The Immortalized UROtsa Cell Line as a Potential Cell Culture Model of Human Urothelium

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The UROtsa cell line was isolated from a primary culture of normal human urothelium through immortalization with a construct containing the SV40 large T antigen. It proliferates in serum-containing growth medium as a cell monolayer with little evidence of uroepithelial differentiation. The working hypothesis in the present study was that this cell line could be induced to differentiate and express known features of *in situ* urothelium if the original serum-containing growth medium was changed to a serum-free formulation. We demonstrated that the UROtsa cells could be successfully placed into a serum-free growth medium consisting of a 1:1 mixture of Dulbeco's modified Eagle's medium and Ham's F-12 supplemented with selenium (5 ng/mL), insulin (5 µg/mL), transferrin (5 µg/mL), hydrocortisone (36 ng/mL), triiodothyronine (4 pg/mL), and epidermal growth factor (10 ng/mL). Under serum-free growth conditions, confluent UROtsa cells were shown by light microscopy to produce raised, three-dimensional structures. Routine ultrastructural examination disclosed these three-dimensional areas to consist of a stratified layer of cells that strongly resembled *in situ* urothelium. The cells displayed numerous desmosomal connections, complex interactions of the lateral membranes, and abundant intermediate filaments within the cytoplasm. Freeze fracture analysis demonstrated that the cells possessed tight-junction sealing strands and gap junctions. The overall morphology was most consistent with that found in the intermediate layers of *in situ* urothelium. The basal expression patterns of the metallothionein (MT) and heat shock proteins 27, 60, and 70 were determined in these cells, and expression was in agreement with that known to occur for *in situ* urothelium. The cells were also successfully tested for their ability to be stably transfected using expression vectors containing the MT-3 or MT-2A genes. The findings suggest that the UROtsa cells grown with a serum-free medium could be a valuable adjunct for studying environmental insult to the human urothelium in general and for the stress response in particular. *Key words*: bladder, bladder cancer, bladder cell line, heat shock proteins, immortalization, interstitial cystitis, metallothionein isoforms, stress response, urothelium. *Environ Health Perspect* 109:801–808 (2001). [Online 10 August 2001]
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Disorders of the bladder have a strong environmental base. Epidemiologically, bladder cancer represents one of the first cancers in which environmental carcinogens were found to play the major role. This association was first observed in 1895, when Rehn (*1*) demonstrated the link between exposure to aromatic amines and development of bladder cancer in factory workers. This association has been confirmed in both animal models and in humans working in industries that involve exposure to aromatic amines (*2*,*3*). Recently, many studies have defined an association between cigarette smoking and bladder cancer, with some reports suggesting a 2- to 4-fold increased risk and that 50% of the bladder cancers in men would not occur in the absence of cigarette smoking (*4*,*5*). The majority of the remaining bladder cancers are believed to be caused by industrial or agricultural carcinogens. The number of cigarettes smoked, degree of inhalation, type of tobacco, use of filters, and smoking cessation have all been shown to have specific relationships with the development of bladder cancer (*6*).

The strong environmental base of bladder cancer and the lack of biomarkers for diseases of the bladder led us to define the expression of the metallothionein (MT) and heat shock protein (hsp) genes in formalin-fixed, paraffin-embedded biopsy specimens from patients with bladder cancer and other diseases using a combination of immunohistochemical and reverse transcription-polymerase chain reaction (RT-PCR) techniques (*7*–*11*). The expression of these genes was chosen for analysis because they are members of the stress response gene superfamily, which are widely accepted as being major weapons in the cell's armamentarium for protection against and recovery from both physical and chemical environmental insult (*12*–*15*). For bladder cancer, it was demonstrated that MT-3 might be a potential biomarker because it was not expressed in normal urothelium but was overexpressed in all bladder cancers, with expression correlating directly to increasing tumor grade (*7*). It was also shown that the MT-1 and MT-2 isoforms had a low level of expression in normal urothelium and were overexpressed in many bladder cancers, with overexpression correlating to tumor grade (*8*). Furthermore, overexpression of the MT-1/2 protein in bladder cancer correlated with the increased expression of mRNA from the MT-1X isoform gene (*8*). For samples from patients with interstitial cystitis and related disorders, it was demonstrated that expression of the hsp 60 and hsp 70 was reduced compared to that found in the urothelium of the normal bladder, whereas hsp 27 and hsc 70 expression were unaltered (*9*–*11*).

While these empirical observations are interesting, an opportunity to gain a mechanistic insight into the underlying alterations in stress gene regulation in the bladder is extremely limited due to the complexities of acquiring human tissue—much less the use of formalin-fixed, paraffin-embedded biopsy material. Because of this limitation, we initially searched for cell culture models of human urothelium that would help define the alterations of metallothionein gene expression that occurs in human bladder cancer. Although highly characterized human bladder cancer-derived cell lines were easy to identify (*16*), the same was not true for cell lines that retain features of normal human urothelium. There are several requirements and desired features of a cell culture model of normal urothelium in general and, in particular, for use in studies that may involve MT. First, the cell culture has to be of human origin because the gene organization and regulation of the MT gene family are more complex in humans than in rodent animal models (*17*). Second, the resulting cell culture needs to retain known differentiated features of normal urothelium and the gene expression patterns identified *in situ* in normal urothelium. Additionally, the cell culture should be immortal, but not tumorigenic, to avoid the complexities of human tissue acquisition that are inherent in

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the isolation and propagation of primary cell cultures. Immortalization also allows the stable transfection of the cell culture with vectors containing gene sequences of interest, and the cell culture should be tested to assure it is receptive to transfection protocols. In the present study, we tested the UROtsa cell line to determine if it would fulfill the above requirements as a cell culture model of human urothelium. The UROtsa cell line was derived from the normal urothelium lining the ureter and was immortalized using the simian virus 40 (SV40) large T antigen (*17*,*18*). After immortalization, the cells did not acquire characteristics of neoplastic transformation, as noted by lack of colony formation in soft agar and growth of tumors in nude mice. The present study advances this initial characterization, and using light and electron microscopy, shows that propagation of the UROtsa cell line on a serum-free growth medium results in expression of structural features of differentiated urothelium. Furthermore, the resulting cultures retain the MT and hsp expression background expected from normal urothelium and can be stably transfected to overexpress the *MT-3* gene or knock-out *MT* gene expression using an antisense vector. The findings suggest the UROtsa cell line could be a valuable *in vitro* model for studies assessing the effects of environmental agents on human bladder urothelium.

Materials and Methods

Cell culture. Stock cultures of the UROtsa cell line were maintained using the original conditions described by Petzoldt et al. (*18*). Briefly, cells were grown on plastic using Dulbecco's modified Eagle's medium (DMEM) containing 5% v/v fetal bovine serum with incubation at 37°C in a 5% CO_2:95% air atmosphere. Confluent flasks were subcultured at a 1:4 ratio, and the cells were fed fresh growth medium every 3 days. The UROtsa cells normally attained confluency within 7 days after subculture.

Development of serum-free growth conditions. The initial step in development of a serum-free growth formulation was to allow the UROtsa cells to attain confluence in standard serum-containing growth media and then to change the media to a serum-free formulation used previously by this laboratory for the growth of human proximal tubule cells (*19*). The serum-free growth medium was composed of a 1:1 mixture of DMEM and Ham's F-12 supplemented with selenium (5 ng/mL), insulin (5 µg/mL), transferrin (5 µg/mL), hydrocortisone (36 ng/mL), triiodothyronine (4 pg/mL), and epidermal growth factor (EGF; 10 ng/mL). The cells were fed this serum-free growth formulation for two additional feedings at 3-day intervals and then subcultured at a 1:4 ratio into the serum-free formulation. As will be detailed, the UROtsa cells proliferated under these conditions with an indefinite culture life span. The cells were able to proliferate on the standard plastic growth surface. Using these cells, a deletion study was performed to determine the minimal requirements for growth of UROtsa cells in culture. UROtsa cells maintained in complete serum-free growth medium were subcultured in six-well plates at a 1:4 subculture ratio. Each plate contained three control wells and three experimental wells. Control wells were fed with the complete serum-free growth medium. Experimental cultures were fed with serum-free medium that was deficient in one of the supplements described above. All cultures were maintained under standard conditions for 10 days, and the time necessary for each of the cultures to reach confluence was recorded. We monitored cells daily and documented growth with phase-contrast micrographs.

Light, electron, and freeze-fracture microscopy. A record of the light-level morphology of the cultured cells was maintained using an Olympus IX70 inverted microscope with capture of the digital image using a Kontron Prog/Res/3012 camera and the Autocyte Image Management System (Zeiss, Thornwood, NY, USA). For ultrastructural analysis, the cultured cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 30 min *in situ*. The cells were then rinsed two times with phosphate buffered saline (pH 7.4) and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 1 hr. The cells were rinsed, routinely dehydrated in a graded series of ethanol, and infiltrated and embedded in Epon 812 (LADD Research Industries Inc., Burlington, VT, USA). Upon polymerization, portions of the resulting preparation were cut and reembedded in additional Epon 812 in two spatial orientations. In this manner, ultrathin sections were obtained in planes that were parallel and perpendicular to the growth surface. Sections were stained with uranyl acetate and lead citrate and viewed and photographed using an electron microscope.

For freeze-fracture analysis, confluent cultures of cells were subcultured onto mylar strips and fractured as described previously (*20*). Briefly, the cells were fixed by treatment with 2.5% glutaraldehyde in culture medium for 1 hr followed by 25% glycerol in culture medium for 30 min. Using a tuberculin syringe and needle, a small drop of polyvinyl alcohol (Elvanol; E.I. DuPont de Nemours and Co., Wilmington, DE, USA) was placed on a 3-mm gold specimen carrier. A section of mylar with attached cells was drained of excess fluid and placed cell-side up on the polyvinyl alcohol. A second drop of polyvinyl alcohol was placed on the surface of the monolayer. A strip of mylar (2×4 mm) was positioned on the second drop of polyvinyl alcohol so that one end overlapped the edge of the filter and specimen carrier. The resulting assembly was frozen in a liquid–solid nitrogen slush and mounted on a standard three-position Balzers specimen table. Using a Balzers BAF 400T freeze-etch unit (BAL-TEC AG, Balzers, Principality of Liechtenstein), fractures were obtained at –105°C and 10^{-7} mbar by positioning the knife blade beneath the free edge of the mylar strip and raising the knife blade. Following platinum/carbon and carbon deposition, the replicas with attached cells were floated from the specimen carriers in physiologic saline. These were treated with chromic-sulfuric acid and the replicas placed in 50% chromic-sulfuric acid overnight. Replicas were rinsed three times with distilled water, mounted on copper grids, and viewed in an electron microscope.

RNA isolation and RT-PCR analysis of MT and hsp mRNA expression in UROtsa cells. Total RNA was isolated according to the protocol supplied with TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH, USA) as described previously (*21*). We determined the concentration and purity of samples using spectrophotometer scan in the UV region and ethidium bromide (EtBr) visualization of intact 18S and 28S RNA bands following agarose gel electrophoresis. Total RNA (0.5 µg) was reverse transcribed using MuLV (murine leukemia virus) reverse transcriptase (50 U) in 1× PCR buffer (50 mM KCl and 10 mM Tris-HCl, pH 8.3), 5 mM $MgCl_2$, 20 U RNase inhibitor, 1 mM each of the dNTPs, and 2.5 µM random hexanucleotide primers. The samples were reverse transcribed for 20 min at 42°C, followed by a 5-min denaturation step at 99°C using a DNA thermocycler (Perkin-Elmer-Cetus 9600; Perkin Elmer, Foster City, CA, USA). The reverse-transcribed product was used for PCR amplification using the AmpliTaq DNA polymerase enzyme (2.5 U; Perkin Elmer) and the specific upstream and downstream primers. The primers and reaction conditions developed for analysis of each of the active *MT* genes and hsp 27, hsp 60, hsc 70, and hsp 70A,B,C have been previously described (*21–25*). Controls for each PCR included a no-template control where water was added instead of the RNA and a no-reverse-transcriptase control where water was added instead of the enzyme. Samples were removed at 25, 30, 35, and 40 PCR cycles to ensure that the reaction remained in the linear region. The final PCR products were electrophoresed on 2% agarose gels containing EtBr along with DNA markers.

The intensity (integrated optical density; IOD) of the PCR product bands was determined on a Dell workstation (Dell Computer Corporation, Austin, TX, USA) configured with Kontron KS 400 image analysis software (Carl Zeiss Vision, Thornwood, NY, USA).

MT protein determination. The immunoblot protocol used for the determination of the levels of MT-1 and MT-2 and MT-3 protein in cell lysates has been described previously (*26,27*). The MT-1 and MT-2 proteins were detected by immunoblotting using a mouse anti-horse antibody (DAKO-MT, E9, Dako, Carpinteria, CA, USA) as the primary antibody. This antibody detects both the MT-1 and MT-2 isoforms, and the product detected is referred to as MT-1/2 in this report. MT-1/2 protein was quantified by comparing the optical density of the sample dots to that of the standard MT curve using KS 400 image analysis software. Rabbit liver Cd/Zn metallothionein-1 (Sigma Chemical Co., St. Louis, MO, USA) was applied to each blot to generate standard curves. This assay has detection limits in the range of 0.1–0.5 ng MT-1/2 protein. The MT-3 protein was detected using an antibody against human MT-3 that was generated using the dodecapeptide GGEAAEAEAEKC (corresponding to MT-3 amino acids, 53-64, which contains the MT-3 unique amino acid insert) conjugated through the C-terminal cysteine sulfhydryl group to keyhole limpet hemocyanine using maleimidobenzoyl-*N*-hydroxysuccinimide ester. This was used to immunize New Zealand white rabbits. The MT-3 antibody was affinity purified using the dodecapeptide linked to SulfoLink gel (Pierce, Rockford, IL, USA) through the C-terminal cysteine residue. MT-3 protein was quantified by comparing the optical density of the sample dots to the standard MT-3 curve using KS 400 image analysis software. Standard curves were obtained by applying known amounts of the conjugated synthetic peptide to each blot. This assay has detection limits in the range of 0.5–2 pg MT-3 protein.

Western analysis of hsp protein expression. The determination of the hsp 27, hsp 60, hsc 70, and hsp 70 proteins by Western analysis has been described previously (*23–25*). The hsp 27 was detected using a primary mouse monoclonal antibody (SPA-800; StressGen, Victoria, BC, Canada); hsp 60 using a primary mouse monoclonal antibody (STM-806; StressGen); hsc 70 using a primary rat monoclonal antibody (SPA-815; StressGen); and hsp 70 using a primary mouse monoclonal antibody (SPA-810; StressGen). For reactions in the linear range of analysis, we obtained IODs of the samples by inputting the image to a Dell workstation configured with KS400 software using a Kodak DCS 420 CCD camera (Eastman Kodak Co., Rochester, NY, USA).

Transfection of UROtsa cells. The MT-2A and MT-3 coding sequences were cloned from cultured human proximal tubule cell RNA by RT-PCR using primers described previously by this laboratory (*28*). The sequences were blunt-end ligated into the *Eco*R V site of pcDNA3.1/Hygro (+) (Invitrogen, Carlsbad, CA, USA). This vector has a cytomegalovirus immediate-early promoter upstream of the multiple cloning site and a hygromycin B resistance gene driven by an SV40 early promoter. All DNA constructs were linearized by *Fsp* I before transfection. The UROtsa cells were maintained in DMEM with 5% fetal bovine serum and were transfected with the MT-3 plasmid construct in the sense direction and MT-2A in the antisense orientation or the vector alone by using Effectene transfection reagent (Qiagen, Valencia, CA, USA). Briefly, lipid DNA complexes were prepared according to the manufacturer's protocol at a ratio of 1:10 plasmid to Effectene. Lipid complexes were added to cells at 2 µg DNA per 9.6-cm^2 well for 24 hr. The cells were placed in normal media for 48 hr, trypsinized, and seeded at 8% confluency for selection in growth medium containing 30 µg/mL hygromycin B. Clones were selected using cloning rings and propagated in media containing 30 µg/mL hygromycin B. The stable transfectants were identified, recloned, and preserved in liquid nitrogen storage. MT-3 mRNA was identified by RT-PCR using MT-3 specific primers and MT-2 antisense using vector specific primers (CGGATCCACTAGTCCAGTGTG; upstream and ACGGGCCCTCTAGACTCG; downstream).

Results

Morphology of UROtsa cells on serum-containing growth medium. Stock cultures of the UROtsa cells have been maintained on serum-containing growth media for more than 100 population doublings with no alteration in the rate of growth (doubling time of approximately 30 hr) or in the light-level morphology of the cells (data not shown). The light-level morphology of the UROtsa cells on serum-containing growth media have a flattened appearance with an epitheloid morphology at both subconfluent and confluence densities (Figure 1A,B). The UROtsa cells appear to grow as a monolayer and, even when maintained for 14 days at confluence, show no tendency to multilayer or form foci of multilayered cells (Figure 1C). The monolayer growth of the UROtsa cells on serum-containing growth media was confirmed by ultrastructural analysis. Examination of highly confluent cells sectioned perpendicular to the growth surface at low magnification showed that the cells overlap one another in a monolayer arrangement and are connected to one another by frequent desmosomal connections (Figure 2A). The cells had a high nuclear-to-cytoplasmic ratio, and profiles of intermediate filaments were prevalent in the cytoplasm of the cells (Figure 2B,C). The apical surface of the cells possessed occasional microvilli, and the overlapping segments of the cells had moderate lateral interdigitation (Figure 2). The low-magnification micrographs showed no evidence of tight junctions between the

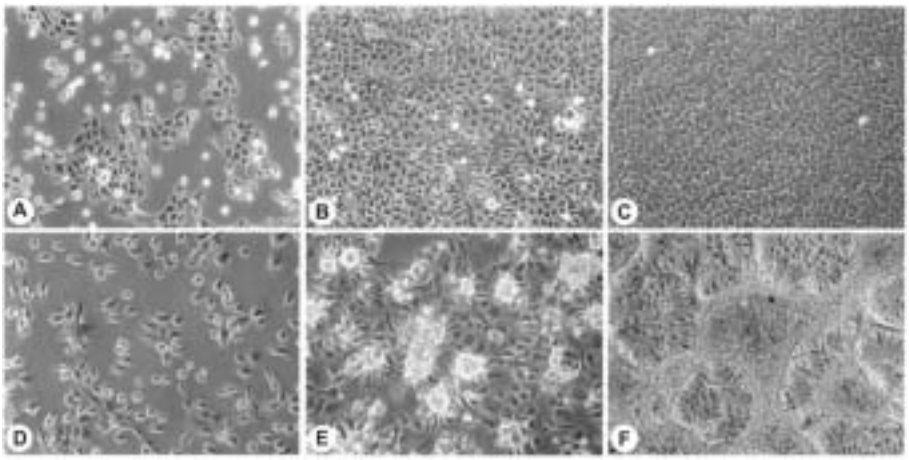

Figure 1. Phase-contrast photomicrographs of UROtsa cultures at various stages of confluency (10×). (*A*) Subconfluent UROtsa (1 day after subculture) and (*B*) confluent UROtsa cells (7 days after subculture) maintained in serum-containing medium exhibit an epithelial morphology. (*C*) UROtsa cells grown in serum-containing medium remain as a monolayer after 14 days in culture. (*D*) Subconfluent UROtsa cells (1 day after subculture) maintained in serum-free medium have an epithelial morphology that gives rise to the development of (*E*) foci of differentiation as the cells achieve confluency (7 days after subculture). (*F*) Postconfluent UROtsa cells (14 days after subculture) have a polarized epithelial morphology with prominent foci projecting from the surface of the growth plane. The UROtsa cells grown with serum-free medium have been subcultured more than 20 times and retained morphology.

apical poles of the cells or the presence of gap junctions. The lack of these structures was confirmed by examination of the apical points of interaction between adjacent cells (Figure 2B,C).

Growth and morphology of UROtsa cells on serum-free growth medium. The UROtsa cell line was successfully placed into a serum-free growth media used previously by this laboratory for the growth of human proximal tubule cells (*19,20*). This serum-free formulation consisted of a 1:1 mixture of DMEM and Ham's F-12 supplemented with selenium (5 ng/mL), insulin (5 µg/ml), transferrin (5 µg/mL), hydrocortisone (36 ng/mL), triiodothyronine (4 pg/mL), and EGF (10 ng/mL). Unlike the human proximal tubule cells, the UROtsa cells needed no matrix other than the plastic growth surface on which to attach and proliferate. The light-level morphology of the UROtsa cells grown on serum-free media was altered from that noted to occur on serum-containing growth media. The UROtsa cells grown on serum-free growth media had a more polarized epithelial morphology when subconfluent and, upon reaching confluency, developed areas of multicellular organoid structures that became even more extensive as the cells were maintained past confluence (Figure 1D,E,F). Ultrastructural examination at low magnification of newly confluent cells sectioned perpendicular to the growth surface confirmed that the raised areas noted on light microscopy were, in fact, areas of cells arranged in a multilayered formation (Figure 2D). The cells contained abundant profiles of intermediate filaments, and there was some stratification of the cells from the bottom to the top of the multilayered structure, with cells located apically having a more differentiated appearance. The cells contained frequent desmosomal connections and the apical aspects of the cells were noted to interdigitate extensively. Higher magnifications demonstrate the extensive connections between cells of the multilayer (Figure 2E,F) and the degree of complexity of the lateral interdigitations between cells and the extensive interaction between cells at the apical surface of adjacent cells. These interactions between cells suggested that the cells might possess gap junctions and tight junctions, but the complexity of the cell-to-cell interactions rendered this hard to determine using routine ultrastructural examination.

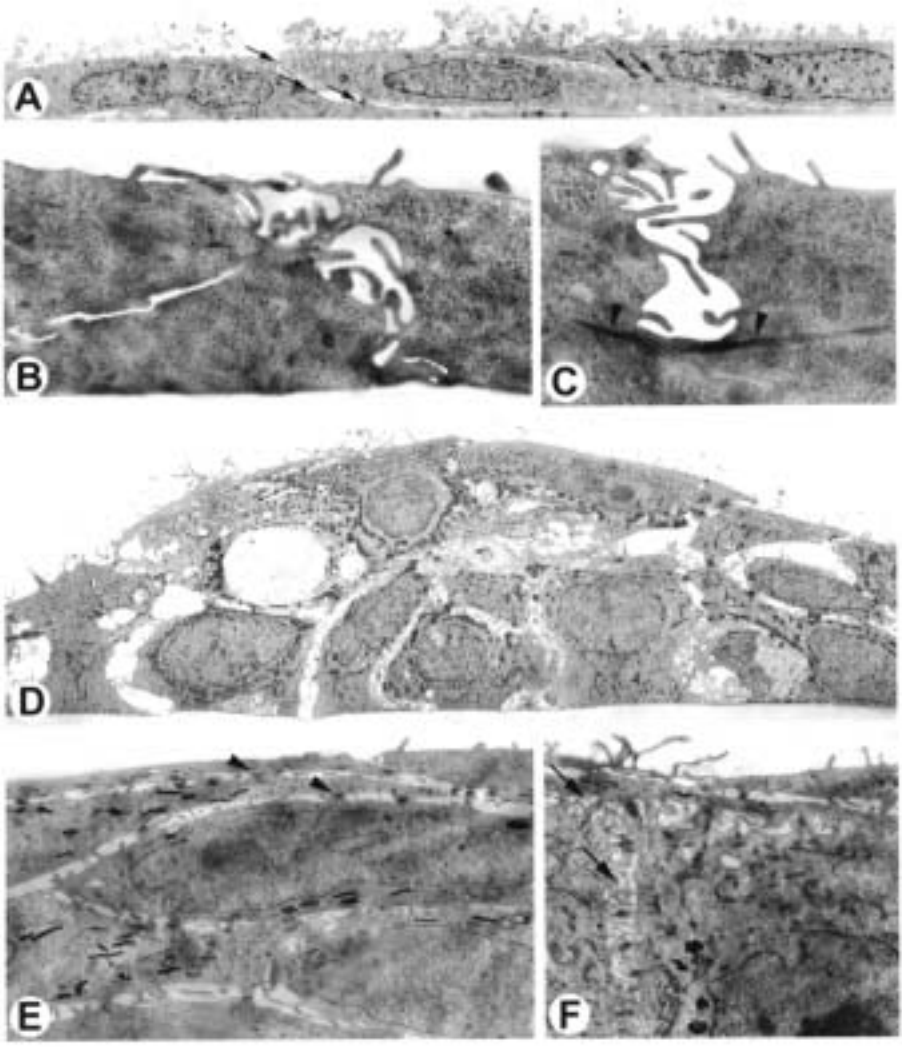

Figure 2. Transmission electron micrographs of postconfluent UROtsa cells sectioned perpendicular to the growth surface. (*A*) Low magnification photomicrograph (5,000×) of UROtsa cells maintained in serum-containing medium shows that these cells have characteristics of undifferentiated basal cells. The cells are arranged in an overlapping monolayer with frequent desmosomal connections (arrows) and have a high nuclear-to-cytoplasmic ratio. (*B*, *C*) Higher magnification photomicrographs (12,500 and 30,000×, respectively) of UROtsa cells grown in serum-containing medium demonstrate that, despite the presence of desmosomes and intermediate filaments (*C*, arrowheads), there is no evidence of tight junctions or gap junctions. (*D*) Low magnification photomicrograph (3,000×) shows that UROtsa cells maintained in serum-free medium have multilayered areas that appear to be representative of transitional urothelium. (*E*, *F*) Higher magnification transmission electron micrographs (10,000 and 20,000×, respectively) of UROtsa cells maintained in serum-free medium demonstrate extensive interactions between cells at the apical surface of adjacent cells (*E*, arrowheads) and complex lateral interdigitation between cells (*F*, arrows).

Because gap junctions are a known feature of urothelium and tight junctions are a feature of the apical-most cells, we used the technique of freeze fracture to confirm the presence of these structures. Freeze-fracture analysis disclosed the presence of both gap junctions and tight-junction sealing strands in the UROtsa cells cultured on serum-free growth medium (Figure 3 A,B,C). The low power micrograph of the replica demonstrates the presence of gap junctions on the surface of the cells and profiles of desomosomal connections and tight junctional sealing strands between the cells (Figure 3A). The higher power micrograph of the fracture replicas provides details of the complexity of the tight-junction sealing strands (Figure 3 B,C).

We performed a deletion study on the serum-free growth medium to determine which components were essential for the growth and differentiation of the UROtsa cells (data not shown). The results of this study demonstrated that only EGF was required for the immediate growth and differentiation of the cells. The absence of EGF resulted in cells that could neither proliferate nor differentiate after subculture. The absence of EGF had no effect on the ability of the UROtsa cells to remain viable and differentiated once a high level of confluence had been attained. The only other component of the growth medium that had an effect on cell growth was insulin, and a slowing of cell growth was only noticeable after insulin was absent from the growth media for several passages. The other components of the

serum-free medium had no noticeable positive or negative contribution to the growth or differentiation of the UROtsa cells.

Basal expression of hsp 27, hsp 60, hsp 70, and hsc 70 mRNA and protein by UROtsa cells. Total RNA was isolated from confluent UROtsa cells grown on both serum-containing and serum-free growth medium and used to determine the basal expression of the respective heat shock protein genes relative to the glyceraldehyde 3-phosphate dehydrogenase (*g3pdh*) housekeeping gene using RT-PCR technology as described previously (*23–25*). The expression of g3pdh mRNA was identical for the UROtsa cells grown on serum-containing and serum-free growth media, as noted by product bands of equal intensity at 30 reaction cycles using a total RNA input of 500 ng (Figure 4). The basal expression of hsp 27 mRNA was also identical for the UROtsa cells grown on both serum-containing and serum-free growth media (Figure 4). In both cases, the basal level of hsp 27 mRNA expression were high relative to the *g3pdh* housekeeping gene as evidenced by the fact that an hsp 27 reaction product of approximate equal intensity could be detected after only 20 cycles of the PCR at a 500 ng total RNA input.

There was no difference in the basal expression of hsp 60 mRNA between the UROtsa cells grown on serum-containing and serum-free media (Figure 4). A positive reaction product for hsp 60 mRNA was routinely detected after 40 cycles of the PCR using 500 ng total RNA from UROtsa cells grown on either serum-containing or serum-free growth media.

As shown in Figure 4, the basal level of hsc 70 mRNA was identical for the UROtsa cells regardless of growth media composition, and relative expression was below that of the *g3pdh* housekeeping gene (35 vs. 30 cycles of PCR, respectively). No basal expression of mRNA for the hsp 70B or hsp 70C isoform genes were detected in the UROtsa cells regardless of growth media when using a 500 ng total RNA input and 40 cycles of the PCR (Figure 4). In contrast, basal expression of mRNA for the hsp 70A isoform gene was detected in the UROtsa cells grown on both media, but basal expression was much higher in cells grown on the serum-containing growth media (Figure 4). For UROtsa cells grown on serum-containing media, basal expression of hsp 70A mRNA was routinely detected at 35 PCR cycles, whereas only a marginal reaction product was demonstrated following 35 PCR cycles using equal total RNA from UROtsa cells grown on serum-free media. Thus, UROtsa cells express basal levels of mRNA for the *hsp 27*, *hsp 60*, *hsc 70*, and *hsp 70A* genes regardless of growth media composition, but demonstrate reduced relative levels of mRNA expression for the *hsp 70A* gene when grown on serum-free media.

In agreement with the basal expression of hsp 27 mRNA, an analysis of hsp 27 protein expression by Western blotting demonstrated that the basal expression of the hsp 27 protein was approximately equal when the cells were propagated on either growth media (Figure 5). This agreement between mRNA and protein expression was also demonstrated for the hsp 60 and hsc 70 protein (Figure 5). In contrast, the reduction in hsp 70A mRNA expression noted for UROtsa cells grown on serum-free media was enhanced at the level of protein expression, with hsp 70 protein being undetectable for cells grown in serum-free growth media, even at 10-µg inputs of total protein (Figure 5). Thus, UROtsa cells express basal levels of the hsp 27, hsp 60, and hsc 70 proteins regardless of growth media composition but demonstrate expression of hsp 70 protein only on serum-containing growth media.

Basal expression of metallothionein isoform-specific mRNA and protein by UROtsa cells. We also used the total RNA isolated from confluent UROtsa cells grown on both serum-containing and serum-free growth media to determine the basal expression of the MT isoform-specific mRNAs relative to the *g3pdh* housekeeping gene using RT-PCR and gene-specific primers, as described previously (*21,22,28*). Using a total RNA input of 500 ng and 40 cycles of PCR, it was demonstrated that there was no expression of mRNA representing the *MT-1A*, *MT-1B*, *MT-1F*, *MT-1G*, *MT-1H*, *MT-3*, or *MT-4* genes in UROtsa cells grown on either serum-containing or serum-free growth media (Figure 6). In contrast, mRNA representing the *MT-1E*, *MT-1X*, and *MT-2A* genes was expressed in

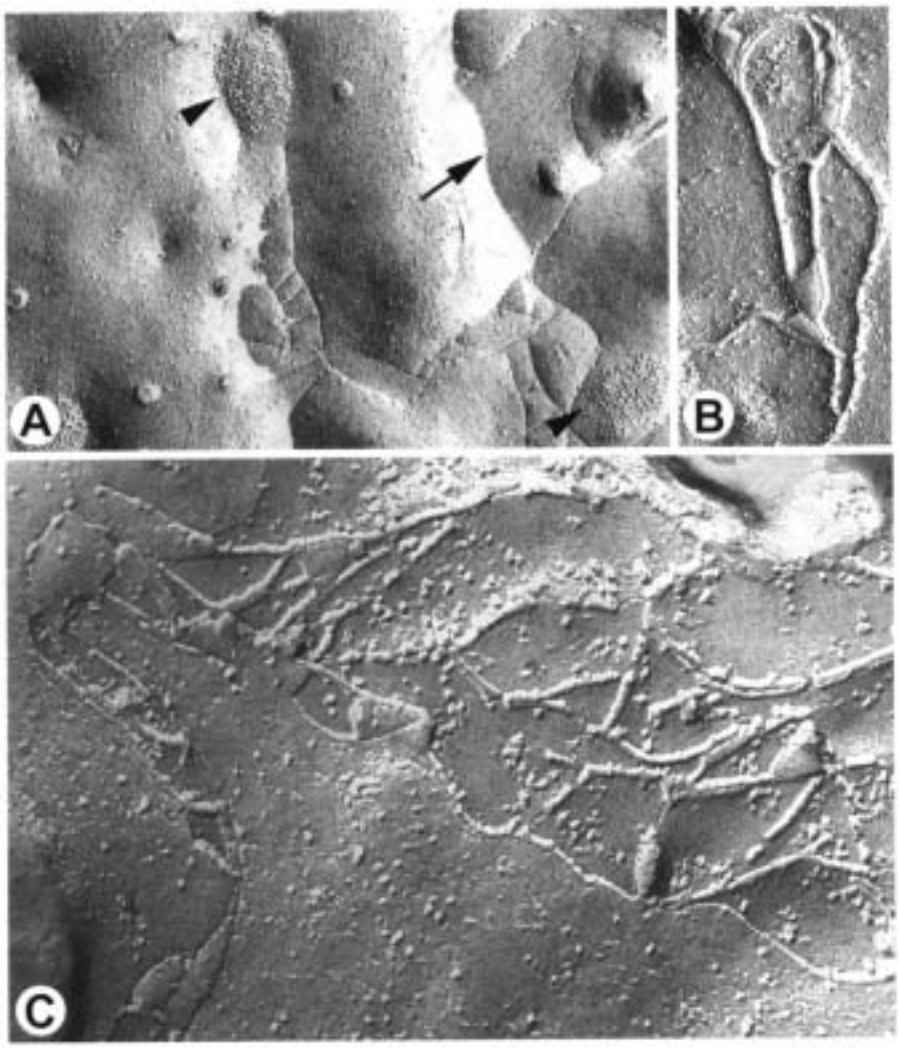

Figure 3. Freeze-fracture transmission electron micrographs of UROtsa cells maintained on serum-free medium. (*A*) Freeze-fracture replica (40,000×) of UROtsa cells grown in serum-free medium shows tight-junction sealing strands, desmosomes (arrowheads), and a prominent gap junction (arrow). Additional replicas (*B*, *C*) highlight the complexity of the tight-junction sealing strands in these cultures (both at 150,000×).

UROtsa cells grown on either serum-containing or serum-free growth media following 35, 30, and 35 cycles of PCR (at 500-ng total RNA inputs), respectively (Figure 6).

Immunoblotting with an MT antibody that recognizes both the MT-1 and MT-2 isoforms, but not the MT-3 or MT-4 isoforms, demonstrated that the cells expressed 0.5 ng of MT-1/2 protein/µg total protein when grown on either growth media (data not shown). Immunoblotting with an MT-3-specific antibody demonstrated no MT-3 protein in the UROtsa cells (limit of detection 0.5–2 pg/µg total protein).

Stable transfection of the UROtsa cell line with vectors overexpressing the MT-3 *gene and an MT-2A isoform antisense sequence.* The coding sequence of the *MT-3* gene was obtained from human proximal tubule cell RNA by RT-PCR, blunt-end ligated into the *Eco*RV site of pcDNA3.1/Hygro(+), and linearized by *Fsp* I before transfection of the UROtsa cells. The UROtsa cells were transfected with the MT-3 plasmid construct in the sense direction or by the vector without insert using the Effectene protocol. After selection in hygromycin B-containing growth medium, four clones were selected for further characterization. It was demonstrated using RT-PCR that each of the four clones overexpressed MT-3 mRNA when compared to wild-type UROtsa cells or UROtsa cells containing the pcDNA3.1 vector without the MT-3 sequence (Figure 7). Expression of MT-3 protein went from nondetectable levels in the wild-type UROtsa cells and the vector controls to a level of 0.3 ng/µg total protein in the MT-3 stably transfected clones (range 0.14–0.30 ng/µg; data not shown). The level of MT-3 mRNA and protein in the stable transfected clones was not affected by growth medium composition, nor did overexpression change the light level morphology of the cells (data not shown).

The coding sequence of the *MT-2A* gene was obtained from human proximal tubule cell RNA by RT-PCR, blunt-end ligated into the *Eco*RV site of pcDNA3.1/Hygro(+), and linearized by *Fsp* I before transfection of the UROtsa cells. The UROtsa cells were transfected with the MT-2A plasmid in the antisense direction or by vector without MT-2A insert using the Effectene protocol. After selection in hygromycin B-containing growth medium, four clones were selected for further characterization. The presence of the antisense MT-2A sequence was confirmed by RT-PCR using vector-specific primers, and it was demonstrated that all four clones overexpressed mRNA for the insert when compared to the vector only control or wild-type UROtsa cells (Figure 8). That the MT-2 antisense sequence was productive in inhibiting MT-1 and MT-2 isoform-specific mRNA expression was confirmed by the finding that no MT-1/2 protein was detected in any of the four clones. The inhibition of MT-1/2 protein expression by MT-2A antisense overexpression had no effect on the light-level morphology of the cells (data not shown).

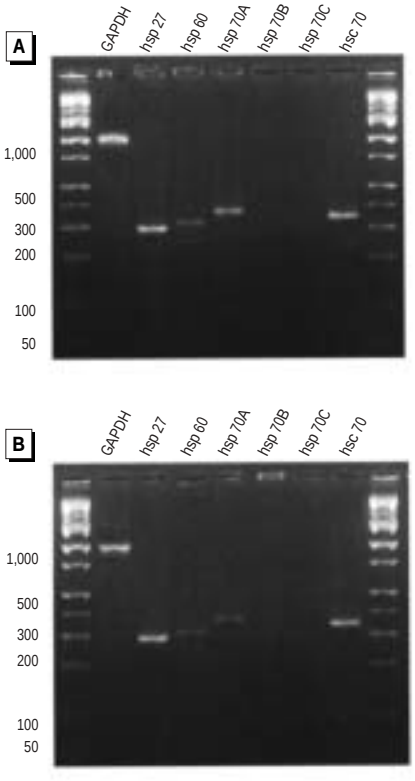

Figure 4. Basal heat shock protein gene expression for UROtsa cells maintained in serum-containing and serum-free media. (*A*) RT-PCR of serum UROtsa mRNA shows basal expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at 30 cycles, hsp 27 (25 cycles), hsp 60 (40 cycles), hsp 70A (35 cycles), and hsc 70 (35 cycles). Gene expression for hsp 70B and hsp 70C was not evident at 40 cycles. (*B*) RT-PCR of serum-free UROtsa mRNA shows a similar expression pattern as that described for serum UROtsa cultures.

Discussion

The goal of the present study was to determine if the UROtsa cell line might serve as a model to study human bladder urothelium in general and the stress response in particular. The study was motivated by the apparent lack of a widely used human-derived cell culture model for studying urothelium that retains important features of urothelial cell differentiation. A major factor inhibiting development of a widely used human urothelial cell culture model probably involves the complexity of acquiring human tissue, because primary cell culture models have been developed that appear to recapitulate the major features of the urothelium. An inherent requirement of a primary (single-use) culture system is that one has access to a constant supply of appreciable amounts of viable human bladder tissue.

Previously developed primary culture systems for studying urothelium have been summarized in a recent report that details a primary urothelial cell culture system derived from rabbit bladder that mimics most, if not all, of the major characteristics of urothelium found *in vivo* (*29*). These cultures consisted of an underlying cell layer that interacts with a collagen substratum, an intermediate cell layer, and an upper cell layer of large, superficial cells. These superficial cells were identified as umbrella cells based on their ability to form junctional complexes, possession of an asymmetric unit membrane, expression of

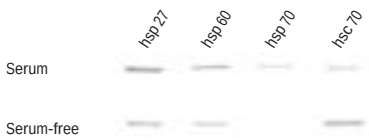

Figure 5. Basal heat shock protein expression for UROtsa cells maintained in serum-containing and serum-free media. UROtsa total protein extracts show basal expression of hsp 27, hsp 60, and hsc 70. Western analysis demonstrated the presence of hsp 70 in serum UROtsa extracts, but not in serum-free extracts. Total cell protein analyzed was 5 µg for hsp 27 and hsp 60, and 10 µg for hsp 70.

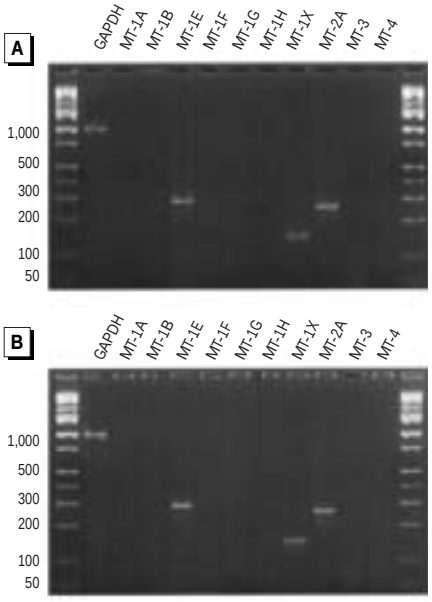

Figure 6. Basal metallothionein gene expression for UROtsa cultures maintained in serum-containing and serum-free media. (*A*) RT-PCR of serum UROtsa cells shows basal expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at 30 cycles and metallothionein isoforms 1E (35 cycles), 1X (30 cycles), and 2A (35 cycles). Other metallotionein isoforms (1A, 1B, etc.) were not expressed at 40 cycles. (*B*) RT-PCR of serum-free UROtsa cells show an identical expression pattern as that described for serum UROtsa cells.

uroplakins and a 27-kDa urothelial cell-specific antigen that assembled into detergent-resistant asymmetric unit membrane particles. The multilayered cultures were also shown to have low diffusive permeabilities for water and urea and high transepithelial resistance when the calcium concentration of the growth medium was increased to 1 mM. For all parameters tested, these primary cultures exhibited hallmark characteristics of *in situ* urothelium.

In an attempt to overcome the problems associated with acquiring human tissue, several investigators have attempted to immortalize primary cultures of human urothelium using SV 40 or a construct containing the SV 40 large T antigen (*18*,*30*). The first of these efforts (*30*) resulted in an immortal epithelial cell culture that was easy to grow on serum-containing growth medium, required no substrate other than plastic for attachment, required no irradiated feeder cell layer, and had a generation time similar to that of primary cultures of human urothelial cell cultures. Unfortunately, the cells lacked one of the major differentiated properties of urothelium: they had monolayer cell growth instead of cell stratification—cell stratification being a hallmark of urothelium. The cells were able to grow in soft agar but were unable to form tumors in athymic mice. The second effort used a construct containing the SV40 large T antigen and produced a similar immortal cell culture (UROtsa) that was easy to use and proliferated on serum-containing growth medium and required no special matrix for medium additions. The UROtsa cells did not grow in soft agar or form tumors in athymic mice. As confirmed by ultrastructural examination in the present study, the UROtsa cells also grew as a monolayer of cells and did not stratify, as would be expected to occur from primary cultures and *in situ* morphology. Thus, currently available culture models for human urothelium are at the two extremes of the technique: highly differentiated primary cultures with no serial growth potential and undifferentiated cultures with unlimited serial growth potential.

The working hypothesis in the current study was that the failure of the UROtsa cells to differentiate might be due to the serum-containing growth medium and not due to an inherent inability of the immortalized cells to differentiate. This hypothesis was tested by adapting the cells to a serum-free growth formulation used successfully for the growth of human proximal tubule cells (*19*). When confluent, UROtsa cells were changed to this growth formulation and then subcultured further using the serum-free medium, and the result was a cell culture that possessed an altered morphology compared to the same cells grown on serum. When examined by light microscopy, preconfluent cells had a similar morphology regardless of growth medium composition. However, once the cells were confluent, the serum-free cultures continued to proliferate and formed raised, three-dimensional interactive structures with one another in many areas of the flask. Routine ultrastructural examination disclosed that the raised areas of the cell cultures displayed the stratification expected of differentiated urothelial cells. Also displayed were the numerous desmosomal connections between cells and abundant cytoplasmic intermediate filaments expected of such cells. There was no evidence of terminal keratinization in the cell cultures, and cells could be serially passaged indefinitely using standard cell culture methods. A comparison of the ultrastructural morphology of the cells with that of *in situ* urothelium suggested that the cultured cells were similar in differentiation to the intermediate layer of the bladder uroepithelium (*31*–*33*). The finding on freeze fracture analysis that the cultured cells possessed tight junctional sealing strands further suggested a level of differentiation similar to the apical intermediate layer of the bladder urothelium. However, the organization of the sealing strands was not complex enough, nor did the cells possess an asymmetric unit membrane, to suggest that the UROtsa cultures have characteristics similar to that of the most apically located umbrella cells.

At the current level of cell culture development, the UROtsa cells grown on the described serum-free growth medium appear to provide a valuable new model that retains important features of urothelial cell differentiation on which to base studies regarding human bladder urothelium. Although outside the scope of the present study, findings with the rabbit primary culture system (*29*) suggest that further manipulation of the cell culture technique could modify the level of differentiation of the UROtsa cell cultures toward either the highly differentiated umbrella cell or the undifferentiated basal cell. This assumption is based on findings in the rabbit primary culture system which showed that for the urothelium to achieve maximal differentiation (from an undifferentiated basal cell layer to a high resistance apical umbrella cell layer) required growth of the cells on a permeable support, careful attention to seeding densities, and manipulation of the calcium concentration of the growth medium (*29*). Likewise, these same studies demonstrated that lowering the calcium concentration of the growth medium resulted in the proliferation of undifferentiated basal cells. Although none of these manipulations were tested in the present study, there is no reason to anticipate that they should not prove operable for the UROtsa cell culture system. The ability to manipulate the degree of differentiation of an easy-to-grow immortal human urothelial cell line by cell culture conditions would have enhanced value for modeling the response of human urothelium to environmental agents.

An additional goal of this study was to determine if the UROtsa cells would have basal patterns of MT and heat shock gene expression similar to those found in both fresh and formalin-fixed normal human urothelium (*7*–*11*). Complete agreement was found between the expression patterns of the MT isoform-specific genes and MT proteins between the UROtsa cells grown on either growth medium and that found *in situ* urothelium. Specifically, expression of the *MT-1E*, *MT-1X*, and *MT-2A* genes and MT-1 and 2 proteins were found in both the cultured cells and *in situ* urothelium (*7*,*8*).

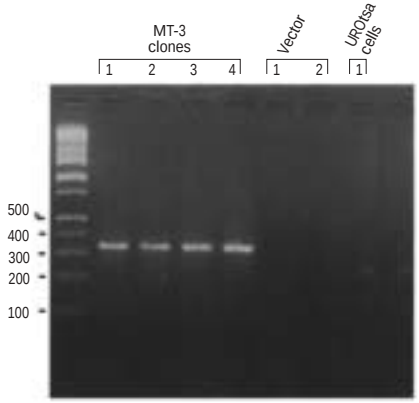

Figure 7. Stable overexpression of MT-3 in UROtsa cells. RT-PCR for MT-3 was performed on RNA from nontransfected UROtsa cells and clones of MT-3 or control vector-transfected cells at 30 cycles with MT-3 specific primers. Reaction products were electrophoresed on 2% agarose gels containing 0.5 µg/mL ethidium bromide.

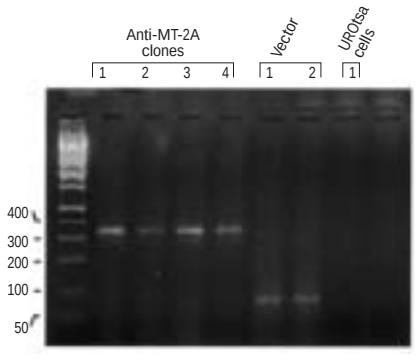

Figure 8. Stable overexpression of MT-2A antisense. RT-PCR was performed on RNA from nontransfected UROtsa cells and clones expressing MT-2A in the reverse orientation or vector-only sequences at 30 cycles with vector-specific primers. Reaction products were electrophoresed on 2% agarose gels containing 0.5 µg/mL ethidium bromide.

No expression of MT-3 mRNA or protein was detected in the UROtsa cells. The lack of MT-3 expression is significant because MT-3 has been shown to be overexpressed in transitional cell carcinoma of the bladder and in associated areas of dysplasia (*7*). A similar analysis of hsp 27, hsp 60, hsc 70, and hsp 70 expression demonstrated complete agreement in the basal patterns of expression of hsp 27, hsp 60, and hsc 70 between the cultured cells and *in situ* urothelium (*9–11*). The expression of hsp 70 mRNA and protein was different between the UROtsa cells cultured on serum-free and serum-containing growth media, but this difference correlated to the localization of hsp 70 protein within *in situ* urothelium. For *in situ* urothelium, the expression of hsp 70 was localized using immunohistochemistry only in the lower basal cells of the intact urothelium, with the more differentiated upper layers of the urothelium being devoid of hsp 70 immunoreactivity (*10*). We found hsp 70 expression only for cells grown on serum-containing growth medium. This pattern of expression correlated with the degree of differentiation of the UROtsa cells grown in the respective growth media.

Finally, for an immortal cell culture model to have full utility and ease of use, it needs to be receptive to stable transfection by expression vectors. This was tested successfully for the UROtsa cells using both sense and antisense sequences. The first was stable transfection of the coding sequence of the *MT-3* gene under the control of the CMV promoter. Stable clones that overexpressed both MT-3 mRNA and protein were obtained, purified, and serially subcultured without difficulty. The second test was the stable transfection of an antisense sequence of the coding region of the *MT-2A* gene that, due to sequence homology among the *MT* genes, would be expected to knock out the mRNA expression of any *MT* gene. Again, stable clones that overexpressed RNA for the expression vector plus insert and underexpressed the MT protein were obtained, purified, and serially subcultured. In conclusion, the findings of this study indicate that the UROtsa cells can serve as a valuable adjunct for studying the human urothelium in general and the stress response in particular.

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