High levels of gene expression give many advantages including early and easy detection of recombinant viruses. Protein purification protocols, such as immunoaffinity chromatography, perform best with a high ratio of the protein compared to total protein in the starting material. *Do* isolate the best expressing recombinant viruses and *do* handle the Sf9 cells at optimal growth conditions, giving yourself enormous potential for maximal yields of purified protein in high concentration with minimal handling.

Acknowledgments

I wish to thank Robert Lanford for numerous discussions. He has been working with the baculovirus system in close association with Max Summers and has, as we have, constructed and tested SV40 T-Ag and small t-Ag recombinant insect viruses.[62] Others with whom I have shared both good and bad research findings include Beth Weiner, Ellen Fanning, Cheryl Murphy, and Nidhi Williams.

[62] R. Lanford, *Virology* **167,** 72 (1988).

[11] Secretion of Heterologous Proteins in *Escherichia coli*

By I. Barry Holland, Brendan Kenny, Boris Steipe, and Andreas Plückthun

Secretion of heterologous proteins from *Escherichia coli* into the culture medium is a useful means of protein purification that avoids the difficulties associated with renaturation and purification of proteins from inclusion bodies. In principle, secretion to the medium rather than export into the periplasmic space should have some advantages in ease of recovery. In addition, since in many cases export of foreign proteins across the inner membrane via the *secA*,*secY*-dependent pathway is rather inefficient, the use of an alternate system may be desirable. The *E. coli* hemolysin (Hly) secretory process, which involves a completely novel translocation mechanism, and apparently promotes high levels of secretion, is described in this chapter.

Secretion from *E. coli* of heterologous proteins via fusion to the C-terminal Hly signal is still a developing technology and its full potential is still to be explored. This chapter is of necessity, therefore, a presentation of the state of the art technology.

The Hly system has the merit of being endogenous to *E. coli* and as such should be amenable to considerable refinement and exploitation in

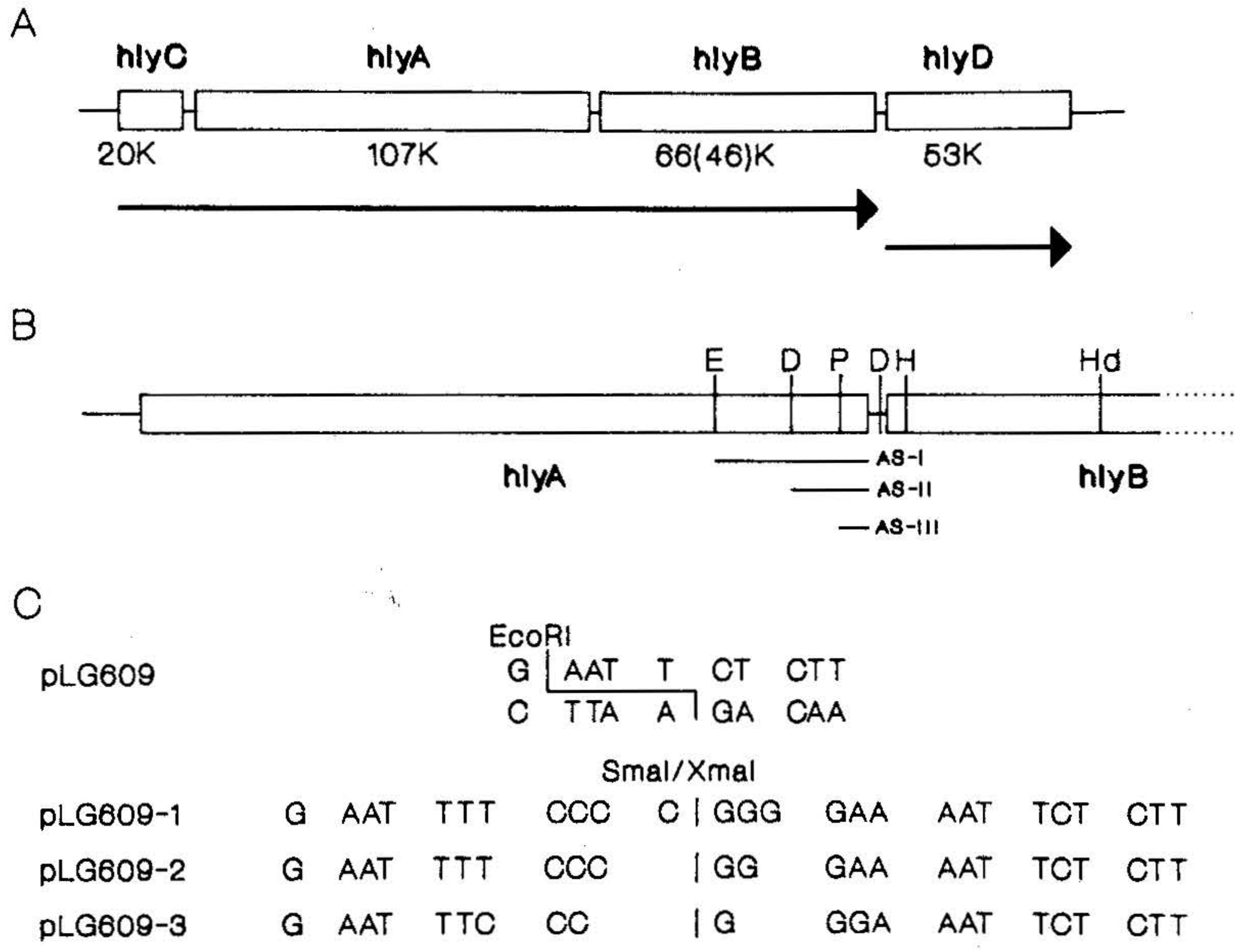

FIG. 1. Organization of the Hly 2001 cassette. (A) Molecular weights of the Hly proteins are indicated and arrows show probable transcription units. (B) The major restriction sites in *hlyA* and *hlyB* referred to in the text: E, *Eco*RI; D, *Dra*I; P, *Pvu*II; H, *Hpa*I; Hd, *Hin*dIII. AS-I, II, III indicates the fragments carrying the HlyA signal domains of 23 kDa, 12 kDa, and approximately 4 kDa (40 terminal amino acids), respectively. (C) See text.

the future as basic knowledge of the secretion mechanism itself is acquired.

We shall describe in this chapter the essential features of the hemolysin transport process and then the strategies, procedures, and vectors currently available, or under development, for achieving secretion of foreign polypeptides. In addition, some limitations still associated with this system will be discussed.

Hemolysin (HlyA) is secreted from certain pathogenic strains of *E. coli* or from laboratory strains carrying the cloned Hly determinant (see Fig. 1). Secretion occurs continuously throughout most of the growth phase and is not accompanied by cell lysis.[1,2] Secretion in this case is dependent on a novel targeting sequence which has been localized to the

[1] N. Mackman, J.-M. Nicaud, L. Gray, and I. B. Holland, *Curr. Top. Microbiol. Immunol.* **125,** 159 (1986).

[2] N. Mackman, K. Baker, L. Gray, R. Haigh, J.-M. Nicaud, and I. B. Holland, *EMBO J.* **6,** 2835 (1987).

last 27–40 amino acids at the extreme carboxy-terminus of HlyA.[2] Secretion is independent of the secA protein export pathway, but instead specifically requires the products of *hlyB* and *hlyD*.[2,3] Transport to the medium does not involve any detectable periplasmic intermediate and it is suggested that HlyB and HlyD form part of a specific translocation complex, spanning the inner and outer membranes, allowing direct secretion to the medium.

Secretion of the hemolysin toxin normally occurs with a $t_{1/2}$ of approximately 2–3 min at 30° (our unpublished data). Secretion levels in *E. coli* K12 strains carrying a single copy of the wild-type Hly determinant are equal to about 0.02% of total cell protein.[4] However, at least 100-fold higher secretion levels of the C-terminal, 23-kDa fragment alone can be achieved when this peptide is expressed from a *lac* promoter on a multicopy plasmid (see Fig. 3).

The HlyA secretion signal is not processed during translocation. Heterologous proteins bearing the Hly targeting sequence are therefore recovered as fusion polypeptides, although protease-sensitive cleavage sites can be introduced into the constructions. C-terminal fragments of HlyA containing either 210 (AS-I), 120 (AS-II), or 40 (AS-III) amino acid residues have so far been used to promote secretion of heterologous polypeptides. Secretion efficiency is, however, considerably reduced with the shorter targeting fragments. Other structural features upstream of the minimal targeting signal may therefore be important in determining the final level of secretion (see below).

General Procedures for Secretion of Fusion Proteins

As illustrated in Fig. 2 the protein of interest is first expressed in *E. coli* from an appropriate expression vector. Suitable restriction sites may then be selected or engineered to generate a blunt cleavage site at the C-terminus which may subsequently be ligated to the unique *Sma*I/*Hpa*I fragment encoding the appropriate reading frame of AS-I from the pLG609 series (Fig. 1C), generating an in-frame C-terminal fusion. pLG609 [6.1 kilobases (kb)] was constructed by insertion of the *Eco*RI–*Hin*dIII fragment of the Hly determinant downstream of the *tac* promoter of pTTQ18, which carries the *lacI*^{q} gene.[5] *Sma*I linkers were inserted into the *Eco*RI site, generating fusions in the three reading frames encoded by pLG609-1, 2, and 3, respectively. pLG609 may occasionally give rise to

[3] L. Gray, K. Baker, B. Kenny, N. Mackman, R. Haigh, and I. B. Holland, *J. Cell Sci., Suppl.* **11,** 45 (1989).

[4] J.-M. Nicaud, N. Mackman, and I. B. Holland, *J. Biotechnol.* **3,** 175 (1985).

[5] M. R. J. Stark, *Gene* **51,** 255 (1987).

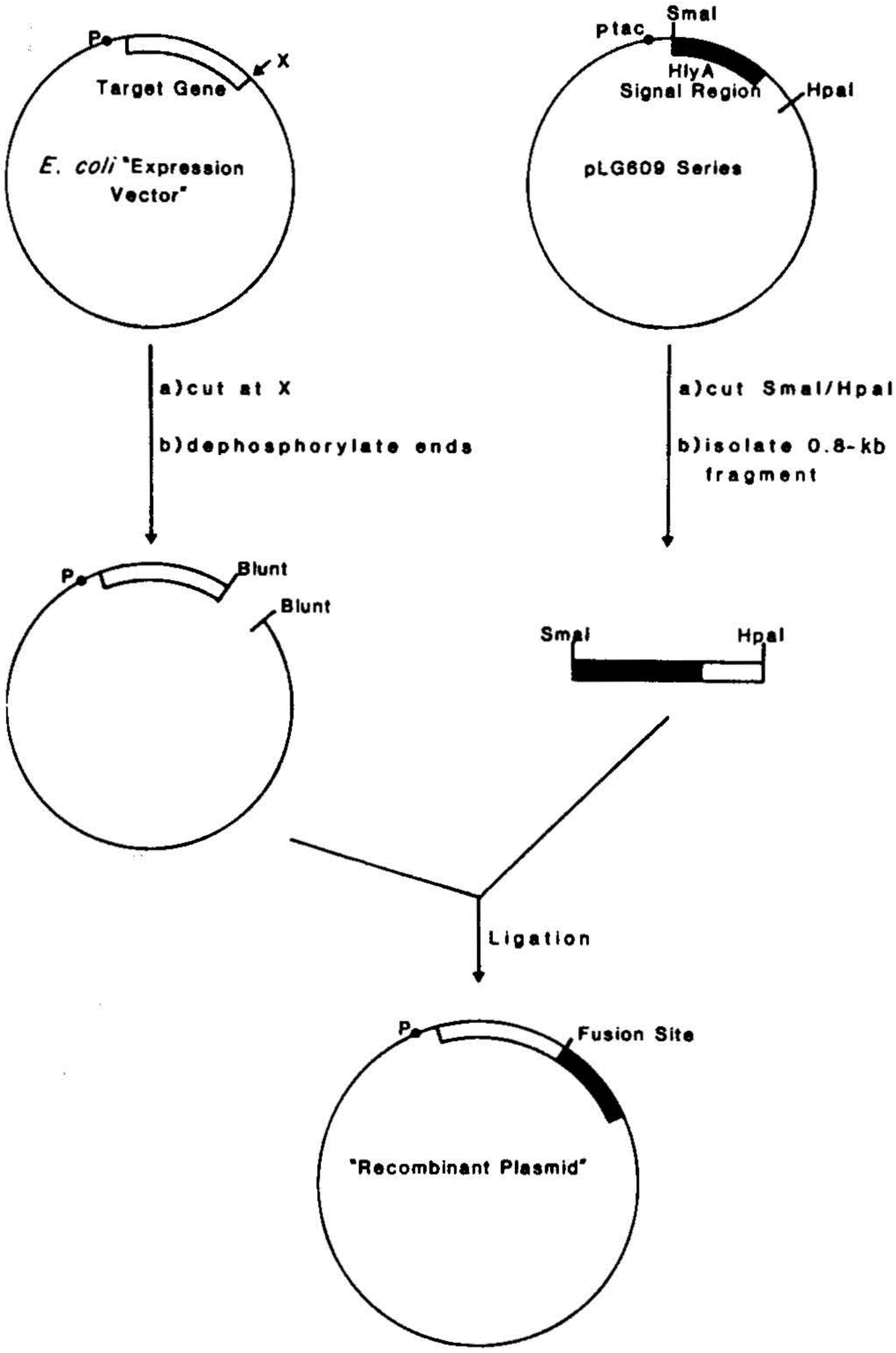

FIG. 2. Strategy for the construction of hybrid genes bearing the AS-I, HlyA signal domain at the 3′ terminus (see also Ref. 2).

internal deletions, eliminating the Hly fragments and generating a smaller, 2.1-kb miniplasmid. In particular, therefore, when preparing plasmid stocks checks should be carried out to ensure that the donor clones do not contain the miniplasmid, which can be readily distinguished from the parent plasmid on agarose gels.

In preparing the appropriate DNA fragment from pLG609 it is most convenient to cut with *Sma*I and *Hpa*I, followed by insertion downstream

of the gene of interest. Alternatively, the *Sma*I–*Hin*dIII fragment may be used or the *Sma*I–*Hpa*I fragment in pLG609 can first be subcloned into, for example, pUC18 and then reexcised using a variety of 3′ restriction sites.

For production of fusions to the 12-kDa (AS-II) signal domain the corresponding *Dra*I fragment (Fig. 1B) can also be removed from the pLG609 vector. In this case alternative reading frames would have to be generated using linkers.

Escherichia coli strains carrying the fusions are then transformed with pLG575 (carrying *hlyB*, *hlyD* cloned into the *tet* gene of pACYC184[6]). Induction of the synthesis of fusion polypeptides is then carried out both in the presence and in the absence of pLG575. Analysis of supernatant fractions should then allow the unequivocal identification of fusion proteins specifically secreted via the Hly system.

Confirmation that the secreted protein is the required fusion can be obtained by Western blotting using antibodies to either the protein of interest or to the HlyA signal region.

Examples of Use of Hly Secretion System

Secretion of OmpF–AS-I Fusion

We have reported previously that fusion of the *E. coli* outer membrane porin, OmpF, to the HlyA C-terminal 23-kDa signal peptide (AS-I) leads to its efficient secretion in an HlyB, HlyD-dependent manner.[2] In this case the fusion protein was expressed from a *lac* promoter on a multicopy pUC vector, following the addition of IPTG. Secretion of the fusion protein was not accompanied by detectable release of either cytoplasmic or periplasmic markers. In addition, no intracellular hybrid protein was detected when *hlyB* and *hlyD* were present, indicating that secretion was extremely efficient. Similar results were obtained when the OmpF porin was fused to the AS-II signal peptide.

Secretion of Calf Prochymosin Using AS-I and AS-II Signal Peptides

Varying portions of calf prochymosin have been fused to AS-I and AS-II and successfully secreted in an HlyB,HlyD-dependent manner[6a] (see Fig. 3). In this case, expression was obtained from a *trp* promoter carried on the dual-origin, temperature-amplifiable vector described by

[6] N. Mackman, J.-M. Nicaud, L. Gray, and I. B. Holland, *Mol. Gen. Genet.* **201,** 282 (1985).

[6a] B. Kenny and B. Holland, submitted.

A

Summary of Quantification Data

	VECTOR	EFTU as %CELL	HYBRID BAND as % CELL	SECRETED BAND as % INTERNAL HYBRID BAND	% EFTU	% CELL	mg/liter/OD SECRETED	M_r
1	23K pUC	5.0	–	–	59	2.95	1.8	23
2	23K pUC (a)	6.3	–	–	51	3.22	2.3	23
3	23K B/D pUC	6.7	–	–	27	1.82	1.5	23
4	800–1 DOV	4.4	25.9	2.84	18	0.93	0.69	40
5	801 DOV	5.3	6.3	11.9	14.3	0.59	0.66	50
6	802 DOV	6.0	9.6	6.42	9.9	0.44	0.52	60
7	806 DOV	5.2	18.6	0.9	3.35	0.174	0.15	33

B

Prochymosin-Hlya Fusions

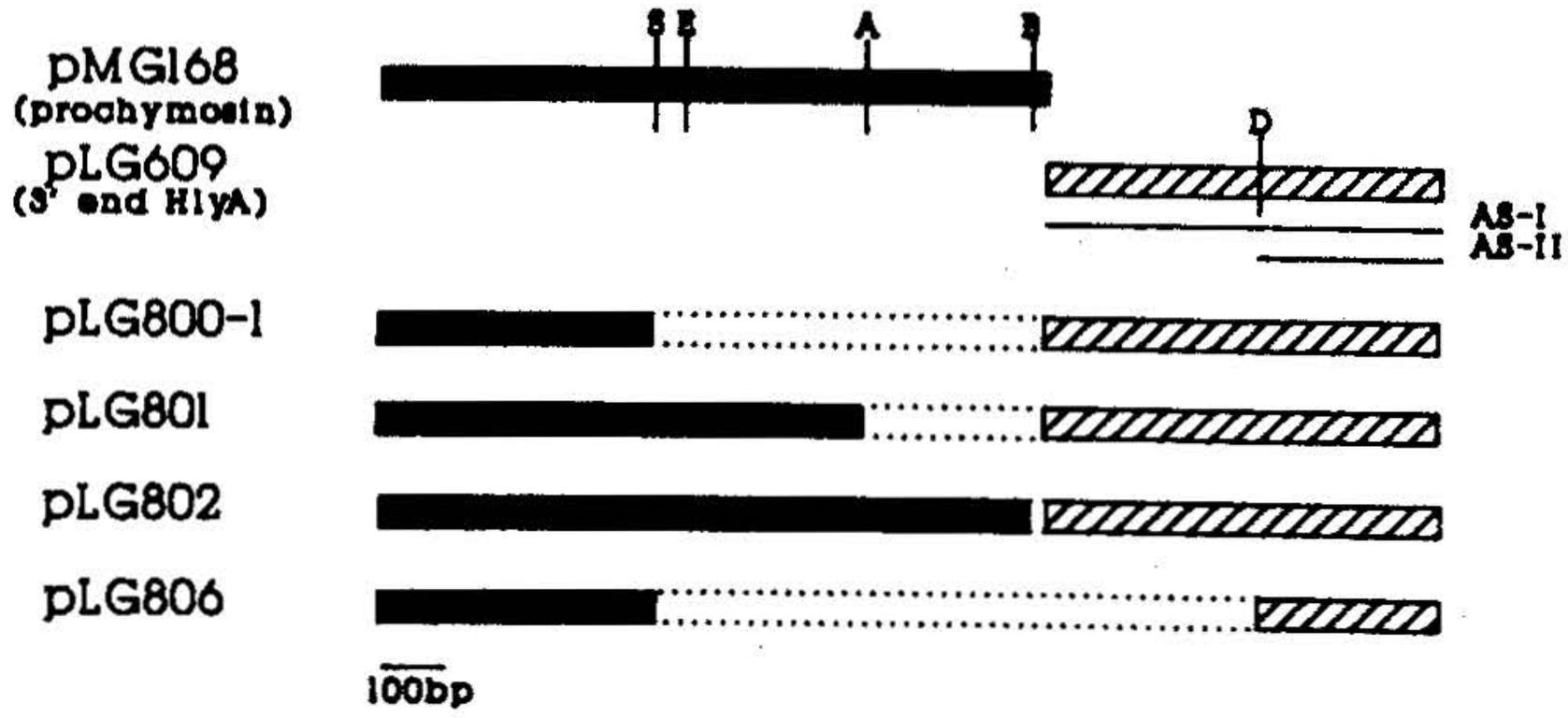

FIG. 3. Secretion levels of the AS-I peptide and different prochymosin-Hly-AS-I, AS-II hybrids. *Escherichia coli* strains (carrying recombinant plasmids) were grown in Difco nutrient medium and induced by addition of IPTG (125 μg/ml) or temperature shift, at $A_{450} = 0.4$ and $A_{450} = 0.7$, respectively. Synthesis of the hybrid proteins was continued for 3 hr when the $A_{450} = 3$ to 4. Samples of cells and culture supernatants were analyzed by SDS–PAGE (11% acrylamide) and the intensity of the Coomassie Blue-stained bands measured using an LKB Bromma Ultrascan XL laser densitometer. (A) Amounts of elongation factor (EFTu), and the intracellular fusion band when present, are expressed as percentage total protein. The level of secreted protein in specific bands is expressed as the percentage of the intracellular form, or of EFTu, or of total cell protein, respectively. The amounts of secreted protein are also given as mg/liter/A_{450} unit. In lines 1–3, the AS-I peptide was expressed from a *lac* promoter in strain NM522 or JM101 [strains carrying an F′ *lacI*^{q} (Ref. 2)] with export functions provided on plasmid pLG575. Alternatively, as shown in line 3, *hlyB,hlyD* were incorporated into the same pUC vector. (a) indicates that in this construct the Ser residue at position 996 in HlyA was replaced by Pro in order to disrupt the putative α helix in this region. As indicated, this had no inhibitory effect on secretion. In lines 4–7 *E. coli* SE5000[2] carried the dual origin vector (DOV)[7] and expression was obtained by heat induction. pLG575 provided the export functions *hlyB,hlyD*. Vectors 800-1, 801, and 802 encoded increasing portions of prochymosin fused to AS-I; 806 encoded approximately the first one-third of prochymosin fused to AS-II. Molecular weights of secreted proteins are given on the right. (B) Details of hybrid constructions indicated above. S, *Sma*I; E, *Eco*RI; A, *Apa*I; B, *Bal*I; D, *Dra*I.

Wright *et al.*[7] With this system total levels of the expressed hybrid protein can reach 25% of total cell protein. However, most of this protein remains in inclusion bodies within the cells. Nevertheless, secreted levels of fusion proteins equivalent to up to 0.8% of total cell protein have been obtained when the Hly export functions are present.

Varying the portion of the calf prochymosin present within the AS-I hybrid protein has little effect upon secreted levels and the largest fusion protein of 60 kDa lacks only the C-terminal three amino acid residues of prochymosin. In this case acidification of culture supernatants containing the fusion protein leads to the formation of a faster running protein in SDS-acrylamide gels,[7a] suggesting that some autocatalytic proteolysis may be taking place as in the case of authentic prochymosin.

Secretion, Purification, and Dimerization of Variable Domains of Antibody McPC603 Using Hemolysin A Signal

To investigate the expression and secretion of antibody domains using the hemolysin export pathway, fusions were constructed between the variable domains of an antibody and the AS-I peptide of HlyA. We used the variable domains V_H and V_L of McPC603, a particularly well-characterized murine immunoglobulin A which binds phosphorylcholine. It has been shown previously that the FV fragment[8,9] and the Fab fragment[10] can be secreted to the periplasm of *E. coli*, using bacterial N-terminal signal sequences, which are correctly processed by the bacterial signal peptidase and that the two chains then associate to form antigen-binding fragments that are fully functional.

Construction of Plasmids. The genes for V_H and V_L were obtained by chemical synthesis and placed under the control of the *tac* promoter of pAP10,[11] a derivative of pKK223-3[12] containing the *lacI*^{q} gene of pJW271.[12a] The AS-I HlyA export signal was obtained as the *Eco*RI–*Hin*dIII fragment from pLG609 (Fig. 1). It was connected in frame to the C-terminus of either V_L or V_H via a synthetic linker encoding the tetrapep-

[7] E. M. Wright, G. O. Humphreys, and G. T. Yarranton, *Gene* **49,** 311 (1986).
[7a] B. Kenny and B. Holland, unpublished observations.
[8] A. Skerra and A. Plückthun, *Science* **240,** 1038 (1988).
[9] A. Plückthun, A. Skerra, R. Glockshuber, and J. Stadlmüller, *in* "Protein Structure and Protein Engineering" (E. L. Winnacker and R. Huber, eds.), p. 123. Springer-Verlag, Berlin, 1988.
[10] A. Plückthun and A. Skerra, this series, Vol. 178, in press.
[11] A. Plückthun, R. Glockshuber, I. Pfitzinger, A. Skerra, and J. Stadlmüller, *Cold Spring Harbor Symp. Quant. Biol.* **52,** 105 (1987).
[12] J. Brosius and A. Holy, *Proc. Natl. Acad. Sci. U.S.A* **81,** 6929 (1984).
[12a] J. Wang, Harvard University, unpublished observations.

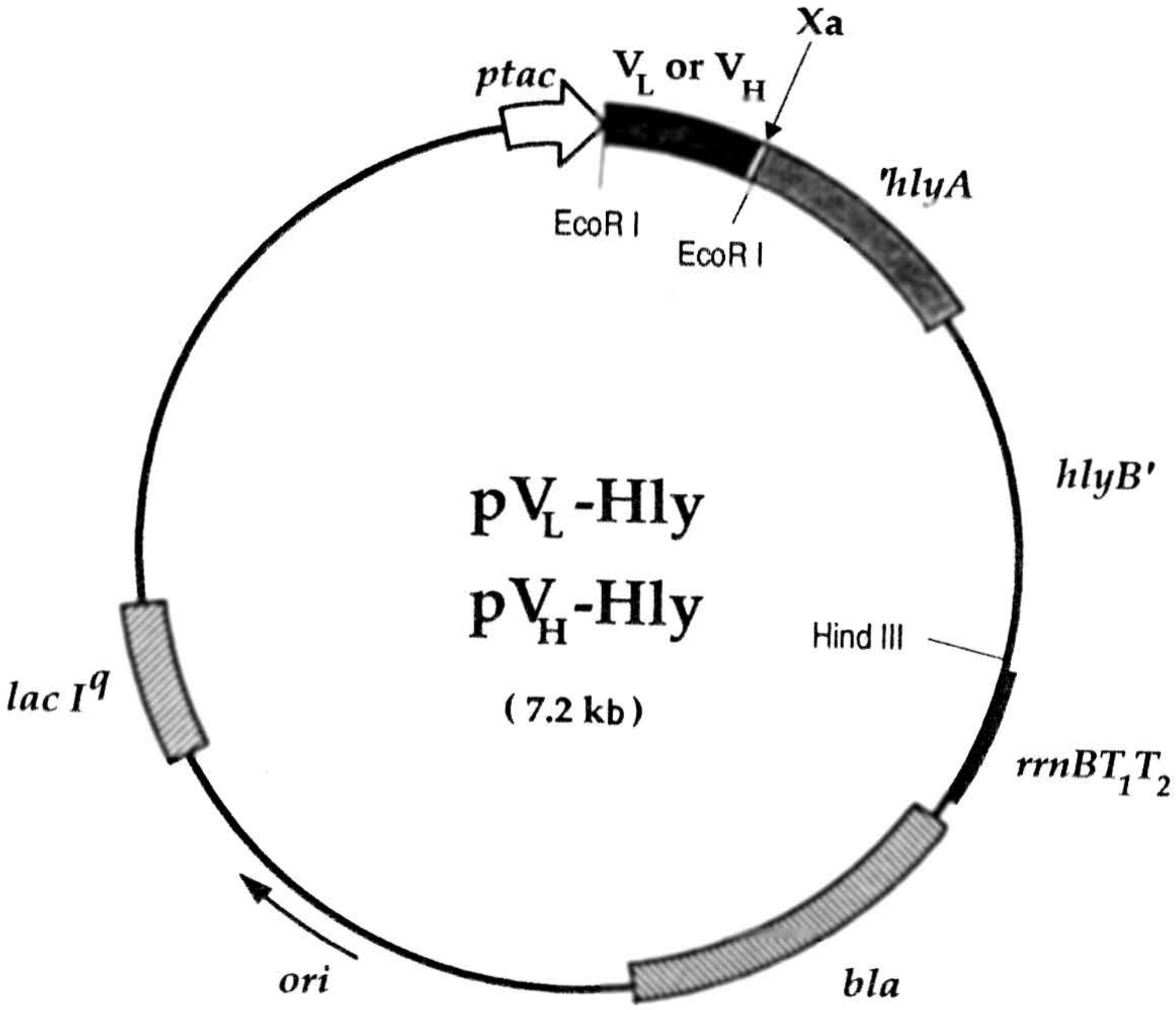

FIG. 4. Plasmids pV_L-*hly* and pV_H-*hly* for the expression of fusion proteins. V_L or V_H designates the segment coding for the respective antibody variable domain, Xa indicates the factor Xa cleavage site connecting the antibody domain and the secretion signal. *'hlyA* is the AS-I fragment of hemolysin A, and *hlyB'* is the N-terminal region of hemolysin B, both encoded on the 1.6-kb *Eco*RI–*Hin*dIII fragment from pLG609.[2] p*tac*, *lacI*^{q}, *rrnBT*$_1$*T*$_2$, *bla*, and *ori* are the *tac* promoter, the gene coding for its repressor, the *rrnB* terminator of transcription, the selectable marker, and the origin of replication, respectively, from pAP10.

tide recognition sequence (Ile-Glu-Gly-Arg) for factor Xa,[13–15] a highly specific protease of the blood clotting system. This linker allows the cleavage of the variable domain from the fusion protein (Fig. 4). Each of the resulting expression plasmids confers ampicillin resistance. Bacteria harboring the plasmids pV_L–Hly and pV_H–Hly can be transformed with pLG575 (*hlyB*, *hlyD*).

Expression and Secretion of V_L-Hly and V_H-Hly Fusions. Both V_L AS-I and V_H AS-I fusions are secreted into the medium in a manner completely dependent on the presence of HlyB and HlyD. The appearance of the fusion proteins in the culture medium is not correlated with cell lysis.

[13] K. Nagai and H. C. Thogersen, this series, Vol. 153, p. 461.
[14] K. Fujikawa, M. E. Legaz, and E. W. Davie, *Biochemistry* **11,** 4882 (1972).
[15] K. Fujikawa, M. E. Legaz, and E. W. Davie, *Biochemistry* **11,** 4892 (1972).

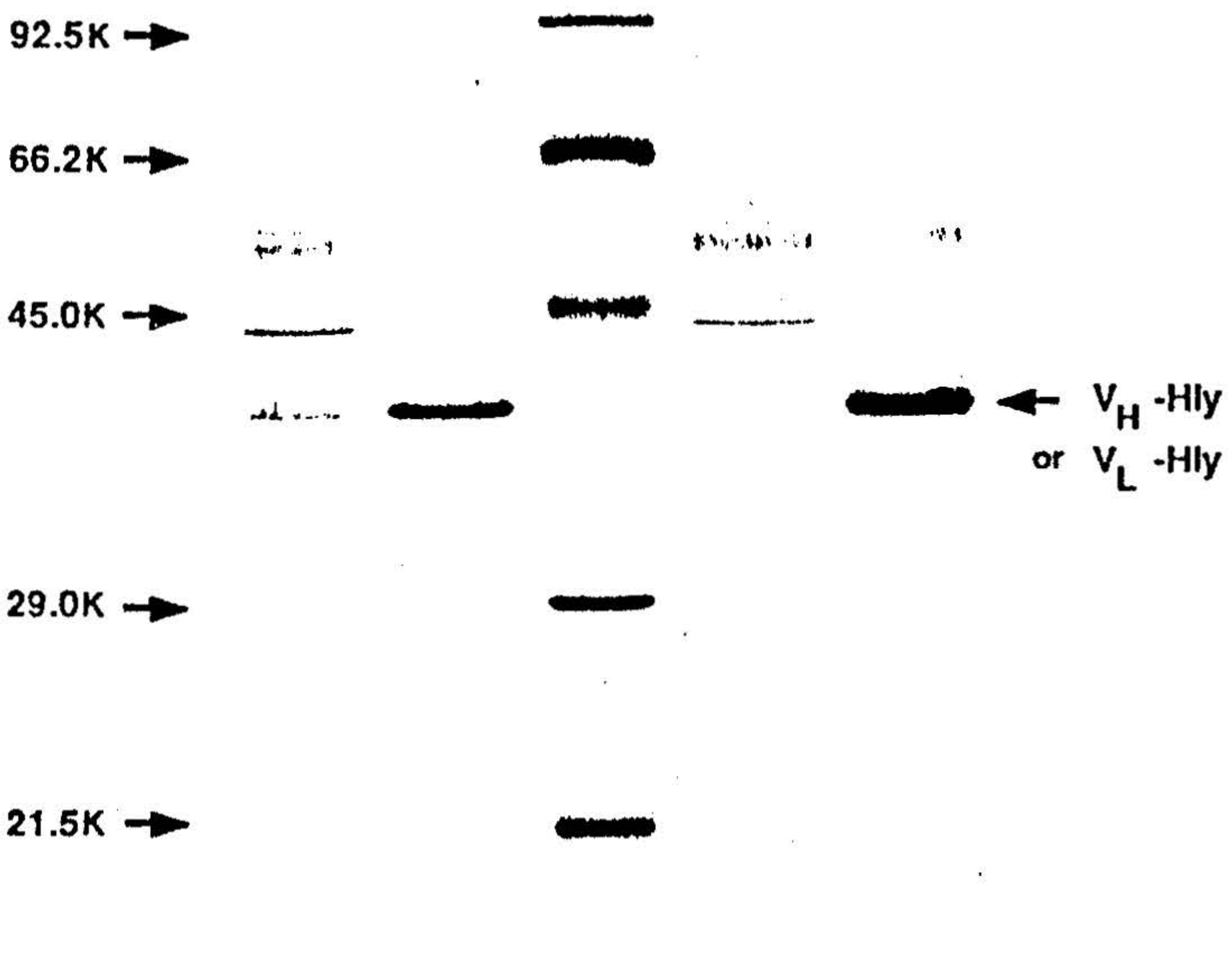

FIG. 5. Secretion of V_L–Hly and V_H–Hly fusions: An SDS–PAG stained with Coomassie Brilliant Blue is shown. The culture medium (10 ml) of the strain SE5000, harboring both pV_L–Hly and pLG575 (lanes A and B) or pV_H–Hly and pLG575 (lanes D and E), was precipitated with 40 ml of methanol. Lanes A and D: before induction; lanes B and E: 60 min after induction with 30 μM IPTG; lane C, molecular weight standard.

Some lysis is, however, observed when expression is induced at high levels. The optimal concentration of IPTG in the culture medium was found to be only 30 μM. The level of secretion of the fusion proteins is somewhat strain dependent. Best results were obtained with W3110[16] and SE5000[2] as hosts, resulting in levels of about 100 μg of fusion protein per liter of culture medium.

The secreted fusion proteins migrate as a single band on SDS–PAGE and no band indicating processing or degradation can be found by staining for total protein or Western blot (Fig. 5). Furthermore, in strains DH1,[17] JM109,[18] or W3110, no inducible protein is detected in the absence of the export functions. The situation is less clear in strain SE5000, where we could detect some intracellular fusion protein either in the presence or absence of the export functions.

[16] B. J. Bachmann, *Bacteriol. Rev.* **36,** 525 (1972).
[17] D. Hanahan, *J. Mol. Biol.* **166,** 557 (1983).
[18] C. Yanisch-Perron, J. Vieira, and J. Messing, *Gene* **33,** 103 (1985).

In all cases, these fusion proteins seem to be targets for rapid degradation by cytoplasmic proteases if they cannot be exported. Thus, we clearly find that secretion into the culture medium protects the fusion proteins.

Purification and Cleavage of Antibody Fusion Proteins. Purification of these fusion proteins has proved difficult. They tend to aggregate and can be partially pelleted from the medium by centrifugation. Even though the proteins are hydrophobic, the capacity of phenyl- or octyl-Sepharose columns is limited for these fusion proteins, and bound protein cannot be quantitatively eluted. The fusion proteins can, however, be readily extracted from the medium by stirring with 4% (w/v) CDR [Cell Debris Remover, Whatman (DEAE-derivatized Cellulose)]. Neither 150 m*M* NaCl nor a pH shift to pH 10 elutes the fusion protein from CDR. The fusion proteins can be efficiently eluted with ionic and nonionic detergents. Unfortunately, detergents that are normally dialyzable cannot be removed by ultrafiltration or dialysis in the presence of the eluted material. This suggests that micelles or other aggregates of a large size are formed.

The most convenient way to concentrate the fusion proteins from the medium is by precipitation with $(NH_4)_2SO_4$ at 90% of saturation and subsequent dialysis. The fusion proteins are obtained in soluble form, but a large amount of colored material is also present.

Both fusion proteins can be cleaved with the highly specific protease factor Xa, yielding bands of the expected molecular weights on Western blot. This cleavage reaction can be carried out directly with the $(NH_4)_2SO_4$ precipitate of the culture medium after dialysis against factor Xa reaction buffer (150 m*M* NaCl, 50 m*M* Tris–HCl, pH 8.0, 1 m*M* $CaCl_2$). Cleavage is slow and not quantitative, requiring an incubation with high amounts of factor Xa overnight. It remains to be determined whether this reflects the existence of an accessible cleavage site only in a subpopulation of the fusion protein or interference by the high concentration of membrane components still present in the dialysate. While the designed cleavage site is clearly the most susceptible, there is apparently a secondary site within V_H that makes it necessary to avoid overdigestion.

Optimization of Secretion System

The levels of secretion under given conditions for a number of prochymosin hybrids and the C-terminal AS-I peptide itself are given in Fig. 3. Highest levels of secretion are obtained in rich medium (Difco nutrient broth) and for best results, with minimum amounts of background proteins in the culture supernatants, we recommend that prototrophic strains

such as MC4100[4] be used as hosts. This could avoid or minimize lysis of cells which accompanies the high-level production of the antibody fusions described here. Although in the case of MC4100 this strain is also poorly transformable by conventional methods, high efficiencies are obtained with electroporation.

Preliminary results also indicate that higher secretion levels are obtained at 37 than at 30°. Other factors including salt concentration, and triethylamine (a membrane perturbant to increase envelope permeability) do not appear to have major effects on the secretion efficiency.

In contrast, the positioning of the HlyA signal in the fusion can have substantial effects upon the level of secretion. Thus, placing the signal domain at the N-terminus of prochymosin completely inhibits secretion. On the other hand, while we have shown that internal deletions within the AS-I peptide (leaving intact the terminal 27 amino acids of HlyA) can promote good secretion in certain fusions, studies with other fusions suggest that sequences immediately upstream of this region or between residues 857 and 904 can be important for efficient secretion.[3]

Further Developments

Attempts are being made to increase the efficiency of secretion through, for example, enhanced expression of *HlyB*, which is probably a limiting factor in the process. Other studies will involve the optimization of the efficiency of the HlyA secretory signal, including the role of sequences at the N-terminus of AS-I and the introduction of mutational changes within the signal region itself. The feasibility of developing a single secretion vector incorporating the export functions is also being explored.

To facilitate purification of secreted polypeptides, it would be highly advantageous to reduce the hydrophobic properties of at least some fusion proteins by further shortening of the secretion signal without losing the transport information. Some preliminary data suggest that this problem might be avoided by the use of the AS-II signal peptide. The identity of the amino acid sequences within the 23-kDa protein responsible for this phenomenon and purification procedures which might avoid it will also be investigated. Further construction of the fusion proteins for this system may also involve improved designs of the factor Xa site,[19] or cleavage by other enzymatic or chemical methods. This particular problem will have to be addressed individually for each protein.

[19] S. Ellinger, R. Glockshuber, G. Jahn, and A. Plückthun, *J. Clin. Microbiol.* in press (1989).

Perspectives

The Hly system has now been demonstrated to promote the efficient secretion from *E. coli* of at least four heterologous polypeptides. Although the precise mechanism of translocation of polypeptides through the cell envelope by the HlyB,HlyD complex is still unclear, these results suggest that a broad range of proteins might be secreted by this means.

In other studies, we have observed that the cytoplasmic protein CAT (chloramphenicol acetyltransferase) fused to the AS-I signal region, is also secreted from *E. coli*. In contrast, β-galactosidase is not secreted and accumulates intracellularly (unpublished data). In this latter respect the Hly system may be subject to the same limitation as the *secA*,*secY*-dependent export system.

In spite of the problems involved, the method holds promise for the secretion of foreign proteins in sufficient quantity and possibly in native conformation after cleavage from the HlyA signal domain. This system may be especially useful if the proteins involved are rapidly degraded in the cytoplasmic compartment. The HlyA secretion mechanism may also be particularly appropriate when expressed in avirulent strains as delivery mechanisms for certain oral vaccines.

Acknowledgments

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