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A TRANSFORMED HUMAN EPITHELIAL CELL LINE THAT RETAINS TIGHT JUNCTIONS POST CRISIS

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SUMMARY

The successful establishment of a postcrisis SV-40 T antigen transformed epithelial cell line, 1HAEo-, which retains tight junctions and vectorial ion transport, is described. Immunocytochemical analysis of 1HAEo- cells shows a defined pattern of cytokeratin staining and a characteristic pericellular localization of the adhesion molecule cellCAM 120/80, indicating the presence of junctional complexes. The presence of both tight junctions and desmosomes has been confirmed by electron microscopy. Cell monolayers have good transepithelial resistance measured in Ussing chambers. Cells increase chloride ion transport in response to addition of agents that raise either intracellular cAMP or calcium, measured either by $^{36}Cl^-$ efflux or whole-cell patch clamp. An increase in short-circuit current, in response to these agents, can be measured in Ussing chambers. The presence of a depolarization-induced outwardly rectifying 45 pS chloride channel has been demonstrated in single cell detached membrane patches. In addition, the cells have been found to express mucin mRNA. These cells therefore demonstrate that it is possible to select transformed cell clones with particular morphologic characteristics, i.e. the presence of tight junctions and cell polarity, which also retain useful epithelial cell-specific functions, including vectorial ion transport. They also provide a major resource for the study of the structure and function of human epithelia.

Key words: SV-40; chloride ion transport; cAMP; calcium-activated channels; cystic fibrosis.

INTRODUCTION

Human epithelia consist of highly organized layers of differentiated, polar cells. The task of reproducing this system in vitro constitutes a considerable challenge. Epithelial cells are linked by junctional complexes, providing a tight barrier between the lumen (e.g., of the airways, gut, or gland ducts) and the underlying tissues. These tight junctions separate the apical and basolateral membranes, which contain different populations of proteins, thereby allowing for net transfer of ions across the epithelium (37,49). There is evidence that not only is the junctional complex adhesion molecule cellCAM 120/80 (E-cadherin, uvomorulin) a necessary component of the junctional complex, but also that transfection with a cDNA encoding this protein is sufficient to cause nonpolarized fibroblast cells in culture to form both tight junctions (35,36) and polarized membranes (34).

Human epithelial cells have been isolated and cultured in vitro, but even under optimal culture conditions such cells have a limited life span and senesce or terminally differentiate (16,31). To circumvent this problem, we (7,15) and others (4,25,26,38,41) have immortalized epithelial cells from airways or sweat glands or both by transfection with DNA encoding the SV-40 T antigen. During early passages, such cells retain tight junctions. Active secretion can be measured in Ussing chambers and is stimulated with β-adrenergic agonists (4,26,41). However, after about 15 to 20 passages in culture, immortalized epithelial cells enter crisis (14). Usually, cells that recover from crisis no longer express the cellCAM 120/80 antigen and do not maintain tight junctions (15). Some tumor-derived colon carcinoma cell lines, e.g., T84 (9), retain tight junctions in culture, but a cell line (CFPAC-1) derived from a pancreatic adenocarcinoma in a cystic fibrosis donor (42), does not.

In this paper we describe the properties of a human epithelial cell line that has tight junctions and retains directional ion transport. The cells have a human karyotype. They are almost certainly tra-

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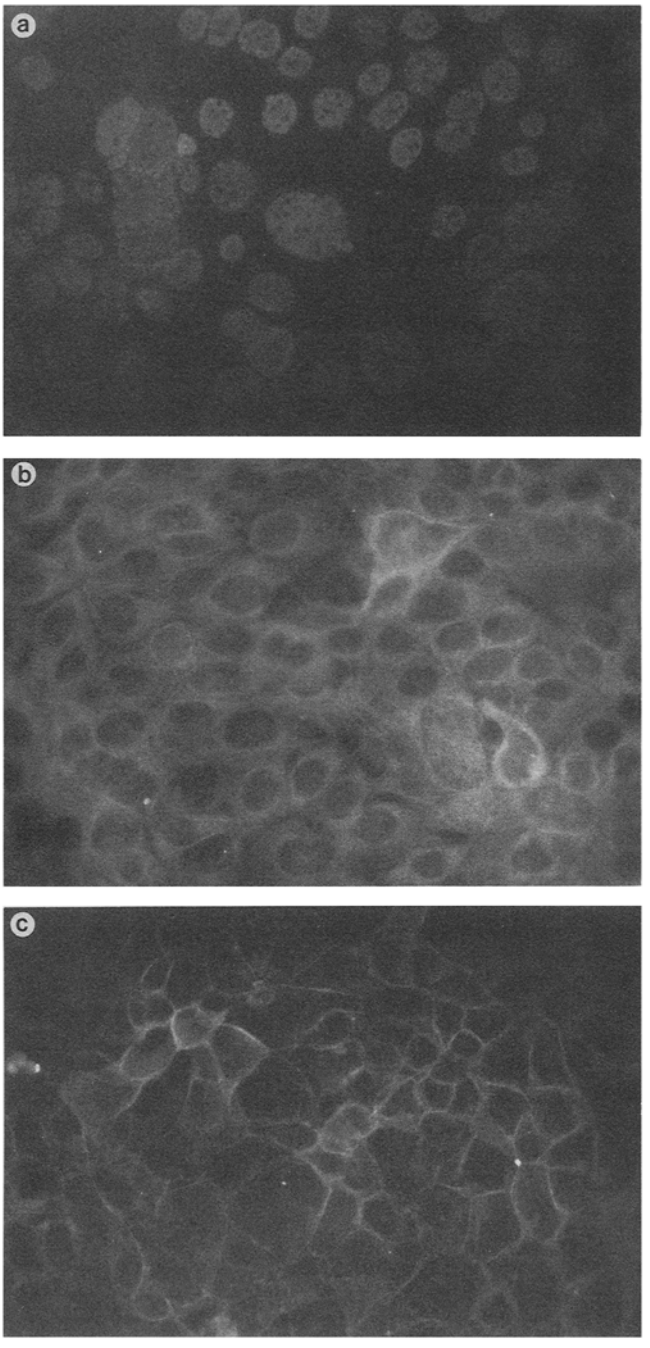

FIG. 1. Immunocytochemical staining of 1HAEo- cells. Cells were stained with primary antibodies raised against *a*, SV40 T antigen; *b*, cytokeratin; or *c*, adhesion molecule cellCAM 120/80.

cheobronchial in origin, but there is a small possibility that they were derived from sweat gland tissue. They may have been derived from either adult or fetal trachea. It is unknown whether the donor did or did not have cystic fibrosis, but the cells do not have the most common cystic fibrosis transmembrane conductance regulator (CFTR) mutation, ΔF508, or other CFTR mutations representing about 98% of all cystic fibrosis mutations (27). Despite this uncertainty as to their origin, these cells have features that should prove useful in studying the cell biology of human epithelia. More importantly, they demonstrate that it is possible to select specific transformed cell clones that retain epithelial morphology and functions postcrisis, perhaps indefinitely. A brief description of this cell line has been given previously (7).

MATERIALS AND METHODS

Cells and culture conditions. Pure cultures of epithelial cells were established either from explant or enzymatic dissociation of native epithelium (16). Epithelial cell cultures were transformed by transfection with the pSVori$^-$ plasmid as described previously (15). Precrisis, they were grown in modified serum-free LHC8 medium, MLHC8e (16,31). To avoid the cell death seen in many other epithelial cell lines at crisis (7), the cells were transferred to modified Eagle's medium (MEM) with 10% fetal bovine serum (FBS) before crisis. A clone that exhibited the characteristic "cobblestone" appearance of freshly isolated epithelial cells was isolated and grown in this medium thereafter. Both before and after crisis, cells were plated on tissue culture plasticware coated with fibronectin, vitrogen (collagen), and bovine serum albumin (16,31).

Immunocytochemical staining. Cells were grown to various stages of confluence on well slides (Lab-Tek) coated with fibronectin, vitrogen (collagen), and bovine serum albumin. After washing, fixing, and drying, the slides were rehydrated and stained as described previously (15). Primary antibodies used in these studies were the L19 monoclonal antibody raised against the SV-40 T-antigen (20), the AE1/AE3 anti-cytokeratin antibody (Boehringer Mannheim, Indianapolis, IN), and the E9 monoclonal against the junctional complex adhesion protein, cellCAM 120/80 (52). SV-40 T-antigen is expressed only in successfully immortalized cells. Both cytokeratin and cellCAM 120/80 are expressed only in epithelial cells.

Electron microscopy. Cells were prepared for transmission electron microscopy as described previously (11,15,56). Cultures were grown to confluence on fibronectin, vitrogen (collagen), and bovine serum albumin-coated glass cover slips and were fixed with glutaraldehyde, postfixed with osmium tetroxide, rinsed, and stained with uranyl acetate. The cells were embedded in polymer and removed from the surface of the cover slip. Sections (0.5 μm) were cut with an ultramicrotome, mounted on microscope slides, stained with toluidine blue, and examined under a light microscope. Specific areas were selected and thin sections cut, mounted on Formvar-coated copper slot grids, stained with uranyl acetate and lead acetate, and examined with a Zeiss EM10 electron microscope.

cAMP analysis. Cells were seeded into 35-mm dishes containing MLHC-8e medium, or MEM supplemented with 10% FBS, and were grown to confluence. The cells were rinsed 3 times with LHC basal or MEM without serum, respectively, and then were incubated in this medium for 30 min at 37° C. The production of cAMP was then stimulated with isoproterenol (10^{-5} *M*) or forskolin (10^{-5} *M*). Cells were incubated at 37° C for 1 min. The medium was removed and replaced with 1 ml of ice-cold 10% trichloroacetic acid (TCA). Dishes were scraped to remove the cells and were rinsed twice with 1 ml TCA. Triplicate fractions were pooled and the cell suspension sonicated at power setting 40 for 20 s with a Branson Sonifier Cell Disruptor 350. The sonicate was centrifuged for 25 min at 5000 *g* at 4° C. The supernatant was removed and placed on ice. The pellet was dried and total protein content measured (32). A 1-ml sample of the supernatant was extracted 4 times with 5 vol water-saturated ether and was evaporated to dryness using a Meyer N-EVAP analytical evaporator at 75° C with continuous air flow. The dried sample was stored at 4° C, the remainder of the supernatant at −20° C. The cAMP content of each sample was measured with a Dupont RIANEN cAMP [^{125}I]radioimmunoassay (RIA) kit. Results were adjusted to account for the efficiency of recovery of cAMP and were measured by the addition of tritiated cAMP to a test sample. The observed recovery of the added cAMP was 95%.

Ussing chamber measurements. Cells were plated in millicell-HA inserts (Millipore, Bedford, MA) on collagen gels (26) at confluence (1 to 2 × 10^6 cells/cm^2). Measurements were taken 5 to 6 days postplating. Inserts were mounted in Ussing chambers as described previously (55). These were bathed in phosphate buffered Krebs-Henseleit solution (pH 7.4), which was bubbled with 95% O_2:5% CO_2, at 37° C. Transepithelial potential difference, and short-circuit current (I_{sc}) were measured. Transepithelial resistance was determined from the size of current pulses (20 ms duration, 20 s apart) passed across short-circuited cell monolayers so as to displace

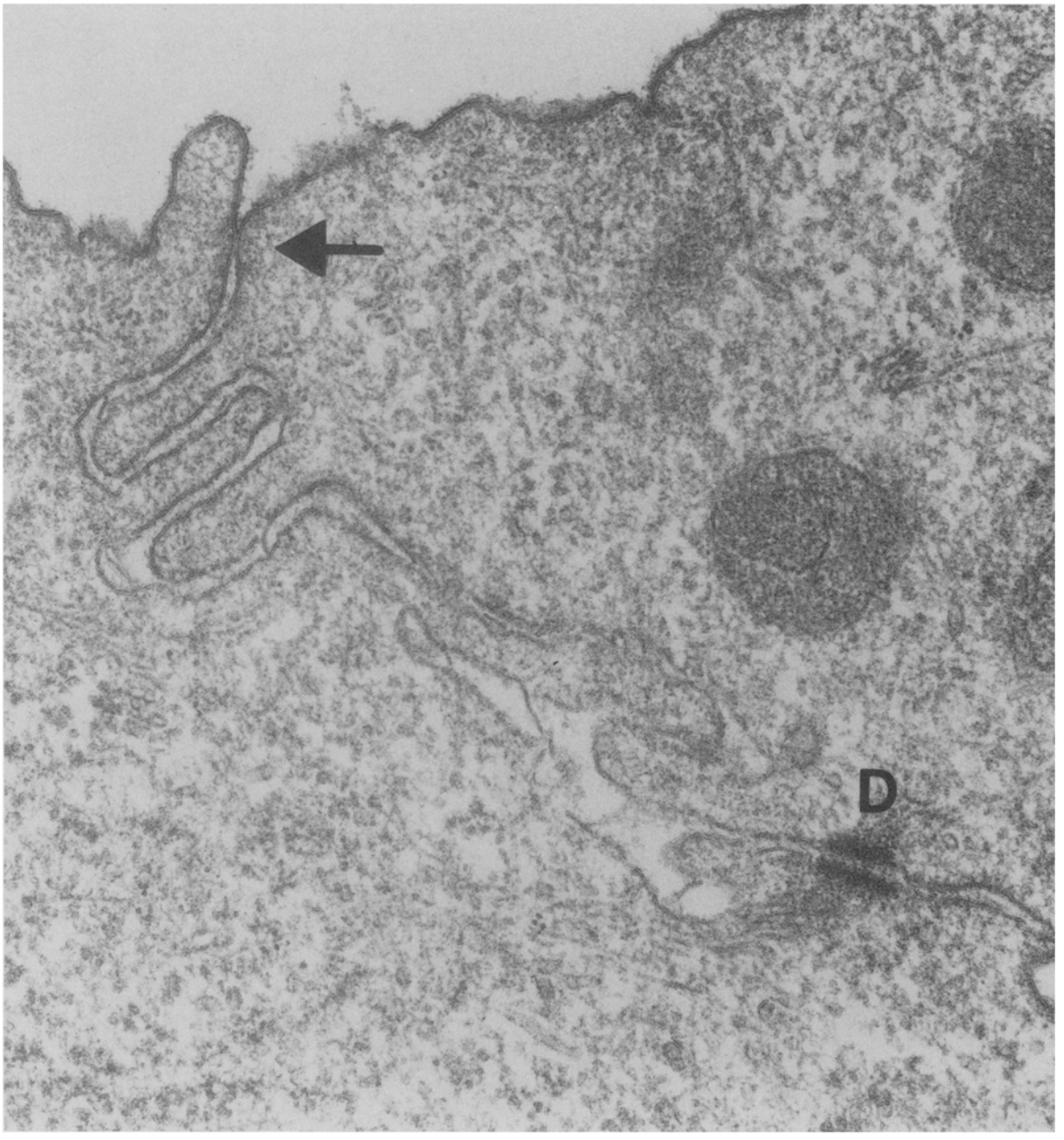

FIG. 2. Electron micrograph of 1HAEo- cells, showing both a tight junction (*arrow*) and a desmosome (*D*).

the voltage by a set amount. Isoproterenol (10^{-5} *M*) was added to the serosal (basolateral) side of the monolayer, bradykinin (10^{-7} *M*), diphenylamine-2-carboxylate (10^{-3} *M*), or amiloride (10^{-5} *M*) to the mucosal (apical) side.

Chloride efflux. Cells were seeded into 35-mm dishes in MEM with 10% FBS and grown to confluence. Cultures were rinsed twice with 2 ml efflux buffer (140 m*M* NaCl, 3.3 m*M* KH_2PO_4, 0.83 m*M* K_2HPO_4, 1 m*M* $CaSO_4$, 1 m*M* $MgSO_4$, 10 m*M* HEPES, pH 7.4, 10 m*M* glucose). Fresh efflux buffer (1 ml) containing 2 μCi/ml $^{36}Cl^-$ was then added to each dish and incubated for 2 h at 37° C. The dishes were washed by dipping each into two beakers of 200 ml efflux buffer for a total wash time of 8 to 10 s. After washing, 1 ml fresh efflux buffer was added, and the cells were again incubated at 37° C. Samples were removed at 1-min intervals and were replaced with fresh buffer. After 3 min, efflux buffer containing 10^{-5} *M* isoproterenol, forskolin, or A23187 was added, and additional samples were removed and replaced at 1-min intervals. At the end of the experiment, the $^{36}Cl^-$ remaining in the cells was extracted with 1 ml of 0.1 *N* HCl overnight at 4° C. Samples were added to 4 ml scintillation cocktail and were counted. Efflux was expressed as percent total counts lost per minute (5).

Whole-cell patch clamp. Cells were studied 2 to 3 days after plating on glass cover slips. Cover slips were placed in a 1-ml acrylic chamber on the stage of a Nikon Diaphot inverted microscope and were washed 6 times with bath solution (170 m*M* Tris-HCl, 1 m*M* $MgCl_2$, 2.5 mm $CaCl_2$, 5 m*M* HEPES, 10 m*M* glucose, pH 7.4, 325 (mOsM/kg). Micropipettes for whole-cell configuration patch-clamp recordings were made by the method of Hamill et al. (19) and had a tip resistance of 3 to 4 MΩ when filled with pipette solution (140 m*M* CsCl, 1 m*M* $MgCl_2$, 0.5 m*M* [ethylene bis (oxyethylenenitrilo)]-tetraacetic acid (EGTA), 2 m*M* MgATP, 5 m*M* HEPES, 10 m*M* glucose, pH 7.35, 298 mOsM/kg). The difference in osmolarities between bath and pipette solutions was required to prevent a volume-induced chloride current (57). Whole-cell voltage clamp recordings were

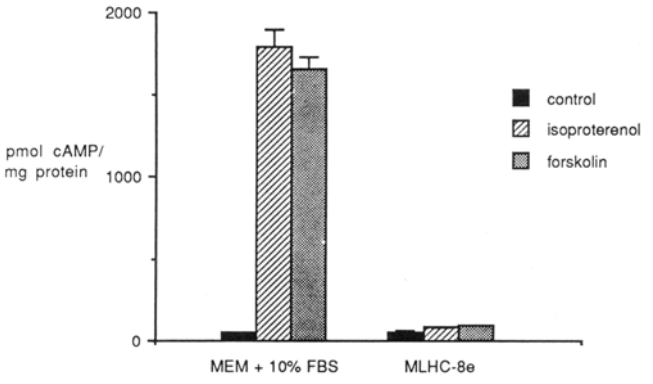

FIG. 3. Accumulation of intracellular cAMP in 1HAEo- cells grown either in serum-containing medium (MEM + 10% FBS) or serum-free medium (MLHC-8e), after a 1-min stimulation with either isoproterenol (10^{-5} *M*), forskolin (10^{-5} *M*), or calcium ionophore (10^{-5} *M*). cAMP was measured by RIA and normalized to cell protein content, assuming a recovery of 95%.

made using a model 1B Axopatch amplifier (Axon Instruments, Burlingame, CA). The membrane was voltage clamped to a holding potential of −70 mV and was stepped to levels between −100 and +100 mV using a Tecmar 12-bit A/D-D/A converter (Scientific Solutions, Cleveland, OH) and an IBM-AT computer. Signals were filtered at 1 kHz and were stored on a strip chart recorder and directly on floppy disc for later analysis. Cell capacitance measurements were determined as total capacitance compensation in the whole-cell configuration minus the capacitance compensation of the glass pipette alone during square-wave voltage steps. All experiments were performed at 30° ± 1° C. Baseline voltage-clamp protocols were obtained between 2 and 7 min after obtaining whole-cell configuration. Forskolin (Calbiochem, La Jolla, CA) was added at 7 min to a final bath concentration of 10 μ*M*. Voltage-clamp protocols were obtained 2 to 10 min after addition of forskolin. The voltage-clamp protocol resulting in maximum baseline or forskolin-stimulated outward current was used to calculate current-voltage relations using the clampfit analysis program (Axon Instruments). Data are expressed as mean ± SD and are divided by cell capacitance to standardize responses.

Single-channel patch-clamp measurements. Outwardly rectifying Cl^- channels were activated and recorded using the standard inside-out patch clamp recording configuration (19). Cl^- channel activity was elicited after excising a patch of membrane and holding at a depolarized voltage for a prolonged period of time (e.g., +80 mV for 1 to 6 min). Experiments were performed with a SSI 11/73 (Digital Equipment Corporation, Maynard, MA) based minicomputer system (Indec Systems, Sunnyvale, CA). The output of an EPC-7 patch clamp amplifier (List-Medical Systems, Greenvale, NY) was low pass filtered through an 8-pole Bessel filter (Frequency Devices Inc., Haverhill, MA) and digitized as indicated. Junctional potential was nulled immediately before obtaining a seal. Records were not corrected for potential junctional errors (≤5 mV).

Electrophysiologic data were obtained and analyzed as described by Hoshi and Aldrich (21,22). Briefly, amplitude histograms were constructed from nonleak subtracted, nonidealized traces. Peak P_o (probability of opening) was determined from ensemble averages of single channel "idealized" traces. (Traces were "idealized" after leak subtraction). Opening and closing transitions were detected using as criterion a level of 50% of the unitary current amplitude at a particular voltage. The pipette contained 154 m*M* NaCl, 1 m*M* $CaCl_2$, 2 m*M* $MgCl_2$, 10 m*M* HEPES-NaOH, pH 7.3. Bath solution was either the same as in the pipette or 140 m*M* KCl, 1 m*M* $CaCl_2$, 2 m*M* $MgCl_2$, 11 m*M* EGTA, 10 m*M* HEPES-NaOH, pH 7.3 Recordings of channel activity in inside-out patches were made in symmetrical Cl^- concentration solutions, unless otherwise indicated. Channel activity was not markedly affected by the composition of the bath solution.

CFTR and mucin mRNA analysis by polymerase chain reaction (PCR) amplification of first-strand cDNA. Cytoplasmic RNA was prepared from 10^6 to 10^8 cells (30). RNA (1 μg) was denatured by heating to 95° C for 2 min. This was reverse transcribed using the PCR RNA GeneAmp kit, according to the manufacturer's instructions (Perkin Elmer Cetus, Emeryville, CA). The first-strand cDNA was amplified in the polymerase chain reaction, using the same kit, in the DNA thermal cycler (Perkin Elmer Cetus). To assay for the presence of CFTR, oligonucleotide primers were designed using the published CFTR cDNA sequence (39), to span intron-exon boundaries, and thereby eliminate any possibility of the amplified DNA having arisen from contaminating genomic DNA. The primers were 5'ACTTTAAAGCTGTCAAGCCGTG-3' and 5'-CTGTATTTTGTTTATTGCTCCAA-3'. These give a predicted product of 627 bp (nucleotide positions 622–1248 of the CFTR gene). These primers were kindly provided by F. Chehab, U.C.S.F. Thirty-five cycles of polymerase chain reaction amplification were used, with cycle times of: denaturation 30 s at 94° C, annealing 30 s at 55° C, and extension 1 min at 72° C. The extension time was increased by 5 s per cycle. The amplification products were separated by electrophoresis on 1.4% agarose gels.

Mucin cDNA was selectively amplified using 7.5 μl from each sample cDNA by two mucin-specific primers taken from an intron flanking, nonrepetitive region of a human intestinal mucin cDNA, SMUC 41 (17). It has been previously shown that human bronchus and intestine express the same mucin gene (24). After an initial amplification of 25 cycles, two mucin primers internal to the first (also from the nonrepetitive region) were used for a second round of amplification (20 cycles). A 1-μl aliquot of a 1:10 dilution of the first round amplification. This two-step, nested primer PCR method (48) was chosen for its high degree of sensitivity and specificity. The PCR reaction mixture included 10 pmol 5' and 3' mucin primers, 200 μmol each dNTP, 3.75 m*M* $MgCl_2$, 10× PCR buffer, and 2.5 U of Taq polymerase, in a total of 100 μl. Each cycle was as follows: denaturation, 94° C, 1 min; annealing, 51° C, 1 min; and extension, 72° C, 1 min.

Haplotype analysis and mutation analysis of CFTR exons. Haploytpe was measured as described previously (1). DNA was PCR amplified for restriction fragment length polymorphic regions KM19 and XV2c. The primers used to generate the XV2C fragment were 5'-CAGGCGCATTCATGAGGCATT-3'(sense) 5'-CCTCCCAGGTTCACGCTAT-3'(antisense). Those for the KM19 fragment were 5'GCTGCATCATATAAGTTGCC-3'-(sense) and 5'-AAGGCTACACTGTTAATTTT-3'(antisense). The digested DNA fragment was analyzed by agarose gel electrophoresis.

Single exons of CFTR were PCR amplified from 500 ng of genomic

TABLE 1

USSING CHAMBER MEASUREMENTS OF TRANSEPITHELIAL RESISTANCE (R_{te}), BASELINE SHORT-CIRCUIT CURRENT (I_{SC}), AND CHANGES IN I_{SC} IN RESPONSE TO ADDITION OF ISOPROTERENOL (10^{-6} *M*) (ΔI_{SC} ISOPROTERENOL) OR BRADYKININ (10^{-7} *M*) (ΔI_{SC} BRADYKININ)[a]

Passages Posttransformation	*n*	Baseline R_{te}, Ω · cm²	Baseline I_{SC}, μA · cm⁻²	ΔI_{SC} Isoproterenol, μA · cm⁻²	ΔI_{SC} Bradykinin, μA · cm⁻²
37	5	151 ± 61	0.3 ± 0.2	0.1 ± 0.1	2.5 ± 2.9
40	7	292 ± 44	1.7 ± 0.6	1.5 ± 1.7	4.7 ± 2.6
44	11	249 ± 108	4.0 ± 1.2	0.2 ± 0.3	3.6 ± 2.6
51	7	86 ± 57	0.9 ± 0.6	0.3 ± 0.2	0.6 ± 0.4
53	6	257 ± 132	0.6 ± 0.5	0.1 ± 0.2	0.7 ± 1.1
56	4	268 ± 325	0.5 ± 0.4	0.1 ± 0.1	0.03 ± 0.05

[a] All values are mean ± SD.

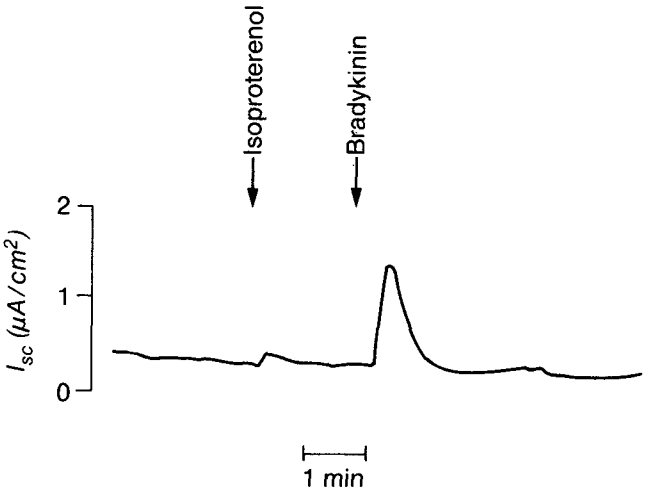

FIG. 4. Representative Ussing chamber record showing stimulation of chloride secretion after stimulation with isoproterenol (10^{-5} *M*) and bradykinin (10^{-7} *M*) (*arrows*). Confluent cell sheets were pretreated with amiloride, which had no effect on I_{sc} or transepithelial resistance.

DNA, using primers selected from intron sequences flanking exons, 1, 2, 4, 9–12, and 19–23. One-tenth of the product was sequences using ^{32}P-end labeled primers and dideoxy-chain terminating method. Genomic DNA from the cell was also screened for two mutations (R334W and R347P) in exon 7 by restriction digestion. Primers, PCR, sequencing and restriction digestion methods were as previously described (8).

RESULTS

A postcrisis transformed human epithelial cell line, which grows well in MEM containing 10% FBS, has been isolated. All the experiments described here were carried out with cells grown in MEM in the presence of 10% FBS, except where noted. Immunocytochemical staining of these cells demonstrated that they produce SV-40 T antigen, localized to the nucleus (Fig. 1 *a*). DNA hybridization analysis indicated that only one or two copies of the pSVori$^-$ plasmid had integrated into the human genome (L. C., unpublished observations).

The postcrisis cells retained the characteristic "cobblestone" appearance of epithelial cells. Immunocytochemical staining confirmed the presence of well-organized cytokeratin filaments (Fig. 1 *b*). Staining with monoclonal antibodies to the cellCAM 120/80 adhesion molecule (52) showed localization of this molecule, a component of the *zonula adherens*, to the cell periphery (Fig. 1 *c*). Electron microscopy confirmed that these cells clearly maintain tight junctions and desmosomes (Fig. 2) and may therefore be expected to retain the compartmentalized membranes characteristic of cells in the native epithelium.

To test whether 1HAEo- cells were able to respond to β-adrenergic agonists, the production of intracellular cAMP in response to incubation with isoproterenol was measured. Production of cAMP in response to forskolin, which can cross the plasma membrane and act directly upon adenylyl cyclase, was also measured (Fig. 3). Stimulation of cAMP production was comparable to that observed with fresh tissue (54). After 1 min of stimulation with isoproterenol, the intracellular cAMP concentration was 1792.9 ± 99.6 pmol/mg protein (n = 3); after 1 min of stimulation with forskolin, it was 1652.1 ± 77.8 pmol cAMP/mg protein (n = 3). In untreated cells, the intracellular accumulation of cAMP after 1 min was only 51.8 ± 5.1 pmol cAMP/mg protein (Fig. 3). However, when the cells were grown in serum-free medium MLHC-8e, which contains 2.7 μ*M* epinephrine, the responses to isoproterenol and forskolin were severely attenuated. Baseline levels were not elevated.

Chloride ion transport in 1HAEo- cells was measured by four complementary assays: transepithelial current in Ussing chambers, $^{36}Cl^-$ efflux, whole-cell patch clamp, and single-channel patch clamp. Transepithelial currents were measured in postcrisis 1HAEo- cells grown on collagen gels. Monolayers had a resistance of 219 ± 21 Ω·cm^2 and a baseline I_{sc} of 1.8 ± 0.2 μA/cm^2 (n = 42). Isoproterenol (10^{-6} *M*) and bradykinin (10^{-7} *M*) gave transient increases in I_{sc} of 0.3 ± 0.08 μA/cm^2 (n = 33) and 2.5 ± 0.4 μA/cm^2 (n = 37), respectively. Bradykinin is believed to increase chloride secretion by raising the intracellular calcium ion concentration (2). Transepithelial resistance, baseline I_{sc}, and responses to addition of isoproterenol and bradykinin were monitored over the course of 10 mo., using cells from 37 to 56 passages posttransfection with pSVori$^-$. All four parameters varied greatly, but no consistent pattern could be seen (Table 1). In almost every batch of cells there were some in which no response to isoproterenol, bradykinin, or both could be observed. The mean value ± SD is given in each case. A representative trace is shown in Fig. 4. Pretreatment with mucosal amiloride, which inhibits the passage of sodium ions across the apical membranes of airway epithelial cells (3), had no effect on transepithelial resistance, baseline I_{sc}, or the responses to either isoproterenol or bradykinin (n = 3). In contrast, the addition of the chloride channel inhibitor diphenylamine-2-carboxylate (33) reduced the isoproterenol-induced change in I_{sc} from 0.7 ± 0.01 to 0.13 ± 0.03 μA/cm^2 and the bradykinin-induced change in I_{sc} from 5.9 ± 1.1 to 0.8 ± 0.2 μA/cm^2 (n = 3). These results show that the 1HAEo- cell line forms high-resistance monolayers and retains the agonist-induced chloride secretion seen in native airway epithelia. The response to isoproterenol is similar to results obtained with primary cultured airway tissue (54) or subcultured airway epithelial cells (16).

The chloride efflux assay utilizes radiolabeled $^{36}Cl^-$ tracer to measure the rate of chloride transport from populations of cells grown as monolayers in tissue culture dishes (5,40,45). The rate of loss of radiolabeled $^{36}Cl^-$ was measured. 1HAEo- cells showed a background $^{36}Cl^-$ efflux rate of about 5%/min (Fig. 5), very similar

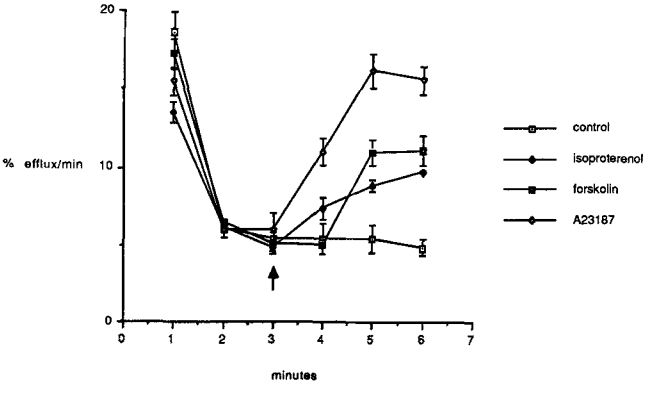

FIG. 5. Chloride ion transport, measured by $^{36}Cl^-$ efflux, in 1HAEo- cells. Forskolin (10^{-5} *M*), isoproterenol (10^{-5} *M*), or calcium ionophore A23187 (10^{-5} *M*) were added after 3 min (*arrow*).

TABLE 2

CHLORIDE EFFLUX IN 1HAEo- CELLS AT DIFFERENT PASSAGES. ALL DATA ARE TAKEN AT THE 5-MIN TIME POINT, I.E. 2 MIN AFTER ADDITION OF ISOPROTERENOL (10^{-5} *M*), FORSKOLIN (10^{-5} *M*), OR A23187 (10^{-5} *M*)[a]

Passages Posttransformation	Background % Efflux/min	Response to Isoproterenol % Efflux/min	Response to Forskolin % Efflux/min	Response to A23187 % Efflux/min
24	5.33 ± 0.87	8.79 ± 0.37	10.88 ± 0.82	16.15 ± 1.08
35	7.79 ± 0.84	10.14 ± 0.85	8.55 ± 1.57	17.48 ± 1.03
35	5.37 ± 1.29	6.11 ± 1.08	9.11 ± 4.67	17.58 ± 1.93

[a] All values are means ± SD; $n = 3$ in each case.

to that observed in dog tracheal cells (5) or T84 colon carcinoma cells (45) (A. L. C. and D. C. G., unpublished results). This efflux rate was increased from 5.37 ± 0.98%/min to 7.33 ± 0.69%/min 1 min after addition of isoproterenol (10^{-5} *M*); to 10.88 ± 0.82 2 min after addition of forskolin (10^{-5} *M*); and to 16.15 ± 1.08%/min 1 min after addition of the calcium ionophore A23187 (10^{-5} *M*) ($n = 3$ for each treatment) (Fig. 5). Note that the forskolin effect is delayed for 1 min after addition of the agonist. This is believed to be due to the time taken for the added forskolin to diffuse through the membrane and activate adenylyl cyclase.

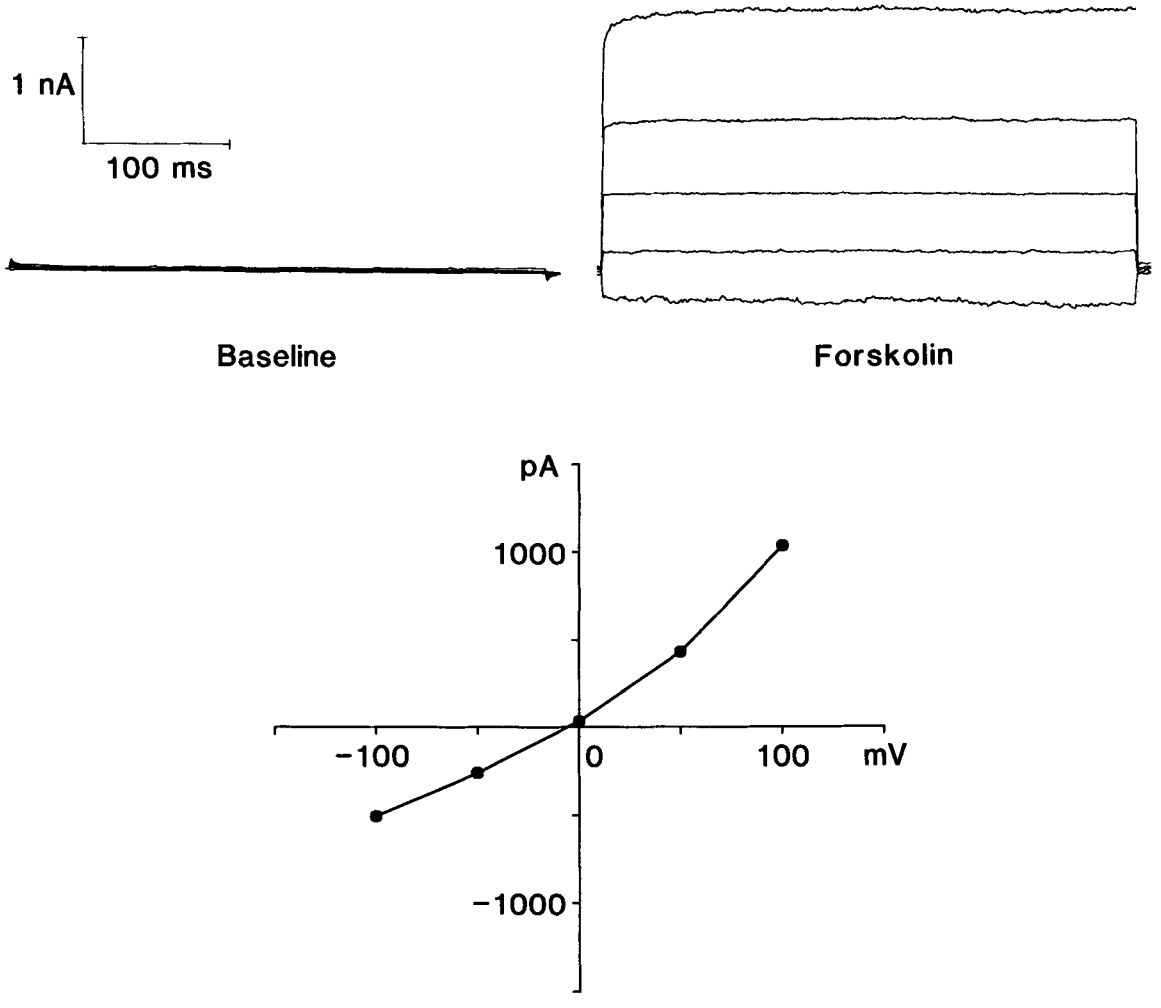

FIG. 6. Representative whole cell patch clamp of a 1HAEo- cell. Cell capacitance was 22.6 pF. Voltage clamp protocol stepped membrane potential from a holding potential of −70 mV to voltages between −100 and +100 mV in 50 mV steps (*inset*). Voltage steps were 500 ms with 500 ms between steps. *Upper left*, baseline currents before addition of forskolin. *Upper right*, currents after addition of forskolin (10^{-6} *M*). *Below*, current-voltage relationship after treatment with forskolin (10^{-6} *M*) (baseline current-voltage relationship is not distinguishable from the x-axis in this figure).

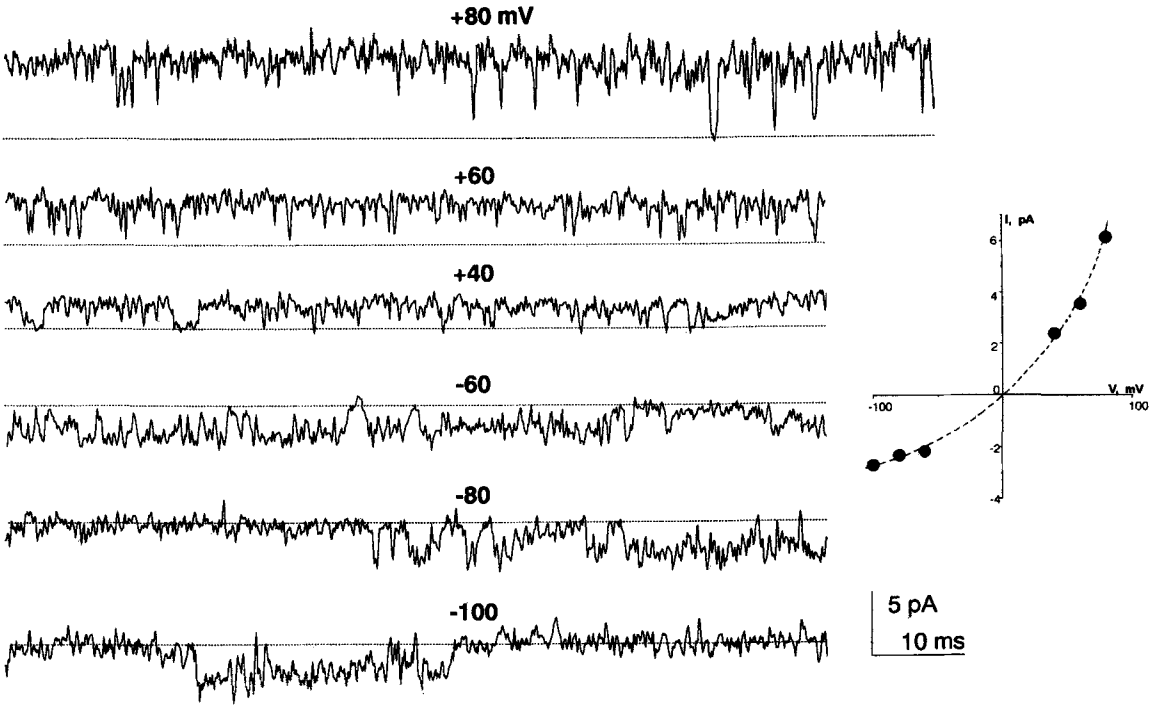

FIG. 7. Representative traces of single chloride channel activity in response to indicated steady-state holding voltages. All traces were recorded from the same patch in the respective cell types. *Dashed lines* indicate closed, nonconducting level. *Inset* shows the rectifying current/voltage relationship (*dashed line* drawn by eye). Traces were recorded at 30° C an 2000 Hz.

Isoproterenol acts more quickly by binding to the β-adrenergic receptor on the outside of the cell. Efflux was measured on two further occasions, and showed considerable variation in response to isoproterenol, forskolin, or calcium ionophore (Table 2). Efflux was also measured at room temperature, and using $^{125}I^-$ in place of $^{36}Cl^-$. In each case, a small but consistent increase in efflux was observed upon addition of isoproterenol, forskolin, or calcium ionophore.

Whole-cell patch-clamp analysis was used to measure chloride currents in single 1HAEo- cells, according to the method of Hamill et al. (19). The mean cell capacitance was 17 ± 5.0 pF (n = 6). Figure 6 displays results from a representative patch-clamp protocol. Baseline inward chloride current at −100 mV was −0.48 ± 0.53 pA/pF. Baseline outward chloride current at +100 mV was 1.3 ± 0.68 pA/pF. Forskolin treatment induced an increase in both inward and outward chloride current. Forskolin-stimulated inward current at −100 mV was −54 ± 31 pA/pF. Forskolin-stimulated outward current at +100 mV was 96 ± 28 pA/pF. The mean reversal potential of the forskolin-stimulated current was −6.0 ± 5.1 mV in close accordance with the chloride reversal potential of −5 mV calculated with the Nernst equation. The current-voltage relationship for the forskolin-stimulated chloride current was nearly linear (with slight outward rectification) between −100 and +100 mV and showed little or no time dependence, similar to results obtained in whole-cell recordings of T84 human intestinal adenocarcinoma cells (6). Chloride currents produced in response either to cell swelling or to elevation of intracellular [Ca^{2+}] have also been demonstrated in this cell line (J. A. W. and P. G., unpublished results).

The single-channel patch-clamp method was used to identify specific ion currents in the 1HAEo- cell line. An outwardly rectifying Cl^- channel of about 45 pS, which is thought to be regulated abnormally in cystic fibrosis cells, has been described in other epithelial cell lines (e.g., T84 cells) using this type of analysis (17) as well as in native airway tissue (50). A Cl^- channel with similar properties is present in 1HAEo- cells (Fig. 7). Channel activity was elicited by excision and voltage activation as described in Methods. The channel becomes irreversibly activated using this method. The current at +80 mV is 6.0 ± 0.3 pA (n = 3). The ratio of current flowing through the channel at +80 and −80 mV is 2.4 ± 0.8 pA (n = 3). The single-channel conductance, measured as the slope conductance through 0 mV, is 42 to 48 pS. The channel exhibits alternate conductance states (Fig. 7 *top trace*). The probability of being open is also voltage dependent such that the channel is almost always open at voltages above −40 mV (13).

Expression of CFTR mRNA was demonstrated by polymerase chain reaction amplification of first strand cDNA prepared from 1HAEo- cells. Primers within exons 5 and 7 of the CFTR gene were used, yielding a product of the correct size, predicted from the published sequence (27) of 627 bp (Fig. 8). Mucin mRNA expression was determined from gel electrophoresis of the PCR products from the second round amplification. A fragment of the expected length (305 bp) for the mucin cDNA was detected (Fig. 9) (43).

A thorough genetic analysis of the 1HAEo- cell line was undertaken. Karyotype analysis of these cells confirmed that they are human, containing 70 to 100 chromosomes per cell. The chromosome number is variable and chromosomes are highly rearranged, but there are consistently at least two copies of chromosome 7. The cystic fibrosis gene is located on chromosome 7 (28,47,53). Genomic DNA was prepared from 1HAEo- cells and amplified in the polymerase chain reaction, using the CFTR exon 10 specific

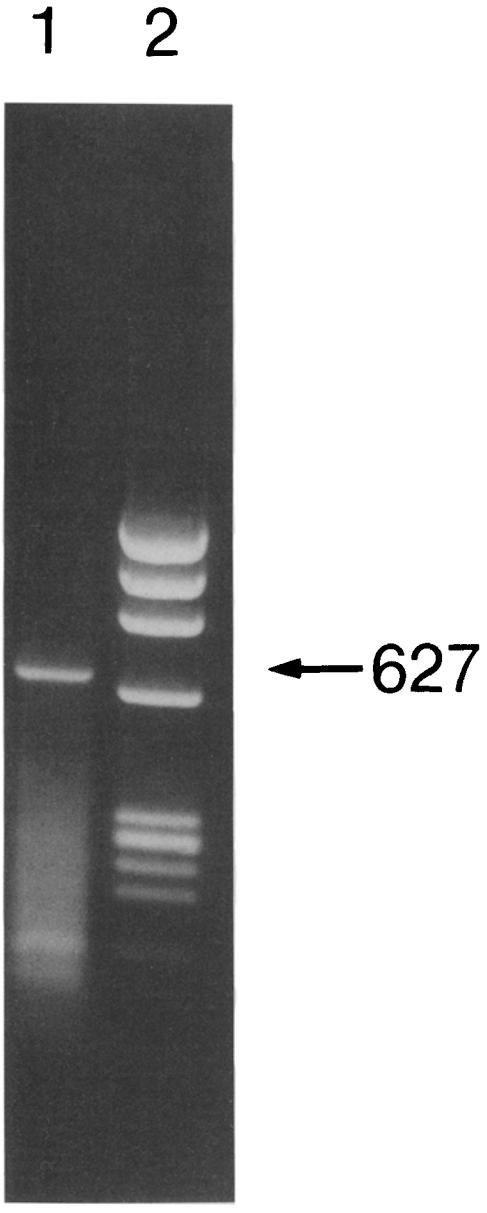

FIG. 8. Expression of CFTR-specific mRNA in 1HAEo- cells. First-strand cDNA was prepared and amplified by PCR, using primers from exons 5 and 7 of the CFTR gene. Amplification products were separated by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. *Arrow* indicates the predicted product of 627 bp, spanning nucleotides 622–1248 of the CFTR gene.

primers C16B and C16D (27). The amplification products were run on an agarose gel, transferred to Genescreen Plus membrane (NEN Dupont), and hybridized to oligonucleotide probes specific for either the normal CFTR gene or the gene containing the ΔF508 mutation. 1HAEo- cells do not contain the ΔF508 mutation (A. L. C., unpublished observations). CFTR sequence analysis of exons 1, 2, 4, 5, 9–12, 19–23, and exon 7 mutations (R347P and R334W) revealed only wild-type CFTR sequences. Haplotype analysis was also conducted. DNA around the marker loci XV2c and KM19 was amplified in the polymerase chain reaction, digested with restriction enzymes (Taq I for XV2c, Pst I for KM19) and analyzed on 1.5% agarose gels. 1HAEo- cells have the A/C haplotype (Table 3) (1).

DISCUSSION

The 1HAEo- cell line should prove useful in the study of epithelial ion transport, secretion, and biochemistry. The isolation of a human epithelial cell line, probably of airway origin, that retains tight junctions and vectorial ion transport in vitro, is important. Tight junctions may be considered the hallmark of epithelial cells, the property which defines cell polarity and allows them to regulate movement of ions, water, and macromolecules in a defined direction across an epithelium. 1HAEo- cells can transport ions across a cell monolayer, in a regulated manner, suggesting that the differentiation of the apical from the basolateral membranes remains intact. Regulation of chloride ion transport by agents that raise either cAMP (forskolin, isoproterenol) or intracellular calcium (calcium ionophores, bradykinin) is retained. This shows that the cells still express receptors for both isoproterenol and bradykinin, and that both cAMP and calcium-dependent second messenger systems are intact. It also confirms that the 1HAEo- cell line was probably derived from a non-cystic fibrosis donor, because cystic fibrosis (CF) epithelia are unable to increase chloride transport in response to agents that raise cAMP (12,51,54). Genetic analysis so far agrees with the functional data, supporting the conclusion that these

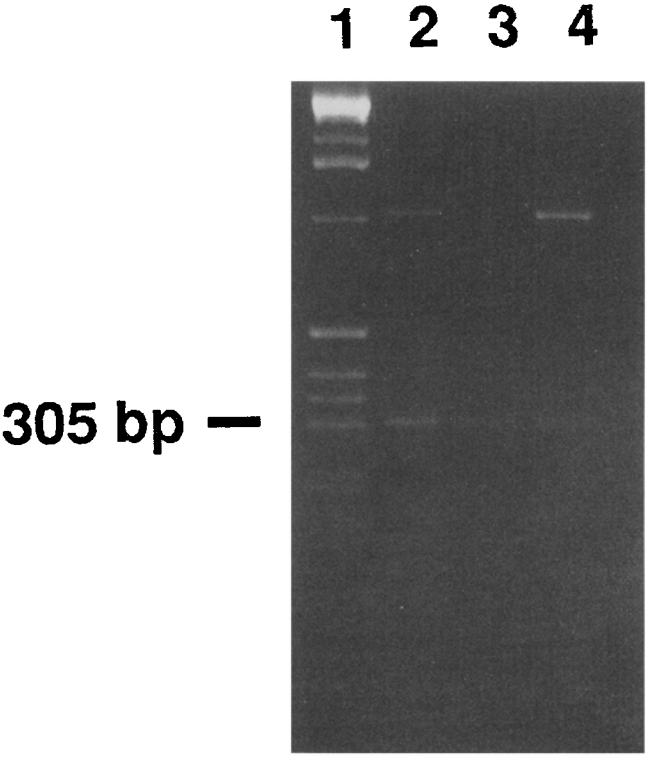

FIG. 9. Mucin-specific mRNA expression after nested-PCR of first-strand cDNA. Primers used generate a 305 bp fragment (*arrow*). *Lane 1*, 1 kb DNA ladder; *lane 2*, 1HAEo- total cytoplasmic RNA, 36 passages posttransformation; *lane 3*, 1HAEo- poly (A^+) RNA, 39 passages posttransformation; *lane 4*, 1HAEo- total cytoplasmic RNA, 15 passages posttransformation.

TABLE 3

HAPLOTYPE ANALYSIS OF NORMAL CELL LINES PREVIOUSLY REPORTED[a]

Cell Lines	XV2C (TaqI)	KM19 (PstI)	Haplotype	Reference
3HTEo-	−/−	−/−	AA	15
9HTEo-	−/−	−/−	AA	15
4AHTE-2o-	+/+	−/−	CC	15
56FHTE-8o-	−/+	−/−	AC	23
1HAEo-	−/+	−/−	AC	—

[a] DNA fragments were generated by PCR. Primers used to generate the XV2C fragment were 5′-CAGGCGCATTCATGAGGCATT-3′ (sense) 5′-CCTCCCAGGTTCACGCTAT-3′ (antisense). Those for the KM19 fragment were 5′-GCTGCATCATATAAGTTGCC-3′ (sense) and 5′-AAGGCTACACTGTTAATTTT-3′ (antisense). Haplotype was determined after restriction enzyme (TaqI and PstI) digestion to detect for the presence (+) or absence (−) of the restriction enzyme site within the fragment from each allele. Digested DNA fragment was analyzed by agarose gel electrophoresis.

cells were derived from normal (i.e. non-cystic fibrosis) tissue. The cell line does not have a mutation in the 12 exons of the CFTR analyzed. Approximately 86% of CF alleles in the Caucasian population occur in these exons. Because CF is caused by mutations in each CFTR gene, 98% of individuals with CF would have at least one mutation detected in the regions examined thus far. Only 1/80 000 North American individuals with the A/C haplotype have cystic fibrosis (1) but we cannot rule out the possibility that these cells originated from a donor with an unusual mutation in the CFTR gene. The possibility that the 1HAEo- cell line has been described previously (15,23) is unlikely. Only one cell line described has the same haplotype (A/C) and this cell line does not maintain tight junctions or cell polarity. It is, however, possible that the 1HAEo- cell line is an independent clone derived from the same individual.

The cells are almost certainly of airway origin. Early passage (< Passage 15) cells have been stained successfully with monoclonal antibodies raised against airway secretions (11), whereas cells at a later passage (Passage 50 posttransformation) showed staining only when exposed to retinoic acid (D. C. G. and W. E. Finkbeiner, in preparation). This result is not unexpected because cells in culture can lose the expression of secretory antigens after multiple subcultures, and retinoic acid has been shown to induce differentiation in airway epithelial cells. In further support of the notion that the cells are airway and not sweat gland in origin is the finding that the cells express mRNA for mucin which has been detected in both airway and intestine epithelium (24).

The response of 1HAEo- cells to agents which raise cAMP is somewhat reduced when compared with native tissue or primary cultured cells. However, this is often true of both cultured primary cells and of immortalized cell lines, and may be due to the culture conditions rather than to any permanent change in cell phenotype caused by the immortalization process. For example, it has been possible to culture primary airway epithelial cells under conditions where they retain highly differentiated morphology and ion transport properties very similar to those of native airway tissue (29,44).

A major advantage of transformed cell lines is the abundance of material available for experimentation. The cells grow quickly, with a generation time of about 30 h. They have been maintained in culture for greater than 60 passages posttransfection, at least 180 generations, and large stocks of cells at earlier passages are available. Such cells provide a source of relatively homogeneous material, avoiding both the genetic variability associated with the use of different individual donors and some of the complexity inherent in the use of freshly isolated epithelial tissue, which may contain both different cell types and cells in different stages of differentiation. Thus, in spite of the uncertainty as to its origin, these cells have been extensively characterized and are a useful addition to the tools available for the study of epithelia in vitro.

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