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Biosynthesis and periplasmic segregation of human proinsulin in *Escherichia coli*

(precursor processing/hybrid leader sequence/iodination/micro-sequence-analysis/secretion)

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ABSTRACT **A plasmid containing human preproinsulin cDNA inserted into the endonuclease *Pst* I site of the ampicillinase gene of plasmid pBR322 was modified by excision of large portions of the ampicillinase-coding region to produce a variety of gene fusion combinations, many of which generated proteins detectable with antisera to insulin or human C peptide. In one case a perfect hybrid of the NH_2-terminal half of the leader sequence of ampicillinase (residues −23 to −12) with the human preproinsulin prepeptide beginning at residue −13 was formed; the result was the synthesis and secretion of human proinsulin into the periplasmic space. We have characterized this protein immunologically and also by labeling it biosynthetically or by iodination followed by immunoprecipitation and automated amino acid sequence analysis. It contains the A and B chain regions of insulin as well as specific human C peptide immunodeterminants and is convertible to an insulin-like component by tryptic digestion. These results demonstrate that human proinsulin can be produced by bacteria and that this biosynthetic approach should prove feasible for the production of adequate amounts of human proinsulin for a variety of clinical studies and human insulin for therapeutic purposes.**

The development of recombinant DNA technology has led to the demonstration that bacteria can be genetically engineered to produce eukaryotic proteins, such as human insulin and interferons, for research and therapeutic purposes. In the case of insulin, Goeddel *et al.* (1) chemically synthesized DNA sequences encoding the A and B peptide chains and ligated these separately to the 3′ end of the *Escherichia coli* β-galactosidase gene. The expected fusion proteins were obtained, and from them the A and B chains were released by chemical degradation (with CNBr), purified individually, and recombined to form biologically active insulin (2).

An alternative approach to the above procedures, however, would be to achieve the biosynthesis of proinsulin, the immediate precursor to insulin. Previous studies have shown that proinsulin will oxidize spontaneously to form the correct disulfide bonds and can then be quantitatively converted to insulin by controlled digestion with trypsin and carboxypeptidase B (3, 4). The feasibility of this approach was recently demonstrated by Gilbert and coworkers (5), who constructed plasmids in which most of the coding sequence of rat preproinsulin was fused to portions of the prepeptide region of ampicillinase (penicillinase, EC 3.5.2.6). When used to transform *E. coli*, some of these constructs produced fused preproteins that were correctly cleaved to rat proinsulin and segregated into the periplasmic space. We report here that human preproinsulin containing a hybrid (prokaryotic and eukaryotic) prepeptide sequence can also be secreted and correctly processed to proinsulin in *E. coli*. We have characterized the secreted form by a highly sensitive technique involving iodination, immunoprecipitation, and sequence analysis on a micro scale. The results confirm the integrity of the protein and the accuracy of the cleavage process.

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MATERIALS AND METHODS

Plasmid Construction. Recombinant DNA was handled as prescribed by the National Institutes of Health guidelines. The parental plasmid, pHn677, was obtained by cDNA cloning of mRNA isolated from a human insulinoma and inserted into the endonuclease *Pst* I site of pBR322 by oligo(dC)·oligo(dG) tailing.

pHn677 contains the coding sequence for human preproinsulin (6) minus the NH_2-terminal seven amino acids of the prepeptide. To construct expression plasmids, most of the *amp* gene upstream from the preproinsulin sequence was removed in the following manner: (*i*) pHn677 was linearized with *Pvu* I, subjected to limited digestion with *Bal* 31, and cleaved with *Hin*dIII, and the large fragment was purified by agarose gel electrophoresis; (*ii*) pBR322 was cleaved with *Hin*cII, similarly digested with *Bal* 31, and cleaved with *Hin*dIII, and the DNA fragment containing the *amp* promoter was isolated; (*iii*) the two fragments were ligated with T4 DNA ligase and used to transform *E. coli* strain K-12 CS412; (*iv*) colonies were scored for expression by an *in situ* radioimmunoassay procedure (8).

Preparation of Osmotic Shock Fluids. Strain K-12 CS412 (auxotrophic for vitamin B1, threonine, proline, and leucine) (9) harboring recombinant plasmids was grown exponentially in Bonner–Vogel medium (10) containing glucose and supplemented with vitamin B1, threonine, proline, leucine, and tetracycline at 4 μg/ml. Cultures were centrifuged, and the cells were washed twice in 10 mM Tris·HCl buffer, pH 8.0/30 mM NaCl and then resuspended in 20% sucrose/30 mM Tris·HCl, pH 8.0/1 mM EDTA. After incubation at room temperature for 10 min the cells were centrifuged and resuspended in distilled H_2O. After 10 min at 4°C the cells were removed by centrifugation, yielding the osmotic shock fluid as a supernatant. For assay of total insulin-related peptides aliquots of cultures were disrupted with a Branson 350 sonicator for 1 min at full power. In these experiments insulin and human C peptide immunoassays were performed with commercial kits supplied by Amersham and Calbiochem/Boehringer, respectively.

Iodination and Immunoprecipitation. Iodination was performed by using an adaptation of the method of Freychet *et al.* (11). Protein samples (3–5 μg) were dissolved in 20 μl of 0.3 M sodium phosphate buffer, pH 7.4 followed by 7 μl of 0.2 M monobasic sodium phosphate and 5–10 μl (2.5–3.0 mCi; 1 Ci = 3.7×10^{10} becquerels) of carrier-free $Na^{125}I$ in water. The reaction was initiated by adding 15 μl of chloramine T (30 μg/ml). The samples were allowed to stand several hours in a hood and were then diluted to 0.5 ml with TAS buffer (0.1 M

Abbreviation: TAS buffer, Tris/albumin/sodium chloride buffer.

Tris·HCl, pH 7.8/0.05 M NaCl/2.5 mg of bovine serum albumin per ml). To reduce nonspecific reactions, 3 μl of normal guinea pig serum was added and the samples were incubated for 30 min at room temperature. Then 75 μl of a 10% suspension of formalin-fixed heat-killed *Staphylococcus aureus* (Cowan I strain) (Miles Laboratories) was added and after 30 min at 2°C the bacteria were removed by centrifugation. The supernatants were brought to 0.5% with Triton X-100, 3 μl of guinea pig anti-insulin serum was added, and the tubes were incubated 30 min at room temperature and then overnight at 4°C. Immune complexes were collected as above by addition of 75 μl of 10% *S. aureus* suspension followed by centrifugation and were either eluted with 50% (vol/vol) acetic acid or taken up in 100 μl of urea-containing buffer for reduction and carboxymethylation (see below).

Reduction and Carboxymethylation. For this procedure we used the method of Crestfield *et al.* (12), except dithiothreitol was substituted for 2-mercaptoethanol. After the coupling reaction was complete the protein and *S. aureus* mixture was precipitated by the addition of 5 vol of cold 20% trichloroacetic acid. After centrifugation the pellet was washed once with absolute ethanol, partially dried, and dissolved in 50% acetic acid. The *S. aureus* cells were removed by centrifugation and the supernatant solution was stored at −18°C.

RESULTS

Description of the Plasmids and Preliminary Analyses of Their Secretory Products. We initially inserted human preproinsulin cDNA, constructed from an insulinoma mRNA, into the *Pst* I site of pBR322 located within the ampicillinase gene (7). DNA sequence analysis of pHn677 determined that the preproinsulin cDNA was transcribed in the same direction as the ampicillinase gene but in a different reading frame. In order to achieve expression and to remove most of the ampicillinase coding sequence, we treated pHn677 with limited nuclease digestion as described in *Materials and Methods*. A number of recombinants were obtained that produced material reactive with anti-insulin or anti-C-peptide sera or both, and two of these, pJW1272 and pJW1452, were selected for further study. It was found that in both cases, the insulin and C peptide immunodeterminants were secreted into the periplasmic space (Table 1). Fig. 1 shows the distribution of both forms of immunoreactivity on gel filtration of the osmotic shock fluids prepared from small bacterial cultures. In the case of plasmid pJW1452 the major immunoreactive component was larger than proinsulin (Fig. 1A), with a molecular mass of about 15,000 daltons. A smaller peak of immunoreactive material similar in size to proinsulin was also detected (Fig. 1A). Treatment with trypsin generated a component having the exclusion characteristics of the free human C peptide, while the insulin immunoreactivity created a broad smear, suggesting that additional amino acid sequences might be attached to the NH_2 terminus of the B chain (data not shown). These results suggested that plasmid

Table 1. Insulin immunoreactivity and its secretion in plasmid-containing *E. coli*

Plasmid	Insulin immunoreactivity, ng/mg protein in whole cell sonicate	% secreted*
pHn677	0	—
pJW1452	9.4	87
pJW2172	2.2	91

* Calculated from the ratio of insulin content of osmotic shock fluid vs. whole cell sonicate.

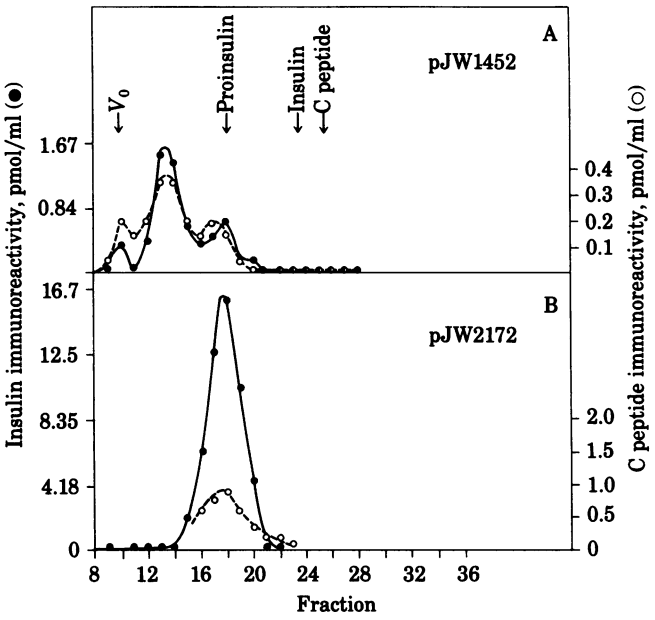

FIG. 1. Gel chromatography [1 × 50 cm Bio-Gel P60 (Bio-Rad); 2.5 M propionic acid] of proteins soluble in 2.5 M propionic acid from osmotic shock fluids of cultures containing plasmids pJW1452 (*A*) or pJW2172 (*B*). Fractions were dried under reduced pressure, redissolved in TAS buffer, and assayed for insulin (13) or human C peptide immunoreactivity (14). For antibody precipitation, formalin-fixed heat-killed *S. aureus* was used (5 μl of 10% suspension) (15). Elution positions of standard proteins are indicated in *A*. V_0, void volume.

pJW1452 generates a fusion protein containing 40–50 amino acids in addition to human proinsulin, as well as small amounts of a proinsulin-like component.

In contrast, gel filtration of the concentrated osmotic shock fluid from cultures of bacteria containing plasmid pJW2172 (Fig. 1*B*) gave rise to a single homogeneous peak of C peptide and insulin immunoreactivity that coeluted at the position of the proinsulin standard. Moreover, the ratio of C peptide to insulin immunoreactivity of this component (approximately 1:15) corresponded well to the known crossreactivity of human proinsulin in the highly specific human C peptide immunoassay currently in use in our laboratories (14), while this was not the case with the larger fusion product derived from plasmid pJW1452, which was less reactive (about ⅓ normal) with insulin antisera (data not shown).

These findings are readily explained by DNA sequence analyses of the fused regions of both plasmids. In pJW1452 the coding sequence indicated that the junction was between residue 65 of the ampicillinase sequence and residue −13 of the prepeptide region of human preproinsulin (Fig. 2). This particular arrangement would generate a fusion protein containing the complete ampicillinase prepeptide sequence followed by 42 residues of the mature ampicillinase protein and then by the sequence of human preproinsulin extending from residue −13 in the prepeptide to the normal termination site at the COOH-terminus of the A chain. Cleavage after the ampicillinase prepeptide sequence during secretion would generate a 14,600-dalton protein corresponding to the major immunoreactive peak seen in Fig. 1A. The size of this protein also has been confirmed by filter affinity transfer after $NaDodSO_4$ gel electrophoresis of the osmotic shock fluid (data not shown).

DNA sequence analysis of plasmid pJW2172 (Fig. 2) revealed that fusion had occurred between residue L12 of the ampicillinase leader peptide sequence (7) and residue −14 of the human prepeptide sequence, creating a perfect hybrid leader se-

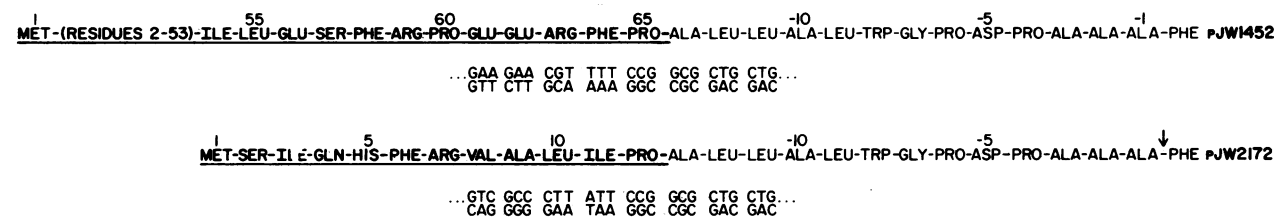

FIG. 2. Predicted amino acid sequences in the NH_2-terminal regions of ampicillinase fusion proteins coded by plasmids pJW1452 and pJW2172 derived from DNA sequence analysis (16). The underlined portions of the sequences are from the ampicillinase and are numbered according to Sutcliffe (7). The preproinsulin sequence is numbered from the cleavage site (arrow) for maturation to proinsulin. The DNA sequence in the junctional region is shown below each amino acid sequence.

quence containing roughly half of each presequence without any substitutions or modifications. This fused leader sequence preserves all of the structural features believed to be required for export and cleavage (17) and would be expected to generate a correctly cleaved secreted product. The experiments that follow demonstrate that this is the case.

Characterization of the pJW2172 Secreted Product. In order to further characterize the proinsulin-like material generated by plasmid pJW2172, a sample of the osmotic shock fluid containing about 0.05 nmol of the peptide was extracted with acid/ethanol and the extract was then chromatographed on a column of Bio-Gel P60 eluted with 2.5 M propionic acid (18). The fraction containing the peak insulin immunoreactivity (approximately 0.01 nmol) was dried under reduced pressure and divided into two equal aliquots, one of which was labeled by iodination and then immunoprecipitated with insulin antiserum. The resultant immunoprecipitate (containing 2.7×10^6 cpm), when examined on a Bio-Gel P30 column eluted with 3 M acetic acid, gave a single peak at the position of proinsulin, and on tube gel NaDodSO$_4$ electrophoresis it migrated as a single component with the same mobility as a bovine proinsulin standard (Fig. 3).

To determine whether cleavage of the presequence had occurred at the first residue of human proinsulin, a portion of the above immunoprecipitate (0.5 pmol), after reduction and carboxymethylation, was subjected to automated Edman degradation. The results are shown in Fig. 4*A*. Significant amounts of labeled tyrosine were found only at positions 16 and 26, as expected for human proinsulin. There was no indication of heterogeneity at the NH_2 terminus. Further corroboration on this point was obtained when the protein in the osmotic shock fluid from a culture grown in the presence of $^{35}SO_4$ was similarly immunoprecipitated, gel filtered, reduced, carboxymethylated, and then sequenced. The results shown in Fig. 5 demonstrate the presence, in correct register, of the sulfur-labeled B7 and B19 *S*-carboxymethylcysteine residues.

To further characterize this proinsulin-like material, we performed three additional experiments. First, an aliquot of the reduced and carboxymethylated iodinated immunoprecipitate was digested with trypsin and then submitted to automated

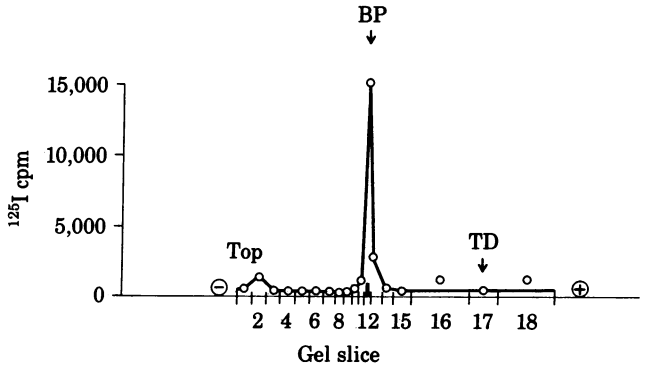

FIG. 3. Tube gel NaDodSO$_4$ electrophoresis of an aliquot of ^{125}I-labeled immunoprecipitated pJW2172 product. The graph represents the radioactivity measured in slices of the gel. The vertical bar indicates the position of the stained band of bovine proinsulin (BP). TD, tracking dye.

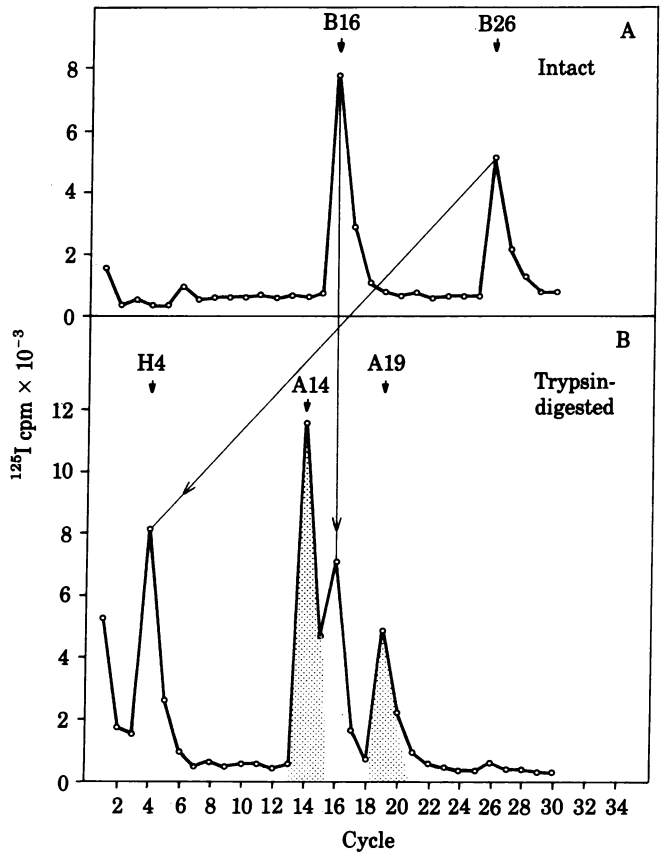

FIG. 4. Automated sequential Edman degradation of ^{125}I-labeled pJW2172 product. Each panel represents the analysis of approximately 0.5 pmol of immunoprecipitated protein, after reduction and carboxymethylation. For *B* the reduced and carboxymethylated protein was dissolved first in 100 μl of 0.2 M Tris·HCl, pH 8.6, and digested for 1 hr at 37°C with 20 μg of trypsin (treated with diphenylcarbamoyl chloride; Sigma) and then acidified with 50% acetic acid for transfer to the Beckman 890 sequencer. An aliquot of this digest was checked for completeness of digestion by gel filtration over a Bio-Gel P60 column in 2.5 M propionic acid. Over 95% of the protein had been cleaved to smaller fragments. The dimethylallylamine fast peptide program 102974 was used for sequence determination. The radioactivities of products from each cycle of Edman degradation were measured in a gamma counter. H designates the tryptic heptapeptide generated by cleavage at positions 22 and 29 in the B chain region.

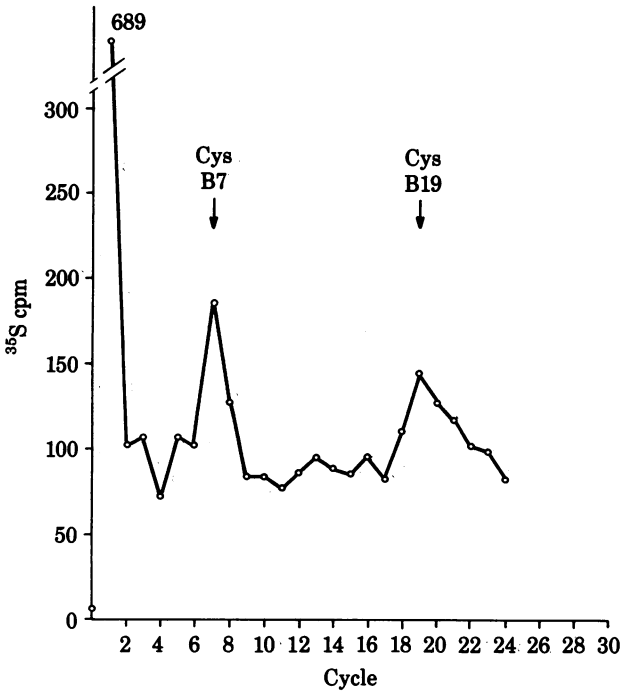

FIG. 5. Automated sequential Edman degradation of ^{35}S-labeled pJW2172 product. Cultures (13 ml) were allowed to incorporate ^{35}S for two generations while growing from a density of about 0.2 to 0.8 OD_{680} units; 7.5 mCi of ^{35}S was added to each medium and about 50% was incorporated. Osmotic shock fluids were prepared as described above and aliquots were used for immunoprecipitation studies. Immunoprecipitation was carried out on the unfractionated osmotic shock fluid and the products were reduced, carboxymethylated, and further purified by gel filtration. The radioactivities of the products from each cycle of degradation, dissolved in 3 ml of butyl chloride plus 10 ml of Aquasol (New England Nuclear), were measured in a liquid scintillation counter.

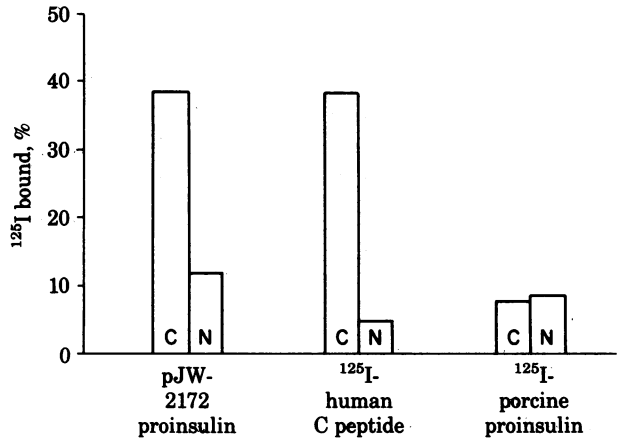

FIG. 6. Binding of ^{125}I-labeled pJW2172 immunoprecipitated proinsulin-like material to a human C peptide antiserum. The final dilution of the antiserum was 1:500. Small aliquots of the iodinated reduced and carboxymethylated protein, ^{125}I-labeled human C peptide with tyrosine added at the NH_2 terminus, or ^{125}I-labeled porcine proinsulin were tested with either normal rabbit serum (N) or human C peptide antiserum (C). Incubation was for 48 hr at 4°C. Precipitation was carried out with *S. aureus* (see Fig. 1 legend).

Edman degradation. The results shown in Fig. 4*B* demonstrate unequivocally that the protein contained the normal A chain, having tyrosines at positions 14 and 19, and that trypsin had also cleaved the B chain region at residue 22 (arginine) to generate a heptapeptide (19) which now contained the B26 tryosine residue at position 4. Second, the presence of C peptide immunodeterminants in this material (which had originally been obtained by immunoprecipitation with an insulin antiserum) was assesed by binding to an antiserum against human C peptide. The results shown in Fig. 6 demonstrate that the carboxymethylated bacterial protein reacted as well as authentic iodinated human C peptide, whereas porcine proinsulin did not bind significantly to the antiserum. Finally, we found that, when treated with trypsin and carboxypeptidase B (4), the pJW2172 proinsulin was converted to a component eluting at the position of insulin on gel filtration (data not shown). There was no indication of the release of free A or B chain material. On the basis of all the above evidence it is clear that the product generated by pJW2172 is intact human proinsulin.

DISCUSSION

The results presented here extend and amplify observations made previously by Talmadge *et al.* (5), who studied several ampicillinase–rat preproinsulin hybrid preprotein sequences. In their plasmids the fusions involved portions of both the ampicillinase and the preproinsulin leader sequences, usually connected by short interposed sequences derived from the construction process and unrelated to either presequence. In the two plasmids we have studied here the method of construction has produced fused proteins that are not substituted or modified.

In the case of plasmid pJW1452 half of the human presequence has been fused to a 65-residue NH_2-terminal coding segment of the preampicillinase gene (7). The product of this plasmid appears to be cleaved predominantly after the ampicillinase leader sequence to generate a 14,600-dalton fused protein, although we also found a small amount of proinsulin-like material on gel filtration. The corresponding plasmid of this type constructed by Talmadge *et al.* (5) contained only a short intervening segment of foreign peptide material between the two presequences, and its product was processed correctly after the rat presequence; whether it was also processed after the ampicillinase presequence was not reported.

A possible explanation for a low cleavage rate in the modified preproinsulin leader sequence in plasmid pJW1452 may be the relocation of a proline residue from its normal location at position −16 in the human prepeptide to position −14, as well as other more polar substitutions upstream (Fig. 2), thus possibly reducing the length of a potential non-polar β-sheet structure‡ that can be formed within the cell membrane by the central hydrophobic core of this prepeptide-like region (17). Alternatively, membrane recognition of the first leader sequence may preclude processing of the second, which is then transferred across the membrane via the attached segment of mature ampicillinase.

‡ If the structure formed by the prepeptide in the bacterial membrane is a *β strand* involving the central hydrophobic region of the prepeptide and a preexisting membrane "receptor" protein, as we have proposed (17), then proline could participate in this structure, but only when its carbonyl oxygen (because it lacks an amide function) is oriented so as to form a hydrogen bond with the opposing peptide strand. This orientation could be achieved only at every other position in the chain. On the other hand, a *β sheet* structure (prepeptide sandwiched between two other peptide chains), or any kind of helical structure, would tend to be disrupted by proline residues anywhere within the hydrophobic sequence. It is interesting to note that in pJW2172 the proline residue at position −14 is shifted by two positions, which keeps it "in register" for β strand formation relative to its prior position in the human prepeptide (6).

In plasmid pJW2172 the prepeptides of both proteins were joined in a hybrid structure consisting of almost equal halves of each presequence. In this hybrid presequence the proline residue normally at position −16 also is shifted to position −14. The protein produced by this plasmid is intact human proinsulin. However, the yield of insulin-like immunoreactivity from this plasmid is approximately 0.1–0.2 that produced by plasmid pJW1452 (Table 1). Whether this difference truly reflects a reduced efficiency of the hybrid prepeptide in export or is due to other factors such as differences in the stability of proinsulin versus the fusion protein within the periplasmic space, is not yet known. The only evidence in the literature bearing indirectly on this point comes from work of Bedouelle *et al.* (20) on fusions or mutations within the leader sequence of the maltose-binding protein that alter its secretion. Both a hybrid protein (PB4-81) and a point mutation (10-1) resulted in the appearance of a proline residue within the hydrophobic segment of the presequence. In the case of PB4-81 the defect in export cannot be ascribed solely to the intrusion of proline because 12 additional residues in the downstream portion of the prepeptide were also altered. However, in point mutant 10-1 the replacement of leucine by proline significantly reduced the export of the maltose-binding protein. It should be noted, however, that the normal ampicillinase leader sequence contains a proline residue at position −12. Whether its presence affects the relative efficiency of export of ampicillinase under normal conditions of expression is not known.‡ Clearly, much further work will be required to clarify the rules for recognition and function of signal sequences. Nonetheless, our results clearly underscore the relative functional universality of the presequences of prokaryotes and eukaryotes.

The method we have used for the characterization of human proinsulin in these experiments may have wider application for other proteins and peptides containing tyrosine residues in view of its ease and great sensitivity. Thus by the simple expedient of iodination with carrier-free $Na^{125}I$ followed by immunoprecipitation we were able to detect and characterize amounts of protein in the range of a few picomoles. Using this technique, we were able to demonstrate that the human proinsulin generated by *E. coli* containing pJW2172 was (*i*) correctly cleaved from preproinsulin by the bacteria, (*ii*) contained normal A and B chain segments, (*iii*) contained specific human C peptide immunodeterminants, (*iv*) contained disulfide bridges, and (*v*) could be converted to insulin-like material by digestion with trypsin and carboxypeptidase B.

Because of the species specificity and the immunogenicity of animal proinsulins in man, it has hitherto been impossible to study the possible role of proinsulin in normal physiology in man. Previous studies have demonstrated that the normal human pancreas secretes about 5% proinsulin and intermediate cleavage products along with insulin, and that, due to its slower disappearance rate, proinsulin constitutes up to 50% of the insulin-like material in the blood in the basal state (21). With the advent of these methods of bacterially assisted synthesis it should soon be possible to produce sufficient quantities of human proinsulin to begin to investigate its normal role in metabolic regulation and its possible application in the therapy of insulin-dependent forms of diabetes.

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