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# Cloning and expression of a hepatic microsomal glucose transport protein

## Comparison with liver plasma-membrane glucose-transport protein GLUT 2

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Antibodies raised against a 52 kDa rat liver microsomal glucose-transport protein were used to screen a rat liver cDNA library. Six positive clones were isolated. Two clones were found to be identical with the liver plasma-membrane glucose-transport protein termed GLUT 2. The sequence of the four remaining clones indicates that they encode a unique microsomal facilitative glucose-transport protein which we have termed GLUT 7. Sequence analysis revealed that the largest GLUT 7 clone was 2161 bp in length and encodes a protein of 528 amino acids. The deduced amino acid sequence of GLUT 7 shows 68% identity with the deduced amino acid sequence of rat liver GLUT 2. The GLUT 7 sequence is six amino acids longer than rat liver GLUT 2, and the extra six amino acids at the *C*-terminal end contain a consensus motif for retention of membrane-spanning proteins in the endoplasmic reticulum. When the largest GLUT 7 clone was transfected into COS 7 cells the expressed protein was found in the endoplasmic reticulum and nuclear membrane, but not in the plasma membrane. Microsomes isolated from the transfected COS 7 cells demonstrated an increase in their microsomal glucose-transport capacity, demonstrating that the GLUT 7 clone encodes a functional endoplasmic-reticulum glucose-transport protein.

## INTRODUCTION

Blood glucose concentrations in mammals are normally restricted within a very narrow range, even though there can be very large variations in both the availability of exogenous glucose and its utilization. The homeostatic regulation of blood glucose is achieved by balancing the rates of flux of blood glucose into all tissues, and into and out of the liver (Ferrannini *et al.*, 1985). The first step in the uptake of glucose from the blood by tissues is the transport of glucose across the plasma membrane of cells. In recent years a combination of biochemical and molecular studies have demonstrated the existence of a family of facilitative glucose-transport proteins which have very different properties but related primary sequences (e.g. Wheeler & Hinkle, 1985; Simpson & Cushman, 1986; Kayano *et al.*, 1988; Thorens *et al.*, 1988, 1990*a*,*b*; Bell *et al.*, 1990; Johnson *et al.*, 1990). To date, five plasma-membrane facilitative glucose transporters (GLUTs 1–5) and a sixth pseudogene (*GLUT 6*) have been identified (Kayano *et al.*, 1990). In contrast, the final steps in the release of glucose from the liver into the bloodstream to maintain normoglycaemia are less well understood. In the liver, glucose is produced by both glycogenolysis and gluconeogenesis. The terminal step of both is catalysed by glucose-6-phosphatase (EC 3.1.3.9) (Nordlie, 1985; Ashmore & Weber, 1959). Glucose-6-phosphatase is a multi-component enzyme comprising at least six different polypeptides [see Burchell (1990, 1992) and Burchell & Waddell (1990, 1991) for recent reviews]. In order for the glucose produced inside the lumen of the endoplasmic reticulum to be released into the blood, it must first cross the endoplasmic-reticulum membrane. Nevertheless, until very recently the existence of a liver endoplasmic-reticulum glucose-transport protein had not been demonstrated. Recently we identified and purified a rat hepatic microsomal glucose-transport protein (Waddell *et al.*, 1991). Antibodies raised against the protein inhibited glucose-6-phosphatase activity in intact microsomal vesicles, indicating that it was the glucose-transport protein, $T_3$, of the glucose-6-phosphatase system (Waddell *et al.*, 1991). Here we describe how this antibody was used to screen a rat liver cDNA library and isolate cDNAs coding for a liver endoplasmic-reticulum facilitative glucose-transport protein which, because of its similarity to GLUTs 1–5, we have termed GLUT 7. Transient expression of the GLUT 7 clone in COS 7 cells results in increased levels of endoplasmic-reticulum-specific glucose transport.

## MATERIALS AND METHODS

### Materials

Biotinylated anti-sheep antibody and a streptavidin-linked horseradish peroxidase detection system were purchased from Amersham International, Amersham, Bucks., U.K., as were Hybond C and $N^+$ membranes. The rat liver $\lambda$ ZAP II cDNA library and R408 helper phage were obtained from Stratagene, Cambridge, U.K. M13 mp 18 and mp 19 replicative form, and 17-mer universal primer were obtained from Pharmacia, Uppsala, Sweden, as were restriction endonucleases and enzymes. Fluorescent primers for sequencing were obtained from Applied Biosystems, Warrington, Cheshire, U.K. Anti-(GLUT 7) serum was prepared as reported by Waddell *et al.* (1991). Nitrocellulose was obtained from Schleicher und Schüll, Dassell, Germany. COS 7 cells (Gluzman, 1981) were kindly provided by Clare Madin (School of Pathology, University of Oxford, U.K.), pc DNAI/NEO was obtained from AMS Biotechnology, Oxford, U.K. RPMI 1640 media and fetal-bovine serum were obtained from ICN–Flow, High Wycombe, Bucks., U.K. 3-*O*-Methyl-D-[U-$^{14}$C]glucose (> 1.85 GBq/mmol) was from Amersham International. All other chemicals were of the highest grade available.

### cDNA library immunoscreening

For screening of the rat liver $\lambda$ ZAP II cDNA library, phage were plated on a lawn of XL-1 blue cells at a density of $2 \times 10^4$ plaque-forming units/140 mm-diameter Petri dish (Sambrook *et*

The nucleotide sequence reported has been submitted to the EMBL and associated databases under the accession no. X66031.

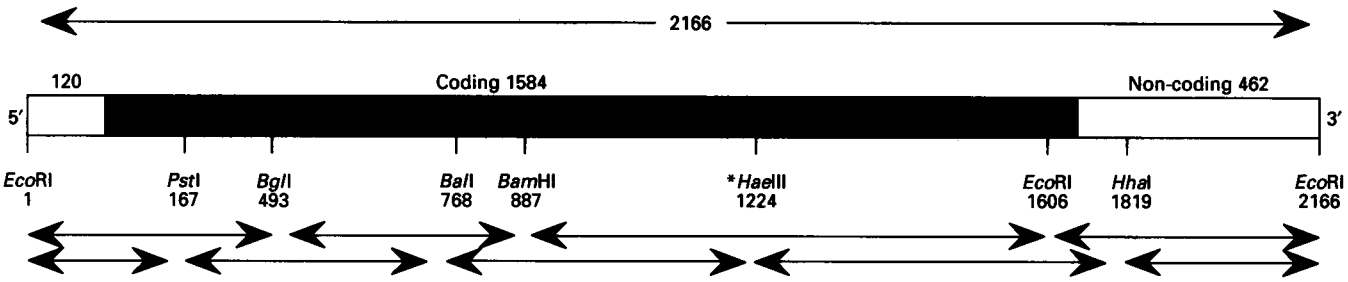


**Fig. 1. Partial restriction map and nucleotide sequencing strategy of the cDNA insert in λzrlglu 2**

The extent of sequencing is shown by horizontal arrows. Each fragment was completely sequenced on both DNA strands from the unique restriction site (except *) indicated.

*al.*, 1989). After 5 h growth at 42 °C, plates were overlaid with isopropyl thiogalactoside-impregnated nitrocellulose filters and growth continued at 37 °C for a further 4 h. Filters were blocked in 3 % (w/v) BSA/0.1 % Marvel (dried milk) each for 1 h, then incubated in affinity-purified anti-(rat liver microsomal 52 kDa protein) antibody for 1 h at ambient temperature. Positive clones were detected by means of a biotinylated secondary antibody and a streptavidin-linked horseradish peroxidase detection system with 4-chloro-1-naphthol as substrate. Putative positive clones were then purified by three additional cycles of plaque purification. Phage clones were amplified. The bluescript plasmid containing the clones was excised from the λ ZAP II vector by the method described in Short *et al.* (1988) and using R408 helper phage (Russell *et al.*, 1986).

### DNA sequencing

Sequencing of restriction fragments subcloned into M13 mp 18 or 19 replicative form was performed by the dideoxy-chain-termination technique (Sanger *et al.*, 1977) on an Applied Biosystems automated DNA sequencer using the Klenow fragment of DNA polymerase, primed by the 17-mer universal fluorescent primer.

### Construction of recombinant plasmids for GLUT 7 expression

A rat liver GLUT 7 cDNA was subcloned into the *Hin*dIII/*Pst*1 site on the expression vector pcDNA1/NEO (Lubon *et al.*, 1989). Recombinant plasmids were grown in *Escherichia coli* and plasmids with the cDNA insert in the correct orientation with respect to transcription from the CMV promotor were confirmed by restriction mapping.

### Transfection of COS 7 cells

COS 7 monkey kidney cells were cultured in RPMI 1640 medium containing 10 % (v/v) dialysed fetal-bovine serum at 37 °C in air/$CO_2$ (19:1). Plasmid DNA (40 mg) was used to transfect each semiconfluent dish (150 $cm^2$) of COS 7 cells, using the calcium phosphate/glycerol shock procedure (Parker & Stark, 1979). The cells were harvested 72 h later and assayed for glucose transport.

### Assay of GLUT 7

Cell pellets were collected by centrifugation at 500 *g* for 5 min and washed twice with 0.5 M-NaCl/5 mM-Tris/HCl buffer, pH 7.4 (TBS), and stored frozen at −80 °C. Cell pellets were thawed and gently hand-homogenized in a ground-glass homogenizer in TBS. Wistar-rat liver and kidney microsomes were prepared as previously described (Waddell *et al.*, 1991).

### Immunocytochemistry

Transformed COS 7 cells grown on glass slides were fixed by immersion in acetone. The slides were incubated for 1 h in TBS/1 % BSA, then for 1 h with the first antibody [affinity-purified anti-(52 kDa polypeptide) antibody; 50 mg/ml in TBS/1 % BSA]. After incubation, the slides were washed five times for 5 min each with TBS and then incubated for 30 min with a 1:75 dilution (in TBS/1 % BSA) of fluoresceinated rabbit anti-(sheep immunoglobulin) antibody. The slides were finally washed five times for 5 min in TBS and mounted in 1 % *p*-phenyldiamine chloride/90 % glycerol/TBS, pH 8.3. Fluorescence microscopy was then performed the same day using a Leitz Orthoplan photomicroscope equipped for epi-illumination using an HBO-200 mercury light source and Phaco NPL objectives. Samples were examined by means of the green fluorescence spectral setting for fluorescein isothiocyanate (excitation wavelength 495 nm; emission wavelength 525 nm). Excitation was performed with a BP-490 nm exciter filter together with centre wavelength chromatic beam splitter and an additional barrier filter (K-510). The preparations were initially photographed on Fujichrome 64 Professional T film.

## RESULTS

A λ ZAP II rat liver cDNA library was screened by using affinity-purified anti-(rat microsomal 52 kDa protein) antibody. Screening of $2 \times 10^6$ individual recombinants resulted in six positive clones λzrlglu 1–6. Initial sequence analysis revealed that four clones, λzrlglu 1, 2, 4 and 5, encoded proteins with strong sequence similarity to rat liver plasma-membrane facilitative glucose-transport protein termed GLUT 2 (Thorens *et al.*, 1988), but were not identical. The remaining clones contained regions of > 100 bp at the C-terminal end and in the 3′ untranslated region which were 100 % identical with GLUT 2. For the purposes of this investigation, λzrlglu 3 and 6 were discarded.

The four remaining clones were sized by agarose-gel electrophoresis and the largest, λzrlglu 2, fully sequenced. Fig. 1 shows the sequencing strategy adopted, whereas Fig. 2 reveals the full-length nucleotide sequence. The cDNA is 2.16 kb long and contains a 1.58 kb open reading frame coding for a 528 amino acid protein; the calculated mass of the protein is 53 kDa. The 5′ untranslated region is 120 bp long and contains no in-frame stop codons upstream of the initiator ATG. The 3′ non-coding region is 462 bp long and contains two putative polyadenylation sites (Fig. 2) (Wickens & Stephenson, 1984). The coding region is 75 % identical with that of the rat liver GLUT 2 and 67 % with that of the human protein. This sequence similarity is not uniform and is accounted for by three in-frame regions of almost 100 % similarity that are underlined in Fig. 3. The first region begins at nucleotide 39 and runs to nucleotide 525 and is completely identical with the same region of GLUT 2,

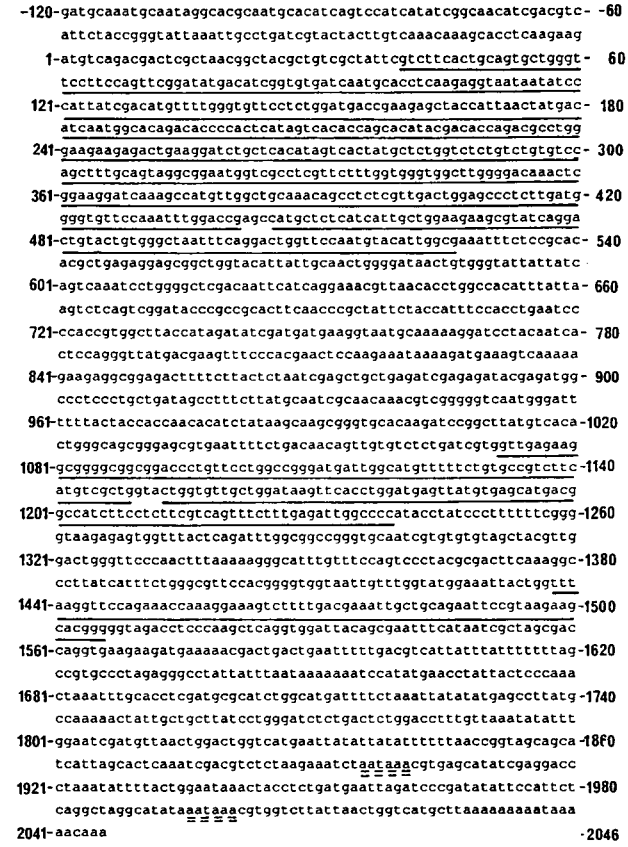

**Fig. 2. Nucleotide sequence of the full-length clone λrlGLUT 7**

Areas of 100% similarity to the nucleotide sequence of the rat liver GLUT 2 are underlined, and putative polyadenylations are indicated by broken underlining.

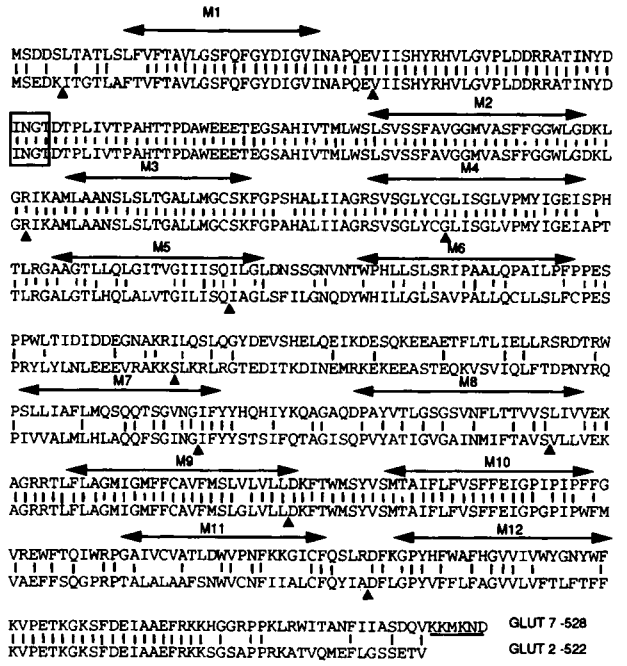

**Fig. 3. Protein-sequence comparison of the rat liver microsomal GLUT 7 with rat liver plasma-membrane GLUT 2**

The arrows indicate the positions of the 12 putative transmembrane segments. The boxed residues are the *N*-glycosylation sites. The consensus motif for retention of transmembrane proteins in the endoplasmic reticulum is underlined. The rat liver GLUT 2 sequence is taken from Thorens *et al.* (1988), the putative intron/exon boundaries for GLUT 2 (Bell *et al.*, 1990) are indicated. Triangles (▲), between residues indicate that intron interrupts gene between codons for adjacent amino acids; a ▲ symbol directly below an amino acid indicates where the intron interrupts the codon for that residue.

except for one in-frame codon beginning at nucleotide 441. These three nucleotides show no conservative changes. A similar pattern is observed in the second identical region, which runs from nucleotide 1071 to nucleotide 1239, with a single non-conserved codon at 1149. The final region is a classical stretch of 69 bp which is 100% identical, running from nucleotide 1437 to nucleotide 1506.

### Amino acid sequence comparison

Fig. 3 indicates that GLUT 7 and GLUT 2 have predicted amino acid sequences which are 68% identical in rats. The twelve putative transmembranous regions of GLUT 2 are indicated as M1–M12. The amino acid similarity between GLUT 7 and GLUT 2 within these regions varies greatly. M1-4, 9 and 10 are almost 100% identical, as was expected, whereas in M5–8, 11 and 12 there is less well marked similarity. However, when these predicted amino acid sequences were used to prepare hydropathy plots (Kyte & Doolittle, 1982) (Fig. 4), it can be clearly seen that GLUT 7 also contains 12 potential membrane-spanning regions and that these line up well with those of GLUT 2. The exoplasmic glycosylated 'loop' between M1 and M2 is identical and contains the single asparagine-linked glycosylation site. These proteins must have very similar secondary and tertiary folding within lipid bilayers. Perhaps the most striking structural difference is that GLUT 7 contains an extra six amino acids at the *C*-terminal end.

### Functional assay

To test whether the hepatic cDNA we have isolated was indeed a glucose transporter, we subcloned the cDNA into the tissue-

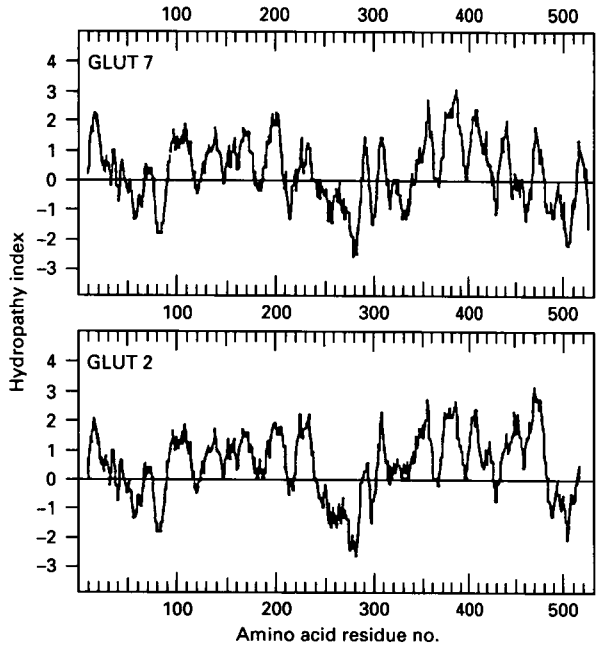


**Fig. 4. Hydropathy plots of GLUT 7 and GLUT 2**

The hydropathy plots were derived according to the algorithm of Kyte & Doolittle (1982) using a 19-amino-acid window.

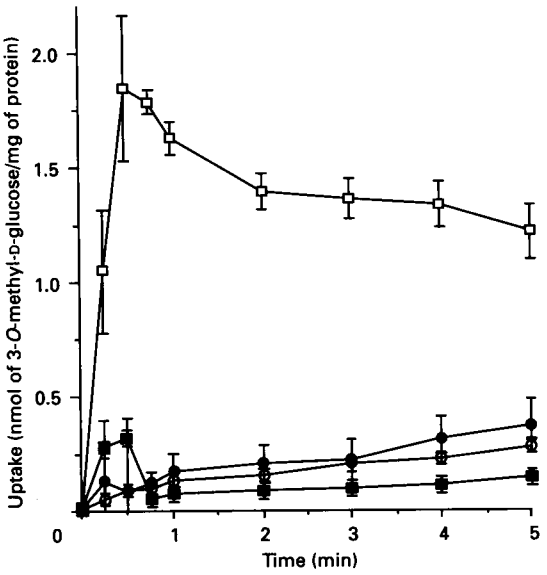


**Fig. 5. Functional expression of GLUT 7**

COS 7 cells were transformed with either a pcDNAI/NEO plasmid containing no insert (■), or a plasmid containing the hepatic GLUT 7 (□). Uptake studies were carried out as described in the Materials and methods section, using a final concentration of 1 mM-3-*O*-methyl-D-[U-$^{14}$C]glucose. Also plotted are the uptake into microsomes from COS 7 cells transformed with pcDNAI/NEO GLUT 7 and treated with either 0.5 mM-phloretin (●) or a 100-fold excess of L-glucose (○).

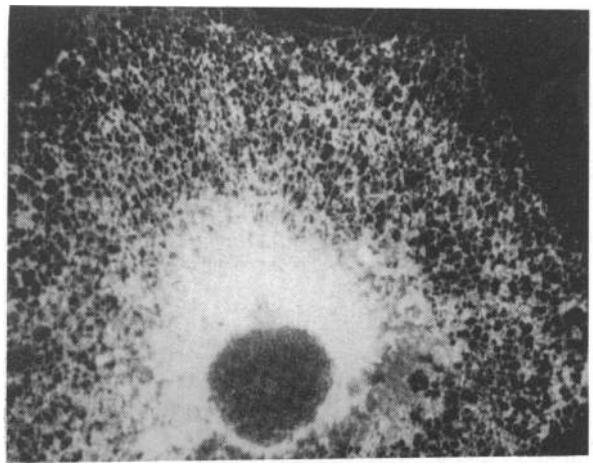

**Fig. 6. Immunofluorescence on COS 7 cells**

COS 7 cells transformed with pcDNAI/NEO containing the hepatic GLUT 7 stained with anti-(52 kDa polypeptide) antibody and a fluorescein-labelled rabbit anti-sheep secondary antibody.

culture vector pcDNA I NEO, downstream of the cytomegalovirus promotor. This construct was used to transform COS 7 cells, which were allowed to transiently express the inserted DNA (48–60 h) before being disrupted and subjected to subcellular localization to isolate the microsomal fraction. Microsomes were assayed for their ability to transport 3-*O*-methyl-D-glucose. Microsomal uptake by transformed cells (Fig. 5) was some 5-fold greater than by control cells, was stereoselective and could be inhibited by phloretin, a potent glucose-transporter inhibitor. Cells transformed with the cDNA in reverse orientation took up glucose to the same low extent as cells containing control plasmids without the cDNA.

### Immunocytochemical localization of GLUT 7 in COS 7 cells

The subcellular localization of GLUT 7 transiently expressed in COS 7 cells was determined by indirect immunofluorescence (Fig. 6). Importantly GLUT 7 was observed in the endoplasmic reticulum and nuclear envelope, but not the plasma membrane.

## DISCUSSION

In the last 3 years there have been major advances in our understanding of many of the protein components of glucose-6-phosphatase (Burchell, 1990, 1992; Burchell & Waddell, 1990, 1991), but the mechanism by which glucose produced by glucose-6-phosphatase exited from the lumen of the endoplasmic reticulum remained unclear. In contrast, there have been many advances in the field of glucose transport across the plasma membranes of many tissues, including liver (e.g. see Thorens *et al.*, 1988; Bell *et al.*, 1990; Gould & Bell, 1990). Recently we isolated a 52 kDa putative rat liver microsomal glucose-transport protein and antibodies raised against the protein inhibited both glucose-6-phosphatase activity in intact microsomes and microsomal output of glucose (Waddell *et al.*, 1991) indicating that the protein was either $T_3$ of the glucose-6-phosphatase system or a component of $T_3$. This antibody was used to screen a rat liver cDNA library, and the sequence of the isolated clone and the protein that it encodes can be seen in Figs. 2 and 3 respectively. That this was isolated using the anti-(52 kDa microsomal) antibody and its sequence similarity to the GLUT superfamily (Bell *et al.*, 1990) indicates that it is most likely a member of the same family of proteins as the plasma-membrane facilitative glucose-transport protein. That GLUT 7 encodes a functional glucose-transport protein was confirmed by expression studies in COS 7 cells (Fig. 5). We have therefore termed the 52 kDa protein 'GLUT 7'.

Of this family of proteins, GLUT 7 is most similar to GLUT 2, the liver plasma-membrane glucose-transport protein (see Figs. 2 and 3). The fact that there are three regions where the cDNA sequence is identical with that of rat liver GLUT 2 presumably explains why, in addition to the four GLUT 7 clones, we also isolated two GLUT 2 clones. Surprisingly, none of the ends of the regions of 100% identity with GLUT 2 correspond to the proposed intron/exon boundaries (Fig. 3) for GLUT 2 (Bell *et al.*, 1990), indicating that GLUT 7 is not a simple splice variant of GLUT 2. However, the lack of third-base drift in the codons of the identical regions suggests the possibility of more complex splicing. Mueckler *et al.* (1985) presented a model for the topology of GLUT 1 including 12 hydrophobic presumed membrane-spanning domains. Similar models have been proposed for GLUTs 2–5. Fig. 4 demonstrates that the hydrophobicity plot of GLUT 7 is very similar to that of GLUT 2, including the 12 hydrophobic, presumably membrane-spanning, domains. The sequences of GLUTs 1–5 contain many conserved invariant amino acids (Bell *et al.*, 1990). GLUT 7 also contains many of the invariant amino acids. Because of the very marked similarity of GLUTs 2 and 7, including three regions where the sequences are the same at both nucleotide and amino acid levels, it seems most probable that many past studies of GLUT 2 using either antibodies or full-length cDNAs as probes may well have also been measuring both GLUT 7 and GLUT 2 expression. In the future, care will have to be taken to use only probes specific for either GLUT 7 or GLUT 2.

The most obvious differences between GLUT 7 and GLUT 2 is that GLUT 7 is six amino acids larger than GLUT 2. The sequence of the last six amino acids of GLUT 7 (KKMKND) is very similar to the last six amino acids of some of the microsomal UDP-glucuronosyltransferase isoenzymes; for example, rat liver microsomal UDP-glucuronosyltransferase R38 contains the se-

quence KKMKNE (Harding *et al.*, 1987). The extra six amino acids at the *C*-terminus contain a KK–K-- consensus motif for retention of transmembrane proteins in the endoplasmic reticulum (Jackson *et al.*, 1990). Unlike the better known KDEL motif, which is a characteristic feature of soluble proteins residing in the endoplasmic-reticulum lumen (Munro & Pelham, 1987), the KK–K-- sequence is not highly conserved, requiring only two lysine residues, one of which must be positioned three residues from the *C*-terminus and the second either four or five residues from the *C*-terminal end (Jackson *et al.*, 1990). As expected, the KK–K-- endoplasmic-reticulum consensus motif is not present in any of the plasma-membrane glucose-transport proteins (GLUTs 1–5). The presence of the endoplasmic-reticulum consensus motif in GLUT 7 strongly suggests that GLUT 7 is an endoplasmic-reticulum protein. This was confirmed by two lines of evidence: firstly, immunoblotting experiments, which showed that the GLUT 7 protein was present in isolated microsomes (that were plasma-membrane-free) and not in plasma membranes (Waddell *et al.*, 1991) and, secondly, immunocytochemical studies published here (Fig. 6) demonstrate that GLUT 7 is expressed in the endoplasmic reticulum and not in the plasma membrane. This is in complete contrast with previous immunochemical data obtained with anti-(GLUT 2) *C*-terminal affinity-purified antibody, showing that GLUT 2 is in plasma membranes and not in intracellular membranes (Thorens *et al.*, 1988, 1990*b*).

More work will be needed to determine whether GLUT 7 is the only hepatic endoplasmic-reticulum glucose-transport protein and hence whether it is all or part of the $T_3$ glucose-transport protein of the glucose-6-phosphatase system. The availability of cDNAs coding for GLUT 7 means that for the first time it may now be possible to develop probes for use in diagnosis of type 1d glycogen storage disease ($T_3$ deficiency). In addition, it will now be possible to study the role of GLUT 7 in the regulation of hepatic output of glucose.

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