Sp1 and Sp3 Regulate Transcriptional Activity of the Facilitative Glucose Transporter Isoform-3 Gene in Mammalian Neuroblasts and Trophoblasts*

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The murine facilitative glucose transporter isoform 3 (Glut 3) is developmentally regulated and is predominantly expressed in neurons and trophoblasts. Employing the primer extension and RNase protection assays, the transcription start site (denoted as +1) of the murine Glut 3 gene was localized to 305 base pairs (bp) 5’ to the ATG translation start codon. Transient transfection assays in N2A, H19-7 neuroblasts, and HRP.1 trophoblasts using sequential 5’-deletions of the murine Glut 3-luciferase fusion gene indicated that the −203 to +237 bp region with reference to the transcriptional start site contained promoter activity. Repressor function was limited to the −137 to −130 bp region within the transcriptional activation domain. The nuclear factors Sp1 and Sp3 bound this GC-rich region in N2A, H19-7, and HRP.1 cells. Diphosphorylation of Sp1 was essential for Glut 3 DNA binding. The related Sp3 protein also bound this same region of mouse Glut 3 in all three cell lines. Mutations of the Sp1-binding site employed in transient transfection and mobility shift assays confirmed the nature of the DNA-binding proteins, whereas supershift assays with anti-Sp1 and anti-Sp3 IgGs characterized the differences in the two DNA-binding proteins. Co-transfection of the Glut 3-luciferase fusion gene with or without mutations of the Sp1-binding site along with the Sp1 or Sp3 expression vectors in Drosophila SL2 cells confirmed a reciprocal effect, with Sp1 suppressing and Sp3 activating Glut 3 gene transcription.

Facilitative glucose transporters (Glut)1 are a family of structurally related, membrane-spanning glycoproteins that mediate the transport of glucose across bilipid layers of cell membranes along a concentration gradient (1, 2). Of the six major isoforms cloned to date, Glut 3, the most efficient isoform with a Km of 1.8 mM (1), is predominantly expressed in cells with a high energy requirement. These cells include neurons of the brain (3) and retina (4) and placental trophoblasts (5). Various studies have examined the response of Glut 3 secondarily to differing perturbations. In the rat brain, there is an increase in Glut 3 expression under chronic insulin-induced glucose deprivation (6), hyperglycemia of diabetes mellitus (7, 8), water deprivation (7), hypoxic ischemia (9), and depolarization (10). In streptozotocin-induced diabetes, an increase in rat placental Glut 3 levels (11) has also been demonstrated.

Cell-specific localization has revealed that Glut 3 is predominantly expressed in neuronal processes at a stage in development when most neuronal cells are fully differentiated (3). Prior to the mature stages of development, little to no Glut 3 is noted in cells with a neuronal phenotype (3). In placental trophoblasts, Glut 3 is primarily localized to the cells on the fetal surface of the materno-fetal barrier (5), and the levels of this protein increase during late gestation when fetal growth is maximal (12). In both cell types, Glut 1, the isoform that is ubiquitously found in all proliferating cells (1, 2), is replaced by Glut 3 when the cells begin differentiating to attain their mature functional form (3, 5, 12). This developmental expression of Glut 3 in brain and placenta is not unlike the other glucose transporter isoforms, namely Glut 4 in insulin-sensitive tissues (13) and Glut 2 in the liver (13), which replace Glut 1 when the cells in these tissues stop proliferating and begin differentiating into the adult phenotype.

Recent studies using antisense oligoprobes in producing a Glut 3 null mutant mouse resulted in abnormal blastocyst differentiation leading to an arrest in embryonic development (14). This observation attests to the critical need for Glut 3 during the early stages of embryonic differentiation. Thus Glut 3 at this stage of development cannot be effectively replaced by any other related glucose transporter isoform (Glut 1), supporting its indispensable role in cell development, particularly in differentiation of high energy-requiring cell types (3, 5, 14).

To date, the promoter region and the transcriptional machinery of the Glut 3 gene have not been characterized. Cis-elements and trans-activating factors that confer tissue specificity have been identified in the case of other facilitative glucose transporter isoforms such as Glut 1 (15), Glut 2 (16), and Glut 4 (17). We undertook the present study to identify cis-elements and transactivating factors involved in regulating Glut 3 expression in neuroblasts and trophoblasts. We employed the murine Glut 3 genomic sequences and two cell lines arising from different species that express the neuroblastic phenotype along with an embryonic trophoblastic cell line (positive control) and COS-7 cells (negative control). We identified the cis-elements (bp −203 to +237), which activate Glut 3 transcription, and further demonstrated that suppression of the Glut 3 transcriptional activity resided (bp −137 to −130) within this activating region. These suppressive cis-elements bound Sp1 and Sp3 in neuroblasts and trophoblasts. Both of these nuclear factors reciprocally modify Glut 3 gene transcription in the Sp-deficient Drosophila SL2 cells. Thus, in cultured proliferating neuroblasts and trophoblasts, Sp1 possibly represses while Sp3 activates murine Glut 3 transcription.
EXPERIMENTAL PROCEDURES

Cells—N2A murine neuroblastoma cells (American Tissue Culture Collection, Rockville, MD) were grown at 37 °C with 95% air, 5% CO2 in 10 μg/ml poly-l-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, COS-7 cells (monkey kidney fibroblasts) (American Tissue Culture Collection) were grown directly on unmodified culture flasks under similar conditions. An immortalized rat embryonic hippocampal neural cell line with a neuroblastic phenotype (H19-7) that has previously (18) been characterized with neuronal markers was also maintained under the same culture conditions with the addition of G418 for selection of the immortalized cells. Mid-gestation trophoblastic cells obtained from the rat placenta were grown and maintained in RPMI medium with 10% fetal bovine serum (Bio-Rad dye-binding assay (23)). Western blot analysis was carried out as described previously (19).

RNA Studies—Poly(A)^+ enriched RNA was extracted from confluent cultured N2A, COS-7, H19-7, and HRP-1 cells obtained from a single 175-cm tissue culture flask (~1 × 10^7 cells) as per the manufacturer's instructions using the MiniRiboSep extraction kit (Collaborative Biomedical Products, Bedford, MA). The extracted RNAs were subjected to Northern blot analysis as described previously (20). A ^32P-randomly primed 1.5 kb XhoI and XbaI fragment of the murine Glut 3 cDNA (21) (Oligolabeling kit, Amersham Pharmacia Biotech) served as the probe. Interlane loading variability was standardized by rehybridization of the primed 1.5 kb fragment to the same blot. The hybridization between the mRNA and the labeled oligonucleotide was quantified by a phosphorimager (Molecular Dynamics). Western blot analysis was carried out as described previously (23).

Protein Studies—Thirty to fifty μg of either cellular homogenate or extracellular medium was solubilized in 50 μl Tris, pH 7.5, containing 2% SDS, and the protein concentration was determined by the Bio-Rad dye-binding assay (23). Western blot analysis was carried out as described previously (23). The primary antibody consisted of an affinity-purified rabbit anti-mouse Glut 3 IgG, which was generated against the keyhole limpet-linked terminal 17 amino acids of the mouse Glut 3 protein. Following characterization of tissue and isofrom specificity (data not shown), we carried out immunoblotting of protein samples transferred to the nitrocellulose filters. The primary anti-mouse Glut 3 antibody was used at 1:500 dilution, and the incubation was carried out at room temperature for 16 h. Mouse Glut 3 peptide (0.1 μg) preabsorbed rabbit anti-mouse Glut 3 antibody served as the negative control. To detect nuclear proteins, the rabbit anti-synthetic human Sp1 peptide (amino acids 436–454, anti-synthetic human Sp3 (amino acids 676–685 in the C-terminal region) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-rat CAMP response element-binding protein (Upstate Biotechnology, Inc., Lake Placid, NY), and anti-mouse c-Jun (amino acids 247–263) (Santa Cruz Biotechnology) IgGs were used at a 1:500 dilution. The corresponding peptides (2 μg) (Santa Cruz Biotechnology) were used to preabsorb and saturate the IgGs, where indicated. ^32P-labeled goat anti-rabbit secondary antibody (NEN Du Pont) (1:500) was used to indirectly label the primary antigen-antibody complex. Autoradiography of the filters was carried out for optimal lengths of time to maintain linearity of the signal.

Primer Extension Assay—Primer extension was carried out as described previously (24). An antisense oligonucleotide (Cruchem Inc., Dulles, VA) complementary to bp 299–320 of the murine Glut 3 mRNA (CTTCGTTGCTCCCATGTTGCCCA) was end-labeled with [γ-^32P]ATP (Du Pont) and T4 polynucleotide kinase. −5 × 10^6 cpm (50 fmol) of the labeled oligonucleotide and five μg of poly(A)^+ mRNA were dissolved in 1 μl of the hybridization buffer (80% formamide, 0.4 M NaCl, 40 mM NaCl, 40 mM PIPES, pH 6.8, and 1 mM EDTA) at 95 °C for 10 min. Hybridization between the mRNA and the labeled oligonucleotide was accomplished for 16 h at 42 °C. Reverse transcription was initiated by adding 30 units of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) to the mRNA/oligonucleotide mixture in 20 μl of a mixture consisting of 50 μM Tris, pH 8.3, 6 mM MgCl2, 40 mM KCl, 10 units of RNasin, 0.625 mM dNTPs, and the reaction was carried out at 42 °C for 2 h. The primer extended products were purified using Jetsorb (Genomed Inc., Research Triangle Park, NC) and separated on 8% polyacrylamide gels.

To demonstrate that the transcription start site did not differ in the Glut 3 5'-flanking sequence-luciferase fusion gene, primer extension was carried out with mRNA extracted from N2A cells transfected with the −203 to +305 bp Glut 3-Luc construct (Fig. 2A) and ^32P-labeled luciferase gene-specific primer GL2 (5' -CTTATGGTTTGTGGCCT-GT-TCCA) (Promega).

RNAse Protection Assay—RNAse protection assay was carried out as per the manufacturer's instructions (Ambion, Austin, TX). A 440 bp fragment spanning from bp −203 to +237 of the mouse Glut 3 gene was amplified by polymerase chain reaction and inserted into pGEM3zf(+) at KpnI and HindIII sites. ^32P-Labeled antisense RNA probe was synthesized using the Sp6 polymerase and a riboprobe kit (Promega). About 50,000 cpmp (~0.5 fmol) of gel-purified riboprobe was hybridized overnight at 45 °C to 5 μg of mRNA from N2A, HRP-1, and H19-7 cells in a hybridization buffer containing 80% formamide, 100 mM sodium citrate, pH 6.4, 300 mM sodium acetate, pH 6.4, and 1 mM EDTA. The hybrid was digested with 200 μl of a 1:50 dilution of RNase A (250 units/ml) and labeled T1 (10,000 units/ml) for 90 min at 37 °C prior to analysis on a 5% polyacrylamide gel.

Isolation of the Murine Glut 3 Genomic Clone—0.5 × 10^6 phage of a mixture consisting of 50 mM Tris, pH 8.3, 6 mM MgCl2, 0.4 M NaCl, 40 mM PIPES, pH 6.8, and 1 mM EDTA (15 s) in 5% polyacrylamide gel.

Transient Transfection and Reporter Expression Assays—A 1.8-kb fragment of the mouse Glut 3 gene spanning the bp −1553 to +237 region was amplified by polymerase chain reaction and subcloned into an enhancerless and promoterless luciferase reporter gene-containing vector (pGGL2-basic; Promega). Subsequently, serial 5'-deletional mouse Glut 3-Luc fusion gene constructs were created by using a polymerase chain reaction-based strategy employing primers outlined in Table I. The sequence and orientation of the individual clones was confirmed by the dideoxynucleotide chain termination sequencing method (25). The sequence information was managed using the MacVector version 5.0 sequence analysis program (Oxford Molecular Group, Campbell, CA).

Transient transfection of cultured cells was carried out by the liposome-mediated technique (26). Essentially, 5 μg of the pGGL2 vector containing the different 5'-deletional DNA constructs was incubated at room temperature for 30 min with Lipofectin (25 μl) (Life Technologies, Inc.) and 200 μl of serum-free Dulbecco's modified Eagle's medium. After thorough washing with Dulbecco's modified Eagle's medium, the cells were exposed to this preincubated DNA-lipofectin complex. pGL-Tk plasmid DNA (thymidine kinase promoter-driven Renilla luciferase, 0.5 μg; Promega) was cotransfected with each individual transfectant to standardize the results for transfection efficiency.

The luciferase reporter activity was assessed by the dual luciferase assay (Promega). Briefly, 36–48 h post-transfection, the cells were washed with phosphate-buffered saline and lysed using 0.5 ml of lysis buffer (Promega). The supernatant was removed, and 10,000 rpm for 10 min was moved at −70 °C until analysis. Twenty μl of this cell lysate was mixed with 100 μl of the luciferase assay buffer, and the luciferase activity was measured as light output (15 s) in a Monolight 2000 luminometer (Analytical Luminescence, San Diego, CA) (27). 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 luciferase activity as a percentage of the SV40 promoter-driven luciferase activity that served as the positive control in every transfection experiment.

Electromobility Shift Assays—Nuclear extracts from the N2A, H19-7, and HRP-1 cells were prepared as described by Wildeman et al. (28). Routinely, 5 × 10^5 cells were retrieved by a rubber policeman and

| DNA construct | Sequence information | Nucleotide (bp) | Orientation |
|---------------|----------------------|---------------|------------|
| −1553G3-Luc   | GCCATCGTTAGATGCTCG  | −1553 to −1537 |sense       |
| 203G3-Luc     | CACTAAAGGCAGACTGA   | −203 to −186   |sense       |
| −177G3-Luc    | CTGGTGTAGACGTTATG   | −177 to −160   |sense       |
| −104G3-Luc    | TCTGTAACAAAAAGGCTG  | −104 to −85    |sense       |
| +221G3-Luc    | AGGCGGCTGGCGGCGG    | +221 to +236   |sense       |
| +100G3-Luc    | GTTACCCTGCCAGAGAG   | +100 to +104   |sense       |
| +331G3-Luc    | CAGGATGAAGGGCTTG    | +331 to +315   |Antisense   |

**Table I**

Primers used in polymerase chain reactions to amplify portions of constructs used in the transient transfection experiments.
suspended in 10 mM Hepes, pH 7.8, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, and 0.5 mM phenethylsulfonyl fluoride at 4 °C. The cells were homogenized using an all glass Dounce homogenizer with pestle B. The disruption was monitored microscopically, and the isolated nuclei were collected as a pellet upon centrifugation at 10,000 rpm for 30 min. The nuclear pellet was resuspended and incubated on ice for 15 min in four volumes of a high salt buffer (20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 mM phenethylsulfonyl fluoride, 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 (0.33 g/ml). The nuclear protein extract pellet was resuspended in a minimal volume of 10 mM Hepes (pH 7.9), 100 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 mM phenethylsulfonyl fluoride, and 17% (v/v) glycerol and dialyzed at 4 °C against 50 volumes of the 10 mM Hepes buffer. The nuclear proteins were separated from the (NH₄)₂SO₄ by centrifugation at 15,000 rpm for 15 min, and the supernatant was stored in aliquots at −70 °C. The concentration of the solubilized nuclear protein was measured by the method of Bradford (23).

Synthesized double-stranded oligonucleotides were end-labeled with [γ-³²P]ATP and T4 polynucleotide kinase. Approximately 6 fmol of the labeled DNA oligonucleotide was added to 5 μg of nuclear extract in a final volume of 20 μl containing 1 μg of poly(dI-dC), 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 1 mM MgCl₂, 4% glycerol, and 1 mM dithiothreitol and incubated for 15 min at room temperature. Subsequently, the DNA-protein complexes were separated from the free DNA by electrophoresis through a 5% nondenaturing polyacrylamide gel in a 90 mM Tris borate, 2 mM EDTA buffer (29). The gels were dried and subjected to autoradiography in the presence of intensifying screens (DuPont) at −80 °C. Competition experiments included the addition of a 10–100-fold excess of unlabeled DNA oligonucleotides, while supershift analysis included the addition of 2 μg of the respective antibody to the luciferase reporter assays. In another set of experiments, the N2A nuclear extracts were incubated with okadaic acid (50, 100, or 200 nM) (Life Technologies, Inc.) for 5 min prior to the addition of recombinant human Sp1 (0.25 footprint unit) followed by the mobility shift assay (Table II).

Okadaic Acid Experiments—N2A cell nuclear extracts (5 μg) and human recombinant Sp1 (1 footprint unit) were incubated together or separately with the radiolabeled bp −149 to −124 region of the mouse Glut 3 gene prior to analysis by the mobility shift assays. In a separate set of experiments, the N2A nuclear extracts were incubated with okadaic acid (50, 100, or 200 nM) (Life Technologies, Inc.) for 5 min prior to the addition of recombinant human Sp1 (0.25 footprint unit) followed by the mobility shift assay.

NDAse I Footprinting Analysis—The linearized bp −203 to +320 of the 5′-flanking region of the mouse Glut 3 gene was end-labeled with [γ-³²P]ATP and T4 polynucleotide kinase. Approximately 50,000 cpm (0.5 fmol) of the end-labeled mouse Glut 3 probe was preincubated with 25–30 μg of the nuclear protein extract in the mobility shift buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 1 mM dithiothreitol, 4% glycerol), and the DNA-protein complex was subjected to partial DNase I digestion (25 ng) (30). The G and G + A ladders were generated by the Maxam-Gilbert chemical sequencing method (31).

Co-transfection Experiments in the Drosophila Schneider Cells—Co-transfections were carried out in Drosophila Schneider cells (American type culture Collection), which were maintained at room temperature in modified Schneider’s Drosophila medium (Life Technologies) supplemented with 10% fetal bovine serum and antibiotics. Cells were seeded at 2 × 10⁶/cm² and then transfected with DNA parasites. The transfection efficiency was determined by the luciferase reporter gene. A 440-bp fragment of the 5′-flanking region of the Glut 3 gene, devoid of the exon 1-intron 1 junction was inserted immediately upstream of the luciferase reporter gene contained in the promoterless expression vector pGL2basie. This fusion construct (−203G3-Luc) exhibited significant luciferase activity when transiently transfected into the N2A, HRP-1, and H19-7 cells. We then confirmed the transcriptional start site to be similar in the transiently transfected mouse Glut 3-luciferase fusion constructs to that of endogenous mouse Glut 3 (Fig. 1E) by primer extension.
Computer analysis of the bp -203 to +237 fragment of the mouse Glut 3 gene revealed an array of putative nuclear factor binding sites, which include four AP1 sites, a single Sp1 binding site, four CAAT boxes, and three CTF/NF1 binding sites (Fig. 2A). No putative TATA box was detected 5' to the transcription start site.

Whereas the -203G3-Luc fusion construct (-203 to +237 bp) demonstrated promoter activity, deletion of 26 nucleotides from bp -203 to -177 created the -177G3-Luc fusion construct (-177 to +237 bp), which led to a significant decline in the transcriptional activity. However, this loss of transcriptional activity was reversed in the -177G3-Luc construct, thereby indicating that the bp -177 to -104 region of the mouse Glut 3 gene possessed repressor function (Fig. 2B). Fig. 2C depicts the results of transient transfection experiments using the same deletional constructs in H19-7, HRP.1, and COS-7 cell lines. The promoter activity and repressor function carrying DNA sequences were identical to that noted in N2A, with the existence of minor inter-cell type variability in the level of luciferase activity observed, with high levels of activation noted in the N2A and HRP.1 cells and lower activity in the rat embryonic hippocampal H19-7 cells. These results paralleled the cell-specific levels of Glut 3 mRNA and protein (Fig. 1, A and B).

Computer analysis of the bp -177 to -104 nucleotide sequence revealed the presence of a single consensus binding site for Sp1 (bp -137 to -130). A role for factors binding the Sp1-binding site in suppressing mouse Glut 3 promoter activity was revealed by observing a restoration of the transcriptional activity in the absence of the bp -203 to -177 region with a mutated Sp1-binding region (-177Sp1-M). However, the inclusion of the bp -203 to -177 region along with a mutant Sp1 binding region (-203Sp1-M) demonstrated the opposite effect, i.e. a 50% suppression in Glut 3 transcription (Fig. 2D). These observations
FIG. 2. Transient transfection assays. A, top panel, sequence information of the 5' flanking region of the mouse Glut 3 (bp −203 to +320) gene. The major transcription start site is shown as +1, and the Sp1 DNA-binding site is underlined and in boldface type. The four AP1 sites are italicized. Bottom panel, a schematic representation of the putative binding sites for DNA-binding proteins in the 5' flanking region of the mouse Glut 3 gene extending from bp −2203 to +1320. The bp −2203 to +1320 region was analyzed for potential putative protein binding motifs by the MacVector software (Oxford Molecular Group, Campbell, CA). These sites are presented in reference to the major transcriptional start site, which is shown as +1. The filled boxes represent consensus AP1-binding sites, while the single Sp1 binding site is shown as an open box. B, sequential 5' deletions created by polymerase chain reaction using primers depicted in Table I followed by subcloning these deletions 5' to the enhancerless and promoterless luciferase gene (pGL2-B). The luciferase activity in N2A cells following correction for transfection efficiency (pRL-Tk co-transfections) was expressed as a percentage of the luciferase gene activity driven by the SV40 promoter (positive control) and is depicted as the mean ± S.E. of three independent experiments performed in duplicate. The arrows indicate the orientation of Glut 3 DNA relative to the direction of Glut 3 gene transcription; exon 1 is indicated by the stippling and the luciferase gene by the crisscross pattern. *p < 0.05 when compared with background (pGL2-basic); †p < 0.05 when compared with DNA constructs −203G3-Luc and −104G3-Luc. C, sequential 5' deletions created by...
collectively confirm that the Sp1-binding region possesses a suppressive function in Glut 3 gene transcription in the absence of the activating bp –203 to –177 DNA elements, while elements in the bp –203 to –177 region activate Glut 3 transcription.

To identify and characterize potential protein binding activity associated with the bp –137 to –130 region, an electromobility shift assay was used. An oligonucleotide encompassing the putative Sp1 binding sequence of the mouse Glut 3 gene (bp –149 to –124) was used as the probe in the mobility shift assay. Nuclear extracts from the three cell types (i.e. H19-7, N2A, and HRP.1) led to three band shifts (Fig. 3A). Competition studies in the presence of unlabeled oligonucleotides corresponding to known consensus DNA-binding regions revealed specific displacement with the bp –149 to –124 region of the mouse Glut 3 gene and the Sp1 consensus sequence (Fig. 3B). A dose-dependent displacement of the band shifts with the unlabeled bp –149 to –124 region of the mouse Glut 3 established a relatively high affinity binding of nuclear factor(s) to this region (Fig. 3C). No such displacement occurred with an excess of unlabeled CTF/NF1, AP-1, cAMP response element, OCT-1, TFIID, GRE, and NF-κB consensus sequences (Fig. 3B).

Since the Sp1 consensus sequence is known to bind Sp1 and other related proteins, particularly Sp3, we attempted to further confirm the identity of the protein(s) that bound the bp –149 to –124 sequence by supershift assays. While anti-Sp1 antibody supershifted upper band shift (I; Fig. 4), the anti-Sp3 antibody led to a supershift of the lower band shift (2; Fig. 4) in all three cell types. These reactions were specific, since there was no shift in the presence of either corresponding peptide preabsorbed antibodies or anti-cAMP response element-binding protein or anti-c-Jun/AP1 antibodies (Fig. 4). When mutant oligonucleotides in which the Sp1 binding site was altered were used, such band shifts were not observed. Anti-Sp1, anti-Sp3, anti-cAMP response element-binding protein, or anti-c-Jun/AP1 antibodies failed to demonstrate any supershifts in this case (data not shown).

The DNA binding ability of Sp1 present in the N2A nuclear extracts was compared with that of the human recombinant Sp1 by mobility shift assays. While the Sp1 present in the N2A nucleus demonstrated distinct band shifts and a supershift in the presence of the anti-Sp1 IgG, the recombinant Sp1 did not bind the Sp1-binding site of the mouse Glut 3 gene at all. In contrast, the addition of recombinant Sp1 along with N2A nuclear extracts enhanced the density of the band shifts (Fig. 5A) that were noted with N2A nuclear extracts alone. Using the Sp1 consensus DNA sequence as the probe, human recombinant Sp1 and N2A nuclear extracts demonstrated DNA binding as evidenced by band shifts. The added presence of recombinant Sp1 and N2A extracts demonstrated maximal binding to the consensus sequence (Fig. 5B). Mobility shift assays with the radiolabeled –149 to –124 bp region of the mouse Glut 3 gene in the presence of recombinant Sp1 and N2A nuclear extracts when incubated with okadaic acid (a phosphatase 2A inhibitor) demonstrated a dose-dependent decline in the density of the band shifts, with maximal inhibition observed at 200 nM (Fig. 5C). These results support the necessity of Sp1 dephosphorylation for Glut 3 DNA binding.

Further DNase I footprinting assays confirmed that the Sp1 sequence of the mouse Glut 3 gene was protected from partial DNase I digestion by N2A nuclear extracts (Fig. 6A). Western blots with nuclear protein extracts from all three cell types revealed the presence of the 95–105-kDa Sp1 protein (Fig. 6B) and the 97- and 67-kDa Sp3 proteins (Fig. 6B).
In incubation with the recombinant human Sp1 protein ( footprinting assay using the bp 149 to 124 region of the mouse Glut 3 as the oligoprobe alone (lane 1), incubated with the N2A nuclear extract (5 µg) in the absence (lane 2) or presence (lane 3) of the anti-Sp1 IgG, incubated with the recombinant human Sp1 protein (1 footprinting unit) or -177G3-Luc constructs yielded divergent results in the case of Sp1 and similar results in the case of Sp3. When the effecter DNA encoding Sp1 was used with the -203G3-Luc, a suppression of Glut 3 transcription in Drosophila Schneider cells was noted, while no additional change was observed with the -177G3-Luc construct. These results indicate that the suppressive effect of Sp1 is dependent on the presence of putative trans-acting factors interacting with the DNA sequence between bp -203 and -177. In contrast, in the presence of the Sp3-encoding sequences, an activation of the -203G3-Luc and -177G3-Luc target DNA transcription occurred (Fig. 6C), suggesting that for Sp3 to act as an activator the bp -203 to -177 sequence is not essential. Co-transfection experiments with target DNA containing mutations of the Sp1-binding site (-203Sp1-M or -177Sp1-M) in the presence of effecter DNA encoding Sp1 led to no modification of the basal transcriptional activity, confirming that Sp1 acts via the Sp1 binding sequence. In contrast, effecter DNA encoding Sp3 did not further alter the transcriptional activity of the -177Sp1-M target DNA but led to a 3-fold increase in transcription with the -203Sp1-M DNA construct (Fig. 6D). These results indicate that the activity of Sp3 is dependent on the trans-acting factor interacting with the DNA sequence between bp -203 and -177, that Sp3 does not act through the Sp1 binding site, and that interaction with Sp1 is not essential for Sp3's activity.

**DISCUSSION**

In the present study, we have identified certain cis-elements involved in activating the Glut 3 murine gene; these elements extend from -203 to +237. Similar to other genes that are devoid of a TATA box in the conventional site, the murine Glut 3 demonstrates GC-rich sequences in the regulatory region (34–36). The sequences between bp -203 and -177 demonstrate gene transcriptional activity, and so did the sequences between bp -103 and +237.

Amid the mouse Glut 3 gene transcriptional activation domain lies a cis-element that exhibits suppression of gene transcription. This repressor domain has been identified to be the only Sp1-binding region present within 130 bp from the transcription start site. Sp1, the ubiquitous nuclear factor that consists of zinc finger domains that bind guanine-rich DNA-binding sites (5’-GGGCGGGGCGG-3’) (37, 38), transactivates gene transcription in most proliferating cells examined, by acting as a promoter-selective transcription initiation factor in vitro (34–36, 39–41). It was initially identified as a factor from HeLa cells that selectively activates in vitro transcription from the SV40 early promoter (42) and binds to the multiple GC boxes in the SV40. While suppression of Sp1 activity by forming inactive non-DNA-binding complexes with other nu-

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**Fig. 5. Sp1 binding mobility shift assays.** A, mobility shift assay (left panel) using the bp -149 to -124 region of the mouse Glut 3 gene as the oligoprobe alone (lane 1), incubated with the N2A nuclear extract (5 µg) in the absence (lane 2) or presence (lane 3) of the anti-Sp1 IgG, incubated with the recombinant human Sp1 protein (1 footprinting unit) in the absence (lane 4) or presence of the anti-Sp1 IgG (lane 5), or incubated with a combination of the N2A nuclear extract and the recombinant human Sp1 (lane 6). Densitometric quantitation of the DNA-protein complex seen as band shifts is depicted on the right, * p < 0.05 when compared with shift seen with N2A alone. B, mobility shift assay (left) using the Sp1 consensus sequence as the probe alone (lane 1), incubated with the recombinant human Sp1 (lane 2), incubated with the nuclear extracts from N2A cells (5 µg) (lane 3), or incubated with a combination of recombinant human Sp1 (1 footprinting unit) and the N2A nuclear extract (5 µg) (lane 4). The right part demonstrates densitometric quantitation of the Sp1-bound complex seen as a band shift. *, p < 0.05 versus N2A-induced band shift. C, mobility shift assay (left panel) using the bp -149 to -124 region of the mouse Glut 3 as the probe (lane 1), incubated with the N2A nuclear extract (5 µg) along with 0 (lane 2), 50 (lane 3), 100 (lane 4), and 200 nM (lane 5) okadaic acid, and incubated with a combination of the N2A nuclear extract (5 µg) and the recombinant Sp1 (0.25 footprinting unit) in the absence of okadaic acid (lane 6). D, densitometric quantitation of the DNA-bound complex seen as the band shift. *, p < 0.05 versus the band shift seen with the N2A nuclear extract alone.
clear factors such as Sp1-I (43) or p107 (44) or disruption of Sp1 binding (45) has been reported, Sp1 has recently also been noted to be involved in the suppression of gene transcription. Direct binding of Sp1 to the Sp1 DNA-binding region in itself has also been observed to suppress gene transcription. Examples of Sp1-directed gene suppression include the human adenine nucleotide translocase 2 gene in human JEG3, HeLa, and mouse NIH3T3 cells (46), the murine Vγ1.1 T cell receptor (47), the g-globin gene upon site-specific cytosine methylation in murine erythroleukemia cells (48), and smooth muscle myosin heavy chain gene in rat aortic smooth muscle cells (49). All of these genes, where transcriptional activity is suppressed by Sp1, were examined in vitro in proliferating cells that had not fully differentiated. In the present study, similar to the myosin heavy chain gene, suppression of the mouse Glut 3 gene is mediated by the Sp1-DNA binding site, which is situated between two gene-activating domains (49).

Recent studies in Drosophila SL2 cells that possess the Drosophila genes but not the mammalian Sp1-related proteins, demonstrated Sp1 to activate transfected genes, while Sp3, by competing for DNA binding with Sp1, brought about the suppression of genes (50). Further, the amino-terminal region of Sp3 tethered to a promoter DNA by connecting to a heterologous DNA-binding protein domain was observed to repress transcriptional activation by different positive regulators (51). Moreover, Sp3 targeted to a promoter-proximal RNA sequence acted as a transcriptional repressor (51). Sp1 and Sp3 were demonstrated to interact with each other in the case of the neuronal nicotinic acetylcholine receptor β4 subunit gene and regulate gene transcription (32). Thus, it appears that while Sp1 can directly suppress gene transcription by DNA binding (46, 48, 49), Sp3, by competing for Sp1 binding or by interacting...
with other nuclear factors, leads to repression of gene transcription (52, 53). In contrast, in the case of the mouse Glut 3 gene in Drosophila cells, Sp1 represses, while Sp3 activates, transcription. Both transcription factors bind the Sp1-binding site directly \textit{in vitro}; however, our studies involving the constructs containing mutations of the Sp1-binding site indicate that the Sp1 binding site is essential for Sp1’s but not for Sp3’s functional activity. Our studies also indicate that interaction with nuclear factors that bind the upstream sequences (bp −203 to −177) appears necessary for the Sp1-mediated repression and Sp3-mediated activation of the mouse Glut 3 gene transcription. Whether a similar interaction and function exist in the neurons and trophoblasts is unknown. Depending upon the levels of endogenous Sp1 versus Sp3, Glut 3 transcription can be modified. Since transient transfection with the bp −177 bp to +1 region of Glut 3 in neuroblasts and trophoblasts reveals transcriptional suppression, Sp1 appears to exert a dominant effect in these cell types.

While Sp1 and Sp3 are ubiquitous nuclear factors, the differences in the level of expression during different stages of development (54, 55) or in varying cell types (55) along with specific post-translational modifications (56) are responsible for altering gene transcription in a development-specific and cell-specific manner. In the murine brain, Sp1 is expressed in the mature motor neurons, in the choroid plexus, in the granular layer of the cerebellum, and in oligodendrocytes within the white matter. Since the brain cellular distribution of Sp1 is not limited to Glut 3-expressing neurons, Sp1-mediated transcriptional modification by itself does not explain the cell specificity of Glut 3 expression (55). In contrast, in the murine embryo, Sp1, Sp3 and other Glut 3 gene transactivating nuclear factors, and other Glut 3 gene responsive factors, and further resolving the role of Sp1 and/or Sp3 in modifying Glut 3 expression during normal development and in disease states.

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