Differential Expression of Na:K:2Cl Cotransporter, Glucose Transporter 1, and Aquaporin 1 in Freshly Isolated and Cultured Bovine Corneal Tissues

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Little is known about whether culturing corneal limiting layers causes changes in the expression of their membrane transporter proteins from those present in fresh tissues. Accordingly, we compared mRNA abundance of three well-described types of transporters: water channel aquaporin 1 (AQP1), glucose transporter (GLUT1), and Na:K:2Cl cotransporter (NKCC), as well as NKCC protein levels in fresh bovine corneal epithelium and endothelium with those in their cultured counterparts. Abundance of mRNA encoding AQP1, GLUT1, and NKCC was quantified by a lysate nuclease protection assay. NKCC transcription was further characterized by Northern blotting. All data were normalized to cell DNA and protein contents. In the fresh epithelium, in all three cases mRNA levels were two to four times higher than in the endothelium. Expression of AQP1 and GLUT1 was 10 to 12 times higher than that of NKCC. After the third passage, the endothelial cell mRNA abundance in each case decreased 2- to 3-fold. Passage-dependent decreases were also observed in NKCC protein expression in the epithelial cells. In both corneal layers, there was a qualitative correlation between NKCC mRNA and protein levels. Both in fresh and cultured epithelial and endothelial cells, a shark NKCC1 DNA probe hybridized with mRNAs of two different lengths (about 5.0–5.5 and 7.0–7.5 kb). An anti-NKCC T4 monoclonal antibody recognized two major proteins with apparent molecular masses of 190 to 200 and 150 to 160 kDa. In summary, membrane transporter function in culture may not be always indicative of their role in fresh tissue since in cultured cells AQP1, GLUT1, and NKCC mRNA levels declined. Furthermore, in both epithelial and endothelial cells, there is expression of two different proteins and mRNAs that possibly encode for secretory (NKCC1) and absorptive (NKCC2) isoforms.

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Key words: corneal epithelium; corneal endothelial cells; Na:K:2Cl cotransporter; gene expression; cell passage

A substantial portion of our understanding of membrane ion transport physiology is based on studies in cell cultures. However, the precise quantitative relevance of some transport parameters thus obtained will depend on whether or not gene and protein expression is similar to that *in situ*. One way to make such an assessment is to compare key membrane transporter gene and protein expression in freshly isolated tissue with those in its cultured cell counterparts. We have not identified any study in which such a determination has been done for the transporters we studied here, aquaporin 1 (AQP1), glucose transporter 1 (GLUT1), and Na:K:2Cl cotransporter 1 (NKCC1). However, it has been shown in cultured bovine corneal endothelial cells that Na^+/H^+ exchange activity is only about 50% of its value in its freshly isolated counterpart (1).

A common characteristic of corneal epithelial and endothelial transport physiology is that each of these tissues exhibits secondary active chloride transport, which is in part dependent on the parallel activity of the Na:K pump and an NKCC. In the epithelium, expression of these transporters results in Cl secretion from the stromal to the tear side, whereas in the endothelium this process presumably contributes to net Cl flux from the stroma towards the anterior chamber. In other tissues mediating either absorptive or secretory chloride transport, a specific NKCC isoform is usually expressed in connection with each type of these processes. In a secretory tissue, the isoform is designated NKCC1. The gene-specific transcript encoding for these proteins is about 7.4 kb (2). On the other hand, in the medullary regions of the kidney absorptive chloride transport is mediated by another isoform, NKCC2. The mRNA encoding this protein is about 5 kb. These two isoforms have an

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overall amino acid identity of 55% to 65%, whereas in the highly conserved transmembrane regions it ranges between 75% to 90% (3). In freshly isolated bovine corneal epithelial and endothelial cells, an NKCC protein has been detected and its expression is apparently higher in the fresh cells than in their cultured counterpart (4). However, there is no information regarding possible expression of two different NKCC proteins and gene-specific encoding mRNA transcripts in either corneal epithelial or endothelial cells; and whether NKCC gene and protein expression is different in fresh tissue than in their cultured counterpart.

Glucose availability plays a critical role in the maintenance of numerous corneal endothelial and epithelial functions. It is well established in these tissues that glucose uptake occurs through a facilitative transport process involving the GLUT. After corneal epithelial wounding, mRNA and protein expression is rapidly enhanced for one of the GLUT isoforms, GLUT1 (5). This effect indicates that increases in intracellular glucose levels are needed to meet the energetic requirements of cell migration and proliferation. In addition, GLUTs may be involved in the maintenance of cell homeostasis because in the presence of an osmotic gradient, facilitative GLUTs act as a minor transmembrane pathway for water flow (6). As with the NKCC, there is no information comparing GLUT1 gene expression levels in freshly isolated corneal tissue with its tissue culture counterpart.

Corneal transparency maintenance depends on appropriate levels of transendothelial fluid transport. In addition, there is some evidence that the epithelium may play a minor role in dehydrating the stroma (7). In each of these tissues, specific aquaporin isoforms serve as conduits for osmotic fluid flows and perhaps for CO_2 flows (8–10). Five homologs of aquaporin (AQP1–5) have been identified in various mammalian tissues. In rat cornea, RT-PCR studies show that AQP1 expression is approximately 3-fold higher than AQP3 and 2.5-fold higher than AQP5 (11). In the rat corneal endothelium, AQP1 expression has been identified, whereas in its epithelial layer AQP3 and 5 are prominently expressed (12). However, there is no information regarding AQP isoform mRNA expression in bovine corneal layers.

Here we compare the levels of AQP1, GLUT1, and NKCC mRNA and NKCC protein expression in freshly isolated bovine corneal epithelium and endothelium with those in cultured cells. As a function of cell passage, these levels declined, suggesting that mRNA expression levels in cell culture may not be reflective of those in these freshly isolated tissues. In addition, some suggestive evidence is provided that there are two different NKCC mRNAs that encode for two NKCC protein isoforms. Finally, a novel approach is described, allowing measurement of mRNA abundance in small amounts of tissues, which avoids reliance on cell cultures to make such a determination.

Material and Methods

Cell Culture. Bovine corneal epithelial (CBCEp) and endothelial (CBCEn) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum with antibiotics (Gibco-BRL/Life Technologies, Grand Island, NY). This culture system optimizes their growth and therefore is used in many different studies. The cells were cultured in an atmosphere of 5% CO_2, 95% ambient air at 37°C. Cultures that had reached confluence after 7 days were used in the experiments.

Materials. Bovine eyes obtained from a local slaughterhouse were placed on ice and were washed with physiological saline, after which their corneas were excised. Only one of the two limiting corneal layers was harvested from a given cornea. Endothelial layers were harvested at 25°C by placing the corneas epithelial side down on watchglasses, after which the endothelial layer was exposed for 2 min to 200 μl of lysate solution (LS) (see below). Epithelial layers were harvested by gently everting the corneas, placing them endothelial side down in the same watchglasses, and exposing them similarly to LS. For a given experimental series, we pooled five preparations. CBCEn cells were from the third to fifth passages. These cells were harvested by lysing them with 1 ml of LS; the contents of one flask sufficed for an experimental series. Lysates were analyzed immediately or stored at −70°C in the same LS.

Solutions. LS was 5 M guanidine thiocyanate (GuSCN) and 0.1 M EDTA; hybridization buffer (HB) was 2× standard saline citrate (SCC) plus 0.1 M EDTA. They were sterilized by filtering through a 0.2-μm Sterile Acrodisc (Gelman Sciences, Ann Arbor, MI), and were stored at room temperature. To protect against RNase contamination, water for all solutions was treated with 0.2% diethyl pyrocarbonate (Sigma, St. Louis, MO), followed by stirring overnight and autoclaving. For the S1 nuclease buffer, a stock solution was made containing 0.56 M NaCl, 9 mM $ZnSO_4$, and 0.1 M sodium acetate (pretitrated to pH 4.5), sterilized as above, and stored at 4°C. Immediately prior to an experiment, 2000 units/ml S1 nuclease (Sigma) and 20 μg/ml denatured salmon sperm DNA (Sigma) were added.

Determination of Cell Lysates DNA Contents. As standards, DNA from ~5 million CBCEn cells was purified using the Puregene kit (Gentra Systems Inc., Minneapolis, MN) and the sample DNA concentration was determined with a spectrophotometer (Gene-Quant, Pharmacia Biotech, Piscataway, NJ). DNA from 20-μl samples of cell lysates was similarly purified. Several dilutions of cell lysates and of standard DNA sample were denatured with 0.1N NaOH and placed on a nylon membrane (Hybond N^+, Amersham, Arlington Heights, IL). After cross-linking by UV, the samples were hybridized with random primer extension-generated (Megaprime DNA labeling system, Amersham) ^{32}P-labeled (with α-^{32}P dATP, New England Nuclear, Boston, MA) total DNA probes. Dots were visualized (Fig. 1A) by exposure to X-ray film (Kodak XAR-2, Eastman Kodak, Rochester, NY), after which the dots were cut and their radioactivity was quantitated by scintillation counting. The radioactivity of the DNA standards was plotted against their DNA amounts, and was fitted to a function

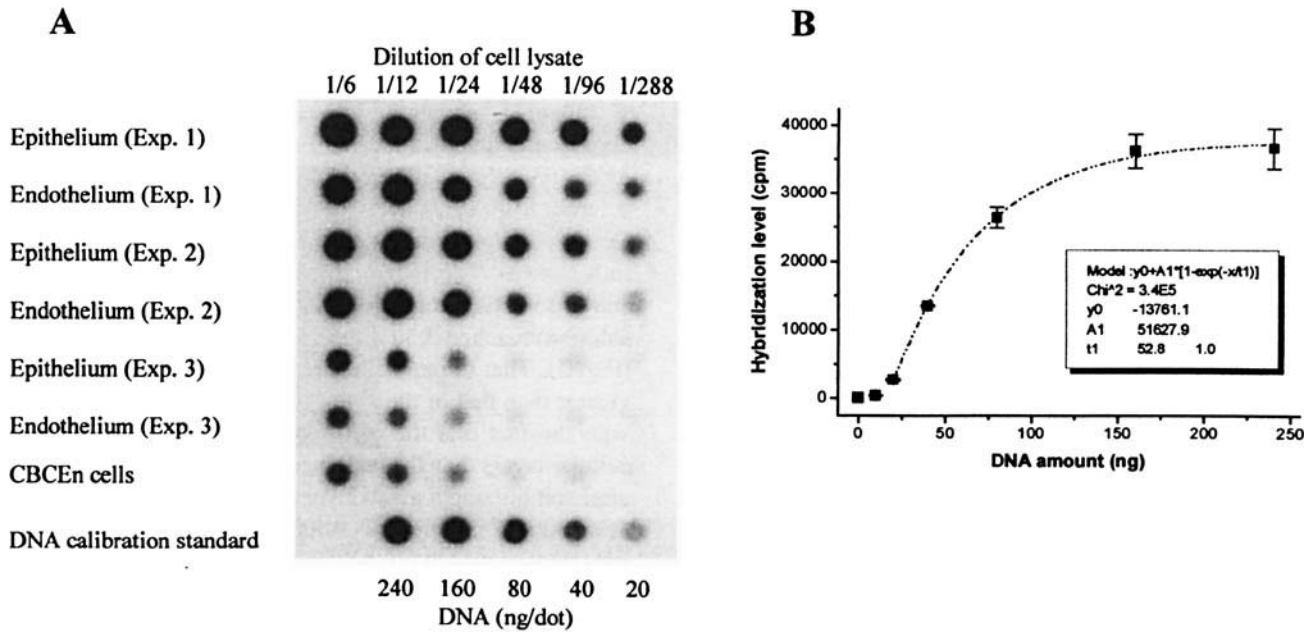

Figure 1. Determination of DNA contents in bovine corneal epithelium and endothelium cell lysates and CBCEn cells. (A) Dot-blot of different dilutions of lysates of corneal layers and of standard DNA hybridized to a ^{32}P-labeled total DNA probe. Cells were lysed in 200 µl of GuSCN/EDTA. DNA was isolated from 20-µl lysate aliquots and was diluted as shown. Epithelium lysates were prediluted 5-fold. Typical data sets are indicated as Experiments 1 and 2. Individual dots were cut out and their radioactivity was quantified by scintillation counting. (B) An exponential build-up fit function provided in Origin™ yields a calibration curve to read the DNA contents of given samples. Convergence was reached when the parameters values shown in the box were used.

(Fig. 1B). The procedure was repeated with similar results, enabling such a calibration curve to be used to quantify the DNA contents in 20-µl aliquots. As an additional precaution, use of the calibration curve was limited to that in its near-linear segment. The protein content of these aliquots was determined by Coomassie Protein Assay (Pierce, Rockford, IL). The protein/DNA ratios in each of the tissues were determined so as to use a constant number of cells in all the different experiments.

Northern Blot Analysis. Poly(A)$^+$ RNA was extracted with a polyRNAsol kit (Biotex Lab., Houston, TX), electrophoresed through 1.2% formaldehyde denaturing gels, and eluted by capillary transfer (7.5 mM NaOH) to a Hybond-N$^+$ nylon membrane. Transferred RNA was crosslinked to the membrane by UV-light and was prehybridized for ≥2 hr in Rapid-Hyb Buffer (Amersham)/50% formamide at 45°C. Overnight hybridization was carried out under the same conditions with random primer extension-generated shark NKCC1 probe (~5 × 10^6 cpm/ml). To evaluate the specificity of our NKCC1 probe as well as to discriminate between NKCC1 and its homologues that are possibly expressed and hybridize with this probe, we used both mid- and high-stringency washes. The membranes were washed by shaking as follows: (A) Mid-stringency, 2× SSC/0.5% SDS, 10 min at room temperature, twice; 1× SSC/0.5% SDS, 15 min at 65°C, twice; 0.7 SSC/0.1 SDS, 15 min at 65°C; 0.1 SSC, 5 min; (B) high-stringency: 2× SSC/0.5% SDS, 10 min at room temperature, twice; 1× SSC/0.1% SDS, 2% (w/v) sodium pyrophosphate, 15 min at 65°C, twice; 0.1 SSC/0.1 SDS, 15 min at 65°C; 0.1 SSC, 5 min. Results were visualized by exposure at −70°C to RE-FLECTION Film and Intensifying Screens (DuPont-NEN).

Probes for Specific mRNA. To generate a bovine NKCC fragment for the nuclease protection assay, the Genbank cDNA sequences for rat, human, and rabbit were analyzed, and the two flanking segments for a 529-bp region of high homology were selected as primers. These primers correspond to rat NM_019134 sequence and were: sense, 1650 TTTCAGGTCATGAGCATGGTGTC 1672; and antisense, 2155 CCCCGAGGAGGTGTGTCCGAGAC 2177.

PCR was done using total bovine DNA as template and the primers above. The reaction generated a 529-bp fragment, which was extracted from agarose slices (QIAEXII Agarose Gel Extraction Kit, Qiagen, Chatsworth, CA). Fragments for bovine AQP1 and rat GLUT1 were in plasmids with a 372-bp insert of AQP1 (13) and a full-length rat cDNA insert of GLUT1 (both in pBS Bluescript® II).

^{32}P-labeled, single-stranded DNA probes having a specific activity of 2 to 4 × 10^9 cpm/µg DNA for AQP1, GLUT1, and NKCC1 were generated by asymmetric PCR utilizing SK/KS-specific sense/antisense primers on the corresponding homologous DNA fragments referred to above. Unreacted nucleotides were removed by spin chromatography through Sephadex G-50. The probes were purified by 1% agarose gel electrophoresis. After identification of radioactive band locations by autoradiography, probes were extracted from agarose slices.

Nuclease Protection Assay. Twenty microliters of cell lysate containing 150,000 cells was mixed with 5 µl of probe contained in 2× SSC/0.1 M EDTA and was incu-

bated at 30°C for 20 hr. After hybridization, 2.5 μl of 10 mg/ml proteinase-K (Sigma) and 2.5 μl of 10% SDS were added. The mixture was incubated at 37°C for 30 min. Nucleic acids were extracted with one volume of a 1:1 mixture of buffer-saturated phenol:chloroform and isolated by spin chromatography through Sephadex G-50. An equivalent volume of S1 nuclease buffer was added, and the mixture was incubated at 45°C for 30 min. After digestion, samples and a 100-bp DNA ladder ^{32}P-labeled with 5′ DNA terminus labeling kit (BRL, Gaithersburg, MD) were electrophoresed in a 2% agarose gel at 100 V for ~3 hr (4°C). The gels were dried and the positions of the RNA-DNA duplexes were determined by autoradiography as described above. The radioactivity of given bands associated with full-length RNA-DNA duplexes were quantified with scintillation counting. The number of mRNA molecules per cell was calculated with the following formula (14):

$$\frac{(\text{cpm hybridized}) \times (6.02 \times 10^{23}\ \text{molecules/mole})}{(\text{probe cpm/g}) \times (320 \times \text{probe nucleotide length}) \times (\text{cell number})}$$

Cpm hybridized obtained from scintillation counting were corrected for assay noise (cpm hybridized in the absence of target). Probe cpm/g was calculated from the manufacturer's ^{32}P-α-dATP specification and was approximately 10^{15} cpm/g. Probe length was the number of nucleotides of full-length probe complementary to target.

Western Blot Analysis. The initial steps for this analysis were performed at 4°C. Bovine or rabbit corneal layers or their cultured counterparts were washed twice with PBS and were then scraped with a steel blade in 0.5 ml of HB containing: 50 mM β-glycerophosphate (pH 7.3), 1.5 mM EGTA, 1 mM EDTA, 0.1 mM Na-vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 mM benzamidine, and 10 μg/ml aprotinin. Using a 1-ml syringe, the suspension was passed through a 26-gauge one-half-inch long needle until easy flow was achieved (three to eight times). Otherwise, all procedures were the same as those described by Bildin *et al.* (15) and a 1:10,000 dilution was used of T4 monoclonal antibody (DSHB, Iowa City, IA) to evaluate NKCC protein content.

Data Analysis. All experimental data are expressed as mean ±SEM. Statistical significance was determined with the Student's *t* test ($P < 0.05$).

Results

Isolation of Corneal Limiting Layers. Concentrated solutions of GuSCN rapidly solubilize cells, inactivate ribonucleases, and provide a suitable solution for efficient nucleic acid hybridization (16). However, such solutions were never used to separate corneal limiting layers from the underlying stroma. We determined whether a GuSCN solution could be applied to intact corneas to separate epithelial and endothelial layers from the underlying stromal tissue in a single step rather than performing surgical isolation followed by tissue lysis. Such a test was done to decrease the time required for tissue isolation, as well as to minimize the possibility of tissue cross contamination.

Figure 2 shows the kinetics of lysis of fresh bovine corneal endothelial and epithelial layers in 5 M GuSCN/0.1 M EDTA solution. The total amount of protein that can be solubilized in each layer reached a plateau after 30 sec for the endothelial layer and after ~2 min for the epithelial layer (not shown). The maximal levels of solubilized epithelial and endothelial protein (micrograms per microliter of lysate solution) reached 3.30 ± 0.17 and 0.69 ± 0.01, respectively (Fig. 2). That the epithelial protein mass was about 5-fold greater than that of the endothelial counterpart is consistent with the fact that the corneal epithelium has 5-fold more cellular layers than the endothelium. As a control procedure, after solubilizing a given layer and aspirating the mixture, a new 200-μl aliquot of solubilizing solution (GuSCN/EDTA) was placed on the denuded stroma and left to stand for 2 to 3 min. This yielded only a negligible amount of protein. Hence, solubilization of stromal proteins must have been much slower than that for the cell layers, and did not play a part in our results. Based on these results, we chose a 2-min interval for the lysis of both corneal layers.

Northern Blot Analysis of NKCC Expression. Figure 3A (agarose gel) shows bands corresponding to total RNA isolated from bovine epithelium, endothelium, and CBCEn cells (third and fifth passages). The bands are sharp and fairly intense, suggesting that isolation has not degraded the material.

Figure 3, B and C, shows Northern blots of NKCC mRNA expressed in corneal tissues and cultured cells. In Figure 3B, all data were obtained using a mid-stringency wash and reveal two bands of ~7.0 to 7.5 and 5.0 to 5.5 kb. In contrast, the data in Figure 3C were obtained with a high-stringency wash. This procedure eliminates the interaction of the probe with the shorter transcript. As can be

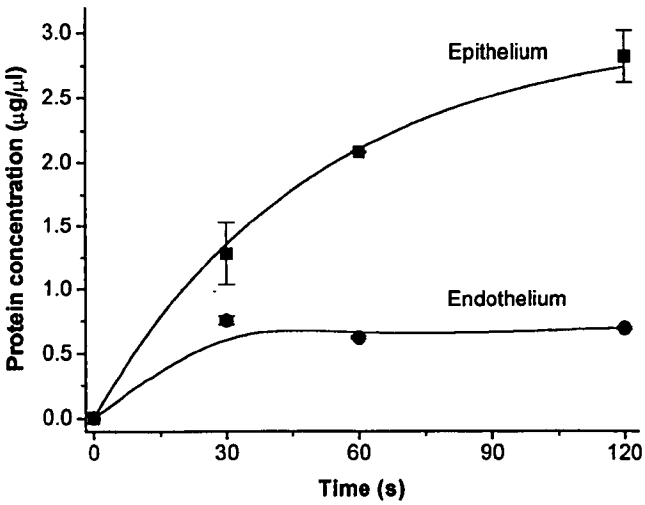

Figure 2. Kinetics of protein release resulting from the lysis of corneal endothelial and epithelial cell layers in GuSCN/EDTA solution. Each experimental point represents the average of eight different determinations. Time denotes exposure to GuSCN/EDTA solution.

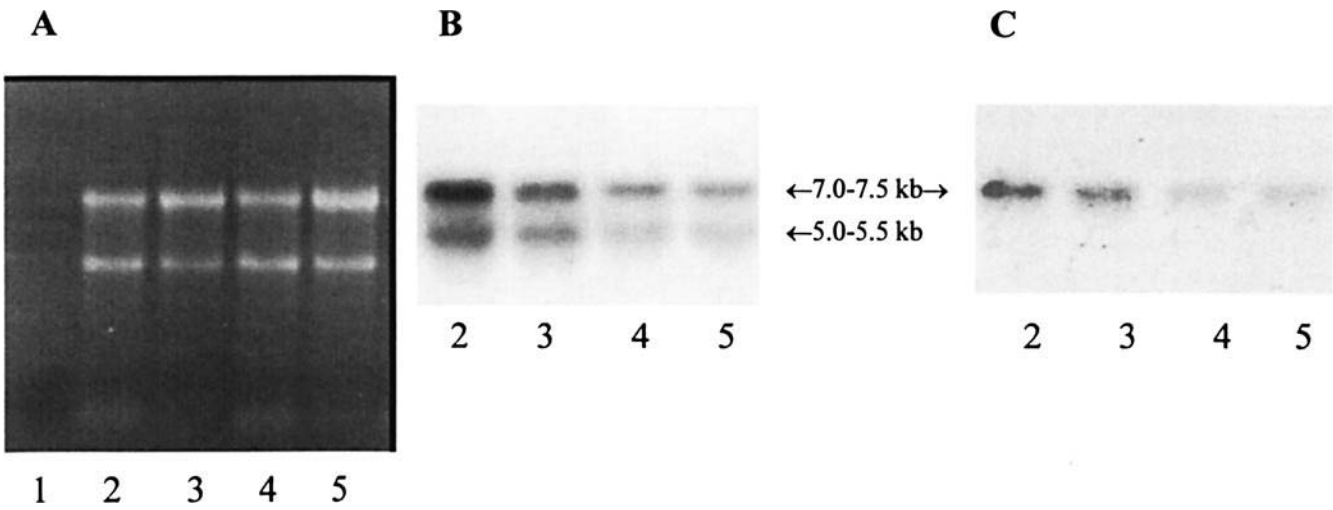

Figure 3. (A) Gel electrophoresis of 5 μg of total RNA isolated from bovine corneal layers and visualized by staining with 0.5 μg/ml ethidium bromide. 28S and 18S bands of ribosomal RNA and a tRNA band are visible. Lanes are: 1) RNA molecular weight marker; 2) corneal epithelial layer; 3) corneal endothelial layer; 4) CBCEn cells of 3rd passage; 5) as in 4, but 5th passage. (B) Northern blot analysis of NKCC mRNA expression after mid-stringency wash; lanes as above. Two micrograms of poly(A)$^+$ RNA per slot was loaded: 14-hr X-ray film exposure. C as in B, but after high-stringency wash.: 36-hr X-ray film exposure.

seen in Figure 3, B and C, the levels of NKCC expression are higher in epithelium than in endothelium, and are lower in CBCEn cells of the third passage, with a further moderate decrease at the fifth passage.

Nuclease Protection Assay of AQP1, GLUT1, and NKCC Expression. Northern blotting is, of course, useful in analyzing the presence and size of mRNA, but it is limited in its ability to quantify messenger levels. To determine more precisely the levels of specific mRNAs, we performed lysate nuclease protection assays. Figure 4, A through C, shows the results of representative S1-nuclease protection assay experiments. In all three panels, labeled bands appear in lanes 3 through 5 that represent the RNA/DNA protected duplexes. As shown on the histogram below (Fig. 4D), the level of NKCC mRNA in bovine epithelium is low (5.0 ± 0.1) molecules/cell. Even lower values (~1 molecule/cell) are found in fresh and CBCEn cells, with cultures (third passage) having almost 2-fold lower NKCC mRNA levels. These corneal layers express sizably more (10–12 times) AQP1 and GLUT1 mRNA (Fig. 4D). However, their comparative patterns of expression are qualitatively similar to those seen for NKCC (fresh epithelium > fresh endothelium > CBCEn cells).

Western Blot Analysis of NKCC Expression. To determine whether the level of NKCC in cell membranes correlates with the abundance of mRNA encoding NKCC, Western blots were done. Figure 5, A and B, demonstrates representative data of experiments in which membrane-enriched cellular fractions of bovine and rabbit corneal tissues and cultured cells were electrophoresed and probed with anti-NKCC T4 antibody. It should be noted that in both corneal tissues, the T4 monoclonal antibody interacts with two proteins with apparent molecular masses of 190 to 200 and 150 to 160 kDa. This observation is in an agreement with the Northern blot data showing that two mRNA species hybridized with a probe to NKCC (Fig. 3B).

Curiously, densitometry of the major 190 to 200 kDa bands shows that the levels of NKCC in rabbit corneal epithelial and endothelial layers were significantly (about 7-fold) higher than in the corresponding bovine layers (histograms, Fig. 5, B and D). It is unlikely that this could be due to a difference in interspecies antibody specificity. Nevertheless, this result is in agreement with the fact that active transepithelial Cl transport is described only in the rabbit (15). Within each species, the amount of normalized epithelial NKCC protein surpassed that in the endothelium by about 2-fold. In addition, in CBCEp and CBCEn, the NKCC levels were lower than in their fresh counterparts (Fig. 5A, lanes, 1, 2, and 5, 4, respectively). A small amount of NKCC protein was found in rabbit stroma, but none could be detected by this method in bovine stroma. The results demonstrate that there is a correlation between NKCC membrane protein amounts and messenger levels.

Discussion

Meaningful comparisons of gene expression between different tissues or even between the same tissue in different species can be problematic if it is normalized to protein content. Such an uncertainty exists because protein content per cell can be variable and also includes any secreted extracellular matrix. Accordingly, we chose to normalize gene expression to DNA content because this is a more direct measure of cell number. DNA binding dyes were used in some studies for this purpose. However, their use may not accurately measure DNA content for at least two reasons: 1) dye selectivity is not absolute for DNA because it binds to ribonucleic acids; and 2) their signal output can be affected by changes in DNA conformation. To avoid these possible

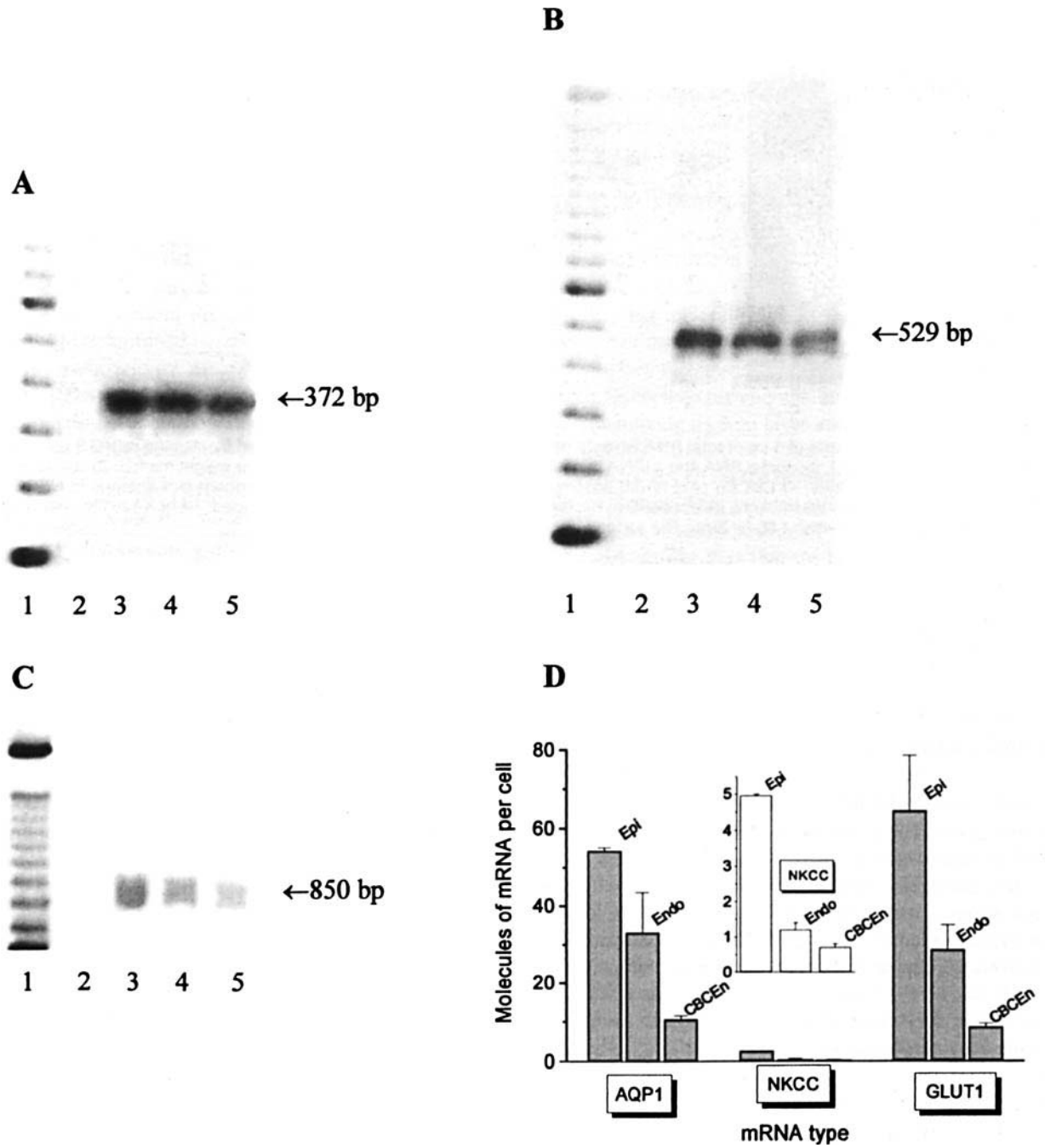

Figure 4. Levels of AQP1 (A), NKCC (B), and GLUT1 (C) mRNAs expression in corneal epithelium (Epi), corneal endothelium (Endo), and cultured bovine corneal endothelial cells (CBCEn) determined with lysate nuclease protection assay. Samples were resolved with 2% agarose gel electrophoresis. Lanes are: 1) 100-bp DNA ladder; 2) probe without target; 3) bovine corneal epithelium; 4) bovine corneal endothelium; 5) CBCEn cells (3rd passage). Same amount of cell lysate containing 150,000 cells was placed in each lane. (D) mRNA abundance per cell: The number of RNA molecules was calculated using the procedure of Haines and Gillespie, as described in Materials and Methods. In all cases, the differences in normalized mRNAs content are statistically significant ($P < 0.01$).

errors in expression evaluation we instead measured DNA content based on its hybridization with a random primer extension ^{32}P-labeled DNA probe (Fig. 1A). As can be seen in Figure 1B, this technique is sensitive enough to detect with excellent reproducibility DNA at a level as low as 20 ng that was obtained from about 5000 cells.

As has been reported, application of a concentrated GuSCN/EDTA solution provides a convenient and rapid method for lysis and extraction of nuclear contents (17). Our use of this procedure appears to be effective because the results shown in Figure 2 reveal that the epithelial protein content is about 5-fold greater than that of the endothelium. This difference corresponds to the histological observation that the epithelium has five cellular layers, whereas the endothelium is a monolayer.

We obtained some suggestive evidence that in both

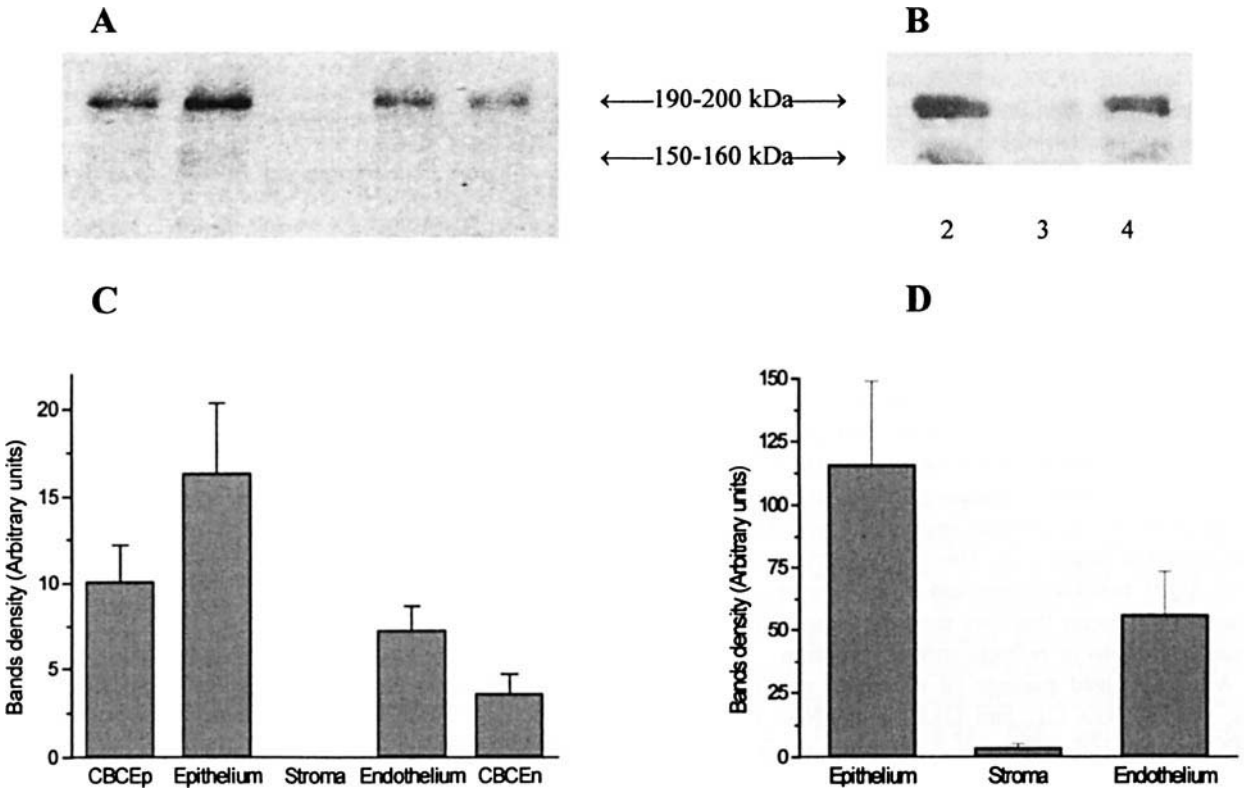

Figure 5. NKCC Western blot analysis in membrane fractions of bovine (A and C) and rabbit (B and D) corneal tissues. Lanes are: 1) cultured bovine corneal epithelial cells (CBCEp), 3rd passage; 2) fresh epithelium; 3) stroma; 4) fresh endothelium; 5) CBCEn cells, 3rd passage. Ten micrograms of membrane-enriched protein fraction was placed in each lane. The imaging films were exposed for 5 min (A) and 2.5 min (B). In all cases, the differences in NKCC content are statistically significant ($n = 3$–4, $P < 0.05$).

epithelial and endothelial layers there is expression of two different NKCC isoforms. The results shown in Figure 3, B and C, indicate that the probe to NKCC could hybridize with two different transcripts of 7.0 to 7.5 and 5.0 to 5.5 kb that are expressed in corneal tissues and cultured cells. The larger one is similar in size to the gene-specific transcript identified in a number of organs that encodes for the secretory NKCC isoform, NKCC1 (2, 18). On the other hand, the smaller sized transcript is more similar in size to the NKCC2 (absorptive) isoform found in the medullary regions of the kidney of many species (18, 19). Another suggestion for two isoforms is that high-stringency washing eliminated the shorter band (Fig. 3C). This could have occurred because under high stringency conditions, the NKCC1 probe that hybridized with putative NKCC2 transcript can be more readily removed. Its dissociation is favored because, as mentioned above, the homology between NKCC1 and NKCC2 is estimated to be in the range between 55% and 65% (20). Western blotting with the anti-T4 antibody identified two protein bands with an apparent molecular mass of about 195 and 155 kDa in both bovine and rabbit corneal tissues and cultured cells (Fig. 5, A and B). The apparent molecular mass of NKCC1 varies from 175 to 205 kDa in different species and tissues, whereas with NKCC2 it is about 150 kDa (2, 18, 19). These results also suggest that both the NKCC1 and NKCC2 isoforms may be expressed in both cornea layers. On the other hand, affinity-purified polyclonal anti-NKCC1 antibody identified proteins of 145 to 155 kDa in rat brain (21). This observation, together with the fact that in embryogenesis the eye develops from the brain, further suggest that in our study the smaller protein could also be a variant of NKCC1. Such a possibility could also account for a finding in mouse kidney where several alternatively spliced cDNAs encoding mNKCC2 were identified. Furthermore, selective antibodies detected proteins of approximately 150 and 120 kDa, respectively (22). Taken together, these results suggest that two different NKCC isoforms may be expressed in both corneal limiting layers. However, other explanations cannot be excluded, such as the existence of other transport proteins with adequate homology to NKCC at the RNA and amino acid levels. Sequencing will distinguish whether other transport proteins are being detected, or whether there are two specific gene transcripts expressing NKCC isoforms in both corneal tissues.

To examine the relative amounts of mRNAs encoding for NKCC, GLUT1, and AQP1 in corneal epithelium and endothelium and CBCEn cells, we used a S1 nuclease protection assay that we have independently developed. This method utilizes asymmetric PCR-generated ^{32}P probes labeled to a high specific activity. This has the advantage of enabling the analysis of very small quantities of RNA. It increases the level of sensitivity by up to 100 times over that of Northern blot analysis. Furthermore, hybridization occurs

in guanidine thiocyanate cell lysates, obviating the need for membrane transfer from gels.

The level of NKCC mRNA was five times higher in bovine epithelium than in endothelium (Fig. 4D). However, the epithelial NKCC protein level was only 2.5-fold higher than that in the endothelium (Fig. 5B). This dissociation may be due to differences in mRNA translation rate, stability, and/or variations in NKCC protein distribution between the plasma membrane and cytosol.

Our finding that there is AQP1 gene expression in the bovine cornea is in agreement with a previous study that used PCR to demonstrate that there is high level of expression of this isoform in the rat cornea (11). However, its origin is open to question because their preparation contained a mixture of the epithelium, stroma, and endothelium. In another study employing various immunodetection techniques on the rat corneal epithelium, no AQP1 protein expression was found (12). This discrepancy with our finding of AQP1 mRNA expression in the bovine cornea epithelium could mean that this message is not translated in normal conditions or reflects species variation.

After the third passage of epithelial and endothelial cells, AQP1, NKCC1, and GLUT1 mRNAs levels and NKCC protein expression fell 2- to 3-fold (Figs. 4 and 5). These observations are in agreement with other data that AQP1 is downregulated in vascular smooth muscle cells after several passages (23), and in cultured human insulinoma cells, there is GLUT gene expression in the early passages, but it is not seen at later passages (24). However, during differentiation of a human intestinal cell line, up to 3-fold increases in NKCC mRNA levels were accompanied by 7-fold increases in NKCC protein expression and approximately 2.5-fold increases in co-transporter functional activity (25).

In summary, our results suggest expression of both NKCC1 and NKCC2 isoforms in freshly isolated and cultured corneal epithelial and endothelial cells; and passage-dependent decreases occurred in mRNA that encode for the membrane transport proteins, NKCC, GLUT1, and AQP1 in cultured versus fresh endothelial cells. These declines suggest that studies with cultured cells may not always yield transport parameters similar to those in their parent tissue. In addition, an approach is described to measure specific mRNA abundance in small amounts of corneal tissue that may make it possible to avoid the use of cell culture to characterize gene expression.

1. Bonanno JA, Giasson C. Intracellular pH regulation in fresh and cultured bovine corneal endothelium. I. Na^+/H^+ exchange in the absence and presence of HCO_3^-. Invest Ophthalmol Vis Sci **33**(11):3058–3067, 1992.
2. Xu JC, Lytle C, Zhu TT, Payne JA, Benz E Jr., Forbush B III. Molecular cloning and functional expression of the bumetanide-sensitive Na-K-Cl cotransporter. Proc Natl Acad Sci U S A **91**:2201–2205, 1994.
3. Park JH, Saier MH Jr. Phylogenetic, structural and functional characteristics of the Na-K-Cl cotransporter family. J Membr Biol **149**:161–168, 1996.
4. Jelamskii S, Sun XC, Herse P, Bonanno JA. Basolateral Na^+-K^+-$2Cl^-$ cotransport in cultured and fresh bovine corneal endothelium. Invest Ophthalmol Vis Sci **41**:488–495, 2000.
5. Takahashi H, Kaminski AE, Zieske JD. Glucose transporter 1 expression is enhanced during corneal epithelial wound repair. Exp Eye Res **63**:649–659, 1996.
6. Loike JD, Cao L, Kuang K, Vera JC, Silverstein SC, Fischbarg J. Role of facilitative glucose transporters in diffusional water permeability through J774 cells. J Gen Physiol **102**:897–906, 1993.
7. Klyce SD. Enhancing fluid secretion by the corneal epithelium. Invest Ophthalmol Vis Sci **16**:968–973, 1977.
8. Cooper GJ, Boron WF. Effect of PCMBS on CO_2 permeability of *Xenopus* oocytes expressing aquaporin 1 or its C189S mutant. Am J Physiol **275**:C1481–C1486, 1998.
9. Nakhoul NL, Davis BA, Romero MF, Boron WF. Effect of expressing the water channel aquaporin-1 on the CO_2 permeability of *Xenopus* oocytes. Am J Physiol **274**:C543–C548, 1998.
10. Prasad GV, Coury LA, Finn F, Zeidel ML. Reconstituted aquaporin 1 water channels transport CO_2 across membranes. J Biol Chem **273**:33123–33126, 1998.
11. Patil RV, Saito I, Yang X, Wax MB. Expression of aquaporins in the rat ocular tissue. Exp Eye Res **64**:203–209, 1997.
12. Hamann S, Zeuthen T, La Cour M, Nagelhus EA, Ottersen OP, Agre P, Nielsen S. Aquaporins in complex tissues: distribution of aquaporins 1-5 in human and rat eye. Am J Physiol **274**:C1332–C1345, 1998.
13. Li J, Kuang K, Nielsen S, Fischbarg J. Molecular identification and immunolocalization of the water channel protein aquaporin 1 in CB-CECs. Invest Ophthalmol Vis Sci **40**:1288–1292, 1999.
14. Haines DS, Gillespie DH. RNA abundance measured by a lysate RNase protection assay. BioTechnique **12**:736–741, 1992.
15. Bildin VN, Yang H, Crook RB, Fischbarg J, Reinach PS. Adaptation by corneal epithelial cells to chronic hypertonic stress depends on upregulation of Na:K:2Cl cotransporter gene and protein expression and ion transport activity. J Membr Biol **177**:41–50, 2000.
16. Klyce SD, Neufeld AH, Zadunaisky JA. The activation of chloride transport by epinephrine and Db cyclic-AMP in the cornea of the rabbit. Invest Ophthalmol **12**:127–139, 1973.
17. Thompson J, Gillespie D. Molecular hybridization with RNA probes in concentrated solutions of guanidine thiocyanate. Anal Biochem **163**:281–291, 1987.
18. Russell JM. Sodium-potassium-chloride cotransport. Physiol Rev **80**:211–276, 2000.
19. Payne JA, Xu JC, Haas M, Lytle CY, Ward D, Forbush B III. Primary structure, functional expression, and chromosomal localization of the bumetanide-sensitive Na-K-Cl cotransporter in human colon. J Biol Chem **270**:17977–17985, 1995.
20. Park JH, Saier MH Jr. Phylogenetic, structural and functional characteristics of the Na-K-Cl cotransporter family. J Membr Biol **149**:161–168, 1996.
21. Plotkin MD, Kaplan MR, Peterson LN, Gullans SR, Hebert SC, Delpire E. Expression of the Na^+-K^+-$2Cl^-$ cotransporter BSC2 in the nervous system. Am J Physiol **272**:C173–C183, 1997.
22. Mount DB, Baekgaard A, Hall AE, Plata C, Xu J, Beier DR, Gamba G, Hebert SC. Isoforms of the Na-K-2Cl cotransporter in murine TAL I: Molecular characterization and intrarenal localization. Am J Physiol **276**:F347–F358, 1999.
23. Weissberg PL, Cary NR, Shanahan CM. Gene expression and vascular smooth muscle cell phenotype. Blood Press **2**(Suppl):68–73, 1995.
24. Baroni MG, Cavallo MG, Mark M, Monetini L, Stoehrer B, Pozzilli PJ. β-Cell gene expression and functional characterization of the human insulinoma cell line CM. Endocrinol **161**:59–68, 1999.
25. Moore-Hoon ML, Turner RJ. Increased expression of the secretory Na^+-K^+-$2Cl^-$cotransporter with differentiation of a human intestinal cell line. Biochem Biophys Res Commun **244**:15–19, 1998.