Construction of Chimeric Phages and Plasmids Containing the Origin of Replication of Bacteriophage Lambda

Abstract. *Segments of the replication control region of bacteriophage lambda (λ) and λ mutants defective in replication were attached in vitro to the φ80 phage vector Charon 3 and to the plasmid vector* mini Col *El (pVH51). The chimeric phages and plasmids have been used to localize the origin of λ DNA replication and to facilitate a structural analysis of the λ replicator.*

Bacteriophage lambda (λ) provides a classic example of a replicon—a contiguous DNA segment whose replication is under unitary genetic control (*1*). The regulatory elements postulated in the replicon model are a diffusible positive factor, the initiator, and the target on the DNA molecule at which it acts, the replicator. The individuality of replicons resides in the specificity of interaction between initiator and replicator.

Autonomous replication of λ can be viewed within this framework. Synthesis of λ DNA initiates at a unique origin. Direct observation by electron microscopy of sets of replicating molecules reveals that replication forks progress in each direction from a single region of the chromosome (*2*). Evidence that this region contains a site essential for initiation of DNA replication comes from genetic studies. Extensive deletions of λ prophage that eliminate the replication control region prevent autonomous replication of the residual prophage DNA, even when all diffusible elements are provided by a helper phage (*3*). Furthermore, localized *cis*-dominant mutations defective for replication, termed *ori*$^-$, have been isolated (*4, 5*). The *ori*$^-$ mutations show that there is a single replicator, and provide a means to identify its components. Both the starting point (or points) for DNA synthesis and the essential site for λ replication defined by genetic experiments are located in the vicinity of 80 percent on the λ physical map (Fig. 1, top line). In fact, all of the phage-coded elements essential for replication must lie between 78 and 83 percent on the physical map because this region, when cyclized, can replicate autonomously as a plasmid (*6*).

Replication of λ requires most of the proteins essential for replication of its host, *Escherichia coli* (*1*). However, as is predicted by the replicon hypothesis, control of initiation is determined by the phage genome. Two λ proteins, the products of genes *O* and *P*, are absolutely required for autonomous replication (*7*). Studies of λ and several closely related temperate phages suggest that each encodes an O protein that can function only with its cognate replicator (*8*). Thus, the *O* gene product behaves as a specific initiator.

Genes *O* and *P* lie together in the major rightward operon controlled by promoter p_R. The repressor encoded by gene *c*I prevents expression of this operon, and so acts indirectly as a negative regulator of λ replication. Surprisingly, rightward transcription in the origin region appears to be directly required for initiation of replication, even in the presence of all diffusible gene products (*4, 9*). Because of this requirement for transcriptional activation of the origin, the *c*I repressor regulates λ replication in a direct way not predicted by the replicon model.

The minor leftward RNA molecule, also termed *oop*, is transcribed leftward from the general vicinity of the replication genes (*10, 11*). It has been postulated that this transcript controls λ replication by serving as a primer for initiation of DNA synthesis (*11*). However, detailed mapping shows that this RNA is encoded outside of the region determining the type specificity of replication control (*12*). Moreover, most of the *oop* coding region and its promoter have now been deleted without impairing function of the λ replication origin (*13*).

In order to understand the complex system of λ replication control, it is necessary to determine the structure of the *ori* region, to dissect it into component parts, and to study the interactions be-

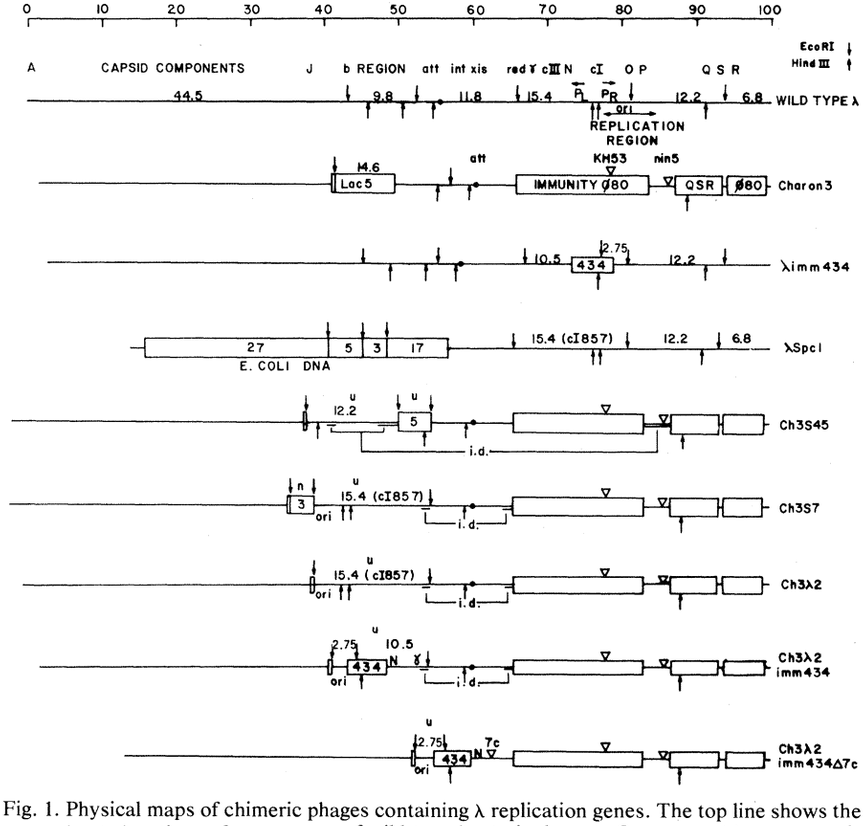

Fig. 1. Physical maps of chimeric phages containing λ replication genes. The top line shows the approximate locations of some genes of wild-type bacteriophage λ. One percent λ corresponds to about 494 base pairs (*14*). The Charon 3 vector phage (*14*) is illustrated beneath λ. The λ*spc* 1, which has the λ type-specific replication genes, was used as the source of DNA for insertion into Charon 3 (*17*). The λ*imm*434 differs from λ only in the immunity control region. The chimeric phages Ch3S45, Ch3S7 (*17*), and Ch3λ2 carry Eco RI fragments from the replication region. The orientation of an inserted fragment is indicated as u (inverted) or n (normal) (*14*). The phages Ch3λ2*imm*434 and Ch3λ2*imm*434Δ7c illustrate modifications of Ch3λ2 described in the text. Regions of DNA which are heterologous to λ are illustrated with boxes. Deletions are shown by inverted triangles. Lines labeled *i.d.* refer to inverted duplications, resulting from homology between vector and cloned fragment. The *att* is the site on λ of integration into the *E. coli* chromosome. Downward arrows represent sites of cleavage by Eco RI. Sizes of some Eco RI fragments are indicated in percent λ units. Upward arrows indicate sites of cleavage by Hind III.

tween the numerous components of the replication system. For this purpose we need a way to propagate segments of the replication region, even if defective, while retaining the ability to assay their biological function. The way we have developed is to attach the λ *ori* region to a nonessential region of a heterologous phage vector, the φ80 derivative Charon 3 (*14*). To facilitate the preparation of large quantities of DNA, we have also incorporated fragments carrying the *ori* region into the plasmid vector *mini Col* EI (pVH51) (*15*).

The contruction of chimeras was divided into stages. First, Charon 3 phages carrying different DNA fragments from the vicinity of the λ origin were analyzed for *ori* function. Conventional genetic crosses were then performed to introduce an *ori*$^-$ mutation [*r*99 (*5*)] into a Charon 3 chimera found to carry a functional λ origin. Quantities of wild-type and mutant *ori* DNA fragments were isolated for physical characterization, and these DNA fragments were inserted into pVH51 (*15*). Finally, for comparative studies, *ori* DNA fragments from seven additional strains were cloned directly into Charon 3 and thence into pVH51.

Within the replication region, near 81 percent on the λ map, is a site of cleavage for the restriction endonuclease Eco RI (*16*). The starting point for our investigation was to locate the origin of replication with respect to this site. For this purpose we used the chimeric phages Ch3λ2 and Ch3S45 (*17*), each of which contains one of the λ Eco RI DNA fragments, 15.4 or 12.2 percent, adjacent to the Eco RI site in the λ replication region (Fig. 1). Furth *et al.* (*18*) have shown that an intact λ origin is present in Ch3λ2 but not in Ch3S45; thus, the fragment to the left of the Eco RI site carries the λ origin. This fragment also carries the immunity control region of λ (Table 1). Eco RI fragments carrying at least part of the immunity region of λ or of the heteroimmune phage λ*imm*434 and the intact λ replication origin are designated *imm*λ-*ori*λ and *imm*434-*ori*λ, respectively. The chimeric phage Ch3S7 (Fig. 1) is identical to Ch3λ2 except that it contains an extra fragment of *E. coli* DNA, which was a useful marker for heteroduplex analysis (*17*) (Fig. 3).

To provide a counterselective marker to guide the introduction of the *ori*$^-$ mutations (see below), the *imm*434 substitution was crossed into the cloned *imm*λ-*ori*λ fragment of Ch3λ2 (*19*). This substitution also introduces an Eco RI site which reduces the size of the Eco RI fragment containing the λ *ori* region to 2.75 percent λ (*20*).

To facilitate purification of the 2.75 percent *imm*434-*ori*λ DNA fragment of Ch3λ2*imm*434 (Fig. 1), we found it desirable to eliminate the adjacent 10.5 percent Eco RI fragment by deleting the Eco RI site defining its right end. This was readily accomplished since the Charon 3 vector, like λ, undergoes spontaneous deletion of DNA near the λ attachment site (*att*) (*21*). Because deletions render λ relatively resistant to inactivation by heating in the presence of a chelating agent, these deletion mutants could be selected by treatment of Ch3λ2*imm*434 with citrate (*21*). Ch3λ2*imm*434Δ7c (Fig. 1), an example of such a deletion mutant, lost the ability to express gene *gam* from the inserted fragment while retaining the ability to express *c*I of *imm*434 (Table 1) (*22*). A similar deletion derivative, Ch3S7Δ2h, was isolated from Ch3S7.

λ*r*99 is one of the *ori*$^-$ mutants isolated by Rambach (*5*). To introduce the *r*99 mutation into the inserted *imm*-*ori*λ fragment in Ch3λ2, λ*N*am7*N*am53*c*I857*r*99 was crossed with Ch3λ2*imm*434. Progeny of the cross were screened for replacement by *imm*λ of the *imm*434 immunity region of the cloned fragment by testing for the ability to help λ*c*I$^-$ to lysogenize (*22*). Of about 300 such *imm*λ progeny, one failed to produce *ori*$^+$ recombinants in a test cross with the λ*r*99 parent, and was designated Ch3λ2*r*99. As expected, this recombinant failed to replicate in the functional origin test (*18*).

The *imm*434 substitution was reintroduced into Ch3λ2*r*99 (*19*). The resulting phage, Ch3λ2*r*99*imm*434, retained the *ori*$^-$ defect. Finally, we isolated a spontaneous deletion mutant which had lost the rightmost Eco RI site (*21*), Ch3λ2*r*99*imm*434Δ8a, to facilitate quantitative purification of the *imm*434-*ori*$^-$*r*99 fragment.

The structure of each of the chimeric phages was verified by agarose gel electrophoresis of the DNA fragments produced by digestion with the restriction endonucleases Eco RI and Hind III (*23, 24, 25*). The Eco RI digestion patterns of DNA from Ch3Δ*lac*, Ch3λ2, Ch3λ2*imm*434, and Ch3λ2*imm*434Δ7c (Fig. 2) illustrate the introduction of the 15.4 percent *imm*λ-*ori*λ fragment, its replacement by two smaller fragments (2.75 and 10.5 percent) after introduction

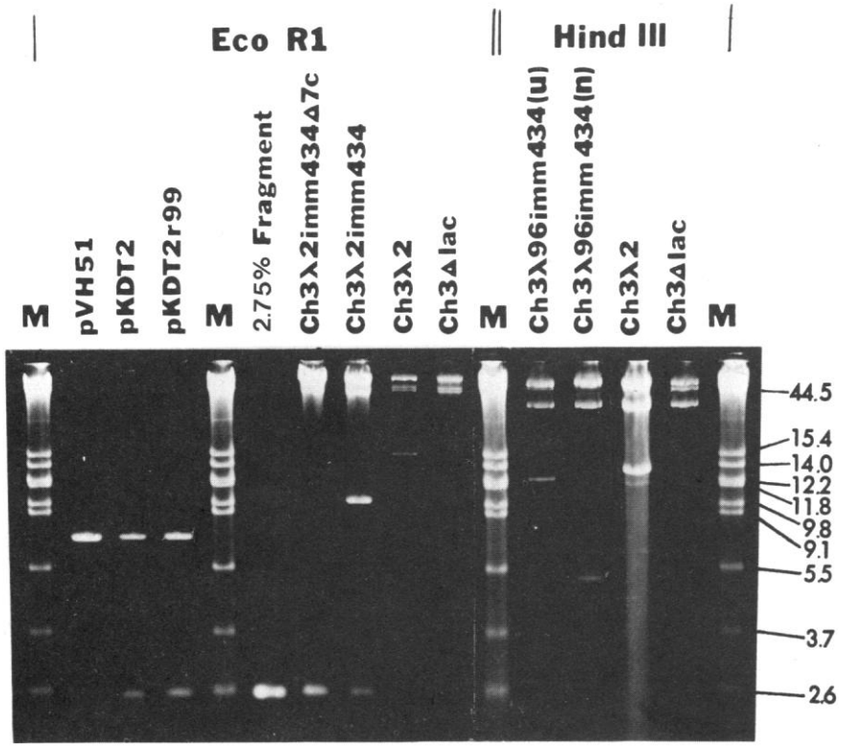

Fig. 2. Agarose gel electrophoresis of Eco RI and Hind III digests of chimeras. Fragments produced by Eco RI and Hind III digestion of purified phage (*23*) and plasmid (*28*) DNA's were separated by electrophoresis through 1.0 percent agarose gels as described by Shinnick *et al.* (*24*). Plasmids pVH51, pKDT2, pKDT2*r*99, and phages Ch3λ2*imm*434Δ7c, Ch3λ2*imm*434, and Ch3λ2 are described in the text. Ch3Δ*lac* was derived from Charon 3 by deletion of the *lac*-containing Eco RI fragment (see Fig. 1). Ch3λ96*imm*434(u) and Ch3λ96*imm*434(n) are chimeric phages which contain identical inserted DNA in opposite orientations, as described in the text. M indicates a mixture of fragments of known sizes produced by Eco RI digestion of three different phage DNA's. The sizes are given in percent λ. The 2.75 percent *imm*434-*ori*λ Eco RI fragment was purified by differential centrifugation (*26*).

of *imm*434, and the elimination of the 10.5 percent fragment by the Δ7c deletion. The 2.75 percent *imm*434-*ori*λ fragment, purified by differential centrifugation (*26*), is shown for comparison.

The orientation of the inserted *imm*λ-*ori*λ fragment of Ch3λ2 was determined by analysis of the digestion pattern obtained with restriction endonuclease Hind III. Since the two closely spaced Hind III sites in the λ immunity region (Fig. 1) are asymmetrically located within the 15.4 percent fragment, their distance from the Hind III site near *att* in Charon 3 depends on the orientation of the inserted fragment. The observed Hind III fragment of 12.4 percent is diagnostic of insertion in the inverted "u" orientation (*14*) (Fig. 2).

The structures of the chimeric phages were further verified by heteroduplex analysis (*27*). In Fig. 3A a single strand of Ch3λ2 is shown. Characteristically, these strands were observed to fold back upon themselves, forming a hairpin loop due to the presence of an internal duplication. This would be expected if the *imm*λ-*ori*λ fragment were cloned in the u orientation, since there is a short region of homology between the left end of that fragment and DNA to the left of the φ80 immunity region (Fig. 1). A heteroduplex between Charon 3 and Ch3λ2 (Fig. 3B) reveals the substitution bubble expected due to replacement of the *lac* fragment with inserted DNA.

In Fig. 3C the *imm*434 substitution and deletion of Ch3λ2*imm*434Δ7c are shown in a heteroduplex with Ch3S7. The small, single-stranded loop is the bacterial DNA from Ch3S7 and lies adjacent to the *ori* end of the cloned *imm*-*ori*λ DNA fragment. The bubble corresponds to the *imm*434 substitution, while the larger loop corresponds to the deletion Δ7c. Figure 3D shows a magnified view of the *ori* region of Ch3λ2*r*99*imm*434Δ8a paired with Ch3S7. Again, the small loop is the bacterial DNA of Ch3S7, while the large loop corresponds to the deletion Δ8a. The *ori* region is located between the small loop and the *imm*434 bubble. Inspection of this region of the heteroduplex shows that *r*99 does not introduce any perceptible (>100 base pairs) heterology into the *ori* region. These heteroduplexes also confirm the proposed structures for these phages (Fig. 1).

To aid purification of milligram quantities of DNA, the *imm*434-*ori*λ fragments were next transferred into the plasmid vector pVH51. Such fragments from Ch3λ2*imm*434Δ7c and from Ch3λ2*r*99*imm*434Δ8a were purified by differential centrifugation (*26*) (Fig. 2). Covalently closed circular DNA of the plasmid pVH51 (*28*), which contains a single Eco RI restriction site (*15*), was digested with Eco RI. The wild-type *imm*434-*ori*λ and the *imm*434-*ori*$^-$*r*99 DNA fragments were then joined to the plasmid DNA by T4 DNA ligase. The *E. coli* strain GM33 (*29, 30*) was transformed with the resulting chimeric DNA molecules. From each experiment a colicin-resistant transformant (*31*) containing a plasmid substantially larger than pVH51 (*32*) was chosen for further study. These isolates were designated GM33/pKDT2 and GM33/pKDT2*r*99. The plasmids were subsequently transferred into *E. coli* MO to obtain larger yields of plasmid DNA (*28*). Test crosses with λ*O*am and *ori*$^-$ mutants confirmed the presence of the expected *imm*434-*ori*λ fragment in each of the chimeric plasmids pKDT2 and pKDT2*r*99.

Plasmid DNA was isolated from strains carrying pVH51, pKDT2, and pKDT2*r*99, and analyzed by digestion with restriction endonucleases. Agarose gel electrophoresis of Eco RI digests (Fig. 2) confirmed that a fragment of the same length as the *imm*434-*ori*λ fragment was present in each chimeric plasmid. The identity of the inserted fragments was further confirmed by comparing the Hpa II subfragments of the purified

Table 1. Gene expression from fragments inserted into Charon 3. Complementation tests to assess the genetic content of fragments inserted in Charon 3 were performed as described (*22*). The + sign indicates that the fragment inserted in Charon 3 can provide a function to the mutant tester phage; the − sign indicates that it cannot. ts indicates that the cloned *imm*λ-*ori*λ fragment carries the *c*Its857 allele, and can help λ*c*I60 to lysogenize at 30°C but not at 39°C. The wild-type allele of *O*am29 can be rescued by genetic recombination from all clones carrying the *imm*-*ori*λ fragment, except Ch3λ29 (*18*), but under recombination-deficient conditions no complementation of λ*O*am29 is observed. Ch3λ3, Ch3λ12, Ch3λ93, Ch3λ96, and Ch3λ99 behaved identically to Ch3λ2*r*99 in these tests.

Chimeric phage	*gam*	*N*	λ*c*I	*imm*434*c*I	λ*cro*	*c*II	*O*
Charon 3	−	−	−	−	−	−	−
Ch3S45	−	−	−	−	−	−	−
Ch3S7	+	+	ts	−	+	+	−
Ch3λ2	+	+	ts	−	+	+	−
Ch3λ2*r*99	+	−	ts	−	+	+	−
Ch3λ2*imm*434	+	+	−	+	−	+	−
Ch3λ2*imm*434Δ7c	−	+	−	+	−	+	−
Ch3S7Δ2h	−	−	ts	−	+	+	−
Ch3λ29	+	+	+	−	+	+	−

Table 2. Chimeric phages and plasmids carrying λ*ori* region. Chimeric phages and plasmids were constructed as described in the text. All except Ch3λ2*r*99 were obtained by insertion in vitro of an Eco RI fragment into the phage vector Charon 3 or the plasmid vector pVH51. Ch3λ2*r*99 was derived from Ch3λ2 by genetic recombination. The orientation of the inserted fragment with respect to the pVH51 vector is designated n or u (*36*).

Name of phage	*ori* region of cloned *imm*λ-*ori*λ Eco RI fragment (15.4 percent λ, u orientation)	Source of cloned fragment
Ch3λ2*	*ori*$^+$	λ*spc*1 (*c*I857)
Ch3λ2*r*99*	*ori*$^-$*r*99	See text
Ch3λ3	*ori*$^+$	λ*N*am7*N*am53 *c*I857†
Ch3λ12	*ori*$^-$*ti*12	λ*N*am7 *c*I857 *ti*12 *S*am7
Ch3λ29	*ori*$^+$ *O*am29	λ*O*am29
Ch3λ93	*ori*$^-$*r*93	λ*N*am7*N*am53 *c*I857 *r*93
Ch3λ96	*ori*$^-$*r*96	λ*N*am7*N*am53 *c*I857 *r*96
Ch3λ99	*ori*$^-$*r*99	λ*N*am7*N*am53 *c*I857 *r*99

Name of plasmid		*ori* region of cloned *imm*434-*ori*λ Eco RI fragment (2.75 percent λ)	Source of cloned fragment
pKDT2	(n)	*ori*$^+$	Ch3λ2*imm*434Δ7c
pKDT2*r*99	(u)	*ori*$^-$*r*99	Ch3λ2*r*99*imm*434Δ8a
pKDT3	(u)	*ori*$^+$	λ*imm*434†
pKDT12	(n)	*ori*$^-$*ti*12	Ch3λ12*imm*434
pKDT29	(n)	*ori*$^+$*O*am29	Ch3λ29*imm*434
pKDT93	(n)	*ori*$^-$*r*93	Ch3λ93*imm*434
pKDT96	(n)	*ori*$^-$*r*96	Ch3λ96*imm*434
pKDT905	(n)	*ori*$^+$*O*am905	λ*imm*434*c*I$^-$*O*am905*nin*5

*A deletion removing the rightmost Eco RI site of the *imm*434 derivative has been isolated. †The phage λ*N*am7*N*am53 *c*I857 is the direct parent of the *ori*$^-$ mutants *r*93, *r*96, and *r*99 (*5*). The replication region of this phage was transferred into an *imm*434 background by crossing with λ*imm*434 *rep*P22 *12*am*N*14, to permit construction of the plasmid pKDT3.

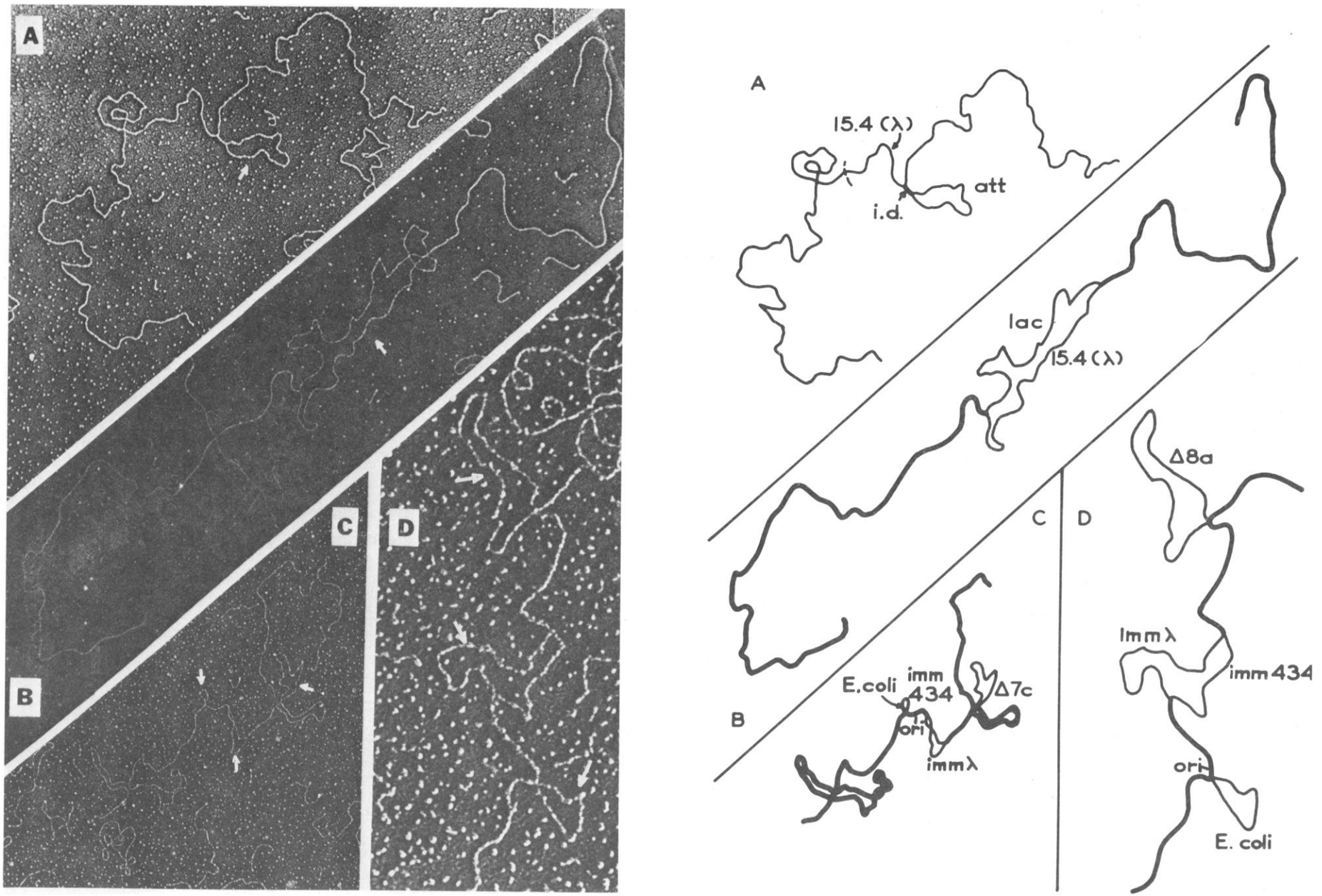

Fig. 3. Heteroduplex analysis of chimeric phages. Heteroduplexes made by annealing DNA strands from genetically different phages were examined by electron microscopy (*27*). Singlestranded loops and substitution bubbles are marked by arrows. Interpretations of the photographs are indicated in the accompanying line diagrams. Thin lines represent single strands and thick lines represent duplex segments. Loops and bifurcations where DNA did not pair are labeled to show the source of heterology. (A) A single strand of Ch3λ2. (B) Ch3λ2/Charon 3. (C) Ch3S7/Ch3λ2*imm*434Δ7c. (D) A magnified view of the *imm-ori*λ region of Ch3S7/Ch3λ2*r99imm*434Δ8a.

*imm*434-*ori*λ fragment with those produced by cleaving the plasmid DNA's with Eco RI and Hpa II (*30*).

For comparative studies, chimeric derivatives of Charon 3 carrying *imm*λ-*ori*λ fragments with several additional *ori*$^-$ mutations (*4*, *5*, *33*) and *O* gene amber mutations (*34*) were constructed in vitro (*35*). These phages are listed in Table 2 along with the sources of the cloned DNA fragments. In each case we chose for further study a chimeric phage shown by deletion analysis to carry the inserted fragment in the u orientation. Next, *imm*434 derivatives were constructed, and the orientation of the inserted fragment was confirmed by means of agarose gel electrophoresis of Hind III digests. The Hind III fragment pattern of Ch3λ96*imm*434 (u) is shown in Fig. 2, along with the corresponding pattern of a chimeric phage with the identical *imm*-*ori*$^-$*r*96 fragment inserted in the n orientation.

Finally, chimeric plasmids were constructed containing each of the desired *imm*434-*ori*λ fragments (*35*). We had observed that *E. coli* strains carrying pKDT2 or pKDT2*r*99 restrict the growth of λ*imm*434, presumably because these strains overproduce the 434-specific *cro* protein. All additional pVH51–*imm*434-*ori*λ chimeric plasmids were therefore detected by screening for resistance to λ*imm*434 among colicin-resistant transformants of *E. coli* MO. This screening procedure obviated the need to purify the *imm*434-*ori*λ fragments before joining to pVH51. In some cases the fragment was obtained directly from a standard *imm*434 derivative, without prior cloning in Charon 3. The chimeric plasmids and the sources of the inserted fragments are listed in Table 2.

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22. Spot tests for genes carried by a λ fragment inserted into Charon 3 were done by infecting an *E. coli* (φ80) host with an appropriate λ mutant, plating with soft agar, and spotting with the Charon 3 derivative to be tested. Portions (0.25 ml) of stocks of the test Charon 3 phages (at 10^8 to 10^9 phage/ml) were distributed into the central 60 wells of a microtiter dish and were transferred to the bacterial lawns with a mechanical replicator. Test plates were incubated overnight. Tests for function of genes (*N*, *O*, *cro*, *gam*) involved in lytic growth were done by infecting a nonpermissive host with a defective λ mutant and scoring for lysis in the area of the test spot. Tests for function of genes involved in lysogenization (*c*I, *c*II) were done by infecting with a sufficient number of the clear-plaque phage to cause confluent lysis of the bacterial lawn, and scoring for bacterial growth (a turbid patch) in the area of the test spot. The indicator strains generally used were 594(φ80)*rec*$^+$*str*A*su*^{0} and N100(φ80)*rec*A*str*A*su*^{0}. To 0.1 ml of a cell suspension at 2×10^9 cell/ml, 10^7 to 10^8 plaque-forming units (pfu) of a defective phage or about 5×10^5 pfu of a clear mutant were adsorbed. Addition of an equal volume of indicator cells not lysogenic for φ80 improves the sensitivity of the tests for *c*I and *c*II functions. Conditions used for particular tests were: *N*, infection of 594(φ80) with λ*N*am7*N*am53*c*I857*r*14 at 39°C; *O*, infection of N100(φ80) with λ*int*6*red*3*imm*434*c*1*O*am29 at 37°C; *cro*, infection of 594(φ80) with λ*c*I857*cro*27 at 42°C; *gam*, infection of N100(φ80) with λ*c*I857*pbio*1 at 39°C; λ*c*I, *imm*434*c*I, and *c*II, infection of 594(φ80) plus 594 with λ*c*I60, λ*imm*434*c*16T, λ*c*II68, or λ*imm*434*c*II2002 at 37°C or 30°C (if the cloned *imm*λ-*ori*λ fragment carries the *c*Its857 mutation).
23. Phage lysates were grown (*14*) and concentrated. Phage were precipitated by addition of 7 percent polyethylene glycol-6000, centrifuged at low speed, and resuspended in φ80 buffer (0.01*M* tris-HCl, *p*H 7.4, 0.1*M* NaCl). The density of the phage suspension was adjusted to 1.4 g/ml with CsCl. The suspension was layered onto a preformed step gradient (CsCl at 1.45 g/ml, 1.5 g/ml, and 1.7 g/ml in φ80 buffer) and centrifuged at 30,000 rev/min (Sorvall T865 rotor) for 3 hours. The phage band (density, 1.5 g/ml) was collected from the top, mixed with CsCl in φ80 buffer (1.5 g/ml), and centrifuged to equilibrium at 30,000 rev/min (Beckman SW50.1 rotor). Purified phage were dialyzed against buffer (0.1*M* tris-HCl, *p*H 7.9, 0.3*M* NaCl) and extracted three times with water-saturated phenol. Residual phenol was removed by dialysis, and phage DNA was stored in 0.01*M* tris-HCl, *p*H 7.9, 0.01*M* NaCl, 0.001*M* EDTA.
24. Agarose gel electrophoresis was as described by T. Shinnick, E. Lund, O. Smithies, F. Blattner, *Nucleic Acids Res.* **2**, 1911 (1975).
25. Digestions with Eco RI and Hind III were carried out at 37°C. Eco RI buffer: 90 m*M* tris-HCl, *p*H 7.9, 10 m*M* $MgCl_2$. Hind III buffer: 6 m*M* tris-HCl, *p*H 7.9, 6 m*M* $MgCl_2$, 6 m*M* dithiothreitol. For sources of enzyme, see (*30*).
26. Large quantities of the 2.75 percent *imm*434-*ori*λ Eco RI fragment were purified by differential centrifugation as described (*14*, p. 166), except that the sample was centrifuged for 4 hours at 47,000 rev/min, 15°C (SW50.1 rotor).
27. B. C. Westmoreland, W. Szybalski, H. Ris, *Science* **163**, 1343 (1969); R. Davis, M. Simon, N. Davidson, in *Methods Enzymol.* **21**, 413 (1971).
28. Plasmid DNA was isolated by the method of G. Humphreys, G. Willshaw, E. Anderson, *Biochim. Biophys. Acta* **383**, 457 (1975). A culture of *E. coli* MO containing the plasmid was grown to an absorbancy at 575 nm of 0.5, chloramphenicol was added to a final concentration of 100 μg/ml, and the culture was incubated overnight at 37°C. The cells were centrifuged, resuspended in a solution of 0.05*M* tris-HCl, *p*H 8.0, and 25 percent sucrose, and lysed by the sequential addition of lysozyme (500 μg/ml), EDTA (50 m*M*), and Triton X-100 (5 percent by volume). Chromosomal DNA was removed by centrifugation at 25,000 rev/min for 30 minutes at 4°C (Beckman type 30 rotor). The supernatant was gently decanted, and nucleic acids were precipitated by the addition of one-half volume of 30 percent polyethylene glycol-6000 in 1.5*M* NaCl. The precipitate was then centrifuged and resuspended in a solution consisting of 0.1*M* tris-HCl, *p*H 8.0, 0.001*M* EDTA, and 500 μg of ethidium bromide per milliliter. DNA was centrifuged to equilibrium (Sorvall T865.1 rotor, 42,000 rev/min) for 48 to 60 hours. The band of plasmid DNA (average density, 1.57 g/ml) was collected from the top of the gradient, extracted with isopropanol, and dialyzed against buffer (0.01*M* tris-HCl, *p*H 7.9, 0.01*M* NaCl, and 0.001*M* EDTA) and stored at 4°C. We thank W. Reznikoff for suggesting the use of strain MO to obtain greater yields of plasmid DNA.
29. M. Marinus and N. Morris, *J. Mol. Biol.* **85**, 209 (1976).
30. K. Denniston-Thompson, D. D. Moore, K. E. Kruger, M. E. Furth, F. R. Blattner, *Science* **198**, 1051 (1977).
31. A culture of *E. coli* MO was grown to $A_{575} = 0.6$ and treated with calcium [S. Cohen, A. Chang, C. Hsu, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2110 (1973)]. A 0.2-ml portion of cells was mixed with 0.2 μg of DNA from the ligation mixture (*35*); the cells were heated for 30 seconds at 37°C and incubated at 0°C for 90 minutes. The mixture was then diluted into 4.0 ml of Luria broth and incubated at 37°C with agitation. Transformants were selected by treating 0.2 ml of each culture with colicin E1 for 30 minutes at 37°C and then plating on Luria broth agar containing 0.5 per-

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