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Regulation of Late Functions in *Salmonella* Bacteriophages P22 and L Studied by Assaying Endolysin Synthesis

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The rate of endolysin synthesis in *Salmonella typhimurium* cells infected by bacteriophage P22 or L was taken as a measure for the activity of 23 gene product (the positive regulator for the "late" genes of P22 and L). Endolysin is coded for by gene 19. The amber mutations in gene 23 of P22 and L, used in this study, reduced the rate of endolysin synthesis by a factor of ca. 90 for P22 and of ca. 20 for L. In mixed infections with *19*$^-$ and *23*$^-$ mutants the 23 gene products of P22 and L act as positive regulators for the respective gene 19 in *cis* and in *trans*. Cross-specificity of the 23 gene products, i.e., turning on expression of gene 19 on a chromosome of the other species, could not be demonstrated.

Bacteriophages P22 and L are temperate phages of *Salmonella typhimurium*. They can be distinguished by immunity and restriction specificity, density, and host range (1). However, they show the same particle morphology and very similar serological properties. Crosses between P22 and L yielded recombinants for immunity specificity, host range, and density (2), and complementation tests with 17 amber mutants from 15 genes revealed cross-activity in 14 genes (16), suggesting a high degree of genetic homology.

The genetic map of phage P22, as determined from vegetative crosses, is circular (11, 17). Genes that determine related functions or are members of the same regulatory unit will be found as neighbors on the genetic map. Based on these criteria, four regions can be defined: one early and one late region and the immunity C and I regions (2). Gene 24 codes for the positive regulator of the early region (13), which in addition to the genes for replication, recombination, and integration/excision also includes gene 23, which in turn determines the positive regulator of the late genes (7, 19, 22). The gene products 23 and 24 have been assigned the role of positive regulators mainly because of the pleiotropic behavior of their mutants in various assays: DNA synthesis, phage maturation, lysis, and protein band patterns in sodium dodecyl sulfate-gel electrophoresis. Studies comparing genes *N* and *Q* of λ with genes 24 and 23 of P22 revealed close functional and genetical similarities, even cross-activity (6). Thus, the model proposed for the mechanism of action of the λ *N* and *Q* genes (24) has been extended to genes 24 and 23 of P22 (25). Roberts (24) proposed that the *N* and *Q* gene products act by preventing termination of transcription. The termination sites are placed in front of the first gene in the early and late regions, which is in accordance with the pleiotropic nature of mutants of the regulatory genes.

In the course of studying the properties of gene 23 of P22 and L, transactivation experiments (28) seemed to suggest that gene 23 of P22 may also act in *trans*, not only on P22 but also on L chromosomes (23). Cells lysogenic for phage L were superinfected with P22 defective in the late gene 19 (endolysin) (4, 5). Cell lysis, detected by the release of phage progeny, was taken to indicate endolysin activity, which in turn was attributed to the gene 23 product of P22 turning on expression of the endolysin gene of prophage L.

In this communication it will be shown that cell lysis is not a reliable indicator of endolysin activity. Therefore, the question of whether gene 23 of P22 is able to act in *trans* on prophage L has not yet been answered.

Throughout this work endolysin is assumed to be a suitable probe for gene 23 product activity. A reproducible and sensitive assay for endolysin activity was developed.

The results in this paper show that endolysin alone cannot cause lysis; the cooperation of at least one other late function (gene 13) is necessary. In both phages P22 and L, gene 23 can act in *trans* and in *cis* only if the other phage in *trans* is of the same species.

MATERIALS AND METHODS

Bacteria. *Salmonella typhimurium* strains LT2 (collection of M. Demerec) and *su*$^+$*am*527 [suppresses

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hisC527(Am) (29)] were used for experiments and for growing lysates. *Escherichia coli* BA (collection of R. S. Edgar) provided the substrate for the lysozyme assay.

Phages. All phages used in this communication are listed in Table 1. Standard phage techniques and media were as described by Prell (21). In addition, the following media were used: tryptone medium—10 g of tryptone (Difco), 5 g of NaCl, and 1 liter of demineralized water; M9-tryptone medium—M9 and tryptone media combined (1:1); and M9CC(vfc)—M9 buffer (21), containing 0.2% glucose, 0.04% sodium citrate, and 0.2% vitamin-free Casamino Acids (Difco).

Measurement of DNA synthesis. LT2 cells growing in M9CC(vfc) at 33°C were infected with phage at a multiplicity of infection of 10 when cell concentration had attained 10^8 cells per ml. Samples of 0.1 ml removed at intervals were added to tubes standing at 33°C and containing 0.1 ml of [*methyl*-^{3}H]thymidine (20 μCi/ml) and unlabeled thymidine (1 μg/ml). Three minutes later, the pulses were terminated by adding 4 ml of chilled 5% trichloroacetic acid containing 1 mg of unlabeled thymidine per ml. The tubes were kept on ice for at least 1 h, and the precipitates were collected on nitrocellulose filters (0.45-μm pore size), washed, dried, and counted in a scintillation spectrometer. The method of measuring [^{3}H]thymidine incorporation into P22 DNA differs from the original one described by Smith and Levine (26), in that the final concentration during the pulses was 10 μCi of [^{3}H]-thymidine and 500 ng of "cold" thymidine per ml (4.8 Ci/mmol). Smith and Levine (26) used 0.9 μCi and 344 ng/ml (640 mCi/mmol).

Under my conditions, in wild-type infections the increase in the rate of [^{3}H]thymidine incorporation stops several minutes before lysis commences and falls ca. 15 min later, probably when a large enough fraction of cells has lysed (Fig. 1) or has ceased functioning. Cells infected with *19*$^-$ mutants (no endolysin) do not lyse but show the same pattern of [^{3}H]thymidine incorporation as a wild-type infection (unpublished data).

Lysozyme assay. (i) Preparation of lyophilized *E. coli* for lysozyme substrate. *E. coli* BA were grown in M9-tryptone medium with aeration to about 3×10^8 cells per ml, chilled, centrifuged, and suspended in 1/10 original volume of 0.1 M EDTA (pH 8.0). After 5 min at room temperature, the cells were centrifuged again, and the pellet was suspended in 1/10 original volume of 5 mM phosphate buffer (pH 5.8)–86 mM NaCl and stored for at least 2 h at +4°C. The cells were concentrated by centrifugation and resuspension in 1/80 original volume of 0.05 M Tris-hydrochloride (pH 7.4). The cell slurry was distributed into small glass tubes, 0.5 ml each, and lyophilized. The tubes were sealed under vacuum. One tube delivered substrate sufficient for at least 80 tests. The sealed tubes were stored at room temperature.

(ii) Enzyme assay. Substrate was prepared only from tubes that had remained air-tight. The cell powder of one tube was suspended in 10 ml of 5 mM phosphate buffer (pH 7.0)–0.1 mM $CaCl_2$–17 mM NaCl and incubated at room temperature for 30 min. The cell suspension was then centrifuged and suspended in 4 ml of 5 mM phosphate buffer (pH 7.0)–1.0 mM $CaCl_2$–17 mM NaCl. The concentration was adjusted to yield an optical density at 350 nm of 0.4 when diluted to 1/10 in Tris buffer. When stored on ice this cell suspension could be used as a substrate for the lysozyme assay over a period of several hours. For measuring lysozyme activity, 50 μl of substrate, 50 μl of sample, and 500 μl of 50 mM Tris-hydrochloride (pH 7.9)–10 mM $CaCl_2$–5 mM NaCl were mixed in a cuvette. The optical absorbance at 350 nm was measured at room temperature in a recording spectrophotometer (Gilford 250). If necessary, the samples were diluted to remain within the limits where the slope (change of absorbance versus time) is proportional to enzyme concentration. The assay was regularly stand-

TABLE 1. *Phage strains*

Genotype	Source
P22 c1-7	Levine (18)
P22 *19*$^-$ *am*H1006 c1-7	This paper
P22 *23*$^-$ *am*H79 c1-7	This paper
P22 *19*$^-$ *am*H100623$^-$ *am*H79 c1-7	This paper
L cI-40	Bezdek et al. (3)
L *19*$^-$ *am*8 cI-40[a]	This paper
L *23*$^-$ *am*23 cI-40[a]	This paper
L *19*$^-$ *am*8 23$^-$ *am*23 cI-40[a]	This paper
P22 *19*$^-$ *am*H1006[b]	Prell (23)
P22 *23*$^-$ *am*79[b]	Kolstad and Prell (17)
L *19*$^-$ *am*8[a,b]	J. Soska
L *23*$^-$ *am*23[a,b]	J. Soska

[a] The genes affected by the amber mutations were assigned according to the results described in this paper.

[b] Phage strains from which mutant alleles were derived.

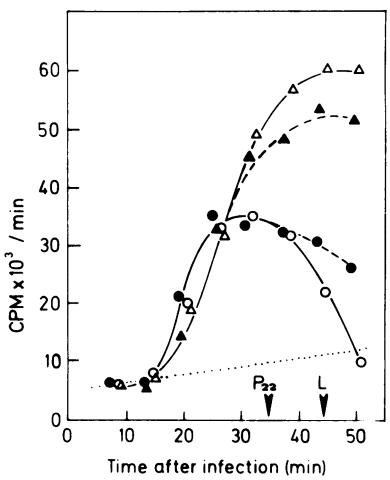

FIG. 1. *Rate of thymidine incorporation after infection with L or P22. LT2 cells growing in M9CC(vfc) at 33°C were infected at a multiplicity of 10 phage per cell. Three-minute pulses of [^{3}H]thymidine were administered at various times after infection, as described in the text. LT2 cells were infected by P22 c1-7 (○), P22 c1-7 23*$^-$ *am79 (△), L cI-40 (●), or L cI-40 am23 (▲). The dotted line shows the rate of incorporation in an uninfected control culture. Arrowheads indicate beginning of lysis of cultures infected by P22 c1-7 or L cI-40.*

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ardized with 1 μg of egg white lysozyme per ml. Phage endolysin activity is expressed in units equivalent to 1 μg of egg white lysozyme per ml.

RESULTS

Characterization of L mutants. A set of P22 mutants, mapped and functionally characterized, was available for studying the role of gene 23 in regulating expression of gene 19 (Table 1). A comparable set of L mutants did not exist. Several lysis-negative L amber mutants, kindly provided by J. Soska, Czechoslovak Academy of Sciences, Brno, were studied in an attempt to associate their defects with functional groups known from P22 (Table 2).

Amber mutants that do not lyse under restrictive conditions may be defective in (i) an early function, e.g., DNA replication, (ii) endolysin activity (gene 19), (iii) sensitizing the cell envelope for the action of endolysin (gene 13?) or (iv) positive regulation of expression of the late genes (gene 23). All five L amber mutants in Table 2 were shown to incorporate [^{3}H]thymidine into acid-precipitable material (unpublished data), indicating that DNA replication is unaffected by these mutations.

In Table 2 mutants of gene 19 can be easily recognized (*am*8 and *am*76). They have no lytic activity but produce progeny like wild-type phage.

The L mutants *am*43 and *am*53 produce large numbers of phage progeny and show endolysin activity, but are not able to lyse the host cells. These mutants might be defective in a gene equivalent to gene 13 of P22 (4).

Table 2 shows that the L amber mutant 23 is of pleiotropic nature because endolysin activity and phage production (burst size) are both severely reduced. In P22 amber mutant 79 of gene 23, which codes for the positive regulator of the late functions (7, 19, 23), is also pleiotropic in the same way, suggesting that *am*23 of L and *am*79 of P22 are equivalent. Further properties were compared to verify this conjecture: (i) the [^{3}H]-thymidine incorporation rate and pattern of both phages L *am*23 and P22 *23*$^-$ *am*79 were similar (Fig. 1); and (ii) mapping by two- and three-factor crosses placed *am*23 at the end of the early and the beginning of the late region (Table 3). The *23*$^-$ *am*79 mutation of P22 is found at the same position on the P22 map.

The data suggest that L *am*23 carries a mutation in the gene coding for a positive regulator of late gene expression, which corresponds to gene 23 in P22.

Is gene product 23 able to act in *trans*? The synthesis of endolysin in LT2 was measured after single or mixed infection with P22 or L mutants (Table 4).

In P22-infected cells, full expression of the gene for endolysin depends on the presence of a functional gene 23. With a defective 23 gene the rate of endolysin synthesis reaches ca. 1/90 of that found in P22 wild-type-infected cells (Table 4, experiments 1 and 7). In comparison, the L *23*$^-$ *am*23 mutant appears to be extremely leaky

TABLE 2. *Lysis-negative amber mutants of phage L in host bacteria LT2*[a]

Mutant	Burst size (phage/cell)		Endolysin activity		
	Spontaneously lysed cells	Sonicated cells	Spontaneously lysed cells (I)	Sonicated cells (II)	Total I + II
L *am*$^+$	168	13	94.9	5.1	100
L *am*8	0.16	221	<1	<1	
L *am*76	0.17	303	<1	1.7	1.7
L *am*23	0.04	0.7	3.4	8.0	11.4
L *am*43	0.67	319	5.7	22.2	27.9
L *am*53	0.12	371	<1	23.9	23.9

[a] LT2 cells growing logarithmically were infected with 5 phage per cell. After incubation in nutrient broth at 37°C for 50 min, 10-ml samples were centrifuged at low speed. The supernatants were tested for phage and endolysin activity ("Spontaneously lysed cells"). The pellets were resuspended, ultrasonicated for 2 × 15 s, and centrifuged. The supernatants were tested as before ("Sonicated cells"). Total endolysin activity of L *am*$^+$ is given a value of 100, and the activities observed in the mutants are expressed relative to this value.

TABLE 3. *Mapping of am23 phage L*[a]

Cross	Selected marker	Unselected marker[b]	No. of recombinants	Frequency recombination between *c*I-40 and *am* sites (%)
L *c*I-40 *am*23	*am*$^+$	*c*$^+$	755	2.3
× L *c*$^+$		*c*I-40	18	
L *c*I-40 *am*8	*am*$^+$	*c*$^+$	719	2.6
× L *c*$^+$		*c*I-40	19	
L *c*I-40 *am*23	*am*$^+$	*c*$^+$	628	
× *c*$^+$ *am*8[c]		*c*I-40	99	

[a] Standard phage crosses were carried out in *su*$^+$ at a multiplicity of seven of each parent (Botstein et al. [5]). The lysates were plated on *su*$^+$ for total progeny and on LT2 (*su*$^-$) for *am*$^+$ progeny.

[b] Frequency of C-type mutants among progeny of L *c*$^+$ infection: 10^{-3}.

[c] Total progeny, 2.0×10^6 per ml; *am*$^+$ recombinants, 1.2×10^4 per ml; frequency of recombination between *am*23 and *am*8, 1.2%. Deduction: *am*23 lies between *c*I-40 and *am*8 (gene 19) as shown:

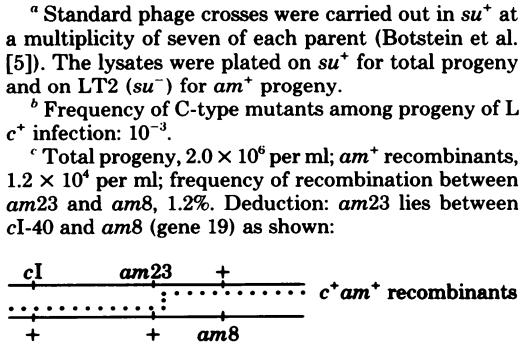

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TABLE 4. *Synthesis of endolysin in LT2 infected with P22 or L*[a]

Expt no.	Mutant or cross	Rate of endolysin synthesis (U/min)	Endolysin activity, 52 min p.i.	Burst size	OD_{578} at 65 min p.i.
1	P22 *c1*	0.85	20	180	0.05
2	P22 *c1* × P22 *c1 23⁻ 19⁻*	0.47	8	170	0.05
3	P22 *c1 23⁻* × P22 *c1 19⁻*	0.32	5	100	0.57
4	L *cI*	0.78	28	240	0.14
		0.85	28	220	0.10
5	L *cI* × L *cI 23⁻ 19⁻*	0.47	13	250	0.13
6	L *cI 23⁻* × L *cI 19⁻*	0.51	14	240	0.16
		0.45	15	230	0.15
7	P22 *c1 23⁻*	ca. 0.01	<1	<1	0.71
8	L *cI 23⁻*	0.05	2	<1	0.80
9	L *cI 23⁻* × P22 *c1 19⁻*	0.03	1	210	0.11
10	L *cI 23⁻* × P22 *c1 23⁻ 19⁻*	0.03	1	<1	0.65
11	P22 *c1 23⁻* × L *cI 19⁻*	ca. 0.01	<1	190	0.70
12	L *cI 23⁻* × L *cI 19⁻*	0.26	9	250	0.10
13	L *cI 23⁻* × L *cI 19⁻* × P22 *c1 19⁻*	0.17	6	260	0.08
14	L *cI 23⁻* × L *cI 19⁻* × L *cI 23⁻ 19⁻*	0.35	11	250	0.18
15	L *cI 23⁻* × L *cI 19⁻* × P22 *c1 23⁻ 19⁻*	0.29	9	240	0.27

[a] Logarithmically growing LT2 (nutrient broth, 37°C), when cell density had reached 2×10^8 cells per ml, were infected with 5 of each phage per cell, except for experiment 12, in which L *cI 19⁻* was used at a multiplicity of infection of 10. Samples of 1 ml were removed at 10, 16, 24, 32, 42, 52, and 62 min postinfection (p.i.). Protein synthesis was halted by adding chloramphenicol (300 μg/ml) and cooling on ice. The samples were centrifuged at low speed, and the resulting supernatant and the resuspended cell sediment fractions were frozen. After 2 h the samples were allowed to thaw at room temperature, and the cells were disintegrated by ultrasonication (Branson Microtip, 2 × 15 s at setting 5). Before endolysin activity was determined (see the text), the samples were purified by centrifugation (20,000 × *g*, 5 min). Because of the large number of infections necessary the experiments were performed in two groups on different days. Infections shown in lines 4 and 6 were present in both groups; their data show that the day-to-day variation was small. OD_{578}, Optical density at 578 nm.

(Table 4, experiments 4 and 8), since it synthesizes ca. 1/20 the endolysin activity of wild-type-infected cells. Cells infected by L have a longer latent period (data not shown) and reach a higher level of endolysin activity than P22-infected cells.

cis-trans experiments were employed to establish whether or not gene 23 product is able to turn on expression of gene 19 located on a different chromosome. Mixed infections of *S. typhimurium su⁻* (LT2) were carried out as follows. In *cis* experiments (Table 4, experiments 2 and 5), the wild-type genes of 23 and 19 were both carried on the same chromosome and the mutant genes were carried on the other one; in *trans* experiments (Table 4, experiments 3 and 6), the wild-type gene 23 was located with a mutant gene 19 on one chromosome, and the wild-type gene 19 was located with the defective gene 23 on the other.

Wild-type chromosomes generated by recombination could give misleading results in the *trans* experiments. However, the fraction of recombinants was found to be ca. 1%, too small to contribute measurably to endolysin activity. To ensure lytic development all the phage strains involved were made *c1*, thereby drastically reducing *c2*-repressor synthesis (12). The phage progeny of *cis-trans* experiments were checked for deviation from the 1:1 ratio of the parental phage types. The maximum deviation found was 1.1:0.9. *cis-trans* experiments with P22 and L were performed three times. Statements about the results presented in the following discussion pertain to all three sets of experiments. The P22 *trans* experiments yielded less endolysin than the *cis* experiments, but also fewer phage particles, and the onset of phage and endolysin production was delayed by about 10 min (results not shown). Because of this general weakness of phage activity, the low endolysin production cannot be attributed solely to the *trans* arrangement of the 23 and 19 wild-type genes.

The results presented in Table 4 (experiments 1 to 6) indicate that the 23 gene of L and P22 acts as a positive regulator for gene 19 in *cis* and in *trans*.

Is the product of gene 23 of P22 (or L) an effective positive regulator for expression of late L (or P22) functions in *trans*? The results presented in the preceding section indicate that gene 23 of both P22 and L can turn on expression of a late gene located in *trans* on a second chromosome. One might expect that positive regulation by gene 23 also operates between the different species P22 and L, since mixed

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infections with P22 and L mutants seemed to reveal efficient complementation of most of the functions tested (1, 3, 16). Genetical homology (base sequence homology) might also exist, as indicated by recombinants found by Bezdek and co-workers in P22 × L crosses (1, 3). Unfortunately, the experiments did not distinguish between recombinational events inside the genes, indicating gene-to-gene homology and exchange of whole groups of genes (14) through sequence homology of regions flanking the genes examined.

The experiments to be presented were designed to probe for functional homology of gene 23 of P22 and of L.

The rate of endolysin synthesis in cells infected by P22 *c1 19*$^-$ + L *cI 23*$^-$ is 1/11 as compared with P22 *c1 19*$^-$ + P22 *c1 23*$^-$ and 1/16 as compared with L *cI 23*$^-$ + L *cI 19*$^-$ infections (Table 4, experiments 9 and 3, and 9 and 6, respectively). The reciprocal experiment, P22 *c1 23*$^-$ + L *cI 19*$^-$ (Table 4, experiment 11), produced even less endolysin.

These results suggest that the gene 23 products of P22 and L have little or no cross-specificity. However, this apparent lack of functional homology may result from other factors, for example, from exclusion. Exclusion occurs in mixed-infection experiments where, for reasons often unknown, one of the phage types is disadvantaged. The results show that P22 reduces the synthesis of L endolysin by only a factor of ca. 1.5 (Table 4, experiments 12 and 13) or ca. 1.2 (experiments 14 and 15). P22 *c1 19*$^-$ in mixed infection with L *cI 23*$^-$ does not seem to contribute any 23 function to L *cI 23*$^-$ (Table 4, experiments 8 and 9). The infection L *cI* 23$^-$ × P22 *c1 23*$^-$ *19*$^-$ (Table 4, experiment 10) yields the same amount of endolysin as L *cI 23*$^-$ × P22 *c1 19*$^-$. Therefore the endolysin produced by L *cI 23*$^-$ × P22 *c1 19*$^-$ probably originates from gene 23-independent expression of gene 19 of L (leakiness). Surprisingly, 1/5 of the progeny in this experiment were of L immunity. Therefore the synthesis of a substantial amount of structural L proteins could be suspected. This is difficult to reconcile with the observation that L gene 19 cannot be turned on by product 23 of P22. However, the high yield of phage of L immunity type (chimeras) in these two experiments is the product of phenotypic mixing (1); these chimeras are composed of L DNA and P22 coats. No L phage were produced when the coinfecting phage P22 *c1 19*$^-$ were also defective in gene 5 (unpublished data). Botstein et al. (7) have shown that gene 5 codes for the major head protein of P22.

Endolysin by itself is not sufficient for lysing cells. During the course of this study it became apparent that cell lysis is not merely a function of endolysin concentration. Experiments with L *cI 23*$^-$ illustrated this point and indicated that another function in addition to endolysin is necessary to effect lysis. In LT2 cells infected by L *cI 23*$^-$ only, endolysin is synthesized at a low rate (leakiness) and the cells do not lyse (Table 4, experiment 8). This inability to lyse is not due to inadequate endolysin activity. In L *cI 23*$^-$ + P22 *c1 19*$^-$ infection (Table 4, experiment 9), where even less endolysin is produced, the cells lyse as is shown by the drop of optical density. P22 *c1 19*$^-$ probably provides a late function which, in concert with endolysin from L *cI 23*$^-$, effects lysis. Gene product 13 seems to be this auxiliary lytic agent, since LT2 cells infected by L *cI 23*$^-$ × P22 *c1 13*$^-$ *19*$^-$ accumulate endolysin but do not lyse (unpublished data).

DISCUSSION

The products of gene 23 in P22 and L and of gene *Q* in λ cannot be assayed directly. Therefore the measurement of endolysin synthesis in λ, which is synthesized under positive control of gene *Q*, has been used by several authors as an indirect method to determine the activity of gene product *Q*, for example, by Dambly and Couturier (9), Couturier et al. (8), and Echols et al. (10).

This indirect assay was here used with phage P22 and L to study the effect of gene 23 on expression of gene 19 with respect (i) to *cis-trans* action of 23 product and (ii) to the degree of functional homology of the 23 products of P22 and L.

The previously used methods of determining endolysin activity suffered from the lack of a substrate that is sufficiently sensitive and yields reproducible results. This drawback could be overcome by dividing a large batch of sensitized *E. coli* cells in per-day portions and lyophilizing them.

Unfortunately, for both P22 and L only one amber mutant each of gene 23 was available. The L amber 23 mutant was found to resemble the P22 *23*$^-$ amber 79 in all but one of the properties checked: the leakiness of L *am*23 was much higher than that of P22 *23*$^-$ *am*79 but of the same magnitude as that observed for all of the lambda *Q* mutants studied by several authors (for references see Dambly and Couturier [9]). Since the lower leakiness of the P22 *23*$^-$ amber mutant could be caused by concomitant inhibition of DNA replication, [^{3}H]thymidine incorporation kinetics of both mutants were determined and found to be practically identical. The different levels of leakiness observed may

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be typical only for the two mutants tested and need not reflect a genuine difference in the mechanism of late regulation in P22 and L. Experiments with other independently isolated 23^- mutants are necessary to distinguish between these alternatives.

The gene 23 products of P22 and L might act as an antiterminator for transcription of the late genes, a role suggested for the λ *Q* gene product (for discussion and references see Roberts et al. [25] and Susskind and Botstein [27]). The sites of formation and of action of product 23 (and of *Q*) lie very close together on the bacteriophage chromosome. In bacteriophage λ a significant difference between the endolysin synthesis rate in *cis* and *trans* experiments has been described (10). Since Echols et al. employed a special method of preparing and infecting the host cells, the P22 and L *cis-trans* experiments were repeated using their method. Under these experimental conditions our results remained unaltered (data not shown). Therefore, the restriction placed on *trans* activity of the late regulatory system in λ appears not to occur in P22 and L.

The gene 23 products of P22 and L appear to be active only on chromosomes of their own species. With the methods and mutants employed, no cross-specificity could be detected. Therefore the genes themselves, and also the termination sites of P22 and L, are probably not homologous. It would be of interest to compare the base sequences of the genes and sites involved. Very small divergences in the base sequence of the regulatory system may lead to complete functional incompatibility. Outside of these regulatory chromosomal regions, complementation may proceed unhampered, and recombination between P22 and L could occur in all regions with frequencies typical of intraspecies crosses. Consequently, evolution of new phage species would be promoted (i) by diverging forces originating in the incompatibility of a regulatory system and (ii) by the optimizing possibilities provided by the exchange of genetic material coding for nonregulatory proteins.

It could be shown that endolysin alone is not sufficient to cause lysis of the infected cells. Another late function, coded for by gene 13, is necessary to effect lysis at the end of the latent period (5). Also, in λ and T4 the lysis mechanism seems to be a sequential process of at least two steps: in λ-infected cells the action of the *S* gene product has to precede the endolysin from *R* gene for lysis to occur (20). In T4, functionally very similar roles are played by the products of genes t and e (15). In P22 in the presence of gene product 13, 1/30 (or less) of the wild-type endolysin activity suffices for the successful lysis of infected cells (Table 4, experiments 4 and 9). Hence the concentration of gene product 13 required seems to be more critical. Preliminary experiments indicate that gene 13, or the gene product 13, determines the time of lysis, independent of the concentration of endolysin.

These results show clearly that cell lysis or the release of phage progeny are not adequate parameters for the assay of endolysin activity. Endolysin must be assayed directly.

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