Physical Mapping and Complete Nucleotide Sequence of the *denV* Gene of Bacteriophage T4

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Phage T4 deletion mutants that are folate analog resistant (*far*) and contain deletions in the region of the T4 genome near *denV* have been isolated previously. We showed that one of these mutants (T4*far*P12) expressed normal *denV* gene activity, whereas another mutant (T4*far*P13) was defective in the *denV* gene. The *rII*-distal (right) physical endpoints of these deletions defined the limits of the interval in which the *rII*-proximal (left) endpoint of the *denV* gene should be located. The deletion endpoints were identified by restriction and Southern hybridization analyses of phage derivatives containing deoxycytidine instead of hydroxymethyldeoxycytidine in their DNAs. The results of these analyses localized the *rII*-proximal (left) end of the *denV* gene to a region between 62.4 and 64.3 kilobases on the T4 physical map. *denV*⁺ phage resulted from marker rescue with two of five *denV*⁻ alleles tested, using plasmids containing a 1.8-kilobase fragment from this region or a 179-base-pair terminal fragment derived from it. Sequencing of the 179-base-pair fragment from wild-type DNA showed a 130-base-pair open reading frame with its termination codon at the *rII*-proximal end. Confirmation that this open reading frame is part of the *denV* coding sequence was obtained by identifying a TAG amber codon in the homologous DNA derived from a *denV* amber mutant strain. This mutant strain rescued the *denV*⁺ allele from plasmids containing the wild-type sequence. An adjacent overlapping restriction fragment was also cloned, permitting determination of the remaining *denV* gene sequence. Based on these results, the 3' end of the coding region of the *denV* locus was mapped to kilobase position 64.07 on the T4 physical map, and the 5' end was mapped to position 64.48.

Bacteriophage T4 is markedly resistant to inactivation by UV radiation relative to other T-even phages (15). This phenotype is determined by a single genetic locus originally designated as *u* (32), then as *v* (7), and currently as *denV* (39). The *denV* gene product (so-called T4 UV endonuclease [5] or endonuclease V of T4 [40]) has recently been shown to contain a pyrimidine dimer DNA glycosylase activity both in vivo (27) and in vitro (4, 6, 26, 30, 37) as well as an activity that in vitro hydrolyzes the phosphodiester bond 3' to the apyrimidinic site created by the DNA glycosylase (17, 23, 36). The latter activity is presumed to be a biologically relevant apyrimidinic endonuclease; however, its involvement in the excision repair of phage T4 DNA has not been demonstrated in vivo.

The *denV* gene product is a small protein with an apparent *M_r* of ca. 16,000 (19, 24) and, hence, represents a particularly attractive system for investigating the structural basis of DNA damage recognition and catalysis by two important classes of DNA repair enzymes. One approach to such studies would be to infer structural attributes of both wild-type and various mutant proteins that are defective in pyrimidine dimer-DNA glycosylase, apyrimidinic endonuclease, or both, by determining the nucleotide sequence of the corresponding genes. However, the *denV* gene has not yet been cloned as a recombinant DNA molecule suitable for experiments of this kind. Using complementation of UV-sensitive *Escherichia coli* mutants as a selection procedure, Lloyd and Hanawalt (14) reported the cloning of the *denV*

gene on fragments of T4 DNA that are far too large (~15 kilobases [kb]) to readily facilitate such DNA sequencing studies. In addition, plasmids containing these fragments are highly unstable when propagated in *E. coli* (P. C. Hanawalt, personal communication).

Several investigators have been unsuccessful in attempts to directly clone the *denV* gene as a small DNA fragment that enhances the UV resistance of *E. coli* mutants defective in excision repair (K. Valerie, E. E. Henderson, and J. K. de Riel. *Fed. Proc.* 42:1985, 1983; J. D. Love and E. C. Friedberg, unpublished data; M. Sekiguchi, personal communication). Such difficulties could arise from disruption of the gene by the restriction endonucleases used in these cloning attempts, particularly if the physical location of the gene is not coincident with its genetically mapped position. We therefore carried out detailed physical mapping of the *denV* locus in the T4 genome by determining the physical endpoints of appropriate T4 deletion mutants, using restriction and hybridization analyses. The results presented here show that the *denV* gene is located at a considerably more *rII*-distal (rightward) position on the physical map than would be expected based on its previously determined genetic map position (34, 39). Restriction fragments located by these analyses were shown to contain the 3' part of the *denV* gene as evidenced by the results of marker rescue tests and by DNA sequencing of wild-type and amber mutant alleles. An overlapping adjacent fragment was shown to contain the remainder of the gene and facilitated sequencing of the entire coding region as well as some of the 5' noncoding region.

MATERIALS AND METHODS

Bacterial and phage strains and plasmids. The bacteria, phage, and plasmids used are described in Table 1.

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TABLE 1. Bacterial and phage strains and plasmids

Strain	Relevant genotype	Source
<i>E. coli</i>		
CR63	<i>Su</i> ⁺	B. Alberts
ED8689	<i>sup</i> ^o <i>hsdR</i> ⁻	B. Alberts
B	<i>F</i> ⁻ <i>sup</i> ^o	S. Wallace
JM103	$\Delta lac \rightarrow pro$ <i>F'</i> <i>traD36 proAB lacI</i> ^q $\Delta M15$ <i>hsdR</i> ⁻	New England Biolabs
T4 phage		
D	Wild type	Laboratory collection (E.C.F.)
<i>alcW7</i>	<i>g56amE51, g42amC87, \Delta denB \rightarrow rIIINB5060 alcW7</i>	E. Kutter
<i>farP12</i>	$\Delta rI \rightarrow regB$ <i>denV</i> ⁺	Laboratory collection (D.H.H.)
<i>farP13</i>	$\Delta nrdC \rightarrow denV$	Laboratory collection (D.H.H.)
<i>farP12-dC</i>	$\Delta rI \rightarrow regB$ <i>denV</i> ⁺ , markers of T4 <i>alcW7</i>	This study
<i>farP13-dC</i>	$\Delta nrdC \rightarrow denV$, markers of T4 <i>alcW7</i>	This study
<i>uvs13-dC</i>	<i>denV uvs 13</i> , markers of T4 <i>alcW7</i>	This study
<i>uvs106</i>	<i>denV</i> ⁻	J. Childs
<i>uvs107</i>	<i>denV</i> ⁻	J. Childs
<i>uvs110</i>	<i>denV</i> ⁻	J. Childs
<i>uvs13</i>	<i>denV</i> ⁻ (amber mutant)	M. Sekiguchi
<i>v₁</i>	<i>denV</i> ⁻	Laboratory collection (E.C.F.)
Plasmids		
pBR322	<i>Amp</i> ^r	Laboratory collection (E.C.F.)
M13mp10		Laboratory collection (E.C.F.)
p30	pBR322::1.8-kb T4 wild-type <i>EcoRI</i> fragment	W. M. Huang
p1800	M13mp10::1.8-kb T4 wild-type <i>EcoRI</i> fragment	This study
p1800 <i>am13</i>	M13mp10::1.8-kb T4 <i>uvsam13 EcoRI</i> fragment	This study
p179	M13mp10::179-bp T4 wild-type <i>TaqI</i> $\rightarrow$ <i>EcoRI</i> fragment	This study

Media and antibiotics. Indicator bacteria and T4 phage were propagated in L broth (16). *E. coli* JM103 infected with phage M13mp10 or its recombinant derivatives was propagated in 2 $\times$ yeast-tryptone broth (16). All phages were plated on L broth containing 1.5% agar in the underlay and 0.7% agar in the overlay. Plasmids p30 and pBR322 were propagated in *E. coli* ED8689 grown in L broth supplemented with ampicillin (50 μ g/ml). Phage buffer consisted of 10 mM Tris-hydrochloride (pH 8.0), 80 mM NaCl, and 10 mM MgCl₂.

Phage strain constructions. Phages T4*farP12-dC* and T4*farP13-dC* (containing deoxycytidine [dC] in their DNAs after propagation on nonsuppressing hosts) were constructed by mixed infection of *E. coli* CR63 with T4*alcW7* (38) (multiplicity of infection, 10) and T4*farP12* or T4*farP13* (multiplicity of infection, 1), respectively. After incubation for 90 min at 37°C, cultures were lysed with chloroform and plated on *E. coli* CR63. Single plaques with the *rI* morphology were picked and screened for the presence of dC-containing DNA after propagation on *E. coli* ED8689 as described by Kutter and Snyder (12). T4*uvs13-dC* was constructed by the same approach, using the genetic cross T4*farP13-dC* $\times$ T4*uvs13* and picking plaques with *rI*⁺ morphology. After selection of the T4 dC-DNA phenotype, UV resistance or UV sensitivity of all phages was confirmed with *E. coli* CR63 (*Su*⁺) or *E. coli* ED8689 (*sup*^o) indicator strains.

Preparation of phage and plasmid DNAs. DNA containing hydroxymethyldeoxycytidine (HMdC) was prepared from lysates of phage propagated in *E. coli* CR63 in L broth as described for phage λ by Maniatis et al. (16). DNA preparations included banding of the phage on cesium chloride step gradients. DNA containing dC instead of HMdC was prepared in the same way from the appropriate phages propagated twice in *E. coli* ED8689 grown in L broth. T4 dC-DNA was used in most experiments; the use of HMdC-DNA is specifically indicated where appropriate. Plasmid p30 was isolated from its original host strain by using an alkaline lysis

procedure (16) and transformed into *E. coli* ED8689. Plasmid DNA was purified by cesium chloride-ethidium bromide gradient ultracentrifugation (16).

Recombinant DNA procedures and nucleotide sequencing. The T4 15-kb *SaII* dC-DNA fragment was isolated as follows. DNA (20 μ g) from T4*alcW7* was cut with *SaII*, and aliquots containing 2 μ g of DNA were loaded onto each of 10 lanes of a 0.5% agarose TBE (Tris-borate, EDTA) gel (16). Electrophoresis was carried out at 2 V/cm for 20 h. The 15-kb band was identified by long-wave UV illumination and transferred to a dialysis membrane by electrophoresis after the membrane was placed in a narrow trough cut in the gel. The electrophoretic field was briefly reversed, and the DNA was recovered from the trough by aspiration. The DNA was extracted with buffer-saturated phenol, precipitated with ethanol, and resuspended in buffer (16).

For isolation of the 1.8-kb *EcoRI* fragment from plasmid p30 and from T4*uvs13-dC*, DNA (3 μ g) was cut with *EcoRI* and electrophoresed at 2 V/cm for 16 h in a 1.0% agarose TAE (Tris-acetate, EDTA) ethidium bromide gel (16). Bands were identified by long-wave UV illumination and recovered directly from gel slices by the NaI-glass bead method (35).

Restriction mapping and Southern hybridization were performed by using standard procedures (16). Hybridization probes were prepared by nick translation (16), using [α -³²P]dCTP (3,000 Ci/mmol). The specific activity of the probes was typically in the range of 10⁷ cpm/ μ g of DNA. Restriction endonucleases were purchased from Bethesda Research Laboratories. Restriction of phage and plasmid DNAs was carried out as suggested by the manufacturer, using 0.5 to 2 μ g of DNA per 50 μ l of reaction volume. Agarose gel electrophoresis was carried out by using the agarose concentrations and buffer compositions indicated for specific experiments.

For DNA sequencing, fragments of T4 DNA were ligated into M13mp10 (double-stranded form) and transfected into *E. coli* JM103 or TG1. The latter strain has the same relevant

genotype as JM103 and was constructed by T. Gibson, MRC Unit, Cambridge, England. White M13 plaques were picked from X-gal plates and plaque purified twice. The isolated 1.8-kb *EcoRI* insert from plasmid p30 was cloned into the *EcoRI* site of M13mp10, and phages containing the insert in either orientation were isolated. Plasmid p1800 was identified by DNA sequencing as a recombinant phage with the orientation of the 1.8-kb fragment such that the *HindIII* and *TaqI* sites (see Fig. 4D) were closest to the M13 priming site. The same procedure was used for isolation and cloning into M13 of the 1.8-kb *EcoRI* fragment from T4 ν s13-dC to generate the recombinant phage p1800am13, except that clones were identified by screening with p30 DNA radiolabeled by nick translation. To clone the 179-base-pair (bp) *EcoRI*-*TaqI* fragment, the 1.8-kb *EcoRI* insert in p30 was isolated and digested with *TaqI*. The mixture was cloned into M13mp10 that had been cut with *EcoRI* and *AccI*. The appropriate insert was identified by its size, by hybridization to p1800, and by DNA sequencing. The orientation of the insert in this

recombinant plasmid (p179) allowed sequencing of the strand complementary to that sequenced in p1800. An 800-bp *HindIII* fragment located between 64.17 and 65.0 kb on the T4 physical map (see Fig. 3) was identified by Southern hybridization, cloned into M13, and sequenced to confirm a 30-bp overlap with the *EcoRI* 1.8-kb fragment containing the 3' end of the gene. Single-stranded DNA from recombinant M13 phage was sequenced by the chain termination method (29), using dideoxynucleotides and [α - 35 S]ATP.

Marker rescue of the *denV* gene. Mutant (*denV*⁻) phage ($\sim 10^4$ PFU) was plated with 10^7 CFU of the indicated host strain carrying a recombinant plasmid or M13 phage (or the vector alone as a control) and incubated at 37°C for 12 to 16 h. Ampicillin (50 μ g/ml) was included in the bottom agar to select pBR322 or p30 plasmid-transformed cells. After incubation, the surface of the agar was rinsed briefly with chloroform, and phage was harvested by elution into 4 ml of phage buffer for 3 h at room temperature. The resulting eluates typically had a phage titer of 10^{10} to 10^{11} PFU/ml.

UV survival studies. Phages were diluted to ca. 10^7 PFU/ml in phage buffer and were UV irradiated while being stirred, using a calibrated 15-W germicidal lamp. At this dilution, the phages typically had negligible absorbance at 254 nm; however, where necessary, a correction for absorbance (no more than 5%) was made as described by Morowitz (20). Irradiated phages were immediately plated on bacteria under yellow (nonphotoreactivating) light at a multiplicity of <0.01 and incubated in the dark at 37°C for 12 to 16 h. Incident dose rates were determined by using a photometer (International Light, model IL254). For irradiation of UV-enriched lysates, plates containing phage that survived irradiation at the indicated dose were rinsed with chloroform and eluted into phage buffer as described above. The resulting lysates typically had a titer of 2×10^9 PFU/ml.

Pyrimidine dimer DNA glycosylase activity in vivo. Oligonucleotides containing pyrimidine dimers excised in vivo were recovered from the acid-soluble fraction of phage-infected UV-irradiated *E. coli* cells labeled in their DNAs with [3 H]thymidine. The oligonucleotides are analyzed for photoreversal-dependent release of free [3 H]thymine as previously described (27).

RESULTS

UV sensitivity of T4 ν far deletion mutants. Chace and Hall (3) isolated and characterized a series of spontaneous folate analog-resistant (*far*) T4 mutants that contain deletions of the *regB* locus located near the *denV* gene at ca. 61 kb on the T4 genetic map (Fig. 1A). We identified two of these mutants as potentially useful for the physical mapping of the *denV* gene. Based on the reported frequency of recombination with the *e* gene (3), the *rII*-distal (right) end of the deletion in one of these mutants (T4 ν farP12) should extend through the *denV* gene (Fig. 1A). However, when we examined T4 ν farP12 for this phenotype directly, we found that it displayed wild-type levels of UV resistance (Fig. 2). Furthermore, cells infected with T4 ν farP12 demonstrated excision of pyrimidine dimers mediated by T4 pyrimidine dimer-DNA glycosylase activity (Table 2). The latter result indicated that the UV-resistant phenotype of T4 ν farP12 resulted specifically from the expression of a functional *denV* gene rather than from a nonspecific phenotypic effect of the deletion. (Deletion of the *regB* locus causes changes in the level of expression of several T4 gene products involved in nucleotide metabolism [3] and could result in a UV-resistant phenotype in a *denV* mutant.) The T4 ν farP12 mutant was not UV sensitive at elevated temperatures or in high-salt medium, conditions

FIG. 1. Restriction endonuclease analysis of T4 wild-type and deletion mutants containing dC in DNA. (A) Partial genetic map of phage T4 showing the genetic endpoints of the deletions in the mutants T4 ν farP12 and T4 ν farP13 determined from recombination frequencies (3) and the four-parameter switch function of Stahl et al. (31) (We used a value of 166 kb for the total length of the T4 genome rather than the value of 200 kb used by Stahl et al. [31].) Gene positions are those of Wood and Revel (39). Below the genetic map is a partial physical map of wild-type T4 DNA showing the location of *SalI* and *XhoI* sites in this region of the genome and the sizes of the DNA fragments generated by restriction with these enzymes (11). (B and C) Agarose gel electrophoresis of restricted T4 ν alcW7 (wild-type) and T4 ν far deletion DNAs containing dC. (B) Restriction with *SalI*. (C) Restriction with *XhoI*.

FIG. 2. UV sensitivity of T4farP12 and T4farP13 deletion mutants. Phages were irradiated as described in the text and plated in triplicate.

that might destabilize a truncated or fused protein (data not shown). Thus, the *rII*-distal (right) end of the deletion in T4farP12, in fact, either does not extend into the *denV* gene, or if it does, the extent to which the gene is deleted is insufficient to detectably alter expression of enzymatic activity or phage viability after UV irradiation. The observation that the T4farP12 strain is phenotypically *denV*⁺ may reflect anomalous recombination in this region of the genome rather than an error in the genetic map data (see below).

In contrast to T4farP12, another deletion mutant in this region of the T4 genome (T4farP13) was abnormally sensitive to UV radiation (Fig. 2). Furthermore, T4farP13-infected cells did not display measurable pyrimidine dimer DNA glycosylase activity in vivo (Table 2). Hence, the deletion in this mutant presumably does extend into or beyond the *denV* gene (Fig. 1). Given these results, it is most likely that the physical *rII*-distal (rightward) endpoints of these two deletions define the boundaries of the interval in which the *rII*-proximal (left) end of the *denV* gene is located. Since the gene is known to encode a protein of ca. 16 kilodaltons (19, 24), the coding region must be about 0.4 kb. Hence, the accurate localization of the left end of the gene could reasonably be expected to define a localized segment of the T4 genome for possible molecular cloning of the *denV* gene without flanking genes.

Restriction enzyme analysis of T4far mutants. A preliminary localization of the physical endpoints of the deletions in the two T4far mutants was made by restriction enzyme analysis of wild-type and deletion mutant DNAs. The *farP12* and *farP13* deletion mutations were genetically crossed into the T4alcW7 background (see above) to yield strains that incorporate and retain dC in their DNAs during propagation in nonsuppressing hosts (12). DNAs from these strains (designated T4farP12-dC and T4farP13-dC [for dC-containing DNA], respectively) are cut normally by restriction endonucleases, unlike T4 DNA containing HMdC (12).

Previous genetic analysis of the T4farP12 and T4farP13

mutants (3) indicated that both deletions should span the *SalI* site at position 57.9 kb on the T4 map and should terminate within a 15-kb *SalI* fragment between the *tk* and *e* genes (Fig. 1A). Thus, the deletions should create fusions between the 15- and 24-kb *SalI* fragments flanking the *SalI* site at position 57.9 kb (Fig. 1A). Restriction enzyme analysis with *SalI* showed that the 15- and 24-kb fragments in T4alcW7 were indeed missing in the deletion mutants and that novel fragments were generated (Fig. 1B). For T4farP12, the novel fragment had the same electrophoretic mobility as that of a 28-kb λ DNA marker. Since this novel fragment represents the fusion of the 24- and 15-kb fragments minus the length of the deletion, the deletion in T4farP12 was calculated at ca. 11 kb. By the same analysis, the deletion in T4farP13 must be greater than 15 kb since the novel fragment generated by digestion of DNA from this mutant was shorter than the 24-kb fragment present in restricted T4alcW7 DNA (Fig. 1B).

Similar experiments were carried out with the enzyme *XhoI*. The deletions should occupy much of the 15-kb *XhoI* fragment spanning ca. 49 to 64 kb in the wild-type T4 genome (Fig. 1A). Restriction analysis of DNA from T4alcW7 and from both deletion mutants confirmed the loss of a ca. 15-kb fragment in the latter (Fig. 1C). The deletion in T4farP13-dC must include the *XhoI* site at 64.3 kb since it also resulted in loss of a 4-kb *XhoI* fragment present in T4alcW7 and T4farP12-dC DNAs (Fig. 2C). These results, together with those obtained with other enzymes that generate relatively few cuts in T4 dC-containing DNA (data not shown), showed that the *rII*-distal (right) end of the deletion in T4farP12-dC ends between the *SalI* site at 57.9 kb and the *XhoI* site at 64.3 kb, while that in T4farP13 ends within the 4-kb *XhoI* fragment (Fig. 1A).

Southern hybridization analysis. Refined localization of the *rII*-distal (rightward) endpoints of the deletions was determined by Southern hybridization analysis, using the ³²P-labeled 15-kb *SalI* fragment from T4alcW7 DNA as a probe. When T4alcW7 DNA was restricted with *EcoRI*, the results obtained corresponded closely to those previously reported (18). Thus, fragments of ca. 6, 5, 4, 1.9, 1.8, and 1.7 kb were detected (Fig. 3A and 3D). Our data indicate, however, that the previously published (18) order of *EcoRI* fragments that placed the 1.9 kb fragment to the right of the 1.8 kb fragment is reversed (Fig. 3D; see below). As expected, we also

TABLE 2. Demonstration of PD-DNA glycosylase activity in T4-infected, UV-irradiated *E. coli*^a

T4 strain	³ H]Thymine radioactivity in acid-soluble extracts (cpm) ^b	
	Without photoreversal	With photoreversal
<i>farP12</i> -dC	27	316
<i>farP13</i> -dC	13	17
<i>v</i> ₁	14	22
D	48	589

^a *E. coli* *uvr* mutant cells were radiolabeled in their DNAs with [³H]thymidine, UV irradiated as described previously (27), and infected with the indicated phage at a multiplicity of 10. After incubation at 37°C, the acid-soluble fractions of the phage-infected cells were isolated and analyzed for the photoreversal-dependent release of free radiolabeled thymine base (27). Detection of free thymine demonstrates the in vivo hydrolysis of the 5' glycosyl bond in thymine-containing pyrimidine dimers and constitutes evidence for excision repair mediated by pyrimidine dimer-DNA glycosylase activity (26, 27).

^b Chromatographic fractions were counted for radioactivity until the two standard-deviation error was $\pm 2\%$. Duplicate measurements agreed within 10% and were averaged.

observed two fragments of ca. 0.6 kb (Fig. 3, insert, and 3D). A band representative of a DNA fragment of >10 kb was also detected (Fig. 3A). This DNA was apparently present in nonstoichiometric amounts since it hybridized less intensely than did adjacent smaller fragments. It did not represent a partial restriction fragment since its presence was not affected by more extensive digestion with *EcoRI* (data not shown). We therefore concluded that this band reflects persistence of the original parental HMdC-DNA that cannot be further restricted with *EcoRI*. In agreement with the previously established restriction map (11), the *EcoRI* fragments of 4.0

and 1.7 kb contained *XhoI* sites (Fig. 3D) since the positions of these fragments shifted after double digestion with *EcoRI* and *XhoI* (Fig. 3B).

Analysis of T4farP12-dC DNA restricted with *EcoRI* (Fig. 3A) showed the absence of the 5-, 1.9-, 1.8-, and one of the 0.6-kb fragments present in T4alcW7 DNA, all of which were located to the left of the *EcoRI* site at 64.2 kb on the wild-type T4 map (Fig. 3D). However, the 6-, 4-, and 1.7-kb fragments as well as another ca. 0.6-kb fragment, all of which were derived from the region to the right of this site (Fig. 3D), were still present (Fig. 3A). Based on these results, we concluded that T4farP12-dC contained a simple deletion in the region of the *rII*-proximal (leftward) end of the 15-kb *SalI* fragment (Fig. 3D) that extended in the *rII*-distal (rightward) direction beyond the *EcoRI* site at 62.4 kb and ended within the adjacent 1.8-kb *EcoRI* fragment (Fig. 3D). A novel *EcoRI* fragment created by the deletion was also detected (Fig. 3A). This fragment was ca. 10 kb and apparently represented a fusion of the remnant of the 1.8-kb fragment to that of an 11.8-kb fragment spanning positions 42.2 to 54.0 kb on the standard map (11) (see below).

The results obtained after digestion of T4farP13-dC DNA with *EcoRI* differed from those for T4farP12-dC DNA only by the additional loss of a 1.7 kb fragment (Fig. 3B). Thus, the deletion in T4farP13-dC included the *EcoRI* site at position 64.2 kb and ended within the adjacent 1.7-kb fragment (Fig. 3D). This conclusion was consistent with the observation described above that the deletion also included the *XhoI* site at position 64.3 kb. With respect to the three small *HaeII* fragments that hybridized to the *SalI* probe, the pattern for T4farP13-dC DNA (Fig. 3C) did not differ from that observed with T4alcW7 DNA, indicating that the deletion in T4farP13-dC did not extend to the *HaeII* site at position 65.1 kb (Fig. 3D). Thus, the physical *rII*-distal (rightward) endpoint of the deletion in T4farP13-dC was located between 64.3 and 65.1 kb on the T4 genetic map (Fig. 3D).

It should be noted that the results presented above were consistent only with the two deletions having their *rII*-distal (rightward) ends in adjacent *EcoRI* fragments. If this were not the case, *EcoRI* fragments in addition to the 1.7-kb fragment should have been present in T4farP12-dC DNA digests and absent in T4farP13-dC DNA digests. This confirmed our assignment of the 1.8-kb *EcoRI* fragment to position 62.4 to 64.2 kb on the T4 map, rather than to a position to the left of this, as reported previously (11, 18).

Localization of the T4farP12 deletion endpoint within the 1.8-kb *EcoRI* fragment. The 1.8-kb *EcoRI* fragment that included the *rII*-distal (rightward) endpoint of the deletion in T4farP12-dC (Fig. 3D) corresponded to a DNA fragment previously identified by King and Huang (9) as containing an origin of replication of T4 DNA. They cloned this fragment into the vector pBR322, yielding a recombinant plasmid designated as p30 (9). The 1.8-kb insert in p30 hybridized to the 15-kb *SalI* probe (Fig. 3A). Furthermore, it comigrated with (Fig. 3A and 4A) and hybridized to (Fig. 4A) the 1.8-kb fragment present in *EcoRI* digests of T4alcW7 DNA and absent in digests of both deletion mutants. Additionally, the 1.8-kb fragment from p30 hybridized to a novel *EcoRI* fragment of >10 kb in restricted T4farP12-dC DNA (Fig. 4A), confirming the results of the restriction analysis (Fig. 3A). The probe also hybridized to a 0.6-kb *HindIII* fragment present in digests of T4alcW7 and T4farP12-dC DNAs (Fig. 4B), demonstrating that the deletion in the T4farP12-dC mutant does not include the *HindIII* site at position 63.6 kb (Fig. 3D and 4D and E). Furthermore, comparison of the

FIG. 3. Analysis of wild-type and deletion mutant T4 DNAs containing dC by Southern hybridization. DNA was restricted with *EcoRI* (A) (electrophoresis in 1.2% TBE agarose gel at 3 V/cm for 24 h), *EcoRI* plus *XhoI* (B) (electrophoresis as for A) or *HaeII* (C) (electrophoresis in 1% TBE agarose gel at 2 V/cm for 8 h). The insert shows details of a blot of DNA cut with *EcoRI* and run as described for C. DNA fragment size standards are indicated by the arrows. (D) Partial physical map of the wild-type T4 genome showing *EcoRI* (E), *XhoI* (X), *SalI* (S), and *HaeII* (H) restriction sites and the *EcoRI* and *HaeII* DNA fragments hybridized by the 15-kb *SalI* probe. The map also shows the location of a *HindIII* (Hn) fragment located between 64.17 and 65.0 kb that was used to determine the sequence of a part of the *denV* gene. The *HindIII* site at 64.17 kb was separated from the *EcoRI* site by only 30 bp. The restriction sites are in the main derived from the data of Kutter and Rüger (11), with slight modifications of the locations of the *EcoRI* and *XhoI* sites to account for the fragment sizes observed in this study (see text). Also shown (D) are the inferred physical *rII* distal (rightward) endpoints of the deletions in T4farP12-dC and T4farP13-dC. The bars define the intervals in which the deletions end, with the endpoints arbitrarily assigned to the midpoints of the intervals.

FIG. 4. Southern hybridization of restricted wild-type (T4alcW7) and deletion mutant DNA, using plasmid p30 as a probe. dC-containing phage and plasmid DNA were restricted with *EcoRI* (A) or *HindIII* (B). HMdC-DNA was restricted with *TaqI* (C). The 1-kb band seen with *TaqI*-restricted T4D HMdC-DNA is a partial restriction fragment not observed after more extensive enzyme digestion (data not shown). (D) Partial physical map of the wild-type T4 genome showing *EcoRI* (E), *HindIII* (H), and *TaqI* (T) sites determined in this study as well as the *HindIII* and *TaqI* fragments hybridized by the probe. (E) Inferred physical *rII*-distal (rightward) T4farP12-dC deletion endpoint. (F) The 179-bp *TaqI*-*EcoRI* fragment derived from the right end of the 1.8-kb *EcoRI* fragment (Fig. 3D) and the 130-bp region that contains the 3' end of the *denV* gene.

hybridization bands after *TaqI* digestion of the 1.8-kb insert in p30, T4farP12 HMdC-DNA and T4D HMdC-DNA (Fig. 4C), showed that the deletion in T4farP12 did not include the *TaqI* site at position 63.4 kb (Fig. 4D and E). We thus localized the right end of the deletion in T4farP12 within the 1.8-kb *EcoRI* fragment between 62.4 and 63.4 kb on the T4 physical map (Fig. 4D and E). As expected from the extent

of the deletion in T4farP13, no hybridization of the p30 probe to *EcoRI* digests of T4farP13-dC DNA was observed (Fig. 4A).

It should be noted (Fig. 4C) that all of the *TaqI* fragments from the dC-containing 1.8-kb DNA insert in p30 (except the two *TaqI*-*EcoRI* fragments derived from its ends) corresponded in size to *TaqI* fragments generated from HMdC-containing wild-type T4 DNA. Hence, there was no detectable rearrangement within the cloned 1.8-kb *EcoRI* fragment resulting from its cloning or from propagation of phage with dC-containing DNA.

Identification of the *denV* gene by marker rescue. The physical mapping of the deletion endpoints in the T4farP12-dC and T4farP13-dC mutants described above indicated that the *rII*-proximal (left) end of the *denV* gene must be located at a more *rII*-distal (rightward) position on the physical T4 map than indicated by previous genetic mapping (Fig. 1A). Specifically, our data predicted that the 1.8-kb *EcoRI* fragment (Fig. 3A) could contain part (and conceivably all) of the *denV* gene. Hence, at least some *denV*⁻ strains might rescue the *denV*⁺ allele from plasmids containing this DNA fragment after infection of plasmid-bearing cells with different *denV*⁻ phages.

The feasibility of detecting marker rescue of the *denV*⁺ allele by demonstrating enhanced UV resistance of a phage population was established in preliminary experiments. A mixed population of *denV*⁺ and *denV*⁻ phages displayed a characteristic biphasic survival curve after exposure to UV radiation (Fig. 5A). The presence of the *denV*⁺ subpopulation resulted in an inflection of the curve, producing a tail with a slope characteristic of the inactivation of a pure population of *denV*⁺ phages (34). The inflection in the survival curve occurred at a UV dose at which *denV*⁺ and

FIG. 5. Detecting *denV*⁺ phage subpopulations by enhanced UV survival. (A and C) T4v₁ or T4v₁ mixed with T4D was irradiated and plated as described in the text. Infection at a multiplicity of 0.1 was effected by preadsorbing UV-irradiated phage for 10 min at 37°C to exponentially growing indicator bacteria previously concentrated 10-fold by centrifugation. Infected cells were diluted and plated for plaque formation. (B and D) UV-enriched lysates were prepared from the phages irradiated with 15 J/m² (A and C) and reirradiated as described in the text. All manipulations were carried out under nonphotoreactivating light.

denV⁻ phages were about equally represented among the survivors. At UV doses higher than this, the survival of the mixed population was greater than that of a pure *denV*⁻ population since proportionately more *denV*⁺ phage survived.

If a subpopulation of *denV*⁺ phage were generated as the result of marker rescue by some fraction of *denV*⁻ phage of a plasmid containing all or part of the *denV* gene, it might be possible to detect this by demonstrating an inflection of the survival curve or, equivalently, by demonstrating enhanced survival relative to that of pure *denV*⁻ phage at a selected UV dose. However, care must be exercised in interpreting the results of such experiments since multiplicity reactivation of UV-irradiated *denV*⁻ phage can also produce enhanced survival and hence an inflection of the survival curve (2). The UV dose at which the reactivation phenomenon manifests is sensitive to the multiplicity of infection in a particular experiment and in practice limits the sensitivity with which a *denV*⁺ subpopulation can be reliably detected. Figure 5A shows that 1% *denV*⁺ phage in a mixed (with T4v₁) phage population resulted in a 2.5-fold increase in survival at a UV dose of 15 J/m².

That this enhanced UV survival reflects the presence of *denV*⁺ phages in a mixed population rather than multiplicity reactivation of *denV*⁻ phage can be shown by taking advantage of the fact that phages in a mixed population that survive an initial UV irradiation are enriched for the more UV-resistant *denV*⁺ subpopulation. One can therefore harvest the total population of phages that survive initial irradiation by eluting the agar plates en masse (see above) and determine the UV sensitivity of this "UV-enriched" lysate by a second irradiation. A large increase in UV resistance confirms the presence of *denV*⁺ phage in the original population. When the survivors of a mixed population originally containing 1% *denV*⁺ phage were isolated as a UV-enriched lysate and subjected to a second irradiation, a considerable enhancement of survival was observed, reflecting the presence of about 60% *denV*⁺ phage in the enriched population (Fig. 5B). In contrast, when the survivors generated by multiplicity reactivation of a pure population of *denV*⁻ phage were reirradiated and then plated at a lower multiplicity of infection, no enhanced survival was observed (Fig. 5B). Note that revertant *denV*⁺ phage was not present

TABLE 3. Marker rescue of the T4 *denV*⁺ allele from the plasmid p30^a

Mutant <i>denV</i> allele	Initial UV resistance	UV Resistance after marker rescue with plasmid:			
		p30		pBR322	
		Marker rescue lysate	UV-enriched marker rescue lysate	Marker rescue lysate	UV-enriched marker rescue lysate
v ₁	-3.8	-3.7	-3.8	ND ^b	ND
<i>uvs106</i>	-4.0	-3.9	-4.0	ND	ND
<i>uvs107</i>	-4.2	-4.2	-4.2	ND	ND
<i>uvs110</i>	-3.2	-2.7	-2.0	-3.3	-3.4
<i>uvs13</i>	-4.0	-3.4	-2.2	-3.9	-3.9

^a The UV sensitivity of the various phage lysates are reported as the log of the surviving fraction at UV radiation of 15 J/m². Marker rescue lysates were prepared after parallel infections of *E. coli* ED8689 transformed with plasmid p30 or with plasmid pBR322. Stocks of the *denV* mutant phages used for infection were propagated from single plaques. UV-enriched marker rescue lysates (see text) were prepared from phage that had been irradiated at 15 J/m².

^b ND, Not determined.

TABLE 4. Demonstration of PD-DNA glycosylase activity in cells infected with marker rescue lysates^a

T4 strain	Rescuing plasmid	[³ H]Thymine radioactivity in acid-soluble extracts (cpm) ^b	
		Without photoreversal	With photoreversal
<i>uvs13</i>	p30	20	93
<i>uvs13</i>	pBR322	11	15
<i>uvs110</i>	p30	17	70
<i>uvs110</i>	pBR322	15	18

^a The UV-enriched marker rescue lysates prepared as described in Table 3, footnote^a, assayed for pyrimidine dimer-DNA glycosylase activity in vivo (Table 2). The relatively low titers of these lysates precluded infection of *E. coli* at multiplicity of >1. This presumably accounts for the smaller amount of free thymine detected relative to the results shown in Table 2.

^b Chromatographic fractions were counted for radioactivity until the two-standard-deviation error was $\pm 2\%$. Duplicate measurements agreed within 10% and were averaged.

in pure *denV*⁻ populations at levels that interfered with the interpretation of these experiments (Fig. 5B).

Analysis of UV-enriched phage also facilitated the detection of *denV*⁺ phage in a mixed population when their level was too low to detect an enhancement of survival with a single irradiation. Addition of 0.3% *denV*⁺ phage to a *denV*⁻ population did not lead to detectably greater survival at 15 J/m² than that observed with pure *denV*⁻ phage (Fig. 5C). However, when the UV-enriched lysate prepared from survivors of the first irradiation was reirradiated, the *denV*⁺ phages were readily detected (Fig. 5D).

A 1.8-kb *EcoRI* DNA fragment supports marker rescue by some *denV* mutant strains. The plasmid p30 was transformed into *E. coli* ED8689, a strain previously shown to support efficient marker rescue of phage T4 (D. H. Hall, unpublished data). Stocks from single plaques of various *denV*⁻ strains were propagated in p30-transformed cells, or as a control, on *E. coli* ED8689 carrying plasmid pBR322. Survival of the lysates was increased three- to fivefold (as determined by initial UV irradiation at 15 J/m²) in the case of the *denV* mutant strains T4*uvs110* and T4*uvs13* propagated on the host carrying plasmid p30 (Table 3). The corresponding UV-enriched lysates displayed an even greater level of survival after a second irradiation (Table 3). In contrast, in the control experiments in which rescue was attempted with plasmid pBR322, no significantly enhanced survival was noted. To prove that the enhanced UV resistance observed specifically reflected rescue of the *denV*⁺ allele by the strains *uvs110* and *uvs13*, the appropriate lysates were examined for pyrimidine dimer-DNA glycosylase activity in vivo. *E. coli* cells infected with UV-enriched lysates containing phage propagated with plasmid p30 displayed such activity, whereas control lysates did not (Table 4).

Three of the five *denV* mutant alleles tested failed to rescue the *denV*⁺ allele from plasmid p30 (Table 3). A likely explanation for this observation is that the 1.8-kb DNA insert in p30 includes only part of the *denV* gene. If so, the incomplete gene should be located at the *rII*-distal (right) end of the insert since, as shown above, the deletion in T4*farP12*-dC extended into the *rII*-proximal (left) end of this fragment (Fig. 3D and 4F) and yet did not alter T4 *denV* gene activity (Fig. 2 and Table 2). To test this assertion we attempted marker rescue by the *denV* mutant strains *uvs110* and *uvs13* using a plasmid containing only the 179-bp *TaqI*-*EcoRI* fragment derived from the extreme right end of the 1.8-kb *EcoRI* fragment (Fig. 4F). Figure 6 shows that this small

FIG. 6. Marker rescue of the *denV*⁺ allele by T4 strains *uvs110* and *uvs13*, using plasmid p179. (A and C) T4*denV* mutant phage was propagated on *E. coli* JM103 transformed with either p179 or M13mp10 and assayed for UV sensitivity as described in the text. (B and D) UV-enriched lysates prepared by using the phages shown in A (UV dose, 15 J/m²) and C (UV dose, 20 J/m²), respectively.

fragment allowed rescue of the *denV*⁺ allele by these strains, indicating that it contains the same part of the coding region of the *denV* gene present in plasmid p30. The reduced level of marker rescue by p179 (Fig. 6) relative to that observed with p30 (Table 3) likely reflects the relatively smaller region of homology in the former plasmid for recombination with the infecting T4 *denV*⁺ mutant alleles during marker rescue.

DNA sequencing. Sequencing of the 179-bp *TaqI-EcoRI* fragment referred to above revealed a 130-bp incomplete open reading frame with a translational stop codon near its left end (Fig. 4F). The T4 strain *uvs13* contains an amber mutation in the *denV* gene (34). Since this strain rescued the *denV*⁺ allele from plasmid p179, the amber mutation should reside in the region of the mutant T4 genome homologous to the wild-type 179-bp *TaqI-EcoRI* fragment. Identification by DNA sequencing of a nucleotide change resulting in the amber TAG codon in the *uvs13* allele would thus clearly establish that this fragment is derived from the *denV* coding region and would also establish the correct reading frame and direction of transcription of the gene. We therefore crossed the *uvs13* mutation into the T4*alcW7* background and cloned the 1.8-kb *EcoRI* fragment from the resulting strain into M13mp10 (see above). Comparison of the nucleotide sequences of the wild type and amber mutant fragments revealed a single base change resulting in the amber codon in the latter (Fig. 7).

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The open reading frame of the wild-type *denV* gene identified by the amber codon in *uvs13*-dC extended for 130 bp, starting at the *EcoRI* site at 64.2 kb and continuing 30 nucleotides beyond the site of the amber codon (Fig. 7). We therefore placed the *rII* proximal or 3' end of the coding region of the *denV* gene at 64.07 kb on the physical map (Fig. 3D and 4F). The remainder of the *denV* gene was identified by sequencing the left end of the 800-bp *HindIII* fragment situated between 61.47 and 65.0 kb on the T4 physical map

(Fig. 3D). This sequence contained a 30-bp overlap with the incomplete open reading frame present in the 179-bp *TaqI-EcoRI* fragment, thus establishing its identity as part of the *denV* gene. Analysis of the entire sequence (Fig. 7) showed that the presumed coding region of the *denV* gene contained 138 codons. The 5' noncoding region contained a consensus "-35 region" (TTTACA), a Pribnow box (TATAGA), and a Shine-Dalgarno putative ribosome-binding site (TACAGGAG) (see reference 13 for a review of these sequences), with the appropriate intervals between each of these elements.

DISCUSSION

Deletion mutants that inactivated (T4*farP13*) or did not inactivate (T4*farP12*) the *denV* gene of phage T4, were crossed into the T4*alcW7* background to obtain derivative strains that incorporated and retained dC in their DNAs under appropriate growth conditions. DNA isolated from these strains was subjected to restriction endonuclease and Southern hybridization analyses to locate the physical *rII*-distal (right) endpoints of the deletions and hence define the location of the *rII*-proximal (left) end of the *denV* gene on the T4 physical map. The *rII* distal end of the deletion in T4*farP12*-dC was mapped within the interval 62.4 to 63.4 kb (Fig. 3D); thus, the *rII*-proximal end of the *denV* gene must be located no further to the left than 62.4 kb. The T4 *ipII* and *ipIII* genes mapped between the *denV* and *e* genes (39) (Fig. 8), and the size of the coding regions of *ipII* and *ipIII* could be estimated from the known amino acid compositions of the proteins they encode (8, 33) (Fig. 8). In addition, the physical location of the *e* gene and of the right end of the *ipIII* gene can be precisely specified from nucleotide sequence data (25) (Fig. 8). This information together with our finding that the *rII*-distal (rightward) end of the deletion in T4*farP13*-dC is in the interval of 64.3 to 65.1 kb (Fig. 3D) placed the most rightward location of the left end of the *denV* gene between 64.3 and 64.4 kb on the T4 physical map.

The region of the T4 genome between 62.4 and 64.4 kb is largely represented on a 1.8-kb *EcoRI* DNA fragment (Fig. 3D) that was previously shown to contain an origin of T4 DNA replication and was cloned as an insert in the recombinant plasmid p30 (9). This plasmid was shown to support marker rescue of the *denV*⁺ allele by some, but not all, *denV* mutant strains tested. The simplest explanation for this result is that the *denV* gene spans the 1.8- and 1.7-kb *EcoRI* fragments such that only those sequences required for marker rescue of the *denV*⁺ allele by T4*uvs110* and T4*uvs13* (but not T4*uvs106*, T4*uvs107* and T4*v₁*) are located on the 1.8-kb fragment. This interpretation is consistent with the observation that a plasmid bearing the terminal 179 bp from the right end of the 1.8-kb *EcoRI* fragment retained the ability to support marker rescue by the two *denV*⁺ strains.

We determined the nucleotide sequence of the *rII*-distal (right) end of the 1.8-kb *EcoRI* fragment cloned from a T4 strain carrying an amber mutation known to be rescued from the 179-bp fragment derived from a wild-type strain. Comparison of this sequence with that of the putative partial wild-type allele revealed a single nucleotide change leading to the formation of the amber TAG codon, hence further establishing the identity of the *denV* gene. Analysis of the nucleotide sequence of the gene showed that it contained 138 codons and could encode a protein with a calculated molecular weight of 16,081, a value in excellent agreement with that of ~16,000 previously determined from studies on purified T4 pyrimidine dimer-DNA glycosylase (24). The 5' noncod-

FIG. 7. Complete nucleotide sequence of the *denV* gene. The coding region and presumed amino acid sequence are identified. A consensus Pribnow (TATA) box beginning at position 39 is identified. A so-called -35 sequence is present beginning at position 14, and a consensus Shine-Dalgarno sequence is present at position 54. The replacement of the normal TGG codon by the TGA amber codon is also shown at position 454. The lower part of the figure diagrammatically illustrates the strategy used for sequencing. Note that the orientation of the *TaqI*-*HaeIII* fragment is reversed from that of the T4 physical map shown in earlier figures, since the DNA sequence is presented in the 5'→3' direction.

ing region of the *denV* gene contained the appropriate regulatory elements identified in most procaryotic genes: hence, the gene is clearly not part of a polycistronic operon. The direction of transcription of the gene was counterclockwise on the T4 circular map, the same as that of most early-expressed T4 genes (39). Our success in cloning discrete fragments containing portions of the entire *denV* gene provides a rational strategy for attempting to clone the intact gene and tailoring it for regulated expression in both procaryotic and eucaryotic host cells. Such studies are currently in progress.

These results positively identify the location of the 3' end of the *denV* gene at position 64.07 kb on the T4 physical map (Fig. 8). This location places the gene at least ca. 2.5 kb to the right of the position determined from recombination frequencies (Fig. 8, top), using map functions that assume a constant number of recombinational events per bp throughout the T4 genome. This discrepancy is highly significant since the agreement between the physical and genetic map locations of the T4 *e* gene, the center of which is located 1.8 kb from the physical left (3') end of the *denV* gene, was good (Fig. 8). To reconcile these results, we suggest that the frequency of recombinational events in the vicinity of the *denV* gene may be considerably greater than the average per unit length of T4 DNA. What might account for such enhanced recombinogenicity? Since DNA replication is intimately associated with recombination in phage T4 (22), it is possible that the putative recombinogenic element is (or is related to) the origin of DNA replication identified on the 1.8-kb *EcoRI* fragment (9). In this regard, it is of interest that an origin of DNA replication has been identified in a different

region of the T4 genome between genes 34 and 35 (10), in which enhanced recombination has been well described (1, 21, 28).

It is paradoxical that the physical and genetic endpoints of the deletions in the two T4_{far} mutants are in good agreement despite the disparity observed for the *denV* gene (Fig. 8). It is therefore possible that the deletions result in the loss of a putative recombinogenic element and hence abolish the apparent genetic map distortion in this region. As the sequences of additional *denV* point mutants become available, it should be possible to test this model by precisely measuring the frequency of genetic recombination in T4 wild-type and in T4_{far}P12 strains by intragenic crosses.

The initiation of DNA replication in phage T4 is both complex and controversial as there is considerable disagreement on both the operational definition and the localization of replication origins (9, 10, 22). This situation may be complicated in part by the apparently intimate association between DNA replication and recombination during propagation of this phage. Thus, Mosig (22) has recently suggested that recombinational hot spots in phage T4 might represent replication origins if, during recombination, invasion of the 3' end of a DNA strand primes new DNA synthesis. In this study we have presented data suggestive of an interaction between replication initiation and recombination in one region of the T4 genome and have noted an apparently similar phenomenon in a second region. Careful comparisons in future studies between origins that are or are not associated with regions of enhanced recombination may thus provide insights into the nature of replication initiation in phage T4 and its relationship to recombination.

FIG. 8. Comparison of the T4 partial genetic (A) and physical (B) maps. The genetic map positions of genes *e* and *denV* are those of Wood and Revel (39). This position for the *denV* gene (right end at 62.0 kb) is probably the most rightward possible one: in a series of five crosses between two different *denV* gene alleles (*uvs5* and *v₁*) and the *e* gene, van Minderhout et al. (34) observed a minimum recombinant frequency of 10%. By using the four-parameter switch function of Stahl et al. (31) and a total T4 genome length of 166 kb (39), this recombinant frequency corresponds to a T4 genetic map distance of 4.2 kb between the *denV* and *e* genes. The recombinant frequencies observed in the other four crosses were 11, 14, 20, and 24% (34), thus placing the *denV* gene further to the left. Two additional crosses between the *denV* and *rI* genes gave a recombinant frequency of 11% (34), again placing the *denV* gene at 60 to 61 kb on the T4 genetic map. The *rII*-distal deletion genetic endpoints were determined as described in the legend to Fig. 1; error bars were calculated from the uncertainty of previous recombination data (3). The number line is the standard T4 genetic map (39). The physical map represents a summary of the results of this study for the location of the *denV* gene and the *rII*-distal endpoints of the T4 *far* deletions. The locations of gene *e* and the *rII*-distal end of the *ipIII* gene are those of Owen et al. (25). The lengths of the *ipII* and *ipIII* structural genes were calculated from amino acid sequence (8) and amino acid composition (33) data, respectively. We assume the absence of nonstructural DNA sequences between these two genes. Number line is the standard T4 physical map (11).

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