The Rex system of bacteriophage λ: tolerance and altruistic cell death

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The *rexA* and *rexB* genes of bacteriophage λ encode a two-component system that aborts lytic growth of bacterial viruses. Rex exclusion is characterized by termination of macromolecular synthesis, loss of active transport, the hydrolysis of ATP, and cell death. By analogy to colicins E1 and K, these results can be explained by depolarization of the cytoplasmic membrane. We have fractionated cells to determine the intracellular location of the RexB protein and made RexB–alkaline phosphatase fusions to analyze its membrane topology. The RexB protein appears to be a polytopic transmembrane protein. We suggest that RexB proteins form ion channels that, in response to lytic growth of bacteriophages, depolarize the cytoplasmic membrane. The Rex system requires a mechanism to prevent λ itself from being excluded during lytic growth. We have determined that overexpression of RexB in λ lysogens prevents the exclusion of both T4 *r*II mutants and λ *ren* mutants. We suspect that overexpression of RexB is the basis for preventing self-exclusion following the induction of a λ lysogen and that RexB overexpression is accomplished through transcriptional regulation.

[*Key Words*: Bacteriophage λ; Rex exclusion; transmembrane domains; gene fusions; alkaline phosphatase; cell death]

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The *rexA* and *rexB* genes of bacteriophage λ encode a two-component system that aborts lytic growth of bacterial viruses (Benzer 1955; Howard 1967; Toothmann and Herskowitz 1980; Matz et al. 1982). The stringency of Rex exclusion depends on the level of *rex* gene expression. Whereas λ lysogens restrict *r*II mutants of bacteriophage T4, but not T4 wild type, overexpressing the *rex* genes restricts T4 wild-type and all other tested phages, except λ wild type (Toothmann and Herskowitz 1980a; Landsmann et al. 1982; Shinedling et al. 1987). In a real sense, Rex exclusion is a defense mechanism, analogous to the plant hypersensitivity response, in which individual cells die rather than propagate an infection that threatens the whole organism (Maclean et al. 1974).

Rex exclusion is characterized by termination of macromolecular synthesis, loss of membrane potential, hydrolysis of intracellular ATP, and cell death (see Results; Garen 1961; Sekiguchi 1966; Sauerbier et al. 1969; Colowick and Colowick 1983; Snyder and McWilliams 1989). This physiology resembles the response of cells that are sensitive to colicins E1 and K (for review, see Luria and Suit 1987). Importantly, an exclusion-like response can be elicited in uninfected cells by overexpressing RexA in the presence of RexB (Snyder and McWilliams 1989).

rexA and *rexB*, together with the *c*I (repressor) gene, reside in the immunity region of λ (Fig. 1). Shortly after λ phages infect a sensitive host, these three genes are transcribed as a polycistronic message from the promoter for repressor establishment, p_{RE} (Fig. 1; Astrachan and Miller 1972; Reichardt 1975; Belfort 1978; Lieb and Talland 1981). In a lysogen, the polycistronic message is initiated at p_{RM}, the promoter for repressor maintenance (Fig. 1; Hayes and Szybalski 1973a; Hayes and Hayes 1978; Landsmann et al. 1982). Regulation of transcription from p_{RE} and p_{RM} and their role in the λ life cycle have been studied intensely and reviewed eloquently (Ptashne 1986).

A third, enigmatic transcript in the immunity region, LIT RNA, has been described (Fig. 1; Hayes and Szybalski 1973a). The LIT transcript is a monocistronic *rexB* mRNA that is initiated at a promoter, p_{LIT}, that appears to be in the *rexA*-coding sequence (Hayes and Szybalski 1973a; Landsmann et al. 1982). After induction of a lysogen, transcription from p_{RM} is shut off and transcription from p_{LIT} is activated. Ten minutes after thermal induction, the rate of *rexB* transcription is about 2-fold higher than in a lysogen, and by 15–20 min it is 5- to 10-fold higher.

The regulation of LIT transcription is complex and not thoroughly understood. LIT transcription in an induced lysogen requires the λ *O* and *P* gene products (Fig. 1) and

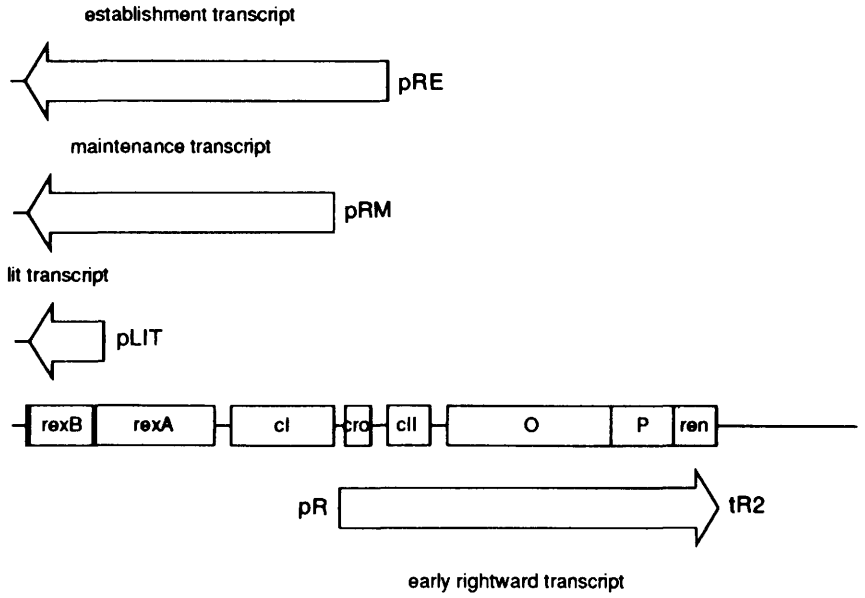

Figure 1. Map of bacteriophage λ, drawn to physical scale, showing relevant genes, transcripts, promoters, and transcriptional terminators.

the *Escherichia coli dnaB* and *dnaG* functions (Hayes and Szybalski 1973a), but plasmid-based, *O*- and *P*-independent LIT transcription has been inferred (Pirrotta et al. 1980; Landsmann et al. 1982). LIT transcription may occur in an uninduced lysogen (Matz et al. 1982) but at a level that is undetectable by classic hybridization techniques (Hayes and Szybalski 1973a). It is not known whether LIT transcription occurs during λ lytic infection, nor has the precise location of the LIT promoter been determined. Pirrotta et al. (1980) have shown that the 150-nucleotide *Hae*III–*Hin*fI restriction fragment that includes the carboxyl terminus of the *rexA* gene and the amino terminus of the *rexB* gene has promoter activity when cloned in a plasmid and a strong RNA polymerase-binding site in vitro. Nuclease protection experiments place the binding site within the carboxyl terminus of *rexA*. However, the fragment does not promote in vitro transcription, which is consistent with the reported requirement for activation of LIT transcription in a lysogen.

Landsmann et al. (1982) concluded that "the observed temporal shift from coordinate to discoordinate expression of *rexB* relative to *rexA*, together with an elaborate structural organization (especially the p_{LIT} promoter) to make such a shift possible, argues strongly that *rexB* has some additional function, independent of *rexA*." The recent observation that RexB stablizes the λ O protein (Schoulaker-Schwarz et al. 1991) may be a manifestation of the mechanism for amplifying LIT transcription.

In an effort to decipher the enigma of Rex exclusion, we have focused our attention on the *rexB* gene product, investigating its cellular location, its topology, and effects of its overexpression. The predicted RexB protein (Landsmann et al. 1982) has a molecular mass of 16 kD with alternating hydrophilic and hydrophobic domains and, by inference, is thought to be an integral membrane protein. Further examination of the predicted amino acid sequence suggests that the *rexB* gene encodes a polytopic transmembrane protein.

Cell fractionation and alkaline phosphatase fusion technology show that RexB is a transmembrane protein with four transmembrane domains. Overexpression of RexB in λ lysogens prevents the exclusion of T4 rII mutants and λ *ren* mutants. This result suggests that the postulated "additional function" of RexB (Landsmann et al. 1982) is to prevent λ from excluding itself (self-exclusion) following induction of a lysogen (Toothman and Herskowitz 1980a).

Results

Physiology of Rex exclusion

In T4 wild-type infections of the Rex overproducing strain NAPIV/prex20, RNA, protein, and DNA synthesis abort ~7–9 min postinfection, as measured by the incorporation of radioactive precursors into TCA-precipitable material (Fig. 2a–c). Correlated with the termination of macromolecular synthesis is the loss of the ability of the infected cells to actively transport β-D-thiomethylgalactopyranoside (TMG; Fig. 2d). Active transport of TMG is dependent on the electochemical potential of the inner membrane (Kashet and Wilson 1973); the inability of induced cells to accumulate TMG is indicative of a loss of membrane potential. These results resemble the response of sensitive cells to colicin E1 and K (Luria and Suit 1987). By analogy, they can be explained by the formation of ion channels that depolarize the inner membrane (Crammer et al. 1990).

It is unlikely that the physiology of exclusion in NAPIV/prex20 is an artifact of membrane damage resulting from overexpression of the Rex proteins. The growth rate and colony morphology of NAPIV/prex20 are essentially identical to that of NAPIV, and its physiology of

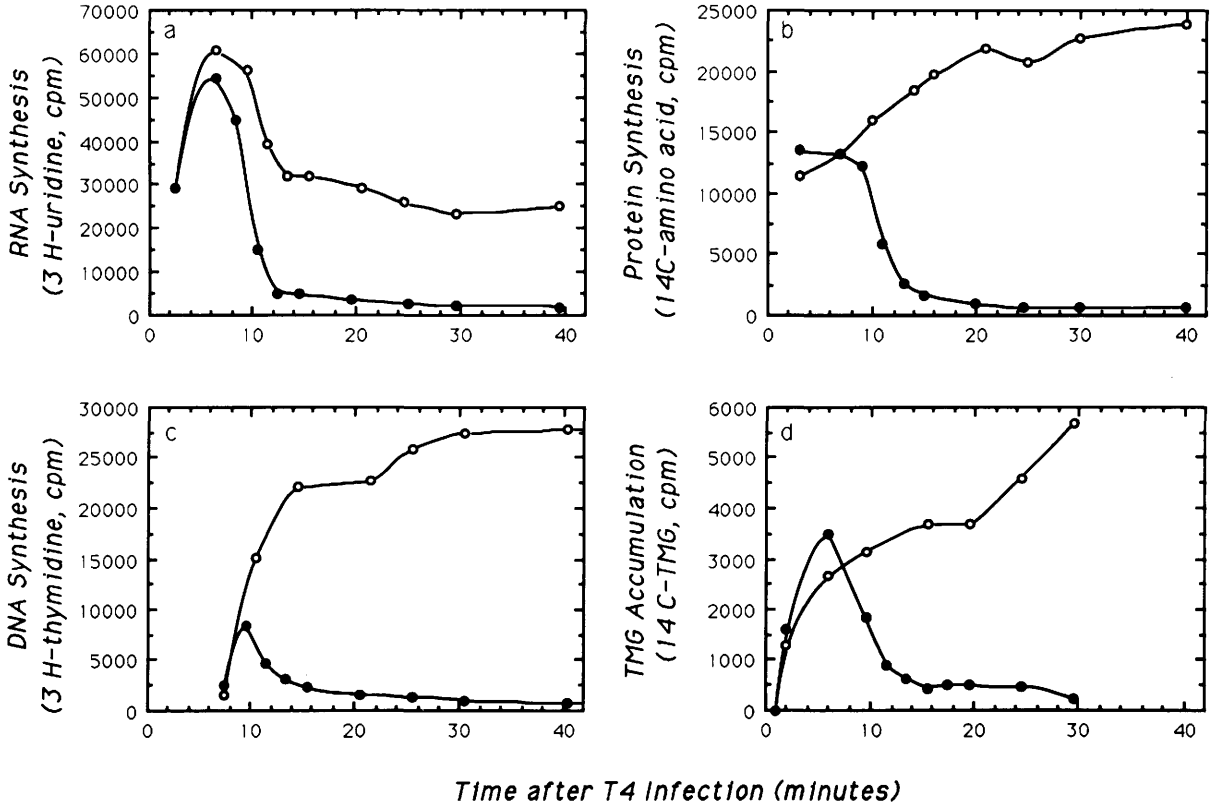

Figure 2. (*a–d*) Macromolecular synthesis and active transport in T4 wild-type infections. RNA, DNA, and protein synthesis in T4 wild-type infections of NAPIV and NAPIV/prex20 were measured by following the incorporation of radioactive precursors, from 1-min pulses, into TCA-precipitable material and measuring (see Materials and methods). Counts are plotted at the mid-point of their pulse. Active transport was monitored by measuring the intracellular accumulation of ^{14}C-labeled TMG that was added to the culture 1 min after infection (see Materials and methods). The TMG data are plotted at the time of sampling. (○) T4 wild-type infections of NAPIV; (●) T4 wild-type infections of NAPIV/prex20.

exclusion is very similar to that in a lysogen where the *rex* gene is expressed at normal levels.

Hydropathy analysis of the RexB protein

The hydropathy profile of the predicted RexB protein contains four hydrophobic peaks that are characteristic of the membrane-spanning segments of transmembrane proteins (Fig. 3). Charged amino acids are restricted, almost exclusively, to hydrophilic regions (Fig. 3). These two observations, coupled with von Heijne's (1986) "positive inside" rule for the distribution of charged amino acids among the hydrophilic domains of transmembrane proteins, suggest a topological model. RexB is an inner membrane protein with four transmembrane domains; the amino and carboxyl termini, as well as the central hydrophilic segment, are cytoplasmic, and the second and fourth hydrophilic sequences are periplasmic (Fig. 4).

Cellular location of the RexB protein

We partitioned cells into soluble and membrane fractions and analyzed the distribution of RexB protein by

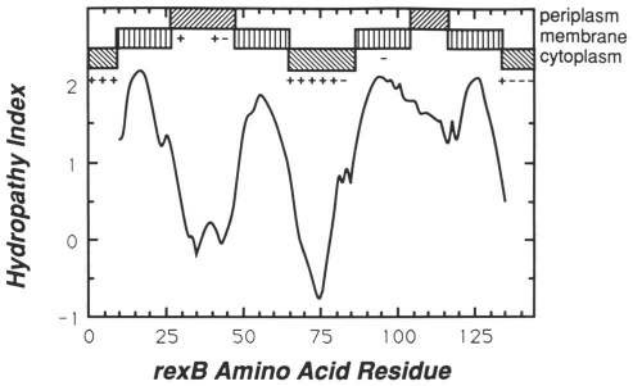

Figure 3. Hydropathy profile of the RexB protein. The hydropathy profile was calculated according to Kyte and Doolittle (1982), using 19-amino-acid residue windows. (*Top*) The positions of charged amino acids (Arg, Lys, Glu, Asp, and amino and carboxyl residues) are indicated by + and − signs, and the deduced cytoplasmic, transmembrane, and periplasmic domains are represented as hatched rectangles. For modeling, transmembrane domains are assumed to be 19 amino acids in length and to be centered at the maxima of the profile (von Heijne 1986).

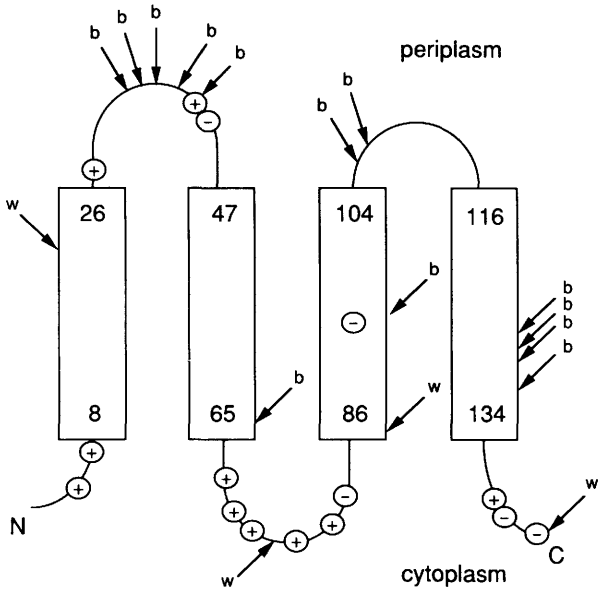

Figure 4. Predicted topology of the RexB protein. Transmembrane domains are shown as rectangles; cytoplasmic and periplasmic domains are lines. Junctions of in-frame RexB–PhoA′ fusions are marked with arrows; their color on X-phosphate plates is indicated: (b) Blue, active; (w) white, inactive. Charged amino acids are depicted by + and − signs. The amino and carboxyl termini are designated by N and C, respectively.

SDS-PAGE (Fig. 5). The RexB protein runs as a broad band from ~15 to 19 kD and, as expected, partitions completely in the membrane fraction. We have not yet determined the basis of the heterogeneity in its apparent molecular mass.

Isolation of rexB–phoA′ *fusions*

To investigate the membrane topology of the RexB protein, we constructed a series of *rexB–phoA′* gene fusions in which an amino-terminal fragment of *rexB* is joined to *phoA′*, a cassette of the alkaline phosphatase gene that lacks a ribosome-binding site, initiation codon, and signal sequence (Hoffman and Wright 1985; see also Materials and methods). Fusions were constructed in vitro by a modification of the procedure of Steggles (1989). In essence, the plasmid pBS rexB phoA′ was digested with exonuclease III and mung bean nuclease (see Materials and methods) to create a set of nested deletions extending into the *rexB* gene from the *Pst*I site at the amino terminus of *phoA′* (Fig. 6). Digested plasmids were religated and used to transform the *phoA* deletion strain AW1061.

Forty-six fusions were isolated by picking random blue and white colonies on ampicillin, X-phosphate plates. Fusion junctions were identified by DNA sequencing (Table 1). The junction of 1 fusion is located in the segment between *rexB* and *phoA′* (Fig. 6), 41 are within the *rexB* gene, and 4 are in the carboxyl terminus of *rexA*. Twenty-five of the *rexB–phoA′* fusions, constituting 14 different types, are in-frame. The *phoA′* nucleotide sequence in 45 of the 46 fusions begins GCCTCAG, as expected; one begins CCTCAG (Table 1). Three additional in-frame fusions were made by modifying in- or out-of-frame fusions (Table 1; see Materials and methods).

Alkaline phosphatase activity of RexB–PhoA′ fusions

In vivo, alkaline phosphatase must be exported to the periplasm to be enzymatically active (Hoffman and Wright 1985; San Millan et al. 1989). When alkaline phosphatase lacking a signal sequence is fused in-frame to a periplasmic domain of an inner membrane protein, the phosphatase moiety of the fusion is exported and displays high levels of phosphatase activity. Fusions to cytoplasmic domains are enzymatically inactive, as they are not exported.

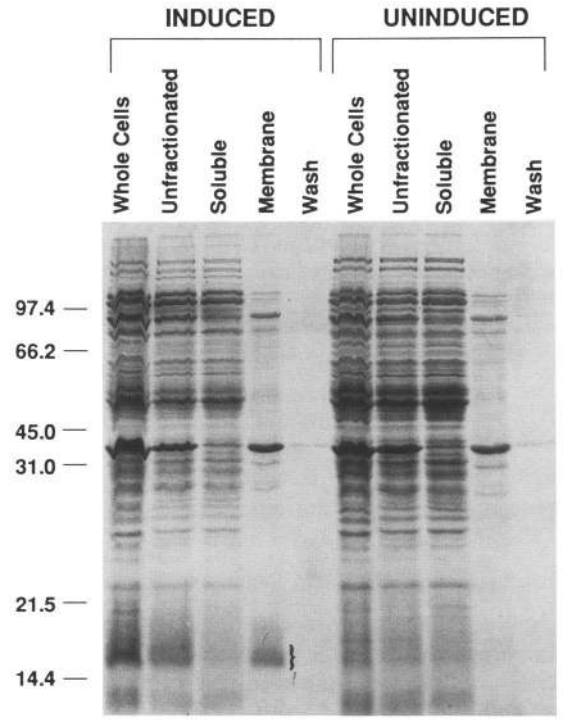

Figure 5. Cellular location of the RexB protein. Induced and uninduced samples of an *E. coli* BL21 (DE3) pDIP19D *Bgl*II$^-$ SD8 *rexB* culture were pulse-labeled with ^{14}C-labeled amino acids for 2 min from 11 to 13 min after induction. The cells were spheroplasted and lysed by osmotic shock (see Materials and methods). Membranes were isolated by centrifugation. Volumes of each fraction, representing equivalent quantities of cells, were analyzed by SDS-PAGE on 12.5% gels. (Whole cells) An aliquot of the labeled cells that was not spheroplasted; (unfractionated) spheroplasted and lysed but not centrifuged; (soluble) supernatant from the first centrifugation; (wash) supernatant from the second centrifugation; (membrane) the centrifugation pellet. (Lanes *1–5*) Induced culture; (lanes *6–10*) uninduced control. The RexB protein (bracket) runs as a broad band from ~15 to 19 kD.

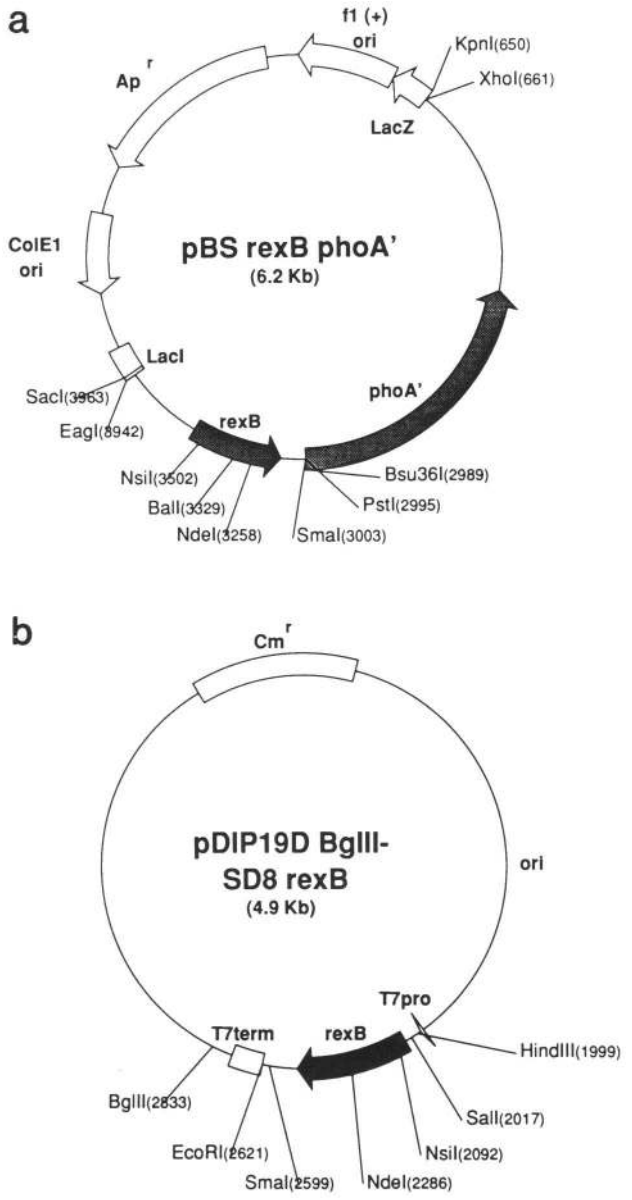

Figure 6. Structure of *rexB* expression vectors. (*a*) pBS rexB phoA′ is Bluescript SK(+) with the *rexB* and *phoA′* genes inserted at the *Eag*I–*Sma*I and *Pst*I–*Xho*I restriction sites, respectively. (*b*) pDIP19D *Bgl*II$^-$ SD8 *rexB* is pDIP19D (Singer and Gold 1991) with a blunted *Bgl*II site and *rexB* with a synthetic ribosome-binding site inserted at the *Sal*I–*Sma*I restriction sites. Relevant restriction sites and their locations are indicated.

The alkaline phosphatase activities of in-frame RexB–PhoA′ fusions are graphed in Figure 7. These data show that (1) fusions to predicted periplasmic domains exhibit high levels of alkaline phosphatase activity (48–462 units), (2) fusions to predicted cytoplasmic domains have low levels of activity (0.6–2 units), (3) fusions to the first periplasmic domain display a gradient of enzymatic activity, and (4) fusions to transmembrane domains display a wide range of activities, from highly active to completely inactive.

Results 1 and 2 verify the predicted topology and orientation of the RexB protein. Although we have not investigated the basis of quantitative differences in active fusions, results 3 and 4 indicate the limits of resolution of alkaline phosphatase fusion technology and suggest that information in the first periplasmic and perhaps the central and carboxyl cytoplasmic domains is necessary for the stable topology of the preceding transmembrane domain (Boyd et al. 1987; San Millan et al. 1989).

Bypass of Rex exclusion: overexpression of RexB

Overexpression of RexA in the presence of RexB produces an exclusion-like response in uninfected cells (Snyder and McWilliams 1989), suggesting that the ratio of RexA to RexB may be important in determining Rex exclusion. To examine the consequence of overexpressing RexB, we transformed the λ lysogen, CR63(λ), with pBS rexB phoA′ and assayed exclusion of *r*638, an *r*II deletion. Both T4 wild-type and T4 *r*II mutants grow on nonlysogens, but only T4 wild type grows on λ lysogens.

Figure 8 shows a surprising result; overexpression of the wild-type *rexB* gene (pBS rexB phoA′) in a lysogen prevents Rex exclusion of *r*II mutants. As expected, vectors lacking the *rexB* gene (pBS and pBS phoA′) do not interfere with exclusion. All eight transformants shown in Figure 8 are *rex*$^+$ lysogens; cultures of each produce λ phage that are capable of lysogenizing C600. As expected, these latter lysogens exclude *r*II mutants (data not shown). Each transformed strain contains a plasmid of the expected size. Sequencing and in vivo tests for Rex function confirm that the *rexB* cistron in the overexpression vector is wild type (data not shown).

Bypass of Rex exclusion of lambdoid phages

Although it seems unlikely that overexpression of RexB is the biological mechanism of T4 for bypassing Rex exclusion, it may be that of λ. Toothman and Herskowitz (1980a,b) observed that heteroimmune λ hybrid phages that lack a functional *ren* gene are sensitive to exclusion by *c*I857 λ lysogens. We transformed their *c*I857 lysogen M5222 with our RexB overexpression vector, pBS rexB phoA′, and assayed exclusion of the hybrid phages, λ*imm*434 (OP)P22 and λ*imm*434 *nin*5, both of which are *ren*$^-$. The results in Table 2 show clearly that RexB overexpression prevents Rex exclusion of sensitive lambdoid phages.

Discussion

We have used the alkaline phosphatase activities of a set of RexB–PhoA′ fusion proteins to analyze the membrane topology of the RexB protein. The results support strongly our model of the membrane topology of the

Table 1. rexB–phoA′ *fusions*

Isolate	Fusion[a]	Fusion junctions amino acid and nucleotide sequence[b]
180.3B	21.2IM	Tyr Val Ile Val Ser Met *Pro Gln Gly Asp Ile* TAC CTT ATC GTA AGC ATG *CCT CAG GGC GAT ATT*
180.4B	32.3G	Arg His Tyr Ile Pro Gly *Pro Gln Gly Asp Ile* CGC CAC TAC ATT CCT GGG *CCT CAG GGC GAT ATT*
45.11B3	35.1SC	Pro Gly Val Ser Phe **Cys Leu** *Gln Gly Asp Ile* CCT GGT GGT TCT TTT TGC CTC *CAG GGC GAT ATT*
180.1B	37.2HQ	Val Ser Phe Ser Ala Gln *Pro Gln Gly Asp Ile* GTT TCT TTT TCA GCT CAG *CCT CAG GGC GAT ATT*
180.2B	38.2R	Ser Phe Ser Ala His Arg *Pro Gln Gly Asp Ile* TCT TTT TCA GCT CAT AGG *CCT CAG GGC GAT ATT*
240.5B	64.2L	Ala Ala Leu Thr Phe Leu *Pro Gln Gly Asp Ile* GCT GCC TTG ACG TTT CTG *CCT CAG GGC GAT ATT*
6.36.W6	73.1AV	Arg Thr Arg Arg Leu Val *Pro Gln Gly Asp Ile* AGA ACG CGC CGA CTG GTG *CCT CAG GGC GAT ATT*
180.1W	86.2IM	Met Thr Ser Val Val Met *Pro Gln Gly Asp Ile* ATG ACA TCG GTA GTT ATG *CCT CAG GGC GAT ATT*
9.38B	95.2L	Leu Ser Phe Val Glu Leu *Pro Gln Gly Asp Ile* CTT AGT TTT GTT GAG CTG *CCT CAG GGC GAT ATT*
10.3B	95.3L	Leu Ser Phe Val Glu Leu *Pro Gln Gly Asp Ile* CTT AGT TTT GTT GAG CTT *CCT CAG GGC GAT ATT*
10.2B	106.2L	Cys Gly Leu Leu Leu Leu *Pro Gln Gly Asp Ile* TGC GGG TTA TTG CTT CTG *CCT CAG GGC GAT ATT*
9.26B	108.2SR	Leu Leu Leu Leu Ser Arg *Pro Gln Gly Asp Ile* TTA TTG CTT CTT TCC AGG *CCT CAG GGC GAT ATT*
8.42B	127.2IM	Ala Ser Ala Ser Phe Met *Pro Gln Gly Asp Ile* GCC TCT GCA TCG TCC ATG *CCT CAG GGC GAT ATT*
7.41	128.2HQ	Ser Ala Ser Phe Ile Gln *Pro Gln Gly Asp Ile* TCT GCA TCG TTC ATT CAG *CCT CAG GGC GAT ATT*
11.41	129.2IM	Ala Ser Phe Ile His Met *Pro Gln Gly Asp Ile* GCA TCG TTC ATT CAT ATG *CCT CAG GGC GAT ATT*
6.36B	131.2IM	Phe Ile His Ile Cys Met *Pro Gln Gly Asp Ile* TTC ATT CAT ATA TGC ATG *CCT CAG GGC GAT ATT*
45.1/8	144.3E	Arg Glu Gln Glu **Ala Ser Arg Ile Arg** *Gln Gly* AGA GAA CAA GAA *GCC* **TCA CGG ATC CGT** *CAG GGC*

[a]Fusions are identified by the *rexB* codon in which the fusion occurs, the last *rexB* nucleotide within the fusion codon, the amino acid corresponding to the codon in the wild-type *rexB* gene, and the amino acid specified by the fusion codon.
[b]*rexB* sequences are in plain type; PhoA′ sequences are italicized. Nucleotides of other origin (i.e., linkers) and the corresponding amino acid sequence are in boldface type.

RexB protein (Figs. 4 and 7). Fusions to both predicted periplasmic domains display high levels of alkaline phosphatase activity. Fusions to two of three predicted cytoplasmic domains were also isolated and, as expected, show low levels of alkaline phosphatase activity. The range of activities for active fusions and the occurrence of periplasmic fusions with lower activity than fusions to transmembrane domains are comparable to results obtained with LacY–PhoA fusions (Calamia and Manoil 1990).

The gradient of alkaline phosphatase activity exhibited by fusions to the first periplasmic domain of RexB suggests that this domain contains topological determinants (Boyd et al. 1987; San Millan et al. 1989). The nature of this information is not obvious, but it does not appear to be a simple balance of charges (von Heijne 1986; Nilsson and von Heijne 1990; for review, see Boyd and Beckwith 1990). Nor is it clear whether the orientation, efficiency of transfer, or stability of the fusion proteins is affected (San Millan et al. 1989).

Fusions to transmembrane domains

Calamia and Manoil (1990) have formulated a first approximation of the rules governing the behavior of fusions to transmembrane domains and suggest that the rules are different for fusions to incoming transmembrane segments (those with a periplasmic amino end) and for those to outgoing transmembrane segments (those with a cytoplasmic amino end). This approximation is hampered by a small data base and by the uncertainty domain boundaries [many putative transmembrane domains (Boyd et al. 1987; San Millan et al. 1989; Calamia and Manoil 1990; Lloyd and Kadner 1990) are longer than necessary for merely crossing the membrane (von Heijne 1986; Crammer et al. 1990)].

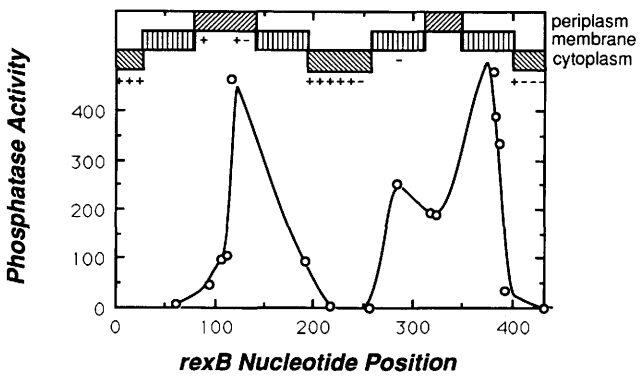

Figure 7. Alkaline phosphatase activity of RexB–PhoA′ fusions. Alkaline phosphatase activity (see Materials and methods) of RexB–PhoA′ fusions is plotted against the *rexB* nucleotide of the fusion junction. Each activity is the mean of at least three independent determinations. The mean activities of the negative controls, AW1061 pBS and AW1061 pBS rexB phoA′, were −0.1 and 2.3 units, respectively, whereas that of the positive control, AW1061 pCH40, was 600.0 units. Proposed topological domains and charged amino acid residues are shown at *top* (see Fig. 2).

The outgoing rule, based on the activities of LacY–PhoA fusions to two different outgoing transmembrane segments, is that 11, but not 8, amino acid residues of the transmembrane sequence are sufficient to translocate alkaline phosphatase to the periplasm. The outgoing fusions MalF 0–55 (Boyd et al. 1987), UphT 335 and UphT 342 (Lloyd and Kadner 1990), and RexB 86.2IM, 95.2L, and 95.3L (Table 1; Figs. 4 and 7) are consistent with this rule. Because 9–11 transmembrane residues apparently suffice for translocation to the periplasm, Calamia and Manoil (1990) speculate that the additional hydrophobic residues in a transmembrane segment "are needed for stable anchoring of the segment in the bilayer." Exceptions to the outgoing rule can be expected for membrane spanning domains whose stable topology is strongly influenced by determinants in the following periplasmic domain. The first transmembrane domain of the RexB protein is of this sort (according to the criteria of Boyd et al. 1987) and RexB fusion 21.2IM is an exception to the rule (Figs. 4 and 7).

The rule for fusions to incoming transmembrane segments is less clear, but these fusions appear to require more transmembrane residues for stable insertion. LacY–PhoA fusions with 10 or 11 residues of an incoming transmembrane segment, UhpT–PhoA fusions with 13 or fewer, and RexB fusions (127.2IM, 128.2HQ, and 129.2IM) with 12, 13, and 14 residues, respectively, exhibit high levels of alkaline phosphatase activity. Although UhpT fusions with 16 and 19 residues have low activity, incoming fusions with junctions very near the carboxyl end of the transmembrane sequence (MalF 17-1, LacY 12, RexB 64.2L, and RexB 131.2IM) often have moderately high levels of activity. The apparent difficulty in stably inserting incoming fusions may be the result of a peculiarity of alkaline phosphatase, elimination of determinants of stable topology in the transmembrane domain or in the adjacent cytoplasmic domain (MalF 17-1), or insufficiently hydrophobic hybrid domains.

Hydropathy analysis of RexB fusions, 127.1IM, 128.2HQ, and 129.2IM (Table 1), within the sequence Ile-His-Ile-Cys-Ile of the fourth transmembrane domain of RexB suggests that their hybrid domains may be insufficiently hydrophobic to act as stable transmembrane domains, which would account for their high alkaline phosphatase activity (Fig. 7). The hybrid domain of fusion 131.2IM (Table 1), which eliminates only the final Ile of the pentapeptide, is appreciably more hydrophobic, allowing it to function fairly well as a stable transmembrane sequence and explaining the approximate 10-fold reduction in its alkaline phophatase activity (Fig. 7).

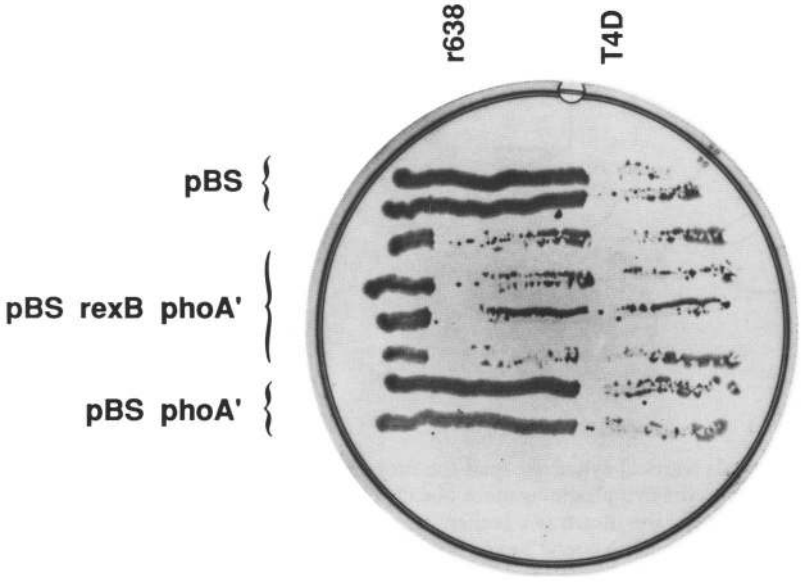

Figure 8. Prevention of Rex exclusion by overexpressing RexB. Independent, single colonies of CR63(λ) transformed with pBS, pBS phoA′, and pBS rexB phoA′ were cross streaked against the *r*II deletion, *r*638, and T4 wild type (see Materials and methods). Growth of *r*638 is restricted by the pBS and pBS phoA′ transformants (no RexB overexpression) but is permitted by pBS rexB phoA′ transformants (RexB overexpression). T4 wild type grows on all strains, as expected. The plasmid carried by each transformant is indicated to the *left* of the bacterial streaks. The genotype of the streaked phages is indicated at *top*.

Table 2. *Effect of RexB overexpression on Rex exclusion*

Plasmid expression	Efficiency of plating[a]				
	nonlysogen[b]		lysogen[c]		
	none[d]	RexB[e]	none[d]	vector[f]	RexB[e]
λ*imm*434 *c*IT6	100	92	24	88	45
λ*imm*434 *c*IT6 (OP)P22	100	76	<0.0008	<0.0008	26
λ*imm*434 *c*I60	100	82	2.5	43	28
λ*imm*434 *c*I60 *ren20*	100	71	<0.0008	<0.0008	31

[a]100 × (titer on given strain)/(titer on M72 without plasmid).
[b]Strain M72.
[c]Strain M5222.
[d]No plasmid.
[e]pBS rexB phoA′ transformant.
[f]pBS transformant.

RexB overproduction and the mechanism of exclusion

The physiology of Rex exclusion (that can be attributed to depolarization of the cytoplasmic membrane), the dependency of Rex exclusion on the RexA/RexB ratio, and the topology of the RexB protein [which resembles that of gap junction proteins (Betz 1990)] suggest a Rex exclusion model. The RexB protein forms ion channels (Unwin 1986) that depolarize the cytoplasmic membrane, causing exclusion. The channels are activated by RexA in response to a signal generated by lytic growth. In the absence of RexA protein, the channels are inactive. We imagine that the glutamic acid residue in the third transmembrane domain may function in ion translocation. Although we do not yet know how the channels are activated, the sensitivity of exclusion to the RexA/RexB ratio suggests that a RexB channel interacts with multiple RexA molecules. Formally, the effect of lytic growth of bacteriophages is equivalent to increasing the RexA/RexB ratio.

A version of this model is depicted in Figure 9; RexB proteins form closed channels (Fig. 9a), which are opened by binding RexA molecules (Fig. 9c). (Although stoichiometry is of crucial importance, it is not in question at our current level of understanding.) The prevailing conditions in a lysogen ensure that channels have at least one unfilled RexA-binding site (Fig. 9a,b). Overexpressing RexA fills all of the binding sites on some channels, opening the channels and causing exclusion (Fig. 9c). However, when RexB is overexpressed, channels remain closed because they have unfilled binding sites (even during phage lytic growth).

An implication of the model is that λ must possess at least one mechanism to avoid excluding itself during lytic growth. We suggest that the mechanism employed

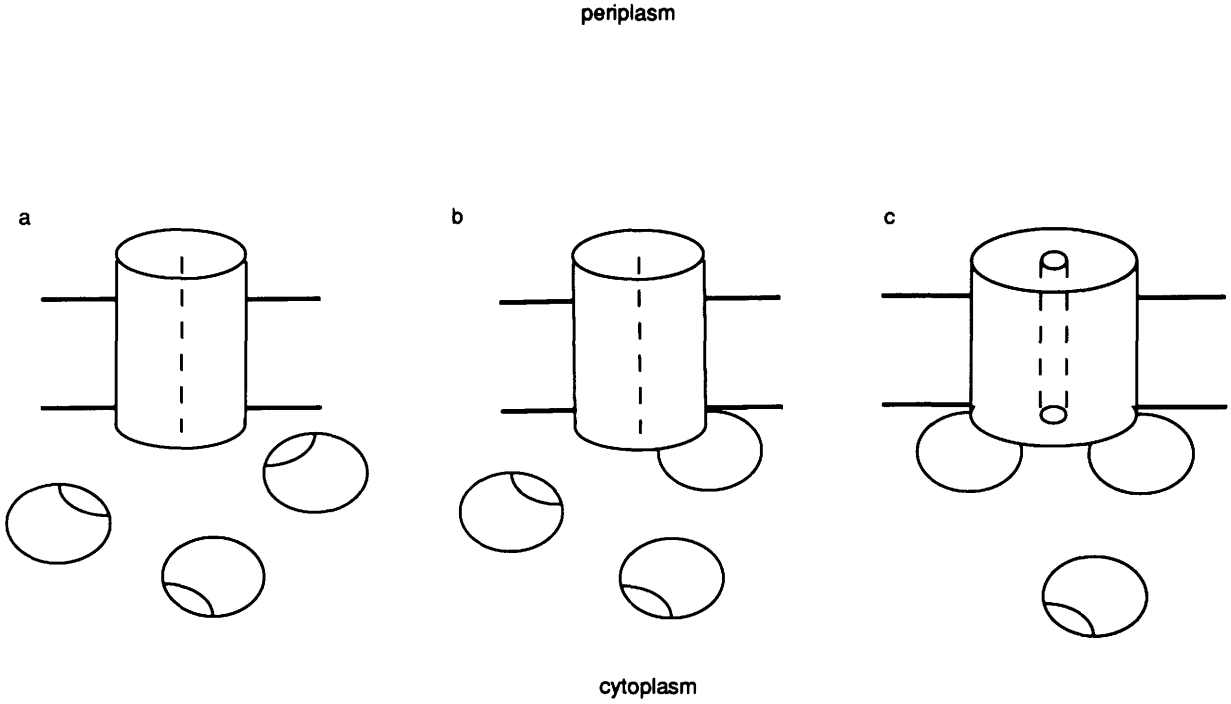

Figure 9. A channel model of Rex exclusion. RexB channels (vertical cylinders) span the cytoplasmic membrane (heavy horizontal lines). RexA molecules are ovals in the cytoplasm or bound to the cytoplasmic surface of a channel. The lumen of closed or inactive channels is depicted as a vertical broken line; for open channels, the lumen is a broken cylinder. In this representation, two bound RexA molecules are necessary to open a channel. (*a*) Closed channel, no bound RexA; (*b*) closed channel, one bound RexA molecule; (*c*) open channel, two bound RexA molecules. By hypothesis, open channels are responsible for Rex exclusion.

by an induced lysogen is based on the overexpression of the *rexB* gene, which is accomplished through LIT transcription. The model and the membrane topology of the RexB protein account for many arcane observations in the Rex literature in a simple way.

pH dependence of Rex exclusion

Rex exclusion is sensitive to extracellular pH and to the concentration of other monovalent cations (Garen 1961; Sekiguchi 1966). If the concentration of monovalent cations is low (<1 mM), Rex exclusion occurs at pH 6, but not at neutral pH (Sekiguchi 1966). The topology of the RexB protein provides a plausible basis for dependence on extracellular pH. The first periplasmic domain contains two histidine residues (residues 27 and 38), whose ionization is expected to be affected strongly in this pH range and may have significant effects on RexB conformation and function. [An alternative, that the RexB channels simply have greater selectivity for protons (μM) than for other monovalent cations (mM), is not mutually exclusive, i.e., the ionization of histidines might influence ion selectivity.]

Hydrolysis of ATP

Even though *uncA* mutations of ATP synthase prevent exclusion-associated hydrolysis of ATP, they do not prevent exclusion (Colowick and Colowick 1983). ATP synthase couples the synthesis and hydrolysis of ATP to the translocation of protons across the inner membrane. An influx of protons through ATP synthase results in the synthesis of ATP. The reverse reaction hydrolyzes ATP and pumps protons out of the cell and thereby can establish a membrane potential. The hydrolysis of ATP during Rex exclusion is the response by ATP synthase to a deenergized cytoplasmic membrane. Without an active ATPase moiety (*uncA*$^-$ mutants), such a response is not possible and ATP is conserved, but the primary defect, loss of membrane potential, is not corrected.

Self-exclusion

We expect that a λ mutant, defective only in LIT transcription, will be able to grow lytically in nonlysogens, be capable of lysogeny, be phenotypically *rex*$^+$, and will exclude itself following induction. Toothman and Herskowitz (1980a) observed that λ *ren* mutants have exactly this phenotype.

In principle, two classes of self-excluding mutations may occur: inactivators of the p_{LIT} promoter (Fig. 1), and inactivators of a gene whose product is required for LIT transcription. Both the location of the *ren* gene and its activity in *trans* (Toothman and Herskowitz 1980b; Kroger and Hobom 1982) suggest that the *ren* gene product may be an activator of LIT transcription. This idea is consistent with two common genetic strategies: physical linkage (Fig. 1) and common regulation of related functions (*O*, *P* and, by hypothesis, *ren* are required for LIT transcription) and phenotypic reversion by second-site mutation in functionally related genes (Floor 1975; *ren*$^-$ suppressors occur in *O* and *P*; Toothman and Herskowitz 1970b). Direct transcription studies on Ren-activated promoters are in progress.

Decay of exclusion following induction of a lysogen

The decay of exclusion after induction of a lysogen is expected to be dependent on the pattern of postinduction expression of the *rex* genes. Continued coordinate expression of the *rex* genes will slow the loss of exclusion, whereas LIT transcription will accelerate it. As expected, Rex exclusion decays more rapidly following induction of a lysogen (*cro*$^+$ *O*$^-$) that is incapable of both coordinate and LIT transcription than it does following induction of a lysogen (*cro*$^-$ *O*$^-$ *c*II$^-$) that is capable of coordinate but not LIT transcription (Hayes and Szybalski 1973a; Mark and Szyblski 1973; Reichardt 1975; Hayes and Hayes 1978). We expect that exclusion will decay most rapidly in an induced lysogen (*cro*$^+$ *O*$^+$) that is capable of only LIT transcription. Unfortunately, the published data for *cro*$^+$ *O*$^+$ lysogens (Mark and Szybalski 1973; Toothman and Herskowitz 1980a) are inconclusive.

However, the kinetics of LIT transcription appear to be compatible with the requirement that the capacity to exclude lambdoid phages must be lost before the onset of self-exclusion (~20 min postinduction; Toothman and Herskowitz 1980c); compared with a lysogen, *rexB* is transcribed in 2-fold excess by 10 min postinduction and in 5- to 10-fold excess by 15–20 min. The decay of Rex exclusion for lambdoid phages has not been reported.

Exclusion of heteroimmune phages

Heteroimmune lambdoid phages are *rex*$^-$ (Kaiser and Jacob 1957; Fiandt et al. 1971; Simon et al. 1971; Landsmann et al. 1982; Matz et al. 1982). We expect that a superinfecting heteroimmune phage or hybrid phage will grow lytically in a *c*I857 defective lysogen only if it can *trans*-activate LIT transcription from the prophage genome and if the *ren* gene is required for *trans*-activation.

Heteroduplex mapping (Fiandt et al. 1971; Simon et al. 1971) and DNA sequencing (Kroger and Hobom 1982; Backhaus and Petri 1984) suggest that phages 434, 21, and 82 have *ren* genes that are homologous to those of λ and that the *ren* gene of Φ80 may be altered or deleted as is that of P22. The ability of heteroimmune phages and hybrids to bypass exclusion is correlated with their *ren* gene. Those with a *ren* gene that is homologous to that of λ (434, 21, and 82) are not excluded, whereas those with a nonhomologous (P22 and Φ80) or defective λ *ren* gene (*ren*$^-$) are excluded (Toothman and Herskowitz 1980b; Landsmann et al. 1982).

Establishment of Rex exclusion

After λ lytic infection of a sensitive host, Rex exclusion of *r*II mutants is established rapidly (Astrachan and Miller 1972; Lieb and Talland 1981). Clearly, LIT tran-

scription does not occur after lytic infection at a level sufficient to bypass exclusion of heterologous phages. However, it is not clear how λ avoids self-exclusion in a lytic infection. Obvious possibilities are (1) the level of Rex proteins may be sufficient to exclude heterologous but not lambdoid phages (Toothman and Herskowitz 1980a; Matz et al. 1982), (2) LIT transcription may be sufficient to bypass exclusion of lambdoid, but not heterologous phages, or (3) the mechanism may be kinetic.

RexB and the UncI proteins

There is a remarkable similarity in the size and hydropathy profile (Fig. 10a–f), distribution of charged amino acid residues, and net charge (data not shown) between the RexB protein and the UncI (gene 1) proteins of ATP synthase of cyano- and nonphotosynthetic bacteria. [Note that the increased size of the RexB protein is due primarily to the larger size of its periplasmic domains (Fig. 6), a suggestive result in view of the sensitivity of Rex exclusion to extracellular monovalent cation concentrations (Sekiguchi 1966).]

Although there is only limited sequence identity among UncI proteins (*Bacillus megaterium* and PS3 are 58.3%, and *Synechococcus* PCC 6301 and *Anabaena* PCC 7129 are 56.0% identical; the extent of identity between these two pairs and with the *E. coli* UncI protein is not significant), it has been suggested that the similarities in physical properties reflect a similarity in structure (Falk and Walker 1988). The similarity may be reasonably expected to extend to function, especially because all of the *uncI* genes have genetically homologous locations.

The striking resemblance of the RexB and UncI proteins suggests that the similarity in structure and perhaps function may extend to RexB. Both Rex exclusion (Sekiguchi 1966) and overexpression of the *E. coli uncI* gene (Schneppe et al. 1990) can be interpreted as uncoupling oxidative phosphorylation.

Rex exclusion: altruistic cell death

Rex exclusion is an example of alturistic cell death; aborting lytic growth may kill the infected cell, but it prevents spreading the infection. The data suggest that lambdoid and unrelated phages present a similar signal during lytic growth. Lambda apparently avoids self-exclusion after induction by overexpressing RexB through LIT transcription. This mechanism (controlling a phenotype by regulating the ratio of components of a structural complex) is novel. Heteroimmune phages may use a similar mechanism by activating LIT transcription from the λ prophage. Many phages have evolved mechanisms to counter exclusion, but it seems unlikely that overexpression of RexB is the mechanism employed by heterologous phages.

Although the large lytic phages can counter the Rex system in the laboratory, overproduction of the Rex proteins at their lysogen ratio causes restriction of many wild-type viruses (such as T4). It seems possible, or even likely, that in the wild, λ lysogens are better able to restrict other viruses than has been imagined previously. Both the common signal that calls for altruistic cell

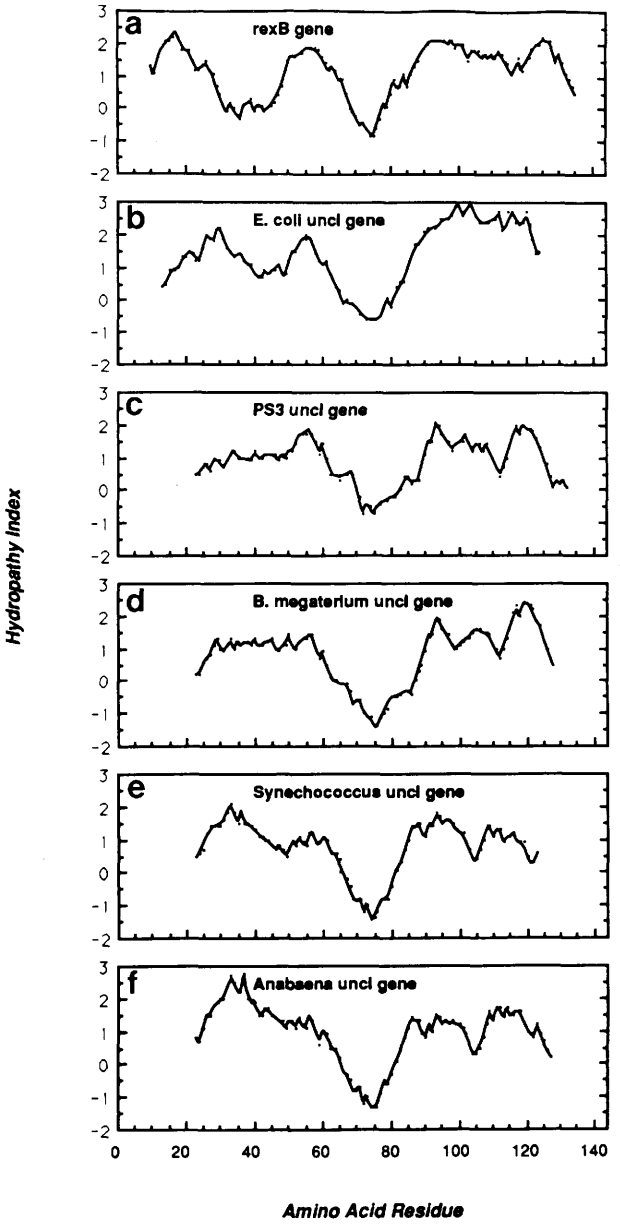

Figure 10. Hydropathy profiles of RexB and UncI proteins. Hydropathy profiles of the RexB and UncI proteins were calculated as described in the legend to Fig. 2. The profiles are aligned at the minimum value of the central hydrophilic domain. (*a*) The RexB protein of bacteriophage λ; UncI protein from *b*, *E. coli* (Gay and Walker 1981); (*c*) thermophilic bacterium PS3 (Ohta et al. 1988); (*d*) *B. megaterium* (Brusilow et al. 1989); (*e*) cyanobacterium *Synechococcus* 6301 (Cozens and Walker 1987); (*f*) cyanobacterium *Anabaena* PCC 7120 (McCarn et al. 1988). Note that the hydropathy profile and charge distribution of the UncI protein of the purple nonsulfur photobacterium *Rhodospirillum rubrum* is radically different (Falk and Walker 1988).

death and the lytic phage components that temper Rex restriction require further investigation.

Materials and methods

Bacteria, phages, and plasmids

NAPIV and NAPIV/prex20 (Shinedling et al. 1987) were the host strains for physiological studies on exclusion of T4 wild type. The *phoA* deletion strain AW1061 (Hoffman and Wright 1985) was used for blue/white, X-phosphate colony screening and for quantitative alkaline phosphatase assays of *rexB–phoA′* fusions. BL21(DE3) (Studier et al. 1990) was used in protein-labeling experiments. JM103 was utilized for blue/white, X-gal colony screening. M72 is a nonlysogen. M5222 is a defective *c*I857 lysogen derived from M72 (Toothman and Herskowitz 1980a). CR63(λ) is a su^+ λ lysogen.

T4 wild-type phage and the *r*IIB deletion, *r*638, are from the Gold laboratory collection. The lambdoid hybrid phages λ*imm*434 *c*IT6, λ*imm*434 *c*IT6 (OP) P22, λ*imm*434 *c*I60, and λ*imm*434 *c*I60 *ren20* carry the immunity region of phage 434 (Toothman and Herskowitz 1980a,b).

The plasmid prex20 is pACYC184, with the *rex* genes inserted at the unique *Bam*HI site and transcribed from the *tet* promoter (Shinedling et al. 1987). The parent plasmid of *rexB–phoA′* fusions, pBS rexB phoA′, was constructed from Bluescript (SK+) (Strategene). To create pBS phoA′, the *Pst–Xho*I segment of the pBS polylinker was replaced with the *Pst*I–*Xho*I *phoA′* cassette containing fragment of pCH40 (Hoffman and Wright 1985). The *rexB*-containing *Eag*I–*Sma*I fragment of pTQ7R (Snyder and McWilliams 1989) was then inserted at the *Eag*I–*Sma*I sites of the polylinker (Fig. 6a) to make pBS rexB phoA′.

To identify the RexB protein, we replaced the natural ribosome-binding site of RexB with an efficient synthetic one (SD8) (Gold and Stormo 1990) and eliminated the promoter, p_{LIT} (Hayes and Szybalski 1973; Landsmann et al. 1982), which is located in the carboxyl terminus of the RexA-coding sequence. A polymerase chain reaction (PCR)-amplified DNA molecule corresponding to coding nucleotides 1–392 of the *rexB* gene was synthesized with upstream *Eag*I, *Sal*I, and SD8 ribosome-binding sites by using an amino-terminal PCR primer 5′-AAA-AACGGCCGTCCGACTAAACTAAACTAAGGAAGGAAA-AAAAAA TGCCGGAACAGAATCATGCCTGG-3′ and a carboxy-terminal primer 3′-GTAGCAAAAGTTAAAGGTTA-ATATACGTAACCTTAGGAAAAAA-5′. The synthetic DNA was digested with *Eag*I and *Nde*I and inserted into the unique *Eag*I and *Nde*I sites of pBS rexB phoA′ to produce pBS SD8 rexB phoA′. This plasmid was digested with *Sal*I and *Sma*I, and the band-purified *Sal*I–*Sma*I fragment containing SD8 *rexB* was then subcloned into pDIP19D *Bgl*II$^-$, producing pDIP19D *Bgl*II$^-$ SD8 *rexB* (Fig. 6b). pDIP19D *Bgl*II$^-$ was made by cutting, filling, and blunt-end-ligating the unique *Bgl*II site of pDIP19D (Singer and Gold 1991). The resulting reading frameshift eliminates a 151-amino-acid peptide that is produced by a downstream open reading frame in pDIP19D and that is nearly the same size as the RexB protein (144 amino acid residues). In this construct, *rexB* is transcribed from the T7 gene 10 promoter. Cloning procedures were adapted from Maniatis et al. (1982) and Sambrook et al. (1989).

Chemicals, media, and plating conditions

The chromogenic substrate 5-bromo-4-chloro-3-indolyl-phosphate (X-phosphate) was used to detect colonies producing active alkaline phosphatase, whereas ρ-nitrophenylphosphate (Sigma 104) was used for quantitative assays of alkaline phosphatase activity (Brickman and Beckwith 1975). 5-Bromo-4-chloro-3-indoly-β-D-galatopyranoside (X-gal) was prepared and used as described by Sambrook et al. (1989).

EHA top and bottom agar (Steinberg and Edgar 1962) was used for plating bacteriophage T4, whereas H broth and M9 media were used for liquid cultures (Nelson et al. 1982). For assays of bacteriophage λ, bacteria were grown in L broth plus 0.2% maltose with vigorous aeration at 30°C. Lambda phages (0.1 ml) in MS (Maniatis et al. 1982) were mixed with 0.1–0.2 ml of bacteria at room temperature for 20 min, plated in 2.5 ml of λ top agar on λ plates, and incubated overnight at 30°C.

Cross streaking

Parallel streaks of *r*638 and T4 wild type (at 10^8–10^9/ml) were made on EHA/ampicillin plates with a 0.2-ml pipette. After the phage streaks dried, single colonies or liquid cultures of the desired bacteria were streaked across the phage (first *r*638, then T4 wild type) with a sterile loop. Cross-streaked plates were incubated overnight at 33°C. Lysis at the intersection of bacterial and phage streaks indicates that the bacterium permits phage growth. Bacterial growth at the intersection indicates that phage growth is restricted. Lysogens are expected to restrict *r*638, but T4 wild type is expected to grow on both lysogens and on nonlysogens.

Radioactive labeling

Cultures of NAPIV and NAPIV/prex20 growing exponentially in M9 (0.02% CAA, 1 μg/ml of B1, 10 μg/ml of tryptophan) at 30°C (2×10^8 cells/ml) were infected with T4 wild type or *rim2* phages at a m.o.i. of 10. Ninety-microliter aliquots of the infected cultures were pulse-labeled for 1 min with aeration by transferring to a 10-ml culture tube containing 10 μl of radioactive label, prewarmed to 30°C. RNA was labeled with 2 μCi of [5-^{3}H]uridine, DNA with 1 μCi of [^{3}H]thymidine, and proteins with 1 μCi of ^{14}C-labeled mixed amino acids. Pulses were terminated by the addition of 5 ml of ice-cold 5% TCA and placed on ice. Samples were filtered on GF/C filters and rinsed with 15 ml of 5% TCA and 5–10 ml of 95% ETOH.

TMG transport

Assays for TMG were derived from Maloney et al. (1975). Cultures of NAPIV and NAPIV/prex20 growing exponentially in M9 glycerol (0.4% glycerol, 0.02% CAA, 1 μg/ml of B1, 1 mM IPTG at pH 7.5) at 30°C (OD_{600} = 0.3) were chilled and centrifuged (5 min at 6000 rpm). The pelleted cells were washed once with an equal volume of M9 buffer. The final pellet was resuspended in M9 glycerol on ice, without IPTG at OD_{600} = 0.4. The cells were warmed to 30°C with aeration and infected with the desired phage at a m.o.i. of 7–10. At 1 min postinfection, 40× ^{14}C-labeled TMG (16 μCi/ml, 16 mM TMG; 1/40 the volume of infected cells) was added. Samples (500 μl) were withdrawn periodically, filtered rapidly on nitrocellulose filters, washed with 5 ml of M9 buffer at room temperature, dried, and counted.

Overexpressing and labeling RexB protein

BL21 (DE3) pDIP19D *Bgl*II$^-$ SD8 *rexB* was grown aerobically at 37°C in M9 plus 0.02% casamino acids to OD_{600} of 0.5. The culture was divided and one-half was induced by adding IPTG to a final concentration of 1 mM. Ten milliliters of induced and

uninduced cultures was pulse-labeled with ^{14}C-labeled amino acids (1 μCi) for 2 min, from 11 to 13 min after induction. Pulses were terminated by adding 1 ml of 5% ice-cold casamino acids and placing the cultures on ice.

Spheroplasting, osmotic shock, and fractionation

The procedure was adapted from Koshland and Botstein (1980). Ten milliliters of labeled cells was centrifuged for 5 min at 6000 rpm. The pellet was resuspended in 1 ml of 0.1 M Tris-HCl (pH 8), transferred to an Eppendorf tube, and microcentrifuged for 2 min. The cells were washed two additional times. The final wash pellet was resuspended in 500 μl of 0.1 M Tris-HCl (pH 8), 0.5 mM EDTA, 0.5 M sucrose; 50 μl of 2 mg/ml of lysozyme and 500 μl of ice-cold water were added sequentially. The cells were then incubated at room temperature for 25 min. Spheroplasting was monitored by phase-contrast microscopy and was >99% complete. Spheroplasts were pelleted by microcentrifuging for 5 min. The supernatant was removed and the cells were lysed by resuspending the pellet vigorously in 1 ml of ice-cold water. Any unlysed cells were removed by microcentrifuging for 10 min at 1000*g*. The supernatant was removed to a new Eppendorf tube, an aliqot was removed for the unfractionated control, and membranes were pelleted by microcentrifuging for 30 min. The supernatant was removed and saved as the soluble fraction. The pellet was resuspended in 1 ml of ice-cold water and remicrocentrifuged. The supernatant was removed and saved as the wash fraction, and the pellet was resuspended in 250 μl of ice-cold water for the membrane fraction.

Volumes of each fraction, representing cell equivalents, were analyzed by SDS-PAGE. Sample preparation, electrophoresis, staining, and autoradiography were as described by O'Farrell and Gold (1973), except that separating gels were 12.5% acrylamide.

Construction of rexB–phoA′ *fusions*

Fusions of the *phoA* cassette, derived from pCH40 (Hoffman and Wright 1985), to random nucleotides in the *rexB* gene were generated by a modification of the exonuclease III mung bean nuclease protocol of Steggles (1989): 15 μl of pBS rexB phoA′ DNA (digested previously with *Pst*I and *Sma*I; Fig. 6), 4 μl of 10× exonuclease buffer, and 17 μl of water were mixed on ice and warmed to 30°C, and 4 μl (400 units) of exonuclease III was added (time zero). At 15, 30, 45, 60, 90, 180, and 240 sec, 5-μl aliquots were removed to 14 μl of 1.4× mung bean buffer, frozen in dry ice/ethanol, heated to 68°C for 15 min and, finally, cooled to 30°C. Mung bean nuclease, 1 μl (8 units) was added to each sample. The samples were incubated for 30 min at 30°C, precipitated with 95% ethanol, washed once with 70% ethanol, dried, and resuspended in 8 μl of water at 30°C; 1 μl of 10× Klenow mix (Steggles 1989) was added, and the mix was incubated for 4 min without added dNTPs. The incubation was continued for another 5 min after adding 1 μl of 0.6 mM dNTPs. Each sample was added to 40 μl of 1.25× ligase buffer and 1 μl (40 units) of T4 DNA ligase. After overnight incubation at 16°C, aliquots of competent AW1061 were transformed with 10 μl of each sample. Transformants were selected on ampicillin, X-phosphate plates.

The in-frame fusion 45.1/8 was derived from the out-of-frame fusion 45.1 (Table 1) by cutting the unique *Bsu*36I site, which is located within the amino terminus of phoA′, inserting and ligating the linker 5′-TCACGGTCCG-3′, which contains a *Bam*HI site and destroys the *Bsu*36I site, redigesting with *Bsu*36I, and transforming AW1061. Two blue-colony-forming transformants were picked, and their DNAs were sequenced. Both contained multiple copies of the linker. To obtain fusions with a single copy of the linker, they were digested with *Bam*HI and religated, and the DNA of a single blue colony transformant of each was sequenced.

The out-of-frame fusion 45.11W was converted to an in-frame fusion, 45.11 B (Table 1), by digesting with *Bsu*36I and filling the A and (G/C) sites of the overhang by incubating with the large Klenow fragment and 0.125 mM dTTP, dCTP, and dGTP, removing the T of the overhang with mung bean nuclease, religating, and sequencing the DNA of a blue-colony-forming transformant of AW1061.

The in-frame fusion 6.36B (Table 1) was converted to an inactive in-frame fusion, 6.36W6, by cutting the unique *Bal*I and *Nsi*I sites, band-purifying the doubly digested DNA, removing the 3′ overhang of the *Nsi*I site by digestion with T4 DNA polymerase in the presence of 2 mM dNTPs, ligating, and transforming AW1061. The DNAs of eight white transformants were digested with *Sty*I and analyzed by agarose gel electrophoresis. The restriction fragments of one, 6.36W6, were of the expected size and number for a *Bal*I–*Nsi*I blunt-end fusion, which was confirmed by DNA sequencing.

Alkaline phosphatase assays

Alkaline phosphatase assays were derived from Brickman and Beckwith (1975) and Hoffman and Wright (1985). Fresh overnight cultures in L broth plus 100 μg/ml of ampicillin (15 μg/ml of tetracycline for AW1061/pCH40) were diluted 50-fold in fresh media without IPTG and aerated at 37°C to OD_{600} = 0.3. The cultures were iced, a 1.4-ml sample of each was microcentrifuged for 30 sec, the pellet resuspended in 1.4 ml of Tris-HCl (pH 8.0), and 250-μl aliquots were distributed in quadruplicate to the wells of a 96-well microtiter plate. Twenty-five microliters of *p*-nitrophenylphosphate (0.4% in 1 M Tris-HCl at pH 8.0) was added to each well with a multichannel pipetter to start the assay. Measurements were made at room temperature using a Titertek Multiscan MC. The phosphatase activity was calculated from the equation Activity = $1200 \times ([OD_{414}/\text{time (min)}]/OD_{620})$.

For a given fusion on a given day, the S.D. of the quadruplicate determinations was <10% of the mean activity. The data reported in Figure 5 are the means of at least three independent assays and have not been corrected for background activity of the negative controls. For mean activities of 7 or greater, the S.E.M. is <25% of the mean.

Sequencing

The location of *rexB–phoA′* fusion junctions was determined by dideoxy sequencing (Gauss et al. 1987) using a primer, phoA410 (5′-CACCCGTTAAACGGCGAGCAC-3′), whose 3′ end anneals 28 bp downstream from the unique *Pst*I restriction site at the amino end of the *phoA′* cassette and forms the *phoA′* half of the fusion junctions.

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