Trans-activators Regulating Neuronal Glucose Transporter Isoform-3 Gene Expression in Mammalian Neurons*

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The murine facilitative glucose transporter isoform 3 is developmentally regulated and is predominantly expressed in neurons. By employing the primer extension assay, the transcription start site of the murine Glut 3 gene in the brain was localized to −305 bp 5′ to the ATG translation start codon. Transient transfection assays in N2A neuroblasts using murine GLUT3-luciferase reporter constructs mapped enhancer activities to two regions located at −203 to −177 and −104 to −29 bp flanking a previously described repressor element (−137 to −130 bp). Diphosphorylated Sp1 and Sp3 proteins from the 1- and 21-day-old mouse brain nuclear extracts bound the repressor elements, whereas both diphosphorylated and phosphorylated cAMP-response element-binding protein (CREB) in N2A, 1- and 21-day-old mouse brain nuclear extracts bound the 5′-enhancer cis-elements (−187 to −180 bp) of the Glut 3 gene, and the Y box protein MSY-1 bound the sense strand of the −83- to −69-bp region. Sp3, CREB, and MSY-1 binding to the GLUT 3 DNA was confirmed by the chromatin immunoprecipitation assay, whereas CREB and MSY-1 interaction was detected by the co-immunoprecipitation assay. Furthermore, small interference RNA targeted at CREB in N2A cells decreased endogenous CREB concentrations, and CREB-mediated GLUT 3 transcription. Thus, in the murine brain similar to the N2A cells, phosphorylated CREB and MSY-1 bound the Glut 3 gene trans-activating the expression in neurons, whereas Sp1/Sp3 bound the repressor elements. We speculate that phosphorylated CREB and Sp3 also interacted to bring about GLUT 3 expression in response to development/cell differentiation and neurotransmission.

Glucose, an essential substrate for brain oxidative metabolism, is transported across the blood-brain barrier and into neurons and glia by a family of structurally related membrane-spanning glycoproteins termed the facilitative glucose transporters (1, 2). Of the 14 major isoforms cloned to date (1–9), GLUT 1 and GLUT 3 are the isoforms predominantly expressed in the brain (10, 11). Whereas GLUT 1 is expressed by endothelial cells lining the microvasculature and glial cells, which are components of the blood-brain barrier (10), GLUT 3 is the predominant neuronal isoform (11). We and others have reported previously that although the spatial distribution of GLUT 3 in brain is not age-dependent (12), a temporal distribution exists with low amounts noted during the embryonic/fetal and early postnatal stages and peak amounts at day 14–21 (13), which coincides with the timing of synaptogenesis (14–16). In addition, GLUT 3 localization to the synaptic region and its vesicular trafficking, which involves SNAP-25 and syntaxin-1, proteins of the SNAP complex present in synaptic vesicles, supports a role for GLUT 3 in neurotransmission (17). Brain 2-deoxyglucose uptake serves as a surrogate marker for neuronal activity (18); thus GLUT 3, which mediates this glucose uptake, must play a major role in fueling neurotransmission (19). Depolarization of neurons in vitro by the presence of extracellular K+ ions or N-methyl-d-aspartate led to an increase in GLUT 3 concentrations, providing credence to this concept (20).

Both the processes of neuro-development and depolarization of cultured neurons cause a pre-translational increase in neuronal GLUT 3 expression (13, 20). Furthermore, conditions of substrate deficiency such as chronic hypoglycemia or hypoxic ischemia, which depolarize neurons, also pre-translatationally increase neuronal GLUT 3 concentrations (21, 22). By determining transcriotinal mechanism(s) underlying the pre-translational increase in neuronal GLUT 3 expression, we had previously characterized the murine Glut 3 promoter. We demonstrated that the murine Glut 3 promoter activity resides in the −203- to +237-bp region of the gene, with reference to the transcriptional start site (23). Additionally, we demonstrated nuclear factors Sp1 to repress and Sp3 to activate Glut 3 gene transcription in cultured murine neuroblasts (23).

In the present study, we extended these observations by determining whether Sp1 and Sp3 present in postnatal murine brain nuclear extracts could bind to the identified Glut 3 promoter cis-elements. We have also confirmed that by in vitro Sp3, and we established that in vitro and in vivo phosphorylated cyclic AMP-regulatory element-binding (pCREB)1 protein and the mouse Y box-binding protein-1 (MSY-1) bind the Glut 3 promoter region and activate GLUT 3 expression in neurons. We speculate that although Sp1/Sp3 along with MSY-1 may regulate the transcriptional activation of GLUT 3 during neuro-
development, trans-activation of GLUT 3 expression by pCREB may mediate the processes of neuronal synaptic activity and neuro-protection under conditions of substrate deficiency.

EXPERIMENTAL PROCEDURES

Oligonucleotides—Synthetic oligonucleotides (Invitrogen) were used in these experiments (nucleotides altered in mutant oligonucleotides are indicated by boldface type). Double-stranded oligonucleotides were generated by annealing the synthetic oligonucleotides with respective complementary sequences.

Animals—Balb/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in cages. The protocol for the care and use of animals was approved by the Animal Care and Use Committee of the Magee Women’s Research Institute in accordance with the guidelines set by the National Institutes of Health. Mice were allowed access to laboratory chow and water ad libitum and were maintained in 12-h light-dark cycles.

Cells—N2A mouse neuroblastoma cells (American Tissue Culture Collection, Manassas, VA) were grown at 37 °C with 95% air, 5% CO₂ in poly-1-lysine-coated culture flasks and maintained in Dulbecco’s modified Eagle’s medium supplemented with 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 units/ml), and 10% fetal bovine serum (23).

RNA Studies—Poly(A)+-enriched RNA was extracted as per the manufacturer’s instructions using the miristrue extraction kit (Collaborative Biomedical Products, Bedford, MA) from confluent cultured N2A cells (~1 × 10⁶ cells) or whole brains from the 1- and 21-day-old mice. The extracted RNA was subjected to Northern blot analysis as described previously (23). A 32P-labeled 1.5-kb XhoI-XbaI fragment of the murine GLUT 3 cDNA served as the probe (24). Inter-lane loading variability was standardized by re-hybridization of the stripped filters with a32P-labeled rat 18 S rRNA probe (25).

Protein Studies—Thirty to fifty μg of either cellular or brain homogenate or extracted nuclear protein (26) were solubilized in 50 mM Tris, pH 6.8, containing 2% SDS and the protein concentration determined by the Bio-Rad dye-binding assay (27). Western blot analysis was carried out as described previously (23). The primary antibody consisted of antibodies raised in rabbit against a keyhole limpet-linked terminal 17 amino acids of the mouse GLUT 3 protein and the isoform specificity previously characterized by us (23). The primary anti-mouse GLUT 3 antibody was used at a 1:500 dilution, and the incubation with filters containing the transferred proteins was carried out at room temperature for 16 h. To detect nuclear proteins, the rabbit anti-synthetic human Sp3 peptide (436–454 amino acid region), anti-synthetic human Sp3 peptide (676–695 amino acids at the C-terminal region) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-rat CREB (Upstate Biotechnology, Inc., Lake Placid, NY), and anti-mouse c-Jun (247–283 amino acids) (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were employed. Antibodies were raised in rabbit against the MSY-1 peptide sequence NH2-DPPAENSSAPEAEGGCGGCOOH (28) were affinity-purified by using an Amino-link Immobilization kit ( Pierce). In certain instances 125I-labeled goat anti-rabbit secondary antibody (50,000 cpm/sample, PerkinElmer Life Sciences) was used to detect the primary antigen-antibody complex. Autoradiography of the filters was carried out for optimal lengths of time to maintain linearity of the signal. Detection of protein bands for MSY-1 was carried out by subjecting the immunoblots to the chemiluminescent method (Amersham Biosciences) of detection.

Primer Extension Assay—Primer extension was carried out as described previously (29). Briefly, an antisense oligonucleotide (Cruachem Biotechnology, Inc., Dulles, VA) complementary to the reporter in the absence of a promoter region was used as a negative control. To assess the transfection efficiency, 0.25 μg of Lipofectin plasmid DNA (thymidine kinase promoter-driven Renilla luciferase, 0.5 μg (Promega, Madison, WI) was transiently co-transfected with each individual transfectant to standardize the results (29, 30). PGL2 basic consisting of the firefly luciferase as the reporter and the SV40 promoter-driven luciferase activity that served as the positive control. To assess the transfection efficiency, the dual luciferase assay (Promega, Madison, WI). Briefly, 36–48 h post-transfection, the cells were washed with PBS and lysed using 0.5 μl of passive lysis buffer (Promega, Madison, WI). The supernatant on centrifugation at 10,000 rpm for 10 min was stored at −70 °C until analysis. Twenty μl of this cellular extract was mixed with 100 μl of the luciferase assay buffer, and the firefly luciferase activity was measured as light output (15 s) in a Monolight 2010 luminometer (Analytical Luminescence, San Diego, CA) (23). Subsequently, the Renilla luciferase activity was estimated after the addition of 100 μl of the Stop and Glo reagent, and the light output (15 s) was measured separately. The Renilla-driven luciferase activity was used to standardize the Glut 3 promoter-driven firefly luciferase activity for transfection efficiency. The corrected Glut 3 promoter-driven luciferase activity was expressed as a percentage of the SV40 promoter-driven luciferase activity that served as the positive control in every transfection experiment.

Small Interfering (Si) RNA Transfection Experiments—siRNA was constructed to target the mouse CREB (Bharacoa Inc., Dallas, TX) sequence between the +810- to +830-bp coding region by using the following oligonucleotides: sense, 5′-GAAGAGAGGCCGCGGACUAAUUCU-3′, and antisense, 5′-CAUUAAGCGGACCUCUCUCUUCU-3′ (29). Complementary oligonucleotides were converted to a 2′-hydroxy-annealed and desalted duplex strands with a 9-base spacer, thereby creating a short hairpin RNA that was driven by the RNA polymerase III promoter followed by a (T₆) RNA poly III transcriptional stop signal. Co-transfections into N2A cells were performed by using the −203-bp GLUT3-luciferase DNA construct (2 μg) and the constructed siRNA plasmid into 6-well plates using Trans-It FX transfection reagent (Mirus Corp., Madison, WI) as transfection reagents, respectively (29, 30). PGL2 basic consisting of the firefly luciferase as the reporter in the absence of a promoter region was used as a negative control. To assess the transfection efficiency, 0.25 μg of pRL-TK plasmid was co-transfected with each individual transfectant to standardize the results for transfection efficiency.

| Primer name | 3′ sequence | 5′ sequence |
|-------------|-------------|-------------|
| Nontargeting | 5′-CCG ACT GGT AGT TGT G | Sense |
| Nontargeting | 5′-CTG AGG CAG CAC GAG GT | Sense |
| Nontargeting | 5′-TCT GTG ACG TTA GTT G | Sense |
| Nontargeting | 5′-CAG GAT CAT AAG AGT CAC | Sense |
| Nontargeting | 5′-AGG CTG GGC TCG CGG CTG | Sense |
| Nontargeting | 5′-GGG CCC ACG CTA GTC CAA GC | Sense |

| Sequence information | Nucleotide orientation |
|----------------------|-----------------------|
| −1553                | 5′-CCC AGT CGT ATG TGC G | Sense |
| −857                 | 5′-CCC AGG CAG CAC GAG GT | Sense |
| −573                 | 5′-CCC AGA GCC GCC GGC AGT G | Sense |
| −372                 | 5′-CTG TGT ACG TCA GAT CCT | Sense |
| −203                 | 5′-CAG CTG GTG TTA TCG | Sense |
| −177                 | 5′-CTG CTT GTC AAA AAA CCC G TG | Sense |
| −104                 | 5′-CAG GAG GAT GAT GTG GTA AAA AG | Sense |
| −29                  | 5′-CAG GAG GAT GAT GTG GTA AAA AG | Sense |
| +58                  | 5′-GCA GAT CAT AAG AGT CAC | Sense |
| −158                 | 5′-AGG CTG GGC TCG CGG CTG | Sense |
| −305                 | 5′-GGG CCC ACG CTA GTC CAA GC | Sense |

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mid DNA (thymidine kinase promoter-driven Renilla luciferase; Promega, Madison, WI) was co-transfected as well. Luciferase activity was measured in 10 μl of the N2A cellular extract (Zylink FB 15 tube luminometer, Fisher) at 48 h post-transfection by the dual luciferase assay system (Promega, Madison, WI). In addition, 30 μg of protein from SiRNA-transfected N2A cell lysates was subjected to SDS-PAGE, and the separated proteins were transferred onto nitrocellulose membranes. Each nitrocellulose membrane was washed twice for 10 min in distilled water. The membranes were then blocked by incubating with freshly prepared 5% bovine milk solution in PBS for 1 h at room temperature under constant agitation. The membranes were then washed twice with distilled H2O and incubated with the secondary antibody that was diluted in a PBS, 1% milk solution for 1.5 h at room temperature. The membranes were blocked by incubating with 1:1000 dilution of the anti-CREB (Upstate Biotechnology, Inc.) antibody that was diluted in PBS containing 1% bovine milk solution. The incubation with primary anti-CREB (Upstate Biotechnology, Inc.) antibody that was diluted in PBS for optimal lengths of time to maintain linearity of the signal (31).

Electromobility Shift Assay (EMSA)—Nuclear extracts from the N2A cells, 1- and 21-day-old mouse brains were prepared as described by Wildeman et al. (26). Briefly, brain tissue was obtained, or 5 × 106 cells were retrieved by a razor policeman and suspended in 10 mM Hepes, pH 7.8, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 25% glycerol to extract the nuclear proteins. The extracted nuclear proteins were collected as the supernatant after centrifugation at 10,000 rpm for 30 min and precipitated with solid (NH4)2SO4 by centrifugation at 15,000 rpm for 15 min, and the supernatant was diluted in aliquots at −70 °C. The concentration of the solubilized nuclear protein was measured by the method of Bradford (27), and the separated proteins were transferred onto nitrocellulose membranes (Bio-Rad) and subjected to Western blot analysis using the antibody raised against MSY-1 (28).

Confirmation of the Sp3, CREB, and MSY-1 DNA Bindability by the Chromatin Immunoprecipitation Assay—Chromatin immunoprecipitation (ChIP) assay was performed as described by Boyd and Farnham (33). N2A murine neuroblastoma cells (~2 × 106 in a 150-mm culture dish) were fixed with 1% formaldehyde for 15 min at room temperature. The cell pellet was resuspended in cell lysis buffer (5 mM PIPES, pH 8.0, 8.5 mM KCl, 0.5% Nonidet P-40) containing protease inhibitors and homogenized with a type B Dounce homogenizer. The nuclei were lysed in the nuclear lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS) containing protease inhibitors. The chromatin was sonicated (Fisher model 60 Sonic Dismembrator) on ice with three pulses of 15 s each at setting 3 with a 1-min interval. The sonicated chromatin was added to Eppendorf centrifuge (10 min at 14,000 rpm) to remove the cell debris from crude chromatin lysate. Ten percent of the lysate was set aside as the input chromatin. The sheared chromatin from 106 cells was pre-cleared at 4 °C for 15 min with 10 μl of staphylococcus A cells (Sigma) which were blocked with 10 μl of herring sperm DNA and 10 μl of bovine serum albumin both at a concentration of 10 mg/ml. One hundred μl of pre-cleared chromatin lysate was incubated with 1 μg of either Sp3 (Santa Cruz Biotechnology, Santa Cruz, CA), CREB, or pCREB polyclonal antibody (Upstate Biotechnology, Inc.) and shaken on a nutator at 4 °C. Then 1 μg of a rabbit secondary antibody (Sigma) was added to the sample and incubated at room temperature for an additional hour. Ten μl of blocked staphylococcus A cells was added to the sample and incubated at room temperature for 15 min. The sample was centrifuged at 14,000 rpm for 15 min at room temperature. The supernatant was removed, and the pellet was washed twice in 1.4 ml of a dialysis buffer (100 mM Tris-HCl, pH 8.9, 500 mM LiCl, 1% Nonidet P-40, 0.1% deoxycholic acid). Antibody-protein-DNA complexes were eluted with an elution buffer (50 mM NaHCO3, 1% SDS) by vortexing the sample for 30 min at setting 3. The staphylococcus A cells were separated as a pellet, and the supernatant was collected. The elution process was repeated once more, and the combined supernatants were incubated at 65 °C for 5 h with 1 μl of RNase A (10 mg/ml) for reversal of the formaldehyde cross-link. Precipitation of RNA in the supernatant was carried out overnight at −20 °C in 2.5 volumes of absolute ethanol. The precipitated pellet was air-dried, subsequently dissolved in Tris/EDTA buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA), and treated with proteinase K at 45 °C for 2 h. The DNA was purified once.

**TABLE II**

| Primers for gel-shift analysis | 5'-AGACGCACTACGTCTGCGG-sense | 5'-GGCGAGTGATAGCCTCTGCGG-antisense | 5'-CAGAGTTGAGGCGGCAGGTTTTG-antisense | 5'-AGACGCACTAAGAACATCGG-sense | 5'-GGCGAGTGATAGCCTCTGCGG-antisense | 5'-CAGAGTTGAGGCGGCAGGTTTTG-antisense |
|------------------------------|--------------------------------|----------------------------------|--------------------------------------|--------------------------------|--------------------------------|--------------------------------|

| Primers used for gel-shift analysis | 195-1 | 195-1 | 195-1 | 195-1 | 195-1 | 195-1 |
|-----------------------------------|------|------|------|------|------|------|

**Note:** bp, and oligo 180, 5′-AGAGCCCGACGACCT-antisense, corresponded to the sense strand between −76 and −90 bp. In separate 500-μl reactions, 100 μg of N2A nuclear protein was mixed with 100 pmol of biotinylated oligonucleotide plus 50 μg of poly(dA) in EMSA binding buffer and incubated for 20 min at room temperature. The protein bound to the biotinylated oligo was then purified by using streptavidin-conjugated paramagnetic beads according to the manufacturer’s instructions (Dynal Inc., Lake Success, NY). The bound protein was eluted by resuspending the beads in 40 μl of Laemmli buffer, incubating for 3 min at 65 °C, then adding 2 μl of 14.3 μl of mercaptoethanol, and heating for 5 min at 90 °C. The eluted protein was then analyzed by Western blot analysis by polyclonal antibody against MSY-1.

Identification of MSY-1 as a Glut 3 Promoter Binding Protein by Western-Analysis—Because the MSY-1 antibody available for the current studies did not supershift MSY-1 protein when complexed with single-stranded DNA (data not shown), the presence of MSY-1 in the protein-DNA complex was ascertained by the shift-Western technique (28).

Briefly, following a preparative electromobility shift analysis, the location of the DNA-protein complex was determined by using a PhosphorImager. The portion of the gel containing the shifted band was excised and transferred to nitrocellulose membrane (Bio-Rad) and subjected to Western blot analysis using the antibody raised against MSY-1 (28).

Conformation of the Sp3, CREB, and MSY-1 DNA Bindability by the Chromatin Immunoprecipitation Assay—Chromatin immunoprecipitation (ChIP) assay was performed as described by Boyd and Farnham (33). N2A murine neuroblastoma cells (~2 × 106 in a 150-mm culture dish) were fixed with 1% formaldehyde for 15 min at room temperature. The cell pellet was resuspended in cell lysis buffer (5 mM PIPES, pH 8.0, 8.5 mM KCl, 0.5% Nonidet P-40) containing protease inhibitors and homogenized with a type B Dounce homogenizer. The nuclei were lysed in the nuclear lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS) containing protease inhibitors. The chromatin was sonicated (Fisher model 60 Sonic Dismembrator) on ice with three pulses of 15 s each at setting 3 with a 1-min interval. The sonicated chromatin was added to Eppendorf centrifuge (10 min at 14,000 rpm) to remove the cell debris from crude chromatin lysate. Ten percent of the lysate was set aside as the input chromatin. The sheared chromatin from 106 cells was pre-cleared at 4 °C for 15 min with 10 μl of staphylococcus A cells (Sigma) which were blocked with 10 μl of herring sperm DNA and 10 μl of bovine serum albumin both at a concentration of 10 mg/ml. One hundred μl of pre-cleared chromatin lysate was incubated with 1 μg of either Sp3 (Santa Cruz Biotechnology, Santa Cruz, CA), CREB, or pCREB polyclonal antibody (Upstate Biotechnology, Inc.) and shaken on a nutator at 4 °C. Then 1 μg of a rabbit secondary antibody (Sigma) was added to the sample and incubated at room temperature for an additional hour. Ten μl of blocked staphylococcus A cells was added to the sample and incubated at room temperature for 15 min. The sample was centrifuged at 14,000 rpm for 15 min at room temperature. The supernatant was removed, and the pellet was washed twice in 1.4 ml of a dialysis buffer (100 mM Tris-HCl, pH 8.9, 500 mM LiCl, 1% Nonidet P-40, 0.1% deoxycholic acid). Antibody-protein-DNA complexes were eluted with an elution buffer (50 mM NaHCO3, 1% SDS) by vortexing the sample for 30 min at setting 3. The staphylococcus A cells were separated as a pellet, and the supernatant was collected. The elution process was repeated once more, and the combined supernatants were incubated at 65 °C for 5 h with 1 μl of RNase A (10 mg/ml) for reversal of the formaldehyde cross-link. Precipitation of RNA in the supernatant was carried out overnight at −20 °C in 2.5 volumes of absolute ethanol. The precipitated pellet was air-dried, subsequently dissolved in Tris/EDTA buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA), and treated with proteinase K at 45 °C for 2 h. The DNA was purified once.
with phenylchloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol and subsequently precipitated overnight at ~20°C in ethanol with 5 μg of RNA and 5 μg of glycogen. The DNA concentration was determined by the Dip Stick kit (Invitrogen). DNA (~4 ng) that was complexed with the immunoprecipitated protein was used as a template in each PCR.

Our initial attempts at cross-linking with formaldehyde did not immunoprecipitate MSY-1-associated DNA. We speculated that this may be due to MSY-1 being tethered to chromatin by another protein co-factor. Thus to increase the efficiency of cross-linking the MSY-1 complex to its DNA target sites in vivo, we treated the cells first with dimethyl adipimide (Pierce), a protein-protein cross-linking agent, followed by formaldehyde as per the method of Kurdistani and Grunstein (34) with some modifications. After washing the adherent N2A cells once with ice-cold PBS, 10 μl of a fresh solution of 10 mM dimethyl adipimide in ice-cold PBS containing 0.25% dimethyl sulfoxide (Me2SO, Sigma) was added and incubated for 45 min with gentle shaking on a rotary platform, followed successively by an ice-cold PBS wash and a final concentration of 1/5 (w/v) formaldehyde in PBS at room temperature for 3 h on a rotary platform. The reaction was stopped by adding glycine, and the subsequent experimental steps of the ChIP assay as outlined above were undertaken employing an affinity-purified rabbit anti-MSY-1-(242–267) antibody (32). The primers used in the ChIP assay were designed and synthesized by Retrogen Inc. (San Diego, CA). The sequence of the forward primer for detecting Sp3 and CREB/pCREB-binding sites was 5’-aggcagcactctactctgcg-3’ extending from ~192 bp of the mouse Glut 3 gene, and the reverse primer for detecting Sp3 and CREB/pCREB-binding sites was 5’-ttactatctctctctggc-3’ beginning at ~7 bp of the mouse Glut 3 gene. The intervening sequence that would be amplified contained the MSY-1-binding site as well. The sequence of the forward primer for detecting the MSY-1-binding site was 5’-aggcagcactctactctgctggc-3’ extending from ~89 bp of the mouse Glut 3 gene, and reverse primer for detecting the MSY-1-binding site was 5’-gtactaatcactctactctggtggc-3’ starting from the +260 bp of the mouse Glut 3 gene. The intervening sequence that would be amplified excluded the Sp3- and the CREB/pCREB-binding sites.

The PCR employed in the ChIP assay consisted of 50 μl of the PCR mix containing 5 μl of the DNA template, 0.25 μl of each primer (0.5 μM), 5.0 μl of PCR buffer (10 times) with 15 mM MgCl2, 4 μl of 2.5 mM dNTPs, and 0.25 μl of Taq polymerase (5 units/μl), which was subjected to amplification in a TA thermocycler (Biometra). The PCR parameters for the Sp3 binding region or the CREB/pCREB-bound AP-1 region of the Glut 3 gene were initially at 95°C for 2 min, followed by 25 cycles at 95°C for 30 s to denature, 55°C for 30 s to anneal, and 72°C for 90 s to extend the DNA. The PCR parameters for amplifying the MSY-1-binding sequence of the Glut 3 gene were 30 cycles at 95°C for 30 s to denature, 56°C for 30 s to anneal, and 72°C for 30 s to extend the DNA. The final PCR-amplified product was purified and identified by the 100-bp DNA ladder (Bayou BioLabs, Los Angeles, CA). In addition, co-immunoprecipitation of MSY-1 with CREB was accomplished by immunoprecipitating chromatin from N2A with 1 μg of the MSY-1 antibody as described above. The immunoprecipitated antibody-antigen-DNA complex was subjected to a reversal of the cross-link followed by Western blot analysis as described above by using the anti-CREB antibody (dilution 1:1000) as the primary antibody, with the HeLa cell nuclear extract serving as the antigen (CREB)-positive control.

Data Analysis—All data are depicted as mean ± S.E. Differences between two groups were validated by the Student’s t test, and differences between more than two groups were determined by the one-way analysis of variance, and inter-group differences were validated by the Neuman-Kuel’s test.

RESULTS

Postnatal Expression of GLUT 3 in Mouse Brain—The GLUT 3 expression in mouse brain was profiled by both Northern and Western blot analyses. These experiments revealed that GLUT 3 protein (Fig. 1A) and mRNA (Fig. 1B) demonstrated a distinct increase in expression postnatally, with the amount of GLUT 3 protein in the brain of the 21-day-old mouse being significantly higher than in the 1-day-old mouse as shown previously (13). Based on the observation that the changes in GLUT 3 mRNA levels paralleled changes in protein expression, we reasoned that transcriptional control of the Glut 3 gene could play a role in the postnatal increase in brain GLUT 3 expression. To begin to investigate this hypothesis, we mapped the transcription start site of the Glut 3 gene in mouse brain. Primer extension assays (Fig. 1C) and ribonuclease protection assays (Fig. 1D) revealed that the transcriptional start site resides 305 bp 5’ to the ATG translational start site, a position similar to that noted previously in N2A neuroblastoma cells (23).

Characterization of the Mouse Glut 3 Gene Promoter—Our previous studies (23) had identified a cis-element in the promoter of the murine Glut 3 gene that bound Sp1 and Sp3. To determine the presence of other regulatory regions, we conducted a deletional analysis of the 5’-flanking region of the Glut 3 gene. For this purpose we created luciferase-fusion constructs containing progressively shorter fragments of the GLUT 3 5’-flanking DNA. Transient transfection of these constructs into N2A cells revealed that deletion of the −1553 to −203 bp did not result in a significant alteration in promoter activity. In contrast, removal of −203 to −177 bp region (region A, Fig. 2A) resulted in complete abrogation of activity indicating the presence of positively acting cis-element(s) within this region. Further deletion of −177 to −104 bp (region B, Fig. 2A) resulted in a restoration of promoter activity, suggesting the presence of a repressor element in region B (Fig. 2A).

Sp Family of Transcription Factors from Postnatal Mouse Brain Binds the −149- to −119-bp Region of the Glut 3 Gene—We demonstrated previously (23) that the Sp family of...
transcription factors from N2A, H19-7 neuroblasts, and HRP-1 trophoblastic cells interacted with the Glut 3 gene. In the present study we observed that Sp1 and Sp3 present in 1- and 21-day-old mouse brains demonstrated a similar interaction with the Glut 3 gene. Our previous transient transfection studies in N2A, H19-7 neuroblasts, and HRP-1 trophoblasts using sequential 5'-deletions of the murine GLUT-3-luciferase fusion gene revealed that the 5'-flanking region of the murine Glut 3 gene exhibited promoter activity, and deletional analysis had established that the region −203 to +237 bp from the transcription start site contained an enhancer element. Further experiments previously established (23) that the −137- to −130-bp region encompassed a repressor element. Computer analysis of the DNA sequence of this region suggested the presence of a GC box motif that is a canonical binding site for the Sp family of factors. In order to find out whether the Sp family of proteins has a role in transcriptional activation of the Glut 3 gene, we performed EMSA with the 32P-labeled −137- to −130-bp region and nuclear protein extracts from the 1- and 21-day-old mouse brains and N2A neuroblastoma cells (Fig. 3A). These experiments revealed that both Sp1 (Fig. 3A, lanes 3, 6, and 9) and Sp3 (Fig. 3A, lanes 4, 7, and 10) participated in the formation of the protein-DNA complex at the −137- to −130-bp site. Western blot analyses confirmed the presence of Sp1 (Fig. 3B, upper panel) and Sp3 (Fig. 3B, lower panel) in nuclear extracts of brain tissues from 1- and 21-day-old mouse brains and N2A cells. In our previous studies, we had established that Sp1 had to be de-phosphorylated in order to bind the Sp1 consensus sequence in the −149- to −119-bp region of the murine Glut 3 gene (23). Thus, although nuclear extracts that contain the de-phosphorylated Sp1 bound both the consensus Sp1 sequence (Fig. 3C, lanes 1 and 2) and the −149- to −119-bp region of the Glut 3 gene that contained the Sp1-binding site (Fig. 3C, lanes 6 and 7), the human recombinant Sp1 protein failed to bind the same region of the Glut 3 gene (Fig. 3C, lane 5) in our present study.

**CREB Binds the −187- to −180-bp Region of the Glut 3 Gene—Deletional analysis of the 5'-flanking region of the murine Glut 3 gene indicated that the region of −203 to −177 bp possessed regulatory activity (region A, Fig. 2A). Computer analysis of this region demonstrated a putative AP-1 site in this region at −187 to −180 bp. Mutation of the AP-1 site significantly reduced the promoter activity (Fig. 2B), indicating the functional significance of the −187- to −180-bp region of the Glut 3 gene to its expression.

To test the putative AP-1 site for protein binding activity, an oligoprobe encompassing the −187- to −180-bp region was used in gel-shift assays with nuclear proteins from N2A cells. These experiments revealed the presence of four protein-DNA complexes (Fig. 4A). Competition experiments using unlabeled oligoprobe established that there was a dose-dependent abrogation of DNA binding of protein complexes 1–3 with complex 4 deemed to be nonspecific because of lack of competition (Fig. 4B, lanes 5–7). To establish the identity of the proteins that
anti-dephosphorylated CREB antibody alone (Fig. 4D), the supershift was more prominent when compared with the anti-phospho-CREB (Ser-133) antibodies revealed CREB expression in the 21- and 1-day-old mouse brain extracts as well. The identity of complexes 1 and 3 remain to be determined. Western blot analysis, using the anti-CREB antibody, and anti-phospho-CREB (Ser-133) antibodies revealed CREB expression in the 21- and 1-day-old mouse brains similar to the N2A cells (Fig. 4D).

In transient transfection assays, when a mutant CREB construct was used, a significant decline in the transcriptional activity of the Glut 3 gene was observed (Fig. 2B). Co-transfection experiments employing a CREB expression vector and the −203-bp GLUT 3-Luc construct were unsuccessful because of high levels of endogenous CREB present in most cell lines investigated. However, co-transfection experiments employing the siRNA against the CREB sequence and the −203-bp GLUT 3-Luc DNA construct revealed a decline in endogenous CREB protein amounts (Fig. 5A), which was associated with a suppression of the luciferase activity driven by the −203- to +237-bp DNA region of the Glut 3 gene (Fig. 5C). No similar decline in pCREB protein amounts (Fig. 5B) was detectable perhaps related to the small amounts present in the native state, so that further suppression by the siRNA was imperceptible. Another interpretation is that the siRNA would not affect the post-translational modification of CREB present in the nuclear extract of the N2A cells.

Characterization of MSY-1 Binding to the Glut 3 Gene Promoter Region—Transient transfection studies using the −104-bp GLUT 3-Luciferase construct showed significant promoter activity in N2A cells (region C, Fig. 2A). Sequence analysis revealed potential binding sites for the single-stranded DNA-binding protein MSY-1. Mobility shift analysis using the sense strand oligoprobe −89 to −66 bp and nuclear extracts from N2A cells showed a doublet DNA:protein band (Fig. 6A, lane 2). This complex was specifically competed out by increasing concentrations of the unlabeled −89- to −66-bp oligoprobe (Fig. 6A, lanes 3–5), whereas the Sp1 oligoprobe did not affect the binding (Fig. 6A, lanes 6 and 7). Similar mobility shift using the antisense probe, −66 to −89 bp, failed to produce this DNA:protein complex. Because the MSY-1 antibody failed to supershift the MSY-1-DNA complex, the specificity of binding was shown by shift-Western blot analysis. After the mobility shift assay, the DNA-protein complex was transferred to a nitrocellulose membrane and probed for MSY-1 with the antibody (28). The presence of MSY-1 in the shifted complex is shown in Fig. 6B.

Single-stranded binding of MSY-1 was further confirmed by affinity purification using biotinylated oligonucleotides, two sense strand probes (oligos 179 and 177), and two antisense probes (178 and 180), spanning the −89- to −66-bp region of the GLUT-3 proximal promoter. Eluted proteins were subjected to Western blot analysis using the MSY-1 antibody (Fig. 6C, lanes 1–4, oligos 177–180, respectively). These results indicate that MSY-1 preferentially binds to the sense strand (oligos 179 and 177). Increasing amounts of MSY-1 expression vector were employed in co-transfection experiments along with the −104-bp GLUT 3-Luciferase construct. A dose-dependent increase in luciferase reporter activity was observed (Fig. 6D) indicating that MSY-1 plays a role in activating Glut 3 gene expression.

In addition, transient transfection experiments utilizing deletion luciferase-fusion constructs containing the region between −104 and −63 bp of the Glut 3 gene indicated the presence of a regulatory element (Fig. 7A). EMSA experiments participated in the formation of complexes 1–3, we performed competition experiments with unlabeled oligonucleotides containing canonical binding sites for common DNA-binding proteins. An oligonucleotide containing the AP-1 consensus sequence competed for the binding proteins to this region (Fig. 4A). In contrast, oligoprobes representing CTF/NF1, C/EBP, Sp1, GRE, and TFIID consensus binding sites failed to abrogate formation of the protein-DNA complexes at this site (Fig. 4A). When a mutant oligoprobe was employed, it did not show the band that was displaced by AP-1 and CREB sequences (Fig. 4A, lane 11). To determine the identity of the proteins in the protein-DNA complex, supershift assays were performed. These experiments revealed a supershift in the presence of the anti-CREB antibody (Fig. 4, B, lane 3, and C, lanes 3, 7, and 11) with no change in the presence of the anti-c-Jun antibody (Fig. 4, B, lane 4, and C, lanes 4, 8, and 12). In the presence of an antibody targeted toward the phosphorylated CREB (Ser-133), the supershift was more prominent when compared with the anti-dephosphorylated CREB antibody alone (Fig. 4C, lanes 5, 9, and 13). This pattern was evident in N2A cells, and the 1- and 21-day-old mouse brain nuclear extracts as well. The identity of complexes 1 and 3 remain to be determined. Western blot analysis, using the anti-CREB antibody, and anti-phospho-CREB (Ser-133) antibodies revealed CREB expression in the 21- and 1-day-old mouse brains similar to the N2A cells (Fig. 4D).
Fig. 4. CREB binds the −194- to −173-bp region of the Glut 3 gene. A, mobility shift assay. 32P-Labeled −194- to −173 bp region (dAGCAGACTGACTCTACTCTGCG) of the mouse Glut 3 gene was employed in binding reactions along with nuclear extracts from N2A cells. Various unlabeled nonspecific oligonucleotides for known nuclear factors were used in competition studies (lanes 3–9). A mutant oligo of the −194 to −173 bp (dAGCAGACTGTTTTGACTCTGCG) was labeled (lane 10) and was used in binding studies with nuclear extracts from N2A cells (lane 11). B, competition studies and the supershift assay demonstrating CREB binding. CREB binds the −194- to −173-bp region of the Glut 3 gene. GLUT 3 oligoprobe −194 to −173 bp was 32P-labeled and was used in supershift assay and competition assay. Addition of the anti-CREB antibody (Upstate Biotechnology, Inc.) to the mobility shift (lane 2) assay with N2A nuclear extracts causes a supershift (lane 3). The anti-c-Jun antibody did not cause a supershift (lane 4). Cold oligoprobe (194 to −173 bp) was used in lane 1 and 10 and 100x excess in competition studies in lanes 5–7, respectively. C, supershift assay demonstrating pCREB and CREB binding. Nuclear extracts from N2A cells and 1- and 21-day-old mouse brains (1-day > 21-day) specifically bound to this region of the Glut 3 promoter (Fig. 7B). The precise identity of the protein(s) in this DNA-protein complex remains to be determined.

Confirmation of Interaction between Sp3, CREB, or MSY-1 and the Glut 3 Promoter Region—The ChIP assay demonstrated that chromatin obtained from the N2A cells that contained the −192- to −7-bp region of the Glut 3 gene, which was detected by PCR, interacted with Sp3 (Fig. 8A), CREB, and pCREB (Fig. 8B). In contrast, no GLUT 3 DNA sequences were detected by PCR from COS-7 cellular chromatin that had been immunoprecipitated with CREB/pCREB antibodies (Fig. 8C). Similarly, N2A cellular chromatin that contained the −89- to +260-bp region of the Glut 3 gene as detected by PCR interacted with the MSY-1 protein following some protein-protein interactions. It is not clear if the proteins that were bound to the −192- to −7-bp region of the Glut 3 gene in N2A chromatin were Sp3, CREB, or MSY-1. The precise identity of the protein(s) in this DNA-protein complex remains to be determined.
Fig. 6. MSY-1 binds the −89- to −66-bp region of the Glut 3 gene. A, mobility shift and competition assays. 32P-Labeled −89- to −66-bp sense (lanes 2–7) and antisense (lanes 9–14) (sense, 5′-AGGC-TGTCGCTCTTGAAAAAGGGAAAGGAA-3′; antisense, 5′-TCGCACA-GCCCGAGACTTCCCTCCCTCCTT-3′) probes from the mouse Glut 3 gene were employed in binding reactions along with nuclear extracts from N2A cells. Various unlabeled nonspecific oligonucleotides of the same probes (sense, lanes 3–6; antisense, lanes 10–12) or the Sp1 binding region (sense, lanes 6 and 7; antisense, lanes 13 and 14) were used in competition studies (lanes 4–9). Free probe is seen in lanes 1 and 8. B, a shift-Western analysis was carried out using a 32P-end-labeled oligonucleotide spanning from −89 to −66 bp of the Glut 3 promoter region. After incubating with N2A nuclear extracts, the DNA-protein complexes were separated by electrophoresis on a 5% acrylamide non-denaturating gel and transferred to nitrocellulose membranes. Immunoblot analysis was carried out with an affinity-purified MSY-1 antibody. C, affinity purification of the MSY-1 protein. MSY-1 protein was affinity-purified using biotinylated oligonucleotides spanning the −89- to −66-bp region of the Glut 3 gene. The sequence and designation of the probes are shown below. 100 picomoles of either sense or antisense biotinylated oligonucleotides were incubated with 100 μg of N2A nuclear extracts. The DNA-protein complex was captured with streptavidin-conjugated paramagnetic beads and the bound protein eluted and analyzed by Western blot analysis. Lane 1, eluate from oligo 177; lane 2, eluate from oligo 178; lane 3, eluate from oligo 179; lane 4, eluate from oligo 180; lane 5, 25 μg of total nuclear extract from N2A cells. D, transient transfection and luciferase activity assay. Luciferase activity from transient co-transfection assays are shown (n = 6) with increasing concentrations of the MSY-1 expression vector (0.3–1.25 μg) along with 2 μg of the −104GLUT3-Luciferase construct. A dose-dependent increase in luciferase reporter activity is seen with peak activity with 1.25 μg of the MSY-1 expression vector.

DISCUSSION

In our previous study, by using neuronal and trophoblastic cell lines we had partially characterized the cis-elements involved in murine GLUT-3 gene activation. We had characterized the −203- to +237-bp region and found two regions with promoter activity (−203 to −177 bp, region A, and −104 to +237 bp, region C) and a single repressor region (−177 to −104 bp, region B). Sp nuclear family members Sp1 and Sp3 bound this repressor element (−137 to −130 bp of region B) in the de-phosphorylated state with Sp1 acting as the repressor and Sp3 as the activator. Our previous studies had determined that whereas Sp3 could displace Sp1 from its DNA-binding site and function as an activator, the more seminal observation was

Fig. 7. Characterization of the −104- to −63-bp region of the Glut 3 promoter. A, transient transfection and luciferase reporter activity assay. Luciferase reporter constructs were made using the −104-bp region and −63-bp region of the Glut 3 promoter and were employed in transfection in N2A cells (n = 5). The bar graph illustrates the reporter activity. B, mobility shift assays. Oligonucleotides were synthesized for the region −83 to −69 bp and were used in EMSA study. Lane 1, free probe. Nuclear extracts from N2A cells (lane 2), 1-day (lane 3), and 21-day (lane 4) brain extracts. Lane 5–7 is the competition assay using cold probe in 1000, 100, and 1X, respectively.
amplification product is seen when the CREB and MSY-1-binding sites containing region of the Glut 3 gene (185 bp) was amplified from chromatin that was immunoprecipitated with either the CREB (lane 2) or MSY-1 antibody (lane 3) against Sp3 (lanes 2 and 3) revealed a 354-bp region. Negative control consisted of no primary antibody for MSY-1 but only the MSY-1 binding domain (354 bp) (lane 4). A 185-bp amplification product is seen in the 2% agarose gel as a 185-bp DNA fragment detected in the N2A cellular chromatin complexed with proteins that were immunoprecipitated with an antibody (Ab) against Sp3 (lane 3) using primers containing the Sp3-binding region of the Glut 3 gene (189 to +260 bp) and the mouse GLUT 3 (−1553 to +331 bp) gene (−control, lane 4). Positive controls consisted of 10% of the total chromatin in the absence of immunoprecipitation (lane 2, positive control for PCR) and the mouse GLUT 3 (−1553 to +331 bp) gene (−control, lane 4). The negative control consisted of no primary antibody but only the secondary antibody (−Ab, lane 5). The positive control consisted of 10% of the total chromatin in the absence of immunoprecipitation (lane 6, positive control for PCR). Lane 1, DNA ladder. A 2% agarose gel demonstrates no CREB/pCREB binding to the Glut 3 promoter in COS-7 cells. PCR amplification product is seen in the 2% agarose gel as a 185-bp DNA fragment detected only in the positive DNA control (+control) which consisted of the mouse GLUT 3 (−1553 to +331 bp) gene (lane 2, positive control for PCR), whereas no amplification product was noted in chromatin obtained from COS-7 (monkey kidney fibroblasts, which served as a negative cellular control) cells and immunoprecipitated with either the CREB (lane 3) or the pCREB (lane 4) antibodies or in 10% of the total chromatin in the absence of immunoprecipitation (lane 5). Lane 1, DNA ladder. C, ChIP assay demonstrates that MSY-1 binds to the Glut 3 promoter region. A 2% agarose gel demonstrates PCR amplifications that were performed on immunoprecipitated chromatin from N2A cells. PCRs targeted at amplifying DNA containing the MSY-1-binding site on the Glut 3 gene (−89 to +260 bp) from immunoprecipitated protein complexed to chromatin in the presence of an anti-MSY-1 antibody (MSY-1 Ab, lane 4) revealed a 354-bp region. Negative control consisted of no primary antibody for MSY-1 but only the secondary antibody (−Ab, lane 5). Positive controls consisted of 10% of the total chromatin (input, lane 3) and the mouse Glut 3 (−1553 to +331 bp) gene (+control, lane 2). Lane 1, DNA ladder. D, ChIP assay to demonstrate an interaction between CREB and MSY-1. PCR amplifications were performed on immunoprecipitated chromatin from N2A cells. A 2% agarose gel demonstrating PCR-amplified products containing the CREB and MSY-1 binding domains (185 bp) (lanes 2, 4, and 6) and only the MSY-1 binding domain (354 bp) (lanes 3, 5, and 7) of the Glut 3 gene obtained by immunoprecipitating proteins complexed to chromatin with either the CREB antibody (lanes 2 and 4) and the MSY-1 antibody (lanes 4 and 5) but not with the IgG which served as the negative antibody control (lanes 6 and 7). Lane 1, DNA ladder. A 185-bp amplification product is seen when the CREB and MSY-1-binding sites containing region of the Glut 3 gene (−192 to −7 bp) was amplified from chromatin that was immunoprecipitated with either the CREB (lane 2) or MSY-1 antibodies (lane 4). A 354-bp amplification product is seen when only the MSY-1-binding site containing region of the Glut 3 gene (−89 to +260 bp) was amplified from chromatin that was immunoprecipitated with the MSY-1 antibody (lane 5) but not when the CREB antibody was used (lane 3). E, co-immunoprecipitation assay demonstrates an interaction between MSY-1 and CREB. Chromatin from N2A cells was immunoprecipitated with the MSY-1 antibody (lane 2), and this immunoprecipitated antibody-antigen-DNA complex and HeLa cell nuclear extract (lane 1, positive control) were subjected to Western blot analysis employing the CREB antibody (1:1000) which demonstrated an ~43-kDa band.
that it had to interact with a trans-activator that bound DNA sequences between −203 and −177 bp (region A) to bring about gene activation. Furthermore, we observed that the repressor function of the Glut 3 gene was situated between two gene-activating domains (23). Hence, the present study was carried out to extend our previous findings. First, we confirmed in vitro that Sp1 and Sp3 present in the postnatal day-1 and −21 mouse brain mimicked the Sp1 and Sp3 of the neuroblastic and trophoblastic cell lines and bound the Glut 3 gene. Next we characterized the trans-activator protein that bound to the −203- to −177-bp activating region (region A) of the Glut 3 gene that could potentially interact with Sp3 that binds downstream, thereby activating gene expression. In addition, we characterized the trans-activating protein that bound the Glut 3 gene downstream from the repressor sequences and activated gene expression (region C).

It is very well established that genes lacking a typical “TATA” box in their promoter sequence depend on multiple upstream regulator sequences for their activation (35). The murine Glut 3 gene does not possess a TATA box but has a GC-rich promoter region (23), suggesting the possibility that the Sp family of proteins may interact with this promoter region (36–42). Our present investigation demonstrates that both Sp1 and Sp3 are present in nuclei obtained from the 1- and 21-day-old mouse brains, at a developmental stage beyond neurogenesis or neuronal proliferation (14–16). Both of these nuclear proteins bind the Glut 3 gene exactly in the same manner as described previously (23) in N2A neuroblastoma cells. Although both Sp1 and Sp3 bind the Glut 3 promoter, both of these proteins demonstrate opposing effects on GLUT 3 expression in neurons/brain (23). Various other systems have demonstrated that Sp1 can function as an activator of genes, whereas Sp3 competing for the same DNA-binding site represses gene activation (43, 44). Yet in other systems, Sp3 has been observed to be the activator, being synergistic or antagonistic to the action of Sp1 (45–47). Whether the Sp family of proteins function as gene activators or repressors depends on the number of Sp-binding sites present in the promoter region. The presence of a single Sp1 site that is capable of interacting with Sp3 (45, 48) results in Sp3 not functioning as a gene repressor, whereas multiple sites result in Sp3 acting as a repressor (44, 47). In the case of GLUT 3, a single Sp-binding site capable of interacting with Sp3 is present in the immediate 5'-flanking region. Thus, Sp3 functions as an activator, whereas Sp1 functions as a repressor of the Glut 3 gene expression (23). Sp1 suppresses other genes as well, such as the γ-globin gene activation when a site-specific cytosine in the transcription factor-β) similar to the induction by insulin observed in other cell systems (56, 57).

CRED, a basic leucine zipper transcription factor, binds the cAMP-response element sequence or occasionally the AP-1 site to activate gene expression (58–60). On binding of cAMP, protein kinase A translocates to the nucleus and phosphorylates CREB on serine 133, which can result in transcriptional activation of CREB. In addition, phosphorylation of serine 133 can occur by Ca2+-activated calmodulin kinase, ribosomal S6 kinase 2, or mitogen-activated protein kinase. Thus numerous upstream signaling factors can lead to the phosphorylation of CREB. Although many factors phosphorylate CREB at serine 133, calcium influx phosphorylates serine 133 and two additional sites in mice (143, 144, and 145 (61)). Phosphorylation of CREB at these different sites may have important physiological consequences. Phosphorylation at the serine 133 leads to the recruitment of the CREB-binding protein (CBP) to the genome; however, the subsequent phosphorylation of serine 142 and serine 143 via the calcium-activated pathways prevents the interaction of CREB with CBP (58, 61). Variance in how CREB is being phosphorylated determines those co-regulatory proteins that are recruited to the transcriptional complex. The formation of unique transcriptional complexes may be a mechanism by which CREB can induce different patterns of gene transcription within the brain (58, 61–64). CBP also increases genomic activity by acetylation histones, leading to a reduction in higher order chromatin structures and making DNA more accessible to transcription factors (65, 66). CBP also enhances transcription by bridging transcriptional factors, such as CREB, to the basal transcriptional complex (65, 66). Although we did not specifically investigate the role of CBP in regulating GLUT 3 transcription, we observed that Ser-133 phosphorylation led to enhanced binding of CREB to the enhancer AP-1 site of the Glut 3 gene (within a linearized DNA fragment and chromatin), thereby trans-activating gene expression. Furthermore, employing siRNAs against CREB in transient transfection experiments resulted in a suppression of endogenous CREB protein concentrations and decreased GLUT 3 transcription in neurons.

CREB is believed to play a vital role in signal transduction that promotes survival and differentiation of neurons (67). β-Adrenergic receptors that mediate the action of dopamine and norepinephrine in neurons increase intracellular cAMP concentrations that activate protein kinase A. In contrast, peptides such as nerve growth factor and brain-derived neurotrophic factor (68, 69), N-methyl-D-aspartate, or the excitatory γ-aminobutyric acid neurotransmitters (69) trans-activate CREB by phosphorylation via the calcium/calmodulin-dependent kinase IV and a mitogen-activated protein kinase (68, 69). Phosphorylation of CREB leads to induction of downstream early genes such as c-fos (70), zif/268 (71), hsp70 (72), and bcl-2 (73) that may either increase biosynthesis of neurotrophic factors or block cell death pathways (69, 73). c-Fos induces biosynthesis of neurotrophic factors by increasing activator protein-1 (AP-1) binding activity (75). Heat shock protein 70 acts as a molecular chaperone that prevents the aggregation of denatured proteins and promotes the refolding of damaged polypeptides. Bcl-2 is an anti-apoptotic protein that blocks the mitochondrial permeability transition pore (73). Formation of mitochondrial permeability transition under adverse conditions, such as mitochondrial calcium overload and reactive oxygen species, permits the release of cytochrome c and apoptotic inducing factor into the cytoplasm that subsequently trigger cell death pathways, eventually causing cell death either by apoptosis or by necrosis (73, 74).

Phosphorylation of CREB is also proposed as a "memory
molecule.” Disruption of the regulatory subunit of cAMP-dep-
dendent protein kinase specifically impairs late phase tran-
scription-dependent long term potentiation and the perform-
ance in learning tasks (75), whereas conditional knock out of
CREB has led to some contradictory results due to redundancy
because of the presence of closely related isoforms (76). Regard-
less, phosphorylated CREB is a key molecule in activating gene
expression in neurons that mediate critical physiological proc-
esses. CREB is a transcription factor in control of many genes
related to synaptic plasticity, long term potentiation, cell sur-
vival, and differentiation (73–75). In the present report we
have established that CREB binds GLUT 3 DNA and enhances
its expression in neurons both in vitro and in vivo. This is the first
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exists at both postnatal stages examined, namely day 1 and 21.
Thus, pCREB-activated GLUT 3 in neurons may play a role in
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The protein that binds the downstream Glut 3 gene-activat-
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MSY-1, which is developmentally regulated and functions in
regulating storage and translation of germ cell RNAs (79, 80).
MSY-1 is responsible for binding to a single strand of DNA in
the 5′-flanking region of a gene and thereby enhancing the
ability of this region to bind other transcription factors (28).
MSY-1 demonstrates an affinity to asymmetric polypurine-
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