

- W. Jelkmann, A. Pfuhl, K. Malmstrom, C. Bauer, *Endocrinology* **118**, 567 (1986); J. B. Sherwood, E. R. Burns, D. Shouval, *Blood* **69**, 1053 (1987).
7. M. Ueno, J. Brookins, B. S. Beckman, J. W. Fisher, *Biochem. Biophys. Res. Commun.* **154**, 773 (1988).
 8. L. Berk, J. H. Burchenal, W. B. Castle, *N. Engl. J. Med.* **240**, 754 (1949).
 9. E. Goldwasser, L. O. Jacobson, W. Fried, L. F. Plzak, *Blood* **13**, 55 (1958).
 10. J. W. Fisher and J. W. Langston, *Ann. N.Y. Acad. Sci.* **149**, 75 (1968).
 11. A. P. Yastrebov, *Fed. Proc.* **25** T630 (1966).
 12. E. Necas and E. B. Thorling, *Am. J. Physiol.* **222**, 1187 (1972).
 13. G. Jasmin and B. Solymoss, *Proc. Soc. Exp. Biol. Med.* **148**, 774 (1975); E. E. Morse, T.-Y. Lee, R. F. Reiss, F. W. Sunderman, Jr., *Ann. Clin. Lab. Sci.* **7**, 17 (1977).
 14. M. F. Perutz, *Nature* **228**, 726 (1970); H. F. Bunn and B. G. Forget, *Hemoglobin: Molecular, Genetic, and Clinical Aspects* (Saunders, Philadelphia, 1986).
 15. T. Yonetani, H. Yamamoto, G. V. Woodrow III, *J. Biol. Chem.* **249**, 682 (1974).
 16. N. Shibayama, H. Morimoto, G. Miyazaki, *J. Mol. Biol.* **192**, 323 (1986).
 17. M. R. Waterman and T. Yonetani, *J. Biol. Chem.* **245**, 5847 (1970); B. M. Hoffman *et al.*, *Ann. N.Y. Acad. Sci.* **244**, 174 (1975); T. Yonetani and T. Asakura, *J. Biol. Chem.* **244**, 4580 (1969).
 18. F. De Matteis and A. H. Gibbs, *Biochem. J.* **162**, 213 (1977); J. Igarashi, N. Hayashi, G. Kikuchi, *J. Biochem. (Tokyo)* **84**, 997 (1978); P. Sinclair, A. H. Gibbs, J. F. Sinclair, F. De Matteis, *Biochem. J.* **178**, 529 (1979).
 19. P. R. Sinclair, J. F. Sinclair, H. L. Bonkowsky, A. H. Gibbs, F. De Matteis, *Biochem. Pharmacol.* **31**, 993 (1982).
 20. M. A. Goldberg *et al.*, unpublished data.
 21. W. S. Caughey, *Ann. N.Y. Acad. Sci.* **174**, 148 (1970).
 22. R. F. Coburn, *Prev. Med.* **8**, 310 (1979).
 23. S. I. Shedlofsky *et al.*, *Biochem. J.* **248**, 229 (1987).
 24. D. P. Tschudy, R. A. Hess, B. C. Frykholm, *J. Biol. Chem.* **256**, 9915 (1981).
 25. N. Schoenfeld, Y. Greenblat, O. Epstein, A. Atsmon, *Biochim. Biophys. Acta* **721**, 408 (1982).
 26. We thank C. Shoemaker for the human Epo cDNA clone (E49F), a polyclonal rabbit antiserum to human Epo, and advice and encouragement. K. Bridges advised us on the manipulations of intracellular heme synthesis. Mouse β -actin cDNA was a gift from B. Spiegelman. Supported in part by NIH grant DK01401. M.A.G. is the recipient of an NIH Physician Scientist Award.

29 July 1988; accepted 27 September 1988

Human Insulin-Degrading Enzyme Shares Structural and Functional Homologies with *E. coli* Protease III

JOSEPH A. AFFHOLTER, VICTOR A. FRIED, RICHARD A. ROTH*

A proteinase with high affinity for insulin has been proposed to play a role in the cellular processing of this hormone. A complementary DNA (cDNA) coding for this enzyme has been isolated and sequenced. The deduced amino acid sequence of the enzyme contained the sequences of 13 peptides derived from the isolated protein. The cDNA could be transcribed *in vitro* to yield a synthetic RNA that in cell-free translations produced a protein that coelectrophoresed with the native proteinase and could be immunoprecipitated with monoclonal antibodies to this enzyme. The deduced sequence of this proteinase did not contain the consensus sequences for any of the known classes of proteinases (that is, metallo, cysteine, aspartic, or serine), but it did show homology to an *Escherichia coli* proteinase (called protease III), which also cleaves insulin and is present in the periplasmic space. Thus, these two proteins may be members of a family of proteases that are involved in intercellular peptide signaling.

DESPITE EXTENSIVE STUDIES, THE mechanism by which insulin elicits its varied responses remains unknown. Recent data implicate the intrinsic tyrosine kinase activity of the insulin receptor in the initiation of many of the biological responses to insulin (1). However, other proposed pathways include the generation of a novel inositol phosphate glycan (2), effects via Ca^{2+} (3), or possibly a direct effect of insulin on subcellular organelles (4). Also controversial has been the subsequent processing of insulin by cells. Although it is known that the same cells that respond to insulin also degrade the hormone (5), the fate of the internalized insulin has been difficult to determine. For example, several reports have indicated that the internalized insulin is transported directly to lysosomes where it is degraded (6); others indicate that most of the insulin is first cleaved in a

nonlysosomal pathway at several specific sites (6).

One enzyme that has been proposed to play a role in the cellular processing of insulin is a proteinase (called insulin-degrading enzyme or IDE) with high affinity for insulin ($K_d \approx 100$ nM). Although this enzyme was first described over 40 years ago (7), its purification has proven very difficult and considerable uncertainty still exists as to its properties (size, specificity, and metal- and thiol-dependence) (6). Evidence that this proteinase participates in the intracellular degradation of insulin includes the following: (i) inhibitors of the purified proteinase inhibit insulin degradation in cells (8); (ii) the insulin cleavage sites observed *in vitro* with the purified proteinase are also observed in insulin extracted from cells (9); (iii) microinjection of monoclonal antibodies to this proteinase inhibits cellular insulin degradation (10); and

(iv) insulin can be cross-linked in intact cells to this proteinase (11). However, since IDE is primarily cytosolic, it has remained unclear how the enzyme could come in contact with endocytosed insulin.

In the current studies, we have used an affinity column composed of monoclonal antibodies to IDE (10) to purify 200 μg of the enzyme from 12 liters of packed human red blood cells (12). The purified protein, which gave a single band on SDS gels of $M_r \approx 110,000$, was found to have a blocked NH_2 -terminus, a characteristic of many cytosolic proteins. The enzyme was therefore digested with trypsin, the resulting peptides were isolated by high-performance liquid chromatography (HPLC), and the sequences of 13 peptides were determined by automated Edman degradation (12). Six different synthetic oligonucleotides were designed on the basis of five of these peptide sequences and were used to screen various cDNA libraries (12). A partial clone was identified and used to obtain a full-length cDNA from a $\lambda\text{gt}10$ human hepatoma library, and its insert was subcloned and sequenced (Fig. 1).

The sequence of this cDNA clone (3048 bp) contains a single open reading frame (ORF) of 952 amino acids with an initiator methionine codon at nucleotide 0 and a terminator codon at nucleotide 2855 (Fig. 1). The predicted polypeptide encoded by this cDNA has a calculated M_r of 110,453 and contains the sequences of all 13 previously determined tryptic peptides. Codon usage analysis of the ORF reveals a strong preference for the "least common" codons (13) at positions encoding highly hydrophilic amino acids (Glu, Asp, Lys, and Asn), which may decrease the efficiency of translation *in vivo*. This is consistent with the low levels of this protein observed in most cells. Sequence analysis reveals one possible glycosylation site (Asn-X-Thr) at amino acids 732 to 734. A Kyte-Doolittle hydrophobicity plot (14), however, shows no obvious NH_2 -terminal signal peptide sequence, suggesting that the protein is not processed and therefore not glycosylated. Finally, blot analyses of total RNA from human lymphocytes (Fig. 2) and human hepatoma cells (HepG2) reveal message sizes of 3.1 and 3.5 kb; perhaps this is because of differences in the processing of the RNA, but possibly because of different isozymes.

J. A. Affholter and R. A. Roth, Department of Pharmacology, Stanford University School of Medicine, Stanford, CA 94305.

V. A. Fried, Department of Pharmacology, Stanford University School of Medicine, Stanford, CA 94305, and Department of Biochemistry, St. Jude Children's Research Hospital, 332 North Lauderdale, Memphis, TN 38105.

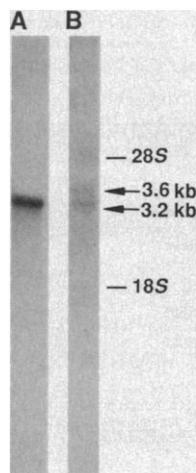
*To whom correspondence should be addressed.

To confirm that the isolated cDNA codes for IDE, we used in vitro transcription and translation systems (Fig. 3). Greater than 98% of the in vitro transcribed RNA is contained in the "full-length" 3.1-kb band that coelectrophoreses with the smaller IDE message on an RNA blot (Fig. 2). In vitro translation of this synthesized RNA gives a major band of $M_r \approx 110,000$ and two smaller bands of $M_r \approx 50,000$ and $60,000$ (Fig. 3B). Monoclonal antibodies to IDE but not control antibodies precipitated the

<p>157 CCGGCTCGAAGCGCAACGAGGAGCTTTGGCGTGATCCCGGCGACTGGCTGGCTA ATGGGGTACCGGCTAGCGTGG 28 H R Y R L A W</p> <p>21 CTCTGCACCCCGGACTGCCAGGACCTTCGGCTCAGTCTCGGCGCCGCGCTCGGCGCTCGGAGCGCTGTGGTTTCAAAAAAG 118# 8 L L H P A L P S T F A S V L G A R L P P P E A R L C G F Q K K 37 M P R S T V F K A L L L L V A L W A P L S Q A E T G V Q . .</p> <p>111 ACTTACAGCAAAAATGAATAATCCAGCCATCAAGAGAATAGGAAATCAGTATACCAAGTCTCTGAAGACAGCGAGAATTCGAGGGCTA 288# 38 T Y S K M N N P A I K R I G N H I T K S P E D K R E Y R G L 67 P I O E T I R K S D K D N R Q V O A I</p> <p>281 GACCTGGCAATGATATAAGATCTCTTATCAAGTATCCACCAAGATAGTATCAGGACCACTTATGTCACATATGTTTCAATG 292# 68 E L C A G D R K F A D F F L C P I F D E S C A A L D V H I G S L 97 R L D R G M V V L L V S D P G A V F S L S A L V V P V G S L</p> <p>291 TCGGATCTCCAAATATTGCTGGCTAAATCATTTTGTGAACATATGCTTTTGTGGAACAAGAAATACCTTAAAGAAAATGAATAC 389# 96 S D P P N I A G L S H F C E H M L P L S T K K Y P K E N E Y 127 E G P E A Y Q C L A H Y L E H M S L M G S K K Y P Q A O S L</p> <p>281 AGCCAGTTTCTCAGTACGATCGCAAGTTCAAATGCCTTTACTAGTGGAGCAGCATACTAATCTATTTGATGTTTCTCATGAACG 478# 128 S D F L S E H A G S S N A F T S G E H T N Y Y F D V S H E H 157 A E V L K M H G G S H N A S T A P Y R T A F V L E V E N D A</p> <p>471 CTAGAAGTCCCTAGACAGGTTTGCAGAGTTTCTGTCGCCCTTGTTCGATGAAGTTCGAAAGCAGACAGGTTGAAATCGACTGAT 568# 158 L E G A L D R F A D F F L C P I F D E S C A A L D V H I G S L 187 L P G A V D R L A D A I A E P L L D K K Y A E R E R N A V N</p> <p>561 TCAGAACATGAGAAGATGTGATGAATGATCCCTGGAGACTCTTCAATTCGAAAGAACTACAGGAACTTAAACACCCCTTCATGAA 658# 188 S E H E K N V M N D A V R L F L E K A T G M T K H P T S K 217 A E L T M A R T R D G M R M A O V S A E T I N F A N P G S K</p> <p>651 TTTGGACAGGTAACAATATACTCTGGAGACTAGCCAAAGCAAGGCACTTATGAAACAAGGACTACTGAAATTCATCTGCT 748# 218 T G T G K K Y T L E T A P R G E G I D V R D E L L K F H S A 247 T S G R K . . . L E T L S D K P G H P V O D A L K D F R E K</p> <p>741 TACTATATCCAAATTAAGTCTGTTTGTGTTTGTAGTGGAGACTCTTATGATGACTTACTGCTGCTGGTAAAGTATTTTCTGAA 838# 246 Y Y S A N L M K A V I Y S N K P L P E L A K M A A D T V G R 277</p> <p>931 GTAGAGAACAATAATGTCATGCGAATTCCTGAACACCCCTTCCAAAGAAACAATCTTAAACAATTTCAAAAATAGTACCCATT 928# 278 V E N K K N V P L P E F P E H P F Q E E H L K O L Y K I V P I 387 V P N K E S K K P E I T V P V V T D A O K G H Y H S L R P W</p> <p>921 AAAGATATTAGCAATCTCTATGTGACATTTCCATACCTGACCTTCAGAAATACACAATCAAACTGCTGATCATCTTGGTCACTC 1818# 388 K D I R N L Y V T F P I P D L D K Y Y K S N P G H Y L G H L 337 L P R K V L R V E F R I D N N S A K F R S K T D E L I T Y L</p> <p>1811 ATTGGCATGAGGCTCTGGAAGTCTGTTATCAGAACTTAAGTCAAGGCTGGGTTAATCTCTGTTGGTGGGCAAGGAAGGAGCC 1188# 336 I G H E G P G S L L S E L K S G V N R T L V G G D K E G A 367 I G N R S P G T L S D W L O K Q G L V E G I S A N S D P I V</p> <p>1181 CGAGGTTTATGTTTTTATCATTAAATGTCGACTGACCGAGGAAGGATATTACATGTTGAAGATATAATTTGCACATGTTCAATAC 1198# 360 R G . F M F F I I N V D L T E E G L L H V E D I I L H M F Q Y 397 N G N S G V L A I S A S L T D K G L A N R D Q V V A A I F S Y</p> <p>1191 ATTCAAGCTTACGTCAGAAAGGACTCAAGATGGGTTTCCAAAGTCCAGGACTTGAATCTGCTTCTTATGCTTTAAAGACAAA 1288# 398 I O K L R A E G P O E W V F Q E C K O L N A V A F R F K D K 427 L N L L R E K G I D K O Y F D E L A N V L D I D F R Y P S I</p> <p>1281 GAGAGCCACCGGCTATACATCTAAGATTCAGGAATATGCAATATTTCCCTAGAAGAGGCTGCTCAGCGGAATATTTACTGAA 1378# 428 E R P R G Y T S K I A G I L H Y V L E E V L T A E Y L L E 457 T R D M D V Y V E V L A D T M I R V V P V E H T L D A V N I A D</p> <p>1371 AAATTAGACTGACTTAATAGAGATGGTCTCGATAAATCAGACCGAAGAAATGTCGGTTCGCCATGTTTCTAAATCTTTTGAAGGA 1468# 450 E F R P D L I E H V L D K L R P E N V R V A I V S K S F E G 487 R Y D A K A V K E R L A M H T P O M A R I V Y I S P . . K E</p>	<p>1461 AAAAATGATGGCAGAGAAGTGGTATGGAAACCCAGTACAAAACAAGAAGCTATACCGGATGAAGTCATCAAGAAATGGCAAAATGCTGAC 1558# 388 K T D R T E E V V G T D V K Q E A I P D E V I K K V G O N A D 517 P H N K T A Y F V D A P Y Q V D K I S A O T F A D U C O K K A</p> <p>1551 CTGAATGGGAAATTTAAACTTCCTACAAAGAATGAATTTATCTACGAATTTTGGAGATTTACCGTTAGAAAAAGGGCCAGCACCATAC 1648# 518 L N G K F K L P T K N E F I P T M F E I L P L E K E A T P Y 547 A D I A L S L P E L N P Y I P D D F S L I K S E K K Y D H P</p> <p>1641 CCTGCTCTTATTAAGATACAGTCTAGGCAAACTTTGGTTCAAACAAGATGATAAGAAAAAGGCGAAGGCTTGTCTCAACTTTGAA 1738# 548 P A L I K D T V M S K L W F K O D D K K K K P K A C L N F E 577 E L I V D E S N L R V V Y A P S R Y F A S E P K A D V S L I</p> <p>1731 TTTTTCAGCCCTTTGCTTATGTGGACCCCTTGCACGTAAACATGCCCATTGTACCTTGAGCTCCTCAAGACTCAGTCAACAGGAT 1828# 578 L R N P K A M D S A R N O V M F A L N D Y L A G L A L D O L 687</p> <p>1821 GCATATGACAGCAGAGCTAGCAGGCTTGAGCTATGATCTCAAATAACCACTATGGGATGATCTTTCAGTGAAGGTTACAATGACAAG 1918# 688 A Y A A E L A G L S Y D L O N T I Y G M Y L S V K G Y N D K 637 S N G A S V G G I S F S . T N A N N G L M V N A N G Y T O R</p> <p>1911 CAGCCAATTTACTAAGAAGATATTAAGAATAAGGCTCCTTTGAGATGATGAAAAAGATTGAAATTTCAAGAAAGCATATATG 2888# 538 Q P I L L K K I I E K H A T F E I D E K R F E I I K E A Y M 567 L P O L F O A L L E G Y F S Y T A T E D O L E O A K S V Y N</p> <p>2881 CGATCTTAAACAATTTCCGGGCTGAACAGCCTCAGCAGATGCCATGACTACCTCCGCTTGCTGATGACTGAAGTGGCTGGACTAAA 2898# 668 R S L N N F R A E O P H O H A Y I V L R L L M H T E V A L T K 697 O M M D S A E K G K A F E O A I M P A Q M L S O V P Y F S R</p> <p>2891 GATGAGTAAAGAAGCTGGATGATGAAACCTTCCCTCCGCTTAAGGCTTCACTACCTCAGCTCCTGTCCAGGCTGCACATTGAAGCC 2188# 698 D E L K E A G L D V T L P R L K A F I P O L L S R L H I E A 727 D E R R K I L P S I T L K E V L A Y R D A L K S G A R P E F</p> <p>2181 CTCTCCATGAAACAATAAAGCAGGCTGCATAGGAATATGACAGATGGTTGAAGACACCCCTCATTGAACATGCTCATCAACAACCT 2278# 728 L L H G N I I K G A A L G I H O H V E D T L I E H A H T K P 757 M V I G N M T E A O A T T L A R D V O K O L G A D G . . .</p> <p>2271 CTCTTCCAAAGTCAAGTCTGCGTATAGAAAGTTCAGCTCCCTGACAGAGGATGGTTTGTATCAGCAGAGAAATGAAGTCCACAAT 2368# 758 L L P S O L V R Y R E V O L P D R G Q W F V Y O O R H E V H N 787 . . . S E W C R N K D V V V D K O S V I F E K A G N S T D</p> <p>2361 AACTGGGCTCGAGATATCTACCAACAAGACATGAAAGCAGCTCAGAGAAATGTTTCTGGAGCTTCTGTGAGATATCTCGGAA 2458# 788 N C G I E I Y Y O T D M O S T S E N H F L E L F C O I I S E 817 S A L A A V F V P T G Y D E Y T S S A V S S L L G O I V O P</p> <p>2451 CCTTCTCAACACCTTCCCAACAGCAGTTCGGCTATACCTTCTCAGCGGCCAGCTGAGCTAATGGCATACAGAGCTTGAGAA 2548# 818 P C F W T T L I T T K E D L G Y I V F S G P R R A N G I O S L R 847 V F Y N D L R T E E O L G Y A V F A F P M S V G R O U G H G</p> <p>2541 TTCATCATCCAGTCAGAAAACCCCTCACTACCTAGAAAAGCAGAGTGAAGTCTTCTTAATTACCATGGAAGTCCATAGAGGACATG 2638# 848 F I I O S . E K P P H Y L E S R V E A F L I T H E K S I E D M 877 F L L O S N D K O P S F L W E R Y K A F F P T A E A K L R A N</p> <p>2631 ACAGAAGGCTTCCAAAACAACATTCAGGCTATGCAATTCGTCAGCTAGCAAAACAAGAAGCTATCTGCTGAGTGTGCTAAATAC 2728# 878 T E E A F Q K H I Q A L A I R R L D K P K L S A E C A R Y 927 K P D E F A Q I O Q A V I T O M L O A P O T L G E E A S K Y</p> <p>2721 TGGGAGAAATCATCTCCAGCAATAAATTTGACAGAGATAACACTAGGCTTGCATATTTAAAGACACTTACCAAGGAAGATATCATC 2818# 908 V G E I I S Q Q Y N F D R D N T E V A V L K T L I T K E D I I 937 S K D F I R G N M R F D S R D K I V A O I K L L T P O K L A</p> <p>2811 AAATTACAGAAAATGTTGGCAGTAGATGCTCAAGGAGCAGATAAGGATTCGGTCCATGTTCTTGCAGGGAATGATGCTTGTCT 2988# 938 K F Y K E M L A V D A P R R R * 952 D F F H Q A V V E P O G H</p> <p>2981 CTGTTGAGAGTCCCATGTCAAAATGACATAAATTTGTCACAAGCAGGCTTGCACAACCTGAAGTATTCAAGAACATGAGGC 2998#</p>
---	--

Fig 1. (Facing page) Nucleotide sequence and deduced amino acid sequence of human IDE (GenBank accession number M21188) and *E. coli* protease III. Nucleotides and amino acids corresponding to IDE are numbered. The amino acid sequence of *E. coli* protease III (16) is aligned with that of IDE and regions of >50% identity are shaded. A 35-bp oligonucleotide probe (12) was labeled with [γ - 32 P]ATP with the use of T4 polynucleotide kinase (23) and was used to screen cDNA libraries. Hybridization with this oligonucleotide was for 14 hours at 42°C in 20% formamide, 5 \times Denhardt's solution, 6 \times saline sodium citrate (SSC) (1 \times SSC = 105 mM NaCl and 15 mM sodium citrate), yeast RNA (100 μ g/ml), 10 mM sodium phosphate, pH 6.5, and 0.1% SDS, and cells were washed at 50°C for 1 hour in 5 \times SSC. A 2.5-kb cDNA was isolated from a λ gt-11 human lymphocyte library (24) and subcloned into pUC19; the 5' 1400-bp (Eco RI-Sac I) fragment was used to screen a λ gt-10 human hepatoma library (25). Hybridization with this cDNA probe (labeled with random priming (23) was for 14 hours at 42°C in 50% formamide, 5 \times Denhardt's solution, 5 \times SSC, yeast RNA (100 μ g/ml), and 1% SDS; washing was at 50°C for 1 hour in 0.1 \times SSC and 1% SDS. A single full-length (3.1 kb) cDNA was identified from this screen. Suitable restriction fragments and exonuclease III fragments were subcloned into pUC19 and Bluescript (26) and sequenced by the dideoxy double-stranded method (27). The sequences of the IM-9 and HepG2 cDNAs were identical over the overlapping region. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

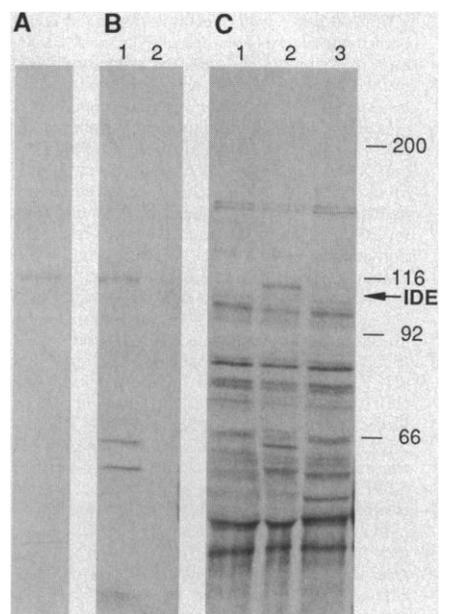
Fig 2. RNA blot analysis of in vitro-transcribed RNA (A) and 20 μ g of total IM-9 RNA (B). Total RNA was isolated from IM-9 cells (23), separated by electrophoresis, and transferred to a NYTRAN membrane (Schleicher and Schuell) as described (28). Hybridization with random-prime (23) labeled cDNA was exactly as described in Fig. 1.



$M_r \approx 110,000$ band and the two smaller fragments (Fig. 3C). The bands of lower M_r therefore appear to be fragments of IDE, whereas the upper band is the fully translated product.

To determine whether IDE shares sequence similarity with other known proteinases, we performed homology searches of the National Biomedical Research Foundation and Protein Information Resource Databanks using either the Intelligenetics, Inc., Bionet system or the VAX/VMS facility of the Stanford cell biology department. Manual comparisons with known proteinase

Fig 3. SDS-PAGE analysis of purified IDE and in vitro-translated IDE. (A) SDS-PAGE analysis of IDE purified from human red blood cell lysates. The gel was stained with Coomassie blue. (B) SDS-PAGE analysis of the products after translation in a rabbit reticulocyte system of either 1 μ g (lane 1) transcribed IDE RNA or a control with no added RNA (lane 2). (C) SDS-PAGE analysis of the immunoprecipitated products. The in vitro-translated proteins were precipitated with either monoclonal antibodies to IDE (lane 2) or control monoclonal antibodies (lane 3). Lane 1 shows the anti-IDE-precipitated proteins from a control in vitro translation without IDE RNA. Bluescript vector (Stratagene) containing the 3.1-kb IDE DNA segment was linearized with restriction enzyme Xho I, extracted with phenol-chloroform, and precipitated with ethyl alcohol. For transcription and translation we used the procedures described by the suppliers (Stratagene for transcription; ProMega Biotec for translation). Briefly, the linearized plasmid was transcribed by T7 RNA polymerase and translated in a micrococcal nuclease-treated rabbit reticulocyte lysate in the presence of 50 μ Ci of [35 S]cysteine and [35 S]methionine (1000 Ci/mmol; ICN). A fraction (1/20) of the radioactive products was separated by electrophoresis on a 7.5% SDS-polyacrylamide gel, dried, and exposed to autoradiographic film (Kodak XAR-5). The remainder was immunoprecipitated for 2 hours at 4°C with protein A Sepharose coated with either control immunoglobulin or monoclonal antibodies directed against IDE. Immunoprecipitated products were separated by electrophoresis with the same method. Markers are shown as $M_r \times 10^3$.



consensus sequences (15) were also done. These analyses revealed no significant homologies between IDE and the classical metallo, cysteine, aspartic, or serine proteinase.

Surprisingly, the sequence of IDE is homologous to that of *Escherichia coli* protease III (16) (Fig. 1). Overall sequence identity is 26%, but when conserved amino acid changes are included as matches, the similarity increases to 46%. Three regions of higher homology are readily visible: amino acids 69 to 121, 208 to 254, and 821 to 834 share 57, 54, and 80% sequence identity, respectively (Fig. 1). These regions may be conserved because they play an important role in the active site and catalytic mechanism of IDE and protease III. Furthermore, these high-homology regions may serve as consensus sequences for identifying related members of this family.

In addition to sharing sequence identity, both proteinases are remarkably similar in molecular mass (110,000 versus 107,000) and both are dependent on divalent cations for activity (6, 7, 17). Both enzymes hydrolyze the insulin B chain at two of the same sites, and both proteinases also cleave glucagon (17, 18). *E. coli* protease III differs from IDE in not being sensitive to sulfhydryl-modifying agents and in having an NH₂-terminal signal sequence that directs the enzyme to its periplasmic location (16, 17). A proteinase similar in its properties to IDE and protease III was also recently isolated from *Drosophila melanogaster* (19).

The availability of a cDNA clone encoding for human IDE should allow additional tests of the role of this enzyme in insulin signaling in mammalian cells. Moreover, this cDNA should allow one to determine whether the elevated synthesis of this enzyme is responsible for the increased degradation observed in certain patients with insulin resistance (20). Finally, this cDNA should allow us to test whether this proteinase has other roles; for example, similar proteinases have been proposed to function in membrane fusion (21), muscle differentiation (22), and intracellular turnover of proteins (18).

REFERENCES AND NOTES

- O. M. Rosen, *Science* **237**, 1452 (1987).
- M. G. Low and A. R. Saliel, *ibid.* **239**, 268 (1988).
- C. B. Graves, R. R. Goewert, J. M. MacDonald, *ibid.* **230**, 827 (1985).
- D. S. Miller, *ibid.* **240**, 506 (1988).
- S. Terris and D. F. Steiner, *J. Biol. Chem.* **250**, 8389 (1975).
- W. C. Duckworth and A. E. Kitabchi, *Endocr. Rev.* **2**, 210 (1981); W. C. Duckworth, *ibid.* **9**, 319 (1988).
- I. A. Mirsky, *Recent Prog. Horm. Res.* **13**, 429 (1957); M. Vaughan, *Biochim. Biophys. Acta* **15**, 432 (1954).
- W. C. Duckworth *et al.*, *Endocrinology* **108**, 1142 (1981); B. J. Goldstein and J. N. Livingston, *ibid.*, p. 953; G. T. Hammons, R. M. Smith, L. Jarett, *J. Biol. Chem.* **257**, 11563 (1982).
- R. K. Assoian and H. S. Tager, *J. Biol. Chem.* **257**, 9078 (1982); W. C. Duckworth *et al.*, *ibid.* **263**, 1826 (1988).
- K. Shii and R. A. Roth, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4147 (1986).
- J. Hari *et al.*, *Endocrinology* **120**, 829 (1987).

12. IDE (200 μ g) was purified at 4°C from 12 liters of packed human red blood cells in batches of 1.6 liters each, as follows. Cells were centrifuged at 1,500g, washed with cold phosphate-buffered saline (PBS), lysed in cold distilled-deionized water containing 10 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF), and centrifuged at 13,000g for 45 min. The supernatant was filtered, mixed with 550 ml of DEAE A-50 ion-exchange resin (equilibrated in 10 mM sodium phosphate, pH 7.4), and stirred for 90 min. The supernatant was removed by filtration and, the resin was washed with 1 liter of cold sodium phosphate, pH 7.4. Resin was eluted by stirring in 550 ml of 100 mM sodium phosphate, pH 7.4, and 0.5M KCl at 4°C. The supernatant was filtered and loaded onto the immunoaffinity column (5 ml). The column was washed with 50 mM Hepes, pH 7.6, 150 mM NaCl [Hepes-buffered saline (HBS)] and 50 mM Hepes, pH 7.6, and 500 mM NaCl. Elution was with 6M urea in HBS. Twenty 2-ml fractions were dialyzed against 3 liters of HBS overnight and assayed by electrophoresis through a 7.5% SDS-polyacrylamide gel. Peak fractions were pooled and concentrated through Centricon 30 (Amicon) filtration membranes. The IDE peak was then applied to preparative SDS-polyacrylamide gels, and the 110-kD band was selectively eluted. Attempts at NH₂-terminal sequencing were negative; therefore the protein was exhaustively digested with trypsin. The resulting tryptic peptides were isolated by HPLC and sequenced by automated Edman degradation on a gas-phase sequencer [Y. Yarden *et al.*, *Nature* **323**, 226 (1986)]. One of the tryptic peptides (WGEIIS QQYNFD, in single-letter code) was used to design the 35-base oligonucleotide probe IDE-5 (TCAAAGTT A/G TACTGCTGG G/C A/T GATGAT T/C TC G/C CCCC) with the use of the codon usage table of Lathe (13).
13. R. Lathe, *J. Mol. Biol.* **183**, 1 (1985).
14. J. Kyte and R. F. Doolittle, *ibid.* **157**, 105 (1982).
15. A. J. Barrett, in *Proteinase Inhibitors*, A. Barrett and G. Salvesen, Eds. (Elsevier Science, New York, 1986), pp. 3–22; B. Malfroy *et al.*, *Biochem. Biophys. Res. Commun.* **144**, 59 (1987); A. Devault *et al.*, *J. Biol. Chem.* **263**, 4033 (1988).
16. P. W. Finch *et al.*, *Nucleic Acids Res.* **14**, 7695 (1986).
17. Y.-S. E. Cheng and D. Zipser, *J. Biol. Chem.* **254**, 4698 (1979).
18. R. J. Kirschner and A. L. Goldberg, *ibid.* **258**, 967 (1983).
19. J. V. Garcia *et al.*, *Biochemistry* **27**, 4237 (1988); J. B. Garcia *et al.*, *J. Cell Biol.* **105**, 449 (1987).
20. A. E. Kitabchi, F. B. Stentz, C. Cole, W. C. Duckworth, *Diabetes Care* **2**, 414 (1979); A. McEl-duff, C. J. Eastman, S. P. Haynes, K. M. Bowen, *Aust. N.Z. J. Med.* **10**, 56 (1980); G. R. Freiden-berg *et al.*, *N. Engl. J. Med.* **305**, 363 (1981); G. F. Maberly *et al.*, *Diabetologia* **23**, 333 (1982); B. R. Blazar *et al.*, *Diabetes* **33**, 1133 (1984).
21. D. I. Mundy and W. J. Strittmatter, *Cell* **40**, 645 (1985); H. A. Farach, Jr., D. I. Mundy, W. J. Strittmatter, W. J. Lennarz, *J. Biol. Chem.* **262**, 5483 (1987).
22. E. Baldwin and C. Kayalar, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8029 (1986).
23. T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning. A Laboratory Handbook* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982).
24. C. Weinberger *et al.*, *Science* **228**, 740 (1985).
25. D. O. Morgan *et al.*, *Nature* **329**, 301 (1987).
26. L. Davis, M. D. Dibner, J. F. Battey, *Basic Methods in Molecular Biology* (Elsevier Science, New York, 1986), pp. 136–139.
27. R. J. Zagursky *et al.*, *Gen. Anal. Tech.* **2**, 89 (1985).
28. R. M. Fourny *et al.*, *Focus* **10**, 5 (1988).
29. We thank R. Evans, J. Edman, and W. Rutter for the cDNA libraries from IM-9 and HepG2 cells, respectively, A. Belland and M. Ando for technical assistance, A. Payne and S. Leff for a critical reading of this manuscript, and K. Bird for preparation of this manuscript. This work was supported by NIH grants DK34926 and DK01393, a grant from the American Lebanese Syrian Associated Charities, and CORE grant CA21765.

19 August 1988; 11 October 1988

Many Transcription Factors Interact Synergistically with Steroid Receptors

ROLAND SCHÜLE, MARC MULLER, CHRISTIAN KALTSCHMIDT, RAINER RENKAWITZ

Progesterone (PRE) or glucocorticoid receptor (GRE) DNA binding sites are often found clustered with binding sites for other transcription factors. Individual protein binding sites were tested without the influence of adjacent factors by analyzing isolated combinations of several transcription factor binding sites with PREs or GREs. All show strong synergistic effects on steroid induction. The degree of synergism is inversely related to the strength of the GRE. Thus, a steroid responsive unit can be composed of several modules that, if positioned correctly, act synergistically.

ONE OF THE FIRST SYSTEMS IN which regulatory sequences and the corresponding trans-acting factors were characterized was that of steroid-inducible gene expression (1). Active steroid-receptor complexes bind with high affinity to sites in the chromatin and, by an unknown process, regulate transcription of specific genes. Progesterone or glucocorticoid receptor binding sites (PRE/GRE) in several cases are tightly clustered with other regulatory sequences: upstream of the chicken lysozyme gene (2, 3) and the human metallothionein II_A gene (4) and in the long terminal repeats (LTRs) of murine mammary tumor virus (MMTV) (5, 6), murine sarcoma virus (MSV) (7), and Moloney murine leukemia virus (Mo-MuLV) (8). A

computer search revealed many more similarities to transcription factor binding sites in the direct vicinity of PREs and GREs (9). We thus hypothesized that the DNA-bound progesterone or glucocorticoid receptor can cooperate with other transcription factors. This idea is supported by the fact that some receptor binding sites seem to be nonfunctional when analyzed individually (5, 10–12). Because single interactions between pairs of bound transcription factors cannot be analyzed within a complex cluster of binding sites, we have tested combinations of several transcription factor binding sites with binding sites for the progesterone or

Max-Planck Institut für Biochemie, Genzentrum, D 8033 Martinsried, Federal Republic of Germany.

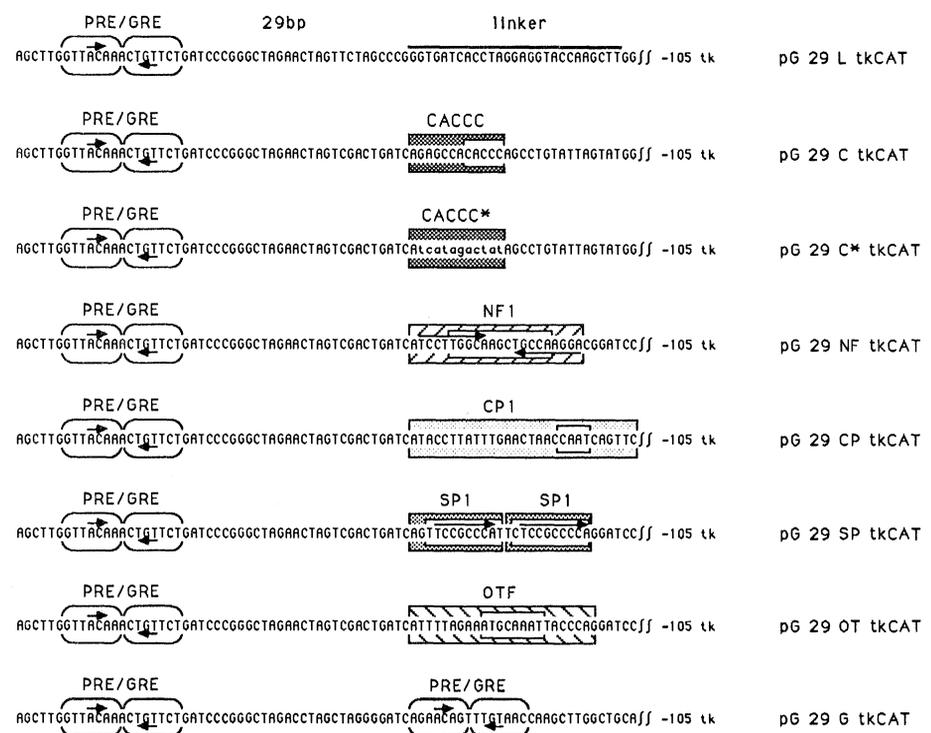


Fig. 1. Different combinations of PRE/GRE transcription factor binding sites were cloned in front of the fusion gene tkCAT. Binding sites for CACCC-factor, NF1, CP1, SP1, and OTF were synthesized and cloned at a distance of 29 bp to the PRE/GRE. As a control the mutagenized CACCC-box (CACCC*) and an unrelated linker sequence were also cloned at the same distance.