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Molecular cloning and sequence analysis of cDNA encoding human prostatic acid phosphatase

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λgt11 clones encoding human prostatic acid phosphatase (PAP) (EC 3.1.3.2) were isolated from human prostatic cDNA libraries by immunoscreening with polyclonal antisera. Sequence data obtained from several overlapping clones indicated that the composite cDNAs contained the complete coding region for PAP, which encodes a 354-residue protein with a calculated molecular mass of 41 126 Da. In the 5'-end, the cDNA codes for a signal peptide of 32 amino acids. Direct protein sequencing of the amino-terminus of the mature protein and its proteolytic fragments confirmed the identity of the predicted protein sequence. PAP has no apparent sequence homology to other known proteins. However, both the cDNA clones coding for human placental alkaline phosphatase and PAP have an *alu*-type repetitive sequence about 900 nucleotides downstream from the coding region in the 3'-untranslated region. Two of our cDNA clones differed from others at the 5'-ends. RNA blot analysis indicated mRNA of 3.3 kb. We are continuing to study whether acid phosphatases form a gene family. as do alkaline phosphatases.

cDNA; Acid phosphatase; mRNA; Prostatic cancer; Sequence analysis; (Human prostate)

1. INTRODUCTION

Acid phosphatases (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) are a group of isoenzymes that hydrolyze phosphate esters under acidic conditions to yield P_i. Human prostatic acid phosphatase (PAP) was the first urological tumor marker, and its primary clinical importance still lies in the staging and monitoring of patients with prostatic adenocarcinoma [1]. The physiological function of PAP has not been clarified. The epithelial cells of the prostate synthesize PAP and secrete it into the seminal fluid (to approx. 1 mg/ml) [2]. This enzyme is under androgen regulation. PAP is a glycoprotein containing two subunits of identical size (48 kDa) [3,4]. The enzyme exhibits electrophoretic heterogeneity partly due to variable sialic acid substitution in the carbohydrate side chains [5]. This can also be ascribed to differences in amino acids in the polypeptide chains [6,7].

PAP has been suggested to possess antigenic properties which are present in the other acid phosphatases that originate from the pancreas [8], spleen [9], kidney [10], placenta 11, and neutrophil [12] and eosinophil granulocytes [13]. However, monoclonal antibodies to PAP have shown that it has an antigenically unique region, not present in acid phosphatases from other sources [14]. When polyclonal antisera to PAP were used, an immunoreactive acid phosphatase from normal embryonic lung cells (WI-38 cells) was detected as a high molecular mass precursor of 112 kDa [10]. In another study, such a precursor was not observed in an in vitro translation assay with WI-38 cells [15]. The authors concluded that the acid phosphatases from prostate and embryonic lung cells are immunologically related, but distinct proteins encoded by different genes.

Despite the fact that the prostatic acid phosphatase has now been used for 50 years as a marker

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of prostatic cancer, the physiological importance and structure of this protein remain largely unknown. For further understanding of the clinical significance of PAP and the contradictory results obtained for heterogeneity and immunoreactivity of acid phosphatases, the best approach is to resolve the structure of PAP and other acid phosphatase isoenzymes. Because PAP is an androgen-regulated protein of the prostate, its gene is suitable for further studies of androgen regulation. This could provide important information on the androgen dependency of prostatic cancer.

Here, we have cloned and sequenced the cDNA encoding human PAP, in order to study its biosynthesis and androgen regulation.

2. MATERIALS AND METHODS

2.1. *Peptide sequence analyses*

Purification of PAP was performed as in [16]. Tryptic peptides of PAP were purified by HPLC using a Vydac 218 TO 54 column (4.6 × 250 mm). Elution (1.0 ml/min) was achieved using a linear gradient of acetonitrile (0–60% in 30 min) in 0.1% trifluoroacetic acid, and tryptic peptides were detected at 218 nm.

For determination of the amino-terminal sequence of the HPLC-purified tryptic peptides of PAP (24 μg), the purified peptides and the protein were applied directly to a Polybrene/NaCl-treated glass fiber filter and degraded with a gas-phase sequencer (model 470A, Applied Biosystems) [17,18]; amino acid phenylthiohydantoin derivatives were analyzed by HPLC on a Spherisorb column (S5 ODS 2, 4.6 × 250 mm) using a gradient of acetonitrile in 30 mM sodium acetate, pH 4.8 [19].

2.2. *Construction and screening of cDNA libraries*

Total cellular RNAs were isolated from human normal prostatic tissue (cystoprostatectomy for bladder cancer), human benign prostatic hyperplasia tissue and human prostatic carcinoma tissue as described [20]. Poly(A)$^+$ RNAs were separated by chromatography on oligo(dT)-cellulose as described by Aviv and Leder [21]. Double-stranded cDNA was synthesized using a cDNA synthesis kit (Amersham) and cloned into a λgt11 expression vector. Two libraries were constructed, one from human prostatic carcinoma tissue (CA I), the other from human benign prostatic hyperplasia tissue (BPH). In addition, we have used a prostatic carcinoma tissue cDNA library (CA II) constructed from our mRNA by Clontech laboratories (Palo Alto).

The cDNA libraries were screened with polyclonal antibodies raised in rabbits against the pure PAP protein [22], using the Promega immunoscreening system. Putative positive clones were carried through two or three rounds of screening until all phages produced positive signals.

2.3. *Characterization of recombinant clones*

The subclonings were carried out using pBR322 (New England Biolabs) and pUC18 (Boehringer Mannheim) vectors. Plasmid DNAs were amplified in *E. coli* MC1061 and *E. coli* JM109 cells, respectively, and purified by a modified CsCl gradient centrifugation method [23]. Southern analysis was carried out according the standard procedures [23,24].

The *Eco*RI-digested inserts of recombinant λgt11 cDNA clones were subcloned into *Eco*RI-cut M13 mp18 vector [25]. DNA sequence determination was done by the dideoxy chain-termination method [26] with Sequenase^{TM} enzyme [27], 17-mer universal primer and specific synthetic oligonucleotides. The sequencing strategy is indicated in fig.1. All sequences were determined at least twice. Computer analyses of DNA sequences were performed with the Microgenie^{R}-sequence analysis program distributed by Beckman Instruments (Palo Alto).

2.4. *Hybridization of cDNA to RNA blots*

3 μg of each poly(A)$^+$ RNA was electrophoresed on 0.7–1.0% agarose gels containing formaldehyde (2.2 M), and transferred to a nylon membrane (Hybond, Amersham). Hybridizations and washes were performed under stringent conditions according to the manufacturer's recommendations. The double-stranded cDNA probe was labeled by nick-translation with [α-^{32}P]dCTP to a specific activity of 5×10^8 cpm/μg.

3. RESULTS

3.1. *Peptide sequences*

The amino-terminus of pure PAP is: Lys-Glu-Leu-Lys-Phe-Val-Thr-Leu-Val-Phe-Arg-His-Gly-(x)-Arg-Ser. The sequences of three tryptic peptides are: (I) Met-Leu-Pro-Gly-x-Ser-Pro-Ser-x-Pro, (II) Phe-Glu-Lys-Gly-Glu-Tyr-Phe-Val-Glu-Met-Tyr, (III) Phe-Ala-Glu-Leu-Val-Gly-Pro-Val-Ile-Pro-Gln-Asp.

3.2. *Screening of libraries and characterization of clones*

To clone the cDNA of PAP we screened three different prostatic cDNA libraries with polyclonal PAP-antiserum. Some of the positive clones were also confirmed as coding for PAP protein by immunoscreening with different PAP antisera. A total of $\approx 1.1 \times 10^6$ λgt11 recombinants were screened. From the CA I library we found several positive clones of sizes ≈250 bp. These short clones hybridized to each other and therefore we chose only our PAP-9 clone for further characterization. The sizes of several positive clones from the BPH library were determined, of which two, PAP-113 and PAP-123, both containing an insert of about 1.6 kb, were selected for further characterization. From the prostate CA II library, recombinant clones PAP-1004, PAP-1005

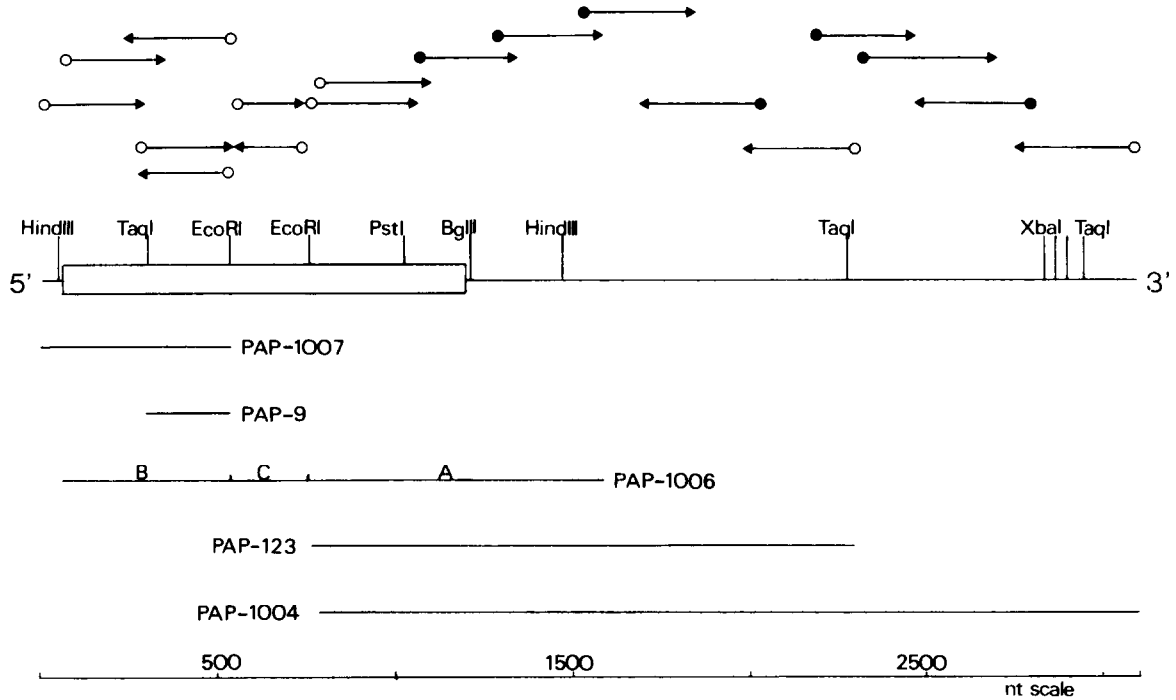


Fig.1. Partial restriction map and sequencing strategy of cDNAs encoding human PAP. Direction and extent of sequence determinations are indicated by arrows. The priming of sequence determinations is indicated by an open circle when the universal primer has been used, and synthetic oligonucleotide primed determinations are preceded by a solid circle. The protein coding region is shown by the open box.

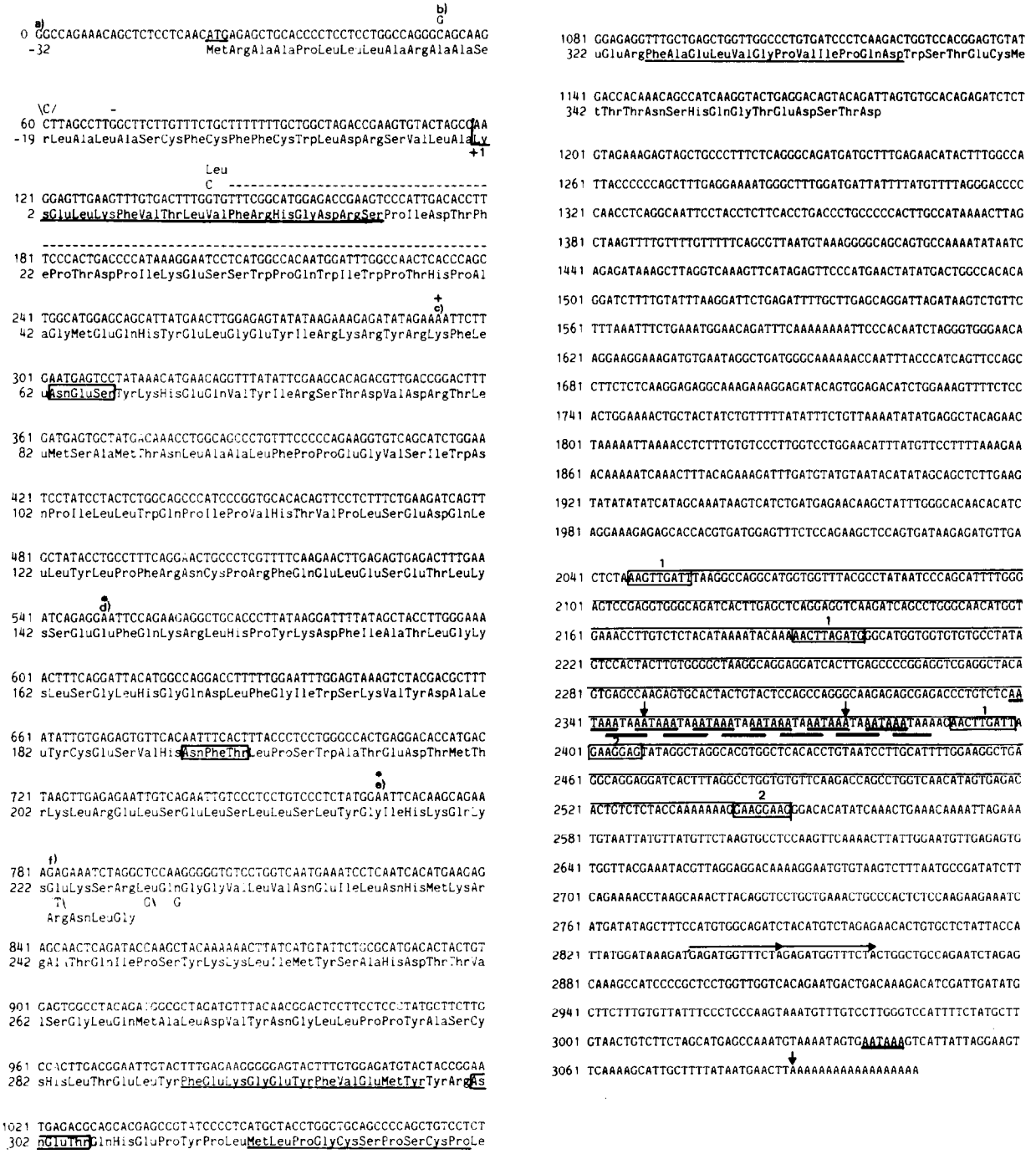
and PAP-1007, containing inserts of 2.3, 1.6 and 0.6 kb, respectively, were chosen for sequence analysis. An additional clone, PAP-1006, was characterized from this library. When digested with *Eco*RI, it released three fragments of 0.4 (PAP-1006B), 0.2 (PAP-1006C) and 0.8 kb (PAP-1006A).

To determine the respective orientation of our cDNA clones (fig.1), Southern analysis was used. A cDNA clone, PAP-9, was subcloned into pBR322 plasmid and used as a hybridization probe. It hybridized only with PAP-1006 and PAP-1007. On the other hand, a 290 bp *Eco*RI-*Pst*I fragment from PAP-1004 in pUC18 recognized PAP-123, PAP-1005 and PAP-1006. Because PAP-1006 hybridized with two independently obtained cDNA clones (PAP-9 and PAP-1004), it is evident that its *Eco*RI-digested multiple inserts were not a cloning artefact, but the result of internal *Eco*RI sites. This could also explain our difficulties in obtaining longer cDNA clones from the prostate library CA I, as apparently the methylation step used to protect internal *Eco*RI sites had been ineffective.

3.3. *Sequence analysis of cDNA clones*

Clones PAP-9, PAP-123, PAP-1004, PAP-1006 and PAP-1007 were sequenced entirely. Partial DNA sequences of PAP-113 and PAP-1005 revealed that they were identical to PAP-123. The combined nucleotide sequence of the overlapping cDNA clones was 3088 bp, containing 1905 bp of 3'-untranslated and 23 bp of 5'-untranslated sequences (fig.2).

All tryptic peptides and the amino-terminal peptide were identified in the protein sequence deduced from the cDNA. The amino-terminal peptide of 16 amino acids was coded by nucleotides 119–187. All the tryptic peptides resided in the carboxy-terminal part of PAP protein: nucleotides 980–1012 (amino acids 324–335); nucleotides 1049–1078 (amino acids 311–320) and nucleotides



1088–1123 (amino acids 288–298).

The combined cDNA clones encode a 354 amino acid polypeptide. A predominantly hydrophobic 32-residue sequence, presumably the signal peptide, beginning with Met, precedes the amino-terminal end of PAP obtained by protein sequencing.

The clone PAP-1004 was sequenced and found to be identical to PAP-123 except for an additional 839 nucleotides of 3′-non-coding sequences and some minor differences at the 5′-end.

The nucleotide sequence of clone PAP-1006B revealed a deletion of 96 bp, and some minor differences at the 5′-end compared with PAP-1007 as depicted in fig.2.

The stretch of 96 nucleotides missing in PAP-1006B, corresponding to 32 amino acid residues, could represent an alternatively spliced exon, although no consensus splicing sequence could be found bordering the deletion. On the other hand, the deletion, the discrepancies between PAP-1006B and PAP-1007, as much as those between the 5′-end of PAP-123 and PAP-1004, could be due to reverse transcriptase errors during cDNA synthesis. Finally, at this stage we cannot exclude possible genetic polymorphism of the PAP-gene locus as the cause of these dissimilarities.

The 3′-non-translated region of PAP-cDNA contains two human *alu*-type repetitive sequences [28,29]. The first repeat extends from nucleotide 2046 to 2398 and is composed of two *alu* monomers. It is flanked by a directly repeated sequence $AA^{G}/_{C}TTGATT$ at both ends. Immediately following the first *alu* repeat there is an *alu* monomer starting at nucleotide position 2401 and ending at nucleotide position 2548. The latter *alu* repeat is surrounded by the sequence GAAGGA(A)G at extreme ends. The *alu* repeats share 81 and 73% homologies with the *alu* consensus sequence of Schmid and Jelinek [28]. The A-rich 3′-flanking sequence of the first *alu* repetitive element is composed of 12 (TAAA) repeats constituting a series of overlapping polyadenylation signals (AATAAA). In clone PAP-123, the poly(A) tail begins after two (TAAA) repeats after nucleotide 2349, whereas in an otherwise identical clone, PAP-1005, the 3′-untranslated sequence extends to nucleotide 2377, thus containing 9 (TAAA) repeats (see fig.2). It is possible that the 3′-end of these cDNA clones may reflect heterogenity in PAP mRNAs caused by differen-

Table 1

Total amino acid composition of PAP

Amino acid	Residues per polypeptide deduced from cDNA	mol% predicted from cDNA	mol% [3]	mol% [4]
Alanine	12	3.4	3.5	3.5
Arginine	15	4.2	4.0	4.3
Asparagine and aspartate	25	7.0	7.0	8.6
Cysteine	6	1.7	2.0	2.0
Glutamate and glutamine	45	12.7	13.0	13.9
Glycine	17	4.8	5.0	6.7
Histidine	14	4.0	3.5	3.7
Isoleucine	15	4.2	3.5	3.7
Leucine	42	11.9	12.0	12.2
Lysine	19	5.4	5.0	5.5
Methionine	10	2.8	2.6	4.0
Phenylalanine	14	4.0	4.0	4.4
Proline	26	7.3	7.0	6.9
Serine	27	7.6	7.0	6.8
Threonine	24	6.8	7.6	6.8
Tryptophan	8	2.3	2.0	ND
Tyrosine	19	5.4	5.0	2.1
Valine	16	4.5	4.0	4.3
Total	354			

ND, not determined. The amino acid composition derived from cDNA is as depicted in fig.2

Fig.2. DNA sequence and deduced amino acid sequence of the PAP cDNA clones. Numbering of nucleotide positions and amino acid residues are given at the beginning of each line. A vertical line precedes amino acid + 1, the most amino-terminal residue of native PAP. The sequenced peptides are underlined with a thin solid line and potential glycosylation sites Ans-X-Thr/Ser are boxed. Two alu-type repeats in the 3′ untranslated region are indicated by a solid line above the sequence and the direct repeats bordering them are boxed and numbered. Polyadenylation sites AATAAA are underlined with a thick solid line. A 13 bp direct repeat is indicated by arrows. The 5′-ends of several clones are given: (a) PAP-1007, (b) PAP-1006B, (c) PAP-9, (d) PAP-1006C, (e) PAP-123, (f) PAP-1004. Vertical arrows mark the beginning of the poly(A) tract in various clones. Two internal *Eco*RI sites are indicated by * and a site closely resembling the *Eco*RI site by $^{+}$. Differences in sequence between PAP-1006B and PAP-1007 are shown above and those between PAP-123 and PAP-1004 beneath the assembled sequence. Deletions are marked with a broken line; V denotes insertions and substituted bases are written over the sequences.

tial use of poly(A) addition sites. Furthermore, at nucleotide position 3038–3044, 44 nucleotides upstream of the polyadenylation site of clone PAP-1004 there is a canonical polyadenylation signal AATAAA.

3.4. *Analysis of PAP protein*

Our cDNAs encode 354 amino acids, corresponding to a molecular mass of 41126 Da. In previous studies the molecular mass of PAP has been determined to range from 46 to 52 kDa [3,4,30]. In vitro translation assays of human prostatic poly(A)$^+$ RNA demonstrate that PAP is synthesized as a 43 kDa preprotein that is processed to a 41 kDa protein [15]. This is in agreement with our results. The difference of 5–11 kDa in molecular mass between the 41 kDa protein and native PAP is explained by glycosylation of PAP. NMR studies have revealed that three oligosaccharide side chains are linked to asparagine residues on PAP [5]. Three putative asparagine-linked glycosylation sites were found in our PAP sequence: Asn-Glu-Ser (residues 62–64), Asn-Phe-Thr (residues 188–190) and Asn-Glu-Thr (residues 301–303). The first and third sites are in hydrophilic regions whereas the second is located in slightly hydrophobic environment. The amino acid distribution as calculated from the deduced sequence is in agreement with published results [3,4] (table 1). Although over a third of PAP is composed of hydrophobic residues, no evident membrane-anchoring domain [31] can be found in the coding region.

3.5. *RNA blot analysis*

Poly(A)$^+$ RNA obtained from human BPH tissue, CA tissue and normal prostatic tissue (cystoprostatectomy for bladder cancer) was subjected to RNA blot analysis. As a control we used poly(A)$^+$ RNA from human placenta. The total RNA yields were about 200–500 μg RNA/g in prostatic tissues and about 1000 μg RNA per g in placenta. After two cycles of oligo(dT)-cellulose chromatography, poly(A)$^+$ RNA yields ranged from 1.5 to 5.0% of total RNA. The hybridization probe was an *Eco*RI-*Pst*I fragment of our PAP-1004 clone (fig.1). This probe recognized a discrete mRNA band of 3.3 kb in all prostatic poly(A)$^+$ RNAs. It did not hybridize to any placental mRNA (fig.3). The PAP-9 insert in pBR322 recognized an mRNA of the same size (not shown). In the BPH poly(A)$^+$ mRNA sample (fig.3, lane 3), a diffuse area of hybridization centered at 2.8 kb can be seen. This could correspond to some other species of PAP mRNA, or simply to its degradation products.

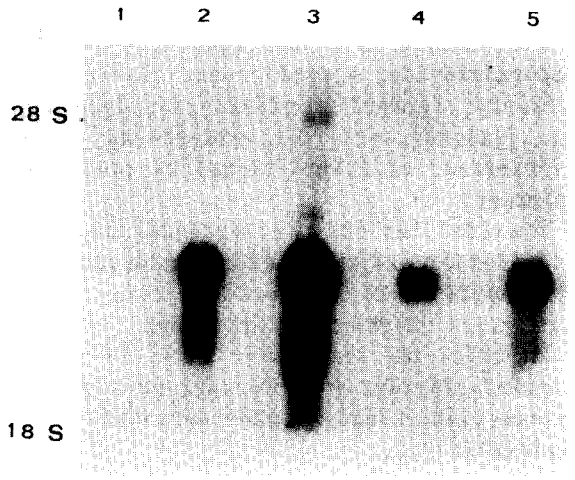


Fig.3. Autoradiogram of RNA blot analysis. Human poly(A)$^+$ RNA from prostatic tissues and placenta were electrophoresed in a 0.7% agarose gel containing 2.2 M formaldehyde, blotted onto nylon, and hybridized with ^{32}P-labeled PAP-1004 fragment. Lanes: 1, placenta (3 μg); 2, normal prostate (1 μg); 3, BPH (3 μg); 4, CA (3 μg); 5, normal prostate (3 μg). Positions of 28 S and 18 S rRNAs are indicated.

4. DISCUSSION

Several overlapping cDNA clones encoding human PAP were isolated from human prostatic cDNA libraries. The size of our PAP cDNA was 3088 nucleotides, covering the entire coding region of PAP mRNA plus 1905 nucleotides of 3′-untranslated regions. The 1065 nucleotides of the coding region give rise to a polypeptide of 354 amino acids. The molecular mass of this non-glycosylated protein is 41 kDa. An amino-terminal peptide of 16 residues and three tryptic peptides were found in the protein sequence.

Recently Yeh and co-workers [32] reported cDNA clones from a liver cDNA library which they assumed to code for prostatic acid phosphatase. However, the restriction map of their clone contains an *Xba*I site about 300 bp upstream

of a *Bgl*II site, while in our cDNA clones there are only three *Xba*I sites at the 3′-end. Furthermore, they reported additional *Bgl*II sites, where we found only one site for *Bgl*II (see fig.1). Although we cannot compare the sequences of their clones (no published data available) with our cDNA sequence, it seems that their clones correspond to some other mRNA species rather than the PAP mRNA of our clones.

A homology search of our cDNA and derived protein sequence was carried out against the Genetic Sequence Data Bank (Gen Bank, release no. 52 of 30.9.1987) and The National Biomedical Research Foundation (NBRF) Protein Data Bank (release no. 13 of 30.9.1987). No significant homologies were found. The overall structure of placental alkaline phosphatase cDNA [33] closely resembles that of PAP cDNA. Both contain an *alu*-type repetitive sequence in the 3′-untranslated region, about 900 nucleotides downstream from the coding region. However, when PAP cDNA was compared with alkaline phosphatase genes, it was interesting to note that no homology at the nucleotide level could be found, except for the *alu* repeats.

PAP protein is reported to consist of two subunits [3,4]. It is not known whether the subunits are identical [7,8]. Two of our cDNA clones, PAP-1006B and PAP-1004, isolated from the carcinoma cDNA library, differed from other cDNAs at their 5′-ends. One possible explanation for the differences is that they might code for slightly different subunits of PAP. This can be answered by further studies of the PAP gene. It will be interesting to clarify the biosynthesis of different PAP isoenzymes and discover whether acid phosphatases form a gene family, as do alkaline phosphatases [34].

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