Expression characteristics and relevance of sodium glucose cotransporter-1 in mammalian renal tubulogenesis

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The subject of renal development has been under intense investigation for several decades (2, 14, 33, 36, 38). The characteristics of renal development include intercalation of the ureteric epithelial bud into mesenchyme with induction of a phenotypic change, whereby the mesenchymal cells acquire the properties of epithelial cells. Certain epithelial cells differentiate into podocytes of the glomerulus, and others form cells that line the various segments of the renal tubules. While the glomerulus is being vascularized (2, 32), the differentiated tubular epithelial cells undergo membrane specialization, acquire intercellular junctional complexes, and thus become polarized with apical and basolateral domains (33, 38). Conceivably, the differentiation of the epithelial cells and the acquisition of the highly specialized membrane domains are governed by a number of factors or macromolecules, including the basement membrane proteins and their receptors, i.e., integrins (14). The latter apparently play a role in the anchorage of the cells onto the basal lamina and, in concert with the junctional complexes, serve to determine the polarity characteristics of the lining epithelial cells. Besides integrins and their ligands, basement membrane proteins and other cell adhesion molecules, like E-cadherin, the appearance of which coincides with expression of laminin, guide the establishment of polarity characteristics of certain membrane proteins, e.g., Na⁺ and K⁺-ATPase (38).

During this time frame, that is, about day 15 or 16 of mouse gestation, when these polarity characteristics are being established, the glomeruli and tubules begin to mature and acquire filtration and reabsorptive transport properties (3, 20, 37). The latter are believed to influence the handling of water, ions, amino acids, and sugars by the renal tubular epithelia (6, 7, 28, 41). This would indicate that many transporters and channels that are required to handle these molecules begin to be expressed at that stage of embryonic life. Although, the biology of numerous transporters has been extensively investigated in mature mammals, information relevant to embryonic life is rather limited (3, 10, 13, 20, 44). In view of these considerations, the developmental biology of one of the transporters that is involved in the transport of sugar as well as ions, i.e., the sodium glucose transporter (SGLT-1), was investigated, and its expression characteristics and relevance to tubulogenesis in metanephrin development are described in this communication.

MATERIALS AND METHODS

Isolation of mouse SGLT-1 cDNA clones. First, SGLT-1 cDNA (347 bp) was isolated by PCR using sense (5'-AGGCCACGACCTCTCTACGATCC-3') and antisense (5'-CTGGTTCC ATGCAGCTCCCGGTCCATAGG-3') primers, and it was used for screening the mouse cDNA library.
Three nylon membrane blots were prepared and hybridized to treatments with denaturing and neutralizing solutions (42). Gels were then treated with 0.2 N HCl, followed by successive visualizations of the intact 18S and 28S bands on the nylon membrane stained with methylene blue. The genomic DNA were subjected to gel electrophoresis. The same nylon filter was hybridized with a radioactive probe. This region exhibits the most diversity among 1- and 3-wk-old mice was extracted (42). After digestion with EcoRI, BamHI, HindIII, and SalI, aliquots of the genomic DNA were subjected to gel electrophoresis. The gels were then treated with 0.2 N HCl, followed by successive treatments with denaturing and neutralizing solutions (42). Three nylon filter membranes were prepared and hybridized with a radiolabeled β-actin probe. The integrity of RNA was monitored by visualization of the intact 18S and 28S bands on the nylon membrane stained with methylene blue.

For genomic analysis, DNAs from adult mouse, rat, and human kidneys were prepared (42). After digestion with EcoRI, XbaI, PstI, BamHI, HindIII, and SalI, aliquots of the genomic DNA were subjected to gel electrophoresis. The gels were then treated with 0.2 N HCl, followed by successive treatments with denaturing and neutralizing solutions (42). Three nylon filter membranes were prepared and hybridized with the [α-32P]dCTP-labeled mouse SGLT-1 cDNA, prepared by PCR using the following sense and antisense primers: 5'-CCAGATCATCTGTGGGGTC-3' and 5'-CTTCCCTCTCTTCCTTAGGC-3'. The 330-bp PCR product generated corresponds to the 3' end of the mouse SGLT-1 cDNA. This region exhibits the most diversity among the various Na+-coupled transporters (23), and BLAST analyses revealed no significant homology (<15%) of the 330-bp SGLT-1 cDNA with other known sequences. The hybridization of the filter was carried out under high-stringency conditions, i.e., at 65°C. The filter was also washed under high-stringency conditions, and autoradiograms were prepared. The same nylon filter was hybridized with a radiolabeled β-actin probe. The integrity of RNA was monitored by visualization of the intact 18S and 28S bands on the nylon membrane stained with methylene blue.

For tissue expression of SGLT-1 in the developing metanephros. For mRNA expression, in situ hybridization experiments, using the 330-bp PCR product employed in Northern blot analysis, were performed. The PCR product was ligated into pCR2.1 TA cloning vector. After the insert from the construct with EcoRI I was released, it was subcloned into pBluescript II KS(+) and used as a template for generating sense- and antisense-riboprobes by employing riboprobe in vitro transcription system (Promega, Madison, WI). The SGLT-1 cDNA was linearized, and the riboprobes were synthesized by incorporating [α-32P]UTP (Amersham, Piscataway, NJ), using T3 and T7 RNA polymerase. They were then used for in situ hybridization with kidney tissue sections (22, 42). For protein expression studies, a polyclonal antibody specific for SGLT-1 was used. The antibodies were generated by using SGLT-1 as an antigen. The antisense antibody was also tested for its ability to block the expression of SGLT-1 in the developing metanephros. The antibodies were affinity purified on 0.2 N HCl, followed by successive treatments with denaturing and neutralizing solutions (42). Three nylon filter membranes were prepared and hybridized with the [α-32P]-radiolabeled mouse SGLT-1 probe under high-stringency conditions, and autoradiograms were developed.

Mouse kidneys, harvested at day 13 of gestation, were maintained in culture for 4 days, and the ODNs were added to the media daily at a concentration range of 0.5 to 1.5 μM, after which they were processed for various studies. Up to a concentration of 2.5 μM, the ODNs usually retain the translational blockade specificity with no discernible cytotoxic effects (9, 42).

SGLT-1 mRNA expression in antisense ODN-treated metanephiroid. To assess the effect of antisense ODNs on mRNA expression, competitive RT-PCR analyses were carried out (42). The sense and antisense primers were: 5'-AGGGCCAGACCCCTCTTCACTAGG-3' and 5'-CTTGAGTTCCATGTAGGCTTGAGTCCCGTCTGTTCCGTTGATGAG-3'. Using these primers and "wildtype" renal cDNA, the expected PCR product sizes would be 347 bp for mouse SGLT-1. A competitive "mutant" DNA construct containing SGLT-1 sequences was synthesized by PCR using the following sense and antisense primers, 5'-CCAGAAATGACTTCTCAGAGTATCCAGCAAGGCGGCGCGG-3' and 5'-CCAGAAATGACTTCTCAGAGTATCCAGCAAGGCGGCGCGG-3'. Using these primers, the expected size of the competitive SGLT-1 DNA product is 215 bp. The nucleotide sequences upstream of the SGLT-1 primers are derived from glomerular epithelial protein-1 (GLEPP-1), which is expressed in the renal glomerulus (40). The GLEPP-1 mRNA expression served as a control, with an expected 467-bp PCR product when wild-type renal cDNA is used, while the expected size of the GLEPP-1 PCR product would be 271 bp when the competitive mutant DNA template is used. The sequence of the PCR product generated by SGLT-1/GLEPP-1 primers was confirmed, and it was then purified by gel electrophoresis and ligated into pCR2.1 cloning vector (Invitrogen, San Diego, CA). This modified minigene construct was used for the mRNA analyses of the SGLT-1 and GLEPP-1. The unmodified original minigene construct, containing primer sequences for β-actin, was used as the competitive mutant DNA template with an expected 224-bp size of the PCR product (22). Finally, the RT-PCR analyses were carried out as previously described (42).

SGLT-1 protein expression in antisense ODN-treated metanephiroid explants. To assess the translational blockade of SGLT-1, immunoprecipitation and -fluorescence studies were performed. For immunoprecipitation experiments, antisense-treated explants were labeled with 35S-methionine, and membrane proteins were extracted (22, 42). The extracts were then immunoprecipitated with the anti-SGLT-1 antibody (Chemicon, Temecula, CA). The controls included the untreated metanephiroid and those treated with sense or nonsense ODNs. To ensure the effect of antisense ODN on the translational blockade of mouse SGLT-1, double the amount of immunoprecipitated radioactivity was used for SDS-PAGE analyses. Finally, the tissue expression of SGLT-1 in antisense and sense or nonsense ODN was assessed by immunofluorescence microscopy.

In situ tissue expression of wheat germ agglutinin and GLEPP-1. To evaluate the status of glomeruli in the antisense-treated explants, expression of wheat germ agglutinin (WGA) and GLEPP-1 was assessed (40). Cryostat sections stained with anti-SGLT-1 antibody were concomitantly stained with WGA conjugated with rhodamine (Sigma, St. Louis, MO). The sections derived from the midplane of the explant were examined. Finally, GLEPP-1 mRNA expression was determined in the antisense-treated explants by in situ
tissue autoradiography. The 467-bp product generated above by PCR was used to prepare the GLEPP-1 riboprobe. The conditions for hybridization of GLEPP-1 riboprobe and preparation of the tissue autoradiograms were the same as previously described (21).

\[ \text{\textsuperscript{6}C} \text{glucose uptake studies.} \]

Glucose uptake experiments were carried out to assess whether there is a correlation of functional activity of the SGLT-1 with its developmental expression. Intact day 13 metanephroi and slices of day 16 and newborn kidneys were maintained in culture for three days. The antisense or sense ODNs were added into the culture medium at a final concentration of 1.5 \( \mu \text{M} \). Ninety minutes prior to the termination of the culture, the explants were transferred to culture media, composed of equal volumes of DME and F-12 (Sigma), and the final concentration of glucose and sodium was adjusted to 5 and 100 mM, respectively. An analog of glucose, \( \text{\textsuperscript{14}C} \text{methyl-d-glucopyranoside (MeGlc, 25 \mu Ci/ml, specific activity = 300 GBq/mmol)} \) (17), was added to the culture medium. To evaluate the specificity of the MeGlc uptake, either 1 mM \( \text{d-galactose} \) or \( l\)-galactose was added to some of the cultures. After the termination of the culture, the explants were rinsed with ice-cold Na\(^+\)-free medium and processed for tissue autoradiography (21, 22, 42).

**RESULTS**

Characterization of mouse newborn SGLT-1 cDNA clones. By combining the overlapping sequences of 5 clones, an open reading frame consisting of 1,995 nucleotides was obtained, which had a deduced translated product of 665 amino acids (Fig. 1). Mouse SGLT-1 had \( \sim 95 \) and \( \sim 86\% \) sequence homology with rat and human forms (18, 24), respectively. In the mouse newborn cDNA, an A(U)nA mRNA instability motif was present at 818 bp downstream from the 3' end of the termination codon. The amino acid sequence had structural domains similar to those of the human and rat. They included a six-residue motif (RFGGKR) located in the extracellular domain of the SGLT-1, which is involved in the binding and translocation of glucose. The protein kinase C (PKC) motifs were also similar to other species; however, a protein kinase A (PKA) motif, like that in the rat, was absent in mouse SGLT-1. There were 2 potential N-linked glycosylation sites (NXT/NXS) at the 248- and 306-amino acid residues, which seem to be conserved among mammalian species. The N-linked glycosylation site at Asn \({565}\) was seen in rat was absent in mouse SGLT-1. The hydrophatic analysis suggested that the N-glycosylation site at the 248 residue be located in the extracellular domain, a finding consistent with previous studies (24).

Northern and Southern blot analyses. An evaluation of SGLT-1 mRNA expression in human, mouse, and rat was carried out in view of the differences in the transcript size and their number reported for various species (16, 18, 24, 30). In mouse, three major mRNA transcripts of \( \sim 4.7, \sim 3.2 \) and \( \sim 2.3\)-kb size were seen (Fig. 2A, lane 2). The midsize transcript (\( \sim 3.2 \)) seems to be a doublet; thus it appears that the mouse SGLT-1 has four transcripts (Fig. 2A, white dots in lane 2). In human SGLT-1, three distinct transcripts were also observed; however, the transcript size of the high- and intermediate-molecular-weight bands differed from the mouse SGLT-1 (Fig. 2A, lane 1, asterisks). An additional high-molecular-weight transcript was also discernible (Fig. 3A, lane 1, small asterisk); however, its size was different from the mouse transcript. In rat, four mRNA transcripts were also present (Fig. 3A, lane 3, asterisks). The high- and low-molecular-weight transcripts were identical to that of the mouse. The intensity of the bands of various transcripts in both human and rat was less compared with that of mouse, which may be related to the fact that the blot was hybridized with a mouse SGLT-1 probe. The amount of RNA loaded, most likely, was comparable among the three species since the intensities of the 28S and 18S bands (Fig. 2B), and of the \( \beta\)-actin (Fig. 2C) were similar. Like the mRNA transcripts, Southern blot analyses revealed differences in the intensity and size of the bands, suggesting a different genomic organization of the SGLT-1 gene among the three species (Fig. 2, D, E and F).
Developmental expression of SGLT-1 in the mouse kidney. The SGLT-1 mRNA expression was detectable at day 13 of gestation (Fig. 3A). Three major transcripts of ∼4.7, ∼3.2, and ∼2.3 kb can be observed in the mouse kidney (A, lane 2), and they are indicated by the white dots on the bands. The middle band appears to be a doublet with transcripts' size of ∼3.2 and ∼3.5. In human, there are also three transcripts (A, lane 1, asterisks), but their size differs from that of the mouse, except for the ∼2.3-kb transcript. An additional high molecular transcript is also seen, and this band is faint and is highlighted by a small asterisk. In rat, four mRNA transcripts are present (A, lane 3, asterisks). The loading of equal amounts of the RNA in various species is indicated by the similar densities of 28S and 18S bands in the blot stained with methylene blue dye (B). The β-actin expression is similar in all of the three species (C). D-F: Southern blot analyses of the SGLT-1 genomic DNA isolated from human (D), mouse (E), and rat kidneys (F), and subjected to digestion with restriction endonucleases. Different banding pattern is observed in blots of various species hybridized with mouse SGLT-1 cDNA probe, suggesting that the genomic organization of SGLT-1 gene is variable among these species.
RNA were similar in all the 5 lanes (Fig. 3B). The β-actin mRNA expression in the mouse kidneys remained essentially constant (Fig. 3C).

By in situ hybridization, a mild SGLT-1 mRNA expression was observed at day 13, and it was localized to the ureteric bud and its branches (Fig. 4A). At day 16,
The mRNA expression was seen in the tubules of the inner cortex and outer medulla (Fig. 4B), and, the signal was absent in the inner medulla. The signal was not uniformly distributed but was “clustered” in certain tubules of the inner cortex and outer medulla (Fig. 4B, arrowheads). In the newborn or day 19, the expression was also seen in tubules confined to the inner cortex and outer medulla in the form of “clusters” (Fig. 4C, arrowheads), while it was not discernible in inner medulla. In kidneys of 1- and 3-wk-old mice, the mRNA expression was confined to the inner cortex and outer medulla (Fig. 4D and E), whereas, no mRNA expression was observed in the glomeruli, and it remained absent in the inner medulla.

The immunofluorescence studies confirmed the SGLT-1 gene expression findings. At day 13, a mild anti-SGLT-1 immunoreactivity was seen in the ureteric bud branches and a few developing tubules (Fig. 4F). At day 16 and 19 (newborn), it was seen mainly in the tubules of the inner cortex and outer medulla (Fig. 4, G and H). A few inner medullary tubules also exhibited weak immunoreactivity; however, no glomerular reactivity was observed. During the postnatal period, i.e., at 1 and 3 wk, the reactivity intensified, and it was mainly confined to the inner cortex and outer medulla (Figs. 4, I and J).

**Role of SGLT-1 in tubulogenesis of the mammalian metanephros (antisense experiments).** The embryonic renal explants treated with 1.5 μM of nonsense or sense ODN did not reveal any major morphological changes (Fig. 5, B vs. A, and F vs. E). The ureteric bud branches exhibited normal iterations, and the glomeruli and tubules were well developed. The explants treated with antisense ODN exhibited an overall reduction in their size (Fig. 5, C and D). At a concentration of 0.5 μM, nephron population was reduced, and the mesenchyme was expanded (Fig. 5, C and G). The reduction was confined to the tubules, although a mild...
decrease in the number of glomeruli was observed as well. At 1.0 μM, a further reduction in the number of nephrons was observed. At 1.5 μM concentration of the antisense ODN, the size of explants was significantly reduced (Fig. 5D), and the number of the tubules was remarkably decreased (Fig. 5H). The glomerular population was largely unaffected (Fig. 5H). The ureteric bud branches were rudimentary, and their normal dichotomous iterations were not seen.

Immunofluorescence studies were performed on the explants treated with nonsense- and antisense ODN to confirm that the development of nascent tubules and not of the glomeruli or the precursor of collecting ducts, i.e., ureteric bud branches, was affected. The sections stained with WGA did not reveal any decrease in its reactivity with the glomeruli in explants treated with 1 μM SGLT-1 antisense compared with the ones treated with nonsense/sense ODN (Fig. 6, G vs. E and H vs. F, arrowheads). Also, enumeration of WGA-stained glomeruli did not reveal any decrease in their population. A marked decrease in the number of tubules was observed in the antisense-treated explants (Fig. 6, G vs. E). Also, anti-SGLT-1 antibody reactivity with the remaining tubules was mildly decreased compared with the control (Fig. 6, C vs. A and D vs. B). Immunoreactivity of anti-SGLT-1 in the ureteric bud branches was slightly decreased as well (Fig. 6, C and D).

Fig. 6. Immunofluorescence photomicrographs of day 13 explants treated with 1.5 μM nonsense-ODN (A, B, E, and F) and SGLT-1 antisense ODN (C, D, G, and H). The photographs in B, D, F and H are high magnifications of A, C, E and G, respectively. The tissue sections in A-D were stained with anti-SGLT-1 antibody, while the adjacent sections in E-H were stained with rhodamine-conjugated wheat germ agglutinin (WGA), the latter being a marker for the renal glomerular podocytes or glomeruli. The immunoreactivity of anti-SGLT-1 is mildly reduced in the remaining tubules (t) and the ureteric bud branches (U) (C vs. A and D vs. B). The WGA expression and the population of the glomeruli (arrowheads) are unaffected by the antisense ODN treatment (G vs. E and H vs. F).
Gene and protein expression studies in antisense-treated metanephric explants. In the nonsense-ODN-treated (control), a linearity in the ratios of wild to mutant SGLT-1 DNA could be maintained when plotted against the $10^{-1}$ to $10^{-7}$ serial logarithmic dilutions of the competitive (mutant) template DNA. Within this range of dilutions, the bands of wild-type and mutant DNA were discernible for densitometric analyses to obtain a ratio. The densitometric graphic plots are not included here because they have been repeatedly shown in our several previous publications (21, 22, 42), and thus only the raw data, i.e., the electrophorograms, are shown in Fig. 7. A ratio of 1 was obtained at dilutions of $10^{-4}$ to $10^{-5}$ of the competitive mutant DNA for control group (Fig. 7A, lanes 4 and 5). For the antisense-treated group, a ratio of 1 was obtained at dilutions of $10^{-5}$ to $10^{-6}$ of the competitive DNA (Fig. 7A, lanes 5 and 6), suggesting a decrease in the order of about one to two logs of the mRNA expression in the antisense-treated explants. However, for the β-actin, no significant differences in the linearity relationship in the range of logarithmic dilutions of the competitive DNA between the two groups (control and antisense) were observed (Fig. 7B). Another control included the determination of mRNA expression of GLEPP-1, a marker for glomeruli, particularly the glomerular podocytes (40). No significant differences were observed between the control and SGLT-1 antisense-treated explants, and a ratio of 1 was observed at a dilution of $10^{-4}$ of the competitive mutant DNA in both the groups (Fig. 7C, lane 4). To confirm that the GLEPP-1 mRNA was unaffected by the antisense treatment, in situ tissue autoradiograms were prepared. No significant reduction in mRNA expression and the population of renal glomeruli was observed between the antisense (Fig. 7E) and the control groups (Fig. 7D).

For translational blockade studies, untreated, antisense and nonsense or sense ODN-treated explants were radiolabeled with $^{35}$S-methionine, and extracts were immunoprecipitated with anti-SGLT-1 antibody. Under reducing conditions, the SDS-PAGE autoradiographic analyses of the immunoprecipitated proteins revealed a major ~73-kDa band (Fig. 7F, lane 1). Minimal reduction in the autoradiographic intensity of the 73-kDa band was noted in the sense-nonsense-treated groups (Fig. 7F, lane 2). However, a reduction in the intensity of the 73-kDa band was observed in the antisense-treated explants, suggesting a blockade in the translation of SGLT-1 (Fig. 7D, lane 3).

Glucose uptake by the developing mammalian metanephros. The uptake of MeGlc was minimal in day 13 kidneys, and a mild concentration of radioactivity was seen over the ureteric bud branches and tubular segments (Fig. 8A). In day 16 explants, an increase of MeGlc uptake was observed (Fig. 8E). The high concentration of radioactivity, associated with MeGlc, was seen as clusters in the outer medulla and inner cortex (Fig. 8E, arrowheads). Interestingly, this pattern of radioactivity, confined to the clusters of tubules, simulated that observed for the SGLT-1 mRNA expression in day 16 explants (Fig. 4B). While most of the medulla was devoid of radioactivity associated with MeGlc, a mild uptake was seen in the inner medulla. In day 19 or newborn explants, the clusters of radioactivity became prominent and enlarged (Fig. 8I, arrowheads), suggesting increased uptake by the tubules of outer medulla and inner cortex. Like in day 16, most of the medulla was devoid of radioactivity, but foci of uptake were seen in the inner medulla (Fig. 8I). To verify the specificity of the MeGlc uptake antisense- and galactose-inhibitory experiments were carried out. Inclusion of 1 mM d-galactose in the media decreased the radioactivity associated with MeGlc in day 13 (Fig. 8B), 16 (Fig. 8F), and 19, or newborn (Fig. 8J) explants. The clusters of radioactivity, reflecting the uptake by the tubules of the inner cortex and outer medulla, were fewer and reduced in their size in explants from day 16 and 19 kidneys (Fig. 8, F and J, arrowheads). The radioactivity in the inner medulla was also reduced. Inclusion of 1 mM l-galactose in the media did not decrease the MeGlc uptake and the autoradiograms were indistinguishable from the controls, i.e., Figs. 8, A, E, and I. Treatment of explants with SGLT-1 antisense ODN reduced the radioactivity associated with the MeGlc uptake in day 13 (Fig. 8C), 16 (Fig. 8G), and 19 (Fig. 8K) explants. The clusters of radioactivity were very few and small, and the radioactivity in the medulla was lost (Fig. 8, G and K). The tissue autoradiograms, prepared from the explants treated with sense/nonsense ODNS, were indistinguishable from the controls.

DISCUSSION

In the mammalian metanephros, glucose transport across the plasmalemma is mediated by a number of integral membrane proteins known as specific carriers. They are involved in the reabsorption of the glucose from the glomerular ultrafiltrate that is in transit through the proximal tubular segment of the nephron. The reabsorbed glucose is returned to the circulation to maintain its blood levels and to provide other cells with a source of energy for various metabolic requirements. By homology cDNA cloning techniques, a number of specific carrier proteins or transporters have been identified since a majority of them reveal substantial similarities in their structural and functional motifs (6, 7, 28, 41). At present about five facilitative-diffusion glucose transporters (GLUT-1 to GLUT-4 and GLUT-7) are believed to exist in mammalian cells. In addition, there are 3 more transporters involved in glucose uptake that are energized by an electrochemical gradient for Na$^+$ ions across the cell membrane. They are known as sodium glucose cotransporters (SGLTs) since they couple Na$^+$ and glucose during transport across the cell membrane. Although they (SGLT-1-SGLT-3) have similar, but not identical, structural motifs, their functional capacities and expressions expectedly vary in the different segments of the nephron (7, 41). Interestingly, a given cotransporter, e.g., SGLT-1, may exhibit minor differences in its structural motif across...
Fig. 7 A-C: Competitive RT-PCR analyses of SGLT-1 (A), β-actin (B), and glomerular epithelial protein-1 (GLEPP-1) (C) cDNAs, prepared from nonsense-treated control (CON) and SGLT-1 antisense-treated (ANTISENSE) metanephroi. A: In the control, a ratio of 1 is observed between the "wild-type" and mutant DNA at $10^{-4}$-$10^{-5}$ log dilutions of the competitive (mutant) DNA (lanes 4 and 5), while in the antisense group, it is at $10^{-5}$-$10^{-6}$ log dilutions (lanes 5 and 6). This indicates 10- to 100-fold reduction in the SGLT-1 mRNA expression in the antisense ODN-treated metanephroi (A). No significant differences in the wild-type vs. mutant ratios are observed in the β-actin (B) and GLEPP-1 (C) cDNAs, derived from nonsense CON and antisense ODN (ANTISENSE)-treated explants. D and E: In situ tissue autoradiograms of day 13 explants treated with either 1.5 μM nonsense ODN (D) or SGLT-1 antisense ODN, and hybridized with GLEPP-1 riboprobe (E). The GLEPP-1 mRNA is localized to the renal glomeruli, and no decrease in their population or GLEPP-1 expression is observed. F: SDS-PAGE autoradiogram of the de novo synthesized SGLT-1. The untreated and treated metanephroi were labeled with [35S]-methionine, and the labeled proteins were extracted and immunoprecipitated with anti-SGLT-1 antibody. The immunoprecipitated products were subjected to SDS-PAGE and the autoradiograms developed. A major band of ~73 kDa is seen (arrow) in the untreated control (CON, lane 1) and nonsense-treated (NS, lane 2) groups. The band intensity is notably reduced in the antisense ODN-treated group (AS, lane 3), suggesting a translational blockade in the synthesis of SGLT-1. The arrowhead indicates the point of application of the samples.
species lines, i.e., human vs. porcine or rabbit vs. rat; however, considerable variation in its mRNA transcript size and number has been reported (16, 18, 24, 30, 31). Along these lines a high sequence homology of the mouse SGLT-1 with that of the rat (95%) and human (86%) was observed in the present investigation (Fig. 1). Also, most of the structural motifs (glucose binding motif, glycosylation and phosphorylation sites, and leucine zipper) were conserved, except that, as in the rat, the PKA site was absent. Intriguingly, significant differences in their mRNA transcript size and number were observed (Fig. 2). Such variations have been thought to be related to the cross-hybridization of the selected DNA probe with other SGLTs. The fact that a SGLT-1-specific DNA probe and high-stringency conditions were used in this study for Northern blot hybridization makes it unlikely that the differences observed here among various species were related to

Fig. 8. Uptake of glucose by day 13 (A-C), day 16 (E-G), and day 19 (newborn) (I-K) metanephric explants. Ninety minutes prior to the termination of the culture, [14C]methyl-α-D-glucopyranoside (MeGlc) was added into the media. After which, the untreated explants (A, E, and I), and those treated with 1 mM of D-galactose (B, F, and J) and 1.5 μM of SGLT-1 antisense ODN (C, G, and K) were processed for tissue autoradiography. In control, the uptake of MeGlc by the explants is minimal at day 13 and is confined to the ureteric bud branches or developing tubules (A). The uptake is notably increased at day 16 (E) and day 19 (I), especially in tubules of the inner cortex (C) and outer medulla (M), where the radioactivity is heavily clustered (arrowheads in E). These clusters of radioactivity simulate the mRNA expression seen confined to the tubules in Fig. 4B. The MeGlc uptake is accentuated in day 19 explants (I). Although, most of the medulla (M) is devoid of radioactivity, some uptake of MeGlc is seen in the inner portion of medulla. Inclusion of D-galactose in the culture medium caused a moderate decrease in the MeGlc uptake by day 13 (B), 16 (F), and 19 (J) explants. The medullary uptake is also reduced. The antisense treatment caused a marked reduction in the MeGlc uptake by all the metanephric explants (C, G, and K). Also, the inner medullary expression is abolished, and the clusters of radioactivity confined to the tubules are reduced in their size, number, and autoradiographic intensity (G and K). D, H, and L: Photomicrographs depicting the morphological features of day 13 (D), 16 (H), and 19 (L) renal explants corresponding to the in situ autoradiograms included in each column of this Figure.
cross-hybridization with other SGLTs. Conceivably, these differences may have originated during transcription. However, the possibility that they may also be related to the differential genomic organization of the SGLT-1 should be considered as well in view of the results of the Southern blot analysis of the restriction digests of the DNA, where notable differences among various species were observed (Fig. 2). Nevertheless, substantial homology of the SGLT-1 at the amino acid level would suggest similar functions that were acquired sometime during embryonic, neonatal, or postnatal life.

The data suggest that the SGLT-1 begins to be expressed in the metanephros during midgestation, i.e., day 13 (Fig. 3), the period ensuing the epithelial-mesenchymal interactions with the formation of nascent nephrons (2, 14, 33, 36, 38). It should be noted here that some of the facilitative glucose transporters, i.e., GLUT-1, are expressed as early as the 2-cell stage in the preimplantation mouse embryo (29). In the rat metanephros, SGLT-1 mRNA expression seems to occur late during gestation, where a single mRNA transcript of 4 kb has been described (44). In contrast, mouse SGLT-1 has at least three mRNA transcripts that are consistently observed quite early in the course of development. During gestation, not only the expression of these three transcripts progressively increased, but a fourth transcript also begins to be expressed at day 19 or in the newborn kidney. These findings suggest that SGLT-1 is developmentally regulated in the metanephros. Such a developmental regulation has also been elegantly demonstrated by in situ tissue autoradiography for various GLUTs, and interestingly, their highly restricted differential spatiotemporal distribution was observed in the embryonic, newborn, and adult rat kidney (10). The mouse SGLT-1 also seems to exhibit a restricted spatiotemporal expression in the embryonic and adult kidneys. By tissue autoradiography and immunofluorescence microscopy, at day 13, a mild expression could be seen in the ureteric bud branches, the precursor of the collecting ducts (Fig. 4A and F). Whereas, at day 16, a dramatic increase in expression was seen in tubular segments of the inner cortex and outer medulla, and an intense radioactivity was observed in the form of clusters (Fig. 4B). Similarly, protein expression was seen in the corresponding tubular segments (Fig. 4G). In addition, a very mild protein expression in the medullary collecting ducts was observed. In subsequent stages, the SGLT-1 expression increased but remained confined to the tubular segments of the inner cortex and outer medulla. Conceivably, these tubular elements represent the S3 segments of the proximal tubules, a finding that is consistent with earlier observations in the rat (24). In the same investigation, the authors also observed that by SGLT-1, mRNA is expressed throughout the cortex, suggesting its distribution may extend beyond the S3 segment of the proximal tubule. Such a diffuse distribution of the SGLT-1 in the cortex has also been seen in other studies where immunohistochemical techniques were employed (11, 39). To this end, an argument was made that the antibodies raised were against the synthetic peptide with sequences homologous with other SGLTs (24). This explanation may be plausible. However, the antibody used in this investigation was raised against the synthetic peptide derived from the portion of the SGLT-1 that exhibits considerable diversity among the various SGLT isoforms; thus the findings reported are most likely an accurate reflection of the localization of SGLT-1 in the mouse metanephros. Moreover, congruence in the findings of the mRNA and protein expression, consistently observed throughout the course of mouse metanephric development, supports the fact that SGLT-1 is principally expressed in the S3 segment of the renal proximal tubule.

Since SGLT-1 seems to be developmentally regulated, the next question which obviously needs to be addressed is whether it is involved in the differentiation and maturation of the mammalian metanephros. The functional activity of SGLT-1 is dependent on its phosphorylation mediated by PKC, after which it is targeted at the apical membrane domain of the cells undergoing differentiation (12). Thus it seems that PKC is involved in the morphogenesis and differentiation of the polarized epithelial phenotype associated with the functional activity of the SGLT-1 transporter. Other support for the notion that SGLT-1 may be involved in the morphogenetic developmental process comes from the fact that a potential binding site for the hepatocyte nuclear factor 1 (HNF-1) has been found in the promoter region of the rat SGLT-1 (34). HNF-1 is a developmentally regulated transcription factor responsible for the tissue-specific spatiotemporal expression of several genes, and induction of the latter during organogenesis and their sustained expression throughout embryonic life extending into the postnatal period are well known (26). In keeping with the above discussion, the relevance of this cotransporter in renal tubulogenesis was investigated by employing antisense technology. This technology has been successfully used in our laboratory and by other investigators to study various embryonic developmental processes (8, 22, 42). The treatment of SGLT-1-specific antisense ODN resulted in dysmorphogenesis of the embryonic metanephros. Intriguingly, tubulogenesis was preferentially affected (Fig. 5). The fact that glomerulogenesis was largely intact would suggest that the antisense ODN effect most likely was specific. Furthermore, the fact that the binding of WGA to glomeruli (Fig. 6) and the mRNA expression of GLEPP-1 and β-actin (Fig. 7) were unaffected would support the specificity of the SGLT-1 antisense ODN. The translational studies, where de novo synthesis of SGLT-1 protein was selectively affected (Fig. 7), would indicate that it is the deficiency of the SGLT-1 that led to an arrest in renal tubulogenesis. The latter may be due to the apoptosis of the renal tubular epithelial cells that are still in the process of being differentiated like that observed under hyperglycemic condition (21). Usually, the differentiation of the tubules continues even after the formation of glomeruli, and perhaps because of this lag period SGLT-1 antisense effect is selectively tar-
geted at the tubules. In this scenario, the glomeruli may continue to mature while the formation of nascent tubules would undergo remarkable regression. Interestingly, it is worth mentioning here that a selective role in tubulogenesis has been previously described for proteins that are exclusively expressed in the tubules, i.e., tubulointerstitial nephritis antigen (TIN-ag) (22). TIN-ag is one of the extracellular matrix proteins, and the latter are known as morphogenetic modulators that exert considerable influence on embryonic development (15). Nevertheless, in view of the data of antisense experiments and the fact that SGLT-1 has a consensus binding site for HNF-1 in its promoter region (34), it is reasonable to assume that it may be involved in the differentiation of the renal tubules. Moreover, the fact that it has been shown to be involved in the differentiation of intestinal epithelia (12), would strengthen the contention that SGLT-1 is relevant to the differentiation of the renal tubules as well.

Since SGLT-1 seems to be potentially involved in renal-tubular differentiation its functional properties with respect to embryonic development need to be discussed since they play a vital role in glucose reabsorption. To study the uptake of glucose one may employ the traditional methods, e.g., brush-border membrane vesicles (5, 25), or isolated tubular fragments (4); although, more sophisticated methods, e.g., the Xenopus laevis oocyte system, are also available at present (16, 17, 44). These methods have yielded excellent results in terms of the kinetics of uptake, i.e., Michaelis-Menten constant and maximum velocity values; however, their applicability in the embryonic systems may be difficult, especially if one has to directly localize or visualize the uptake into a given compartment of the developing kidney. Studies in rabbits (5), lambs (35), and guinea pigs (27) suggest that the renal tubular epithelium of the fetal kidney is capable of glucose transport, at least during the late stages of gestation. Studies regarding murine fetal kidneys, especially for the earlier stages of gestation, have not been described in the literature. Although the data from other species, i.e., rabbits, lambs, and guinea pigs, can be extrapolated for mice and rat kidney, one has to exercise a certain degree of caution, since kinetic and specificity differences of the SGLT-1 have been reported among different mammalian species (19). The kinetic properties of the cotransporter may be related to the degree of phosphorylation of SGLT-1, which apparently may vary depending on the presence or absence of PKC and PKA motifs in various species (43). In any instance, one of the objectives of this investigation was to establish a correlation between the functionality of SGLT-1 with its developmental expression, and due to technical constraints, the method of in situ tissue autoradiography was chosen. In this method, intact or slices of embryonic metanephroi (days 13, 16, and 19) were incubated in culture with MeGlc, an analog of glucose that is handled by the Na⁺-glucose cotransporter (17). Such a method to measure the uptake of glucose has been described for other tissues, e.g., preimplantation embryos (29), and thus it seemed to us that these experiments would be feasible in embryonic kidneys as well. In day 13 metanephroi, minimal uptake was observed (Fig. 5A). A high glucose uptake by the tubules, reflected by the clusters of radioactivity in the inner cortex was observed in day 16 (Fig. 5B) and 19 (Fig. 5I) explants. This uptake was inhibited by D-galactose (Fig. 5B, F, and J), which is believed to be a specific inhibitor of glucose uptake mediated by SGLT-1 (24). The specificity of glucose uptake was further established by the antisense experiments, where a marked decrease in uptake was observed (Fig. 8C, G, and K). Conceivably, the mechanism(s) by which antisense oligonucleotide exerted its inhibitory effect may partly be similar to those of the hybrid-depletion experiments (44) and partly due to the inhibition of renal tubulogenesis. The latter possibility would be more applicable to day 13 metanephroi, while the former may be relevant to the experiments with day 16 and 19 explants. Since no discernible decrease in uptake was observed with l-galactose and sense- or nonsense- oligonucleotides, it is reasonable to assume that the effects observed are reflective of the SGLT-1-mediated glucose uptake. Regarding the SGLT-1-mediated effect, the issue of uptake in the medulla (Fig. 8, E and I) warrants some discussion. Since uptake in the medulla was inhibited with D-galactose and by SGLT-1-specific antisense oligonucleotide, it is likely that the uptake is mediated by SGLT-1. In support of this notion are two previous studies in which SGLT-1 mRNA and protein expression were localized to the inner medulla of the porcine and rat kidneys, respectively (31, 39). As already indicated, one certainly can argue about the validity of the findings of previous investigations on the grounds of cross-hybridization and reactivity of cDNA probes and of the antibodies. Alternatively, the uptake of Na⁺-dependent glucose in the renal medulla may be due to another closely related SGLT-1 isofrom that is expressed in the medullary tubules and is yet to be identified.

In summary, it seems that SGLT-1 is developmentally regulated in the mouse metanephros, maintains renal tubulogenesis, and the degree of SGLT-1-mediated glucose uptake correlates with its developmental expression. Finally, it is anticipated that availability of mouse SGLT-1 cDNA should give impetus for the future in vivo genetic experiments to elucidate the role of this cotransporter in normal as well as abnormal growth related processes, i.e., metanephric development and diabetic nephropathy (1).

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REFERENCES


