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Viable Molecular Hybrids of Bacteriophage Lambda and Eukaryotic DNA

(*Eco*RI restriction endonuclease/DNA joining/calcium transfection/electron microscopy)

MARJORIE THOMAS, JOHN R. CAMERON, AND RONALD W. DAVIS

Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

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ABSTRACT **A bacteriophage λ strain has been constructed and a method developed by which DNA from potentially any source can be covalently inserted through *Eco*RI cohesive ends into the middle of the λ DNA. These hybrid DNAs can infect nonrestricting *Escherichia coli* cells and can then propagate as plaque-forming phage. A unique feature of this λ strain is that extra DNA in the middle of its genome is required for plaque formation. A large number of such phages have been produced with *E. coli* DNA and *Drosophila melanogaster* DNA.**

Hybrid DNA molecules can be constructed *in vitro* by joining any two or more DNA molecules through the cohesive ends that are generated by cleavage with *Eco*RI endonuclease (1). This discovery has made possible the formation of hybrid DNA molecules that can be propagated in *Escherichia coli*. Such hybrids may be formed between a vector molecule, which can be any molecule cleaved by *Eco*RI endonuclease capable of self propagation in *E. coli*, and potentially any other DNA molecule with cohesive ends generated by *Eco*RI endonuclease.

There already exist in bacteriophage λ (Fig. 1a) viable deletion mutants that lack most of the center two *Eco*RI-endonuclease-generated B and C fragments (2, 3). It was established that fragments B and C are in fact not required for lytic growth by cleaving λ DNA with *Eco*RI endonuclease, randomly rejoining the fragments with DNA ligase, and, after DNA infection, selecting viable phage that lack the B and C fragments (Abraham, Thomas, and Davis, in preparation). Therefore, it should be possible to construct viable hybrid DNA molecules by inserting foreign DNA into λ DNA in place of the B and C fragments. To facilitate such an insertion, the two rightmost *Eco*RI restriction sites were eliminated by mutation.

An advantage of λ DNA as a vector molecule is that an insertion of foreign DNA can be made to be essential for plaque formation. The shortest λ DNA molecules that produce plaques of nearly normal size are 25% deleted (J. S. Parkinson, unpublished observation). Apparently, if too much of even nonessential DNA is deleted from the λ genome, it cannot be packaged into virus particles. The *Eco*RI-B and -C fragments represent 21.1% of the λ DNA (Thomas and Davis, submitted to *J. Mol. Biol.*). Deletion of the nonessential *nin*5 region (6.1% of λ DNA) (2) as well as the *Eco*RI-B and -C fragments yields a DNA that would not be expected to produce plaque-forming phage even though no essential genes have been deleted. However, this deleted λ DNA can be made to produce plaques if a new DNA segment is inserted into the remaining central *Eco*RI restriction site. This constitutes a positive selection for λ phage carrying a DNA insertion. The λ strain used for the construction of these hybrid phages, hereafter referred to as λgt-λC, contains the λ *Eco*RI-C fragment in this central region in order to retain sufficient DNA that it may be propagated. This segment is removed after *Eco*RI endonuclease cleavage, and *Eco*RI-endonuclease-cleaved foreign DNA is inserted.

MATERIALS AND METHODS

Terminology. The term λgt (generalized transducer) designates that portion of the λ genome that is common to all of the hybrid DNA molecules and contains all of the essential genes for plaque formation. Following λgt and separated by a dash is a term referring to the origin of the DNA that is inserted. Following this term is an isolation number or capital letter(s) referring to an identified and ordered *Eco*RI-endonuclease-generated fragment(s). Prime letters indicate a fragment inserted in an inverted direction. Thus, λgt-λ*Eco*RI · C′ contains an inverted *Eco*RI-C fragment from λ DNA. For this report, the *Eco*RI term will be dropped. Thus, λgt-λ*Eco*RI · C′ becomes λgt-λC′.

Phages, Bacteria, DNAs, and Enzymes. λ *c*I857 was obtained from A. D. Kaiser, λ*bio*69*c*I857*P*$^-$ and λ*N*$^-$*N*$^-$ *c*I857*nin*5 from A. Campbell, and λb189*c*I$^-$ from J. S. Parkinson. *E. coli* strains C600, 594, PolA, and W3110 were obtained from A. D. Kaiser, C600 rK$^-$mK$^-$ was obtained from M. Meselson, *E. coli* rB$^+$mB$^+$ and *E. coli* rB$^+$mB$^+$ carrying the RI plasmid were obtained from J. Morrow. The DNAs and *Eco*RI endonuclease preparations have been described (refs. 5 and 7; Thomas and Davis, submitted to *J. Mol. Biol.*). *Drosophila melanogaster* DNA, bacteriophage P4 DNA, and *E. coli* DNA ligase were kindly supplied by R. Karp, R. Calendar, and P. Modrich, respectively.

Enzyme Reactions. *Eco*RI endonuclease reactions were performed in 0.1 M Tris·HCl (pH 7.5), 0.01 M $MgSO_4$, and 0.1 mM EDTA at 37° for 10 min as described by Mertz and Davis (1). *E. coli* DNA ligase reactions were in 20 mM Tris·HCl (pH 8.0), 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 0.1 M KCl, 100 μg/ml of gelatin, and 100 μM NAD at 10° for 6 hr. These conditions result in more than 50% joining of *Eco*RI ends and less than 10% joining of the λ ends. The DNA is heated to 70° for 2 min immediately before the ligase reaction to dissociate the λ cohesive ends and to inactivate excess endonuclease.

Phage Crosses and Mutagenesis. Phage crosses were performed as described by Parkinson (6). The desired recombinant phage was selected by pyrophosphate killing (7). The

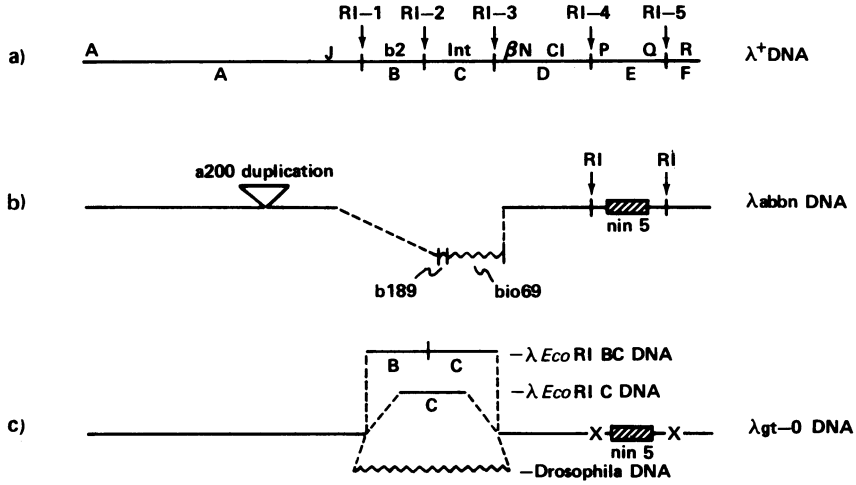

FIG. 1. Construction of λgt-λC. (a) *Eco*RI sites in λ DNA from Thomas and Davis (submitted to *J. Mol. Biol.*). This map differs from that of Allet *et al.* (4). *Eco*RI fragments are lettered A to F below the line. λ genes are above the line. (b) Structure of λ a200 b189 *bio*69 *c*I857 *nin*5 DNA called λabbn DNA. (c) Structure of λgt-λBC, λgt-λC, and λgt-Dm DNAs.

λa200 b189 *bio*69 *c*I857 *nin*5 phage was mutagenized in 0.1 M Na acetate buffer (pH 4.6) containing 50 mM $NaNO_2$ for 80 min at 25°, which reduced its viability by 10^3 (10).

Selection of EcoRI Restriction Site Mutants. The *Eco*RI restriction site mutants were enriched first by growth of 5×10^6 mutagenized phage on an *E. coli* strain containing no restriction system and then by growth on one containing the *Eco*RI restriction systems. This process was repeated about eight times until the efficiency of plating on the *Eco*RI strain increased.

RESULTS

Elimination of EcoRI Restriction Sites 4 and 5 in λ DNA. *Eco*K restriction sites in λ DNA can be removed by genetic selection for a nonrestricted λ phage (8). In the case of the *Eco*RI restriction sites, the selective pressure was desired against sites 4 and 5 rather than against 1, 2, or 3 (Fig. 1). It was determined that the λb189 substitution removes sites 1 and 2 and that the λ *bio*69 substitution removes site 3 without adding new restriction sites. Therefore, in the recombinant λ b189 *bio*69 *c*I857 *nin*5, sites 1, 2, and 3 should be absent and only sites 4 and 5 available for restriction (Fig. 1). But since this phage would have a 25% DNA deletion and therefore would not be readily propagated, it was necessary to select a duplication in the left arm of λ DNA (designated a200) in order to restore a suitable total DNA length (Emmons and Thomas, submitted to *J. Mol. Biol.*). The structure of this phage, illustrated in Fig. 1b, was determined by heteroduplex analysis (9) (Fig. 2a). Agarose gel electrophoresis of the *Eco*RI

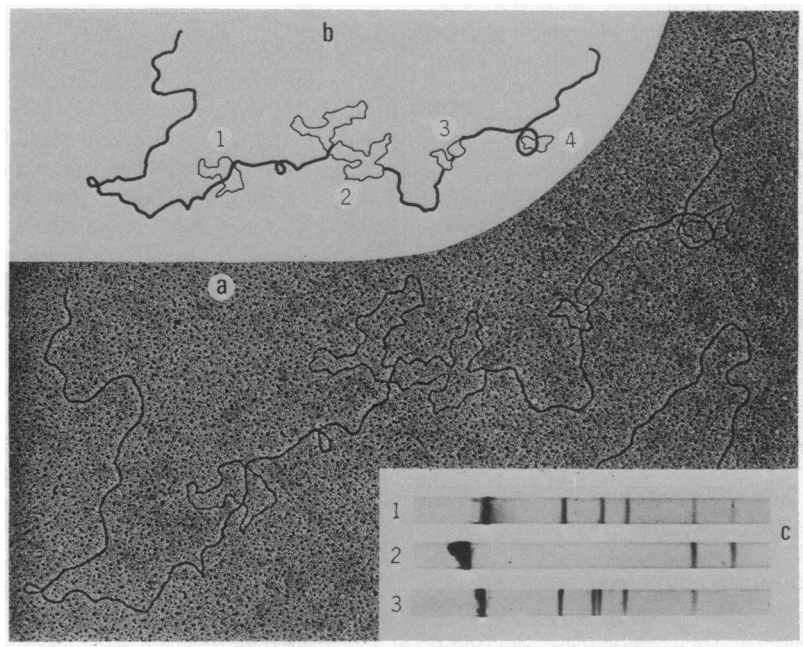

FIG. 2. Characterization of λa200 b189 *bio*69 *c*I857 *nin*5. (a) Heteroduplex with λ*imm*434 mounted for electron microscopy in 40% formamide and shadowed with platinum–paladium (9). DNA length measurements were performed as described by Mertz and Davis (1). (b) Illustration of micrograph in (a). Region 1 = a200 duplication; region 2 = b189 *bio*69/λ nonhomology; region 3 = *imm*434/λ nonhomology; region 4 = *nin*5 deletion. (c) Agarose gels of *Eco*RI endonuclease digests of (gel 3) λcI857, (gel 2) λa200 b189 *bio*69 *c*I857 *nin*5, and (gel 1) λcI857 *nin*5. The gels were prepared as described in the legend of Fig. 3.

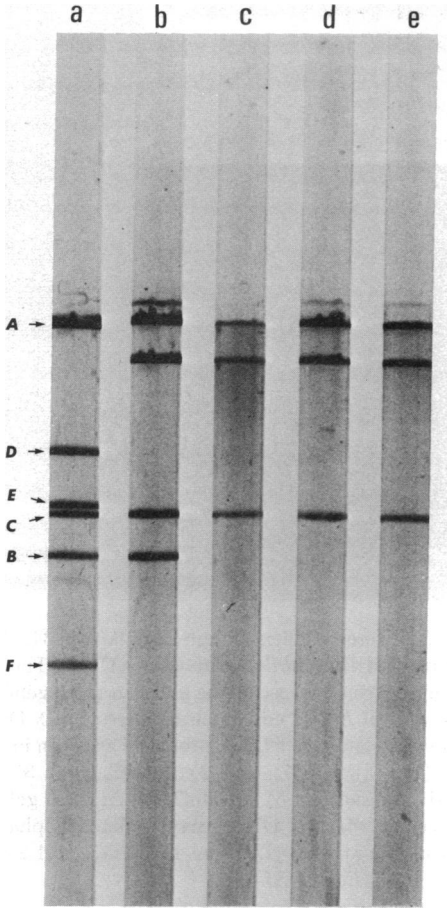

FIG. 3. Agarose gel electrophoresis of an *Eco*RI enconuclease digest of DNA from (a) λ *c*I857, (b) λgt-λBC, (c) λgt-λC, (d) λgt-λC′, and (e) mixture of λgt-λC and λgt-λC′. The λ *Eco*RI endonuclease fragments are lettered A through F. The right- and left-end fragments from the λgt strains are lettered R and L, respectively. The faint band above the left-end fragment band is from left- and right-end fragments hydrogen-bonded through the λ cohesive ends. Agarose gel electrophoresis was performed as described by Thomas and Davis (submitted to *J. Mol. Biol.*), using Tris-borate buffer and 0.5 μg/ml of ethidium bromide. Preparative gels were performed with 5 μg of DNA per tube. The DNA bands, made visible with a long-wave-length UV light (transilluminator C50 Ultra-Violet Products, Inc., San Gabriel, Calif.), were cut from the gel and eluted by electrophoresis. There was no loss in λ DNA infectivity when the DNA was treated in this manner. The gels were photographed with short-wave-length UV light.

endonuclease cleavage products of this DNA (Fig. 2c) shows that it contains only sites 4 and 5, as expected.

To select for the loss of one of two restriction sites, the efficiency of restriction must be a function of the number of restriction sites. The efficiency of plating of a number of unmodified λ deletion and substitution mutants was determined on *E. coli* cells containing only the *Eco*RI restriction system. These mutants contained from one to five *Eco*RI restriction sites. It was found that the logarithm of the efficiency of plating (E) is linearly related to the number of *Eco*RI restriction sites (R) according to the relationship log E = −0.88R. Therefore, restriction sites 4 and 5 can be sequentially eliminated and do not have to be eliminated simultaneously. λa200b189*bio*69c1857*nin*5 was mutagenized with HNO_2 (10), and mutations in the restriction sites were selected by infection of an *Eco*RI-restricting bacterium. Eight sequential selections were required to obtain a mutation in site 4. Five additional selections were required to obtain a further mutation in site 5.

Construction of λgt-λBC and λgt-λC. The a200b189*bio*69 region was removed from the strain lacking sites 4 and 5 and replaced with wild-type λ DNA by crossing to λN^- and selecting for a recombinational event between the β and the *N* genes. The resulting phage, called λgt-λBC, illustrated in Fig. 1c, yields 4 fragments after *Eco*RI endonuclease cleavage at *Eco*RI sites 1, 2, and 3 (Fig. 3b). The center two fragments, B and C, carry no genes essential for lytic growth. It is not necessary that both of these fragments be in the original DNA for propagation. The retention of only one of these fragments adds sufficient DNA to allow plaque formation. Therefore, the *Eco*RI-B fragment was biochemically removed. This was accomplished by first isolating the left and right DNA end fragments and the *Eco*RI-C fragment from the cleavage of λgt-λBC, by preparative electrophoresis on agarose gels (Fig. 3b). A mixture of these fragments was then covalently joined by DNA ligase and used to infect *E. coli* (nonrestricting C600 rK^-mK^-) cells treated with $CaCl_2$ (11). Several of the resulting phage were plaque-purified and characterized by heteroduplex analysis. Two types of phages were found: those that contained the *Eco*RI-C fragment oriented in the same direction as in λ$^+$, called λgt-λC, and those that contained the *Eco*RI-C fragment inverted, called λgt-λC′. Agarose gels of the cleavage products of λgt-λC and λgt-λC′ are indistinguishable, as shown in Fig. 3c, d, and e. These reconstructed *Eco*RI restriction sites are still cleaved by the *Eco*RI endonuclease.

λgt-λC′ is *red*$^-$ and gives a smaller plaque than λgt-λC. The *red*$^-$ genotype (12) results from the inversion at *Eco*RI restriction site 3, which probably splits the *exo* gene.

Inserted DNA is Required in λgt-O for Plaque Formation. Covalent joining of the *Eco*RI-endonuclease-generated DNA end fragments from any of the λgt strains will produce a DNA molecule with all of the essential lytic genes (Fig. 1). This type of DNA molecule is termed λgt-O since it does not contain an inserted DNA fragment (Fig. 1c). λgt-O DNA should not produce plaques after DNA infection since it has lost 27% of its DNA. Extra DNA must be inserted between these two *Eco*RI end fragments to produce DNA that is capable of generating plaques. These predictions have been substantiated by covalently joining the agarose-gel-purified end fragments from λgt-λBC through their *Eco*RI cohesive ends. (Substantial covalent joining was verified by electron microscopy.) The infectivity of this preparation of λgt-O DNA was less than 1% of that of uncleaved λgt-λBC DNA that had been subjected to the same process (Table 1). All 50 plaques from this infection were pooled, and the DNA from the phages was analyzed by observation of heteroduplexes with λ*imm*434. No DNA molecules were found that did not contain a segment of DNA between the λgt end fragments. Most of the plaques resulted from λgt-λBC DNA that had not been completely digested with *Eco*RI endonuclease. The remaining plaques resulted from DNA molecules created by the insertion of contaminating *Eco*RI-B and -C fragments that were not removed by electrophoresis on agarose gels. Further evidence that λgt-O DNA does not yield plaque-forming phage is seen in the 10-fold increase in the number of

TABLE 1. *Hybrid phage formation*

Vector	DNA concentration, μg/ml	Insert	DNA concentration, μg/ml	Plaques/plate‡ ($\times 10^{-2}$)	Plaques/ng of vector
λgt ends	50	None	—	0.5	1
λgt ends	50	*Eco*RI-C	10	5	10
λgt ends	50	λCI857	50	7	13
λgt ends	50	P4	50	10	20
λgt ends	50	*Drosophila*	50	6	11
λgt-λBC*	—	—	—	40	400
λb2c†	—	—	—	12	1200
λgt-λC (*Eco*RI cleaved)	20	None	—	1	2
λgt-λC (*Eco*RI cleaved)	20	*E. coli*	20	2	4
λgt-λC (*Eco*RI cleaved)	20	*E. coli*	50	5	10
λgt-λC*	—	—	—	20	200
λb2c†	—	—	—	10	1000

* DNA used as a control was treated as in the formation of the hybrids, but no *Eco*RI endonuclease was added.

† λb2c DNA was used untreated as a control of transfection efficiency.

‡ The DNA infections were carried out by the procedure of Mandel and Higa (11).

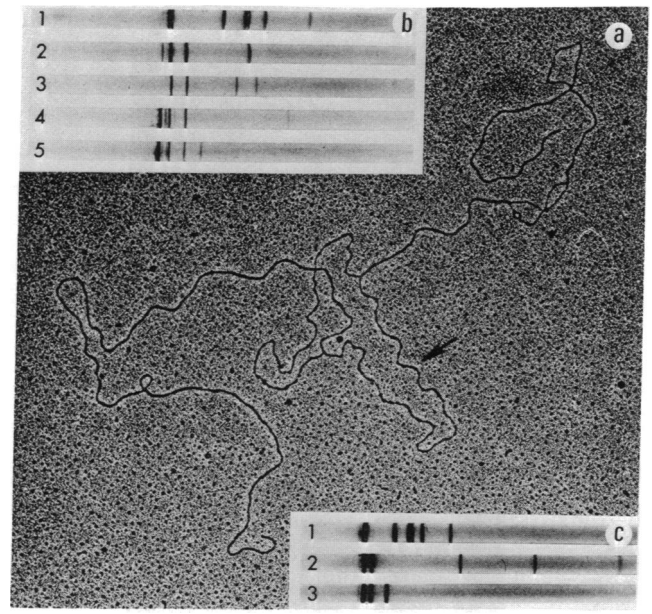

FIG. 4. (a) Heteroduplex of λgt-Dm7/λ*imm*434. The arrow shows the inserted *Drosophila melanogaster* DNA. The DNA was prepared for electron microscopy as given in the legend of Fig. 2. (b) Agarose gels of *Eco*RI endonuclease digests of λ DNA carrying *Drosophila melanogaster* DNA prepared as given in the legend of Fig. 3. (Gel 1) λ*c*I857, (gel 2) λgt-λC, (gel 3) λgt-Dm8, (gel 4) λgt-Dm9, and (gel 5) λgt-Dm7. (c) Agarose gels of *Eco*RI endonuclease digests of λ DNA carrying bacteriophage P4 and *Drosophila melanogaster* DNA. (Gel 1) λ*c*I857, (gel 2) λgt-Dm3, and (gel 3) λgt-P4*Eco*RI·AD.

plaques observed when the λ *Eco*RI-C fragment was added to the ligase reaction with the purified end fragments.

Although the joined λgt DNA end fragments from λgt-λC DNA are viable only if they acquire an additional segment of DNA, there is a residual infectivity from uncleaved λgt-λC DNA. A plaque produced by a hybrid phage can be identified easily in this residual background by the EMBO test for lysogeny (13) in which some of the cells from each plaque are spotted onto an EMBO (eosin and methylene blue with no sugar) plate (at 32°) overlayed with 10^9 λb2c*I*$^-$ phage. Plaques from phage whose DNA does not contain the λ *Eco*RI-C fragment (the only extra λ *Eco*RI fragment present) must contain the foreign *Eco*RI-endonuclease-cleaved DNA added to the reaction mix. Phage containing the *Eco*RI-C fragment (λgt-λC or λgt-λC′) have the λ attachment site, the *int* and *xis* genes, and form stable lysogens which give a light-colored colony in the EMBO test. Hybrid phages containing foreign DNA in place of the *Eco*RI-C fragment can only form abortive lysogens, which give a dark-colored colony in the EMBO test. It is interesting to note that λgt-λC′, which has the λ *att* site and the *int* and *xis* genes inverted, still forms a stable lysogen, with the prophage presumably integrated in an inverted order. This inverted lysogen can be induced to yield phage.

Construction of Hybrid Phages with the λgt DNA End Fragments. Hybrid phages have been formed by two procedures. The first involves preparative agarose gel electrophoretic separation of the λgt DNA end fragments and the *Eco*RI-C fragment after complete (99%) digestion of λgt-λC DNA with *Eco*RI endonuclease. The addition of *Eco*RI-endonuclease-cleaved λ, bacteriophage P4, or *Drosophila melanogaster* DNAs to the λgt end fragments before ligase action increased the infectivity of the product about 10 times (Table 1). These DNAs were only partially cleaved (about 1/3 of the sites) so that larger fragments could be inserted. Ninety to 95% of the plaques were judged to contain hybrid DNAs by the EMBO test. A number of these hybrids were grown and studied by heteroduplex analysis and gel electrophoresis. Fig. 4a shows a heteroduplex between λgt-Dm7 (*Drosophila melanogaster* hybrid 7) and λ*imm*434 DNAs. Fig. 4b and c show the electrophoretic separation on agarose gels of *Eco*RI endonuclease digests of λgt hybrids containing *Drosophila* DNA and P4 DNA. λgt-Dm7 has a single large *Eco*RI DNA fragment inserted (Fig. 4b, gel 5). Also shown (Fig. 4c, gel 2) is λgt-Dm3 in which the inserted *Drosophila* DNA is cleaved to give three very small *Eco*RI fragments. The two *Eco*RI end fragments of bacteriophage P4 (72% of the P4 genome) are in λgt-P4*Eco*RI·AD (Fig. 4c, gel 3). This hybrid phage contains an origin of DNA replication in the P4 DNA insert as well as in the λ DNA (Goldstein, Thomas, and Davis, in preparation).

The second approach is to use the cleavage products of λgt-λC DNA directly without the removal of the λ *Eco*RI-C fragment. The experiment shown in the second part of Table 1 used a relatively low concentration of λgt-λC DNA with two concentrations of *Eco*RI-endonuclease-cleaved *E. coli* DNA. The *E. coli* DNA fragments were in large numerical excess over the λ *Eco*RI-C fragment (about 10 and 25 times). Two-fold and 5-fold increases in the infectivity of the cleaved and rejoined λgt-λC DNA were obtained; the percentages of hybrid plaques, as measured by the EMBO test, were 50% and 80%, respectively. Higher concentrations of cleaved λgt-λC gave a smaller yield of hybrids.

DISCUSSION

This report illustrates the use of λ DNA in a generalized technique for the construction of hybrid phages containing DNA from potentially any source. In fact, hybrid phages carrying DNA from eukaryotic cells can be constructed. A plasmid DNA system for the formation and propagation of hybrid molecules has also recently been described (ref. 14; Wensink, Donelson, Finnegan, and Hogness, in preparation; Glover, White, Finnegan and Hogness, in preparation). With these λgt strains, approximately 10^4 independently generated hybrid phages containing segments of DNA up to 17,000 base pairs in length can be generated with 1 μg of foreign DNA. DNA from a eukaryotic source, *Drosophila*, gives the same yield of hybrids as DNA from *E. coli*. Thus, a sizeable proportion of *Drosophila* DNA can be propagated in *E. coli* cells. Whether there are specific eukaryotic DNA sequences that cannot be propagated in *E. coli* remains an intriguing question. At present, about 10^3 independently generated λgt-Dm phages prepared with *Drosophila melanogaster* DNA have been produced.

Insertion of DNA between the *Eco*RI endonuclease-generated λgt DNA end fragments requires two bimolecular coupling reactions. The competing nonproductive coupling reactions involving the *Eco*RI-endonuclease-generated cohesive ends are the bimolecular joining of these λgt DNA end fragments, the bimolecular oligomerization of the DNA to be inserted, and the unimolecular circularization of the DNA to be inserted. Joining of the λ DNA cohesive ends has a high activation energy and is minimized by the low temperature used for the ligase reaction. The rate of the bimolecular *Eco*RI DNA coupling reactions is determined by the concentrations of the λgt DNA end fragments and of the DNA to be inserted, while the rate of the competing unimolecular circularization reaction is determined by the length of the DNA to be inserted (2). Therefore, insertion of DNA between the *Eco*RI λgt DNA end fragments is promoted by higher concentrations and higher molecular weights of the DNA to be inserted.

A unique feature of the system described here is that it provides a selection for hybrid DNAs based upon the minimal DNA length for an infective λ particle. Also, the EMBO test readily distinguishes hybrid phage from the small background due largely to uncleaved λgt-λC DNA, since only the latter are capable of forming lysogens. Use of these phages allows easy manipulation of the hybrid DNA. For example, cells can be coinfected with two hybrids to carry out experiments involving recombination and complementation. Other possibilities for the use of lambda as a vector are being explored: for instance, a phage has been constructed that can also enter into hybrid formation but is capable of forming stable lysogens in *E. coli*. The present experimental design and its variations should, therefore, prove to be a useful tool in the study of both prokaryotic and eukaryotic DNA. Unique DNA segments from any organism can be produced in large quantities for physical and biological studies.

Note Added in Proof. The elimination of *Eco*RI restriction sites in λ DNA has been independently accomplished by N. Murray and K. Murray (15) and by A. Rambach and P. Tiollais (16).

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