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## Androgen regulation of canine prostatic arginine esterase mRNA using cloned cDNA

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### Summary

Canine prostatic arginine esterase complementary DNA has been cloned in pPBS27, a new cloning vector. The relative abundance of androgen-regulated mRNA in intact dog prostate was reflected by the finding that a high proportion of the clones in the cDNA library hybridized strongly by plaque or colony hybridization with a poly(A)<sup>+</sup> RNA probe from intact dog prostate but not with a poly(A)<sup>+</sup> RNA probe from castrated dog prostate. One clone carrying a 400 base pairs cDNA insert was selected for further studies. Translation of the hybrid-selected RNA in a cell-free system resulted in the production of a 31 kDa peptide immunoprecipitable by antibodies against arginine esterase. This identification was confirmed by partial sequence analysis of the cDNA revealing an encoding protein with high homology to known kallikreins. Northern blot analysis of poly(A)<sup>+</sup> and total RNA showed that arginine esterase mRNA had an approximate size of 1.0 kb which corresponded to a major androgen-regulated RNA species that could be observed after denaturing agarose gel electrophoresis of prostatic poly(A)<sup>+</sup> RNA from intact dogs. Dot-blot analysis showed that dogs which had been castrated 3 weeks before had more than 100-fold lower arginine esterase mRNA level than intact dogs or castrated dogs treated with Depo-testosterone.

### Introduction

The canine prostate secretes under the influence of androgens large amounts of a protein which contains two chains having respective molecular weights of 15 and 12–14 kDa and which

constitutes 90% of total seminal plasma proteins (Isaacs and Shaper, 1983). This protein was recently identified as an arginine esterase (Chapdelaine et al., 1984). Partial amino acid sequencing revealed a high degree of homology with other proteins belonging to the kallikrein family (Lazure et al., 1984). Arginine esterase was found to be synthesized as a single chain pre(pro)protein post-translationally modified by proteolytic cleavages and glycosylation (Chapdelaine et al., 1985). The function of this enzyme as well as the nature of its *in vivo* substrate are presently unknown.

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Several other proteases have been described in the mammalian genital tract secretions (Zaneveld et al., 1975). In man, in particular, the prostate-specific antigen of 34 kDa originally described by Wang et al. (1979) has been found to present some structural and enzymatic similarities with the canine protein (Dubé et al., 1986). Since the prostate-specific antigen has been shown to be one of the most sensitive markers of prostate cancer (Kuriyama et al., 1982), it becomes important to study in an animal model the expression of a related gene and in particular the fine details of the effects of hormones. Indeed, hormonal therapy has become the treatment of choice for prostatic carcinoma since the discovery of Huggins et al. (1941) of the beneficial effect of castration on this disease. To better define the effects of hormones, we have cloned the cDNA for canine prostate arginine esterase. Using this probe, we report some of the characteristics of its regulation by androgenic hormones.

## Materials and methods

### *Animals*

Adult mongrel dogs weighing 20–30 kg were obtained from a local supplier through the Laboratory Animal Service of Laval University in Quebec City. Castration was performed via a scrotal incision under nembutal anesthesia. In addition, two castrated animals received three injections of 100 mg of Depo-testosterone at weekly intervals beginning 3 weeks after castration.

### *Isolation of RNA*

Total RNA was isolated by the method of Chirgwin et al. (1979) and poly(A)<sup>+</sup> RNA was obtained after two passages of total RNA over an oligo-dt cellulose column (Aviv and Leder, 1972).

### *RNA analysis*

Total and poly(A)<sup>+</sup> RNAs were analyzed by electrophoresis on horizontal 1.5% agarose gel slabs containing 2.2 M formaldehyde and 0.02 M sodium phosphate buffer pH 7.0 as described by Lehrach et al. (1977).

### *Cell-free translation and immunoprecipitation*

Cell-free translation was carried out in rabbit

reticulocyte lysates as previously described (Chapdelaine et al., 1985). In order to achieve immunoprecipitation of radioactive arginine esterase, we mixed together 30  $\mu$ l of translation medium, 10  $\mu$ l of purified arginine esterase (10  $\mu$ g), 50  $\mu$ l of rabbit anti-arginine esterase antiserum and 1.0 ml of 10 mM Tris-HCl buffer containing 0.15 M NaCl, 1 mM EDTA and 0.2% of Triton X-100. This mixture was first incubated for 1 h at 37°C and then overnight at 4°C. The immunoprecipitate was centrifuged and washed twice with buffer. Total translation products and immunoprecipitates were analyzed by polyacrylamide gel electrophoresis as described by Laemmli et al. (1970). The gels were impregnated with En<sup>3</sup>Hance (New England Nuclear). They were dried in vacuo and exposed to Kodak X-omat AR XR2 film at –70°C for several days.

### *cDNA cloning*

A cDNA library was prepared from 5  $\mu$ g of canine prostatic poly(A)<sup>+</sup> RNA using a new cloning vector, pPBS27, and a procedure recently described by Bellemare et al. (1987).

### *Screening of the cDNA library*

100 bacterial recombinants were chosen at random from the cDNA library. Single-stranded phage recombinants were produced by mean of helper phage M13K07 (Pharmacia). The phages were spotted on Hybond-N membranes (Amersham) which were placed for 1 min in a solution containing 0.5 M NaOH and 1.5 M NaCl and then for 3 min in 1.0 M Tris-HCl buffer pH 8.0 containing 1.5 M NaCl. The filters were dried and the DNA was covalently cross-linked by ultraviolet light during 5 min. Screening for the presence of cDNA for androgen-dependent mRNA was done by in situ hybridization with  $2 \times 10^6$  cpm/ml of <sup>32</sup>P-labeled poly(A)<sup>+</sup> RNA from intact or castrated canine prostate. Labeling of the RNA was done with [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol) and T<sub>4</sub> polynucleotide kinase from New England Biolabs as described by Smith et al. (1979).

Hybridization conditions were similar to those of Hanahan and Meselson (1983). Hybridization solution contained 0.2% SDS in 6  $\times$  SET buffer (SET = 0.15 M NaCl, 30 mM Tris-HCl pH 8.0, 1

mM EDTA), 50% formamide,  $1 \times$  Denhardt's, 150  $\mu\text{g}/\text{ml}$  of *E. coli* tRNA and 10  $\mu\text{g}$  of poly(A) (Boehringer Mannheim) in a total volume of 50 ml. The membranes were then washed 3 times for 10 min at  $68^\circ\text{C}$  in  $2 \times$  SET solution containing 0.2% SDS. The filters were air-dried and deposited on Kodak X-omat AR films at  $-70^\circ\text{C}$  for autoradiography.

The cDNA size of the positive recombinants was estimated by agarose gel electrophoresis after treatment of plasmids with endonucleases *Pst*I and *Bam*HI. Large-scale preparation of some recombinants was done as described by Micard et al. (1985). The cDNA was labeled to high specific activity ( $10^7$  cpm/ $\mu\text{g}$  DNA) by nick translation as described by Meinkoth and Wahl (1984). Screening of transformants with this probe was done by in situ colony hybridization as described by Hanahan and Meselson (1983).

#### *Nucleotide sequence analysis*

The nucleotide sequences of the cloned arginine esterase cDNA were done by the dideoxy chain termination procedure (Sanger et al., 1977) using [ $^{35}\text{S}$ ]dATP. The template was single-stranded DNA of clone pAgE400 annealed with reverse sequencing primer from Pharmacia.

#### *Primer extension analysis*

The heptadecamer oligonucleotide primer 5'-CCCTGGGTGAAAAATCG-3' was chemically synthesized using Biosearch Model 8700 DNA synthesizer (New Brunswick Sci. Co.). This primer was complementary to the mRNA sequence coding for Thr-Ile-Phe-His-Pro-Gly (residues 140-145) deduced from the cDNA sequence of pPBS clone 10 (see Results section). The mRNA sequence was done using 2  $\mu\text{g}$  of prostatic poly(A) $^+$  RNA annealed with 1 ng of the specific oligonucleotide primer in the presence of AMV reverse transcriptase from BRL as described by Geliebter et al. (1986).

#### *Hybrid selection of mRNA*

AgE400 single-stranded DNA was immobilized on Hybond-N as described by the manufacturer (Amersham.). Hybridization with prostate poly(A) RNA from normal dog, and elution of the selected mRNA were done according to Ricciardi et al.

(1979). Cell-free translation of the selected mRNA was performed as described above.

#### *Dot hybridization and Northern blot*

Dot hybridization of polyadenylated RNA on Hybond-N was performed as suggested by the supplier (Amersham, 1985). Northern blots were done as described by Maniatis (1982) after the electrophoresis of RNA in agarose gel in presence of 2.2 M formaldehyde and its transfer to the Hybond-N membrane. Hybridization washing conditions were the same as described above for the screening of recombinants. Radioactive probes were prepared by nick translation of the cDNA.

## **Results**

Screening by plaque hybridization of 100 recombinants of the canine prostatic cDNA library with RNA probes from intact and castrated dog prostates showed that a large proportion of the clones were coding for androgen-dependent mRNAs. Following this preliminary screening, we selected one 350 bp recombinant clone that gave a strong signal. The cDNA from this clone was used for testing 500 recombinant clones by in situ colony hybridization. The radiolabeled cDNA hybridized with 12% of the spotted clones. The size of the DNA insert for all these clones was determined by agarose gel electrophoresis and was found to be small in most cases. Four clones contained inserts of approximately 400 bp. One of them, clone 10, was selected for further study. It was first characterized by in vitro translation of the mRNA selected by hybridization of dog prostate poly(A) $^+$  RNA to isolated single-stranded DNA of pPBS clone 10. This experiment showed that the hybrid-selected RNA directed the synthesis of a 31 kDa protein that could be immunoprecipitated with antiarginine esterase antibodies (Fig. 1). The in vitro translation of unselected poly(A) $^+$  RNA from the prostates of intact, castrated and castrated-Depo-testosterone-treated dogs is also shown for comparison. The endogenous 45 kDa band was not immunoprecipitated with this antiserum. A control hybrid selection experiment using pPBS27 DNA without insert did not give rise to any specific translation product. This constituted the first evidence that

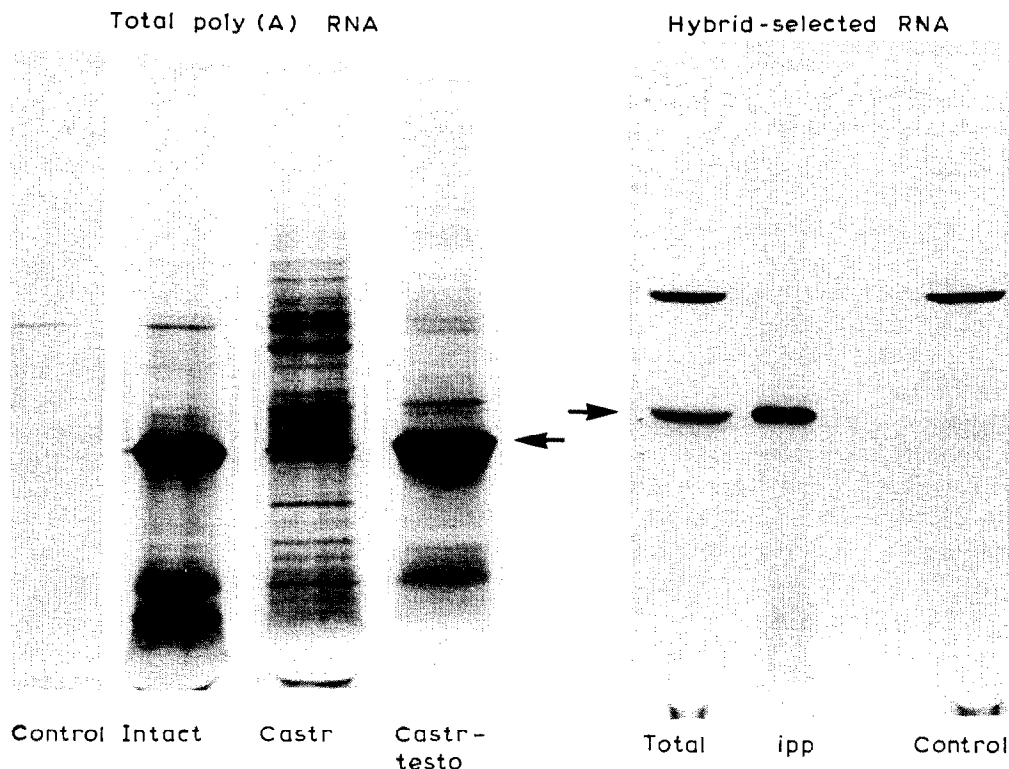


Fig. 1. SDS-polyacrylamide gel analysis of proteins labeled by [ $^{35}\text{S}$ ]methionine and obtained after *in vitro* translation in the rabbit reticulocyte lysate. In the left panel, the translation assay contained total poly(A) $^{+}$  RNAs from the prostates of intact, castrated (castr) or castrated-Depo-testosterone-treated dogs (castr-testo). Control translation contained no added exogenous RNA. The right panel shows the translational products of poly(A) $^{+}$  RNA hybrid-selected with pPBS clone 10 (total products and products obtained after immunoprecipitation (ipp) with arginine esterase antiserum). In that experiment, the control contained poly(A) $^{+}$  RNA hybrid-selected with pPBS27 without insert. The arrows indicate the position of the abundant 31 kDa arginine esterase translation product (Chapdelaine et al., 1985). Non-radioactive molecular weight standards were included in parallel lanes and stained with Coomassie blue (results not shown).

clone 10 contained a cDNA for arginine esterase. For this reason, clone 10 was designated thereafter as clone pAgE400.

In order to confirm this identification, we determined the nucleotide sequence of a portion of the mRNA by a strategy depicted in Fig. 2 using clones 10 and 5 and primer extension with a synthetic oligonucleotide. The nucleotide sequence and the deduced amino acid sequence are shown in Fig. 3. The sequence contains 471 nucleotides (including the stop codon) coding for the carboxy terminal of arginine esterase and 46 nucleotides before the poly(A) stretch in the 3' non-coding portion for a total of 517 nucleotides. Peculiar features of this sequence include the presence of a polyadenylation site consisting of AGUAAA instead of usual AAUAAA. The sequence AGUAAA

has also been found recently in a human prostatic mRNA for a 94 amino acid protein secreted by human prostate (Mbikay et al., 1987), in prostate-

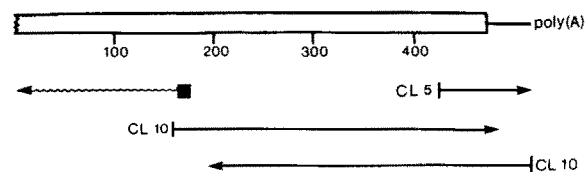


Fig. 2. Diagram of sequencing strategy for clone pAgE400. The sequence was determined in both directions for clone 10 (CL 10) or pPBS400 and in one direction for the smaller clone 5 (CL 5). The open box contains the protein-encoding region. The broken left side of the box indicates that the sequence is not complete in the 5' region. The black box and the wavy line indicate respectively a synthetic oligonucleotide probe used for primer extension and a primer-extended cDNA transcript.



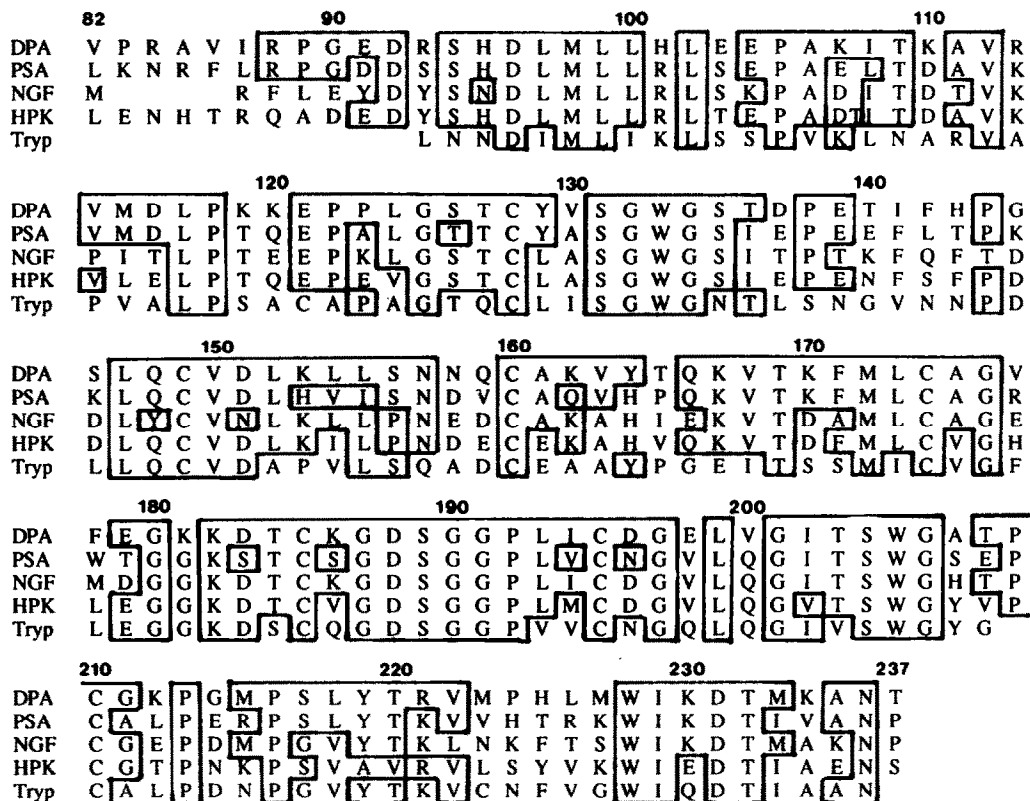
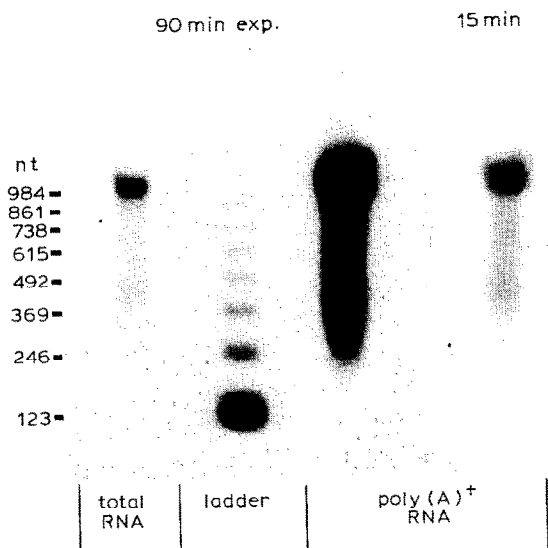


Fig. 4. Amino acid sequence comparison of dog prostate arginine esterase (DPA), prostate-specific antigen (PSA) (Lundwall and Lilja, 1987), human pancreas kallikrein (HPK) (Fukushima et al., 1985), mouse nerve-growth factor (NGF) gamma subunit (Thomas et al., 1981), and rat trypsin (MacDonald et al., 1982). Identical residues are enclosed in boxes.



esterase mRNA levels, we used a dot-blot assay. Serial concentrations of poly(A)<sup>+</sup> RNA from the prostates of intact dogs, of 3-week-castrated dogs and of 3-week-castrated dogs treated with Depo-testosterone were spotted on Hybond filters that were then hybridized with the [<sup>32</sup>P]cDNA probe. This experiment showed that arginine esterase mRNA levels in castrated dogs were at least 100-

Fig. 5. Northern blot analysis for arginine esterase mRNA in total RNA and poly(A)<sup>+</sup> RNA extracted from the prostate of an intact dog. Ladder DNA (123 bp) was included as a size marker. The quantity of RNA was 10 µg in each lane. On the right portion of the figure, the result of a shorter exposure of the poly(A)<sup>+</sup> RNA on X-ray film is shown. It indicates that the cDNA probe hybridization with the poly(A)<sup>+</sup> RNA is also mainly localized on the 1 kb band.

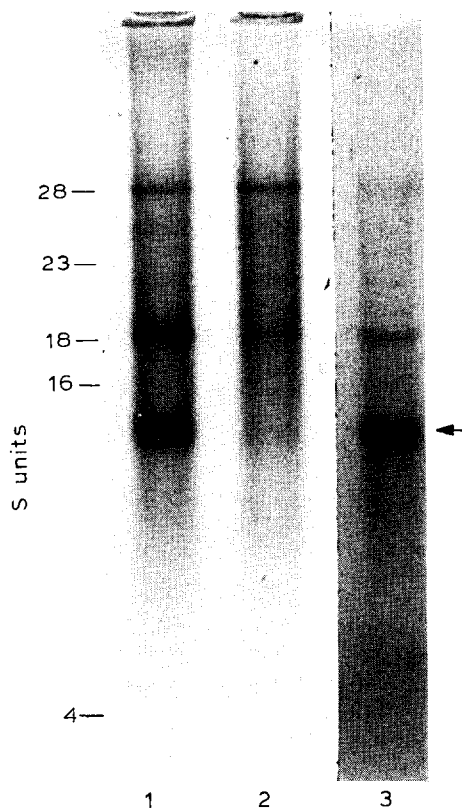


Fig. 6. Agarose-formaldehyde gel electrophoresis of poly(A)<sup>+</sup> RNA from the prostates of intact dogs (1), castrated dogs (2) and castrated-Depo-testosterone-treated dogs (3). This figure is the negative image of the ethidium bromide staining of the RNA. Standard 23, 16 and 4S RNAs were electrophoresed on adjacent lanes while 28 and 18S positions are those of endogenous ribosomal RNA still present as contaminants. The arrowhead indicates the migration of a major androgen-dependent RNA.

fold lower than in intact animals (Fig. 7). Furthermore, after three injections of 100 mg of Depo-testosterone at weekly intervals to castrated animals, the arginine esterase mRNA levels had returned to precastration levels.

## Discussion

We have cloned complementary DNA from canine prostate arginine esterase mRNA. The identification of the clone was based on the hybrid select translation and on the immunoprecipitation of the 31 kDa product with antiarginine esterase antibodies. The nucleotide sequence and the de-

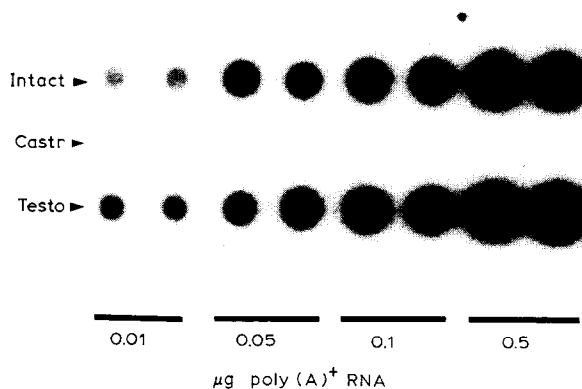


Fig. 7. Dot hybridization of increasing concentrations (in duplicate) of poly(A)<sup>+</sup> RNAs isolated from the prostates of intact, castrated and castrated-Depo-testosterone (Testo)-treated dogs.

duced amino acid sequence confirmed this identification and showed that arginine esterase had a high homology with members of the serine-protease class and in particular with PSA and with kallikrein-related enzymes.

Our dot hybridization results (Fig. 7) showed that arginine esterase mRNA was highly concentrated in normal dog prostates and are thus in accord with the results of Fig. 1 which demonstrated that arginine esterase was the only major protein translated in vitro with poly(A)<sup>+</sup> RNA extracted from this tissue. These results are also compatible with the fact that this protein represents more than 90% of total prostatic secretory proteins (Isaacs and Shaper, 1983).

Arginine esterase mRNA levels after castration with or without androgen administration were parallel to the in vitro translation of this protein using poly(A)<sup>+</sup> RNA. Therefore, we can conclude that the decrease of arginine esterase synthesis after androgen withdrawal is really due to a decrease of mRNA levels and not to inactivation of still present mRNAs. The situation is thus similar to the one observed in rat prostate where the levels of mRNAs coding for prostatic binding protein subunits fall dramatically after castration (Parker et al., 1980). However, the exact mechanisms involved in the regulation of these levels are still not completely understood and may involve regulation at various steps including transcription rate, RNA elongation rate and nuclear stability or processing of nuclear RNA (Zhang and Parker,

1985). Since the rat is the only species in which gene expression for an androgen-dependent prostatic protein has been extensively studied, there is an obvious need for similar studies in other mammalian species. Successful cloning of canine prostate arginine esterase constitutes a first step in that direction.

Since we have recently shown that arginine esterase is related to the kallikrein family by its amino acid sequence (Lazure et al., 1984) and by its nucleotide sequence in this study, it is also interesting to note that two submaxillary gland kallikrein mRNAs are present at high levels in rat prostate (Ashley and MacDonald, 1985). These mRNAs are approximately the same size (1.0 kb) as canine prostate arginine esterase mRNA. Furthermore, human prostatic tissue and its secretion contain high levels of a protein designated as prostate-specific antigen (PSA) and shown to be a proteolytic enzyme (Ban et al., 1984) structurally and functionally related to serine-proteases and kallikrein (Watt et al., 1986). The cDNA for this protein has been cloned recently (Lundwall and Lilja, 1987). Preliminary experiments in our laboratory indicate that the arginine esterase cDNA probe hybridized slightly with human prostate poly(A)<sup>+</sup> RNA immobilized on nylon membranes. Therefore, the dog should constitute an excellent model for studying the hormonal control of this important prostatic marker.

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