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30 November 1983; accepted 12 January 1984

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 Hl (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

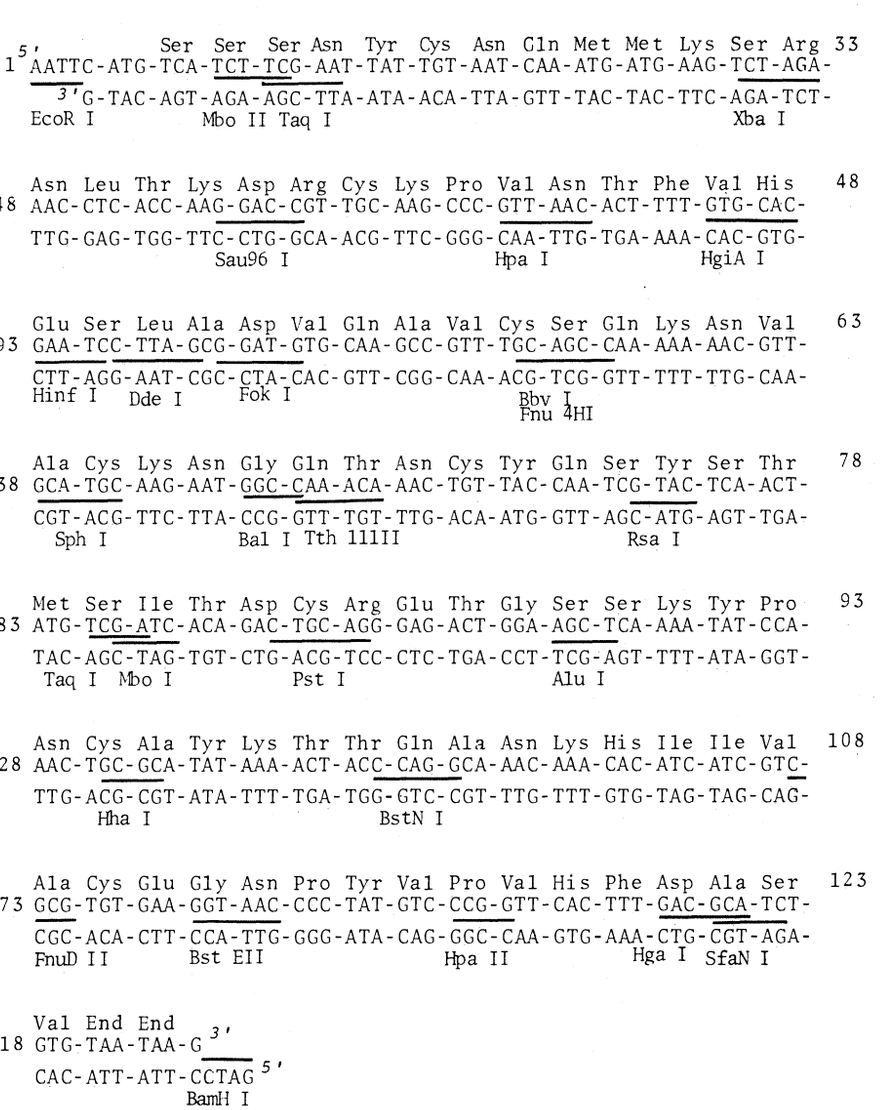

Fig. 1. Sequence of the synthetic gene coding for the ribonuclease S protein, containing about 330 base pairs. The numbering of nucleotides in the sequence is on the left; numbering of the amino acids is on the right, and corresponds to the numbering in native bovine ribonuclease. Restriction sites are 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.

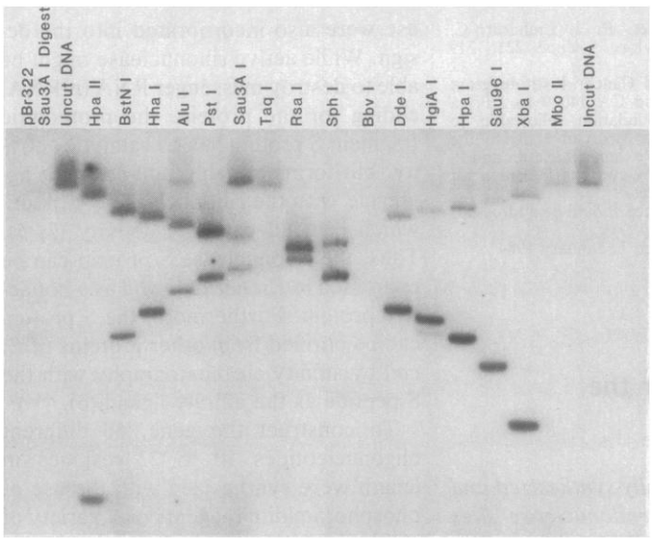

Fig. 2. Autoradiograph of a restriction digest map of the gene for ribonuclease, excised by digestion with Bam HI and Eco RI endonucleases, labeled with ^{32}P at the 5′ ends, and subjected to electrophoresis on an acrylamide (15 percent) gel.

to the support through a 3′-succinamido group. The coupling cycle consisted of detritylation with $ZnBr_2$ in a mixture of CH_3NO_2 and CH_3OH (95:5), washing with CH_3OH and CH_3NO_2 and then anhydrous CH_3CN, drying at 50°C at reduced pressure, addition of tetrazole in anhydrous CH_3CN and solid-protected 5′-dimethoxytrityl-2′deoxynucleosidyl-3′-phosphoramidite, incubation, hydrolysis [in a mixture of tetrahydrofuran, lutidine, and water (2:1:2)], oxidation with I_2 in the same solvent, capping in acetic anhydride and dimethylaminopyridine in tetrahydrofuran, and washing with tetrahydrofuran and then CH_3NO_2. Cycle times were typically 1 hour (*10*).

Synthetic oligonucleotides were released from the supports (*7*); purified by thin-layer chromatography on silica gel with a mixture of *n*-propanol, ammonia, and water (55:35:10) as eluent; 5′-phosphorylated; and sequenced (*11*) prior to subsequent ligation. Oligomers to be ligated were mixed and treated with γ-^{32}P–labeled adenosine triphosphate (ATP) (30 to 60 Ci/mmole) in the presence of polynucleotide kinase; oligomers forming the overlapping ends were treated in the same way, except that the specific activity of the ATP was tenfold higher, and added to the ligation mixture. The oligomer mixture was heated at 90°C for 5 minutes and annealed; dithiothreitol, unlabeled ATP, and T4 DNA ligase (final concentrations 20 m*M*, 0.1 to 1 m*M*, and 200 U/ml, respectively) were added. The final DNA concentration was never less than 10 μM per oligomer. The oligomers destined to become the 5′ overhanging Bam HI and Eco RI ends were added but they were not treated with the kinase in order to prevent these from self-ligating.

Oligomers were convergently ligated in eight groups, each group containing eight to ten oligomers, to yield DNA duplexes that were 40 to 50 base pairs in length, with single-stranded segments five bases in length at each of the 5′ ends (*12*). Oligomers were isolated by electrophoresis on 15 percent acrylamide gels (a mixture of acrylamide and bisacrylamide, 20:1, with 89 m*M* tris-borate buffer, *p*H 8.3, 2.5 m*M* EDTA), and sequenced (*11*). The eight DNA duplexes were joined to form the gene in two parts (*12*). Ligations of the DNA duplexes were performed under the same conditions as for the oligomers, but samples were not heated. The DNA concentration was 0.1 to 1 n*M* per duplex. The complete gene was assembled by ligating these two parts with a pBR322 plasmid that had been cut to remove the small region of DNA between Bam HI and Eco RI sites.

Transformation was effected by treatment with calcium (calcium shock) in the presence of sulfolane (*13*), and transformants were selected for ampicillin resistance. Of the clones isolated, several contained plasmids having the expected restriction digest map (Fig. 2). The structure of the cloned gene was verified by DNA sequencing (*11*).

While a number of different enzymes are being altered by means of recombinant DNA techniques, ribonuclease has certain advantages as a system for study. Six crystal structures of ribonuclease and its variants are now available (*14*), including three-dimensional structures of ribonuclease bound to reactants, products, and transition state analogs. Primary sequences for more than 40 analogous mammalian ribonucleases, mostly from herbivores, are known, indicating positions where alteration is possible and potentially interesting (*15*). There is a considerable amount of bioorganic and chemical information on ribonuclease available (*2*). Kinetic and thermodynamic methods are available making catalysis by ribonuclease subject to rigorous physical organic analysis.

In addition to being a system well suited to the study of catalysis, ribonuclease is appropriate for the study of many other important problems in biological chemistry. This enzyme represents a process that models virtually every step in protein folding. The aggregation between the S peptide and the S protein is an excellent model for chain association and has been studied by systematic alteration of S peptides (*16*). Ribonuclease is believed to have a hydrophobic nucleation site for folding (*17*). *Cis-trans* isomerization of proline is believed to determine the rate of folding (*18*). Ribonuclease is a conventional system for studying the formation of disulfide bonds (*19*). Furthermore, the protein is suited for detailed studies on the chemical nature of thermal stability of proteins (*20*).

Information from the primary amino acid sequences further guides research on this enzyme. Beintema has deduced the primary structures of ribonuclease from extinct fossil organisms that appear to be ancestral ruminants (*15*). It therefore now seems possible to prepare a series of ribonucleases that are intermediates in its recent evolutionary history. From this series, it should be apparent whether the evolution of ribonuclease is most accurately described as a gradual improvement in the protein, as a development characterized by major alterations in the protein's physical and catalytic properties, or as the random fixation of "neutral" mutations (*21*). Finally, certain ribonucleases show activity against tumors both in vitro and in vivo (*22*), and studies on the relation between the structure of the protein and its anticancer properties promises to be pharmacologically interesting.

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23. Supported by the Dreyfus Foundation, the Milton Fund, the Petroleum Research Fund, Alfred Bader, Harvard University, NSF grants PCM-8111659 (D.M.S.) and CHE 8213267 (J.K.E.), NIH grant 1 RO1 GM 30110-01A2 (J.S. and K.P.N.), and the Anna Fuller Fund (W.P.K.). We thank R. Meyers, J. Lingappa, and T. Maniatis for gifts of reagents, F. H. Westheimer and K. E. Bloch for equipment, and E. Brown and O. Makabe for helpful discussions. Address correspondence to S.A.B.

8 November 1983; accepted 27 January 1984

Insulin Receptor Phosphorylation May Not Be a Prerequisite for Acute Insulin Action

Abstract. *An antiserum to the insulin receptor mimicked insulin's acute actions on glucose transport, phosphorylation of integral membrane proteins, and internalization of the insulin receptor in isolated rat adipose cells. These insulinomimetic actions of the antiserum occurred without the equivalent increase in phosphorylation of the β subunit of the insulin receptor observed with insulin. Thus, a role of receptor phosphorylation in acute insulin action is now questioned.*

Since the initial observations by Kasuga *et al.* (*1*) that insulin can induce the phosphorylation of the β subunit (molecular weight, ≃ 95,000) of its own receptor in both IM-9 lymphocytes and H-35 hepatoma cells, there has been considerable speculation about the potential role of this phosphorylation in insulin action. Further studies have now extended the number of cell types in which this phenomenon can be shown (*2–5*). In addition, in common with the receptors for epidermal growth factor (EGF) (*6, 7*) and several other growth factors (*8, 9*), the partially purified insulin-receptor complex itself appears to mediate both the specific autophosphorylation of a tyrosine residue in its own β subunit (*3*) and the phosphorylation of tyrosine residues in exogenous protein substrates (*10, 11*). However, in the intact H-35 hepatoma cell, insulin induces multisite phosphorylations of the β subunit, with serine residues predominating (*1*).

Häring *et al.* (*2*) have demonstrated an insulin-induced increase in the phosphorylation of the β subunit of the insulin receptor in both intact rat adipose cells and a crude plasma membrane fraction prepared from these cells. This cell type is exquisitely sensitive to insulin and, consequently, has been extensively used for studies of insulin action.

In the present study, we used the rat adipose cell in an attempt to correlate insulin action with receptor phosphorylation. More specifically, we compared the action of insulin with that of a polyclonal antiserum (B-10) against the insulin receptor. This antiserum was derived from a patient with severe insulin resistance and acanthosis nigracans. Such antisera have pronounced insulinomimetic actions including both acute actions, such as inhibition of lipolysis and stimulation of glucose transport in adipose cells, and more long-term actions, such as stimulation of protein, RNA, and DNA synthesis (*12*). In contrast to agents such as H_2O_2, vitamin K_5, and spermine, these antisera appear to exert their effects through direct interactions with the insulin receptor. Although the immunoglobulin G (IgG) fraction from a rabbit polyclonal antiserum against the insulin receptor in rat liver membrane has been shown to stimulate insulin receptor autophosphorylation in a partially purified receptor preparation from 3T3-L1 cells (*4*), none of these antisera has been investigated with respect to insulin receptor phosphorylation in intact cells.

Isolated rat adipose cells were prepared as described (*13*). Glucose transport activity was measured by the 3-*O*-methylglucose uptake technique (*13*) and insulin binding, by the method of Cushman *et al.* (*14*). To assess the effects of insulin and antiserum B-10 to the insulin receptor on insulin receptor phosphorylation, cells were incubated for 2 hours at 37°C with [^{32}P]orthophosphate in a phosphate-free Krebs-Ringer buffer containing bicarbonate (10 m*M*), Hepes (30 m*M*), and 1 percent untreated bovine serum albumin. The cells were then incubated for 1 hour at 37°C with either no addition (basal) insulin or with heat-inactivated antiserum B-10. After incubation the cells were washed and homogenized, and plasma membranes and high- and low-density microsomes were prepared as described (*13*). The membrane fractions were solubilized in 1 percent Triton X-100, and the receptors quantitatively immunoprecipitated with antiserum B-2 to the insulin receptor (1:200 dilution) and staphylococcal protein A (*15*). The immunoprecipitates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (*15*). Control precipitations were performed with pooled normal human serum.

At the highest concentration of antiserum tested (1:10 dilution), glucose transport was stimulated to 93 ± 3 percent of the value obtained with a saturating concentration of insulin (Fig. 1). At this same concentration of antiserum, insulin binding was also completely inhibited. Half-maximal effects were observed at antiserum dilutions of 1:200 to 1:100 for both parameters. Figure 2A shows that both insulin and antiserum B-10 increased the phosphorylation of proteins of molecular weight ≃ 115,000 (115K), 43K, and 35K in the low-density microsomes and decreased the phosphorylation of a 26K protein in the plasma membranes. In fact, all of the changes in protein phosphorylation induced by insulin were mimicked by the antiserum, although quantitative differences be-