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Factors Involved in *GLUT-1* Glucose Transporter Gene Transcription in Cardiac Muscle*

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Glucose constitutes a major fuel for the heart, and high glucose uptake during fetal development is coincident with the highest level of expression of the glucose transporter GLUT-1 during life. We have previously reported that GLUT-1 is repressed perinatally in rat heart, and GLUT-4, which shows a low level of expression in the fetal stage, becomes the main glucose transporter in the adult. Here, we show that the perinatal expression of GLUT-1 and GLUT-4 glucose transporters in heart is controlled directly at the level of gene transcription. Transient transfection assays show that the −99/−33 fragment of the *GLUT-1* gene is sufficient to drive transcriptional activity in rat neonatal cardiomyocytes. Electrophoretic mobility shift assays demonstrate that the transcription factor Sp1, a *trans*-activator of *GLUT-1* promoter, binds to the −102/−82 region of *GLUT-1* promoter during the fetal state but not during adulthood. Mutation of the Sp1 site in this region demonstrates that Sp1 is essential for maintaining a high transcriptional activity in cardiac myocytes. Sp1 is markedly down-regulated both in heart and in skeletal muscle during neonatal life, suggesting an active role for Sp1 in the regulation of *GLUT-1* transcription. In all, these results indicate that the expression of GLUT-1 and GLUT-4 in heart during perinatal development is largely controlled at a transcriptional level by mechanisms that might be related to hyperplasia and that are independent from the signals that trigger cell hypertrophy in the developing heart. Furthermore, our results provide the first functional insight into the mechanisms regulating muscle *GLUT-1* gene expression in a live animal.

Facilitative glucose uptake in mammalian cells is mediated by a family of glucose transporter proteins (GLUT-1 to GLUT-5) (1). The pattern of expression of these proteins is very complex: GLUT-1 is found in virtually all tissues and seems to be responsible for the basal glucose uptake (2), whereas GLUT-4 is mainly expressed in the peripheral insulin-sensitive tissues (skeletal and cardiac muscle and brown and white adipose tissue) (1). In insulin-sensitive-tissues, insulin is able to induce a rapid increase in glucose uptake, and this effect is due to the recruitment of GLUT-4 glucose transporters from an intracellular pool to the plasma membrane (3). Insulin-sensitive tissues express both GLUT-4 and GLUT-1, although GLUT-4 is the major glucose transporter isoform. Thus, in the rat adipose tissue, 90% of glucose transporters expressed are GLUT-4 (4), and a similar percentage has been observed in skeletal muscle (5). A higher relative expression of GLUT-1 has been observed in rat cardiomyocytes, where GLUT-1 expression accounts for 30% of the total glucose transporters (6).

There are several reports (7, 8) indicating that in the fetal heart, and also in other muscles, glucose consumption is very high during the late fetal stage and that the response to insulin increases postnatally (7). Moreover, the glycogen content in fetal heart and other tissues is higher in near-term fetuses than in the mature rat (9). This may constitute an adaptive trait that would confer protection against hypoxic stress in the fetus during delivery (10). Given that glucose transport is a rate-limiting step for glycolysis (11), it is feasible that maintaining a high level of expression of GLUT-1 would be crucial for the fetus, because a constitutive high glucose transport rate would be ensured this way and would help to overcome any hypoxic events that may arise during birth. Thus, anaerobic metabolism of glucose would fulfill the ATP demand of the fetus during the hypoxic episode. Furthermore, the relevance of maintaining appropriate expression levels of GLUT-1 *in vivo* has been recently highlighted by Seidner *et al.* (12); they attribute the cause of a severe human brain disorder to the existence of mutations in *GLUT-1* gene that reduce the expression of this transporter, which is specially detrimental to the energy metabolism of brain. It has also been pointed out that an increase in glucose availability may be beneficial for reducing the stress during cardiac ischemic episodes (13–15).

The expression of GLUT-1 and GLUT-4 glucose transporters is strongly regulated during the perinatal development of rat heart, skeletal muscle, and brown adipose tissue. We have described (16) that around birth and during the first weeks of neonatal life, glucose transporter expression is characterized by a dramatic change in the accumulations of GLUT-1 and GLUT-4, both mRNA and protein, in rat heart, in skeletal muscle, and in brown adipose tissue. Regarding the signals regulating these processes, we have previously shown (17) that thyroid hormones have an essential role in the maintenance of the postnatal induction of GLUT-4 and the repression of

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GLUT-1 in rat heart. Whether this effect is direct or not is still unknown. In further studies performed in the L6E9 skeletal muscle cell line, we have observed that the differentiation of myoblasts into myotubes is associated with the repression of the expression of GLUT-1 and the induction of GLUT-4 (18). In these cells, the −99/−33 region of the *GLUT-1* proximal promoter drives transcriptional activity of *GLUT-1* and participates in the reduced transcription after muscle differentiation. Furthermore, we have shown that the Sp1 zinc-finger transcription factor is able to bind to a putative binding site in −91/−86 of the *GLUT-1* promoter. Sp1 is able to *trans*-activate the *GLUT-1* promoter in L6E9 cells, and its own expression undergoes down-regulation during muscle cell differentiation and in response to overexpression of the myogenic basic helix-loop-helix factor MyoD (18). Here we show that the changes in GLUT-1 and GLUT-4 expression in rat heart are due, at least in part, to alterations in the transcriptional rate of both genes. Moreover, the −99/−33 region of the *GLUT-1* promoter is also essential for the transcription of *GLUT-1* in rat neonatal cardiomyocytes in primary culture and that mutation of the Sp1 site at −91/−86 region compromises the transcriptional activity. Furthermore, binding of Sp1 to this site can be detected in fetal heart and skeletal muscle nuclear extracts but not in extracts from adult heart and muscle. We also show that this reduced binding is due to a reduced Sp1 protein abundance in nuclear extracts from heart and muscle in the adult. Together, these findings suggest that Sp1 contributes to the high level of expression of GLUT-1 in the fetal heart.

EXPERIMENTAL PROCEDURES

Materials—[α-^{32}P]dCTP, [α-^{32}P]UTP, and [γ-^{32}P]ATP were purchased from ICN, NEN Life Science Products, and Amersham Pharmacia Biotech, respectively. Hybond N+ was from Amersham Pharmacia Biotech, and random primed DNA labeling kit was from Roche Molecular Biochemicals. Immobilon was obtained from Millipore. Most commonly used chemicals were from Sigma. Dulbecco's modified Eagle's medium, fetal bovine serum, glutamine, and antibiotics were obtained from Whittaker (Walkersville, MD). Human recombinant Sp1, a double-stranded oligonucleotide containing an Sp1 consensus binding site, and rRNasin were obtained from Promega (Madison, WI).

Clones prGT3 (which contains the 2572-bp[1] *Eco*RI rat GLUT-1 cDNA insert) and pSM111 (containing the 2470-bp *Eco*RI rat GLUT-4 cDNA insert) were kindly provided by Dr. Morris Birnbaum (University of Pennsylvania, Philadelphia, PA) (19, 20).

Nuclear Run-on—The protocol for the isolation of nuclei was adapted from one previously described (21), although we introduced several modifications, described below. Nuclei were isolated from pooled hearts removed from several litters of anesthetized fetuses or after decapitation of neonates and washed in ice-cold saline. 0.25 g of rat ventricular muscle was homogenized in 25 ml of NA buffer (1 mM Tris-Cl, pH 8, 300 mM sucrose (Merck), 2.5 mM magnesium acetate, 3 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.25% (v/v) Triton X-100, and 40 units of hrRNasin/ml of buffer), with 15 strokes in a motor-driven Potter-Elvehjem homogenizer in the cold-room. The homogenate was filtered through four layers of cheesecloth and mixed with one volume of NB buffer (1 mM Tris-Cl, pH 8, 2.4 M sucrose, 2.5 mM acetate, 3 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.1% (v/v) Triton X-100, and 40 units of rRNasin/ml of buffer). This mixture was layered onto 12-ml cushions of NB buffer and ultracentrifuged in a SW28 Beckman rotor at 27,000 rpm for 60 min at 4 °C. The supernatant and the interphase were discarded, and the nuclei pellet was dislodged with a spatula in 20 ml of NA buffer (without Triton X-100). The nuclear suspension was centrifuged in a SS34 Sorvall rotor for 10 min at 2000 × *g*, and the nuclei resuspended in 1.5 ml of NA buffer (without Triton X-100). Nuclei were counted in a hemocytometer and pelleted at 4000 rpm for 5 min in a microcentrifuge. The nuclei pellet was resuspended in Keller storage buffer (22) containing 200 units of rRNasin/ml, frozen in liquid nitrogen, and stored in 200-μl aliquots of approximately 2 × 10^7 nuclei each at −80 °C until used.

Nuclear run-on reactions were carried out by incubating approximately 2 × 10^7 heart nuclei in a mixture containing 0.625 mM ATP, 0.312 mM CTP, 0.312 mM GTP, 0.625 μM UTP, 0.5 mCi of 800 Ci/mmol [α-^{32}P]UTP, 300 units of hrRNasin/ml, 40 mM Tris-Cl, pH 8.3, 150 mM NH_4Cl, and 12.5 mM $MgCl_2$, in a final volume of 400 μl, for 20 min at 27 °C. 2-μl samples were removed from the reactions at 0, 5, 10, and 20 min in order to calculate the incorporation of [α-^{32}P]UTP into RNA. DNA was digested after the addition of an additional 80 units of hrRNasin to each reaction, with 75 μl of RQ1 RNase-free DNase (Promega) for 20 min at 27 °C, prior to isolation of RNA. This was isolated by pelleting the nuclei at room temperature for 2 min at low speed. The supernatant was discarded, and the nuclei were resuspended in 500 μl of GuSCN solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% *N*-laurylsarcosyne, 0.1 M β-mercaptoethanol). Next, 50 μl of 2 M sodium acetate were added to the nuclei, prior to extracting RNA with 500 μl of water-equilibrated acid phenol and 100 μl of chloroform: isoamyl alcohol (49:1) solution. RNA was precipitated with 1 volume of isopropanol and centrifuged for 5 min at full speed. The RNA pellet was washed in 70% ethanol and resuspended in GuSCN solution, followed by another precipitation in 100 μl of isopropanol. The final RNA pellet was resuspended in 100 μl of TE, pH 8, and 2 μl was counted by liquid scintillation, in order to calculate the activity of the RNA solution.

Detection of GLUT-4 and GLUT-1 newly transcribed RNA was carried out by hybridization of the labeled RNA to membranes onto which the plasmids containing the cDNAs for GLUT-1 and GLUT-4 (10 μg of each per slot) had been previously slot-blotted. Hybridization and washes were performed as described (21). Data are expressed in ppm as described previously (23, 24)

GLUT-1 CAT Reporter Constructs, Transient Transfection, and CAT Assays—Preparation of enriched rat neonatal cardiomyocyte culture has been described previously (25). Briefly, ventricular myocardium of 1–2-day Sprague-Dawley rat neonates was minced and digested with collagenase and pancreatin. Isolated cells were collected by centrifugation, pooled, and then preplated for 30 min on plastic dishes (Primaria, Becton Dickinson) to remove non-myocytes, which attach rapidly to plastic. The supernatant, which contains >95% cardiomyocytes as determined by immunohistochemistry with an anti-sarcomeric actin antibody (26)[2] was used to seed 0.5 × 10^6 cells/well in 35-mm gelatin-coated plastic tissue culture dishes in culture medium (10% horse serum, 5% fetal bovine serum in 4:1 Dulbecco's modified Eagle's medium/199 medium plus 1% (v/v) antibiotics (10,000 units/ml penicillin G and 10 mg/ml streptomycin), 2 mM glutamine, 25 mM HEPES, pH 7.4) containing bromodeoxyuridine (100 μM), in order to stop proliferation of non-myocytic cells.

A series of CAT reporter constructs containing different 5′ deletions of rat GLUT-1 promoter extending to a common 3′-end point at +134 (18) were transfected into cardiomyocytes (10 μg each). Site-directed mutagenesis of the −99/+134 GLUT-1 CAT construct was performed with the QuickChange™ kit from Stratagene, according to the manufacturer's instructions. The oligonucleotide used for generating the mutant construct was 5′-CCTCAGGCCCCGTACCCCGGCCCACC-3′, which contains a two-nucleotide substitution (underlined) in the core of the Sp1 site (see below). The test plasmids were co-transfected with 7.5 μg of β-galactosidase expression plasmid pON239 (27) to normalize for the efficiency of transfection. Cardiomyocytes were transfected on the day following isolation by a calcium phosphate protocol (25, 28) and harvested 2–3 days later for CAT assay (29). For the harvesting of cardiomyocytes and preparation of cytoplasmic extracts, cells were washed twice in phosphate-buffered saline and then lysed in 300 μl of reporter lysis buffer (Promega) according to the manufacturer's instructions. After centrifugation in a microcentrifuge for 5 min at 4 °C, the supernatants were stored at −80 °C.

CAT activity was measured by incubating 75 μl of cytoplasmic extract with 0.1 μCi of [^{14}C]chloramphenicol, 1.3 mM acetyl-CoA, 200 mM Tris-HCl, pH 7.5, for 3.5 h at 37 °C. At the end of the incubation, extraction into ethyl acetate and thin layer chromatography (29) were performed. The CAT activity was quantitated using an InstantImager (Packard Instrument Co.). β-Galactosidase activity was measured as described (30). Data are expressed as percentage of maximum expressing construct and represent the average of at least three independent rounds of transfection.

Electrophoretic Mobility Shift Assays (EMSAs)—Nuclei were isolated from adult rat ventricular muscle for the preparation of nuclear extracts essentially as described above (under "Nuclear Run-on"), although a different homogenization procedure was used, given the par-

[1] The abbreviations used are: bp, base pair(s); hrSp1, human recombinant Sp1; EMSA, electrophoretic mobility shift assay.

[2] P. Burton, personal communication.

ticular features of the tissue. Hearts were removed from 20 male Wistar rats after cervical dislocation and were washed in ice-cold saline. Atria were removed from the ventricles, and three sets of ventricular tissue of 4 g each were finely minced with scissors in 35 ml of NA buffer. The tissue was then homogenized with three strokes of 12 s each at 3000 rpm in a Polytron homogenizer (Kynematica, Littau, Switzerland). The homogenate was centrifuged at 2600 × *g* for 10 min, and the pellets were resuspended in another 35 ml of fresh NA buffer. The suspension was further homogenized in a Potter-Elvehjelm homogenizer with eight strokes of the pestle and then filtered through four layers of cheesecloth. The filtered homogenate was centrifuged again at 2600 × *g* for 10 min. 25 ml of NA buffer supplemented with Triton X-100 was used in order to resuspend the pellet, and the suspension was centrifuged again at 2600 × *g* for 10 min. After this last centrifugation, all three pellets were resuspended in the same 35 ml of NB buffer (see the protocol of the nuclear run-on), and this volume was ultracentrifuged as described under "Nuclear Run-on". The nuclei pellet was saved and processed for the nuclear extraction.

Nuclei from fetal hind limb skeletal muscle were isolated as described under "Nuclear Run-on." However, 2.2 M sucrose was used for making NB buffer. As to adult rat skeletal muscle, nuclei were isolated according to Zahradka *et al.* (31) and Neufer *et al.* (32). Nuclei from L6E9 myoblasts and myotubes were isolated as described (33).

Nuclear extract preparation and EMSAs were performed as described previously (18). The −102/−37 probe was obtained by digesting with *Ava*II the −201/+134 CAT construct, purifying the 66-bp fragment, and filling in the cohesive ends with the Klenow fragment of the DNA polymerase and [α^{32}P]dATP. Both a wild type (wtG1Sp1, 5′-CCTCAGGCCCCGCCCCCCG-3′) and a mutated (mutG1Sp1, 5′-CCTCAGGCCCCGTACCCCG-3′; mutation underlined) oligonucleotide encompassing positions −100 to −82 in the rat GLUT-1 proximal promoter were used as competitors in EMSA. When oligonucleotide wtG1Sp1 was used as a probe, 20 pmol of double stranded oligonucleotide was end-labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (Promega), and 10,000 cpm of the probe was incubated with 5 μg of the corresponding nuclear extracts as described by Viñals *et al.* (18). All competitor oligonucleotides were used at 100-fold molar excess. Supershift experiments were performed as described (18).

Electrophoresis and Immunoblotting—SDS-polyacrylamide gel electrophoresis was performed in accordance with the method of Laemmli (34). Proteins were transferred to Immobilon as reported (35). Transfer was confirmed by Coomassie Blue staining of the gel after the electroblot. An anti-Sp1 affinity-purified rabbit polyclonal antibody (PEP-2, Santa Cruz Biotechnology) was used at a 5 mg/ml dilution in 1% nonfat dry milk, 0.02% sodium azide in phosphate-buffered saline and incubated overnight at 4 °C. Detection of the immune complexes with the rabbit polyclonal antibody was accomplished using the ECL Western blot detection system (Amersham Pharmacia Biotech). Immunoblots were performed under conditions in which autoradiographic detection was in the linear response range.

RESULTS

GLUT-1 and GLUT-4 Transcriptional Activity Is Regulated during Development—We have previously shown (16) that both GLUT-4 and GLUT-1 glucose transporters are strongly regulated during perinatal development in heart, skeletal muscle, and brown adipose tissue. This results in changes to both mRNA and protein accumulation. The above observations led us to consider that the induction of GLUT-4 mRNA and repression of GLUT-1 during perinatal development may be due to transcriptional control. To test this hypothesis, we carried out a series of nuclear run-on experiments. These experiments were performed on nuclei isolated from fetal and neonatal rat heart, and the transcriptional rate of both *GLUT-1* and *GLUT-4* genes was analyzed over a period of time spanning from fetal day 19 to postnatal day 20. The transcriptional run-on reactions were linear throughout the experiment (Fig. 1), indicating that the incubation conditions were appropriate during the reaction, and substrate was not limiting for the elongation of nascent transcripts. The rate of incorporation of labeled UTP into RNA inversely correlated with the age of the animals from which nuclei were prepared. Thus, transcriptional activity was higher in nuclei from fetal heart than in 15- or 20-day-old neonates. Such a postnatal decrease of general

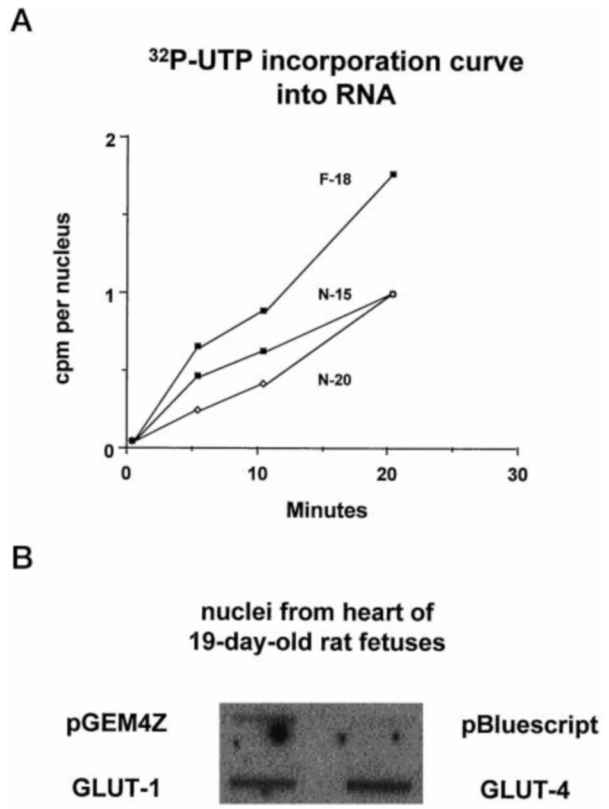

FIG. 1. **The transcriptional activity of nuclei isolated from rat ventricle decreases with development.** Nuclei isolated from pooled hearts of between 18-day-old fetuses and 15- and 20-day-old neonates were used in nuclear run-on reactions. Reactions were carried out as described under "Experimental Procedures," and the amount of [^{32}P]UTP incorporated into total RNA was measured during the course of each reaction. Data are represented as cpm incorporated per nucleus (*A*) (*F,* fetal; *N,* neonatal). *B* shows a representative autoradiograph of a single run-on experiment, corresponding to a reaction in which 2.2 × 10^7 nuclei isolated from heart of 19-day-old fetuses were used. 2.3 × 10^6 cpm of total ^{32}P-labeled RNA were added to the hybridization solution. The slots corresponding to pGEM4Z and pBluescript plasmids are included as negative controls and show nonspecific hybridization. This membrane was exposed to an X-OMAT AR Kodak film for 1 week.

transcription in rat ventricle agrees with previous observations (36). Fig. 1*B* shows an autoradiograph of a run-on reaction performed with nuclei isolated from 19-day fetal heart. The signals corresponding to the hybridization of newly transcribed GLUT-1 and GLUT-4 RNAs were well above that of background (pBluescript and pGEM), thus indicating that transcription of both genes was active at this developmental stage.

The data corresponding to the transcriptional activity of *GLUT-1* and *GLUT-4* genes during rat heart perinatal development are shown in Fig. 2. Transcription of *GLUT-1* was maximal in 19-day fetal heart and had decreased by 50% at birth; in 20-day-old neonates, *GLUT-1* transcription was lowest and accounted for <25% of the maximal values (Fig. 2). In contrast, *GLUT-4* transcription increased markedly between the late fetal stage and 20-day-old neonates (a nearly 4-fold increase). *GLUT-4* transcription levels increased very rapidly after birth, and in 5-day-old neonates were nearly 2-fold greater than those in fetal heart nuclei. As a control, total RNA was prepared from hearts saved from the same litters as the ones used in the nuclear run-on experiments and used to detect the mRNA levels of GLUT-1 and GLUT-4 glucose transporters (data not shown). Thus, GLUT-1 mRNA levels in heart were highest in the fetal stage (18-day-old fetuses) but steadily decreased after birth (1- and 20-day-old rats showed 34 and 10% of the level in 18-day-old fetal heart, respectively), which is in

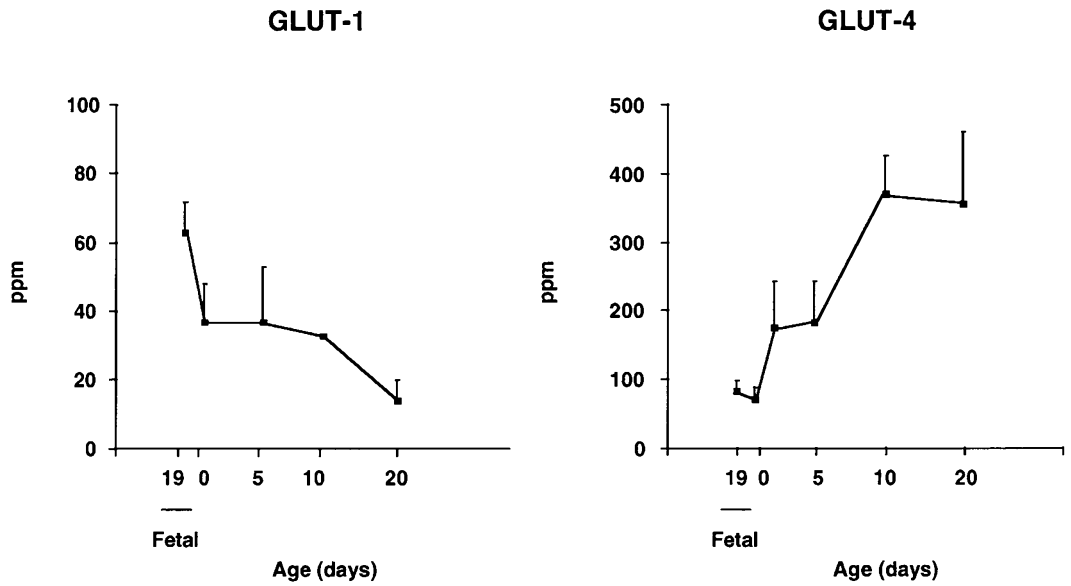

FIG. 2. **The transcriptional activity of *GLUT-1* and *GLUT-4* genes is regulated during rat heart perinatal development.** Run-on reactions were performed, as described under "Experimental Procedures," on nuclei isolated from pooled hearts from animals with ages in a range between embryonic day 19 to neonatal day 20. Results are expressed in ppm (background value previously subtracted) of the RNA species of interest relative to newly transcribed total RNA. Data are represented as mean ± S.E. of 3–8 observations of each sample.

contrast to the increase in GLUT-4 mRNA abundance observed over the same period of time (in 17-day-old fetal heart, expression levels accounted for only 35% of those observed in 20-day-old rat heart, whereas 5-day-old rat heart contained mRNA levels similar to those observed in 20-day-old rat heart). Given the similarity observed between the expression profiles of protein, mRNA, and transcriptional activity of GLUT-1 and GLUT-4 transporters in rat ventricle during perinatal development, the regulation of these two genes in development appears to be regulated chiefly at the level of transcription.

The 99-bp Region Upstream of GLUT-1 Transcription Initiation Site Is Sufficient to Drive Transcription in Isolated Cardiac Myocytes—Our next goal was to identify the *cis*-elements that may regulate *GLUT-1* transcription and to understand how GLUT-1 expression was repressed postnatally in heart and skeletal muscle. In a previous report we showed that the −99/−33 fragment in *GLUT-1* promoter drives transcriptional activity of the CAT reporter gene in the L6E9 rat muscle cell line (18). These findings prompted us to consider that maybe this region would be also important for the transcription of GLUT-1 in rat cardiomyocytes. This hypothesis was tested through the transfection of a series of 5′ deletion constructs of *GLUT-1* promoter containing various lengths of the proximal and 5′ upstream sequence through to +134 fused to the CAT gene, into primary cultures of rat neonatal cardiomyocytes prepared from 1–2-day-old neonates (Fig. 3). These experiments showed that transcription was maintained to a high level in the constructs containing deletions from −812 to position −99 of the GLUT-1 promoter, but deletion of the region between −99 and −33 relative to *GLUT-1* transcription initiation site resulted in an 80% decrease of CAT transcriptional activity relative to the −99/−33 CAT construct. As observed for the skeletal muscle cell lines, this region, which contains putative binding sites for known transcription factors, such as Sp1, AP2, and a CAAT box, (18) appears to be crucial for maintaining basal transcription in rat neonatal cardiomyocytes.

Sp1 Transcription Factor Is Present in Fetal Heart and Muscle Nuclei Extracts and Binds to a Region Essential for the Function of the GLUT-1 Promoter—We have reported previously that Sp1 is able to *trans*-activate a chimeric construct containing the rat *GLUT-1* promoter inserted upstream of the

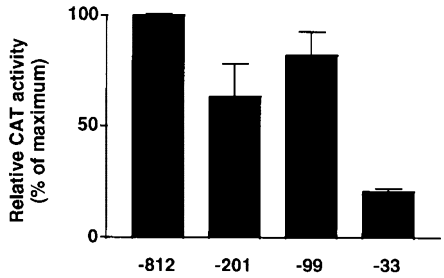

FIG. 3. **Expression of *GLUT-1* promoter 5′ deletion constructs in rat cardiac myocytes.** A series of 5′ deletions of the upstream region of the *GLUT-1* gene from −812 to −33 bp relative to the transcription start site were generated as indicated under "Experimental Procedures." Rat neonatal cardiomyocytes were transiently transfected with the indicated constructs and cells were harvested 72 h after transfection. The data are expressed as relative CAT activity/β-galactosidase activity ±S.E. from three experiments, performed in duplicate, using at least two preparations of DNA, with the −812/+134 CAT construct being set to a value of 100.

CAT reporter gene in L6E9 cells (18), and it binds to a probe encompassing positions −102 to −37 of *GLUT-1* promoter. Given the drop in basal transcription of the *GLUT-1* promoter in cardiomyocytes when 5′ sequence is deleted from −99 to −33, we used the −102/−37 fragment of *GLUT-1* promoter as a probe to determine whether Sp1 protein was present in nuclear extracts prepared from fetal or adult rat heart and skeletal muscle and able to bind to the −91/−86 Sp1 binding site in the *GLUT-1* promoter, previously shown to be functionally relevant in skeletal muscle cells (18). EMSA experiments showed that both fetal heart and muscle nuclei extracts generated a series of protein-DNA complexes when incubated with the −102/−37 probe (Fig. 4*A*). Most of these bands are not present in adult heart and skeletal muscle nuclear extracts. The *arrows* in Fig. 4*A* indicate a band with a slow mobility, which is specific to fetal heart and skeletal muscle, and also present in L6E9 myoblast nuclear extracts. This band in the L6E9 myoblast nuclei extracts was shown in our previous report to be due to the binding of Sp1 factor (18) and disappears with differentiation of L6E9 myoblasts into myotubes. Therefore, fetal heart and muscle extracts, but not their adult coun-

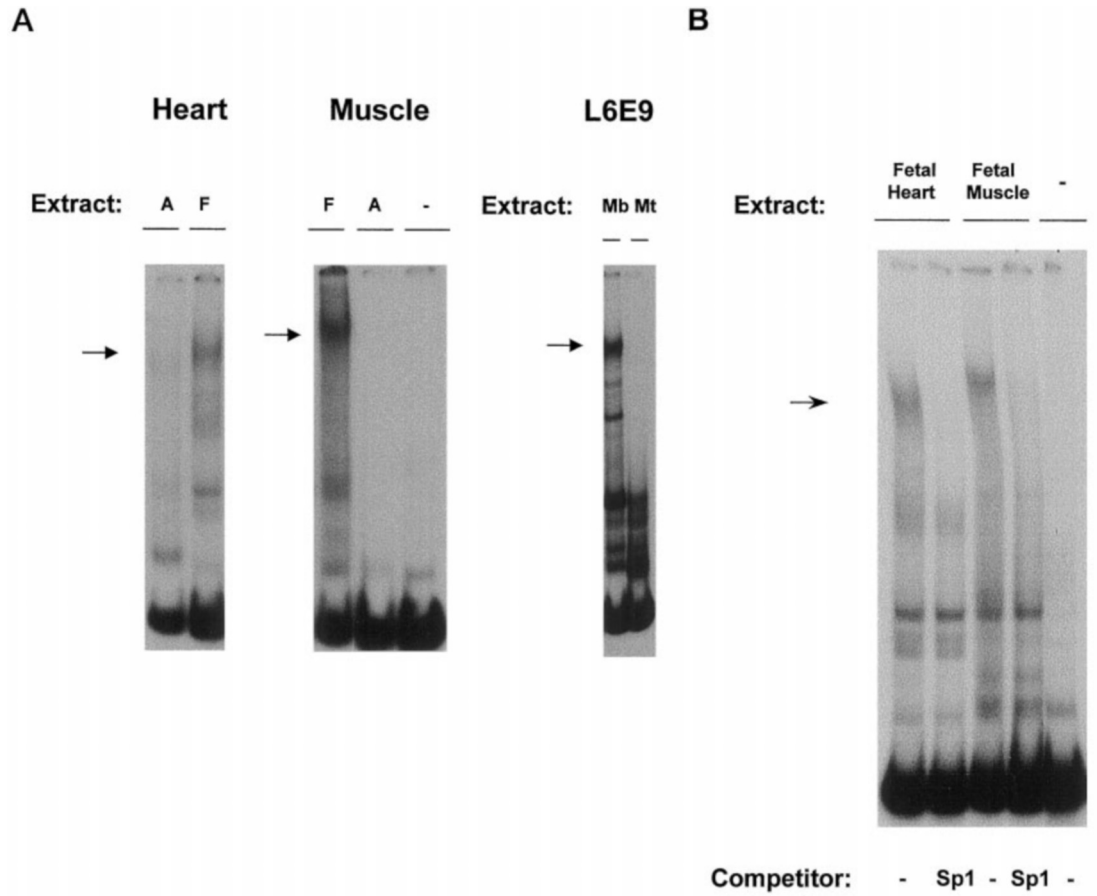

FIG. 4. **The −102/−37 probe and a Sp1 consensus oligonucleotide compete for the binding of a specific protein present in fetal heart and muscle nuclear extracts.** *A,* 5 μg of protein nuclear extract from fetal (*F*) and adult (*A*) heart or skeletal muscle, as well as from L6E9 myoblast (*Mb*) and myotube (*Mt*) nuclear extracts, were incubated as described under "Experimental Procedures" with 10,000 cpm of the radiolabeled −102/−37 fragment of *GLUT-1* promoter. Samples were loaded on a nondenaturing 7% polyacrylamide gel and run at 4 °C and 325 V for 90 min. The gel was then dried and exposed to an AGFA CURIX film. *Arrows* point to a specific band obtained after incubation with nuclear extracts from fetal heart, fetal muscle, and L6E9 myoblasts, which was attributed to the binding of Sp1 in L6E9 myoblasts extracts. *B,* EMSA experiments were performed by incubating 5 μg of protein from fetal heart or muscle nuclear extracts with 10,000 cpm of the radiolabeled GLUT-1 promoter −102/−37 probe. *Arrows* point to a specific band obtained after incubation with fetal heart or fetal muscle extracts that was prevented when the Sp1 consensus oligonucleotide was included as a competitor in some of the reactions (100-fold molar excess).

terparts, may exhibit binding of Sp1 to the *GLUT-1* core promoter. To ascertain whether that band in fetal heart and skeletal muscle extracts corresponds to the Sp1 transcription factor, we performed competition EMSA experiments by using a commercial oligonucleotide containing a consensus binding site for Sp1. This oligonucleotide was able to compete with the −102/−37 probe for the binding of the protein producing the slow-migrating band, indicating that Sp1 protein may be responsible for this DNA-protein complex (Fig. 4*B*). In order to provide more direct evidence that the Sp1 transcription factor present in fetal heart and skeletal muscle nuclear extracts binds to this site in *GLUT-1* promoter, we used a polyclonal antibody against human Sp1 in supershift EMSA (according to the supplier, this antibody cross-reacts with rat Sp1 and was therefore appropriate for these studies). When an oligonucleotide encompassing positions −102 to −82 in GLUT-1 promoter was radiolabeled and used as a probe, it proved to be able to bind human recombinant Sp1 protein (hrSp1). This protein was recognized specifically by the anti-Sp1 antibody, which produced a decrease in the apparent mobility of the complex formed by this factor bound to the probe (Fig. 5, *left panel*). This supershift was specific, because an irrelevant antibody (IgG) had no effect on the mobility of the complex (Fig. 5, *left panel*). Fetal heart and L6E9 myoblast nuclear extracts were incubated with the same probe and produced several bands (*arrows*), one of which had the same relative mobility as the one produced by hrSp1 (Fig. 5, *right panel*). This band could also be supershifted by the anti-Sp1 antibody, and this supershift was observed both in fetal heart and in L6E9 myoblasts and was specific because the irrelevant antibody was not able to produce them. These data show that Sp1 is present in fetal heart nuclear extracts and can bind to the Sp1 site located at −91/−86 in the GLUT-1 promoter. We performed other supershift experiments with an antiserum against Sp3 (gift of Dr. Guntram Suske, Phillips-Universität, Marburg, Germany) that showed this other member of the Sp family of transcription factors was present in the fetal heart nuclear extracts and able to bind to the same site in *GLUT-1* promoter (data not shown).

In order to analyze the functional significance of the −91/−86 Sp1 site in *GLUT-1* promoter, we tested the effect that a point mutation introduced into the −91/−86 Sp1 site would have in (*a*) the binding of Sp1 to the *GLUT-1* promoter, and (*b*) the transcriptional activity of the −99/+134 *GLUT-1* CAT construct transfected into rat neonatal cardiomyocytes. First, a mutant version of the oligonucleotide encompassing positions −102 to −82 in GLUT-1 promoter was used in competition EMSA experiments. The ability of this oligonucleotide to compete for the binding of Sp1 to the −102/−37 probe was compared with that of the wild type oligonucleotide (Fig. 6*A*). Thus, whereas the wild type competitor (wtG1Sp1) was able to compete with the probe, a two-nucleotide substitution in the −91/−86 site (GC → TA) abolished the ability of the oligonucleotide

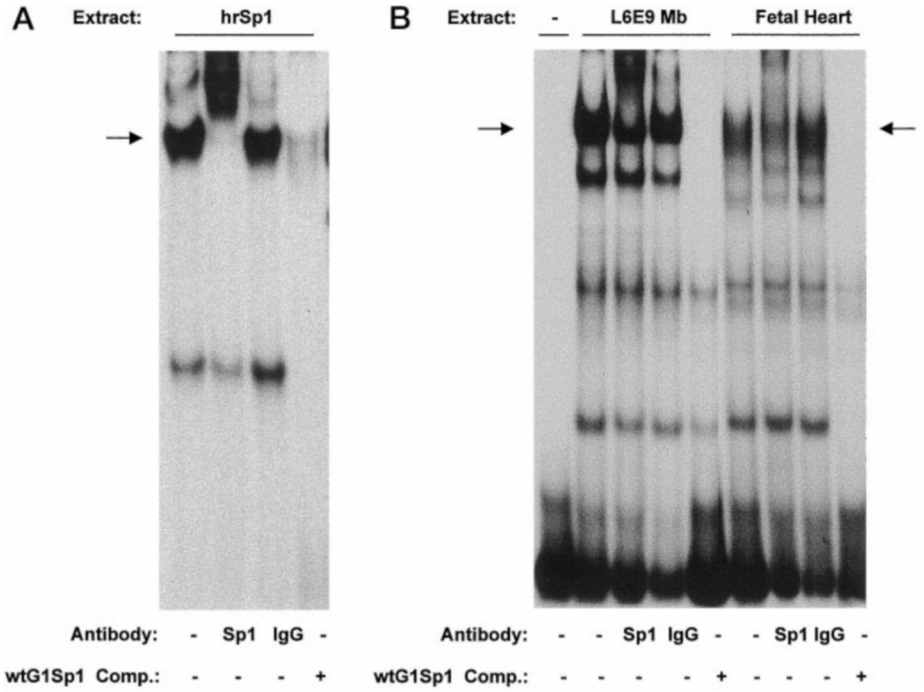

FIG. 5. **The Sp1 transcription factor present in the nuclear extracts from fetal heart binds to the Sp1 site.** Supershift experiments were carried out by incubating 5 μg of protein of fetal heart nuclear extract or L6E9 myoblast (*Mb*) extract with 10,000 cpm of the radiolabeled oligonucleotide wtG1Sp1 (−102/−82 fragment of *GLUT-1* promoter), which contains the putative Sp1 site present in this region (*right panel*). One μl of a commercial solution of human recombinant Sp1 transcription factor (hrSp1) was also used in the supershift experiments as a control (*left panel*). *Sp1* and *IgG* antibodies stand for a polyclonal antibody raised against human Sp1 and MANRX, a polyclonal antibody raised against rat rBAT amino acid transporter, respectively. The latter was used as an irrelevant antibody in these experiments (negative control). *Arrows* point to the bands specifically displaced by the addition of the antibody against Sp1.

to compete in EMSA (Fig. 6*A, mutG1Sp1*). The same two-nucleotide substitution was introduced into the core of the Sp1 site in the −99/+134 *GLUT-1* CAT construct, and the transcriptional activity of this DNA construct was then compared with that of the wild type after transfection into rat neonatal cardiomyocytes. Fig. 6*B* shows that the mutation induced a reduction of transcriptional activity of the −99/+134 CAT construct to a value that was 38% of the activity observed in the wild type. Interestingly, this reduced level of activity is similar to that of the −33/+134 CAT construct when compared with the activity of the −99/+134 CAT construct (the activity of the mutant −99/+134 CAT construct accounts for 75% of the drop observed when the sequence between −99 and −33 is deleted). These data indicate the functional significance of this site in the maintenance of the transcriptional activity of *GLUT-1* in neonatal cardiomyocytes.

Sp1 Levels Are Down-regulated in Adult Cardiac and Skeletal Muscle—Additional data were obtained by Western blot carried out with fetal rat heart, 21-day-old rat heart, and fetal and adult rat skeletal muscle nuclear extracts. Sp1 protein was detected in these samples with the same antibody used in the supershift experiments. This antibody detected a band with an apparent molecular mass of 106 kDa, which co-migrated with human recombinant Sp1 (Fig. 7*A*). Notably, both fetal heart and muscle extracts contained greater levels of Sp1 protein than 20-day-old neonatal heart and adult muscle extracts. In comparison, 21-day-old rat heart extracts only contained 5% of the levels observed in fetal muscle, and Sp1 protein was barely detected in the adult skeletal muscle extract under these conditions. These results confirm that Sp1 levels decrease in both skeletal muscle and heart during perinatal development. Indeed, strong variations in Sp1 protein expression in nuclear extracts along with perinatal development have been previously reported in heart (37), and aortic smooth muscle cells (38). Moreover, Saffer *et al.* (39) have reported a wide variation in Sp1 mRNA levels between tissues in adult mice (in which they detected extreme differences in Sp1 mRNA levels of up to 100-fold), as well as a decrease in Sp1 mRNA abundance during postnatal development in most of the tissues examined. We also measured Sp3 protein levels in fetal and adult heart nuclear extracts with the antiserum against Sp3. Fetal heart extracts contained high levels of the two reported Sp3 polypeptides (40), whereas Sp3 expression was barely detectable in adult heart extracts, paralleling the pattern of expression observed for Sp1(data not shown).

DISCUSSION

The expression of GLUT-1 and GLUT-4 mRNA levels is regulated during rat heart perinatal development. Furthermore, GLUT-1 and GLUT-4 mRNA expression in heart correlates with the pattern of protein expression during perinatal development, which had been reported previously (16). We show here that these changes in expression represent direct alterations in the transcriptional activity of *GLUT-1* and *GLUT-4* genes, as deduced from nuclear run-on experiments. We have analyzed the DNA region upstream of the *GLUT-1* transcription initiation site in order to look for regions responsible for the control of GLUT-1 transcription in cardiac muscle. To this end, we have performed transient transfection experiments in rat neonatal cardiomyocytes, with a series of *GLUT-1* promoter-CAT chimeric constructs spanning positions −812 to −33 relative to *GLUT-1* transcripition initiation site. These experiments showed that the 99-bp region upstream of *GLUT-1* transcription site are essential for the transcription of *GLUT-1* in rat cardiomyocytes. We also showed by electrophoretic mobility shift analysis that the zinc finger transcription factor Sp1 is present in fetal heart and skeletal muscle nuclear extracts and can bind to a site present in the −102/−37 fragment of *GLUT-1* promoter. This was further demonstrated by supershift experiments, using an oligonucleotide encompassing the Sp1 site located at −91/−86 and an antibody raised against rat Sp1. Furthermore, disruption of the Sp1 site produced a remarkable decrease in the transcriptional activity of a *GLUT-1* promoter-driven CAT construct in neonatal cardiomyocytes, showing a functionally essential role for Sp1 in the transcription of *GLUT-1* in those cells. Finally, we have observed that the levels of expression of Sp1 in nuclear extracts from heart or muscle in the fetus are higher than those in the adulthood. Together, these data indicate that the expression of GLUT-1 and GLUT-4 in the developing heart is largely regulated at a transcriptional level and that Sp1 plays a pivotal role in the regulation of the expression of GLUT-1 in heart and muscle.

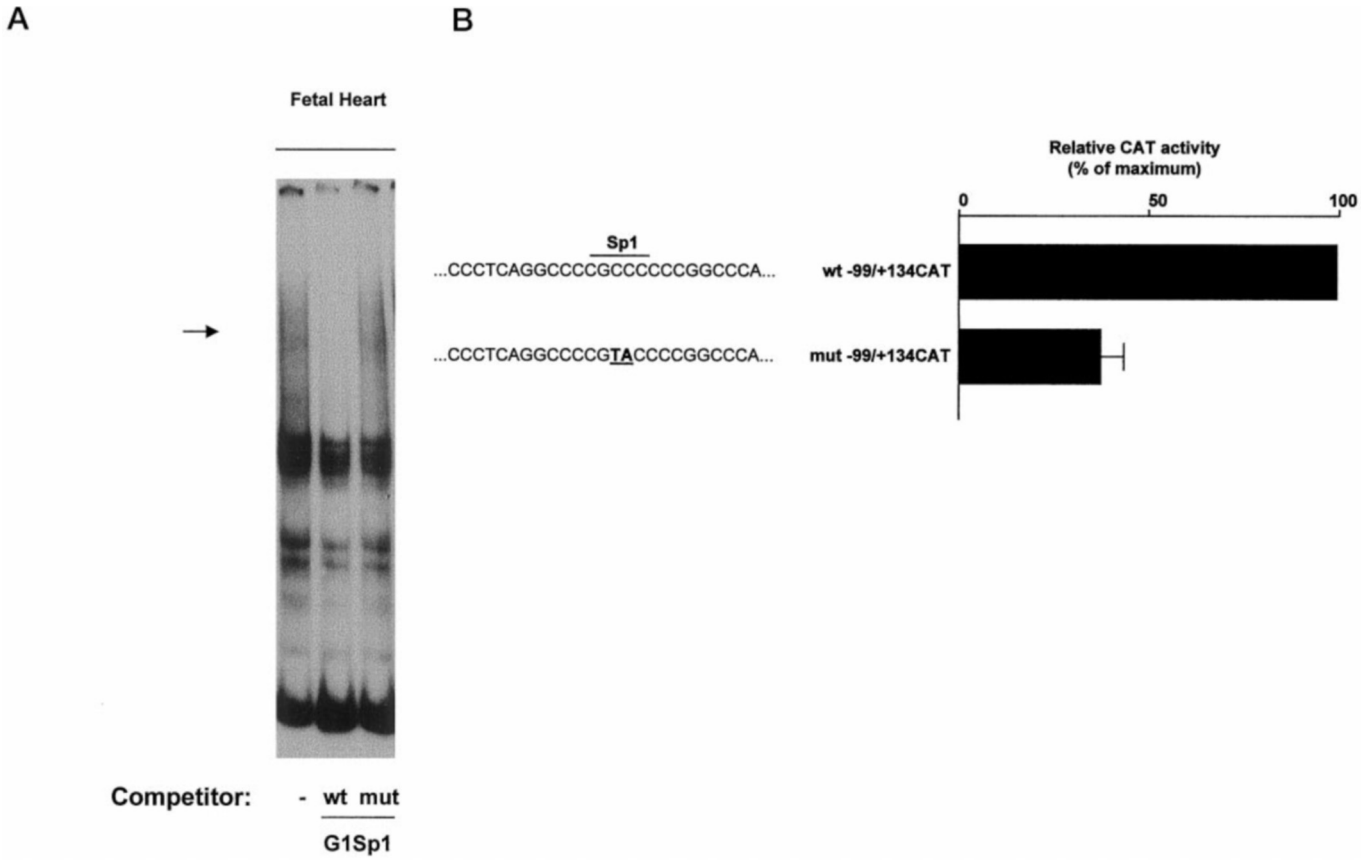

FIG. 6. **Disruption of the Sp1 binding site in *GLUT-1* promoter compromises the binding of Sp1 and the transcription of the −99/+134 GLUT-1 CAT construct in cardiac myocytes.** *A,* 5 μg of protein nuclear extract from fetal heart were incubated as described under "Experimental Procedures" with 10,000 cpm of the radiolabeled −102/−37 fragment of *GLUT-1* promoter. A wild type (wtG1Sp1) and a mutated version (mutG1Sp1) of an oligonucleotide containing the −91/−86 Sp1 site in *GLUT-1* promoter were included as competitors (100-fold molar excess). The *arrow* points to a band that was competed only by the wtG1Sp1 oligonucleotide and not by the mutant version, in which the consensus Sp1 site was disrupted. *B,* either a wild type or a mutated version of −99/+134 GLUT-1 CAT were transiently transfected into rat neonatal cardiomyocytes as described in Fig. 3 and under "Experimental Procedures." The sequence at the Sp1 site is shown for both the wild type and the mutant construct. Note that the same point mutation was included in the mutant competitor oligonucleotide (mutG1Sp1) shown in *A*. The data are expressed as relative CAT activity/β-galactosidase activity ± S.E. from four experiments, with the wild type −99/+134 CAT construct being set to a value of 100.

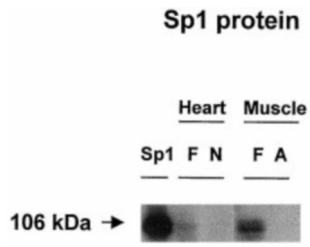

FIG. 7. **High abundance of Sp1 transcription factor in fetal heart and muscle nuclear extracts.** Twenty-five μg of protein from nuclear extracts obtained from fetal heart and muscle, 20-day-old neonatal heart, and adult skeletal muscle were loaded together with a 1:5 dilution of hrSp1 (human recombinant Sp1) on a 7.5% acrylamide minigel, and after electrotransfer onto an Immobilon membrane, Sp1 protein was detected by using PEP2 polyclonal antibody against human Sp1 factor (see under "Experimental Procedures"). Immunoblots were autoradiographed and subjected to scanning densitometry in order to quantify the relative amount of Sp1 in these samples.

Using nuclear run on assays, we were able to observe a decrease in the transcriptional activity of *GLUT-1* during the perinatal development, which explains the decrease in the GLUT-1 mRNA and protein levels observed in the same period (16). The changes in GLUT-1 mRNA expression in rat heart are probably due to the regulation of the expression of this transporter in cardiomyocytes and not in other cell types, because *in situ* hybridization experiments reveal that the expression of GLUT-1 mRNA in the late fetal heart is higher in the ventricular cardiac muscle than in any of the surrounding tissue.[3] We do not know, however, whether the levels of transcriptional activity of this gene were higher in earlier stages of rat heart development prior to embryonic day 17, because isolation of fetal nuclei from earlier time represented a technically unaffordable task. However, we cannot discard this possibility because the fetal period is characterized by a high glucose consumption rate, which is typical of cells in an active proliferative state.

Although both GLUT-1 protein and mRNA are still detectable in the adult rat heart (16), we found that the postnatal transcriptional activity of *GLUT-1* was very low. These data suggest potential posttranscriptional regulatory mechanisms. Such a mechanism has already been postulated for the Na^+/Ca^{2+} exchanger (24) and SR Ca^{2+}-ATPase (41). The Na^+/Ca^{2+} exchanger in rat heart is also down-regulated perinatally, at the levels of both protein and mRNA. However, even though the protein is still detectable in adult rat heart samples, the transcriptional rate and mRNA expression of this gene fall below detection levels soon after birth, suggesting a long half-life for the Na^+/Ca^{2+} exchanger protein in the adult rat heart (24). It is unclear whether the half-life of GLUT-1 is also developmentally regulated in rat heart. Nevertheless, the sta-

[3] P. Thomas, personal communication.

bility of the GLUT-1 mRNA in L6 myoblasts (42), or of the GLUT-1 protein in 3T3-L1 adipocytes (43), is up-regulated by oxidative stress and glucose deprivation, respectively. Therefore, it is likely that posttranscriptional and/or translational regulatory mechanisms are occurring in addition to the transcriptional regulation we have described.

Heart growth is characterized by a fetal and early neonatal hyperplastic phase (cell division), which is followed by the withdrawal of rat cardiomyocytes from the cell cycle (a fact that is particularly relevant because in adulthood, dead cardiac myocytes cannot be replaced), and the onset of a hypertrophic phase of growth (increase in cell mass). Thus, the repression of GLUT-1 occurs when myocardial cells are still involved in a period of active replication but are starting to withdraw from the cell cycle. Although cardiac fibers are fully functional, cardiomyocytes retain the ability to divide during the hyperplastic growth phase. This is in contrast to skeletal muscle, where myoblasts withdraw irreversibly form the cell cycle prior to differentiation (44). In fact, the incorporation of [^{3}H]thymidine into DNA in heart is much higher in the late fetal stage than later on (45), and it decreases rapidly after birth (46, 47), ceasing completely by the 17th day of neonatal life. From this moment on, the growth of heart depends exclusively on the hypertrophy of preexisting cardiomyocytes (48). It is possible that the signal regulating the perinatal repression of GLUT-1 and induction of GLUT-4 is in some way related to the withdrawal from the cell cycle. In fact, the expression of GLUT-1 is rapidly induced by proliferative stimuli, such as growth factors (49), and is high in transformed cells (50). Moreover, in the L6E9 muscle cell line, cells exit the cell cycle prior to differentiating into myotubes (44), and this is accompanied by the parallel repression of GLUT-1 and the induction of GLUT-4 (18).

GLUT-4 transcription is mainly induced postnatally, as deduced from our transcriptional activity data. This transporter is characteristically expressed in differentiated skeletal muscle and heart. However, the rat heart is already pumping blood as early as in the ninth day of gestation, and therefore is an organ with a precocious differentiation. Nevertheless, *GLUT-4* transcriptional activation does not occur until the late fetal or early postnatal phase. This fact indicates that, although the differentiation of cardiac fibers is necessary for the induction of GLUT-4 expression, this may not be sufficient, and other factors may be required in order to achieve high GLUT-4 expression levels. One candidate may be thyroid hormone, because the responsiveness to this hormone is elicited at the end of the fetal stage. We have previously shown (17) the pivotal role of this hormone in the perinatal induction of GLUT-4. A recent report (51) presents some evidence for the existence of a low-affinity binding site in the *GLUT-4* promoter, in a region that had been shown as functionally important for the thyroid hormone-dependent regulation of GLUT-4 in the skeletal muscle cell line C2C12 (52). Nevertheless, we cannot exclude the importance of other transcription factors, such as those facilitating the establishment of the differentiated muscle-fiber phenotype, in the onset of *GLUT-4* transcription. In keeping with this, it has been shown that MEF2A and/or C transcription factors are able to bind to the human *GLUT-4* promoter (53), to a site that is functionally important for the muscle-specific expression of this transporter in the C2C12 skeletal muscle cell line (54).

As a first step to characterize the transcription factors regulating the transcriptional activity of *GLUT-1*, we looked for *cis*-acting regions that may account for the transcriptional activity of *GLUT-1* in cardiac cells. Our experiments (Fig. 3) show that the 99 bp upstream of the *GLUT-1* transcription initiation site were essential for basal transcriptional activity in primary cultures of rat neonatal cardiomyocytes. Next, we tested whether Sp1 transcription factor, which regulates, to a certain extent, *GLUT-1* transcription in the skeletal muscle cell line L6E9 (18), might be responsible for the high level of expression of this glucose transporter in fetal heart. Results from EMSA experiments indicate that Sp1 binds to a region in the *GLUT-1* promoter that we have shown by mutational analysis to be essential for *GLUT-1* transcription in neonatal cardiomyocytes. Although Sp1 factor was initially considered to have a function related to the maintenance of the basal transcription of many genes, an increasing number of studies, including ours, demonstrate that Sp1 regulates the transcription of some genes by changes in binding activity or expression levels (55, 56), and also through the interaction with transcription factors, such as Egr-1 (57), MEF2C (58), and Smad family members (59). In the case of GLUT-1, Sp1 protein levels in fetal heart and muscle nuclear extracts, together with its binding activity, correlate with the high levels of expression observed for GLUT-1 glucose transporter in the late fetal stage in both tissues. In a recent report (60), an indispensable role for Sp1 in the development of the mouse embryo has been shown, given that Sp1$^{-/-}$ homozygous embryos die *in utero* at about day 11 of gestation. These embryos show a delayed development although the absence of Sp1 did not impair the early steps on the formation of heart, neither of other organs. However, the early death of these embryos prevents further study about the effects of such a mutation in the perinatal expression of GLUT-1 in heart.

Our results show that Sp1 expression in heart nuclear extracts is down-regulated around birth. Little is known about the regulation of the expression of Sp1. However, in our previous study (24), we showed that MyoD may be a regulator of Sp1 expression because in cells overexpressing this myogenic factor, Sp1 levels are dramatically reduced. This suggests the participation of a myogenic factor in the regulation of the expression of a gene that may, in turn, be responsible for the down-regulation of *GLUT-1* transcription during terminal differentiation of muscle that takes place during perinatal development. However, MyoD cannot be exerting this effect on Sp1 during heart development, because neither this myogenic factor nor its relatives myogenin, myf-5, and MRF4 are expressed in this organ, but only in developing skeletal muscle. We therefore postulate that another unidentified factor might be partially mediating the GLUT-1 repression in cardiac muscle, through the down-regulation of the levels of expression of Sp1. Given the parallel between the postnatal reduction of Sp1 levels of expression in heart and the progressive cell cycle withdrawal experienced by cardiac myocytes along the same period, it is intriguing to think that cell cycle regulatory proteins may have a role in the perinatal down-regulation of Sp1. In keeping with this, a tight control of the Sp1-mediated activation of transcription by Rb and other cell cycle regulatory proteins has been shown (61–63). However, whether Rb or any other protein involved in cell cycle control is able to regulate the expression of Sp1 itself remains unknown. Sp3, another member of the Sp family of zinc-finger transcription factors, has been shown to have opposite roles in the regulation of the transcription of a number of genes. Thus, Sp3 has been attributed a role as an activator for the transcription of genes such as SIS/platelet-derived growth factor-B (64), the neuronal nicotinic acetylcholine receptor 4 subunit (65), and *GLUT-3* (66), but it has also been well characterized as an inhibitor of Sp1-mediated transcativation in other genes (40, 67). We have found that Sp3 is more abundant in extracts from fetal heart than in adult heart and that the Sp3 transcription factor present in fetal extracts is able to bind to the −91/−86 Sp1 site in GLUT-1 promoter. Therefore, Sp3, which is subject to a strong

regulation during heart development, may have a role in the regulation of GLUT-1 transcription in heart. In keeping with this, unpublished data from our laboratory,[4] show that, in the L6E9 myoblasts, Sp3 acts as a negative regulator of GLUT-1 transcription. Moreover, despite both Sp1 and Sp3 being down-regulated in L6E9 myoblasts along differentiation, alterations in the relative Sp1/Sp3 ratio at early stages of differentiation (through a temporary increase in Sp3 abundance) may trigger the down-regulation of GLUT-1 observed in those conditions. However, investigating whether a similar mechanism operates during the down-regulation of GLUT-1 expression in heart perinatal development and in parallel to the exit of the cardiac myocytes from the cell cycle lies beyond the scope of the present study. The identification of the nature of the signals responsible for the triggering of the perinatal repression of Sp1 and Sp3 in rat heart constitutes an important issue and will be addressed in future studies.

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