Volume 14 Number 20 1986 Nucleic Acids Research

Isolation and sequence of a human gene encoding a potent inhibitor of leukocyte proteases

Gary Stetler, Michael T.Brewer and Robert C.Thompson

Synergen, Inc., 1885 33rd Street, Boulder, CO 80301, USA

Received 14 July 1986; Revised and Accepted 23 September 1986

ABSTRACT

We report the isolation of the human gene encoding an inhibitor of neutrophil elastase and cathepsin G. We have sequenced the gene and a cDNA clone isolated from human parotid tissue. The protein encoded by this gene appears to contain two functional domains, one having a trypsin inhibitory site and the other an elastase inhibitory site. The two-domain structure of the protein is reflected in the organization of the gene, with each domain represented by a separate exon. We have also noted that the intervening sequence separating the trypsin-inhibitor-exon and the elastase-inhibitor-exon is flanked by eleven base-pair direct repeats, suggesting that this intron may have been generated by a transposition-type event.

INTRODUCTION

The pathologies of several chronic and acute diseases of the respiratory tract involve an imbalance between the proteases of cells involved in inflammatory responses (primarily neutrophils) and the inhibitors of these proteases. The paradigm for diseases of this nature is emphysema, in which a major factor in the development of the disorder is the presence of mutations in the gene encoding alpha-1-antiprotease and the concomitant lower levels of active circulating alpha-1-antiprotease (1,2). The inflammation-mediated release of neutrophil elastase in the lungs of patients whose levels of active alpha-1-antiprotease are compromised by genetic background, cigarette smoking, air pollutants, or a combination of all three can result in severe lung damage and a decreased lifespan. An effective treatment for this disease does not yet exist. However, one treatment that is currently under study attempts to increase the basal levels of alpha-1-antiprotease by the administration of human alpha-1-antiprotease purified from blood or produced in microorganisms by recombinant DNA technology (3). Thompson and Ohlsson have recently reported the purification and complete primary structure of another human

inhibitor of leukocyte elastase and cathepsin G. This inhibitor was purified from human parotid secretions and was named SLPI for: secretory leukocyte protease inhibitor (4). SLPI is a non-glycosylated cysteine-rich polypeptide of 107 amino acids. The relatively small size of SLPI, its lack of glycosylation and its stability make this protein a candidate for use as a therapeutic agent in diseases mediated by leukocyte elastase-antielastase imbalances.

Using oligonucleotide hybridization probes based on the protein sequence, we have cloned and determined the nucleotide sequence of the human SLPI gene and a SLPI cDNA clone isolated from a parotid gland cDNA library. The organization of the SLPI gene reveals a close correspondence of exon boundaries with the three functional domains of the protein: secretion signal, trypsin inhibitory domain, and elastase inhibitory domain.

MATERIALS AND METHODS

Preparation of Oligonucleotide Probes and Hybridization Conditions

Each oligonucleotide probe (figure 1) was synthesized on an Applied Biosystems DNA synthesizer and consists of a pool of all possible DNA sequences for a given amino acid sequence. Probe 5 contains 512 sequences; probes 29 and 78 each contain 256 sequences. The hybridization temperatures were chosen to be 2°C below the calculated T_m of the most AT-rich member of each pool (5). The hybridization buffer contained 1.0 M NaCl, 0.10 M sodium citrate, 2x Denhardt's solution (6), 0.1% SDS, 0.05% sodium pyrophosphate, and 0.1 mg/ml yeast tRNA. Filters were hybridized for 12-16 hours with 0.2 pM/ml of oligonucleotide that had been labeled with ^{32}P ATP (Amersham) and T4 polynucleotide kinase (Pharmacia) to a specific activity of $6\text{-}8 \times 10^6$ cpm/pM. After hybridization, the filters were washed at ambient temperature for 45 minutes with three changes of 1 M NaCl, 0.1 M sodium citrate, and 0.1% SDS. A final 8-10 minute wash was done at the calculated T_m for the most AT-rich member of each set of probes (i.e., 2°C above the hybridization temperature). These conditions gave a high degree of specificity and autoradiographic signals that were readily visible in 16-24 hours.

DNA Sequencing

The sequence of the genomic clone and the cDNA clone was determined by the method of Sanger and Coulson (7). Both strands

of the genomic and cDNA clones were sequenced utilizing subcloned fragments in the sequencing vectors Mp18 and Mp19 (8) with the standard sequencing primers (Pharmacia). As sequence data were obtained, we routinely synthesized new oligonucleotide primers to complete the sequence with a minimum of subcloning. The sequence beginning at nucleotide 1 and ending at the EcoRI site of the genomic clone (figure 2) was obtained using the lambda genomic clone as template and oligonucleotides derived from the SLPI sequence as primers.

The cDNA clone was sequenced in both orientations using the universal primers and two pairs of primers synthesized to match residues 100-122 (both orientations) and 265-279 (also both orientations) (figure 3).

Construction of a Parotid cDNA Library

Messenger RNA was purified from human parotid glands essentially as described by Chirgwin et al. (9) and poly-A containing mRNA purified by chromatography on oligo-dT cellulose (10). Using 5 ug of poly-A mRNA, we synthesized six ug of double-stranded DNA with the Amersham cDNA synthesis kit. EcoRI sites in the cDNA were protected by methylation with EcoRI methylase (New England Biolabs) and EcoRI linkers (New England Biolabs) attached with T4 DNA ligase (New England Biolabs). Excess linkers were removed by digestion with EcoRI and chromatography on a 1 ml C16B spin column (11). Eighty ng of cDNA was ligated to EcoRI digested and alkaline phosphatase-treated lambda gt10 and packaged in vitro (Vector Cloning Systems). The packaged cDNA yielded 7.7×10^5 recombinant phage when titered on E. coli C600 hflA. We screened this library with a ^{32}P-labeled (12) 3.8 kb EcoRI fragment from the genomic clone. Plaque lifts and hybridization conditions were as described by Benton and Davis (13).

Electrophoretic Analysis of Nucleic Acids

RNA isolated from tissue culture cells (10,11) or from human tissues (9,10) was denatured and analyzed by electrophoresis in agarose gels containing 6% formaldehyde (14). After electrophoresis, RNA was transferred to Zetaprobe membranes (BioRad) in 0.33 M NaCl, 0.03 M sodium citrate, and hybridized (13) to a ^{32}P-labeled (12), 3.8 kb EcoRI fragment containing the SLPI genomic clone.

High-molecular-weight human DNA, prepared from whole blood, was digested with the indicated restriction endonucleases under

conditions suggested by the supplier, electrophoresed in a 0.65% agarose gel, and transferred to nitrocellulose (11) and hybridized to the SLPI cDNA clone as described above for the RNA blots.

RESULTS

Isolation of a Genomic SLPI Clone

Based on the protein sequence of SLPI (4), we synthesized three degenerate oligonucleotide hybridization probes representing amino acid residues 5-11, 29-36, and 78-84. Initially, these probes were used to screen 10^7 members of the well-characterized human genomic library constructed by Lawn et al. (15). In this screen of more than ten human genome equivalents, we were unable to isolate a clone corresponding to SLPI protein sequence. It has become apparent that certain kinds of DNA sequences are not stably maintained by many of the common lambda-phage-based cloning vectors when they are propagated on specific E. coli host strains. Palindromic sequences in particular seem to be unstable in recBC+ host strains and either are rapidly lost or undergo extensive rearrangements (16). For this reason, we obtained a library constructed by Wyman in lambda Charon 30 (Ch30) (17). This library was constructed by cloning size-selected, partial Sau3A1 fragments into the BamHI site of Ch30. At the time we used this library, it had undergone a single amplification on the recBC sbc host, E. coli CES200 (A. Wyman, personal communication). We screened $8x10^5$ members of this library on duplicate nitrocellulose filters with probe 29 as described in Methods and Materials. Thirteen phage were identified that reproducibly hybridized to this probe; of these 13, six also hybridized to probes 5 and 78 (figure 1). The phage were plaque-purified and DNA prepared (18) from two of the six clones. Both phage contained a 3.8 kb EcoRI fragment that hybridized to all three oligonucleotide probes. The sequence presented in figure 2 was derived by utilizing the restriction sites indicated in this figure to subclone fragments into the sequencing vectors Mp18 and Mp19. None of these isolates was capable of growth on E. coli AB1157 (recBC+, sbc+). The inability of these clones to grow on AB1157 suggests the reason for the absence of these clones from Lawn's library, which was amplified on a recBC+ sbc+ host.

Sequence of the Human Genomic SLPI Clone

The sequence of the genomic clone revealed two major exons that

Probe	Amino Acid[1] Residues	Sequence[2]	Hybridization Temperature
5	5-11	5'-GG$^{A}_{G}$CANACNCCNGC$^{C}_{T}$TT$^{A}_{G}$AA-3'	54°C
29	29-36	5'-TT$^{C}_{T}$TTNCCNGG$^{A}_{G}$CA$^{C}_{T}$TGCCA$^{A}_{G}$TC-3'	62°C
78	78-86	5'-CA$^{C}_{T}$TGNCC$^{A}_{G}$TCCAT$^{C}_{T}$TC$^{A}_{G}$CA$^{A}_{G}$AA$^{A}_{G}$TT-3'	66°C

Figure 1. Hybridization Probes
[1]Refers to amino acid residues in the mature polypeptide represented by the probe, numbered from the N-terminus.
[2]N, G, A, T, or C.

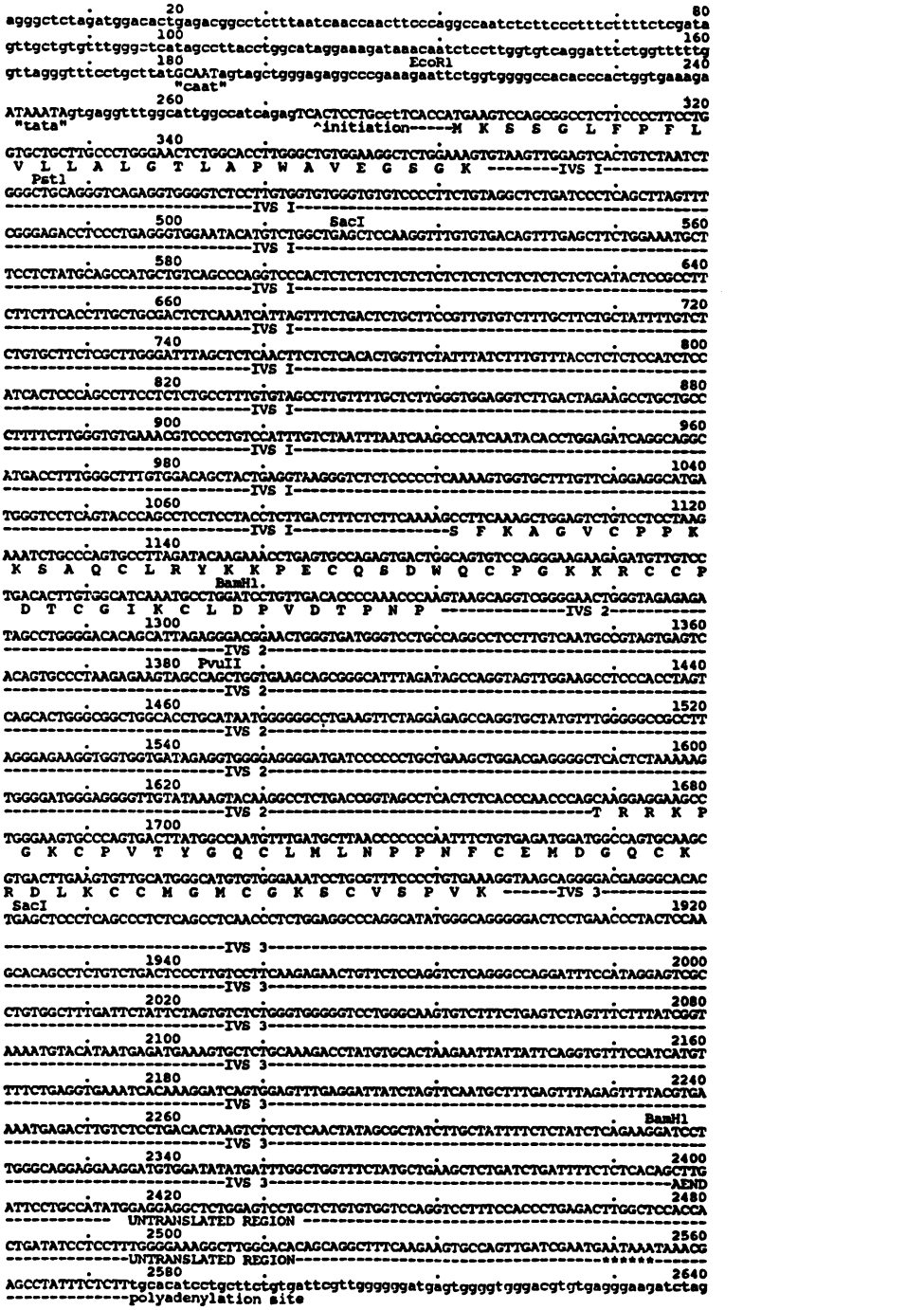
encode amino acids 4-54 and 55-106 (exons II and III) of the mature SLPI protein. The isolation of a cDNA clone was essential to determine the complete sequence of the SLPI gene and to confirm that our assignments of exon/intron junctions were correct. To this end, we used the 3.8 kb EcoRI fragment (probe 3.8) of the genomic SLPI clone as a hybridization probe to identify a tissue containing SLPI mRNA. Because SLPI represents about 0.1% of the protein secreted by the parotid (4), this gland seemed a likely source of SLPI mRNA, and, indeed, a northern blot of mRNA isolated from this tissue, hybridized with probe 3.8, confirmed the presence of SLPI mRNA in the parotid. SLPI mRNA was not detectable in RNA isolated from pancreas, fibroblasts, or hepatoma cells (figure 3).

Isolation of a SLPI cDNA Clone

We constructed a cDNA library using mRNA isolated from human parotid glands as described in Methods and Materials. We screened $8x10^5$ members of this library with probe 3.8 and isolated five clones containing DNA homologous to the genomic clone. Two of the five cDNA clones also hybridized to an oligonucleotide derived from sequences near the 5' end of exon II, indicating that they contained full-length or nearly full-length cDNA.

DISCUSSION

A comparison of the cDNA and genomic sequences confirmed the boundaries of exons II and III and defined the locations of exons I and IV. The sequence of the cDNA clone also revealed that the primary translation product of SLPI mRNA is most probably 132 amino


"caat"
"tata"
^initiation-----M K S S G L F P F L
V L L A L G T L A P W A V E G S G K --------IVS 1----------
PstI
SacI
BamH1
PvuII
EcoR1
AEND
UNTRANSLATED REGION
polyadenylation site

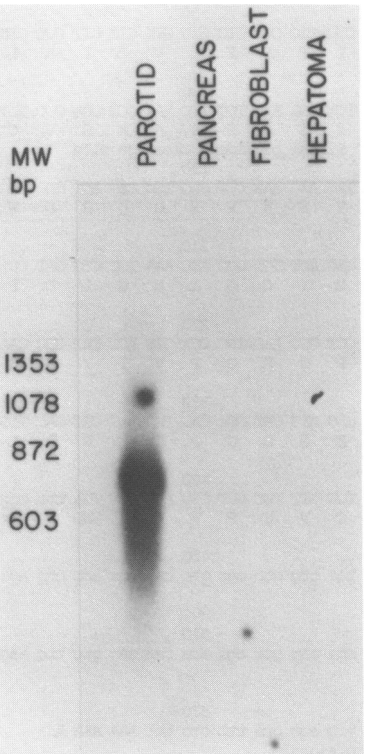


Figure 3. Parotid specific expression of SLPI.
PolyA$^+$ RNA (10 ug/lane) from human parotid, pancreas, lung fibroblasts, and the SK-hepatoma cell line were hybridized to a 3.8 kb EcoRI fragment of the genomic SLPI clone as described in Materials and Methods. The autoradiograph was made by a 72-hour exposure of the filter at -70°C with an intensifying screen.

acids long with a 25 amino acid secretory signal sequence. We believe that the cDNA sequence presented in figure 4 includes the complete protein coding region of the SLPI gene for the following reasons: first, the sequence adjacent to and including the N-terminal methionine codon in the genomic sequence of SLPI agrees with the consensus sequence derived for translation initiation signals ($CC\frac{A}{G}CCAUGG$) (19). Second, no methionine codons are found between a pair of upstream termination codons and the proposed translational

Figure 2. DNA sequence of the human SLPI gene. ^ initiation, putative transcription start, ***, consensus polyadenylation signal; IVS, intron sequences.**

```
1                                                          30                                                          60
ATG AAG TCC AGC GGC CTC TTC CCC TTC CTG GTG CTG CTT GCC CTG GGA ACT CTG GCA CCT
 M   K   S   S   G   L   F   P   F   L   V   L   L   A   L   G   T   L   A   P

                                                           90                                                         120
TGG GCT GTG GAA GGC TCT GGA AAG TCC TTC AAA GCT GGA GTC TGT CCT CCT AAG AAA TCT
 W   A   V   E   G   S   G   K   S   F   K   A   G   V   C   P   P   K   K   S
                   ^ Signal peptidase cleavage site
                                                          150                                                         180
GCC CAG TGC CTT AGA TAC AAG AAA CCT GAG TGC CAG AGT GAC TGG CAG TGT CCA GGG AAG
 A   Q   C   L   R   Y   K   K   P   E   C   Q   S   D   W   Q   C   P   G   K

                                                          210                                                         240
AAG AGA TGT TGT CCT GAC ACT TGT GGC ATC AAA TGC CTG GAT CCT GTT GAC ACC CCA AAC
 K   R   C   C   P   D   T   C   G   I   K   C   L   D   P   V   D   T   P   N

                                                          270                                                         300
CCA ACA AGG AGG AAG CCT GGG AAG TGC CCA GTG ACT TAT GGC CAA TGT TTG ATG CTT AAC
 P   T   R   R   K   P   G   K   C   P   V   T   Y   G   Q   C   L   M   L   N

                                                          330                                                         360
CCC CCC AAT TTC TGT GAG ATG GAT GGC CAG TGC AAG CGT GAC TTG AAG TGT TGC ATG GGC
 P   P   N   F   C   E   M   D   G   Q   C   K   R   D   L   K   C   C   M   G

                                                          390                                                         420
ATG TGT GGG AAA TCC TGC GTT TCC CCT GTG AAA GCT TGA ttc ctg cca tat gga gga ggc
 M   C   G   K   S   C   V   S   P   V   K   A  END

                                                          450                                                         480
tct gga gtc ctg ctc tgt gtg gtc cag gtc ctt tcc acc ctg aga ctt ggc tcc acc act

                                                          510                                                         540
gat atc ctc ctt tgg gga aag gca agc aca cag cag gtc ttc aag aag tgc cag ttg atc

                                                          570
gaa tgT AAT AAA taa acg agc cta ttt ctc ttt AAA AAA A
     polyadenylation signal
```

Figure 4. Sequence of the parotid SLPI cDNA clone.

initiation site, nor is there a potential intron/exon junction in this region (20). Third, the secretion signal sequence follows the established pattern for eukaryotic signal sequences. A charged amino acid (Lys) follows the initiator methionine; there is a hydrophobic core and a proline residue located 6 amino acids upstream of the putative processing site. Comparison of the SLPI (4) protein sequence with the DNA sequence suggests that the signal peptidase removes the leader peptide by cleaving at a gly-ser bond. The gly-ser cleavage site is relatively rare, however (21,22), and a second, more commonly processed site (ala-val) is found just upstream of the gly-ser pair. Our data do not allow us to rule out the possibility that the signal peptidase cleaves the ala-val bond and that additional processing occurs to generate the amino terminus of the mature protein.

SLPI is Encoded by a Single Gene

Genomic DNA blots hybridized with the SLPI cDNA clone (figure 5)

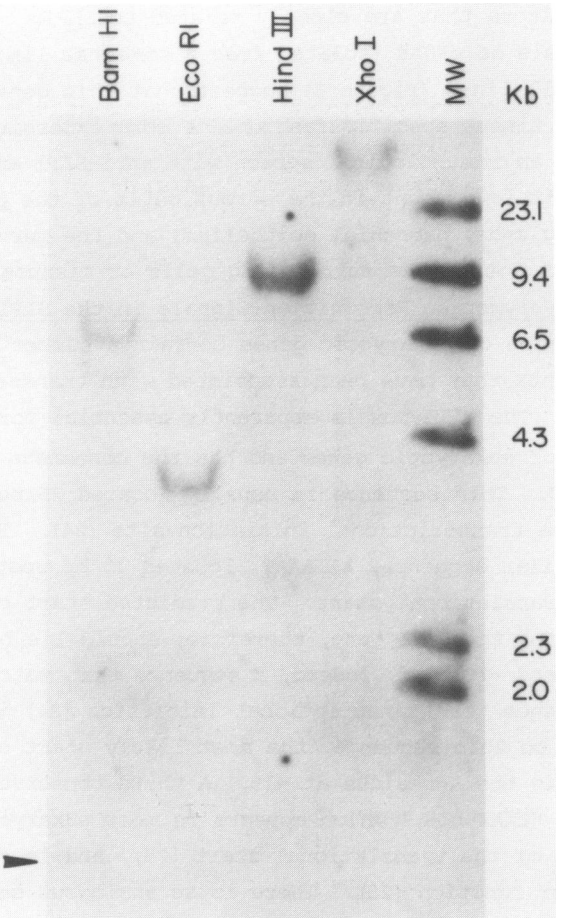


Figure 5. Hybridization of human genomic DNA to the SLPI cDNA clone. Human DNA (10 ug/lane) isolated from a single individual was digested with the enzymes indicated and hybridized to the complete SLPI cDNA clone (Materials and Methods). The arrow indicates the position of a third BamHI fragment which could be seen on the original autoradiograph.

show the hybridization patterns expected for a single gene. The genomic sequence data show that the coding region of SLPI is contained within a single 3.8 kb EcoRI fragment, which contains two BamHI sites and does not have internal XhoI sites or HindIII sites (the HindIII site in the cDNA clone is generated by mRNA splicing). In addition to showing that SLPI is encoded by a single gene, these results indicate that the human genome does not code for a family of

protease inhibitors that are closely related to SLPI.

Our analysis of mRNAs isolated from a somewhat limited number of tissues and cell lines (figure 3) suggest that this gene is expressed in a tissue specific fashion. A more extensive survey of tissues, using an immunological screen with anti-SLPI antibody has shown antigen to be present in the serous cells of the parotid, submandibular glands, bronchial epithelium, and the cervical epithelium, but not in the surrounding cells or tissues (23).

Potential RNA Polymerase Recognition Signals in the SLPI Gene

The 5' region of eukaryotic genes contain a number of conserved sequence elements that have been associated with transcription by RNA polymerase II. The TATA-box is apparently essential for transcription of eukaryotic genes and has the consensus sequence: 5'- TATA$\begin{smallmatrix}A\\T\end{smallmatrix}A\begin{smallmatrix}T\\A\end{smallmatrix}$ -3'. This sequence is usually located 25 to 32 bp upstream of the transcriptional initiation site (24). The SLPI gene contains a similar sequence, AATAAAT, located 43 bp upstream (-43) of the proposed translational start. The predicted start of transcription for the SLPI gene, therefore, should lie between positions -11 and -18, and, indeed, a sequence that matches the consensus sequence for transcriptional initiation (24) is found at -7 to -18. Based on this sequence, the most likely start of transcription is the A-residue at -15. A third conserved sequence element is the "CAAT-box," which appears in most eukaryotic genes 60-80 bp upstream of the translational start (25), and is essential for normal promoter function (26). There is an analogous sequence in the 5' region of the SLPI gene located 82 bp upstream of the proposed transcriptional initiation site.

The Exon-intron Organization Reflects the Structural and Functional Domains of the Inhibitor

The protein sequence data of Thompson and Ohlsson (4) and of Seemuller et al. (27) suggest a two-domain structure for SLPI. The N-terminal domain (I) consisting of amino acids 1-58 and containing 8 cysteines most probably is the trypsin inhibitory region. The C-terminal, elastase inhibitory domain includes residues 59-107 and, like domain I, contains 8 cysteines. The spatial organization of the cysteines in each domain is nearly identical. If one includes the secretion signal sequence, SLPI contains three structural-functional domains. The organization of the exons and introns in the SLPI gene

precisely reflects the domain structure of the protein (figure 6). Exon I contains the signal sequence and the first three amino acids of the mature polypeptide. Exon II contains coding information for the trypsin inhibitor domain (residues 4-56). The third domain, corresponding to the elastase inhibitory region, is contained within exon III. The fourth exon departs from this pattern and contains only two nucleotides of coding information, the last two nucleotides of the final alanine codon. Also included within exon IV are the 3'-untranslated region and the polyadenylation recognition signal. The correlation of protein structural and functional regions with discrete exons has become a consistent theme in the organization of eukaryotic genes (28,29). This had led to the speculation that exons represent the coding information for ancestral polypeptides that have, in some cases, been duplicated or recombined to generate new proteins with new functions (30,31). Although the spatial arrangement and the number of cysteines in the two domains of SLPI are highly conserved, the overall amino acid sequence homology between the two is relatively low (35%). This suggests that if the two domains were generated by a gene duplication, the duplication occurred long enough ago to allow considerable divergence in amino acid sequence, with selective pressure to maintain the number and arrangement of the cysteine residues, and presumably the disulfide bonds formed by these cysteines. A second possibility is that the two exons represent the joining of two ancestral genes whose apparent conservation of cysteine arrangement is a consequence of convergent evolution.

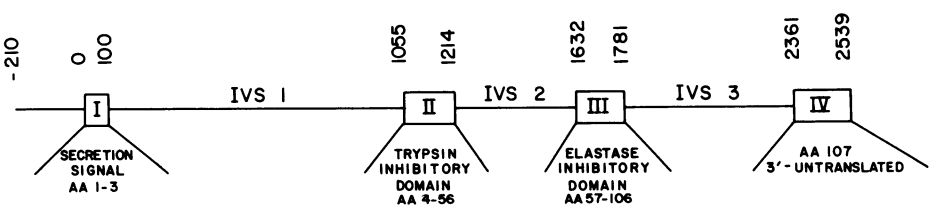


Figure 6. Correlation of the functional domains of the SLPI protein with the organization of the SLPI gene.

The top line shows the distance in base pairs from the proposed transcriptional initiation site. Each of the exons is contained within the boxed regions. IVS, intervening sequence; AA refers to the amino acid positions in the mature protein. The precise length of the gene 5' to the start of the protein coding region is, as yet, undetermined.

Two other proteins have been described that share some features with SLPI. One is a protease inhibitor isolated from red sea turtles and the other a phosphoprotein isolated from rat milk (32). Both of these proteins contain two structural domains. The turtle protein contains one domain that resembles the Kazal class of protease inhibitors (33) and a second domain that contains 8 cysteines and inhibits subtilisin. The rat protein contains two domains with eight cysteine residues each and has no known protease inhibitor activity. The two domains of SLPI, both domains of the rat milk protein, and the subtilisin inhibitory domain of the turtle protein share some protein sequence homology, with precise conservation of six cysteine residues and about 40% homology over the region defined by the cysteine residues. The conservation of the number and position of the cysteine residues in these proteins contrasts with the relatively low degree of overall sequence homology, and implies that the location of the cysteines confers a favored structure that is independent of function. In this regard, we note that SLPI is very resistant to proteolysis and to chemical or thermal denaturation.

Intron Generation by Transposition?

An interesting feature of the SLPI gene is the presence of a short, direct repeat that occurs at the 3' end of exon II and at the 3' end of IVSII. This creates an 11 bp imperfect repeat:

5' - CCCCAAACCCA - 3' (exon)

5' - CACCCAACCCA - 3' (intron)

This sort of direct repeat is a common feature of the recombinational events involved in insertion of transposable elements in prokaryotes and in eukaryotes (34) and with events involved in the generation of pseudogenes (35). If IVSII was generated by a transposition, the target sequence would have been within the sequences encoding the last three amino acids of the trypsin inhibitory domain which may have been near the 3' end of the "proto-SLPI" gene. If the putative transposon were a member of a repeated sequence family, such an event would generate a sequence adjacent to the ancestral SLPI gene with homology to other regions in the ancestral genome. Subsequent recombination could have led to the formation of exon III from a preexisting, convergently evolved protease inhibitor. Alternatively, the proposed transpositional event could have occurred after an original gene duplication event and could represent a relatively

recent insertion. The latter hypothesis would explain the high degree of conservation within the direct repeats relative to the homology between exons II and III of the gene.

ACKNOWLEDGMENTS

We thank Tom Gleason for synthesis and purification of oligonucleotides; Sandra Martin, Steve Eisenberg, and Mike McDonell for critical reviews of the manuscript; and Arlene Wyman for the human genomic library.

Abbreviations: cDNA, complementary DNA synthesized from messenger RNA with reverse transcriptase; pM, 10^{-12} M; cpm, counts per minute.

REFERENCES

1. Carrell, R.W. (1982) Nature (London) 298, 329-333.
2. Travis, J. and Salvesen, G.S. (1983) Ann. Rev. Biochem. 52, 655-709.
3. Glaser, C. (1983) Ann. Rev. Respir. Disease 127, S47-53.
4. Thompson, R. and Ohlsson, K. (1986) Proc. Natl. Acad. Sci (USA), in press.
5. Suggs, S.V. (1981) In Brown, D.D. and Fox, C.F. (eds), Developmental Biology Using Purified Genes, Academic Press, New York, pp. 683-693.
6. Denhardt, D.T. (1966) Biochem. Biophys. Res. Commun. 23, 641-646.
7. Sanger, F. and Coulson, A.R. (1975) J. Mol. Biol. 94, 441-448.
8. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Gene 33, 103-119.
9. Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979) Biochemistry 18, 5294-5299.
10. Aviv, H. and Leder, P. (1972) Proc. Natl. Acad. Sci. (USA) 69, 1408-1412.
11. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular Cloning, Cold Spring Harbor Laboratory, New York, pp. 466-467.
12. Rigby, P.W.J., Dieckmann, M., Rhodes, C., and Berg, P. (1977) J. Mol. Biol. 113, 237-247.
13. Benton, W.D. and Davis, R.W. (1977) Science 196, 180-182.
14. Jensen, R., Sprague, G.F., Jr., and Herskowitz, I. (1983) Proc. Natl. Acad. Sci (USA) 80, 3035-3039.
15. Lawn, R.M., Fritsch, E.F., Parker, R.C., Blake, G., and Maniatis, T. (1978) Cell 15, 1157-1174.
16. Wyman, A.R., Wolfe, L.B., and Botstein, D. (1985) Proc. Nat. Acad. Sci. (USA) 82, 2880-2884.
17. Rimm, D.L., Horness, D., Kucera, J., and Blattner, F.R. (1980) Gene 12, 301-309.
18. Davis, R.W., Botstein, D., and Roth, J.R. (1980) Advanced Bacterial Genetics: A Manual for Genetic Engineering, Cold Spring Harbor Laboratory, New York.
19. Kozak, M. (1984) Nucleic Acids Res. 12, 857-870.

20. Breathnach, R. and Chambon, P. (1981) Ann. Rev. Biochem. 50, 349-383.
21. Watson, M.E.E. (1984) Nucleic Acids Res. 12, 5145-5165.
22. Von Heijne, G. (1983) Eur. J. Biochem. 133, 17-21.
23. Casslen, B., Rosengren, M., and Ohlsson, K. (1981) Hoppe-Seyler's Z. Physiol. Chem. 362, 953-961.
24. Corden, J. (1980) Science 209, 1406-1414.
25. Benoist, C., O'Hare, K., Breathnach, R., and Chambon, P. (1980) Nucleic Acids Res. 8, 127-142.
26. Graves, B.J., Johnson, P.F., and McKnight, S.L. (1986) Cell 44, 565-576.
27. Seemuller, U., Arnhold, M., Fritz, H., Wiedenmann, K., Machleidt, W., Heinzel, R., Appelhans, H., Gassen, H.G., and Lottspeich, F. (1986) FEBS Lett. 199, 43-48.
28. Lonberg, N. and Gilbert, W. (1985) Cell 40, 81-90.
29. Ny, T., Elgh, F., and Lund, B. (1984) Proc. Natl. Acad. Sci. (USA) 81, 5355-5359.
30. Lonberg, N. and Gilbert, W. (1985) Cell 40, 81-90.
31. Rogers, J. (1985) Nature 315, 458-459.
32. Dandekar, A.M., Robinson, E.A., Appella, E., Qasba, P. (1982) Proc. Natl. Acad. Sci (USA) 79, 3987-3991.
33. Laskowski, M. and Kato, I. (1980) Ann. Rev. Bioch. 49, 593-626.
34. Calos, M.P. and Miller, J.H. (1980) Cell 20, 579-595.
35. Rogers, J.H. (1985) Internatl. Rev. Cytol. 93, 187-279.