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The role of the GLUT 4 transporter in regulating rat myoblast glucose transport processes

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Abstract

Previous studies revealed an inverse relationship between *GLUT 1* and *GLUT 4* expression in rat myoblasts [L. Xia, Z. Lu, T.C.Y. Lo, J. Biol. Chem., 268 (1993) 23258–23266]. It was not clear whether these were coincidental or causal occurrences. To examine the regulatory roles of the GLUT 4 isoform, rat L6 myoblasts were transfected with full length *GLUT 4* cDNAs (2.5 kb) in the sense or antisense orientation. L6 myoblasts transfected with the *GLUT 4* sense cDNA (L6/G4S transfectants) possessed much elevated levels of both endogenous *GLUT 4* transcripts (1.4 kb and 2.8 kb). Transport and immunofluorescence studies showed that this *GLUT 4* sense cDNA was responsible for a functional GLUT 4 transporter. L6 cells transfected with the *GLUT 4* antisense cDNA (L6/G4A transfectants) possessed only 6% of the L6 level in day 6 cultures. These antisense transfectants were essentially devoid of any functional GLUT 4 transporter. The activation of transcription of the endogenous *GLUT 4* gene in L6/G4S myoblasts suggested auto-regulation of *GLUT 4* expression. *GLUT 3* expression and activity were not altered in both sense and antisense GLUT 4 transfectants. More interestingly, *GLUT 1* expression was reduced in L6/G4S myoblasts, whereas it was elevated in L6/G4A myoblasts. This was the first direct evidence indicating GLUT 4 might play an important role in suppressing *GLUT 1* expression.

Keywords: GLUT 1; GLUT 3; GLUT 4; Transfection; Transport mutant; Myoblast; (Rat)

1. Introduction

Glucose transporters (GLUT) are known to play pivotal roles in energy metabolism. At least six different eukaryotic GLUT isoforms have been identified [1–3]. They possess 12 transmembrane helical segments with the N- and C-termini and a large central loop exposed to the cytoplasm. GLUT transporters have distinct antigenic C-terminal regions, and are subject to different metabolic and developmental regulations [1–3]. They are responsible for the facilitative uptake of D-glucose and its analogues. GLUT 1 is most abundant in erythrocytes and brain microvessels, and is present in most tissues and cell cultures. GLUT 2 is expressed mainly in pancreatic β cells, liver, and enterocytes basolateral membranes. GLUT 3 is found in brain neurons, fetal muscle, skeletal and cardiac myoblasts [4,1–3,5]. GLUT 4 is present in insulin-sensitive tissues, such as brown and white adipose tissues, cardiac and skeletal muscles [1–3,5,6]. GLUT 5 is actually a fructose transporter

Abbreviations: dGlc, 2-deoxy-D-glucose; GLUT, glucose transporter; MeGlc, 3-*O*-methylglucose; PBS, phosphate-buffered saline

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detected in human enterocyte luminal membranes, adipocytes, skeletal muscle and sperm. GLUT 7 is located in liver microsomes [1–3].

We have recently demonstrated the presence of the GLUT 1, 3, and 4 isoforms in rat L6 skeletal myoblasts [4]. The rat myoblast glucose transport properties have been examined by transport kinetic analysis, cytochalasin B binding and photolabelling studies, and by inhibitor studies [7–15]. Transport, and not phosphorylation, is the rate-limiting step in the uptake of 2-deoxy-D-glucose (dGlc), even at very high substrate concentrations [7]. A high (HAHT) and a low (LAHT) affinity hexose transport processes are present in undifferentiated glucose-grown myoblasts [7]. The transport affinities for HAHT and LAHT are around 0.6 mM and 3.5 mM, respectively. While dGlc can be taken up by both processes, 3-*O*-methylglucose (MeGlc) is taken up primarily by LAHT in glucose-grown myoblasts [14]. These two transport processes are regulated differently during metabolic and developmental changes [4]. The GLUT 3 and GLUT 4 transporters are responsible for the HAHT and LAHT processes, respectively [4,13,14].

Glucose transport activity is affected by changes in the intrinsic activity, intracellular trafficking, and stability of the GLUT transporters [1–3]. Studies using myoblast mutants possessing only the GLUT 1 transporter (mutants M1 and M3) indicate that the apparent K_m values of this transporter for dGlc and MeGlc were around 0.7 mM and 20 mM, respectively [14]. More interestingly, this transporter exists in a cryptic form in glucose-grown cells [14]. It can be activated by glucose starvation, cholera toxin, protein synthesis inhibitors, energy poisons, and cadmium [13,14,16–18]. Acute insulin treatment of adipocytes and myoblasts activates not only glucose transport activity, but also the translocation of the GLUT 1, 3 and 4 transporter from intracellular sites to the plasma membrane [19–23]. Insulin has a greater effect on GLUT 4 translocation than on GLUT 1 translocation [22–26]. Chronic insulin treatment results in a greater increase in glucose transport activity than in the amount of GLUT 1 and GLUT 4 proteins in the plasma membrane [27]. These studies show that GLUT transport activity can be modulated, directly or indirectly, by protein–protein interaction, post-translational modification of the transporter, and/or changes in cellular environment [16,28,29].

A number of mechanisms are involved in regulating the expression of GLUT transporters. GLUT expression can be regulated by the transporter itself, or by other GLUT transporters. Auto-regulation of the GLUT 3 isoform was recently observed in cells transfected with the *GLUT 3* sense cDNA [30]. An inverse relationship between *GLUT 1* and *GLUT 4* expression has been observed in cells undergoing developmental and physiological changes, or in cells treated with insulin, cAMP, tumor necrosis factor or monokines [13,22,31–36]. While chronic insulin treatment increases *GLUT 1* transcript level in L6 muscle cells, 3T3-L1 adipocytes and human fibroblasts, it reduces *GLUT 4* transcript level in these cells [36–38]. The depletion of *GLUT 4* transcript and protein observed in arachidonic acid treated 3T3-L1 adipocytes was accompanied by stabilization of *GLUT 1* transcript and increased GLUT 1 transporter levels [39]. It is not clear whether the observed inverse relationships are coincidental or causal occurrences. There is no direct evidence on whether one or both transporters are responsible for maintaining this inverse relationship.

In view of the observation that *GLUT 1* expression was elevated in mutants devoid of the GLUT 4 transporter, it was suggested that *GLUT 1* expression was suppressed by the GLUT 4 transporter [13]. An innate problem associated with mutant studies is the inability to distinguish mutation of the GLUT 4 transporter itself from alterations of regulatory components. To circumvent this problem, we have transfected rat myoblasts with either the *GLUT 4* sense or antisense cDNAs. This investigation examined the properties of these *GLUT 4* transfectants.

2. Experimental procedures

2.1. Cell cultures

Rat L6 myoblast was originally isolated by Yaffe [40]. L6/CMV, L6/G4A (clones 3, 7 and 11), L6/G4S (clones 2, 8 and 9) myoblasts are stable transfectants harbouring the pRc/CMV vector, *GLUT 4* antisense cDNA, and *GLUT 4* sense cDNA, respectively. Mutant M1 is a GLUT 3^-GLUT 4^- myoblast originally isolated from rat L6 myoblasts [4,13]. M1/CMV (clones 1), M1/G4A (clones 7 and 11)

and M1/G4S (clones 8 and 9) are stable transfectants harbouring the pRc/CMV vector, *GLUT 4* antisense cDNA, and *GLUT 4* sense cDNA, respectively. Myoblasts were grown in Alpha Minimal Essential Medium (Life Technologies), supplemented with 50 μg/ml of gentamicin sulphate (Life Technologies) and 10% v/v horse serum (Hyclone). Cells were routinely seeded at a density of 1×10^6 cells per 150 mm Nunc plates and subcultured every 3 days (before fusion), cells were detached using 0.4% trypsin [4,13].

2.2. Plasmids and culture media

The pRc/CMV expression vector was purchased from Invitrogen. Human *GLUT 1* and *GLUT 3* cDNAs were from the Repository of Human and Mouse DNA Probes and Libraries, ATCC. The *GLUT 4* cDNA used was isolated from rat adult skeletal muscle (clone pSM1-1-2), and was a generous gift from Birnbaum [41]. The *β2-microglobulin* cDNA was a gift from Daniel et al. [42]. Qiagen DNA purification kit (Qiagen) was used to purify plasmid DNAs used in transfection studies and in generating cDNA probes.

Bacterial cultures were grown in TB (Terrific Broth) medium containing carbencillin or tetracycline depending on the resistance markers of the plasmids. For transformation studies, cells were grown in SOC medium (SOB + 20 mM glucose) [43].

2.3. Transfection of rat L6 myoblast with GLUT 4 sense or antisense constructs

CMV vectors containing the *GLUT 4* insert were constructed using *GLUT 4* cDNAs in the sense or antisense orientation relative to the CMV promoter. The rat *GLUT 4* cDNA used was a full length *GLUT 4* cDNA (2.5 kb) including a 5′untranslated region of 105 bp and a 3′untranslated sequence of 800 bp; and it was inserted into the *Eco*RI site of the pBluescript KS + vector [41]. In this study, *GLUT 4* cDNA was digested with *Eco*RI, and then blunt-ended with the large Klenow fragment of DNA polymerase I at 20°C for 30 min. This was then ligated with the phosphorylated *Hin*dIII linker using T4 DNA ligase. The pRc/CMV vector was linearized by digesting with *Hin*dIII and dephosphorylated with calf intestinal alkaline phosphatase; this was then ligated to the *GLUT 4* cDNA with *Hin*dIII-recessed ends using T4 DNA ligase. Digestion with *Hin*dIII and *Bgl*II was used to determine the presence and orientation of the insert, respectively. The *GLUT 4* sense (clones 2, 8 and 9) and antisense (clones 3, 7 and 11) constructs were transfected into L6 or M1 myoblasts using the $CaPO_4$ precipitation method [43]. As a control, the pRc/CMV vector was also transfected into L6 or M1 myoblasts. Stable transfectants were selected and cloned by their resistance to geneticin (Life Technologies). At least five clones of each type of transfectants were isolated.

2.4. Southern blot analysis

Genomic DNA was isolated from L6 transfectants as previously described [44]. Fifteen micrograms of DNA were digested with *Hin*dIII to determine the incorporation of the *GLUT 4* cDNA into the genomic DNA. Another digestion using *Bgl*II was carried out to verify the orientation of *GLUT 4* inserts in the transfectants. The digests were run on a 0.8% agarose gel and transferred to an ICN Biotrans nylon membrane. The sizes of the DNA fragments were determined using the 1 kb DNA ladder (GibcoBRL). The blot was then washed in 2× SSC for 10 min and air-dried for 30 min. After pre-hybridization for 1 h at 42°C, the blot was hybridized with $^{32}P-$ labelled *GLUT 4* cDNA overnight at 37°C. The resulting blot was exposed to Kodak X-OMAT AR film.

2.5. Northern blot analysis

Poly $(A)^+$ RNA was extracted from myoblasts using the Invitrogen Fast Track™ kit. Northern blot studies were carried out as previously described [4,13], using 1 μg mRNA per lane. The sizes of the transcripts were determined through the use of the 0.24–9.5 RNA ladder (GibcoBRL). The blot was initially probed with labelled *β2-microglobulin* cDNA to determine sample loading. It was then probed with *GLUT 1*, *GLUT 3* and *GLUT 4* cDNAs. The blot was stripped with 60% formamide at 65°C for 2 h before hybridizing with a new probe. After exposure to Kodak X-OMAT AR film, the autoradiogram was analysed using the JAVA Video Analysis Software (Jandel Scientific). Measurements were

made in the linear range of the optical density. Two poly $(A)^+$ RNA preparations were made from each cell type, samples from each preparation were probed at least three times. The amount of mRNA present in each lane was normalized according to the amount of *β2-microglobulin* mRNA present in each sample. In calculating the relative transcript levels, the amount in day 2 L6 culture was used as 100%.

2.6. Immunofluorescence studies

Immunofluorescence studies were conducted using rabbit anti-rat GLUT 4 antibodies (East Acres Biologicals, Southbridge, MA) [45,46]. The secondary antibody was rhodamine-labelled TRITC-conjugated Affini Pure goat anti-rabbit IgG (H + L) (Jackson Immunoresearch Lab.). Rabbit IgG was used as a control. Myoblasts were grown at low density on acid-washed sterile coverslips in Falcon six-well plates (35×15 mm) for two days in Alpha medium. After washing twice with ice cold phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, and 0.5 mM $MgCl_2$), cells were fixed with Carnoy's Fix (30% methanol and 10% glacial acetic acid) for 2 min followed by 100% methanol for 10 min. After washing twice with PBS, cells were permeabilized using 0.1% Triton X-100 in PBS for 5 min, and then blocked with 1% BSA in PBS for 30 min. They were then washed three times with PBS at room temperature. Each coverslip was then incubated for 1 h with 100 μl of primary antibody diluted 1:30 in cPBS containing 0.2% gelatin, and 0.05% Tween 20. This was followed by two 5-min wash with PBS. Each coverslip was then incubated with 100 μl of rhodamine-labelled secondary antibody for 1 h at room temperature. After washing twice with PBS, the coverslips were examined under a fluorescence microscope. Pictures of representative cells were taken using Kodak Tri-X film. All pictures were taken at the same exposure time, and were printed under the same conditions to allow comparison.

2.7. Whole cell transport studies

Transport studies were carried out using six-well Falcon plates [47,48]. Cells were routinely plated at a density of 1.5×10^5 cells/well. Medium was aspirated and each well was washed with 10 ml of PBS. Nine hundred microlitres of uptake buffer (PBS containing 1 mg/ml bovine serum albumin) were added to each well. Transport studies were carried out at 23°C and were initiated by adding 100 μl of radioactive substrate to the desired final concentration. At appropriate times, uptake was terminated by rapidly washing the cells twice (less than 15 s) with 10 ml of ice-cold PBS. In the case of MeGlc uptake, cells were washed with cold PBS containing 1 mM mercuric chloride. Samples were taken at 15, 30, 45 and 60 s after the addition of radioactive substrate. Cells were solubilized with 1 ml of 0.1% Triton X-100, and 0.8 ml aliquots were counted in 10 ml of scintillation fluid. Under these conditions, the uptake of MeGlc and dGlc was linear with time, and over 95% of the internalized dGlc were phosphorylated [7]. Cells in two wells from each plate were detached with 0.1% trypsin, and counted using a Coulter counter. Studies were carried out in duplicate and each experiment was repeated at least twice. Results were consistent in all cases. Data were analysed by a linear least squares regression fit program (SlideWrite Plus™, version 4.0, Advanced Graphics Software, Sunnyvale, CA), and by a non-linear regression data analysis program (Enzfitter program, Biosoft, Cambridge, UK).

2.8. Materials

[α-^{32}P]-dCTP, 2-deoxy-D-[1,2-^{3}H]glucose and 3-*O*-[*methyl*-^{3}H]methyl-D-glucose were purchased from ICN Biochemicals. Reagents used in bacterial cultures were from Difco. Restriction and modifying enzymes were purchased from Pharmacia, whereas λ DNA *Hin*dIII digest and λ DNA *Bst*EII digest were from New England Labs. All other chemicals were purchased from commercial sources and were of the highest available purity.

3. Results

3.1. Transfection of rat L6 myoblast with GLUT 4 sense or antisense cDNA

To examine the regulatory roles of the GLUT 4 transporter, rat L6 myoblasts were transfected with

GLUT 4 cDNAs in the sense or antisense orientation. The presence of the *GLUT 4* cDNA insert was determined using genomic DNAs from day 2 cultures of L6, L6/CMV, L6/G4S (clones 2, 8 and 9) and L6/G4A (clones 3, 7 and 11) myoblasts. After digestion with *Hin*dIII, these genomic DNAs were probed with ^{32}P – labelled *GLUT 4* cDNA. The exogenous 2.5 kb *GLUT 4* cDNA was detected as a very intense and broad band in L6/G4S and L6/G4A myoblasts, but not in L6 and L6/CMV myoblasts (Fig. 1A). The fairly faint 3.1 kb band detected in all cell lines was likely the endogenous *GLUT 4* gene. Orientation of

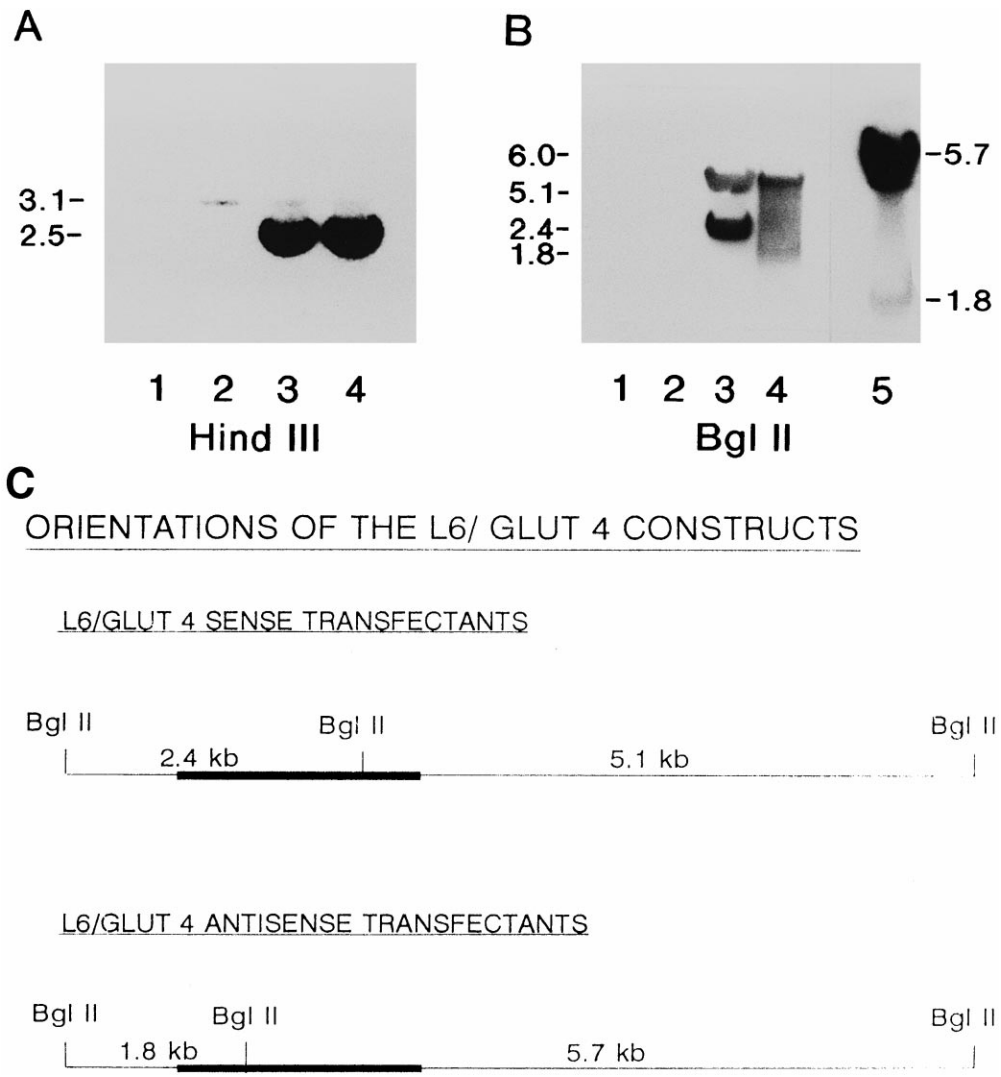

Fig. 1. Southern blot analyses of rat L6 myoblasts transfected with *GLUT 4* sense or antisense cDNAs. Genomic DNAs were isolated from L6 myoblasts, control transfectant (L6/CMV), *GLUT 4* antisense transfectant (L6/G4A), and *GLUT 4* sense transfectant (L6/G4S). Southern blot analyses were carried out as described in the text. Panel A indicates digestion of 10 μg of genomic DNA with *Hin*dIII. This demonstrates the presence of the 2.5 kb *GLUT 4* cDNA insert in L6/G4A and L6/G4S myoblasts. Panel B indicates digestion of 10 μg of genomic DNA with *Bgl*II. This indicates the orientation of the *GLUT 4* cDNA insert. Lanes 1, 2, 3, and 4 in Panels A and B denote genomic DNAs from L6, L6/CMV, L6/G4S and L6/G4A myoblasts, respectively. Lane 5 in Panel B indicates the presence of the 5.7 kb and 1.8 kb fragments when a 0.8% agarose gel was overloaded with a *Bgl*II digested genomic DNA from L6/G4A myoblast, and run for a longer time to achieve better separation. The numbers beside each panel indicate DNA fragment sizes in kb. Panel C is the linear orientation CMV/GLUT 4 *Bgl*II restriction map. The thick line denotes the *GLUT 4* cDNA, and the thin line represents the pRc/CMV vector.

the *GLUT 4* cDNA insert was determined using genomic DNAs digested with *Bgl*II (Fig. 1B). As expected from the *CMV/GLUT 4* restriction map (Fig. 1C), the *GLUT 4* cDNA probe was able to recognize a 2.4-kb and a 5.1-kb band in L6/G4S myoblasts (Fig. 1B, lane 3), and a 1.8-kb and 5.7-kb band in L6/G4A myoblasts (Fig. 1B, lanes 4 and 5), and it could hardly label any DNA bands in L6 (lane 1) and L6/CMV (lane 2) myoblasts (Fig. 1B). Thus, the *GLUT 4* cDNA inserts were present in the expected orientations in L6/G4S and L6/G4A myoblasts. The GLUT 3^{-}GLUT 4^{-} mutant, M1, was also transfected with pRc/CMV vector, the *GLUT 4* sense and antisense cDNAs. The presence and orientation of these exogenous cDNAs in various M1 transfectants were confirmed using similar approaches (data not shown).

3.2. GLUT 4 transcript levels in L6 transfectants

Northern blot studies were carried out using poly $(A)^{+}$ RNAs from days 2, 4 and 6 cultures of L6 transfectants. Similar to previous observations [4,27], a 1.4-kb and a 2.8-kb transcript were recognized by the *GLUT 4* cDNA probe in all cell types examined (Figs. 2 and 3). These two *GLUT 4* transcripts were thought to arise from differential RNA processing [4,27]. L6 and L6/CMV myoblasts possessed similar levels of the 1.4-kb and 2.8-kb *GLUT 4* transcripts; about a threefold increase was observed between day 2 and day 6 cultures (Fig. 3). More interestingly, L6/G4S transfectants possessed relatively constant levels of the endogenous 1.4-kb and 2.8-kb *GLUT 4* transcripts, and they were maintained at about three times higher than those in day 2 L6 myoblasts (Fig. 3). It was important to note that a transcript corresponding to the size of the exogenous *GLUT 4* cDNA (2.5 kb) was hardly detectable in these transfectants (Fig. 2, lane 3). This showed that transcription of the endogenous *GLUT 4* gene was activated, directly or indirectly, by the exogenous *GLUT 4* sense cDNA; this suggested auto-regulation of *GLUT 4* expression. Similar auto-regulation of the GLUT 3 transporter was also observed in rat myoblast transfectants harbouring the *GLUT 3* sense cDNA [30].

In the case of L6/G4A myoblasts, about 15% and 12% of the L6 1.4-kb and 2.8-kb *GLUT 4* transcripts were detected in day 2 culture, respectively (Fig. 3). These transcripts remained at relatively low levels during growth. Day 6 culture of L6/G4A myoblasts possessed only 8% and 4% of the 1.4-kb and 2.8-kb *GLUT 4* transcripts present in day 6 L6 cultures,

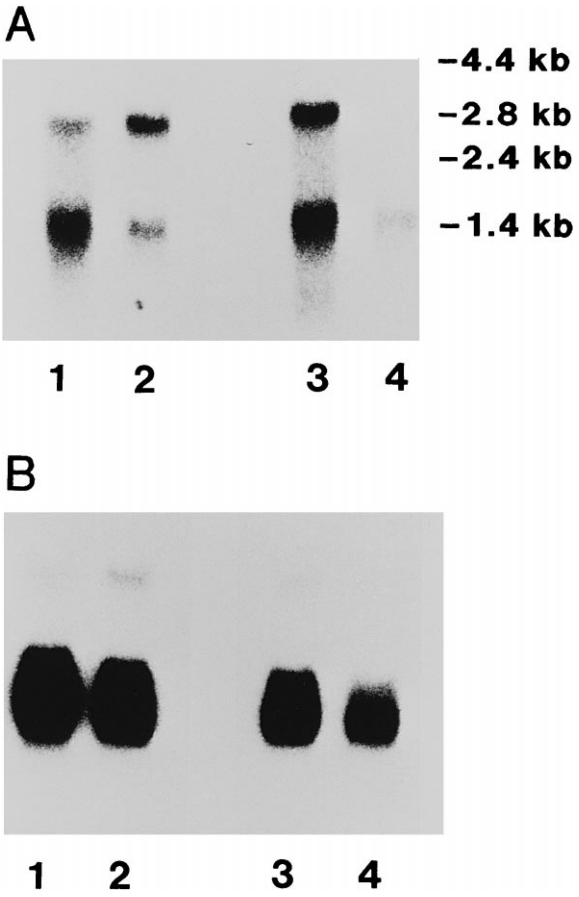

Fig. 2. Autoradiogram showing the levels and sizes of the *GLUT 4* transcripts. Poly $(A)^{+}$ RNAs were isolated from day 2 culture of different clones of L6, L6/CMV, L6/G4A and L6/G4S myoblasts. Two preparations were made from each cell type and samples from each preparation were probed at least three times. Northern blot analyses were carried out as described in the text. The 0.24–9.5 kb RNA ladder (GibcoBRL) was used to determine transcript sizes. Panel A is an autoradiogram indicating the levels and sizes of the transcripts labelled by the *GLUT 4* cDNA. The number beside the panel indicates sizes of transcripts and standards in kb. Panel B shows the Northern blot probed with the *β2-microglobulin* cDNA. Lanes 1, 2, 3 and 4 denote L6, L6/CMV, L6/G4S, and L6/G4A myoblasts, respectively. It is important to note that a transcript corresponding to the size of the exogenous 2.5 kb *GLUT 4* cDNA could not be detected in both L6/G4A and L6/G4S transfectants.

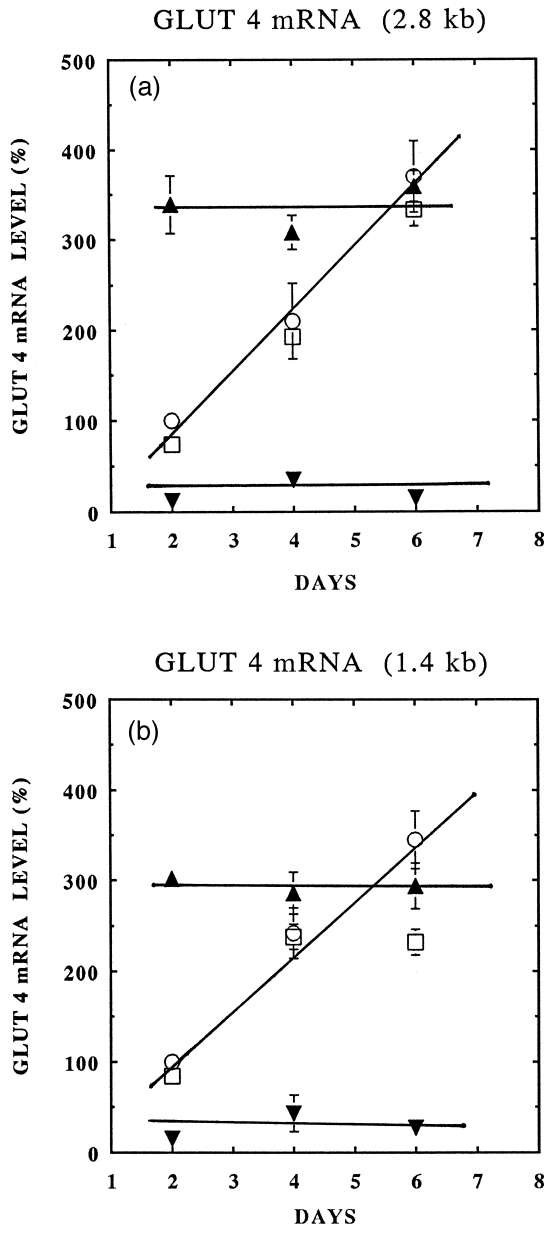

Fig. 3. *GLUT 4* transcript levels in *GLUT 4* transfectants. Poly $(A)^+$ RNAs were isolated from day 2 to day 6 cultures of different clones of L6, L6/CMV, L6/G4A and L6/G4S myoblasts. Northern blot analyses were carried out as described in the text. Two preparations were made from each cell type and samples from each preparation were probed at least three times. *GLUT 4* transcript levels in each culture were normalized according to the amount of *β2-microglobulin* mRNA present in each preparation. Transcript levels of day 2 L6 culture were taken as 100%. ○, □, ▼ and ▲ denote L6 and L6/CMV, L6/G4A and L6/G4S myoblasts, respectively. Panels A and B indicate the *GLUT 4* (2.8 kb) and *GLUT 4* (1.4 kb) transcript levels, respectively.

respectively (Fig. 3). Thus, expression of the endogenous *GLUT 4* gene was suppressed in cells harbouring the *GLUT 4* antisense cDNA.

3.3. GLUT 4 transport activities of L6 transfectants

Although 3-*O*-methyl D-glucose (MeGlc) has been shown to be taken up by both GLUT 1 and GLUT 4 transporters [14], it is taken up primarily by the GLUT 4 transporter in glucose-grown myoblasts, as the GLUT 1 transporter is not functional in glucose-grown cells [14]. This sugar analogue was therefore used to examine the GLUT 4 transport properties of various transfectants. Analysis of transport data by the Lineweaver–Burk plot indicated that both L6/CMV and L6/G4S transfectants had similar GLUT 4 transport kinetic properties (Fig. 4). Analy-

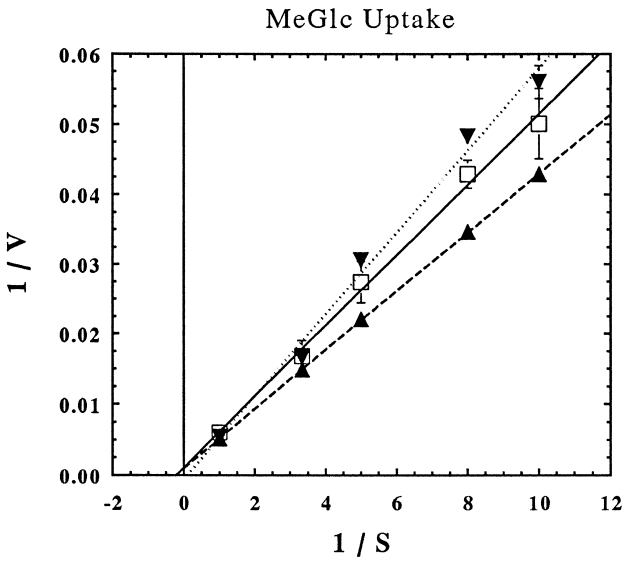

Fig. 4. Kinetics of 3-*O*-methylglucose (MeGlc) uptake by L6 transfectants. L6 transfectants were grown in Alpha medium containing 25 mM D-glucose. Transport studies were carried out as described in the text. Studies were carried out in duplicate and each experiment was repeated at least twice. Initial rates of uptake were calculated from the amount of MeGlc taken up at 23°C in 15, 30, 45 and 60 s. Velocity (V) is expressed as nmoles of MeGlc taken up per min per 1×10^5 cells. S refers to the concentration of MeGlc (in mmoles per litre) used. An equal amount of radioactivity was added to each MeGlc concentration. Data were analysed by the Lineweaver–Burk plot. The lines through the symbols were drawn by a linear least squares regression fit program (SlideWrite Plus™). □, ▲, ▼ denote MeGlc uptake by L6/CMV, L6/G4S and L6/G4A myoblasts, respectively.

sis of data by a linear regression program indicated that intercepts on the *X*-axis were −0.2057, and −0.24748 for L6/CMV, and L6/G4S cells, respectively. Thus, the apparent K_m values of MeGlc uptake by L6/CMV and L6/G4S were 4.86 mM and 4.04 mM, respectively.

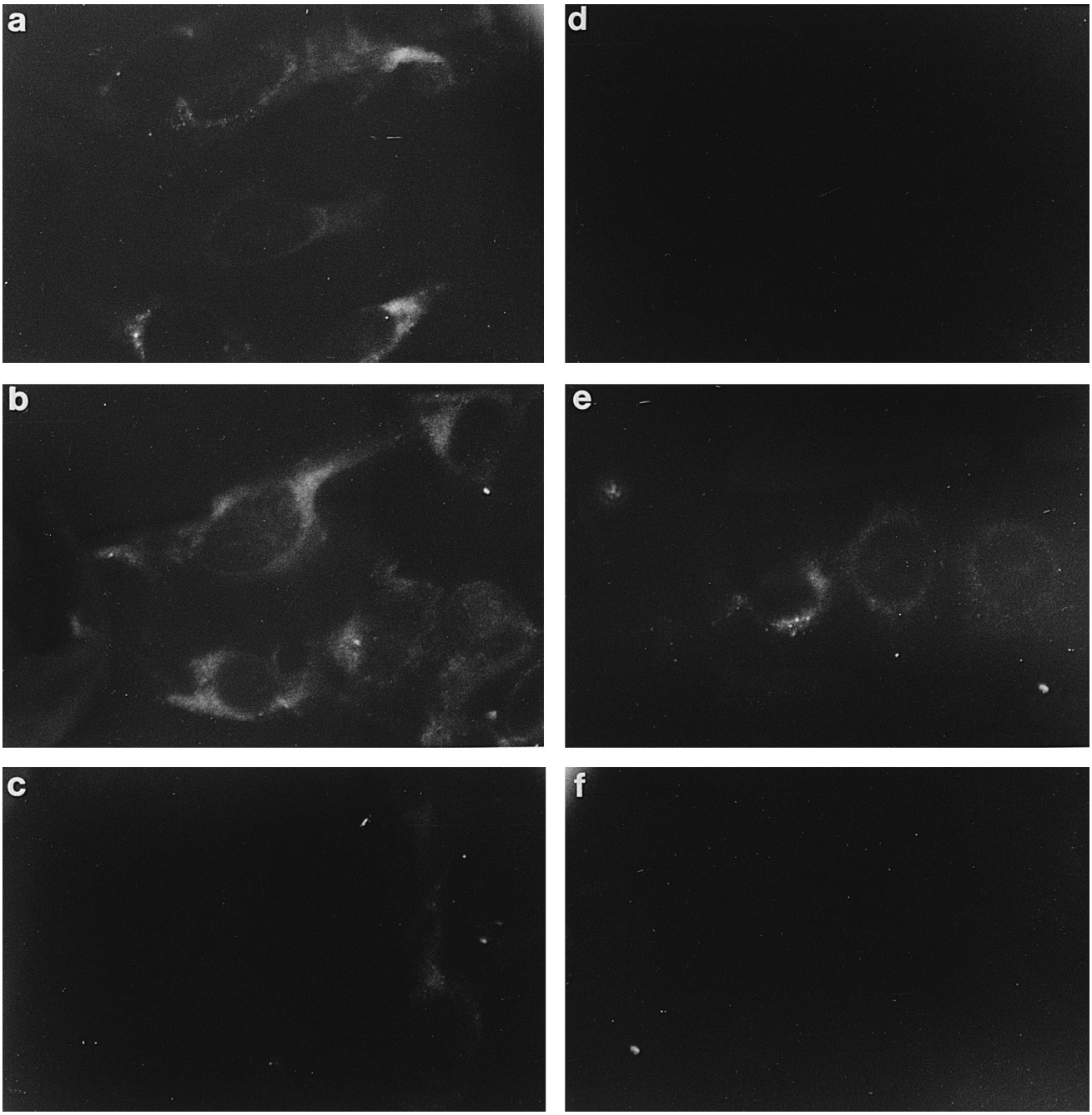

Fig. 5. Immunofluorescence studies using anti-GLUT 4 antibodies. Immunofluorescence studies were carried out as described in the text. These studies were carried out using rabbit anti-rat GLUT 4 antibodies (East Acres Biologicals). Rhodamine-labelled TRITC-conjugated AffiPure goat anti-rabbit IgG (Jackson Immunoresearch Lab.) was used as the secondary antibody. Panels a, b, and c denote L6/CMV, L6/G4S, and L6/G4A transfectants, respectively; whereas, panels d, e, and f denote M1/CMV, M1/G4S, and M1/G4A transfectants, respectively.

Analysis of data by a non-linear regression fit to the Michaelis–Menten hyperbola revealed that the apparent K_m values of MeGlc transport by L6/CMV and L6/G4S cells were 4.68 ± 1.09 mM and 4.13 ± 0.09 mM, respectively. These were similar to those obtained by the Lineweaver–Burk analysis (Fig. 4).

As shown in Fig. 4, L6/G4S transfectants exhibited increases in the rates of MeGlc uptake when compared with those of L6/CMV transfectants. This was probably due to an increase in the number of GLUT 4 transporters. The intracellular volume of rat L6 myoblasts was previously determined to be around 0.22 ml per 10^5 cells [7,14]. When the external MeGlc concentrations were 0.125 mM, 0.2 mM and 0.3 mM, the intracellular MeGlc concentrations in L6/CMV myoblasts were calculated to be around 0.106 mM, 0.17 mM, and 0.28 mM, respectively; whereas those in L6/G4S myoblasts were 0.136 mM, 0.21 mM, and 0.316 mM, respectively (Fig. 4). This showed that equilibration of MeGlc was readily achieved in L6/CMV and L6/G4S myoblasts. The MeGlc transport capacities of these two cell lines were around 900 pmol/min/10^5 cells.

Unlike L6/CMV and L6/G3S transfectants, L6/G4A transfectants were unable to take up MeGlc via an efficient transport process. In Fig. 4, the line denoting the L6/G4A transport kinetic properties was drawn by a linear least squares regression fit program (SlideWrite Plus™). The intercept of this line on the *X*-axis was calculated by a linear regression program to be at +0.00484. Since this line extrapolated close to zero, it might be surmised that MeGlc entered the cells via an extremely low affinity transport process, or simple diffusion. Similar conclusions were also made when data were analysed by a non-linear regression fit to the Michaelis–Menten hyperbola (Enzfitter program, Biosoft). This lack of a functional GLUT 4 transporter was expected as these cells contained very low *GLUT 4* transcript level (Fig. 3). Unlike L6/CMV and L6/G4S myoblasts, only residual amount of the GLUT 4 transporter was detected in L6/G4A myoblasts by immunofluorescence studies (Fig. 5).

3.4. GLUT 4 transport activities in M1 transfectants

Mutant M1 is a GLUT 3^-GLUT 4^- mutant with a similar genetic background as rat L6 myoblast [4,13]. Even though its GLUT 1 level was three times higher than that in L6 myoblast, glucose-grown M1 myoblast was devoid of any carrier-mediated glucose transport processes [13,14]. Despite this deficiency, this glucose-grown mutant could still take up a substantial amount of glucose via simple diffusion [14]. When grown in the presence of 25 mM D-glucose, the doubling times of M1 and L6 myoblasts were 25 and 22 h, respectively [14]. The absence of any functional GLUT transporters in glucose-grown M1 myoblasts makes this mutant an ideal tool to examine the functional states of the protein encoded by the *GLUT 4* sense cDNA.

M1 myoblasts were first transfected with the *GLUT 4* sense or antisense cDNA. Immunofluorescence studies showed that the GLUT 4 transporter was detected in M1 transfectants harbouring the *GLUT 4* sense cDNA (M1/G4S) (Fig. 5e), but not in transfectants harbouring the pRc/CMV expression vector (M1/CMV) (Fig. 5d) or the *GLUT 4* antisense cDNA (M1/G4A) (Fig. 5f). Thus, the protein encoded by

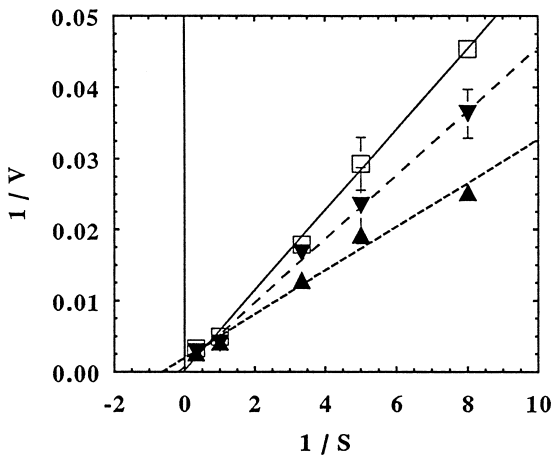

Fig. 6. Kinetics of 2-deoxy-D-glucose (dGlc) uptake by M1 transfectants. M1 transfectants were grown in Alpha medium containing 25 mM D-glucose. Transport studies were carried out as described in Fig. 4, except that dGlc was used as the transport substrate. Studies were carried out in duplicate and each experiment was repeated at least twice. Velocity (V) is expressed as nmoles of dGlc taken up per min per 1×10^5 cells. *S* refers to the concentration of dGlc (in mmoles per litre) used. An equal amount of radioactivity was added to each dGlc concentration. Data were analysed by the Lineweaver–Burk plot. The lines through the symbols were drawn by a linear least squares regression fit program (SlideWrite Plus™). □, ▲, ▼ denote dGlc uptake by M1/CMV, M1/G4S and M1/G4A transfectants, respectively.

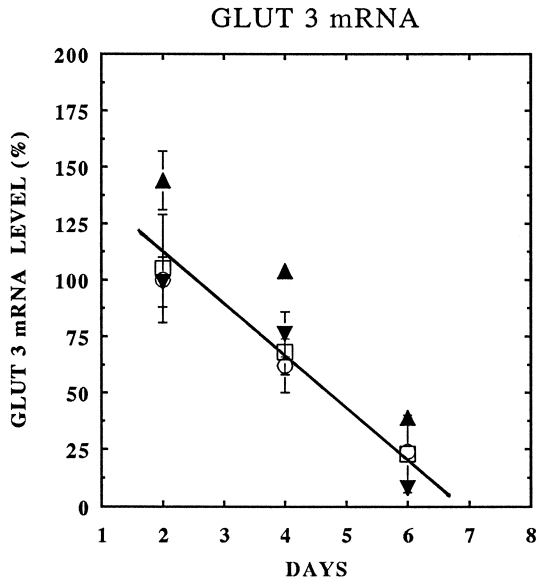

Fig. 7. *GLUT 3* transcript levels in L6 transfectants. Northern blot analysis and quantification of transcript levels were performed as described in Fig. 3. Two preparations were made from each cell type and samples from each preparation were probed at least three times. *GLUT 3* transcript level was determined by probing with the *GLUT 3* cDNA. Transcript levels in each culture were normalized according to the amount of *β2-microglobulin* transcript present in each preparation. Transcript levels of day 2 L6 culture were taken as 100%. ○, □, ▼ and ▲ denote L6 and L6/CMV, L6/G4A and L6/G4S myoblasts, respectively.

the *GLUT 4* sense cDNA was recognized by the rabbit anti-GLUT 4 antibody.

To examine the functional states of GLUT 1 and 4 transporters in M1 transfectants, transport kinetic studies were carried out using 2-deoxy-D-glucose (dGlc), which is taken up by the GLUT 1, 3 and 4 transporters. The apparent transport affinities of GLUT 1, 3 and 4 for dGlc are around 0.7 mM, 0.6 mM, and 3.5 mM, respectively [13,14]. As shown in Fig. 6, dGlc was taken up by glucose-grown M1/CMV and M1/G4A myoblasts via a very inefficient transport process, thus confirming the absence of any functional GLUT transporters. On the other hand, dGlc was taken up by M1/G4S myoblasts with an affinity of around 1.7 mM (Fig. 6). This indicated that the protein encoded by the *GLUT 4* sense cDNA was able to function as a GLUT transporter. The transport capacity of M1/G4S myoblasts for dGlc was around 576 pmol/min/10^5 cells.

3.5. GLUT 3 transcript level and transport activity in L6 transfectants

Previous studies using glucose transport mutants did not allow one to examine the effect of GLUT 4 on *GLUT 3* expression, as it was not possible to isolate mutants defective only in the GLUT 4 isoform [13,14]. The availability of transfectants over- or under-expressing *GLUT 4* provided an excellent opportunity to examine the effect of GLUT 4 on *GLUT 3* expression and activity.

Similar levels of the *GLUT 3* transcript (4.1 kb) were present in L6, L6/CMV, L6/G4A and L6/G4S transfectants (Fig. 7). The levels of these transcripts declined upon prolonged growth. Since L6/G4A and L6/G4S myoblasts were impaired in myogenic differentiation, whereas L6 and L6/CMV myoblasts were not (unpublished observation), the observed decline in *GLUT 3* transcript was not likely due to

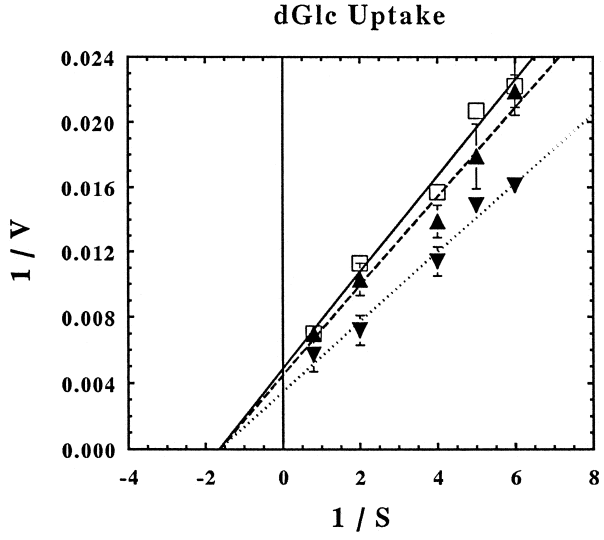

Fig. 8. Kinetics of 2-deoxy-D-glucose uptake by L6 transfectants. L6 transfectants were grown in Alpha medium containing 25 mM D-glucose. Transport studies were carried out as described in Fig. 4, except that dGlc was used as the transport substrate. Studies were carried out in duplicate and each experiment was repeated at least twice. Velocity (V) is expressed as nmoles of dGlc taken up per min per 1×10^5 cells. S refers to the concentration of dGlc (in mmoles per litre) used. An equal amount of radioactivity was added to each dGlc concentration. Data were analysed by the Lineweaver–Burk plot. The lines through the symbols were drawn by a linear least squares regression fit program (SlideWrite Plus™). □, ▲, ▼ denote dGlc uptake by L6/CMV, L6/G4S and L6/G4A transfectants, respectively.

myogenic differentiation. This decline was probably brought about by increases in cell density during growth [4].

Even though dGlc is taken up by GLUT 1 and GLUT 3 transporters with similar transport affinities, the GLUT 3 transport property can be examined by using glucose-grown cells, in which the GLUT 1 transporter is not functional [13,14]. Transport kinetic analysis revealed that all three types of glucose-grown L6 transfectants took up dGlc with a similar transport affinity (Fig. 8). Analysis of data by a non-linear regression fit to the Michaelis–Menten hyperbola indicated that the apparent K_m values of dGlc uptake by L6/CMV, L6/G4S and L6/G4A myoblasts were 0.64 ± 0.07 mM, 0.51 ± 0.06 mM and 0.45 ± 0.09 mM, respectively. Thus changes in GLUT 4 levels had no effect on the *GLUT 3* transcript level and its transport affinity.

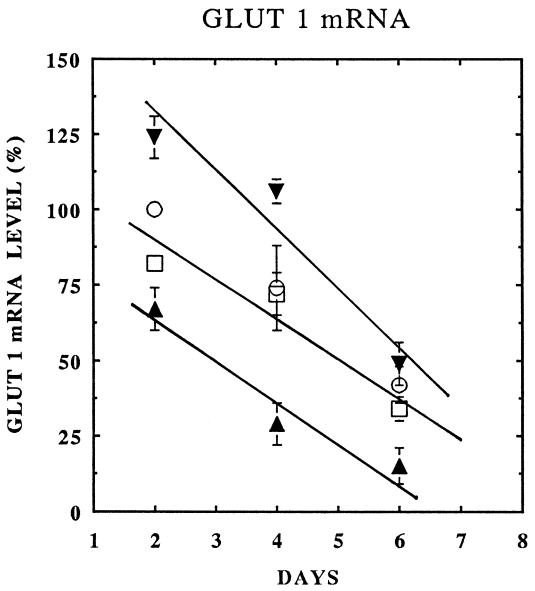

Fig. 9. *GLUT 1* transcript levels in L6 transfectants. Northern blot analysis and quantification of transcript levels were performed as described in Fig. 3. Two preparations were made from each cell type and samples from each preparation were probed at least three times. *GLUT 1* transcript level was determined by probing with the *GLUT 1* cDNA. Transcript levels in each culture were normalized according to the amount of *β2-microglobulin* transcript present in each preparation. Transcript levels of day 2 L6 culture were taken as 100%. ○, □, ▼ and ▲ denote L6 and L6/CMV, L6/G4A and L6/G4S myoblasts, respectively.

3.6. GLUT 1 transcript and transport activity in L6 transfectants

The effects of over- or under-expression of the GLUT 4 isoform on *GLUT 1* expression were also examined. *GLUT 1* transcript levels in days 2, 4 and 6 cultures of L6/G4A transfectants were about 147%, 146% and 150% of the corresponding L6 and L6/CMV levels, respectively (Fig. 9). This showed that increases in *GLUT 1* expression were observed at all stages of growth. Conversely, *GLUT 1* transcript levels were reduced in transfectants harbouring much elevated level of the GLUT 4 isoform. *GLUT 1* transcript levels in days 2, 4 and 6 L6/G4S cultures were about 69%, 56% and 20% of the corresponding values in L6 and L6/CMV cells, respectively (Fig. 9). This study showed that the *GLUT 1* transcript levels in L6/G4S myoblasts were distinctly lower than those in L6/G4A myoblasts at all stages of growth. Increases in *GLUT 1* transcript and transporter levels were also observed in mutants deficient in the GLUT 4 transporter [4,13]. Our present transfection studies show that the GLUT 4 isoform may play a key role in maintaining the inverse relationship between *GLUT 4* and *GLUT 1* expression.

4. Discussion

Considerable efforts have been focused on the mechanisms by which glucose transport (GLUT) processes can be regulated in eukaryotic cells. Studies on the effects of insulin, tumor necrosis factor, glucose starvation, exercise and development on the expression, intracellular location, and activities of the GLUT 1 and GLUT 4 transporters have yielded valuable information on the molecular mechanisms and the signal transduction pathways by which glucose transport processes can be regulated [1–3,31–35,49]. Not much is presently known on how the expression and activity of one GLUT isoform can be affected by itself, or by other GLUT isoforms.

Studies using mutants deficient in the GLUT 4 isoform have generated valuable information on the effects of under-expression of this transporter [13]. However, these studies were unable to ascertain whether the observed changes were due to mutations

in the transporter itself, or in some regulatory components. One approach to tackle this problem is to transfect cells with the *GLUT 4* sense or antisense cDNAs. This will enable one to examine the effects of over- and under-expression of the GLUT 4 isoform on its own expression, and on the expression of the GLUT 1 and/or GLUT 3 isoforms under identical conditions.

L6 transfectants harbouring the *GLUT 4* sense cDNA (L6/G4S) possessed much elevated levels of both endogenous 1.4 kb and 2.8 kb *GLUT 4* transcripts at all stages of growth; the levels of these transcripts were about three times higher than those in day 2 L6 myoblasts (Fig. 3). Conversely, L6 transfectants harbouring the *GLUT 4* antisense cDNA (L6/G4A) possessed much reduced level of the endogenous *GLUT 4* transcripts; L6/G4A myoblasts had around 6% of the L6 level in day 6 cultures (Fig. 3). The observed activation of transcription of the endogenous *GLUT 4* gene by the *GLUT 4* sense cDNA therefore suggests auto-regulation of the *GLUT 4* gene. This was similar to the auto-regulation of the GLUT 3 isoform observed in cells transfected with an exogenous *GLUT 3* sense cDNA [30].

Not much is known on the mechanism(s) that bring about auto-regulation. We have shown in this study that the *GLUT 4* sense cDNA codes for a functional GLUT 4 transporter (Figs. 5 and 6). It is possible that the GLUT 4 transporter may modulate the level of functional transcription factor(s) required for regulating *GLUT 4* expression. This may be brought by interaction of the GLUT 4 transporter with specific cytoplasmic proteins. We have recently observed that the central cytoplasmic loop of the GLUT 4 transporter can interact directly with several cytoplasmic proteins. Attempts are being made to determine the involvement of these components in regulating *GLUT 4* expression.

The availability of L6/G4S and L6/G4A transfectants provides a unique opportunity to examine the effect of GLUT 4 on *GLUT 3* expression. Despite differences in their *GLUT 4* transcript levels, both types of transfectants possessed similar amount of the *GLUT 3* transcript (Fig. 7). Transport kinetic analyses revealed that the apparent K_m values of dGlc uptake by glucose-grown L6/CMV, L6/G4S and L6/G4A were 0.64 ± 0.07 mM, 0.51 ± 0.06 mM and 0.45 ± 0.09 mM, respectively. This showed that changes in GLUT 4 level had no effect on the expression and kinetic properties of the GLUT 3 transporter.

While there are ample indirect evidence on the inverse relationship between *GLUT 1* and *GLUT 4* expression [13,14,22,31–36], it is not clear whether these are coincidental or causal occurrences. The following observations indicate that the GLUT 4 transporter may play a crucial role in suppressing *GLUT 1* expression. (i) Increases in *GLUT 4* expression occurred before the major decline of the *GLUT 1* transcript during myogenic differentiation [13]. Similar temporal order of expression was also observed in adipose tissues [38]. Thus *GLUT 4* expression was altered before any observed changes in *GLUT 1* expression. (ii) Both cytochalasin B binding and immunoblotting studies using plasma membrane and microsomal preparations revealed that mutants deficient in the GLUT 4 transporter possessed 2–3 times the amount of GLUT 1 transporter present in their parental L6 myoblasts [4,13]. Similarly, the *GLUT 1* transcript levels in GLUT 4^- mutants were about twice that present in L6 myoblasts [4,13]. These studies clearly indicated that both GLUT 1 transporter and expression were increased in the absence of the GLUT 4 isoform. (iii) Even though *GLUT 1* transcript level was doubled in glucose-starved L6 myoblasts, their *GLUT 4* transcript level remained unaltered [13]; thus, the GLUT 1 isoform was not likely to play a role in modulating *GLUT 4* expression.

The availability of GLUT 4 transfectants allowed one to examine directly the relationship between these two GLUT isoforms. We have shown in this study that the L6/G4S myoblasts harboured substantial amount of the *GLUT 4* cDNA in the correct orientation (Fig. 1), and their *GLUT 4* transcripts were maintained at a level about three times higher than those in day 2 L6 myoblasts (Fig. 3). Both immunofluorescence and transport studies showed that the protein encoded by the *GLUT 4* sense cDNA was not only recognized by the rabbit anti-GLUT 4 antibody (Fig. 5b), but also able to function as a transporter (Fig. 6). More importantly, overexpression of *GLUT 4* in L6/G4S myoblasts was accompanied by a reduction of *GLUT 1* expression (Fig. 9). Conversely, L6/G4A transfectants were found to possess about 13% of the L6 *GLUT 4* transcript

levels (Fig. 3); hardly any GLUT 4 transporter (Fig. 5c) and GLUT 4 transport activity (Fig. 6) could be detected in these transfectants. More importantly, these transfectants possessed much elevated levels of the *GLUT 1* transcript at all stages of growth (Fig. 9). This is the first direct evidence indicating the role of the GLUT 4 transporter in modulating *GLUT 1* expression. We are currently studying the mechanisms involved.

In summary, studies using transfectants harbouring the *GLUT 4* sense or antisense cDNAs revealed that the GLUT 4 transporter might play important roles in regulating its own expression and the expression of the GLUT 1 transporter. The GLUT 4 transporter may interact with appropriate signal transducers; this interaction will in turn regulate the level of functional transcription factors. While some of these transcription factors may function by suppressing *GLUT 1* expression, others (which may or may not be the same transcription factors) may serve by activating *GLUT 4* transcription.

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