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Research Article

A novel muscle DNA-binding activity in the GLUT1 promoter

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Abstract. GLUT1 glucose transporters are highly expressed in proliferating and transformed cells and serum and cAMP or the transcription factor Sp1 induce GLUT1 gene transcription. Here we identified a cis element situated at –46/–37 (MG1E – muscle-specific GLUT1 element) to which muscle-specific nuclear factors bind, and the DNA-protein complexes showed electrophoretic mobility of 41 and 32 kDa. MyoD over-expression induced the generation of MG1E-protein complexes characteristic of myoblast cells. MG1E does not bind any known factors defined in databases. Mutation of the MG1E sequence impaired transcriptional activity of the GLUT1 promoter specifically in skeletal or cardiac muscle cells. The transcriptional activity of the GLUT1 promoter induced by either Sp1, cAMP or serum was markedly reduced when MG1E was inactivated. We propose that the MG1E sequence permits the binding of muscle-specific nuclear factors and a maximal transcriptional activity in muscle cells in response to Sp1, cAMP or serum.

Key words. Glucose transporter; GLUT1; gene expression; muscle cell; Sp transcription factor; cAMP.

GLUT1 is a ubiquitous glucose transporter that mediates basal glucose transport into cells. GLUT1 is highly expressed in conditions with a high demand for glucose, as in proliferating cells or in fetal tissues [1]. In adult life, GLUT1 is ubiquitously expressed, although usually at low levels; however, GLUT1 levels are exceptionally high in endothelial cells from the blood-brain barrier, in epithelial cells from the mammary gland during pregnancy and lactation, and in primate erythrocytes, and they are also substantially elevated in cardiac myocytes from adult rats [2–5]. The importance of GLUT1 is highlighted by the observation that some patients show GLUT1 deficiency syndrome caused by haploinsufficiency due either to hemizygosity of GLUT1 or to nonsense mutations resulting in truncation of the GLUT1 protein [6]. These patients have infantile seizures, delayed development, acquired microcephaly, normal blood glucose, low-to-normal cerebrospinal fluid lactate, persistent low concentrations of glucose in cerebrospinal fluid and diminished hexose transport into isolated red blood cells [6].

GLUT1 gene expression is controlled by the activity of the core promoter and two distinct enhancers [7–9]. We have previously identified the core promoter of the rat GLUT1 gene in the –99/–33 region [7, 8, 10]. This promoter drives transcriptional activity of GLUT1 in a vari-

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ety of non-muscle and muscle cells and is responsible for the down-regulation of GLUT1 during myogenic differentiation of L6E9 cells [7]. A GC box has been identified at −91/−86 in the GLUT1 core promoter that binds Sp1 and Sp3. Sp1 acts as a transcriptional activativator and Sp3 as a repressor [7, 8, 10]. Sp1 expression is down-regulated in L6E9 myotubes concomitantly with GLUT1 gene repression [7]. In addition, Sp3 undergoes transient up-regulation in L6E9 cells after induction of differentiation, which may explain the initial GLUT1 repression that takes place in the presence of normal levels of Sp1 [8]. Furthermore, Sp1 also acts as a transactivator in neonatal rat cardiomyocytes and Sp1 levels are high in fetal heart and low in the adult state, which correlates with the rate of GLUT1 gene transcription [10].

There is evidence that the −91/−86 GC box is not the only element contributing to the transcriptional activity of the GLUT1 promoter. Thus, inactivation of the GC box in L6E9 muscle cells or in neonatal rat cardiomyocytes does not completely inhibit transcriptional activity of the GLUT1 promoter, despite the abolition of Sp1 binding [8, 10]. On the basis of these observations, we searched for other elements contributing to the transcriptional activity of the GLUT1 promoter. We identified a novel activating element, which binds muscle-specific nuclear factors.

Material and methods

Materials

[γ-^{32}P]-ATP was purchased from ICN (Irvine, Calif.). Gamma-globulin, 8-bromo-cAMP and most commonly used chemicals were from Sigma (St. Louis, Mo.). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, pancreatin, glutamine and antibiotics were obtained from BioWhittaker (Walkersville, Md.). Fugene transfection reagent and CAT Elisa kit were from Roche (Basel, Switzerland). A double-stranded oligonucleotide containing an Sp1 consensus binding site and the pCAT-Basic vector were obtained from Promega (Madison, Wis.). The plasmid containing the −2106/+134 region of the rat GLUT1 genomic sequence was obtained from Dr M. Birnbaum (University of Pennsylvania, Philadelphia, Pa.). CMV-Sp1 plasmid was a generous gift of Dr R. Tjian (University of California, Berkeley, Calif.). Constitutively active Ras (RasVal12) was kindly given by Dr P. Ruiz-Lozano (University of California, San Diego, La Jolla, Calif.). Polyclonal antibody against Sp1 and against NF-YB (C-20) were from Santa Cruz Biotechnology (Santa Cruz, Calif.). Antiserum against rat Sp3 was a gift from Dr G. Suske (Philipps-Universität Marburg, Germany) and polyclonal antibody against GLUT1 was from Diagnostic International (Karlsdorf, Germany).

Cell culture and preparation of cell extracts

Skeletal muscle L6E9, C3H10T1/2 fibroblasts and C3H10T1/2 cells stably transfected with MyoD were grown as reported elsewhere [7]. Preparation of enriched neonatal rat cardiomyocyte culture has been described previously [10]. Membrane proteins were obtained as reported in Viñals et al. [7].

Site-directed mutagenesis

GCbox mutants (mut1 and mut2) and MG1E mutant were constructed as described elsewhere [8].

GLUT1 CAT reporter constructs, transient transfection and CAT assays

The GLUT-1 CAT constructs containing various 5′ deletions of the 2240-bp fragment of the rat GLUT1 promoter region from positions −2106 to +134 (relative to the transcription start site) and the mutations −99 mut1 (5′-CCTCAGGCCCCGTACCCCGGCCCACC-3′), −99 mut2 (5′-CCTCAGGCCCCGTACGTCGGCCCACC-3′) have been previously reported [7, 8]. The mutations −2106 MG1Emut and −99 MG1Emut (5′-CGGGCCAATGGCATCGGTCCTATAAAAAG-3′) were generated as previously reported [8].

A series of CAT reporter constructs were transfected into L6E9 myoblasts, C3H10T1/2 cells or neonatal rat cardiomyocytes as previously reported [7, 11]. The test plasmids were co-transfected with β-galactosidase expression plasmid pON239 [12] to normalize for the efficiency of transfection. After harvesting, cells were lysed. The CAT activity was measured either by incubation with ^{14}C-chloramphenicol, further extraction into ethyl acetate and thin-layer chromatography or by using a CAT-ELISA method, in accordance with the manufacturer's instructions (Roche).

Electrophoretic mobility shift assays

The nuclear protein extracts from cultured cells were prepared as described by Ausubel et al. [13]. Nuclei were isolated from adult rat ventricular muscle or fetal rat hearts for the preparation of nuclear extracts as described elsewhere [10]. Nuclear extract preparation was performed as described previously [7].

Electrophoretic mobility shift assays (EMSA) were performed as described previously [7, 8]. A consensus Oct-1 probe (5′-GGGATCCATATGCAAATCAATT-3′) and a series of wild-type and mutant oligonucleotides corresponding to short sequences in the rat GLUT1 proximal promoter were used as probes in the EMSA: −48/-35 (5′-CCAATGGCGGCGGT-3′), −100/−82 (5′-CCTCAGGCCCCGCCCCCCG-3′), −60/−41 (CTGCGGCGCGGGCCAATGGC) and (−40/−39) mut (5′-CCAATGGCATCGGT-3′) (mutation underlined). When oligonucleotides were used as probes, 20 pmol of double stranded oligonucleotide were end-labeled with [γ-^{32}P]-ATP using T4

polynucleotide kinase (Promega), and 10,000 cpm of the probe was incubated with 5 µg of the corresponding nuclear extracts as described in Viñals et al. [7]. An oligonucleotide containing the consensus site for Sp1 (Promega) was also used as a competitor. Supershift experiments were performed as described elsewhere [7].

UV cross-linking assay

Five micrograms of nuclear protein were incubated with 0.1 pmol of the biotinylated probes –48/–35 or (–40/–39)mut, in the same conditions as in EMSAs. The reaction mixure was then maintained on ice in Eppendorf tubes and irradiated using a 254-nm UV lamp (UV Stratalinker 1800; Stratagene) placed at a distance of 4 cm from the sample for 10 min. Cross-linked samples were subjected to electrophoresis in SDS-12% PAGE and transferred to Immobilon (same buffer as electrophoresis and immunoblotting). The membrane was blocked in PBS/0.05% (v/v) Tween 20 plus 3% (w/v) bovine serum albumin (BSA) overnight at 4 °C. Filters were then incubated with streptavidin-conjugated peroxidase (Roche) diluted 1:5000 in blocking buffer for 1 h at room temperature, washed three times with PBS/0.2% (v/v) Tween 20 BSA and detection was performed with the ECL chemiluminiscence system.

Results

Nuclear proteins bind to a conserved –48/–35 fragment of the GLUT1 promoter

Several results suggest the operation of elements in the core promoter of the rat GLUT1 gene other than the –91/–86 GC box, previously reported and known to bind Sp1 and Sp3 [7, 8, 10]. Thus, inactivation of the –91/–86 GC box in L6E9 muscle cells or in neonatal rat cardiomyocytes, in conditions known to abolish Sp1 binding, did not cause the complete inhibition of transcriptional activity. Thus, the transcriptional activity of the –99/+134 mut1 construct (construct where the –91/–86 GC box is mutated) was greater than the –33/+134-CAT construct (that only contains the GLUT1 TATA box and 5′ downstream of the TATA) (fig. 1). Based on these observations, we searched for additional elements, other than the GC box, that could contribute to the transcriptional activity of the proximal GLUT1 promoter. Analysis of the sequence of the proximal promoter of the rat GLUT1 gene using the TESS program from the TRANSFAC database [14] revealed a putative protein-binding element at –48/–35, near the TATA box, which corresponds to a conserved region in the rat and mouse genes. Band-shift analysis using the oligonucleotide –48/–35 (5′-CCAATGGCGGCGGT-3′) as a labeled probe revealed two retardation complexes, showing similar mobility profiles in nuclear extracts obtained from L6E9 myoblasts (fig. 2, see arrows); the unlabeled –48/–35 oligonucleotide competed these complexes (fig. 2). In contrast, the unlabeled –55/–42 oligonucleotide (5′-GCGCGGGCCAATGG-3′) did not compete, and the mutant form (–40/–39)mut (5′-CCAATGGCATCGGT-3′) competed less for the formation of the two complexes (fig. 2). In addition, the labeled oligonucleotide –60/–41 (CTGCGGCGCGGGCCAATGGC) did not generate any retardation band when incubated with nuclear extracts from L6E9 myoblasts (data not shown). This suggests that the 3′ end of the –48/–35 oligonucleotide is required for complex formation.

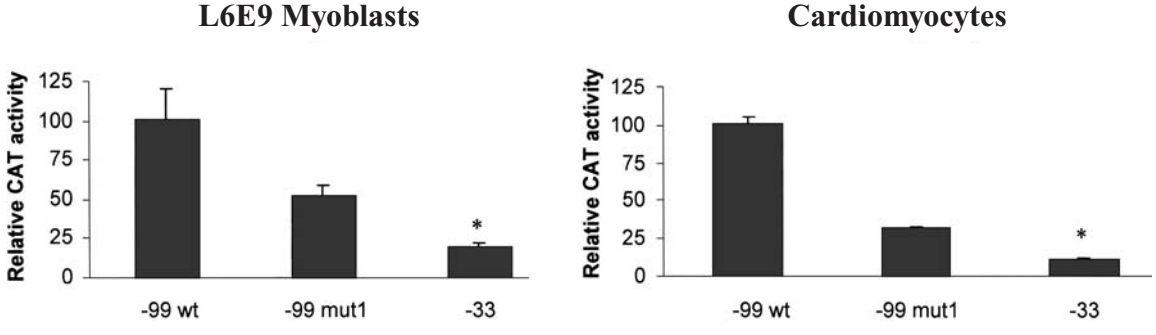

Figure 1. Inactivation of the –91/–86 GC box does not abolish transcriptional activity of the GLUT1 gene promoter. L6E9 myoblasts or neonatal rat cardiomyocytes were transiently transfected with either the wild-type –99/+134-CAT construct, the mut1 –99/+134-CAT construct (mutation of the –91/–86 GC box) or the –33/+134-CAT construct. Left, L6E9 myoblasts were transfected with 10 µg of CAT constructs and 5 µg of β-galactosidase plasmid (control of transfection efficiency). Ninety-six hours after transfection, cells were harvested and lysed and CAT and β-galactosidase activity was determined. Right, neonatal rat cardiomyocytes were transfected with 8 µg of CAT constructs and 250 ng of β-galactosidase plasmid. Ninety-six hours after transfection, cells were harvested and processed for the analysis of enzymatic activity. The data are expressed as relative CAT activity/β-galactosidase activity (mean ± SE) from six to eight experiments in L6E9 myoblasts and from a representative experiment in cardiomyocytes. * indicates a significant difference compared with the –33/+134-CAT group, at $p < 0.05$.

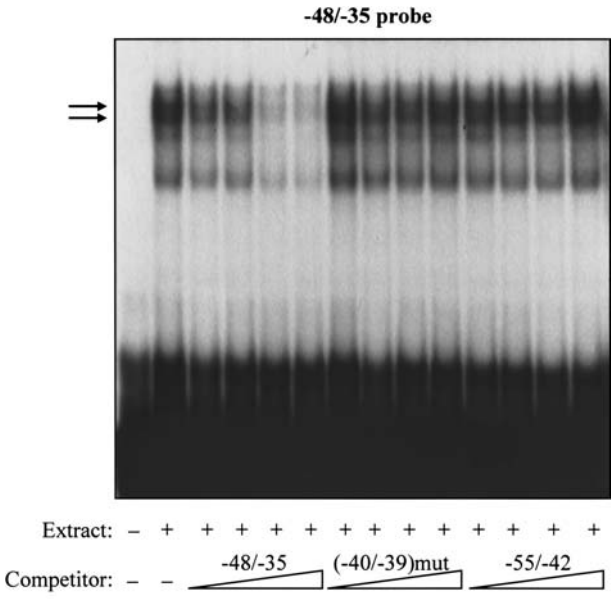

Figure 2. Nuclear factors bind to the –48/–35 fragment of the GLUT-1 promoter. The –48/–35 oligonucleotide was labeled and incubated with 5 µg of nuclear extracts from L6E9 myoblasts, and EMSA was performed. Two retarded bands with different electrophoretic mobility appeared, and a molar excess of the same unlabeled oligonucleotide –48/–35, –55/–42 oligonucleotide or a mutant form (–40/–39)mut of the –48/–35 oligonucleotide (ranging from 10- to 100-fold molar excess) were used as competitors.

Muscle-specific nuclear factors bind to the –48/–35 sequence

Next, we assessed whether the –48/–35 sequence bound ubiquitous factors. We incubated the –48–35 oligonucleotide with nuclear factors from L6E9 myoblasts and myotubes, neonatal rat cardiomyocytes, 10T1/2 fibroblasts or 10T1/2-MyoD cells (after stable transfection with MyoD), and from fetal, neonatal or adult vat heart. There were no retardation bands in nuclear extracts derived from 10T1/2 fibroblasts or from fetal rat hearts (fig. 3). No complexes were detected after incubation of the –48/–35 oligonucleotide with nuclear extracts from either NIH-3T3 or rat liver (data not shown). In contrast, nuclear extracts from L6E9 myoblasts, 10T1/2-MyoD myoblasts or neonatal rat cardiomyocytes generated a doublet of strongly retarded bands (fig. 3). Furthermore, L6E9 myotubes, 10T1/2-MyoD myotubes or adult rat heart showed two distinct retardation bands that migrated faster than the bands seen in myoblasts (fig. 3). Competition of band-shift performed in the presence of increasing concentrations of wild-type –48/–35 oligonucleotide revealed that the bands were specific both in myoblast and in myotube nuclear cell extracts (figs 2, 3 A).

The acquisition of the retardation complexes characteristic of myoblasts or myotubes was dependent upon the culture conditions. Thus, when L6E9 myoblasts became confluent, they showed the characteristic strongly retarded bands as well as the faster retarded bands of the myotubes (fig. 5 B). This situation was also observed when myotubes co-existed with a large myoblast population (data not shown).

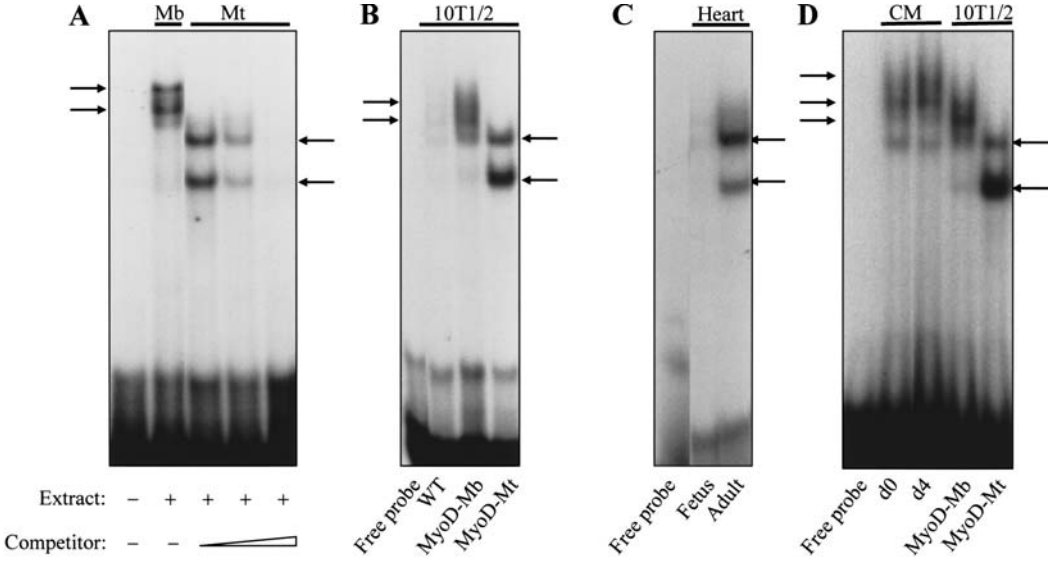

Figure 3. The –46/–37 fragment binds to nuclear factors present in cardiac and skeletal muscles in a differentiation- and development-dependent manner. EMSA experiments using the –48/–35 probe and 5 µg of different nuclear extracts. (*A*) L6E9 myoblasts and myotubes (competition with a 50- and 500-fold excess of the unlabeled nucleotide). (*B*) 10T1/2 fibroblasts, 10T1/2MyoD-Myoblast and 10T1/2 MyoD-Myotube. (*C*) Fetal heart and adult heart. (*D*) Freshly isolated neonatal cardiomyocytes (d0), cardiomyocytes cultured for 4 days (d4) and 10T1/2-MyoD-myoblasts and 10T1/2-MyoD-myotubes.

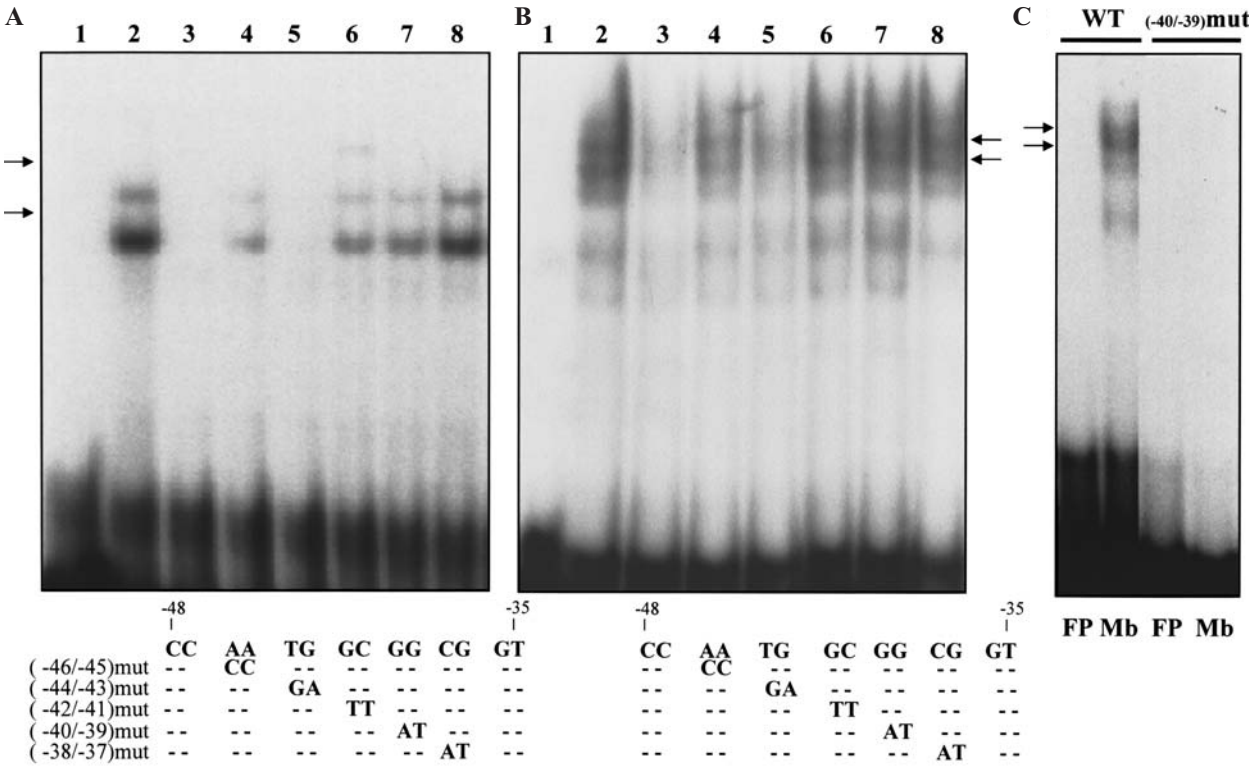

Figure 4. Determination of the specific bases in the –48/–35 oligonucleotide that confer capacity for competition in band-shift assays. (*A*, *B*) The –48/–35 oligonucleotide was labeled and incubated with 5 µg of nuclear extracts from 10T1/2 myotubes (*A*) or L6E9 myoblasts (*B*). Bands were competed with a 50-fold molar excess of unlabeled oligonucleotide. Lane 1, probe without nuclear extracts; lane 2, probe without competitor; from lane 3 to lane 8, sequences of unlabeled oligonuclotides used for competition are specified in the figure: lane 3, competition with wild-type –48/–35; lane 4, competition with (–46/–45)mut; lane 5, competition with (–44/–33)mut; lane 6, competition with (–42/–41)mut; lane 7, competition with (–40/–39)mut; lane 8, competition with (–38/ –37)mut. (*C*) EMSA experiment using two different labeled probes: wild-type –48/–35 and (–40/–39)mut and using 5 µg of nuclear extracts from L6E9 myoblasts.

Together, these data indicate that the –48/–35 sequence binds to nuclear factors that are present in cardiac and skeletal muscles. Accordingly, we named this element MG1E (muscle-specific GLUT1 element)

Characterization of the MG1E element

More insight into the specific bases involved in the competition in band-shift analyses was obtained by incubating the labeled –48/–35 oligonucleotide probe with nuclear extracts from L6E9 myoblasts or 10T1/2-MyoD myotubes either in the absence or presence of an array of unlabeled mutant –48/–35 oligonucleotides (fig. 4A, B). The (–46/–45)-mutant oligonucleotide (5′-CCCCTGGCGGCGGT-3′) competed to a large extent but not completely (fig. 4A, B), and in keeping with this, the labeled (–46–45) mutant oligonucleotide did not generate any retardation band when incubated with nuclear extracts from L6E9 myotubes (data not shown). In addition, the (–44/–43) mutant oligonucleotide (5′-CCAAGAGCGGCGGT-3′) caused a competition similar to that detected with the wild-type oligonucleotide (fig. 4A, B). In contrast, the mutant forms (–42/–41) (5′-CCAATGTTGGCGGT-3′), (–40/–39) (5′-CCAATGGCATCGGT-3′) and (–38/–37) (5′-CCAATGGCGGATGT-3′) competed less or did not compete for the formation of the retardation complexes (fig. 4A, B). In keeping with these observations, the labeled (–40/–39) mutant oligonucleotide did not generate any retardation band when incubated with nuclear extracts from L6E9 myoblasts or myotubes (fig. 4C, and data not shown). Further evidence of the specificity of this element was obtained with more subtle mutations of the sequence; thus, the mutant oligonucleotide (5′-CCAATGGCCGGGGT-3′) neither competed for the formation of band-shift complexes nor generated any retardation bands when used as a labeled probe (data not shown). All these observations suggest that the MG1E element that binds nuclear factors from myoblasts or myotubes requires the sequence 5′-AANNGCGGCG-3′ located at –46/–37 in the GLUT1 promoter.

Novel factors bind to MG1E

Both the TESS and the Signal Scan programs from the TRANSFAC database [14, 15] recognized a number of putative factors that could bind the –48/–35 element: they include CCAAT-binding factors, YY1 and Sp1. To determine whether any of these factors participated in the

binding to MG1E, we performed band-shift studies in which the formation of complexes with the −48/−35 oligonucleotide was prevented by increasing amounts of consensus oligonucleotides that bind to the above-mentioned factors. Consensus oligonucleotides corresponding to CCAAT-binding proteins such as NF-YB/CP1 (5′-GGAACCAATGAAATGCGAGG-3′) [16], CP2 (5′-GAGCAAGCACAAACCAGCCAA-3′) [17], CTF (5′-TTTTGGATTGAAGCCAATATGATA-3′) [18] or C/EBP (5′-AATTCAATTGGGCAATCAGG-3′) [19] did not compete for the retardation bands formed from L6E9 myoblasts (data not shown). Negative results were obtained in supershift assays for the presence of the NFY-B subunit. In addition, the unlabeled −55/−42 oligonucleotide (5′-GCGCGGGCCAATGG-3′) did not compete for the band-shift complexes (fig. 2), which further supports the absence of CCAAT-binding proteins in the MG1E retardation complexes. We also analyzed whether YY1 or Sp1 factors bind the MG1E element. We incubated the −48/−35 oligonucleotide with L6E9 nuclear extracts and with either the YY1 (5′-GTTTTGCGACATTTTGCGACAC-3′) [20] or Sp1 consensus oligonucleotides (5′-ATTCGATCGGGGCGGGGCGAGC-3′); in these conditions, no competition was detected. Additional evidence that neither Sp1 nor YY1 binds to the MG1E element is the finding that they bind to DNA in a Zn^{2+}-dependent manner [21, 22] and in our experimental conditions, retardation bands were independent of Zn^{2+} or EDTA. Further demonstration that Sp1 does not bind to MG1E is the fact that Sp1-binding activity is abolished in nuclear extracts from L6E9 myotubes [7, 8], whereas MG1E-binding activity is apparent (fig. 3). Factors binding to MG1E were heat sensitive, since treatment of nuclear factors from L6E9 myoblasts at 80 °C for 5 min prevented the formation of complexes (data not shown). Heat sensitivity further supports the view that C/EBPs are not involved in binding to MG1E [23].

Partial characterization of MG1E-binding proteins

EMSA is limited in its ability to resolve the size(s) of the specific nuclear protein(s) that interact with a DNA element. We therefore performed experiments in which the DNA-protein complexes were UV cross-linked. DNA-protein complexes were allowed to form in the same conditions as in EMSA, and after probe incubation, the binding reaction was either left untreated or cross-linked by exposure to UV light at 254 nm at 4 °C for 10 min. The binding reactions were subjected to SDS-PAGE to resolve DNA-protein complexes.

Nuclear extracts from confluent myoblasts showed the characteristic strongly retarded bands as well as the faster retarded bands of the myotubes in EMSA assays (fig. 5A). Under these conditions, two major UV-dependent polypeptide bands that bind specifically to the wild-type probe (−48/−35) migrating at a position corresponding to approximately 41 and 32 kDa were detected in nuclear extracts from myoblasts (fig. 5B). Nuclear extracts from L6E9 myotubes and from adult rat heart preferentially showed the 32-kDa band (figs 6B, C and data not shown). The detection of the 41- and 32-kDa bands was dependent on the integrity of the MG1E sequence and when the (−40/−39)mut oligonucleotide (mutation that inhibits protein binding to MG1E) was used as a probe, the detection of these bands was markedly reduced (figs 5B, C). In addition, excess of the unlabeled −48/−35 oligonucleotide competed the two bands, whereas no competition was observed using the (−40/−39)mut oligonucleotide (data not shown).

MG1E maintains high rates of promoter activity in skeletal muscle and in cardiac myocytes

To determine the precise role of the MG1E situated between −46 and −37 in the proximal GLUT1 promoter, we mutated this element and analyzed the impact on transcriptional activity. We incorporated two mutations at positions −40 and −39, thus changing the sequence from 5′-CCAATGGCGGCGGT-3′ to 5′-CCAATGGCATCGGT-3′ (MG1E mutant). This mutation cancels the binding of nuclear factors to MG1E (fig. 4C) and the mutant competes poorly for the generation of retardation complexes (fig. 2). In these conditions, transient transfection analysis indicated that basal transcriptional activity was markedly reduced in MG1E mutant constructs in L6E9 myoblasts (33% and 47% reduction using the −2106/+134 and −99/+134 constructs, respectively), in L6E9 myotubes (37% reduction) and in neonatal rat cardiomyocytes (50% reduction) (fig. 6). In contrast, we found no significant effects of the MG1E mutant on GLUT1 transcriptional activity when 10T1/2 fibroblasts were transiently transfected (fig. 6). The transcriptional activity of the MG1E mutant construct was greater than the activity displayed by the −33/+134-CAT construct (fig. 6), which is consistent with the activity of the −91/−86 GC box reported in those different cell types [7, 8].

Based on the reduction of the transcriptional activity in MG1E mutants, we next analyzed whether agents that are known to stimulate transcriptional activity of the GLUT1 promoter in muscle cells depend upon the integrity of MG1E. We analyzed the effect of cAMP analogues, which stimulate transcriptional activity of the GLUT1 promoter and GLUT1 expression in L6E9 muscle cells [24]. The stimulatory effect of 8-bromo-cAMP on the GLUT1 promoter is not mediated by the −91/−86 GC box and the cAMP analogue caused a nearly complete reduction of Sp1 and Sp3 binding to the GC box, which was due to Sp1 and Sp3 repression (fig. 7A). The generation of Sp1 and Sp3 complexes was demonstrated by supershift assay (fig. 7A). Transient transfection studies indicated that 8-bromo-cAMP caused a 2.7-fold stimulation

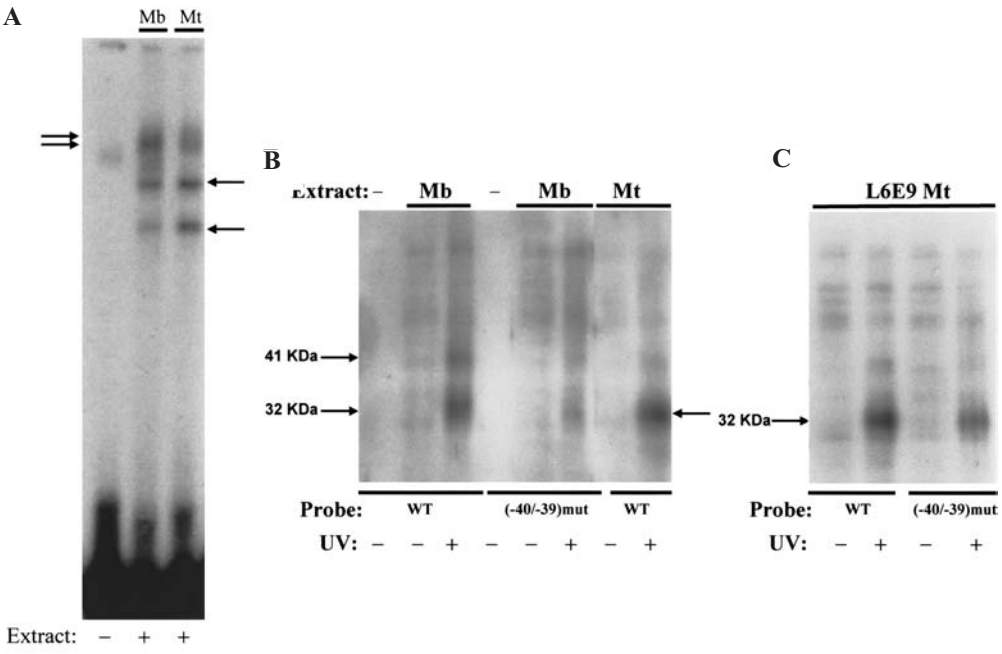

Figure 5. Determination of the molecular masses of the DNA-protein complexes. (*A*) EMSA experiment using the –48/–35 probe and 5 µg of L6E9 myoblast and myotube nuclear extracts. (*B, C*) UV cross-linking assay. 5 µg of L6E9 myoblast or myotube nuclear extracts was incubated with 0.1 pmol of biotinylated probes [(–48/–35) or (–40/–39)mut] in the same conditions as EMSA assays and irradiated at 254 nm. Cross-linked samples were subjected to electrophoresis to resolve DNA-protein complexes. The nuclear cell extracts samples used in panels *A* and *B* were the same as in the EMSA assay shown in *A*.

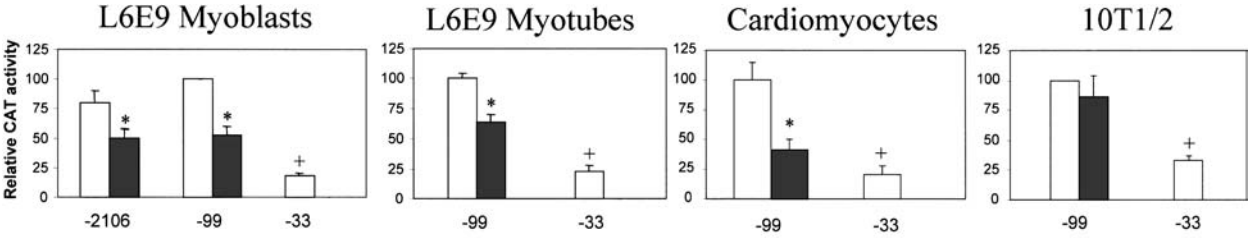

Figure 6. Inactivation of the MG1E reduces basal transcriptional activity of the GLUT1 promoter in skeletal muscle cells and cardiomyocytes. Comparison of CAT activity in wild-type (white bars) and MG1E mutant (black bars) constructs (–2106/+134-CAT, –99/+134-CAT or –33/+134-CAT) in different cell types. L6E9 myoblasts, L6E9 myotubes and 10T1/2 fibroblasts were transfected with CAT constructs (10 µg in L6E9 cells and 5 µg in 10T1/2 cells) and 5 µg of β-galactosidase plasmid (control of transfection efficiency). Ninety-six hours after transfection, cells were harvested and enzymatic activity was determined. In cardiomyocytes, 1 µg of CAT construct and 250 ng of β-galactosidase were used for transfection in a depleted serum medium. Enzymatic activity was determined after 72 h. Data are expressed as CAT activity corrected per micrograms of protein and per β-galactosidase activity (mean ± SE) from six to ten independent experiments in L6E9 myoblasts and from a representative experiment in cardiomyocytes, L6E9 myotubes and 10T1/2 fibroblasts with the wild type CAT set to a value of 100. * indicates a significant difference compared with the wild-type group, at $p < 0.05$. + indicates a significant difference between the –33/+134-CAT construct and the MG1E mutant group, at $p < 0.05$.

of transcriptional activity of the GLUT1 promoter when using the –99/+134 construct in L6E9 myoblasts (fig. 7C); in these conditions, the maximal transcriptional activity of the MG1E mutant construct was reduced in the presence of 8-bromo-cAMP and this analogue only caused a 2-fold stimulation of the transcriptional activity (fig. 7C). However, mutation of the GC box did not alter 8-bromo-cAMP-induced transcriptional activity (fig. 7C). A 5′ deletion construct that only contains the TATA box (–33/+134-CAT) still responded to cAMP (data not shown), which indicates that MG1E is not the only cis-element responsible for the effects of cAMP. In keeping with the view that MG1E participates in the effects of cAMP, treatment with 8-bromo-cAMP enhanced formation of the MG1E retardation complexes in L6E9 myoblasts (fig. 7B).

Sp1 transactivates the GLUT1 promoter after binding to the -91/-86 GC box [7, 8, 10]. Thus, we also tested whether the effects of Sp1 on transcriptional activity required an intact MG1E. L6E9 myoblasts were co-trans-

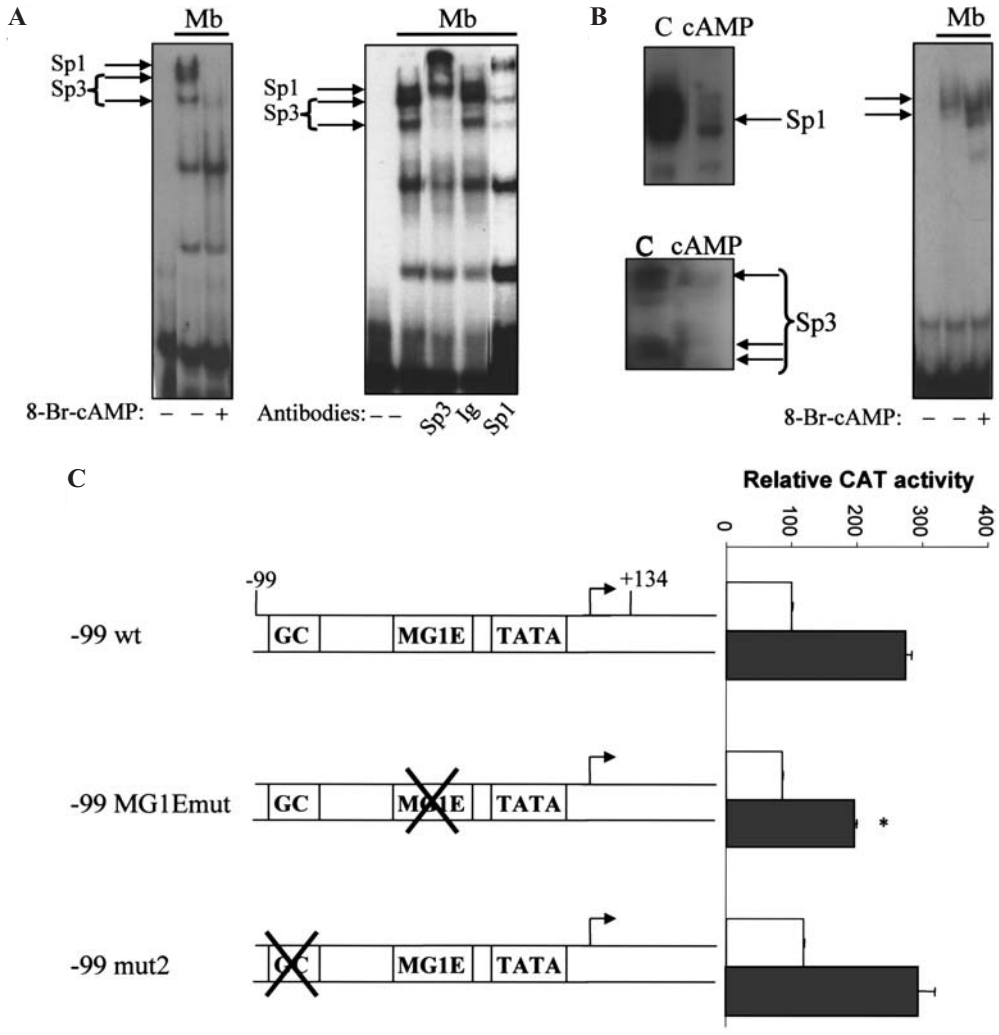

Figure 7. The maximal response of the GLUT1 promoter to cAMP requires the integrity of MG1E in L6E9 muscle cells. (*A*) The –100/–82 oligonucleotide was labeled and incubated with 5 µg of nuclear extracts from control or 8-bromo-cAMP-treated L6E9 myoblasts (1 mM 8-bromo-cAMP for 48 h) (left panel). Incubations were performed in the absence or presence of an anti-Sp1, an anti-Sp3 or an irrelevant antibody (Ig) (middle panel). Sp1 and Sp3 levels were analyzed by Western blot in nuclear extracts from each condition (right panels). (*B*) The –48/–35 oligonucleotide was labeled and incubated with 5 µg of nuclear extracts from control or 8-bromo-cAMP-treated L6E9 myoblasts. (*C*) Effect of 8-bromo-cAMP on the expression of wild-type, MG1E-mutant or GC-mutant –99/+134-CAT GLUT1 constructs in L6E9 myoblasts transient transfections. After transfection, cells were incubated in 10% FBS medium for 48 h in the absence (white bars) or presence of 1 mM 8-bromo-cAMP (black bars) and harvested in the same way as myoblasts. The data are expressed as the relative CAT activity/µg of protein ± SE from a representative experiment performed in triplicate. * indicates a significant difference between the 8-bromo-cAMP-treated wild-type and MG1E mutant groups, at $p < 0.05$.

fected with the –2106/+134 wild-type or the MG1E mutant construct together with Sp1. Sp1 over-expression caused a three-fold stimulation of GLUT1 transcriptional activity (fig. 8A). On the other hand, transcription from the MG1E mutant was only stimulated two-fold after Sp1 over-expression and the maximal transcriptional activity was reduced 33% in the MG1E mutant construct compared to the wild type (fig. 8A). Thus, maximal activation induced by Sp1 is dependent on the integrity of MG1E. Other stimulatory agents do not show sensitivity to the MG1E box; thus, RasVal12, a constitutively active form of Ras, stimulates GLUT1 promoter activity in L6E9 myoblasts and this effect was not reduced in the MG1E mutant construct (fig. 8B).

MG1E also plays a role in maintaining high rates of transcriptional activity in neonatal rat cardiomyocytes. Cardiomyocytes responded to the addition of serum in the culture medium by enhancing the expression of GLUT1 protein (fig. 9A) and the transcriptional activity of the GLUT1 promoter (fig. 9B). Transfection of cardiomyocytes with the MG1E mutant or the GC mutant constructs in the presence of serum caused a substantial reduction (nearly 50%) in the transcriptional activity of the GLUT1 promoter (fig. 9B). Serum addition increased the

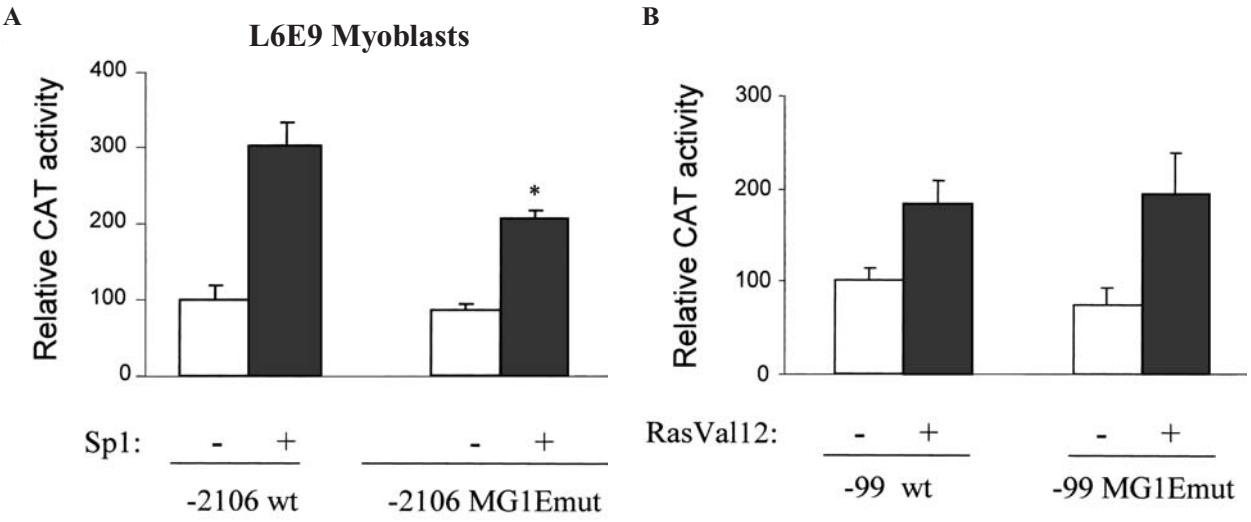

Figure 8. The maximal response of the GLUT1 promoter to Sp1 but not to RasVal12 requires the integrity of MG1E in L6E9 muscle cells. (*A*) Effect of Sp1 transcription factor on the expression of wild-type and MG1E mutant –2106/+134-CAT GLUT1 constructs in L6E9 myoblasts. Cells were transfected without (white bars) or with (black bars) Sp1 expression vector (10 µg) and after 96 h, cells were harvested and homogenized and CAT activity was determined. Data are expressed as CAT activity per microgram of protein ± SE from a representative experiment performed in triplicate. Differences between control and Sp1-transfected groups were significant at $p < 0.05$. * indicates a significant difference between wild-type and MG1E mutant groups after Sp1 transfection, at $p < 0.05$. (*B*) Effect of RasVal12 on the expression of wild-type and MG1E mutant –2106/+134-CAT GLUT1 constructs in L6E9 myoblasts. Cells were transfected without (white bars) or with (black bars) RasVal12 expression vector (10 µg) and after 96 h, cells were harvested and homogenized and CAT activity was determined. Data are expressed as CAT activity per microgram of protein ± SE from a representative experiment performed in triplicate. Differences between control and Sp1-transfected groups were significant at $p < 0.05$. Differences between wild-type and MG1E mutant groups were statistically non-significant.

binding of Sp1 and Sp3 to the –91/–86 GC box; under these conditions, the formation of MG1E was reduced, due to an unspecific event as revealed by the parallel reduction in the formation of Oct-1 complexes (fig. 9C). In keeping with the observations in L6E9 myoblasts, stimulation of the GLUT1 promoter by RasVal12 in neonatal cardiac myocytes was independent of the MG1E box (data not shown).

Discussion

In this study, we identified a novel cis-acting element in the mouse and rat GLUT1 glucose transporter gene situated at –46/–37, overlapping with a potential TFIIB recognition element, and which drives transcriptional activity of the GLUT1 proximal promoter. This element operates in skeletal and cardiac muscle cells and not in fibroblasts; in addition, this element binds nuclear factors in a differentiation-dependent and development-dependent manner and we have named it MG1E (muscle specific GLUT1 element). Furthermore, MG1E permits maximal transcriptional activity in skeletal and cardiac muscle cells in response to the transcription factor Sp1 or in response to serum or cAMP. Based on EMSA competition assays, MG1E does not seem to be present in the human GLUT1 gene.

MG1E was found to bind muscle-specific factors. Thus, robust band-shift complexes were detected in nuclear extracts from myoblasts, and a marked change in mobility was detected after myogenic differentiation. Band complexes were also detected in nuclear extracts from rat heart but only in neonatal and adult samples, not in fetal samples. Furthermore, a marked change in mobility was detected in the transition between neonatal and adult life in heart. In contrast, MG1E did not bind nuclear factors from 10T1/2 or NIH-3T3 fibroblasts or from rat liver. Thus, the MG1E element may permit muscle-specific regulation of GLUT1 gene transcription.

Our results indicate that the commitment of cells to the myogenic lineage is associated with the induction of nuclear MG1E-binding protein and that myogenic differentiation leads to the generation of faster complexes. MyoD over-expression in 10T1/2 fibroblasts induced the formation of protein-MG1E complexes characteristic of myoblast cells, supporting the view that muscle determination is associated with the formation of protein-MG1E complexes. In addition, cell differentiation into myotubes generates the faster complexes. In parallel with these findings, muscle-specific complexes are found in nuclear extracts from adult rat heart or in neonatal rat cardiomyocytes in culture. Furthermore, a pattern of complexes similar to that found in myoblasts was detected in nuclear extracts from day 1 neonatal rat hearts; hardly any com-

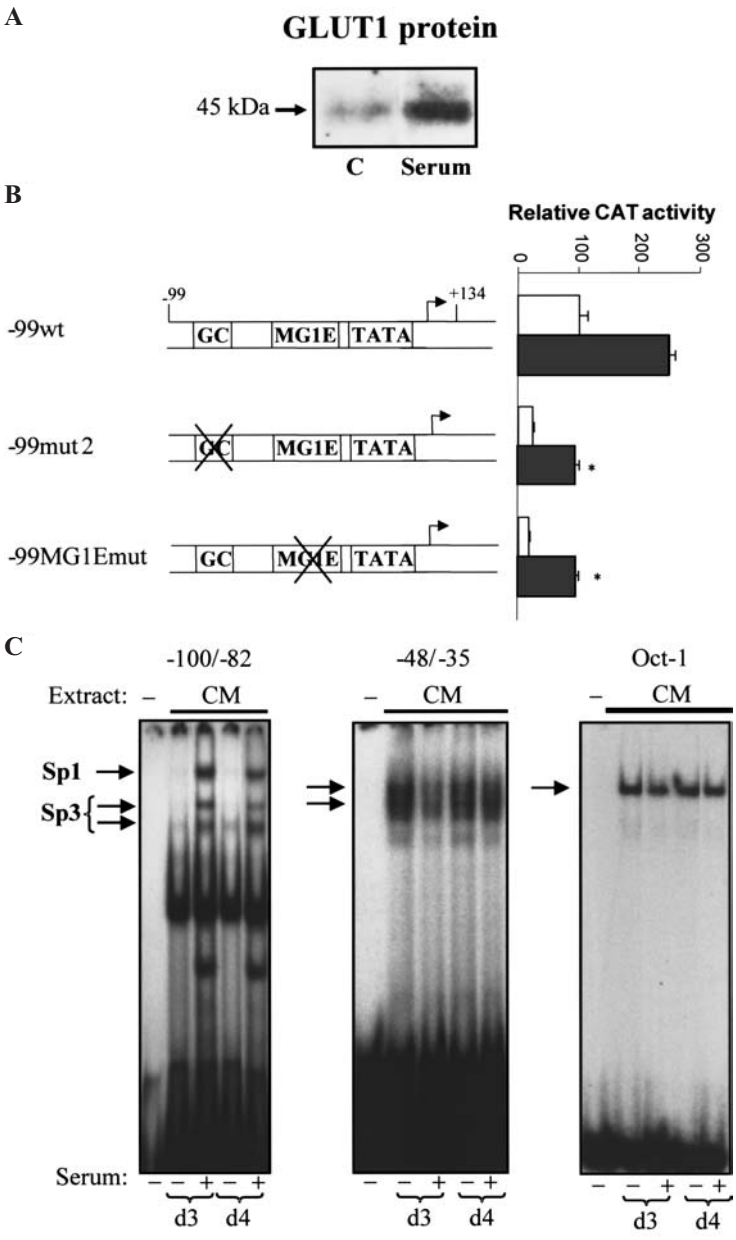

Figure 9. Effect of serum on GLUT1 expression and GLUT1 promoter activity in neonatal rat cardiomyocytes. (*A*) Effect of stimulation with serum on the expression of GLUT1 protein. Cardiac myocytes were cultured either in a serum-depleted medium or in a completed medium for 72 h before harvesting. GLUT1 protein levels were analyzed by Western blot in total membrane extracts from each condition. (*B*) Effect of stimulation with serum on the expression of wild-type –99/+134-CAT, GC mutant –99/+134-CAT or MG1E mutant –99/+134-CAT GLUT1 constructs in neonatal rat cardiomyocytes. Cells were transfected, and after washing the precipitates, they were cultured either in a serum-depleted medium (white bars) or in a complete medium (black bars) for 72 h before harvesting. Data are expressed as CAT activity per microgram of protein ± SE from a representative experiment performed in triplicate with the wild-type CAT in the absence of serum set to a value of 100. * indicates a significant difference compared with the serum-treated wild-type group, at $p < 0.05$. (*C*) The –100/–82 oligonucleotide (left panel), the –48/–35 oligonucleotide (middle panel) or an Oct-1 consensus oligonucleotide (right panel) were labeled and incubated with 5 µg of nuclear extracts from day 3 (d3) or d4 cardiomyocytes. d3 cells were cultured in a serum-depleted medium or in a complete medium for 48 h before harvesting. d4 cells were cultured in a serum-depleted medium or in a complete medium for 72 h before harvesting.

plexes were detected in nuclear extracts obtained from fetal rat hearts.

The factors that bind to MG1E are heat sensitive and do not require Zn^{2+}. Furthermore, we excluded the participation of putative transcription factors found in databases as MG1E-binding proteins: (i) CAAT-binding factors such as CP1 (NFY), CP2, CTF or C/EBP, based on band-shift competition experiments, super-shift assays for CP1 and the fact that MG1E-binding proteins are heat sensitive, in contrast to C/EBPs which are heat resistant; (ii) Sp1 based on band-shift competition assays, lack of correlation with bona fide Sp1-binding activity and Zn^{2+}/EDTA insensitivity, and (iii) YY1, also based on band-shift competition assays and Zn^{2+}/EDTA insensitivity. We identified two polypeptide bands by DNA-protein cross-linking assays as putative MG1E-binding proteins with approximate molecular masses of 41 and 32 kDa. The 32-kDa band was only detected in nuclear extracts from myotubes, whereas the two bands were detected in nuclear extracts from myoblasts. We propose that in the proliferative state, the 41-kDa is the main MG1E-binding protein whereas after confluency and/or early differentiation, the 32-kDa is the major MG1E-binding protein.

MG1E is located in the proximal promoter relatively near a GC box (–91/–86) that binds Sp1 and Sp3 factors and controls GLUT1 gene transcription in cells [7, 8, 10]. MG1E must play a pivotal role maintaining the activity of the GLUT1 promoter in conditions in which the GC box is inactive, as in terminally differentiated muscle cells. In addition, MG1E also permits maximal stimulation of transcriptional activity in response to a variety of regulators such as cAMP, Sp1 or serum. Thus, the stimulation of GLUT1 transcriptional activity in response to the cAMP analogue 8-bromo-cAMP in L6E9 myoblasts is markedly reduced after inactivation of the MG1E. We have also identified a functional cooperation between Sp1 and MG1E in the GLUT1 promoter. Sp1 binds GC box of GLUT1 gene and activates the GLUT1 promoter in muscle cells [7, 8] and inactivation of MG1E markedly reduces the maximal transcriptional activity induced by over-expression of Sp1 in L6E9 myoblasts. This suggests functional cooperativity between the GC box at –91/–86 and the MG1E box at –46/–37.

Finally, we found that both basal and maximal activity of the GLUT1 promoter due to serum is markedly reduced after inactivation of MG1E in neonatal rat cardiomyocytes. Furthermore, we have seen that re-addition of serum to the culture medium was associated with enhanced binding of Sp1 and Sp3 to the GC box of the proximal promoter and normal MG1E-binding activity. We suggest that serum stimulates GLUT1 promoter activity by a mechanism that entails activation of the GC box by Sp1 and Sp3, and functional cooperation between the GC box and the MG1E box.

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