

Cyclic Adenosine 3',5'-Monophosphate Regulates GLUT4 and GLUT1 Glucose Transporter Expression and Stimulates Transcriptional Activity of the GLUT1 Promoter in Muscle Cells*

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ABSTRACT

We have previously reported that innervation-dependent basal contractile activity regulates in an inverse manner the expression of GLUT1 and GLUT4 glucose transporters in skeletal muscle. Based on the facts that muscle innervation decreases and muscle denervation increases cAMP levels, we investigated whether cAMP might mediate the effects of innervation/denervation on glucose transporter expression. Treatment of L6E9 myotubes with 8-bromo-cAMP, forskolin, or monobutyl-8-bromo-cAMP led to a marked decrease in GLUT4 protein levels; 8-bromo-cAMP also diminished GLUT4 messenger RNA (mRNA), suggesting pretranslational repression. In contrast, L6E9 myoblasts and myotubes responded to 8-bromo-cAMP or forskolin by increasing the cell content of GLUT1 protein. Induction of GLUT1 protein was a consequence of the activation of different mechanisms in myoblast and myotube cells; whereas 8-bromo-cAMP treatment

caused a substantial increase in GLUT1 mRNA in myoblasts, no change in GLUT1 mRNA was detected in myotubes. The increase in GLUT1 mRNA in L6E9 myoblasts induced by 8-bromo-cAMP was the result of transcriptional activation, as concluded from transfection analysis of 2.1 kilobases of the rat GLUT1 gene promoter fused to the bacterial chloramphenicol acetyltransferase gene. Furthermore, the stimulatory effect of 8-bromo-cAMP on the transcriptional activity of the GLUT1 promoter required a 33-bp sequence lying 5' upstream of the transcription start site. In all, cAMP inversely regulates GLUT4 and GLUT1 glucose transporter expression in muscle cells. Furthermore, our results suggest that down-regulation of GLUT4 expression and up-regulation of GLUT1 expression in muscle associated with denervation are partly attributable to cAMP. (*Endocrinology* 138: 2521–2529, 1997)

SKELETAL MUSCLE is the main tissue responsible for insulin-induced glucose utilization in humans and rodents (1, 2), and glucose transport in this tissue is rate limiting for glucose disposal under most physiological conditions (3). Skeletal muscle expresses both GLUT4 and GLUT1 glucose transporters (4, 5) in a highly regulatable manner. Streptozotocin-induced diabetes leads to a reduction of GLUT4 expression (6–8), which seems to affect red and white muscle in different ways (9). Experimental models of type II diabetes, such as the Zucker diabetic rat or the SHR/N-cp diabetic rat, also display a reduction of GLUT4 expression in skeletal muscle (10–12).

The expression of GLUT1 and GLUT4 genes in skeletal muscle and heart is also developmentally regulated (13–15).

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Thus, during fetal and early postnatal life, GLUT1 is the predominant glucose transporter expressed in muscle (14). Postnatal life is characterized by GLUT1 repression in muscle concomitant with an increase in GLUT4 expression (14). The expression of glucose transporter isoforms during perinatal life fits well with the observations of a high rate of glucose uptake by fetal and early neonatal rat heart and diaphragm (16, 17) and a moderate stimulatory effect of insulin on glucose transport by early neonatal diaphragm (17). Regarding the nature of the factors that trigger GLUT4 induction and GLUT1 repression during perinatal development, it has been reported that perinatal hypothyroidism greatly impairs the normal transition of GLUT4 and GLUT1 glucose transporters from fetal to neonatal levels in heart (18), suggesting that thyroid hormones play a critical role in the regulation of glucose carriers during perinatal life in muscle tissues.

Furthermore, during embryonic life, there is a similar timing for nerve-muscle connections and GLUT4 induction and GLUT1 repression in rat skeletal muscle (15, 19, 20). These findings suggest that the regulation of glucose carriers during perinatal life might be related to skeletal muscle innervation (15). Muscle denervation is characterized by an impairment of insulin action (21, 22) and a parallel modification of glucose transporter expression. Thus, GLUT4 markedly decreases and GLUT1 is enhanced in muscles after resection of sciatic or peroneal nerves in rats and rabbits (15, 23–25),

which seems to be a consequence of altered muscular contractile activity (15). The precise intracellular signals that are triggered by muscle denervation and cause repression of GLUT4 expression and activation of GLUT1 expression are unknown. However, there is information indicating that muscle denervation causes accumulation of intracellular levels of cAMP in muscle and stimulates protein kinase A activity (26–28). To determine whether cAMP has a regulatory role in the expression of glucose carriers in muscle, we have investigated the effects of cAMP analogs on GLUT1 and GLUT4 expression in L6E9 muscle cells. These muscle cells were selected for this study because they express glucose carriers in a differentiation-dependent manner: myoblasts mainly express GLUT1, and muscle cell differentiation into myotubes induces GLUT4 expression (29). Our results indicate that cAMP inversely regulates GLUT1 and GLUT4 expression in muscle cells and causes a transcriptional activation of the rat GLUT1 promoter.

Materials and Methods

Materials

[¹²⁵I]Protein A and [³²P]deoxy (d)-CTP were purchased from ICN (Irvine, CA). Hybond-N was obtained from Amersham (Arlington Heights, IL), and the random primed DNA labeling kit was purchased from Boehringer Mannheim (Indianapolis, IN). Immobilon was obtained from Millipore Corp. (Bedford, MA). All electrophoresis reagents and mol wt markers were obtained from Bio-Rad (Richmond, CA) and BRL (Gaithersburg, MD). γ -Globulin, 8-bromo-cAMP, forskolin, 2'-O-monobutyl-8-bromo-cAMP, T₃, and most commonly used chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). The L6E9 rat skeletal muscle cell line was provided by Dr. B. Nadal-Ginard (Harvard University, Boston, MA). DMEM, FBS, glutamine, and antibiotics were obtained from BioWhittaker (Walkersville, MD).

The plasmid containing the –2212/+164 region of the rat GLUT4 genomic sequence was obtained from Dr. J. Pessin (University of Iowa, Ames, IA). The plasmid containing the –2106/+134 region of the rat GLUT1 genomic sequence was obtained from Dr. M. Birnbaum (University of Pennsylvania, Philadelphia, PA). pCAT-Basic and pSV- β -galactosidase (pSV- β -gal) vectors were obtained from Promega (Madison, WI). pMT2TR α_1 vector containing the thyroid hormone receptor α_1 was obtained from Dr. V. Mahdavi (Harvard University) (30).

Cell culture

Rat skeletal muscle L6E9 myoblasts were grown in monolayer culture in DMEM supplemented with 10% (vol/vol) FBS, 1% (vol/vol) antibiotics (10,000 U/ml penicillin G, and 10 mg/ml streptomycin), 2 mM glutamine, and 25 mM HEPES (pH 7.4). Confluent myoblasts were differentiated by lowering FBS to a final concentration of 2% (vol/vol).

Preparation of membrane fractions

Cells were washed twice in PBS, scraped, and homogenized in 2 ml ice-cold buffer containing 25 mM HEPES, 250 mM sucrose, 4 mM EDTA, 1 trypsin inhibitor units/ml aprotinin, 25 mM benzamidine, 0.2 mM phenylmethylsulfonylfluoride, 1 μ M leupeptin, and 1 μ M pepstatin, pH 7.4, using a Dounce homogenizer (Kontes Co., Vineland, NJ). After homogenization, the cell suspension was centrifuged for 10 min at 750 \times g at 4 C. The pellet of the 750 \times g centrifugation showed low contamination with cell surface or intracellular membranes based on the scarce abundance of glucose transporters. The supernatants were then centrifuged at 200,000 \times g for 90 min at 4 C to obtain the membrane fractions. The membrane pellets were resuspended in homogenization buffer and repeatedly passed through a 25-gauge needle before storage at –20 C. Proteins were measured by the method of Bradford (31) using γ -globulin as a standard.

Electrophoresis and immunoblotting of membranes

SDS-PAGE was performed on membrane protein in accordance with the method of Laemmli (32). Proteins were transferred to Immobilon (Millipore, Bedford, MA) as previously reported (8) in buffer consisting of 20% methanol, 200 mM glycine, and 25 mM Tris, pH 8.3. After transfer, the filters were blocked with 5% nonfat dry milk and 0.02% sodium azide in PBS for 1 h at 37 C and incubated with antibodies. Transfer was confirmed by Coomassie blue staining of the gel after the electroblot. Polyclonal antibody OSCRX (raised against the 15C-terminal peptide from GLUT4) (33) was used at a 1:400 dilution in 1% nonfat dry milk and 0.02% sodium azide in PBS overnight at room temperature to immunoblot GLUT4. Rabbit Bb antiserum raised against the purified human erythrocyte glucose transporter (a gift from Dr. C. Carter-Su, University of Michigan) was used at a 1:400 dilution and was incubated with transferred protein overnight at room temperature in 1% nonfat dry milk and 0.02% sodium azide in PBS to detect GLUT1. Detection of the immune complex with the rabbit polyclonal antibodies was accomplished using [¹²⁵I]protein A for 4 h at room temperature. The autoradiograms were quantified using scanning densitometry. Immunoblots were performed under conditions in which autoradiographic detection was in the linear response range.

RNA isolation and Northern blot analysis

Total RNA was extracted using the acid guanidinium thiocyanate-phenol-chloroform method as described by Chomczynski and Sacchi (34). All samples had a 260/280 absorbance ratio above 1.8.

After quantification, total RNA (30 μ g) was denatured at 65 C in the presence of formamide, formaldehyde, and ethidium bromide (35) to allow the visualization of RNA. RNA was separated on a 1.2% agarose/formaldehyde gel and blotted on Hybond-N filters. The RNA in gels and filters was visualized with ethidium bromide by UV transillumination to ensure the integrity of RNA, to check the loading of equivalent amounts of total RNA, and to confirm proper transfer. RNA was transferred in 10 \times SSC (1 \times SSC is 0.15 NaCl and 0.015 M sodium citrate, pH 7.0).

Blots were initially prehybridized for 4 h at 45 C in 50% formamide, 5 \times Denhardt's solution (1 \times Denhardt's solution is 0.02% polyvinylpyrrolidone, 0.02% Ficoll, and 0.02% BSA), 0.1% SDS, 5 \times SSPE (1 \times SSPE is 0.15 M NaCl, 1 mM EDTA, and 10 mM NaH₂PO₄, pH 7.4), and 0.2 mg/ml denatured salmon sperm DNA. The blots were then hybridized to the complementary DNA (cDNA) probes for 12 h at 42 C in 50% formamide, 5 \times Denhardt's solution, 0.1% SDS, 5 \times SSPE, 10% dextran sulfate, and 0.2 mg/ml denatured salmon sperm DNA. The rat cDNA probe for GLUT1 was a 2521-bp fragment, and the rat cDNA probe for GLUT4 was a 2470-bp EcoRI fragment (both cDNA probes were obtained from Dr. M. Birnbaum, University of Pennsylvania). The cDNA probes were labeled with [³²P]dCTP by random oligonucleotide priming. The probes were included at 2 \times 10⁶ cpm/ml. Filters were washed for 15 min in 2 \times SSC at room temperature, then for 20 min in 0.4 \times SSC–0.1% SDS at 55 C, and finally for 30 min in 0.1 \times SSC–0.1% SDS at 55 C. The abundance of specific glucose transporter message was quantified by scanning densitometry of autoradiograms, as described above.

GLUT4 and GLUT1 chloramphenicol acetyltransferase (CAT) reporter constructs

Plasmid –2106/G1CAT was constructed by inserting a 2240-bp EcoRI-XhoI fragment containing the rat GLUT1 promoter region from positions –2106 to +134 (relative to the transcription start site) into the XbaI site of pCAT-basic vector (Promega). pCAT-basic was digested with XbaI, and the ends were filled with dNTPs in the presence of the Klenow fragment and treated with alkaline phosphatase. The GLUT1 promoter DNA fragment was filled in the presence of dNTPs and Klenow fragment and ligated to the pCAT-basic vector. 5'-Deletions were generated by cleavage with SmaI at –201 (–201/G1CAT) or with Acl at –99 (–99/G1CAT), and adding Klenow and T4 DNA ligase. The –33/G1CAT construct was generated by obtaining a 28-bp HindIII-BssHII DNA containing the fragment –38/–15 from a 106-bp AvaII fragment (position –38/+68) of the GLUT-1 promoter subcloned in Bluescript. The 28-bp fragment was subcloned into the BssHII site of the BssHII-G1CAT construct (–15/+134).

Plasmid -2212/G4CAT was generated by subcloning a 2400-bp *EcoRI-BamHI* fragment containing the rat GLUT4 promoter region from positions -2212 to +152 into the *XbaI* site of pCAT-basic vector.

Transient transfection

L6E9 cells ($n = 250,000$) were grown in 10-mm diameter plates for 2 days in DMEM with 10% FBS. Monolayers were washed in PBS, and DNA transfection was performed by using the CaPO_4 coprecipitation procedure (36). One milliliter of calcium phosphate DNA precipitate containing 10 μg of various deletion promoter-CAT constructs, 5 μg pSV- β -gal control vector (Promega), and 20 μg Bluescript DNA (pSK⁺, Stratagene, La Jolla, CA) was added dropwise to the plate. After 16 h, the cells were washed and incubated with 1 ml 15% glycerol in HEPES-buffered saline for 3 min, washed in DMEM, and incubated in fresh complete medium for 72 h. For the myotubes, the medium was changed to differentiation medium (DMEM supplemented with 2% FBS) after incubation with the DNA precipitate. 8-Bromo-cAMP (1 mM) was added 48 h before cells were harvested.

Preparation of cell extracts and measurement of CAT activity

The cells were washed twice in PBS and harvested by scraping in 1 ml STE (10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl and 1 mM EDTA). The cells were collected by centrifugation in a microcentrifuge for 10 min, and the pellet was resuspended in 200 μl 0.25 M Tris, pH 7.5. The cells were lysed by three cycles of freezing and thawing at 37°C. After centrifugation in a microcentrifuge for 5 min at 4°C, the supernatant was stored at -20°C.

The CAT activity of 75 μl cytoplasmic extract was measured by incubating 0.1 μCi [¹⁴C]chloramphenicol, 1.3 mM acetyl coenzyme A, 200 mM Tris-HCl (pH 7.5), and the soluble extract for 3.5 h at 37°C. At the end of the incubation, extraction into ethyl acetate and TLC (37) were performed. CAT activity was quantitated using an InstantImager (Packard, Downers Grove, IL). β -Gal activity was measured as previously described (38). The CAT activity was expressed as a function of β -gal activity in the same volume of cell lysate.

Results

cAMP analogs repress GLUT4 expression in muscle cells

To test the hypothesis that raising the intracellular cAMP level causes down-regulation of GLUT4 and up-regulation of GLUT1, myoblast and myotube L6E9 cells were treated with cAMP analogs or forskolin. In parallel and as a control, studies were also performed in the presence of insulin, which has been reported to regulate GLUT4 and GLUT1 expression in L6 muscle cells in culture (39).

L6 and L6E9 muscle cells in culture express GLUT4 after muscle cell differentiation, and this parallels a greater stimulatory effect of insulin (29, 40). In consequence, L6E9 myotubes were treated with 1 mM 8-bromo-cAMP. Twenty-four and 48 h after treatment, cells were processed to obtain total membrane proteins, and GLUT4 protein levels were determined. Western blot assays indicated that 8-bromo-cAMP treatment for 24 or 48 h caused a substantial progressive decrease in GLUT4 proteins levels (near 25% and 57% decreases after 24 or 48 h of treatment, respectively; Fig. 1A). Similar results were obtained after treatment for 24 h with 50 μM forskolin (27% decrease) or treatment for 48 h in the presence of monobutyl-8-bromo-cAMP (62% decrease) (data not shown). Treatment with cAMP analogs or forskolin did not alter the yield of total membrane protein, and in fact, similar effects were observed when the abundance of GLUT4 was expressed per total membrane fraction (data not shown).

Northern blot assays also revealed a 39% decrease in

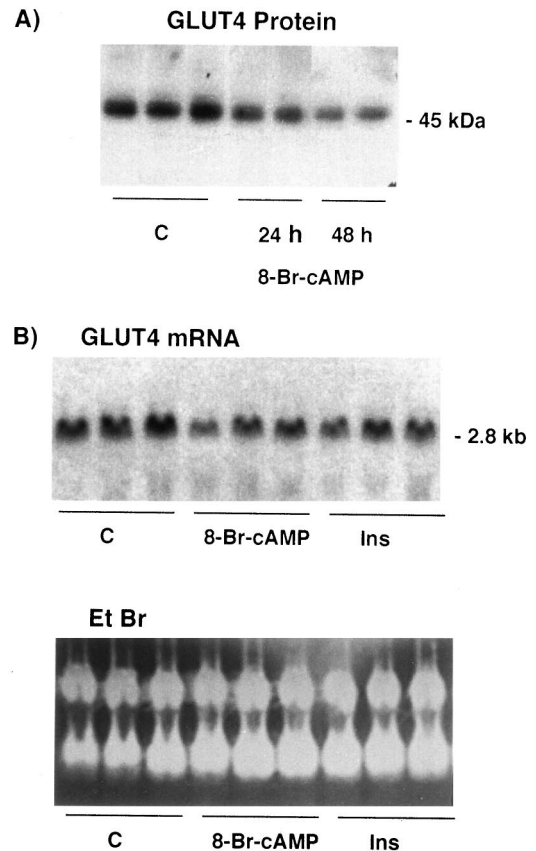


FIG. 1. Effect of 8-bromo-cAMP or insulin on the expression of GLUT4 in L6E9 muscle cells. L6E9 myotubes either were not treated (C) or were treated with 1 mM 8-bromo-cAMP for the indicated times (for 48 h if not indicated) or with 1 μM insulin for 24 h. After this time, membrane proteins or total RNA were obtained. A, Twenty-five micrograms of membrane proteins from the different experimental groups were applied to the gels. After blotting, GLUT4 protein was detected by incubation with polyclonal antibody OSCRX. B, Thirty micrograms of total RNA from the different experimental groups were applied to the gels. The integrity and the relative amounts of RNA in each sample were checked by ethidium bromide staining on the same gel (Et Br). After blotting, GLUT4 mRNA was detected after hybridization with a 2470-bp *EcoRI* fragment as a cDNA probe, as described in *Materials and Methods*. Autoradiographs were subjected to scanning densitometry. Representative autoradiographs from three independent experiments are shown. The effects of 8-bromo-cAMP or insulin were statistically significant ($P < 0.05$).

GLUT4 messenger RNA (mRNA) levels after 24 h of treatment in the presence of 8-bromo-cAMP (Fig. 1B). Under these conditions, insulin treatment for 24 h also decreased GLUT4 mRNA levels by 37% (Fig. 1B), which is in agreement with previous observations obtained in L6 muscle cells (39). This pattern of changes in response to 8-bromo-cAMP treatment occurred in the absence of alterations in the yield of total RNA (data not shown). In all, these results indicate that 8-bromo-cAMP causes a pretranslational repression of GLUT4 expression in L6E9 myotubes. We next attempted to determine whether 8-bromo-cAMP treatment modified the half-life of GLUT4 mRNA in muscle cells. To this end, cells were treated with actinomycin D (10 $\mu\text{g}/\text{ml}$) for up to 8 h, and GLUT4 mRNA was determined in control cells or in cells previously treated with 8-bromo-cAMP. Under our experi-

mental conditions, GLUT4 mRNA levels were stable, and even after 8 h in the presence of actinomycin D no decrease in GLUT4 mRNA levels was detectable in control and 8-bromo-cAMP-treated groups (data not shown). Longer treatment of cells with actinomycin D resulted in decreased viability, so measurements of GLUT4 mRNA levels were not performed. These results, although not providing the precise half-life of GLUT4 transcripts, suggest that 8-bromo-cAMP does not substantially modify the stability of GLUT4 mRNA in muscle cells.

To determine whether the effect of 8-bromo-cAMP involved repression of GLUT4 gene transcription, the 5'-regulatory region of the rat GLUT4 gene, containing 2212 bp of upstream GLUT4 sequences relative to the transcription start site and fused to the CAT reporter gene (–2212/G4CAT), was transiently transfected to L6E9 cells and allowed to differentiate into multinucleated myotubes. This fragment of the rat GLUT4 promoter contains the elements necessary for both myotube-specific GLUT4 expression and thyroid hormone responsiveness (41). Transfection of –2212/G4CAT led to a 5-fold increase in CAT activity compared with that in cells transfected with the promoterless pCAT-basic vector. In keeping with previous findings (41), the transcriptional activity of the GLUT4 gene was highly sensitive to thyroid hormone. Thus, transfection of –2212/G4CAT in the absence of thyroid hormones caused a 64% decrease in CAT activity (Fig. 2). Furthermore, cotransfection of –2212/G4CAT and thyroid hormone receptor α_1 led to a 3.8-fold increase in CAT activities in the presence of T_3 for 48 h (Fig. 2). In parallel assays, L6E9 cells at different cell densities were transfected with –2212/G4CAT and treated or not with 1 mM 8-bromo-cAMP for different periods. No change in CAT activity due to 8-bromo-cAMP treatment was detected under any of these conditions (Fig. 2 and data not shown). These results suggest that if 8-bromo-cAMP induces GLUT4 gene repression, this requires the participation of *cis* elements located outside the 2212-bp fragment of the rat GLUT4 promoter used in this study.

8-Bromo-cAMP enhances GLUT1 expression in muscle cells

We also assessed the effect of 8-bromo-cAMP, forskolin, or insulin on the expression of GLUT1 in L6E9 muscle cells. As both L6E9 myoblasts and myotubes express GLUT1, we studied the effects of the above-mentioned agents on GLUT1 expression in L6E9 cells before and after myogenic differentiation. We should note that the results obtained in myotubes correspond to the same samples as those shown in Fig. 1.

In L6E9 myoblasts, treatment with 8-bromo-cAMP (1 mM), forskolin (50 μ M), or insulin (1 μ M) led to a marked increase in GLUT1 protein content (from 2- to 3.6-fold increase; Fig. 3A and data not shown). Similarly, treatment of L6E9 myotubes in the presence of 8-bromo-cAMP for 24 or 48 h or in the presence of insulin for 24 h caused a substantial increase in GLUT1 protein levels (1.6- and 1.7-fold increase after 24 or 48 h of 8-bromo-cAMP treatment, respectively, and 3.1-fold increase after insulin treatment; Fig. 3B). The increase in GLUT1 protein content in response to 8-bromo-cAMP or forskolin treatment occurred in the presence of a similar yield

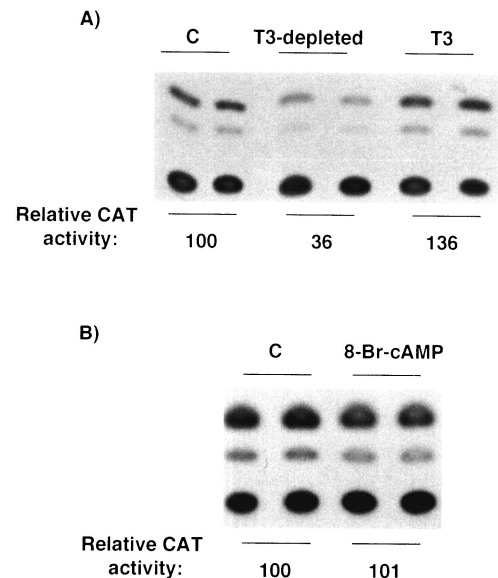


FIG. 2. Effect of 8-bromo-cAMP or thyroid hormones on CAT activity driven by GLUT4 promoter in transfected L6E9 myotubes. A, L6E9 myotubes were transiently cotransfected by calcium phosphate precipitation with the –2212/G4CAT construct (10 μ g), pMT2TR α_1 encoding the rat thyroid hormone receptor α_1 (10 μ g) and with pSV- β -gal (10 μ g) as indicated. Cells were allowed to differentiate in T_3 -depleted medium in the absence or presence of 50 nM T_3 . In parallel, cells maintained in normal medium were transfected with the –2212/G4CAT construct (10 μ g) or with pSV- β -gal (10 μ g; C). B, L6E9 myotubes were transiently cotransfected by calcium phosphate precipitation with the –2212/G4CAT construct (10 μ g) and with pSV- β -gal (5 μ g) as indicated. Sixteen hours after transfection, cells were transferred to differentiation medium for 48 h in the absence (C) or presence of 1 mM 8-bromo-cAMP and harvested as myotubes. Autoradiographs were subjected to scanning densitometry, and representative autoradiograms are shown. Data are also presented as the relative CAT activity/ β -gal activity (mean) in four to six experiments performed in duplicate using at least two preparations of each DNA construct.

of membrane protein, and in consequence, similar effects were observed when the abundance of GLUT1 was expressed per total membrane fraction (data not shown).

To determine the mechanism for the enhanced GLUT1 expression, we obtained total RNA from L6E9 cells treated or not with 8-bromo-cAMP or insulin, and Northern blot assays were performed with a probe specific for GLUT1. The results shown in Fig. 4 reveal an enhanced GLUT1 mRNA expression after treatment with 8-bromo-cAMP (2.2-fold increase) or insulin (4-fold increase) in L6E9 myoblasts (Fig. 4A). Insulin treatment for 24 h led to a 40% increase in GLUT1 mRNA levels in L6E9 myotubes (Fig. 4B). In contrast, no stimulatory effect of 8-bromo-cAMP on GLUT1 mRNA levels was detected in L6E9 myotubes (Fig. 4B).

These results suggest a different pattern of effects of 8-bromo-cAMP in myoblasts and myotubes; whereas 8-bromo-cAMP induces GLUT1 expression in myoblasts at a pretranslational level, the 8-bromo-cAMP-induced GLUT1 expression in myotubes is a consequence of activation at translational or posttranslational steps. To assess whether the stimulatory effect of 8-bromo-cAMP on GLUT1 mRNA levels in myoblasts results from changes in the rates of gene transcription, the 5'-regulatory region of the rat GLUT1 gene

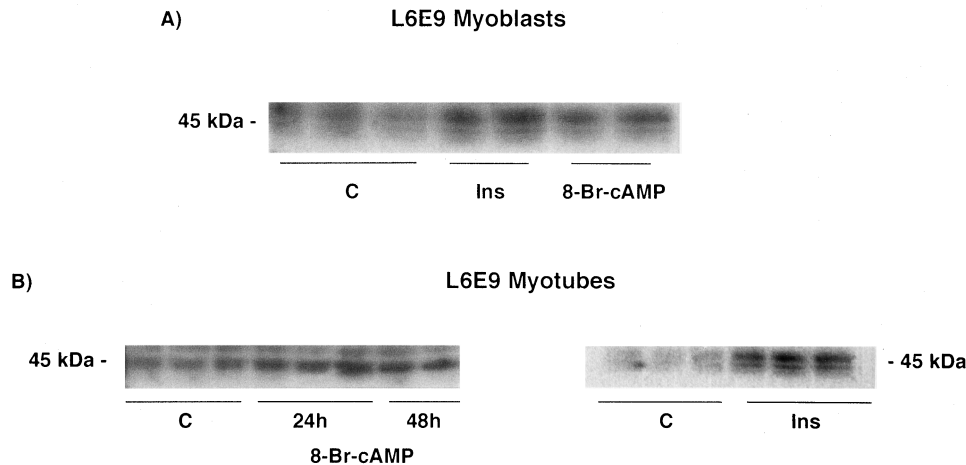


FIG. 3. Effect of 8-bromo-cAMP or insulin on the expression of GLUT1 protein in L6E9 muscle cells. L6E9 myoblasts (A) or myotubes (B) either were treated with 1 mM 8-bromo-cAMP for the indicated times (for 48 h if not indicated) or with 1 μ M insulin for 24 h, after which total membrane proteins were obtained. Twenty-five micrograms of membrane proteins from the different experimental groups were applied to the gels. After blotting, GLUT1 protein was detected by incubation with a polyclonal antibody. Autoradiographs were subjected to scanning densitometry. Representative autoradiographs from three independent experiments are shown. The arrows indicate the bands that correspond to GLUT1 protein. Values for the 8-bromo-cAMP and insulin groups were statistically significant ($P < 0.05$).

containing 2106 bp upstream GLUT1 sequence relative to the transcription start site and fused to the CAT reporter gene (–2106/G1CAT) was transiently transfected to L6E9 myoblasts. This transfection led to an 11.5-fold increase in CAT activity in soluble extracts compared with that in cells transfected with the promoterless pCAT-basic vector (Fig. 5). These cells were treated or not with 8-bromo-cAMP for 48 h or with insulin for 24 h, and thereafter CAT activity was determined. The results shown in Fig. 5 also indicate that CAT activity was markedly enhanced (1.9-fold increase) in myoblast L6E9 cells treated with 8-bromo-cAMP. Under these conditions, insulin did not affect the transcriptional activity of the GLUT1 gene (data not shown). These results indicate a regulatory element within the 2.1 kilobases (kb) of the GLUT1 promoter that confer sensitivity to 8-bromo-cAMP by stimulating transcriptional activity in L6E9 myoblasts. The extent of stimulation caused by 8-bromo-cAMP on the transcriptional activity of the rat GLUT1 gene promoter was very similar to the enhancement of GLUT1 mRNA levels.

Identification of a promoter region of GLUT1 gene required for up-regulation of transcription in response to 8-bromo-cAMP

The regulatory element(s) within the GLUT1 gene responsible for cAMP-induced transcriptional activation in L6E9 myoblasts was dissected out by 5'-deletions of the rat GLUT1 promoter and transfected into L6E9 myoblasts. Deletion from –2106 to –812 of the GLUT1 gene caused no significant alterations in CAT activity (data not shown), and deletion from –812 to –201 caused nearly a 3.8-fold stimulation of CAT activity (data not shown). The transcriptional activity of the –201 construct was maximal, and only a slight decrease was noted after deletion from –201 to –99 (Fig. 6). However, deletion of an additional 66 bp (from –99 to –33) led to a marked reduction (80%) in the transcriptional activity (Fig. 6).

To assess the effect of truncating the GLUT1 promoter on cAMP-induced transcriptional induction, myoblasts transiently transfected with a series of GLUT1 promoter-CAT 5'-deletion mutants were treated or not with 8-bromo-cAMP for 48 h and further analyzed. The results shown in Fig. 6 demonstrate that CAT activity was markedly activated by cAMP in cells transfected with all of the constructs studied. The extent of the activation ranged from a 1.8- to 2.3-fold increase (Fig. 6). The maximal stimulatory effect of 8-bromo-cAMP was found in the –201CAT and –99CAT constructs (Fig. 6); however, the effect of 8-bromo-cAMP was still clearly detectable in –33CAT, a construct that contains the TATA box of the GLUT1 promoter and the transcription start site. Deletions of up to 151 bp in the 3'-end of the fragment –2106/+134 of the –2106/G1CAT construct did not eliminate the stimulatory effect of 8-bromo-cAMP (data not shown), suggesting that the cAMP response elements do not lie 3' of the transcriptional start site.

In all, these results indicate a fragment located between –99 and –33 bp of the initiation transcription site that confers transcriptional activity of the rat GLUT1 gene in myoblast cells. Furthermore, our data indicate that 8-bromo-cAMP stimulates the transcriptional activity of the rat GLUT1 promoter, which requires the 33 bp lying 5' upstream of the transcription start site.

Discussion

The results of the present study indicate that cAMP has a marked influence on the expression of glucose carriers in muscle cells. Thus, treatment with cAMP analogs or the activator of adenylate cyclase, forskolin, reduces GLUT4 expression and increases GLUT1 expression in L6E9 muscle cells. In myoblasts, cAMP stimulates the transcriptional activity of the rat GLUT1 promoter and a 33-bp sequence lying 5' upstream of the transcription start site is required for the effects of 8-bromo-cAMP. In general, the effects of cAMP on glucose transporter expression are similar to the effects of

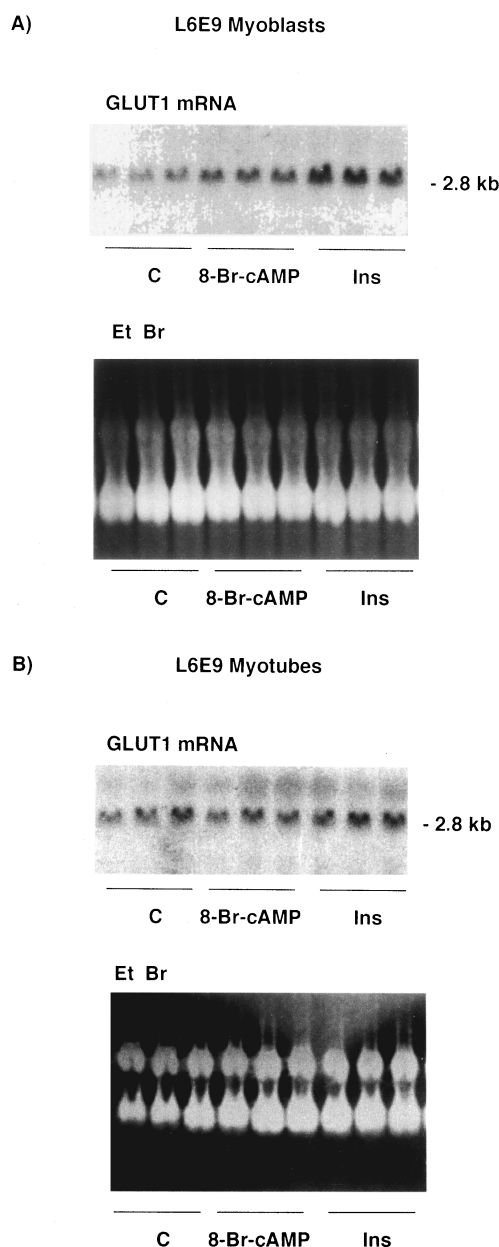


FIG. 4. Effect of 8-bromo-cAMP or insulin on the expression of GLUT1 mRNA in L6E9 muscle cells. L6E9 myoblasts (A) or myotubes (B) were not treated (C) or were treated with 1 mM 8-bromo-cAMP for 48 h or with 1 μ M insulin for 24 h, after which total RNA was isolated. Thirty micrograms of total RNA from the different experimental groups were applied to the gels. The integrity and the relative amounts of RNA in each sample were checked by ethidium bromide staining on the same gel (Et Br). After blotting, GLUT1 mRNA was detected after hybridization with a 2521-bp *Eco*RI fragment as a cDNA probe, as described in *Materials and Methods*. Autoradiographs were subjected to scanning densitometry. Representative autoradiographs from three independent experiments are shown. Treatment of 8-bromo-cAMP in myoblasts and treatment of insulin in myoblasts and myotubes caused statistically significant effects on GLUT1 mRNA levels ($P < 0.05$).

prolonged administration of insulin in muscle cells (Refs. 39, 42, and 43 and this study). Nevertheless, the mechanisms that activate GLUT1 in response to cAMP and insulin are differ-

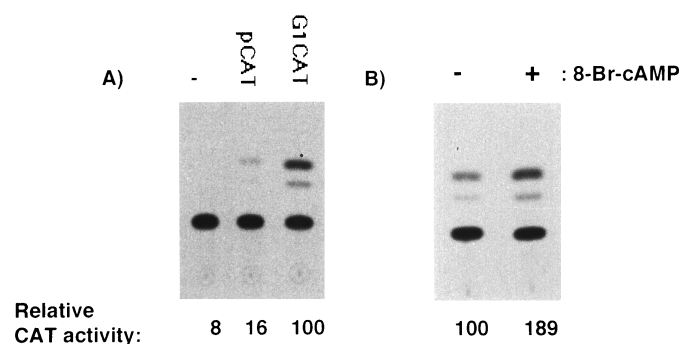


FIG. 5. Effect of 8-bromo-cAMP or insulin on CAT activity driven by GLUT1 promoter in transfected L6E9 myoblasts. A, L6E9 myoblasts were untransfected (-) or transiently cotransfected by calcium phosphate precipitation with the -2,106/G1CAT promoter construct (10 μ g) or with the promoterless pCAT-basic vector (10 μ g) and with pSV- β -gal (5 μ g) as indicated. B, L6E9 myoblasts were transfected with the -2,106/G1CAT construct, and then incubated in growth medium for 48 h in the absence (-) or presence of 1 mM 8-bromo-cAMP (+) and harvested as myoblasts. Autoradiographs were subjected to scanning densitometry. Representative autoradiographs are shown. The data are also expressed as the relative CAT activity/ β -gal activity (mean) in four to six experiments. These results were obtained from transfection experiments, performed in duplicate, using at least two preparations of DNA.

ent, as cAMP, but not insulin, increases the transcriptional activity of 2.1 kb of the rat GLUT1 promoter.

Here we found that GLUT4 expression is markedly inhibited by 8-bromo-cAMP, monobutyl-8-bromo-cAMP, or forskolin. Regarding the mechanisms involved, we detected reduced levels of GLUT4 mRNA that were already apparent after 24 h of 8-bromo-cAMP treatment, indicating changes at a pretranslational step. Furthermore, no modification of GLUT4 mRNA levels was detected even after 8 h of treatment with actinomycin D in control or 8-bromo-cAMP-treated myotubes, which indicates that the stability of the GLUT4 transcripts is high in myotubes under both conditions and, therefore, favors an action of 8-bromo-cAMP at the level of gene transcription. We also analyzed the effect of 8-bromo-cAMP on the transcriptional activity of a 2.2-kb fragment of the rat GLUT4 gene, which contains the elements necessary for both myotube-specific GLUT4 expression and thyroid hormone responsiveness in muscle cells (41, 44) and response to streptozotocin-induced diabetes in transgenic mice (45). Our results did not reveal any inhibitory effect of 8-bromo-cAMP under conditions in which we substantiated sensitivity to thyroid hormones. If indeed cAMP induces GLUT4 gene repression, the *cis*-acting elements involved in the response to cAMP must lie in a fragment other than the 2.1-kb 5' flanking the rat GLUT4 gene. A cAMP-responsive fragment of the mouse GLUT4 gene located between positions -469 and -78 has been identified after transfection into 3T3-L1 adipocytes (46). This suggests a tissue-specific mechanism involved in the regulation of GLUT4 in response to cAMP.

In this study, we have shown that insulin enhances GLUT1 expression and represses GLUT4 in L6E9 muscle cells. These effects are in keeping with previous findings reported in L6 muscle cells (39) and 3T3-L1 adipocytes (47). These results are in contrast to *in vivo* studies in which the acute infusion

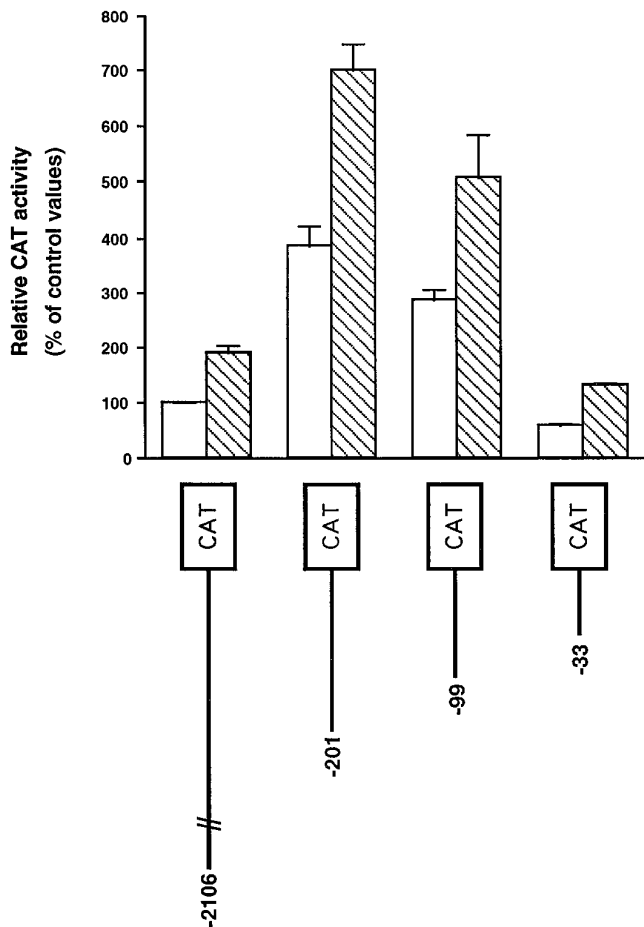


FIG. 6. Effect of 8-bromo-cAMP on the expression of GLUT1 5'-deletion constructs in L6E9 myoblasts. A series of 5'-deletions of the upstream region of the GLUT1 gene from -2106 to -33 bp relative to the transcription start site was generated as indicated. L6E9 myoblasts were transiently transfected with the various constructs. After transfection, cells were incubated in growth medium for 48 h in the absence (open bars) or presence of 1 mM 8-bromo-cAMP (hatched bars) and harvested as myoblasts. The data are expressed as the relative CAT activity/ β -gal activity \pm SEM from four to six experiments, with the $-2016/G1CAT$ construct being set at a value of 100. These results were obtained from transfection experiments performed in duplicate using at least two preparations of DNA. Differences between control and 8-bromo-cAMP groups were statistically significant ($P < 0.05$).

of insulin enhances GLUT4 mRNA levels in human muscle (48). It is likely that these contradictory data are explained by a different effect of insulin in cultured myotubes and muscle fibers or by possible differences due to acute or chronic exposure to insulin.

cAMP exerts a stimulatory effect on GLUT1 expression in L6E9 muscle cells as a consequence of the triggering of separate mechanisms in myoblasts and myotubes. Thus, cAMP stimulates GLUT1 protein and mRNA and the transcriptional activity of rat GLUT1 promoter in myoblasts; however, cAMP up-regulates GLUT1 protein in the absence of changes in mRNA levels, suggesting stimulation at a posttranscriptional level. Furthermore, we identified a promoter region of GLUT1 gene that contains *cis* elements responsible for cAMP responsiveness in myoblast cells. Studies of transient transfection with 5'-deletion constructs of the rat GLUT1 gene

indicate that cAMP stimulates the transcriptional activity of the rat GLUT1 promoter through 33 bp lying 5' upstream of the transcription start site. This fragment of the rat GLUT1 promoter contains the TATA box and shows very low transcriptional activity. This suggests that cAMP might stimulate GLUT1 gene transcription through enhancement of the basal transcriptional machinery. Similar to our data, it has been reported that a fragment ($-38/+138$) located in the proximal promoter of the carbamoyl-phosphate synthetase I gene, which contains only the TATA box motif, is capable of responding to cAMP by enhancing transcriptional activity (49). Furthermore, it was recently reported that myogenic regulatory factors seem to interact with the basal transcription machinery of the skeletal muscle myosin heavy chain gene (50). Whether the effect of cAMP on transcriptional activation of GLUT1 requires the TATA motif of the rat GLUT1 gene should be determined.

We have also identified a fragment located between -99 and -33 bp of the initiation start site that confers transcriptional activity in the rat GLUT1 promoter. Whether this fragment is also involved in the response to 8-bromo-cAMP remains to be determined. Although the specific DNA elements involved in the response to cAMP are unknown, inspection of the 99 bp lying 5' upstream of the transcription start site reveals the absence of cAMP-regulatory elements and the presence of some putative *cis* elements (51). Thus, there is an Sp1 element in the 5'-end of this fragment and two separate activating protein-2-like elements as well as a CAAT box element toward the 3'-end. Whether some of these elements, namely the activating protein-2-like elements that are found in a number of cAMP-responsive genes that lack cAMP regulatory elements (52), are involved in the effect of cAMP remains unknown. Gel retardation analysis are underway to identify the specific DNA sequences involved and the possible regulatory proteins responsible for activation of GLUT1 gene transcription. Based on studies performed with the mouse GLUT1 gene, the activities of two different enhancers of the GLUT1 gene have been reported (53). These two enhancers have not been characterized in the rat GLUT1 gene, and therefore, their participation in the response to cAMP remains to be determined. In this regard, it has been reported that the stimulatory effect of insulin on GLUT1 gene expression in NIH/3T3 fibroblasts indeed requires the presence of these two enhancers (54).

The effects of cAMP on glucose transporter expression are similar in adipocytes and muscle cells. Thus, it has been reported that exposure to 8-bromo-cAMP enhances GLUT1 and represses GLUT4 expression in 3T3-L1 adipocytes (55), in agreement with this work. The role of cAMP controlling glucose transporter expression in adipocytes explains the changes in glucose transporter expression in adipose tissue during experimental diabetes, in which enhanced cAMP levels have been detected (56) in parallel with a marked depletion of GLUT4 content (8, 57–60). The effect of cAMP on the regulation of glucose transporter expression may be relevant to skeletal muscle *in vivo*, where GLUT4 is the major glucose transporter isoform. Thus, cAMP levels rise after denervation in skeletal muscle (26–28) a situation in which GLUT1 expression increases and GLUT4 is repressed (15, 23–25); similarly, cAMP concentrations are high in skeletal muscle at

birth and drop during neonatal life (61), when there is a transition regarding the expression of glucose carriers in muscle, so GLUT1 is repressed and GLUT4 is induced (14, 15). Our results together with the above-mentioned observations favor the view that cAMP plays an important role in regulating GLUT1 and GLUT4 expression after denervation or during perinatal life in skeletal muscle. Furthermore, it has been reported that increased cAMP concentrations inhibit myogenic differentiation and the activity of the muscle-specific helix-loop-helix transcription factors in C2C12 and L6 cells (62). In this regard, it will be of interest to assess whether myogenic factors are involved in the cAMP-induced regulation of GLUT1 and GLUT4 expression.

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