beled at the 5′ end were purified by Sephadex G-50 chromatography in a 0.3*M* sodium acetate, 0.01*M* tris-HCl, *p*H 7.9, 0.001*M* EDTA system, and concentrated by precipitation with ethanol.
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32. This is paper 2172 from the Laboratory of Genetics of the University of Wisconsin and paper No. 7 in the series "Charon phages for DNA Cloning." Paper No. 6 is (*2*). Supported by NIH grant GM21812 to F.R.B., NIH grant CA-07175 to McArdle Laboratory (W. F. Dove), an NSF predoctoral fellowship and an NIH training grant to McArdle Laboratory (M.E.F.), NIH training grant T32 CA09075 (K.D.-T.), and NIH training grant 144-J825 (D.D.M.). We thank W. F. Dove for support and advice, Nigel Godsen for critical reading of the manuscript, Ed Kopetsky and Brenda Dierschke for technical assistance, R. Roberts for hospitality in his laboratory, A. Maxam for providing the detailed sequencing procedures and A. Honigman for discussions concerning AvaX. This work was done under NIH guidelines, which call for EKI, PI.

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Expression in *Escherichia coli* of a Chemically Synthesized Gene for the Hormone Somatostatin

Abstract. *A gene for somatostatin, a mammalian peptide (14 amino acid residues) hormone, was synthesized by chemical methods. This gene was fused to the* Escherichia coli *β-galactosidase gene on the plasmid pBR322. Transformation of* E. coli *with the chimeric plasmid DNA led to the synthesis of a polypeptide including the sequence of amino acids corresponding to somatostatin. In vitro, active somatostatin was specifically cleaved from the large chimeric protein by treatment with cyanogen bromide. This represents the first synthesis of a functional polypeptide product from a gene of chemically synthesized origin.*

The chemical synthesis of DNA and recombinant DNA methods provide the technology for the design and synthesis of genes that can be fused to plasmid elements for expression in *Escherichia coli* or other bacteria. As a model system we have designed and synthesized a gene for the small polypeptide hormone, somatostatin (Figs. 1 and 2). The major considerations in the choice of this hormone were its small size and known amino acid sequence (*1*), sensitive radioimmune and biological assays (*2*), and its intrinsic biological interest (*3*). Somatostatin is a tetradecapeptide; it was originally discovered in ovine hypothalamic extracts but subsequently was also found in significant quantities in other species and other tissues (*3*). Somatostatin inhibits the secretion of a number of hormones, including growth hormone, insulin, and glucagon. The effect of somatostatin on the secretion of these hormones has attracted attention to its potential therapeutic value in acromegaly, acute pancreatitis, and insulin-dependent diabetes.

The overall construction of the somatostatin gene and plasmid was designed to result in the in vivo synthesis of a precursor form of somatostatin (see Fig. 1), expected to have biological activity, but could be converted to a functional form by cyanogen bromide cleavage (*4*) after cellular extraction. The synthetic somatostatin gene was fused to the lac operon because the controlling sites of this operon are well characterized.

Given the amino acid sequence of somatostatin, one can design from the genetic code a short DNA fragment containing the information for its 14 amino acids (Fig. 2). The degeneracy of the code allows for a large number of possible sequences that could code for the same 14 amino acids. Therefore, the choice of codons was somewhat arbitrary except for the following restrictions. First, amino acid codons known to be favored in *E. coli* for expression of the MS2 genome were used where appropriate (*5*). Second, since the complete sequence would be constructed from a number of overlapping fragments, the fragments were designed to eliminate undesirable inter- and intramolecular pairing. And third, G·C-rich (guanine-cytosine) followed by A·T-rich (adenine-thymine) sequences were avoided since they might terminate transcription (*6*).

Eight oligonucleotides varying in

in Fig. 2 as A through H, were synthesized by the triester method (*7*). In addition to the 14 codons for the structural information of somatostatin, several other features were built into the nucleotide sequence. First, to facilitate insertion into plasmid DNA, the 5′ ends have single-stranded cohesive termini for the Eco RI and Bam HI restriction endonucleases. Second, a methionine codon precedes the normal NH_2-terminal amino acid of somatostatin, and the COOH-terminal codon is followed by two nonsense codons.

In the cloning and expression of the synthetic somatostatin gene we used two plasmids. Each plasmid has an Eco RI substrate site at a different region of the β-galactosidase structural gene (see Figs. 3 and 4). The insertion of the synthetic somatostatin DNA fragment into the Eco RI sites of these plasmids brings the expression of the genetic information in that fragment under control of the lac operon controlling elements. After the insertion of the somatostatin fragment into these plasmids, translation should result in a somatostatin polypeptide preceded either by ten amino acids (pSOM1) or by virtually the whole β-ga-

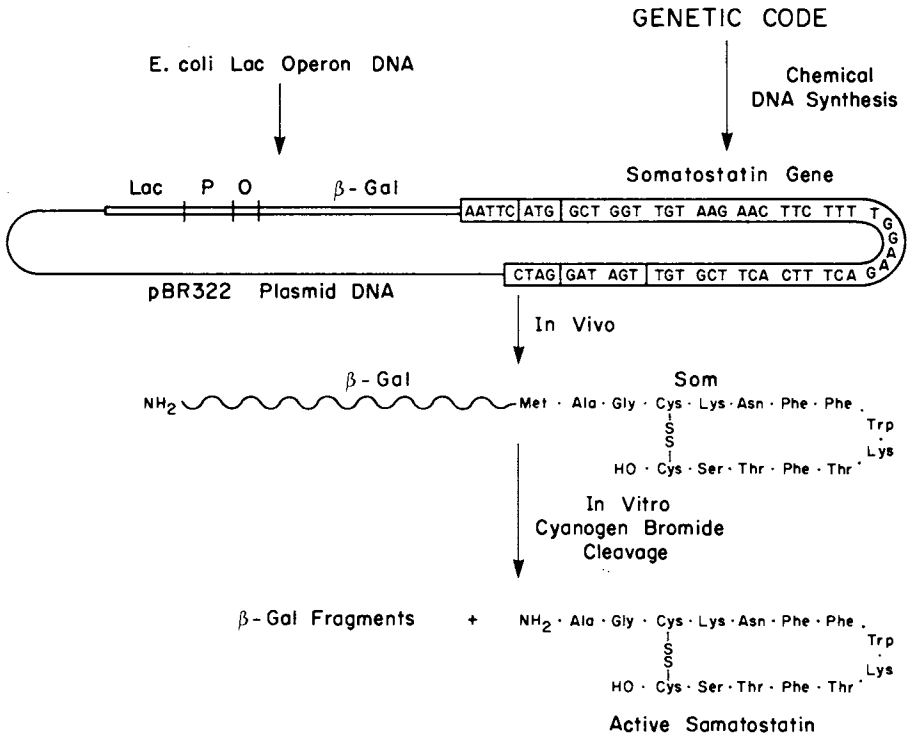


Fig. 1. Schematic outline of the experimental plan. The gene for somatostatin, made by chemical DNA synthesis, was fused to the *E. coli* β-galactosidase gene on the plasmid pBR322. After transformation into *E. coli*, the chimeric plasmid directs the synthesis of a chimeric protein that can be specifically cleaved in vitro at methionine residues by cyanogen bromide to yield active mammalian peptide hormone.

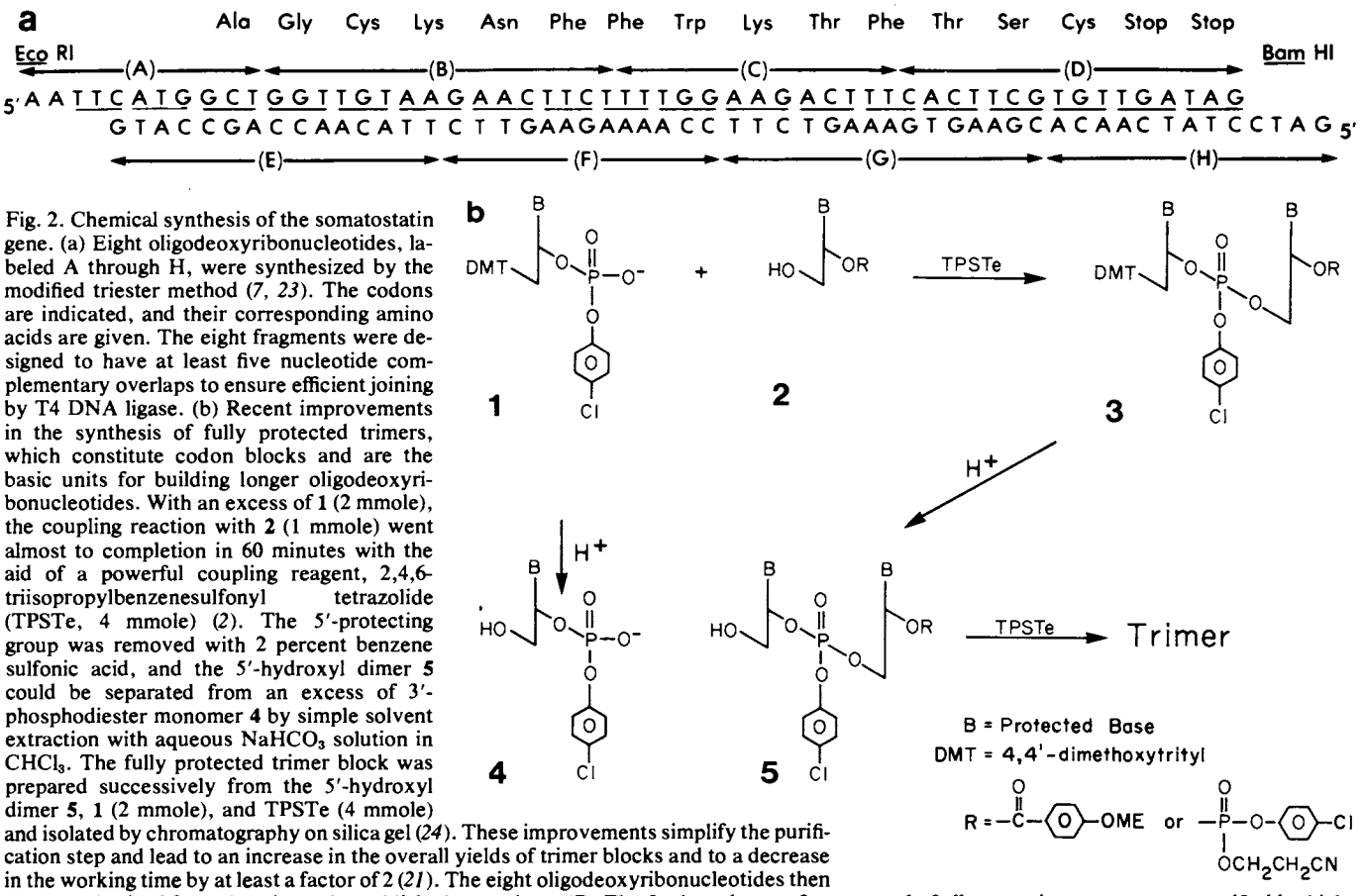
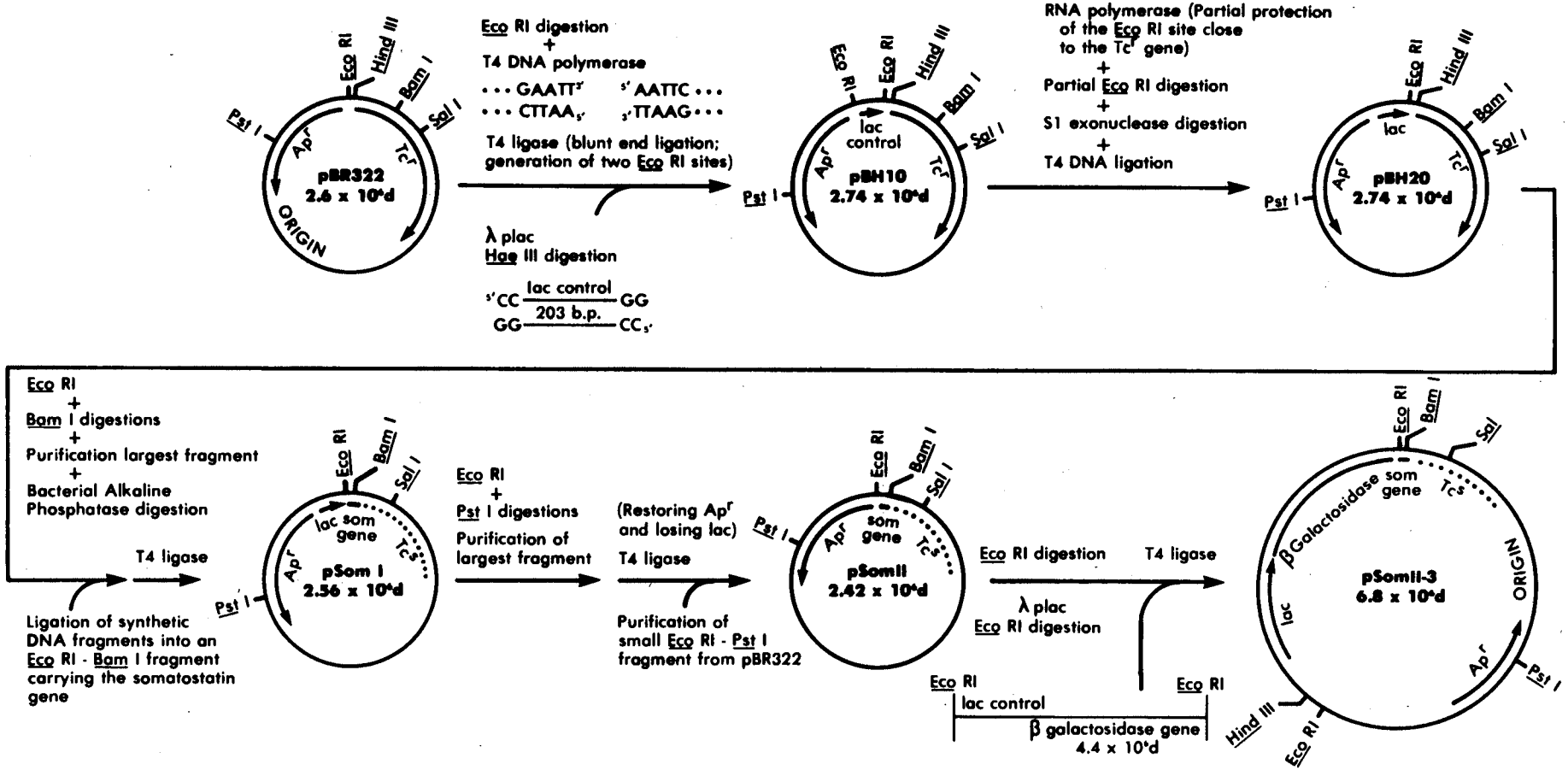
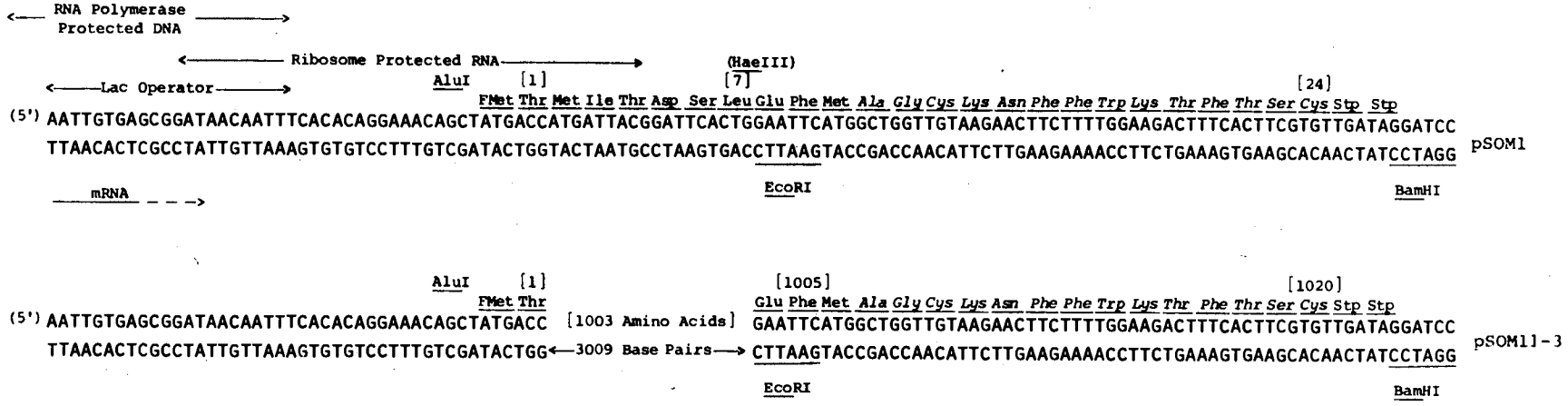


Fig. 2. Chemical synthesis of the somatostatin gene. (a) Eight oligodeoxyribonucleotides, labeled A through H, were synthesized by the modified triester method (*7*, *23*). The codons are indicated, and their corresponding amino acids are given. The eight fragments were designed to have at least five nucleotide complementary overlaps to ensure efficient joining by T4 DNA ligase. (b) Recent improvements in the synthesis of fully protected trimers, which constitute codon blocks and are the basic units for building longer oligodeoxyribonucleotides. With an excess of **1** (2 mmole), the coupling reaction with **2** (1 mmole) went almost to completion in 60 minutes with the aid of a powerful coupling reagent, 2,4,6-triisopropylbenzenesulfonyl tetrazolide (TPSTe, 4 mmole) (*2*). The 5′-protecting group was removed with 2 percent benzene sulfonic acid, and the 5′-hydroxyl dimer **5** could be separated from an excess of 3′-phosphodiester monomer **4** by simple solvent extraction with aqueous $NaHCO_3$ solution in $CHCl_3$. The fully protected trimer block was prepared successively from the 5′-hydroxyl dimer **5**, **1** (2 mmole), and TPSTe (4 mmole) and isolated by chromatography on silica gel (*24*). These improvements simplify the purification step and lead to an increase in the overall yields of trimer blocks and to a decrease in the working time by at least a factor of 2 (*21*). The eight oligodeoxyribonucleotides then were synthesized from the trimers by published procedures (*7*). The final products, after removal of all protecting groups, were purified by high-pressure liquid chromatography on Permaphase AAX (*25*). The purity of each oligomer was checked by homochromatography on thin-layer DEAE-cellulose and also by gel electrophoresis in 20 percent acrylamide (slab) after labeling of the oligomers with [γ-^{32}P]ATP in the presence of





lactosidase subunit structure (pSOM11-3).

The plasmid construction scheme (Fig. 3) begins with plasmid pBR322, a well-characterized cloning vehicle (*8*). The lac elements were introduced to this plasmid by insertion of an Hae III restriction endonuclease fragment (203 nucleotides) carrying the lac promoter, catabolite-gene-activator-protein binding site, operator, ribosome binding site, and the first seven amino codons of the β-galactosidase structural gene (*9*) (Figs. 3 and 4). The Hae III fragment was derived from λplac5 DNA. The Eco RI–cleaved pBR322 plasmid, which had its termini repaired with T4 DNA polymerase and deoxyribonucleotide triphosphates, was blunt-end ligated to the Hae III fragment to create Eco RI termini at the insertion points. Joining of these Hae III and repaired Eco RI termini generate the Eco RI restriction site (Figs. 3 and 4) at each terminus. Transformants of *E. coli* RR1 (*8*) with this DNA were selected for resistance to tetracycline (Tc) and ampicillin (Ap) on 5-bromo-4-chloro-indolylgalactoside (X-gal) medium (*10*). On this indicator medium, colonies constitutive for the synthesis of β-galactosidase by virtue of the increased number of lac operators titrating repressor, are identified by their blue color. Two orientations of the Hae III fragment are possible, but these were distinguished by the asymmetric location of an Hha restriction site in the fragment. Plasmid pBH10 was further modified to eliminate the Eco RI endonuclease site distal to the lac operator (pBH20).

The eight chemically synthesized oligodeoxyribonucleotides (Fig. 2) were labeled at the 5′ termini with [γ-^{32}P]ATP (adenosine triphophatase) by T4 polynucleotide kinase and joined with T4 DNA ligase. Through hydrogen bonding between the overlapping fragments, the somatostatin gene self-assembles and eventually polymerizes into larger molecules because of the cohesive restriction site termini. The ligated products were treated with Eco RI and Bam HI restriction endonucleases to generate the somatostatin gene (Fig. 2).

The synthetic somatostatin gene fragment with Eco RI and Bam HI termini was ligated to the pBH20 plasmid, previously treated with the Eco RI and Bam HI restriction endonucleases and alkaline phosphatase. The treatment with alkaline phosphatase provides a molecular selection for plasmids carrying the inserted fragment (*11*). Ampicillin-resistant transformants obtained with this ligated DNA were screened for tetracycline sensitivity, and several were examined for the insertion of an Eco RI–Bam HI fragment of the appropriate size.

Both strands of the Eco RI–Bam HI fragments of plasmids from two clones were analyzed by a nucleotide sequence analysis (*12*) starting from the Bam HI and Eco RI sites. The sequence analysis was extended into the lac-controlling elements; the lac fragment sequence was in-

Fig. 3 (facing page, left). Construction of recombinant plasmids. Plasmid pBR322 was used as the parental plasmid (*8*). Plasmid DNA (5 μg) was digested with the restriction endonuclease Eco RI. The reaction was terminated by extraction with a mixture of phenol and chloroform; the DNA was precipitated with ethanol and resuspended in 50 μl of T4 DNA polymerase buffer (*26*). The reaction was started by the addition of 2 units of T4 DNA polymerase. The reaction (held for 30 minutes at 37°C) was terminated by extraction with phenol and chloroform and precipitation with ethanol. The λplac5 DNA (3 μg) was digested with the endonuclease Hae III (*8*). The digested pBR322 DNA was blunt-end ligated with the Hae III–digested λplac5 DNA in a final volume of 30 μl with T4 DNA ligase (hydroxylapatite fraction) (*27*) in 20 m*M* tris-HCl (*p*H 7.6), 10 m*M* $MgCl_2$, 10 m*M* dithiothreitol, and 0.5 m*M* ATP for 12 hours at 12°C. The ligated DNA mixture was dialyzed against 10 m*M* tris-HCl (*p*H 7.6) and used to transform *E. coli* strain RR1 (*8*). Transformants were selected for tetracycline resistance (Tc^r) and ampicillin resistance (Ap^r) on antibiotic (20 μg/ml) minimal X-gal (40 μg/ml) medium (*10*). Colonies constitutive for the synthesis of β-galactosidase were identified by their blue color. After 45 independently isolated blue colonies were screened, three of them were found to contain plasmids with two Eco RI sites separated by approximately 200 base pairs (*28*). Plasmid pBH10 was shown to carry the fragment in the desired orientation, that is, lac transcription going into the Tc^r gene of the plasmid. Plasmid pBH10 was further modified to eliminate the Eco RI site distal to the lac operator and plasmid pBH20 was obtained (*29*). The nucleotide sequence from the Eco RI site into the lac-control region of pBH20 (data not shown), was confirmed. This plasmid was used for cloning the synthetic somatostatin gene. Plasmid pBH20 (10 μg) was digested with endonucleases Eco RI and Bam HI and treated with bacterial alkaline phosphatase (0.1 unit of BAPF, Worthington), and incubation was continued for 10 minutes at 65°C. The reaction mixtures were extracted with a mixture of phenol and chloroform, and the DNA was precipitated with ethanol (*30*). Somatostatin DNA (50 μl of a solution containing 4 μg/ml) was ligated with the Bam HI–Eco RI, alkaline phosphatase–treated pBH20 DNA in a total volume of 50 μl with the use of 4 units of T4 DNA ligase for 2 hours at 22°C (*31*). In a control experiment, Bam HI–Eco RI alkaline phosphatase–treated pBH20 DNA was ligated in the absence of somatostatin DNA under similar conditions. Both preparations were used to transform *E. coli* RR1. Transformants were selected on minimal X-gal antibiotic plates. Ten Tc^s transformants were isolated. In the control experiment no transformants were obtained. Four out of the ten transformants contained plasmids with both an Eco RI and a Bam HI site. The size of the small Eco RI–Bam HI fragment of these recombinant plasmids was in all four indesired somatostatin DNA fragment inserted (data not shown). Because of the failure to detect somatostatin activity from cultures carrying plasmid pSOM1, a plasmid was constructed in which the somatostatin gene could be located at the COOH-terminus of the β-galactosidase gene, keeping the translation in phase. For the construction of such a plasmid, pSOM1 (50 μg) was digested with restriction enzymes Eco RI and Pst I. A preparative 5 percent polyacrylamide gel was used to separate the large Pst I–Eco RI fragment that carries the somatostatin gene from the small fragment carrying the lac control elements (*12*). In a similar way plasmid pBR322 DNA (50 μg) was digested with Pst I and Eco RI restriction endonucleases, and the two resulting DNA fragments were purified by preparative electrophoresis on a 5 percent polyacrylamide gel. The small Pst I–Eco RI fragment from pBR322 (1 μg) was ligated with the large Pst I–Eco RI DNA fragment (5 μg) from pSOM1. The ligated mixture was used to transform *E. coli* RR1, and transformants were selected for Ap^r on X-gal medium. Almost all the Ap^r transformants (95 percent) gave white colonies (no lac operator) on X-gal indicator plates. The resulting plasmid, pSOM11, was used in the construction of plasmid pSOM11-3. A mixture of 5 μg of pSOM11 DNA and 5 μg of λplac5 DNA was digested with Eco RI. The DNA was extracted with a mixture of phenol and chloroform; the extract was precipitated by ethanol, and the precipitate was resuspended in T4 DNA ligase buffer (50 μl) in the presence of T4 DNA ligase (1 unit). The ligated mixture was used to transform *E. coli* strain RR1. Transformants were selected for Ap^r on X-gal plates containing ampicillin and screened for constitutive β-galactosidase production. Approximately 2 percent of the colonies were blue (such as pSOM11-1 and 11-2). Restriction enzyme analysis of plasmid DNA obtained from these clones revealed that all the plasmids carried a new Eco RI fragment of approximately 4.4 megadaltons, which carries the lac operon control sites and most of the β-galactosidase gene (*13*, *14*). Two orientations of the Eco RI fragment are possible, and the asymmetric location of a Hind III restriction in this fragment can indicate which plasmids had transcription proceeding into the somatostatin gene. The clones carrying plasmids pSOM11-3, pSOM11-5, pSOM11-6, and pSOM11-7 contained the Eco RI fragment in this orientation.

Fig. 4 (facing page, right). Nucleotide sequences of the lac-somatostatin plasmids. The nucleotide sequence of the lac control elements, β-galactosidase structural gene, and the synthetically derived somatostatin DNA, are depicted (*9*, *14*, *27*) along with the restriction endonuclease substrate sites. The nucleotide sequence of pSOM1, as depicted, was confirmed (legends to Figs. 3 and 5). The nucleotide sequence of pSOM11-3 was inferred from published data (*9*, *13*, *14*, *27*).

tact, and in one case, pSOM1, the nucleotide sequence of both strands were independently determined, each giving the sequence shown in Fig. 3. In the other case, the sequence was identical except for a base pair deletion (A·T) at a position equivalent to the junction of the B-C oligonucleotides in the original DNA fragment. The basis for the deletion is unclear.

The standard radioimmune assays (RIA) for somatostatin (*2*) were modified by decreasing the assay volume and by using phosphate buffer (Fig. 6). This modification proved suitable for the detection of somatostatin in *E. coli* extracts. Bacterial cell pellets, extracts, or cultures were treated overnight in 70 percent formic acid containing cyanogen bromide (5 mg/ml). Formic acid and cyanogen bromide were removed under vacuum over KOH before the assay. Initial experiments with extracts of *E. coli* strain RR1 (the recipient strain) (*10*) indicated that less than 10 pg of somatostatin could easily be detected in the presence of 16 μg or more of cyanogen bromide–treated bacterial protein. More than 2 μg of protein from formic acid–treated bacterial extracts interfered somewhat by increasing the background, but cyanogen bromide cleavage greatly reduced this interference. Reconstruction experiments showed that somatostatin is stable in cyanogen bromide–treated extracts.

The DNA sequence analysis of pSOM1 indicated that the clone carrying this plasmid should produce a peptide containing somatostatin. However, to date all attempts to detect somatostatin radioimmune activity from extracts of cell pellets or culture supernatants have been unsuccessful. Negative results were also obtained when the growing culture was added directly to 70 percent formic acid and cyanogen bromide. We calculate that *E. coli* RR1 (pSOM1) contains less than six molecules of somatostatin per cell. In a reconstruction experiment we have observed that exogenous somatostatin is degraded very rapidly by *E. coli* RR1 extracts. The failure to find somatostatin activity might be accounted for by intracellular degradation by endogenous proteolytic enzymes.

If the failure to detect somatostatin activity from pSOM1 was due to proteolytic degradation of the small protein (Fig. 4), attachment to a large protein might stabilize it. The β-galactosidase structural gene has an Eco RI site near the COOH-terminus (*13*). The available data on the amino acid sequence of this protein (*13*, *14*) suggested that it would be possible to insert the Eco RI–Bam HI somatostatin gene into the site and maintain the proper reading frame for the correct translation of the somatostatin gene (Fig. 4).

The construction of this plasmid is outlined in Fig. 3. The Eco RI–Pst fragment of the pSOM1 plasmid, with the lac-controlling element, was removed and replaced with the Eco RI–Pst fragment of pBR322 to produce the plasmid pSOM11. The Eco RI fragment of λplac5, carrying the lac operon control region and most of the β-galactosidase structural gene, was inserted into the Eco RI site of pSOM11. Two orientations of the Eco RI lac fragment of λplac5 were expected. One of these orientations would maintain the proper reading frame into the somatostatin gene, the other would not.

A number of independently isolated clones (with plasmid designations pSOM11-2 and pSOM11-3) were analyzed for somatostatin activity, as described above. In constrast to the results of experiments with pSOM1, four clones

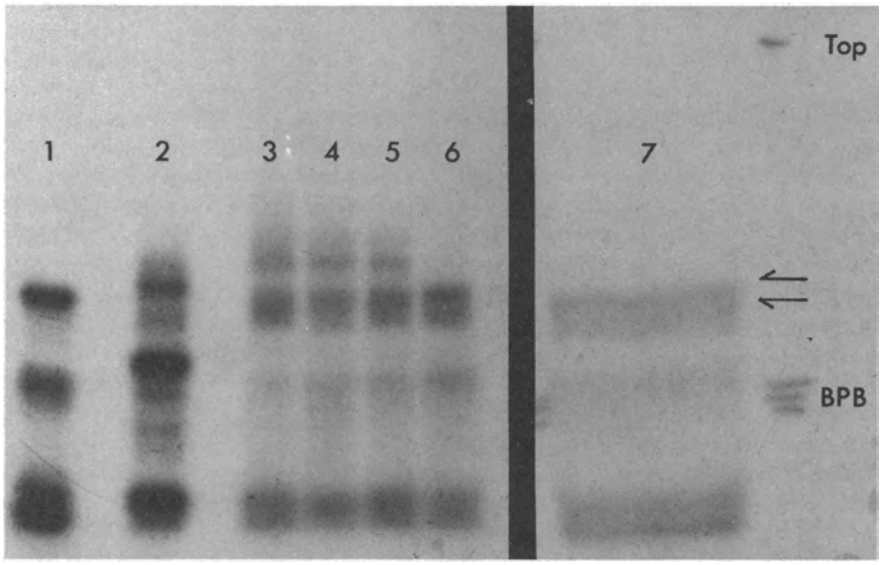


Fig. 5. Ligation and acrylamide gel analysis of somatostatin DNA. The 5′-OH termini of the chemically synthesized fragments A through H (Fig. 2a) were labeled and phosphorylated separately. Just prior to the kinase reaction, 25 μc of [γ-^{32}P]ATP (~ 1500 c/mmole) (*12*) was evaporated to dryness in 0.5-ml Eppendorf tubes. The fragment (5 μg) was incubated with 2 units of T4 DNA kinase (hydroxylapatite fraction, 2500 unit/ml) (*26*), in 70 m*M* tris-HCl, *p*H 7.6, 10 m*M* $MgCl_2$, and 5 m*M* dithiothreitol in a total volume of 150 μl for 20 minutes at 37°C. To ensure maximum phosphorylation of the fragments for ligation purposes, 10 μl of a mixture consisting of 70 m*M* tris-HCl, *p*H 7.6, 10 m*M* $MgCl_2$, 5 m*M* dithiothreitol, 0.5 m*M* ATP, and 2 units of DNA kinase were added, and incubation continued for an additional 20 minutes at 37°C. The fragments (250 ng/μl) were stored at −20°C without further treatment. Kinase-treated fragments A, B, E, and F (1.25 μg each) were ligated in a total volume of 50 μl in 20 m*M* tris-HCl (*p*H 7.6), 10 m*M* $MgCl_2$, 10 m*M* dithiothreitol, 0.5 m*M* ATP, and 2 units of T4 DNA ligase (hydroxylapatite fraction, 400 unit/ml) (*26*), for 16 hours at 4°C. Fragments C, D, G, and H were ligated under similar conditions. Samples (2 μl) were removed for analysis by electrophoresis on a 10 percent polyacrylamide gel and subsequent autoradiography (*16*) (lanes 1 and 2, respectively). The fast migrating material represents unreacted DNA fragments. Material migrating with the bromophenol blue dye (*BPB*) is the monomeric form of the ligated fragments. The slowest migrating material represents dimers, which form by virtue of the cohesive ends, of the ligated fragments A, B, E, and F (lane 1) and C, D, G, and H (lane 2). The dimers can be cleaved by restriction endonuclease Eco RI or Bam HI, respectively (data not shown). The two half molecules (ligated A + B + E + F and ligated C + D + G + H) were joined by an additional ligation step carried out in a final volume of 150 μl at 4°C for 16 hours. A sample (1 μl) was removed for analysis (lane 3). The reaction mixture was heated for 15 minutes at 65°C to inactivate the T4 DNA ligase. The heat treatment does not affect the migration pattern of the DNA mixture (lane 4). Enough restriction endonuclease Bam HI was added to the reaction mixture to cleave the multimeric forms of the somatostatin DNA in 30 minutes at 37°C (lane 5). After the addition of NaCl to a concentration of 100 m*M*, the DNA was digested with Eco RI endonuclease (lane 6). The restriction endonuclease digestions were terminated by phenol-chloroform extraction of the DNA. The somatostatin DNA fragment was purified from unreacted and partially ligated DNA fragments by preparative electrophoresis on a 10 percent polyacrylamide gel. The band indicated with an arrow (lane 7) was excised from the gel, and the DNA was eluted by slicing the gel into small pieces and extracting the DNA with elution buffer (0.5*M* ammonium acetate, 10 m*M* $MgCl_2$, 0.1 m*M* EDTA, and 0.1 percent sodium dodecyl sulfate) overnight at 65°C (*12*). The DNA was precipitated with two volumes of ethanol, centrifuged, redissolved in 200 μl of 10 m*M* tris-HCl (*p*H 7.6), and dialyzed against the same buffer, resulting in a somatostatin DNA

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