mas can be the progeny of normal cells that bear antigen-specific T-cell receptors for engaging viral envelope antigens. Binding of a retrovirus to these cells is postulated to provide both a portal of infection and a continuous mitogenic signal. Such events could have played a role in the emergence of the human T-cell clone K7 in the current studies, although the receptor-mediated leukemogenesis theory per se would not explain the inhibitory effects of exogenous HTLV after the clone emerged.

The mechanisms for the specific inhibition of this clone by its antigen HTLV are not known. Normal human T-cell clones that are specific for discrete peptides of influenza A virus can be rendered unresponsive to antigen by incubation with high concentrations of the appropriate peptide (*22*). This state of specific unresponsiveness is defined by a failure to mount a proliferative response to antigen in vitro and is perhaps akin to certain forms of immunologic tolerance. Perhaps clone K7 represents HTLV-induced transformation and expansion of a T cell that had already been programmed for such unresponsiveness. In this context, K7 cells might serve as an in vitro model for immunologic tolerance based on a mechanism of clonal deletion. Alternatively, the insertion of HTLV (or a mutant strain of the virus) into a critical domain within the genome could induce or predispose reactive T cells to somatic errors that cause them to recognize exogenous viral antigens as a negative signal. These are topics for future research that could have implications in understanding the relation between retroviruses in T-cell neoplasms and acquired immunodeficiency disease states.

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References and Notes

1. B. J. Poiesz, F. W. Ruscetti, A. F. Gazdar, P. A. Bunn, J. D. Minna, R. G. Gallo, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7415 (1980).
2. B. J. Poiesz, F. W. Ruscetti, M. S. Reitz, V. S. Kalyanaraman, R. C. Gallo, *Nature (London)* **294**, 268 (1981).
3. R. C. Gallo, A. Sliski, F. Wong-Staal, *Lancet* **1983-II**, 962 (1983).
4. Y. Hinuma *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6476 (1981).
5. M. Popovic *et al.*, *Nature (London)* **300**, 63 (1982).
6. R. C. Gallo *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5680 (1982).
7. M. Popovic, P. S. Sarin, M. Robert-Guroff, V. S. Kalyznaraman, D. Mann, J. Minowada, R. C. Gallo, *Science* **219**, 856 (1983).
8. M. Popovic, G. Lange-Wantzin, P. S. Sarin, D. Mann, R. C. Gallo, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5402 (1983).
9. T. Uchiyama, K. Sagawa, K. Takatsuki, H. Uchino, *Clin. Immunol. Immunopathol.* **10**, 24 (1978).
10. T. Hattori, T. Uchiyama, T. Toibana, K. Takatsuki, H. Uchino, *Blood* **58**, 645 (1981).
11. P. A. Bunn, Jr., *et al.*, *N. Engl. J. Med.* **309**, **257** (1983); R. C. Gallo, unpublished data.
12. Y. Hinuma *et al.*, *Int. J. Cancer* **29**, 631 (1982).
13. R. C. Gallo *et al.*, *Science* **220**, 865 (1983); E. P. Gelmann *et al.*, *ibid.*, p. 862; M. Essex *et al.*, p. 859; *ibid.* **221**, 1061 (1983).
14. M. Essex, W. D. Hardy, Jr., S. M. Cotter, R. M. Jakowski, A. Sliski, *Infect. Immun.* **11**, 470 (1975); W. D. Hardy, Jr., *et al.*, *Cancer Res.* **36**, 582 (1976); S. M. Cotter, W. D. Hardy, Jr., M. Essex, *J. Am. Vet. Med. Assoc.* **166**, 449 (1975); Z. Trainin, D. Wernicke, H. Unger-Waron, M. Essex, *Science* **220**, 858 (1983).
15. H. Mitsuya *et al.*, *J. Exp. Med.* **158**, 994 (1983). Patient M.J. is a 54-year-old white male with circulating antibodies against internal structural proteins of HTLV. In 1977 he was diagnosed as having cutaneous T-cell lymphoma, and he eventually became a complete responder to a regimen of chemotherapy, whole-body electron beam irradiation, and topically applied nitrogen mustard. The patient's disease was in clinical remission when these studies were initiated in January 1983.
16. F. Wong-Staal *et al.*, *Nature (London)* **302**, 626 (1983).
17. V. Manzari *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1574 (1983).
18. M. F. Clarke, E. P. Gelmann, M. S. Reitz, Jr., *Nature (London)* **305**, 60 (1983).
19. After approximately 1 year in culture (50 passages), some K7 cells were found to express p19 and p24 proteins as well as virus particles, confirming the presence of a complete provirus and indicating that the restriction of HTLV expression was a reversible phenomenon.
20. I. C. Weissman and M. S. McGrath, *Curr. Top. Microbiol. Immunol.* **98**, 103 (1982).
21. J. N. Ihle and J. C. Lee, *ibid.*, p. 85.
22. J. R. Lamb, B. J. Skidmore, N. Green, J. M. Chiller, M. Feldmann, *J. Exp. Med.* **157**, 1434 (1983).
23. We thank R. C. Gallo for helpful advice.
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Heparin Affinity: Purification of a Tumor-Derived Capillary Endothelial Cell Growth Factor

Abstract. *A tumor-derived growth factor that stimulates the proliferation of capillary endothelial cells has a very strong affinity for heparin. This heparin affinity makes it possible to purify the growth factor to a single-band preparation in a rapid two-step procedure. The purified growth factor is a cationic polypeptide, has a molecular weight of about 18,000, and stimulates capillary endothelial cell proliferation at a concentration of about 1 nanogram per milliliter.*

The proliferation of capillary endothelial cells is a key component of angiogenesis (*1*). It has been postulated that in tumor-induced angiogenesis tumor cells produce growth factors that stimulate the proliferation of capillary endothelial cells (*2*). A class of compounds of very low molecular weights (200 to 1000) that can stimulate endothelial cell proliferation in vitro and angiogenesis in vivo have been isolated from tumor cells (*3, 4*) but are as yet uncharacterized. Two recent developments have helped considerably in efforts to purify and characterize tumor-derived endothelial cell mitogens of larger molecular weights. The first is the availability of cultured capillary endothelial cells (*5*). Capillary endothelial cells differ structurally and functionally from aortic endothelial cells (*6*) and respond differently to tumor-derived factors. For example, tumor-derived factors have been shown to stimulate proliferation (*3*), motility (*7*), and collagenase production (*8*) in capillary but not in aortic endothelial cells. Thus capillary endothelial cells appear to be the target cell of choice in the analysis of growth factors that may be involved in capillary growth.

The second development, described in this report, is the finding that capillary endothelial cell growth factors derived from tumors have a strong affinity for heparin. This affinity enabled us to develop a rapid, two-step procedure for purifying a capillary endothelial cell growth factor found in chondrosarcoma. The chondrosarcoma-derived growth factor is a cationic polypeptide of about 18 kilodaltons (kD) that stimulates capillary endothelial cell proliferation at a concentration of about 1 ng/ml.

Growth factor isolated from extracts of chondrosarcoma extracellular matrix (ECM) bound tightly to columns of heparin-Sepharose. The growth factor activity, as measured by the ability to stimulate DNA synthesis in BALB/c mouse 3T3 cells (*10*), eluted at about 1.5*M* NaCl (Fig. 1A). The strong affinity of chondrosarcoma-derived growth factor for heparin was not shared by other growth factors that stimulate 3T3 cell DNA synthesis, such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). The EGF did not bind at all; PDGF bound but eluted at about 0.5*M* NaCl (Fig. 1A). The much tighter binding of chondrosarcoma-derived growth factor to heparin than PDGF is surprising. Both growth factors are cationic polypeptides of similar charge (isoelectric points between 9.5 and 10), and both adhered to the cationic exchange resin Bio-Rex 70 and eluted at about 0.5*M* NaCl (Fig. 1B). From the similarities in positive charge it might be expected that

the two growth factors would bind equally well to heparin, a highly anionic glycosaminoglycan. The much stronger binding of chondrosarcoma-derived growth factor suggests that it has a specific affinity for heparin. Another piece of evidence suggesting this is that the growth factor does not bind to columns of chondroitin sulfate–Sepharose.

Heparin affinity chromatography greatly facilitated purification of the growth factor. A very highly purified preparation was obtained from crude chondrosarcoma ECM by two chromatographic cycles. The elution profile of the second heparin-Sepharose column is exactly the same as that for the first cycle shown in Fig. 1A. The results of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (*11*) and silver staining (*12*) of the peak of growth factor activity obtained after the second cycle on heparin-Sepharose are shown in Fig. 1C (lane 1). Very few polypeptide bands are detectable. A prominent band of about 18 kD is the major polypeptide species observed. As discussed below, this polypeptide appears to be chondrosarcoma-derived growth factor.

Chondrosarcoma-derived growth factor, unlike PDGF and EGF, is a potent stimulator of capillary endothelial cell proliferation and could be purified to a single-band preparation with an assay that measures proliferation of these cells (Fig. 2). Only two steps were required. In the first step, crude chondrosarcoma ECM was applied to a Bio-Rex 70 cation-exchange column (Fig. 2A). All the growth factor activity but less than 0.01 percent of the protein adhered to the column. Growth factor activity eluted at about 0.5*M* NaCl. In the second step the growth factor activity peak obtained by Bio-Rex 70 chromatography was applied to heparin-Sepharose (Fig. 2B). All the growth factor activity adhered to the column and eluted at 1.5*M* NaCl. Only one polypeptide band was observed after SDS-PAGE and silver staining of this growth factor activity peak (lane 2 in Fig. 3A). This 18-kD band is the same one shown in Fig. 1C (lane 1) to be the major polypeptide species purified by heparin-Sepharose chromatography alone. When the single-band preparation (lane 2 in Fig. 3A) was analyzed by high-performance liquid chromatography (HPLC) on TSK size-exclusion columns, the growth factor activity eluted with a molecular weight of about 18 kD (Fig. 3B). The close correlation between the molecular weights determined by SDS-PAGE and by measurement of growth factor activity on size-exclusion columns is strong presumptive evidence that the 18-kD polypeptide band is chondrosarcoma-derived growth factor. The growth factor stimulated both capillary endothelial cell proliferation and DNA synthesis in 3T3 cells in a dose-dependent manner (Fig. 3C). Half-maximal stimulation (1 unit of activity) was induced by growth factor concentrations of about 1 ng/ml. In the two-step purification procedure the recovery of activity was about 5 percent and the yield of pure growth factor was about 1 μg from 5 g of crude chondrosarcoma ECM.

The question arises as to whether chondrosarcoma-derived growth factor is also a stimulator of angiogenesis in vivo. Preliminary results indicate that pure growth factor is angiogenic when applied to the chorioallantoic membrane. About 600 units of growth factor activity (120 ng) was required to induce strong angiogenesis within 24 hours on the 9-day chick chorioallantoic membrane. Histological sections reveal that neovascularization took place in the virtual absence of inflammatory cells.

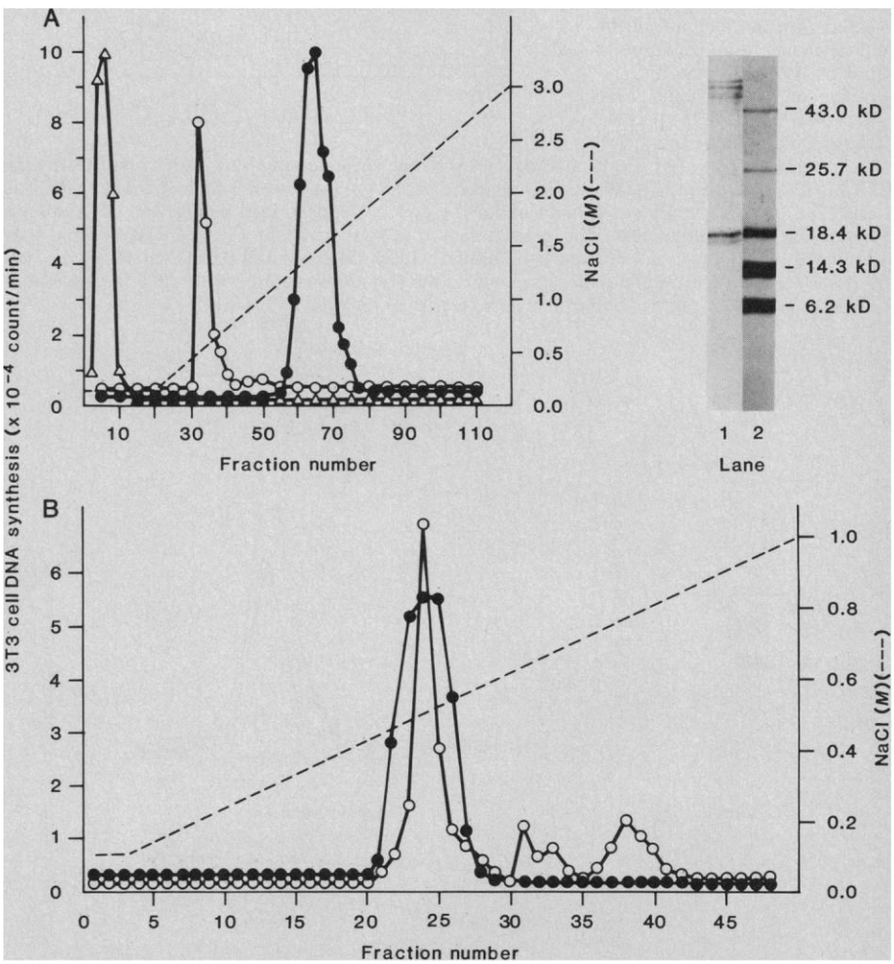

Fig. 1. (A) Heparin-Sepharose chromatography of chondrosarcoma-derived growth factor. A transplantable rat chondrosarcoma was digested with collagenase to produce ECM and cellular fractions (*9*). Crude ECM was used as the source of growth factor. PDGF was obtained from H. Antoniades and EGF from Collaborative Research. Growth factor activity was tested by measuring [^{3}H]thymidine incorporation into the DNA of quiescent monolayers of BALB/c mouse 3T3 cells (*10*). One unit of activity is defined as the amount of growth factor required to induce half-maximal incorporation of labeled thymidine into 3T3 cell DNA. We applied 1000 units of chondrosarcoma-derived growth factor (●), 800 units of PDGF (○), and 600 units of EGF (△) dissolved in 20 ml to columns (1 by 8 cm) of heparin-Sepharose (Pharmacia) equilibrated with 0.1*M* NaCl and 0.01*M* tris-HCl (*p*H 7.0). After a wash with 20 ml of equilibration buffer, growth factor activity was eluted with a 300-ml gradient of 0.1*M* to 3.0*M* NaCl in tris buffer at a flow rate of 30 ml/hour. (B) Bio-Rex 70 cation exchange chromatography. Crude chondrosarcoma ECM (●) and PDGF (○) (1000 units each) in 50 ml were applied to 2.5 by 20 cm columns of Bio-Rex 70 (200 to 400 mesh; Bio-Rad) equilibrated with 0.1*M* NaCl and 0.1*M* tris-HCl (*p*H 7.0). After a wash with 50 ml of equilibration buffer, growth factor activity was eluted with a 300-ml gradient of 0.1*M* to 1.0*M* NaCl at a flow rate of 30 ml/hour. (C) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Chondrosarcoma ECM (500 mg; 6000 units) was applied to a heparin-Sepharose column and the peak of growth factor activity (A) was applied to a second heparin-Sepharose column. This growth factor peak (250 ng; 1000 units) was analyzed by SDS-PAGE (*11*) with silver staining (*12*) (lane 1). Molecular weight markers (Bethesda Research Laboratories) are shown in lane 2.

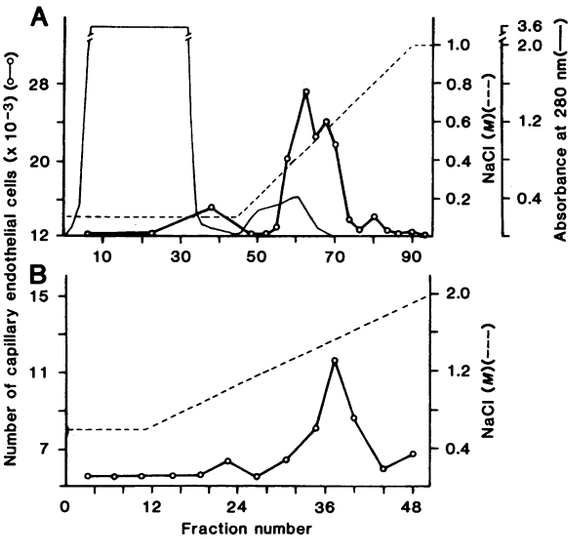

Fig. 2. Purification of chondrosarcoma-derived growth factor. Bovine capillary endothelial cells were grown as described by Folkman *et al.* (*5*). The cells (passage 4 to 14) were resuspended in Dulbecco's modified Eagle's medium supplemented with 10 percent calf serum and plated sparsely into 24-well plates 16 mm in diameter (Costar) at a concentration of 10,000 cells per 0.5 ml per well. On the following day unattached cells were removed and attached cells (plating efficiency, approximately 50 percent) were incubated with the medium in the absence or presence of growth factor. After 72 hours of incubation at 37° the cells were detached with 0.25 percent trypsin and counted in a Coulter particle counter. (A) Bio-Rex 70 cation exchange chromatography. Crude chondrosarcoma ECM (1.35 g per 75 ml; 10,000 units) prepared from six tumors about 3 cm in diameter each was analyzed on a 2.5 by 30 cm column under the conditions described in Fig. 1B. (B) Heparin-Sepharose chromatography. The growth factor activity peak in Fig. 2A (3000 units) was analyzed on a 1 by 15 cm column equilibrated with 0.6*M* NaCl and 0.01*M* tris-HCl (*p*H 7.0). After a wash with 50 ml of equilibration buffer, the column was eluted with a 300-ml gradient of 0.6*M* to 2.0*M* NaCl in tris buffer at a flow rate of 30 ml/hour.

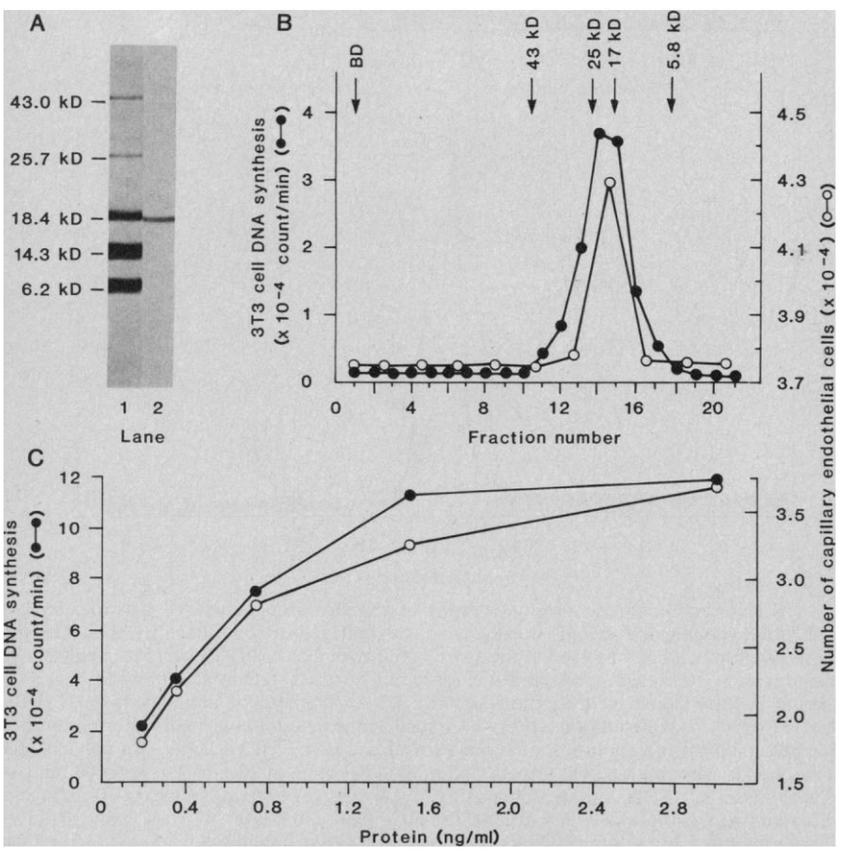

Fig. 3. Characterization of purified chondrosarcoma-derived growth factor. (A) SDS-PAGE. (Lane 1) Molecular weight markers; (lane 2) peak fraction of growth factor activity (200 ng; 1000 units) obtained when crude chondrosarcoma ECM was purified by Bio-Rex 70 chromatography (Fig. 2A) followed by heparin-Sepharose chromatography (Fig. 2B). (B) Size exclusion chromatography. About 5000 units of single-band preparation [lane 2 in (A)] was analyzed by HPLC (Beckman model 332 gradient liquid chromatography system) at room temperature on a TSK 2000 column (60 cm by 7.5 mm inner diameter; Altex) equilibrated with 0.6*M* NaCl and 0.02*M* tris-HCl (*p*H 7.0). The flow rate was 0.5 ml/min. Fractions were tested for growth factor activity on bovine capillary endothelial cells (○) and 3T3 cells (●). *BD*, blue dextran. (C) Pure growth factor [lane 2 in (A)] was added at various concentrations to cultures of bovine capillary endothelial cells (○) and 3T3 cells (●).

The affinity of chondrosarcoma-derived growth factor for heparin is significant because purification is so greatly facilitated that growth factor can be almost totally purified by chromatography on heparin-Sepharose alone and because the affinity of growth factor for heparin may have implications for the mechanisms of endothelial cell proliferation. Exogenous heparin binds avidly to endothelial cells (*13, 14*). Heparan sulfate, a glycosaminoglycan that is structurally (*15*) and at high concentrations biologically similar (*16*) to heparin, is the major glycosaminoglycan species on the endothelial cell surface (*17*). It is conceivable that heparin-like molecules associated with endothelial cells bind specifically those growth factors that have a high affinity for heparin. Binding of growth factor by heparin could potentiate growth factor activity. A recent report (*18*) indicates that the activity of endothelial cell growth factor is greatly potentiated by heparin but not by other glycosaminoglycans, such as chondroitin sulfate.

Heparin binding of growth factor may also have implications for understanding the mechanism of angiogenesis. Heparin has been shown to potentiate tumor-induced angiogenesis on the chorioallantoic membrane (*19*). However, when heparin is administered to animals in conjunction with cortisone, tumor-induced angiogenesis is inhibited (*20*). The mechanism underlying these phenomena may involve a three-way interaction between capillary endothelial cells, heparin, and endothelial cell growth factors with high affinity for heparin.

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References and Notes

1. T. Cavallo, R. Sade, J. Folkman, R. S. Cotran, *J. Cell Biol.* **54**, 408 (1972).
2. J. Folkman, *Adv. Cancer Res.* **29**, 331 (1974).
3. A. Keegan *et al.*, *J. Cell Sci.* **55**, 261 (1982).
4. A. Fenselau, S. Wald, R. J. Mello, *J. Biol. Chem.* **256**, 9605 (1982).
5. J. Folkman, C. C. Haudenschild, B. R. Zetter, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5217 (1979).
6. B. R. Zetter, *J. Am. Diabetes Assoc.* **30**, 24 (1981).
7. ———, *Nature (London)* **285**, 41 (1980).
8. D. B. Rifkin, J. L. Gross, D. Moscatelli, E. Jaffe, in *Pathobiology of the Endothelial Cell*, H. Nossel and H. J. Vogel, Eds. (Academic Press, New York, 1982), p. 191.
9. J. C. Azizkhan, R. Sullivan, R. Azizkhan, B. R. Zetter, M. Klagsbrun, *Cancer Res.* **43**, 3281 (1983).
10. M. Klagsbrun, R. Langer, R. Levenson, S. Smith, C. Lillehei, *Exp. Cell Res.* **105**, 99 (1977).
11. U. K. Laemmli, *Nature (London)* **227**, 680 (1970).
12. B. R. Oakley, D. R. Kirsch, N. R. Morris, *Anal. Biochem.* **105**, 361 (1980).

13. L. Hiebert and L. Jaques, *Thromb. Res.* **8**, 195 (1976).
14. C. Busch, C. Ljungman, C. M. Heldin, E. Waskson, D. Obrink, *Haemostasis* **8**, 142 (1979).
15. P. M. Kramer, *Biochemistry* **10**, 1445 (1971).
16. A. Wasteson *et al.*, *Thromb. Res.* **11**, 309 (1977).
17. V. Buonassissi and M. Root, *Biochem. Biophys. Acta* **385**, 1 (1975).
18. S. C. Thornton, S. N. Mueller, E. M. Levine, *Science* **222**, 623 (1983).
19. S. Taylor and J. Folkman, *Nature (London)* **297**, 307 (1982).
20. J. Folkman, R. Langer, R. J. Linhardt, C. Haudenschild, S. Taylor, *Science* **221**, 719 (1983).
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Total Synthesis and Cloning of a Gene Coding for the Ribonuclease S Protein

Abstract. *A gene for ribonuclease S protein, has been chemically synthesized and cloned. The gene is designed to have 25 specific restriction endonuclease sites spaced at short intervals, permitting its structure to be rapidly modified. This flexibility facilitates tests of hypotheses relating the primary structure of the enzyme to its physical and catalytic behavior.*

Systematic variation of the amino acid sequence of enzymes promises to be useful for developing an understanding of structure, physical behavior, and catalysts in proteins (*1*). Such a tool is likely to be most productive when the enzymes being studied have structures known to atomic detail, catalyze reactions with rates measurable at the level of the individual reaction step, and have thermodynamic properties that can be determined at the level of microscopic reaction intermediates.

We describe here the chemical synthesis and cloning of a gene coding for the ribonuclease S protein (*2*), engineered to contain more than 25 specific restriction sites at short intervals. These restriction sites permit rapid modification of the synthetic gene by replacing sections of the gene with synthetic duplex DNA. By expression of altered genes, ribonucleases having multiple alterations in their amino acid sequences may be prepared. This altered gene has been engineered to permit rapid mutation by design, and appears to be the first synthetic gene for any enzyme (*3*).

The designed sequence of the gene for ribonuclease is shown in Fig. 1. The specific restriction sites are underlined. Multiple restriction sites and homologous sequences were removed. Sites for Eco R1 and Bam HI (restriction endonucleases) were incorporated at the ends of the gene to facilitate introduction into cloning and expression vectors (*4*). Provisions for the expression of ribonuclease were also incorporated into the design. While active ribonuclease might be able to destroy messenger RNA (mRNA) coding for the protein, the proteolytic fragment S protein has no catalytic activity, but forms a stable non-covalent aggregate with the ribonuclease S peptide, which has full catalytic activity (*2, 5*). Thus, the ribonuclease S protein can be expressed in *Escherichia coli* as a nonactive protein. Furthermore, the S protein can be purified from other proteins of *E. coli* by affinity chromatography with the S peptide as the affinity ligand (*6*).

To construct the gene, 66 different oligonucleotides 10 to 22 residues in length were synthesized with the use of phosphoramidite reagents on a variety of solid supports (*7*), including colloidal suspensions of magnetic particles developed as supports for oligonucleotide synthesis (*8, 9*). Adapting the procedures of Beaucage and Caruthers (*8*), we prepared supports that were functionalized with 3-aminopropyltrimethoxysilane; the appropriate protected 5′-dimethoxytrityl-2′-deoxynucleoside was then linked

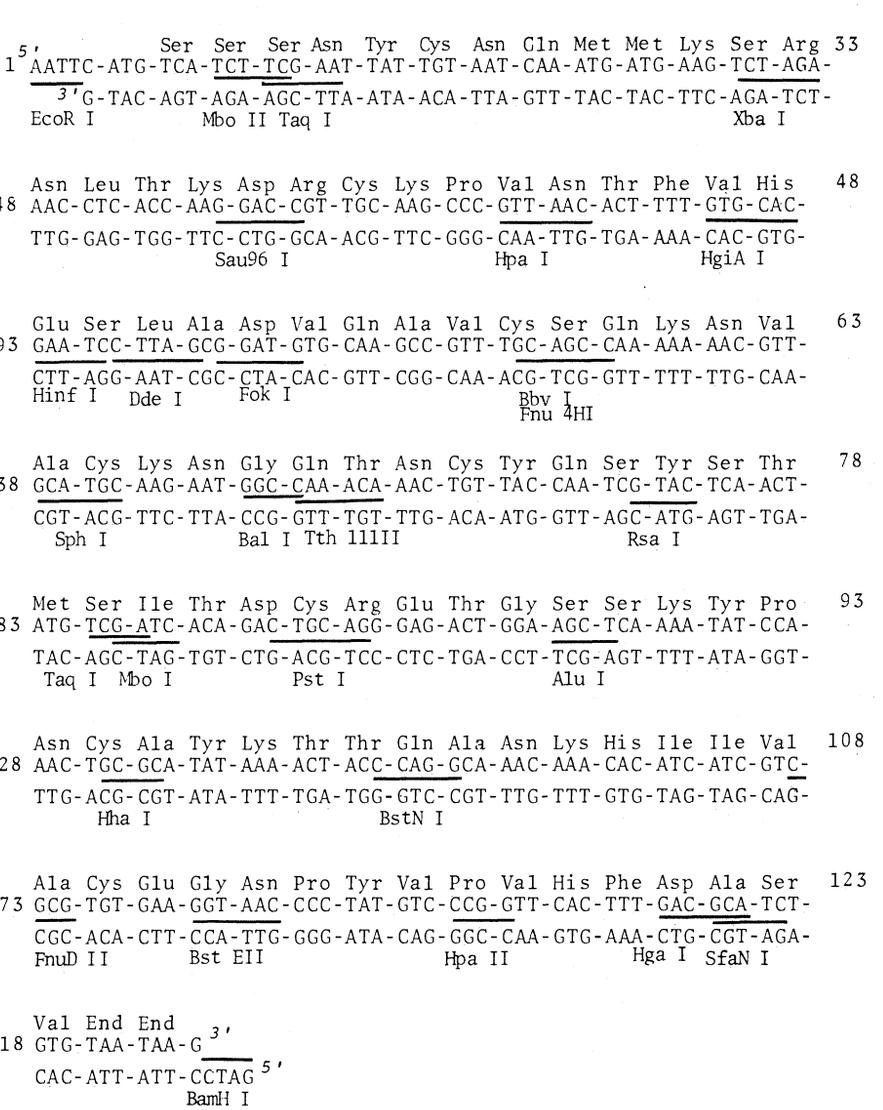

Fig. 1. Sequence of the synthetic gene coding for the ribonuclease S protein, containing about 330 base pairs. The numbering of nucleotides in the sequence is on the left; numbering of the amino acids is on the right, and corresponds to the numbering in native bovine ribonuclease. Restriction sites are underlined and labeled. Some restriction sites are not underlined, as their location adjacent to other sites makes them redundant for the purpose of cutting small segments from the gene.