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Properties of Polylysogens Containing Derepressed λN^- Prophages

III. A Large Number of Intergrated λ Prophages

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Stable λN^-cI^- lysogens contain 20–25 copies of the λ genome per bacterial genome. Less than 10% of the λ genomes occur as closed circular plasmids. The remaining λ genomes can not be separated from the bacterial DNA by centrifugation in neutral sucrose. We suggest that the bacterial chromosome contains several tandemly integrated prophages which replicate at a higher rate than the remainder of the chromosome.

INTRODUCTION

The prophage state of the temperate bacteriophage λ in a lysogen is maintained by action of a repressor, the product of the *c*I gene (Kaiser, 1957). The product of gene *N* has an important role in both lysogenization and lytic development (for review, see Dove, 1968). Most of the bacteria infected with λN^- mutants become carriers in which λ genomes replicate as plasmids, but are eventually lost from the culture (Signer, 1969; Lieb, 1970). However, stable lysogens can be obtained using high multiplicities of infection (Brooks, 1965). Phages having mutations in both the *N* gene and the *c*I gene can also become established as prophages in stable lysogens (Lieb, 1971). These lysogens exhibit the converted phenotype which was previously described in the case of derepressed pm$^-$ (λ*Nsus53 cIts*A) lysogens (Lieb, 1967). The converted lysogens carrying λN^- cI^- prophage contain about 20–25 copies of the λ genome per bacterial genome (Lieb, 1972b). It was not known whether all of these λ genomes or only a fraction are integrated. We now report the results of biophysical studies to answer this question.

MATERIALS AND METHODS

Materials

Bacterial stocks	Alternate designation	Reference
C600	*pm*$^+$	Cross and Lieb, 1970
594	*pm*$^-$	Cross and Lieb, 1970
B583 [F′450]		Friefelder *et al*, 1971
pm$^-$ (λ*Nsus7sus53 c*I60)	*pm*$^-$ (λN^-cI^-)	Lieb, 1972
W3101 *rec* A13 *tryp*$^-$	recA$^-$	from N. Franklin
Phage stocks		
*imm*434cI^-		from W. Dove
*imm*λ *int*102 *red*3 *Nsus7 cIts2*		prepared here
*imm*λ *Nsus7sus53 cIsus6*	λN^-*cIsus*	prepared here

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Media and solutions. The formula for tryptone broth (TB) and tryptone broth with maltose (TBM) are in Lieb (1970). Media for assay of phage are described in Cross and Lieb (1970) and Lieb (1970). *K buffer*: 0.001% Gelatin; 0.74% $Na_2HPO_4 \cdot 12\ H_2O$; 0.15% KH_2PO_4; 0.4% NaCl; 0.5% K_2SO_4; 0.001 *M* $MgSO_4$; 0.0001 *M* $CaCl_2$. *TES buffer*: 0.05 *M* Tris-HCl, pH 8.0; 0.005 *M* EDTA; 0.05 *M* NaCl. *SSC*: 0.15 *M* NaCl; 0.015 *M* sodium citrate.

Methods

Multiple carrier lysogens. The *rec*A$^-$ strain was lysogenized with λ*int*$^-$ *red*$^-$ *Nsus*7 c*Its*2 using *imm*434c*I*$^-$ as helper phage. The *rec*$^-$ (λ*int*$^-$ *red*$^-$ *N*$^-$ c*Its*) lysogen was superinfected for 15 min at 25°C with λ*int*$^-$ *red*$^-$ *N*$^-$ c*Isus* phage at a multiplicity of 15. The superinfected lysogen was diluted 1:100 in TB, heated for 60 min at 45°C, and plated. The derepressed prophage (which cannot be excised) kills all lysogens except those containing a large number of *N*$^-$ c*Isus* genomes (Lieb, unpublished). Bacteria in converted colonies among the surviving bacteria contain the genetic markers of both the original (derepressed) prophage and the superinfecting λ. These lysogens are referred to as "stable carrier lysogens" in the text.

λ *labeled with* ^{14}C. About 1×10^5 phage particles and 5×10^7 plating bacteria were added to 2 ml of soft agar containing 12 μCi of [^{14}C]thymidine (50 μCi/mmole) and poured on a tryptone agar plate. After phage plaques became confluent, 3 ml of broth was added. The phage in the eluate was then precipitated at 12,000 rpm, washed once with K buffer, and finally resuspended in K buffer.

Preparation of labeled cultures. Bacteria were grown in TB to about 10^8 cells/ml the day before labeling and stored at 4°C. The next day, the culture was diluted 1:5 or 1:10 in TB containing 250 μg/ml of uridine and the labeled thymidine (either ^{3}H or ^{14}C) at the desired concentration. To grow B583 [F'450], 0.01% yeast extract was included in the TB. The diluted culture was then grown at 34°C with forced aeration. At a bacterial concentration of between 5×10^7 and 2×10^8/ml, the culture was chilled in ice. All the subsequent operations were done at 0–4°C unless otherwise stated.

Collection of gradient fractions. After gradient centrifugation, fractions were collected from the bottom of the tube by inserting a capillary micropipette from the top and pumping out the liquid fractions by means of a polystaltic pump. Whenever necessary, fractions were stored at −20°C before counting.

Determination of radioactive counts. Equal aliquots from fractions were precipitated in the presence of 20 μg carrier DNA with 10% trichloroacetic acid (TCA) at 0°C. The precipitates were then collected on Millipore filters and washed with 5% TCA, dried, and counted in 5 ml Omniflour–toluene scintillation fluid.

DNA–DNA hybridization. For the hybridization of DNA from gradient fractions, the fractions were incubated overnight at room temperature in the presence of 0.1–0.2 *N* NaOH, followed by neutralization of excess alkali with diluted HCl. The neutralized solution was then dialyzed against a large volume of 0.01 × SSC containing 0.001 *M* EDTA in the cold, with two changes over a period of 20 hr. The hybridization was done according to the method of Denhardt (1966). A description of the details of the procedure may be found elsewhere (Lieb, 1970). In each determination, less than 0.25 μg of labeled DNA was hybridized to 2.5 μg λDNA fixed to a filter (Schleicher and Schuell, type B-6). The (control) hybridization of λ DNA to λ DNA on filters was used to correct experimental data in each experiment. Hybridization in controls varied from about 60 to 70% in independent experiments.

RESULTS

There are three possible configurations for the large number of λ copies in a converted lysogen.

(I) All λ genomes are present in the plasmid state as nonintegrated cytoplasmic elements. The fact that both the λ*att* site and the *int* gene are required for the formation of converted lysogens argues against this hypothesis (Lieb, 1971).

(II) All λ genomes are integrated into the host chromosome.

(III) Some λ genomes are integrated and the rest are plasmids.

Alkaline Sucrose Gradients

When λ superinfects an immune λ lysogen, the superinfecting genomes remain in the cytoplasm as plasmids (Bode and Kaiser, 1965). A large fraction of plasmid DNA is present as covalently closed circles, which can be separated from the bulk of the chromosomal DNA by alkaline sucrose sedimentation (Freifelder *et al.*, 1971).

To show that we could detect λ plasmid DNA in the converted lysogen, we superinfected an [^{3}H]thymidine-labeled converted lysogen with [^{14}C]thymidine-labeled $\lambda N^- cI^-$ phage and centrifuged a lysate of the superinfected culture on alkaline sucrose as described in Fig. 1. About 35% of the ^{14}C radioactivity of the superinfecting λ DNA was recovered in the faster-sedimenting component, but under the same conditions, no peak of ^{3}H label could be detected in this region. Thus, it is evident that little or no closed circular DNA of a size comparable to λ DNA exists in the converted lysogen. It is possible that λ genomes exist as very large plasmids, i.e., covalent circles of polymeric λ DNA. A large plasmid might have been pelleted during the longer time of sedimentation. So the alkaline sucrose sedimentation was done for a shorter time, using as a control a strain carrying plasmids of a size larger than λ. In B583 [F′450], the plasmid DNA is 3 times as large as λ DNA (Freifelder *et al.*, 1971). B583 [F′450] was labeled with [^{14}C]thymidine, pm^- ($\lambda N^- cI^-$) was labeled with [^{3}H]thymidine, and the two were mixed before lysis. From Fig. 2, it can be seen that about 1.8% of the total [^{14}C]DNA was recovered in fast-sedimenting peak II. Peak III, which constitutes about 1% of total DNA, may represent a dimeric form of the F′ plasmid. The converted lysogen gave a very small ^{3}H peak at the same position as peak III of B583 [F′450]. Even if this peak represents λ DNA, the amount is very small in comparison with the amount of λ DNA present in the converted lysogen.

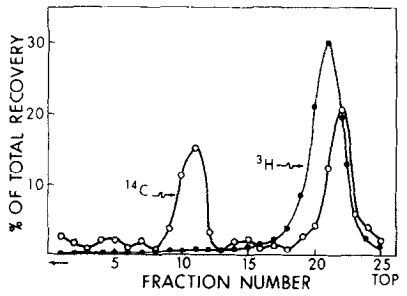

FIG. 1. Alkaline sucrose sedimentation of pm^-($\lambda N^- cI^-$) superinfected with $\lambda N^- cI^-$ phage. A culture of pm^-($\lambda N^- cI^-$) was grown and labeled in the presence of 20 μCi/ml of [^{3}H]thymidine. Thirteen milliliters of labeled cells (5.7×10^7/ml), in which the radioactivity was diluted by adding 2 μg/ml of cold thymidine, was then infected with ^{14}C-labeled $\lambda N^- cI^-$ phage at a multiplicity of 420. After allowing the phage to adsorb for 10 min at 36°C, the culture was aerated for 20 min at the same temperature, followed by chilling in ice. The chilled cells were then vortexed for 30 sec, centrifuged, and the pellet washed with 12 ml of buffer containing 0.01 *M* KH_2PO_4, 0.001 *M* $MgSO_4$, 0.0001 *M* $CaCl_2$, and 0.1 *M* NaCl, pH 7.0. Twenty per cent of the phages remained in the supernatant. The cells were suspended in 0.5 ml lysis buffer containing 0.05 *M* NaCl, 0.005 *M* EDTA, and 0.02 *M* Tris-HCl, pH 9.1. Cell suspension (0.1 ml) was put in a 0.5 dram shell vial (12 x 35 mm), 10 μl Antifoam (Corning, 1:10 dil.) added, and 50 μl of alkaline sodium dodecyl sulfate (1% in 0.8 *M* NaOH) added over a period of 1 min with slow stirring. The lysate was then sheared by vortexing for 30 sec at maximum speed. The lysate was centrifuged on alkaline sucrose according to the method of Freifelder *et al.* (1971). Total cpm recovered after centrifugation: ^{3}H, 2.96×10^6; ^{14}C, 650.

CsCl–Ethidium Bromide Gradients

The failure to detect λ plasmids in polylysogens by the alkaline sucrose sedimentation procedure led us to use CsCl–ethidium bromide equilibrium density gradient centrifugation. Using the procedures of Bazaral and Helinski (1968), and Clewell and Helinski (1969), we did not detect any heavier peak of (circular) DNA after centrifuging the DNA from the converted lysogen. We considered the possibility that the λ plasmids, if they exist in this lysogen, might be nicked during the lysis of the cell. Clark and Walker (1972) used ethidium bromide to protect the mitochondrial circular DNA in yeast from being nicked during the

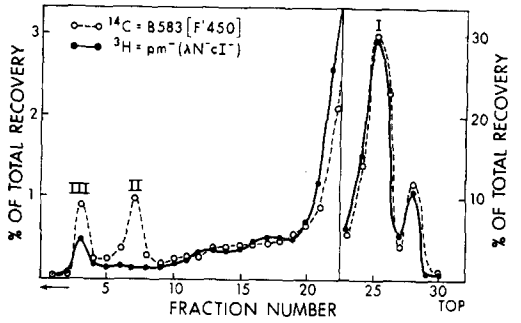

FIG. 2. Alkaline sucrose sedimentation of pm^{-}(λN^{-}cI^{-}) and B583 [F'450]. pm^{-}(λN^{-}cI^{-}) cells were labeled in the presence of 50 μCi/ml of [^{3}H]-thymidine, and B583 [F'450] cells were labeled in the presence of 10 μCi/ml of [^{14}C]thymidine (see Methods). Labeled cells (about 2.72 × 10^{8} and 8.0 × 10^{8}, respectively) were separately centrifuged and washed and resuspended as described for Fig. 1. Each cell suspension (0.1 ml) was mixed and lysed with alkaline SDS, sheared and centrifuged, according to the procedure of Freifelder *et al.*, (1971). The volume of the gradient was 11 ml with a cushion of 0.5 ml "Angioconray" (Mallinckrodt Pharmaceuticals). Total cpm recovered after centrifugation: ^{3}H, 3.25 × 10^{6}; ^{14}C, 3.7 × 10^{4}.

preparation of the mitochondrial fraction. So we included 1 mg of ethidium bromide per ml of cell suspension during the spheroplast formation; subsequent lysis and CsCl–ethidium bromide equilibrium centrifugation was done as described in *Methods*. The results are depicted in Fig. 3a. With this modified procedure, we recovered 3.2% of the total radioactivity in the heavier peak. The heavier peak from the converted lysogen is not so sharp as that from B583 (F'450]; in Fig. 3b, the heavier peak represents about 4.6% of the total DNA. The results in Table 1 show that the percent of DNA from converted lysogens or B583 [F'450] that is recovered in the heavier peak may be affected by the concentration of dye used during spheroplast formation, but that we consistently detected less circular DNA in the converted lysogen than in the F' strain. Assuming that F'450 DNA constitutes between 2 and 4% of the total cellular DNA (Freifelder *et al.*, 1971), it can be concluded that by our modified lysis procedure, we recovered 80–100% of F'450, assuming all the radioactivity recovered in the heavier peak with B583 [F'450] to be contributed by this DNA species only. Thus, we conclude that covalently closed circular DNA in 594 (λN^{-} cI^{-}) represents only 3–3.6% of total cellular DNA. But the hybridization data in Table 1 show that the DNA in the peak fraction of the heavy peak from the converted lysogen is not 100% λ DNA. If we correct for non-λ DNA in the heavy peak, we get a maximum recovery of about 2% of the DNA as λ circles. If all of the λ DNA were in plasmids, about 25% of the counts would be in the heavy peak.

Neutral Sucrose Gradients

From the results of CsCl–ethidium bromide density gradient centrifugation, it is apparent that most of the λ genomes in this converted lysogen are not present in the form of covalently closed circular DNA. The question arises as to whether these λ genomes are present as extrachromosomal nicked circles that become linear during our extraction or sedimentation procedures. In this case, we would not get any separation of λ DNA from the bulk of the cellular DNA by either of the procedures used above. For any such nicked DNA pieces to be detected and separated from the chromosomal DNA, the bacterial chromosome must remain as intact as possible during the experiment. Petes and Fangman (1972) have described a method for the study of sedimentation properties of intact chromosomal DNA of yeast. In their procedure, they first converted the yeast cells into spheroplasts and then lyzed the spheroplasts directly on the top of a neutral sucrose gradient containing 1% sarcosyl, so that the DNA released after lysis was not subjected to any shear. Instead of 1% sarcosyl, we used 1% sodium dodecyl sulphate and 0.4% deoxycholate for better lysis and to have more effective dissociation of the bacterial DNA from the membrane.

In the conditions described in Fig. 4, species of DNA sediment according to their molecular size. So if the λ genomes in the converted lysogen are present in the non-integrated state, they should be resolved

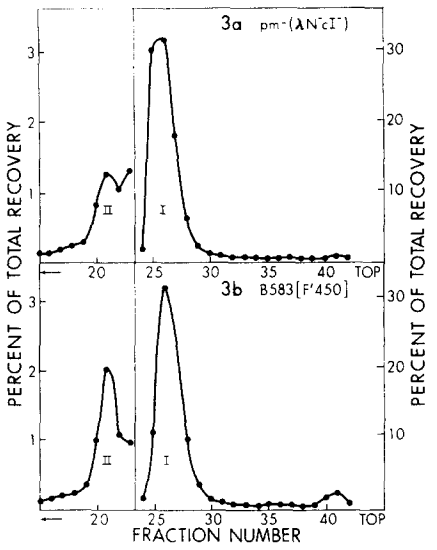

FIG. 3. CsCl-ethidium bromide buoyant density centrifugation. Both pm⁻(λ*N*⁻*c*I⁻) and B583 (F'450] were grown and labeled in the presence of 50 μCi/ml [³H]thymidine by the procedures described in Methods. Labeled cells (about 4.0×10^8 and 7.4×10^8, respectively) were separately centrifuged, and the pellets were washed once with TES buffer and then suspended in 0.9 ml TES buffer containing 25% sucrose. To 0.4 ml of each cell suspension was added 0.1 ml of a mixture containing 5 mg/ml of lysozyme and 5 mg/ml of ethidium bromide in TES buffer. The cell–lysozyme mixture was then kept at 0°C for 30 min, 0.5 ml of 2% sarcosyl (NL-30) was added and mixed, and the mixture was kept at room temperature for 5 min. After the addition of 1 ml of TES buffer, the mixture was passed through a 1-ml pipette 10 times at the approximate rate of 5 ml/min.

Cesium chloride (10.596 g) was dissolved in a mixture of 5.2 ml distilled water and 4 ml of an ethidium bromide solution containing 700 μg/ml of the dye in 0.1 *M* sodium phosphate buffer, pH 7.0. Then 1.8 ml lysate was added, and the liquids were mixed by gentle inversion. Twelve milliliters from this mixture was centrifuged for 48 hr at 40,000 rpm in a 50 TI rotor in the Spinco L ultracentrifuge at 13°C. Input (cpm) in the gradient was approximately 5.28×10^8 for pm⁻·(λ*N*⁻*c*I⁻) and 4.15×10^6 for B583 [F'450]. All the operations up to collection of fractions were done in dim light. Fractions 1–14 are intentionally omitted from the figure, since these fractions altogether constitute less than 1% of the total cpm recovered.

TABLE 1

DETECTION OF CIRCULAR DNA IN CsCl–ETHIDIUM BROMIDE GRADIENTS[a]

Expt	Lysogen	EtBr concn during lysis (mg/ml)	% of total ³H in heavy peak	% of ³H hybridizing to λ[b]
1	B583 [F'450]	0.5	2.9	—
	pm⁻ (λ*N*⁻*c*I⁻)	0.5	1.65	79
2	B583 [F'450]	0.5	3.6	—
	pm⁻ (λ*N*⁻*c*I⁻)	0.5	1.65	60
3	B583 [F'450]	1.0	4.6	—
	pm⁻ (λ*N*⁻*c*I⁻)	1.0	3.2	—
4	pm⁻ (λ*N*⁻ *c*I*ts*2)[c]	1.0	3.6	58

[a] All the procedures were done as in Fig. 3.

[b] Only the peak fraction of the heavy peak was hybridized, using the procedure described in Methods.

[c] This converted lysogen also contains about 25 copies of λ genomes per copy of bacterial genome (Lieb, 1972).

from the host chromosomal DNA. As shown in Fig. 4a, about 89% of the total ³H radioactivity was recovered in the unique peak (Fractions 3–9), while the added ¹⁴C-labeled λ DNA remained near the top (Fraction 21) of the gradient. But with ³H-labeled DNA from the stable carrier lysogen which carries λ plasmids (see *Methods*), three peaks were observed (Fig. 4b). The major peak (peak I) can account for about 72%, peak II about 10%, and peak III about 13.5% of the total ³H radioactivity. When the samples from each peak were pooled and the [³H] DNA was hybridized with λ DNA filters, about 89% of the radioactivity from the peak III fraction hybridized with λ DNA, and the corresponding values for peak II and peak I were 35.5% and 8.5%, respectively. This plasmid-carrying strain contains about 23% of the total cellular DNA as λ DNA (calculated from the data in Fig.

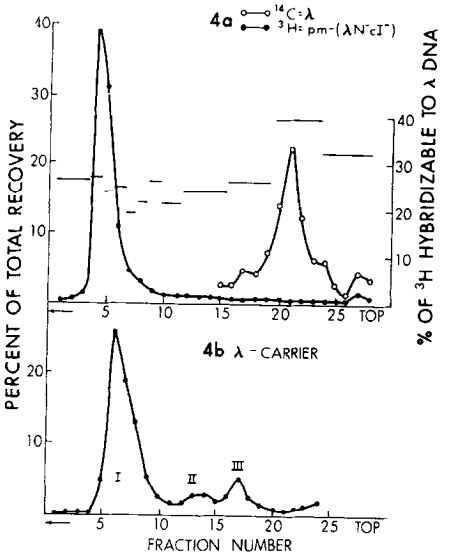

FIG. 4. Density gradient centrifugation on neutral sucrose in the presence of ionic detergent. pm⁻(λ*N*⁻*c*I⁻) and the multiple carrier lysogen (described in the *Methods* section) were labeled in the presence of 50 μCi/ml of [^{3}H]thymidine. Labeled cells (about 4.6 × 10^{7} and 1.54 × 10^{7}, respectively) were centrifuged, and the pellets were washed once with a buffer containing 0.05 *M* Tris-HCl and 0.05 *M* NaCl, pH 8.0, and finally suspended in 0.2 ml of same buffer containing 5% sucrose and 0.01 *M* EDTA. Lysozyme was added to a final concentration of 1.6 mg/ml, and the suspension kept 15 min in ice to give spheroplasts. Spheroplasts (0.1 ml) were layered very slowly on the top of a 15–30% sucrose (5 ml with a cushion of 0.2 ml "Angioconray") gradient containing 0.05 *M* NaCl, 0.05 *M* EDTA, 0.4% deoxycholate, 1% SDS, and 0.01 *M* Tris-HCl, pH 8.0. For an internal marker, 0.1 ml of ^{14}C-labeled λ phage was heated to 60°C for 15 min in 1% SDS and was then layered above the 594 (λ*N*⁻*c*I⁻) spheroplasts on the gradient using a wide-mouthed Pasteur pipet. The spheroplasts were completely lysed by keeping the gradient tube at room temperature for 20–30 min. Centrifugation was then done at 20°C for 3.5 hr using the SW-50 rotor at 25,000 rpm in the Spinco L ultracentrifuge. After centrifugation, fractions were collected, counted, and hybridized by the procedure described in Methods. In Fig. 4a, the bars cover the fraction or pooled fractions that were hybridized to λDNA. Total TCA-precipitable cpm recovered after centrifugation was (a) for the pm⁻(λ*N*⁻*c*I⁻) gradient: ^{3}H = 6.4 × 10^{5}, ^{14}C = 4.4 × 10^{3}; (b) for the multiple carrier lysogen: ^{3}H = 4.5 × 10^{5}.

4b); about 50% of the λ genomes were recovered in peak III. Although we did not get any minor peak from the converted lysogen in the corresponding region, we decided to hybridize the DNA in various fractions to determine the λ content. The results are represented by small bars parallel to the horizontal axis in Fig. 4a. There is very slight enrichment of λ-DNA-hybridizable ^{3}H radioactivity near the top of the gradient at the region of marker λ DNA. However, the total % recovery in this region (fractions 20–28) has been calculated to be only about 3.4%. Thus, there is no evidence that an appreciable fraction of the λ genomes in the N⁻ cI⁻ lysogen are separable from the host chromosome.

DISCUSSION

The formation of a stable λ*N*⁻ *c*I⁻ lysogen requires a λ attachment site on the bacterial chromosome and the product of the λ *int* gene (Lieb, 1971). Therefore, there was little doubt at the start of this study that λ*N*⁻ *c*I⁻ genomes could, indeed, be carried in an integrated state. The fact that λ*N*⁻ *c*I⁻ lysogens contain 20–25 λ genomes per host genome (Lieb, 1972a) suggested that some of these genomes might exist in the plasmid state. Unlike stable λ*N*⁻ *c*I⁻ lysogens, cultures containing only λ*N*⁻*c*I⁻ plasmids segregate noncarriers at a high frequency (Lieb, 1970). However, it was conceivable that the presence of one or more prophages might allow a high concentration of plasmids to be maintained.

We have now shown that only a small proportion (less than 10%) of the λ genomes in λ*N*⁻ *c*I⁻ polylysogens can be detected as closed circular plasmids. The circular DNA detected in alkaline sucrose gradients was at the position corresponding to a plasmid the size of an F′ dimer. Centrifugation of gently lysed cells on neutral sucrose did not allow the separation of 90% of the λ genomes from the bulk of the bacterial DNA. In control experiments, we established our ability to separate closed circular plasmids and λ-sized pieces of DNA from bacterial DNA. Thus, it is very likely that the majority of λ genomes in polylysogens are covalently bound to bacterial DNA.

How do we imagine 20–25 λ genomes to be arranged in the bacterial chromosome? An obvious possibility is a long end-to-end tandem sequence. However, another observation suggests that the number of λ genomes in a contiguous tandem sequence may be only 6–8 per bacterial chromosome. When immune lysogens containing 6–8 λ*N*⁻ *cIts* prophages are derepressed, they become converted to bacteria having the same phenotype as λ*N*⁻ *c*I⁻ lysogens (Lieb, 1972b). The number of λ genomes per bacterium increases rapidly (within 1 hr) to 20–25 per host genome (Lieb, unpublished results). No increase has been detected in the number of closed circular λ genomes per bacterium in these experiments, and λ genomes cannot be separated from bacterial DNA on neutral sucrose gradients (Mandal, unpublished).

As a tentative model, we suggest that derepressed λ*N*⁻ prophages may undergo replication without excision from the host chromosome, forming "bubbles" or "puffs". Each of the prophages is assumed to be capable of initiating its own replication, resulting in a large number of initiation sites in the prophage region of the bacterial chromosome. As soon as a prophage has replicated, a new replication fork can appear in each of the progeny prophages. Our data suggest that in a λ*N*⁻ *c*I⁻ lysogen, each prophage is replicated two and one-half times while the bacterial chromosome undergoes one round of replication. Thus, although there may be only 6–8 tandemly integrated prophages, three or more copies of each are always present, attached to bacterial DNA.

Replication of λ prophage *in situ* has been proposed to account for the increase in the adjacent bacterial *gal* DNA observed in λ*N*⁻ lysogens after λ prophage derepression (Imae and Fukasawa, 1970). Since the derepression of *N*⁻ prophages generally leads to loss of viability, it has been supposed that replication of integrated λ is lethal to the cell (Eisen *et al.* 1968). However, we have shown that the presence of a *large number* of prophages (e.g., 6–8) allows λ*N*⁻ *cIts* lysogens to survive after derepression (Lieb, 1972b). Thus, a large number of derepressed genomes is not as deleterious as a small number.

A consideration of the properties of λ*N*⁻ *c*I⁻ polylysogens may help to resolve this paradox. The "immunity" of λ*N*⁻ *c*I⁻ lysogens to superinfection is attributable to the presence in such bacteria of an immunity-specific repressor that acts at the leftward and rightward early promotors (Lieb 1972a). A similar "immunity" is observed in bacteria carrying multiple copies of an *N*-deleted *c*I⁻ λ plasmid (Matsubara, 1972). This immunity is attributable to the product of the *tof* (*cro*, *fed*) gene, which does not require gene *N* product for its expression (Pero, 1970; Eisen *et al.*, 1970). The *tof* product represses the expression of the "early" genes whose products are required for λ recombination, integration, excision, and replication. It is probable that one or more of these "early" gene functions is potentially lethal to the λ*N*⁻ lysogen. We propose that several molecules of *tof* product act cooperatively at each operator site to block transcription. Thus, the concentration of "active" *tof* repressor would increase in a nonlinear fashion with the number of *tof* genomes. In normal lytic development, λ genome replication assures the production of sufficient *tof* product for effective repression of early functions. In *N*⁻ lysogens, many prophage copies are required to produce the same effect.

Friedman and Yarmolinsky (1972) observed that λ*N*⁻ *cIts* dilysogens had a higher probability than monolysogens of surviving after derepression. They suggested that λ genomes were excised from the dilysogens, and that these plasmid genomes then competed with the prophage for replication sites or products. We have shown tht derepressed λ*N*⁻ lysogens do not contain a significant number of plasmids, indicating that cytoplasmic λ is probably not required for the survival of such lysogens.

It is to be expected that λ genomes are occasionally excised from λ polylysogens by the *rec* system of the host (Kellenberger-Gujer and Weisberg, 1971). This should lead to a gradual diminution of the number of λ prophages per bacterium. Presumably,

bacteria in which the concentration of *tof* product falls below a minimum concentration are no longer viable. The low growth rate and relatively high rate of DNA degradation in pm$^-$ ($\lambda N^- cI^-$) cultures (unpublished data) suggests that nonviable bacteria appear at a significant rate.

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