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Alteration of myogenic regulatory components in a rat myoblast GLUT 3$^-$ mutant

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Abstract

Myogenesis is a complex process characterized by both biochemical and morphological differentiation. Recent transfection studies suggested a close relationship between the GLUT 3 transporter and the myogenic ability of rat skeletal L6 myoblast. In this study, the myogenic properties of GLUT 3$^-$ mutants were examined. Studies using three different GLUT 3$^-$ mutants (D2, D9 and D23) revealed that these mutants were defective not only in the GLUT 3 transporter, but also in the expression of myogenesis-associated genes. The properties of mutant D23 were further characterized. Overexpression of an exogenous functional GLUT 3 transporter was unable to restore the myogenic defects of this mutant. This mutant was subsequently found to be altered in components acting on at least two different sites of the L6 myogenic pathway. Transfection studies revealed that mutant D23 was deficient in component(s) essential for the *myogenin* promoter activity. The second component was required for the transcription of muscle-specific protein genes, as overexpression of *myogenin* was unable to rescue the inability of this mutant to express muscle-specific genes and to form myotubes. Mutant D23 was therefore thought to be deficient in a regulatory component which controlled the expression of *GLUT 3*, *myogenin* and muscle-specific genes.

Keywords: Glucose transporter; Myogenin; Muscle-specific expression; Myoblast; Myoblast mutant; (*Rat*)

1. Introduction

Rat L6 myoblast is a permanent cell line originally isolated from embryonic skeletal muscles [1]. It is characterized by its ability to form multinucleated myotubes. Myogenesis is a complex process consisting of both biochemical and morphological differentiation [2]. A family of structurally related basic helix-loop-helix (bHLH) transcription factors, MyoD, Myf-5, Myf-4/myogenin, and Myf-6/MRF-4/herculin, is closely associated with myogenic regulation [3–5]. Inhibition of *myf-5* translation blocks *myogenin* expression and myogenic differentiation [6,7]. *Myogenin* expression is normally repressed in proliferating myoblasts, and is up-regulated rapidly during myogenesis [8–10]. Transcription of *myogenin* and muscle-specific contractile protein genes (such as myosin heavy chain (*MHC*), myosin light chain (*MLC*) and troponin T (*TnT*)) is dependent on the binding of the myogenin oligomeric complex to the E box (CANNTG) and the AT-rich sequence motifs present in these muscle-specific genes [3,4,11].

Eukaryotic glucose transport systems have been examined through a combination of biochemical,

Abbreviations: dex, dexamethasone; dGlc, 2-deoxy-D-glucose; GLUT, glucose transporter; MHC, myosin heavy chain; MLC, myosin light chain; TnT, troponin T

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molecular biological, and biophysical approaches [12–18]. All mammalian cells contain one or more members of the glucose transporter (GLUT) superfamily. This GLUT family consists of six functionally distinct proteins (GLUT 1, 2, 3, 4, 5 and 7) that share significant sequence similarity. The GLUT 1 transporter is comprised of 12 transmembrane α-helical segments (TM) with the amino and carboxyl termini and a large connecting loop between TM6 and TM7 oriented intracellularly [12–18]. While other GLUT isoforms exhibit similar structural properties as the GLUT 1 transporter, they also contain a considerable divergence in their amino acid sequences. These isoform-specific amino acid sequences may play important roles in determining the intrinsic activities of specific glucose transporters [13].

The rat myoblast glucose transport processes have been examined through a variety of different approaches [19–28]. Rat L6 myoblasts possess the GLUT 1, 3 and 4 transporters [27,28]. The GLUT 1 transporter is mainly responsible for glucose transport activity in proliferating glucose-starved myoblasts, the GLUT 3 transporter is the predominant transporter in proliferating glucose-grown myoblasts, and the insulin-responsive GLUT 4 transporter is present in a very high level in myotubes.

Studies on the physiological roles of the rat myoblast GLUT 3 transporter revealed a close correlation between its expression and the cells' myogenic ability. First, all independently isolated GLUT 3^- mutants of rat skeletal myoblast (i.e. those harboring less than 20% of the L6 GLUT 3 level) are impaired in myogenesis [19,27]. Second, GLUT 3^- mutants of rat cardiac myoblast are also defective in myogenesis [29]. Third, myotube formation is always preceded by a dramatic decrease of the *GLUT 3* transcript and transport activity [27,30]. Fourth, cells retaining relatively high levels of the *GLUT 3* transcript and transport activity are unable to form myotubes [27,30]. The relationship between GLUT 3 and myogenesis was also examined by transfecting L6 myoblast with the *GLUT 3* sense or antisense cDNA [31]. Overexpression of the GLUT 3 transporter (3–5-fold of the L6 level) suppressed expression of *myogenin* and muscle-specific genes, and myotube formation. On the other hand, diminished *GLUT 3* expression (to around 39% of the L6 level) reduced only *myogenin* expression, but not the rates of fusion and expression of muscle-specific genes. These studies showed that a critical level of the GLUT 3 transporter was essential for myogenesis. GLUT 3 was thought to regulate myogenesis by modulating the levels of signal transducers involved in the myogenic pathway [31].

In this study, the properties of a rat myoblast GLUT 3^- mutant, D23, were examined. This mutant was thought to be altered in regulatory component(s) which modulate not only GLUT 3 expression, but also the transcription of *myogenin* and muscle-specific genes. More importantly, this study revealed that different components were involved in regulating the *myogenin* promoter activity and the expression of muscle-specific genes.

2. Methods and materials

2.1. Cell culture

Rat L6 skeletal myoblast was originally isolated by Yaffe [1]; clones D2, D9 and D23 were independent GLUT 3^- mutants isolated from L6 cells [19,25]. Myoblasts were grown in Alpha Minimal Essential Medium (Life Technologies), supplemented with 50 μg/ml of gentamicin sulfate (Life Technologies) and 10% v/v horse serum (Hyclone) [32,33]. Cells were seeded at a density of 1×10^6 cells per 150 mm Nunc plate, and were subcultured every 3 days.

2.2. Plasmids and culture media

Human *myf-4*, *myf-5* and *GLUT 3* cDNAs were purchased from the Repository of Human and Mouse DNA Probes and Libraries, ATCC. The *GLUT 3* cDNA contains the complete *GLUT 3* coding sequence and its 3'UTR (115–2742 bp). *MLC*, *MHC* and *TnT* cDNAs were gifts obtained originally from B. Nadal Ginard [34]. The β_2-*microglobulin* cDNA was a gift from F. Daniel [35]. The mouse *myogenin* promoter constructs, pGSC and pGXC, were generous gifts from S.P. Yee [36]. Both constructs consist of a *chloramphenicol acetyltransferase* (CAT) gene (1.6 kb) and a SV40 t-antigen sequence with poly(A)$^+$ signal. The pGSC construct contains the full-length (1092 bp) *myogenin* promoter, whereas the pGXC construct contains only the first 133 bp of

the *myogenin* promoter [36]. The *PGK-myogenin* construct and the PKJ1ΔR vector were gifts from I.S. Skerjanc [37,38]. *PGK-myogenin* contains the 1.5 kb *myogenin* coding sequence placed under the control of the *phosphoglycerate kinase* promoter (PGK) [37,38].

2.3. Southern blot analysis

Genomic DNAs were isolated from L6 and D23 transfectants using a previously described procedure [2,31]. Ten μg of genomic DNA were digested with appropriate restriction enzymes and analyzed on a 0.8% agarose gel. The gel was then transferred to an ICN Biotrans positive nylon membrane, cross-linked, probed with an [α-^{32}P]dCTP-labeled cDNA probe, and exposed to Kodak X-OMAT AR film.

2.4. Northern blot analysis

Poly(A)$^+$RNAs were extracted from myoblasts using the Invitrogen FastTrack kit. Northern blot analysis was carried out as described previously, using 1 μg mRNA per sample [2,27]. The blot was probed initially with labeled β_2-*microglobulin* cDNA to determine sample loading, and then with *myf-4*, *MHC*, *MLC* and *TnT* cDNAs. The blot was stripped with 60% formamide at 65°C for 2 h before hybridizing with a new probe. This was then exposed to Kodak X-OMAT AR film; the autoradiogram was analyzed using the JAVA Video Analysis software (Jandel Scientific). Measurements were made in the linear range of optical density. In some studies, band intensity from the phosphorimage was measured using Image Quant Software (Molecular Dynamics, Sunnyvale, CA). Two different poly(A)$^+$RNA preparations were used in each study. Samples from each preparation were probed at least twice. The amount of mRNA present in each lane was normalized according to the amount of β_2-*microglobulin* mRNA present in each sample. In calculating the relative transcript levels, the amount in day 2 L6 culture was used as 100%.

2.5. Whole cell transport studies

Transport studies were carried out using six-well Falcon plates [22,23]. 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 μl 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. At appropriate times, uptake was terminated by rapidly washing the cells twice (less than 15 s) with 10 ml of ice-cold PBS. Samples were taken at 15, 30, 45 and 60 s after 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 this condition, the uptake of 2-deoxy-D-glucose (dGlc) was linear with time and over 95% of the internalized dGlc were phosphorylated [22]. Cells in two wells from each plate were detached with 0.1% trypsin and counted using a Coulter counter. Studies were carried out in quadruplicate. Results were consistent in all cases.

2.6. Enzyme assays

β-Galactosidase activity was measured according to the standard procedure [39]. Optical density of the solution was determined at a wavelength of 420 nm; readings were within the linear range (0.2–0.8) of the assay.

Chloramphenicol acetyltransferase (CAT) assays were carried out by first heating the sample at 65°C for 10 min to inactivate deacetylases, after which samples were centrifuged at $12\,000\times g$ at 4°C for 10 min. The supernatant was then used in the CAT assay [39]. 87.5 μl of the cell extract were incubated with 27 μl of 1 M Tris-HCl (pH 7.8), 64 μl of 5 mM chloramphenicol in water, and 1.5 μl of [^{3}H]acetyl coenzyme A (ICN) for 2 h at 37°C. The reaction was terminated by transferring the sample to ice. Each sample mixture was extracted twice with 200 μl ice-cold ethyl acetate, mixed thoroughly by vortexing for 1 min. Samples were centrifuged for 5 min at $12\,000\times g$. The amount of radioactivity present in the upper (organic) phase was then determined using Non-Aqueous Scintanalyzed Scintilene (Fisher).

2.7. Fusion index determination

Fusion index was used to determine the extent of myotube formation [19]. Five fields (at least 100 nu-

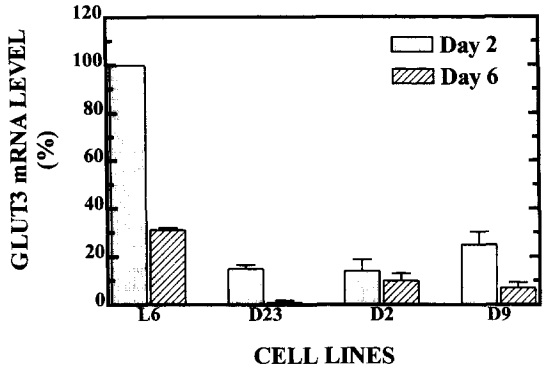

Fig. 1. *GLUT 3* transcript levels in GLUT 3$^-$ mutants. Poly-(A)$^+$RNA was isolated from day 2 and day 6 cultures of GLUT 3$^-$ mutants (D23, D2 and D9), and their parental L6 myoblast. Northern blot studies were carried out as described in the text. Band intensities from the phosphorimage were measured using Image Quant Software (Molecular Dynamics, Sunnyvale, CA). *GLUT 3* transcript levels were normalized according to the β_2-*microglobulin* transcript level in each sample. The day 2 L6 *GLUT 3* transcript level was taken as 100%. The dotted bar and slashed bar denote samples from day 2 and day 6 cultures, respectively. Standard deviations were calculated for three different samples.

clei per field) per well were chosen to determine the extent of fusion. A myotube was scored only if it contained at least three nuclei [19].

2.8. Immunofluorescence studies

Immunofluorescence studies were conducted using mouse monoclonal anti-myogenin (F5D) and anti-MHC (MF-20) antibodies [40]. The former was a generous gift from W.E. Wright [37], whereas the latter was obtained originally from the Developmental Studies Hybridoma Bank. The rabbit anti-mouse CY3 antibody was purchased from Jackson Laboratories. Samples were examined using a Zeiss Axioplot Immunofluorescence microscope. Pictures were captured using Northern Exposure Software, cropped and resized by Adobe Photoshop Software and imported into Corel Draw for final placement before printing.

2.9. Materials

[α-^{32}P]dCTP and [^{3}H]acetyl coenzyme A were purchased from ICN. Reagents used in bacterial cultures were obtained from Difco. Restriction and modifying enzymes were from Pharmacia, whereas λDNA *Hin*dIII digest and λDNA *Bst*EII digest were from New England Labs. All other chemicals were from commercial sources and were of the highest available purity.

3. Results

3.1. Myogenic ability of GLUT 3$^-$ mutants

The myogenic ability of three independent GLUT 3$^-$ mutants of rat skeletal myoblast was examined in this study. Mutant D2 was isolated from ethyl methanesulfonate-mutagenized L6 myoblast by its ability to grow in the presence of 0.1 mM 2-deoxy-D-glucose (dGlc) [19]. D9 and D23 myoblasts were two spontaneous mutants isolated independently from L6 myoblasts by their ability to grow in the presence of 0.05 mM dGlc [19]. *GLUT 3* transcript levels in day 2 cultures of D23, D2, and D9 myoblasts were found to be 15%, 14% and 25% of that in L6 myoblasts, respectively, whereas day 6 cultures of L6, D23, D2 and D9 harbored 31%, 1%, 10% and 7% of the day 2 L6 level, respectively (Fig. 1). This showed that D2, D9 and D23 myoblasts were defective in *GLUT 3* expression. Previous studies also showed that day 2 D23 myoblast possessed around 17% of the L6 GLUT 3 transporter level [27].

The rates of fusion of D2, D9 and D23 myoblasts

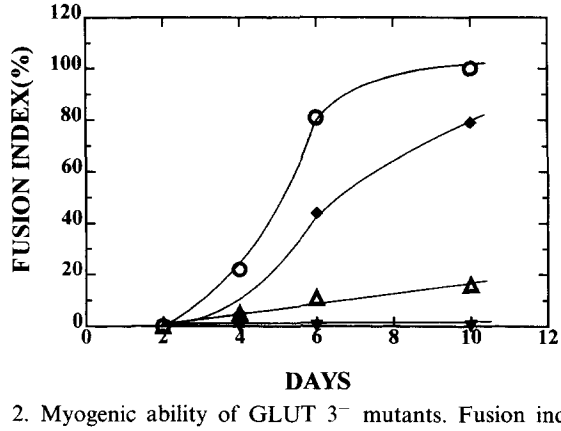

Fig. 2. Myogenic ability of GLUT 3$^-$ mutants. Fusion indices were determined for the GLUT 3$^-$ mutants and their parental L6 myoblast. ○, ♦, △, and ▼ denote the rates of fusion by L6, D2, D9 and D23 myoblasts, respectively. The standard error was calculated for each cell line for each day ($n = 18$). Since these values were less than 2%, they were not apparent on the graph.

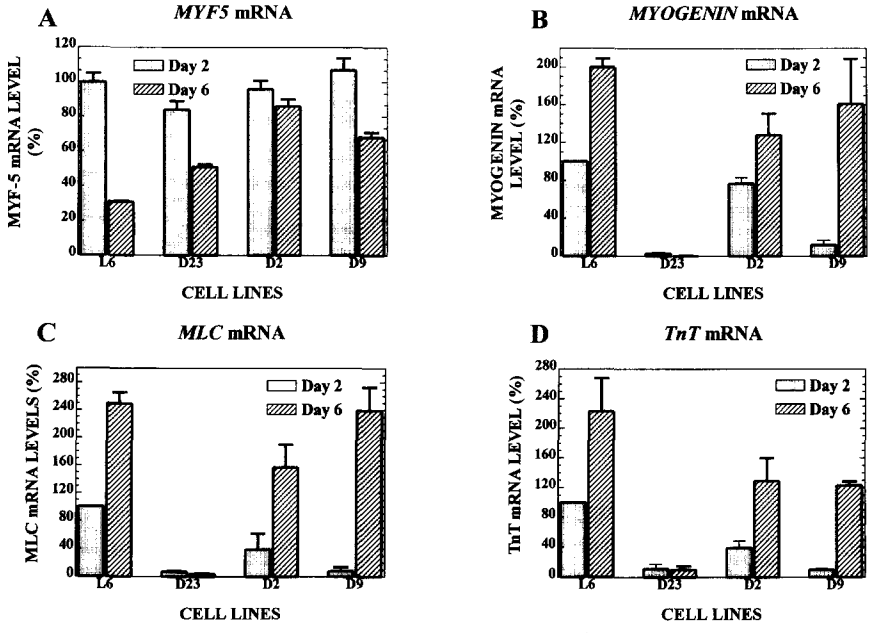

Fig. 3. Expression of myogenesis-associated genes in GLUT 3$^-$ mutants. Poly(A)$^+$RNAs were isolated from day 2 and day 6 cultures of GLUT 3$^-$ mutants (D23, D2 and D9) and their parental L6 myoblast. Northern blot analysis was performed as described in the text. The levels of *myf-5*, *myogenin*, *MLC* and *TnT* mRNAs were determined by probing with their respective cDNAs. The day 2 L6 level was taken as 100% for each probe. A, B, C and D indicate the levels of *myf-5*, *myogenin*, *MLC* and *TnT* transcripts, respectively. The dotted bar and the slashed bar denote samples from day 2 and day 6 cultures, respectively. Standard deviations were calculated for three different samples.

were determined over a 10 day period (Fig. 2). The fusion indices of rat L6 cells were 81% and 100% in day 6 and day 10 cultures, respectively. D2 cultures had reduced rates of fusion; day 6 and day 10 cultures had fusion indices of around 44% and 79%, respectively, whereas the corresponding values for mutant D9 were 11% and 16%, respectively. More interestingly, mutant D23 was unable to form myotubes, even though it had similar *GLUT 3* transcript level as mutant D2. While this study showed that all GLUT 3$^-$ mutants were impaired in myogenesis, it also suggested that these mutants were altered in different myogenic components.

3.2. *Expression of myogenesis-associated genes in GLUT 3$^-$ mutants*

To further explore the myogenic defects of GLUT 3$^-$ mutants, the expression of myogenesis-associated genes was examined. Cells were grown under conditions that promoted myotube formation. Poly(A)$^+$RNAs from these cells were probed with *myf-5*, *myogenin*, *MLC*, and *TnT* cDNAs (Fig. 3). *Myf-5* transcript levels in day 2 GLUT 3$^-$ mutants (D2 (96%), D9 (107%), and D23 (84%)) were similar to that of L6 culture. While the day 6 L6 *myf-5* transcript level was reduced to around 31% of day 2 L6 level, the reduction of this transcript was considerably smaller in GLUT 3$^-$ mutants. *Myogenin* transcript levels in GLUT 3$^-$ mutants were significantly lower than that of day 2 L6 culture; about 7%, 77%, and 12% of the day 2 L6 *myogenin* level were observed in D23, D2, and D9 mutants, respectively (Fig. 3). The day 6 *myogenin* transcript levels in L6, D2 and D9 myoblasts were 200%, 128% and 161% of that in day 2 L6 myoblast, respectively. More importantly, this transcript remained at a very low level in day 6 D23 myoblast. These studies showed that *myogenin* expression was reduced to different extent in these GLUT 3$^-$ mutants.

While both *MLC* and *TnT* transcripts were present in fairly low levels in day 2 cultures of D2 and D9 myoblasts, they were elevated in day 6 cultures of both mutants (Fig. 3). On the other hand, only residual levels of these two transcripts were present in day 2 and day 6 D23 cultures. The fact that mutant

D23 was essentially devoid of transcripts for *myogenin* and muscle-specific genes made this mutant an ideal cell line for studying the GLUT 3-associated myogenic events.

3.3. Transfection of D23 myoblast with a GLUT 3 cDNA

If the GLUT 3 transporter is solely responsible for the myogenic defect in mutant D23, then expression of an exogenous GLUT 3 transporter may restore the myogenic ability of this mutant. In this study, the *GLUT 3* cDNA was placed under the control of a dexamethasone-inducible MMTV promoter [41]. The concentration of dexamethasone (dex) used for induction was 0.1 μM; this concentration should not affect endogenous metabolic processes, as it altered myoblast metabolism only when used at concentrations above 12 μM [42].

Stable D23 transfectants harboring the MMTV-GLUT 3 construct (D23/GLUT 3 myoblast) or the pMAMneo expression vector (D23/MMTV myoblast) were selected using G418. Genomic DNAs from these transfectants were digested with *Hin*dIII and *Eco*RV, and then probed with ^{32}P-labeled *GLUT 3* cDNA. A DNA fragment similar in size (1.5 kb) to the *GLUT 3* cDNA was detected in different clones of D23/GLUT 3 transfectants, but not in D23, or D23/MMTV myoblasts (Fig. 4). More importantly, *GLUT 3* expression was induced upon incubation of D23/GLUT 3 myoblasts (clones 2-4 and 2-6) with

Fig. 4. Southern blot analysis of D23/GLUT 3 transfectants. Genomic DNAs from D23 transfectants were digested with *Eco*RV and *Hin*dIII. After electrophoresis and transfer to an ICN Biotrans membrane, the blot was probed with the *GLUT 3* cDNA. No band was detected in the controls: D23 myoblast (lane 1) and D23/MMTV transfectant (lane 2). A 1.5 kb band was observed in D23/GLUT 3 transfectant clones 2-1, 2-2, 2-4, 2-6, 2-7, 2-8, 2-9, 2-11 and 2-12 (lanes 3, 4, 6, 8, 9, 10, 11, 13 and 14, respectively); this band was not detected in D23/GLUT 3 clone 2-3, 2-5 and 2-10 transfectants (lanes 5, 7 and 12, respectively).

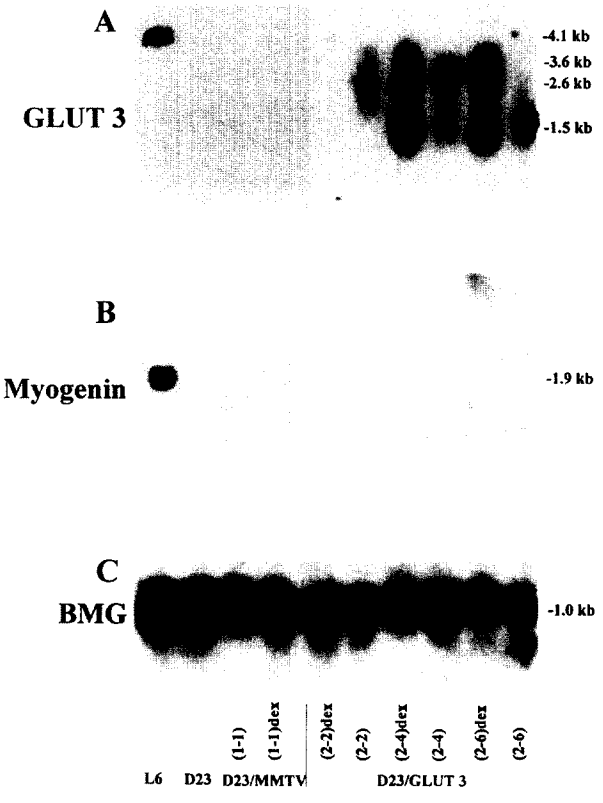

Fig. 5. Northern blot analysis of D23/GLUT 3 transfectants. Northern blot analysis was carried out as described in the text. D23 transfectants were treated with or without 1×10^{-7} M dexamethasone (dex). Two μg of poly(A)$^+$RNA were loaded into each lane. After electrophoresis and transfer to Biotrans membrane, the blot was probed with *GLUT 3*, *myogenin*, and β_2-*microglobulin* (*BMG*) cDNAs (A, B and C, respectively). The 4.1 kb endogenous *GLUT 3* transcript was evident in L6 myoblast, but not in other cell lines (A). A transcript corresponding to the size of the exogenous *GLUT 3* cDNA (1.5 kb) was detected in dex-induced D23/GLUT 3 transfectants 2-4 and 2-6, but not in uninduced cells. The other bands observed in the dex-induced transfectants were probably transcripts from genes that had integrated the exogenous *GLUT 3* cDNA (A). The endogenous *myogenin* transcript level (1.9 kb) was evident only in L6 myoblast, but not in other cell lines (B). RNA loading was indicated by probing with the *BMG* cDNA (C).

0.1 μM dex (Fig. 5); their *GLUT 3* transcript levels were at least 3–5 times higher than that of L6 cells.

The ability of D23 transfectants to take up 2-deoxy-D-glucose (dGlc) was examined (Fig. 6). Similar to D23 myoblast, D23/MMTV and uninduced D23/GLUT 3 transfectants were unable to take up dGlc (0.06 mM). However, dex-induced D23/GLUT 3 transfectants were able to take up dGlc at a rate slightly higher than that for the wild type L6 myo-

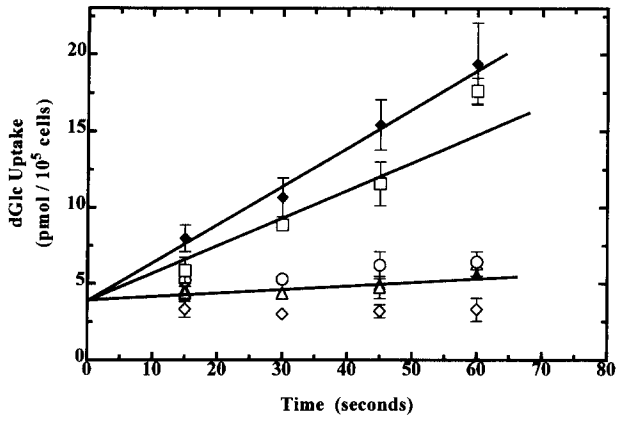

Fig. 6. Uptake of 2-deoxy-D-glucose by D23 transfectants. Transport studies were carried out as described in the text. Where applicable, cells were induced with 10^{-7} M dexamethasone (dex) for 24 h. The uptake of 0.06 mM 2-deoxy-D-glucose was monitored over a 1 min period. □, ○, △, ▲, ◇, and ◆ denote the uptake by day 2 cultures of L6, D23, D23/MMTV (1-1), dex-induced D23/MMTV (1-1), D23/GLUT 3 (2-4) and dex-induced D23/GLUT 3 (2-4) myoblasts, respectively. The error bars denote standard deviations for a sample size of 3.

blast. This clearly demonstrated the presence of functional GLUT 3 transporters in dex-induced D23/GLUT 3 transfectants. These transfectants were therefore used to study the role of GLUT 3 in myogenic differentiation.

3.4. *Biochemical and morphological differentiation of dex-induced D23/GLUT 3 transfectants*

Poly(A)$^+$RNAs from day 2 and day 6 cultures of dex-induced and uninduced transfectants were probed with *myogenin*, *MHC*, or *TnT* cDNAs. Induction of GLUT 3 was found to have no effect on *myogenin*, *MHC* and *TnT* expression (Fig. 5 and data not shown). Immunofluorescence studies also failed to demonstrate the presence of myogenin and MHC in dex-induced or uninduced day 6 D23 and D23/GLUT 3 transfectants (Figs. 7 and 8, data not shown). These studies showed that overexpression of *GLUT 3* did not activate *myogenin* or *MHC* expression. An examination of cell morphology also failed to reveal the presence of myotubes in dex-induced D23/GLUT 3, D23 and D23/MMTV myoblasts (Figs. 7 and 8). Previous studies showed that about 4–6% fusion was observed in day 6 cultures of dex-induced D23/GLUT 3 myoblasts [31].

Since overexpression of GLUT 3 also suppressed myogenesis [31], it was difficult to determine whether the myogenic defect of the dex-induced D23/GLUT 3 transfectants was due to the much elevated GLUT 3 transporter level, or some GLUT 3-independent events. Whatever be the case, this study suggests that mutant D23 is defective in regulatory component(s) essential for the expression of *myogenin* and muscle-specific genes, and these defects could not be restored by overexpression of the GLUT 3 transporter.

3.5. *Myogenin promoter activities in L6 and D23 myoblasts*

Another approach to characterize the myogenic defect of mutant D23 was to determine why D23 myoblast was impaired in *myogenin* expression. To this end, *myogenin* promoter activity was examined by transfecting D23 and L6 cells with constructs containing the full-length (pGSC) or the truncated (pGXC) *myogenin* promoter [36]. In both constructs, a *chloramphenicol acetyltransferase* (*CAT*) gene was placed under the control of a *myogenin* promoter; the activity of which could then be monitored by the CAT activity. Cells were also co-transfected with a construct containing a *lacZ* gene placed under the control of a constitutive CMV promoter (pRc/CMV-βgal), this enabled one to determine the efficiency of transfection by measuring β-galactosidase activity.

Transient transfectants of L6 cells harboring the full-length *myogenin* promoter (pGSC) were found

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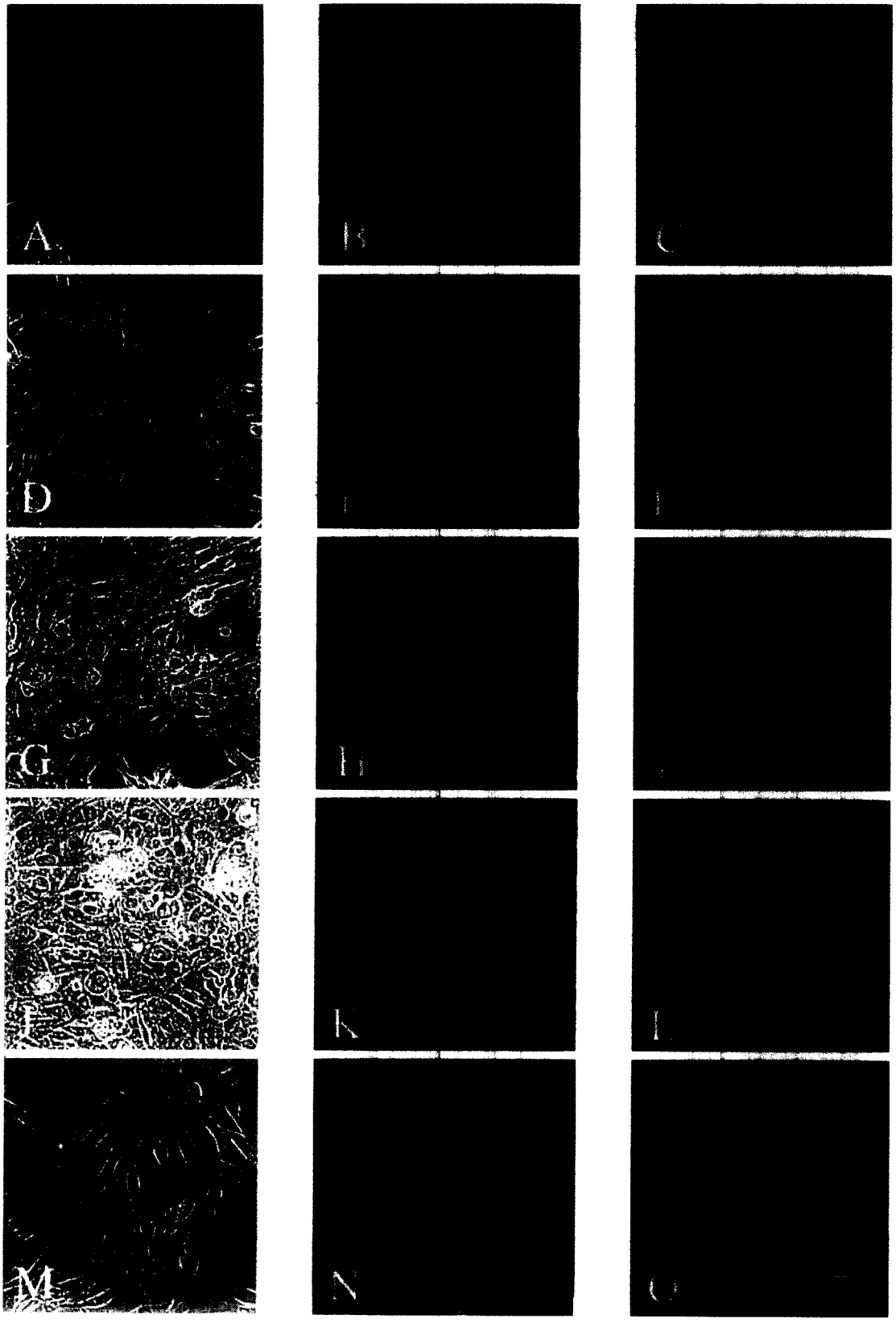
Fig. 7. Immunofluorescence staining for myogenin in day 6 dex-induced cultures. Immunofluorescence studies were carried out as described in the text. Day 6 cultures of L6 (A–C), D23 (D–F), D23/MMTV (G–I), D23/GLUT 3 clones 2-4 (J–L) and D23/GLUT 3 clone 2-6 (M–O) were induced with 10^{-7} M dex on day 1 for 24 h. A, D, G, J, and M indicate cell morphology as viewed by the phase contrast microscope. B, E, H, K and N indicate cells stained with Hoechst DNA stain. C, F, I, L and O indicate staining of myogenin by an anti-myogenin antibody (F5D). Myogenin staining was observed only in the nuclei of L6 myotubes (C), and not in other cultures (F, I, L, and O).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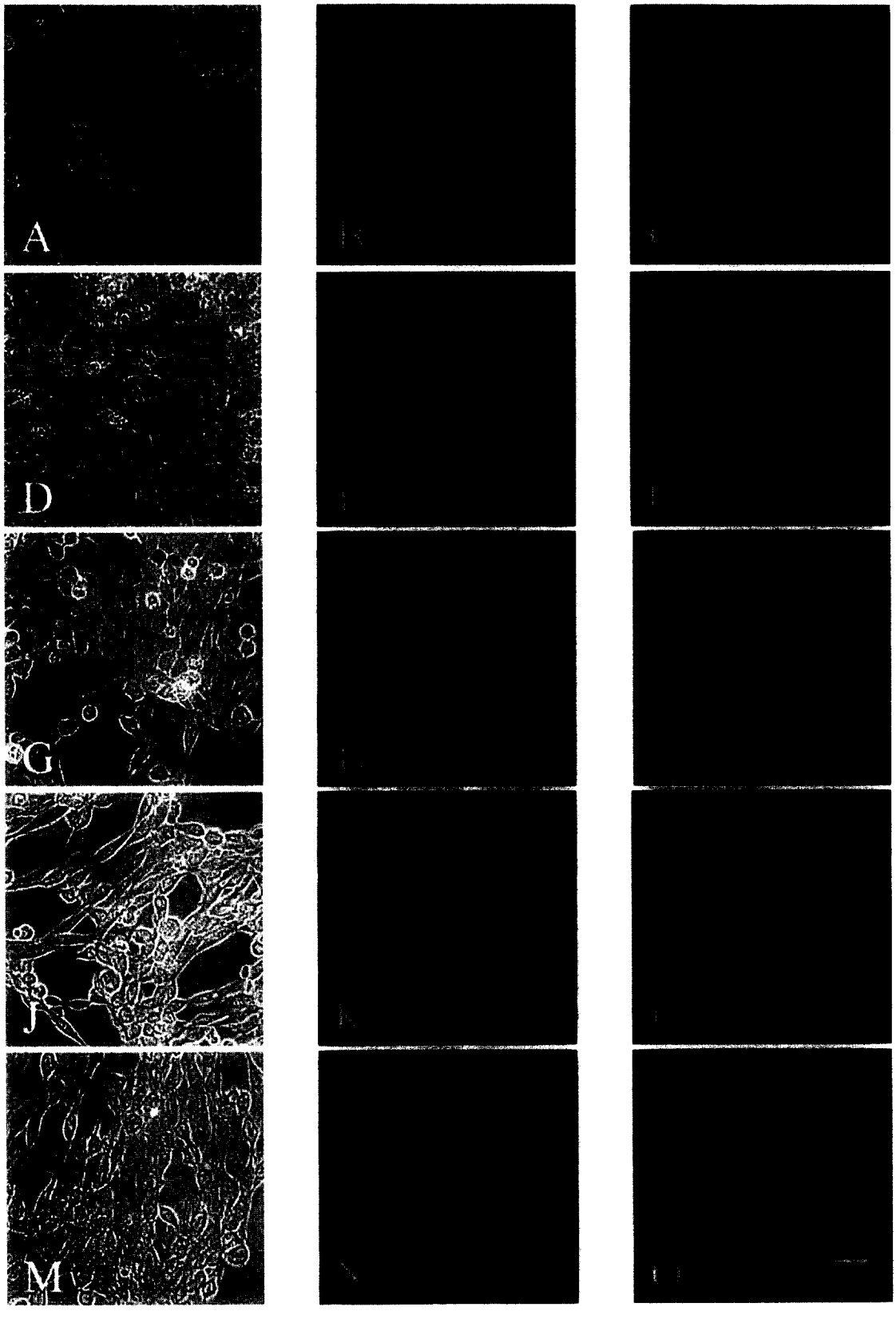
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Fig. 8. Immunofluorescence staining for myosin heavy chain in day 6 dex-induced cultures. Day 6 cultures of L6 (A–C), D23 (D–F), D23/MMTV clone 1-1 (G–I), D23/GLUT 3 clones 2-4 (J–L) and D23/GLUT 3 clone 2-6 (M–O) were induced with 10^{-7} M dex on day 1 for 24 h. A, D, G, J, and M indicate cell morphology as viewed by the phase contrast microscope. B, E, H, K and N indicate cells stained with Hoechst DNA stain. C, F, I, L and O indicate staining of MHC by the anti-MHC antibody (MF-20). MHC staining was observed only in L6 myotubes (C), and not in other cultures (F, I, L, and O).
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to possess significant levels of the *myogenin* promoter activity; only 35% of this activity was observed in L6 cells containing the truncated *myogenin* promoter (pGXC) (Fig. 9). This was similar to the observation made by Yee et al. [36]. More importantly, less than 10% of the *myogenin* promoter activity was observed in D23 myoblasts harboring similar constructs. This indicated that mutant D23 was defective in component(s) required for the *myogenin* promoter activity.

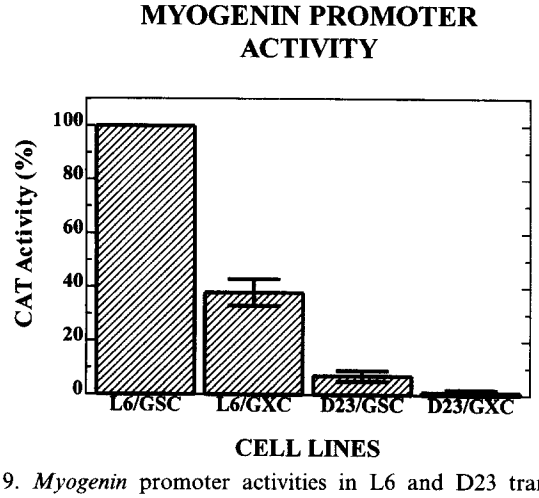

Fig. 9. *Myogenin* promoter activities in L6 and D23 transfectants. Chloramphenicol acetyltransferase (CAT) and β-galactosidase (βgal) activities were measured in L6 and D23 transient transfectants harboring a full-length (GSC) or truncated (GXC) *myogenin* promoter construct along with a pRc/CMV-βgal vector. L6 and D23 transfectants containing the expression vector pRc/CMV were used as negative controls in assessing β-galactosidase activity. Cells transfected with the Bluescript KS^+ vector were used as negative controls for measuring CAT activity. To normalize transfection efficiencies, the *myogenin* promoter activity was measured as a ratio of the CAT and the βgal activities. The *myogenin* promoter activity of L6/GSC transfectant was taken as 100%. The error bars denote standard deviations for a sample size of 4.

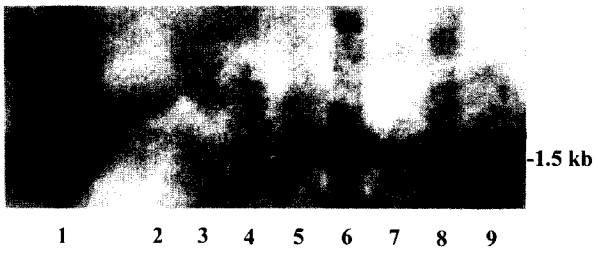

Fig. 10. Southern blot analysis of D23/myogenin transfectants. Genomic DNAs from D23/myogenin transfectants were digested with *Xho*I and *Bam*HI. Southern blot studies were carried out as described in Fig. 4. Lanes: 1, *myogenin* cDNA digested with *Xho*I and *Bam*HI; 2 and 3, genomic DNAs of D23 and D23/PKJ1ΔR clone 1-6, respectively; 4–9, DNAs from D23/myogenin transfectants clones 2-5, 2-3, 1-6, 1-5, 1-4, and 1-2, respectively.

3.6. *Transfection of D23 myoblast with a myogenin cDNA*

If the deficiency in *myogenin* expression is solely responsible for the myogenic defect in D23 myoblast, then expression of an exogenous *myogenin* cDNA may restore its myogenic ability. To examine this possibility, mutant D23 was transfected with a construct (PGK-myogenin) containing the *myogenin* coding sequence placed under the control of a constitutive *phosphoglycerate kinase* promoter (PGK) or with its control vector (PKJ1ΔR). These cells were co-transfected with the pRc/CMV-βgal vector (which contained the *neomycin* gene) to allow selection of stable transfectants using G418.

Genomic DNAs from D23 transfectants were prepared and digested with *Xho*I and *Bam*HI. Significant levels of the exogenous *myogenin* cDNA (1.5 kb) were detected in a number of D23/myogenin transfectants (clones 1-2, 1-6, 1-10, 2-3, 2-5 and 2-7), but not in D23 or D23/PKJ1ΔR myoblasts (Fig. 10). More importantly, *myogenin* expression was detected in day 4.5 cultures of L6 cells and in D23/myogenin transfectants, but not in D23 or D23/PKJ1ΔR myoblasts (Fig. 11). Despite their much elevated *myogenin* transcript levels, these D23/myogenin transfectants were still devoid of the *MLC*, *MHC* and *TnT* transcripts, which were present in very high levels in L6 cultures (Fig. 11 and data not shown). These studies therefore showed that overexpression of *myogenin* in D23/myogenin transfectants was not accompanied by increased expression of muscle-specific genes.

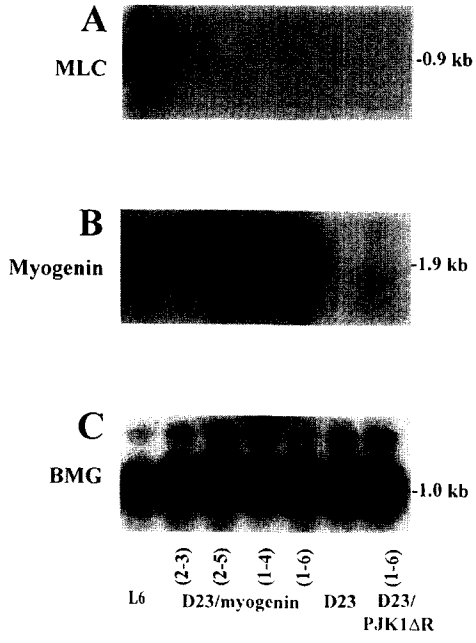

Fig. 11. Northern blot analysis of D23/myogenin transfectants. Northern blot analysis was carried out as described in the text. Poly(A)$^+$RNAs were extracted from day 4.5 cultures. Two μg of sample were loaded onto each lane. The blots were probed with ^{32}P-labeled *MLC* (A), *myogenin* (B) and β_2-*microglobulin* (*BMG*) cDNAs (C).

Immunofluorescence studies also revealed the presence of myogenin in day 6 cultures of L6 and D23/myogenin cells, but not in D23 or D23/PKJ1ΔR myoblasts (Fig. 12). More importantly, MHC could not be detected in D23/myogenin transfectants, even though this protein was readily detected in L6 myoblasts (Fig. 13). An examination of cell morphology also failed to detect myotubes in day 6 cultures of D23/myogenin, D23 or D23/PKJ1ΔR myoblasts, whereas these structures were clearly visible in L6 cultures (Figs. 12 and 13). The inability of D23/myogenin transfectants to express *MHC*, *MLC* and *TnT* genes, and to form myotubes suggests that mutant D23 is also defective in component(s) essential for the transcription of muscle-specific genes. These component(s) are required even in the presence of excess amount of myogenin. They may act in conjunction with myogenin to activate muscle-specific gene expression.

4. Discussion

Myogenic differentiation is controlled by very complex regulatory events. Studies using myogenesis-defective myoblast mutants suggested that the temporal order of in vitro expression of myogenic components was Myf-5, myogenin and muscle-specific contractile proteins [2]. Overexpression of *GLUT 3* in L6 myoblast (as in L6 GLUT 3 sense transfectants) suppressed not only expression of *myogenin* and muscle-specific genes (*MLC*, *MHC*, and *TnT*), but also myotube formation [31]. Reduction of *GLUT 3* expression to about 39% of the L6 level (as in L6 GLUT 3 antisense transfectants) also inhibited *myogenin* expression; however, this had no effect on the expression of muscle-specific genes and on the rates of fusion [31].

To further investigate the relationship between GLUT 3 and the myogenic pathway, the myogenic properties of three independently isolated GLUT 3$^-$ mutants (D2, D9 and D23) were first examined (Figs. 1–3). Even though these mutants possessed similarly reduced *GLUT 3* transcript levels, they differed considerably in the expression of myogenesis-associated genes and in the rate of myotube formation. These mutants were therefore likely altered in different myogenic regulatory components. Since mutant D23 is essentially devoid of transcripts for *myogenin* and muscle-specific genes and is unable to form myotubes, it can serve as a useful tool to study the GLUT 3-associated myogenic pathway.

To explore the role of GLUT 3 in myogenic differentiation, mutant D23 was transfected with a *GLUT 3* cDNA placed under the control of a dexamethasone-inducible MMTV promoter. Elevated levels of the *GLUT 3* transcript and functional GLUT 3 transporter were observed upon induction of the D23/GLUT 3 transfectants with dexamethasone

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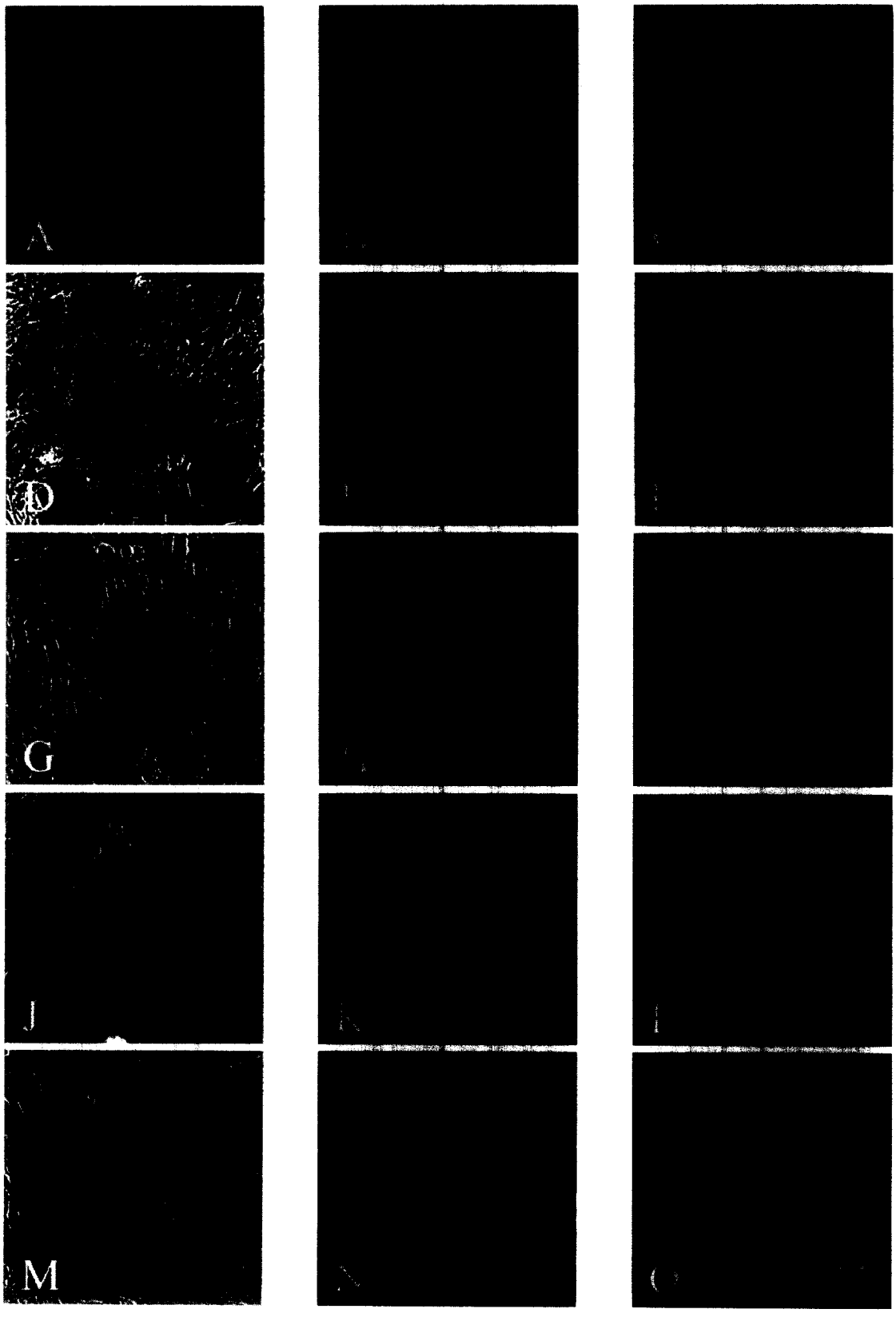
Fig. 12. Immunofluorescence staining of myogenin in day 6 D23/myogenin transfectants. Day 6 cultures of L6 (A–C), D23 (D–F), D23/PKJ1ΔR (G–I), D23/myogenin clone 1-6 (J–L), and D23/myogenin clone 2-5 (M–O) were examined in this study. A, D, G, J, and M indicate cell morphology as viewed by the phase contrast microscope. B, E, H, K and N indicate cells stained with Hoechst DNA stain. C, F, I, L and O indicate staining of myogenin by the anti-myogenin antibody (F5D).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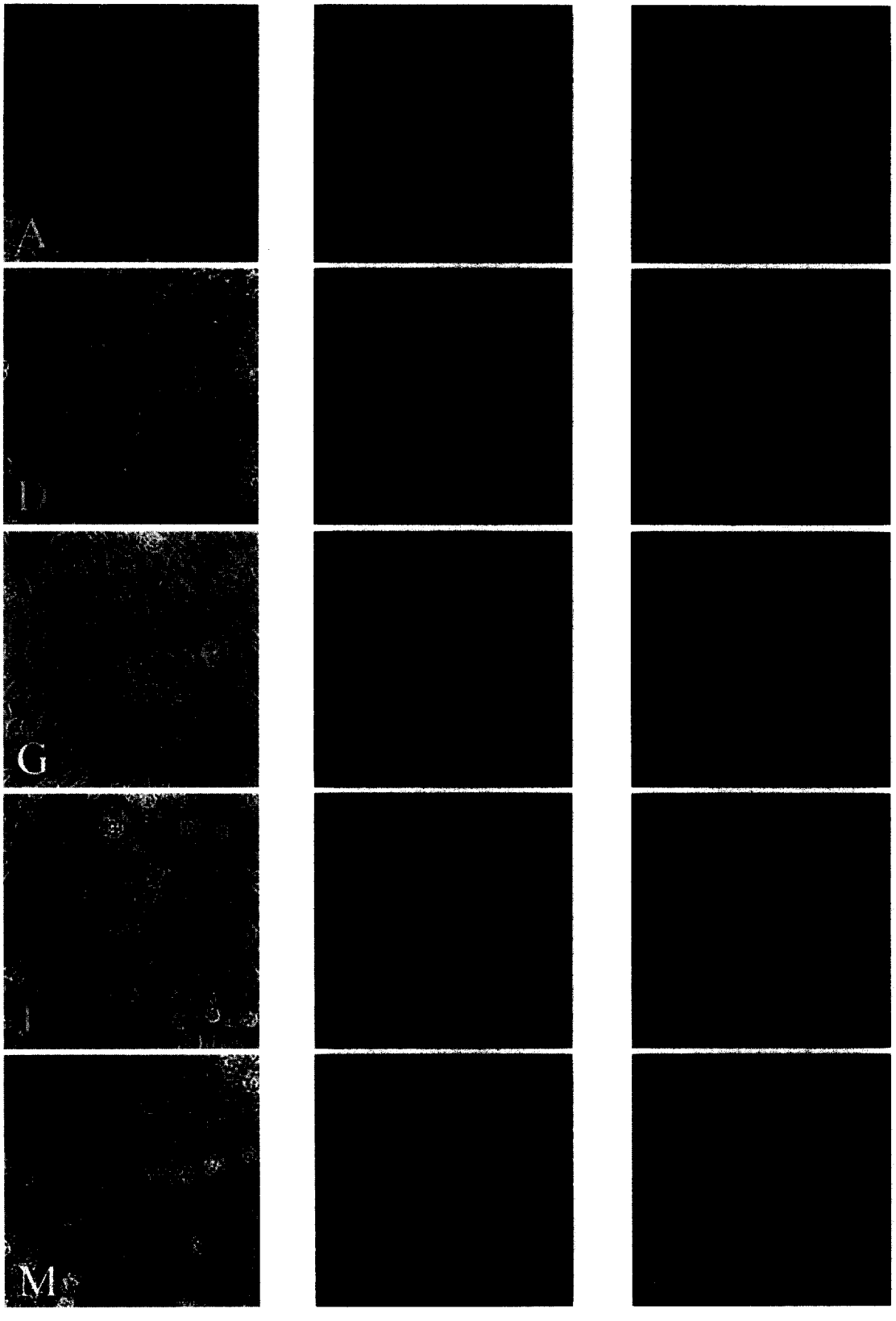
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(Figs. 5 and 6). It was important to note that these changes were not accompanied by increases in myogenin or MHC levels (Figs. 5, 7 and 8). Since overexpression of GLUT 3 in L6 myoblasts also suppressed myogenesis [31], it was difficult to determine whether the myogenic defect of the dex-induced D23/GLUT 3 transfectants was due to elevated GLUT 3 level, or some GLUT 3-independent events.

To gain more insight into the inability of mutant D23 to express *myogenin*, its *myogenin* promoter activity was examined by transfection studies using constructs containing a *chloramphenicol acetyltransferase* (*CAT*) gene placed under the control of a *myogenin* promoter (Fig. 9). Unlike L6 transfectants, *myogenin* promoter activity was hardly detectable in D23 transfectants. This suggested that mutant D23 was deficient in component(s) (hereafter referred to as Factor M) required for the *myogenin* promoter activity. Since *myogenin* expression was also reduced in L6 transfectants over- or underexpressing the GLUT 3 transporter [31], this factor might also be altered in these L6 transfectants. If so, then the levels and/or functional states of Factor M were probably dependent on a critical level of the GLUT 3 transporter.

If the inability of D23 myoblast to express muscle-specific transcripts was solely due to the absence of myogenin, then increased levels of myogenin should restore its myogenic ability. To explore this possibility, mutant D23 was transfected with a construct containing the *myogenin* coding sequence. Despite overexpression of *myogenin* in these cells, these D23/myogenin transfectants were still unable to express *MLC*, *MHC* and *TnT* genes and to form myotubes (Figs. 11–13, and data not shown). Thus mutant D23 might also be defective in a component (Factor S) required for activating *MLC*, *MHC*, and *TnT* transcription. This factor is required for myogenesis even in the presence of an excess amount of myogenin. Since the myogenic defect of L6 GLUT 3 sense transfectants was due to the inability to express muscle-specific genes, and not due to insufficient myogenin [31], Factor S might also be absent or defective in these L6 GLUT 3 sense transfectants.

We have shown in this study that mutant D23 is deficient in components responsible for *myogenin* expression and for the transcription of muscle-specific genes. Even though all GLUT 3^- mutants possessed similar GLUT 3 levels, mutant D23 had more reduced *myogenin* and muscle-specific transcripts than D2 and D9 myoblasts (Fig. 3). This together with the inability of GLUT 3 to restore myogenesis suggested that GLUT 3 was not solely responsible the myogenic defects in mutant D23 (Figs. 5, 7 and 8). This mutant is probably deficient in a regulatory factor (R), which controls the expression of GLUT 3, Factor M and Factor S. On the other hand, D2 and D9 myoblasts may be mutated in Factor R or in GLUT 3 itself, such that only *GLUT 3* expression is directly affected, whereas expression of Factor M and Factor S is still possible. The observed reduced expression of *myogenin* and muscle-specific genes could be due to reduced GLUT 3 level in these mutants [31]. This may explain why these mutants still possess *myogenin*, *MLC*, and *TnT* transcripts and the ability to form myotubes (Figs. 2 and 3).

An important assumption of this model is that the observed changes are due to mutation of a single regulatory gene in mutant D23, namely the one coding for Factor R. Since mutant D23 is a spontaneous mutant [19], the chances of this mutant acquiring simultaneous mutations in GLUT 3, Factor M, and Factor S are fairly remote. Since the frequency of spontaneous mutation is around 10^{-6}, the frequency of three simultaneous mutations should then be around 10^{-18}. In other words, it is virtually impossible to obtain mutants acquiring three simultaneous mutations.

In summary, we have shown in this study that the rat myoblast GLUT 3^- mutant, D23, is deficient in factors required for the *myogenin* promoter activity, and for the transcription of muscle-specific contractile protein genes. These changes may be brought

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Fig. 13. Immunofluorescence staining of MHC in day 6 D23/myogenin transfectants. Day 6 cultures of L6 (A–C), D23 (D–F), D23/PKJ1ΔR (G–I), D23/myogenin clone 1-6 (J–L) and D23/myogenin clone 2-5 (M–O) were examined in this study. A, D, G, J, and M indicate cell morphology as viewed by the phase contrast microscope. B, E, H, K and N indicate cells stained with Hoechst DNA stain. C, F, I, L and O indicate staining of an anti-MHC antibody (MF-20).

about by reduced level of a regulatory component, which also controls the expression of the GLUT 3 transporter.

Acknowledgements

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