AN INVESTIGATION OF THE ROLE OF INTERLEUKIN-6 IN SYSTEMIC-ONSET JUVENILE CHRONIC ARTHRITIS

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ABSTRACT

Serum IL-6 levels in the acute phase of systemic-onset juvenile chronic arthritis (S-JCA) are markedly elevated, with additional peaks and troughs that exactly parallel the rise and fall of the fever characteristic of this condition. One explanation for this unique feature is that the control of expression of IL-6 in S-JCA patients is different to that in normal people. This thesis discusses research conducted to examine the hypothesis that this is due to polymorphisms in the region of the IL-6 gene controlling its expression.

Single-strand conformational polymorphism analysis of a PCR-generated fragment from the 5' flanking region of the IL-6 gene (-550 to +61) indicated the existence of polymorphisms. Sequencing of this region from 57 individuals revealed two novel polymorphisms: a G/C polymorphism at position -174; and a variation in the number of A and T bases in an A_nT_n tract between positions -392 and -373.

RFLP analysis of the -174 polymorphism in 59 Caucasian S-JCA patients demonstrated that they had a lower C allele frequency than a group of healthy Caucasians (n=72) (0.35 vs 0.53). The overall genotype distribution between the groups was significantly different (χ^2=4.15, p=0.0002). Moreover, the patients had a significantly lower CC genotype frequency (0.033 vs 0.32, p<0.0001).

Luciferase reporter constructs containing either allele were transiently transfected into HeLa cells. The $C(A_8T_{12})$ construct showed a 0.624 $\pm$ 0.15 fold lower (p<0.005) basal expression of luciferase, compared to the $G(A_8T_{12})$ construct. Following stimulation with lipopolysaccharide or interleukin-1, luciferase expression from the $C(A_8T_{12})$ construct did not change. However, luciferase expression from the $G(A_8T_{12})$ construct increased by 2.35 $\pm$ 0.10 fold and 3.60 $\pm$ 0.26 fold, respectively (both p<0.001). Deletion of the sequence upstream of the AP-1 site (at -283 ~ -277) resulted in loss of this differential response and an overall reduction in expression.

These results suggest that there is a genetically determined difference in the degree of the IL-6 response to stressful stimuli between individuals, and that the CC genotype may be protective against the development of S-JCA.

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ABBREVIATIONS

ACR	American College of Rheumatology
AP-1	Activator protein-1
A_nT_n	Polyadenine/polythymidine tract
AOSD	Adult-onset Still's Disease
APP	Acute phase protein
APR	Acute phase reaction
APS	Ammonium persulphate
AUBP	AU-region binding protein
BSF-2	B-cell stimulatory factor-2
cAMP	Cyclic-adenosine monophosphate
CAT	Chloramphenicol acetyltransferase
CBF-1	C-promoter binding factor-1
CBP	CREB-binding protein
CIA	Collagen-induced arthritis
CNFT	Ciliary neurotrophic factor
cPCR	Colony polymerase chain reaction
CRE	cAMP response element
CREB	cAMP response element binding protein
CRP	C-reactive protein
CT-1	Cardiotropin-1
EDTA	Ethylene diamine tetraacetate
EGF	Epidermal growth factor
EPO	Erythropoietin
ER	Estrogen receptor
ESR	Erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
FGF	Fibroblast growth factor
FLS	Fibroblast-like synoviocyte

fra	Fos-related antigen
G-CSF	Granulocyte-colony stimulating factor
GH	Growth hormone
GM-CSF	Granulocyte-macrophage-colony stimulating factor
GR	Glucocorticoid receptor
HBS	Hepes buffered saline
HIV1	Human immunodeficiency virus 1
HSF	Hepatocyte stimulating factor
IFN-β2	Interferon-β2
IFN-γ	Interferon-γ
IGF-1	Insulin-like growth factor-1
IKK	IκB-kinase α-subunit
IL	Interleukin
IL-1RA	Interleukin-1 receptor antagonist
IL-6R	Interleukin-6 receptor
Inr	Initiator region
IP-1	Inhibitor protein-1
IRF-1	Interferon regulatory factor-1
JCA	Juvenile chronic arthritis
JRA	Juvenile rheumatoid arthritis
KID	Kinase inducible domain
LB	Luria-Bertani
LIF	Leukaemia inhibitory factor
LPS	Lipopolysaccharide
LTB_4	Leukotriene B_4
MCB	Multicore buffer
MEKK1	Mitogen-activated protein kinase/extracellular signal-related kinase 1
MHC	Major histocompatibility complex
MRE	Multiple response element
NIK	NF-κB-inducing kinase
NLS	Nuclear localisation signal

NRD	Negative regulatory domain
OD	Optical density
OM	Oncostatin M
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PKC	Protein kinase C
PMA	Phorbol myristate acetate
pp1	Primer pair 1 (for amplifying the 611bp fragment of the IL-6 gene)
pp2	Primer pair 2 (for amplifying the 860bp fragment of the IL-6 gene)
RA	Rheumatoid arthritis
RB	Retinoblastoma
RBP-Jκ	Recombination Signal Sequence Binding Protein-Jκ
RCE	Retinoblastoma control element
RFLP	Restriction fragment length polymorphism
RHD	Rel homology domain
SAA	Serum amyloid-A
SDW	Sterile distilled water
sIL-6R	Soluble interleukin-6 receptor
S-JCA	Systemic-onset juvenile chronic arthritis
SLE	Systemic lupus erythematosus
SP	Substance P
SRE	Serum response element
SRF	Serum response factor
SSc	Systemic sclerosis
SSCP	Single-strand conformational polymorphism
STAT	Signal transduction and activation of transcription
TAE	Tris acetic acid EDTA
TBE	Tris boric acid EDTA
TBP	TATA Binding Protein
TCF	Ternary complex factor
TEMED	N, N, N', N' -tetramethlyethylene diamene

TG	Transgenic
TGF-β	Transforming growth factor-β
TIMP-1	Tissue inhibitor of metalloproteases-1
TNFα	Tumour necrosis factor-α
TPA	Tetradecanoyl phorbol-13-acetate
TRAF-2	TNF-receptor associated factor 2
UTR	Untranslated region
VNTR	Variable number of tandem repeats

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DEDICATION

To Corinne, without whose help, advice, support and understanding,
this thesis could never have been done.
Also to Jessica and Rachel,
my two most successful sub-clones.

CHAPTER 1

INTRODUCTION

1.1 Juvenile Chronic Arthritis

1.1.1 *Introduction: 'JCA' defined*

Juvenile chronic arthritis (JCA) is one of the commonest forms of chronic childhood disability. The most recently published epidemiological data, based on the diagnostic register of the British Paediatric Rheumatology Group, suggests an annual incidence of 10 new cases of 'juvenile arthritis' presenting to hospital per 100,000 children under 16 years of age (Symmons, 1996). As many as 58% of these patients will still have active disease after ten years (Wallace and Levinson, 1991). In order to control their disease, and prevent disabling joint damage, young children are often exposed to potentially toxic therapies for many years. Even with the use of potent immunosuppressive drugs some children experience early joint destruction, necessitating surgical replacement. In addition to the obvious adverse effects that the disease has on the patient, the care of a child with such a potentially disabling condition as JCA places enormous strain on the parents and siblings.

JCA itself is an umbrella term which includes several distinct rheumatic diseases. Differences exist both in the nomenclature of these individual diseases, and whether certain ones can be classed as JCA. The European League Against Rheumatism (EULAR) currently defines JCA as 'arthritis of at least three months duration, in a child under the age of 16 years'. The American College of Rheumatology (ACR) definition is frequently used outside of Europe, and permits the diagnosis to be made after six weeks of arthritis.

EULAR and the ACR subdivide JCA into its component diseases according to a combination of clinical and serological markers. The following subgroups are currently recognised by EULAR: Pauciarticular JCA (four or less joints involved); Polyarticular JCA (five or more joints involved); Pauciarticular JCA extending to Polyarticular JCA; Systemic-onset JCA; Rheumatoid-factor positive polyarthritis; the seronegative spondyloarthropathies; and Psoriatic arthritis. It is generally considered that these diseases are distinct entities, with individual aetiologies but similar pathological features and modes of treatment. The ACR definition excludes the seronegative spondyloarthropathies and uses the general term 'Juvenile Rheumatoid Arthritis' ('JRA'). EULAR restricts the use of the term JRA to those patients who have juvenile-onset rheumatoid factor positive polyarthritis.

The inconsistent and confusing nature of these definitions has undoubtably resulted in the previous mis-classification of patients. In fact, it is still common to observe studies in which all patients with JCA are combined together and analysed as if they formed a homogeneous group. This has an important bearing on the interpretation of studies in the field of JCA. The need to resolve these problems has led to the development of a unified, internationally acceptable and applicable set of diagnostic criteria for the idiopathic arthritides of childhood which are currently being evaluated. (Woo, 1996).

1.1.2 *Systemic-onset JCA*

Of the different sub-types of JCA, the systemic-onset form (S-JCA) is perhaps the most unusual and interesting. Unlike the other diseases that comprise JCA, the diagnosis of S-JCA does not rely on the number or pattern of affected joints, nor on the presence or absence of serological features. Hence it is less likely to be affected by the classification problems that affect the other childhood arthritides. Within the new ILAR classification criteria S-JCA will be known as Systemic Arthritis. Its diagnosis will be considered definite in those children under 16 years of age, with at least 6 weeks of arthritis, a documented quotidian fever for at least 2 weeks, and an evanescent, non-fixed erythematous rash (Woo, 1996). Although there are no diagnostic tests for S-JCA, which remains a diagnosis of exclusion in most cases, the disease is clinically homogeneous and can usually be diagnosed with a fair degree of certainty. This makes obtaining a uniform patient group for the purposes of analysis much easier. There are several unique features about S-JCA - especially the associated fever and cytokinaemia - which make it a particularly interesting disease to study.

Patients with JCA were originally considered to have a relatively benign disease, whose period of activity was confined to childhood. However, it has become increasingly apparent that even though many patients with JCA have a disease of only mild to moderate severity, their long-term outcome with respect to disability is frequently poor. This is especially true of those patients with S-JCA. Although only about 10% of the incident cases of JCA - about 1 new case per 100,000 children per year - suffer from S-JCA (Symmons *et al.*, 1996), it is the subgroup most likely to be associated with severe, debilitating extra-

articular features and occasionally fatal complications (Laxer and Schneider, 1993).Indeed, follow-up studies have revealed that up to 58% of patients with S-JCA still have active disease after ten years (Wallace and Levinson, 1991).

S-JCA is a clinically homogeneous and quite unique illness. It is equally prevalent in both sexes, with a mean age of onset between four and six years. There are no consistent HLA associations, with DR4, DR5 and Dw7 all having been reported to occur in a greater frequency in S-JCA patients (Nepom, 1991). Many of these studies only looked at small numbers of patients. However, in a cohort of 108 patients no increased HLA antigen frequencies were detected, and no HLA-DR associations were identified as predictors of chronic polyarthritis (Desaymard *et al.*, 1996).

In the active disease, patients display a typical spiking fever, an evanescent salmon-pink macular rash, lymphadenopathy, hepatosplenomegaly, serositis, myalgia and arthritis. This final symptom may be absent while the 'systemic' features of fever and rash are highly active. The arthritis is frequently aggressive, leading to early joint damage and often requiring the young patient to undergo joint replacement surgery.

Laboratory investigations reveal an anaemia with raised neutrophil and platelet counts; a high erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP); an elevated fibrinogen; a polyclonal hypergammaglobulinaemia; and sometimes mildly deranged liver function tests. In common with other inflammatory arthritides, such as rheumatoid arthritis, high levels of the inflammatory cytokines are found in the serum and synovial fluid during active disease (De Benedetti *et al.*, 1991, Lepore *et al.*, 1994, Rooney *et al.*, 1995).

The particularly unusual feature of the acute stage of S-JCA is the unique pattern of fever (Figure 1.1). Once, or occasionally twice, a day the patient's temperature rapidly rises, often to more than 40^0C, and remains elevated for several hours before returning to normal or sub-normal. In some cases the fever spike may occur at the same time each day. During the spike the patient feels extremely unwell, with chills and myalgia. The cause of the fever spike is unknown. However, it has been observed that the already elevated serum concentration of interleukin-6 (IL-6) that is present during active S-JCA undergoes a further significant rise. In fact, the serum IL-6 concentration rises and falls in parallel with the fever course over a 24 hour period (Rooney *et al.*, 1995, Prieur *et al.*, 1996). This observation is unique to this illness and has, to date, remained unexplained. It is possible that the regulation

of IL-6 expression in these patients is somehow different to unaffected individuals. This is the hypothesis upon which this thesis is based. In the late-onset form of the disease ('Adult-onset Still's disease', AOSD) serum IL-6 levels are also significantly elevated and fall with treatment (Scheinberg *et al.*, 1996). However, no 24 hour cytokine/fever profiles have been published for these patients.

In the following sections there is a more detailed discussion of the background to this hypothesis. This is followed by a review the relevant literature appertaining to the importance of cytokines in the aetiopathogenesis of inflammatory arthritis, with special attention given to IL-6 and S-JCA. Following this is a synopsis of the literature relating to the biological functions of IL-6 and the control of its expression. Finally there is a description of some of the polymorphisms known to exist in the genes for IL-6 and the other inflammatory cytokines, and their possible influence on disease.

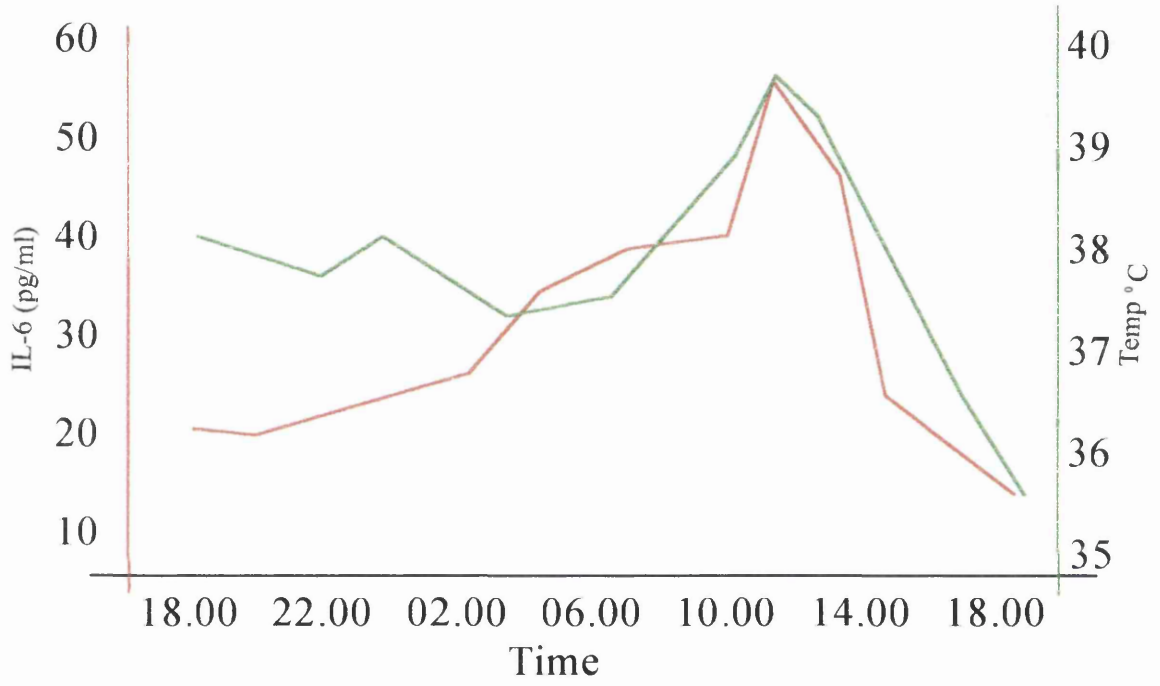


Figure 1.1: Serum IL-6 concentration during a period of fever in a patient with active S-JCA. A 24 hour cytokine profile encompassing a fever spike, showing the relationship of IL-6 to the fever. The IL-6 concentration rises and falls in exact parallel with the fever curve. This is in contrast to the serum concentrations of TNF-α and IL-1β which are shown in Figure 1.2.

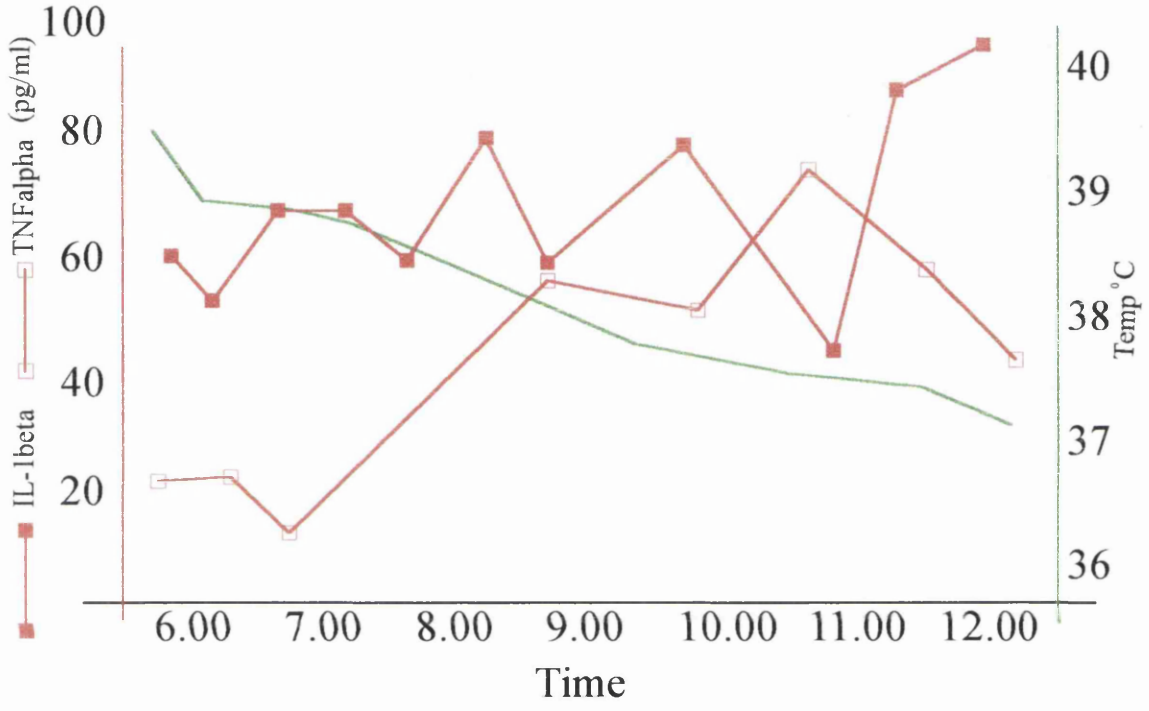


Figure 1.2: Serum TNF-α and IL-1β concentration during the resolution phase of a fever spike in a patient with active S-JCA. The TNF-α peak is out of phase with the fever. The IL-1β concentration is depressed during the fever peak and elevates once the fever has abated. (Modified from Rooney *et al.*, 1995).

1.2 Hypothesis: IL-6 is overexpressed in S-JCA

The hypothesis on which this investigation is based is that there is overexpression of IL-6 in patients with S-JCA, compared to unaffected individuals. This may be due to a difference in the control of the expression of IL-6 in these patients. There are several possible points in the pathway of IL-6 expression, from the initial stimulus for expression to the factors directly influencing its bioactivity in the serum, where differences between normal individuals and S-JCA patients may arise:

1.2.1 *Antigen driven release of IL-6*

There are several reasons why this is unlikely to be the cause of the elevation of IL-6 in S-JCA. Despite frequent investigation, no pathogen has been implicated in the aetiology of S-JCA. This does not rule out the possibility that a viral agent, for instance, is responsible for the disease induction. Intercurrent viral illnesses are recognised to act as triggers which can induce a flare in the disease or precipitate severe extra-articular complications (Fishman *et al.*, 1995).

If antigen driven release of IL-6 was occurring, large amounts could certainly be produced via its principal stimulating factors, TNFα and IL-1. However, IL-1β is not elevated in active S-JCA. Moreover, it is very difficult to detect at all during the fever spike, probably due to IL-1 receptor antagonist (IL-1ra), its specific inhibitor, the concentration of which also reaches a peak during the fever spike (Rooney *et al.*, 1995, De Benedetti *et al.*, 1995). Both Rooney *et al.* and De Benedetti *et al.* concluded that IL-1 is not as important in S-JCA as IL-6. The IL-1ra peak is likely to be due to IL-6, which can rapidly induce the expression of IL-1ra (Tilg *et al.*, 1994).

TNF-α is also elevated in S-JCA, with additional peaks and troughs through the fever curve (Rooney *et al.*, 1995). However, the peak of TNF-α serum concentration occurs about five hours after the fever spike, which is also after the IL-6 peak (Figure 1.2). This suggests no direct association between the fever and TNF-α. Although the precise sequence of cytokine activation is unknown, it is possible that - in a situation analogous to that postulated for rheumatoid arthritis - TNFα is the initial cytokine produced, leading to the eventual

stimulation of IL-6 expression (Feldmann, 1996). Although it has not been possible to correlate TNF-α and IL-6 levels in any of the previous S-JCA studies to date, it would be consistent with the current hypothesis to suggest that the observed IL-6 peak is an aberrant response to the TNF-α stimulus.

Although it is inappropriate to draw too many conclusions from a single case, it is interesting to note the effects of administering humanised monoclonal anti-TNF-α antibody (cA2) to a patient with severe active S-JCA (Elliott *et al.*, 1997). The first dose induced a rapid reduction in the patients fever, lasting several days. A second infusion ten days later resulted in only brief control of the fever. At no point did the patient's arthritis respond, but the anorexia and serositis did temporarily resolve. These results contrast to the excellent response of the arthritis and systemic features to cA2 in patients with severe rheumatoid arthritis (Elliott *et al.*, 1995). In this situation, where TNF-α is postulated to have a pivotal role, cA2 efficiently blocks the activity of TNF-α for some time. The brief effect of cA2 on S-JCA suggests that it is not the most important cytokine in this disease.

1.2.2 *Increased transcriptional induction*

The presence of different or altered repressors and/or inducers of IL-6 transcription in S-JCA patients could give rise to an altered rate of IL-6 transcription for a given stimulus. There is evidence to suggest that such variations in the control of IL-6 transcription do occur and are associated with disease. Many tumours, for example colonic tumours, display mutations within p53, a transcriptional repressor. Within these tumours it is possible to detect large amounts of IL-6 by immunostaining (Baker *et al.*, 1989). Myeloma cells produce IL-6 as an autocrine growth factor and one series found deletion of Rb-1, another repressor of IL-6 transcription, in more than 50% of patients (Dao *et al.*, 1994). A similar Rb-1 deletion is associated with autocrine production of interleukin-6 by, and hence autonomous growth of, acute myeloid leukemia blast (Zhu *et al.*, 1994).

Hence, Rb-1 gene deletion, and mutations leading to the production of p53 protein with reduced or absent repressor activity, may result in dysregulation of IL-6 expression. Mutated p53 protein has been found in RA synovium (Firestein *et al.*, 1997), but no studies on JCA patients have been undertaken so far.

1.2.3 *Mutations in regions of the IL-6 gene controlling its expression*

The principal control of expression of the majority of eukaryotic genes lies at the level of gene transcription (Latchman, 1995). Transcription is in turn mainly regulated by the activity of a large family of nuclear proteins - transcription factors - which bind to specific regions on the gene. These regions are usually located immediately 5' to the start site for gene transcription (promoters), but they may also lie much further upstream, downstream or even within the coding region of the gene itself (enhancers). Once bound, these factors interact with the basal transcription machinery to either increase or decrease the rate of transcription of the gene. Each binding site is specific for a transcription factor, although the factors may bind to regions with small differences in different genes, providing that the site still conforms to a consensus sequence. If a base change occurs within the binding site it is possible that the binding characteristics of the transcription factor will alter. This alteration will then effect the interaction between the transcription factor and the basal transcriptional machinery. The end result of these changes may be an altered transcriptional rate for the gene, producing an otherwise normal protein.

It is now well recognised that the human genome contains sites where the base sequence differs between individuals. These polymorphic regions do not necessarily alter the coding sequence of a gene, but they may occur within its promoter or enhancer sites. If this is the case, then the polymorphism may have the effect of influencing the degree of transcription of the gene following a given stimulus. This is not without precedent, as several important examples of polymorphisms in cytokine genes are recognised. The examples are described in more detail in section 1.5. Prior to the commencement of this study it therefore appeared possible that a promoter/enhancer region polymorphism could give rise to an allele in patients with S-JCA permitting greater expression of IL-6 for a given stimulus. There are no published studies in which the 5' flanking region of the IL-6 gene has been systematically examined for the presence of polymorphisms. As described later, a series of RFLPs have been described within the 3' flanking region among the general population (Bowcock *et al.* 1988). However, they do not affect the regulation of the IL-6 gene (Sehgal,1992).

1.2.4 *Post-transcriptional and post-translational alterations*

It is possible that a considerable degree of post-transcriptional regulation of IL-6 is occurring during the active systemic phase of S-JCA, to an extent that the serum levels vary greatly. However, the all of the available experimental evidence to date (reviewed in 1.4.4) suggests that IL-6, like the majority of eukaryotic genes, is regulated primarily at the level of transcription (Latchman, 1995). This is capable of producing the variations in the plasma concentrations of IL-6 usually observed in disease. However, additional post-transcriptional mechanisms, such as an alteration in the IL-6 mRNA half-life may also be important factors in increasing the serum concentration of IL-6. This is discussed further in section 1.4.6.1. However, there is no *in vivo* evidence to support this process as an important factor in the aetiology of the elevated IL-6 levels seen in S-JCA. There is evidence that the binding of IL-6 to specific anti-IL-6 antibodies might be significant (Hansen *et al.*, 1991), as this appears to increase the biological half-life of the cytokine, although there are no published reports of IL-6 antibodies in S-JCA.

The soluble IL-6 receptor (sIL-6R) also increases the effective concentration of bioactive IL-6 by enhancing the presentation of IL-6 to its cell surface signaling component, gp130. Significantly lower levels of sIL-6R are present in the serum of patients with active S-JCA than in normal controls (De Benedetti *et al.*, 1994). However, whereas little or no sIL-6R/IL-6 complex is normally detectable, in the patients this complex was frequently measurable. It was estimated that there was twenty times more IL-6 within the complex than free in the serum. As the correlation between this complexed IL-6 and the CRP was greater than that between the free IL-6 and CRP, it was hypothesised that this complexed IL-6 was important in stimulating the APR in S-JCA.

1.2.5 *Conclusion*

Although other factors are potentially involved in the abnormal cyclical pattern of IL-6 expression observed during active S-JCA, the available evidence suggests that an investigation looking for differences in gene expression between patients and controls should begin at the point at which IL-6 expression is predominantly controlled - ie within the region which determines the rate of gene transcription.

In common with many of the other inflammatory cytokine genes, the control of IL-6 transcription is facilitated by sequences which lie within the first few hundred bases immediately 5' to the coding region (described herein as the 5' flanking region). A more detailed description of these sequences is given in a subsequent chapter. The search for polymorphisms was therefore restricted to this region, specifically the 611 bases 5' to the translation start point.

1.3 Cytokines and Arthritis

1.3.1 *Introduction*

Cytokines are polypeptide mediators with diverse biological functions. They modulate the development, migration and differentiation of cells; the inflammatory response to injury; and the immune response to infection. They generally act over short intercellular distances. In some circumstances they may have an autocrine effect but in others they may be systemically active. It is possible to categorise cytokines broadly into families on the basis of similar functions: the interleukins, the growth factors (which are mainly responsible for bone marrow stem cell differentiation and maturation); and the transforming growth factors and bone morphogenic proteins (important in extracellular matrix formation and regulation) (Woo, 1993). The main sources of interleukins (IL) are monocytes and lymphocytes. The major monocyte-derived interleukins - interleukin-1α (IL-1α), IL-1β, IL-6, IL-8, Tumour Necrosis Factor-α (TNF-α) and Granulocyte/Monocyte-Colony Stimulating Factor (GM-CSF) - have predominantly proinflammatory effects. Under normal, healthy circumstances serum inflammatory cytokines are undetectable or found at very low levels. However, in infectious, inflammatory and immunological diseases their concentrations increase considerably, followed by a reduction during recovery. The elevated concentrations frequently correlate with disease activity. Their presence in large amounts in the serum, synovial fluid and synovial tissue from humans with inflammatory arthritis, and laboratory animals with experimentally-induced arthritis, implicates them as mediators of inflammation in these diseases.

The inflammatory cytokines induce both local and systemic responses which are important in the repair and regeneration of damaged tissue, processes that are collectively known as the Acute Phase Response (APR) (Banks *et al.*, 1993). The effect of the APR includes fever, leucocytosis, muscle proteolysis, endocrine changes and the marked upregulation of several liver-derived plasma proteins. The functions of many of these acute phase proteins (APP) are still unclear (such as serum amyloid A [SAA]). However, others such as opsonisation (by complement and C-reactive protein [CRP]), coagulation (by fibrinogen), haemoglobin scavenging (by haptoglobin) and serine protease inhibition (by α_1-

antitrypsin) are well defined. IL-6 is the major stimulus of this 'hepatic' APR. The APP can be divided into two classes (Baumann *et al.*, 1989): Class 1 proteins (including α_1-acid glycoprotein, CRP, haptoglobin and SAA) are induced by IL-1 or IL-1 and IL-6 together; Class 2 proteins (which include α_2-macroglobulin, α_1-antichymotrypsin and fibrinogen) are regulated mainly by IL-6. Individually, IL-1α and TNF-α can also stimulate some components of the APR *in vitro*, but not to the same extent as IL-6 (Castell *et al.*, 1988). The increase in serum level of an individual APP correlates with an increase in the rate of transcription of its gene. Many of the APP genes are regulated by the same transcription factors that are responsible for controlling the expression of IL-6 from its source, eg. fibroblasts (see below). IL-6 is also responsible for the down-regulation of the 'negative' APP such as albumin and transferrin (Castell *et al.*, 1988).

The consequences of inflammatory cytokine activity and the APR are normally beneficial to the host. They facilitate the removal of dead tissue, the elimination of infecting organisms and the promotion of healing. However, the outcome of such activity is not always benign. For example, the APR might become persistent, such as in severe cases of rheumatoid arthritis or S-JCA refractory to treatment, or in patients with chronic infections such as osteomyelitis. In these instances many of the APR proteins become chronically elevated, the most important of which under these circumstances is SAA, the precursor of the insoluble amyloid protein. Amyloidosis is the clinical syndrome arising from the deposition of amyloid protein in visceral organs, leading to impairment of their function and eventual failure. Patients with S-JCA are at particular risk of amyloidosis as their disease is often difficult to control and their SAA levels may remain elevated for some time (Laxer and Scheider, 1993).

Other examples of the deleterious effects of the inflammatory cytokines occur in pathological conditions where they are overproduced. A poor outcome from cerebral malaria is associated with the amount of TNF-α produced, which is in turn related to the individuals TNF-α genotype (1.5.3) Similarly, survival from septic shock is also related to serum TNF-α levels, although to date this has not been correlated with the patients genotype. Numerous tumours - squamous cell, adenocarcinoma, sarcoma and lymphoma - produce IL-6 (Tabibzadeh *et al.*, 1989). These conditions may display several common clinical features: fever, lymphadenopathy, arthritis and a persistent APR. The systemic illness in plasma cell-type Castleman's Disease is associated with the production of exceptionally large amounts

of IL-6 by plasma cells infiltrating the affected lymph nodes (Kinney *et al.*, 1994). It is likely that IL-6 is responsible for the systemic illness associated with all these conditions, as many of these features can be reproduced by its administration (Sehgal, 1990). Additionally, following resection of the tumour or Castleman's node, IL-6 levels return to normal and the systemic features resolve.

1.3.2 *Inflammatory Cytokines in Arthritis*

Rheumatoid arthritis (RA) is the archetypal rheumatic disorder in which the concentrations of inflammatory cytokines have been investigated. It is a chronic inflammatory disease of synovial joints which eventually leads to permanent damage. The pathological RA joint demonstrates a hypertrophied synovium, with a considerable invasion of T-cells and mononuclear leucocytes, producing large amounts of inflammatory cytokines. Quantisation of cytokines in synovial fluid and *in situ* hybridisation has demonstrated the presence of protein and/or mRNA for: TNF-α and -β, IL-1α and -β, IL-2, IL-6, IL-8, GM-CSF, TGF-β, FGF and PDGF-A and -B in the RA joint (Firestein *et al.*, 1990, Brennan *et al.*, 1991). Additionally, TNFα, IL-1 and IL-6 levels correlate with disease activity (Eastgate *et al.*, 1988, Houssiau *et al.*, 1988, Saxne *et al.*, 1988).

IL-1 and TNF-α are probably among the first cytokines to be produced by activated macrophages (Brennan *et al.*, 1991). They stimulate the production of each other and of a wide range of other inflammatory cytokines, including IL-2, IL-6, IL-8, PDGF and GM-CSF. In addition, both have broad pro-inflammatory effects, by upregulating expression of MHC molecules, activating macrophages, stimulating synovial hypertrophy and fibrosis, increasing endothelial cell adhesion molecule expression and inducing metalloprotease synthesis by articular chondrocytes, synoviocytes, fibroblasts, invading macrophages and neutrophils (Walport and Duff, 1993). Metalloproteinases are the major factors responsible for the systematic degradation of cartilage and bone and are thus likely to be responsible for the joint damage observed in inflammatory arthritis. It is noteworthy that this effect is downregulated by IL-6, which stimulates the production of inhibitors, such as tissue inhibitor of metalloprotease-1 (TIMP-1) from rheumatoid fibroblasts (Ito *et al.*, 1992). Individually, and through their induction of IL-6, IL-1 and TNFα also upregulate the hepatic APP. IL-8 is a

major chemoattractant for neutrophils, T-cells and macrophages, and activates neutrophils (Walport and Duff, 1993). Platelet-derived growth factor (PDGF) stimulates fibroblast proliferation and release of monocyte chemoattractants from endothelial cells, and fibroblast growth factor (FGF) enhances metalloprotease-induced cartilage degradation (Remmers *et al.*, 1991). GM-CSF may be the agent inducing HLA class II expression within the rheumatoid joint, an important step in generating the immune response here, and is also a potent macrophage activator (Maini and Feldmann, 1993).

IL-6 is an important mediator of systemic inflammation in its own right, through its strong induction of the APP, its activity as a potent B-cell growth factor, producing more antibody forming plasma cells, and also its stimulation of haemopoietic stem cell proliferation (Brandt *et al.*, 1990). Additionally, IL-6 has been shown to stimulate multi-nucleated cell formation *in vitro* and bone resorption in fetal rodent organ cultures containing primitive cells (Kurihara *et al.*, 1990, Lowick *et al.*, 1989). This suggests that IL-6 has permissive effects towards bone resorption by maintaining adequate numbers of early osteoclast precursors. Periarticular osteoporosis is an early feature of RA, and generalised osteoporosis in those with longstanding disease can be a considerable problem. It has been shown that IL-6 plays an important role in female menopausal osteoporosis. IL-6 knockout mice do not develop osteoporosis when ovariectomised, an effect which is reversed with exogenous IL-6 (Poli *et al.*, 1994). Similarly, male IL-6 knockouts are protected from senile osteoporosis mediated by androgen loss (Bellido *et al.*, 1995). The functions of IL-6 are further discussed below.

1.3.3 *Inflammatory Cytokines in S-JCA*

Single measurements of serum IL-6 in S-JCA patients with active disease have revealed that background IL-6 levels are significantly elevated (Lepore *et al.*, 1993a, Lepore *et al.*, 1994, Madson *et al.*, 1994, Mangge *et al.*, 1995). Additionally, there is a significant correlation between serum IL-6 levels and joint involvement and thrombocytosis during active disease (De Benedetti *et al.*, 1991). However, the highly unusual cyclical fever which characterises the active 'systemic' phase of the disease has prompted investigators to examine serum cytokine profiles. Serial cytokine measurements have been analysed over a 24 hour period during active S-JCA in two separate studies (Rooney *et al.*, 1995, Prieur *et al.*, 1996). They

both demonstrate that IL-6 levels rise significantly in conjunction with the fever spike, and then fall in parallel with the return of body temperature to normal. The first of these studies (Rooney *et al.*, 1995) also showed that TNFα levels are only moderately elevated and are dissociated from the fever peak, occurring about five hours later. Additionally, IL-1β concentrations were shown to be consistently low to normal. The level of IL-1 receptor antagonist also parallels the fever curve, but with a lag phase of about one hour. The second study (Prieur *et al.*, 1996) confirmed the elevation of IL-1ra with the fever spike, although the IL-6 peak preceded that of IL-1ra. In addition, IL-6 and IL-1ra were significantly correlated at the time of the fever spike ($r=0.927$, $p>0.001$), but not at any other time. An earlier report found that IL-1ra levels correlated with IL-6 levels to a significant degree (De Benedetti *et al.*, 1995). Prieur *et al.* (1996) also demonstrated a correlation between TNF-α and TNF-sR75 levels during the fever spike; an association between TNF-α and the fever spike itself was not reported.

A circadian rhythm has been demonstrated for many of the circulating mediators found in normal, healthy subjects, including IL-6. Sothern *et al.* (1995 a and b) demonstrated that IL-6 levels peaked at 01.00 am, with a nadir at 10.00 am. In contrast, Arvidson *et al.* (1994) found that in patients with RA, peak values appeared in the morning and low values in the afternoon and evening. However, they were unable to detect IL-6 in healthy controls and did not measure IL-6 levels during the night. These findings are at a variance with the observed patterns during the S-JCA fever spike, which often occurs in the afternoon or evening. Thus, the unique circadian rhythm of IL-6 observed in S-JCA patients with active disease suggests that there may be an alteration in the homeostatic mechanisms that usually control IL-6 levels.

There is no literature concerning the effect of fever itself on IL-6 levels. *In vitro* studies on IL-1α suggest that, in the presence of supraphysiological temperatures, mononuclear cells from S-JCA patients have a reduced capacity to produce this, and probably other, cytokines (Rooney pers. comm.). It is therefore unlikely that fever *per se* is responsible for inducing the observed pattern of IL-6 rise and fall. This cyclical pattern of fever is seen universally in different ethnic groups and irrespective of their drug therapy. IL-6 receptors (IL-6R) are found throughout the brain, including the medial preoptic nucleus and medial hypothalamus, structures that control body temperature and food intake. Direct infusion of IL-6 into the rat cerebroventricular system induces fever and reduces locomotor activity and

food intake (Schobitz *et al.*, 1992). Infusion of sIL-6R before IL-6, potentiates these effects.

One of the major complications of longstanding, active JCA is growth retardation, a feature often seen in children with S-JCA. In these cases, growth hormone (GH) profiles are normal, but there is a reduction of insulin-like growth factor-1 (IGF-1) which can be corrected by administration of therapeutic amounts of GH (Davies *et al.*, 1997). Mice transgenic for IL-6 are also growth retarded and display low IGF-1 levels, as do non-transgenic mice treated with IL-6 (De Benedetti *et al.*, 1997)

Cytokine measurements in other forms of JCA have also been made and it is clear that within affected joints in pauciarticular JCA, IL-6 (and TNF-α) are also raised (Lepore, 1993b, Lepore *et al.*, 1994). There is also evidence that sIL-6R is elevated in active polyarticular and pauciarticular disease. In the systemic disease the levels are lower than normal and inversely related to IL-6 concentrations, but directly related to IL-6 in poly- and pauciarticular disease (De Benedetti *et al.*, 1994). In S-JCA synovial fluid, IL-6 is also significantly elevated (Lepore, 1993b) and to a greater extent than in patients with pauci-articular JCA or adults with RA (De Benedetti *et al.*, 1993). There have also been reports of increased levels of IL-6 in synovial fluid and serum of pauciarticular JCA and serum IL-6 levels correlating with disease activity in polyarticular JCA (Lepore, 1993b, De Benedetti *et al.*, 1992). More recently, a close correlation between IL-6 and several clinical, functional and laboratory parameters has been reported for children with active pauciarticular and polyarticular JCA (Spadaro *et al.*, 1996).

The source of IL-6 in the active S-JCA is unknown. Inflamed synovium is a major source of IL-6 and other inflammatory cytokines in RA (Okamoto *et al.*, 1997). However, S-JCA patients with active systemic features (fever, lymphadenopathy, serositis, hepatosplenomegaly and an APR) are often encountered who have no clinical joint pathology. It is likely that circulating monocytes and/or macrophages that are the major source of IL-6 in this instance, as in healthy subjects. In support of this is the observation that patients with severe disease or intercurrent infection may exhibit features of the Haemophagocytic Syndrome (Fishman *et al.*, 1995). This results from over-activation of macrophages, which may already be in an activated state as a result of the S-JCA disease process. Although this does not exclude there being a source of IL-6 or other inflammatory cytokines within the joint, it makes the synovium an unlikely site for the primary pathology in S-JCA.

1.4 Interleukin-6

1.4.1 *Introduction*

IL-6 is the consensus name given to the lymphokine described independently by several groups as: B-cell stimulatory factor-2 (BSF-2); Interferon-β_2 (IFN-β_2); 26 kD protein; hepatocyte stimulating factor (HSF); hybridoma growth factor; T-cell activating factor; and colony-stimulating factor 309. Cloning and sequencing has demonstrated all these to derive from the same gene, now termed IL-6 (Ray *et al.*, 1988).

Human IL-6 is a 184 amino acid glycoprotein with a core molecular weight of 20 kDa. Post-translational *N* - and *O*-linked glycosylation and phosphorylation varies between cellular sources, hence the final molecular weight lies between 21 and 26 kDa. However, the glycosylation status of the final peptide appears unimportant for its biological activity. IL-6 belongs to the Group 1 cytokine family, members of which share a four-α-helix bundle in common, and include GH, Erythropoietin (EPO), Granulocyte-colony Stimulating Factor (G-CSF), Oncostatin M (OM), IL-11, Ciliary Neurotrophic Factor (CNTF), Leukaemia Inhibitory Factor (LIF) and Cardiotropin-1 (CT-1).

1.4.2 *Biological Functions*

IL-6 is a highly pleiotropic cytokine, with effects on numerous cell lines. In the immune system it has an important role in stimulating B-cell differentiation into antibody-secreting plasma cells, in cytotoxic T-cell differentiation, T-cell activation, growth and differentiation (Lotz *et al.*, 1988). Additionally it upregulates IL-2Rα chain expression and IL-2 production in T-cells (Tosato and Pike, 1988). In haemopoiesis, IL-6 can synergise with IL-3 to shorten the G_0 of early haemopoietic precursors, and also to stimulate megakaryocyte development, inducing increased platelet numbers *in vivo* (Ishibashi *et al.*, 1993). IL-6 is the only cytokine able to induce the synthesis of all the APR proteins. (Heinrich *et al.*, 1990). IL-6 has marked growth-factor activity for hybridomas (the basis of the IL-6 bioassay), plasmacytomas, myelomas, sarcomas, carcinomas, EBV-transformed B-cells, keratinocytes and mesangial cells (Sehgal, 1990). In contrast, it is also inhibitory to a number of leukaemia and carcinoma

lines. Miscellaneous bioactivities include: the induction of terminal differentiation of M1 myeloid leukaemic cells (Shabo et al., 1988); the differentiation and survival of neuronal cells (Satoh *et al.*, 1988); and the activation of osteoclasts (de la Mata *et al.*, 1995, Ishimi *et al.*, 1990, Lowick *et al.*, 1989). The original reports which ascribed antiviral activity to IL-6 have not been replicated (Akira *et al.*, 1993).

1.4.3 *The IL-6 gene*

The gene for human IL-6 is located on chromosome 7p14-21. It is 6 kB long and consists of five exons and four introns (Figure 1.3). This gives rise to an mRNA encoding a 212 amino acid precursor protein with a 28 amino acid hydrophobic signal sequence. In comparison with murine IL-6, human IL-6 shares a 65% sequence homology at the nucleotide level, and a 42% homology at the amino acid level. The area of closest homology between the two genes lies in a region of 400 bases 5' to the transcription start site. Here the sequences are nearly identical, but further upstream all similarity is lost. Although human and mouse IL-6 are equally active on murine cells, murine IL-6 is not active on human cells.

The sequence of the IL-6 gene, as deposited in GenBank (accession number Y00081) is shown in Appendix A. Regions highlighted are the sequences relevant to this research.

S1 nuclease mapping and primer extension analyses have demonstrated the presence of two potential transcription initiation sites (C2 and C3), in addition to the one predicted from the nucleotide and cDNA sequences (C1) (Figure 1.4). By consensus, the C1 site is defined as base +1 in the IL-6 nucleotide sequence. C2 is located 25 bases upstream of C1, and C3 is located approximately 113 bases upstream of C1. The C2 and C3 sites each have a corresponding TATA box and ATG translation initiation codon. The majority of cell lines preferentially use the major (C1) site, although some lines - such as fibroblasts - appear to be capable of utilizing the C2 site *in vitro* to produce a variant IL-6 mRNA, which has not been extensively studied (Yasukawa *et al.*, 1987). Other than in these S1 nuclease mapping and primer extension experiments, it is not known whether the C3 site is ever utilised *in vitro* to produce a transcript, or if a protein is ever translated from transcripts starting at any site other than C1. If the C2 transcript were ever translated, the resulting protein is predicted to contain an additional 25 amino acids, giving this version of IL-6 a hydrophilic sequence preceding its

hydrophobic signal sequence. If the hydrophobic region remained membrane-embedded, the protein could form a membrane-bound version of IL-6.

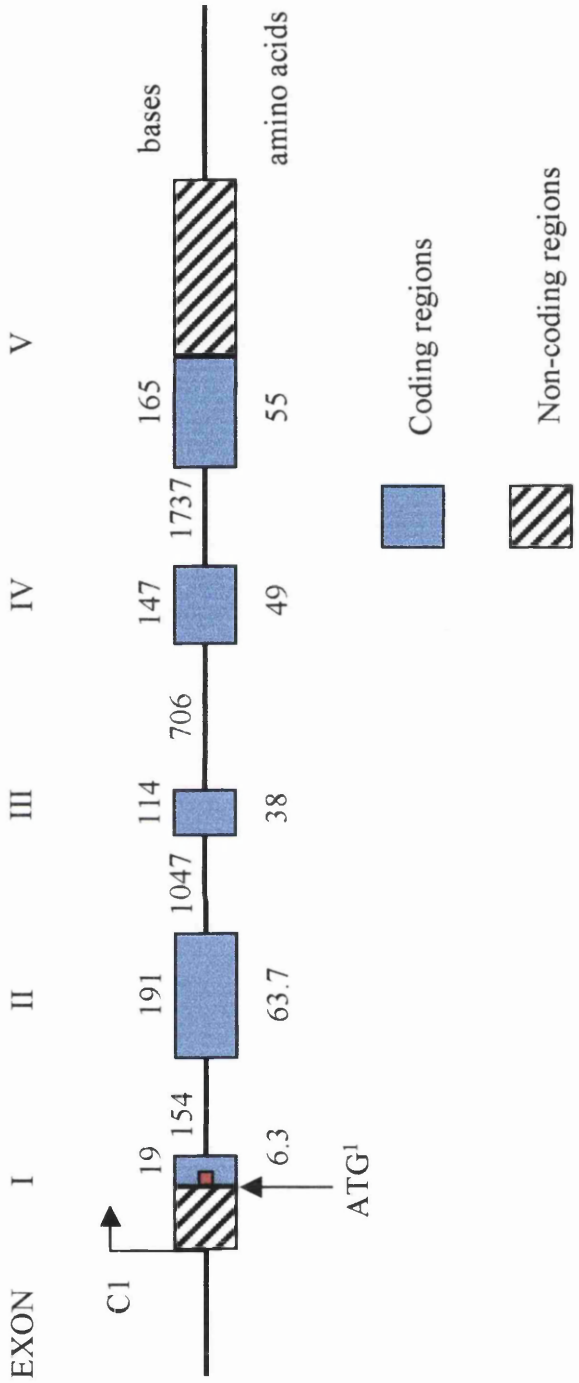


Figure 1.3 An intron and exon map of the Interleukin-6 gene. This was characterised by Yasukawa *et al.*, by sequencing plasmid vectors containing genomic DNA subcloned from clones which hybridized with IL-6 cDNA, then comparing this sequence with that of the cDNA (Yasukawa *et al.*, 1987).

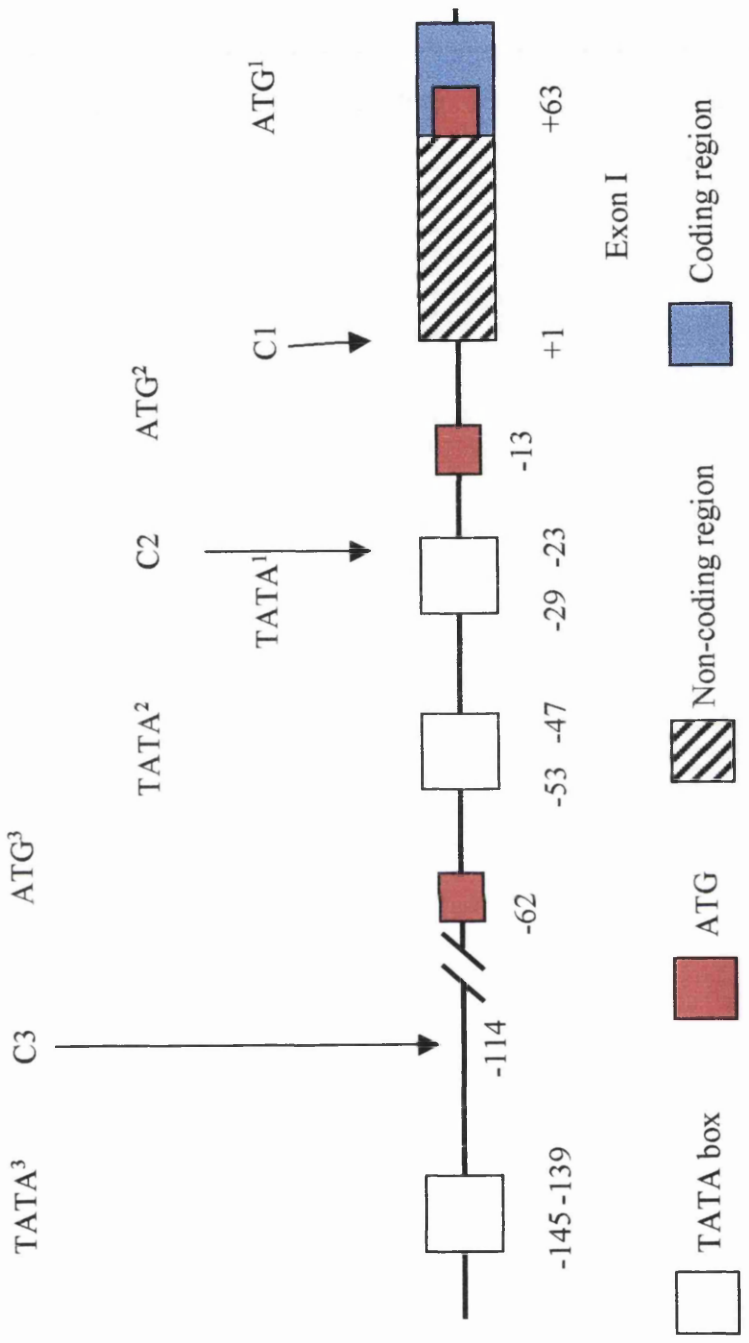


Figure 1.4 A summary of the location of transcription initiation sites, with their appropriate TATA boxes and translation initiation codons, within the 5' flanking region of the IL-6 gene. Only the transcription product from C1 - the major transcription start site in most cell lines - is shown. (Adapted from Yasukawa *et al.*, 1987).

1.4.4 *Transcriptional Control of IL-6*

In common with the majority of eukaryotic genes control of transcription of IL-6 is the primary method of regulating IL-6 expression. Extensive studies have identified several transcription factor binding sites within the region immediately 5' of the transcriptional start site (the 5' flanking region) (Figure 1.5). There is no evidence to suggest that sequences elsewhere than in this region play an active role in controlling the rate of gene transcription.

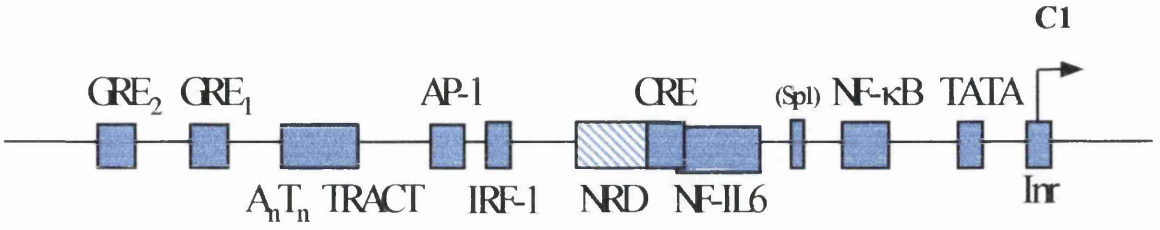
1.4.4.1 TATA Box and the Inr

The four-base consensus sequence lies at -25 to -21. As with other TATA boxes, this is the region to which TATA Binding Protein (TBP) binds during the complex process of initiation of transcription. The IL-6 gene also contains the consensus initiator (Inr) sequence at the transcription start point.

1.4.4.2 NF-κB

The Molecular Biology of NF-κB.

NF-κB is a transcription factor originally described as a nuclear factor from B cells binding to the B motif in the intronic κ light chain gene enhancer. It is now recognised to be an important transcription factor in numerous mammalian cell types. It is expressed both constitutively and following induction. The inducible form is usually a heterodimer of two of members of the Rel family of proteins, p50 (NF-κB1) and p65 (RelA) (Grilli *et al.*, 1993). The other members of the mammalian Rel family - p52 (NF-κB2), Rel (c-Rel) and RelB, can also form homo- and heterodimers. All the members of the Rel family share the common Rel homology domain (RHD), a highly conserved region of 300 amino acids containing the DNA-binding, dimerisation and nuclear localization signal (NLS) regions. All except p50 and p52 also contain one or more transcriptional activating domains. These two proteins also differ from the others in that they are produced as precursor proteins, p105 and p100, respectively. They contain multiple copies of ankyrin repeats and bind DNA weakly. After proteolysis they



Inr	-2 ~ +2	IRF-1	-267 ~ -254
TATA	-25 ~ -21	AP-1	-283 ~ -277
NF-κB	-73 ~ -64	A_nT_n Tract	-392 ~ -373
NF-IL6	-158 ~ -146	GRE_1	-466 ~ -461
CRE	-163 ~ -158	GRE_2	-557 ~ -552
NRD	-225 ~ -164		

(Sp1 - murine gene, 3 consensus sites found in the human IL-6 gene between -125 and -94)

Figure 1.5 Putative and functional transcription factor binding sites in the 5' flanking region of the IL-6 gene.

contain only the Rel homology and bind strongly to DNA. These differences define the two classes of Rel proteins. All mature Rel proteins bind to specific DNA target sites (κB sites) dependant on the dimer composition. Generally they act as transcriptional activators, although p50 dimers may repress transcription (Pan and McEver, 1995).

Prior to its translocation to the nucleus, NF-κB is sequestered in the cytoplasm in an inactive form, complexed to IκB-α, a member of the IκB family of inhibitor proteins (which includes IκB-β, IκB-γ, IκB-ϵ, Bcl-3, p100 and p105). The members of this family share the characteristic 30-33 amino acid ankyrin repeat motif required for interaction with the Rel proteins (Grilli *et al.*, 1993). Mature Rel protein, eg p65, is also held in the cytoplasm bound to the Rel precursors p105 or p100 (Baeuerle and Henkel, 1994). The specific interaction between the ankyrin repeats of the IκB proteins and the RHD of NF-κB masks the nuclear localisation signal of the latter, preventing its nuclear translocation.

Activation of NF-κB and its translocation to the nucleus begins following stimulation of the cell by agents such as TNFα , IL-1, bacterial lipopolysaccharide (LPS), double stranded RNA or phorbol myristate acetate (PMA/phorbol ester). This stimulation signals phosphorylation of IκBα by at least two kinases, IκB-kinase α-subunit (IKKα) and β-subunit (IKKβ) (DiDonato *et al.*, 1997). Phosphorylation of IκB-α takes place at serine residues 32 and 36 (Whiteside *et al.*,1996), and it is the ability of both IKK to dual phosphorylate that is sets these kinases apart from others. They are 52% homologous (Mercurio *et al.*, 1997), are exquisitely specific serine kinases but they phosphorylate IκB-α and IκB-β to different degrees (DiDonato *et al.*, 1997). They are unable to phosphorylate other IκB isoforms, which (together with the unknown effects on other IκBs) possibly provides for the levels of differential regulation of NF-κB induced by differing initial stimuli. A second, ubiquitin-dependent, IκB kinase system has also been described, and its relationship to the IKKs is as yet unclear (Chen *et al.*, 1996)

Following phosphorylation of the IκB protein at its N-terminal serines it is ubiquitinated at lysine residues 21 and 22 (Baldi *et al.*, 1996). Ubiquitinated IκBα - which remains associated with NF-κB - is then targeted for proteolytic degradation by the 26S proteosome, resulting in the release of NF-κB (Chen *et al.*, 1995). Ubiquitination and proteolysis are both ATP-dependant (Li *et al.*, 1995).

The upstream control of the function of the IKKs remains to be fully elucidated. MEKK1 (mitogen-activated protein kinase/extracellular signal-related kinase 1) is a potential candidate in the chain of activation from the cell surface receptor (eg the TNF-α receptor) to the phosphorylation of IκB (Mercurio *et al.*, 1997). Other factors, such as NIK (NF-κB-inducing kinase) can associate with TNF-receptor associated factor 2 (TRAF-2) to stimulate IκB-α degradation (Malinin *et al.*, 1997), probably by an association with IKKα and IKKβ (Regnier *et al.*, 1997).

A further manner in which the regulation of NF-κB is controlled is through the roles played by the other IκB proteins. For instance, where IκB-α and IκB-β associate predominantly with p50:p65 and p50:c-rel heterodimers (Zhong *et al.*, 1997) IκB-ϵ binds to p65 and c-Rel heterodimers (Sun *et al.*, 1993) and Bcl-3 interacts with p50 and p52 homodimers (Franzoso *et al.*, 1992). All known inducers of NF-κB cause degradation of IκB-α, but IκB-β is only affected by LPS, IL-1 and HIV Tax protein, and the subsequent NF-κB activation is persistent (McKinsey *et al.*, 1996). The mechanism of this action is complex. After it is activated NF-κB, binds to κB sites in the IκB-α promoter, leading to an upregulation of IκB-α which binds to any free cytoplasmic NF-κB, completing a negative feedback loop for NF-κB regulation (Brown *et al.*, 1993). However, IκB-β that is newly synthesised by this process, and that is not basally phosphorylated, complexes with free NF-κB and prevents it from interacting with IκB-α (Suyang *et al.*, 1996). Unphosphorylated IκB-β does not mask the NLS or DNA-binding domains of NF-κB, allowing it to enter the nucleus and stimulate transcription. This form of IκB-β is therefore acting to chaperone NF-κB, permitting its activity to continue long after IκB-α is produced.

Phosphorylation of the tyrosine at amino acid position 42 (Tyr42) in IκB-α inhibits phosphorylation at Ser32 and Ser36 by IKKs (Singh *et al.*, 1996), which allows for further specific regulation of the activation of NF-κB as there are no analogous tyrosine residues in IκB-β or IκB-ϵ.

The catalytic subunit of PKA (PKAc) has been demonstrated to associate with IκB-β. In addition there is a phosphorylation site for PKA in p65 which does not affect NF-κB-IκB complex dissociation, but does increase the *trans*-activating activity of DNA-bound NF-κB. (Zhong *et al.*, 1997).

Using deletion mutants of the 5' flanking region of IL-6 in reporter gene constructs, it has been demonstrated that a region containing a consensus κB binding site between positions -73 and -64 (NF-κB) is important for the induction of IL-6 expression by LPS, IL-1 and TNFα (Matsusaka *et al.*, 1993, Shimizu *et al.*, 1990, Zhang *et al.*, 1994, Sanceau *et al.*, 1995). It is of considerable interest that Shimizu *et al.* (1990) found that the region immediately upstream of the NF-κB site repressed expression in the reporter construct. Additionally, they noticed little reduction in expression of the reporter gene after IL-1 induction when the AP-1 and CRE further upstream were removed from the construct. Sanceau *et al.* (1995) showed that p50 and p65 subunits accumulate after LPS stimulation in THP-1 cells, whereas the p65 subunit is the predominant species observed following TNF-α or TNF-α and IFNγ stimulation.

NF-κB has been demonstrated to mediate IL-6 expression following stimulation with Leukotriene B_4 (LTB4) (Brach *et al.*, 1992). This is inhibited in the presence of a hydrogen peroxide scavenger, supporting a role for hydrogen peroxide as a possible intermediary conferring the LTB4 message. It is also noteworthy that the half-life of the induced IL-6 mRNA is considerably longer in LTB4 treated monocytes (15 hours) than usually observed in these cells (Brach *et al.*, 1990). Thus, LTB4 may additionally be altering mRNA stability to upregulate IL-6 expression.

Ionizing radiation causes a range of acute and chronic changes to mammalian tissues. The mechanism of initiation of these changes is not well characterised, but activation of NF-κB in irradiated myeloid leukaemia cells has been reported (Brach *et al.*, 1993). The authors demonstrated that x-ray treatment causes increased expression of IL-6 in human lung fibroblasts, mediated via increased NF-κB binding. A small proportion of increased IL-6 expression was due to activation of the AP-1 site. These results are in contrast to the observation that photodynamic therapy (the application of a photosensitising agent that accumulates within skin tumours and is activated by visible light to produce damaging oxygen radicals) will induce IL-6 expression from HeLa cells via the AP-1 site rather than the NF-κB site (Kick *et al.*, 1995). Hypoxia is also able to induce the generation of reactive oxygen intermediates, which causes a degradation of IκBα and thus stimulates IL-6 expression via

NF-κB (Muroaka *et al.*, 1997)

In the majority of previous investigations of IL-6 gene regulation, either heterologous promoter constructs or deletion mutants have been used. However, one study looked at the function of the entire 1.3 kB 5' flanking region in a reporter gene construct with specific mutations in one or more transcription factor binding sites (Dendorfer *et al.*, 1994). The NF-κB site was found to be crucial for induction of IL-6 by LPS. NF-κB binding is also regulated by cAMP, although this increases IL-6 expression by inducing binding of nuclear factors at several other sites as well. NF-κB is also solely responsible for induction of IL-6 expression by LIF (Gruss *et al.*, 1992).

Fibroblasts from patients with Systemic Sclerosis (SSc) constitutively produce large amounts of IL-6. This has been shown to be due to increased mRNA production, mediated by constitutive binding of NF-κB (and NF-IL6) to the promoter (Feghali *et al.*, 1994). Astrocytes and human intestinal epithelial cells are other cell lines whose IL-1β-stimulated release of IL-6 is dependant on NF-κB (Lieb *et al.*, 1998 and Parikh *et al.*, 1997 respectively). Astrocytes also respond to stimulation by adenosine - produced during focal cerebral ischaemia - by expressing IL-6 in a manner dependant on NF-κB and NF-IL6 (Schwaninger *et al.*, 1997).

The fibroblast-like synoviocyte (FLS) is considered to be the major source of IL-6 in RA, and inducible binding of NF-κB following IL-1β stimulation has recently been demonstrated (Miyazawa *et al.*, 1998a). Constitutive NF-IL6 binding produces basal IL-6 expression from these cells. In addition to NF-κB binding in the downstream 5' flanking region, a recognition sequence for CBF1 has been identified which overlaps the NF-κB site at -67 to -59 (see 1.4.4.5). CBF1 (Epstein-Barr virus [EBV] C-promoter binding factor-1, also known as RBP) acts as a repressor of IL-6 expression in murine carcinoma cell lines, but in FLS it is a constitutive component of the NF-κB binding complex (Miyazawa *et al.*, 1998a). It is as important as NF-κB in conveying the IL-1β-stimulated increase in IL-6 expression, and the two transcription factors act additively to mediate the IL-1β signal. Some rheumatoid FLS clones maintain their upregulated IL-6 expression *in vitro*. The constitutive binding of NF-κB and CBF1 is greater in these cells than in those producing low levels of IL-6, irrespective of IL-1 or TNF-α (Miyazawa *et al.*, 1998b). This suggests that spontaneous activation of these transcription factors may play a role in the increased expression of IL-6

in RA. The tissue-specific activity of CBF1 is also interesting, but not unusual for a transcription factor.

NF-κB is also relevant in the *inhibition* of IL-6 expression. The presence of IL-4 results in a downregulation of the amount of NF-κB in nuclear extracts from THP-1 and U937 cells following inflammatory stimuli, which results in a lower IL-6 expression in this circumstance (Takeshita *et al.*, 1996). IL-10, on the other hand, reduces IL-6 expression from these cells by reducing IL-6 mRNA half-life.

1.4.4.3 CRE, NF-IL6 and the Multiple Response Element (MRE)

The molecular biology of CRE and NF-IL6

As described in the AP-1 section below, stimulation by cAMP results in the PKA-induced phosphorylation of CREB at serine-133, a critical step in cAMP-induced gene expression (Gonzalez and Montminy, 1989). CREB, and the related proteins ATF-1 and CREM, bind to DNA via a leucine zipper, although unlike other members of the bZIP family they bind predominantly as homodimers. The PKA phosphorylation site lies within the kinase inducible domain (KID), which is highly conserved between the three family members. CREB-binding protein (CBP) specifically associates with CREB through the KID, and also with RNA polymerase II and possibly TFIID, thereby forming the connection between CREB activation and increased gene transcription (Montminy, 1997).

NF-IL6 contains a region highly homologous to the C-terminal portion of the leucine zipper transcription factor C/EBP, hence its alternate name, C/EBPβ (Akira *et al.*,1992). The highly homologous region contains a basic domain and the leucine zipper structure, essential for DNA binding and dimerization. Although they recognise the same nucleotide sequences, the tissue distribution of C/EBP and NF-IL6 varies: the former is expressed predominantly in hepatic and adipose tissue, whereas the latter can be found in all normal tissues (Akira *et al.*, 1995). Stimulation with LPS, TNF-α, IL-1α and IL-6 induces increased NF-IL6 mRNA levels in all tissues (Akira *et al.*, 1992). Conversely, hepatic C/EBP mRNA levels fall following IL-6 induction, a possible mechanism for the induction of the positive APP and repression of the negative APP by IL-6 (Isshiki *et al.*, 1991).

NF-IL6 is rapidly activated by post-translational phosphorylation, at Serine-231 and Threonine-235 within the serine-rich domain adjacent to the bZIP (basic domain), and at Serine-325 within the leucine zipper. Surrounding the Thr-235 region is a consensus sequence for MAP kinase. There is good evidence that it is MAP kinase that is responsible for NF-IL6 activation (see 'IL-6 signal transduction' below) (Peleche *et al.*, 1992). Following this phosphorylation, the DNA transactivating domains become exposed, thus NF-IL6 is activated by 'derepression' (Kowenz-Leutz *et al.*, 1994).

NF-IL6 binding sites are also present in the promoters of other cytokines, such as TNF-α, IL-1, IL-8 and G-CSF, in addition to the promoters of many other genes, such as the IL-6-induced APP (eg SAA) and the hsp90 gene (Stephanou *et al.*, 1998). Many of these promoters require both NF-IL6 and NF-κB for maximal expression. There is good evidence that NF-IL6 and NF-κB synergise to enhance expression of several genes, including SAA (Betts *et al.*, 1993). The physical interaction between the leucine zipper of NF-IL6 and the Rel homology domain of NF-κB may be the explanation for this synergy (Stein *et al.*, 1993).

CRE, the MRE, NF-IL6 and IL-6

Early experiments with large 5' flanking region constructs demonstrated the ability of several agents to induce IL-6 expression: IL-1, TNF-α, serum, Sendai virus, pseudorabies virus, poly(I).poly(C), activators of protein kinase C (PKC) (eg 1,2-dioctanolyglycerol, PMA), forskolin (which activates adenylate cyclase via protein kinase A - PKA) and calcium ionophore (Ray *et al.*, 1988). Chimeric genes containing deletion mutants of the IL-6 5' flanking region linked to chloramphenicol acetyltransferase (CAT) reporter gene were generated by truncating the 5' flanking region at restriction fragment sites. This demonstrated that the region 5' to -225 was partly responsible for responsiveness to PMA, serum and Sendai virus. However, deletion of the region -225 to -112 led to a marked reduction in responsiveness to all inducers.

The nucleotide sequence from -170 to -124 shows 70% homology to the Serum Response Element (SRE) within the c-fos gene promoter. The SRE is responsible for conferring inducability to serum, epidermal growth factor (EGF), tetradecanoyl phorbol-13-acetate (TPA) and insulin via the binding of the serum response factor (SRF) (Treisman,

1992). The regulation of c-fos and IL-6 is similar: the transcriptional activation of both occurs rapidly after exposure to an inducer, and both share the same group of factors capable of inducing transcription, although c-fos transcription is shut off after an hour, whereas IL-6 transcription can continue for four to six hours.

Using further deletion mutants linked to CAT reporter genes, Ray *et al.* (1989) demonstrated that the region -164 to -111 had a greater enhancing capability than -225 to -111. This implicated the region -225 to -165 as having a negative regulatory effect on IL-6 expression (the 'Negative Regulatory Domain'- NRD). They also showed that an intact CGTCA motif is essential within the c-fos SRE homology to maintain binding for serum/TPA/IL-1 induced nuclear extracts. They generated an artificial 23 bp oligonucleotide homologue of the area around the motif (-173 to -151) and showed that it conferred responsiveness to serum, IL-1α, TNF-α, TPA, and forskolin. This sequence - and the region within the c-fos SRE that it represented - therefore became known as the Multiple Response Element (MRE). The motif CGTCA was shown to be essential for inducability by TPA and forskolin, with a different area within the MRE being responsible for conferring serum, IL-1β and TNF-α inducability. The motif has been extended to GGACGTCA, which corresponds to the consensus sequence for the cAMP Response Element (CRE), TGACGTCA (Dendorfer *et al.*, 1994). This is likely to be the means by which IL-6 responsiveness to cAMP is mediated, stimulated *in vitro* by agents such as TPA and forskolin. Competition analysis has shown that the factors interacting specifically with the IL-6 MRE are identical to those binding classical CREs in other cAMP-responsive genes. Mutation of the motif had little effect on LPS responsiveness in the cell line studied, confirming the importance of the κB site in mediating this effect.

Transcriptional control mechanisms can vary considerably between different cell lines, as shown by the work of Isshiki *et al* on the IL-1 responsive element within the c-fos SRE homology (Isshiki *et al.*, 1990) of the IL-6 promoter. In a glioblastoma cell line, a reporter construct containing the -122 to +12 region was unresponsive to IL-1 stimulation. Instead they identified a constitutive and inducible nuclear factor they named NF-IL6, which bound to a domain further upstream to the NF-κB site. This domain is a 14 bp palindromic repeat sequence (ACATTGCACAATCT) that was responsible for conferring IL-1 inducability. The NF-IL6 sequence overlaps the MRE, which explains some of the overlap in response noted

in other studies. What is more, NF-IL6 and the NF-κB p65 subunit directly associated via their basic leucine-zipper domains and Rel homology domains respectively. This is the origin of the synergistic activation of the IL-6 promoter by NF-IL6 and NF-κB (Matsusaka *et al.*, 1993). NF-IL6 complexes with HIV Tat protein to upregulate IL-6 expression, an effect thought to be of relevance in the association between HIV infection and psoriasis, B cell lymphomas and Kaposi's sarcoma (Ambrosino *et al.*, 1997).

Further examples of tissue specificity in IL-6 transcriptional control relate to Protein kinase C (PKC). When investigated in human monocytes, the PKC activator phorbol-12-myristate 13-acetate (PMA) did not increase IL-6 expression (Gross *et al.*, 1993). Moreover, PKC activation resulted in the reduction of IL-1-induced IL-6 mRNA expression. However, in HEp-2 cells IL-6 expression was induced by PMA. In an astrocyte cell line, inhibition of PKC by GF109203X strongly inhibited IL-6 induction by substance P (SP) and histamine (Lieb *et al.*, 1998). The PKC_{α} and PKC_{β} isoform inhibitor Gö6917 only weakly inhibited this stimulation, suggesting that other PKC isoforms were responsible for mediating the intracellular signal cascade. Using deletion mutant reporter constructs it was shown that an intact NF-IL6 site is required for maximal SP and histamine stimulation.

IL-6 is able to induce its own expression: when in combination with sIL-6R it stimulates further IL-6 expression from osteoblastic cells (in this case rat Ob cells) through activation of NF-κB, NF-IL6 and the CRE (Franchimont *et al.*, 1997). This autoregulatory feature has important implications, in osteoporosis for instance, where increased local concentrations of IL-6 are implicated in activating osteoclasts (discussed below).

1.4.4.4 AP-1

The molecular biology of AP-1

Activator protein 1 (AP-1) was originally identified as a DNA binding activity in nuclear extracts from HeLa cells recognising the sequence TGACTCA present in the SV40 and human metallothionein II_A enhancers (Lee *et al.*, 1987). Several proteins, belonging to two families, are now recognised as components of the AP-1 transcription factor. c-Jun, the product of the c-*jun* proto-oncogene (the cellular counterpart of v-*jun*, the avian sarcoma

virus oncogene) was the first to be identified. Other members of the *jun* family are *jun*B and *jun*D. Similarly *c-fos*, the cellular counterpart of the v-*fos* oncogene carried by the FBJ and FBR osteogenic sarcoma viruses, encodes c-Fos. The *fos* family consists of *fos*-related antigen-1 (*fra*-1), *fra*-2, *fos*B and its naturally truncated form *fos*B2. It is now recognised that the various Fos and Jun family members are able to form dimeric complexes with one another and bind to the AP-1 consensus sites that are present in numerous cellular and viral genes (Halazonetis *et al.*, 1988). Highly specific dimer formation occurs between the α-helical leucine-zipper regions (heptad repeats containing 4-6 leucine residues) of the proteins. Thus, Fos proteins cannot homodimerise or demerise with other Fos proteins, but Jun proteins can form homo-and heterodimers with each other. However, the most stable complexes are Fos/Jun family member heterodimers (O'Shea *et al.*, 1989).

Contiguous with the leucine zipper dimerisation domain is another α-helical region, the DNA recognition domain. When the proteins dimerise these domains, which are rich in basic amino acids, become juxtaposed into a Y-shape, between which the major groove of the DNA strand fits. Each constituent of the AP-1 complex contributes equally to the DNA site recognition and binding properties of the dimer (Glover and Harrison, 1995).

Transcription factors with these two α-helical regions form the b-zip protein family, a large class which also includes the cAMP response element binding proteins (CREBs) and the C/EBP family (including NF-IL6). Members of this class can also recognise AP-1-like sequences and AP-1 half-sites. AP-1 family members are also able to complex with non-b-zip proteins, such as the glucocorticoid receptor (GR), NF-κB p65 and the TATA binding protein (TBP) .

The control of AP-1 transcriptional regulation is primarily at the level of transcription of the AP-1 proteins themselves. *Fos* and *jun* are among the first genes transcribed when a cell is stimulated, but only transiently so and without the requirement for *de novo* protein synthesis. The c-*fos* promoter is the best characterised, with sites for CRE, the SRE and a Sis-inducible enhancer. This latter site is induced by growth factor and cytokine stimulation through the JAK/STAT pathway (Darnell *et al.*, 1994). cAMP and calcium-dependant pathways signal via the CRE. CREB is constitutively bound, but phosphorylation at serine 133 permits the binding of CREB-binding protein (CBP), which itself binds to TFIIB and facilitates gene transcription (Sheng *et al.*, 1991). The SRE site is constitutively occupied by

a serum response factor (SRF) dimer, to which is bound the ternary complex factor (TCF). UV, growth factors and cytokines stimulate MAK-induced phosphorylation of the TCF, which induces transcription (Price *et al.*, 1995). The TCF interacts with CBP to transactivate the c-*fos* promoter, especially following MAPK phosphorylation (Janknecht and Nordheim, 1996). Thus CBP appears to integrate different signaling pathways to the c-*fos* promoter.

AP-1 protein function is also regulated by post-translational methods. Phosphorylation of N-terminal sites of Jun augments its DNA binding and transcriptional activity (Derijard *et al.*, 1994). Inhibitory protein-1 (IP-1) binds AP-1 and prevents it from binding to DNA. When phosphorylated, IP-1 releases AP-1 in both the nucleus and cytosol, allowing it to locate to DNA-binding sites (Auwerx and Sassone-Corsi, 1992). The redox potential of transcription factors, such as AP-1 and NF-κB, also influences their DNA binding properties. For example, reduction of the single conserved cysteine in the Fos and Jun DNA binding domain increases the DNA binding affinity of the Fos-Jun heterodimer (Walker *et al.*, 1993). Redox regulation of AP-1 is controlled by the Ref-1 protein, which is also a DNA repair enzyme.

AP-1 sites may be many kilobases upstream of the transcription start point. Their interaction with the basal transcription machinery is mediated via CBP, whose CREB-binding domain interacts with the N-terminal activation domain of Jun to activate AP-1-directed transcription (Bannister *et al.*, 1995). Interactions with other proteins also influences AP-1 binding to DNA. In the presence of AP-1-associated factor (AF-1) the DNA binding affinity of JunD increases over 100-fold (Powers *et al.*, 1996), although the interaction of AF-1 with other AP-1 complexes varies, providing a degree of differential control to AP-1 mediated transcription.

AP-1 and IL-6

In the original investigation of the 5' flanking region of the IL-6 gene, individual sequences from the region were cloned into reporter plasmids and tested for transcriptional activity (Ray *et al.*, 1988). From these experiments it appeared that the AP-1 site at -280 to -274 was unimportant in regulating IL-6 expression. However, when the entire 5' flanking region (containing mutations in either the AP-1, MRE, NF-IL6, or NF-κB sites) was tested in a

reporter plasmid, it was shown that AP-1 is important for mediating the cAMP response (Dendorfer *et al.*, 1994). Additionally, elimination of the AP-1 site in combination with a mutation in a second regulatory element almost completely prevents IL-6 activation. These results demonstrate the limitations of deletion-construct investigations, where deletion of the AP-1 region might also remove a negative regulatory element, thereby compensating for a loss of activity.

1.4.4.5 A/T Tract

This is a stretch of 20 adenine and thymine bases between -392 and -373 which has not previously been commented upon. A long adenine repeat tract has an unusual shape, inducing a bend in double helical DNA (Price and Tullis, 1993). Greater bending occurs if the adenine tracts are regularly phased through the DNA region. In addition, (A+T)-rich regions induce a local helical destabilisation and an AT tract can undergo cruciform extrusion at elevated temperature (greater than 24^{0}C), in low salt conditions and when negatively supercoiled (Bowater *et al.*, 1994). Thus, such sections of DNA may exert a destabilising effect on nearby DNA sequences. Gene transcription is known to require strand opening as a fundamental event (Siebenlist *et al.*, 1979). Strand opening is linked to DNA transitions such as cruciform extrusion, which suggests that the AT tract could also influence transcriptional control.

1.4.4.6 Glucocorticoid Response Element

The molecular biology of the Glucocorticoid Receptor

Glucocorticoids (GC) are a group of endogenous steroid hormones with powerful anti-inflammatory and immunosuppressant functions which act via several mechanisms to reduce the expression of inflammatory and immunoactive mediators. The intracellular human glucocorticoid receptor (GR) exists in two forms, the 777-amino acid ligand-binding GRα, and the 742-amino acid GRβ which does not bind active GC. In the unliganded state, GRs are associated with several chaperone proteins including HSP 90 and HSP 56, which maintain the receptor in an inactive form with a high affinity for hormone (Funder, 1997). Following

ligand binding the chaperone proteins dissociate to expose the GR nuclear localization signals which allow its translocation to, and retention in, the nucleus.

The glucocorticoid response element (GRE) is a 15-nucleotide DNA sequence (AGAACAnnnTGTTCT) that represents a highly degenerate recognition sequence present in a large number of genes, including IL-6. Liganded GRs bind to this sequence as homodimers or potentially as heterodimers with mineralocorticoid, progesterone or androgen receptors (Wang, 1995). Several co-activator proteins, such as SRC-1 and CBP, have been identified which mediate the *trans*-activating effect of glucocorticoids on genes whose expression they induce (McEwan *et al.*, 1997). The co-activators bind both to the τ1 and τ2 transactivating domains of the GR and to elements of the transcriptional machinery (TBP, TFIIB) in a manner analogous to AP-1-stimulated transcriptional induction. Another important mechanism whereby the GR can activate transcription is its ability to disrupt nucleosomes. In the case of the tyrosine aminotransferase gene, this disruption removes the repressive effect of the nucleosome on gene transcription by permitting access to enhancer regions by other transcription factors (Reik *et al.*, 1991).

GR and IL-6

Although two consensus GR binding sites upstream of the AP-1 site (GRE_1 and GRE_2) are frequently shown in published schematics of the IL-6 5' flanking region, they have not as yet been shown to have a functional significance. However, the negative effect on transcription produced by binding of the GR to other transcription factors and to sections of the 5' flanking region is important in the regulation of IL-6 expression and is discussed below.

1.4.4.7 Sp1

Although not yet reproduced in human cell lines, there is good evidence to show that in the murine IL-6 gene, the C2H2 zinc-finger transcription factor Sp1 binds to three CCACC motifs between the NF-κB and NF-IL6 sites and is responsible for mediating basal and inducible IL-6 expression (Kang *et al.*, 1996). Deletion experiments demonstrated that this binding site is necessary for IL-6 transcription via NF-κB and NF-IL6. It is therefore possible

that the bound Sp1 transcription factor provides a framework on which NF-κB and NF-IL6 synergise.

On the other hand, there is evidence that Sp1 is involved in the repression of the IL-6 gene in the human THP-1 cell, through its interaction with the retinoblastoma control element (RCE) (Sanceau *et al.*, 1995)(discussed below).

1.4.4.8 Interferon Regulatory Factor (IRF)

Interferon-γ (IFN-γ) stimulation of HeLa cells induces the expression of IL-6 via the phosphorylation of STAT1 which binds as a homodimer to the IFN-γ activation site sequence on the interferon regulatory factor-1 (IRF-1) gene. This is a transcription factor that has been demonstrated to bind to the IL-6 5' flanking region and leads to upregulation of IL-6 expression in S3 HeLa cells (Faggioli *et al.*, 1997). The IRF binding site is located at position -267 to -254, but there is an absolute requirement for the co-presence of NF-κB (p65) before IFN-γ can *trans*activate the IL-6 gene. This is a further example of the requirement for co-operation between transcription factors for the stimulation of the IL-6 gene.

Similar results have been demonstrated in the human THP-1 cell, where IFN-γ is an essential co-signal for the TNF-α-induced expression of IL-6 (Sanceau *et al.*, 1995). This synergism involves cooperation between IRF-1 and NF-κB p65 homodimers with concomitant removal of the negative effect of the RCE. This removal occurred by activation of the constitutive Sp1 factor, whose increased binding activity and phosphorylation were mediated by IFN-γ.

1.4.5 *Repression of IL-6 Expression*

1.4.5.1 Glucocorticoid Receptor

There are several factors whose binding to various regions of the IL-6 5' flanking region results in downregulation of transcription, one of the most important of which is the GR. GCs enter the cytoplasm and bind to the GR, after which the complex translocates to the nucleus. Here the complex is able to bind across important DNA sequences and suppress IL-6

transcription. Ray *et al.* (1990) reported significant repression of IL-6 transcription from promoter deletion mutant-CAT reporter constructs transfected in HeLa cells. Dexamethasone (a potent synthetic GC) attached to the GR was found to bind across the MRE, TATA box and Inr site (the 'Initiator': an RNA polymerase start site, an octomeric motif, straddling the C1 site, which is the most basic type of promoter that RNA polymerase will recognise in the absence of the TATA motif). Within the MRE, it was the NF-IL6 site which was occluded. Imperfect palindromic repeat sequences homologous to the consensus GRE are present at the MRE, TATA and Inr sites.

Physical association between the GR (mediated by its DNA-binding domain) and NF-κB p65 subunit has also been demonstrated as a mechanism for GR-induced suppression of IL-6 expression (Ray *et al.*, 1994).

GC also down regulates cytokine gene expression by inducing the expression of the IκBα gene, resulting in the formation of new IκB protein (Scheinman *et al.*, 1995). This then re-associates with NF-κB released from previously synthesised cytoplasmic IκB following stimulation with inflammatory mediators such as TNF-α, preventing NF-κB it activating its numerous target genes, such as IL-6. In addition, newly synthesised IκBα can enter the nucleus and inhibit DNA binding of NF-κB (Arenzana-Seisdedos *et al.*, 1995).

1.4.5.2 p53 and RB

The p53 protein and the product of the retinoblastoma gene (RB) are considered to be tumour repressors. Mutant proteins are frequently found in tumours where increased IL-6 immunostaining is also present. Both p53 and RB are repressors of IL-6 transcription (Santhanam *et al.*, 1991). p53 binds to the NF-IL6 recognition sequence in the IL-6 5' flanking region, strongly repressing transcription (Margulies and Sehgal, 1993).

RB binds to the RB-control element (RCE) of the *c-fos* promoter. There is a region strongly homologous (21 of 26 bases) to the RCE in the IL-6 5' flanking region at -126 to -101 which may represent the domain of functional IL-6 repression by RB.

IFNγ-stimulated THP-1 cells contain a nuclear protein complex that binds to the RCE and contains the transcription factor Sp1 (Sanceau *et al.*, 1995), although direct DNA binding by this factor has not yet been shown in human cells. Interestingly, RB protein was not a part

of this DNA binding complex. This suggests that RB regulates transcriptional control partly by interacting with other RCE-binding proteins.

1.4.5.3 Oestrogens and Androgens

IL-6 transcription is efficiently repressed by the estrogen receptor (ER). This is mediated via an interaction between the D region of the ER and both the Rel domain of NF-κB and the bZIP domain of C/EBPβ (Stein and Yang, 1995, Ray *et al.*, 1997). The low serum estrogen concentrations found in postmenopausal women may therefore enable increased IL-6 expression. As IL-6 can activate immature osteoclasts to resorb bone, the loss of this repression of IL-6 expression is a potentially significant component in the development of postmenopausal osteoporosis. This hypothesis is supported by the observation that IL-6 knockout mice are protected from osteoporosis after ovariectomy (Poli *et al.*, 1994). However, it was not possible to detect increased amounts of IL-6 mRNA or protein in bones or bone marrow cells up to 3 weeks after ovariectomy in a mouse model (Vargas *et al.*, 1996). It is possible that the increase in IL-6 levels does not occur for some weeks after ovarian failure, as other inhibitory mechanisms are active, or the IL-6 increase occurs locally, and at levels difficult to detect.

Androgens are also capable of repressing the IL-6 gene, a possible mechanism for the reciprocal serum concentrations of testosterone and IL-6 (which fall and rise, respectively) with increasing age. This increase in serum IL-6 levels may be a factor in male senile osteoporosis (Bellido *et al.*, 1995). In support of this is the observation that following orchiectomy in mice, bone marrow IL-6 mRNA and protein levels both increase, the bone marrow becomes hypercellular and the mice develop splenomegaly (Zhang *et al.*, 1998). Unlike oestrogens, androgens suppress IL-6 expression not through a direct interaction with NF-κB, but rather through the maintenance of intracellular IκBα levels, which prevents the nuclear localisation of NF-κB (Keller *et al.*, 1996).

1.4.5.4 The 'Negative Regulatory Domain'

In the early studies of the 5' flanking region it was common to employ deletion mutants of varying length, linked to reporter genes (frequently chloramphenicol acetyltransferase), to obtain information about regions of importance to transcription. In this manner it was observed that the region -225 to -164 conveyed a negative effect on transcription, and was termed the Negative Regulatory Domain (NRD) (Ray *et al.*, 1989). In separate experiments the GR was observed to bind within this region (-210 to -201) (Ray *et al.*, 1990) but no repressor transcription factors have been demonstrated to bind within the NRD.

1.4.5.5 Recombination Signal Sequence Binding Protein Jκ (RBP-Jκ or CBF1)

RBP (also known as RBP-Jκ or CBF1) functions as a transcriptional repressor in most mammalian cells. During adenoviral or EBV infection the respective viral proteins pIX and EBNA2 associate with RBP to inhibit its repressive function (Kannabiran *et al.*, 1997). A consensus binding sequence for RBP (TGGGAAA) lies on the antisense strand within the NF-κB site of the IL-6 gene (Figure 1.6).

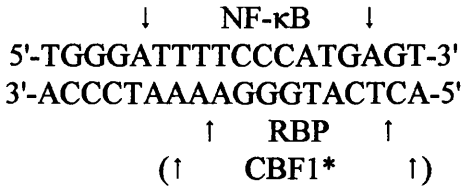


Figure 1.6 RBP binding site within the NF-κB site on the IL-6 5' flanking region. (*CBF1 consensus binding sequence, after Miyazawa *et al.*, 1998a).

RBP represses IL-6 expression in a variety of cell lines by binding adjacent to the NF-κB site and interfering with the cooperation between NF-κB-p65 and NF-IL6 (Kannabiran *et al.*, 1997). The NF-IL6 binding site lies immediately 5' to the NF-κB site, but it is not functional as an inducer of IL-6 expression alone (unlike the main NF-IL6 site at -158 ~ -146). RBP is present within the nucleus in excess to NF-κB, but the DNA binding affinity of NF-κB is ten times greater (Plaisance *et al.*, 1997). Together this suggests a constitutive, physiological repression of the IL-6 gene by RBP, which is displaced when NF-κB becomes activated.

1.4.6 *Post-transcriptional Regulation*

1.4.6.1 The role of mRNA stability

In addition to transcriptional control, post-transcriptional processes are also of importance in regulating gene expression. Cytokines, proto-oncogenes and growth factors are frequently encoded by mRNA that exhibits rapid cytoplasmic turnover. The mRNA transcripts of many cytokine genes have highly homologous AU-rich sequences within their 3' untranslated regions (UTR). Reiterated AUUUA pentamers within the UTR of these intrinsically labile mRNAs have been shown to confer mRNA lability using heterologous gene constructs (Shaw and Kamen, 1986). In contrast, where certain malignancies are accompanied by elevated cytokine levels, deletion of some of the pentamers has resulted in abnormal stability of the cytokine mRNA (Henics *et al.*, 1994).

Associated with the pentamers are other longer AU-rich domains which are also important in conferring instability. Mutations within these sites also affect mRNA stability, as does varying the number of AUUUA motifs (Akashi *et al.*, 1994).

It is likely that the pentameric motifs exert their destabilising effect by acting as recognition sites for ribonucleases, which cleave mRNA immediately upstream of the motif (Gillis and Malter, 1991). The pentamers have also been demonstrated to be binding sites for a family of nuclear and cytoplasmic AU-binding proteins (AUBP) (Ross, 1995). Once bound, the mRNA/AUBP complex is highly resistant to ribonuclease. AUBP is activated, by

phosphorylation and a redox switch, by phorbol esters and calcium ionophore (Malter and Hong, 1991). For example, the induction of IL-1 by TNFα is mediated in part by activation of protein kinase C and a resultant increase in IL-1 mRNA stability (Gorospe *et al.*, 1993).

For AUBP to bind there must be at least two pentamers, or one and an AU-rich domain. The secondary structure of the mRNA molecule produced by the combination of pentamers and pentamer/AU-rich domains is likely to be an important regulator of AUBP binding and mRNA turnover (Hennics *et al.*, 1994, Gillis and Malter, 1991).

IL-6 mRNA has a half-life of approximately 1 hour in fibroblasts (Elias and Lentz, 1990) and monocytes (Brach *et al.*, 1990, Rola-Pleszczynski and Stankova, 1992). It contains an AU-rich region with six AUUUA conserved sequences in the 3' UTR. Deletion of this region from an IL-6 expression plasmid transfected into NIH3T3 cells resulted in a more than tenfold increase in mRNA levels compared to controls (Tonouchi *et al.*, 1989). IL-6 protein production was threefold greater than controls in this system. As discussed below (Chapter 1.5.1), the *Bst*N1 restriction enzyme polymorphic alleles result from insertions/deletions within this region of the 3' flanking region (Bowcock *et al.*, 1988). This, and the other restriction fragment length polymorphisms, do not alter the coding sequence for the IL-6 protein (Sehgal, 1992).

There are several examples of variations in expression of IL-6 resulting from altered IL-6 mRNA stability. IL-1 and TNF-α act synergistically to up regulate fibroblast IL-6 expression, partly mediated by increased mRNA stabilisation and partly by an increase in gene transcription (Elias and Lentz, 1990). Leukotriene B_4 has a similar effect (Rola-Pleszczynski and Stankova, 1992), possibly related to its activity as a calcium ionophore and subsequent upregulation of AUBP. LTB_4 also induces IL-6 by activating NF-κB (see earlier). TNF-α up regulates IL-6 by stabilising its mRNA, whereas TNF-β (Lymphotoxin) reduced its half-life by a process requiring protein synthesis (Brach *et al.*, 1990). However, other research has suggested that TNF-β does stabilise IL-6 mRNA, increasing fibroblast-derived mRNA half-life, in this case, from 0.3 to 0.85 hours (Akashi *et al.*, 1990). This work also demonstrated that the stabilising effect was potentiated by stimulators of protein kinase C, and also by inhibitors of protein synthesis. IL-10, but not IL-4, reduces IL-6 mRNA half-life in stimulated THP-1 and U937 cells, resulting in a reduced IL-6 expression (Takeshita *et al.*, 1996). Histamine potentiates the expression of IL-6 by PBMC stimulated by IL-1, mediated by an

increase in steady-state mRNA levels (Vannier and Dinarello, 1994). There was actually a small reduction in mRNA half-life, but overall expression was increased as a result of increased transcription. Mutations in the *ras* oncogene are often associated with tumours secreting cytokines (Demetri *et al.*, 1990). Normal human fibroblasts transfected with a mutant H-*ras* oncogene demonstrate an increase in IL-6 expression, due to increased mRNA stability. Finally, treatment of human thymic epithelial cells with EGF or TGF-α increases IL-6 protein and mRNA levels, not through increased transcription of the IL-6 gene, but rather both EGF and TGF-α increase IL-6 mRNA stability (Le *et al.*, 1991).

The actual mechanism by which IL-6 mRNA stabilisation occurs is unknown. Reduced production of ribonuclease, or its increased degradation, the production of a ribonuclease inhibitor or an altered sensitivity of IL-6 mRNA to degradation have all been proposed (Elias and Lentz, 1990). An additional hypothesis concerns the two potential poly(A) addition sites, spaced 72 bases apart. It is assumed that the distal one is used during routine gene transcription (May *et al.*, 1989) but if the proximal one were employed, the AU-rich region would be missing from the mRNA transcript (Elias and Lentz, 1990, Rola-Pleszczynski and Stankova, 1992). No specific studies of this feature have been published to date.

The *in vivo* situation with respect to the control of IL-6 (and the other inflammatory cytokine mRNAs) is likely to involve a combination of mechanisms. For example, the initial decay step for many mRNAs is deadenylation, a process modified by a specific binding protein (Ross, 1995). Other *trans*-acting mediators include estrogen and glucocorticoids, both of which can act to stabilise some mRNAs and destabilise others (Ross, 1995). Despite these various effects, the overall degree of IL-6 expression is still regulated at other levels, such as the rates of gene transcription and mRNA translation, which can negate the effect of a large increase in mRNA stability.

1.4.6.2 Alternative mRNA splicing

The IL-6 gene contains a 636bp coding region comprised of five exons. In addition to the predominant mRNA transcript of 1.3-1.4kB there is evidence for the existence of additional IL-6 transcripts and also multiple transcriptional start sites (Figure 1.4) (Yasukawa *et al.*, 1987, May *et al.*, 1989). Although the alternatively spliced mRNAs have been reported to be

minor and infrequent species, Kestler *et al.* consistently found a 0.45kB RT-PCR product, in addition to the expected 0.64kB product, in stimulated PBMC, monocytes and lymphocytes (Kestler *et al.*, 1995). Sequencing demonstrated that this smaller product derived from alternative splicing of IL-6 mRNA, with exon II spliced out. The authors also reported an inducible 17 kD protein, consistent with a protein translated from the alternatively spliced mRNA, in detergent lysed LPS-stimulated PBMC. A fibroblast-derived IL-6 polypeptide of a similar size has previously been attributed to post-translational modification. (Santhanam *et al.*, 1989).

The most interesting feature of this work is that the exon II deficient protein would be lacking helix A, which contains the majority of site 2, the gp130 interactive domain (discussed in 'IL-6 Signal Transduction', below). In theory, this alternative IL-6 could bind IL-6R but could not interact with gp130. Hence it might be a natural antagonist of IL-6. This new IL-6 polypeptide is currently being further characterised.

1.4.6.3 mRNA translation

Patients infected with the human immunodeficiency virus 1 (HIV1) have high levels of plasma IL-6, and HIV1 infection is associated with severe psoriasis, B cell lymphoma, and Kaposi's sarcoma (Scala *et al.*, 1994). HIV Tat, a protein with increases both viral and cellular gene transcription, binds to a short sequence in the 5' untranslated region of IL-6 mRNA. This UCU sequence forms a stem-loop to which Tat binds, leading to a *trans*-activation of the promoter (Ambrosino *et al.*, 1997). In addition there is evidence that Tat increases both the amount of activated NF-IL6 and also its transcriptional activity (Ambrosino *et al.*, 1997). These mechanisms are likely to be responsible for the observed high levels of IL-6 in HIV infection, but are probably not of relevance in other clinical situations.

1.4.7 *IL-6 Signal Transduction*

The intracellular pathways by which IL-6 signals the induction or suppression of target genes within multiple cell lines has been the source of much recent investigation. IL-6 shares a functional redundancy with the other group 1 four-α-helix cytokines: IL-11, LIF, OM and

CNTF. This is explained by the observation that they all share gp130 as a signal transducing receptor component (Taga and Kishimoto, 1995). Each binds to its own specific receptor. In the case of IL-6 this is the IL-6Rα (Yamasaki *et al.*, 1988), which triggers the association of the receptor with the non-IL-6-binding signal transducer, gp130 (Taga *et al.*, 1989), at their extracellular region, allowing gp130 homodimerisation (Murakami *et al.*, 1993). This results in the formation of further high-affinity IL-6 binding sites, and the homodimerised gp130 generates the IL-6 signal. The cytokine receptor domain of the IL-6Rα, not the immunoglobulin-like domain, is required for both IL-6 binding and signal transduction through gp130 (Yawata *et al.*, 1993). Because of this 'delivery' of IL-6 to gp130, sIL-6Rα enhances rather than abrogates the effect of IL-6.

The binding of IL-6 to an IL-6Rα chain eventually results in the formation of a hexameric complex containing two molecules of each component: IL-6, α receptor chain and gp130 (Murakami *et al.*, 1993). In this complex each IL-6 molecule contacts the IL-6Rα, through site 1, and both gp130 chains, mediated via sites 2 and 3. Site 1 has been mapped, both by molecular modeling and functionally by engineering base substitutions to produce increased binding to the IL-6Rα ('superbinder'), to the A-B loop (Toniatti *et al.*, 1996). Site 2 is formed by exposed residues in the A and C helices, and is a conserved receptor binding site for several cytokines (Sporeno *et al.*, 1996). Site 3 is composed of residues from helix D and from the A-B loop. Super-antagonists of the IL-6Rα have been developed which employ substitutions in these three binding sites (Sporeno *et al.*, 1996).

Homodimerisation of gp130 chains juxtaposes their cytoplasmic components, triggering the activation of members of the JAK family of tyrosine kinases - Jak1, Jak2 and Tyk2 - which are bound to the cytoplasmic domains of gp130 (Yawata *et al.*, 1993). The juxtaposition, or increase in local concentration, results in JAK kinases phosphorylating each other, as well as the receptor components. With respect to IL-6 signal transduction, the active JAKs are then able to tyrosine-phosphorylate the latent transcription factors STAT3 (signal transduction and activation of transcription three, previously called acute phase response factor [APRF]) and STAT1 (Lutticken *et al.*, 1994). This process allows STAT3 to form stable homodimers, through interactions between intermolecular SH2 domains and phosphotyrosine, giving the STAT3-homodimer DNA binding properties. The IL-6 -mediated JAK-STAT pathway has been demonstrated to activate several genes, including *junB*

(Nakajima *et al.*, 1993) and several of the acute phase reactant genes, such as α_2-macroglobulin, α_1-acid glycoprotein and fibrinogen (Wegenka *et al.*, 1993).

A second intracellular pathway for IL-6 signaling involves the phosphorylation of NF-IL6. There is evidence that MAP kinase can phosphorylate NF-IL6 *in vitro* (Nakajima *et al.*, 1993) but the precise kinase responsible is still under investigation. The activation of the MAP kinase itself occurs through the Ras-Raf-MAPKK(MEK)-MAP kinase cascade (Pelech and Sanghera. 1992). The activation of this cascade also occurs by phosphorylation, induced by activation of gp130-associated tyrosine kinases.

It is possible that other pathways also lead to the phosphorylation of NF-IL6. Mouse and rat homologues of NF-IL6 (C/EBPβ and LAP respectively) have been shown to be phosphorylated by a calcium-calmodulin kinase and PKC respectively; cAMP-induced phosphorylation is associated with nuclear translocation and transcriptional activation (Akira *et al.*, 1995)

1.4.8 *IL-6 Knockout and Transgenic animals*

A detailed discussion of these two large fields of research is outside of the remit of this introduction. However, a brief mention of the impact that these two techniques have had on the understanding of the functional importance of IL-6 in development, disease and animal models of arthritis is appropriate.

1.4.8.1 IL-6 Knockout Animals

Targeted disruption of the IL-6 gene results in mice that develop normally, but which fail to generate normal titres of neutralising IgG to, or cytotoxic T cell activity against, vaccinia virus infection (Kopf *et al.*, 1994). Additionally they are unable to clear intracellular infection with *Listeria monocytogenes*. The critical role for IL-6 in the stimulation of the hepatic APR is underlined by the absence of increased acute phase proteins after tissue damage or infection in these mice. In more recent studies, IL-6-/- mice have been shown to express no STAT3 after localised tissue damage (subcutaneous turpentine oil injection), and they do not show

an APR in this situation, whereas following systemic inflammatory stimulus (intraperitoneal LPS injection) both STAT1 and STAT3 are expressed at almost normal levels (Alonzi *et al.*, 1998a).

The involvement of IL-6 in Castleman's Disease (CD) is also emphasised by experiments with C/EBPβ /IL-6 double knockout mice (Screpanti *et al.*, 1996). These animals are protected from the development of a disease phenotypically like CD, which occurs in single knockout C/EBPβ-/- mice as their IL-6 levels increase with age (Screpanti *et al.*, 1995). That the expression of IL-6 should increase in the absence of a transcription factor documented to be important in its normal positive control underlines the complexity and redundancy in the control of IL-6 expression.

Knockout studies have demonstrated the importance of IL-6 in liver regeneration following hepatic damage (experimental partial hepatectomy), predominantly through a reduced STAT3 expression in the remaining liver tissue (Cressman *et al.*, 1996).

There is increasing evidence that IL-6 has potent anti-inflammatory activity (as will be discussed in Chapter 4) including work by Di Santo *et al.* (1997) who showed that anti-TNF-α activity is reduced in IL-6-/- mice, and that intrapulmonary proinflammatory cytokines and serum TNF-α are increased during *Streptococcus pneumoniae* infection (van der Poll *et al.*, 1997). These findings are at odds with the demonstration that the development of collagen-induced arthritis (CIA) is completely inhibited in susceptible DBA/1J mice who are IL-6-/-, but the inflammatory polyarthritis of TNF-α transgenic mice is unaffected by the coincident absence of IL-6 (Alonzi *et al.*, 1998b). This intriguing observation has yet to be fully explained.

1.4.8.2 IL-6 Transgenic animals

Transgenic (TG) mice, expressing high levels of human IL-6 in various tissues (by placing the expression of the transgene under the control of a tissue specific promoter) have been widely utilised to demonstrate the effects of overexpression of IL-6. Many of these studies do not provide further insights into the normal physiological functions of IL-6, but they do detail the effects of chronic IL-6 overexpression on previously normal tissue. For instance, renal

damage and myeloma kidney (but not liver damage) in hepatic overexpression (Fattori *et al.*, 1994); gliosis and astrocytosis (with increased central nervous system TNF-α, IL-1β and IL-6 expression after LPS stimulation) in central nervous system overexpression (Di Santo *et al.*, 1996).

It is particularly relevant that mice transgenic for human IL-6 under the control of a neurone specific promoter (rat neurospecific enolase) express high levels of neuronal and circulating IL-6 from soon after birth and become significantly growth retarded compared to non-transgenic littermates (De Benedetti *et al.*, 1997). Their levels of circulating insulin-like growth factor 1 (IGF-1) are significantly reduced (~50% of non-TG mice) in a manner analogous to that of children with active S-JCA, who are also markedly growth stunted, have normal levels of GH but reduced IGF-1 (Davies *et al.*, 1994). In these children administration of recombinant human GH leads to an increase in height velocity in short term treatment.

1.5 Cytokine Gene Polymorphisms

1.5.1 *Interleukin-6*

Two groups have published details of IL-6 restriction fragment length polymorphisms (RFLP). Fugger *et al.* (1989a) showed that *Msp*I and *Bgl*II both identify (different) bi-allelic polymorphisms, neither of which show an association to RA, SLE or pauci-articular JCA (Fugger *et al.*, 1989b). Bowcock *et al.*, (1988) demonstrated RFLPs with *Msp*I, *Bst*NI and *Bgl*I, using a probe containing the fifth exon. All the phenotypes occurred in a variety of combinations, and in differing relative frequencies, in five separate populations tested, indicating no connection between them. They concluded that the sites of the first two polymorphisms lay in, or near, the fifth exon. There are no *Bgl*I sites within the IL-6 gene, only a single site in the 3' flanking region and one or more sites in the 5' region. They conclude that the *Bgl*I site lies in the 5' region. Fugger *et al.* (1989a) also concluded that their *Bgl*II site lay here.

As the *Msp*I and *Bgl*I RFLPs were not detected by other enzymes, they are likely to be due to base pair substitutions. However, the *Bst*NI polymorphism was detected by ten other enzymes, indicating that insertion/deletion events had occurred, producing four alleles. The region containing the RFLP is located within 1kb of the 3' end of the fifth exon, an extremely AT rich region. This has been further examined by PCR (Bowcock *et al.*, 1989). This method successfully amplified the polymorphic 3' flanking region of the IL-6 gene from human and primate subjects, which was then sequenced to provide confirmation that the polymorphism is due to a variation in the length of the AT rich sequence. This variation was the result of a variable number of tandem repeat (VNTR) sequences. Some differences were also observed due to occasional loss of one or two bases in the AT region. This loss may affect mRNA half-life, as the presence of AUUUA motifs in the 3' end of cytokine mRNA confers a reduced half-life, proportional to the number of motifs present (Akashi *et al.*, 1994, Saini *et al.*, 1990). Hence, differing lengths of the 3' AT rich region may produce a variation in the number of AUUUA motifs and thus variable mRNA stability. This has not been explored further for IL-6, although two recent papers describe possible disease associations for these 3' polymorphisms.

Linker-Israeli *et al.* (1996) report an increase in the number of allelic higher molecular weight bands following digestion of PCR products with *Xba*I, in patients with SLE compared to controls. They report the *Xba*I site to lie 500bp downstream of the second polyadenylation site in the AT-rich 3' flanking region. It is therefore highly likely to be identifying the same polymorphic region as described originally by Bowcock *et al.* (1988). Previous reports have found an increased IL-6 expression in SLE, and Linker-Israeli *et al.* (1996) postulate that this is associated with the increased variability in the 3' end of the IL-6 gene in SLE patients.

The PCR method of Bowcock *et al.*(1989) was used to demonstrate that the spinal bone mineral density measured in a cohort of 200 women was higher in those with the *C/F* genotype than those with the *F/F* genotype (Murray *et al.*, 1997). These two genotypes were the commonest observed, where 6 alleles (*A-F*) were identified. They differed slightly in size from the original description, probably because they were identified using polyacrylamide gel electrophoresis rather than agarose. Murray *et al.* (1997) concluded that the peak bone mass of women in this cohort was related to their IL-6 genotype.

The PCR method has also been used to examine the inheritance of the polymorphisms in a group of Spanish control subjects (Arroyo *et al.*, 1997). Five alleles were detected in this group, and no deviation from Hardy-Weinberg equilibrium was detected.

Recently, a polymorphic dinucleotide (CA) sequence has been described in the IL-6 gene, mapped to 7p21. (Tsukamoto *et al.*, 1998) As yet, there is no known association with the previously described RFLP.

1.5.2 *Interleukin-1 Gene Cluster*

The IL-1 gene cluster is located on the long arm of the second chromosome (2q) and contains the genes for IL-1α, IL-1β and IL-1ra. Several different polymorphisms have been investigated in this region. The IL-1α gene demonstrates two different types of polymorphism. Firstly, a C/T transition at position -889 within the 5' regulatory region. An increased frequency of this rare allele (IL-1A2) was found in Norwegian children with pauci-articular JCA (McDowell *et al.*, 1995). The association with IL-1A2 was even stronger for those children within this group who developed chronic iridocyclitis compared to those without this complication. However, these findings have not been reproduced in a cohort of

UK Caucasian patients (Donn *et al.*, 1998).

Secondly, the VNTR within intron six is also polymorphic, with a 46bp stretch of DNA repeated between five and 18 times (Bailly *et al.*, 1993). No disease associations are yet known for this or the single base substitution polymorphism in the IL-1β gene (Di Giovane *et al.*, 1992).

Within the IL-1ra gene there is a VNTR polymorphism in intron 2 (Tarlow *et al.*, 1993). It comprises an 86bp tandem repeat and displays five alleles. As with the IL-1α VNTR, the tandem repeat contains potential transcription factor binding sites. One of the rarer alleles is associated with increased production of IL-1ra protein, and also reduced production of IL-1α protein by monocytes (Danis *et al.*, 1995b). Associations between various alleles of IL-1ra and several diseases have been reported: SLE (Blakemore *et al.*, 1994), although not consistently (Danis *et al.*, 1995c); Graves Disease (Blakemore *et al.*, 1995); Lichen sclerosus (Clay *et al.*, 1994); Ulcerative colitis (Mansfield *et al.*, 1994) and Alopecia areata (Tarlow *et al.*, 1994).

1.5.3 *Tumour Necrosis Factor*

The genes for TNFα and -β lie within a 7kb stretch of DNA in the class III region of the Major Histocompatibility Complex (MHC). TNFα is an important mediator of the inflammatory response, and so considerable effort has been made in investigating polymorphisms discovered in this area. Probably the most clinically significant is the bi-allelic G/A transition polymorphism at position -308 in the TNFα promoter (Wilson *et al.*, 1992). This lies within a consensus sequence for the transcription factor AP-2. Functional studies have demonstrated higher constitutive and inducible levels of TNFα produced by reporter constructs containing the rarer, TNF2 (also known as TNFA2 and TNF-308.2) allele compared to the common (TNF1) allele (Wilson *et al.*, 1994a).

Possession of the TNF2 allele is strongly associated with the 'autoimmune' haplotype HLA A1, B8, DR3, DQ2 (Wilson *et al.*,1993). This haplotype is associated with increased production of TNFα by cultured lymphocytes. Consistent with this is the observation that diseases associated with this haplotype are also associated with TNF2, such as Systemic Lupus Erythematosus (SLE). Here there is a strong association between the allele and anti-Ro

and anti-La antibody production (Wilson *et al.*, 1994c). This finding was not reproduced in another study, looking at the frequency of TNF2 and the -238 TNF-α polymorphism in Caucasian and black South African SLE patients, although the numbers were small (Rudwaleit *et al.*, 1996). The TNF2 allele is also strongly associated with the presence of anti-PM-Scl antibodies in Systemic Sclerosis (Wilson *et al.*, 1994b). An association between TNF2 and RA could not be found in two studies (Wilson *et al.*, 1995a and Brinkman *et al.*, 1997), but was in one, small study (Danis *et al.*, 1995a).

In these studies there is a stronger association between HLA-DR3 and the disease than between TNF and the disease. This suggests that the observed association results from linkage disequilibrium between the TNF locus and the nearby DR3 locus. Similarly, the strong association of TNF2 with Dermatitis herpetiformis (an autoimmune skin disease with strong associations to HLA A1, B8, DR3, DQ2) was weaker than its association to the class II loci. Hence TNF2 is not a major disease susceptibility marker for DH, but may be a factor in disease severity (Wilson *et al.*, 1995b).

TNF2 may also be directly associated with disease outcome, as Gambian children who are homozygous for this allele have a relative risk of seven for death or severe neurological sequelae due to cerebral malaria (McGuire *et al.*, 1994). As TNF2 is associated with a greater expression of TNF-α, this might explain the very high levels found in cerebral malaria (Wilson *et al.*, 1997. However, in other studies there was no difference in the expression of a reporter gene from constructs containing either allele of this polymorphism (Brinkman *et al.*, 1995/6). Additionally, high levels of TNF-α are found in patients with Mucocutaneous Leishmaniasis. TNF2 is found more frequently in these patients and is associated with a relative risk of 3.5 for the disease, even in heterozygotes (Cabrera *et al.*, 1995). It is also more common in lepromatous than tuberculoid leprosy, where the serum levels of TNF-α are higher in the lepromatous form of the disease (Roy *et al.*, 1997) Thus, a polymorphism in the regulatory region of an inflammatory cytokine can influence the outcome of infectious diseases.

Other TNF-α G/A transition polymorphisms have also been reported at positions -376, -238, and -163 (Brinkman *et al.*, 1997). Following LPS stimulation of monocytes *in vitro* no difference in TNF-α production was observed between the -238GG and -238GA genotypes (Pociot *et al.*, 1995). Recently it has been reported that the -238GA genotype is associated with a reduced degree of radiological progression in rheumatoid arthritis

(Brinkman *et al.*, 1997). Finally for TNF-α, a C insertion polymorphism at position +70 has also been reported (Brinkman *et al.*, 1995).

In addition to TNF-α, a polymorphism within the promoter of TNF-β has been identified (Messer *et al.*, 1991a). Unlike TNF-α, this allele is not related to a reduced rate of TNF-β production. It could not be associated with the low level of TNF-β produced by patients with primary biliary cirrhosis (Messer *et al.*, 1991b) nor to the development of ankylosing spondylitis (Verjans *et al.*, 1994).

1.6 Specific aims and outline of this research

The specific aims of this research were to demonstrate and characterise polymorphisms within the 5' flanking region of the IL-6 gene and investigate their frequency in S-JCA.

The region investigated was a portion of the 5' flanking region from the translation initiation codon (+61) up to a point mid-way between the two putative glucocorticoid receptor elements (GRE_1 and GRE_2, position -550). This includes all of the transcription factor binding sites currently recognised to be important in the control of IL-6 expression. It was generated using PCR primer pair 1 (pp1) and is thus referred to as the pp1 fragment in this thesis. A longer region was also amplified (by primer pair 2, the pp2 fragment) in some instances during the population screening when no suitable pp1 fragment could be amplified. The overall composition of the IL-6 gene, and the region studied, is shown in Appendix A.

The initial screening for polymorphisms was conducted by SSCP (single-strand conformational polymorphism) of first *Hin*fI or *Mnl*II digested pp1 fragment PCR samples, and then by *Mae*III digested samples. Following the observation that differences in SSCP band appearance occurred between individuals, the pp1 fragment from a large number of subjects was sequenced. At first this was done by cloning the fragment into the pCRII vector. Later direct sequencing of the fragment was undertaken with an automated sequencer. Once the polymorphisms had been identified, an RFLP method was developed to assess the frequency of one of them - the -174G/C polymorphism - in patients and controls. The functional significance of this polymorphism was then assessed by transient transfection experiments using THP-1, and then HeLa, cells with luciferase reporter constructs containing both the full length, or length deletion mutant, pp1 fragment, as either of the -174 alleles.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Buffers, Gels and Chemicals

In all cases, buffers were made up with reverse osmosis purified water. Where necessary this was first sterilised by autoclaving. In addition some buffers were autoclaved or sterilised by filtration through a 0.2μ filter before use. For methods requiring highly purified, sterile distilled water (SDW), RO water was further purified by an Elga water purifier (Elga Water Systems, UK) and sterilised by autoclaving. All chemicals were obtained from Sigma, UK or BDH, UK.

2.1.1 *TAE* (50x stock): (per litre):

2M Tris base	242g
Glacial acetic acid	57.1ml
0.05M EDTA	100ml 0.5M EDTA

2.1.2 *TBE* (10x stock):

0.89M Tris base	108g
0.89M Boric acid	55g
20mM EDTA	40ml 0.5M EDTA

2.1.3 *TE*:

10mM Tris-HCl
1 mM EDTA
adjusted to pH 7.5 or 8.0 with 10M NaOH

2.1.4 *STE*:

10mM Tris-HCl
1mM EDTA, pH8.0

100mM NaCl

adjusted to pH 8.0 with 10M NaOH

2.1.5 *TEN*:

40mM Tris-HCl, pH 7.5

1mM EDTA, pH 8.0

150mM NaCl

2.1.6 *Phosphate buffered saline*

PBS was obtained by dissolving 1 tablet of pre-prepared PBS component (Sigma, UK) in 100ml of SDW.

2.1.7 *10X Hepes Buffered Saline (HBS)*

8.18% NaCl (w/v)

5.94% HEPES (w/v)

0.2% Na_2HPO_4 (w/v)

used at 2X concentration, pH 7.12

2.1.8 *Phenol:Chloroform solution*

Phenol solution containing 8-hydroxyquinoline to 0.1% (w/w) was firstly equilibrated with an equal volume of 1M Tris-HCl, pH 8.0. The phenol and Tris were allowed to separate, the upper (Tris) layer being removed and replaced twice with equal volumes of fresh 100mM Tris-HCl, pH 8.0. An equal volume of chloroform:iosamyl alcohol (24:1) was then added.

2.1.9 *Ethanol precipitation*

To precipitate DNA dissolved in water or TE, 0.1 volumes of 3M sodium acetate and a volume of absolute ethanol twice that of the DNA/sodium acetate solution were added. This solution was chilled at -70^{0}C for 20 minutes and then centrifuged at 14,000 rpm in a bench top centrifuge for 15 minutes at 4^{0}C. The pelleted DNA was washed twice in chilled 70% ethanol, followed each time by a 10 minute centrifuge at 14,000 rpm at 4^{0}C. The ethanol was removed, the pellet dried in air and then resuspended in an appropriate volume of SDW or TE.

2.1.10 *Phenol:Chloroform extraction*

To remove protein contamination from DNA samples, the solution of DNA was mixed with an equal volume of Tris-equilibrated phenol:chloroform:isoamyl alcohol (25:24:1) solution and vortexed. The phases were separated by centrifugation at 14,000 rpm for 5 minutes at room temperature and the top phase removed. DNA was precipitated from this layer by ethanol precipitation.

2.1.11 *4.5% SSCP polyacrylamide gel*

5.63ml	40% polyacrylamide gel solution (39:1 acrylamide to bis-acrylamide)
5.0ml	10X TBE
39.37ml	SDW

Polymerised with:	50µl fresh 10% ammonium persulphate (APS)
	35µl N, N, N', N' -tetramethlyethylene diamene (TEMED)

2.1.12 *0.5X MDE SSCP Gel*

18.75 ml 2X MDE gel solution
4.5ml 10X TBE
51.75ml SDW

Polymerised with: 300µl 10% APS and 30µl TEMED.

2.1.13 *8% Polyacrylamide gel for manual sequencing*

15ml 40% acrylamide solution
37.5g urea
22.5ml SDW
7.5ml TBE

Polymerised with: 400µl 10% APS and 40µl TEMED.

2.1.14 *5% polyacrylamide gel for Vistra sequencer*

Each gel was made immediately prior to use from 30ml of a stock of mixture prepared within the previous two weeks and kept at 4^0C. The mixture comprised:

15ml 10X Sequagel XR (National Diagnostics, USA)
55g Urea
87ml SDW
15ml TBE
5g Amberlite IRN-1500 (BDH)

Sequagel is a high grade, ultrapure, polyacrylamide gel solution which has a low inherent fluorescence, necessary for fluorescence sequencing. The urea/polyacrylamide/water solution was deionised with Amberlite resin whilst the urea was dissolving and filtered out

before the TBE was added.

The solution was de-gassed under vacuum for five minutes prior to use. Polymerisation was achieved by the addition of 200µl of 10% APS and 20µl of TEMED.

2.1.15 *Agarose Gel Electrophoresis*

Agarose gels of 1-2.5% were made by dissolving the appropriate weight of agarose (Promega, UK) in 50 or 100ml of 1X TAE by boiling. Once cool 2µl or 4µl of ethidium bromide solution (10mg/ml) were added and the gel cast in a horizontal gel tray with well-formers. When electrophoresing large volumes (~25µl) a deeper gel was poured (100~200ml volume, depending on the tray size).

2.2 DNA Samples

2.2.1 *Sources of DNA*

The DNA samples examined during this work were obtained from three sources. The initial source was a stock of genomic DNA samples in storage from the time that the department had been located at Northwick Park Hospital. A large number of samples had been collected from patients with several different subtypes of JCA, and a careful cross-check was made to ensure that only samples from patients with S-JCA were included (although samples from 15 subjects with other types of JCA were also sequenced). Informed consent had been obtained prior to the collection of blood for this DNA extraction, which was originally done for other research purposes within the department. Control DNA from healthy Caucasian personnel working in the MRC Clinical Research Centre at Northwick Park was also obtained and stored during this period.

A second, and more recent, source of genomic DNA was that extracted from blood samples collected from patients with S-JCA under the care of the Paediatric Rheumatology Unit, attending either Great Ormond Street Hospital for Sick Children or University College London Hospital. In all cases, these samples had been taken solely for the purpose of routine full blood count analysis. DNA was extracted from residual blood before the sample was

discarded, after storage at 4°C for up to 1 week.

A third source of genomic DNA samples was the ARC DNA repository at the University of Manchester, who kindly provided S-JCA samples that had been previously prepared by an automated DNA extraction process.

2.2.2 *DNA preparation*

The original Northwick Park genomic DNA samples were extracted from 10mls of blood taken into EDTA (Kunkel *et al.*, 1977). Briefly, one volume of whole blood was diluted with 9 volumes of cold buffer (0.32M sucrose, 5mM $MgCl_2$, 1% Triton and 0.01M Tris-HCl pH 7.6) and centrifuged to produce a nuclear pellet. This was resuspended in 25ml of cold buffer containing 75mM NaCl and 24mM NaEDTA, pH 8.0, 1.25ml of 20% sodium dodecylsulphate and Protease. The DNA was extracted from the solution by phenol: chloroform extraction and ethanol precipitation at -20°C. It was washed in 70% ethanol, dried and resuspended in TE buffer in which it was stored at 4°C.

Genomic DNA was extracted from the blood samples of patients from Great Ormond Street and University College London Hospitals using the Nucleon BACC Kit (Scotlabs, UK). The extraction process was carried out according to the manufacturer's protocol, by a member of Prof. Woo's research staff, as the DNA was intended for use in several ongoing projects in her laboratory.

Briefly, the blood sample was diluted by 4 times its own volume with buffer containing 10mM Tris-HCl, 320mM sucrose, 5mM $MgCl_2$ and 1% Triton X-100 at a final pH of 8.0, for 4 minutes at room temperature. The cells were pelleted by centrifugation at 7500rpm for five minutes. The pellet was resuspended in 350µl of lysis solution containing 400mM Tris-HCl, 60mM EDTA, 150mM NaCl and 1% SDS at pH 8.0. After deproteinisation with 100µl of 5M sodium perchlorate, 600µl of chloroform were added and the sample mixed to emulsify the phases. 150µl of Nucleon Resin were then added and the sample centrifuged at 2000rpm for 2 minutes. The DNA was precipitated from the aqueous phase with 900µl of cold absolute ethanol and then pelleted out, washed in cold 70% ethanol and redissolved in TE buffer, in which it was stored at 4°C.

2.3 Polymerase chain reaction

2.3.1 *Reaction buffers* (shown as the concentration in a PCR reaction volume of 50µl):

PCR buffer B1 (used predominantly with PCR primer pair 1 [primers 5/2 and 3/2]):

10mM each deoxynucleotide triphosphate (dNTP)
2.5 mM $MgCl_2$
60 mM Tris-HCl, pH 8.0
15mM NH_4SO_4

PCR buffer B2 (used with PCR primer pair 2 [primers DF20 and DF21] and for generating the length deletion fragments) was the Promega Thermocycling Buffer:

2.5 mM $MgCl_2$
50 mM KCl
10mM Tris-HCl, pH 9.0
0.1% Triton X-100

2.3.2 *Oligonucleotide primers*

Oligonucleotide primers for PCR were obtained from the Genosys Biotechnologies, Cambridge, UK. Resuspension at a concentration of 1µg/ml in SDW was confirmed by spectrophotometry. Aliquots of the oligonucleotide were stored at -20^{0}C at 250ng/µl, 1µg/ml and lyophilised. Oligonucleotide primers used for manual sequencing were stored at a concentration of 1.3pmol/µl.

5' Texas Red labeled primers for automated sequencing were obtained from Oswell DNA Service, Southampton, UK and stored at -20^{0}C in transfer buffer and at 1.3pmol/µl in SDW.

The sequence of each oligonucleotide primer and the cycling conditions used for PCR

or sequencing is given in the appropriate section.

2.3.3 *PCR Equipment*

Initial PCR of the IL-6 5' flanking region fragment with primer pair 1 was carried out in a Biometra Trio-Block PCR machine (Biometra, Germany). This machine was also used for colony PCR, to carry out the thermocycle sequencing reactions on the plasmid clones containing the pp1 fragment and to maintain an overnight temperature of 14^0C for ligation reactions. For all the remaining PCR experiments (production of fragments for directional cloning, for RFLP analysis, for direct sequencing and the sequencing reactions) a Perkin-Elmer 480 DNA Thermocycler machine was used. Both machines use 500µl PCR tubes where, with the exception of ligation reactions, the samples were overlaid with 2 drops of sterile mineral oil (Sigma, UK) to prevent evaporation.

2.3.4 *Visualisation, confirmation and quantification*

Successful and specific amplification of a PCR product was confirmed by electrophoresis of a 5µl aliquot of the reaction mixture, in a final volume of 10 µl containing 1X Type II Ficol loading buffer. Samples were run in a horizontal 1% agarose gel (Biorad, UK) and visualised under UV light. The size of the product was estimated by comparison against a ladder of DNA fragments of known sizes. Ladders used were : ΦX174, λHindIII and a 100 base ladder (all from Promega, UK).

2.3.5 *PCR product isolation*

Where a strong, single band of PCR product was visible, the PCR sample was purified without prior agarose gel isolation. The sample was made up to 100µl with SDW and then mixed with 1ml of 'Wizard' PCR Clean-up Resin ('Wizard' PCR Clean Up Kit, Promega, UK). Where contaminating bands were present in the PCR mixture, or where a linearised plasmid was being purified away from any cut out fragments prior to cloning, the correct band was isolated by first running the entire 50µl sample in a 1% agarose gel and cutting out the

required band visualised under UV light. This agarose chip was then dissolved in the resin which contains 6M Guanidine thiocyanate, to which the DNA binds.

The resin/DNA mix was run into a Wizard Minicolumn, attached to a vacuum manifold, where it became bound to a filter within the minicolumn. This was then washed with 2ml of 80% isopropanol and 2ml of 70% ethanol. The solubility of DNA is greater in water than in the resin. Hence the DNA was eluted in 30-50µl of SDW at 65^{0}C.

2.3.6 'Basic' (Primer pair 1; pp1) PCR reaction

The PCR used to generate the pp1 fragment (the 'basic' PCR reaction) from genomic stock (250ng-1µg template) or plasmid vectors (250ng template or less) was carried out using primer pair 1 and the PCR buffer described in 2.3.1. The primer sequences and reaction conditions were:

5/2 5'-CAG AAG AAC TCA GAT GAC TGG-3'
3/2 5'-GCT GGG CTC CTG GAG GGG-3'

a) Denaturation at 95^{0}C for 4 minutes;
b) Addition of 0.5U *Taq* polymerase in 1µl of 1X buffer whilst samples maintained above 72^{0}C;
c) Further denaturation at 95^{0}C for 1 minute;
d) Annealing at 63^{0}C for 1 minute;
e) Extension at 72^{0}C for 2 minutes;
f) 25 cycles of c-e;
g) Final extension at 72^{0}C for 10 minutes;

The location of the 5/2 and 3/2 primers and the pp1 fragment within the published sequence of the IL-6 gene is shown in Appendix A. The specificity of the PCR was checked by running 5-10µl of the sample on a 1% agarose gel against a DNA size ladder.

When the pp1 product could not be generated, despite alterations in the concentration of template DNA and/or alterations in the primer annealing temperature, primer pair 2 (DF20

and DF21) sometimes successfully amplified the 860bp pp2 fragment:

DF20 5'-GGA GTC ACA CAC TCC ACC T-3'
DF21 5'-GTG ACT GAC AGC ACA GCT-3'

The reaction parameters were similar to those for pp1, with the exception that the annealing temperature was 62^{0}C and 35 cycles were run. The pp2 primers and fragment is also shown in Appendix A.

2.3.7 *3' Flanking region (3' FR) PCR*

PCR of the 3' FR was carried out according to the method of Bowcock *et al* (1989) using the following primers:

DF9 5'-GCA ACT TTG AGT GTG TCA CG-3'
DF10 5'-GAC GTG ATG GAT GCA ACA C-3'

The cycling conditions were similar for pp1, with the exception that the annealing temperature was 55^{0}C and no 72^{0}C final extension was carried out after the 25 cycles had been completed. To obtain adequate separation of the PCR fragments, the samples were run overnight (15 hours) on a 2.5% agarose gel at 40V. The gel was then stained with ethidium bromide (10mg/ml) for 5 minutes in a shaking bath of TBE before visualisation under UV light.

The location of the primers relative to the IL-6 gene is shown in Figure 2.1.

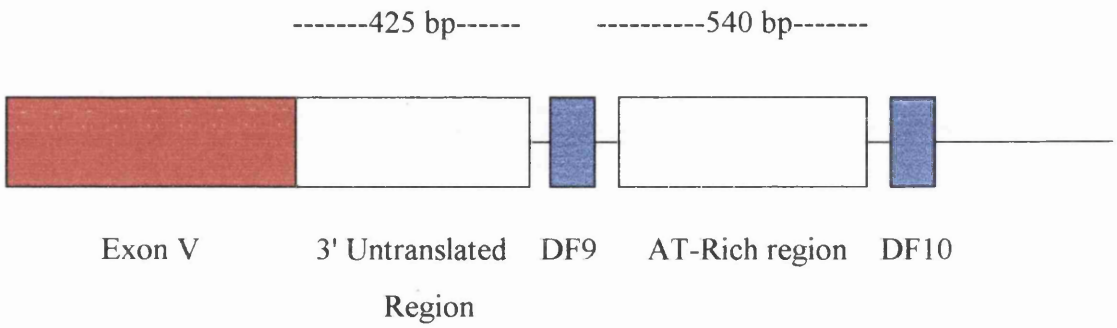


Figure 2.1 The location of the 3' FR PCR primers relative to the 3' flank of the IL-6 gene. The distances in base pairs represent the published sequence (Yasukawa *et al* 1987).

2.4 Restriction enzyme digestion

2.4.1 *Enzymes*

The following restriction enzymes (with their appropriate buffers, which are shown below) were obtained from Promega, UK:

*Aat*II (Buffer J)
*Bgl*II (Buffer D or Multicore Buffer (MCB))
*Eco*RI (MCB)
*Kpn*I (Buffer J)
*Nhe*I (Buffer B or MCB)
*Not*I (Buffer D)
*Sac*I (Buffer J)
*Xba*I (Buffer D or MCB)
*Xho*I (Buffer D or MCB)

The following restriction enzyme was obtained from Boehringer Mannaheim:

*Mae*III (Buffer SuRE Cut M)

The following restriction enzyme was obtained from New England Biolabs:

*Sfa*NI (Buffer NEB3)

2.4.2 *Buffers*

Promega (1X):

	pH	Tris-HCl (mM)	$MgCl_2$ (mM)	NaCl (mM)	KCl (mM)	DTT (mM)
B	7.5	6	6	50	-	1
D	7.9	6	6	150	-	1
J	7.5	10	7	-	50	1

Multicore Buffer (MCB) (1X):

25 mM Tris-Acetate, pH 7.8 (at 25^0 C);
100 mM potassium acetate;
10 mM magnesium acetate;
1 mM DTT

Boehringer Buffer: SuRE Cut M (2X)

40 mM Tris-HCl;
550 mM NaCl;
12 mM $MgCl_2$;
14 mM 2-mercaptoethanol;
(pH 8.2 at 55^0C)

NEB Buffer: NEBuffer 3 (1X)

50 mM Tris-HCl;
10 mM $MgCl_2$;
100 mM NaCl;
1 mM DTT
(pH 7.9 at 25^0C)

2.4.3 *Analysis of sequences for restriction enzyme sites.*

An automated analysis of the restriction enzyme sites present in a given sequence was obtained by entering the sequence data into the 'Webcutter', an on-line program available at: http://www.medkem.gu.se/cutter. The database for Webcutter contains >400 restriction enzyme recognition sequences. The program scanned both the positive and negative DNA strands (in both directions) to look for matches. Sequence data were pasted into the program from the GenBank sequence file, thus removing the risk of erroneous data entry.

2.5 Single-strand Conformational Polymorphism

2.5.1 *Initial SSCP method*

Single-strand Conformational Polymorphism (SSCP) was chosen as the initial method to screen the pp1 fragment. This method relies on the ability of single-strand DNA to adopt a secondary conformation, by self-annealing, when run in a non-denaturing gel. Originally described by Orita *et al.*(Orita *et al.*, 1989) this method is able to detect changes down to the single nucleotide level, as even this small alteration in the nucleotide sequence will result in an altered conformation and thus an altered mobility within the gel.

To generate the pp1 fragment suitable for SSCP analysis, the basic PCR reaction (2.3.6) was carried out, with the addition of 3μCi [^{33}P]-dATP per reaction. Following confirmation, 8μl of product were digested with 1U of either *Hin*fI or *Mnl*I. 5 μl of digested PCR product were made up to 10μl with SSCP running buffer (10nM NaOH, 80% formamide, 1mM EDTA and 0.1% each BPB and XC) and denatured at 95 ^{0}C for 5 minutes, after which the sample was placed on ice for 5 minutes. Each sample was run at 45W for four hours on a standard 0.4mm 4.5% non-denaturing polyacrylamide gel (2.1.12) at 4^{0}C. Nondenatured, double-stranded DNA was also run as a control. The gel was dried without prior fixation and exposed for 3-5 days against Kodak film at room temperature.

2.5.2 *Modified method*

Initial experiments established that at least one SSCP-band difference existed between individuals. The original SSCP method was then modified to produce better definition and fewer bands: 6μCi of [^{33}P]-dATP were used per reaction; the PCR was purified with a Wizard DNA clean-up kit prior to digestion; samples are digested with *Mae* III to yield only four possible fragments (two of 119 bp, one of 161 bp and one of 201 bp) which are better suited to SSCP analysis; a commercially formulated 6% agarose gel specifically designed for SSCP was used (MDE Agarose, Anachem, UK); 15μl of PCR product were digested and 9μl of this run on the gel overnight (18 hours) at 5W at room temperature. Thereafter the gel was processed in the same way as previously.

2.6 Bacterial Culture

2.6.1 *Competent E. coli*

The bacteria used throughout this study were commercially prepared, competent *E. coli* ('One-Shot', Invitrogen, Netherlands), stored at -70^{0}C until required.

2.6.2 *Bacteriological media*

All reagents were obtained from Difco, UK.

LB broth	per litre
Bacto-tryptone	10g
Yeast extract	5g
Sodium chloride	10g
adjusted to pH 7.0 with 10M KOH	

LB-agar: LB broth plus Bacto-agar (10g/l)

The solutions were prepared in small aliquots, autoclaved and stored at room temperature until required. LB agar plates were prepared by adding the appropriate antibiotic (ampicillin or kanamycin, final concentrations 50mg/ml) and X-gal(1mg/ml) to the solution whilst warm, and then pouring 20ml per culture plate. The plates were allowed to solidify and were either used immediately or stored at 4^0C for up to 1 week. Alternatively, solidified LB-agar was remelted in a microwave, allowed to cool and then prepared as above.

2.6.3 *Glycerol and bacterial stocks*

Transformed bacteria remaining after an overnight culture for miniprep plasmid preparation were stored at -70^0C in 20% glycerol. Larger volumes of bacteria, remaining after midi- or maxiprep cultures, were pelleted in an SS-34 rotor at 6000 rpm for 15 minutes at 4^0C and stored at -20^0C. These stocks were used directly for plasmid preparation.

2.7 Plasmid Production

2.7.1 *Small-scale plasmid preparation - 'Miniprep'*

Small quantities of plasmid DNA (approximately 50µg) were prepared by the Wizard Miniprep DNA Purification system (Promega, UK) according to the manufacturer's protocol. 4ml of LB media containing ampicillin at 50mg/ml were incubated overnight in a rotary-shaking incubator at 37^0C. The inoculated bacteria originated from colonies grown from transformants, isolated on agar plates by antibiotic ± blue/white selection or colonies from glycerol stocks of previously identified single clones grown on antibiotic agar plates to ensure continued presence of the target plasmid. After overnight culture, bacteria were pelleted from 2.5ml of culture medium by centrifugation at 3000rpm for 5 minutes at 4^0C. The media was fully drained away and the cells resuspended in 200µl of Resuspension solution containing 50mM Tris-HCl, pH 7.5, 10mM EDTA and 100µg/ml RNase A.

The cells were lysed with 200µl of Lysis solution (0.2N NaOH and 1% SDS). Precipitation of protein, cell wall debris and genomic DNA was achieved by addition of 200µl Neutralisation solution (1.32M potassium acetate, pH 4.8).

The resulting solution was cleared by centrifugation at 14,000rpm for 5 minutes at room temperature. The cleared lysate was mixed with 1ml of Wizard DNA purification resin (containing 7M Guanidine HCL) and drawn into a minicolumn by vacuum. Plasmid DNA in the lysate bound to the resin, which in turn was bound to the minicolumn filter. The minicolumn was washed with 2ml of Column Wash solution containing 190mM potassium acetate, 20mM Tris-HCl, pH 7.5 and 1mM EDTA in 55% ethanol. In addition, the columns were washed with 2ml of 70% ethanol. The columns were cleared of wash solution by applying a vacuum to them for 30 seconds, by centrifugation at 14,000 rpm for 25 seconds and by allowing them to air dry for 10 minutes. The plasmid was then eluted in 50µl of SDW, passed through the column by centrifugation at 14,000 rpm for 25 seconds and stored at -20^0 C until required.

2.7.2 *Large-scale plasmid preparation - 'Maxiprep'*

In order to obtain sufficient quantities of high quality plasmid suitable for transfection, two methods were used:

2.7.2.1 Standard Alkaline Lysis Maxiprep

A loop of bacterial glycerol stock of the plasmid required was first plated out on an appropriate antibiotic agar plate and cultured overnight at 37^{0}C. A discrete colony was then picked and grown in 5ml of selective media (LB with ampicillin at 50mg/ml) for 6-8 hours at 37^{0}C. This starter culture was then added to 500ml of the same media and cultured overnight in a rotary shaking incubator at 37^{0}C.

The next day the cultures were centrifuged at 8000 rpm for 5 minutes in a GS3 rotor using a Sorvall RC 5C Plus centrifuge at 4^{0}C. The medium was removed, the pellet was resuspended in 100 ml of cold STE, and the centrifuge step repeated to ensure complete removal of medium. The bacterial pellet was resuspended in 18ml of cold Solution 1 (50mM glucose, 25mM Tris, pH 8.0 and 1mM EDTA, pH8.0). After the addition of 36ml of Solution 2 (0.2mM NaOH and 1% SDS) the suspension was incubated at room temperature for 10 minutes. 20ml of cold Solution 3 (60ml 5M KOAc, 11.5ml glacial acetic acid and 28.5ml

SDW) were then added, the mixture shaken to produce a floccular precipitate and then incubated on ice for 20 minutes. The sample was centrifuged in a SS34 rotor at 11,000 rpm for 15 minutes at 4°C to pellet the bacterial cell wall, protein and chromosomal DNA. The supernatant was transferred to a fresh bottle through a fine mesh to remove any remaining debris. The plasmid was precipitated by adding 0.6 volumes (44ml) of isopropanol, incubating for 10 minutes at room temperature and then centrifuging at 10,000 rpm for 15 minutes at room temperature in an SS34 rotor. The resulting plasmid was washed with 70% ethanol and allowed to dry.

The plasmid was resuspended in 8ml of TE with RNase (10μg/ml) and transferred to a plastic Corex tube, to which was added 8g of caesium chloride ($CsCl_2$) and 175μl of ethidium bromide (40 mg/ml). Once the $CsCl_2$ was fully dissolved the sample was centrifuged at 5000rpm for 5 minutes at 18°C to pellet any bacterial debris. The sample was transferred to an ultracentrifuge tube (Beckman Quick Seal, Beckman, USA) which was topped up with a solution of $CsCl_2$ in TE (1.05 g/ml). Paired tubes were balanced to within 0.01g and then centrifuged overnight (at least 14 hours) at 45,000 rpm in a Beckman L8-70M Ultracentrifuge at 20°C.

The band of closed, circular plasmid was recovered using a 14G needle and transferred to a fresh tube. This was topped up with $CsCl_2$ in TE (1.05g/ml) and recentrifuged overnight at 45,000 rpm as before. The 'double banded' closed circular plasmid was recovered and the ethidium bromide removed by extracting with water-saturated butanol. The sample was transferred to a fresh tube and the plasmid precipitated by adding 1 volume of water, 2 volumes of 1M ammonium acetate and ethanol to a final concentration of 70%. The plasmid DNA was spooled out of the solution, washed three times in fresh 70% ethanol, air dried and dissolved in TE.

2.7.2.2 Qiagen Midiprep Kit

The midiprep manufacture was carried out according to the manufacturer's protocol, with one exception. A 500 ml culture of the appropriate plasmid was prepared as described in 2.7.2.1. Bacteria from 100ml of media were harvested by centrifugation in the Sorvall RC Plus centrifuge using an SS34 rotor at 6000 rpm and 4°C for 15 minutes. The remaining 400ml of

bacterial culture were spun down, and the cells stored dry at -20^{0}C for future plasmid preparation. The medium from the harvested cells was completely removed and the pellet of cells resuspended in 4ml of buffer P1 (50 mM Tris-HCl, pH 8.8, 10 mM EDTA and 100 µg/ml RNase). The sample was then transferred to a polypropylene centrifugation tube. The bacteria were lysed by incubation for exactly 5 minutes (to minimise plasmid nicking) with 4ml of room temperature buffer P2 (200 mM NaOH and1% SDS). Bacterial protein, cell wall debris, SDS and genomic DNA were precipitated by incubating on ice for 15 minutes with 4ml of buffer P3 (3.0 M potassium acetate, pH 5.5). The lysate was cleared by centrifugation in the SS-34 rotor, at 13,000 rpm and 4^{0}C, for 30 minutes.

The Qiagen system purifies plasmid by selectively binding it to a resin packed into a plastic column. This column was equilibrated immediately prior to use by applying 4ml of buffer QBT (750 mM NaCl, 50 mM MOPS, pH7.0, 15% isopropanol and 0.15% Triton X-100). The cleared lysate was then applied to the column. The resin-bound plasmid within the column was washed free of any remaining contaminants (such as RNA fragments or protein) with 20 ml of medium-salt buffer QC (1.0 M NaCl, 50 mM MOPS, pH 7.0 and 15% isopropanol). The pure plasmid was then eluted from the column into a glass Corex tube, using a high-salt buffer QF (1.35M NaCl, 50 mM Tris-HCl, pH 8.5 and 15% isopropanol).

The DNA was precipitated by adding 0.7 volumes (3.5ml) of room temperature isopropanol, followed by centrifugation in an HB-4 swinging bucket rotor, at 10,000 rpm for 1 hour at 4^{0}C. (The 30 minutes recommended in the manufacturers protocol did not result in plasmid precipitation). Once precipitated, the plasmid was washed twice with 2 ml 70% ethanol, followed each time by a 20 minute centrifugation at 4^{0}C in the HB-4 rotor at 10,000 rpm. The plasmid was then air-dried in a fume cupboard (to minimise the risk of bacterial contamination) and redissolved in 2ml of sterile TE to ensure complete dissolution of plasmid precipitated along the sides of the tube and in its base. Once fully dissolved, each plasmid sample was reduced in volume by ethanol precipitation in aliquots, according to the protocol described in 2.1, and redissolved in a small volume of sterile TE to give a final total volume of 500µl. The plasmid concentration was calculated from its UV absorption at 260nm, as described in 2.7.3 below.

2.7.2.3 Wizard Midiprep Kit

This kit uses the identical reagents and protocol as the miniprep kit but is designed for bacterial cultures of 50-100ml. It yielded ~200µg of plasmid, suitable for ligation/sequencing but not for eukaryotic transfection.

2.7.3 *Spectrophotometric determination of DNA concentration*

A 1:100 dilution of the DNA sample to be quantified was made and the optical density (OD) for ultraviolet light at 260nm and 280nm was measured in a Spectrophotometer. An OD of 1 corresponds to a double-stranded DNA concentration of 50µg/ml (Sambrook *et al.*, 1987), thus the plasmid concentration was calculated for each sample. Comparing the OD_{260}/OD_{280} ratio provided a measure of the purity of the sample: a ratio significantly lower than 1.8 implied contamination with phenol or protein, so the sample underwent a further round of Phenol:Chloroform extraction and ethanol precipitation.

2.8 Cloning the pp1 fragment

2.8.1 *Introduction*

In order to obtain DNA of a quality high enough for it to be an efficient sequencing template it was necessary to produce a clone of the pp1 fragment in a plasmid vector, and then manufacture a large quantity of pure plasmid. The cloning of PCR products into a vector prior to further manipulation is now a well recognised procedure. During this study the pCRII vector (TA Cloning Kit, Invitrogen, Netherlands) was used. This vector is provided in a linearised form with cohesive ends, comprising a single deoxythymidine (dT) overhanging residue. This facilitates simple ligation of PCR products which, by virtue of the non-template-dependant activity of *Taq* polymerase, have a single overhanging deoxyadenosine (dA) residue present at their 3' end. These A overhangs degrade rapidly, so the use of fresh PCR product is important - in these experiments PCR products were ligated immediately after confirmation.

2.8.2 *Cloning into the pCRII vector*

A circle map of the pCRII vector is shown in Figure 2.2. Once ligated within the polylinker, the PCR fragment is flanked on either side by *Eco* R1 restriction sites. Sequences recognised by the M13 forward (-20 and -40) and reverse oligonucleotide primers are present for sequencing the ligated PCR fragment. Successful cloning of a PCR fragment disrupts the close association between the *lac* promoter and *lacZα* fragment, which lie on either side of the multicloning site, preventing the expression of β-galactosidase. This feature enables screening of positive clones by blue/white selection on agar containing X-gal (colonies of bacteria containing successfully ligated vector appear white or have very light blue centres). The vector also contains kanamycin and ampicillin resistance genes.

2.8.3 *Ligation Protocol*

Genomic DNA samples from normal control subjects and patients with S-JCA were used as templates to produce the pp1 fragment as described in 2.3.6. The presence of a PCR product of the correct size was established by electrophoresis as described in 2.3.4. The ligation protocol was carried out in accordance with the manufacturer's instructions.

A 1:1 (vector:insert) ratio was used, with the amount of PCR product required calculated by the following formula:

X ng PCR product = (Y bp PCR product)(50 ng pCRII vector)/3900*

(*the size of the TA vector).

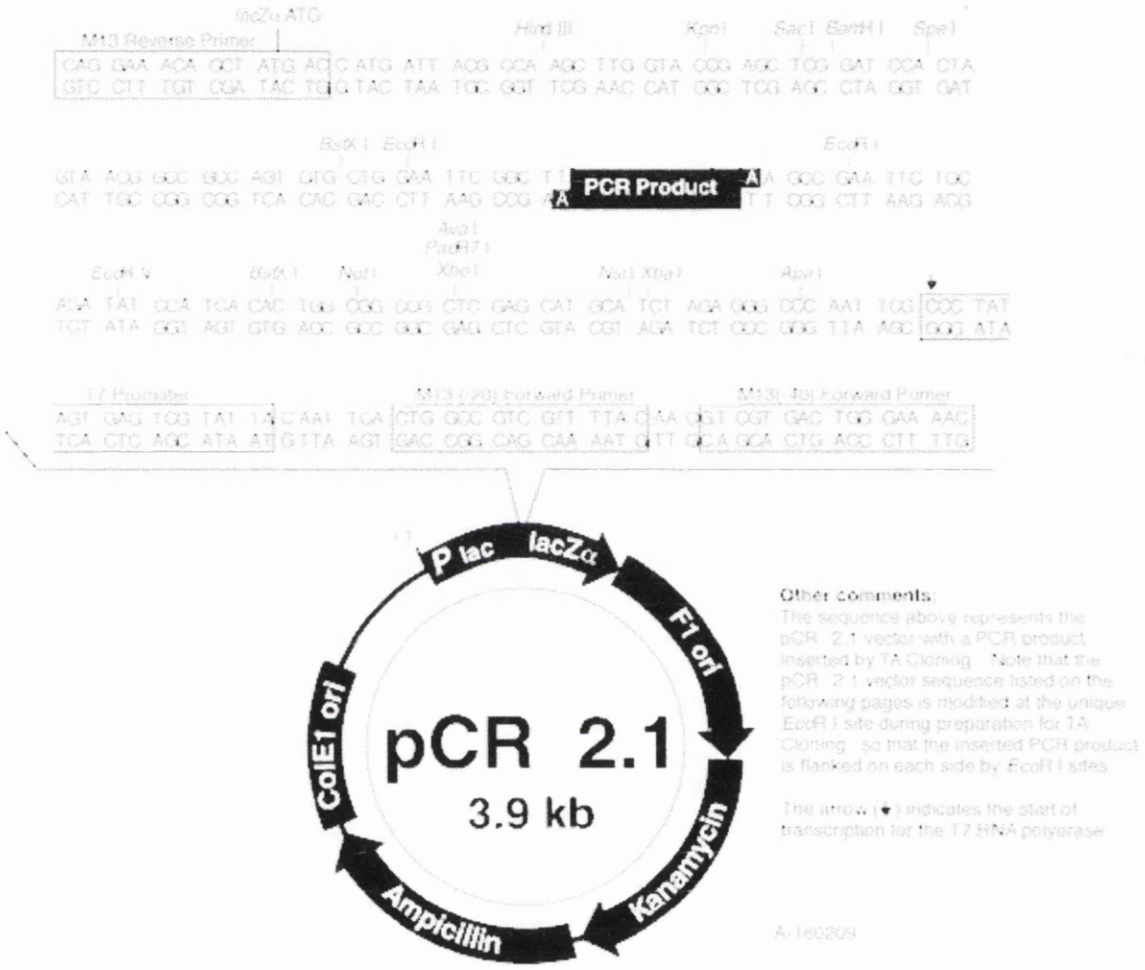


Figure 2.2 The pCRII vector circle map. This figure shows the PCR fragment insertion site, the positions of the restriction enzyme sites, the primer recognition sequences and the major components of the plasmid. (Reproduced from the company Web page).

X frequently approximated to 1µl of PCR product, the amount used in most of the 1:1 ligation reactions. Where a positive clone was difficult to achieve, an increased ratio was used, commonly 2.5 µl of PCR product. The PCR product was added to a 10µl solution containing:

4U T4 DNA ligase
6.0mM Tris-HCl, pH 7.5
6.0mM $MgCl_2$
5.0mM NaCl
0.1mg/ml bovine serum albumin
7.0mM β-mercaptoethanol
0.1mM ATP
2.0mM dithiothreitol
1.0mM spermidine
2µl linearised pCRII vector

Ligation was carried out overnight (for at least 14 hours) at 14°C. If not used the next day, the ligation was stored at -20°C.

2.8.4 *Transformation Protocol*

The competent *E. coli* strain used for all the transformation experiments in this project were commercially prepared INVαF'bacteria ('One-Shot' cells). The competent cells were available in 50µl aliquots, which were successfully used for five transformations each. Prior to dividing into five Eppendorf tubes, 2µl of 0.5M β-mercaptoethanol were gently mixed into the cell suspension. 1µl of the ligation mixture was then added to each tube of 10µl of competent cells, which were then incubated on ice for 30 minutes. The bacteria were heat-shocked by floatation in a 42°C water bath for 20 seconds before being returned to ice for a further 2 minutes. 450µl of SOC medium were then added to each tube, which was next incubated at 37°C for 1 hour in a shaking incubator (at 225 rpm). Depending on the success of previous cloning experiments for that ligation, between 50 and 250µl of the transformed bacterial

culture were plated onto agar plates containing 50μg/ml kanamycin and X-gal (1mg/ml). The plates were incubated for at least 18 hours in a 37^{0}C incubator. If only white colonies of a reasonable size had developed by this stage the plates were transferred to 4^{0}C to encourage proper colour development. The appearance of a light blue centre to a colony suggested partial disruption of β-galactosidase expression by the ligated PCR fragment.

On optimisation of this procedure, the test plasmid provided with the kit (pUC18, which contains the ampicillin resistance gene) was used to transform bacteria to test their efficiency. With each experimental ligation re-ligated plasmid (ie. pCRII with no PCR fragment in the ligation mixture) and sham-transformed bacteria were plated.

2.8.5 *Screening for positive clones*

Screening of candidate colonies was done by colony PCR (cPCR), followed by restriction enzyme digestion of plasmid produced by miniprep, which was then used for sequencing if the clone was correct.

2.8.5.1 Colony PCR

Colony PCR (cPCR) was carried out as described for the basic PCR reaction (2.3.6) with the following modifications: A candidate bacterial colony was picked from the agar plate and briefly dipped into a PCR tube containing all the reaction components apart from the *Taq* polymerase. A small quantity of bacterial material was allowed to float into the PCR mixture and the remaining colony material was placed in an Eppendorf tube containing LB medium with ampicillin at 50mg/ml and stored at 4^{0}C. 0.5U of *Taq* polymerase was then added to each PCR reaction, which then underwent the same thermocycling conditions as the basic PCR.

If the PCR produced a correctly sized band, the stored colony material was added to a further 3 ml of LB medium (with 50mg/ml ampicillin) and cultured overnight at 37^{0}C in a shaking incubator. Those colonies that yielded bands of incorrect size on cPCR were rejected. Where no band was visible the colony was still processed to the next stage, as on occasion the correct insert was present but could not be amplified by cPCR.

2.8.5.2 Mini-prep plasmid manufacture and restriction enzyme digestion

Following overnight culture of candidate colonies, 2.5 ml of bacterial culture were processed by the 'Wizard' Mini-prep Kit (2.7.1). This yielded an abundant amount of plasmid for further analysis. The remaining culture was stored as a glycerol stock at -70^0 C.

To confirm correct cloning, 0.5µl (approximately 250ng) of each plasmid was digested with the restriction enzymes *Aat*II (which linearises the plasmid through a single restriction site within the pp1 fragment) and *Eco*R1, two sites for which are present on either side of the ligated fragment. This later digest confirmed the size of the ligated PCR product. The digestions were carried out as detailed in 2.4. After digestion, the two reaction mixtures were run on an agarose gel, together with an uncut portion of the plasmid. When the result of restriction digestion was not clear, the plasmid was used as a template for PCR using primer pair 1 and the standard PCR reaction conditions (with 0.1µl of plasmid as template). The generation of a PCR product of correct size confirmed the presence of a correct ligation, although this did not exclude the presence of a concatemeric ligation. However, this was very unlikely given the nature of the cloning system.

TA cloning is bidirectional. Thus the orientation of the insert was determined prior to sequencing. A pair of *Xho*I sites, one at the 3' end of the fragment and the other within the plasmid polylinker, downstream of the insertion site, were utilised. *Xho*I digestions were carried out in parallel with the *Aat*II and *Eco*R1 digestions.

2.9 Sequencing the pp1 fragment

In view of the large number of clones to be sequenced, a rapid sequencing method was required. Initially a manual sequencing system, the 'Circumvent' Dideoxy DNA Thermal Cycle Sequencing (TCS) (New England Biolabs, Herts, UK), was used. Later on during this study, the Vistra Automated sequencing system became available, and was used to sequence the pp1 fragment both as a pCRII clone and directly from the PCR mixture.

2.9.1 *Thermocycle Sequencing of pCRII clones*

TCS utilises the properties of $Vent_R$ (exo^-) DNA polymerase, a highly thermostable DNA polymerase, to generate polynucleotides which are labeled by the addition of radioactive deoxyadenosine to the reaction mixture. Chain termination occurs following the random incorporation of a dideoxynucleotide during the chain elongation phase. The reactions are run in quadruplicate, with a different dideoxynucleotide in each tube. The reaction proceeds through multiple cycles, with an exponentially increasing number of molecules of each terminated chain, in a manner analogous to PCR. The sequence is then read as a ladder on a sequencing gel.

0.5µl of plasmid (250ng) was mixed with 1.3pmol of oligonucleotide primer, Sequencing buffer, 30X Triton-X100 solution, 2µl of fresh [α-^{33}P] dATP and 2 units of $Vent_R$ (exo^-) DNA polymerase. The final concentration of buffer in this 15 µl master solution was:

10 mM KCl
10 mM $(NH_4)_2SO_4$
20 mM Tris-HCl, pH 8.8
5 mM $MgSO_4$
0.2 % Triton-X100

3.2µl of this master solution was then distributed to four PCR tubes containing 3µl of Circumvent deoxy/dideoxy sequencing mixtures - A, C, G or T. The relative final concentrations (in µM) of each component of the mixes is given over the page:

	A mix	C mix	G mix	T mix
ddATP	900	-	-	-
ddCTP	-	420	-	-
ddGTP	-	-	360	-
ddTTP	-	-	-	720
dATP	30	30	30	30
dCTP	100	41	100	100
dGTP	100	100	37	100
dTTP	100	100	100	33

The samples were denatured for 20 seconds at 95^{0}C, annealed at a temperature appropriate for the particular primer (Table 2.1), and extended at 72^{0}C for 20 seconds. Twenty cycles were run, after which the reaction was stopped by adding 4µl of Stop solution containing 0.3% Xylene Cyanol FF, 0.3% Bromophenol Blue and 0.37% EDTA (pH 7.0), in deionized formamide. The samples were stored at -20^0 until used.

5'-TTC ACA CAG GAA ACA G-3' (M13 Reverse, within pCRII vector)	45^{0}C
5'-GTC AGA GGA AAC TCA GTT CAG-3' (DF3)	55^{0}C
5'-GTG CAT GAC TTC AGC TTT AC-3' (DR4)	55^{0}C
5'-GTG GTT CTG CTT CTT AGC-3' (DF5)	55^{0}C
5'-GGA CGT CAC ATT GCA C-3' (DF6)	55^{0}C
5'-GAG TCT CAA TAT TAG AGT CT-3' (DF7)	55^{0}C
5'-GTC GTG ACT GGG AAA AC-3' (M13 Forward, within pCRII vector)	50^{0}C
5'-CAG AAG AAC TCA GAT GAC TGG-3' (5/2)	56^{0}C
5'-GCT GGG CTC CTG GAG GGG-3' (3/2)	63^{0}C

Table 2.1 Sequence and sequencing reaction annealing temperature for oligonucleotide primers used to sequence the cloned pp1 fragment within the pCRII vector. The position of each primer within the pp1 fragment in shown in Appendix A.

To analyse the sequence, the reactions were run on a 0.4mm 8% polyacrylamide sequencing gel. Prior to loading each sample was denatured at 80^{0}C for 5 minutes then placed on ice. The samples were run at 40W for 4-8 hours on a Flowgen sequencing gel system. The gel was fixed for 5 minutes in solution of 40% glacial acetic acid/40% absolute ethanol before drying and then exposed to Kodak film for 1-2 days at room temperature with an enhancing screen.

The sequence was recorded manually on to a template containing the published sequence to allow rapid identification of sequence variations.

2.9.2 *Automated sequencing*

2.9.2.1 Introduction

The Vistra 725 automated DNA sequencer uses the properties of a fluorescent dye attached to the sequencing primer, to detect the passage of dideoxynucleotide-terminated DNA strands through a 5% polyacrylamide/6M urea sequencing gel. The system is based around a 2.5mW helium-neon laser generating light at 594.1nm in the yellow region of the visible spectrum. The laser passes through a transparent spacer and traverses the entire width of the sequencing gel 30 cm from the sample wells. The dideoxynucleotide-terminated DNA chains are illuminated as they pass through the laser because of the presence of Texas Red which is covalently coupled to the 5' end of the sequencing primer. This fluorochrome has an absorption maximum at 596nm and an emission maximum at 615nm. Fluorescence from the labeled DNA passes through a window behind the gel and is focused through a band-pass filter on to a 512-element photodiode array detector. The signal from the detector is digitised and transmitted to a Power Macintosh 7200/90 computer which controls the sequencer, using a serial interface.

Once the sequencing run was completed the data were down loaded from the controlling computer via an Internet link to a Power Macintosh in the department office, where the data were analysed. Each sequence reaction lane was identified manually and then the computer calculated the base sequence ('base calling routine') from the pattern and

sequence of peaks of fluorescence in each of the four lanes (A, C, G and T) run for each sample. It automatically corrected for 'smiling', peak space variations and compressions. In addition, the peak profiles were enhanced to resolve partially 'buried' peaks. The data was displayed as a fluorogram image of the entire gel (analogous to the standard sequencing gel appearance) and individual tracks showing both the actual fluorogram image of each band and its corresponding fluorescence peak. The computer calculated base sequence was displayed above the peaks.

During this work, all the sequences were analysed semi-automatically and displayed on the computer screen. Each sequence was then read and checked by manually comparing the computer sequence to the band fluorogram image and fluorescence peaks. These data were then added onto a template of the known sequence (as done for thermocycle sequencing).

2.9.2.2 Sequencing reaction

The sequencing reactions were carried out in accordance with the manufacturer's protocol (Vistra Thermo Sequenase RPN 2444, Amersham, UK). The reaction components were supplied in a pre-mixed form, with the correct ratio of deoxy- to dideoxynucleotide in each tube, which also contained the correct amount of 'Thermo Sequenase', an exonuclease-free heat stable DNA polymerase derived from T7 DNA polymerase. The mixtures also contained Tris-HCl (pH9.5), magnesium chloride, Tween20, Nonidet P-40, 2-mercaptoethanol, thermostable pyrophosphatase, and 7-deaza-dGTP to eliminate compression artefacts. The concentrations of each component of the pre-mix are not available from the manufacturer.

For the sequencing reaction, 2pmol of each Texas Red primer (1µl) were mixed with 200fmol of template: for pCRII plasmid this was equivalent to 1µl of a 1:10 dilution of the miniprep; for the PCR this varied between 2µl and 4µl of a 1:5 direct dilution of the PCR. These components were made up to 25µl with SDW. 6µl of this mixture were then placed into each of 4 PCR tubes, to which were added 2µl of the A, C, G or T pre-mix. The samples were covered with sterile mineral oil and thermocycled in the Perkin-Elmer thermocycler as follows:

a) 94^{0}C for 3 minutes,

b) 94^{0}C for 30 seconds,

c) annealing for 15 seconds (see below),

d) 72^{0}C for 30 seconds

e) steps b-d repeated 25 times,

f) 4^{0}C soak.

Annealing temperatures and primer sequences:

A) pCRII Plasmid template sequencing: this utilised the M13 recognition sequences within the pCRII plasmid on either side of the cloned fragment:

M13- (Reverse)	5'-GGA AAC AGC TAT GAC CAT G-3'	50^{0}C
M13+ (Forward)	5'-GTA AAA CGA CGG CCA GT-3'	50^{0}C

B) Direct sequencing of PCR product: Sequencing primers were nested within the original PCR primers 5/2 (DF13 at the 5' end) and 3/2 (DF14 at the 3' end):

DF13	5'-CCT TCT TCA TAA TCC AGG-3	51^{0}C
DF14	5'-GAG CTT CTC TTT GCT TCC-3'	50^{0}C

The reaction was terminated by adding 3µl of stop solution containing 90% (v/v) formamide and loading dye. The sample was recovered free from mineral oil by running it down a slope of Nescofilm, to which the mineral oil adheres. The sample was reduced to 3µl by placing it on a heated block in a vacuum desiccator before loading onto the sequencing gel.

The sequencing gel was prepared as described earlier (2.1.14). Once fully polymerised, the gel was loaded into the sequencer, running with TBE buffer. The gel was pre-run at 1000V for 15 minutes, during which time any potential problems with the gel, such as fluorescent dust on the plates over the photodiode window, were identified and corrected.

The samples are not denatured prior to loading, as the products were single-strand and in vast excess to the template. The sequencer was run at 1400V, 0-30 mA with data collection

starting at 55 minutes into the run. The 614 bases of interest were all read by 4 hours.

2.10 Screening the Control and SA Populations for the -174 Polymorphism

The control and S-JCA populations were genotyped for the -174 polymorphism by RFLP analysis of genomic PCR products generated with primer pair 1 (2.3.6). When repeated attempts at generating a pp1 fragment failed (possibly as a result of the poor quality of some of the oldest DNA samples), the use of primer pair 2 with their specific conditions was often successful. This fragment was processed in an identical manner to the pp1 fragment. After confirming the presence of a correct, single PCR product, 0.5U of *Sfa*N1 was mixed directly with 2.5-10µl of the reaction, in a total volume of 10-25µl, including 1X restriction enzyme buffer. Larger volumes of the PCR mixture were required when the reaction produced only a low concentration of product.

Following restriction at 37^{0}C for 2.5-3 hours the samples were denatured at 65^{0}C for 5 minutes and then placed on ice for 5 minutes before electrophoresis of the entire sample on a 1% agarose gel. Digested samples were run alongside a sample of equal volume, containing the same concentration of PCR product, which had been processed in an identical manner to the digested sample, but which did not contain the restriction enzyme.

The method was validated by comparison of the first dozen samples with their previously obtained sequence. In all cases the genotypes were read directly from the gel picture and ambiguous cases were repeated.

2.11 Production of reporter constructs for functional studies

2.11.1 *Luciferase Vectors*

The luciferase reporter vector is one of the commonest systems currently used to investigate the potential promoter or enhancer activity of a DNA sequence. The vectors used in this study were the pGL2-Control (containing the SV40 promoter and enhancer sequences, resulting in strong expression of luciferase activity), pGL2-Basic (lacking any eukaryotic promoter and

enhancer sequences, therefore ideal for assessing the promoter activity of a ligated sequence) and pGL3-Basic (also lacking any promoter or enhancer sequences) vectors (Promega, Maddison, WI. USA) (Figure 2.3). pGL2-Control vectors were used in the initial assessment of transfection into THP-1 cells. The luciferase enzyme produced by these vectors is encoded by the *luc* gene (or the *luc*+ gene, in the case of pGL3) of the firefly *Photinus pyralis*. The enzyme is a monomer of 60.7 kDa which catalyses the oxidation of beetle D-luciferin using ATP in the presence of oxygen and Mg^{2+}, with the concomitant production of a photon. The incorporation of coenzyme A into the reaction allows oxidation to occur through the intermediary luciferyl-CoA, which produces a long, sustained light reaction. The reaction is quantitated in a luminometer, the intensity of light emitted is proportional to the amount of luciferase reporter activity in the sample. This in turn is a function of the transcriptional activity of the *luc* gene, driven by the promoter under investigation, in this case the two different alleles of the IL-6 5' flanking region. At the time when this method was changed in favour of calcium-phosphate transfection into HeLa cells, the pGL3 vector had become available. This is a development of the pGL2 vector and contains a modification of the *luc* gene (*luc*+), which reduces the risk of the *luc* gene itself contributing spurious transcriptional start signals. Modifications to the vector backbone were also made, to achieve a greater efficiency of luciferase translation initiation. The pGL3 vector lacks any eukaryotic promoter or enhancer elements, hence the expression of luciferase is dependent on the sequence inserted upstream of *luc*+ functioning as a promoter.

2.11.2 *Subcloning from pCRII into pGL2*

1µg of pCRII plasmid was digested with 0.5U each of *Sac*I and *Xho*I to yield a portion of the plasmid containing the pp1 fragment, with short flanking sections of residual pCRII polylinker. The fragment was isolated from the plasmid by electrophoresing the restriction reaction mixture on a 1% agarose gel, removing the band containing the fragment by UV visualisation, and purifying it from the agarose with the Wizard clean up kit (2.3.5).

5µl of a midiprep (2.7.2.3) of the pGL2-Basic vector were digested with 1.0U each *Sac*I and *Nhe*I as standard and the linearised vector purified by agarose gel electrophoresis and Wizard purification (2.3.5). 0.5-1.5µl of the subcloned pp1 fragment was ligated with 1µl

of the purified, linearised vector and competent *E. coli* were transformed, both as described above. Colonies were screened for the presence of correct clones by cPCR using PCR primers specific for the pGL2 plasmid (GL1: 5'-TGT ATC TTA TGG TAC TTG TAA CTG-3'; and GL2: 5'-CTT TAT GTTT TTG GCG TCT TCC A-3'. The annealing phase of the cPCR was at 60^{0}C, otherwise the reaction parameters were the same as for the basic pp1 PCR given in 2.3.6.) (Figure 2.4, panel A). Further confirmation was made by restriction digestion of an aliquot of the plasmid, amplified by the Wizard miniprep method, with *Aat*II (which cuts only within the ligated PCR fragment) and with *Kpn*I plus *Xba*I, which remove a section of the plasmid containing most of the cloned insert. (Figure 2.4, panel B).

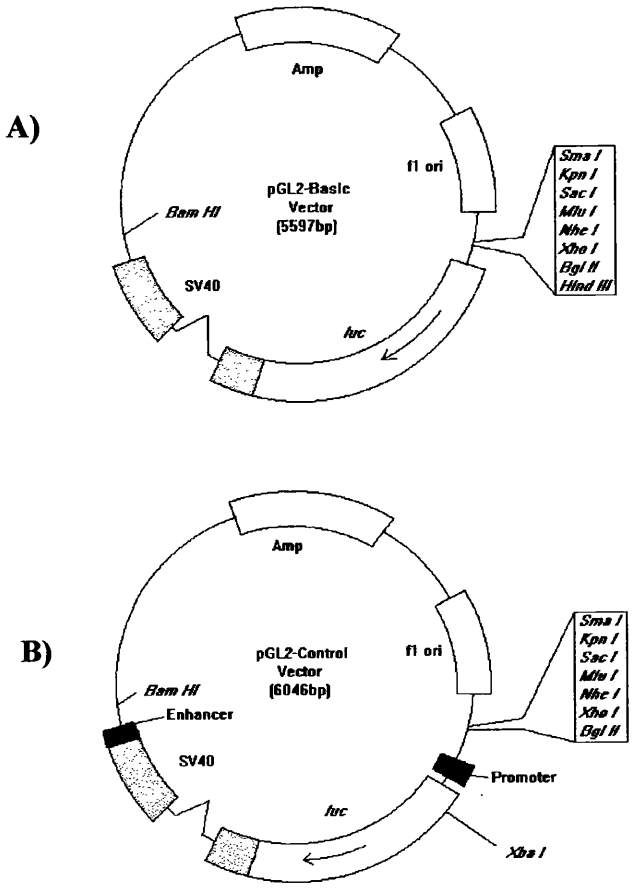


Figure 2.3 Circle maps of the Promega luciferase vectors used in this thesis. A) pGL2-Basic, B) pGL2-Control, and (on the following page) C) pGL3-Basic. The diagrams show the major components of the vectors, including the location of restriction enzyme sites.

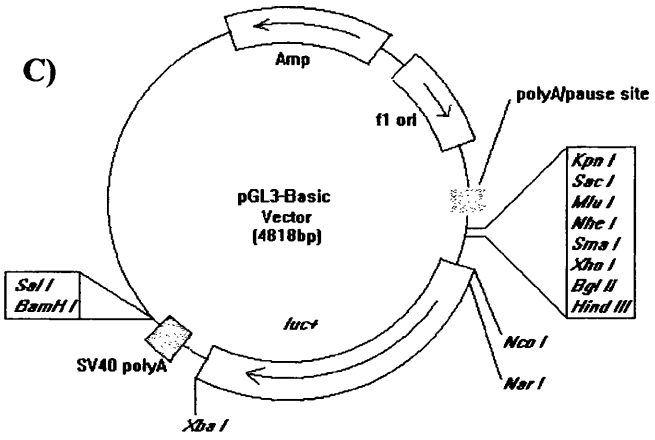


Figure 2.3 (continued) Circle map of the pGL3-Basic luciferase vector. The diagram shows the major components of the vector, including the location of restriction enzyme sites.

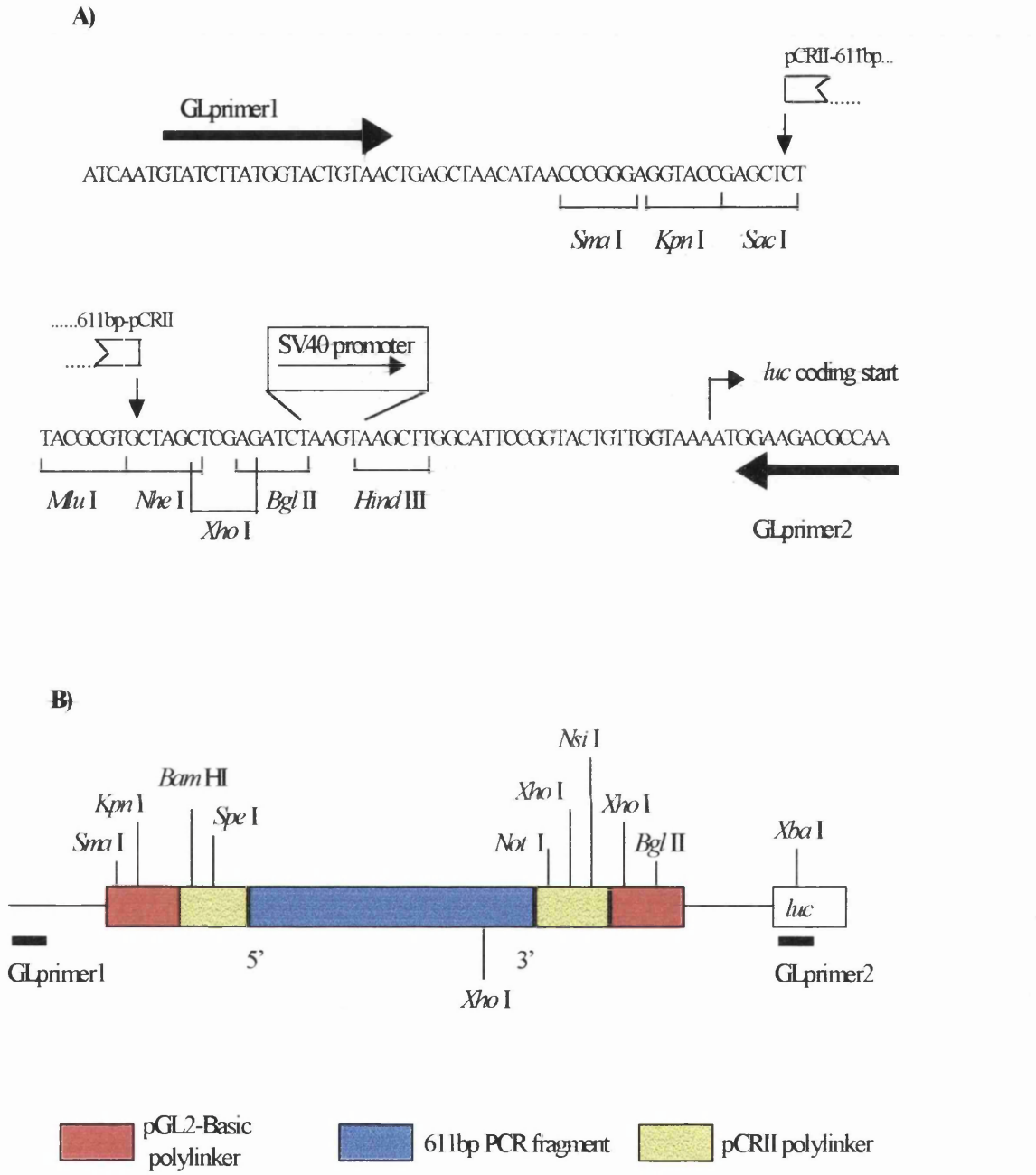


Figure 2.4 Schematic representation of the pGL2-Basic polylinker containing the pp1 fragment, subcloned from the pCRII plasmid. A) The location of the GL1 and GL2 cPCR primers and the polylinker restriction enzyme sites. B) The restriction enzyme sites within the subcloned sequence.

2.11.3 *Directional cloning of PCR products into pGL3*

A second protocol was developed to obviate the requirement for subcloning the pp1 fragment from the pCRII construct into the newer pGL3 plasmid. This method also ensured that the PCR product was ligated into the pGL3 luciferase vector in the correct orientation. PCR primers were designed that contained the restriction sites for *Kpn*I in the 5' end of the upstream primer (DF5/2D) and *Nhe*I in the 3' end of the downstream primer (DF3/2D). The primers were otherwise identical to the original 5/2 and 3/2 primers. Thus, the subsequent PCR product derived from an identical region of the IL-6 5' flanking region as previously examined (the pp1 fragment). Shorter fragments which lacked specific domains of the 5' flanking region were generated using additional directional primers located further downstream, with DF3/2D These were also ligated into pGL3-Basic and used in transient transfection experiments. The sequence of each of these directional primers is given in Table 2.2 below. A schematic of the size and composition of the derived PCR fragment is shown in the Results section, Figure 3.34.

DF5/2D	5'-**TATGCGAGGTAC***CAGAAGAACTCAGATGACTGG-3'
DF3/2D	5'-**AGTTAAT$^{\nabla}$CTAGATATGCTGGGCTCCTGGAGGGG-3'**
DFΔAT	5'-**ACTGGTGGTAC***GTCAAGACATGCCAAAGTG-3'
DF5/2DS	5'-**GACCTGTAGGTAC***CTCAATGACGACCTAAGCTG-3'
DFΔATGC	5'-**ACTGGTGGTAC***CATGCTAAAGGACGTCACATTG-3'

pGL3-Basic multicloning site:

5'....GGTAC*C.....TG$^{\nabla}$CTAGCTC....3'

Table 2.2 The sequences of the primers used for directional cloning of the full pp1 fragment and deletion length mutants. The portions of each primer not complementary to the genomic sequence (and containing the restriction site) is shown in **bold** type. *- *Kpn*I restriction site; $^{\nabla}$- *Nhe*I and *Xba*I restriction sites.

PCR was carried out as described in 2.3, with the exception that the annealing temperature used was 67^0C. The templates used for PCR were pCRII plasmids of known sequence. All had the A_8T_{12} tract, in order to ensure that the G and C constructs were directly comparable. These are notated: G(A_8T_{12}) and C(A_8T_{12}). 1µl of a 1:10 dilution of the original miniprep stock was used in the PCR reaction. Correct amplification was confirmed by agarose gel electrophoresis of a 5µl aliquot of the PCR reaction. To remove any residual *Taq* polymerase, the entire PCR sample underwent Phenol:Chloroform extraction and ethanol precipitation. The PCR product was resuspended in 30µl of SDW and 5µl run on a gel to confirm the fragment had not been lost. The fragments were prepared for ligation into the pGL3-Basic vector, previously linearised with *Kpn*I and *Nhe*I, by digestion with 0.5U each of *Kpn*I and *Xba*I. 5µl of a midiprep (2.7.2.3) of the pGL3-Basic vector were digested with *Kpn*I and *Nhe*I as standard and the linearised vector purified by agarose gel electrophoresis and Wizard purification (2.3.5). 0.5-1.5µl of the digested PCR product was ligated with 1µl of the purified, linearised vector and competent *E. coli* were transformed, both as described above. Colonies were screened for the presence of correct clones by cPCR using the original PCR primers. Further confirmation was made by restriction digestion of an aliquot of the plasmid, amplified by the Wizard miniprep method, with *Aat*II (which cuts only within the ligated PCR fragment) and with *Not*I plus *Bgl*I.

Once the constructs were confirmed to be correct they were amplified to produce large quantities of transfection-grade DNA by the standard alkaline lysis maxiprep and Qiagen Midiprep methods. Prior to transfection all the constructs were sequenced using the Vistra system as a final confirmation of their content.

2.12 Tissue Culture

All tissue culture was undertaken in a dedicated tissue culture laboratory, using full sterile technique in a Class 1 hood. All tissue culture media components were filtered through a 0.2μm filter prior to use. An aliquot of freshly prepared media was cultured on each occasion to exclude infection and unused media was stored at 4^0C for no longer than 1 week. Cellular viability was estimated by Trypan Blue exclusion.

THP-1 cells were cultured at 37^0C in a 5% CO_2 enriched atmosphere in vertical T75 flasks in 10~30ml of RPMI 1640 culture media (Sigma, UK), containing 10% heat-inactivated foetal calf serum, $5x10^{-5}$M β-mercaptoethanol, 10mmol glutamine, non-essential amino acids and penicillin (50U/ml)/streptomycin (50μg/ml) (THP-1 'complete media'). Cells were split every 3 days by removing 3ml of cells, washing them in fresh RPMI and diluting them in a further 7ml of fresh RPMI.

HeLa cells were cultured at 37^0C in a 5% CO_2 enriched atmosphere in horizontal T75 flasks in 15ml Dulbeccos Modified Eagles Medium (GibcoBRL,UK), supplemented with 10% heat-inactivated foetal calf serum, non-essential amino acids and penicillin (50U/ml)/streptomycin (50μg/ml) (HeLa 'complete media'). The culture was split 1:5 every 3 days. To achieve this the media was removed and the adherent cells washed twice with 5ml of Hanks Balanced salt solution (Sigma). The cells were loosened from the flask by incubating for 2 minutes in 2ml trypsin/EDTA solution. The suspended cells were diluted with 8ml of fresh complete media, then pelleted by centrifugation at 100rpm for 5 minutes. The pelleted cells were resuspended in 10 ml of complete media, and 2ml of this were added to 13ml of complete media in the T75 flask. The cells were usually 95% confluent again within 3 days.

2.13 Transient Transfection

2.13.1.1 Transfection protocol: THP-1 Cells

A protocol for the electroporation of undifferentiated THP-1 cells was developed, based on the method of Pahl *et al.* (1991). Cells within log phase were harvested, washed, pooled and then resuspended in serum-free RPMI media at 4 x 10^7 /ml. 0.5ml of cell suspension was placed in a 0.4cm electroporation cuvette, to which was added up to 60µg of pGL2-Control plasmid, diluted in 50µl of RPMI. After incubation at room temperature for 5 minutes, the sample was electroporated at 960µF, between 220 and 350V, by a Gene Pulser (Bio-Rad, UK). The samples were immediately placed on ice for 15 minutes, after which they were incubated in 10ml of fresh complete media at 37^{0}C in a 5% CO_2 -enriched atmosphere for 6 hours. The cells were then harvested, washed in PBS, and assessed for luciferase activity following lysis for 15 minutes in 125µl of lysis solution. Because a satisfactory protocol for transfecting the pGL2-Control vector into THP-1 cells could not be developed, no transfections using the pGL2-Basic constructs containing the -174 alleles were made.

Alternatively, 4 x 10^5 of THP-1 cells were differentiated to become adherent by culturing in 6 well plates for 72 hours, in the presence of 50mg/ml of vitamin D_3. Once confluent the cells were transfected with 20µg of pGL2-Control plasmid by the calcium phosphate/DNA precipitation method, exactly as described below for HeLa cells (2.13.1.2).

2.13.1.2 Transfection protocol: HeLa Cells

When several flasks of HeLa cells had achieved 95% confluence they were harvested, pooled and the cells resuspended at a concentration of 2 x 10^5/ml. 2ml of this suspension were added to each well of a six well plate and the cells grown to 95% confluence over 2 days. On the day of transfection, the media was replaced with 2ml of fresh media and 4 hours later the cells were transfected - in duplicate - by the calcium phosphate/DNA precipitation method: 10µg of each reporter plasmid, and 3µg of transfection control plasmid containing the β-galactosidase gene (RSV-β-gal, which lacks a cytokine responsive promoter, see 2.13.5

below), were diluted to a final volume of 175µl in TE and mixed with 25µl of 2M $CaCl_2$. This was added dropwise to 200µl of 2X HBS, pH 7.12 and mixed by bubbling with air from the pastette. The DNA precipitate solution was incubated at room temperature for 30 minutes before being added dropwise onto the HeLa cells. After 18 hours incubation the medium was replaced and the cells stimulated with either IL-1 (10U/ml) or LPS (10µg/ml) in fresh, complete media. After a further 24 hours the medium was removed with a suction pipette and the cells washed twice with 2ml of phosphate buffered saline. This was completely removed and 250µl of 1X cell lysis buffer were added for 30 minutes, after which the cells were harvested with a plastic cell lifter. The lysate was transferred to a 1.5ml centrifuge tube and frozen at -70^{0}C for 30 minutes. This single freeze-thaw cycle improves the luciferase yield. Before being assayed for reporter activity the lysate was warmed to room temperature and cleared by centrifugation at 14,000 rpm for 30 seconds.

2.13.2 *Luciferase assay*

A 20µl aliquot of cleared, room temperature lysate (all 125µl for the THP-1 cells) was mixed with 100µl (200µl for the THP-1 cells) of room temperature Luciferase Assay Reagent (Promega, UK) and placed in a BioOrbit 1253 luminometer (BioOrbit, Finland). After a 2 second delay the luminescence was read for 5 seconds. This was repeated 5 times and the average reading recorded. Background luminescence was subtracted from the readings by the luminometer.

2.13.3 *RSV β-gal transfection control plasmid and the β-Galactosidase assay*

The use of a control gene vector to normalise for transfection efficiency and cell lysate recovery between samples is common. In these experiments a vector based on pKS containing the β-galactosidase gene (*lacZ*), under the control of the RSV promoter, was used (the RSV β-gal plasmid). A full circle map of the vector was not available, as it was originally a gift from another lab (Dr G. Nabel, University of Michigan Medical Centre, Ann Arbor, USA). The RSV promoter does not respond to the cytokines used to stimulate reporter gene transcription in these experiments. Therefore β-galactosidase production should be

comparable between samples.

The Promega cell lysis buffer was suitable for β-galactosidase estimation as well as luciferase. Thus, a 20μl aliquot of the cleared, room temperature lysate was placed in a luminometer tube, and mixed with 200μl of β-galactosidase reaction buffer (GalactoLight Plus, Tropix, USA). After exactly 1 hour incubation at room temperature 300μl of light emission accelerator were added. The luminescence was measured after a 5 second delay for 5 seconds. The average luminescence of 5 readings, minus background, was recorded. Correction for variations in transfection efficiency and cell lysate recovery was achieved by dividing the luciferase luminescence value by that for β-galactosidase.

2.14 Data collection and Statistical Methods

The sequence of each PCR product examined was recorded on to a template to rapidly identify differences from the published sequence.

Genotyping data were collated and analysed by the χ^2 test, using Yates' correction. A p value of <0.05 was considered to be significant. The differences in reporter gene expression between constructs were compared using the t-test. Statistical analysis was carried out with the Arcus program running on a PC.

CHAPTER 3

RESULTS

3.1 Investigation of the IL-6 5' Flanking Region by Single-strand Conformational Polymorphism

The experiments carried out at the start of this study demonstrated differences in the SSCP banding pattern between normal control samples. (Figure 3.1 and 3.2). The method was subsequently refined to reduce the number of bands on the gel by purifying the pp1 fragment from any possible contaminating PCR fragments prior to restriction. In addition the restriction enzyme was changed to *Mae*III, which produced 4 fragments (two of 119bp, one of 150bp and one of 223bp) as compared to the 7 from *Hin*f1 and 8 from *Mnl*I (Figure 3.3). An increased concentration of isotope in the PCR also helped to increase the SSCP band definition. With this modified protocol it was possible to confirm that significant inter-individual differences in SSCP banding patterns occurred in several regions of the gel (Figure 3.4). These features were highly suggestive of the presence of variations in the nucleotide sequence of the pp1 fragment. However it was not possible to predict where these were located, as the identity of a restriction digest product cannot be inferred from its position on an SSCP gel. Although SSCP is a powerful screening method for both unknown and recognised polymorphisms, to confirm the presence of a new polymorphism, and to exclude other undetected sequence differences between individuals, the region required formal sequencing.

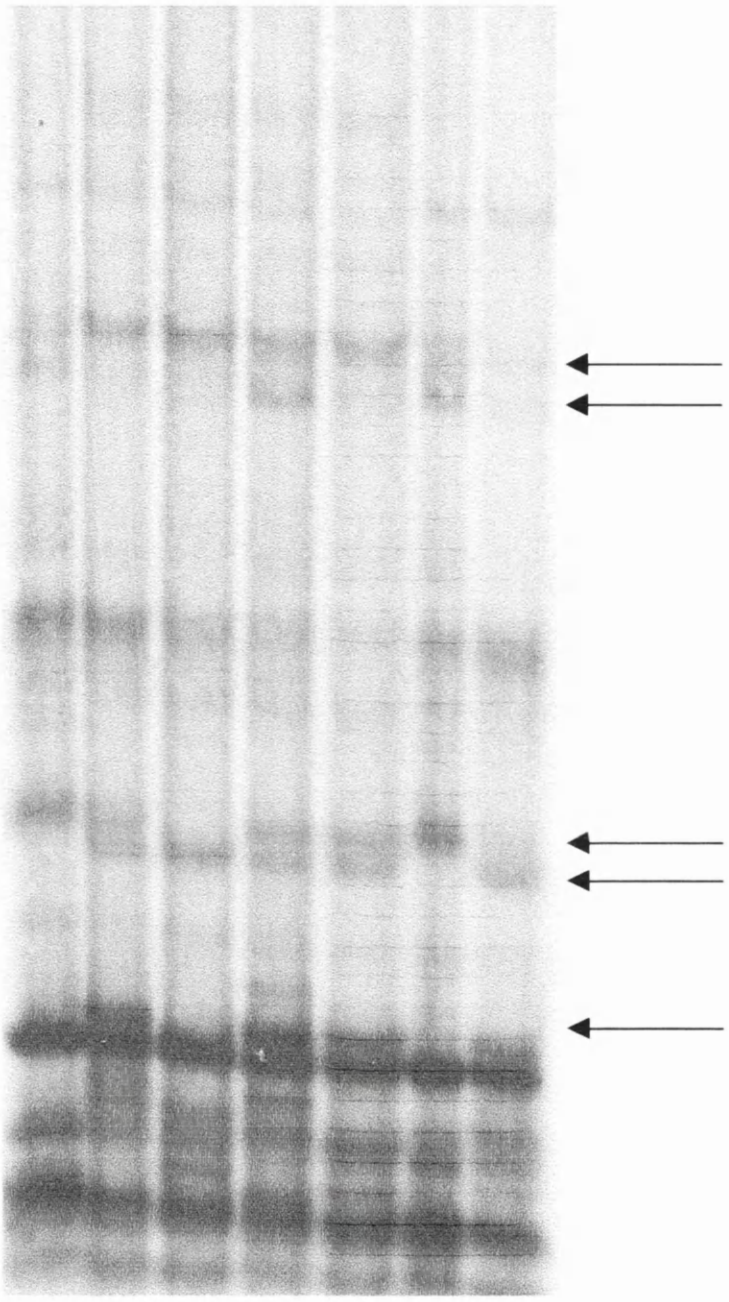


Figure 3.1 SSCP autoradiograph of samples processed using the initial SSCP method- a) *Hin*FI digested fragments. The regions where the band pattern differs between individuals (each lane represents a different individual) are indicated by arrows.

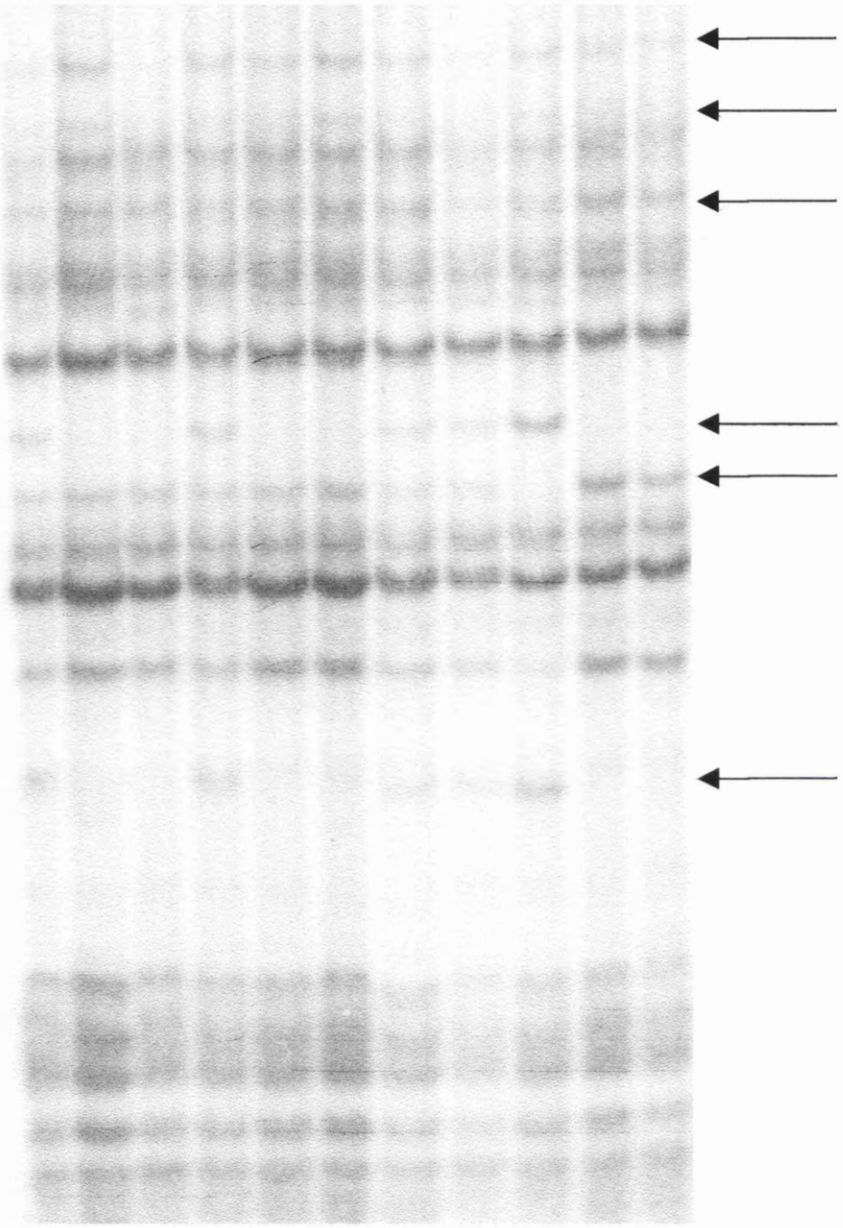


Figure 3.2 SSCP autoradiograph showing samples processed using the initial SSCP method- b) *Mnl*I digested fragments. The regions where the band pattern differs between individuals (each lane represents a different individual) are indicated by arrows.

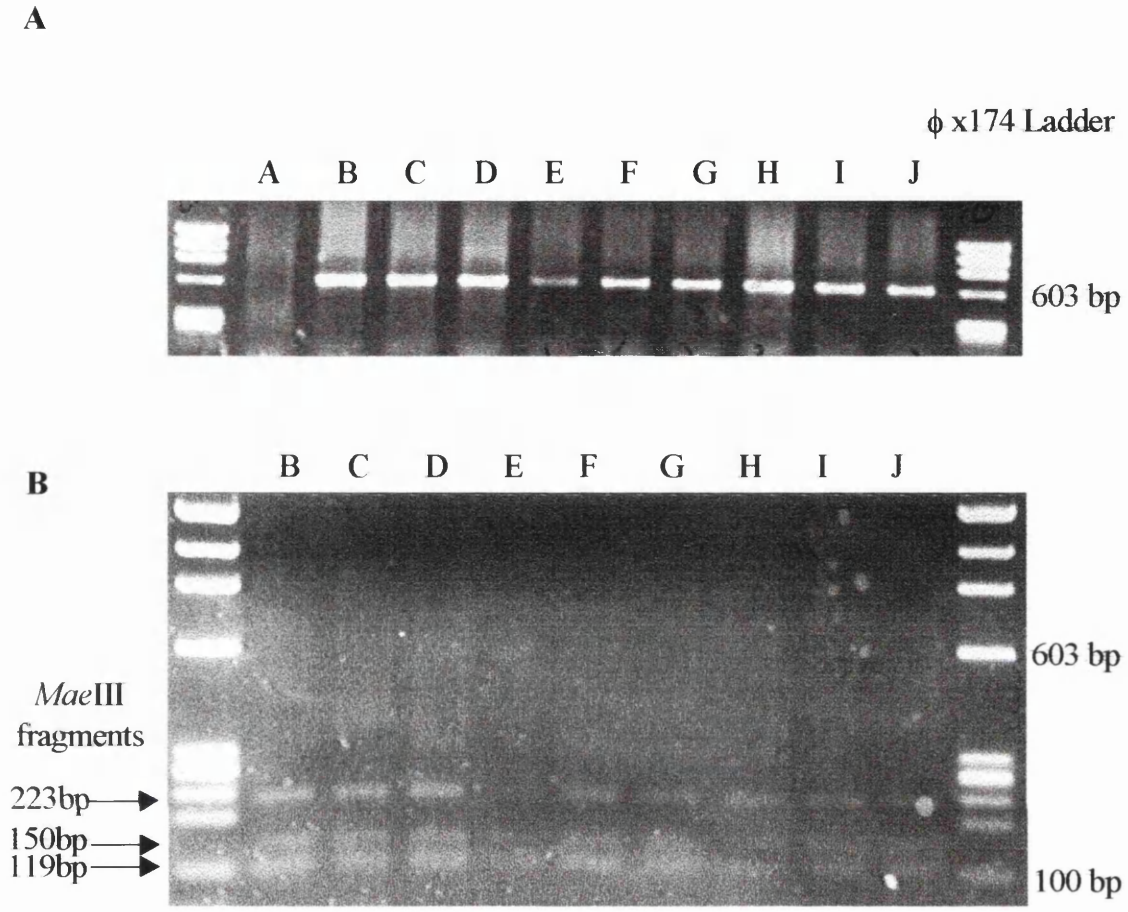


Figure 3.3 PP1 PCR products from different individuals prior to SSCP: A) Aliquots of each sample run on a 1% agarose gel to confirm correct amplification; B) Aliquots of each sample run on a 1% agarose gel to confirm complete *Mae*III digestion.

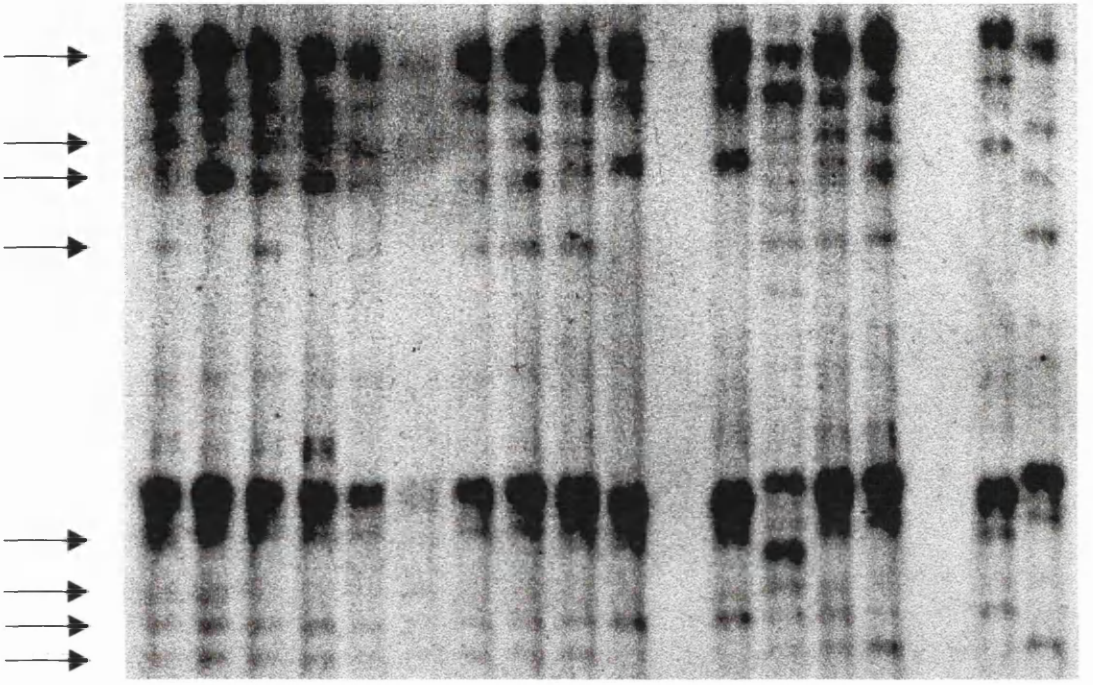


Figure 3.4 SSCP autoradiograph of samples processed using the modified method, including *Mae*III digestion. Arrows indicate the regions where the band pattern differs between individuals.

3.2 Sequence Analysis of the IL-6 5' Flanking Region

In all the sequencing studies, it was the fragment amplified by primer pair 1 (the pp1 fragment; the primer sequence is given in 2.3.6) of the IL-6 5' flanking region (predicted to be 611bp), that was analysed. In order to obtain reasonable concentrations of DNA of a purity suitable for it to be used as a sequencing template, it was at first necessary to clone the pp1 fragment into a plasmid vector, which was then grown in *E. coli*. At a later stage in this work an automated sequencer became available, in addition to a Perkin-Elmer PCR machine. Using this equipment, very satisfactory sequences were obtained from both the cloned fragment and also directly from PCR products obtained from both plasmid and genomic DNA templates.

3.2.1 *Cloning results*

Using the previously described methods, 57 plasmids containing the pp1 fragment were constructed. The genomic DNA source of these fragments were normal controls (n=29), patients with S-JCA (n=13) and also some patients with other forms of JCA (seropositive RA n=2; pauciarticular n=4; polyarticular n=2; unclassified JCA n=7). Although it was not originally intended that patients with JCA sub-types other than systemic were to be included, it was decided that examination of a few subjects was appropriate in case a major sequence difference was detected.

Figure 3.5 shows examples of pp1 (and pp2) PCR products generated from genomic DNA stocks which were used for cloning, and as sequencing templates. Samples frequently failed to amplify with pp1 the primers, in which case pp2 (as described in 2.3.6) were used, but on several occasions a sample could not be amplified despite alterations in all of the PCR parameters. This was most frequently the case for the older S-JCA patient and Northwick Park Hospital control samples.

Confirmation of correct pp1 cloning into the pCRII plasmid was initially achieved by cPCR on the colonies grown after transformation, followed by restriction enzyme digestion of the subsequent miniprep with *Aat*II and *Eco*R1 (Figure 3.6), and then *Xho*1 (which demonstrates the orientation of the fragment) (Figure 3.7). Finally, the pCRII miniprep plasmids were used as a template for the pp1 PCR.

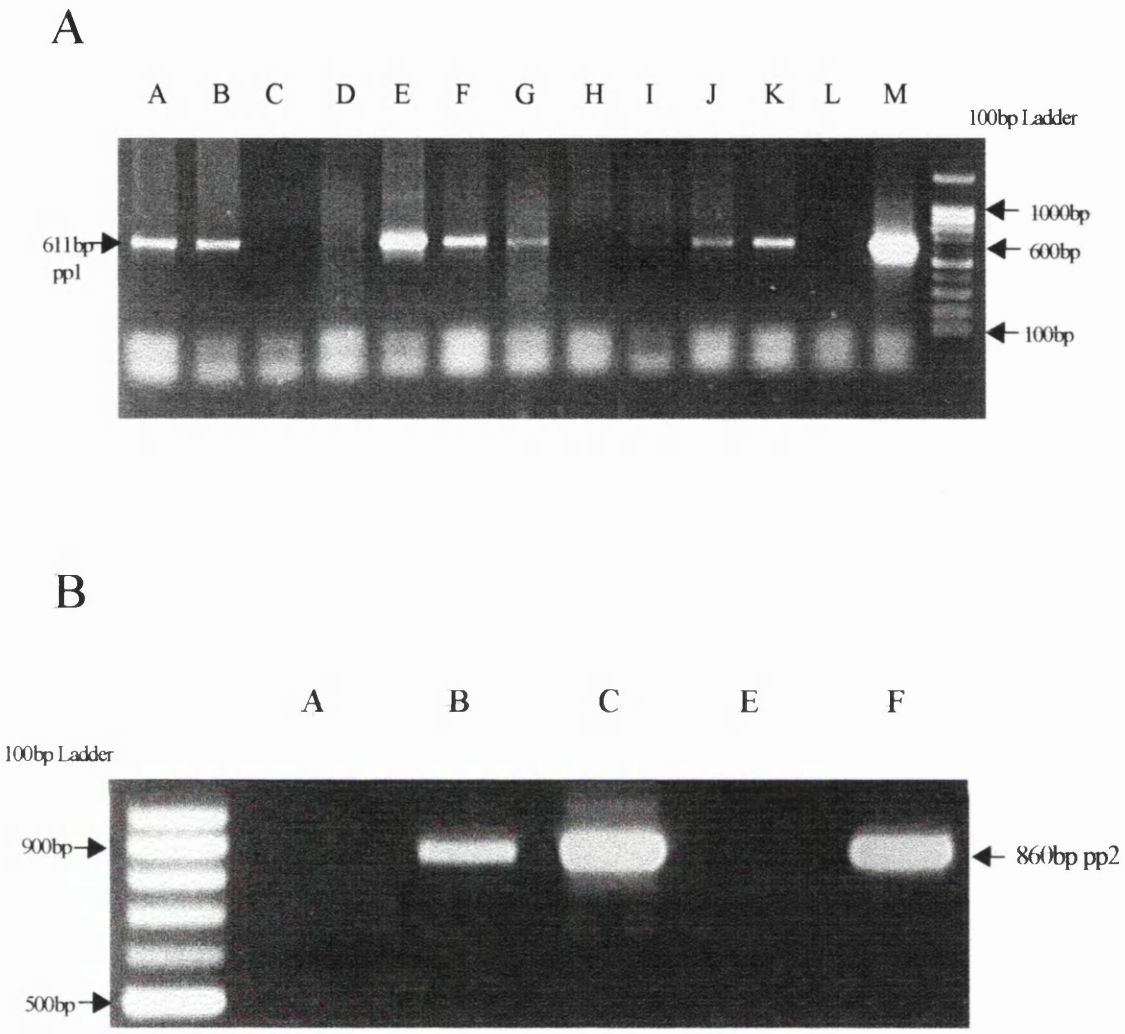


Figure 3.5 1% agarose gels of PCR products generated from genomic DNA stocks to be used for cloning and as sequencing templates. A) The pp1 (611bp) fragment generated from several samples (A-K), four of which failed to amplify. Lanes L and M are the negative and positive (pp1-ligated pCRII plasmid) controls respectively. B) The pp2 (860bp) fragment from three samples (A-C), one of which failed to amplify. Lanes D and E are the control positive and negative (pp2-ligated pCRII plasmid) controls respectively.

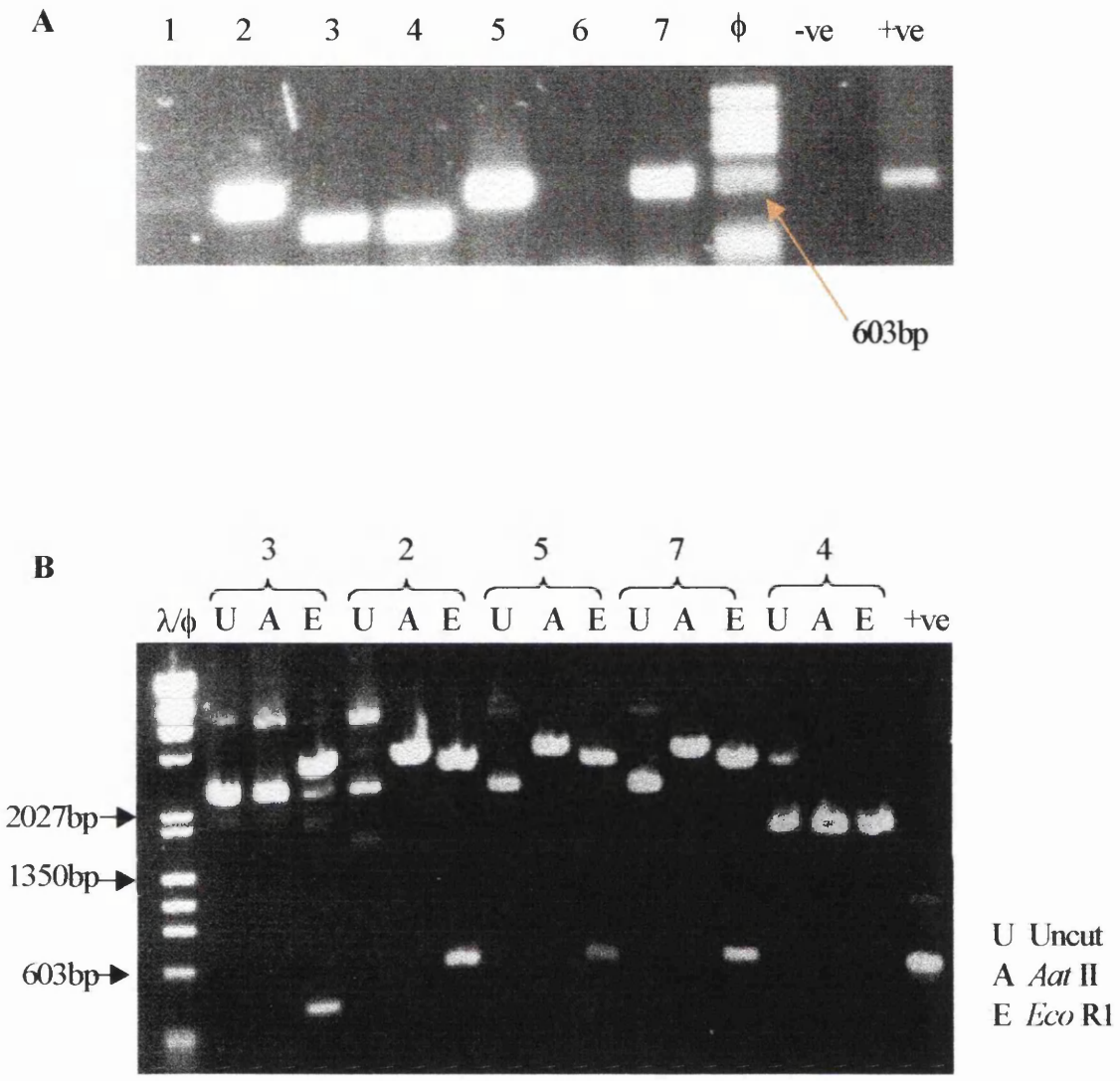


Figure 3.6 Confirmation of correct pp1 cloning into pCRII- 1) cPCR and miniprep digests. A) 1% agarose gel showing the results of cPCR of seven samples, with positive and negative controls. Samples 2, 5 and 7 gave bands of appropriate size. B) Following cPCR, the plasmids were expanded by the miniprep method. This 1% agarose gel shows the previous samples (correct and incorrect) after incubation with *Aat*II (which linearises the plasmid with a single cut) and *Eco*R1 (which cuts out the cloned fragment). Samples 2, 5 and 7 linearised correctly and contain the appropriate size fragment.

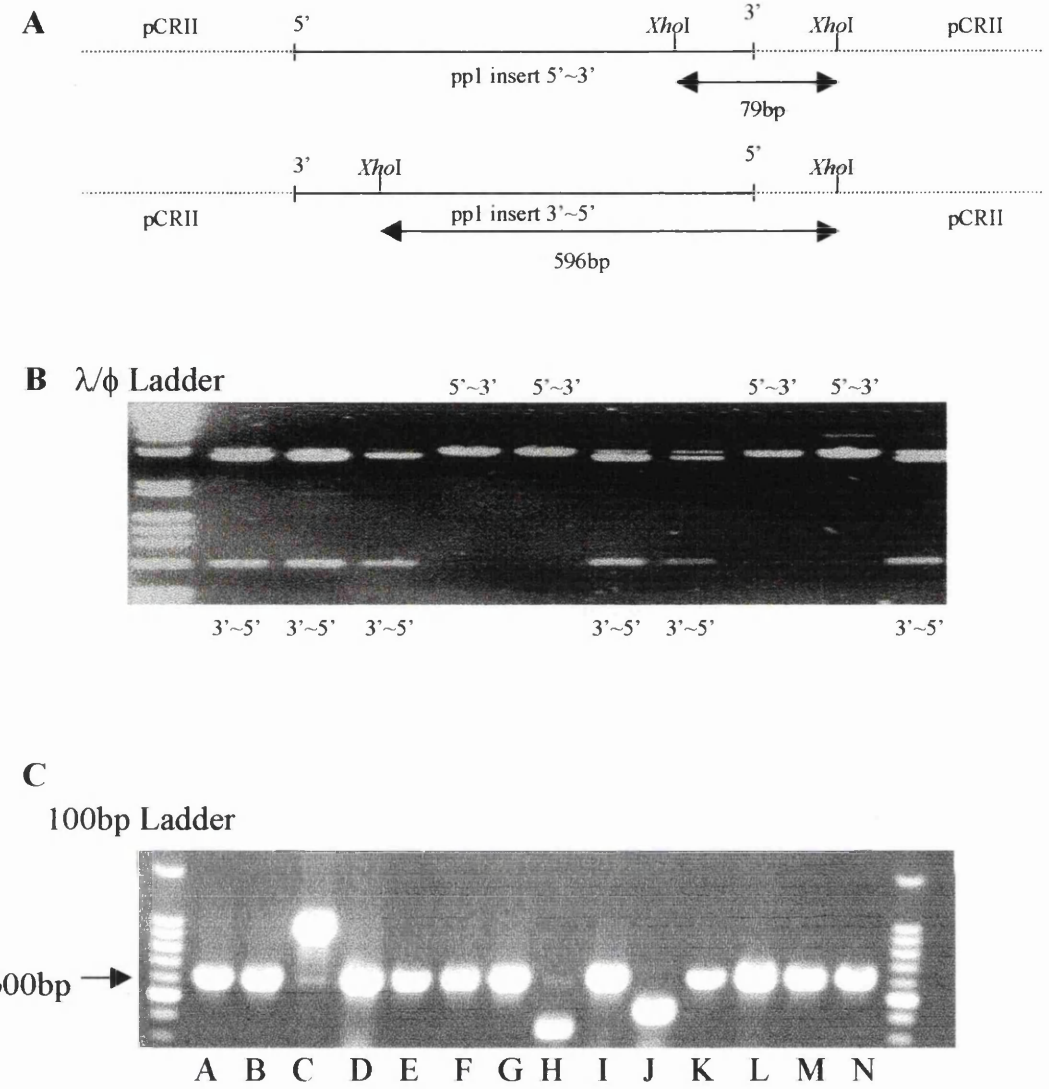


Figure 3.7 Confirmation of correct pp1 cloning into pCRII- 2) *Xho*1 digestion and PCR using the pCRII miniprep plasmids as template. A) Diagrammatic representation of pp1 orientation within the pCRII vector: The position of the pp1 insert is shown by the red line. The distance between the two *Xho*I restriction sites is shown by the arrow-headed line. The relative positions of these two *Xho*I sites within the insert and vector produce a large fragment on restriction of a 3'~5' ligated construct, and a small fragment from the 5'~3' ligated construct (which is not visible on 1% agarose gel electrophoresis). B) Restriction digestion with *Xho*1 demonstrates the orientation of the fragment (useful information prior to sequencing). C) The plasmids provide a large concentration of template DNA, hence the resulting PCR product is also very concentrated. The plasmids shown to have an incorrect insert on cPCR and plasmid restriction also have an incorrect insert for PCR template purposes (C, H and J). These were therefore rejected from sequencing.

3.2.2 *Sequencing Results*

A direct comparison of the nucleotide sequence (obtained by TCS and/or automated sequencing of the cloned plasmid) of the pp1 fragment from these 57 subjects with the published sequence of the entire IL-6 gene (Yasukawa *et al.*, 1987) revealed them to be in agreement, with the exception of four regions:

3.2.2.1 G/C Polymorphism at position -174

At position -174 of the published sequence a G to C transversion polymorphism was detected. This was initially demonstrated by TCS, and representative sections of the sequence around this position on TCS gels of both alleles are shown in Figure 3.8. The polymorphism was then confirmed using the Vistra automated fluorescent sequencing system. Sequences representing the two alleles detected in PCR fragments cloned into the pCRII plasmid vector are presented here. Figure 3.9 clearly demonstrates the -174G allele when sequenced from the positive strand. The -174C allele, however, is less well defined when using the positive strand as a template in this method, because the two adjacent cytosine peaks at positions -175 and -174 tend to merge into one large peak (Figure 3.10). However, the presence of two adjacent cytosines is clearly visualised when the negative strand is used as a template for sequencing (Figure 3.11).

Sequence data obtained directly from pooled PCR products (direct sequencing) shows the appearances of heterozygous individuals: Figures 3.12 and 3.13 display data obtained when using the positive and negative strands as templates respectively .

Note: During the semi-automated analysis of negative strand fluorescence sequences, the bases were automatically renamed to their complementary positive strand bases. Thus the sequences shown correspond to that of the positive strands, but are in the reverse orientation, ie. reading 3' to 5', left to right.

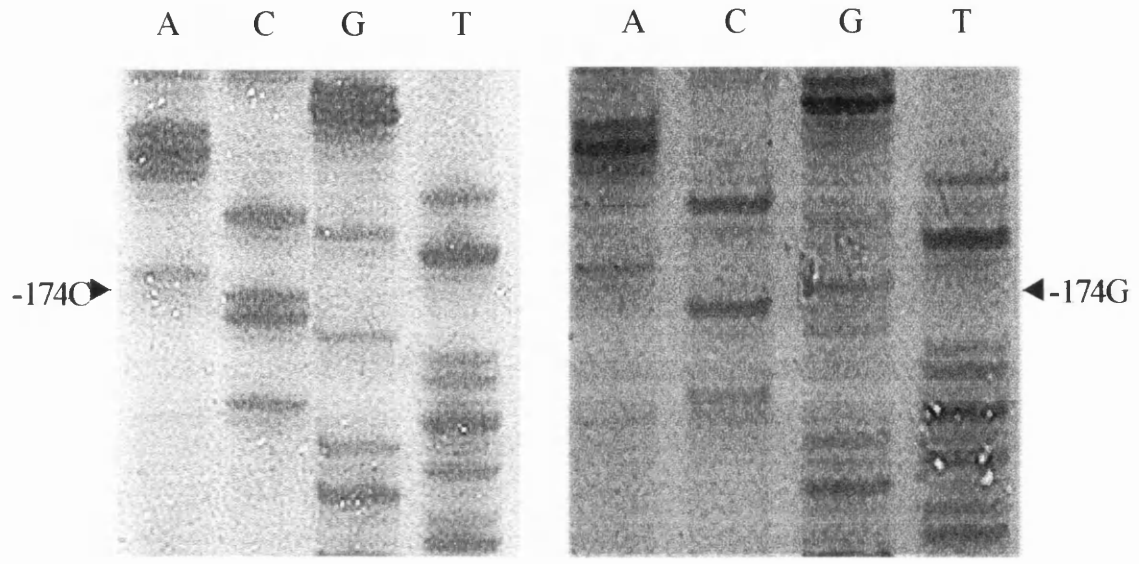


Figure 3.8 Sections from TCS autoradiographs of cloned pp1 fragments, demonstrating the G/C polymorphism at the -174 position. The gel on the left displays the -174C allele. The one to its right is the corresponding sequence for the -174G allele. (Positive strand sequenced; 5' to 3', reading upwards)

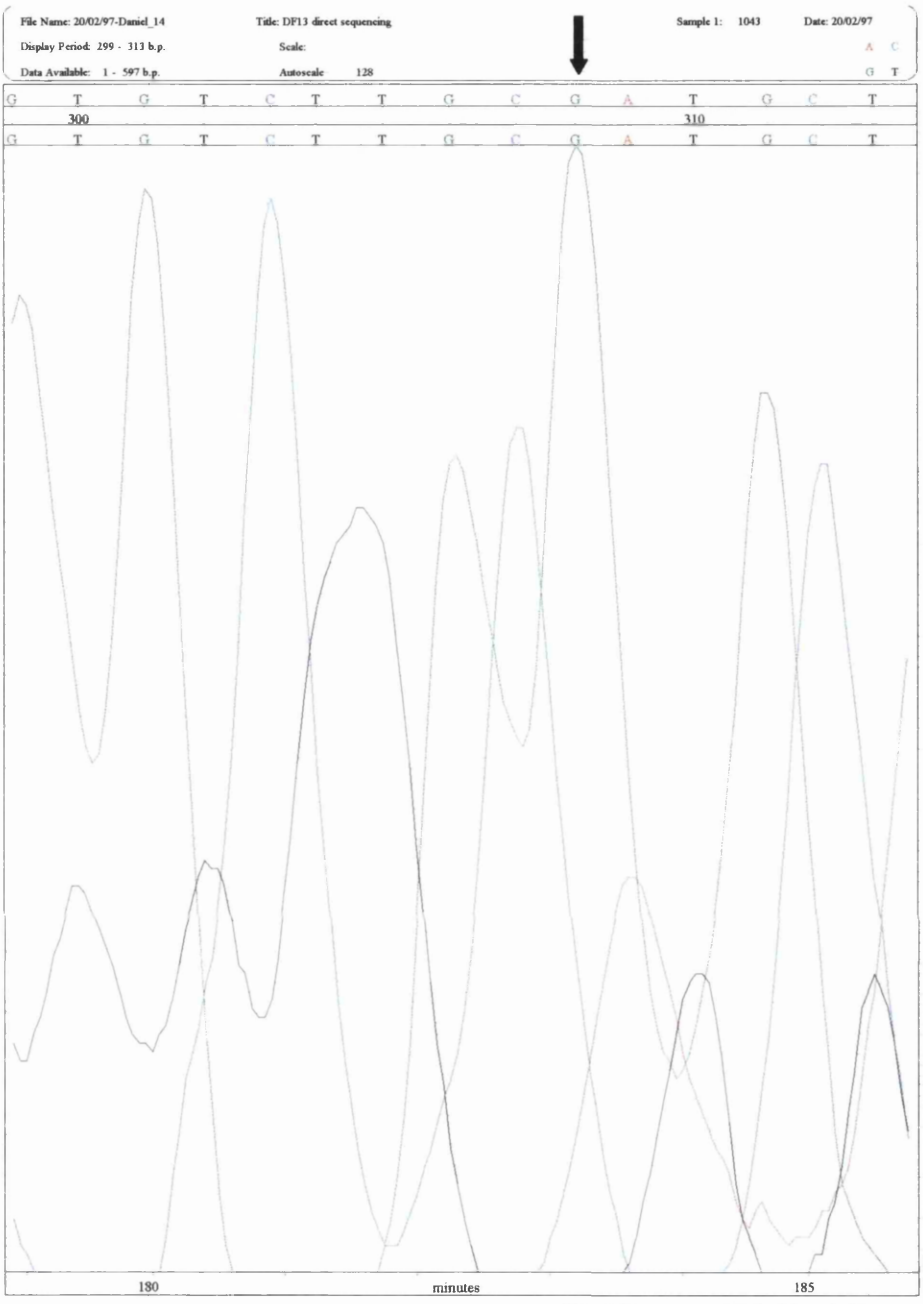


Figure 3.9 Fluorescence sequence data - a) -174G allele visualised from the positive strand of the cloned pp1 fragment. In this, and subsequent figures, the -174 position is indicated by an arrow; the presence of an adenine, cytosine, guanine or thymidine base in the sequence is indicated by a peak in the red, blue, green or black traces respectively (5' to 3', reading left to right).

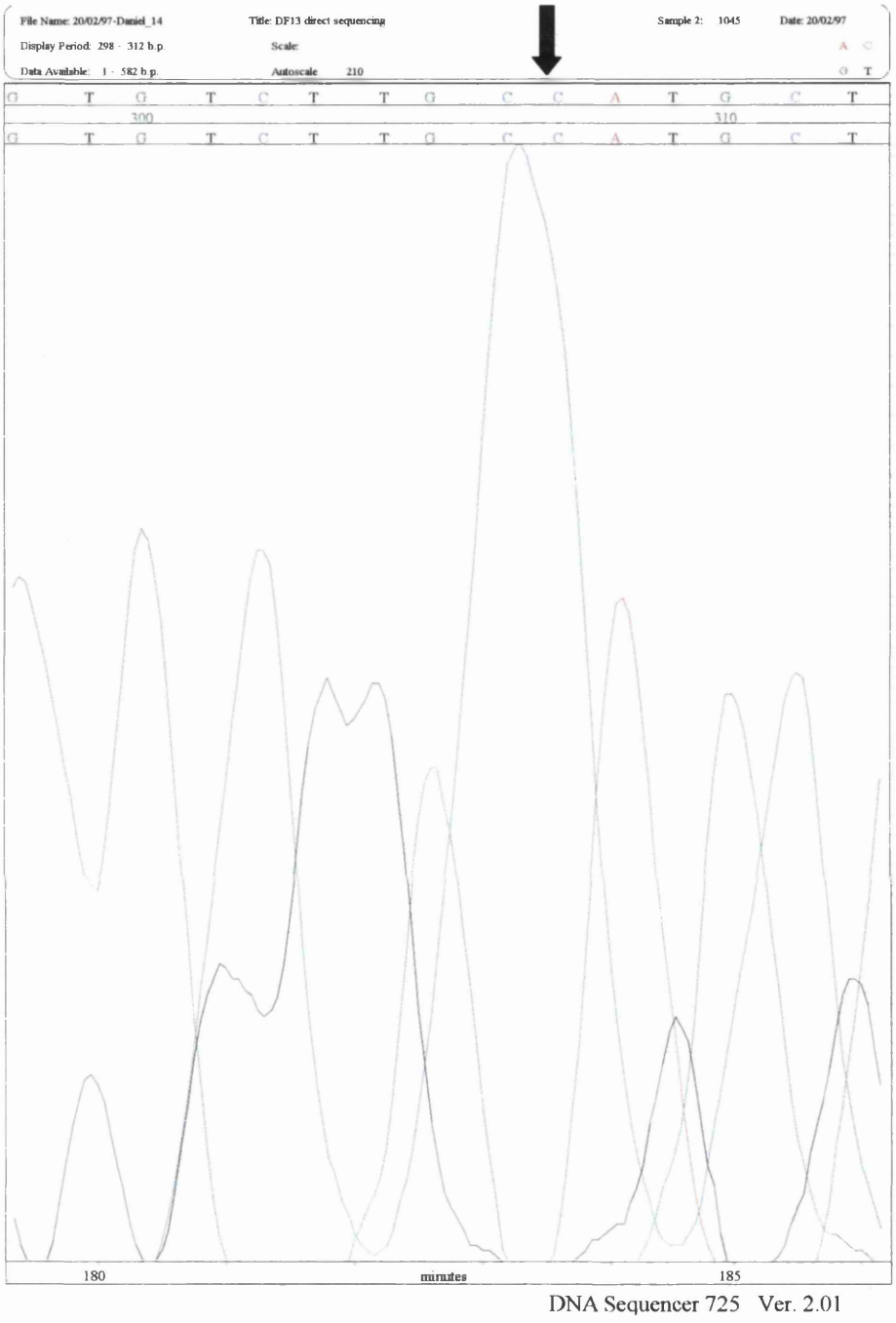


Figure 3.10 Fluorescence sequence data -b) The -174C allele visualised from the positive strand of the cloned pp1 fragment. The two adjacent cytosine bases are represented by a very large peak in the blue (cytosine) trace (5' to 3', reading left to right).

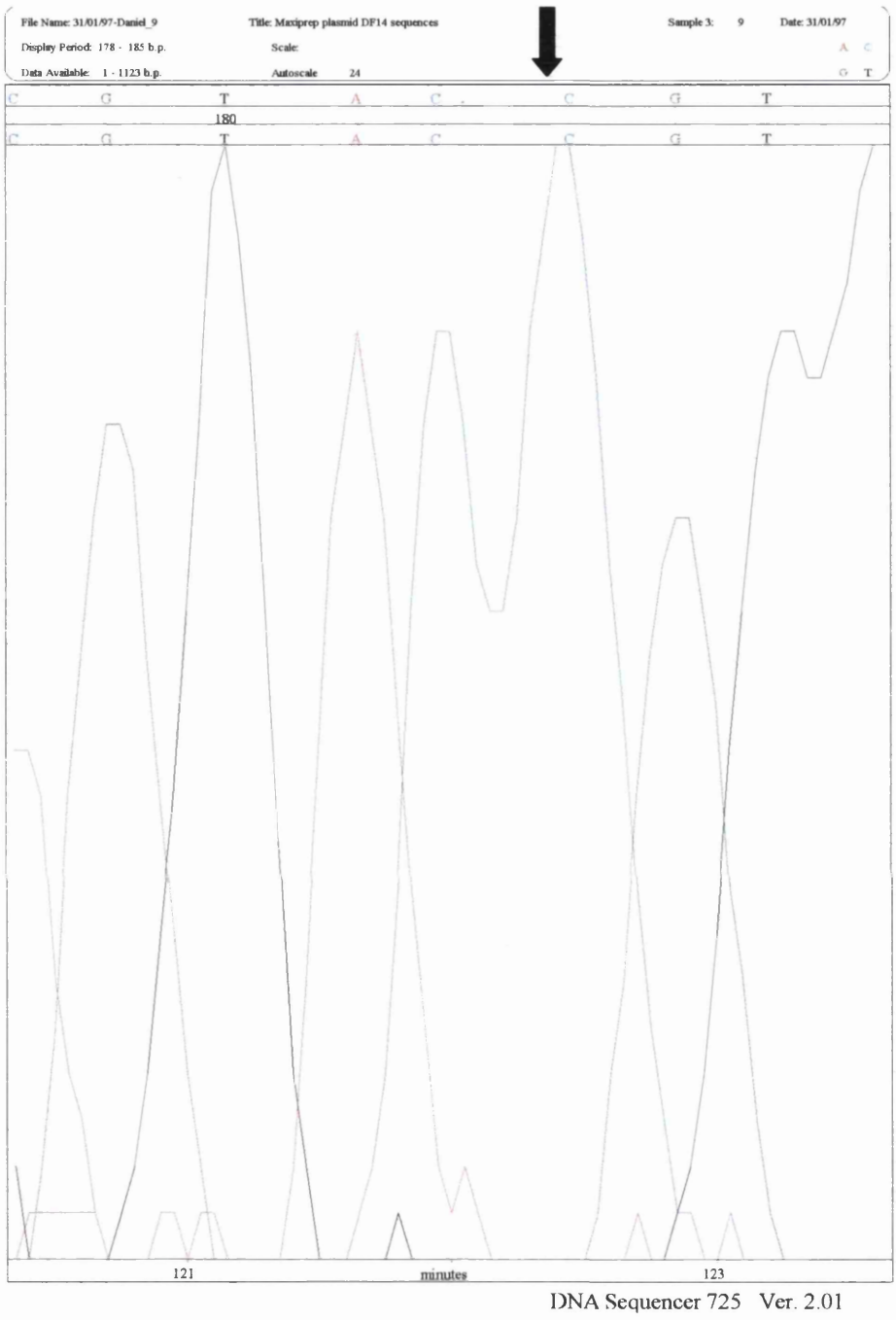


Figure 3.11 Fluorescence sequence data -c) The -174C allele visualised from the negative strand of the cloned pp1 fragment. A better differentiation between the adjacent cytosine bases is observed by sequencing the negative strand, compared to the positive (3' to 5', left to right).

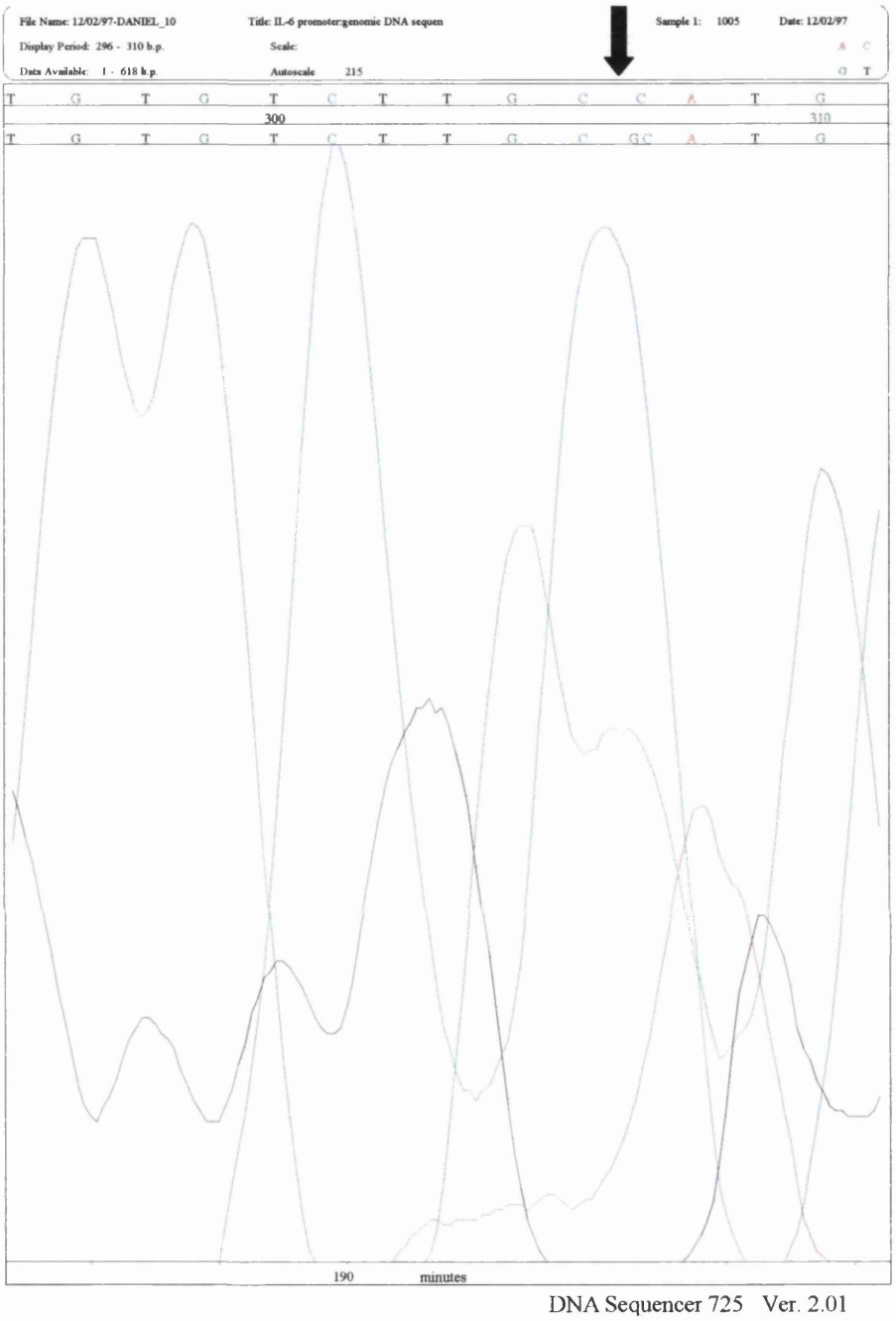


Figure 3.12 Fluorescence sequence data -d) The -174G/C heterozygous genotype visualised directly from the positive strands of pooled PCR products. The presence of a cytosine at position -174 in 50% of the PCR samples produces an expansion of the blue peak from -175 to this point, with a coincident reduction in the -174 green (guanine) peak (5' to 3', left to right).

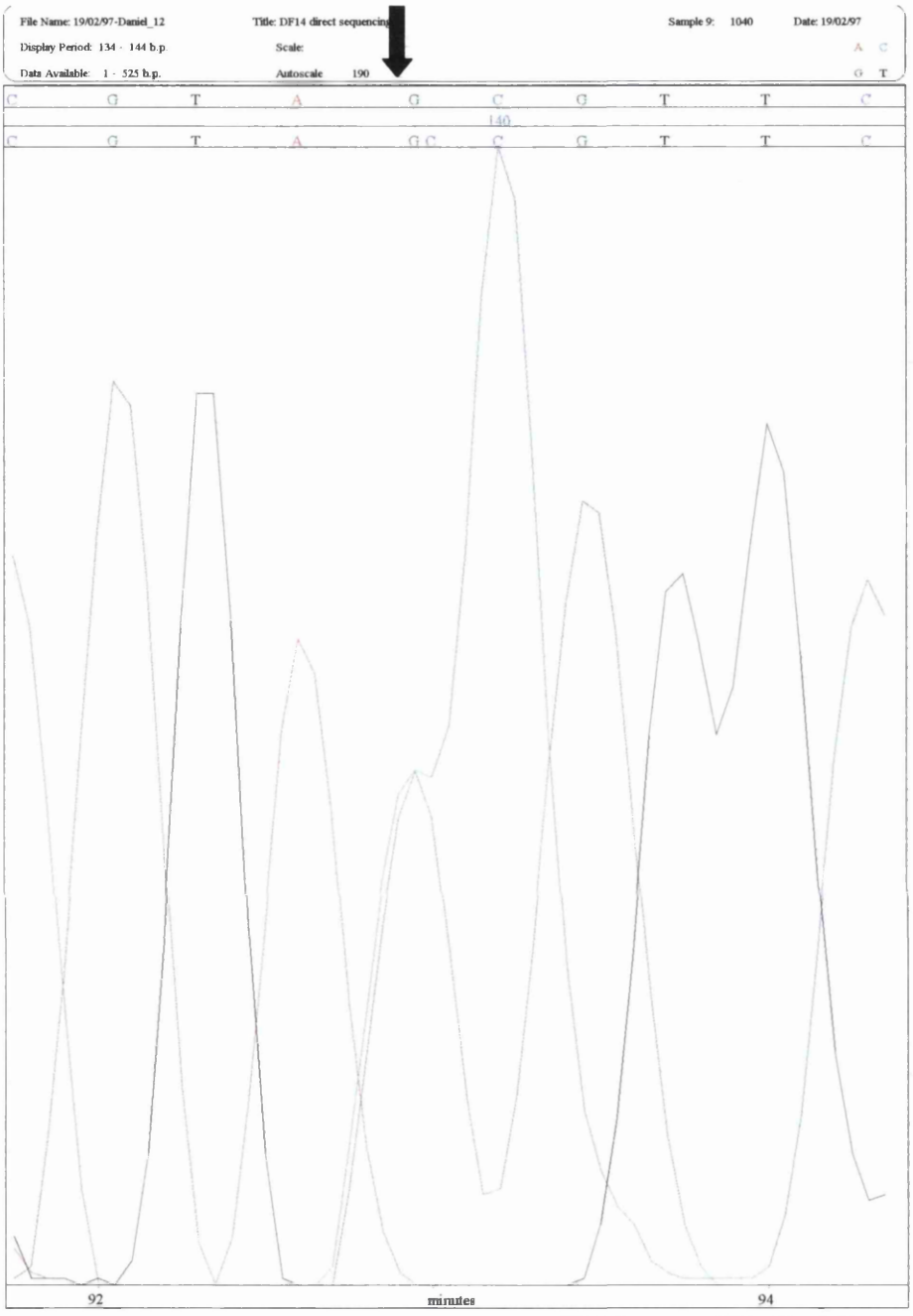


Figure 3.13 Fluorescence sequence data -e) The -174G/C heterozygous genotype visualised directly from the negative strands of pooled PCR products. The presence of both a cytosine and a guanine at -174 (only possible if the sample was heterozygous) is more obvious when the negative strands are used as sequencing templates (3' to 5', left to right).

From a combination of thermocycle and automated fluorescence sequencing of cloned pp1 fragments, the -174G/C allele frequency was determined for the control population and each disease subgroup. This is shown in Table 3.1.There was no significant difference between the allele frequencies of the (combined) patient and control groups (χ^2=0, p=1, OR 1.05). These values are unlikely to be representative of the true population allele frequencies because the number of subjects in each group was small. More importantly however, as this method relied on selecting a single clone to sequence, only one of the individuals pair of IL-6 genes could be analysed. Thus the true population frequency could not be determined.

Heath Status	G allele (frequency)	C allele (frequency)
S-JCA (n=13)	8 (0.62)	5 (0.38)
Rheumatoid factor positive, poly-JCA (n=2)	2 (1.0)	0 (0)
Pauci-JCA (n=4)	3 (0.75)	1 (0.25)
Poly-JCA (n=2)	2 (1.0)	0 (0)
Unclassified JCA (n=7)	3 (0.43)	4 (0.57)
All above patients (n=28)	18 (0.64)	10 (0.36)
Controls (n=29)	19 (0.66)	10 (0.34)

Table 3.1 The distribution of -174G and -174C alleles in the control and patient populations. This was determined by sequencing the cloned pp1 fragment by either thermocycle or the automated, fluorescent method. Only a few samples were examined by direct automated sequencing of PCR products. They were used to confirm the thermocycle sequence data and were therefore not included here.

3.2.2.2 A_nT_n Polymorphism at -392 to -373

There was a variation in the number of adenine and thymine residues which formed an A_nT_n tract between positions -392 and -373 (of the published sequence, which is A_9T_{11}). In this, study three configurations of 20 bases were observed (Figures 3.14~3.18; and Tables 3.2 and 3.3). These were:

I) an A to T transition polymorphism at -384 was observed, which produced an A_8T_{12} configuration (Figures 3.14 and 3.15). This was the commonest configuration encountered and notably the -174C allele was almost exclusively associated with this (Tables 3.2 and 3.3);

ii) the published A_9T_{11} configuration was the next commonest observed (Figures 3.14~3.16), and was always observed in association with the -174G allele. However, this allele unlike the -174C allele, was also found in association with other A_nT_n configurations (Tables 3.2 and 3.3).

iii) a T to A transition polymorphism was observed at -383, creating an $A_{10}T_{10}$ configuration (Figures 3.17 and 3.18). This was also almost exclusively associated with the -174G allele (Tables 3.2 and 3.3).

The consistency of these three configurations suggests that their observation was not due to a PCR or sequencing artefact.

iv) three additional configurations of 19 or 21 bases were also observed, although less frequently than the 20 base configurations (Figures 3.17, 3.19 and 3.20). The base changes resulting in these configurations are less clear and their presence as a result of a PCR or sequencing artefact cannot be fully excluded.

The distribution of the A_nT_n polymorphisms, with respect to their -174 polymorphism status, is shown in Table 3.2, and a summary of the data (a combination of all the cases analysed together) is shown in Table 3.3.

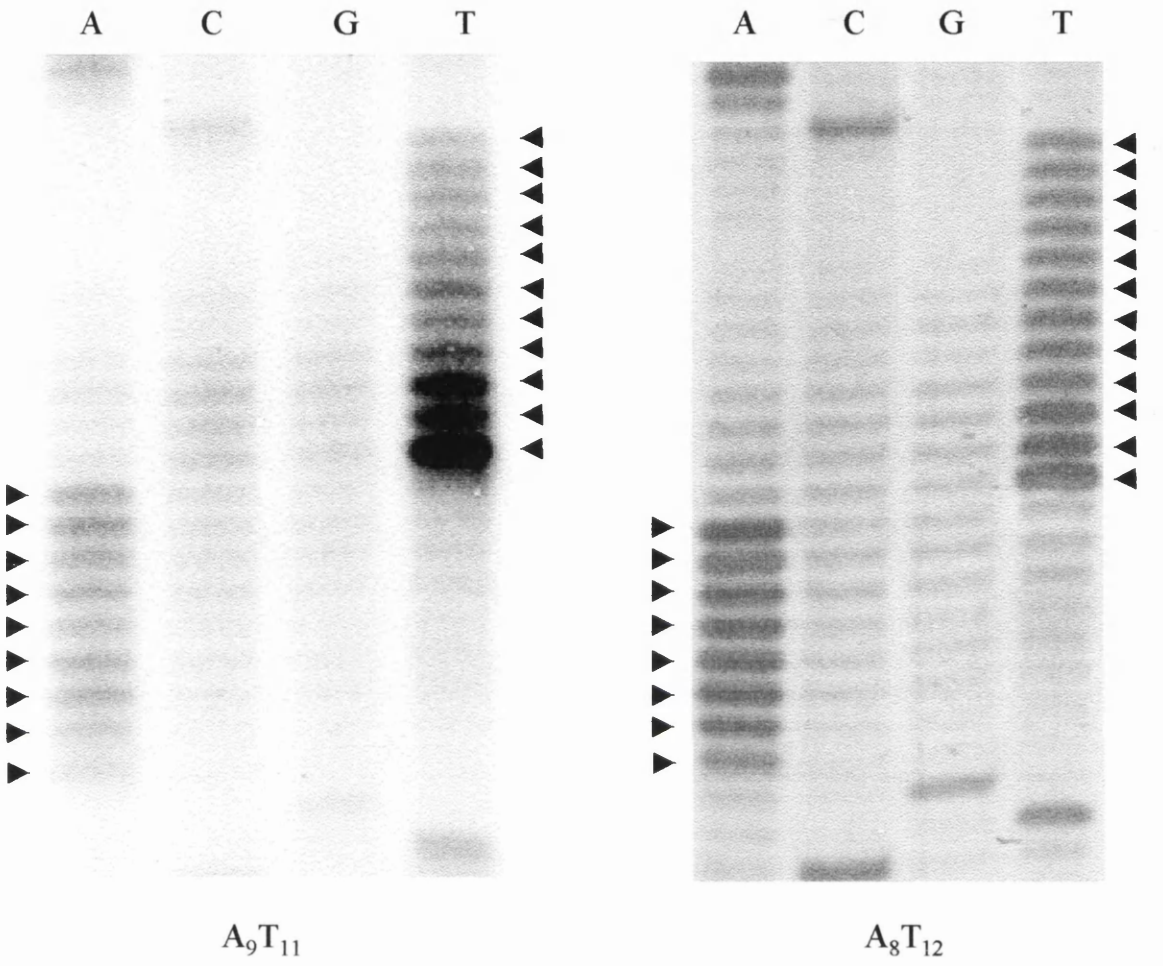


Figure 3.14 Sections from thermocycle sequencing autoradiographs showing the A_nT_n tracts of two cloned pp1 PCR products; A) a comparison of the A_9T_{11} and A_8T_{12} compositions. (Positive strand sequenced; 5' to 3' reading upwards). Typically for the thermal cycle sequencing method, the first adenine in a tract is pale, whereas the first thymidine is dark.

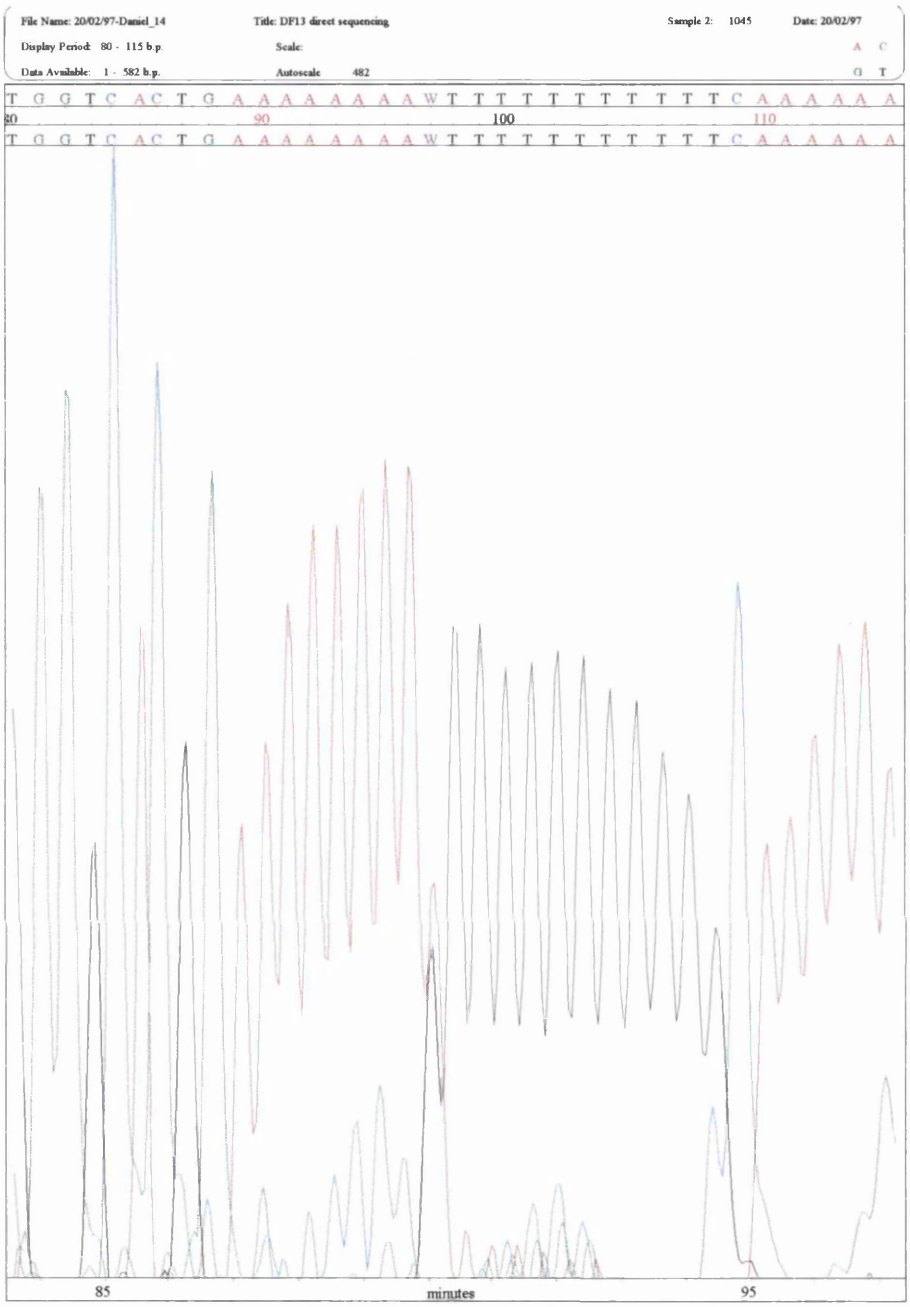


Figure 3.15 Fluorescence sequence data from the A_nT_n tract - a) An A_8T_{12} /A_9T_{11} heterozygous sequence. The presence of an adenine and thymidine base at the same position in the centre of the A_nT_n tract is due to the subject being heterozygous for the A_nT_n tract polymorphism. (Directly sequenced from pooled pp1fragment PCR products; 5' to 3', reading left to right).

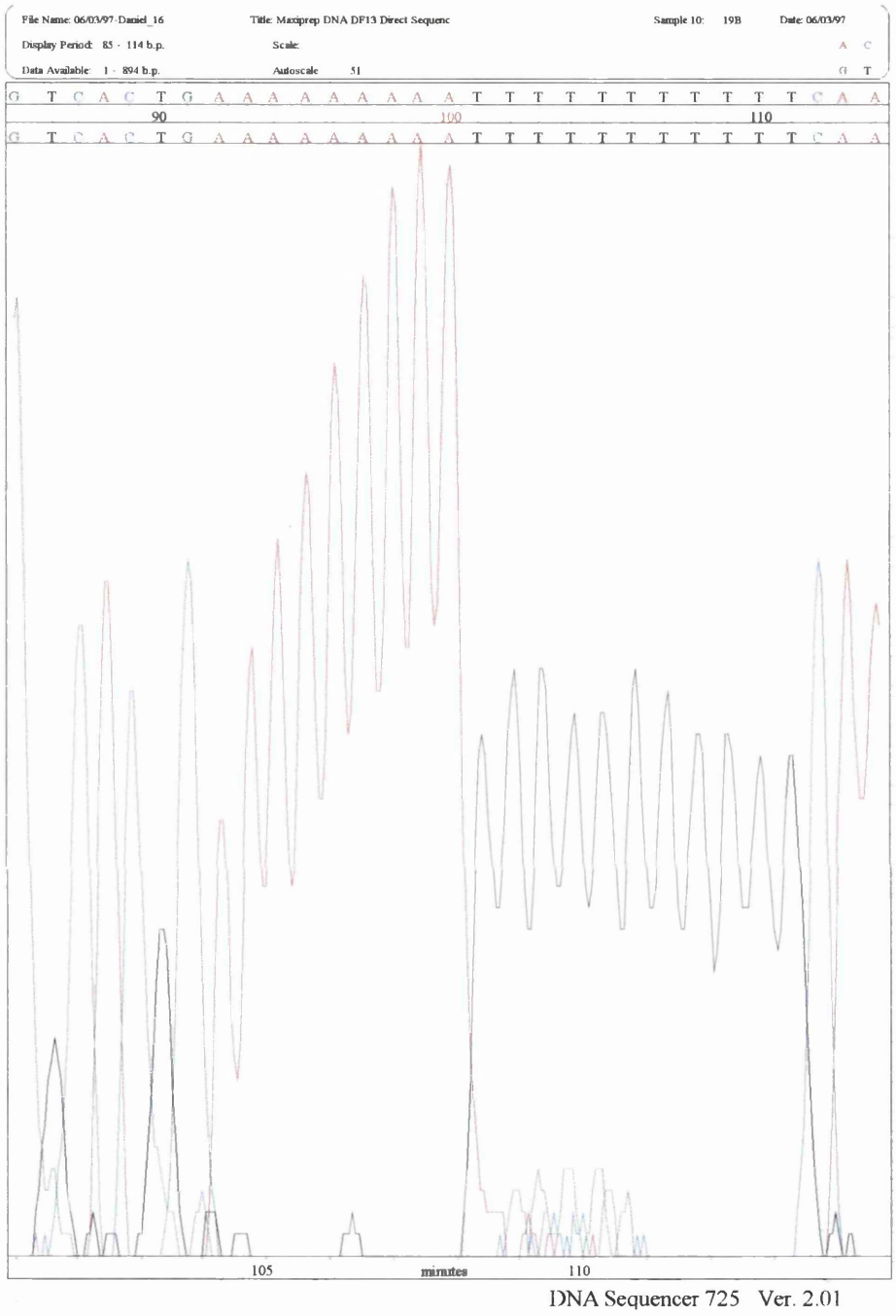


Figure 3.16 Fluorescence sequence data from the A_nT_n tract - b) An A_9T_{11} sequence from a cloned pp1fragment (5' to 3', reading left to right).

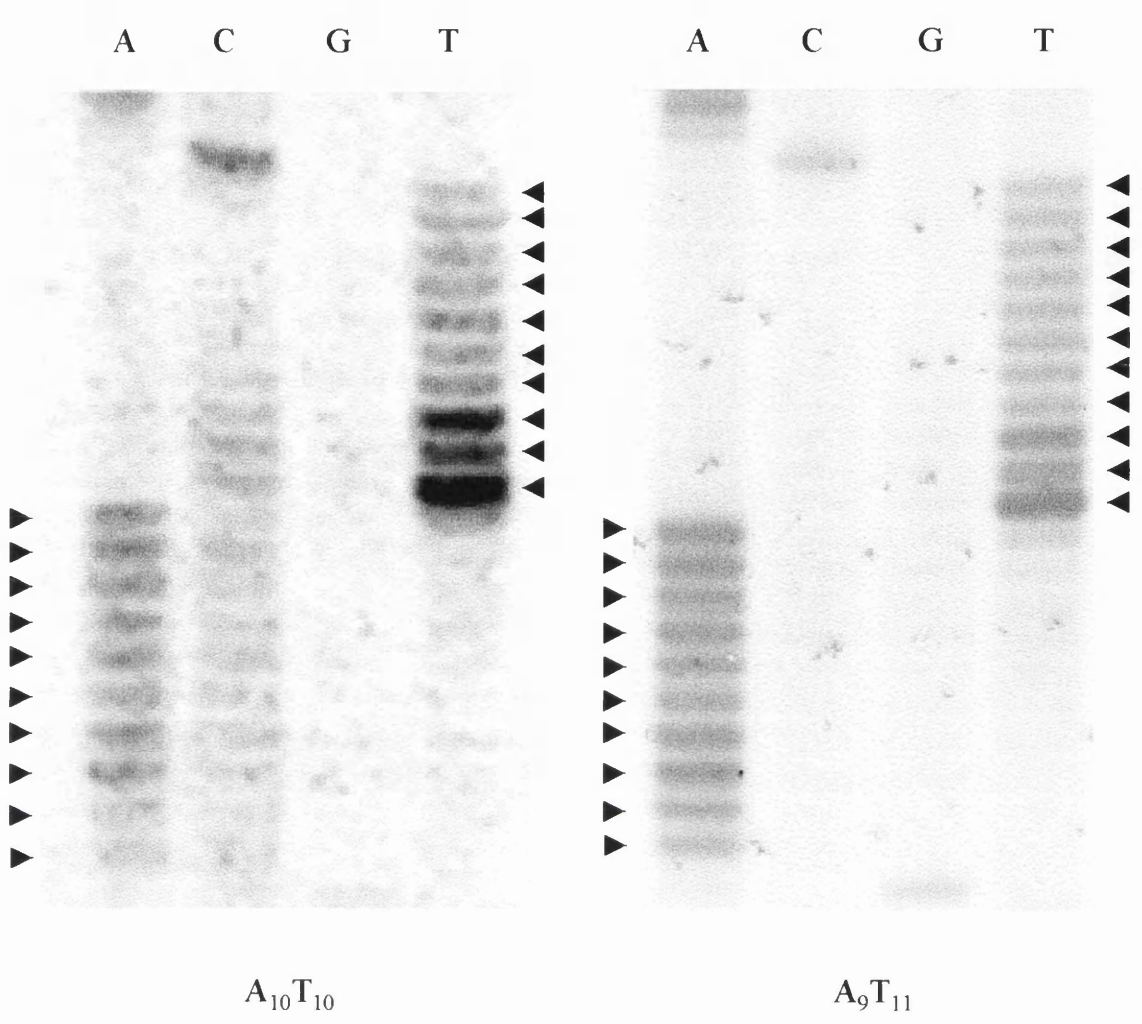


Figure 3.17 Sections from thermocycle sequencing autoradiographs showing the A_nT_n tracts of two cloned pp1 PCR products; B) a comparison of the $A_{10}T_{10}$ and $A_{10}T_{11}$ compositions. (Positive strand sequenced; 5' to 3' reading upwards).

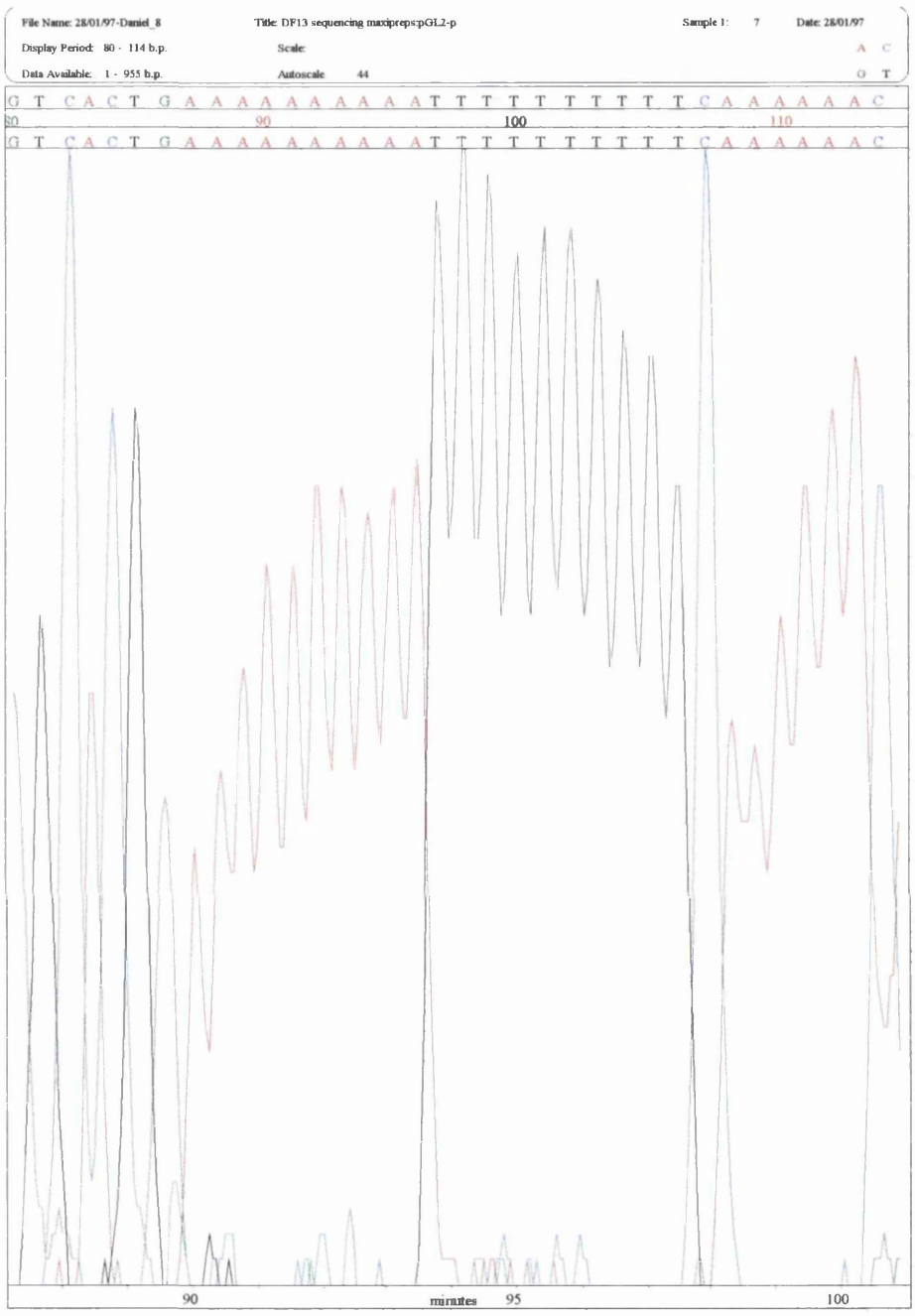


Figure 3.18 Fluorescence sequence data from the A_nT_n tract - c) An $A_{10}T_{10}$ sequence from a cloned pp1fragment (Positive strand sequenced; 5' to 3', reading left to right).

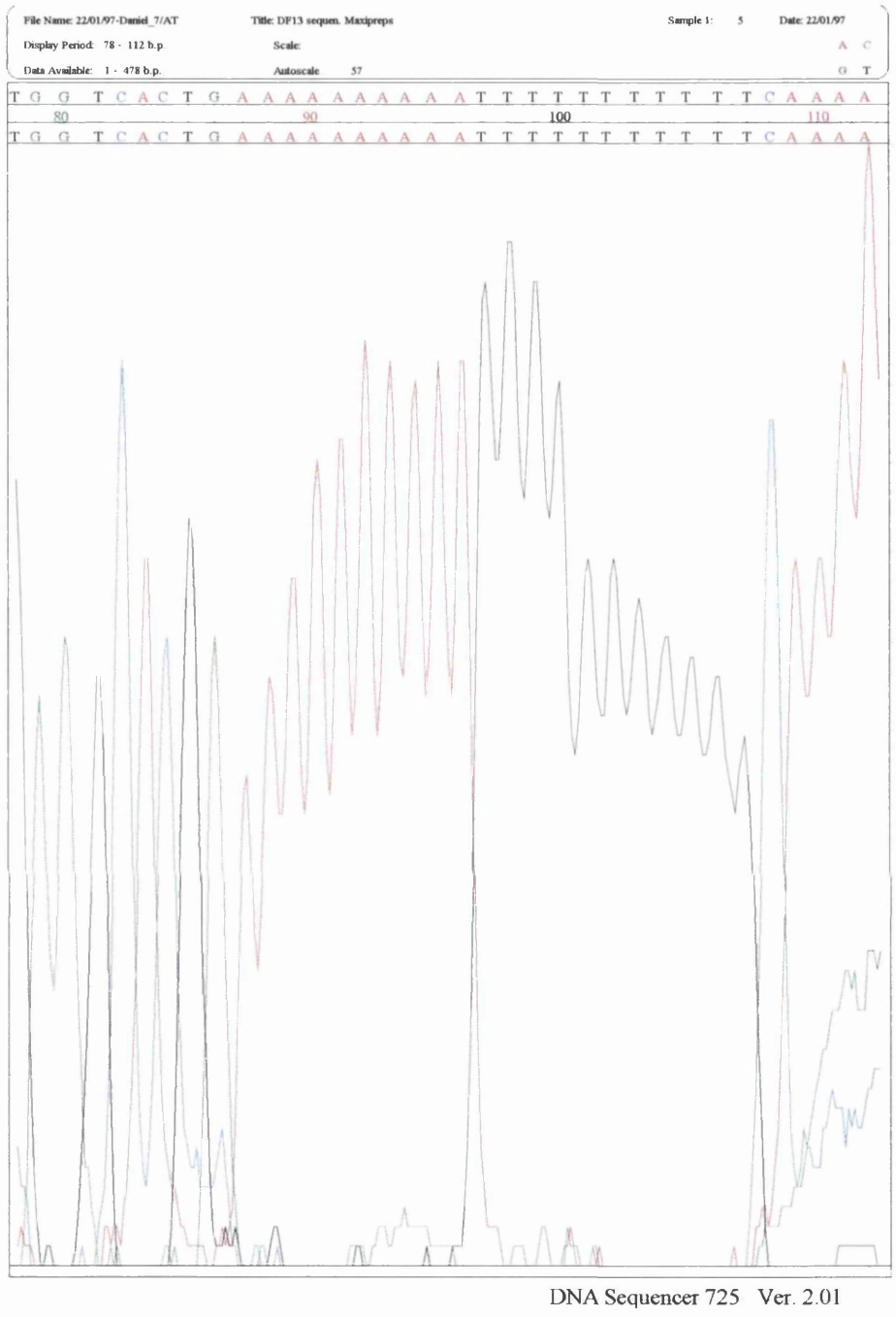


Figure 3.19 Fluorescence sequence data from the A_nT_n tract - d) An $A_{10}T_{11}$ homozygous sequence obtained directly from pooled pp1 fragment PCR products. (Positive strand sequenced; 5' to 3', reading left to right).

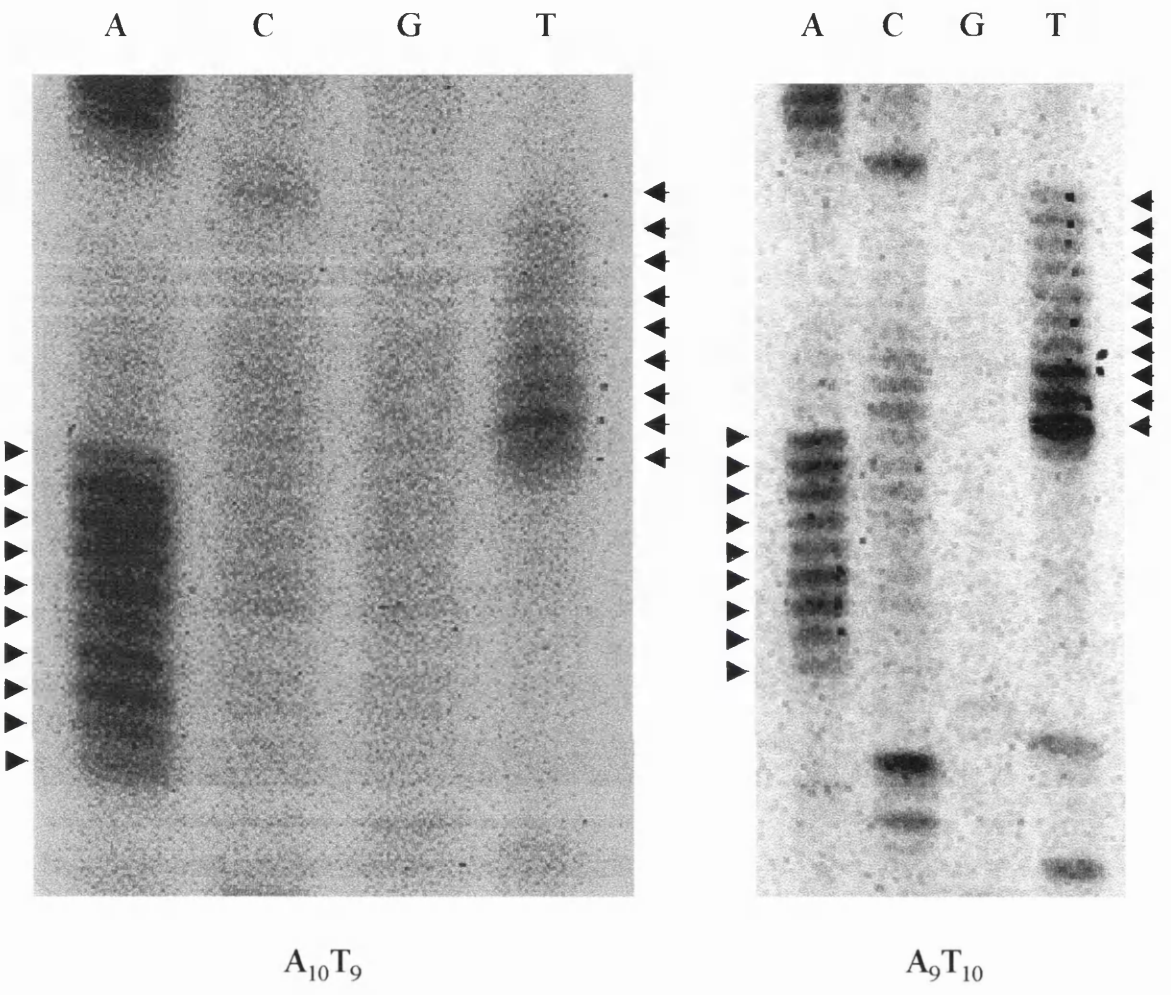


Figure 3.20 Sections from thermocycle sequencing autoradiographs showing the A_nT_n tracts of two cloned pp1 PCR products; C) a comparison of the $A_{10}T_9$ and A_9T_{10} compositions. (Positive strand sequenced; 5' to 3' reading upwards).

Health status	-174 allele	A_8T_{12}	A_9T_{11}	$A_{10}T_{10}$	A_9T_{10}	$A_{10}T_{11}$	$A_{10}T_9$
Control	G	4	5	5	2	3	
Control	C	9		1			
S-JCA	G		4	2	1	1	
S-JCA	C	5					
Sero+ve	G	1	1				
Sero+ve	C						
Pauci	G		2			1	
Pauci	C	1					
Poly	G			1			1
Poly	C						
Unclass.	G			1		2	
Unclass.	C	4					
Health status	**-174 allele**	A_8T_{12}	A_9T_{11}	$A_{10}T_{10}$	A_9T_{10}	$A_{10}T_{11}$	$A_{10}T_9$

Table 3.2 Distribution of A_nT_n configurations according to their associated patient or control -174 allele.

	A_8T_{12}	A_9T_{11}	$A_{10}T_{10}$	A_9T_{10}	$A_{10}T_{11}$	$A_{10}T_9$
-174G	5	12	9	3	7	1
-174C	19	0	1	0	0	0

Table 3.3 Distribution of A_nT_n configurations, according to their associated -174 allele, in the control and patient groups combined.

As with the data obtained for the -174 polymorphism, these results are representative of only one of the individuals pair of IL-6 genes. Hence an extrapolation to the population level would not be appropriate. However, these preliminary data clearly show that there is a trend towards the development of a haplotype, with the -174C allele being almost exclusively associated with the A_8T_{12} tract. The -174G allele, on the other hand, is associated with all of the different variations of the A_nT_n tract, frequently the A_9T_{11} one.

3.2.2.3 Additional cytosine at position -508

At position -508 of the published sequence an extra cytosine was present forming a triplet of C bases, where the published sequence had only a pair of C bases (Figure 3.21). The consistent nature of this result is likely to represent a technical error in the published sequence.

3.2.2.4 Additional cytosine at position +15

In similar circumstances to the additional cytosine observed at position -508, another additional cytosine base was also observed at +15, producing a further C triplet (Figure 3.22).

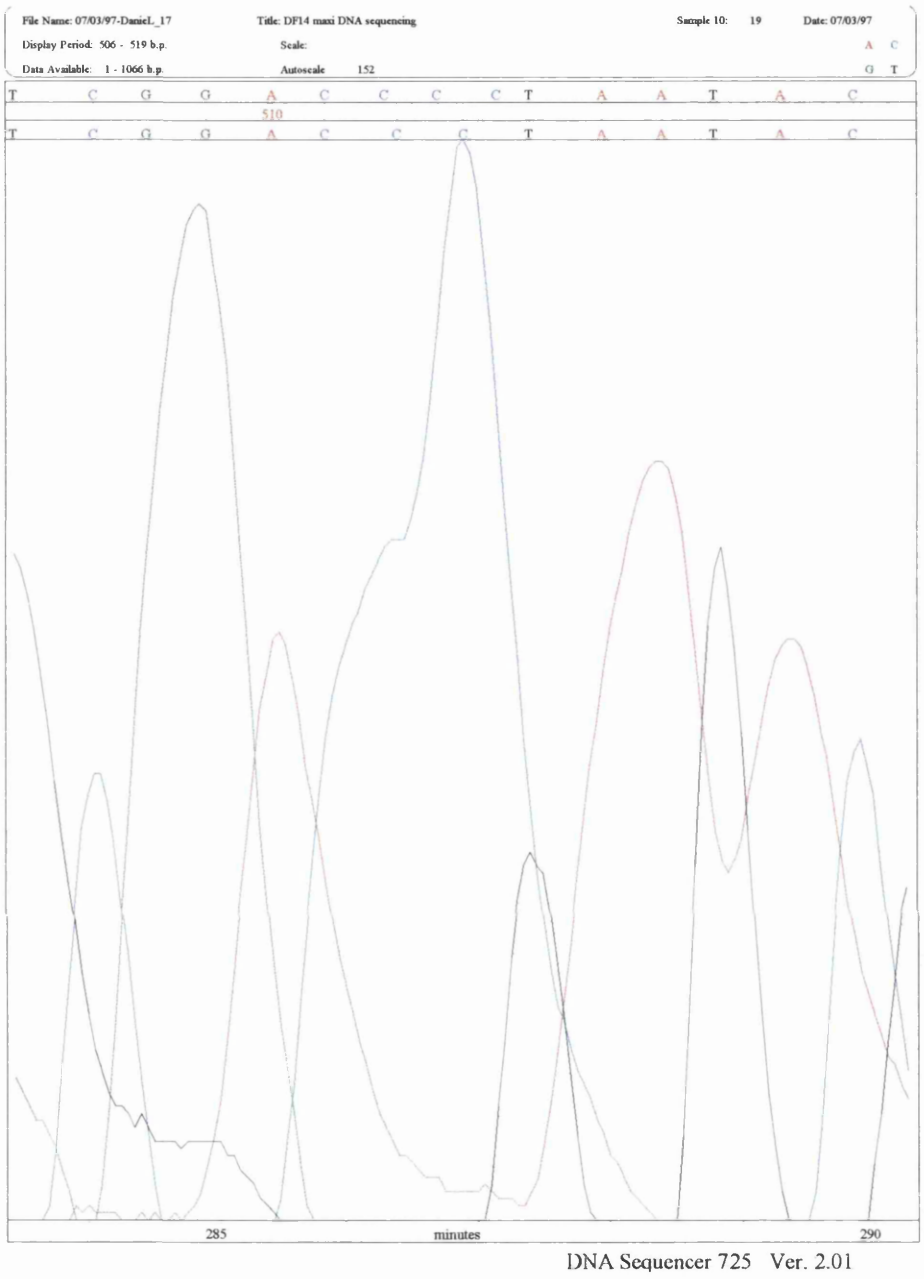


Figure 3.21 Fluorescence sequence data for the region flanking the extra cytosine base at position -508. The published sequence is a doublet of cytosines here, but the extended fluorescent intensity peak, and the spacing of all of the bases, suggests that this should be a triplet. (Negative strand sequenced; base sequence shown corresponds to the positive strand in the reverse orientation, reading 3' to 5', left to right).

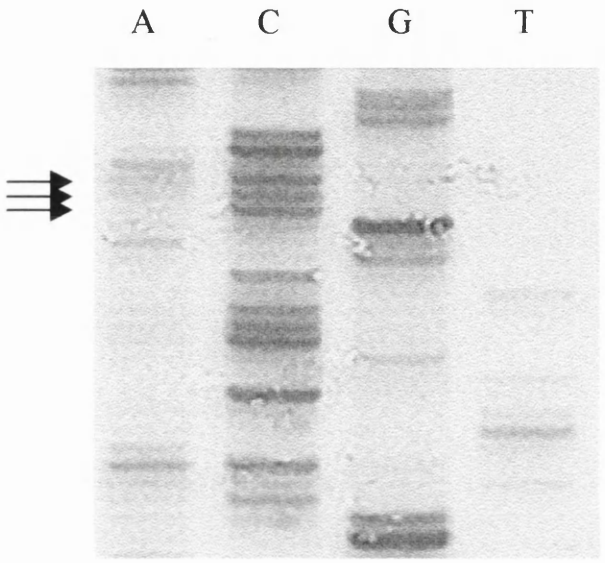


Figure 3.22 A section from a thermocycle sequencing autoradiograph showing the region around the extra cytosine at position +15. The triplet of cytosine bases is indicated by the arrows. (Positive strand sequenced; 5' to 3' reading upwards).

In this study, thermal cycle sequencing produced very good results, often with 125 bases readable from the primer in a single run. However, this method was impractical for screening large numbers of individuals as multiple reactions and sequencing gel runs were required to cover the entire cloned fragment. Using the Perkin-Elmer PCR machine to run sequencing thermocycling reactions for the Vistra automated sequencing system allowed complete sequence analysis of a cloned pp1 fragment in one run. Moreover, it was possible to sequence genomic PCR products using this system, which obviated the need to produce clones from each subject. However, as sequencing was still impractical as a method of population screening for the -174 polymorphism, an RFLP method was developed.

Due to the complexity of the polymorphism which resulted in the different A_nT_n tract configurations, RFLP was not possible and this region of the IL-6 gene was not analysed further during this study.

3.3 Polymorphism Screening of Control and Systemic-JCA Populations

3.3.1 *RFLP design*

Following the discovery of the -174 polymorphism, a search for suitable restriction enzymes to perform an RFLP analysis was made. The results of the Webcutter on-line restriction enzyme database analysis of a 30 base oligonucleotide sequence encompassing the G/C polymorphism are shown in Figures 3.23 and 3.24.

*Hsp*92II and *Nla*III both cut three times within the -174G pp1 fragment (yielding fragments of 229, 28, 232 and 119 bases) and four times within the -174C fragment (yielding fragments of 229, 28, 121, 110 and 119 bases). Separating these products on a 1% agarose gel would have been very difficult. A shorter PCR product only encompassing the RFLP site could have been used for screening, but this would have required designing (and optimising the use of) new PCR primers. As *Sfa*NI cut only once within the -174G pp1 fragment, and not at all within the -174C pp1 fragment, this enzyme was used for screening.

As predicted, GG homozygous pp1 products were completely digested by *Sfa*NI into two fragments of 381 and 230 base pairs. pp1 products homozygous for the C allele were resistant to digestion and heterozygous subjects yielded 3 fragments of 611, 381 and 230 base pairs (Figure 3.25).

PCR of the pp2 fragment was highly specific (as shown in Figure 3.26). *Sfa*N1 digestion of GG homozygous pp2 products produced three fragments of 482, 332 and 46 bases, although the latter was too small to be observed on the standard 1% agarose gel electrophoresis used. *Sfa*N1 digestion of CC homozygous subject pp2 products yielded the 46 base fragment only, which was not visualised; the remaining 812 base fragment appeared almost identical to the undigested 858bp fragment on these gels. (Figure 3.27).

BfaI -174G

*Mae*III ↓

5'- CCT AGT TGT GTC TTG CGA TGC TAA AGG ACG-3'

3'- GGA TCA ACA CAG AAC GCT ACG ATT TCC TGC-5'

*Sfa*NI

Enzyme:	Recognition sequence:
*Bfa*I	c/tag
*Mae*III	c/tag
*Sfa*NI	gcatcnnnnn/n

Figure 3.23 Restriction enzyme sites in the sequence flanking the -174G allele.

BfaI -174C

*Mae*III ↓

5'- CCT AGT TGT GTC TTG CCA TGC TAA AGG ACG-3'

3'- GGA TCA ACA CAG AAC GGT ACG ATT TCC TGC-5'

*Hsp*92II

*Nla*III

Enzyme:	Recognition sequence:
*Bfa*I	c/tag
*Mae*III	c/tag
*Hsp*92II	catg/
*Nla*III	catg/

Figure 3.24 Restriction enzyme sites in the sequence flanking the -174C allele.

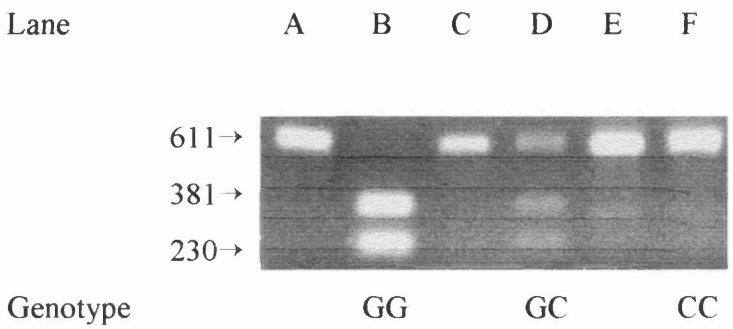


Figure 3.25 pp1 PCR product restriction digest pattern for the -174G/C genotypes. PCR products from three individuals incubated with *Sfa*NI plus buffer (lanes B, D and F) or buffer alone (lanes A, C and E) run together for comparison on a 1% agarose gel. (Size of fragments indicated in base pairs).

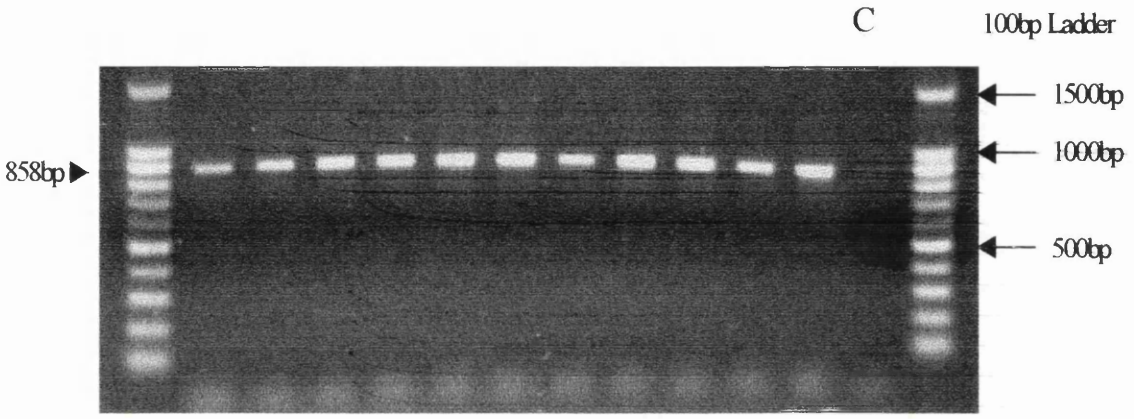


Figure 3.26 pp2 PCR products from genomic DNA run on a 1% agarose gel. Compared to the pp1 PCR (Figure 3.5) the pp2 PCR amplified a greater percentage of samples, especially those which had initially failed to amplify by pp1 PCR.

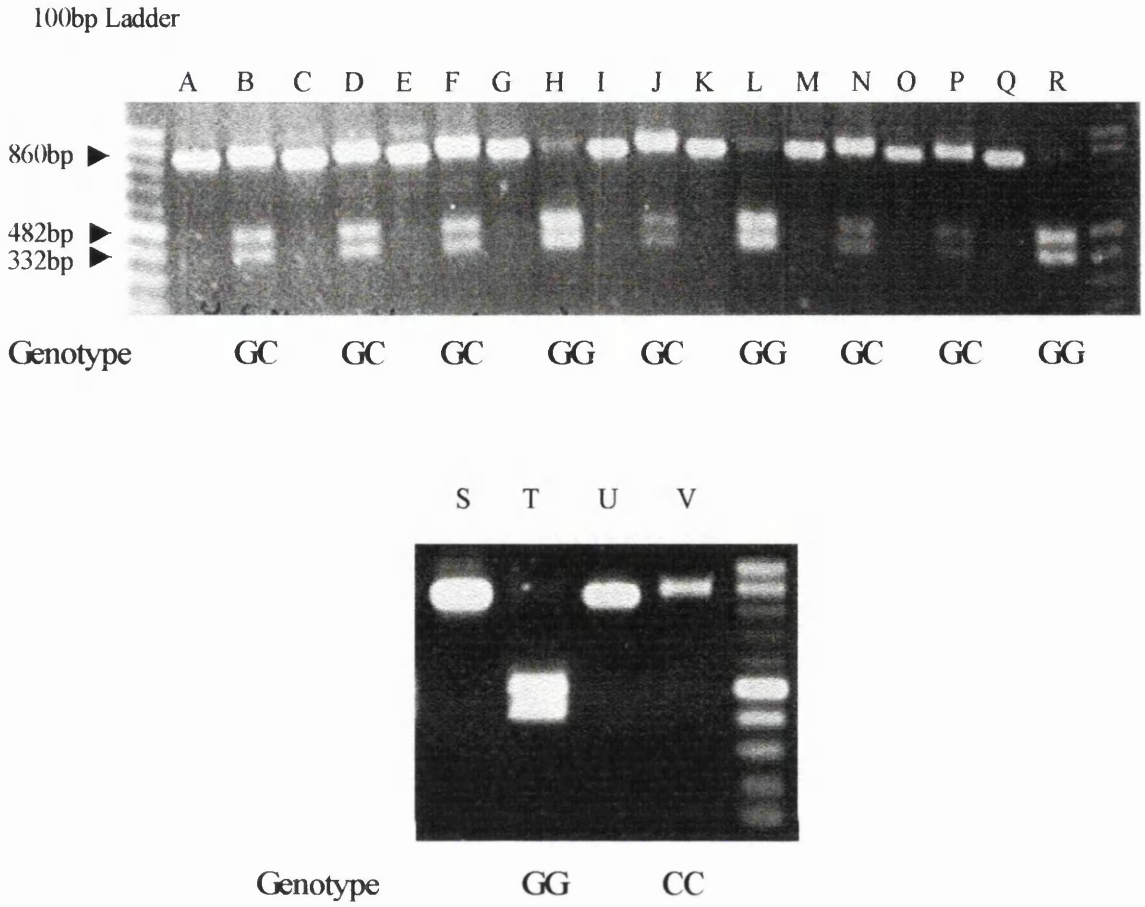


Figure 3.27 pp2 PCR product restriction digest pattern for the -174G/C genotypes. PCR products from eleven individuals incubated with *Sfa*NI plus buffer (lanes B, D, F, H, J, L, N, P, R, T and V) or buffer alone (lanes A, C , E, G, I, K, M, O, Q, S and U) run together for comparison on a 1% agarose gel. (Size of fragments indicated in base pairs). When run with *Sfa*NI plus buffer, the 860bp fragment left undigested runs slower than its counterpart in the enzyme-free lane. This may be due to the enzyme storage buffer or the formation of concatemers with the digested fragments which were resistant to heating. The 482bp and 332bp restriction fragments also ran slower than expected, possibly for similar reasons.

3.3.2 *Frequencies of -174 alleles*

The results of screening 72 Caucasian control subjects and 59 Caucasian (including non-Anglo-Saxon) S-JCA subjects for the -174 polymorphism are shown in Table 3.4.

Although there was no significant difference in the C allele frequency between the two groups (χ^2=3.2, p=0.07, OR=0.49, 95% CI 0.25~1.0) a comparison of the genotype frequencies of the two groups as a whole, using a 3 x 2 χ^2 test, shows them to be significantly different (χ^2=4.15, p=0.0002). This difference is principally due to the marked reduction in the CC genotype frequency, and increase in the GC genotype frequency, in the S-JCA patients.

These differences are highlighted by a comparison of the groups by their CC genotype frequency (using a 2 x 2 χ^2 test), which shows that the reduction in CC genotype frequency in the patient group is highly significant: χ^2= 15.3, p<0.0001, OR 13.4 (95% CI 3.00~59.6). A comparison of the groups by their GC genotype frequency also reveals a significant difference: χ^2=4.94, p=0.02, OR 2.35 (95% CI 1.16~4.77). A similar comparison for the frequency of the GG genotype is not significant: χ^2=0.55, p=0.45, OR=0.70 (95% CI 0.33~1.48).

The observed genotype frequencies in the control group did not differ significantly from those predicted by the Hardy-Weinberg equation:

$p^2+2pq+q^2 = 1$; where p = G allele frequency and q = C allele frequency

when calculated using a 3 x 2 χ^2 test for the genotype distribution within the groups as a whole (controls p=0.6; χ^2=1.02; S-JCA p=0.07; χ^2=5.07). However, the Hardy-Weinberg equation assumes that the genotypes are evenly distributed within a large population. If this is not the case, then the expected genotype frequency may be influenced by sampling error. Sampling error is a function of the allele frequencies and the population size and is given by the equation:

$\text{sampling error}^2 = (p)(q)/2N$ (N = population size)

In this part of the study the sample error only surpassed the difference in frequencies of the control GC genotype, it was equal to the differences in GG and CC frequencies. This suggests that the size of the group was only just adequate. The genotype distribution of the S-JCA samples did not follow the Hardy-Weinberg prediction. However, the frequency differences for all of the genotypes exceeded the sampling error, suggesting that the patient sample was too small for the purposes of accurately calculating the Hardy-Weinberg equilibrium.

In summary, there was a significant reduction in the CC genotype frequency (and to a lesser extent in the GC genotype frequency) in Caucasian patients with S-JCA, compared to controls. This finding is of particular importance when considered in the light of the functional studies of the polymorphism, which are discussed later.

	Controls (n=72)		S-JCA (n=59)	
	C allele frequency 0.53		C allele frequency 0.35	
Genotype	Observed	Expected	Observed	Expected
GG‡	19 (0.26)	15.8 (0.22)	20 (0.34)	24.9 (0.42)
GC†	30 (0.42)	36.0 (0.50)	37 (0.63)	26.9 (0.46)
CC*	23 (0.32)	20.2 (0.28)	2 (0.03)	7.2 (0.12)
Difference between observed and expected genotype frequencies for each group	NS ($p=0.6$; $\chi^2=1.02$)		NS ($p=0.07$; $\chi^2=5.07$)	
Sampling error	0.04		0.04	

(Significance for the difference in observed genotype frequencies between the control and S-JCA groups: * $p<0.0001$; † $p=0.02$; ‡ $p=0.45$ NS).

Table 3.4 -174C allele frequency and -174G/C polymorphism genotype distribution from the *Sfa*NI RFLP study. The allele frequency was derived from the observed genotype distribution (gene counting method). A full discussion of the statistical difference in the frequency difference between each observed genotype in the control and S-JCA groups is given in the text.

The observed frequencies are also compared to the expected distribution of the genotypes, calculated by the Hardy-Weinberg equation ($p^2+2pq+q^2 = 1$; where p = G allele frequency and q = C allele frequency). There was no statistical difference between the observed and expected genotype frequencies for either the control or the S-JCA groups (as measured by a 3 x 2 χ^2 test). However, whereas in the control group the difference in C allele frequency is equal to the expected sampling error (sampling error^2 = (p)(q)/2N ; where N = population size) for the GG and CC genotypes, and only slightly higher than that for the GC genotype, in the S-JCA group it was always much higher than the sampling error.

3.4 Collaborative study results

Following the completion of the experimental work which is presented in this thesis, and my departure from Professor Woo's laboratory, this project was extended by several members of her group, in collaboration with Dr Gary Faulds in Professor Steve Humphries' laboratory in the Rayne Institute, UCL. I was closely involved in this work, as I assisted in the supervision of the Ph.D. student directly involved in the majority of the experimental work in Professor Woo's laboratory, and also held regular discussions with her, our collaborators and Professor Woo. I collated the extended S-JCA patient data, which contained the S-JCA patient data presented in this thesis, and analysed all of the patient and control data obtained. I was the lead author of the published collaborative paper which confirms and extends the findings reported in this thesis (Fishman *et al.*, 1998). The relevant parts of the paper are discussed here, and it is attached to this thesis as Appendix C.

The -174 polymorphism was confirmed in Professor Humphries' laboratory using both *Hsp*92II or *Nla*III in the MADGE PCR-RFLP system (Day and Humphries, 1994). Caucasian control DNA from 383 healthy men and women aged 40 - 75 years, recruited originally from a general practice in North London (the 'Goodinge' sample), was analysed. In addition, 115 Gujarati Indian and 101 Afro-Caribbean DNA samples, also obtained from North London, were analysed. DNA samples from 3 orangutans, 3 chimpanzees and 3 gorillas were also examined.

The previously described RFLP display considerable inter-ethnic variation in their frequencies (Bowcock *et al.*, 1988). This is also true for the -174C allele which is considerably rarer in Gujarati Indians and Afro-Caribbeans (C allele frequency 0.15 and 0.05 respectively), compared to UK Caucasians (C allele frequency 0.403) (Table 3.5). This compares with the C allele frequency for the control sample of 0.53 reported in this thesis. There are no published data concerning the incidence of S-JCA in non-Caucasians, and given the low C allele frequencies in the Gujarati Indians and Afro-Caribbeans, it would be very interesting to investigate the genotypes of S-JCA patients in these groups.

All of the non-human primates examined were GG homozygotes, and so it is possible that this allele is ancestral, with the C allele representing a relatively recent change in the IL-6 5' flanking sequence. This conclusion is, however, based on a very small sample size.

Sample size is likely to be the explanation for the very different genotype frequencies encountered in the larger (Goodinge) UK Caucasian control population, compared to the 72 control subjects examined in this thesis. There was still a significant difference in the overall genotype distribution between the control and S-JCA groups (which was increased to 97 in the collaborative study) but it was not as great as in the thesis (χ^2=7.00, p=0.03) (Table 3.6). The CC genotype frequencies were not significantly different. However, when the S-JCA group was divided by age of onset (as less than or equal to 5 years and greater than or equal to 6 years), a significant reduction in CC genotype frequency compared to the controls was found, but only for the younger age of onset group (χ^2=4.14, p=0.04, OR 0.39, 95% CI 0.15-0.99). This age of onset division was based on the distribution of the age of onset in this cohort, where the majority of patients had a disease onset of below 5 years of age (Figure 3.28). The overall age of onset distribution was skewed, where two overlapping curves could be fitted to the data. The peak age of onset for the majority of the patients was at two years, which is consistent with previous epidemiological studies in the UK, Europe and North America (Laxer and Scheider, 1993).

Our collaborators also detected a G to A transition polymorphism at position -597. However, no data was available at the time of writing to provide a detailed assessment of the frequency of this additional polymorphism, which I was not involved in the discovery of, and report it here only for completeness.

	UK Caucasian male and female controls (n=383)	Gujarati Indians (n=115)	Afro-Caribbeans (n= 101)
Genotype	Observed Expected*	Observed Expected*	Observed Expected*
GG	144 (0.38) 137 (0.36)	85 (0.73) 84 (0.72)	92 (0.91) 91 (0.90)
GC	169 (0.44) 184 (0.48)	28 (0.24) 29 (0.25)	9 (0.09) 10 (0.10)
CC	70 (0.18) 62 (0.16)	2 (0.03) 2 (0.03)	0 0
C allele frequency (95% CI)	0.403 (0.37-0.44)	0.150 (0.094-0.184)	0.05 (0.016-0.072)
Overall difference obs/expt.	NS (p=0.53)	NS (p=0.98)	NS (p=0.97)
Sampling error	0.02	0.02	0.01

*As expected by the Hardy-Weinberg equation.

Table 3.5 Results from the collaborative study: 1- Population genotype and allele frequencies of the -174 polymorphism observed by MADGE PCR-RFLP, in subjects of different ethnic origin. In all three racial groups the genotype frequencies were in Hardy-Weinberg equilibrium, ie the observed and expected frequencies were not significantly different.

	All S-JCA patients (n=97)	S-JCA Onset ≤5 years old (n=63)	S-JCA Onset ≥6 years old (n=34)	UK Caucasian male and female controls (n=383)
Genotype	Observed Expected*	Observed Expected*	Observed Expected*	Observed Expected*
GG	29 (0.30) 34 (0.36)	18 (0.29) 23 (0.36)	11 (0.32) 11 (0.33)	144 (0.38) 137 (0.36)
GC	57 (0.59) 47 (0.48)	40 (0.63) 30 (0.48)	17 (0.50) 17 (0.49)	169 (0.44) 184 (0.48)
CC	11 (0.11) 16 (0.16)	5 (0.08) 10 (0.16)	6 (0.18) 6 (0.18)	70 (0.18) 62 (0.16)
C-allele frequency (95% CI)	0.407 (0.34-0.48)	0.397 (0.31-0.48)	0.426 (0.35-0.52)	0.403 (0.37-0.44)
Comparison of overall genotype distribution with controls	p=0.03 χ^2=7.00	p=0.01 χ^2=8.98	p=0.79 χ^2=0.48	

Table 3.6 Results from the collaborative study: 2- Population genotype and allele frequencies of the -174 polymorphism in the S-JCA patients, compared to the UK control Caucasian group. The S-JCA patients as a whole, and both of the subgroups, were in Hardy-Weinberg equilibrium (ie. differences between observed and expected* genotype frequencies were not significant: All S-JCA patients p=0.32; S-JCA Onset ≤5 years p=0.16; S-JCA onset ≥6 years p=1.0). There was a significant difference in the overall genotype distribution between the controls and the entire S-JCA patient group (p=0.03, χ^2=7.00). This difference was even greater when the controls were compared to S-JCA patients with a disease onset of ≤5 years of age (p=0.01, χ^2=8.98).

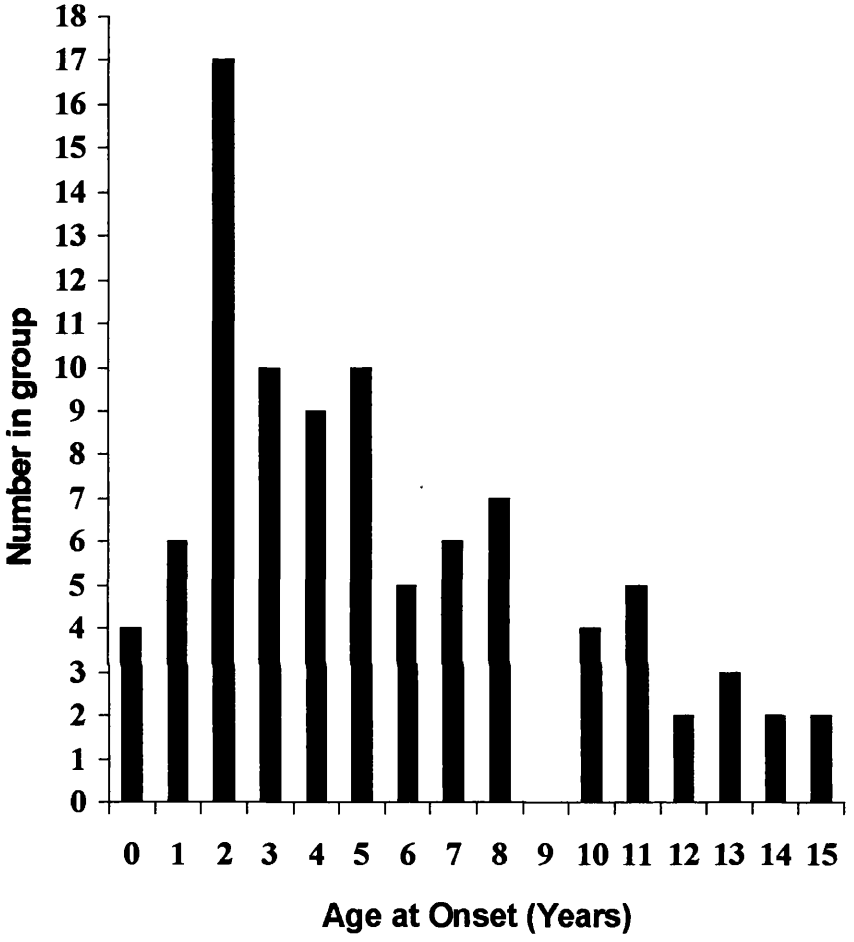


Figure 3.28 Distribution of S-JCA patients in the collaborative study by age of disease onset.

3.5 Functional Assessment of the IL-6 5' Flanking Region Polymorphisms

Once the allele frequencies for the G to C polymorphism at position -174 within the 5' flanking region of the IL-6 gene had been investigated, and the genotype frequencies in the control and S-JCA populations compared, a functional difference resulting from the polymorphism was sought. The polymorphism lies within the region of the gene controlling transcription, but more importantly it is located within a section previously suggested to have a negative effect on gene transcription (Ray *et al.*, 1989). Thus, there was the possibility that the two alleles could differ in their ability to induce the expression of IL-6 under the same circumstances.

3.5.1 *Plasmid Construction*

Correct orientation of the pp1 product and its truncated analogues within the luciferase reporter vector was vital for the correct interpretation of the functional comparison of the two -174 alleles. The success of sub-cloning from pCRII into pGL2 was limited, as many of the pCRII constructs contained the pp1 fragment cloned in the reverse (3' to 5') orientation (the TA cloning method is non-directional). As the subcloning method was directional, the resulting pGL2-Basic luciferase reporter constructs would have contained incorrectly orientated inserts. In order to avoid the necessity of screening a large number of constructs to exclude these incorrect ones (especially as the desired pp1 fragment was frequently not available as a 5' to 3' clone within pCRII), a directional PCR-based cloning protocol was developed. This method used primers with a redundant sequence which contained the appropriate restriction enzyme site. With these primers, the correct orientation of inserts of varying lengths (and transcription factor binding site compositions) was almost certain. Additionally, the pGL2 plasmid was abandoned in favour of the pGL3 plasmid, which had been modified to increase the efficiency with which it could express luciferase. Therefore the opportunity was taken to create new inserts, rather than subclones, for these new plasmids.

At each stage of the manufacture of the plasmid constructs they were analysed to confirm that they contained the correct pp1 sequence, in the correct orientation. Aliquots of the samples were analysed by electrophoresis on a 1% agarose gel to confirm their correct

size both before, and after phenol:chloroform purification (Figure 3.29). Following ligation, the miniprep plasmid underwent PCR for the pp1 inserts and restriction digestion (Figure 3.30). Finally, the maxiprep/midiprep plasmids were sequenced by the Vistra system. All these stages ensured that incorrectly cloned pp1 fragments were not used in the transfection experiments, that each construct was correctly identified as -174G or-174C and that all the constructs were A_8T_{12}.

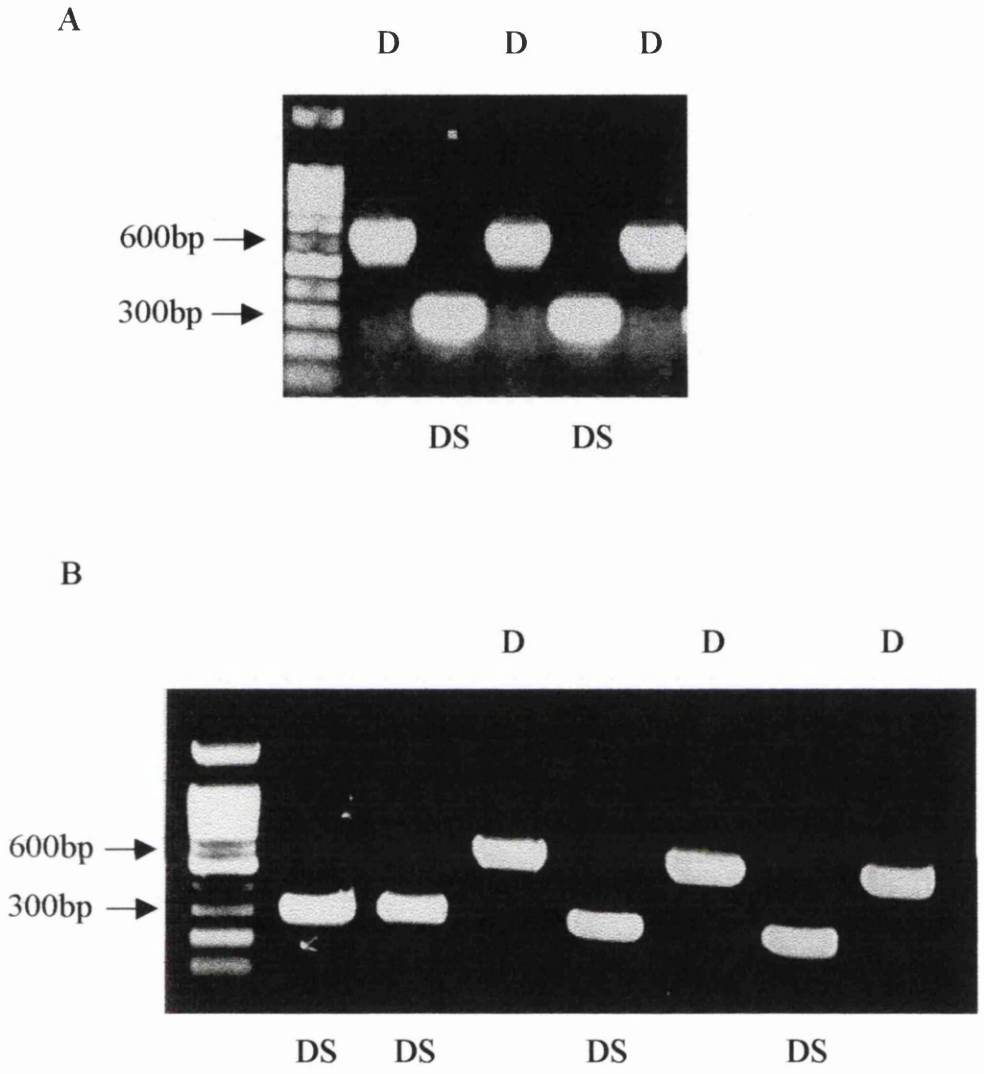


Figure 3.29 Confirmation of correct amplification of the full length construct insert prior to ligation into pGL3. A) 1% agarose gel electrophoresis of PCR fragments showing correct length (samples 'D'[611bp long]; 'DS' length deletion products [280bp long] are also shown on this gel, for ligating as 'ΔATAP-G or -C'). B) After phenol:chloroform purification an aliquot of the samples was run on a 1% agarose gel to check the sample was not lost or contaminated.

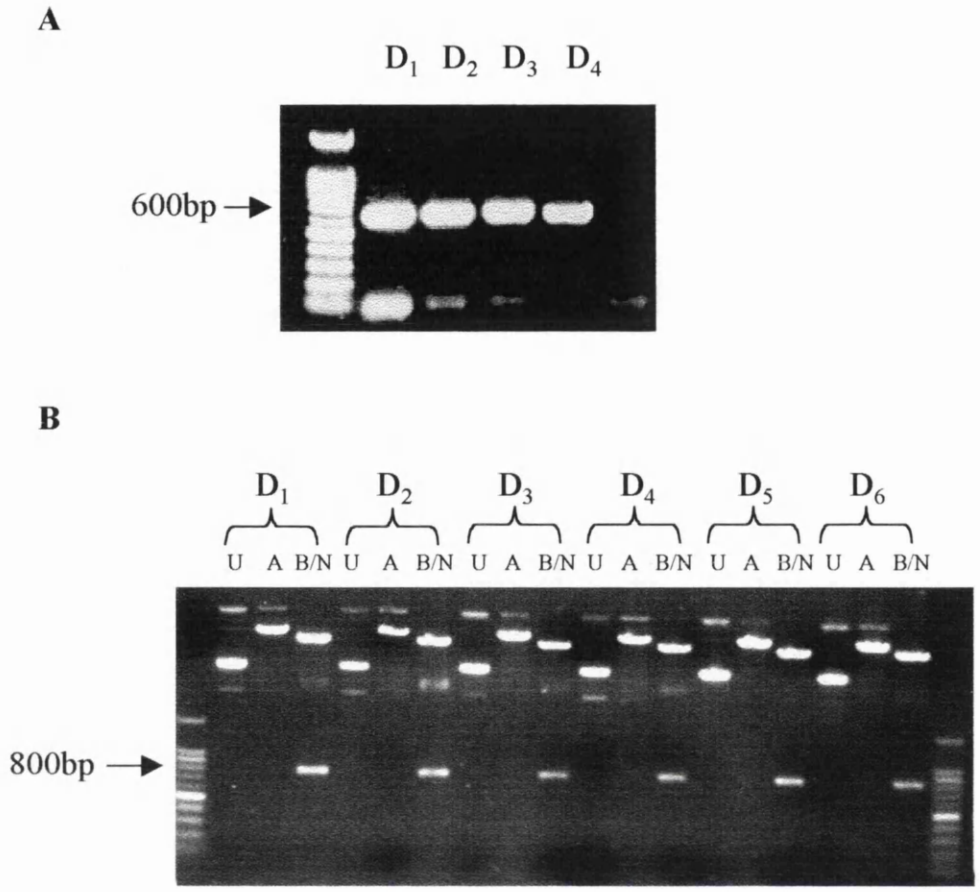


Figure 3.30 Confirmation of correct ligation of the full length insert into pGL3. A) PCR amplification of the full length (611bp, pp1) insert (samples D_1 to D_4) from four different pGL3 plasmids (aliquots run on a 1% agarose gel). B) Restriction enzyme digestion of samples D_1 to D_6 from six different pGL3 midi/maxipreps with *Aat*II (which cuts only within the ligated pp1 fragment), and with *Not*I plus *Bgl*I, which removes the ligated fragment with a small amount of flanking DNA from the pGL3 polylinker (producing an 814bp fragment from a construct containing the correctly sized pp1 insert).

As discussed above, a variation in the relative base composition and overall length of the A_nT_n tract was discovered prior to the commencement of the functional studies. In order to control for this variation, all the constructs were A_8T_{12}. Although this was not the most frequent pattern overall, it had to be used, as the -174C allele was associated with this pattern in 19/20 cases.

Milligram amounts of transfection-grade plasmid were prepared by the traditional alkaline lysis maxiprep method, and a commercial midiprep kit method. In the former, each plasmid sample was subjected to two rounds of caesium chloride gradient centrifugation ("double-banding") in order to completely remove incomplete DNA fragments and nicked plasmid, and to clean the plasmid DNA. The use of the midiprep kit reduced the time taken to prepare the plasmids, removed the necessity for using ethidium bromide with the corresponding risk of it contaminating the plasmid if not fully removed, and also allowed comparison of two different methods of plasmid production, in case the results of the transfection experiments were influenced by one particular method. However, certain problems were encountered with the commercial kit: the manufacturer's recommendation for the duration of centrifugation during the ethanol precipitation was not of adequate length to precipitate most of it on to the Corex tube base, and it therefore had to be doubled. In addition their recommendation for a maximum volume of bacterial culture to be processed could not be approached without significantly contaminating the eluent containing the plasmid with debris. This was likely to have contributed to the failure of several transfection experiments.

Initially, the THP-1 cell was chosen to be the recipient of the reporter constructs. This is a human monocytic cell line, derived from a child with acute monocytic leukaemia (Tsuchiya *et al.*, 1980) which does not constitutively express IL-6. The difficulties encountered in achieving a significant level of luciferase after transfection of the pGL2-Control constructs (see below, 3.5.2.1) lead to a change to using the HeLa cell as the recipient cell line.

HeLa cells were used in some of the first published studies that analysed the 5' flanking region of the IL-6 gene to delineate transcriptionally important sequences (for example Ray *et al* 1990) and were therefore considered to be an ideal cell line for these

transient transfection experiments. They are known to produce IL-6 following stimulation with pro-inflammatory mediators such as IL-1 and LPS. Moreover, from a practical perspective, HeLa cells were readily available in the laboratory, grow easily and quickly and are recognised to be efficiently transfected by the calcium phosphate DNA precipitation method. This method had been previously optimised for HeLa cells in the laboratory, and was the method used to transfect the HeLa cells in the early published studies (eg, Ray *et al.*, 1988). IL-1 and LPS were also chosen to stimulate the cells because of their previous use in the published studies (eg, Ray *et al.*, 1988) and because it is known that the IL-6 promoter is strongly activated by these mediators (as described in 1.4.4).

3.5.2 *Transfection Results*

3.5.2.1 Reporter constructs containing the full length pp1 fragment: a) THP-1 cells

Initial experiments employing the calcium phosphate/DNA precipitation method of transfecting THP-1 cells were not successful. The method relies on cells being adherent to the base of the plastic tissue culture wells. This was achieved by differentiating them into macrophage-like cells by culturing them with vitamin D_3. However, after 4 hours incubation with the calcium phosphate/DNA precipitate, the cells floated free from the base of the wells and were impossible to separate from the precipitate. A suitable modification of the method could not be found and this protocol was not pursued.

Transfection by electroporation of the THP-1 cells was more successful, but to a limited extent. No significant luminescence could be detected when transfecting with less than 40μg of plasmid DNA, and then all of the cell lysate had to be used for the analysis in order to achieve this result. The optimum electroporation voltage was determined to be 350V, with the maximum luminescence achieved by transfecting with 60μg of pGL2-Control plasmid DNA (Figure 3.31 and 3.32). These results were determined for the absolute luminescence detected. When the cells were co-transfected with the RSV-β-galactosidase expressing plasmid, in order to correct for transfection efficiency, no discernable luciferase activity could be detected. This was probably due to the toxicity of such a large amount of plasmid DNA applied to the cells. Therefore it would not have been possible to correct for transfection

efficiency by any method currently available in the laboratory. Moreover, this relatively poor luciferase expression was achieved from the control plasmid, which should have been able to express large amounts of luciferase through its component SV40 promoter and enhancer sequences. The likelihood was, therefore, that the degree of expression from the pGL2-Basic constructs would have been even less, and probably not enough to observe a difference between the various alleles. It was decided that (in this instance) the THP-1 cell line was not an appropriate cell line in which to test the pGL2 constructs for differences in promoter strength between the two -174 alleles. This cell line was therefore abandoned in favour of the HeLa cell, and at the same time a change to the more advanced pGL3 plasmid was made.

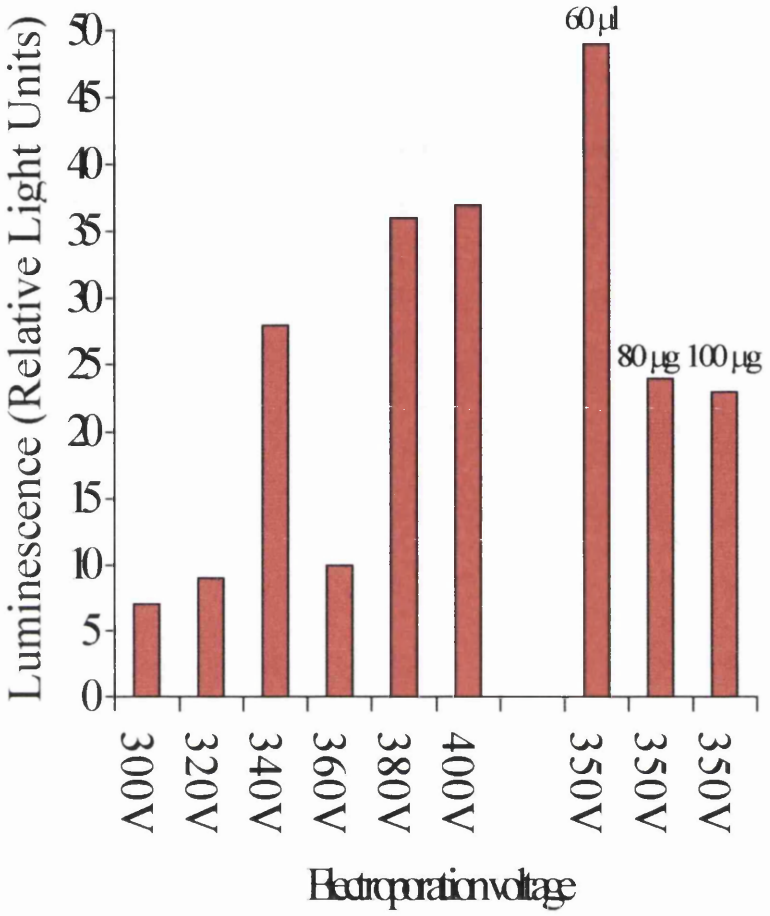


Figure 3.31 THP-1 transfections by electroporation-1: Plasmid concentration and voltage dose/response. In these experiments, 40µg of pGL2-Control plasmid was transfected by electroporation at the voltages given, except where shown, when 60µg, 80µg or 100µg was transfected by electroporation at 350V. (The 40µg/360V experiment probably suffered from a poor transfection efficiency, compared to the experiments for other parameters).

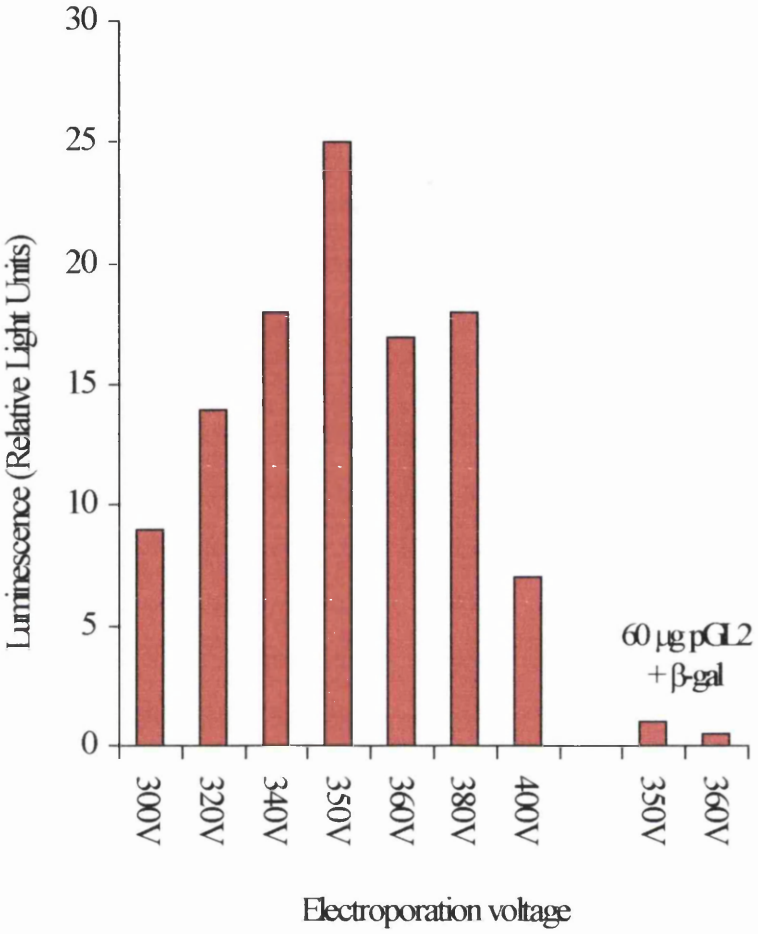


Figure 3.32 THP-1 transfections by electroporation-2: Voltage dose/response for a fixed plasmid concentration, and co-tranfection with the β-galactosidase-expressing plasmid. This experiment shows that the maximal luciferase expression occurs with 60µg of pGL2-Control plasmid, transfected with a 350V electroporation. Co-transfection with the β-galactosidase-expressing plasmid results in a drastic reduction in transfection efficiency, probably related to the toxic effect of such a large amount of DNA applied to the cells.

3.5.2.2 Reporter constructs containing the full length pp1 fragment: b) HeLa cells

The results of transient transfection of the pGL3-Basic constructs, containing each of the two alleles of the -174 polymorphism, into HeLa cells are shown in Figure 3.33. This graph represents the combined data from two separate transient transfection experiments (each performed in duplicate). In the first experiment, the transfected plasmids had been isolated by standard alkaline lysis maxiprep. In the second experiment they were isolated using the Qiagen midiprep kit. The cells were cultured for 24 hours post-transfection, after which they were harvested and the luciferase activity within a 20µl aliquot of the cell lysate was measured.

These experiments were a direct comparison between the responses of the -174G and -174C constructs (both A_8T_{12}) to stimulation with either LPS or IL-1. The results are expressed as the fold change in corrected luciferase expression, relative to the corrected luciferase expression from the unstimulated -174G construct. Correction for transfection efficiency and lysate recovery was made by dividing the luciferase luminescence by the β-galactosidase luminescence from the same cells.

In unstimulated cells, the -174C construct showed 0.62 ± 0.15 fold lower expression ($p<0.005$) than the -174G construct. Following stimulation with lipopolysaccharide (LPS) or IL-1, expression from the -174G construct increased by 2.35 ± 0.10 fold and 3.60 ± 0.26 fold respectively, compared to the unstimulated level (both $p<0.001$). However, expression from the -174C construct did not change significantly following these pro-inflammatory stimuli ($p=0.5$ and $p=0.46$ respectively). Overall, LPS- and IL-1-induced luciferase expression from the -174G construct was, respectively, 4.55 ± 0.39 fold and 4.38 ± 0.54 fold that induced from the -174C construct (both $p<0.0001$). Untransfected cells showed negligible luciferase or β-galactosidase expression. The pGL3-Basic plasmid directed very low levels of luciferase expression, which did not change significantly when stimulated with LPS (0.6 ± 0.1 fold to 0.5 ± 0.2 fold, NS) or IL-1 (0.6 ± 0.1 fold to 0.8 ± 0.3 fold, NS).

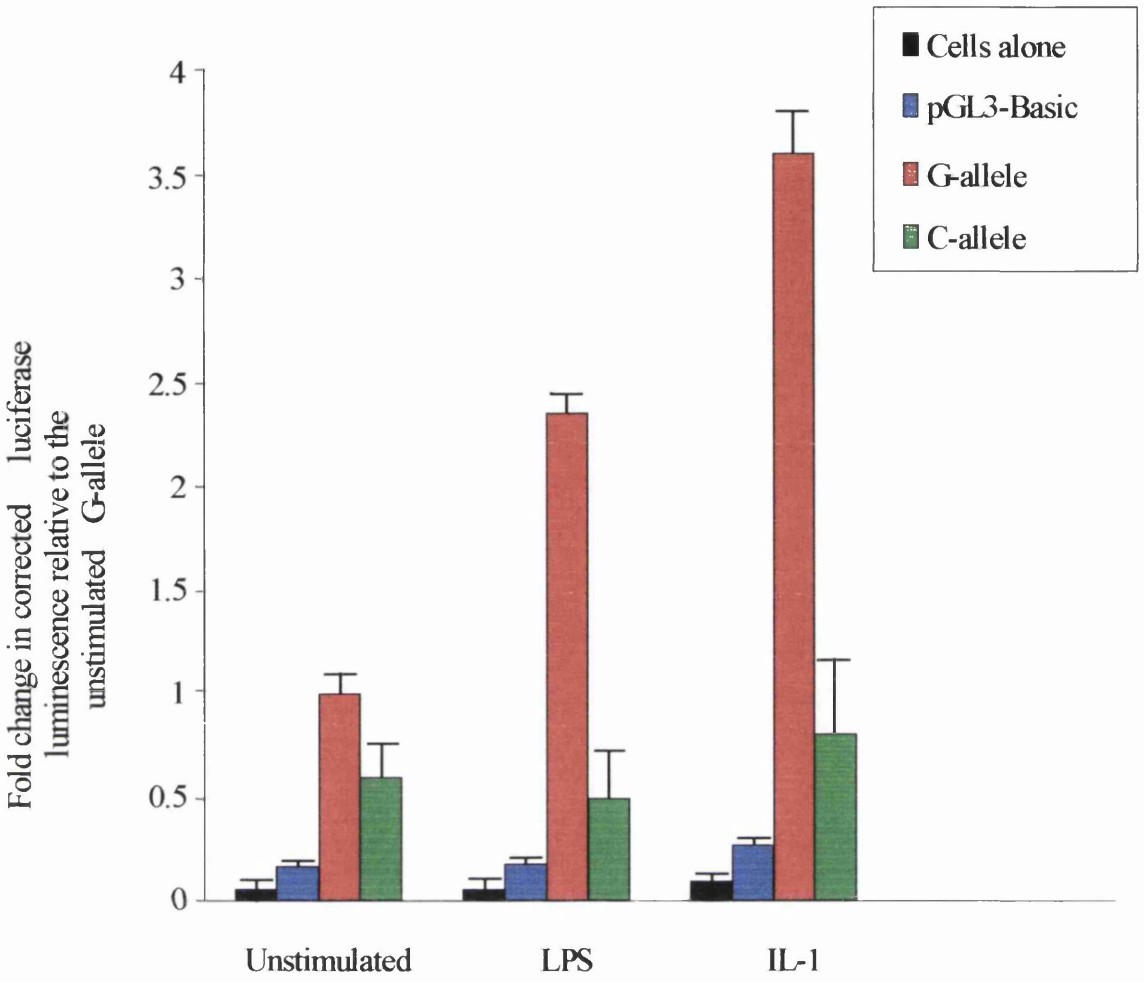


Figure 3.33 Functional comparison of the -174G and -174C alleles: full length constructs. The fold difference in corrected luminescence (24 hours post-transfection) between samples of untransfected cells; cells transfected with the pGL3-Basic plasmid (which does not contain a functional eukaryotic promoter or enhancer sequence); cells transfected with the -174G allele in pGL3-Basic; and cells transfected with the -174C allele in pGL3-Basic. The cells were stimulated with either IL-1 (10U/ml) or LPS (10μg/ml). Correction for transfection efficiency was made by dividing the luminescence value obtained for luciferase by that obtained for β-galactosidase, which was expressed by the RSV β-gal plasmid co-transfected with the pGL3-Basic construct. The fold change in corrected luminescence is shown relative to the unstimulated -174G allele.

3.5.2.3 Reporter constructs containing length deletion mutants of the pp1 fragment

The second series of transfection experiments investigated the effect of deleting specific sections of the pp1 fragment. These length deletions removed specific transcription factor binding sites. The resulting changes in luciferase expression were compared between the length deleted constructs containing either of the two -174 alleles. These constructs were all A_8T_{12} and their composition is shown in Figure 3.34. The composition of these constructs was confirmed by 1% agarose electrophoresis post-PCR, followed by *Sfa*NI digestion of an aliquot of the PCR product (Figure 3.35). The samples were then sequenced by the Vistra system as a final confirmation of their correct composition.

Following IL-1 stimulation, the greatest luciferase expression was observed from the full length G-construct, as was expected, because it contained all of the important transcription factor binding sites. Therefore, the expression from the remaining constructs is shown as a fold decrease in expression relative to the expression from this construct (Figure 3.36). The expression from the full length C-construct was 0.25 ± 0.17 of that of the G-construct, which is consistent with the results obtained in the previous experiments. The differential response to IL-1 was no longer present for the ΔAT-G and ΔAT-C constructs, which demonstrated a 0.68 ± 0.03 and 0.64 ± 0.17 respective reduction in expression compared to the full length G-construct. Further deletion of the sequence resulted in a relative expression of only 0.11 ± 0.06 and 0.19 ± 0.14 for the ΔATAP-G and ΔATAP-C constructs respectively. Finally, the shortest construct (ΔATAPGC) demonstrated only baseline expression (comparable to transfection with the pGL3 plasmid without a cloned insert). A statistical analysis of this data was not possible because of the small number of experiments.

In summary, there is a significant difference in the effectiveness of the two alleles of the -174 polymorphism in inducing expression of the luciferase gene, when cloned into the pGL3 plasmid vector. This difference becomes less apparent when the cloned insert containing the polymorphic region was truncated. The expression from the constructs also fell as the insert became shorter. These findings are discussed in 4.2.2.

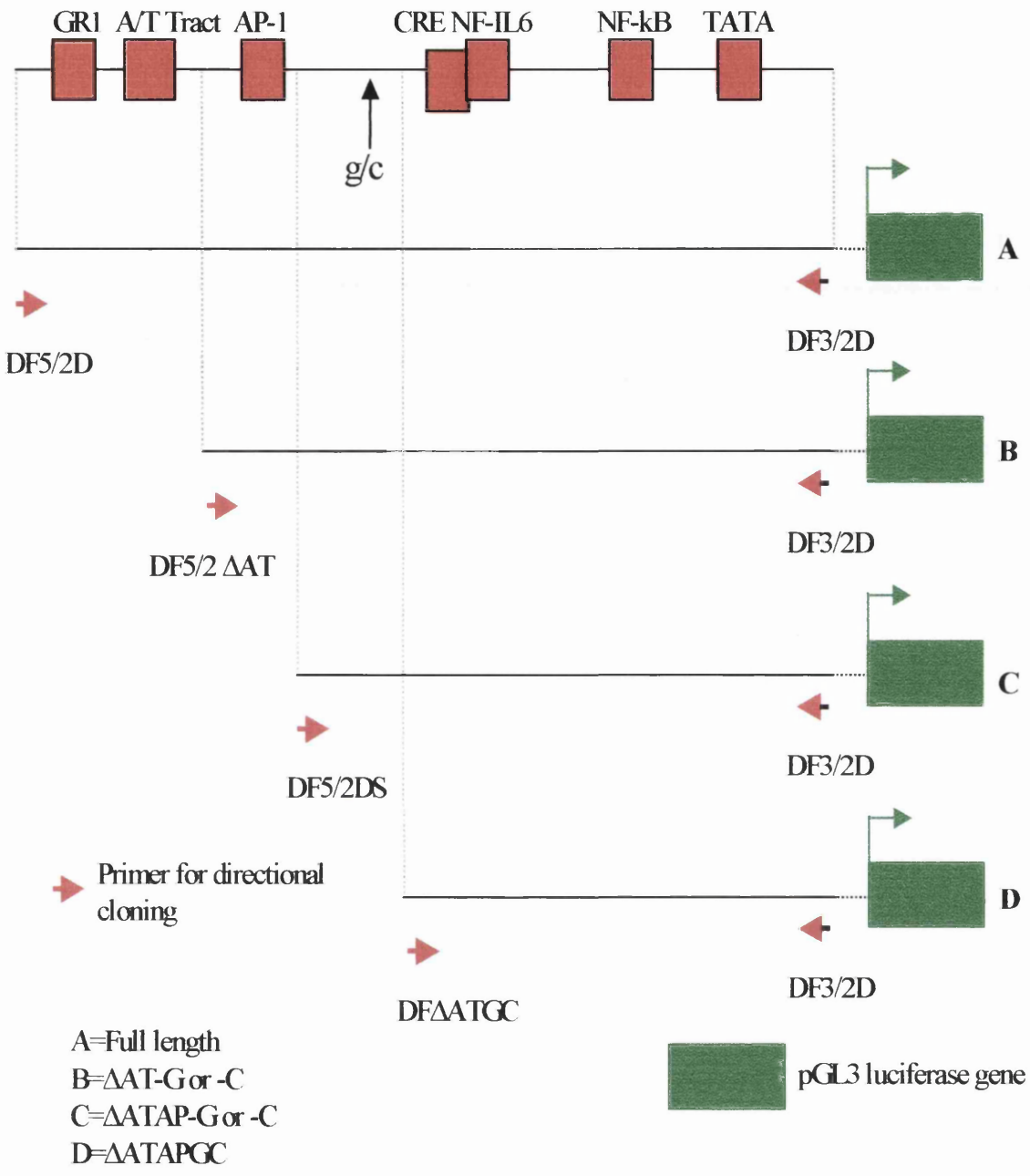


Figure 3.34 Composition of length deletion mutants derived from the IL-6 5' flanking region. Each mutant is shown with the primer used for its manufacture and the transcription factor binding sites it contains.

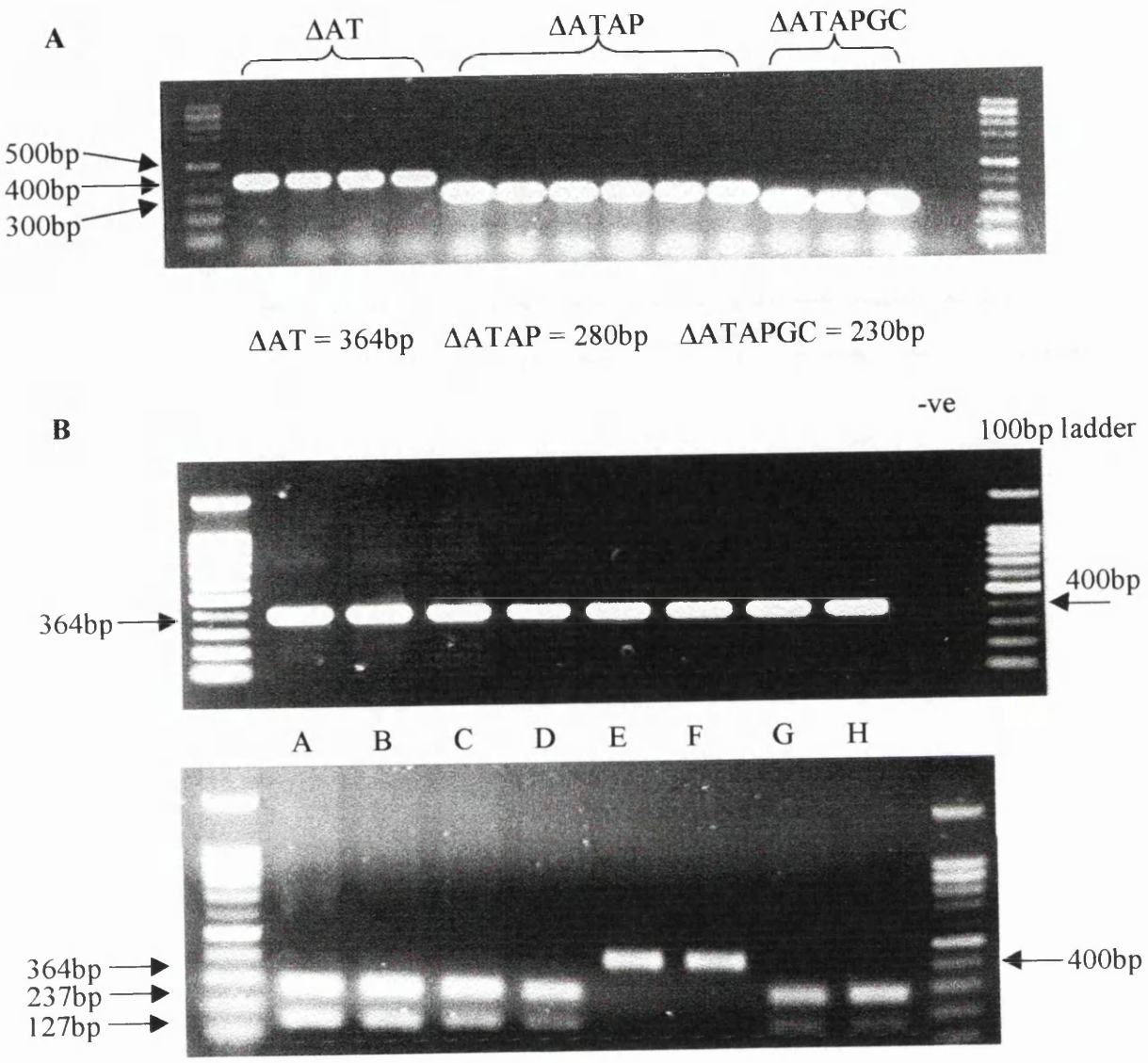


Figure 3.35 Confirmation of correct amplification of the length deletion construct inserts prior to ligation into pGL3. A) 1% agarose gel electrophoresis of several examples of each of the three length deletion mutant PCR products confirming their correct length. B) Following PCR confirmation (upper gel) the eight ΔAT samples (in this example) were digested with *Sfa*N1 in order to ensure their correct -174 allele composition (lower gel). In this example samples E and F were -174C, the rest were -174G.

No further confirmatory plasmid preparation digests/PCR's were undertaken, as the samples were all sequenced prior to consideration of their use in transfection experiments.

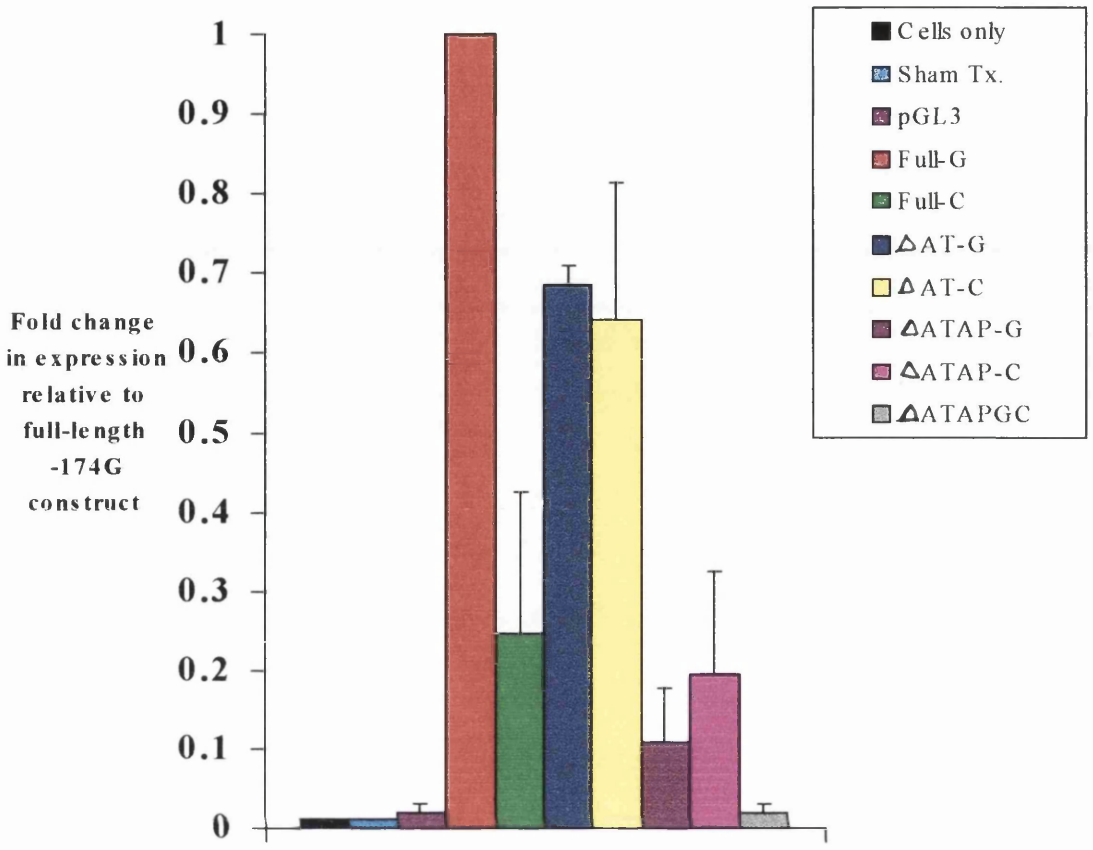


Figure 3.36 Functional comparison of the -174G and -174C alleles: length deletion constructs. The results of transfecting HeLa cells with constructs containing the various length deletion mutants of the 5' flanking region, as either the G- or C-alleles. The results are corrected for transfection efficiency by co-transfecting with the β-galactosidase plasmid and dividing the luciferase luminescence result by that for β-galactosidase. The expression of luciferase is shown relative to the full length G-allele. (Full: entire pp1 insert; ΔAT: no A_nT_n tract; ΔATAP: no A_nT_n tract or AP-1 site; ΔATAPGC: no A_nT_n tract, AP-1 site or -174G/C polymorphism site).

3.5 3' Flanking Region Polymorphism

The region of the IL-6 gene immediately downstream of the coding sequence is recognised to contain several RFLP which are also detectable by PCR (Bowcock *et al.*, 1989). Although the investigation of these 3'flanking region polymorphisms in S-JCA was not a primary goal in this study, the availability of suitable numbers of DNA samples from patients and controls, the relative simplicity of the previously published method and the connection between previously recognised IL-6 polymorphisms in the 3' flanking region (3'FR) and this current search for 5' polymorphisms, all suggested that a brief examination of the S-JCA samples during the course of this study was not inappropriate. The only difficulty encountered with the method was in adequately separating each PCR band. This was achieved after electrophoresis overnight through a 2.5% agarose gel, at low voltage to reduce the 'smile' distortion effect (Figure 3.37). Even then, the fragments did not run at their expected sizes, compared to the markers in the 100bp ladder. This is not unexpected, as this marker was not designed to run at these electrophoresis parameters. An exact sizing of the fragments by comparison to the 100bp markers was therefore impossible, but they were compared in size to the pp1 fragment (Figure 3.37, lower gel, sample '608'). Three fragments were produced by this PCR method. The smallest fragment was the commonest, and because it ran close to the 611bp pp1 marker, it was assumed to be the 610bp fragment described by Bowcock *et al.* (1989). A second fragment, assumed to be the 640bp fragment, was observed less frequently and an assumed 680bp fragment was observed rarely. Table 3.7 shows the distribution of the genotypes for the subjects studied.

There was no significant difference between the groups when compared with respect to the frequency of the three commonest genotypes (610bp, 640bp, 610bp/640bp) ($p=0.9$, $\chi^2=0.13$). When the groups were compared by the frequency of heterozygosity for their 3' FR polymorphism there was also no significant difference between the groups ($p=0.9$; $\chi^2=0.009$).

In summary, on the basis of the allele frequencies obtained from the limited cohort of patients and controls tested here, the 3'FR polymorphisms do not appear to be associated with the development of S-JCA.

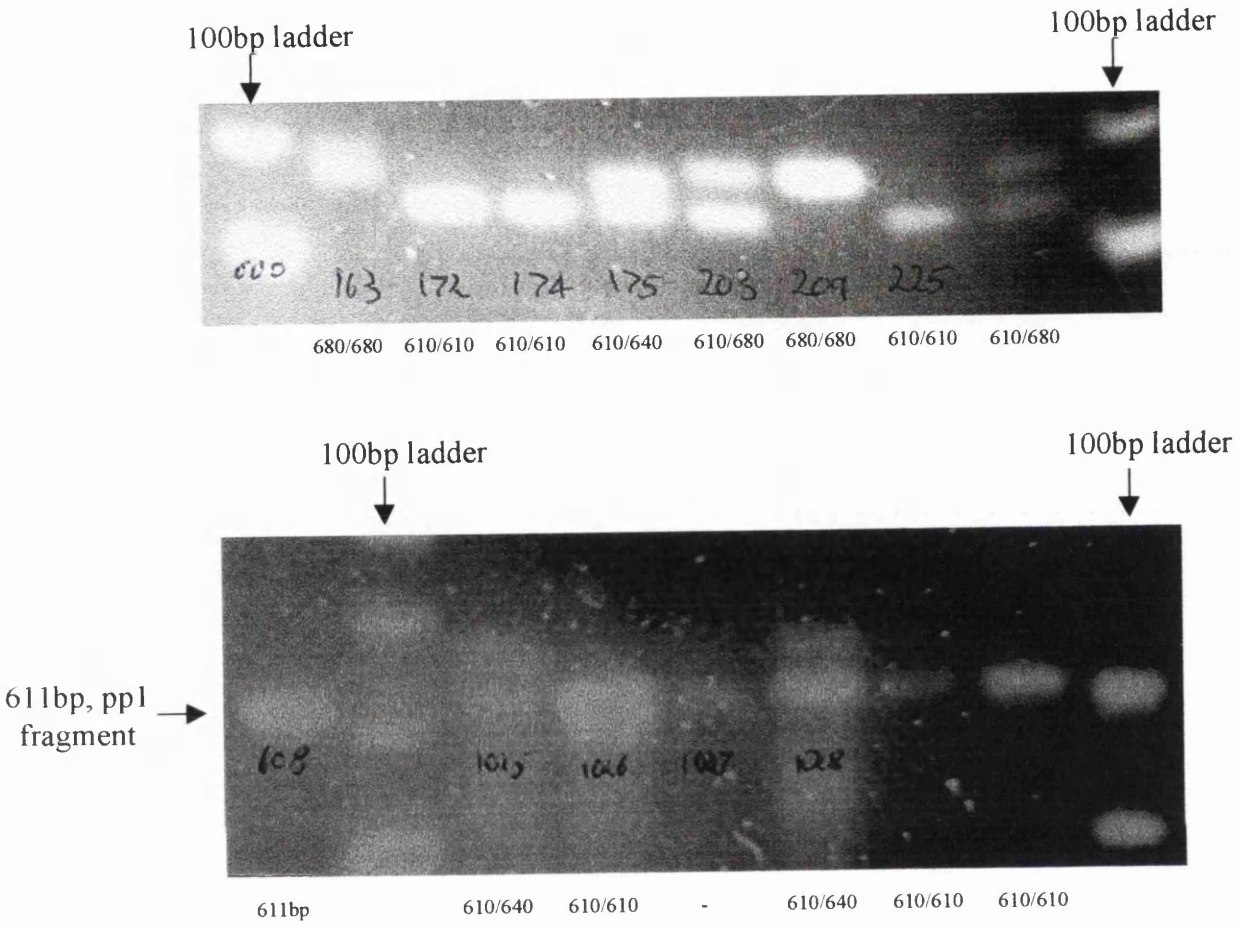


Figure 3.37 3' flanking region PCR products run on 2.5% agarose gels. The lower gel shows the samples run against the 100bp ladder and the 611bp pp1 PCR product. The smallest 3'flanking region PCR product corresponds to the 610bp product described by Bowcock *et al.* (1988), the others being 640bp and 680bp. The genotypes is shown below each sample. (The numbers below each band correspond to the individuals laboratory number).

Genotypes Subjects	610/610	640/640	610/640	680/680	610/680
Controls (n=69)	37	6	23	2	1
SA patients (n=25)	13	2	10	0	0

Table 3.7 IL-6 3' flanking region polymorphism genotypes for a cohort of S-JCA patients and controls.

CHAPTER 4

GENERAL DISCUSSION and FUTURE WORK

This research has yielded a number of novel and potentially very important findings. The implications of these discoveries are discussed later in this section, after some general points concerning methodology.

4.1 Methodology

4.1.1 *SSCP*

Single-strand conformational polymorphism analysis is frequently employed in studies which examine a known gene sequence for polymorphisms. The sensitivity of the method is mainly determined by the length of the fragment studied. In one analysis 70% of known mutations in the p53 gene were identified in 200bp fragments, but up to 97% were detected when the fragments were only 150bp long (Sheffield *et al.*, 1993). The sequence flanking the mutation does exert a small influence over the sensitivity of the SSCP assay, as do the electrophoresis parameters, but the type of mutation (base transition or transversion), is less important.

There are now several developments of the original protocol that use non-radioactive detection methods, such as silver staining, to rapidly reveal SSCP band patterns. Because of its relative complexity compared to RFLP analysis, SSCP is usually limited to an initial search for the presence of polymorphisms in a sequence, and is not usually employed to examine the population allele frequency. However, where a suitable RFLP is not present, or where a complex polymorphism is known to exist as a haplotype, it has been used successfully.

There are several potential problems associated with SSCP analysis. It is feasible that the polymorphism may not produce enough of a conformational difference between the fragments to reveal itself. Where more than one polymorphism exists in the sequence it may provide conflicting information. Most importantly, the finding of one pattern of band differences does not rule out the possibility that more than one polymorphism is present in the sequence. What is more, it is not possible to predict the location of a given polymorphism, as the identity of the restriction digest product cannot be inferred from its position on the gel.

This last feature of SSCP could potentially have been resolved in this research by

isolating the restriction products and then analysing them separately on the gel. Alternatively, the whole sequence under investigation could have been broken down into smaller sections by designing PCR primers which amplified slightly overlapping sequences. These could then have been analysed by SSCP individually and the source of the altered band pattern would have been immediately obvious. In addition, it would have been necessary to sequence only that fragment with the altered gel banding position. This was not done, as it was considered possible that more than one polymorphism existed in the pp1 fragment. This turned out to be the case. Thus, SSCP was used here solely to confirm the hypothesis that polymorphism(s) existed in the IL-6 gene. Moreover, the resolution obtained on the SSCP gels was only good enough to identify the presence of band pattern differences. This was despite an adaptation of the original protocol, whereby a different restriction enzyme was used (*Mae*III) and the amount of radioactivity was increased. Undoubtedly, the protocol could have been further refined in order to obtain a method suitable for screening a large population. However, for the reasons already stated this was not pursued.

Previous RFLP studies, which examined the gene using only enzymes commercially available a decade ago, had overlooked what turned out to be a very common polymorphism. This was the first study to examine systematically the 5' flanking region of the IL-6 gene for polymorphisms. Overall this SSCP method can therefore be judged to have been highly successful, as it demonstrated the presence of at least one previously unrecognised polymorphism in this area. However, in order to fully characterise this polymorphism, to confirm the actual number of polymorphisms in the region and to obtain a preliminary estimate of the frequency of the polymorphisms, I went on to sequence the entire pp1 fragment.

4.1.2 *Sequencing Protocols*

A considerable amount of time was spent in sequencing the normal control and S-JCA patient DNA samples. Initially, very poor results were obtained, mainly because of the poor quality and low concentration of these original DNA samples. In order to obtain template DNA of a standard suitable for sequencing it was necessary to firstly clone the individuals' pp1

fragments into a suitable vector (pCRII), and then to amplify and purify these samples as plasmid minipreps. From here the samples were successfully sequenced. There are several problems which are inherent in this approach. First, there is the potential for the introduction of random errors into the sequence during its manipulation from genomic DNA to sequenced strand. Although endogenous bacterial DNA polymerases have a high level of fidelity (estimated at one error per million bases) that of the PCR *Taq* polymerase is around one error per 10,000 bases. PCR base incorporation errors are more common in large PCR products. As the pp1 fragment is only 613 bases long, it is unlikely that a random error would be incorporated into the sequence. Moreover, the consistency with which the polymorphism at -174 was observed makes it highly unlikely that it could be due to a PCR error. Moreover, the same (-174G/C) polymorphism was observed at a high frequency in this study, and in samples sequenced directly from the PCR reaction and not subjected to further potential sources of error during the cloning and plasmid miniprep stages. No other single base polymorphisms were observed (with the exception of the A_nT_n tract), and these would be expected if random PCR errors were frequent enough to influence the findings of this study, since base insertion errors should occur at random throughout the amplified sequence.

The above arguments hold true if it is assumed that the fidelity of *Taq* polymerase is independent of the sequence of the DNA template. This is not always the case, as it has been recognised, for example, that amplification of sequences rich in GC residues is more error-prone. Thus, it is possible that the sequence around an observed polymorphism may be responsible for its appearance. Although this is unlikely to be the case for the -174G/C polymorphism, the observed A_nT_n tract polymorphisms may arise from slippage of *Taq* polymerase when attempting to reproduce the long repeats of adenine and thymine bases.

Another problem inherent in sequencing a cloned PCR product is that the resulting sequence represents only one of the pair of chromosomes from the original genomic DNA sample. Thus, this method cannot be used for assessing the true population frequencies of the -174 alleles, as 50% of the IL-6 genes from the total population are unrepresented. In addition there is also the potential for missing another polymorphism altogether.

From a practical point of view, PCR amplification of the genomic pp1 (or pp2) sequence, followed by its cloning and then sequencing was not a viable method for

determining the frequency of the -174G/C polymorphism in a large population. This was despite the good results obtained from each of the component steps of this method. The initial PCR-amplification stage was usually the 'rate-limiting' step in this process, as it was frequently impossible to amplify a clean band from the genomic DNA source despite altering the various PCR reaction parameters. This problem was not unique to the older genomic samples. DNA produced using the Nucleon Kit was often highly insoluble, making it difficult to produce aliquots of the samples which actually contained DNA. Those that did were not necessarily good as PCR templates. Poor solubility of the DNA, rather than its degradation, was often suspected to be the cause of problems with the older samples, as they did not reveal fragmentation of DNA when aliquots were run on 1% agarose gel.

The TA cloning kit was a highly efficient method of producing clones containing the pp1 fragment. In combination with the use of cPCR as a screening method, it was rare that a cloning experiment did not produce at least one positive clone. cPCR was found to be a highly specific method of detecting positive clones. Initially, those clones which yielded cPCR bands of incorrect length were still processed through the miniprep stage and, when checked by restriction digestion and PCR, were always found to contain truncated or incorrect inserts. Clones correct by cPCR were always correct when the miniprep plasmid insert was assessed by restriction digestion and PCR. False negative cPCR results sometimes occurred, often when a large amount of colony material was deposited in the reaction mixture. Thus the sensitivity of the cPCR method was not as high as its specificity.

The plasmid miniprep kit consistently yielded large amounts of high quality plasmid which was used to generate sequence data both by thermal cycle sequencing and using the Vistra automated sequencer. The former method was overshadowed by automated sequencing in most respects: the entire cloned pp1 fragment could be sequenced in a single run; data could usually be obtained from 10 bases downstream of the sequencing primer site; the quality of the sequence data was often higher by the automated method, with fewer compression artefacts and 'stops' (one area in particular, around base -194, consistently failed

to sequence correctly by the TCS method). Although it was not possible to sequence directly from the genomic stock by either method, a highly successful protocol for sequencing the pp1 PCR fragment was developed for the Vistra system. The entire fragment could be sequenced using the downstream and upstream primers in combination, with a large area of overlap that encompassed the -174G/C polymorphism. However, the quality of sequence data varied, with the negative strand frequently producing longer sequences of a generally higher quality than sequences of the positive strand. This may have been a sequencing reaction annealing temperature problem, but it was never successful resolved. The Vistra method was also liable to other problems: excess primer within the sample led to a 'smile' effect on the sequencing gel, which caused the outer 2-3 sample lanes on both sides to drift away from the midline at the start of the sequencing run. This led to difficulties with the automated base-calling routine, so the data from several samples had to be analysed repeatedly over short sections of the total run, in order to achieve a full sequence. These disadvantages were minor, as the Vistra system is non-radioactive and results were obtained several days ahead of the comparable TCS-based sequencing method. Overall, therefore, the automated method was preferred for screening small numbers of individuals for the presence of polymorphisms.

4.1.3 *RFLP Analysis*

Despite the advantages of the automated sequencing system when applied to direct sequencing of PCR products, this method was still too impractical to be applied to the screening of a population for the -174G/C polymorphism. Fortunately, this polymorphism resulted in an RFLP, which was potentially detectable using one of three restriction enzymes. *Sfa*N1 was chosen, as this gave the most appropriate restriction digest pattern.

The protocol for the PCR-RFLP was straightforward, although at first it proved very difficult to distinguish between the digested fragments, as they appeared to form concatemers within the reaction solution. This was overcome by briefly heating, and then cooling, the reaction mixture prior to loading onto the agarose gel. Overall, the method was appropriate to quickly, easily and reproducibly screen the patient and control populations for the -174 allele frequency.

4.1.4 *Functional Studies*

This section of the thesis produced probably the most interesting results, showing for the first time that a polymorphism in its 5' flanking region could influence the expression of the IL-6 gene. HeLa cells proved to be a very efficient cell line for these transient transfections, as opposed to THP-1 cells, which were initially tried, but without success. The reasons for this may be related to the age of the batch of THP-1 cells available in the laboratory. It is recognised that transfection efficiency falls in cells which have been through many passages, as these had been. In addition, the paucity of publications in which the THP-1 cell line is used as a recipient for reporter constructs would tend to suggest that it is a difficult cell line to transfect.

IL-1 and LPS were chosen as stimuli for the HeLa cells because of their known strong, positive effect on IL-6 gene transcription. There are several other stimuli that can be used (eg TNF-α, cAMP or LTB_4), and it might be interesting to confirm that they also result in a differential response between the two -174 alleles. The implications of the results of this section are discussed below (4.2.2).

4.2 Implications of results of this study

4.2.1 *A_nT_n tract Polymorphisms*

The relative composition of the IL-6 5' flanking region adenine-thymine (A_nT_n) tract in the published sequence is A_9T_{11} (Yasukawa *et al.*, 1987), but in this study a total of six different configurations were observed. The significance of these different configurations remains to be ascertained. However, it is recognised that A_nT_n tracts (of at least four contiguous adenines) cause bending of B-DNA (right hand coiled DNA - the general structure of DNA found in the living cell, with a major and a minor groove) especially when they are phased every 10-11 bases. Consistent measurements of the amount of bending are difficult to obtain, but it is known that an A_6 tract induces a 17-21^0 bend in the DNA double helix (Koo *et al.*, 1990).

The sequence context within which the adenine tract occurs also influences the degree to which the DNA strand is bent. For instance the oligonucleotide $(CGA_4T_4)_n$ runs on a polyacrylamide gel as if it were bent, whereas the $(CGT_4A_4)_n$ oligonucleotide migrates with straight DNA (Hagerman, 1986). In addition, this bending cannot form at the TA step (ie. within the sequence 'TTAA' or 'TTTAAA', Price and Tullis, 1993). Outside of this central 'core' the normal adenine tract structure can form to cause a global bend in the DNA, provided that it is phased correctly.

There is no information in the literature on the global effect on DNA of a single A_nT_n tract. It seems likely, however, based on observations made on artificially generated A_nT_n tracts, that a significant amount of DNA bending will occur around the location of the tract. As the model proposed by Price and Tullis (1993) suggests that the core sequence is protected from bending, it is possible that the relative effect of an alteration of one or two adenines to thymines will not effect the localised bending of the tract. However, increasing the overall length of the tract may alter the degree to which it is bent. Alternatively, the relative composition of the tract may be of vital importance in determining its overall bend, but this remains to be demonstrated. The findings discussed in this study, that the core sequence of a naturally occurring A_nT_n tract is polymorphic, may stimulate further research

in this field. For instance, it might be possible to identify the various A_nT_n tract configurations by their mobility on an SSCP gel. It should be possible to predict the amount of bend of the A_nT_n tract by modeling its three dimensional structure or by x-ray crystallography.

The functional significance of bending the DNA strand in the context of gene transcription is that it facilitates an apposition between transcription factors bound to sequences which are otherwise separated by large distances along the DNA strand. There is evidence that altering the degree of DNA bending between transcription factor binding sites in an artificial promoter model has a marked effect on the degree of synergistic activation of the reporter gene by the two transcription factors (Kim *et al.*, 1996). This work is supported by the observation that DNA bending is induced following the binding of AP-1 (Rajaram and Kerpolla, 1998), and that the base sequence flanking the binding site has an important influence on the amount of induced DNA bending. Any change in the overall amount of DNA bending brought about by a polymorphism in the A_nT_n tract (either by altering its overall constituency or its length) may therefore alter the degree to which disparate sequences in the promoter region are apposed, which may eventually alter the rate of gene transcription. In the 5' flanking region of the IL-6 gene the A_nT_n tract lies upstream of the AP-1 site. There are no other recognised transcription factor binding sites upstream of the A_nT_n tract, thus the influence of this region on synergism between transcription factors remains to be demonstrated. The putative glucocorticoid receptor binding site GR_1 does lie upstream of the A_nT_n tract, but again no definitive GC binding has been demonstrated here.

Further research in the area of the consequences of a variation in the relative composition of the A_nT_n tract is warranted. The use of reporter constructs containing the various A_nT_n tract configurations, with the -174G or -174C allele, would demonstrate any influence that the A_nT_n tract polymorphisms have on gene transcription.

Another intriguing feature of the A_nT_n tract polymorphism reported here is the association of the -174C allele with the A_8T_{12} tract, albeit in the relatively small number of subjects studied. No disease associations were observed, but again this may be due to the small sample size for each subgroup. The A_nT_n tract association for the -174G allele was less clear cut, with the majority of subjects *not* possessing the A_8T_{12} tract configuration. There is therefore the suggestion that a haplotype exists as A_8T_{12} /-174C. As the two polymorphic

regions are located very close to one another, recombination events would almost certainly carry the two polymorphisms together.

The identification of this potential haplotype was only made possible by sequencing cloned PCR products, in order to ensure the sequence only represented that on a single chromosome. Direct PCR sequencing demonstrated individuals who were heterozygous for both the A_nT_n tract and -174 polymorphisms, thus identifying a haplotype in these circumstances would not have been possible. In this respect, the more labour intensive method of sequencing cloned PCR products was an important step in the characterisation of this region.

4.2.2 *-174G/C polymorphism*

4.2.2.1 Frequency of the polymorphism

The frequency of the -174G/C polymorphism in the S-JCA patients, as assessed by PCR-RFLP, was less than in the control population, but not statistically significantly so: C allele frequency 0.35 vs. 0.53 respectively; $\chi 2 = 0.292$, $p=0.088$, OR=1.54, 95% CI 0.97~2.38. When comparing the two populations by their genotype frequencies, there was a large overall difference between them ($\chi 2 = 4.15$, $p=0.0002$). This was even more significant when the two groups were compared by the frequencies of the separate genotypes, with the CC genotype being extremely rare in the S-JCA group ($\chi 2 = 15.3$, $p<0.0001$, OR=13.4, 95% CI 3.00~59.6) and the GC genotype being more common ($\chi 2 = 4.94$, $p=0.02$, OR=2.35, 95% CI 1.16~4.77).

Although the frequency of the genotypes within the control group is in agreement with that predicted by the Hardy-Weinberg equation, this is not the case for the S-JCA patients. This is probably because the Hardy-Weinberg equation assumes that the genotypes are distributed evenly within a large population. When the population is small, as with the S-JCA patients, sampling error can change allele frequencies even if there is no increased mutation rate, no assortive mating, no selection, or no migration from the group. It is unlikely that assortive mating is functioning to influence the genotype frequencies in this group, as S-JCA patients are less likely to marry and have children than healthy individuals (Wallace and

Levinson, 1991). The degree to which an otherwise unrecognised negative selection bias for the CC genotype, and migration out of the study group, is influencing the results is unknown. Overall, however, the considerable reduction in CC genotype frequency in the S-JCA group suggests that this genotype confers a degree of protection against the development of the disease. This is discussed again below.

The reduced CC genotype frequency in S-JCA patients was confirmed in the collaborative study, using larger patient and control groups. The value of the statistical difference for this reduction was less than in the thesis study. However, within this larger population of S-JCA patients the significance of the reduced CC genotype was greatest in those children with a disease onset of 5 years or below. This could not be confirmed for the thesis S-JCA population as details of the age of onset was not available for several patients.

4.2.2.2 Functional significance of polymorphism

Information from experiments using transfection with the full length constructs

Following the discovery of this bi-allelic polymorphism, which lies within 200bp of the coding region of the IL-6 gene, it was of considerable interest to know whether or not a functional difference existed between the two alleles, in terms of their relative strengths as promoters of IL-6 transcription. Three observations suggested that such a difference might exist. Firstly, the portion of the IL-6 5' flanking region between bases -225 and -164 has previously been reported to convey a negative regulatory effect on reporter gene expression (Ray *et al.*, 1990). This study, on the repression of the IL-6 promoter, employed co-transfection of length deletion reporter constructs into HeLa cells along with a glucocorticoid receptor-expressing plasmid. Progressive shortening of the 5' flanking region segment cloned into the reporter plasmid resulted in reducing amounts of the reporter gene being expressed, up until the point where the section -225 to -164 was removed, when the reporter gene expression increased. This observation suggested that the section -225 to -164 was inhibitory to reporter gene expression. DNA footprinting analysis demonstrated binding of glucocorticoid receptor to multiple regions of the IL-6 promoter, such as the Inr, TATA box and CRE/NF-IL6 sites, and

an area between bases -201 to -210. The polymorphism at -174 is close enough to this area for it to potentially influence the binding of the glucocorticoid receptor and thus its ability to repress transcriptional activation. This could be tested by comparing the DNA footprint for GR binding at -201 to -210 between the two alleles. An effect from the -174 polymorphism should be apparent as a difference in this footprint.

The second observation is the creation of a potential binding site for the transcription factor NF-1 in the -174C allele. As discussed below, NF-1 is a potential inhibitor of gene transcription in HeLa cells.

Finally, at least one other polymorphism in the 5' flanking region of a cytokine gene (the -308 TNF-α polymorphism) has been demonstrated to convey an altered promoter activity (Wilson *et al.*, 1997).

Analysis of the difference in the expression of luciferase induced by IL-1 and LPS from the -174G and -174C constructs shows that the latter did not respond to these pro-inflammatory stimuli. There are several potential causes for this lack of stimulation of the luciferase reporter gene. Firstly, the difference may be an artefact. This is unlikely, as the results were consistent between the experiments, and also for both of the plasmid preparations. It is also unlikely that the construct was defective, as baseline (unstimulated) expression was still significantly greater than background (although it was still only 0.62 ± 0.15 of the -174G unstimulated expression) and the sequence of the plasmid insert was confirmed prior to transfection.

Secondly, the presence of a cytosine at position -174 may have an adverse affect on the binding of a positive transcription factor in this region. However, there have been no published reports to date which would support this explanation, especially since this region appears to confer a negative effect on reporter gene transcription (see above, Ray *et al.*, 1988). Similarly, there is no evidence to suggest that a base change at this position, which lies away from the other recognised positive transcription factor binding sites, could result in a reduction in the capacity of the whole 5' flanking region to respond to pro-inflammatory stimuli.

The final explanation as to why the -174C construct is not as efficient at promoting luciferase gene transcription in this situation as the -174G construct may be because a

negative transcription factor is able to bind to the sequence around -174C. This explanation is supported by the fact that the change from a guanine to a cytosine at position -174 creates a potential binding site for the transcription factor NF-1. (Figure 4.1)

A) 5' - TGG C/ANN NNN GCC A - 3'

B) 5' - TT**G** T **GT CTT GCC A** - 3' -174C

C) 5' - TT**G** T **GT CTT GC**G **A** - 3' -174G

↑

-174

Figure 4.1 NF-1 binding site - consensus sequence. The upper sequence (A) is the consensus binding sequence for NF-1 (after Lui *et al.*, 1997). Below this is the positive strand DNA sequence around the site of the -174C (B) and -174G alleles (C). Bases matching the consensus are underlined and in bold. Although there is only a single base difference between the alleles, it is in the location of the downstream hemi-palindrome sequence, which is more important for binding than the redundant central sequence, which remains unchanged.

NF-1 comprises a family of structurally related transcription factors active in many cell types (Rein *et al.*, 1995, Liu *et al.*, 1997). Although this factor has been shown to have varying effects on transcription, in HeLa cells it has been demonstrated to be a repressor of reporter gene expression (Rein *et al.*, 1995). However, it as been shown to be a stimulator of transcription in some cell lines other than HeLa's (Rein *et al.*, 1995). It is therefore possible that in other cell lines the transcriptional response from a -174C construct could be very different. It was perhaps fortuitous that HeLa cells were chosen for this study, as the consequence of the -174G/C polymorphism might be different when tested by transient transfection in cell lines which do not express NF-1 or where it is stimulatory. The consequence of the inhibitory NF-1 site on IL-6 transcription and S-JCA is discussed below. The *in vivo* situation is likely to be complex, where IL-6 is expressed from several different tissues in which the possession of an NF-1 site has varying effects on the rate of IL-6 expression.

The mechanism by which NF-1 is inhibiting IL-6 gene transcription is unknown. The IL-6 gene is typical in that it contains a TATA-box promoter, upstream promoter and regulatory elements such as NF-κB, NF-IL6 and the CRE, and also upstream enhancer elements such as AP-1. The binding of the various transcription factors responsible for the induction of the expression of IL-6 (as discussed in 1.4.4) is a co-operative event, resulting in the up-regulation of the activity of the stable transcriptional complex of RNA polymerase II and its numerous associated nuclear proteins.

The earliest step in the formation of this complex is the binding to the TATA box of TFIID, which is facilitated by TFIIA. This factor binds to TFIID and overcomes the DNA-binding inhibitory effect of Dr1 and Dr2 (Drapkin *et al.*, 1993). Thereafter, TFIIB binds to TFIID, an important stage as TFIIB can bind RNA polymerase II itself. TFIIE, TFIIH and TFIIJ then rapidly associate with the complex, TFIIH unwinding the DNA helix, allowing it to be copied to RNA. TFIIH then also phosphorylates the now bound RNA polymerase II, allowing it to progress to transcriptional elongation of the RNA product.

Like the other TFII factors, TFIID comprises a complex of associated proteins, which includes most importantly the TATA-binding protein (TBP), which is directly responsible for

binding the complex to the TATA box. The remaining components of TFIID are TBP-associated factors (TAFs) which modulate the response of TFIID to transcription factors (Reviewed in Latchman, 1998).

Repression of transcription may occur through several mechanisms, such as a repressor interfering with the normal binding site of the activator of transcription, sequestering of the activator in solution by the repressor, binding to and degradation of the activator in solution by the repressor, or neutralization of the activators' activity when the repressor binds next to it. There is no evidence, in the case of NF-1, that these mechanisms are occurring. It is more likely that direct repression of transcription, following binding of NF-1 to a specific site, is the mechanism.

In a manner analogous to the process of direct enhancement of transcription by positive transcription factors binding upstream of the basal transcriptional complex and interacting with it, direct repression of transcription also occurs. The repressor can interact with the complex, inhibiting its assembly or reducing its activity and/or its stability after its assembly. As mentioned above, the activity of inhibitors such as Dr1 is via the interference with the ordered assembly of the basal transcriptional complex. There is no published information on the mechanism by which NF-1 inhibits transcription, such as whether it does interact with the basal transcriptional complex, or with other, bound, transcription factors. However, a further possible mechanism exists, which is discussed in the next section.

Information from experiments using transfection with the length deletion constructs

The results of the deletion construct transfections were also very interesting. As expected, the two full length constructs ('Full-G' and 'Full-C') displayed a differential response to inflammatory stimuli. However, when the sequence upstream of the AP-1 site was deleted for the constructs ΔAT-G and ΔAT-C, this differential response was lost (an effect also observed in the shorter constructs, ΔATAP-G and ΔATAP-C).This may have been due to a technical error, as this result was based on one (duplicated) transfection experiment. However, if this result is real, it suggests that this upstream region influences both the overall control of transcription (as the luciferase expression from the constructs was below that of the full length

constructs), and possibly binding of NF-1.

As discussed previously, the A_nT_n tract can have an effect on DNA bending. In this instance its presence may be necessary to allow the GR1 (or another, as yet unidentified transcriptional enhancer) to positively influence the transcription of the IL-6 gene by bringing it into contact with the other bound transcription factors and RNA polymerase. Alternatively, the A_nT_n tract may not be vital for this purpose, the GR1 and/or other enhancers coming into contact with the transcriptional machinery by other methods, such as DNA 'looping out' (Latchman, 1990)

The loss of differential expression in constructs not containing the sequences upstream of AP-1 suggests that this region contains a sequence which binds an enhancer that interacts with NF-1. It is possible that this putative enhancer (GR1 or another, unidentified one) is the same enhancer required for maximal transcription, as suggested by the first part of this deletion transfection experiment. This, or perhaps a second and unrelated sequence, is also required for the binding and negative transcriptional effect of NF-1. Alternatively, NF-1 may still bind in these constructs, but it requires an interaction with a factor bound upstream of AP-1 to have its full, negative effect on transcription. Thus, the removal of the sequence upstream of AP-1 allows the -174C constructs to respond in the same manner as the -174G constructs. This requirement of NF-1 for a second factor in order for it to exert its inhibitory effect on transcription is the final mechanism mentioned at the end of the previous sub-section. This second factor may be the Glucocorticoid Receptor. The binding of steroid receptors to DNA sequences (1.4.4.6) is associated with the binding of co-activators, such as Steroid Receptor Co-activator-1, which has histone acetyltransferase activity (Spencer *et al.*, 1997), leading to an alteration in the chromatin structure, allowing a second transcription factor (in this case NF-1) to bind.

Deletion of the AP-1 site produces a further major reduction in transcriptional response to inflammatory stimuli, underlying the importance of this site for control of expression (as shown by Dendorfer *et al.*,1994, with CAT reporter constructs and site directed mutagenesis of various transcription factor binding sites). The construct containing only the CRE, NF-IL6 and NF-κB sites displayed a luciferase expression comparable to that

of the unligated pGL3 plasmid, suggesting that in this system it is the AP-1 site that is most important for transcriptional control. However, in most other studies the CRE, NF-IL6 and NF-κB sites still significantly contribute to the expression of the reporter gene constructs. The efficiency of this reporter system may be lower than those previously reported, and not enough to show an effect on luciferase expression from the ΔATAPGC construct. Therefore, the relative differences in expression from the other constructs may be greater in other, more efficient, systems.

These transfection experiments require confirmation, both in HeLa cells and in other cell lines, where the results (like those for the full length constructs) may be different to those obtained here. Electromobility shift assays using nuclear extracts from different cell lines and the various length deletion constructs could be used to assess whether there is a transcription factor binding upstream of AP-1 and possibly influencing NF-1.

In summary, this phase of the study has demonstrated the first example of a functionally significant IL-6 polymorphism. There are no published reports on the transcriptional influence of the previously described RFLP. Their location outside of the regions currently known to control transcription would tend to suggest that they are not important, but this remains to be formally confirmed.

4.2.2.3 Implication of polymorphism on the aetiopathogensis of S-JCA

Until now there have been no known disease associations with any polymorphisms in the 5' flanking region of the IL-6 gene. An increased frequency of an *Xba*I RFLP, probably due to 3' flanking region insertions, has been described in some patients with SLE and elevated IL-6 levels (Lineker-Israeli *et al.*, 1996). Using PCR-RFLP and sensitive polyacrylamide gel electrophoresis, an association between the 3' flanking region polymorphism genotype and peak bone mineral density in women has been demonstrated (Murray *et al.*, 1997). However, none of these RFLP are located in areas known to be responsible for inducible transcriptional control. A brief investigation of the IL-6 3' flanking region in this thesis did not reveal any significant differences between controls and the S-JCA patients.

The -174 polymorphism genotype frequencies in the thesis S-JCA patient group are

significantly different from those in the thesis control population. The inference from the *in vitro* data showing that reporter expression from the -174C allele is repressed following stimulation with IL-1 and LPS, suggests that the presence of the -174C allele (which is forming an NF-1 binding site and thus inhibiting IL-6 transcription)- and more so the CC genotype - might result in a lower IL-6 expression following a given inflammatory stimulus compared to the GG genotype. Along with the recognised importance of IL-6 in the pathogenesis of S-JCA (evidence which was thoroughly reviewed in a recent editorial [De Benedetti and Martini, 1998], and discussed in here in Chapter 1), these *in vitro* functional studies (albeit in one cell line) support the suggestion that the CC genotype confers a potential protective effect against the development of S-JCA.

The corollary of this suggestion would therefore be that the serum level of IL-6 during active S-JCA would vary according to the patient's genotype, as would the level of IL-6 in the pre-fever, febrile and post-fever state. However, these findings are likely to be very difficult to interpret for several reasons. Firstly, IL-6 levels may be influenced by the patient's concurrent medication. Secondly, there is a large variation in the absolute IL-6 level between children acutely ill with S-JCA (Rooney *et al.*, 1995). Thirdly, other factors, such as the 'severity' of their disease (for which there is no agreed, objective or validated scale), their age, disease duration as well as the duration of their current flare and their nutritional state are also likely to influence the serum IL-6 levels.

Although it would therefore appear that an individuals' -174 genotype influences his or her IL-6 response to a stressful stimulus in a manner analogous to that of the TNF-α -308 polymorphism (Wilson *et al.*, 1997), it is highly unlikely that the -174 polymorphism alone represents the susceptibility gene for the development of S-JCA. It is more likely to represent a severity marker, with a reduced IL-6 expression in the CC individual reducing their risk of developing S-JCA, but not removing it. As the G allele appears to respond appropriately to *in vitro* inflammatory stimuli, it would seem unlikely that the GG (or GC) genotypes actually predispose the individual to S-JCA. However, it is still possible that they interact with other genetic and/or environmental triggers to produce the disease. It still remains to be shown what, if any, effect the individual's A_nT_n genotype has on their predisposition to S-JCA.

4.2.2.4 Implication of the IL-6 polymorphisms on the aetiopathogensis of other diseases

Perhaps the most important implication of the discovery of these polymorphisms is their potential to affect the individual's predisposition to several other conditions, all commoner than S-JCA. Increased serum and synovial fluid IL-6 has been described most of the inflammatory arthropathies, frequently in good correlation with the disease activity (discussed in Chapter 1.3.2). Elevated serum IL-6 levels in AIDS and multiple myeloma suggest that this cytokine at least plays a part in the symptomatology of these conditions, and is potentially relevant to their aetiopathogenesis. As the only cytokine capable of inducing the production of all the hepatic acute phase reactants, IL-6 is vitally important in the body's response to injury and infection. If an individual's IL-6 genotype determines their response to these insults, it is likely that their recovery may be affected. Although there is considerable redundancy in the cytokine network, such that the relative reduction of one cytokine is offset by the effects of others, it is possible that one function may not be adequately covered, resulting in an adverse outcome. Whilst this may not be immediately obvious, over time it could play an important role in predisposition to certain conditions which have a long evolution, such as atherosclerosis and coronary heart disease.

IL-6 is likely to have a role in the development of atherosclerosis, as increased amounts of protein are found in atherosclerotic plaques, where it is thought to represent a local response to injury (Rus *et al.*, 1996). Increased serum IL-6 and acute phase reactants are also observed in patients with non-insulin dependant diabetes mellitus who have hypertriglyceridaemia, low HDL cholesterol, hypertension, glucose intolerance, insulin resistance and accelerated atherosclerosis (Pickup *et al.*, 1997). The initial triggers to these conditions are unknown, but if they are dependant on IL-6 it is conceivable that the CC genotype may also be protective. This is discussed further in the last section.

Over the last ten years, there have been numerous papers detailing the transcriptional control of IL-6, and the effects of exogenous mediators on IL-6 expression. The constructs used for these experiments were initially derived from the same clones used to sequence the IL-6 gene. Thereafter, investigators used their own clones, or artificially-produced

oligonucleotides based on the published sequence. It is therefore highly unlikely that the -174 genotype was the same in all published experiments. It is even possible that genotypes varied between constructs within the same experiment. This probably explains the observed differences in transcriptional response reported between papers and is also a potential explanation for some of the reported tissue specificity (eg the absence of LPS-induced expression from IL-6 promoter reporter constructs in HeLa cells, Ray *et al,* 1988). The implication is therefore that these previous publications will have to be re-assessed in the light of the -174 genotype of the reporter constructs used. It is unlikely that these data will be available, but the conclusions of these papers may only hold true for one genotype. The effect of the A_nT_n genotype, including the possible existence of an A_8T_{12} /-174C haplotype, on these results is as yet unknown.

IL-6 is frequently referred to as one of the pro-inflammatory cytokines, but an alternative way of viewing this cytokine is now emerging. With its ability to promote the expression of the acute phase reactants (which promote healing and repair), to stimulate the production of glucocorticoids (which are anti-inflammatory) and cytokine inhibitors (such as IL-1ra and soluble TNF-receptor p55) and also to induce the expression of TIMPs in the inflamed joint, IL-6 is now being proposed as an *anti*-inflammatory cytokine by some authors (Tilg *et al.,* 1994; Silacci *et al.,* 1998). This 'new' role for IL-6 is supported by some experimental evidence, for instance increased levels of TNF-α in the lungs of IL-6-/- mice treated with aerosol endotoxin (Xing *et al.,* 1998). Intraperitoneal delivery of endotoxin to these mice also resulted in higher levels of TNF-α, GM-CSF, and IFN-γ in IL-6-/- mice than in IL-6+/+ mice. IL-11, which is related to IL-6 by virtue of its use of the same signal-transducing gp130 molecule, also has anti-inflammatory properties. This was demonstrated recently in the murine collagen-induced arthritis model, where exogenous administration of recombinant human IL-11 significantly ameliorated the disease severity (Walmsley *et al.,* 1998).

If the proposed anti-inflammatory properties of IL-6 can be extrapolated to humans, there is the potential for the IL-6 genotype to modify disease outcome in a manner analogous to the TNF-α genotypes and infectious diseases (eg malaria; McGuire *et al.,* 1994). However,

in the case of S-JCA it is low levels of IL-6, associated with the CC genotype, that may be protective (ie. anti-inflammatory). Thus, in this disease (and in the normal, *in vivo* situation), the separation of IL-6 into either a pro- or an anti-inflammatory cytokine may not be as clear cut as some of the experimental data suggests. Undoubtably the body's eventual response to the presence of increased levels of IL-6 will be influenced by numerous other factors, such as other cytokines. Therefore a strict extrapolation of the results of *in vitro* transfection experiments or animal gene knockout studies, to human disease states is inappropriate.

4.3 Advances made since my work described in this thesis, and their relevance to these results

As discussed in 3.4, I was closely involved in the extension of this project by members of Professor Woo's group, in collaboration with Dr Gary Faulds in Professor Steve Humphries' laboratory in the Rayne Institute, UCL. The presence of the -174 polymorphism was confirmed in that institution. The C allele frequency in a large cohort of Caucasians was found to be 0.403, compared to 0.15 and 0.05 in Gujarati Indians and Afro-Caribbeans respectively, and 0.53 for the Caucasian controls investigated in this thesis. The difference in the C allele frequencies between the two Caucasian control groups may be due to the selected nature of the control group investigated in this thesis, and its smaller size. In addition, although both groups were Caucasian, there is still a potential for considerable genetic differences between individuals within this group, which might include individuals who were as diverse as being of Northern European or Southern Mediterranean descent. These effects were minimised by including in the study only those with Anglo-Saxon surnames, and also using a large number of individuals in the groups. This later precaution reduces the error inherent when comparing genetically heterogeneous populations. The size of the S-JCA cohort was increased as much as possible (n=97) which represented a significant proportion of the cases of S-JCA currently recorded in Britain (Symmons *et al.*, 1996).

This larger study revealed that there was still a significant difference in the overall genotype distribution between the control and S-JCA groups, but it was not as great as in the thesis (χ^2=7.00, p=0.03). Unlike this thesis results, the overall CC genotype frequencies were

not significantly different. However, when the S-JCA group was divided by age of onset (as less than or equal to 5 years or greater than 6 years) a significant reduction in CC genotype frequency compared to the controls was found, but only for the younger age of onset group (χ^2=4.14, p=0.04, OR 0.39, 95% CI 0.15-0.99). The expanded S-JCA group was now in Hardy-Weinberg equilibrium with respect to its genotype distribution.

The division of the S-JCA patients by their age of onset may at first appear arbitrary. However, it is recognised that the younger patients often have the most aggressive and recalcitrant disease. This sub-division is also of relevance in other JCA subgroups, in particular the young onset oligoarticular JCA subgroup who have strong HLA associations that are not seen in older oligoarticular onset children (Murray *et al.*, 1997).

The transfection experiments were not extended, but their conclusions are supported by data from 102 of the Goodinge control subjects who were recalled for measurement of their fasting plasma IL-6. In this subset, the GG homozygotes had circulating IL-6 concentrations approximately twice as high as those homozygous for the C allele (p=0.02), with GC heterozygous subjects midway between these two (Fishman *et al.*, 1998.).

This additional work therefore strengthens the conclusions of this thesis, that the CC genotype is protective against the development of S-JCA, but mainly for children whose disease began under 5 years. This difference demonstrates the importance of having a large group of patients and controls, but also highlights the difficulties inherent in this type of research. Obtaining a true control population is very difficult. Population mixing is common, but can be partially controlled for by excluding non-Caucasians. However, even within this group there is still considerable ethnic diversity. Selection bias operates when obtaining the samples in the first place. Although the thesis control group was a 'randomly' selected group of Caucasians, they all worked in the same institution. Similar bias may operate when individuals are collected from a general practice list, as ethnic minorities tend to group together in small areas and the GP's list may therefore not be truly representative of the population as a whole. Even blood donors are a self-selected group. The best way to overcome these areas of bias is to obtain the largest group of patients and controls as possible. An even larger control population was available for analysis in the collaborative study, but it was composed of only males and was therefore inappropriate for comparison

with a male and female disease group. With respect to the S-JCA patients, there are only around 200 cases currently recorded in this country, thus it is unlikely that a much larger group will be available in the near future.

A comprehensive analysis of the frequency of the -597 polymorphism, and an assessment of the possible existance of a -174/-597 haplotype, is underway in the two laboratories. In addition, the functional studies are being extended, to include an assessment of the possible differential response between the -174G and C alleles when transfected into adipose cells.

4.4 Further research

The research described in this thesis has revealed several previously unknown features of the 5' flanking region of the IL-6 gene, and an extended investigation of their properties would be very interesting. For instance, the A_nT_n tract in all of the reporter constructs used to compare the two alleles of the -174 polymorphism was A_8T_{12} . The effect, if any, of varying the A_nT_n tract length and composition on promoter function would indicate that this region may have an influence on the control of IL-6 expression. Additionally, developing a method for delineating the A_nT_n tract that did not involve sequencing would make genotyping a population for the alleles of this region a realistic possibility. SSCP would be a good place to start, using relatively small PCR products containing the entire A_nT_n tract. Such a method should demonstrate a single base difference between samples, although it remains to be seen whether it is sensitive enough to detect a base change within the territory of the A_nT_n tract.

It would be very worthwhile investigating the possibility that the differential effect between the two -174 alleles observed in the functional experiments is due to binding of a nuclear protein to DNA in this region, which is then acting as a transcriptional repressor in HeLa cells. As discussed earlier, NF-1 is a potential candidate in this respect, as the -174C allele contains the downstream hemi-palindrome of the binding site for this transcription factor (Figure 4.1). The binding of NF-1 to this region could be investigated using electromobility shift assays (with a radiolabelled oligonucleotide containing the sequence in question and nuclear extracts of HeLa - or other - cells stimulated with LPS or IL-1) and

DNA footprinting. This could be combined with an analysis of the binding of the glucocorticoid receptor, to assess the possibility that this is a 'co-factor' in the negative effect on transcription resulting from the binding of NF-1. The results of transfection into other cell lines, such as macrophages (which are a major source of IL-6 in human inflammatory diseases), fibroblasts (which are also a major source of IL-6) and adipose cells (which secrete IL-6, an observation which may be of relevance in the development of diabetes [Mohamed-Ali *et al,* 1997]) would also be very interesting. This is because NF-1 has been shown to have varying effects in different cell lines, and outside of HeLa cells it may be stimulatory. Thus, the *in vivo* consequence of the -174 polymorphism may be more difficult to interpret and may not be as simple as the -174C allele associating with a reduced IL-6 expression. Extending the transfection data therefore has an important role to play in validating the results of this thesis.

If the polymorphism is truly a susceptibility factor for S-JCA, linkage between it and the disease is amenable to analysis by the transmission disequilibrium test, for which families are currently being collected by Professor Woo's group.

There is no published data concerning the incidence of S-JCA in non-Caucasians, and given the low C allele frequencies in the Gujarati Indians and Afro-Caribbeans, it would be very interesting to investigate the genotypes of S-JCA patients in these groups.

Finally, a direct correlation between genotype and IL-6 expression (at least *in vitro/ ex vivo)* would further support a potentially important role for the -174 polymorphism in disease. The measurement of IL-6 production by PBMC from healthy donors with different genotypes, to assess their IL-6 response to inflammatory stimuli, is likely to provide very interesting results.

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APPENDICES

Appendix A: IL-6 sequence from GenBank (Y00081, Yasukawa *et al* 1987). Nucleotides displayed in CAPITALS represent the locations of the PCR primers: pp1 fragment primers and sequence; pp2 fragment primers and additional bases flanking the pp1 sequence which together form the pp2 sequence.

ggatcctcct gcaagagaca ccatcctgag ggaagagggc ttctgaacca gcttgaccca ataagaaatt cttgggtgcc gacgcggaca gagattcaga gcctagagcc gtgcctgcgt ccgtactttc cttctagctt cttttgattt caaatcaaga cttagaggga gagggagcga taaacacaaa ctctgcaaga tgccacaagg tcctcctttg acatccccaa caaagaggtg agtagtaatc tccccctttc tgccctgaac caagtgggct tcagtaattt cagggctcca ggagactggg catgcaggtg ccgatgaaac agtggtgaag agactcagtg gcagtgggga gagcactggc agcacaggca aacctctggc acaagagcaa agtcctactg gagattccaa gggtcacttg ggagagggca ggcagcagcc aacctcctct aagtgggctg aagcaggtga agaaatggca gacaagcgcg gtggcaaaaa GGAGTCACAC ACTCCACCTg gagacgcctt gaagtaactg cacgaaattt gagggtggcc aggcagtcta caacagccgc tcacagggag agccagaaca CAGAAGAACT CAGATGACTG Gtagtattac cttcttcata atccaggctt ggggggctgc gatggagtca gaggaaactc agttcagaac atctttggtt tttacaaata caaattaact ggaacgctaa attctagcct gttaatctgg tcactgaaaa aaaaattttt ttttttcaaa aaacatagct ttagcttatt ttttttctct ttgtaaaact tcgtgcatga cttcagcttt actctttgtc aagacatgcc aaagtgctga gtcactaata aaagaaaaaa agaaagtaaa ggaagagtgg ttctgcttct tagcgctagc ctcaatgacg acctaagctg cacttttccc cctagttgtg tcttgcgatg ctaaaggacg tcacattgca caatcttaat aaggtttcca atcagcccca cccgctctgg cccccccctc accctccaac aaagatttat caaatgtggg attttcccat gagtctcaat attagagtct caacccccaa taaatatagg actggagatg tctgaggctc attctgccct cgagccaccg ggaacgaaag agaagctcta tctCCCCTCC AGGAGCCCAG Ctatgaactc cttctccaca agtaagtgca ggaaatcctt agccctggaa ctgccagccg gtcgagccct gtgtgaggga ggggtgtgtg gcccagggat gcggggcgcc agcagcagag gcaggctccc AGCTGTGCTG TCAGTCACcc ctgcgctcgc tcccctccgg cacaggcgcc ttcggtccag ttgccttctc cctggggctg ctcctggtgt tgcctgctgc cttccctgcc ccagtacccc caggagaaga ttccaaagat gtagccgccc cacacagaca gccactcacc tcttcagaac gaattgacaa acaaattcgg tacatcctcg acggcatctc agccctgaga aaggaggtgg gaaggcttgg cgatggggtt gaagggcccg gtgcgatgcg tctcccctcc ctgcgtgtgg gggggctgcc tgcataagga ggtctttgct gggttctaga gcactgtaga tttgaggcca acgacctaga ctgacttctg tatttatcct ttgctggtgt caggaggttc ctttcctttc tggaaaatgc agaatgggtc tgaaatccat gcccaccttt ggcatgagct gagggttatt gcttctcagg gcttcctttt cccttccaa aaaattaggt ctgtgaagct ccttttgtc ccccgggctt tggaaggact agaaaagtgc cacctgaaag gcatgttcag cttctcagag cagttgcagt acttttggt tatgtaaact caatggttag gattcctcaa agccattcca gctaagattc atacctcaga gcccaccaaa gtggcaaatc ataaataggt taaagcatct ccccacttte aatgcaaggt attttggtcc tgtttggtag aaagaaaaga acacaggagg ggagattggg agcccagact cgaattctgg ttctgccaaa ccagccttgt gatcttgggt aaattcccta ccacctctgg actccatcag taaaattggg ggtggactag gtgatctcat agatccttcc tgctggaaca ttctatggct tgaattatat tctcctaatt attgtcaaaa ttgctgttat taagtatcta ctgtgtgcca ggcactttaa ataaatattg tgtctaatct tcaaaacaaa tttgcaagga aggttttgg agataaggaa actgagactc aggattaagt aacacaccta aagtcaaagg tgagcttgga actgaaccca agtgtgcccc cactccactg gaatttgctt gccaggatgc caatgagttg tagcttcatt tttcttagag actttcctgg ctgtggttga acaatgaaaa ggccctctag tggtgtttgt tttagggaac ttaggtgata acaattctgg tattctttcc cagacatgta acaagagtaa catgtgtgaa agcagcaaag aggcactggc agaaaacaac ctgaaccttc caaagatggc tgaaaaagat ggatgcttcc aatctggatt caatgaggta ccaacttgtc gcactcactt ttcactattc cttaggcaaa acttctccct cttgcatgca gtcctgtata catatagatc caggcagcaa caaaaagtgg gtaaatgtaa agaatgttat gtaaatttca tgaggaggcc aagttcaagc ttttttaaag gcagtttatt cttggacagg tatggccaga gatggtgcca ctgtggtgag attttaacaa ctgtcaaatg tttaaaactc ccacaggttt aattagttca tcctgggaaa ggtactcgca gggcctttte cctctctggc tgcccctggc agggtccagg tctgccctcc ctccctgccc

agctcattct ccacagtgag ataacctgca ctgtcttctg attattttat aaaaggaggt tccagcccag cattaacaag ggcaagagtg caggaagaac atcaaggggg acaatcagag aaggatcccc attgccacat tctagcatct gttgggcttt ggataaaact aattacatgg ggcctctgat tgtccagtta tttaaaatgg tgctgtccaa tgtcccaaaa catgctgcct aagaggtact tgaagttctc tagaggagca gagggaaaag atgtcgaact gtggcaattt taacttttca aattgattct atctcctggc gataaccaat tttcccacca tctttcctct taggagactt gcctggtgaa aatcatcact ggtcttttgg agtttgaggt atacctagag tacctccaga acagatttga gagtagtgag gaacaagcca gagctgtgca gatgagtaca aaagtcctga tccagttcct gcagaaaaag gtgggtgtgt cctcattccc tcaacttggt gtgggggaag acaggctaaa gacagtgtcc tggacaactc agggatgcaa tgccacttcc aaaagagaag gctacacgta aacaaaagag tctgagaaat agtttctgat tgttattgtt aaatcttttt ttgtttgttt ggttggttgg ctctcttctg caaaggacat caataactgt attttaaact atatattaac tgaggtggat tttaacatca atttttaata gtgcaagaga tttaaaacca aaggcggggg ggcgggcaga aaaaagtgcc atccaactcc agccagtgat ccacagaaac aaagaccaag gagcacaaaa tgattttaag attttagtca ttgccaagtg acattcttct cactgtggtt gtttcaattc ttttttcctac cttttaccag agagttagtt cagagaaatg gtcagagact caagggtgga aagaggtacc aaaggctttg gccaccagta gctggctatt cagacagcag ggagtagact tgctggctag catgtggagg agccaaagct caataagaag gggcctagaa tgaaacccct ggtgctgatc ctgcctctgc catttctact taagccaggg tttctcatat gttaacatgc tagggaattc cctgggcatc ttcttgtggt gtggagtctg acttagcaag cctcgggtgg gtttgagggt caaatttcta ccaggcttat atccctggtg atgctgcaga attccaggac cacacttgga ggtttaaggc cttccacaag ttacttatcc catatggtgg gtctatggaa aggtgtttcc cagtcctctt tacaccacca gatcagtggt ctttcaacag atcctaaagg gatggtgaga gggaaaactgg agaaaagtat cagatttaga ggccatgaag aacccatatt aaaatgcctt taagtatggg ctcttcattc atatactaaa tatgaactat gtgccaggca ttatttcata tgacagaata caaacaaata agatagtgat gctggtcagg cttggtggct catgcctgta ttccctaaac tttgggagcc taaggtgaga actccttgaa ctcctaaggc caggagttca agaccagcct ggataacata gcaagacccc atctctacaa aaaaccaaaa ccaaacaaac aaaaatgata gtggtgcttc cctcaggatg cttgtggtct aatgggagac agaacagcaa agggatgatt agaagttggt tgctgtgagc caggcacagt gctatataat cccagcgcta tgggaggctg aggtgggtgg atcatttagg ccaggagttt aagaccagcc tggtcaacat ggtaaaaccc catcttactt aaaaatacaa aaaagttagc caggcatggt ggcatacacc tgtaacccag ctactcagga ggctgaggca catgaatcac ttgaacccag gaggcagagg ttgctgtgca ccactgcact ccagcctggg tgacagaacg agaccttgac tcaaaaaaaa aaaaaagaag tttgttgcta tggaagggtc ctactcagag caggcacccc agttaatctc attcacccca catttcacat ttgaacatca tcccatagcc cagagcatcc ctccactgca aaggatttat tcaacattta aacaatcctt tttactttca ttttccttca ggcaaagaat ctagatgcaa taaccacccc tgacccaacc acaaatgcca gcctgctgac gaagctgcag gcacagaacc agtggctgca ggacatgaca actcatctca ttctgcgcag ctttaaggag ttcctgcagt ccagcctgag ggctcttcgg caaatgtagc atgggcacct cagattgttg ttgttaatgg gcattccttc ttctggtcag aaacctgtcc actgggcaca gaacttatgt tgttctctat ggagaactaa aagtatgagc gttaggacac tattttaatt atttttaatt tattaatatt taaatatgtg aagctgagtt aatttatgta agtcatattt atattttaag aagtaccact tgaaacattt tatgtattag ttttgaaata ataatggaaa gtggctatgc agtttgaata tcctttgttt cagagccaga tcatttcttg gaaagtgtag gcttacctca aataaatggc taacttatac atatttttaa agaaatattt atattgtatt tatataatgt ataaatggtt tttataccaa taaatggcat tttaaaaaat tcagcaactt tgagtgtgtc acgtgaagct t

Appendix B: Base sequence of the 'pp1' fragment showing the location of the PCR primers (in **bold**; normal typeface representing sequences complemetary to the positive strand, those in *italics* for the negative strand) and transcription factor binding sites (in CAPS). Additional bases the location of the polymorphisms are shown in red.

c----------5/2----------g ctt------DF13------ggc

cagaagaact cagatgactg gtagtattac cttcttcata atcccaggctt

g----------DF3----------Ag-GR1-A

gggggggctgc gatggagtca gaggaaactc agttcagaac atctttggtt tttacaaata

caaattaact ggaacgctaa attctagcct gttaatctgg tcactgaaaa aaaaattttt

g--------

ttttttcaaa aaacatagct ttagcttatt ttttttctct ttgtaaaact tcgtgcatga

---DF4-------c T--AP1-A G-IRF-1---

cttcagcttt actctttgtc aagacatgcc aaagtgctga gtcactaata aaagaaaaaa

-------G **g----------DF5---------c**

agaaagtaaa ggaagagtgg ttctgcttct tagcgctagc ctcaatgacg acctaagctg

ACA-NFIL-6-ATC

ATG---------MRE--------GCA

cacttttccc cctagttgtg tcttgcgatg ctaaaggacg tcacattgca caatcttaat

c **gg--------DF6---------c**

aaggtttcca atcagcccca cccgctctgg ccccaccctc accctccaac aaagatttat

GGG-NF-kB-TCC TATA

caaatgtggg attttcccat gagtctcaat attagagtct caacccccaa taaatatagg

gag-------DF7-------tct

actggagatg tctgaggctc attctgccct cgagcccaccg ggaacgaaag agaagctcta

cct------DF14------gag

c------- ----3/2---------c

tctcccctcc aggagcccag c

Appendix C: Publication

Fishman D, Faulds G, Jeffery R, Mohamed-Ali V, Yudkin JS, Humphries S, Woo P. Novel polymorphisms in the Interleukin-6 gene: their effect on IL-6 transcription, plasma IL-6 levels and an association with Systemic-onset Juvenile Chronic Arthritis. J Clin Invest 1998;102 (7): 1369-1376.

Paper appended over.

The Effect of Novel Polymorphisms in the Interleukin-6 (IL-6) Gene on IL-6 Transcription and Plasma IL-6 Levels, and an Association with Systemic-Onset Juvenile Chronic Arthritis

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Abstract

During active disease, patients with systemic-onset juvenile chronic arthritis (S-JCA) demonstrate a rise and fall in serum interleukin-6 (IL-6) that parallels the classic quotidian fever. To investigate the possibility that this cytokine profile results from a difference in the control of IL-6 expression, we examined the 5′ flanking region of the IL-6 gene for polymorphisms. A G/C polymorphism was detected at position −174. In a group of 383 healthy men and women from a general practice in North London, the frequency of the C allele was 0.403 (95% confidence interval 0.37–0.44). In comparison, 92 patients with S-JCA had a different overall genotype frequency, especially those with onset of disease at < 5 yr of age. This was mainly due to the statistically significant lower frequency of the CC genotype in this subgroup. When comparing constructs of the 5′ flanking region (−550–+61 bp) in a luciferase reporter vector transiently transfected into HeLa cells, the −174C construct showed 0.624±0.15-fold lower expression than the −174G construct. After stimulation with LPS or IL-1, expression from the −174C construct did not significantly change after 24 h, whereas expression from the −174G construct increased by 2.35±0.10- and 3.60±0.26-fold, respectively, compared with the unstimulated level. Plasma levels of IL-6 were also measured in 102 of the healthy subjects, and the C allele was found to be associated with significantly lower levels of plasma IL-6. These results suggest that there is a genetically determined difference in the degree of the IL-6 response to stressful stimuli between individuals. The reduced frequency of the potentially protective CC genotype in young S-JCA patients may contribute to its pathogenesis. Similarly the individual's IL-6 genotype may be highly relevant in other conditions where IL-6 has been implicated, such as atherosclerosis. (*J. Clin. Invest.* 1998. 102:1369–1376.) Key words: cytokine • SSCP • JCA • genetics • transfection

Introduction

Juvenile chronic arthritis (JCA)[1] is one of the commonest forms of chronic childhood disability, with an annual UK incidence of 10 cases per 100,000 children under 16 yr of age (1). Approximately 11% of patients with JCA suffer from the systemic-onset form (S-JCA), which is the subgroup most likely to be associated with severe, debilitating, extra-articular features and occasionally fatal complications (1, 2). To control their disease, young children with S-JCA are often exposed to potentially toxic therapies for many years. However, many children still experience early joint destruction, necessitating surgical replacement. Moreover, up to 48% of these patients will still have active disease after 10 yr (3).

S-JCA is a clinically homogeneous and quite unique illness. When the disease is active, patients display a typical quotidian spiking fever, an evanescent macular rash, lymphadenopathy, hepatosplenomegaly, serositis, myalgia, and arthritis. They are frequently anemic with markedly raised neutrophil and platelet counts; they have a high erythrocyte sedimentation rate, C-reactive protein, and serum fibrinogen. Patients also have a polyclonal hypergammaglobulinaemia and in severe cases raised liver enzymes and a coagulopathy (4). Additionally, inflammatory cytokines, such as tumor necrosis factor (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) have been shown to be elevated in the serum and synovial fluid of inflamed joints (5–8).

The particularly unusual feature of acute S-JCA is the unique pattern of fever. Once, or occasionally twice a day—and sometimes at the same time each day—the patient's temperature rapidly rises, often to more than 40°C. Patients may remain febrile for several hours before their temperature returns to normal, or subnormal. 24-h serum cytokine profiles have demonstrated that the serum IL-6 concentration rises significantly in conjunction with the fever spike, and then falls in parallel with the return of body temperature to normal (9, 10). Over this period TNFα levels are only moderately elevated and reach their maximum level ~ 5 h after the peak of fever.

The first two authors contributed equally to this study.

During the preparation of this manuscript, the −174G/C polymorphism was independently submitted to GenBank (GenBank accession number AF005485, authors Olomolaiye, O.O., N.A.P. Wood, and J.L. Bidwell, unpublished). We have submitted the A_nT_n polymorphism and corrections to the original sequence to GenBank (accession numbers AF039224–8 inclusive).

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1. *Abbreviations used in this paper:* JCA, juvenile chronic arthritis; S-JCA, systemic-onset juvenile chronic arthritis; SSCP, single-strand conformational polymorphism.

The level of interleukin-1 receptor antagonist also parallels the fever curve but with a lag phase of ~ 1 h. This is consistent with the fact that IL-1 receptor antagonist expression is rapidly induced by IL-6 (11, 12), and this probably contributes to the consistently low interleukin-1β (IL-1β) concentrations found during the fever curve.

Many of the clinical and laboratory features of S-JCA are a reflection of a vigorous hepatic acute phase response, the chief stimulus of which is IL-6. Furthermore, the systemic features resemble those of other conditions where there is overexpression of IL-6, such as Castleman's disease (13) and multiple myeloma (14). IL-6 has been shown to mediate fever induced by IL-1 in animals (15) and to induce fever when administered to patients with malignancy (16). Although TNF-α and IL-1 may also have an important role in S-JCA, the temporal patterns of increase in the serum concentration of these two cytokines are unlikely to be the cause of the cyclical levels of IL-6 or the characteristic fever of S-JCA (9). The central role of TNF-α in the pathogenesis of RA was demonstrated by the beneficial effect of humanized anti–TNF-α monoclonal antibody therapy on disease activity (17). However, the pathogenesis of S-JCA is likely to be different, since treatment with this antibody in one patient resulted in the abolition of fever for a few days, but the arthritis and malaise did not respond (18).

To explain the unique cyclical pattern of IL-6 serum concentration in active S-JCA, we have proposed that the regulation of the expression of IL-6 in these patients differs from unaffected individuals, possibly as the result of 5′ flanking region polymorphisms. There are now several well-documented instances where nucleotide polymorphisms occur within the regulatory region of cytokine genes, and some of these are associated with an altered rate of expression of the gene (19–21). In addition, some examples of these polymorphisms have been associated with autoimmune diseases, such as the combination of oligoarthritis with uveitis and a polymorphism of IL-1α in Norwegian patients (22) (although this is not confirmed for UK oligoarthritis patients [23]) and infectious diseases (24–26).

In this paper, we report the presence of polymorphisms within the 5′ flanking region of the IL-6 gene. One of these lies in an area previously reported to have a negative effect on gene transcription. We demonstrate that it affects the transcription rate of a reporter gene in transient transfection studies and is associated with lower plasma IL-6 levels in normal individuals. Furthermore, there was a significant difference in the frequency of genotypes between patients with S-JCA and the controls.

Methods

Patients and controls. Genomic DNA from Anglo-Saxon Caucasian patients with S-JCA was extracted as previously described (27) from blood samples collected at Northwick Park Hospital and from Great Ormond Street Hospital for Sick Children and University College London Hospitals. In all cases, samples were obtained from blood taken during routine clinical analysis. The ARC DNA Repository at the University of Manchester, England also kindly provided us with genomic DNA samples from other Anglo-Saxon Caucasian S-JCA patients. All patients had an age of onset of disease below 16 yr and had a persistent polyarthritis for at least 5 yr. 53% of the patients were female. Genomic DNA was also collected from healthy members of staff at Northwick Park Hospital but used only for sequencing and RFLP analysis during the search for polymorphisms, and not for assessing genotype frequencies.

Caucasian control DNA was obtained from 383 healthy men and women aged 40–75 yr recruited originally from a general practice in North London as part of the Goodinge Study, which was designed to investigate associations of urinary albumin excretion rate with cardiovascular disease in nondiabetic subjects. Full details of these subjects have been described elsewhere (28, 29).

The characteristics of the subjects of Gujarati Indian and Afro-Caribbean origin from the Brent and Harrow study have also been described previously (30). DNA was extracted by the salting-out method (31). DNA samples from three orangutans, three chimpanzees, and three gorillas (a kind gift of Dr. David Hunt, Institute of Ophthalmology, London, UK) was also used.

Polymerase chain reaction. The sequence of each PCR primer set is given in Table I. Primer set 1 amplified a region of 611 bp, starting upstream of the proximal putative glucocorticoid response element and finishing immediately adjacent to the main translation start site. The cycling conditions were: 95°C for 5 min, followed by the addition of 0.5 U of *Taq* polymerase (Promega, UK), then 25 cycles of 95°C for 1 min, 63°C for 1 min, and 72°C for 2 min, followed by a terminal 10-min extension phase at 72°C. Primer set 2 generated a PCR fragment encompassing the product of set 1 but extending 100 bases upstream and 148 bases downstream. Cycling conditions were similar to set 1, except that an annealing temperature of 62°C was used; 35 cycles were carried out.

PCR for single-strand conformational polymorphism (SSCP) and MADGE system RFLP (protocols developed by G. Faulds and S.

Table I. Sequences of Primers Used to Generate PCR Products for the Analysis of the IL-6 5′ Flanking Region

Name of primers	Nucleotide sequence of primers
Set 1	5′-CAG AAG AAC TCA GAT GAC TGG-3′ (5/2)
	5′-GCT GGG CTC CTG GAG GGG-3′ (3/2)
Set 2	5′-GGA GTC ACA CAC TCC ACC T-3′ (DF20)
	5′-GTG ACT GAC AGC ACA GCT-3′ (DF21)
Set 3	5′-TGA CTT CAG CTT TAC TCT TGT-3′
	5′-CTG ATT GGA AAC CTT ATT AAG-3′
Set 4	5′-CTG GTA GTA TTA CCT TCT TCA-3′
	5′-TTC TTT ATT AGT GAC TCA GCA-3′
DF13	5′-CCT TCT TCA TAA TCC AGG-3′
DF14	5′-GAG CTT CTC TTT GCT TCC-3′
DF5/2D	5′-TAT GCG AGG TAC CAG AAG AAC TCA GAT GAC TGG-3′
DF3/2D	5′-AGT TAA TCT AGA TAT GCT GGG CTC CTG GAG GGG-3′

Upstream primer of each pair is described first.

Humphries) were carried out using primer sets 3 and 4. The cycling conditions were: five cycles: of 96°C for 9 min, 95°C for 1 min, and 72°C for 3 min, followed by 30 cycles of: 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Primer sets 3 and 4 were part of a group of six primer pairs (others not shown) used by two of us (G. Faulds and S. Humphries) to screen 1179 bp of the 5′ flanking region by SSCP. Only products from these two primer pairs displayed SSCP changes in this study and were further investigated by automated sequencing.

SSCP. SSCP was carried out according to the method of Orita et al. (32) with some modifications. ^{33}P-dATP–labeled primer set 1 product was digested with Mae III. 9 μl of digested PCR product was diluted with SSCP running buffer (10 nM NaOH, 80% formamide, 1 mM EDTA, and 0.1% each BPB and XC). The sample was heated to 95°C for 5 min and then placed on ice. 10 μl of each sample was run at 5 W overnight (18 h) on a 6% nondenaturing polyacrylamide gel (MDE; Anachem, Luton, UK) at room temperature. Alternatively, 2 μl of PCR product generated by primer sets 3 or 4 was added to 6 μl of a 7:5 ratio of formamide dye mix (95% formamide, 10 mM EDTA, and 0.02% wt/vol each of BPB and XC) and a solution of 0.1% wt/vol SDS and 10 mM EDTA. The DNA was denatured at 95°C for 5 min, and then placed on ice. 4 μl of each sample was run overnight at 300 V on a 7.5% nondenaturing polyacrylamide gel with 5% vol/vol glycerol and 10 mM EDTA at room temperature (33).

Sequencing of the IL-6 5′ flanking region. Initially, 1 μl of PCR product generated from genomic DNA from S-JCA patients and staff controls by using primer pair 1 was ligated into the pCRII vector (Invitrogen, Groningen, Netherlands). This was then used to transform competent *Escherichia coli* (Invitrogen). Plasmid DNA prepared by the Wizard Miniprep kit (Promega, Southampton, UK) was sequenced with *Vent* polymerase (ThermoCycle Kit; NEB, Hitchin, UK) by using the primers shown in Table II. Subsequently, the same PCR product was sequenced using the Vistra 725 automated sequencing system (Amersham, Little Chalfont, UK). 5′-Texas Red–labeled sequencing primers (DF13 and DF14; Oswell, Southampton, UK) were nested within the location of the original PCR primers. The reaction parameters were: 95°C for 3 min, followed by 25 cycles of 95°C for 30 sec, 51°C (DF13) or 50°C (DF14) for 15 sec, and 72°C for 30 sec.

PCR products from Caucasian, Gujarati, and Afro-Caribbean control DNA samples generated using primer sets 3 and 4 were sequenced using the ABI Prism 377 system, using 3.2 pmol of the 5′ primer from the initial PCR and 0.1 pmol/μl PCR product. Cycle sequencing was: 96°C for 30 sec, 50°C for 15 sec, and 60°C for 4 min, for 25 cycles.

Restriction fragment length polymorphisms. 10 μl of PCR product generated from S-JCA patient genomic DNA by using primer sets 1 or 2 was digested with Sfa NI. After digestion the sample was denatured at 65°C for 5 min, then placed on ice for 5 min before electrophoresis on a 1% agarose gel. PCR reactions and restriction digests were carried out in duplicate. 12 μl of primer set 3 PCR product, generated from the Caucasian, Gujarati Indian, and Afro-Caribbean control sample genomic DNA, was digested with either Hsp 92II or Nla III. Analysis of the fragments was undertaken using the 96-well MADGE system (34). Restriction enzymes were obtained from Promega and New England Biolabs, Hitchin, UK.

Reporter gene constructs. PCR primers annealing to the same sequences as primer set 1 but containing recognition sites for Kpn I in the upstream primer (DF5/2D) and Xba I in the downstream primer (DF3/2D) were used to produce a 614-bp product (−550–+61 bp) from pCRII clones containing either a G or a C at the −174 position. For consistency, both clones contained the A_8T_{12} allele, allowing a direct comparison between the G and C alleles to be made without the confounding effects of a different A_nT_n allele between the two reporter constructs. This allele was chosen because it represented the commonest observed allele associated with the −174C allele. PCR conditions were the same as for primer set 2, with the exception that the primer annealing temperature was 67°C and 25 cycles were carried out. After restriction enzyme digestion, the products were directionally ligated into the pGL3 Basic Luciferase vector (Promega), linearized by digestion with Kpn I and Nhe I. Transfection-grade plasmid was prepared using the standard alkaline lysis "maxiprep" procedure, followed by two caesium chloride centrifugation gradients. Alternatively, plasmids were prepared using the Qiagen Midiprep system (Qiagen, UK). Before transfection, the sequence of each cloned product was confirmed by automated sequencing.

Transient transfections. 4×10^5 HeLa cells (ATCC, Rockville, MD) were seeded into each well of a six-well plate and grown to confluence (2 d) in Dulbecco's modified Eagle's medium (GIBCO BRL, Paisley, UK), supplemented with 10% heat-inactivated fetal calf serum, nonessential amino acids, and penicillin (50 U/ml)/streptomycin (50 μg/ml), all obtained from Sigma (Poole, UK). The HeLa cells were transfected with 10 μg of each reporter plasmid and 3 μg of a control plasmid containing the β-galactosidase gene (RSV-β-gal), which lacks a cytokine responsive promoter, by the calcium phosphate method (35). After 18 h, the cells were stimulated with either IL-1 (10 U/ml) or LPS (10 μg/ml). After a further 24 h, the cells were harvested. Luciferase and β-galactosidase expression were measured using commercial assay kits (Luciferase Assay; Promega and GalactoLight Plus; Tropix, Bedford, MA) in a BioOrbit 1253 luminometer (Labtech, Uckfield, UK). Each transfection was carried in duplicate, and transfections were done twice each.

Measurement of plasma IL-6. Plasma IL-6 levels were measured from blood samples obtained in the early morning, after an overnight fast. All the subjects were healthy at the time of venesection. The samples were analyzed in duplicate using a commercially available ELISA kit (R & D Systems, Oxon, UK) as described previously (36).

Statistical methods. The gene-counting method with a χ^2 test with Yates correction was used to compare the frequency of the C allele between the different groups. All other tests and transformations were performed using the SPSS/PC+ statistical package. The *t* test was used to compare levels of luciferase between constructs. Statistical significance was considered to be at the 0.05 level. ANOVA was used to compare concentrations of plasma IL-6 between individuals with different IL-6 genotypes. To test differences in IL-6 concentrations, values were log-transformed before statistical analysis. Multiple linear regression was used to adjust for the effects of age, body mass index, gender, and smoking on plasma levels of IL-6 and the adjusted values used in the ANOVA. Original rather than log transformed values are presented for clarity.

able II. Sequence of Primers Used for Manual Sequencing

Nucleotide sequence of primers	Name of primers
·CAG AAG AAC TCA GAT GAC TGG-3′	5/2
GCT GGG CTC CTG GAG GGG-3′	3/2
TTC ACA CAG GAA ACA G-3′	M13 reverse, within pCRII vector
GTC AGA GGA AAC TCA GTT CAG-3′	DF3
GTG CAT GAC TTC AGC TTT AC-3′	DF4
GTG GTT CTG CTT CTT AGC-3′	DF5
GGA CGT CAC ATT GCA C-3′	DF6
GAG TCT CAA TAT TAG AGT CT-3′	DF7
GTC GTG ACT GGG AAA AC-3′	M13 forward, within pCRII vector

Results

SSCP analysis. The region of the IL-6 gene investigated is shown in Fig. 1. Significant interindividual differences in SSCP banding patterns were observed for Mae III digested primer set 1 PCR products and for the nondigested primer set 3 and 4 products.

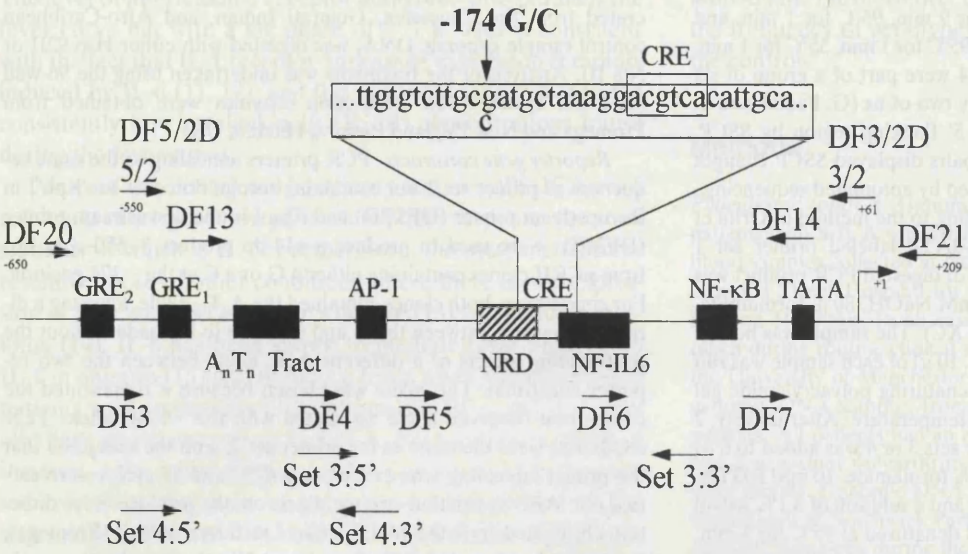

Figure 1. A schematic representation of the 5′ flanking region of the IL-6 gene identifying the 174G/C polymorphism, transcription factor binding sites, and the position and orientation of the oligonucleotide pairs used in the SSCP, RFLP, and sequencing analysis. Locations of the binding sites are relative to the major transcription start site: TATA box −27 to −24; NF-κB −73 to −64; NF-IL-6 (C/EBPβ) −158 to −145; CRE (cAMP responsive element) −163 to −158; NRD (negative regulatory domain) −225 to −164; AP-1 −283 to −277; A_nT_n tract −392 to −373; GRE_1 and GRE_2 (glucocorticoid responsive elements 1 and 2) −466 to −461 and −557 to −552, respectively.

Overall, these features were highly suggestive for the presence of variations in the nucleotide sequence in these regions.

G to C polymorphism at position −174. Initially the 611-bp primer set 1 PCR product from 41 staff controls and 19 patients with S-JCA was cloned into the pCRII vector and sequenced by a combination of manual and automated sequencing. Compared with the published sequence of Yasukawa et al. (37), a G to C polymorphism was identified at position −174 in 14 of these controls and 7 of the S-JCA cases (35% of the total samples). It was independently confirmed (by G. Faulds and S. Humphries) by automated sequencing of 12 examples of the primer set 3 PCR product, 6 with each SSCP appearance, corresponding to either allele. The polymorphism produced an RFLP that was identified by three restriction enzymes, Sfa NI, Hsp 92II, and Nla III. The sensitivity and specificity of each RFLP method was demonstrated as each was able to correctly recognize the genotype identified by sequencing (data not shown).

The C allele and genotype frequencies of the 383 Caucasian controls, 115 Gujarati Indians, and 101 Afro-Caribbeans analyzed by MADGE system RFLP are shown in Table III. The frequency of the C allele in the different racial groups was 0.403 (0.37–0.44), 0.150 (0.094–0.184), and 0.05 (0.016–0.072), respectively.

Table III. Results of MADGE PCR-RFLP for the −174 Polymorphism, Showing the Genotype Distribution and C Allele Frequency (95% confidence interval) in Subjects of Different Ethnic Origin

Genotype	UK Caucasian male and female controls (n = 383)	Gujarati Indians (n = 115)	Afro-Caribbeans (n = 101)
GG	144	85	92
GC	169	28	9
CC	70	2	0
C allele Frequency (95% CI)	0.403 (0.37–0.44)	0.150 (0.094–0.184)	0.05 (0.016–0.072)

All three racial groups were in Hardy-Weinberg equilibrium. χ^2 values were: Caucasians 2.7 (P = 0.10), Gujaratis 0.03 (P = 0.86), and Afro-Caribbeans 0.22 (P = 0.64).

The distribution of the age of onset of S-JCA in our sample is shown in Fig. 2. The peak age of onset of disease in this group was at 2 yr. Table IV shows the genotype and C allele frequencies for the S-JCA patients (both as a whole and divided into onset at or before 5 yr and onset at or after 6 yr of age), compared with Caucasian controls. There was no significant difference between the C allele frequencies in the controls and any of the S-JCA patient groups. However, the overall genotype distribution of the whole patient group was significantly different than the controls (P = 0.03, χ^2 = 6.82). In the ≤ 5-yr-old onset group, this difference was even greater (P = 0.01, χ^2 = 8.94). When the genotype frequencies of the S-JCA patients and the controls were compared, there was a statistically significant reduction in CC genotype frequency in the S-JCA patients with age of onset ≤ 5 yr (P = 0.04, χ^2 = 4.32, OR 0.34, 95% CI 0.12–0.98).

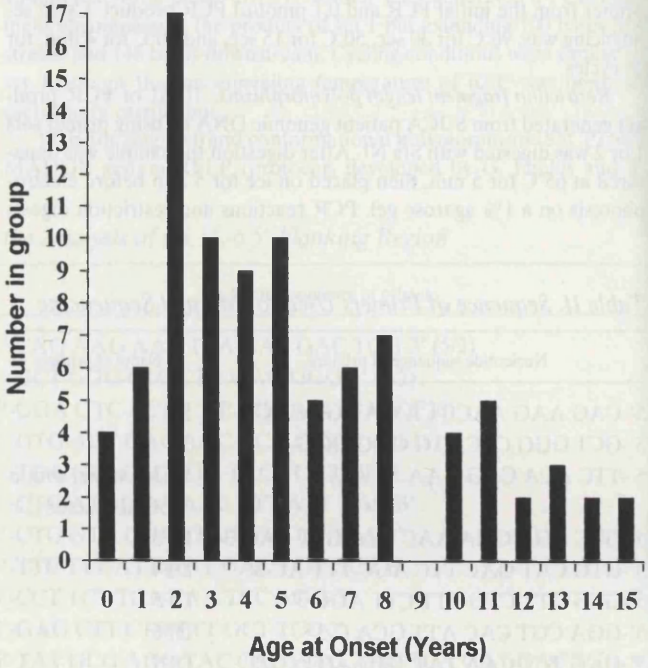

Figure 2. Age of onset distribution for patients with S-JCA. The peak age of onset in the group studied here was 2 yr. 61% of the patients had an age of onset of 5 yr or below.

Table IV. Results of the RFLP Analysis of Patients with S-JCA, Showing the Genotype and C Allele Frequencies, Compared with the UK Control Caucasian Group

Genotype	All S-JCA patients (n = 92)	S-JCA Onset ≤ 5 yr old (n = 56)	S-JCA Onset ≥ 6 yr old (n =36)	UK Caucasian male and female controls (n = 383)
GG	28 (0.30)	16 (0.29)	12 (0.33)	144 (0.38)
GC	54 (0.59)	36 (0.64)	18 (0.50)	169 (0.44)
CC	10 (0.11)	4 (0.07)	6 (0.17)	70 (0.18)
C-allele	0.402	0.393	0.417	0.403
Frequency*	(0.33–0.47)	(0.30–0.48)	(0.30–0.53)	(0.37–0.44)
Patients** vs. controls	$P = 0.03$	$P = 0.01$	$P = 0.79$	
	$\chi^2 = 6.82$	$\chi^2 = 8.94$	$\chi^2 = 0.46$	

* 95% CI. **Overall genotype distribution analyzed by χ^2 test.

Other polymorphisms. We also observed a variation in the composition of the A_nT_n tract at the published positions −392 to −373, the published nucleotide sequence being A_9T_{11} (37). We assessed the composition of the A_nT_n tract in a total of 57 subjects (32 staff controls and 25 S-JCA patients) by sequencing of cloned PCR products. Three patterns of 20 base length were observed: A_8T_{12} (n = 24), A_9T_{11} (n = 12), and $A_{10}T_{10}$ (n = 10). Two patterns of 19 bases: A_9T_{10} (n = 3) and $A_{10}T_9$ (n = 1) and one pattern of 21 bases: $A_{10}T_{11}$ (n = 7) were also seen. In all but one case, the −174C allele was associated with the A_8T_{12} pattern (Table V).

In addition, we consistently observed that the published sequence had underestimated two triplets of C residues, at positions −508 and +15, as pairs of C residues. This is likely to represent a technical error in the reported sequence.

Functional studies. Fig. 3 shows the results of transient transfection experiments in HeLa cells by using constructs containing either allele of the −174 5′ flanking region polymorphism. Correction for variations in transfection efficiency and cell lysate recovery was achieved by dividing the luciferase luminescence value by that for β-galactosidase control (38). The results of two separate experiments (each performed in

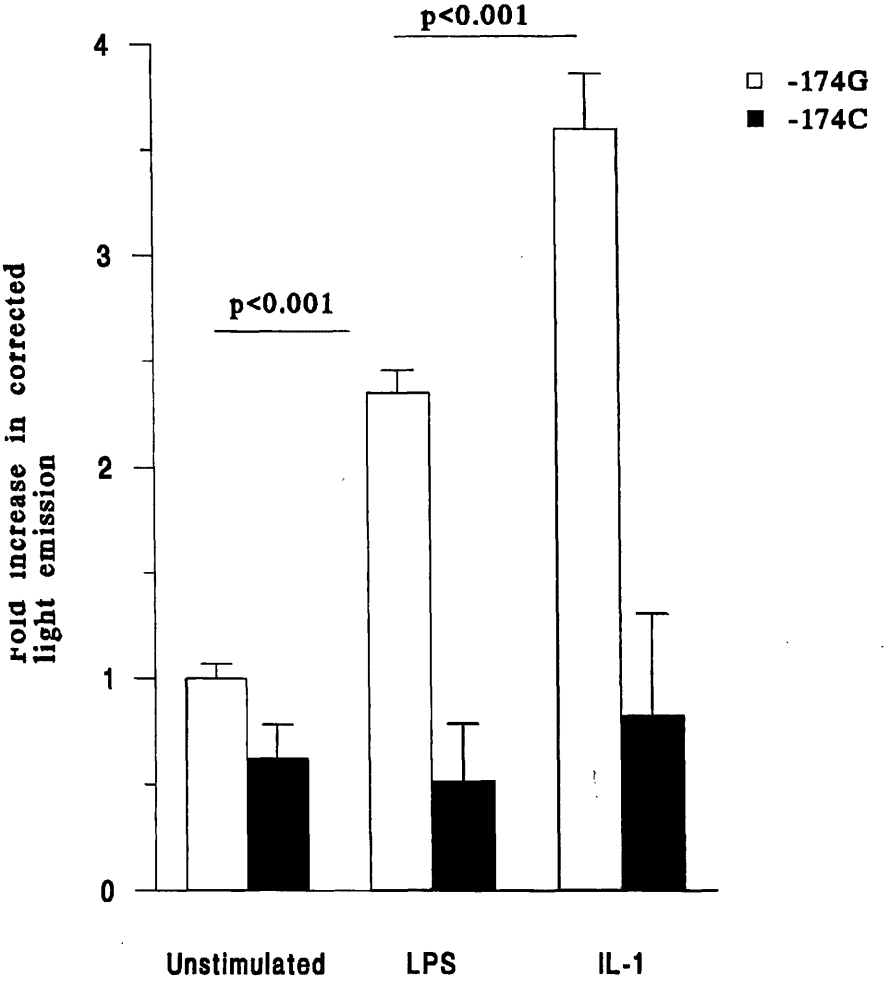

Figure 3. Luciferase activity (SD), measured as the fold increase in light emission relative to the unstimulated G(A_8T_{12}) construct, of G(A_8T_{12}) and C(A_8T_{12}) constructs expressed in HeLa cells. Results are the mean of four transfections, corrected for protein content and efficiency of transfection by β-galactosidase cotransfection.

duplicate), where the plasmids were prepared by caesium chloride gradient separation or Qiagen preparation, were combined by calculating the fold change in corrected luciferase expression relative to the unstimulated −174G allele. In unstimulated cells, the C(A_8T_{12}) construct showed 0.62±0.15-fold lower ($P < 0.005$) expression than the G(A_8T_{12}) construct. After stimulation with lipopolysaccharide or IL-1, expression from the G(A_8T_{12}) construct increased by 2.35±0.10-fold and 3.60±0.26-fold, respectively, compared with the unstimulated level (both $P < 0.001$). However, expression from the C(A_8T_{12}) construct did not change significantly ($P = 0.5$ and 0.46, respectively). LPS- and IL-1–induced luciferase expression from the G(A_8T_{12}) construct was, respectively, 4.55±0.39-fold and 4.38±0.54-fold that induced from the C(A_8T_{12}) construct (both $P < 0.0001$).

Plasma IL-6 levels. From the 383 healthy Caucasians, a subset of 102 subjects were recalled for measurement of their fasting plasma IL-6. In this subset, the GG homozygotes had circulating IL-6 concentrations approximately twice as high as those homozygous for the C allele ($P = 0.02$) (Fig. 4).

Discussion

There are now several examples of polymorphisms occurring within cytokine genes, some of which are associated with disease. A polymorphism in the promoter region of the IL-1α gene has been found to be associated with pauci-articular JCA in a cohort of Norwegian children, especially in those with chronic iridocyclitis (22), but not in UK children (23). There is a strong association between a worse outcome from malaria or mucocutaneous leishmaniasis and the rare allele of the −308 TNF-α promoter polymorphism (TNFA2) (24, 25). This allele has been shown to be a stronger inducer of reporter gene expression than the common allele (19). IL-6 polymorphisms have previously been reported: Msp I and Bgl I identify probable base substitutions around the fifth exon and in the 5′ flanking region, respectively (39). In addition, Bst NI and at least nine other enzymes identify an RFLP in an AT-rich area of the 3′ flanking region, representing insertions of varying sizes. A PCR method to screen this region has been developed (40). Considerable interethnic variation in the frequencies of these polymorphisms has been demonstrated (39), which is consistent with our data for the −174C allele, which is considerably rarer in Gujarati Indians and Afro-Caribbeans, compared with UK Caucasians. As all of the primates examined were GG homozygotes, it is likely that this allele is ancestral and that the C allele represents a relatively recent change in the IL-6 5′ flanking sequence.

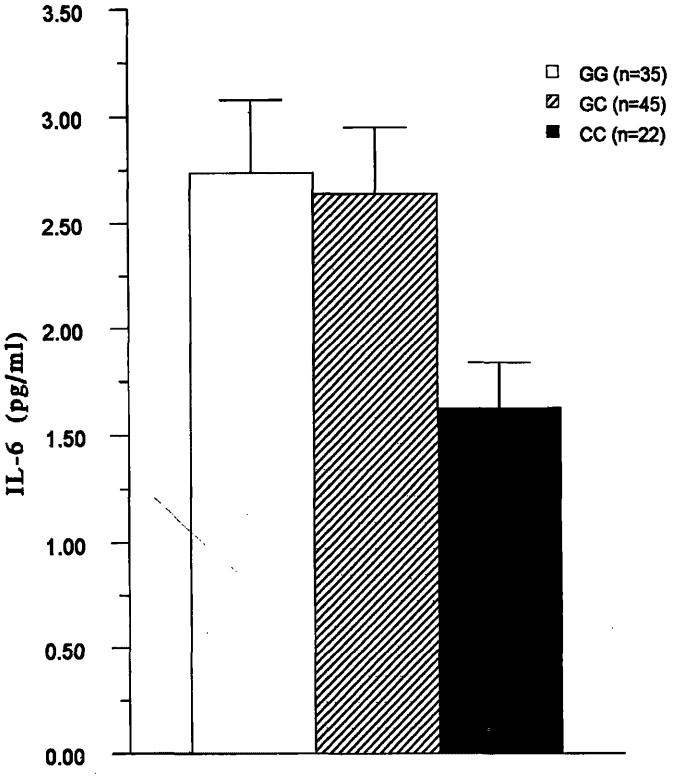

Figure 4. Mean plasma IL-6 levels in healthy men and women with different IL-6 G/C genotypes. Mean values (adjusted for age, body mass index, gender, and smoking) were GG: 2.74 (95% CI 2.43–3.10); GC: 2.64 (2.35–2.97); CC: 1.63 (1.44–1.86). ($P = 0.02$ by ANOVA).

Table V. Combined Results for Sequencing A_nT_n Tract from Patient and NPH Controls

	A_8T_{12}	A_9T_{11}	$A_{10}T_{10}$	A_9T_{10}	$A_{10}T_{11}$	$A_{10}T_9$
−174G	5	12	9	3	7	1
−174C	19	0	1	0	0	0

In all but one case, the −174C allele was associated with the A_8T_{12} tract configuration. Considerably more variation was observed for the A_nT_n tract association with the −174G allele.

Until now, there have been no disease associations with any polymorphisms in the 5′ flanking region of the IL-6 gene. The Msp I and Bgl I RFLP are not associated with RA, SLE, or pauci-articular JCA (41). An increased frequency of an Xba I RFLP, also likely to be due to 3′ flanking region insertions, has been described in some patients with SLE and elevated IL-6 levels (42). By using PCR-RFLP and sensitive polyacrylamide gel electrophoresis, an association between genotype for the 3′ flanking region polymorphism and peak bone mineral density in women has been demonstrated (43). However, none of these RFLP are located in areas known to be responsible for inducible transcriptional control. This current report is the first description of a polymorphism in the 5′ flanking region that alters the transcriptional response to stimuli such as LPS and IL-1, results that have since been repeated consistently in both our laboratories.

The region between −225 to −164 containing the G to C polymorphism has previously been reported to demonstrate a negative regulatory effect on reporter gene expression (44). Studies on the repression of the IL-6 promoter by using DNA footprinting have demonstrated binding of the glucocorticoid receptor to a region around −201. The polymorphism at −174 is close enough to this site for it to potentially influence the binding of the glucocorticoid receptor and thus its ability to repress transcriptional activation. It is of considerable interest that the change from a G to a C at position −174 creates a potential binding site for the transcription factor NF-1. NF-1 comprises a family of structurally related transcription factors active in many cell types (45). Although this factor has been shown to have varying effects on transcription, in HeLa cells it has been demonstrated to be a repressor of gene expression (46). Our work has shown that reporter gene expression from the C allele is repressed in HeLa cells. It is possible that in

other cell lines this response is very different. Thus, the in vivo situation is likely to be complex, where IL-6 is expressed from several different tissues. We are currently investigating the possibility that this effect is due to binding of DNA in this region by a nuclear protein acting as a repressor in HeLa cells, by using electromobility shift assays and DNA footprinting. We are also investigating whether this reduced expression occurs in other tissue cell lines, by transient transfection experiments and if different A_nT_n patterns influence this differential expression.

The genotype frequencies in our S-JCA patient group is significantly different from that in a large control population. The reduction in the prevalence of the CC genotype in patients with S-JCA, especially those with disease onset at or before 5 yr, is a very interesting finding. This is the first time that a significant genetic association has been demonstrated for S-JCA. The in vitro data showing that reporter expression from the C allele is repressed after stimulation with IL-1 and LPS, whereas the G allele induces reporter expression as would be predicted, suggest that the presence of the C allele—and more so the CC genotype—would result in a lower IL-6 expression after a given inflammatory stimulus compared with the GG genotype. This is supported by our in vivo observation that IL-6 levels are lower in normal subjects with the CC genotype, compared with GC or GG subjects.

The reduction in the frequency of the CC genotype in S-JCA patients therefore suggests that this genotype confers a protective influence against the development of the disease. This implies that an individual's genotype influences his or her IL-6 response to a stressful stimulus in a manner analogous to that of the TNF-α −308 polymorphism (19). It is highly unlikely that the -174 polymorphism alone represents the susceptibility gene for the development of S-JCA. Rather, the GG (or GC) genotypes may be interacting with other genetic and/or environmental triggers to produce the disease. Linkage between the polymorphism and S-JCA is amenable to analysis by the transmission disequilibrium test, for which we are currently collecting simplex families.

Although there is no validated method for assessing disease severity in S-JCA, the disease course of persistent arthritis in all of these patients demonstrates that they are of the moderate to severe spectrum. There is no published data concerning the incidence of S-JCA in non-Caucasians, and given the low frequency of the C allele in Gujarati Indians and Afro-Caribbeans, it would be very interesting to investigate the genotypes of S-JCA patients in these groups. The reduction in CC genotype frequency in the ≤ 5-yr-old onset group is very interesting. These children are frequently very unwell, with chronic disease that is difficult to suppress. The subdivision into onset before or after 5 yr of age is also of relevance in other JCA subgroups. In particular, the young onset oligoarticular JCA subgroup is often defined by onset age 5 or below, and they have strong HLA associations that are not seen in older oligoarticular onset children (47).

Although it would be of interest to determine the association between a patient's −174 genotype and their serum IL-6 levels during active disease, and also to compare the level of IL-6 in the prefever, febrile, and postfever state between patients with different genotypes, these findings are likely to be very difficult to interpret for several reasons. First, IL-6 levels may be influenced by medication. Second, we have shown a large variation in the absolute IL-6 level between children acutely ill with S-JCA (9). Third, other factors, such as the "severity" of disease (for which there is no agreed, objective scale), age, disease duration as well as the duration of current flare, and nutritional state are also likely to influence the serum IL-6 levels. However, the association between the C allele and lower levels of IL-6 in healthy fasting men and women strongly supports the in vitro finding of reduced promoter strength. We are also in the process of measuring IL-6 production by PBMC from healthy donors with different genotypes to assess the in vitro IL-6 response to inflammatory stimuli.

In summary, we have demonstrated novel polymorphisms in the 5′ flanking region of the IL-6 gene, an area important in the regulation of gene expression. A single nucleotide change from G to C at position −174 results in suppression of IL-6 transcription in response to LPS or IL-1 in HeLa cells. Furthermore, the CC genotype is significantly less common in S-JCA than in Caucasian control subjects and may play a protective role against the development of the disease.

Acknowledgments

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