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Biochemistry

Production in *Escherichia coli* of biologically active secretin, a gastrointestinal hormone

(synthetic gene/27-desamidosecretin/radioimmunodetection/minicell/pancreatic secretion)

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ABSTRACT **A synthetic gene for porcine secretin was ligated with *Pst* I-cleaved pBR322 by its flanking synthetic *Pst* I linkers to produce a fused protein consisting of an amino-terminal portion of β-lactamase and an entire secretin molecule. The hybrid plasmid was transferred into competent *Escherichia coli* cells. The plasmids, which were proved in our investigation to contain the secretin gene in the desired orientation, were then screened for the production of secretin. This was shown by radioimmunoassay and gel electrophoretic analysis of the polypeptides that were synthesized in *E. coli* minicells transformed with the hybrid plasmids. Secretin so produced, whose carboxy terminal residue may not be amidated in contrast with natural porcine secretin, showed the same activity in stimulating pancreatic secretion in a bioassay with anesthetized rats as does natural secretin.**

Recombinant DNA technology already has yielded a few hormones such as somatostatin (1), insulin (2), growth hormone (3), β-endorphin (4), thymosin α_1 (5), and interferon (6). In the case of a small peptide hormone whose amino acid sequence has only been determined, a synthetic nucleotide coding sequence can be used in place of a natural gene (1, 2, 5). We applied this technique to the production of secretin, one of the gastrointestinal hormones. Secretin shows various physiological activities, such as inhibition of gastric acid secretion, stimulation of pancreatic secretion of water and bicarbonate, and potentiation of the stimulating action of cholecystokinin on pancreatic enzyme secretion (7). The amino acid sequences are known as yet only for secretins from three different animal species—i.e., pig, cow, and chicken (8–10). Clinically, porcine secretin has been used for diagnosis of pancreatic disease (11) and the Zollinger–Ellison syndrome (12). Its use in the treatment of duodenal ulcer disease also has been suggested (13). Porcine secretin consists of 27 amino acids, and its carboxyl-terminal residue, valine, is in amide form. We describe here the cloning and expression of the synthetic 27-desamidosecretin gene (14) in bacteria. The microbially-synthesized secretin, like natural secretin, showed activity in stimulating pancreatic secretion.

MATERIALS AND METHODS

Bacteria and DNA. *Escherichia coli* C 600 (m_K^-, r_K^-, *leu, thr, thi, lacY, supE 44, F*$^-$, *recBC, tonA*) was initially obtained from H. Boyer. *E. coli* carrying plasmid pBR322 (15) was provided by W. Schumann. pBR322 (a certified B2 vector in Japanese guidelines for recombinant DNA experiments), after amplification with chloramphenicol, was purified in two ethidium bromide/CsCl gradients from cleared lysates. Organic synthesis of the gene that specifies 27-desamidosecretin has been reported (14). *E. coli* Km 1196 (*F*$^-$, *thr, ara, leu, azi*^{s}, *tonA, minA, minB, gal,* λ$^-$, *str*^{r}, *malA, xyl, mtl, thi, sup*) (16), a minicell-producing strain, was provided by K. Matsubara.

Enzymes. Restriction enzyme *Hae* II was purchased from Bethesda Research Laboratories, *Hae* III and *Pst* I were from Takara (Kyoto, Japan), and T4 DNA ligase was from New England BioLabs. Egg white lysozyme, pancreatic DNase I, and RNase I were from Sigma.

Construction of a Hybrid Plasmid Consisting of *Pst* I-Cleaved pBR322 and a Synthetic Secretin Gene. Synthetic 27-desamidosecretin gene segment (0.2 μg, 33 pmol) was digested with 8 units of *Pst* I in 15 μl of 100 mM Tris·HCl, pH 7.3/50 mM NaCl/5 mM $MgCl_2$. pBR322 (16.5 μg, 6.3 pmol) was cleaved with 24 units of *Pst* I in 40 μl of the same buffer. The *Pst* I-cleaved secretin gene segment (3.3 pmol) was ligated to *Pst* I-cleaved pBR322 (1.6 pmol) with 600 units of T4 DNA ligase (one unit is the amount required to give 50% ligation of *Hin*dIII-cleaved λ DNA in 30 min at 16°C in a 20-μl final volume and 0.12 μM 5′-terminal end) at 14°C for 20 hr in 30 μl of 20 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/10 mM dithiothreitol/0.5 mM ATP. The ligation reaction mixture (5 μl) was used for transformation of *E. coli* C 600 (m_K^-, r_K^-). Transformation was carried out by the method of Kushner (17) with some modifications. After heat shock at 43.5°C, 4.8 ml of Z broth (1.6% Bacto-nutrient broth/1% Bacto-tryptone/0.2% glucose, pH 7.5) was added to the transformation mixture (≈200 μl), and the mixture was incubated at 37°C for 1 hr without shaking. Aliquots (0.1 ml) of the mixture were plated on L plates (1% Bacto-tryptone/0.5% yeast extract/0.1% glucose/0.5% NaCl/1.5% agar, pH 7.2) containing 10 μg of tetracycline per ml. Tetracycline-resistant (Tet^r) transformants that appeared after a 48-hr incubation at 37°C were transferred to L plates containing 10 μg of ampicillin per ml and incubated as described. Forty-five of 500 Tet^r colonies tested were ampicillin sensitive (Amp^s). The presence of hybrid plasmids of pBR322 and 27-desamidosecretin gene in these clones was checked by *Pst* I or *Hae* III cleavage of the plasmids prepared from each of them. Almost all of the Tet^{r}Amp^s clones had hybrid plasmids containing 27-desamidosecretin gene, and 15 of those were chosen for determination of the orientation of the secretin gene insert.

Preparation of Bacterial Extracts for Radioimmunoassay (RIA) and Bioassay of 27-Desamidosecretin. *E. coli* C 600 cells harboring pBR322 or hybrid plasmids were grown to 3 × 10^9 cells/ml in 3 liters of L broth containing 10 μg of tetracycline per ml and were harvested by centrifugation. The pellets were resuspended in 100 ml of 10 mM Tris·HCl, pH 8.0/1 mM phe-

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Abbreviations: bp, base pair(s); kDal, kilodaltons; RIA, radioimmunoassay; Tet^r, tetracycline resistant; Amp^s, ampicillin sensitive.

nylmethylsulfonyl fluoride. The suspension was incubated with 2.0 ml of 0.1 M Na_2EDTA (unbuffered) at 0°C for 5 min and then with 4.0 ml of egg white lysozyme (5 mg/ml) at 0°C for 30 min. The resulting spheroplasts were disrupted by ultrasonication, and the supernatants that were obtained by centrifugation were treated with pancreatic DNase I (100 μg/ml) and RNase I (100 μg/ml) at 37°C for 30 min. After centrifugation, solid ammonium sulfate was added to the supernatants to achieve 75% of saturation. The resulting precipitates were dissolved in 40 ml of Tris·HCl, pH 8.0/1 mM phenylmethylsulfonyl fluoride and dialyzed against the same buffer. Acetone was mixed with the protein solution (50 ml) to 75% (vol/vol). The resultant acetone-insoluble powder was dissolved in 30 ml of formic acid and incubated with cyanogen bromide (1 g) overnight at room temperature. Partial purification of the released 27-desamidosecretin followed the procedure for that of natural secretin from porcine upper intestine (18). After evaporation, peptides were extracted with isopropanol and methanol, followed by evaporation. Peptides were then extracted with 0.1 M acetic acid and applied to a Sephadex G-25 column. Peptides were eluted from the column with H_2O, and the eluent was divided into three fractions and lyophilized. These fractions were designated as SS-1, -2, and -3.

RIA. RIA of secretin was carried out with the Secretin Kit "Daiichi" (Daiichi Radioisotope Laboratories, Tokyo, Japan) by following the instructions supplied. Standard solutions (200 μl) containing synthetic porcine secretin (0–3,200 pg/ml) or serial dilutions of the SS-1, SS-2, or SS-3 fractions with 0.1 M phosphate buffer, pH 7.4/25 mM Na_2EDTA/1% bovine serum albumin were mixed with 1.0 ml of anti-secretin antibody that had been raised in rabbits against synthetic secretin, and the mixtures were incubated at 4°C for 4 days. ^{125}I-Labeled [Tyr^1]-secretin solution (100 μl, 0.02 μCi; 1 Ci = 3.7×10^{10} becquerels) was added to the reaction mixture and incubated at 4°C for 1 day. The solution (100 μl) of the second antibody, anti-rabbit γ-globulin antibody raised in goats against rabbit γ-globulin, was added and the incubation was continued 1 day more at 4°C. After centrifugation at 2,000 × *g* for 30 min, the resulting supernatants were removed, and the radioactivity in the pellets was measured.

Physical and Biological Containments. Experiments with recombinant DNA were performed under P3-B1 containment conditions as described by Japanese Guidelines for Recombinant DNA Experiments issued by the Japanese Prime Minister.

RESULTS

Construction of a Hybrid Plasmid Containing a Synthetic Secretin Gene. The synthetic 27-desamidosecretin gene comprising 81 nucleotide pairs is flanked by a 13-nucleotide segment (containing a *Pst* I recognition site and an ATG sequence coding for methionine at its 5′ terminus) and a 16-nucleotide segment [containing a *Pst* I site and two stop signals (TGA and TAG) at its 3′ terminus of the coding strand] (Fig. 1). First, the synthetic polynucleotide was cleaved with *Pst* I, and the cleavage product was directly ligated to *Pst* I-cleaved pBR322 with T4 DNA ligase. The resultant hybrid plasmids were transferred into competent *E. coli* C 600 (m_K^-, r_K^-) cells. Because most of the Tet^rAmp^s clones would contain only one 27-desamidosecretin gene insert in a correct or wrong orientation, only a small number (45 clones) of such clones was examined for the presence of the hybrid plasmid.

Pst I-cleavage analysis of the plasmids prepared from each of the Tet^rAmp^s clones showed that almost all of the clones contained the pBR322–27-desamidosecretin gene hybrid plasmids. Fifteen of these clones were chosen for examination of the orientation and number of the secretin gene inserts and were des-

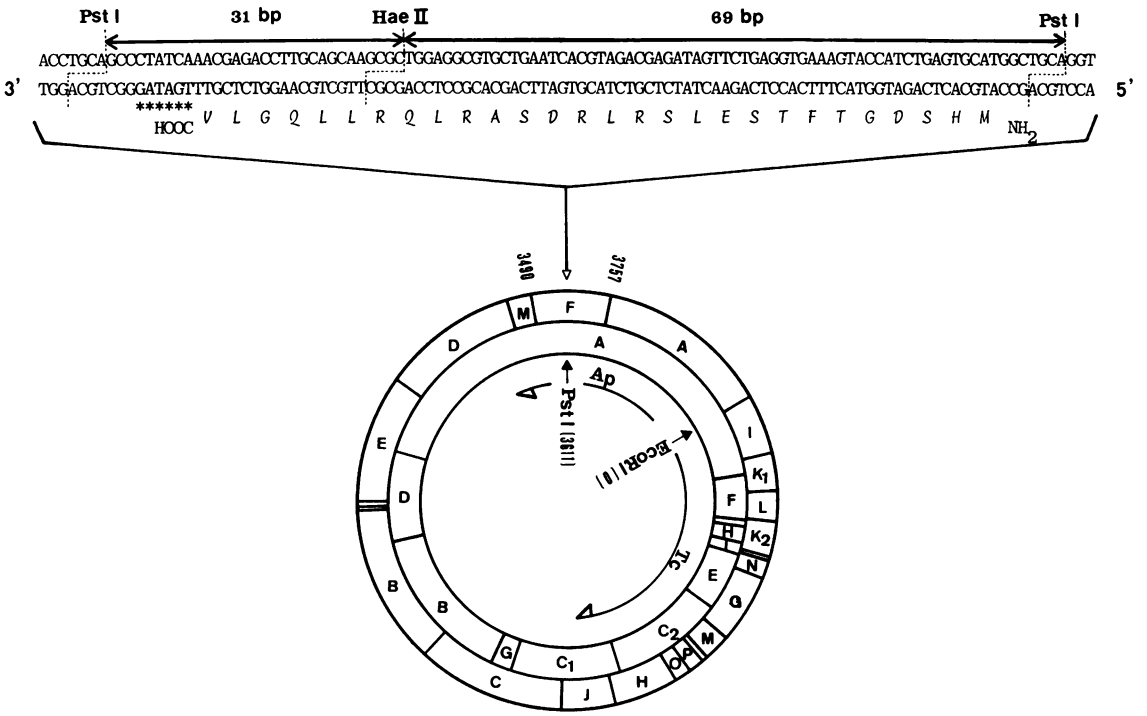

FIG. 1. Nucleotide sequence of a synthetic 27-desamidosecretin gene segment and its insertion site in pBR322. (*Upper*) Two stop signals for opal and amber nonsense codons are indicated with asterisks. The one-letter abbreviations recommended by the International Union of Pure and Applied Chemistry-International Union of Biochemistry Commission on Biochemical Nomenclature (19) are used for the amino acid sequence. (*Lower*) Restriction sites in pBR322 are indicated with their distance in base pairs measured clockwise from position 0 (*Eco*RI site). Inner circle, *Hae* II restriction fragments; outer circle, *Hae* III restriction fragments; Ap and Tc, regions of the plasmid conferring resistance to ampicillin and tetracycline, respectively.

ignated *E. coli* C 600 (pMG101) to *E. coli* C 600 (pMG115). The *Pst* I site was located in the *Hae* III fragment F [267 base pairs (bp)] in pBR322, dividing it into 145- and 122-bp segments, and insertion of the secretin gene resulted in the production of a new *Hae* III fragment of 367 bp (Fig. 1). The secretin gene insert had one recognition site for *Hae* II (between nucleotides 69 and 70 from the 5′ terminus of the coding strand); thus, in the event of the secretin gene being inserted in a correct orientation, the two fragments of 214 and 153 bp should have been generated by *Hae* II cleavage of the 367-bp *Hae* III fragment. Fig. 2 shows an example of such an orientation analysis. Clearly pMG103 contains a 27-desamidosecretin gene insert in a desired orientation, whereas pMG109 has a 27-desamidosecretin gene that is wrongly inserted. Four of the 15 clones examined contained a 27-desamidosecretin gene insert in a correct orientation. Because *E. coli* C 600 (pMG103) produced the largest number of the hybrid plasmid copies among all of the clones tested, it was chosen for further experiments of gene expression.

Radioimmunodetection of Secretin Produced by *E. coli*. To examine the expression of 27-desamidosecretin gene in *E. coli*, we chose three different approaches—i.e., RIA, electrophoretic analysis of the polypeptides produced in minicells, and bioassay. The 27-desamidosecretin was partially purified. RIA was carried out with the three fractions obtained by gel chromatography on a Sephadex G-25 column by the double-antibody method (20). Only the SS-2 fraction competitively inhibited the binding of ^{125}I-labeled [Tyr^1]secretin to the rabbit secretin antibody (Fig. 3). On the basis of the immunological activity in the fraction SS-2, it is estimated that 7 ng of the released 27-desamidosecretin can be obtained from a 3-liter culture (containing $\approx 9 \times 10^{12}$ cells), based on the assumption that its reactivity in the RIA is equal to that of natural porcine secretin. However, this value seems to be an underestimation because some bacterial component greatly interferes with this RIA system. It was found that the addition of a small amount of the extracts obtained from *E. coli* containing or lacking pBR322 into the reaction mixture for RIA containing the standard secretin greatly increased the radioactivity in the pellets obtained by centrifugation. In contrast with this, the extracts that had been prepared in the same manner from *E. coli* C 600 cells harboring pBR322 plasmid showed no immunological reactivity against secretin antibody (Fig. 3).

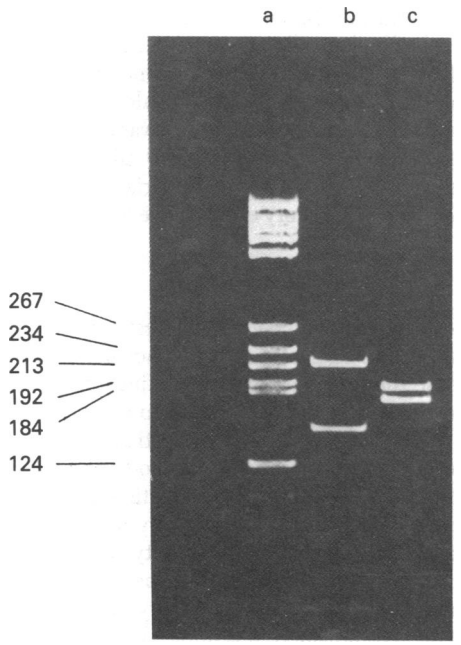

FIG. 2. Determination of the orientation of a 27-desamidosecretin gene insert. Hybrid plasmids (pMG101 to pMG115) were prepared from each of 15 Tet^{r}Amp^s clones of *E. coli* C 600 by the conventional cleared-lysate technique. Twenty micrograms of hybrid plasmids were digested with 60 units of *Hae* III in 240 μl of 6 mM Tris·HCl, pH 7.5/6 mM $MgCl_2$/6 mM NaCl. From the cleavage products, the 367-bp fragment (1.6 μg) was purified and cleaved with 24 units of *Hae* II in a 20-μl reaction mixture containing 10 mM Tris·HCl, pH 7.5/7 mM $MgCl_2$. After incubation at 37°C for 3 hr, the reaction mixture was heated at 68°C for 10 min, loaded on a 5% polyacrylamide gel, and electrophoresed. Lanes: a, *Hae* III-cleavage products of pBR322; b, *Hae* II-cleavage products of the 367-bp *Hae* III fragment of pMG103; c, *Hae* II-cleavage products of the 367-bp *Hae* III fragment of pMG109.

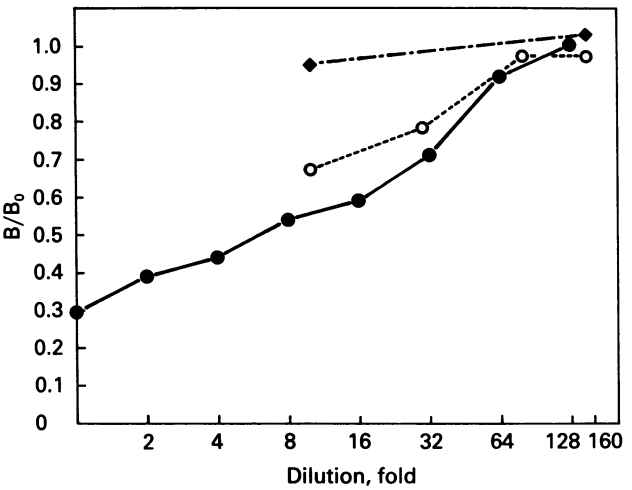

FIG. 3. RIA of 27-desamidosecretin produced in bacteria. Standard synthetic porcine secretin was dissolved initially at 3,200 pg/ml (nondiluting sample) and then was diluted serially with 0.1 M phosphate buffer, pH 7.4/25 mM Na_2EDTA/1% bovine serum albumin. SS-2 fraction obtained by gel filtration through a Sephadex G-25 column was lyophilized and redissolved in the dilution buffer, followed by serial dilution with the same buffer. B/B_0 = cpm in the pellet of the experimental sample/cpm in the pellet obtained with buffer only. ●, Standard synthetic porcine secretin; ○, SS-2 fraction obtained from *E. coli* C 600 (pMG103); ◆, SS-2 fraction obtained from *E. coli* C 600 (pBR322).

Electrophoretic Analysis of Polypeptides Produced in *E. coli* Minicells Harboring pMG103. To analyze the polypeptides encoded by pMG103, we transferred the hybrid plasmid into the minicells of *E. coli* Km 1196, and the polypeptides that were synthesized *de novo* were labeled with [^{35}S]methionine: β-lactamase-secretin fused protein would have seven methionine residues. The extracts from *E. coli* minicells lacking plasmids did not contain any detectable proteins (Fig. 4, lane d). Minicells harboring pBR322 produced five discrete, heavily labeled polypeptides (Fig. 4, lane c) of 31, 29 (these two discrete bands are not clear in Fig. 4, lane c), 26, 20, and 16 kilodaltons (kDal).

The electrophoretic pattern of polypeptides synthesized in minicells harboring pMG103 was markedly different (Fig. 4, lane b). Insertion of a synthetic secretin gene into the *Pst* I site of pBR322 resulted in the loss of the 31-, 29-, and 20-kDal polypeptides and induced the production of new 23-, 19.5-, and 16.5-kDal polypeptides, indicating that they are controlled by the segment of DNA located at the region of *bla* gene. It has been known that β-lactamase is first produced in bacterial cells in the premature form. A signal peptidase located in the bacterial membrane removes the amino-terminal 23-amino acid residues from the premature form to give the mature β-lactamase (22). The molecular mass of premature and mature β-lactamase can now be calculated precisely on the basis of their

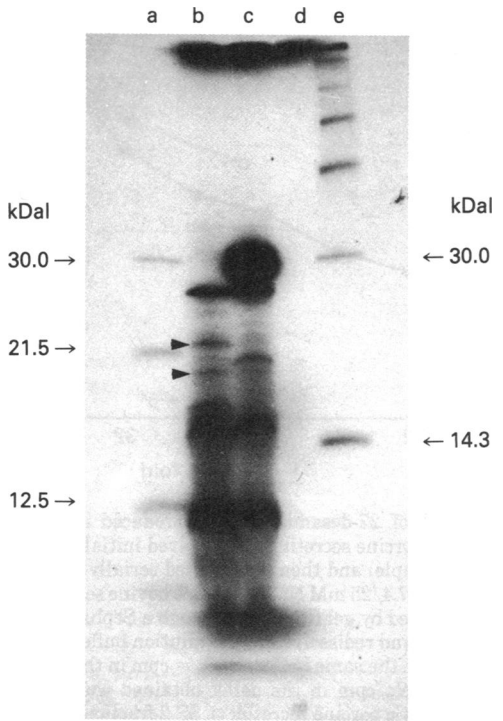

FIG. 4. Electrophoresis of polypeptides encoded by a hybrid plasmid containing a 27-desamidosecretin gene. After minicell-producing *E. coli* strain Km 1196 was transformed with pMG103 or pBR322, the transformants exhibiting Tet^r were selected and cultivated overnight in 500 ml of Davis' minimal medium (0.2% glucose/0.5% Casamino acids/5 mM $MgSO_4$/0.5% K_2HPO_4/0.1% ammonium sulfate/0.05% sodium citrate) supplemented with thiamine hydrochloride (20 μg/ml, final concentration) and thymine (100 μg/ml, final concentration) at 37°C. Preparation of minicells and labeling with [^{35}S]methionine of polypeptides synthesized *de novo* in the minicells were performed by the procedures of Roozen *et al.* (21) with some modifications, and the details will be described elsewhere. After ^{35}S labeling, the minicells were pelleted, resuspended in 100 μl of a sample buffer (20% glycerol/4% $NaDodSO_4$/0.125 M Tris·HCl, pH 6.8/10% 2-mercaptoethanol/0.04% bromophenol blue), and boiled. The aliquots (20 μl) were electrophoresed on a 15% polyacrylamide/$NaDodSO_4$ gel. Lanes: a, molecular mass standards: aprotinin (6.5 kDal), cytochrome *c* (12.5 kDal), soybean trypsin inhibitor (21.5 kDal), and carbonic anhydrase (30 kDal); b, extracts from *E. coli* Km 1196 (pMG103) minicells; c, extracts from *E. coli* Km 1196 (pBR322) minicells; d, extracts from *E. coli* Km 1196 minicells lacking plasmid; e, molecular mass standards: lysozyme (14.3 kDal), carbonic anhydrase (30 kDal), ovalbumin (46 kDal), bovine serum albumin (69 kDal), phosphorylase *b* (92.5 kDal), and myosin (200 kDal). The two triangles indicate unprocessed β-lactamase-secretin and processed β-lactamase-secretin fused proteins.

amino acid sequences deduced from the nucleotide sequence of pBR322 (23), and they are 31.56 and 28.93 kDal, respectively. From the above electrophoretic data, it can be concluded that the two major polypeptides of 31 and 29 kDal, which are found in *E. coli* Km 1196 (pBR322) cell extracts, are premature and processed forms of β-lactamase, respectively. It should be noted that most of the β-lactamase was detected as the mature enzyme rather than as the precursor (Fig. 4, lane c). This indicates that processing of β-lactamase can efficiently occur in *E. coli* minicells.

Because the secretin gene insert is linked in phase with regard to a coding frame to pBR322 at its *Pst* I site, and because the two stop signals are added to the 3′ terminus of the insert, the minicells containing the hybrid plasmid should produce a fused protein (23.45 kDal on the basis of the amino acid sequences of β-lactamase and secretin) consisting of (*i*) a 182-amino acid portion of unprocessed β-lactamase and a 27-desamidosecretin gene or (*ii*) a processed β-lactamase-27-desamidosecretin fused protein of 20.83 kDal, or both. Their calculated molecular masses were fairly close approximations of those estimated for the two new polypeptides that were detected in the extracts of minicells harboring pMG103. In view of our observation that no corresponding polypeptides can be produced in minicells containing a hybrid plasmid (such as pMG109) in which the secretin gene segment is inversely inserted (data not shown), it can be concluded that the two polypeptides having apparent molecular masses of 23 and 19.5 kDal are unprocessed β-lactamase-secretin and processed β-lactamase-secretin fused proteins, respectively. By comparison of the electrophoretic patterns in Fig. 4, lanes b and c, it is clear that the artificial fusion of the secretin sequence—therefore, the shortening of β-lactamase sequence itself—interferes with processing of the β-lactamase signal peptide. Derivation of the 16.5-kDal polypeptide found in *E. coli* Km 1196 (pMG103) extracts is unknown.

Bioassay. The practical method that has been used for bioassay of secretin is the stimulation of pancreatic secretion with anesthetized rats (24). The SS-2 fraction was lyophilized, and the dried material was dissolved in physiological salt solution. When aliquots of the solution were intravenously administered into rats, the stimulation of pancreatic secretion was clearly observed with all five rats used, as was observed with the standard porcine secretin. By comparing the increase in the volume of secreted pancreatic juice with that caused by the administration of the standard secretin whose activity was known, it was estimated that the activity corresponding to ≈0.58 Crick–Harper–Raper units of porcine secretin could be obtained from 9×10^{12} *E. coli* C 600 (pMG103) cells. It has been reported that the specific activity of thoroughly purified porcine secretin is 16,000 Crick–Harper–Raper units/mg (18). Therefore, 36 ng or 11.6 pmol of biologically active 27-desamidosecretin can be obtained from the culture, if one assumes that 27-desamidosecretin has the specific activity equivalent to that of natural porcine secretin. A control experiment was carried out with the preparation obtained from *E. coli* C 600 (pBR322) by the same procedures as were used for the bacteria bearing the recombinant DNA. Any stimulatory effect, however, was not observed.

DISCUSSION

We successfully synthesized a gastrointestinal hormone in bacterial cells. Studies on gastrointestinal hormones are of importance because they regulate gastrointestinal motor activity and gastric juice secretion and are related to gastric and duodenal ulcerations (25). Because all gastrointestinal hormones characterized are peptides, our present method can be extended to produce any and all of these gastrointestinal hormones.

It has been believed that the entirety of secretin molecule is indispensable for its biological activity because various derivatives and fragments of natural porcine secretin show either greatly reduced or practically no activity (26, 27). Although it has not yet been established, it is unlikely that the carboxyl-terminal residue of presently synthesized secretin is specifically amidated in *E. coli* cells. Thus, demonstration of a physiological activity of microbially synthesized secretin is somewhat of a surprise. However, the data on bioassay of microbially synthesized 27-desamidosecretin suggest that it might be more unstable than natural porcine secretin (data not shown). The low yield of biologically active secretin (equivalent to 2.3 molecules of porcine secretin per one bacterial cell) may be due to an instability of the 27-desamidosecretin. Even in the form of β-lactamase chimeric proteins, 27-desamidosecretin could be subjected to an intensive proteolytic degradation. The lability of natural porcine secretin also has been suggested because its

half-life in human body has been estimated to be only 4.06 min (28).

As a natural porcine secretin, carboxyl-terminal residues of many peptide hormones are in amide forms, but their roles in hormonal actions have hardly been investigated. Our finding suggests the availability of molecular cloning technology to investigate the relationship between the structures and functions of such peptide hormones. In this connection, the production of biologically active N^{α}-desacetylthymosin α_1 in *E. coli* is noteworthy because the amino-terminal group of thymosin α_1 isolated from calf thymus is acetylated (5).

It was found that the amount of microbially-produced secretin estimated by RIA was different from that obtained by bioassay, but this difference does not seem to indicate a difference in the biological activity between natural porcine and bacterial secretin. It was suggested that the underestimation in RIA may be attributable to the inhibitory actions of bacterial component(s). With regard to the secretin RIA, the presence of nonspecific interfering substance(s) in human serum has been suggested (29, 30).

β-Lactamase-secretin fused protein can be efficiently synthesized in *E. coli* minicells harboring pMG103, indicating the applicability of β-lactamase promoter for the expression of a synthetic gene. Whereas free β-lactamase can be efficiently processed in *E. coli* minicells, its signal peptide cannot readily be removed from β-lactamase-secretin fused protein (see Fig. 4, lanes b and c). This result suggests that efficient processing of β-lactamase precursor requires the translation of the carboxyl-terminal region of the enzyme protein. In agreement with this, Koshland and Botstein (22) have reported that β-lactamase is processed posttranslationally and that secretion of this protein requires the carboxyl-terminal amino acid sequence.

There are now two major questions to be raised: (*i*) whether microbially synthesized 27-desamidosecretin has a specific biological activity equivalent to that of natural porcine secretin and (*ii*) whether there is a difference in stability between the two types of secretin.

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