EXPRESSION AND CHARACTERIZATION OF THE HUMAN ERYTHROCYTE GLUCOSE TRANSPORTER IN INSECT CELLS USING A RECOMBINANT BACULOVIRUS

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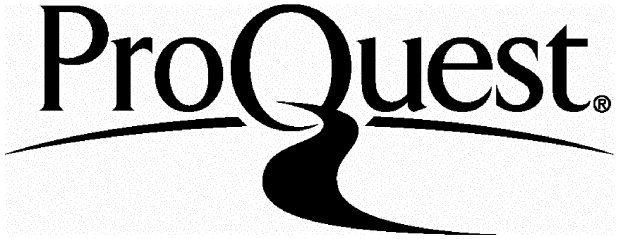

To my mother

Expression and Characterization of The Human Erythrocyte Glucose Transporter in Insect Cells Using a Recombinant Baculovirus

ABSTRACT

The GLUT1 isoform of the mammalian passive glucose transporter family plays a physiologically vital role in catalysing the uptake of sugar by a variety of tissues, including human erythrocytes and the brain. Although the transporter can be purified from natural sources in small amounts, investigation of its structure and function, by techniques such as site-directed mutagenesis and by biophysical methods such as crystallization, would be greatly facilitated by development of heterologous expression systems capable of producing large amounts of the recombinant protein. To this end, a recombinant baculovirus was produced by inserting cDNA encoding human GLUT1 into the genome of the *Autographa californica* nuclear polyhedrosis virus, under the control of the polyhedrin promoter.

Insect cells infected with the recombinant virus were found to synthesise high levels of recombinant transporter (up to 8% of total membrane protein). The expressed protein was recognised by a range of polyclonal, site-directed antibodies against GLUT1, indicating that it corresponded to a full-length version of GLUT1. However, its electrophoretic mobility on SDS gels was substantially greater than that of the natural erythrocyte protein, probably as

a result of the recombinant protein being less heavily and heterogeneously glycosylated. Four days after infection with the virus, the insect cell-surface and intracellular membranes exhibited >200 pmol cytochalasin B binding sites per mg protein, which bound the transport inhibitor with characteristics identical to those of the erythrocyte protein. The inhibitor also photolabelled the expressed protein in a D-glucose sensitive manner. Thus, it appears that expression of GLUT1 in insect cells yields a protein that is functionally similar to its erythrocyte counterpart. This is the first demonstration of overexpression of a mammalian facilitative glucose transporter in functional form.

An attempt was also made to develop procedures for the rapid isolation of the recombinant transporter in functional form from insect cells, by producing a fusion protein containing a polyhistidine domain: the high affinity of the latter for metal ions should facilitate isolation of such fusion proteins by chromatography on columns of immobilised Ni^{2+}. However, the low level of expression and apparent instability of the recombinant fusion protein encoding the transporter unfortunately precluded its purification by this approach.

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LIST OF ABBREVIATIONS

A_{260}	absorption at 260 nm
AcNPV	*Autographica californica* nuclear polyhedrosis virus
Ala	alanine (A)
Amp	ampicillin
Arg	arginine (R)
ASA-BMPA	2-*N*-(4-azidosalicoyl)-1,3-bis-(D-mannos-4-yloxy)-2-propylamine
Asn	asparagine (N)
Asp	aspartic acid (D)
ATB-BMPA	2-*N*-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(D-mannos-4-yloxy)-2-propylamine
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BmNPV	*Bombyx mori* nuclear polyhedrosis virus
bp	base pairs
BSA	bovine serum albumin
CD	circular dichroism
cDNA	complementary DNA
$C_{12}E_8$	octaethylene glycol dodecyl ether
CHO	chinese hamster ovary
Ci	curie (3.7×10^{10} Bq)
CIP	calf intestinal phosphatase
cpm	counts per minute
CsCl	cesium chloride
Cys	cysteine (C)
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanidine triphosphate
dTTP	deoxythymidine triphosphate
2dGlc	2-deoxy-D-glucose

DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTPs	deoxynucleoside triphosphates
dpm	disintegrations per minute (60 Bq)
DTT	dithiothreitol
ECL	enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
ER	endoplasmic reticulum
EtBr	ethidium bromide
EV	extracellular budded virus
FP	few polyhedra
FTIR	Fourier transform infrared
GET	glucose, EDTA and Tris-HCl
Gln	glutamine (Q)
x g	centrifugal force (x unit gravitational field)
HA	haemagglutinin
HCl	hydrochloric acid
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
[^{125}I]IAPS-foskolin	3-[^{125}I]iodo-4-azidophenethylamido-7-*O*-succinyldeacetyl-forskolin
IPTG	isopropyl β-D-thiogalactoside
Kb	kilobases
K_d	dissociation constant
KDa	kilodaltons
K_i	inhibition constant
K_m	the half saturation concentration of substrate
LB	Luria-Bertaini broth
LCMV	lymphocytic choriomeningitis virus

Leu	leucine (L)
Lys	lysine (K)
MNPV	mutiple nuclear polyhedrosis virus
MOI	multiplicity of infection
MOPS	3-(*N*-Morpholino)propanesulfonic acid
M	molarity
mRNA	messenger RNA
NBT	nitro blue tetrazolium
NE	sodium acetate and EDTA
NPV	nuclear polyhedrosis virus
NTP	(unspecified)nucleoside triphosphate
ORF	open reading frame
Ori	origin of replication
OV	occluded virus
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PBSA	Dulbecco 'A' phosphate-buffered saline
PCR	polymerase chain reaction
pfu	plaque forming unit
p.i.	post-infection
Phe	phenylalanine (F)
PMSF	phenylmethanesulphonyl fluoride
RF	replicative form
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolution per minute
SDS	sodium dodecyl sulphate
Sf	*Spodoptera frugiperda*
SNPV	single nuclear polyhedrosis virus
TAE	tris-acetate buffer
TBE	tris-borate buffer
TBS	tris-buffered saline

TE	Tris-EDTA buffer
TEMED	tetramethyl ethylene diamine
Tris	tris(hydroxymethyl)-aminomethane
Trp	tryptophan (W)
TTBS	TBS containing 0.2 % Tween-20
UV	ultraviolet
V_{max}	the maximum velocity of transport
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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CHAPTER 1. GENERAL INTRODUCTION

1.A. MAMMALIAN FACILITATIVE GLUCOSE TRANSPORTERS

1.A.1. INTRODUCTION

Glucose is one of the most abundant natural organic compounds and is also a major source of metabolic energy for most mammalian cells. For example, the energy supply for oxidative metabolism in the human brain is almost entirely dependent on a sustained availability of glucose (Lund-Andersen, 1979). However, the cell cannot absorb glucose by simple diffusion. The entry of such a hydrophilic molecule across the hydrophobic lipid bilayer of the membrane requires the aid of a special carrier protein, termed a glucose transporter.

Transport across the cell membrane may be passive or active. Passive transport, often termed facilitative diffusion, is a type of diffusion in which an ion or molecule crossing the membrane moves down its electrochemical or concentration gradient through mediation of a transport protein, requiring no metabolic energy. In contrast, active transport uses metabolic energy to move an ion or molecule against its electrochemical gradient. Such import or export of a small molecule against a concentration gradient may be achieved by coupling its flux to that of another molecule or ion, usually H^+ or Na^+, either in the same or in opposing direction to that of the first molecule, these processes being termed symport or antiport respectively. In mammals, active, sodium-linked symport systems for glucose occur in only a few tissues, such as the

small intestine and kidney, where transepithelial transport against a concentration gradient is necessary (Hediger *et al.*, 1987). However, most mammalian cells take up glucose by passive facilitated diffusion (Elbrink and Bihler, 1975).

1.A.2. FACILITATIVE GLUCOSE TRANSPORTERS

The purification and characterization of the human erythrocyte glucose transporter (Kasahara and Hinkle, 1977; Baldwin *et al.*, 1982) provided the basis for the isolation of human and rat glucose transporter cDNA clones from HepG2 hepatoma cell and rat brain cDNA libraries, respectively (Mueckler *et al.*, 1985; Birnbaum *et al.*, 1986). These cDNA clones were subsequently used as probes to screen cDNA libraries, derived from other mammalian tissues, at low hybridisation stringency. With these procedures, five homologous facilitative glucose transporter isoforms have now been identified and characterized (Table 1.1, reviewed in Gould and Bell, 1990; Lienhard *et al.*, 1992; Baldwin, 1993). Each glucose transporter (GLUT, the gene symbol for facilitative glucose transporter) isoform is numbered in the order of its cDNA discovery, i.e. GLUT1-GLUT7 (the terminology of Fukumoto *et al.*, 1989). GLUT6 represents a glucose transporter pseudogene-like sequence which is part of a mRNA that appears to be expressed in all human tissues (Kayano *et al.*, 1990). However, this sequence cannot encode a functional glucose transport protein due to the presence of multiple stop codons and frame shifts.

The facilitative glucose transporter isoforms display a marked tissue-specific pattern of expression and their kinetic and biochemical properties differ.

Table 1.1. Properties of the mammalian passive glucose transporters

Type	M_r+ (residues)	Expression in Tissues & Cells	Chromosomal Location+
GLUT1 (erythrocyte/brain, HepG2)	54117 (492)	erythrocyte (human), blood-brain barrier,placenta, fetal tissues in general	1
GLUT2 (liver)	57000 (524)	liver,pancreatic β-cell, kidney,small intestine	3
GLUT3 (brain)	53933 (496)	brain(neurones)	12
GLUT4 (adipocyte/muscle, insulin-regulatable)	54797 (509)	brown & white adipocytes, heart and skeletal muscle	17
GLUT5 (small intestine)	54983 (501)	small intestine	1
GLUT7 (hepatic microsomal)	53000 (528)	endoplasmic reticulum of hepatocytes	N.D.

+ M_r values, number of residues and chromosomal locations of genes are given for the human glucose transporters, except for GLUT7 where the values for the rat protein are indicated. (Adopted from Baldwin, 1993).

This diversity of the transport proteins allows precise control of blood glucose concentration to be maintained in mammals over a range of physiological conditions. The transporter isoforms, which vary in size from 492 to 528 residues, exhibit considerable similarity in their amino acid sequences with ≥40% of the residues being identical (Mueckler *et al.*, 1985; Fukumoto *et al.*, 1988; Kayano *et al.*, 1988; James *et al.*, 1989; Kayano *et al.*, 1990; Waddell *et al.*, 1992). This degree of sequence similarity suggests that the membrane topologies of these isoforms are essentially identical to that proposed for GLUT1 (Mueckler *et al.*, 1985) in which the protein spans the plasma membrane 12 times, with its *N*- and *C*-termini cytoplasmically oriented (see Section 1.A.5.2). Greatest sequence divergences are found in the cytoplasmic domains at the *N*- and *C*-termini, the hydrophilic central loop, and the extracellular loop between the first two putative, transmembrane regions, implying that these regions may contribute to the unique features of these proteins including their intrinsic activities and subcellular localization. Following their expression in heterologous expression systems, including bacteria, *Xenopus* oocytes, or transfected mammalian cells (discussed in Section 1.A.8.1), each of the transporter isoforms has been shown to be capable of transporting D-glucose. Furthermore, in each case glucose transport has been found to be inhibitable by the fungal metabolite cytochalasin B. It is noteworthy that in addition to functioning as sugar carriers, the facilitative glucose transporters may play a role in transport of nicotinamide (Sofue *et al.*, 1992) and vitamin C (Vera *et al.*, 1993), and may also serve as membrane water channels (Fischbarg *et al.*, 1990).

1.A.2.1. GLUT1

The GLUT1 is also known as the HepG2 or erythrocyte/brain type transporter, because the isolation of cDNAs encoding human and rat GLUT1 was achieved by screening human hepatoma carcinoma cell line (HepG2) and rat brain libraries using antibodies raised against the purified erythrocyte glucose transporter (Mueckler *et al.*, 1985; Birnbaum *et al.*, 1986). cDNAs encoding the transporter isoform have also been isolated from other species (see Baldwin, 1993).

Analysis of the predicted amino acid sequence of the GLUT1 family indicates that the protein is composed of 492 amino acid residues, and that the sequences are highly conserved with more than 97 % identity, implying the importance of all domains of the polypeptide. GLUT1 has a high degree of stereospecificity for pentose and hexose monosaccharides in the pyranose ring form (LeFevre, 1961; Barnett *et al.*, 1973). For example, the K_m for D-glucose of zero-trans uptake at 37°C is approximately 7mM (Lowe and Walmsley, 1986), whereas for L-glucose the K_m is greater than 3 M (Carruthers, 1990). However, GLUT1 has a very low affinity for fructose (K_m = about 1.5 M, LeFevre and Marshall, 1958). The highest levels of polypeptide expression of GLUT1 are found in fetal tissues, including placenta and brain (Asano *et al.*, 1988; Santalucia *et al.*, 1992). In many of the adult tissues in which it is expressed, the GLUT1 isoform is particularly abundant in cells of blood-tissue barriers (Takata *et al.*, 1990). Since low levels of GLUT1 mRNA or protein can be detected in most tissues, this transporter isoform may be responsible, at least in part, for constitutive glucose uptake. Interestingly, studies of the

developmental regulation of GLUT1 mRNA in rat tissues have shown that the transporter is also highly expressed in tissues such as heart, liver, skeletal muscle and brown fat during fetal life, but its expression in these tissues diminishes rapidly after birth, implying the existence of a circulating factor responsible for the enhanced expression of GLUT1 during fetal life (Santalucia *et al.*, 1992). However, the nature of this signal involved in the developmental regulation of GLUT1 remains unknown.

1.A.2.2. GLUT2

The liver is an important site for glucose uptake and plays a vital role in the maintenance of blood glucose levels. The finding that only very low levels of mRNA for the ubiquitous GLUT1 transporter isoform are detectable in adult liver (Birnbaum *et al.*, 1986; Flier *et al.*, 1987) suggested the existence of another glucose transporter in this tissue. Clones encoding the latter, the GLUT2 or liver-type transporter, have now been isolated by low stringency hybridisation screening, using a human GLUT1 cDNA probe, from human (Fukumoto *et al.*, 1988), rat (Thorens *et al.*, 1988), and mouse (Asano *et al.*, 1989a; Suzue *et al.*, 1989) cDNA libraries. The encoded proteins are predicted to contain 522, 523 and 524 amino acids, respectively, and share 55 % sequence identity with GLUT1. However, the amino acid sequence of GLUT2 is not as highly conserved among species as that of GLUT1. The most divergent regions of GLUT2 are the extracellular loop and the intracellular *C*-terminal domains. The extracellular loop of 65 (GLUT2) or 33 (GLUT1) amino acids that connects putative transmembrane helices 1 and 2 contains a

potential site for asparagine-linked glycosylation.

Interestingly, the GLUT2 isoform has an 15-fold higher affinity (K_m = 100 mM) for fructose compared to GLUT1 (Okuno and Gliemann, 1986). Furthermore, it exhibits higher K_m values for glucose and its analogues than do the other isoforms (Craik and Elliot, 1979; Johnson *et al.*, 1990a; Gould *et al.*, 1991). For example, when expressed in *Xenopus* oocytes human GLUT2 exhibits a K_m for 3-*O*-methyl-D-glucose equilibrium exchange of about 42 mM, which is significantly higher than the value of about 17 mM measured for GLUT1 under the same circumstances (Gould *et al.*, 1991). This high K_m value implies that glucose flux through the GLUT2 isoform at physiological glucose concentrations would be changed in a virtually linear fashion with glucose concentration. Consequently, it would prevent the transport of glucose from becoming rate limiting for either metabolism or supply to the blood. It has also been shown that the affinity for cytochalasin B of GLUT2 is 10-fold lower than that of GLUT1, and that GLUT2 is only poorly photoaffinity-labelled by this ligand (Axelrod and Pilch, 1983).

Analysis of protein and mRNA distribution shows that the GLUT2 isoform is expressed in the liver, kidney, small intestine and insulin-producing β-cell of the endocrine pancreas (Thorens *et al.*, 1988). The presence of GLUT2 in the first three tissues suggests that it mediates the uptake and release of glucose by the liver, and that it participates in the transepithelial transport of absorbed and reabsorbed glucose by the small intestine and kidney, respectively. As described in the Introduction, the absorption of glucose from the lumen of the small intestine and proximal tubule of the kidney into the epithelial cells is

mediated by a Na^+/glucose cotransporter (Hediger *et al.*, 1987). In addition to these roles in liver, kidney and intestine, the presence of GLUT2 in the β-cell also suggests that it may play a role in the regulation of glucose-stimulated insulin secretion. This possibility is supported by the finding that a decrease in the level of the GLUT2 isoform in the pancreatic β-cell precedes the development of type II noninsulin-dependent diabetes mellitus (Johnson *et al.*, 1990b).

1.A.2.3. GLUT3

cDNA clones encoding the GLUT3 isoform, the brain-type transporter, were originally isolated from a human fetal skeletal muscle library (Kayano *et al.*, 1988). The encoded protein of 496 amino acids has 64 and 52 % sequence identity with human GLUT1 and GLUT2, respectively. Recently, Nagamatsu *et al.* (1992) have identified and characterized a cDNA clone encoding mouse GLUT3, which contains 493 amino acids having 83 % identity with the sequence of human GLUT3. The sequence of GLUT3 is not as highly conserved among species as that of the GLUT1 family. The greatest sequence divergence is found in the regions of the extracellular loop that connects transmembrane helices 1 and 2 and in the hydrophilic *C*-terminal domain of the protein.

RNA blotting studies have shown that GLUT3 mRNA is found at various levels in most adult human tissues but is most abundant in brain, kidney and placenta (Kayano *et al.*, 1988). Similar studies in monkeys, rabbits, rats and mice have indicated that the GLUT3 mRNA has a more restricted tissue distribution in these species compared to humans, and that it is expressed at

high levels only in the brain (Nagamatsu *et al.*, 1992; Maher *et al.*, 1992; Yano *et al.*, 1991). Furthermore, *in situ* hybridisation studies have demonstrated that GLUT3 mRNA is widely distributed in mouse brain and is present at the highest levels in the hippocampus, cerebellum and cerebral cortex (Nagamatsu *et al.*, 1992). The GLUT3 isoform is also expressed in primary cultured rat cerebellar neurones and neuronal cell lines (Maher *et al.*, 1992). It is therefore possible that the physiological role of GLUT3 is the transport of glucose into neuronal cells, whereas GLUT1 contributes to the uptake of glucose across the blood-brain barrier (Takata *et al.*, 1990). Further support for this hypothesis is provided by the kinetic properties of the two isoforms. When expressed in *Xenopus* oocytes, human GLUT1 and GLUT3 have K_m values for 3-*O*-methyl-D-glucose equilibrium exchange of about 17 and 10 mM, respectively (Gould *et al.*, 1991). This higher affinity for sugar of GLUT3 would assure efficient uptake of glucose by neuronal cells at the extracellular glucose concentrations which prevail in the brain. The latter are known to be lower than the glucose concentrations found at the blood side of the blood-brain barrier.

1.A.2.4. GLUT4

The existence of a novel insulin-regulatable glucose transporter was suggested by the poor cross-reactivity between antibodies specific for GLUT1 and rat adipocyte glucose transporter (Lienhard *et al.*, 1982; Wang, 1987; Oka *et al.*, 1988). The insulin-regulatable glucose transporter, termed GLUT4 or muscle/fat type transporter, was first identified using a monoclonal antibody raised against a low-density microsomal fraction of rat adipocytes (James *et al.*,

1988). Subsequently, cDNA clones encoding the GLUT4 isoform have been isolated from human (Fukumoto *et al.*, 1989), rat (Birnbaum, 1989) and mouse (Kaestner *et al.*, 1989) cDNA libraries.

The human and rat GLUT4 contain 509 amino acids, whereas the mouse protein is composed of 510 amino acids. They have 95 % sequence identity among species and share 65 % identity with human GLUT1. The major differences are in the first extracellular region, the central cytoplasmic loop and the *C*-terminus. RNA blotting studies have shown that the highest levels of GLUT4 mRNA are found in insulin-responsive tissues such as brown and white fat, and cardiac and skeletal muscle (Fukumoto *et al.*, 1989; James *et al.*, 1989; Birnbaum, 1989; Charron *et al.*, 1989; Kaestner *et al.*, 1989). The apparent K_m values for 3-*O*-methyl-D-glucose of GLUT4 and GLUT1 under equilibrium exchange conditions have been estimated to be about 2 and 21 mM, respectively (Keller *et al.*, 1989). The major mechanism by which insulin regulates transport in these tissues is by stimulating the translocation of glucose transporters from an intracellular membranous pool (the so-called low-density microsomal pool) to the plasma membrane of the cell (Cushman and Wardzala, 1980).

1.A.2.5. GLUT5

cDNA clones encoding the GLUT5 isoform, the small intestine type transporter, have been isolated from a human small intestine cDNA library by low stringency screening with GLUT1 cDNA (Kayano *et al.*, 1990). The encoded protein comprises 501 amino acids sharing only about 40 % identity

with the other isoforms. The GLUT5 isoform is expressed predominantly in the small intestine and at low levels in kidney, skeletal muscle and adipose tissue (Kayano *et al.*, 1990). Immunohistochemical studies have shown that it is located mainly at the luminal surface of mature small intestinal enterocytes, where the active Na^+/glucose cotransporter SGLT1 is also found (Davidson *et al.*, 1992). Thus, the precise role of this protein in the small intestine remains unclear. Furthermore, its role in glucose transport is at present uncertain. A recent study based on the substrate specificity of GLUT5 has in fact suggested that the GLUT5 isoform functions as a fructose transporter (Burant *et al.*, 1992). When expressed in *Xenopus* oocytes it mediates only very low levels of glucose uptake, but transports fructose with a high affinity (K_m = 6 mM). It is therefore possible that GLUT5 may subserve the role of a fructose transporter in tissues, such as intestine, kidney and sperm that are known to use fructose (Kayano *et al.*, 1990; Harris *et al.*, 1992; Burant *et al.*, 1992). Interestingly, Mantych *et al.*, (1993) have very recently demonstrated that the GLUT5 isoform is also expressed in the endothelial cells of human brain microvasculature. The presence of the GLUT5 isoform in the brain, where fructose is not used as a substrate, leaves the physiological role of GLUT5 unclear.

1.A.2.6. GLUT7

Glucose release from the liver plays a crucial role in the maintenance of constant glucose concentrations in blood. The final step in glucose production by both glycogenolysis and gluconeogenesis occurs within the lumen of the

endoplasmic reticulum (ER), catalysed by the enzyme glucose 6-phosphatase (reviewed in Nordlie, 1985). Recently, the existence of a novel glucose carrier for the transport of glucose across the ER membrane has been demonstrated (Waddell *et al.*, 1991). cDNA clones encoding the liver microsomal glucose transporter, termed GLUT7, have been isolated from rat liver cDNA library (Waddell *et al.*, 1992). Sequence analysis of the 528-residue protein shows that GLUT7 shares 68 % identity with the sequence of rat GLUT2, which catalyses glucose flux across the hepatocyte plasma membrane. The major difference between two transporter isoforms is the presence of an extra six residues at the *C*-terminus of GLUT7, which include a consensus motif for retention of membrane-spanning proteins in the ER (Waddell *et al.*, 1992). Expression of GLUT7 in COS 7 cells has shown that the protein is indeed a glucose transporter, and that it is localized to the ER membrane rather than the plasma membrane. The latter finding has been confirmed by Western blotting studies on subcellular membrane fractions of liver, which show that the transporter isoform is present in the microsomes (plasma-membrane free) and not in the plasma membrane (Waddell *et al.*, 1991).

1.A.3. KINETICS OF GLUCOSE TRANSPORT IN HUMAN ERYTHROCYTE

The human erythrocyte possesses a specialized transport system for glucose, a classic example of a passive facilitated diffusion system that has been subjected to a more intense kinetic analysis than perhaps any other transporter system. The relative simplicity of the erythrocyte in its structure and metabolism has made an important contribution to such intensive kinetic

studies. However, despite of all these studies, the essential kinetic mechanism of glucose transport is still subject to controversy.

1.A.3.1. General properties

Firstly, glucose enters the human erythrocyte at rates several orders of magnitude faster than would be predicted for simple diffusion of the sugar (i.e. diffusion is facilitated). For example, the estimated permeability coefficients for D-glucose fluxes across the human erythrocyte membrane and across artificial lipid membranes (protein-free) are in the order of 2×10^{-5} and $2\text{-}4 \times 10^{-10}$ cm sec^{-1}, respectively (Jung, 1971a,b). Secondly, transport of sugar is passive and selective, that is, net flux only occurs in the presence of a concentration gradient, from high to low sugar concentration (Widdas, 1952) and shows a high degree of specificity for the D-stereoisomers of hexose sugars in the pyranose ring form (LeFevre, 1961; Barnett *et al.*, 1973). In addition to this, the transport system is characterised by hyperbolic kinetics (i.e. it is saturable) under all conditions (Widdas, 1952; Lieb and Stein, 1972). Thirdly, hexose transport is inhibited competitively by a number of sugars and by other substances such as cytochalasin B and phloretin (Barnett *et al.*, 1975; Devés and Krupka, 1978; Krupka and Devés, 1981).

1.A.3.2. Methods of investigation

The kinetics of glucose transport, which obey the Michaelis-Menten equation, can be described in terms of two kinetic parameters V_{max} (the maximum velocity of transport) and K_m (the half saturation concentration of

glucose). These parameters for glucose transport have been determined in a number of different ways (Sen and Widdas, 1962; Levin and Stein, 1966; Miller, 1968; Lowe and Walmsley, 1986; Wheeler, 1986; Wheeler and Whelan, 1988; Appleman and Lienhard, 1989). However, the estimated values of V_{max} and K_m have been reported to differ greatly according to the experimental procedures employed. Four main types of experimental situation used are zero-trans procedure, equilibrium exchange procedure, infinite-trans procedure, infinite-cis procedure (reviewed in Eilam and Stein, 1974). In order to account for differences in kinetic data for glucose transport obtained under different conditions, several models have been proposed, including the alternating conformation and two-site carrier model.

1.A.3.3. Kinetic models for the transport of glucose

The inability of simple diffusion to explain the qualitative and quantitative circumstances associated with glucose transport in mammals led a simple, symmetric carrier model to be proposed (LeFevre, 1948; Widdas, 1952). This model was based on the assumption such that glucose association with and dissociation from the carrier on both sides of the membrane was diffusion-limited, and thus that rate constants (a,b,e and f, Fig. 1.1) were very much higher than those governing reorientation of the carrier between the two sides of the membrane (c,d,g and h). It was also predicted that V_{max} and K_m for zero-trans glucose influx (entry into glucose-free cells) would be equal to those for efflux. However, such is not the case for glucose transport in human erythrocyte. The human erythrocyte exhibits asymmetric kinetics: the K_m and

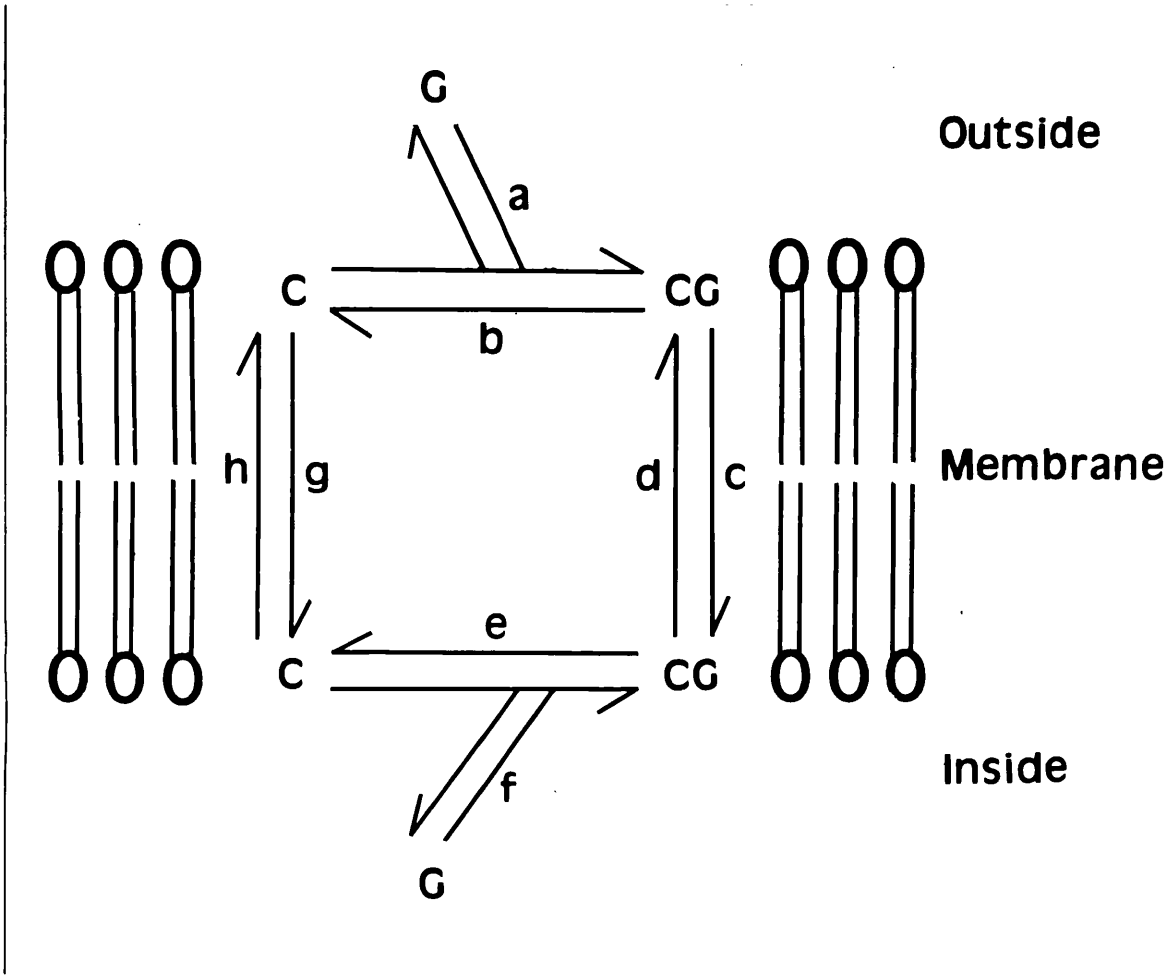


Fig. 1.1. The simple asymmetric carrier model for glucose transport. The hexose (G) binds to the carrier protein (C) in the lipid bilayer. It is then transported across the membrane as a sugar-carrier complex (CG). Dissociation constants for sugar binding are b/a and e/f. The letters (c,d,g and h) represent rate constants for carrier re-orientation. This model is a development of the earlier symmetric carrier model of Widdas (1952).

V_{max} values for zero-trans influx are significantly lower than those observed for zero-trans efflux (exit into glucose-free medium). For example, zero-trans influx has a K_m of 1.6 mM (Lacko *et al.*, 1972), similar to the K_m for infinite-cis exit (Sen and Widdas, 1962) and entry (Hankin *et al.*, 1972), and a V_{max} of 36 mmol/cell unit/min. Zero-trans efflux has much higher values of K_m and V_{max}, 25 mM and 130 mmol/cell unit/min, respectively (Karlish *et al.*, 1972). The parameters for equilibrium exchange, 20 to 38 mM and 260 to 360 mmol/cell unit/min are even higher compared to those derived under zero-trans conditions (Miller, 1968; Eilam and Stein, 1972; Lacko *et al.*, 1972; Eilam, 1975). These findings are incompatible with the simple symmetric model. However, Carruthers and Melchior (1983) have suggested that such an asymmetry results from the complex interaction of transporter with cytosolic modulatory factors, perhaps ATP, and that glucose transport in the erythrocyte is in fact intrinsically symmetric. Another feature of the erythrocyte transport that could not be explained by the symmetric carrier model is the trans-acceleration phenomenon. For example, the efflux of labelled glucose from preloaded cells is accelerated by the presence of increasing concentrations of unlabelled glucose outside the cell, suggesting that the loaded carrier is translocated much faster than the unloaded carrier (Levine *et al.*, 1965; Lieb and Stein, 1974). This phenomenon can never be observed in the simple symmetric model.

In order to resolve the above contradictions, Geck (1971) has proposed a simple, asymmetric carrier model. The model predicts that the dissociation constants (b/a and e/f) for glucose binding at the two sides of the membrane can differ, and that the rate constants (g, h) for translocation of the unloaded

carrier are lower than those (c, d) of the loaded carrier (Fig. 1.1). Such a kinetic model is compatible with a mechanism for transport in which a single sugar-binding site of the transporter is present alternately at either the inner or outer side of the membrane, but not at both sides simultaneously (Vidaver, 1966; Widdas, 1952; Barnett *et al.*, 1975; Baldwin and Lienhard, 1981). However, the simple asymmetric or alternating conformation model is kinetically equivalent to a mobile carrier model in which no assumptions are made regarding symmetry or equal mobility of loaded and unloaded carriers (Regan and Morgan, 1964). Although the asymmetry and trans-acceleration phenomena of glucose transport in human erythrocytes could be explained by the alternating conformation model, some aspects of the kinetics appear to be inconsistent with this model. For example, several researchers (Hankin *et al.*, 1972; Ginsburg and Stein, 1975; Baker and Naftalin, 1979) have suggested that there are sites with both high and low operational affinities for D-glucose at the inner surface of the membrane.

Because of this and other discrepancies between the measured and predicted kinetics of the simple asymmetric carrier model (Hankin *et al.*, 1972; Dustin *et al.*, 1984; Karlish *et al.*, 1972; Baker and Naftalin, 1979), several alternative models have been proposed. Ginsburg (1978) has predicted that transport is mediated by two asymmetric carriers, oppositely directed across the membrane. However, evidence for the postulated two sites could not be found in a later study by Weiser *et al.* (1983). In contrast, Naftalin and Holman (1977) have described a symmetric, two-site carrier model involving rate-limiting formation and breakdown of a complex between glucose and

haemoglobin. This has been rejected by the studies of Challiss *et al.* (1980), which have demonstrated that transport occurs asymmetrically in ghosts with most of the haemoglobin removed. Subsequently, Holman (1980) has proposed an asymmetric, allosteric pore model which involves three subunits and negative cooperativity. This model is supported by Holman *et al.* (1981), but conflicting results regarding negative cooperativity of equilibrium exchange have been reported (Weiser *et al.*, 1983). An additional view is provided by the allosteric two-site carrier model proposed by Carruthers's group (Carruthers, 1986a,b; Helgerson and Carruthers, 1987). This model predicts that two sugar binding sites are exposed simultaneously on the two sides of the membrane. Evidence for this two-site model has come from fluorescence quenching (Carruthers, 1986a,b) and ligand binding (Helgerson and Carruthers, 1987) studies. Recent studies of purified GLUT1 by the same group suggest that the transport protein may exist as a dimer as well as a tetramer, and thus that the individual subunits of the dimer mediate transport of glucose independently but show cooperative effects promoted by intersubunit disulphide bonds when in the tetrameric form (Hebert and Carruthers, 1991, 1992).

Particular early kinetic findings that led to rejection of the simple asymmetric carrier model by some researchers (Stein, 1986; Helgerson and Carruthers, 1987) were the observation of a lower than predicted K_m for infinite-cis uptake (Hankin *et al.*, 1972; Dustin *et al.*, 1984) and a higher than predicted K_m for zero-trans efflux (Karlish *et al.*, 1972; Baker and Naftalin, 1979). However, the results of some later studies of these parameters are in good agreement with the asymmetric model (Brahm, 1983; Lowe and

Walmsley, 1986; Wheeler, 1986; Wheeler and Whelan, 1988). For example, using initial rate kinetics Wheeler and Whelan (1988) have reported a much higher K_m value (11 mM) for infinite-cis uptake for outdated blood at room temperature than those previously estimated using a time course method (2 - 3 mM, Hankin *et al.*, 1972; Dustin *et al.*, 1984). In addition, Lowe and Walmsley (1986) have determined the initial rate constants governing reorientation of the loaded and unloaded carrier, using rapid reaction techniques. The results show that at 0°C the rate constants for translocation of the loaded carrier (c and d) are much higher than those for the unloaded carrier (g and h, Fig. 1.1). They have also described that the affinity for glucose at the external membrane site is about 2.5 times greater than that at the internal membrane site. These findings account for two characteristic features of glucose transport in human erythrocytes and are consistent with the simple asymmetric carrier model. Although much of the present kinetic data for the transport support strongly the alternating conformation model, the compatibility of kinetic data with one type of transport model does not constitute definite proof that glucose transport is mediated by such a mechanism. Furthermore, the apparent discrepancies in the kinetic studies of erythrocyte glucose transport may result from technical difficulties in measuring transport (Lowe and Walmsley, 1986; Wheeler, 1986; Naftalin, 1988). It is, therefore, unlikely that further kinetic approaches will reveal the mechanism (physical basis) for glucose transport. However, kinetic analyses are valuable as descriptive, predictive formalisms.

1.A.4. PURIFICATION AND CHARACTERIZATION OF THE HUMAN ERYTHROCYTE GLUCOSE TRANSPORTER

The transport of glucose across the human erythrocyte membrane is inhibited by a variety of protein-modifying reagents (Smith and Ellman, 1973; Bloch, 1974; Krupka and Devés, 1980), indicating the involvement of an intrinsic membrane protein. A number of studies have provided strong evidence that a protein, designated as Band 4.5 in the electrophoretic profile of the erythrocyte membrane proteins (Steck, 1974), is responsible for sugar transport and contains the binding site for the potent inhibitor of transport, cytochalasin B (Lienhard *et al.*, 1977; Kasahara and Hinkle, 1977; Baldwin *et al.*, 1979; Baldwin and Lienhard, 1980; Carter-su *et al.*, 1982; Shanahan, 1982; Deziel and Rothstein, 1984). Using the reconstitution of D-glucose transport as an assay, Kasahara and Hinkle (1977) first purified the glucose transporter in functional form from human erythrocytes. Subsequently, Baldwin *et al.* (1982) have further purified the protein to near homogeneity, using the dialysable detergent octyl glucoside. The protein is found to constitute about 5 % of the total membrane proteins of the erythrocyte (Allard and Lienhard, 1985). The isolated transporter is a glycoprotein, containing almost 15 % carbohydrate by weight (Sogin and Hinkle, 1978) and migrates as a very broad band of average M_r 55,000 on SDS-polyacrylamide gels due to heterogeneous glycosylation (Gorga *et al.*, 1979). Complete deglycosylation can be achieved by treatment with endoglycosidase F (Elder and Alexander, 1982), resulting in a sharp band of apparent M_r 46,000 or 38,000 for unheated or boiled gel samples, respectively (Lienhard *et al.*, 1984; Haspel *et al.*, 1985). The purified protein

has similar kinetic parameters to those in the intact erythrocytes (Wheeler and Hinkle, 1981) and also binds cytochalasin B with a stoichiometry of about 1 molecule per polypeptide chain (Baldwin *et al.*, 1982). It is also found that the isolated protein is slightly contaminated with the nucleoside transporter (Jarvis and Young, 1981; Wu *et al.*, 1983).

1.A.5. STRUCTURE OF GLUT1

1.A.5.1. Cloning and sequencing

The isolation of a cDNA encoding a 492 amino acid polypeptide of M_r 54117 was described in Section 1.A.2.1. Comparison of the predicted amino acid composition of the HepG2 protein with amino sequence data reported for the purified erythrocyte transporter indicated that these proteins are highly similar, if not identical (Mueckler *et al.*, 1985). The identity of the cDNA clone was further confirmed by structural analysis of the latter protein, using fast atom bombardment mass spectrometry and gas phase sequencing (Mueckler *et al.*, 1985). The analysis also revealed that no proteolytic processing occurs at the *N*-terminal or *C*-terminal sequences of the protein.

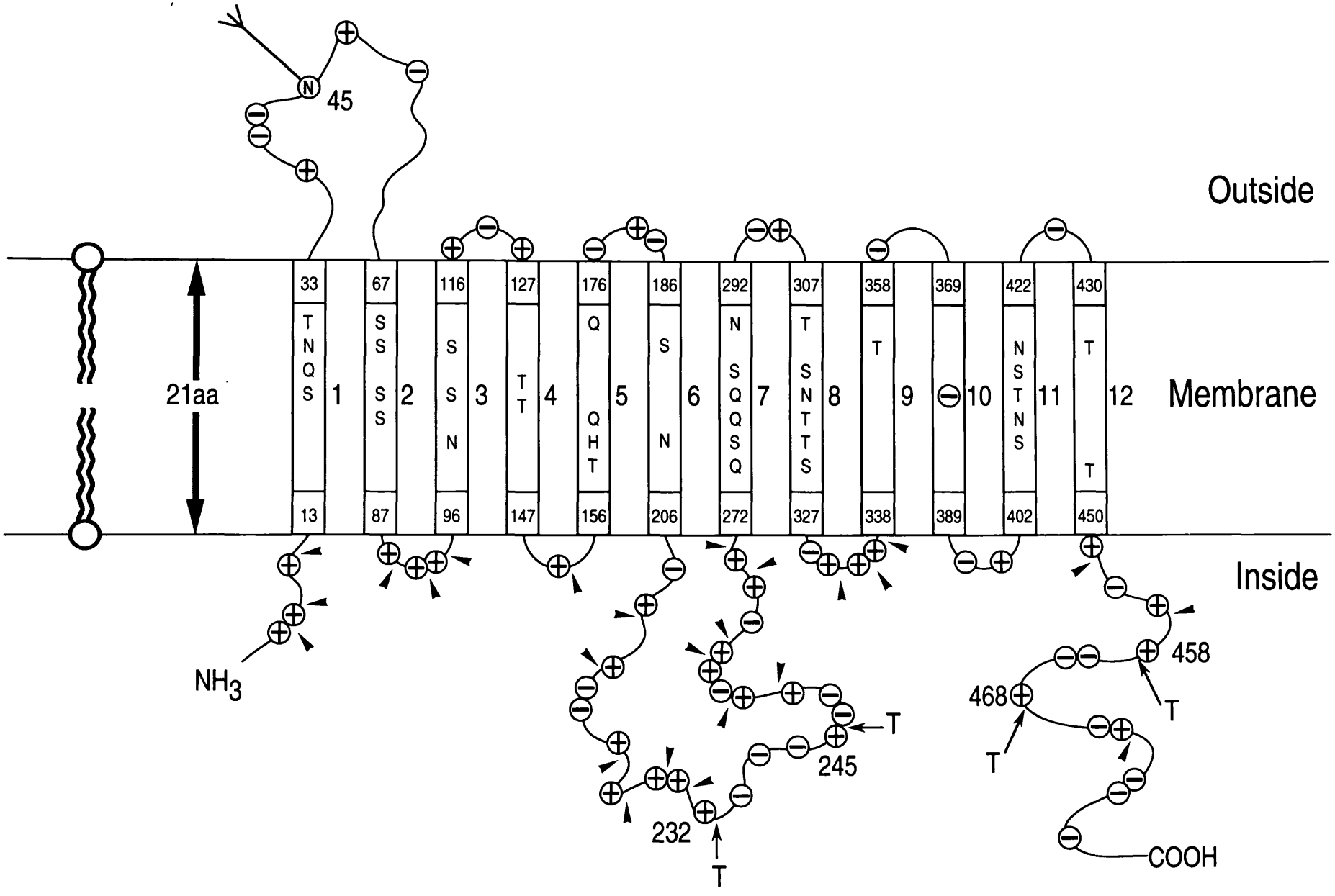
1.A.5.2. Membrane topology and structure of GLUT1

Using hydropathic and secondary structure predictions as a guide, Mueckler *et al.* (1985) have proposed a model for the two-dimensional arrangement of the GLUT1 protein in the plasma membrane in which the protein spans the membrane 12 times in the form of α-helices of 21 amino acid residues each, accounting for about 50 % of the 492 amino acid residues of the

polypeptide (Fig. 1.2). The *N*-terminus (residues 1 - 12), *C*-terminus (residues 451 - 492), and a very hydrophilic region (residues 207 - 271) in the centre of the protein are all predicted to lie on the cytoplasmic side of the membrane. Putative transmembrane domains 1 and 2 are connected by an extracellular segment of 33 amino acids (residues 34 - 66) and the remaining membrane-spanning domains by short regions of 7 - 14 amino acids. There are two potential *N*-linked glycosylation sites in the GLUT1 at Asn_{45} and Asn_{411}. However, the latter site is predicted to be in transmembrane domain 11 and is therefore unlikely to be modified. In addition, the model predicts that several of the putative transmembrane domains (3, 5, 7, 8 and 11) may form amphipathic α-helices and contain abundant hydroxyl and amide side chains that could participate in glucose binding or line a transmembrane pore through which the sugar moves.

Several features of the model for the orientation of GLUT1 have been confirmed. The glycosylation of Asn_{45} has been shown by expressing GLUT1 *in vitro* in the presence of pancreatic microsomes (Mueckler and Lodish, 1986). This finding is in good agreement with proteolytic digestion studies of the human erythrocyte glucose transporter, which have localized the site of glycosylation to the amino terminal half of the protein (Cairns *et al.*, 1987; Davies *et al.*, 1990). Cleavage of the transport protein with trypsin yields two large membrane-bound fragments in addition to a number of small, water-soluble peptide fragments (Cairns *et al.*, 1984; Deziel and Rothstein, 1984). The *N*-terminal fragment (residues 1 - 212) migrates as a broad band of apparent M_r 23,000 - 42,000 on SDS-polyacrylamide gels, indicative of

Fig. 1.2. Proposed orientation of the human erythrocyte glucose transporter in the plasma membrane (Mueckler *et al.*, 1985). The 12 putative membrane-spanning segments, usually of 21 amino acid residues, are depicted as rectangles with their end residues numbered from the N-terminus. Amino acid residues are indicated by their single letter code. Symbols (+) and (-) represent basic (Lys, Arg) and acidic (Glu, Asp) residues, respectively. The Asn_{45} is the predicted glycosylation site. Large arrows (T) indicate initially known tryptic cleavage sites. Indicated by the small arrows are the location of potential tryptic cleavage sites revealed by recent studies (Cairns *et al.*, 1987; Davies *et al.*, 1990). Diagram taken from Mueckler *et al.* (1985).


Outside
Membrane
Inside
21aa
45
33
67
116
127
176
186
292
307
358
369
422
430
TNQS
SSSS
SSN
TT
QQHT
SN
NSQQSQ
TSNTTS
T
NSTNS
T
13
87
96
147
156
206
272
327
338
389
402
450
1
2
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4
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6
7
8
9
10
11
12
NH3
232
T
245
T
458
T
468
T
COOH

glycosylation. The other fragment (residues 270 - 456) containing the potential site of glycosylation at Asn_{411} is not glycosylated and migrates as a sharp band of apparent M_r 18,000 (Cairns *et al.*, 1984,1987; Davies *et al.*, 1987,1990). Therefore, Asn_{45} must be the site of glycosylation. Direct evidence for the cytoplasmic orientation of the hydrophilic central and *C*-terminal regions of the GLUT1 has also been obtained by using vectorial proteolytic digestion and site-specific peptide antibodies (Cairns *et al.*, 1987; Davies *et al.*, 1987, 1990; Haspel *et al.*, 1988b).

Biophysical studies of the human erythrocyte transporter provide indirect evidence in support of the proposed model. On the basis of circular dichroism (CD) spectroscopy, Chin *et al.* (1987) have estimated the secondary structural composition of the purified erythrocyte transport protein in reconstituted liposomes. The results show that the transporter is composed of approximately 82 % α-helices, 10 % β-turns, and 8 % random coil structure. The fact that the erythrocyte transporter contains predominantly α-helical structure is support by the Fourier transform infrared (FTIR) spectroscopic studies of Alvarez *et al.* (1987). But the detection of putative β-strands in the protein in the latter study conflicts with the failure to detect such structures in the CD spectroscopic studies (Alvarez *et al.*, 1987; Chin *et al.*, 1987). The predominant α-helical structure may be a common feature of many membrane proteins with transport function. For example, CD spectroscopy of the lactose permease of *E. coli*, a protein of similar molecular weight and related function to the glucose transporter, has shown that the permease is also largely composed of α-helices which account for about 85 % of the total mass (Foster *et al.*, 1983).

However, membrane proteins with a predominant β-structure like porin, a major envelope protein of *E.coli*, have also been reported (Chen *et al.*, 1979). The presence of a significant amount of α-helical structure in the extramembranous domain of the erythrocyte transporter has also been suggested (Mueckler *et al.*, 1985; Chin *et al.*, 1986, 1987; Alvarez *et al.*, 1987). Direct evidence for this was obtained by FTIR spectroscopy of the transporter before and after trypsin digestion (Cairns *et al.*, 1987). The analysis showed that both the membrane embedded domains and the central, hydrophilic and *C*-terminal domains of the protein contain α-helical structure.

Evidence obtained from deuterium and tritium exchange studies suggests the existence of an intraprotein aqueous channel which penetrates the membrane (Alvarez *et al.*, 1987; Jung *et al.*, 1986). The hydrogen exchange experiments reveal that a large proportion of the transport protein is readily accessible to solvent, and that about 20 - 25 % of the peptide amide hydrogens appear to be free and in contact with water. Furthermore, the high deuterium exchange rate of the glucose transporter contrasts with the behaviour of other membrane proteins like rhodopsin, bacteriorhodopsin, or sarcoplasmic reticulum Ca^{2+}-ATPase which exchange much more slowly (Osborne and Nabedryk-Viala, 1977; Downer et al., 1986). Considering the fact that the glucose transporter is a transmembrane protein with most of its mass embedded in a hydrophobic environment (Mueckler *et al.*, 1985), the extent and rapidity of hydrogen exchange strongly support the existence of a water-filled channel in the transport protein. These findings are compatible with the proposed model of Mueckler *et al.* (1985) which predicted the presence of amphipathic helices.

Additional support for the presence of an aqueous channel is provided by the polarized FTIR spectroscopic studies of Chin *et al.* (1986). These studies suggest that the α-helices in the glucose transporter are preferentially orientated perpendicular to the membrane plane. Thus, it might be possible that some of these helices, which are predicted to be amphipathic, participate in forming a hydrophilic channel across the lipid bilayer. It is noteworthy that a water channel protein has recently been cloned and expressed in *Xenopus* oocytes (Preston *et al.*, 1992).

In the absence of a known 3-dimensional structure for the transport protein, models proposed for the tertiary arrangement of its 12 putative transmembrane helices can only be speculative. However, the possible arrangements of such helices are dictated by the apparent shortness of the loops that connect them (Fig. 1.2), thus suggesting that the helices adjacent in the sequence are likely to be adjacent in the tertiary structure. An additional constraint may apply if the present structure of the facilitative glucose transporters is a consequence of duplication of a gene encoding a protein with six membrane-spanning helices (Maiden *et al.*, 1987). Based on this hypothesis, models for the 3-dimensional arrangement of the 12 putative helices have been described (Baldwin, 1993). For example, the transport protein is predicted to be composed of 2 bundles of 6 helices, and a channel may lie within the C-terminal bundle.

1.A.5.3. Oligomeric state

Despite many investigations, it still remains unclear whether GLUT1

exists as oligomers and whether the functional properties of the transporter are determined by its monomeric or oligomeric structure. Several studies on the size of GLUT1, measured by irradiation inactivation of either D-glucose flux (Cuppoletti *et al.*, 1981) or cytochalasin B binding (Jung *et al.*, 1980; Jarvis *et al.*, 1986) and by freeze-fracture electron microscopy (Sogin and Hinkle, 1978) have yielded estimates of molecular size that are compatible with either a dimer (Jarvis *et al.*, 1986; Sogin and Hinkle, 1978) or a tetramer (Cuppoletti *et al.*, 1981; Jung *et al.*, 1980). Recent studies by hydrodynamic techniques have also shown that the transporter exists as an oligomeric structure in a cholate solution (Hebert and Carruthers, 1991, 1992). In contrast, analyses of octyl glucoside-solubilized GLUT1 by high performance molecular-sieve chromatography have shown that the transporter exists as a monomer in this detergent immediately after ion-exchange chromatography (Lundahl *et al.*, 1991). A number of kinetic studies have also suggested that glucose transporters may function as oligomers (Rampal *et al.*, 1986; Hebert and Carruthers, 1991, 1992). Additional evidence for this has come from the studies of Pessino *et al.* (1991). They have expressed chimeric GLUT1 molecules bearing the *C*-terminal region of GLUT4 in chinese hamster ovary (CHO) cells. Immunoprecipitation of these recombinant transporters from detergent-solubilized membranes with anti-GLUT4 *C*-terminal peptide antibody also precipitated endogenous CHO cell GLUT1, indicating that functional GLUT1 exists as oligomers. Interestingly, Burant and Bell (1992) have recently studied the transport properties of oocytes coexpressing two glucose transporter isoforms (GLUT2 and GLUT3) having distinct kinetic parameters and found that

these isoforms do not form heteromultimers with altered kinetic properties. They have also demonstrated that oocytes coexpressing different amounts of normal and functionally-inactive forms of GLUT3 show no inhibition of transport activity, indicating that when expressed in *Xenopus* oocytes, the monomer is a sufficient unit for functional activity of the glucose transporter.

1.A.6. LOCATION OF THE BINDING SITE(S) FOR SUBSTRATES AND INHIBITORS

Although the precise localization of the binding sites in GLUT1 for glucose and its transport inhibitors awaits further investigation, the use of selective inhibitors in conjunction with chemical and enzymic digestion has provided valuable information regarding the topography of the transporter (Cairns *et al.*, 1984, 1987; Deziel and Rothstein, 1984; Karim *et al.*, 1987; Davies *et al.*, 1987, 1990; Wadzinski *et al.*, 1990). Treatment of the erythrocyte transporter with trypsin inactivates its hexose transport (Baldwin *et al.*, 1980) and removes most of the hydrophilic regions of the polypeptide facing the cytoplasm (Cairns *et al.*, 1987). Despite the loss of these residues, the remaining membrane-embedded domains of the transporter are still able to bind cytochalasin B. Furthermore, the binding is D-glucose inhibitable, but with reduced affinity (Cairns *et al.*, 1987; Karim *et al.*, 1987). These findings indicate that the substrate-binding is primarily retained in the membrane-embedded regions of the protein. Based on the fragmentation pattern of GLUT1 photolabelled with [^{3}H] cytochalasin B, it has been suggested that the cytochalasin B binding site is located in the *C*-terminal half of the protein

comprising residues 270 - 456 (Cairns *et al.*, 1984,1987; Davies *et al.*, 1987,1990). In addition, similar studies by Holman and Rees (1987) have shown that the binding site of an exofacial inhibitor ASA-BMPA [2-*N*-(4-azidosalicoyl)-1,3-bis-(D-mannos-4-yloxy)-2-propylamine] may be located at the extracellular end of helix 9, and that the photolabelling site for the endofacial ligand cytochalasin B may be located in the internal region of helix 10. Furthermore, specific cleavage of the cytochalasin B- and/or ASA-BMPA-photolabelled transporter at its cysteine residues with 2-nitro-5-thiocyanobenzoic acid and at tryptophan residues with N-bromosuccinimide has narrowed the binding site of the endofacial ligand possibly to the region between Phe_{389} and Trp_{412}, and the labelling site of the exofacial inhibitor to somewhere between Cys_{347} (in helix 9) and Trp_{388} (in the extracellular loop connecting helices 9 and 10) (Cairns *et al.*, 1984; Holman and Rees, 1987). Based on the pattern of recognition of labelled fragments by site-directed anti-peptide antibodies, it has been also suggested that the site of labelling by another bis-mannose derivative ATB-BMPA (2-*N*-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(D-mannos-4-yloxy)-2-propylamine) lies within the membrane-spanning helix 8, possibly between Ala_{301} and Arg_{330} (Davies *et al.*, 1992). Furthermore, evidence, based on microsequencing of a small fragment of GLUT1 labelled with another endofacial ligand, 3-[^{125}I]iodo-4-azidophenethylamido-7-*O*-succinyldeacetyl-forskolin ([^{125}I]IAPS-forskolin), has indicated that the photoactivable forskolin derivative appears to bind at a site near or within putative transmembrane helix 10 (Wadzinski *et al.*, 1990). From the evidence described above, it is quite possible that while putative-membrane

spanning helices 8 and 9 are involved in the exofacial substrate-binding site, the transmembrane helices 10 and 11 are involved in the endofacial sugar binding site. However, definitive identification will require further investigation of the labelled fragments themselves.

Analyses of the aligned sequences of the mammalian facilitative transporters and other homologous members of the sugar transporter super family (Baldwin, 1993) provide further information on functionally important residues. Trp_{412} in helix 11 is conserved among all facilitative glucose transporter isoforms. Mutation of this highly-conserved residue to Leu results in a significant decrease in transport activity and interferes with the binding of the endofacial inhibitor cytochalasin B (Katagiri *et al.*, 1991; Garcia *et al.*, 1992). But there is no apparent effect upon the binding of the exofacial inhibitors ATB-BMPA and 4,6-*O*-ethylidene-D-glucose. Thus, it appears that the helix 11 is involved in the endofacial sugar binding site of the transporter. In contrast, substitution of Leu for Trp_{388}, located in helix 10 of GLUT1, significantly reduces the affinity of cytochalasin B (Garcia *et al.*, 1992) but has a minor effect upon transport activity (Garcia *et al.*, 1992; Schürmann *et al.*, 1993). Furthermore, photolabelling of the mutant with IAPS-forskolin, a derivative of the inhibitor forskolin, is 70 % less effective as compared with that of the wild-type GLUT1. Thus, the helix 10 also appears to be involved in the endofacial binding site because the forskolin resembles cytochalasin B in being an endofacial ligand.

1.A.7. CONFORMATIONAL CHANGE AND THE MECHANISM OF TRANSPORT

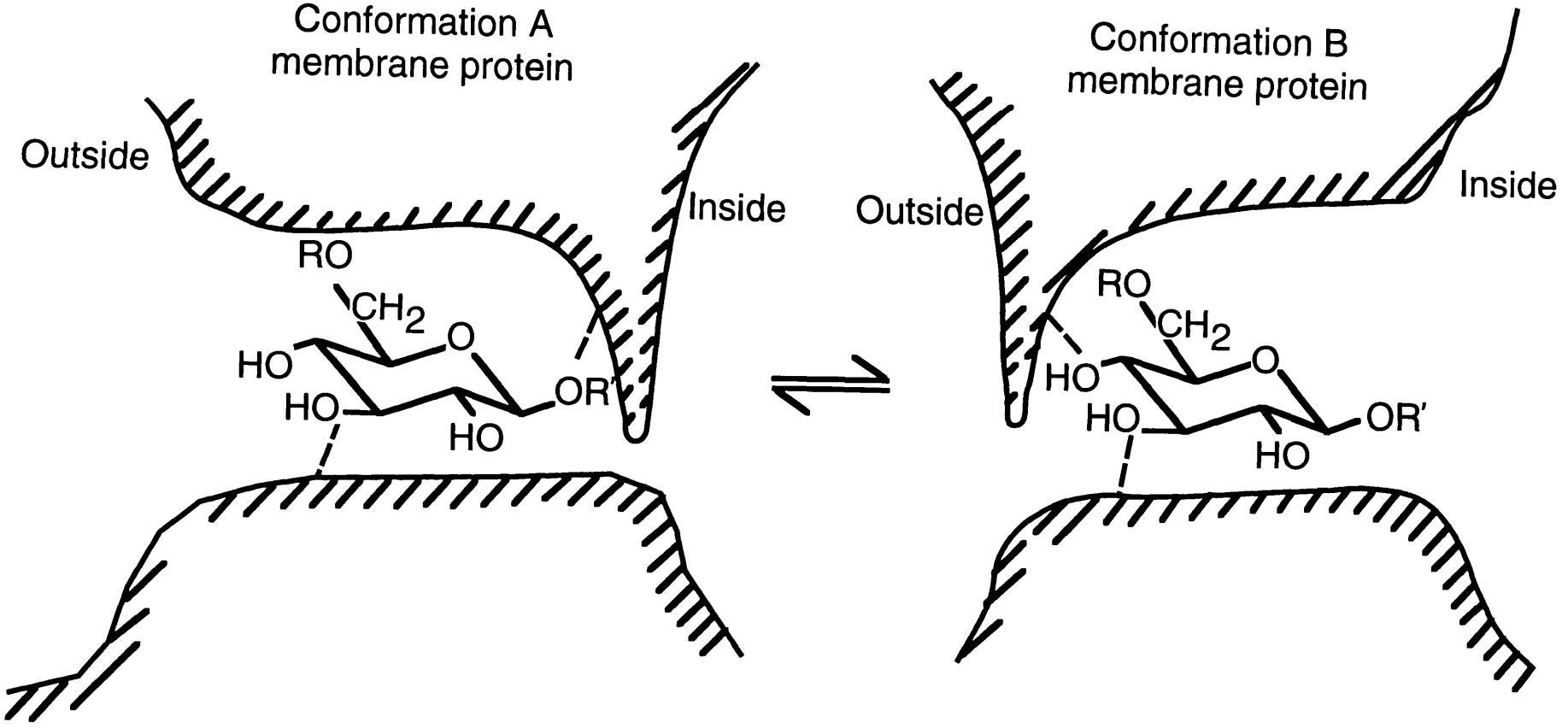
Although the two main types of kinetic model for glucose transport (the alternating conformation and two-site carrier models) remain controversial, both imply that substrate-induced conformational changes are central to the molecular mechanism of transport. Several lines of evidence support such changes in the transporter including binding studies of side-specific inhibitors of transport, chemical modification studies, fluorescence spectroscopy of the transporter, and studies of susceptibility of conformers to proteolysis. However, a detailed structural description of the proposed conformational change is yet to be established.

As discussed in Section 1.A.3.3, a reasonable kinetic model for glucose transport is the alternating conformation model, in which the substrate binding site is exposed on only one side of the transporter at any instant (Vidaver, 1966; Baldwin and Lienhard, 1981). This model suggests that the change of the substrate-bound transporter from one conformation to the other is the mechanism by which a sugar molecule is translocated. Studies of the interaction of various side-specific transport inhibitors and nontransported sugar analogues have provided strong evidence for the proposed alternating conformations of the transport protein (Barnett *et al.*, 1975 ; Baker *et al.*, 1978; Krupka and Devés, 1980, 1981; Gorga and Lienhard, 1981; May, 1988; King *et al.*, 1991). For example, Barnett *et al.* (1975) have suggested that the substrate specificities of GLUT1 differ at the cytoplasmic and extracellular surfaces of the membrane. Sugar derivatives with alkyl groups at the C-6 position of the sugar pyranose ring, such as 6-*O*-n-propyl-D-glucose, inhibit

sugar transport when they are present at the outside of the cell, but not when present only on the inside. The converse situation is found for sugars with bulky groups at C-1, such as n-propyl-β-D-glucopyranoside. To interpret these findings, Barnett *et al.* (1975) have proposed a structural model for hexose transport (Fig. 1.3). According to the model, the C-1 end of the sugar interacts with the transporter at the outward-facing binding site to enter the cell. The converse is predicted for sugar exit with the C-6 end of the molecule interacting at the inward-facing site. In addition to this, bonding of glucose with the binding site is found to involve the hydroxyl groups at the C-1, C-3 and C-4 positions, with some hydrophobic interactions involved at the C-6.

One clear approach to investigating the occurrence of conformational change in the transporter is to determine whether inhibitors known to act at different sites on the membrane can bind simultaneously to the transporter, or whether binding at one site inhibits binding of a second type of inhibitor at the other site. For example, Gorga and Lienhard (1981) have examined the sugar transport system using side-specific inhibitors such as cytochalasin B, n-propyl-β-D-glucopyranoside and 4,6-*O*-ethylidene-D-glucose. The first two compounds bind to the inner face of the membrane, whereas the latter binds to the outer face. The binding of cytochalasin B was completely inhibited by the two sugar derivatives. These results strongly suggest that binding of inhibitor at one surface of the membrane prevents binding of another inhibitor that binds at the other surface, and thus that ligands cannot bind simultaneously. These findings are consistent with the single-site alternating conformation model. Similar conclusions have been reached from another inhibitor study using the inhibitor

Fig. 1.3. Proposed model for hexose transport in human erythrocytes (Barnett *et al.*, 1975). The sugar binding site is depicted as a cleft in the carrier protein, and can exist in an outward-facing (the left) or an inward-facing (the right) conformation. R and R′ represent more or less bulky substituents on the substrate glucose, and are present in the C6- and C1-position, respectively (taken from Stein, 1986).


Conformation A
membrane protein
Outside
Inside
RO
CH2
O
HO
HO
HO
OR'
Conformation B
membrane protein
Outside
Inside
RO
CH2
O
HO
HO
HO
OR'

pair cytochalasin B and phloretin, which bind at the cytoplasmic and extracellular surfaces of the membrane respectively (Krupka and Devés, 1981). Furthermore, studies of cytochalasin B binding in the presence of sugar analogues (Gorga and Lienhard, 1981; May, 1988; King *et al.*, 1991) have shown that substrate and inhibitor do not form a ternary complex with the transporter, as opposed to two-site models (Holman, 1980; Carruthers, 1986a,b) in which the transporter forms a ternary complex with two sugars bound simultaneously on both surfaces of the membrane. The ^{1}H NMR studies of Wang *et al.* (1986) have given additional support for the absence of ternary complexes in the transport system. They have shown that treatment of ghost membranes with cytochalasin B prevents D-glucose from binding to either side of the membrane.

GLUT1 contains six sulphydryl groups (Mueckler *et al.*, 1985), one of which is known to be exofacial (Abbott *et al.*, 1986). Since irreversible (covalently-reacting) inhibitors, such as 1-fluoro-2,4-dinitrobenzene (Edward, 1973) and maleimide derivatives (May, 1987, 1988, 1989a,b), react in a highly specific manner with the thiol (-SH) group, inactivation studies of the transporter with a variety of sulphydryl agents provide useful information in support of the alternating conformation model. According to this model, the exofacial thiol group is exposed only when the transporter is orientated with the substrate binding site facing outward (Krupka and Devés, 1986). Additional support for this comes from the following studies: Pretreatment of the transporter in intact cells with membrane-impermeant inhibitors such as maltose and phloretin accelerates transport inactivation by impermeant thiol-reagents

(Krupka and Devés, 1986; May, 1988, 1989b). In contrast, the endofacial inhibitor cytochalasin B decreases the rate of transport inactivation. Such protection of the thiol group from reaction (Batt *et al.*, 1976; Roberts *et al.*, 1982; Krupka and Devés, 1986; May, 1988, 1989b) probably stems from stabilization of the transporter in an inward-facing conformation by cytochalasin B.

Another line of evidence in support of the conformational change comes from studies of the intrinsic fluorescence characteristics of GLUT1. The intrinsic fluorescence properties, resulting from the presence of six tryptophan residues in the protein (Mueckler *et al.*,1985), could be altered if any of these residues takes part in direct substrate binding and/or conformational change. Binding of some substrates and inhibitors to the transporter does in fact quench its intrinsic fluorescence, and this phenomenon has been used to probe the conformational changes induced by the ligands (Gorga and Lienhard, 1982; Carruthers, 1986b; Appleman and Lienhard, 1989; Pawagi and Deber, 1990). The intrinsic fluorescence of tryptophan is sensitive to the polarity of the surrounding environment, having an emission peak at 350 nm in water and at 310-324 nm in nonpolar protein regions (Konev, 1967). In contrast, the fluorescence emission spectrum of GLUT1 has a maximum at around 336 nm, indicating that the six tryptophan residues of the transporter (Mueckler *et al.*, 1985) are distributed in both polar (exposed to the aqueous medium) and nonpolar (located within the hydrophobic region of the membrane) environments (Gorga and Lienhard, 1982; Pawagi and Deber, 1990; Chin *et al.*, 1992). Recently, Chin *et al.* (1992) have shown that binding of D-glucose causes an

increase in non-polar tryptophan fluorescence (at 320 nm) with a reduction in polar fluorescence (at 350 nm), and suggested that glucose binding shifts a tryptophan residue from a polar to a nonpolar environment. Similar findings have been reported by Pawagi and Deber (1990). Although identification of the tryptophan residue(s) involved in such conformational changes needs to be confirmed, studies of the effect of pH on the intrinsic fluorescence of the transporter suggest that Trp_{388} and Trp_{412} in the *C*-terminal half of GLUT1 may be involved in such changes (Chin *et al.*, 1992). This conclusion is supported by recent mutagenesis studies (Katagiri *et al.*, 1991; Garcia *et al.*, 1992).

Additional evidence for the conformational changes has come from proteolytic cleavage studies of the transporter. For example, Gibbs *et al.* (1988) have studied the effect of substrates and inhibitors of transport on the rate of cleavage by the protease trypsin, an enzyme which is known to cleave the transporter solely at the cytoplasmic side of the membrane (Deziel and Rothstein, 1984; Lienhard *et al.*, 1984; Cairns *et al.*, 1984). In the presence of transport inhibitors such as phloretin or 4,6-*O*-ethylidene-D-glucose, which bind preferentially at the extracellular surface of the transporter, the rate of tryptic cleavage at the cytoplasmic surface is substantially reduced. Such a finding must result from conformational changes, presumably involving stabilization of the transporter in an outward-facing conformation that is less accessible to trypsin, rather than from steric hinderance. Similar findings have been reported for susceptibility of the transporter to thermolysin (Holman and Rees, 1987; King *et al.*, 1991). If the alternating conformation model is correct, the converse effect is expected for inhibitors such as cytochalasin B,

which acts specifically at the inner binding site of the transporter. However, the endofacial inhibitor has no effect on the rate of either tryptic or thermolytic cleavage (Gibbs *et al.*, 1988; King *et al.*, 1991). It is therefore concluded that the conformational changes induced by cytochalasin B are not the same as those caused by binding of sugar analogues to either sugar site. Interestingly, the presence of D-glucose increases the rate of proteolytic cleavage (Gibbs *et al.*, 1988).

1.A.8. HETEROLOGOUS EXPRESSION AND STRUCTURE/FUNCTION STUDIES OF THE GLUT1 FAMILY

1.A.8.1. Heterologous expression of the transporter isoforms

A number of different expression systems have been exploited in the study of members of the GLUT family of transporters. Two of the transporter isoforms, GLUT1 and GLUT2, have been expressed in an *E. coli* mutant defective in glucose uptake (Sarkar *et al.*, 1988; Thorens *et al.*, 1988). However, the low levels of expression precluded their detailed characterization. Another potentially useful expression system employs the oocytes (precursor cells of the mature egg cells) of the African clawed toad *Xenopus laevis*. These oocytes have been exploited very usefully both for functional studies on expressed, foreign proteins and for studies of cell development (see Old and Primrose, 1985). The *Xenopus* oocyte system is particularly well suited for the study of facilitative glucose transporters, since uninjected oocytes exhibit only low levels of endogenous glucose transport activity. So far, five of the facilitative glucose transporter isoforms (GLUT1 to GLUT5) have been

expressed in *Xenopus* oocytes (Birnbaum, 1989; Gould and Lienhard, 1989; Keller *et al.*, 1989; Vera and Rosen, 1989; Permutt *et al.*, 1989; Kayano *et al.*, 1990; Gould *et al.*, 1991; Burant and Bell, 1992; Thomas *et al.*, 1993). The proteins expressed in this system show similar kinetic properties to those of their native counterparts and can be distinguished on the basis of their affinity (K_m) for nonmetabolized glucose analogues, such as 2-deoxy-D-glucose and/or 3-*O*-methyl-D-glucose. Moreover, sugar transport in this system is inhibited by cytochalasin B. However, the oocyte system can not be used to express heterologous DNAs on a scale large enough for biochemical and biophysical analyses of the expressed proteins. Similarly, although functional expression of facilitative glucose transporters has also been achieved in transfected mammalian cells, only relatively small amounts of the transport proteins can be produced in this way. Examples of such expression include GLUT1 (Gould *et al.*, 1989; Asano *et al.*, 1989b; Harrison *et al.*, 1990a,b), GLUT3 (Asano *et al.*, 1992), and GLUT7 (Waddell *et al.*, 1992). In addition, the GLUT4 isoform has been expressed in transgenic mice (Liu *et al.*, 1992).

1.A.8.2. Site-directed mutagenesis

Analysis of the structure-function relationships of facilitative glucose transporters has been greatly facilitated by site-directed mutagenesis studies. For example, Baldwin and colleagues have suggested that the *C*-terminus of GLUT1 is not directly involved in substrate or inhibitor binding, but it is essential for glucose transport itself (Cairns *et al.*, 1987). Indeed, Oka *et al.* (1990) have exploited the techniques of site-directed mutagenesis to confirm this suggestion

by showing that deletion of most of the *C*-terminal domain (37 out of 42 amino acids) of GLUT1 abolishes sugar transport activity, possibly by locking the glucose binding site into an inward-facing form. Truncation of the protein in this way does not, however, affect the ability of the protein to be photoaffinity-labelled with cytochalasin B. These finding imply that although *C*-terminal domain probably plays an essential role in the conformational changes that accompany sugar transport, it is not directly required for ligand binding. Additional support for this conclusion comes from recent studies of Katagiri *et al.* (1992). These authors expressed in CHO cells a mutant GLUT1 whose *C*-terminal domain had been replaced with the corresponding domain of GLUT2, a transporter isoform that has a higher K_m for glucose transport and a much lower affinity for cytochalasin B than GLUT1 (see Section 1.A.2.2). The mutant was found to have an affinity for cytochalasin B similar to that of GLUT1, but had a higher K_m value than that of GLUT1. These results indicate that the cytoplasmic *C*-terminal domain of GLUT1 may play an important role in determination of the affinity for glucose. It is noteworthy that the *C*-terminal region is most diverse in amino acid sequence and size among the GLUT family (discussed in Section 1.A.2).

It has been also suggested that putative transmembrane helices 10 and 11, which include two tryptophan residues (Trp_{388} and Trp_{412}) possibly involved in ligand binding, play an important role in the transport activity of GLUT1 (Cairns *et al.*, 1987; Holman and Rees, 1987). As described in Section 1.A.7, substitution of Leu for Trp_{412}, which is located in helix 11 and is a highly conserved residue among the GLUT family, dramatically decreases the transport

activity of the GLUT1 but the effect on cytochalasin B binding remains unclear (Katagiri *et al.*, 1991; Garcia *et al.*, 1992). In contrast, mutation of the helix 10 residue Trp_{388} to Leu results in a minor reduction in intrinsic activity of the transporter but significantly decreases the affinity of the transporter for cytochalasin B. In addition, substitution of the highly conserved residue Asn_{415}, located in helix 11 of GLUT1, by Asp gives similar results to those obtained in the Trp_{412} mutant (Ishihara *et al.*, 1991). Furthermore, Hashiramoto *et al.* (1992) have shown that transmembrane helix 7 constitutes part of the outward-facing binding site of GLUT1. They mutated residues Gln_{282} (in helix 7) to Leu and Asn_{288} (in helix 7) and Asn_{317} (in helix 8) to Ile, respectively. The results showed that mutations at Asn_{288} and Asn_{317} had little effect upon transport activity or upon labelling by the exofacial ligand ATB-BMPA or the endofacial ligand cytochalasin B. However, substitution at Gln_{282} strongly decreased the affinity for exofacial ligands such as ATB-BMPA and 4,6-*O*-ethylidene-D-glucose, while having little effect upon transport activity or cytochalasin B binding.

1.B. THE BACULOVIRUS EXPRESSION SYSTEM

1.B.1. INTRODUCTION

Baculoviruses have been of interest for many years as effective insecticides. However, the recent popularity of these viruses stems from their usefulness as helper-independent viral vectors for the high level expression of foreign genes in a eukaryotic environment. Since the introduction by Summers and colleagues of an expression vector that uses a powerful baculovirus gene (polyhedrin) promoter to express foreign genes (Smith *et al.*, 1983), a wide variety of eukaryotic proteins have been successfully produced using recombinant baculovirus vectors (reviewed in Luckow and Summers, 1988). For a fuller understanding of the baculovirus/insect expression system that has been used in the present study, the biology and molecular biology of baculoviruses are discussed below.

1.B.2. BIOLOGY OF BACULOVIRUSES

1.B.2.1. Structure and classification

Baculoviruses are viruses that are specific and limited in their host range to arthropods and arthropod cell cultures (Gröner, 1986). Over 400 baculovirus isolates have been described, the majority coming from lepidopteran (moth) species. The genome of baculoviruses consists of 80 to 220 kilobases (kb) of closed circular double-stranded DNA, depending on the trophic species (Burgess, 1977). The DNA is packaged as rod-shaped nucleocapsids, which acquire membrane envelopes either by budding through the plasma membrane

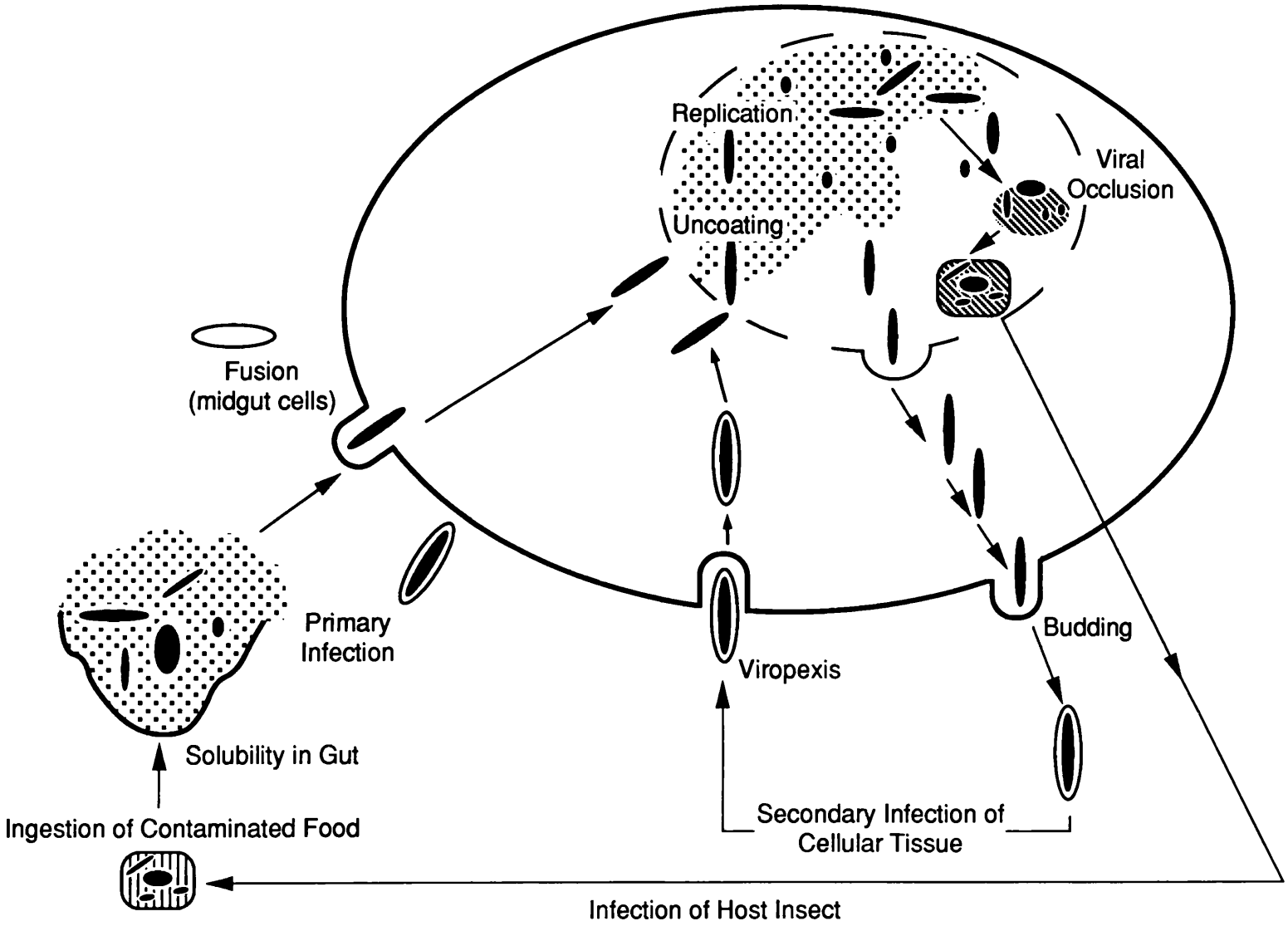
of the cell or by a nuclear envelopment process (Granados and Williams, 1986). The *baculo* portion of the name refers to the rod-shaped capsids of the virus particle. The family Baculoviridae can be divided into three subgroups, according to the nature of the structures embedding virions (Matthews, 1982): the nuclear polyhedrosis viruses (NPVs), the granulosis viruses, and the nonoccluded viruses.

NPVs have a unique property of producing proteinaceous nuclear occlusion bodies (polyhedra) in which progeny virions are embedded at a late stage of infection (Harrap, 1972a). The NPVs can be further distinguished on the basis of whether they contain a single nucleocapsid (SNPV) or multiple nucleocapsids (MNPV) per envelope. The polyhedrosis viruses form large polyhedral occlusion bodies that may be up to 5μm in diameter and comprise many virions, whereas the granulosis viruses have only a single virion embedded in a very small occlusion body. In contrast, the nonoccluded viruses do not embed virus particles during infection. Among these viruses, it is the subgroup NPV that has been intensively exploited as a source of expression vectors.

1.B.2.2. Life cycle *in vivo*

Two distinctive forms of an NPV are the occluded virus (OV) and the extracellular budded virus (EV) (Volkman *et al.*, 1976). The viral occlusions are an important part of the natural virus cycle, providing the means for horizontal transmission of the virus from insect to insect (Fig. 1.4). They protect the embedded virus particles from inactivation by environmental factors that would

Fig. 1.4. Schematic representation of baculovirus life cycle. The life cycle of the baculovirus occurs in a biphasic manner with the formation of polyhedrin inclusion bodies and budded virus. Polyhedra are ingested by a susceptible insect and solubilised by the high pH of the insect midgut. Released virions from polyhedrin inclusion bodies invade the gut cells, penetrate to the nucleus and uncoat. The occluded virus is responsible for horizontal transmission among susceptible insects and the extracellular virus is responsible for secondary and cell to cell infection in cultured cells or in the insect host. Some progeny nucleocapsids bud through the nuclear membrane and are transported to the plasmid membrane but apparently lose the nuclear-derived envelope in the cytoplasm. A second group of progeny nucleocapsids become occluded by polyhedrin protein within the nucleus. Upon insect death and cell lysis, the polyhedra are released into the environment. Diagram taken from Summers and Smith (1987).


Replication
Viral
Occlusion
Uncoating
Fusion
(midgut cells)
Primary
Infection
Viropexis
Budding
Solubility in Gut
Ingestion of Contaminated Food
Secondary Infection of
Cellular Tissue
Infection of Host Insect

otherwise rapidly inactivate EV. When the occlusion bodies are ingested by host insect larvae, the polyhedrin protein, the paracrystalline matrix of OVs, is dissolved in the alkaline environment (pH 10.5) of the insect gut, releasing embedded virions (Harrap and Longworth, 1974). The enveloped virions then enter the midgut cells by fusion and the viral DNA is uncoated at the nuclear pore or in the nucleus (Granados and Williams, 1986). DNA replication occurs at about 6 hours post infection (p.i) and by 10 hours p.i. extracellular virus is released from the cell by budding. The EV is responsible for the secondary infection of other insect tissues as well as the infection in cell cultures. At a late stage of infection, numerous occlusion bodies containing mature virions are produced in the infected cell nuclei (Granados and Williams, 1986).

1.B.3. MOLECULAR BIOLOGY OF AcNPV

Autographa californica multiple nuclear polyhedrosis virus (AcMNPV or AcNPV), originally isolated from *Autographa californica* (alfalfa looper), is the prototype virus of the subgroup NPV and has been the baculovirus of choice for exploitation as an expression vector (Smith *et al.*, 1983), although the NPV of the silk worm, *Bombyx mori*, (BmNPV) has also been used for this purpose (Maeda *et al.*, 1985).

AcNPV has a 128 kb double-stranded, closed circular DNA genome with a biphasic life cycle (Smith and Summers, 1978a,b). Following adsorption of the virus to a susceptible cell, the viral genes are expressed in an ordered fashion (Adang and Miller, 1982; Kelly, 1982). They are designated "early", "late" and "very late". The factors that regulate their expression are little

known. The first 6 hours of infection constitutes the early phase, which precedes viral DNA replication. The late phase extends from 6 hours p.i. to approximately 20 hours p.i. and is characterized by extensive replication of viral DNA and the formation of progeny EV. In the very late phase, from approximately 20 through 72 hours p.i., two new genes are expressed: the polyhedrin gene and the p10 gene. The polyhedrin protein crystallizes around viral nucleocapsids in the cell nucleus to produce a polyhedron, i.e. the formation of OV within the nucleus (Rohrmann, 1986). By 70 hours p.i., polyhedrin becomes the most abundant protein of the cell, and represents up to 50 % of the total stainable cellular protein (Summers and Smith, 1987). The function of the p10 protein is less clear but it has been suggested to be involved in the lysis of host cells (Williams *et al.*, 1989).

AcNPV has several features which make this virus particularly attractive as an expression vector (Miller, 1981). It has a capacity to accommodate large foreign DNA inserts in its expandable nucleocapsids and possesses a strong promoter which allows for high level expression of foreign genes. Furthermore, the genes for occlusion are not essential and thus provide an ideal site for passenger gene insertion.

1.B.4. BACULOVIRUSES AS EXPRESSION VECTORS

The development and use of baculoviruses as expression vectors are based on the fact that the polyhedrin and p10 genes are not required for the synthesis of infectious virus particles. Therefore, a foreign gene can be substituted for the coding sequences of the polyhedrin gene while maintaining

the integrity of the polyhedrin promoter. The techniques were first described by Smith *et al.* (1983), who reported the expression of human β-interferon by inserting the gene in place of the polyhedrin gene in a recombinant baculovirus. The system is based on a cell line established from ovarian cells of the moth *Spodoptera frugiperda* (Vaughn *et al.*, 1977).

A wide variety of transfer vectors suitable for production of nonfused or fused proteins have been constructed. pEV55 (Miller *et al.*, 1986), pAc373 (Summers and Smith, 1987) and pAcYM1 (Matsuura *et al.*, 1987) are commonly used vectors for the high-level expression of a single gene. All three are very similar in their basic design and have portions of the polyhedrin gene deleted. In phase of these deletions, multicloning sites have been inserted. In addition to the vectors constructed for expression of single genes, multiple expression vectors such as pAcVC1 and pAcVC3 have been designed to allow high level coexpression of two or more genes (Bishop and Emery, 1988, 1989). Fusion vectors are also available for genes lacking their own "ATG" initiation codon. Such examples are pAc311, pAc360, pAc401, pAc436 (Summers and Smith, 1987) and pAcRP14 (Overton *et al.*, 1989).

1.B.5. ADVANTAGES AND DISADVANTAGES OF THE EXPRESSION SYSTEM

The baculovirus expression system has the potential to produce a desired protein in large quantities that will facilitate its purification and characterization. Certain proteins have been expressed to very high levels equating to polyhedrin itself (Matsuura *et al.*, 1987). Scale-up facilities are available for insect cell culture. The successful use of insect larvae for production purposes has also

been reported (Maeda *et al.*, 1985; Miyajima *et al.*, 1987). In addition, baculoviruses are not pathogenic to vertebrates or plants and do not employ transformed cells or transforming elements as do mammalian expression systems (Tjia *et al.*, 1983). Furthermore, the ability of baculovirus-infected insect cells to perform many of the post-translational modifications of higher eukaryotes, including phosphorylation (Miyamoto *et al.*, 1985), glycosylation and signal peptide cleavage (Smith *et al.*, 1983), makes it a valuable tool for heterologous production of biologically active proteins.

However, the baculovirus expression system also has certain drawbacks. It is not possible to predict the absolute level of synthesis of a particular protein *per se* prior to expression. The capacity of the baculovirus system to secrete proteins appears to be limited. Lastly, expression is necessarily transient, the cells dying within a few days of infection.

1.C. AIMS OF THE STUDY

For detailed studies of structure/function relationships in the human erythrocyte glucose transporter, and in particular for the investigation of its structure by crystallization, it would be desirable to have a high-level expression system. Unfortunately, expression of eukaryotic membrane proteins such as GLUT1 in functional form in traditional bacterial expression systems provides inadequate amounts for such studies. The aim of my study was therefore to develop a means of producing large amounts of the human erythrocyte glucose transporter by recombinant techniques using the baculovirus/insect cell expression system.

If transporter produced in this way was found to be biologically active and properly inserted into cell membrane, the next step was to determine the kinetic properties of the expressed protein. Interference from the endogenous sugar transporters of the insect cells used for expression represented a potential problem for such kinetic assessments, and so it was necessary first to characterize the properties of these transport proteins. Furthermore, overexpression of other membrane proteins has been reported to lead to their accumulation in highly proliferated intracellular membranes rather than solely at the cell surface (Matsuura *et al.*, 1987). Thus, I also aimed to explore procedures for the rapid purification of recombinant transporters in functional form from the expression system, so that their properties could be studied in isolation after reconstitution.

Finally, it was hoped that these studies could make a useful contribution towards the ultimate goal of work in our group, which is to elucidate the

molecular mechanism of glucose transport of the human erythrocyte transporter by determining its 3-dimensional structure.

CHAPTER 2. MATERIALS AND METHODS

2.1. MATERIALS AND SUPPLIERS

Chemicals, enzymes and other materials used in this study were supplied by the following companies:-

ALDRICH ; Aldrich Chemical Company Ltd.,
The Old Brickyard, New Road Gillingham,
Dorset, SP8 4JL.

AMERSHAM ; Amersham International PLC.,
Lincoln Place, Green End, Aylesbury,
Buckinghamshire, HP20 2TP.

BDH ; BDH Laboratory Supplies Merck Ltd.,
Hunter Boulevard, Lutterworth,
Leicestershire, LE17 4XN.

BECKMAN ; Beckman Instruments Ltd.,
Progress Road, High Wycombe,
Buckinghamshire, HP12 4JL.

BIO-RAD ; Bio-Rad Microscience Ltd.,
Maylands Avenue, Hemel Hemstead,
Hertfordshire HP2 7TD.

FLOW ; ICN Biochemicals Ltd.,
Gomm Road, High Wycombe,
Buckinghamshire, HP13 5BR.

FLUKA ; Fluka Chemicals Ltd.,
Peakdale Road, Glossop,
Derbyshire, SK13 9XE.

GIBCO-BRL ; Life Technologies Ltd.,
PO BOX 35, Renfrew Road,
Paisley, PA3 4EF

HYBAID ; Hybaid Ltd.,
111-113 Waldegrave Road, Teddington,
Middlesex, TW11 8LL.

INVITROGEN ; British Bio-technology Ltd.,
4-10 The Quadrant, Abingdon,
Oxon, OX14 3YS.

LKB ; LKB Instruments Ltd.,
232 Addington Road, South Croydon,
Surrey, CR2 8YD.

MSE ; Fisons Ltd.,
Bishop Meadow Road, Loughborough,
Leicester, LE11 0RG.

NBL ; Northumbria Biologicals Ltd.,
South Nelson Industrial Estate, Cramlington,
Northumberland, NE23 9HL.

NEN ; New England Nuclear Ltd.,
2 New Road,
Southampton, SO2 0AA.

OXOID ; Oxoid Ltd.,
Wade Road, Basingstoke,
Hampshire, RG24 0PW.

PACKARD ; Brook House, 14 Station Road,
Pangbourne,
Berkshire, RG8 7DT.

PHARMACIA ; Pharmacia Biotech,
Davy Avenue, Knowlhill,
Milton Keynes, MK5 8PH.

PHARMINGEN ; AMS (UK) Ltd.,
6 Tannery Yard, Burford,
Oxon, OX8 3DN.

POLAROID ; Genetic Research Instrumentation Ltd.,
Dunmow Road, Dunmow,
Essex, CM6 3LD.

PROMEGA ; Promega Ltd.,
Epsilon House, Chilworth Research Centre
Southampton, SO1 7NS.

QIAGEN ; Hybaid Ltd.,
111-113 Waldegrave Road, Teddington,
Middlesex, TW11 8LL.

SIGMA ; Sigma Chemical CO. Ltd.,
Fancy Road, Poole,
Dorset, BH17 7NH.

SCHLEICHER &

SCHUELL ; Anderman and Co. Ltd., Central Ave.

East Molesey,

Surrey, KT8 QQ2.

SORVALL ; Du Pont (UK) Ltd.,

Wedgwood way, Stevenage,

Hertfordshire, SG1 4QN.

STRATAGENE ; Stratagene Ltd.,

Cambridge Science Park, Milton Road,

Cambridge, CB4 4GF.

TECHNE ; Techne (Cambridge) Ltd.,

Duxford,

Cambridge, CB2 4PZ.

USB ; Cambridge Bioscience Ltd.,

25 Signet Court, Newmarket Road,

Cambridge, CB5 8LA.

WHATMAN ; Whatman Scientific Ltd.,

St Leonard's Road, Maidstone,

Kent, ME14 2LE.

2.2. TECHNIQUES FOR PROTEIN CHEMISTRY

2.2.1. SDS-polyacrylamide gel electrophoresis

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a powerful method for the separation of proteins according to size and was carried out using a discontinuous buffer system essentially as described by Laemmli (1970). Protein samples were routinely run on 10 or 12 % polyacrylamide (acrylamide:bis-acrylamide = 37:1, w/w) slab gels. The slab gel comprised a 2 cm stacking gel of high porosity and a 10 cm separating gel of low porosity. The latter contained 10 or 12 % polyacrylamide in 375 mM Tris-HCl (pH 8.8), 0.1 % (w/v) SDS, 0.1 % (w/v) ammonium persulphate and 0.016 % (v/v) tetramethyl ethylene diamine (TEMED). The stacking gel contained 3 % polyacrylamide in 125 mM Tris-HCl (pH 6.8), 0.1 % SDS and was polymerised by addition of 0.1 % ammonium persulphate and 0.05 % TEMED. Prior to loading the proteins were solubilized in a loading buffer [40 mM Tris-HCl, pH 6.8, 0.8 mM ethylene diamine tetraacetic acid (EDTA), 0.8 % SDS, 4 mM dithiothreitol (DTT), 10 % (v/v) glycerol, and 0.12 % (w/v) pyronin Y]. Between 5 and 50 μg of each of the solubilized samples was then loaded into each track of a 1.5 or 3 mm-thick gel. Low molecular weight range markers (M_r 14,400 -97,400, Bio-Rad) were run alongside the sample tracks. Coomassie blue-prestained molecular markers (M_r 18,500 - 106,000) from Bio-Rad were also used when the samples were to be blotted onto nitrocellulose or nylon membranes. Electrophoresis was carried out using a Bio-Rad protein MK I electrophoresis cell and Pharmacia EPS 500/400 power supply. A constant current of 30 mA was applied for two, 1.5 mm thick gels, during migration of

the tracking dye through the stacking gel, and then the current was increased to 60 mA during passage of the proteins through the separating gel. These values were doubled when 3 mm gels were employed. The gels were run until the pyronin Y marker had migrated about 9 cm from the top of the separating gel. The gel running buffer used was 25 mM Tris, 190 mM glycine and 0.1 % SDS, pH 8.3.

Following electrophoresis the gels were either stained with coomassie blue or subjected to electrotransfer for Western blotting. For the coomassie staining, the gels were fixed overnight in 10 % (v/v) acetic acid, 25 % (v/v) isopropanol, and then soaked for 8 hours in staining solution 1 [10 % acetic acid, 25 % isopropanol, 0.025 % (w/v) coomassie blue R 250]. The gels were then stained for a further 16 hour in staining solution 2 (10 % acetic acid, 10 % isopropanol, 0.0025 % coomassie blue R250) followed by destaining in 10 % acetic acid.

2.2.2. Immunoblot (Western blot) analysis

The immunoblot analysis of proteins comprises four main stages:-

a) Separation of the protein samples by SDS-PAGE (Section 2.2.1)

b) Electrophoretic transfer of the separated proteins from the gel to a membrane

c) Immunoreaction of primary antibody with the proteins bound to the membrane

d) Detection of the specifically bound primary antibody

2.2.2.1. Electrotransfer

Following SDS-PAGE, the proteins were electrophoretically transferred from the gels onto nitrocellulose or nylon membranes such as 'Hybond-N' (Amersham) essentially according to the methods described by Towbin *et al.* (1979). After removal of the stacking gels, the separating gels were soaked in transfer buffer [39 mM glycine, 48 mM Tris, 0.0375 % SDS, 20 % (v/v) methanol] at room temperature for 20 min with gentle shaking. A piece of the membrane (15 x 9.2 cm), wet with the transfer buffer, was placed on top of 9 stacked filter papers soaked in the buffer, avoiding air bubbles. Each gel was then laid on top of the membrane and sandwiched by another 9 wet filter papers. The gel and the membrane were held in intimate contact by the sandwich of filter papers held under pressure between the electrode plates of a LKB Multiphor II semi-dry blotter. The blotter was run at a constant current of 1.6 mA per cm^2 of gel for 60 to 90 min, using a LKB Macrodrive I power supply. Following electrotransfer, the efficiency of the blotting was checked by staining the gel as described in Section 2.2.1. To visualize the molecular weight markers, a piece of the membrane containing the markers was briefly stained in 0.1 % (w/v) amido black, 25 % isopropanol, 10 % acetic acid and then destained in 10 % acetic acid.

2.2.2.2. Immunostaining

After electrotransfer of the proteins, the blot was washed in Tris-buffered saline (TBS, 20 mM Tris-HCl, 500 mM NaCl, pH 7.5) for 10 minutes. The membrane was then placed in a 150 ml Sterilin bottle containing blocking buffer

[5 % (w/v) dried skimmed milk powder in TTBS (TBS containing 0.2 % Tween-20)] and incubated for 2 hours on a roller mixer to block non-specific binding sites on the membrane. The nitrocellulose was washed twice for 5 min with 100 ml TTBS with gentle agitation and then incubated overnight at room temperature with 15 to 20 μg of relevant affinity-purified antibody or 20 μl of antiserum in 10 ml of antibody buffer (1 % dried milk powder in TTBS) with rolling. Following incubation, the membrane was washed three times for 15 min in TTBS and immunoreactive bands were then detected in one of three ways:-

1) Alkaline phosphatase method: The membrane was transferred into a 150 ml roller bottle (Sterilin) containing 10 ml antibody buffer and 3.3 μl (1:3,000 dilution) of an alkaline phosphatase conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Bio-Rad) and incubated for 1 to 2 hours at room temperature with gentle agitation. The nitrocellulose was washed three times for 15 min in TTBS and twice for 10 min in TBS to remove Tween, which might otherwise form a precipitate with the colour development reagents. The membrane was then incubated in colour development solution [0.37 mM nitro blue tetrazolium (NBT), 0.35 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 100 mM sodium bicarbonate, 1 mM $MgCl_2$, pH 9.8) until strong purple bands were present. The reaction was stopped by washing the membrane several times in distilled water. The membrane was then photographed in a wet condition.

2) ^{125}I-labelled antibody method: The nitrocellulose was incubated in 10 ml of

the antibody buffer containing 2.5 μCi of donkey anti-rabbit IgG ^{125}I-F(AB')$_2$ conjugate (500 - 2,000 Ci/mmol) for 2 hours at room temperature on a roller mixer. Following incubation, the membrane was washed three times for 30 min in TTBS and dried in filter papers for at least 2 hours. The dried membrane was mounted on 3MM filter paper (Whatman), wrapped in cling film and exposed to Kodak X-Omat AR film with a high speed X-ray intensifying screen at -70°C for 16 to 48 hours using a light-resistant cassette. The autoradiogram was then developed using facilities in the X-ray Department of the Royal Free Hospital. Spectrophotometric scanning of the immunoblot was performed using a Chromoscan Scanner (Joyce Loebl).

3) Enhanced Chemiluminescence (ECL) method: Bound primary antibody was detected according to the protocol supplied with the ECL detection Kit (Amersham). The nitrocellulose was incubated for 1 hour at room temperature on the roller mixer with 1 μl of donkey anti-rabbit IgG peroxidase-conjugate in 10 ml antibody buffer (1:10,000 dilution). The membrane was washed three times for 30 min in TTBS and then incubated for precisely 1 min with a mixture of equal volumes of detection solution 1 and 2 (0.125 ml/cm^2). The membrane was blot-dried with paper towelling, mounted on a 3MM filter paper, covered with Saran wrap, placed under a sheet of Hyperfilm-ECL (Amersham) and exposed for 30 sec at room temperature. The film was then developed immediately. A second piece of film was then exposed to the blot for a period which varied from 1 min to 1 hour, depending upon the intensity of the bands seen on the first film. However, appreciable loss of luminescence occurred by

30 min.

2.2.3. Protein determination

The concentrations of proteins were determined by the method of Lowry *et al.* (1951). This assay relied on the formation of a protein-copper complex (Biuret reaction) and the reduction of Folin reagent by the tyrosine and tryptophan residues of the protein.

The assay required a standard curve, which was established with duplicate samples of 0, 12.5, 25, 37.5 and 50 μg of bovine serum albumin (BSA). Solutions used in the assay were as follows; 1) Solution A: 2 % (w/v) sodium carbonate in 0.1 M NaOH, 2) Solution B: 1 % (w/v) copper sulphate (pentahydrate) in water, 3) Solution B*: 2 % (w/v) sodium/potassium tartrate in water, 4) Solution C: 0.4 ml of solution B and 0.4 ml of solution B* mixed with 39 ml of solution A and 1 ml of 20 % SDS, and 5) Solution D: 2 ml of Folin's reagent plus 2 ml of water. The quantity of the above solutions were enough for 40 samples.

Protein standards (0 - 50 μg of BSA) or membrane samples (5 - 20 μl) that had been saved from the preparations prior to storage at -70°C, were made up to a volume of 0.2 ml with distilled water. The samples were mixed with 1 ml of solution C, vortexed, and then incubated for 20 min at room temperature. Following incubation, 0.1 ml of solution D was added to each sample with immediate vortexing. After incubating for 30 min at room temperature, the absorbances of the samples at 750 nm were measured using the solution of standard (0 μg of BSA) as blank. The quantity of proteins in the

membrane samples was then determined from comparison with the standard curve.

2.2.4. Trypsin digestion

Trypsin cleaves the human erythrocyte glucose transporter, in the absence of substrates or inhibitors of transport, only at the cytoplasmic surface of the membrane (Lienhard *et al.*, 1984). Tryptic digestion of GLUT1 expressed in insect cell membranes was performed, essentially as described previously (Cairns *et al.*, 1987). The digestion was carried out at 25°C in 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.4. The concentrations of GLUT1 in the membranes and of trypsin were 145 μg/ml and 2.5 μg/ml, respectively. Fresh trypsin was added after 2 hours to ensure the completeness of digestion. At various periods of time, samples were taken and the enzyme reaction was terminated by the addition of a 2-fold excess (by weight) of bovine lung aprotinin.

2.2.5. Cytochalasin B binding assay

The binding of cytochalasin B was used as a functional assay for the glucose transporter expressed in insect cells and was measured by equilibrium dialysis using [4-^{3}H] cytochalasin B essentially according to the methods described by Zoccoli *et al.* (1978).

The binding of cytochalasin B to the glucose transporter can be expressed by the following equations:-

$$R + L \rightleftharpoons RL$$

$$[R]_T = [RL] + [R] \qquad (1)$$

$$K_d = [R]\,[L] / [RL] \qquad (2)$$

where R = Free glucose transporter

L = Free cytochalasin B

RL = Bound cytochalasin B

K_d = Dissociation constant

$[R]_T$ = Total glucose transporter concentration

Under equilibrium conditions, both bound cytochalasin B and free cytochalasin B can be measured as a bound-to-free ratio:-

$$\text{Bound/Free (B/F)} = [RL]/[L] \qquad (3)$$

Substituting from (2) for [L] we get:-

$$B/F = [RL][R] / K_d[RL] = [R] / K_d \qquad (4)$$

Substituting from (1) for [R] we have:-

$$B/F = [R]_T / K_d - [RL] / K_d \qquad (5)$$

If the binding of cytochalasin B is measured at a single low concentration (eg. 40 nM), which is sufficiently below the K_d of 120 nM (Zoccoli *et al.*, 1978), [RL] can never be greater than 0.25 $[R]_T$ and so equation 5 can be simplified further to the following:-

$$B/F = [R]_T / K_d \qquad (6)$$

Since K_d is a constant, the ratio of bound to free cytochalasin B is proportional to $[R]_T$. Thus, the B/F value measured under these conditions is a good approximation of the concentration of functional transporter.

The apparatus used in the binding assay was a simple microdialysis rack, similar to that described by Uhlenbeck (1972) and Zoccoli *et al.* (1978). This apparatus comprised two pieces of rectangular perspex held together by wing nuts. Each piece had six circular, shallow chambers of 50 μl volume, pairs of which were separated in the assembled racks by small pieces of dialysis membrane (BDH). The latter were boiled in 20 mM Na_2CO_3, 1 mM Na_2EDTA before use, to remove heavy metals.

[4-^{3}H]-labelled cytochalasin B (specific radioactivity 7-15 Ci/mmol, NEN) and unlabelled cytochalasin B (Aldrich) were diluted to 8 μM and 4 mM with ethanol, respectively, and stored as stock solutions at -20°C. Solutions of the desired concentration and radioactivity were prepared with the same buffer as

the protein samples to be assayed and contained a final concentration of 0.5 % (v/v) ethanol. A typical procedure involved in binding experiments was as follows: Using a 50 μl Hamilton syringe (Aldrich), a 40 μl sample of a membrane suspension was loaded into one chamber and a 40 μl sample containing 80 nM [4-^{3}H] cytochalasin B was placed in the opposing chamber. The entry holes of the racks were covered with tape to prevent evaporation and the racks were then placed on an orbital shaker overnight at room temperature for the establishment of equilibrium. On the following day, 25 μl samples of the liquid were removed from each chamber and placed into mini vials containing 4 ml of Optima gold (Packard) scintillation cocktail. The radioactivity of samples was then counted using a Beckman liquid scintillation counter (LS 5,000 CE). To prevent non-specific binding of cytochalasin B to cytoskeletal elements such as actin filaments, cytochalasin E was also added to the membrane samples at a final concentration of 10 μM.

The B/F values of cytochalasin B binding were calculated as outlined below: Let X be the counts of the radioactivity (in cpm) on the protein side, which will represent bound plus free ligand. Thus:-

$$X = RL + L$$

Let Y be the counts for the buffer side, which will represent free ligand alone. Under equilibrium conditions, the concentration of free ligand is the same on both sides of the membrane. Thus:-

$$Y = L$$

For the conversion of cpm counts into concentration, the total added cytochalasin B must be considered.

$$[RL] + [L] = [\text{cytochalasin B}] \bullet X / (X + Y) \quad (7)$$

$$\text{and } [L] = [\text{cytochalasin B}] \bullet Y / (X + Y) \quad (8)$$

substituting equation (8) into (7) we get:-

$$[RL] = [\text{cytochalasin B}] \bullet (X - Y) / (X + Y) \quad (9)$$

If we divide equation (9) by (8), we obtain:-

$$[RL] / [L] = B/F = (X - Y) / Y$$

$$= X/Y - 1$$

Therefore, the B/F value (a measure of the concentration of functional transporter) can be obtained by dividing the count on the protein side by the count on the ligand side and subtracting 1.

For routine binding assay, the binding of cytochalasin B to protein-depleted erythrocyte membranes (Gorga and Lienhard, 1981) or insect cell membranes (Section 2.2.7) was measured by equilibrium dialysis using a single, final concentration of 40 nM [4-^{3}H] cytochalasin B in the absence and presence of 400 mM D-glucose. In order to construct Scatchard plots for the more accurate determination of the concentration and affinity of cytochalasin B-binding sites, binding was measured at a number of concentrations (50 nM - 7.55 μM) of the ligand.

2.2.6. Photoaffinity labelling

The photoaffinity labelling of GLUT1 expressed in insect cells was performed using tritiated cytochalasin B, essentially as described previously (Kasanicki *et al.*, 1987). Insect membrane samples at 1 mg protein/ml in 50 mM sodium phosphate, pH 7.4, 100 mM NaCl, 1 mM EDTA and 500 mM D- or L-Glucose, were incubated with 0.51 μM [^{3}H]-cytochalasin B on ice for 30 minutes to allow the attainment of binding equilibrium. Cytochalasin E (10 μM) was also included to inhibit cytochalasin B binding to cytoskeletal elements not associated with glucose transport. The samples were transferred to 1 ml quartz cuvettes, flushed with N_2, stoppered and then irradiated on ice for 10 minutes with a 100W UV lamp (Model R-52, Ultraviolet Products Inc., San Gabriel, C.A., U.S.A.) at a distance of 10 cm. The irradiated samples were then transferred to ultracentrifuge tubes (Beckman) and washed twice with 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA pH 7.4, containing 20 μM unlabelled cytochalasin B by centrifugation at 126,000 x g for 10 min at 4°C

to remove non-covalently-bound [^{3}H]-cytochalasin B. The supernatants were discarded each time. After washing, samples (100 μg) were electrophoresed on a 3 mm thick, SDS/12 % polyacrylamide gel. The gel was then fixed, stained with Coomassie Blue and then destained. Relevant tracks were cut out to a width of 1 cm using a long blade and then cut into 2 mm slices with a gel slicer. Four blank slices were also taken from non-radioactive part of the gel to determine backgrounds. The slices were then placed in scintillation vials and solubilized by incubation with 1 ml 50 % (v/v) Solvable (Du Pont GMBH, Hamburg, Germany) for 3 hours at 50°C. Finally, 4 ml of scintillation fluid was added to each vial and the radioactivity was counted by using a Beckman LS 5,000 CE scintillation counter.

2.2.7. Preparation of plasma membranes from Sf9 cells

Plasma membranes were prepared from insect cells using a method obtained from Mr. V. Skinner (Department of Biochemistry, RFHSM), which was originally developed for the preparation of membranes from hepatoma cells. Culture media were discarded from confluent monolayers of Sf9 cells in three 75 cm^2 flasks. The monolayers were then washed three times with Dulbecco's phosphate-buffered saline (PBSA, Oxoid). The cells were scraped from the flasks using a rubber cell scraper and then transferred to a universal tube containing PBSA. They were then harvested by centrifugation at 80 x g for 10 min at 20°C. The cell pellet was resuspended in 10 ml of ice-cold 10 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4, containing protease inhibitors [2 mM iodoacetamide, 0.2 mM phenylmethanesulphonyl fluoride (PMSF), 10 μg/ml pepstatin, Sigma].

All subsequent manipulations were performed at 4°C. Clumps of cells were broken up by drawing the suspension through a 21 gauge needle several times. The cells were then disrupted by sonication (using an MSE sonicator, model 1-73, fitted with a 10 mm probe) at medium power and amplitude 5 (meter reading 10 microns), using a regime of 5 sec on and 5 sec off for a total sonication time of 30 sec. This procedure was performed in a 30 ml universal tube, kept cold by being in a 100 ml beaker of ice. The probe was dipped deeply into the suspension (to about 0.5 cm from the bottom of the universal). Then, the broken cells were transferred to a 50 ml Sorvall (Du Pont) tube and topped up with the Tris/magnesium buffer, containing protease inhibitors, to just below the shoulder. The broken cells were then pelleted by centrifugation at 18,000 x g for 20 min at 4°C. The pellet was resuspended in 5.5 ml of the Tris/magnesium buffer containing protease inhibitors by drawing through a 19 gauge needle attached to a 10 ml syringe until homogeneous. This homogenate was then gently mixed with 3.5 ml Percoll. The mixture was transferred to a 10 ml plastic Sorvall tube and centrifuged at 18,000 x g for 1 hour at 4°C, using an SS34 rotor. Following this centrifugation step a band of plasma membranes, that formed at about 1.5 cm from the top of the tube, was carefully removed using a pastette. Then, the Percoll was removed by making the samples up to a volume of about 40 ml with PBSA and then centrifuging at 18,000 x g for 20 min at 4°C. This step was repeated another 2 times. Finally, the pellet was resuspended in 1 ml of PBS using a 1 ml of syringe and a 19 gauge needle. Duplicate 5 to 20 μl samples of the membranes were taken for protein estimation (Section 2.2.3) prior to storage at -70°C.

2.3. TECHNIQUES FOR CLONING

The methods used in DNA work were essentially as described by Sambrook *et al.* (1989) with some modifications. Plasticware, glassware, and media were routinely sterilized by autoclaving for 15 - 20 min at 121°C (15 pounds/inch^2). Chemicals were either autoclaved or filter sterilized and the highest grades available were used.

2.3.1. Phenol extraction and precipitation of nucleic acids

Phenol extraction is a rapid method for purification of nucleic acids from cellular extracts. The removal of proteins was achieved by extracting aqueous solutions of nucleic acids with phenol:chloroform and chloroform. This procedure could also be used to inactivate and remove enzymes. Stocks of phenol equilibrated with Tris-HCl (pH 8.0) contained 0.1 % (w/v) 8-hydroxyquinoline as an antioxidant. Stocks of chloroform contained 4 % (v/v) isoamylalcohol. While the phenol and chloroform extracted lipids and denatured proteins, the isoamylalcohol reduced foaming during the extraction and facilitated the separation of the aqueous and organic phases. An equal volume of phenol:chloroform (1:1) was added to a DNA preparation and mixed thoroughly by vortexing. The mixture was then centrifuged to separate the phases at 12,000 x g in a microfuge for 2 min. The top (aqueous) layer containing DNA was carefully removed and re-extracted with chloroform as before. The recovery of nucleic acids was then achieved by ethanol precipitation.

The ethanol precipitation was carried out by the addition of 0.1 volume

of 3 M sodium acetate (pH 4.8) and 2 volumes of cold, absolute ethanol. The mixture was routinely held at -20°C for 30 min to 16 hours or -70°C for 15 to 20 min. The precipitate was collected by centrifugation at 12,000 x g for 10 min in a microfuge. The pellet was then washed with 70 % (v/v) ethanol and dried either by inversion for 20 min at room temperature or under vacuum for a few minutes. Finally, the nucleic acids were dissolved in either TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) or sterile distilled water.

2.3.2. Plasmid DNA preparations by the alkaline lysis method ("Minipreps")

A tube containing 5 ml of LB [1 % (w/v) tryptophan (Oxoid), 0.5 % yeast extract (Oxoid), 0.5 % NaCl, pH 7.0] or 2TY (1 % tryptophan, 1 % yeast extract, 0.5 % NaCl, pH 7.0) medium was inoculated with a single bacterial colony from a freshly streaked LB or 2TY plate and grown overnight at 37°C with constant shaking. Appropriate antibiotics were present in the media and plates, depending on the plasmids being prepared (e.g. 50 - 100 μg/ml of ampicillin). Samples (1.5 ml) of each overnight culture were then transferred to 1.5 ml eppendorf tubes and centrifuged at 6,000 x g in a microfuge for 30 sec. The pellets were each resuspended in 250 μl of GET buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0) and held for 5 min on ice. The cells were then lysed by the addition of 250 μl of a solution of 0.2 M NaOH, 1 % SDS and incubated for 5 min on ice. Neutralization was achieved by adding 200 μl of 3 M sodium acetate (pH 4.8) and incubating on ice for 10 to 60 min. The precipitate of cellular DNA and debris was removed by centrifugation at 12,000 x g for 10 min in a microfuge. The supernatants

containing plasmid DNA were transferred to fresh 1.5 ml eppendorf tubes. To each supernatant was then added 0.9 ml of cold ethanol, followed by incubation at -20°C for 30 min or 15 min at -70°C to precipitate nucleic acids. The precipitate was collected by centrifugation at 12,000 x g for 10 min and resuspended in 200 μl of NE (0.3 M sodium acetate, pH 7.0, 1 mM EDTA). After holding for 15 min at room temperature, the suspension was vortexed and extracted with a 1:1 phenol:chloroform mix. The sample was then re-precipitated by the addition of 2 volumes of cold ethanol. After standing at -20°C or -70°C as before, the plasmid DNA was pelleted by centrifugation at 12,000 x g for 10 min, washed with 70 % ethanol, dried and resuspended in 20 to 50 μl of TE or sterile water, depending on the plasmid copy number. The plasmid DNA was stored at -20°C.

2.3.3. Large scale preparations of plasmid DNA ("Maxipreps")

A 5 ml overnight culture was used as inoculum for 500 ml of 2TY or LB medium containing ampicillin (50 - 100 μg/ml) in a 2 L flask and incubated at 37°C with shaking for 16 hours. The cells were harvested by centrifugation at 6,000 x g for 10 min at 4°C, resuspended with 4 x 10 ml chilled GET buffer and held for 5 min on ice. To this was added 10 ml of 0.2 M NaOH, 1 % SDS, it was mixed thoroughly and incubated for a further 5 min on ice. The cell lysate was then mixed with 8 ml of 3 M sodium acetate (pH 4.8). Following incubation on ice for 1 hour, the mixture was centrifuged at 12,000 x g for 10 min at 4°C. The supernatants were then carefully transferred to fresh tubes and centrifuged again as before to remove any debris remaining in the

suspension. The clear supernatants were then mixed with 2 volumes of cold ethanol and incubated for 30 min at -20°C. The precipitate of nucleic acids was collected by centrifugation at 12,000 x g for 10 min at 4°C and redissolved in 600 μl of NE at room temperature for 20 min. The sample was then extracted with an equal volume of phenol and chloroform. The nucleic acids were again precipitated with 2 volumes of ethanol as before. The precipitate was collected by centrifugation, washed with 70 % ethanol, dried, and resuspended in 400 μl of TE. The quality of the DNA prepared in this way was suitable for routine use such as restriction digestions.

2.3.4. Removal of RNA from preparations of plasmid DNA

DNA obtained by the alkaline lysis method also contained a large amount of RNA. To remove this, the DNA was treated with the enzyme RNase A (Sigma) that had been heated for 10 min at 100°C to prevent any DNase activity. The RNase A was added to a DNA preparation at a concentration of 50 μg/ml and incubated for 30 to 60 min at 37°C. The reaction was terminated by phenol/chloroform extraction and the DNA was recovered by ethanol precipitation (Section 2.3.1). However, if the DNA was used for monitoring restriction digestions, the RNase was simply added to the restriction reactions and no further steps required.

2.3.5. Digestion of DNA with restriction endonucleases

Restriction endonucleases type II are DNases that recognize specific oligonucleotide sequences. These enzymes cleave double-stranded DNAs and

produce unique, equal molar fragments of a DNA. Restriction enzymes were purchased from NBL or Promega. The restriction reaction was typically composed of the substrate DNA incubated for at least 1 hour at 37°C in a solution buffered near pH 7.5, containing Mg^{2+}, frequently Na^{+}, and the desired restriction enzyme. Digestions were carried out where possible in their appropriate 'Reaction buffer' (supplied with the enzymes). Enzymes were used at a concentration of 2 to 5 units per μg of DNA. The enzyme reactions were monitored by agarose gel electrophoresis (Section 2.3.7) whenever possible and terminated by phenol/chloroform extraction (Section 2.3.1). The digested DNA was then recovered by ethanol precipitation as described before (Section 2.3.1).

2.3.6. Conversion of 3'recessed to blunt ends

The 3'recessed termini created by digestion of DNA with restriction enzymes were blunted by using the Klenow fragment of *E. coli* DNA polymerase I. The enzyme works quite well in most restriction enzyme buffers but, if necessary, DNA samples can be resuspended in Klenow enzyme buffer (50 mM Tris-HCl, pH 7.2, 10 mM $MgSO_4$, 0.1 mM DTT). The Klenow polymerase (1 unit) was added to 20 μl of DNA fragment samples (0.1 to 0.5 μg) containing all four deoxyribonucleoside triphosphates (dNTPs) at 300 μM and then incubated at 33°C for 30 min. The enzyme reaction was terminated by the addition of 2 μl of 0.5 M EDTA, followed by either phenol:chloroform extraction or heat inactivation at 65°C for 10 min.

2.3.7. Agarose gel electrophoresis

Separation of DNA fragments generated after restriction digestion was achieved by agarose gel electrophoresis, using a horizontal submarine gel apparatus (Bio-Rad). Gel concentrations used were dependent on the size of the DNA fragments to be analyzed and the degree of separation required. Most of the DNA fragments used in this study for further manipulations were of such a size that they were usually resolved in agarose gels with concentrations between 0.7 and 1.0 %. Gels were prepared by microwaving the required amount of agarose (Sigma) in 1 x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) or 0.5 x TBE (45 mM Tris-borate buffer, 1 mM EDTA, pH 8.0). Ethidium bromide, which intercalates between the bases of DNA and emits fluorescent radiation on UV illumination (302 nm recommended), was added to the melted agarose (cooled to 45°C) at a final concentration of 0.5 μg/ml. The agarose was then poured into a gel former and allowed to set for about 1 hour at room temperature. Prior to loading onto the gel, DNA samples were mixed with 0.1 volume of 10 x loading buffer [0.4 % bromophenol blue and 67 % (w/v) sucrose in water] to increase their density and so make them sink into the wells. Electrophoresis was typically carried out in 1 x TAE or 0.5 x TBE buffer at a constant voltage of 60 to 100 V for 20 to 60 min. Molecular weight markers were provided by lambda DNA restricted with Hind III (0.5 to 23 kb, NBL). DNA bands were visualized using a LKB UV transilluminator (2011 Macrovue) and photographed using a Polaroid land camera and Polaroid type 667 film.

2.3.8. Quantification of DNA

DNA concentrations were accurately measured by spectrophotometric absorbance readings of diluted DNA solutions at 260 nm. An absorbance of 1.0 at 260 nm was taken to be equivalent to a concentration of 50 μg/ml for double-stranded DNA. The purity of DNA preparations could be estimated by the ratio between the absorbances at 260 nm and 280 nm. Pure DNA preparation should have an A_{260}/A_{280} ratio of $\geq$ 1.8. Amounts of DNA fragments in agarose gels were estimated by visual comparison of their fluorescence intensities with those of known amounts of lambda molecular markers.

2.3.9. Purification of DNA fragments from low-melting point agarose gels

Purification of DNA by preparative electrophoresis is greatly facilitated by the use of chemically modified forms of agarose that gel and melt at low temperature without loss in the strength of the hardened gel. Such properties provide a simple way for the recovery and purification of DNA fragments before and after enzymic modification, ligation with other fragments, or sequencing. For such purifications, samples of DNA were electrophoresed at 4°C, typically at 30 - 40 V, following which the DNA bands were visualized using UV transillumination and excised. The gel piece of interest was transferred to a 50 ml polypropylene tube containing 5 volumes of TE and then melted at 65°C in a water bath for 15 to 45 min, depending on the size of the agarose piece. One volume of phenol equilibrated with 0.1 M Tris-HCl (pH 8.0) was added to the agarose solution and vortexed for 2 min. The upper phase was then

recovered by centrifugation at 6,000 x g for 10 min at room temperature and then extracted once more with phenol. The aqueous phase was then re-extracted with phenol:chloroform and with chloroform. DNA was recovered by ethanol precipitation at -20°C overnight. Finally, the DNA was resuspended in an appropriate volume of TE.

2.3.10. Preparation of competent cells and transformation

In order to introduce DNA into cells, competence was artificially induced in *E. coli* cells (e.g. JM109 and DH1) by treating them with calcium chloride prior to adding DNA. This was performed by the method of Cohen *et al.* (1972) with some modifications.

A single colony of JM109 from a fresh, streak plate was picked into 2 ml of 2TY or LB medium and grown overnight at 37°C with constant shaking. The overnight culture (0.5 ml) was then used to inoculate 50 ml of 2TY in a 250 ml flask. The cells were grown at 37°C with moderate agitation (800 rpm) for 2 to 3 hours until the absorbance reading at 600 nm (A_{600}) reached 0.4 to 0.5. Then, the cells were chilled on ice for 10 to 60 min and collected by centrifugation at 800 x g for 10 min at 4°C. The pellet was resuspended in 10 ml of ice-cold, 100 mM $CaCl_2$. After holding on ice for 30 min, the cells were pelleted again as before and then resuspended by gentle swirling with 2.5 ml of ice-cold 100 mM $CaCl_2$. At this point, the cells either were dispensed into 0.2 ml aliquots including 15 % (v/v) glycerol that allows snap freezing and storage at -70°C or could be used immediately for transformation. But, a higher efficiency of transformation was achieved when the cells were stored overnight

at 4°C.

For transformation, up to 100 ng of DNA in a volume of less than 10 μl was added to a 200 μl aliquot of competent cells. After incubating on ice for 45 min with occasional mixing for every 15 min, the cells were heat-shocked at 42°C in a circulating water bath for exactly 90 sec and then immediately returned to an ice bath and held for 1 min. A small volume (0.8 ml) of LB or 2TY medium prewarmed at 37°C was added to each tube and then incubated at 37°C for 30 to 45 min to allow the expression of the antibiotic resistance gene. Following incubation, 100 μl of the transformed cells were plated out onto prewarmed LB or 2TY agar plates containing ampicillin (100 μg/ml) and grown overnight at 37°C. The rest of the cells were microfuged briefly and resuspended in 100 μl of LB prior to plating.

2.3.11. Probe preparation

The probe used in the DNA dot blot hybridisation experiments (Section 2.5.3) was prepared according to the protocol supplied with the USB Random Primed DNA labelling Kit. A sample (50 ng) of the 1922 bp BamH1/HindIII fragment of plasmid pSGT, containing the coding region of the GLUT1 cDNA (Section 4.2.1), was first denatured by heating for 10 min at 100°C and then chilled on ice immediately. To a 0.5 ml eppendorf tube on ice, the following constituents were added: 25 ng of the denatured DNA, 3 μl of dNTP mixture (dATP:dGTP:dTTP = 1:1:1), 2 μl of random hexanucleotides in 10 x reaction mixture (2 M N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid (HEPES), pH 6.6, 2 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, 4 mg/ml BSA), 5 μl of [α-^{32}P]

dCTP (50 μCi, 3,000 Ci/mmol), sterile distilled water to yield a total volume of 19 μl, and then 1 μl of Klenow enzyme (2 units). The mixture was incubated for 30 min at 37°C. Then, the reaction was terminated by heating at 65°C for 10 min. Finally, the probe was stored at -20°C until required. It was normally not necessary to remove non-incorporated [α-^{32}P] dCTP when using labelled DNA for hybridisation, although this could be done by chromatography on Sephadex G-50 or by ethanol precipitation.

2.4. METHODS FOR INSECT CELL CULTURE

2.4.1. Insect cells

Spodoptera frugiperda (Sf) cells are commonly employed as the host permissive cell line to support AcNPV replication and protein synthesis. Two common cell lines used for AcNPV-based vectors are Sf9 and Sf21-AE (AE stands for adapted in England). They were both derived from IPLB-Sf21 cells, which were originally isolated from the pupal ovarian tissue of Sf cells (the alfalfa looper) at the USDA Insect pathology Laboratory (IPLB) at Beltsville, Maryland, USA (Vaughn *et al.*, 1977). The cell line used in this study was the Sf9 line, which is a clonal cell line isolated from Sf21 AE. The Sf9 cells were a kind gift from Dr. V. Emery (RFHSM). The cells were maintained according to the method described by Summers and Smith (1987), with some modifications.

2.4.2. Monolayer culture

Sf9 cells exhibited a doubling time of approximately 18 to 24 hours in

complete TC 100 medium [TC 100 medium (Gibco-BRL), 10 % (v/v) fetal calf serum (Flow), 1 % of antibiotics (penicillin 5,000 units/ml + streptomycin 5,000 μg/ml, Gibco-BRL)] at 28 ± 1.0°C. The Sf9 cells did not require CO_2, and neither trypsin nor other enzymes were used during subculturing. Subculturing in tissue culture flasks was performed as follows: The old culture medium in a 75cm^2 flask was discarded. Then, the cells were gently resuspended by tapping the side of the flask 3 to 5 times and rapidly pipetting fresh medium across the monolayer, avoiding excessive foaming. The cell suspension was split (typically 1:4) and then transferred into new flasks containing a suitable quantity of complete TC 100 medium (10 ml for a 75 cm^2 or 5 ml for a 25 cm^2 flask) and incubated at 28°C. Cell viability was checked by adding 0.1 ml of trypan blue (0.4 % stock, pH 7.3) to 1 ml of cells and examining under a microscope. The cells that took up trypan blue were considered non-viable. The viability should be more than 97 % for healthy log-phase cells. The morphology of the healthy cells appeared to be nicely rounded but not granular. When the Sf9 cells were overgrown or in older culture, they began to float in the medium.

2.4.3. Suspension culture

Sf9 cells were not anchorage dependent and could be transferred between monolayer and suspension cultures without significant losses in growth rate or viability. The cells were seeded at a density of about 1 x 10^6 cells/ml into a 1 litre spinner flask (Techne) containing 250 ml of the complete medium. The spinner flask was placed in a waterbath-type incubator containing

magnetic stir plates and incubated at 28°C with constant stirring at 60 rpm. For routine maintenance the cells were subcultured, when the cell density reached about 2.5 x 10^6 cells/ml, by removing 80 % of the suspension and replacing it with fresh complete medium. To prevent the accumulation of cell metabolic byproducts and potential contaminants, the suspension culture was concentrated by gentle centrifugation and resuspended in fresh medium in a new sterile vessel.

2.4.4. Freezing and storage of insect cells

Cells used for freezing were healthy log-phase cultures (>97 % viability). They were pelleted by centrifugation at 1,000 x g for 5 min and resuspended in fresh complete medium at a density of 4 to 5 x 10^6 cells/ml. The cell suspension was diluted with an equal volume of fresh freezing medium [20 % (v/v) dimethyl sulfoxide (DMSO) in complete TC100] to yield a final DMSO concentration of 10 % and maintained on ice. The diluted cell suspension was then dispensed into 1 ml aliquots. The cells were frozen slowly by placing freezing vials in an insulated container at -20°C for 1 hour and then at -70°C overnight. Finally, the cells were stored in liquid nitrogen.

2.4.5. Thawing cells

A vial was removed from liquid nitrogen and thawed rapidly with gentle agitation in a 37°C waterbath. The outside of the vial was decontaminated quickly with 70 % ethanol. Cells were placed into a 25 cm^2 flask containing 5 ml of fresh complete medium. The cells were then incubated at 28°C. After

1 day incubation, the old medium was discarded and replaced with fresh complete TC100.

2.5. GENERAL VIROLOGY

2.5.1. Infection of Sf9 cells with AcNPV

2.5.1.1. Monolayer culture

Several genotypic variants of wild type AcNPV are available and used by different laboratories, but all are derived from one original isolate (Vail *et al.*, 1971). A clonal variant of AcNPV, C-6, was used in this study and was received from Dr. V. Emery (RFHSM). The virus was stored at 4°C for routine use.

The Sf9 cells were counted using a haemocytometer (BDH) and seeded into flasks or dishes at the appropriate density (eg. 6-8 X 10^6 cells/75 cm^2). The cells were then allowed to attach by leaving the dishes for 1 hour in a laminar flow cabinet. Following attachment, the medium was removed and the appropriate amount of virus was added to the cells. Unless a specific multiplicity of infection (MOI) was desired, the minimum volume of virus stock necessary to cover the cells was used (eg. 1 ml inoculum for a 75 cm^2 flask). After incubating for 1 hour at 28°C or room temperature, the inoculum was removed. Fresh complete medium (e.g. 10 ml for a 75 cm^2 flask) was then added to the cells, followed by incubation at 28°C for 2 to 4 days. The infected cells were visually examined daily for cytopathic effects under a microscope. Following incubation, the culture medium was collected and centrifuged to remove residual cells at 1,000 x g for 10 min. The supernatant,

i.e. the extracellular virus, was then stored at 4°C. For routine use, virus stocks should not be frozen.

2.5.1.2. Suspension culture

The number and viability of Sf9 cells were determined as described in Section 2.4.2. The amount of virus required was calculated as follows: The amount of virus required = MOI x number of cells / titer of virus. The suspended cells were pelleted and resuspended in the calculated amount of the virus inoculum. The cells were then diluted to an initial density of 1 x 10^7 cells/ml by adding complete medium. Following incubation at 28°C or room temperature for 1 hour, the concentration of cells was re-adjusted to a density of 1 to 5 x 10^6 cells/ml by adding more fresh medium and then the incubation at 28°C was continued for 2 to 4 days. The virus infection process was monitored by transferring 5 ml of the cell/virus suspension to a 25 cm^2 flask and examining the cells under a microscope.

2.5.2. Plaque assay

The plaque assay was used either to purify a virus stock or to determine the number of infectious virus particles (i.e. the titer) in a stock. The assay was carried out essentially according to the methods described in Summers and Smith (1987) and Emery (1991).

Sf9 cells were seeded into 35 mm tissue culture dishes (1.5 x 10^6 cells/dish) and allowed to attach for 1 hour at room temperature; the cells should not be confluent in the dishes, since they will grow over the course of

the plaque assay. Serial (10-fold) dilutions of virus inoculum were prepared ranging from 10^{-2} to 10^{-6}. Following incubation, the medium was removed from the attached cells and then 100 μl of the diluted virus was added to each plate, ensuring even distribution of the inoculum. The dishes were then incubated for a further 1 hour at room temperature or 28°C. While incubating, equal volumes of 3 % (w/v) "Seaplaque (FMC)", a low melting point agarose, and complete TC100 medium were mixed to yield a final concentration of 1.5 % agarose. The agarose/TC100 mixture was kept at 45°C in a water bath until required. The TC100 medium was not added until the agarose had cooled to about 45°C. The mixture should not be re-autoclaved. Following incubation, the inoculum was removed thoroughly from each dish. Immediately, 2 ml of the agarose/TC100 mixture was added slowly to the edge of each dish, ensuring even spreading and held at room temperature until the agarose hardened. Following solidification, 1 ml of complete medium was added to each dish and incubated for 5 to 7 days at 28°C in a humidified container. Plaques appeared as clear, circular areas approximately 1 to 3 mm in diameter.

2.5.3. Limiting dilution and DNA dot blot hybridisation

Detection and partial purification of recombinant viruses by limiting dilution coupled with the dot blot hybridisation was based on the procedure described by Fung *et al.* (1988). Sf9 cells (1.5×10^4 cells in 80 μl of TC100 per well) were seeded into each well of a 96 well plate (Flow) and allowed to attach for at least 30 min. While incubating, 10 fold serial dilutions (10^{-1} to 10^{-8}) of a virus stock were prepared. Following incubation, the culture medium

was removed from the wells in the plate and replaced with 50 μl of suitably diluted viruses. After incubating the plate at 28°C for 6 to 8 days in an humidified container, the supernatants of each well were transferred to a new 96-well plate using a multi channel pipette and stored at 4°C to serve as a master plate.

The infected cells in the original plate were then lysed by adding 200 μl of 0.2 M NaOH to each well and mixing with a pipette. The plate was then incubated for 15 min at room temperature. Pieces of nylon membrane (Hybond N, Amersham) and Whatman 3MM filter paper were cut to fit the dimensions of a dot blot apparatus (Schleicher & Schuell). They were wet with 10 x SSPE (1.8 M NaCl, 0.1 M $NaH_2PO_4 \bullet H_2O$, 0.01 M EDTA), and placed into the dot blot apparatus according to the manufacturer's instructions. The membrane was supported from below by the filter paper. Each well of the blotting apparatus was rinsed with 0.4 ml of 2 M sodium chloride followed by 0.2 ml of the lysed cells, aspirating the solutions through the membrane via a vacuum pump. The membrane was soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 minutes and then transferred to neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2, and 1 mM EDTA) for 1 min. The membrane was blot-dried with filter paper, air-dried for 10 min, and then wrapped with Saran-wrap. The DNA-side of the nylon was placed down on a transilluminator and exposed to UV light (312 nm recommended) for 5 min to fix the DNA.

The fixed membrane was placed into a small hybridisation bottle (Hybaid) containing an appropriate amount (eg. 1 ml / 25 cm^2) of prehybridisation solution [5 x SSPE, 5 x Denhardt's solution (0.1 %, w/v, BSA fraction V

(Sigma), 0.1 %, w/v, Ficoll type 400 (Pharmacia), 0.1 %, w/v, Polyvinylpyrrolidone (Sigma)), 0.5%, w/v, SDS], plus denatured salmon sperm DNA (20 μg/ml). The denatured salmon DNA was prepared by heating 0.5 ml of sonicated salmon sperm DNA (1 mg/ml, Sigma) to 100°C for 5 min and chilling on ice immediately. The membrane was then prehybridized at 65°C for 1 hour with gentle rotation. Following prehybridisation, 5 μl of ^{32}P-labelled GLUT1 cDNA probe (Section 2.3.11) was added to the hybridisation bottle. After incubating overnight at 65°C, the membrane was washed twice in 2 x SSPE, 0.1 % SDS for 10 min at room temperature and then once in 1 x SSPE, 0.1 % SDS for 15 min at 65°C. Then, the membrane was washed twice in 0.1 x SSPE, 0.1 % SDS at 65°C for 10 min. The nylon was briefly air dried, covered with Saran-wrap, and then autoradiographed overnight at -70°C.

2.5.4. Preparation of infectious AcNPV DNA

Confluent monolayers of Sf9 cells were infected with AcNPV at a MOI of about 1 (Section 2.5.1.1). Three to five days after infection, the cells were removed by centrifugation at 1000 x g for 10 min. The supernatants containing extracellular virus particles were then subjected to ultracentrifugation at 100,000 x g for 1 hour at 4°C. The viral pellet was resuspended in 1 ml of TE and then laid onto a gradient consisting of equal volumes of 10 and 50 % sucrose in TE (w/w). Following centrifugation in a swing-out rotor for 90 min at 100,000 x g, the band of virus at the 10 - 50 % sucrose interface was carefully removed, diluted with TE to 50 ml, and pelleted at 100,000 x g in a fixed angle rotor for 1 hour at 4°C. The viral pellet was then resuspended in 1

ml of TE. To this was added 0.6 ml of lysis buffer [10 % (w/v) sodium *N*-lauryl sarcosinate, 10 mM EDTA] and incubated at 60°C for 20 min. Following incubation, the mixture was laid onto a 54 % (w/w) cesium chloride/TE gradient containing 200 μl of ethidium bromide (10 mg/ml) and subjected to ultracentrifugation at 200,000 x g for 18 hours at 20°C. Using UV light, the viral DNA bands corresponding to supercoiled and open circular DNA were collected. Then, the sample was extracted with water-saturated butan-1-ol to remove the ethidium bromide and then dialysed overnight against sterile TE. Finally, the viral DNA was stored at 4°C.

2.5.5. Handling of bacteriophage M13

M13 is a filamentous bacteriophage that contains a circular single-stranded DNA. The virus can infect only cells having F pili (eg. TG1 and TG2 strains of *E. coli*), to which it binds for entering the cell, and is extruded from infected cells without lysis.

2.5.5.1. Preparation of plating bacteria

A M9 minimal agar plate [0.6 % (w/v) Na_2HPO_4, 0.3% KH_2PO_4, 0.05 % NaCl, 0.1 % NH_4Cl, 1.5 % agar, 0.2 % glucose, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 mM thiamine-HCl] was streaked with a culture of TG2 strain and incubated for 1 to 2 days at 37°C. A single well-isolated colony was then picked into 5 ml of LB or 2TY medium. The liquid culture was incubated with vigorous shaking for 6 to 8 hours at 37°C. Then, the culture was either used immediately or stored at 4°C for later use for up to 1 week.

2.5.5.2. Plaque assay for bacteriophage M13mp18

A series of labelled tubes (13 mm x 100 mm) containing 3 ml of melted top agar [0.7 % (w/v) agar in LB medium] were prepared and kept at 50°C in a heating block. Serial ten-fold dilutions of M13mp18 stock were prepared in LB medium. Samples (20 or 80 μl) of each dilution were then dispensed into each of two labelled tubes. To each of these 200 μl of plating bacteria (Section 2.5.5.1) was added, followed by vortex mixing. To each of another set of the tubes containing top agar, 40 μl of 5-bromo-4-chloro-3-indoxyl β-D-galactoside [X-gal, 20 mg/ml in dimethylformamide (DMF), Stratagene] and 4 μl of isopropyl β-D-thiogalactoside (IPTG, 200 mg/ml, Sigma) were added. The contents of such a tube were immediately transferred into one of the tubes containing plating bacteria and diluted viruses, mixed by vortexing, and then poured onto a labelled LB agar plate, ensuring an even distribution of the mixture. Following solidification of the top agar, the plates were inverted and incubated overnight at 37°C.

2.5.5.3. Preparation of the M13mp18 virus stocks

A plaque, freshly picked using a sterile pasteur pipette from a plate such as that described above, was expelled from the pipette into 1 ml of LB medium. The suspension was left for 1 hour at room temperature to allow the virus particles to diffuse out of the agar. Then, one fifth of the phage suspension was added to 4 ml of LB medium containing 150 μl of plating bacteria (Section 2.5.5.1). The infected cells were grown for 6 to 8 hours at 37°C with vigorous agitation. Following incubation, the culture was centrifuged at 12,000 x g for

5 min at room temperature in a microfuge. The supernatants containing the phage particles were then saved and stored at 4°C or -20°C, indefinitely. The bacterial pellet was used to prepare double-stranded replicative form (RF) of M13 DNA by the method described for the preparation of plasmid DNA (Section 2.3.2).

2.6. OTHER TECHNIQUES

2.6.1. Hexose transport assay

Tritiated sugars used were 2-[1,2-^{3}H]-deoxy-D-glucose (30.2 Ci/mmol) and L-[1-^{3}H(N)]-glucose (10.7 Ci/mmol) and were supplied by NEN. Prior to 2-deoxy-D-glucose (2dGlc) uptake measurements, Sf9 cells were washed twice with phosphate-buffered saline (PBS, 10 mM sodium phosphate, 150 mM NaCl, pH 7.4) to remove glucose. The cells were resuspended to a density of 1 x 10^6 cells per ml in PBS and then stored on ice until required for the experiment (up to 30 min). Assays were performed in triplicate, with 1 x 10^6 cells per assay. The hexose transport assay was performed as follows: 1 ml of the cell suspension was centrifuged at 6,000 x g for 15 sec in a microfuge and resuspended to a volume of 150 μl in PBS. The cells were then incubated at 27°C for 2 min. The transport was initiated by the addition of 100 μl of 2.5 mM 2dGlc or L-glucose containing 1 μCi tritiated sugar to give a final concentration of 1 mM sugar. Following incubation for 1 min at 27°C, the assay was terminated by adding 1 ml of ice-cold PBS containing 10 μM cytochalasin B and 0.1 mM phloretin, the potent glucose transport inhibitors, and then by centrifuging at 12,000 x g for 20 seconds in a microfuge. The

cells were washed in this fashion a further 2 times and then solubilized in 200 μl of 10 % SDS by vortexing. Finally, 150 μl of the solubilized cells were dispensed to a vial containing 4 ml of scintillant using a Microman pipette and radioactivity was then determined by liquid scintillation counting (Beckman LS 5,000 CE). All results were expressed as the means of triplicate estimations, which routinely differed from the mean by less than 10 %.

2.6.2. Electron microscopy

To examine the ultrastructure of infected or non-infected insect cells, electron microscopy was performed with help from the Electron Microscopy Unit of the Royal Free Hospital. Infected cells were harvested 2 days post infection, transferred to a small specimen bottle containing 2 ml of 3 % glutaraldehyde (pH 7.4) and fixed for 8 hours at 4°C. The cells were rinsed three times with distilled water at 4°C for 15 min by gentle agitation and then post-fixed in 1 % osmium tetroxide, which binds to and stabilizes lipid bilayers as well as proteins. The fixed cells were rinsed twice with distilled water for 15 min at 4°C and twice at 15°C with agitation and then subjected to dehydration. The samples were dehydrated through a standard graded ethanol series for each step at 15°C for 30 minutes, involving 30 %, 50 %, 70 %, 90 %, 100 % ethanol. The specimens were placed twice for 5 hours in pure ethanol-resin (Lemix, Biorad) mixture (1:1) and then incubated in fresh pure resin overnight on a roller mixer for infiltration. The samples were then incubated for 5 hours at 70°C for polymerization. The embedded samples in Lemix were sectioned with a Reichert ultracut ultramicrotome (C. Reichert AG,

Wien, Austria) and stained with uranyl acetate and lead citrate. The sections were then placed on a small circular nickel grid for viewing in a Philips 201 transmission electron microscope.

CHAPTER 3. INVESTIGATION OF THE NATURE OF THE ENDOGENOUS GLUCOSE TRANSPORTER(S) IN INSECT CELLS

3.1. INTRODUCTION

Passive glucose transport in the human erythrocyte can be reversibly inhibited by the presence of various hexose and pentose sugars and their analogues (LeFevre, 1961; Barnett *et al.*, 1973, 1975). These compounds bind noncovalently to the carrier protein, each preventing the binding of the others. The degree of transport inhibition is a function of the affinity of the individual sugars, relative to that of D-glucose, for the carrier protein. Some non-transported natural sugars, such as the disaccharides maltose and cellobiose, also bind to the transporter in competition with smaller sugar substrates (Lacko and Burger, 1962). This property of binding to and inhibiting the transporter without being themselves transported is shared by a number of sugar analogues bearing bulky substitutions, such as the 6-*O*-propyl, 6-*O*-pentyl, and 6-*O*-benzyl derivatives of galactose, and 4,6-*O*-ethylidene-D-glucose (Barnett *et al.*, 1975; Baker and Widdas, 1973). However, the presence of smaller substitutions can be tolerated, as evidenced by 3-*O*-methyl-D-glucose, which exhibits an apparent affinity for the transporter comparable to that of glucose and which is transported at rates up to 50 % those shown by glucose itself (Baker and Widdas, 1988).

A second group of transport inhibitors consists of chemical reagents that attack the amino acid side chains of the transport proteins. These irreversible inhibitors form covalent bonds with the transporter. Examples of such reagents

include 1-fluoro-2,4-dinitrobenzene (Lienhard *et al.*, 1977), which attacks the ϵ-amino groups of lysine residues but reacts also with the imidazole group of histidine and the thiol of cysteine, and sulphydryl reagents such as *N*-ethyl maleimide (Deziel *et al.*, 1985; May, 1989a) and glutathione-maleimide (Batt *et al.*, 1976; May, 1988), which attack the thiol groups of cysteine residues. The use of these irreversible, protein-modifying reagents has provided strong evidence concerning the topology and the proteinaceous nature of the transport protein (Baldwin, 1992).

It is known from inhibition studies of enzymatic reactions that competitive inhibitors usually have a structural resemblance to the substrate. Thus, they can compete with it for binding to the active site of the enzyme. A similar mechanism is likely to be responsible for inhibition of glucose transport by competitive inhibitors in the human erythrocyte. However some substances, including a number of steroids (Krupka and Devés, 1980), diphenolic compounds (Jung *et al.*, 1971c) and cytochalasin B (Jung and Rampal, 1977), act as competitive inhibitors of transport while exhibiting no apparent structural similarity to glucose. Cytochalasin B is a metabolite of the fungus *Helminthosporium dematioideum* (Aldridge *et al.*, 1967) and is amongst the most potent inhibitors of glucose transport. Devés and Krupka (1978) have shown by kinetic analysis that cytochalasin B potently inhibits the transport protein by competing with glucose for a site accessible from the cytoplasmic side the human erythrocyte membrane, while no such inhibitor-binding site is associated with the extracellular surface of the membrane. To explain the specific inhibition of D-glucose transport by cytochalasin B, Griffin *et al.* (1982)

have proposed a model for isosteric binding of this inhibitor and D-glucose on the basis of structure-activity relationships. According to the model, the spatial relationships of three groups in the cytochalasin B molecule potentially involved in hydrogen bonding to the transporter are the same as those of the hydroxyl groups present on carbon atoms 1, 3 and 6 of β-D-glucose. Involvement of these groups in the formation of hydrogen bonds between sugar and carrier protein has been demonstrated (Barnett *et al.*, 1973).

The fact that the cytochalasin B binds to the transporter much more tightly than glucose (Bloch, 1973; Sogin and Hinkle, 1978) has been exploited in a number of important ways. For example, the number of sugar transport sites per erythrocyte has been estimated at approximately 1.3×10^5 by measuring the amount of the cytochalasin B bound to the cell membranes (Jung and Rampal, 1977). The transporter-associated, D-glucose-sensitive binding sites account for two-thirds of the total erythrocyte membrane cytochalasin B binding sites and have an apparent dissociation constant (K_d) of $3\text{-}5 \times 10^{-7}$M, whereas the remaining D-glucose-insensitive sites, which are unrelated to sugar transport, exhibit a K_d of $1\text{-}1.25 \times 10^{-7}$M (Jung and Rampal, 1977). The latter binding sites are thought to be associated with cytoskeletal components of the erythrocyte membrane such as actin and are inhibitable with cytochalasin E, which does not bind to the glucose transporter (Lin and Snyder, 1977). In addition, Baldwin and colleagues have used cytochalasin B to monitor the isolation of the glucose transport protein from erythrocyte membranes (Baldwin *et al.*, 1979, 1980, 1982). The glucose transporter, purified from membranes solubilized in the detergent octyl glucoside, was reconstituted into vesicles

containing erythrocyte phospholipids. The vesicles exhibited stereospecific glucose transport activity and bound the inhibitor with a stoichiometry of about 1 cytochalasin B molecule per polypeptide chain (Baldwin *et al.*, 1982, Cairns *et al.*, 1984).

Like cytochalasin B, phloretin is a potent, competitive inhibitor of glucose transport and demonstrates asymmetry of inhibition. However, phloretin binds exclusively on the extracellular surface of the cell membrane, unlike cytochalasin B which binds only at the cytoplasmic surface (Krupka, 1985). Similar asymmetries of binding can also be found in a number of steroids that inhibit the transport (Krupka and Devés, 1980). The diterpene forskolin is another potent inhibitor of glucose transport in the human erythrocyte (Sergeant and Kim, 1985). Binding of the diterpene appears to occur, like cytochalasin B, at the substrate-binding site of the transporter, since it is inhibited by glucose and cytochalasin B (Joost and Steinfelder, 1987). As with cytochalasin B (Shanahan, 1982; Cater-Su *et al.*, 1982), and 2-N-(4-azidosalicoyl)-1-3-bis(D-mannos-4'-xyloxy) propyl-2-amine (ASA-BMPA, Holman and Rees, 1987), the erythrocyte transporter can be covalently labelled by photolysis of the membrane in the presence of tritiated forskolin (Shanahan *et al.*, 1987) or the forskolin derivative 3-[^{125}I]iodo-4-azidophenylamido-7-O-succinyldeacetyl-forskolin (IAPS-forskolin, Wadzinsky *et al.*, 1987).

In the project described in this thesis, insect cells were chosen as the host for heterologous expression of a mammalian glucose transporter using the baculovirus expression system. Characterization of the expressed protein was expected to include assay of its function, including its ability to transport sugars

and to bind inhibitory ligands such as cytochalasin B. It was therefore very important first to establish the transport characteristics and other properties of the endogenous sugar transport proteins of the host insect cells. Little was known of the transport characteristics of Sf9 cells, although their ability to grow on TC-100 medium strongly suggested the presence of endogenous glucose transporters: this medium contains 0.1 % D-glucose as the major carbon source. Given the known sequence similarities of sugar transporters found in many different eukaryotes and prokaryotes, it was also important to establish whether antibodies raised against the human glucose transporter cross-reacted with endogenous proteins, since these antibodies were to be used to quantify expression of the mammalian protein. In the work described in this chapter the kinetic properties, and sensitivity to inhibitors, of the insect cell transporter(s) were characterised. In addition, their ability to be recognised by anti-human glucose transporter antibodies was examined.

3.2. KINETIC ANALYSIS OF SUGAR TRANSPORT IN INSECT CELLS

3.2.1. Time course of hexose uptake

The hexose transport characteristics of Sf9 cells were investigated using 2-[1,2-^{3}H]-deoxy-D-glucose (2dGlc, NEN). This sugar was chosen because in most mammalian cells it is phosphorylated by hexokinase but not further metabolised. It is also a good substrate for mammalian GLUT1. Although hexokinase activity in the insect cells was not investigated in the present study, several lines of evidence indicated that transport rather than phosphorylation of the sugar was rate-limiting for its uptake by the cells, and thus that true rates of transport were being observed. However, before analysis of the substrate and inhibitor specificity of transport could be performed, it was first necessary to determine the time period over which uptake of sugar into the cells was linear, so that a true measure of the initial rate of uptake could be obtained. To this end, the uptake of [^{3}H]2dGlc from both low (100 μM) and high (10 mM) extracellular concentrations was measured over periods ranging from 30 sec to 30 min, using the assay described in Section 2.6. As shown in Figures 3.1 and 3.2, the uptake was linear for at least 2 min at both concentrations, suggesting that measurements made over a 1 min time course would reflect initial rates of the hexose uptake. Additional support for the premise that 1 min uptake experiments provided a measure of the initial rate of uptake was provided by comparison of the intracellular concentration of sugar (calculated from the uptake of radioactivity and the estimated intracellular volume of Sf9 cells) with that outside the cells. For example, when the uptake of 0.1 mM sugar was investigated, the calculated intracellular concentration of sugar corresponded

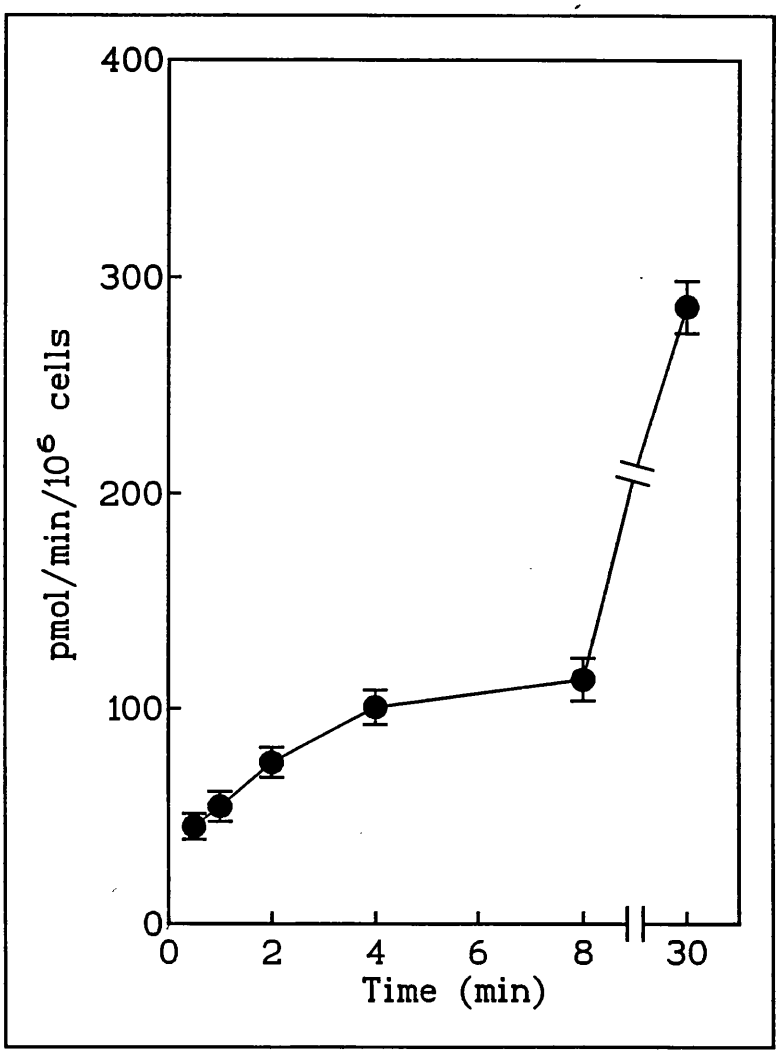


Fig. 3.1. Time course of 2-deoxy-D-glucose uptake by Sf9 cells from a low extracellular concentration of sugar. Transport was carried out in the presence of 100 μM 2dGlc, as described in Section 2.6.1. After the times indicated, uptake was terminated and the radioactivity accumulated was determined by scintillation counting. Each data point on the graph represents the mean of triplicate estimations. The data was corrected for the zero time uptake and converted into pmol/min/10^6 cells.

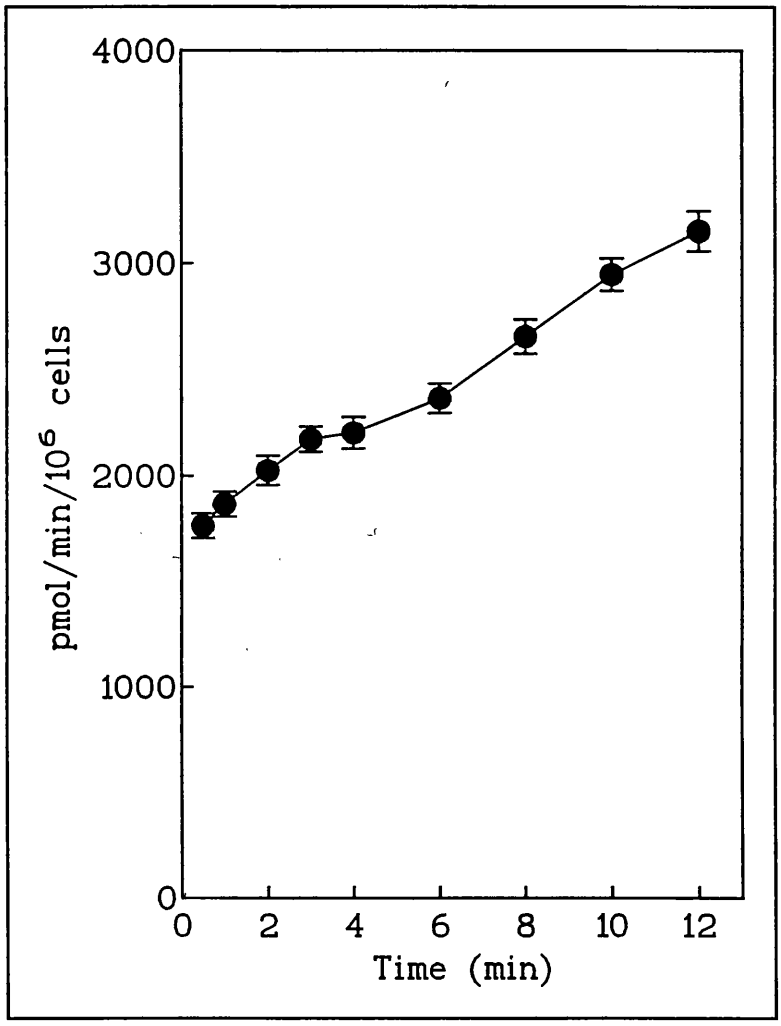


Fig. 3.2. Time course of 2-deoxy-D-glucose uptake by Sf9 cells from a high extracellular concentration of sugar. Transport was carried out in the presence of 10 mM 2dGlc, as deseribed in the legend to Fig. 3.1. Each data point on the graph represents the mean of triplicate estimations. The data was corrected for the zero time uptake and converted into pmol/min/10^6 cells.

to less than 15 % of the extracellular concentration as much as 12 min after uptake had been initiated. Thus uptake periods of 1 min were used for all further experiments.

3.2.2. Kinetic parameters of the insect cell transporter

To determine K_m (the half-saturation concentration) and V_{max} (the maximum velocity) of the endogenous glucose transporter(s) in the insect cells, the uptake of 2dGlc was measured as described in Section 2.6.1, over a range of substrate concentrations (50 μM - 10 mM). As can be seen from the data presented in Figures. 3.3 and 3.4, 2dGlc uptake by the insect cells appeared to involve both saturable and non-saturable (or very low affinity) components. In contrast, uptake of [1-^{3}H]L-glucose, which is not a substrate for the hexose transporters of mammalian cells, appeared to involve only a non-saturable process (Fig. 3.5). However, the chemical differences between 2dGlc and L-glucose, including the greater hydrophilicity of the L-sugar, precluded use of the latter as a means of correcting the 2dGlc transport data for the nonsaturable component of uptake. Instead, the K_m and V_{max} for transport were estimated by using the non-linear regression program ENZFITTER (Elsevier Biosoft, Cambridge, UK) to fit the uptake data to a 2-component model described by the following equation:

$$V = \frac{V_{max} \bullet S}{K_m + S} + M \bullet S \qquad (3.1)$$

where V = the rate of uptake, S = substrate concentration and M = a

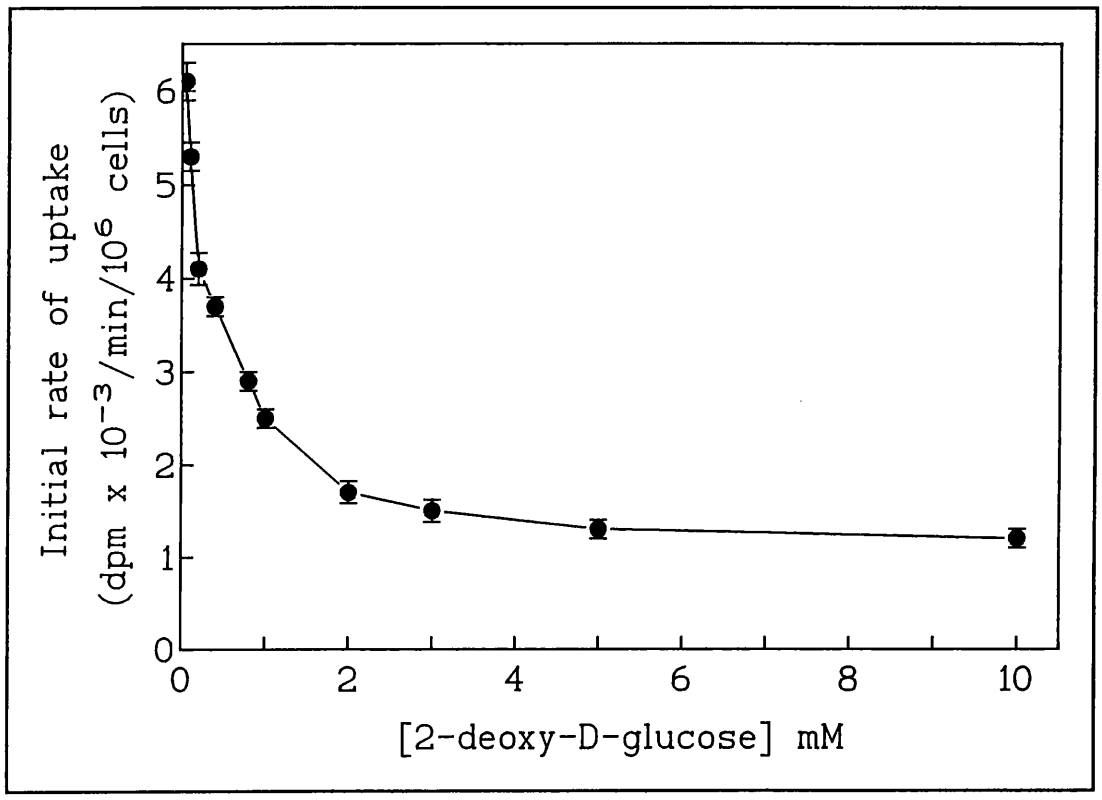


Fig. 3.3. Transport of 2-deoxy-D-glucose in Sf9 cells. Uptake was carried out as described in Section 2.6.1. Shown is a representative experiment in which increasing concentrations of 2dGlc were added to the extracellular medium of the cells. The concentration of radiolabelled 2dGlc used was held constant. Each data point is the mean of triplicate estimations. The K_m for 2 dGlc transport was determined with the aid of ENZFITTER as described in the text.

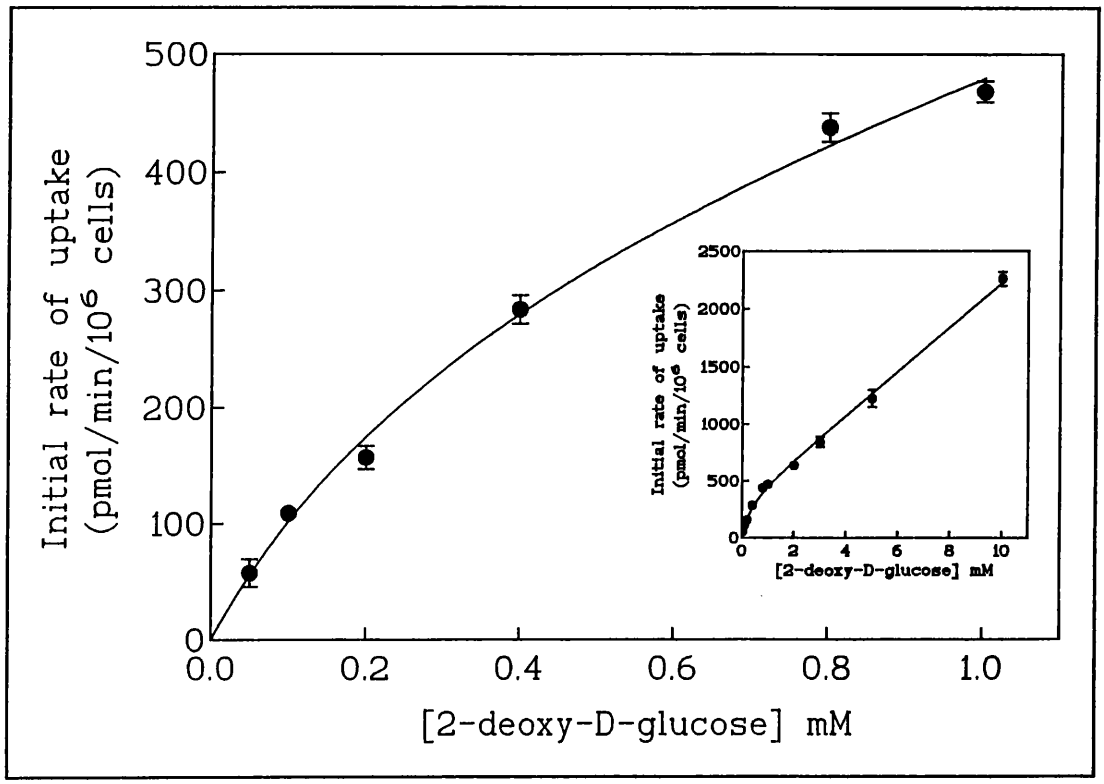


Fig. 3.4. Determination of the maximal rate of transport (V_{max}). The V_{max} was estimated by using the non-linear regression program ENZFITTER to fit the data to equation 3.1, as described in the text. The curve was produced by using the data points upto 1 mM of 2dGlc, which approach saturation. As shown in the insert, the line drawn through experimental points at high concentrations (>1 mM) of substrate is straight and represents the non-saturable component.

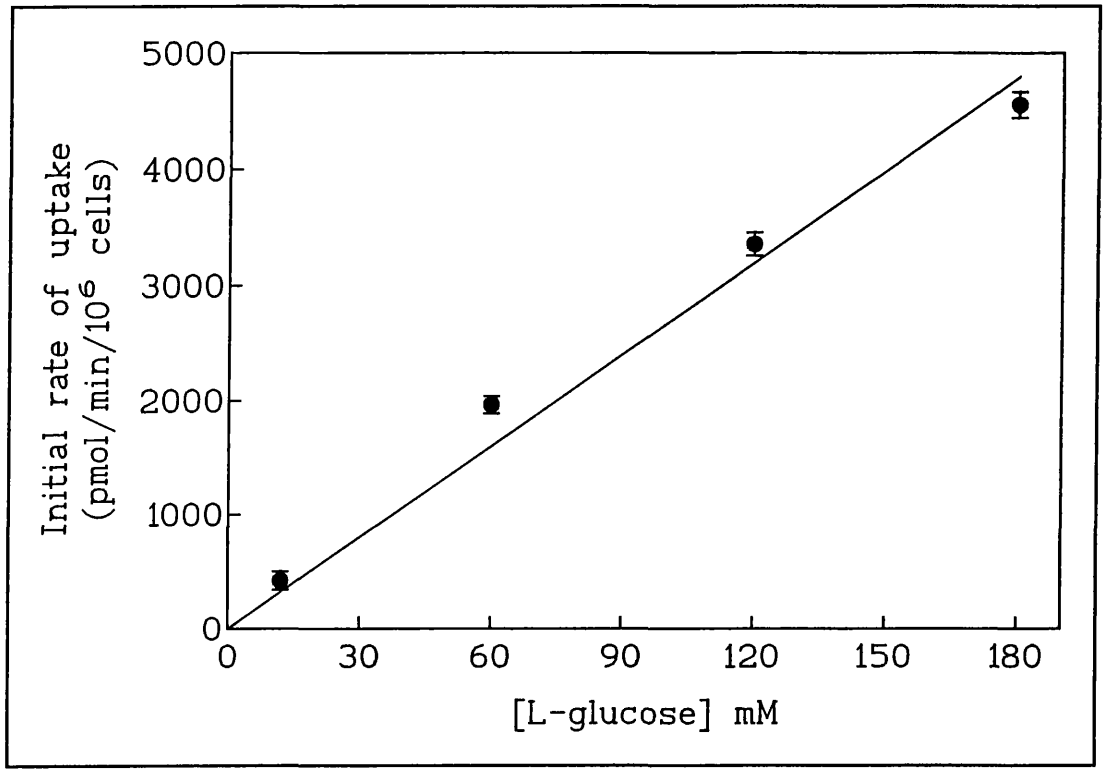


Fig. 3.5. Uptake of [^{3}H]L-glucose by Sf9 cells. Transport was carried out as described in Section 2.6.1. Shown is a representative experiment in which increasing concentrations of L-glucose were added to the extracellular medium of the cells. Each data point is the mean of triplicate estimations.

constant describing the non-saturable component of the uptake process. In the uptake experiments, the concentration of radiolabelled sugar was held constant while the concentration of unlabelled sugar was varied. The resultant plot of radiolabel uptake versus concentration of unlabelled sugar is shown in Figure 3.3. The K_m for transport was estimated by using ENZFITTER to fit these data to equation 3.2 (see below). This procedure yielded an apparent K_i for the unlabelled sugar, which corresponds to the K_m value for 2dGlc uptake in this case, of 378 ± 42 μM. The K_m value was calculated from the raw uptake data (dpm) in this way, rather than by fitting the initial rate data to a hyperbola as described for V_{max} below, in order to avoid undue biasing of the calculation by the uptakes measured at high sugar concentrations. Such biasing would result from the fact that the specific radioactivity of the 2dGlc was not held constant over the range of sugar concentrations used in the present experiment. The V_{max} for transport was then estimated by using ENZFITTER to fit the data from a plot of initial rate of 2dGlc uptake versus substrate concentration (Fig. 3.4) to equation 3.1, using the K_m value estimated as described above. This procedure yielded a V_{max} value of 415 ± 37 pmol/min/10^6 cells. In addition, the value of M (218) calculated from the computer fit to equation 3.1 for the non-saturable component of the uptake process corresponded closely to that (209) directly estimated from the slope of the curve shown in Figure 3.4 at high 2dGlc concentrations. For comparison, the V_{max} of the endogenous transporter in insect cells for 2dGlc was about 9.7-fold higher than that calculated for Chinese hamster ovary (CHO) cells which contain a GLUT1 homologue, whereas the K_m value of the insect transporter for 2dGlc was approximately

1.4-fold lower than that reported for the latter cells. Thus, it appeared that the transport activity of the insect cells was very high.

3.2.3. Studies of the inhibition of 2dGlc transport in insect cells by hexoses, phloretin and cytochalasin B

The kinetic experiments described above had revealed the existence of a saturable transport system for hexose uptake by insect cells. The apparent affinity of this transporter(s) for 2-deoxyglucose was relatively high, the K_m for uptake being < 0.5 mM. To investigate further the substrate and inhibitor recognition properties of the insect cell transporter, the ability of other sugars and of drugs to inhibit 2-deoxy-D-glucose transport was examined by measuring inhibition constants (K_i). Transport assays were carried out essentially as described in Section 2.6.1. To initiate transport, cells were added to 100 μl of PBS buffer containing radiolabelled substrate and an appropriate concentration of inhibitor. After the required time of 1 min, the transport was terminated, and radioactivity was determined.

The effect of a competitive inhibitor on the uptake of 2dGlc can be described by the following equation:-

$$V_I = \frac{V_{max} \bullet S}{K_m \bullet (1 + I/K_i) + S} + M \bullet S \qquad (3.2)$$

where V_I = the rate of uptake in the presence of inhibitor,

I = inhibitor concentration,

and the other terms are as described for equation 3.1.

The K_i's for reversible inhibitors were determined from plots of uptake versus inhibitor concentration. For each inhibitor illustrated in Figures 3.6 - 3.12, the curve through the data points represents the best-fit of the data to equation 3.2. Using this approach, 2dGlc uptake in the insect cells was found to be most potently inhibited by phloretin, the aglucone of phlorizin, which inhibited with an apparent K_i of 0.77 ± 0.10 μM (Fig. 3.6). Next in order of inhibitory potency was cytochalasin B (apparent K_i = 9.8 ± 7.1 μM, Fig. 3.7). The cytochalasin B was dissolved in ethanol and the concentration of the ethanol was kept constant in all the samples. Of the hexoses tested, L-glucose had the least effect on 2dGlc transport in the insect cells (apparent K_i = 50.1 ± 32.7 mM, Fig. 3.8), indicating that the transport is stereoselective. D-mannose had the highest apparent affinity for the insect cell transporter, exhibiting a K_i = 1.24 ± 0.23 mM (Fig. 3.9). Unlike the situation for the human erythrocyte glucose transport system, where the K_d for mannose is somewhat greater than that for glucose, this apparent affinity of the C-2 epimer for the insect cell transporter was similar to that of D-glucose itself (K_i = 1.46 ± 0.28 mM, Fig. 3.10). However, as shown in Figure 3.11, epimerization at the C-4 position of D-glucose resulted in a dramatic decrease in affinity of the hexose for the insect cell transporter, the apparent K_i for inhibition of 2dGlc transport by D-galactose being 24.5 ± 6.8 mM. Interestingly, the insect cell sugar transporter also showed high affinity for D-fructose, which inhibited 2dGlc transport with an apparent K_i = 4.33 ± 0.90 mM (Fig. 3.12). In contrast the reported K_m of the human erythrocyte transporter for the ketose is 1.5 M (LeFevre and Marshall, 1958).

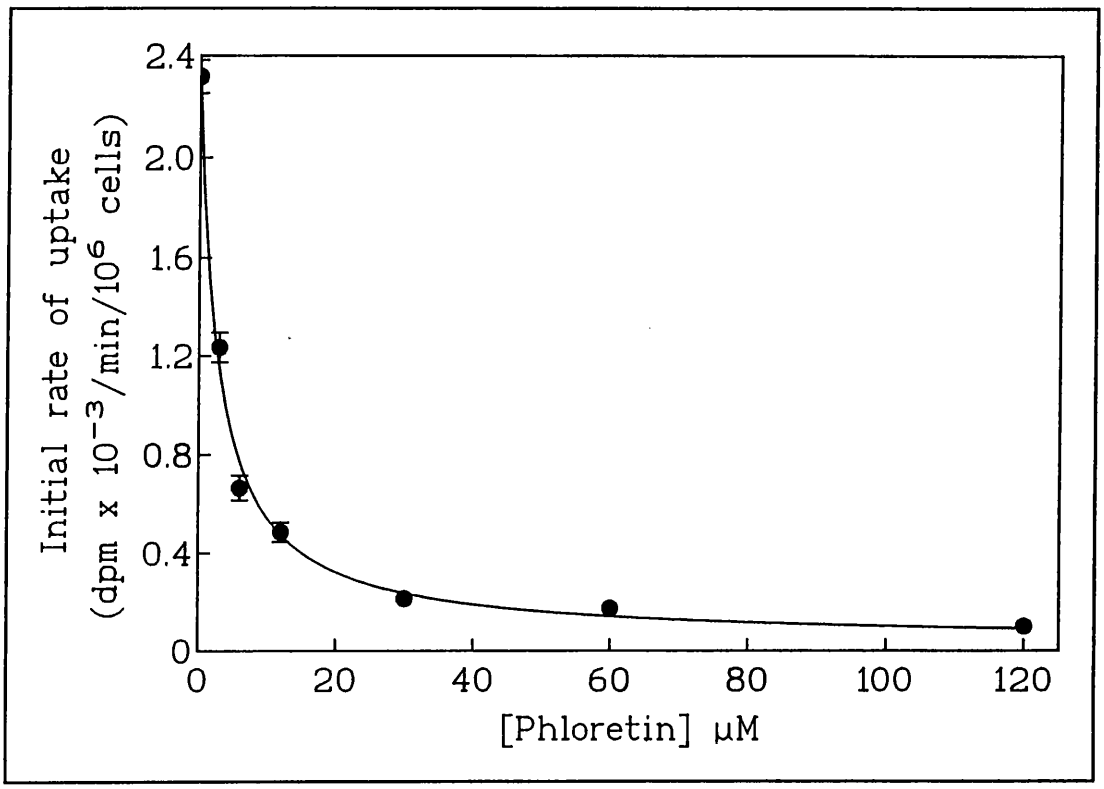


Fig. 3.6. Effect of phloretin on the initial rate of 2-deoxy-D-glucose uptake. Transport was initiated by the addition of 100 μl of PBS buffer containing [^{3}H]2dGlc and an appropriate concentration of phloretin. After 1 min, the transport was terminated by addition of ice-cold stop solution. Cell were then solubilized and radioactivity was determined as described in Section 2.6. The data were analysed with the help of ENZFITTER. The curve represents the best-fit of the data to equation 3.2. Each data point represents the mean of triplicates.

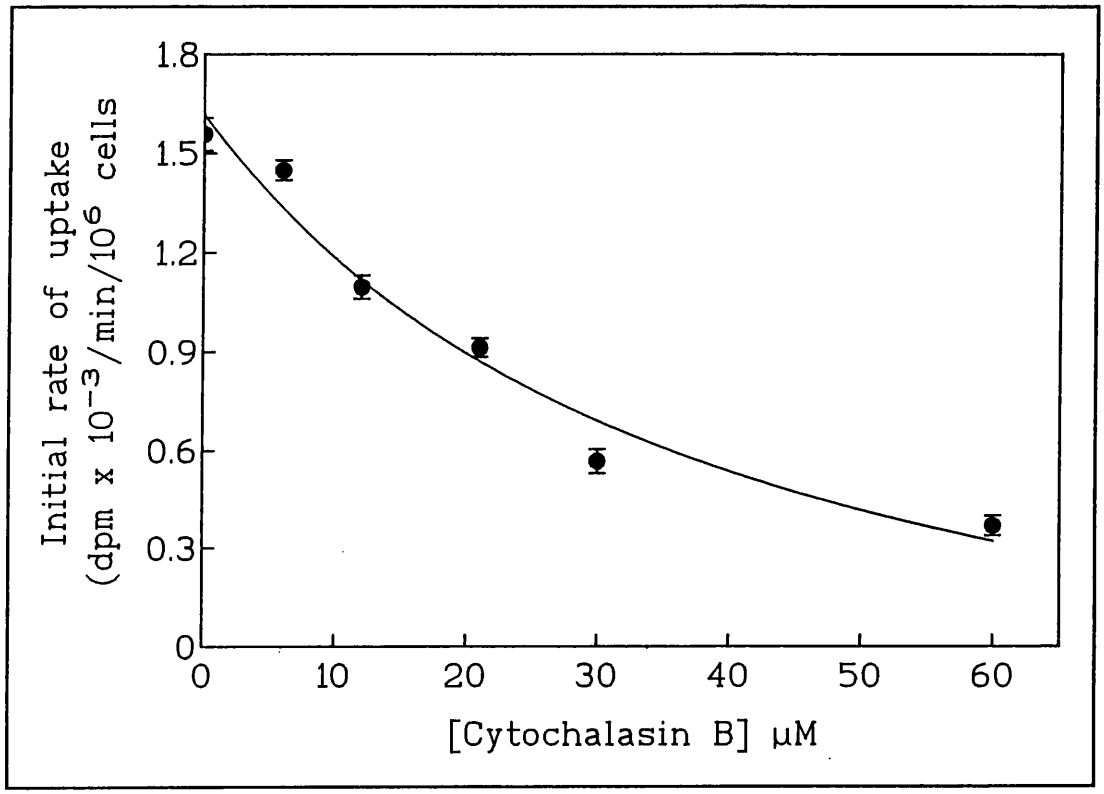


Fig. 3.7. Effect of cytochalasin B on 2-deoxy-D-glucose transport. Transport assay was carried out essentially as described in Section 2.6. The ability of cytochalasin B to inhibit the uptake of 2dGlc was determined as described in the legend to Fig.3.6. The curve represents the best fit of the data to equation 3.2. Each data point is the mean of triplicate estimations.

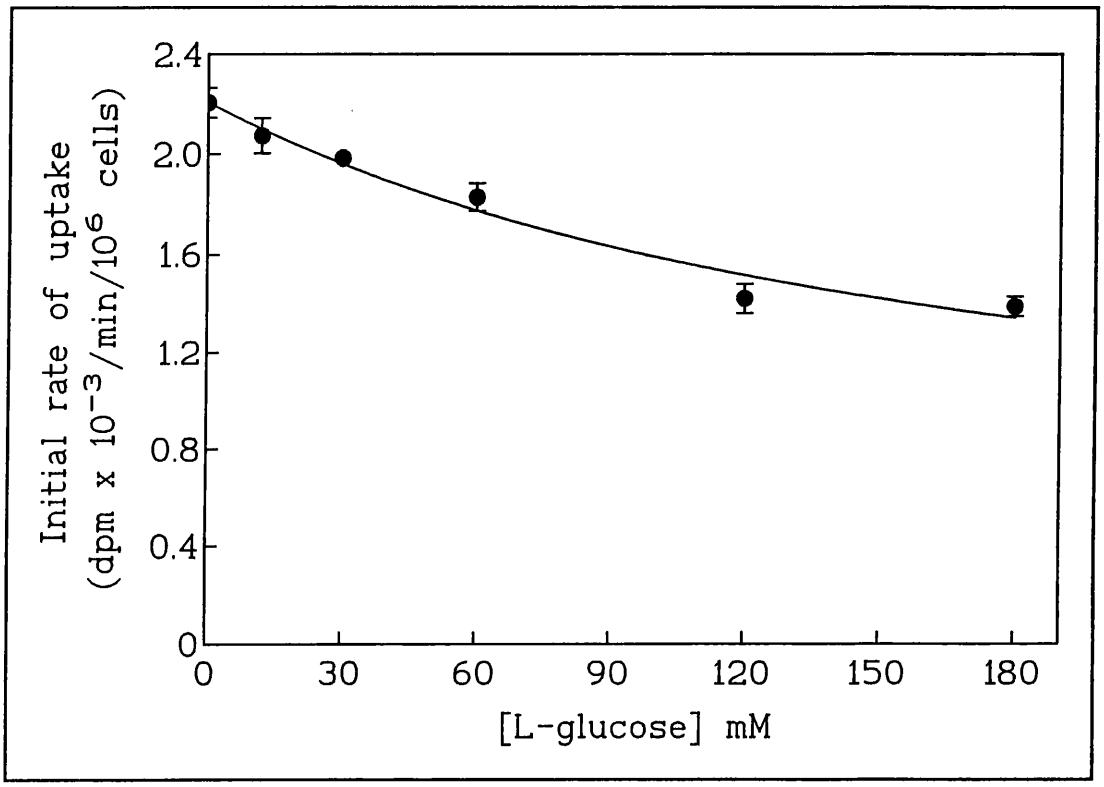


Fig. 3.8. Effect of L-glucose on 2-deoxy-D-glucose transport. Transport assay was carried out essentially as described in Section 2.6. The ability of L-glucose to inhibit the uptake of 2dGlc was determined as described in the legend to Fig.3.6. The curve represents the best fit of the data to equation 3.2. Each data point is the mean of triplicate estimations.

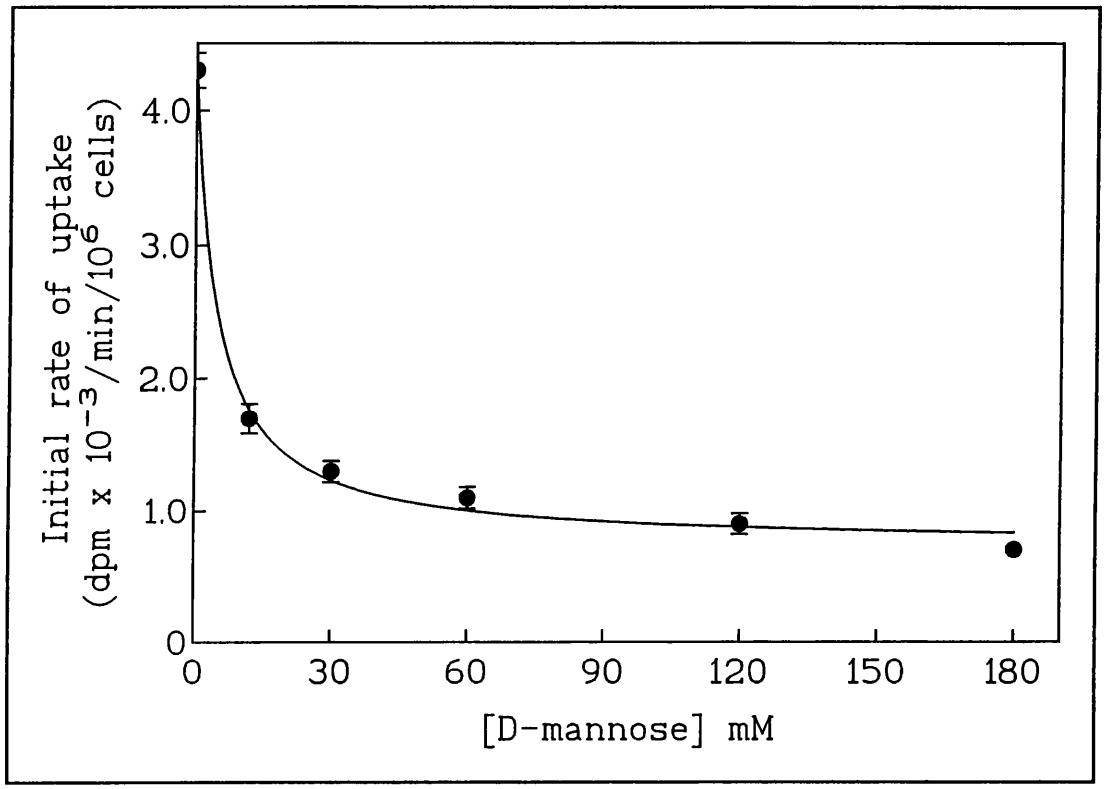


Fig. 3.9. Effect of D-mannose on 2-deoxy-D-glucose transport. Transport assay was carried out essentially as described in Section 2.6. The ability of D-mannose to inhibit the uptake of 2dGlc was determined as described in the legend to Fig. 3.6. The curve represents the best fit of the data to equation 3.2. Each data point is the mean of triplicate estimations.

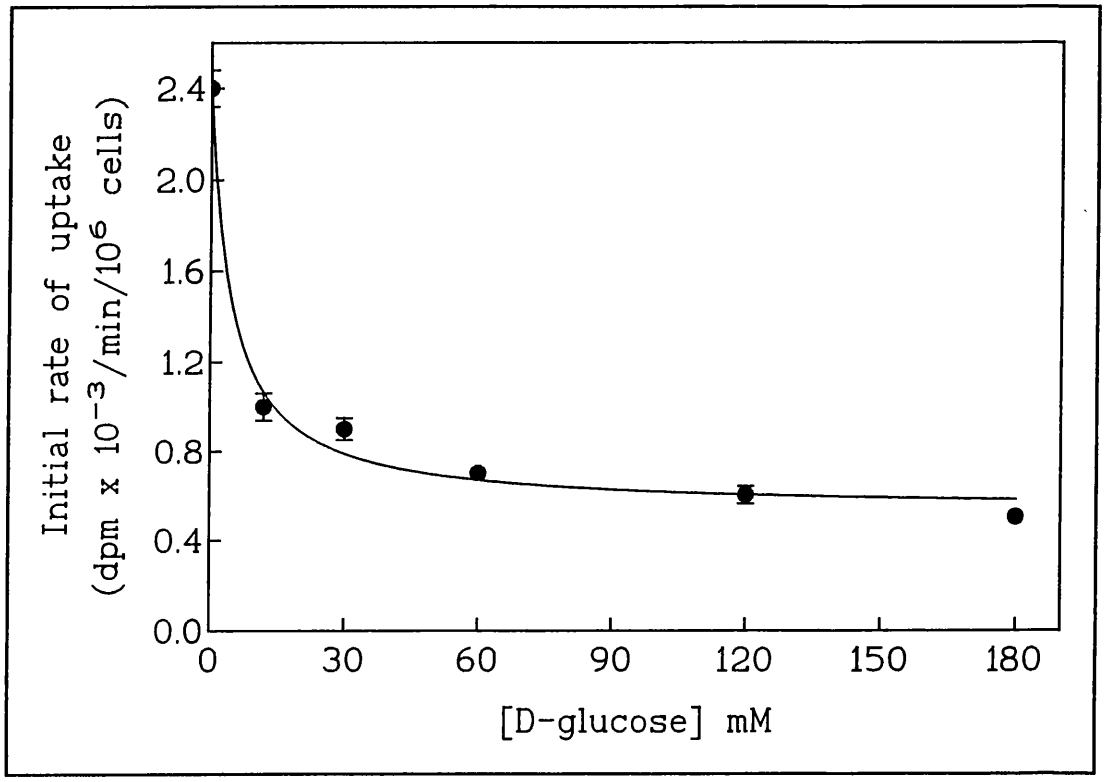


Fig. 3.10. Effect of D-glucose on 2-deoxy-D-glucose transport. Transport assay was carried out essentially as described in Section 2.6. The ability of D-glucose to inhibit the uptake of 2dGlc was determined as described in the legend to Fig.3.6. The curve represents the best fit of the data to equation 3.2. Each data point is the mean of triplicate estimations.

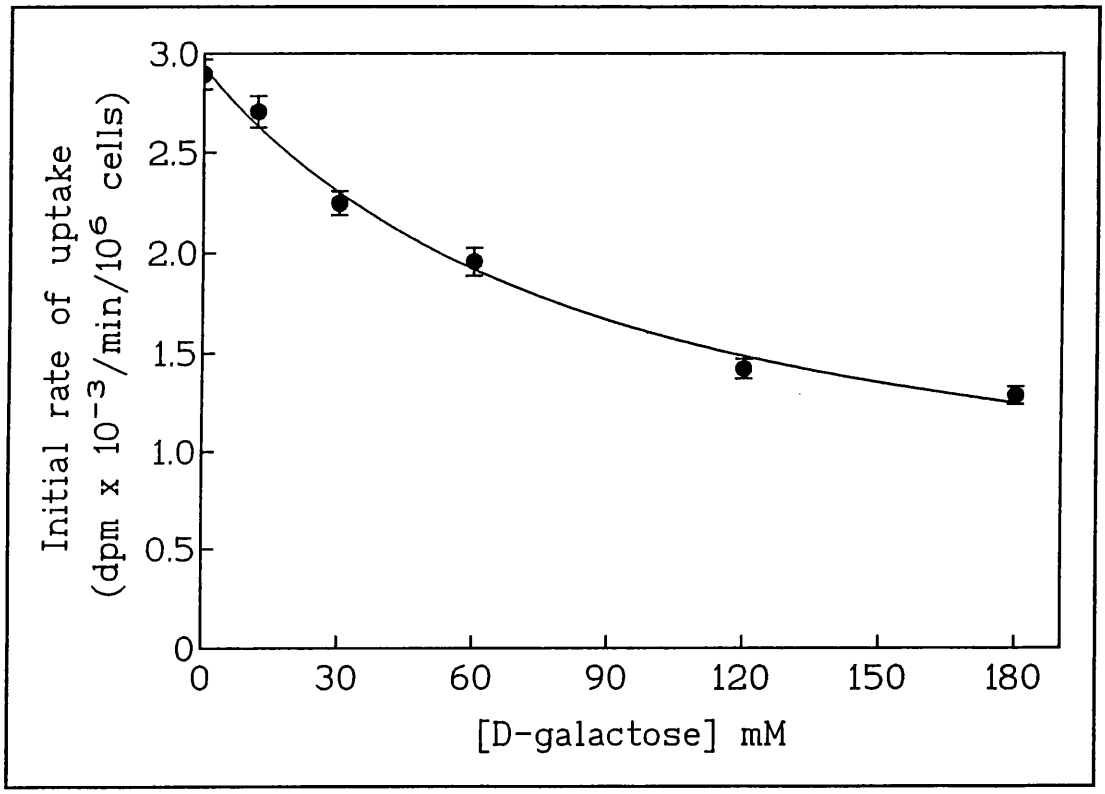


Fig. 3.11. Effect of D-galactose on the initial rate of 2-deoxy-D-glucose uptake. Transport assay was carried out essentially as described in Section 2.6. The ability of D-galactose to inhibit the uptake of 2dGlc was determined as described in the legend to Fig.3.6. The curve represents the best fit of the data to equation 3.2. Each data point is the mean of triplicate estimations.

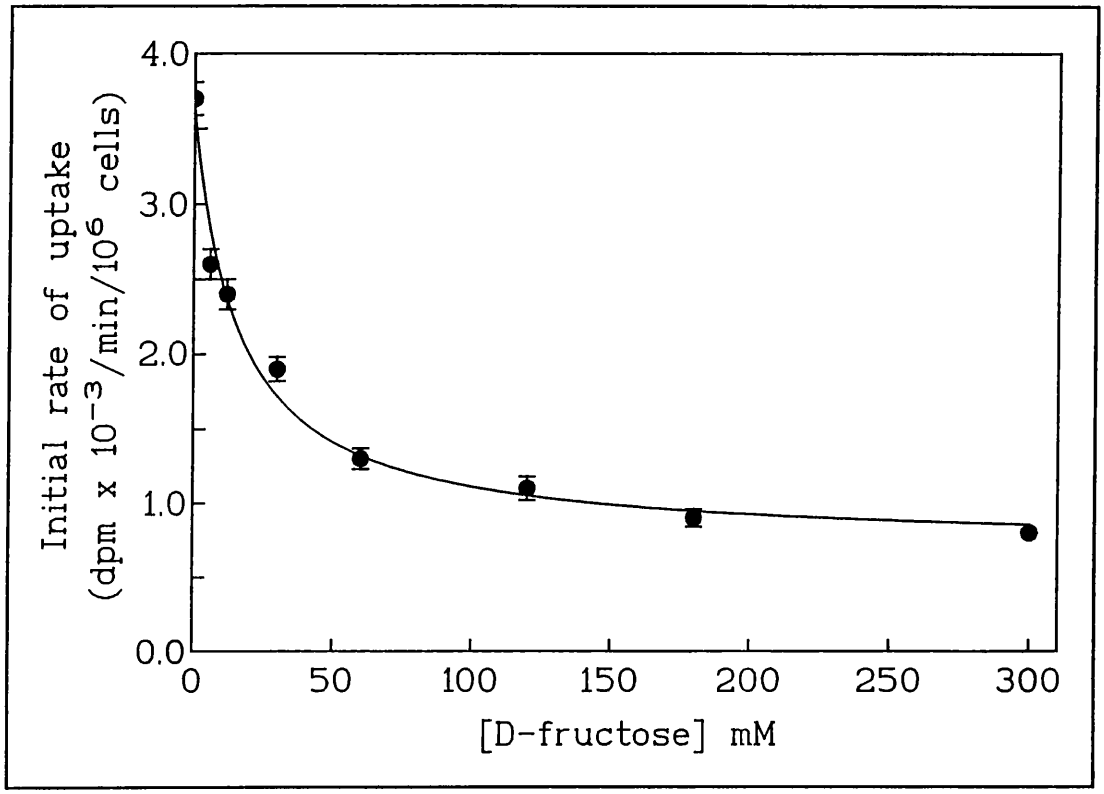


Fig. 3.12. Effect of D-fructose on 2-deoxy-D-glucose transport. Transport assay was carried out essentially as described in Section 2.6. The ability of D-fructose to inhibit the uptake of 2dGlc was determined as described in the ligend to Fig.3.6. The curve represents the best fit of the data to equation 3.2. Each data point is the mean of triplicate estimations.

3.3. CYTOCHALASIN B BINDING TO INSECT CELL MEMBRANES

Cytochalasin B is a potent inhibitor of the human erythrocyte glucose transporter, to which it binds with a K_d of approximately 0.12 μM (Zoccoli *et al.*, 1978). The experiments described above had shown that it was a much less potent inhibitor of the hexose transport in insect cells. However, it was not clear whether this lower potency resulted from a lower affinity of the transporter for the inhibitor, or from a lesser effect of bound inhibitor on the transport activity of the protein. In order to resolve this question, the ability of cytochalasin B to bind to the insect cell transporter was directly examined in a ligand binding assay. Cytochalasin B binding activity was assayed by equilibrium dialysis using a single, final concentration of [4-^{3}H] cytochalasin B of 40 nM, in the absence and presence of 400 mM D-glucose (Section 2.2.5). The insect cell membranes used were prepared as described in Section 2.2.7. Alkali-stripped human erythrocyte membranes (Gorga and Lienhard, 1981) were used as a positive control. Cytochalasin binding activity was calculated by subtracting the value of the ratio of bound cytochalasin B to free cytochalasin B obtained in the presence of D-glucose from the equivalent value obtained in the absence of D-glucose (Section 2.2.5). The corrected bound-to-free ratio is approximately equal to the ratio of the concentration of cytochalasin B binding sites on glucose transporters to the dissociation constant for cytochalasin B. Thus, it is proportional to the concentration of binding sites (Zoccoli *et al.*, 1978). The cytochalasin B binding activity for the insect cell and erythrocyte membranes at the concentration of 1 mg per ml were 0.001 and 7.538, respectively (Table 3.1). From the results, it may be concluded that the

Table 3.1

Cytochalasin B binding to Sf9 cell membranes

Sample (1 mg/ml)	Cytochalasin B Binding (B/F)		
	(-)D-Glucose	(+)-D-Glucose	*Specific B/F
Sf9 cells membranes	0.049	0.048	0.001
Erythrocyte membranes[a]	8.055	0.517	7.538

The binding of cytochalasin B was measured at a single low concentration (40 nM), in the absence (-) and presence (+) of 400 mM D-glucose, as described in Section 2.2.5. Cytochalasin B binding activity (*) was calculated as described in the text. Human erythrocyte membranes (a) were prepared as described previously (Gorga and Lienhard, 1981). B/F = [bound cytochalasin B]/[free cytochalasin B].

absence of the binding activity in the former results either from the low concentration of binding sites on the insect transporter(s), or from the low affinity for the transporter, or both. However, judging from the effects on transport (Section 3.2.3), it is probably because the affinity is low. Therefore, the presence of endogenous transporters should not interfere with the assay of the cytochalasin B binding properties of human GLUT1 expressed in insect cells.

3.4. IMMUNOLOGICAL EXAMINATION OF THE INSECT CELL TRANSPORTER WITH ANTIBODIES TO THE MAMMALIAN TRANSPORTER

Antibodies are very powerful tools for detecting and quantifying the expression of proteins in heterologous expression systems. A number of antibodies against the human erythrocyte glucose transporter were available that could potentially be used for this purpose in the present project. However, the successful use of such antibodies for quantification of GLUT1 expression in insect cells relied upon their specificity for the human protein and lack of cross-reaction with endogenous transporters. The hexose transport activity of insect cells described above suggested that endogenous transporters were abundant, and the occurrence of sugar transporters homologous to GLUT1 in a wide range of organisms (Baldwin, 1993) indicated a likelihood that the insect cell transporter(s) might exhibit sequence similarity to the human protein. Therefore, before expression of the human protein was attempted, the potential cross-reactivity of the antibodies with the endogenous insect cell glucose transporters was first examined. Insect cell membranes (Section 2.2.7) were analysed by SDS-polyacrylamide gel electrophoresis using a 10 %

polyacrylamide gel (Laemmli, 1970) and then transferred to nitrocellulose for immunoblotting as described in Section 2.2.2. The blots were immunostained with antisera raised against intact GLUT1 or against the *C*-terminus of GLUT1 (Davies *et al.*, 1987). The bound primary antibodies were then detected with a secondary antibody labelled with ^{125}I.

As shown in Figure 3.13, neither the antibodies against intact GLUT1 nor those against the *C*-terminus labelled any band migrating in the region expected for a protein of M_r comparable to GLUT1 (lanes C and E), but some cross-reactive bands with higher M_r were detected in the insect cell membranes by the antibody against the whole protein. One of these bands was also present when pre-immune serum was used to stain the blot. However, such bands were not detected when the antibodies against the *C*-terminus of GLUT1 were used. The specificity of the antibodies used was confirmed by the fact that both, as expected, recognised a broad band of apparent M_r 45,000 - 65,000 on blots of protein-depleted human erythrocyte membranes. Thus, it appears that uninfected insect cell membranes do not contain transport proteins immunologically cross-reactive with antibodies directed against the *C*-terminus of the mammalian glucose transporter, although the nature of the bands of higher M_r, that were recognised by antibodies against whole GLUT1, remains unclear. Lack of immunological cross-reactivity suggests that even if the endogenous insect cell glucose transporter is homologous to GLUT1, the mammalian and insect cell proteins share only very limited sequence similarity. Consequently, the presence of endogenous glucose transporter(s) should not interfere with the immunological detection of human GLUT1 expressed in insect

cells, at least if anti-*C*-terminal antibodies are used to probe for the expressed protein.

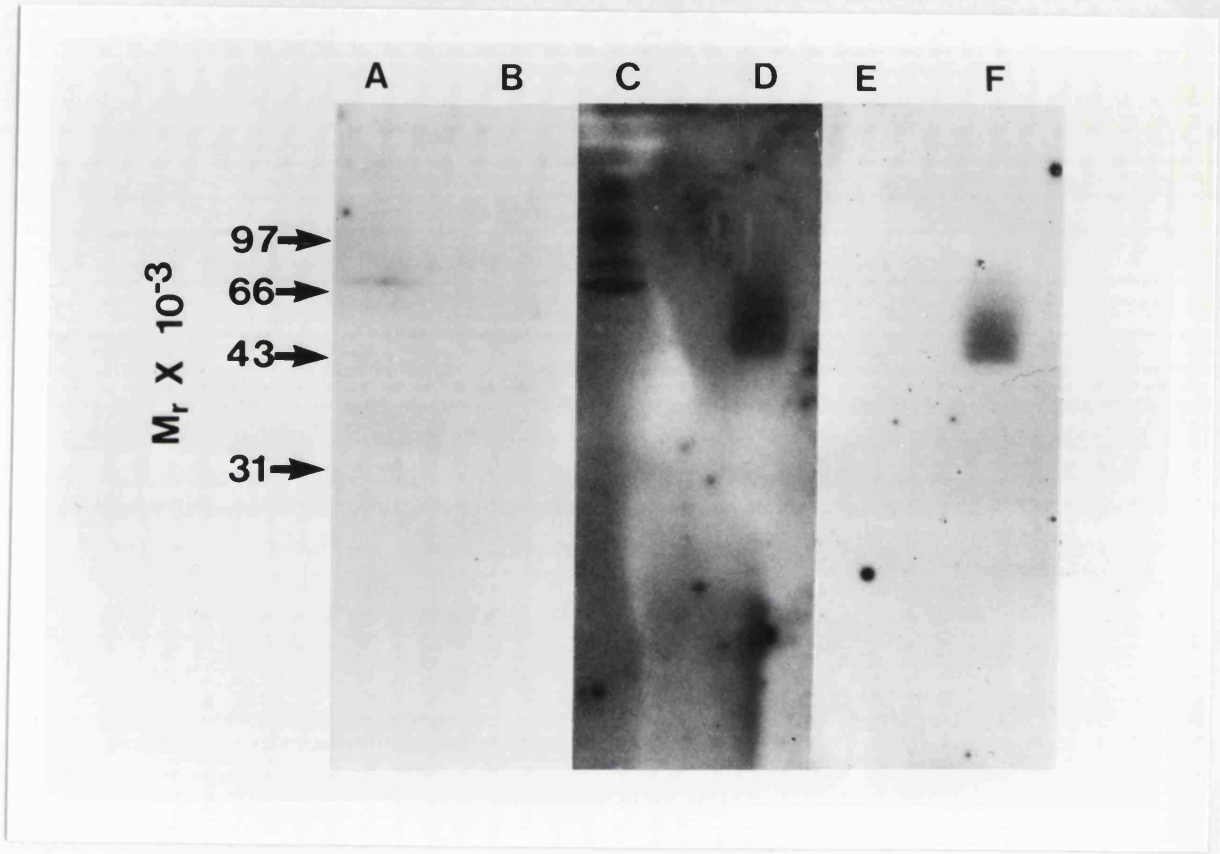


Fig. 3.13. Western blotting of insect cell membrane proteins using antibodies against the mammalian glucose transporter GLUT1. Samples containing 5 μg of Sf9 cell membranes (A, C, E) or of protein-depleted human erythrocyte membranes (B, D, F) were electrophoresed on an SDS/10 % polyacrylamide gel, transferred to nitrocellulose and stained with either antibodies against intact GLUT1 (C and D), against the *C*-terminus (E and F) or pre-immune (A and B) as described in Section 2.2.2. The positions of M_r markers are indicated.

3.5. DISCUSSION

The erythrocyte glucose transporter has a broad substrate specificity, a large number of simple sugars being substrates. The rank order of apparent affinities is as follows: 2-deoxy-D-glucose > D-glucose > D-mannose > D-galactose > D-xylose > L-arabinose > D-fucose >> L-fucose > L-rhamnose >> L-glucose (LeFevre, 1961). As is apparent from this rank order, the transporter displays a particularly strong specificity for D-stereoisomers of sugars. To investigate the presence and direction of hydrogen bonds between sugar and transport protein, Barnett *et al.* (1973) have studied the ability of various sugars to inhibit sorbose equilibrium exchange transport in human erythrocyte by replacing the hydroxyl groups of sugar with hydrogen and fluorine atoms. Their investigation is based on the prediction that if at any position hydrogen bonding plays an important role in sugar binding to the active site of the transporter, and the sugar acts as oxygen donor, then replacement of the sugar hydroxyl group by hydrogen atom should decrease the affinity of the sugar analogue for the transporter. Similarly, substitution of a fluorine atom for the hydroxyl group would restore the affinity if it is the transport protein that donates the hydrogen atom of the hydrogen bond. In accordance with the prediction, removal of the oxygen at C-1 to form 1-deoxy-D-glucose decreases affinity for the transporter, suggesting the presence of a hydrogen bond. But the affinity is restored by substitution of a fluorine atom in the β-configuration for the hydroxyl at this position. The binding affinity is not restored when the fluorine is introduced into the α-configuration, suggesting that the β-form of the sugar is the substrate for the transporter. However, the discrimination between

the two anomers of D-glucose by the transporter still remains unclear.

In contrast, replacement of the hydroxyl group at C-2 by a hydrogen atom has no effect on inhibition of sorbose transport, apart from a marginal increase in binding affinity. This suggests that there is no hydrogen bonding at this site. At the C-3 position, 3-deoxy-D-glucose has a lower affinity for the transporter than D-glucose, but substitution of a fluorine atom for the hydroxyl at this site restores the high affinity, indicating that the C-3 oxygen may act as an hydrogen bond acceptor. Unlike the hydroxyl groups at C-1 and C-3, the role of the hydroxyl group at C-4 is not clear. D-galactose has a lower affinity for the transporter than D-glucose, but C-4 substituents of D-glucose such as 4,6-*O*-ethylidene-D-glucose and 4-*O*-propyl-D-glucose have affinities for the transport system comparable with that of D-glucose. Thus, the low affinity shown by galactose may be due to the loss of a hydrogen bond at this position. Unfortunately, 4-deoxy-D-glucose is not available. The situation at the C-6 position is also uncertain. 6-Deoxy-D-glucose binds effectively to the transporter, indicating no involvement of a hydrogen bond at this site. But replacement of the C-6 hydroxyl with a fluorine atom leads to an enhanced binding affinity, suggesting the involvement of hydrogen bonding. In addition to contributions from these hydroxyl groups for binding, the pyranose ring oxygen of D-glucose is also thought to be required for optimal binding (Kahlenberg and Dolansky, 1972). Replacement of the ring oxygen with sulphur to form 5-thio-D-glucose results in a marked reduction in affinity for the transporter.

However, unlike the mammalian transporter little is known about the

nature of the sugar transporter(s) in insect cells. In order to establish whether the insect cells would be a suitable host for expression of the human glucose transporter, it was first necessary to investigate the transport characteristics of the insect cell transporter(s). The affinities of the insect cell transporter for different hexoses were assessed by examining the ability of these sugars to inhibit 2-deoxy-D-glucose (2dGlc) transport in Sf9 cells. Since the same substrate (2dGlc) was used for all experiments of this type, then the apparent K_i values for each sugar should provide a measure of their relative affinities. The uptake of 2dGlc in the Sf9 cells followed a simple linear function of time for at least 2 min. The transport was inhibited by D-mannose, D-glucose and D-fructose. However, the apparent affinity of the C-4 epimer, D-galactose, for the *Spodoptera* transporter was relatively low, implying that the hydroxyl group at C-4 position may play a role in the strong binding of glucose and mannose to the transporter. The results also showed that transport was stereoselective, being inhibited by D-glucose, not by L-glucose. Unlike the human erythrocyte glucose transport system, which has a very low affinity for fructose (LeFevre and Marshall, 1958), the insect cell transporter appeared to have high affinity for D-fructose (K_i = approximately 5mM). Indeed, the K_i for fructose was only about 3-fold lower than that for D-glucose, suggesting that the insect cell transporter may also be capable of transporting fructose, although this suggestion would have to be confirmed by transport experiments using radiolabelled fructose itself. Whether or not fructose is in fact transported, its affinity for the transporter suggested that it might be possible to use the ketose as an inhibitor for blocking the endogenous glucose transport activity of insect

cells when the functional activity of heterologously expressed human erythrocyte glucose transporter was being assessed. The insect cell transporter was also found to differ from the human protein in being much less sensitive to inhibition by cytochalasin B. However, it was inhibited by the diphenolic compound phloretin with a K_i (approximately 3 μM) similar to the value of about 2 μM reported for inhibition of glucose transport in the human erythrocyte (LeFevre and Marshall, 1959).

The importance of molecular conformation in sugar transport across the lipid bilayer of the human erythrocyte was first recognised by LeFevre (1961), who demonstrated a correlation between affinity and propensity to exist in the C1 conformation. It is now thought that the affinity of sugars for the transporter is probably determined by their possession of suitable equatorial hydroxyl groups to form hydrogen bonds with the transporter. Binding affinity is also likely to be influenced by axial hydroxyls that may sterically hinder sugar binding to the transporter. Thus, of the natural hexose and pentose sugars, the β-anomers of D-glucose and D-xylose, which are completely free of axial substituents in the C1 conformation, show the highest affinities for the transport protein, whereas their enantiomers (mirror images), L-glucose and L-xylose, have extremely low affinities for the protein. In addition, epimerization of the equatorial hydroxyl groups at carbons 2, 3 or 4 of D-glucose to an axial orientation is known to result in a marked decrease in the affinity of the corresponding D-glucose analogue for the binding site of the erythrocyte transporter (LeFevre, 1961; Barnett *et al.*, 1973). For example, D-galactose (the C-4 epimer of D-glucose) inhibits sorbose transport (via the glucose

transporter) with 13-fold lower affinity than does D-glucose. Similarly, the K_i of the C-4 epimer is 16-fold greater than for D-glucose in the insect cell transport system. Such a lowering of affinity might be the result either of the involvement of the C-4 hydroxyl in hydrogen bonding or of steric hinderance from the axial hydroxyl group. Interestingly, unlike the human erythrocyte glucose transport system, the C-2 epimer (D-mannose) has a somewhat greater affinity for the insect cell transporter than D-glucose. In addition, the kinetic results suggest that 2-deoxy-D-glucose binds more tightly than does D-glucose. This finding implies that the hydroxyl group at C-2 position is not required for a strong binding.

In conclusion, insect cells were found to contain an endogenous glucose transport activity that in several aspects resembled the mammalian passive glucose transporter GLUT1. The presence of such an activity might interfere with assessment of the kinetic properties of the mammalian transporter expressed in the insect cells, although the mammalian and insect transporters do differ in some of their kinetic properties, namely their affinities for fructose and for cytochalasin B. Therefore, it would probably be necessary to purify the expressed protein away from the endogenous transporter and then reconstitute it into lipid vehicles before detailed kinetic studies could be performed. However, if expression of the mammalian transporter could be achieved in this system, its functionality might initially be assessed in other ways. For example, binding of cytochalasin B could be used as a measure of the biological activity of the expressed protein, since the insect cell transporter was found to bind this transport inhibitor only very poorly. An alternative approach would be to exploit

the finding that the insect cell transporter(s) is not recognised by specific antibodies raised against the human erythrocyte transport protein. Therefore, the technique of photoaffinity labelling with [^{3}H]cytochalasin B followed by immunoprecipitation could be applied to label functionally intact, native protein. Hence, the insect cell/baculovirus expression system does appear to be a good candidate to explore for expression of large amounts of biologically active mammalian glucose transporters.

CHAPTER 4. CONSTRUCTION OF RECOMBINANT TRANSFER VECTOR AND GENERATION OF RECOMBINANT BACULOVIRUS

4.1. INTRODUCTION

Recent advances in recombinant DNA technology have greatly enhanced our ability to manipulate organisms and have made it possible to express cloned genes in heterologous living organisms. One of the most important elements in gene cloning is the vector, which, in conjunction with the DNA molecule to be cloned, forms the recombinant DNA molecule. A vector is a DNA molecule that is capable of replication in certain host cells due to the presence of a specific DNA structural element known as the origin of replication. In the absence of such replication, fragments of foreign DNA introduced into host cells would be diluted out and thus lost as the latter divided and multiplied. The commonest vectors in current use are derived from bacterial plasmids and from *E. coli* phage λ. In addition to these, a variety of viruses have also served as vectors because they are, in nature, highly efficient in transporting their own genome into susceptible cells. Examples of such viral vectors are simian virus 40 (Goff and Berg *et al.*, 1979), bovine papilloma virus (Lowy *et al.*, 1980), adenoviruses (Mansour *et al.*, 1985), vaccinia virus (Panicali and Paoletti, 1982), and retroviruses (Dick *et al.*, 1986).

The production of proteins from recombinant DNA molecules requires a special class of vectors which have been designated as expression vectors. Such vectors not only provide the structural prerequisites for the cloning, transfer, and multiplication of recombinant DNA, but also allow this DNA to be

transcribed and translated into protein, through provision of the necessary expression signals. In recent years baculoviruses have received considerable attention as expression vectors because of their potential for high level expression of foreign proteins. The development and use of baculoviruses as expression vectors is based on the fact that two of the viral proteins, p10 and polyhedrin, that are very abundantly expressed during the normal life cycle of the virus, are not in fact essential for the synthesis of lipid-enveloped, infectious virus particles (Smith *et al.*, 1983; Williams *et al.*, 1989). It is therefore possible to exploit the very strong promoters of these genes, by substituting foreign genes for the coding regions of either the p10 or polyhedrin gene, without preventing viral replication (see chapter 1.B).

In order to take advantage of the baculovirus expression system, foreign genes need to be positioned immediately downstream of the strong polyhedrin or p10 gene promoters. However, baculovirus genomes are very large (128 kb for AcNPV) and suitable restriction sites for cloning are lacking at the desired positions (Smith and Summers, 1978a,b). Consequently, recombinant viruses containing foreign genes cannot be obtained by a direct cloning route. Instead, the foreign gene has to be first subcloned into a baculovirus transfer vector. Recombinant viruses can then be obtained by cell-mediated allelic replacement of the target gene in the wild type baculovirus genome with the plasmid-borne foreign gene (Smith *et al.*, 1983; Maeda *et al.*, 1985; Matsuura, *et al.*, 1987). The plasmids used in this allelic replacement reaction are referred to as transfer plasmids.

Most transfer vectors developed to date contain sequences from AcNPV

including the promoter of the polyhedrin gene and a site for heterologous gene insertion, as well as varying amounts of flanking viral sequences, which provide homologous 5′ and 3′ sequences for recombination (Smith *et al.*, 1983; Miller *et al.*, 1986; Matsuura *et al.*, 1987, Summers and Smith, 1987). The transfer vector pAcYM1, utilised in the present study, was developed in Oxford by Bishop and associates to improve the level of foreign gene expression (Matsuura *et al.*, 1987). Using suitable restriction enzymes and Bal31, the complete coding region of the polyhedrin gene was removed from the EcoRI "I" fragment of AcNPV and a BamH1 restriction site placed after the A of the polyhedrin ATG translation initiation codon. The vector pAcYM1 contains all of the polyhedrin 5′ leader sequence up to, and including, the "A" of the normal polyhedrin ATG translation initiation codon, but lacks the rest of the polyhedrin coding sequence plus 13 nucleotides downstream from the translation termination codon of the gene. The insertion site for pAcYM1 is a BamHI restriction site (Fig. 4.1).

In order to insert a foreign gene into a baculovirus transfer vector, the latter must first be linearized by appropriate restriction endonuclease digestion followed by phosphatase treatment to minimise re-ligation of its complementary ends. The foreign gene, manipulated such that it is flanked by compatible restriction sites, can then be inserted into the restriction site of the vector by ligation. Finally, the orientation of foreign DNA insert must be determined either by standard restriction endonuclease mapping and/or DNA sequencing. The resultant baculovirus transfer vectors containing foreign DNA can be amplified and purified by standard DNA techniques (Sambrook *et al.*, 1989). After

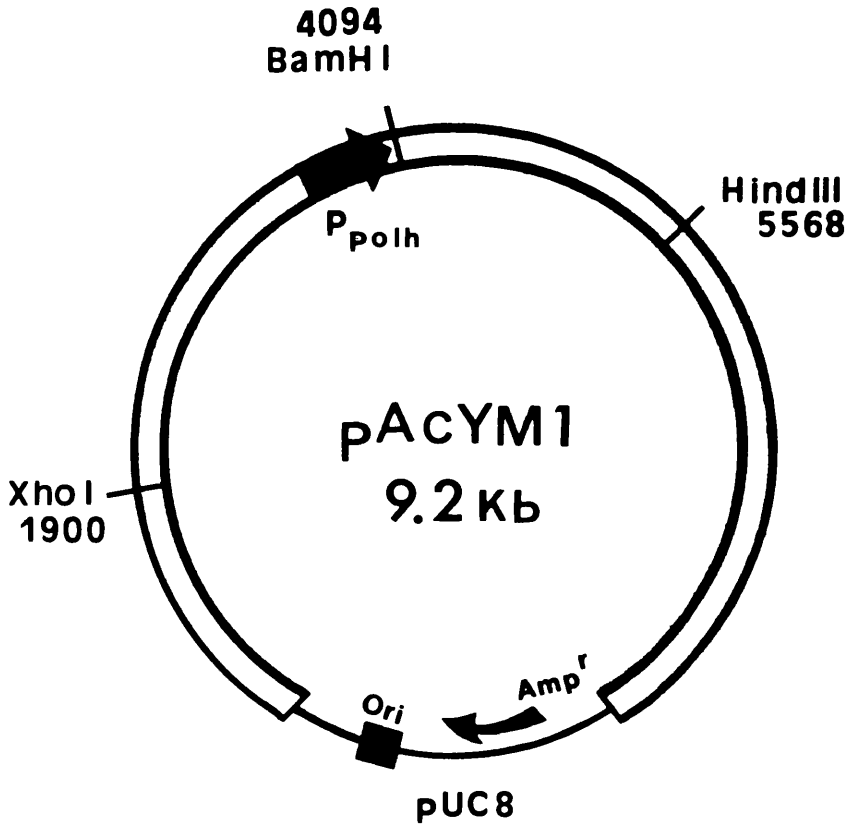


Fig. 4.1. A restriction enzyme map of transfer vector pAcYM1. The plasmid pAcYM1 contains all the upstream sequences of the polyhedrin gene and the A of the initiating ATG codon but lacks the rest of the polyhedrin coding sequences and 13 3'downstream nucleotides (Matsuura *et al.*, 1987). In place of this deletion, the unique BamH1 cloning site has been inserted. The region containing the EcoRI-'I' fragment of AcNPV is shown as a double-lined plasmid segment. Key restriction sites in the plasmid are indicated.

cloning a foreign gene into an appropriate transfer vector, recombinant baculoviruses can then be generated by following each of the steps shown schematically in Figure 4.2. Each stage involved is described in detail in this chapter.

4.2. CLONING GLUT1 cDNA INTO THE TRANSFER VECTOR pAcYM1

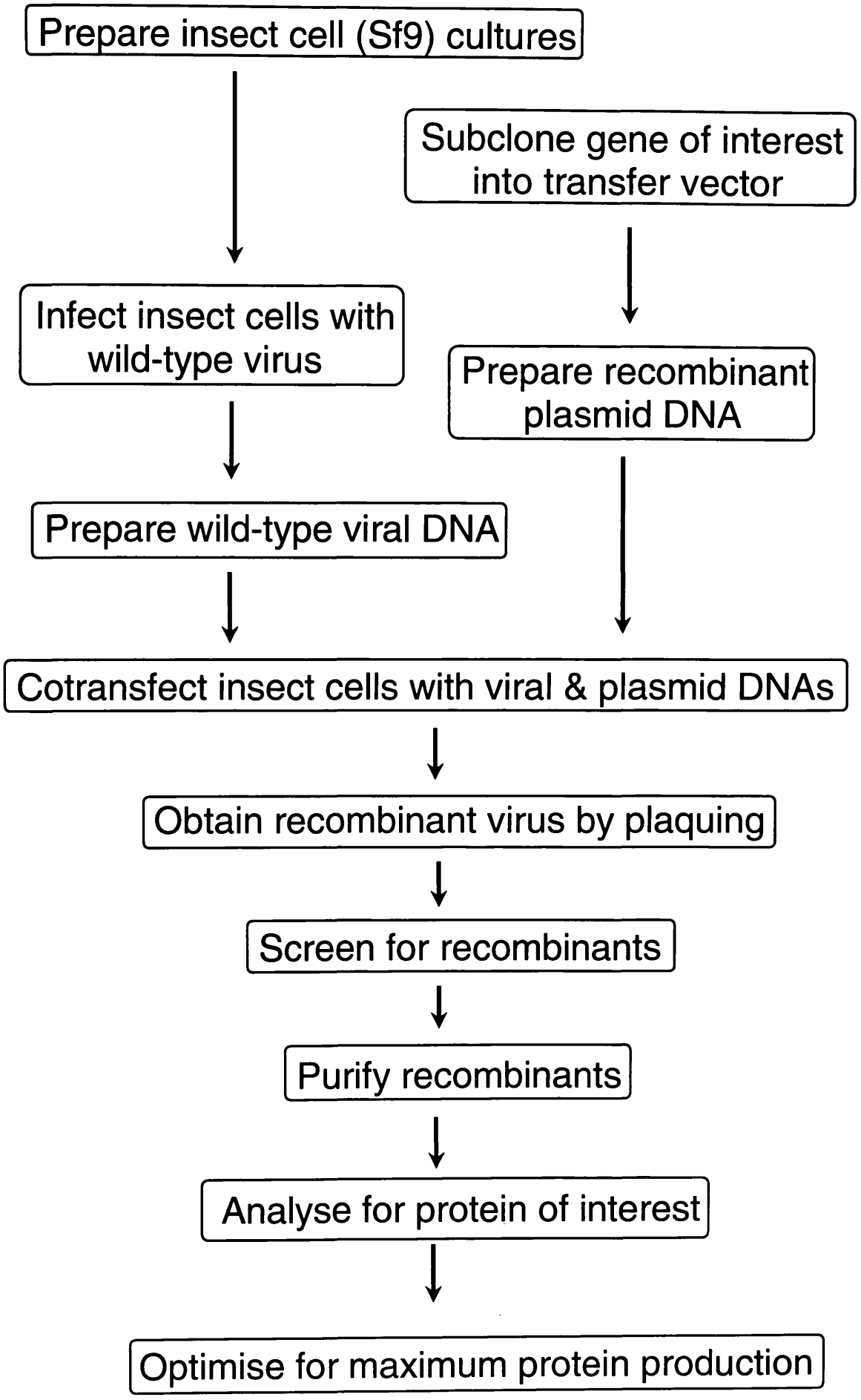
For the successful expression of a foreign gene using the baculovirus expression system, it is necessary to place the gene under the control of the powerful polyhedrin promoter. To achieve this, the coding sequence of GLUT1 cDNA was first subcloned into the transfer vector pAcYM1. Standard recombinant DNA techniques were used throughout, as described by Sambrook *et al.* (1989) with some modifications.

4.2.1. Preparation of plasmid DNAs

The plasmid pSGT (Fig. 4.3) contains the entire coding region of the human HepG2 glucose transporter mRNA (Mueckler and Lodish, 1986) and was gratefully received from Dr. M. Mueckler (Washington University, St. Louis, MO, U.S.A.). The *E. coli* strain JM109 was used as the recipient for transformation with the plasmid. Plasmid DNA was subsequently "maxi-prepared" from the transformed cells by the alkaline lysis method described in Section 2.3.3.

The coding region of GLUT1 cDNA was excised from the plasmid by double restriction digestion with BamHI and HindIII. Although the GLUT1 cDNA could be easily obtained by a single restriction digestion with BamHI, the BamHI/HindIII fragment contained 560 bp less sequence in the 3′-untranslated

Fig. 4.2. Flow diagram of the steps involved in the generation of a recombinant baculovirus.


Prepare insect cell (Sf9) cultures
Subclone gene of interest into transfer vector
Infect insect cells with wild-type virus
Prepare recombinant plasmid DNA
Prepare wild-type viral DNA
Cotransfect insect cells with viral & plasmid DNAs
Obtain recombinant virus by plaquing
Screen for recombinants
Purify recombinants
Analyse for protein of interest
Optimise for maximum protein production

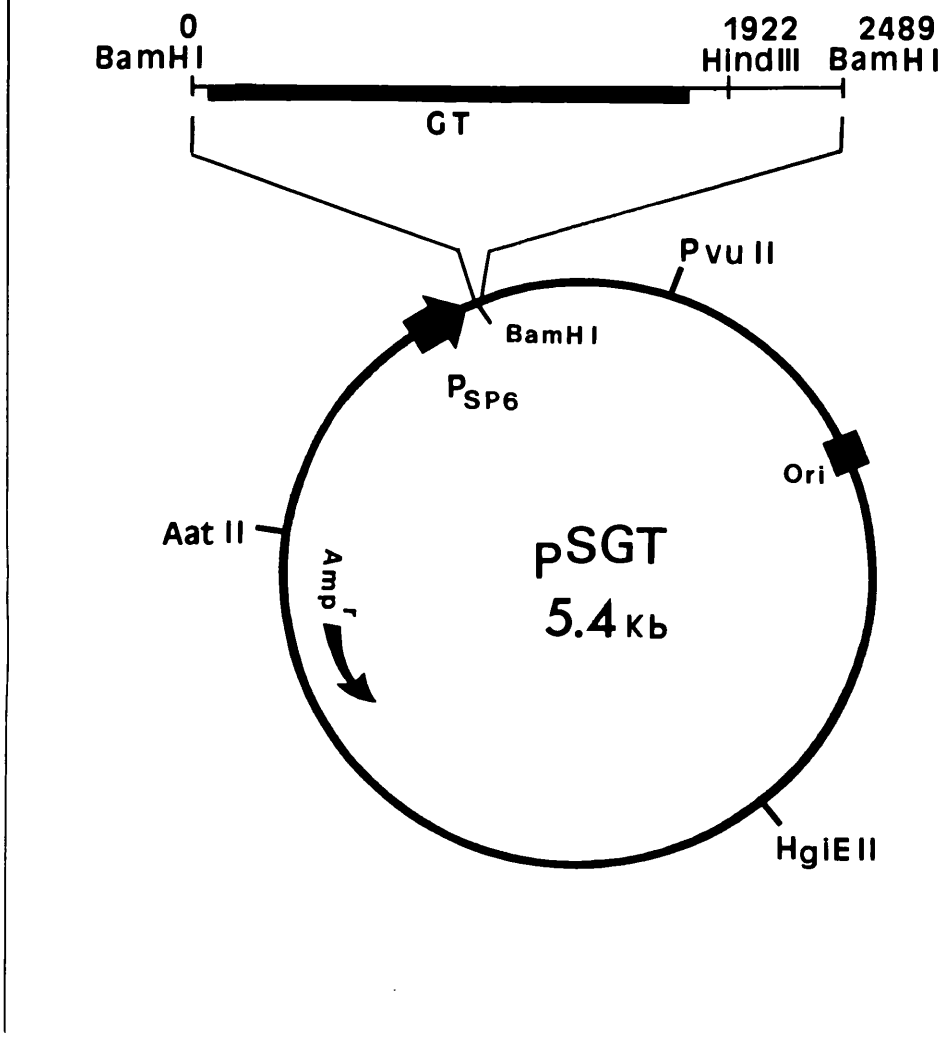


Fig. 4.3. A restriction enzyme map of the plasmid pSGT. The plasmid pSGT contains the entire coding region of GLUT1 cDNA under the control of an SP6 promoter (Mueckler and Lodish, 1986). Key restriction sites in the plasmid are indicated.

region of GLUT1 cDNA than the BamHI fragment. The plasmid was first digested to completion with the restriction enzyme HindIII, which requires a low salt concentration (50 mM NaCl) in the reaction buffer for optimal activity. Completeness of digestion was assessed by analysing a small sample (2 μl) of the reaction mixture by gel electrophoresis on a 1 % agarose minigel. The ionic strength of the incubation buffer was next adjusted to 100 mM NaCl, which is optimal for the activity of the enzyme BamHI. Digestion was then continued with this enzyme at 37°C until it was complete, as assessed by gel electrophoresis of a small sample of the reaction mixture. To isolate the restriction fragment containing the coding region of GLUT1 cDNA, the entire digest was then electrophoresed at 50V for 5 hours at 4°C on a 0.8 % low melting point agarose gel. Following electrophoresis, DNA fragments were visualised under U.V. light and the 1992 bp BamHI/HindIII fragment containing the GLUT1 cDNA was excised from the gel. The DNA was then recovered and purified according to the procedure described in Section 2.3.9.

The transfer vector pAcYM1 was kindly donated from Dr. V. Emery (Division of Communicable Diseases, RFHSM, University of London). The vector DNA was prepared by the alkaline lysis procedure (Section 2.3.2) and linearised by restriction digestion with the enzyme BamHI (Section 2.3.5). The strain JM109 was used for propagation of the pAcYM1, according to the methods described in Section 2.3.10.

4.2.2. Modification of DNA termini

In order to obtain a high efficiency of correct ligation of DNA fragments,

it was necessary to modify the termini both of the vector DNA and of the fragment encoding GLUT1. The modifications involved were blunting of 3' recessed ends and dephosphorylation.

4.2.2.1. Blunting of 3' recessed ends

Since the linearized vector DNA and the 1922 bp GLUT1 cDNA to be cloned possessed different and non-compatible molecular ends, the latter had to be modified to facilitate ligation of the vector and insert DNAs. To achieve this, the protruding ends of the plasmid DNAs were filled-in using the Klenow fragment of *E. coli* polymerase I and complementary deoxy NTPs as described in Section 2.3.6. The enzyme reaction was stopped by the addition of 0.5 M EDTA, followed by the standard phenol/chloroform extraction as described in Section 2.3.1.

4.2.2.2. Dephosphorylation

Self-annealing of the vector DNA cut with a single restriction enzyme was minimised by treatment of the DNA with calf intestinal alkaline phosphatase (CIP). This treatment removes the terminal 5' phosphate groups from linear DNAs and so prevents re-ligation of the two ends. The missing 5' phosphate residues required in the ligation reaction can be provided only by the insert DNA, thus favouring the intermolecular joining event for the blunted ends.

To improve the efficiency of dephosphorylation of the blunted molecules, 3 μl of CIP (1 unit/μl) were added to 5 μg of the linearized vector DNA in a total volume of 30 μl of 10 mM Tris, 1 mM EDTA, pH 8.0, and the mixture was

incubated at 37°C for 15 min. At the end of this period more enzyme (3 μl) was added and incubation was continued for a further 15 min at 57°C. The enzyme was then inactivated by extraction with phenol and chloroform as described in Section 2.3.1.

4.2.3. Ligation

The linearized, modified transfer vector and the blunted cDNA insert were ligated at various molar ratios (1:1, 1:2, 1:3) for 4 hours at 23°C using bacteriophage T4 DNA ligase. This enzyme, unlike *E. coli* DNA ligase, can catalyze the ligation of blunt-ended fragments of DNA. Hexamminecobalt chloride (final concentration 1 mM) was also included in the ligation mixture, because it is known to increase the efficiency of blunt-end ligation about 50-fold (Rusche and Howard-Flanders, 1985).

4.2.4. Transformation

Freshly prepared, competent DH1αF′ cells which are non-recombigenic and transformed with high efficiency by most transformation methods were transformed with a small portion (0.5 μl) or with the remainder (4.5 μl) of the ligation reaction, respectively, according to the method described in Section 2.3.10. The transformation mix was then plated onto agar plates containing ampicillin (100 μg/ml) and incubated at 37°C overnight. Table 4.1 shows the results of the transformation experiment, in terms of the numbers of colonies obtained. The efficiency of transformation obtained using a control plasmid (pSP64T) was in the region of 1 x 10^6 colonies per μg of DNA. This value is

Table 4.1

Transformation results of cloning the 1.9 kb BamHI/HindIII fragment of GLUT1 cDNA into pAcYM1

Ligation Conditions		No. of Transformants A		B	
		Portion$^+$	Rest$^-$	Portion$^+$	Rest$^-$
Vector:Insert (molar ratios)	1:1	0	0	3	7
	1:2	0	0	0	12
	1:3	0	2	6	24
Vector only		0	1	0	2
Insert only		0	0	0	1
Uncut, circular pSP64T				98	confluent

Competent *E. coli* (strain DH1αF') were transformed according to the procedures described in Section 2.3.10, using 0.5 μl (A) or 4.5 μl (B) of the ligation reaction. Following transformation 100 μl (+) of the transformed cells were plated out onto LB plates containing ampicillin. The rest (-) of the cells were pelleted and resuspended in 100 μl of LB medium before plating.

comparable with those reported for other strains of *E. coli*, which are transformed with efficiencies in the region of 1 x 10^5 to 1 x 10^7 colonies per μg of DNA when made competent by the calcium chloride method (Hanahan, 1985). In contrast to this were the total numbers of colonies obtained on each plate containing cells transformed with the GLUT1 cDNA-vector ligation mixture. The efficiency of transformation was very low, being in the region of 100 - 300 colonies per μg of DNA. Such a low apparent efficiency, in comparison to the control, probably stemmed from inefficiency of the blunt-ended ligation reaction. The linearised vector or insert alone produced no significant numbers of transformants as expected.

4.2.5. Identification and characterization of recombinant plasmids

Although the frequency of apparent transformants obtained was not higher than the background level seen with unligated controls, 12 colonies were nonetheless selected for further characterization, in order to see whether any did in fact contain a recombinant plasmid. Plasmid DNA was prepared from each colony by the alkaline lysis "mini-prep" procedure described in Chapter 2, and was then characterised by restriction digestion with the enzyme EcoRI. The results of this restriction mapping, which are illustrated in Figure 4.4, suggested that clones numbered 1, 2, 3, 4, 6, 9 and 11 constituted wild-type transfer vector (pAcYM1). Clones 7 and 10 appeared to contain inserts corresponding to dimers of the 1922 bp GLUT1 fragment. However, one colony (clone 8, lane 8 in Fig. 4.4) appeared to represent the desired recombinant in that it contained an insert of the appropriate size. The nature

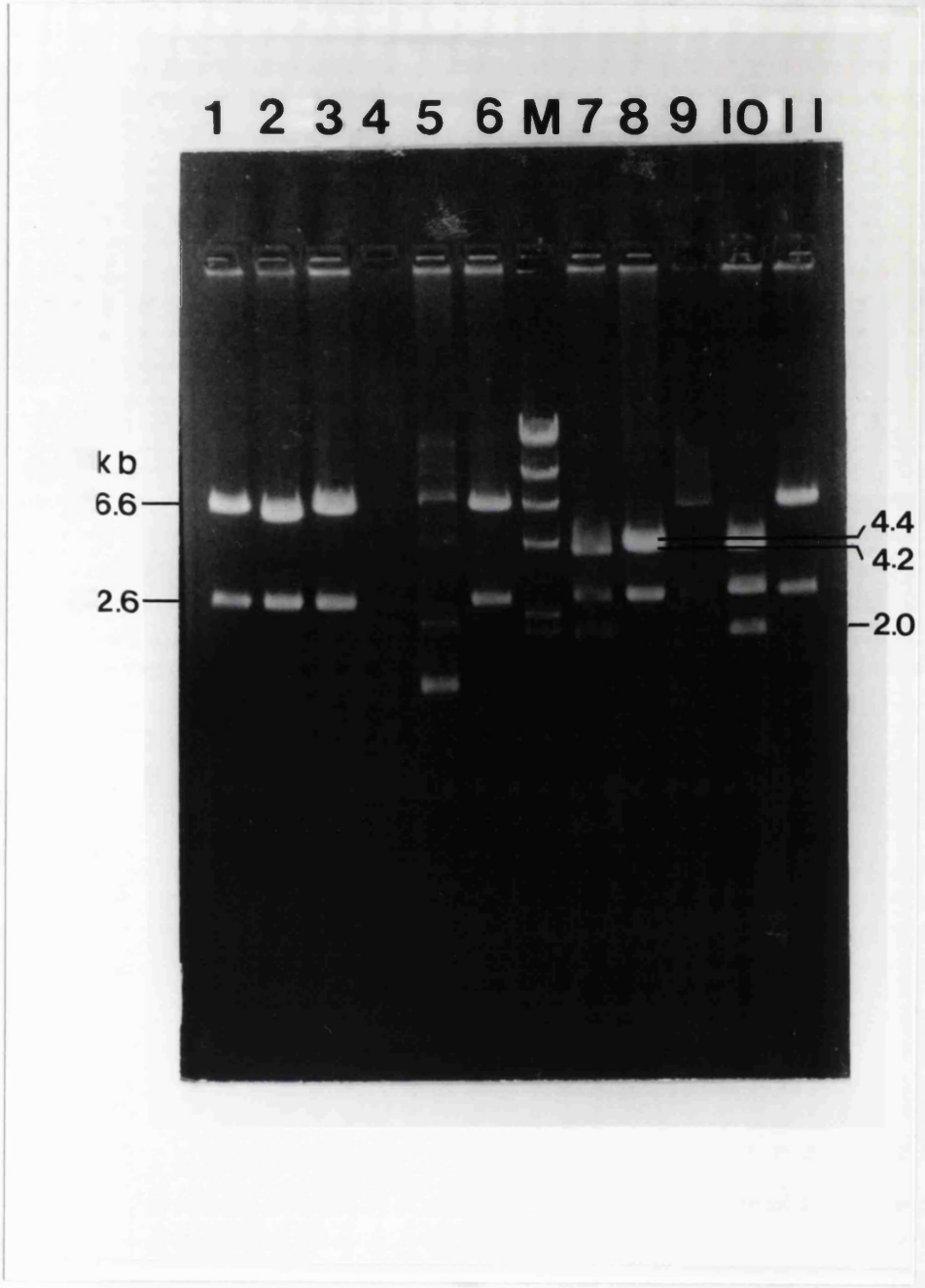


Fig. 4.4. Agarose gel electrophoresis of EcoRI-cleaved products of plasmid preparations isolated from different clones. The digestion products were resolved by electrophoresis through a TAE-buffered 0.8 % (w/v) agarose gel containing ethidium bromide. Lane M: λ DNA/Hind III size markers (from top: 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56 Kb). Lane 8 represents the desired recombinant of pAcYM1 with a 1.9 kb GLUT1 cDNA insert.

of this recombinant was further examined by restriction digestion of the plasmid with various restriction enzymes (EcoRV, HindIII, EcoRV plus EcoRI, HindIII plus EcoRI). The digests were then electrophoresed on 1 % agarose gels and visualised under U.V. light. Although EcoRI and HindIII digestions did not proceed to completion, the restriction maps confirmed that this clone was the desired recombinant, and that the GLUT1 cDNA was ligated into pAcYM1 in the correct orientation for subsequent expression (Fig. 4.5). The recombinant plasmid was designated as pAcYM1-GT (Fig. 4.6).

4.2.6. Large-scale preparation of recombinant plasmid DNA

In order to produce sufficient plasmid DNA for preparation of recombinant baculovirus, clone 8 was used to inoculate 5 ml of LB broth, which was subsequently incubated overnight at 37°C in the presence of ampicillin (100 μg/ml). The resultant suspension was used as inoculum for 500 ml of LB medium containing the same antibiotic (100 μg/ml). Amplification of the plasmid was achieved by adding chloramphenicol (final concentration 170 μg/ml) when the absorbance of the culture at 600 nm had reached 0.4, followed by incubation at 37°C overnight.

The plasmid DNA was then prepared using a Qiagen plasmid preparation kit according to the manufacturer's protocol (Diagen GmbH, Düsseldorf, Germany). Cells were first harvested by centrifugation at 8,000 x g for 10 min. The bacterial pellet was resuspended in 10 ml of a cold solution of RNase A (100 μg/ml) in 50 mM Tris/HCl, 10 mM EDTA, pH 8.0. Cell lysis was then achieved by adding 10 ml of 200 mM NaOH, 1 % SDS and incubating at room

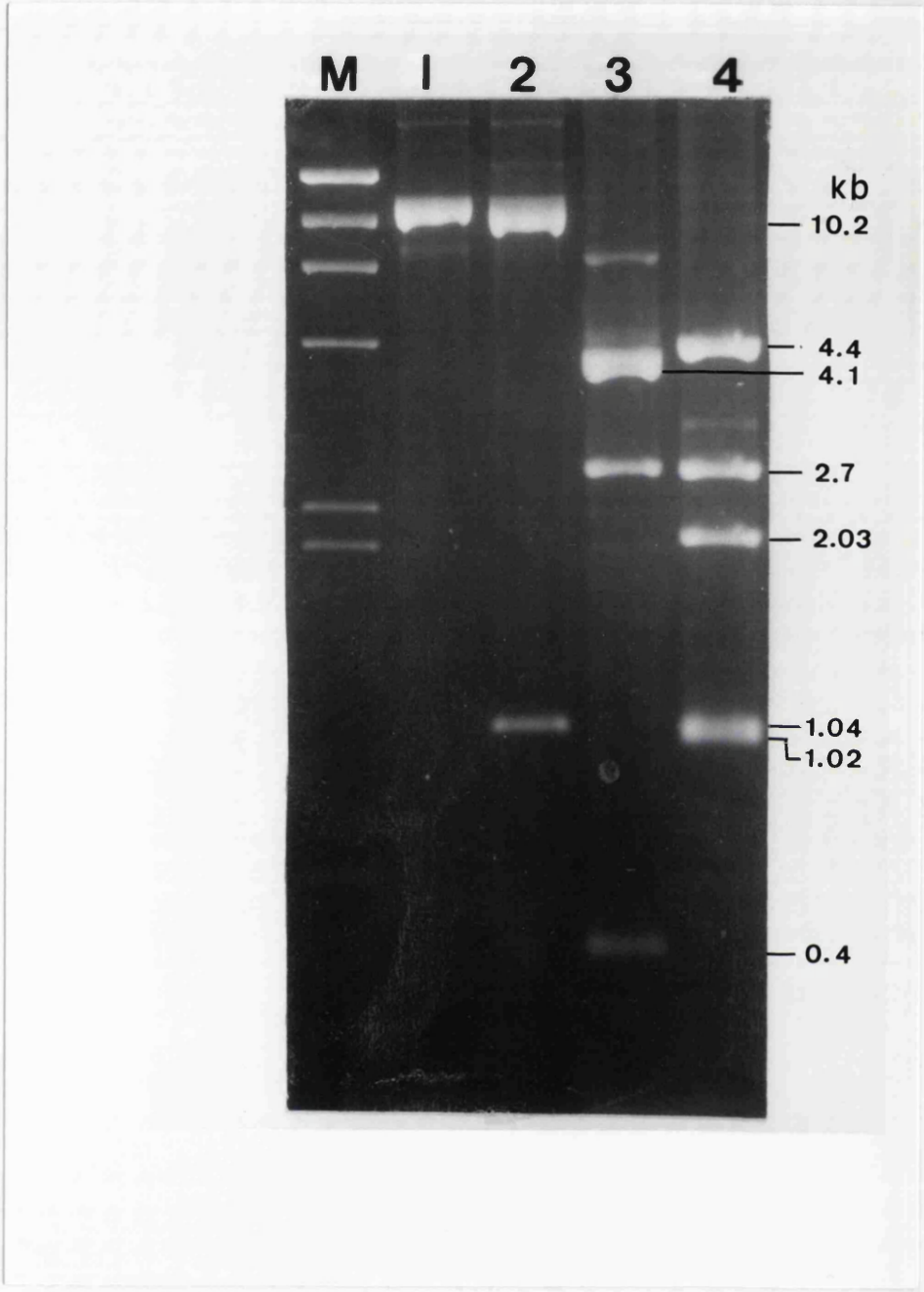
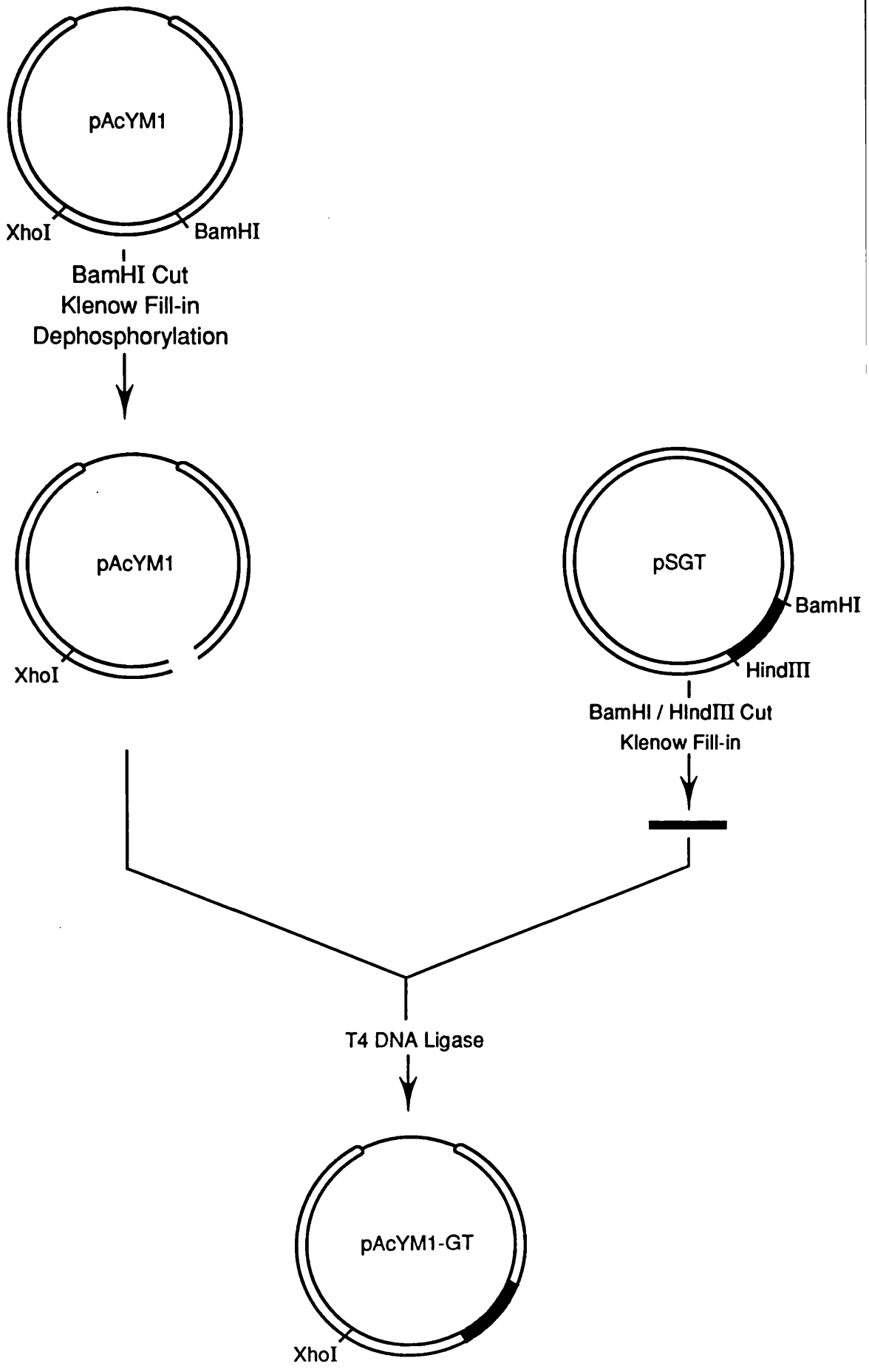


Fig. 4.5. Restriction enzymic analysis of the putative recombinant plasmid containing GLUT1 cDNA. Nucleic acid fragments were separated by electrophoresis through a TAE-buffered, ethidium bromide-stained 1 % agarose gel. All samples (except Lane M) consisted of the plasmid pAcYM1-GT incubated with restriction enzyme as indicated below: Lane 1; -EcoRV, Lane 2; -Hind III, Lane 3; -EcoRV plus EcoRI, Lane 4; -Hind III plus EcoRI. Lane M represents λ DNA digested with Hind III (from top to bottom; 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56 kb).

Fig. 4.6. A schematic diagram showing the construction of the recombinant transfer vector pAcYM1-GT.


pAcYM1
XhoI
BamHI
BamHI Cut
Klenow Fill-in
Dephosphorylation
pAcYM1
XhoI
pSGT
BamHI
HindIII
BamHI / HIndIII Cut
Klenow Fill-in
T4 DNA Ligase
pAcYM1-GT
XhoI

temperature for 5 min. The mixture was neutralised by adding 10 ml of 2.55 M potassium acetate, pH 4.8. It was then clarified by centrifugation at 4°C for 30 minutes at 22,000 x g. The supernatant was applied onto a Qiagen-tip 500 equilibrated with 10 ml of 750 mM NaCl, 50 mM 3-[N-Morpholino]propanesulfonic acid (MOPS), 15 % ethanol, pH 7.0 and was allowed to enter the resin by gravity flow. The tip was then washed with 3 x 10 ml of 1.0 M NaCl, 50 mM MOPS, 15 % ethanol, pH 7.0 before elution of DNA with 15 ml of elution buffer (1.2 M NaCl, 50 mM MOPS, 15 % ethanol, pH 8.0). Eluted DNA was precipitated with 0.7 volumes of isopropanol and then pelleted by centrifugation as described before. The pellet was washed with 70 % (v/v) ethanol, dried briefly, and redissolved in 400 μl of 10 mM Tris-HCl, 1mM EDTA, pH 8.0. The plasmid DNA was then stored at -20°C and used for cotransfection of Sf9 cells.

4.3. PREPARATION OF WILD-TYPE AcNPV DNA

Wild-type AcNPV DNA was prepared from extracellular viruses as previously described by Emery (1991). The sucrose gradient-purified virus was lysed by incubating at 60°C in lysis buffer (10 % w/v sodium *N*-lauryl sarcosinate, 10 mM EDTA). DNA was then purified by using CsCl/EtBr density gradient centrifugation as described in Section 2.5.4. Since the AcNPV genome is quite large (128 kb) and thus is sensitive to damage by shearing, vortexing was avoided during the preparation of the AcNPV DNA.

4.4. TRANSFERRING GLUT1 cDNA INTO THE BACULOVIRUS GENOME

4.4.1. Transfection of Sf9 cells

To introduce the mammalian glucose transporter gene into the AcNPV genome, the permissive insect cell line Sf9 was cotransfected with wild-type viral DNA and recombinant transfer vector DNA (pAcYM1-GT), using the calcium phosphate precipitation technique. Insertion of the GLUT1 cDNA into the baculovirus genome was then achieved via a cell-mediated homologous recombination between the flanking sequences of the vector and the wild-type viral DNA. The methods used were modifications of those previously described by Summers and Smith (1987).

Sf9 cells were seeded onto 35 mm tissue culture dishes at a density of 1.2×10^6 cells per dish as detailed in Chapter 2 and allowed to attach for at least 30 minutes. While the cells were incubating, the following constituents were mixed in an eppendorf tube: 0.475 ml of 2 x HEPES-buffered saline (40 mM HEPES pH 7.05, 2 mM Na_2HPO_4, 10 mM KCl, 280 mM NaCl), 1 μg of the purified wild-type AcNPV DNA, 12.5 or 25 μg of the purified pAcYM1-GT DNA, 0.1 ml of filter-sterilised 100 mM glucose, and sterile water to a final volume of 0.95 ml. To the above transfection mixture, 50 μl of filter-sterilised 2.5 M $CaCl_2$ was added dropwise, with vortexing. The mixture was then left at room temperature for 30 min. During this stage the $CaCl_2$ and the sodium phosphate in the medium formed a calcium phosphate which co-precipitated with the DNA, resulting in the appearance of a slight opaqueness. This precipitate facilitates subsequent adsorption of the DNA to the cells.

The TC100 medium from the Sf9 cells seeded in the dish was aspirated,

replaced with the transfection mixture containing the precipitate, and incubated further for 1 hour at room temperature. Following incubation, the transfection mixture was removed and replaced with 1 ml of complete TC100, and then incubated at 28°C in a humidified container. After 3 days, the cells were examined under an inverted light microscope. Many cells contained visible polyhedra confirming that the transfection was successful. Figure 4.7 shows a typical example of the appearance of infected cells under the microscope. The culture supernatant containing the extracellular virus was harvested by briefly centrifuging at 6,000 x g in a microcentrifuge and stored at 4°C for screening of recombinant baculoviruses.

4.4.2. Detection of recombinant baculoviruses

Recombinant baculoviruses were identified and partially purified by limiting dilution and DNA dot blot hybridisation as previously described by Fung *et al.* (1988).

4.4.2.1. Probe preparation

The 1922 bp BamHI/HindIII fragment of plasmid pSGT containing the entire coding region of GLUT1 cDNA (Section 4.2.1) was used as a probe. It was radiolabelled with ^{32}P, according to the methods described in Section 2.3.11. A mixture of all possible hexanucleotides was hybridized to the denatured cDNA and the complementary strand was synthesized from the 3'-hydroxyl termini of the hexanucleotide primers using Klenow enzyme. ^{32}P-Labelled dCTP present in the reaction mixture was thus incorporated into the

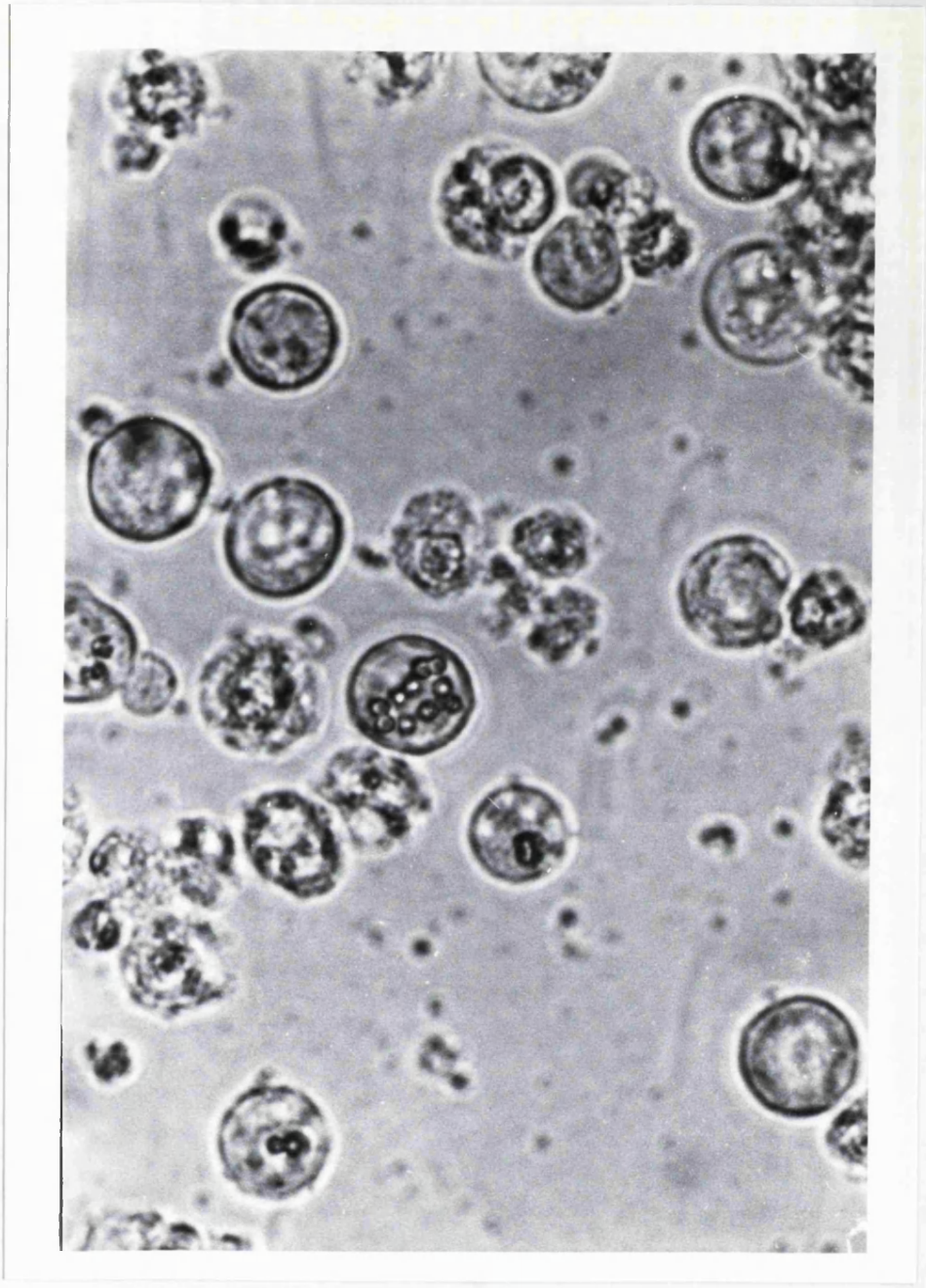


Fig. 4.7. *Spodoptera frugiperda* (Sf9) cells showing polyhedra as a result of wild-type AcNPV infection.

newly-synthesized complementary strand. The probe was stored at -20°C.

4.4.2.2. Limiting dilution and DNA dot-blot hybridisation

In order to isolate recombinant baculoviruses, limiting dilution of the virus (the supernatant from co-transfected, cultured insect cells, obtained from Section 4.4.1) was performed in conjunction with DNA dot-blot hybridisation as described in detail in Section 2.5.3. Sf9 cells were seeded into the wells of a 96 well tissue culture plate. The cells were infected with 10-fold serial dilutions of the cotransfection experiment supernatants, ranging from 10^{-1} to 10^{-8}. The plate was then incubated at 28°C for 8 days. At the end of this period, the supernatants from each well were removed and saved, and then the cells attached to the plate were lysed by mixing with 0.2 M sodium hydroxide solution. Total viral and cellular DNA obtained from the lysed, infected cells in each well was then transferred to a nylon membrane as detailed in Section 2.5.3. The immobilised DNA was prehybridised and hybridised with the ^{32}P-labelled probe representing the GLUT1 cDNA prepared from Section 4.4.2.1, according to the standard methods described in Section 2.5.3.

From two cotransfection experiments performed, positive hybridisation signals were only detected from the second experiment. In the first experiment 1 μg of AcNPV viral DNA and 12.5 μg of plasmid pAcYM1-GT DNA were used, whereas 1 μg of the viral DNA and 25 μg of the plasmid DNA were employed in the second cotransfection. Three wells (2B, 2C and 7F) yielded positive hybridisation signals, indicating that they contained cells which had been infected by recombinant viruses encoding GLUT1 (Fig. 4.8). A second round

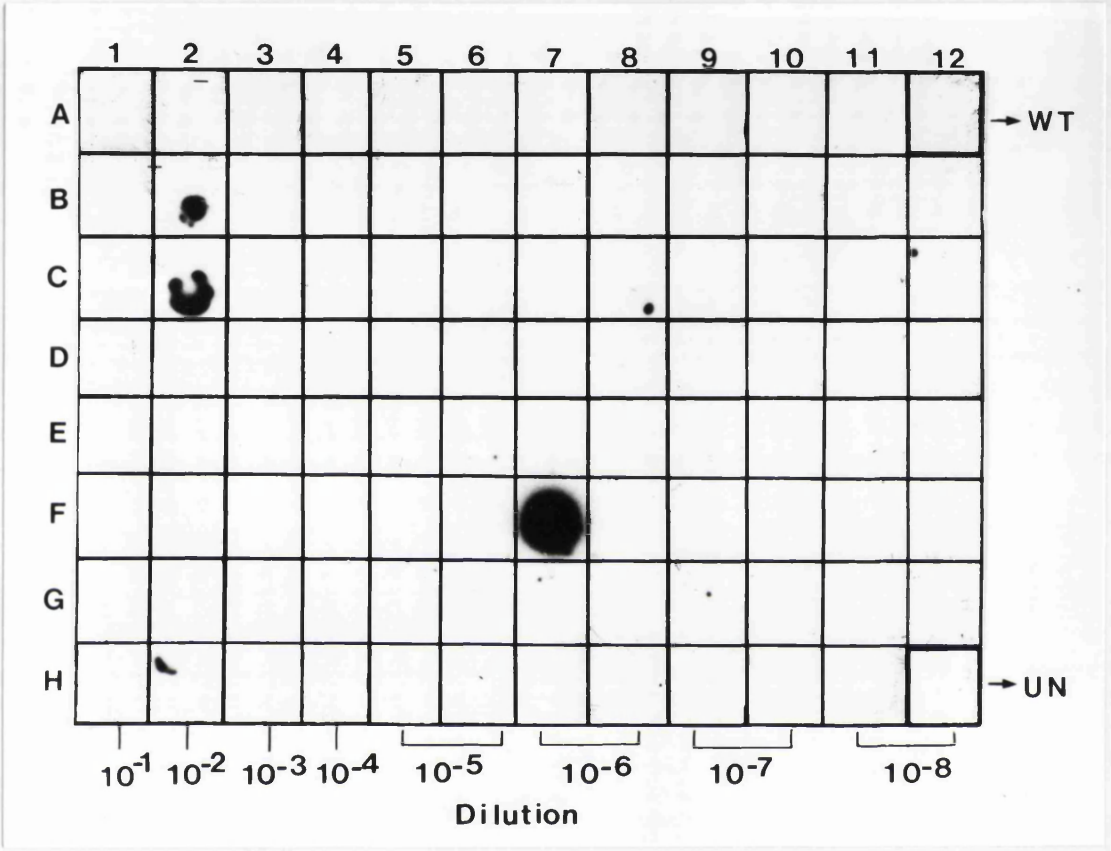


Fig. 4.8. Primary screening for the recombinant viruses by limiting dilution and DNA dot-blot hybridisation. Sf9 cells infected with dilutions of the transfection mixture ranging from 10^{-1} to 10^{-8} were lysed with 200 mM NaOH, transferred to the dot-blot apparatus and screened as described in Section 2.5.3, using ^{32}P-labelled probe representing GLUT1 cDNA. Wells 12A and 12H were infected with wild-type AcNPV and no virus, respectively.

of dilution and screening was then carried out to enrich the viral population in recombinants, starting with the viral stock from the well 7F that had yielded the strongest signal. The intensity of the positive signals yielded by wells from this round increased with dilution of the viral stock over the range from 10^{-2} to 10^{-4} dilution (Fig. 4.9). However, although it was clear that the viral populations in these positive wells were highly enriched in recombinant virus, the presence of cells with occlusion bodies indicated that there was still contamination with wild-type virus. Therefore, a third round of dilution and screening was carried out using the viral stocks from wells 9B and 12C of the second round (Fig. 4.9). These viral stocks were used at dilutions ranging from 10^{-3} to 10^{-8}. Much stronger signals were detected at higher than at lower dilutions (Fig. 4.10). Of these high dilutions, four wells (10B, 10E, 10G, and 11F) were chosen and thoroughly examined for the presence of polyhedrin using phase-contrast microscopy. They appeared to contain no wild-type virus as occlusion bodies were absent. Viral stocks from wells 10B and 11F (Fig. 4.10) were used for further studies.

4.4.2.3. Western blotting

At this stage, it was of great interest to determine whether the recombinant viruses that had been shown by DNA hybridisation to encode the human erythrocyte glucose transporter could also bring about expression of this protein in insect cells. In order to discover whether or not this was so, Western blotting was performed using the same membranes that had been used for the DNA hybridisation experiments described above.

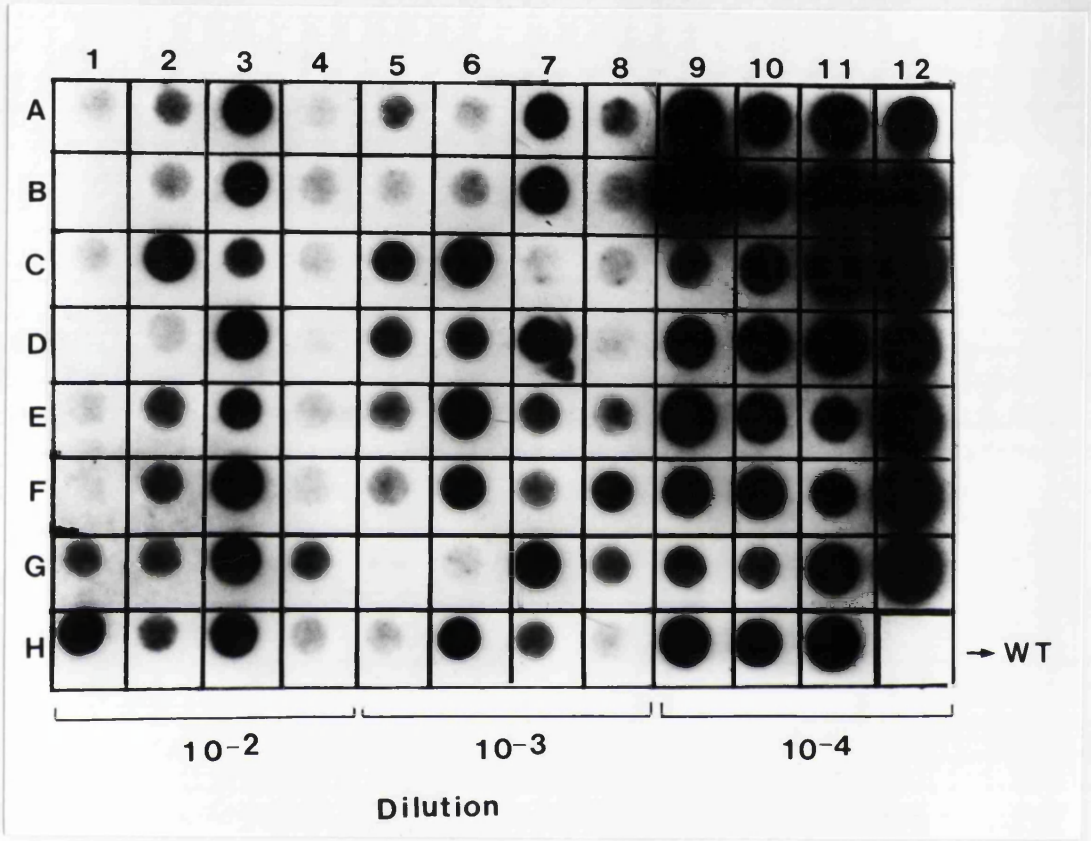


Fig.4.9. Secondary screening of the recombinant baculoviruses. The viral stock from well 7F (Fig.4.8) was diluted from 10^{-2} to 10^{-4}. Sf9 cells seeded in a 96-well plate were infected with 50 μl of virus from each dilution. Screening was carried out as described in the legend to Fig. 4.8. Well 12 H was infected with wild-type AcNPV.

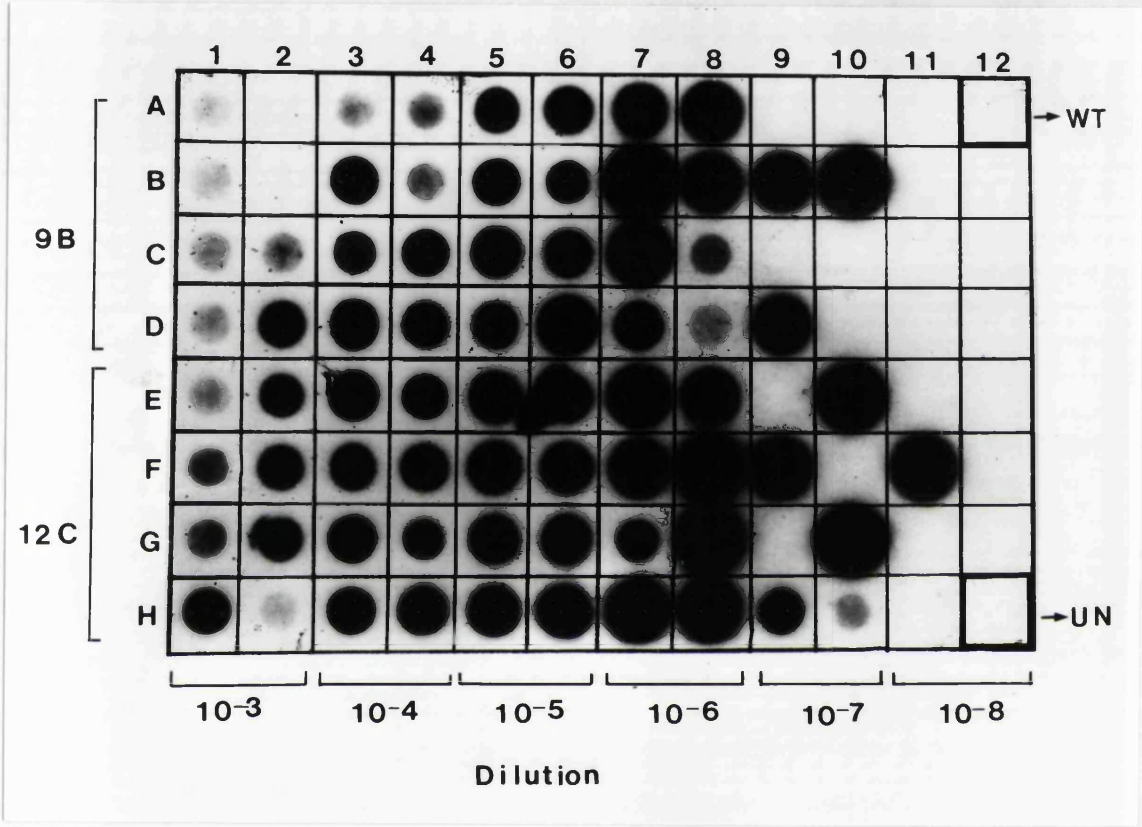


Fig. 4.10. Tertiary screening of the recombinant baculoviruses. Viral stocks of well 9B and 12C (Fig.4.9) were diluted and 50 μl of virus dilutions ranging from 10^{-3} to 10^{-8} were used to infect the cells in a 96-well plate. Screening was performed as described in the legend to Fig. 4.8. Wells 12A and 12H were infected with wild-type AcNPV and no virus, respectively.

Since nylon membrane has greater avidity for proteins than that of nitrocellulose membrane, more thorough blocking of non-specific sites was required. The nylon membranes (Section 4.4.2.2) were therefore blocked at 37°C overnight in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) containing 10 % (w/v) dried skimmed milk and 0.04% (w/v) sodium azide. The membranes were then washed in TTBS (TBS containing 0.2 % (v/v) Tween-20) for 5 minutes on an orbital shaker. Immunoblotting was then performed as described in Section 2.2.2 by using an affinity-purified antibody against the *C*-terminus of GLUT1 (Davies *et al.*, 1987) and an alkaline phosphatase conjugate of goat anti-rabbit IgG (Bio-rad). The results showed that all the wells that exhibited positive signals in the DNA hybridisation assays also exhibited positive signals when probed with antibody (Fig. 4.11). This finding indicated that not only did the recombinant viruses contain the GLUT1 cDNA sequence, but that they were also capable of directing synthesis of the human protein in insect cells.

4.4.2.4. Plaque assay

The recombinant viral stocks purified by the limiting dilution approach described above appeared to be free of wild-type virus. However, to ensure their purity, they were subjected to a final purification step by the plaque purification procedure described by Emery (1991). Plaque purification was performed using the virus stock from the well 11F (Fig. 4.10). Sf9 cells seeded into 35 x 10 mm tissue culture dishes were infected with appropriate serial dilutions of virus (10^{-1} to 10^{-4}) as described in Section 2.5.1. After allowing the virus to adsorb for 1 hour at 28°C, the inoculum was removed and the cells

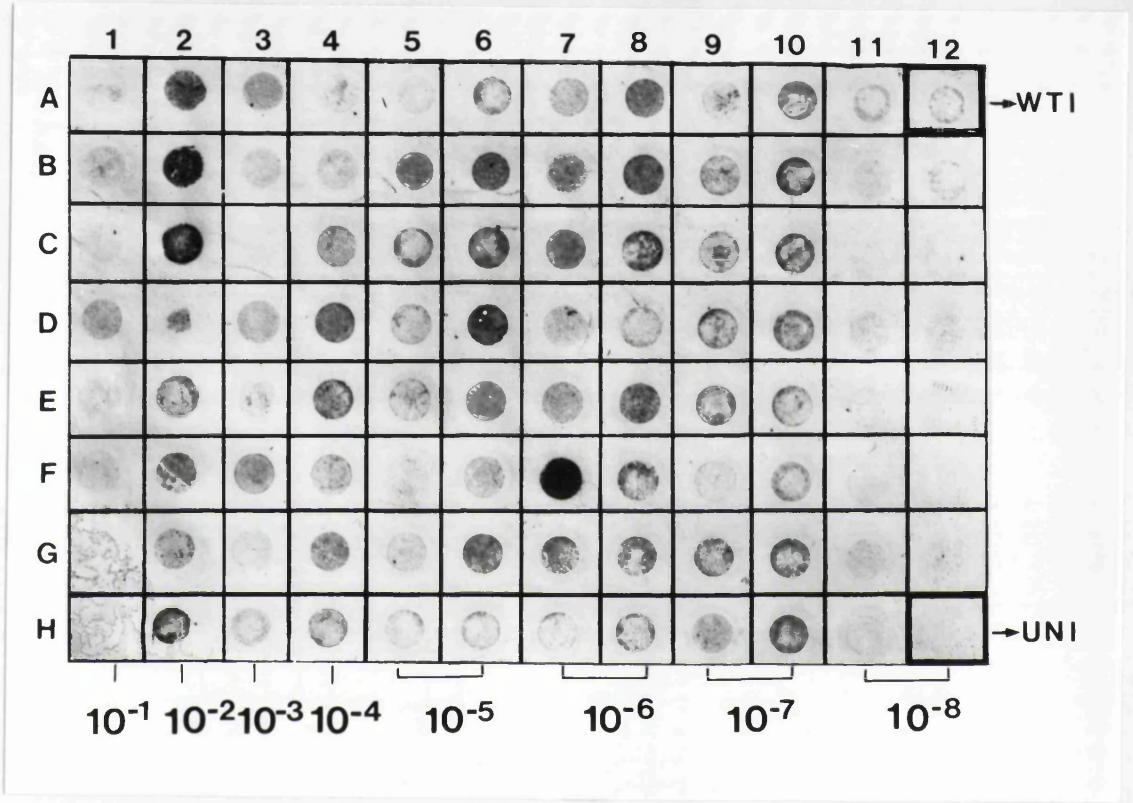


Fig. 4.11. Dot-blot analysis of GLUT1 expression in insect cells. The membrane used for the primary screening (Fig.4.8) was further subjected to Western blot analysis using an affinity-purified antibodies against the *C*-terminus of GLUT1. Bound primary antibody was then detected using an alkaline phosphatase conjugated goat anti-rabbit IgG as described in Section 2.2.2.2. The wells 2B, 2C, 7F that hybridised positively (in Fig.4.8), were the only ones to yield a strong, purple staining, as expected (The apparent staining seen in the other wells was brownish rather than purple in colour).

were then overlaid with agarose/TC 100 mixture, which limits the spread of virus particles. Following solidification, fresh TC100 medium was added and the cells were incubated in a humid condition at 28°C for 4 days.

To examine for the presence of polyhedrin, the medium from the dishes was discarded and the dishes were inverted over a light box, to which a piece of blackened X-ray film had been attached. When viewed using this means of providing contrast, wild-type AcNPV plaques appear refractile, with a white or yellow colour. Six well-separated plaques, which appeared less refractile than expected for wild-type virus, were chosen and circled with a marker pen to indicate their identity as putative recombinant viral clones. The circled plaques were then examined under an inverted phase-contrast microscope at 400 x magnification. One plaque was found to contain polyhedrin occlusion bodies. The remaining plaques, which were free of polyhedrin, were picked using sterile Pasteur pipets and ejected into 0.5 ml of complete TC100 medium. The plaques were then vortexed, allowed to diffuse for one hour at room temperature and stored at 4°C.

Selected plaques containing recombinant viruses were then subjected to two more rounds of plaque purification by visual screening for the occlusion-negative phenotype. By these means three clones of recombinant baculoviruses were isolated. One of them was designated as AcNPV-GT and served as a master stock for amplification of the virus. The propagation of the AcNPV-GT was achieved as follows: The plaque containing the virus was picked, transferred into 0.8 ml of TC100 medium and then vortexed vigorously. Subsequently, four 25 cm^2 flasks, each containing 2 x 10^6 Sf9 cells, were

infected with 0.2 ml of the virus as described in Section 2.5.1. Five days post infection the supernatants were collected and then subjected to a brief centrifugation to remove residual cells. The extracellular virus-containing supernatant was subsequently stored at 4°C. All experiments described in this thesis employed this clone, unless otherwise indicated.

4.4.2.5. Determining virus titer

Since each plaque derives from a single infectious virus particle, the concentration of infectious units (i.e. the titer) in a virus stock can be determined by counting the number of plaques formed by different dilutions of the virus on the plates and is expressed in plaque forming units per ml (pfu/ml). Titer of the AcNPV-GT virus obtained from Section 4.4.2.4 was determined by performing plaque assays as described in Chapter 2. Sf9 cells were infected with 0.1 ml of serial dilutions of the virus ranging from 10^{-1} to 10^{-8}. Duplicate dishes were used for each dilution. The titer (pfu/ml) was calculated as follows (Summers and Smith, 1987): pfu/ml = 1/dilution x number of plaques x 1/(ml inoculum/dish). As an average of 25 plaques were present on each of the plates infected with a 10^{-5} dilution, the titer of AcNPV-GT was estimated to be 2.5×10^7 pfu/ml.

4.5. DISCUSSION

An important factor affecting the expression of a foreign genes in heterologous expression systems is the nature of the promoter employed. A promoter is a short nucleotide sequence that is recognised by an RNA polymerase enzyme as a point at which to bind to DNA in order to begin transcription. Studies of promoter efficiency have shown that this varies greatly, with consequent variation in the rate of expression of the corresponding genes (Hawley and McClure, 1983). A particularly powerful promoter is the baculoviral promoter that is normally responsible for the synthesis of polyhedrin, the nonessential viral matrix protein that forms occlusion bodies in infected cells (discussed in Chapter 1.B). This promoter is tightly regulated during a baculovirus infection. It is inactive in the early stages of the infection and exhibits low activity during the late phase (i.e. 6 - 18 hours post infection) but becomes very active beginning about 20 hours p.i. By 48 hours p.i., 20 % of the total polyadenylated RNA in the cell is polyhedrin mRNA (Adang and Miller, 1982; Rohel *et al.*, 1983). For the present study it was therefore decided to place the mammalian GLUT1 cDNA under the control of the baculovirus polyhedrin promoter, in the hope of enabling large-scale expression of the transport protein in insect cells.

The large size of the AcNPV genome makes it impossible to construct a recombinant baculovirus by standard *in vitro* methods. In order to introduce the coding sequence of GLUT1 cDNA into the genome of wild-type AcNPV, a recombinant transfer vector, pAcYM1-GT, was therefore first constructed (Fig. 4.6). The transfer vector contained the entire coding region of the GLUT1

cDNA, flanked by 15 nucleotides of the 5′ untranslated leader and 431 nucleotides of the 3′ untranslated region, under the control of the strong polyhedrin promoter.

Although the factors determining the level of expression of a foreign gene in the baculovirus expression system are not yet well characterised, one factor that affects the efficiency of the production of foreign gene products is the length and nature of the leader sequence preceding the foreign gene (Matsuura *et al.*, 1987; Horiuchi *et al.*, 1987). Matsuura *et al.* (1987) examined the effect of the integrity of the polyhedrin leader sequence by deletion experiments. A series of AcNPV expression vectors carrying either the influenza virus haemagglutinin (HA) gene or lymphocytic choriomeningitis virus (LCMV) gene in lieu of the polyhedrin gene were created such that the lengths of the polyhedrin leader sequence differed. Highest levels of expression for HA protein were observed for vectors in which no more than 14 bases had been deleted from the leader sequence immediately upstream from the polyhedrin ATG initiation codon. A significant reduction in HA activity was observed in the recombinants lacking between 16 and 27 bases immediately upstream from the polyhedrin ATG. Furthermore, the highest levels of expression of LCMV genes were obtained using recombinant viruses derived from the transfer vector pAcYM1, which contains the entire 5′ leader sequence including the 'A' of the original polyhedrin ATG. It is therefore suggested that the sequences immediate upstream from and possibly including the polyhedrin ATG codon are important for the high-level expression of foreign genes. The importance of the corresponding sequence of BmNPV was also demonstrated by Horiuchi *et al.*

(1987). By contrast, none of the coding sequence of the polyhedrin gene or of the 13 bases immediately downstream of this coding region appeared to be required for high level expression of a foreign protein (Matsuura *et al.*, 1987). Because of these considerations, the transfer vector pAcYM1 was chosen for the present experiments.

Sequence analysis of the polyhedrin gene of AcNPV has revealed that the polyhedrin leader is very AT-rich (Hooft van Iddekinge *et al.*, 1983). Therefore it has been suggested that the presence of GC-rich sequences in the leader of the foreign gene to be expressed should be minimised, if possible, to optimise expression. For example, Pendergast *et al.* (1989) reported that the deletion of GC-rich 5′ untranslated sequences significantly improved the level of expression of the foreign gene. Furthermore, since the size of the polyhedrin promoter appears to be rather small, encompassing the region from -1 to -60 from the translation start site of polyhedrin (Hooft van Iddekinge *et al.*, 1983) and the effect of addition of nucleotides to the leader sequence on the expression of foreign genes is not yet known, it is generally recommended that the length of the 5′ untranslated leader sequences from a foreign gene to be cloned should be minimised whatever their base composition. For the work described in this chapter, the plasmid pSGT was chosen as the source of GLUT1 cDNA because in this plasmid most of the 3′ untranslated region of the native GLUT1 mRNA has been deleted.

In the present study recombinant baculoviruses were generated by introducing DNA from the recombinant transfer vector pAcYM1-GT into the permissive cell line Sf9 together with the wild-type AcNPV viral DNA by calcium

phosphate transfection (Summers and Smith, 1987). The AcNPV sequences present in the transfer vector underwent homologous recombination with the wild-type AcNPV genomic DNA and gave rise to recombinant AcNPV-GT baculoviruses. The frequency of recombination is known to be very low, in the order of 0.1 % - 5 % (Summers and Smiths, 1987), and so it was necessary to purify the recombinant virus from a background of a large excess of wild-type virus. Since Smith *et al.* (1983) first introduced a visual screening method based on plaque phenotype for identifying the recombinant baculovirus, this method has been used by many workers. It is based on the fact that viruses lacking a polyhedrin gene form occlusion body-negative plaques, whereas wild-type AcNPV produces occlusion body-positive plaques. Although this approach to detecting recombinants is rapid, it is tedious and can present difficulties to inexperienced workers. In addition, "few polyhedra" (FP) mutants of wild-type AcNPV produce a plaque phenotype containing only a few polyhedral occlusion bodies in infected cells and so may be mistaken as recombinant viruses during plaque assay (Frazer *et al.*, 1983). In order to avoid these difficulties, Pennock et al. (1984) described the use of "wild-type" virus encoding the enzyme β-galactosidase. These virus form blue plaques in the presence of an appropriate chromogenic indicator. However, in recombinant virus the β-galactosidase gene is replaced by the foreign gene, and so colourless plaques are formed in infected cells, thus providing a means of visual screening. The disadvantage of this method is that it requires a particular parent virus and transfer vector.

An alternative way for detection of recombinants is to use plaque hybridisation or DNA dot-blot hybridisation (Summers and Smith, 1987; Jeang

et al., 1987). In the plaque hybridisation procedure, the agarose overlay from a plaque purification step is removed and saved and the cells blotted onto a membrane. The immobilised DNA on the membrane is hybridised with a suitable probe. Positive signals are lined up with the agarose overlay and the putative recombinant-containing agarose plugs are picked and subjected to further screening and purification. The plaque hybridisation method may be used with any transfer vector. However, the major drawback is that it is frequently difficult to achieve any effective purification because the cells tend to smear during blotting, enlarging the apparent diameter of the plaque. As a result, larger plugs of agarose have to be picked unnecessarily. In contrast, the dot-hybridisation procedure depends on the quality and intensity of the hybridisation signal. A positive signal only indicates the presence of the foreign gene in the baculovirus genome but does not reflect the contamination with wild-type virus. Therefore, the occlusion-negative plaques that might be contaminated with wild-type virus would not be detected by using the dot-blot hybridisation procedure alone. To overcome these problems, Fung *et al.* (1988) suggested a method which combines limiting dilution and DNA dot-blot hybridisation. This approach allows the detection and purification of a recombinant virus directly from transfection mixtures of wild type and recombinant virus.

Recombinant baculoviruses can also be identified by immunological means (Domingo and Trowbridge, 1988; Capone, 1989). However, this approach depends on the availability of specific antibody probes. Recently, the polymerase chain reaction (PCR) procedure, that has proven to be extremely

useful for the amplification of specific fragments of DNAs (Saiki *et al.*, 1985), has also been introduced as a method to screen for recombinant viruses (Malitschek and Schartl, 1991; Webb *et al.*, 1991). This technique may be used with any transfer vector provided that the appropriate primers are used. It allows the rapid identification of recombinants and can differentiate them from contaminating wild type virus. However, contamination of the PCR reaction often causes problems and generates false signals. If a small amount of amplifiable template is present in the reaction, it may result in artifactual amplified products. Therefore, extreme care to avoid contamination is required if this method is employed.

In the present study a combination of methods was employed. Recombinant virus was first identified and partially purified by limiting dilution of the transfection mixture, combined with DNA dot-blot hybridisation using a GLUT1-specific probe. Of the 10^6 fold-dilutions of the transfection supernatant screened, one out of 16 wells showed a very strong positive signal (Fig. 4.6). In order to obtain the fully-purified recombinant baculovirus AcNPV-GT, a further two rounds of the limiting dilution procedure (Fung *et al.*, 1988) were followed by three rounds of visual screening using plaque phenotype (Summers and Smiths, 1987). The fact that the recombinant virus encoded the human erythrocyte glucose transporter was then confirmed by Western blotting techniques using anti-peptide antibodies directed against the *C*-terminus of GLUT1 (Davies *et al.*, 1987).

CHAPTER 5. PRODUCTION AND CHARACTERIZATION OF HUMAN GLUCOSE TRANSPORTER EXPRESSED IN INSECT CELLS

5.1. INTRODUCTION

One of the major reasons for attempting the expression of a protein in a heterologous system that in its natural state the protein is in limited supply, either because it is normally expressed in trace amounts, because its natural source is of limited availability, or because its purification presents other practical problems. In addition, the ability to modify the protein by genetic means before expression can be of great utility. Up till the present the bacterium *Escherichia coli* has been the most commonly used prokaryotic host system for foreign gene expression. The advantages of this system are the ease of growing bacteria on a large scale, and the relative cheapness of the materials required for such growth. These bacteria by now have a well-proven ability for the high level expression of many prokaryote and eukaryote proteins. The wealth of genetic information on *E. coli* also renders it a good host for genetic manipulation experiments. Sugar transport proteins are physiologically and clinically important in humans but their low natural abundance in cell membranes renders their isolation and study difficult. Thus, their production in large amounts by genetic means has been an important goal of many research groups throughout the world. Expression of the GLUT1 and GLUT2 isoforms of the mammalian passive glucose transporter family in *E. coli* was reported several years ago by the groups of Kaback and Lodish (Sarkar *et al.*, 1988; Thorens *et al.*, 1988). However, use of this expression system yielded only

very small amounts of functional protein, and other researchers have subsequently been unable to reproduce even these results.

Functional expression of a foreign gene requires that the gene be both transcribed and translated. In many cases, post-translational modifications and compartmentalization of the nascent polypeptide are also required. A failure to perform correctly any one of these processes can result in the failure of a gene to be expressed. Despite the recent development of a wide variety of prokaryotic expression vectors, there are still numerous difficulties associated with the production of protein from eukaryotic genes cloned into *E. coli*. The particular problems are as follows: firstly, expression signals are different in different organisms. Hence, the bacterial RNA polymerase may not recognize the eukaryotic promoter. The mRNA transcribed from the eukaryotic genes also lacks a specific nucleotide sequence needed for binding to bacterial ribosomes. Secondly, most eukaryotic genes contain introns that must be excised from the initial transcript in order to produce the mature, translatable mRNA molecule. The necessary processing machinery is absent from bacteria. Thirdly, post-translational processing such as glycosylation, proteolysis, phosphorylation, accurate disulphide bond formation and oligomerisation, do not occur properly in prokaryotic systems due to the lack of the necessary enzymatic machinery. Lastly, the expressed eukaryotic proteins are often recognized by bacterial proteases as foreign and are cleaved. To overcome these problems, considerable efforts have been made to develop systems to express eukaryotic proteins in a eukaryotic environment. Examples of such expression systems are yeast (Gunge, 1983), insect (Summers and Smith, 1987), and cultured

mammalian cells (Gorman, 1985) as well as in whole organisms by the generation of transgenics (Palmiter *et al.*, 1982).

Recently, successful expression has been reported for several mammalian glucose transporter isoforms in a variety of eukaryotic expression systems. These systems have included *Xenopus* oocytes [GLUT1-5 (Birnbaum, 1989; Gould and Lienhard, 1989; Keller *et al.*, 1989; Vera and Rosen, 1989; Permutt *et al.*, 1989; Kayano *et al.*, 1990; Gould *et al.*, 1991)], mammalian cells [GLUT1 (Gould *et al.*, 1989; Asano *et al.*, 1989b; Harrison *et al.*, 1990a,b); GLUT3 (Asano *et al.*, 1992)] and transgenic mice [GLUT4 (Liu *et al.*, 1992)]. However, for detailed studies of structure and function relationships in these proteins, and in particular for the investigation of their structures by crystallization, high levels of expression are a prerequisite. Recently, baculovirus expression of foreign genes has gained wide popularity for the study of a large variety of proteins from numerous organisms (reviewed in Luckow and Summers, 1988). Expression of proteins in this system has several advantages over either prokaryotic or transfection-based eukaryotic expression systems (discussed in Chapter 1). The most attractive is the ability of the system to produce biologically active proteins in substantial amounts. I therefore chose the baculovirus system for my attempt at expression of large amounts of the human erythrocyte-type glucose transporter isoform, GLUT1.

5.2. PRODUCTION AND CHARACTERIZATION OF HUMAN GLUCOSE TRANSPORTER EXPRESSED IN INSECT CELLS

5.2.1. Immunological characterization of the expressed protein

The preliminary dot-blot experiments described in Chapter 4 had shown that insect cells infected with the recombinant baculovirus AcNPV-GT contained a protein(s) recognised by antibodies against human GLUT1. In order further to characterize this putative glucose transporter, and to establish its location within the insect cell, Western blotting experiments were carried out, as described in Section 2.2.2. Sf9 cells were seeded and infected with recombinant virus AcNPV-GT, wild type AcNPV or no virus, as described in Section 2.5.1. Virus was added to a multiplicity of infection (MOI) of 5. Four days post infection the cells were harvested in Dulbecco's phosphate buffered-saline (PBSA) and washed twice with PBSA. The cells were resuspended in ice-cold Tris/magnesium buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4) containing protease inhibitors (2 mM iodoacetamide, 0.2 mM PMSF, 10 μg/ml pepstatin), and then disrupted by sonication as described in Section 2.2.7. The broken cells were pelleted by centrifugation at 400 x g for 10 min at 4°C. The pellet containing cell debris as well as nuclei was discarded. The remaining supernatant was then fractionated by centrifugation at 300,000 x g for 30 min at 4°C. The resultant pellet was resuspended in Tris/magnesium buffer and considered as the crude membrane fraction, the supernatant as the soluble fraction.

The membrane and soluble fractions derived from equal numbers of cells

(2×10^6) were electrophoresed on an SDS/10 % polyacrylamide gel, electrophoretically transferred to nitrocellulose paper, and immunolabelled with an affinity-purified antibody against the *C*-terminus of GLUT1. The immunoreactive bands were then detected with alkaline phosphatase conjugated goat anti-rabbit IgG. As presented in Figure 5.1, a single fairly sharp immunoreactive band of apparent M_r 45,000 was detected only in the cells infected with the recombinant virus containing GLUT1 cDNA (lane E). In comparison, a sample of alkali-stripped human erythrocyte membranes electrophoresed on the same gel yielded a broad band of staining, corresponding to an apparent M_r 45,000 - 65,000 (lane G), as expected for the natural erythrocyte glucose transporter (Gorga and Lienhard, 1981). However, such bands were not detected when pre-immune serum was used, giving evidence for the specificity of the antibodies (data not shown). The expressed protein was present in the membrane fractions of the insect cell but was absent from the soluble fraction (lanes E and F, Fig. 5.1). No immunostaining was observed in cells infected with either the wild type AcNPV (lanes C and D) or no virus (lanes A and B).

The results described above indicated that a protein of approximately the expected size, and which cross-reacted with antibodies against the *C*-terminus of the human erythrocyte glucose transporter, was expressed insect cells infected with the recombinant baculovirus AcNPV-GT. However, the electrophoretic mobility of the expressed protein was substantially greater than the average value for the natural erythrocyte protein. In order to determine whether the transporter expressed in AcNPV-GT-infected Sf9 cells

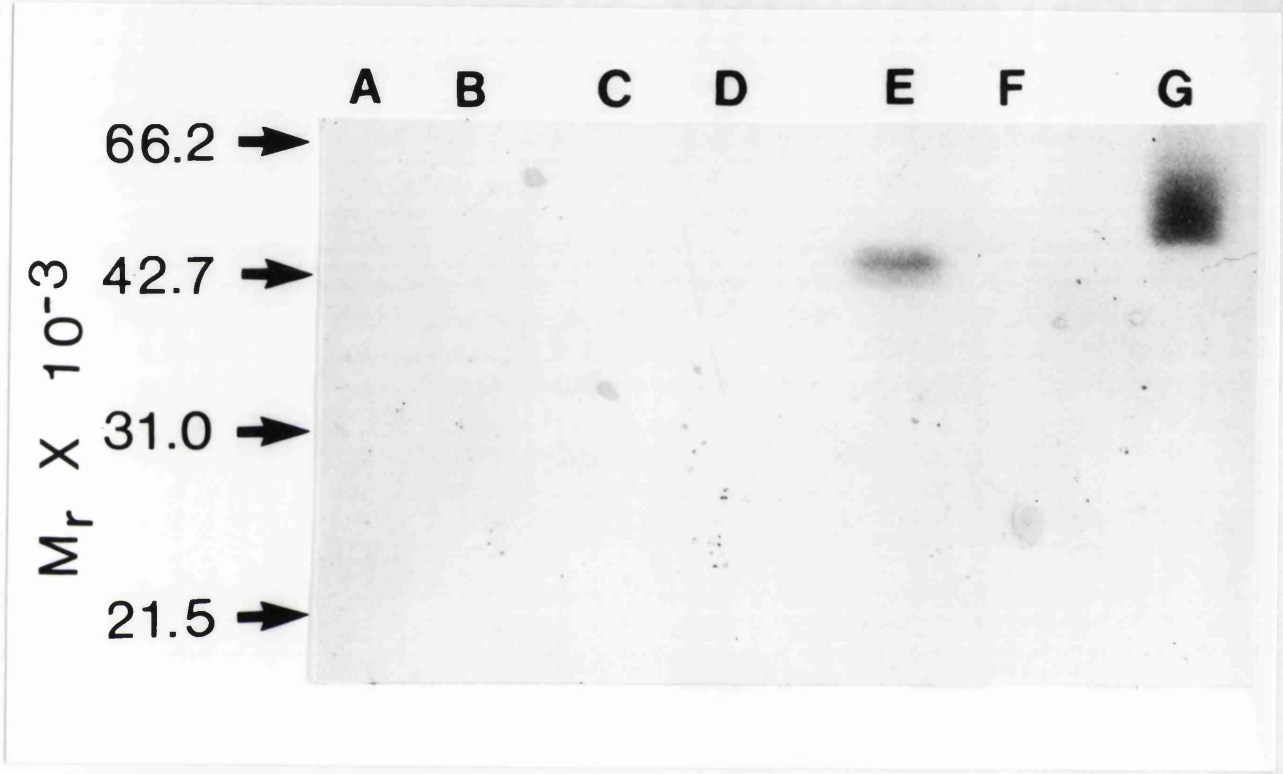


Fig. 5.1. Western blot analysis of GLUT1 expression in insect cells. Cells were grown in the absence of virus (lanes A & B), infected with wild-type AcNPV (C &D) or with AcNPV-GT (E & F). After 4 days they were harvested, sonicated and then separated into membranous (A, C and E) and soluble (B, D and F) fractions. Samples derived from equal numbers of cells (2 x 10^6 cells) were then electrophoresed on a 10% SDS/polyacrylamide gel and subjected to Western blotting using affinity-purified antibodies against the *C*-terminus of GLUT1, and alkaline phosphatase conjugated goat anti-rabbit IgG as described in Section 2.2.2.2. G = human erythrocyte membrane control. The positions of proteins used as M_r markers are indicated.

corresponded to a full-length version of the GLUT1 protein, and that no frame-shifts had occurred during construction of the recombinant virus, the cross-reactivity of the protein towards a range of polyclonal, site-directed antibodies (Davies *et al.*, 1987, 1990) was examined by Western blotting. For these experiments alkaline-phosphatase conjugates of goat anti-rabbit IgG, and a chromogenic substrate, were employed to detect bound rabbit IgG, as described in Section 2.2.2.2. Antisera raised against intact GLUT1 (Fig. 5.2, lane B, residues 1 - 492), against the *C*-terminal peptide (lane C, residues 477 - 492), the *N*-terminal peptide (lane E, residues 1 - 15) and against the central cytoplasmic loop of GLUT1 (lane D, residues 240 - 255), all recognised the recombinant protein on Western blots. However, no immunostaining was seen with pre-immune serum (lane A). These results suggested that despite its greater than expected mobility on SDS/polyacrylamide gels the recombinant transporter expressed in insect cells corresponded to a full-length form of GLUT1.

5.2.2. Endoglycosidase F treatment

A possible explanation for the unexpected electrophoretic mobility of the expressed GLUT1 in insect cells might be a difference in its glycosylation state, compared to the natural erythrocyte protein. Therefore experiments were performed to ascertain whether the protein expressed in insect cells was being post-translationally modified by the addition of an *N*-linked carbohydrate moiety, as occurs in mammalian tissues. To this end, membranes were prepared from Sf9 cells 4 days post-infection with recombinant AcNPV-GT, as described in

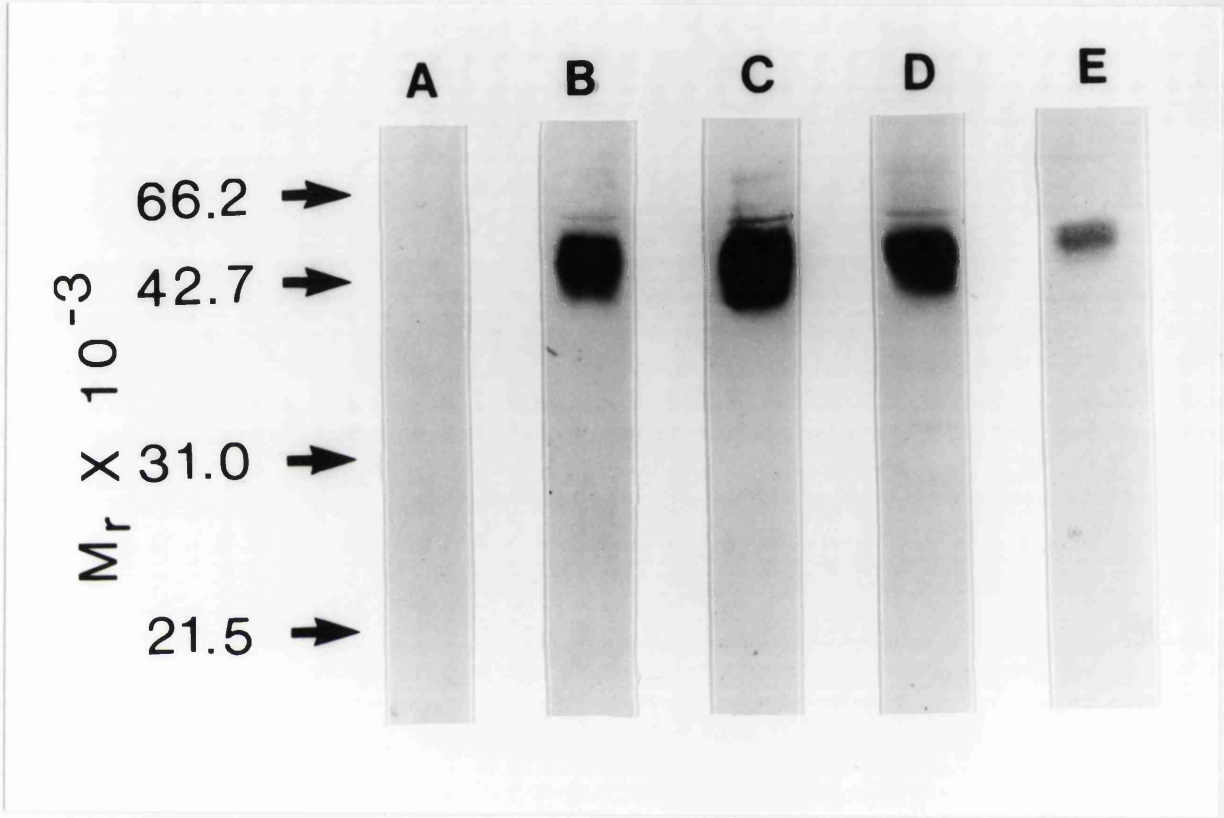


Fig. 5.2. Cross-reaction of the protein expressed in insect cells with a range of polyclonal antibodies against human GLUT1. Samples (5 μg) of AcNPV-GT-infected cell membranes were electrophoresed on an SDS/10%-polyacrylamide gel and subjected to Western blotting using anti-transporter antibodies and alkaline phosphatase-conjugated goat anti-rabbit IgG as described in Section 2.2.2.2. The primary antisera used were: Lane A (pre-immune serum), B (anti-intact GLUT1), C (anti-C-terminus of GLUT1, residues 477-492), D (anti-middle loop of GLUT1, residues 240-255), E (anti-N-terminus of GLUT1, residues 1-15).

Section 2.2.7. Prior to digestion with endoglycosidase, 20 μg of the insect cell membranes and, for comparison, of protein-depleted human erythrocyte membranes (Gorga and Lienhard, 1981), were dissolved in 100 μl of digestion buffer by vortexing (100 mM sodium phosphate, 50 mM EDTA, 75 mM 2-mercaptoethanol, 0.05 % (w/v) SDS, 1 % Triton X-100, 0.1 mM PMSF, pH 6.1). Samples were then incubated with 5 μl of water or with 5 μl (0.25 units) of endoglycosidase F (Boehringer), an enzyme which catalyses the hydrolysis of the glycosidic bond between two *N*-acetylglucosamine residues linked to asparagine in glycoproteins with both high mannose and complex carbohydrates (Elder and Alexander, 1982). After incubating at 22°C for 18 hours, the reaction was terminated by denaturing the proteins with a final concentration of 3 % SDS in preparation for polyacrylamide-gel electrophoresis. Subsequently samples (2 μg) were run on a 12 % polyacrylamide gel (Laemmli, 1970). Following electrophoresis, polypeptides were electrophoretically transferred to nitrocellulose and stained with affinity-purified antibodies against the *C*-terminus of GLUT1. The bound primary antibody was detected with an ^{125}I-labelled $F(AB')_2$ fragment of donkey anti-rabbit IgG, as described in Section 2.2.2.2.

As shown in Figure 5.3 (lane D), the native erythrocyte glucose transporter migrates on SDS-polyacrylamide gels as a broad band of apparent M_r 45,000 - 65,000. This behaviour is known to result from heterogeneity in the oligosaccharide chains attached to Asn_{45} of the protein (Sogin and Hinkle, 1978; Gorga *et al.*, 1979; Lienhard *et al.*, 1984). However, as previously reported by Lienhard *et al.* (1984), treatment with endoglycosidase F converted the transporter to a species that migrated as a sharp band of apparent M_r

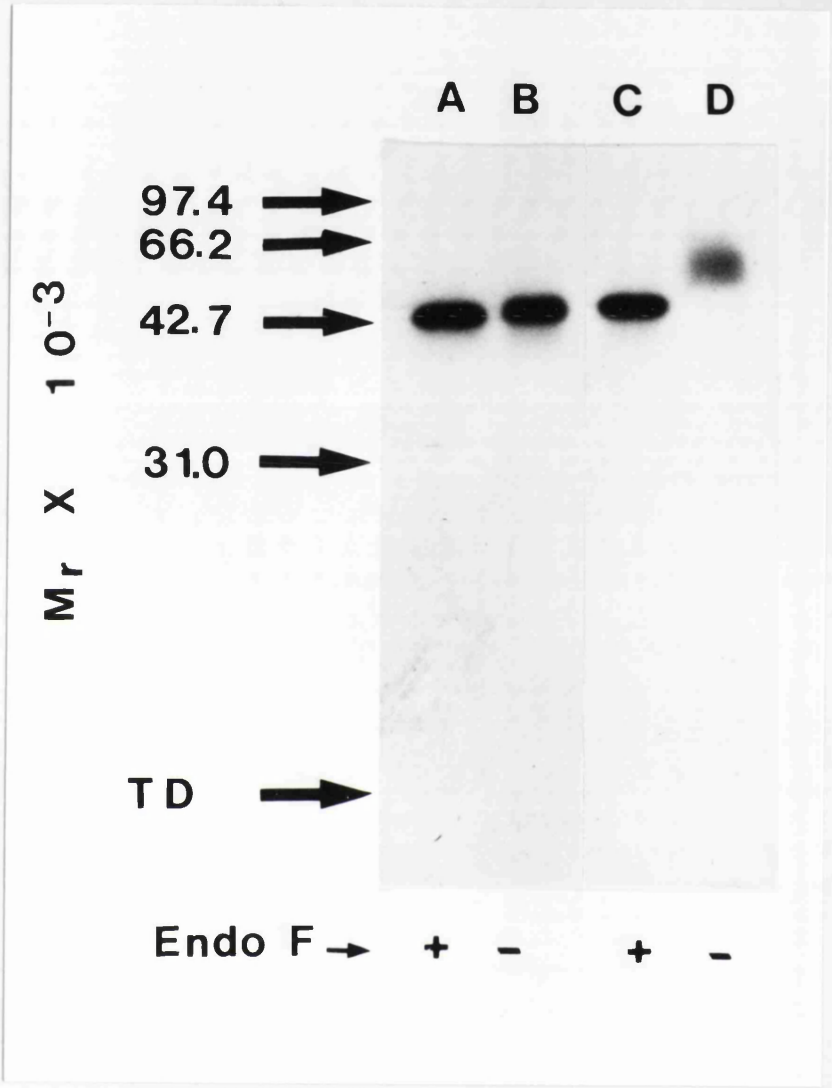


Fig. 5.3. Endoglycosidase F treatment of the glucose transporter expressed in Sf9 cells. Samples (20μg) of insect cell membranes (A and B) and of alkali-stripped human erythrocyte membranes (C and D) were incubated with (+) or without (−) 0.25 units endoglycosidase F for 18h at 25°C. Samples (2μg) were then electrophoresed on an SDS/12% polyacrylamide gel and subjected to Western blotting using anti-transporter antibodies as described in Section 5.2.2. The positions of proteins used as M_r markers are indicated.

46,000 on an SDS-polyacrylamide gel, indicative of its complete deglycosylation (lane C). By contrast, the transporter expressed in insect cells migrated as a single, fairly sharp band of apparent M_r 45,000 (lane B), almost identical to that of its deglycosylated erythrocyte counterpart. This observation suggests that the recombinant transporter is much less heavily and heterogeneously glycosylated than the erythrocyte protein. Nonetheless, treatment with endoglycosidase F did result in a slight but reproducible increase in the electrophoretic mobility of the protein on SDS-polyacrylamide gels, indicating that it was *N*-glycosylated by the insect cell glycosylation machinery, presumably at Asn_{45} (lane A).

5.2.3. Tryptic digestion of GLUT1 expressed in insect cells

The number of sites at which a protein can be readily cleaved by a proteolytic enzyme is greatly influenced by its three-dimensional structure: for native, properly-folded proteins both the rate of cleavage and number of sites at which cleavage takes place are usually much less than for the denatured protein. In order to compare the tertiary structure of the recombinant GLUT1 with that of its native counterpart in the erythrocyte, the pattern of tryptic cleavage of the protein expressed in insect cell membranes was therefore examined. Tryptic digestion was carried out as described in Section 2.2.4. Membranes were prepared from Sf9 cells infected with the recombinant baculovirus AcNPV-GT (MOI = 5) 4 days post infection (Section 2.2.7). The membranes were then digested with trypsin at 25°C. Samples (10 μg) were analysed by SDS/polyacrylamide gel electrophoresis and Western blotting

(Section 2.2.2), using affinity-purified antibody against intact GLUT1 and ^{125}I-labelled donkey anti-rabbit IgG. Figure 5.4 shows the fragments of the glucose transporter, expressed in insect cells, generated by limited tryptic digestion over a time course of up to 4 hours. Shown in lane A is a band of apparent M_r 45,000 corresponding to the intact, expressed glucose transporter. After 30 min digestion, a fragment of approximate M_r 19,000 - 21,000 was generated. In addition to this, there were two less intensely stained fragments of apparent M_r 28,000 and 17,000. The pattern of labelling was similar up to 2 hours of digestion (Lanes B - D). However, the fragments of M_r 19,000 - 21,000 and M_r 17,000 were no longer detectable after 4 hours digestion (lane E).

Trypsin is known to cleave the human erythrocyte glucose transporter only at the cytoplasmic surface of the membrane (Lienhard *et al.*, 1984; Cairns *et al.*, 1984). Cleavage occurs at several sites in the hydrophilic central loop of the sequence between Arg_{212} and Arg_{269}, and in the *C*-terminal region of the sequence, following Lys_{456} (Cairns *et al.*, 1987). After short periods of tryptic digestion the protein is therefore cleaved primarily into two large, membrane-bound fragments. The *N*-terminal half of the protein, comprising residues 1 - 212, migrates as a broad, poorly staining band of apparent M_r 23,000 - 42,000 on SDS/polyacrylamide gels due to heterogeneous glycosylation. It does not contain any epitopes recognised by the anti-transporter antibodies (Davies *et al.*, 1987) and so would not have been detectable in the present Western blotting experiments. However, tryptic fragments derived from the *C*-terminal half of the protein are recognised by the anti-transporter antibodies. After very short times of digestion the erythrocyte protein preferentially yields such a *C*-

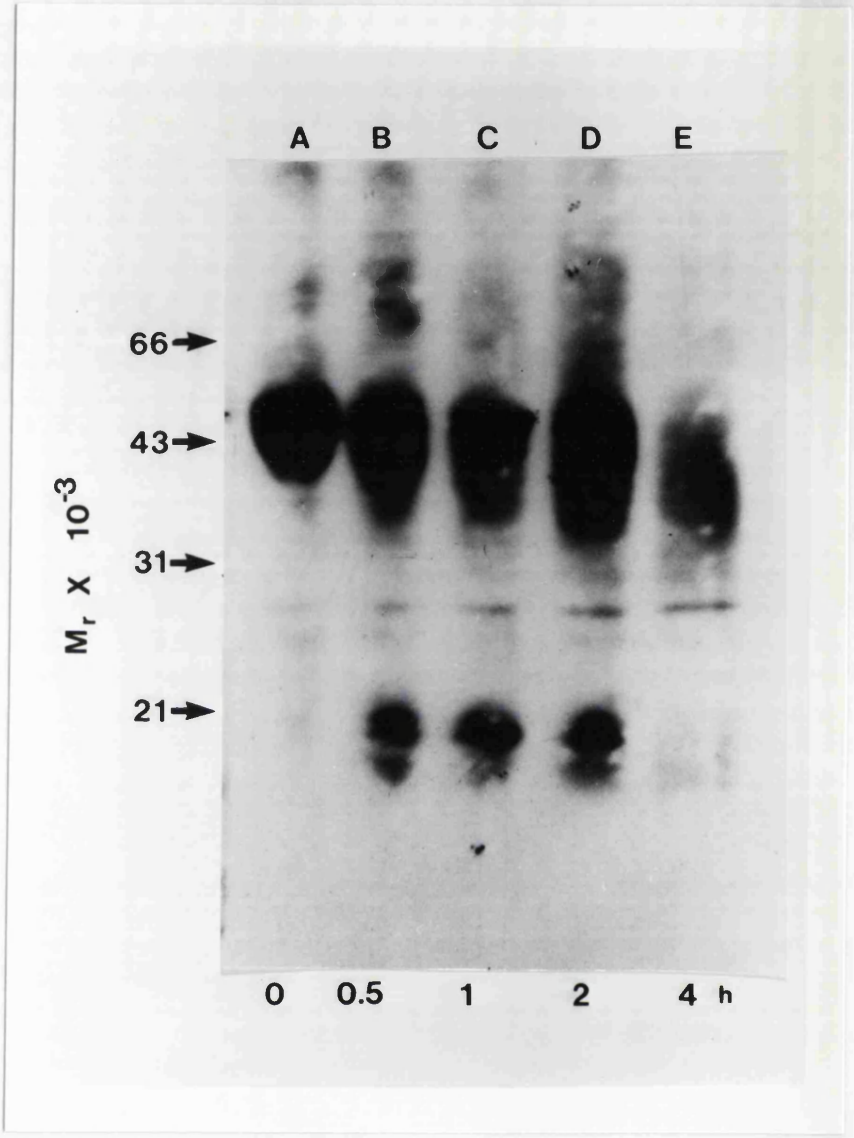


Fig. 5.4. Proteolytic fragmentation pattern of human GLUT1 expressed in insect cells. Samples (10 μg) of insect cell membranes digested with trypsin (2.5 μg/ml) for the times indicated were electrophoresed on a SDS/12 % polyacrylamide gel and then subjected to Western blotting, using affinity-purified antibody against intact GLUT1. The bound primary antibody was detected with ^{125}I-labelled donkey anti-rabbit IgG as described in Section 2.2.2. The positions of M_r markers are indicated.

terminal fragment of apparent M_r 25,000, which is subsequently converted via a fragment of apparent M_r 23,000 to a stable form of apparent M_r 18,000, although the order in which these cleavage events occur is not obligatory (Cairns *et al.*, 1984, 1987; Davies *et al.*, 1987). Of particular importance for the present experiments is the fact that the M_r 18,000 fragment (residues 270 - 456) contains many potential sites of tryptic cleavage which remain cryptic in the native, membrane-bound protein. The observation of a very similar pattern of fragments yielded by tryptic digestion of GLUT1 expressed in insect cells suggests that the recombinant protein exhibits a tertiary structure similar if not identical to that of its erythrocyte counterpart.

5.2.4. Time course for expression of GLUT1 in insect cells

In order to establish the optimum conditions for the large-scale production of GLUT1 using the baculovirus system, it was important to determine the time course of the protein's expression following infection of insect cells with the recombinant baculovirus, AcNPV-GT. Sf9 cells were infected with the recombinant AcNPV-GT or the wild type AcNPV, at a MOI of 5, and harvested at different times after infection. The cells were washed three times in PBSA and then solubilized in 1 % SDS. Subsequently, samples (10 μg) of the total lysates of the infected cells were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting using anti-transporter antibodies that recognise the *C*-terminal peptide of GLUT1 (Davies *et al.*, 1987) and the ^{125}I-labelled donkey anti-rabbit IgG second antibody, as detailed in Section 2.2.2. As shown in Figure 5.5, recombinant glucose transporter was detectable in

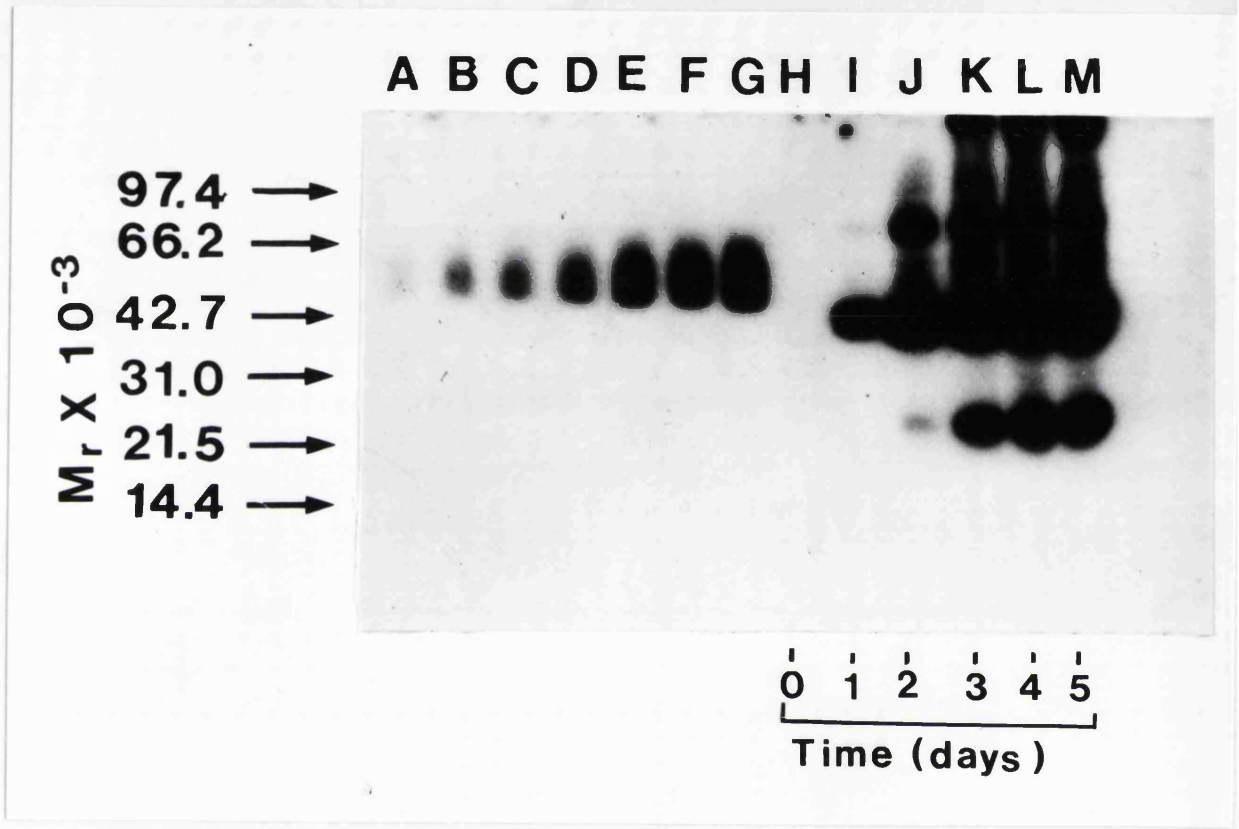


Fig. 5.5. Immunoblot analysis of recombinant GLUT1 production in baculovirus-infected insect cells. Sf9 cells were infected with recombinant AcNPV-GT (at MOI = 5) as described in Chapter 2, harvested at various intervals after infection, and solubilized in 1 % SDS. Samples (10 μg) of the total cell lysates (lanes H-M) were electrophoresed on a SDS/10 % polyacrylamide gel and then assayed for GLUT1 by Western blotting as described in the text. Samples of protein-depleted human erythrocyte membranes containing known amounts of GLUT1 (lanes A-G, 25-250 ng GLUT1) were also included on the same gel. The positions of proteins used as M_r markers are indicated.

AcNPV-GT-infected cells within 24 hours of infection and the intensity of the band stained by the antibodies continued to rise until the cells began to lyse, 4 - 5 days after infection. However, no detectable immunostaining was seen in cells infected with the wild type AcNPV through the time period examined (Fig. 5.6). The time course curve shown in Figure 5.7 was obtained by measuring the radioactivity of the labelled bands using the procedures described in Madon *et al.* (1990). Values are expressed as a percentage of the maximal value obtained at the end of the time course. Accumulation of the transporter, and the lack of visible degradation products on the blots, suggest that the protein is reasonably stable after it has been synthesised. As cells harvested 4-5 days post-infection contained the largest amounts of recombinant glucose transporter, this incubation period was thereafter routinely used for large-scale expression of the protein.

5.2.5. Subcellular location of GLUT1 expressed in insect cells

In most mammalian cells that express GLUT1, this transporter isoform is predominantly located at the cell surface, although many cells in addition contain an intracellular pool of transporters. To examine the subcellular location of the human transporter when expressed in insect cells, indirect immunofluorescence studies were carried out. The method used was obtained from Mr. M. Kids (Department of Virology, RFHSM). It was originally developed for the staining of respiratory tract infectious viruses, and was slightly modified. Sf9 cells were infected with recombinant AcNPV-GT or with wild-type virus at a MOI of 5, or were not exposed to viral infection. They were then incubated

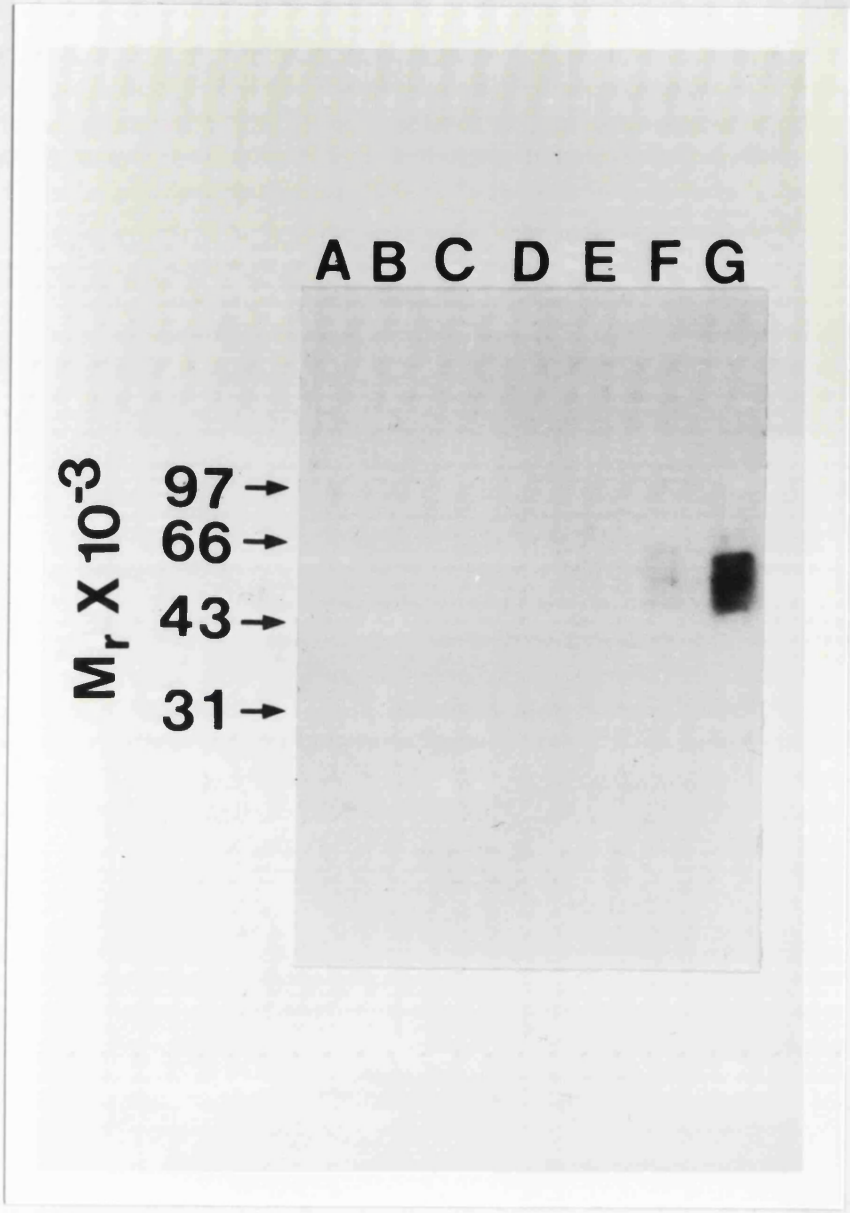


Fig. 5.6. Western blot analysis of insect cells infected with wild-type AcNPV. Sf9 cells were infected with the wild-type virus (at MOI = 5), collected at various intervals after infection (1-5 days, lanes A-E). Samples (10 μg) of the total cell lysates were then assayed for GLUT1 by SDS/polyacrylamide gel electrophoresis and Western blotting as described in the legend to Fig. 5.5. Samples of protein-depleted human erythrocyte membranes containing known amounts of GLUT1 (25 and 50 ng GLUT1 in lanes F & G, respectively) were also included on the same gel as a control. The positions of proteins used as M_r markers are indicated.

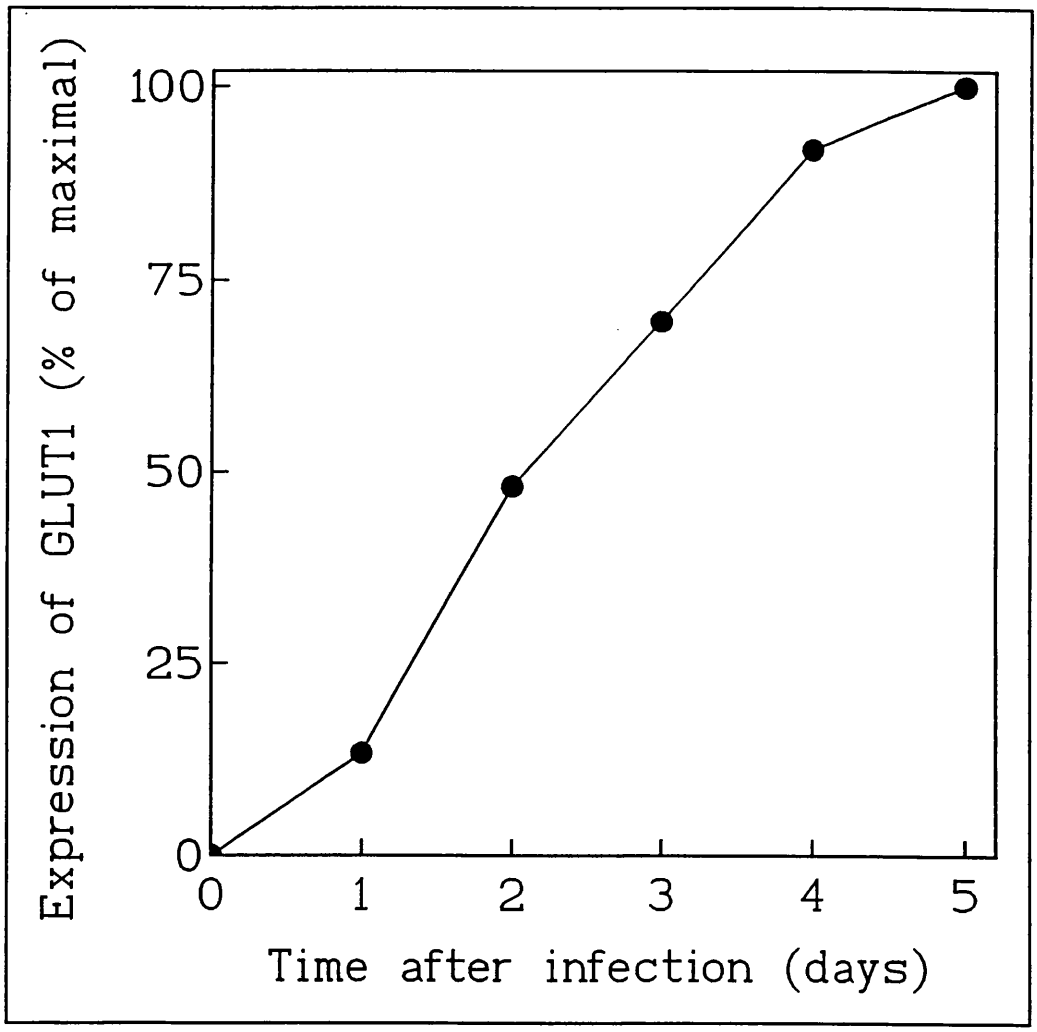
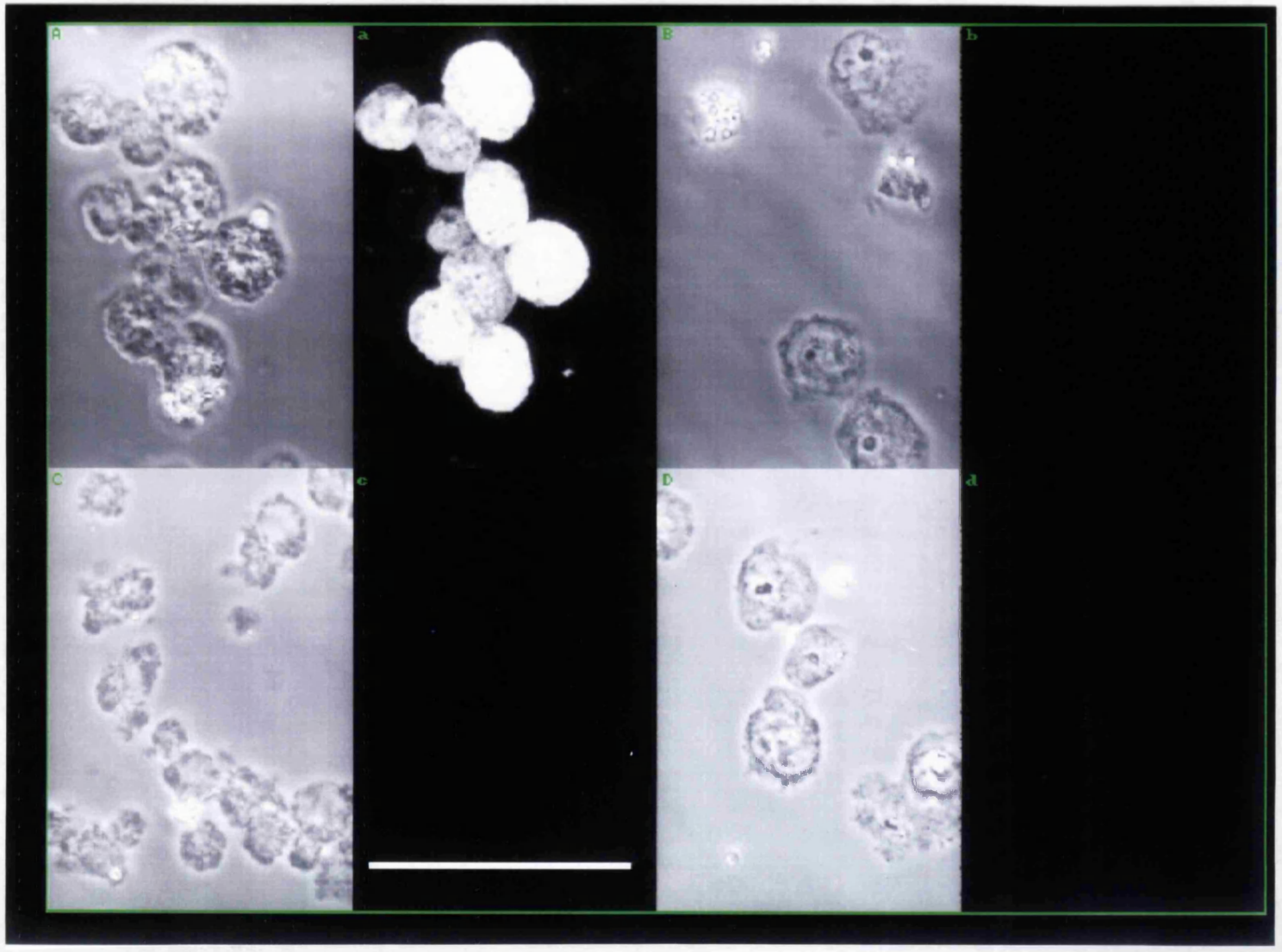


Fig. 5.7. Time course of GLUT1 expression in insect cells. Sf9 cells were infected with recombinant AcNPV-GT (MOI = 5), then collected and lysed at various intervals after infection. Samples (10 μg) of the resultant cell lysates were assayed for GLUT1 by Western blotting as described in the text. Maximal levels of GLUT1 expression typically corresponded to approximately 1.4 nmol/mg of protein.

at 28°C. Two days post infection the cells were collected and resuspended in TC100 medium at a density of 4 x 10^4 cells/ml. Then, 25 μl samples of the cell resuspension were spotted onto multispot microscope slides (Hendley, Essex, U.K.) and allowed to dry in a current of air using a laminar flow cabinet. The cells were then fixed in acetone at -20°C for 2 min and briefly dried in the laminar flow cabinet. This procedure not only fixes the cells, but permeabilises the cell membrane, allowing access of antibodies to the interior. Subsequently, the cells were stained at 37°C for 1 hour in a humid condition, with 25 μl of affinity-purified antibody (50 μg/ml) raised against the *C*-terminus of GLUT1 (Davies *et al.*, 1987). Following incubation, the cells were washed three times in PBS for 10 min duration each with light stirring and then immunolabelled with 25 μl of diluted (1:80) goat anti-rabbit IgG conjugated to fluorescein (Pierce, Chester, U.K.). After incubating at 37°C for 1 hour, the cells were examined using a confocal laser scanning microscope.

As shown in Figure 5.8, only cells infected with the recombinant AcNPV-GT exhibited a bright fluorescence (panel a), whereas no fluorescent staining was observed in wild type AcNPV-infected cells (panel b). Successful infection of the latter was clearly demonstrated by their expression of substantial amounts of polyhedrin, as evidenced from the viral occlusion bodies visible in Figure 5.8 (panel B). Similarly, non-infected cells showed no fluorescence (panel c). The specificity of the labelling was demonstrated by omitting the primary antibody during staining of another sample of cells infected with the recombinant AcNPV-GT: in this case no fluorescent labelling of the cells was seen (panel d). These results confirmed the finding of the Western blotting

Fig. 5.8. Confocal microscopy of non-infected and baculovirus-infected insect cells. Two-days post-infection cells were collected, fixed and incubated first with affinity-purified anti-glucose transporter antibody (50μg/ml) and then a fluorescein conjugate of goat anti-rabbit IgG, before examination using a confocal laser scanning microscope. The panels show immunofluorescent images of cells infected with (a) recombinant AcNPV-GT, (b) wild-type AcNPV (c) no virus or (d) cells infected with recombinant AcNPV-GT and stained as for the other panels except with omission of the primary antibody. The corresponding phase-contrast images are shown in panels A- D. Scale bar 50μm.


A
a
B
b
C
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D
d

experiments described in Section 5.2.1, namely that cells infected with the recombinant virus abundantly expressed the glucose transporter. However, it was possible to gain further information by exploiting the ability of the confocal microscope optically to section a thick tissue or cell sample. By this means a series of optical images was obtained at section planes spaced regularly (0.6 μm) through the fluorescently-labelled, AcNPV-GT-infected cells. These serial "sections" of a labelled cell are illustrated in Figure 5.9. They illustrate the presence of substantial cell-surface fluorescence, indicating that expressed GLUT1 is indeed located at the plasma membrane. However, in addition intense fluorescent labelling is evident throughout the cytoplasm, except for the region occupied by the enlarged nucleus of the infected cell. It was therefore clear that in addition to its cell surface expression, large amounts of GLUT1 were present in membranous structures throughout the cytoplasm.

5.2.6. Ultrastructure of baculovirus-infected insect cells

As shown in previous sections, infection of Sf9 cells with the recombinant baculovirus AcNPV-GT resulted in expression of large amounts of glucose transporter within the cell. It had been reported that heterologous expression of other membrane proteins using the baculovirus/insect cell system induced proliferation of cytoplasmic membranes (Matsuura *et al.*, 1987). Thus, it was of interest to see if this was also the case for the AcNPV-GT-infected cells. To this end, electron microscopy was performed as described in Section 2.6.2. Cells were infected with the AcNPV-GT, the wild type or no virus at a MOI of 5. Two days after infection the cells were harvested, fixed in

Fig. 5.9. Sequential display of confocal optical sections through AcNPV-GT-infected Sf9 cells. Sf9 cells were infected with the recombinant virus. At 2 days after infection, the cells were collected, fixed and then fluorescently-labelled as described in the legend to Fig. 5.9. A series of optical images were obtained at section planes spaced regularly (0.6 μm) through the fluorescently-labelled, AcNPV-GT-infected cells, using a confocal laser scanning microscope. Some of these images were then recalled individually from an image file.

glutaraldehyde, and post-fixed in osmium tetroxide. The fixed cells were then dehydrated through a standard graded ethanol series at 15°C for 30 min duration each. The samples were then transferred into resin and the latter was polymerised. Following these embedding procedures, the embedded samples were sectioned, stained and then examined using a transmission electron microscope.

Electron micrographs of cells infected with the recombinant virus, with the wild-type virus, or with no virus can be compared in Figures 5.12, 5.11 and 5.10 respectively. Budded viruses are visible in the extracellular space of infected cells (Figs. 5.11 and 5.12), whereas no virus was detectable in the uninfected cells (Fig. 5.10). Infection of cells with either AcNPV-GT or wild type AcNPV resulted in an increase in cell diameter due to enlargement of the nucleus (Figs. 5.11 and 5.12). The very late phase of wild-type AcNPV infection is characterized by the production of polyhedrin which accumulates within the nucleus (Harrap, 1972a). Figure 5.11 shows clearly the synthesis of polyhedrin in the nucleus of a cell infected with wild-type virus, although the polyhedra appear to be immature as enveloped virions were not observed. However, cells infected with the recombinant virus did not produce polyhedrin (Fig. 5.12). This was expected because insertion of the GLUT1 cDNA into the polyhedrin locus by homologous recombination completely deletes the coding region for this viral protein. Another abundant protein in the very late phase is 10 kDa polypeptide known as p10 (Van Der Wilk *et al.*, 1987). It accumulates primarily in the nucleus but sometimes in the cytoplasm as well. The large arrays of fibrous material visible in Figure 5.12 in the nucleus of a cell infected

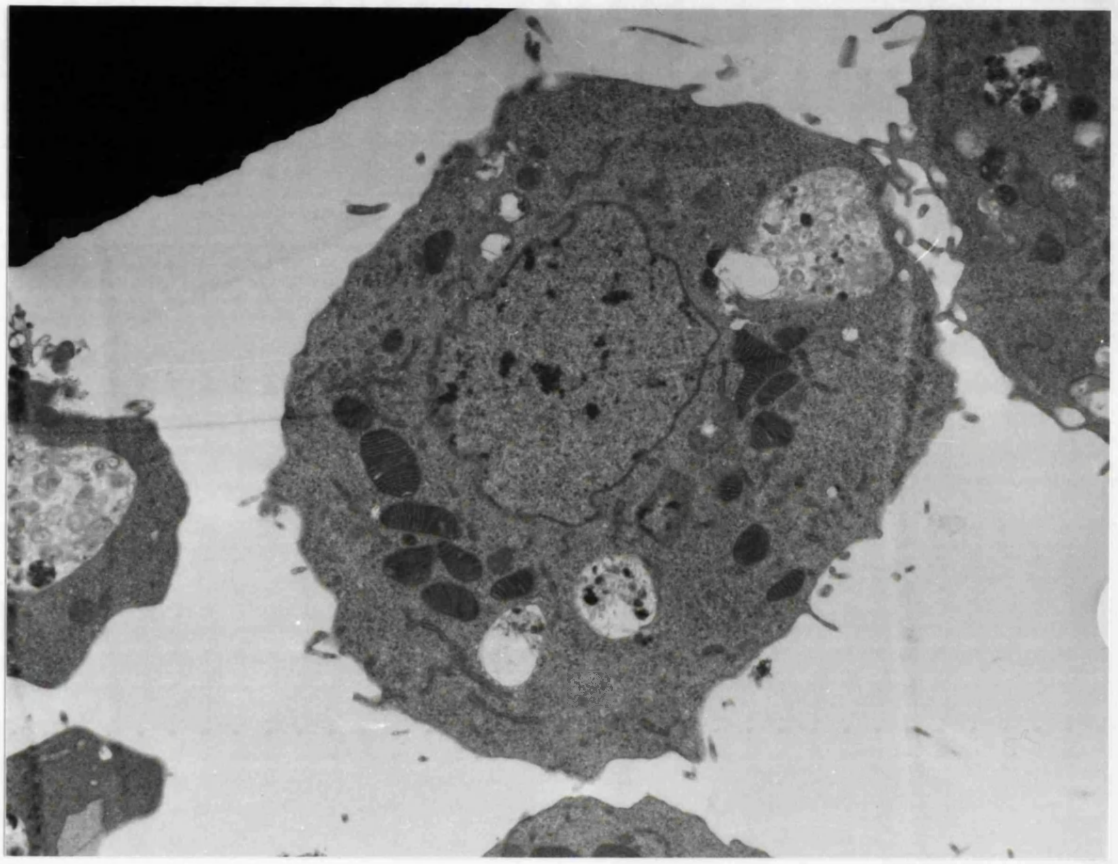


Fig. 5.10. Electron micrograph of uninfected *S. frugiperda* cells. Sf9 cells were post-fixed with osmium tetroxide as described in Section 2.6.2. Signs of viral infection (e.g. the enlarged nucleus) are not observed, as expected. Magnification X 8,800.

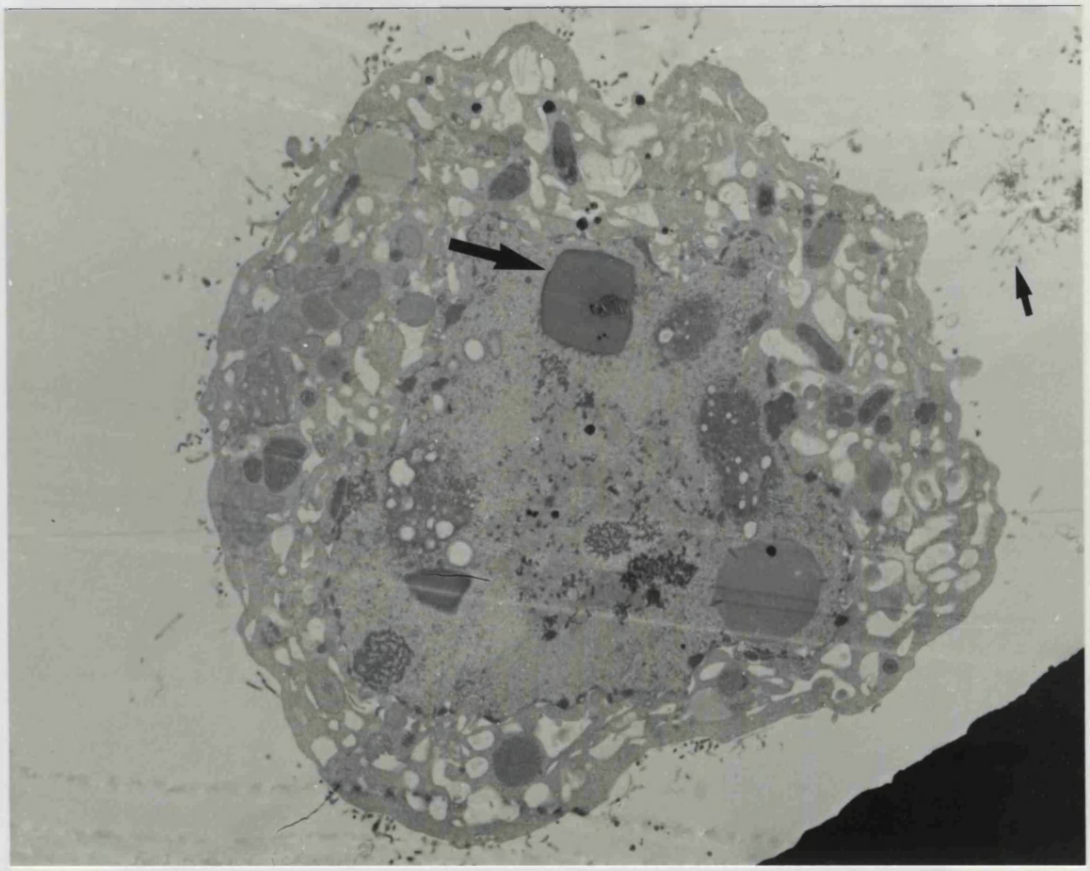


Fig. 5.11. Electron micrograph of Sf9 cells infected with wild-type AcNPV. The cells were post-fixed with osmium tetroxide as described in Section 2.6.2. Polyhedral inclusions are clearly visible within the enlarged cell nucleus (Large arrow). Budded virus can be seen in the extracellular space (small arrow). Magnifications X 8,800.

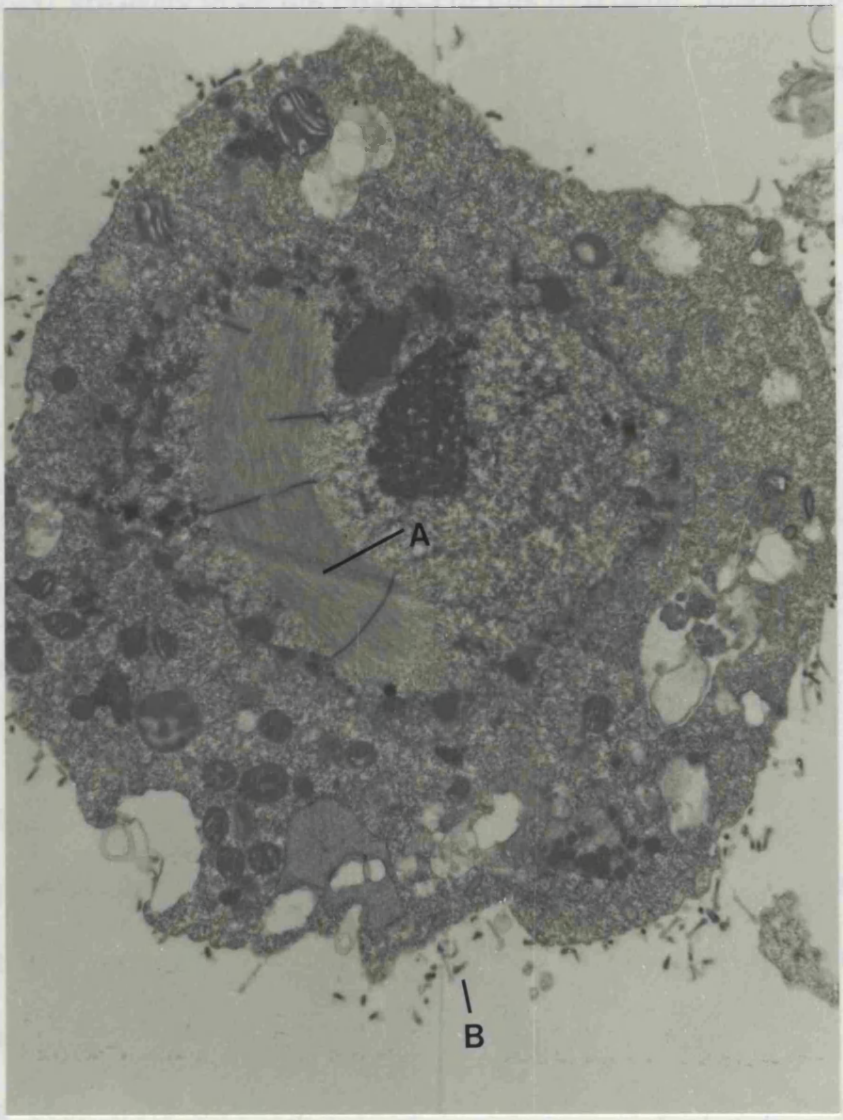


Fig. 5.12. Electron micrograph of *S. frugiperda* cells infected with recombinant AcNPV-GT. The cells were post-fixed with osmium tetroxide. A large array of fibrillar structures, the product of p10 gene, is visible in the enlarged nucleus (A). Also visible are budded viruses (B), but not polyhedra. Magnifications X 13,250.

with AcNPV-GT are likely to be the product of this p10 gene. Disruption of the p10 gene has been reported to cause the disappearance of such fibrous material without affecting formation of polyhedra containing enveloped virions (Williams *et al.*, 1989). Although signs of successful infection by viruses were evident in the electron micrographs of the infected cells (Figs. 5.11 and 5.12), the phenomenon of interest, i.e. an extensive cytoplasmic vacuolation, was not observed in the cells infected with the AcNPV-GT that expressed large quantities of glucose transport proteins. This finding contrasts with the extensive vacuolation seen in insect cells expressing other membrane proteins (Matsuura *et al.*, 1987).

5.2.7. Quantification of GLUT1 expressed in insect cells

For quantification of the erythrocyte glucose transporter expressed in Sf9 cells, quantitative immuno-blotting was carried out, essentially as described by Madon *et al.* (1990). Membrane samples (0.4 - 4 μg) prepared from AcNPV-GT-infected cells were electrophoresed on SDS/10 % polyacrylamide gels. Proteins were then electrophoretically transferred to nitrocellulose and immunolabelled with affinity-purified antibodies against residues 477 - 492 of human GLUT1. The bound primary antibody was detected by incubation with ^{125}I-labelled donkey anti-rabbit IgG as described in Section 2.2.2.2. The blots were then dried and autoradiographed for 16 hours at -70°C (Fig. 5.13). To estimate the amount of the expressed protein, samples of protein-depleted human erythrocyte membranes were included on the same gel to act as standards. These contained known amounts (25 - 200 ng) of glucose

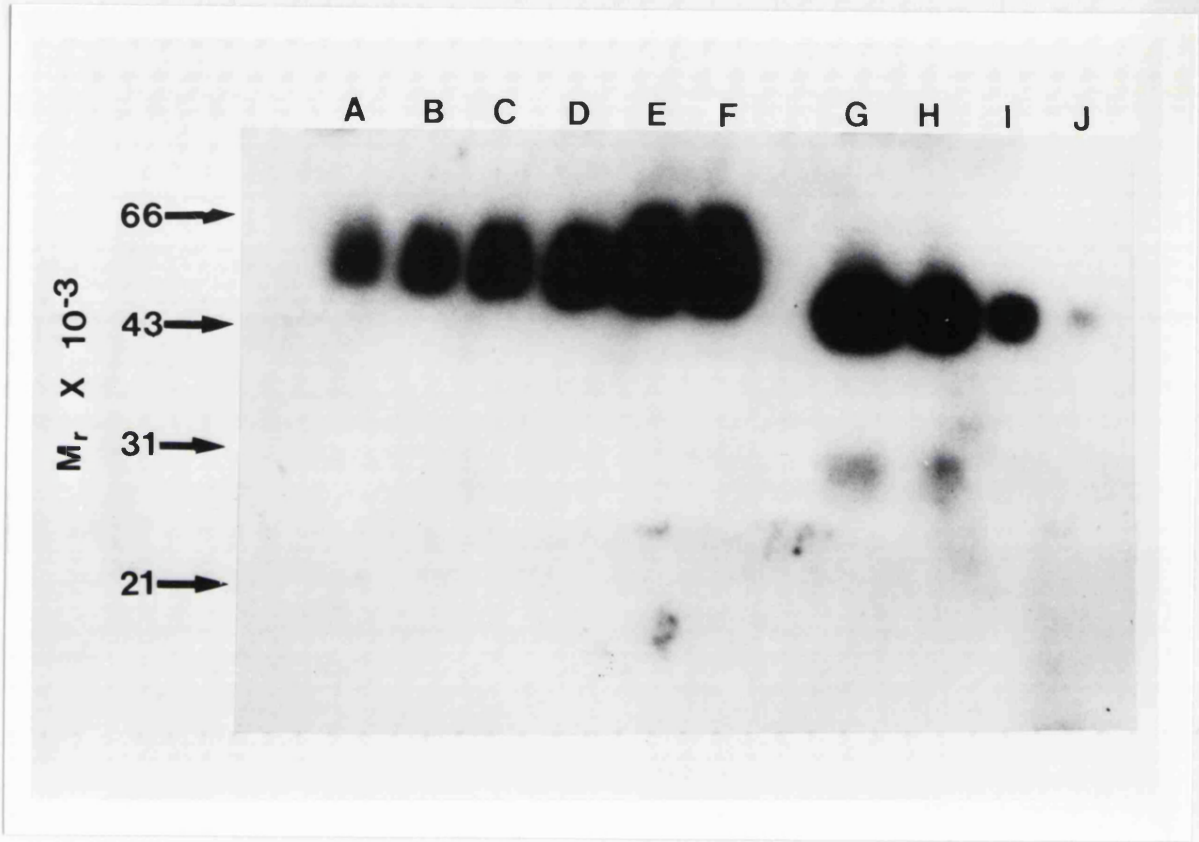


Fig. 5.13. Autoradiograph for quantification of GLUT1 expressed in insect cells. Samples (4 - 0.4 μg) of AcNPV-GT-infected cells membranes (G-J) and of protein-depleted human erythrocyte membranes containing known amounts of glucose transporter (A-F) were electrophoresed on a SDS/polyacrylamide gel and subjected to Western blotting as described in the text. The blot was dried and then autoradiographed for 16 hours at -70°C.

transporter, as measured by Scatchard analysis of cytochalasin B binding, performed as described in Section 2.2.5 (Fig. 5.14). The autoradiographs were then aligned over the nitrocellulose and radioactive bands were cut out and counted in a *γ*-radiation counter. A piece of nitrocellulose of equal area to the radioactive bands was also cut from a non-radioactive part of the blot to determine backgrounds. As the amount of ^{125}I specifically bound to the erythrocyte transporter band was linearly proportional to the amount of transporter standard applied to the gel, a calibration curve could be created from the human erythrocyte standard data (Fig. 5.15) and used to determine the amount of glucose transporter expressed in Sf9 cells by employing linear-regression analysis. The different glycosylation state of the standards and the expressed proteins is unlikely to affect the results, because the site of glycosylation is remote from the *C*-terminus. The results of the quantitative Western blotting analysis of two separate preparations presented in Table 5.2 showed that 4 days after infection 1.41 and 1.47 nmol of transporter respectively were present per mg of membrane protein, values which represent almost 8 % (w/w) of the total membrane protein (calculated on the basis of the predicted M_r for GLUT1 of 54,117). However, when samples (5 *μ*g) of AcNPV-GT-infected cell membranes were examined by SDS-polyacrylamide gel electrophoresis with Coomassie blue staining as described in Section 2.2.1, the expressed protein was not detectable as a separate sharp band on the gels (lane A, Fig. 5.16).

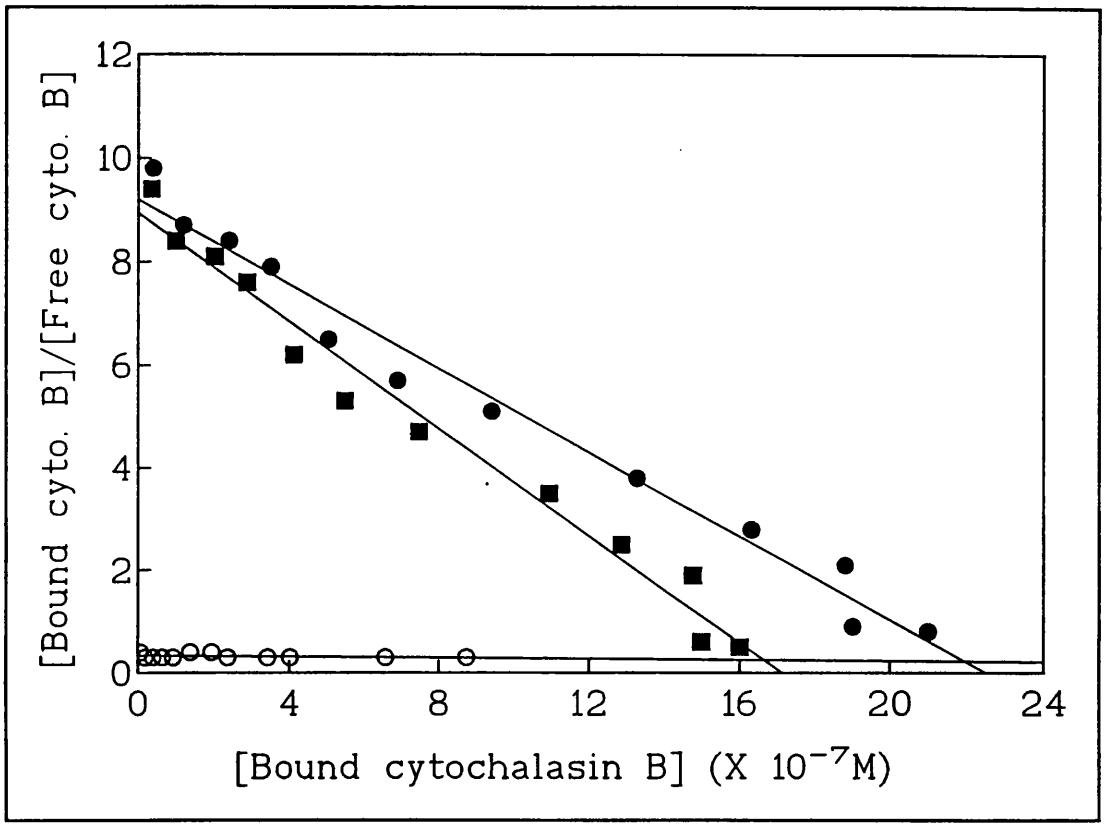


Fig. 5.14. Scatchard-plot analysis of cytochalasin B binding to protein-depleted human erythrocyte membranes. Binding of cytochalasin B to the erythrocyte membranes (1 mg/ml) was measured over a range of cytochalasin concentrations (0.5 - 75.5 x 10^{-7} M) in the presence (○) or absence (●)of 400 mM-D-glucose, as described in Section 2.2.5. Each point represents the mean of triplicate determinations. A derived Scatchard-plot (■) was generated by subtracting, along radial axes, the curve obtained in the presence of D-glucose from that obtained in its absence. The straight lines are the best fits derived by linear regression analysis of the data.

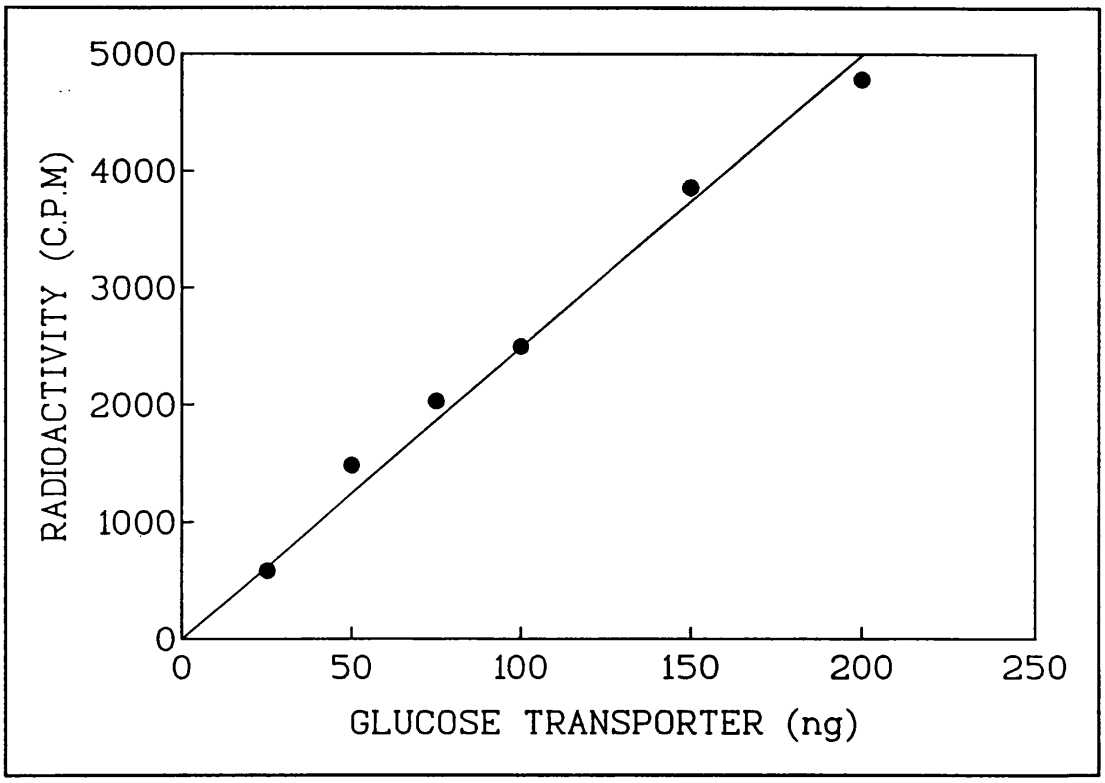


Fig. 5.15. A typical example of a calibration curve for quantification of GLUT1 expressed in insect cells. For quantification of immunoblots, autoradiographs were aligned over the nitrocellulose and radioactive bands were cut out and counted in a *γ*-radiation counter as described in the text. Linear-regression analysis was applied to create the calibration curve from the human erythrocyte standard data.

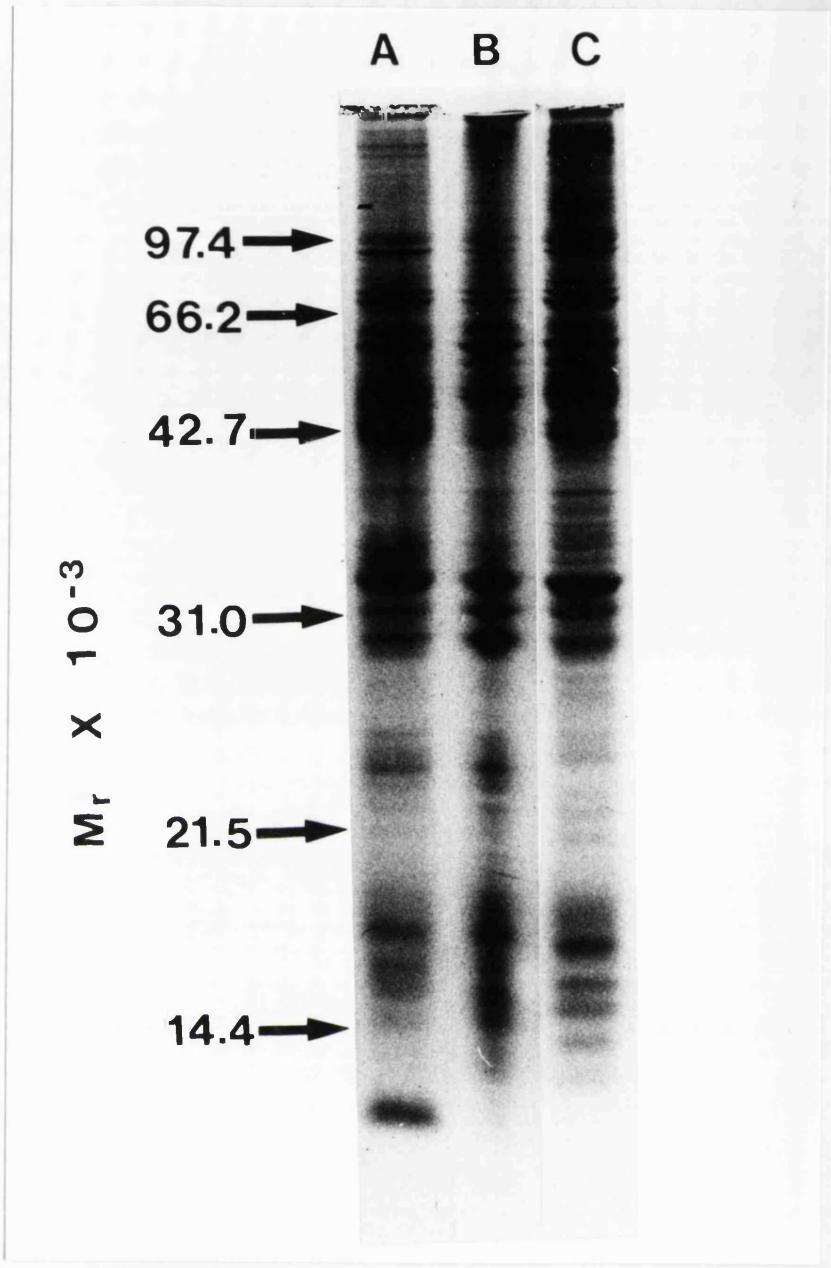


Fig. 5.16. Coomassie blue-stained gel of noninfected and infected Sf9 cells. Cells were infected with recombinant (A), wild-type (B) or no virus (C) at a MOI of 5, as described in Section 2.5.1. After 4 days infection the cells were harvested and solubilised in 1% SDS. Samples (5 μg) of total cell lysates were electrophoresed on a SDS/10% polyacrylamide gel and coomassie blue-stained as described in Section 2.2.1.

5.2.8. Functional characterization of the expressed protein

5.2.8.1. Ligand binding

The experiments described in Sections 5.2.1 and 5.2.5 had shown the abundant expression of GLUT1 in insect cells infected with recombinant baculovirus. The protein was shown to be present in its full-length, undegraded form, and was *N*-glycosylated, albeit less heterogeneously than the natural erythrocyte transporter. It was also wholly membrane-bound, and at least a proportion was located at the cell surface. The pattern of fragments produced by tryptic digestion of the protein also suggested that it was correctly folded, but it was not yet known whether the protein was functionally active. As described in Chapter 3, *Spodoptera* cells possess an abundant endogenous glucose transport activity, which complicates measurement of transport activity resulting from expression of exogenous glucose transporters following infection with recombinant baculovirus. However, unlike the mammalian passive glucose transporters, the *Spodoptera* transporter(s) are relatively insensitive to cytochalasin B. The latter is a potent inhibitor of GLUT1 and binds to a site at the cytoplasmic surface of the membrane with a dissociation constant in the region of 0.1 μM. Binding is competitively inhibited by the substrate D- but not by L-glucose. Cytochalasin B is commercially available in tritiated form, and its binding can conveniently be measured by equilibrium dialysis (Baldwin & Lienhard, 1989). Measurement of the extent and D-glucose sensitivity of cytochalasin B binding is therefore a very convenient means of assessing the functional integrity of mammalian glucose transporters, which unlike transport measurements does not require either intact cells or sealed transporter-

containing vesicles. Furthermore, the insensitivity of insect cell glucose transport to inhibition by cytochalasin B, as demonstrated in Section 3.3, suggested the absence of endogenous binding sites. Therefore cytochalasin B binding measurements were used to assess the function of GLUT1 after its expression in these cells.

Routine measurements for the cytochalasin B binding activity of membrane samples were performed in triplicate by equilibrium dialysis using a single, low concentration of cytochalasin B (40 nM). The membranes used were prepared from cells 4 days post infection with wild-type virus, with AcNPV-GT or with no virus. As described in Section 2.2.5, under these conditions the ratio of bound to free cytochalasin B (B/F) is a good measure of the concentration of functional transporter (Zoccoli *et al.*, 1978). The results summarized in Table 5.1 show that as expected from the transport measurements, there is little or no D-glucose-inhibitable binding in cells infected with the wild type or no virus. Only AcNPV-GT-infected cells exhibit specific binding, which is inhibitable by D- but not by L-glucose. More accurate estimation of the concentration and affinity of cytochalasin B binding sites was obtained by measuring in triplicate over a range of cytochalasin concentrations (0.5×10^{-7} - 75.5×10^{-7} M), in the presence or absence of 400 mM D-glucose. The data were treated by the method of Scatchard (1949) with correction for the non-specific binding to membrane lipids. A derived Scatchard plot was obtained by subtracting, along radial axes, the curve obtained in the presence of D-glucose from that obtained in its absence (Fig. 5.17). Linear regression was used to fit the resultant data points to the best straight line. Scatchard

Table 5.1

Assay of cytochalasin B binding to non-infected and baculovirus-infected cells

Sample (1 mg/ml)	Cytochalasin B Binding (B/F)		
	(-)D-Glucose	(+)-D-Glucose	*Specific B/F
Sf9 cells infected with			
-No virus	0.053	0.052 (0.051)	0.001
-Wild-type virus	0.054	0.053 (0.053)	0.001
Recombinant virus (AcNPV-GT)	1.088	0.160 (1.041)	0.928

Cells were harvested 4 days after infection and membranes prepared as described in Section 2.2.7. The assay for cytochalasin B-binding activity of membrane samples was performed by equilibrium dialysis using 40 nM-[^{3}H]cytochalasin B, in the absence (-) or presence (+) of 400 mM D-glucose, as described in Section 2.2.5. Cytochalasin B binding activity (*) was calculated as described in the text. The values shown in parenthesis were obtained by performing the binding experiments in the presence of 400 mM L-glucose. B/F = [bound cytochalasin B]/[free cytochalasin B].

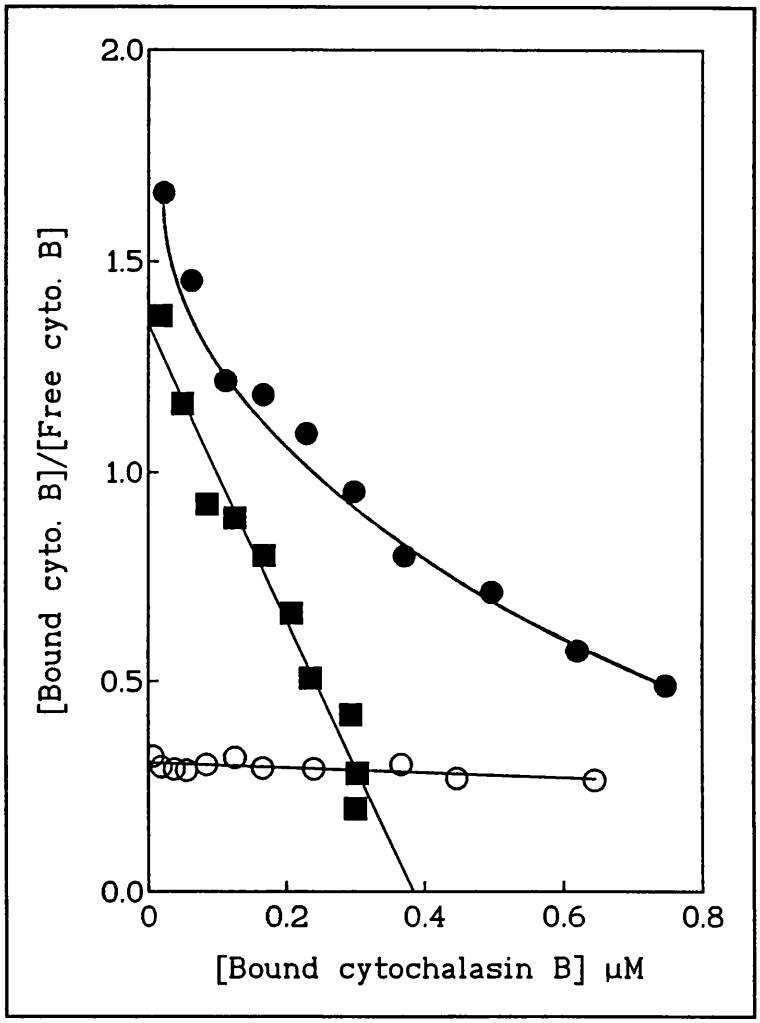


Fig. 5.17. Representative Scatchard-plot analysis of D-glucose-inhibitable cytochalasin B binding to recombinant GLUT1 expressed in Sf9 cells. Binding of cytochalasin B to infected cell membranes (3 mg/ml) was measured in the presence (○) or absence (●) of 400 mM D-glucose as described in Section 2.2.5. A derived Scatchard plot (■) was generated by subtracting, along radial axes, the curve obtained in the presence of D-glucose from that obtained in its absence. Linear regression analysis was used to fit the resultant data points to the best straight line.

plot analysis showed the presence of a single class of binding sites in the membranes prepared from AcNPV-GT-infected insect cells. The dissociation constant (K_d) of 284 nM obtained was almost identical to that previously reported for the erythrocyte transporter, 282 nM (Madon *et al.*, 1990). Binding was completely inhibited by the presence of 400 mM-D-glucose but not by L-glucose (Fig. 5.18). It was therefore clear that the expressed protein bound not only the inhibitor, but also the transported substrate. However, the concentration of cytochalasin B binding sites detectable in the AcNPV-GT-infected cell membranes, up to 290 pmol/mg protein (corresponding to 2 % of the total membrane protein, assuming M_r = 54,117), was less than the concentration of immunologically cross-reactive protein (up to 1470 pmol/mg protein, see Table 5.2).

5.2.8.2. Photoaffinity labelling

From the ligand binding study described in Section 5.2.8.1, it was evident that there were D-glucose-sensitive cytochalasin B binding sites in cells infected with recombinant AcNPV-GT. In order to assess whether these binding sites actually corresponded to the protein immunologically identified on Western blots as the expressed GLUT1 transporter (Section 5.2.1), photoaffinity labelling experiments with [^{3}H] cytochalasin B were carried out according to the methods described previously (Kasanicki *et al.*, 1987). Cytochalasin B binding to the transporter in the human erythrocyte is normally reversible. However, exposure of the protein to ultraviolet light in the presence of tritiated cytochalasin B results in covalent binding to the transporter (Carter-su *et al.*, 1982; Shanahan,

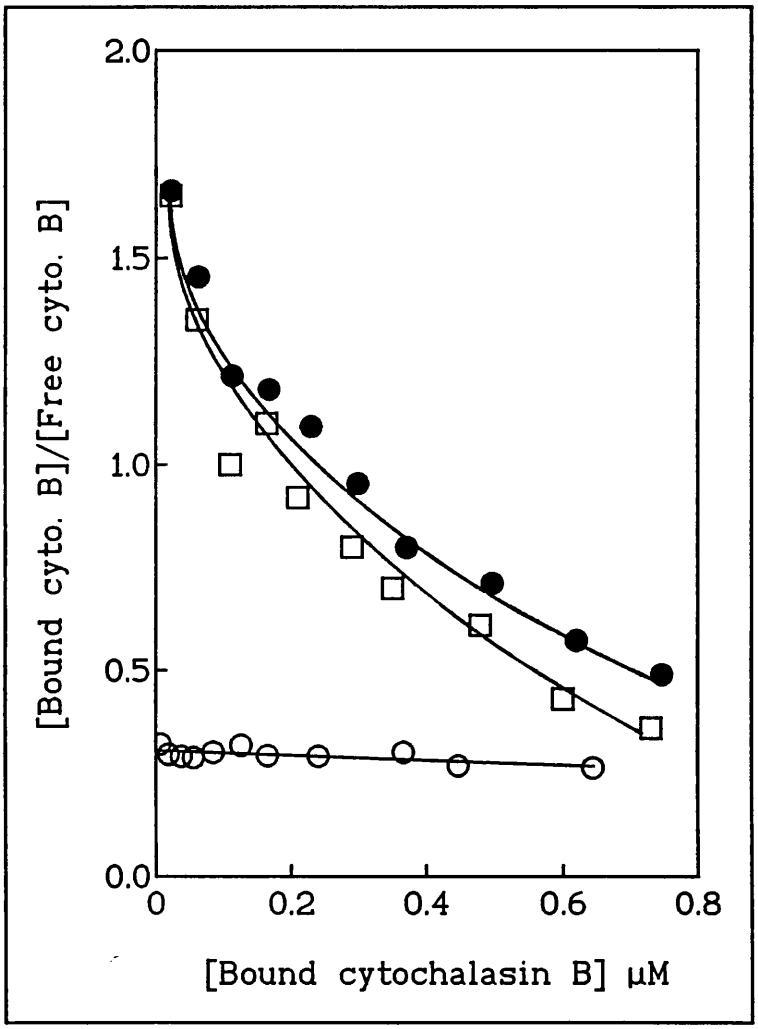


Fig. 5.18. Scatchard-plot analysis of stereo-specific cytochalasin B binding to recombinant GLUT1 expressed in Sf9 cells. Binding of cytochalasin B to infected cell membranes (3 mg/ml) was measured over a range of cytochalasin concentrations (0.5 - 75.5 x 10^{-7} M) in the presence of 400 mM D-(○) or L-glucose (□), as described in the legend to Fig. 5.17. (●) represents the data obtained in the absence of glucose. Each point is the mean of triplicate determinations. Linear regression analysis was used to fit the resultant data points to the best straight line.

Table 5.2

A typical example of quantification of baculovirus-expressed GLUT1

Sample (1 mg/ml)	Cytochalasin B Binding		Western Blotting
	*B/F	+pmol/mg	=pmol/mg
Sf9 cells infected[a] with			
-Recombinant virus[o]	0.940	290	1470
-Recombinant virus[o]	0.709	226	1410

*B/F = [Bound cytochalasin B]/[Free cytochalasin B], measured using 40 nM cytochalasin B and corrected for non-specific binding to membrane lipids as described in Section 2.2.5.

+Calculated from Scatchard analysis.

=Calculated from the intensity of staining with anti-*C*-terminal peptide antibodies on a Western blot, in comparison with a standard curve constructed using human erythrocyte membranes of known cytochalasin B binding site content.

[a]Cells were harvested 4 days after infection and membranes prepared as described in Section 2.2.7.

[o]Two separate preparations were examined.

1982). Membranes were prepared from Sf9 cells infected with the recombinant AcNPV-GT or the wild-type AcNPV (MOI=5) 4 days post infection. The membrane samples were then photolabelled with [^{3}H]cytochalasin B in the presence of either 500 mM-D-or L-glucose, as detailed in Section 2.2.6. Subsequently, the labelled proteins were analysed by SDS-polyacrylamide gel electrophoresis. The position of the radiolabelled peak was compared with the measured migration distance of the molecular weight markers. Membranes labelled with [^{3}H] cytochalasin B in the presence of L-Glucose yielded a single sharp peak of labelling of apparent M_r 45,000 on SDS/polyacrylamide gels (Fig 5.19). The mobility of this peak corresponded exactly to that of the band detected by anti-glucose transporter antibodies on Western blots of membranes prepared from insect cells infected with recombinant virus (discussed in Section 4.2.2). Furthermore, the incorporation of label into this peak was completely inhibited by the presence of 500 mM-D-Glucose during the photolabelling procedure, showing the stereoselectivity of the labelling. No peak of labelling was seen with the membranes prepared from non-infected Sf9 cells (Fig. 5.20). In addition, the sharpness of the radioactive peak shown in Figure 5.19 provides further evidence for the conclusion that the expressed protein is much less heavily and heterogeneously glycosylated than its erythrocyte counterpart. In case of photolabelled human erythrocyte membranes, it is reported that the labelling occurs as a broad band of apparent M_r 45,000 - 70,000 on SDS/polyacrylamide gels (Madon *et al.*, 1990).

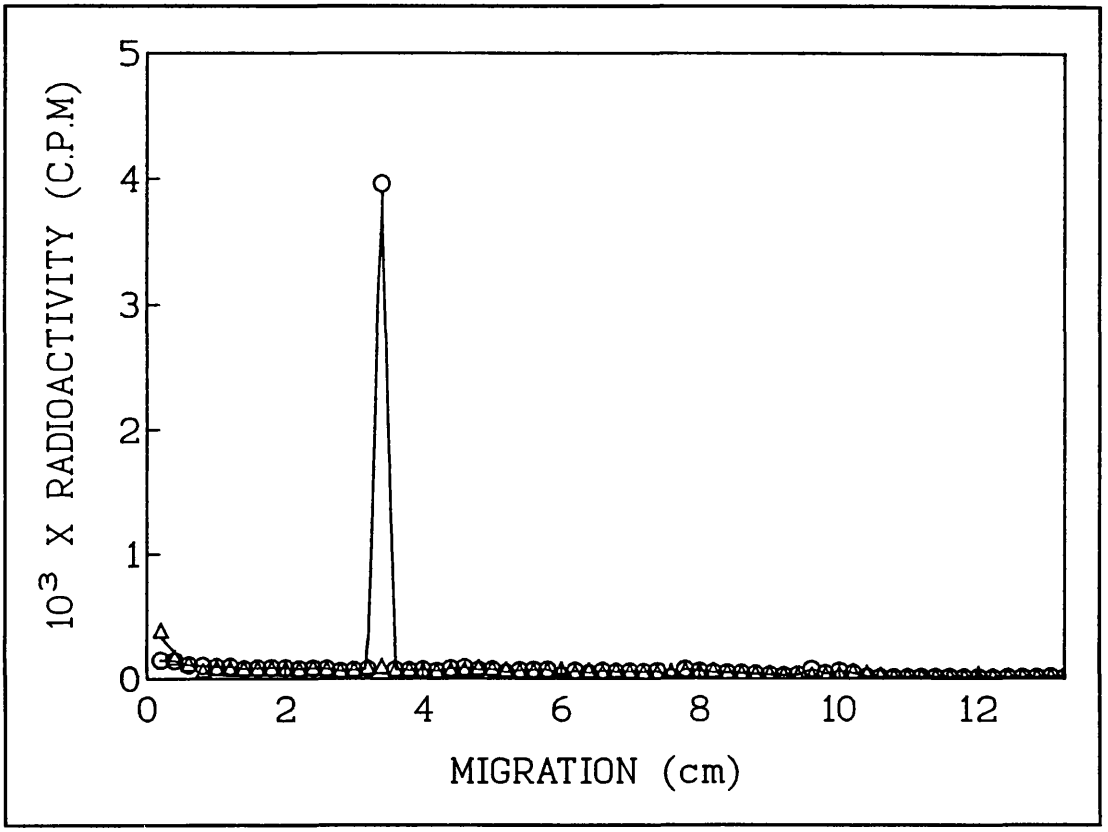


Fig. 5.19. Electrophoretic profile of photoaffinity-labelled GLUT1 expressed in insect cells. Membranes were labelled with [^{3}H]cytochalasin B in the presence of 500 mM-L-glucose (○) or 500 mM-D-glucose (△), as described in Section 2.2.6. Samples were electrophoresed on a SDS/12 % polyacrylamide gel. The radioactivity of 2 mm gel slices were determined by liquid scintillation counting. Arrows indicates the positions of M_r markers.

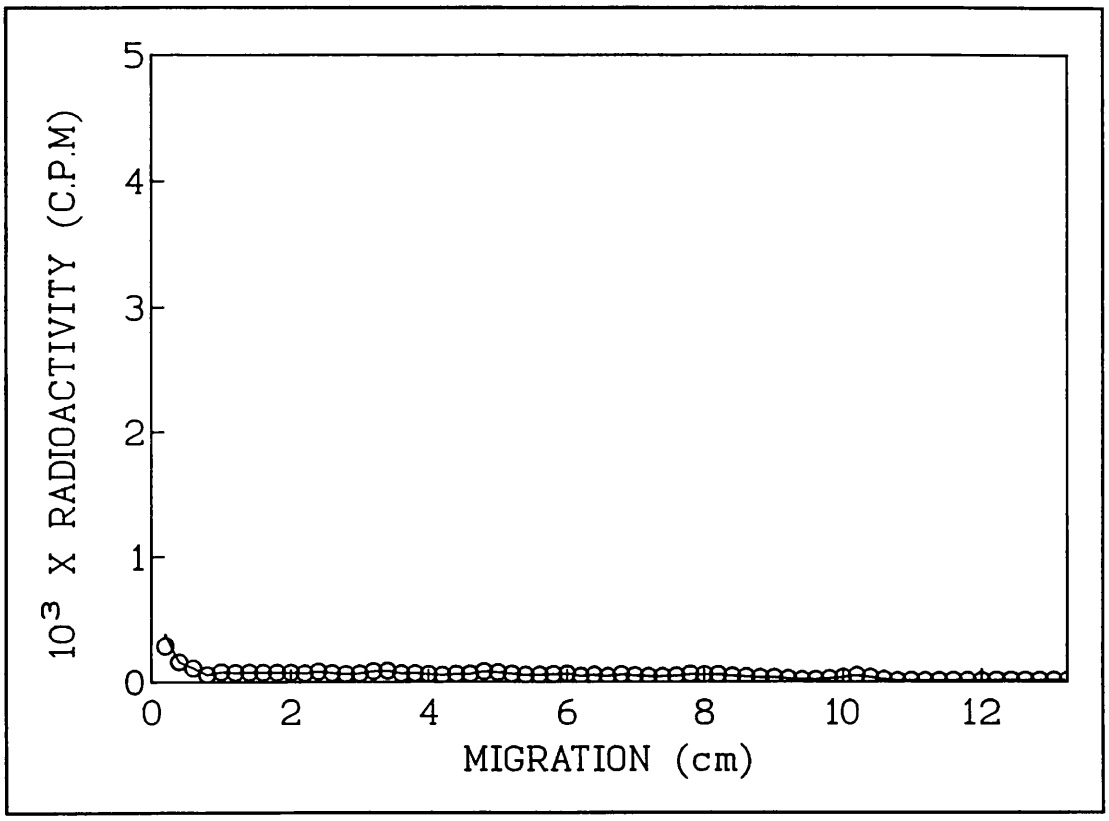


Fig. 5.20. Electrophoretic profile of photoaffinity labelling of uninfected insect cells. Non-infected Sf9 cell membranes were photoaffinity-labelled with [^{3}H]cytochalasin B in the presence of 500 mM-L-glucose, as described in the legend to Fig. 5.19.

5.2.8.3. Transport activity

As demonstrated in Sections 5.2.8.1 and 5.2.8.2, the characteristics of ligand binding to, and photoaffinity labelling of, GLUT1 expressed in insect cells were similar to those of its human erythrocyte counterpart. In order to determine whether the expressed protein also had similar transport function, measurements of 2-deoxy-D-glucose uptake were performed essentially as described in Section 2.6.1. Sf9 cells were infected with the recombinant virus AcNPV-GT or no virus, at a MOI of 5. They were harvested for use in transport experiments two days after infection, because infected cells become "leaky" at the time of maximal transporter expression (4 - 5 days post infection; data not shown). As described in Chapter 3 and as reported by others (Dr. G.D. Holman, personal communication), fructose is a good inhibitor of the endogenous insect cell sugar transporter, whereas it is not an effective inhibitor of human GLUT1. At the suggestion of Dr. Holman high concentrations of unlabelled fructose were therefore included in some 2dGlc uptake assays in order to suppress the activity of the endogenous insect cell transporters and therefore enhance detectability of GLUT1-catalysed transport in infected cells. Advantage was also taken of the differential susceptibility of the insect and mammalian transporters to inhibition by cytochalasin B - expression of human GLUT1 should give rise to a component of the 2dGlc uptake activity of the cells that is more sensitive to inhibition by this ligand. Infected cells were therefore resuspended in PBS plus and minus 300 mM fructose, and plus and minus 20 μM cytochalasin B for use in transport assays. Uptake was measured at 28°C for 1 min, with final concentration of 1 mM deoxy-D-glucose, 2-[1,2-^{3}H]- or

glucose, L-[1-^{3}H]-, used at a specific radioactivity of 4 Ci/mol. The assay was terminated by the addition of the ice-cold stop solution, plus or minus fructose as appropriate. As shown in Figures 5.21 and 5.22, the sugar uptake in uninfected cells was stereospecific, and was strongly inhibited by fructose but only poorly inhibitable by cytochalasin B, as expected from the known properties of the endogenous insect cell transporter (Chapter 3). Surprisingly, the AcNPV-GT-infected cells showed an essentially identical pattern of transport inhibition, and the rate of 2dGlc uptake was in fact somewhat less than that seen for the uninfected cells. There was thus no evidence for any contribution of expressed GLUT1 to 2dGlc uptake by the infected cells; in particular there was no appearance of a novel cytochalasin B-sensitive but fructose-insensitive component. The origins of the apparent lack of activity of the expressed protein are not clear - Western blotting showed that the transporter was abundant in the cells 2 days after infection. One possibility is that the infected cells are heterogeneous, those expressing the most GLUT1 being the most leaky. Another is that the level of cell-surface GLUT1 transporter in cells 2 days after infection is too low for its transport activity to be detectable in the face of the very high levels of endogenous hexose transport activity. In either case, assessment of the transport activity of the expressed protein would be likely to require its purification, free of endogenous insect cell transporters, followed by reconstitution into lipid vesicles suitable for sugar uptake experiments.

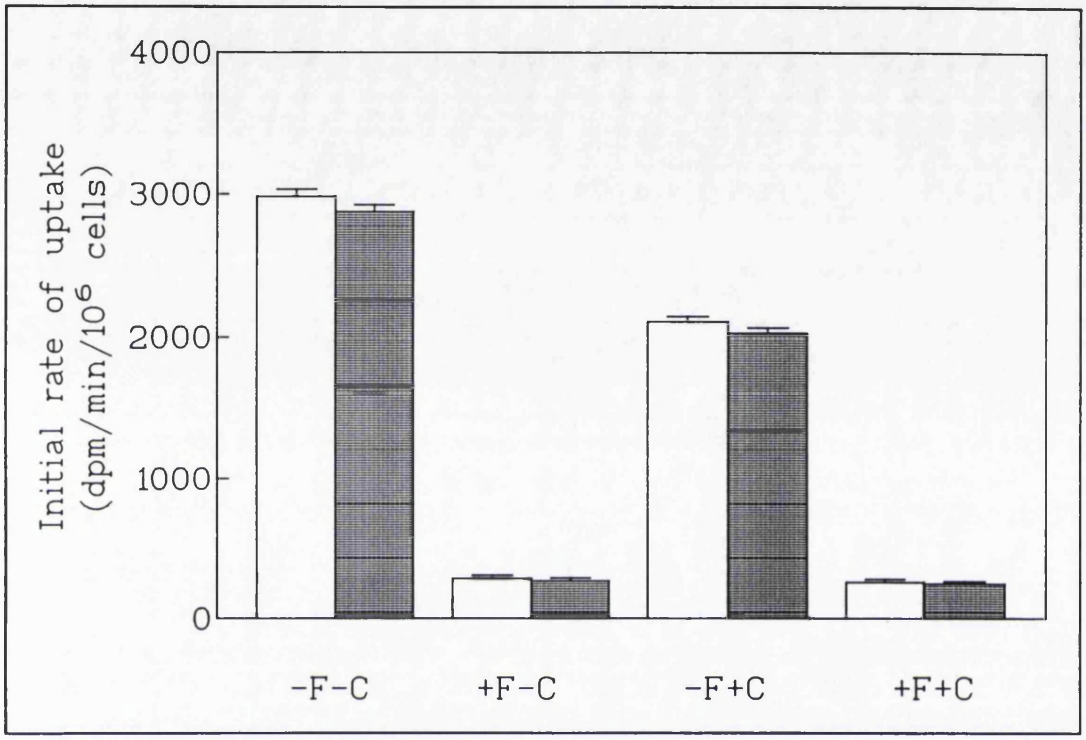


Fig. 5.21. Uptake of 1 mM 2-deoxy-D-glucose by noninfected- and recombinant virus infected Sf9 cells. Cells were infected with recombinant AcNPV-GT or no virus (MOI = 5) as described in Section 2.5.1. After 2 days infection cells were collected and resuspended in PBS plus(+) and minus (-) 300 mM fructose, and plus (+) and minus (-) 20 μM cytochalasin B. Transport was carried out as described in Section 2.6.1. Bars represent mean ± SEM for three measurements assayed in triplicate. Open bar: noninfected cells. Speckled bar: recombinant AcNPV-GT-infected cells. F: fructose. C: cytochalasin B.

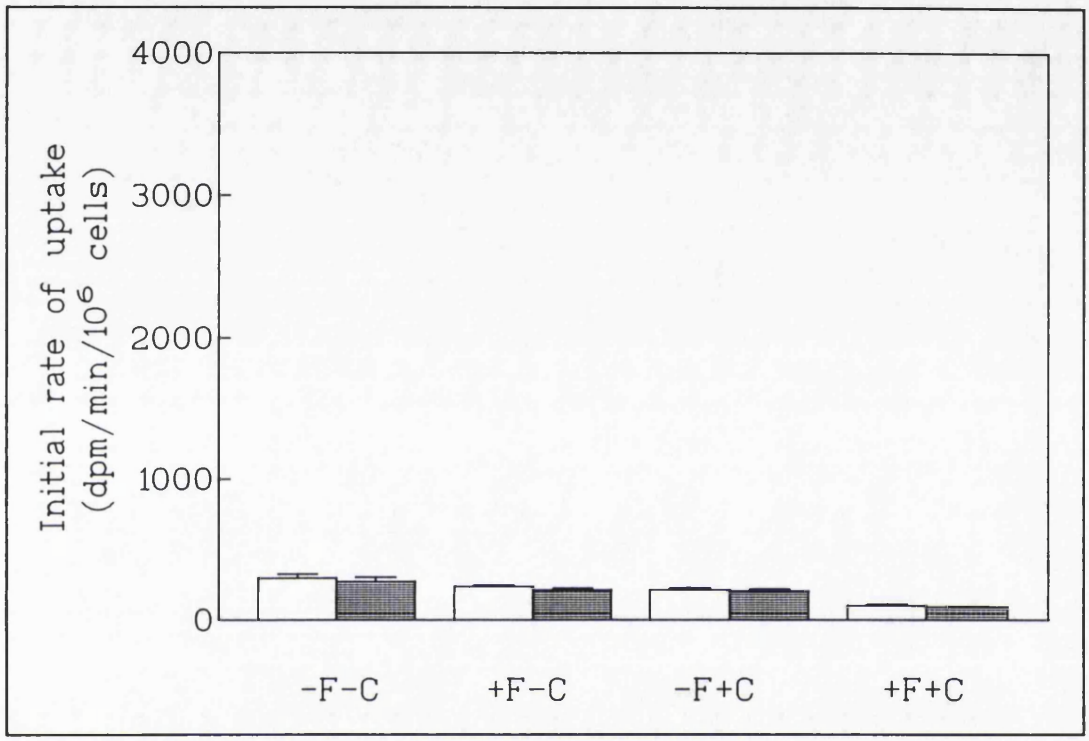


Fig. 5.22. Uptake of 1 mM L-glucose by noninfected- and recombinant virus infected Sf9 cells. Cells were infected with recombinant AcNPV-GT or no virus (MOI = 5) as described in Section 2.5.1. After 2 days infection cells were collected and resuspended in PBS plus(+) and minus (-) 300 mM fructose, and plus (+) and minus (-) 20 μM cytochalasin B. Transport was carried out as described in Section 2.6.1. Bars represent mean ± SEM for three measurements assayed in triplicate. Open bar: Noninfected cells. Speckled bar: recombinant AcNPV-GT-infected cells. F: fructose. C: cytochalasin B.

5.3. DISCUSSION

In order to establish a system useful for the large scale production of normal and mutant mammalian passive glucose transporters for structural and functional studies, an attempt was made to express a human glucose transporter cDNA in insect cells using a recombinant baculovirus. The work described in this chapter has clearly demonstrated that the human erythrocyte transporter can be successfully overexpressed in the insect cell-baculovirus expression system. To my knowledge, this is the first demonstration of functional expression of a mammalian facilitative glucose transporter in substantial amounts using the baculovirus expression system.

The nature of the human glucose transporter expressed in insect cells was analysed by Western blotting using antibodies against residues 477 - 492 of GLUT1. The Western analysis revealed that a single immunoreactive band was present in cells infected with a recombinant baculovirus, but not in the negative controls (uninfected cells and those infected with the wild-type virus). No labelling of the infected or non-infected cells was seen with pre-immune serum. The recombinant polypeptide was also recognized specifically by antisera raised against the central and *N*-terminal regions of the transporter sequence, indicating that the expressed protein corresponded to a full-length rather than truncated form of GLUT1.

The native erythrocyte transporter migrates as a broad band of average apparent M_r 55,000 on SDS/polyacrylamide gels as a result of heterogeneous glycosylation (Sogin and Hinkle, 1978; Gorga *et al.*, 1979). By contrast, the recombinant glucose transporter expressed in Sf9 cells migrated as a sharper

and faster band than its erythrocyte counterpart. It was initially thought that this difference was due to either a difference in glycosylation or truncation. However, the evidence shown in Section 5.2.1 excluded a difference in length of the polypeptide chain. Thus, enzymic deglycosylation was carried out to determine whether the reason for the apparent mass difference was a difference in glycosylation. Treatment of the native erythrocyte transporter with endoglycosidase F, which cleaves both high mannose and complex *N*-linked glycans (Elder and Alexander, 1982), results in its migrating as a much sharper band of apparent M_r 46,000 on SDS/polyacrylamide gels (Lienhard *et al.*, 1984). By contrast, the same enzyme treatment of the expressed transporter caused only a slight increase in the electrophoretic mobility of the protein, suggesting that it is either less-extensively or more homogeneously glycosylated.

N-linked glycosylation is prevalent in mammals. Insect cells are also known to be capable of performing *N*-linked glycosylation of expressed proteins at authentic sites. Recently, Kuroda *et al.* (1990) characterized and compared the chemical structures of the oligosaccharides of baculovirus-produced and vertebrate-produced influenza hemaglutinin. They showed that the sites that are targeted for glycosylation in insect cells are the same as those targeted in mammalian cells. However, the study also indicated that some of the mechanisms of glycosylation in insects differ from those in mammals. Their findings were consistent with earlier studies that mosquito cells lack the galactosyl- and sialyl- transferases required for complex glycosylation (Butters and Hughes, 1981). Thus, the conversion of high-mannose to complex *N*-linked

oligosaccharides known to occur in mammals does not appear to take place in those insect cells. In contrast, mammalian cells extensively modify the core oligosaccharide in terminal glycosylation events involving the transfer of glucosamine, galactose and sialic acid residues to form complex oligosaccharides. Hence, baculovirus-expressed glycoproteins are generally less glycosylated than those produced by mammalian cells. Examples of this phenomenon include beta interferon (Smith *et al.*, 1983), influenza haemaglutinin (Possee, 1986; Kuroda *et al.*, 1990), LCMV GPC protein (Matsuura *et al.*, 1987) and human EGF receptor (Greenfield *et al.*, 1988). However, most studies concerning glycosylation of foreign proteins produced in baculovirus-infected *Spodoptera frugiperda* cells have used indirect approaches, such as assessment of sensitivities to endoglycosidases, the effect of addition of the glycosylation inhibitor, tunicamycin, or the incorporation of radiolabelled sugars. Hence the glycosylation events occurring in the *Spodoptera frugiperda* cell lines used for baculovirus propagation remain to be fully characterized at the molecular level.

Recent structural studies of the carbohydrate chains attached to the human erythrocyte transporter have revealed the presence of high-mannose type oligosaccharides and biantennary complex-type oligosaccharides as their cores and poly-*N*-acetyllactosamine structures as their outer chains (Endo *et al.*, 1990). However, the significance of oligosaccharide chains in glycoprotein structure and function in mammals remains in most cases unclear, and the biological roles of glycosylation of the human glucose transporter also remain controversial. It has been reported that inhibition of protein glycosylation by

tunicamycin led to a decrease in the V_{max} value for hexose uptake in Swiss 3T3 cells without a significant change in the K_m (Kitagawa *et al.*, 1985). Feugeas *et al.* (1990) showed that removal of the carbohydrate moiety of GLUT1 with *N*-glycanase resulted in a decrease of the glucose transport activity. On the other hand, Wheeler and Hinkle (1981) showed that the release of the carbohydrate of the human erythrocyte transporter with endo-β-galactosidase did not alter its transport activity. Similarly, treatment of the transporter with the same enzyme had little effect on cytochalasin binding activity (Gorga *et al.*, 1979). It has been demonstrated by Haspel *et al.* (1986) that extended exposure of fibroblasts to glucose deprivation depleted the transporter of asparagine-linked oligosaccharides and led to an increase in transport activity. The same group later reported that a glucose transporter produced by a cell mutant, which expressed a transporter protein with a high mannose glycosidic chain instead of the complex-type carbohydrate chain present in wild type, exhibited glucose transport activities similar to those observed in normal cells (Haspel *et al.*, 1988a). Recently, Feugeas *et al.* (1991) reported that a minimum glycosidic structure obtainable by partial deglycosylation of the erythrocyte transporter with endo-β-galactosidase is required to maintain glucose transport activity. Evidence that *N*-glycosylation may be important in stabilising the transporter in a form with high affinity for glucose has been provided by Asano *et al.* (1991), who used oligonucleotide-directed mutagenesis of GLUT1 to delete the site of glycosylation at Asn_{45}. Although the transport activity of the baculovirus-encoded transporter remains to be demonstrated, other experimental evidence in the present study suggests that

the difference in glycosylation compared to transporter expressed in mammalian cells is not critical for function. Similar conclusions have been reported for other membrane proteins expressed in the baculovirus/insect cell system (Smith *et al.*, 1992).

Infection of insect cells with a recombinant baculovirus encoding the human erythrocyte glucose transporter resulted in the expression of considerable quantities of the transport protein. The protein was clearly detectable by Western blotting 24 hours after infection and synthesis of the polypeptide became significant 2 days post infection, about 50 % of maximal expression being achieved. The transporter was stable and accumulated until the cells lysed, 4 - 5 days infection. This time course resembled that for the synthesis of polyhedrin protein in wild type AcNPV-infected insect cells (Summers and Smith, 1987), a finding that probably reflected the use of polyhedrin promoter in this expression system. In order to estimate the amount of transporter expressed, cells infected with recombinant baculovirus were subjected to quantitative Western analysis based on the loaded material and the intensities of autoradiographic signals. The study showed that 4 days post infection up to 1.47 nmol of transporter was present per mg of membrane protein, equivalent to almost 8 % (w/w) of the total membrane protein assuming M_r = 54,117. However, it was not possible to distinguish the protein as a separate sharp band on Coomassie blue-stained gels. Comparable levels of expression have been reported in the baculovirus system for some other membrane glycoproteins (Klaiber *et al.*, 1990), although the mammalian Na^+/glucose cotransporter was found to be expressed at a somewhat lower

level, corresponding to 5 % of the membrane protein (Smith *et al.*, 1992). Some non-membrane proteins have been expressed to levels equating to polyhedrin itself, i.e. 35 - 50 % of total cell protein (Emery, 1991). Thus, it is difficult to generalize how efficiently foreign genes will be expressed in the baculovirus expression system. The level of synthesis of a particular protein must be determined empirically, because the factors determining the level of expression of a foreign gene in the system have yet to be characterized. However, in most cases the expression rates in established cell lines are strongly dependent on the details of construction of the vectors and the intrinsic properties of the foreign gene product and its acceptability in the host-cell (Matsuura *et al.*, 1987; Luckow and Summers, 1988). How a cell responds to the synthesis of large amounts of a glycoprotein could be an interesting area for future investigation. Insect cells infected with a recombinant baculovirus containing a gene for a glycoprotein exhibited an extensive cytoplasmic vacuolation due to overproduction of the glycoproteins (Matsuura *et al.*, 1987). In case of the human multidrug transporter expressed in insect cells, the glycoprotein was also found within the nucleus envelope which is not the location for its native counterpart (Germann *et al.*, 1990).

Immunofluorescence microscopy has provided further evidence for the expression of human glucose transporter in insect cells. Although the level of expression of the glucose transporter was higher at 4 - 5 days post infection, cells were examined 2 days after infection because infected cells eventually lyse at the time of maximal protein expression. The lysis process is likely to be dependant on the health of the cells and the freshness of the media and oxygen

levels. Without changing media or oxygenating cells, lysis usually begins about 60 hours p.i. and by 72 hours p.i., most cells are in the process of dying or lysing (O'Reilly *et al.*, 1992). A high level of fluorescence displayed in cells infected with AcNPV-GT virus indicated that the transporters are expressed abundantly and present on the surface of infected Sf9 cells. The evidence for the specificity of the immunostaining was strengthened by the negative results shown in the negative controls. These observations suggest that insect cells synthesise and process the glucose transporter protein in a manner similar to human cells. Thus, the protein is correctly inserted into the plasma membrane.

Distribution of the transporter protein expressed in insect cells was revealed by making a series of optical sections through an AcNPV-GT-infected cell using a confocal microscope, which permits optical sectioning of cell sample. These optical sections of the recombinant baculovirus infected cells displayed intense cytoplasmic immunofluorescence surrounding the region occupied by the enlarged nucleus, indicating that the expressed protein was present not only at the cell surface but also throughout the cytoplasm. However, the presence of the protein was confined to the internal membranes but not in the cytosol, as the immunologically cross-reactive band was detected only in the membrane fractions of the cells (Fig. 5.1). Previous studies have shown that high level of synthesis of glycoproteins results in highly vacuolated cytoplasmic membranes (Matsuura *et al.*, 1987). Although more precise localization of the expressed protein could have been obtained by electron microscopic immunocytochemistry, this approach was not adopted in the present study. But electron microscopy was performed to examine

ultrastructures of infected cells. It is noteworthy that Sf9 cells undergo important morphological modifications after infection by baculoviruses (Harrap, 1972b). First, a nuclear expansion characteristic of cytopathic effects results in an increase of the cellular diameter. Second, a large network of densely stained material, at electron microscopic level, known as virogenic stroma is formed often in a less well stained matrix. From the electron micrographs of uninfected and infected cells, it was clearly shown that the nuclei of infected cells were much larger than those of uninfected cells indicating a successful viral infection, although the presence of virogenic stroma was not obvious in the micrographs. However, proliferation of internal membranes was not observed.

Three attempts have been made to demonstrate whether the transporter expressed in insect cells is biologically active. Firstly, by performing binding assays with a radiolabelled specific inhibitor of the GLUT1. Secondly, by photolabelling of the expressed protein with [^{3}H] cytochalasin B. Lastly, by measuring the glucose transport activity of the recombinant protein expressed in Sf9 cells. However, it was not possible to determine the transport function of the expressed protein in the insect cells, because of the presence of highly active, endogenous transport systems which allow the cells to grow on glucose. Fortuitously, however, the insect-cell transporter(s) was found neither to cross-react with antibodies to the mammalian protein nor to bind cytochalasin B, a potent competitive reversible inhibitor of glucose transport. The binding of this inhibitor could therefore be used as a measure of the biological activity of the expressed protein. Studies of reversible cytochalasin

B binding showed that the recombinant protein expressed in Sf9 cells not only bound the cytochalasin B with characteristics identical to the native erythrocyte protein, but also bound the transported substrate D-glucose. The functional activity of the expressed transporter was confirmed by the ability of the 45 kDa protein, recognised by antibodies against GLUT1 on Western blots, to be photoaffinity-labelled by cytochalasin B. The mechanism of photolabelling is not yet understood, but it appears to proceed via activation of an aromatic amino acid residue on the transporter rather than by photoactivation of the ligand itself (Deziel *et al.*, 1984). D-glucose and other transported sugars, but not L-glucose, have been reported to inhibit photolabelling of the erythrocyte protein by displacing non-covalently-bound cytochalasin prior to photolabelling (Shanahan, 1982,1983; Carter-su *et al.*, 1982). A similar inhibition was seen in the present study, and confirmed the ability of the expressed protein to bind its substrate, D-glucose.

It is therefore concluded that human glucose transporter expressed in insect cells exhibits native-like biological activity, although purification and reconstitution will be required to demonstrate that it can actually transport glucose. However, the concentration of cytochalasin B-binding sites obtained by ligand binding assay was less than the concentration of immunologically cross-reactive protein. A phenomenon similar to this has been reported for other membrane glycoproteins (Germann *et al.*, 1990; Klaiber *et al.*, 1990). Although the reason for this discrepancy is not clear, it may stem from the high level of expression of the transport protein. As shown in Figure 5.9, not all the transporters expressed in insects cells were located in the plasma membrane,

many of them were distributed in internal membranes as revealed by the series of confocal sections of the AcNPV-GT infected cell. Due to overproduction of the glycoprotein, the efficiency of the translocation process bringing newly-synthesised protein to the plasma membrane might have been limiting and so some protein might have been retained in the Golgi stack membranes. In addition, one can not rule out the effect of the less extensive glycosylation of the expressed protein compared to its erythrocyte counterpart. The possibility that alterations in asparagine-linked glycosylation may change the cell surface localization or acquisition of a functional conformation of the glucose transporter has been suggested (Haspel *et al.*, 1988a).

In conclusion, the work described in the present chapter clearly demonstrates that the aim of my study, to develop a means of producing large amounts human GLUT1 by recombinant DNA techniques using the baculovirus expression system, has been successfully achieved. Although some non-functional protein is apparently present, expression of biologically active homogeneous human glucose transporter in the baculovirus system is nonetheless abundant, amounting to between 1 and 2 % of the total membrane protein. In addition, it may be possible to produce the erythrocyte glucose transporter without contamination by constructing recombinant fusion proteins that could facilitate purification. Preparations of the human GLUT1 are known to contain the erythrocyte nucleoside transporter as a minor component (Lin and Snyder, 1977; Jarvis and Young, 1981). Therefore, the baculovirus system will not only provide a means of obtaining large quantities of human erythrocyte glucose transporter for biochemical and mechanistic studies, but it

will also be a system for the study of structure-function relationships of the protein by site-directed mutagenesis in the absence of other contaminating mammalian proteins.

CHAPTER 6. ATTEMPTED PURIFICATION OF RECOMBINANT PROTEIN

6.1. INTRODUCTION

In the work described in the previous chapters, it was demonstrated that biologically active human glucose transporter could be expressed in substantial amounts by using the baculovirus-insect cell expression system. However, it was not possible to show that the expressed transporter could actually transport glucose. The probable reason for this was that the activity of the endogenous insect glucose transporter was extremely high and so rendered transport activity resulting from the expression of exogenous transporter very difficult to detect. Therefore, it was necessary to develop procedures for the rapid isolation of recombinant transporter in functional form from the expression system.

To achieve this, several potential approaches were considered. One of them was an affinity chromatographic technique based on a tight-binding inhibitor such as cytochalasin B. However, the non-ionic detergents required for transporter solubilization are known to be inhibitors of cytochalasin B binding (Zoccoli *et al.*, 1978). The use of highly specific monoclonal antibodies that were available in our laboratory was also considered. Binding of the transporter to these antibodies is not affected by non-ionic detergent. Unfortunately, isolation of functional membrane transport proteins by immuno-affinity chromatography is frequently unsuccessful, because the harsh conditions required to elute the proteins from the column of immobilised antibody denature them. The interaction between the available monoclonal

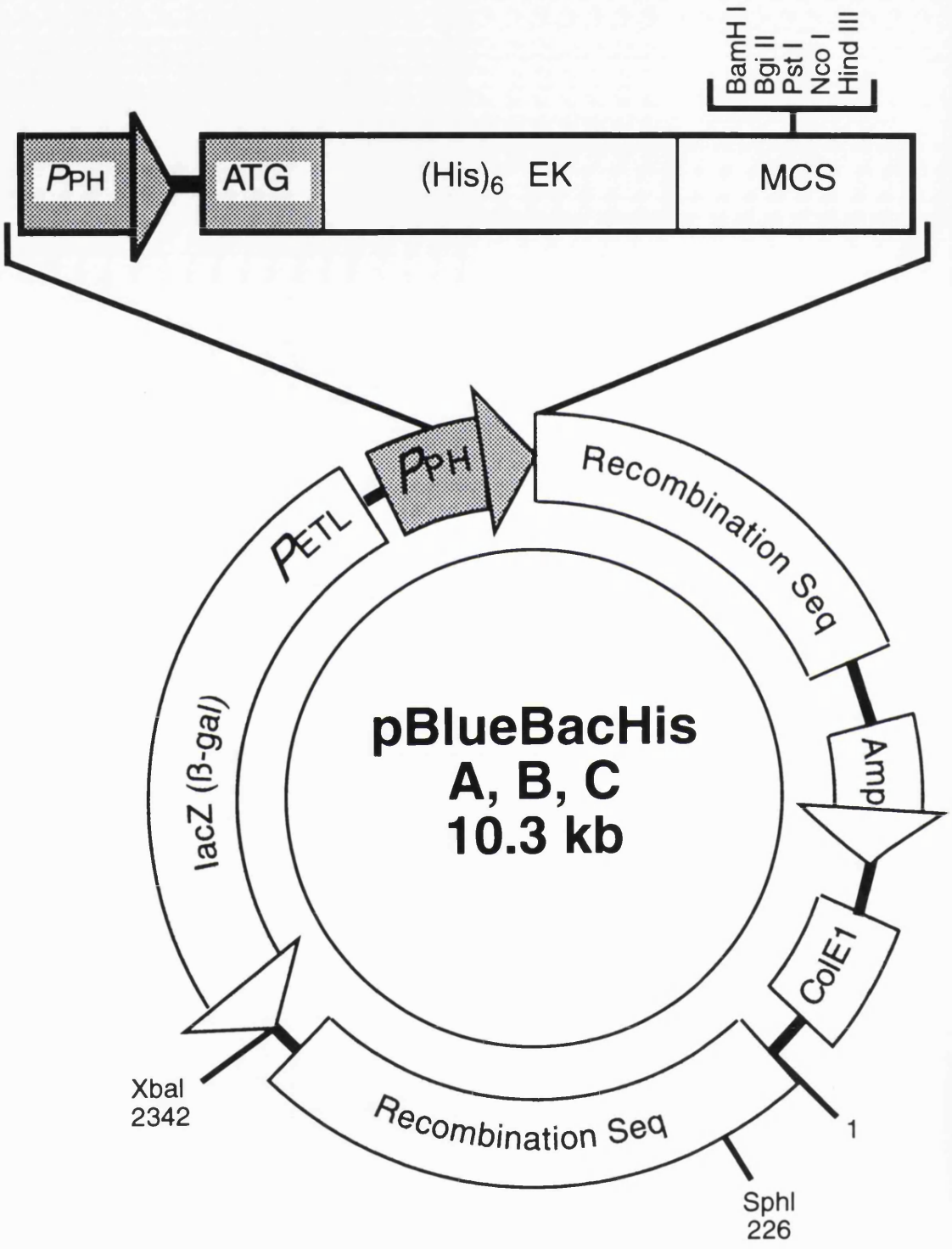
antibodies and the glucose transporter is known to be reversible only by extremes of pH (S.A. Baldwin, personal communication). An alternative approach was to exploit gene fusion techniques. Such a strategy has already been successfully used for the purification of another polytopic membrane protein, the Na^+/proline co-transporter from *Escherichia coli* (Hanada *et al.*, 1987, 1988). For this purification procedure, recombinant DNA technology was used to make a construct that encoded the proline carrier joined via a collagen "linker" sequence to β-galactosidase. After expression of the fusion protein in *E. coli*, cytoplasmic membranes containing the protein were solubilized in detergent. Then the fusion protein was specifically adsorbed onto an anti-β-galactosidase IgG-Sepharose column. The target protein was finally eluted under mild, non-denaturing conditions by site-specific proteolysis of the linker with collagenase.

In mid-1992, Invitrogen introduced the pBlueBacHis vectors, which were designed for high level production and direct purification of recombinant fusion proteins from insect cells. The vectors were derived from pJVETL-Z (Vialard *et al.*, 1990) and contain twin promoters derived from the polyhedrin and ETL genes of AcNPV. The polyhedrin promoter is utilised to direct the synthesis of foreign gene products, whereas the ETL promoter directs expression of vector-encoded β-galactosidase and thus provides a marker for visual identification of blue recombinant viral plaques. The pBlueBacHis vector contains the natural polyhedrin leader sequence followed by sequences encoding an ATG translation initiation codon, a tract of six histidine residues that function as a metal binding domain in the translated protein, a transcript-stabilizing sequence from gene 10

of phage T7, and an enterokinase cleavage recognition sequence. Next is a multiple cloning site that contains unique BamHI, BstII, PstI, NcoI and Hind III sites (Fig. 6.1).

The rationale behind the use of this type of expression vector is that it produces recombinant proteins with a short *N*-terminal leader peptide from the bacteriophage T7 gene 10 protein that can be removed by cleavage with enterokinase. This leader peptide contains a polyhistidine sequence that has a high affinity for divalent metal ions and an enterokinase-specific cleavage site. The metal binding domain of the fusion peptide allows purification of recombinant proteins by immobilized metal affinity chromatography using, for example, a Ni^{2+}-charged affinity resin. The polyhistidine-containing fusion proteins bind with high affinity to the metal-charged resin under normal binding conditions (200 mM phosphate buffer, 500 mM NaCl, pH 7.8), while most of the host cell proteins do not bind to the resin. Any host cell proteins that may also have some intrinsic affinity for the Ni^{2+} on the resin can usually be selectively removed by washing the protein-bound resin with a lower pH buffer (eg. pH 6.0). The polyhistidine-containing recombinant protein can then be eluted from the column by lowering the pH of the buffer (eg. to pH 4.0) or application of imidizole buffer (pH 6.0), which competes with the recombinant protein for the Ni^{2+} ions on the resin. Elution with imidizole buffer provides a particularly mild procedure for protein purification. Following elution, the leader peptide can be removed from the fusion protein by cleavage with porcine enterokinase, which cleaves the protein immediately after the lysine residue in the recognition sequence (asp-asp-asp-asp-lys-asp) located *N*-terminal to the

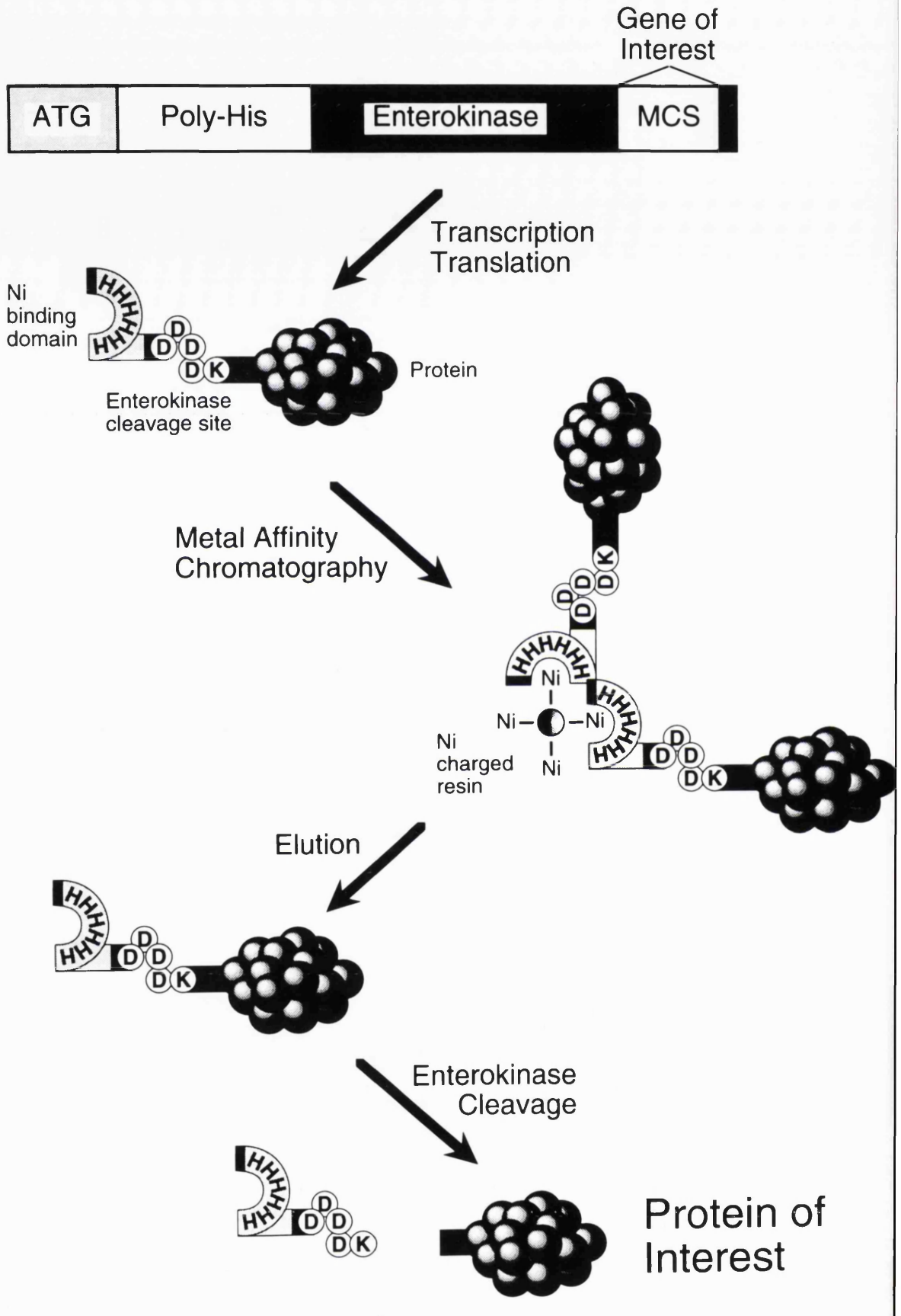
Fig. 6.1. Schematic of pBlueBacHis transfer vector. The vector provides translation start site and leader peptide from the T7 gene 10 protein plus a polyhistidine metal-binding domain and an enterokinase cleavage site. The vector also express β-galactosidase from the ETL promoter to aid visual screening for recombinant viruses.


BamH I
Bgl II
Pst I
Nco I
Hind III
PPH
ATG
(His)6 EK
MCS
PPH
PETL
Recombination Seq
lacZ (β-gal)
pBlueBacHis
A, B, C
10.3 kb
Amp
ColE1
Xbal
2342
Recombination Seq
1
SphI
226

junction of the leader peptide and the protein of interest. Separation of the recombinant protein from the leader peptide is then achieved by passing the digest once more through the metal-charged resin under binding conditions. The methodology is summarised in Figure 6.2.

In the work described in this chapter, I used a pBlueBacHis vector for the construction of the recombinant transfer vector, pBacHis-GT, in an attempt to facilitate purification of the recombinant protein. Recombinant baculoviruses were then generated by allelic exchange following transfection of insect cells with "wild-type" virus and the recombinant transfer plasmid (Summers and Smith, 1987). To improve the efficiency of such recombination events, "BaculoGold DNA" from PharMingen was used as "wild-type" virus. This viral DNA bears a lethal deletion of a gene, adjacent to the polyhedrin locus, that is essential for virus viability (Possee *et al.*, 1991). Use of this viral DNA selects for recombinants because viable viruses can be produced only by recombination of the DNA with the transfer vector. The latter includes sequences complementary to the lethal deletion and so recombination will restore the integrity of the essential viral gene (Fig. 6.3).

Fig. 6.2. Schematic representation of strategies involved in the expression and purification of a recombinant fusion protein. Following expression of recombinant fusion protein with a metal-binding domain and enterokinase cleavage site, the fusion protein is recovered by competition with imidizole using immobilized metal charged resin. The leader peptide is then removed from the fusion protein by cleavage with porcine enterokinase. Final separation of the recombinant protein of interest is achieved by passage over Ni^{2+}-charged resin under binding conditions.


Gene of Interest
ATG
Poly-His
Enterokinase
MCS
Transcription
Translation
Ni binding domain
Protein
Enterokinase cleavage site
Metal Affinity Chromatography
Ni charged resin
Elution
Enterokinase Cleavage
Protein of Interest

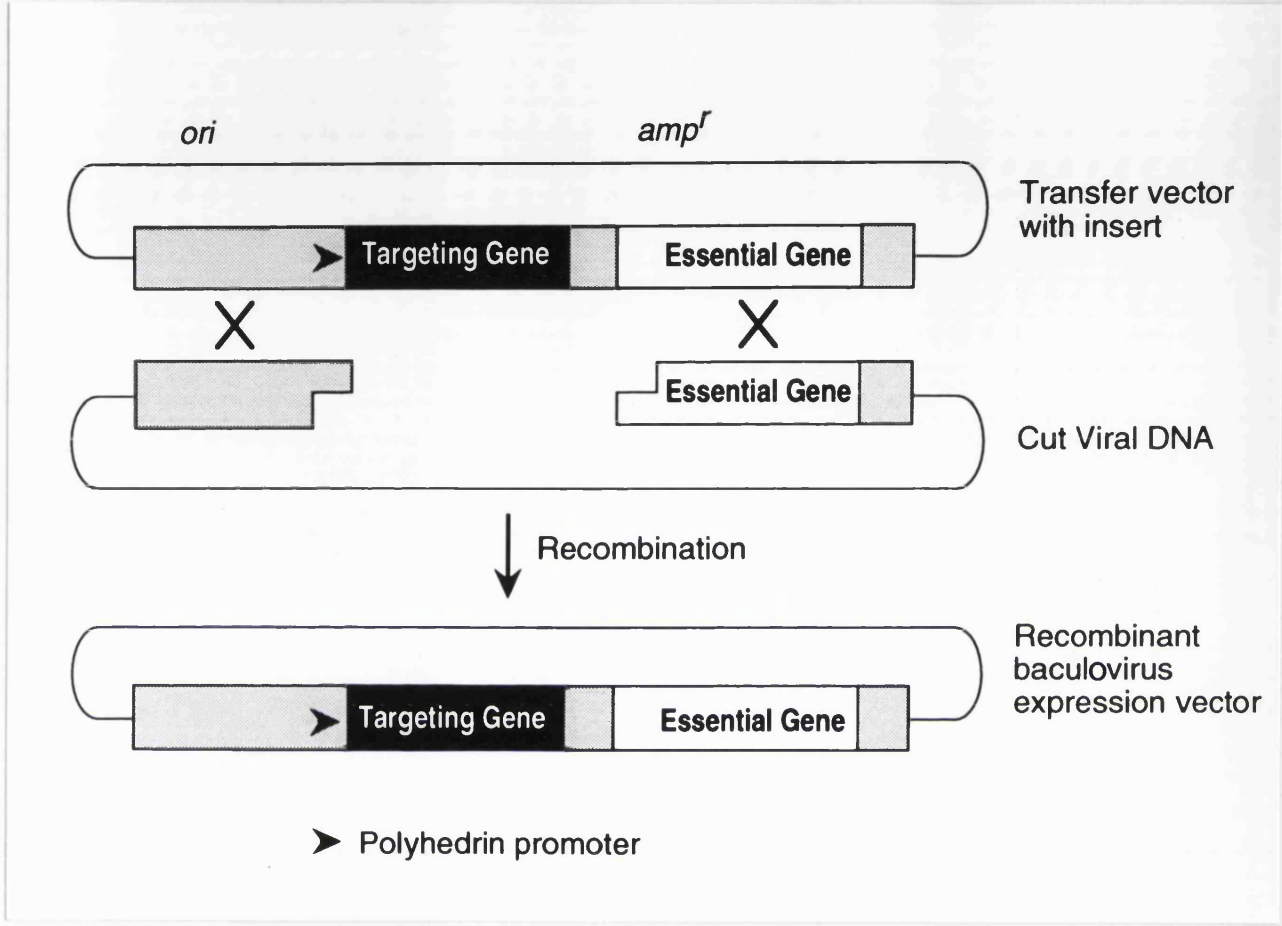


Fig. 6.3. Illustration of allelic replacement reaction between recombinant transfer vector and viral DNA containing the lethal deletion.

6.2. M13 SUBCLONING

Expression of GLUT1 in cells infected with AcNPV-GT, described in Chapter 4, exploited the natural initiation codon of the transport protein. However, expression of GLUT1 from cDNA inserted into the BamHI site of pBlueBacHis utilised an ATG initiation codon contained within the vector sequence. Three versions of pBlueBacHis are commercially available, such that any BamHI insert can be placed in frame with this vector-encoded ATG. However, in order to choose the appropriate pBlueBacHis vector for expression of GLUT1 it was first necessary to sequence the region between the natural initiation codon of the transporter and the adjacent BamHI site in pSGT, because this sequence had not been reported in the literature. The BamHI insert of pSGT was therefore subcloned into M13mp18 for sequencing. The reason for cloning into M13 was that the phage could produce single-stranded DNA templates which would be useful in future studies of site-directed mutagenesis as well as sequencing. Many aspects of the handling, storage, propagation and manipulation of M13 vectors for use as cloning vehicles have been described in Section 2.5.5. The BamHI fragment of GLUT1 cDNA was inserted into the bacteriophage M13mp18 vector in the same way that it was inserted into plasmid vectors.

To use M13 as a cloning vector, it was necessary to prepare the double-stranded M13 replicative form (RF), because only double-stranded DNA could be cut with restriction enzymes. RF DNA was linearized by cleavage with BamHI restriction enzyme, and then treated with phosphatase (CIP) to reduce the background of non-recombinant molecules formed by recircularization of the

vector. The linear vector was then ligated with an excess of a GLUT1 cDNA fragment that had been generated by BamHI digestion of the plasmid pSGT. The ligation mix was then used to transform competent *E. coli* (TG2 strain) cells as described in Section 2.3.10. Bacteriophages carrying the insert of interest were identified by their ability to form colourless plaques on LB plates containing IPTG and X-gal.

Twenty-four colourless plaques were selected and examined by restriction digestion with Hind III, followed by agarose gel electrophoresis. The restriction analysis revealed that all the bacteriophage plaques appeared to contain recombinants in which the cDNA insert was present in the same orientation. A typical result is represented in Figure 6.4. One of these recombinants (designated M13 GTW) was chosen for further analysis by restriction digestion with BamHI. The presence of a 2.5 kb fragment within the digest confirmed the presence of the BamHI insert of GLUT1 cDNA with the expected size (Fig. 6.5). This recombinant was used for sequencing.

6.3. PARTIAL SEQUENCING OF GLUT1 cDNA

Sequence analysis of double stranded DNA was carried out by the chain termination method (Sanger *et al.*, 1977), using Sequenase Version 2 (a variant of T7 DNA polymerase with no 3'-5' exonuclease activity) in a kit from United States Biochemical (USB). Although the chain-termination method is known to work best when using single-stranded templates, many laboratories have begun using double-stranded DNA directly for sequencing because of simplicity and convenience of the method. In order to be able to read sequence very close to

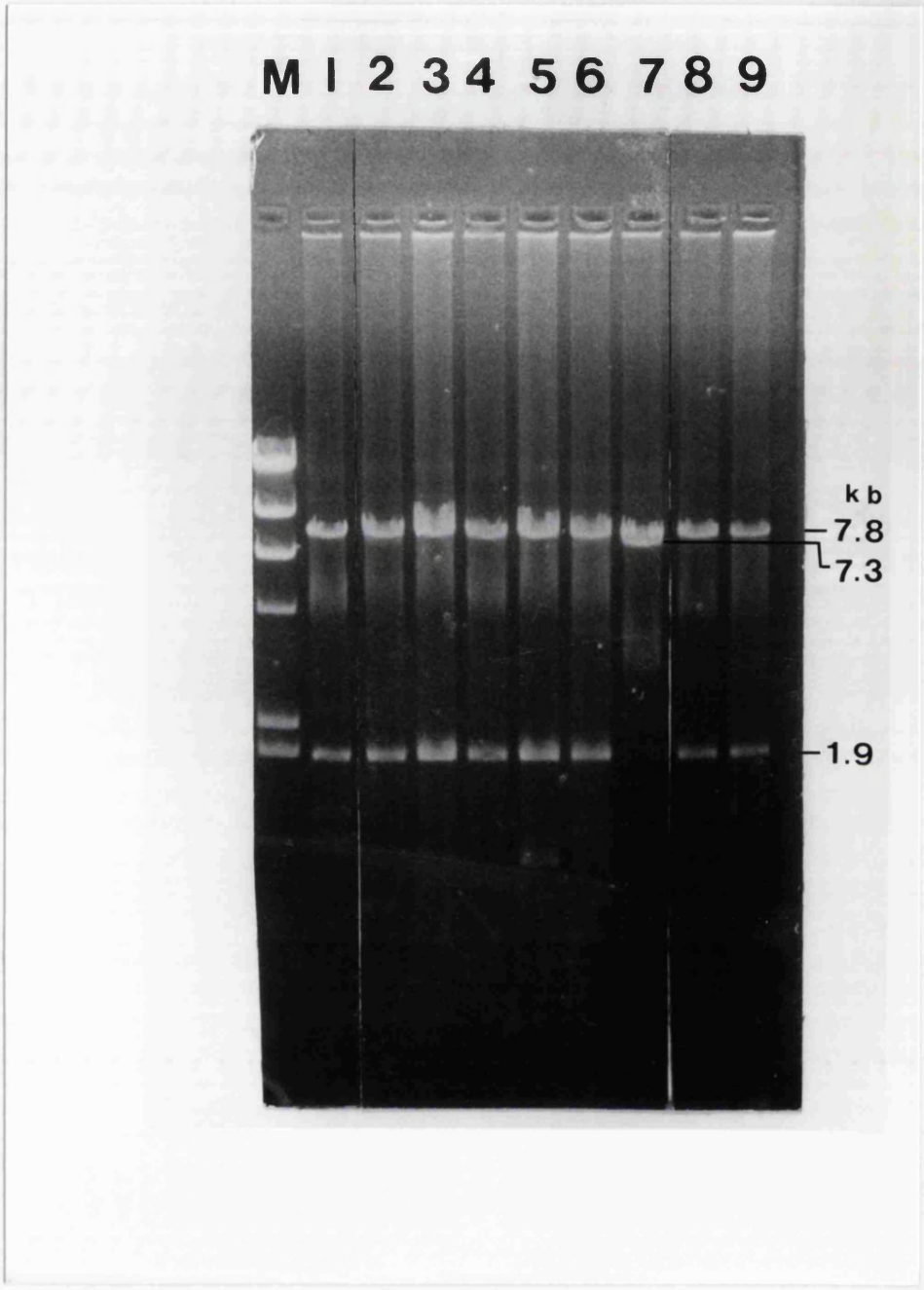


Fig. 6.4. Agarose gel electrophoresis of Hind III-cleaved products of plasmid preparations isolated from colourless M13 plaques. The plasmid DNAs were digested with Hind III and then analyzed in a 0.8 % agarose gel as described in Chapter 2. Lane M shows λ DNA/Hind III size markers (from top: 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56 kb). Lane 7 represents wild-type M13mp18 without insert (7.3 kbp).

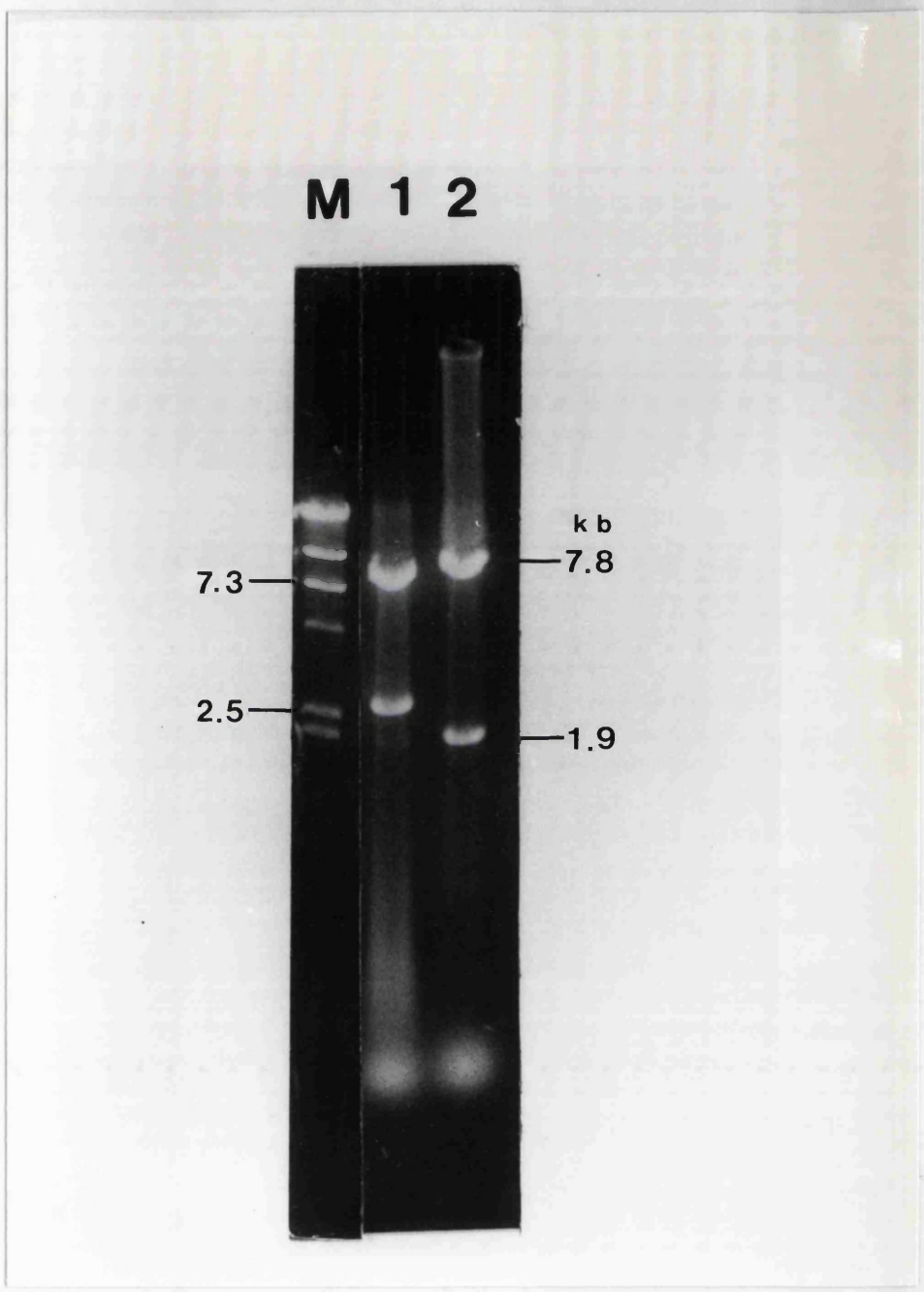


Fig. 6.5. Restriction enzyme analysis of plasmid M13-GTW. The plasmid was digested with BamH1 or HindIII. The digest products were resolved by electrophoresis through a TAE-buffered 0.8 % agarose gel containing ethidium bromide. Lane M: Lambda DNA digested with Hind III (from top: 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56 kb). Lanes 1 and 2 show the digestion of M13-GTW with BamH1 and Hind III, respectively.

the primer, low concentrations of nucleotide were used in the labelling reactions and Mn, which affects the termination step, was included in the reaction buffer, as described by the manufacturers and detailed below.

6.3.1. Preparation of template DNA

The DNA of M13 GTW was extracted by the alkaline lysis method, RNase-treated, and purified by phenol and chloroform extraction as detailed in Chapter 2. Before sequencing, the ds DNA was alkali-denatured by adding 6μl of 1 M NaOH, 1 mM EDTA to 5 μg of M13 GTW DNA dissolved in 25 μl of TE and incubating at room temperature for 10 min. The DNA was then separated from alkali by passage through a "Spin-Column." Such columns were prepared by piercing the bottom of a 0.5 ml eppendorf tube with the tip of a 21 gauge needle and then adding 20 μl of glass beads (Sigma G-1145, 150-212μm) followed by 500 μl of Sepharose CL-6B equilibrated in TE. The 0.5 ml tube was mounted in a 1.5 ml eppendorf tube, which was also perforated with the needle. The eppendorf tubes, supported in a glass test tube, were centrifuged in a bench centrifuge at 3,000 rpm for 5 min to remove the sepharose buffer. Following alkali denaturation, the DNA was transferred to a column prepared as above and centrifuged at 3,000 rpm for 3 min.

6.3.2. Annealing, labelling and termination reactions

In order to anneal the template DNA to the sequencing primer, 6 μl of the denatured template DNA was mixed with 2 μl of 5 X annealing buffer (100 mM $MgCl_2$, 200 mM Tris-HCl, pH 7.5, 250 mM NaCl) and 2 μl (3 ng/μl) of M13

sequencing Primer (-20 forward, USB). The mixture was heated to 65°C for 2 min and then allowed to cool slowly to room temperature over a period of about 30 min. After cooling, the mixture was placed on ice and then used within 4 hours. For labelling, to the annealed primer-template mixture was added 1 μl of 100 mM DTT, 2 μl of diluted labelling mix (0.75 μM dGTP, dCTP and dTTP), 1 μl of [α-^{35}S]dATP at 10 μCi per μl and 10 μM (1000 Ci/mmol), 1 μl of Mn buffer (0.15 M sodium isocitrate, 0.1 M $MnCl_2$) and 2 μl of Sequenase Version 2.0 enzyme (diluted 1:8 in 10 mM Tris-HCl, pH 7.5, 5 mM DTT, 0.5 mg/ml BSA). Following mixing, the labelling reaction was incubated at room temperature for 2 min and then immediately placed on ice.

A sample (3.5 μl) of the above labelling mix was transferred to each of 4 eppendorf tubes (labelled G, A, T and C), and 2.5 μl of the appropriate, prewarmed termination buffer was spotted on to the side of each tube. The compositions of the buffers were shown in Table 6.1. The reactions were then started simultaneously by brief centrifugation in a microfuge, followed by incubation at 37°C for 5 min. They were stopped by the addition of 4 μl of stop solution (95 % formamide, 20 mM EDTA, 0.05 % bromophenol blue, 0.05 % xylene cyanol FF) and stored on ice until ready to load the sequencing gel. Sequencing mixtures were denatured at 90°C for 5 min before loading onto 6 % polyacrylamide, 8 M urea gels (0.4 mm thick), prepared and pre-run in 1 X Tris-borate buffer (TBE, 0.08 M Tris-borate, 0.02 M EDTA, pH 8.0). Gels were electrophoresed at a constant power (35 watts) until the bromophenol blue dye had run about 80 % of the length of the gel. Following electrophoresis, the gel was fixed in 10 % (v/v) acetic acid for 30 min, dried onto Whatman 3 MM

Table 6.1

Composition of sequencing termination mixes

Termination Mix	Composition
ddG	8 μM ddGTP, 80 μM dNTPs*, 50 mM NaCl
ddC	8 μM ddCTP, 80 μM dNTPs*, 50 mM NaCl
ddT	8 μM ddTTP, 80 μM dNTPs*, 50 mM NaCl
ddA	8 μM ddTTP, 80 μM dNTPs*, 50 mM NaCl

* 80 μM each of four respective dNTPs (dATP, dCTP, dGTP, dTTP)

paper and autoradiographed overnight at room temperature (Fig. 6.6)

6.3.3. Sequence data

The sequence was read directly into a computer data base using a sonic digitizer and analysed using the DNAStar program. The sequence data of interest was the sequence between the BamHI site and the initiation codon ATG, because it was not known which linker had been used for construction of the plasmid PSGT. The sequence obtained is shown below and underlined, with the BamHI site and ATG in bold:

.....CAGGTCGACTCTAGA**GGATCC**GCGTTGCC**ATG**GAG.....

6.4. CLONING OF GLUT1 cDNA INTO TRANSFER VECTOR

The sequence between the proximal BamHI site and the first codon of GLUT1 in pSGT, determined as described above, enabled the appropriate pBlueBacHis vector for GLUT1 expression to be chosen. Detailed sequence maps of the vectors provided by Invitrogen indicated that insertion of the GLUT1 BamHI fragment into pBlueBacHis C would place the coding region in frame with the ATG initiation codon provided by the vector, and so pBlueBacHis C was used for the construction of a recombinant transfer vector (pBacHis-GT). Figure 6.7 illustrates the nucleotide sequence and the corresponding amino acid sequence between the vector ATG and the GLUT1 ATG.

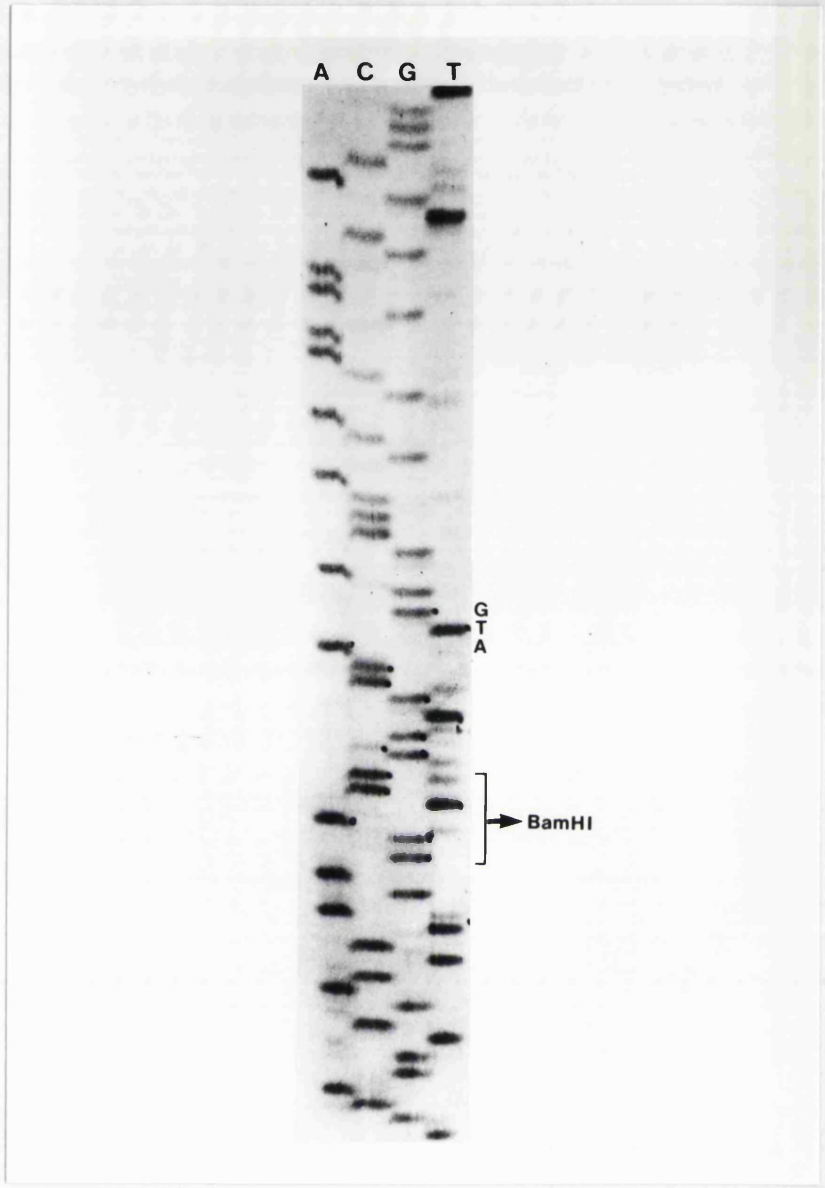


Fig. 6.6. Autoradiogram of a dideoxy sequencing gel. Sanger sequencing gel (6 % , w/v) shows the partial sequence of the GLUT1 cDNA cloned into M13 mp 18 (M13-GTW). The order of the sequencing tracks is A, C, G, and T, reading from the left-hand side. The arrow points to the cloning site (BamH1) in M13mp18.

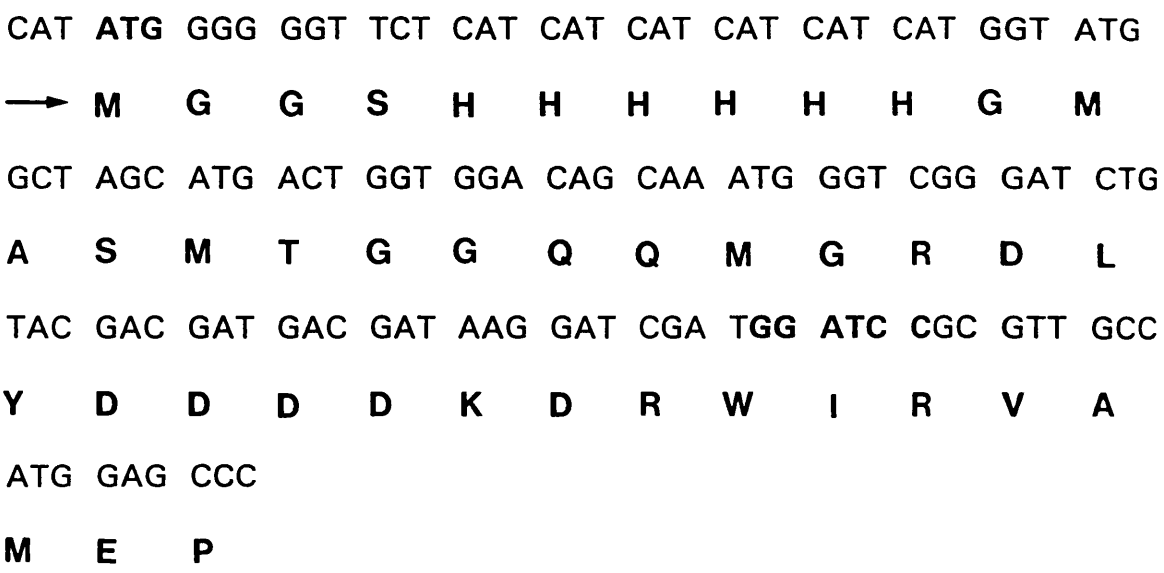


Fig. 6.7. Nucleotide sequence and predicted polypeptide sequence between the initiation codon of pBlueBacHis C and of GLUT1. Amino acid residues are shown using the single-letter amino acid convention. The restriction enzyme BamHI cleavage site is underlined.

6.4.1. Preparation of vector and insert DNA

The plasmid pBlueBacHis C was propagated by transforming 100 ng of the plasmid DNA into competent *E. coli* (Top 10 strain) cells. Since the pBlueBacHis C plasmid contains the PUC origin of replication and an ampicillin resistance gene, the transformants were grown overnight on LB plates containing ampicillin (100 μg/ml). A small volume (5 ml) of LB broth was inoculated with a single Top 10 colony and grown at 37°C overnight in the presence of the antibiotic (50 μg/ml). Plasmid DNA was then prepared by using a Magic Miniprep DNA preparation kit from Promega. Cells from 2 ml of the overnight-grown culture were pelleted by centrifugation using a microfuge and resuspended in 20 μl of Resuspension Solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 μg/ml RNase A). The cells were lysed by adding 200 μl of Lysis Solution (0.2 M NaOH, 1 % SDS), mixed by inverting the tube several times, and neutralised by the addition of 200 μl of Neutralisation Solution (2.55 M potassium acetate, pH 4.8). The mixture was then clarified by centrifugation for 5 min at 12,000 x g. The supernatant was mixed well with 1 ml of the Magic Minipreps DNA purification Resin, and then passed through a Magic Minicolumn. The column was washed with 2 ml of column wash solution (200 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA) and centrifuged for 30 seconds at 12,000 x g in a microfuge to dry the resin. The DNA was then eluted by applying 40 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) onto the column and centrifuging it for 20 seconds at 12,000 x g. The DNA was then analysed by restriction digestion with BamHI and EcoRI enzymes, which yielded fragments of the expected size. Following confirmation of its

identity in this way, the plasmid DNA was prepared on a large scale from 40 ml of an overnight-grown Top 10 culture by using the Magic Minipreps System. To obtain a fragment containing the GLUT1 cDNA, plasmid pSGT DNA was prepared from a 5 ml of overnight culture by the alkaline lysis method described in Chapter 2, treated with RNase, and digested with BamHI restriction enzyme. The digest was then electrophoresed on a 1 % (w/v) low melting point agarose gel as described in Chapter 2. Following electrophoresis, a 2.5 kb fragment containing the entire coding sequence of the GLUT1 cDNA was excised from the gel and purified by the standard phenol/chloroform extraction procedure.

6.4.2. Phosphatase treatment

The pBlueBacHis C vector DNA prepared from Section 6.4.1, was linearized by digestion with BamH1 (3 units/μg), and then phosphatase-treated to prevent recircularisation of the vector during the ligation reaction. Dephosphorylation was carried out by incubation with 0.1 unit of CIP per μg of DNA at 37°C for 30 min in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. The reaction was terminated by phenol/chloroform extraction followed by ethanol precipitation as previously described in Chapter 2.

6.4.3. Ligation and transformation

Ligation of the GLUT1 cDNA into the de-phosphorylated, BamHI-digested pBlueBacHis C vector was carried out, essentially as described in Section 4.2.3, in the absence of hexamminecobalt chloride. The reaction was incubated at 12°C for 16 h. Following ligation, 8 μl of the ligation mixture was used to

transform competent *E. coli* (strain Top10) cells which were then plated onto LB plates containing ampicillin (50 μg/ml). The results of transformation are shown in Table 6.2, in terms of number of colonies. Since a very large number of colonies were produced on plating out Top 10 strain transformed with undigested, circular vector, it could be assumed that the potential transformation efficiency of the cells was high. The cells transformed with the BamH1-digested, de-phosphorylated vector DNA alone also produced some colonies, probably as the result either of incomplete dephosphorylation or incomplete restriction digestion.

6.4.4. Identification of recombinant plasmids

Ten well-separated transformants were picked, and each was then inoculated into 5 ml of LB medium containing 100 μg/ml ampicillin and grown overnight at 37°C. The plasmid DNAs were prepared by using the Magic Miniprep system (Section 6.4.1) and characterized by restriction enzyme digestion with Hind III (Section 2.3.5). Recombinants were expected to contain two sites for cleavage by this enzyme, one in the multi-cloning region of the vector and one in the GLUT1 cDNA insert. Six colonies appeared to contain recombinants with an insert in the desired orientation, by virtue of their yielding Hind III fragments identical in size to those expected (12.3 kb and 0.5 kb) (Fig. 6.8). These putative recombinants were further examined by restriction analysis with BamHI and EcoRI enzymes. The EcoRI digests gave the three bands (6.8 kb, 3.8 kb and 2.2 kb), that were expected for the correct construction (Fig. 6.9). Furthermore, the presence of the 2.5 kb insert of

Table 6.2

Transformation results of cloning the 2.5 kbp BamHI fragment of GLUT1 cDNA into pBlueBacHis C.

Ligation Conditions		No. of Transformants	
		Portion^{+}	Rest^{-}
Vector:Insert (molar ratios)	1:1	16	70
	1:3	36	>200
	3:1	7	32
Modified vector only		0	12
No DNA		0	0
100 ng Uncut, circular Vector		>100	confluent

Competent *E. coli* (strain Top 10) were transformed according to the procedures described in Section 2.3.10, using 8 μl of the ligation reaction. Following transformation, 100 μl (+) of the transformed cells were plated out onto LB plates containing ampicillin. The rest (-) of the cells were pelleted and resuspended in 100 μl of LB medium before plating.

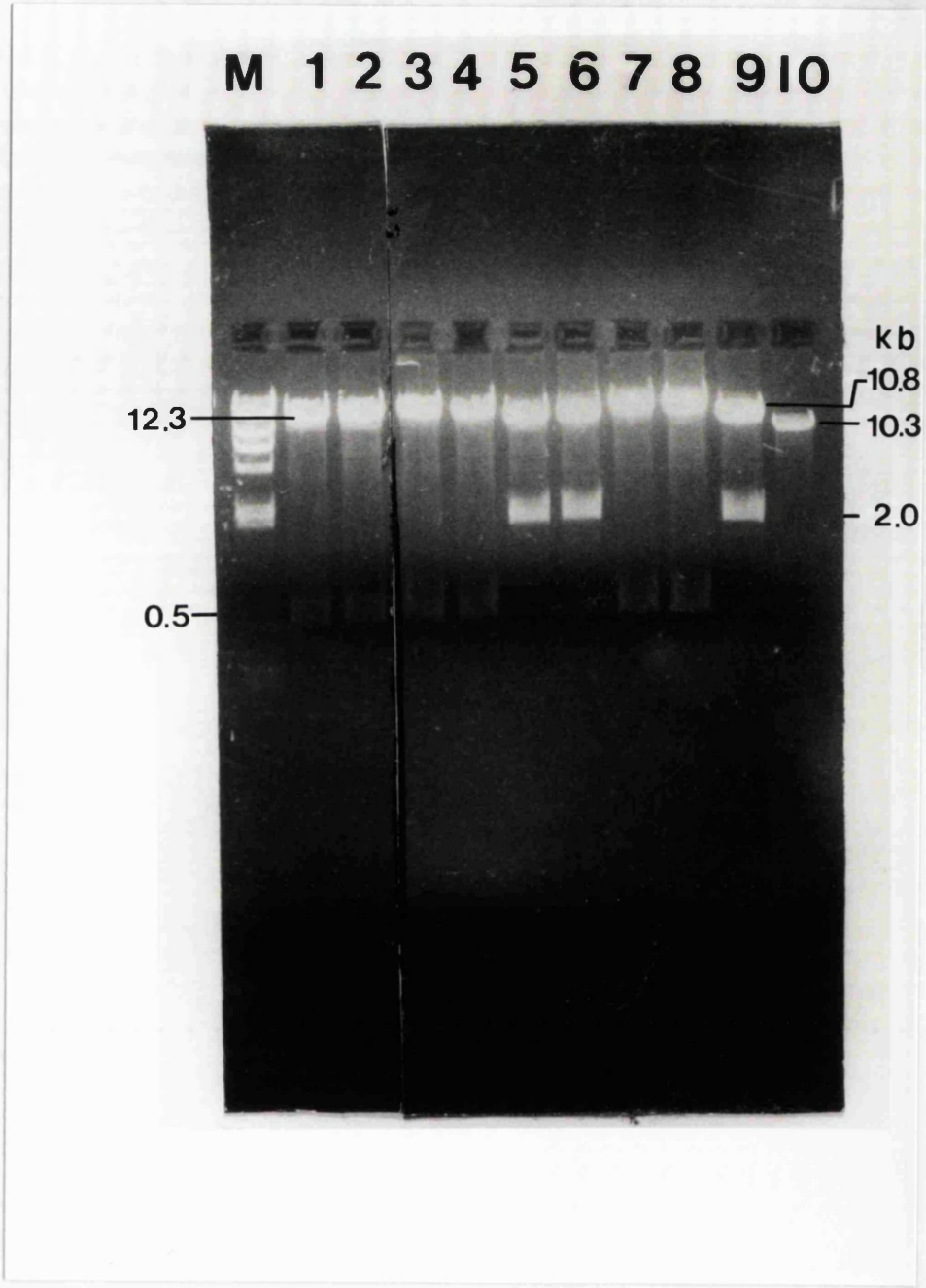


Fig. 6.8. Agarose gel analysis of Hind III-digested products of plasmid preparations. GLUT1 cDNA was ligated to prepared vector, and then transformed into competent DH1αF' *E. coli* as described in the text. Individual colonies picked at random were digested with Hind III, and analyzed in a 0.8 % agarose gel containing ethidium bromide. Lane 10: linearised pBlueBacHis C vector (10.3 Kb). Six clones (lanes 1,2,3,4,7 and 8) appear to be recombinants with insert GLUT1 cDNA in the desired orientation. Lane M: λ DNA/Hind III size markers (from top: 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56 Kb).

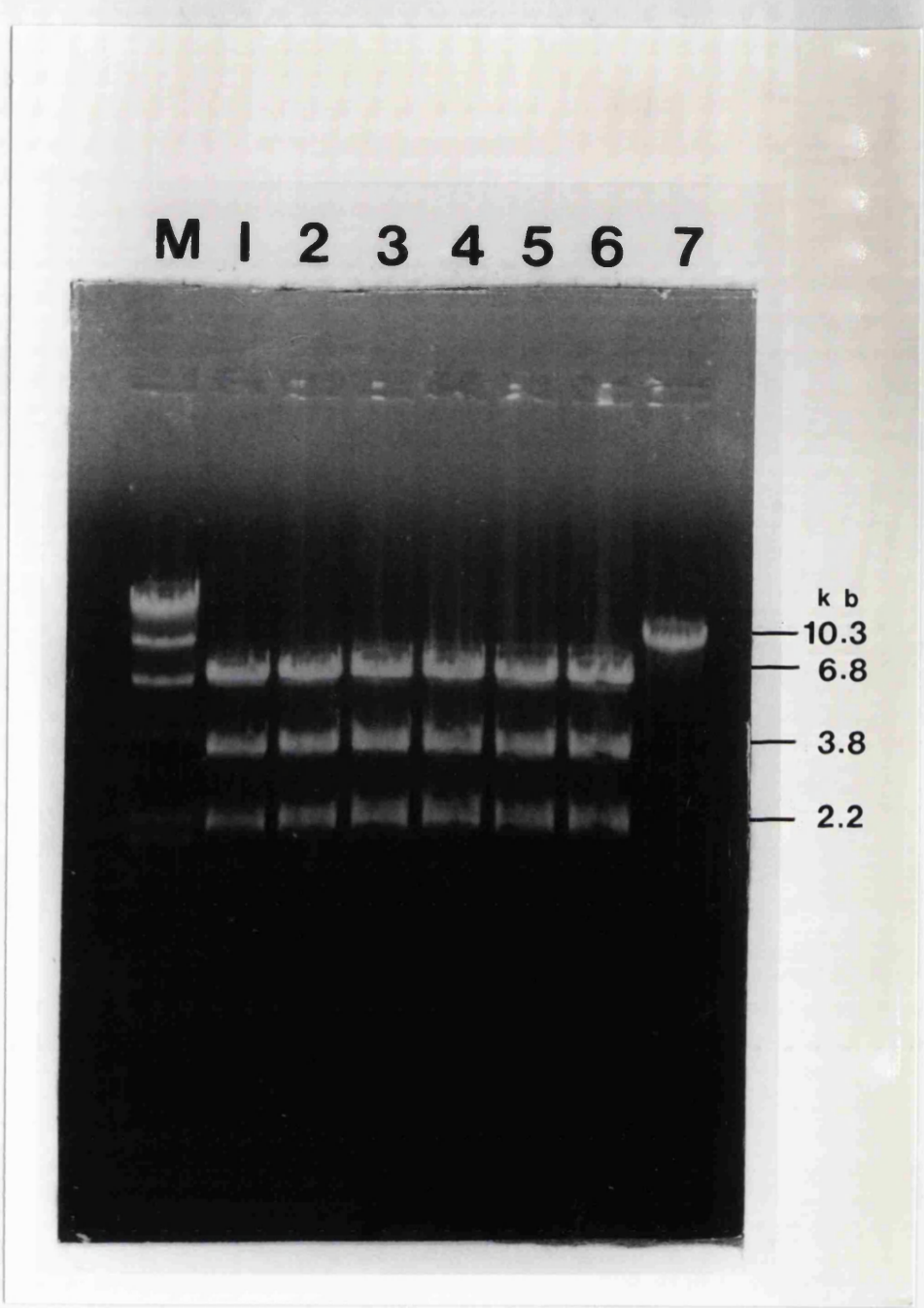


Fig. 6.9. Restriction digests (EcoRI) of putative recombinant plasmids obtained by cloning GLUT1 cDNA into the plasmid pBlueBacHis C. The putative six recombinants (Fig. 6.8) were digested with EcoRI (lanes 1-6) and electrophoresed on a 0.8 % agarose gel containing ethidium bromide. Lane M; - λ DNA/Hind III size markers (from top: 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56 Kb). Lane 7; linearized plasmid pBlueBacHis C.

interest in the construct was confirmed by the BamHI digestion (Fig. 6.10). It was therefore concluded that all six transformants corresponded to the desired recombinants. One of them (Clone No. 1) was chosen for large-scale preparation of recombinant transfer vector DNA. LB medium (40 ml) containing ampicillin (100 μg/ml) was inoculated with the Clone No.1 and grown overnight at 37°C. The plasmid DNA was then prepared using the Magic Miniprep System (Section 6.4.1). The plasmid was designated pBacHis-GT.

6.5. GENERATION OF RECOMBINANT BACULOVIRUS

6.5.1. Cotransfection and plaque assay

Recombinant baculoviruses were obtained by cotransfection of Sf9 cells with the plasmid pBacHis-GT DNA and the viral DNA "BaculoGold," using the BaculoGold Transfection Kit from PharMingen, following the protocols provided by the manufacturers. Sf9 cells (3×10^6) were seeded onto a 60 mm tissue culture plate and allowed to attach for 15 min at room temperature. The culture medium was then replaced with 1 ml of BaculoGold Transfection Buffer A (composition not described by the manufacturer). Linearized BaculoGold viral DNA of (0.5 μg, 0.1 μg/1 μl) was mixed with 2 μg (1 μg/3 μl) of the recombinant plasmid DNA in a sterile 1.5 ml eppendorf tube. The mixture was incubated at room temperature for 5 min and then 1 ml of BaculoGold Transfection Buffer B (composition not described by the manufacturer) was added. The Transfection Buffer B/DNA solution (1 ml) was then added dropwise to the tissue culture plate, gently rocking it back and forth to mix with the Transfection Buffer A. After 4 hours incubation at 27°C, the transfection

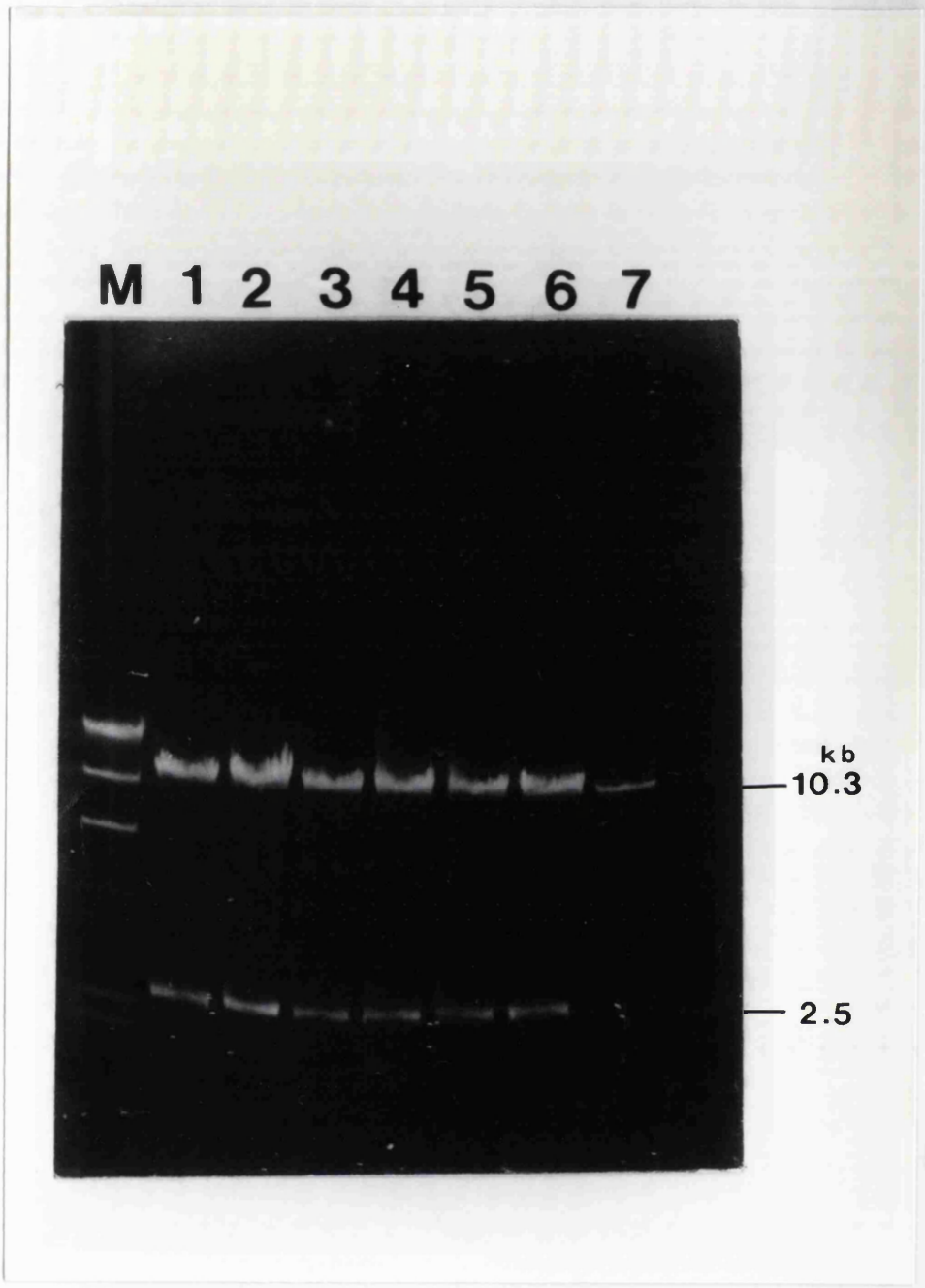


Fig. 6.10. Agarose gel electrophoresis of BamH1-digested products of putative recombinant plasmids. The putative six recombinants (Fig.6.8) were digested with BamH1 and analyzed in a 0.8 % agarose gel containing ethidium bromide. Lane M: - λ DNA/Hind III size markers (from top: 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56 Kb). Lane 7: -linearized plasmid pBlueBacHis C. Lanes 1-6 show a band at 2.5 Kb representing the BamH1 fragment of GLUT1 cDNA in addition to the pBlueBacHis vector of 10.3 Kb.

solution from the plate was replaced with 3 ml of complete TC100 medium and the plate was incubated for a further 4 days in a humid environment at 27°C. Following incubation the virus-containing supernatant was freed from cellular debris by brief centrifugation in a microfuge and then stored at 4°C.

Plaque assay was performed according to the method as described in Section 2.5.2, with some modifications. Sf9 cells (2 x 10^6) were seeded into 60 mm tissue culture plates. Following attachment, the cells were infected by incubation for 1 hour at room temperature with 0.3 ml samples of serial dilutions (10^3- to 10^8-fold) made from the virus-containing supernatant. Following incubation the inoculum was completely removed from the plates and the cells were then overlaid with 3 ml of 1.25 % agarose/complete TC100 medium mixture, maintained at 50°C, containing X-gal (150 μg/ml) and incubated at 27°C. After 6 days incubation, 16 blue plaques were visible on the plate that had been infected with a 10^7-fold dilution of the virus (Fig. 6.11). Eight of these plaques were picked at random and then each was separately stored at 4°C in 0.5 ml of TC 100 medium.

6.5.2. Identification of recombinant viruses by limiting dilution and immunostaining

Recombinant viruses containing GLUT1 cDNA were identified and purified by using the limiting dilution procedures and immunological screening methods previously described (Section 2.5.3). Sf9 cells (1.5 x 10^4 cells/well) were seeded into the wells of a 96-well plate and infected with 50 μl of serial dilutions (10^2- to 10^5-fold) of virus prepared from the 8 individual plaques

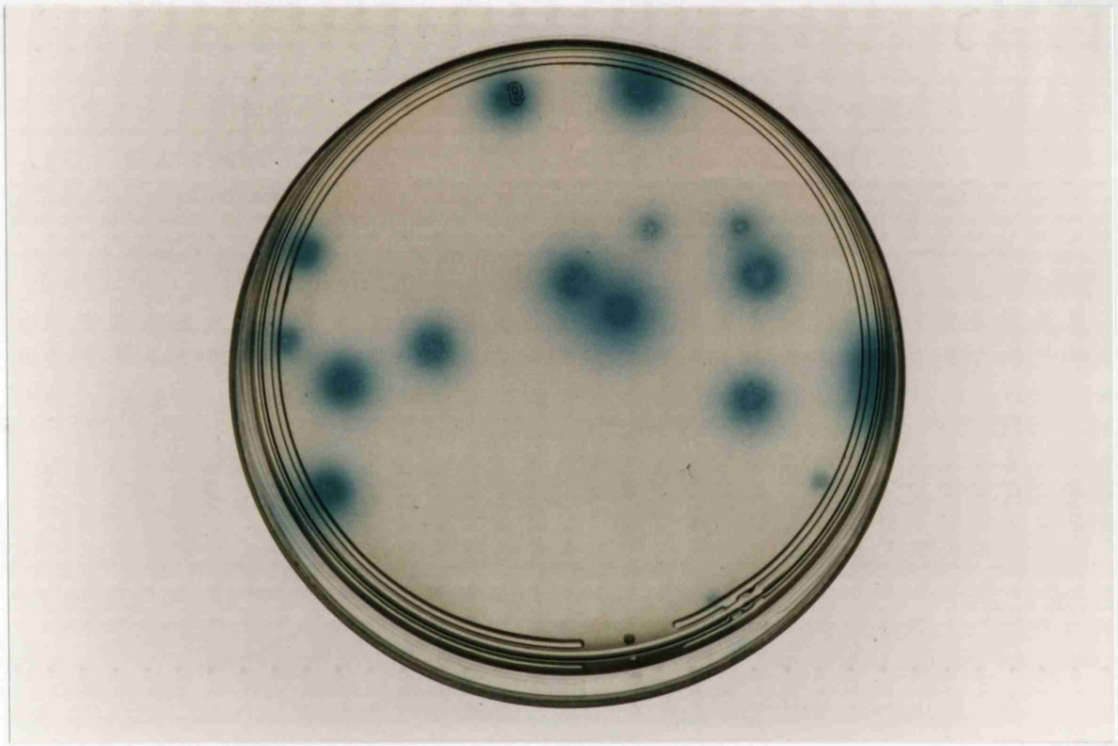


Fig. 6.11. Plaques containing infected cells that have expressed β-galactosidase. Since the lethal 'BaculoGold' baculovirus contains a lacZ gene that is replaced after plasmid rescue by the foreign gene of a complementing plasmid, all recombinant plaques are expected to be white on X-gal plates. However, the transfer vector pBlucBacHis C also contains the cloned lac Z gene. Therefore, plaques containing recombinant viruses turn blue.

described in Section 6.5.1. As controls, cells in some wells were either not infected or were infected with wild-type virus. Following 5 days incubation at 28°C, the cells were lysed with sodium hydroxide and then transferred onto nitrocellulose C-extra (Amersham) using a dot blot apparatus (Schleicher & Schuell) as described before (Section 2.5.3). The nitrocellulose was immunostained with an affinity-purified antibody raised against the *C*-terminus of GLUT1 (Davies *et al.*, 1987). The bound primary antibody was then detected with an alkaline phosphatase conjugate of goat anti-rabbit IgG (Bio-Rad) as described in Chapter 2.

As shown in Figure 6.12, all the cell samples that had been infected with viruses from the 8 blue plaques exhibited a positive reaction in the immunoassay, demonstrating expression of the glucose transporter. It was therefore concluded that these viruses contained the GLUT1 cDNA in the correct reading frame relative to the initiation codon provided by the transfer vector. In contrast, no colour development in the immunoassay was observed for cells infected with the wild-type virus or no virus. The specificity of the antibody used was confirmed by the strong purple colour yielded in the immunoassay by a dot containing 4 μg of purified human erythrocyte glucose transporter (Baldwin *et al.*, 1982) as a positive control.

6.5.3. Propagation of recombinant AcNPVHIS-GT

In order to ensure that the recombinant virus stock used for subsequent experiments was derived from a single clone, one round of plaque purification was carried out, using the virus-containing supernatant from the well that had

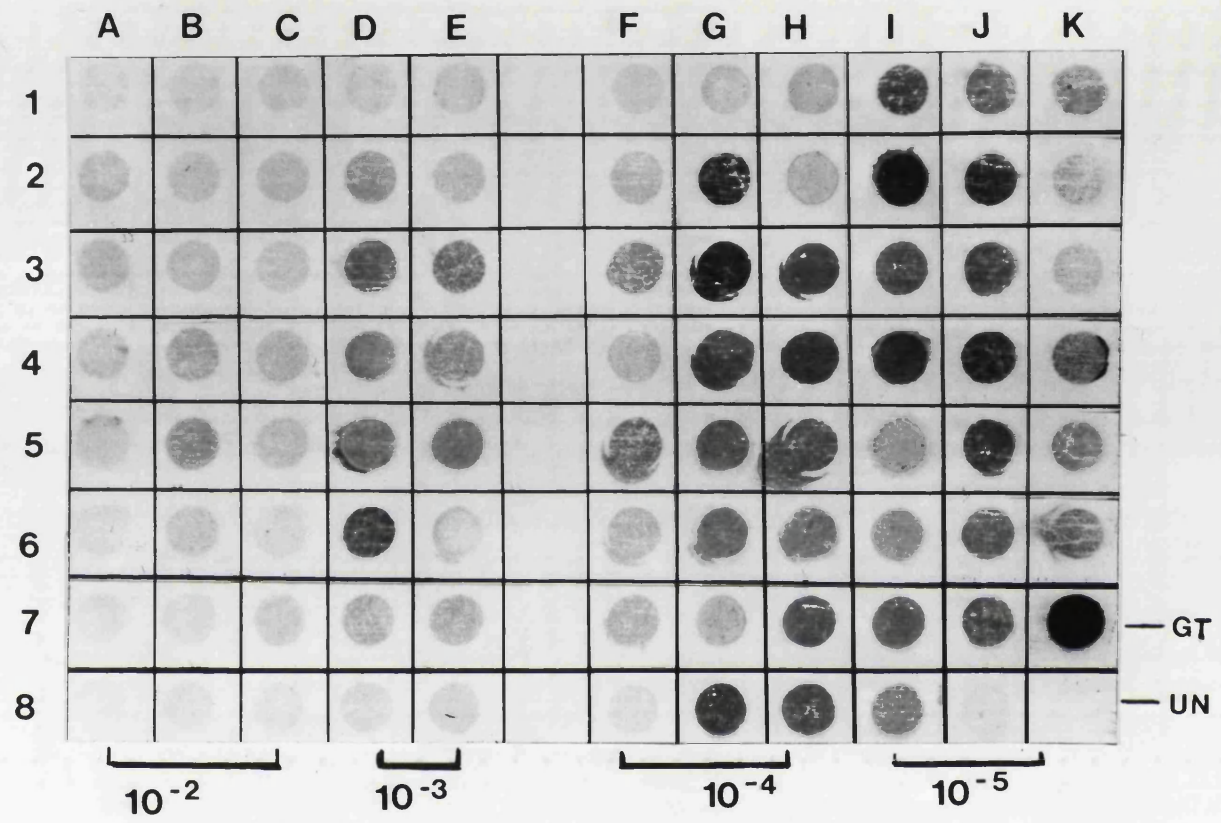


Fig. 6.12. Detection of recombinant viruses by limiting dilution and immunostaining. Sf9 cells were infected with 50 μl of 10-fold dilutions of virus prepared from the eight individual plaques (Section 6.5.1), ranging from 10^{-2} to 10^{-5}. The assay was performed as described in Section 2.5.3. Viruses expressing GLUT1 were detected by Western blotting using antibodies against the *C*-terminus of GLUT1 and alkaline phosphatase-conjugated goat anti-rabbit IgG as described in Chapter 2. GT: purified erythrocyte glucose transporter as a positive control. UN: uninfected insect cells.

exhibited the strongest positive signal on the immunoblot (Fig. 6.12). Subsequently, a well-developed single plaque was picked, placed in 1 ml of TC 100 medium, and then vortexed thoroughly to release the budded virus particles embedded in the agar plug. This recombinant viral clone was designated AcNPVHIS-GT and this initial stock is referred to hereafter as P1. Propagation of the virus was achieved by infecting five 25 cm^2 flasks, each containing 2 x 10^6 Sf9 cells, with 200 μl of the P1 virus as described in Chapter 2, followed by incubation at 27°C for 5 days (until the cells were 90 % lysed). The virus-containing supernatant was then collected and stored at 4°C. It is hereafter referred to as the P2 viral stock. The titer of the P2 virus was determined by performing plaque assay (Section 4.4.2.5) and was 2 x 10^8 pfu/ml.

6.6. IMMUNOFLUORESCENCE MICROSCOPY

The immunoblotting experiment described in Section 6.5.2 indicated that the recombinant virus was capable of directing the synthesis of immunologically-reactive GLUT1 fusion protein in insect cells. In order to confirm this result, and to localise the expressed protein, indirect immunofluorescence staining of viable Sf9 cells infected with AcNPVHis-GT was performed as described in Section 5.2.5, but with the following modifications. Sf9 cells infected with the recombinant virus P2 (MOI = 5) were harvested 2 days post infection. Following fixation in acetone at room temperature for 2 min, the cells were incubated with 10 % (v/v) foetal calf serum for 1 h at 37°C, washed in Dulbecco's phosphate buffered saline (PBSA), and then incubated with affinity-purified antibodies against the *C*-terminus of

GLUT1 for 90 min at 37°C. Then the cells were washed in PBSA and incubated for 1 h at 37°C with affinity-purified swine anti-rabbit IgG conjugated to rhodamine (1:40 dilution, Dako). The cells were washed again in PBSA and visualized using a Bio-Rad confocal microscope. Figure 6.13 shows that many cells infected with AcNPVHIS-GT exhibited bright fluorescence, indicating the presence of the recombinant transporter at the surface and interior of the infected cell (panel A). However, some cells showed little or no staining above background. No immunofluorescence was observed when cells infected with the AcNPVHIS-GT were stained with the rhodamine-conjugated second antibody alone (data not shown). The specificity of the staining was further demonstrated by the negative results obtained with cells infected with wild-type virus (panel B).

6.7. WESTERN BLOTTING

The immunofluorescence data presented in Section 6.6 confirmed the presence of immunologically-reactive GLUT1 in insect cells infected with AcNPVHIS-GT, and showed that the subcellular distribution of the expressed protein resembled that for cells infected with AcNPV-GT, as described in Chapter 5. In order to compare the expression level of the polyhistidine-containing GLUT1 recombinant with that for normal-length GLUT1 expressed in insect cells, Western blotting was performed as described in Section 2.2.2. Sf9 cells (2.2×10^6) were infected with the P2 recombinant virus at a MOI of 5. Four days after infection, the cells were harvested and solubilized in 100 μl of 1 % SDS containing protease inhibitors (2 mM iodoacetamide, 0.2 mM

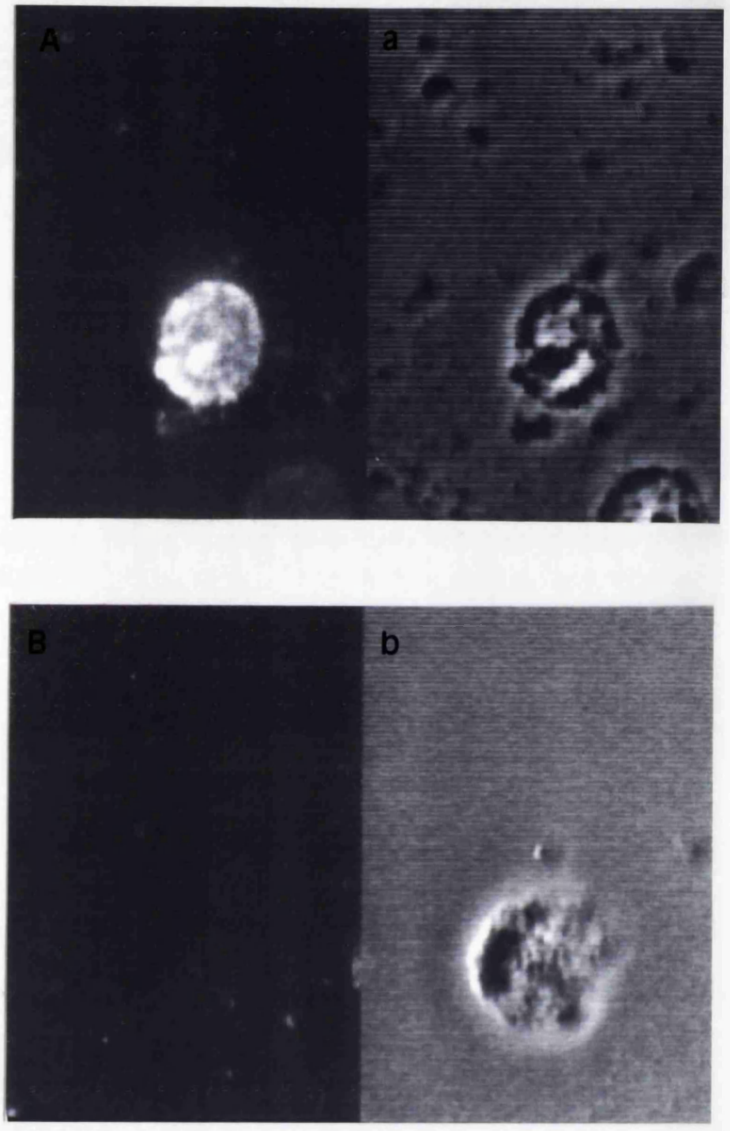


Fig. 6.13. Immunofluorescence analysis of *S. frugiperda* cells. Two days post infection, cells were collected, fixed and subjected to indirect immunofluorescent staining as described in the text. The panels A and B show immunofluorescent images of cells infected with recombinant AcNPVHIS-GT and no virus,respectively. The corresponding phase-contrast images are shown in panels a and b.

PMSF, 10 μg/ml pepstatin). The total cell lysates were electrophoresed on SDS/10 %(w/v) polyacrylamide gels (Laemmli, 1970), and then subjected to the immunoblotting, using the anti-GLUT1 *C*-terminal peptide antibody previously described. The blot was probed with an alkaline-phosphatase conjugate of goat anti-rabbit IgG (Bio-Rad). As shown in Figure 6.14, a major immunoreactive band of apparent M_r 43,000 - 44,000 was evident in the lysate from cells infected with AcNPVHIS-GT (lane A). This apparent M_r is smaller than that of GLUT1 found in the human erythrocyte (lane C), but almost identical to that of 45,000 seen for recombinant GLUT1 in cells infected with AcNPV-GT (lane B). It is therefore likely that the polyhistidine-containing protein resembles the latter in lacking complex oligosaccharides of the structure found on the naturally occurring human erythrocyte-type transporter, if indeed it is glycosylated at all (see Section 5.3). Several minor bands at lower molecular mass were also detected in the immunoblot and may reflect degradation of the expressed protein. The expression level of the fusion GLUT1 induced by AcNPVHIS-GT was compared with that of the previous, non-fused, recombinant transporter (Chapter 5) by spectrophotometric scanning of the immunoblot. Figure 6.15 shows that the total integral obtained for the former (lane A, Fig. 6.14) was approximately 1.1-fold less than that of the latter (lane B), although 50 times greater amounts of proteins (250 μg) were used for the lane A. This finding indicated that the level of expression of the polyhistidine-containing protein in insect cells was at least 50-fold lower than that previously seen for unmodified GLUT1. Furthermore, membrane preparations from the AcNPVHIS-GT-infected cells did not react at all with anti-GLUT1 antibodies on Western blots, an

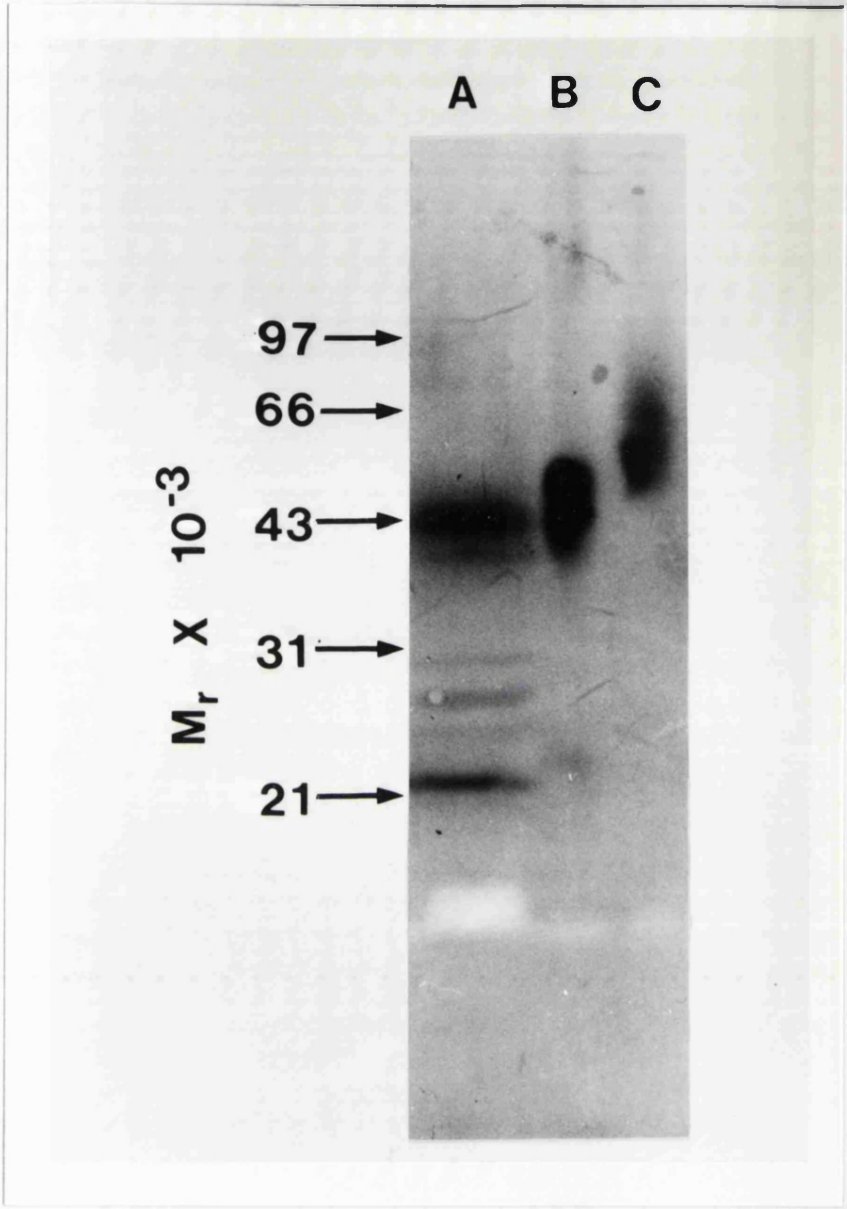


Fig. 6.14. Immunoblot analysis of insect cells infected with the recombinant virus AcNPVHIS-GT. Cells were grown and infected as described in Chapter 2 and harvested at 4 days after infection. They were solubilized in 1 % SDS, electrophoresed on a SDS/10 % polyacrylamide gel, and then subjected to Western blotting as described in the legend to Fig. 6.12. Lane A: Total lysates of AcNPVHIS-GT infected cells (250 μg). Lane B: Total lysates of AcNPV-GT infected cells (5 μg). Lane C: Alkali-stripped human erythrocyte membrane (5 μg). The molecular mass markers are indicated.

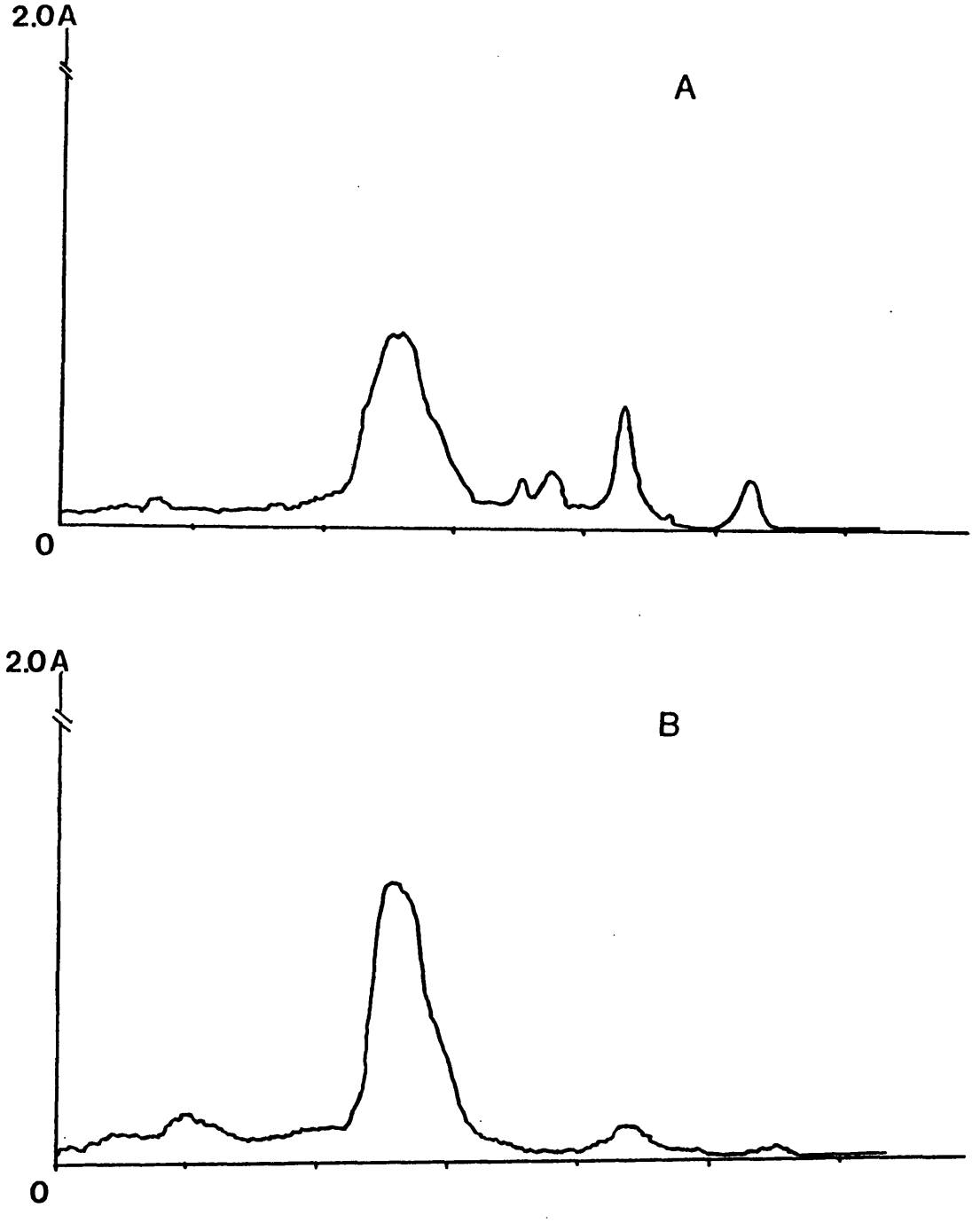


Fig. 6.15. Spectrophotometric profiles of a Western blot. Samples were electrophoresed on a SDS/10 % polyacrylamide gel, electrophoretically transferred to nitrocellulose, and then subjected to Western blotting as described in the legend to Fig. 6.14. The immunoblot described in Section 6.7 was then used for the spectrophotometric scanning using a Chromoscan 3 densitometer (Joyce Loebl, U.K). Total integrals of the lysates from cells infected with AcNPVHIS-GT (A: lane A in Fig. 6.14) or AcNPV-GT (B: lane B in Fig. 6.14) were 18616 and 20928, respectively.

observation that suggests the expressed protein may be inordinately sensitive to proteolytic degradation during membrane preparation (lane A, Fig. 6.16).

6.8. LARGE SCALE INFECTION FOR PRODUCTION OF RECOMBINANT PROTEIN

The Western blotting experiments described above showed that the level at which the polyhistidine-containing GLUT1 recombinant was expressed in insect cells was much lower than for the normal-length GLUT1 molecule. It was therefore decided that a large-scale culture of insect cells should be infected with AcNPVHIS-GT in order to provide sufficient expressed protein for attempts at purification. Infection of Sf9 cells in suspension culture was performed as described in Chapter 2. Sf9 cells (8×10^8) were pelleted by gentle centrifugation (at 800 x g for 10 min) using a benchtop clinical centrifuge and resuspended in 20 ml of the P2 virus inoculum to achieve a MOI of 5. The suspension was then diluted to an initial density of 1×10^7 cells/ml by adding 60 ml of fresh complete TC 100 medium and incubated at 28°C for 1 hour. Following incubation, 40 ml portions of the infected cell suspension were transferred to each of two 1-litre spinner culture flasks containing 210 ml of fresh complete medium to yield a density of 1.6×10^6 cells/ml. The resultant suspensions were then incubated at 28°C in a waterbath for 4 days with constant stirring at 70 rpm. The spinner flasks were siliconized with dimethyldichlorosilane solution (BDH), rinsed thoroughly and sterilized by autoclaving for 20 min before use. After 4 days, the cells were pelleted, resuspended in 50 ml of "Native Binding Buffer" (20 mM sodium phosphate,

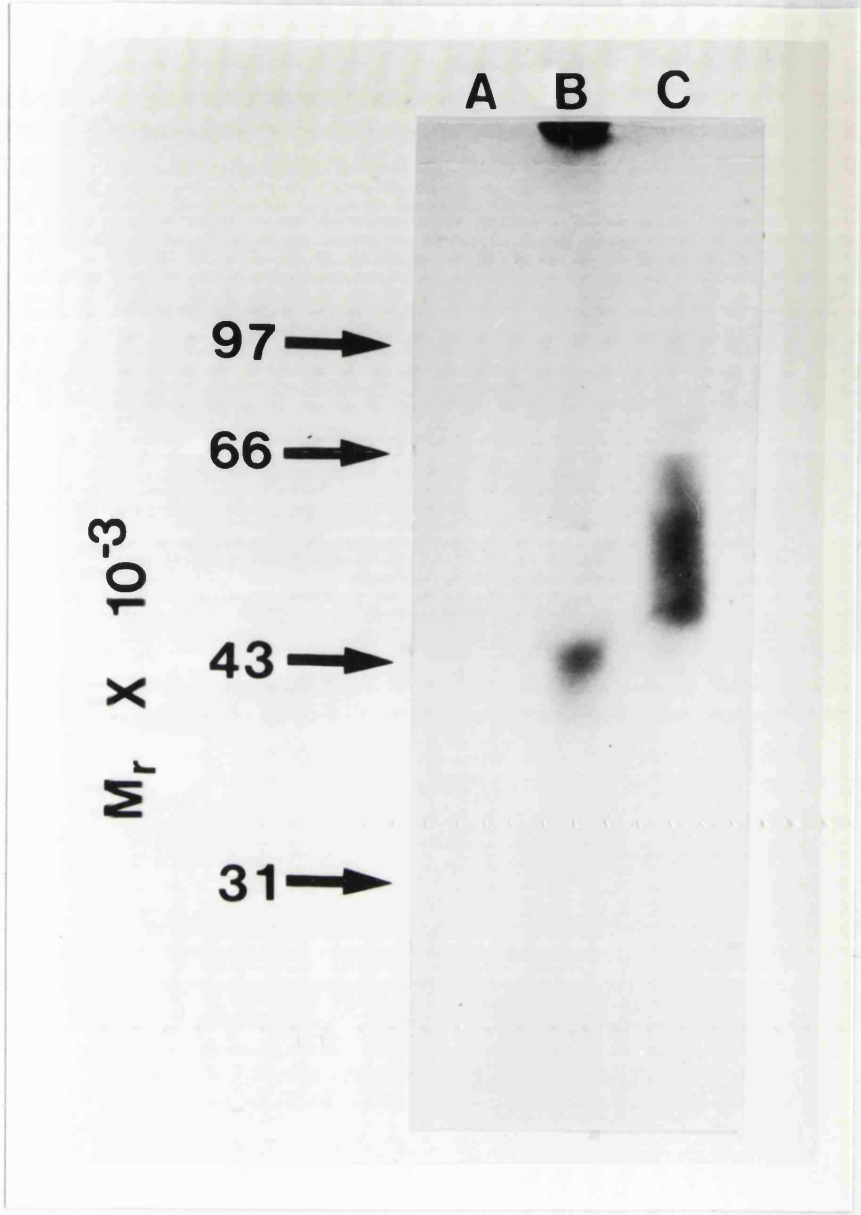


Fig. 6.16. Western blot analysis of AcNPVHIS-GT-infected Sf9 cell membranes with anti-peptide antibodies against GLUT1. Cells were infected with recombinant virus encoding the polyhistidine-containing GLUT1 (AcNPVHIS-GT). After 4 days infection, the cells were harvested and cell membranes were prepared as described in Section 2.2.7. Membrane samples (5 μg, Lane A) were electrophoresed on an SDS/10% polyacrylamide gel, transferred electrophoretically to nitrocellulose and then detected with antibodies against the *C*-terminal peptide of GLUT1 and alkaline phosphatase-conjugated second antibody, as described in Section 2.2.2. Lane B: membrane proteins (5 μg) from Sf9 cells infected with AcNPV-GT encoding non fusion recombinant GLUT1 (see Chapter 5). Lane C: 5 μg of protein-depleted human erythrocyte membranes. The positions of Mr markers are indicated.

500 mM sodium chloride) for subsequent purification attempts, divided into five aliquots and then stored at -70°C. The supernatant was saved at 4°C and designated P3 virus.

6.9. ATTEMPTED PURIFICATION OF RECOMBINANT TRANSPORTER

The recombinant glucose transport protein expressed in Sf9 cells (Sections 6.6 and 6.7) contained polyhistidine residues in its N-terminus, which should endow it with a high affinity for metal ions such as nickel. Thus, purification of the fusion protein was attempted by chromatography on a nickel-charged resin (Probond resin, Invitrogen), according to the manufacturer's protocol.

6.9.1. Preparation of column

A sample of immobilised Ni^{2+}-charged affinity resin (2 ml), in a spin column (0.8 x 4 cm), was resuspended by gentle inversion. The column was then clamped in a vertical position and the resin allowed to settle completely by gravity. The buffer was carefully removed and 7 ml of sterile water was added to the column. The resin was then resuspended and allowed to settle as before. This water wash was repeated one additional time. The column was then washed once with 7 ml of "imidizole elution buffer" (20 mM sodium phosphate; 500 mM NaCl, pH 6.3, containing 300 mM imidizole) and then twice with 7 ml of native binding buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.8). The buffers were sterilized by passage through 0.45 μm filters before use.

6.9.2. Preparation of cell lysates and sample application

A sample (9 ml) of the infected cell suspension prepared as described in Section 6.8 was used as the starting material for the purification attempt. Protease inhibitors were added (0.1 mM E-64, 0.2 mM PMSF, 1 μg/ml pepstatin A) and then the cells were solubilised by adding 1 ml of 20 % (w/v) octaethylene glycol dodecyl ether ($C_{12}E_8$, Calbiochem) to achieve a final concentration of 2 % and gently shaking for 20 min at 4°C. The mixture was next centrifuged at 100,000 x g for 1 h using a Beckman Ultracentrifuge. The solubilised cell supernatant containing the membrane proteins was then loaded onto a resin column that had been equilibrated, just before use, with 8 ml of the native binding buffer containing 1 % $C_{12}E_8$. After loading the column was washed with 8 ml of the native binding buffer containing 1 % $C_{12}E_8$ and protease inhibitors, and then with 8 ml of "Native Washing Buffer" (20 mM sodium phosphate, 500 mM NaCl, pH 6.3) containing 1 % $C_{12}E_8$ and protease inhibitors, to remove loosely-bound protein. Tightly-bound protein was finally eluted by applying 8 ml of the native washing buffer containing 300 mM imidizole and 1 % of the detergent plus protease inhibitors. The imidizole elution procedure was used to avoid denaturation of the recombinant protein at low pH. Fractions (1 ml) of the eluate were collected throughout loading and elution of the column, and their absorbances at 280 nm measured. The peak absorbances (0.13 - 0.40) shown in Figure 6.17, were obtained from the second and third fractions of the imidizole eluent. The fractions that contained the peak absorbance were pooled together and then dialysed against two 2-litre volumes of 50 mM Tris-HCl, 1 mM EDTA, pH 6.8 at 4°C for 2 days to remove

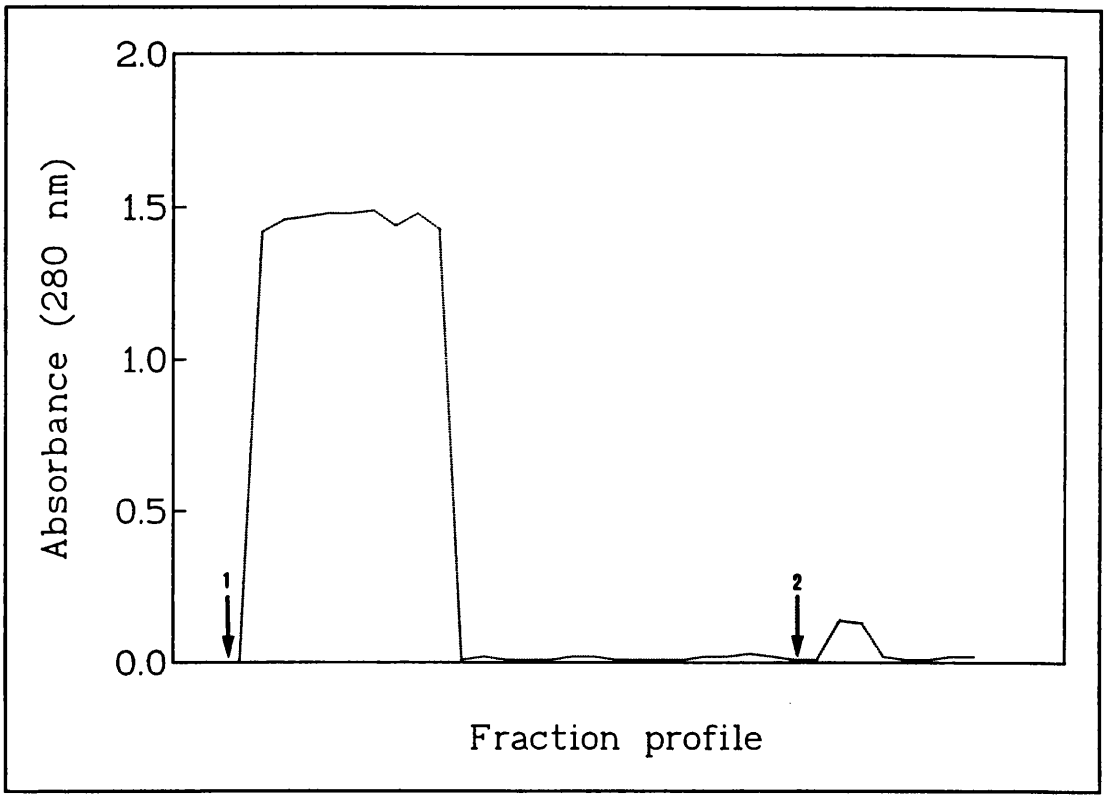


Fig. 6.17. Imidizole elution of polyhistidine-containing GLUT1-fusion protein. AcNPVHIS-GT-infected Sf9 cells (1.1 x 10^8) were solubilised in $C_{12}E_8$ and the supernatant (9 ml) containing the membrane proteins was passed over a Ni^{2+}-charged resin column as described in the text. After washing, the column was eluted with 8 ml of the native imidizole elution buffer at pH 6.0. Fractions (1 ml) were collected and their absorbance was measured at A_{280} nm. Arrow 1 indicates the application of sample. Arrow 2 indicates the addition of imidizole elution buffer.

the high concentration of salt.

6.9.3. Western blot analysis of the peak fractions

Samples (10 μg) of the dialysed eluate from Section 6.9.2 were electrophoresed on a SDS-10 % (w/v) polyacrylamide gel as described in Section 2.2.1. An excess of SDS (3 %, w/v) was included in the gel samples to swamp out the effect of the non-ionic detergent ($C_{12}E_8$). Following electrophoresis, the proteins were electrophoretically transferred to nitrocellulose membrane. The latter was then subjected to immunostaining with antibodies against the *C*-terminal peptide of GLUT1 and then an alkaline phosphatase conjugate of goat anti-rabbit IgG as previously described. However, the results were rather disappointing because no immunostaining was detectable in the eluates (lane D, Fig. 6.18). Staining was similarly not found in the original cell suspension (Section 6.8) nor the $C_{12}E_8$ solubilised membranes (lanes A and B). The negative result was also seen in the material that had not bound to the column (lane C). However, staining was clearly seen in the positive controls as expected: the pAcYM1-produced, nonfusion recombinant GLUT1 (lane E, see chapter 5) and alkali-stripped human erythrocyte membrane (lane F, Gorga and Lienhard, 1981).

To find out what had gone wrong, the natures of the P1, P2 and P3 viruses were examined using the same antibodies by the limiting dilution and immunostaining procedures described in Section 6.5.2. As shown in Figure 6.19, all the viruses exhibited positive signals. The result produced by the P2 virus clearly indicated that the virus used as the inoculum for the large scale

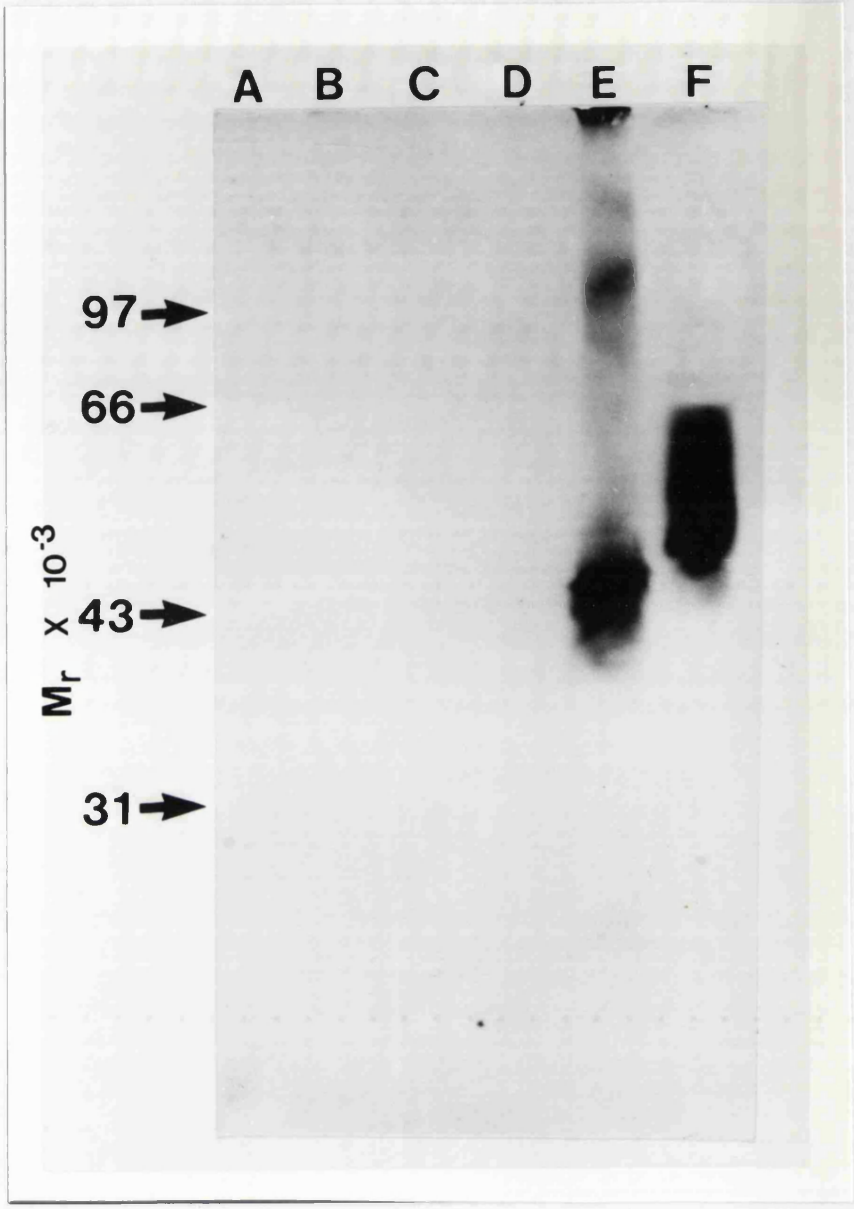


Fig. 6.18. Immunoblot analysis of peak fractions. Samples (10 μg) from each purification step, as described in the text, were electrophoresed on an SDS/10 % polyacrylamide gel and then subjected to Western blotting as described in the legend to Fig. 6.16. Lane A and B contain whole cell lysate and $C_{12}E_8$ solubilised membrane proteins, respectiverly. Lane C shows the material that was not bound to column. Lane D displays 10 μg of protein from the peak fractions dialysed. Lane E and F, 10 μg of Sf9 cell membranes infected with AcNPV-GT and protein-depleted human erythrocyte membrane, respectively.

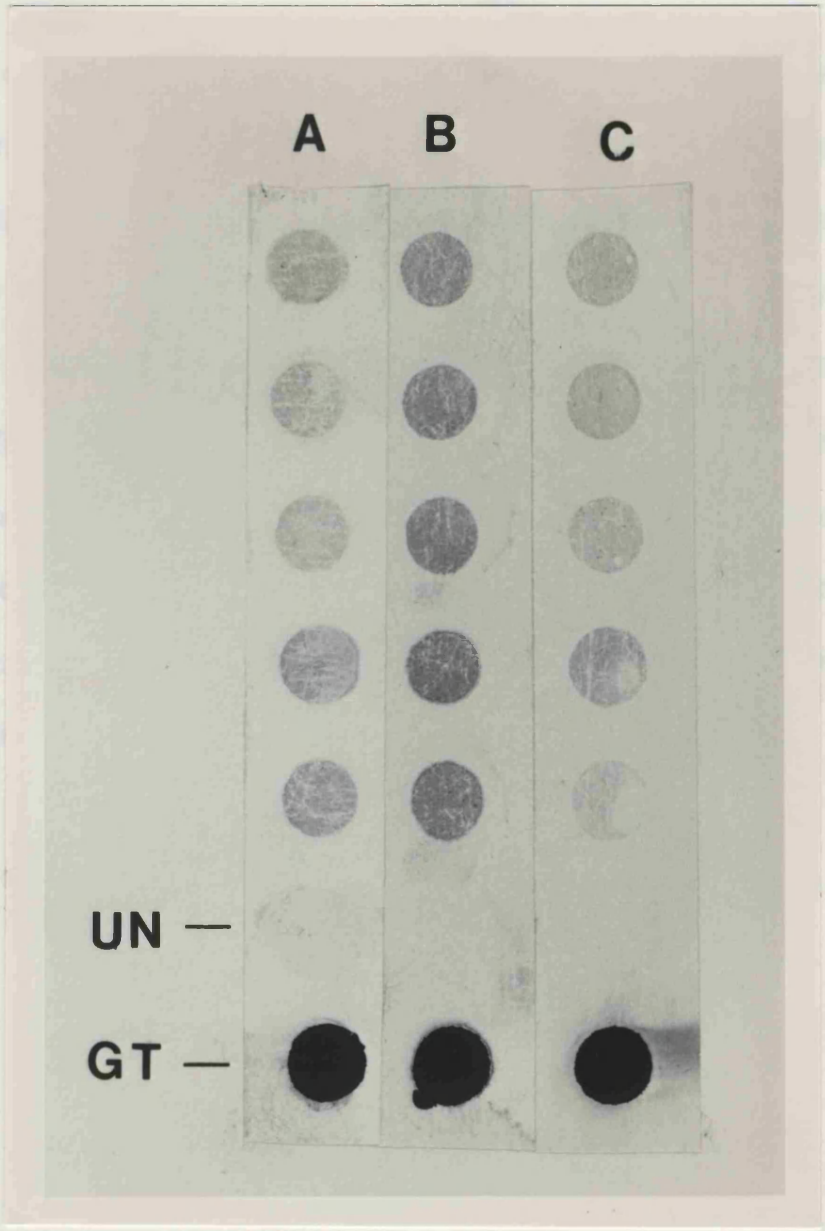


Fig. 6.19. Examination of the nature of P1, P2 and P3 viruses by limiting dilution and immunostaining. Sf9 cells were infected with either each recombinant or no virus. At 4 days after infection, the cells were lysed and then subjected to the limiting dilution and immunostaining as described in the legend to Fig. 6.12. A: cells infected with the P1 virus, B: cells infected with the P2 virus, C: cells infected with the P3 virus, UN: uninfected cells, GT: purified human erythrocyte glucose transporter (4μg) as positive control.

infection of suspension culture (Section 6.8), was indeed capable of synthesizing the glucose transporter in insect cell. This was further supported by the finding that the P3 virus also produced the positive signals, since the P3 virus was the resultant extracellular virus generated from the large scale infection. A possible explanation for the failure to detect GLUT1 in any of the fractions from the affinity column might be that the polyhistidine-containing protein was not only expressed at low levels but was also unstable and underwent rapid degradation. Such a conclusion is supported by the fact that it was necessary to load whole cell lysates onto the gels to see any of the polyhistidine-containing transporter (Section 6.7).

6.10. DISCUSSION

The Xpress System marketed by Invitrogen provides three different versions of the pBlueBacHis transfer vector - pBlueBacHis A, pBlueBacHis B, and pBlueBacHis C. These vectors differ only in the spacing between the sequences that code for the *N*-terminal leader peptide of the fusion protein and the multiple cloning sites. To facilitate the positioning of foreign genes in the same translational reading frame as the *N*-terminal peptide, the multiple cloning site of each vector is positioned in a different reading frame. Therefore, it was necessary first to decide which restriction site within the multi-cloning region was the most appropriate to use, and then which vector would preserve the reading frame between the 5′ vector sequence and the fragment encoding the GLUT cDNA when ligated into that site. Since a fragment containing the entire coding region of GLUT1 cDNA could readily be excised from the plasmid pSGT by a single restriction digestion with BamHI enzyme, the BamHI site of the pBlueBacHis vector was selected for cloning of the fragment into the vector. However, the details of the BamHI linker used in the construction of the plasmid pSGT were not available (Mueckler and Lodish, 1986). It was therefore necessary to perform partial sequencing of the plasmid to obtain sequence data on the region containing the BamHI restriction site, so that the GLUT1 cDNA could be positioned in frame with the leader peptide of the pBlueBacHis vector. To achieve this, the BamHI fragment of pSGT, containing the coding sequence of the GLUT1 cDNA, was first subcloned into M13mp18. Subsequent sequence analysis revealed that the BamHI linker used for the construction of the pSGT plasmid must have been a hexamer (GGATCC). From this sequence

data and the detailed sequence map of pBlueBacHis vectors provided by Invitrogen, it was concluded that the pBlueBacHis C was the only vector that could preserve the reading frame between the 5′ leader sequence and the GLUT1 cDNA insert when ligated into the BamHI cloning site.

One of the most laborious steps in the construction of baculovirus expression vectors is the identification of a virus that has incorporated foreign DNA by recombining with a transfer vector. This is because the frequency of such recombination events is typically less than 1 % (Miller *et al.*,1986; Fraser, 1989). Historically, recombinant baculoviruses were isolated on the basis of plaque morphology (Luckow and Summers, 1988). This process might involve several rounds of plaque purification, requiring inspection of hundreds of individual plaques. Thus, the identification of a recombinant virus against a background of contaminating wild-type viruses could be very time-consuming and tedious.

To facilitate screening of recombinant viruses, Pennock *et al.* (1984) suggested the use of the *lacZ* gene encoding *E.coli* β-galactosidase as a marker. A number of transfer vectors that contain the marker gene adjacent to the gene of interest have been constructed (Zuidema *et al.*, 1990; Vialard *et al.*, 1990). Recombinant viruses produced using these transfer vectors can be identified because they give rise to blue plaques in agar containing X-gal. In contrast to this, Summers and Smith (1987) have described the construction of a virus containing the lacZ gene, and its use as parental virus. In this case recombinants lack the gene and so produce white plaques, in contrast to the blue plaques produced by wild-type virus. Examples of this approach include

the parent viruses AcAs3 (Vlak *et al.*, 1990), AcUW1-lacZ (Weyer *et al.*, 1990), and vDA26Z (O'Reilly *et al.*, 1990). However, although both approaches facilitate the detection of recombinants, the problem of the low recombination rate remains.

As an alternative approach, Kitts *et al.*(1990) have described linearisation of the viral DNA before use to increase the proportion of recombinant viruses. They constructed a parental virus that contained a unique restriction site for Bsu36I, allowing it to be linearized by digestion with this enzyme. The use of the linearised viral DNA greatly increased the frequency of recombinants to around 30 %. This was achieved due to a large reduction in the background of wild-type viruses rather than to an increase in the absolute number of recombinant viruses produced. The circularity of the viral DNA could be restored by double recombination with a transfer vector carrying DNA homologous to the viral sequences on either side of the break. However, a disadvantage of this approach is the recircularisation of linear viral DNA by either self-ligation of the molecule or illegitimate recombination between viral sequences on either side of the break. The resultant viruses cannot be visually distinguished from the recombinants. Therefore transfer plasmids with marker genes must still be used or genetic analysis of plaques conducted to assure isolation of recombinants.

Very recently, PharMingen has introduced "BaculoGold DNA". This viral DNA includes a lethal deletion, which removes a cloned lacZ gene present in the parent virus, together with part of ORF 603 and ORF 1629. The ORF 1629 gene, which is essential for virus replication, lies downstream of the polyhedrin

promoter and in the opposite orientation (Possee *et al.*, 1991). Therefore the linearized viral DNA cannot infect insect cells and does not survive in tissue culture. Infectious virus can only be produced by co-transfection of cells with a transfer plasmid that can, by recombination, provide complementing sequences to the ORF 1629 deletion. If the linear viral DNA recircularizes by itself, the resulting viral DNA is still missing an essential part of the virus genome and so cannot survive. Therefore, the use of this type of viral DNA should minimize the proportion of non-recombinant plaques obtained.

In the present study baculoviruses expressing recombinant transporters, produced by cotransfection of Sf9 cells with the BaculoGold viral DNA and the transfer plasmid pBacHis-GT, were identified by their reactivity with specific polyclonal antibodies. The analysis showed that the frequency of the recombinants could be dramatically improved by the use of the viral DNA containing the lethal deletion. Most of the fusion protein expressed in Sf9 cells infected with the recombinant baculovirus AcNPVHIS-GT exhibited an apparent M_r of 43,000 - 44,000, a value essentially identical to that found for GLUT1 expressed in insect cells infected with AcNPV-GT. This finding suggests that in both cases the expressed protein was much less extensively glycosylated than its native erythrocyte counterpart, which migrates as a broad band of higher apparent M_r on SDS/polyacrylamide gels (see Section 5.3). However, the levels of GLUT1 expression yielded by the two recombinant viruses clearly differed. The first indication of this difference was provided by indirect immunofluorescence staining of viable Sf9 cells, with the antibody raised against the *C*-terminal of GLUT1, 2 days after infection with AcNPVHIS-GT.

The recombinant virus-infected cells clearly displayed high immunofluorescence. However, in all experiments, there was striking heterogeneity in the intensity of staining, with some cells showing little or no staining above background. The intensity of staining was also much less than that seen for cells infected with the recombinant virus AcNPV-GT, described in Chapter 5. This apparently lower level of expression was confirmed by the results of Western blot analysis of whole cell lysates using the same antibody. Spectrophotometric scanning of the immunoblots revealed that the level of recombinant transporter expression in insect cells infected with the AcNPVHIS-GT virus was approximately 60-fold lower than that induced by the AcNPV-GT virus. Such lower levels of expression could stem from the presence of the *N*-terminal leader peptide of the polyhistidine-containing fusion protein, which might render it susceptible to endogenous proteases or hinder its correct insertion into the endoplasmic reticulum membrane. Interestingly, immunoreactive bands of lower apparent M_r than the full-length transporter, which probably corresponded to degradation products, were visible on blots of cells expressing the fusion protein but not on blots of cells infected with AcNPV-GT. Alternatively, the presence of an additional 560 bp of 3′ non-coding sequence from GLUT1 cDNA in AcNPVHIS-GT (produced using a BamHI fragment of GLUT1 cDNA) as compared to AcNPV-GT (produced using a BamHI/Hind III fragment of the GLUT 1 cDNA), might affect the level of mRNA synthesis, its stability or the efficiency of its translation.

In conclusion, in the present chapter I have described another example of the successful expression of human erythrocyte-type glucose transporter in

insect cells, this time in the form of a fusion protein. Construction of a recombinant virus was aided by use of viral DNA containing a lethal deletion. Use of this viral DNA in cotransfection experiments with transfer vector resulted in a much higher proportion of recombinants, thus minimizing the time required for isolating and purifying recombinant viruses. Although an initial attempt at purifying the expressed transporter was not successful, this failure can probably be ascribed to the low levels of expression of the fusion protein. In the future, purification might be achieved if the expression level of the fusion protein could be improved. One reasonable approach might be to create a fusion protein in which the polyhistidine domain is *C*-terminal rather than *N*-terminal, in case the presence of the domain in the latter position renders the protein unstable. Alternatively, higher levels of expression might be achieved if the unnecessary 560 bp sequence in the 3'-untranslated region of the cloned BamHI fragment of GLUT1 cDNA were eliminated. Lastly, as shown by the results of immunofluorescence microscopy (Fig. 6.13), the expression of the human glucose transporter as a fusion protein in infected insect cells was very heterogeneous. Identification of the factors that contribute to this heterogeneity might suggest means of increasing the amounts of transporter produced.

CHAPTER 7. OVERVIEW AND FUTURE INVESTIGATIONS

7.1. OVERVIEW

The structure and function relationship of the human erythrocyte glucose transporter is not fully understood. An essential step in our progress towards an understanding of this relationship is likely to be the ability to produce large amounts of the protein. For example, elucidation of the structure of the transporter by biophysical techniques such as x-ray crystallography would be greatly facilitated by development of a system for the large-scale expression of the recombinant protein. Towards this end, the baculovirus/insect cell expression system was investigated in the present study in an attempt to overexpress the erythrocyte transporter by genetic means. This expression system offers a number of important practical advantages for a study of this kind, as described in Chapter 1. The aims and the corresponding findings of the four main parts of the present study are summarized below.

Insect cells (Sf9) were employed in the present study as the host for heterologous expression of the mammalian glucose transporter using a recombinant baculovirus. However, at the onset of the study little was known of the endogenous transport systems which allow the insect cells to grow on glucose. The nature of the lepidopteran transporter(s) was therefore investigated in Chapter 3. Firstly, the hexose transport characteristics of Sf9 cells were examined using 2-[1,2-^{3}H] deoxyglucose as substrate. It was found from kinetic analysis that the insect cells contained an endogenous glucose transport activity that in several aspects resembled the mammalian passive

glucose transporter GLUT1. The presence of this endogenous activity is likely to complicate assessment of the activity of heterologously expressed transporters in insect cells. Thus, it would probably be necessary to purify the expressed protein away from the endogenous transporter and then reconstitute it into lipid vehicles before detailed kinetic studies could be performed. However, interestingly some of the kinetic properties of the insect transporter(s) were very different from those of its human erythrocyte counterpart. For example, the insect transporter appeared to have high affinity for D-fructose (apparent K_i approximately 5 mM), unlike human GLUT1 (K_m approximately 1.5 M, LeFevre and Marshall, 1958). This property suggested that it might be possible to use this ketose as an inhibitor for blocking the endogenous glucose transport activity of insect cells when the functional activity of recombinant GLUT1 was being determined. In addition, glucose transport in insect cells was much less potently inhibited than mammalian glucose transport by cytochalasin B, which binds to the human GLUT1 transporter with a K_d of about 0.12 μM (Zoccoli *et al.*, 1978). Subsequent ligand binding assay of uninfected insect cell membranes by equilibrium dialysis using a low concentration of [4-H^3] cytochalasin B, directly revealed that the insect transporters had low affinity for the inhibitor. Thus, it was unlikely that the presence of endogenous transporters would interfere with the assay of the cytochalasin B binding properties of human GLUT1 expressed in insect cells. Furthermore, no insect cell proteins of M_r comparable to that of GLUT1 were recognised by antibodies against the mammalian transporter, although the nature of some bands of higher apparent M_r that were detected in insect cell membranes by antibodies

against GLUT1 remains unclear. These findings suggested that there is only very limited sequence similarity, if any, between the mammalian and insect proteins, and thus that the presence of endogenous glucose transporter(s) would not be a problem for the immunological detection of human GLUT1 expressed in insect cells, at least if the available anti-peptide antibodies against GLUT1 were employed.

The genome of the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) is a double-stranded circular DNA of 128 kb (Smith and Summers, 1978). The size of the viral DNA makes it difficult to manipulate by standard recombinant DNA methods. Thus, to take advantage of the baculovirus expression system, cDNA encoding human GLUT1 was first inserted into the transfer vector pAcYM1 which was designed to position the cloned cDNA immediately downstream of the powerful baculovirus polyhedrin gene promoter (Matsuura *et al.*, 1987). The vector also contained the 5'- and 3'- flanking regions of the polyhedrin gene to direct its integration into the genome of the wild-type AcNPV. The resultant recombinant plasmid (pAcYM1-GT) was then cotransfected into cultured insect cells together with the viral DNA. A double recombination between viral sequences flanking the cDNA in the recombinant transfer vector and the corresponding sequences in the viral DNA introduced the GLUT1 cDNA into the genome of the baculovirus. The recombinant baculoviruses were initially identified and partially purified by limiting dilution coupled with DNA dot hybridisation analysis using a radiolabelled GLUT1 cDNA probe. Confirmation that the recombinant virus encoded the human erythrocyte transporter was obtained by Western blot

analysis. The recombinant virus (AcNPV-GT) was plaque-purified to homogeneity by visual screening for the absence of polyhedrin.

Chapter 5 describes the first demonstration of overexpression of a mammalian facilitative glucose transporter in functional form. Infection of insect cells with recombinant baculovirus encoding the human erythrocyte transporter resulted in the expression of considerable quantities of the transport protein. All the expressed protein was found in the membrane fractions of the cells, and none in the cytosol, as immunologically cross-reactive protein was detected only in the membrane fractions of the cells. The protein was detectable in the cells as early as 24 hours post-infection and was stable, accumulating in amount until the cells lysed, 4-5 days post-infection. Immunofluorescence microscopy has provided further evidence for the expression of human glucose transporter in insect cells. Only cells infected with the recombinant baculovirus AcNPV-GT exhibited bright fluorescence, indicative of an abundance of transporter. In addition, a series of optical sections through the fluorescently labelled, infected cells, made using a confocal microscope, revealed that the recombinant transporter was present not only at the cell surface but also in membranous structures throughout the cytoplasm, except for the region occupied by the nucleus. The expressed protein was recognised by anti-peptide antibodies directed against various regions of the human erythrocyte transporter including the *N*- and *C*-termini. The results not only suggested that the expressed protein was genuinely the transporter, but also that it was not subject to proteolytic degradation. The expressed protein migrated as a fairly sharp band of apparent M_r 45,000 on

SDS/polyacrylamide gels, almost identical to that of the enzymatically deglycosylated form of its human erythrocyte counterpart (Lienhard *et al.*, 1984). Thus, the recombinant GLUT1 appeared to be much less heavily and heterogeneously glycosylated than the erythrocyte protein. This was confirmed by the fact that treatment with endoglycosidase F only slightly increased the electrophoretic mobility of the recombinant polypeptide. Nontheless, the effect of endoglycosidase F did indicate that the expressed protein was *N*-glycosylated, presumably at Asn_{45}. Despite the differences in glycosylation it appeared that the protein expressed in insect cells had a tertiary structure similar if not identical to that of its erythrocyte counterpart, as revealed by the pattern of tryptic cleavage of the recombinant GLUT1.

To estimate the amount of the expressed protein, quantitative immunoblotting was performed. The analysis showed that 4 days after infection up to 1.4 nmol transporter was present per mg membrane protein, a value which represents almost 8 % (w/w) of the total membrane protein. It has been reported that high level of synthesis of glycoproteins led to a proliferation of cytoplasmic membranes (Matsuura *et al.*, 1987). However, electron microscopy of the AcNPV-GT-infected cells did not show such a phenomenon.

It was not possible to determine the transport activity of the expressed protein in the insect cells, both because of the existence of an abundant endogenous glucose transport activity, and because infected cells became leaky at the time of maximal transporter expression. However, unlike the mammalian facilitative glucose transporters, the insect transporter(s) were relatively insensitive to inhibition of cytochalasin B, a potent inhibitor of GLUT1. The

binding of this inhibitor could be therefore used as a measure of the biological activity of the expressed protein. Scatchard plot analysis of the binding showed that at 4 days after infection with the recombinant AcNPV-GT, infected cell membranes exhibited >200 pmol cytochalasin B binding sites per mg protein, which bound the transporter inhibitor with characteristics identical to those of the erythrocyte protein. Furthermore, cytochalasin B was found to photolabel, in a D-glucose-sensitive manner, a protein in the infected insect cell membranes that had a mobility identical to the band identified as GLUT1 on Western blots. Thus, it was clear that GLUT1 expressed in the baculovirus system exhibited native-like biological activity, although purification and reconstitution would be required to demonstrate that it could actually transport glucose. However, the concentration of cytochalasin B binding sites detectable in infected cell membranes, up to 290 pmol/mg protein, was less than the concentration of immunologically cross-reactive protein (up to 1,470 pmol/mg protein) and the origin of this discrepancy requires further investigation. A phenomenon similar to this has been reported for other membrane glycoproteins (Germann *et al.*, 1990; Klaiber *et al.*, 1990).

Despite the abundant expression of the recombinant glucose transporter, it was not possible to demonstrate that the transporter expressed in insect cells could actually transport glucose. This was largely because of the very high activity of the endogenous glucose transporter(s) present in the lepidopteran cells. Therefore, in Chapter 6 an attempt was made to develop procedures for the rapid isolation of the recombinant transporter in functional form away from the endogenous sugar transporter. Towards this end, I constructed a

recombinant baculovirus encoding a polyhistidine-containing fusion protein which might facilitate purification of GLUT1 expressed in insect cells. Viral DNA containing a lethal deletion was used in the construction of the recombinant virus. Only homologous recombination between the recombinant transfer vector carrying the missing sequence and the viral DNA could produce viable recombinant viruses. The use of such a viral DNA dramatically lowered the fraction of non-recombinant viruses, thus minimizing the time spent for screening and purifying recombinants. The fusion protein encoding the GLUT1 was designed to contain a polyhistidine sequence that had a high affinity for metal ions, thus theoretically allowing purification of the recombinant proteins by immobilized metal affinity chromatography using Ni^{2+} charged affinity resins. Unfortunately, the low level of expression and apparent instability of the recombinant fusion protein precluded its purification by this approach in the present study.

7.2. FUTURE INVESTIGATIONS

As demonstrated in the present study, baculovirus expression is a powerful system for producing large amounts of recombinant sugar transporter in functional form. One can envisage future further exploitation of the baculovirus expression system in the following ways: -

1) The baculovirus system should be of great value in the study of structure-function relationships in GLUT1 and other transporter isoforms by site-directed mutagenesis. A number of mutations of GLUT1 have already been carried out in our laboratory and elsewhere, with the aim of identifying the

function of individual residues in the transport mechanism. These include deletion, insertion and replacement of specific residue(s) of the transport protein. The resulting mutants may be examined by using an established expression system such as the *Xenopus* oocyte. However, for a number of reasons, including the low levels of expression, detailed kinetic analyses of the mutant transporters will not be easy. Furthermore, application of the *Xenopus* system is very labour intensive. These types of difficulties could be overcome by use of the baculovirus expression system. The latter system may also allow us to design mutants proteins specifically tailored for mechanistic studies of structure and function using techniques such as NMR and fluorescence spectroscopy (eg. by mutagenesis of Trp residues of GLUT isoforms). Furthermore, the purification approach outlined in Chapter 6 may provide a rapid and efficient means for isolation and subsequent study of recombinant transporters if the expression level of the fusion protein can be improved by adopting the suggestions discussed in Chapter 6.

2) The baculovirus system may also prove to be useful in future crystallization trials of the transport protein. Molecular cloning studies have shown the existence of a family of homologous sugar transport proteins in organisms ranging from bacteria to humans (Baldwin, 1993). The amino acid sequence similarities between the prokaryotic and eukaryotic members of the family, as revealed by sequence analysis of the members, suggest that their molecular mechanisms share many features at the molecular level. Therefore, the acquisition of information about the three-dimensional structure of any member of the transporter family should facilitate future progress in

understanding solute translocation mechanisms in the sugar transporter family in general. However, progress in determining the three-dimensional structures of membrane-spanning proteins such as GLUT1 has been slow, because the hydrophobic nature of these proteins makes it difficult to obtain crystals. In this respect, the baculovirus system might provide a useful genetic means for synthesizing fusion proteins that have been designed to enhance their susceptibility to crystallization. Furthermore, the native transporter isolated from human erythrocyte is heavily and heterogeneously glycosylated (Gorga *et al.*, 1979). This type of glycosylation may interfere with crystallization, in particular with formation of 3-D crystals. By contrast, GLUT1 expressed in insect cells was found to be less heavily and more homogeneously glycosylated than its native erythrocyte counterpart. Thus, the baculovirus system could be employed to produce a less-glycosylated protein that might be more suitable than the native transporter for crystallization trials.

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RESEARCH COMMUNICATION

Characterization of functional human erythrocyte-type glucose transporter (GLUT1) expressed in insect cells using a recombinant baculovirus

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The human erythrocyte-type glucose transporter (GLUT1) has been abundantly expressed in insect cells by using a recombinant baculovirus. At 4 days after infection with the virus, the insect cell-surface and intracellular membranes were found to contain > 200 pmol of D-glucose-sensitive binding sites for the transport inhibitor cytochalasin B per mg of protein. The characteristics of binding were identical with those of the erythrocyte transporter, although the two proteins differed substantially in apparent M_r, probably as a result of glycosylation differences.

INTRODUCTION

The uptake of glucose into most mammalian cells is mediated by passive transport proteins in the plasma membrane. Recent cDNA cloning studies have revealed that these proteins are members of a large family of homologous sugar transporters present in both prokaryotes and eukaryotes [1]. The best known of these proteins is the human erythrocyte glucose transporter (GLUT1), which has been purified to near homogeneity and extensively characterized [2–4]. Functional expression of small amounts of the transporter has been achieved in *Xenopus* oocytes [5] and in mammalian cells [6]. However, for detailed studies of structure–function relationships in this protein, and in particular for the investigation of its structure by crystallization, higher levels of expression would be desirable. Recently, the baculovirus expression system has been utilized to produce biologically active recombinant proteins from a variety of eukaryotic genes, including several that cannot be efficiently expressed in lower eukaryotic or prokaryotic systems [7]. In the present study we describe the successful use of this system to produce substantial amounts of functionally active erythrocyte glucose transporter suitable for structural and functional studies.

EXPERIMENTAL

Materials

Wild-type *Autographa californica* (alfalfa looper) nuclear polyhedrosis virus (AcNPV), the transfer vector pAcYM1 [8] and *Spodoptera frugiperda* Clone 9 (Sf9) cells were kindly given by Professor D. H. L. Bishop (NERC Institute of Virology and Environmental Microbiology, Oxford, U.K.). The plasmid pSGT, which contains the entire coding region of the human GLUT1 transporter [9], was gratefully received from Dr. M. Mueckler (Washington University, St. Louis, MO, U.S.A.). New England Nuclear (U.K.) supplied [4-^{3}H]cytochalasin B, and endoglycosidase F was from Boehringer.

Construction of recombinant baculovirus

Standard recombinant DNA techniques were used throughout. The plasmid pSGT was restriction-digested with *Bam*H1 and *Hin*dIII, and a 1922 bp fragment containing the entire coding region of the GLUT1 cDNA was isolated. Protruding ends were filled in and then the fragment was ligated into the blunt-ended unique *Bam*H1 site of pAcYM1 to produce a recombinant (pAcYM1-GT). Recombinant baculoviruses carrying the GLUT1 cDNA were then produced by co-transfection of Sf9 cells with pAcYM1-GT and wild-type AcNPV viral DNA using the procedure of Summers & Smith [10]. Recombinants were identified and partially purified as described in [11]. Selected recombinants were then plaque-purified to homogeneity [10]. These procedures yielded three clones of recombinant virus, one of which, AcNPV-GT, was used for all the experiments described.

Culture and infection of insect cells, and preparation of membrane fractions

Sf9 cells were cultured and infected as described in [10]. For production of membranes, cells were harvested 4–5 days after infection [multiplicity of infection (MOI) 5–10] and washed three times at 20 °C with 10 mM-sodium phosphate/150 mM-NaCl, pH 7.2. They were then resuspended in 10 mM-Tris/5 mM-$MgCl_2$, pH 7.4, containing proteinase inhibitors [2 mM-iodoacetamide, 0.2 mM-phenylmethanesulphonyl fluoride and pepstatin A (10 μg/ml)] and sonicated on ice for 1 min. Membranes were separated from soluble components by centrifugation for 1 h at 117000 $g_{av.}$. For examination of the time course of GLUT1 expression, washed cells were not separated into membrane and soluble fractions before analysis by Western blotting. Alkali-stripped human erythrocyte membranes were prepared by the method of Gorga & Lienhard [12].

Western blotting

Antibodies against the human erythrocyte glucose transporter

Abbreviations used: GLUT1, human erythrocyte-type glucose transporter; AcNPV, *Autographa californica* nuclear polyhedrosis virus; Sf9, *Spodoptera frugiperida* Clone 9; MOI, multiplicity of infection.

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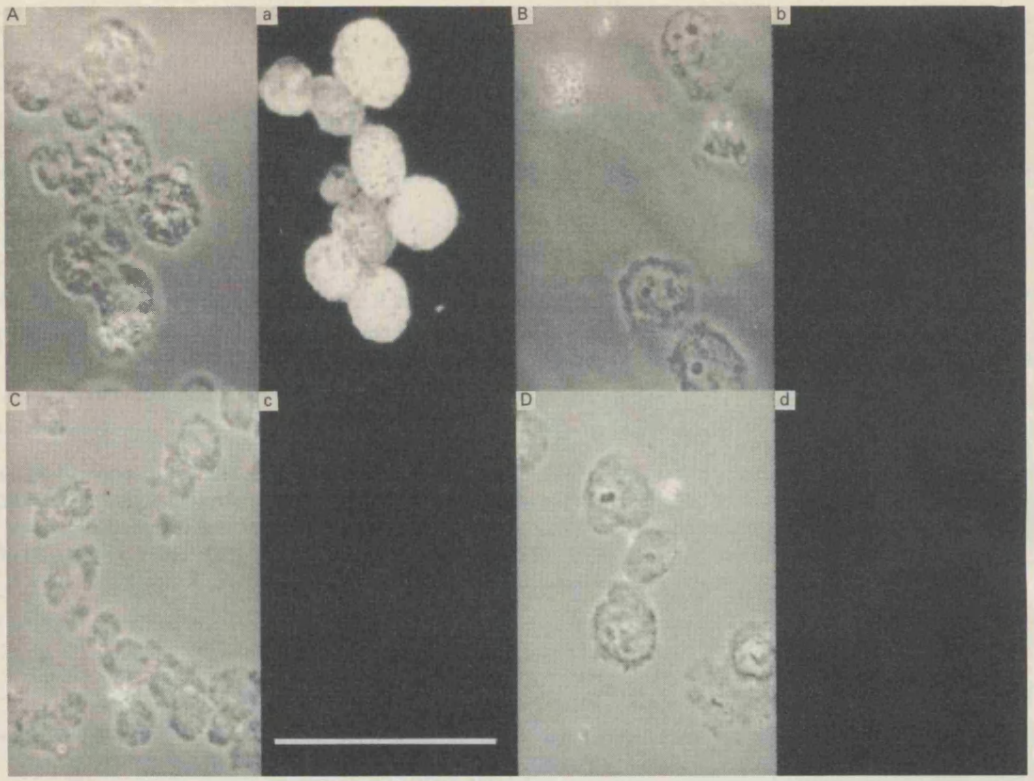

Fig. 1. Confocal microscopy of non-infected and baculovirus-infected insect cells

At 2 days after infection, cells were collected, fixed and incubated first with affinity-purified anti-(glucose transporter) antibody (50 μg/ml) and then with a fluorescein conjugate of goat anti-rabbit IgG, before examination using a confocal laser scanning microscope. The panels show immunofluorescent images of cells infected with recombinant AcNPV-GT (panel a), wild-type AcNPV (panel b), no virus (panel c) or cells infected with recombinant AcNPV-GT, but omitting the primary antibody (panel d). The corresponding phase-contrast images are shown in panels A–D. The scale bar represents 50 μm.

were raised in rabbits against a synthetic peptide corresponding to the *C*-terminal sequence (residues 477–492) as detailed in [13]. Western blotting was performed as previously described, by using either an alkaline phosphatase conjugate of goat anti-rabbit IgG [13] or ^{125}I-F(ab′)$_2$ donkey anti-rabbit IgG [14] as the second antibody. The concentration of transporter within membrane samples was determined by γ-radiation counting of the ^{125}I-labelled bands and comparison with human erythrocyte membrane standards of known cytochalasin B-binding-site content run on the same gel, as previously described [14].

Other methods

Routine assays for cytochalasin B-binding activity of membrane samples were performed by equilibrium dialysis using 40 nM-[^{3}H]cytochalasin B [3]. Correction for non-specific binding to membrane lipids was made by performing the binding experiments in the presence or absence of 400 mM-D-glucose. More accurate estimation of the concentration and affinity of cytochalasin B-binding sites was obtained by measuring binding over a range of cytochalasin concentrations by the method previously described [3]. Glycosylation analysis was carried out as previously described [14]. The procedure used for indirect immunofluorescence staining was also as described [15], except that samples were examined using a Bio-Rad MRC600 confocal laser scanning microscope. The same anti-transporter antibodies were used as for Western blotting.

RESULTS AND DISCUSSION

The expression of human glucose transporter in insect cells was examined by confocal immunofluorescence microscopy using anti-transporter antibodies and a fluorescein-labelled secondary antibody. Fig. 1 shows that, 2 days after infection with recombinant baculovirus, the cells exhibited bright fluorescence, indicative of an abundance of transporter. In contrast, non-infected cells and those infected with wild-type virus showed no fluorescent staining. Serial confocal 0.6 μm optical sections through the fluorescently labelled infected cells showed that the expressed transporter was present not only at the cell surface but also in membranous structures throughout the cytoplasm, except for the region occupied by the nucleus (results not shown). Previous studies have shown that heterologous expression of other membrane proteins using the baculovirus/insect-cell system induces a proliferation of cytoplasmic membranes [8].

Western-blot analysis using the same anti-transporter antibodies confirmed the presence of immunologically cross-reactive transporter in cells infected with recombinant baculovirus, but not in non-infected cells or those infected with wild-type virus (Fig. 2). Expressed transporter was confined to the membrane fraction of the infected cells and was absent from the soluble fraction (Fig. 2). The native erythrocyte protein is heterogeneously glycosylated and migrates on SDS/polyacrylamide gels as a broad band of apparent M_r 45000–65000, which is converted into a sharp band of M_r 46000 upon enzymic deglycosylation [3,16] (Fig. 3). By contrast, the transporter expressed in insect cells migrated as a single fairly sharp band of M_r 45000, almost identical with that of its deglycosylated erythrocyte counterpart. The presence of less-extensive and more homogeneous glycosylation was confirmed by the fact that treatment with endoglycosidase F only slightly increased the

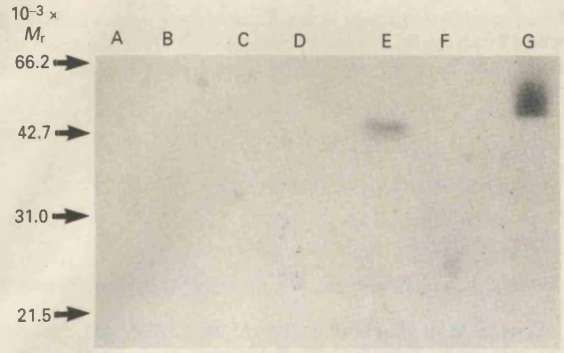

Fig. 2. Western-blot analysis of GLUT1 expression in insect cells

Sf9 cells were grown in the absence of virus (mock infection; lanes A and B), infected with wild-type virus (lanes C and D) or with AcNPV-GT (lanes E and F). After 4 days they were harvested, sonicated, and then separated into membranous (lanes A, C and E) and soluble (lanes B, D and F) fractions. Samples derived from equal numbers of cells (2×10^6 cells) were then electrophoresed on an SDS/10 %-polyacrylamide gel and subjected to Western blotting using affinity-purified antibody against the *C*-terminus of GLUT1, and alkaline phosphatase-linked second antibody as described in [13]. G, human erythrocyte membrane control. The positions of proteins used as M_r markers are indicated.

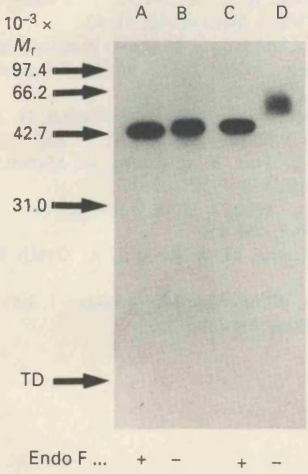

Fig. 3. Endoglycosidase F treatment of the glucose transporter expressed in Sf9 cells

Samples (20 μg) of insect cell membranes (lanes A and B) and of alkali-stripped human erythrocyte membranes (lanes C and D) were incubated with (+) or without (−) 0.25 unit of endoglycosidase F (Endo F) for 18 h at 25 °C as described in [14]. Samples (2 μg) were then electrophoresed on an SDS/12 %-polyacrylamide gel and subjected to Western blotting, using anti-transporter antibodies as described in [14]. The positions of proteins used as M_r markers are indicated.

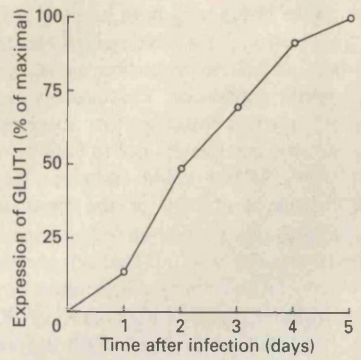

Fig. 4. Time course of GLUT1 expression in insect cells

Sf9 cells were infected at a MOI of 5–10 with the recombinant virus, collected at various intervals after infection and sonicated. Samples (10 μg) of the resultant cell lysates were assayed for GLUT1 by Western blotting as described in the Experimental section. Maximal levels of GLUT1 expression typically corresponded to approx. 1.4 nmol/mg of protein.

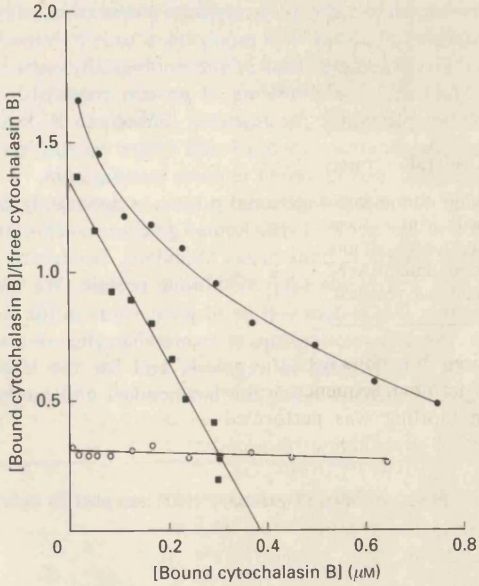

Fig. 5. Scatchard-plot analysis of D-glucose-inhibitable cytochalasin B binding to recombinant GLUT1 expressed in Sf9 cells

Binding of cytochalasin B to infected cell membranes (3 mg/ml) was measured in the presence (○) or absence (●) of 400 mM-D-glucose as described in the Experimental section. A derived Scatchard plot (■) was generated by subtracting, along radial axes, the curve obtained in the presence of D-glucose from that obtained in its absence. Linear regression analysis was used to fit the resultant data points to the best straight line.

electrophoretic mobility of the recombinant protein (Fig. 3). The insect cell is known to perform *N*-linked glycosylation of expressed proteins at authentic sites. However, it lacks galactosyl- and sialyl-transferases [7] and so cannot process the *N*-linked high-mannose oligosaccharides to the complex carbohydrate chains known to occur on the erythrocyte protein [17]. It should be noted that the transporter possesses a single glycosylation site (Asn^{45}) [4].

Infection of insect cells with a recombinant baculovirus encoding the human erythrocyte glucose transporter led to the expression of considerable quantities of the transport protein. The transporter was stable and accumulated until the cells lysed, 4–5 days after infection (Fig. 4). Quantitative Western blotting of two separate preparations showed that, 4 days after infection, 1.41 and 1.47 nmol of transporter respectively were present per mg of membrane protein, values which represent almost 8 % (w/w) of the total membrane protein. Comparable levels of expression have been reported in the baculovirus system for other membrane glycoproteins, although higher levels, equivalent to that of polyhedrin itself, i.e. 35–50 %, have been reported for some non-membrane proteins [7].

It was not possible to determine the transport activity of the

expressed protein in the insect cells, both because of the presence of endogenous transport systems which allow the cells to grow on glucose, and because infected cells become leaky at the time of maximal transporter expression. Fortuitously, however, the insect-cell transporter(s) was found neither to cross-react with antibodies to the mammalian protein nor to bind cytochalasin B. The binding of this inhibitor could therefore be used as a measure of the biological activity of the expressed protein. Scatchard-plot analysis, after correction for non-specific binding to membrane lipids, showed that infected cell membranes contained a single class of specific sites which bound cytochalasin with a K_d of 284 nM, almost identical with the K_d of 282 nM which we have previously reported for the native erythrocyte transporter [14]. Binding was completely inhibited by 400 mM-D-glucose (Fig. 5), but not by L-glucose (results not shown), indicating that the expressed protein bound not only the inhibitor, but also the transported substrate. Non-infected cells and cells infected with the wild-type virus exhibited no D-glucose-inhibitable binding of cytochalasin B (results not shown). Thus it is clear that human glucose transporter expressed in the baculovirus system exhibits native-like biological activity, although purification and reconstitution will be required to demonstrate that it can actually transport glucose. However, the concentration of cytochalasin B-binding sites detectable in two separate preparations of infected cell membranes (0.23 and 0.29 nmol/mg of protein respectively), was less than the concentration of immunologically cross-reactive protein (1.41 and 1.47 nmol/mg of protein respectively). This phenomenon resembles the reported differences in functional activity of baculovirus-expressed and native human multidrug transporter [18], and its origin requires investigation.

Although some non-functional protein is apparently present, expression of *biologically active* human glucose transporter in the baculovirus system is nonetheless abundant, amounting to between 1 and 2% of the total membrane protein. We therefore conclude that this system will be of great value in the study of structure–function relationships in mammalian glucose-transport proteins by site-directed mutagenesis and for the large-scale production of these proteins for biochemical and mechanistic studies.

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