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Changing Patterns of Gene Expression Identify Multiple Steps During Regression of Rat Prostate *in Vivo*

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ABSTRACT

The rat ventral prostate is an androgen-dependent organ that undergoes dramatic cell death upon removal of testosterone by surgical castration. Several well characterized criteria, such as nuclear condensation, organelle blebbing, and DNA fragmentation, have been used to demonstrate that most of this cell loss is due to programmed cell death, or apoptosis, of the secretory epithelial cells. In addition to changes in morphology, it is well known that cells undergoing apoptosis show alterations in gene expression, and it is widely assumed that many of these genes are directly involved in the mechanism of programmed cell death. Using poly A^+ RNA derived from normal rat prostate as well as from the regressing prostates of castrated rats, we have used a PCR-based subtractive hybridization approach to generate complementary DNA (cDNA) libraries greatly enriched in cDNAs strongly regulated during rat prostate regression. Several hundred of the genes represented in these libraries appear to be strongly regulated during prostate regression and most of these are prostate specific. Sequence analysis indicates that up to 30% of these clones are similar or identical to genes of known function, approximately 20% are similar to expressed sequence tags (ESTs), and as many as 50% of these clones have not been characterized previously. Analysis of selected clones using *in situ* hybridization indicates that they are expressed specifically in prostate epithelial cells, and that certain of these clones are regulated temporally in a pattern consistent with apoptosis. The patterns of gene expression include: 1) genes whose expression decreases uniformly after removal of androgen, indicative of androgen sensitive genes; 2) genes whose expression increases in apoptotic prostate cells and in other tissues, suggesting a class of genes generally involved in apoptosis; 3) and genes whose expression increases in individual regressing prostate epithelial cells, suggesting a class of prostate specific genes associated with apoptosis. (*Endocrinology* **139:** 2935–2943, 1998)

PROPER regulation of the growth and death of individual cells is fundamental for the development and normal function of complex organisms. An understanding of cell growth control is emerging from genetic, molecular and biochemical studies in many organisms, leading to a detailed mechanistic description of the cell cycle and its regulation (see Refs. 1 and 2 for review). The realization that, similar to cell growth, cell death is an orderly process that is critical for organogenesis (3, 4), and that abnormal regulation of cell death can be a critical initiating event in human disease (5), has stimulated a great deal of interest in discovering its mechanistic basis. Genetic studies of the stereotyped death of individual cells during development of *Caenorhabditis elegans (C. elegans)* have led to the identification of several genes that are regulators of programmed cell death and have provided a gross outline for the cell death pathway (6, 7). Convincing evidence that some of these molecules are fundamental participants in the cell death pathway in all metazoans has emerged from parallel studies of mammalian genes first identified in the context of oncogenesis (8, 9) and later shown to allow abnormal cell expansion due to failures in programmed cell death (10, 11). For example, the *C. elegans* ced 9 gene and the mammalian bcl-2 gene are demonstrated functional homologues that can prevent programmed death in both insect and mammalian cells (12, 13). The mammalian ICE like proteases and *C. elegans* ced 3 genes are also functionally homologous, although in this case their role is to induce apoptotic death (14, 15). While definition of the specific mechanisms through which these molecules act is an area of intense investigation, an appreciation that the program mediating cell death may be as complex as that regulating cell growth is also emerging. The aim of this study is to identify additional components of the mammalian programmed cell death pathway that can provide an avenue toward further mechanistic exploration of this pathway.

Given the complexity of the cell death pathway and the possibility that it may not be accurately reflected in established tissue culture cell lines, we have chosen to analyze gene expression during prostate regression in castrated male rats (16). In this system, there is a dramatic programmed death of epithelial and stromal cells in the ventral lobe during the first several days following androgen depletion (17). That this cell death occurs by apoptosis has been extensively documented histologically (17), and by *in situ* analysis of DNA fragmentation (18). Furthermore, some molecules that are differentially regulated during prostate regression have been cloned (19, 20), and it is clear that their regulation is sensitive to androgen withdrawal (20), and these molecules can provide early markers for cell death in several tissues (21). In this study, we employed PCR-based subtraction hybridization

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methodology (22) to prepare complementary DNA (cDNA) libraries that are very highly enriched in clones whose cognate messenger RNAs (mRNAs) are strongly regulated during prostate regression. Characterization of a large number of these clones demonstrates that at least several hundred genes are strongly regulated during regression and that these include many known genes that have been previously implicated in cell death. Thus, these novels cDNAs provide a rich resource for the initiation of molecular and genetic studies of prostate cell growth and death, and for the identification of new molecules associated with the programmed cell death pathway.

Materials and Methods

Library construction and subtractive hybridization

Total RNA was prepared from the prostate glands of control rats as well as from the prostate glands of rats 12, 24, 48, and 72 h after castration using the method of Chomzynski and Sacchi (23). After selection over an oligo(dt) column (Pharmacia, type 7) (24), 5 μg of poly(A+) RNA from control and pooled regressing prostates was used to synthesize double-stranded cDNA using a cDNA synthesis kit (Stratagene, LaJolla, CA). Half of this cDNA was used to construct a library in zap express (Promega, Madison, WI) according to manufacturers' protocols, and the other half was used for the subtractive hybridization protocol of Wang and Brown (22).

For the subtractive hybridization procedure, the cDNA from control (−cDNA) and regressing (+cDNA) prostate was digested with *Alu*I and *Rsa*I, blunt end cutters, and ligated to a double-stranded phosphrorylated oligonucleotide linker containing an *Eco*RI site and having one blunt end and one end with a 4-base 3′ overhang (CTCTTGCTTGAATTCGGACTA and TAGTCCGAATTCAAGCAAGAGCACA). After low melting agarose gel purification of the linker ligated cDNAs, both the −cDNA and +cDNA were amplified by PCR and digested with *Eco*RI. Driver cDNA was biotinylated twice with Photoprobe biotin (Vector Laboratories) to increase the amount of biotinylation.

Biotinylated driver (100 μg) and nonbiotinylated cDNA (5 ug) were mixed, precipitated, and resuspended in 10 ul of HE buffer (10 mM HEPES, pH 7.3, 1 mM EDTA). The DNA was boiled for 3 min and mixed with 10 μl of 2 × hybridization buffer (1.5 M NaCl, 50 mM HEPES, pH 7.3, 10 mM EDTA, and 0.2% SDS). After overlaying with a drop of mineral oil, the cDNA was boiled again for 3 min and hybridized at 68 C for 20–35 h (long hybridization). The concentration of NaCl in the hybridization mixture was reduced to 0.1 M by adding HE buffer, and strepavidin was added to the mixture. After 20 min at room temperature, the mixture was extracted several times with phenol/chloroform to remove the biotin/strepavidin/DNA complexes. The aqueous phase (subtracted +1 or −1 cDNA) was precipitated and mixed with additional biotinylated driver cDNA, hybridized for 2 h (short hybridization), reacted with strepavidin, and extracted as before. This cDNA, the subtracted +2 or −2 cDNAs, was PCR amplified and used for both driver and tracer in the next round of subtraction, a long hybridization, to yield +3 and −3 cDNA. Following PCR amplification of the +3 and −3 cDNA, a short hybridization with biotinylated +1 or −1 cDNA as driver resulted in +4 or −4 cDNA. Several such rounds of subtraction were performed to yield +8 and −8 cDNAs. At this stage, biotinylated SGP-2 cDNA was used as a driver in two rounds of subtraction to remove this abundant clone from the +8 cDNA. The resulting cDNA libraries are the +10 cDNAs.

DNA and RNA analysis

During each round of subtraction, the enrichment of cDNAs for differentially expressed clones was monitored by Southern blot analysis as previously described (22). Individual clones from the +10 cDNA were analyzed on Northern blots of control and regressing prostate RNA, as well as on blots with RNA from various tissues. For Northerns, approximately 10 μg of total RNA from various tissues was loaded per lane in a formaldehyde gel, electrophoresed, and transferred to nylon membranes. Northern blots were hybridized to ^{32}P-labeled probes from individual clones, washed, and exposed for autoradiography. Northern blot loading was normalized with glyceraldehyde phosphate dehydrogenase (GAPDH) hybridization.

Cell and tissue preparation

Male Sprague-Dawley rats (250–300 g) were used to isolate prostate glands for *in situ* hybridization and TUNEL labeling studies. Animals were anesthetized with 80 mg/kg pentobarbitol for castration and sham operation. For castration, a scrotal incision was made, the spermatic cord and arteries were isolated and securely tied off, and the testes and epididymus were excised. After 24 or 48 h, control and castrated animals were killed by CO_2 asphyxiation and their ventral prostate glands were removed. Tissue was either used for RNA extraction or frozen in O.C.T. freezing compound. Fifteen-micrometer sections were cut on a cryostat for *in situ* hybridizations and TUNEL labeling.

Various rat tissues were isolated for the Northern blot analysis, including embryonic limb bud tissue. Rat granulosa cells were isolated from developing follicles as previously described (25) then cultured in the absence or presence of serum to promote *in vitro* apoptosis. Prostate stromal cells were isolated from 20-day-old rat ventral prostates as previously described (26) with an enzymatic digestion procedure. Prostate stromal cells were cultured in the absence or presence of serum to promote *in vitro* apoptosis. After 72 h of culture, cells were harvested for RNA isolation. Normal and apoptotic thymus were collected after dexamethasone treatment as previously described (27).

In situ labeling of DNA

Labeling of degraded DNA in dying cells was performed according to the protocol from Boehringer Mannheim (Indianapolis, IN). Terminal deoxynucleotidyl transferase (TdT) was used to incorporate fluorescein conjugated nucleotides into the 3′-OH termini of DNA strand breaks. The florescein was then detected with antifluorescein Fab fragments conjugated to horseradish peroxidase. Tissue sections were then incubated with a diamino benzidine (DAB) substrate to complete the reaction.

In situ hybridization

Digoxegenin (dig)-labeled RNA probes were synthesized according to the protocol from Boehringer. Plasmids containing the various cDNA clones were linearized by restriction digest for sense and antisense orientations and the appropriate RNA polymerase (T3, T7, or SP6) was used to incorporate dig-labeled UTP into the RNA. After synthesis of dig-labeled RNA probes, plasmid DNA was digested with DNaseI. The probe was precipitated and resuspended in 100 μl of 20 mM DTT. Hybridizations to tissue sections with these probes were performed with a modified protocol from Schaeren-Wiemers and Gerfin-Moser (28). Fresh-frozen sections were fixed for 10 min in 4% paraformaldehyde/PBS (PFA/PBS), washed three times with PBS, and acetylated for 10 min in 0.1 M triethanolamine (pH 8.0), 0.25% acetic anhydride. After acetylation, sections were permeabilized for 10 min at room temperature in 0.05% Triton X-100/PBS and washed with PBS. Dig-labeled probes were added to preheated hybridization solution (50% formamide, 5 × SSC, 5 × Denhart's reagent, 250 μg/ml yeast RNA, and 500 μg/ml salmon sperm DNA), denatured at 80 C for 5 min, and placed on ice. Probes typically were used at 1:250 to 1:500 dilution. Tissue sections were incubated with diluted probes in a sealed humidified chamber overnight at 72 C. After hybridization, sections were washed for 5 min with 5 × SSC at 72 C, then with 0.2 × SSC for 1 h at 72 C and blocked for at least 1 h with 1% heat inactivated goat serum (HIGS) in buffer B1 (0.1 M Tris, pH 7.5, 0.15 M NaCl). Sections were then incubated overnight at 4 C with a 1:2000 dilution of an alkaline phosphatase conjugated antidigoxegenin in buffer B1/1% HIGS. After three washes in buffer B1, slides were incubated in buffer B3 (0.1 M Tris, pH 9.5, 0.1 M NaCl, 50 mM MgCl) for 5 min before developing with nitrotetrazolium blue chloride (0.34 mg/ml) and bromochloroindolylphosphate (0.18 mg/ml) in buffer B3 with levamisole added. Development was stopped with TE, and the sections were coverslipped with aquamount (Lerner Laboratories, Pittsburgh, PA) mounting medium.

Results

Preparation of libraries enriched in cDNAs regulated during prostate regression

To decide on time points from which to isolate RNA in preparation for the subtractive hybridization analysis, genomic DNA from rat prostate 12, 24, 48, and 72 h post castration was isolated and analyzed by Southern blot (data not shown). In agreement with previously published data (17), DNA degradation was first observed 24 h post castration, peaked at 48 h, and slowly decreased thereafter. Because we are most interested in changes in gene expression occurring early during prostate regression, RNA preparations from the 12, 24, and 48 h time points were pooled for use in the subtractive hybridization procedure.

Our initial attempt to identify cDNAs from genes that are strongly regulated during prostate regression were based on the cDNA display procedure of Liang and Pardee (29), and on the arbitrarily primed PCR fingerprinting protocol of McClelland and colleagues (30). The results of these analyses were disappointing, yielding only a small number of abundant regulated transcripts (data not shown). To improve the recovery of cDNAs representing mRNAs that are either induced or repressed during prostate regression, we turned to the PCR-based subtractive hybridization procedure of Wang and Brown (31–33).

To search for mRNAs that are both repressed and induced during prostate regression, we have used cDNA from normal prostate mRNA (minus cDNA) and cDNA from the pooled regressing prostate mRNA (plus cDNA) as drivers in the subtraction protocol. To monitor the enrichment of regulated mRNAs during the individual rounds of subtraction hybridization, Southern blots of the resulting amplified cDNAs were probed with a cDNA (−8 cDNA) that was isolated as part of the arbitrarily primed cDNA screen mentioned above (data not shown), with SGP-2 cDNA, an abundant late stage marker of apoptosis in ventral prostate (18) and with cDNA from the glyceraldehyde phosphate dehydrogenase (GAPDH) gene (34). As shown in Fig. 1, the −8 cDNA is difficult to detect in the original amplified cDNA preparations and is present only in the minus cDNA. In each round of subtraction, the representation of this cDNA increases in the minus cDNA and decreases in the plus cDNA preparation such that by the eighth cycle of subtraction, this −8 cDNA is highly specifically enriched in the minus cDNA population. In contrast, SGP-2 cDNA is abundant, although differentially expressed in the plus side of the initial amplified cDNA preparations (Fig. 1). Its representation is significantly enriched in the plus cDNA preparations in each cycle of subtraction, and it is effectively removed from the minus cDNA samples. GAPDH cDNA is present in the amplified cDNAs from both control and regressing prostate, and it is effectively removed from both subtractive cDNA preparations as the experiment progresses. These results demonstrate that significant enrichment for cDNAs differentially represented in the two initial cDNA populations occurs using this methodology, and that common cDNAs are lost during the successive cycles of subtraction. They also demonstrate that, as observed by Brown and colleagues (31), both relatively rare and highly abundant cDNAs can be successfully enriched using this protocol.

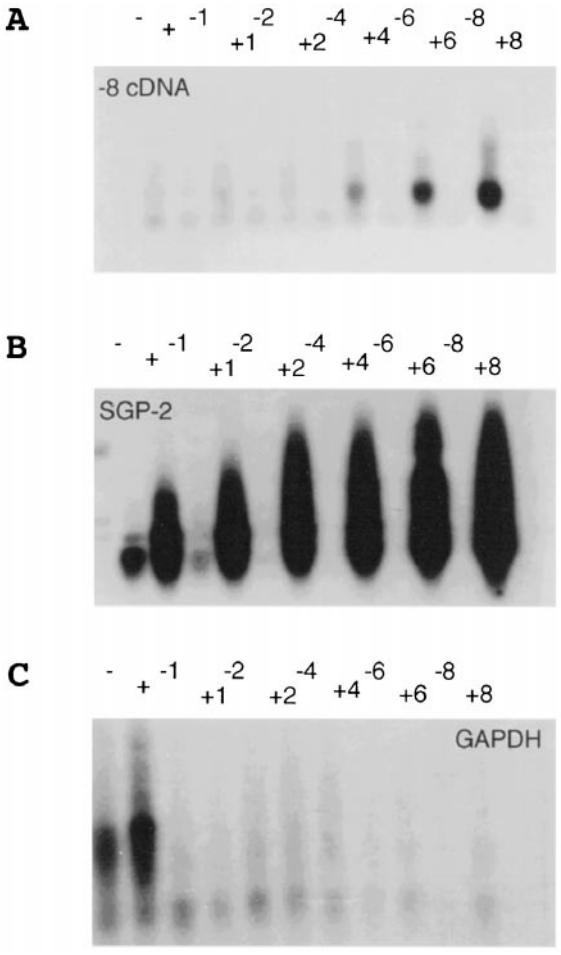

FIG. 1. Enrichment of plus and minus cDNA by subtractive hybridization. A. Minus cDNA enrichment as demonstrated by cDNA display clone. B, Plus cDNA enrichment as demonstrated by SGP2 hybridization. C, Removal of housekeeping cDNAs as demonstrated by GAPDH hybridization.

The very strong hybridization of SGP-2 in the plus cDNA preparation suggested that its strong representation could be problematic in our efforts to identify additional clones that are induced during prostate regression. To overcome this problem, SGP-2 cDNA was employed as driver to subtract the SGP-2 cDNAs from the +8 cDNA preparation. The results of this subtraction are shown in Fig. 2. Thus, after two cycles of subtraction with SGP-2 driver, Southern blot analysis indicates that no significant SGP-2 cDNA remains in the +10 cDNA preparation. However, using the entire +10 cDNA as a probe yielded signal specifically in the plus cDNA populations (panel A), indicating that the +10 cDNA retained differential cDNA. To confirm these results, cDNA libraries were prepared from the +8 and +10 cDNA populations in λZAP and screened using both probes. Approximately 40% of the cDNAs detected using the +8 cDNA as probe on the +8 cDNA library were also detected using SGP-2 as probe (compare panels B and C of Fig. 2). In contrast, none of the clones detected using +10 cDNA as probe on the +10 library were detected using SGP-2 as probe (com-

pare panels D and E of Fig. 2). While these results indicate that SGP-2 cDNA has been completely removed from the +10 cDNA pool and library, they also indicate that some relatively abundant cDNAs may be retained in these preparations because some plaques hybridize significantly more strongly to the complex probe than others (Fig. 2D).

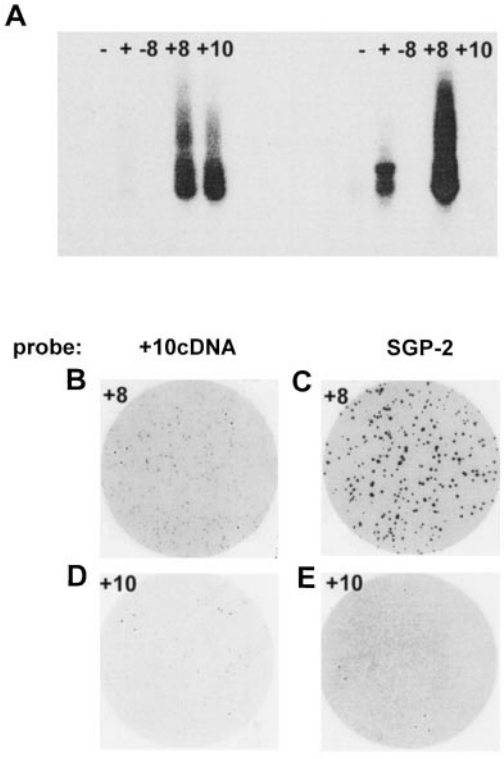

FIG. 2. Removal of SGP-2 cDNA from the +8 cDNA by subtractive hybridization. A. Southern blots showing plus and minus cDNA hybridized to total +10 cDNA and SGP-2 cDNA. B–E, Individual plates of +8 and +10 cDNA libraries hybridized to total +10 cDNA and SGP-2 cDNA.

Screening individual clones induced during prostate regression

To begin to assess the complexity of the +10 cDNA library, and to determine whether it contains cDNAs representing novel, strongly regulated mRNAs, thirty individual plaques (designated 10.1–10.30) were isolated, plasmid rescued, and the encoded cDNAs further analyzed (Table 1). Cross-hybridization analysis of each of these cDNAs to filters containing all thirty established that there are twenty different cDNAs present in this collection. Four clones are represented twice, and two clones represented four times in these thirty isolates.

As an initial screen to determine whether these twenty cDNAs are differentially represented in the subtracted cDNA populations, Southern blots similar to those shown in Fig. 1 were performed for each clone. As indicated in Table 1, eighteen of the twenty clones (90%) were enriched during the cycles of subtractive hybridization. It was immediately apparent from this analysis that the representation of specific cDNAs within the subtracted pools varied greatly. To test the utility of this type of prescreen for regulated cDNAs, we compared the intensity of hybridization to the plus and minus cDNA pools with Northern blots from normal and regressing prostate. As shown in Fig. 3, the abundance of the cDNA fragment as represented in the initial and final subtracted cDNA pools generally reflects both the abundance and the degree of regulation of that mRNA in the tissue as assayed by Northern blot hybridization. Thus, relatively rare mRNAs such as 10.19 are not strongly represented in the initial cDNA preparations, although longer exposures reveal their presence and relative enrichment in the plus cDNA. Abundant mRNAs such as that encoded by the 10.3 cDNA are easily detected even at short exposure times, and the small percentage of cDNAs representing mRNAs that do not change in abundance during prostate regression are not differentially represented in the initial cDNA pools. These results indicate that hybridization to the initial amplified cDNA pools can be a very rapid and fairly accurate screen for cDNAs representing regulated mRNAs.

TABLE 1. Encoded cDNA analysis

Clone	cDNA Blot	Northerns	Tissues	Similarity
10.1	Differential	Differential	Prostate, others	GST
10.2, 10.3, 10.6, 10.7	Differential	Differential	Prostate	Novel lipase
10.5, 10.9	Differential	Differential	Prostate	Novel cystatin
10.8	Unregulated	Unregulated	General	rp S27
10.10	Differential	ND	ND	None
10.11	Differential	ND	ND	None
10.12	Differential	Differential	Prostate	Vacuolar ATPase
10.13	Differential	ND	ND	None
10.15	Differential	Differential	Prostate	None
10.16, 10.21	Differential	Differential	Prostate	None
10.17, 10.25	ND	Differential	Prostate	None
10.18	Unregulated	Unregulated	General	None
10.19	Differential	Differential	Prostate	None
10.20, 10.22, 10.24, 10.26	Differential	Differential	Prostate	None
10.24, 10.26, 10.23	Differential	Differential	General	rp S18
10.27	Differential	Differential	General	Thymosin B-4
10.28	Differential	Differential	Prostate, others	None
10.29	Differential	Differential	Prostate	None
10.30	Differential	Differential	Prostate	None

ND, Not determined.

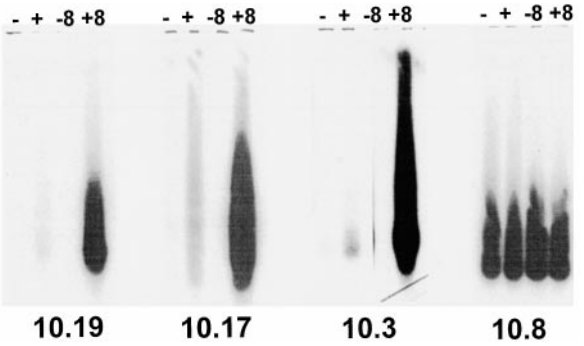

FIG. 3. Abundance and regulation of individual cDNAs as indicated by hybridization of probes 10.19, 10.17, 10.3, and 10.8 to plus and minus cDNA preparations after 0 and 8 rounds of subtractive hybridization.

To determine whether these randomly chosen clones represent previously characterized cDNAs, the nucleotide sequence of each of them was determined and used to search GenBank with the BlastN and BlastX search programs (NCBI). The results of these searches are shown in Table 1. Of the twenty different cDNA sequences analyzed, seven (32%) are highly similar to previously characterized cDNAs. Four of these (10.1, 10.8, 10.23 and 10.27) are identical to known genes. Three of these (10.8, 10.23, 10.27) encode abundant, generally expressed genes that are only marginally regulated in regressing prostate. Their presence in the cDNA pool represents the small number of cDNAs that are not significantly differentially regulated in regressing prostate, yet remain present after repeated cycles of subtraction. Clone 10.1 is identical to rat glutathione-S-transferase, which has been shown to be strongly regulated during prostate regression in response to castration (35) and during apoptosis of lymphocytes in response to steroids (36). Three additional cDNAs have significant homology to previously characterized genes (Table 1). Clone 10.3 is approximately 60–70% identical to members of the acid lipase family, which includes the lysosomal acid lipases (37), and gastric (38) and lingual lipases (39). Clone 10.5 is approximately 80% identical to the rat cystatin S gene and likely represents a novel member of the cystatins, a large family of cysteine proteinase inhibitors, some of which are abundant and androgen regulated in normal rat ventral prostate (40). Finally, clone 10.12 encodes a vacuolar ATPase with approximately 55% homology to both the bovine and human vacuolar (H+)-ATPase C subunit mRNAs (41, 42). None of the remaining thirteen cDNAs are strongly similar to any known cDNA (Table 1).

Organ specificity of +10 cDNAs

To assess the magnitude of regulation of individual cDNAs, and determine whether they are expressed predominantly in the prostate or are also expressed in other tissues containing apoptotic cells, Northern blots were performed. Results from ten representative blots are shown in Fig. 4. All clones were analyzed by Northern blots with data from representative clones being presented. Northern blot loading was normalized with GAPDH hybridization. Cells and tissues were obtained as outlined in the *Materials and Methods* section, and the apoptotic status of the cells was confirmed

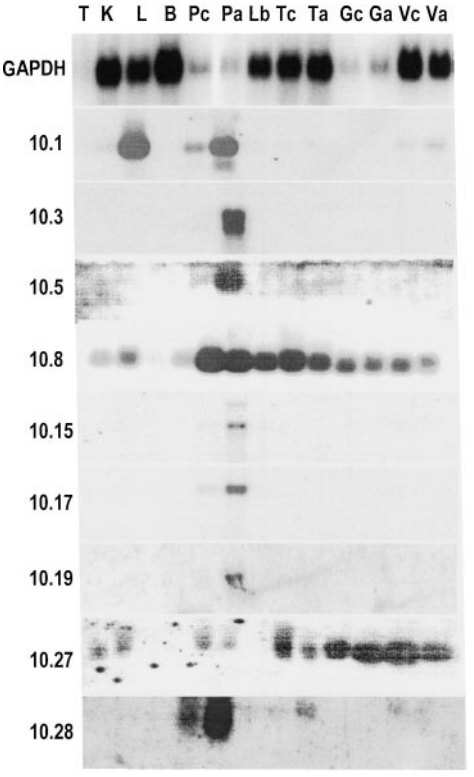

FIG. 4. Northern blots showing regulation of individual representative cDNAs in regressing prostate and abundance in several other tissues containing significant numbers of dying cells: (T) testis; (K) kidney; (L) liver; (B) adult brain; (Pc) control prostate; (PA) prostate 24 h post castration; (Lb) e12.5–e14.5 limb bud; (Tc) control thymus; (Ta) dexamethasone treated thymus; (Gc) control granulosa cells in culture; (GA) granulosa cells undergoing apoptosis in culture; (Vc) ventral prostate stromal cells in culture; (Va) apoptotic ventral prostate stromal cells undergoing apoptosis in culture. Each probe was used on at least three different blots to confirm results.

on isolated cells and tissue (data not shown). All samples indicated as apoptotic were found to have DNA fragmentation, whereas nonapoptotic cells or tissue did not (data not shown). Results confirm those obtained in the prescreen using amplified cDNA to assess regulation (Fig. 3) and those obtained through DNA sequence analysis. Thus, clones that were not strongly regulated in the prescreen or that were homologous to generally expressed mRNAs (10.8, 10.27) appear to be both generally expressed and minimally regulated when analyzed by Northern blot. All other clones, while varying substantially in abundance, are strongly induced in regressing prostate by 24 h post castration (Fig. 4). Inspection of these Northern blots demonstrates that six of the eight strongly regulated prostate mRNAs are expressed predominantly or exclusively in prostate tissue undergoing regression, whereas two of the clones appear to be expressed in other tissues containing apoptotic cells. For example, glutathione-S-transferase mRNA (clone 10.1) is known to be induced in both regressing prostate (35) and thymus from dexamethasone treated mice (36). Our Northern analysis confirms these results and extends them to include ventral prostate stromal cells undergoing apoptosis *in vitro* (44), as well as limb buds isolated from e12.5–e14.5 mouse embryos containing dying interstitial epithelial cells (44, 45). Whereas

GST mRNA expression is correlated with cell death in these situations, its very abundant expression in liver indicates that it is also present in tissues that do not contain significant numbers of apoptotic cells (Fig. 4). Clone 10.28, which encodes an mRNA that has not been previously reported, is also expressed in several instances of programmed cell death. Thus, its expression is induced in regressing prostate, in apoptotic primary prostate stromal cells, in dexamethasone treated thymus and in limb bud (Fig. 4). It is not present at significant levels in adult brain, liver, kidney, or testis. The expression of 10.28 mRNA, therefore, closely correlates with cell death in the tissues that we have examined. These results indicate that the vast majority of the cDNAs present in the +10 cDNA library are both strongly regulated in regressing prostate, and specifically expressed in that tissue. They also suggest that a relatively small percentage of these genes (10–15%) are of general interest for exploration of the fundamental programmed cell death pathway (Table 1 and Fig. 4).

In situ localization of mRNAs regulated during prostate regression

The system we have chosen for this analysis is complicated by the fact that the physiologic stimulus causing prostate regression is the severe drop in androgen levels following castration (15). As a consequence, we expect at least two classes of regulated genes: those that are simply responsive to androgen levels, irrespective of the status of the cell with regard to programmed death, and those whose expression is intimately associated with the cell death process itself. These two classes of regulated genes can be identified if assayed in individual cells by *in situ* hybridization (Figs. 5–7). To gain an appreciation of the number and types of cells in later stages of programmed death in regressing prostate, *in situ* labeling of DNA degradation was performed at 24 and 48 h post castration. As shown in Fig. 5, the gross morphology of ventral prostate is not dramatically altered during the first 48 h post castration, although at high magnification one can appreciate that distortion and condensation of epithelial cell nuclei is beginning to occur at 24 h and is clearly apparent by 48 h post castration. *In situ* analysis of DNA fragmentation indicates that only a very small percentage of cells in the ventral prostate are in late stages of cell death by 24 h post castration, whereas this number significantly increases by 48 h (Fig. 5). These observations are in close agreement with those of Isaacs and colleagues (17), although in our experiments the number of stromal cells revealed by *in situ* DNA fragmentation assays at both time points is very small (Fig. 5).

These results suggest that genes whose regulation is directly responsive to circulating androgen levels will be induced or repressed in most prostate epithelial cells. In contrast, those genes regulated as a consequence of programmed cell death will be regulated at the single cell level with the regulation being observed in increasing numbers of cells between 24 and 48 h post castration. Analysis of gene expression is a reflection of mRNA transcription, mRNA levels, and mRNA stability. The experiments in the current study do not distinguish how mRNA expression is influenced, and this needs to be considered in any data interpretation. As shown in Fig. 6, the *in situ* hybridization analysis suggests some of the clones are regulated in most cells. Clones 15.8–5, PBK4, and PBK14 are each strongly down-regulated after castration when analyzed by Northern hybridization (data not shown). For each of these clones, *in situ* hybridization indicates that the mRNA is specific for prostate epithelial cells and that they disappear from the entire epithelial cell population in synchrony (Fig. 6). The disappearance of

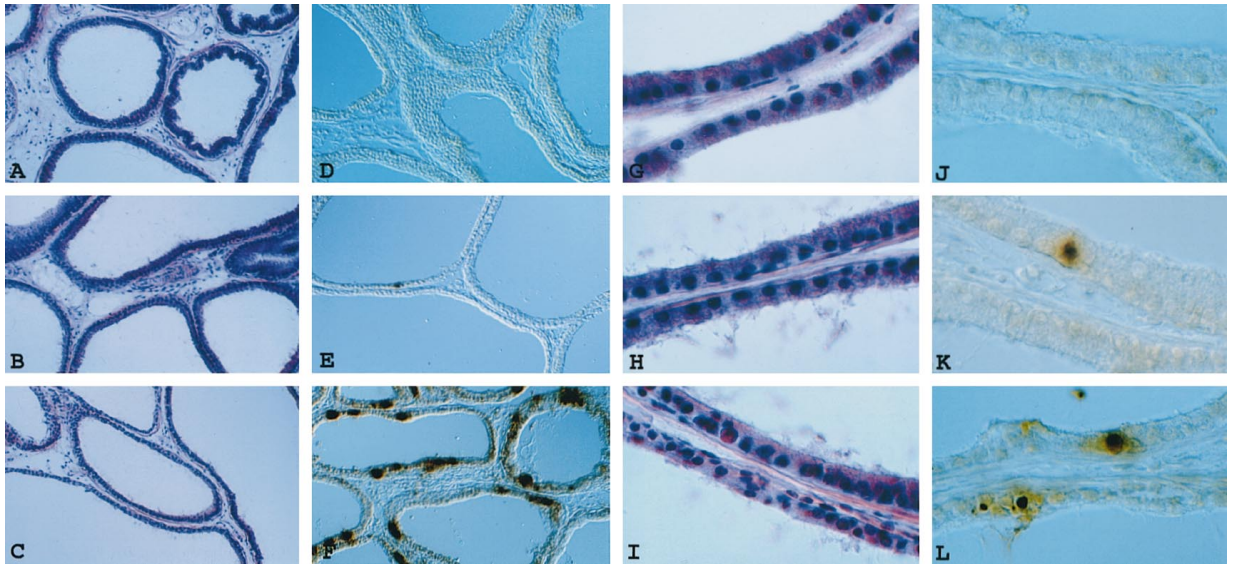

FIG. 5. Cell death in regressing prostate. A–C, Low power (50×) images of hematoxylin and eosin (H&E) stained normal (A), 24 h castrate (B), and 48 h castrate (C) rat ventral prostate sections. D–F, Low power (50×) images of TUNEL-labeled normal (D), 24 h castrate (E), and 48 h castrate (F) prostate sections. G–I, High power (100×) images of H&E stained normal (G), 24 h castrate (H), and 48 h castrate (I) ventral prostate sections. Forty-eight hours after castration, cell size has decreased and membrane blebbing and nuclear distortion are apparent. J–L, High power (100×) images of TUNEL-labeled normal (J), 24 h castrate (K), and 48 h castrate (L) ventral prostate. Similar results were obtained for TUNEL labeling on 8–10 sections from each time point.

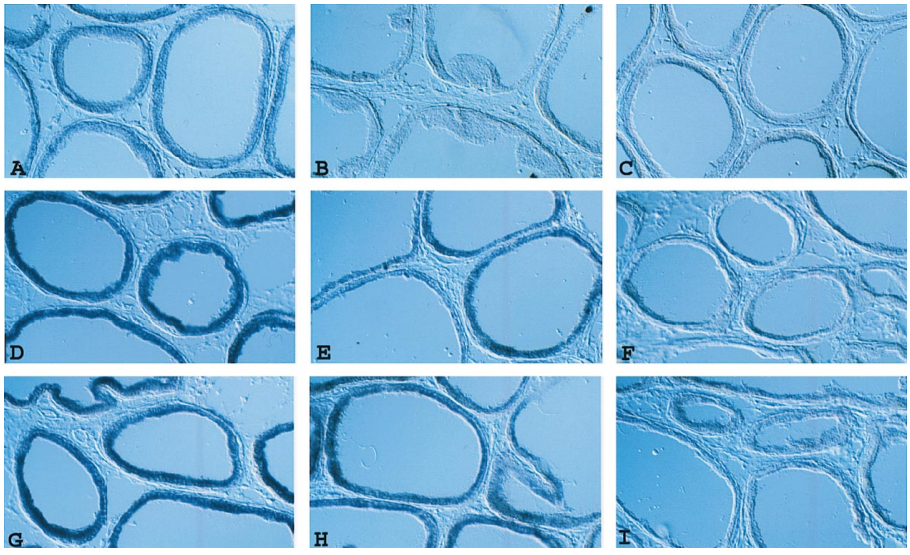

FIG. 6. All prostatic epithelial cells respond to changing hormone levels. *In situ* hybridization analysis of clones 15.8–5 (A–C), PBK4 (D–F), and PBK14 (G–I). Each clone is expressed throughout the epithelial sheet in normal rat ventral prostate (A, D, G). For each clone, expression in the prostate uniformly decreases 24 h. (B, E, H) and 48 h. (C, F, I) after castration. *In situ* hybridization for each probe was repeated 2–3 times. Magnification, 50×.

15.8–5 mRNA clearly precedes that of PBK4 and PBK14, both of which are still detectable 24 h after castration. However, by 48 h after castration these clones are only weakly detected if at all by *in situ*. The fact that all of the cells lining the ducts behave similarly with respect to these changes in gene expression demonstrates that there is a rapid and synchronous response of this cell population to changing hormone levels (Fig. 6). These results clearly document that the entire population of epithelial cells in ventral prostate responds to castration by altering gene expression patterns. Analysis of the clones isolated from the +10 cDNA library indicates that some but not all of the clones are regulated synchronously in regressing prostate epithelial cells. The regulation of several of these clones evidently occurs independently in single cells as regression proceeds. The results for several clones from the +10 cDNA library (10.17, 10.1, 10.3) are shown in Fig. 7. Like clones 15.8–4, PBK4, and PBK14, clone 10.17 mRNA appears to be regulated synchronously in all prostate epithelial cells after castration. Expression of 10.17 is initially seen weakly at 24 h post castration. Forty-eight hours after castration, 10.17 is expressed uniformly throughout the epithelial sheet (Fig. 7). These results support the conclusion that all epithelial cells respond to a decrease in androgen levels by changing gene expression. In contrast, clone 10.1, which encodes rat glutathione-S-transferase, is not detectable in control prostate tissue by *in situ* hybridization, but by 24 h post castration, individual cells that are strongly positive are evident, with the number of positive cells significantly increasing between 24 and 48 h post castration (Fig. 7). Similar results are obtained with clone 10.3, which encodes a protein with very high similarity to pregastic lipase (38). However, Northern blot analysis revealed a strongly regulated mRNA that is not expressed at detectable levels in other tissues (Fig. 4).

Discussion

Genetic characterization of programmed cell death during *C. elegans* (6, 7) and *Drosophila melanogaster* (46, 47) development have provided a framework for further investigation of cell death and its regulation. Consideration of these studies in the light of more recent studies of molecular mechanisms of cell death in vertebrates (3, 4) have revealed a complex program that involves an array of both regulatory and effector genes. Several clear conclusions can be drawn from this work that are relevant to the present study. First, a wide array of proteins can induce or suppress cell death if ectopically activated in a particular cell type. Second, programmed cell death in a given cell type may be regulated by pathways that are specific to that cell type. Third, several classes of molecules appear to be fundamental to the effector mechanisms for cell death in a wide variety of circumstances. Fourth, extracellular ligands can have a crucial initiating role in selecting cells for an apoptotic fate. Finally, it is not yet possible to delineate rate limiting steps for the cell death pathway in many well characterized paradigms for programmed cell death in vertebrates.

In the present study, we have cloned a large number of cDNAs whose cognate mRNAs are very strongly regulated in prostate epithelial cells following castration and have screened among them for molecules that either appear to be specifically induced in dying prostate epithelial cells or that are more generally expressed in tissues containing significant numbers of apoptotic cells. Our detailed analysis of the plus cDNA library has demonstrated that it is highly enriched in cDNAs representing mRNAs that are strongly induced during prostate regression: 85–90% of the clones are regulated when assayed by Northern blot or *in situ* hybridization. Cross-hybridization studies and DNA sequence analysis indicates that the number of genes that are induced during prostate regression is large. In fact, extensive DNA sequence analysis (performed in collaboration with Dr. J. Trent and colleagues, NIHGR) has demonstrated the presence of at least several hundred different cDNAs in the plus cDNA library and has confirmed their distribution into groups of novel genes (~50%), expressed sequence tags (~20%), and known genes or closely related to genes of known function (~30%).

The identity of the known genes found in this screen supports the conclusion that many of these genes could have a direct role in death of prostatic epithelial cells. Thus, SGP-2

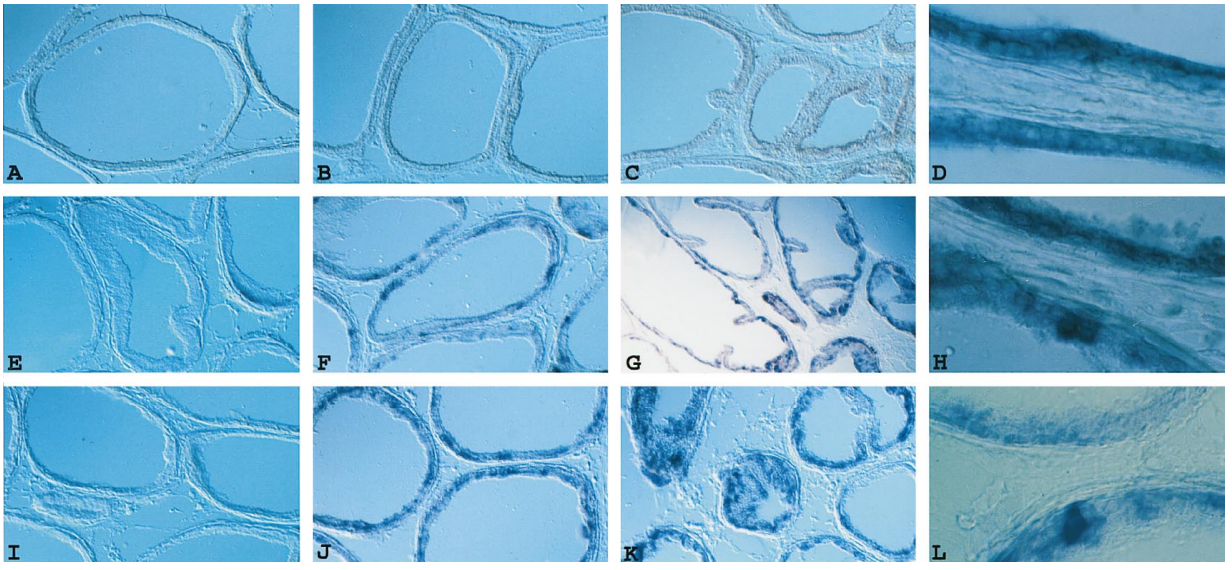

FIG. 7. Differential regulation of clones from the +10 cDNA library by *in situ* analysis. A–D, Clone 10.17, a novel sequence, is up-regulated in all epithelial cells after castration. E–H, Clone 10.1, glutathione-S-transferase, is up-regulated strongly in individual epithelial cells, and is found in other tissues. I–L, Clone 10.3, a novel lipase, is also up-regulated strongly in single epithelial cells, and is prostate specific. Little or no expression of each of these clones is seen in normal prostate (A, E, I); 24 h after castration clone 10.17 is weakly expressed throughout the epithelial sheet (B), whereas clone 10.1 (F) and 10.3 (J) are strongly up-regulated in individual epithelial cells. Expression of 10.17 (C), 10.1 (G), and 10.3 (H) further increases 48 h. after castration. High power (100×) images of *in situ* shows uniform expression of clone 10.17 (D), whereas clones 10.1 (H) and 10.3 (L) demonstrate up-regulation in individual cells. Each probe was used for *in situ* hybridization 2–3 times.

(18, 20), GST (35), vacuolar ATPase (48, 49), and thymosin B4 (50) have all been implicated in apoptosis. Whereas no direct evidence has been reported to implicate cystatin S (40) or pregastric lipase (35) in cell death, a role for these molecules in tissue regression can be envisioned. The only genes identified in this screen that are not easily incorporated into our thoughts concerning prostate regression are the two ribosomal genes represented in this collection of cDNAs (Table 1). Because these cDNAs are only marginally regulated during prostate regression and are generally expressed, they represent the small background of irrelevant cDNAs that appear to contaminate this library. Our evidence suggests, therefore, that this library is highly enriched in cDNAs representing genes that are strong candidates for a direct role in prostate physiology, and that a small percentage of these genes may have a direct role in a fundamental cell death program.

The *in situ* hybridization studies reveal several features of prostate regression that have not been explicitly demonstrated in previous studies. First, it is clear that there is a rapid and synchronous response of all epithelial cells in the rat ventral prostate following castration. Several genes are repressed (15.85, pBK4, pBK14) and at least one gene is induced (10.17) in nearly all prostatic epithelial cells within the first 2 days of castration. We anticipate that these genes may be direct targets of androgen action rather than participants in or markers of programmed cell death. Second, the strong induction of several genes (*e.g.* 10.1, 10.3, SGP-2) in a significant number of individual epithelial cells in ventral prostate at 24 h post castration, before significant labeling of cells using the DNA fragmentation assay, suggests that the initial stages of cell death in this tissue are marked by changes in gene expression that occur in single cells. This is consistent with the asynchronous appearance of late stage apoptotic cells as revealed in the DNA fragmentation assays, supporting the view that it is the asynchronous initiation of cell death in individuals cells rather than a varying temporal progression in the death program that is responsible for the disappearance of cells from ventral prostate over time. Furthermore, the differences in the number of cells expressing these markers at 24 and 48 h post castration *vs.* the number of cells containing fragmented DNA strongly suggests that many hours must pass between the initiation of the death pathway in individual cells and their eventual demise by DNA fragmentation. Whereas we believe that there is also a temporal progression in the expression of these genes in regressing prostate, in depth quantitative studies have not yet been performed. Third, comparison of the *in situ* hybridization and Northern blot results clearly indicates that both prostate specific (*e.g.* 10.3) and general (*e.g.* 10.1, 10.28) markers of cell death are present within this library. It will be of great interest to begin functional analysis of these genes to discover molecular mechanisms underlying both the tissue specific and general features of apoptotic death in this system.

The identification of a large number of novel cDNAs from regressing prostate provides an important avenue toward deepening our understanding of the physiology of prostate growth and death. We anticipate that some of these genes will provide excellent markers for the events underlying abnormalities in prostate growth, and that some may encode critical regulatory molecules that play direct roles in benign prostate hyperplasia or prostate cancer. In addition, it is probable that among these genes are as yet unrecognized regulators of programmed cell death that may play critical roles in other tissues.

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