

Characterization and Expression of the Prostatic Arginine Esterase Gene, a Canine Glandular Kallikrein

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ABSTRACT

The prostatic arginine esterase gene was isolated from a genomic library prepared with dog liver DNA in λ EMBL3. The selected clone contained an insert of approximately 17 kb which included the whole coding portion of arginine esterase mRNA (5 exons plus 4 introns), 2 kb upstream from the initiation site and 12 kb downstream from the polyadenylation site. The intron-exon boundaries were identical to all known mammalian kallikrein genes. The deduced amino acid sequence indicated a high degree of identity (51–61%) with other kallikreins expressed not only in the prostate but also in the pancreas of various animal species. The 5'-flanking sequences contained potential regulatory elements such as a variant TATA box (TTTAAA), a CCAAT box, a SP1 transcriptional factor binding site (GGGCGG), and two TGTCCT motifs resembling glucocorticoid response elements. Southern blot analysis with an amplified cDNA fragment of 487 bp corresponding to the 5' portion of the mRNA and with a DNA probe from a different portion of the arginine esterase gene indicated the presence of two to three homologous genes in the canine genome while in a previous study a single band was detected using a 400-bp arginine esterase cDNA corresponding to the 3' portion of the mRNA. These results suggest that the arginine esterase gene belongs to a small kallikrein gene family. Arginine esterase mRNA is expressed primarily in the prostate but also at an extremely low level (approximately a thousandfold less) in several other tissues including the liver, the gracilis thigh muscle, the kidney, and the pancreas.

INTRODUCTION

DETAILED KNOWLEDGE of the expression of glandular kallikreins, a subfamily of serine-proteases, in the mouse, rat, and human species (for review, see Clements, 1989 and MacDonald *et al.*, 1988) is increasing rapidly. The high degree of sequence similarity and the close proximity of these genes on given chromosomes or loci (Mason *et al.*, 1983; Riegman *et al.*, 1989; Wines *et al.*, 1989) indicate that all of these enzymes are members of the same family, even though most of them do not conform to the classical definition of kallikreins, *i.e.*, enzymes having the ability to generate vasoactive kinins, bradykinin, and kallidin from kininogen (Fiedler, 1979). Indeed some kallikreins are able to cleave other substrates such as hormone precursors, but the exact physiological role of many of the gene products remains unknown. In addition to classical

kallikrein-containing tissues such as the submaxillary glands, the kidney, and the pancreas, kallikrein-related enzymes are also present in many other tissues and in particular in the prostate of several (if not all) mammalian species. The human prostate is rich in a proteolytic enzyme called prostate-specific antigen (PSA) (Wang *et al.*, 1979), whose function appears to hydrolyze seminal vesicle proteins forming the seminal coagulum (Lilja, 1985). It also expresses human glandular kallikrein-1 (hGK-1) mRNA (Chapdelaine *et al.*, 1988a) whose protein has not yet been isolated. Rat prostate expresses kallikreins S3 and P1, which are at least partly regulated by androgens (Clements *et al.*, 1988). The prostates of two other species, guinea pig and dog, are extremely rich sources of arginine esteroprotease activity (Chapdelaine *et al.*, 1984; Dunbar and Bradshaw, 1987). The canine enzyme represents more than 90% of whole seminal plasma proteins (Isaacs and

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Shaper, 1983), and its expression is dependent on androgenic steroids (Chapdelaine *et al.*, 1988b). In spite of its abundance, the enzyme has no known physiological substrates. It is not involved in coagulum hydrolysis because semen coagulation does not occur in the dog. Furthermore, arginine esterase is unable to generate vasoactive kinins from kininogen (Frenette *et al.*, 1985). Another area of uncertainty about the dog prostate kallikrein-like enzyme is its relationship with kallikreins present in other tissues. We have shown that with the use of a cDNA probe representing about half of the arginine esterase mRNA in its 3' portion only one band is detected by Southern blot (Chapdelaine *et al.*, 1988c). However, it is unlikely that the dog has only one kallikrein gene because two additional arginine esterases (including true glandular kallikrein) have been isolated from dog urine (Murthy and Scicli, 1988). As a first step in the characterization of the kallikrein gene family in the dog, we have isolated and characterized the prostatic arginine esterase gene.

MATERIALS AND METHODS

Isolation and characterization of dog arginine esterase gene

A genomic library prepared with dog liver DNA in λ EMBL 3 vector was purchased from Clontech Laboratories Inc. Phage plaques on agar plates were transferred to Hybond-N membranes (Amersham) as recommended by the manufacturer and were prehybridized for 2–5 hr at 42°C in 50% formamide hybridization solution (0.9 M NaCl, 0.18 M Tris-HCl pH 8.0, 6 mM EDTA, 1× Denhardt's solution, 0.1% NaDodSO₄ and 150–200 µg/ml of salmon sperm DNA). Hybridization was done for at least 18 hr at 42°C in the presence of 2×10^6 cpm/ml of pAgE-400 cDNA insert (Chapdelaine *et al.*, 1988a) ³²P-labeled to a specific activity of 4.2×10^9 cpm/µg with the multiprimer labeling system of Amersham. After the hybridization period, the filters were washed three times at room temperature and twice at 68°C in a solution containing 1× STE and 0.2% NaDodSO₄. One positive plaque after autoradiography was subjected to five rounds of purification. The positive clone was designated λ DAG-1. Its DNA was prepared by the procedure of liquid lysates and polyethylene glycol precipitation (Miller, 1987) followed by rapid purification through a DE-52 DEAE-cellulose column (Reddy *et al.*, 1988).

The localization of mRNA coding sequences on restriction fragments of the genomic clone was done by Southern blot analysis with synthetic oligonucleotide probes. The oligodeoxyribonucleotides used were: TrB11 (5'-CAGGAGTGCTGGGAGCTGGCAGACAGTGAG-3') complementary to nucleotides 1–30; TrB4 (5'-GCCTCTAATGATCCGGGGCTG-3') complementary to nucleotides 98–118 and TrB9 (5'-ACAGCATGAACTTTGTACCT-3') complementary to nucleotides 604–624 of the mRNA sequence (Chapdelaine *et al.*, 1988b). In mouse, rat, and human kallikrein genes, the corresponding sequences are localized, respectively, in exons I, II, and IV. These oligo-

nucleotides were synthesized using a Biosearch Model 8700 DNA synthesizer (New Brunswick Scientific). Hybridization with oligonucleotide probes was done in a solution of 6× SSC (0.9 M NaCl, 0.09 M sodium citrate), 5× Denhardt's solution, 0.5% NaDodSO₄, 20 mM Tris-HCl pH 7.4, 100 µg/ml of *E. coli* MRE 600 tRNA from Boehringer Mannheim, and 10% dextran sulfate from Pharmacia (omitted in the prehybridization solution). The hybridization temperatures for TrB4 and TrB9 were determined by the formula: $t(^{\circ}\text{C}) = 4(\text{G} + \text{C}) + 2(\text{A} + \text{T}) - 5$ while TrB11 (30-mer) was hybridized at 60°C. After overnight hybridization, the filters were washed at 5°C lower than the hybridization temperatures for 1 hr in 2× SSC, 10 mM Tris-HCl pH 7.4 and 0.5% NaDodSO₄ followed by an additional hour in 1× SSC, 10 mM Tris-HCl pH 7.4, and 0.5% NaDodSO₄. The oligonucleotide probes were labeled at their 5' end with T4 polynucleotide kinase from Bethesda Research Laboratories and [γ -³²P]ATP from Amersham and they were purified according to our recently published procedure (Chapdelaine *et al.*, 1990).

DNA sequence

The nucleotide sequence of the arginine esterase gene was determined using two fragments of λ DAG-1 subcloned into the multifunctional plasmids pTZ18R and pTZ19R obtained from Pharmacia. The fragments of 2.8 and 2.9 kb were generated by digestion with *Eco* RI and *Kpn* I, respectively. The sense and antisense strands of particular subclones could be identified with oligonucleotide probes complementary to one of the two strands. Nucleotide sequencing was done by the dideoxy chain-termination method (Sanger *et al.*, 1977) using T7 DNA polymerase from Pharmacia and 40 different synthetic oligonucleotides as primers on single-stranded DNA which was produced by co-infection of the recombinant pTZ DNA with M13K07 helper phage.

Southern blot analysis

Canine blood DNA prepared according to Gustafson *et al.* (1987) was digested to completion with *Eco* RI, *Bam* HI, *Hind* III, *Pst* I, or *Kpn* I. Samples containing 10 µg of DNA were electrophoresed through 1% agarose gels and transferred to Hybond-N membranes (Southern 1975). The filters were prehybridized and hybridized with specific probes as described above.

Northern blot analysis

Total RNA was isolated from various canine tissues as described by Chomczynski and Sacchi (1987). Poly(A)⁺ RNA fractions were obtained after one passage of total RNA over a small oligo(dT)-cellulose column. Aliquots of RNA were resolved in 1.2% agarose gels containing formaldehyde and 1× MOPS as described by Khandjian (1986). After the electrophoresis, the RNA was transferred directly to filters. In all the experiments described in this paper, sexually mature uncastrated dogs were used. Sexual maturity was ascertained by visual inspection of the size of

the testes and by determining the prostate weight. In all cases, the weight was greater than seven grams.

Primer extension analysis

Synthetic oligonucleotide TrB24 (5'-TCCACCCCAGG-GACATT-3') (30 ng) complementary to nucleotides 62-78 of the mRNA sequence (Chapdelaine *et al.*, 1988b) was labeled at its 5' end and was hybridized with 0.5 μ g of prostatic poly(A)*RNA for 10 min at 70°C and then for 4 hr at 48°C. The primer extension reaction was carried out in the presence of RAV-2 reverse transcriptase from Amersham as described by Calzone *et al.* (1987). The reaction products were electrophoresed on a 5% sequencing gel. To localize exactly the chain termination(s), the neighboring wells contained the dideoxy chain-termination ladders obtained by doing a sequence reaction with the single-stranded 2.8-kb *Eco* RI fragment of λ DagE1 and TrB24 as primer.

Amplification of cDNA corresponding to the 5' portion of the mRNA

Poly(A)*RNA preparations from the prostate (2.5 ng), the gracilis thigh muscle (2.5 μ g), or the liver (2.5 μ g) were mixed separately in a total volume of 20 μ l with a solution containing 100 ng of oligo-[dT18], PCR buffer 1 $\times$ [10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin (wt/vol)], 1 mM each of all four deoxyribonucleotides from Pharmacia, 40 units of RNasin from Promega,

and 20 units of RAV 2 reverse transcriptase from Amersham. The reaction was allowed to proceed for 15 min at room temperature and for 45 min at 42°C. Thereafter, polymerase chain reaction (PCR) was performed as follows: to the 20- μ l reverse transcriptase reaction mixture were added 180 μ l of a solution containing 200 μ M each of dATP, dCTP, dGTP, and dTTP, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 0.01% gelatin, and 100 ng each of 21-mer primers TrB6 (5'-TCTCAGCCCTGG-CAGGTGGCT-3') corresponding to positions 138-158 of the mRNA sequence (Chapdelaine *et al.*, 1988b) and TrB9 (see above). The mixture was heated at 95°C for 5 min and then 5 units of *Taq* DNA polymerase from Perkin-Elmer Cetus were added. The amplification was done for 35 cycles as follows: denaturation at 95°C for 30 sec, cooling at 50°C for 1 min, annealing of primers at 50°C for 30 sec, heating at 72°C for 45 sec, extension of the primers at 72°C for 2 min, heating at 95°C for 1 min, and so on. Analysis of amplification products was done on 1.5% agarose gel made in TBE buffer using as a probe oligonucleotide TrB3 (5'-CCTCATAATTACTGTTTGAC-3') which is complementary to nucleotides 232-252 of the mRNA sequence (Chapdelaine *et al.*, 1988b).

RESULTS

Identification of prostatic arginine esterase DNA clone

The use of pAgE400 cDNA probe of 400 bp coding for the 3' portion of arginine esterase mRNA and hybridizing

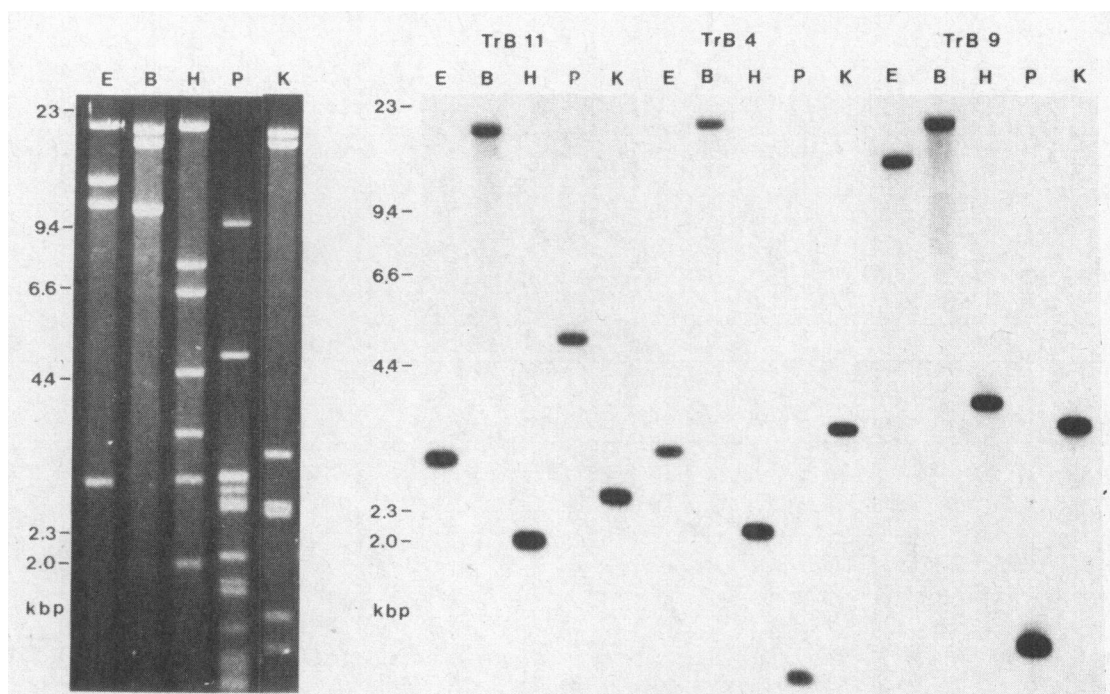


FIG. 1. Southern blot analysis of the genomic clone λ DagE1 containing the dog prostate arginine esterase gene. The genomic clone was digested to completion with *Eco* RI (E), *Bam* HI (B), *Hind* III (H), *Pst* I (P), or *Kpn* I (K). Left panel. Ethidium bromide staining of the restriction fragments and the other panels represent the autoradiographs of the hybridizations with oligonucleotides TrB11, TrB4, and TrB9 which are complementary, respectively, to nucleotides 1-30, 98-118, and 604-624 of the mRNA sequence (Chapdelaine *et al.*, 1988b).

to a single gene at high stringency conditions (Chapdelaine *et al.*, 1988c) made it relatively easy to select a specific arginine esterase genomic clone from the canine library. One positive clone was obtained from a total of 1.5×10^6 plaques screened. The genomic insert had an approximate size of 17 kb. It was characterized by Southern blotting after digestion with restriction enzymes. The fragments were detected by ethidium bromide staining and hybridization with pAgE400 cDNA and three synthetic oligonucleotides coding for exons I, II, and IV (Fig. 1). Fragments of 2.8 and 3.0 kb after digestion with *Eco* RI or *Kpn* I were subcloned in pTZ18R. These two subclones covered the whole coding portions of arginine esterase mRNA and approximately 2 kb upstream from the initiation site. They were used for sequence determination by primer extension with a collection of synthetic oligonucleotides according to the strategy illustrated in Fig. 2.

Gene structure and characteristics

The arginine esterase gene included five exons and four introns (Fig. 3). The exons coded for an mRNA containing 873 nucleotides. This mRNA is translated into a protein of 260 amino acids which has the classical features of serine proteases as previously described (Chapdelaine *et al.*, 1988a,b). The four introns that begin with dinucleotide GT and end with dinucleotide AG contain respectively 288, 1070, 184, and 750 nucleotides. The intron-exon boundaries respective to amino acid sequence were identical to those of all known members of the kallikrein family in the

mouse (Mason *et al.*, 1983), human (Schedlich *et al.*, 1987), and rat (Wines *et al.*, 1989).

The 5'-flanking sequences of the arginine esterase gene contained potential regulatory elements such as a variant TATA box (TTTAAA) at 2,369–2,374, a CAT box (CCAAT) at 2,261–2,265, and a SP1 transcriptional factor binding site (GGGCGG) at 2,340–2,345. In addition to these customary elements, the 5'-flanking sequences showed the presence of two potential glucocorticoid receptor binding sites TGTCTT at 2,182–2,177 and 2,435–2,430. The latter site was immediately adjacent to initiation codon ATG. Two other TGTCTT sequences were also found in intron 2 at positions 3,047–3,042 and 3,163–3,168. Another feature in the 5' sequence was a 19-bp palindromic sequence GTTCCCCTAGTAGGGGAAC at 1,856–1,874. We could also observe the presence of a long repeat of dinucleotide GT from 1,442–1,489.

The site of transcription initiation was determined by primer extension. Figure 4 shows that one major and two minor bands were present. They corresponded to positions 2,396, 3,003, and 3,004 of the sequence. The nucleotide sequence downstream from the stronger signal at 2,396 was highly similar to the sequence adjacent to the initiation site in rat glandular kallikreins (Chen *et al.*, 1988; Wines *et al.*, 1989).

Number of homologous genes

Southern blot analysis was used to estimate the number of homologous genes hybridizing with DNA probes prepared from two different portions of the arginine esterase

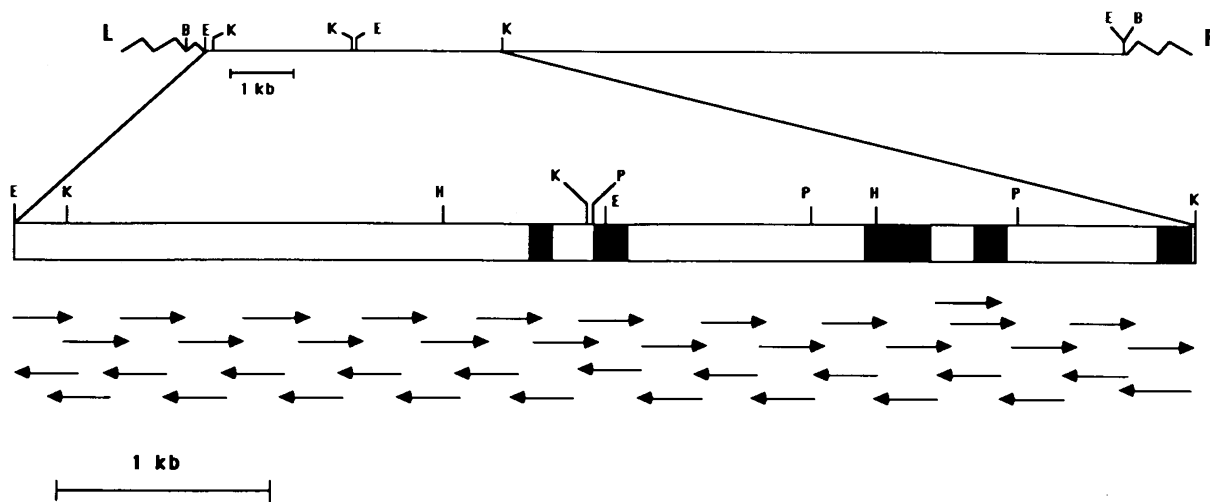


FIG. 2. Sequencing strategy of the dog arginine esterase gene. Restriction endonucleases were *Eco* RI (E), *Bam* HI (B), *Hind* III (H), *Kpn* I (K), and *Pst* I (P). Arrows indicate the direction and extent of each sequencing reaction. The positions of the five exons along the gene are indicated by black boxes.

4141 CTCCATTGGGGAGTACCTGCTATGTCCTCTGGATGGGGACGCACTGATCCAGAAACGAGTATGCTGGGGCTAGACCATATATCCTGGAGC
roProLeuGlySerThrCysTyrValSerGlyTrpGlySerThrAspProGluThrI

4231 CAGACTTGGGTGTAGGGAGGAGGGTGTCTGAGGACCTGGACTTCTGGGCCTGAGAGAGGAGGGGCTGGGGCCTGGGCTCTGGGTCTGAG

4321 GGAGGAGGGCTGAGGAGCCACCTGGGCTCTGGCCACAGACCTTTTCTCTCTGGCTTGTAGTTTTCACCCAGGGAGTCTCCAGTGTGTG
 Exon 4
 1ePheHisProGlySerLeuGlnCysVal
 140

4411 GACCTCAAACCTCCTGTCCAAATAATCAATGTGCAAAAGTATACACTCAAAGGTGACAAAGTTCATGCTGTGTGCTGGTGTCTTGGAGGGT
 AspLeuLysLeuLeuSerAsnAsnGlnCysAlaLysValTyrThrGlnLysValThrLysPheMetLeuCysAlaGlyValLeuGluGly
 150 160 170

4501 AAAAAAGATACCTGCAAGGTGAGCCAGGTTTGCTTGCTTGCTTGCTTGTCTTTAAAGATTTTATTTAGGGACATTGGGGTGGCTTAGTGG
 LysLysAspThrCysLys
 180

4591 TTGAGCATCTGCCTTCAGCTCAGGGCGTGGTCTGGGGTCAGGGATCGAGTCCCGCATCAGGCATCCTGCAGGGAGCCTGATGTGGGACT
 4681 CGATTCCAGGATATCAGGATCATGACCTGATCCAAAGGTAGATGCTCAACCACTGAGCCAACCAAGGTGCCCAATGAGCCAATCTTTCAC
 4771 TTCAAGGCCCTTGAAGGAAAGAAAGGAGATGACTATGGTTAATGCTGGAACCTAAATGCTGTTTTATCCCCCTCCTTGTGACTTCTTCAA
 4861 TATACGGTGTGACCTTATTTCTTCTGCTTACCCCTCTTCCAGGGCTCCCTAGGGCCTAGAGGGAGTGCCTACTCAGAAAAAAACT
 4951 CTGGGAGAGTTAATATCTGAGATGAGCTGTGATGGTGGGAGGTCCCTTAAATGGTCTCTCCTCCTGCTGACCCATCTGTAGGGAGT
 5041 TCCTTCTGGACTCTGAAGTGCCATGTCTAGTAGCCAGGACTTGAGACACTCTCCTCTGTGTGGAATTTCCACCTACCTTGTGGGGAGAG
 5131 GACTCAGGCAATGCTTCTTTCCCCATCACCCTATTGCCGTGTGAGATACGCAAGTCCAGGTTTGACAGCTTGGGTCCCCAGGCAGAA

5221 TTAGGGCTGGCCTTCCTCACCCTCTGTCTCTATCTCTTTCACTTGCAAGGTGACTCAGGAGGCCCTCTGATATGTGATGGTGAATTGGTA
 Exon 5
 GlyAspSerGlyGlyProLeuIleCysAspGlyGluLeuVal
 190

5311 GGTATCACATCATGGGGGCTACTCCATGTGGCAAGCCTCAAATGCCATCCTTGTATACCAAGGTGATGCCTCATCTGATGTGGATCAAG
 GlyIleThrSerTrpGlyAlaThrProCysGlyLysProGlnMetProSerLeuTyrThrArgValMetProHisLeuMetTrpIleLys
 200 210 220

5401 GACACCATGAAGCCAACACCTGAATGTCCCTTTCTACTCTCTACTCCTAGTAAATGAGTATACATCAAGTTGTGGAATCATCTGGT
 AspThrMetLysAlaAsnThr***
 230 236

5491 CTTTCTGGATACAGGTACC

FIG. 3. Nucleotide sequence of the dog arginine esterase gene. The numbers on the left represent the nucleotide residues. The beginning and the end of introns are indicated by dashes above the GT and AG splice site sequences. The large and small vertical arrowheads above the nucleotide sequence indicate major and minor transcription initiation sites. The deduced amino acid sequence is shown, and the numbering refers to the position relative to the amino acid terminus of the mature protein (Lazure *et al.*, 1984). The three stars indicate the stop codon.

gene. Figure 5 shows the results obtained with exon-specific probe PCR 487, which covers two-thirds of exon 2, all of exon 3, and two-thirds of exon 4, and with probe F3, which covers part of the 5' portion of the arginine esterase gene to a position located at the beginning of exon 2. Probe PCR 487 itself contained one restriction site for *Hind* III but not for *Bam* HI, *Kpn* I, and *Eco* RI. It should also be mentioned that a *Pst* I restriction site is present in intron 2 of the arginine esterase gene (Fig. 2). Probe F3 contained a *Pst* I site able to generate a minor fragment of 67 bp along with a major fragment of 0.6 kb. It contained no restriction sites for *Eco* RI, *Bam* HI, and *Hind* III. Probe PCR 487 labeled two to three strong bands along with additional minor bands with each of the restriction enzymes tested while probe F3 gave two strong bands with each of the four enzymes tested. Thus, these results are different from the ones observed previously when pAgE-400 cDNA (covering the 3' portion of mRNA) used as probe detected a single band by Southern blot (Chapdelaine *et al.*, 1988c). These results suggest that at least two to three genes highly similar to some portions of the arginine esterase gene are present in the canine genome.

Arginine esterase mRNA expression

The expression of arginine esterase in a few selected tissues of the dog was studied by Northern blot analysis using pAgE400 cDNA as a probe (Fig. 6). This probe was used because of its high specificity to the arginine esterase gene (Chapdelaine *et al.*, 1988c). At low stringency conditions, strong hybridization signals around 1.0 kb were detected not only in the prostate but also in the pancreas, liver, kidney, gracilis muscle, and salivary glands when higher amounts of poly(A)⁺RNA were electrophoresed. This band was absent from the testis. At high stringency conditions, the signal was still present in the muscles, liver, and kidney. It was barely detectable in the pancreas and it was no longer observed in the salivary glands. To confirm that the signal observed in muscles and liver was really due to arginine esterase mRNA, poly(A)⁺RNA was first transcribed with reverse transcriptase and the product was amplified with two oligonucleotide primers designed from two regions that are usually divergent between kallikreins. Figure 7 showed that a fragment of the expected size was amplified in the three tissues examined, prostate, muscle, and liver. Furthermore, this fragment from all three tissues was

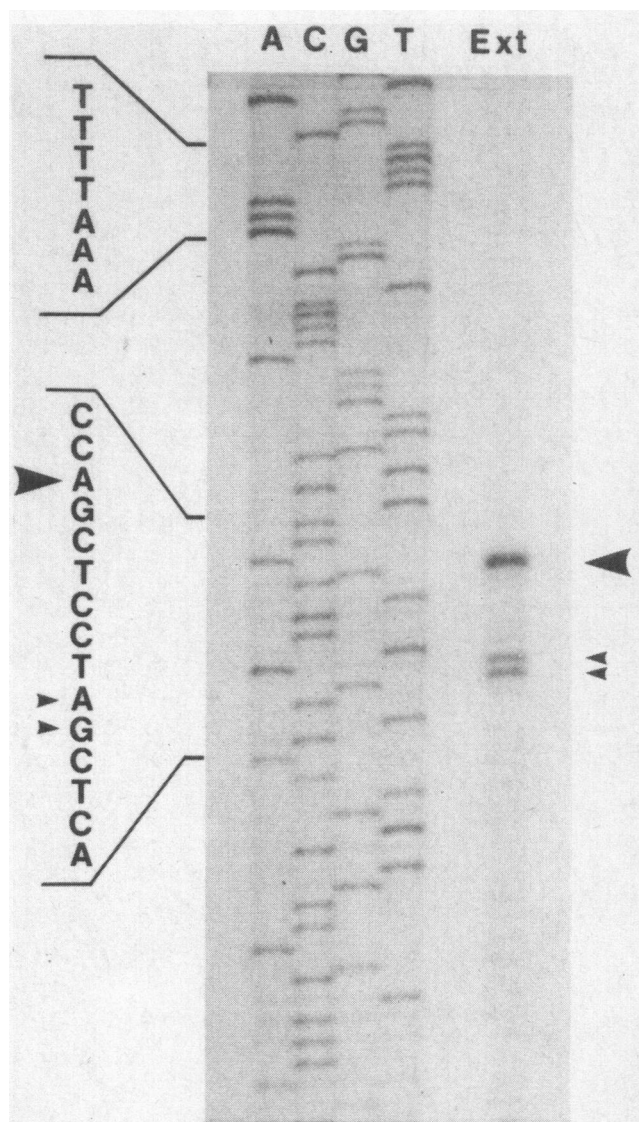


FIG. 4. Primer extension (Ext) analysis of the 5' end of prostatic arginine esterase mRNA using nucleotide TrB24 complementary to positions 62–78 of the mRNA sequence (Chapdelaine *et al.*, 1988b). These positions correspond to nucleotides 2,482–2,466 in Fig. 3. The neighboring wells contained the dideoxy chain-termination ladders of a sequence reaction using the same oligonucleotide primer and the single-stranded 2.8-kb *Eco* RI fragment of the genomic clone. For the sake of clarity, the strand corresponding to mRNA is shown. The large and small arrowheads indicate major and minor initiation sites. Note the proximity of the TTTAAA box.

labeled with another synthetic oligonucleotide probe, TrB3, complementary to the middle portion of the arginine esterase-amplified fragment. One should note, however, that a thousandfold more mRNA from muscle and liver was required to yield similar levels of the amplified fragment. In other experiments, we found that arginine esterase mRNA levels were extremely variable in the muscles and liver of different dogs. In fact in one dog (out of

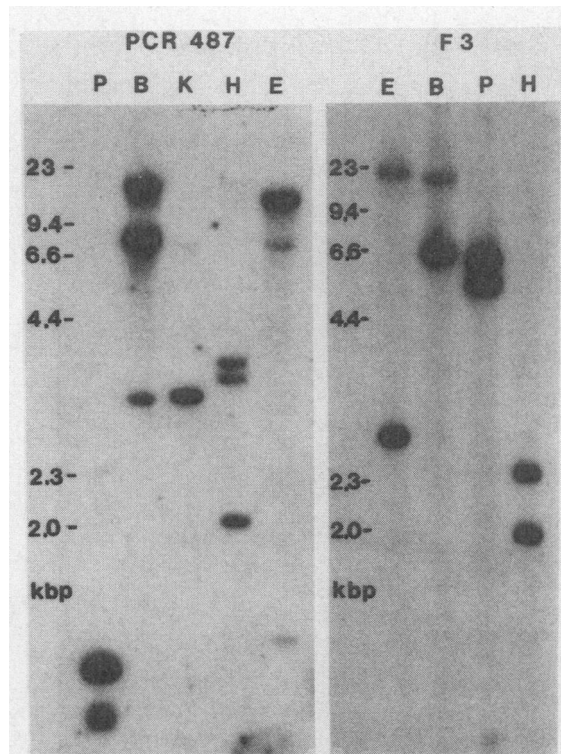


FIG. 5. Southern blot analysis of canine blood DNA with DNA probes PCR 487 and F3. Probe PCR 487 was obtained by PCR using primers TrB6 and TrB9 and prostatic cDNA. The exon-specific probe covers 487 nucleotides (138–624) of the arginine esterase mRNA sequence (Chapdelaine *et al.*, 1988b). Probe F3 covers nucleotides 2,073–2,736 of the gene sequence and includes part of 5' upstream sequences, all of exon 1 and intron 1, and about one-third of exon 2. It was obtained by digestion with *Hind* III and *Eco* RI. The DNA (10 μ g per sample) was digested with *Eco* RI (E), *Bam* HI (B), *Pst* I (P), *Hind* III (H), or *Kpn* I (K).

6), no hybridization could be detected at all in the gracilis muscle. This variability made it impossible to study the androgen dependence of arginine esterase mRNA levels in these tissues. Furthermore, arginine esterase mRNA was not found in other muscles such as the diaphragm, the heart, the quadriceps, and the abdominal muscles (results not shown).

DISCUSSION

The coding part of the genomic sequence presented here differs at four positions with respect to previously published cDNA sequence (Chapdelaine *et al.*, 1988c). Three of the differences occur in exon 2 at positions 2,688, 2,802, and 2,803 in which T, T, and A have become, respectively, C, A, and C. The replacement of an A by a C at 2,803 changed amino acid asparagine to an histidine while the two other changes were silent. Another silent modification was found at the extreme end of exon 4 at position 4,518

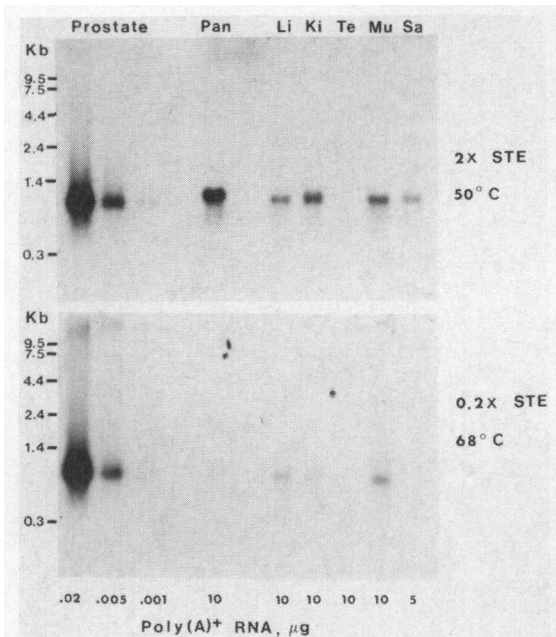


FIG. 6. Northern blot analysis of poly(A)⁺RNA from canine prostate, pancreas (Pan), liver (Li), kidney (Ki), testis (Te), gracilis thigh muscles (Mu), and salivary glands (Sa). The hybridization was done with pAgE400 cDNA of 400 bp at low stringency (upper panel) and at high stringency (lower panel). In all tissues examined, with the exception of the testis, the cDNA probe hybridized to a 1.0-kb band. Note that much lower concentrations of prostatic (0.02–0.001 μ g) RNA than of muscle (10 μ g) were used in this experiment.

where an A has become a G. These differences can probably be explained by errors due to the use of reverse transcriptase in the previous study (Chapdelaine *et al.*, 1988c) although the presence of allelic variations is not excluded.

The results presented in this study clearly show that canine prostate arginine esterase is a member of the kallikrein gene family. This conclusion is based on the striking similarity of the arginine esterase gene structure to the one of kallikrein family members in the mouse (Mason *et al.*, 1983), the human (Schedlich *et al.*, 1987), and the rat (Wines *et al.*, 1989). In addition to this structure similarity, arginine esterase demonstrates high amino acid similarities not only with kallikreins expressed in the prostate of man, rat, and guinea pig but also with renal/pancreatic glandular kallikreins having kininogenase activity. Indeed, the overall identity between canine arginine esterase and human prostate-specific antigen (Lundwall and Lilja, 1987), human glandular kallikrein-1 (Schedlich *et al.*, 1987), rat kallikrein S3 (Ashley and MacDonald, 1985), rat kallikrein P1 (Brady *et al.*, 1989), guinea pig prostate kallikrein (Dunbar and Bradshaw, 1987), human renal/pancreatic kallikrein (Fukushima *et al.*, 1985), and porcine pancreatic kallikrein (Tschesche *et al.*, 1979) is 58, 61, 51, 53, 57, 58, and 58%, respectively. This homology can also be extended to the noncoding portions of the nucleotide sequence. In particular, the first 120 nucleotides upstream from the cap site in the only kallikrein genes reported to be expressed exclusively or specifically in the prostate (PSA, hGK-1, and arginine esterase) are remarkably similar to the corresponding portion of the renal/pancreatic gene (Fig. 8). Immediately before these sequences, the renal/

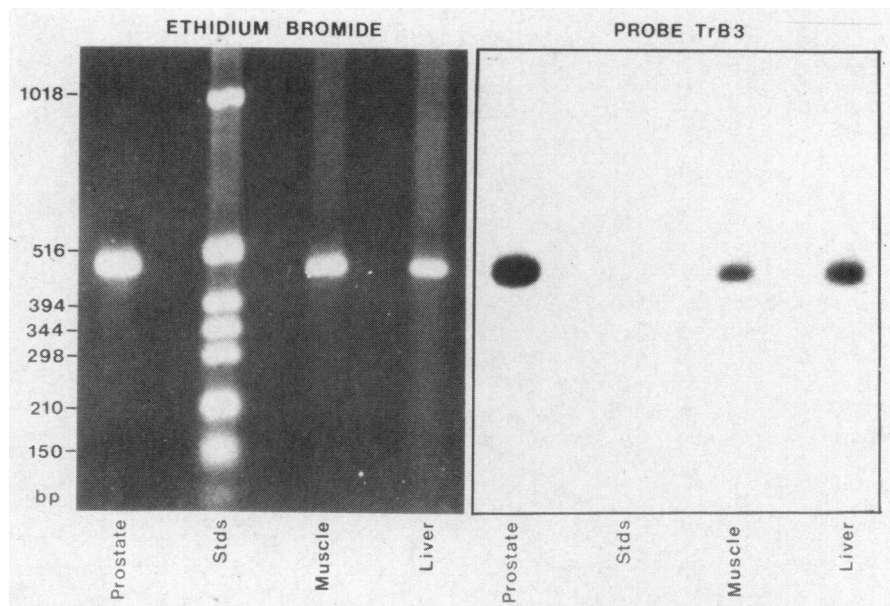


FIG. 7. Detection of arginine esterase transcripts in prostate, gracilis thigh muscle, and liver of a male dog after enzymatic amplification of cDNAs (see Materials and Methods and the legend of Fig. 5). The cDNA preparations were done with 2.5 ng of prostatic poly(A)⁺RNA and 2.5 μ g of muscle or liver poly(A)⁺RNA. The PCR products were visualized by ethidium bromide staining and hybridization with oligonucleotide TrB3 after transfer on Hybond-N membrane. Oligonucleotide TrB3 codes for sequences located between the two primers. The corresponding sequences in various kallikrein genes show large variations even within the same species.

	-225		-200	
dAE	GGGCTATAGG	TCCTGTCCCA	TTTGGCCTCA	GACCTTGAAA
hPSA	tGtCccTAGA	TgaAGTctCc	aTgaGCTaCA	.Agggcctgg
hGK-1	GatCcatAGG	cCTGcCCaA	TCTGaCCTCA	cACCGTgGAA
hRPK	aGagccacaG	cCTGcCCCA	ccTGGCtTCA	acCCcAGgAA
	-175		-150	
dAE	ATGATCAAGG	ACCT..AGGA	CAACAGGTGT	TTGCTTTTTC
hPSA	gTGATctAGt	AatTgcAGaA	CAGCAAGTGC	TAAGTcTccC
hGK-1	ctGATctAGt	AtgTgtgGaA	CAGCAAGTGC	TgGCTcTccC
hRPK	gTGATcAGG	gCCT...GcA	gAACAGGTGc	TgGtTcTccC
	-125		-100	
dAE	TAGCCTGGG	GGT.....	GGGGG	CTGTCCA..C
hPSA	cAGCtCTGGG	tGT.....	gggaGGGGG	tGTGTCCAG.C
hGK-1	cAGCtCTGGG	tGT.....	gggaGGGGG	tGTGTCCAG.C
hRPK	cAGCtCTGGG	GaTcggaggc	ggggggGGcaa	tGTGTCCAGc
			-75	-50
dAE	A..GGGCCAG	CCTTGGCCAG	CTTCTGGGT.	GGCAGCAGAG
hPSA	AtggGGgagg	CCTTGGtCAG	CcTCTGGGTc	GcCAGCAGgG
hGK-1	AtggGGgagg	CCTTGGtCAG	CaTCTAGGT.	GcCAaCAGgG
hRPK	AggGgaagg	CCTTGGCCA	CaTCTGGGTg	GcCAGCAGgG
			-25	-1
dAE	GCCCTGGGG	TGTATGTAGG	CTTTTAAAGG	.CTCCCCAGG
hPSA	GtCCTGGG..	.GaATGaAGG	.TTTTAtAGG	gCTCctggGg
hGK-1	GtCCTGGa..	.GaATGaAGG	C.TTTAtAGG	gCTCctCAGG
hRPK	GCTCTGaGGG	.G.ATAaggG	CTTTTAAAGG	cCTCCCCaGg

FIG. 8. Comparison of early promoter regions of canine arginine esterase (dAE) and three human prostatic genes, members of the kallikrein family: prostate-specific antigen (hPSA) (Lundwall, 1989), human glandular kallikrein-1 (hGK-1) (Schedlich *et al.*, 1987), and human pancreatic renal kallikrein (hRPK) (Evans *et al.*, 1988). The sequence flanking the 5' end of the arginine esterase gene was aligned with corresponding parts of the human genes. Identities with the arginine esterase gene are indicated by capital letters while lowercase letters indicate nonidentity. A minimal number of gaps (represented by dots) were introduced into the sequences. The numbering of the nucleotide sequence is relative to the major transcription initiation site of the canine arginine esterase gene.

pancreatic kallikrein gene contains an additional G-rich stretch of 8–12 nucleotides by comparison with the human and canine prostatic genes. Sequences further upstream (up to about 200 nucleotides from the cap site) also show highly significant similarity in all four kallikrein genes. Thus, it appears that, except for the G-rich insertion in the renal/pancreatic gene, early promoter sequences in kallikrein genes are neither tissue- nor species-specific. Another point of resemblance with kallikrein genes is the variant TATA box (TTTAAA), which is found in the rat renal kallikrein gene RSKG-7 (Chen *et al.*, 1988), the mouse glandular kallikreins mGK-1 (Mason *et al.*, 1983), and mGK-5 (Drinkwater and Richards, 1987), the γ -subunit of mouse nerve growth factor (Evans and Richards, 1985), and rat tonin (Shai *et al.*, 1989). For all these reasons and despite the fact that arginine esterase has no apparent kininogenase activity (Frenette *et al.*, 1985) like true glandular kallikreins according to classical definition, the canine prostate enzyme is by all evidence a member of this family.

We wished to determine the number of kallikrein genes in the dog genome. In the mouse and rat, the number of such related genes can be as high as 20–30 (Mason *et al.*, 1983; Gerald *et al.*, 1986), while this number may be restricted to three to four genes in man (Schedlich *et al.*, 1987) and in the hamster (Howles *et al.*, 1984). In the dog, evidence points to a limited number (two to three) or homologous kallikrein genes (Fig. 5). However, these are only minimal estimates because it could be argued that a number of additional less homologous family members

could be present. The genes for two additional arginine esterases isolated from dog urine by Murthy and Scicli (1988) may be part of this family. It also remains to be determined whether all these genes are clustered on the same chromosome as in mouse (Mason *et al.*, 1983), rat (Wines *et al.*, 1989), and man (Riegman *et al.*, 1989).

The canine arginine esterase is expressed in tissues other than the prostate. Previous experiments from our laboratory (Frenette *et al.*, 1987) had suggested that the prostate was the only tissue that contained detectable amounts of arginine esterase by radioimmunoassay. However, the present study shows that at least small amounts of arginine esterase mRNA are also present in various tissues including skeletal muscles, liver, kidney, and pancreas (Fig. 6). The significance of this finding remains unexplained and can be placed in parallel with other studies showing that transcripts previously thought to be expressed in a tissue-specific manner could be detected in several additional, if not all, tissues (Chelly *et al.*, 1988, 1989). In many instances, genes that are expressed in several tissues are differentially regulated. In the case of nonprostatic arginine esterase, any regulation by testicular steroids appears doubtful since in one normal uncastrated adult dog no arginine esterase mRNA could be detected in gracilis muscle despite normal arginine esterase mRNA levels in the prostate (results not shown). The factors involved in this variability are unknown. However, it is clear that prostatic arginine esterase mRNA levels are at least a thousandfold higher than in any other tissues examined. Therefore, we must conclude that some tissue-specific factors control mRNA levels and that the presence of androgens and their receptors is not sufficient to account for the observed variability.

Despite increasing knowledge on the mechanisms of transcription activation by glucocorticoids, progesterone, and estradiol (for reviews, see Schütz, 1988; Beato, 1989), the picture for androgen-regulated genes is less clear. One study (Parker *et al.*, 1988) concluded that upstream sequences of the C3 subunit of the prostatic steroid binding protein gene do not function as androgen-response elements in prostate cell lines, although they selectively bind androgen receptors in cell-free systems. By contrast, promoter sequences of mouse mammary tumor virus can mediate induction not only by glucocorticoids and progesterone but also by androgens (Darbre *et al.*, 1986). The regulatory elements in these sequences are four repeats of the TGTYCT motif (Cato *et al.*, 1987). Such elements are also present in upstream sequences and/or in introns of the various subunits of prostatic steroid binding protein (Claessens *et al.*, 1989). The arginine esterase gene contains four TGTCCT motifs, two of which are located in the 5' upstream sequences and the two others in intron 2. Further experiments will be necessary to determine whether these or other sequences can bind androgen receptor complexes and function as hormone-responsive elements.

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