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Transfection Alters Ion Transport in MDCK Cells

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Abstract. In the course of an investigation into the effect of Tamm-Horsfall protein (THP) on ion transport, we performed stable transfection of THP into MDCK cells using the SV40 or the cytomegalovirus (CMV) promoter. As controls, we transfected MDCK cells with an ''empty'' plasmid containing SV40 or CMV promoter but without THP cDNA. In another set of controls, we subjected cells to transfection procedures without DNA (mock transfection). K influx was not altered in cells subjected to mock transfection procedures without DNA, but both ouabain sensitive (OS) and ouabain resistant (OR) components of K influx were diminished in cells transfected with THP cDNA using either SV40 or CMV promoter. However, K influx was also reduced in cells transfected with a control plasmid containing either the SV40 promoter alone, or the CMV promoter alone, without the THP cDNA. Thus, the transport alterations were caused by transfection and not by THP. The reduction in ouabain-sensitive K influx was accompanied by a proportional reduction in the abundance of Na-K pump units as assessed by [^{3}H] ouabain binding. [^{3}H] bumetanide binding, a measure of the number of functioning NaK2Cl cotransporter sites, was reduced pari passu with the reduction in bumetanide-sensitive K influx. These results highlight the possibility that alterations in properties of transfected cells may not be solely due to the presence of transfected protein, but the result of some process associated with transfection itself. Without appropriate controls to evaluate this possibility, results of transfection studies are subject to potentially faulty and misleading interpretation.

Key words: Uromodulin — Cation transport — Na-K-ATPase — Anion-dependent cotransport — MDCK — mTAL

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Introduction

cDNA sequences for several transport proteins have been determined in recent years. A natural step in investigation of the properties of the protein under study is to transfect the cDNA for the protein being studied into cells not naturally endowed with that protein. Conclusions about the function of the protein can then be derived from a comparison of properties of the transfected cells with those of the untransfected cells. Thus, transfections of Na-K-ATPase [7], Na-glucose transporter [12], Na^+/H^+ exchanger [25–27], CFTR [20], MDR [21] and Na-K-2Cl cotransporter [6, 29] into deficient cells have been used to derive conclusions about the properties of those transporters.

Tamm-Horsfall protein (uromodulin, THP) is the most abundant protein in urine of all placental mammals [17]. THP is synthesized and expressed exclusively in the thick ascending limb (TAL) in the kidney [16,17], and has been postulated to be involved in specific transport properties of TAL [8, 11]. To examine the specific transport function for THP, we made a model of THP production by stable transfection of THP cDNA into a well-characterized kidney epithelial cell line (Madin Darby Canine Kidney, MDCK, Ref. 24) and examined cation transport in these cells.

In evaluating the properties of cells transfected with a new protein, it is tempting to attribute the differences between the properties of transfected and nontransfected cells solely to the presence of transfected protein. The possibility of the process of transfection itself altering the transport function has not been examined exhaustively. During the course of investigating the properties of THP by transfection of THP cDNA into MDCK cells, we observed results that suggest that the process of transfection may itself alter transport properties. The results of those investigations form the basis for this report. A

preliminary report of these findings was published as an abstract [15].

Materials and Methods

Cell Culture

MDCK cells, obtained from American Type Culture Collection (ATCC, Bethesda, MD) were grown on plastic in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 1-glutamine 2 mM, penicillin 50 U/ml and streptomycin 50 mg/liter at 37°C. The medium was changed every 2–3 days as required. Transport studies were performed on cells in six well plates when cells were about 90% confluent.

Transfection of MDCK Cells

Full length human uromodulin cDNA was obtained from Cathy Hession [10]. Plasmid pSV2neo [22], containing SV40 promoter and neomycin phosphotransferase cDNA was obtained from ATCC. Expression vectors were prepared as follows: (1) pSV-THP containing SV40 promoter and THP cDNA, (2) pCMV-THP-neo containing CMV promoter, THP cDNA and neomycin phosphotransferase cDNA, (3) pSV2-neo containing SV40 promoter and neomycin phosphotransferase cDNA but no THP cDNA, and (4) pCMV-neo containing CMV promoter and neomycin phosphotransferase cDNA but no THP cDNA. Expression vectors were transfected into MDCK cells by lipofectio [5]. DNA-liposome mixture was prepared by mixing 0.1 ml of liposomes (Lipofectin, Gibco/BRL, Gaithersburg, MD) with 50 mg plasmid DNA containing the appropriate expression vector. This plasmid DNA-liposome mixture was added to medium over confluent monolayers of MDCK cells grown on 10 cm diameter plastic dishes. After 24 hr, the medium was changed and a new medium containing genetecin (G418, 0.8 mg/ml) was applied. Genetecin-resistant colonies were picked 10–14 days later and cloned by limiting dilution. Selected colonies were allowed to expand and THP expression was assessed at mRNA and protein levels as described later.

Nontransfected wild-type MDCK cells were designated MDCK-WT. MDCK cells successfully transfected with pSV-THP (THP cotransfection using the SV-40 promoter and pSV2neo) were designated MD + 1. MDCK cells transected with pCMV-THP-neo (THP transfection using CMV promoter) were designated MD + 2. Cells transfected with pSV2-neo were designated MD − 1. Cells transfected with pCMV-neo (control plasmid containing CMV promoter but no THP) were designated MD − 2. In addition, some MDCK monolayers were subjected to the transfection procedure but without DNA using the lipofection procedure or the calcium phosphate procedure (mock-transfected MDCK).

Expression of THP was assessed by (i) Northern blot, (ii) immunocytochemistry, and (iii) Western blot for THP. RNA was isolated by a single step method using guanidinium thiocyanate-phenol-chloroform extraction [4]. RNA (12 μg/lane) was run on 1% agarose gel, blotted on to a nylon membrane (Genescreen Plus, Dupont-NEN, Boston, MA) and hybridized with a radiolabeled, full length human THP cDNA probe [10]. For direct visualization of THP, transfected cells grown on chamber slides were stained for THP with mouse monoclonal antibodies [16] to human THP (Vectastain, Vector, Burlingame, CA) using an avidin-biotin sandwich immunoperoxidase technique according to the protocol from the manufacturer. For Western-blot studies, cell lysates were prepared by sonication for 20 sec at 4°C in a buffer containing 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM EDTA, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 100 μg/ml phenylmethylsulfonylfluoride, and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Pefabloc-SC, Boehringer Mannheim, Indianapolis, IN). The protein concentration was measured by a bicinchoninic acid assay (BCA protein kit, Pierce Chemical, Rockford, IL). Equal amounts of protein were loaded and run on SDS-PAGE using a 10% separating gel and a 4% stacking gel. The gel was blotted on to a nylon membrane (Immun-Lite, Bio-Rad, Hercules, CA) and probed with a mouse monoclonal antibody against human THP (1:1000, Accurate, Westbury, NY) using a biotinylated anti-mouse goat IgG (1:1000, Pierce), a streptavidin-horse radish peroxidase conjugate (2 μg/ml, Pierce) and a chemiluminescence detection system (Amersham, Arlington Heights, IL).

Transport Studies

Unidirectional K influx was measured using ^{86}Rb as described previously [14]. MDCK were washed 3 times with transport assay buffer (TAB) containing (in mM): NaCl 145, KCl 5, dextrose 5, Na phosphate 2.5, pH 7.4 at 37°C and allowed to equilibrate with this medium for 15 min at 37°C. ^{86}Rb (1 μCi/ml) was added at zero time and the transport was continued in the absence or presence of 0.1 mM ouabain or 20 μM bumetanide. K uptake was terminated at 5 min by washing the cells four times with ice-cold 0.115 M $MgCl_2$. Preliminary results revealed that uptake was linear for at least 10 min. The cells were solubilized wtih 2 ml of 1% sodium dodecyl sulfate with 0.1 N NaOH. Cell extracts (0.5 ml aliquots in duplicate) were counted for Cerenkov radiation in 10 ml water in a scintillation counter. K(Rb) uptake was expressed as μmol.mg protein^{-1}.hour^{-1}. Protein content of cell extracts was measured by the method of Avruch and Wallach [1] which utilizes the intrinsic fluorescence of tryptophan, using bovine serum albumin as the standard. This method gave results which were nearly identical to those obtained by using the BCA assay. The Na-K pump mediated K influx was defined as the difference in K uptake in absence of 0.1 mM ouabain. The NaK2Cl cotransport activity was defined as the difference in K uptake in the presence and absence of 20 μM bumetanide.

[^{3}H]Ouabain Binding

Ouabain binding was measured as described by Manuli and Edelman [19]. The K_i for ouabain in dog kidney is 1 nM [3], which predicts that >99% of the binding sites will be occupied at a ouabain concentration of 100 nM. This prediction has been confirmed in MDCK cells [3]. Cells were incubated with 100 nM [^{3}H] ouabain with and without excess (0.1 mM) unlabeled ouabain for 1 hr at 37°C in TAB. The binding assay was terminated by washing the cells four times rapidly with ice-cold 115 mM $MgCl_2$. Cell extracts were prepared with SDS-NaOH and protein was measured as described above. Radioactivity of cell extract (1 ml) was counted with 10 ml of scintillation cocktail (Formula 989) in a scintillation counter (LS 7000, Beckman Instruments, Fullerton, CA). Nonspecific binding was 10 ± 1% of the total binding and was subtracted from total binding to obtain the specific ouabain binding.

[^{3}H] Bumetanide binding

Cell monolayers were incubated in TAB (transport assay buffer, for full composition see above) with 31 nM [^{3}H] bumetanide (sp. act. 80 Ci/mmol) and various concentrations of unlabeled bumetanide (0 to 2 μM) for 30 min at 37°C. In preliminary studies, we determined that (a) steady state binding was complete by 15 min and (b) the K_d for bumetanide binding in MDCK cells was 0.20 ± 0.02 μM. Therefore, [^{3}H] bumetanide binding studies were performed in the presence of 1 μM bumetanide for 30 min at 37°C. Cells were washed four times rapidly

with ice-cold 0.115 M $MgCl_2$. Cell extracts were prepared with SDS-NaOH and protein was measured as described above. Radioactivity of cell extract (1 ml) was counted with 10 ml of scintillation cocktail (Formula 989) in a scintillation counter (LS 7000, Beckman Instruments, (Fullerton, CA). Nonspecific binding was measured in the presence of 100 μM unlabeled bumetanide and subtracted from total bumetanide binding to obtain specific bumetanide binding. Nonspecific binding was about 50% of the total binding. It was essential to repurify the radioligand frequently (~2–3 months) by HPLC. Failure to repurify the radioligand resulted in an increase in nonspecific binding to unacceptably high levels (>50%), presumably because of breakdown of bumetanide into degradation products.

Solutions Chemicals and Drugs

[^{3}H] ouabain, [^{3}H] bumetanide and ^{86}Rb were purchased from Amersham. Formula 989 was obtained from Dupont-NEN (Boston, MA). Unlabeled bumetanide was a gift from Dr. Peter Sorter, Hoffman La Roche, Nutley, NJ. Culture media were obtained from Gibco/BRL (Grand Island, NY). Flasks, petri dishes, multiwell plates and filter supports were from Falcon Labware (Becton Dickinson, Lincoln Park, NJ). All other reagents were from Sigma (St. Louis, MO), Alfa Products (Ward Hill, MA), or J.T. Baker (through WWR Scientific, Piscataway, NJ).

Presentation of Data

Results are presented as means ± SD (rather than SE) of experiments on different days. Fluxes were measured in at least two wells (duplicate samples per well) on each day, and the results on each day were averaged and expressed as *n* of one. The intra-day variation was <12%. The differences in results between groups were analyzed by unpaired *t* test, and the null hypothesis was rejected when *P* was <0.05. *NS* = not significant.

Results

THP mRNA and Protein Expression

MDCK cells transfected with THP cDNA showed mRNA for THP, whereas untransfected cells did not (Fig. 1). On immunocytochemistry (Fig. 2), MDCK-WT and MD − 1 (cells transfected with control plasmid) showed no staining for THP, whereas positive staining for THP was evident in MD + 1 (cells transfected with THP cDNA using SV40 promoter, not shown) and MD + 2 (cells transfected with THP cDNA using CMV promoter, Fig. 2). Confocal microscopy showed (*data not shown*) that THP was expressed predominantly on the apical cell surface. There was cell to cell variability in expression of THP despite the fact that the cells have been cloned by limiting dilution. By immunoperoxidase staining, about 75% of the cells expressed THP in each of the transfected cell lines. By Western blot, THP produced in the transfected cells was of the same molecular size as the native THP isolated from human urine (Fig. 3), suggesting that the full-length protein was synthesized in the transfected cells.

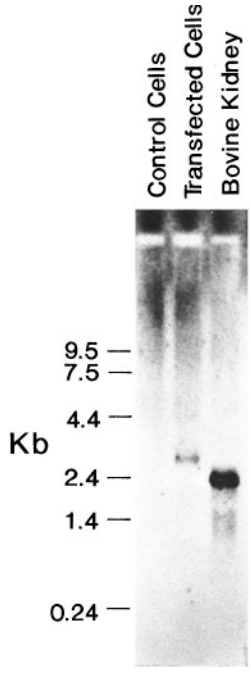

Fig. 1. Northern blot showing expression of THP mRNA in cells transfected with MD + 1 plasmid. Untransfected, wild-type MDCK cells are negative. The THP transcript in transfected cells has a slightly higher molecular weight than that in control bovine kidney due to extra vector sequences contained in the transfection vector.

K Influx

Total K influx, expressed as $\mu mol.mg^{-1}.min^{-1}$ was reduced in the two THP-transfected cell lines MD + 1 (4.33 ± 0.50, n = 6) and MD + 2 (4.00 ± 0.49, n = 6) compared to MDCK-WT (11.23 ± 0.83, n = 11, $P < 0.005$ for both, Fig. 4, top panel). However, total K influx was also diminished in the two cell lines transfected with control plasmid without the cDNA for THP. Thus, total K influx was reduced in MD − 1 cells (3.32 ± 0.25, n = 5, $P < 0.005$), and in MD − 2 cells (4.83 ± 0.25, n = 5, $P < 0.001$) compared to MDCK-WT (11.23 ± 0.83, n = 11). In contrast, mock-transfected cells (cell subjected to the lipofection transfection procedure without DNA) did not show any alteration in K influx compared to MDCK-WT (10.26 ± 0.73 *vs.* 11.23 ± 0.83, NS). Similarly, no alteration in transport was detected in MDCK cells subjected to mock transfection with the calcium phosphate method (10.33 ± 1.29, n = 3, *not shown*). The cells subjected to calcium-phosphate mock transfection were not studied further because, (*a*) no alteration in transport was noted in these cells, and (*b*) other cell lines were transfected with the lipofection procedure. Therefore, only the cells subjected to mock transfection with the lipofection procedure were used as controls in subsequent studies.

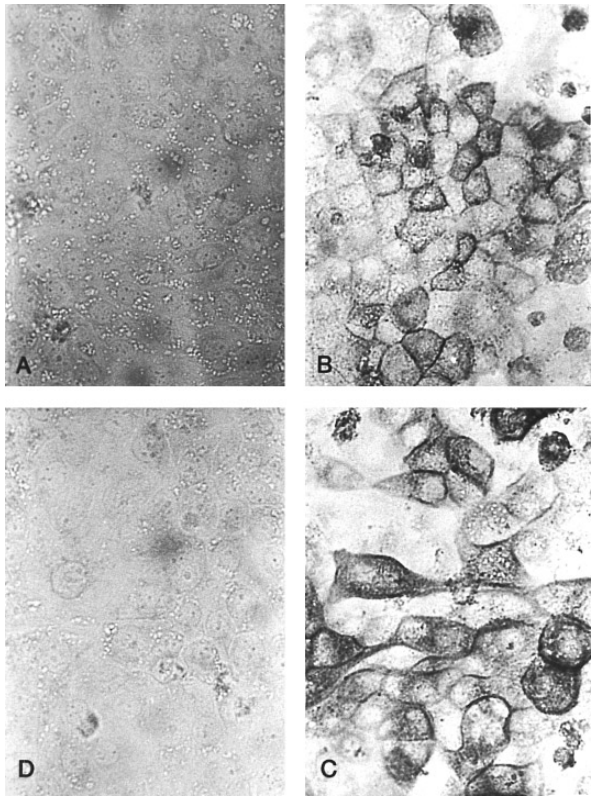

Fig. 2. Immunoperoxidase staining for THP in control and THP producing cell lines. (*A*) Wild type, untransfected MDCK cells, (*B*) MD + 1 (MDCK cells transfected with THP cDNA using SV40 promoter, (*C*) MD + 2 (MDCK cells transfected with THP cDNA using CMV promoter), (*D*) MD − 1 (MDCK cells transfected with a control plasmid containing CMV promoter but no THP cDNA. Cells in panels *B* and *C* show positive staining for THP, whereas those in panels *A* and *D* do not.

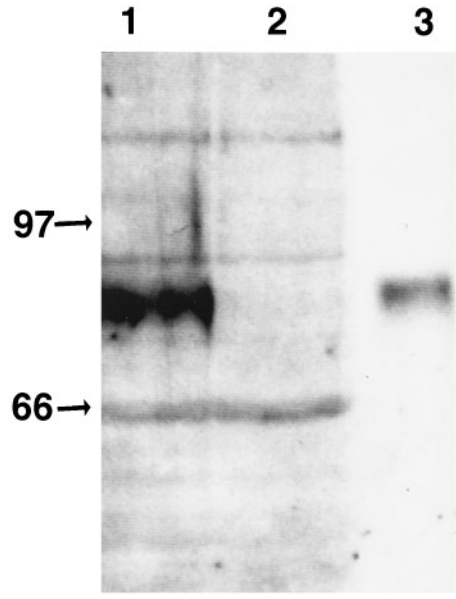

Fig. 3. Western blot showing that the TH protein in transfected cells is of the same molecular size as the native protein isolated from human urine. Lane 1, cell lysate from transfected (MD + 2) cells; lane 2, cell lysate from control (MD − 2) cells; lane 3, THP isolated from human urine (25 ng).

The decrease in total K influx in transfected cells was due to reduction in both the Na-K pump and the NaK2Cl cotransport activities. Ouabain-sensitive component of K influx, mediated by Na-K pump, was significantly decreased in cells transfected with THP using SV40 (MD + 1, 2.67 ± 1.00, n = 6, $P < 0.005$) or CMV promoter (MD + 2, 1.83 ± 0.17, n = 6) compared to MD-WT (5.67 ± 0.75, n = 11, $P < 0.005$, Fig. 4, middle panel). The ouabain-sensitive component of K influx was also reduced in MD − 1 (SV40 promoter alone, 3.83 ± 0.17, n = 6, $P < 0.005$ *vs.* MDCK-WT) and in MD − 2 cells (CMV promoter alone, 1.56 ± 0.15, $P < 0.005$, n = 6 *vs.* MDCK-WT). Mock transfected cells did not show any alteration in ouabain-sensitive K influx (5.89 ± 0.45 *vs.* 5.67 ± 0.83, NS, Fig. 4, middle panel).

Similarly, the bumetanide-sensitive component of K influx was significantly reduced in all four transfected cell lines whether or not they contained THP cDNA (1.50 ± 0.60 for MD + 1, 1.50 ± 0.67 for MD + 2, 1.36 ± 0.61 for MD − 1 and 2.17 ± 0.33 for MD − 2) compared to MDCK-WT (3.84 ± 0.50, but was not reduced in mock transfected cells (3.74 ± 0.2, Fig. 3, bottom panel).

There were no statistically significant differences in residual, ouabain- and bumetanide-resistant K influx among the different groups of transfected, mock transfected and untransfected wild type MDCK cells (*not shown*).

To assess whether the decrease in ouabain-sensitive K influx was due to a decrease in the abundance of Na-K pump units, a decrease in the ion turnover per pump site, or both, we estimated the abundance of Na-K pump units estimated from [^{3}H] ouabain binding. The number of Na-K pump sites, estimated by specific maximum [^{3}H] ouabain binding, was 33 ± 4 × 10^{12} molecules/mg protein in MDCK-WT, in good agreement with results obtained by previous workers [3, 19]. The number of Na-K pump sites was also estimated in two transfected cell lines, one with THP cDNA (MD + 2) and the other with control plasmid but no cDNA for THP (MD − 1). Ouabain binding was significantly diminished in both MD + 2 and MD − 1 (Table 1) compared to the value in MDCK-WT ($P < 0.005$). In contrast, cells subjected to mock transfection procedure using lipofectin did not show any decrease in the ouabain binding (Table 1). The decrease in ouabain binding was proportional to the decrease in ouabain sensitive flux, so that the K ion turnover number was not different in the four cell lines tested.

Similarly, the [^{3}H] bumetanide-binding sites were decreased in MD + 1 and MD + 2 cells, cells transfected

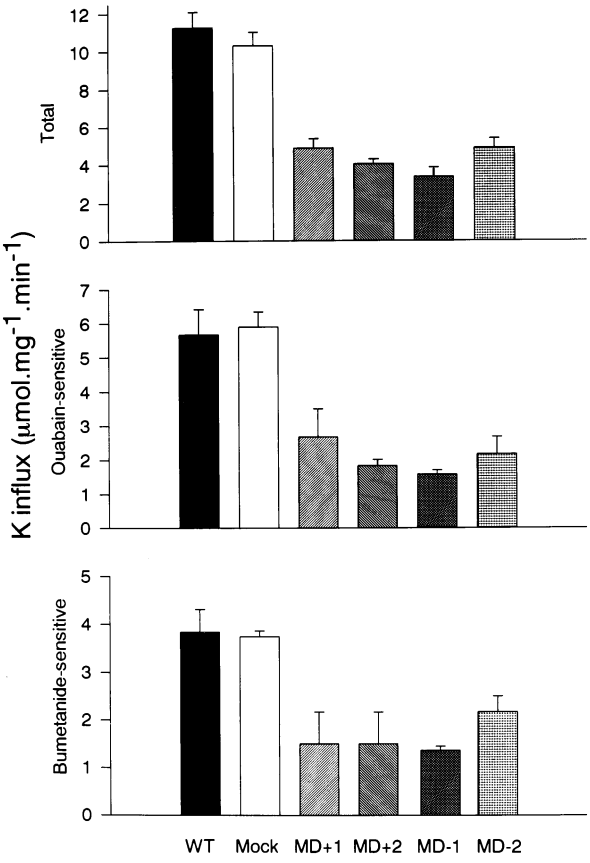

FIG. 4. K influx in wild type, transfected and mock-transfected MDCK cells. Total (top panel), ouabain-sensitive (middle panel) and bumetanide-sensitive (bottom panel) components of K influx were measured in MDCK-WT, mock transfected, MD + 1, MD + 2, MD − 1, and MD − 2 cell lines, shown in bars from left to right on each panel. WT refers to wild type MDCK cells and mock refers to mock-transfected cells using the lipofection procedure.

Table 1. Effect of transfection on the number of Na-K pump sites in MDCK cells

	Specific [^{3}H] ouabain binding (molecules × 10^{12}/mg protein)
MDCK-WT	33.25 ± 4.56
MD + 2	11.24 ± 0.34*
MD-1	10.23 ± 0.16*
Mock-transfected	34.23 ± 3.42

* $P < 0.005$ compared to MDCK-WT. Mock transfection was performed without DNA using the lipofectin procedure.[^{3}H] ouabain binding refers to the binding in the presence of 100 nM ouabain for 1 hr. Nonspecific binding, which was ~10% of the total, was subtracted to obtain the specific ouabain binding.

with THP cDNA (Table 2). [^{3}H]bumetanide binding sites were also decreased in MD − 1 and MD − 2 cells, cells transfected without THP cDNA. [^{3}H]bumetanide binding was not decreased in mock transfected cells.

Table 2. Maximum specific [^{3}H] bumetanide binding in wild type, transfected and mock transfected MDCK cells

	[^{3}H] bumetanide binding (fmol/mg)	*P* value (*vs.* MDCK-WT)
MDCK-WT	218 ± 26	
MD + 2	85 ± 14	<0.005
MD − 1	70 ± 15	<0.005
Mock-transfected	203 ± 29	NS

Discussion

Our findings of reduced Na-K pump and cotransport activity in MD + 1 and MD + 2 cells compared to wild type MDCK cells (MDCK-WT) initially led us to the conclusion that THP, the transfected protein, is important in modulating cation transport. However, when we compared these results with those in MD − 1 and MD − 2 cells, cells transfected with control plasmids without the cDNA for THP, we concluded that the process of transfection itself may alter transport properties. Our findings do not support the suggestion of Greven et al. (7) that THP may be a component of the NaK2Cl cotransporter.

To examine the mechanism of transfection-induced alteration in ion transport, we examined the various components of K influx. Both the Na-K pump-mediated (ouabain-sensitive) and NaK2Cl cotransport-mediated (bumetanide-sensitive) components of K influx were depressed in transfected cells. The decrease in pump-mediated transport can be the result of an alteration of the affinity for substrates, a decrease in the ion turnover rate or a decrease in the number of Na-K pump units. The number of Na-K pump units, assessed by the specific [^{3}H] ouabain binding, was decreased (Table 1) and the reduction of the ouabain binding in transfected cells appeared to be proportional to the decrease in pump-mediated K influx (Fig. 2). Similarly, [^{3}H] bumetanide binding was also diminished in transfected cells (Table 2). Thus, the decrease in cotransport-mediated ion flux could be explained by the decrease in the number of functioning cotransporter sites. The finding of proportional reductions in bumetanide-sensitive ion flux and [^{3}H] bumetanide binding makes it unlikely that transfection-mediated alteration in transport were caused by altered substrate affinity for the cotransporter, or a decreased ion turnover number. Thus, transfection appears to alter the transport properties of the host cell by altering the number of functioning transporter sites at the cell surface. Whether the decrease in the number of transporter sites was the result of decreased synthesis of new transporter units, decreased insertion of existing transporter sites to the membrane surface, or both, is not yet known.

The presence of endogenous protein in host cells has

been a complicating factor in many studies using expression systems. Apparently, it is not easy to recognize the presence of endogenous transporter in the host cell selected for transfection. Thus, endogenous Na-glucose [28] and NaK2Cl cotransport [23] were described in cells initially believed to be lacking these transport proteins [2, 12]. The possibility of transfection affecting the endogenous transport pathways of recipient cells has not been evaluated extensively.

The results of our study indicate that the process of transfection itself may alter the properties of the transfected cells. We found altered Na-K pump and NaK2Cl cotransport in MDCK cells transfected with control plasmid. Others have reported that transfection of either the anion exchanger or control plasmid into human 293 cells led to altered intrinsic buffering capacity of the transfected cells (18). Similar alterations in buffering capacity were noted in transfected CHOP cells (13). Thus it appears that the process of transfection itself may lead to altered properties in the host cell. If such transfection-induced alterations lead to an increase in the activity of an endogenous transport protein of the host cell, it may be difficult to distinguish that effect from the transport mediated by the transfected protein.

Thus, the findings in the present study suggest that future studies utilizing transfection techniques to study properties of transfected proteins in general, and transport properties in particular may be interpreted with greater confidence if appropriate controls are employed. Such controls would include cells transfected with plasmids containing all the promoter and polyadenylation sequences without the cDNA for the test protein so that the effects of transfection could be studied independently of the effects of the transfected protein.

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