Eur. J. Biochem. *171*, 541 – 549 (1988)

Production in *Escherichia coli* and one-step purification of bifunctional hybrid proteins which bind maltose

Export of the Klenow polymerase into the periplasmic space

Hugues BEDOUELLE[1] and Pascale DUPLAY[2]

[1] Unité de Biochimie des Régulations Cellulaires (Centre National de la Recherche Scientifique Unité Associée 1129)
[2] Unité de Programmation Moléculaire et Toxicologie Génétique (Centre National de la Recherche Scientifique Unité Associée 271, Institut National de la Santé et de la Recherche Médicale Unité 163), Institut Pasteur, Paris

(Received August 31/October 22, 1987) – EJB 87 0997

Two enzymes, the secreted *Staphylococcus aureus* nuclease A and the Klenow fragment of the cytoplasmic *Escherichia coli* DNA polymerase I, were fused, at the genetic level, to MalE, the periplasmic maltose-binding protein of *E. coli*, or to a signal-sequence mutant. The hybrid proteins were synthesized in large amounts by *E. coli* under control of promoter *malEp*. The synthesis was repressed with glucose and could be totally switched off in a *malT* mutant strain. The hybrid between MalE and the nuclease was exported into the periplasmic space. Several criteria demonstrated that a fraction of the hybrid chains with the Klenow polymerase was exported to the periplasm in a signal-sequence-specific manner and ruled out the possibility of a membrane leakage. The hybrid with the Klenow polymerase was not exported and remained in the cytoplasm when carrying a tight signal-sequence mutation in its MalE portion. The hybrid proteins were purified in one step by affinity chromatography on cross-linked amylose. Most of the hybrid chains in the periplasm but only a fraction of those in the other cell compartments had their MalE portion correctly folded. The nuclease and the Klenow polymerase had their full specific activities in the purified hybrids. The potential of MalE as a vector for the production, export and purification of desirable proteins in *E. coli* is discussed.

Expression of foreign (i.e. heterologous, engineered or synthetic) genes in *Escherichia coli* meets multiple problems. The foreign polypeptide may be toxic to the host cell, degraded *in vivo*, difficult to purify or uncorrectly folded. Toxicity can be avoided by placing the coding gene under control of a strong promoter which is normally repressed and only derepressed during the terminal phase of cellular growth, in order to induce the polypeptide synthesis. Degradation can be overcome by fusing the foreign polypeptide to a stable protein which serves as a vector. Fusion of a polypeptide to a vector, via their coding genes, can also facilitate the purification of the polypeptide if the vector keeps its activity in the hybrid and if it is easy to purify, for example by affinity chromatography. Export of a foreign polypeptide outside the bacterial cytoplasm, by fusion to a signal peptide, can avoid its toxicity, favor its correct folding in an environment which is less reducing than the cytoplasm and facilitate its purification. However, export of a normally cytoplasmic protein can, in itself, be toxic to the host cell.

Protein MalE seems a good candidate as a vector to express, export and purify foreign polypeptides. It is an abundant, medium-sized (370 residues) protein which is encoded by gene *malE* of *E. coli* [1]. Its expression is controlled by *malEp*, a strong promoter which is activated by protein MalT in the presence of inducing maltose or maltodextrins and repressed by glucose [2 – 4]. These controls are still operative when *malE* and its promoter are carried by a multicopy plasmid, as pPD1 [1, 2]. MalE is exported into the bacterial periplasmic space by means of an N-terminal signal sequence which has a length of 26 residues and is cleaved during the export process. Mutations in the signal sequence can prevent MalE export and provoke its accumulation within the cytoplasm [5]. MalE is a protein binding maltose and maltodextrins which is necessary for the transport of these sugars across the bacterial envelope (reviewed in [1]). MalE and its precursor can be purified by affinity chromatography on amylose, cross-linked with epichlorohydrin. The proteins can be eluted by competition with 10 mM maltose [6, 7].

Duplay et al. have constructed a set of *MalE* mutants by random insertion of a *Bam*HI linker into pPD1. Two insertions, *malE91* and *malE140*, change the reading frame and lead to the synthesis of mutant proteins, MalE91 and MalE140, having respectively the 362 and 369 N-terminal residues of the mature protein MalE and a C-terminal extension of respectively 12 and 27 residues (see legend to Fig. 3 for details). Characterisation of the mutants has shown that short polypeptides can be fused at the C-terminal of MalE without greatly affecting its properties. A third insertion, *malE127*, occurs in the signal sequence and prevents export of the mutant protein, MalE127, into the periplasmic space [8].

To test the potential of protein MalE as a vector, we constructed hybrids between MalE or MalE127 and the secreted *Staphylococcus aureus* nuclease A or the Klenow fragment of the cytoplasmic *E. coli* DNA polymerase I. We

Correspondence to H. Bedouelle, Unité de Biochimie des Régulations Cellulaires, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cédex 15, France

purified these hybrids by affinity chromatography on cross-linked amylose and determined their activities.

MATERIALS AND METHODS

Media, bacterial strains, plasmids and phages

The media, strains PD28 [8] and DH1 [9], plasmid pPD1 (which carries gene *malE* under control of its own promoter, *malEp*) [1], its mutant derivatives, pPD91, pPD127 and pPD140 [8], and the vectors pBR322, pUC9 and M13mp8 [10–12] have all been described previously. We isolated HB2200, a *malT* derivative of DH1, as a spontaneous, lambda-vir-resistant clone that did not revert to a Mal^+ phenotype on McConkey maltose agar. The DNA region, *polAk*, coding for the Klenow portion of *E. coli* DNA polymerase I was excised from a pUC9 recombinant as a *Bam*HI fragment [13]. The *S. aureus* nuclease A coding region, *nuc*, was derived from an M13mp8 clone containing a *Sau*3A fragment of *S. aureus* DNA inserted into the *Bam*HI site [14].

Plasmid constructions

The double-stranded DNAs of recombinant plasmids and M13 phages were prepared from 1.5-ml cultures by a modification of the alkaline lysis technique [15]. Restriction fragments were purified by electrophoresis through an agarose gel, cutting out from the gel and extrusion of the DNA from the gel slices by freezing and centrifugation in the presence of phenol [16]. Prior to use, the DNA preparations were microdialysed on 0.05-μm Millipore VS filters [16].

Growth and induction of the PD28 derivatives

The PD28 derivatives, harboring the recombinant plasmids, were grown at 30 °C with ampicillin. They were kept in conditions where promoter *malEp* is repressed, i.e. on plates of ML agar supplemented with 2% glucose. A repressed preculture was prepared by inoculation of an isolated colony into 5–25 ml ML medium supplemented with 0.4% glucose and overnight growth. The preculture was centrifuged at room temperature, the cell pellet washed with and finally resuspended into 1 vol. ML medium. The cell suspension was diluted into fresh ML medium, supplemented with 0.4% maltose if the gene *malE* derivative carried a wild-type signal sequence. The derepressed culture was grown from a starting absorbance (1-cm path length) $A = 0.1$ at 600 nm to $A = 1.0$, chilled on ice and further processed at 4 °C unless otherwise stated. In the following, the fractions of volume refer to the original culture volumes.

Preparation of periplasmic extracts

The periplasmic extracts were prepared by an adaptation of a published procedure [17]. The cells were pelleted by centrifugation, washed with 0.2 vol. 10 mM Tris/Cl pH 8.0 and resuspended in 0.05 vol. 20% sucrose, 30 mM Tris/Cl pH 8.0. EDTA was added to a concentration of 1 mM and the suspension gently stirred for 10 min. The cells were again pelleted; they were shocked by resuspending and gently stirring them for 10 min in 0.05 vol. 0.5 mM $MgCl_2$. The shocked cells were pelleted; the supernatant (shock fluid or periplasmic extract) was taken and the pellet (shocked cells) frozen at −20 °C then processed as described in the next paragraph. For small quantities, the shocked cells were directly dissolved in 0.1 vol. protein loading buffer [18]. The periplasmic extract was diluted with an equal volume of 2× loading buffer; 20-μl samples were analysed by polyacrylamide gel electrophoresis (PAGE) [18].

Preparation of shocked-cell and whole-cell extracts

The shocked cells were prepared as described in the previous paragraph. For the preparation of the whole-cell extracts, the culture was centrifuged, the cells were washed with 0.5 vol. 50 mM Tris/Cl pH 7.5 and frozen at −20 °C as a pellet. From this point, the protocols were identical for the whole-cell and shocked-cell extracts. The cells were thawed on ice and resuspended in buffer A (50 mM Tris/Cl pH 7.5, 0.1 mM phenylmethylsulfonyl fluoride, 2.5 mM 2-mercaptoethanol) containing 1 mg/ml of freshly dissolved lysosyme. The cell suspension was incubated 20 min at room temperature, chilled on ice, sonicated briefly and the debris were pelleted by centrifugation (15 min at 15000 × *g*). The supernatant was diluted to 0.05-fold of the initial volume with buffer A.

Purification of MalE hybrids

The various extracts were filtered through 0.45-μm Millex-HA filters, prerinsed with water. The column of cross-linked amylose [6] (6 ml for a culture of 500 ml) was equilibrated with buffer A, charged with the extract (in 25 ml), washed with five column volumes of buffer A then eluted with buffer A + 10 mM maltose. The absorbance of the eluate was monitored at 280 nm; 10-μl samples of the primary extracts and flow-through fractions were mixed with an equal volume of 2× protein loading buffer and analysed by PAGE [18].

Protein and enzyme assays

The protein gels were 10% in polyacrylamide (acrylamide/bisacrylamide = 19:1). The protein concentration in individual fractions was measured by the Bradford's method [19], using bovine serum albumin as standard. The concentration of the purified wild-type MalE was higher than 1 mg/ml in the best fractions. When necessary, the purified hybrids were concentrated by precipitation with 5% trichloroacetic acid before gel electrophoresis. The purified hybrids were kept in elution buffer containing 45% glycerol at −20 °C. The DNA polymerase and nuclease assays were performed as described [20, 21].

RESULTS

Construction of hybrids

To test the potential of protein MalE as a vector, we constructed three hybrid proteins. A hybrid between mutant MalE91 and the Klenow fragment of *E. coli* DNA polymerase I (MalE91-PolAk); a derivative of the previous one, harboring mutation MalE127 in its signal sequence (MalE127/91-PolAk), and a hybrid between mutant MalE140 and the *S. aureus* nuclease A (MalE140-Nuc). The three hybrid proteins were constructed at the genetic level, by recombining their

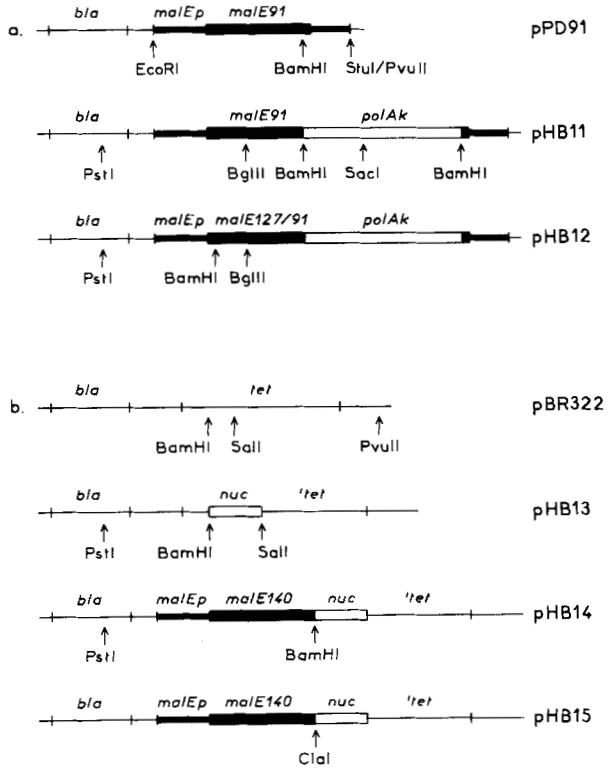

Fig. 1. *Recombinant plasmids used for the hybrid constructions.* (a) Hybrids between gene *malE* and the Klenow polymerase coding region, *polAk*. The *Bam*HI fragment that contains *polAk* was excised from a pUC9 vector (Materials and Methods) and ligated into the *Bam*HI site of plasmid pPD91, which carries mutation *malE91*. We determined the orientation of *polAk* in the recombinant plasmids by analysis of a *Bgl*II-*Sac*I double digest and named the resultant plasmid that codes for hybrid MalE91-PolAk, pHB11. We subsequently introduced a signal sequence mutation by exchanging the *Pst*I-*Bgl*II fragments of plasmids pPD127, which harbors the signal sequence mutation, *malE127*, and pHB11. We named the resultant plasmid that codes for hybrid MalE127/91-PolAk, pHB12. (b) Hybrid between *malE* and the *S. aureus* nuclease A gene, *nuc*. We excised the *Bam*HI–*Sal*I fragment that contains *nuc* from an M13mp8 vector (Materials and Methods) and ligated it between the *Bam*HI and *Sal*I sites of vector pBR322, to obtain plasmid pHB13. We exchanged the *Pst*I–*Bam*HI fragments of pHB13 and pPD140, which carries mutation *malE140*, to obtain pHB14. We subsequently put the *malE* and *nuc* genes in phase by filling the cohesive ends of the unique *Bam*HI site in pHB14 and ligation, to obtain pHB15. In pHB15, the *Bam*HI site is replaced by a *Cla*I site. pHB15 codes for hybrid MalE140-Nuc. The deduced sequences, at the junctions of the fused proteins, were: Lys^{362}-**Asp-Gly-Ser-Gly-Ala-Thr**-Val^{1} for MalE91-PolAk and Thr^{369}-**Arg-Ile-Asp-Pro-Thr-Val-Tyr-Ser**-Ala^{1} for MalE140-Nuc, where the linker polypeptide is printed in bold-face and the numbers refer to the positions of the last and first residues in the two partners [8, 43, 44]. The deduced lengths for MalE140-Nuc and MalE91-PolAk were 526 and 973 amino acid residues, respectively, in their mature forms and that for MalE127/91-PolAk was 996 residues

genes on plasmid vectors as described in Fig. 1 and legend. In the resultant constructs, the hybrid genes were under control of promoter *malEp*.

Expression of hybrids

To investigate the activity of promoter *malEp* in various *E. coli* host strains and growth conditions, when it is carried by a multicopy plasmid, we analysed the amounts of protein

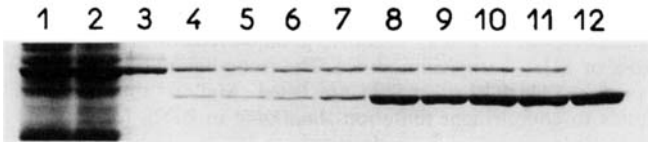

Fig. 2. *Expression of MalE from plasmid pPD1.* The shocked-cell (lanes 1, 2) and periplasmic (lanes 3–11) extracts were prepared as described in Materials and Methods. The host strains were HB2200 (lanes 1 and 3) and PD28 (lanes 2 and 4–11). The bacteria were grown in ML medium supplemented as follows (P = preculture, C = culture, O = no addition, Glu = 0.4% glucose, Mal = 0.4% maltose): lanes 1 and 3, P = 0, C = 0; lanes 2 and 4, P = Glu, C = Glu; lanes 5–7, P = Glu, C = Glu + 0.5, 1.0 and 5.0 mM cyclic AMP, respectively; lane 8, P = Glu, C = 0; lane 9, P = Glu, C = Mal; lane 10, P = 0, C = 0; lane 11, P = 0, C = Mal; lane 12: 2 μg pure wild-type MalE

MalE that were synthesized from plasmid pPD1, by polyacrylamide gel electrophoresis of cell extracts (Fig. 2). To avoid the genetic instability that often results from the toxic expression of recombinant genes, we used HB2200, a *malT recA* strain which has a defective activator of the maltose regulon, as a host for all the genetic constructions (Materials and Methods). Promoter *malEp* was totally inactive in HB2200 (Fig. 2, lanes 1 and 3). We used PD28, a *ΔmalE444 malT*^{c}*1 recA* strain, as a host for the expression of the hybrid genes. The deletion, *ΔmalE444*, of gene *malE* avoided the expression of wild-type protein MalE from the bacterial chromosome and its copurification with the MalE hybrids. Due to *ΔmalE444*, PD28 cannot transport and therefore cannot be induced with maltose [22] but mutation *malT*^{c}*1* enables a constitutive expression of the maltose regulon [23]. In PD28, promoter *malEp* was active in the absence of inducing maltose (Fig. 2, lanes 8 and 10). Because the constitutive expression of hybrid proteins may be toxic or lethal to the cell, we grew the PD28 derivatives in complete medium supplemented with glucose (Materials and Methods). In these conditions, *malEp* was strongly repressed (Fig. 2, lanes 2 and 4). A simple wash of the cells and their growth in complete medium without glucose was sufficient to trigger the full activity of *malEp* (Fig. 2, lanes 8 and 9) as soon as the first generation. Alternatively, the activity of *malEp* could be tuned by the addition of increasing concentrations of cyclic AMP to a glucose-containing medium (Fig. 2, lanes 5–7). When they were introduced in strain PD28, the plasmids listed in Table 1 directed the expression of the corresponding MalE mutant or hybrid proteins in large quantities (see Fig. 4).

Study of hybrids in vivo

To investigate whether the MalE portions of the hybrid proteins were functional for maltose transport, we performed a genetic complementation assay. Due to deletion *ΔmalE444*, strain PD28 cannot ferment maltose. Because *ΔmalE444* is not polar on the expression of the distal genes in the *malE-malF-malG* operon [22], a gene *malE* derivative that is carried by a plasmid can complement *ΔmalE444* for maltose fermentation if it directs the expression of a functional (and therefore exported) protein MalE. Table 1 shows the complementation pattern of *ΔmalE444* by the various recombinant plasmids. The PD28 derivatives that harbored the signal sequence mutation, *malE127*, on their recombinant plasmids were totally unable to ferment maltose while the other strains could ferment maltose.

Table 1. *Phenotypes of the PD28 derivatives at 30°C and 42°C*
Colonies were streaked at 30°C and 42°C onto prewarmed McConkey maltose or ML + ampicillin plates. The recombinant plasmids and the proteins which they encode are listed. Mal = ability of the plasmids to complement mutation *ΔmalE444* in PD28 for maltose fermentation: +++ = dark-red colonies indicating full complementation; − = white colonies, no complementation [1]. Sick = morphology of the colonies: ++ = small and very flat colonies, growth in sectors; − = normal *E. coli* colonies

Plasmid	Protein	At 30°C		At 42°C	
		Mal	Sick	Mal	Sick
pPD1	MalE	+++	−	++	−
pPD91	MalE91	++	−	+	−
pPD127	MalE127	−	−	−	−
pPD140	MalE140	+++	−	++	−
pHB11	MalE91-PolAk	+++	+	−	++
pHB12	MalE127/91-PolAk	−	+	−	++
pHB15	malE140-Nuc	+++	−	++	−
pBR322		−	−	−	−

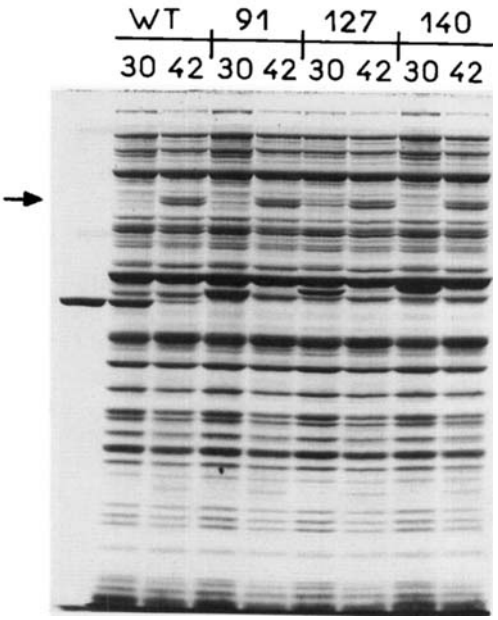

Fig. 3. *Effect of temperature on MalE expression in strain PD28.* The strains were grown as described in Materials and Methods except that, after derepression, the cultures were done in parallel at 30°C and 42°C. The cells were then centrifuged, resuspended into 0.1 vol. protein loading buffer [18], boiled for 5 min and 20 µl of the resultant total extract loaded on a gel. The lengths of MalE and its mutants, predicted from the DNA sequences [1, 8] are: MalE wild type (WT) = 370, MalE91 = 374, MalE127 = 387, MalE140 = 396 residues. These lengths were calculated in the hypothesis that the wild-type MalE, MalE91, and MalE140 were in mature form whereas MalE127 was in precursor form. First lane on the left: 2 µg pure wild-type MalE. The arrow, in the left margin, indicates a prominent doublet of protein bands, with a relative molecular mass around 60500, that was present at 42°C but absent at 30°C [except for PD28 (pPD127) in which it was present at both temperatures]. Other bands, with lower molecular masses, follow the same pattern

The phenotypes were different at 30°C and 42°C, especially for strain PD28 (pHB11) which synthesized hybrid MalE91-PolAk. Analysis of the total extracts from cultures of the PD28 derivative strains, by polyacrylamide gel electrophoresis, showed that the syntheses of MalE and its mutants were strongly decreased at 42°C relative to 30°C (Fig. 3). In addition, the expressions of both Klenow polymerase hybrids were toxic to the cell and toxicity was higher at 42°C than at 30°C (Table 1).

To test the expression of DNase by the PD28 derivatives, we grew them as colonies on complete medium with ampicillin and replicated them onto toluidine blue-O DNA agar. This agar diffusion assay scores DNase activity as a pink zone of clearing on a dark blue background [24]. PD28 (pHB15) scored as strongly positive in this assay whereas PD28 (pPD1) and PD28 (pPD140) scored as negative. PD28 (pHB14) showed a faint DNase activity (not shown). The results indicated that in pHB15 but not in pHB14, the *malE140* and *nuc* genes were in phase and that pHB15 directed the expression of active nuclease A.

Cellular location of hybrids

The periplasmic proteins can usually be released from the *E. coli* cells by the cold osmotic shock procedure of Neu and Heppel [17]. They are found in the shock fluid (periplasmic extract) whereas the cytoplasmic and membrane proteins remain associated with the shocked cells. To determine whether the MalE hybrid proteins were exported into the periplasmic space, we submitted cultures of the PD28 derivative strains to this procedure and analysed the cell extracts by polyacrylamide gel electrophoresis (Fig. 4). We found the wild-type MalE, MalE91 and MalE140 in the periplasmic extracts whereas MalE127 remained associated with the shocked cells, as previously described [8]. No protein corresponding either to MalE127 or to a maturation product was found in the periplasmic extract from strain PD28 (pPD127) (Fig. 4, lane 3). Thus, the signal sequence mutation, *malE127*, totally blocked MalE export as judged by this experimental criterion. We found the major part of hybrid MalE140-Nuc in the periplasmic extract (Fig. 4, lanes 5 and 12). An additional amount of the hybrid could be released from the surface of the shocked cells by a high-salt wash (not shown, see [25]). Hybrid MalE127/91-PolAk and most of MalE91-PolAk remained in the shocked cells (Fig. 4, lanes 6, 7 and 13, 14). However, a faint protein band, with the apparent molecular mass of MalE91-PolAk, was present in the periplasmic extract from strain PD28 (pHB11) (Fig. 4, lane 6) but absent from those of the other strains, suggesting that low amounts of MalE91-PolAk might be exported into the periplasmic space. We found most of proteins MalE127, MalE91-PolAk and MalE127/91-PolAk in whole-cell extracts prepared by sonication of whole cells and centrifugation of the debris for 15 min at $15000 \times g$ (Materials and Methods; not shown). This result showed that the hybrids did not precipitate as aggregates within the cell.

Purification of hybrids

Proteins MalE, MalE91, and MalE140 can be purified in large amounts from periplasmic extracts of cells harboring the multicopy plasmids pPD1, pPD91 and pPD140, respectively, by affinity chromatography on cross-linked amylose [8]. Table 2 and Fig. 5 show the results of the purifications for the wild-type MalE, MalE127 and the three hybrids from various cellular extracts by the same technique.

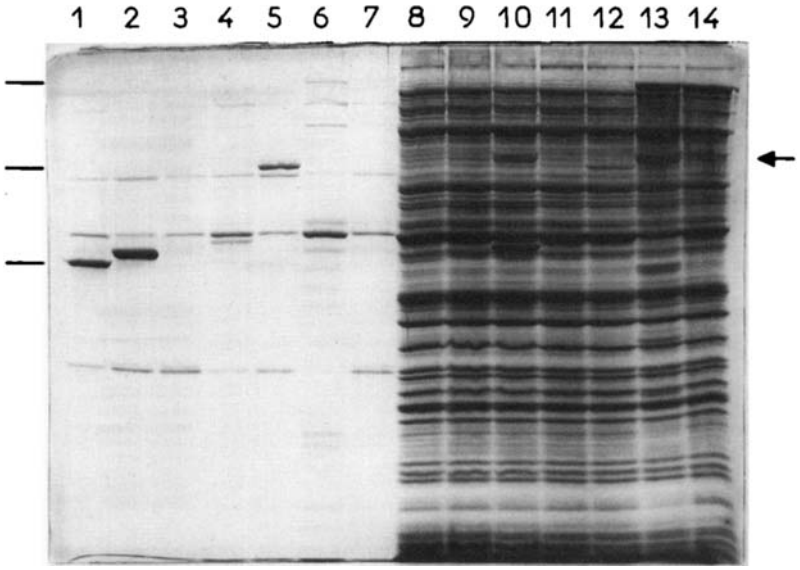

Fig. 4. *Cellular location of the hybrid proteins.* Lanes 1–7, periplasmic extracts; 8–14, shocked-cell extracts of strain PD28 derivatives overproducing: lanes 1 and 8, MalE (wild type); lanes 2 and 9, MalE91; lanes 3 and 10, MalE127; lanes 4 and 11, MalE140; lanes 5 and 12, MalE140-Nuc; lanes 6 and 13, MalE91-PolAk; lanes 7 and 14, MalE127/91-PolAk. The extract in each lane was prepared from 200 µl culture at $A_{600\ nm} = 1.0\ cm^{-1}$. The horizontal lines in the left margin give, from bottom to top, the positions of the wild-type MalE, MalE140-Nuc and MalE91-PolAk (or MalE127/91-PolAk). For the positions of MalE91, MalE127 and MalE140 relative to the wild-type MalE, see Fig. 3. The arrow in the right margin indicates a doublet of protein bands that was induced by the expression of MalE127, MalE91-PolAk and MalE127/91-PolAk or by a shift from 30 °C to 42 °C (see Fig. 3)

When the periplasmic extracts were loaded onto the amylose column, the wild-type MalE and MalE140-Nuc were absent from the fractions flowing through and therefore totally retained by the amylose (Fig. 5, lanes 1, 2 and 9, 10). Likewise, little of MalE91-PolAk in the periplasmic extract from strain PD28 (pHB11) seems to have flowed through the column (Fig. 5, lanes 13 and 14; also see the figure legend). Consistently, the yield of MalE91-PolAk purified from this periplasmic extract (344 µg, see Table 2) was close to its total amount in the same extract as estimated from band intensities in protein gels (about 250 µg/l culture at $A_{600\ nm} = 1.0\ cm^{-1}$). About a third of MalE127 in a whole-cell extract flowed through the column (Fig. 5, lanes 5 and 6). Consistently, the yield of MalE127 after purification was about two-thirds of that for the wild-type MalE (2.32 vs 3.52 mg, see Table 2). In contrast, most of the MalE91-PolAk and MalE127/91-PolAk hybrid chains in the shocked-cell and whole-cell extracts, respectively, flowed through the column. From protein gels, we estimated the amounts of the PolAk hybrids in the shocked-cell or whole-cell extracts at more than 5 mg/l culture at $A_{600\ nm} = 1.0\ cm^{-1}$ (for example, compare lanes 17 and 22 to lane 4 in Fig. 5). From the yields of the purified proteins (Table 2), one can thus conclude that less than 5% of the MalE91-PolAk and MalE127/91-PolAk chains, in the shocked-cell or whole-cell extracts, were retained by the amylose column. Furthermore, when the flow-through fractions from the MalE127/91-PolAk purification were loaded back onto the amylose column, the yield of purified hybrid in this second passage was less than 14% of the yield in the first passage.

The elution profiles of the MalE derivatives from the amylose column were very similar and consisted of a unique, sharp peak, except for the purification of MalE91-PolAk from the shocked cells. In this last case, there was a large shoulder in the peak and elution began much earlier (not shown).

Activity of hybrids

The specific activity of MalE140-Nuc in a nuclease assay was 2530 U/mg hybrid, i.e. 8940 U/mg of the nuclease A portion in the hybrid (Table 2). This value is similar to that obtained for authentic nuclease A [21]. Similarly, the specific activities of hybrids MalE91-PolAk and MalE127/91-PolAk, purified from the periplasmic and whole-cell extracts, respectively, show that their polymerase portions were fully active [20]. The value for MalE91-PolAk was slightly lower than for MalE127/91-PolAk (3100 vs 4514 U/mg). This might be due to the greater degradation of MalE91-PolAk (Fig. 5, lanes 16 and 24). In contrast, the specific activities of MalE91-PolAk purified either from the shocked-cell or periplasmic extracts were very different, with the former only 10% of the latter.

DISCUSSION

The results reported in this paper show that gene *malE* and its promoter, *malEp*, can be used for the efficient expression, export and purification of foreign proteins in *E. coli*, in an active form.

Expression under control of malEp

The properties of promoter *malEp* are convenient for the expression of foreign genes on multicopy plasmids in *E. coli*. (a) *malEp* activity is completely switched off in a *malT* strain such as HB2200. This enables the safe construction and stable preservation of recombinant plasmids where a potentially harmful gene, as *malE91-polAk* or *malE127/91-polAk*, is under control of *malEp*. (b) In *malT*$^+$ or *malT*^{c}*1* strains, the *malEp* activity can be strongly repressed with glucose. We have continuously maintained PD28 derivatives, harboring deleterious *malE* hybrid genes, on plates of complete medium

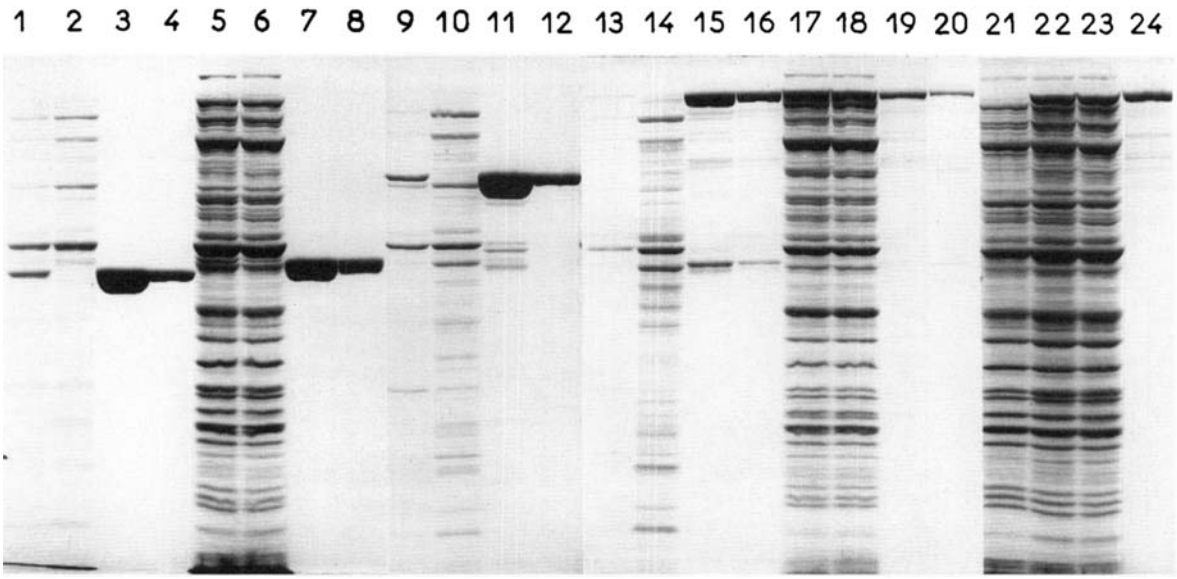

Fig. 5. *Purification of the hybrid proteins.* For each purification, the primary extract, the flow-through fraction and 6 μg and 3 μg of the purified fractions were loaded on the gel (for the purified MalE127/91-PolAk, a single load of 3 μg was performed). For P, SC, WC, see legend to Table 2. Lanes 1–4, MalE (wild type) (P); lanes 5–8, MalE127 (WC); lanes 9–12, MalE140-Nuc (P); lanes 13–16, MalE91-PolAk (P); lanes 17–20, MalE91-PolAk (SC); lane 21, shocked-cell extract of strain PD28 (pPD1); lanes 22–24, MalE127/91-PolAk (WC). The flow-through fraction corresponds to the fraction with the highest $A_{280\ nm}$ during the loading and washing of the column. Because the periplasmic extracts were loaded in a low-salt buffer (0.5 mM $MgCl_2$) and washed with a medium salt buffer (buffer A), most of the proteins flowed through the column as a sharp peak. Consequently, the flow-through fractions were overloaded relative to the periplasmic extracts on the gel (for example, see lanes 13 and 14)

Table 2. *Purification and activity of the MalE hybrids*
P, SC and WC = periplasmic, shocked-cell and whole-cell extract, respectively, i.e. the cell extracts from which the proteins were purified. Load = mass of protein in the extract prepared from 1 l culture at $A_{600m} = 1.0\ cm^{-1}$. Yield = mass of purified protein obtained from the same extract. The DNA polymerase and nuclease assays were performed with the purified hybrids (Materials and Methods). The specific activities were calculated per mass of hybrid and per mass of the enzyme portion (Nuc or PolAk) in the hybrid

Plasmid	Protein	Fraction	Load	Yield	Specific activity	
					hybrid	enzyme portion
			mg		U/mg	
pPD1	MalE	P	30.0	3.52		
		SC	182			
pPD127	MalE127	WC	280	2.32		
pHB11	MalE91-PolAk	P	12.8	0.344	3100	5103
		SC	200	0.498	306.5	504.6
pHB12	MalE127/91-PolAk	WC	296	0.136	4514	7431
pHB15	MalE140-Nuc	P	25.0	3.28	2533	8942
		SC	206			

with glucose during a year without stability problems. (c) Full derepression of *malEp* can be achieved by a simple wash of the cells; we used centrifugation but filtration might be an alternative. The activity of *malEp* could also be tuned by the addition of cyclic AMP to a glucose-containing medium.

The quantities of the MalE derivatives that we purified (Table 2) give lower limits to the levels of expression that can be achieved under control of *malEp*. Indeed, because we expected toxicity problems, we used standard, non-optimal growth conditions (Materials and Methods). Under such conditions, we purified 3.5 mg wild-type MalE/l culture while 50 mg/l and more can be achieved (P. D., unpublished results). Moreover, the Klenow fragment of DNA polymerase I is a large polypeptide (605 residues). The purification of hybrids between MalE and shorter proteins, from either the cytoplasm or the periplasm, will probably be more efficient. Nevertheless, the activity of *malEp* might be limited by the cellular amount of activator MalT (see [1–4] for a discussion of and solutions to this problem).

The cellular amounts of the MalE derivatives were lower at 42 °C than at 30 °C. This decrease occurred for both exported and non-exported derivatives and therefore was unlinked to export. A likely explanation is that the constitutive activator of transcription, $MalT^c1$, was thermosensitive.

Hybrids MalE91-PolAk and MalE127/91-PolAk were toxic to the cell. The toxicity did not appear to be simply linked to the export of hybrid MalE91-PolAk, as reported for MalE-LacZ hybrids [26]. Indeed, (a) toxicity was not

abolished by the signal sequence mutation, *malE127*, which is a tight mutation (see below) and (b) it was stronger at 42 °C than at 30 °C, i.e. in conditions where the syntheses of MalE91-PolAk and MalE127/91-PolAk were decreased. Moreover, the toxicity was not due to the polymerase activity of the hybrids since overproduction of the Klenow polymerase is not toxic to the cell [27]. It is possible that the large amounts of the PolAk hybrids that were synthesized were toxic because they were uncorrectly folded (see below) and competed with other proteins either for degradation or for renaturation. A shift of the temperature from 30 °C to 42 °C may have increased the amount of abnormally folded proteins and, as a consequence, the toxicity of the hybrids to the cell. It is known that the production of abnormal proteins in *E. coli* or a heat-shock stimulate the expression of the *lon* protease and other heat-shock proteins [28] and it has been speculated that heat-shock proteins may have a general role in protein folding or solubilization of aggregates [29]. In Figs 3 and 4, we have pointed out a prominent doublet of protein bands that was induced by a shift in temperature from 30 °C to 42 °C and by the expression of MalE127, MalE91-PolAk or MalE127/91-PolAk at 30 °C.

A vector system for cytoplasmic and secreted proteins

Hybrid MalE140-Nuc was found in and purified from a shock fluid, as the wild-type MalE and contrarily to the signal-sequence mutant, MalE127. These results show that MalE140-Nuc was exported into the *E. coli* periplasmic space and that MalE can be used as a vector to export foreign secreted proteins. Takahara et al. have shown that the *S. aureus* nuclease A can be exported into the *E. coli* periplasmic space by fusion to the signal peptide of the outer-membrane protein OmpA. In that case, the nuclease remains stuck to the outer surface of the shocked cells and must be released by a high-salt wash [25]. In contrast, the major part of MalE140-Nuc was directly recovered from the shock fluid. The MalE portion probably helped to solubilize the nuclease portion of the hybrid.

Strains PD28 (pPD127) and PD28 (pHB12) overproduced proteins MalE127 and MalE127/91-PolAk from multicopy plasmids. Yet, these strains were unable to ferment maltose, the two proteins were totally absent from their periplasmic extracts and MalE127 was only present in precursor forms. By analogy with other MalE signal-sequence mutants for which the ability to ferment maltose correlates with the residual export of MalE and which accumulate MalE within the cytoplasm in precursor form [5, 30], we concluded that MalE127 is a tight mutation, that MalE127 and MalE127/91-PolAk remained in the cytoplasm and thus, that foreign proteins can be synthesized in the *E. coli* cytoplasm after fusion to MalE127.

Export of the Klenow polymerase into the periplasm

Williams and Neuberger have shown that a hybrid between an immunoglobulin Fab fragment and the Klenow fragment of DNA polymerase I can be secreted in good yield from myeloma cells [13]. It was therefore interesting to analyse the cellular location of hybrid MalE91-PolAk. The following results show that a fraction of MalE91-PolAk was exported into the periplasmic space of strain PD28 (pHB11) in a signal-sequence-specific manner and rule out a non-specific membrane leakage. The demonstration is partly based on results showing that the MalE91-PolAk chains had different properties when they were purified from the periplasmic and from the shocked-cell extracts.

a) For similar amounts of proteins in the shocked-cell extracts, there was less protein in the periplasmic extract of strain PD28 (pHB11) than in those of PD28 (pPD1) or PD28 (pHB15) (Table 2).

b) 40% of hybrid protein MalE91-PolAk that could be purified from PD28 (pHB11) came from the periplasmic extract while the total amount of protein in this extract represented less than 6% of the total in a whole-cell extract (Table 2).

c) A fraction of MalE91-PolAk but no trace of MalE127/91-PolAk was in the periplasmic extract, which showed that export into the periplasm depended on the integrity of the signal peptide.

d) Most of the MalE91-PolAk chains in the periplasmic extract of PD28 (pHB11) but less than 5% of its chains in the shocked-cell extract were retained by the amylose column.

e) The elution profiles of MalE91-PolAk from the amylose column were different when the hybrid was purified from the shocked-cell and periplasmic extracts, suggesting different affinities of the MalE portion for amylose.

f) There was a 10-fold difference in the specific activities of the polymerase portion of MalE91-PolAk when the hybrid was purified from the whole-cell and from the periplasmic extracts. Thus about 7% of the MalE91-PolAk chains were exported into the periplasmic space. To our knowledge, this is the first demonstration that a normally cytoplasmic protein can be exported, in an active form, to the *E. coli* periplasm. Copeland et al. have reported that, in *E. coli*, a fragment of the α subunit of the tryptophan synthase can be secreted from spheroplasts by fusion to the leucine-specific binding protein. However, in that case, the hybrids were not purified and no activity was assayed after export [31].

A general system for the purification of active proteins

From Fig. 5 and Table 2, we have deduced what percentages of the hybrid chains, in the various extracts, were retained by the amylose column and what percentages flowed through it. These results gave indications on the activity, i.e. amylose binding, of the MalE portions in the hybrids and thus on their folding state. The wild-type protein MalE and the MalE portion of hybrid MalE140-Nuc were correctly folded. Likewise, about two-thirds of the MalE127 chains were folded into an amylose binding conformation. Thus, the signal peptide mutation, in MalE127, prevented the export but not the correct folding of the mutant precursor. In contrast, less than 5% of the MalE127/91-PolAk hybrid chains had their MalE portion in a correct fold. Thus, the polymerase portion of MalE127/91-PolAk interfered with the folding of the MalE127 portion. The polymerase portion of MalE91-PolAk had different effects according to its final location: most of the MalE91-PolAk chains in the periplasmic extract but less than 5% of them in the shocked-cell extract had their MalE portion correctly folded. Although explanations for these effects of the PolAk portion abound, we favor the hypothesis that, when MalE91-PolAk is exported, its MalE portion has time to fold in the periplasm before its PolAk portion is extruded from the membrane, without interference between the foldings of the two portions.

The specific activities listed in Table 2 show that the enzyme portions of MalE140-Nuc and MalE91-PolAk had their full activities when the hybrids were purified from the periplasmic extracts. Likewise, the PolAk portion of

MalE127/91-PolAk had its full activity when the hybrid was purified from a whole-cell extract. Thus, in the above cases, a functional MalE portion was associated with a fully functional enzyme portion and affinity chromatography on cross-linked amylose enabled those hybrid chains that had both their MalE and enzyme portions correctly folded to be selectively purified. When hybrid MalE91-PolAk was purified from the shocked-cell extracts, its elution profile from the amylose column was abnormal and suggested a lower affinity of the MalE portion for amylose. The specific activity of its PolAk portion was 10 times lower than normal. Further experiments will be needed to determine (a) whether only part of the MalE91-PolAk chains, purified from the shocked-cell extract, had a normal activity or the folding of all the chains was incorrect, resulting in different enzyme kinetics, and (b) whether the hybrid chains, purified from this extract, were in a soluble state or associated with the membrane, as occur for MalE-LacZ hybrids [32].

After purification, the MalE derivatives were slightly degraded. MalE127 was represented by three protein bands upon gel fractionation. The three bands probably corresponded to proteolytic cleavages of the N-terminal signal peptide, as occur for the wild-type precursor MalE [7]. The degradation seemed more important for the hybrids purified from the periplasmic extracts than for those from the shocked-cell or whole-cell extracts and, in the former cases, the main degradation products had an apparent molecular mass around that for mature MalE. The linker polypeptides between the MalE and enzyme portions of the hybrids might have been more susceptible to proteolysis in the periplasm than in the cytoplasm. Alternatively, the differences might come from the protocols used to prepare the extracts (Materials and Methods). Because of these proteolytic cleavages, the complementation experiments were insufficient to conclude firmly that the MalE portions of MalE140-Nuc and MalE91-PolAk were active for maltose transport in the hybrids (see Results).

Comparison with other systems

At least five systems have been described that enable the purification of hybrid proteins by affinity chromatography. They are based on the following polypeptides: β-galactosidase with the fusion point either at its N-terminal [33] or C-terminal [34]; a phosphate-binding protein [35]; the protein A from *S. aureus* [36]; a C-terminal poly(arginine) tail [37] and the Fab fragment of an immunoglobulin [13, 14]. The system based on MalE has the following advantages. Because the foreign protein is fused at the C-terminal of MalE, the genetic signals that control the expression and eventually the export of the hybrid are brought with the vector and always identical. The host cell is *E. coli*, promoter *malEp* is repressed with glucose, induced by a simple wash of the cells and the resin for chromatography is cross-linked amylose. Therefore, all the reagents have very low costs; in addition, cross-linked amylose seems very stable and indefinitely reusable. The hybrid can be either exported or synthesized in the cytoplasm if a signal-sequence mutation is introduced into its MalE portion. We have demonstrated that a fraction of the hybrid between MalE and the Klenow polymerase was exported into the periplasmic space. Thus, MalE may enable the export of large cytoplasmic enzymes. MalE is a monomer, at least in the presence of maltose [38], so that there is no constraint of quaternary structure on the hybrid. The efficiency of the chromatographic purification is very good, at least 85% (see Results). The chromatographic purification is done in very mild, physiological conditions, with elution being performed with 10 mM maltose. This enables the foreign proteins to keep their full activity. In contrast, purification by fusion to protein A necessitates the pH to be decreased to 3.0 for elution, with the risk of inactivation [36]. Finally, methods exist to separate a foreign protein from a vector after purification. In the case of MalE, the following methods seem appropriate: (a) cleavage at a cysteine after cyanylation since MalE does not contain any cysteine [39]; (b) cleavage at a dipeptide Asp-Pro by treatment with formic acid since MalE only contains one such dipeptide that could be removed by mutagenesis [40]; (c) cleavage to the tetrapeptide Ile-Glu-Gly-Arg by blood-clotting factor X_a [41]; (d) cleavage to a pentapeptide Pro-Xaa-Gly-Pro-Yaa with collagenase [42]. However, in many applications, cleavage is not required.

We thank Maurice Hofnung for his interest in this work and Michael Neuberger for the gift of plasmids and phages. Our laboratories were supported by grants from the *Centre National de la Recherche Scientifique*, the *Ministère de la Recherche et de l'Enseignement Supérieur*, the *Université Paris-7*, the *Institut Français du Pétrole*, the *Fondation pour la Recherche Médicale*, the *Association pour le Développement de la Recherche sur le Cancer* and the *Ligue Nationale Française contre le Cancer*.

A summary of this work has been published in: H. B., P. D. & M. Hofnung (1987) *C.R. Acad. Sci. Paris 305 série III*, 623–626.

REFERENCES

1. Duplay, P., Bedouelle, H., Fowler, A. V., Zabin, I., Saurin, W. & Hofnung, M. (1984) *J. Biol. Chem. 259*, 10606–10613.
2. Bedouelle, H., Schmeissner, U., Hofnung, M. & Rosenberg, M. (1982) *J. Mol. Biol. 161*, 519–531.
3. Bedouelle, H. (1983) *J. Mol. Biol. 170*, 861–882.
4. Bedouelle, H. (1984) *Bull. Inst. Pasteur 82*, 91–145.
5. Bedouelle, H., Bassford, P. J. Jr, Fowler, A. V., Zabin, I., Beckwith, J. & Hofnung, M. (1980) *Nature (Lond.) 285*, 78–81.
6. Ferenci, T. & Klotz, U. (1978) *FEBS Lett. 94*, 213–217.
7. Ito, K. (1982) *J. Biol. Chem. 257*, 9895–9897.
8. Duplay, P., Szmelcman, S., Bedouelle, H. & Hofnung, M. (1987) *J. Mol. Biol. 194*, 663–673.
9. Hanahan, D. (1983) *J. Mol. Biol. 166*, 557–580.
10. Backman, K. (1986) *Nucleic Acids Res. 14*, 1542.
11. Vieira, J. & Messing, J. (1982) *Gene 19*, 259–268.
12. Messing, J. & Vieira, J. (1982) *Gene 19*, 269–276.
13. Williams, G. T. & Neuberger, M. S. (1986) *Gene 43*, 319–324.
14. Neuberger, M. S., Williams, G. T. & Fox, R. O. (1984) *Nature (Lond.) 312*, 604–608.
15. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular cloning*, Cold Spring Harbor Laboratory, New York.
16. Silhavy, T. J., Berman, M. L. & Enquist, L. W. (1984) *Experiments with gene fusions*, Cold Spring Harbor Laboratory, New York.
17. Neu, H. C. & Heppel, L. A. (1965) *J. Biol. Chem. 240*, 3685–3692.
18. Laemmli, U. K. (1970) *Nature (Lond.) 227*, 680–685.
19. Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.
20. Richardson, C. C. (1966) *Procedures in Nucleic Acid Research*, vol. 1 (Cantoni, G. L. & Davies, D. R. eds) pp. 263–276, Harper and Row, New York.
21. Heins, J. N., Taniuchi, H. & Anfinsen, C. B. (1966) *Procedures in Nucleic Acid Research*, vol. 1 (Cantoni, G. L. & Davies, D. R. eds) pp. 79–84, Harper and Row, New York.
22. Shuman, H. A. (1982) *J. Biol. Chem. 257*, 5455–5461.
23. Débarbouillé, M., Shuman, H. A., Silhavy, T. J. & Schwartz, M. (1978) *J. Mol. Biol. 124*, 359–371.
24. Lachica, R. V. F., Genigeorgis, C. & Hoeprich, P. D. (1971) *Appl. Microbiol. 21*, 585–587.

25. Takahara, M., Hibler, D. W., Barr, P. J., Gerlt, J. A. & Inouye, M. (1985) *J. Biol. Chem. 260*, 2670–2674.
26. Bassford, P. J. Jr, Silhavy, T. J. & Beckwith, J. R. (1979) *J. Bacteriol. 139*, 19–31.
27. Joyce, C. M. & Grindley, N. D. F. (1983) *Proc. Natl Acad. Sci. USA 80*, 1830–1834.
28. Goff, S. A. & Goldberg, A. L. (1985) *Cell 41*, 587–595.
29. Pelham, H. R. B. (1986) *Cell 46*, 959–961.
30. Emr, S. D. & Bassford, P. J. Jr (1982) *J. Biol. Chem. 257*, 5852–5860.
31. Copeland, B. R., Su, T. Z. & Oxender, D. L. (1986) *Microbiology*, pp. 266–269, American Society for Microbiology, Washington DC.
32. Rasmussen, B. A., Bankaitis, V. A. & Bassford, P. J. Jr (1984) *J. Bacteriol. 160*, 612–617.
33. Ullmann, A. (1984) *Gene 29*, 27–31.
34. Rüther, U. & Müller-Hill, B. (1983) *EMBO J. 2*, 1791–1794.
35. Anba, J., Baty, D., Lloubès, R., Pagès, J. M., Joseph-Liauzin, E., Shire, D., Roskam, W. & Lazdunski, C. (1987) *Gene 53*, 219–226.
36. Nilsson, B., Abrahmsén, L. & Uhlén, M. (1985) *EMBO J. 4*, 1075–1080.
37. Sassenfeld, H. M. & Brewer, S. J. (1984) *Biotechnology 2*, 76–81.
38. Richarme, G. (1982) *Biochem. Biophys. Res. Commun. 105*, 476–481.
39. Stark, G. R. (1977) *Methods Enzymol. 47*, 129–132.
40. Szoka, P. R., Schreiber, A. B., Chan, H. & Murthy, J. (1986) *DNA 5*, 11–20.
41. Nagai, K. & Thogersen, H. C. (1984) *Nature (Lond.) 309*, 810–812.
42. Germino, J. & Bastia, D. (1984) *Proc. Natl Acad. Sci. USA 81*, 4692–4696.
43. Shortle, D. (1983) *Gene 22*, 181–189.
44. Joyce, C. M., Kelley, W. S. & Grindley, N. D. F. (1982) *J. Biol. Chem. 257*, 1958–1964.