Restoration by Chloramphenicol of Bacteriophage Production in *Escherichia coli* B Infected with a Ligase-Deficient Amber Mutant

ANDRZEJ W. KOZINSKI AND MARILYN MITCHELL

Department of Medical Genetics, University of Pennsylvania, Philadelphia, Pennsylvania, 19104

Received for publication 30 June 1969

The addition of chloramphenicol (CM) 5 min after infection of the nonpermissive host *Escherichia coli* B with the ligase-negative T4 amber, T4 AmH39X, allowed replication of parental deoxyribonucleic acid (DNA) and the production of high-molecular-weight progeny DNA, composed mostly of subunits with a D_2/D_1 of 0.6. When CM was removed after the accumulation of a large pool of this DNA, most of the infected bacteria were able to produce viable progeny phage, with an average yield of approximately 15 bacteriophage per bacterium. This phenomenon is called CM rescue of the ligase-negative T4 Am. CsCl and sucrose gradient analyses showed both the resulting phage and DNA extracted from them to be similar to the phage and DNA produced on the permissive host. The total transfer of the parental label to progeny phages was as high as 20%. In contrast, in bacteria not treated with CM or in bacteria to which CM was added after phage-coded nucleases had already been synthesized, both parental and progeny (newly synthesized) DNA was composed of very short fragments. Phage which are produced under conditions other than those of CM rescue are dead, light in CsCl, and contain only very short fragments of DNA. Parent-to-progeny transfer in this case is below 1%. When light radioactive parental DNA was used to infect heavy bacteria, DNA replicating in the CM rescue conditions assumed only a hybrid density. After removal of CM and maturation, the parental DNA was incorporated into progeny molecules in fragments constituting approximately 7 to 10% of its mass. This pattern of distribution is essentially what is observed in similar experiments in the permissive host. The role of ligase as an enzyme which compensates for the lethal action of phage-coded nuclease and which is stringently required for the repair of single-stranded nicks is emphasized. The possibility of specific sites for a unique cutting enzyme is discussed in connection with the hypothesis of a circularly permuted assembly of sets.

The ligase-deficient mutant T4 AmH39X is capable of inducing limited synthesis (approximately 0.5 phage-equivalent units per minute per bacterium) of DNA after infecting the nonpermissive host *Escherichia coli* B23 (11). When analyzed in sucrose gradients, this newly synthesized deoxyribonucleic acid (DNA) is seen to be very small; in native or denatured form it bands close to the top of the gradient as a rather inhomogeneous peak of a fractional molecular size between 5 and 0.5% phage-equivalent units (11). In view of the known ligase deficiency of this particular mutant, the small sizes of the progeny DNA could be interpreted as corroboration of the hypothesis that polymerase works in a discontinuous manner, i.e., synthesizing short fragments of oligonucleotides attached to a matrix strand by hydrogen

bonding. According to this hypothesis, ligase would follow polymerase, repairing the discontinuities, and thus allow the production of integral progeny strands. However, in a previous paper (11), we showed that soon after infection parental DNA undergoes an extensive breakdown to fragments of approximately the same molecular weight as that of newly produced progeny. We suggested that one could not visualize polymerase first destroying the parental DNA into short fragments and then synthesizing a product longer than the existing primer.

Moreover, we observed that chloramphenicol (CM) added between 3 and 5 min after infection (when the greatest rate of synthesis is achieved) allows the production of large progeny DNA and preserves the integrity of the parental DNA. This

newly synthesized DNA contains equal proportions of both *l* and *r* strands (10). Both the progeny and parental molecules under these conditions can be as large as one phage-equivalent unit, but more typically (which is relevant for further discussion) belong to a class of fragments of the molecular size, assuming their homogeneity, of approximately 15 million to 20 million, which is several orders of magnitude higher than the so-called Okazaki fragments (14). The interpretation of these results is obvious: CM added at 5 min allows the expression of an enzyme causing replication of phage DNA. Neither in the past nor present experiments have we actually measured synthesis of T4 polymerase in the same experiments in which radioactivity of the parental or progeny molecules was assayed. However, previously published data (1, 17) indicate that, by 5 min, a sizeable amount of T4 polymerase is synthesized. Thus, the most reasonable assumption is that the observed replication of T4 DNA in bacteria in which CM was added at 5 min is indeed caused by T4 polymerase. The fact that the addition of CM at times later than 5 min after infection allows a sizeable destruction of parental DNA into short fragments, and allows production of only very short fragments of progeny molecules, is interpreted (1, 11) as proof that CM added at 5 min prevents expression of an endonuclease which we called "nickase" (12). As pointed out by Cohen (2), the expression of what we call "nickase" and the appearance of an endonuclease described by Horwitz (5) coincide remarkably well.

It was concluded, therefore, that ligase is an enzyme which repairs nicks inflicted by phage-coded nickases, thus compensating for the evolutionary handicap in the production of endonucleases. From this point of view, ligase should be stringently required only if endonucleases are present; in their absence, ligase deficiencies should not be lethal. Indeed, some of the apparently wild revertants in the ligase-negative (*Lig*⁻) amber mutants are actually double mutants, being both ligase- and nickase-negative phage. Those mutants were proven to be both *rII*⁻ and *Lig*⁻ (H. Berger and A. W. Kozinski, Proc. Nat. Acad. Sci. U.S.A., *in press*).

Noting the good production of phage DNA of integral or nearly integral size in the presence of CM added at 3 or 5 min after infection, and knowing that both the parental DNA (transferred to the progeny phage under any of the experimental conditions) and progeny DNA contain equal proportions of *l* and *r* strands (10), we hoped that, after an accumulation of such integral DNA inside the infected *E. coli* B and the subsequent withdrawal of CM, there might be

maturation of some viable phage containing integral DNA. [Annealing experiments showed the presence of equal amounts of right (*r*) and left (*l*) strands. Although this definitely excludes the possibility of preferential replication of only *l* or *r* strands in every bacterium, it does not bear on the possibility that each bacterium independently replicates only one or the other of the strands. In this latter case, a population of bacteria replicating *l* and *r* at random would result in equal numbers of *l* and *r* strands which naturally occur as single-stranded molecules. However, CsCl density profiles show that newly synthesized DNA cobands with native reference double-stranded DNA (11). [In CsCl, single-stranded T4 DNA is denser than double-stranded DNA by 0.02 g/cm³ and would be displaced under our conditions of fractionation by at least two fractions (11).] We have found this, in fact, to be the case by proving that under those conditions nearly all infected bacteria yield a sizeable crop of progeny phage. We have termed this phenomenon "CM rescue" (10; Berger and Kozinski, *in press*).

The objectives of this paper are to describe in greater detail the phenomena of CM rescue, replication, and recombination in the presence of CM, the percentage of parent-to-progeny transfer, and the integrity of DNA enclosed within the bacteriophage which mature from the nonpermissive host under variable rescue conditions.

MATERIALS AND METHODS

Most of the analytical methods used in this report were previously described (11).

The strains used were *E. coli* B23 as the nonpermissive host, *E. coli* Cr63 as the permissive host, and phage T4 AmH39X, a *Lig*⁻ mutant (3), sometimes referred to as *L*⁻, which plates on *E. coli* B with an efficiency of around 10⁻⁶. The wild phage used was T4BO₁r, and is referred to as *Lig*⁺ or *L*⁺.

The plating efficiency of the *Lig*⁻ phage on *E. coli* B, which is low, does not necessarily reflect an absolute incapability of producing some progeny phage on the nonpermissive host. An average of 1:100 of infected *E. coli* B produces a very limited yield of phage (Table 1). Thus, infecting *E. coli* B and plating it either on *E. coli* B or Cr63 gives different results. The chances of releasing a single phage are 10⁻² for each infected *E. coli* B. This low probability continues throughout, causing, in effect, a lack of detectable plaques. If, on the other hand, infected *E. coli* B cells are plated on Cr63, even a single released phage would successfully produce a plaque. In summary, plating T4 AmH39X on a lawn of B23 yields a plating efficiency of 10⁻⁶; however, plating infected *E. coli* B on Cr63 yields a plating efficiency of approximately 10⁻².

Since a CsCl fractionation of the *Lig*⁻ phage was performed in this investigation, it is necessary to comment on the peculiar density characteristics of this phage. T4 AmH39X is not an osmotic, shock-resistant

mutant (as is T_4BO_1 , which is commonly used in this laboratory) and achieves equilibrium in CsCl at a slower rate. It was shown, however, that equilibrium can be achieved within 20 hr of centrifugation if the phage suspension in CsCl is warmed at 45°C for 20 min prior to centrifugation. It is also important to note (see the CsCl fractionation data, Fig. 2) that the Lig^- phage is endowed with a density marker; in equilibrium runs it does not coband with the reference wild phage but is slightly displaced toward the heavier side. The increased density of this phage, which is approximately 0.01 to 0.02 g/cm³ greater than that of wild phage, is not caused by a larger size of DNA, as DNA of this phage cobands perfectly with that of wild phage in either neutral or alkaline sucrose gradients, nor is it caused by a difference in glucosylation, as the Lig^- DNA cobands in CsCl and CsSO₄ gradients with wild phage DNA labeled with a different isotope.

A third phage used to a limited extent was a double amber mutant, Lig^- and polymerase-negative ($L-P^-$), defective in genes 30 and 43, respectively. This was obtained in this laboratory by crossing $T_4AMH39X$ with T_4AMB22 . After infection of *E. coli* B, its reversion rate is approximately 10^{-8} , (compared to 10^{-6} for the Lig^- phage). Moreover, there is no observed leakiness such as that described for the Lig^- phage; i.e., *E. coli* B infected with this mutant and plated on Cr63 does not give rise to plaques.

Details of sucrose gradient and CsCl centrifugations, the estimation of phage viability, and the efficiency of parent-to-progeny transfer are discussed below.

RESULTS

Fate of light parental phage DNA in light *E. coli* B (experiment A). Light *E. coli* B was grown in TCG medium (13) to 3×10^8 cells/ml; this was confirmed by plating. The cells were infected with a multiplicity of 3.0 light ³²P-labeled (specific activity 5.0 mc/mg of phosphorus) Lig^- phage per bacterium. CM (100 µg/ml) was added to samples of the infected suspension at 0, 3, 5, 10, and 13 min after infection. Part of the original suspension remained untreated with CM. At 5 min after infection, infective centers were plated from the untreated portion by using the standard procedure of incubation in T4 antiphage serum. The suspensions treated with CM were incubated for a total of 30 min after infection. Samples were withdrawn at this time for plating of infective centers and for sucrose gradient analysis (Fig. 1). The rest of the suspension was sedimented at 5°C, washed once, and resuspended in TCG without CM. The incubation was continued for a total of 120 min after infection for both the untreated suspensions and those treated with CM. At this time, a sample of the suspension was transferred to medium containing chloroform and indole, for estimating phage production. The rest of the suspension was supplemented with carrier phage to a concentration of 10^{10} /ml, and was then purified.

The results of the platings are summarized in Table 1. All platings for infective centers and for phage production were performed on the permissive host Cr63. Simultaneous platings on *E. coli* B, not documented in Table 1, revealed no plaque production at the given dilutions, proving that both the observed infective centers and phage produced after treatment with CM are of an amber type.

Table 1 reveals [confirming previous data (10; Berger and Kozinski, *in press*)] that the addition of CM at around 5 min after infection, followed by incubation for a total of 30 min, allows infected *E. coli* B to express itself as an infective center if plated on the permissive detector. Moreover, after the withdrawal of the antibiotic and further incubation, a sizeable crop of viable phage is produced on those bacteria. This is in contrast to bacteria not treated with CM or treated with the antibiotic very early or late after infection, when most of the bacteria produce neither plaques after plating nor a sizeable burst of progeny phage. We call the observed phenomenon "CM rescue" of the Lig^- mutant in the nonpermissive host.

The intracellular samples of infected bacteria withdrawn after 30 min of incubation were used for analysis in sucrose gradients. Part of the results are shown in Fig. 1. Note that, with the exception of the early addition of CM, the parental molecule undergoes extensive degradation. Also, the addition of CM at 5 min, which correlates with the best yield of progeny phage, allows a sizeable number of parental molecules to be degraded into a unique class of subunits banding at a D_2/D_1 of 0.6 and called FSBP (11).

The purified progeny phage were supplemented with reference ³H-labeled wild phage and analyzed in a CsCl equilibrium run, or after lysis with 0.2 M KOH were analyzed in an alkaline sucrose gradient (Fig. 2).

The top row of Fig. 2 represents CsCl gradient analysis of progeny phage produced under variable conditions of rescue. Panel 3 (cm 5') reveals a striking similarity in the pattern of

TABLE 1. Phage production in *Escherichia coli* B23^a

Time of addition of CM	Infective centers	Final yield
No CM	4.0×10^6	1.8×10^7
CM, 0 min	2.0×10^6	1.4×10^6
CM, 3 min	5.0×10^7	1.9×10^9
CM, 5 min	2.1×10^8	3.5×10^9
CM, 10 min	1.0×10^7	4.8×10^7
CM, 13 min	9.0×10^6	4.0×10^7

^a 3×10^8 *E. coli* cells infected with phage T4 AMH39X at a MOI of 3.

FIG. 1. Integrity of parental ^{32}P -labeled DNA at 30 min after infection. Intracellular DNA was extracted from samples of infected bacteria 30 min after infection (the time at which the various groups of bacteria which received CM were sedimented, rendered free of CM, and resuspended in fresh, CM-free medium). Sampling was performed by transferring one volume of bacterial suspension into 3 volumes of ice-cold ethylenediaminetetraacetate (EDTA) solution. Bacteria were sedimented and resuspended in salt-EDTA solution [0.015 M EDTA-0.05 M NaCl; 0.5 M tris(hydroxymethyl)aminomethane, pH 8.0] to which muramidase at a concentration of 100 $\mu\text{g}/\text{ml}$ was added. They were kept on ice for 10 hr, and supplemented with Triton X-100 to a final concentration of 1%. After 10 min of incubation in ice, KOH was added to a final concentration of 0.2 M. The suspensions were then incubated for 20 min at 37 C. Omission of the muramidase-Triton step causes, at later times after infection, some random losses of intracellular DNA. Prior to alkaline digestion, ^3H -labeled reference phage was added. The lysates were layered on the top of 5 to 20% alkaline sucrose gradients and centrifuged in the SW39L head of a Spinco centrifuge for 3 hr at 28,000 rev/min at 15 C. At the conclusion of the run, fractions were collected from the bottom of the tube and counted in a scintillation spectrometer, adjusted for ^3H - ^{32}P counting.

distribution of reference phage and the progeny phage endowed with the parental label. In contrast, phage produced without CM rescue and with CM added at 3 or 10 min produce strongly bimodal distributions of label consisting of a very-low-density band and a moiety which partially cobands with reference ^3H -labeled phage. To prove that both ^{32}P -containing moieties, those represented by peaks roughly coinciding with the reference phage and those appearing near the top of the gradient, are indeed DNA molecules enclosed in phage-specific proteins, a parallel experiment was performed in which "dense" and "light" moieties were preparatively isolated, dialyzed against saline to remove CsCl, supplemented with ^3H -labeled reference phage, and used for the determination of the rate of adsorption to *E. coli* and the rate of precipitation

with antiphage sera. The results of this experiment indicated no difference between adsorption or serum precipitation characteristics for the ^{32}P -containing moieties as compared with ^3H -labeled reference, indicating clearly that the light moieties contain DNA in phage-specific capsomer. The results of analysis in alkaline sucrose gradients of DNA extracted from the progeny phage are shown in the lower panel of Fig. 2. Again, examining the "cm-5'" panel first, it is obvious that parental fragments in the rescued system reside in molecules showing the best fitness in cobanding with reference ^3H -labeled integral DNA.

Fate of light parental DNA in heavy *E. coli* B (experiment B). In experiment A, the fate of parental DNA was followed without the use of a density label. It provided information on the extent of degradation, parent-to-progeny transfer,

FIG. 2. *CsCl* and sucrose gradient analysis of progeny phage. After the removal of CM at 30 min after infection, suspensions of bacteria were incubated for a total of 120 min. The samples of bacteria not receiving CM were also sedimented at 10 min and washed in a manner similar to that used with the CM group. This was done partly to provide the same treatment, but most importantly, to eliminate an unadsorbed moiety of parental phage. Lysis was induced with chloroform in the presence of indole, a sample was withdrawn for the estimation of the final yield, and then carrier phage (to 10^{10} /ml) was added to the remaining lysate. After digestion with deoxyribonuclease, phage was purified by the conventional method of filtration followed by two high-speed and one low-speed sedimentations. Part of the purified phage was precipitated with trichloroacetic acid, and the recovered radioactivity was compared with the input radioactivity. The figures for parent-to-progeny transfer obtained for this experiment were: 0.2% transfer for phages not treated with CM, 1.5% for the samples treated for 3 to 30 min, 18% for those treated with CM from 5 to 30 min after infection, and less than 5% for those treated with CM for 10 to 30 min. (The pattern of profiles both in *CsCl* and sucrose gradients are reasonably similar for both CM-rescued DNA and the resulting progeny phages, but tends to display a sizable fluctuation for progeny phage obtained without the addition of CM, or from bacteria treated with CM added at late times. This is obvious when one considers the extremely low transfer of DNA pool to progeny.) The summary of the plating part, i.e., the number of infective centers and final production is presented in Table 1. Purified phage were supplemented with ^3H -labeled reference phage (osmotic shock-resistant mutant T4BO $^+$) and either analyzed in a *CsCl* density gradient (upper panels) or lysed with alkali and analyzed in alkaline sucrose gradients (lower panels). The ligase-negative mutant is a density marker phage and, when analyzed simultaneously with T4BO $^+$, it bands at a slightly denser position in the gradient. Phages resulting from bacteria treated with CM at 5 min after infection resemble normal parental phage in exhibiting the same density displacement from the peak locations of wild-type phage. Thin arrows indicate this displacement.

and the density of progeny phage inheriting the parental label. This last point could only be considered in a situation where the absence of a DNA density label made any observed change in the density of the progeny phage meaningful. In experiment B, the parental DNA was allowed to replicate in a heavy host, and the resulting progeny phage DNA was analyzed in CsCl. On the intracellular level alone, the data presented here will merely provide confirmation of previously published data (9, 11). However, these experiments also allow a comparison with the DNA extracted from progeny phage which, *as must be emphasized*, incorporate variable, and often very small amounts of DNA from the replicative pool. The difference between this and experiment A is that *E. coli* B23 was pregrown in heavy medium for two generations prior to infection with a multiplicity of infection (MOI) of 5 of light, radioactive T4 AmH39X (viability estimated for those was 89%). The remaining procedural details are identical to those of experiment A: CM was added at intervals and the suspensions were incubated for 30 min. Part of each suspension was then ice-chilled and saved for physicochemical analysis. Samples taken at this time for platings of infective centers gave results which were similar to those in Table 1, and therefore are not shown here. The remaining suspensions were washed free of the antibiotic and were resuspended in fresh heavy medium. At 120 min, bacteria were lysed and the progeny phage were purified.

The replicated intracellular DNA extracted from infected bacteria at 30 min was subjected to analysis in sucrose gradient (Fig. 3). The patterns resulting from sucrose gradient analysis of intracellular DNA resemble those observed in experiment A. CsCl gradient analysis of native DNA (Fig. 4, top row) revealed that the addition of CM at 5 min after infection allows parental DNA to replicate only to the hybrid density. The addition of CM at later times allows extensive recombination to occur, resulting in the further displacement of the parental label from the light toward the heavy progeny density. Replication is very efficient and most of the injected DNA assumes a dense location. The nonreplicated moiety corresponds to the fraction of dead phage determined from the viability calculation.

Denaturation of replicated moieties revealed that the parental strands are predominantly not covalently joined with the heavy moieties in those bacteria which are treated with CM at 5 min after infection. In bacteria treated with CM at 10 min, there is a significant degree of covalent repair in the recombinant moieties. These results are similar to those previously published (9, 11).

The analysis of progeny phage in CsCl (Fig. 5)

FIG. 3. Comparison of intracellular integrity of injected light ^{32}P -labeled phage DNA in heavy, non-permissive *E. coli* B at 30 min. Heavy bacteria, pregrown in heavy 5-bromodeoxyuridine medium, were infected with a multiplicity of 5.0 light radioactive phage per bacterium. The viability of phage as calculated from the ^{32}P content was 89%. The infected suspension was divided into three groups: not treated with CM, treated with CM at 5 min, and treated with CM at 10 min. At 30 min; all suspensions were sedimented, washed, and resuspended in fresh heavy medium. The results of plating at this stage of the experiment resembled closely the data presented for the light part of the experiment summarized in Table 1. At 30 min, samples were taken for DNA extraction while the remainder of the resuspension was further incubated for the production of progeny phage. From the withdrawn samples, DNA was extracted by the Pronase-phenol method, supplemented with integral ^3H -labeled reference DNA, and analyzed either in CsCl or alkaline sucrose gradients. This figure shows the results of sucrose gradient analysis. The addition of CM at 5 min assures the preservation of FSBP subunits (D_2/D_1 0.6). DNA extracted from the other two samples reveals much more extensive breakdown.

revealed that phage which are rescued efficiently by the addition of CM at 5 min are extensively recombined, i.e., they contain, on the average, 7 to 10% light parental contribution per phage. This result is similar to that obtained with the permissive host.

Quite different patterns were revealed for

FIG. 4. *CsCl* analysis of intracellular DNA at 30 min (light ^{32}P -labeled parental phage DNA in heavy host). Samples of the intracellular DNA which were analyzed for its integrity in Fig. 3 were analyzed also in *CsCl* in native and denatured form. The ^3H label represents a mixture of light and heavy reference DNA added prior to the run. Note an extensive recombination and covalent repair in the moieties not treated with CM or treated with CM at 10 min. The addition of CM at 5 min (condition of rescue) allows only semi-conservative replication—a hybrid location—in which progeny and parental moieties are mostly not covalently associated.

progeny phage obtained without CM rescue. Those band mostly as two moieties: one larger, partially coinciding with reference light phage, and one moiety floating close to the top of the gradient. Aside from a small peak at the heavy location, the pattern observed for those phage resembles closely the pattern observed in experiment A.

DNA was extracted from progeny phage with phenol and analyzed in native and denatured form in both *CsCl* and sucrose gradient. The results of *CsCl* gradient analysis are presented in Fig. 6. The results of sucrose gradients, not documented here, resembled reasonably well the results obtained for the progeny in experiment A.

Fate of newly synthesized intracellular DNA and the resulting progeny. The conclusion from experiments A and B is that CM added at 5 min

preserves to a large extent the integrity of the parental DNA and allows production of viable phage upon withdrawal of the antibiotic. Curiously, the best phage production is obtained when the integrity of the parental molecule is not perfect, but the molecules have undergone reduction to subunits which band at a unique location in the sucrose gradient, at a D_2/D_1 of 0.6. These fragments do not have internal nicks and, according to our previous data (11), normally appear at about 10 min after infection and are not a result of random breaks. We have called these fragments FSBP, for first-step breakage product. The mechanism leading to their production is still under investigation. We now deal with the fate of

FIG. 5. *CsCl* analysis of progenyphage (light ^{32}P -labeled parental phage DNA in heavy host). Phage produced in experiment B were purified and analyzed in *CsCl*. Examination of the graphs indicates a sizeable transfer of parental light material into recombined phages. The extent of displacement here is approximately 7 to 10%. The values for parent-to-progeny transfer were similar to those presented in Fig. 2. Final yields of viable phage were significantly smaller, owing to the fact that heavy Lig^- phage is mostly uninformative. In the portion with no CM, the moiety of phage which partially coincides with light ^3H -labeled reference were preparatively isolated. Their DNA was extracted with phenol and analyzed in a *CsCl* gradient. The result of this analysis (not documented here) proved that phage coinciding with reference contain unreplicated, conservative, pure light DNA. Thus, parent-to-progeny transfer in the absence of CM should be considered to be much smaller than this estimated by the total ^{32}P transfer, by roughly a factor of three, as it could be appreciated when comparing the total mass of ^{32}P at the heavy as compared with the light location.

FIG. 6. CsCl analysis of the DNA extracted from progeny phage (light parental ^{32}P -labeled phage DNA in heavy host). Samples of the DNA prepared as described in Fig. 5 were analyzed in both native (upper) and denatured (lower) form. Thermal denaturation was used, and 1% formaldehyde was added into the CsCl solution. The arrow between the reference peaks indicates the density location of hybrid DNA consisting of one light strand and one heavy strand. The brackets with the per cent figures represent the summed per cent of recovered activity within the indicated areas of the gradient.

newly synthesized DNA in the resulting progeny phage produced both without and after preincubation in CM.

E. coli B was infected with the Lig^- phage with an MOI of 7.0, and, at intervals, samples were transferred to CM. At 10 min after infection, ^3H -thymidine (specific activity 20 mc/mg of thymidine) was added to all flasks of infected bacteria. 5-Fluorodeoxyuridine (5 $\mu\text{g}/\text{ml}$) was also

added at this time. Incubation was continued to 30 min after infection, at which time all bacterial suspensions were sampled for the analysis of the integrity of intracellular DNA (in alkaline sucrose gradient).

Part of the intracellular DNA derived from this experiment was purified and used for annealing experiments against preparatively separated left and right strands of T4 DNA. These results, published elsewhere (10), show that under these conditions both progeny strands are produced in equal quantities.

The remaining bacterial suspensions, including those not treated with CM, were sedimented, washed, and resuspended in fresh medium free of CM. Bacteria were further incubated for up to 120 min and lysed. After purification, the resulting progeny phage were used for analysis of phage and its extracted DNA in CsCl and sucrose gradients.

Results of the sucrose gradient analysis of intracellular DNA at 30 min are shown in Fig. 7. Although the fates of the parental and progeny intracellular DNA are similar (compare Fig. 1 and 7), there is somewhat more integral DNA with the parental label than with the progeny. This could be due to the moiety of bacteria which did not participate in replication and produced no progeny phage. DNA synthesized in bacteria treated with CM at 5 min is not completely integral but is composed of fragments of the FSBP type, which, as known from previous experiments, can replicate autonomously (8, 11).

The results of analyses of these phage and the DNA are presented in Fig. 8, 9, and 10. It is clear that the phage which matures from a pool produced in the presence of CM added at 5 min bands in a normal location (Fig. 9) and contains a large amount of integral DNA (Fig. 8). This is not true for the phages not treated with CM; in this case, most of the phage band close to the top of the gradient (Fig. 9), whereas sucrose gradient analysis of such a population of phage reveals mostly broken DNA (Fig. 8). Thus, the picture is consistent with the assumption that FSBP subunits can be assembled quite efficiently into mature phage.

Since the length of the FSBP is approximately 0.15 to 0.2 phage-equivalent units, it would take somewhere between five and seven subunits to complete one phage-equivalent unit. Assembly of phages containing integral DNA from autonomously replicating FSBP subunits is also supported by a density labeling experiment (experiment B) in which the parental label resides in hybrid fragments of the FSBP size which mature

FIG. 7. Integrity of newly synthesized (^3H -thymidine-labeled) progeny DNA inside infected bacteria at 30 min after infection. *E. coli* B was grown to 3×10^8 in light TCG medium, infected with an MOI of 7.0, and divided into flasks which received no CM, CM at 5 min, and CM at 10 min after infection. At 10 min after infection, all three bottles received radioactive ^3H -thymidine (specific activity 20 mc/mg; 2 μg of thymidine/ml, 5 μg of fluorodeoxyuridine/ml, and 20 μg of uracil/ml). Incubation was continued for 30 min, at which time bacteria were sedimented, washed, and suspended in fresh, nonradioactive, CM-free medium. At this time, samples were taken for the analysis of intracellular DNA in a manner similar to that described in Fig. 1. A small quantity of (integral) ^{32}P -labeled DNA was added as a reference. Most of the newly synthesized DNA, except for the samples receiving CM at 5 min, is quite small and bands close to the top of the tube. DNA synthesized in CM added at 5 min bands at a D_2/D_1 of 0.63, thus belonging to a class of FSBP subunits. In a similar experiment in which the label was attached to the parental DNA, the overall pattern of events was similar, with the exception that when CM was added at 5 min there was some intact DNA apart from FSBP subunits. This phenomenon is generally true, indicating that the parental label undergoes less synchronous replication as compared with progeny. The estimation of the molecular sizes of fragments banding close to the top of the gradient, compared with integral T4 DNA, cannot be precise, and would, rather, demand different speeds and different reference DNA such as ϕX174 . It is, however, adequate to estimate that they are at least $10\times$ smaller than FSBP subunits. Newly synthesized DNA was extracted from infected bacteria at 30 min and analyzed in both CsCl and CsSO_4 . The results of this analysis are not documented here, but it was shown that newly synthesized DNA coincided perfectly with ^{32}P -labeled reference DNA.

into progeny phage and contribute approximately 7 to 10% of its mass, or 14 to 20% of the length.

Part of the progeny phage was preparatively purified in CsCl , and the fractions indicated with

arrows in Fig. 9 were isolated. DNA of these phages was analyzed in alkaline sucrose gradients. The results are shown in Fig. 10, which shows a good correlation between the observed density

FIG. 8. Sucrose gradient analysis of progeny DNA labeled on progeny strands. After removal of CM, the suspension described in Fig. 7 was further incubated and lysed, and phage was purified as described in Fig. 2. The percentage of transfer of the intracellular label to the final progeny was estimated by comparing the total amount of trichloroacetic acid-insoluble label at 30 min with that recovered in the final progeny. Significantly, the percentage of transfer is similar to that observed for transfer of the parental label, ranging from 0.5 to 1.0% for phage resulting from bacteria not treated with CM to 20% for those treated with CM at 5 min, and from 1 to 5% for bacteria treated with CM between 10 and 30 min. DNA was extracted from the resulting progeny phages with phenol and analyzed in sucrose gradients with the addition of ^{32}P -labeled integral reference DNA. The upper panel gives the results from neutral and the lower panel from alkaline sucrose gradients. The most integral DNA resides in phage derived from bacteria treated with CM at 5 min.

FIG. 9. *CsCl* analysis of progeny phages. Samples of the progeny phage analyzed in Fig. 8 were fractionated preparatively in *CsCl*. ^{32}P -labeled phage (wild type) was added as a reference. After collection, part of each fraction was counted. As in Fig. 2, phage produced from the suspension treated with CM between 5 and 30 min are slightly more dense than reference phage. Phage derived from the suspension not treated with CM or treated with CM at 10 min display a sizeable amount of label which is lighter than reference phage. The groups of fractions indicated by the numbered arrows were further analyzed as described in Fig. 10.

and the size of the fragments included in the progeny phage. DNA extracted from the moiety of very light phage appeared very short, whereas DNA extracted from phage of nearly normal density is only slightly smaller than reference. Due to a large inhomogeneity of fragments in sucrose gradient and densities in *CsCl*, there was no attempt to calculate the absolute amount of DNA per phage, or to compare the result with the fractional size in the sucrose gradient. We consider the present data satisfactory to state that the reason for assuming a light location in *CsCl* is a small net amount of DNA per resulting phage. In view of the very small fraction of intracellular DNA pool, maturing into complete phage, a comparison of the intracellular fate of DNA with DNA extracted from progeny phage is un-

fair, since one cannot exclude a likely possibility of the maturation of some of the progeny phage from a nonrepresentative minority of intracellular DNA which was subjected to less extensive breakdown. This minority might actually be confined to a class of bacteria which, for this or another reason (mostly asynchrony of infection), are delayed.

Lack of semiconservative replication and CM rescue with a double amber mutant defective in ligase and polymerase production. The explanation of the results of the previous experiments was that the addition of CM at 5 min after infection inhibited the production of phage-coded nucleases (which normally cause the breakdown of both parental and progeny DNA), and thus allowed the production of integral DNA by phage polymerase. One could challenge this hypothesis, though, by assuming (what we consider to be an unlikely hypothesis) that the addition of CM at 5 min actually causes inhibition of phage polymerase. One would have to make the additional assumption that host polymerase would now take over and be complemented by the host ligase, following "in its footsteps," repairing the product of its discontinuous action and thus allowing production of large phage DNA. If this were the case, one would expect that similar CM experiments performed with a double mutant, defective in the production of both T4 ligase and T4 polymerase, would allow semiconservative replication of phage DNA.

A double mutant was obtained by crossing *Lig*⁻ phage, defective in gene 30, with *Am* B22, a

FIG. 10. Sucrose gradient analysis of various density classes of progeny phage. The density classes indicated by the numbered arrows in Fig. 9 were isolated and dialyzed against citrate-salt. Their DNA was extracted, supplemented with ^{32}P -labeled integral reference molecules, and analyzed in alkaline sucrose gradients.

polymerase-negative mutant (17) defective in gene 43. The resulting double mutant was used in a test for semiconservative replication by using differential density labeling. The shift in density of parental label is considered to be more sensitive than the estimate of net synthesis in that there are fewer possibilities for experimental error. In the past we have shown, for instance, that Am453, defective in gene 32 and considered DNA-negative, does replicate semiconservatively, but just once (6). Therefore, an experiment was performed in which heavy, 5-bromodeoxyuridine-substituted *E. coli* B was infected with an MOI of 3.0 light ^{32}P -labeled parental phage. Samples were transferred to CM at 0, 3, 5, and 10 min; a fifth sample was included which was not treated with CM. At 30 and 60 min, samples of infected bacteria were ice-chilled in ethylenediaminetetraacetate. Their DNA was extracted and analyzed in a CsCl gradient together with ^3H -labeled light reference DNA. Even partial replication of the parental molecule should cause the parental label to be noticeably displaced from its original light location in CsCl. The reciprocal experiment was also performed with the labels reversed, that is, heavy radioactive phage and cold, light medium. In both experiments, infective centers were estimated at 5, 30, and 60 min, and the samples were assayed for the possible production of progeny phage.

We do not report any specific results of this experiment; however, there was no production of progeny phage, no infective center above the expected frequency, and no semiconservative replication of the injected parental DNA. We consider this evidence sufficient to disprove the unlikely hypothesis of replication of phage DNA by a team of host, polymerase, and ligase.

Effect of CM on DNA-deficient T4 amber mutants. In the previous paper it was demonstrated that most of the DNA-deficient amber mutants do not replicate in the nonpermissive host but undergo extensive breakages of their DNA (6). This breakdown was proven to be a prerequisite for recombination between parental molecules. It was demonstrated, in addition, that CM added at 0 time after infection, in any of the amber mutants analyzed, prevented completely the breakdown of the injected parental DNA. At this time, CM was added at different times after infection, and it was demonstrated that adding CM at 7 to 9 min does not inhibit endonucleotic breakdown. As controls for these experiments, plating for phage production and infective centers was routinely performed, but these results were not reported in the previous paper (6). In the

present investigation, however, plating results performed in the absence and presence of CM, added at variable times to *E. coli* B infected with variable DNA-deficient amber mutants, are of value as a control for CM rescue of the Lig⁻ mutant. We do not report specific data, because the results were all negative. *E. coli* B23 cells were infected with the following DNA-deficient amber mutants: Am453, gene 32; N122, gene 42; B22, gene 43; 82, gene 49. The amber mutants were usually ^{32}P -labeled. The infected suspensions were divided: some received no CM, and some received CM at 0, 5, 7, and 9 min after infection. Plating of infective centers revealed that, irrespective of the treatment with CM, all infective centers plated as expected for the frequency of revertants. There was no production of phage. Invariably, the addition of CM at 0 time preserved the integrity of the parental molecule. The addition of CM at 7 min and later allowed a sizeable breakdown of parental molecules. Under these conditions, there was no replication of parental DNA, the exception being AM453 which replicated once.

DISCUSSION

It had been observed that CM added to a nonpermissive host bacterium infected with a Lig⁻ amber mutant permits the synthesis and accumulation of a large pool of DNA molecules of approximately 20×10^6 daltons (11). The experiments described above were based on the assumption that, after the removal of CM, there would be a competition between two phenomena, (i) the synthesis of nicking enzymes and the resulting degradation of DNA and (ii) the synthesis of structural proteins and subsequent maturation of viable phage, and that under favorable conditions one would be able to detect the latter. This has been shown to be the case (10; Berger and Kozinski, *in press*).

The experiments described demonstrated a dramatic difference between the control group and the bacteria treated with CM at 5 min. In the latter case, a large part of the parental DNA remained integral, and the newly synthesized progeny are of a size of approximately 15 million to 20 million. In this case, almost every infected bacterium yielded a burst of viable phage ranging from 5 to 30. (Control experiments revealed that no other DNA-deficient amber mutants tested can be rescued in this manner.) The total parent-to-progeny transfer of DNA increased from a value of less than 1% for phage produced without CM to a value as high as 20%. We have reported the results of analyses of the physicochemical properties of phage produced with or without CM rescue

and, in somewhat greater detail, the mode of parent-to-progeny transfer.

Phage produced under rescue conditions coband in CsCl gradients with reference phage added prior to centrifugation. Similarly, DNA extracted from these phage cobands fairly well in sucrose gradients with integral reference phage DNA, although there are some shorter fragments of DNA revealed in alkaline sucrose gradients.

In contrast, similar analyses show that phage produced without CM rescue or treated with CM at improper times (too early or too late) after infection are mostly light; i.e., they do not coband with normal phage reference but band near the top of the tube. However, there is always a moiety of progeny phage containing the parental label which cobands with reference phage.

Density labeling experiments with a light parental radioactive phage revealed efficient semi-conservative replication which, in bacteria not treated with CM or treated with CM at 10 min, is followed by an extensive recombination between short fragments. Large fractions of such recombinants are covalently repaired. After removal of CM (under rescue conditions), DNA produced in the presence of CM matures and contributes approximately 7 to 10% of parental label to each progeny phage. Considering that the FSBP hybrid has a molecular weight of approximately 20 million, and that it is 15% of the length of the molecule, transfer to the progeny of 7 to 10% of parental material per phage indicates that hybrid FSBP subunits are simply inserted into the progeny phage, mostly as single fragments, thus contributing 15 to 20% of the length of the molecule and 7 to 10% of light material (only one strand light). In the case of no CM addition, the density labeling experiments have proven that most of these progeny phage contain parental DNA in an unreplicated form. Although these progeny phage have the density of the original parental phage, alkaline sucrose gradients reveal a sizeable number of single-stranded breaks in their DNA; this is not true of the parental DNA. On the basis of the present experiments, we cannot conclusively state whether the parental label is transferred into the progeny without further replication or whether the process of parent-to-progeny transfer allows enrichment with a nonrandom moiety of non-injecting, highly nicked parental phage. This question could, in principle, be resolved by labeling proteins of the coat, but, owing to the extremely small number of phage in this class and the small (approximately 0.2%) parent-to-progeny transfer, this type of experiment presents significant difficulties.

The moieties of phages banding close to the top of the tube contain only very short fragments of

DNA. By isolating DNA from phage moieties banding at different densities, a suggestive correlation was shown between the observed density and the size of the fragments recovered from the bacteriophage: the lighter the phage the shorter the DNA fragments.

The fact that, in the Lig⁻ mutant, almost all of the progeny DNA matures into dead particles endowed with only short "chunks" of DNA might provide a very useful tool for testing the hypothesis that the amount of DNA determines the size of the head.

So far, then, we can state that, by inhibiting phage-coded nucleases by adding CM at around 5 min after infection, we are able to produce a sizeable pool of relatively large phage DNA from which, after removal of CM, a good crop of viable progeny phage are able to mature.

It is notable that the best CM rescue was achieved when CM was added at 5 min after infection, when most of the parental and almost all of the progeny DNA belong to a class of large fragments which we call FSBP. This seems to indicate the FSBP subunits are suitable precursors for mature viable phage. We have previously demonstrated (9, 11) that there is a covalent repair between light and heavy moieties in recombinants in Lig⁻ phage. It is quite feasible, then, that FSBP subunits can be assembled and covalently joined, thus producing viable progeny. These lesions are in contrast to nicks which are inflicted by an enzyme "nickase" which is inhibited by the addition of CM at 5 min. Richardson et al. (15) showed that nicks remain unrepaired but can be repaired by ligase *in vitro*. There are obviously a number of experiments required to explain the phenomenon of CM rescue.

In particular, if CM can inhibit the production of an enzyme (nickase) which introduces ligase-reparable lesions, one should also be able to find such an inhibition produced genetically; i.e., one should be able to isolate a nickase-deficient mutant which, occurring simultaneously with the ligase deficiency, removes the stringent requirement for ligase. Indeed, we have recently discovered (Berger and Kozinski, *in press*) that an enzyme whose expression is suppressed by ligase is controlled by the rII locus. A number of Lig⁻ rII⁻ mutants have been isolated and shown to produce average bursts of 20 phage from each infected *E. coli* B cell. This is strikingly similar to the figure produced for CM rescue of the Lig⁻ phage; it is also interesting, in this context, that both parental and progeny DNA of such a double mutant in the nonpermissive (*E. coli* B23) host are relatively large fragments of FSBP size (Berger and Kozinski, *in press*).

We present here a hypothesis according to which DNA of T4 contains a limited number of determinant groups, possibly short runs of specified base sequence, recognized by a specific "cutting" enzyme which is produced relatively early, and prior to the nicking enzyme. This enzyme cuts the molecule [as a result of either a single- or double-hit phenomenon (11)] into fragments whose terminals are always specific and are likely to join with each other in a sequential manner. These subunits (FSBP) are able to replicate autonomously (8). Since there are at least six such subunits, it is suggested that mature T4 DNA could arise by assembly of the subunits in an orderly prearranged manner. Orderliness of arrangement might be determined by base composition of the ends of the subunits. Provided one starts with an initially terminally redundant molecule, it is possible that subsequent arrangements of subunits could be circularly permuted. For instance, they might be arranged from 1 through 10 to 1, or from 5 through 10 to 6, etc., thus generating a molecule which is terminally redundant and circularly permuted.

Even the requirement of terminal redundancy of subunits can be relaxed when one realizes that inherent in the process of assembly is a recognition process, presumably based on regions of complementarity, which serves to order the subunits. This may well be similar to the sticky ends of lambda (18). The necessary terminal redundancy in a complete genome in this case is only that of the recognition area, i.e., about 10 to 20 bases. The correlation between the size of FSBP and the size of the parent-to-progeny transferred subunit (7, 16) is interesting, also, although there is increased dispersion of the parental material after the second generation transfer, indicating that the subunits participating in material recombination may be subject to further breakdown.

Circular permutation in phage in this case should not be considered a true permutation in which the ends of the molecules might be represented by any base within the molecule, but rather a circular permutation of sets of bases such that the terminals of the molecule are just as likely to be those of any of the subunits. The assumption that those subunits can be arranged in a circularly permuted manner would also make possible the assembly of subunits into a structure of a length far exceeding one phage-equivalent unit. Theoretically, all vegetative DNA, if residing in this type of subunit, could be assembled into one linear structure. In our previous paper (12), we postulated "that recombinational events, occurring at later times after infection might lead to the

formation of a very large structure. Reduction in the size of this hypothetical structure could easily produce circularly permuted, mature phage molecules." Indeed, since that time a large single-stranded DNA has been observed by Frankel (4) at later times after infection, or in bacteria to which CM was added at the times which, according to our previous estimates (12), should allow efficient parent-to-progeny recombination.

This model makes it clear that there may well be two types of recombinational breakages in T4 phage: one occurring at predetermined spots and one truly at random between any bases. Owing to the high frequency of nicking and concomitant repair by ligase, this type of recombination might escape observation during the performance of genetic crosses in which both nickase and ligase are present. If recombination would occur mostly by exchanging subunits, the linkage map of T4 phage would be ruined, and to a large extent terminal heterozygosity would be restricted to only a small number of possible markers. However, considering that frequent breakages at non-random locations are accompanied almost equally by frequent instantaneous repair, the longer the distance the more likely that some of the breakages will remain unrepaired, thus, in essence, providing for a linear linkage map of T4, despite the possibility that certain recombinational breaks do occur at nonrandomly located points.

We stress, once more, that the subunit hypothesis and its relevance for the theory of circular permutation of sets will remain only as a working hypothesis, and the results presented do not prove it but rather encourage its formulation.

ACKNOWLEDGMENTS

This investigation was supported by National Science Foundation grant GB 13048 and by Public Health Service grant CA 10055 from the National Cancer Institute. The computer evaluation of data was performed in the University of Pennsylvania Medical School Computer Center, supported by National Institutes of Health grant FR 15-06.

We acknowledge the excellent editorial assistance of Ezra Shahn.

LITERATURE CITED

1. Aposhian, H. V., and A. Kornberg. 1962. Enzymatic synthesis of deoxyribonucleic acid. IX. The polymerase formed after T₂ bacteriophage infection of *E. coli*: a new enzyme. *J. Biol. Chem.* 237:519.
2. Cohen, S. S. 1968. Virus induced enzymes, p. 135. Columbia Univ. Press, New York.
3. Fareed, G. C., and C. C. Richardson. 1967. Enzymatic breakage and joining of deoxyribonucleic acid. II. The structural gene for polynucleotide ligase in bacteriophage T4. *Proc. Nat. Acad. Sci. U.S.A.* 58:665.
4. Frankel, F. 1968. Evidence for long DNA strands in the replicating pool after T4 infection. *Proc. Nat. Acad. Sci. U.S.A.* 59:131.

5. Hurwitz, J. A., M. L. Becker, M. Gefter, and M. Gold. 1961. Enzymatic reaction at termini of DNA. *J. Cell Physiol. Suppl.* 1:70, 181-200.
6. Kozinski, A. W., and Z. Z. Felgenhauer. 1967. Molecular recombination in T4 bacteriophage deoxyribonucleic acid. II. Single-strand breaks and exposure of uncomplemented areas as a prerequisite for recombination. *J. Virol.* 1:1193-1202.
7. Kozinski, A. W., and P. B. Kozinski. 1963. Fragmentary transfer of ^{32}P -labeled parental DNA to progeny phage. II. The average size of the transferred parental fragment. Two cycle transfer. Repair of the polynucleotide chain after fragmentation. *Virology* 20:213.
8. Kozinski, A. W., and P. B. Kozinski. 1968. Autonomous replication of short DNA fragments in the ligase negative Tr Am H39X. *Biochem. Biophys. Res. Commun.* 33:670.
9. Kozinski, A. W., and P. B. Kozinski. 1969. Covalent repair of molecular recombinants in the ligase-negative amber mutant of T4 bacteriophage. *J. Virol.* 3:85-88.
10. Kozinski, A. W. 1969. Unbiased participation of T4 phage DNA in replication. *Biochem. Biophys. Res. Commun.* 35:294.
11. Kozinski, A. W. 1968. Molecular recombination in the ligase negative T4 amber mutant. *Cold Spring Harbor Symp. Quant. Biol.* 33:375.
12. Kozinski, A. W., P. B. Kozinski, and R. James. 1967. Molecular recombination in T4 bacteriophage deoxyribonucleic acid. I. Tertiary structure of early replicative and recombining DNA. *J. Virol.* 1:758-770.
13. Kozinski, A. W., and W. Szybalski. 1959. Dispersive transfer of the parental DNA molecule to the progeny of phage X174. *Virology* 9:260.
14. Okazaki, R. T. Okazaki, K. Sakabe, K. Sugimoto, and A. Sugino. 1968. Mechanism of DNA chain growth. I. Possible discontinuity and unusual secondary structure of newly synthesized chains. *Proc. Nat. Acad. Sci. U.S.A.* 59:598.
15. Richardson, C. C., Y. Masamune, T. R. Live, A. Jacquemin-Sablon, B. Weiss, and G. C. Fareed. 1968. Studies on the joining of DNA by polynucleotide ligase of phage T4. *Cold Spring Harbor Symp. Quant. Biol.* 33:151.
16. Shahn, E., and A. W. Kozinski. 1966. III. Incorporation of a single parental fragment to the progeny molecule. *Virology* 30:455.
17. Wiberg, J. S., M. L. Dirksen, R. H. Epstein, S. E. Luria, and J. M. Buchanan. 1962. Early enzyme synthesis and its control in *E. coli* infected with some amber mutants of bacteriophage T4. *Proc. Nat. Acad. Sci. U.S.A.* 48:293.
18. Wu, R., and A. D. Kaiser. 1968. Structure and base sequence in the cohesive ends of bacteriophage λ DNA. *J. Mol. Biol.* 35:523.