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Induction of the *TRPM-2* Gene in Cells Undergoing Programmed Death

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RNA and protein products encoded by the testosterone-repressed prostate message-2 gene (*TRPM-2*) are induced to high levels, coordinate with the onset of cell death, in numerous rodent models of inducible tissue damage. These models include cell death initiated by hormonal stimuli (prostate regression), pressure insult (renal atrophy after ureteral obstruction), developmental stimuli (necrosis of interdigital tissue), and cytotoxic injury (chemotherapeutic regression of a tumor). Sequence analysis of cDNA encoding TRPM-2 revealed its close homology with a product referred to as SGP-2 or clusterin expressed constitutively by Sertoli cells; however, the immunologically related polypeptides expressed in regressing tissues differ in molecular mass from the forms secreted by the testis. Although the function(s) of the products encoded by the *TRPM-2* gene remains unclear, their presence provides a remarkable and early indicator of programmed cell death in many types of mammalian cells.

Ontogeny of multicellular organisms requires the atrophy and death of specific cells at temporally distinct periods of development (17). Juvenile cells die, allowing for outgrowth of adult tissue during insect metamorphosis (29). Likewise, in higher organisms, entire populations of embryonic cells die during morphogenesis. For example, regression of interdigital tissue occurs during limb bud formation (36), Wolffian or Müllerian duct regression accompanies sexual development (32), and some neurons in both the central and peripheral nervous systems die during embryogenesis (20). Cells undergoing death in response to developmental signals can be identified by specific cytological characteristics and can be distinguished by these markers from cells that die as the result of traumatic injury (45). Histologically, this process is termed apoptosis, and we will refer to it here as programmed cell death (17, 41). Characteristic biochemical changes are also shared by different types of cells undergoing programmed death. Most notably, the nuclear DNA is rapidly fragmented into oligomers of nucleosome-sized pieces (14, 25, 44).

Programmed death can also be induced in adult tissues by manipulating the hormone levels of an organism. This can be accomplished in some instances by hyperphysiological levels of hormones. For example, a high dose of glucocorticoids given to adolescent mammals will stimulate the death of thymocytes (12). Alternatively, hormone depletion can result in the active regression of hormone-dependent glands and tissues, as evidenced by the involution of sexual accessory tissue after castration (4, 25).

The strict timing and evolutionary conservation of programmed cell death during ontogeny, in addition to the ability of normal and malignant cells to develop insensitivity to the signals for cell death (23, 40), clearly indicate the genetic nature of the programmed death response. In lower organisms, distinct gene loci that regulate the ability of cells to undergo programmed death have already been identified (15). Other experimental data showing that synthetic activity is required for cells to die in this manner would be consistent with the requirement of specific gene products for the onset and progression of this process. Novel RNAs and proteins are induced in regressing tissues during the period of cell death (27, 42), and the expression of several constitutive gene products becomes markedly enhanced (5). The importance of synthetic activity is further shown by experiments in which either RNA or protein synthesis inhibitors can delay or prevent programmed death (37, 39).

In this study of gene activity during programmed cell death, we examined the pattern of expression and sequence of the testosterone-repressed prostate message-2 gene (*TRPM-2*) that was originally cloned from regressing rat ventral prostate tissue (28, 30). We show that the expression of this gene is associated with the onset of cellular atrophy and death in many other rodent tissues. This gene is unique in that its induced expression during tissue regression is caused by a multiplicity of environmental or hormonal stimuli. Sequence analysis of a partial cDNA encoding TRPM-2 revealed very close homology with the sulfated glycoprotein-2 gene (*SGP-2* or clusterin gene), expressed constitutively by rodent Sertoli and epididymal epithelial cells. Although the function(s) of the products encoded by *TRPM-2* gene remains unclear, their presence provides a remarkable and early indicator of programmed cell death in many types of mammalian cells.

MATERIALS AND METHODS

Animals and tissue. Male Sprague-Dawley rats (250 to 300 g) were obtained from Camm, Inc., Wayne, N.J. All surgical procedures, when indicated, were performed under sodium pentobarbital anesthesia. Castration was performed by scrotal incision. The testis and epididymus were exised after ligation of the spermatic cord and arteries. Acute unilateral ureteral obstruction was performed by lateral incision and ligation of the proximal ureter with surgical thread. At indicated times, rats were sacrificed by lethal overdoses of sodium pentobarbital and select tissues were excised and

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immediately frozen in liquid nitrogen for RNA extraction or fixed in paraformaldehyde for in situ analysis. Mouse bladder tumors (MBT-2) (43) were maintained as subcutaneous implants in female C3H/He mice. At indicated times after intraperitoneal injection of cyclophosphamide (100 mg/kg; Neosar [Adria Laboratories, Dublin, Ohio]), tumor-bearing mice were sacrificed by cervical dislocation and tumors were excised and frozen in liquid nitrogen. Developing limb buds from rat embryos obtained at day 14.5 after fertilization were frozen in liquid embedding resin. A cryostat was used to produce 8-μm-thick sections, which were affixed to poly-L-lysine-coated slides for in situ analysis.

RNA extraction and Northern (RNA) blot analysis. Polyadenylated mRNA was extracted from frozen tissue by the method of Cathala et al. (6), as described previously (5). Samples of RNA (10 μg) were electrophoresed on 1.2% denaturing agarose gels, then transferred, and fixed to nylon filters. Blots were hybridized to a denatured ^{32}P-labeled probe for *TRPM-2* (pG21-04) (28) or for β-actin (pAT-1) (10) and then were exposed to X-ray film for autoradiography. When indicated, autoradiographs were analyzed by a Joyce-Loebl scanning densitometer to determine relative band density.

In situ hybridization analysis for expression of *TRPM-2*. Tissues for in situ hybridization were fixed overnight in 4% paraformaldehyde-saline at 4°C. By standard dehydration and paraffin embedding procedures, 6-μm-thick sections were cut and hybridized to sense or antisense *TRPM-2* probes by the procedure of Mutter and Wolgemuth (31). Tissue sections affixed to poly-L-lysine-coated slides were rehydrated, then prehybridized for 4 h at 50°C under cover slips in a solution containing 50% formaldehyde, 0.6 M NaCl, 10 mM Tris hydrochloride (pH 7.5), 1 mM EDTA, 1× Denhardt solution (0.5% Ficoll, 0.5% polyvinylpyrrolidine, 0.5% bovine serum albumin), 10% dextran sulfate, 10 mM dithiothreitol, 500 μg of yeast tRNA per ml, and 10 μg of denatured salmon sperm DNA per ml. The prehybridization solution was replaced with hybridization solution, as described above, containing 300 ng of ^{35}S-labeled RNA probe per ml. RNA probes were prepared from a plasmid vector (pG21-04) (10) containing cDNA homologous to *TRPM-2* inserted between SP6 and T7 RNA polymerase promoter sites. The orientation of the insert allows antisense RNA to be transcribed from the SP6 promoter, whereas sense RNA is transcribed from the T7 promoter. In vitro transcription was performed on linearized plasmid in a buffer containing 50 mM Tris hydrochloride (pH 7.9); 6 mM $MgCl_2$; 2 mM spermidine; 10 mM NaCl; 10 mM dithiothreitol; 0.5 mM ATP, 0.5 mM CTP, and 0.5 mM GTP for 1 h at 37°C with SP6 polymerase or at 42°C with T7 polymerase (Promega Biotec, Madison, Wis.) in the presence of 1.2 μM [^{35}S]UTP (Amersham Corp., Arlington Heights, Ill.) and 1,000 U of RNasin per ml. Labeled RNA transcripts were extracted with phenol-chloroform (1:1) and were precipitated twice with 0.35 M sodium acetate (pH 5.5) in 3 volumes of ethanol.

After hybridization, slides were washed to remove unhybridized probe. All washes were done at 50°C. Slides were washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 30 min and then incubated in 2× SSC containing 50 μg of pancreatic RNase (Sigma Chemical Co., St. Louis, Mo.) per ml for 30 min. Finally, slides were washed in a successive series of solutions containing decreasing concentrations of SSC (from 2× to 0.1×), 15 mM 2-mecaptoethanol, 0.1% sodium PP_i, and 0.1% sodium thiosulfate for 30-min periods. Tissue sections were dehydrated, then dipped in melted NBT-2 autoradiographic emulsion (Eastman Kodak Co., Rochester, N.Y.) and exposed at 4°C for various periods of time. Slides were developed, then stained with hematoxyin and eosin, and dehydrated for microphotography.

Cloning of *TRPM-2* cDNA and DNA sequence analysis. A cDNA library was constructed from poly(A)$^+$ mRNA extracted from the kidneys of rats 48 h after ureteral obstruction, as described by Gubler and Hoffman (19). Poly(A)$^+$ mRNA was transcribed from DNA with avian myeloblastosis virus reverse transcriptase (Stratagene, Inc., La Jolla, Calif.) after hybridization to an oligo(dT) primer. Second-strand synthesis was accomplished with DNA polymerase I after treatment of the RNA-DNA duplex with RNase H. After the attachment of *Eco*RI linkers, the cDNA was cloned into the *Eco*RI site of the lambda ZAP vector (Stratagene). The recombinant library was screened with a ^{32}P-labeled probe for *TRPM-2* (pG21-04), and plaques showing positive hybridization were picked and rescreened. The inserts in the final seven candidate bacteriophages were recombined into a phagemid vector as prescribed by the supplier (pBluescript, Stratagene). The recombinant plasmid containing the largest cDNA insert, p1321, and a series of vectors in which various portions of the insert had been deleted by using exonuclease I and mung bean nuclease (21) (Stratagene) were sequenced by a dideoxynucleotide termination procedure (35) using T7 polymerase (Pharmacia Fine Chemicals, Piscataway, N.J.) extension of T3 or T7 primers in the presence of [^{35}S]ATP (Amersham).

Western blot (immunoblot) analysis of proteins in regressing rat tissues. The frozen rat epididymides and ventral prostate glands removed from control rats or castrated rats 3 days after castration and the kidneys removed from control rats or ureterally obstructed rats after 48 h were pulverized under liquid nitrogen. The powder was homogenized in a buffer containing 25 mM Tris hydrochloride (pH 7.5), 1 mM EDTA, and 0.5% Triton X-100 for 2 min with a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, N.Y.). The homogenate was centrifuged at 10,000 × *g* for 15 min, and the supernatants were recovered and stored at −85°C. Proteins in the extracts were measured by a modified version of the Bradford assay (3). Aliquots of extracts containing 50 μg of protein were electrophoresed on a sodium dodecyl sulfate–12% polyacrylamide gel by the procedure of Laemmli (26). The proteins in the gel were transferred to a nitrocellulose filter at 125 mA overnight in a buffer containing 25 mM Tris hydrochloride, 0.2 M glycine, and 20% methanol. The Western blot was blocked in a 10% solution of powdered milk and then incubated with rabbit serum against rat Sertoli cell clusterin (7) at a 1:100 dilution in 10 mM Tris hydrochloride (pH 7.4)–150 mM NaCl. Secondary goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) was used to detect binding of the rabbit antibodies in the presence of 5-bromo-4-chloro-3-indolyl-phosphate-p-toluidine and Nitro Blue Tetrazolium chloride at pH 9.5.

RESULTS

In situ analysis of *TRPM-2* expression in regressing rat ventral prostate tissue. *TRPM-2* cDNA was originally cloned from regressing rat ventral prostate tissue. Quantitation of *TRPM-2* transcript levels in ventral prostates removed at various times after castration showed that few, if any, transcripts were present in the normal gland; however, after

castration, transcript expression increased rapidly, reaching a peak at 5 to 6 days, and then declined to low levels again after 10 days (30). The temporal pattern of *TRPM-2* expression corresponds with the peak period of cell death in this gland (34). We performed in situ hybridization to identify which of the cell types in the regressing ventral prostate express *TRPM-2* transcripts. Secretory epithelial cells represent the majority of cells in the gland and are the most sensitive to androgen withdrawal (16, 24). Hybridization of tissue sections of ventral prostate from a castrated rat after 3 days with ^{35}S-radiolabeled antisense *TRPM-2* RNA showed that the atrophic epithelial cells expressed *TRPM-2* at extremely high levels (Fig. 1). The epithelial cells along a cross section of any given duct showed a gradient of expression, with the cells closest to the ductile tips having the highest level of *TRPM-2* transcripts. This is consistent with the heterogeneous sensitivity of ductile epithelial cells to androgen withdrawal (38) and clearly demonstrates that the atrophic acinar epithelial cells are the source of *TRPM-2* expression in the regressing rat ventral prostate gland. In contrast to the epithelial cells, few interstitial stromal or vascular cells in the regressing gland expressed *TRPM-2*. Concurrent hybridization of normal prostate tissue sections on the same slide did not detect *TRPM-2* expression (Fig. 1B), whereas hybridization of consecutive sections with radiolabeled sense *TRPM-2* probe showed a low background of silver grains (Fig. 1C).

Induction of *TRPM-2* transcripts in an atrophic renal unit during unilateral ureteral obstruction. Since *TRPM-2* expression was associated with the death of prostatic epithelial cells, we began to probe other experimental systems of inducible cell atrophy and death for *TRPM-2* expression. Initially, we analyzed rat renal tissue damaged by acute ureteral obstruction. Ligation of the proximal ureter causes urine retention in the obstructed kidney. The resulting pressure of accumulated fluid damages epithelial cells lining the renal tubules (46). Continuous obstruction leads to progressive atrophy of renal cells starting from the renal medullary area, and histological examination of acutely obstructed kidneys shows the accumulation of apoptotic bodies, characteristic of cells undergoing programmed cell death (18). After unilateral ureteral obstruction, the contralateral (unobstructed) kidney experiences progressive hypertrophy. The gene activity that accompanies both degenerative atrophy and hypertrophic renal growth after unilateral ureteral obstruction has been studied previously (22). In cells of the obstructed kidney, c-*fos*, c-*myc*, and heat shock 70K transcripts are rapidly induced, as in atrophic prostatic epithelial cells (5). When we examined mRNA extracted from either the ureter-obstructed or contralateral rat kidney, we found that 2-kilobase *TRPM-2* transcripts were expressed only in the obstructed kidney. Northern blot analysis of mRNA from kidneys taken at various times after unilateral ureteral ligation showed that *TRPM-2* transcripts appeared in mRNA from obstructed kidneys as early as 10 h after the ligation procedure (Fig. 2), significantly earlier than histological evidence of cell death (18). At 48 h after ureteral ligation, we estimate that the *TRPM-2* transcript makes up approximately 0.5% of the total mRNA content of the organ. The abrupt appearance of this transcript constitutes a minimum of 1,700-fold induction, as determined from densitometric analysis of the autoradiogram of the Northern blot. *TRPM-2* transcripts were not detected in mRNA extracted from normal, sham-operated or contralateral kidneys at any of the time points (Fig. 2), even after long-term exposure of the blot. Since the expression of *TRPM-2* in the ventral prostate was initially linked to the androgenic status of the rat, we also analyzed mRNA from kidney tissue of castrated rats at 3 days for *TRPM-2* expression. Northern blot hybridization showed no evidence for transcripts homologous to

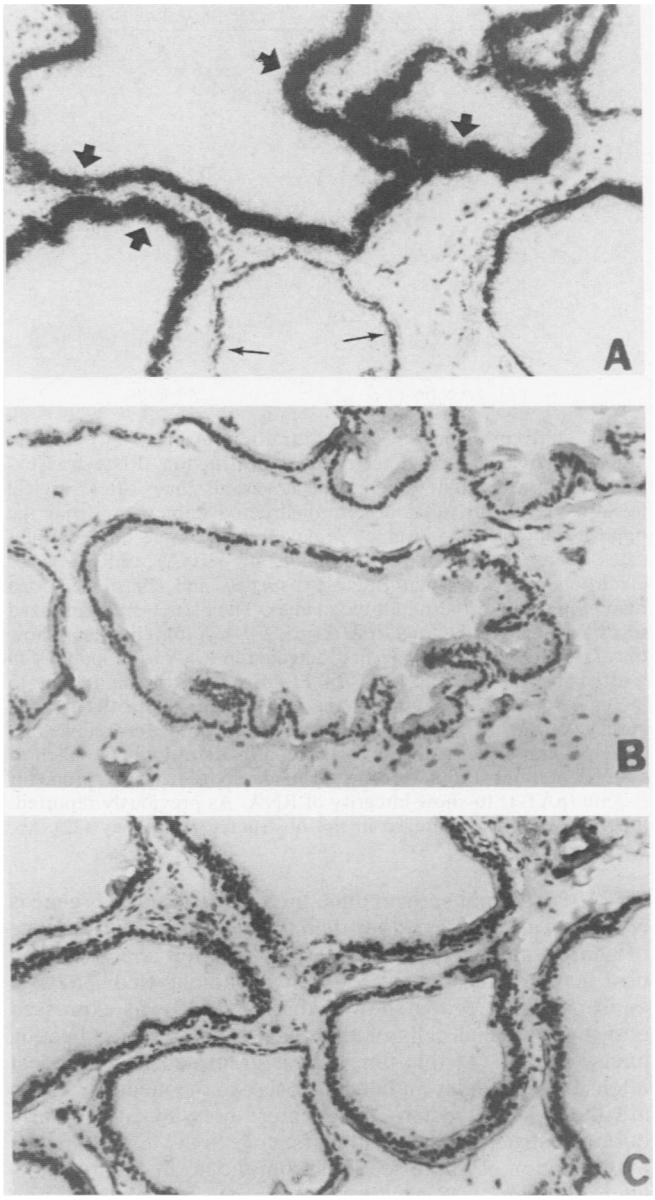

FIG. 1. Expression of *TRPM-2* transcripts in dying epithelial cells during regression of the rat ventral prostate. Thin sections (6 μm thick) of a fixed rat ventral prostate (A) removed 3 days after castration of the rat or of a fixed normal ventral prostate (B) were hybridized to an ^{35}S-labeled antisense RNA probe for *TRPM-2* (pG21-04). Slides were coated with photographic emulsion, developed for in situ localization of *TRPM-2* expression, and counterstained with hematoxylin. Intense distribution of silver grains over the secretory epithelial cells lining the tips of the prostatic ducts (large thick arrows) indicate that they are the source of *TRPM-2* expression 3 days after castration. In contrast, epithelium lining a more internal region of a duct (small thin arrows) shows no labeling above background. Concurrent hybridization of the ventral prostate section of a castrated rat at day 3 with ^{35}S-labeled sense RNA probe (C) also showed no specific hybridization; the dark-staining nuclei of the epithelial cells, but no silver grains, are observed in panels B and C.

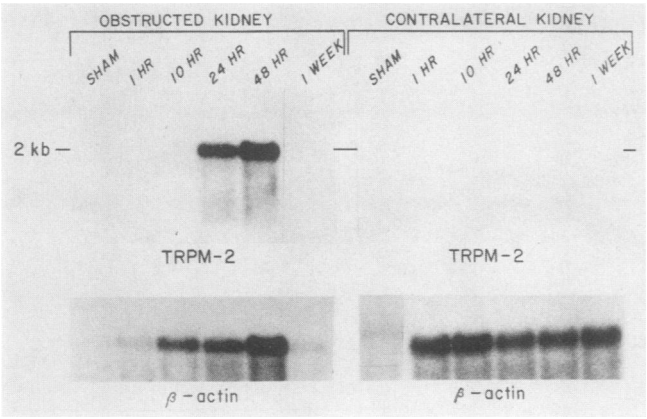

FIG. 2. Expression of *TRPM-2* transcripts in an atrophic rat kidney after acute unilateral ureteral obstruction. RNA was extracted from rat kidneys removed at various times after surgical ligation of the proximal ureter (obstructed kidney) or from the opposite, unobstructed kidney removed at the same time, as indicated (contralateral kidney). Aliquots of poly(A)$^+$ mRNA were electrophoresed in denaturing agarose gels and transferred to a nylon filter by Northern blot techniques. The blots were hybridized to a ^{32}P-labeled probe for *TRPM-2* (pG21-04). Autoradiograms show that *TRPM-2* transcripts are first detected in RNA from kidneys 10 h after obstruction. The levels of *TRPM-2* transcripts continue to increase for up to 2 days after the surgical procedure. RNA from kidneys of rats at 48 h after a sham operation procedure showed no *TRPM-2* expression, similar to the contralateral kidneys taken at equivalent times. Blots were cleaned and rehybridized to a probe for β-actin (pAT-1) to show integrity of RNA. As previously reported, β-actin levels also increase in the obstructed rat kidney (22). kb, Kilobase.

TRPM-2 (data not shown) thus, the expression of this gene is related to cell death, rather than to the presence or absence of androgenic steroids. In situ hybridization analysis of an obstructed kidney at 48 h with a radiolabeled *TRPM-2* antisense RNA probe showed that *TRPM-2* was expressed only by epithelial cells of the distal tubules and collecting ducts (Fig. 3). At this time, silver grain density was most intense over tubules and ducts closest to the medulla. These are the cells subject to the greatest pressure stress early during obstruction and are the first cells to die. Simultaneous hybridization of tissue sections from the normal and contralateral kidney at 48 h with the antisense probe showed no sign of *TRPM-2* expression. Hybridization of consecutive sections with an RNA sense probe for *TRPM-2* showed few background autoradiographic grains and confirmed the positive localization of *TRPM-2* signal (Fig. 3).

Expression of *TRPM-2* identifies embryonic rat tissue undergoing programmed cell death. Fetal development is accompanied by the programmed death of large populations of embryonic cells (20, 32, 36). This process permits the morphologic restructuring of the embryo to the adult form. One of the better-characterized regions of programmed death in fetal tissue occurs during limb bud formation (36). Interdigital tissue dies, allowing for the separation and definition of distinct digits. Tissue sections of developing fetal rat limb buds taken at day 14.5 after fertilization were hybridized to a radiolabeled antisense probe for *TRPM-2*. The regions of regressing interdigital tissue were distinctively marked by *TRPM-2* expression (Fig. 4). In addition, in situ hybridization analysis of sections of rat embryos at day 14 has allowed us to detect *TRPM-2* expression in other fetal cell populations that will die, for example, chondrocytes in the forming forelimbs and hindlimbs.

Induction of *TRPM-2* transcripts during chemotherapeutic regression of a mouse bladder tumor. *TRPM-2* expression has already been demonstrated to accompany the regression of an androgen-dependent tumor after hormone withdrawal therapy (33). A tumor model of cell death resulting from a cytotoxic agent further confirms an association between *TRPM-2* expression and the onset of programmed cell death. Although the mechanism of chemotherapeutic action remains a complex subject, evidence is accumulating to indicate that at least some of the effects of cytotoxic chemicals result from the initiation of programmed death in proliferating cells (1). To determine whether *TRPM-2* expression is induced during chemotherapeutic tumor regression, we analyzed mRNA extracted from a transplantable mouse bladder tumor (MBT-2) and from a series of bladder tumors excised from mice at sequential daily intervals after treatment of the host with cyclophosphamide in a dose (100 mg/kg) sufficient to cause tumor regression. Hybridization of a Northern blot prepared using mRNA from these sources showed that *TRPM-2* transcripts were present in all samples of the regressing (cyclophosphamide-treated) tumors but were undetectable in mRNA extracted from untreated tumors (Fig. 5). These results show that *TRPM-2* transcripts are induced in tumors during effective chemotherapy. Therefore, *TRPM-2* expression may ultimately provide an index of tumor response to chemotherapeutic agents.

DNA sequence homology and antibody recognition identifies the *TRPM-2* gene with a product expressed constitutively by mammalian Sertoli cells. Analysis of *TRPM-2* expression during acute ischemic injury to the testis led us to discover that transcripts homologous to this gene were constitutively expressed in rodent testis. Hybridization of Northern blots containing mRNAs from mouse testis at various stages of postnatal development showed the distinct presence of a 2-kilobase transcript hybridizing to the *TRPM-2* probe (Fig. 6). This data was confirmed, both by in situ hybridization of mature rat testis tissue with a *TRPM-2* antisense probe (results not shown) and also by sequence analysis of a plasmid vector containing cDNA homologous to *TRPM-2* (p1321). A cDNA library constructed from poly(A)$^+$ mRNA of obstructed rat kidneys at 48 h was screened with the prostate-derived probe for *TRPM-2* (pG21-04) (10). Seven different cDNA clones with homologous inserts were isolated and the clone containing the largest insert, p1321 (approximately 1,400 base pairs [bp]), was partially sequenced, using dideoxynucleotide chain termination procedures. The sequences obtained from three different regions of the p1321 insert were matched by computer analysis with a gene previously shown to be expressed constitutively by mammalian Sertoli cells, the sulfated glycoprotein-2 (SGP-2 or clusterin) gene (8, 9, 11). Sequence information from two regions of the p1321 insert near the 5′ end of the cDNA (a total of 400 bp) had total identity with the published sequence of SGP-2 (11), whereas a 200-bp region of p1321 adjacent to the poly(A) site demonstrated six nucleotide deviations from the published sequence [a G-C-G insert next to the poly(A) site and three additional cytosine residues in positions 1430, 1516, and 1555]. In our analysis, all of the differences we found were in the 3′-untranslated region of the cDNA, and these alterations would not influence the amino acid sequence of the product. This identity is further strengthened by an recent report of the cloning and sequencing of *SGP-2* cDNA from regressing rat ventral prostate tissue (2).

The sequence homology of *TRPM-2* cDNA with *SGP-2* prompted us to test whether antiserum made against SGP-2

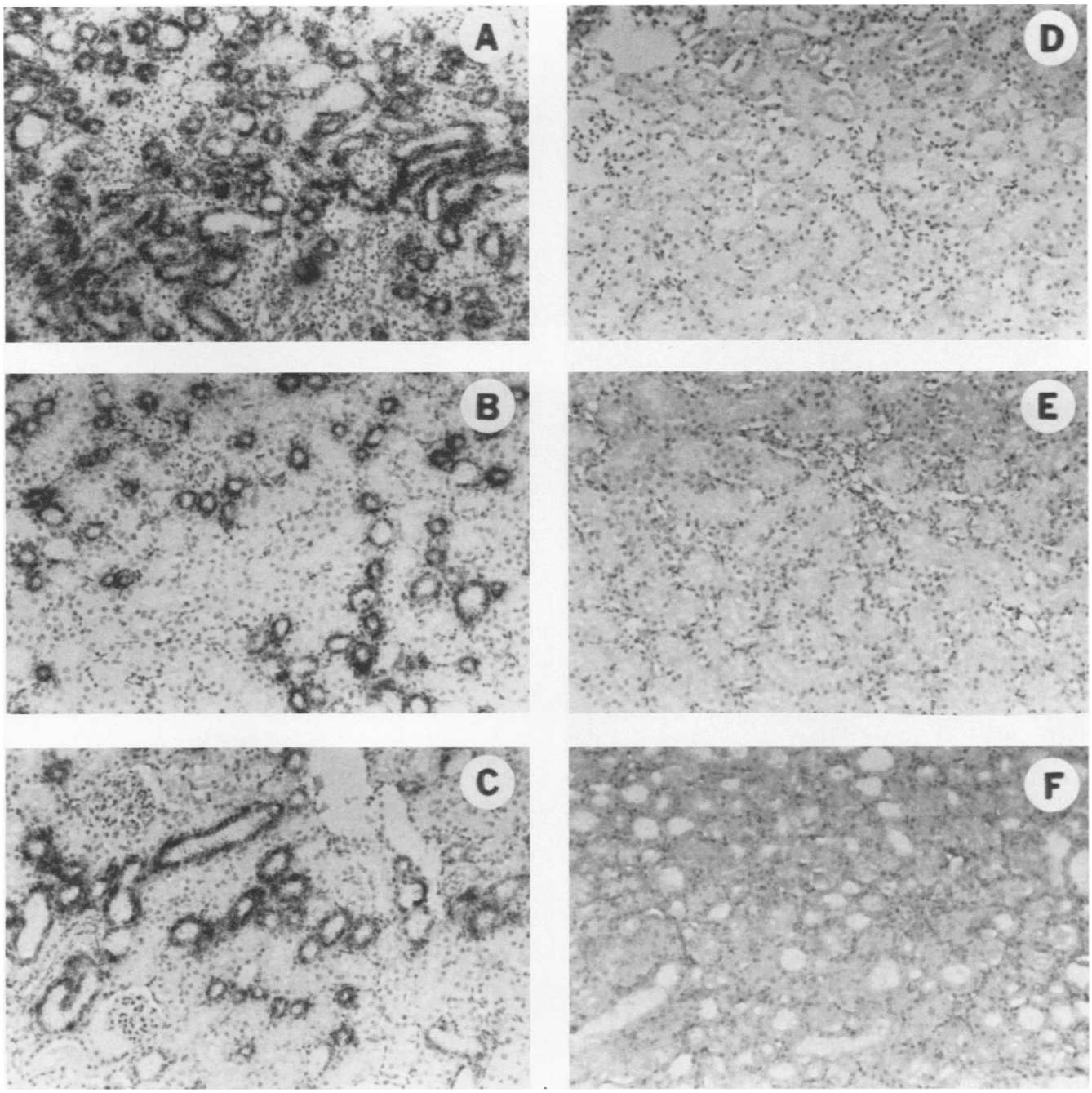

FIG. 3. In situ localization of *TRPM-2* expression in ureterally obstructed rat kidney. Longitudinal sections of a rat kidney taken 48 h after surgical ligation of the proximal ureter (A through C) and sections of sham-operated rat kidney (E) or of the contralateral rat kidney recovered 48 h after ureteral obstruction (D) were hybridized to an ^{35}S-labeled antisense probe for *TRPM-2*. The tissues were coated with photographic emulsion, developed to localize cellular expression of *TRPM-2*, and stained with hematoxylin. Panels A through C show regions of the obstructed kidney at increasing distances from the renal medulla. *TRPM-2* is intensely expressed in epithelial cells of collecting ducts and distal tubules. Hybridization of an obstructed kidney section at 48 h with an ^{35}S-labeled sense probe for *TRPM-2* (F) shows no specific hybridization; dark-staining nuclei, but no silver grains, are observed in panels D, E, and F.

could detect related proteins in regressing tissues. Under normal conditions, SGP-2 is secreted in large amounts by Sertoli cells so that it becomes a major component of epididymal seminal fluid. The secreted testicular protein is glycosylated and sulfated and demonstrates a lectinlike ability to aggregate cells in suspension (9). For this reason, SGP-2 has also been designated clusterin (8). As described, the Sertoli cell-secreted SGP-2 or clusterin molecule consists of two monomeric subunits (α and β) which are processed from a propeptide of approximately 64 kilodaltons (kDa). Although their source is not yet defined, small amounts of clusterin-related polypeptides can be detected in normal rat serum (7; M. G. Bandyk, I. S. Sawczuk, C. A. Olsson, A. E. Katz, and R. Buttyan, submitted for publication). Figure 7 shows that antiserum made against purified clusterin from rat Sertoli cells (7) recognizes three different polypeptides in

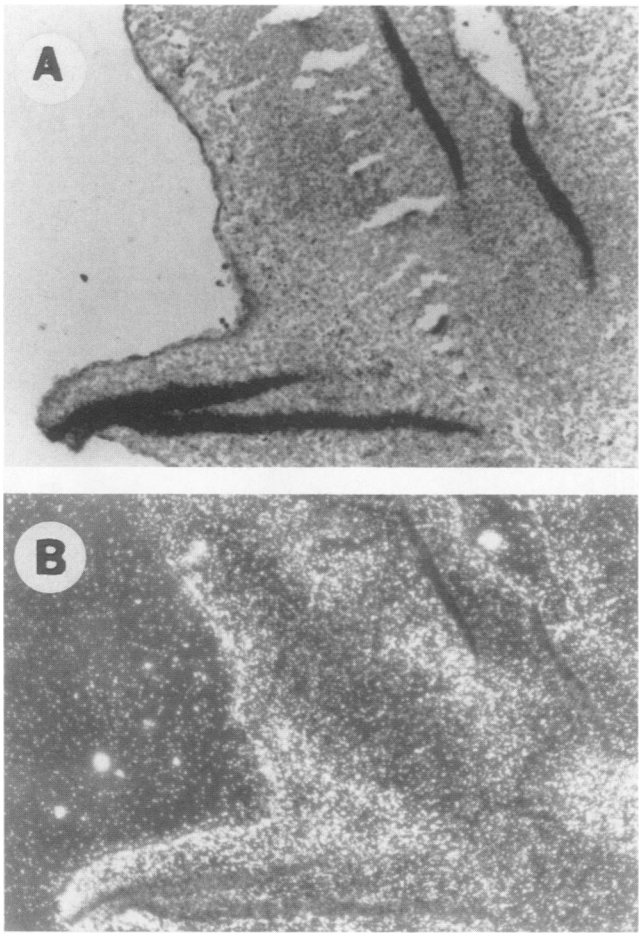

FIG. 4. Expression of *TRPM-2* transcripts in necrotic interdigital tissue of a developing mouse limb bud. Longitudinal sections through the limb bud from a rat embryo at day 14.5 were hybridized with an ^{35}S-labeled antisense probe for *TRPM-2*. Tissues were coated with photographic emulsion and developed to show embryonic regions expressing *TRPM-2*. Dense pyknotic nuclei in the hematoxylin-eosin-stained limb bud (A) identify regions of interdigital necrosis at this time. These regions showed an intense and more extensive expression of *TRPM-2* transcripts, as noted in the dark-field photomicrograph of this same limb bud (B).

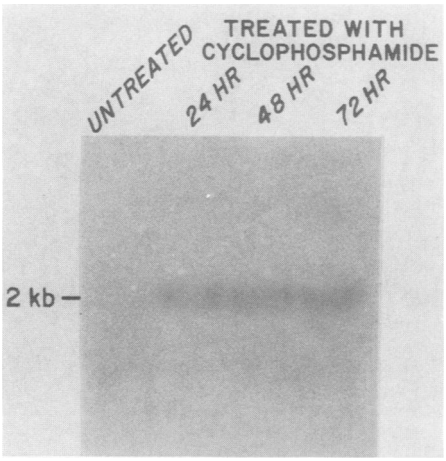

FIG. 5. Expression of *TRPM-2* transcripts during cyclophosphamide-induced regression of a mouse bladder tumor. RNA was extracted from a mouse bladder tumor (MBT-2) and from tumors at sequential daily intervals after treatment of the host with cyclophosphamide (100 mg/kg). Aliquots of total RNA were electrophoresed in denaturing agarose gels and were transferred to a nylon filter for Northern blot analysis. This blot was hybridized to a ^{32}P-labeled probe for *TRPM-2*. Autoradiography shows the expression of transcripts for *TRPM-2* in all RNAs from cyclophosphamide-treated tumors, but not in RNA from the untreated tumor. kb, Kilobase.

an extract of rat epididymus (lane A) on a Western blot, as expected. The high-molecular-mass band at 62 kDa would correspond with the estimated molecular mass of the precursor propeptide, and the bands at 39 and 29 kDa would correspond with the estimated sizes of the α- and β-subunits. Although we cannot detect *TRPM-2* (*SGP-2* or clusterin) transcripts by Northern blot analysis of normal rat ventral prostate or kidney RNA, the antiserum recognizes small amounts of immunologically related polypeptides in extracts from these same tissues (lanes B and D). We suspect that serum perfusion may be the source of this low-level background in the undamaged tissues. Both regressing ventral prostate (lane C) and obstructed kidney (lane E) show greatly enhanced levels of polypeptides recognized by this antiserum compared with the polypeptide levels of normal control tissues. Although the polypeptides in the high-molecular-mass region (62 kDa) of all tissues appear to be similar in size, the smaller subunits expressed in regressing tissues differ in apparent molecular mass from the subunits in the epididymal extract. The α-subunit expressed by re-

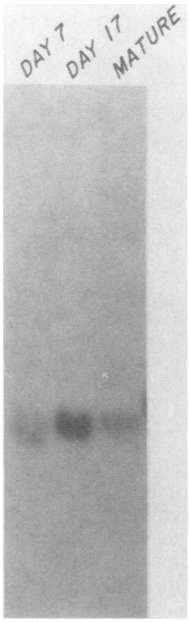

FIG. 6. Constitutive expression of TRPM-2 transcripts in mouse testes. RNA was extracted from the mouse testis at day 7 or 17 after birth or from 3.5-month-old mature mice, as indicated. Aliquots of the RNA were electrophoresed in denaturing agarose gels and were transferred to a nylon filter for Northern blot analysis. This blot was hybridized to a ^{32}P-labeled probe for *TRPM-2*. Autoradiography shows the constitutive expression of 2-kilobase transcripts related to *TRPM-2* in RNAs from mice at all these ages. In situ hybridization (results not shown) of thin sections through a rat testis identifies Sertoli cells as the source of the constitutive expression of *TRPM-2*.

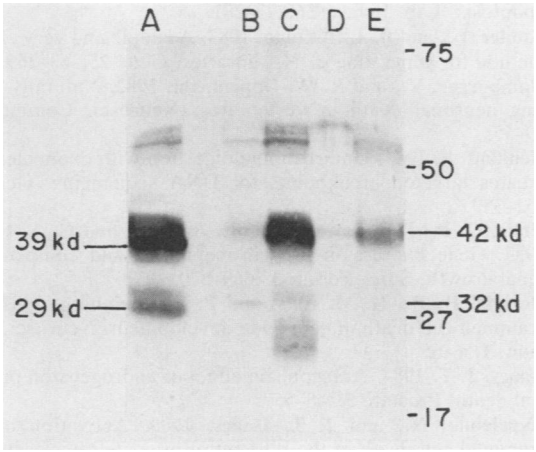

FIG. 7. Induction of clusterin-related proteins in regressing rat tissues. Cytosol extracts containing 50 μg of protein from a rat epididymis (lane A), a rat ventral prostate (lane B), a rat kidney (lane D), or a ventral prostate gland of a castrated rat at day 3 (lane C) or a kidney from a ureterally obstructed rat at 48 h (lane E) were electrophoresed on a sodium dodecyl sulfate–12% polyacrylamide gel. The proteins in the gel were electroeluted onto a nitrocellulose filter for Western blot analysis. Incubation of the filter with primary rabbit anticlusterin serum and secondary alkaline phosphatase detection allowed for the identification of the precursor for rat clusterin (above 50 kDa) and the two clusterin subunits in the epididymal extract (α at 39 kDa and β at 29 kDa). Extracts from regressing prostate or obstructed kidney also contain the high-molecular-mass precursor and an immunologically related polypeptide migrating slower than the α-subunit (42 kDa). Regressing prostate and kidney extracts also contain a small amount of polypeptide migrating slower than the epididymal β-subunit (at 32 kDa). In addition, the prostates from castrated rats show a series of related polypeptides running at 27 kDa (kd) and lower.

gressing prostate and kidney is most distinct and shows a sharp band higher (42 kDa) than the corresponding subunit in the epididymus (39 kDa). The β-subunit is much less distinct in regressing tissues but may also be higher in molecular mass than the corresponding band in the epididymus (32 versus 29 kDa). In addition, we detected several immunologically related bands at 27 kDa and lower in the regressing rat ventral prostate. Altered processing or enhanced proteolytic activity of dying cells may play a role in the appearance of these altered molecular mass forms. Alternatively, the substantial difference in molecular sizes of the subunits synthesized by regressing tissues compared with those of the subunits synthesized by Sertoli cells allow for the possibility that the proteins made during cell death serve a function distinct from that in the normal male reproductive tract.

DISCUSSION

TRPM-2 was originally cloned as a major gene product of the regressing rat ventral prostate. This product was of particular interest because its synthesis was not detected in the normal rat ventral prostate gland. Therefore, its synthesis after androgen withdrawal suggested the possibility that androgenic steroids could simply suppress its expression. As we have shown here, however, *TRPM-2* expression is induced in many regressing or dying systems we have examined to date, regardless of the androgenic milieu of the organism. These systems include human, rat, and mouse cells, benign or malignant. Although other genes show increased activity during programmed cell death (5), *TRPM-2* is unique in that it has not yet shown activity during proliferation or other cellular processes. Even more remarkable is the high level to which *TRPM-2* gene products are induced in the various systems studied.

Both sequence characterization and antibody recognition studies confirm that the *TRPM-2* gene products are homologous, if not identical, to the products of a gene expressed constitutively by mammalian Sertoli cells, the *SGP-2* or clusterin gene. Southern blot analysis of *Eco*RI-digested rat DNA with the probe for *TRPM-2* has identified the presence of three distinct bands with the combined total of approximately 27 kbp of information (R. Buttyan, unpublished observations). This would allow for the possibility of having two nonallelic genes encoding similar products, one expressed by Sertoli cells and another expressed during programmed cell death. Although we are currently investigating the number and structure of the gene(s) encoding these products, we do not yet have the information that would allow us to address this question. In fact, at the present time, we know very little about the product encoded by this gene in dying cells. The related protein synthesized by Sertoli cells is sulfated, highly glycosylated, and secreted into the lumen of the seminiferous tubules and epididymis. As we have shown by Western blot analysis, an immunologically similar protein is induced to high levels in dying prostate and renal cells and traces of this protein appear in the serum (Bandyk et al., submitted). Whether the function of the protein expressed during cell death differs from its function in the male reproductive tract remains to be determined.

Several alternative hypotheses could be considered for the function of a gene which is induced in many different types of cells as they are about to undergo programmed death. First, this gene could be expressed in an attempt to protect cells against the noxious conditions which cause their death. Although this proposed function would mimic putative functions of heat shock genes, we have not been able to induce *TRPM-2* expression by transient heat shock stresses on cultured L cells. Second, the association of this gene with programmed cell death suggests that its products might participate in the mechanism by which cells are killed. In this sense, expression of this gene could activate a suicide mechanism. Although we have not yet been able to test whether non-Sertoli cells are irreversibly committed to cell death by expression of *TRPM-2*, we have been able to show that drugs which slow the onset of cell death in prostate tissues coordinately reduce expression of *TRPM-2* transcripts (13). Further experiments in which *TRPM-2* cDNA is linked to an inducible promotor and transfected to cultured cells should allow us to test this hypothesis. Finally, the product(s) of this gene may serve either to attract lymphocytic cells in an attempt to rid the body of the damaged tissue or to enhance the degradation of the dying cell in order to make easier its removal from the system. Sequence analysis of TRPM-2 (SGP-2 or clusterin), however, does not show significant homology to known proteases or nucleases. Until its function is better understood, TRPM-2 might serve as an unusually sensitive molecular marker of the onset of programmed cell death in many kinds of mammalian cells.

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