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A secretory protease inhibitor requires androgens for its expression in male sex accessory tissues but is expressed constitutively in pancreas

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A full length cDNA clone encoding a mouse prostatic secretory glycoprotein (p12) whose synthesis is dependent upon testicular androgens has been cloned and characterized. The predicted amino acid sequence of p12 shares extensive homology with several members of the Kazal family of secretory protease inhibitors, in particular the pancreatic secretory trypsin inhibitors. In agreement with sequence data, prostatic secretory p12, purified from mouse ventral prostate secretion, exhibits anti-trypsin activity. Steady-state levels of protease inhibitor mRNA in ventral prostate are reduced from ~0.06% in normal mice to undetectable after androgen withdrawal but are inducible within 4 h by re-administration of testosterone. Androgen-dependent expression of the secretory protease inhibitor mRNA was also observed in coagulating gland and seminal vesicle. In seminal vesicle, a tissue of different embryonic origin to the prostate, the kinetics of secretory protease inhibitor mRNA loss after castration are not as rapid as in the ventral prostate and coagulating gland. Low-level androgen independent expression was also observed in the pancreas. There appears to be a single gene for this secretory protease inhibitor and yet expression is markedly stimulated by testosterone in the sex accessory tissues and unaffected by this hormone in the pancreas.

Key words: testosterone/prostate/protease inhibitor/seminal vesicle/pancreas

Introduction

Steroid hormones control many aspects of cellular development and differentiated function, primarily by regulating the rates of transcription of specific genes. It has been proposed (Jensen *et al.*, 1968; Yamamoto, 1985; Parker, 1987) that such steroid hormone mediated regulation is achieved by the binding of hormone receptor complexes to specific DNA sequences, termed hormone response elements, which upon activation can function as transcriptional enhancers. One such glucocorticoid response element in the long terminal repeat (LTR) promoter of mouse mammary tumour virus (MMTV) has been extemely well characterized by *in vitro* binding studies with purified glucocorticoid-receptor complexes (Payver *et al.*, 1983; Scheidereit and Beato, 1984) and by gene transfer studies *in vivo* (Buetti and Diggleman, 1983; Chandler *et al.*, 1983; Hynes *et al.*, 1983; Ponta *et al.*, 1985). In addition to glucocorticoids, this hormone response element has been found to mediate the effects of progestins (Cato *et al.*, 1986) and androgens (Darbre *et al.*, 1986; Cato *et al.*, 1987; Parker *et al.*, 1987) resulting in the stimulation of MMTV promoter activity.

We have been analysing the effects of androgens on the expression of the C1, C2 and C3 rat prostate genes which encode prostatic steroid binding protein (PBP) (Parker *et al.*, 1978; Hurst and Parker, 1983; Parker *et al.*, 1983). When the binding of purified androgen receptors to the C3(1) gene was examined, two regions were shown to selectively bind receptor, one upstream of the promoter and one within the first intron (Perry *et al.*, 1985; Rushmere *et al.*, 1987). The upstream region, however, failed to function as an androgen response element in transfection experiments with rat prostate cell lines, whereas the MMTV LTR in similar experiments retained its androgen responsiveness (Parker *et al.*, 1987).

In view of the contrast between the effects of androgens on the promoters of MMTV and for the rat C3(1) gene, we initiated a study to investigate genes in the mouse ventral prostate whose expression was stimulated by testosterone but which were not related to the PBP genes. Clearly, the mouse ventral prostate is a target tissue for androgens since its growth, morphogenesis and cytodifferentiation depends on circulating androgen (reviewed in Cunha *et al.*, 1983). To date, we have described two proteins whose synthesis is dependent upon testicular androgens (Mills *et al.*, 1987a) and these have now been identified by molecular cloning as a secretory protease inhibitor, which is the subject of this paper, and a spermine binding protein (Mills *et al.*, 1987b). We have found that the secretory protease inhibitor is expressed in the seminal vesicle, coagulating gland and the pancreas as well

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                                                                    54
GCA CCC TGT ATA GTT CTT CTG GCT TTT GCA CCC AGA TCT TCG ACA ATG AAG GTG
                                          BglII      MET Lys Val
                                                      1
                                                                   108
GCT GTC ATC TTT CTT CTC AGT GCT TTG GCC CTG CTG AGT TTA GCA GGT AAC ACT
Ala Val Ile Phe Leu Leu Ser Ala Leu Ala Leu Leu Ser Leu Ala Gly Asn Thr
                        10                                      20
                                                                   162
TTT TCA GCT AAG GTG ACT GGA AAA GAG GCT AGT TGC CAT GAT GCA GTG GCG GGA
Phe Ser Ala Lys Val Thr Gly Lys Glu Ala Ser Cys His Asp Ala Val Ala Gly
                              30
                                                                   216
TGT CCC AGA ATT TAT GAT CCT GTG TGT GGG ACT GAC GGA ATT ACT TAT GCC AAT
Cys Pro Arg Ile Tyr Asp Pro Val Cys Gly Thr Asp Gly Ile Thr Tyr Ala Asn
40                                    50
                                                                   270
GAA TGT GTT CTG TGC TTT GAA AAC AGG AAA CGC ATA GAG CCT GTC CTC ATT CGA
Glu Cys Val Leu Cys Phe Glu Asn Arg Lys Arg Met Glu Pro Val Leu Ile Arg
        60                                          70
                                                                   324
AAA GGT GGG CCT TGC TGA AAG TCA AGA TTT GAA CTC TGT TAT GGC TAC CGT AAT
Lys Gly Gly Pro Cys
               80
                                                                369
GAA AGG CAA CGT GGG TTC GTT GAA TAA ATC GCT TCA TGA ACA CTT...poly(A)
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Fig. 1. Nucleotide sequence and predicted amino acid sequence of MP12. The numbers on the right-hand side refer to the nucleotide sequence. Numbering of amino acid residues is given below the sequence starting from number 1 at the first ATG. A potential signal peptide at the amino terminus (amino acid residues 1–23) and a polyadenylation signal (AATAAA) at nucleotides 349–354 are underlined. Pertinent restriction emzyme sites are indicated.

Fig. 2. Alignment of homologous secretory protease inhibitors. Comparison of the amino acid sequence of MP12 from mouse ventral prostate (**top line**) with the amino acid sequence of boar seminal plasma acrosin inhibitor, the human, porcine, bovine and ovine PSTIs and with the three domains of the Japanese quail ovomucoid secretory protease inhibitor. The amino acid sequences are taken from Tschesche *et al.* (1976) and references therein. Gaps (indicated by dashes) have been included to maximize the alignment. The cysteine residues conserved between the nine sequences are framed in heavy lines. Other amino acids which exhibit the greatest degree of conservation between the different proteins are boxed.

as in the ventral prostate. Interestingly, expression of this gene is unaffected by testosterone in the pancreas, but is absolutely dependent upon this hormone for expression in the sex accessory glands.

Results

Characterization of cDNAs derived from androgen-dependent mRNA

Poly(A)$^+$ mRNA from normal mouse ventral prostate was used to generate a cDNA library (Materials and methods). Duplicate filters of approximately 7000 colonies were hybridized with single-strand ^{32}P-labelled cDNA probes derived from ventral prostate RNA of normal and 3-day castrate mice. After re-screening, 24 differentially hybridizing clones were identified which could be divided into three distinct groups on the basis of cross-hybridization analyses by Southern blotting (data not shown).

This paper deals with one group of four overlapping clones whose corresponding mRNA represents ~0.06% of ventral prostate poly(A)$^+$ mRNA. The nucleotide sequence of one cDNA clone (MP12) which is full length (see below) is shown in Figure 1. The sequence encodes an open reading frame of 80 amino acids where the ATG at nucleotides 46–48 represents the first methionine residue. MP12 has 5′ and 3′ untranslated regions of 45 and 84 nucleotides respectively. A consensus polyadenylation signal (AATAAA) (Proudfoot and Brownlee, 1976) is located 17 nucleotides upstream of the poly(A) tail (nucleotides 349–354). The amino terminal portion of the MP12 open reading frame contains many hydrophobic residues, a characteristic feature of signal peptides found at the N-terminus of most secretory proteins (Blobel and Dobberstein, 1975). Although the amino-terminal sequence of the mature protein encoded by MP12 has not been directly determined, the signal peptide cleavage site can be tentatively assigned. On the basis of (i) comparisons with secretory proteins closely related to that encoded by MP12 (see below) and (ii) predictions based on the (−3, −1) rule and weight matrix analysis (von Heijne, 1986), the cleavage site is likely to be between amino acid residues 23 (Ser) and 24 (Ala). No consensus sequences corresponding to potential N-glycosylation sites (Asn-X-Ser or Thr) are evident within the MP12 open reading frame.

Sequence homologies between MP12 and secretory protease inhibitors

Nucleotide and protein databases were searched for homologies to MP12. At the amino acid level, highly significant homologies (>50%) were found with several proteins of the secretory protease inhibitor (Kazal) family (reviewed in Laskowski and Kato, 1980). The degree of homology between MP12 and eight such secretory protease inhibitors is shown in Figure 2. These secretory protease inhibitors, which include the human, bovine and porcine pancreatic secretory trypsin inhibitors (PSTI), the boar seminal acrosin inhibitor and the Japanese quail ovomucoid inhibitor are members of a single family of genetically related proteins of which the bovine PSTI was the first representative to be identified. The greatest degree of overall homology (67% at this amino acid level) is shared between MP12 and the ovine PSTI and this homology is 87% in the region between the 2nd and 5th cysteine residues of these proteins. Furthermore, the spatial position of five out of the six cysteine residues is precisely conserved between MP12 and the eight secretory protease inhibitors in Figure 2. Nucleotide sequence data is only available for the human PSTI (Yamamoto *et al.*, 1985) and this enzyme shares 67% nucleotide sequence homology and 62% amino acid homology with MP12.

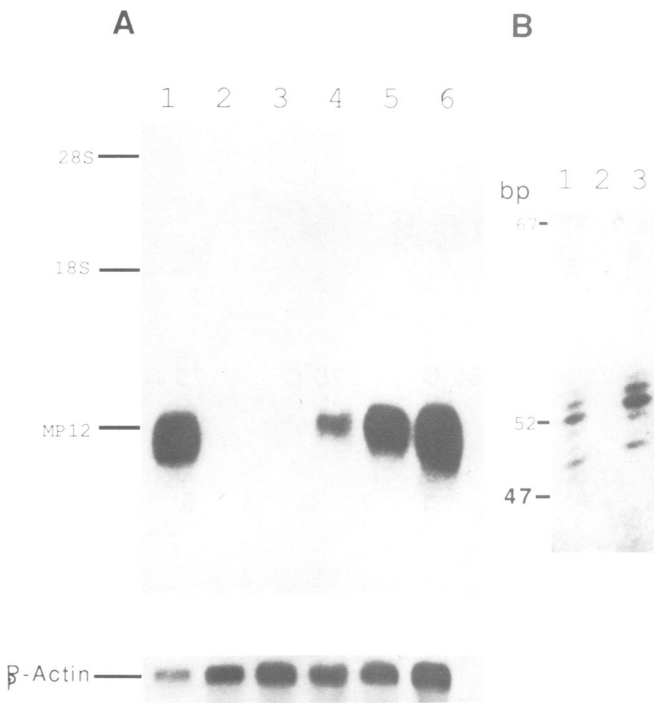

Fig. 3. Androgen dependence of MP12 mRNA in ventral prostate. **Panel A.** Northern blot hybridization analysis. Total ventral prostate RNA (10 μg) was fractionated by electrophoresis on a 1.5% gel under denaturing conditions and transferred to nitrocellulose paper. RNA was isolated from mice subsequent to (**Lane 1**) no treatment, (**Lane 2**) 3 days castration, (**Lane 3**) 3 days castration and 4 h testosterone treatment (**Lane 4**) 3 days castration and 16 h testosterone treatment, (**Lane 5**) 3 days castration and 48 h testosterone treatment and (**Lane 6**) 3 days castration and 96 h testosterone treatment. The nictrocellulose filter was hybridized with an antisense RNA probe corresponding to MP12 (**upper panel**) and subsequently with an antisense probe for mouse β-actin (**lower panel**). Filters were exposed to X-ray film for 4 h without intensifying screens. The migration of 28S and 18S rRNAs are indicated on the left. **Panel B.** Primer extension analysis. Total ventral prostate RNA (10 μg) from (**Lane 1**) normal mice, (**Lane 2**) 3-day castrates and (**Lane 3**) 3-day castrates treated with testosterone for 48 h at daily intervals, was annealed with a 20 bp oligonucleotide primer (Materials and methods) and extended with reverse transcriptase. The major extended product of 52 bp and the position of DNA mol. wt markers are indicated.

Androgen induction of MP12 mRNA in ventral prostate

We next studied the effects of androgens on the steady-state level of MP12 mRNA in mouse prostate. Total cellular RNA was isolated from the ventral prostates of mice of defined androgen status, that is, normals, 3-day castrates and mice subjected to androgen therapy subsequent to 3 days castration. Results of a Northern analysis of these mRNAs using a ^{32}P-labelled MP12 antisense RNA probe are presented in Figure 3A. Messenger RNA of 400–500 bp is observed in prostate RNA from normal mice, but is not apparent in prostate RNA from 3-day castrates. Re-administration of testosterone to 3-day castrates results in the reappearance of MP12 mRNA within 4 h. Normal levels of MP12 mRNA are attained after 48 h of daily testosterone injections. A control experiment shows that the concentration of β-actin mRNA in the castrate ventral prostate is unaffected by testosterone (Figure 3A, lower panel) but the amount of RNA from normal mice appears to have been under-loaded.

The 5′ end of MP12 mRNA was analysed by primer extension (Figure 3B). Primer extension products were detected in RNA from normal and testosterone-treated mice, but not in castrates, confirming the androgenic regulation of this mRNA.

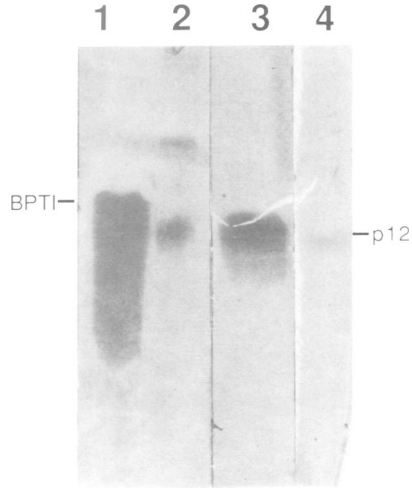

Fig. 4. Inhibition of protease activity by prostatic secretory p12. Protein samples (1 μg) were electrophoresed at 20 mA on a 12% SDS–polyacrylamide gel co-polymerized with 0.1% gelatin. Samples were treated with β-mercaptoethanol or heated prior to loading, unless stated otherwise. The gel was washed and incubated with 64 000 BAEE units of trypsin, re-washed and stained as described in Materials and methods. The stained bands represent regions of the gel where the co-polymerized gelatin has not been solubilized by trypsin-medicated proteolysis. The samples are: (**Lane 1**) bovine pancreatic trypsin inhibitor (0.04 BAEE units), (**Lane 2**) mouse ventral prostate secretion, (**Lane 3**) purified prostatic p12, (**Lane 4**) purified prostate p12 heated for 5 min at 100°C prior to electrophoresis.

The most prominent product is 52 bp, equal to the number of base pairs from the 5′ end of the oligonucletide primer to the 5′ end of MP12, thus confirming that the MP12 cDNA represents the entire length of its corresponding cellular mRNA at least with respect to its 5′ end. The presence of a number of other extension products, both shorter and longer, may result from genuine microheterogeneity in the start site for RNA polymerase (Ziff and Evans, 1978) or be experimental artefacts arising from premature termination of the reverse transcriptase due to mRNA secondary structure (Williams and Mason, 1985).

Identity of MP12 and p12: demonstration of anti-proteolytic activity

We have previously identified and characterized two mouse ventral prostate secretory proteins of mol. wt 12 kd (p12) and 25 kd (p25) whose synthesis is dependent upon testicular androgens (Mills *et al.*, 1987a). Three lines of evidence support the assumption that the MP12 cDNA corresponds to the previously identified p12 secretory protein. First, the expression of p12 and mRNA corresponding to MP12 are both dependent upon testicular androgens. Second, p12 is a secretory protein, likewise the predicted protein sequence of MP12 has a potential signal peptide at its amino-terminus, indicative of secretory function and, furthermore, MP12 is homologous to a known family of secretory proteins—the secretory (Kazal) protease inhibitors. Third, as expected from the sequence data analysis of MP12, the mouse prostate p12 secretory protein was found to exhibit an anti-protease activity (Figure 4). Proteins to be tested for protease inhibitor activity were electrophoresed on SDS–polyacrylmide gels co-polymerized with gelatin and then assayed for their ability to inhibit the proteolysis and subsequent solubilization of the gelatin by trypsin. With purified bovine pancreatic trypsin inhibitor, a distinct zone of inhibitory activity is observed (Figure 4, Lane 1). Two inhibitory bands of ~30 kd and 12 kd are also obtained when mouse ventral prostate secretion is assayed (Figure 4, Lane 2). To demonstrate that the inhibitory band with the lower

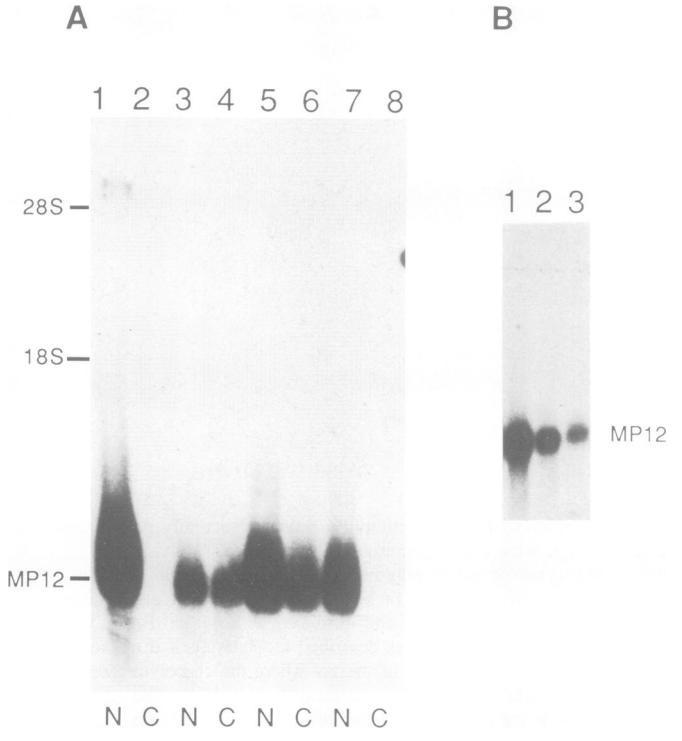

Fig. 5. Tissue distribution and androgen dependence of secretory protease inhibitor (MP12). Northern blot hybridization analysis. Nitrocellulose filters were probed with an antisense RNA probe corresponding to MP12 and exposed to X-ray film for 24 h without intensifying screens. **Panel A.** Total RNA (5 μg) from (**Lanes 1** and **2**) ventral prostate, (**Lanes 3** and **4**) pancreas, (**Lanes 5** and **6**) seminal vesicle, (**Lanes 7** and **8**) coagulating gland, from (**N**) normal mice and (**C**) 3-day castrates. **Panel B.** Total seminal vesicle RNA (5 μg) from (**Lane 1**) normals, (**Lane 2**) 3-day castrates and (**Lane 3**) 7-day castrates.

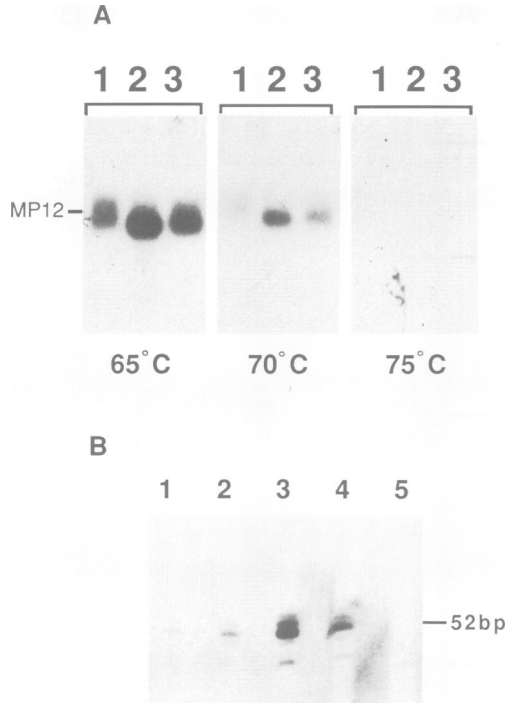

Fig. 6. Analysis of protease inhibitor expression. **Panel A.** Northern blot hybridization analysis. Total RNA samples from (**Lane 1**) ventral prostate (0.1 μg), (**Lane 2**) pancreas (5 μg), and (**Lane 3**) seminal vesicle (0.1 μg) were electrophoresed through a 1.5% formaldehyde gel and transferred to nitrocellulose. After hybridization with a ^{32}P-labelled antisense RNA probe corresponding to MP12, the filters were washed for 2 h (Materials and methods) at the temperature indicated. **Panel B.** Primer extension analysis. Total RNA from (**Lane 1**) pancreas (20 μg), (**Lane 2**) ventral prostate (10 μg), (**Lane 3**) seminal vesicle (20 μg), (**Lane 4**) coagulating gland (20 μg) and (**Lane 5**) liver (20 μg) was annealed with a 20 bp oligonucleotide primer and extended with reverse transcriptase (Materials and methods). The major extension product of 52 bp is indicated.

mol. wt in the prostatic secretion is due to p12 activity, a sample of purified p12 was assayed. Secretory p12 was purified from non-denatured ventral prostate secretion after electrophoresis on an SDS gel and electro-elution from the appropriate gel slice. Given the anomalous electrophoretic mobility of some proteins when electrophoresed on SDS–polyacrylamide gels without prior heat or β-mercaptoethanol treatment of the samples (Heussen and Dowdle, 1980), an aliquot of the purified protein was denatured and assayed on a standard protein gel to confirm that the appropriate protein had been isolated (data not shown). In the protease inhibitor assay, the purified p12 gives rise to a distinct inhibitory band (Figure 4, Lane 3). Furthermore, when p12 is heated to 100°C for 5 min prior to electrophoresis, the protein retains some ability to inhibit the proteolytic activity of trypsin (Figure 4, Lane 4) demonstrating the heat-stable nature of the protein—a feature shared by all low mol. wt secretory protease inhibitors of the Kazal family (reviewed by Laskowski and Sealock, 1971).

Tissue distribution of secretory protease inhibitor

In view of the homology between the prostatic secretory protease inhibitor and that of certain pancreatic enzymes (Figure 2), we investigated the tissue distribution of MP12 mRNA in the mouse. Northern blots indicate that the protease inhibitor is expressed in the seminal vesicle and coagulating gland (anterior prostate) as well as in the pancreas (Figure 5A). The approximate concentration of protease inhibitor mRNA in these tissues relative to those in the ventral prostate are: seminal vesicle 20%, coagulating gland 20% and pancreas 2%. Expression in the seminal vesicle and coagulating gland is testosterone dependent (Figure 5A), although the kinetics of mRNA loss after castration is more rapid in the ventral prostate and coagulating gland than in the seminal vesicles (Figure 5B). In contrast, expression of the protease inhibitor in the pancreas is androgen independent (Figure 5A, Lanes 3 and 4). No hybridization was detected between the protease inhibitor and RNA from mouse liver, kidney, spleen and salivary gland or with RNA from rat ventral prostate and human benign prostatic hypertrophy tissue (data not shown).

We further investigated whether the protease inhibitor is expressed from a single gene or from different genes expressed in a tissue-specific manner. Firstly, we examined the effect of stringency on the hybridization of MP12 with RNA from ventral prostate, pancreas and seminal vesicle (Figure 6A). As the Northern hybridization washing temperature was increased from 65°C to 75°C, equivalent loss of hybridization signal was observed for all three RNA species, suggesting that they are highly homologous. Secondly, primer extension analysis indicates that the 5′ termini of the protease inhibitor mRNAs from the ventral prostate, pancreas, seminal vesicle and coagulating gland are identical (Figure 6B). Thirdly, on the basis of restriction enzyme mapping six independent protease inhibitor genomic clones, isolated from a non-amplified mouse DNA library, are identical (data not shown) and fourthly, the size and pattern of hybridizing bands obtained on Southern blots of genomic DNA are consistent with the restriction enzyme map of the protease inhibitor genomic clones (Figure 7) indicating that it is a single copy gene.

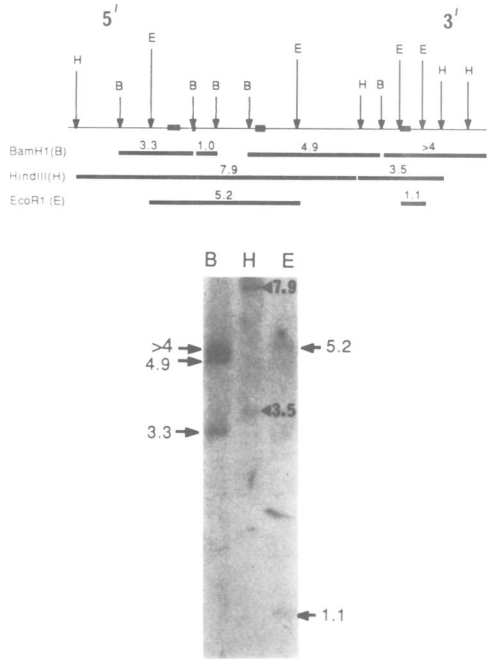

Fig. 7. Organization of the protease inhibitor gene. The restriction enzyme map of a protease inhibitor genomic clone (λMP12) shows the position of exons as blocks and the DNA fragment sizes in kb generated with *Bam*HI (**B**), *Hin*dIII (**H**) and *Eco*RI (**E**). The autoradiograph shows the sizes of hybridizing fragments generated in mouse DNA with these enzymes after Southern blotting using the MP12 cDNA as probe.

Discussion

We have isolated and sequenced a full length cDNA molecule (MP12) from the mouse ventral prostate which corresponds to an mRNA whose synthesis is dependent upon testicular androgens. Sequence analysis reveals that MP12 shares extensive homology with members of the Kazal family of secretory protease inhibitors (Figure 2). In agreement with this observation, we find that a previously identified prostatic secretory protein of mol. wt 12 kd (p12) whose synthesis is induced by androgens (Mills *et al.*, 1987a) corresponds to the MP12 cDNA and exhibits anti-trypsin activity.

Surprisingly, within the Kazal family, the prostatic protein secretory protease inhibitor exhibits greater homology at the amino acid level with the human, bovine, porcine and ovine PSTIs than with the secretory protease inhibitors found in the seminal fluid of these species. Comparison of the amino acid sequences of the porcine and bovine seminal fluid Kazal inhibitors with their corresponding PSTIs (Cechova and Meloun, 1979), for example, clearly demonstrates that the seminal and pancreatic proteins are members of the same family, but are not products of the same gene. These proteins arise from different genes which have evolved independently subsequent to the duplication of an ancestral gene. In contrast to this situation, we have several lines of evidence which suggest that the prostatic secretory protease inhibitor and the PSTI in mouse are products of the same gene. First the protease inhibitor mRNAs in these tissues hybridize the prostate cDNA to the same extent at different stringencies (Figure 6A). Secondly, the 5′ termini of the mRNAs in the different tissues are identical (Figure 6B). Thirdly, analysis of genomic DNA and genomic clones corresponding to MP12 indicate that there is a single gene. Finally, the mouse prostatic protease inhibitor exhibits a high degree of homology with the pancreatic trypsin inhibitor from a number of species, but shares less homology with the seminal fluid protease inhibitors which represent a more divergent family of proteins; a reflection of their apparent broader substrate specificity (Schiessler *et al.*, 1976).

The mouse prostate secretory protease inhibitor has a mol. wt of 12 kd, whereas the 80 amino acid open reading frame of MP12 should generate a primary translation product of 9 kd, which would decrease in size to approximately 7 kd after removal of the presumptive signal peptide. This has been confirmed by *in vitro* translation of RNA derived from the *in vitro* transcription of the cDNA (J.S.Mills, unpublished). This discrepancy presumably can be accounted for by secondary modification of the primary translation product, since the secretory protease inhibitors found in the seminal fluid of bulls, boar and humans (reviewed by Fritz *et al.*, 1975) are known to have apparent mol. wt of 11−13 kd on SDS−polyacrylamide gels and these represent the glycosylated form of a 7-kd core protein. The absence of Asn-X(Ser or Thr) sequences in the mouse prostatic protease inhibitor suggests that the carbohydrate residues are not N-linked, but they may well be O-linked to Ser and/or Thr as in the boar seminal secretory protease inhibitor (Tschesche *et al.*, 1976).

The identification of an androgen-dependent secretory protein in the sex accessory tissues which can function as a protease inhibitor raises questions as to its physiological role. With the exception of the rat seminal vesicle secretory proteins which are involved in the formation of the copulatory plug (Fawell *et al.*, 1986), the biological and physiological function of the major androgen-dependent secretory protein of the male sex accessory tissues is far from clear. The secretions of the mouse seminal vesicles, ventral and anterior prostate are known to contribute to the seminal fluid (Price and Williams-Ashman, 1961) and it is clear that the surgical removal of these mouse sex accessory tissues does result in lower fertility and fecundity (Pang *et al.*, 1979). The secretory protease inhibitor described in the mouse sex accessory tissues may have one of several potential physiological roles. The Kazal family of secretory protease inhibitors is typified by the pancreatic secretory trypsin inhibitors found in all vertebrates examined (reviewed in Laskowski and Kato, 1980), which inactivates trypsin derived from the premature activation of trypsinogen. In this way the premature trypsin-mediated activation of other pancreatic proteinase zymogens is prevented and the tissues of the organism are protected. One of the most potent proteases in the seminal fluid is acrosin which has trypsin-like specificity and is known to play an important role in the penetration of the zona pellucida of the female ovum by the spermatozoa. The secretory protease inhibitor identified in this paper may, therefore, be involved in the enzymatic regulation of the acrosome reaction at fertilization or in the neutralization of acrosin when the enzyme is prematurely activated; for example, before mating or before capacitation of the spermatozoa has occurred in the female genital tract. Alternatively, the secretory protease inhibitor may play a role in regulating the coagulation of the seminal fluid which is known to occur in many rodent and non-human primate species (Williams-Ashman, 1983).

Whilst secretory protease inhibitors have been identified in many species, the effect of hormones on their expression has received little attention. To our knowledge, the only other example of a hormone-regulated secretory protease inhibitor is α-antichymotrypsin, a member of the Kunitz family whose expression is induced by oestradiol in the human breast cancer cell lines MCF7 and T47D (Massot *et al.*, 1985).

Analysis of the tissue distribution and androgen dependence of expression of the secretory protease inhibitor demonstrated the differential promoter activity of this gene. Expression is clear-

ly androgen dependent in the sex accessory tissues (Figure 5A) such that MP12 mRNA is undetectable in the ventral prostate of 3-day castrates. In pancreas, however, expression is constitutive at a level of ~2% of that in ventral prostate. Such tissue-specific effects of steroids have also been observed with the chicken transferrin gene, which is strongly stimulated by oestradiol in the oviduct, but is expressed constitutively in liver (Lee *et al.*, 1978) and with the rabbit uteroglobin gene (reviewed by Beato *et al.*, 1983). One possibility to explain the apparent differential regulation of the secretory protease inhibitor gene is that the mRNA is initiated at two distinct tissue-specific promoters; however, the mRNA does appear to be produced from a single promoter in pancreas and the sex accessory tissues, since the 5′ terminus of the transcript in the different tissues is identical (Figure 6B).

Whilst the androgen-independent nature of expression in pancreas may be expected, given that this organ is not a target tissue for androgens, such a level of expression is not maintained in the prostate after androgen withdrawal, suggesting either that (i) there are positive regulatory transcription factors in pancreas cells that are absent in prostate and/or (ii) there are regulatory factors which repress transcription in the prostate in the absence of steroids. Alternatively, steroid-mediated changes in chromatin structure in prostate may exclude the necessary factors required for the maintenance of transcription after withdrawal of circulating testosterone.

The hormonal and tissue-specific regulation of secretory protease inhibitor gene expression should clearly be an attractive system for studying the mechanisms responsible for differential gene expression in differentiated tissues and we are currently investigating these mechanisms using the promoter of the cloned secretory protease inhibitor gene in gene transfer experiments.

Materials and methods

Animals

Ventral prostate tissue was isolated from young adult male mice (C57/BT, ICRF 'conventionalized'). Androgens were withdrawn by castration and, where indicated, testosterone was injected (Androject) s.c. in arachis oil in the flank region at a dose of 5 μg/g body weight.

Isolation and manipulation of nucleic acids

Total cellular RNA was isolated by the guanidium thiocyanate extraction procedure of Chirgwin *et al.* (1979) and enrichment for poly(A)$^+$ containing sequences by oligo-dT-cellulose chromatography was as described by Parker and Mainwaring (1977). Standard methods for the isolation and manipulation of plasmid DNAs were as described by Maniatis *et al.* (1982). The mouse β-actin cDNA was a gift from Dr K.Willison (Chester Beatty Research Institute, London).

Construction and screening of cDNA and genomic libraries

Poly(A)$^+$ RNA from mouse ventral prostate was used as template for the synthesis of double-stranded cDNAs by reverse transcriptase (Northumbria Biologicals Ltd) primed by oligo-dT following the method of Gubler and Hoffman (1983). Oligo-dC tails were added to mouse ventral prostate cDNA using terminal transferase (PL-Biochemicals) and annealed with oligo-dG-tailed pUCK8, a derivative of pUC8 (Vieira and Messing, 1982) in which the *Bam*HI site has been replaced with a *Kpn*I linker. The method of Hanahan (1983) was then used to transform *Escherichia coli* DH1 cells. Colony filter hybridizations were carried out following the methods of Maniatis (1982). A genomic library was constructed in λ EMBL 4 (Frischauf *et al.*, 1983) using mouse seminal vesicle DNA which had been partially cleaved with *Sau*3A and size-selected on sucrose gradients. Genomic clones were isolated using a full length cDNA clone (MP12) as probe.

DNA sequencing

Appropriate cDNA fragments were subcloned into M13 mp18 and mp19 to facilitate nucleotide sequencing analysis by the dideoxy chain termination method of Sanger *et al.* (1977) using [α-^{35}S]ATP (400 Ci/mmol, Amersham International plc). dNTPs were obtained from Sigma Chemical Company and the 'Klenow' enzyme was from BCL Ltd. Nucleotide and predicted amino acid sequence data was compared with the EMBL, Doolittle and Los Almos databases using the local optimal alignment procedure of Wilbur and Lipman (1983).

Northern hybridization analysis

RNA samples were analysed by electrophoresis through 1.5% agarose gels containing 2.2 M formaldehyde as described by Lehrach *et al.* (1977). The 28S and 18S RNAs were used as size markers and were visualized by ethidium bromide staining. The RNA was transferred to nitrocellulose paper (Thomas, 1980) and baked *in vacuo* for 2 h at 80°C. For the synthesis of RNA probes, appropriate cDNA fragments were subcloned into the SP6 vectors (Melton *et al.*, 1984). The recombinant pSP6 plasmids were linearized and incubated with 100 μCi [α-^{32}P]GTP (400 Ci/mmol, Amersham International plc) and SP6 RNA polymerase (Boehringer-Mannheim) according to the supplier's instructions, to produce RNA probes of ~6 × 10^8 d.p.m./μg. Filters were prehybridized for 4 h at 60°C in 50% formamide, 50 mM sodium phosphate buffer (pH 6.5), 0.8 M NaCl, 1 mM EDTA, 0.1% SDS 2.5 × Denhardt's, 250 μg/ml denatured salmon sperm DNA and 500 μg/ml yeast RNA and hybridized for 16 h at 60°C in the same buffer containing 1−5 × 10^6 d.p.m./ml of ^{32}P-labelled RNA probe. Filters were then washed at 65°C for four 30-min periods in 50 mM NaCl, 10 mM sodium phosphate buffer (pH 6.5), 1 mM EDTA 0.1% SDS and exposed for autoradiography to XAR-5 film (Kodak). Probes were subsequently removed by boiling filters for 20 min in 0.1% SDS, 1 mM EDTA.

RNA mapping by primer extension

The method used for primer extension was essentially that described by Green *et al.*, 1986. Briefly, a 20 bp oligonucleotide primer (5′ CTTCATTGTCGAAGATCTGG 3′) corresponding to nucleotides 32−52 (Figure 1) was end-labelled with [α-^{32}P]ATP (3000 Ci/mmol, Amersham International plc) using polynucleotide kinase (Pharmacia) and purified on a 20% acrylamide gel. Labelled primer (5 × 10^4 d.p.m. at 2 × 10^5 d.p.m./pmol) was annealed to 10 μg of total RNA in 40 mM Pipes pH 6.4, 40 mM NaCl and 10% formamide at 85°C for 2 min and 42°C overnight. After ethanol precipitation the annealed primer was extended using 10 units of reverse transcriptase (NBL Enzymes Ltd) in 50 mM Tris-Cl pH 8.3, 40 mM KCl, 8 mM $MgCl_2$, 0.5 mM dNTPs and 0.4 mM DTT at 42°C for 40 min. The RNA was then digested by treatment with RNase-A (Sigma) at 20 μg/ml and RNase-T1 (Cal-Biochem) at 1 μg/ml for 30 min at room temperature. The samples were then phenol:chloroform extracted prior to ethanol precipitation and electrophoresis on a standard 6% sequencing gel.

Protein analysis

To obtain secretory proteins, excised ventral prostate glands were minced finely in 10 vol of ice-cold 0.25 M sucrose. Insoluble proteins were removed by centrifugation. Proteins were analysed by SDS−PAGE (Laemmli, 1970) and visualized by Coomassie blue staining. The ventral prostate secretory protein (p12) was purified by SDS−PAGE and electroelution from the appropriate gel slice (Fawell *et al.*, 1986). Assays for protease inhibitor activity were carried out following the method of Hanspal *et al.* (1983). Briefly, SDS−polyacrylamide gels containing a polymerized substrate (0.1% gelatin) were prepared as described by Heussen and Dowdle (1980). Samples to be assayed for protease inhibitor activity were dissolved in an aqueous solution of 2.5% SDS, 10% glycerol and bromophenol blue (0.01%) and were applied to the gels without heating. Electrophoresis was carried out at room temperature at 20 mA constant current. When the tracking dye was 1 cm from the bottom of the gel they were washed with gentle rocking in 500 ml of 2.5% (w/v) Triton X-100 with three consecutive changes of 45 min each. After a 45 min wash in distilled water, the gels were incubated in 500 ml of 0.1 M glycine−NaOH buffer (pH 8.3) containing 64 000 BAEE units of trypsin (Sigma Chemical Co.) for 16 h at 37°C. Gels were subsequently washed as above and stained in a 0.1% solution of amido black in methanol:acetic acid:water (30:10:60) and destained in the same buffer without amido black. Bovine pancreatic trypsin inhibitor was obtained from Sigma Chemical Co.

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