Differential screening of a PCR-generated mouse embryo cDNA library: glucose transporters are differentially expressed in early postimplantation mouse embryos

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Summary

Differential screening of a cDNA library constructed using PCR amplification techniques from RNA isolated from the distal portion (embryonic ectoderm, mesoderm and visceral endoderm) of 7.5 days post coitum (dpc) mouse embryos led to the isolation of two cDNA clones expressed at higher levels in 7.5 dpc embryos than 12.5 dpc embryos. Nucleotide sequence analysis revealed that each of these clones was a different member of the family of facilitative glucose transporters (Glut genes). The differentially expressed cDNA clones represent mouse Glut-1 and Glut-3. Levels of the Glut-3 mRNA declined 14-fold between days 7.5 and 12.5 of gestation, and were under our limits of detection by 14.5 dpc. The levels of the Glut-1 mRNA declined about 3-fold between days 7.5 and 12.5 of gestation.

Analysis of the expression of these genes by in situ hybridization revealed striking differences in transcript localization in early postimplantation mouse embryos. At 7.5 dpc, both transporters were expressed more strongly in extraembryonic tissues than in the embryo proper. While both transporters were expressed in the amnion and chorion, only Glut-1 was expressed in the ectoplacental cone. In the yolk sac, Glut-3 appeared to be expressed only in the endoderm while Glut-1, although expressed in both layers, was expressed more strongly in the mesoderm layer. Thus, the two transporters have relatively reciprocal sites of expression in the developing extraembryonic membranes. Expression of Glut-1 was fairly widespread in the embryo at 8.5 dpc, but by 10.5 dpc expression was down-regulated and was observed in the eye and the spinal cord. Expression of Glut-3 was largely confined to non-neural surface ectoderm and was also substantially down-regulated by 10.5 dpc.

These results prompted an examination of the RNA expression pattern of two other glucose transporter isoforms, Glut-2 and Glut-4. We did not detect Glut-4 expression, while Glut-2 expression was largely confined to extraembryonic visceral yolk sac endoderm. These data suggest differential roles for these glucose transporter family members during early postimplantation development of mice.

Key words: glucose transporter, PCR, postimplantation mouse embryo, glucose utilization.

Introduction

A variety of experimental strategies have been utilized to isolate genes whose expression is important for early postimplantation mouse development. These strategies include the isolation of mouse homologs of genes known to be important for development in other organisms and the production of developmental mutations by insertional mutagenesis (for reviews, see Gridley, 1991; Kessel and Gruss, 1990). Another approach to the identification of genes important for early postimplantation mouse development is the isolation of genes differentially expressed during these stages. Recent developments in the construction of cDNA libraries from small numbers of cells using PCR techniques (Belyavsky et al., 1989; Brady et al., 1990; Welsh et al., 1990) have made feasible the production of cDNA libraries from defined regions of early postimplantation mouse embryos. One developmental stage of particular interest is the 7.5 dpc mouse embryo. At this time, the embryonic axis is established and mesoderm formation is well underway (Rugh, 1990; Theiler, 1989). Over the succeeding 24 hours, extensive changes will take place in the developing embryo. The heart primordium and the foregut begin to develop, the neural plate forms, the neural folds start to close and somites begin to condense in the paraxial mesoderm. In order to examine genes that might be differentially expressed at this important developmental stage, we have used PCR amplification techniques to construct a cDNA library from the distal portion (embryonic ectoderm, mesoderm and visceral endoderm) of 7.5 dpc mouse embryos. This library was differentially screened with labelled cDNA from 12.5 dpc embryos. Two clones were isolated that were
more abundant in 7.5 dpc embryos than 12.5 dpc embryos. Nucleotide sequence analysis of these clones revealed that each was a different member of the family of facilitative glucose transporters.

The facilitative glucose transporters are a small multigene family of transmembrane proteins that equilibrate sugar across cell membranes by a passive system of facilitated diffusion (for reviews, see Mueckler, 1990; Pessin and Bell, 1992; Silverman, 1991). Five family members (Glut-1 through Glut-5) have been described to date and their cDNAs have been cloned and sequenced. The sizes of the facilitative glucose transporters vary from 492 to 524 amino acids, with between 40 and 65% amino acid identity between any two family members. The hydrophatic profiles of the different family members are virtually superimposable and the current model for the structure of a facilitative glucose transporter predicts that the polypeptide chain spans the cell membrane twelve times. The twelve membrane-spanning regions presumably form a channel or pore through which glucose moves.

While the sites of expression of different Glut family members have been extensively analyzed in adults of a variety of species, very little is known about embryonic sites of expression, particularly during postimplantation development. We used RNA blot analysis and in situ hybridization to analyze the spatial and temporal localization of transcripts from the two Glut genes (Glut-1 and Glut-3) isolated by differential hybridization from our 7.5 dpc embryo cDNA library, as well as the glucose transporter isoforms Glut-2 and Glut-4. This analysis revealed striking differences in localization of these transcripts in early postimplantation mouse embryos.

**Materials and methods**

**Embryo isolation**

Embryos were obtained from natural matings of C57Bl/6 mice. The day on which the vaginal plug was detected was designated 0.5 dpc. For construction of the PCR-amplified cDNA library, twenty five 7.5 dpc embryos were dissected from the uterus in phosphate-buffered saline. Embryos were dissected from the decidua and Reichert’s membrane was removed using #5 forceps. The embryos were then fixed at the level of the amnion with fine glass needles. The distal portions (consisting primarily of embryonic ectoderm, mesoderm and visceral endoderm) of the dissected embryos and total RNA were isolated by the acid guanidinium thiocyanate-phenoI-chloroform technique (Chomczynski and Sacchi, 1987).

**Library construction**

First strand cDNA synthesis was performed using the entire RNA sample from the dissected 7.5 dpc embryos. Primer 1 (GGCTCAGGCC[T][T][G]) was used to prime first strand synthesis using Moloney-Murine Leukemia Virus reverse transcriptase (BRL) according to the manufacturer’s recommendations. After completion of first strand synthesis, primer was removed by spin dialysis using Centricon-100 spin filters (Amicon). The sample was extracted with phenol/chloroform and precipitated with ethanol. The sample was then tailed with dGTP using terminal transferase (Pharmacia) according to the manufacturer’s recommendations, extracted and precipitated.

PCR amplification of this material was performed in a 100 µl reaction using primer 1, primer 2 (ATATCGATT[C][T][G]) and Taq polymerase (Perkin-Elmer) using the manufacturer’s buffer conditions. Amplification parameters were 94°C, 1 minute 30 seconds; 60°C, 1 minute 30 seconds; 72°C, 4 minutes for 20 cycles. After completion, the product was diluted into four fresh 100 µl reactions and 6 more cycles were performed using the above amplification parameters. The product was then extracted with phenol/chloroform and precipitated with ethanol.

The next step involved differential PCR based on the size of the products from the first round of PCR (Belyavsky et al., 1989). One half of the product from the first set of PCR reactions was electrophoresed on a 1% agarose gel. The gel was stained with ethidium bromide and the electrophoresed product was visualized under long-wave UV light. The gel was cut into 5 slices containing product ranging in size from 400 bp (slice 1) to >4 kb (slice 5). The amplified product was recovered from the gel slices by centrifugation through a plug of siliconized glass wool. Differential PCR was then performed as follows. Denaturation and annealing conditions were identical to the first set of PCR amplifications, but cycle number and extension times were altered, depending on the size of the product. The altered amplification parameters for the DNA isolated from the gel slices were: slice 1, 4 cycles, 2 minutes extension; slice 2, 10 cycles, 2 minutes extension; slice 3 through 5, 20 cycles, 4 minutes extension. Upon completion of differential PCR, samples were pooled, the primers were removed by spin dialysis and the samples were extracted with phenol/chloroform and precipitated with ethanol. For cloning, PCR products >400 bp in length were size-selected on a 1% agarose gel as described above. EcoRI adapters were ligated to the PCR product and it was cloned into λZap II phage arms (Stratagene) using standard procedures (Sambrook et al., 1989).

**Library screening, northern analysis and nucleotide sequencing**

For differential screening of the library, approximately 2,000 phage were plated and plaque lifts were performed according to standard procedures (Sambrook et al., 1989). The lifts were hybridized with 32P-cDNA made from day 12.5 p.c. embryo poly(A)* RNA. From 100 non-hybridizing plaques, phage inserts were purified from 50 by in vivo excision and were used as probes on northern blots containing 20 µg total RNA isolated from 7.5 dpc and 12.5 dpc embryos. Northern blotting was performed as described (Gavin et al., 1990). Inserts isolated from two phage clones (clones 103 and 106) reproducibly gave greater hybridization to RNA from 7.5 dpc embryos than 12.5 dpc embryos. Quantitation of northern blot results was performed on a Betascope 603 gel analyser (Betagen). The signal (counts per minute) from clones 103 and 106 were normalized to the GAPDH signal and the normalized signal at 7.5 dpc was arbitrarily set at 100 units. The inserts from clones 103 and 106 were subcloned and sequenced on both strands using the Sequenase kit (US Biochemical) as described (Gridley et al., 1991). Northern blots hybridized with the Glut-1 and Glut-3 probes were exposed for 4 hours, while blots hybridized with the Glut-2 probe were exposed for 2 days.

Probes for the Glut-2 (Asano et al., 1989) and Glut-4 (Charron et al., 1989) genes were isolated by PCR amplification, as described (Hogan et al., 1991). PCR reactions were performed with specific primer pairs using first strand cDNA synthesized from total RNA isolated from 8.5 dpc mouse embryos (Glut-2) or genomic mouse DNA (Glut-4). PCR amplification products were subcloned and sequenced as described above.

**In situ hybridization**

In situ hybridization was performed essentially as described by Wilkinson and Green (1990). Briefly, C57Bl/6 embryos were dis-
sected and fixed at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight. The fixed embryos were dehydrated, embedded in paraffin and 6 μm sections were cut and floated onto 3-aminopropyltriethoxyxilene-coated slides. For hybridization, slides were dewaxed in xylene, hydrated in an ethanol series and fixed in fresh 4% paraformaldehyde in PBS. Sections were treated with 20 μg ml⁻¹ proteinase K in 50 mM Tris-HCl, 5mM EDTA (pH 8.0), washed in PBS and postfixed in 4% paraformaldehyde in PBS. Sections were then treated with acetic anhydride, washed and dehydrated. [³²S]UTP-labelled single-stranded sense and antisense RNA probes were prepared by standard procedures (Sambrook et al., 1989). The probe was hydrolyzed to an average length of 100 bases, unincorporated nucleotides were removed by chromatography on a Nick column (Pharmacia) and the probe was ethanol-precipitated. The probe was resuspended at a concentration of 2 ng μl⁻¹ kb⁻¹ in 100 mM DTT. The probe was then diluted 1:10 in hybridization solution (50% formamide, 0.3 M NaCl, 20 mM Tris-HCl, 5 mM EDTA (pH 8.0), 10% dextran sulphate, 5× Denhardt’s, 0.5 mg ml⁻¹ yeast RNA), giving a final probe concentration of 0.2 ng μl⁻¹ kb⁻¹. After sections were hybridized overnight at 55°C, they were treated with ribonuclease A, washed at high stringency (50% formamide, 2× SSC, 10 mM DTT at 65°C) and dehydrated. Slides were dipped in NTB2 emulsion (Eastman Kodak). Exposure times were 6 days for the Glut-1 and Glut-3 probes and two weeks for the Glut-2 probe. Exposed slides were developed in D19 (Kodak), stained in 0.5% toluidine blue and mounted with Permount (Fisher). Double exposure (dark-field with red filter, bright-field with blue filter) photomicrographs were taken on a Zeiss Axioplan microscope.

Results

Construction and screening of a PCR-amplified cDNA library from dissected 7.5 dpc mouse embryos

In order to search for genes that might be differentially expressed in early postimplantation mouse embryos, we constructed a PCR-amplified cDNA library from dissected 7.5 dpc embryos. Twenty five 7.5 dpc embryos were dissected from the deciduum, Reichert’s membrane was removed and the embryos were transected at the level of the amnion with fine glass needles. The distal portions (consisting primarily of embryonic ectoderm, mesoderm and visceral endoderm) of the dissected embryos were pooled and total RNA was isolated. First strand cDNA prepared from this RNA was amplified by differential PCR amplification and was cloned into λZap II (see Materials and Methods).

Approximately 2,000 phage from this library were plated for differential screening. Plaque lifts of these phage were hybridized with ³²P-labelled cDNA prepared from 12.5 dpc mouse embryos. Two hundred phage which did not hybridize with the 12.5 dpc cDNA probe were picked and grown up for further analysis. cDNA inserts were prepared from 50 of these phage and tested by northern analysis for differential hybridization to 7.5 dpc embryo RNA versus 12.5 dpc embryo RNA. Two of these 50 phage clones reproducibly showed higher levels of hybridization to RNA isolated from 7.5 dpc embryos. The inserts of these two phage were subcloned and analyzed further.

Expression and sequence analysis of the differentially expressed cDNA clones

The inserts of these two cDNA clones, clones 103 and 106, were used as probes on a developmental northern blot (Fig. 1A). This blot was also hybridized with a probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to control for RNA integrity and loading. The insert from clone 103 hybridized to a 4.0 kb message which exhibits strong differential hybridization to RNA of early postimplantation embryos. The insert from clone 106 hybridized to a 2.5 kb message which is also differentially expressed, although not as strongly as clone 103. When the data from this northern blot was quantitated and normalized to the GAPDH signal, it could be seen that the levels of the clone 103 RNA declined 14-fold between days 7.5 and 12.5 of gestation.

![Fig. 1. Developmental northern blot analysis of differentially expressed cDNA clones. (A) 20 μg of total RNA isolated from embryos at days 7.5 through 12.5 of gestation was electrophoresed, blotted and hybridized with the inserts from the cDNA clones 103 and 106. To control for RNA integrity and loading, the blot was also hybridized with a probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (B) Quantitation of northern blot hybridization data. The Signal (counts per minute of ³²P) for each lane of the blot depicted in A was determined (see Materials and methods), normalized to the GAPDH signal and the normalized signal at 7.5 dpc was arbitrarily set at 100 units.]
The nucleotide and predicted amino acid sequences of the inserts of clones 103 and 106 are presented in Fig. 2. Database searching revealed that both of these clones encode facilitative glucose transporters. Clone 106 is identical to the mouse Glut-1 cDNA (Reed et al., 1990), while clone 103 is identical to the mouse Glut-3 cDNA (Nagamatsu et al., 1992). These results are in good agreement with the sizes of the transcripts detected on northern blots. Mouse Glut-1 is encoded by a 2.5 kb mRNA (Reed et al., 1990), while mouse Glut-3 is encoded by a 4.0 kb mRNA (Nagamatsu et al., 1992). Both of these clones are derived from the same region of the glucose transporter gene. Clone 106 is identical to nucleotides 565-825 of the mouse Glut-1 cDNA, except for one nucleotide change in the second nucleotide of clone 106. Since this change results in an alanine to glycine substitution at an amino acid conserved in several glucose transporter family members, it probably resulted from misincorporation during the PCR reaction, rather than a genetic polymorphism. This region encodes amino acids 153-238 of the GLUT1 protein, including the membrane spanning regions 5 and 6 as well as part of the hydrophilic intracellular loop. Clone 103 is identical to nucleotides 538-785 of the mouse Glut-3 cDNA, encoding amino acids 153-235, which includes the membrane spanning regions 5 and 6. Only the first 249 base pairs of clone 103 are identical to mouse Glut-3. After nucleotide 249 the sequences of clone 103 and mouse Glut-3 diverge entirely. To determine if this might be an artifact of the PCR amplification or cDNA cloning procedure, the insert from clone 103 was digested with the restriction enzyme PstI, which separated the insert sequence encoding mouse Glut-3 from the unique sequence (Fig. 2B). Each part of clone 103 was then used as a probe on a northern blot. The portion of clone 103 encoding mouse Glut-3 hybridized to the 4.0 kb mRNA that we had previously detected using the entire insert as a probe (data not shown; see Fig. 1A). The unique portion of clone 103 did not detectably hybridize to any RNA. We conclude, therefore, that the 3′ end of clone 103 consists of sequences that were joined to mouse Glut-3 sequences as an artifact of the PCR amplification or cDNA cloning procedure.

**In situ hybridization**

To gain some idea of the spatial and temporal localization of these two glucose transporters during early postimplantation mouse development, we hybridized 35S-labelled single-stranded antisense riboprobes to sections of embryos from 7.5 dpc to 10.5 dpc. This analysis revealed striking differences in the localization of the mouse Glut-1 and Glut-3 transcripts in early postimplantation mouse embryos. For analysis of Glut-3 transcription only the portion of clone 103 identical to mouse Glut-3 was used to hybridize to 4.0 kb mRNA that we had previously detected using the entire insert as a probe (data not shown; see Fig. 1A). The unique portion of clone 103 did not detectably hybridize to any RNA. We conclude, therefore, that the 3′ end of clone 103 consists of sequences that were joined to mouse Glut-3 sequences as an artifact of the PCR amplification or cDNA cloning procedure.
Fig. 3. Expression of Glut-1 and Glut-3 RNA in 7.5 dpc embryos. (A) Sagittal section of 7.5 dpc mouse embryo hybridized with an antisense riboprobe of clone 106 (Glut-1). High levels of expression are observed in the ectoplacental cone, amnion, chorion and mesodermal layer of the yolk sac. Lower levels of expression are observed in the embryonic ectoderm and the endodermal layer of the yolk sac. (B) Sagittal section of 7.5 dpc mouse embryo hybridized with an antisense riboprobe of clone 103 (Glut-3). High levels of expression are observed in the amnion, chorion and endoderm layer of the yolk sac. Expression is also observed in isolated cells in the decidua (arrows). Abbreviations: a, amnion; c, chorion; ec, ectoplacental cone; es, endodermal layer of the visceral yolk sac; ms, mesodermal layer of the visceral yolk sac.
Fig. 4. Expression of Glut-1 and Glut-3 RNA in 8.5 dpc embryos. (A) A frontal section of a 8.5 dpc embryo hybridized with an antisense riboprobe of clone 106 (Glut-1). Widespread expression of Glut-1 is observed in both extraembryonic and embryonic tissues. (B) A higher magnification view of the section shown in A. High levels of Glut-1 expression are observed in the neural tube and in non-neural surface ectoderm. No expression is observed in the somites or in somatic or splanchnic mesoderm. (C) An oblique transverse section of a 8.5 dpc embryo hybridized with an antisense riboprobe of clone 103 (Glut-3). High levels of expression are observed in the amnion, yolk sac and non-neural surface ectoderm. No expression is observed in neuroepithelium or mesenchyme. (D) A near-adjacent section of the embryo shown in A and B hybridized with an antisense riboprobe of clone 103 (Glut-3). High levels of expression are observed in surface ectoderm, while weak levels of expression are seen in endoderm (arrow). No expression is seen in the neural tube, somite, or somatic or splanchnic mesoderm. Abbreviations: a, amnion; ne, neuroepithelium; nt, neural tube; s, somite; se, non-neural surface ectoderm; so, somatopleure; sp, splanchnopleure; ys, yolk sac.
Fig. 5. Expression of Glut-1 and Glut-3 RNA in 9.5 dpc embryos. (A) Parsagittal section of 9.5 dpc mouse embryo hybridized with an antisense riboprobe of clone 106 (Glut-1). High levels of expression are observed in the pericardium and mesocardium of the heart and in the spinal cord. Lower levels of expression are observed in neuroepithelium of the optic lobe. (B) A near-adjacent section of the embryo shown in A hybridized with an antisense riboprobe of clone 103 (Glut-3). High levels of expression are observed in the pericardium of the heart. Expression is also observed in isolated patches of surface ectoderm (arrow). (C) Another view of the section shown in B. Glut-3 expression is observed in surface ectoderm of the head. Abbreviations: h, heart; o, neuroepithelium of the optic lobe; sc, spinal cord; se, surface ectoderm.
Fig. 6. Expression of Glut-1 in eye and spinal cord at 10.5 dpc. (A) Section (parasagittal through head, transverse through spinal cord) hybridized with antisense riboprobe of clone 106 (Glut-1). Expression is observed in eye and spinal cord. (B) Higher magnification view of the section shown in A. Glut-1 expression is observed in the lens and in the pigment layer of the retina, but not in the sensory layer of the retina. (C) Higher magnification view of the section shown in A. Glut-1 expression in the spinal cord is spatially restricted. The white arrows indicate the dorsal and ventral limits of expression of Glut-1 in the spinal cord. No expression is observed in the roof or floor plates, in neuroepithelial cells adjacent to these structures or in the notochord. Abbreviations: f, floor plate; l, lens; n, notochord; p, pigment layer of the retina; r, roof plate; s, sensory layer of the retina.

Fig. 8. Expression of Glut-2 RNA in early postimplantation embryos. All sections were hybridized with a Glut-2 antisense riboprobe. (A) Parasagittal section of 7.5 dpc mouse embryo. (B) Sagittal section of 8.5 dpc mouse embryo. Expression is observed only in the endodermal layer of the extraembryonic visceral yolk sac. No expression is observed in yolk sac endoderm which is in contact with the embryo. (C) Parasagittal section of 10.5 dpc mouse embryo. Expression is observed in the liver primordium. Abbreviations: da, dorsal aorta; h, heart; es, endodermal layer of the visceral yolk sac; pa, posterior amniotic fold; sc, spinal cord.
as in isolated cells in the decidua. Clone 106 (Glut-1) was expressed at varying levels throughout the embryonic and extraembryonic tissues, with highest levels of expression in the ectoplacental cone, amnion, chorion and mesodermal layer of the yolk sac (Fig. 3A). Lower levels of expression were seen in the embryonic ectoderm, embryonic mesoderm and endoderm layer of the yolk sac. Expression of clone 103 (Glut-3) at 7.5 dpc appeared to be restricted to extraembryonic tissues, with the exception of embryonic ectoderm immediately adjacent to the amnion (Fig. 3B). High levels of expression of clone 103 were seen in the ectoderm layer of the amnion, chorion and endoderm layer of the yolk sac, while no expression was observed in the ectoplacental cone, extraembryonic mesoderm and embryonic mesoderm or ectoderm (with the exception noted above).

At 8.5 dpc, both clones continued to be expressed at high levels in the yolk sac (although not in the hematopoietic cells of the blood islands) and the amnion. Clone 103 (Glut-3) showed high levels of expression in non-neural surface ectoderm and in the endoderm layer of the yolk sac, but was not detected in other embryonic tissues (Fig. 4C,D). Clone 106 (Glut-1) had a broader distribution at 8.5 dpc, and expression could be detected in the amnion, yolk sac mesoderm and neural folds but not in the somites or somatic or splanchic mesoderm (Fig. 4A,B). By 9.5 dpc, expression of clone 103 (Glut-3) could be detected in the pericardium (Fig. 5B), cells lining the pharynx (not shown) and surface ectoderm (Fig. 5B,C). Expression of clone 106 (Glut-1) was detected primarily in heart and spinal cord at this time (Fig. 5A).

By 10.5 dpc, only very limited expression of clone 103 (Glut-3) could be detected. This abrupt decline in the level of Glut-3 transcripts between 9.5 dpc and 10.5 dpc was also detected by the northern blot analysis (Fig. 1). Sporadic expression of Glut-3 at this time was detected in surface ectoderm (not shown). At 10.5 dpc clone 106 (Glut-1) was expressed in the developing eye, where expression was seen in the lens and in the pigment layer of the retina, but not in the sensory layer of the retina (Fig. 6A,B). Expression of clone 106 was also detected in the spinal cord at this time. Expression in the spinal cord was spatially restricted; expression was not observed in the floor and roof plates or cells adjacent to these structures (Fig. 6A,C).

RNA expression analysis of Glut-2 and Glut-4

Since our differential screen of the PCR-generated cDNA library had led to the isolation of two different glucose transporter isoforms, we were interested in determining if other glucose transporter isoforms might also be differentially expressed during these embryonic stages. Probes for Glut-2 and Glut-4 were cloned by PCR (see Materials and methods) and were used as probes on developmental northern blots and for in situ hybridization. By northern blot analysis, Glut-2 RNA was detected at fairly high levels in 7.5 and 8.5 dpc embryos and was barely detectable in RNA isolated from 10.5 and 12.5 dpc embryos (Fig. 7). Glut-4 RNA was not detected at any embryonic stage (data not shown). In situ hybridization analysis with the Glut-2 probe revealed that expression was confined to visceral yolk sac endoderm of 7.5 and 8.5 dpc embryos (Fig. 8A,B). Glut-2 expression at these stages was observed in extraembryonic visceral endoderm; endoderm that was in contact with the embryo did not express Glut-2. No localized Glut-2 RNA expression was observed in the embryo proper at 7.5 and 8.5 dpc. At 10.5 dpc Glut-2 expression was observed in the liver primordium (Fig. 8C).

Discussion

Library construction and screening

In this study, we have described the construction and screening by differential hybridization of a cDNA library derived from the distal portion (embryonic ectoderm, mesoderm and visceral endoderm) of 7.5 dpc mouse embryos. In the construction of this library, PCR amplification techniques were used to increase the amounts of cDNA available for cloning (Belyavsky et al., 1989; Brady et al., 1990; Welsh et al., 1990). During the initial round of differential screening approximately 5% (100/2000) of the phage plaques from the library did not hybridize to 32P-labelled cDNA made from 12.5 dpc embryo poly(A)+ RNA. These non-hybridizing phage could represent phage without cDNA inserts, cDNAs representing low abundance RNA species present in 12.5 dpc embryos, or cDNAs that were not expressed in 12.5 dpc embryos. When inserts from 50 of these phage were tested by a more sensitive technique (i.e., using 32P-labelled insert from the phage as probes on developmental northern blots), two clones reproducibly gave greater hybridization to RNA isolated from 7.5 dpc embryos than 12.5 dpc embryos. Inserts from the other 48 clones either failed to detect an RNA band on the northern blots, or detected an RNA species that gave approximately equal hybridization signals at days 7.5 and 12.5 of gestation.
Nucleotide sequence analysis of these two differentially expressed clones revealed that each was a different member of the family of facilitative glucose transporters (Glut-1 and Glut-3). It is perhaps not surprising that glucose transporters would be differentially expressed in early postimplantation embryos. Glucose homeostasis is a matter of critical importance for early embryonic development in rodents, and glucose utilization has been extensively studied during in vitro culture of mouse and rat embryos. While fertilized one-cell mouse eggs cultured in vitro have an absolute requirement for pyruvate to complete the first cleavage division (Biggers et al., 1967), preimplantation mouse embryos switch from a pyruvate to a glucose-based metabolism at around the time of compaction (Gardner and Leese, 1986; Leese and Barton, 1984). Recently glucose was shown to be essential for the morula-to-blastocyst transition during in vitro culture (Brown and Whittingham, 1991). Gardner and Leese (1988) demonstrated the presence of a facilitated transport system for glucose entry into preimplantation mouse embryos, and indirect evidence from the in vitro culture of early postimplantation rat embryos also supports the existence of a carrier-mediated transport system (Ellington, 1987b). In postimplantation embryos, the major energy source of rat embryos cultured in vitro from the early headfold to the 25-somite stage is glucose (Cockroft, 1979). The concentration of glucose present during in vitro culture can have a profound effect on development, as in vitro culture of rodent embryos in conditions of either glucose deficiency (Ellington, 1987a) or glucose excess (Cockroft and Coppola, 1977; Sadler, 1980) has been shown to have teratogenic effects on the developing embryos.

**Glucose transporter expression in extraembryonic membranes**

Analysis of the spatial and temporal localization of the Glut-1 and Glut-3 genes by in situ hybridization revealed striking differences between the two clones. In the construction of the PCR-amplified cDNA library, we attempted to enrich for genes expressed in the embryo proper, as opposed to extraembryonic tissues. However, the starting material for the library construction did contain a portion of the visceral yolk sac. Therefore genes expressed in this extraembryonic tissue would be represented in the library. Both the Glut-1 and Glut-3 genes were expressed at high levels in the visceral yolk sac, relative to the embryo proper, at 7.5 dpc. In the yolk sac, Glut-3 appeared to be expressed only in the endoderm while Glut-1, although expressed in both layers, appeared to be expressed more strongly in the mesoderm layer. While both transporters were expressed in the amnion and chorion, only Glut-1 was expressed in the ectoplacental cone. Thus, the two transporters have relatively reciprocal sites of expression in the developing extraembryonic membranes. Another glucose transporter isoform, Glut-2, is also highly expressed in the endoderm layer of the extraembryonic visceral yolk sac. However, unlike Glut-3, Glut-2 was not expressed in the amnion and chorion. Glut-4 was not detectably expressed in extraembryonic membranes. Thus, all of the glucose transporter isoforms analyzed have at least partially non-overlapping expression patterns in extraembryonic membranes.

Considering the metabolic requirements of the developing embryo, it is not surprising that glucose transporters would be highly expressed in extraembryonic membranes. Upon implantation, trophoblast cells of the blastocyst invade and subsequently erode, the uterine epithelium (Rugh, 1990; Theiler, 1989). Large, glycogen-rich decidual cells appear in the vicinity of the implantation site and, after the degeneration of the uterine epithelium, the embryo becomes bathed in maternal blood. The glucose transporters in the extraembryonic tissues would then allow delivery of maternally derived glucose to the rapidly growing embryo.

**Glucose transporter expression in the embryo**

At 7.5 dpc, none of the glucose transporter isoforms assayed (Glut-1, -2, -3 or -4) was expressed at high levels in embryonic ectoderm or mesoderm, leaving open the question as to which glucose transporter family member is functioning in these cells. At 8.5 dpc, expression of Glut-1 was fairly widespread in the embryo while expression of Glut-3 was confined primarily to non-neural surface ectoderm. By 10.5 dpc, expression of both genes had been down-regulated. Glut-3 expression was observed only in isolated patches of surface ectoderm, while Glut-1 expression was observed in the eye and a spatially restricted region of the spinal cord. In the adult, the Glut-2 gene encodes the isoform that mediates glucose transport in hepatocytes and pancreatic \( \beta \)-cells (Thorens et al., 1988). We detected Glut-2 expression at 10.5 dpc in the liver primordium. We did not detect Glut-4 expression through 10.5 dpc. Both our results with Glut-2 and Glut-4 are in agreement with the results of Hogan et al. (1991), who detected Glut-2 but failed to detect Glut-4 expression through 9.5 dpc using a reverse transcriptase-PCR assay. GLUT1 and GLUT2 proteins have also been detected in preimplantation embryos by immunofluorescence and immunoelectron microscopy (Aghyan et al., 1992; Hogan et al., 1991).

Recent results have demonstrated that Glut-3 encodes the glucose transporter isoform of adult mouse neuronal cells (Nagamatsu et al., 1992), while Glut-1 is expressed in brain but is largely confined to the microvasculature of the blood-brain barrier (Pardridge et al., 1990). Our results demonstrate that Glut-3 is not detectably expressed in neuroepithelium at 8.5 dpc or in the developing brain at 9.5 and 10.5 dpc. Thus, Glut-3 transcription must be turned on in the brain sometime subsequent to 10.5 dpc. Glut-1, however, is expressed in regions of the brain, spinal cord and eye prior to 10.5 days of gestation.

Glut-4 encodes the major transporter responsible for the insulin-stimulated transport of glucose in muscle and adipose tissue in adults. Our results demonstrated that none of the glucose transporter isoforms assayed (Glut-1, -2, -3 or -4) was expressed at levels detectable by in situ hybridization in somites (Fig. 4, Fig. 8 and data not shown), the paired blocks of mesodermal cells that give rise to the axial skeleton, the muscles and the dermal layer of the skin. That would leave only expression of Glut-5 (among the presently known facilitative glucose transporters) as candidate genes encoding the transporter functioning in the somites. It is also possible that additional members of the facilitative glucose transporter family have yet to be cloned. Further experiments will be required to address these questions.
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References


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