*ISOLATION OF THE λ PHAGE REPRESSOR**

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A bacterium can carry within it, integrated in its chromosome, the genome of a potentially lethal phage (prophage), because the genes of the prophage are prevented from functioning by a repressor. This lysogenic bacterium, as it is called, will lyse and produce phage if the repressor is inactivated. This same repressor, which is made by a gene on the prophage, is also responsible for the immunity of lysogenic cells to superinfection by phages similar to the prophage. Such superinfecting phages inject their DNA but the newly introduced phage genes neither function nor replicate.

These facts were elucidated largely by studies of the phage λ which grows on *E. coli*. In 1957, Kaiser and Jacob showed that the prophage gene C_1, required for the maintenance of lysogeny, is also responsible for the immunity against superinfecting phages.[1] On the basis of this and other observations, Jacob and Monod[2] proposed that the C_1 gene produces a repressor molecule (often referred to as the immunity substance) which blocks lytic development of both prophage and superinfecting phages by selectively repressing the expression of one or more phage genes. This is exactly analogous to their model for the action of the *i* gene of the lactose operon. Although both the *lac* and λ repressors have been the objects of extensive studies *in vivo*, their mechanism of action is unknown. Only recently has a repressor, the *lac* repressor, been detected *in vitro*.[3] This paper describes the specific labeling and partial purification of the C_1 product of phage λ.

Preliminary Results and Considerations.—In an ordinary λ-lysogen, phage repressor synthesis probably constitutes on the order of only one part in 10^4 of the cell's total protein synthesis. In order to label the λ phage repressor specifically, I sought conditions in which the rate of synthesis of repressor is a significant fraction (5–10%) of the total protein synthesis of the cell. To achieve the necessary differential increase in repressor synthesis, I attempted (1) to inhibit the synthesis of host proteins while maintaining the capacity to synthesize phage proteins; (2) to inhibit the synthesis of most or all of the phage proteins other than that of the repressor; and (3) to maximize the number of functioning λ repressor genes in the cell. The first objective was achieved by irradiating cells with massive doses of ultraviolet (UV) light, a treatment which damages the host DNA and dramatically decreases the level of cellular protein synthesis. Figure 1 shows that lytic infection of phage in sufficiently damaged cells stimulates about tenfold the incorporation of labeled leucine. This incorporation presumably represents the synthesis of numerous phage proteins, only one of which is the C_1 product. The second objective is achieved in principle by infecting λ-lysogens with other λ phages of the same immunity. Although most of the genes of these superinfecting λ phage chromosomes are repressed, one newly injected gene which *does* function is the C_1 gene itself.[4] The third objective might be achieved simply by increasing the multiplicity of λ phages infecting the lysogenic cell. However, at multiplicities above 10–15, immunity to superinfection breaks down, and phage proteins other than the repressor are syn-

thesized. I have found that in lytic infection the production of many of the proteins of phage λ, perhaps all of them except for the C_1 product, is blocked if the phage carries a mutation in the early gene N, a result consistent with results of others.[5] Phages carrying mutations in gene N fail to stimulate the large increase in labeled leucine incorporation when added to UV-irradiated cells. This result is specifically confirmed by visualization of the labeled λ phage proteins made in irradiated cells by electrophoresis and autoradiography in polyacrylamide gels.[6] Although 8–10 prominent phage bands appear in extracts of λ-sensitive irradiated cells infected with wild type λ, these bands all disappear when the infecting λ phage carries a mutation in gene N. We know from genetic experiments that mutations in gene N do not block synthesis of the repressor. Therefore, high multiplicities of these mutant phages should stimulate the production of C_1 product without stimulating the production of most of the other λ proteins. This greatly simplifies the task of finding the C_1 product itself.

These considerations lead to the following experiment: a strain of *E. coli* lysogenic for λ is subjected to heavy UV irradiation. (The prophage used is λ*ind*$^-$, a mutant of λ which, unlike wild type, is not inducible by UV light.) One portion of the cells is infected with 30–35 λ phages carrying mutations in gene N, the other with λ phages carrying in addition a mutation in the C_1 gene which prevents synthesis of the C_1 product. One culture then receives H^3-leucine, the other C^{14}-leucine. After a period of labeling, the cells are mixed and sonicated, and the extracts are fractionated to look for a single protein marked with one label but not with the other. Figure 2 shows the location and names of the C_1 mutants used in these experiments. At each stage, I ran parallel experiments with the labels reversed to avoid being misled by the artifacts which can arise in double-label experiments.

The block provided by single mutations in gene N is noticeably incomplete on some bacterial strains, and so, in the experiments described below, *all* the phages carry double suppressor-sensitive mutations (ambers) in the N gene. Only the name of the C_1 gene, be it wild type, amber mutant, or temperature-sensitive mutant, will be explicitly used.

TABLE 1

DETECTION OF THE C_1 PRODUCT BY RATIO COUNTING

Cell fraction	H^3 cpm/-0.1 ml	C^{14} cpm/-0.1 ml	Ratio H^3/C^{14}
Sonicate	14,653	7,290	2.01
	15,079	7,654	1.97
Supernatant	6,852	3,201	2.14
	6,853	3,248	2.11
Pellet	7,007	3,747	1.87
	7,641	3,589	1.85

Counts and ratios are given for duplicate samples from each fraction. See text and *Materials and Methods* for experimental details.

Detection of the C_1 product: When a double-label experiment is performed comparing wild-type λ with the amber C_1 mutant C_1*sus*34, a differential fractionation of the labels can be observed following sonication and high-speed centrifugation. Table 1 shows the results of a typical experiment. In this case the cells infected with wild-type phage were labeled with H^3-leucine, the others with C^{14}-leucine. There is approximately a 15 per cent increase in the ratio of H^3 to C^{14} in the supernatant fraction compared to that found in the pellet. This means that there is a component which is labeled with H^3 but not with C^{14} comprising about 15 per cent of the H^3 label in the supernatant. A ratio difference of 10–15 per cent is consistently observed when wild-type λ is compared with this amber mutant, whether the labels are in the configuration described in Table 1 or reversed.

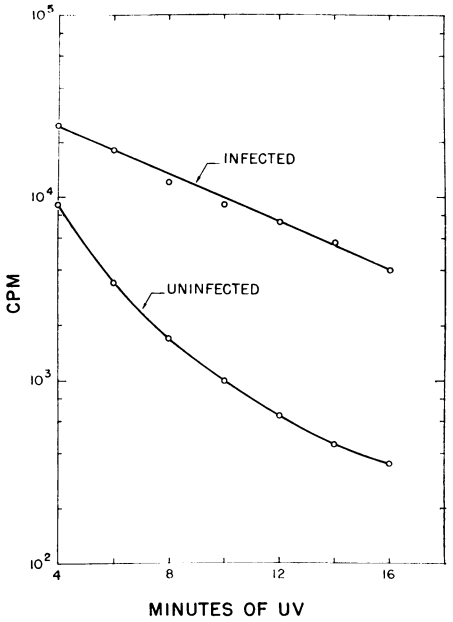

FIG. 1.—Stimulation of labeled leucine incorporation into irradiated cells by lytic phage infection. The cells were grown and irradiated as described in *Materials and Methods*. One-ml portions were labeled with $^1/_2$ μc C^{14}-leu for 1 hr with or without added phage at a multiplicity of 30 phage/cell. The cells are lysogenic for λ*ind*$^-$. In this particular experiment the infecting phage is λ*imm*^{434}, a phage similar to λ but of different immunity which grows normally on λ-lysogens. The incorporation was terminated by the addition of TCA. The precipitated cells were collected and washed on a Millipore filter and counted in a gas-flow counter. These curves do not extrapolate to the correct unirradiated values. The uninfected cell synthesis is decreased about 5,000-fold at 14 min of irradiation.

DEAE chromatography: The ratio counting experiment suggests that the supernatant fraction contains a protein made by the wild-type repressor gene but not by the amber C_1. This protein is isolated by fractionation on a DEAE column. The results of a typical experiment are presented in Figure 3. In this case the H^3 label is again in cells infected with λ wild type, and the C^{14} label in cells infected with the C_1 amber mutant C_1sus10. The gradient elution profile shows a distinct peak present in the H^3 label with no corresponding peak in the other label. This experiment has also been performed using the amber C_1 mutants C_1sus34, C_1sus14, and C_1sus80. In each case the results are as seen in Figure 3, that is, none of these mutants produces detectable amounts of the major protein peak. These mutants are also lacking the much smaller peak which is usually seen immediately following the major one. Although other minor peaks preceding and trailing the major peak at tube 20 often appear, there are no other major peaks (excluding the flowthrough) in either label throughout a gradient run from 0.07 *M* KCl to 0.5 *M* KCl. Furthermore, there is no increase in the ratio of H^3/C^{14} in the flowthrough nor in the acid and base washings of the column, nor is there any significant differential loss of label. Finally, the percentage of supernatant counts appearing in the major peak is large enough to account for the 10–15 per cent ratio difference described in Table 1. The remaining 85–90 per cent of the label in the supernatant represents

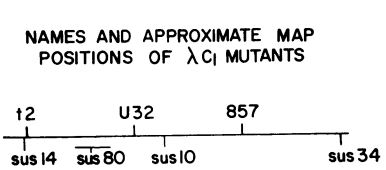

FIG. 2.—Mutants shown above the line are temperature sensitives (C_1ts), and those below the line are ambers (C_1sus). Mutants C_1sus34 and C_1sus14 are located at the extremities of the C_1 gene. Mutant C_1sus80 is only known to lie between C_1sus14 and C_1ts857. This map is a composite of two maps constructed by Drs. M. Lieb and F. Jacob. (These C_1sus mutants should not be confused with amber mutants of the same *sus* numbers located in other λ genes.)

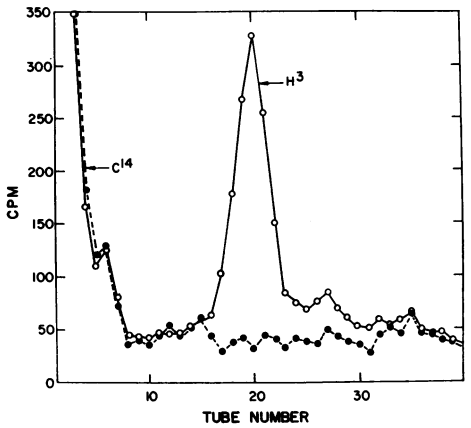

FIG. 3.—DEAE elution profile of an extract of a mixture of H^3-leu-labeled λ wild-type infected cells and C^{14}-leu-labeled λC_1-*sus*10 infected cells. The cells are lysogenic for λ*ind*$^-$. The gradient begins around tube 4. The counts are normalized by multiplying the measured C^{14} values by the ratio of H^3/C^{14} in the sample applied to the column.

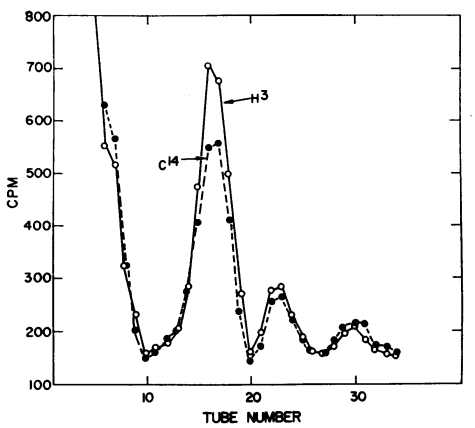

FIG. 4.—DEAE elution profile of an extract of H^3-labeled λC_1*ts*857 infected cells and C^{14}-labeled λ wild-type infected cells. Reconstituted protein hydrolysate is the label used in this experiment. Other details as in Fig. 3.

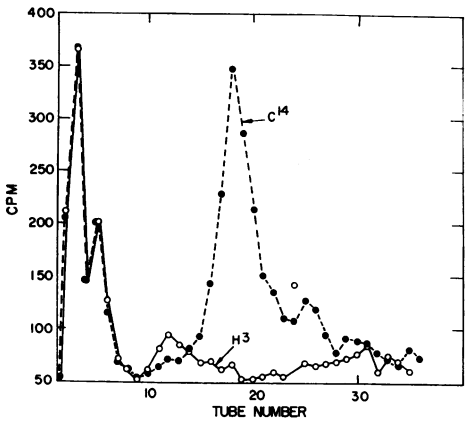

FIG. 5.—DEAE elution profile of a mixture of H^3-leu-labeled λC_1*ts*U32 infected cells and C^{14}-leu-labeled λ wild-type infected cells. Other details as in Fig. 3.

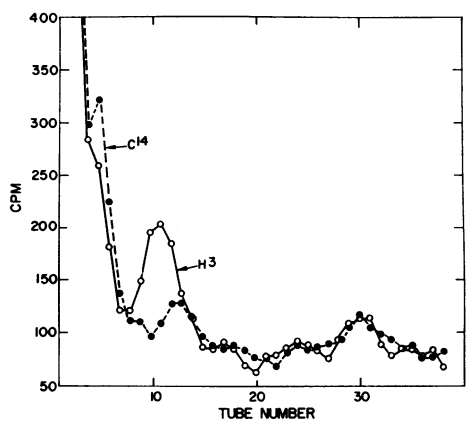

FIG. 6.—DEAE elution profile of a mixture of H^3-leu-labeled λC_1*ts*U32 infected cells and C^{14}-leu-labeled λC_1*sus*34 infected cells. Other details as in Fig. 3.

the synthesis of some bacterial proteins and possibly the limited synthesis of a few phage proteins other than the repressor.

Temperature-sensitive C_1 mutants: The absence of the isolated protein from C_1 amber mutant infected cells strongly suggests, but does not prove, that the structural gene for this protein is the C_1. To confirm this directly, three temperature-sensitive C_1 mutants (C_1*ts*857, C_1*ts*U32, and C_1*ts*t2) have been examined to determine whether a modified form of the protein is produced by these mutants. In each case the repressors are labeled at temperatures at which they are functional *in vivo*.

Figure 4 shows the DEAE elution profile of a mixture of H^3-labeled C_1*ts*857

TABLE 2

DIFFERENTIAL FRACTIONATION OF λC1*ts*2 AND WILD-TYPE C1 PRODUCTS

Fraction	Ratio H^3/C^{14}	Fraction	Ratio H^3/C^{14}
(1) Sonicate	2.39	(2) Sonicate	3.03
	2.39		3.00
Supernatant	2.27	Supernatant	3.13
	2.30		3.19
Pellet	2.52	Pellet	2.85
	2.49		2.78

In (1), H^3-leu-labeled λC1*ts*2 infected cells were mixed with C^{14}-leu-labeled λC1*sus*34 infected cells. In (2), H^3-leu-labeled wild-type infected cells were mixed with an aliquot of the same C^{14}-leu-labeled λC1*sus*34 infected cells used in (1). Ratios are given from duplicate samples from each fraction. About half the label appears in the pellet following centrifugation. The experiment was performed as described in *Materials and Methods*, except that the infected cells were labeled at 32°C.

repressor and C^{14}-labeled wild-type repressor. The mutant C1*ts*857 repressor chromatographs identically to wild type. The result of a similar experiment with mutant C1*ts*U32 gives a strikingly different result (Fig. 5). No peak corresponding to the C1*ts*U32 repressor is seen under the major C^{14} peak. A much smaller H^3 peak of variable height which is probably the C1*ts*U32 gene product is observed around tube 12. This small peak is seen more easily in an experiment in which the C1*ts*U32 and C1*sus*34 gene products are chromatographed together (Fig. 6). The most likely reason that only a small amount of the C1*ts*U32 gene product is seen is that this material is partially insoluble. Another temperature-sensitive mutant, C1*ts*t2, produces an even less soluble product. No significant differentially labeled H^3 peak is seen when the experiment described in Figure 6 is performed with this mutant. The C1*ts*t2 repressor is labeled in these experiments, but it precipitates and appears in the pellet upon sonication and subsequent centrifugation (Table 2). Although H^3-labeled wild-type repressor produces a H^3/C^{14} ratio increase in the supernatant when tested against C^{14}-labeled C1*sus*34, H^3-labeled C1*ts*t2 repressor produces a ratio increase in the pellet when tested against this mutant. The fact that the magnitude of the ratio changes is about equal in the two cases suggests that C1*sus*34 produces only a small C_1 fragment, if any.

The important finding is that two mutations which produce modified repressors *in vivo* also alter the major protein synthesized in the present experiments.

Electrophoresis of the C_1 product: The migration of the wild-type C_1 product in a 7.5 per cent polyacrylamide gel is shown in Figure 7. The material applied to the gel was collected and concentrated from the peak fractions of a DEAE run. A single major H^3 band is observed, with no corresponding C^{14} peak. This shows that the differentially labeled peak recovered from DEAE consists of a single-labeled protein of high isotopic purity. The C_1 product migrates toward the anode at pH 8.7 as would be expected for an acidic protein.

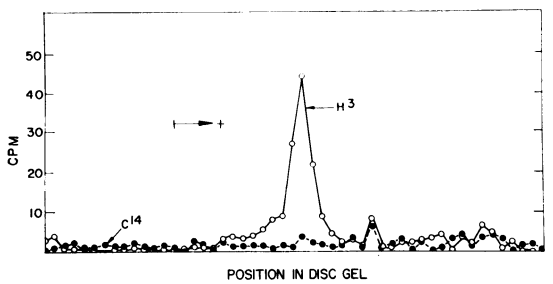

FIG. 7.—Polyacrylamide gel electrophoresis of the protein isolated from DEAE columns. See *Materials and Methods* for experimental details.

A mixture of H^3-labeled C1*ts*857 repressor and C^{14}-labeled wild-type repressor was isolated on

DEAE and subjected to electrophoresis as in Figure 7. A single superimposable major band appears in both labels, showing that the C$_1$*ts*857 product bears the same charge as the wild-type C$_1$ product.

Sedimentation of the C$_1$ product: Sedimentation of the wild-type C$_1$ product from the major DEAE peak was followed on sucrose gradients. Figure 8 shows the approximate *s* value of the C$_1$ product as 2.7–2.8*S*. This corresponds to a molecular weight of approximately 30,000 if the label is in a spherical protein.

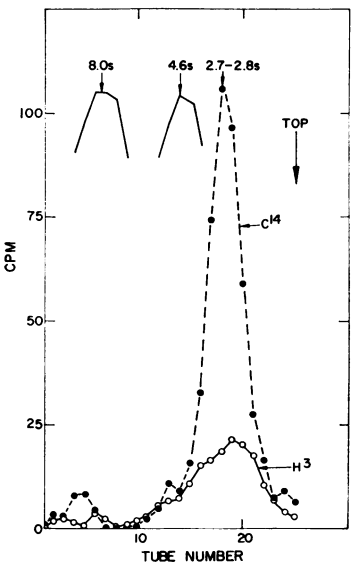

FIG. 8.—Sedimentation of the protein isolated from DEAE columns. The peak fractions from a DEAE run were pooled and concentrated, and a sample was layered on a 5–30% sucrose gradient in T-M buffer plus 0.15 *M* KCl. The gradient was spun 60,000 rpm for 10.5 hr at 8°C. Parallel tubes were run carrying a mixture of the markers aldolase and hemoglobin. Fractions were collected and assayed for radioactivity or for absorbance at 230 and 410 mμ.

Discussion.—The behavior of the temperature-sensitive mutants is particularly interesting because of the observation[7,8] that, *in vivo*, temperature-sensitive mutations mapping on the left side of C$_1$ cause a more drastic alteration of the repressor than do temperature-sensitive mutations mapping on the right side of C$_1$. It has been suggested that right-hand side mutants such as C$_1$*ts*857 reversibly denature when heated; repression is restored when the temperature is lowered. In contrast, left-hand side mutants, such as C$_1$*ts*t2 and C$_1$*ts*U32, apparently are irreversibly denatured by a pulse of heat. Attempts to attribute this difference to the existence of two C$_1$ products have so far failed: no complementation has been detected between amber mutants from opposite sides of the C$_1$ gene, nor between any amber mutants and temperature-sensitive mutants located anywhere in the gene.[9] In experiments reported here, no difference has yet been detected between the wild type and C$_1$*ts*857 repressors *in vitro*, but the C$_1$*ts*t2 and C$_1$*ts*U32 repressors are found to differ markedly from wild type. Under standard conditions, the unmutated repressor appears in the supernatant following high-speed centrifugation, the C$_1$*ts*t2 repressor invariably precipitates, and the C$_1$*ts*U32 repressor shows an intermediate solubility. The fraction of C$_1$*ts*U32 repressor which remains in the supernatant following centrifugation chromatographs separately from the wild type on DEAE columns. Furthermore, the *in vitro* distinction between the C$_1$*ts*t2 and C$_1$*ts*U32 repressors is consistent with the observation that *in vivo* the latter is more heat-stable than the former.

The repressor isolated from DEAE has an apparent molecular weight of approximately 30,000. The molecular weight of another repressor, the *lac i*-gene product, has been estimated at 150,000–200,000.[3] There is indirect evidence that the functional *i*-gene product is an oligomer.[10] It is possible that the λ repressor can also exist as an oligomer, and that I have isolated it in some subunit form. In this regard it may be significant that the sedimentation of the *lac* repressor was effected at high concentrations (about 10^{-6} *M*), whereas in the experiments de-

scribed here, the concentrations are much lower (about 10^{-8} to 10^{-9} M). There is a small peak of high s value in the sedimentation profile shown in Figure 8, but I have not attempted to characterize this fraction or the small peak which consistently emerges immediately behind the major peak on DEAE columns. Either of these components might represent alternate forms of the repressor.

Summary.—A product of the λC_1 (repressor) gene has been labeled with amino acids and isolated free from any other labeled components of the cell. The identification is based on the fact that this molecule is not made by amber mutants and is made in a modified form by temperature-sensitive mutants of the C_1 gene. The product electrophoreses in a single band, moving as an acidic protein. It sediments at 2.8S, which corresponds to a molecular weight of approximately 30,000.

Materials and Methods.—Bacteria: The bacterial strain used in all these experiments is a UV-sensitive mutant isolated by Dr. M. Meselson from the *E. coli* strain W3102.[11] This strain is prototrophic and nonpermissive (su^-) for amber mutants. The UV^s locus mutated in this strain is unknown. This strain is used because, as with some other UV^s strains,[12] its capacity to support λ phage growth is higher than that of wild-type strains following exposure of the host to the same physical dose of UV irradiation.

Bacteriophages: All the λ phages carrying double N mutations were prepared by recombination with the phage $\lambda N o sus_7 sus_{53}$ isolated by Dr. D. Hogness. The derivative of phage λimm^{434} used here was described previously.[13] Phage λind^- was isolated and described by Jacob and Campbell.[14] Two amber mutants,[15] $C_1 sus34$ and $C_1 sus80$, were supplied by Dr. F. Jacob. One of the temperature-sensitive mutants, $C_1 ts857$, was isolated and described by Sussman and Jacob.[16] The other temperature-sensitive[7] and amber mutants were isolated and donated by Dr. M. Lieb. All the phages used in these experiments carry the h marker from the phage λh of Kaiser[17] to ensure efficient absorption. Phage stocks were grown in strain C600 by the agar-overlay method, pelleted, resuspended in phage buffer, and dialyzed against the same buffer.

Media and buffers: Cells were grown in A medium[18] with 4 gm/liter maltose as carbon source. Preconditioned medium was prepared by filtration of A medium which had supported the growth of cells to 2–3 $\times$ 10^8 cells/ml. T-M buffer is 0.01 M Tris pH 7.4, 0.005 M $MgSO_4$. Phage buffer is T-M buffer made 0.1 M NaCl.

Radioactivity: H^3- and C^{14}-labeled leucine and reconstituted protein hydrolysates were purchased from Schwartz BioResearch, Inc., and from New England Nuclear Corp. These labeled compounds were used without further dilution with cold amino acids. Numerous different batches of isotopes were used during the course of these experiments. The specific activity of the C^{14}-leu is about 200 mc/mmole, and that of the H^3-leu is about 2.0 c/mmole. All radioactive isotope counting, unless specified otherwise, was performed according to the method described by Fox and Kennedy,[19] except that the ethanol wash was replaced with another TCA wash.

Electrophoresis: Disc electrophoresis was performed at pH 8.7 as described by Davis.[20] After electrophoresis the gels were frozen and cut into slices 1 mm thick using a slicing apparatus designed and built by Dr. C. Levinthal. The slices were distributed into counting vials and dissolved in $^1/_2$ ml of 15% hydrogen peroxide by heating at 60–70°C for a few hours. One-half ml of hydroxide of hyamine (Packard Inst. Co.) was added to the vials, and the mixture was incubated at 57°C for 3 min. After cooling, 10 ml of Bray's[21] solution was added, and the samples were counted in a Packard scintillation counter.

Chromatography: DEAE-cellulose chromatography was performed using a 15-ml column, 1 cm in diameter, with Whatman DEAE-cellulose #DE52. After applying the sample and washing the column with about 10 ml of T-M buffer containing 0.07 M KCl, a 150-ml linear KCl gradient was run to 0.2 M KCl, and fractions containing about 4 ml were collected. Aliquots from each fraction were assayed for radioactivity.

Irradiation: Cells were irradiated at a distance of 30 cm from 2 G.E. 15-w germicidal lamps. The incident dose at this distance is about 75 ergs mm^{-2} sec^{-1}. Eighty ml of cells at concentration 3 $\times$ 10^8 cells/ml were irradiated at 0°C in a Petri dish of diameter 11 cm, with swirling.

A typical experiment: Cells grown to a concentration of 10^9 cells/ml in medium A are chilled and diluted to a concentration of 3 $\times$ 10^8 cells/ml with cold preconditioned medium. The cells

are irradiated for 12 min, and the $MgSO_4$ concentration is then raised from 10^{-3} *M* to 2×10^{-2} *M*. Two 40-ml portions are distributed into flasks, and phage are added at a multiplicity of 30–35 phage/cell. After swirling 5 min at 37°C, 0.4 mc H^3-leu is added to one flask, and 0.04 mc C^{14}-leu to the other, and the cells are aerated for 1 hr at 37°C. The cells are then chilled, centrifuged, mixed together, washed twice in T-M buffer, and finally resuspended in 2 ml of T-M buffer containing 0.4 *M* KCl and a few micrograms of DNase. The cells are disrupted while on ice by several 30-sec pulses delivered by an MSE sonicator. Small aliquots are taken to determine the original ratio of H^3/C^{14}, and the extract is immediately spun at 350,000 *g* for 36 min in an International B-60 centrifuge. The supernatant is withdrawn and the pellet is resuspended in 2 ml of T-M buffer. After removing samples for ratio counting, the supernatant is dialyzed overnight against T-M buffer plus 0.07 *M* KCl.

It is of critical importance that the Mg^{++} concentration be raised before addition of the phage. Phage λ absorbs well to cells grown in medium A with maltose as the carbon source, even at low Mg^{++} concentrations. However, if the experiment described in Figure 1 is performed in the presence of only 10^{-3} *M* Mg^{++}, no stimulation of labeled leucine incorporation is observed. In fact, even at high UV doses, the addition of 35 phage/cell causes a further 10–20-fold decrease in the incorporation of labeled leucine. Others have noted a strong inhibition in protein and RNA synthesis following infection of unirradiated cells with high multiplicities of phage λ.[22] The discovery that high concentrations of Mg^{++} can reverse this inhibition, at least in the irradiated strain used here, made possible the success of these experiments.

Throughout the course of this work I have had the advice and encouragement of Walter Gilbert and James Watson, for which I am very grateful. I would also like to thank Matthew Meselson for providing laboratory space and many facilities necessary for these experiments; Nancy Hopkins and Louise Rogers for excellent assistance; and Margaret Lieb, Racquel Sussman, and François Jacob for phage mutants.

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