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CHARACTERIZATION AND CLONING OF ANDROGEN-REPRESSED mRNAs FROM RAT VENTRAL PROSTATE

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The involution of the prostate that occurs after castration is thought to be an active process, requiring protein synthesis. A number of "castration-induced" proteins which might be involved in this process have been identified. We recently described a group of "testosterone-repressed" mRNA sequences in the prostate which could code for these proteins. Because of their potential importance in the autophagic response we have cloned these sequences, and we report here the characterization of the most abundant of these sequences (TRPM-2), and the kinetics of the induction of this gene in the prostate after castration. TRPM-2 is induced to a maximum level of approximately 1440 ppm of total RNA six days after castration, by which time the androgen dependent, prostate steroid binding protein (PSBP) mRNA sequences have diminished to undetectable levels. The translation product of TRPM-2 is a protein of approximately 46,000 daltons, with a pI of 5.9-6.3. Since this gene is expressed in other involuting tissues, it may play an important role in the process of tissue regression. © 1987 Academic Press, Inc.

The rat ventral prostate has been used for a number of years as a model system for analyzing the effects of androgens on gene expression and replication (1). The synthesis and activity of a number of proteins such as prostate steroid binding protein (PSBP)(2, 3) and secretory acid phosphatase (4, 5) are absolutely dependent on the continuous supply of androgens. In the case of PSBP it has been shown that androgens are required for the normal transcription of the genes and for the stability of the individual hnRNA transcripts (6, 7). These androgen dependent enzymes have been used to monitor the effects of castration on the function of the prostate during involution, and most are undetectable four to six days after castration.

The process of involution is more complex than the simple loss of androgen dependent proteins of the prostate and involves a combination of cellular regression and cell death. The regression of the gland is accompanied by a loss

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of DNA, RNA and protein which occurs primarily between the third and seventh day after castration and which can be partially blocked by the inhibition of protein synthesis (8, 9). At the same time there is a rise in a number of unidentified "castration-induced proteins" (10, 11) and an increase in the levels of several degradative enzymes, particularly ribonuclease (12), cathepsin D (13) and plasminogen activator (14). These results have led to the suggestion that the process of cell death in the prostate and other organs is an active one, which is repressed in the presence of androgens (15). Consistent with the requirement of protein synthesis for prostate regression is the observation of the expression of specific castration-induced mRNA sequences in the prostate (16, 17) suggesting that prostatic regression is associated with the activation of specific genes in prostatic cells. We report here the characterization and cloning of the most abundant of these sequences (TRPM-2) and demonstrate that this gene, within the limits of detection, appears to be repressed by androgens in the normal prostate.

MATERIAL AND METHODS

Animals Male Sprague-Dawley rats were obtained from Charles River Inc. (Montreal). The rats were castrated via the scrotal route under light Innovar-Vet (fentanyl citrate/Droperidol) anesthesia. At indicated times after castration, the rats were sacrificed by cervical dislocation, and the prostates excised and pooled.

Extraction of RNA Total RNA was extracted from the ventral prostate by the LiCl/ urea procedure (18). Poly(A)$^+$ RNA was isolated using oligo-(dT) cellulose chromatography (19), and aliquots were electrophoresed on 1.5% denaturing agarose slab gels (20). The RNA was also characterized by *in vitro* translation in a commercially available rabbit reticulocyte lysate (Promega-Biotec; Mississauga, Ontario) using [^{35}S]-methionine (800 Ci/mmol, Amersham Co.; Oakville, Ontario). The translation products were analyzed by two dimensional gel electrophoresis (21).

Preparation of cDNA clone library from castrated rat prostate Double stranded cDNA was prepared from poly(A)$^+$RNA extracted from the prostates of rats castrated 8 days previously using the method outlined by Rutledge et al. (22). The size fractionated cDNA molecules were ligated into Sma-1 digested, calf intestinal phosphatase treated pUC8. The recombinant plasmids were used to transform *E. coli* DH1 cells. Plasmids containing cDNA inserts were identified by Grunstein-Hogness hybridization (23). Plasmids of interest were prepared using a modification of the method described by Birnboim and Doly (24), and restriction mapped. The cDNA containing plasmids were characterized by differential Northern hybridization to poly(A)$^+$RNA isolated from the prostates of rats castrated 8 days previously (25). Plasmids characterized as androgen-repressed were nick translated in the presence of [^{32}P]- dCTP (3000 Ci/mmol, Amersham Co.; Oakville, Ontario) following a standard procedure (26).

Northern hybridization analysis Poly(A)$^+$RNA from intact rats and from castrated 4 days rats was transferred to nylon filters as described by Thomas (25) for nitrocellulose filters, and hybridized to nick translated plasmids (average specific activity: 4×10^7 cpm/µg). The filters were prehybridized at 42°C for a minimum of 6 hours in 50% formamide, 5X SSC, 5X Denhardt's solution (0.1%

albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidine), and 100 µg/ml calf thymus DNA. The filters were hybridized with [^{32}P]-labelled plasmids in the same buffer at 42°C for 48 hours. They were washed twice in 2x SSC, 0.1% SDS at room temperature for 5 to 10 minutes, then twice in 0.1X SSC, 0.1% SDS at 42°C for 5 to 10 minutes. Dot blot hybridization assays were performed as outlined by Thomas (27), using isolated PSBP (pA34) or TRPM-2 (p21-04) inserts as standards to estimate the relative levels (in ppm poly(A)$^+$RNA) of the two messages. Each time point is the mean of triplicate analyses from one experiment.

RESULTS AND DISCUSSION

The comparison of the *in vitro* translation products of the poly(A)$^+$RNA extracted from the prostates of intact (Fig. 1a) and castrated rats (Fig. 1b) reveals a number of striking differences. The PSBP subunits are the most abundant androgen dependent proteins in the prostate, and are clearly visible in the translation products of the poly(A)$^+$RNA extracted from the prostate of intact rats (Fig 1a). Although this gel system does not adequately resolve proteins with molecular weights less than 18,000 daltons, the prostate binding proteins are visible as a group at an apparent molecular weight of approximately 14,000 daltons with isoelectric points ranging from 5.5 to 6.8. These proteins are conspicuously absent in the translation products of the RNA of the castrated rats (Fig 1b), which makes them an excellent control for androgen dependent gene expression in the prostate (2). There are also a

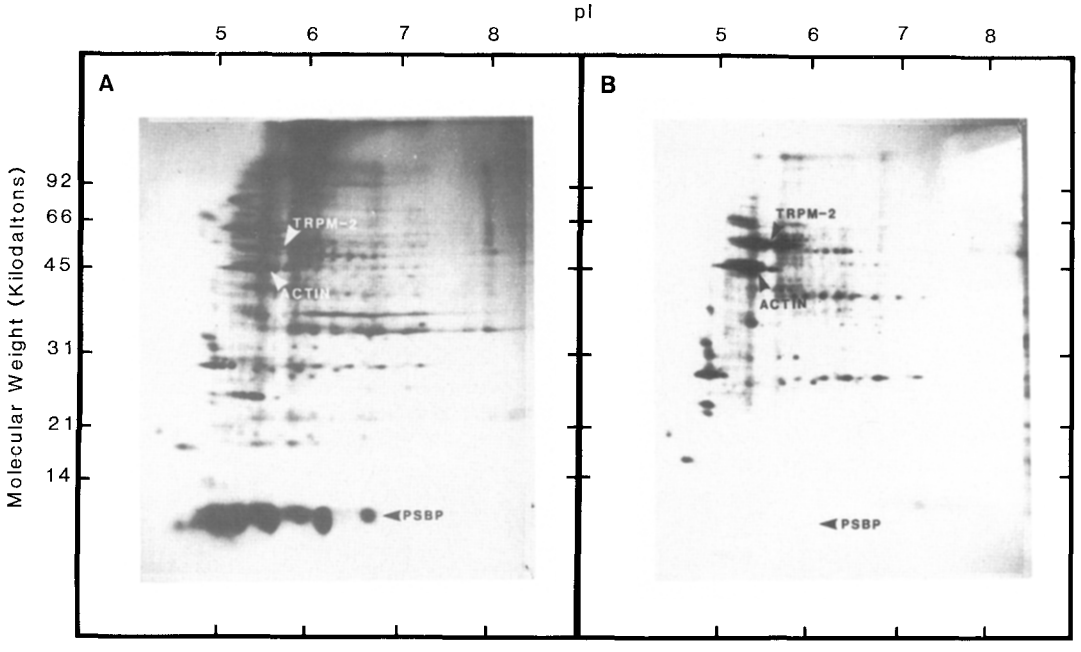

Fig 1. Two dimensional gel electrophoresis of *in vitro* translation products Panel A: Translation products of poly(A)$^+$RNA extracted from the normal rat prostate. Panel B: Translation products of poly(A)$^+$RNA extracted from the prostate of rats castrated 8 days previously.

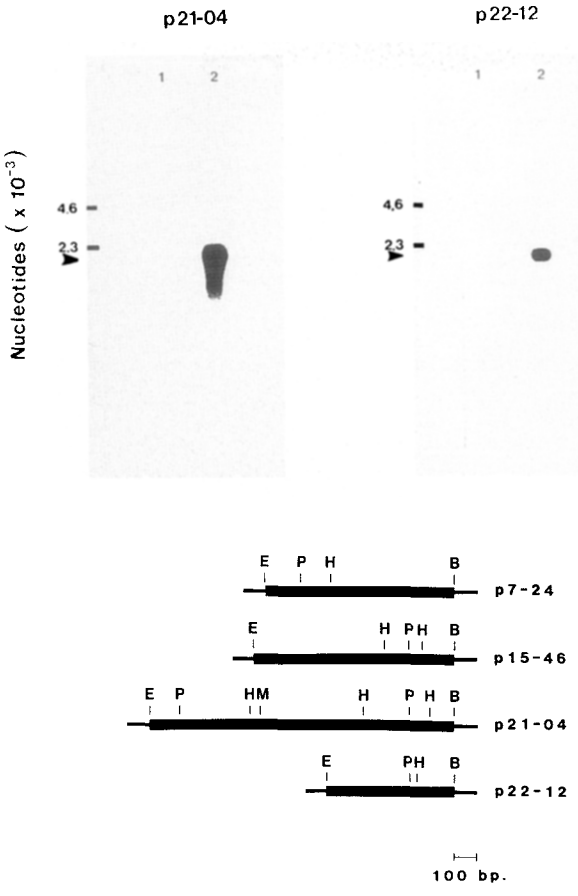

Fig. 2. Characterization of TRPM-2 plasmids

Panel A: Replicate samples of 2µg poly(A)$^+$ RNA from intact (lane 1) and castrated (lane 2) rat prostate were electrophoresed on 1.5% agarose gels, and transferred to nylon membrane filters by Northern transfer. Individual plasmids were nick translated and hybridized to individual replicates of the Northern blots.

Panel B: Restriction maps of recombinant plasmids.
B: Bam HI; E: Eco RI; P: Pst I; H: HaeIII; M: Msp I.

considerable number of unidentified proteins which show a similar degree of androgen dependence. On the other hand there are a number of proteins which are more prominent in the translation products of the castrated rats, most notably a protein band with a molecular weight of approximately 46,000 daltons and an isoelectric point between 5.9 and 6.3. We have previously suggested that this protein is coded for by an RNA sequence of approximately 2000 nucleotides, which we have called TRPM-2 (16). We have analyzed 4400 clones from a cDNA library prepared from poly(A)+RNA from 8 day castrated rats, of which only 35 sequences (or less than 0.8% of the clones) were found to represent androgen repressed sequences. Eight of these were shown to hybridize to an RNA species of 2000 nucleotides (Fig. 2a), present in the poly(A)+RNA from castrated rats.

No signal was detected in the lane containing poly(A)$^+$RNA from intact rats, even after extensive over exposure (results not shown). The PSBP mRNA sequences are the most abundant mRNA species in the prostate. Since they are completely androgen dependent they are not represented in the poly(A)$^+$RNA used to prepare the clone bank. This suggests that the TRPM-2 mRNA sequence represents approximately 0.2% of the poly(A)$^+$RNA (8 of 4400 clones) in the prostate of rats castrated 8 days previously, and if TRPM-2 sequences are expressed at all in the normal prostate, they must be present at even lower levels. Dot blot analysis of RNA extracted from a number of other tissues has established that the TRPM-2 gene does not appear to be expressed in the liver, kidney, adrenals, or seminal vesicles, either before or after castration. TRPM-2 is expressed at low levels in the testes and in the dorsolateral prostate after castration and in the rat uterus both before and after ovariectomy (J. Léger, unpublished observations).

The restriction maps of the TRPM-2 specific plasmids show many similarities, including common Hae 111, Msp 1 and Pst 1 sites (Fig. 2b). Plasmid p21-04, the largest clone contains 75% of the mRNA sequence. By selective cross hybridization we have demonstrated that plasmid p7-24, which does not share the same restriction sites, is an overlapping segment of the same gene. Thus p21-04 and p7-24 span virtually 100% of the mRNA sequence. Both p21-04 and p7-24 hybrid select a protein of approximately 44,000-46,000 daltons, the size of the abundant translation product shown in Fig. 1b (Tenniswood, unpublished observations). Analysis of preliminary sequence data suggests that TRPM-2 is not homologous to other sequences in the Microgenie^{TM} data bank (J. Léger, in preparation).

To analyze the time course of induction of TRPM-2 poly(A)$^+$RNA was extracted from the prostate at various times after castration. Two cDNA plasmids were used to probe the RNA: pA-34, specific for the C2 subunit of prostate steroid binding protein; and p22-12, specific for TRPM-2 (Fig. 3). Northern analysis reveals that the PSBP sequences disappear by day four, as previously reported (inset) (3, 6). TRPM-2 specific sequences are first readily detectable on day two after castration and the steady state levels of the mRNA reach a peak between days four and six (inset). The relative levels of these two sequences were quantitated by dot blot hybridization, using the plasmid inserts as standards to estimate the relative abundance of the two messages in the prostate. Figure 3 shows the steady state levels of TRPM-2 RNA reach a maximum of 1440 ppm of poly(A)$^+$RNA on day six after castration, almost two orders of magnitude lower than the steady state levels of PSBP mRNA in the intact rat prostate (approximately 80,000 ppm). The maximum steady state levels of TRPM-2 mRNA are achieved at the time when the rate of cell death in the prostate is

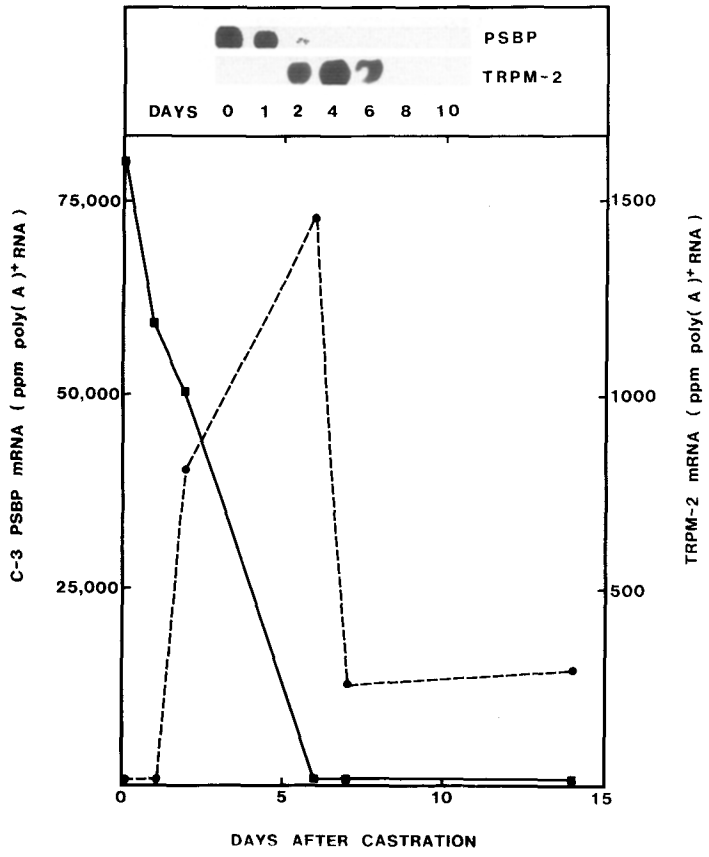

Fig 3. Analysis of TRPM-2 induction after castration by dot blot hybridization

One µg poly(A)$^+$RNA, extracted from the prostate at the indicated times after castration was blotted in triplicate onto duplicate nylon membranes. One membrane was hybridized to a TRPM-2 specific probe (p21-04), the other to a PSBP specific probe (pA34). The steady state levels of each mRNA sequence (ppm of poly(A)$^+$RNA) were calculated from standard curves established for each of the sequences.

(■——■) Steady state level of C-3 mRNA.
(●- - -●) Steady state level of TRPM-2 mRNA

Inset: Northern hydridization of p21-04 and pA34 to poly(A)+RNA extracted from the prostate at the indicated times after castration.
3µg poly(A)$^+$RNA, extracted from the prostate at the indicated times after castration, was electrophoresed on a 1.5% agarose gel and transferred to nylon membrane. The same filter was hybridized sequentially to a TRPM-2 specific probe (p22-12, $5x10^5$cpm/ng) and to a PSBP specific probe (pB44, $2.9x10^5$ cpm/ng). The autoradiographs were exposed for 5 days (TRPM-2), and 18 hours (PSBP).

maximum (15). It is of interest that this gene is also expressed in the uterus both before and after ovariectomy and thus appears to be involved in the active processes of tissue regression and cell death. Other androgen-repressed genes have recently been identified and cloned in both the prostate (17) and the liver (28). Taken together, these results suggest that TRPM-2 is one of a

number of genes, which are normally repressed in the prostate, that are responsible for the active autolysis that occurs after the withdrawal of androgens in the male. In view of the biochemical complexity of the ductal-acinar network in the prostate (29-31), it is still possible that the TRPM-2 gene is expressed at very low levels in one anatomical location within the prostate before castration, and at higher levels elsewhere in the gland after castration. We have suggested that the rapid decline in the steady state levels of TRPM-2 is due to the death of the epithelial cells in the prostate which express the gene after androgen ablation (16). Further analysis using radiolabeled RNA probes for transcription assays and for in situ hybridization will help to establish the mechanism of repression of this gene and may help to clarify the physiological role of the protein.

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