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S Bachmann, ... , D Moser, D H Ellison

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Research Article

A thiazide-sensitive Na-Cl cotransporter contributes importantly to mammalian salt homeostasis by mediating Na-Cl transport along the renal distal tubule. Although it has been accepted that thiazide-sensitive Na-Cl cotransport occurs predominantly along the distal convoluted tubule in rats and mice, sites of expression in the rabbit have been controversial. A commonly accepted model of rabbit distal nephron transport pathways identifies the connecting tubule, not the distal convoluted tubule, as the predominant site of thiazide-sensitive Na-Cl cotransport. The thiazide-sensitive Na-Cl cotransporter has been cloned recently. The present experiments were designed to localize sites of thiazide-sensitive Na-Cl cotransporter mRNA expression along the rabbit distal nephron. Nonradioactive in situ hybridization with a thiazide-sensitive Na-Cl cotransporter probe was combined with immunocytochemistry with an antibody that recognizes distal convoluted tubule cells and with a Na⁺/Ca²⁺ exchanger antibody that recognizes only connecting tubule cells. The results indicate that thiazide-sensitive Na-Cl cotransporter mRNA is highly expressed by cells of the distal convoluted tubule and not by connecting tubule cells. Segments that stain with the Na⁺/Ca²⁺ exchanger antibody (connecting tubules) do not demonstrate thiazide-sensitive Na-Cl cotransporter mRNA expression. We conclude that the predominant site of thiazide-sensitive Na-Cl cotransporter mRNA expression in rabbit distal nephron is the distal convoluted tubule and that sites of mRNA expression of electroneutral Na and Cl transport are similar in rabbits, rats, and mice.

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Expression of the Thiazide-sensitive Na-Cl Cotransporter by Rabbit Distal Convoluted Tubule Cells

Sebastian Bachmann,* Heino Velázquez,† Nicolas Obermüller,* Robert F. Reilly,‡ Doris Moser,* and David H. Ellison‡
 *Department of Anatomy and Cell Biology, University of Heidelberg, D-69120 Heidelberg, Germany; and †Section of Nephrology, Department of Medicine, Yale University, and Veterans Administration Medical Center, West Haven, Connecticut 06516

Abstract

A thiazide-sensitive Na-Cl cotransporter contributes importantly to mammalian salt homeostasis by mediating Na-Cl transport along the renal distal tubule. Although it has been accepted that thiazide-sensitive Na-Cl cotransport occurs predominantly along the distal convoluted tubule in rats and mice, sites of expression in the rabbit have been controversial. A commonly accepted model of rabbit distal nephron transport pathways identifies the connecting tubule, not the distal convoluted tubule, as the predominant site of thiazide-sensitive Na-Cl cotransport. The thiazide-sensitive Na-Cl cotransporter has been cloned recently. The present experiments were designed to localize sites of thiazide-sensitive Na-Cl cotransporter mRNA expression along the rabbit distal nephron. Nonradioactive in situ hybridization with a thiazide-sensitive Na-Cl cotransporter probe was combined with immunocytochemistry with an antibody that recognizes distal convoluted tubule cells and with a Na⁺/Ca²⁺ exchanger antibody that recognizes only connecting tubule cells. The results indicate that thiazide-sensitive Na-Cl cotransporter mRNA is highly expressed by cells of the distal convoluted tubule and not by connecting tubule cells. Segments that stain with the Na⁺/Ca²⁺ exchanger antibody (connecting tubules) do not demonstrate thiazide-sensitive Na-Cl cotransporter mRNA expression. We conclude that the predominant site of thiazide-sensitive Na-Cl cotransporter mRNA expression in rabbit distal nephron is the distal convoluted tubule and that sites of mRNA expression of electroneutral Na and Cl transport are similar in rabbits, rats, and mice. (*J. Clin. Invest.* 1995. 96:2510–2514.) **Key words:** diuretics, thiazide • kidney tubules, distal • sodium chloride • calcium transport proteins • in situ hybridization

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Address correspondence to David H. Ellison, M.D., Section of Nephrology (2074 LMP), Yale School of Medicine, 333 Cedar Street, New Haven, CT 06520-8029. Phone: 203-932-5711x4424; FAX: 203-937-3455; E-mail: Ellison@Biomed.Med.Yale.Edu

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Introduction

A thiazide-sensitive Na-Cl cotransport pathway mediates the majority of Na and Cl transport by the mammalian renal distal tubule. This segment of the nephron is morphologically heterogeneous, comprising the distal convoluted tubule (DCT),¹ connecting tubule (CNT), and initial portion of the cortical collecting tubule (CCT). Evidence from in vivo microperfusion of rat distal tubules (1, 2) and from an immortalized mouse DCT cell line (3, 4) indicates that the thiazide-sensitive Na-Cl cotransporter (TSC) is expressed predominantly by DCT cells in rodents. We recently reported in situ hybridization experiments in rats showing that message for the TSC is highly expressed by DCT cells, but not by thick ascending limb or collecting tubule cells in rats (5). mRNA expression by connecting tubule cells was limited to a few transitional cells adjacent to the DCT (transitional cell types are known to separate distal nephron segments in rat and human [6]). Recently, Lee et al. (7) reported preliminary results of immunohistochemical experiments confirming that expression of the TSC protein within the rat distal nephron is restricted to the DCT.

The site of thiazide-sensitive cotransporter expression in rabbit has been controversial. Autoradiographic studies suggested that [³H]metolazone receptors (believed to be TSCs) are localized along short cortical segments (8) in a pattern indistinguishable from that in rat and mouse; although the labeled segments could not be identified with certainty using these techniques, taken together, the data suggest that the TSC is expressed predominantly by the DCT in all species. Greger and Velázquez (9) reported results of in vitro microperfusion of rabbit DCT segments; the apical conductance of DCT cells was very low, consistent with an electroneutral mechanism mediating the bulk of Na and Cl transport by this nephron segment. In contrast, Imai and co-workers reported that thiazide diuretics had no effect on Na or Cl transport by rabbit DCT perfused in vitro; they found that Na and K were transported across the apical membrane predominantly by electrogenic mechanisms (10). These workers did detect thiazide-sensitive Na and Cl transport by CNT cells (11). These results led to a commonly accepted view that the predominant site of electroneutral Na-Cl cotransport in rabbits is the CNT and that electrogenic mechanisms predominate along the DCT (12, 13). The present experiments were designed to investigate sites of TSC mRNA expression in rabbit distal nephron by in situ hybridization.

1. *Abbreviations used in this paper:* CCT, cortical collecting tubule; CNT, connecting tubule; DCT, distal convoluted tubule; TSC, thiazide-sensitive Na-Cl cotransporter.

Methods

Animals. Male adult New Zealand rabbits (mean body wt 3.5 kg) were used. They were allowed free access to standard laboratory diet and tap water. For perfusion fixation, animals were anesthetized by injection of Nembutal (sodium pentobarbital, 20 mg/kg body wt) through the ear vein. Animals then underwent laparotomy and kidneys were fixed by retrograde perfusion through the abdominal aorta with freshly prepared 3% paraformaldehyde in PBS (pH 7.4) for 5 min at 220 mmHg. To protect tissues from freezing artifacts, kidneys were subsequently rinsed at low perfusion pressure with a sucrose-PBS solution adjusted to 800 mosm/kg. Kidneys were then removed and cut into slices ~3 mm thick. The slices were snap frozen in liquid nitrogen-cooled isopentane.

Molecular cloning and preparation of riboprobes. To prepare the riboprobes, plasmids containing a rabbit or mouse TSC cDNA fragment were used. A 713-bp mouse cDNA has been described previously (14). The rabbit TSC cDNA was generated by PCR from rabbit kidney cortical cDNA using primers based on the mouse TSC cDNA sequence. The PCR product was excised from an agarose gel, ligated into the EcoRV site of pBluescript II KS⁺, and sequenced. The nucleotide sequence of the rabbit cDNA fragment is 93% homologous to the same region of mouse TSC and 92% homologous to the same region of rat TSC.

Riboprobes were synthesized by *in vitro* transcription using digoxigenin-11-dUTP. For generation of antisense riboprobe, plasmid was linearized by HindIII and transcribed by T7 RNA polymerase. For generation of the sense riboprobe (control), plasmid was linearized with EcoRI and then transcribed using T3 RNA polymerase. Because RNA probes for *in situ* hybridization should not exceed 500–1,000 bases, probes were shortened by alkaline hydrolysis to an average length of 200 bases.

***In situ* hybridization.** Cryostat sections from kidneys (5–7 μ m thick) were thawed onto silanized glass slides. For *in situ* hybridization, slides were processed essentially as described previously (15). The optimal concentrations of digoxigenin-labeled sense or antisense riboprobes were between 5 and 10 ng/ μ l of hybridization mixture. Hybridization was performed at 40°C for 16–18 h. Slides were washed at 47°C for 30 min in 2 \times SSC (SSC: 150 mM NaCl, 15 mM Na citrate), followed by two washes (30 min each) in 1 \times SSC containing 50% formamide, two washes for 30 min in 0.5 \times SSC containing 50% formamide, and another wash for 30 min in 0.1 \times SSC containing 50% formamide. Subsequently, slides were rinsed in 0.5 \times SSC at room temperature for 15 min followed by a rinse in 0.2 \times SSC for 10 min. Detection of the signal was performed using an anti-DIG antibody (Boehringer Mannheim Corp., Indianapolis, IN) (Fab fragments, dilution 1:500 in blocking medium containing 1% Boehringer's blocking reagent plus 0.5% BSA dissolved in buffer I [100 mM Tris-HCl, 150 mM NaCl, pH 7.5]), and nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl-phosphate for color development (15).

Combined *in situ* hybridization/immunocytochemistry. To identify the nephron segments that possess the TSC, a combination of *in situ* hybridization with a TSC probe and immunohistochemistry with antibodies specific to defined nephron segments was performed on the same tissue section. Double labeling was done by *in situ* hybridization with TSC probe according to the protocol above combined with antibody labeling. A mouse monoclonal antibody JM5, raised against the high-affinity thiazide-diuretic receptor, recognizes cells along the distal nephron and gives a characteristic staining pattern for DCT cells (16). Instead of applying anti-DIG antibody alone, a mixture of sheep anti-DIG and JM5 at a dilution of 1:200 in blocking medium was applied, as described previously (15). Bound JM5 was detected using Texas red-conjugated secondary antibody.

To identify the transition between DCT and CNT cells, a triple labeling procedure using TSC *in situ* hybridization, antibody JM5, and a polyclonal antibody against the Na⁺/Ca²⁺ exchanger was used (17). The Na/Ca exchanger antibody stains only basolateral membranes of CNT cells in rabbit kidney (17). In these triple label experiments, TSC double labeling was performed first, as described above, and the entire procedure including color development for the *in situ* signal was carried

out. Subsequently, sections were incubated in blocking medium for 30 min followed by antibody to the Na⁺/Ca²⁺ exchanger (dilution 1:50) for 2 h at room temperature and then for 18 h at 4°C. Bound Na⁺/Ca²⁺ antibody was detected by fluorescein isothiocyanate-conjugated secondary antibody (FITC; Sigma, Deisenhofen, Germany, diluted 1:65 in PBS).

Controls. Each of the antibodies used in the present experiments has been characterized extensively (17–19). The specificity of the immunohistochemical staining in the present experiments was tested by replacing the respective antibody with nonimmune serum; in all cases, no signal was obtained under this condition. The specificity of the *in situ* hybridization signal was verified by parallel incubation with sense and antisense riboprobes. Throughout all experiments, the sense probes did not produce any detectable signal.

Results

Fig. 1 A shows that TSC is expressed exclusively along short nephron segments in the kidney cortex. The level of mRNA expression is high, permitting clear differentiation between segments that do and segments that do not express TSC mRNA. Fig. 1 B shows that segments expressing the TSC also stain intensely with monoclonal antibody JM5. In contrast, segments that express message for the TSC are distinct from segments that stain with the Na⁺/Ca²⁺ antibody (Fig. 1 C).

Fig. 2 shows more detail of the onset of TSC mRNA expression. TSC mRNA expression is absent from thick ascending limb cells and from the macula densa region (*between arrows*). mRNA expression begins abruptly at the start of the DCT (Fig. 2, *arrowheads*) following the post-macula densa thick ascending limb and is present in other distal tubule profiles. Cells expressing TSC mRNA (Fig. 3) stain uniformly with antibody JM5 in a predominantly apical distribution. All cells in TSC-positive segments stain with JM5, indicating that intercalated cells, which do not react with JM5 (16), are not present in the TSC-positive regions.

Fig. 4 shows a transition from DCT to CNT (*arrowhead*). A clear transition from a TSC-positive to a TSC-negative region is observed (Fig. 4 A) that corresponds to a transition to less intense and more variegated staining with JM5 (Fig. 4 B). Staining with the Na⁺/Ca²⁺ exchanger antibody (Fig. 4 C) begins precisely at the site in which TSC mRNA expression ceases. The segment that is negative for TSC and positive with JM5 and Na⁺/Ca²⁺ exchanger is the CNT and contains cells that do not stain with either antibody. These cells are probably intercalated cells.

Discussion

The present results indicate that message for the TSC is highly expressed by rabbit DCT cells, in agreement with results reported previously for rat and human (5, 7). TSC mRNA expression was not evident in thick ascending limb cells, CNT cells, CCT cells, or intercalated cells. In the rabbit, TSC mRNA expression begins abruptly at the junction between thick ascending limb and DCT; mRNA expression ceases abruptly at the junction between DCT and CNT.

In this study, several criteria were used to identify cells that express TSC message. First, cells were identified by their morphological and anatomical features (6); thick ascending limb–macula densa–DCT transitions were readily identified by close apposition between tubule and glomerulus and by the characteristic cellular morphology of the macula densa (Fig.

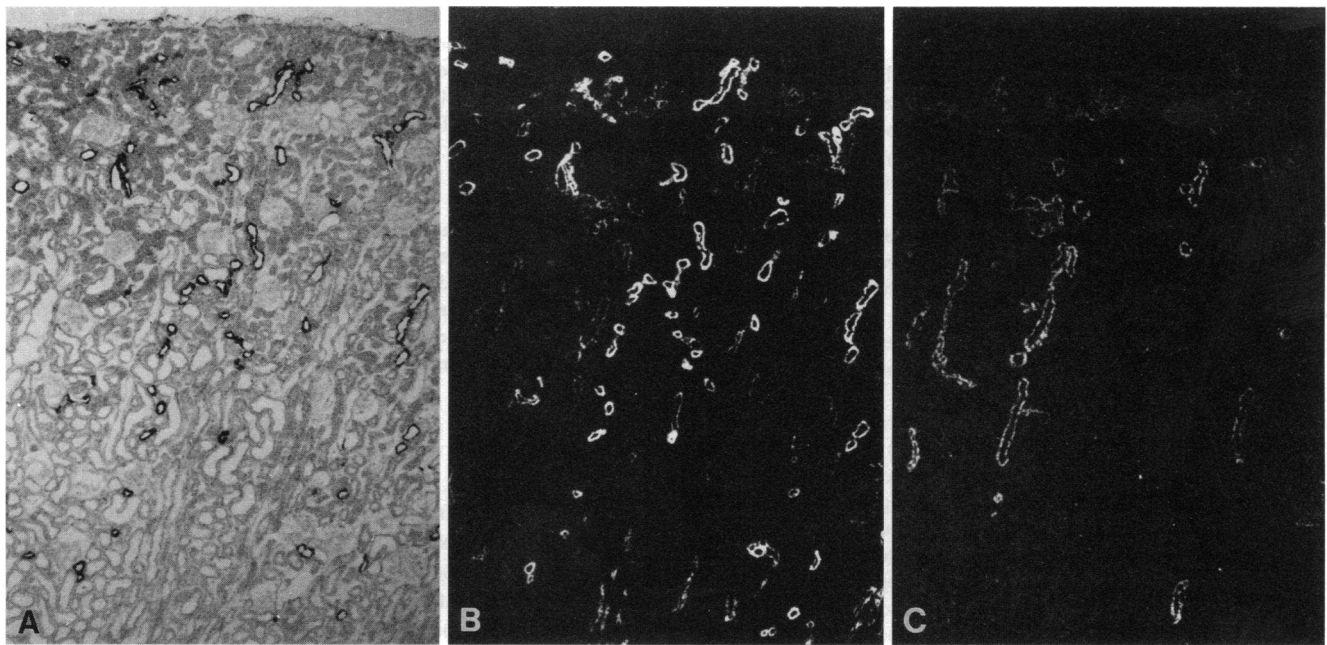


Figure 1. Overview of the distribution of TSC mRNA and immunoreactivity with JM5 and anti- $\text{Na}^+/\text{Ca}^{2+}$ in nephron segments of rabbit renal cortex as revealed by triple labeling on the same section. (A) In situ hybridization for TSC. (B) Immunofluorescence with JM5. (C) Immunofluorescence with antibody to $\text{Na}^+/\text{Ca}^{2+}$ exchanger. TSC mRNA colocalizes with strong JM5 immunoreactivity (compare A and B) whereas TSC mRNA is not detected along $\text{Na}^+/\text{Ca}^{2+}$ exchanger-positive profiles (compare A and C). $\times 43$.

2). Connecting tubules were identified by the appearance of intermingled intercalated cells (which are not present along DCT segments in this species [6]) and by their characteristic association with interlobular arterioles (6). Based on these cri-

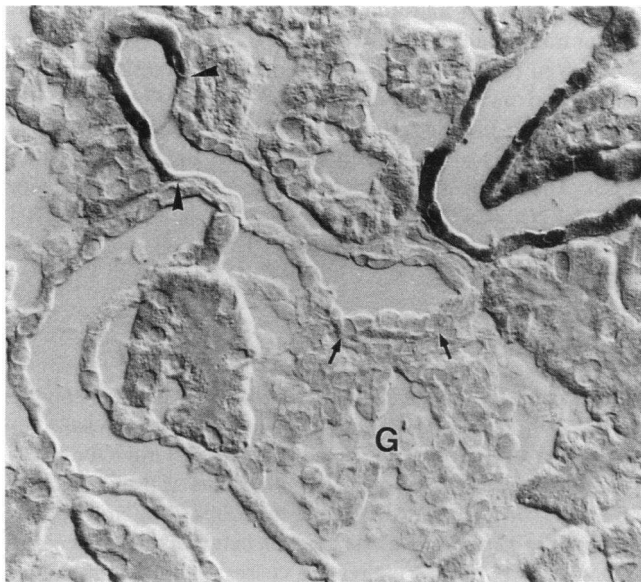


Figure 2. Onset of TSC mRNA expression in rabbit kidney cortex as revealed by in situ hybridization. The thick ascending limb including its post-macula densa segment is nonreactive. The macula densa region is indicated by the arrows. After the long post-macula densa segment, the onset of the DCT (arrowheads) coincides with that of TSC mRNA expression. To the right, another DCT profile that expresses TSC mRNA is seen. G, glomerulus. $\times 360$.

teria, segments expressing TSC mRNA were seen to be distinct from both thick ascending limb and connecting tubule segments.

To provide more definitive identification of TSC-positive segments, two well characterized antibodies that recognize antigens in the rabbit distal tubule were used. Antibody JM5 recognizes antigens predominantly along the DCT (16). Although it stains CNT and some CCT segments, the staining of DCT is most intense, more uniform, and predominates along the apical membrane. These features make it possible to distinguish the DCT from CNT and CCT (reference 16 and see Fig. 1). The antibody does not stain intercalated cells. In the present studies, all cells in nephron segments expressing TSC mRNA demonstrate high intensity staining with JM5 (see Figs. 1 and 3); this pattern is characteristic of DCT segments.

Because staining with JM5 does extend into the CNT, a second antibody was used to define the downstream extent of TSC mRNA expression in distal tubules. The $\text{Na}^+/\text{Ca}^{2+}$ exchanger antibody was shown previously to label basolateral cell membranes of connecting tubule cells; it does not stain DCT cells, CCT cells, or intercalated cells (17). The present results indicate that expression of TSC message and $\text{Na}^+/\text{Ca}^{2+}$ exchanger activity are mutually exclusive; cells that stain positive for $\text{Na}^+/\text{Ca}^{2+}$ exchanger do not express TSC message and vice versa. Fig. 4 indicates that the transition between TSC-positive and $\text{Na}^+/\text{Ca}^{2+}$ exchanger-positive segments is abrupt. Morphological transitions between distal nephron segments in the rabbit are also abrupt (6) and in complete agreement with the transitions observed in the present experiments using molecular probes.

Although these results are consistent with some previous studies localizing TSC in rabbit kidney (8, 9), they are not consistent with results of in vitro perfusion experiments using trichlormethiazide (11, 20). Those studies detected thiazide-

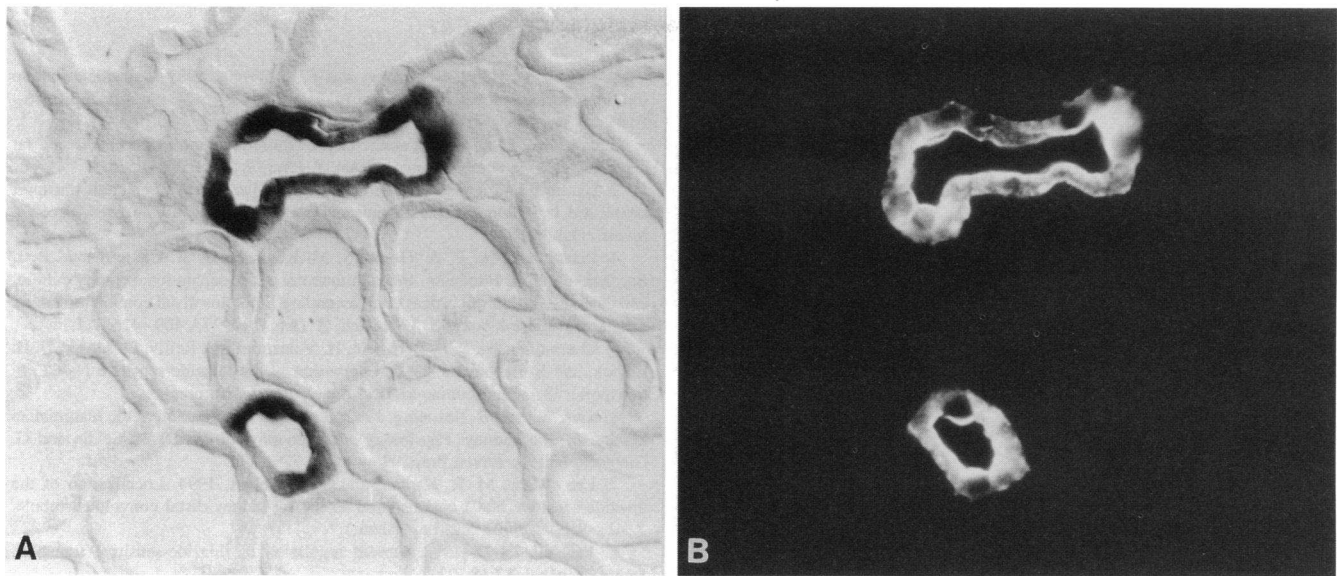


Figure 3. Combined in situ hybridization (A) and fluorescent antibody staining with JM5 (B) in rabbit kidney cortex. $\times 420$.

sensitive Na and Cl transport along CNT segments and not along the DCT. Several reasons for this discrepancy must be considered. First, levels of TSC mRNA expression below those that can be detected by in situ hybridization may be physiologically significant and may mediate ion transport that can be detected by in vitro microperfusion of the CNT. In situ hybridization using radioisotopes or immunohistochemical experiments might be able to demonstrate such mRNA expression by CNT cells. Second, levels of message expression may not correlate with amounts of functional protein. Third, thiazide-sensitive transport pathways that are not homologous to the cloned TSC might mediate thiazide-sensitive Na and Cl transport by CNT cells in rabbit. Such pathways would not be detected by in situ hybridization using probes generated based on homology with the rat TSC. Thus, the present results cannot

exclude a component of thiazide-sensitive Na or Cl transport by CNT cells.

More surprising, however, is that high level expression of TSC mRNA by DCT cells was demonstrated. This pattern of mRNA expression is similar to that observed in rat and human (5). In rat, mRNA expression is present along segments in which physiological experiments have shown that the TSC mediates the majority of transepithelial Na and Cl transport (1). Reasons that high level TSC mRNA expression in rabbit might not have been detected functionally by previous investigators using in vitro microperfusion are not clear. One explanation for a failure to detect thiazide-sensitive transport by DCT segments perfused in vitro is the sensitivity of the TSC to ischemia. Beaumont et al. (21) reported that 10 min of warm ischemia decreases the number of thiazide-sensitive transporters in the

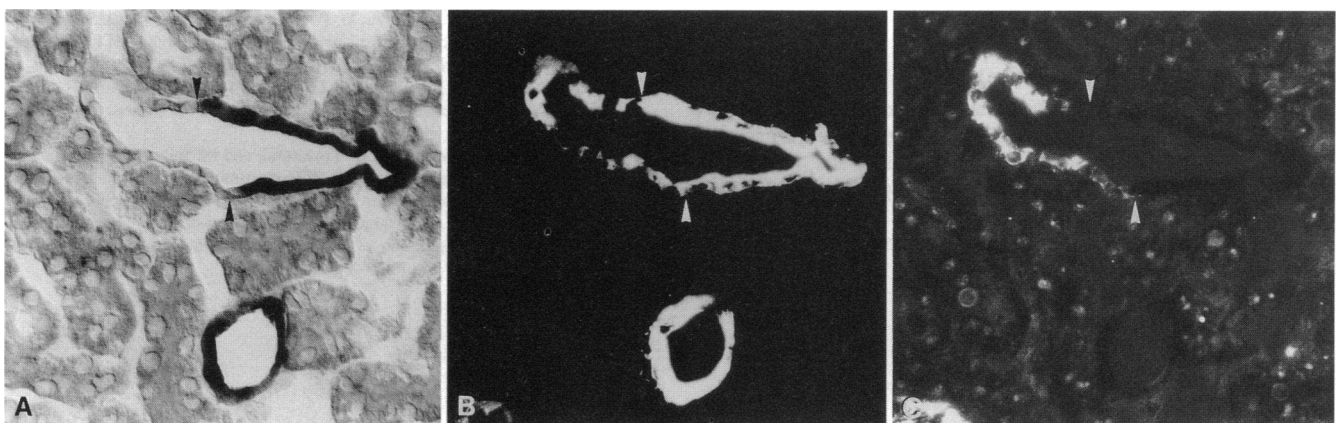


Figure 4. Definition of the downstream extent of TSC mRNA expression along the nephron in rabbit kidney cortex. (A) TSC in situ hybridization. (B) Immunocytochemistry with antibody JM5. (C) Immunocytochemistry with antibody to $\text{Na}^+/\text{Ca}^{2+}$ exchanger. A distal nephron profile shows TSC mRNA along half of a longitudinally sectioned transition between DCT and CNT (arrowheads). Also shown is an entirely TSC-positive DCT profile. Note that the transition to CNT corresponds to a region of more variable staining with JM5 and with the appearance of $\text{Na}^+/\text{Ca}^{2+}$ exchanger-positive cells. The basolateral aspect of staining with Na/Ca antibody is usually discernible, but is less apparent in micrographs from 5–7- μm -thick sections than in thinner sections (17). $\times 330$.

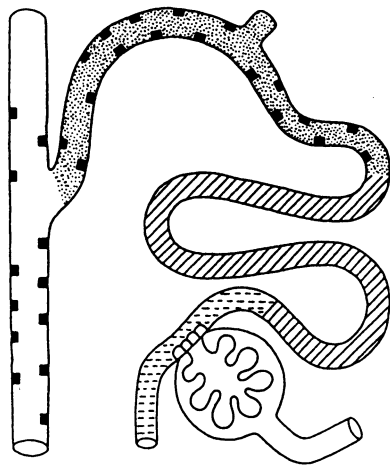


Figure 5. Schematic drawing of rabbit distal nephron as characterized in the present study. *Dashed horizontal lines*, thick ascending limb; *oblique lines*, DCT with expression of TSC mRNA and intense luminal labeling with JM5; *stippling*, CNT with Na/Ca exchanger immunoreactivity, light staining with JM5, no expression of TSC mRNA, and unreactive intercalated cells (*filled boxes*); *white*, cortical collecting duct, with

intercalated cells but without expression of TSC mRNA or Na/Ca exchanger immunoreactivity.

active state by as much as 90%. The conditions of in vitro perfusion, therefore, may activate the same downregulatory mechanisms and may make detection of thiazide-sensitive transport difficult. A final possibility is that nephron segments identified as DCT in the present studies may not be the same as those identified as DCT during some microperfusion experiments. In the present work, DCT segments were identified by their microscopic morphology, by their contiguity with thick ascending limbs and macula densa regions, and by specific immunocytochemical markers. Identification of tubule segments for in vitro perfusion relies on proximity to glomeruli and on appearance when viewed using phase-contrast microscopy.

Fig. 5 summarizes the current results; message for the TSC is highly expressed only along DCT segments of rabbit kidney. Expression of the Na⁺/Ca²⁺ exchanger is limited to CNT cells and does not colocalize with the TSC. Neither TSC nor Na⁺/Ca²⁺ exchanger is expressed by cells of the thick ascending limb or CCT. The results indicate that sites of TSC mRNA expression in rabbit kidney are similar to those in rat, mouse, and human.

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