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 EKl, Pl.

19 July 1977; revised 20 September 1977

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). The precursor protein would not be 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 length from 11 to 16 nucleotides, labeled

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-

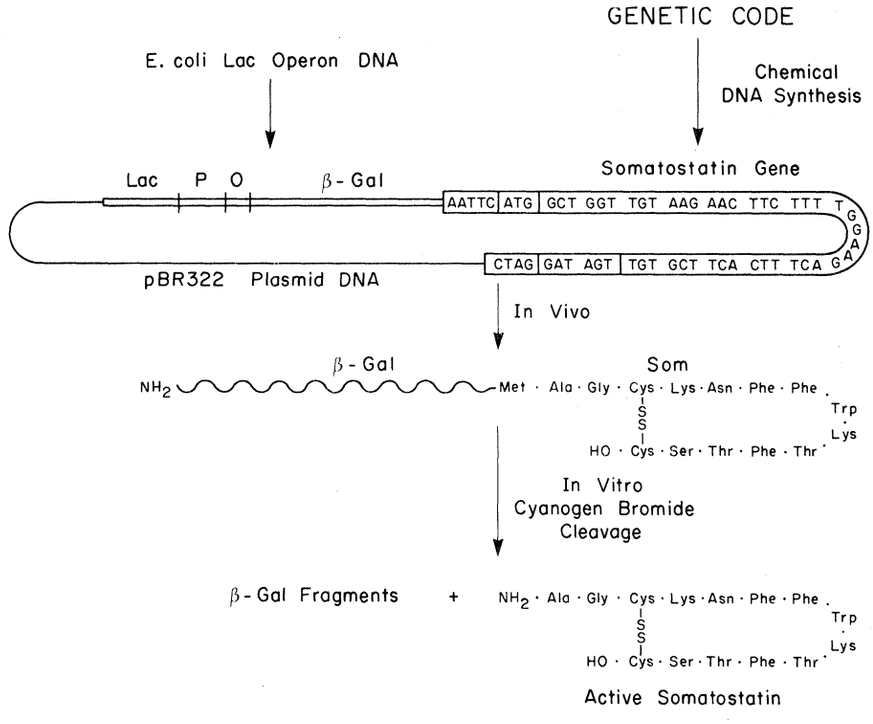


Fig. 1. Schematic outline of the experimental plan. The gene for somatostatin, made by chemical DNA synthesis, was fused to the *E. coli* β-galactosidase gene on the plasmid pBR322. After transformation into *E. coli*, the chimeric plasmid directs the synthesis of a chimeric protein that can be specifically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

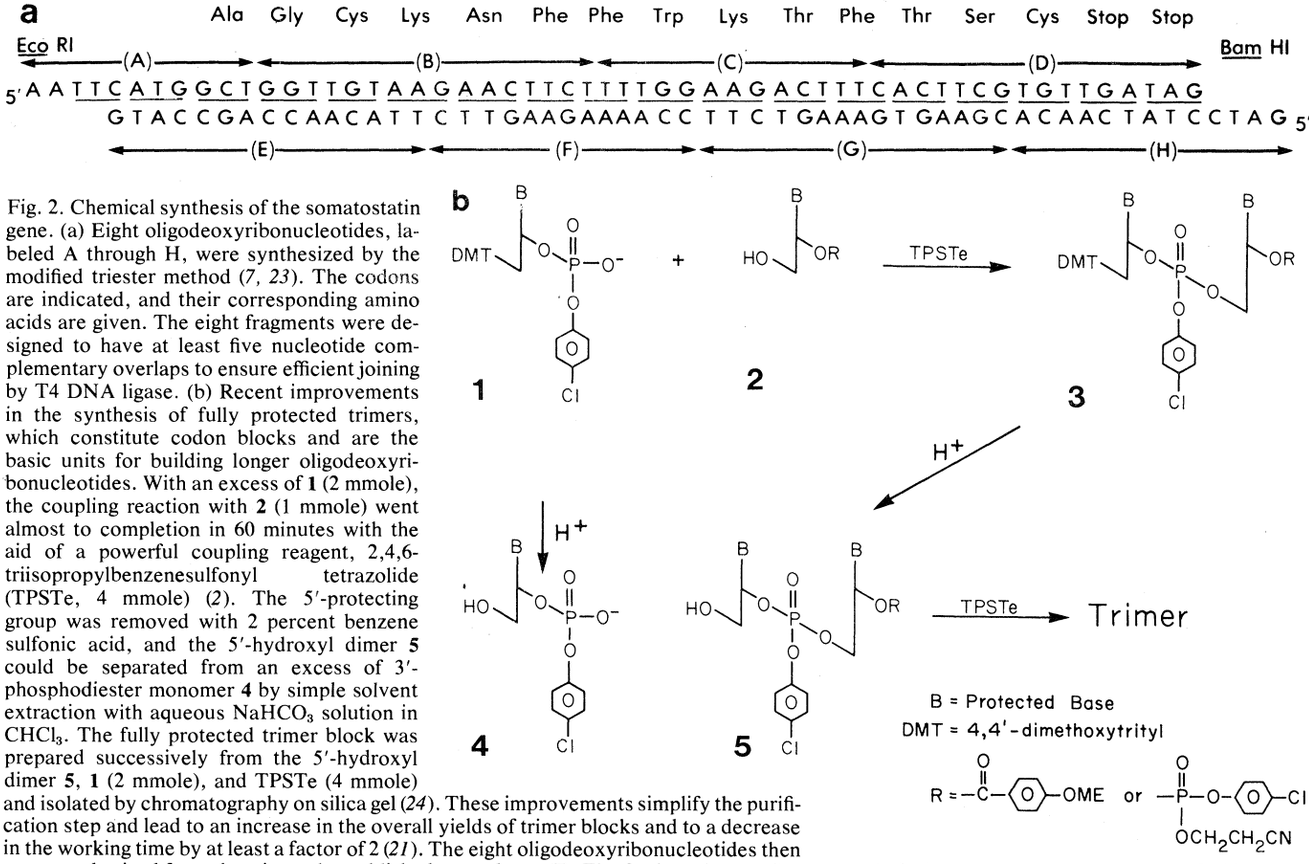
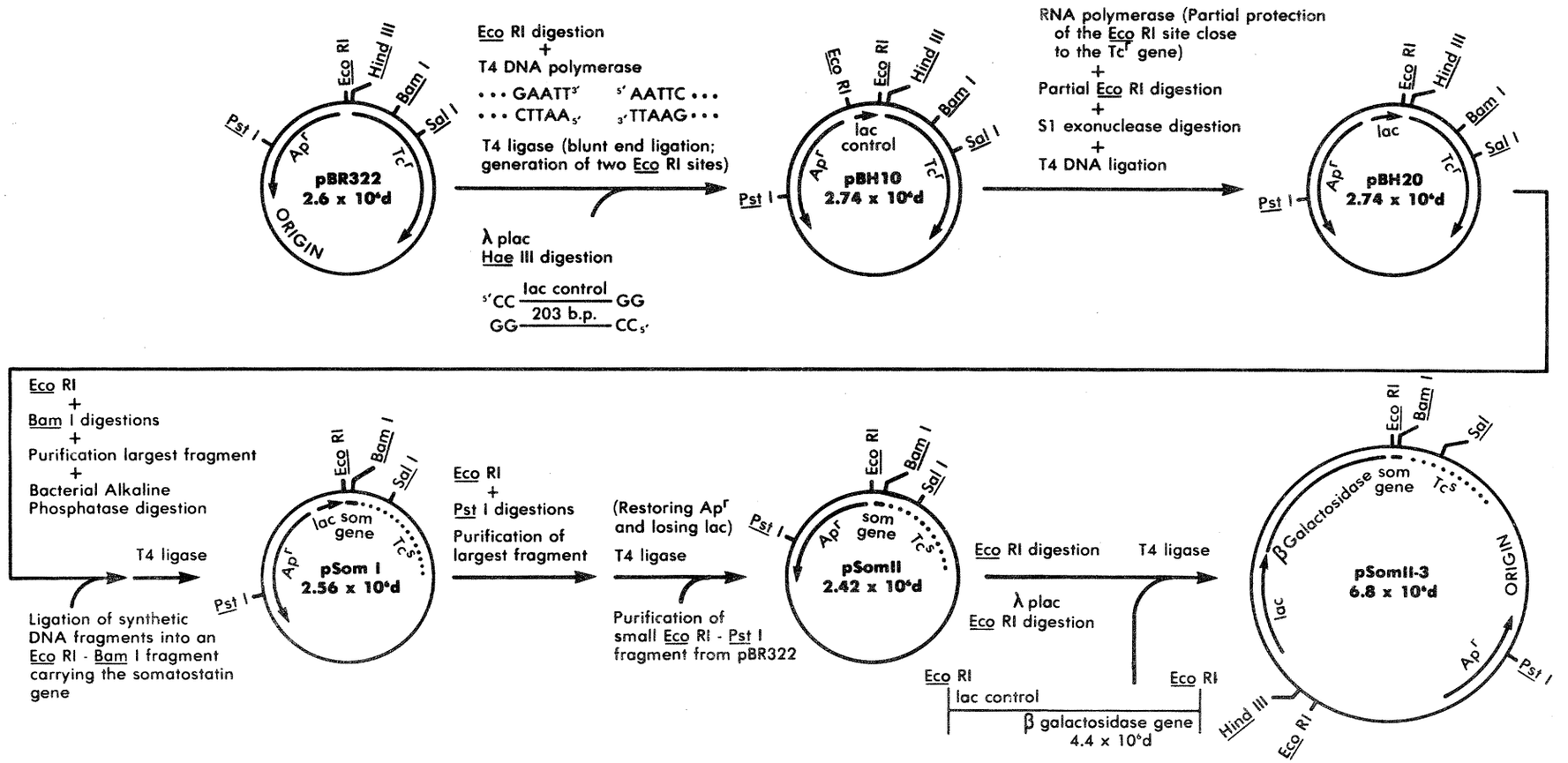
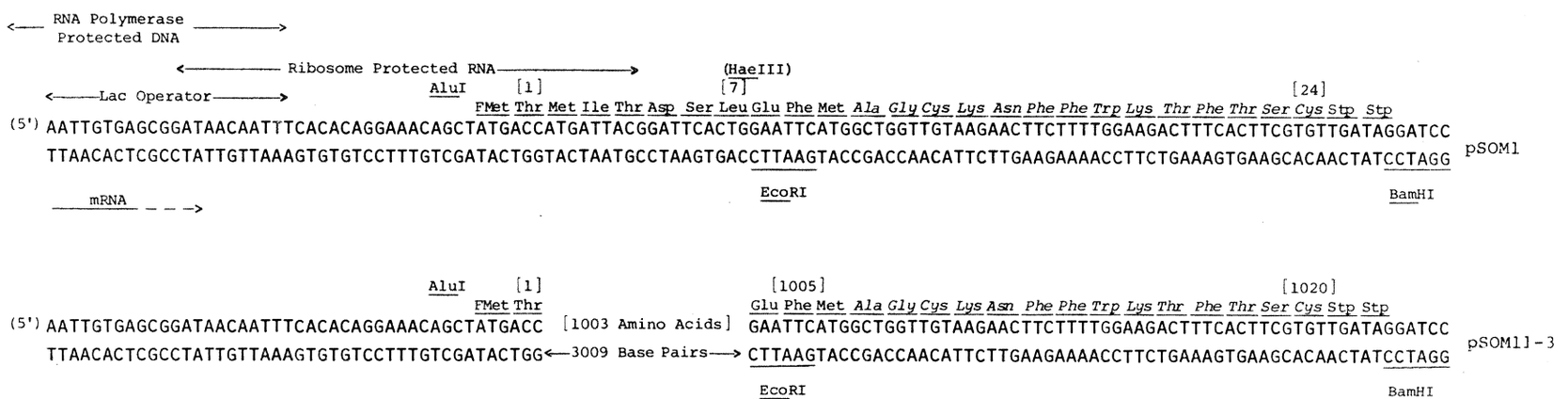


Fig. 2. Chemical synthesis of the somatostatin gene. (a) Eight oligodeoxyribonucleotides, labeled A through H, were synthesized by the modified triester method (*7, 23*). The codons are indicated, and their corresponding amino acids are given. The eight fragments were designed to have at least five nucleotide 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 polynucleotide kinase. One major labeled product was obtained from each DNA fragment.


pBR322
2.6 x 10⁶d
Eco RI digestion
+
T4 DNA polymerase
T4 ligase (blunt end ligation; generation of two Eco RI sites)
λ plac
Hae III digestion
lac control
203 b.p.
pBH10
2.74 x 10⁶d
RNA polymerase (Partial protection of the Eco RI site close to the Tc^r gene)
Partial Eco RI digestion
S1 exonuclease digestion
T4 DNA ligation
pBH20
2.74 x 10⁶d
Eco RI
Bam I digestions
Purification largest fragment
Bacterial Alkaline Phosphatase digestion
T4 ligase
Ligation of synthetic DNA fragments into an Eco RI - Bam I fragment carrying the somatostatin gene
pSom I
2.56 x 10⁶d
Pst I digestions
Purification of largest fragment
(Restoring Ap^r and losing lac)
Purification of small Eco RI - Pst I fragment from pBR322
pSomII
2.42 x 10⁶d
Eco RI digestion
λ plac
pSomII-3
6.8 x 10⁶d
lac control
β galactosidase gene
4.4 x 10⁶d


RNA Polymerase Protected DNA
Ribosome Protected RNA
(HaeIII)
AluI
Lac Operator
mRNA
EcoRI
BamHI
pSOM1
pSOM11-3
3009 Base Pairs
1003 Amino Acids

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 instances similar to the size of the in vitro prepared somatostatin DNA. Base sequence analysis (*12*) revealed that the plasmid pSOM1 had the desired 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*). The amino acid sequence of somatostatin is italicized. The amino acid sequence numbers of β-galactosidase are in brackets.

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 (pSOM11-3, 11-5, 11-6, and 11-7) were

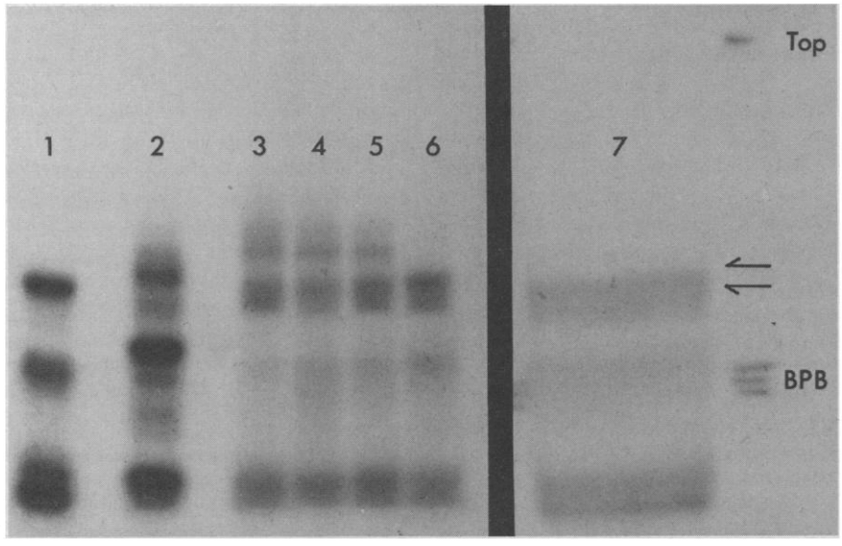
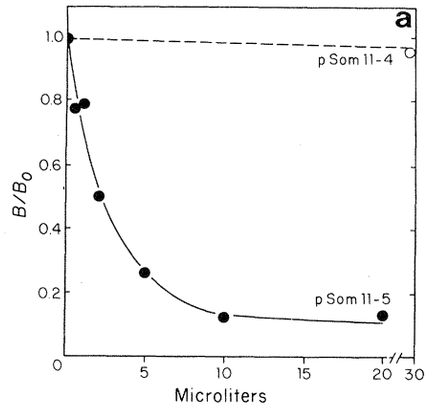


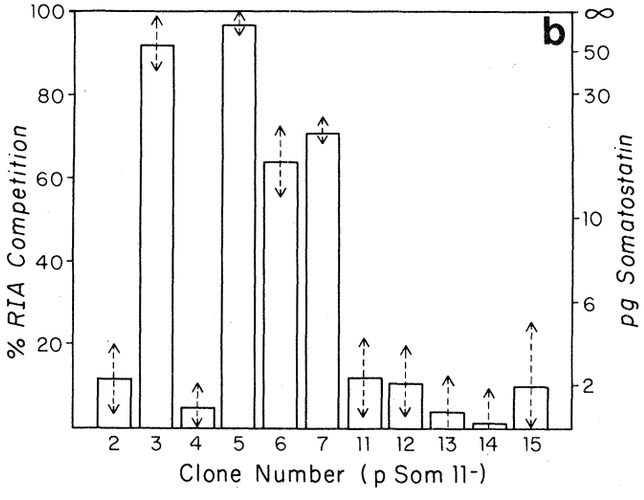
Fig. 5. Ligation and acrylamide gel analysis of somatostatin DNA. The 5′-OH termini of the chemically synthesized fragments A through H (Fig. 2a) were labeled and phosphorylated separately. Just prior to the kinase reaction, 25 μc of [γ-^{32}P]ATP (~ 1500 c/mmole) (*12*) was evaporated to dryness in 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 concentration of 4 μg/ml.

found to have easily detectable somatostatin radioimmune activity (Fig. 6, a and b). Restriction fragment analysis revealed that pSOM11-3, pSOM11-5, pSOM11-6, and pSOM11-7 had the desired orientation of the lac operon, whereas pSOM11-2 and 11-4 had the opposite orientation. Thus, there is a perfect correlation between the correct orientation of the lac operon and the production of somatostatin radioimmune activity.

The design of the somatostatin plasmid predicts that the synthesis of somatostatin would be under the control of the lac operon. The lac repressor gene is not included in the plasmid, and the recipient strain (*E. coli* RR1) contains the wild-type chromosomal lac repressor gene, which produces only 10 to 20 repressor molecules per cell (*15*). The plasmid copy number (and therefore the number of lac operators) is approximately 20 to 30 per cell and complete repression is impossible. The specific activity of somatostatin in *E. coli* RR1 (pSOM11-3) was increased by IPTG, an inducer of the lac operon (Table 1). As expected, the level of induction was low, varying from 2.4- to 7-fold. In experiment 7 (Table 1), the α activity (*14*), a measure of the first 92 amino acids of β-galactosidase, also was induced by a factor of 2. In several experiments (Table 1 and other experiments not shown), no somatostatin radioimmune activity was detected prior to cyanogen bromide cleavage of the total cellular protein. Since the antiserum used in the radioimmune assay, S39, requires a free NH_2-terminal alanine, no activity was expected prior to cyanogen bromide cleavage. After cleavage by cyanogen bromide, cell extracts were chromatographed on Sephadex G-50 in 50 percent acetic acid (Fig. 6c). In this system, somatostatin is well separated from excluded large peptides and fully included small molecules. Only extracts of clones positive for somatostatin exhibited radioimmune activity in the column fractions, and this activity elutes in the same position as chemically synthesized somatostatin.

The strains carrying the Eco RI lac operon fragment (such as pSOM11-2 and pSOM11-3) segregate with respect to the plasmid phenotype. For example, after





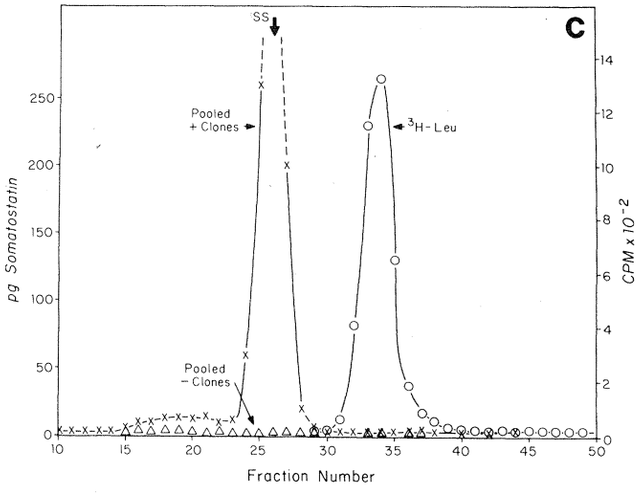


Fig. 6. Radioimmune assay for somatostatin activity. The assay used is a modification of existing methods (*2*). [Tyr[11]]Somatostatin (a gift from W. Vale) was iodinated by a chloramine T procedure (*2*). To assay for somatostatin, the sample, usually in 70 percent formic acid containing cyanogen bromide (5 mg/ml), was dried in a conical polypropylene tube (0.7 ml, Sarstedt) over moist KOH under vacuum. Then 20 μl of PBSA buffer [75 m*M* NaCl; 75 m*M* sodium phosphate (*p*H 7.2; bovine serum albumin (1 μg/ml): and sodium azide (0.2 mg/ml)] was added, followed by 40 μl of a [^{125}I]somatostatin mixture and 20 μl of a 1000-fold dilution in PBSA of rabbit antiserum to somatostatin S39 (*2*) obtained from W. Vale. The [^{125}I]somatostatin mixture contained (per milliliter of PBSA buffer) 250 μg of normal rabbit gamma globulin (Antibodies, Inc.), 1500 units of Trasylol (Calbiochem), and about 100,000 counts of [Tyr^{11}-^{125}I]somatostatin. After at least 16 hours at room temperature, 0.333 ml of goat antibody to rabbit gamma globulin (Antibodies, Inc., P = .03) in PBSA buffer was added to the sample tubes. The mixture was incubated for 2 hours at 37°C, cooled to 5°C, then centrifuged at 10,000*g* for 5 minutes. The supernatant was removed, and the radioactivity in the pellet was counted in a gamma counter. With the amount of antiserum used, 20 percent of the counts was precipitated with no unlabeled competing somatostatin. The background with infinite somatostatin (200 ng) was usually 3 percent. One-half maximum competition was obtained with 10 pg of somatostatin. (a) Competition by bacterial extracts. Strains *E. coli* RR1 (pSOM11-5) and *E. coli* RR1 (pSOM11-4) were grown at 37°C to 5 × 10^8 cell/ml in L broth. Then IPTG was added to a concentration of 1 m*M* and growth continued for 2 hours. Portions (1 ml) were centrifuged for a few seconds in an Eppendorf centrifuge, and the pellets were suspended in 500 μl of 70 percent formic acid containing cyanogen bromide (5 mg/ml). After approximately 24 hours at room temperature, the samples were diluted tenfold in water, and the indicated volumes were assayed in triplicate for somatostatin. B/B_0 is the ratio of [^{125}I]somatostatin bound in the presence of sample to that bound in the absence of competing somatostatin. Each point is the average of triplicate tubes. The protein content of the undiluted samples were determined to be 2.2 mg/ml for *E. coli* RR1 (pSOM11-5) and 1.5 mg/ml for *E. coli* RR1 (pSOM-4). (b) The initial screening of clones for somatostatin. Cyanogen bromide-treated extracts of 11 clones (such as pSOM11-2 and pSOM11-3) were made as described above for (a). A sample (30 μl) of each extract was taken in triplicate for radioimmune assay. The range of assay points is indicated. The values for picograms of somatostatin were read from a standard curve obtained as part of the same experiment. (c) Gel filtration of cyanogen bromide-treated extracts. Formic acid and cyanogen-treated extracts of the positive clones (11-3, 11-5, 11-6, and 11-7) were pooled (total volume, 250 μl), dried, and resuspended in 0.1 ml of 50 percent acetic acid. [^{3}H]Leucine was added, and the sample was applied to a column (0.7 by 47 cm) of Sephadex G-50 in 50 percent acetic acid. Portions (50 μl) of the column fractions were assayed for somatostatin. Pooled negative clone extracts (11-2, 11-4, and 11-11) were treated identically. On the same column known somatostatin (Beckman Instruments, Inc.) elutes as indicated (*SS*).

Table 1. Somatostatin radioimmune specific activity. Abbreviations: LB, Luria broth, IPTG, isopropylthiogalactoside; CNBr, cyanogen bromide; SS, somatostatin. Protein was measured by the method of Bradford (*32*).

Experiment	Strain	Medium	IPTG 1 m*M*	CNBr 5 mg/ml	SS/protein (pg/mg)
1	11-2	LB	+	+	< 0.1
	11-3	LB	+	+	12
	11-4	LB	+	+	< 0.4
	11-5	LB	+	+	15
2	11-3	LB	+	+	12
	11-3	LB	+	−	< 0.1
3	11-3	LB	+	+	61
	11-3	LB	−	+	8
	11-3	LB	+	−	< 0.1
4	11-3	LB	+	+	71
	11-3	VB + glycerol*	+	+	62
5	11-3	LB + glycerol	+	+	250
6	11-3	LB	+	+	320
	11-2	LB	+	+	< 0.1
7	11-3	LB	+	+	24
	11-3	LB	−	+	10

*Vogel-Bonner minimal medium plus glycerol.

about 15 generations, about one-half of the *E. coli* RR1 (pSOM11-3) culture was constitutive for β-galactosidase, that is, carried the lac operator, and about half of the nonconstitutive colonies were ampicillin-sensitive. Strains positive (pSOM11-3) and negative (pSOM11-2) for somatostatin are unstable, and, therefore, the growth disadvantage presumably comes from the overproduction of the large but incomplete and inactive galactosidase. The yield of somatostatin has varied from 0.001 to 0.03 percent of the total cellular protein (Table 1) probably as the result of the selection for cells in culture having plasmids with a deleted lac region. The highest yields of somatostatin have been from preparations where growth was started from a single Ap-resistant, constitutive colony. Even in these cases, 30 percent of the cells at harvest had deletions of the lac region.

Several moderate scale (up to 10 liters) attempts have been made to purify somatostatin from *E. coli* strain RR1 (pSOM11-3). The initial purification scheme was based on known purification properties of β-galactosidase followed by purification of the cyanogen bromide cleavage products of the chimeric protein. However, essentially all of the somatostatin activity found in the crude extract is insoluble and is found in the pellet from the first low speed centrifugation. The activity can be solubilized in 70 percent formic acid, 6*M* guanidinium hydrochloride, 8*M* urea, or 2 percent sodium docecyl sulfate. Somatostatin activity has been enriched approximately 100-fold from the cellular debris by cyanogen bromide cleavage, and subsequent alcohol extraction and chromatography on Sephadex G-50 in 50 percent acetic acid.

Recent improvements in the chemical synthesis of DNA provide the opportunity to synthesize quickly DNA with biological interest for genetic manipulation and experimentation. As illustrated earlier (*16, 17*), in vitro recombinant DNA techniques and molecular cloning enhance the experimental value of chemically synthesized DNA. There are two well-established methods for the synthesis of DNA. The phosphodiester method of Khorana and co-workers (*18*) and the more recently developed modified phosphotriester method (*7*). Both methods are capable of producing functional DNA (*16, 17, 19, 20*); however, the triester method is probably faster. Moreover, a method for rapidly synthesizing trimer blocks (codons) as building units for longer oligodeoxyribonucleotides (*21*) (Fig. 2b) has increased the speed of the triester method. From the trimer block library, a hexadecadeoxyribonucleotide now can be obtained in a week. We have established here that the DNA made with this improvement is functional.

The data establishing the synthesis of a polypeptide containing the somatostatin amino acid sequence are summarized as follows. (i) Somatostatin radioimmune activity is present in *E. coli* cells having the plasmid pSOM11-3, which contains a somatostatin gene of proven correct sequence and has the correct orientation of the lac Eco RI DNA fragment. Cells with the related plasmid pSOM11-2, which has the same somatostatin gene but an opposite orientation of the lac Eco RI fragment, produce no detectable somatostatin activity. (ii) As predicted by the design scheme, no detectable somatostatin radioimmune activity is observed until after cyanogen bromide treatment of the cell extract. (iii) The somatostatin activity is under control of the lac operon as evidenced by induction by IPTG, an inducer of the lac operon. (iv) The somatostatin activity cochromatographs with known somatostatin on Sephadex G-50. (v) The DNA sequence of the cloned somatostatin gene is correct. If translation is out of phase, a peptide will be made which is different from somatostatin at every position. Radioimmune activity is detected indicating that a peptide closely related to somatostatin is made, and translation must be in phase. Since translation occurs in phase, the genetic code dictates that a peptide with the exact sequence of somatostatin is made. (vi) Partially purified samples have been independently assayed by W. Vale (Salk Institute). He has confirmed our radioimmune activity with both antiserum S39, which is directed by the NH_2-terminal, and with antiserum S201 which interacts mainly with somatostatin positions 6 through 14. (vii) Finally, the above samples of *E. coli* RR1 (pSOM11-3) extract inhibit the release of growth hormone from rat pituitary cells, whereas samples of *E. coli* RR1 (pSOM11-2) prepared in parallel and with identical protein concentration have no effect on growth hormone release (*22*).

Our results represent the first success in achieving expression (that is, transcription into RNA and translation of that RNA into a protein of a designed amino acid sequence) of a gene of chemically synthesized origin. The large number of plasmid molecules per cell results in a substantial amount (at least 3 percent) of the cellular protein as the β-galactosidase-somatostatin hybrid. This molecule appears to be relatively resistant to endogenous proteolytic activity. There is evidence that abnormally short β-galactosidase peptides are degraded in *E. coli* (*14*) suggesting that the hybrid protein molecule expected from the first somatostatin-lac plasmid (pSOM1) is also rapidly degraded. The synthesis of many gratuitous proteins in *E. coli*, whether large enzymes or smaller polypeptides, may be undetectable for this reason. In cases where the amino acid composition of the protein is appropriate, the precursor technique described here can be employed. This approach could possibly be extended by taking advantage of proteolytic enzymes with amino acid sequence specificity.

The amount of somatostatin synthesized was variable and about a factor of 10 less than the maximum predicted yield. This variability could be interpreted in several ways. Protein degrada-

tion by endogenous proteases, the inability to fully solubilize the chimeric protein, and the selection of altered plasmids could all be contributing factors to the variability in yield. Although recombinant DNA experiments with chemically synthesized DNA are inherently less hazardous than those with DNA from natural sources, consideration should be given to the possible toxicity of the peptide product. A major factor in the choice of somatostatin was its proven low toxicity (*3*). In addition, the experiment was deliberately designed to have the cells produce not free somatostatin but rather a precursor, which would be expected to be relatively inactive. The cloning and growth of cell cultures were performed in a P-3 containment facility.

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28. The position of an asymmetrically located Hha I site in the 203 base pair Hae III lac control fragment (*9*) allows for the determination of the orientation of the Hae III fragment, now an Eco RI fragment, in these plasmids.
29. This was accomplished by preferential Eco RI endonuclease cleavage at the distal site by partial protection with RNA polymerase of the other Eco RI site localized between the Tc^r and lac promoters, which are only about 40 base pairs apart. After binding RNA polymerase, the DNA (5 μg) was digested with Eco RI (1 unit) in a final volume of 10 μl for 10 minutes at 37°C. The reaction was stopped by heating at 65°C for 10 minutes. The Eco RI cohesive termini were digested with S1 nuclease in a solution of 25 m*M* sodium acetate (p*H* 4.5), 300 m*M* NaCl, and 1 m*M* $ZnCl_2$ at 25°C for 5 minutes. The reaction mixture was stopped by the addition of EDTA (10 m*M*, final) and tris-HCl (p*H* 8) (50 m*M* final). The DNA was extracted with phenol-chloroform, precipitated with ethanol, and resuspended in 100 μl of T4 DNA ligation buffer. The T4 DNA ligase (1 μl) was added and the mixture was incubated at 12°C for 12 hours. The ligated DNA was transformed in *E. coli* strain RR1, and Ap^rTc^r transformants were selected on X-gal–antibiotic medium. Restriction enzyme analysis of DNA screened from ten isolated blue colonies revealed that these clones carried plasmid DNA with one Eco RI site. Seven of these colonies had retained the Eco RI site located between the lac and Tc^r promoters.
30. The alkaline phosphatase treatment effectively prevents self-ligation of the Eco RI–Bam HI treated pBH20 DNA, but circular recombinant plasmids containing somatostatin DNA can still be formed upon ligation. Since *E. coli* RR1 is transformed with very low efficiency by linear plasmid DNA, the majority of the transformants will contain recombinant plasmids (*11*).
31. After 10, 20, and 30 minutes, additional somatostatin DNA (40 ng) was added to the reaction mixture (the gradual addition of somatostatin DNA may favor ligation to the plasmid over self-ligation). Ligation was continued for 1 hour and then the mixture was dialyzed against 10 m*M* tris-HCl (p*H* 7.6).
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Cytidine 3′,5′-Monophosphate (Cyclic CMP) Formation in Mammalian Tissues

Abstract. *Mammalian tissues possess the capacity to synthesize cytidine 3′,5′-monophosphate (cyclic CMP) via the enzymatic conversion of cytidine 5′-triphosphate to cyclic CMP by cytidylate cyclase. Cyclic CMP formation occurs best in the presence of manganese or iron, at neutral* p*H, at 37°C, in the absence of detergents, and with whole tissue homogenate fractions. Thus, mammalian tissues are capable of synthesizing not only cyclic AMP and cyclic GMP, but also cyclic CMP.*

Adenosine 3′,5′-monophosphate (cyclic AMP) and guanosine 3′,5′-monophosphate (cyclic GMP) are purine cyclic nucleotides that are generally thought to influence or regulate numerous cell functions and biological events. In many instances, however, alterations in cell function cannot be accounted for by corresponding or concomitant alterations in the tissue concentrations of either of the two purine cyclic nucleotides. Therefore, the existence of other endogenous regulatory molecules is constantly being sought. Cytidine 3′,5′-monophosphate (cyclic CMP) was first identified in cells (leukemia L-1210) by Bloch, who demonstrated also that the addition of exogenous cyclic CMP to L-1210 cells in culture abolishes the characteristic temperature-dependent lag phase and stimulates the resumption of growth or proliferation of these leukemic cells (*1, 2*). These experimental findings suggest that cyclic CMP, a pyrimidine cyclic nucleotide, may play a biologic role in the control of proliferation of leukemic cells.

Shortly after the discovery of the natural occurrence of cyclic CMP in certain leukemic cells, an enzyme system capable of forming cyclic CMP from its naturally occurring substrate was found in murine myeloid leukemic tumors and in normal mouse liver and spleen (*3*). Thus, our experimental findings on the capacity of mammalian tissues to synthesize cyclic CMP support those of Bloch on the identification of cyclic CMP in malignant cells.

The properties and biologic importance of cytidylate cyclase in normal and malignant mammalian tissues were recently reported briefly (*4*). At the same time Cailla and Delagge reported on the