or weakly (lanes 5, 9-11, 13) with proteins in the region of 100 kD. One (lane 7) also reacted strongly with a 50 kD and weakly with a 30 kD protein, while another (lane 11) reacted strongly with a different 50 kD protein. Supernatants 7G3 (lane 7) and 15C2 (lane 11) were therefore chosen for an immunoblot against affinity purified DRTF-1, because they recognized proteins in the regions of 50 and 30 kD. By that time, the cells had already been re-plated at low density once and reactivity of 15C2 was lost (Fig.10.7b: lanes 5 and 7), presumably because the antibody producing clone(s) had been outgrown by faster growing clones. 7G3, however, still recognized a 50 kD protein in the HS fraction (Fig.10.7b: lane 6), but not in the affinity column fraction (lane 8). This could be explained either by the low amount of antigen in the affinity fraction or by the antibodies in 7G3 being auto-antibodies against a 50 kD protein present in the HS fraction but absent from the affinity fraction. Neither 7G3 nor 15C2 reacted positively in an ELISA assay where the antigen was the same affinity fraction (data not shown). A supernatant (6C3) that did react positively in that assay, was also negative by immunoblotting against an affinity fraction although it did weakly recognize a 50 kD protein in a HS fraction under the same conditions (data not shown).

In summary, the gel retardation assay failed to identify an ELISA-positive hybridoma population producing α-DRTF-1 antibody able to affect the DRTF-1/*E2A* complex, and thus the most sensitive and specific assay available was proven to be unusable. On the other hand, two ELISA-positive supernatants contained antibody that recognized, by immunoblotting, two different 50 kD proteins in a HS but not in an affinity fraction. However, given that affinity purified DRTF-1 was of low concentration and limited in amount, it was not possible to determine the identity of these proteins with certainty, in order to proceed to the cloning of the α-DRTF-1 antibody producing clones. Although other ways of identification were considered (for example, testing the positive supernatants by immunoprecipitation from F9 EC and PE extracts), none seemed to offer a practical basis for cloning to homogeneity the appropriate antibody producing cells. Thus, all positive cells were frozen down and stored until the time when greater availability of purer antigen might offer a feasible means of identifying the clones of interest.

10.7 Discussion

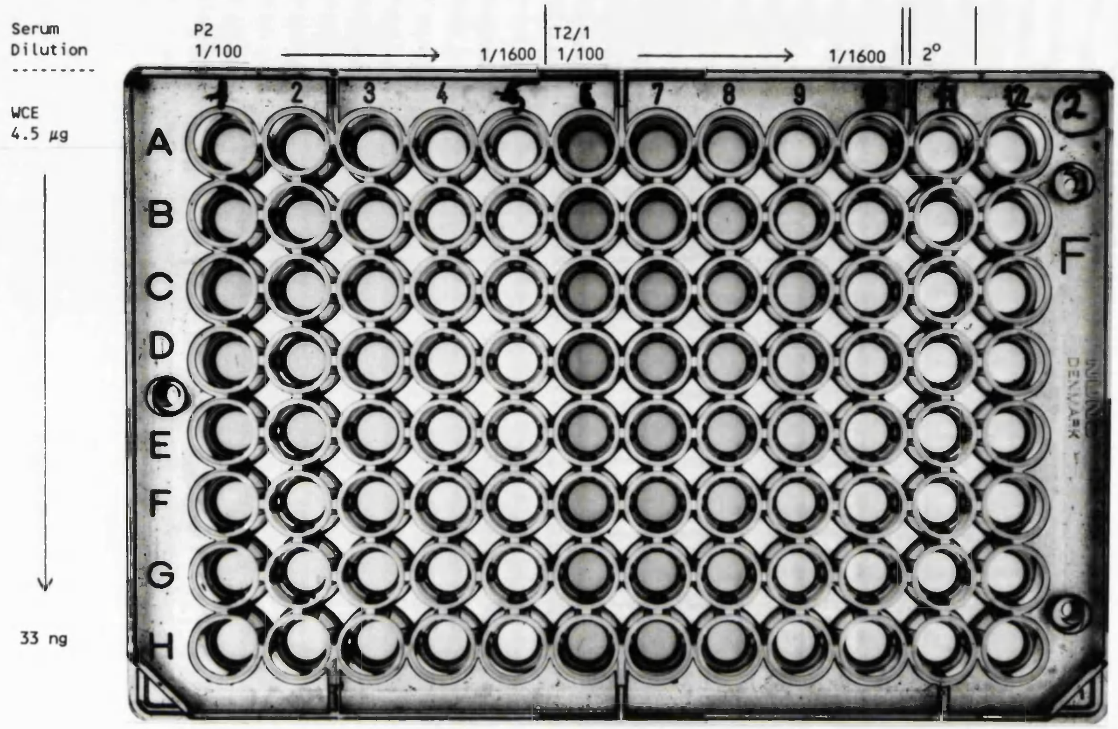
The ELISAs and the immunoblots performed with test sera from the immunised mice indicated that the animals were responding to the injected antigen and producing antibodies that recognized proteins of molecular weights consistent with them being components of DRTF-1 activity as characterised previously (Shivji and La Thangue, 1991). The same sera, when incubated with affinity-purified DRTF-1 and the *E2A* promoter, also "super-shifted" the DRTF-1/E2A complex.

Thus, although no single type of assay revealed an impressively strong immune response, their combined results suggested that the mice, especially 1 and 2, had responded.

None of the ELISA-positive supernatants of hybridomas derived from mouse 2 "super-shifted" or abolished the DRTF-1 "shift". And, although some of them recognized, by immunoblotting of a HS fraction, proteins whose molecular weights were consistent with them being components of DRTF-1 activity, none of them reacted, under the same conditions, with the affinity fraction. This failure to identify a specific reaction between the antibodies produced and DRTF-1, may originate from the weakness of the immune response. The number of α-DRTF-1 antibody producing cells in the animal may not have been high enough to give rise to a detectable number of the appropriate hybridomas. On the other hand, this failure may simply be a problem of detection. For example, the epitopes recognized on the partly denatured antigen on an ELISA plate may not be available for binding in the native protein assayed by gel retardation. At the same time, the antigen concentration in the affinity fraction may be below the level of detection by immunoblotting. A third explanation may be that DRTF-1 is not immunogenic and the antibodies produced were against unrelated contaminating antigens or even auto-antibodies against antigens that happened to be present in the HS fraction used for the screening, but not in the injected affinity fraction. However, given that the test sera indicated that the mice were responding against the right antigen and that at least one supernatant reacted against an affinity fraction in an ELISA assay, the second explanation seems more likely : i.e. that the limiting factor is detection. Since the only functional assay, i.e. gel retardation, failed, there was no way of confirming that any of the proteins detected by immunoblotting was the right one, and therefore it was not judged advisable to continue the analysis. Hopefully, greater availability and purity of antigen in the future, for example after the already successful isolation of a DRTF-1 cDNA (Girling *et al.*, submitted), will provide the means for identifying any α-DRTF-1 antibody producing clones with ease and certainty.

FIG. 10.1 : *ELISAs with test sera from mouse 2*

Examples of ELISAs with test sera from mouse 2. Rows A→H : serial two-fold dilutions of F9 EC whole cell extract (WCE), ranging from 4.5 μg/well (90 μg/ml) to 33 ng/well (660 ng/ml). Columns 1→5 and 6→10 : serial two-fold dilutions of the pre-immune and test sera indicated, ranging from 1/100 to 1/1600 (top), or 1/400 to 1/6400 (bottom). Column 11 : no serum. Column 12 : peroxidase substrate only. The blocking buffer was 3% BSA in PBS.


Serum
Dilution
WCE
4.5 µg
33 ng
P2
1/100
1/1600
T2/1
1/100
1/1600
2°
A
B
C
D
E
F
G
H
1
2
3
4
5
6
7
8
9
10
11
12
F
NUNC
DENMARK

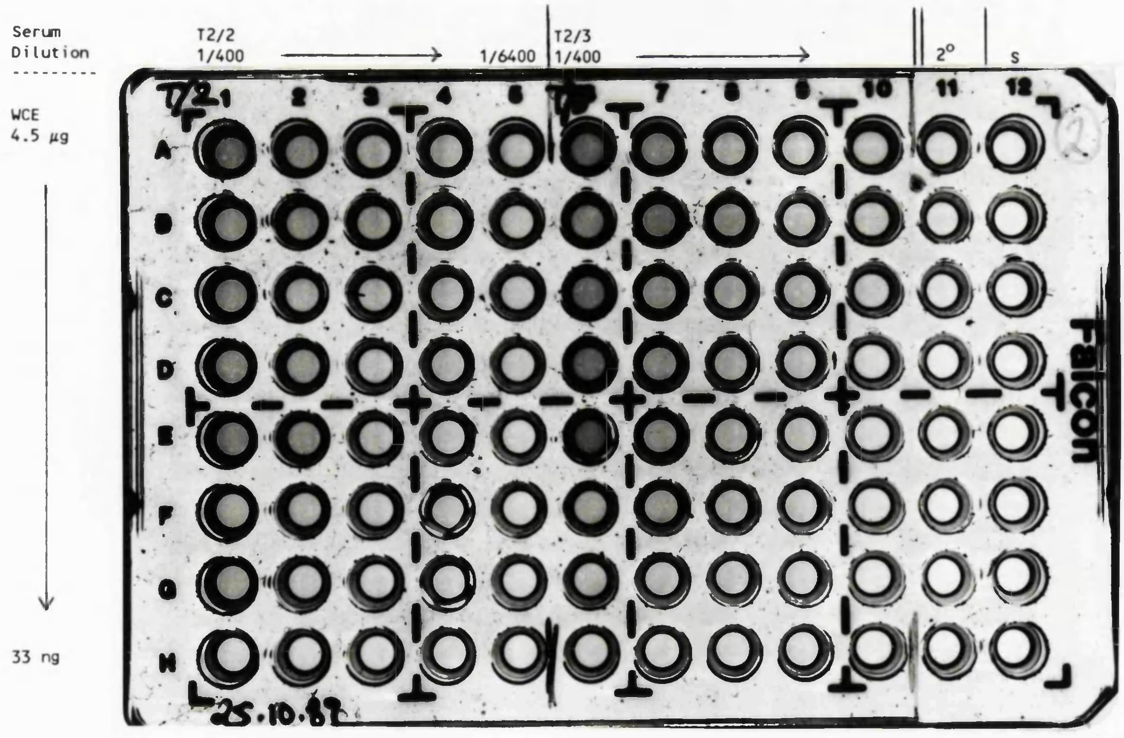

Serum
Dilution
WCE
4.5 µg
33 ng
T2/2
1/400
1/6400
T2/3
1/400
2°
S
T/2
A
B
C
D
E
F
G
H
1
2
3
4
5
6
7
8
9
10
11
12
Falcon
25.10.88

FIG. 10.2 : *Control "super-shift" experiment with α-Sp1 polyclonal antibody*

Gel retardation assay in the presence of polyclonal sera ; Probe : HSV tk promoter (lanes 1-9) ; Extract : F9 EC whole cell extract (lanes 2-9) ; PI : pre-immune rabbit serum ; (2892-pI ; lanes as indicated) ; α : α-Sp1 rabbit polyclonal serum raised against bacterially synthesized Sp1 (2893-E ; lanes as indicated). Volume/μl of serum used : 0.2 (lanes 2,3), 0.5 (lanes 4,5), 1 (lanes 6,7), 2 (lanes 8,9).

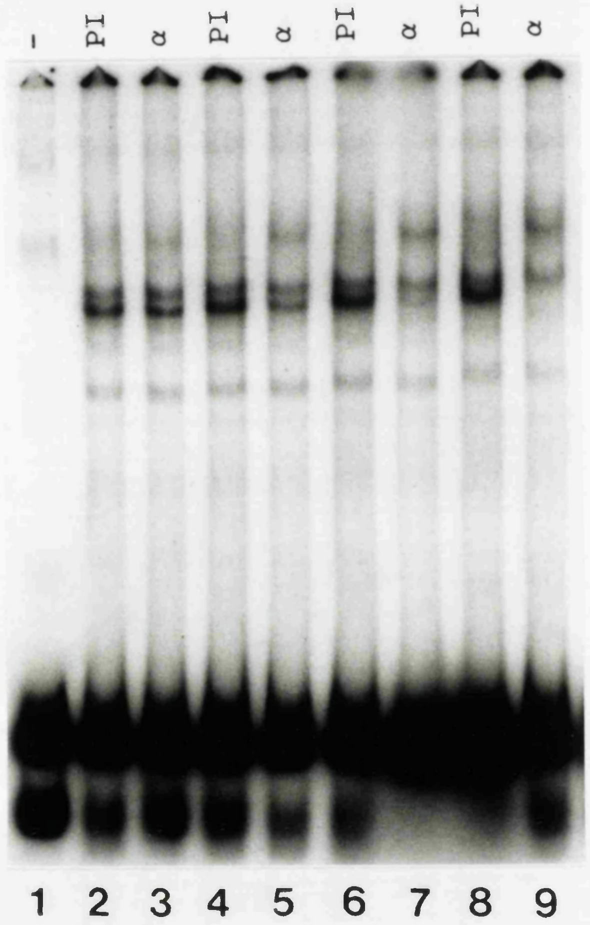

–
PI
α
PI
α
PI
α
PI
α
1
2
3
4
5
6
7
8
9

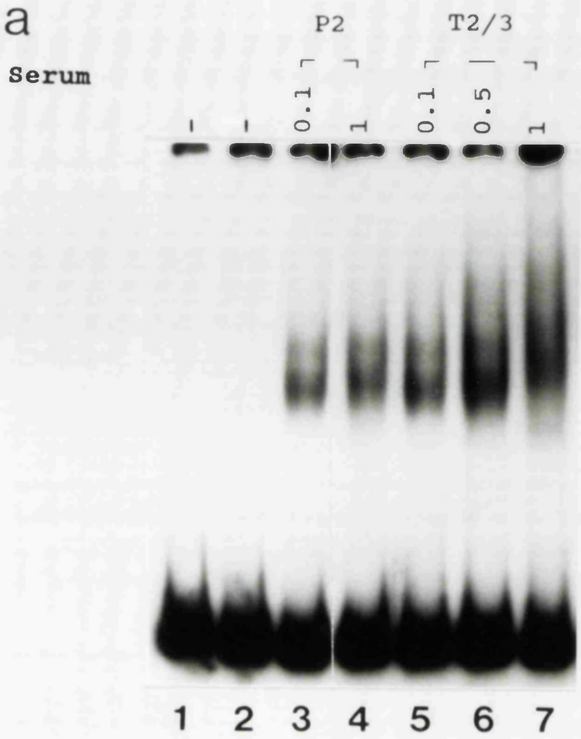
FIG. 10.3 : *a) Titration of test sera in a gel retardation assay*

Gel retardation assay in the presence of pre-immune (P2) and test (T2/3) sera from mouse 2 (volumes/μl as indicated). Probe : *E2A* (lanes 1-7) ; Protein : affinity purified DRTF-1 (0.5 μl ; lanes 2-7).

b) Specificity of "super-shifts" produced by test sera

Gel retardation assay in the presence of pre-immune and test sera (1.5 μl ; origin as indicated). Probe : *E2A* (lanes 1-18) ; Protein : affinity purified DRTF-1 (0.5 μl ; lanes 2-18) ; Oligonucleotide competitors (650-fold molar excess) : 19/20 or 63* (lanes as indicated ; sequences shown below):

19/20	TAGTTTTCGCGCTTAAATTTGA
63*	TAGTTTTCTCGCTTAAATTTGA


a
Serum
P2
T2/3
-
-
0.1
1
0.1
0.5
1
1
2
3
4
5
6
7

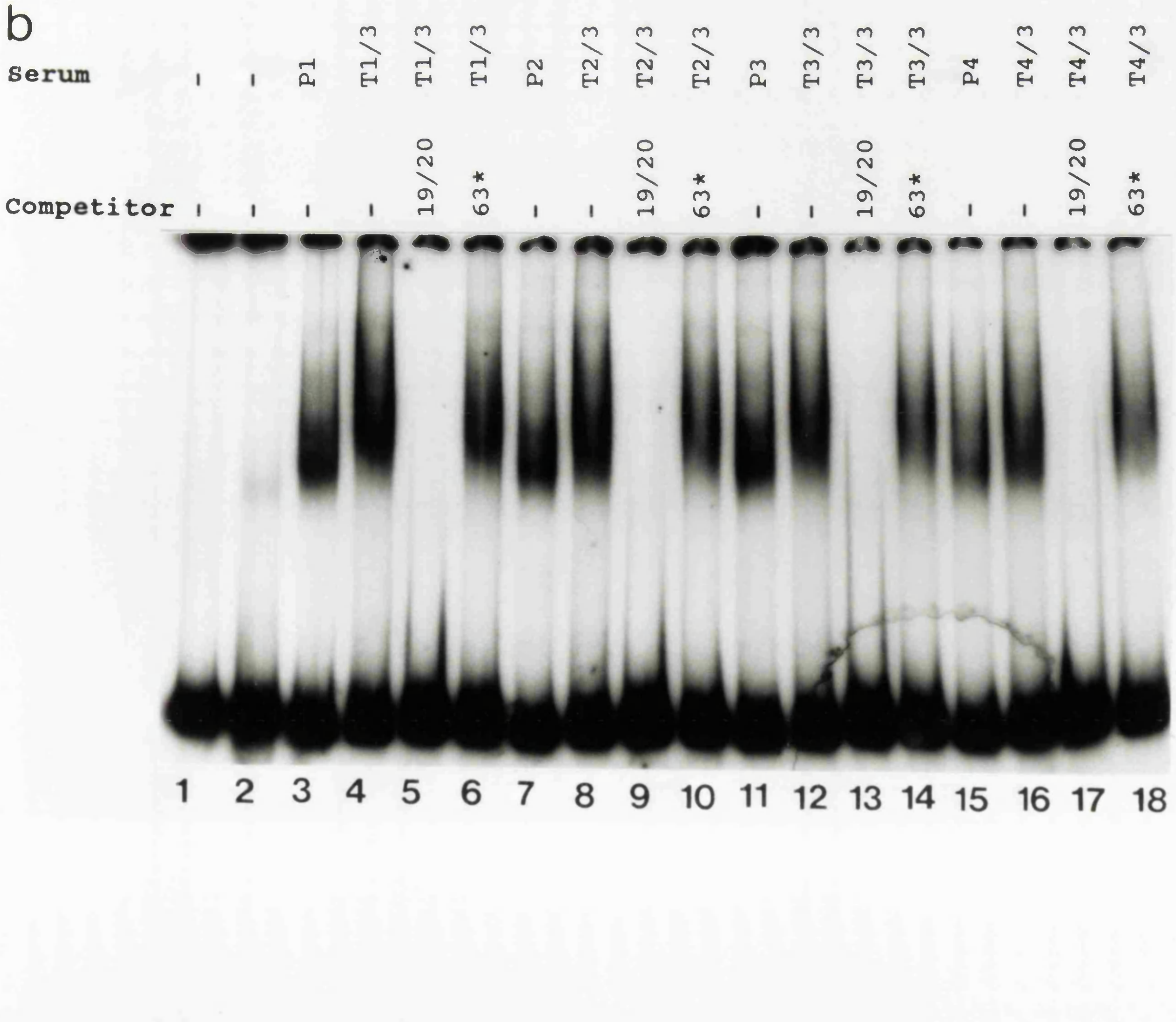
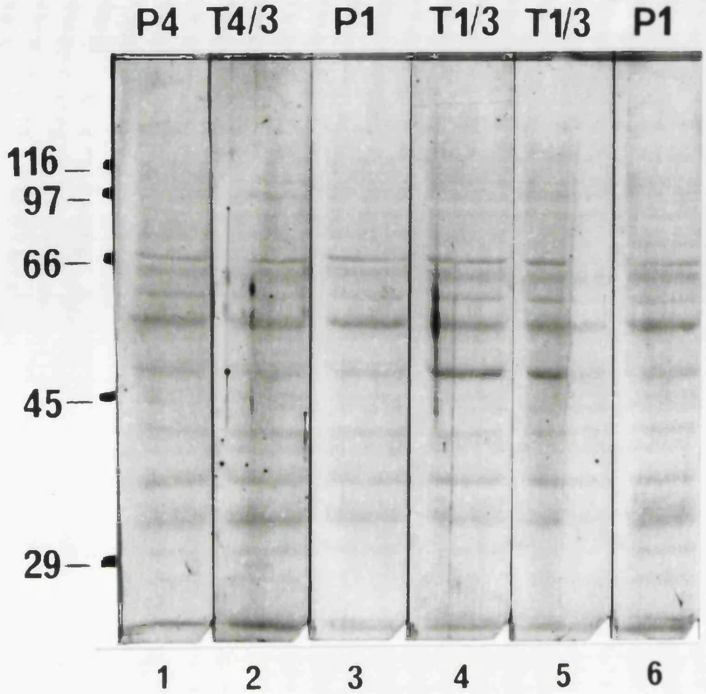

b
Serum
-
-
P1
T1/3
T1/3
T1/3
P2
T2/3
T2/3
T2/3
P3
T3/3
T3/3
T3/3
P4
T4/3
T4/3
T4/3
Competitor
-
-
-
-
19/20
63*
-
-
19/20
63*
-
-
19/20
63*
-
-
19/20
63*
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18

FIG. 10.4 : *Immunoblots with test sera*

a) Protein : heparin Sepharose column fraction (30 μg/lane). The membrane was blocked in 3% BSA. It was first incubated with the indicated pre-immune and test sera (1/100 dilution) for 1 h at r.t., and then with horseradish peroxidase-conjugated rabbit α-mouse immunoglobulins (1/500 dilution) for 1 h at r.t.. The enzyme reaction was stopped after 20 min. Positions of migration and sizes, in kD, of protein molecular weight standards are indicated to the left of the blot.

b) Protein : heparin Sepharose column fraction (30 μg/lane). The membrane was blocked in 3% BSA. It was first incubated with the indicated pre-immune and test sera (1/100 dilution) for 75 min at r.t., then with horseradish peroxidase-conjugated rabbit α-mouse immunoglobulins for 1 h at r.t.. Positions of migration and sizes, in kD, of protein molecular weight standards are indicated to the left of the blot.

a


P4
T4/3
P1
T1/3
T1/3
P1
116
97
66
45
29
1
2
3
4
5
6

b

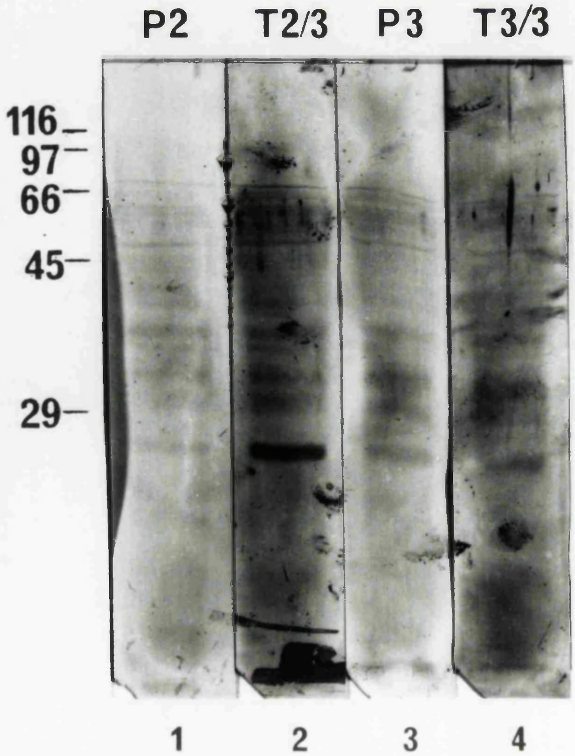

P2
T2/3
P3
T3/3
116
97
66
45
29
1
2
3
4

FIG. 10.5 : *Gel retardation assay in the presence of supernatants from the first screen of hybridomas in 96-well plates*

Probes : *E2A* and SV40 origin of replication (ori ; nucleotides 5175/37), as indicated ; Protein : heparin Sepharose column fraction (HS ; 200 ng) and purified SV40 large T antigen (T ; 150 ng), as indicated (lanes 11-13 also contain 5 mM ATP and 100 μg BSA) ; Supernatants : 419 (lanes 11 and 13 : 2 μl) contains α-T monoclonal antibody 419 (L19 in Harlow *et al.*, 1981) ; 1C2 and 3G6 (lanes 2, 7 and 3, 8, respectively ; 10 μl) came from wells that were positive, 1F2 and 3B6 (lanes 4, 9 and 5, 10, respectively ; 10 μl) from wells that were negative, in the ELISA.

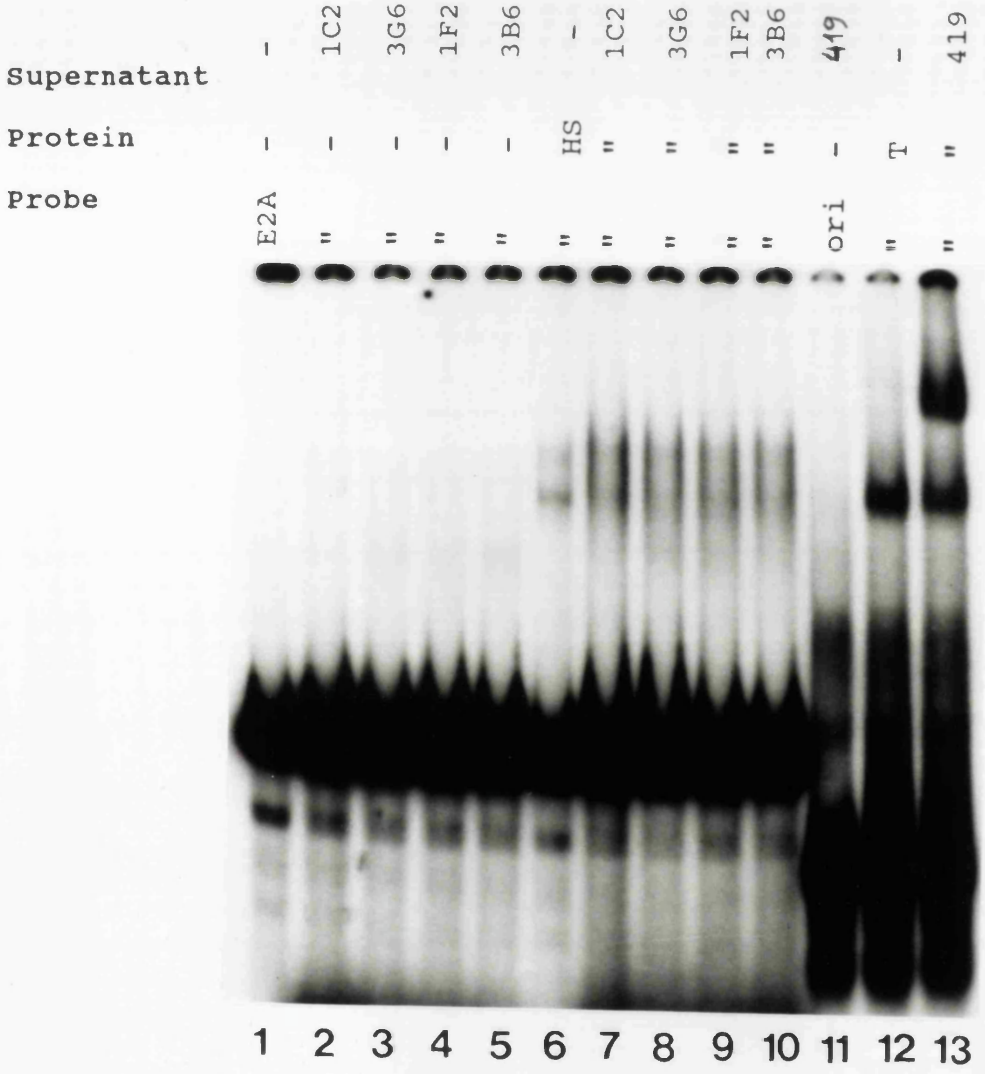
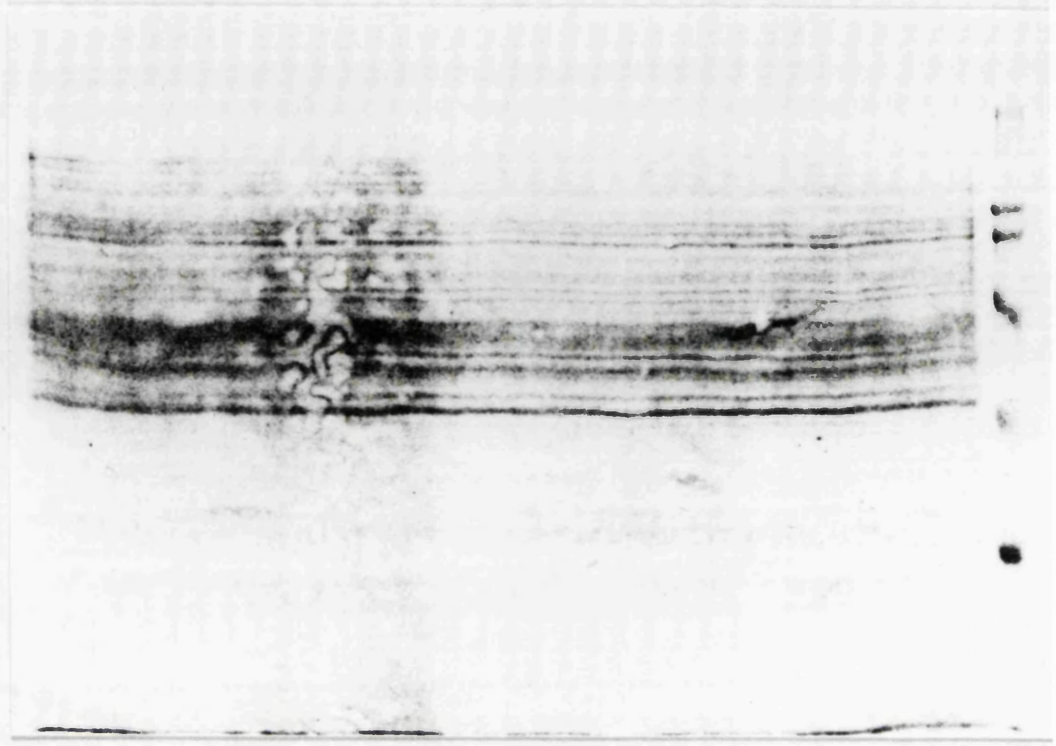

Supernatant
–
1C2
3G6
1F2
3B6
–
1C2
3G6
1F2
3B6
419
–
419
Protein
–
–
–
–
–
HS
"
"
"
"
–
T
"
Probe
E2A
"
"
"
"
"
"
"
"
"
ori
"
"
1
2
3
4
5
6
7
8
9
10
11
12
13

FIG. 10.6 : *Immunoblot with the first supernatants from 24-well plates*

a) Ponceau-S stained protein blot used for immunoblots shown in (b). Protein : heparin sepharose column fraction (5 μg/lane shown in (b)). Protein molecular weight standards are on the right side of the blot. (The "bubbles" seen towards the left of the blot are an artefact which occurred during photocopying of the wet nitrocellulose filter, and do not reflect irregularities in protein transfer.)

b) Immunoblot. The nitrocellulose strips were incubated overnight at 4 °C with the indicated supernatants (419 : α-T monoclonal antibody ; 6A7 : monoclonal antibody against the 70 kD subunit of human DNA polymerase α ; all others are supernatants from 24-well plates), then for 4 h at r.t. with alkaline phosphatase-conjugated rabbit α-mouse immunoglobulins. The enzyme reaction was stopped after 15 min. Sizes, in kD, of protein molecular weight standards (M) in lane 1 are indicated to the left of the figure. The membrane was blocked with foetal calf serum. [This experiment was performed jointly with R.Girling].

a



b

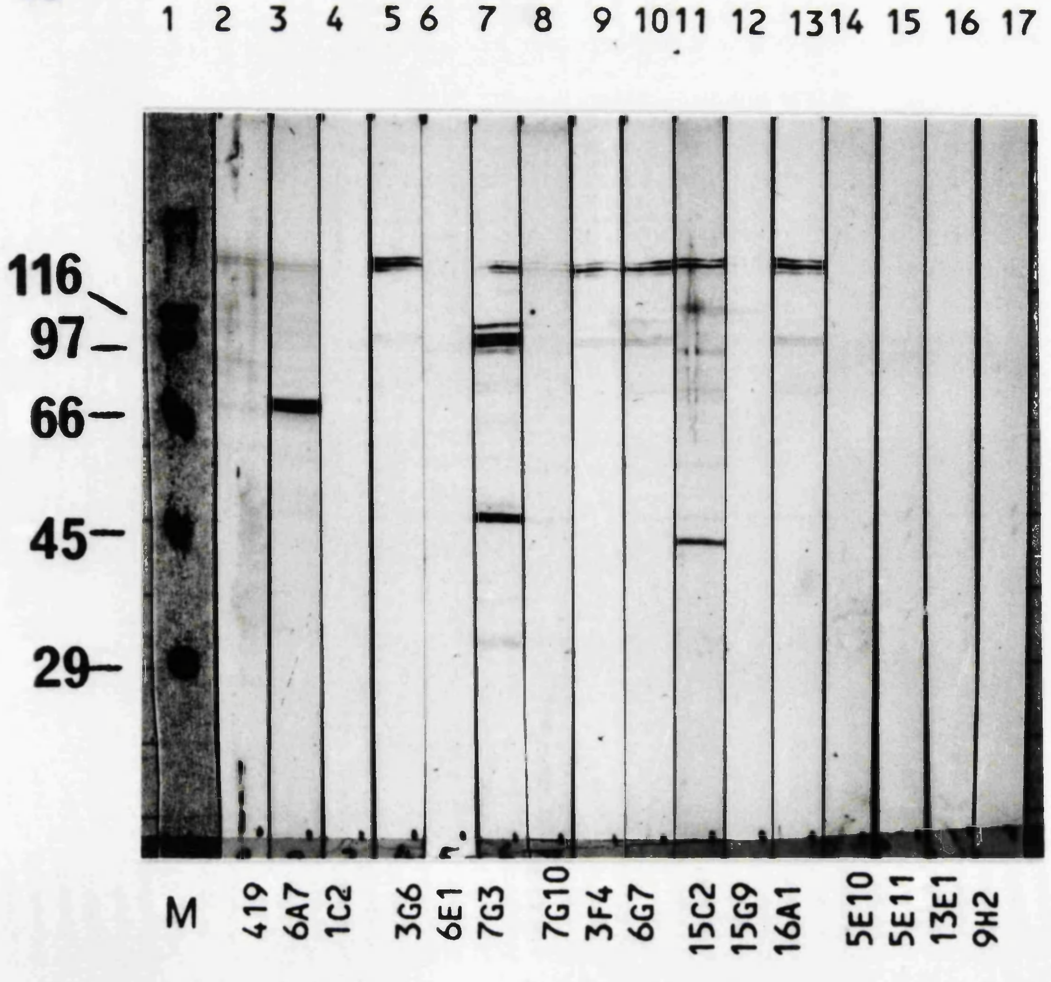
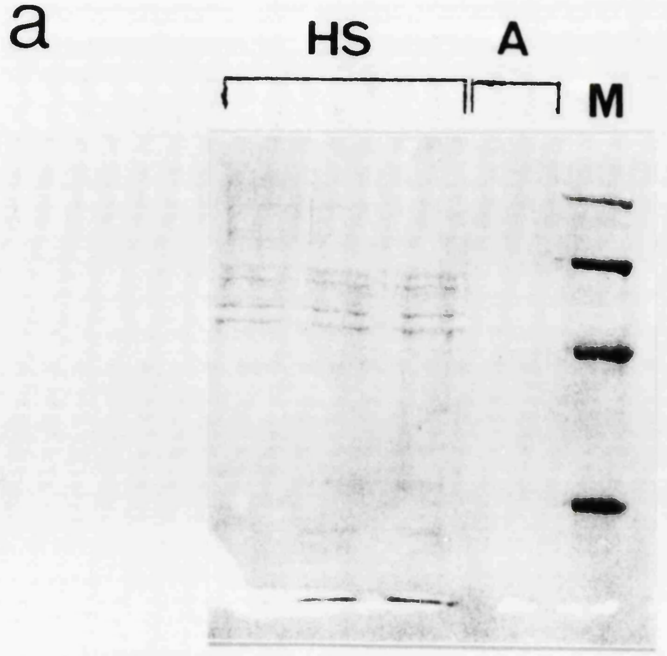
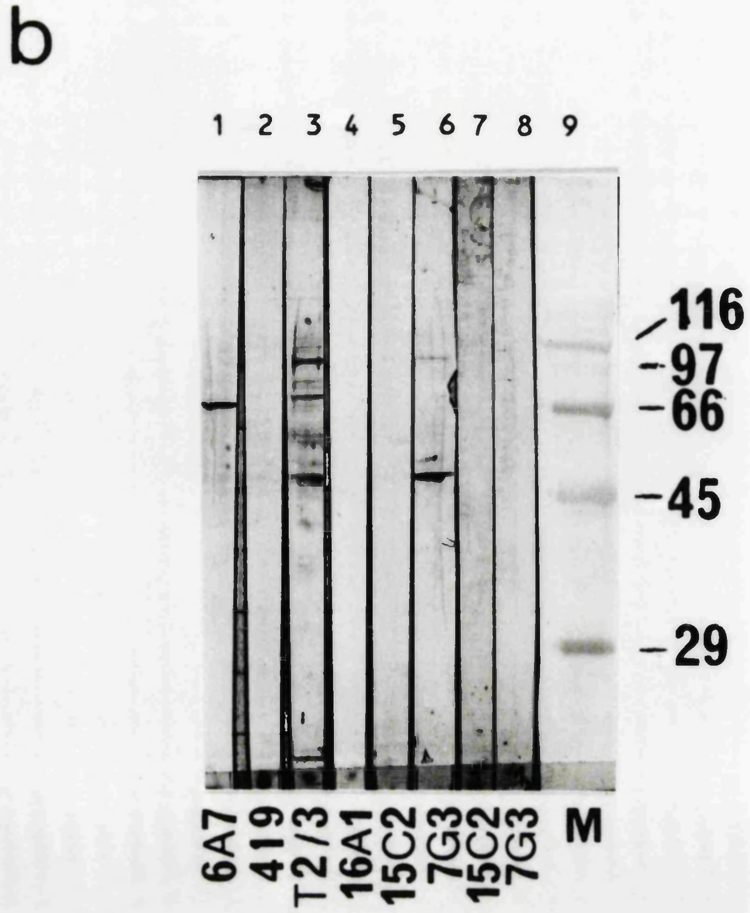

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
116
97
66
45
29
M
419
6A7
1C2
3G6
6E1
7G3
7G10
3F4
6G7
15C2
15G9
16A1
5E10
5E11
13E1
9H2

FIG. 10.7 : *Immunoblot with 24-well plate second supernatants*

a) Ponceau-S stained protein blot used for immunoblots shown in (b). Protein : heparin sepharose column fraction (HS ; 5 µg/lanes 1-6 in (b)) and affinity column fraction (A ; 15 µl/lanes 7, 8 in (b)). M : protein molecular weight standards.

b) Immunoblot. The nitrocellulose strips were incubated overnight at 4 °C with the indicated supernatants (for description of 6A7 and 419 see Fig.10.6 ; T2/3 test serum is diluted 1/200 ; all others are supernatants from 24-well plates), then for 3 h at r.t. with alkaline phosphatase-conjugated rabbit α-mouse immunoglobulins. The enzyme reaction was stopped after 20 min. The sizes, in kD, of protein molecular weight standards (M) in lane 9 are indicated to the right of the figure.


a
HS
A
M


b
1 2 3 4 5 6 7 8 9
116
97
66
45
29
6A7
419
T2/3
16A1
15C2
7G3
15C2
7G3
M

CHAPTER 11

GENERAL DISCUSSION

11.1 Summary and perspectives

The aim of this study was to examine aspects of transcriptional regulation in the context of a differentiating cell system which models early events of murine embryogenesis. A number of interesting observations were made, illustrating the important role that transcription factor families can play during development.

The results presented here have demonstrated that a family of six DNA binding activities, the ECRE family, that recognize the ATF site, exist in murine F9 EC cells and their differentiated derivatives. Members of this family differ in the electrophoretic mobility of their DNA complexes and in their regulation during differentiation of EC cells : ECRE-2, for example, is detectable in both cell types, while DNA binding by ECRE-3 is down-regulated as EC cells differentiate. These ATF site-binding activities are distinguished further by their ability to bind DNA which is mutated within or near the ATF site. ECRE-2, for example, only binds the wild type site, ACGTCA ; ECRE-1 and ECRE-4, on the other hand, also bind the point-mutated site, AC$\underline{\text{A}}$TCA. Differences in binding site preference between ECRE activities extend to their selective binding to promoters which contain ATF sites : ECRE-2 binds to the *VIP* but not the *E4* promoter, while ECRE-1 and ECRE-4 exhibit the converse specificity. Since the most important *cis*-acting elements of these promoters are their ATF sites, it is presumably their differences in ECRE binding that are responsible for their different transcriptional activities *in vivo* : in contrast to *E4*, *VIP* is transcriptionally inactive in F9 EC and PE cells. Similarly, the increased transcription from the *E4* promoter after differentiation correlates with a change in the ECRE activities that bind to it in PE cells compared to EC cells.

The existence of a multiplicity of ATF site-binding activities in F9 EC and PE cells reflects the existence of a mammalian *ATF* gene family, which so far comprises of ten cDNAs isolated by various laboratories. In an attempt to relate the products of these cDNAs to the ECRE activities, the expression of five *ATF* genes was tested. *ATF2*, *ATF4* and *ATF6*, are expressed to the same extent in both EC and PE cells, while *ATF3* RNA levels increase during differentiation, and no *ATF1* RNA is detected in either cell type. These results therefore suggested three candidate genes, *ATF2*, *ATF4* and *ATF6*, that could encode ECRE-2 and ECRE-4/PCRE-1, but no obvious candidates for the down-regulated ECRE activities. However, as experiments with *in vitro* translated ATF polypeptides demonstrated, dimerization can affect DNA binding specificity and, consequently, constant RNA or even protein levels need not necessarily result in constant DNA binding activity. For example, *junD* is expressed in both F9 EC and PE cells. However, the JunD/ATF2 dimer binds to the ATF site, while JunD/cFos binds to the TRE. Assuming, for the sake of argument, that JunD/ATF2 represents an ECRE activity, it is therefore conceivable that cFos, which is synthesized only after differentiation, sequesters JunD away from the ATF site and onto the TRE. This would result in the down-regulation of the ECRE activity, even though neither *ATF2* nor *JunD* RNA levels decrease during differentiation. Anti-peptide polyclonal antibodies were therefore raised against regions of ATF2 and ATF3 in order to investigate further the relationship between these ATF proteins and the ECRE activities. Unfortunately, using a gel retardation assay, no cross-reactivity between unpurified anti-ATF2 or anti-ATF3 sera and the ECRE activities in whole cell extracts was detected. An anti-cJun serum that also reacted with JunD, which is expressed in both EC and PE cells, and can bind to an ATF site as a heterodimer with ATF2 or ATF3, did recognize ECRE-1, however. It is possible, therefore, that JunD is contained in ECRE-1. Despite its failure to react with any of the ECRE activities, anti-ATF2 serum was, nevertheless, successfully used to characterise ATF2 : it is a cluster of nuclear polypeptides, of 41-45 kD, whose levels remain constant during F9 EC cell differentiation, thus reflecting the unchanging *ATF2* RNA levels.

This study has therefore described the regulation and DNA binding specificity of ATF site-binding activities in the F9 EC cell system, and related them to the transcription of ATF site-containing promoters. However, the intended identification of these activities with defined members of the ATF family proved unsuccesssful, since neither the anti-ATF2 nor the anti-ATF3 sera affected the ECRE/DNA complexes in a gel retardation assay. Perhaps the sensitivity of detection could be improved by partial purification of both extracts and sera. It should be noted, though, that an anti-Jun serum did "super-shift" ECRE-1 under the conditions used. An additional problem was that the chosen ATF3 epitope appeared to be denaturation-sensitive, since anti-ATF3 sera reacted with native *in vitro* translated ATF3 but not with denatured *in vitro* translated or cellular ATF3. This problem might perhaps be overcome by raising antisera against the appropriate fusion proteins expressed in bacteria ; such sera should react with a greater variety of epitopes. Alternatively, sera against a number of peptide epitopes from each protein could be raised, in the hope of obtaining some that would recognize the native and/or the denatured polypeptides. The failings of the antisera did not allow the *in vivo* heterodimerization hypothesis to be tested, although the presence of polypeptides that co-immunoprecipitated with ATF2 but were not recognized by anti-ATF2 in an immunoblotting experiment suggests that ATF heterodimerization may indeed occur *in vivo*.

Even though these specific antisera had the mentioned shortcomings, the immunochemical approach still remains the most valid single method for establishing the relationship between ECRE activities and ATF proteins. DNA binding site affinity purification could perhaps be used as an alternative method for eventually obtaining cDNAs for the ECRE activities, though this method may not necessarily be appropriate in all cases, as exhaustive but unsuccessful attempts to purify ECRE-3 have suggested (M.Zamanian, unpublished observations).

Regardless of these problems, the present study has yielded some results that can be readily followed up. For example, it should be feasible to screen a λgt11 expression library with anti-PEP2 in order to clone the cDNA for the ECRE-1 protein that is recognized by that serum. The anti-ATF2 sera, which exclusively stain the nuclei of cells (Tassios and La Thangue, submitted), could also be used in further immunocytochemical experiments, to examine, for example, whether ATF2 and E1A co-localise after adenovirus infection, given that E1A is thought to interact with ATF2 (Liu and Green 1990). The anti-P10 serum, on the other hand, could be used to investigate the differences in structure and stability of different dimers, since it inhibited the DNA binding of ATF3 homodimers but not heterodimers. This inhibition could be due to disruption either of homodimerization or of contact to the DNA. These two possibilities could be distinguished by assessing the efficiency of co-immunoprecipitation of an epitope-lacking but dimerization-competent ATF3 polypeptide with a wild type ATF3 polypeptide. Co-precipitation of the epitope-lacking ATF3 with wild type ATF3 would mean that dimerization was not disrupted by antibody binding and that therefore the inhibitory effect of the anti-serum was on DNA binding. On another level, it would be interesting to establish whether expression of the *ATF2* and *ATF3* genes, whose steady state RNA levels are regulated in distinct fashions during differentiation of F9 EC cells, those of *ATF2* remaining constant while those of *ATF3* are induced, is controlled at the level of transcription, and if so, to make a comparative study of the two genes' transcriptional elements and activities.

In conclusion, ATF site binding activities in F9 EC and PE cells have been shown to constitute a model family of DNA binding proteins, with respect to their complex DNA binding specificities, heterodimerization, regulation during differentiation and binding to diverse promoters.

Such characteristics are shared by transcription factor families in general ; they, and their relevance to transcriptional control during development, are discussed in the sections that follow.

11.2 The versatility of transcription factor families

It is now recognized that transcription factors can be grouped into families, according to sequence similarities in their DNA binding domains and recognition sites. Members of such families are thought to be used during mouse development, for example in transcriptional "cascades" (the myogenic transcription factors ; see below), or for providing an identity code for similar components of an organism (the *Hox* (murine homoeobox) genes and the vertebral column ; Kessel and Gruss, 1991), or fulfilling presumably similar roles in different parts of the organism where they are differentially expressed (the retinoic acid receptors ; see below). Of course, transcription factor families have been extensively studied in other developmental systems, from σ factors during sporulation of *Bacillus subtilis* (reviewed by P.Stragier, 1991), and proteins directing the mating type switch in yeast (reviewed by Herskowitz, 1989), to those *D.melanogaster* homoeodomain proteins which set up the antero-posterior embryonic axis (reviewed by Nüsslein-Vollhard, 1991) or define segment polarity and identity (reviewed by Levine and Harding, 1989). However, there is now such a wealth of examples from murine development that this discussion will mostly focus on them, and specifically on those that display similarities to aspects of the ATF family.

11.2.1 Alternative splicing

Indeed, it seems that all the mechanisms for generating even more diversity from transcription factor families than that already provided by their different member genes, are encountered in the ATF family. One such mechanism is alternative splicing of primary transcripts. Six distinct mouse *CREB* transcripts have been characterised in F9 EC cells (Ruppert *et al.*, 1992), arising from alternative splicing, promoter usage and polyA sequences. The rat and human equivalents of two of these transcripts, *CREBα*, the longest known *CREB* transcript, and *CREBΔ*, lacking the 14 amino acid α exon, have also been characterised (Yamamoto *et al.*, 1990 ; Berkowitz and Gilman, 1990). Reports are conflicting as to their relative *trans*-activating potentials, Yamamoto *et al.* reporting that CREBΔ is 10 times less efficient than CREBα in activating transcription, while the other two groups observe no such difference. In addition, two other transcripts, *CREBαγ* and *CREBγ*, which both lack the DNA binding domain, are present in the primary spermatocytes of the adult testis at much higher levels than in any other tissue (Ruppert *et al.*, 1992) ; their function is unclear. An alternative transcript of *ATF2* is also preferentially expressed in the adult, but not the new-born, thymus or the adult spleen (Georgopoulos *et al.*, 1992). Furthermore, three characterised *ATF2* transcripts have distinct abilities to stimulate transcription upon co-transfection with a T-cell-specific enhancer to which they can all bind *in vitro* (Georgopoulos *et al.*, 1992), a situation reminiscent of the distinct ligand binding and *trans*-activation properties of different thyroid hormone receptor α isoforms (Izumo and Mahdavi, 1988). Similarly, an alternatively spliced form of Pit-1/GHF1, GHF2, which contains an additional twenty-six amino acids in the activation domain, retains the same binding specificity as GHF1, but only activates a subset of the GHF1-inducible genes (Theill *et al.*, 1992). Alternative splicing has also been reported for *ATFa* (Gaire *et al.*, 1990), and *fosB* transcripts (Mumberg *et al.*, 1991 ; Dobrzanski *et al.*, 1991). No functional comparison has been made of the two ATFa proteins ; however, the short isoform of FosB, lacking the 101 carboxy-terminal amino acids of the full length protein, is unable to transform cells and repress the c*fos* promoter, though both long and short isoforms *trans*-activate through a TRE. Distinct mRNA molecules, arising not only from alternative splicing and the use of more than one promoters but also from different translational initiation codons, also give rise to multiple isoforms of the three retinoic acid receptors (RARs) (Zelent *et al.*, 1991 ; Nagpal *et al.*, 1992). Alternative transcripts of transcription factor genes can therefore exhibit not only different temporal and spatial expression patterns and *trans*-activation potentials during development, but also distinct DNA binding specificities, as shown for the two *Drosophila* Tramtrack isoforms which contain alternative sets of zinc fingers (Read and Manley, 1992).

11.2.2 Antagonists of transcriptional activators

Alternatively spliced transcripts of transcriptional activator genes can also give rise to specific transcriptional antagonists, as in the case of the basic domain/helix-loop-helix (bHLH) protein gene *TFE3* (immunoglobulin enhancer μE3 motif-binding transcription factor) : exclusion of an exon generates a shorter form of this transcription factor, which is expressed to varying levels in different mouse tissues (Roman *et al.*, 1991). The resulting polypeptide binds DNA, since it has a fully functional DNA binding domain, but does not stimulate transcription, since it lacks an activation domain. On the other hand, an inhibitor of CREB activity, CREM, is encoded by a separate gene containing two bzip regions. Alternatively spliced transcripts, *CREMα*, containing both bzip's, *CREMβ* containing only the carboxy-terminal one, and *CREMγ*, differing from *CREMβ* by a deletion of 36 nucleotides near the amino-terminus, are differentially expressed in adult mouse tissues (Foulkes *et al.*, 1991). All three resulting CREM proteins contain a fully functional DNA binding domain through which they bind to the ATF site as CREM homodimers or CREM/CREB heterodimers. Co-expression of *CREB* and *CREM* inhibits ATF site-dependent transcription, presumably because CREM lacks an activation domain (Foulkes *et al.*, 1991). However, a different temporally and spatially regulated *CREM* transcript encodes a transcriptional activator, CREMτ, which incorporates two relatively glutamine-rich amino acid stretches in the CREMβ protein and appears in the testis at the onset of spermatogenesis (Foulkes *et al.*, 1992). Conversely, a 21 kD antagonist of CREMτ, lacking one of these two insertions, is produced by translational initiation from an internal AUG in *CREMτ* RNA (Delmas *et al.*, 1992). Most CREM isoforms, therefore, inhibit transcription by sequestering CREB activator molecules into complexes that can bind DNA but cannot *trans*-activate. Id (inhibitor of differentiation) proteins, on the other hand, prevent some bHLH proteins from activating transcription by a different mechanism : they can heterodimerize with, for example, the activator E12, but the resulting dimer cannot bind to a bHLH recognition site ("E-box") since Id lacks the basic domain that is required for DNA binding (Benezra *et al.*, 1990 ; Sun *et al.*, 1991). *Id* RNA levels are regulated during differentiation of a variety of cell types (Benezra *et al.*, 1990 ; Sun *et al.*, 1991). Forced constitutive expression of *Id* can block both differentiation of a myeloid precursor cell line and the consequent appearance of "E-box" binding activity (Kreider *et al.*, 1992). Id also forms inactive complexes with E12 and E47, thus inhibiting the formation of transcriptionally active dimers of, for example, MyoD and E12, and, consequently, not allowing muscle differentiation to proceed (Jen *et al.*, 1992).

Transcription factor families, therefore, increase their versatility by super-imposing on the multiplicity of their member genes alternative splicing, transcriptional and translational starts. Alternative transcripts can include or exclude transcriptional activation or DNA binding domains, giving rise, in the latter case, to transcriptional antagonists belonging to the same family. The expression of both activators and their antagonists is under temporal and spatial control, thus rendering the regulation of transcription from the cognate DNA binding site during development even more flexible.

11.2.3 Different members of transcription factor families display distinct expression patterns and responses to stimuli

As was shown in Chapter 6, RNA levels of *ATF3*, c*jun* and c*fos* differ between undifferentiated and differentiated EC cells. In the mouse embryo also, c*jun* and *junB* show distinct patterns of *in situ* RNA hybridization (Wilkinson *et al.*, 1989), as do members of the RAR gene family and the related RXR (retinoid X receptor) gene family (Ruberte *et al.*, 1991 ; Mangelsdorf *et al.*, 1992). Such differential expression may have functional consequences, since, for example, cJun activates transcription through the c*jun* and collagenase TREs whereas JunB does not (Chiu *et al.*, 1989 ; Schütte *et al.*, 1989). This is presumably due to the lower affinity of the JunB/cFos dimer for the TRE, compared to cJun/cFos (Ryseck and Bravo, 1991), as well as to differences in their activation domains (Chiu *et al.*, 1989). Similarly, DNA binding and RNA levels of different proteins recognizing the octamer motif have overlapping but distinct spatial and temporal distributions in the mouse embryo (Schöler *et al.*, 1989 ; Rosner *et al.*, 1990 ; Stoykova *et al.*, 1992). Therefore, distinct patterns of expression of different family members in the adult organism or during embryogenesis presumably provide another way of expanding the repertoire of *trans*-regulatory effects that can be achieved through a single *cis*-acting element.

Different members of a transcription factor family can respond to different signals. In the ATF family, CREB and ATF1, but not ATF2, positively regulate transcription in response to protein kinase A activation (Yamamoto *et al.*, 1988 ; Gonzalez *et al.*, 1989 ; Liu and Green, 1990 ; Rehfuss *et al.*, 1991). On the other hand, ATF2, but not ATF1 or ATF3, binds to the retinoblastoma gene product (pRb), a negative regulator of proliferation (reviewed by La Thangue, 1992), and activates the transforming growth factor β2 promoter through a pRb-inducible ATF site (Kim *et al.*, 1992). In the AP1 family, c*fos* and *jun*B but not c*jun* RNA levels increase with the rise of intracellular Ca^{+2} ions during membrane depolarization of PC12 cells (Bartel *et al.*, 1989). Furthermore, induction of c*fos* transcription under these conditions has been shown to be mediated by phosphorylated CREB, which is therefore a substrate of calcium-calmodulin-dependent (CaM) protein kinases I and II, as well as of protein kinase A (Sheng *et al.*, 1990). The CaM kinases are activated in response to elevated intracellular calcium concentration after membrane depolarization of neuronal cells (Nairn and Greengard, 1987) and ATF sites have been shown to be responsible for the transcriptional induction of the c*fos* and proenkephalin genes by intracellular calcium and membrane depolarization (Sheng *et al.*, 1988 ; Van Nguyen *et al.*, 1990). Such selective responses to extracellular and intracellular stimuli presumably determine the involvement of transcription factors in specific developmental processes, since, for example, membrane depolarization, by altering short and long term gene expression in neuronal cells, is thought to contribute to the establishment of their identity and position in the synaptic network of the nervous system (reviewed by Sheng and Greenberg, 1990 ; He and Rosenfeld, 1991). The ATF site also plays an important role in a different regulatory network, namely the positive autoregulation of G protein-coupled receptors, such as the β_2 adrenergic receptor, which activate adenylyl cyclase, thus generating high intracellular cAMP levels (reviewed by Collins *et al.*, 1992).

As well as being regulated by different kinases, ATF proteins respond to a number of viral *trans*-activators. E1A can *trans*-activate *via* ATF2 but not ATF1 (Liu and Green, 1990). The human T cell leukaemia virus I (HTLV-I) Tax (transcriptional activator) protein also *trans*-activates its own promoter *via* an ATF site (Fujisawa *et al.*, 1989) by binding to at least three ATF proteins (Yoshimura *et al.*, 1990), while the hepatitis B virus (HBV) X protein can induce binding of ATF2 and CREB to a non-consensus ATF site in the HBV enhancer (Maguire *et al.*, 1991). Proteins binding to the ATF site, therefore, can respond to a variety of stimuli and participate in a number of regulatory networks.

11.2.4 Transcription factor dimerization

Members of the same transcription factor family can also deliver diverse regulatory effects by displaying subtle differences in binding site-, and therefore target gene-specificity, as exemplified by the ECRE activities described in this study. An efficient way of achieving this is through heterodimerization, as demonstrated by the cJun/ATF2 dimer, which binds to the ATF site, compared to cJun/cFos, which binds to the TRE. Similarly, a comparative study of affinity chromatography-purified, SDS-PAGE-separated and renatured CREB/CREB, CREB/ATF1 and ATF1/ATF1 dimers, suggested that they differ in their binding affinities as well as their *trans*-activation efficiencies *in vitro* (Wada *et al.*, 1991). Heterodimers that display increased binding efficiency with respect to homodimers include Jun/Fos compared to Jun/Jun (Halazonetis *et al.*, 1988), and NFκB p50/p65 compared to p50/p50 (Urban and Bauerle, 1991). Significantly, Fos/Jun and Jun/Jun dimers bend DNA in opposite orientations (Kerppola and Curran, 1991). Thus, different dimers could make different contacts with the transcriptional initiation complex, presumably affecting transcriptional initiation in a non-identical fashion. All these aspects of transcription factor activity regulation by heterodimerization are also encountered in the bHLH family. E12, for example, binds DNA only as a heterodimer (Sun and Baltimore, 1991) and different bHLH protein dimers have distinct preferences for variations of the CANNTG (where N is any nucleotide) consensus binding site (Blackwell and Weintraub, 1990). In addition, the full *trans*-activation and myogenic effects of MyoD depend on dimerization with the products of the ubiquitously expressed *E2A* gene (Lassar *et al.*, 1991), while two alternative transcripts of the *max* gene, whose protein products dimerize with Myc (Blackwood and Eisenman, 1991 ; Blackwood *et al.*, 1992), have opposite effects on *myc-ras* cotransformation of primary cells (Mäkelä *et al.*, 1992). Furthermore, DNA binding of Max homodimers but not that of Myc/Max heterodimers is inhibited by casein kinase II *in vitro* phosphorylation (Berberich and Cole, 1992). Thus, heterodimerization, a mechanism that regulates transcription factor activity by controlling the efficiency and specificity of DNA binding, is itself regulated by post-translational modifications. It is also clear that, although heterodimerization is, in principle, an efficient way of enhancing the repertoire of transcriptional regulators and integrating different stimuli, it is not promiscuous (Hai *et al.*, 1989 ; Hai and Curran, 1991 ; Sun *et al.*, 1991) : heterodimerization increases the number of specific responses rather than abolishes specificity altogether. Dimerization between ATF and AP1 proteins, for example, provides one more "cross-talk" point between the PKA and the PKC pathways (Otte *et al.*, 1989), since members of the ATF family respond to PKA (Gonzalez *et al.*, 1991 ; Rehfuss *et al.*, 1991), while members of the AP1 family respond to PKC activation (Lee, W. *et al.*, 1987).

11.2.5. Distinct DNA binding specificities of transcription factor family members

All members of a transcription factor family have, by definition, overall highly similar DNA binding domains and, as a result, can bind to the same DNA site under appropriate conditions *in vitro*. However, it is the differences in these homologous amino acid sequences which determine individual preferences for nucleotides deviating from the consensus recognition site and which will therefore presumably also define distinct *in vivo* targets for each member. In the absence of other cooperative factors, such individual preferences will presumably be guided by the compatibility between protein structure and DNA structure, as well as optimum electrostatic attraction and hydrogen bond formation. It has been shown, for example, that each of the three Krox20 zinc fingers recognizes either a GGG or a GCG triplet within the nonamer recognition site, depending on the amino acids present in two critical positions of each finger (Nardelli *et al.*, 1991). The functional importance of a few amino acids in the interaction of a transcription factor with other proteins and the DNA is borne out most spectacularly in the case of I-POU (inhibitor of POU), a *Drosophila* member of the POU transcription factor family (including Pit-1, Oct proteins and Unc-86) (Herr *et al.*, 1988), which forms non-DNA binding heterodimers with the transcriptional activator Cf1-a (C element binding factor 1a), thus preventing the activation of a neuron-specific gene (Treacy *et al.*, 1991). An alternatively spliced form, "twin of I-POU", however, which differs from I-POU only by two amino acids in the homoeodomain, does not dimerize with Cf1-a but can act independently as a transcriptional activator, though through a different target sequence than Cf1-a (Treacy *et al.*, 1992). By contrast, ECRE-2 presents the converse situation : a single protein surface binds to an isolated ATF site but not to that same site when it is in the context of the *E4* promoter. Though the linear nucleotide sequence remains identical, it is possible that its structure is altered by the presence of neighbouring sequences, rendering it incapable of recognizing a previously compatible protein structure, in an analogous fashion, perhaps, that identical Pit-1 binding site core sequences flanked by different nucleotides differentially bind phosphorylated Pit-1 (Kapiloff *et al.*, 1991).

Starting and evolving from a common DNA binding protein domain and its cognate DNA recognition sequence, a transcription factor family can therefore generate and accommodate diversity of transcriptional regulation through its members. A repertoire based on a number of related but distinct activator genes can be enhanced further by alternative transcripts produced from a single gene, according to a temporal or cell-specific programme, and counter-balanced by the similarly complex expression of antagonists. The differences in the precise amino acid sequence of different members' DNA binding domains presumably dictate precise DNA binding specificities that vary from the consensus, and these can be modified by heterodimerization of activator and inhibitor proteins. Thus, different signals, mediated by the monomers, can be integrated, and specific promoters activated or repressed, according to the affinity of their sites for the expressed transcription factor complexes.

11.3 Transcription factors as instruments of development

Development of the mouse, as of any multicellular organism, relies on the stage-specific expression of genes according to cell lineage and in response to intercellular and hormonal stimuli. Transcription factors, precisely because of their ability to regulate transcription, are the primary instruments of gene expression programmes, and their own expression precedes or coincides with, and reflects the developmental events which they direct.

One of the most interesting characteristics of the ECRE activities and the *ATF* genes is that their DNA binding and RNA levels are regulated during differentiation of EC cells. Similarly, as was mentioned in the previous sections, the expression of other transcription factor genes is regulated both temporally and spatially during embryogenesis proper. This developmental control extends, *via* post-transcriptional and post-translational mechanisms, to their DNA binding and *trans*-activation activities.

11.3.1 Transcription factors as developmental "switches"

The regulated expression of transcription factors can take the form of a transcriptional activation cascade during development, as has been demonstrated for *Drosophila* (reviewed by Nüsslein-Volhard, 1991 ; Levine and Harding, 1989). In the mouse, and on a smaller scale, the sequential though partly overlapping expression of myogenic transcription factors in the developing mouse embryo, culminating with skeletal α-actin expression, may represent a similar phaenomenon (Sassoon *et al.*, 1989 ; Hinterberger *et al.*, 1991 ; Ott *et al.*, 1991). Expression of an exogenous copy of a myogenic gene in muscle cell cultures activates the expression of the endogenous copy as well as other myogenic and muscle structural protein genes (Thayer *et al.*, 1989 ; Braun *et al.*, 1989 ; Yutzey *et al.*, 1990).

Given that the promoters of such myogenic genes as have been characterized contain consensus bHLH protein binding sites (Salminen *et al.*, 1991 ; S.-P.Yee, pers. comm.), it is possible that myogenic factors bind to and activate each other's promoters, and that this transcriptional cascade plays an important role in muscle differentiation. A similar transcriptional activation hierarchy exists in hepatocytes, where the steroid receptor superfamily transcription factor HNF4 (hepatic nuclear factor 4) is required for expression of the homoeodomain transcription factor HNF1α (hepatic nuclear factor 1α) (Kuo *et al.*, 1992). Of course, the question arises : if development relies on a cascade of tissue-specific transcription factors, how is this cascade itself put into motion ? In the case of the pituitary-specific transcription factor Pit-1, it has been suggested that CREB, a less tissue-specific protein (Berkowitz and Gilman, 1990), activates the *Pit-1* gene, which thereafter is autoregulated and is required for the maintenance of three anterior pituitary cell types (McCormick *et al.*, 1990 ; Li *et al.*, 1990). In addition, it is not unreasonable to expect that, as with *Drosophila* (reviewed by Nüsslein-Volhard, 1991), maternally expressed transcription factors that are stored in the oocyte, in RNA or protein form, such as murine Oct-3 (Rosner *et al.*, 1990), also initiate transcriptional cascades during murine development.

Transcription of any given gene during development can be modulated by the stage-specific binding of different transcription factors on the same or overlapping sites. The increase in *E4*Δ promoter activity during F9 EC cell differentiation, for example, coincides with a change in the ECRE activities binding to its ATF site. Similarly, in the skeletal α-actin promoter, the binding site of SRF (serum response factor), a transcriptional activator necessary for muscle-specific expression of α-actin, is occupied by an inactive protein, F-ACT1, in non-muscle cells and proliferating myoblasts. It is only after myoblasts have differentiated to myotubes that F-ACT1 is replaced by SRF and α-actin expression occurs (Lee, T.C. *et al.*, 1991). Even a modulation of transcription factor levels can result in specific activation of genes which respond above a given threshold of transcription factor concentration, as has been demonstrated for Bicoid-dependent activation of *hunchback* expression in *Drosophila* (Struhl *et al.*, 1989). For example, the down-regulation of ρ-globin gene expression during chicken erythroblast differentiation correlates with a ten-fold decrease in Sp1 and GATA1 (GATA motif binding protein 1), whose binding sites are important for ρ-globin transcription (Minie *et al.*, 1992).

11.3.2 Post-translational regulation of transcription factor activity

Alternatively, rather than appear at a specific developmental stage, pre-existing transcription factors can be maintained in an inactive state by post-translational mechanisms. The p50 subunit of NFκB, for example, is translated as a non-DNA binding, cytoplasmic polypeptide of 110 kD molecular weight (Ghosh *et al.*, 1990 ; Kieran *et al.*, 1990). Only after ATP-dependent proteolytic cleavage can the active p50, complexed to the p65 NFκB subunit, migrate to the nucleus and activate transcription (Fan and Maniatis, 1991). However, contrary to mature B cells, where NFκB is nuclear and binds DNA (Sen and Baltimore, 1986), in pre-B cells and other cell types, it is sequestered in the cytoplasm as a non-DNA binding complex with IκB : this complex can be dissociated *in vitro* after phosphorylation of IκB (Bauerle and Baltimore, 1988 ; Ghosh and Baltimore, 1990). It is therefore likely that NFκB becomes activated by a similar post-translational mechanism during B-cell maturation.

Clearly, mechanisms used for the activation of transcription factors during differentiation may be shared with other processes where such activation is required : another instance where phosphorylation is a prerequisite for transcription factor activity is CREB-mediated *trans*-activation (Gonzalez and Montminy, 1989 ; Lee *et al.*, 1990). Phosphorylation can also provide a link between a developmentally regulated transcription factor like DRTF-1 (Partridge and La Thangue, 1991), and the cell cycle. DRTF-1 has the ability to complex with the underphospholylated form of pRb, the form that is most likely to suppress proliferation (Bandara and La Thangue, 1991 ; Ludlow *et al.*, 1990). In the rapidly proliferating F9 EC cells, the levels of DRTF-1 which is not complexed with pRb are high and DRTF-1 binding site-dependent transcription is very efficient. After differentiation, however, the majority of DRTF-1 molecules are complexed with pRb and DRTF-1-

dependent transcription decreases, as a direct consequence of this association (Partridge and La Thangue, 1991 ; La Thangue *et al.*, 1990 ; Zamanian and La Thangue, 1992). Phosphorylation of pRb, and consequently its ability to complex with DRTF-1, is cell cycle-regulated and differentiation-dependent (Chen *et al.*, 1989). Thus, pRb, a suppressor of proliferation, regulates, in a differentiation-dependent manner, the activity of a transcription factor, DRTF-1, binding sites for which are present in a number of promoters which are active in rapidly growing cells (Mudryj *et al.*, 1990). There is evidence that cJun activity is also regulated in a cell cycle-specific manner, since mitogen-activated serine kinases can phosphorylate the amino-terminus of cJun, thus enhancing its *trans*-activation potential (Pulverer *et al.*, 1991). This phosphorylation domain of cJun is an activation domain which is the target for cell-specific repression of cJun activity (Baichwal and Tjian, 1990 ; Baichwal *et al.*, 1992) : as in the interaction of DRTF-1 with pRb, the mechanisms of cell-specific and cell cycle regulation seem to converge.

Another, as yet unstudied, mechanism through which the activity of transcription factors could be regulated during development is the expression of transcriptional co-activators, proteins that do not bind DNA but are required for *trans*-activation above the levels of transcription achieved by the basal initiation complex, and are thought to act as "bridges" between it and DNA bound activators. Their regulation may be particularly relevant to transcriptional control in F9 EC cells and the mouse blastocyst which both contain an "E1A-like" activity, since adenovirus E1A seems to *trans*-activate by "mediating" between specific DNA binding proteins and the initiation complex (Martin *et al.*, 1990 ; Schöler *et al.*, 1991 ; Lillie and Green, 1989). Importantly, activation by transcription factors Oct-4 and RARβ2 can occur in the absence of E1A in F9 EC cells but requires E1A in other cell types (Schöler *et al.*, 1991 ; Berkenstam *et al.*, 1992). Therefore, if adenovirus E1A does indeed function as a co-activator, or if it stabilises the interaction of cellular co-activators with DNA binding transcription factors, it is likely that the "E1A-like" activity of F9 EC cells will function in a similar way. In that case, transcriptional control during differentiation would rely as much on the activity of DNA binding proteins as on that of transcriptional co-activators.

11.4 Conclusions

In conclusion, ATF site binding proteins constitute a model family of transcription factors whose activity is controlled at various levels and which respond to a variety of stimuli. Transcripts of different *ATF* members, as well as alternative transcripts of the same gene, are differentially distributed in different tissues and their levels are regulated during the differentiation of EC cells. Alternative splicing gives rise to ATF isoforms lacking a DNA binding or an activation domain, thus increasing the repertoire of ATF *trans*-activation and DNA binding properties. This functional multiplicity is enhanced further by dimerization. ATF proteins bind DNA as homodimers or heterodimers: the choice of dimerization partner, which can be a member of the ATF or the related AP1 family, determines DNA binding specificity and therefore presumably *trans*-activation potential as well. These are also regulated by phosphorylation and can be further modified by interaction with viral transcriptional activators. It is, presumably, such mechanisms, as well as specific amino acid differences in their DNA binding domains, that result in the ability of distinct ATF site-binding activities to bind preferentially to specific variants of the ATF site and ATF site-containing promoters. Finally, ATF transcription factors seem to be involved in various developmental processes. They activate, for example, the expression of a cell-specific transcription factor in the pituitary, they participate in the electrically induced gene expression changes required for neuronal development, and their DNA binding levels are regulated during differentiation.

The present study has explored the regulation of the ATF family at the level of expression and DNA binding, and related it to the transcriptional activities of ATF site-containing promoters in a cell system that models early differentiation events in the mouse embryo. The results presented, emphasizing the complexity and versatility of this family, are compatible with, and contribute to, the accumulating evidence which indicates that the activity of ATF proteins is under multiple controls and responds to various stimuli, characteristics which make it a useful agent for organising gene expression programmes during development.

REFERENCES

Abate, C., D.Luk, R.Gentz, F.J.Rauscher III and T.Curran (1990) : Expression and purification of the leucine zipper and DNA-binding domains of Fos and Jun : both Fos and Jun contact DNA directly. *Proc. Natl. Acad. Sci. USA* **87** : 1032-1036

Abel, T., R.Bhatt and T.Maniatis (1992) : A *Drosophila* CREB/ATF transcriptional activator binds to both fat body- and liver-specific regulatory elements. *Genes Dev.* **6** : 466-480

Adamkiewicz, J., H.-J.Brüller, H.Gausepohl, R.Frank, and R.Müller (1990) : Mapping of functional domains in Fos and Jun proteins using epitope-specific antibodies. *Oncogene* **5** : 525-533

Akgün, E., M.Ziegler and M.Grenz (1991) : Determinants of retrovirus gene expression in embryonal carcinoma cells. *J. Virol.* **65** : 382-388

Akrigg, A., G.W.G.Wilkinson, and J.D.Oram (1985) : The structure of the major immediate early gene of human cytomegalovirus strain AD169. *Virus Res.* **2** : 107-121

Almendral, J.M., D.Sommer, H.MacDonald-Bravo, J.Burckhardt, J.Perera and R.Bravo (1988) : Complexity of early genetic response to growth factors in mouse fibroblasts. *Mol. Cell. Biol.* **8** : 2140-2148

Andrisani, O.A., D.A.Pot, Z.Zhu, and J.E.Dixon (1988) : Three sequence-specific DNA-protein complexes are formed with the same promoter element essential for expression of the rat somatostatin gene. *Mol. Cell. Biol.* **8** : 1947-1956

Andrisani, O.A., T.E.Hayes, B.Roos, and J.E.Dixon (1987) : Identification of the promoter sequences involved in the cell specific expression of the rat somatostatin gene. *Nucl. Acids Res.* **15** : 5715-5728

Angel, P., E.A.Allegretto, S.T.Okino, K.Hattori, W.J.Boyle, T.Hunter and M.Karin (1988) : Oncogene *jun* encodes a sequence-specific *trans*-activator similar to AP-1. *Nature* **332** : 166-171

Angel, P., M.Imagawa, R.Chiu, B.Stein, R.J.Imbra, H.J.Rahmsdorf, C.Jonat, P.Herrlich, and M.Karin (1987) : Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor. *Cell* **49** : 729-739

Bagchi, S., P.Raichaudhuri and J.R.Nevins (1990) : Adenovirus E1A proteins can dissociate heteromeric complexes involving the E2F transcription factor : a novel mechanism for E1A *trans*-activation. *Cell* **62** : 659-669

Baichwal, V.R., A.Park and R.Tjian (1992) : The cell-type-specific activator region of c-Jun juxtaposes constitutive and negatively regulated domains. *Genes Dev.* **6** : 1493-1502

Baichwal, V.R. and R.Tjian (1990) : Control of c-Jun activity by interaction of a cell-specific inhibitor with regulatory domain δ : differences between v- and c-Jun. *Cell* **63** : 815-825

Bakker, O. and M.G.Parker (1991) : CAAT/enhancer binding protein is able to bind to ATF/CRE elements. *Nucl. Acids Res.* **19** : 1213-1217

Bandara, L.R. and N.B. La Thangue (1991) : Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor. *Nature* **351** : 494-497

Bandara, L.R., J.P.Adamczewski, T.Hunt and N.B.La Thangue (1991) : Cyclin A and the retinoblastoma gene product complex with a common transcription factor. *Nature* **352** : 249-251

Bartel, D.P., M.Sheng, L.F.Lau and M.E.Greenberg (1989) : Growth factors and membrane depolarization activate distinct programs of early response gene expression : dissociation of *fos* and *jun* induction. *Genes Dev.* **3** : 304-313

Bauerle, P.A. and D.Baltimore (1988) : IκB : a specific inhibitor of the NF-κB transcription factor. *Science* **242** : 540-546

Benezra, R., R.L.Davis, D.Lockshon, D.L.Turner, and H.Weintraub (1990) : The protein Id : a negative regulator of helix-loop-helix DNA binding proteins. *Cell* **61** : 49-59

Benbrook, D.M., and N.C.Jones (1990) : Heterodimer formation between CREB and JUN proteins. *Oncogene* **5** : 295-302

Berberich, S.J. and M.D.Cole (1992) : Casein kinase II inhibits the DNA-binding activity of Max homodimers but not of Myc/Max heterodimers. *Genes Dev.* **6** : 166-176

Berger, S.L., B.Piña, N.Silverman, G.A.Marcus, J.Agapite, J.L.Regier, S.J.Triezenberg and L.Guarente (1992) : Genetic isolation of ADA2 : a potential transcriptional adaptor required for function of certain acidic activation domains. *Cell* **70** : 251-265

Berger, S.L, W.D.Cress, A.Cress, S.J.Triezenberg and L.Guarente (1990) : Selective inhibition of activated but not basal transcription by the acidic activation domain of VP16 : evidence for transcriptional adaptors. *Cell* **61** : 1190-1208

Berk, A.J. (1989) : Regulation of eukaryotic transcription factors by post-translational modification. *Biochim. Biophys. Acta* **1009** : 103-109

Berk, A.J. (1986) : Adenovirus promoters and E1A transactivation. *Ann. Rev. Genet.* **20** : 45-79

Berkenstam, A., M. del Mar Vivanco Ruiz, D.Barettino, M.Horikoshi and H.G.Stunnenberg (1992) : Cooperativity in transactivation between retinoic acid receptor and TFIID requires an activity analogous to E1A. *Cell* **69** : 401-412

Berkowitz, L.A. and M.Z.Gilman (1990) : Two distinct forms of active transcription factor CREB (cAMP-responsive-element-binding protein). *Proc. Natl. Acad. Sci. USA* **87** : 5258-5262

Bernstine, E.G., M.L.Hooper, S.Grandchamp, and B.Ephrussi (1973) : Alkaline phosphatase activity in mouse teratoma. *Proc. Natl. Acad. Sci. USA* **70** : 3899-3903

Biggin, M.D. and R.Tjian (1989) : Transcription factors and the control of Drosophila development. *Trends Genet.* **5** : 377-383

Blackwell, T.K. and H.Weintraub (1990) : Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. *Science* **250** : 1104-1110

Blackwood, E.M., B.Lüscher and R.N.Eisenman (1992) : Myc and Max associate in vivo. *Genes Dev.* **6** : 71-80

Blackwood, E.M. and R.N.Eisenman (1991) : Max : a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. *Science* **251** : 1211-1217

Bohmann, D., T.J.Bos, A.Admon, T.Nishimura, P.K.Vogt, and R.Tjian (1987) : Human proto-oncogene c-*jun* encodes a DNA binding protein with structural and functional properties of transcritpion factor AP-1. *Science* **238** : 1386-1392

Bokar, J.A., R.A.Keri, T.A.Farmerie, R.A.Fenstermaker, B.Andersen, D.L.Hamernik, J.Yun, T.Wagner, and J.H.Nilson (1989) : Expression of the glycoprotein hormone α-subunit gene in the placenta requires a functional cyclic AMP response element, whereas a different *cis*-acting element mediates pituitary-specific expression. *Mol. Cell. Biol.* **9** : 5113-5122

Boshart, M., F,Weber, G.Jahn, K.Dorsch-Häsler, B.Fleckenstein, and W.Schaffner (1985) : A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell* **41** : 521-530

Buratowski, S., S.Hahn, L.Guarente and P.A.Sharp (1989) : Five intermediate complexes in transcription initiation by RNA polymerase II. *Cell* **56** : 549-561

Braun, T., E.Bober, G.Buschhausen-Denker, S.Kotz, K.-H.Grzeschik and H.H.Arnold (1989) : Differential expression of myogenic determination genes in muscle cells : possible autoactivation by the *Myf* gene products. *EMBO J.* **8** : 3617-3625

Busch, S.J., and P.Sassone-Corsi (1990) : Dimers, leucine zippers and DNA-binding domains. *Trends Genet.* **6** : 36-40

Carey, M., Y.-S.Lin, M.R.Green and M.Ptashne (1990) : A mechanism for synergistic activation of a mammalian gene by GAL4 derivatives. *Nature* **345** : 361-364

Chatterjee, P.K., M.Bruner, S.J.Flint and M.L.Harter (1988) : DNA-binding properties of an adenovirus 289R E1A protein. *EMBO J.* **7** : 835-841

Chen, P.-L., P.Scully, J.-Y.Shew, J.Y.J.Wang and W.-H.Lee (1989) : Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. *Cell* **58** : 1193-1198

Chiu, R., P.Angel, and M.Karin (1989) : Jun-B differs in its biological properties from, and is a negative regulator of, c-Jun. *Cell* **59** : 979-986

Cohen, D. and T.Curran (1988) : *fra*-1 : a serum-inducible, cellular immediate early gene that encodes a Fos-related antigen. *Mol. Cell. Biol.* **8** :2063-2069

Collins, S., M.G.Caron and R.J.Lefkowitz (1992) : From ligand binding to gene activation : new insights into the regulation of G-protein-coupled receptors. *Trends Biochem. Sci.* **17** : 37-39

Comai, L., N.Tanese and R.Tjian (1992) : The TATA-binding protein and associated factors are integral components of the RNA polymerase I transcription factor, SL1. *Cell* **68** : 965-976

Corden, J.L. (1990) : Tails of RNA polymerase II. *Trends Biochem. Sci.* **15** : 383-387

Cormack, B.P. and K.Struhl (1992) : The TATA-binding protein is required for transcription by all three nuclear polymerases in yeast cells. *Cell* **69** : 685-696

Cormack, B.P., M.Strubin, A.S.Ponticelli and K.Struhl (1991) : Functional differences between yeast and human TFIID are localized to the highly conserved region. *Cell* 65 : 341-348

Cortes, P., L.Buckbinder, M.Aleida Leza, N.Rak, P.Hearing, A.Merino, and D.Reinberg (1988) : EivF, a factor required for transcription of the adenovirus EIV promoter, binds to an element involved in Ela-dependent activation and cAMP induction. *Genes Dev.* 2 : 975-990

Cowell, I.G., A.Skinner and H.C.Hurst (1992) : Transcriptional repression by a novel member of the bZIP family of transcription factors. *Mol. Cell. Biol.* 12 : 3070-3077

Curran, T., C.Van Beveren, N.Ling and I.M.Verma (1985) : Viral and cellular *fos* proteins are complexed witha 39,000-Dalton cellular protein. *Mol. Cell. Biol.* 5 : 167-172

Curran, T., A.D.Miller, L.Zokas and I.M.Verma (1984) : Viral and cellular *fos* proteins : a comparative analysis. *Cell* 36 : 259-268

Cusella-De Angelis, M.G., G.Lyons, C.Sonnino, L.De Angelis, E.Vivarelli, K.Farmer, W.E.Wright, M.Molinaro, M.Bouchè, M.Buckingham and G.Cossu (1992) : MyoD, myogenin independent differentiation of primordial myoblasts in mouse somites. *J. Cell. Biol.* 116 : 1243-1255

Dannenberg, A.M. and M.Suga (1981), in *Methods for Studying Mononuclear Phagocytes*, D.O.Adams, P.J.Edelson and M.S.Koren, Academic Press, New York

Delegeane, A.M., L.H.Ferland, P.L.Mellon (1987) : Tissue-specific enhancer of the human glycoprotein hormone α-subunit gene : dependence on cyclic AMP-inducible elements. *Mol. Cell. Biol.* 7 : 3994-4002

Delmas, V., B.M.Laoide, D.Masquilier, R.P. de Groot, N.S.Foulkes and P.Sassone-Corsi (1992) : Alternative usage of initiation codons in mRNA encoding the cAMP-responsive-element modulator generates regulators with opposite functions. *Proc. Natl. Acad. Sci. USA* 89 : 4226-4230

de Thé, H., M. del Mar Vivanco-Ruiz, P.Tollais, H.Stunnenberg and A.Dejean (1989) : Identification of a retinoic acid responsive element in the retinoic acid receptor β gene. *Nature* 343 : 177-180

Deutsch, P.J., J.P.Hoeffler, J.L.Jameson, J.C.Lin and J.F.Habener (1988) : Structural determinants for transcriptional activation by cAMP-responsive DNA elements. *J. Biol. Chem.* 263 : 18466-18472

Deutsch, P.J., J.L.Jameson and J.F.Habener (1987) : Cyclic AMP responsiveness of human gonadotropin-α gene transcription is directed by a repeated 18-base pair enhancer. *J. Biol. Chem.* 262 : 12169-12174

Devereux, J., P.Haeberli and O.Smithies (1984) : A comprehensive set of sequence analysis programs for the VAX. *Nucl. Acids Res.* 12 : 387-395

Dignam, J.D., R.M.Lebovitz and R.G.Roeder (1983) : Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucl. Acids Res.* 11 : 1475-1489

Dobrzanski, P., T.Noguchi, K.Kovary, C.A.Rizzo, P.S.Lazo and R.Bravo (1991) : Both products of the *fos*B gene, FosB and its short form, FosB/SF, are transcriptional activators in fibroblasts. *Mol. Cell. Biol.* **11** : 5470-5478

Dony, C., M.Kessel and P.Gruss (1985) : Post-transcriptional control of *myc* and p53 expression during differentiation of the embryonal carcinoma cell line F9. *Nature* **317** : 636-639

Dooley, T.P., M.Miranda, N.C.Jones, and M.L.De Pamphilis (1989) : Transactivation of the adenovirus Ella promoter in the absence of adenovirus E1A protein is restricted to mouse oocytes and preimplantation embryos. *Development* 107 : 945-956

Dwarki, V.J., M.Montminy and I.M.Verma (1990) : Both the basic region and the "leucine zipper" domain of the cyclic AMP response element binding (CREB) protein are essential for transcriptional activation. *EMBO J.* 9 : 225-232

Dynan, W.S. (1989) : Modularity in promoters and enhancers. *Cell* **58** : 1-4

Dynan, W.S. and Tjian, R. (1983) : Isolation of transcription factors that discriminate between different promoters recognized by RNA polymerase II. *Cell* **32** : 669-680

Dynlacht, B.D., T.Hoey and R.Tjian (1991) : Isolation of coactivators associated with the TATA-binding protein that mediate transcriptional activation. *Cell* **66** : 563-576

Dzierzak, E.A., T.Papayannopoulou and R.C.Mulligan (1988) : Lineage-specific expression of a human β-globin gene in murine bone marrow transplant recipients reconstituted with retrovirus-transduced stem cells. *Nature* **331** : 35-41

Enoch, T., K.Zinn and T.Maniatis (1986) : Activation of the human β-interferon gene requires an interferon-inducible factor. *Mol. Cell. Biol.* **6** : 801-810

Fan, C.-M. and T.Maniatis (1991) : Generation of p50 subunit of NF-κB by processing of p105 through an ATP-dependent pathway. *Nature* **354** : 395-398

Fahrlander, P.D. and K.B.Marcu (1986) : Regulation of c-*myc* expression in normal and transformed mammalian cells. In *Oncogenes and Growth Control* (ed. P.Kahn and T.Graf), pp. 264-270. Springer-Verlag, Heidelberg.

Felsenfeld, G. (1992) : Chromatin as an essential part of the transcriptional mechanism. *Nature* **355** : 219-224

Ferguson, B., B.Krippl, N.Jones, H.Westphal and M.Rosenberg (1985) : E1A 13S and 12S mRNA products made in *E.coli* both function as nucleus-localized transcription activators but do not directly bind DNA. *Mol. Cell. Biol.* **5** : 2653-2661

Fink, J.S., M.Verhave, S.Kasper, T.Tsukada, G.Mandel, and R.Goodman (1988) : The CGTCA sequence motif is essential for biological activity of the vasoactive intestinal peptide gene cAMP-regulated enhancer. *Proc. Natl. Acad. Sci. USA* **85** : 6662-6666

Flanagan, P.M., R.J.Kelleher III, M.H.Sayre, H.Tschohner and R.D.Kornberg (1991) : A mediator required for activation of RNA polymerase II transcription *in vitro*. *Nature* **350** : 436-438

Flores, O., H.Lu and D.Reinberg (1992) : Factors involved in specific transcription by mammalian RNA polymerase II. *J. Biol. Chem.* **267** : 2786-2793

Foulkes, N.S., B.Mellström, E.Benusiglio and P.Sassone-Corsi (1992) : Developmental switch of CREM function during spermatogenesis : from antagonist to activator. *Nature* **355** : 80-84

Foulkes, N.S., E.Borrelli and P.Sassone-Corsi (1991) : CREM gene : use of alternative DNA-binding domains generates multiple antagonists of cAMP-induced transcription. *Cell* **64** : 739-749

Frankel, A.D. and P.S.Kim (1991) : Modular structure of transcription factors : implications for gene regulation. *Cell* **65** : 717-719

Fujisawa, J., M.Toita and M.Yoshida (1989) : A unique enhancer element for the trans-activator of human T-cell leukemia virus type I that is distinct from cyclic AMP and 12-*O*-tetradecanoyl phorbol-13-acetate-responsive elements. *J. Virol.* **63** : 3234-3239

Gabrielsen, O.S.and A.Sentenac (1991) : RNA polymerase III (C) and its transcription factors. *Trends Biochem. Sci.* **16** : 412-416

Gaire, M., B.Chatton and C.Kédinger (1990) : Isolation and characterization of two novel, closely related ATF cDNA clones from HeLa cells. *Nucl. Acids Res.* **18** : 3467-3473

Gasch, A., A.Hoffmann, M.Horikoshi, R.G.Roeder and N.-H.Chua (1990) : *Arabidopsis thaliana* contains two genes for TFIID. *Nature* **346** : 390-394

Gentz, R., F.J.Rauscher III, C.Abate and T.Curran (1989) : Parallel association of Fos and Jun leucine zippers juxtaposes DNA binding domains. *Science* **243** : 1695-1699

Georgopoulos, K., B.A.Morgan and D.D.Moore (1992) : Functionally distinct isoforms of the CRE-BP DNA-binding protein mediate activity of a T-cell-specific enhancer. *Mol.Cell.Biol.* **12** : 747-757

Ghosh, S., A.M.Gifford, L.R.Riviere, P.Tempst,G.P.Nolan and D.Baltimore (1990) : Cloning of the p50 DNA-binding subunit of NF-κB : homology to *rel* and *dorsal*. *Cell* **62** : 1019-1029

Ghosh, S. and D.Baltimore (1990) : Activation *in vitro* of NF-κB by phosphorylation of its inhibitor IκB. *Nature* **344** : 678-682

Gilardi, P. and M.Perricaudet (1986) : The E4 promoter of adenovirus type 2 contains an E1A dependent cis-acting element. *Nucl. Acids Res.* **14** : 9035-9049

Gilardi, P. and M.Perricaudet (1984) : The E4 transcriptional unit of Ad2 : far upstream sequences are required for its transactivation by E1A. *Nucl. Acids Res.* **12** : 7877-7888

Gill, G. and R.Tjian (1991) : A highly conserved domain of TFIID displays species specificity in vivo. *Cell* **65** : 333-340

Gill, G. and M.Ptashne (1988) : Negative effect of the transcriptional activator GAL4. *Nature* **334** : 721-724

Goding, C., P.Jalinot, D.Zajchowski, H.Boeuf, and C.Kédinger (1985). Sequence-specific *trans*-activation of the adenovirus EIIa early promoter by the viral EIV transcription unit. *EMBO J.* **4** : 1523-1528

Gonzalez, G.A., P.Menzel, J.Leonard, W.H.Fischer and M.R.Montminy (1991) : Characterization of motifs which are critical for activity of the cyclic AMP-responsive transcription factor CREB. *Mol. Cell. Biol.* **11** : 1306-1312

Gonzalez, G.A. and M.R.Montminy (1989). Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* **59** : 675-680

Gonzalez, G.A., K.Y.Yamamoto, W.H.Fischer, D.Karr, P.Menzel, W.Biggs III, W.W.Vale, and M.R.Montminy (1989) : A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. *Nature* **337** : 749-752

Gorman, C.M., L.F.Moffat and B.H.Howard (1982) : Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* **2** : 1044-1051

Gorman, C.M., P.W.J.Rigby, and D.P.Lane (1985) : Negative regulation of viral enhancers in undifferentiated embryonic stem cells. *Cell* **42** : 519-526

Green, M., P.M.Loewenstein, R.Pusztai and J.S.Symington (1988) : An adenovirus E1A protein domain activates transcription in vivo and in vitro in the absence of protein synthesis. *Cell* **53** : 921-926

Green, S. and P.Chambon (1988) : Nuclear receptors enhance our understanding of transcription regulation. *Trends Genet.* **4** : 309-314

Greenblatt, J. (1991b) : RNA polymerase-associated transcription factors. *Trends Biocem. Sci.* **16** : 408-411

Ha, I., W.S.Lane and D.Reinberg (1991) : Cloning of a human gene encoding the general transcription initiation factor IIB. *Nature* **352** : 689-694

Habener, J.F. (1990) : Cyclic AMP response element binding proteins : a cornucopia of transcription factors. *Mol. Endo.* **4** : 1087-1094

Hai, T. and T.Curran (1991) : Cross-family dimerization of transcritpion factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc. Natl. Acad. Sci. USA* **88** : 3720-3724

Hai, T., F.Liu, W.J.Koukos, and M.R.Green (1989) : Transcription factor ATF cDNA clones : an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. *Genes Dev.* **3** : 2083-2090

Hai, T., M.Horikoshi, R.G.Roeder and M.R.Green (1988a) : Analysis of the role of the transcription factor ATF in the assembly of a functional preinitiation complex. *Cell* **54** : 1043-1051

Hai, T., F.Liu, E.Allegretto, M.Karin, and M.R.Green (1988b) : A family of immunologically related transcription factors that includes multiple forms of ATF and AP-1. *Genes Dev.* **2** : 1216-1226

Halazonetis, T.D., K.Georgopoulos, M.E.Greenberg, and P.Leder (1988) : c-Jun dimerizes with itself and woth c-Fos, forming complexes of different DNA binding affinities. *Cell* **55** : 917-924

Han, K., M.S.Levine and J.L.Manley (1989) : Synergistic activation and repression of transcription by Drosophila homeobox proteins. *Cell* **56** : 573-583

Hanaka, S., T.Nishigaki, P.A.Sharp, and H.Handa (1987) : Regulation of *in vitro* and *in vivo* transcription of early-region IV of adenovirus type 5 by multiple *cis*-acting elements. *Mol. Cell. Biol.* 7 : 2578-2587

Handa, H. and P.A.Sharp (1984) : Requirement for distal upstream sequences for maximal transcription *in vitro* of early region IV of adenovirus. *Mol. Cell. Biol.* 4 : 791-798

Harlow, E. and D.Lane (1988) : *Antibodies : A laboratory manual*, Cold Spring Harbor Laboratory

Harlow, E., L.V.Crawford, D.C.Pim, and N.M.Williamson (1981) : Monoclonal antibodies specific for simian virus 40 tumour antigens. *J. Virol.* 39 : 861-869

Hatzopoulos, A.K., U.Schlokat and P.Gruss (1988) : Enhancers and other *cis*-acting regulatory sequences, in *Transcription and splicing*, B.D.Hames and D.M.Glover, eds., IRL Press

Hay, N., M.Takimoto, and J.M.Bishop (1989) : A *FOS* protein is present in a complex that binds a negative regulator of *MYC. Genes Dev.* 3 : 293-303

He, X. and M.G.Rosenfeld (1991) : Mechansims of comples transcriptional regulation : implications for brain development. *Neuron* 7 : 183-196

Helin, K., J.A.Lees, M.Vidal, N.Dyson, E.Harlow and A.Fattaey (1992) : A cDNA encoding a pRB-binding protein with properties of the ttranscription factor E2F. *Cell* **70** : 337-350

Herbomel, P., B.Bourachot, and M.Yaniv (1984). Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. *Cell* 39 : 653-662

Herr, W., R.A.Sturm, R.G.Clerc, L.M.Corcoran, D.Baltimore, P.A.Sharp, H.A.Ingraham, M.G.Rosenfeld, M.Finney, G.Ruvkun and H.R.Horvitz (1988) : The POU domain : a large conserved region in the mammalian *pit*-1, *oct*-1, *oct*-2, and *Caenorhabditis elegans unc-86* gene products. *Genes Dev.* **2** : 1513-1516

Herskowitz, I. (1989) : A regulatory hierarchy for cell specialization in yeast. *Nature* **342** : 749-757

Hinterberger, T.J., D.A.Sassoon, S.J.Rhodes and S.F.Konieczny (1991) : Expression of the muscle regulatory factor MRF4 during somite and skeletal myofiber development. *Dev. Biol.* **147** : 144-156

Hirai, S.-I., R.-P.Ryseck, F.Mechta, R.Bravo and M.Yaniv (1989) : Characterization of *junD* : a new member of the *jun* proto-oncogene family. *EMBO J.* **8** : 1433-1439

Hoeffler, J.P., T.E.Meyer, Y.Yun, J.L.Jameson, and J.F.Habener (1988) : Cyclic AMP-responsive DNA-binding protein : structure based on a cloned placental cDNA. *Science* 242 : 1430-1433

Hoey, T., B.D.Dynlacht, M.G.Peterson, B.F.Pugh and R.Tjian (1990) : Isolation and characterization of the Drosophila gene encoding the TATA box binding protein, TFIID. *Cell* 61 : 1179-1186

Hoffmann, A., E.Sinn, T.Yamamoto, J.Wang, A.Roy, M.Horikoshi and R.G.Roeder (1990) : Highly conserved core domain and unique N terminus with presumptive regulatory motifs in a human TATA factor (TFIID). *Nature* 346 : 387-390

Hogan, B., F.Constantini and E.Lacy (1986) : *Manipulating the mouse embryo - a laboratory manual*, Cold Spring Harbor Laboratory

Hogan, B.L.M., D.P.Barlow and R.Tilly (1983) : F9 teratocarcinoma cells as a model for the differentiation of parietal and visceral endoderm in the mouse embryo. *Cancer Surveys* **2** : 115-140

Hogan, B.L.M., A.Taylor, and E.Adamson (1981) : Cell interactions modulate embryonal carcinoma cell differentiation into parietal or visceral endoderm. *Nature* **291** : 235-237

Hopp, T.P, and K.R.Woods (1983) : A computer program for predicting protein antigenic determinants. *Mol. Immunol.* **20** : 483-489

Horikoshi, M., K.Maguire, A.Kralli, E.Maldonado, D.Reinberg and R.Weinmann (1991) : Direct interaction between adenovirus E1A protein and the TATA box binding transcription factor IID. *Proc. Natl. Acad. Sci. USA* **88** : 5124-5128

Horikoshi, M., T.Yamamoto, Y.Okhuma, P.A.Weil and R.G.Roeder (1990) : Analysis of structure-function relationships of yeast TATA box binding factor TFIID. *Cell* **61** : 1171-1178

Horikoshi, M., C.K.Wang, H.Fuji, J.A.Cromlish, P.A.Weil and R.G.Roeder (1989) : Cloning and structure of a yeast gene encoding a general transcription initiation factor TFIID that binds to the TATA box. *Nature* 341 : 299-303

Horikoshi, M., T.Hai, Y.-S.Lin, M.R.Green, and R.G.Roeder (1988a) : Transcription factor ATF interacts with the TATA factor to facilitate establishment of a preinitiation complex. *Cell* **54** : 1033-1042

Horikoshi, M., M.F.Carey, H.Kakidani and R.G.Roeder (1988b) : Mechanism of action of a yeast activator : direct effect of GAL4 derivatives on mammalian TFIID-promoter interactions. *Cell* **54** : 665-669

Huang, M.-M., and P.Hearing (1989). The adenovirus early region 4 open reading frame 6/7 protein regulates the DNA binding activity of the cellular transcription factor, E2F, through a direct complex. *Genes Dev.* **3** : 1699-1710

Hurst, H.C., N.F.Totty and N.C.Jones (1991) : Identification and functional characterisation of the cellular activating trancription factor 43 (ATF-43) protein. *Nucl. Acids Res.* **19** : 4601-4609

Hurst, H.C., N.Masson, N.C.Jones and K.A.W.Lee (1990) : The cellular transcription factor CREB corresponds to activating transcription factor 47 (ATF-47) and forms complexes with a group of polypeptides related to ATF-43. *Mol. Cell. Biol.* **10** : 6192-6203

Hurst, H.C. and N.C.Jones (1987). Identification of factors that interact with the E1A-inducible adenovirus E3 promoter. *Genes Dev.* **1** : 1132-1146

Imperiale, M.J., H.-T.Kao, L.T.Feldman, J.R.Nevins, and S.Strickland (1984). Common control of the heat shock gene and early adenovirus genes : evidence for a cellular E1A-like activity. *Mol. Cell. Biol.* **4** : 867-874

Inostroza, J.A., F.H.Mermelstein, I.Ha, W.S.Lane and D.Reinberg (1992) : Dr1, a TATA-binding protein-associated phosphoprotein and inhibitor of class II gene transcription. *Cell* **70** : 477-489

Ivashkiv, L.B., H.-C.Liou, C.J.Kara, W.W.Lamph, I.M.Verma, and L.H.Glimcher (1990) : mXBP/CRE-BP2 and c-Jun form a complex which binds to the cyclic AMP, but not to the 12-*o*-tetradecanoylphorbol-13-acetate, response element. *Mol. Cell. Biol.* **10** : 1609-1621

Izumo, S. and V.Mahdavi (1988) : Thyroid hormone receptor α isoforms generated by alternative splicing differentially activate myosin HC gene transcription. *Nature* **334** : 539-542

Jackson, I.J. (1989) : The mouse, in *Genes and embryos*, D.M.Glover and B.D.Hames, eds., IRL Press

Jaynes, J.B. and P.O'Farrell (1988) : Activation and repression of transcription by homoeodomain-containing proteins that bind a common site. *Nature* **336** : 744-749

Jen, Y., H.Weintraub and R.Benezra (1992): Overexpression of Id protein inhibits the muscle differentiation program : in vivo association of Id with E2A proteins. *Genes Dev.* **6** : 1466-1479

Johnson, P.F. and S.L.McKnight (1989) : Eukaryotic transcriptional regulatory proteins. *Annu. Rev. Biochem.* **58** : 799-839

Jones, N.C., P.W.J.Rigby and E.B.Ziff (1989) : *Trans*-acting protein factors and the regulation of eukaryotic transcription : lessons from studies on DNA tumor viruses. *Genes Dev.* **2** : 267-281

Jones, N., and T.Shenk (1979). An adenovirus 5 early gene function regulates expression of other early viral genes. *Proc. Natl. Acad. Sci. USA* 76 : 3665-3669

Kaelin, Jr., W.G., W.Krek, W.R.Sellers, J.A.DeCaprio, F.Ajchenbaum, C.S.Fuchs, T.Chittenden, Y.Li, P.J.Farnham, M.A.Blanar, D.M.Livingston and E.K.Flemington (1992) : Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. *Cell* 70 : 351-364

Kapiloff, M.S., Y.Farkash, M.Wegner and M.G.Rosenfeld (1991) : Variable effects of phosphorylation of Pit-1 dictated by the DNA repsonse elements. *Science* **253** : 786-789

Kara, C.J., H.-C.Liou, L.B.Ivashkiv, and L.H.Glimcher (1990): A cDNA for a human cyclic AMP response element-binding protein which is distinct from CREB and expressed preferentially in brain. *Mol. Cell. Biol.* **10** : 1347-1357

Katagiri, F., E.Lam and N.H.Chua (1989) : Two tobacco DNA-binding proteins with homology to the nuclear factor CREB. *Nature* **340** : 727-730

Kelleher, III, R.J., P.M.Flanagan and R.D.Kornberg (1990) : A novel mediator between activator proteins and the RNA polymerase II transcription apparatus. *Cell* **61** : 1209-1215

Kerppola, T.K. and T.Curran (1991): Fos-Jun heterodimers and Jun homodimers bend DNA in opposite orientations : implications for transcription factor cooperativity. *Cell* **66** : 317-326

Kessel, M. and P.Gruss (1991) : Homeotic transformations of murine vertebrae and concomitant alteration of *Hox* codes induced by retinoic acid. *Cell* **67** : 89-104

Kessel, M. and P.Gruss (1990) : Murine developmental control genes. *Science* **249** : 374-379

Kieran, M., V.Blank, F.Logeat, J.Vandekerchkove, F.Lottspeich, O.Le Bail, M.B.Urban, P.Kourilsky, P.A.Bauerle and P.A.Israel (1990) : The DNA-binding subunit of NF-κB is identical to factor KBF1 and homologous to the *rel* oncogene product. *Cell* **62** : 1007-1018

Kim, S.-J., S.Wagner, F.Liu, M.A.O'Reilly, P.D.Robbins and M.R.Green (1992) : Retinoblastoma gene product activates expression of the human *TGF-β2* gene through transcription factor ATF-2. *Nature* **358** : 331-334

Klemm, D.J., W.J.Roesler, J.Liu, E.A.Park and R.W.Hanson (1990) : In vitro analysis of promoter elements regulating transcription of the phosphoenolpyruvate carboxykinase (GTP) gene. *Mol. Cell. Biol.* **10** : 480-485

Köhler, G. and C.Milstein (1976) : Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. *Eur. J. Immunol.* **6** : 292-295

Kornberg, R.D. and Y.Lorch (1991) : Irresistible force meets immovable object: transcription and the nucleosome. *Cell* **67** : 833-836

Kovesdi, I., R.Reichel, and J.R.Nevins (1986) : Identification of a cellular transcription factor involved in E1A *trans*-activation. *Cell* **45** : 219-228

Kozak, M. (1987) : An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucl. Acids Res.* **15** : 8125-8132

Kreider, B.L., R.Benezra, G.Rovera and T.Kadesch (1992) : Inhibition of myeloid differentiation by the helix-loop-helix protein Id. *Science* **255** : 1700-1702

Kryszke, M.-H., J.Piette, and M.Yaniv (1987) : Induction of a factor that binds to the polyoma virus A enhancer on differentiation of embryonal carcinoma cells. *Nature* **328** : 254-256

Kuo, C.J., P.B.Conley, L.Chen, F.M.Sladek, J.E.Darnell Jr. and G.R.Crabtree (1992) : A transcriptional hierarchy involved in mammalian cell-type specification. *Nature* **355** : 457-461

Kurkinen, M, A.R.Cooper, D.P.Barlow, J.R.Jenkins and B.L.M.Hogan (1983) : Gene expression during parietal endoderm differentiation in mouse embryos and teratocarcinoma cells, in *Teratocarcinoma Stem Cells*, L.M.Silver, G.R.Martin and S.Strickland, eds., Cold Spring Harbor Laboratory.

Kyte, J. and R.F.Doolittle (1982) : A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157** : 105-132

Landschulz, W.H., P.F.Johnson and S.L.McKnight (1989) : The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. *Science* **243** : 1681-1688

Landschulz, W.H., P.F.Johnson. E.Y.Adashi, B.J.Graves and S.L.McKnight (1988a) : Isolation of a recombinant copy of the gene encoding C/EBP. *Genes Dev.* **2** : 786-800

Landschulz, W.H., P.F.Johnson, and S.L.McKnight (1988b) : The leucine zipper : A hypothetical structure common to a new class of DNA-binding proteins. *Science* **240** : 1759-1764

Lassar, A.B., R.L.Davis, W.E.Wright, T.Kadesch, C.Murre, A.Voronova, D.Baltimore and H.Weintraub (1991) : Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. *Cell* **66** : 305-315

La Thangue, N.B. (1992) : The mechanism of action of the retinoblastoma gene product, in *Nucleic Acids and Molecular Biology*, vol. 7, F.Eckstein and D.M.J.Lilley, eds., Springer-Verlag (in press)

La Thangue, N.B., B.Thimmappaya, and P.W.J.Rigby (1990) : The embryonal carcinoma stem cell E1A-like activity involves a differentiation-regulated transcription factor. *Nucl. Acids Res.* **18** : 2929-2938

La Thangue, N.B. and P.W.J.Rigby (1988a) : The regulation of SV40 early gene expression in embryonal carcinoma stem cells - faithful transcriptional regulation *in vitro*. *Nucl.Acids Res.* **16** : 11417-11430

La Thangue, N.B. and P.W.J.Rigby (1988b) : *Trans*-acting protein factors and the regulation of eukaryotic transcription, in *Transcription and splicing*, B.D.Hames and D.M.Glover, eds., IRL Press

La Thangue, N.B., and P.W.J.Rigby (1987) : An adenovirus E1A-like transcription factor is regulated during the differentiation of murine embryonal carcinoma stem cells. *Cell* **49** : 507-513

Laurent, B.C. and M.Carlson (1992) : Yeast SNF2/SWI2, SNF5, and SNF6 proteins function coordinately with the gene-specific transcriptional activators GAL4 and Bicoid. *Cell* **6** : 1707-1715

Laybourn, P.J. and J.T.Kadonaga (1991) : Role of nucleosomal cores and histone H1 in regulation of transcription by RNA polymerase II. *Science* **254** : 238-245

Lee, C.Q., Y.Yun, J.P.Hoeffler and J.F.Habener (1990) : Cyclic-AMP responsive transcriptional activation of CREB-327 involves interdependent phosphorylated subdomains. *EMBO J.* **9** : 4455-4465

Lee, K.A.W., J.S.Fink, R.H.Goodman, and M.R.Green (1989) : Distinguishable promoter elements are involved in transcriptional activation by E1A and cyclic AMP. *Mol. Cell. Biol.* **9** : 4390-4397

Lee, K.A.W. and M.R.Green (1987) : A cellular transcription factor E4F1 interacts with an E1A-inducible enhancer and mediates constitutive enhancer function *in vitro*. *EMBO J.* **6** : 1345-1353

Lee, K.A.W., T.-Y.Hai, L.SivaRaman, B.Thimmappaya, H.C.Hurst, N.C.Jones, and M.R.Green (1987) : A cellular protein, activating transcription factor, activates transcription of multiple E1A-inducible adenovirus early promoters. *Proc. Natl. Acad. Sci. USA* **84** : 8355-8359

Lee, T.-C., K.-L.Chow, P.Fang and R.J.Schwartz (1991) : Activation of skeletal α-actin gene transcription : the cooperative formation of serum response factor-binding complexes over positive *cis*-acting promoter serum response elements displaces a negative-acting nuclear factor enriched in replicating myoblasts and nonmyogenic cells. *Mol. Cell. Biol.* **11** : 5090-5100

Lee, W., P.Mitchell and R.Tjian (1987) : Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell* **49** : 741-752

Lee, W.S., C.C.Kao, G.O.Bryant, X.Liu and A.L.Berk (1991) : Adenovirus E1A activation domain binds the basic repeat in the TATA box transcritpion factor. *Cell* **67** : 365-376

Levine, M. and J.L.Manley (1989) : Transcriptional repression of eukaryotic promoters. *Cell* **59** : 405-408

Levine, M. and T.Hoey (1988) : Homeobox proteins as sequence-specific transcription factors. *Cell* **55** : 537-540

Levine, M.S. and K.W.Harding (1989) : *Drosophila* : the zygotic contribution, in *Genes and embryos*, D.M.Glover and B.D.Hames eds., IRL Press

Lewin, B. (1990) : Commitment and activation at pol II promoters : a tail of protein-protein interactions. *Cell* **61** : 1161-1164

Li, R., J.D.Knight, S.P.Jackson, R.Tjian and M.R.Botchan (1991) : Direct interaction between Spl and the BPV enhancer E2 protein mediates synergistic activation of transcription. *Cell* **65** : 493-505

Li, S., E.B.Crenshaw III, E.J.Rawson, D.M.Simmons, L.W.Swanson and M.G.Rosenfeld (1990) : Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene *pit-1*. *Nature* **347** : 528-533

Licht, J.D., M.J.Grossel, J.Figge and U.M.Hansen (1990) : *Drosophila* Krüppel protein is a transcriptional repressor. *Nature* **346** : 76-79

Lillie, J.W. and M.R.Green (1989) : Transcription activation by the adenovirus Ela protein. *Nature* **338** : 39-44

Lillie, J.W., P.M.Loewenstein, M.R.Green and M.Green (1987) : Functional domains of adenovirus type 5 Ela proteins. *Cell* **50** : 1091-1100

Lin, Y.-S., I.Ha, E.Maldonado, D.Reinberg and M.R.Green (1991) : Binding of general transcription factor TFIIB to an acidic activating region. *Nature* **353** : 569-571

Lin, Y.-S., M.Carey, M.Ptashne and M.R.Green (1990) : How different eukaryotic transcriptional activators can cooperate promiscuously. *Nature* **345** : 359-361

Lin, Y.-S. and M.R.Green (1989a) : Identification and purification of a *Saccharomyces cerevisiae* protein with the DNA binding specificity of mammalian activating transcription factor. *Proc. Natl. Acad. Sci. USA* **86** : 109-113

Lin, Y.-S. and M.R.Green (1989b) : Similarities between prokaryotic and eukaryotic cyclic AMP-responsive elements. *Nature* **340** : 656-659

Lin, Y.-S., M.F.Carey, M.Ptashne and M.R.Green (1988) : GAL4 derivatives function alone and synergistically with mammalian activators in vitro. *Cell* **54** : 659-664

Linney, E., B.Davis, J.Overhauser, E.Chao, and H.Fan (1984). Non-function of a Moloney murine leukaemia virus regulatory sequence in F9 embryonal carcinoma cells. *Nature* **308** : 470-472

Liu, F. and M.R.Green (1990) : A specific member of the ATF transcription factor family can mediate transcription activation by the adenovirus Ela protein. *Cell* **61** : 1217-1224

Loh, T.P., L.L.Sievert and R.W.Scott (1990) : Evidence for a stem cell-specific repressor of Moloney murine leukemia virus expression in embryonal carcinoma cells. *Mol. Cell. Biol.* **10** : 4045-4057

Loh, T.P., L.L.Sievert and R.W.Scott (1989) : Negative regulation of retrovirus expression in embryonal carcinoma cells mediated by an intragenic domain. *J. Virol.* **62** : 4086-4095

Lu, H., L.Zawel, L.Fisher, J.-M.Egly and D.Reinberg (1992) : Human general transcription factor IIH phosphorylates the C-terminal domain of RNA polymerase II. *Nature* **358** : 641-645

Lu, H., O.Flores, R.Weinmann and D.Reinberg (1991) : The nonphosphorylated form of RNA polymerase II preferentially associates with the preinitiation complex. *Proc. Natl. Acad. Sci. USA* **88** : 10004-10008

Lucas, P.C., R.M.O'Brien, J.A.Mitchell, C.M.Davis, E.Imai, B.M.Forman, H.H.Samuels and D.K.Granner (1991) : A retinoic acid response element is part of a pleiotropic domain in the phospho*enol*pyruvate carboxykinase gene. *Proc. Natl. Acad. Sci. USA* **88** : 2184-2188

Luckow, B. and G.Schütz (1987) : CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. *Nucl. Acids Res.* **15** : 5490

Ludlow, J.W., J.A.De Caprio, C.-M.Huang, W.-H.Lee, E.Paucha and D.M.Livingston (1990) : SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. *Cell* **56** : 57-65

Luisi, B. (1992) : Zinc standard for economy. *Nature* **356** : 379-380

Luisi, B.F., W.X.Xu, Z.Otwinowski, L.P.Freedman, K.R.Yamamoto and P.B.Sigler (1991) : Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature* **352** : 497-505

McCormick, A., H.Brady, L.E.Theill and M.Karin (1990) : Regulation of the pituitary-specific homeobox gene *GHF1* by cell-autonomous and environmental cues. *Nature* **345** : 829-832

Macgregor, P.F., C.Abate and T.Curran (1990) : Direct cloning of leucine zipper proteins : Jun binds cooperatively to the CRE with CRE-BP1. *Oncogene* **5** : 451-458

Maekawa, T., H.Sakura, C.Kanei-Ishii, T.Sudo, T.Yoshimura, J.Fujisawa, M.Yoshida, and S.Ishii (1989) : Leucine zipper structure of the protein CRE-BP1 binding to the cyclic AMP response element in brain. *EMBO J.* **8** : 2023-2028

McKnight, S.L. and R.Kingsbury (1982) : Transcriptional control signals of a eukaryotic protein-coding gene. *Science* **217** : 316-324

Maguire, H.F., J.P.Hoeffler and A.Siddiqui (1991) : HBV X protein alters the DNA binding specificity of CREB and ATF-2 by protein-protein interactions. *Science* **252** : 842-844

Mäkelä, T.P., P.J.Koskinen, I.Västrik and K.Alitalo (1992) : Alternative forms of Max as enhancers or suppressors of Myc-Ras cotransformation. *Science* **256** : 373-377

Maldonado, E., I.Ha, P.Cortes, L.Weis and D.Reinberg (1990) : Factors involved in specific transcription by mammalian RNA polymerase II : role of transcription factors IIA, IID and IIB during formation of a transcription-competent complex. *Mol. Cell. Biol.* **10** : 6335-6347

Mangelsdorf, D.J., U.Borgmeyer, R.A.Heyman, J.Y.Zhou, E.S.Ong, A.E.Oro, A.Kakizuka and R.M.Evans (1992) : Characterization of three RXR genes that mediate the action of 9-*cis* retinoic acid. *Genes Dev.* **6** : 329-344

Manley, J.L., A.Fire, A.Cano, P.A.Sharp, and M.L.Gefter (1980) : DNA-dependent transcription of adenovirus genes in a soluble whole-cell extract. *Proc. Natl. Acad. Sci. USA* **77** : 3855-3859

Maniatis, T., S.Goodbourn and J.A.Fischer (1987) : Regulation of inducible and tissue-specific gene expression. *Science* **236** : 1237-1244

Marcu, K.B. (1987) : Regulation of expression of the c-*myc* proto-oncogene. *BioEssays* **6** : 28-32

Martin, K.J., J.W.Lillie and M.R.Green (1990) : Evidence for interaction of different eukaryotic transcriptional activators with distinct cellular targets. *Nature* **346** : 147-152

Masson, N., M.Ellis, S.Goodbourn and K.A.W.Lee (1992) : Cyclic AMP response element-binding protein and the catalytic subunit of protein kinase A are present in F9 embryonal carcinoma cells but are unable to activate the somatostatin promoter. *Mol. Cell. Biol.* **12** : 1096-1106

Meijlink, F., T.Curran, A.D.Miller and I.M.Verma (1985) : Removal of a 67-base-pair sequence in the noncoding region of protooncogene fos converts it to a transforming gene. *Proc. Natl. Acad. Sci. USA* **82** : 4987-4991

Meisterernst, M. and R.G.Roeder (1991) : Family of proteins that interact with TFIID and regulate promoter activity. *Cell* **67** : 557-567

Meisterernst, M., A.L.Roy, H.M.Lieu and R.G.Roeder (1991) : Activation of class II gene transcription by regulatory factors is potentiated by a novel activity. *Cell* **66** : 981-993

Minie, M.E., T.Kimura and G.Felsenfeld (1992) : The developmental switch in embryonic ρ-globin expression is correlated with erythroid lineage-specific differences in transcription factor levels. *Development* **115** : 1149-1164

Mitchell, P.J. and R.Tjian (1989) : Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* **245** : 371-378

Montano, X. and D.P.Lane (1987) : The adenovirus E1a gene induces differentiation of F9 teratocarcinoma cells. *Mol. Cell. Biol.* **7** : 1782-1790

Montminy, M.R. and L.M.Bilezikjian (1987) : Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. *Nature* **328** : 175-178

Montminy, M.R., K.A.Sevarino, J.A.Wagner, G.Mandel, and R.Goodman (1986) : Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. *Proc. Natl. Acad. Sci. USA* **83** : 6682-6686

Moran, E. and M.B.Mathews (1987) : Multiple functional domains in the adenovirus E1A gene. *Cell* **48** : 177-178

Mudryj, M., S.W.Hiebert, and J.R.Nevins (1990) : A role for the adenovirus inducible E2F transcription factor in a proliferation dependent signal transduction pathway. *EMBO J.* **9** : 2179-2184

Mueller, C.R., P.Maire and U.Schibler (1990) : DBP, a liver-enriched transcriptional activator, is expressed late in ontogeny and its tissue specificity is determined posttranscriptionally. *Cell* **61** : 279-291

Müller, H.-P., J.M.Sogo and W.Schaffner (1989) : An enhancer stimulates transcription in *trans* when attached to the promoter via a protein bridge. *Cell* **58** : 767-777

Mumberg,D., F.C.Lucibello, M.Schuermann and R.Müller (1991) : Alternative splicing of *fosB* transcripts results in differentially expressed mRNAs encofing functionally antagonistic proteins. *Genes Dev.* **5** : 1212-1223

Murray, E.J., D.Stott and P.W.J.Rigby (1991) : Sequences and factors required for the F9 embryonal carcinoma stem cell E1a-like activity. *Mol. Cell. Biol.* **11** : 5534-5540

Murthy, S.C.S., G.R.Bhat, and B.Thimmappaya (1985) : Adenovirus EIIA early promoter : transcriptional control elements and induction by the viral pre-early E1A gene, which appears to be sequence independent. *Proc. Natl. Acad. Sci. USA* **82** : 2230-2234

Näär, A.M., J.-M.Boutin, S.M.Lipkin, V.C.Yu, J.M.Holloway, C.K.Glass and M.G.Rosenfeld (1991) : The orientation and spacing of core DNA-binding motifs dictate selective transcriptional responses to threee nuclear receptors. *Cell* **65** : 1267-1279

Nagpal, S., A.Zelent and P.Chambon (1992) : RAR-β4, a retinoic acid reseptor isoform is generated from RAR-β2 by alternative splicing and usage of a CUG initiator codon. *Proc. Natl. Acad. Sci. USA* **89** : 2718-2722

Nairn, A.C. and P.Greengard (1987) : Purification and characterization of Ca^{2+}/calmodulin-dependent protein kinase I from bovine brain. *J. Biol. Chem.* **262** : 7273-7281

Nakabeppu, Y., K.Ryder, and D.Nathans (1988) : DNA binding activities of three murine Jun proteins : stimulation by Fos. *Cell* **55** : 907-915

Nardelli, J., T.J.Gibson, C.Vesque and P.Charnay (1991) : Base sequence discrimination by zinc-finger DNA-binding domains. *Nature* **349** : 175-178

Nevins, J.R. (1991) : Transcriptional activation by viral regulatory proteins. *Trends Biochem. Sci.* **16** : 435-439

Nevins, J.R. and J.Jensen-Winkler (1980) : Regulation of early adenovirus transcription : a protein product of early region 2 specifically represses region 4 transcription. *Proc. Natl. Acad. Sci. USA* **77** : 1893-1897

Nevins, J.R., H.S.Ginsberg, J.-M.Blanchard, M.C.Wilson and J.E.Darnell, Jr. (1979) : Regulation of the primary expression of the early adenovirus transcription units. *J. Virol.* **32** : 727-733

Nichols, M., F.Weih, W.Schmid, C.DeVack, E.Kowentz-leutz, B.Luckow, M.Boshart and G.Schütz (1992) : Phosphorylation of CREB affects its binding to high and low affinity sites : implications for cAMP induced gene transcription. *EMBO J.* **11** : 3337-3346

Nüsslein-Volhard, C. (1991) : Determination of the embryonic axes of *Drosophila*. *Development Supplement* **1** : 1-10

O'Neill, K.T., R.H.Hoess and W.F.DeGrado (1990) : Design of DNA-binding peptides based on the leucine zipper motif. *Science* **249** : 774-

Orkin, S.H. (1990) : Globin gene regulation and switching : circa 1990. *Cell* **63** : 665-672

O'Shea, E.K., J.D.Klemm, P.S.Kim and T.Alber (1991) : X-ray structure of the GCN4 leucine zipper, a two-stranded, parallel coiled coil. *Science* **254** : 539-544

Ott, M.-O., E.Bober, G.Lyons, H.Arnold and M.Buckingham (1991) : Early expression of the myogenic regulatory gene, *myf-5*, in precursor cells of skeletal muscle in the mouse embryo. *Development* **111** : 1097-1107

Otte, A.P., P.van Run, M.Heideveld, R.van Driel and A.J.Durston (1989) : Neural induction is mediated by cross-talk between the protein kinase C and cyclic AMP pathways. *Cell* **58** : 641-648

Partridge, J.F. and N.B.La Thangue (1991) : A developmentally regulated and tissue-dependent transcription factor complexes with the retinoblastoma gene product. *EMBO J.* **10** : 3819-3827

Parvin, J.D., H.T.M.Timmers and P.A.Sharp (1992) : Promoter specificity of basal transcription factors. *Cell* **68** : 1134-1144

Patel, L., Abate, C. and T.Curran (1990) : Altered protein conformation on DNA binding by Fos and Jun. *Nature* **347** :572-575

Pei, R. and A.J.Berk (1989) : Multiple transcription factor binding sites mediate adenovirus E1A transactivation. *J. Virol.* **63** : 3499-3506

Peterson, C.L. and I.Herskowitz (1992) : Characterization of the yeast *SWI1*, *SWI2*, and *SWI3* genes, which encode a global activator of transcription. *Cell* **68** : 573-583

Peterson, M.G. and R.Tjian (1992) : The tell-tail trigger. *Nature* **358** : 620-621

Peterson, M.G., N.Tanese, B.F.Pugh and R.Tjian (1990) : Functional domains and upstream activation properties of cloned human TATA binding protein. *Science* **248** : 1625-1630

Pinto, I., D.E.Ware and M.Hampsey (1992) : The yeast *SUA7* gene encodes a homolog of human transcription factor TFIIB and is required for normal start site selection in vivo. *Cell* **68** : 977-988

Plet, A., D.Evain and W.B.Anderson (1982) : Effect of retinoic acid treatment of F9 embryonal carcinoma cells on the activity and distribution of cyclic AMP-dependent protein kinase. *J. Biol. Chem.* **257** : 889-893

Ptashne, M. (1988) : How eukaryotic transcriptional activators work. *Nature* **335** : 683-689

Ptashne, M. (1986) : Gene regulation by proteins acting nearby and at a distance. *Nature* **322** : 697-701

Pugh, B.F. and R.Tjian (1991) : Transcription from a TATA-less promoter requires a multisubunit TFIID complex. *Genes Dev.* **5** : 1935-1945

Pugh, B.F. and R.Tjian (1990) : Mechanism of transcriptional activation of Sp1 : evidence for coactivators. *Cell* **61** : 1187-1197

Pulverer, B.J., J.M.Kyriakis, J.Avruch, E.Nikolakaki and J.R.Woodgett (1991) : Phosphorylation of c-*jun* mediated by MAP kinases. *Nature* **353** : 670-674

Raychaudhuri, P., S.Bagchi, and J.R.Nevins (1989) : DNA-binding activity of the adenovirus-induced E4F transcription factor is regulated by phosphorylation. *Genes Dev.* **3** : 620-627

Raychaudhuri, P., R.Rooney, and J.R.Nevins (1987) : Identification of an E1A-inducible cellular factor that interacts with regulatory sequences within the adenovirus E4 promoter. *EMBO J.* **6** : 4073-4081

Read, D., and J.L.Manley (1992) : Alternatively spliced transcripts of the *Drosophila tramtrack* gene encode zinc finger proteins with distinct DNA binding specificities. *EMBO J.* **11** : 1035-1044

Reddy, P. and S.Hahn (1991) : Dominant negative mutations in yeast TFIID define a bipartite DNA-binding region. *Cell* **65** : 349-357

Rehfuss, R.P., K.M.Walton, M.M.Loriaux and R.H.Goodman (1991) : The cAMP-regulated enhancer-binding protein ATF-1 activates transcription in response to cAMP-dependent protein kinase A. *J. Biol.Chem.* **266** : 18431-18434

Reinberg, D., M.Horikoshi and R.G.Roeder (1987) : Factors involved in specific transcription by mammalian polymerase II : functional analysis of initiation factors IIA and IID and identification of a new factor operating at sequences downstream of the initiation site. *J. Biol. Chem.* **262** : 3322-3330

Renkawitz, R. (1990) : Transcriptional repression in eukaryotes. *Trends Genet.* **6** : 192-196

Rhodes, D. and J.W.R.Schwabe (1991) : Complex behaviour. *Nature* **352** : 478-479

Rickles, R.J., A.L.Darrow, and S.Strickland (1989) : Differentiation-responsive elements in the 5' region of the mouse tissue plasminogen activator gene confer two-stage regulation by retinoic acid and cyclic AMP in teratocarcinoma cells. *Mol. Cell. Biol.* **9** : 1691-1704

Roberts, S., T.Purton and D.L.Bentley (1992) : A protein-binding site in the c-*myc* promoter functions as a terminator of RNA polymerase II transcription. *Genes Dev.* **6** : 1562-1574

Roeder, R.G. (1991) : The complexities of eukaryotic transcription initiation : regulation of preinitiation complex assembly. *Trends Biochem. Sci.* **16** : 402-408

Roesler, W.J., G.R.Vanderbark, and R.W.Hanson (1988) : Cyclic AMP and the induction of eukaryotic gene transcription. *J. Biol. Chem.* **263** : 9063-9066

Roman, C., L.Cohn and K.Calame (1991) : A dominant negative form of transcription activator mTFE3 created by alternative splicing. *Science* **254** : 94-97

Rooney, R.J., P.Raychaudhuri and J.R.Nevins (1990) : E4F and ATF, two transcription factors that recognize the same site, can be distinguished both physically and functionally : a role for E4F in E1A *trans* activation. *Mol. Cell. Biol.* **10** : 5138-5149

Rosner, M.H., M.A.Vigano, K.Ozato, P.M.Timmons, F.Poirier, P.W.J.Rigby and L.M.Staudt (1990) : A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. *Nature* **345** : 686-692

Roy, A.L., M.Meisterernst, P.Pognonec and R.G.Roeder (1991) : Cooperative interaction of an initiator-binding transcription factor and the helix-loop-helix activator USF. *Nature* **354** : 245-248

Ruberte, E., P.Dollé, P.Chambon and G.Morriss-Kay (1991) : Retinoic acid receptors and cellular retinoid binding proteins : II. Their differential pattern of transcription during early morphogenesis in mouse embryos. *Development* **111** : 45-60

Ruppert, S., T.J.Cole, M.Boshart, E.Schmid and G.Schütz (1992) : Multiple mRNA isoforms of the transcription activator protein CREB : generation by alternative splicing and specific expression in primary spermatocytes. *EMBO J.* **11** : 1503-1512

Ryder, K., A.Lanahan, E.Perez-Albuerne and D.Nathans (1989) : Jun-D : a third member of the Jun gene family. *Proc. Natl. Acad. Sci. USA* **86** : 1500-1503

Ryder, K., L.F.Lau and D.Nathans (1988) : A gene activated by growth factors is related to the oncogene v-*jun*. *Proc. Natl. Acad. Sci. USA* **85** : 1487-1491

Ryseck, R.-P. and R.Bravo (1991) : c-JUN, JUN B, and JUN D differ in their binding affinities to AP-1 and CRE consensus sequences : effect of FOS proteins. *Oncogene* **6** : 533-542

Ryseck, R.-P., S.I.Hirai, M.Yaniv and R.Bravo (1988) : Transcriptional activation of c-*jun* during the G_0/G_1 transition in mouse fibroblasts. *Nature* **334** : 535-537

Salminen, A., T.Braun, A.Buchberger, S.Jürs, B.Winter and H.-H.Arnold (1991) : Transcription of the muscle regulatory gene *MYF4* is regulated by serum components, peptide growth factors and signaling pathways involving G proteins. *J. Cell Biol.* **115** : 905-917

Sambrook, J., E.F.Fritsch and T.Maniatis (1989) : *Molecular cloning - a laboratory manual* (2nd edition). Cold Spring Harbor Laboratory Press.

Sassoon, D., G.Lyons, W.E.Wrught, V.Lin, A.Lassar, H.Weintraub and M.Buckimgham (1989) : Expression of two myogenic regulatory factors myogenin and MyoDl during mouse embryogenesis. *Nature* **341** : 303-307

Sauer, F. and H.Jäckle (1991) : Concentration-dependent transcriptional activation or repression by *Krüppel* from a single binding site. *Nature* **353** : 562-566

Schöler, H.R., T.Ciesiolka, and P.Gruss (1991) : A nexus between Oct-4 and ElA : implications for gene regulation in embryonic stem cells. *Cell* **66** : 291-304

Schöler, H.R., A.K.Hatzopoulos, R.Balling, N.Suzuki, and P.Gruss (1989) : A family of octamer-specific proteins present during mouse embryogenesis : evidence for germline-specific expression of an Oct factor. *EMBO J.* **9** : 2543-2550

Schultz, M.C., R.H.Reeder and S.Hahn (1992) : Variants of the TATA-binding protein can distinguish subsets of RNA polymerase I, II and II promoters. *Cell* **69** : 607-702

Schütte, J., J.Viallet, M.Nau, S.Segal, J.Fedorkr, and J.Minna (1989) : *jun-B* inhibits and c-*fos* stimulates the transforming and *trans*-activating activities of c-*jun*. *Cell* **59** : 987-997

Sen, R. and D.Baltimore (1986) : Multiple factors interact with the immunoglobulin enhancer sequences. *Cell* **46** : 921-928

Seto, E., Y.Shi and T.Shenk (1991) : YY1 is an initiator sequence-binding protein that directs and activates transcription *in vitro*. *Nature* **354** : 241-245

Sharp, P.A. (1992) : TATA-binding protein is a classless factor. *Cell* **68** : 819-821

Sheng, M. and M.E.Greenberg (1990) : The regulation and function of c-*fos* and other immediate early genes in the nervous system. *Neuron* 4 : 477-485

Sheng, M., G.McFadden and M.E.Greenberg (1990) : Membrane depolarization and calcium induce c-*fos* transcription via phosphorylation of transcription factor CREB. *Neuron* 4 : 571-582

Sheng, M., S.T.Dougan, G.McFadden and M.E.Greenberg (1988) : Calcium and growth factor pathways of c-*fos* transcriptional activation require distinct upstream regulatory sequences. *Mol. Cell. Biol.* 8 : 2787-2796

Shi, Y., E.Seto, L.-S.Chang and T.Shenk (1991) : Transcriptional repression by YY1, a human GLI-Krüppel-related protein, and relief of repression by adenovirus E1A protein. *Cell* 67 : 377-388

Shivji, M.K.K. and N.B.La Thangue (1991) : Multicomponent differentiation-regulated transcription factors in F9 embryonal carcinoma stem cells. *Mol. Cell. Biol.* 11 : 1686-1695

Shuman, J.D., C.R.Vinson and S.L.McKnight (1990) : Evidence of changes in protease sensitivity and subunit exchange rate on DNA binding by C/EBP (1990). *Science* 249 : 771-774

Silver, B.J., J.A.Bokar, J.B.Virgin, E.A.Vallen, A.Milsted and J.H.Nilson (1987) : Cyclic AMP regulation of the human glycoprotein hormone α-subunit gene is mediated by an 18-base-pair element. *Proc. Natl. Acad. Sci. USA* 84 : 2198-2202

Sleigh, M.J., T.J.Lockett, J.Kelly and D.Lewy (1987) : Competition studies with repressors and activators of viral enhancer function in F9 mouse embryonal carcinoma cells. *Nucl. Acids Res.* 15 : 4307-4324

Sleigh, M., and T.J.Lockett (1985). SV40 enhancer activation during retinoic acid-induced differentiation of F9 embryonal carcinoma cells. *EMBO J.* 13 : 3831-3837

Smolik, S.M., R.E.Rose and R.H.Goodman (1992) : A cyclic AMP-responsive element-binding transcriptional activator in *Drosophila melanogaster*, dCREB-A, is a member of the leucine zipper family. *Mol. Cell. Biol.* 12 : 4123-4131

Solter, D. and B.B.Knowles (1978) : Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). *Proc. Natl. Acad. Sci. USA* 75 : 5565-5569

Steenbergh, P.H. and J.S.Sussenbach (1979) : The nucleotide sequence of the right-hand terminus of adenovirus type 5 DNA : implications for the mechanism of DNA replication. *Gene* 6 : 307-318

Stern, S., M.Tanaka and W.Herr (1989) : The Oct-1 homoeodomain directs formation of a multiprotein-DNA complex with the HSV transactivator VP16. *Nature* 341 : 624-630

Stoykova, A.S., S.Sterrer, J.R.Erselius, A.K.Hatzopoulos and P.Gruss (1992) : Mini-Oct and Oct-2c : two novel, functionally diverse *oct-2* gene products are differentially expressed in the CNS. *Neuron* 8 : 541-558

Stragier, P. (1991) : Dances with sigmas. *EMBO J.* 10 : 3559-3566

Strickland, S., K.K.Smith and K.R.Marotti (1980) : Hormonal induction of differentiation in teratocarcinoma stem cells : generation of parietal endoderm by retinoic acid and dibutyryl cAMP. *Cell* 21 : 347-355

Strickland, S. and V.Mahdavi (1978) : The induction of differentiation in teratocarcinoma stem cells by retinoic acid. *Cell* **15** : 393-403

Stringer, K.F., C.J.Ingles and J.Greenblatt (1990) : Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID. *Nature* **345** : 783-786

Strubin, M. and K.Struhl (1992) : Yeast and human TFIID with altered DNA-binding specificity for TATA elements. *Cell* **68** : 721-730

Struhl, G., K.Struhl and P.Macdonald (1989) : The gradient morphogen *bicoid* is a concentration-dependent transcriptional activator. *Cell* **57** : 1259-1273

Su, W., S.Jackson, R.Tjian and H.Echols (1991) : DNA looping between sites for transcriptional activation : self-association of DNA-bound Sp1. *Genes Dev.* **5** : 820-826

Suemori, H., S.Hashimoto, and N.Nakatsuji (1988) : Presence of the adenovirus E1A-like activity in preimplantation stage mouse embryos. *Mol. Cell. Biol.* **8** : 3553-3555

Sumimoto, H., Y.Okhuma, T.Yamamoto, M.Horikoshi and R.G.Roeder (1990) : Factors involved in specific transcription by mammalian RNA polymerase II : Identification of general transcription factor TFIIG. *Proc. Natl. Acad. Sci. USA* **87** : 9158-9162

Sun, X.-H and D.Baltimore (1991) : An inhibitory domain of E12 transcription factor prevents DNA binding in E12 homodimers but not in E12 heterodimers. *Cell* **64** : 459-470

Sun, X.-H., N.G.Copeland, N.A.Jenkins and D.Baltimore (1991) : Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix-loop-helix proteins. *Mol. Cell. Biol.* **11** : 5603-5611

Tabata, T., T.Nakayema, K.Mikami and M.Iwabuchi (1991) : HBP-1a and HBP-1b : leucine zipper-type transcription factors of wheat. *EMBO J.* **10** : 1459-1467

Talanian, R.V., C.J.McKnight and P.S.Kim (1990) : Sequence-specific DNA binding by a short peptide dimer. *Science* **249** : 769-771

Tanaka, M. and W.Herr (1990) : Differential transcriptional activation by Oct-1 and Oct-2 : interdependent activation domains induce Oct-2 phosphorylation. *Cell* **60** : 375-386

Tanese, N., B.F.Pugh and R.Tjian (1991) : Coactivators for a proline-rich activator purified from the multisubunit human TFIID complex. *Genes Dev.* **5** : 2212-2224

Tasset, D., L.Tora, C.Fromental, E.Scheer and P.Chambon (1990) : Distinct classes of transcriptional activating domains function by different mechanisms. *Cell* **62** : 1177-1187

Tassios, P.T., and N.B.La Thangue (1990) : A multiplicity of differentiation-regulated ATF site-binding activities in embryonal carcinoma cells with distinct sequence and promoter specificities. *New Biol.* **2** : 1123-1134

Taylor, I.C.A., J.L.Workman, T.J.Schuetz and R.E.Kingston (1991) : Facilitated binding of GAL4 and heat shock factor to nucleosomal templates : differential function of DNA-binding domains. *Genes Dev.* **5** : 1285-1298

Taylor, I.C.A. and R.E.Kingston (1990) : E1a transactivation of human HSP70 gene promoter substitution mutants is independent of the composition of upstream and TATA elements. *Mol. Cell. Biol.* **10** : 176-183

Thayer, M.J., S.J.Tapscott, R.L.Davis, W.E.Wright, A.B.Lassar and H.Weintraub (1989) : Positive autoregulation of the myogenic determination gene MyoD1. *Cell* **58** : 241-248

Theill, L.E., K.Hattori, D.Lazzaro, J.-L.Castrillo and M.Karin (1992) : Differential splicing of the *GHF1* primary transcript gives rise to two functionally distinct homeodomain proteins. *EMBO J.* **11** : 2261-2269

Théveny, B., A.Bailly, C.Rauch, M.Rauch, E.Delain and E.Milgrom (1987) : Association of DNA-bound progesterone receptors. *Nature* **329** : 79-81

Timmers, H.Th.M. and P.A.Sharp (1991) : The mammalian TFIID protein is present in two functionally distinct complexes. *Genes Dev.* **5** : 1946-1956

Tooze, J. (ed.) (1981) : *DNA Tumor Viruses*, Second Edition, Part 2/revised, Cold Spring Harbor Laboratory

Treacy, M.N., L.I.Neilson, E.E.Turner, X.He and M.G.Rosenfeld (1992) : Twin of I-POU : a two amino acid difference in the I-POU homeodomain distinguishes an activator from an inhibitor of transcription. *Cell* **68** : 491-505

Treacy, M.N., X.He and M.Rosenfeld (1991) : I-POU : a POU domain protein that inhibits neuron-specific gene activation. *Nature* **350** : 577-584

Triezenberg, S.J., R.C.Kingsbury and S.L.McKnight (1988) : Functional dissection of VP16, the *trans*-activator of herpes simplex virus immediate early gene expression. *Genes Dev.* **2** : 718-729

Tsai, S.Y., M.-J.Tsai and B.W.O'Malley (1989) : Cooperative binding of steroid hormone receptors contributes to transcriptional synergism at target enhancer elements. *Cell* **57** : 443-448

Tsukada, T., J.S.Fink, G.Mandel, and R.H.Goodman (1987) : Identification of a region in the human vasoactive intestinal polypeptide gene responsible for regulation by cyclic AMP. *J. Biol. Chem.* **262** : 8743-8747

Turner, R. and R.Tjian (1989) : Leucine repeats and an adjacent DNA binding domain mediate the formation of functional cFos-cJun heterodimers. *Science* **243** : 1689-1694

Urban, M.B. and P.A.Bauerle (1991) : The role of the p50 and p65 subunits of NF-κB in the recognition of cognate sequences. *New Biol.* **3** : 279-288

Usheva, A., E.Maldonado, A.Goldring, H.Lu, C.Houbavi, D.Reinberg and Y.Aloni (1992) : Specific interaction between the nonphosphorylated form of RNA polymerase II and the TATA-binding protein. *Cell* **69** : 871-881

Vallée, B.L., J.E.Coleman and D.S.Auld (1991) : Zinc fingers, zinc clusters, and zinc twists in DNA-binding protein domains. *Proc. Natl. Acad. Sci. USA* **88** : 999-1003

Van Beveren, C., F.Van Straaten, T.Curran, R.Müller and I.M.Verma (1983) : Analysis of FBJ-MuSV provirus and c-fos (mouse) gene reveals that viral and cellular fos gene products have different carboxy termini. *Cell* **32** : 1241-1255

Van Nguyen, T., L.Kobierski, M.Comb and S.E.Hyman (1990) : The effect of depolarization on expression of the human proenkephalin gene is synergistic with cAMP and dependent upon a cAMP-inducible enhancer. *J. Neurosci.* **10** : 2825-2833

Vasios, G.W., J.D.Gold, M.Petkovich, P.Chambon and L.Gudas (1989) : A retinoic acid-responsive element is present in the 5' flanking region of the laminin B1 gene. *Proc. Natl. Acad. Sci. USA* **86** : 9099-9103

Velcich, A., F.G.Kern, C.Basilico and E.B.Ziff (1986) : Adenovirus E1a proteins repress expression from polyomavirus early and late promoters. *Mol. Cell. Biol.* **6** : 4019-4025

Velcich, A. and E.Ziff (1985) : Adenovirus E1a proteins repress transcription from the SV40 early promoter. *Cell* **40** : 705-716

Vinson, C.R., K.L.La Marco, P.F.Johnson, W.H.Landschulz and S.L.McKnight (1988) : In situ detection of sequence-specific DNA binding activity specified by a recombinant bacteriophage. *Genes Dev.* **2** : 801-806

Wada, T., H.Watanabe, Y.Usuda and H.Handa (1991) : Different biological activities of the hetero- and homodimers formed by the 47- and 43-kilodalton proteins of transcription factor ATF/E4TF3. *J. Virol.* **65** : 557-564

Wasylyk, B. (1988) : Transcription elements and factors of RNA polymerase B promoters of higher eukaryotes. *CRC Crit. Rev. Biochem.* **23** :77-120

Weih, F., A.F.Stewart, M.Boshart, D.Nitsch and G.Schütz (1990) : In vivo monitoring of a cAMP-stimulated DNA-binding activity. *Genes Dev.* **4** : 1437-1449

White, R.J., S.P.Jackson and P.W.J.Rigby (1992) : A role for the TATA-box-binding protein component of the transcription factor IID complex as a general RNA polymerase III transcription factor. *Proc. Natl. Acad. Sci. USA* **89** : 1949-1953

Wilkinson, D.G., S.Bhatt, R.-P.Ryseck and R.Bravo (1989) : Tissue-specific expression of c-*jun* and *jun*B during organogenesis in the mouse. *Development* **106** : 465-471

Williams, G.T., T.K.McClanahan and R.I.Morimoto (1989) : E1a transactivation of the human *HSP70* promoter is mediated through the basal transcriptional complex. *Mol. Cell. Biol.* **9** : 2574-2587

Williams, T., A.Admon, B.Lüscher and R.Tjian (1988) : Cloning and expression of AP2, a cell-type-specific transcription factor that activates inducible enhancer elements. *Genes Dev.* **2** : 1557-1569

Wobbe, C.R. and K.Struhl (1990) : Yeast and human TATA-binding proteins have nearly identical sequence requirements for transcription in vitro. *Mol. Cell. Biol.* **10** : 3859-3867

Workman, J.L., I.C.A.Taylor and R.E.Kingston (1991) : Activation domains of stably bound GAL4 derivatives alleviate repression of promoters by nucleosomes. *Cell* **64** : 533-544

Workman, J.L., S.M.Abmayr, W.A.Cromlish and R.G.Roeder (1988) : Transcriptional regulation by the immediate early protein of pseudorabies virus during in vitro nucleosome assembly. *Cell* **55** : 211-219

Workman, J.L. and R.G.Roeder (1987) : Binding of transcription factor TFIID to the major late promoter during in vitro nucleosome assembly potentiates subsequent initiation by RNA polymerase II. *Cell* **51** : 613-622

Woychik, N.A. and R.A.Young (1990) : RNA polymerase II : subunit structure and function. *Trends Biochem. Sci.* **15** : 347-351

Yamamoto, K.K., G.A.Gonzalez, P.Menzel, J.Rivier and M.R.Montminy (1990) : Characterization of a bipartite activator domain in transcription factor CREB. *Cell* **60** : 611-617

Yamamoto, K.K., G.A.Gonzalez, W.H.Biggs III, and M.R.Montminy (1988) : Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. *Nature* **334** : 494-498

Yang-Yen, H.-F., R.Chiu and M.Karin (1990) : Elevation of AP1 activity during F9 cell differentiation is due to incresaed c-*jun* transcription. *New Biol.* **2** : 351-361

Yee, A.S., P.Raychaudhuri, L.Jakoi, and J.R.Nevins (1989) : The adenovirus-inducible factor E2F stimulates transcription after specific DNA binding. *Mol. Cell. Biol.* **9** : 578-585

Yoshimura, T., J.-i. Fujisawa and M.Yoshida (1990) : Multiple cDNA clones encoding nuclear proteins that bind to the *tax*-dependent enhancer of HTLV-I : all contain a leucine zipper structure and basic amino acid domain. *EMBO J.* **9** : 2537-2542

Yutzey, K.E., S.J.Rhodes and S.F.Konieczny (1990) : Differential *trans* activation associated with the muscle regulatory factors MyoD1, myogenin, and MRF4. *Mol. Cell. Biol.* **10** : 3934-3944

Zamanian, M. and N.B.La Thangue (1992) : Adenovirus E1a prevents the retinoblastoma gene product from repressing the activity of a cellular transcription factor. *EMBO J.* **11** : 2603-2610

Zelent, A., C.Mendelsohn, P.Kastner, A.Krust, J.-M.Garnier, F.Ruffenbach, P.Leroy and P.Chambon (1991) : Differentially expressed isoforms of the mouse retinoic acid receptor are generated by usage of two promoters and alternative splicing. *EMBO J.* **10** : 71-81

Zerial, M., L.Toschi, R.-P.Ryseck, M.Schuermann, R.Müller and R.Bravo (1989) : The product of a novel growth factor activated gene, *fos B*, interacts with JUN proteins enhancing their DNA binding activity. *EMBO J.* **8** : 805-813