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Cloning and analysis of a cDNA coding for bovine prothrombin

(mRNA enrichment/recombinant DNA/cDNA hybridization/DNA sequence analysis)

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ABSTRACT Poly(A)-RNA enriched for prothrombin was isolated by specific immunoprecipitation of bovine liver polysomes. Prothrombin consisted of about 8% of the cell-free translation products of this RNA. A double-stranded cDNA was synthesized by using reverse transcriptase (RNA-dependent DNA nucleotidyltransferase) and made blunt-ended with nuclease S1. After tailing with dCTP and terminal transferase, the double-stranded cDNA was annealed to pBR322 DNA that had been cleaved previously at the single *Pst* I site and similarly tailed with dGTP. The resulting plasmids were used to transform *Escherichia coli* strain RR1 under P3-EK1 conditions. Sixty-three tetracycline-resistant clones were obtained that hybridized to ^{32}P-labeled cDNA synthesized from prothrombin-enriched mRNA. Recombinants containing cDNA to prothrombin mRNA sequences were screened by a solution hybridization assay with a [^{3}H]cDNA synthesized from mRNA. This enriched mRNA was 50% prothrombin mRNA, as determined by a reticulocyte lysate translation assay. Three positive clones were identified by this assay; they contained bovine DNA inserts of 700, 500, and 400 base pairs. The DNA sequence of the 700-base-pair insert was then determined. This recombinant plasmid contained DNA coding for the carboxyl-terminal 160 residues of bovine prothrombin followed by a noncoding region of 119 base pairs and a poly(A) tail of 60 base pairs.

The coagulation of blood is the result of a series of stepwise reactions in which many of the coagulation proteins in plasma are converted from zymogens to highly specific serine proteases (see ref. 1 for a recent review). In the final stages of the blood coagulation cascade, prothrombin is converted to thrombin by Factor X_a in the presence of phospholipid, calcium ions, and Factor V_a. Thrombin then cleaves fibrinogen to form an insoluble fibrin clot. Although prothrombin is one of the most abundant coagulation proteins present in plasma, its mRNA constitutes only about 1% of the total poly(A)-RNA in bovine liver (2). This level of RNA makes it difficult to purify prothrombin mRNA to the degree of homogeneity required for many biochemical investigations. Recent developments in recombinant DNA techniques, however, permit the isolation of large amounts of homogeneous DNA corresponding to the specific mRNAs. Such cloned DNAs can be used for many different studies, such as the quantitation of cellular mRNA levels in response to various stimuli, the purification of specific mRNA by hybridization to immobilized DNA, and as a hybridization probe to isolate and study genomic DNA.

In this paper, we describe the construction, cloning in *Escherichia coli*, and sequence of a recombinant plasmid containing DNA coding for the carboxyl-terminal portion of bovine prothrombin.

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MATERIALS AND METHODS

Enzymes. Avian myeloblastoma virus reverse transcriptase (RNA-dependent DNA nucleotidyltransferase) was generously supplied by Joseph Beard (Life Sciences, Gulfport, FL). Nuclease S1 was purchased from Miles or was generously provided by Richard Palmiter (University of Washington). Calf thymus terminal deoxynucleotidyltransferase was generously supplied by R. L. Ratliff (Los Alamos Scientific Laboratory, Los Alamos, NM). All restriction endonucleases were purchased from Bethesda Research Laboratories (Rockville, MD) and used according to the manufacturer's recommendations.

Enrichment of mRNA. Polysomes were prepared from bovine liver as described (2). Polysomes synthesizing prothrombin were enriched by specific immunoprecipitation with antiprothrombin prepared in rabbits and either goat anti-rabbit immunoglobulin coupled to *p*-aminobenzyl-cellulose (3) or *Staphylococcus aureus* cells (Calbiochem) (4). After elution of the polysomes from the solid matrix, the RNA was extracted with phenol/chloroform and precipitated with ethanol. Poly(A)-RNA was isolated by chromatography with oligo(dT)-cellulose (5). The degree of enrichment for prothrombin mRNA was estimated by translation of the RNA in an mRNA-dependent cell-free protein-synthesizing system derived from rabbit reticulocytes (6), followed by immunoprecipitation of prothrombin. Further details of the prothrombin mRNA purification will be published elsewhere.

Synthesis of cDNA and Addition of Homopolymeric Tails. Poly(A)-RNA that had been enriched to about 8% prothrombin mRNA was used to synthesize a cDNA by using reverse transcriptase and oligo(dT) as primer (7). After base hydrolysis of the mRNA, double-stranded DNA was synthesized with reverse transcriptase and made blunt-ended with nuclease S1 (7). Poly(C) (approximately nine dCMP residues) was then added to the 3′ ends of the double-stranded DNA by use of terminal deoxynucleotidyltransferase (7).

Construction and Analysis of Recombinant Plasmids. Plasmid pBR322 DNA was cleaved with *Pst* I and tailed with an average of 13 dGMP residues by use of terminal deoxynucleotidyltransferase. The double-stranded DNA tailed with poly(C) was then annealed to an equimolar amount of the linear pBR322 DNA in 10 mM Tris·HCl, pH 7.4/0.10 M NaCl/1 mM EDTA for 2 hr at 43°C. The temperature was then reduced to 25°C over a period of 3 hr. The resulting chimeric plasmids were used to transform *E. coli* strain RR1 in the presence of $CaCl_2$ (8). Transformants were selected for tetracycline resistance. The colonies containing a recombinant plasmid were identified by the method of Grunstein and Hogness (9) by using a [^{32}P]cDNA synthesized from the enriched poly(A)-RNA.

Abbreviation: bp, base pairs.

Plasmids were then isolated from each of the recombinants (10) and purified by CsCl gradient centrifugation (11). Recombinant plasmids containing prothrombin cDNA were identified by a solution hybridization procedure by using a ^{3}H-labeled cDNA synthesized from poly(A)-RNA. This poly(A)-RNA had been enriched such that about 50% of the translation products precipitated by trichloroacetic acid were specifically immunoprecipitated with antiprothrombin. The hybridization assay mixture (20 μl) contained 0.3 M NaCl, 20 mM Tris·HCl (pH 7.4), 2 mM EDTA, 0.1% $NaDodSO_4$, 5 mM 2-mercaptoethanol, 200 ng of plasmid, and 0.2 ng of [^{3}H]cDNA ($\approx$1100 cpm). The reaction mixture was overlaid with oil, heated to 100°C for 2 min, and incubated at 68°C for 16 hr. Radiolabeled DNA that was resistant to nuclease S1 was precipitated with trichloroacetic acid and collected on Whatman GFC filters, and the radioactivity was determined.

Restriction Endonuclease Analysis. Samples of pBR322, the recombinant plasmid, and the *Pst* I-released insert were digested with one or more restriction endonucleases, and the resulting fragments were analyzed by polyacrylamide gel electrophoresis (12).

DNA Sequence Analysis. Restriction fragments were labeled with either [α-^{32}P]dNTPs by using the Klenow fragment of *E. coli* polymerase (Boehringer Mannheim) (13) or [γ-^{32}P]ATP by using T4 kinase (Boehringer Mannheim) after prior treatment of the DNA with bacterial alkaline phosphatase (Worthington) (14). Labeled fragments were subjected to base modification and cleavage as described by Maxam and Gilbert (14) and subjected to electrophoresis on 0.35-mm polyacrylamide gels containing 8.3 M urea (15).

DNA sequences were stored and analyzed by the computer programs of Staden (16, 17). The programs were adapted for use on the local computer facility by Jon Herriott (University of Washington).

Containment. Cloning of recombinant DNA in *E. coli* was carried out under P3-EK1 conditions in accordance with the Revised Guidelines for Recombinant DNA Research (National Institutes of Health, 1978).

RESULTS

Construction of Recombinant Plasmids and Transformation of *E. coli*. Specific immunoprecipitation of polysomes was used to enrich for prothrombin mRNA because this mRNA constitutes only 1% of bovine liver poly(A)-RNA (2). Initially, bovine liver polysomes were incubated with rabbit anti-bovine prothrombin antibodies, followed by goat anti-rabbit immunoglobulin bound to *p*-aminobenzyl-cellulose (3). This procedure resulted in an 8- to 20-fold enrichment for prothrombin mRNA with a 30% yield, as estimated by translation in a reticulocyte lysate. The enrichment for prothrombin mRNA was accompanied by an 80% decrease in albumin mRNA levels (from about 10% to 2%). In the latter part of this study, polysomes were immunoprecipitated with antiprothrombin and *S. aureus* cells as described (4). This procedure resulted in a 40- to 60-fold enrichment of prothrombin mRNA with a 40% yield.

Complementary DNA was synthesized from enriched poly(A)-RNA by using reverse transcriptase and oligo(dT) as primer. The poly(A)-RNA used as template contained about 8% prothrombin mRNA. After base hydrolysis of the mRNA template, reverse transcriptase was used to synthesize a second DNA strand by use of the hairpin portions at the 3′ terminus of the cDNA as primer. Under the conditions used, 47% of the cDNA was primed to produce a double-stranded cDNA. The double-stranded cDNA was made blunt-ended by treatment with nuclease S1, and an average of nine dCMP residues were added to the 3′ termini by terminal transferase. Similarly, plasmid pBR322 was cleaved at its single *Pst* I site (18), and an average of 13 dGMP residues were added to the 3′ termini with terminal transferase. Chimeric plasmids resulting from the reannealing of the tailed DNAs were used to transform *E. coli* strain RR1, and transformants were monitored for their ability to grow in the presence of tetracycline (18). Five hundred sixty-eight transformants were isolated with a transformation efficiency of 2×10^3 transformants per μg of vector DNA. The transformants were replated in duplicate and screened for recombinants by the *in situ* hybridization procedure of Grunstein and Hogness (9). The ^{32}P-labeled cDNA probe was synthesized from the original mRNA preparation. Sixty-three colonies readily hybridized to this probe. Plasmid isolated from each of these colonies was digested with *Pst* I, and the resulting DNA fragments were analyzed by electrophoresis in 1% agarose gels. Construction of chimeric plasmids by G·C tailing at the *Pst* I site of pBR322 results in the regeneration of *Pst* I sites flanking the inserted DNA. Accordingly, the size of the DNA insert was estimated by gel electrophoresis, and these inserts ranged from 150 to 1600 base pairs (bp). About 10% of the recombinants had only one *Pst* I site regenerated, presumably due to exonuclease digestion of the *Pst* I-digested plasmid before tailing. Transfer of the DNA fragments to nitrocellulose (19) and subsequent hybridization to ^{32}P-labeled cDNA showed that only the inserted DNAs contained bovine mRNA sequences.

Screening for Prothrombin cDNA-Containing Plasmids. Because the mRNA used for the synthesis of the double-stranded cDNA was not pure, only about 8% of the recombinant plasmids were expected to contain prothrombin cDNA inserts. To screen for these recombinants, we synthesized a ^{3}H-labeled cDNA ($cDNA_{II}$) from poly(A)-RNA that contained 50% prothrombin mRNA. Translation of the enriched RNA showed that it contained predominantly prothrombin mRNA, with a large number of other mRNA species making up the remaining RNA. Plasmid and $cDNA_{II}$ were denatured and reannealed in a 1000-fold plasmid excess. After hydrolysis with nuclease S1, the radiolabeled $cDNA_{II}$ that was not digested was precipitated with trichloroacetic acid and quantitated in a liquid scintillation counter. The results for some of these plasmids are shown in Fig. 1. The control plasmids, pOV230 (a recombinant plasmid containing an ovalbumin cDNA insert) (20) and pBR322, provided no protection for the $cDNA_{II}$ against digestion by nuclease S1. Sixty of the recombinant plasmids that were screened also showed negligible hybridization to the $cDNA_{II}$ probe. This is illustrated by plasmids pBII1‡ and pBII33 shown in Fig. 1. Three recombinant plasmids, pBII3, pBII4, and pBII21, protected 7.8%, 6.2%, and 5.3% of the $cDNA_{II}$ probe against nuclease S1 digestion, respectively. These three plasmids were shown to contain bovine cDNA inserts of 700 bp, 500 bp, and 400 bp, respectively, when cleaved with *Pst* I and analyzed by agarose gel electrophoresis. Thus, the degree of protection from nuclease S1 correlates reasonably well with the length of the cDNA insert. Although the DNA sequences of pBII4 and pBII21 were not investigated, analysis of these inserts with restriction enzymes was consistent with the fact that they contained DNA corresponding to the 3′ region of pBII3 (with double-stranded DNA, 5′ and 3′ refer to the coding strand). The degree of protection of the radiolabeled $cDNA_{II}$ against nuclease S1 digestion afforded by the plasmids was, however, lower than expected. This was the case even after allowances

‡ With the various recombinant plasmids, such as pBII1–pBII63, the B corresponds to a plasmid containing a bovine cDNA insert and II corresponds to a plasmid derived from mRNA enriched for prothrombin. Prothrombin has been named Factor II by an international nomenclature committee (21).

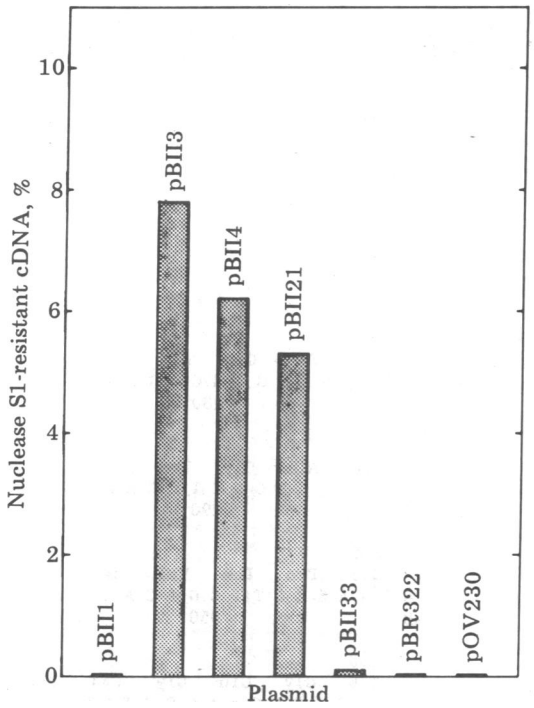

FIG. 1. Hybridization of recombinant plasmids with a [^{3}H]cDNA synthesized from prothrombin-enriched mRNA. See text for details.

were made for the impurity of the cDNA$_{II}$ and the discrepancy of length of prothrombin cDNA [about 2000 bp (unpublished results)] compared to the plasmid inserts. The reasons for this are not known.

Restriction Endonuclease Mapping. The 700-bp insert of pBII3 contains cleavage sites for the restriction enzymes *Ava* II, *Bam*HI, *Hae* III, *Hin*dII, *Hin*fI, *Hpa* II, *Sau* 3A, and *Taq* I (Fig. 2). Many of the restriction enzymes that were tested did not cleave the insert, including *Bgl* I, *Bgl* II, *Bst*EII, *Eco*RI, *Hae* II, *Hha* I, *Hin*dIII, *Hpa* I, *Kpn* I, *Pst* I, *Sal* I, *Sma* I, *Sst* I, *Sst* II, *Tha* I, *Xba* I, and *Xho* I. The only enzymes having six base recognition sequences that cleaved pBII3 were *Bam*HI and *Hin*dII.

DNA Sequence Analysis. The complete amino acid sequence of bovine prothrombin has been determined by Magnusson *et al.* (23). Accordingly, DNA sequence analysis of pBII3 was undertaken to clearly establish whether or not this plasmid contained DNA coding for bovine prothrombin. The strategy used in determining the complete nucleotide sequence of the pBII3 insert is illustrated in Fig. 2. In these studies, the majority of the nucleotide sequence was established by two or more separate experiments, and portions of the DNA were analyzed on complementary strands. These data are shown in Fig. 3. The sequence is presented in the same orientation as the ampicillin resistance gene of pBR322 (22). The insert is comprised of 692 bp and is flanked by G·C tails of 18 and 11 bp at the 5′ and 3′ ends, respectively. Nucleotides 20–499 correspond to amino acid residues 423–582 of bovine prothrombin, with the exception of nucleotides 269–274. These bases correspond to the sequence of Asp-Asn for residues 506 and 507, whereas the protein sequence for these two residues was reported as Asn-Asp. The DNA sequencing gel was of very high quality in this region. Thus, this minor discrepancy is probably due to an incorrect amide assignment made during the protein sequence analysis. The Asp-Asn sequence for residues 506 and 507 in bovine prothrombin is also identical to the corresponding residues in the human molecule (24). However, the presence of genetic variants of bovine prothrombin cannot be ruled out. Nucleotides 500–502 of the pBII3 insert code for the stop codon UAG in the mRNA and are followed by 119 bp of noncoding sequence, a poly(A) tail of 60 bp, and the 3′ G·C tail.

DISCUSSION

In this communication, we report the cloning in *E. coli* of a cDNA that codes for the 3′ region of bovine prothrombin mRNA. The cDNA was synthesized from bovine liver poly(A)-RNA that was enriched for prothrombin mRNA by specific immunoprecipitation of polysomes. The RNA used as template for the reverse transcriptase was enriched about 8-fold for prothrombin mRNA. However, an alternate procedure for immunoprecipitating polysomes resulted in a 50-fold enrichment for prothrombin mRNA, such that 50% of the cell-free translation products of this RNA were specifically immunoprecipitated with antiprothrombin antiserum. This highly enriched RNA was used to synthesize a ^{3}H-labeled cDNA that was predominantly prothrombin cDNA. When this enriched

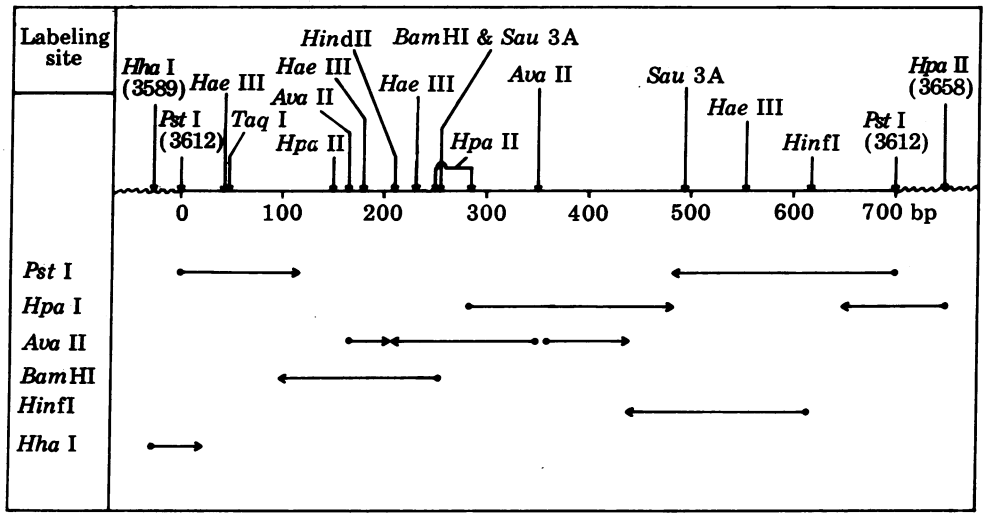

FIG. 2. Restriction endonuclease map and sequencing strategy for the insert of plasmid pBII3. Not all restriction sites are necessarily shown (see *Discussion*). The *Pst* I site corresponds to nucleotide 3612 of pBR322 (22). DNA fragments were labeled at the sites shown and subjected to sequence analysis by the procedure of Maxam and Gilbert (14). The lengths of the arrows show the extent of sequence determined for a particular fragment.

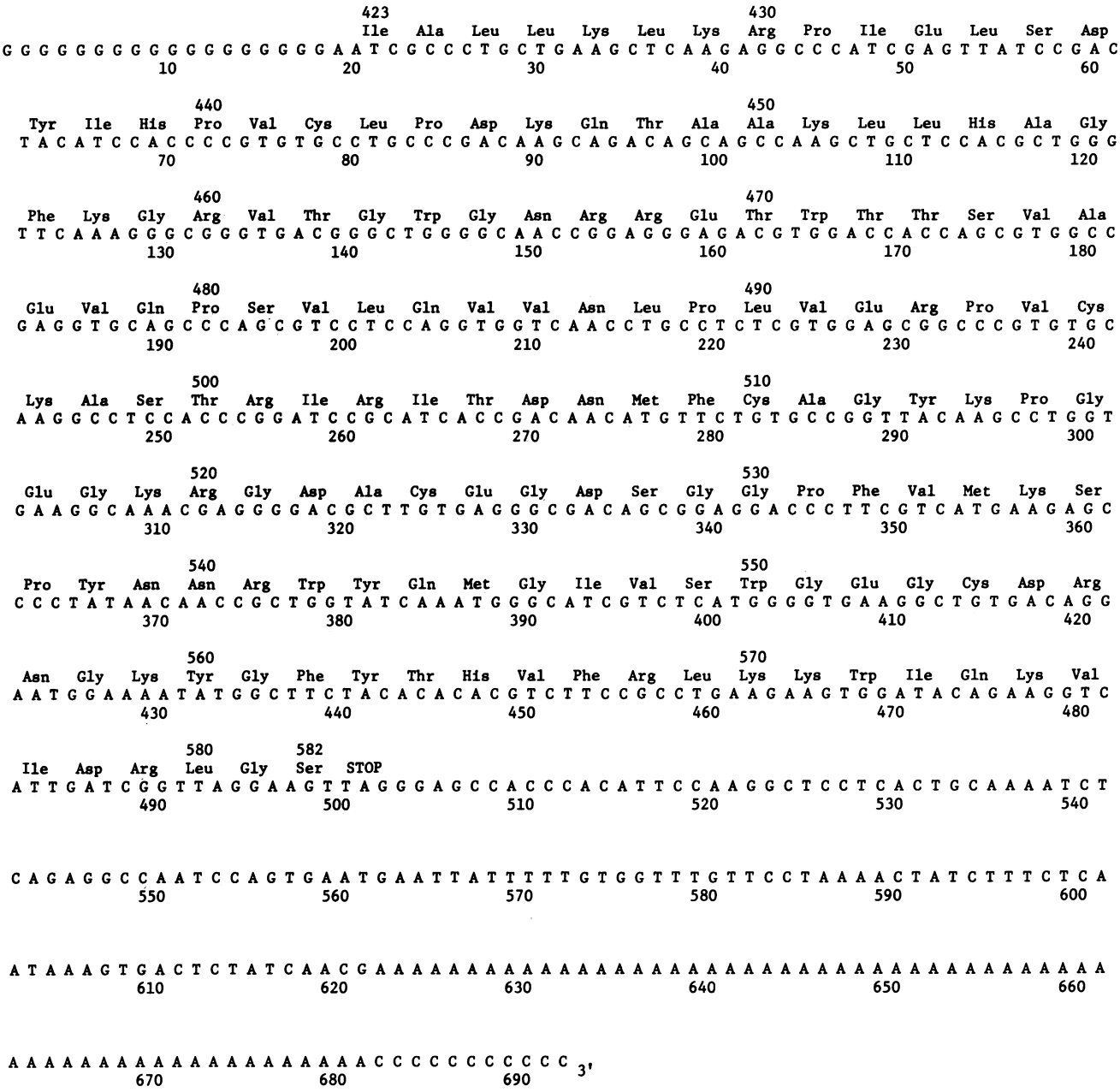

FIG. 3. Complete nucleotide sequence of the insert of plasmid pBII3. Only the coding strand is shown, and it is numbered in the same orientation as the ampicillin resistance gene of pBR322 (22). The amino acid sequence predicted from the DNA sequence has been numbered according to Magnusson *et al.* (23).

cDNA was used to screen the 63 recombinant plasmids by a solution hybridization assay, three of the plasmids protected the cDNA against nuclease S1 digestion, suggesting that they contained prothrombin DNA sequences. This was subsequently confirmed by DNA sequence analysis of the plasmid containing the 700-bp insert. Fewer plasmids containing prothrombin cDNA inserts were isolated than expected from the composition of the mRNA. This may be due in part ot the fact that the prothrombin mRNA content was determined by an assay based on mass (cell-free translation), whereas transformation of bacteria is a reflection of the number of mRNA species present in the preparation.

DNA sequence analysis of pBII3 showed that this recombinant plasmid contained a cDNA coding for the 3′ region of prothrombin mRNA, corresponding to amino acid residues 423–582 of bovine plasma prothrombin (23). Accordingly, this sequence, starting at nucleotide 329, includes the active site region Gly-Asp-Ser-Gly-Gly-Pro, which is common to all serine proteases (1). Whether the nucleotide sequence coding for this region in other serine proteases is highly conserved remains to be determined. The coding sequence of prothrombin mRNA is terminated by the stop codon UAG starting with nucleotide 500. The coding region is followed by a noncoding region of 119 bp and a poly(A) tail of 60 bp at the 3′ end. Because both the exact location of the oligo(dT) primer used in the synthesis of the cDNA and the efficiency of the second-strand synthesis are not known, 60 nucleotides is a minimal estimate of the size of the poly(A) tail on the original mRNA. The sequence A-A-U-A-A-A occurs 15 nucleotides upstream from the poly(A) tail of prothrombin mRNA and does not occur in any other place in the insert of pBII3. This sequence has been found in other eukaryotic polyadenylylated RNA species (25–30) and may be involved in the synthesis or processing of mRNA (31). The sequence immediately surrounding the A-A-U-A-A-A in prothrombin mRNA is very similar to the corresponding sequence of chicken ovomucoid mRNA (30) and MOPC 21 κ light chain

Table 1. Restriction endonuclease cleavage sites in pBII3

Enzyme	Position	Recognition sequence
Alu I*	33, 105	AG/CT
Atu I*	202, 296	CC/$^{A}_{T}$GG
Ava II	165, 341	G/G$^{A}_{T}$CC
*Bam*HI	255	G/GATCC
Dde I*	540	C/TNAG
*Eco*RII*	202, 296	/CC$^{A}_{T}$GG
Hae III	42, 178, 231, 244, 546	GG/CC
*Hin*dII	209	GTYRAC
*Hin*fI	18, 609	G/ANTC
Hpa II	151, 253, 285	C/CGG
Sau 3A	256, 485	/GATC
Sau 961*	42, 165, 231, 341	G/GNCC
Taq I	48	T/CGA

Cleavage sites for enzymes marked with an asterisk were deduced from the DNA sequence only (Fig. 3); other enzyme cleavage sites were deduced from endonuclease digests as well (see text). The position in the sequence corresponds to the first nucleotide in the recognition sequence. The cleavage site, where known, is shown by a slash (/). N, any nucleotide; R, purine; Y, pyrimidine.

mRNA (25), but the homology does not extend to other parts of the 3′ noncoding regions of these RNAs. The 3′ noncoding region of prothrombin mRNA is also somewhat rich in A-T (57%).

The DNA sequence of the pBII3 insert confirms the restriction map (Fig. 2) that was deduced from the size of the various fragments resulting from restriction enzyme digests (Table 1). Additional cleavage sites for the enzymes *Hae* III and *Hin*fI were predicted from the DNA sequence. Fragments resulting from these cleavages were too small to be readily identified on the polyacrylamide gels used in determining the original restriction map. No recognition sites were found in the sequence for enzymes that did not cleave the insert. Analysis of the sequence of pBII3 shows that some codons are preferentially used. For example, seven of the nine proline residues in the sequence are encoded by the codon CCC, and the other two residues are encoded by the codon CCU. Codon preferences have been reported for other mRNA sequences (for review, see ref. 32). Of the prothrombin mRNA codons determined from pBII3, 56% are terminated by a pyrimidine and 81% of these are cytidine. This is consistent with the hypothesis that the preference of cytidine over uridine in the third position of codons minimizes translation errors caused by codon–anticodon base wobble (33). The codon preferences in bovine prothrombin mRNA are generally similar to those preferred in other bovine mRNAs (34, 35). DNA sequence analysis of the flanking region shows that the prothrombin cDNA is inserted in the same orientation as the ampicillin gene of the pBR322 cloning vector. We have not investigated the possibility that the pBII3 insert is expressed in *E. coli*, as observed with some other cDNAs cloned in a similar way (36). Because it is not known whether such a product of pBII3 would contain any bovine prothrombin antigenic determinants, a plasmid bearing a full-length bovine prothrombin cDNA would be preferable for these and other studies.

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