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Cloning and sequence of a cDNA coding for the human β-migrating endothelial-cell-type plasminogen activator inhibitor

(vascular fibrinolysis/serine protease inhibitor/placental cDNA expression library/DNA sequence analysis)

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ABSTRACT **A λgt11 expression library containing cDNA inserts prepared from human placental mRNA was screened immunologically using an antibody probe developed against the β-migrating plasminogen activator inhibitor (β-PAI) purified from cultured bovine aortic endothelial cells. Thirty-four positive clones were isolated after screening 7 × 10^5 phages. Three clones (λ1.2, λ3, and λ9.2) were randomly picked and further characterized. These contained inserts 1.9, 3.0, and 1.9 kilobases (kb) long, respectively. *Escherichia coli* lysogenic for λ9.2, but not for λgt11, produced a fusion protein of 180 kDa that was recognized by affinity-purified antibodies against the bovine aortic endothelial cell β-PAI and had β-PAI activity when analyzed by reverse fibrin autography. The largest cDNA insert was sequenced and shown to be 2944 base pairs (bp) long. It has a large 3′ untranslated region [1788 bp, excluding the poly(A) tail] and contains the entire coding region of the mature protein but lacks the initiation codon and part of the signal peptide coding region at the 5′ terminus. The two clones carrying the 1.9-kb cDNA inserts were partially sequenced and shown to be identical to the 3.0-kb cDNA except that they were truncated, lacking much of the 3′ untranslated region. Blot hybridization analysis of electrophoretically fractionated RNA from the human fibrosarcoma cell line HT-1080 was performed using the 3.0-kb cDNA as hybridization probe. Two distinct transcripts, 2.2 and 3.0 kb, were detected, suggesting that the 1.9-kb cDNA may have been copied from the shorter RNA transcript. The amino acid sequence deduced from the cDNA was aligned with the NH_2-terminal sequence of the human β-PAI. Based on this alignment, the mature human β-PAI is 379 amino acids long and contains an NH_2-terminal valine. The deduced amino acid sequence has extensive (30%) homology with α_1-antitrypsin and antithrombin III, indicating that the β-PAI is a member of the serine proteinase inhibitor (serpin) superfamily.**

The generation of plasmin from plasminogen provides an important source of proteolytic activity in cells, tissues, and biological fluids (1, 2). Precise regulation of plasminogen activator (PA) activity may thus constitute a critical feature of many biological systems (3). Such control may be at the level of the formation and resolution of fibrin itself (4), at the level of the interaction of PAs with cells (5, 6), or by specific PA inhibitors (PAIs; ref. 7).

Available evidence indicates that there are at least three immunologically distinct PAIs, including the placental PAI (8), protease nexin (9), and the endothelial cell-derived PAI (10–12). The PAI synthesized by cultured bovine aortic endothelial cells (BAEs) has been purified and partially characterized (13). It differs from the placental PAI and protease nexin in that it exhibits β-mobility when analyzed by agarose zone electrophoresis (14). Moreover, it inhibits tissue-type PA (tPA) as well as urokinase-type PA (uPA), whereas protease nexin and the placental PAI are primarily uPA inhibitors (15). Antiserum to the β-migrating PAI from BAEs has been developed and employed to show that the β-PAIs from human endothelial cells, plasma, serum, and platelets are immunologically related (16).

The level of β-PAI mRNA produced by cultured BAEs varies depending on culture conditions (17), suggesting that the β-PAI gene is regulated by external factors. The production of β-PAI by human endothelial cells (18) and by rat HTC cells (19) is stimulated several fold by interleukin 1 and dexamethasone, respectively, whereas gonadotropins decrease the β-PAI activity of granulosa cells (20). β-PAI is also subject to regulation at the protein level, since both activated protein C (21) and oxidants (22) directly neutralize its activity.

To facilitate the precise biochemical characterization of β-PAI, and to eventually understand the nature of factors regulating β-PAI gene expression, we have undertaken the molecular cloning of the gene. Here we describe the isolation of β-PAI cDNA from a human placental expression library and demonstrate that the β-PAI is a member of the serine protease inhibitor (serpin) superfamily (23, 24).

MATERIALS AND METHODS

Materials. Restriction enzymes, alkaline phosphatase, bacteriophage T4 DNA ligase, *Escherichia coli* DNA polymerase I, Klenow fragment of DNA polymerase I, and T4 DNA polymerase were purchased from Boehringer Mannheim. [α-^{32}P]dGTP (3000 Ci/mmol) and 5′-[α-[^{35}S]thio]dATP (600 Ci/mmol; 1 Ci = 37 GBq) were purchased from Amersham. Human α-thrombin was a generous gift of J. Fenton (Albany, NY), and fibrinogen was purchased from Calbiochem-Behring. The purification of human plasminogen and urokinase (25) and the purification of BAE β-PAI and the development of antibodies to it (13) were as described. Antiserum to the placental PAI was purchased from American Diagnostica (Greenwich, CT).

Preparation and Analysis of Crude Placental Extract. Frozen placenta (3.5 g) was washed with phosphate-buffered saline (PBS: 0.14 M NaCl/0.01 M sodium phosphate, pH 7.2) and then extracted with 15 ml of PBS containing 0.5% Triton X-100 at 4°C. The tissue was homogenized using a Dounce homogenizer, and cellular debris was removed by centrifugation at 10,000 × *g* for 10 min. The extracts were analyzed for inhibitor activity by reverse fibrin autography (25). Monospecific antisera against human placental-type PAI and

Abbreviations: PA, plasminogen activator; tPA, tissue-type PA; uPA, urokinase-type PA; PAI, PA inhibitor; BAE, bovine aortic endothelial cell; bp, base pair(s); kb, kilobase(s).

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bovine β-PAI were coupled to protein A-Sepharose (Pharmacia) and used as described (13, 17) to immunoprecipitate the PAIs present in the extract. Antiserum against bovine β-PAI recognizes human β-PAI (16).

Immunological Screening of a Human λgt11 cDNA Library. The library, derived from a premature (34 weeks of gestation) human placenta and consisting of 10^6 independent recombinant phages (26), was screened immunologically (27–29) for β-PAI, using the affinity-purified IgG fraction (30) of antibodies to the purified BAE β-PAI as antibody probe (13). To visualize antibody binding, ^{125}I-labeled protein A (55 mCi/mg) was employed. Autoradiography was performed by exposing the filters to Kodak XAR5 film with an intensifying screen at −80°C.

Immunoblot Analysis of *E. coli* Lysates. λgt11 and recombinant lysogens were induced and crude extracts were prepared as described (29). For immunoblot analysis, 50 μl of crude extract was fractionated by $NaDodSO_4$/PAGE (31). The proteins were electrophoretically transferred to nitrocellulose paper and immunoblotted as described (32, 33), using the immunoglobulin fraction of antiserum purified on either β-PAI affinity columns (above) or on the fusion protein. For the affinity purification of antisera on the fusion protein, 900 μl of crude extract from induced *E. coli* lysogens (29) was fractionated by $NaDodSO_4$/PAGE and transferred to nitrocellulose paper. Strips containing proteins of 150–200 kDa were excised from the nitrocellulose sheets and used for the affinity purification of antisera. Blocking of the nitrocellulose filter strips, binding of specific antibodies, and washings were performed as described for the screening of λgt11 libraries with antibody probes (29). To elute bound antibody, the filter strips were incubated twice (3 min each) with 200 μl of 0.1 M glycine/HCl buffer (pH 2.5) containing 0.02% fetal bovine serum. The eluted material was neutralized by the addition of 140 μl of 0.5 M Tris/HCl (pH 8.0), dialyzed overnight, and used as the primary antibody in immunoblot analysis.

Nucleic Acid Methods. Phage particles were prepared by the plate-lysate method and phage DNA was purified by CsCl equilibrium centrifugation (34). Plasmid DNA was isolated by the method of Birnboim and Doly (35), followed by two consecutive ethidium bromide/CsCl equilibrium centrifugations. Enzyme reactions were carried out according to the conditions suggested by the suppliers. Total RNA was prepared (36) from cultured HT-1080 cells (American Type Culture Collection CCL 121), fractionated by agarose gel electrophoresis in the presence of formaldehyde (37), and subjected to blot hybridization analysis (38).

DNA from λgt11 clones was digested with *Eco*RI endonuclease, and the excised cDNA insert was subcloned in bacteriophage M13 cloning vector mp9 (39). M13 clones containing the cDNA insert in both orientations were isolated, and deletion libraries of both strands were constructed using the single-stranded M13 method of Dale *et al.* (40). Before sequencing, the size of the M13 templates was determined by electrophoresis in 0.7% agarose gels, and selected templates were sequenced by the dideoxy chain-termination method (41). Both DNA strands were sequenced and more than 80% of each strand was sequenced two or more times. DNA sequence data was processed using the Staden program (42). Homology searches were done by the Pearson fast protein homology program (43).

RESULTS AND DISCUSSION

Identification of Endothelial Cell-Type β-PAI Activity in Placenta. When 20 μl of the crude placental extract was analyzed for PAI activity by $NaDodSO_4$/PAGE (30) and reverse fibrin autography (25), two inhibitor zones of 50–55 kDa were revealed (data not shown). Immunoprecipitation experiments demonstrated that the two inhibitor zones resulted from the presence of both the placental-type PAI (8) and the endothelial cell-type PAI (10–13). Quantitation by radioimmunoassay (44) indicated that the extract contained 270 ng of β-PAI per ml. Since the placental tissue had been extensively washed before extraction, this β-PAI was most likely synthesized by cells contained in placenta and not a serum contaminant. Placenta was therefore employed as a source for the isolation of a cDNA for β-PAI.

Isolation of Human β-PAI cDNA. Approximately 7 × 10^5 recombinant phages from a λgt11 expression library containing cDNA inserts prepared from human placental mRNA (26) were screened immunologically to obtain cDNAs for the β-PAI. Thirty-four positive clones were obtained, half of which continued to be positive through a second screening. Three positive clones were randomly picked and plaque-purified, and phage DNA was prepared. The phage DNA from the three clones (λ1.2, λ3, and λ9.2) was digested with *Eco*RI and the cDNA inserts were determined to be 1.9, 3.0, and 1.9 kilobases (kb) long, respectively. The 3.0-kb cDNA insert from λ3 was subcloned into a plasmid vector (45), excised with *Eco*RI, and purified from an agarose gel. The cDNA insert was nick-translated and shown to hybridize with λ1.2 and λ9.2 DNA under conditions of high stringency, indicating that the DNA inserts in the three clones were related.

Three lines of evidence support the conclusion that the isolated clones code for the human β-PAI. (*i*) Induction of an *E. coli* lysogenic strain prepared by infecting a high-frequency-of-lysogeny strain (Y1089) with λ9.2 resulted in the expression of a recombinant fusion protein (180 kDa) that was recognized by an affinity-purified IgG from antiserum raised against the purified BAE β-PAI (Fig. 1, lane B). An *E. coli* strain lysogenic for λgt11 and thus lacking the cDNA insert did not produce such an immunoreactive protein (Fig. 1, lane C). (*ii*) The 180-kDa recombinant fusion protein and the BAE β-PAI share antigenic epitopes, since affinity purification of the antiserum on the recombinant fusion protein yielded antibodies that recognized the purified BAE β-PAI on immunoblots (Fig. 1, lane D). (*iii*) Analysis of *E. coli*

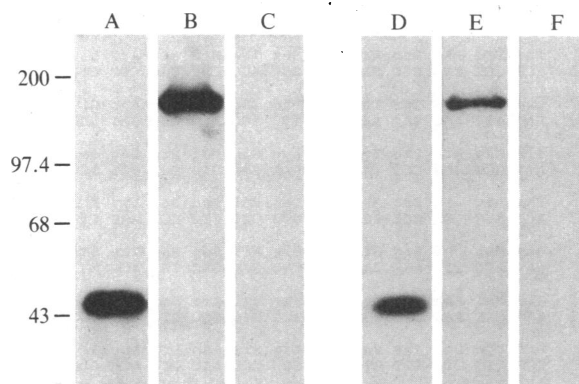

FIG. 1. Immunoblot analysis of *E. coli* crude extracts. Extracts prepared from induced lysogenic *E. coli* strains were fractionated by $NaDodSO_4$/PAGE and analyzed by immunoblotting as described in *Materials and Methods*. Lanes A, D, and F: 300 ng of purified BAE β-PAI. Lanes B and E: 50 μl of extract from strain Y1089 lysogenized with λ9.2. Lane C: 50 μl of extract from strain Y1089 lysogenized with λgt11. For the immunoblotting experiments shown in lanes A–C, affinity-purified IgG against the BAE β-PAI was used as primary antibody. For lanes D–F, the antiserum used was affinity-purified on various proteins bound to nitrocellulose paper: for lane D, antiserum affinity-purified on fusion protein from λ9.2 was used; for lane E, antiserum affinity-purified on the BAE β-PAI; and for lane F, antiserum affinity-purified on proteins of 150–200 kDa from the λgt11 lysogen. The autoradiograms were exposed for 16 hr. Positions of standards (molecular mass in kDa) run in parallel are at left.

FIG. 2. Analysis of inhibitor activity in extracts from induced *E. coli* lysogens. Extracts from lysogenic *E. coli* strains were fractionated by NaDodSO$_4$/PAGE and then analyzed by reverse fibrin autography (lanes A–C) and immunoblotting (lanes D–F). Lanes A and D: 300 ng of purified bovine β-PAI. Lanes B and E: 50 μl of extract from the strain lysogenic for λ9.2. Lanes C and F: 50 μl of extract from the strain lysogenic for λgt11. The autoradiograms for lanes E and F were exposed for 2 weeks.

extracts by NaDodSO$_4$/PAGE followed by reverse fibrin autography revealed that the λ9.2 lysogen containing the 1.9-kb insert, but not the λgt11 lysogen, expresses PAI activity (Fig. 2, lane B). Surprisingly, two PAIs of 180 kDa and 40 kDa, respectively, were present in these extracts. To investigate the relationship of these PAIs, proteins from the lysogens were fractionated by NaDodSO$_4$/PAGE and again analyzed by immunoblotting, but this time the autoradiograms were developed after a longer exposure (Fig. 2, lanes E and F). Two peptides were detected, and these comigrated with the inhibitor activities (compare lanes B and E). The great majority of the β-PAI antigen produced by the λ9.2 lysogen was detected at ≈180 kDa; however, a small amount of antigen was also detected at 40 kDa (Fig. 2, lane E). Since the two PAIs share immunologic and biologic properties, the smaller is most likely derived from the larger by proteolytic processing of the β-galactosidase-PAI fusion protein. The specific activity of the released 40-kDa protein may be higher than that of the larger fusion protein because it is no longer sterically hindered.

Nucleotide Sequence of DNA Coding for Human β-PAI and Assignment of Protein Sequence. The DNA sequence from both strands of the 3.0-kb cDNA insert of clone λ3 was established by sequencing deletion subclones constructed by the method of Dale *et al.* (40). Fig. 3 shows the DNA sequence along with the inferred amino acid sequence. This is not a full-length cDNA, since the untranslated region, the initiation codon, and most of the signal peptide are missing from the 5′ end of the molecule. To identify the codon coding for the NH_2 terminus of the mature protein, we aligned the deduced amino acid sequence with that of the human and bovine β-PAIs (unpublished data). Based on that alignment, the valine designated number 1 in Fig. 3 is the NH_2-terminal residue of the human β-PAI. The β-PAI is secreted and is therefore likely to contain a signal peptide (46). The signal

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        -4          -1  +1
        Glu Gly Ser Ala Val His His Pro Pro Ser Tyr Val Ala His Leu Ala Ser Asp Phe Gly Val Arg Val Phe Gln Gln Val Ala Gln Ala   26
   1    GAA GGG TCT GCT GTG CAC CAT CCC CCA TCC TAC GTG GCC CAC CTG GCC TCA GAC TTC GGG GTG AGG GTG TTT CAG CAG GTG GCG CAG GCC

        Ser Lys Asp Arg Asn Val Val Phe Ser Pro Tyr Gly Val Ala Ser Val Leu Ala Met Leu Gln Leu Thr Thr Gly Gly Glu Thr Gln Gln   56
  91    TCC AAG GAC CGC AAC GTG GTT TTC TCA CCC TAT GGG GTG GCC TCG GTG TTG GCC ATG CTC CAG CTG ACA ACA GGA GGA GAA ACC CAG CAG

        Gln Ile Gln Ala Ala Met Gly Phe Lys Ile Asp Asp Lys Gly Met Ala Pro Ala Leu Arg His Leu Tyr Lys Glu Leu Met Gly Pro Trp   86
 181    CAG ATT CAA GCA GCT ATG GGA TTC AAG ATT GAT GAC AAG GGC ATG GCC CCC GCC CTC CGG CAT CTG TAC AAG GAG CTC ATG GGG CCA TGG

        Asn Lys Asp Glu Ile Ser Thr Thr Asp Ala Ile Phe Val Gln Arg Asp Leu Lys Leu Val Gln Gly Phe Met Pro His Phe Phe Arg Leu  116
 271    AAC AAG GAT GAG ATC AGC ACC ACA GAC GCG ATC TTC GTC CAG CGG GAT CTG AAG CTG GTC CAG GGC TTC ATG CCC CAC TTC TTC AGG CTG

        Phe Arg Ser Thr Val Lys Gln Val Asp Phe Ser Glu Val Glu Arg Ala Arg Phe Ile Ile Asn Asp Trp Val Lys Thr His Thr Lys Gly  146
 361    TTC CGG AGC ACG GTC AAG CAA GTG GAC TTT TCA GAG GTG GAG AGA GCC AGA TTC ATC ATC AAT GAC TGG GTG AAG ACA CAC ACA AAA GGT

        Met Ile Ser Asn Leu Leu Gly Lys Gly Ala Val Asp Gln Leu Thr Arg Leu Val Leu Val Asn Ala Leu Tyr Phe Asn Gly Gln Trp Lys  176
 451    ATG ATC AGC AAC TTG CTT GGG AAA GGA GCC GTG GAC CAG CTG ACA CGG CTG GTG CTG GTG AAT GCC CTC TAC TTC AAC GGC CAG TGG AAG

        Thr Pro Phe Pro Asp Ser Ser Thr His Arg Arg Leu Phe His Lys Ser Asp Gly Ser Thr Val Ser Val Pro Met Met Ala Gln Thr Asn  206
 541    ACT CCC TTC CCC GAC TCC AGC ACC CAC CGC CGC CTC TTC CAC AAA TCA GAC GGC AGC ACT GTC TCT GTG CCC ATG ATG GCT CAG ACC AAC

        Lys Phe Asn Tyr Thr Glu Phe Thr Thr Pro Asp Gly His Tyr Tyr Asp Ile Leu Glu Leu Pro Tyr His Gly Asp Thr Leu Ser Met Phe  236
 631    AAG TTC AAC TAT ACT GAG TTC ACC ACG CCC GAT GGC CAT TAC TAC GAC ATC CTG GAA CTG CCC TAC CAC GGG GAC ACC CTC AGC ATG TTC

        Ile Ala Ala Pro Tyr Glu Lys Glu Val Pro Leu Ser Ala Leu Thr Asn Ile Leu Ser Ala Gln Leu Ile Ser His Trp Lys Gly Asn Met  266
 721    ATT GCT GCC CCT TAT GAA AAA GAG GTG CCT CTC TCT GCC CTC ACC AAC ATT CTG AGT GCC CAG CTC ATC AGC CAC TGG AAA GGC AAC ATG

        Thr Arg Leu Pro Arg Leu Leu Val Leu Pro Lys Phe Ser Leu Glu Thr Glu Val Asp Leu Arg Lys Pro Leu Glu Asn Leu Gly Met Thr  296
 811    ACC AGG CTG CCC CGC CTC CTG GTT CTG CCC AAG TTC TCC CTG GAG ACT GAA GTC GAC CTC AGG AAG CCC CTA GAG AAC CTG GGA ATG ACC

        Asp Met Phe Arg Gln Phe Gln Ala Asp Phe Thr Ser Leu Ser Asp Gln Glu Pro Leu His Val Ala Gln Ala Leu Gln Lys Val Lys Ile  326
 901    GAC ATG TTC AGA CAG TTT CAG GCT GAC TTC ACG AGT CTT TCA GAC CAA GAG CCT CTC CAC GTC GCG CAG GCG CTG CAG AAA GTG AAG ATC

        Glu Val Asn Glu Ser Gly Thr Val Ala Ser Ser Ser Thr Ala Val Ile Val Ser Ala Arg Met Ala Pro Glu Glu Ile Ile Met Asp Arg  356
 991    GAG GTG AAC GAG AGT GGC ACG GTG GCC TCC TCA TCC ACA GCT GTC ATA GTC TCA GCC CGC ATG GCC CCC GAG GAG ATC ATC ATG GAC AGA

        Pro Phe Leu Phe Val Val Arg His Asn Pro Thr Gly Thr Val Leu Phe Met Gly Gln Val Met Glu Pro
1081    CCC TTC CTC TTT GTG GTC CGG CAC AAC CCC ACA GGA ACA GTC CTT TTC ATG GGC CAA GTG ATG GAA CCC TGA CCC TGG GGA AAG ACG CCT

1171    TCATCTGGGACAAAACTGGAGATGCATCGGGAAAGAAGAAACTCCGAAGAAAAGAATTTTAGTGTTAATGACTCTTTCTGAAGGAAGAGAAGACATTTGCCTTTTGTTAAAAGATGGTAA
1291    ACCAGATCTGTCTCCAAGACCTTGGCCTCTCCTTGGAGGACCTTTAGGTCAAACTCCCTAGTCTCCACCTGAGACCCTGGGAGAGAAGTTTGAAGCACAACTCCCTTAAGGTCTCCAAAC
1411    CAGACGGTGACGCCTGCGGGACCATCTGGGGCACCTGCTTCCACCCGTCTCTCTGCCCACTCGGGTCTGCAGACCTGGTTCCCACTGAGGCCCTTTGCAGGACGGAACTACGGGGCTTAC
1531    AGGAGCTTTTGTGTGCCTGGTAGAAACTATTTCTGTTCCAGTCACATTGCCATCACTCTTGTACTGCCTGCCACCGCGGAGGAGGCTGGTGACAGGCCAAAGGCCAGTGGAAGAAACACC
1651    CTTTCATCTCAGAGTCCACTGTGGCACTGGCCACCCCTCCCCAGTACAGGGGTGCTGCAGGTGGCAGAGTGAATGTCCCCCATCATGTGGCCCAACTCTCCTGGCCTGGCCATCTCCCTC
1771    CCCAGAAACAGTGTGCATGGGTTATTTGGAGTGTAGGTGACTTGTTTACTCATTGAAGCAGATTTCTGCTTCCTTTTATTTTTATAGGAATAGAGGAAGAAAGGTCAGATGCGTGCCCA
1891    GCTCTTCACCCCCCAATCTCTTGGTGGGGAGGGGTGTACCTAAATATTTATCATATCCTTGCCCTTGAGTGCTTGTTAGAGAGAAAGAGAACTACTAAGGAAAATAATATTATTTAAACT
2011    CGCTCCTAGTGTTTCTTTGTGGTCTGTGTCACCGTATCTCAGGAAGTCCAGCCACTTGACTGGCACACACCCCTCCGGACATCCAGCGTGACGGAGCCCACACTGCCACCTTGTGGCCGC
2131    CTGAGACCCTCGCGCCCCCCGCGCCCCCCGCGCCCCTCTTTTTCCCCTTGATGGAAATTGACCATACAATTTCATCCTCCTTCAGGGGATCAAAAGGACGGAGTGGGGGGACAGAGACTC
2251    AGATGAGGACAGAGTGGTTTCCAATGTGTTCAATAGATTTAGGAGCAGAAATGCAAGGGGCTGCATGACCTACCAGGACAGAACTTTCCCCAATTACAGGGTGACTCACAGCCGCATTGG
2371    TGACTCACTTCAATGTGTCATTTCCGGCTGCTGTGTGTGAGCAGTGGACACGTGAGGGGGGGGTGGGTGAGAGAGACAGGCAGCTCGGATTCAACTACCTTAGATAATATTTCTGAAAAC
2491    CTACCAGCCAGAGGGTAGGGCACAAAGATGGATGTAATGCACTTTGGGAGGCCAAGGCAGGAGGATTGCTTGAGCCCAGGAGTTCAAGACCAGCCTGGGCAACATACCAAGACCCCCGTC
2611    TCTTTAAAAATATATATATTTTAAATATACTTAAATATATATTTCTAATATCTTTAAATATATATATATATTTTAAAGACCAATTTATGGGAGAATTGCACACAGATGTGAAATGAATGT
2731    AATCTAATAGAAGCCTAATCAGCCCACCATGTTCTCCACTGAAAAATCCTCTTTCTTTGGGGTTTTTCTTTCTTTCTTTTTTGATTTTGCACTGGACGGTGACGTCAGCCATGTACAGGA
2851    TCCACAGGGGTGGTGTCAAATGCTATTGAAATTGTGTTGAATTGTATGCTTTTTCACTTTTGATAAATAAACATGTAAAAATGTTTCAAAAAAA
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FIG. 3. Complete nucleotide sequence of the cDNA insert of λ3. The nucleotide sequence of the coding strand and the corresponding predicted amino acid sequence are shown. The numbers at left refer to the position of the nucleotides, and those at right, to the position of the amino acids. The valine designated as number 1 is the amino terminus, and the amino acid sequence corresponding to the mature protein is numbered 1–379. The amino acid sequence that corresponds to a portion of the signal peptide is represented by the minus numbers in the opposite direction.

peptidase normally cleaves to the carboxyl side of residues with small neutral side chains, such as glycine, alanine, and serine (47). Thus, the alanine at the NH_2-terminal side of the valine designated number 1 may represent the termination of the signal peptide. The reading frame shown in Fig. 3 is the only one without multiple termination codons and codes for 383 residues followed by a TGA stop codon. Removal of the putative signal peptide by cleavage between alanine at position −1 and the valine designated as number 1 would result in a mature β-PAI that is 379 residues long and has a calculated molecular mass for the carbohydrate-free molecule of 42,770 Da. This calculation agrees well with the molecular mass of the unglycosylated form of the BAE β-PAI as determined by *in vitro* translation of its mRNA (17). The β-PAI is glycosylated (13) and the amino acid sequence in Fig. 3 contains three putative glycosylation sites conforming to the canonical Asn-Xaa-Ser/Thr sequence (48) at positions 209–211, 265–267, and 329–331. The 3′ untranslated region of the 3.0-kb cDNA is 1788 base pairs (bp) long, excluding the poly(A) tract. The consensus polyadenylylation sequence AATAAA is found 16 bp upstream from the poly(A) attachment site, in agreement with previous reports that this sequence is generally located 15–25 nucleotides upstream from the polyadenylylation site (49).

The two clones carrying cDNA inserts of 1.9 kb were partially sequenced and appear to be identical. These clones are also identical to the 3.0-kb cDNA except that they are truncated and lack much of the 3′ untranslated region (i.e., they lack the region 3′ from nucleotide 1960). Blot hybridization analysis of electrophoretically fractionated total RNA prepared from the human fibrosarcoma cell line HT-1080, using the 3.0-kb cDNA as probe, indicated the presence of two distinct transcripts, 3.0 and 2.2 kb long (Fig. 4). This observation suggests that the 1.9-kb cDNAs may have been copied from the shorter RNA transcript. A similar size heterogeneity at the 3′ termini of mRNAs has been observed in other systems. It may result from expression of more than one gene, from alternative splicing events, or from the use of multiple polyadenylylation signals (50–52). The mechanism in this case is not clear. Although the only polyadenylylation consensus signal (AATAAA) found in this cDNA sequence is at the 3′ end of the 3.0-kb cDNA (Fig. 3), a similar but slightly

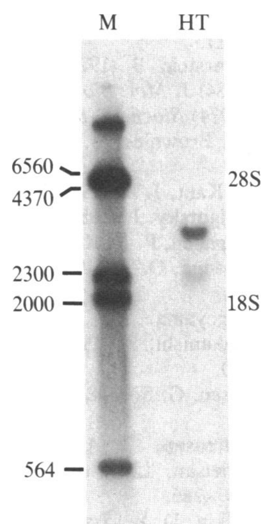

FIG. 4. Detection of β-PAI mRNA. Lane HΓ: total RNA (10 μg) from the fibrosarcoma cell line HT-1080 was isolated and subjected to agarose gel electrophoresis in the presence of formaldehyde and, after blotting to nitrocellulose, was hybridized to ^{32}P-labeled λ3 cDNA. The autoradiogram is shown. Lane M: markers (*Hin*dIII-digested λ DNA; lengths in bp at left). The mobilities of the eukaryotic 28S and 18S ribosomal RNAs are indicated at right.

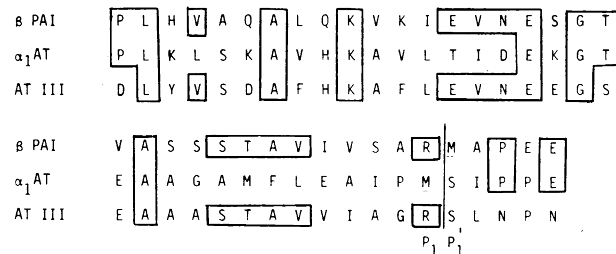

FIG. 5. Comparison of β-PAI, α_1-antitrypsin (α_1AT), and antithrombin III (AT III). Sequences around the reactive centers were aligned according to the fast protein homology program (43). The reactive-site peptide bonds are indicated by the vertical line, and the terminology of the P_1-P_1' reactive-site residues is adopted from Travis and Salvesen (54). The reactive-site methionines are underlined, and homologous residues are boxed. Standard one-letter amino acid abbreviations are used.

modified sequence (AATAAT) was found at positions 1998–2003. If this sequence were used as a signal for poly(A) addition, it could explain the presence of the shorter transcript. In other systems, polyadenylylation has been found to take place in the absence of the AATAAA sequence (53).

Comparison of the deduced amino acid sequence with other proteins, using the fast protein analysis homology program (43), revealed that the β-PAI is 25–30% homologous with antithrombin III, α_1-antitrypsin, α_1-antichymotrypsinogen, and ovalbumin (data not shown) and therefore is a member of the serine proteinase inhibitor superfamily of proteins (serpins; refs. 23 and 24). The serpins have diverged from an ancestral molecule over a 500-million-year period (24) and now represent a diverse group of related proteins that control the major proteolytic cascades of the body (e.g., the coagulation, complement, fibrinolytic, and inflammatory cascades; ref. 24).

The inhibitory specificity of the serpins appears to be defined primarily by a single amino acid in the reactive center, the P_1 residue (24). In general, this amino acid reflects the known specificity of the target proteinase. The reactive center of the serpins is located near the COOH terminus and, because it appears to protrude from the rest of the molecule (24), may represent the ideal substrate or "bait" for the proteinase. Inhibition is associated with the formation of 1:1 complexes between inhibitor and enzyme. The amino acid sequences of the reactive centers of β-PAI, α_1-antitrypsin, and antithrombin III are aligned in Fig. 5. In this alignment, the arginine at position 346 is the P_1 residue of the PAI. Plasminogen activators convert plasminogen into plasmin by cleavage of a single Arg-Val bond (1). Thus, this alignment is consistent with the known arginine-specificity of PAs. The finding that the P_{17} residue is glutamic acid also supports this alignment, since this glutamic acid acts as the "hinge" in serpins and is conserved in all serpins sequenced to date (24). The discussion about the reactive site of β-PAI will remain somewhat speculative until the P_1 and P_1' residues are actually isolated and identified.

The β-PAI is unusually sensitive to oxidants and rapidly loses its activity in the presence of low concentrations of chloramine-T (22). Since there are no cysteines in the deduced protein sequence (Fig. 3), and since the activity of the oxidatively inactivated β-PAI can be restored by treatment with methionine sulfoxide peptide reductase, the loss of activity may reflect the oxidation of a critical methionine (22). The methionine in the reactive center of β-PAI (i.e., at position 347, the inferred P_1' position) is a likely candidate, since α_1-antitrypsin also is sensitive to oxidation and its loss of activity has been related to the oxidation of the P_1 methionine (24). In both cases, the resulting methionine sulfoxide is a bulkier residue and may not readily fit into the pocket of its substrate proteinase.

It has been suggested that the ability to selectively inactivate α_1-antitrypsin by oxidation of its active-site methionine is an important and unique regulatory feature of this system. Activated neutrophils may neutralize the inhibitor by the secretion of oxygen free radicals (24) at inflammatory sites. This additional level of regulation may provide the means by which essential tissue breakdown can take place, even in the presence of inhibitors that normally inhibit neutrophil elastase. Elevated PA activity has been correlated with tissue destruction, tissue remodeling, and the formation of new organs (for review, see ref. 55). The ability to oxidatively inactivate the β-PAI present in these tissues may also be an important regulatory feature of these systems, enabling PAs to function in the presence of their inhibitor. Thus, the local generation of oxidants may inactivate both α_1-antitrypsin and β-PAI and in the process unleash a cascade of proteolytic enzymes including elastase, plasmin, and collagenase (56).

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