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Synthesis and maturation of λ receptor in *Escherichia coli* K-12: *In vivo* and *in vitro* expression of gene *lam*B under *lac* promoter control

(outer membrane protein/phage receptor/protein export/*in vitro* protein synthesis)

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ABSTRACT The λ receptor is an outer membrane protein from *Escherichia coli* K-12. *lam*B, its structural gene, is part of the maltose regulon. We have cloned this gene in a phage so that it is under the control of the *lac* promoter. The phage was devised in such a way that it can infect *lam*B mutants and that chromosomal *lam*B mutations can be transferred to it. *In vivo*, the λ receptor is expressed under *lac* promoter control and is exported normally to the outer membrane, independently of the expression of the other genes of the maltose regulon. *In vitro*, DNA of the phage allows efficient synthesis of the *lam*B product. The protein—or pre-λ-receptor—made *in vitro* contains an NH_2-terminal sequence of about 25 amino acids not found in the λ receptor. We have detected no inactivation of phage λ by the pre-λ-receptor. Conversion of the pre-λ-receptor to a form that has the apparent molecular weight of the mature λ receptor was achieved. A *lam*B mutation that blocks export *in vivo* also blocks conversion *in vitro*.

Bacteria export certain classes of proteins through their cytoplasmic membrane (1). To study the molecular mechanisms of this translocation process, which is similar to that of protein secretion in higher cells (2), it is desirable to have a system that allows both genetic and biochemical analyses. The *lam*B gene (3–5) in *Escherichia coli* K-12 is quite suitable for such a combined approach. The *lam*B product is an outer membrane protein (6) involved in the transport of maltose and maltodextrins (7). It serves as the cell surface receptor for phage λ and other phages (8) and can be assayed in cellular extracts by its ability to inactivate phage λ (6). The *lam*B gene is a part of the *mal*K–*lam*B operon of the *mal*B region of the *E. coli* chromosome (9). Extensive genetic and restriction site analyses of the region have been performed (10–13).

We wanted to develop a system in which the export of an outer membrane protein could be studied *in vitro*. This could allow us to test the current models of protein export, particularly the role of the signal sequence (14–17). To bypass the positive control factors that are required for *mal*B expression (9–18), we cloned *lam*B under the control of the promoter for the *lac* operon on a transducing phage. The DNA of this phage programs the synthesis of the *lam*B product *in vitro* so efficiently that biochemical studies become possible.

MATERIALS AND METHODS

Strains, Media, Bacteriological Techniques, and Chemicals. The bacterial strains used for S30 preparation were pop945:*lac*ZW4680, *rps*L, *mal*Q6, *mal*BΔ7 (λ^+) *rpo*B and pop997:*lac*ZW4680, *rps*L, *mal*AΔ108, *mal*BΔ7 (λ^+) *rpo*B. The relevant markers of the other strains used are given in the text. The phage strains were λYh$\dot{4}$34 (Fig. 1), λCI857h80t68d-*lac*L8UV5 (a gift of Jack Greenblatt), λvh$^+$, λvho (4), and λCI57h434. Strain pop3257 or SE2060 [*ara*D139, Δ(*lac*)U169, *str*A, *rel*A, *thi*, *lam*B S60] which is resistant to λ (λr) and unable to grow on dextrins (Dex$^-$) (S. Emr and T. Silhavy, personal communication) was used to generate, by homogenization, phage [λYh$\dot{4}$34 (1B8) S60].

Media and bacteriological techniques were as described (10). DNA hydrolysis with restriction enzymes, ligation, and transfection were as described (19).

Cell-Free Protein Synthesis. The system described by Zubay (20) was used without major modifications. The pressure on the French press was 1550 psi (10.7×10^6 pascals). Polyethylene glycol 6000 was added (1.5%, wt/vol) to the final mixture. Isopropyl β-thiogalactoside (IPTG), when present, was at 5 mM. For [^{35}S]methionine labeling, the concentration of nonradioactive methionine was decreased to 0.45 mM and [^{35}S]-methionine was present at 0.04 mCi/ml (1 Ci = 3.7×10^{10} becquerels). Formyl[^{35}S]Met-tRNA was synthesized according to Wendell and Stanley (21).

Assay of Pre-λ-Receptor Activity. To assay the activity of the pre-λ-receptor, the *in vitro* synthesis mixture was dialyzed overnight against the assay buffer. Samples (5 μl) were then incubated at 37°C with about 300 plaque-forming units of λho in a total volume of 25 μl (adjusted with buffer). At 30 min, 100 μl of buffer containing strain CR63 (3) (about 2×10^8 cells) was added, and plating was done with top agar on tryptone plates. The experiments were performed with *in vitro* synthesis mixtures programmed with a DNA carrying *lam*B under *lac*L8UV5 promotor control, and the appropriate controls were run in parallel. By adding various concentrations of active receptor it was found that the assay was 1/10th as sensitive in presence of synthesis mixture—i.e., in the presence of synthesis mixture the K value was 1/10th that in the assay buffer. No inactivation of λvho was detected. Because inactivation of 20% of the phage would have been detected, taking into account the decreased sensitivity of the assay, we can calculate that the K value in the *in vitro* synthesis mixture (K_{pre}) is less than 0.5.

Evaluation of the Amount and Relative Specific Activity of the Pre-λ-Receptor. The methionine content per unit molecular weight is approximately the same in β-galactosidase [23 methionine residues, $M_r \approx 116{,}000$ (22)] and pre-λ-receptor [8–9 methionine residues, $M_r \approx 56{,}000$ (ref. 23 and unpublished data)]. Syntheses were performed in parallel with DNA carrying *lam*B or *lac*Z. By comparing the radioactivity incor-

Abbreviations: K_{pre}, K value in *in vitro* synthesis mixture; λr, λ-resistant; λs, λ-sensitive; IPTG, isopropyl β-thiogalactoside.

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porated *in vitro* in the protein bands we could estimate that the amount of pre-λ-receptor protein was generally double that of β-galactosidase per unit volume of synthesis mixture. Because the amount of β-galactosidase calculated from the enzymatic activity was about 1.5 μg/ml, the amount of pre-λ-receptor protein was 3 μg/ml. This is approximately the amount of λ receptor protein found in the extract of 2.5–5 × 10^9 cells (6) and corresponds to a K value of 200–400. Because K_{pre} was found to be less than 0.5, this shows that the specific activity of the pre-λ-receptor is no more than 1/800 to 1/400 of that of the λ receptor.

RESULTS

Cloning and *In Vivo* Expression of Gene *lamB* Under *lac* Promoter Control. Phage λYh434 contains a single *Eco*RI site located early in the *lacZ* gene (19–25) (Fig. 1). Most of *lacZ* is deleted in this phage, but *lacY* is present and is expressed under *lac* promoter control. We assumed that insertion of the *Eco*RI fragment [*lamB* (*Eco*RI)] containing *lamB* but not its natural promoter into the *Eco*RI site of the phage would block *lacY* expression. We transformed a *lacZ*$^+$ *lacY*$^-$ strain (pop265) (10) with a ligated mixture of *Eco*RI-treated λYh434 DNA and *lamB* (*Eco*RI) fragment and plated the cells on MacConkey's lactose medium at 32°C. Of 482 plaques, 450 became red within 2 days; 32 remained white, as expected if no *lacY* complementation occurred. Lysogens were purified from seven white plaques and their progeny phages were characterized.

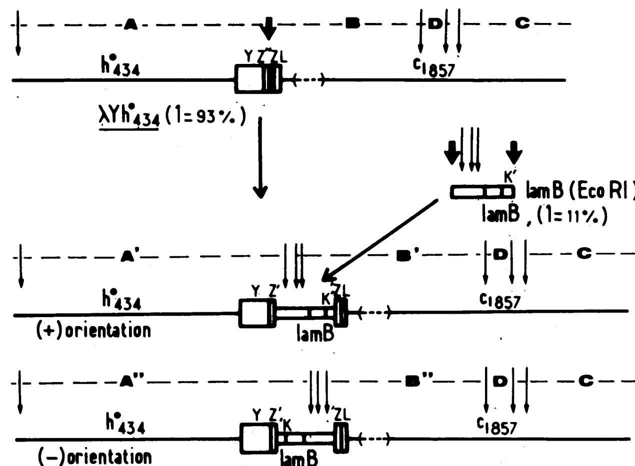

FIG. 1. Cloning of *lamB* under the control of the *lac* promoter on a phage vector. The phage vector λYh434 was constructed by *in vivo* recombination. The late functions give the host range of phage 434 and thus allow growth on λ-resistant strains. They originate from phage λoh434. This hybrid between phage λ and phage 434 was constructed by Irène Mannheimer. λoh434 is free of any *Eco*RI cuts. λYh434 is a recombinant between λoh434 and λY (19). It carries the promoter mutations *lac*L8UV5. *lamB* (*Eco*RI) is a 5.5-kilobase restriction fragment limited by *Eco*RI sites. It contains the distal part of *malK* (≈1 kilobase), gene *lamB* (≈1.6 kilobases), and additional bacterial DNA (≈3 kilobases). It was purified by *Eco*RI hydrolysis of the DNA of phage λap *malB*21 (25) and separation of the restriction fragments by ultracentrifugation on a sucrose gradient. On the lower part of the figure the two possible orientations for the insertion of the *lamB* (*Eco*RI) fragment in the vector have been represented. Expression under *lac* promoter control occurs only in the + orientation. l, DNA length in % of λ wild-type DNA; - - -, *Hin*dIII deletion extending from 52.2% to 57.2% of λ (19); ⬇, an *Eco*RI cut; ↓, a *Bgl* II cut. A, B, A′, B′, A″, and B″ correspond to restriction fragments obtained after *Eco*RI or *Bgl* II hydrolysis (or both) used to determine the orientation of the inserted *lamB* (*Eco*RI) fragment (see Fig. 2). Z is *lacZ*, Y is *lacY*, K is *malK*, and L is the promoter *lac*L8UV5. Relevant gene fragments have been designated by a letter with the symbol prime (′) on the side of the missing piece.

If the *lamB* (*Eco*RI) fragment has been inserted, the phages should carry the distal part of *malK*. Indeed, strain pop1768 having a late mutation in *malK* (*malK*4) (9) yielded Mal$^+$ recombinants when lysogenized with any of the seven phages.

Insertion of the *lamB* (*Eco*RI) fragment into phage λYh434 can occur in either of two possible orientations (Fig. 1). Expression of *lamB* under *lac* promoter control should be possible only in the orientation such that the sense strand of *lamB* can be transcribed from the *lac* promoter (+ orientation). We thus expected that only the phages carrying *lamB* in the + orientation could convert λ-resistant (λr) strains to λ-sensitive (λs). Furthermore, we expected that expression of *lamB* would then be independent of *malT* (9) (the positive regulatory gene of the maltose system) but dependent on derepression of the *lac* promoter. *lamB* expression should also be cyclic AMP independent because of the L8UV5 mutations. This was tested by constructing lysogens of a *lamB recA* strain (pop6510), a *recA* strain carrying a partial deletion of *malK* and *lamB* (pop5208), a strain carrying a deletion in *malT* (pop101), and a strain carrying a *cya* deletion (CA8306). Three of the phages [λYh434(1B8), (1B10), and (1B19)] gave complementation to λs for all of these λr strains and should contain the *lamB* (*Eco*RI) fragment in the + orientation; four [λYh434(1B1), (1B3), (1B5), and (1B30)] did not, suggesting that they contained the fragment in the opposite orientation (−). This was confirmed by restriction endonuclease mapping of two + phages and one − phage (data not shown). Complementation occurred only in the presence of IPTG as expected with a *lacI*$^+$ strain; in particular, lysogenic derivatives of the Dex$^-$ strain (pop6510) became Dex$^+$ only in presence of IPTG.

Table 1. Assay of λ receptor activity extracted from lysogens for phages carrying the *lamB* (*Eco*RI) fragment

malT allele*	*lamB* orientation†	Inducer‡	λ rec. act.§	β-Galase act.¶
+		None	300	—
+		Mal	450	—
+		IPTG	320	13,110
−		IPTG	<0.2	7,420
−	−	None	2.6	12
−	−	IPTG	2.8	6,750
−	+	None	2.1	11
−	+	IPTG	1630	5,700
−	+	None	2.7	10
−	+	IPTG	1910	6,500

* The *malT*$^+$ strain is HfrG$_6$. The *malT* strain is HfrG$_6$ *malA*Δ157; it carries a deletion in *malT*.

† The control strains (first four lines) contained no phage. The phage carrying the *lamB* (*Eco*RI) fragment in the − orientation was λYh434 (1B5); the phages carrying the + orientation were λYh434 (1B8) (lines 7 and 8) and λYh434 (1B10) (lines 9 and 10). λYh434 (1B10) carries mutation *malK*5 (9). No polar effect of this ochre mutation has been detected on *lamB* expression.

‡ Mal, maltose.

§ Cell extracts were prepared as described (6) and were assayed for λ receptor activity by measuring the initial kinetics of λvho inactivation (6). The assay buffer contained 10 mM Tris·HCl at pH 7.5 and 2 mM $MgCl_2$. The kinetics of inactivation follow the relationship $\log(\phi/\phi_0) = -K_d \cdot t$, ϕ_0 and ϕ are plaque-forming units/ml at times 0 and t (min), respectively. K_d is the inactivation constant at dilution d of the extract. The activity is measured by the slope of the curve $K_d = K \times d$. K is directly proportional to the concentration of active receptor in the extract and hence is a measure of the amount of active receptor (24) which is expressed here as K/mg of protein. The relative error is 20%.

¶ β-Galactosidase activity, assayed for comparison, is encoded by the chromosomal *lacZ* gene which is under the control of the wild-type *lac* promoter. The activity is expressed in units/mg of protein. The induction ratio for β-galactosidase here is of the order of 550.

λ receptor activity was assayed in extracts of *lac*I$^+$ strains carrying a *mal*T deletion and lysogenic for the phages having inserted the *lam*B (*Eco*RI) fragment in each orientation (Table 1). As expected, only in the + orientation was the λ receptor activity inducible by IPTG. The *in vivo* induction ratio was about 700–800. The fully induced level was somewhat higher (about 3 times) than the level in the *mal*T$^+$ strain (HfrG$_6$) induced by maltose. Surprisingly, the level of activity in the absence of IPTG was the same in the + and − orientations.

***In Vitro* Synthesis of the *lam*B Product.** DNA from a phage carrying *lam*B inserted in the + orientation was used to program *in vitro* protein synthesis. The S30 extract was prepared from a *lac*I$^+$ Δ*lac*Z Δ*lam*B strain. A major protein band of apparent M_r 56,000 could be detected (Fig. 2*a*, lanes 5 and 6; Fig. 2*b*, lanes 1–4; and Fig. 2*c*, lane 4). This band was not encoded by the vector phage DNA (Fig. 2*a*, lane 4). The intensity of the band was decreased in the absence of IPTG (Fig. 2*a*, lanes 7, 8, and 9) so that this protein is synthesized under *lac* promoter control. Althrough its apparent M_r is about 2000–3000 greater than that of the λ receptor (Fig. 2*b*, lanes 5, 6, and 7) we conclude by the following criteria that it is the product of gene *lam*B. (*i*) The protein made *in vitro* was precipitated with anti-λ-receptor serum (Fig. 2*a*, lanes 3, 8, and 9; Fig. 2*b*, lane 6). (*ii*) When the DNA carried a small deletion, *lam*B S60 [λYh$\mathring{4}$34 (1B8 S60)], which is internal to *lam*B and does not change the coding frame (S. Emr and T. Silhavy, personal communication), the protein made *in vitro* was smaller than that corresponding to *lam*B$^+$ (Fig. 2*c*, lanes 1 and 2; Fig. 3*b*). (*iii*) Partial proteolysis of the protein synthesized *in vitro* and native λ receptor yielded peptides common to both proteins (Fig. 3*a*). However, three peptides (B′, C′, and D′), derived from the λ receptor, were absent from the digest of protein made *in vitro*. They were replaced by peptides B, C, and D, each of which exhibited the same increase in apparent M_r over its counterpart in the λ receptor. This increase is identical to that found between the λ receptor and the protein made *in vitro*. All this is consistent with the presence of the same extra sequence of amino acids in the protein made *in vitro* and in peptides B, C, and D.

Two lines of evidence demonstrate that the extra sequence present in the protein synthesized *in vitro* is located at the NH_2 terminus. (*i*) The difference in apparent M_r between the *lam*B$^+$ and *lam*B S60 proteins made *in vitro* is also found between the peptides B_1, C_1, and D_1 derived from the *lam*B S60 protein and the peptides B, C and D, respectively (Fig. 3*b*). Thus, these peptides contain the amino acid sequence deleted in the *lam*B S60 product. Because the S60 deletion maps in the earliest known segment of the *lam*B gene (S. Emr and T. Silhavy,

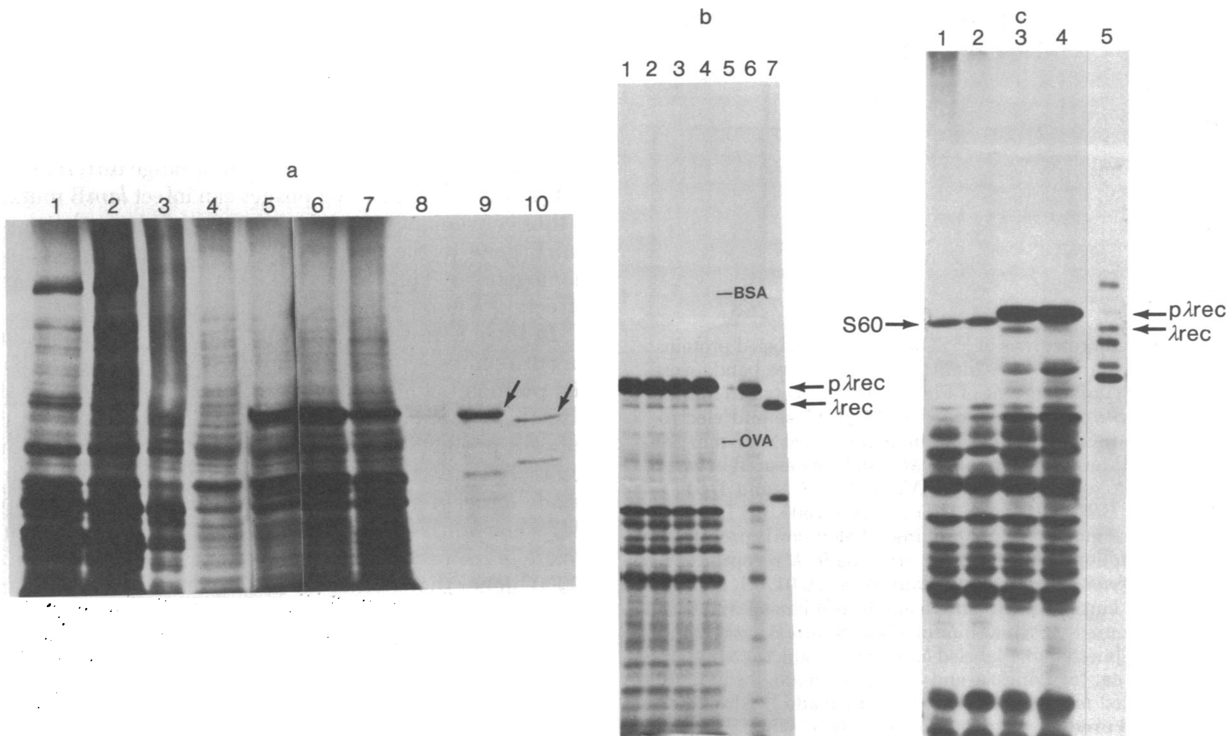

FIG. 2. Gel electrophoresis and autoradiography of [^{35}S]methionine-labeled protein synthesized *in vitro*. Gel electrophoresis and autoradiography were performed as described (26, 27). Nonradioactive protein markers were located by staining with 1% alkylbenzyldimethylammoniumchloride (Merck). When no immunoprecipitation was performed, each lane contained 10 μl of *in vitro* synthesis mixture. Otherwise, the amount of washed immunoprecipitate per lane corresponded to 50 μl of an *in vivo* extract or an *in vitro* synthesis mixture treated with 5 μl of anti-λ-receptor antiserum and with 10 mg of protein A-Sepharose beads. The *in vivo* extracts (6) were obtained from the λs strain pop3 labeled with $^{35}SO_4$. pλrec, *lam*B product made *in vitro*; λrec, λ receptor made *in vivo*. (*a*) A 130 × 75 × 1.5 mm $NaDodSO_4$/10% polyacrylamide gel run for 3.5 hr at 30 mA. Lanes: 1, 2, and 3, λCI857h80t68d*lac*L8UV5 DNA; 2, with IPTG; 3, with IPTG and λ receptor antiserum; 4, λYh$\mathring{4}$34 DNA with IPTG; 5, λYh$\mathring{4}$34 (1B19) DNA with IPTG; 6, 7, 8, and 9, λYh$\mathring{4}$34 (1B10) DNA; 6, with IPTG; 8, with λ receptor antiserum; 9, with IPTG and λ receptor antiserum; 10, immunoprecipitate of λ receptor. Because the S30 contains some repressor of the *lac* operon, induction of genes under *lac* control can be seen *in vitro*. However, the induction ratio is much smaller than *in vivo*. Compare for example, lanes 1 and 2 for β-galactosidase and lanes 6 and 7 or 8 and 9 for *lam*B product. (*b*) a 130 × 300 × 1.5 mm $NaDodSO_4$/10% polyacrylamide gel run for 20 hr at 150 V. Lanes: 1–4, λYh$\mathring{4}$34 (1B10) DNA with IPTG; 5, position of nonradioactive markers used for M_r determination (28) (OVA, $M_r \approx$ 45,000; BSA, $M_r \approx$ 69 000); 6, λYh$\mathring{4}$34 (1B10) DNA with IPTG and λ receptor antiserum; 7, immune precipitate of λ receptor. (*c*) A 130 × 200 × 1.5 mm $NaDodSO_4$/10% polyacrylamide gel run for 18 hr at 140 V. Lanes: 1 and 2, λYh$\mathring{4}$34 (1B8 S60) DNA; 3 and 4, Sam 7 hλ derivative of λYh$\mathring{4}$34 (1B8); 5, nonprecipitated *in vivo* extract. Lanes 1 and 3 had inverted membrane vesicles (19 μl/100 μl of *in vitro* synthesis). Longer times of exposure of the gel confirmed that no band corresponding to mature λ receptor was produced in lane 1.

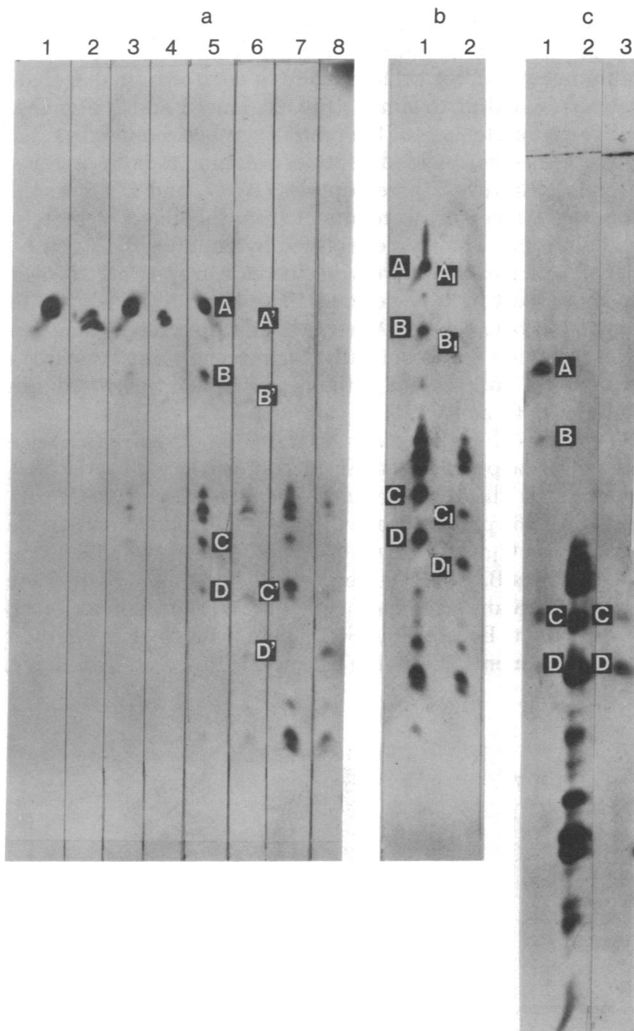

FIG. 3. Limited proteolysis of [35SO]methionine-labeled proteins. After nonlabeled markers were identified, radioactive bands were excised from gels similar to that of Fig. 3*a* and subjected to limited proteolysis with *Staphylococcus aureus* V8 protease and electrophoresis on $NaDodSO_4$/15% polyacrylamide 130 × 300 × 1.5 mm gels (29). A constant difference of apparent M_r can be measured between the following couples of peptides: A/A′, B/B′, C/C′, and D/D′, ≈ 2000–3000; A/A_1, B/B_1, C/C_1, and D/D_1, ≈1200–1500. (*a*) Lanes: 1, 3, 5, and 7, *in vitro* made [^{35}S]methionine-labeled *lam*B product; 2, 4, 6, and 8, immunoprecipitate of *in vivo* made λ receptor. The amounts of *S. aureus* V8 protease per lane were 0, 0.01, 0.1, and 1 μg for lanes 1 and 2, lanes 3 and 4, lanes 5 and 6, and lanes 7 and 8. (*b*) Lanes: 1, *in vitro* made [^{35}S]methionine-labeled *lam*B$^+$ product; 2, *in vitro* made [^{35}S]methionine-labeled *lam*B S60 product. V8 protease per lane was 0.1 μg. (*c*) Lanes: 1 and 3, *in vitro* made formyl[^{35}S]-methionine-labeled *lam*B product; 2, *in vitro* made [^{35}S]methionine-labeled *lam*B product. V8 protease per lane was 0.01 μg in lane 1 and 0.1 μg in lanes 2 and 3.

personal communication), this indicates that peptides B, C, and D must be at or near the NH_2-terminal fragment of the pre-λ-receptor. (*ii*) *In vitro* synthesis was performed in the presence of formyl[^{35}S]methionine-tRNA which labels only the NH_2 terminus of the protein. Partial proteolysis showed that the label is only in the intact protein and in peptides B, C, and D (Fig. 3*c*).

We assume that the *in vitro lam*B product is the precursor of the λ receptor predicted by the signal hypothesis (1, 2) and designate it the "pre-λ-receptor."

The Pre-λ-Receptor: Conversion and Lack of Activity. Addition of inverted membrane vesicles (15) to the synthesis mixture programmed with a *lam*B$^+$ template resulted in the appearance of a protein that had the apparent M_r of the λ receptor (Fig. 2*c*, lane 3). When the *lam*B template carried deletion S60, this protein was not produced (Fig. 2*c*, lanes 1 and 2). This shows that appearance of this protein is due to the conversion of the *in vitro* made *lam*B$^+$ product—i.e., the pre-λ-receptor. In addition, it suggests strongly that the conversion corresponds to the processing of the signal sequence of the pre-λ-receptor (see *Discussion*). Conversion by vesicles was not seen when the vesicles were added after the synthesis is completed (data not shown).

When the ability of the pre-λ-receptor to inactivate phage λvho was assayed, no activity was detected. We can determine that the specific activity of the pre-λ-receptor is no more than 1/800th to 1/400th that of the λ receptor. We did not detect any activity of the conversion product under the conditions of the assay.

DISCUSSION

***In Vivo* Expression of *lam*B Under *lac* Promoter Control.** Insertion of the *lam*B (*Eco*RI) fragment in phage λYh434 in the + orientation results in the synthesis of the *lam*B protein under *lac*L8UV5 promoter control. λ receptor activity is inducible by IPTG in a *lac*I$^+$ background. It is expressed independently of the maltose regulon and of the cyclic AMP-dependent control systems. The fact that λ receptor activity can be obtained in the total absence of the *mal*T product proves that, according to the present criteria, neither the *mal*T product nor any other known gene of the maltose regulon is required for translation, positioning, and maturation of the λ receptor.

Because the phage vector has a host range different from λ (h434), the *lam*B-carrying phages can infect *lam*B mutants. It is thus convenient to transfer mutations from a chromosomal *lam*B gene to the *lam*B copy of the phage by looking for homogenotes. This can be used in particular to compare the effects of a mutation *in vivo* and *in vitro*.

***In Vitro* Expression of *lam*B Under *lac* Promoter Control.** *In vitro* synthesis programmed with DNA carrying *lam*B under *lac* promoter control yields a major protein. We have shown that this protein is the product of the *lam*B gene: (*i*) it is specifically precipitated with anti-λ receptor antiserum; (*ii*) limited proteolysis gives a pattern that is similar to that of the *in vivo* made λ receptor; and (*iii*) it is made with a smaller apparent M_r when *lam*B carries the internal deletion S60. The *in vitro* made *lam*B product has an apparent M_r that is about 2000–3000 higher than that of the *in vivo* made λ receptor, and we assume that it is the pre-λ-receptor (1–2). The existence of a precursor to the λ receptor had already been found by using various systems (5–30). We show here that the pre-λ-receptor carries an extra NH_2-terminal sequence that suffices to account for its higher apparent M_r.

This was confirmed by determination of the partial amino acid sequence of the NH_2-terminal part of the pre-λ-receptor: it corresponds to the DNA sequence of the early part of gene *lam*B (unpublished data).

We have observed conversion of the pre-λ-receptor by inverted vesicles to a form that has the apparent M_r of the λ receptor. This conversion was found to be cotranslational (15).

It is not established at present whether the conversion product obtained *in vitro* corresponds to the processing occurring *in vivo*. However, it is interesting that this conversion is not seen when the *lam*B template carries deletion S60 which prevents export *in vivo* (S. Emr and T. Silhavy, personal communication). This mutation as well as two other mutations (5) that are known to prevent *in vivo* processing are located in the signal

sequence (unpublished data). One possible explanation is that all these signal sequence mutations block a very early step in export so that processing, which is subsequent, cannot take place. This situation may also be the one prevailing in the *in vitro* system.

No ability of the pre-λ-receptor to inactivate phage λvho was found by us. We found also no phage inactivation after synthesis in the presence of inverted vesicles—i.e., when conversion occurred. However, this does not allow us to conclude that the converted product is not active because (*i*) the converted product might be unable to interact with the phage in the conditions of the assay [for example, it could be sequestered in the vesicles (15)], and (*ii*) the yield of conversion being low, the total activity of the converted product might be near the limit of detection of the assay. At any rate, it is not known at present whether processing of the signal sequence is enough to yield λ receptor activity. Further maturation steps, or cofactors, might be required.

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