Translational Regulation of Na,K-ATPase α1 and β1 Polypeptide Expression in Epithelial Cells*

(Received for publication, March 12, 1996, and in revised form, June 6, 1996)

Kent K. Grindstaff, Gustavo Blanco, and Robert W. Mercer‡

From the Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110

To investigate the regulation of the Na,K-ATPase, we have studied the expression of the Na,K-ATPase polypeptides in several mammalian cell lines using the vaccinia virus/T7 RNA polymerase expression system. Infection of several fibroblast-like cell lines with viral recombinants containing the Na,K-ATPase α and β isoforms, the glucose transporters, GLUT 1 and GLUT 4, or the capsid protein of the Sindbis virus all result in the production of the appropriate protein products. However, all epithelial cell lines tested fail to synthesize the Na,K-ATPase viral recombinants, yet they efficiently express the other virally directed polypeptides. While Madin-Darby canine kidney (MDCK) epithelial cells infected with the Na,K-ATPase α1 or β1 recombinant viruses produce both mRNAs, the messages are inefficiently translated. Furthermore, the RNA from infected MDCK cells does not direct the in vitro synthesis of the β1 polypeptide, whereas the message from infected fibroblast-like BSC 40 cells is efficiently translated both in vivo and in vitro. Moreover, the synthesis of the H,K-ATPase α subunit is also limited in MDCK cells, although the H,K-ATPase β subunit is efficiently expressed. Expression of chimeras constructed between the Na+ pump β1 isoform and the H,K-ATPase β subunit indicates that sequences in the 5' coding region of the β1 message have an inhibitory effect; however, the stringent translational regulation of the β1 isoform in MDCK cells requires the 5' and 3' regions of the coding sequence. The ability of the polarized cell lines to limit the synthesis of the Na+ pump polypeptides while expressing other vaccinia recombinants at high levels suggests that the polarized cells possess a stringent mechanism for the specific translational regulation of a select set of messages.

The sodium- and potassium-dependent adenosine triphosphatase (Na,K-ATPase, Na+ pump) is a plasma membrane enzyme that couples the hydrolysis of ATP to the exchange of Na+ and K+ against their respective chemical gradients. The α1 subunit (α1, α2, and α3) and β subunit (β1 and β2) have been identified in mammals, and a third β isofrom has been described in amphibians. While the functional significance for this isoform diversity is not well understood, the tissue-specific and developmentally regulated pattern of expression (7, 8) suggests different roles for the isoforms.

Characterization of different tissues and cell types demonstrate that the Na,K-ATPase isoforms have distinct patterns of expression. In addition, when multiple isoforms are expressed in a single cell type, their pattern of distribution can be disparate suggesting that differences in cellular localization may be important in defining isoform functions (9). The localization of the Na+ pump to specific plasma membrane domains is also important in defining the characteristics of individual tissues and cells. For example, while the Na+ pump is uniformly distributed over the cell surface in most cell types, in polarized epithelial cells the enzyme is restricted to either the apical (10, 11) or basolateral domain (12, 13) where it establishes the transepithelial sodium gradient that drives the vectorial transport of ions and solutes.

There have been numerous studies detailing the tissue-specific modulation of Na+ pump expression through a combination of transcriptional, translational, and post-translational mechanisms (reviewed in Ref. 5). For instance, analysis of the developmental expression of the Na+ pump isoforms in rat tissues demonstrates that the transcripts for the α and β isoforms are coordinately regulated in a distinct tissue-specific manner (7). Likewise, several studies on the effects of hormone treatment on tissue culture cells and whole animals show that Na+ pump expression and activity can be further regulated in a cell-specific manner by a complex series of regulatory mechanisms (14–18).

To investigate the expression of the Na+ pump subunits in a common polarized background, we attempted to express the rodent α1 and β1 subunits in Madin-Darby canine kidney (MDCK)3 cells, a commonly used renal cell culture model. Us-
ing recombinant vaccinia viruses, MDCK cells fail to express virally directed Na\(^+\) pump polypeptides but are able to support the synthesis of several other mammalian and viral membrane proteins from similarly constructed viral recombinants. Identical results were obtained using other epithelial cell lines. In contrast, infection of several fibroblast-like cell lines with the Na\(^+\) pump viral recombinants results in the production of the appropriate proteins. In MDCK cells this regulation is not limited to the α1 and β1 isoforms of the enzyme. The synthesis of the Na\(^+\) pump α2, α3, and β2 isoforms in MDCK cells is also limited, although not as stringently as observed for the α1 and β1 isoforms. In addition, while the α and β subunits of the structurally related H,K-ATPase are also efficiently expressed in BSC 40 cells, in MDCK cells only the β subunit is expressed. Analysis of RNA from MDCK cells infected with α1 or β1 recombinant viruses demonstrates that both mRNAs are produced at significantly higher levels than in infected cells; however, the virally directed α1 and β1 messages are insufficiently translated. The lack of any native 5′-untranslated region (UTR) sequence in the vaccinia recombinants suggests that the epithelial specific regulation lies in the 3′-UTR or coding region of the Na\(^+\) pump isoforms. Infection of MDCK cells or fibroblast-like BSC 40 cells with α1 recombinants either lacking the 3′-UTR or possessing the 3′-UTR of the Sindbis capsid or glucosptide transporter cDNA demonstrates that the regulation of expression resides within the coding region of the message. Because the β subunit of the structurally related H,K-ATPase is efficiently expressed in both BSC 40 and MDCK cells, we constructed chimeras between the Na\(^+\)-K- and H,K-β subunit cDNAs to identify nucleotide sequences important for regulation. Our results indicate that while sequences corresponding to the N terminus of the Na\(^+\) pump β subunit have some inhibitory effect on the expression of the chimeras, the stringent regulation of expression observed in epithelial cells requires both 5′ and 3′ coding regions of the β1 message. The ability of polarized epithelial cells to limit the synthesis of Na\(^+\) pump polypeptides while expressing other vaccinia recombinants at high levels suggests that these cells possess a stringent mechanism for the specific translational regulation of a select set of messages.

MATERIALS AND METHODS

Cell Culture—The MDCK cell line used, except where otherwise indicated, is a type II clonal line provided by W. J. Nelson (Stanford University). The MDCK D7 cell line is a type II clonal line provided by G. Ojakian (State University of New York, Brooklyn). The Caco-2 cell line (human colon carcinoma) and the MDCK (ATCC) cell line, a nonclonal descendant of the original cells, were obtained from the American Type Culture Collection (Rockville, MD). The MDBK (Madin Darby bovine kidney) and LLC-PK1 (pig kidney) cells were provided by S. Gluck (Washington University). The BSC 40 and CV1 (African green monkey kidney) cells were provided by C. Rice (Washington University). L929 (mouse connective tissue) and WISH (human amnion) cell lines were provided by R. Schreiber (Washington University). All viral recombinant stockswere prepared and titrated in BSC40 cells. All viral recombinant stocks were prepared and titrated in BSC40 cells.

Analysis of RNA from MDCK cells infected with α1 isoform (8) was subcloned as a full-length NeuI (initiation site/PstI fragment into pTM3 (VVp1). An α1 isoform was created at the initiation site of pTM3 (VVp1) using PCR. The engineered NeuI site in α2 was filled in with the Klenow fragment of DNA polymerase I, in the presence of deoxynucleotides as suggested by the supplier (Promega Corp., Madison, WI), and the NeuI site in pTM3 was blunt-ended with mung bean nuclelease according to the supplier (New England BioLabs, Beverly, MA). The full-length α2 cDNA was then subcloned as a blunt end/EcoRI fragment into pTM3 (VVp2). For the α3 construct (VVp3), an NeuI site was created at the initiation site of the rat α3 cDNA (8) using PCR. The full-length α3 was then subcloned as an NeuI/EcoRI fragment into pTM3. Similarly, an NeuI site was created at the initiation site of the rat β2 cDNA (24) by PCR and subcloned as an NeuI/EcoRI fragment into pTM3 (VVp2). The α1-Δ3′ end construct was created by using PCR to engineer an XhoI site immediately after the termination sequence of α1. The clone was then digested with PstI removing all but the last 4 bp of 3′-untranslated region from the pTM3 α1 construct (VVp1-Δ3′ end). The entire 3′-untranslated region (135 bp) from the pTM3 Sindbis virus structural protein construct was subcloned 3′ of the coding region in the α1-Δ3′ end construct using the engineered PstI resulting in the α1-SinS clone (VVp1-SinS). The Pcr-generated PstI site in α1 was also used to ligate in frame the engineered 3′ (in α2 and α3) and 5′ (in α1) restriction sites in the Na\(^+\) pump β1 coding sequence of pTM3Δ1 between base pairs 234–239. The 5′ portion of the β subunit (Aur I/AflI fragment) containing the sequences for the cytoplasmic and transmembrane domains was then subcloned into the pTM3 H,K\(^{\alpha}\) construct replacing the corresponding sequences with those of β1. The reverse β chimera was constructed in a similar fashion. An AflII/NcoI fragment from pTM3Δ1, containing the sequences for the extracellular domain, was subcloned into the pTM3 H,K\(^{\alpha}\) construct replacing the corresponding sequences. The fidelity of all PCR products was verified by sequencing. The VVGLUT1 and VVGLUT4 recombinant viruses were provided by M. Mueckler (Washington University). The VVsinsS (Sindbis virus structural proteins cassette) and vTF 7.3 (TT7 RNA polymerase) recombinant viruses were provided by C. Rice.

Homologous recombination of cDNAs in the pTM3 vector was carried out in CV1 cells infected with wild-type vaccinia virus (strain WR) at a multiplicity of infection (m.o.i.) of 0.1 plaque-forming units/cell. After infection for 1 h, the cells were transfected with a calcium phosphate precipitate containing 1 μg of wild-type vaccinia DNA, 5 μg of the appropriate pTM3 construct, and 14 μg of sheared calf thymus DNA. After 36–48 h, the viral stocks were harvested and plaque-purified through three rounds of mycophenolic acid selection using BSC 40 cells. Plaques from the final round of selection were amplified and tested for their ability to direct the synthesis of the appropriate protein in BSC 40 cells. All viral recombinant stocks were prepared and titered in BSC 40 cells. Infections using the recombinant viral stocks were carried out at 25°C in phosphate-buffered saline supplemented with 1% FBS and 1 mg MgCl\(_2\). After 1 h, the virus was removed and normal cell culture medium added back. The cells were then placed at 37°C in a 5% CO\(_2\) atmosphere. After 7 h, the cells were harvested. To ensure optimal infectivity of the polarized cell lines, the polarized cultures were treated with 0.5 mg/ml (without FBS) containing 30 mM EDTA for 30 min at 37°C in a 5% CO\(_2\) atmosphere prior to infection (25).

Antibodies—The α1 subunit of the Na,K-ATPase was identified with a polyclonal antibody raised against the γ-terminal region of α1 (γ-terminal synthetic peptide) and a polyclonal antibody raised against the rat α1γ holoenzyme [poly-α1]. The poly-α1 antibody was also used to identify the β1 subunit. A second polyclonal β1 antibody (provided by A. Askari, Medical College of Ohio, Toledo) was used in the immunoprecipitation experiments. The α2 subunit was identified using the monoclonal antibody MAb2 provided by K. Swain (Massachusetts General Hospital). The α3 subunit was identified with a polyclonal antibody raised against the γ-terminal region of α3 (α3 synthetic peptide). The β2 subunit was identified with an affinity purified polyclonal β2 antibody purchased from United Biotechnology (Lake Placid, NY). The GLUT 1 polypeptide was identified using a monoclonal antibody (37) provided by M. Mueckler. The GLUT 4 polypeptide was identified with the polyclonal antibody R820 provided by D. James (University of Queensland, Australia). The Sindbis virus capsid protein, used to monitor the expression of the Sindbis virus structural proteins, was identified with a polyclonal antibody provided by C. Rice. TT7 RNA polymerase
Regulation of Na\textsuperscript{+} Pump Expression in Epithelial Cells

was identified using a polyclonal antibody provided by W. Studier (Brooks Laboratory). The H,K-ATPase \( \beta \) subunit was identified with either a monoclonal antibody that recognizes the N term-

in (2/2E6) or the C term (2G11) of the polypeptide (provided by G. Forte, University of California, Berkeley).

SDS-PAGE, Immunobots and Immunoprecipitations—Protein content of whole cell homogenates was determined using bicinchonic acid/copper sulfate solution as described by the supplier (Pierce). Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose (Hybond C Plus, Amersham Corp.). Nitrocellulose blots were blocked in Blotto (5\% \( w/ v \) nonfat dry milk, 0.1\% sodium azide in 150 m\( M \) NaCl, 25 m\( M \) HEPES, pH 7.4 (HBS)) overnight at 4\( ^\circ \)C. The indicated specific primary antibodies in 1\% Blotto were bound at 37 \( ^\circ \)C for 2 h on a rocking table. After three 10-min washes in HBS containing 1\% Triton X-100, 1-2 \( \mu l \) of 1\% labeled goat anti-mouse or goat anti-rabbit secondary antibody were added. Blots were incubated for an additional hour at 37 \( ^\circ \)C, washed as above, and exposed for autoradiography. For immunoprecipitations, \([35\text{S}]\)methionine/cysteine-labeled cells were harvested by scraping, and the total protein content determined. For each immunoprecipitation, 100 \( \mu g \) of protein were solubilized in HBS containing 1\% SDS. The supernatant was then centrifuged for 10 min at 4 \( ^\circ \)C in a microcentrifuge. For each sample, 0.8 ml of the resulting supernatant was layered onto a linear 15-50\( \%\) (w/v) sucrose gradient. The gradient solution contained 0.5 m\( M \) KCl, 20 m\( M \) HEPES, pH 7.5, 5 m\( M \) MgCl\(_2\), 1 m\( M \) dithiothreitol, 100 \( \mu g/\mu l \) cycloheximide, 0.5\% (v/v) Triton X-100, and 100 units/ml RNasin (Promega Corp.) and solubilized on ice for 10 min. The lysate was then centrifuged for 10 min at 4 \( ^\circ \)C and the supernatant, containing 1\% SDS at 65 \( ^\circ \)C and exposed for autoradiography. Autoradiograms were quantitated by densitometry using an Epson ES-800C scanner interfaced to a Macintosh 7100 computer. Intensity of the bands was determined using the NIH Image 1.52 software. Multiple exposures were analyzed to ensure that the signals were within the linear range of the film.

RESULTS

Na,K-ATPase Activity—Na,K-ATPase activity was determined in whole cell preparations as described previously (26). Specific hydrolysis was followed by the release of \( ^3\text{P} \) from 1\%-3\%P\( \text{IATP.} \) 50 \( \mu g \) of protein was preincubated for 30 min at 37 \( ^\circ \)C in a reaction medium containing 120 m\( M \) NaCl, 30 m\( M \) KCl, 3 m\( M \) MgCl\(_2\), 0.2 m\( M \) HEPES, pH 7.4, 1 m\( M \) Tris-\( HCl \), 30 m\( M \) Tris-H\( \text{HGA} \), 30 m\( M \) Tris-\( H\text{TGA} \), 30 m\( M \) Tris-\( H\text{UTA} \), 30 m\( M \) Tris-\( H\text{DTA} \), 30 m\( M \) Tris-\( H\text{TDA} \), pH 7.4, in the absence or presence of 3 m\( M \) or 1 m\( M \) ouabain. The assay was started with the addition of ATP (3 m\( M \) final concentration) at 37 \( ^\circ \)C and stopped 1 h later with 5% trichloroacetic acid. The difference in ouabain affinity between the endogenous and rodent Na,K-ATPase (27) was used to determine the activity of the virally directed Na,K-ATPase in BSC 40 cells.

RNA Blot Analysis—RNA was isolated from cells using RNeasyTM according to the suppliers protocol (Tel-Test, Inc., Friendswood, TX). RNA content was quantitated spectrophotometrically. Equal amounts of RNA were separated by electrophoresis in a formaldehyde gel and transferred overnight by capillary action to nitrocellulose (Biotrans, ICN). The blots were UV cross-linked, prehybridized, and hybridized according to Sambrook et al. (28). Labelled probes corresponding to the specific messages were made from cDNA fragments using random primers. For the virally directed messages were made from a fragment corresponding to the eucaryomycarditis virus leader sequence that is added to the 5\' end of each cDNA in the pTM3 vector. Blots were washed in 0.1 \( \times \) SSC containing 1\% SDS at 65 \( ^\circ \)C and exposed for autoradiography. Autoradiograms were quantitated by densitometry using an Epson ES-800C scanner interfaced to a Macintosh 7100 computer. Intensity of the bands was determined using the NIH Image 1.52 software. Multiple exposures were analyzed to ensure that the signals were within the linear range of the film.

RESULTS

Na,K-ATPase vaccinia recombinants were produced using the rat cDNAs encoding the \( \alpha \) and \( \beta \) subunits. For all constructs, the cDNAs were inserted into the vaccinia shuttle vector (pTM3) at the translation initiation site. This removed 9\% untranslated sequences and resulted in recombinants that are directed by the vaccinia early-late RNA polymerase gene under the control of a vaccinia early-late promoter. In this fashion, production of T7 RNA polymerase early in the infective cycle drives the transcription of the gene under control of the T7 promoter. This double viral expression system was used to circumvent the relatively poor efficiency of transient transfection of the epithelial cell lines (results not shown). As shown in Fig. 1, when cells are infected with the T7 RNA polymerase virus, high levels of the polymerase are produced. However, coinfection of polarized MDCK cells with the \( \alpha \) or \( \beta \) recombinants failed to induce the synthesis of the appropriate Na\textsuperscript{+} pump polypeptides. This is not due to the inability of the double recombinant system to direct the expression of exogenous polypeptides in the MDCK cells. While virally directed Na\textsuperscript{+} pump polypeptides are not produced, similarly constructed viral recombinants containing the cDNAs for the glucose transporters (GLUT1 and GLUT4) and Sindbis virus structural proteins are able to induce the expression of the appropriate proteins. The lack of virally directed Na\textsuperscript{+} pump expression does not arise from disproportionate levels of T7 RNA polymerase since an equivalent amount of the enzyme is produced in all infections (Fig. 1). Although the Na\textsuperscript{+} pump recombinant viruses fail to promote the expression of the polypeptides in MDCK cells, these viruses are competent in
directing the synthesis of the appropriate proteins in other cell lines. Infection of the fibroblast-like BSC 40 cell line with the recombinant viruses coding for GLUT1, GLUT4, and Sindbis virus capsid proteins, as well as the α1 and β1 polypeptides, results in the production of the proper protein products (Fig. 2A). Moreover, the virally produced Na⁺ pump polypeptides are catalytically active. As shown (Fig. 2B), the coinfection of BSC 40 cells with the α1 and β1 viral recombinants results in an increase in Na,K-ATPase activity as compared with the uninfected or GLUT1, GLUT4 coinfected cells. Attempts to vary the multiplicities of infection (m.o.i.) or the combination of recombinants used to infect MDCK cells had no effect on virally induced Na⁺ pump expression (results not shown). In addition to the vaccinia viruses using the T7 RNA polymerase promoter, a second vaccinia expression system, not dependent upon T7 RNA polymerase, was used. These α1 and β1 recombinant vaccinia viruses, which utilize a synthetic early-late promoter, were able to direct the production of α1 and β1 polypeptides in BSC 40 cells. However, the recombinant viruses failed to produce Na⁺ pump polypeptides in the MDCK cells (results not shown). Thus, the failure of the recombinant vaccinia viruses to induce the expression of Na⁺ pump polypeptides in MDCK cells is not a result of the different promoters or vector-derived sequences.

To test if the inability to produce vaccinia-directed Na⁺ pump polypeptides is unique to MDCK cells, several other cell

---

**FIG. 1. Immunoblot analysis of virally infected MDCK cells.** Cell homogenates (30 μg/lane) from polarized MDCK cells infected at a multiplicity of infection (m.o.i.) of 10 with the indicated vaccinia recombinants (VVα1, VVβ1, VVSinS, VGGLUT1, VGGLUT4, vTF 7.3) were analyzed using the following antibodies, α1 (α1 synthetic peptide antibody), Rα1 (poly-αA antibody), β1 (poly-αA antibody), SinS (Sindbis virus capsid protein antibody), GLUT1 (37.6 antibody), GLUT4 (R820 antibody), and T7 (T7 RNA polymerase antibody). The poly-αA antibody recognizes only the rodent Na⁺ pump polypeptides. To demonstrate the specificity of the antibodies, protein from rat brain and rat kidney (10 μg/lane) is shown.

**FIG. 2. Immunoblot analysis and Na,K-ATPase activity of virally infected BSC 40 cells.** A, cell homogenates (30 μg/lane) from BSC 40 cells infected at an m.o.i. of 10 with the indicated vaccinia recombinants (VVα1, VVβ1, VVSinS, VGGLUT1, VGGLUT4, vTF 7.3) were analyzed using the following antibodies, α1 (α1 synthetic peptide antibody), Rα1 (poly-αA antibody), β1 (poly-αA antibody), SinS (Sindbis virus capsid protein antibody), GLUT1 (37.6 antibody), GLUT4 (R820 antibody), and T7 (T7 RNA polymerase antibody). The poly-αA antibody recognizes only the rodent Na⁺ pump polypeptides. To demonstrate the specificity of the antibodies, protein from rat brain and rat kidney (10 μg/lane) is shown. B, BSC 40 cells uninfected or infected at an m.o.i. of 10 with the indicated vaccinia recombinants (VVα1, VVβ1, VVSinS, VGGLUT1, VGGLUT4, vTF 7.3) were assayed for Na,K-ATPase activity. Coinfection of α1 and β1 recombinants results in an increase in Na,K-ATPase activity as compared with uninfected or GLUT1, GLUT4 coinfectected cells.
lines were surveyed for their ability to support the synthesis of the α1 and β1 polypeptides. Infection of fibroblast-like cells derived from monkey kidney (CV1), mouse connective tissue (L929), or human amnion (Wish cells) with viral recombinants containing the Na,K-ATPase α1 and β1 isoforms, the capsid protein of the Sindbis virus, and T7 RNA polymerase all result in the production of the appropriate proteins (Table I). However, the polarized epithelial cell lines, Caco-2 (human colon carcinoma), Madin Darby bovine kidney (MDBK), LLC-PK1 (pig kidney), as well as several other clones of MDCK cells fail to synthesize vaccinia-directed α1 and β1 polypeptides, yet they efficiently express the other virally directed proteins (Table I). Thus, the phenotype of the cells appears to correlate with their ability to support the expression of the Na⁺ pump recombinants.

Morphologically, a major difference among the two groups of cell lines is the ability to polarize in culture. The epithelial cells form junctional complexes that effectively separate the apical and basolateral surfaces of the cells. The formation of the junctional complexes in MDCK cells is dependent on the establishment of specific cell-cell contacts initiated by the Ca²⁺-dependent, cell adhesion protein, E-cadherin (30). MDCK cells that have not had time to establish the proper cell-cell contacts or are grown in low Ca²⁺ medium maintain a nonpolarized phenotype. To determine if the lack of expression of Na⁺ pump polypeptides is a consequence of cell polarization, MDCK cells were infected either immediately after plating or at confluence when grown in low Ca²⁺ medium. As the polarized cells, the nonpolarized MDCK cells did not express the recombinant α1 and β1 polypeptides indicating that the lack of expression is not dependent on the polarized state of the cells (data not shown). However, similar to the polarized cells, the nonpolarized MDCK cells efficiently express the virally directed T7 RNA polymerase and the Sindbis capsid protein.

Because the α1 and β1 subunits are the only Na⁺ pump isoforms expressed in the MDCK and BSC 40 cells (31), we wondered if the expression of the Na⁺ pump α2, α3, and β2 isoforms are similarly regulated. Infection of BSC 40 cells with α2, α3, and β2 viral recombinants results in the appropriate protein expression (Fig. 3A). However, infected MDCK cells limit the expression of the α2, α3, and β2 polypeptides (Fig. 3B), although not as completely as the α1 and β1 recombinants. At high multiplicities of infection the α2 recombinant virus is able to direct the expression of a small amount of α2 protein in the MDCK cells. Likewise, attempts to express the α3 isoform show that while MDCK cells support the synthesis of the α3 polypeptide, its expression is substantially lower than in BSC 40 cells (Fig. 3). In Fig. 3B, the protein band seen in the uninfected MDCK lane does not correspond to α3. This cross-reacting protein runs as the upper band of the doublet while the specific α3 band runs as the lower protein band in the infected samples. While β2 expression is seen at low multiplicities of infection in MDCK cells, its expression is substantially less than that in BSC 40 cells (Fig. 3). As shown before, the Sindbis virus capsid protein is efficiently expressed in the BSC 40 and MDCK cells emphasizing the ability of the MDCK cells to restrict the expression of the α2, α3, and β2 subunits (Fig. 3). Therefore, while expression of all the Na⁺ pump isoforms appears to be specifically limited in MDCK cells, there are clearly differences in the extent of the regulation.

### Table I

| Cell line (morphology) | T7 RNA polymerase | Sindbis capsid protein | α1 polypeptide | β1 polypeptide |
|------------------------|------------------|-----------------------|----------------|----------------|
| BSC 40 (fibroblast-like) | Yes              | Yes                   | Yes            | Yes            |
| CV1 (fibroblast-like)   | Yes              | Yes                   | Yes            | Yes            |
| L929 (fibroblast-like)  | Yes              | Yes                   | Yes            | Yes            |
| WISH (fibroblast-like)  | Yes              | Yes                   | Yes            | Yes            |
| MDCK (epithelial-like)  | Yes              | Yes                   | Yes            | Yes            |
| MDCK D7 (epithelial-like) | Yes       | Yes                   | Yes            | No             |
| MDCK (ATCC) (epithelial-like) | Yes     | Yes                   | Yes            | No             |
| Caco-2 (epithelial-like) | Yes              | Yes                   | Yes            | No             |
| MDBK (epithelial-like)  | Yes              | Yes                   | Yes            | No             |
| LLC-PK1 (epithelial-like) | Yes              | Yes                   | Yes            | No             |

### Figure 3

**A** Immunoblot analysis of α2, α3, and β2 virally infected BSC 40 and MDCK cells. Protein from cell homogenates (30 µg/lane) of BSC 40 (A) and polarized MDCK cells (B) infected at an m.o.i. of 10 or 40 with the indicated recombinant viruses (VVα2, VVα3, VVβ2) were analyzed using the indicated antibodies. The viruses for the Sindbis structural proteins and T7 (VVSinS, vTF 7.3) were used at an m.o.i. of 10 in each infection. Antibodies: α2 (Mcβ2), α3 (α3 synthetic peptide antibody), β2 (UBI β2 antibody), SinS (Sindbis virus capsid protein antibody), and T7 (T7 RNA polymerase antibody). To demonstrate the specificity of the antibodies, protein from rat brain (20 µg) is shown.
Fig. 4. RNA hybridization and immunoblot analysis of infected MDCK cells at increasing m.o.i. A, RNA was isolated from polarized MDCK cells infected with vaccinia recombinants (VVα1, VVβ1, VVSinS, vTF 7.3) at an m.o.i. of 1, 5, 10, and 25. 5 μg/lane of RNA was separated on formaldehyde gels and probed with the indicated cDNAs. For comparison, rat kidney RNA (5 μg) is shown. B, proteins from cell homogenates (30 μg/lane) of polarized MDCK cells infected at an m.o.i. of 1, 5, 10, and 25 with the indicated vaccinia recombinants (VVα1, VVβ1, VVSinS, vTF 7.3) were analyzed using the following antibodies: α1 (α1 synthetic peptide antibody), (R)α1 (poly-αA antibody), β1 (poly-αA antibody), SinS (Sindbis virus capsid protein antibody), and T7 (T7 RNA polymerase antibody). The poly-α1 antibody recognizes only the rodent Na⁺ pump polypeptides. To demonstrate the specificity of the antibodies, protein from rat kidney (10 μg) is shown.

To determine if the inability of the MDCK cells to express the vaccinia-directed α1 and β1 polypeptides is due to a lack of transcription from the recombinant viruses, RNA was isolated from MDCK cells coinfected with the recombinant viruses coding for the α1, β1, and Sindbis structural proteins. As shown in Fig. 4A, at increasing multiplicities of infection all three mRNAs are present at significantly higher levels than in uninfected cells. Although not visible in Fig. 4, both endogenous α1 and β1 mRNAs are present in longer exposures. The broad profile of the virally directed messages represents initiation from an upstream promoter and readthrough of the termination sequence. Even at the lowest m.o.i. more α1 and β1 mRNA is present in the infected samples than endogenously expressed in these cells. Moreover, as shown in Fig. 4B, the MDCK cells express Sindbis capsid protein at an m.o.i. of 1, whereas they fail to synthesize α1 and β1 polypeptides over endogenous background even though the level of message for both Na⁺ pump subunits at an m.o.i. of 25 is greater than that of the Sindbis capsid protein at an m.o.i. of 1 or 5.

The apparent lack of expression in the virally infected MDCK cells may be a result of the rapid degradation of the Na⁺ pump polypeptides. To determine if Na⁺ pump polypeptides are being synthesized and rapidly degraded, proteins from MDCK cells infected with either the α1 or β1 recombinant viruses were labeled with [35S]methionine/cysteine for 10 min and immediately immunoprecipitated with either an α1 or β1 antibody, respectively. While the virally directed α1 and β1 subunits are synthesized in infected BSC 40 cells, the α1 and β1 recombinant viruses are unable to direct the synthesis of the appropriate polypeptides in MDCK cells (Fig. 5A). In a similar set of experiments, proteins from cells infected with both the α1 and β1 viruses were labeled with [35S]methionine for 30 min, chased for 15 or 60 min, and immunoprecipitated with the α1-specific antibody. While infected BSC 40 cells efficiently produce α1 and β1 polypeptides, there is little α1 expression in infected MDCK cells (Fig. 5B). Comparison of the levels of α1 and β1 polypeptides in MDCK and BSC 40 cells demonstrates the vastly disproportionate level of virally induced α1 and β1 expression. After a 15-day exposure there is no β1 and only a small amount of α1 polypeptide in the MDCK samples, while a 2-h exposure of BSC 40 samples shows a substantial amount of virally induced α1 and β1 polypeptides (Fig. 5B). Furthermore, there is no decrease in the level of α1 polypeptide expression between the 15- and 60-min chase periods in both infected cell lines. Although we cannot completely discount the possibility that the Na⁺ pump polypeptides are promptly degraded, it seems unlikely since little labeled α1 polypeptide can be immunoprecipitated from the infected MDCK cells. Taken together with the large amount of virally induced Na⁺ pump mRNA, these data strongly suggest that the regulation of Na⁺ pump synthesis is primarily at the level of translation.

Because the α1 and β1 viral recombinants are able to direct the synthesis of the appropriate messages, we examined if the lack of translation in MDCK cells was a result of the inability of these messages to initiate translation. Fig. 6A shows a representative absorbance profile of sucrose gradients containing cytoplasmic extracts from uninfected BSC 40 and MDCK cells. As shown, the RNA separates into specific fractions of the gradient (Fig. 6). In Fig. 6A, fractions 1–5 of the sucrose gradient contain the ribosomal subunits and monosomes, while the bulk of the polysomes are found in fractions 6–11 (29). For each gradient, equivalent volumes of the fractions were transferred to nitrocellulose. To detect the endogenous messages the blots were probed with the Na⁺/K-ATPase α1 and β1 cDNAs. The blots were quantitated and the amount of signal in each fraction plotted as a percentage of the total signal for that message. As shown in Fig. 6B, the endogenous α1 and β1 messages from the uninfected BSC 40 and MDCK cells are distributed throughout the gradient; however, in all cases the majority of the message is in the fractions containing the polysomes. In BSC 40 cells the α1 and β1 messages sediment to sharp peaks; the α1 message is associated with larger polysomes consistent with its greater size. In MDCK cells the α1 and β1 messages are more broadly distributed over several of the polysome-containing fractions, possibly a result of the substantially higher levels of endogenous α1 and β1 messages expressed in MDCK cells as compared with BSC 40 cells. However, as in BSC 40 cells the α1 message is present in the fractions containing the larger polysomes as compared with the β1 message.

To determine if the virally directed α1 and β1 messages from infected BSC 40 and MDCK cells are present in the polysome-containing fractions of the gradient (Fig. 7), blots were probed with the encephalomyocarditis virus leader sequence present at the 5’ end of the recombinant message. Using this probe it is possible to distinguish the virally directed message from the endogenous message. As shown in Fig. 7B, it appears that the expression of the virally induced transcripts results in a broader distribution of the message through the gradient. The appearance of the virally induced message in the lower density
fractions containing ribosomal subunits and monosomes is not surprising given the high level of expression. However, although present in lower density fractions, in all cases a substantial portion of the virally directed messages are present in the fractions containing polysomes (Fig. 7B). The virally directed α1 and β1 messages from MDCK cells have the same general distribution as the virally directed GLUT4 mRNA from MDCK cells and the α1 and β1 mRNA from BSC 40 cells. Although in MDCK cells the virally directed α1 and β1 mes-
nucleotide sequences within the coding region of the message. The Na,K-ATPase belongs to a widely distributed class of E_{1}E_{2} or P-type cation transporting proteins that includes the Ca-ATPase of the sarcoplasmic reticulum and plasma membrane (36, 37), the H,K-ATPase from gastric mucosa (38), the H-ATPase of plants and fungi (39), and the bacterial Cd- and K-ATPases (40). Comparisons of nucleotide sequences show that the Na,K-ATPase is most similar to the H,K-ATPase. Both enzymes consist of an α and β subunit. The α subunit cDNAs share approximately 64% homology, whereas the β subunit cDNAs share 52% nucleotide identity. Because the H,K-ATPase cDNAs exhibit high levels of homology with the Na,K-ATPase, we investigated the expression of H,K-ATPase α and β polypeptides. As with the Na\textsuperscript{+} pump subunits, both the H,K-ATPase α and β viruses direct the expression of the appropriate polypeptides in BSC 40 cells (Fig. 10A). However, similar to the Na,K-ATPase α subunit, the expression of the H,K-ATPase α subunit is stringently regulated in the MDCK cells. In contrast, the H,K-ATPase β subunit is efficiently expressed in the MDCK cells (Fig. 10B). Taking advantage of the ability of the H,K-ATPase β recombinant virus to direct the expression of the β polypeptide in MDCK cells, we constructed chimeras between the H,K- and Na,K-ATPase β subunit cDNAs. As expected, the recombinant viruses for both chimeras direct the expression of the appropriate polypeptides in BSC 40 cells (Fig. 11A). Likewise in MDCK cells, the H/K-Na/K β polypeptide is expressed at the same level as the virally directed wild-type H,K-ATPase β subunit (Fig. 11B). Because the H/K-Na/K β chimera contains the 3′-untranslated region of the Na,K-ATPase β subunit, its expression confirms that this region is not responsible for regulating polypeptide expression. This observation is similar to previous results demonstrating that the 3′-untranslated region of the Na,K-ATPase α subunit does not influence expression (Fig. 9). However, the expression of the Na/K-H/K β recombinant is reduced by approximately 40% as compared with the wild-type H,K-ATPase β recombinant in MDCK cells, yet these recombinants are equivalently expressed in BSC 40 cells (Fig. 11). This inhibition is less than the stringent regulation of the Na\textsuperscript{+} pump β1 and indicates that, while sequences within in the 5′ end of the β1 coding region may have an effect on β1 expression in MDCK cells, the strict translational regulation in the epithelial cells requires the presence of both the 5′ and 3′ coding regions of the message.

**DISCUSSION**

Using a vaccinia virus expression system we have shown that cultured epithelial cells have the ability to limit the synthesis of Na\textsuperscript{+} pump polypeptides while efficiently expressing other virally directed proteins. Although the recombinant viruses fail to direct the expression of Na,K-ATPase α1 and β1 polypeptides in MDCK epithelial cells, these viruses are capable of directing the expression of the appropriate polypeptides in fibroblast-like cell lines. The failure to express Na\textsuperscript{+} pump polypeptides in MDCK cells is not a consequence of the polarized state of the cells. Nonpolarized MDCK cells, as well as fully differentiated cells, limit Na\textsuperscript{+} pump synthesis. Moreover, the regulation of α1 and β1 synthesis in epithelial cells is not restricted to these endogenously expressed isoforms. MDCK cells also limit the synthesis of the virally directed α2, α3, and

![Fig. 8. In vitro translation of RNA from infected BSC 40 and MDCK cells.](http://www.jbc.org/)

RNA was isolated from BSC 40 cells infected at an m.o.i. of 5 and polarized MDCK cells infected at an m.o.i. of 25. RNA from each sample was either translated without further manipulation or size-fractionated on sucrose gradients to enrich the β1 message. For each sample, RNA (3 μg) was incubated in rabbit reticulocyte lysate containing dog pancreatic microsomal membranes and [35S]methionine. Samples from each reaction were separated by SDS-PAGE.
b isoforms of the Na,K-ATPase as well as the α subunit of the homologous H,K-ATPase. As with the α1 and β1 isoforms, the Na,K-ATPase α2, α3 and β2 and the H,K-ATPase α and β viral recombinants directed the expression of the appropriate polypeptides in BSC 40 cells. The inability to express Na\(^+\) pump polypeptides in cultured epithelial cells is not a consequence of the vaccinia expression system. Attempts to express various α and β constructs in MDCK or Caco-2 epithelial cells using recombinant retroviruses or conventional protocols for stable transfection also fail to produce Na\(^+\) pump polypeptides.  

In various tissues and cell types, Na\(^+\) pump expression is regulated through a combination of transcriptional, translational, and post-translational mechanisms (30). Pulse-chase experiments of infected MDCK cells showed no appreciable protein synthesis or turnover of α1 and β1 polypeptides demonstrating that the lack of expression was not a result of the rapid degradation of the proteins. Likewise, analysis of RNA isolated from MDCK cells infected with increasing multiplicities of infection of recombinant α1, β1, and Sindbis capsid viruses demonstrates that all three mRNAs are produced at high levels (Fig. 4). Thus the inability of the MDCK cells to express the vaccinia-directed α1 and β1 polypeptides is in part due to the specific regulation of the translation of the α1 and β1 messages. This regulation, however, does not appear to be due to the inability of the virally directed α1 and β1 messages to initiate translation since both messages appear to be associated with polyribosomes in MDCK cells as other messages that are translated (Figs. 6 and 7). Numerous studies have shown that the regulation of gene expression at the level of translation is an important modulator of protein synthesis (34, 35, 41). The binding of regulatory proteins, specific sequences, or secondary structures within the 5'-untranslated regions of mRNAs can influence the rate of translation (42–44). For example, translation of ornithine decarboxylase in reticulocyte lysate and cultured mammalian cells is notably increased by removal of the 5' leader sequence. This inhibition is largely attributed to the formation of a stem loop structure in the 5'-UTR (35). For the Na,K-ATPase α1 isoforms of the Na,K-ATPase as well as the α subunit of the homologous H,K-ATPase. As with the α1 and β1 isoforms, the Na,K-ATPase α2, α3 and β2 and the H,K-ATPase α and β viral recombinants directed the expression of the appropriate polypeptides in BSC 40 cells. The inability to express Na\(^+\) pump polypeptides in cultured epithelial cells is not a consequence of the vaccinia expression system. Attempts to express various α and β constructs in MDCK or Caco-2 epithelial cells using recombinant retroviruses or conventional protocols for stable transfection also fail to produce Na\(^+\) pump polypeptides.  

In various tissues and cell types, Na\(^+\) pump expression is regulated through a combination of transcriptional, translational, and post-translational mechanisms (30). Pulse-chase experiments of infected MDCK cells showed no appreciable protein synthesis or turnover of α1 and β1 polypeptides demonstrating that the lack of expression was not a result of the rapid degradation of the proteins. Likewise, analysis of RNA isolated from MDCK cells infected with increasing multiplicities of infection of recombinant α1, β1, and Sindbis capsid viruses demonstrates that all three mRNAs are produced at high levels (Fig. 4). Thus the inability of the MDCK cells to express the vaccinia-directed α1 and β1 polypeptides is in part due to the specific regulation of the translation of the α1 and β1 messages. This regulation, however, does not appear to be due to the inability of the virally directed α1 and β1 messages to initiate translation since both messages appear to be associated with polyribosomes in MDCK cells as other messages that are translated (Figs. 6 and 7). Numerous studies have shown that the regulation of gene expression at the level of translation is an important modulator of protein synthesis (34, 35, 41). The binding of regulatory proteins, specific sequences, or secondary structures within the 5'-untranslated regions of mRNAs can influence the rate of translation (42–44). For example, translation of ornithine decarboxylase in reticulocyte lysate and cultured mammalian cells is notably increased by removal of the 5' leader sequence. This inhibition is largely attributed to the formation of a stem loop structure in the 5'-UTR (35). For the Na,K-ATPase α1 isoforms of the Na,K-ATPase as well as the α subunit of the homologous H,K-ATPase. As with the α1 and β1 isoforms, the Na,K-ATPase α2, α3 and β2 and the H,K-ATPase α and β viral recombinants directed the expression of the appropriate polypeptides in BSC 40 cells. The inability to express Na\(^+\) pump polypeptides in cultured epithelial cells is not a consequence of the vaccinia expression system. Attempts to express various α and β constructs in MDCK or Caco-2 epithelial cells using recombinant retroviruses or conventional protocols for stable transfection also fail to produce Na\(^+\) pump polypeptides.  

In various tissues and cell types, Na\(^+\) pump expression is regulated through a combination of transcriptional, translational, and post-translational mechanisms (30). Pulse-chase experiments of infected MDCK cells showed no appreciable protein synthesis or turnover of α1 and β1 polypeptides demonstrating that the lack of expression was not a result of the rapid degradation of the proteins. Likewise, analysis of RNA isolated from MDCK cells infected with increasing multiplicities of infection of recombinant α1, β1, and Sindbis capsid viruses demonstrates that all three mRNAs are produced at high levels (Fig. 4). Thus the inability of the MDCK cells to express the vaccinia-directed α1 and β1 polypeptides is in part due to the specific regulation of the translation of the α1 and β1 messages. This regulation, however, does not appear to be due to the inability of the virally directed α1 and β1 messages to initiate translation since both messages appear to be associated with polyribosomes in MDCK cells as other messages that are translated (Figs. 6 and 7). Numerous studies have shown that the regulation of gene expression at the level of translation is an important modulator of protein synthesis (34, 35, 41). The binding of regulatory proteins, specific sequences, or secondary structures within the 5'-untranslated regions of mRNAs can influence the rate of translation (42–44). For example, translation of ornithine decarboxylase in reticulocyte lysate and cultured mammalian cells is notably increased by removal of the 5' leader sequence. This inhibition is largely attributed to the formation of a stem loop structure in the 5'-UTR (35). For the Na,K-ATPase α1
Regulation of Na⁺ Pump Expression in Epithelial Cells

Subunit, in vitro analysis of mRNA stability and translational efficiency suggests that sequences within the 5'-UTR of the mRNA may reduce α1 translation (45). Because the 5'-UTR is removed from the viral recombinants, the only potential regulation sites outside the coding region lie in the 3'-UTR. α1 recombinants either lacking the 3'-UTR or possessing the 3'-UTR of the Sindbis capsid or GLUT4 cDNAs have no effect on α1 expression in either MDCK cells or BSC 40 cells. In addition, noncoding sequences added to the induced messages are also not responsible for the lack of synthesis in the epithelial cells. α1 and β1 vaccinia viruses using a different promoter and resulting in messages containing different untranslated sequences also failed to induce the synthesis of polypeptides in MDCK cells. However, these recombinant viruses were efficient in directing α1 and β1 polypeptide synthesis in BSC 40 cells. Therefore it appears that the regulation of Na⁺ pump α1 synthesis must reside within the coding region of the message.

Analysis of chimeras constructed between the Na,K-ATPase β1 and the H,K-ATPase β subunits demonstrate that while the 5' end of the β1 coding sequence has an inhibitory effect on the expression of the Na/K-H/K β chimera, the stringent regulation of the β1 polypeptide in MDCK cells requires both the 5' and 3' coding regions of the message. A possible regulatory mechanism consistent with our results is that MDCK cells specifically modify the Na,K-ATPase mRNA to make it a less efficient transcript for translation. Although rare, the tissue-specific editing of mRNA is not novel. For example, the mRNA coding for apolipoprotein B undergoes a single base pair modification that generates an in frame stop codon that results in the formation of protein that is approximately half the size of the protein encoded by the full-length message. This process is tissue-specific and mediated by sequences that lie within the coding region of the message (46). Moreover, the editing process for apolipoprotein B mRNA exists in several cell lines and a wide variety of tissues that do not normally express the protein, suggesting that this process may function in the regulation of other proteins (46, 47). The possibility that such a mechanism for the regulation of the Na,K-ATPase exists in epithelial cells is an intriguing hypothesis that will require further study.

The regulation of the virally directed mRNAs may reflect control mechanisms important in regulating endogenous Na,K-ATPase activity. While uninfected MDCK cells have approximately 10 times the Na,K-ATPase activity and maintain higher levels of α1 and β1 endogenous message and polypeptide than BSC 40 cells, the de novo synthesis of the endogenous Na,K-ATPase polypeptides is similar in the two cell lines (results not shown). Thus, the steady-state translation of the Na,K-ATPase subunits is reduced in relation to the levels of endogenous message in MDCK cells as compared with BSC 40 cells. There are numerous examples of discordant changes in mRNA levels and protein expression in various systems emphasizing the importance of translational mechanisms in regulating Na⁺ pump activity (47-50).

The central role of the Na⁺ pump in maintaining ionic homeostasis necessitates tight regulation of its expression and activity. While it is clear that maintaining sufficient levels of Na⁺ pump activity is critical for all cells, it appears that overexpression is also regulated. Chronic exposure of HeLa cells to low extracellular K⁺ results in the doubling of the number of plasma membrane pump sites and a coordinate increase in pump-mediated K⁺ uptake. Return to normal K⁺ medium rapidly restores the activity and number of pump sites to normal (52). Similarly, Fambrough and colleagues (53) have shown that up-regulation of Na⁺ pump expression in chicken skeletal muscle by increasing intracellular Na⁺ with veratridine results in an approximately 80% increase in cell surface pump sites within 24–36 h. Restoring intracellular Na⁺ concentrations by removing veratridine and blocking Na⁺ channels with tetrodotoxin results in a rapid return of the Na⁺ pump sites to basal levels (53). Thus, it appears that both under- and overexpression of the pump may have deleterious effects on cellular functions. Therefore, Na⁺ pump expression appears to be precisely regulated to maintain the appropriate levels of activity.

Polarized epithelial cells maintain distinct apical and basolateral plasma membrane domains despite extensive vesicular traffic among intracellular organelles and the plasma membrane. Besides its involvement in maintaining cellular ion gra-
Regulation of Na\textsuperscript{+} Pump Expression in Epithelial Cells

...dients, the Na\textsuperscript{+} pump may also have an important role in vesicular transport. The orderly movement and processing of membrane traffic is dependent on the gradual acidification of the endosomal compartments (54). While the acidification of endosomes requires an ATP-driven proton pump (9, 55), it appears that the Na\textsuperscript{+} pump influences the acidic pH of the early endosomes (56, 57). The recycling of the Na\textsuperscript{+} pump out of the endocytic pathway facilitates the acidification of the endosomal compartment. Given the importance of vesicular pH in regulating endocytic membrane traffic, it is feasible that proper Na\textsuperscript{+} pump activity to maintain appropriate vesicular sorting and trans-...
Translational Regulation of Na,K-ATPase α1 and β1 Polypeptide Expression in Epithelial Cells
Kent K. Grindstaff, Gustavo Blanco and Robert W. Mercer

J. Biol. Chem. 1996, 271:23211-23221.
doi: 10.1074/jbc.271.38.23211

Access the most updated version of this article at http://www.jbc.org/content/271/38/23211

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 57 references, 38 of which can be accessed free at http://www.jbc.org/content/271/38/23211.full.html#ref-list-1