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Biochemistry

Regulation of a bifunctional mRNA results in synthesis of secreted and nuclear probasin

(translational regulation/ligand carrier protein)

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ABSTRACT **Probasin, a rat prostatic protein, is statistically related to members of a protein family that includes serum, cellular, and nuclear proteins. *In vivo*, probasin appears both in the secretions and in the nucleus of prostatic epithelial cells. Using primer extension and S1 nuclease protection assays we detected only one probasin mRNA. Thus, the localization of this protein to two separate cellular regions must be encoded by this one mRNA. Furthermore, *in vitro* translation of synthetic probasin mRNA demonstrated that a protein containing a signal peptide and a protein lacking a signal peptide were synthesized by initiation at different AUG codons. These data are consistent with a mechanism of translational regulation of a eukaryotic bifunctional mRNA.**

Eukaryotic translational initiation usually occurs at the first in-frame AUG codon that is in the optimal context, CCACCAUGG (1). However, if the first AUG codon in-frame is not in an optimal context, dual initiation may occur at this site and another, more favorable site downstream. This can generate two proteins that differ only in the sequence between the two AUG start sites. The additional amino acids could be at the N terminus, a domain often associated with signals for intracellular distribution (2). Strubin *et al.* (3) reported that the Ia antigen-associated invariant polypeptides were synthesized by alternative initiation at two in-phase AUG codons. The relative amounts of the two proteins indicated that the second initiation site is preferred as predicted by the favorable context of this AUG. Since then, this mechanism of "leaky scanning" has been documented for a number of oncogenes, hormone receptors, and growth factors (2). Here, the mRNAs often have a termination codon after the first AUG, resulting in a separate short peptide from the 5′ mRNA sequence as well as a major protein resulting from initiation at the second AUG (2). Although the mechanisms regulating translational initiation are not fully understood, it is known that increasing secondary structure within the 5′ noncoding mRNA sequence impedes initiation and thus influences the efficiency of translation (4–6). Further, protein interactions may be involved in this secondary structure. For example, ferritin mRNA contains a stem–loop iron-responsive element in the 5′ untranslated region that binds a cytosolic repressor protein that is removed in the presence of iron (7).

Probasin (PB), isolated originally from dorsolateral prostate nuclei, is an androgen-regulated nuclear protein (8) that increases in concentration with zinc uptake (9). In this report, we show that the translation product corresponding to the cDNA clone pM-40 (10) is PB. PB mRNA expression is regulated by androgen (10) and zinc (11). Within 7 days after castration, the PB mRNA decreases as expected for an androgen-sensitive gene (10), but it will rebound to intact levels by 12 days after castration (12). Thus the gene progresses to a fully induced state in the absence of testicular androgens.

Previous work showed that the pM-40 clone would hybrid-arrest the translation of two proteins (10) and that one of these proteins contained a signal peptide for secretion (11). Now, we present the predicted amino acid sequence of PB,** which indicates that it is related to a gene family of ligand carrier proteins (13, 14). We demonstrate that PB is localized in both the nucleus of epithelial cells and in prostatic secretions. Further, we present evidence that the secreted as well as the nuclear protein can be translated from one bifunctional mRNA. These data are consistent with a mechanism of *in vivo* translational regulation from a bifunctional mRNA.

MATERIALS AND METHODS

Sequencing and Subcloning. The pM-40 cDNA (10) insert was subcloned into the *Pst* I site of the M13 and pUC119 vectors. Sequencing was performed by the method of Sanger *et al.* (15). The pM-40 cDNA was subcloned into the *Pst* I multicloning site of pT7/T3 18U/19U plasmid vector to produce synthetic PB mRNA for *in vitro* translation. Since the original pM-40 cDNA clone was produced by oligo(dG·dC)-tailing, the resulting synthetic mRNA started with 30 guanines at the 5′ end and the PB mRNA sequence began at −8 (Fig. 1). To check whether the oligo(G) tails from the homopolymer at the 5′ end were inhibitory, the *Pst* I–*Hin*fI restriction fragment of the pM-40 cDNA was removed, and the *Sac* I–*Hin*fI fragment from the first exon of the genomic clone was linked to the *Hin*fI site of the full-length cDNA clone. This construction started at the *Sac* I site of exon 1, which is nucleotide 12 of the 5′ untranslated region (Fig. 1), and included 3′-end adenines from the poly(A) tail plus cytosines of the cloning site. This construct was subcloned into the multicloning site (*Sac* I/*Pst* I) of pT7/T3 18U/19U vector. Deletion constructs were prepared by using exonuclease III (16) to remove 5′ untranslated sequences and N-terminal amino acid codons. All constructions were sequenced.

RNase Protection and Primer Extension Analysis. A *Hin*fI genomic clone fragment starting with 5′ flanking sequence and ending within exon 1 was 5′-end-labeled with [γ-^{32}P]ATP. This DNA was cut with *Dra* II to generate a labeled 380-base-pair (bp) flanking/exon 1 *Dra* II–*Hin*fI/fragment for S1 nuclease protection (17). Two transcription start sites 4 nucleotides apart were mapped, as well as a general hetero-

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Abbreviation: PB, probasin.

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**The sequence reported in this paper has been deposited in the GenBank data base (accession no. M27156).

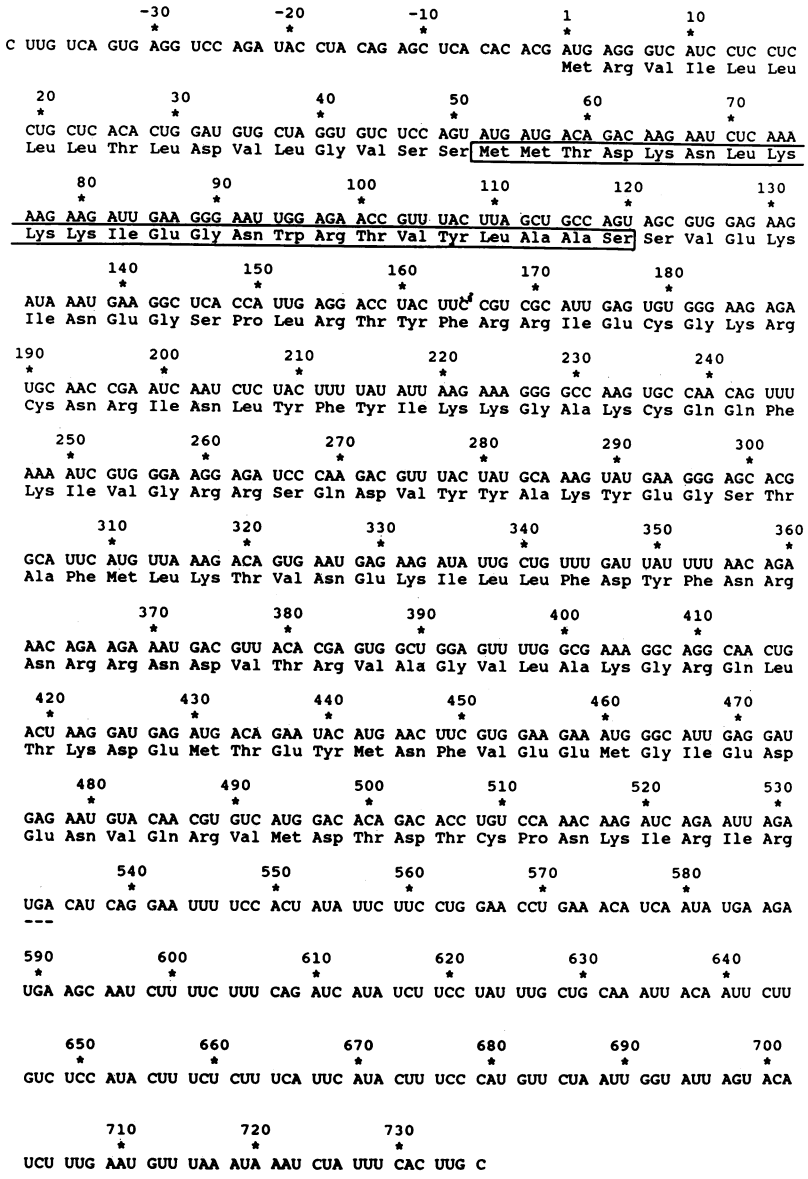

FIG. 1. Sequence of PB. The coding nucleotide sequence of PB mRNA was derived from the cDNA clone (10) that started at −8; the remaining 5′ untranslated sequence was obtained from the genomic clone. Initiation of translation at the first in-frame AUG codon (+1) starts a protein containing a hydrophobic sequence that would be absent if initiation occurred at the second in-frame AUG codon (+52). The remaining amino acid sequence and termination codon (UGA) are the same for both predicted proteins. Sequencing of the N-terminal region of purified PB demonstrated an amino acid sequence identical (boxed sequence) to the mRNA-predicted sequence starting with the second AUG codon.

geneity for the 5′ end of the PB mRNA. For primer extension (18), a 17-nucleotide oligomer ending at the *Hin*fI site of exon 1 was end-labeled. This mapped to the same two start sites of transcription.

Antibody Preparation. Nuclei were prepared from rat dorsolateral prostates (19). The nuclei were resuspended in 0.6 M NaCl/10 mM Tris·HCl, pH 7.5/0.7 mM EDTA/0.1 mM phenylmethanesulfonyl fluoride and centrifuged at 12,000 × *g* for 10 min. The supernatant was made 0.2 M in H_2SO_4, and the acid-insoluble material was removed by centrifugation. The acid extract was dialyzed overnight at 4°C against 0.1 M acetic acid, lyophilized, and redissolved in distilled water. Of the acid-soluble proteins, PB was the most abundant. The acid-soluble proteins were electrophoretically separated in a 5.4% acetic acid/6.7 M urea/0.375% (wt/vol) Triton X-100/15% polyacrylamide gel. The protein band was visualized by staining with 8-anilino-1-naphthalenesulfonic acid (19). The gel slice was neutralized in 0.15 M sodium phosphate (pH 7.4) and the mixture was emulsified by repeated passage through a syringe prior to injection into rabbits.

Cellular Fractionation. Rat ventral, dorsal, or lateral prostate was homogenized in 1 M hexylene glycol/10 mM Pipes, pH 7.0/2 mM $MgCl_2$/1% thiodiglycol/30 mM sodium butyrate/0.2% Nonidet P-40/0.1 mM phenymethanesulfonyl fluoride (19). A portion of the homogenate was centrifuged at 750 × *g* for 10 min. The pellet was resuspended in 50 mM Tris·HCl, pH 7.0/2 mM $MgCl_2$/25 mM KCl, and the suspension was made 0.2 M in H_2SO_4. The remainder of the homogenate (total) and the supernatant were made 0.2 M in H_2SO_4, and acid-soluble proteins were prepared as described above. Nuclei were isolated from rat liver as described (19).

Western Blotting. Acid-soluble proteins were electrophoretically separated in SDS/15% polyacrylamide gels and transferred to nitrocellulose (19). Immunochemical staining for PB was performed with a 1:1000 dilution of antiserum containing polyclonal anti-PB antibody and anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad) at 1:3000 dilution.

Immunohistochemistry. Cryostat-cut (6-μm) fresh frozen sections were lifted onto cold gelatin-coated slides, air-dried, and fixed with 4% paraformaldehyde in 0.05 M phosphate-buffered 0.9% saline (PBS), pH 7.2. The slides were washed with PBS and made permeable in PBS plus 0.3% Triton X-100 for 2 hr. Preincubation was performed in PBS/0.3% Triton X-100/1% bovine serum albumin (BSA) plus 5% normal rabbit serum for 30 min when the monoclonal antibody was used. All reactions involving the polyclonal antibody were performed in PBS/Triton/BSA. The monoclonal antibody was used at 1:300 dilution and the polyclonal at 1:750 or 1:1000 dilution. The slides were incubated with the primary antibody in a moist chamber overnight at 4°C. The slides were

washed in PBS/0.3% Triton twice for 20 min and then either rabbit anti-mouse (1:40 dilution) or goat anti-rabbit (1:20) IgG was applied for 1.5 hr at room temperature for monoclonal or polyclonal reaction, respectively. After PBS/Triton washes, 1:100 peroxidase-anti-peroxidase complex (either mouse monoclonal or rabbit antiperoxidase) was applied for an additional 1.5 hr at room temperature. Other than the primary antibodies, all antibodies were purchased from Sternberger–Meyer Immunocytochemical (Jarrettsville, MD). Staining was accomplished in 0.005% hydrogen peroxide and 0.02% diaminobenzidine tetrahydrochloride (Sigma). Control prostatic sections had equivalent dilutions of normal rabbit serum or mouse serum substituted for the primary antibody.

RESULTS

PB. The rat pM-40 cDNA sequence (Fig. 1) predicts a protein nearly identical in amino acid composition, molecular weight, and pI to PB, a rat dorsolateral prostate nuclear protein (8, 9), suggesting that the pM-40 cDNA encodes PB. This was proven by purification of PB from the prostate and N-terminal sequencing. This sequence shows that PB contains two methionines at the N terminus (Fig. 1).

To determine whether PB was related to any known protein(s) (National Biomedical Research Foundation data base, version 10), computer analysis of the predicted PB amino acid sequence was performed by the DFAST program (20). This program predicts a statistically significant similarity, or Z score, which measures the similarity between two proteins and their relationship to a control, a randomly permuted sequence. A Z score >6 is probably significant, whereas a score >10 is significant. This analysis revealed that PB is statistically related to rat α_2-microglobulin ($Z = 43$), rat odorant-binding protein ($Z = 27$), and bovine β-lactoglobulin ($Z = 8$), members of a ligand carrier family (13, 14) of proteins (Fig. 2).

***In Vivo* Cellular Location.** To determine cellular location, both monoclonal (24) and polyclonal antibodies against PB were used. Acid-soluble proteins of nuclei and cytosolic fractions of dorsal, lateral, and ventral prostate were isolated and assessed by Western blotting. In all prostatic tissues, one 20-kDa protein was present in fractions containing nuclei, while PB also was detected in the lateral fractions enriched for cytosol/secretions (Fig. 3). The Western blots show that the polyclonal antibody does not crossreact with histones.

Initially, Matuo *et al.* (8, 9) reported that PB was an abundant prostatic nuclear protein. Subsequently, they reported that immunofluorescence staining with an anti-PB monoclonal antibody localized the protein within prostatic ducts with hardly any staining in the nuclei (24). This limited nuclear staining has been enhanced by achieving efficient nuclear penetration with the antibody (Fig. 4). Dual cellular localization within the ducts and nucleus has been confirmed with the polyclonal and monoclonal antibodies. Perfused fixed and fresh frozen prostatic tissues were sectioned and immunoperoxidase-stained using primary antibody to PB. Both nuclear and cytosolic staining were visualized. Within the lateral lobe epithelium, staining was concentrated along the border of cells revealing secretory granules, within the secretions of the ducts, and in the nucleus (Fig. 4*A*). Epithelial cells showing nuclear staining, but lacking secretory granules, could be seen in the same ducts and were adjacent to cells showing nuclear and secretory staining (Fig. 4*B*). Morphology of the lateral epithelial cell layers correlated with PB localization in the nucleus and/or secretions. In cells that formed parts of ducts that had highly convoluted structures, the nuclear protein was present, while cells lining ducts that were not convoluted expressed both nuclear and secreted PB (Fig. 4*A*). In the dorsal (Fig. 4*D*) and ventral (Fig. 4*E*) lobes nuclear staining was seen, whereas secretions were negative.

PB mRNA and *in Vitro* Translation Products. The presence of PB in two cellular locations could be achieved by translation from two different mRNAs. Primer extension and S1 nuclease protection analyses demonstrated the presence of only one PB mRNA (Fig. 5). The level of the PB mRNA was ≈100-fold lower in the ventral and 2-fold lower in the dorsal

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PB      MRVILLL----LTLDVLGVS     MMTDKNLK-KKIEGNWRTVYLAASSV   42
A2u-G   MKLLLLLLCLGLTL-VCGHA  EEASSTRGNLDVAKLNGDWFSIVVASNKR
OBP     M-VKFLLIVLALVSC         AHHENLDISPSEVNGDWRILYIVADNV
BLG                           LIVTQTMKGLDIQKVAGTWYSLAMAASDI

PB      EKINEGS-PLRTYFRRIECGKRCNRINLYFYIK-KGAKCQQFKIVGRRSQ-  90
A2u-G   EKIEENG-SMRVFMQHIDVLENSLGFK--FRIK-ENGECRELYLVAYKTPE
OBP     EKVAEGG-SLRAYFQHMECGDECQELKIIFNVK-LDSEC-QTHTVVGKHED
BLG     SLLDAQSAPLRVYV-EELKPTPEGDLEILLQ-KWENGECAQKKIIAEKTKI

PB      DVYYAK-YEGSTAFML--KTVNEKILLFDYFN-RNRRNDVTRVAGVL-AKG  136
A2u-G   DGEYFVEYDGGNTFTIL-KTDYDRYVMFHLINFKNGETFQLMVL---YGRT
OBP     -GRYTTDYSGRNYFHVLKKTDDIIFFHNVNVDESGRRQCDL-VAG----KR
BLG     PAVFKIDALNENKVLVL-DTDYKKYLLFCMENSAEPEQSLACQCL---VRT

PB      RQLTKDEMTEYMNFVEEMGIEDENVQRVMDT-DTCPNKIRIR          177
A2u-G   KDLSSDIKEKFAKLCEAHGITRDNIIDLTKT-DRCLQARG
OBP     EDLNKAQKQELRKLAEEYNIPNENTQHLVPT-DTCNQ
BLG     PEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHI
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FIG. 2. Amino acid sequence alignment with the ligand carrier family. Protein similarity was analyzed by the DFAST program (20) and alignments with three members of the ligand carrier family are presented in ranking order of statistical significance. Rat PB is compared to rat α_2-microglobulin (A2u-G; refs. 21 and 22), rat odorant-binding protein (OBP; ref. 13), and bovine β-lactoglobulin (BLG, ref. 23). The signal-peptide sequences are separated from the mature protein sequences by a space. Inserted gaps are indicated by dashes. Amino acids in PB are numbered at right. The shaded areas represent highly conserved amino acids. Conservative amino acid changes are as follows: acidic, D or E; basic, K or R; aromatic, F or Y; hydrophobic, I = L = M = V or T = S.

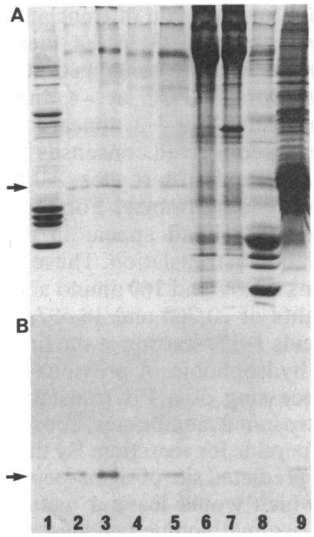

FIG. 3. Distribution of PB. (*A*) Acid-extracted rat liver histones (lane 1; 6 μg), total dorsal prostate extracted protein (lanes 2 and 3; 0.5 and 1 μg), total lateral prostate extrated protein (lanes 4 and 5; 1 and 2 μg), soluble supernatant from dorsal (lane 6; 9 μg), lateral (lane 7; 9 μg), or ventral (lane 8; 9 μg) prostate, and insoluble pellet from ventral prostate (lane 9; 9 μg) were electrophoresed in an SDS/15% polyacrylamide gel. Total extracted protein represents both cytosolic/secretory and nuclear fractions. The enriched cytosolic/secretory fraction was present in soluble supernatant; the nuclear fraction was present in the insoluble pellet. Protein was determined by turbidity assay and bands were detected by staining with Coomassie blue. (*B*) A nitrocellulose Western blot was probed with polyclonal antibody to PB (1:1000 dilution) and the signal was developed with horseradish peroxidase. Arrow identifies the 20-kDa PB. Note that the antibody did not react with histones (lane 1) or overloaded ventral prostate proteins other than PB (lanes 8 and 9).

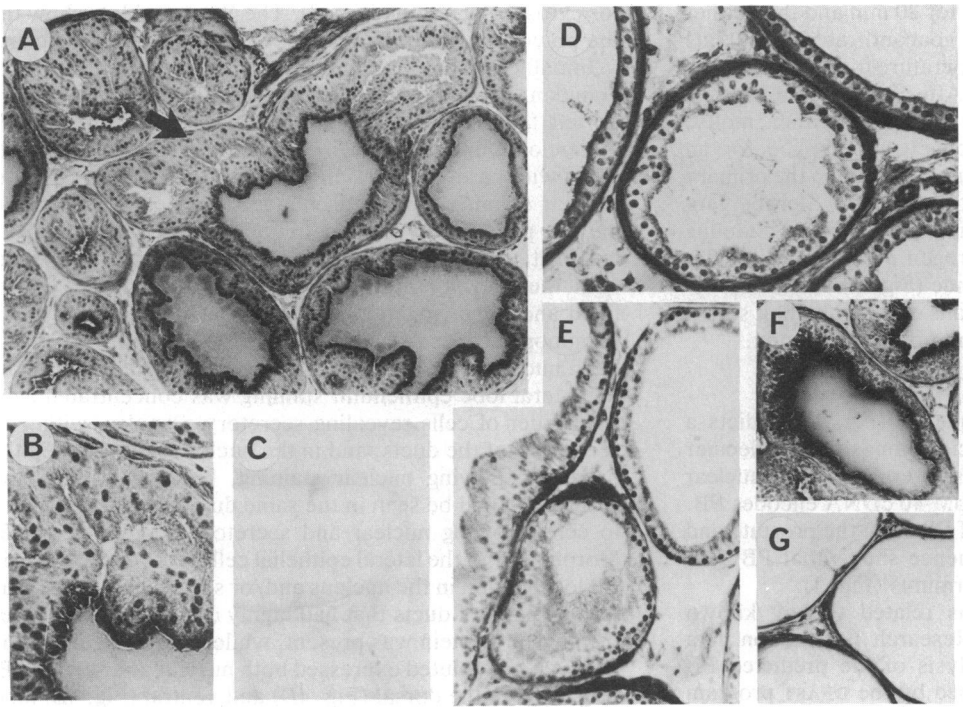

FIG. 4. Immunohistochemical localization of a nuclear and a secreted PB product. Photomicrography to localize PB was performed with both polyclonal and monoclonal antibody detected by peroxidase-antiperoxidase complex. Staining is qualitative with the purpose of showing cellular distribution of PB. (*A*) Polyclonal antibody to PB shows lateral prostate nuclear and secretory staining. Arrow indicates a region of two different epithelia within the same duct. (×90.) (*B*) Enlargement of region noted by arrow in *A*. (×200.) (*C*) Control prostate. (×60.) (*D–F*) Monoclonal antibody to PB shows dorsal prostate nuclear staining (*D*, ×170), ventral prostate nuclear staining (*E*, ×170), and lateral lobe secretions and nuclear staining (*F*, ×90). (*G*) Control prostate. (×80.)

prostate than in the lateral prostate (Fig. 5*C*). Therefore, the presence of a secreted and nuclear protein must arise from one PB mRNA. This can occur if initiation of translation occurs at two sites within the mRNA. In PB mRNA, the first AUG codon is located at nucleotide +1, and the second and third AUG codons are at nucleotides +52 and +55, respectively (Fig. 1). In comparison to the consensus sequence (CCACCAUGG) for initiation of translation (1), where C in positions −1 and −2 and G in +4 facilitate initiation of translation (25), the first site contains C at −2 and at −4, and the second site contains CC at −4 and −5. The highly conserved consensus A at −3 appears for all three sites, whereas the highly conserved consensus G at +4 is replaced by A for all three potential start sites. All three AUG codons begin long open reading frames. For this mRNA, the first AUG and then the second appear in the most favorable context for initiation of translation. These two reading frames code for proteins of 177 and 160 amino acids with calculated molecular weights of 20,760 and 18,597, respectively. Predicted amino acids 1–17, starting at the first AUG codon, are predominantly hydrophobic. A previous report (11) demonstrated the processing of a PB translation product in the presence of microsomal membranes, consistent with the presence of a signal peptide for secretion. By the −3,−1 rule of von Heijne (26), the predicted site of signal-peptide cleavage would be at Ser-17, which would leave a mature protein product beginning with two methionine residues. Sequencing of purified PB showed that two methionine residues (Met-18 and -19) exist at the N terminus of the isolated protein (Fig. 1).

To demonstrate whether a single mRNA can code for two PB proteins, synthetic mRNA transcripts of the pM-40 cDNA from a bacteriophage promoter system were produced with various deletions of the 5′ end. The synthetic PB mRNA transcribed from a series of these constructions and total dorsolateral prostate mRNA were translated in rabbit reticulocyte lysate (Fig. 6). The presence of oligo(G) tails at the 5′ end of the synthetic PB mRNA interfered with translation from the first AUG codon. Removal of this tail was accomplished via a genomic/cDNA construction. This construction produced a mRNA that lacked the leader sequence and was translated into two proteins with a preference for the first AUG initiation codon. Synthetic PB mRNAs that lacked the untranslated region and first AUG, and various portions of the signal sequence, produced a protein that initiated at the second set of AUG codons. Synthetic PB mRNA lacking the second set of AUG codons did not produce an *in vitro* translation product (data not shown). These data confirm that the two proteins are not the result of a possible frameshift.

DISCUSSION

Previous work showed that the pM-40 cDNA clone will hybrid-arrest the *in vitro* translation of two proteins that differ by ≈2 kDa (10). The larger protein contained a signal peptide that was cleaved after microsomal membrane processing of the *in vitro* translation product (11). Both of these proteins were synthesized in the wheat germ *in vitro* translation system (10), which has been shown to initiate only at authentic AUG codons (27, 28). The relationship of these two proteins has now been demonstrated.

We report that the androgen-regulated M-40 gene (10) codes for PB, an androgen-regulated protein (8, 9). Sequence analysis identified PB as a new member of a ligand carrier family of serum, cellular, and nuclear proteins (13, 14, 29, 30). Immunohistologically, PB was detected in the prostate in at least two of these locations, the ducts (as a secreted protein) and the nucleus. Ductal localization is in keeping with the presence of a signal peptide sequence for secretion (11), but the origin of the nuclear protein raised a provocative question.

Primer extension and S1 protection analysis demonstrated the presence of only one PB mRNA, suggesting that both the secreted and the nuclear protein must be encoded by this mRNA. Immunohistochemistry demonstrated that *in vivo*, adjacent epithelial cells exhibit selected expression for a secreted or a nuclear protein. This difference in expression is not due to altered cellular mRNA levels, since *in situ* hybridization histochemistry has shown that all lateral lobe epithelial cells express the PB mRNA equally (12). These data suggest translational regulation of a bifunctional mRNA. In order to demonstrate the feasibility of this mechanism, synthetic PB mRNAs were produced. These PB mRNAs prove that two proteins can be produced *in vitro* by initiating translation with the AUG codon at Met-1 or at Met-18. These data are consistent with an *in vivo* mechanism in which the PB

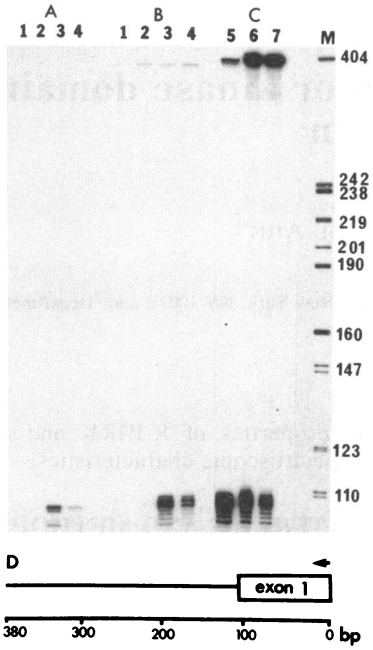

FIG. 5. Primer extension and S1 protection analysis of rat mRNA. (*A*) A 17-nucleotide oligomer starting at nucleotide +54 (see Fig. 1) was used as a primer for extension of mRNA. (*B* and *C*) A 380-bp fragment isolated from the genomic clone was end-labeled at the *Hin*fI site of exon 1 (corresponding to nucleotide +70 of the mRNA, Fig. 1) for S1 protection. The extended/protected products were electrophoresed in a 15% sequencing gel. The S1-protected, reannealed 380-bp genomic fragment appears at the top in *B* and *C*. (*D*) The chosen primer sequence (arrow) was located at the 3′ end of the *Dra*II–*Hin*fI fragment. The primer-extended product mapped to the same start site for transcription as determined by the S1-protected exon 1 region. The major start site for transcription is followed by a minor start site 4 nucleotides downstream. No other forms of the PB mRNA appear. Lanes represent products from total RNA of rat liver (lane 1, 10 μg), ventral prostate (lane 2, 10 μg), dorsal prostate (lane 3, 10 μg), or lateral prostate (lane 4, 5 μg) or from poly(A)$^+$ RNA of rat ventral prostate (lane 5, 10 μg), dorsal prostate (lane 6, 0.2 μg), or lateral prostate (lane 7, 0.1 μg). Markers (lane M) were end-labeled *Hpa* II fragments of pAT153 DNA.

mRNA codes for two proteins. In this mechanism, initiation at the first AUG codon produces a protein containing a signal peptide that directs PB for secretion, whereas initiation at the second AUG codon results in a cellular PB that is translocated to the nucleus. The processed secreted protein would become identical to the nuclear protein.

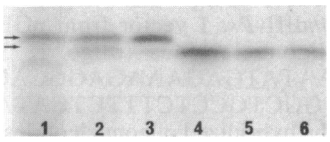

FIG. 6. *In vitro* translation of synthetic PB mRNA. By Kozak's rules, initiation of translation for the bifunctional PB mRNA could occur at AUG codons at +1, +52, and +55 (see Fig. 1). The first two AUG codons appear in the most favorable context, and sequencing of the purified protein confirmed that both Met-18 and Met-19 are present in the mature protein. Total dorsolateral prostate mRNA translated in rabbit reticulocyte lysate resulted in two PB proteins (lane 1, arrows) as previously defined by hybrid-arrested translation (11). Synthetic PB mRNA that contained oligo(G) from the 5′-end cloning site starting at nucleotide −8 produced two PB proteins (lane 2) where initiation of translation at the second start site was markedly higher than seen in lane 1. Synthetic PB mRNA that lacked the 5′-end oligo(G) tail and started at nucleotide −12 produced two PB proteins (lane 3) similar in ratio to those in lane 1. Synthetic PB mRNA that removed the first methionine residue and subsequent residues to position 8 or 11 or 13 (Fig. 1) initiated translation at the second start site (lanes 4, 5, and 6, respectively).

The principle of more than one protein initiating from different AUG codons in higher eukaryotic mRNAs has been established *in vitro* (3, 31). Our data are consistent with a mechanism whereby PB is encoded by a bifunctional mRNA that undergoes translational regulation *in vivo*.

Note Added in Proof. Two forms of the progesterone receptor are produced from one mRNA (32).

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