Proc. Natl. Acad. Sci. USA
Vol. 77, No. 6, pp. 3369–3373, June 1980
Biochemistry

Eukaryotic signal sequence transports insulin antigen in *Escherichia coli*

(rat preproinsulin/hybrid signal sequences/secretion/cloning vectors)

KAREN TALMADGE, STEPHEN STAHL*, AND WALTER GILBERT

Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138

Contributed by Walter Gilbert, March 28, 1980

ABSTRACT We made a series of plasmids with unique *Pst* restriction sites within or near the DNA that encodes the penicillinase signal sequence. Inserted DNA can be read in all three frames both within and immediately after the signal sequence. We cloned *Pst*-terminated DNA copies of the structural information for rat proinsulin and preproinsulin into these plasmids, forming a large number of hybrid penicillinase (bacterial) and insulin (eukaryotic) signal sequences. We then compared the levels of insulin antigen in the *Escherichia coli* periplasm with those inside the cells. We conclude that either the bacterial or the eukaryotic signal is sufficient to transport rat insulin antigen into the periplasmic space.

Proteins must pass through a cell membrane in order to function outside the cell. With one known exception (1), all secreted proteins, both eukaryotic and prokaryotic, have an amino-terminal extension, or pre-sequence, that is removed as or after the protein crosses the membrane (2). The amino acid sequences of more than 30 pre-sequence peptides (3) share one obvious structural feature: a stretch of 5–10 highly hydrophobic residues near the middle of the peptide. The signal hypothesis (2, 4) proposes that these hydrophobic amino-terminal extensions serve to bind the protein to the membrane and then to lead the protein through.

Direct evidence establishes that the bacterial pre-sequence, or signal sequence, has an essential role in protein transport. Emr *et al.* (5) and Bedoulle *et al.* (6) characterized in two cases a number of mutations that lead to the accumulation of the mutant protein in the cytoplasm as the pre-protein. In each case, the mutations result in amino acid replacements in the signal sequence, most of which are changes from a hydrophobic to a charged residue.

Recombinant DNA technology attempts to produce higher cell proteins in bacteria. Such proteins are simpler to detect and purify if they are secreted from the cell. Villa-Komaroff *et al.* (7) cloned a cDNA copy of the rat proinsulin gene into the *Pst* restriction site of plasmid pBR322 (8). The *Pst* site encodes amino acids 181–182 of *E. coli* penicillinase (9), a periplasmic protein with a 23 amino acid signal sequence (9, 10). Thus, when the proinsulin sequence was inserted into the middle of the penicillinase gene, a fused protein was created which served to carry most of the insulin antigen to the periplasmic space (7).

This successful transport of the penicillinase–insulin hybrid molecule prompted two overlapping experimental lines. First, we wanted to use the recombinant DNA techniques to alter pBR322 to create a set of sites for cloning closer to and within the signal sequence region and thus eliminate as much of the extraneous bacterial protein as possible. Second, we wanted to examine altered signal sequences created by such clonings to answer direct questions about the role of those sequences in protein secretion.

The publication costs of this article were defrayed in part by page

MATERIALS AND METHODS

Bacterial Strains. *E. coli* K-12 strain HB101 (*hrs*$^-$, *hrm*$^-$, *recA*$^-$, *gal*$^-$, *str*^{r}, *B*$_1^-$) was obtained from Herb Boyer; *E. coli* K-12 strain FMA10 (F$^-$, su$^-$, *gal*$^-$, *str*^{r}, *thyA*$^-$, *deo*$^-$, *hrs*$^-$, *hrm*$^+$) was provided by Fred Ausubel and lysogenized by Stephanie Broome with λ *c*I$_{857}$; *E. coli* K-12 strain PR13 (*pnp*13, *rna*19, thr1, *leu*B6, *lac* lambda 1, *xyl*7, *mtl*2, *mal*A1, strA132, lambda^R, lambda$^-$) was from the Yale strain library.

Enzymes. Restriction enzymes and DNA polymerase I (Klenow fragment) were purchased from New England BioLabs. Polynucleotide kinase was purchased from Boehringer Mannheim. BAL31 was the gift of Horace Gray. T4 DNA ligase was a gift of A. Poteete and Stewart Scherer.

DNA. pBR322 was obtained originally from Herb Boyer; pI19 and pI47 were the gift of Peter Lomedico. Plasmid was prepared as described (12) and (13). *Pst* linker was purchased from Collaborative Research and treated with kinase under standard conditions (14).

Ligations and Transformations. Ligations (15) and transformations (16) were done under standard conditions.

Exonuclease. The ends of insulin insert 1947 were digested with the double-stranded exonuclease BAL31 in the following mixture (17): 2.5 μg of DNA, 2 units of BAL31 in 150 μl of 20 mM Tris·HCl (pH 8), 12.5 mM $MgSO_4$, 12.5 mM $CaCl_2$, 0.2 M NaCl, and 1 mM EDTA; incubation was for 45 sec at 15°C.

Radioimmunoassays. Two-site solid-phase radioimmunoassays were performed as described (18) with minor modifications (7). FMA10/λ *c*I$_{857}$ was transformed with signal sequence plasmids containing insulin gene inserts and induced at 42°C. Standard liquid radioimmunoassays were performed as described (19). Aliquots of cell fractions were preincubated for 1–3 hr with an amount of guinea pig anti-porcine insulin IgG fraction [prepared as described (18)] sufficient to complex 75% of the added labeled insulin.

Cell Lysis and Fractionation. FMA10/λ *c*I$_{857}$ and PR13 bearing signal sequence plasmids expressing insulin antigen were grown in 100 ml of glucose minimal medium (supplemented with 40 μg of thymine per ml for FMA10) (14) at 34°C to OD_{550} = 0.24–0.4. Harvested cells were washed in 1 ml of 100 mM Tris·HCl, pH 8/20% sucrose. Whole-cell lysis was performed exactly as described for plasmid preparation by the method of Clewell (12). Cell fractionation was by spheroplast formation and lysis (M. Russel, personal communication). Washed cells were resuspended in 900 μl of Tris/sucrose, incubated 30 min with 100 μl of lysozyme (5 mg/ml in 20 mM EDTA), and pelleted. The pelleted spheroplasts were washed gently with Tris/sucrose, resuspended in 100 μl of Tris/sucrose (by using a glass rod), lysed by the addition of 850 μl of 0.3% Triton/150 mM Tris·HCl, pH 8/0.2 M EDTA (12), and centrifuged for 1 hr at 17,000 rpm in a Sorvall SA-600 rotor. A

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

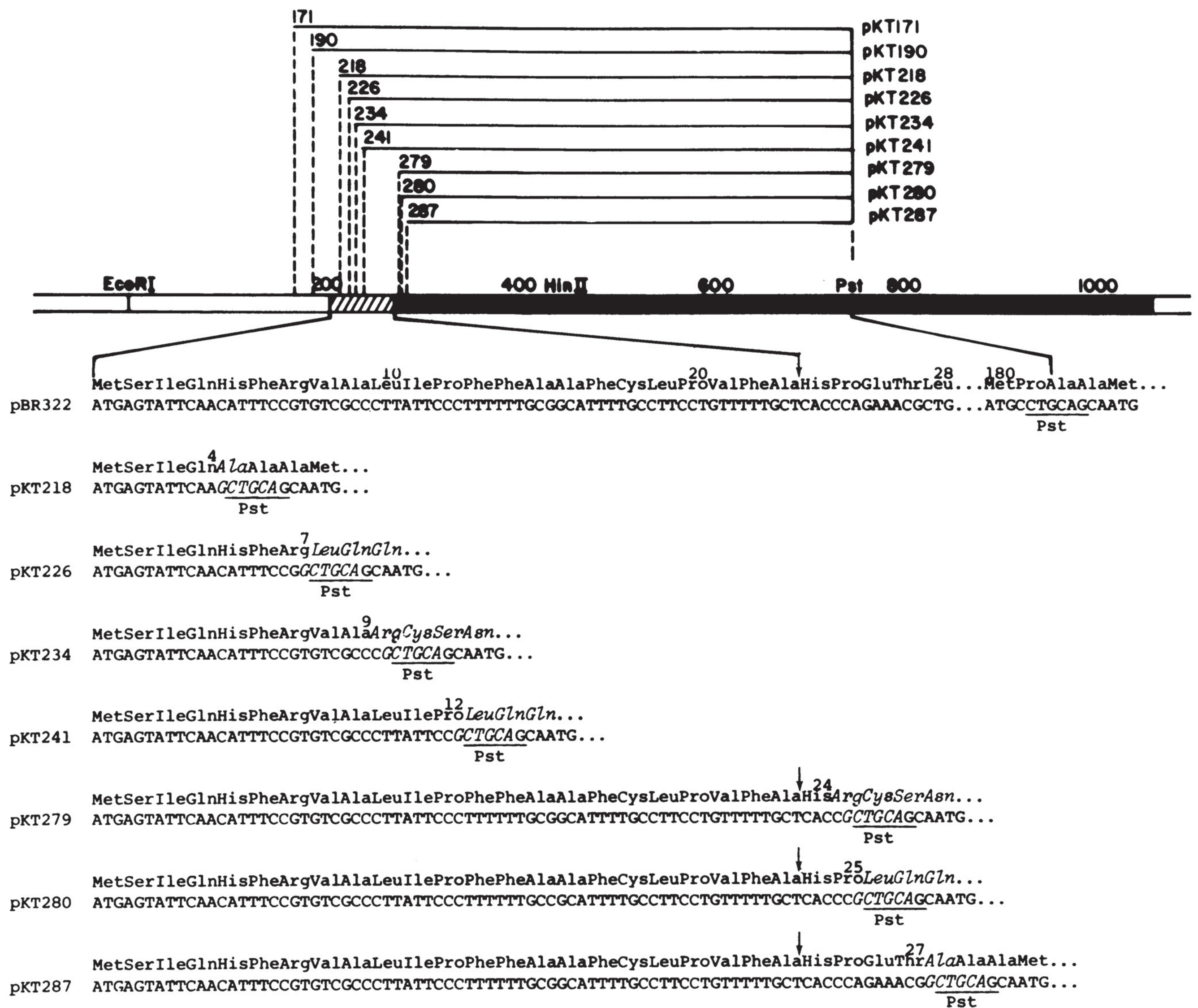


FIG. 1. Deletion map of pBR322 penicillinase gene and sequence of derivative plasmid signal sequence regions (constructions to be described elsewhere). DNA regions that encode protein are represented as follows: penicillinase signal sequence, hatched; mature penicillinase protein, black. The derivatives were deleted from the *Pst* to the signal sequence coding region and the *Pst* site (C-T-G-C-A-G) was re-created by insertion of a *Pst* linker whose sequence is G-C-T-G-C-A-G-C. The bases donated by the linker on that strand are indicated in italics. The last wild-type penicillinase amino acid is indicated by the number of its wild-type position above it. The amino acids encoded by the inserted *Pst* linker are in italics. The arrows indicate the site of cleavage for maturation of wild-type prepenicillinase to penicillinase.

membrane fraction was prepared from pelleted spheroplasts by two methods. Spheroplasts were resuspended in 1 ml of 10 mM Tris·HCl, pH 8/5 mM $MgCl_2$/5 mM dithiothreitol/0.2 M KCl (20) and sonicated three times for 10 sec each on ice. Alternately, the spheroplasts were resuspended in 100 μl of Tris/sucrose and lysed with 900 μl of distilled water (M. Russel, personal communication). Both lysates were centrifuged for 1 hr at 35,000 rpm in a Beckman SW 60 rotor. The membrane pellet was resuspended in Triton buffer with a Dounce homogenizer.

DNA Sequence Analysis. Plasmid prepared from 5 ml of cells was 3′-end labeled in the presence of 20 μM GTP and 2 μM [α-^{32}P]ATP at 15°C for 4 hr (21). Sequence analysis was by the method of Maxam and Gilbert (14).

Recombinant DNA. All manipulations involving cells with insulin-gene plasmids were done under P2 containment according to the National Institutes of Health guidelines issued

RESULTS

Fig. 1 shows a deletion map of nine derivatives of pBR322 and the sequences of seven of these plasmids that have useful *Pst* restriction sites in the penicillinase signal sequence coding region (unpublished data). Four of these plasmids—pKT218, pKT226, pKT234, and pKT241—code for 4, 7, 9, and 12 signal sequence amino acids, respectively. Three of the plasmids—pKT279, pKT280, and pKT287—code for the entire signal sequence as well as for one, two, and four amino acids of the mature penicillinase respectively. Within each group, there are plasmids in all three reading frames.

Fig. 2 shows the restriction maps and 5′-end sequences of the pI19 and pI47 cDNA *Pst*-ended gene fragments of rat preproinsulin and proinsulin isolated and sequenced by Villa-Komaroff *et al.* (7). We used BAL31 to chew back the ends of the pI1947 insert (derivation explained in figure legend), ligated the pieces to kinase-treated *Pst* linker, and cloned *Pst*-diges-

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

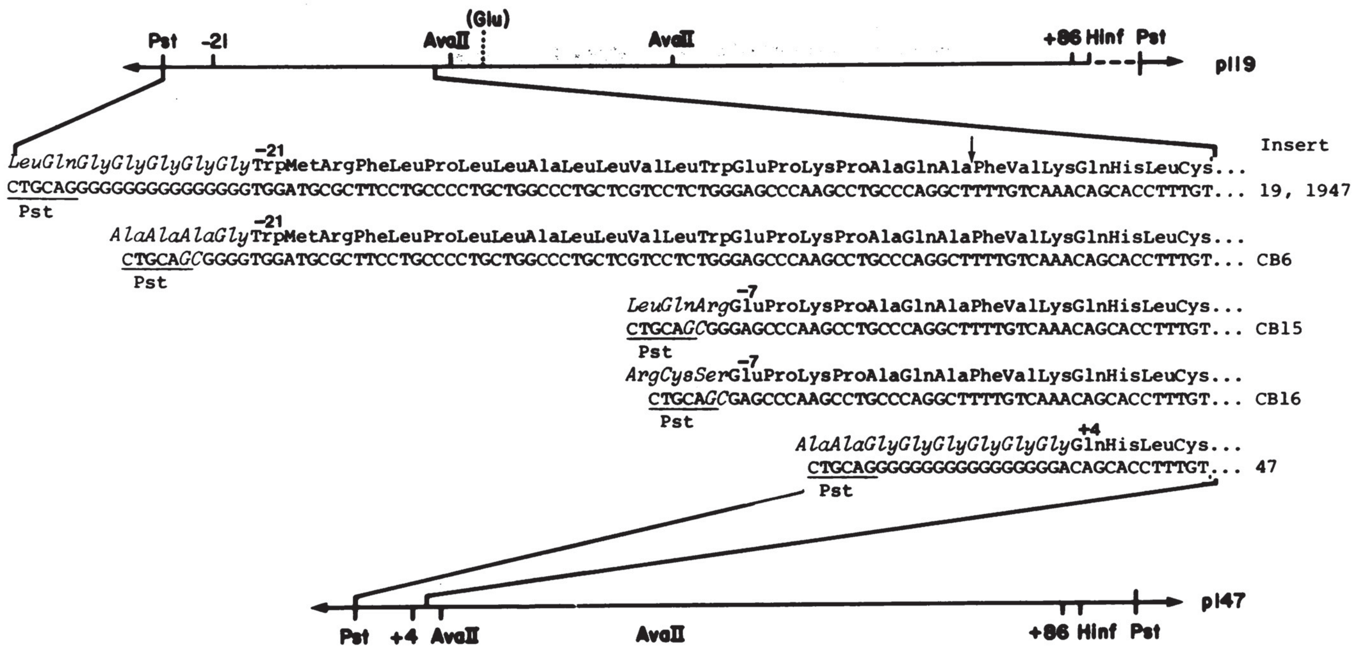


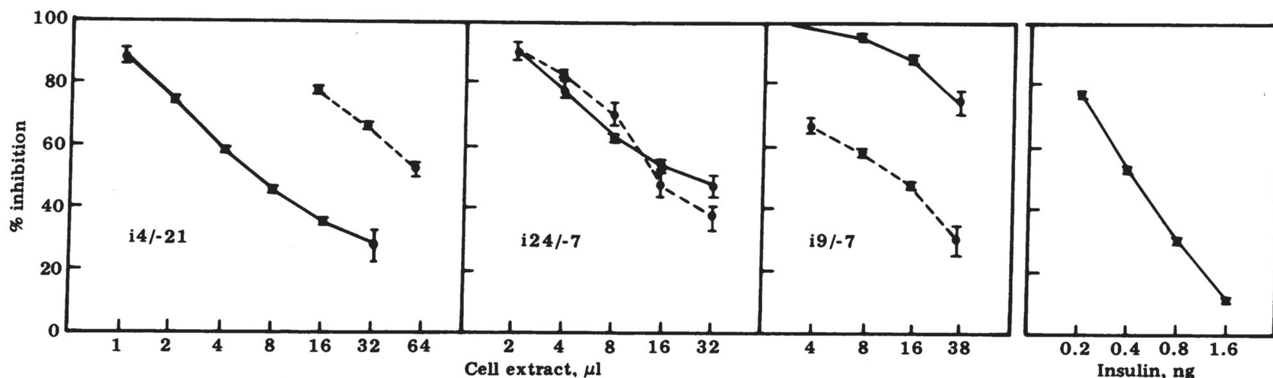
FIG. 2. Restriction map of rat preproinsulin (pI19) and proinsulin (pI47) *Pst* inserts (7) [1947 is a recombinant between the 19-insert 5′ end and the 47-insert 3′ end at the first *Ava* II site to remove a mutant glycine encoded in the 19 insert (22)]; sequences at the 5′ end of these inserts and the digested derivatives of 1947 insert. Bases in the digested 1947 insert sequences in italics have been donated by an inserted *Pst* linker. The first wild-type amino acid is indicated by the number of its wild-type position above it. Amino acids in italics were created by G-C-tailing during the original isolation of pI19 and pI47 (7) or by the insertion of a *Pst* linker. Arrows indicate the site of cleavage for maturation of preproinsulin to proinsulin.

Maxam and Gilbert C+T and G+A reactions (14) to analyze inserts digested with *Ava* II and 3′-end labeled (21). With these *Pst* fragments, we can insert the gene for rat preproinsulin in two reading frames and the gene for rat proinsulin in all three reading frames in the penicillinase sequence.

Table 1 lists the insulin constructions. We name the hybrid protein products with a lower case "i" and a pair of numbers: the first refers to the last prepenicillinase wild-type amino acid before the amino acids encoded by the insertion of the *Pst* site, and the second refers to the first amino acid of the preproinsulin (negative numbers) or proinsulin (positive numbers) sequence. Table 2 shows the amino acid sequence of each hybrid protein from the first methionine to proinsulin amino acid 7. In each case, there is a minimum of three amino acids between the last amino acid of the penicillinase signal sequence and the first amino acid of the insulin portion; these extra amino acids are in italics in Figs. 1 and 2.

Expression of Insulin Antigens. To explore the transport of the various fusions of proinsulin and preproinsulin to the modified signal sequences, we identified clones that were expressing rat proinsulin antigen with a two-site solid-phase radioimmunoassay (18) and then fractionated the cells into a periplasmic and a cytoplasmic/membrane fraction. A lysozyme/EDTA treatment in hypertonic sucrose released the periplasmic proteins. After washing the resulting spheroplasts in Tris/sucrose, we lysed them with Triton (12) and removed the chromosomal DNA by centrifugation at 30,000 × *g*. We assayed the insulin antigen in these two fractions and compared those results to the values found in a Triton lysate of the whole cells. Less than 1% of the cells were lysed during the formation of the spheroplasts, as shown by β-galactosidase assays (23) on cells induced with IPTG.

We measured insulin antigen by a standard radioimmune assay (19). Fig. 3 shows typical assays for each of three different constructions as well as a standard curve. Naturally, the bacterial material is not identical to mature insulin, and the competition tails off earlier. To calculate the number of molecules per cell, we used the amount of cell extract required for 50% inhibition of the binding of radiolabeled antigen, the number of input cells, and the standard insulin curve. Table 1 collects all the results; Table 2 summarizes them. Some curves had to be extrapolated to the 50% point; such data, indicated by pa-



DOCKET ALARM
Find authenticated court documents without watermarks at docketalarm.com.

Table 1. Insulin constructions

Plasmid	Insulin	Host	Molecules per cell P	C/M	WC	% PS
pI47	i181/+4	FMA10	>70*	—	~100†	>70
		PR13	—	—	980	—
p287.47	i27/+4	PR13	—	—	5903	—
			6501	(292)	—	>96
			—	—	1533	—
			1424	(126)	—	>92
			1517	(143)	—	>91
p280.1947	i25/−21	FMA10	124	(18)	—	>88
			132	(10)	—	>93
p241.1947	i12/−21	FMA10	156	(29)	—	>84
			160	(11)	—	>93
		PR13	—	—	1555	—
			1320	(298)	—	>82
			1592	105	—	94
p218.CB6	i4/−21	FMA10	—	—	23	—
			18	(4)	—	>82
		PR13	—	—	368	—
			365	(37)		>91
p280.CB15	i25/−7	PR13	—	—	288	—
			136	132	—	51
			66	77	—	46
p279.CB16	i24/−7	PR13	—	—	446	—
			196	212	—	48
			201	192	—	51
p241.CB15	i12/−7	PR13	—	—	207	—
			(18)	191	—	<9
p234.CB16	i9/−7	PR13	—	—	346	—
			(34)	342	—	<9
			(18)	176	—	<9
p218.47	i4/+4	FMA10	—	—	10	—
			(1)	10	—	<9
			(1)	15	—	<6

Collected data from radioimmunoassays. Insulin antigen content: P, of the periplasm; C/M, of the cytoplasmic/membrane fraction; WC, of the whole cell lysate. The percentage antigen in the periplasmic space (% PS) is $100 \cdot [P \div (P + C/M)]$. Data in parentheses are extrapolated to the 50% inhibition point.

* Stephanie Broome, personal communication.

† Ref. 7.

rentheses in Table 1, represent rough maximal estimates. In an attempt to increase the yield, we moved some of the constructions into a polynucleotide phosphorylase-negative strain, *E. coli* PR13 (11), which carries the *pnp*13 mutation. In a fresh construction, the total number of insulin molecules in each cell was more than 10–15 times higher than that found in other host strains. However, that high level expression (for example, that in i27/+4, construction p287/47) was lost over time. Efforts to maintain stable high level production in the *pnp* host have not yet been successful; we think it likely that the plasmid copy number is elevated in these mutants and gradually decreases over time.

Table 2 shows that hybrid molecules from four different constructions are transported efficiently into the periplasmic space: proinsulin fused at four amino acids after the entire penicillinase signal sequence (i27/+4), preproinsulin fused at two amino acids after the entire penicillinase signal (i25/−12), preproinsulin fused to the first half of the penicillinase signal (i12/−21), and preproinsulin fused to just the first four amino acids of the penicillinase signal (i4/−21). In contrast, three constructions with fragmentary signal sequences did not secrete the insulin antigen. Less than 10% of the material from i12/−7

hybrid proteins produced by two fusions that inserted a charged amino acid after a complete penicillinase signal (i25/−7 and i24/−7) appeared half in the periplasm and half in the cytoplasmic/membrane fraction.

In an attempt to determine whether or not those proteins that were not fully transported were in the membrane, we sonicated the spheroplast pellets (20) and collected the membranes by centrifugation at $100{,}000 \times g$. Only about 10% of the total insulin antigen in these cells was associated with the membrane pellet (resuspended in Triton). In addition, we gently lysed the spheroplast pellet from cells producing i4/+4 by adding water instead of Triton, but less than 5% of the insulin antigen was associated with the membrane pellet. These proteins appear to be cytoplasmic by these tests, but we cannot eliminate the possibility of some recondite membrane interaction (such as an interaction with a protein transport channel).

DISCUSSION

These experiments examined various hybrid signal sequences fused to rat proinsulin. Table 1 shows that, in four different constructions, 90% of the proinsulin appeared in the periplasm when attached either to a bacterial or to a preinsulin signal sequence. In contrast, fusions containing short fragments of the signal sequence did not appear in the periplasm. Neither the first four amino acids of penicillinase nor two hybrid presequences formed from the first half of the bacterial signal and the last third of the rat signal directed secretion. These fusions lack hydrophobic cores, and the last two have added a charged amino acid to that region of the presequence. We conclude that the signal sequence is essential for secretion; the information for secretion cannot reside in the proinsulin moiety alone. Furthermore, because either a prokaryotic or a eukaryotic signal suffices, the transport mechanism must recognize some very general (and very ancient) aspect of structure.

The signal sequence is not sufficient for transport; other regions of the protein have some role. Fusions of β-galactosidase to the signal sequence portion of various secreted bacterial proteins have yielded hybrids that are membrane-bound or remain cytoplasmic (24). However, β-galactosidase is not a secreted protein, and its structure may render transport impossible. Several of our constructions contain the complete penicillinase signal attached to a normally secreted protein but move only 50% of the proinsulin to the periplasm. These fusions contain an extra arginine shortly after the end of the signal sequence; they *are* transported but with a reduced efficiency. We attempted to determine whether or not such partially transported proteins were trapped in the membrane. Sonication released them, so they are not tightly bound, but more subtle experiments, such as proteolytic digestion of the spheroplasts, are required to rule out other membrane associations and to show definitively that these untransported molecules are cytoplasmic.

In these constructions, a few amino acids from the beginning of the penicillinase sequence (and always the fMet) are fused to the rat preproinsulin sequence, which lacks its first three amino acids. Clearly, these first few amino acids do not dominate the transport. They are not sufficient in themselves (as shown by i4/+4), and an examination of the 33 (4) available pre-sequences reveals the presence in this region of every amino acid except tyrosine.

The striking finding is that both a bacterial and a eukaryotic sequence serve to direct efficient transport; this suggests that the two presequences play a similar and interchangeable role. Fraser and Bruce (25) demonstrated that another secreted eukaryotic protein, ovalbumin, is also transported (50%) when cloned and expressed in bacteria. Ovalbumin is unique; it does

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

Table 2. Amino sequence of hybrid signal sequences and summary of transport data

Pen*	MSIQHFRVALIPFFAAFCLPVFA ↓	HPETLVK......				
i27/+4	MSIQHFRVALIPFFAAFCLPVFA	HPET	*AAG*GGGGG		QHLC...	>90%
i25/−21	MSIQHFRVALIPFFAAFCLPVFA	HP	*LQ*GGGGG	WMRFLPLLALLVLWEPKPAQA	FVKQHLC...	>90%
i12/−21	MSIQHFRVALIP		*LQ*GGGGG	WMRFLPLLALLVLWEPKPAQA	FVKQHLC...	>90%
i4/−21	MSIQ		*AAAG*	WMRFLPLLALLVLWEPKPAQA	FVKQHLC...	>90%
i25/−7	MSIQHFRVALIPFFAAFCLPVFA	HP	*LQR*	EPKPAQA	FVKQHLC...	50%
i24/−7	MSIQHFRVALIPFFAAFCLPVFA	H	*RCS*	EPKPAQA	FVKQHLC...	50%
i12/−7	MSIQHFRVALIP		*LQR*	EPKPAQA	FVKQHLC...	<10%
i9/−7	MSIQHFRVA		*RCS*	EPKPAQA	FVKQHLC...	<10%
i4/+4	MSIQ		*AAG*GGGGG		QHLC...	<10%
Preproinsulin				MALWMRFLPLLALLVLWEPKPAQA ↓	FVKQHLC...	

Each sequence begins at the penicillinase fMet and ends at amino acid 7 of proinsulin. Each line represents one continuous sequence which has been grouped to emphasize similarities and differences as follows: first group, penicillinase signal sequence amino acids; second group, matured penicillinase amino acids; third group, amino acids created by the inserted *Pst* linker (italics) or by poly(G,C) tailing (glycines); fourth group, preproinsulin signal sequence amino acids; fifth group, matured proinsulin amino acids through amino acid 7. The arrows above the prepenicillinase and preproinsulin sequences indicate sites of cleavage for maturation. The sequence for prepenicillinase is from refs. 9 and 10; the sequence for preproinsulin is from refs. 7 and 22. A, Ala; R, Arg; C, Cys; Q, Gln; E, Glu; G, Gly; H, His; I, Ile; L, Leu; K, Lys; M, Met; F, Phe; P, Pro; S, Ser; T, Thr; W, Trp; V, Val.

* Penicillinase

may contain an internal hydrophobic region that serves as the signal (26). Whatever the process of secretion may be in this case, again the bacterial and eukaryotic mechanisms respond similarly. We know that cleavage of the signal sequence is not essential to transport because a mutation in the signal sequence blocks the cleavage of the pre-sequence yet permits 45% of the protein to appear in the outer membrane (27). The unifying picture is that a hydrophobic "core" sequence, somewhere in the protein, serves to attach the protein to an element that leads to the passage of the protein across the membrane. The cleavage of a pre-sequence then might be related solely to the overall efficiency, and irreversibility, of secretion.

The similarity in behaviors of the prokaryotic and eukaryotic signal sequences, which must reflect some underlying similarity in structure, raises the question of whether the eukaryotic sequence is processed correctly in bacteria. As we shall show next month, it is.

We thank Peter Lomedico, Roger Brent, Marjorie Russel, and Stephanie Broome for helpful discussions. We thank Stephanie Broome and Ann Forsham for generously sharing antibody and Horace Gray and Stewart Scherer for the gift of enzymes. We thank Jeremy Knowles for being particular. This work was supported in part by National Institutes of Health Grant AM 21240. S.S. was supported by a Damon Runyon Fellowship. W.G. is an American Cancer Society Professor of Molecular Biology.

1. Palmiter, R. D., Gagnon, J. & Walsh, K. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 94–98.
2. Milstein, C., Brownlee, G. G., Harrison, T. M. & Matthews, M. B. (1972) *Nature (London) New Biol.* **239**, 117–120.
3. Blobel, G., Walter, P., Chang, C. N., Goldman, B. M., Erikson, A. H. & Lingappa, V. R. (1979) *Symp. Soc. Exp. Biol.* **33**, 9–36.
4. Blobel, G. & Dobberstein, B. (1975) *J. Cell Biol.* **67**, 835–851.
5. Emr, S. D., Hedgpeth, J., Clement, J. M., Silhary, T. J. & Hofnung, M. (1980) *Nature (London)* **284**, 82–85.
6. Bedoulle, H., Bassford, P., Fowler, A., Zabin, I., Beckwith, J. & Hofnung, M. (1980) *Nature (London)* **284**, 78–81.
7. Villa-Komaroff, L., Efstradiatis, A., Broome, S., Lomedico, P., Tizard, R., Naber, S. P., Chick, W. L. & Gilbert, W. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3727–3731.
8. Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L., Boyer, H. W., Crossa, J. H. & Falkow, S. (1977) *Gene* **2**, 95–113.
9. Sutcliffe, G. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3737–3741.
10. Ambler, R. P. & Scott, G. K. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3732–3736.
11. Reiner, A. (1969) *J. Bacteriol.* **97**, 1522–1523.
12. Clewell, D. B. (1972) *J. Bacteriol.* **110**, 667–676.
13. Zasloff, M., Ginder, G. D. & Felsenfeld, G. (1978) *Nucleic Acids Res.* **5**, 1139–1151.
14. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65**, 499–560.
15. Backman, K., Ptashne, M. & Gilbert, W. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 4174–4178.
16. Mandell, M. & Higa, A. (1970) *J. Mol. Biol.* **53**, 159–162.
17. Legerski, R. J., Hodnett, J. L. & Gray, H. B. (1978) *Nucleic Acids Res.* **5**, 1445–1464.
18. Broome, S. & Gilbert, W. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2746–2749.
19. Makula, D. R., Vichnuk, D., Wright, P. H., Sussman, K. E. & Yu, P. L. (1969) *Diabetes* **18**, 660–689.
20. Osborne, M. J., Gander, J. E., Parisi, E. & Carson, J. (1972) *J. Biol. Chem.* **247**, 3962–3972.
21. Englund, P. (1972) *J. Mol. Biol.* **66**, 209–224.
22. Lomedico, P., Rosenthal, N., Efstradiatis, A., Gilbert, W., Kolodner, R. & Tizard, R. (1979) *Cell* **18**, 545–558.
23. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), pp. 352–355.
24. Bassford, P., Silhavy, T. J. & Beckwith, J. (1980) *J. Bacteriol.* **139**, 19–31.
25. Fraser, T. & Bruce, B. J. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 5936–5940.
26. Lingappa, V. R., Lingappa, J. R. & Blobel, G. (1979) *Nature (London)* **281**, 117–121.
27. Lin, J. J. C., Kanazawa, H., Ozols, J. & Wu, H. C. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4891–4895.

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.