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Isolation of a λdv Plasmid Carrying the Bacterial *gal* Operon

DOUGLAS E. BERG[1], DAVID A. JACKSON[2], AND JANET E. MERTZ

Department of Biochemistry, Stanford University Medical School, Stanford, California 94305 and Université de Genève, Département de Biologie Moléculaire, Sciences II, CH-1211-Genève 4, Switzerland

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A λ*dvgal* plasmid carrying genes for controlled plasmid replication from phage λ and the bacterial *gal* operon was isolated as a deletion mutant of phage λ*galq*4, which carries the *gal* operon between λ genes *P* and *Q*. The plasmid DNA was found in cell extracts as covalently closed circular molecules. The plasmid was characterized by using genetic crosses, digestion with the specific endonuclease *Eco*RI, sucrose gradient centrifugation, and electron microscopy. In one clone analyzed, the plasmid was a complete dimer (O^{λ} P^{λ} *gal* O^{λ} P^{λ} *gal*); in a subclone derived from it, the plasmid was a partial dimer with only one copy of *gal* (O^{λ} P^{λ} O^{λ} P^{λ} *gal*). The partial dimer may be a recombination product of the complete dimer, since test crosses show that the *gal* and λ sequences in the plasmid can be separated by recombination. Analyses of the *Eco*RI digests of plasmid DNAs indicated one cleavage site per λ gene sequence and none in the *gal* operon. A λ*dvgal* monomer was approximately 6.7 × 10^6 daltons and the λ gene and *gal* components were 3.9 × 10^6 and 2.8 × 10^6 daltons, respectively. The λ*dvgal* plasmid can be introduced into a new bacterial host by transfection at an efficiency of 10^{-6} per DNA molecule.

λ*dv* plasmids are deletion mutants of phage λ which retain, in essence, one operon—a segment including replication genes *O* and *P*, the origin of DNA replication, regulatory gene *cro*, and the operator-promoter region controlling transcription of this segment (3, 15; D. Berg, Virology, in press). The plasmid DNA is found as covalently closed circular DNA molecules separate from the bacterial chromosome in extracts of cells carrying λ*dv*; many copies are present per cell (13, 15; G. Hobom and D. Hogness, manuscript in preparation). Electron microscope heteroduplex analysis has shown that λ*dv* plasmids are completely homologous to part of the λ phage genome and need not contain any non-λ DNA sequences (4).

In this report we describe the isolation and genetic and physical characterization of λ*dvgal* 120, a λ*dv* plasmid which carries and expresses the entire *gal* operon of *Escherichia coli*. The DNA of λ*dvgal* 120 can be purified and then reestablished as a plasmid by transfection; it can also be cut specifically by the restriction endonuclease *Eco*RI. This plasmid has already been employed by several groups (10, 16, 19; L. Chow, Ph.D. thesis, California Institute of Technology, Pasadena, 1972; P. Wensink and D. Hogness, personal communication), and we imagine that it will continue to be a useful research tool.

[1] Present address: Université de Genève, Département de Biologie Moleculaire, Sciences II, 30, quai Ernest-Ansermet, CH-1211-Genève 4, Switzerland.

[2] Present address: Department of Microbiology, University of Michigan, Medical Center, Ann Arbor, Mich. 48104.

MATERIALS AND METHODS

Phage and bacterial strains. In phage λ*b*221 *galq*4, the parent of λ*dvgal* 120, the DNA of the entire *gal* operon of *E. coli* is inserted between λ genes *P* and *Q* (see Fig. 1); the size of the bacterial DNA inserted corresponded to approximately 8% of the λ$^+$ genome. A region corresponding to approximately 1% of the λ genome between *P* and *gal* was present again between *gal* and *Q*. (The duplicated sequence is indicated by thickenings in the lines in Fig. 1 and 4.) Recombination can occur between the duplicated sequences to regenerate the ancestral phage λ*b*221, lacking the *gal* insertion (9). λ*b*221 *c*I857 *galq*4 from M. Feiss was crossed with λ *v*2 *v*1*v*3 *O*am29, and the recombinant λ*b*221 *v*2 *v*1*v*3 *galq*4 was selected as the immediate source of λ*dvgal* 120. λ*v*2 *v*1*v*3, λ*imm*21 cI, the amber and deletion derivatives of λ*imm*21 used for marker rescue and λ*dv* size determination, and the *E. coli* bacterial strain DB866 *sup*$^-$ *recA*1$^-$ *gal-att*$\lambda_{\text{deletion}}$, strain 829S, *sup*$^-$ $N^{\lambda+}$ (the λcI857 prophage in this strain is deleted of all λ genes outside the *N-cro* segment of the phage genome), and strain 594, *sup*$^-$ *gal*$^-$ *str*, have been described (3). Bacterial strain B9, *supE*$^+$ *rec*$^+$ *gal*$^-$, was derived from C600 by A. D. Kaiser.

Media. Tryptone broth (10 g of tryptone [Difco], 5 g of NaCl per liter) and L broth (tryptone broth

containing 0.5% yeast extract) were used for liquid media. Tryptone broth with 1% agar (bottom agar) and 0.6% agar (top agar) was used for phage platings. Difco MacConkey galactose agar and TTC gal agar (tryptone broth, 0.05% 2,3,5-triphenyltetrazolium chloride, 0.2% galactose, and 1.5% agar [Difco]) were used to select Gal$^+$ colonies. Minimal salts medium E (21) supplemented with 0.1% Casamino Acids, 0.4% glucose, 1 μg of thiamine-hydrochloride per ml, and 100 μg of adenosine per ml was used for radioactive labeling of bacterial DNA.

EcoRI enzyme. The *E. coli* RI restriction endonuclease, *Eco*RI (P. Greene, M. Betlach, H. Goodman, and H. Boyer, *In* R. Wickner, ed., *Methods in Molecular Biology*, in press), was the same preparation described by Morrow and Berg (17), a generous gift of R. Yoshimori and H. Boyer. *Eco*RI reactions were performed in 0.1 M Tris, pH 7.5, and 0.01 M $MgCl_2$ at 37 C for the times indicated.

Purification of ^{3}H-labeled λdvgal 120 DNA. Strain DB866 (λ*dvgal*120) was grown at 37 C with aeration in supplemented minimal salts medium to a titer of approximately 5×10^8 cells/ml. [^{3}H]Thymidine (18.1 Ci/mmol) was added to a final concentration of 10 μCi/ml, and the growth was continued for one generation. The cells were collected by centrifugation, washed with 0.05 M Tris (pH 8.1) and 0.04 M EDTA (Tris-EDTA), resuspended in 25% sucrose in Tris-EDTA, at a cell density of approximately 10^{10}/ml, and lysozyme was added (final concentration of 0.1 mg/ml). After a 7-min incubation at 25 C, the cells were lysed by the addition of sodium dodecyl sulfate (final concentration 0.6%) and incubated at 50 C for 30 min. One-fifth volume of 5 M NaCl was added, and the lysate was stored overnight at 0 C. The majority of chromosomal DNA was selectively precipitated along with the cell debris and was removed by centrifugation in a Beckman SW27 rotor at 4 C for 30 min at 20,000 rpm. The covalently closed-circular λ*dvgal* 120 DNA was then separated from other DNA remaining in the supernatant by centrifugation in CsCl ethidium bromide (18) in a Beckman type 65 rotor at 4 C for 60 h at 37,000 rpm. Ethidium bromide was removed from the DNA by passing the DNA through a Dowex 50 column; the DNA in the eluate was concentrated by ethanol precipitation. The specific radioactivity of this DNA was 1.9×10^4 counts/min per μg.

Velocity sedimentation analysis of DNA. DNA samples were layered on neutral 5 to 20% sucrose gradients in 0.01 M Tris (pH 7.4), 1 mM EDTA, and 10 mM NaCl and were centrifuged in a Beckman SW56 rotor at 4 C and 55,000 rpm. Fractions were collected onto disks (2.5 cm in diameter) of Whatman 3MM paper, dried without washing, and counted in 2,5-diphenyloxazole-dimethyl 1,4-bis-(5-phenyloxazolyl)benzene toluene scintillator in a Nuclear Chicago Mark II scintillation spectrometer. No correction was made for a 0.4% overlap of ^{32}P into the ^{3}H channel. Simian virus 40 (SV40) DNA from cells infected at low multiplicity with plaque-purified SV40 virus, prepared essentially as described by Morrow and Berg (17), was used as an internal length standard in the centrifugations and in the electron microscopy (see below).

Electron microscopy. Length measurements of DNA were made either by tracing projected molecules on paper and measuring contour lengths with a map measurer, or with a Hewlett-Packard 9864A Digitizer and a 9810A Calculator with a fully smoothed length calculator program.

Transfection by λdvgal 120 DNA. The transfection assay was performed by a modification of the procedure of Mandel and Higa (14). *E. coli* strain B9 was grown at 37 C in L broth to a titer of 5×10^8 cells/ml, collected by centrifugation, resuspended at the same titer in cold 0.01 M $MgSO_4$, and stored at 4 C until used for the assay. (Cells stored in this manner remain competent for transfection for 1 to 2 weeks; R. Davis and M. Thomas, personal communication.) Just before use, the cells were centrifuged, suspended at 10^9 cells/ml in cold 0.05 M $CaCl_2$, kept at 0 C for 15 min, harvested by centrifugation, and then resuspended at 10^{10} cells/min in 0.05 M $CaCl_2$. A portion (0.2 ml) of the cells was added to 0.1 ml of DNA dissolved in 10 mM Tris (pH 7.1), 10 mM $CaCl_2$, and 10 mM $MgCl_2$ and incubated at 0 C for at least 15 min. Cells were then heated at 42 C for 1 to 2 min and immediately diluted and spread on TTC *gal* plates. λ*dvgal* 120 transfectants were scored as large Gal$^+$ (red) colonies emerging from the background Gal$^-$ (white) lawn after 3 to 7 days at 37 C. Representative Gal$^+$ transductants were purified by restreaking and tested by phage λ*v*2 *v*1*v*3 and λ*imm*21 infections to see if they carried λ*dvgal* 120 DNA.

RESULTS

Origin and genetic analysis of λdvgal 120. Cells carrying λ*dv* plasmids can be isolated after infection with phage λ*v*2 *v*1*v*3. They are not killed if reinfected by λ*v*2 *v*1*v*3, but are killed by heteroimmune phage λ*imm*21c. The plasmid retains a segment of λ which always includes the DNA replication genes, and in some cases extends to gene *Q*. Marker rescue tests show that segregants that have lost the λ*dv* plasmid regain sensitivity to λ*v*2 *v*1*v*3 and lose λ genes *O* and *P* (3, 15; D. Berg, Virology, in press).

To isolate a λ*dv* plasmid carrying bacterial genes, we started with phage λ *b*221 *v*2 *v*1*v*3

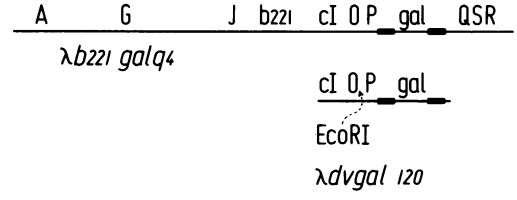

FIG. 1. *Genetic map of phage λb221 galq4, and of λdvgal 120 (1, 4, 9). Thickened lines flanking gal indicate λ sequences that are duplicated on either side of gal and between which generalized recombination can take place to excise gal (9). The site of cleavage by the endonuclease EcoRI is indicated by the arrow.*

*galq*4 (9) which carries the *gal* operon inserted between genes *P* and *Q* (Fig. 1). The phage was irradiated with UV light to a survival of 1% and was used to infect *recA*⁻ *gal*⁻ strain DB866 at a multiplicity of one particle per cell. Gal⁺ bacterial clones were selected on MacConkey galactose agar.

Of the 250 Gal⁺ bacterial clones tested, one had the properties expected of a carrier of a λ*dvgal* plasmid. The isolate was insensitive to phage λ*v*2 *v*1*v*3, but was sensitive to λ*imm21c* and did not produce phage. Gal⁻ segregants from it had regained sensitivity to λ*v*2 *v*1*v*3. Marker rescue tests with amber derivatives of phage λ*imm*21 (3) indicated that the Gal⁺ clone carried λ genes *O* and *P*, but not *A*, *G*, *J*, *Q*, or *S*; Gal⁻ segregants had lost genes *O* and *P*. A single colony isolate of this strain was grown to stationary phase. Part of the culture was stored at 4 C and another part was stored in 50% glycerol at −20 C. Clone A of 866 λ*dvgal* 120 was an inoculum from the frozen culture, whereas clone B was a single colony isolate from one of the cells in the refrigerated culture which survived 0.5 years of storage. Clones A and B differed in genetic and physical properties (see below).

To estimate the size of the λ and *gal* components of λ*dvgal* 120, further test crosses were performed by using the following rationale. Among the *P*⁺ phage recombinants formed after λ*imm*21 *P*⁻ phage infection of a λ*dv* carrier are addition recombinants containing the λ*dv* segment as a tandem duplication in the phage genome (2, 4, 11). A typical addition phage sequence would be *A imm*21 *O P*⁻ *imm*λ *O P*⁺ *Q*, where *imm*λ *O P*⁺ is derived from λ*dv*. In the case of λ*dvgal*, some addition recombinants should have the sequence *A imm*21 *O P imm*λ *O P gal Q*; others might be expected to have the sequence *A imm*21 *O P gal Q* because of the duplicated sequences flanking *gal* in the ancestral λ*galq*4 phage.

To be packaged as a viable plaque-forming particle, however, the net length of the phage genome must be no longer than approximately 106% of λ (3). If the λ*dv* segment to be inserted into the phage genome is quite long, phage with deletions in nonessential regions of the genome can be used to compensate for the added λ*dv* segment. Thus, the size of a new λ*dv* plasmid can be estimated from the minimal size deletion necessary to allow formation of a viable phage genome carrying the added λ*dv* DNA (3).

Clone B of 866 (λ*dvgal* 120) was infected with λ*imm*21 *P*⁻ phage containing deletions of increasing size. The frequencies of addition phage among *P*⁺ recombinants as a function of the genome size of λ*imm*21 *P*⁻ are shown in Table 1. The λ and *gal* segments can be separated by recombination, and their respective sizes estimated independently; the λ segment was estimated to be 11 to 14% of λ⁺ (addition phages formed with an 8% deletion, but not with a 5% deletion), and the *gal* segment was estimated to be less than 11% of λ⁺. A complete λ*dvgal* 120 monomer would be 17 to 27% of λ⁺ in length. These estimations are in agreement with other types of measurements (Table 2, Fig. 2 and 3) (4, 9).

The λ gene *P-gal* linkage (*P*⁺ *gal*/total *P*⁺) in the cross with the 21% deletion phage was 45% for clone B (Table 1, line 4). In comparable crosses with clone A, the *P-gal* linkage was 55% (data not shown).

Physical characterization of λdvgal 120 DNA. Covalently closed circular λ*dvgal* 120 DNA from clone A sedimented at 34*S* (Fig. 2A) in neutral sucrose gradients, which suggests a molecular weight of approximately 14×10^6 (6). Since phage λ is known to have one endonuclease *Eco*RI cleavage site near gene *O* (1), the plasmid DNA was treated with *Eco*RI enzyme, and the digestion products were analyzed by sucrose gradient centrifugation. The partial digest product was composed of two species with sedimentation coefficients of 21*S* and 17*S* corresponding to linear molecules of 14×10^6 and 7×10^6 daltons (Fig. 2B). The limit digest product was a linear molecule with a molecular weight of 7×10^6 (Fig. 2C).

TABLE 1. *Analysis of P⁺ phage recombinants in λimm21 P⁻ × λdvgal 120 clone B crosses*[a]

Deletion in λ*imm*21 P⁻ (%)[b]	*P*⁺ recombinants with added segments of λ*dvgal* 120 (%)				
	λ genes and *gal*	λ genes only	*gal* only	No addition	No. tested
5	0	0	3	97	219
8	0	17	8	75	414
11	0	16	11	72	339
21	35	21	10	34	547

[a] Cells growing exponentially in tryptone broth, 37 C, were infected at a multiplicity of 0.5 phage per cell, diluted 1,000-fold, and incubated at 37 C for 2 h. $CHCl_3$ was added, and the lysates were diluted and plated with *sup*⁻ *gal*⁻ strain 747. All *P*⁺ phage make turbid plaques on this strain. The plaques were picked to MacConkey galactose plates to test for *gal*, and to lawns of 829S on which addition phages carrying a λ*dv* segment make clear plaques (2, 3).

[b] Sizes are expressed as a percentage of λ⁺ DNA. The deletion phage were λ*imm*21 *P*am902 (−5%), λ*b*515 *imm*21 *P*am901 (−8%), λ*b*519 *imm*21 *P*am901 (−11%), and λ*b*538 *imm*21 *P*am901 (−21%).

TABLE 2. *Lengths of λdvgal 120 DNA*[a]

Species	Length relative to SV40 (± standard deviation)[b]	Mol wt[c]
λ*dvgal* 120 clone A closed circular	4.09 ± 0.14	13.9×10^6
λ*dvgal* 120 clone A *Eco*RI treated	2.00 ± 0.04	6.8×10^6
λ*dvgal* 120 clone B closed circular	3.15 ± 0.06	10.7×10^6
λ*dvgal* 120 clone B *Eco*RI treated	1.97 ± 0.04[d] 1.16 ± 0.03[d]	6.7×10^6 3.9×10^6
SV40 open circular	1.00	3.4×10^6
SV40 *Eco*RI treated	1.00 ± 0.03	3.4×10^6

[a] 40 to 224 molecules were measured for the various DNA species.

[b] The contour length of plaque-purified SV40 open-circular DNA is defined as 1.00 U.

[c] Based on the molecular weight of 3.4×10^6 for SV40 DNA (16).

[d] Approximately equal numbers of each size class were seen.

A higher resolution gradient (Fig. 3) indicated that the limit digest product was a single homogeneous species. It sedimented as a symmetrical peak whose width corresponded to that of *Eco*RI-cleaved SV40 linear DNA analyzed under similar conditions. Electrophoresis of the *Eco*RI limit digest product on agarose gels with an SV40 DNA marker also indicated that it was one homogeneous species, 7×10^6 daltons (data not shown).

The lengths of the plasmid DNA molecules were also measured by electron microscopy (Table 2). The results demonstrate that the *Eco*RI cleavage products of λ*dvgal* 120 DNA from clone A were linear molecules one-half the length of the uncleaved circular plasmid DNA. The length of untreated λ*dvgal* 120 plasmid DNA from clone B indicated a molecular weight of 10.7×10^6, three-fourths the length of the plasmid of clone A (Table 2). The limit digest product of clone B DNA was composed of two species. One, 6.7×10^6 daltons, was the same size as the monomer length cleavage product of clone A. The other equally frequent species had a molecular weight of 3.9×10^6.

Chow et al. (4) examined intact λ*dvgal* 120 DNA from clone B by heteroduplex mapping in the electron microscope. They concluded that the plasmid DNA molecule is a partial dimer composed of two identical λ gene components, each 4.3×10^6 daltons, and one *gal* segment, 2.4×10^6 daltons. The data in Table 2 supports that interpretation.

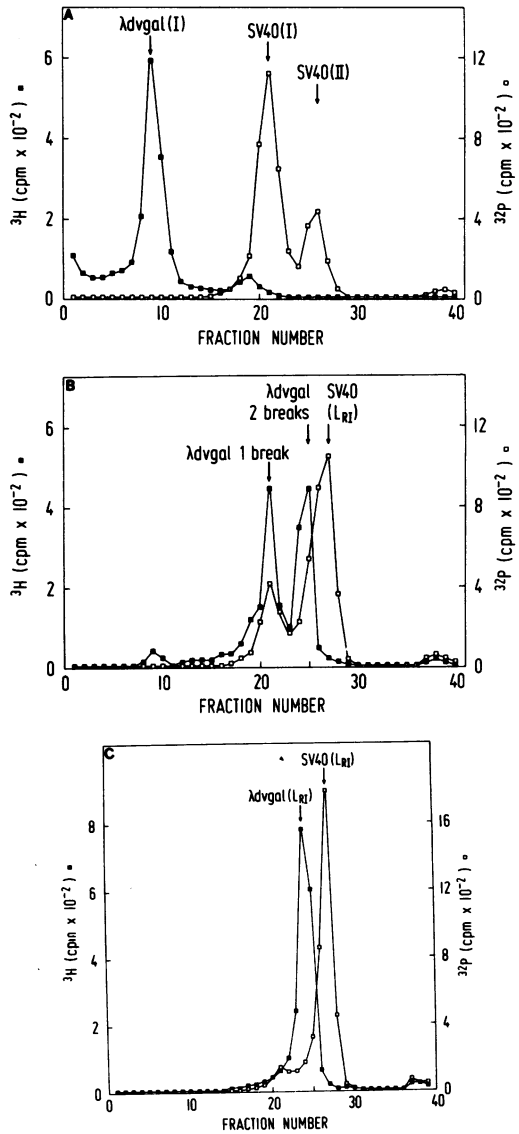

FIG. 2. *Neutral sucrose gradient sedimentation of ³H-labeled DNA of λdvgal 120 of clone A, and ³²P-labeled SV40 DNA. Two micrograms of ³H-labeled λdvgal 120 DNA (I; covalently closed rings) and 1.2 μg of ³²P-labeled SV40 DNA (I and II; covalently closed and nicked rings) were incubated at 37 C in a final volume of 80 μliters in 0.1 M Tris, (pH 7.5) and 10 mM $MgCl_2$ with sufficient EcoRI enzyme to convert the circular DNA to a limit digest product in 2 min. Portions (20 μliters) were removed to 80-μliter portions of 50 mM EDTA prior to addition of the EcoRI enzyme (A), after 30 s of incubation (B), after 2 min (C), and after 15 min (data not shown, but profile identical to panel C). The samples were centrifuged for 2 h as described and analyzed. (I) covalently closed circular DNA; (II) nicked circular DNA; (L_{RI}) linear DNA molecules resulting from EcoRI digestion.*

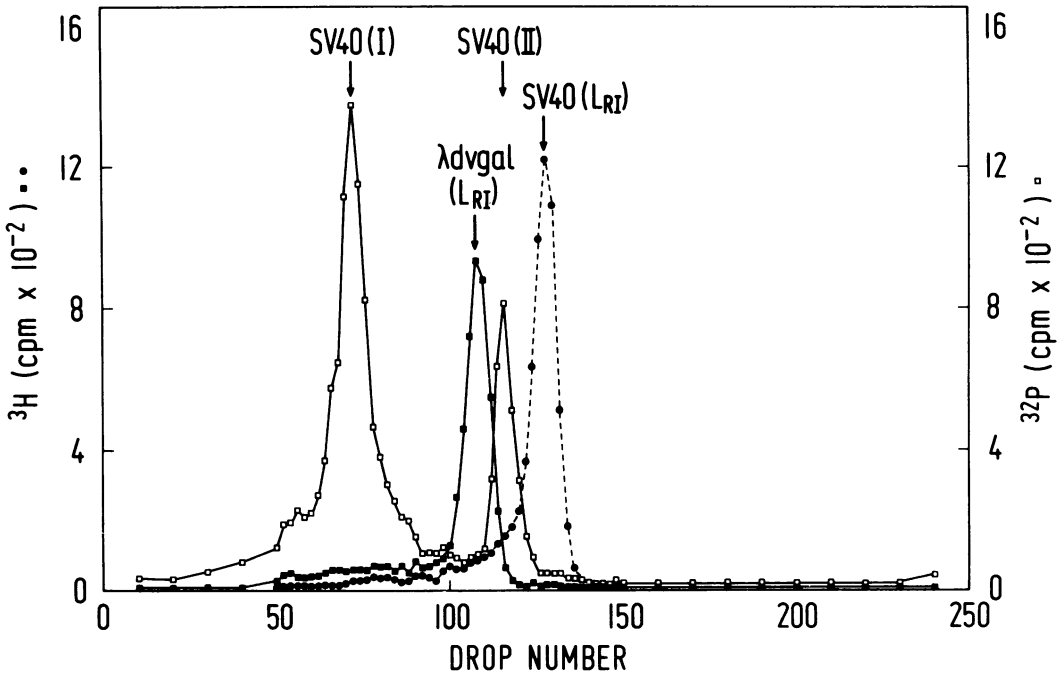

FIG. 3. *Neutral sucrose gradient sedimentation of the limit product of EcoRI digestion of* ^3H*-labeled λdvgal 120 clone A DNA and* ^3H*-labeled SV40 DNA.* ^3H*-labeled λdvgal 120 (I) DNA (0.5 μg) and 0.4 μg of* ^3H*-labeled SV40 DNA were incubated separately for 15 min at 37 C under the same conditions used in Fig. 1. The reactions were stopped with EDTA, and 0.3 μg of* ^{32}P*-labeled SV40 closed and open circular DNA was added to each sample. The samples were centrifuged for 3.5 h as described and analyzed. The results from the gradient containing the* ^3H*-labeled SV40 DNA have been plotted with those of the* ^3H*-labeled λdvgal 120 DNA by aligning the two sets of* ^{32}P*-labeled SV40 marker profiles. I, II, and* L_{RI} are used as in Fig. 2.

Establishment of λdvgal 120 in new bacterial hosts by transfection. Mandel and Higa (14) have shown that *E. coli* cells treated with CaC12 can be transfected with λ DNA and can produce progeny phage. We adapted the Mandel-Higa procedure to reestablish λ*dvgal* 120 in *gal*$^-$ strain B9. The efficiency was 10^{-6} Gal$^+$ transfectants per plasmid DNA molecule (from clone A); in the same assays, 3×10^{-6} infectious centers per phage λ DNA molecule were obtained. Twenty representative Gal$^+$ colonies obtained by transfection were purified and shown to carry the λ*dvgal* 120 plasmid by the following criteria. (i) The Gal$^+$ cells were insensitive to λ*v*2 *v*1*v*3 but sensitive to λ*imm*21*c*. (ii) Genes *O* and *P*, but not *A*, *G*, *J*, *Q*, or *S*, were detected in marker rescue tests. (iii) Phage λ*imm* 21 *P*am$^-$ grown on these cells formed addition recombinant phage as described above. (iv) Most Gal$^-$ segregants had lost λ genes *O* and *P* and regained sensitivity to λ. λ*dvgal* 120 covalently closed-circular plasmid DNA was isolated from the one Gal$^+$ transfectant clone tested (data not shown).

In independent experiments, Cohen et al. (7) have shown that a similar procedure also permits transfection by R factor plasmid DNAs.

DISCUSSION

The λ*dvgal* 120 plasmid may have arisen from phage λ*b*221 *galq*4 as diagrammed in Fig. 4. The product of the initial deletion event was probably a λ*dvgal* monomer. Replication, and then recombination between two daughter molecules (perhaps promoted by phage recombination enzymes before loss of the deleted genome; 12), would have generated a complete dimer such as was found in clone A. The partial dimer (clone B) could have arisen from a complete dimer by a recombination event between the duplicated sequences flanking the *gal* operon (9). Clone B was a single colony isolate from an old culture of clone A. It is reasonable to think that the partial dimer might have a selective advantage under certain conditions, perhaps because of its higher proportion of replication genes.

The analyses of the plasmid DNAs on sucrose gradients, and by electron microscopy, before and after *Eco*RI digestion suggest that (i) the

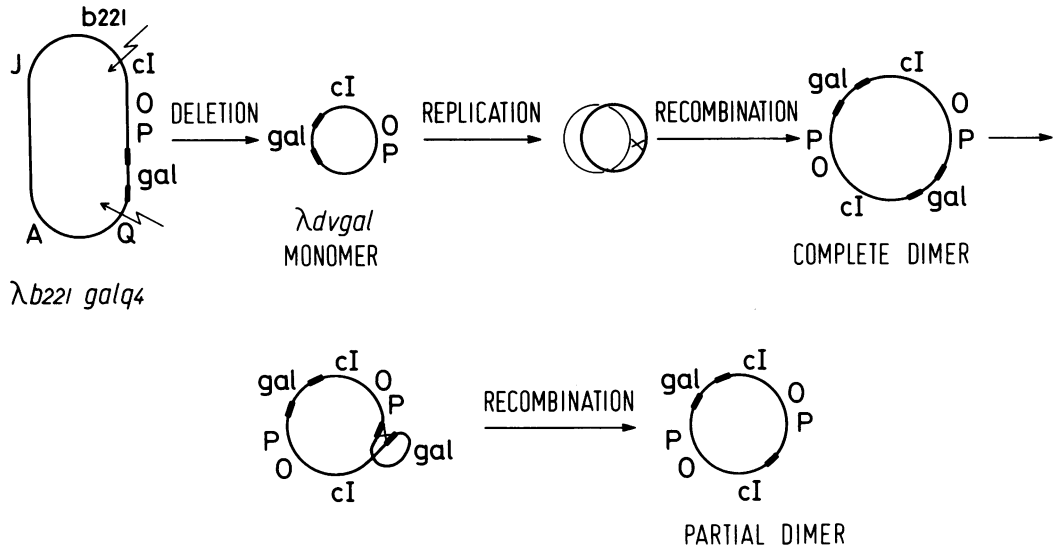

FIG. 4. *A possible origin of the complete and partial dimer forms of the λdvgal 120 plasmid.*

λ*dvgal* 120 plasmid is a complete dimer in clone A, and a partial dimer in clone B (Fig. 4), (ii) the *Eco*RI sites in the dimeric molecule are in the λ sequence, probably the site found near gene *O* by Allet et al. (1), and (iii) the *gal* operon does not carry an *Eco*RI site. The first conclusion is based on the contour lengths of clone A and B plasmid DNAs before and after cleavage by the *Eco*RI endonuclease. Cleavage of clone A plasmid DNA generates linear molecules which are the length of the λ*dvgal* monomer unit and half the length of the plasmid DNA molecule. Clone B plasmid DNA, which is 23% shorter than that of clone A, is cleaved into linear molecules, one of which is the length of the λ*dvgal* monomer, and the other of which is the length of the λ gene segment without *gal* (Fig. 2 and 3, Table 1 and 2; 4). Conclusions ii and iii are indicated by the fact that the plasmid molecules containing two copies of the λ gene sequences are cut twice, regardless of whether *gal* is present in one or two copies per molecule.

Populations of λ*dv* plasmid DNA molecules from individual *recA*⁻ clones, although quite uniform in size, always contain small numbers of molecules of other size classes (4, 12; G. Hobom and D. Hogness, manuscript in preparation). Since they contain integral multiples of the plasmid's monomer length, they have the appearance of recombination products of the major species. The partial dimer from clone B provides one additional example of this phenomenon. The role of the *recA* product in recombination is not yet understood, although it appears to be indispensable for the formation of viable recombinants in bacterial and in *red*⁻ phage crosses (5, 11, 20). It would appear that molecules in the λ*dv* plasmid population can recombine by some mysterious minor pathway which requires neither the phage *red* nor the bacterial *recA* product.

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