# Evidence for the Presence of a Hg-Inhibitable Water-Permeability Pathway and Aquaporin 1 in A5 Salivary Epithelial Cells

KRZYSZTOF W. LAZOWSKI, JUN LI, CHRISTINE DELPORTE, AND BRUCE J. BAUM*

*Clinical Investigations and Patient Care Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892-1190*

The molecular basis of water-permeability in salivary and other exocrine glands is not understood. We have examined two well-studied salivary epithelial cell lines for evidence of a Hg-inhibitable water-permeability pathway. A5 and HSG cells are derived from rat and human submandibular glands, respectively. Only A5 cells demonstrated such a pathway. The rate of A5 cell osmotic shrinkage was inhibited about fivefold in the presence of 300 $\mu$M $HgCl_2$. To determine if this activity was associated with the expression of the prototypical water channel (aquaporin, AQP) AQP1, we used three separate experimental approaches; Northern analysis and reverse transcription-polymerase chain reaction (RT-PCR) analysis of isolated mRNA, and Western analysis of cell membranes. All three methods yielded positive results with A5 cells and negative results with HSG cells. The ~800 bp product of RT-PCR was analyzed further by sequencing and restriction enzyme digestion. The results were consistent with the previously reported coding region sequence for rat kidney AQP1. The aggregate data demonstrate that marked differences in water-permeability and water channel expression exist in these two salivary epithelial cell lines.    © 1995 Wiley-Liss, Inc.**

Considerable progress has been achieved in the last 3 years in understanding the molecular basis of osmotic water permeability across eukaryotic cell membranes (e.g., Verkman, 1992; Agre et al., 1993; Knepper, 1994). Although water can only slowly diffuse across a lipid bilayer, the existence of a water channel was long hypothesized to account for the rapid flux of water known to exist across membranes in many cell types (Verkman, 1992). The first water channel to be isolated was the aquaporin (AQP) CHIP28, now known as AQP1, from the human erythrocyte, whose water-permeability coefficient ($P_f$) was shown to be markedly sensitive to Hg (Preston and Agre, 1991; Preston et al., 1992; Zeidel et al., 1992). Since these reports, at least four additional and homologous mammalian AQPs have been described: AQP2, which is a vasopressin-sensitive, Hg-inhibitable water channel from apical membranes of rat collecting duct (Fushimi et al., 1993); AQP3, a Hg-inhibitable water channel from rat collecting duct basolateral membranes and also permeable to urea (Ishibashi et al., 1994); a Hg-insensitive water channel (AQP4; MIWC) recently suggested to be present in several rat tissues (Hasegawa et al., 1994; Jung et al., 1994); and AQP5, which is a Hg-sensitive water channel recently reported as found in exocrine glands and respiratory parenchyma (Raina et al., 1995).

Salivary glands are a site of substantial, regulated fluid secretion (e.g., Cook and Young, 1989; Turner, 1993; yet at present little is understood about water movement across salivary epithelial cells (e.g., Steward et al., 1992). We have initiated a series of studies aimed at elucidating the molecular basis of water transport in

these exocrine glands (Li et al., 1994). Salivary glands contain several cell types, however, and it is difficult to obtain a uniform preparation of any single cell type from acutely dispersed gland tissue (e.g., Baum et al., 1990; Dehaye and Turner, 1991; Li et al., 1994). Therefore, in addition to studies with isolated gland tissue, we have examined the $P_f$ of two established, well-studied salivary epithelial cell lines, A5 and HSG. A5 cells are derived from rat submandibular gland epithelial cells transformed with 3-methylcholanthrene and share immunological characteristics with intra- and extralobular salivary duct cells (He et al., 1989). HSG cells are derived from an irradiated human submandibular gland and are thought to originate from the pluripotential intercalated duct cell region (Shirashuna et al., 1981). The purpose of the present study was to determine if these cell lines exhibit a Hg-inhibitable water permeability pathway and express AQP1.

## MATERIALS AND METHODS
### Cell culture

HSG cells (a gift of Dr. Mitsunabo Sato, Tokushima University, Tokushima, Japan) were cultured in Ham's F-12: Dulbecco's Modified Essential Medium, 1:1, (NIH

Media Unit, Bethesda, MD) supplemented with 10% fetal bovine serum (Biofluids, Rockville, MD), 2 mM glutamine, 1,000 U/ml penicillin, and 100 μg/ml streptomycin, at 37°C and 5% $CO_2$. A5 cells were grown in McCoy's 5A medium (NIH Media Unit) with the above supplements. Cells were routinely passaged approximately weekly using 0.2 g/l EDTA in phosphate-buffered saline (NIH Media Unit).

## Measurement of osmotic water permeability

$P_f$ (in cm/s) was determined in freshly suspended cells by light scattering using a SLM-Aminco spectrofluorometer (SLM Instruments, Urbana, IL). Near-confluent cell layers were washed with $Ca^{2+}/Mg^{2+}$-free Hank's balanced salt solution (HBSS; Biofluids), then incubated in $Ca^{2+}/Mg^{2+}$-free HBSS containing 4 mM EGTA at 37°C for 3–5 min. Suspended cells were washed three times in $Ca^{2+}/Mg^{2+}$-free HBSS, then diluted to ~$10^5$ cells/ml in this same isosmotic solution.

Thereafter, cells were exposed to an inward osmotic gradient of 91 mM by rapidly mixing 2 ml of the stirred cell suspension with 0.2 ml of 1 M sucrose in $Ca^{2+}/Mg^{2+}$-free HBSS. Upon mixing, the rapid increase in extracellular osmolality caused water efflux from the cells and an increase in the intensity of scattered light, reflecting an increased cell shrinkage. Light scattering data were analyzed with the MathCad software package (MathSoft, Cambridge, MA) and fitted to single exponential curves. $P_f$ was calculated by matching simulated curves with experimental data fit using the basic transport equation:

$$dv(t)/dt = P_f M_w S[C_i(t) - C_o]/V(0) \qquad (1)$$

where $v(t) = V(t)/V(0)$ is the relative volume of a cell at time $t$, $M_w$ is the partial molar volume of water (18 $cm^3$/mol), $S$ is the cell surface area, $V(0)$ is the isosmotic cell volume at $t = 0$, $C_i(t)$ is the concentration of intracellular impermeant solutes at time $t$, and $C_o$ is the concentration of extracellular impermeant solutes. The latter essentially remains constant in time as the total volume of cells is much smaller than the volume of the bathing buffer. The relationship between relative cell volume (v) and extracellular osmolality (measured using a 5500 Vapor Pressure Osmometer, Vescor, Logan, UT) was determined by sizing the cells that had been equilibrated in buffers of different osmolalities for ~30 sec, with a Coulter Counter (Coulter Electronics, Hialeah, FL) connected to an IBM AT computer with PCA-II software (Nucleus, Oak Ridge, TN). Data revealed (see Results) a linear relationship between v and relative reciprocal osmolality,

$$v(t) = C_i(0)/C_i(t) \qquad (2)$$

indicating that the cells behave as "perfect" osmometers over the experimental range of osmolalities. This allows substitution of $C_i(t)$, in equation (1), by the measured variable $C_i(0)/v(t)$. $V(0)$ and $S$ were calculated from the average diameter (13 μm) of cells measured by video light microscopy assuming spherical geometry.

## RNA isolation

Total cellular RNA was obtained from freshly dissected adult rat kidney and confluent cell layers of A5 and HSG cells, using the methods of Chomczynski and Sacchi (1987). Poly (A)$^+$ RNA was obtained from these preparations using oligo (dT)-cellulose columns (Qiagen, Chatsworth, CA).

## Northern blot analysis

Poly (A)$^+$ RNA (10 μg) was fractionated on 1.2% agarose gels containing 0.8 M formaldehyde, then blotted onto nylon membranes (Schleicher & Schuell, Keene, NH) and crosslinked by ultraviolet light (Stratagene, LaJolla, CA). Prehybridization and hybridization of blots were performed essentially as described previously (Li et al., 1994), using as probes either a 1.4 kb rat AQP1 cDNA or a 1.1 kb glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (Clonetech, Palo Alto, CA) labelled by random priming. Blots were exposed to Kodak X-Omat AR film, with intensifying screens, at −70°C.

## RT-PCR and sequencing

mRNA isolated as above was reverse transcribed and subjected to PCR amplification using an RNA PCR kit (Perkin Elmer, Norwalk, CT). PCR amplification utilized 100 pmol primers. The sense primer corresponded to the rat parotid AQP1 cDNA sequence nt 51-71, whereas the antisense primer was derived from nt 843-864. mRNA samples were amplified over 35 cycles of 94°C (1 min), 55°C (1 min), and 72°C (2 min) using a DNA Thermal Cycler (Perkin Elmer). The resulting predicted 813 bp PCR product was isolated following 1% agarose gel electrophoresis and subjected to restriction enzyme digestion to confirm its identity to AQP1 using Pst I and Pvu II (obtained from Boehringer Mannheim, Indianapolis, IN, and New England Biolabs, Beverly, MA, respectively). As additional verification, we generated a 354 bp PCR product from the 813 bp PCR fragment (above) as a template using nested primers based on the human red cell AQP1 cDNA sequence (Preston and Agre, 1991). The sense primer corresponded to nt 288-318, whereas the antisense primer was derived from nt 612-642. This PCR product was sequenced by the dideoxy chain termination method.

## Western blot analysis

Crude plasma membranes were prepared from adult rat kidney, A5 and HSG cells as described (Dai et al., 1991). SDS-PAGE was performed as reported (Li et al., 1994) except that 12% acrylamide gels were used and no β-mercaptoethanol was added to the samples. Proteins were transferred electrophoretically to Imobillon PVDF membranes (Millipore, Bedford, MA) and then immunolabeled for AQP1 using a previously reported antiserum (1:1,000 dilution) obtained from rabbits immunized with AQP1 purified from outdated human red blood cells (Li et al., 1994). Antibody bound to proteins on the transfer membranes was detected using the ECL chemiluminescence method (Amersham, Arlington Heights, IL).

## RESULTS
### Osmotic properties of salivary cell lines

A5 and HSG cells were suspended in HBSS and then mixed with solutions containing different concentrations of the impermeant solute sucrose. Osmotic response was determined by measuring the volume of the cells equilibrated at different osmolalities and plotting
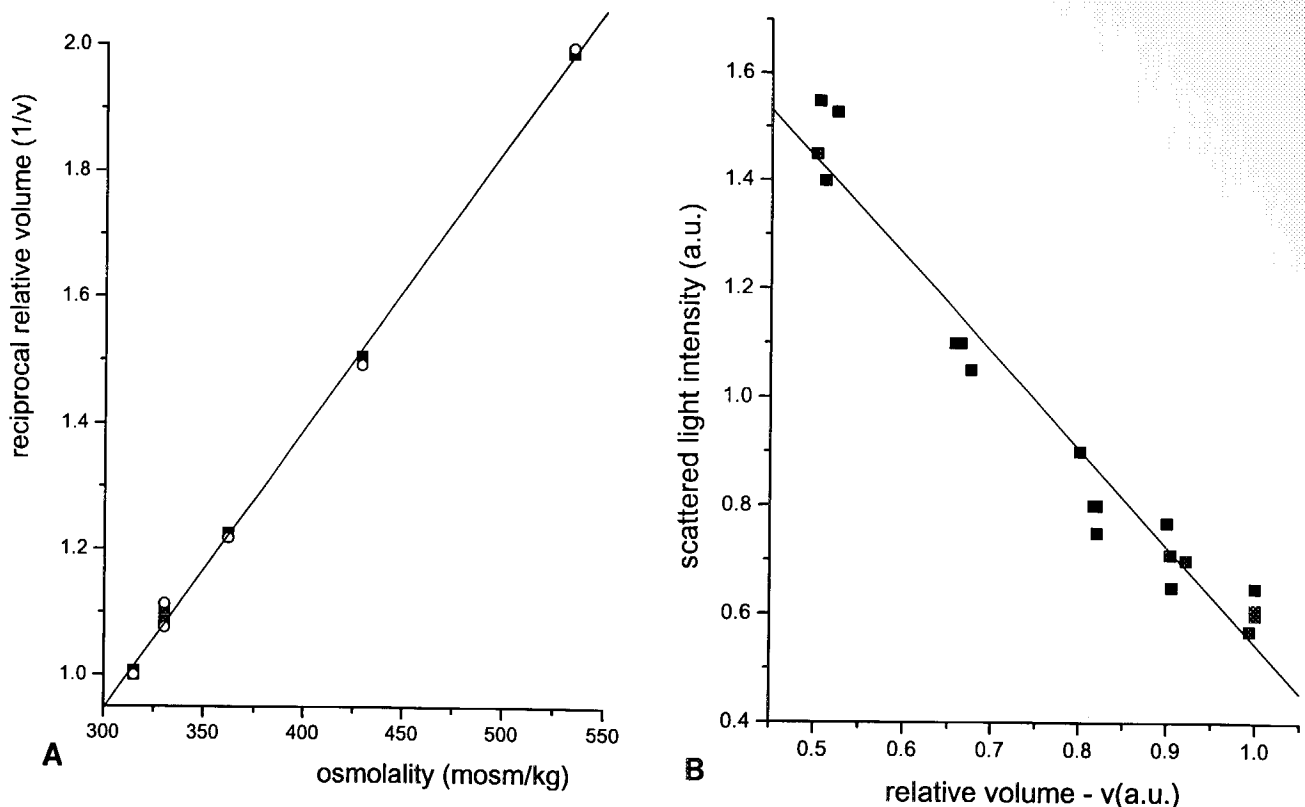
Fig. 1. **A.** Effects of changes in extracellular osmolality on the reciprocal relative volume of A5 and HSG cells. Cells were placed in a stirred Coulter counter cuvette in media with different osmolalities (different concentrations of sucrose as described under Materials and Methods). As the extracellular osmolality increased, the reciprocal relative volume increased due to water efflux. Relative volume was calculated from the maxima on the size distribution plots (see Materials and Methods) and divided by the isosmotic volume. A5 cells (■); HSG cells (○). **B.** Relationship between the intensity of scattered light (a.u., arbitrary units) and relative cell volume in A5 cells exposed to media of different osmolality.

the reciprocal of the relative cell volume (1/v) against the medium osmolality (Fig. 1A). This plot shows a linear relationship between the two variables up to 540 mosm/kg, indicating that in this range of extracellular osmolality both A5 and HSG cells behave as perfect osmometers. Over this same range of osmolality, the intensity of scattered light from the cell suspension was measured revealing a nearly linear relationship with the relative cell volume (Fig. 1B). These two observations validate the assumptions necessary to determine the $P_f$ (see Materials and Methods).

Figure 2 shows the time course of scattered light intensity for A5 cells exposed to a sudden increase in extracellular osmolality of 91 mosm/kg. The inwardly directed osmotic gradient caused water efflux, thus cell shrinkage, and an increase in scattered light intensity. These data also show that osmotically induced cell volume changes were sensitive to the presence of 300 μM $HgCl_2$ in the medium. The cells incubated for 25–30 min in the presence of the mercurial showed much slower kinetics of shrinkage (~5 times) than cells incubated in its absence. The calculated $P_f$ was 0.011 cm/s for A5 cells incubated in HBSS and 0.0022 cm/s for cells incubated in the presence of the mercurial. When we analyzed the responses of HSG cells to similar osmotic

stresses, we observed that the kinetics of cell shrinkage (and $P_f$) were insensitive to the presence of $HgCl_2$ and were in fact of a similar magnitude as observed with A5 cells incubated in $HgCl_2$ ~0.002 cm/s.

## Examination of salivary cells for AQP1 expression

AQP1 is a widely distributed water channel, markedly sensitive to Hg (Agre et al., 1993; Knepper, 1994). Accordingly, we tested the hypothesis that the Hg-sensitive reduction in $P_f$ observed with A5 cells may reflect, at least in part, the expression of AQP1 by these cells. Conversely, we posited that HSG cells would not express this molecule. Initially, we isolated mRNA from both cell types and performed Northern analysis. As shown in Figure 3, rat kidney mRNA strongly hybridizes to a rat AQP1 probe. The size of the transcript detected is ~3kb, the known size of authentic rat AQP1 mRNA (Deen et al., 1992; Li et al., 1994). A similarly sized positive band was detected in mRNA from A5 cells, although it was of much lower intensity. No AQP1 positive hybridization was observed when mRNA from HSG cells was analyzed, even at great excess (see Fig. 3 legend).
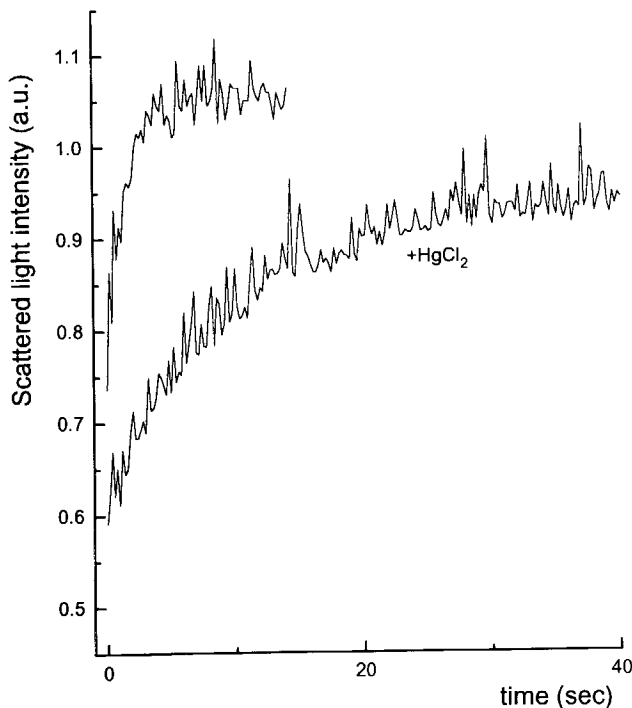
Fig. 2. Time course of A5 cell water transport. Plot shows change in scattered light intensity (a.u., arbitrary units) vs. time for A5 cells in HBSS alone and for cells incubated in HBSS in the presence of 300 μM HgCl$_2$ for 25–30 min. Cells in HBSS (osmolality 310 mosm, pH 7.4, 24°C) were mixed at time 0 with a hyperosmotic solution of sucrose in HBSS, which gave an inwardly directed osmotic gradient of 91 mosm/kg. The results shown are representative of four experiments performed.
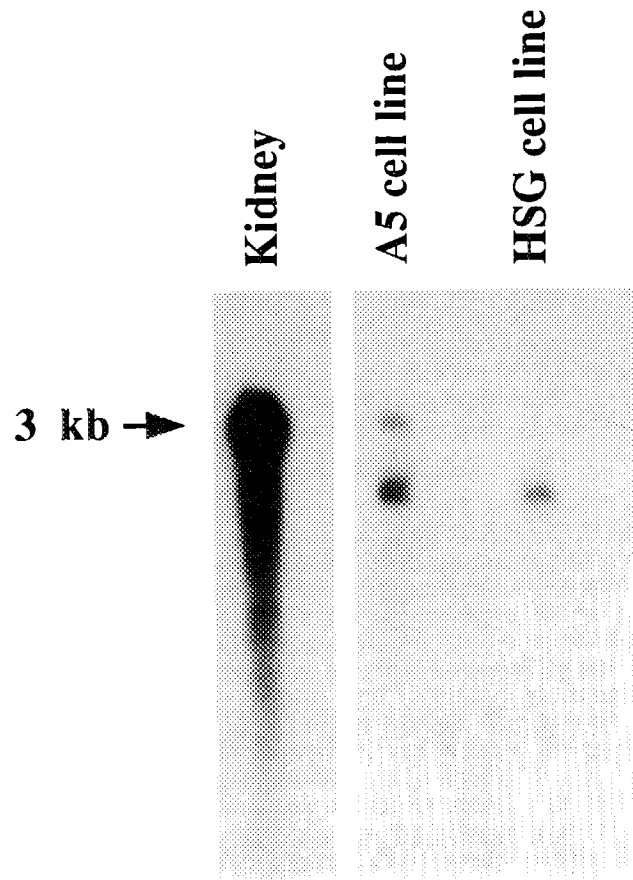


Fig. 3. Northern analysis of mRNA from A5 and HSG cells. mRNA was extracted and subjected to 1.2% agarose gel electrophoresis as described under Materials and Methods. Rat kidney mRNA was used as a positive control for AQP1 hybridization. All samples were also examined for levels of G3PDH mRNA (lower band). Blots were exposed to the x-ray film for 72 hr. Only samples from kidney and A5 cells showed evidence of mRNA for AQP1 (arrow). In separate experiments, up to 10-fold higher amounts of mRNA from HSG cells were used without detection of any AQP1 signal (not shown). The results shown are representative of four experiments performed.

Next, we employed RT-PCR, with primers defining a cDNA fragment of 813 bp including the open reading frame of the AQP1 mRNA (Zhang et al., 1993; Li et al., 1994) to examine the mRNA samples. The results of agarose gel electrophoresis of representative PCR reaction products are shown in Figure 4. As expected, rat kidney mRNA resulted in the amplification of a cDNA band of the predicted size. A similar size band was also detected in the samples containing A5 cell mRNA, whereas no such band was detected in the samples containing HSG cell mRNA. To verify that this predicted cDNA band indeed encoded AQP1, the band was isolated from agarose gels of A5 cell reaction products and its restriction enzyme sensitivity determined. Results indicated the restriction fragments expected from authentic rat AQP1 (Fig. 5; see Deen et al., 1992; Zhang et al., 1993). Additionally, using nested PCR we generated a second fragment corresponding to nt 288-642 (354 bp) of rat AQP1 and subjected the fragment to sequence analysis. The resulting sequence (not shown) was identical to that previously reported for this portion of the rat AQP1 open reading frame (Deen et al., 1992; Zhang et al., 1993).

Finally, we wished to determine if AQP1 protein was expressed by A5 cells. Crude membranes were prepared from rat kidney, as well as both cell lines studied herein. Membranes were subjected to SDS-PAGE and

Western blot analyses. The results (shown in Fig. 6) are consistent with those of mRNA analyses.

Rat kidney membranes exhibited high levels of immunoreactive AQP1. Two prominent immunopositive bands were seen. One, at ~28 kDa, is nonglycosylated AQP1, whereas a higher molecular weight band (~30–40 kDa) likely represents the glycosylated protein (Denker et al., 1988). A5 cell membranes yielded similar, albeit much less intense, results. Note that in the figure shown, 80× more A5 cell membrane protein was subjected to electrophoresis. A 28 kDa band was clearly observed, as was the higher molecular weight glycosylated form of AQP1. No proteins, corresponding to the two immunopositive AQP1 bands, could be detected in HSG membranes. When blots of A5 cell and rat kidney membranes were incubated with the preimmune serum, no immunopositive bands were seen (not shown).

## DISCUSSION

The purpose of the present investigation was to determine if two well-studied salivary epithelial cell lines
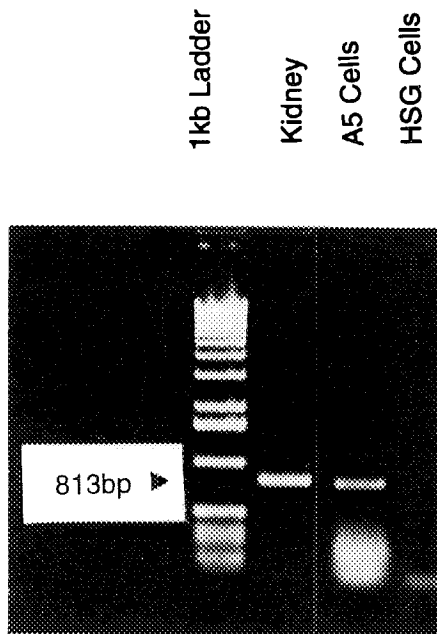
Fig. 4. RT-PCR examination of mRNA for evidence of AQP1 expression. Samples of mRNA from A5 and HSG cells, and from rat kidney, were subjected to RT-PCR analysis as described in Materials and Methods. The predicted product of the PCR was an 813 bp fragment (arrowhead), which included the coding region of the AQP1 mRNA. Samples from kidney and A5 cells resulted in the amplification of such a cDNA product, whereas samples from HSG cells yielded negative results consistently. The experiment shown is representative of at least five separate PCR amplifications performed.
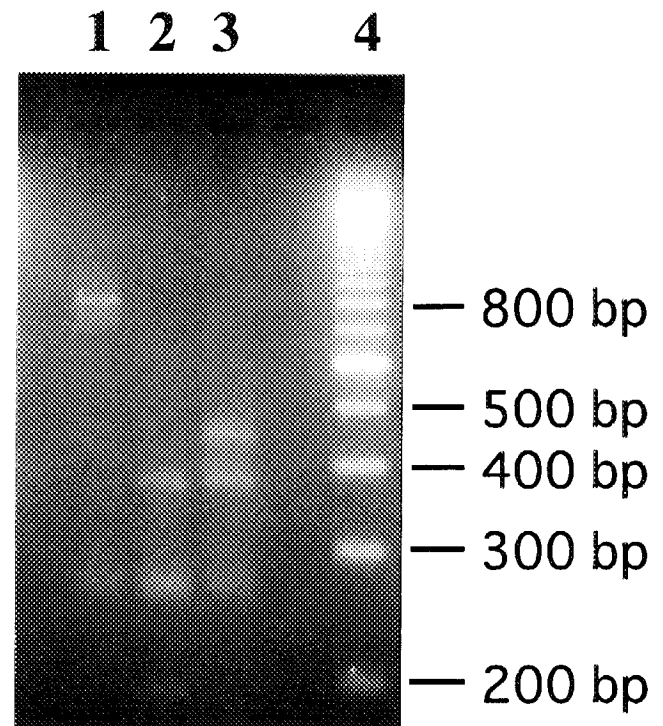


Fig. 5. Restriction enzyme digestion of the 813 bp A5 PCR product. The PCR products generated from experiments such as shown in Figure 4 were isolated and incubated without restriction enzymes (lane 1) or with Pvu II (lane 2) or Pst I (lane 3). Samples were incubated overnight, then subjected to agarose gel electrophoresis. Based on the known nucleotide sequence of AQP1, digestion with Pvu II should yield fragments of 187, 257, and 369 bp, whereas digestion with Pst I should yield two fragments of 443 and 370 bp. Note that a PCR artifact is seen in lane 3 of a similar size to the 257 bp product of Pvu II digestion. DNA size standards are shown in lane 4. The results shown are representative of three experiments performed.

display Hg-sensitive water permeability and express AQP1. As noted earlier, the molecular basis for salivary gland water permeability is not known and as shown recently by us (Li et al., 1994), it is difficult to utilize the mixed cell populations that occur in gland tissue for such studies. The results of our experiments clearly demonstrate that A5 cells display a Hg-sensitive water-permeability pathway and express AQP1, whereas HSG cells are negative for these characteristics. All experiments performed by us consistently supported such a conclusion (i.e., functional studies, Northern and Western blot analyses, RT-PCR). Interestingly, whereas A5 cells exhibit considerable water permeability ($P_f = 0.011$ cm/s), they express a relatively small amount of AQP1 in their membranes (see Fig. 6) compared to whole kidney membranes. It is possible that A5 cells express additional aquaporins, e.g., AQP5 (Raina et al., 1995; pers. comm. by P. Agre). Further studies are necessary to address this possibility.

Although the experimental results are clear, they are also physiologically somewhat surprising. Salivary intra- and extralobular ducts are considered to be water impermeable (e.g., Mangos et al., 1973; Cook and Young, 1989) and A5 cells share immunological characteristics with such ducts in the rat submandibular gland. Further, two studies (Nielsen et al., 1993; Li et al., 1994) have demonstrated that AQP1 is not expressed by any epithelial cells (ductal and acinar) in this gland. Rather, the only site of AQP1 expression in this tissue is by endothelial cells of fenestrated capillar-

ies and venules. Although A5 cells have been well characterized and often used as a model salivary epithelial cell (e.g., He et al., 1989; Lin and Ann, 1992; Yeh et al., 1993), after our initial results we tested them for expression of Von Willenbrand factor (an endothelial cell marker). Although positive immunocytochemical staining was observed with control human umbilical vein endothelial cells, no immunoreactivity was detected in A5 cells (results not shown).

Thus this cell line, which displays numerous salivary ductal cell characteristics, seems to express inappropriately AQP1 and show a high $P_f$. A possible consideration in this regard may lie in the recent findings of Lanahan et al. (1992). While studying changes in the program of gene expression that follows mitogenic stimulation of 3T3 mouse fibroblastic cells, they observed that DER-2 (the murine homolog of AQP1, with 94% amino acid identity) was among a battery of delayed early response genes activated when 20% fetal bovine serum was added to quiescent cells. Further, DER-2 was expressed continuously in cycling mouse L cells during exponential growth. A specific role for AQP1 in the complex process of cellular growth control is not known, but the observations of Lanahan et al. (1992) are suggestive of some (direct or indirect) in-
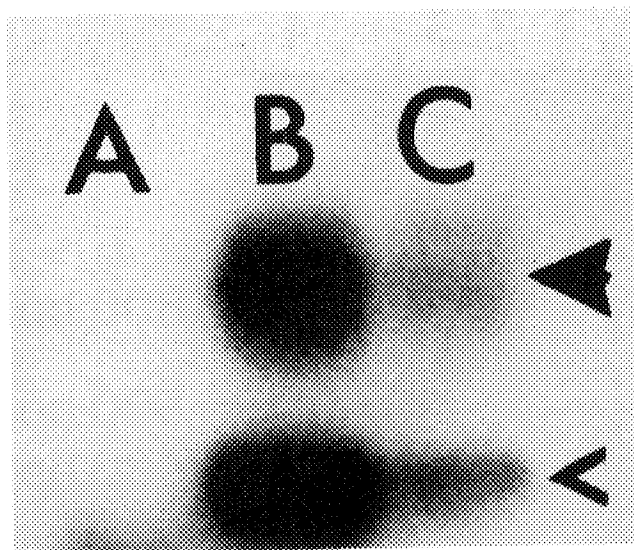
Fig. 6. Immunoblot analysis of cell membranes. Western blots were performed as described under Materials and Methods with membranes prepared from rat kidney (lane B; 0.25 µg of protein was used) as a positive control, and from A5 (lane C) and HSG (lane A) cells (for each, 20 µg of protein was used). Blots were incubated with antiserum to AQP1 and immunoreactive proteins were detected using the ECL chemiluminescence method. Samples from kidney and A5 cells consistently showed two immunopositive bands, one at 28 kDa (arrowpoint) and another, broad higher molecular weight, ~30–40 kDa (arrowhead). In samples from HSG cells, no such bands were detected. The results shown are representative of at least six experiments.

volvement. As noted earlier, A5 cells are derived from salivary epithelial cells chemically transformed with 3-methylcholanthrene (He et al., 1989). The expression of AQP1 found by us in A5 cells may be a reflection of the loss of growth control associated with transformation.

Although our results may appear to lack overt exocrine physiological relevance, they do suggest that it is possible for cells with ductal characteristics to express functional water channels and exhibit considerable water permeability. In particular, these findings may have implications for efforts aimed at repairing irreversibly damaged salivary glands. For example, acinar (water-permeable) cells are lost following therapeutic ionizing radiation for head and neck malignancies, and as part of the autoimmune damage associated with Sjögren's syndrome. The water-impermeable ductal cells preferentially survive these circumstances, but such glands secrete markedly less saliva. We have proposed that the repair of this type of damage, by adenovirus-mediated in vivo gene transfer (Mastrangeli et al., 1994), could at least in part occur by transducing remaining ductal cells with a water channel gene.

The other salivary cell line studied by us, HSG, exhibited only low water permeability. HSG cells are reported to display many characteristics of intercalated duct cells, considered to be a "pluripotent" cell capable of giving rise to acinar, as well as ductal, cells (e.g., Shirashuna et al., 1981; Patton and Wellner, 1993). At a practical level, HSG cells should prove useful for testing in vitro the transfer, expression, and function of water channel genes in an epithelial cell line derived from salivary glands.

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