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Secretion and folding of human growth hormone in *Escherichia coli*

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Introduction

Since the advent of the recombinant DNA era, a large number of heterologous proteins have been produced in *Escherichia coli*. In fact, some proteins such as human insulin and human growth hormone are produced in kilogram quantities for use as human pharmaceuticals. In many cases, the genetic constructions used for producing these proteins have resulted in their high-level expression. The over-production of foreign proteins in the *E. coli* cytoplasm often results in the formation of inclusion bodies or 'granules' which are insoluble precipitates of protein, highly enriched in the protein of interest. The formation of such granules has advantages as well as disadvantages for the isolation and purification of the protein produced in *E. coli*. A significant purification is accomplished by simply collecting the granules and washing them to remove soluble protein contaminants. A disadvantage, however, is that to solubilize the protein in the granule, detergents or denaturing agents are often required and this necessitates a refolding step to obtain the protein with the correct secondary and tertiary structure. Very often, refolding of the protein results in loss of product due to the inefficiency of the folding process, leading to decreased yields in the production process. Another potential disadvantage of the production of proteins as cytoplasmic products is that the endogenous enzyme responsible for the removal of the initiation methionine does not work efficiently for all proteins. Indeed, the penultimate residue of the protein appears to determine whether the cellular methionine aminopepti-

A_{550} or A_{600}: Absorbance of light at 550 or 600 nm, a measure of cell culture density; ATP: adenosine triphosphate; CD: circular dichroism; DEAE: diethylaminoethyl; DP: docking protein; hGH: human growth hormone; HPLC: high performance liquid chromatography; LacZ: β-galactosidase; LamB: λ receptor protein; MalE: maltose-binding protein; RER: rough endoplasmic reticulum; RNA: ribonucleic acid; SRP: signal recognition particle.

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dase will remove the methionine. Certain amino acids such as alanine appear to promote the proteolytic removal of the methionine, whereas others such as phenylalanine are not conducive to its removal. (A discussion of this enzyme and the effect of the various amino acids on its action appears in the literature [Ben-Bassat *et al.*, 1987].) Therefore, using this approach, it is not always possible to produce a protein that does not have an *N*-terminal methionine residue.

For these reasons, there has been considerable interest in developing secretion systems that will result in the deposition of the protein in a compartment where purification might be facilitated, where processing to achieve a desired amino-terminal sequence can occur, and where the polypeptide can fold into its native conformation. While protein secretion has been studied in both Gram-positive and Gram-negative bacteria, the discussion here will be limited to the Gram-negative organism, *Escherichia coli*. The *E. coli* cell has four major components: the cytoplasm, the inner membrane, the outer membrane, and the periplasmic space. In addition, there is the extracellular space, the culture medium, which can be considered when designing protein secretion/excretion systems. For the purposes of this article, *secretion* is used to refer to transport of the protein across the inner membrane to the periplasmic space; *excretion* refers to transport across both the inner and the outer membranes with release into the culture medium.

The purpose of this review is to discuss in detail the engineered secretion of human growth hormone in *E. coli* with the concomitant processing of the protein and folding into its native conformation. Designing a secretion system for a heterologous protein requires an understanding of the mechanisms of *E. coli* protein secretion in general. As has been pointed out in the literature, it is not sufficient simply to attach a signal peptide to assure the secretion of a protein (Moreno *et al.*, 1980). For this reason, we will include a brief overview of protein secretion and transport in general, followed by a more detailed discussion of protein transport in *E. coli*.

Protein synthesis occurs within the cytoplasm of all cells but many of the proteins are required at sites other than the cytoplasm, thereby necessitating a protein translocation system to move the proteins from the cytoplasm to their final destination. This translocation process entails the passage of the protein through one or more membranes. Therefore, a mechanism must be provided for moving the protein through the hydrophobic lipid bilayer of the membrane.

In eukaryotes, protein transport across the membrane of the rough endoplasmic reticulum (RER) is thought to be co-translational and involves a complex secretory machinery. Walter and Blobel (1980) and Meyer, Krause and Dobberstein (1982) isolated two receptors for protein secretion in eukaryotes: the signal recognition particle (SRP) and the docking protein (DP). Both are known to participate in the secretion process. SRP is a complex of six proteins and 7S RNA. It binds to polysomes that synthesize pre-secretory proteins and causes a pause in chain elongation after approximately 80 amino acid residues. The SRP–polysome complex then binds to DP, a 72 kD integral membrane protein of the RER. The SRP is then released and chain elongation is resumed. The growing polypeptide chain traverses the membrane of the

RER into the lumen, where the signal peptide is cleaved and core glycosylation occurs.

There are several common features between protein secretion across the membrane of endoplasmic reticulum in eukaryotes and protein secretion across the cytoplasmic membrane in bacteria. First, in both systems, the protein is synthesized as a precursor with an amino-terminal extension known as the signal peptide or signal sequence. This signal sequence is typically 15–30 amino acids in length and is specifically cleaved during translocation of the protein across the membrane. Bacterial and eukaryotic signal sequences share many structural similarities, including a positively charged amino terminal segment, a hydrophobic core, and a cleavage site. These features, as they apply to *E. coli* signal sequences, are discussed in greater detail later.

Prokaryotic and eukaryotic signal sequences also share functional similarities, as shown by the fact that bacterial signal sequences work in eukaryotes (Talmage, Kaufman and Gilbert, 1980) and eukaryotic signal sequences work in bacteria (Talmadge, Stahl and Gilbert, 1980). There are similarities in the cleavage site of both prokaryotic and eukaryotic signal sequences and, in fact, bacterial signal peptidases have the ability to cleave eukaryotic signal sequences.

PROTEIN SECRETION IN *E. COLI*

Protein secretion in *E. coli* is the subject of extensive investigation in a number of laboratories and has been reviewed in several articles published over the past few years (Michaelis and Beckwith, 1982; Randall and Hardy, 1984; Benson, Hall and Silhavy, 1985; Duffaud *et al.*, 1985; Oliver, 1985; Pugsley and Schwartz, 1985; Randall, Hardy, and Thom, 1987). The following discussion is not intended to be a complete review of protein secretion in *E. coli*, but, instead a general overview of the topic. For additional details, the reader is referred to the review articles listed above.

Three components are necessary for transporting a protein in *E. coli*: (1) the signal sequence; (2) secretion sequences within the mature protein; and (3) the cellular export machinery.

Signal sequence

The signal sequence appears to be an absolute requirement for protein export, although, it is not cleaved from the precursor in all cases. For example, the outer membrane phospholipase A (probably phospholipase A_1, EC 3.1.1.32), which must traverse both membranes of *E. coli* to reach its final destination in the outer membrane, contains a putative signal sequence which does not appear to be removed during transport (de Geuss *et al.*, 1984). Most exported proteins of *E. coli*, however, are synthesized as precursors with cleavable signal sequences ranging from approximately 15 to 30 residues in length. All of the signal sequences thus far described, with the exception of the previously mentioned outer membrane phospholipase A, have common structural features. These common features are (1) the charged segment near the amino

terminus, (2) the hydrophobic core, and (3) the cleavage site. A table listing bacterial signal sequences has been compiled by Oliver (1985). The role of the signal sequence in protein secretion has recently been reviewed by Briggs and Gierasch (1986).

The charged segment is near the amino terminus of the precursor and in all cases is positively charged, due to either arginine or lysine or both. The number of charged amino acids within this segment varies from one residue in proteins such as alkaline phosphatase (EC 3.1.3.1) to as many as three in maltose-binding protein. The hydrophobic core, containing a preponderance of leucine and isoleucine residues, starts after the charged segment and extends to a more polar region containing the cleavage site. The hydrophobic regions of the signal sequences of all *E. coli* proteins appear to have little structural homology. The cleavage site is that peptide bond within the precursor that is proteolytically cleaved by an enzyme, the signal peptidase, to yield the mature protein. Cleavage of the signal sequence typically occurs after an alanine but in some cases can occur after a glycine or a serine.

Sequences within the mature protein

While the signal sequence is necessary for protein export in *E. coli*, for many proteins it is not sufficient. It is now clear that for many secreted proteins in *E. coli* there are sequences that lie within the mature protein that are necessary for directing the protein to its final destination, whether that be the periplasm or one of the membranes (Ferenci and Silhavy, 1987).

Two experimental approaches have been taken to assess the role of mature protein sequences in secretion. The gene fusion approach has been used whereby portions of two genes are fused together to form a hybrid gene. Expression of these genes gives rise to hybrid proteins, the secretion characteristics of which can be studied. The second approach is gene deletion where portions of a gene are removed. Expression of the modified gene results in a protein that has a deletion and the secretion properties of this protein can be studied. These types of experiments have led to the observation that there are two kinds of export information available in mature protein sequences, one causing a secretion incompatibility and the other conferring positive information making export optimal (Ferenci and Silhavy, 1987).

An example of the first kind of export information is found in the protein β-galactosidase (EC 3.2.1.23 (Lac Z)), which is normally a cytoplasmic protein of *E. coli*. Bassford, Silhavy and Beckwith (1979) used the gene fusion approach to study the secretion of a hybrid protein of the *E. coli* maltose-binding protein (MalE), which is normally secreted, and β-galactosidase (LacZ) which is not secreted. Various size hybrids were formed with MalE sequences at the amino-terminal end and LacZ sequences at the carboxy-terminal end. When only a portion of the MalE protein signal peptide is included, the hybrid is not secreted at all but is found in the cytoplasm. Inclusion of an intact MalE signal sequence results in initiation of secretion. However, the hybrid protein does not pass through the membrane but instead becomes stuck in it. Even when the hybrid protein contains almost the entire

maltose-binding protein sequence, it is still not secreted into the periplasm. These results indicate that attachment of a signal peptide is not sufficient to assure secretion of a protein but additional information included in the mature protein sequence is required. Furthermore, the inability to secrete the MalE–LacZ hybrid protein also interfered with the secretion of normal envelope proteins, which accumulated as precursors in the cytoplasm. Thus, it would appear that β-galactosidase contains sequences that render it incompatible for secretion.

Gene deletion experiments have provided examples of positive secretion information residing within mature protein sequences. One important study involving an outer membrane protein of *E. coli*, LamB, revealed that there is information in the first 28 amino acids of the mature protein that affects the secretion of this protein (Rasmussen and Silhavy, 1987). Deletion or replacement of residues within this region significantly decreased the rate and efficiency of secretion. Deletion in other regions of the protein did not lead to this decrease, indicating that the first 28 amino acids of the mature protein have a role in optimizing secretion.

In other proteins, it appears that the carboxy-terminal amino acids can also have a role in secretion. An example can be found in the translocation of leader peptidase in *E. coli* (Dalbey and Wickner, 1986). Leader peptidase is an integral membrane protein found in the cytoplasmic membrane. There is a small amino-terminal region of this protein which is exposed to the cytoplasm and a large carboxy-terminal domain exposed to the periplasm. Deletions were made in this carboxy-terminal domain and assembly of these deletion proteins into the membrane was assessed by protease accessibility. It was found that deletion of a large portion of this domain prevented the remaining protein from being translocated across the membrane. This result suggests that this carboxy-terminal domain contains positive information necessary for membrane assembly.

Other examples of positive secretion information found in the sequences of mature proteins are discussed in the article by Ferenci and Silhavy (1987). These authors also address the question of whether these residues play a passive role in protein secretion or an active role, perhaps as a recognition site. While the question has certainly not been resolved, the available evidence seems to support a passive role. One possibility is that residues at the amino terminus of the mature protein do not assume a highly structured conformation, thereby facilitating an interaction of the signal sequence with the cellular export machinery. Consistent with this possibility is the observation that the *N*-terminal amino acid sequence of the major outer membrane protein of *E. coli* consists of residues not expected to form α-helices or β-sheets (Ferenci and Silhavy, 1987).

Cellular export machinery

As is the case with eukaryotes, prokaryotic protein secretion appears to require several cellular proteins. These components of the cellular export machinery of *E. coli* have not been well characterized biochemically but have been studied

genetically. Benson, Hall and Silhavy (1985) have compiled a list of genetic loci in *E. coli* that may specify components of the cellular export machinery. This list contains the genes *lep* and *lsp* which code for the signal peptidase I (Date, 1983; Silver and Wickner, 1983) and the signal peptidase II (Yamagata *et al.*, 1983; Regue *et al.*, 1984), respectively. In addition there are 18 other chromosomal genes that appear to be involved in protein secretion. These genes have been discussed in detail (Benson, Hall and Silhavy, 1985; Beckwith and Ferro-Novick, 1986) and for the purposes of this review only two well-characterized genes, *secA* and *secY* or *prlA*, are discussed here.

Sec gene mutants were identified and isolated by their phenotype of causing a pleiotropic defect in secretion. Beckwith and co-workers have described a gene, *secA*, which seems to code for a component of the *E. coli* secretion machinery (Oliver and Beckwith, 1981). The *secA* gene product is a 92 kD polypeptide that is a peripheral component of the cytoplasmic membrane (Oliver and Beckwith, 1982a, b). Temperature-sensitive mutations in this gene lead to the cytoplasmic accumulation of precursors of a number of secreted proteins.

The *prlA* (or *secY*) gene was first detected by the restoration of secretion of λ receptor protein (LamB) with mutationally defective signal sequences (Emr, Hanley-Way and Silhavy, 1981). Mutations in the *prlA* gene were also found to suppress the secretion defect in signal sequence mutants of maltose-binding protein (MalE) and alkaline phosphatase (PhoA) (Emr and Bassford, 1982; Michaelis *et al.*, 1983). PrlA (or SecY) protein is probably a component of the normal secretory apparatus because other mutations on the *prlA* (or *secY*) gene can cause a pleiotropic defect in protein export (Ito *et al.*, 1983; Ito, 1984). The fact that *prlA* mutants can restore the secretion of proteins with defective signal sequences argues that a protein component (encoded by the *prlA* or *secY* gene) of the normal secretory apparatus has been altered in such a way as to allow passage of those mutated proteins with defective signal sequences. Furthermore, SecY (or PrlA) protein was found to be an integral membrane protein, the properties of which closely resemble those of another integral membrane protein, lactose carrier (LacY) protein (Akiyama and Ito, 1987).

The roles of other genes (e.g. *secB, C, D* and *prlB, C, D*, etc.) that have been identified await further elucidation. It is likely that the products of these genes will also be important components of the secretory mechanism of *E. coli*.

Biochemical approaches to defining the components of the cellular export machinery have yielded only limited results. Müller and Blobel (1984) have demonstrated the existence of a soluble activity from *E. coli* that is necessary for the export of proteins in a reconstituted cell-free translocation system. This soluble activity sediments in a sucrose gradient at about 12S. The factor has been partially purified by selectively adsorbing 6S RNA to an ω-NH_2-butylagarose column and by salt elution from DEAE-sepharose. The behaviour on the DEAE column suggests that the factor is a complex of molecules. This soluble export factor may be related to one of the proteins defined by genetic methods. The SecA protein may be a part of this export factor complex but definite identification awaits future purification of the components of this complex.

A soluble activity called 'trigger factor' has been reported by Crooke and Wickner (1987) to be necessary for translocation of pro-OmpA in a cell-free *E. coli* translocation system. Trigger factor appears to be a protein with an apparent molecular weight of about 60 000 and is thought to act during the folding of the protein allowing pro-OmpA to fold into an assembly-competent conformation. The apparent molecular weight of trigger factor suggests that it is distinct from either SecA protein which is about 92 000 daltons or the soluble activity of Müller and Blobel (1984).

Coupling of protein synthesis and secretion

Eukaryotic protein secretion has long been thought to be co-translational, i.e. tightly coupled to its synthesis. While this is undoubtedly true in many situations, there are now examples of eukaryotic proteins that can be secreted post-translationally (Schatz, 1986).

In *E. coli*, protein secretion can occur by either a co- or a post-translational mechanism. Josefsson and Randall (1981) were able to demonstrate that processing *in vivo* of the precursor of *E. coli* maltose-binding protein could occur by either mechanism. Similarly, Koshland and Botstein (1982) have presented evidence for the post-translational translocation of β-lactamase (EC 3.5.2.6) across the bacterial inner membrane, and Wickner and his co-workers (Goodman, Watts and Wickner, 1981) have found that M13 procoat protein could be inserted post-translationally into *E. coli* membranes. More recently, Chen, Rhoads and Tai (1985), working with an *in vitro* protein translocation system, have shown that both the *E. coli* OmpA protein and alkaline phosphatase can be translocated across membrane vesicles after protein synthesis has been inhibited. Thus, many secreted *E. coli* proteins, including proteins normally secreted to the periplasm and proteins normally inserted into the outer membrane, are translocated either co- or post-translationally.

ENERGY OF PROTEIN TRANSLOCATION

What is the source of energy for the translocation of proteins across the membranes of the *E. coli* cell envelope? This question has been discussed by Randall (1985), Wickner and Lodish (1985), and more recently by Eilers and Schatz (1988). At one time, translocation across membranes was thought to be driven by elongation of the polypeptide chain during protein synthesis. This energy was derived from the hydrolysis of high-energy phosphate bonds. For this to be true, protein secretion would have to be co-translational. As discussed above, protein export in *E. coli* is not necessarily coupled to protein synthesis, therefore, an alternative source of energy must exist. Recently acquired evidence indicates that two sources of energy are required for the optimal export of proteins in *E. coli*. These sources of energy are ATP and the proton motive force.

The first demonstration of an energy requirement for protein translocation in *E. coli* was by Wickner and his colleagues (Date, Goodman and Wickner, 1980) who showed that depletion of cellular pools of high-energy phosphate

had no effect on the conversion of M13 procoat to mature coat protein. However, use of an uncoupler of the electrochemical potential gradient of protons did result in an accumulation of precursor within the cytoplasm of the cell (Date *et al.*, 1980). Subsequently, others have shown a similar effect with several periplasmic proteins including maltose-binding protein (Enequist *et al.*, 1981; Pages and Lazdunski, 1982), arabinose-binding protein (Enequist *et al.*, 1981), leucine-specific binding protein (Daniels *et al.*, 1981), TEM β-lactamase (Daniels *et al.*, 1981; Pages and Lazdunski, 1982), and alkaline phosphatase (Pages and Lazdunski, 1982). In addition, secretion of the outer membrane proteins OmpF (Enequist *et al.*, 1981; Pages and Lazdunski, 1982), OmpA (Enequist *et al.*, 1981; Pages and Lazdunski, 1982; Zimmerman and Wickner, 1983), and LamB (Enequist *et al.*, 1981; Pages and Lazdunski, 1982) also appears to require the presence of a proton motive force.

A requirement for ATP in protein translocation was demonstrated in an *E. coli* cell-free translocation system (Chen and Tai, 1985). Export of alkaline phosphatase and OmpA in this system was dependent on ATP and stimulated by proton motive force. In a similar system, Wickner and colleagues showed that both ATP and proton motive force were required for the secretion of OmpA (Geller, Movva and Wickner, 1986).

THE ROLE OF PROTEIN CONFORMATION IN EXPORT

From the preceding discussion, it is apparent that export of proteins in *E. coli* is a complex process, involving a multitude of proteinaceous factors, and the expenditure of energy. One fundamental concept is the idea that proteins are competent for secretion only if they have not folded into a stable tertiary conformation. The importance of conformation to protein secretion has been pointed out by several authors (Koshland and Botstein, 1982; Minsky, Summers and Knowles, 1986; Wickner and Lodish, 1985). Indeed, conformational differences between precursors and mature forms have been demonstrated for the leucine-specific binding protein of *E. coli* (Oxender *et al.*, 1980) and two conformationally distinct forms of processed β-lactamase have been demonstrated (Minsky, Summers and Knowles, 1986). Randall and Hardy (1986) have demonstrated a correlation between competence for export and a lack of tertiary structure in maltose-binding protein.

There is now evidence that there are soluble proteins in the *E. coli* cytoplasm that may be involved in maintaining a translocation-competent conformation of precursor proteins. Trigger factor, discussed in an earlier section, is one such protein (Crooke and Wickner, 1987). Trigger factor has been implicated in an *in vitro* system as a component necessary to form or maintain a translocation-competent conformation of pro-OmpA. Whether trigger factor has an *in vivo* role in protein secretion awaits further experimentation.

MODELS OF PROTEIN SECRETION

Over the years, numerous models have been proposed to account for the movement of proteins across biological membranes. The most prominent of

these are: (1) the signal hypothesis (Blobel and Dobberstein, 1975); (2) the membrane trigger hypothesis (Wickner, 1979, 1986); (3) the direct transfer model (von Heijne and Blomberg, 1979; von Heijne, 1980); and (4) the loop model (Inouye and Helegoua, 1980). These models have been instrumental in stimulating investigation into the events leading to translocation of a protein, but none of them adequately accounts for all of the experimental data currently available. Within the past year, a model has been proposed by Randall, Hardy and Thom (1987) that does attempt to bring up to date our ideas concerning the export of proteins. This recent model is the only one discussed here and the reader is directed to the references listed above for details of the earlier models.

In this model, proteins destined for secretion are synthesized as precursors with amino-terminal signal sequences. During synthesis, or after synthesis is complete, the precursor associates with a soluble protein in the cytoplasm. There is evidence that such a factor exists in *E. coli* (Müller and Blobel, 1984; Crooke and Wickner, 1987) and the precursor of maltose-binding protein is reported to associate with a cytosolic factor (Randall, Hardy and Thom, 1987). In eukaryotes, an analogous association of the precursor with a cytosolic complex occurs and this association is thought to lead to translation arrest. However, in *E. coli* there is no evidence for an arrest in synthesis of the precursor.

The complex formed between the precursor protein and the cytoplasmic factor is postulated to have two functions. The factor is thought to bind to the precursor to form a complex which hinders folding of the polypeptide into a tertiary conformation, thereby locking the precursor into a state that is competent for secretion. The second role of this complex is to bind a proteinaceous component of the translocation apparatus in the membrane. The translocation apparatus would be made up of several elements including the signal peptidase and the SecY protein and possibly other proteins. The precursor protein would be transferred from the cytosolic factor to the translocation apparatus. ATP is involved in this process, but its role is unknown. However, Randall and co-workers (1987) speculate that hydrolysis of ATP might occur in a reaction catalysed by the cytosolic factor with an accompanying change in conformation that would allow transfer of the precursor to the translocation apparatus and release of the factor. Another possibility is that hydrolysis of ATP could provide the energy for translocation.

Another possible role for ATP has been suggested by Rothman and Kornberg (1986) who predicted that ATP-dependent 'unfoldases' participate in the post-translational movement of proteins across membranes. These 'unfoldases' would be responsible for unfolding the precursor into the loosely structured conformation necessary for secretion.

The events involved in the actual translocation event itself are even more obscure. Randall, Hardy and Thom (1987) have included a proteinaceous translocation apparatus in their model. However, it is possible that translocation occurs directly through the membrane and that any proteins involved (such as SecY) function only as receptors. What is clear is that at some point during this translocation, the signal peptidase cleaves the signal sequence and the processed protein is deposited on the outer side of the membrane.

The foregoing discussion of protein secretion in *E. coli* was intended to illustrate the complexity of this process. Although considerable progress has been made in the past ten years, there are still many aspects of protein secretion in *E. coli* that are not well understood and undoubtedly many components of the secretory pathways that are yet to be discovered. The remainder of this review turns to a discussion of how these components can be exploited to achieve a goal, i.e. translocation of a protein (specifically human growth hormone (hGH)) from the site of synthesis to a chosen compartment, either the periplasm or the culture medium.

Secretion of higher eukaryotic proteins in *E. coli*

Secretion cloning vectors have been constructed using signal sequences of homologous and heterologous origin to direct the secretion of foreign proteins (Lunn, Takahara and Inouye, 1986a, b). Secretion of mammalian proteins (excluding hGH) and other eukaryotic proteins in *E. coli* using various cloning vectors are not discussed in detail in this review. Instead, data related to this topic are summarized in *Table 1*. The secretion of human growth hormone in *E. coli*, however, is discussed in detail in the following sections.

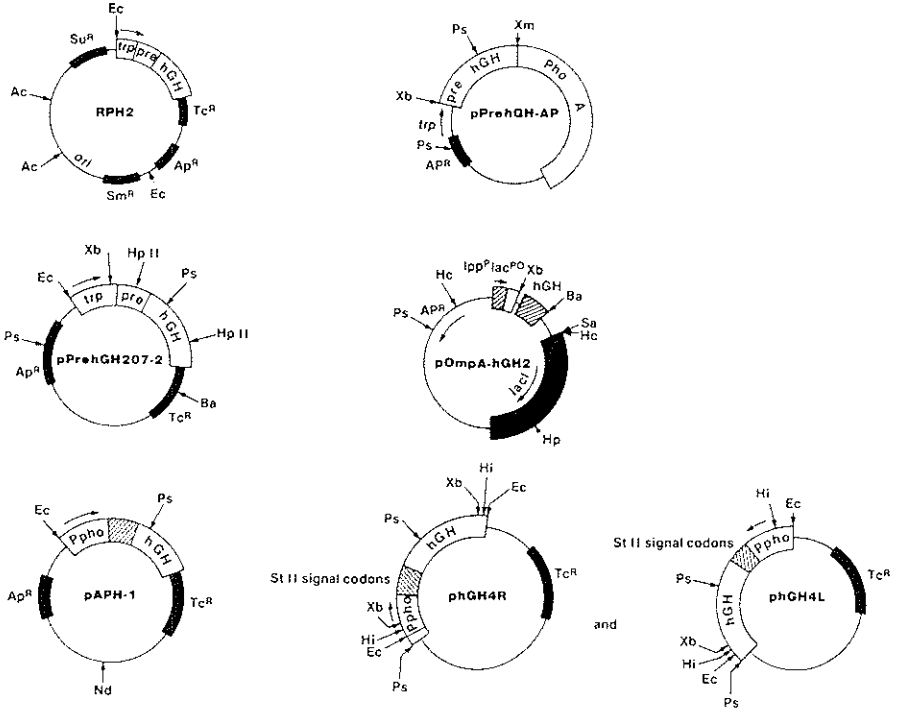

Figure 1. hGH secretion vectors. Abbreviations are hGH, human growth hormone gene; trp, trp promoter; pre, signal or pre-sequence; Tc^R, tetracycline resistance gene; Ap^R, ampicillin resistance gene; phoA, alkaline phosphatase gene; STII, heat-stable enterotoxin II gene; Sm^R, streptomycin resistance gene; Su^r, sulphonamide resistance gene; lac^{PO}, lac promoter and operator gene; lacI, lac repressor gene.

Table 1. Secretion of higher eukaryotic proteins in *E. coli*

Proteins	Strain	Signal sequence	Correct processing and folding	References
Chicken ovalbumin	HB101	Chicken ovalbumin	Unknown	Fraser and Bruce, 1978
Rat proinsulin	PR13	Rat pre-proinsulin	Unknown	Talmage, Kaufman and Gilbert, 1980 Talmage, Brosius and Gilbert, 1981
Human proinsulin	SK107 or MM294	*E. coli* β-lactamase	Unknown	Emerick *et al.*, 1984
Mouse immunoglobin κ light chain L-321	HB101	*E. coli* β-lactamase	Unknown	Zemel-Dreasen and Zamir, 1984
Human epidermal growth factor	C600 or YK537	*E. coli* alkaline phosphatase	Yes	Oka *et al.*, 1985, 1987
Bovine pancreatic trypsin inhibitor	W3110	*E. coli* alkaline phosphatase	Yes	Marks *et al.*, 1986
Interferon-α_2	SE5000 or JA221	*E. coli* OmpA	Incomple processing	Barbero *et al.*, 1986
Human superoxide dismutase	W620 or DHI	*E. coli* OmpA	Yes	Takahara *et al.*, 1988
Human granulocyte–macrophage colony-stimulating factor	JM107	*E. coli* OmpA	Correct processing only	Libby *et al.*, 1987

Table 2. HGH secretion plasmids

Plasmid	hGH gene expressed	Host strain	Fraction	hGH by RIA or Elisa assay	
				(μg/ml/A_{550})	(Percentage of total)
RPH2	pre-hGH	*P. aeruginosa* PA02003	Periplasm	1·49	82
			Spheroplasts	0·13	7
pPreHGH207-2	pre-hGH	*E. coli* 294	Periplasm	0·45	76
			Spheroplasts	0·14	23
pAPH-1	pho-hGH	*E. coli* 294	Periplasm	0·23	82
			Spheroplasts	0·015	5
pOmpA-hGH2	OmpA-hGH	*E. coli* RV308	Periplasm	10–15	>80
			Spheroplasts	N/A	N/A
phGH4L	STII-hGH	*E. coli* 294	Periplasm	5·8	N/A
			Spheroplasts		
phGH4L	STII-hGH	*E. coli* W3110	Periplasm	13·8	90
			Spheroplasts	1·39	9
phGH4R	STII-hGH	*E. coli* W3110	Periplasm	7·4	94
			Spheroplasts	0·3	4

Secretion of human growth hormone in bacteria

The first successful secretion of human growth hormone into the periplasmic space by a micro-organism was achieved by transforming *Pseudomonas aeruginosa* with a plasmid containing a gene for the natural hGH precursor (Gray *et al.* 1984). In order to make our discussion of hGH secretion by heterologous hosts (*P. aeruginosa* and *E. coli*) easier to follow, the structures of hGH secretion vectors are shown in *Figure 1* and hGH secretion data are summarized in *Table 2*. The plasmid (RPH2) used the *E. coli trp* promoter and ribosome-binding site and contained a tetracycline-resistance marker (*Figure 1*). *P. aeruginosa* PA02003 transformed with RPH2 was grown in LB medium supplemented with tetracycline. HGH-related material was detected in total cell extracts at a level of 1.6 μg/ml/A_{550}. About 82% of the hGH-related material was found in the periplasm and was shown to have the correct *N*-terminal sequence of hGH (Phe-Pro-Thr-Ile-).

The first successful secretion of hGH in *E. coli* was achieved using the plasmids (*Figure 1*) constructed by Gray *et al.* (1985). In one plasmid (pPrehGH207-2), the natural hGH precursor (pre-hGH) was expressed under the control of the *E. coli trp* promoter. In another plasmid (pAPH-1), the *E. coli* alkaline phosphatase promoter and signal sequence were fused to the mature hGH coding sequence (pho-hGH). *E. coli* 294 cells harbouring either plasmid were found to secrete hGH into the periplasmic space (*Table 2*); however, the yields were poor: 294[pPrehGH207-2] produced 0.6 μg hGH/ml/A_{550} and 294[pAPH-1] produced 0.28 μg hGH/ml/A_{550}. Most of the mature hGH was recovered from the periplasmic fraction and was found to have the correct *N*-terminus, indicating correct processing during protein

transport. The secreted hGH was monomeric and homogeneous as shown by 12.5% SDS-PAGE either in a reduced or unreduced state.

To increase the yield of hGH secretion in *E. coli*, Matteucci and Lipetsky (1986) mutagenized the hGH signal sequence with nitrous acid. They first constructed an hGH-alkaline phosphatase (hGH-AP) fusion vector that secreted hGH-AP fusion protein into the periplasm. Since the hGH-AP fusion protein possessed phosphatase activity only when it was secreted into the *E. coli* periplasm, an alkaline phosphatase assay was used to screen for the mutants that produced and secreted elevated levels of fusion protein. The signal sequences from these mutants were then transferred into plasmids directing the expression and secretion of mature hGH. Although the phenotypes of enhanced expression of secreted protein were largely preserved, the yield of hGH secretion was still low. One hGH signal sequence mutant (Gly → Asp, amino acid 4) gave 0.3 μg hGH/ml/A_{550} cells, while another mutant (Ala → Thr , amino acid 2) gave 0.45 μg hGH/ml/A_{550} cells.

Ghrayeb *et al.* (1984) constructed a series of pIN-III-OmpA secretion cloning vectors using the OmpA signal sequence to direct secretion of foreign proteins. Since the OmpA protein is the most abundant protein in *E. coli* cells, the OmpA signal sequence was suspected to be highly efficient in directing protein secretion. Hsiung, Mayne and Becker (1986) used the plasmid pIN-III-$OmpA_3$ for construction of an hGH secretion vector. The resulting hGH secretion vector, pOmpA-hGH2 (*Figure 1*), contains a hybrid lpp^p-lac^{PO} promoter and the *lacI* gene. The ompA signal peptide sequence is fused directly to the mature hGH gene (*Figure 1*). *E. coli* RV308 cells harbouring the hGH secretion vector pOmpA-hGH2 were found to secrete mature hGH into the periplasmic space. The level of hGH production was approximately 6% of total cell protein as determined by densitometric scanning of an SDS gel. It was also found (H.M. Hsiung, unpublished results) that growing the cells in rich medium (TY broth) resulted in a higher yield of hGH than growth in a minimal medium (M9). The secreted hGH was released from cells by an osmotic shock procedure and hGH was found to constitute 30% of the periplasmic protein (Becker and Hsiung, 1986). A radioimmunoassay and a micro ELISA assay gave values of 10–15 μg hGH/ml/A_{600} in the periplasmic fraction (*Table 2*). Secreted hGH was purified and subjected to amino-terminal sequence analysis. The first four residues were determined to be Phe-Pro-Thr-Ile with Phe the only residue detected at the amino terminus. This result indicated that the *E. coli* signal peptidase efficiently cleaved the ompA-hGH precursor protein at the correct position to generate mature hGH.

Other hGH secretion vectors (phGH4R and phGH4L, *Figure 1*) have been constructed by fusing the hGH gene to the signal sequence of the *E. coli* heat-stable enterotoxin II (STII) (Chang *et al.*, 1987). This hybrid gene was expressed in *E. coli* under the transcriptional control of the *E. coli* alkaline phosphatase promoter. In low-phosphate growth medium, *E. coli* W3110 cells synthesized 8–15 μg of hGH/ml/A_{550} unit of cells. More than 90% of the hGH produced was also found to be processed correctly and secreted into the periplasmic space. These results further demonstrate that *E. coli* cells are able to synthesize and secrete high levels of a mammalian protein using an *E. coli*

signal sequence. It is difficult to assess the relative efficiencies of hGH secretion by OmpA and STII signal sequences since different promoters and different *E. coli* host strains were used to produce and secrete hGH.

It was observed that *E. coli* host strains have an important role in hGH production, affecting the efficiency of the secretion vectors. The *E. coli* strain W3110 yielded two to three times the amount of hGH compared with the *E. coli* strain 294. Strain W3110 cells harbouring phGH4L yielded the highest amount of hGH, i.e. 15·4 μg of hGH/ml/A_{550} cells (*Table 2*). Furthermore, it was found that the counter-clockwise orientation of the phoA promoter in phGH4L is preferred to the clockwise orientation in phGH4R for optimal hGH synthesis (*Table 2*). The difference in hGH production levels between the two plasmids was due to the difference in plasmid copy number (Chang *et al.*, 1987).

Expression and secretion of hGH in *E. coli* is also dramatically affected by the conditions of cell culture. Chang *et al.* (1986) have distinguished certain profound culture medium effects: (1) culture pH is critical, with neutrality being optimal for hGH production; (2) potassium levels seem to be important, either inhibiting or stimulating hGH production; (3) some poorly defined medium components such as yeast extract and vegetable lecithin are strongly stimulatory for hGH production. These authors suggest that extracellular organic molecules might affect secretion by absorption into, and modification of, the *E. coli* membranes and that K^+ and pH might affect secretion by changing membrane energetics.

Excretion of human growth hormone into the *E. coli* culture medium

There exist even fewer reports in the literature of successful excretion of hGH into the culture medium by *E. coli*. Kato *et al.* (1987) constructed an excretion vector, pEAP8, to facilitate the excretion of foreign protein in *E. coli* (*Figure 2*). This plasmid contained the weakly activated *kil* gene of plasmid pMB9 (Kobayashi *et al.*, 1986) and the penicillinase (β-lactamase; EC 3.5.2.6) promoter and signal sequence from an alkalophilic *Bacillus* sp. were used to direct the expression and excretion of foreign proteins. This vector pEAP8 was then used to construct an hGH excretion plasmid p8hGH1 (*Figure 2*). The *E. coli* cells harbouring the plasmid p8hGH1 excrete mature hGH (11·2 mg/litre culture) into the culture medium through the outer membrane, made permeable by the action of the *kil* gene. However, significant amounts of hGH (8·6 mg/litre or 42%) still remained in the periplasmic fraction. The *N*-terminal amino acid sequence and the biological activity of the excreted hGH were consistent with those of authentic hGH.

Processing and folding of secreted human growth hormone

For secretion into the periplasm to be advantageous over cytoplasmic production of hGH, the secreted hGH must be both correctly processed, i.e. the signal sequence must be cleaved at the appropriate site, and correctly folded. In most of the studies discussed above, these issues have been

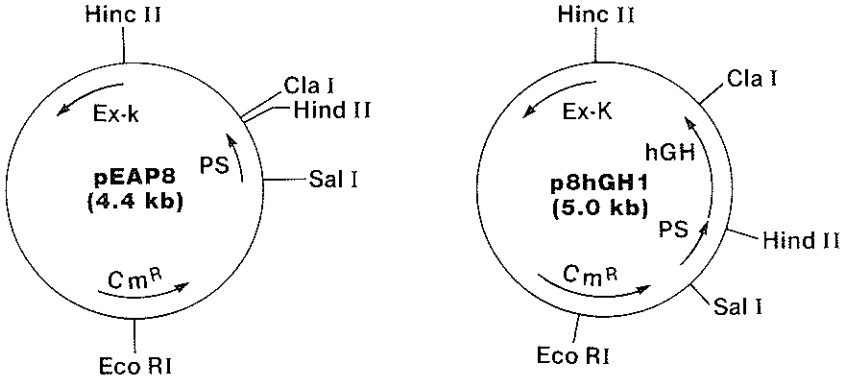

Figure 2. hGH excretion vectors (after Kato *et al.*, 1987). Abbreviations are Ex-k, excretion promoter and *kil* gene region; PS, the penicillinase promoter and signal fragment; Cm^R, chloramphenicol resistance gene.

addressed. Correct processing can be assessed by comparing the mobility on an SDS-polyacrylamide gel with a standard or by actually isolating the secreted hGH and subjecting it to *N*-terminal sequence analysis. Clearly, this latter method is better and has been applied by Gray *et al* (1985), by Hsiung, Mayne and Becker (1986) and by Chang *et al.* (1987) to prove that processing of precursor hGH takes place at the correct position during protein transport across the cytoplasmic membrane.

These results also demonstrate that both the mammalian signal sequence and the bacterial signal sequence can direct hGH secretion in *E. coli*. However, the two most efficient secretion vectors used *E. coli* signal sequences from either the outer membrane *ompA* or the heat-stable enterotoxin II gene products.

The folding of secreted hGH has been studied by several techniques. Disulphide bridge formation was first analysed by Gray *et al.* (1985) who examined the electrophoretic pattern of hGH on SDS-polyacrylamide gels. The met-hGH produced intracellularly in *E. coli* harbouring the expression plasmid pHGH509 was heterogeneous, displaying several electrophoretic forms when the protein was not first reduced. These forms included monomers with different disulphide bridges as well as dimers and higher-order oligomers. It was postulated that the generation of various hGH forms is a consequence of the sudden transfer of met-hGH from the reducing environment of the *E. coli* cytoplasm (Pollitt and Zalkin, 1983) to a more oxidizing environment upon the preparation of cell extracts. If the proteins in the cell extract are first reduced, the hGH is monomeric and has essentially the same mobility as the reduced met-hGH standard on SDS-polyacrylamide gels. In contrast, periplasmic hGH is homogeneous and monomeric in both its reduced and unreduced states. The mobilities of these two states, which differ from each other, are similar to the corresponding states of the purified met-hGH or authentic hGH standard, which are known to contain the correct disulphide bonds (cys53-cys165, cys182-cys189).

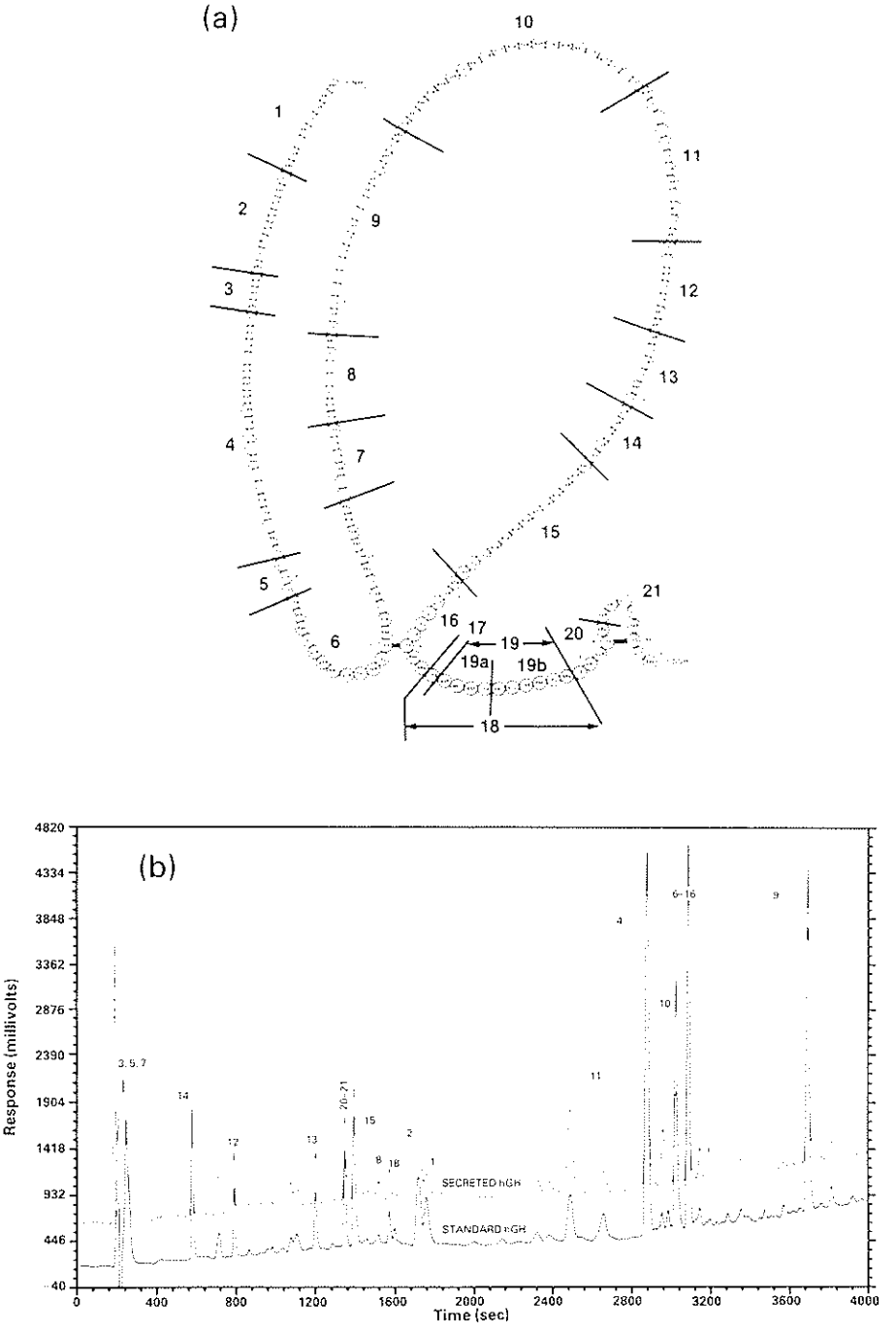

Figure 3. (a) Disulphide bonds in hGH; (b) tryptic maps comparing secreted hGH with hGH standard. Tryptic digests of secreted hGH and standard hGH were prepared as described by Becker and Hsiung (1986) [Courtesy of Elsevier Science Publishers].

The most convincing evidence showing the correct disulphide bond formation in secreted hGH is provided by the tryptic mapping data for hGH shown in *Figure 3* (Becker and Hsiung, 1986). When native hGH is digested with trypsin (EC 3.4.21.4) at pH 8 and the resulting tryptic peptides are separated by reverse-phase HPLC (tryptic mapping), the two disulphide bond-containing peptides can be readily resolved and identified. These two disulphide peptides are labelled 6–16 and 20–21 (*Figure 3a*). The tryptic map of secreted hGH (*Figure 3b*) clearly shows that these two peptides are present. Comparison of the tryptic map of the *E. coli* secreted hGH with that of standard hGH (*Figure 3b*) shows that secreted hGH is indistinguishable from standard hGH.

The conformation of secreted hGH was also investigated using circular dichroism (CD) spectroscopy. The spectra of secreted hGH and standard hGH (*Figure 4*) appeared to be identical, each with a minimum at 208 nm and a shoulder at 222 nm, characteristic of a high α-helical content. The two spectra

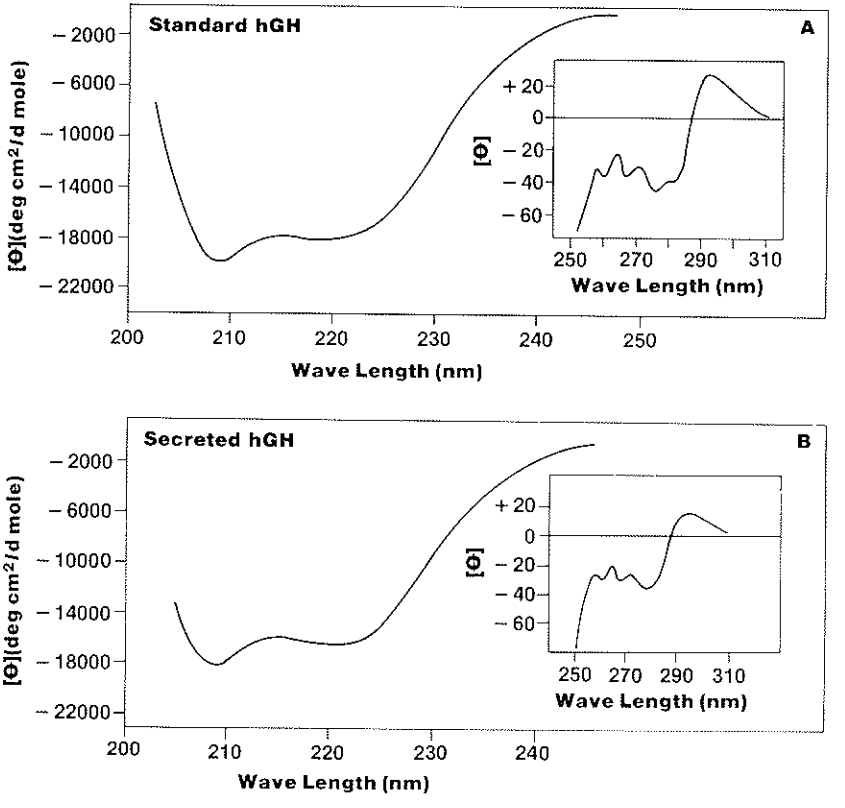

Figure 4. CD spectrum of secreted hGH. The CD spectrum of secreted hGH was scanned from 200 to 350 nm on a Cary 61 spectropolarimeter calibrated with d-10-camphorsulphonic acid. Panel A shows the CD spectrum of standard hGH and Panel B the CD spectrum of secreted hGH (from Becker and Hsiung, 1986) [Courtesy of Elsevier Science Publishers].

are also quite similar in the near-UV region, with a small positive band in the 290 nm range and weak negative dichroic bands in the 260–290 nm region. These two spectra are also identical to the published CD spectra of pituitary hGH (Bewley and Li, 1975). These results indicate that the conformation of secreted hGH, isolated from the *E. coli* periplasm, is indistinguishable from the conformation of authentic native hGH isolated from the pituitary gland. In conclusion, the tryptic mapping and CD studies of the secreted hGH indicate that the hGH protein has a correct secondary and tertiary structure. Furthermore, these results show that the *E. coli* periplasm can provide an environment to facilitate efficient disulphide bond formation and proper folding of hGH.

Conclusions

In this review, we have discussed in general the apparatus and mechanism of *E. coli* secretion systems. We have also described in detail the specific use of *E. coli* secretion systems to secrete a mammalian protein, hGH. Although Gram-negative bacteria, such as *E. coli*, have not been recognized for their secretion capability, we believe that *E. coli* can be an ideal secretion host for a number of commercially important eukaryotic proteins. *E. coli*, unlike *Bacillus* or other Gram-positive bacteria, does not secrete many proteases that may degrade the desirable protein products in the culture medium. Furthermore, several laboratories, including ours, have found that the *E. coli* signal peptidase can correctly process the secreted proteins, thereby producing the authentic natural protein products. The use of authentic natural sequence proteins may be important for reducing the incidence of antibody formation in patients treated with these protein products. Finally, the *E. coli* periplasm is able to provide an appropriate oxidative environment to allow correct folding of secreted proteins, thereby eliminating the often inefficient *in vitro* refolding processes required to generate biologically active proteins.

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