cent deoxycholate. (Colicin El was a gift of M. Nomura and W. Reznikoff.)

32. Cultures (5 ml) of a number of single transformant colonies were grown to 5×10^8 cells per milliliter and incubated with chloramphenicol (100 μg/ml) overnight. The cells were harvested by centrifugation and resuspended in 0.1 ml of 0.16*M* tris acetate, *p*H 8.0, 0.08*M* NaCl, 0.08*M* sodium acetate, 0.008*M* EDTA, 16.5 percent glycerol, 1.7 percent sodium dodecyl sulfate, 0.003*M* EDTA, and bromophenol blue. The suspension was heated to 70°C for 10 minutes, vortexed until the viscosity decreased, and incubated with ribonuclease (100 μg/ml) at 37°C for 1 hour. DNA was examined by agarose gel electrophoresis (*24*).
33. Low titer stocks of the tight *ori*⁻ mutants were obtained by temperature induction of Ymel*su*III(λ*N*am7*N*am53*c*Its857*ori*⁻) lysogens at 42°C for 20 minutes, followed by incubation at 37°C for 4 to 6 hours. Chloroform was added to complete lysis, and cell debris was removed by low-speed centrifugation. Titers of lysates generally ranged from about 10^8 to 10^9 phages per milliliter. Phages were precipitated by addition of polyethylene glycol-6000 (PEG) to 7 percent and NaCl to 1*M*, and collected by centrifugation. The PEG pellet was washed twice by centrifugation in SM buffer [J. Weigle, M. Meselson, K. Paigen, *J. Mol. Biol.* **1**, 379 (1959)], and the phages were recovered in the supernatants. The phage suspension was made 1.3 g/ml with CsCl, layered onto step gradients (*23*), and centrifuged for 2 hours at 30,000 rev/min in an SW41 motor (Beckman). The phage band was collected and concentrated by centrifugation to equilibrium in CsCl (*23*). High titer (5×10^{10} phages per milliliter) stocks of the leakier *ori*⁻ mutant *ti*12 were obtained by induction of the lysogen TC600*su*II(λ*N*am7*c*I857*ti*12*S*am7), followed by incubation at 38°C for 7 to 8 hours. In this strain the *S*am7 mutation is not suppressed, and the resulting defect in cell lysis greatly improves the final yield of the *ori*⁻ phage. Phages were concentrated as described above. DNA was extracted as described (*23*).
34. *O*am29: A. Campbell, *Virology* **14**, 22 (1961); *O*am905: P. Toothman, thesis, University of Oregon (1976).
35. For construction of chimeric phages the ligation mixtures contained Eco RI digested Charon 3 DNA at 150 μg/ml and target DNA at 270 μg/ml. Transfection of spheroplasts was as described by W. Henner, I. Kleber, R. Benzinger [*J. Virol.* **12**, 741 (1973)]. Phages carrying the *imm*λ-*ori*λ fragment were identified by their ability to complement λ mutants defective in *cro*, *c*I, and *c*II (*22*). For plasmid constructions the ligation mixtures contained Eco RI digested pVH51 DNA at 50 μg/ml and Eco RI and Hind III digested target DNA at 200 μg/ml. Hind III was used to prevent insertion of irrelevant Eco RI fragments. Tranformation was as described (*31*). Clones carrying the *imm*434-*ori*λ fragment are resistant to λ*imm*434*c*I but sensitive to λ*c*I and were identified by cross-streaking individual colonies against streaks of these two phages (from suspensions at 10^7 to 10^8 phages per milliliter) on EMB-O plates [M. Gottesman and M. Yarmolinsky, *J. Mol. Biol.* **31**, 487 (1968)]. Ligase reactions contained 160 m*M* tris-HCl, *p*H 7.8, 10 m*M* $MgCl_2$, 25 m*M* dithiothreitol, 0.02 m*M* adenosine triphosphate, and two units of T4 DNA ligase (Miles Research Products or New England Bio-Labs) and were incubated overnight at 4°C. Success of ligation was verified by agarose gel electrophoresis.
36. In the n orientation the standard coordinate systems of vector and inserted fragment increase in the same direction (*14*). We have chosen as the standard coordinate system for pVH51 that described by K. Armstrong, V. Hershfield, D. Helinski [*Science* **196**, 172 (1977)] and J.-I. Tomizawa, H. Ohmori, R. Bird [*Proc. Natl. Acad. Sci. U.S.A.* **74**, 1864 (1977)], in which the origin of *Col* E1 replication lies about 1300 base pairs clockwise from the single Eco RI site. The single Hinc II restriction site in pVH51 lies about 150 base pairs counterclockwise from the Eco RI site. Because the *imm*434-*ori*λ fragment also contains a single Hinc II site, the orientation was determined by digestion with this enzyme.
37. This is paper 2170 from the Laboratory of Genetics of the University of Wisconsin and paper No. 5 in the series "Charon phages for DNA cloning." We thank Ed Kopetsky, Brenda Dierschke, and Carol McLeester for technical assistance and William Dove for critical reading of the manuscript. These experiments were performed under NIH guidelines, which call for EK1, P1. Supported by NIH grant GM21812 to F.R.B. NIH grant CA07175 to the McArdle Laboratory (W. F. Dove), NSF predoctoral fellowship to M.E.F., NIH training grant T32 CA09135 to the McArdle Laboratory, NIH training grant T32 CA09075 to K.D-T, and NIH training grant 144-J825 to D.D.M.

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Genetic Structure of the Replication Origin of Bacteriophage Lambda

Abstract. *A fragment of bacteriophage lambda DNA produced by the restriction endonuclease Eco RI and extending from the immunity region to a point inside gene* O *is found to have a fully functional origin of replication. Seven* ori⁻ *mutations of lambda cluster in a small region just to the left of the Eco RI cleavage site which defines the right end of this fragment. These mutations lie within gene* O.

We have determined the genetic fine structure of the bacteriophage lambda (λ) replicator, *ori*, the DNA target for control of the λ replicon. This regulatory element can be defined by mutations, called *ori*⁻, which prevent initiation of DNA replication, and which are *cis*-dominant to *ori*⁺. The λ*ori*⁻ mutants have been obtained among mutants of λ prophage which fail to kill the *Escherichia coli* host upon induction (*1–4*). The *ori*⁻ mutants remain able to transcribe the λ replication genes *O* and *P*, and all studied here were chosen to retain active O and P proteins. The *ori*⁻ mutants are deficient in DNA replication, even in mixed infection with an *ori*⁺ phage. An *ori*⁻ phage can be replicated extensively only if it is joined in tandem to another replicon.

The λ replicator also can be defined by its specific interaction with the initiator. The replication protein encoded by gene *O* is type specific, so that initiation at the λ replication origin cannot be promoted by the analogous gene product of a related phage such as φ80 or P22 (*5–7*).

The function of the λ replicator appears to be activated by local rightward RNA synthesis. Replication of λ is prevented by repression by the *c*I protein of transcription initiating from promoter p_R (see Fig. 1), even if all essential gene products are provided by a heteroimmune helper phage such as λ*imm*434 (*8*). The inhibition of replication by the *c*I repressor can be overcome by mutations, termed *ri*ᶜ, which permit constitutive rightward transcription in the vicinity of *ori* (*1, 2, 9, 10*).

In our study we first delimited the λ replicator by studying the capacity of cloned DNA restriction fragments to utilize λ proteins to direct the replication of a chimeric phage. We found that all essential components of the replicator lie to the left of an Eco RI restriction site located within the replication region of the λ genome. We then mapped this Eco RI site, and found that it lies within the initiator gene *O*. Finally, we were able to show that the components of the λ replicator defined by *ori*⁻ mutations also lie within *O*, in a small cluster near the Eco RI site.

The insertion of fragments from the left or right side of the Eco RI site in the λ replication region into the φ80 phage vector Charon 3 (*11*) is described by Moore *et al.* (*12*) (Fig. 1).

We have devised a test for the presence of a functional λ replication origin on a DNA fragment cloned in Charon 3. Our strategy was to infect cells with the chimeric phage under conditions that prevent replication from initiating at the vector's φ80-type origin, and to coinfect with a helper phage to supply all diffusible λ gene products. If a functional λ origin is present on the cloned fragment, it should permit replication of the test phage. This can be assayed by measuring the yield of the Charon 3 derivative in the phage burst. We have used two methods to prevent replication from starting at the vector's origin. In some experiments, the test phage carries a mutation in a gene essential for φ80 replication (*13, 14*). In other cases, the host cell carries a φ80 prophage; the φ80-specific immunity repressor prevents expression of the Charon 3 replication genes. Our results show that the only Charon 3 derivatives that can replicate in the functional origin test are those which contain a fragment from the left of the Eco RI site in the λ replication region (an *imm-ori* λ fragment, of either λ or 434 immunity type).

The functional origin test is illustrated in Table 1. A replication-deficient Charon 3 derivative with no inserted fragment yields few progeny phage, even in the presence of λ helper phage. However, a clone carrying the *imm*434-*ori* λ fragment gives a substantial burst in the presence of helper, even when vector replication is doubly blocked. Thus, the cloned *imm-ori*λ fragment appears to contain a functional replication origin.

To exclude the possibility that replication of the chimeric test phage occurs

passively, by formation of a tandem replicon with the helper phage (*15*), we repeated the functional origin test under conditions in which all sources of genetic recombination are eliminated by phage and host mutations. Replication from the vector origin is prevented by ϕ80 repressor. Here the burst size of a Charon 3 derivative carrying an *imm*λ-*ori*λ fragment (Table 2, tests 2, 3, 6, and 11) remains at least 50-fold greater than those of control phages; neither Charon 3 (Table 2, test 1) nor a Charon 3 clone carrying the righthand portion of the λ replication region (Ch3S45, Table 2, test 5, and see Fig. 1) grows under these conditions.

Because the phage yields in the recombination-deficient test system are relatively low, we wished to determine whether the progeny phage contain newly replicated DNA. The extent of replication was assessed by tagging the parental phage with the host-specific modification conferred by a P1 lysogen, and measuring the dilution of this label in progeny phage (*16*). We found that the burst of a Charon 3 derivative carrying an *imm*λ-*ori*λ fragment is comprised of particles of which at least 95 percent contain only newly synthesized DNA (Table 2, test 2). In contrast, the background yields seen in controls represent particles carrying at least one parental DNA strand (Table 2, test 1).

Does an *imm*-*ori*λ fragment direct replication by providing a functional origin, or does it release the block imposed on the vector's replication system? We determined whether in a dominance test an active Charon 3 derivative, carrying an *imm*λ-*ori*λ fragment, can promote in *trans* the replication of Charon 3 lacking an inserted fragment. If cells are infected simultaneously with Ch3S7Δ2h, Charon 3, and λ helper, we find that Ch3S7Δ2h grows but Charon 3 does not (Table 2, test 3). Thus, in the functional origin test a chimeric phage carrying an *imm*-*ori*λ fragment replicates because of the *cis*-dominant action of an element present on the fragment. We take this element to be the λ replication origin.

Further experiments prove that the transposed origin of replication retains the following properties of the normal λ replicator: (i) requirement for replication proteins of the λ type; (ii) dependence upon origin region transcription for replication; and (iii) inactivation by mutations known to block function of the λ origin (*cis*-dominant *ori*$^-$ mutations).

The requirement for replication proteins of the λ type was tested by using as helper a hybrid phage, λ*rep*P22, in which all known λ replication functions are replaced by heterospecific ones from the *Salmonella* phage P22 (*5*). As is shown in Table 2, test 4, this heterospecific helper fails to act on the *ori* target carried on the *imm*λ-*ori*λ fragment in Ch3S7Δ2h.

Stimulation of origin function by local rightward transcription within the cloned *imm*-*ori*λ fragment was studied by an experiment analogous to that of Thomas and Bertani (*8*). With a derivative of Ch3λ2 that carries an *imm*434-*ori*λ fragment (*12*), transcription of the transposed origin region is repressed in cells lysogenic for λ*imm*434. In Table 3 we see that, when repressed, normal λ*imm*434 phage is unable to replicate in the presence of λ helper (test 2 compared to test 1). Similarly, when transcription of the *imm*434-*ori*λ fragment is repressed, Ch3λ2*imm*434Δ7c is unable to replicate in the functional origin test (test 4 compared to test 3). As a control, we see that the effects of repression on the activity of the transposed origin are immunity

Table 1. Test for λ *ori* function of Ch3λ2 derivatives. Two ϕ80 *14*am8 derivatives were used as test phages, namely, Charon 3 and Ch3λ2*imm*434Δ7c. Cells of strain 594*su*^{0} or of the lysogen 594(ϕ80 *14*am8) were grown in tryptone broth with 0.2 percent maltose, harvested by centrifugation, and resuspended in TM buffer (0.01*M* tris-HCl, *p*H 8.0, 0.01*M* $MgSO_4$) at 4×10^8 cells/ml. Cells were infected with the Charon 3 derivative to be tested (test phage) (*12*) at a multiplicity of infection of 0.1 to 0.3. The introduction of *imm*434 and the deletion Δ7c into Ch3λ2 is described by Moore *et al.* (*12*). In the cases indicated, helper phage λ*b*221 *bet*am270 *c*I26 (a gift of D. Berg) was introduced at a multiplicity of infection of 3 to 5. After adsorption for 15 minutes at 37°C, the infected cells were diluted 500-fold into LB broth (1 percent tryptone, 0.5 percent yeast extract, 1 percent NaCl, 0.2 percent glucose), and growth was allowed for 90 minutes at 37°C. The yield of the test phage was ascertained by determining the titer on C600*su*II(λ), while that of the λ helper was measured by determining the titer on Ymel*su*III(ϕ80). The burst size of the test phage is calculated as the ratio of yield to input phage. The yield of the helper averaged 3.6 per input phage.

Test phage, ϕ80 *14*am8 derivative of:	*ori* Region on fragment	Helper, λ*b*221 *red*270 *c*I60	Host	Burst size of test phage
Charon 3	None	−	594	0.2
		+	594	0.2
		+	594(ϕ80 *14*am8)	0.3
Ch3λ2*imm*434Δ7c	*ori*λ	−	594	0.2
		+	594	13
		+	594(ϕ80 *14*am8)	13

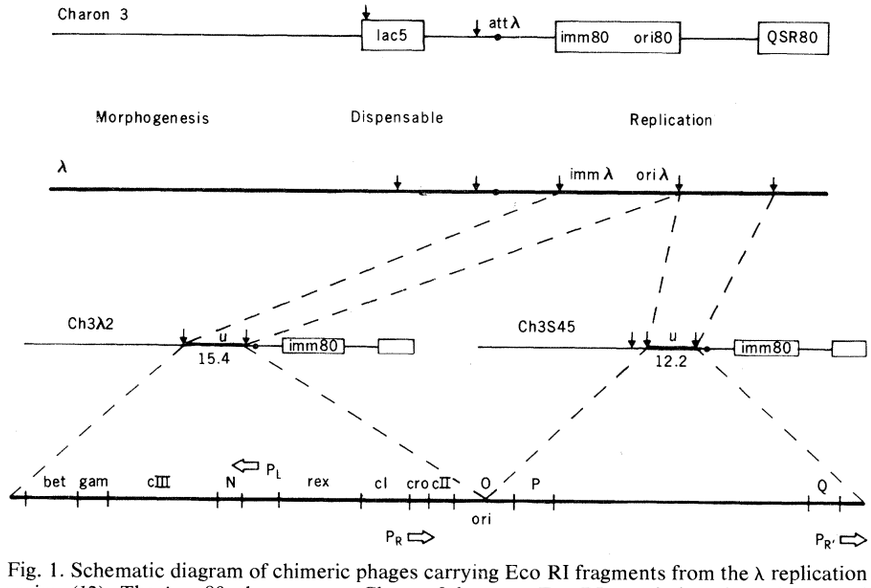

Fig. 1. Schematic diagram of chimeric phages carrying Eco RI fragments from the λ replication region (*12*). The *imm*80 phage vector Charon 3 has two Eco RI restriction sites (downward arrows) flanking a nonessential region, lying to the left of the attachment sequence for integration into the host chromosome (*att*λ), which includes the *lac*5 substitution (*11*). In Ch3λ2 and Ch3S45d, this region has been replaced by an Eco RI fragment of λ DNA from either side of the Eco RI site within the λ replication region. One fragment contains 15.4 percent of the λ genome, and the other 12.2 percent. In each case the λ fragment inserted into Charon 3 is inverted (u) with respect to its usual orientation. The bottom line shows the genetic map of the region spanned by the two cloned fragments, drawn roughly to scale (although some genes in the vicinity of *c*III have been omitted). Large arrows indicate the direction of transcription from each of the major early promoters (p_L and p_R) and the late promoter (p_R').

specific; the replication of Ch3λ2, carrying an *imm*λ-*ori*λ fragment, is unaffected by *imm*434 repression (test 6 compared to test 5). Thus, activity of the transposed λ origin requires transcription from the p_R promoter.

A stringent criterion that the transposed *imm-ori*λ fragment serves as a normal origin of λ replication is that this fragment contains the sites of the *cis*-dominant *ori*$^-$ mutations, which define essential components of normal λ origin function (*1–4*). We find that those Charon 3 derivatives which carry the *imm-ori*λ fragment can recombine with each of seven λ*ori*$^-$ mutants to give λ*ori*$^+$ progeny. In each case, the *ori*$^+$ allele is rescued from the chimeric phage at a level at least 100-fold above the background observed in control crosses with the *imm*80-*ori*80 vector (data not shown).

Not only does the *imm-ori*λ fragment cover the site of each of these *ori*$^-$ mutations, but it is inactivated for its replicator function by the introduction of an *ori*$^-$ mutation. Charon 3 clones carrying each of four representative *ori*$^-$ mutations within the 15.4 percent *imm*λ-*ori*λ Eco RI fragment have been made either by genetic recombination between Ch3λ2*imm*434 and a λ*ori*$^-$ phage, or by direct insertion into Charon 3 of this fragment from DNA preparations of λ*ori*$^-$ phage stocks (*12*). Functional origin tests of these chimeric phages carrying mutant λ replicators are displayed in Table 2, tests 7 to 11. Replication capacity is reduced by about 30- to 70-fold by each of the *ori*$^-$ defects.

We conclude that the translocated *imm-ori*λ Eco RI fragment of λ contains a fully competent replication origin displaying all known specificity requirements of the normal λ origin. Thus, no specific λ DNA sequences outside of this fragment are required for a functional replication origin.

Because the *imm-ori*λ Eco RI fragment contains an intact functional origin, it helps to delimit the λ replicator. It is important, therefore, to localize the Eco RI site in the replication region with respect to the λ genetic map.

Charon 3 clones carrying the *imm-ori*λ Eco RI fragment do not express λ *O* function (*12*). This implies that the end point of this fragment does not lie to the right of gene *O*. The Eco RI site was mapped by crossing each of a set of λ*O*am nonsense mutants with appropriate derivatives of Ch3λ2 and Ch3S45 (*13*). These crosses were analyzed by determining the fraction of *O*$^+$ phage among total λ progeny (marker rescue). The data of Table 4 reveal that a derivative of Ch3λ2, carrying the *imm*λ-*ori*λ Eco RI fragment, recombines productively with *O*am29 but not with *O*am1005. In contrast, a derivative of Ch3S45, carrying the adjacent 12.2 percent Eco RI fragment, rescues *O*am1005 but not *O*am29. Thus, the Eco RI restriction site in the λ replication region must lie between the positions of these two amber mutations in gene *O* (Fig. 2).

We have mapped two additional mutations affecting DNA replication with respect to the Eco RI site in gene *O*. The rightmost of the *ri*^{c} mutations (*ri*^{c}5b and *ri*^{c}D), which permit λ to replicate in the presence of helper phage when transcription from p_R is repressed, have been shown to lie within gene *O* (*2*). Each of these *ri*^{c} mutants contains a new promoter directing constitutive transcription to the right (*1, 2, 10*). Surprisingly, we find that *ri*^{c}5b and *ri*^{c}D lie to the right of the Eco RI site in *O*, and thus outside of the DNA fragment shown here to be sufficient for *ori* function (*17*).

The *ori*$^-$ mutations define essential components of the λ replication origin, and lie to the left of the Eco RI site in structural gene *O*. Previous studies in this laboratory and others have placed *ori*$^-$ mutations to the left of gene *O* markers, and to the right of gene *c*II markers and of the sequence encoding *oop* RNA (*1–5, 18*) (see Fig. 2). The isolation of ad-

Table 2. Test for λ *ori* function in a *recA* host. Cells of strain N100*recA*su^0 (φ80 *8*am2) (*14*) were grown and infected as described in the legend of Table 1. In the cases indicated, test phages and helper were modified by growing stocks on Ymel (Pl). λ*b*221 *bet*am270 *c*I26 was used as helper in all cases except test 4, in which λ*c*Its857 *rep*P22 (*7*) was used. The structures of Charon 3 and Ch3S45 (*12*) are shown in Fig. 1. Ch3S7 is identical to Ch3λ2, except that it carries a small fragment of *E. coli* DNA in addition to the *imm*λ-*ori*λ fragment (*12*). The deletion Δ2h extends from a point to the right of *att* in the vector into the inserted *imm*λ-*ori*λ fragment, and eliminates λ functions *bet*, *gam*, *c*III, and *N*. The total yield of the test phage was measured on Ymel(λ), and the yield of test phage carrying Pl-modified DNA (*16*) was measured on Ymel(λ)(Pl). The yield of helper phage was measured on Ymel(φ80) and Ymel(φ80)(P1). In the dominance test (test 3), cells were mixedly infected with Charon 3 and Ch3S7Δ2h, each at multiplicity of infection 3, and with λ helper at a multiplicity of infection of 5. The progeny Charon 3 and Ch3S7Δ2h were distinguished by plating on XG plates, on which Charon 3 forms blue plaques (*11*). The average helper yield was 2.0 per input phage, and reflected 40-fold replication as judged by dilution of P1-modification.

Test	Phage	Cloned λ fragment	Helper	Burst size of test phage	Fold DNA replication*
1	Charon 3	None	λ	0.02	1.0
2	Ch3S7Δ2h	*imm*λ-*ori*$^+$	λ	1.5	26
3	Charon 3	None		0.01	1.0
	+		λ		
	Ch3S7Δ2h	*imm*λ-*ori*$^+$		1.1	28
4	Ch3S7Δ2h	*imm*λ-*ori*$^+$	λ*rep*P22	0.02	
5	Ch3S45	12.2% (*O*am1005-*P*-*Q*)	λ	0.02	
6	Ch3λ2	*imm*λ-*ori*$^+$	λ	1.5	
7	Ch3λ12	*imm*λ-*ori*$^-$*ti*12	λ	0.05	
8	Ch3λ93	*imm*λ-*ori*$^-$*r*93	λ	0.03	
9	Ch3λ96	*imm*λ-*ori*$^-$*r*96	λ	0.04	
10	Ch3λ99	*imm*λ-*ori*$^-$*r*99	λ	0.02	
11	Ch3λ29	*imm*λ-*ori*$^+$*O*am29	λ	1.4	

*Calculated as the ratio of total phage or P1-modified phage in the yield.

Table 3. Transcriptional activation of the translocated λ *ori* region. Cells of strain N100(φ80 *8*am2) or of N100(φ80 *8*am2) (λ*imm*434 *rep*P22 *12*amN14) were grown and infected as described (Table 1, legend). The helper phage was λ*b*221 *bet*am270 *c*I26. The test phages were Ch3λ2 (*12*) (Fig. 1), its derivative Ch3λ2*imm*434Δ7c (*12*) (Table 1), and λ*imm*434 *c*II2002. The φ80 prophage prevents expression of the φ80-type replication genes of Ch3 test phages. The λ*imm*434 prophage prevents transcription from initiating at p_R of the λ*imm*434 test phage, and of the cloned *imm*434-*ori*λ fragment, but does not affect transcription of an *imm*λ-*ori*λ fragment. The yield of each test phage was measured on 594su^0(λ), while helper yield was measured on 594su^0(λ*imm*434) (tests 1 and 2) or on 594su^0(φ80) (tests 2 to 6). The average helper yield was 1.4 per input phage.

Test	Phage	Helper	Host	Transcription of test *ori* region	Burst size of test phage
1	λ*imm*434	λ	N100(φ80)	+	3.8
2	λ*imm*434	λ	N100(φ80)(λ*imm*434)	−	0.1
3	Ch3λ2*imm*434Δ7c	λ	N100(φ80)	+	2.7
4	Ch3λ2*imm*434Δ7c	λ	N100(φ80)(λ*imm*434)	−	0.1
5	Ch3λ2 (*imm*λ)	λ	N100(φ80)	+	1.8
6	Ch3λ2 (*imm*λ)	λ	N100(φ80)(λ*imm*434)	+	2.3

ditional prophage deletions and nonsense mutations in gene *O* allows us to extend the fine-structure mapping of *ori* and *O* (*19*).

The λ prophage deletions were transferred to a common host genetic background, permissive for the growth of amber mutants (*20*). We determined the fraction of wild-type recombinants among progeny phage issuing from such deletion strains after infection with λ*ori*⁻ and *O*am mutants. The results of these marker rescue experiments are shown in Table 5. Seven representative *ori*⁻ mutations lie in the deletion interval defined by F3 and MJ17 on the left and M25 on the right (see Fig. 2). The site of *O*am29 also lies in this interval (*18*). To our surprise, however, the site of *O*am905 appears to lie to the left of this interval, outside deletions F3 and MJ17. This suggests that the seven λ*ori*⁻ mutations lie within gene *O* (*19*).

The ability of *O*am905 to recombine with F3 and MJ17 was tested more critically by a map-expansion technique. The strategy of these crosses is shown in Fig. 3. Prophage deletion strains were infected with *imm*434 derivatives of *O*am mutants, and recombinant progeny carrying the *imm*λ prophage character were selected. These recombinants were tested for the simultaneous rescue of an *am*⁺ character from the prophage (Table 6). The results of this analysis show that *O*am905 can recombine with deletions F3 and MJ17. This confirms that *ori*⁻ mutations lie within gene *O* (*21*) (see Fig. 2).

Further evidence that at least two *ori*⁻ mutations lie entirely inside gene *O* comes from more recent physical studies. Some of the *ori*⁻ mutations are small deletions of multiples of three base pairs (*22*). It has been found that the *O* gene polypeptide synthesized in vitro from a template bearing the *ori*⁻ deletion *r*99 or *r*93 is shorter than that from λ*ori*⁺ when measured by gel electrophoresis (*23*).

Can the *ori*⁻ mutations be used to define a substructure to the origin region? Rambach distinguished two classes of *ori*⁻ mutants on the basis of details of phenotype, and suggested that these fall into two distinct clusters, separable by genetic recombination (*3, 4, 24*). Further recombinational analysis (Table 7), and nucleotide sequence determination (*22*) lead to a more complete picture of the fine structure of the *ori* region.

The *ori*⁻ mutations *r*93, *r*96, and *r*99 do not overlap (Table 7). We find that each of these mutations is a small deletion and that they lie in the order *r*93, *r*99, *r*96 (*22*).

The *r*99 deletion is separated from *r*93 and *r*96 by six base pairs on either side. The observed recombination frequency of *r*99 with each of the two flanking deletions is very low, but significantly above the background that results from reversion (Table 7) (*24*). The recombination frequency between *r*93 and *r*96 is at least 100-fold greater. These two deletions lie 24 base pairs apart. It is apparent that recombination frequencies observed for

Table 4. Marker rescue of *O* and *P* alleles from Charon 3 clones. λ*imm*434 *rep*P22 *12*am derivatives of Charon 3, Ch3λ2 and Ch3S45 were constructed (*13*) to eliminate homology between the vector and the cloned DNA fragment for the righthand portion of the replication region. Crosses between these derivatives and λ*O*am or *P*am phages were performed in the permissive host C600*su*II at a multiplicity of infection of 3 to 5 for each phage. Recombination was stimulated by ultraviolet irradiation of infected cells (600 erg mm^{-2}) and growth was allowed in LB broth at 37°C for 2 hours. The fraction of *am*⁺ among progeny phage was calculated as the ratio of λ*am*⁺, measured by plating on 594*su*⁰(λ*imm*434 *rep*P22 *12*amN14), to the total λ yield, which was determined on C600*su*II(λ*imm*434).

Source of cloned fragment	Fraction *am*⁺ phage in cross with			
	*O*am905	*O*am29	*O*am1005	*P*am3
Charon 3	2×10^{-6}	1×10^{-6}	3×10^{-7}	1×10^{-6}
Ch3λ2	4×10^{-3}	4×10^{-3}	5×10^{-7}	3×10^{-7}
Ch3S45	3×10^{-6}	2×10^{-6}	2×10^{-3}	4×10^{-3}

Table 5. Test crosses of *ori* and *O* alleles against prophage deletions. Lysogens permissive for growth of amber mutants and carrying deletion prophages (λ*N*am*N*am53 *c*Its 857Δ) (*18–20*) were infected with λ*N*am *c*Its857 *ori*⁻ or with *imm*434 derivatives of the *O*am test alleles at a multiplicity of infection of 0.5 to 1. The prophages were induced by incubation at 42°C for 20 minutes, and growth was allowed at 37°C for 90 minutes. λ*ori*⁺ recombinants were selected at 30°C on lawns of Ymel*su*III. The yields of λ*ori*⁻ phages were estimated as described (Table 7, legend). Crosses with the *ori*⁻ mutants *r*97 and *r*98 gave results very similar to those with *r*93 (data not shown). Most pseudorevertants of *ti*12 and *r*93 form minute plaques at 30°C and could be ignored. *O*am⁺ recombinants were selected at 39°C on the *rho*-negative strain *psu*104 described by Korn and Yanofsky (*28*). On this strain *N* function is not necessary for plaque formation by λ, but amber mutations in other genes are not suppressed. Thus, *O*⁺ recombinants can be scored directly. The fraction of *O*⁺ phage was calculated as the ratio of the titer of progeny on the *psu*104 strain to the titer on Ymel*su*III at 39°C. Background levels due to reversion were determined by infecting a nonlysogenic *su*III derivative of 594 (column 1).

Allele tested	Fraction *O*⁺ or *ori*⁺ phage among progeny				
	None	Prophage			
		ΔF3	ΔMJ17	ΔM25	ΔMJ7
*O*am905	1×10^{-7}	2×10^{-5}	9×10^{-6}	8×10^{-5}	1×10^{-4}
*O*am29	5×10^{-7}	2×10^{-7}	5×10^{-8}	1×10^{-4}	3×10^{-4}
*O*am1005	1×10^{-7}	8×10^{-8}	8×10^{-8}	5×10^{-8}	6×10^{-5}
ori⁻*ti*12	1×10^{-5}	1×10^{-5}	1×10^{-5}	1×10^{-3}	2×10^{-3}
ori⁻*r*93	2×10^{-8}	2×10^{-8}	2×10^{-8}	2×10^{-3}	
ori⁻*r*95	$< 6 \times 10^{-8}$	$< 6 \times 10^{-8}$	$< 1 \times 10^{-7}$	3×10^{-2}	
ori⁻*r*96	$< 1 \times 10^{-7}$	$< 9 \times 10^{-8}$	$< 3 \times 10^{-8}$	3×10^{-3}	
ori⁻*r*99	$< 3 \times 10^{-8}$	$< 7 \times 10^{-8}$	$< 1 \times 10^{-7}$	9×10^{-3}	

Table 6. Joint marker rescue of *imm* and *O* characters from prophage deletions. Crosses with deletion prophages were done as described (Table 5, legend). Total *imm*λ recombinants were selected on Ymel*su*III(λ*imm*434 *rep*P22 *12*amN14), and *imm*λ *O*⁺ recombinants were selected on a λ*imm*434 *rep*P22 *12*amN14 lysogen of the *psu*104 strain described (Table 5, legend). The fraction of *imm*λ was calculated as the ratio of the titer on the Ymel (λ*imm*434) lysogen to the titer on Ymel. The percentage of *O*⁺ was calculated as 100 times the ratio of the titer on the *psu*104(λ*imm*434) lysogen to the titer on the Ymel(λ*imm*434) lysogen. In cases in which the percentage of *O*⁺ was greater than 1 percent, this ratio was determined by testing 100 to 200 individual plaques selected on the Ymel(λ*imm*434) lysogen for growth on the *psu*104(λ*imm*434) lysogen. The strategy of the cross is shown in Fig. 3.

O allele	Prophage							
	ΔF3		ΔMJ17		ΔM25		ΔMJ7	
	Fraction *imm*λ	Percent *O*⁺	Fraction *imm*λ	Percent *O*⁺	Fraction *imm*λ	Percent *O*⁺	Fraction *imm*λ	Percent *O*⁺
*O*am905	7×10^{-5}	42	4×10^{-5}	21	4×10^{-4}	58		
*O*am29	2×10^{-5}	≤ 0.01	2×10^{-5}	≤ 0.02	3×10^{-4}	44	7×10^{-4}	43
*O*am1005					3×10^{-4}	≤ 0.001	8×10^{-4}	5

the small intervals between the deletions that we describe are monotonically increasing but not linearly increasing functions of distance (*25*).

Crosses of *r*93, *r*96, and *r*99 with other *ori*⁻ mutants provide further information on *ori* structure (Table 7, legend). First, we find that three of Rambach's class B strains (*r*93, *r*97, and *r*98) bear mutations that are not separable by recombination. Inspection of the nucleotide sequence in the vicinity of *r*93 (*22*) suggests that this deletion could have arisen by intramolecular recombination between a pair of repeated segments (17 nucleotide pairs, of which 15 are identical, separated from each other by 7 nucleotide pairs). It is plausible that this quasi-duplication is a "hot spot" for deletion formation (that is, a site of recurrent mutations) (*26, 27*). Thus, the class B mutations may be essentially identical. Second, we find that two additional *ori*⁻ mutations lie close to or overlap the *r*99 deletion. The λ*r*95 fails to recombine with *r*99, but differs from *r*99 in that it recombines more efficiently with *r*93. λ*ti*12, an *ori*⁻ mutant with less-pronounced phenotype (*2, 9*), does not give *ori*⁺ recombinants with *r*95 or *r*99 (above the relatively high level of revertants), but recombines efficiently with each of the flanking deletion mutants *r*93 and *r*96. In accord with these genetic mapping data, we find that the *ti*12 mutation is a transversion at a site that is overlapped by the *r*99 deletion (*22*).

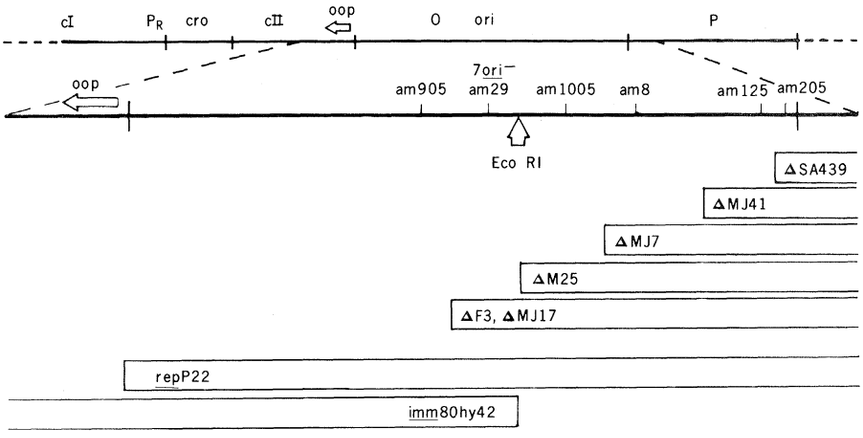

Fig. 2. Genetic map of the replication region of phage λ. Genes *cro*, *c*II, *O*, and *P* are in the operon controlled by promoter p_R. The origin of replication is designated *ori*. The second line shows an expanded map of gene *O*, including the sites of six *O*am and seven *ori*⁻ mutations and a site cleaved by restriction endonuclease Eco RI. The extents of substitutions and prophage deletions are indicated by boxes (*1–4, 18* and Tables 5 and 6). The *rep*P22 substitution covers all known components of the λ replication system, but does not replace the sequence encoding *oop* RNA (*5, 7*). The *imm*80hy42 substitution, present in Charon 3 (*11*), replaces *ori* and the NH_2-terminal region of gene *O* with the analogous φ80 type-specific functions (*6, 7*). The map is drawn roughly to scale.

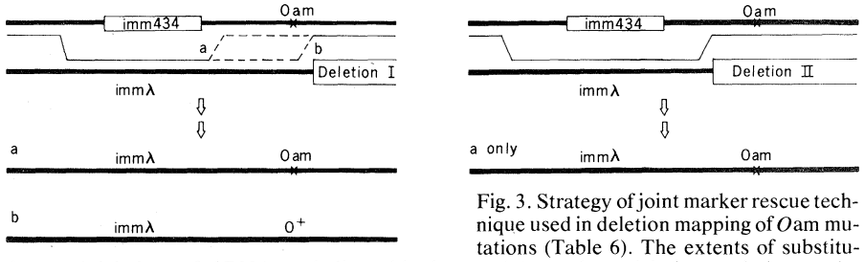

Fig. 3. Strategy of joint marker rescue technique used in deletion mapping of *O*am mutations (Table 6). The extents of substitutions and deletions of λ DNA are indicated by boxes. A λ prophage deletion strain is superinfected with an *imm*434 *O*am mutant, and recombinants which have rescued the *imm*λ region of the prophage are selected. If the *O*am mutation lies to the left of the deletion (deletion I), then a significant fraction of *imm*λ recombinants will be *O*⁺ (class b). If the deletion extends beyond the site of the *O*am mutation (deletion II), then all *imm*λ recombinants will retain the *O*am allele (class a).

The presence of a hot spot for formation of one class of *ori*⁻ deletions, and the nonlinear relation of recombination frequency to distance for deletions separated by very small intervals would be sufficient to account for the apparent separation of *ori*⁻ mutations into two clusters (*4*). We conclude, instead, that seven independent *ori*⁻ mutations lie close together in a single region. Because each of the *ori*⁻ mutants studied here was obtained by a procedure in which *O*⁻ mutants were discarded (*1–4*), and because *O* overlaps *ori*, it must be assumed that these mutants represent a set biased by the constraints of *O* function. An *ori*⁻ mutant which was selected without this bias, λ*t*5, appears to be defective in both *O* and *ori* (*1*). We do not know whether both defects result from a single mutation (*2*).

We find that all essential components of the λ replication origin lie within a small fragment of the genome (*imm-ori*λ) which includes the region between p_R and an Eco RI restriction site near the middle of gene *O*. The ability of this delimited origin to function depends on the products of λ-specific replication genes and on local transcription. The origin is inactivated by *cis*-dominant *ori*⁻ mutations. Although each of the mutants studied was chosen to retain function of replication genes *O* and *P* (*1–4*), these *ori*⁻ mutations lie in a small cluster with-

Table 7. Recombination between *ori*⁻ mutants. Crosses between λ*ori*⁻ mutants were performed in Ymel by coinfection with *ori*⁻ phages at a multiplicity of infection of 1 to 5. Infective centers were irradiated with 600 erg mm^{-2} of ultraviolet light and growth was allowed, in broth, at 37°C for 2.5 hours. λ*ori*⁺ recombinants were titered on Ymel at 30°C. Minute plaque pseudorevertants of λ*r*93 were ignored. The total yield of *ori*⁻ phages was estimated as follows: 594(λ*c*Its857*A*am*B*am) was infected with the lysate containing *ori*⁻ phages, irradiated with 450 erg mm^{-2} of ultraviolet light, heated at 39°C to induce the prophage and plated on 594*su*⁰ to detect λ*am*⁺ *ori*⁺ recombinants. Control experiments with λ*ti*12, an *ori* mutant that can be directly assayed by plating (*2*), showed that the recombination-based assay is about 10 to 15 percent efficient. Fractions of *ori*⁺ recombinants were computed on this assumption. Numbers in parentheses under the fraction of *ori*⁺ recombinants indicate the number of base pairs separating the *ori*⁻ mutations (*22*). We find the *ori*⁻ mutants λ*r*97 and λ*r*98 behave very similarly to λ*r*93 and fail to recombine with it or with each other (*3, 4, 24*). The mutant λ*r*95 fails to recombine with λ*r*99, recombines infrequently with λ*r*96 (8×10^{-7}), and recombines strongly with λ*r*93 (9×10^{-4}). The mutant λ*ti*12 recombines strongly with λ*r*93 and λ*r*96 (1 to 2×10^{-3}) but with λ*r*95 and λ*r*99 at a frequency which is below the level of reversion for this allele ($<1 \times 10^{-5}$).

Mutant	Mutant		
	*r*93	*r*99	*r*96
*r*93	$\leq 4 \times 10^{-7}$	7×10^{-5} (6)	7×10^{-3} (24)
*r*99		$< 3 \times 10^{-7}$	2×10^{-6} (6)
*r*96			$< 3 \times 10^{-8}$

in gene *O*, close to the Eco RI restriction site. Thus, the gene encoding the initiator of the λ replicon overlaps the replicator. Denniston-Thompson *et al.* report the precise localization of the *ori*⁻ mutations, to the level of nucleotide sequence (*22*).

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17. λ*ri*$^+$ recombinants are obtained in crosses of λ*ri*^{c}5b or λ*ri*^{c}D with a derivative of Ch3S45 but not with a derivative of Ch3λ2. [M. E. Furth, in preparation].
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19. *O*am905 was isolated by P. Aoothman [thesis, University of Oregon (1976)] and was identified as an *O* mutant on the basis of complementation tests. We were concerned that this mutation might lie in a gene upstream to *O* and that the failure to express *O* function might be due to nonsense polarity. However, we find that *O*am905 fails to complement with other *O*⁻ mutants even in a host carrying the strong polarity suppressor *psu*104 (*28*) (unpublished data of M.E.F. and C.M.). Thus, *O*am905 must lie within gene *O*. We thank P. Toothman, M. Jones, and I. Herskowitz for their gifts of the *O*am905 mutant and of the MJ series of prophage deletions. We thank N. Franklin for sending us the *psu*104 *E. coli* strain. *O*am1005 was isolated in this laboratory among a set of nitrosoguanidine-induced amber mutants of λ (M. E., Furth, unpublished).
20. The deleted prophages (λ*N*am7*N*am53 *c*Its857 Δ) were introduced by P1 transduction into strain 594 *gal*K⁻T⁻ *trp* : : *Tn*5 *su*° (a gift of D. Berg), by selection of *gal*$^+$ and screening for *iomm*λ at 30°C. The amber suppressor *su*III was then introduced by cotransduction with *trp*$^+$ by phage P1 grown on Ymel.
21. Strictly speaking, the failure of *ori*⁻ mutants to recombine with deleted prophages F3 and MJ17 implies that at least one component of each of the *ori* defects lies to the right of the *O*am905 site. The *ori*⁻ mutants all recombine efficiently with *O*am905 (data not shown). It seems highly unlikely that each of these spontaneous *ori*⁻ mutants could involve multiple defects spanning *O*am905. Although we have assumed that the *ori*⁻ mutations directly inactivate the λ replicator, we cannot completely exclude the formal possibility that they prevent expression of an unknown *cis*-acting replication gene (*2*).
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24. In agreement with Rambach (*4*), we find that the class A mutants, *r*95, *r*96, and *r*99, fail to revert frequently (< 10^{-8} at 30°C) and fail to give minute recombinants with λ*imm*21 *O*⁻ *P*⁻. Class B mutants, including *r*93, *r*97, and *r*98, both revert and give minute recombinants with λ*imm*21 *O*⁻ *P*⁻. The higher reversion rate of class B mutants is probably due to second-site mutations.
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Physical Structure of the Replication Origin of Bacteriophage Lambda

Abstract. *The nucleotide sequence of part of the replication region of wild-type bacteriophage lambda and of four mutants defective in the origin of DNA replication* (ori⁻) *has been determined. Three of the* ori⁻ *mutations are small deletions, and one is a transversion. The sequence of the origin region, defined by these mutations, contains a number of unusual features.*

Moore *et al.* (*1*) and Furth *et al.* (*2*) have described the construction and functional analysis of chimeric phages carrying restriction endonuclease fragments from the replication control region of the bacteriophage lambda (λ) genome. Fragments extending from the immunity control (*imm*) region to the Eco RI site within gene *O* (*imm-ori*λ fragments) have been shown to carry a fully functional origin of replication, displaying all the known requirements of the normal λ replicator. All known mutations (*ori*⁻) that inactivate the λ replication origin, without affecting expression of the structural genes (*O* and *P*) essential for replication, lie in a small portion of this fragment (*2*). We now describe a structural analysis of the λ replication region, including a determination of the changes in nucleotide sequence by which four *ori*⁻ mutations inactivate the replicator.

The first step in this analysis was to determine the locations of restriction endonuclease cleavage sites within the 2.75 percent λ Eco RI fragment from Ch3λ2*imm*434Δ7c (or an identical fragment from phage KK1, Fig. 1; *3*). This short fragment contains *imm*434 genes on the left and λ genes on the right, as shown in Fig. 1. A similar Eco RI fragment from the *imm*λ phage KK2 (Fig. 1) is about 3.7 percent λ in length and contains only λ genes. The two types of fragment were, respectively, designated *imm*434-*ori*λ and *imm*λ-*ori*λ. Many restriction endonuclease cleavage sites (*4*) were located by comparing digestion patterns of the two *imm-ori*λ fragments.

The fragments were purified by differential centrifugation (*1*) and were labeled at the 5′ termini with ^{32}P (*5, 6*). The fragments were then redigested with a second restriction enzyme. The subfragments were separated by electrophoresis on polyacrylamide gels (*7*) and visualized both by staining and by radioautography. Since the right ends of the two *imm-ori*λ fragments are identical, any subfragments with differing