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Enzymic Joining of Polynucleotides

VII.† Role of the T4-induced Ligase in the Formation of Recombinant Molecules

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After mixed infection of *Escherichia coli* strain BB with ^{32}P-labeled- and bromouracil density-labeled T4 *am* EB6 (a T4 mutant defective in the DNA polymerase gene), two kinds of hybrid molecules were isolated. One type contained the ^{32}P- and bromouracil-labeled components linked only by hydrogen bonds (joint molecules); the second type contained the labeled components in covalent linkage (recombinant molecules). Infection with ^{32}P-labeled- and bromouracil-labeled T4 *am* EB6-605 (a T4 mutant defective in both the DNA polymerase and polynucleotide ligase genes) led to the formation of only joint DNA molecules. These results indicate that a functional T4-induced ligase is required for the formation of recombinant DNA molecules *in vivo*.

Conversion of joint to recombinant molecules was achieved *in vitro* and required ligase, DNA polymerase and the four deoxynucleoside triphosphates. This finding suggests that joint molecules are double-stranded structures in which single-strand gaps separate the polynucleotide segments derived from the parental DNA molecules.

1. Introduction

In their studies of genetic recombination in bacteriophage T4, Tomizawa and his colleagues identified two steps in the interaction of parental chromosomes following infection of susceptible host cells. The first consisted of the formation of overlapping "joint" DNA molecules containing components derived from different parental chromosomes joined through hydrogen bonds. In the second step, the joint molecules were converted to forms in which the two components could not be dissociated by procedures known to disrupt hydrogen bonds. These covalently linked structures were termed "recombinant" molecules (Tomizawa & Anraku, 1964; Anraku & Tomizawa, 1965*a*,*b*; Tomizawa, 1967; see also Fig. 1, Anraku, Anraku & Lehman, 1969).

The T4 phage-induced polynucleotide ligase displays the specificity expected of an enzyme which could promote the conversion of joint to recombinant molecules; that is, it catalyzes the synthesis of phosphodiester bonds between DNA chains which are properly aligned in a DNA duplex (Weiss & Richardson, 1967; Gefter, Becker & Hurwitz, 1967; Cozzarelli, Melechen, Jovin & Kornberg, 1967). Recently, Fareed & Richardson have identified gene 30 as the structural gene for the T4 ligase (Fareed & Richardson, 1967). Earlier studies (Hosoda, 1967) had demonstrated that a defect in

† Paper VI in this series is Hall & Lehman, 1969.

gene 30 led to only a very limited synthesis of phage DNA. Moreover, the DNA synthesized under these conditions was of relatively low molecular weight (9 to 11 s) (Sugimoto, Okazaki & Okazaki, 1968; Yudelevich, Ginsburg & Hurwitz, 1968; Newman & Hanawalt, 1968; Masamune & Richardson, 1968). Clearly, a functional T4-induced ligase is necessary for normal T4 DNA synthesis.

The aim of this investigation was to determine whether the T4 ligase is also essential for the conversion of joint to recombinant DNA molecules. The studies described here have shown that, whereas infection of *Escherichia coli* strain BB with a mutant defective in DNA polymerase alone leads to the formation of both joint and recombinant DNA, infection with mutants defective in both the T4-induced ligase and DNA polymerase results in the production almost exclusively of joint structures. This result would suggest that a functional phage-induced ligase is indeed required for the formation of recombinant molecules. We have also examined the structure of the joint DNA molecules with enzymes of known specificity, including DNA polymerase, *E. coli* joining enzyme, polynucleotide kinase and *E. coli* exonuclease III. These studies the details of which are described in the following paper (Anraku, *et al.*, 1969) have demonstrated that in the joint DNA the segments derived from the parental chromosomes are separated from each other in the double-stranded structure, by gaps approximately 300 nucleotides in length rather than by single-strand interruptions ("nicks").

2. Materials

(a) *Bacteriophages and bacterial strains*

The following amber mutants of phage T4 were used: *am* X5 (genes 41 through 45), *am* B22 (gene 43), *am* E4332 (gene 43) and *am* E605 (gene 30). These mutants were kindly provided by Dr R. S. Edgar and by Dr John Drake. *am* EB6 (a double mutant in gene 43) was isolated by Mr Richard Atkinson by recombination of *am* B22 with *am* E4332; *am* EB6-605 (mutant in genes 43 and 30) was obtained by recombination of *am* EB6 with *am* E605. *am* EB6 is defective in the T4-induced DNA polymerase; *am* EB6-605 is defective in both the T4-induced DNA polymerase and polynucleotide ligase (deWaard, Paul & Lehman, 1965; Warner & Barnes, 1966; Fareed & Richardson, 1967). *am* X5 is defective in the T4-induced DNA polymerase, deoxycytidylate hydroxymethylase (Wiberg & Buchanan, 1964) and in three other functions, as yet unidentified, which are required for T4 DNA synthesis.

The permissive host for these mutants is *E. coli* CR63 and the non-permissive host, *E. coli* BB (McFall & Stent, 1958).

(b) *Enzymes*

The polynucleotide joining enzyme of *E. coli* (fraction VII; specific activity 4000 units/mg protein) was prepared by the method of Anraku, Anraku & Lehman (manuscript in preparation). The T4-induced DNA polymerase (fraction VI; specific activity 9900 units/mg protein) was purified by a modification (Hall & Lehman, 1968) of the method of Goulian, Lucas & Kornberg (1968); *E. coli* exonuclease III was purified according to the method of Jovin, Englund & Bertsch (1969) and T4-induced polynucleotide kinase was prepared as described by Richardson (1965). Lysozyme (twice crystallized) and pronase were purchased from the Worthington Biochemical Corp.

(c) *Nucleotides*

[^{3}H]dTTP was purchased from New England Nuclear Corporation and dATP, dTTP, dGTP and dCTP were obtained from Sigma.

Concentrations of DNA are expressed per mole of nucleotide.

3. Methods

(a) *Preparation of labeled bacteriophages, bacteriophage crosses and isolation of DNA*

T4 phages labeled with ^{3}H, ^{32}P or bromouracil, were prepared according to methods already published (Tomizawa & Anraku, 1964; Anraku & Tomizawa, 1965*a*,*b*). Labeled intracellular T4 DNA was isolated in the following way. *E. coli* strain BB was grown to a density of 5×10^8 cells/ml. in Difco peptone broth, and resuspended in broth to a concentration of 0·5 to 1×10^{10} cells/ml. The culture was then infected with an average of two ^{32}P-labeled and eight BU†-labeled phages per cell. After incubation at 37°C for the times indicated, the cells were collected and the DNA extracted by the method of Anraku & Tomizawa (1965*a*) except that 100 μg of pronase/ml. (pretreated by the method of Young & Sinsheimer, 1967) was used instead of trypsin. In a routine centrifugation run, the DNA extracted from 1×10^{10} phage-infected cells was used. Its specific activity was 3000 to 4000 cts/min/mμmole.

(b) *Analysis of DNA by density-gradient centrifugation*

For neutral CsCl density-gradient centrifugation, DNA samples were mixed with CsCl buffered at pH 8·5 with 0·01 M-Tris–HCl and adjusted to a final density of 1·725 g/cm^3. Alkaline CsCl density-gradient centrifugation of the DNA was performed using alkaline CsCl solutions which were 0·04 M in sodium phosphate buffer (pH 12·4) and 0·002 M in EDTA at a final density of 1·755 g/cm^3. The solutions were centrifuged in the SW39 rotor of the Spinco model L centrifuge at 35,000 rev/min for 45 hr at 15°C. The bottoms of the tubes were punctured with a hollow needle and fractions (usually 45 to 60) were collected directly on to Whatman 3 MM filter discs. The discs were washed successively with 7% trichloroacetic acid, 0·25 M-HCl, 95% ethanol and finally absolute ether. After drying, radioactivity was determined by liquid-scintillation counting. When the fractions were to be used for additional experiments, they were collected in tubes and stored at 4°C until needed.

(c) *Treatment of joint T4 DNA with DNA polymerase and joining enzyme*

A fraction of DNA banding at the hybrid density of 1·75 to 1·76 g/cm^3 in neutral CsCl was used as substrate to investigate the conversion *in vitro* of joint to recombinant molecules. ^{3}H-labeled T4 DNA (0·2 μg, 3000 to 4000 cts/min) was added as carrier and the solution was dialyzed against 10 mM-Tris–HCl (pH 8·0), for 16 hr (three changes). The standard reaction mixture (0·3 ml.) contained 0·067 M-Tris–HCl (pH 8·0), 2·3 mM-$MgCl_2$, 10 mM-β-mercaptoethanol, 10 mM-ammonium sulfate and the DNA substrate (approximately 0·5 mμmole of nucleotide). For the reaction with DNA polymerase, dATP, dTTP, dCTP and dGTP were each added at a final concentration of 33 μM. When joining enzyme was used, 50 μM-NAD was added. After incubation at 30°C for 30 min, the reaction was terminated by the addition of EDTA to a concentration of 0·02 M and by chilling to 0°C. The DNA product was then examined by alkaline CsCl density-gradient centrifugation as described above.

(d) *Preparation of infected-cell extracts*

E. coli BB (100 ml.) was grown in peptone broth at 37°C to a cell density of 5×10^8/ml. and infected with T4 phage at a multiplicity of five. After incubation for the time specified, the culture was poured on to crushed ice. The cells were harvested by centrifugation (10 min at 12,000 *g*) and resuspended in 2 ml. of a solution containing 0·05 M-glycylglycine buffer (pH 7·4), 0·01 M-β-mercaptoethanol and 0·002 M-EDTA at 0°C. The cells were disrupted by means of a Mullard Ultra Sonic drill (three 0·5-min bursts), then centrifuged for 10 min at 12,000 *g* to remove cell debris. The extract thus obtained was adjusted to a protein concentration of 12 mg/ml.

(e) *Enzyme assays*

Assay of *E. coli* joining enzyme was performed as described by Olivera & Lehman (1968). For assay of T4-induced ligase activity, NAD was replaced by 0·5 mM-ATP. DNA poly-

† Abbreviation used: BU, bromouracil.

merase activity was measured by the method of Richardson, Schildkraut, Aposhian & Kornberg (1964).

(f) *Other methods*

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Radioactive samples were counted with a toluene-base scintillator using a Nuclear Chicago Mark I liquid-scintillation counter. A Zeiss PMQII spectrophotometer was used for all optical measurements.

4. Results

(a) *Formation of joint and recombinant molecules* in vivo

Cultures of *E. coli* BB were infected at 37°C with ^{32}P-labeled and BU-labeled *am* EB6, *am* EB6-605 or *am* X5. After incubation for 60 minutes (*am* EB6 and EB6-605) or 90 minutes (*am* X5), the cells were harvested, the DNA was extracted, mixed with reference ^{3}H-labeled T4 DNA and subjected to neutral CsCl density-gradient centrifugation as described under Methods.

As shown in Figures 1(a), 2(a) and 3(a), the ^{3}H-labeled T4 DNA marker formed a single band at the buoyant density characteristic of T4 DNA. (Under these conditions T4 DNA fully labeled with bromouracil would be expected to band in fractions 3 to 10.) On the other hand, a significant fraction of the [^{32}P]DNA isolated from cells infected with each of the three mutants was found at a density intermediate between the thymine-containing ^{32}P-labeled DNA and the heavier bromouracil containing material. A portion of the DNA in each intermediate density region was then recentri-

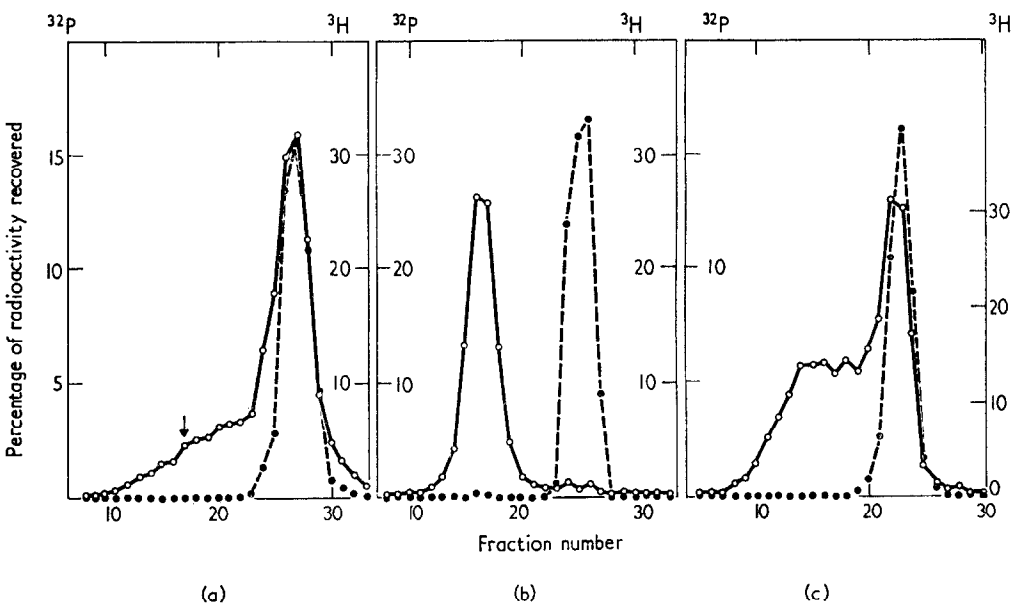

FIG. 1. CsCl density-gradient analysis of DNA from cells infected with ^{32}P-labeled and BU-labeled *am* EB6 (poly^{-}).

(a) The infected cells were incubated for 60 min; the DNA was then extracted and centrifuged at 35,000 rev./min for 45 hr in CsCl solution of density 1·725 g/cm^{-3} at about 15°C; (b) recentrifugation of a portion of fraction 17 of (a); (c) centrifugation of a portion of fraction 17 of (a) in alkaline CsCl solution (density 1·755 g/cm^{-3}) containing 0·04 M-sodium phosphate buffer (pH 12·4) and 0·002 M-EDTA. ^{3}H-labeled T4 DNA was added to each sample as a reference. —○—○—, ^{32}P; --●--●--, ^{3}H.

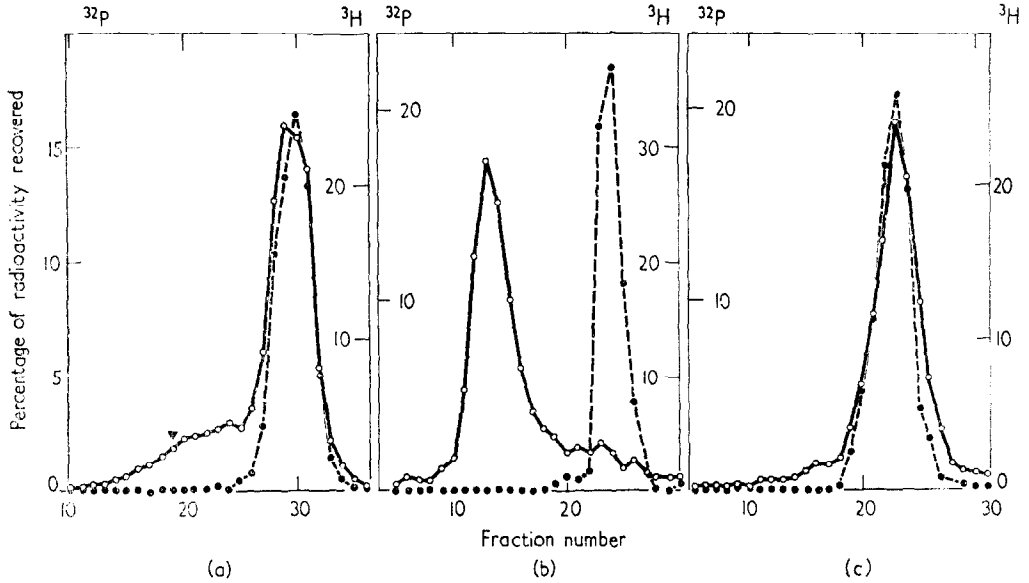

FIG. 2. CsCl density-gradient analysis of DNA from cells infected with ^{32}P-labeled and BU-labeled *am* EB6-605 (poly$^-$, lig$^-$).

(a) The infected cells were incubated for 60 min and the DNA was extracted and centrifuged as described in the legend to Fig. 1; (b) recentrifugation of a portion of fraction 19 of (a); (c) centrifugation of a portion of fraction 19 of (a) in alkaline CsCl solution. —○—○—, ^{32}P; --●--●--, ^3H.

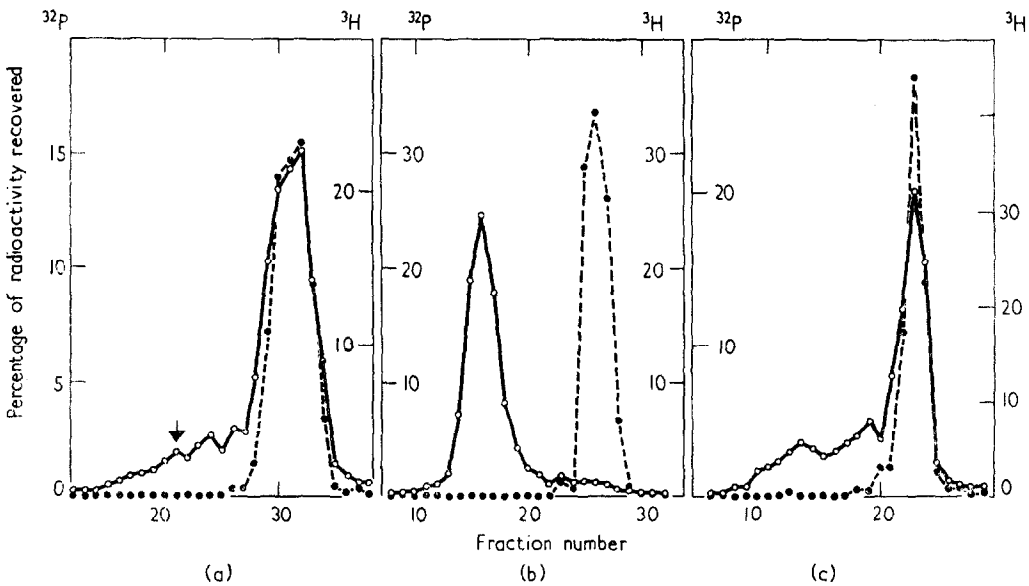

FIG. 3. CsCl density-gradient analysis of DNA from cells infected with ^{32}P-labeled and BU-labeled *am* X5 (genes 41 to 45).

(a) The infected cells were incubated for 90 min and the DNA was extracted and centrifuged as described in the legend to Fig. 1; (b) recentrifugation of a portion of fraction 21 of (a); (c) centrifugation of a portion of fraction 21 of (a) in alkaline CsCl solution. —○—○—, ^{32}P; --●--●--, ^3H.

fuged under the same conditions. The ^{32}P was now found to band as a symmetrical peak at the hybrid density observed in the initial centrifugation (Figs 1(b), 2(b) and 3(b)), thus indicating the presence of ^{32}P-labeled DNA molecules heavier than the input [^{32}P]DNA. To examine further the structure of the molecules with the hybrid density, portions of the same fractions used for recentrifugation (above) were mixed with ^{3}H-labeled T4 DNA and centrifuged to equilibrium in alkaline CsCl density gradients. The data presented in Figure 1(c) show that, after alkaline denaturation of the hybrid DNA extracted from cells infected with *am* EB6, approximately half of the ^{32}P coincided with the denatured [^{3}H]DNA standard; the remainder appeared in fractions of heavier density. This result is in agreement with the finding by Tomizawa (1967) that infection with *am* B22 (which bears a single mutation in the T4 DNA polymerase gene) led to the formation of covalently linked recombinant DNA. On the other hand, when the DNA of intermediate density, obtained from cells infected with *am* EB6-605 was subjected to alkaline CsCl density-gradient centrifugation, essentially all of the ^{32}P banded at the position of the denatured [^{3}H]DNA (Fig. 2(c)). Figure 3(c) shows the results obtained with *am* X5 where approximately 70% of the radioactivity of the ^{32}P was found at the position of the denatured reference [^{3}H]DNA; the remaining 30% banded at the hybrid density.

These experiments indicate that following infection with *am* EB6, defective only in the DNA polymerase, or the multiple mutant, *am* X5, also defective in the polymerase, covalently linked recombinants were formed in addition to the hydrogen-bonded joint molecules. In contrast, infection with the mutant *am* EB6-605, defective in both the polymerase and ligase, led almost exclusively to the formation of joint molecules. Complete cessation of phage DNA synthesis is required for the detection of joint or recombinant hybrids (Tomizawa & Anraku, 1964). Because T4 mutants, defective only in the ligase gene, do promote limited amounts of T4 DNA synthesis (Hosoda, 1967), they are unsuitable for experiments of the type performed with the multiple mutants.

There was no significant effect of the length of time the infected cells were incubated on the relative proportion of joint and recombinant molecules. When the infection with *am* EB6 was carried out for 100 or 120 minutes rather than 60 minutes, there was a small increase in the amount of [^{32}P]DNA which banded at an intermediate density. However, these fractions contained joint and recombinant molecules in approximately the same proportion as observed after 60 minutes of infection. In the case of *am* EB6-605, samples were taken at 20, 40, 60 and 90 minutes after infection. Fractions of intermediate density were observed as early as 20 minutes and increased in amount until 60 minutes after infection, but did not increase further after that time. Each sample was subjected to alkaline CsCl density-gradient centrifugation. Again, almost all of the ^{32}P banded at the position of the denatured ^{3}H-labeled DNA reference, hence was in the form of joint molecules. The fraction of ^{32}P which banded at a buoyant density heavier than the denatured ^{3}H-labeled T4 DNA did not exceed 8% of the radioactivity recovered. At 60 minutes after infection of *E. coli* BB with *am* X5, a small amount of DNA with hybrid density was observed which increased somewhat after 90, 120 and 150 minutes of infection. In each case, the DNA was composed predominantly of joint molecules, however, some covalently linked recombinant forms (15 to 35%) were consistently observed. This is in contrast to the earlier report of Anraku & Tomizawa (1965*b*) where essentially no recombinant molecules were found after infection with *am* X5. We have no explanation for this discrepancy.

(b) *Conversion of joint to recombinant molecules* in vitro

From the experiments described above, it is clear that while the product of gene 43 (the T4-induced DNA polymerase) is not essential for the conversion *in vivo* of joint to recombinant molecules, there is a requirement for the product of gene 30 (polynucleotide ligase).

In vitro, polymerase and ligase were both required for the conversion of joint to recombinant molecules. A fraction of intermediate density obtained from cells infected with *am* EB6-605 and consisting almost entirely of joint DNA molecules (fraction 20 in Fig. 2(a)) was incubated with *E. coli* joining enzyme, with T4 DNA polymerase (and the four deoxynucleoside triphosphates) or with both enzymes and then examined by alkaline CsCl density-gradient centrifugation. Upon incubation with joining enzyme there was no detectable conversion of joint to recombinant molecules (Fig. 4(b)). Similarly, DNA polymerase alone was without effect. However, after incubation with both joining enzyme and DNA polymerase, approximately 50% of the [^{32}P]DNA was converted to a recombinant form as indicated by the ^{32}P which now banded at a hybrid density (Fig. 4(c)). Similar results were obtained with DNA obtained from cells infected with *am* EB6 (Fig. 5) and *am* X5 (Fig. 6). As judged by the amount of ^{32}P which shifted toward a higher density, the extents of conversion were 17% for *am* EB6 and 43% for *am* X5. Here again, joining enzyme alone did not promote conversion of joint to recombinant DNA molecules; both joining enzyme and DNA polymerase were required. These findings are in agreement with those reported

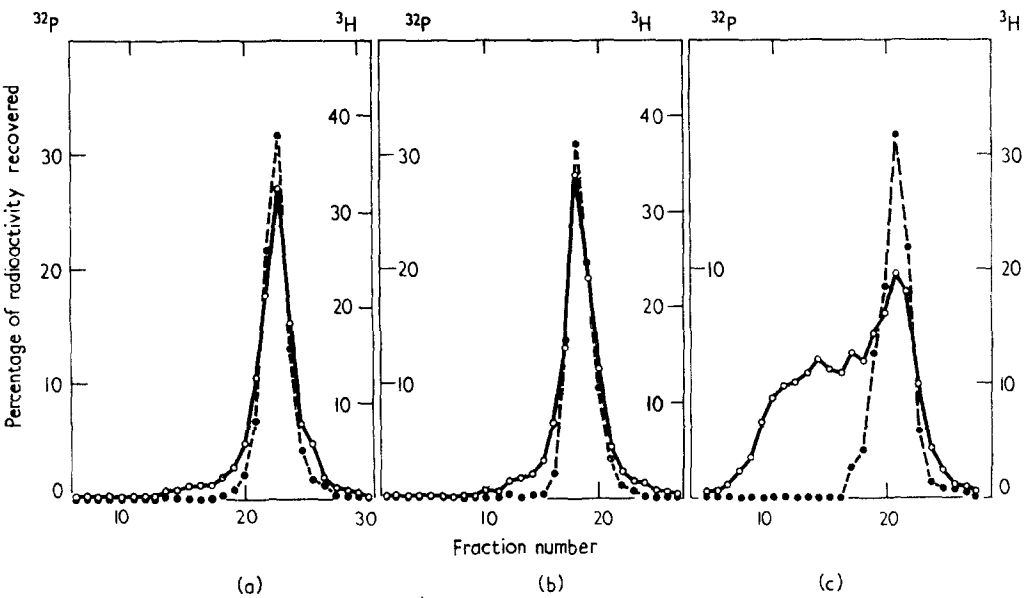

FIG. 4. CsCl density-gradient analysis of T4 *am* EB6-605 DNA (poly$^-$, lig$^-$) after treatment with *E. coli* joining enzyme and T4 DNA polymerase. DNA from *am* EB6-605-infected cells, corresponding to fraction 20 in Fig. 2 (a), was divided into three portions. Each portion was dialyzed, then incubated under standard conditions at 30°C for 30 min (see Methods) with enzymes added as indicated. The products were then analyzed by alkaline CsCl density-gradient centrifugation for 45 hr at 35,000 rev./min.

(a) No enzyme added; (b) *E. coli* joining enzyme (5 units) was added at 0, 10 and 20 min. (c) *E. coli* joining enzyme was added as described in (b) and T4 DNA polymerase (5 units) was added at 0 min. —○—○—, ^{32}P; --●--●--, ^{3}H.

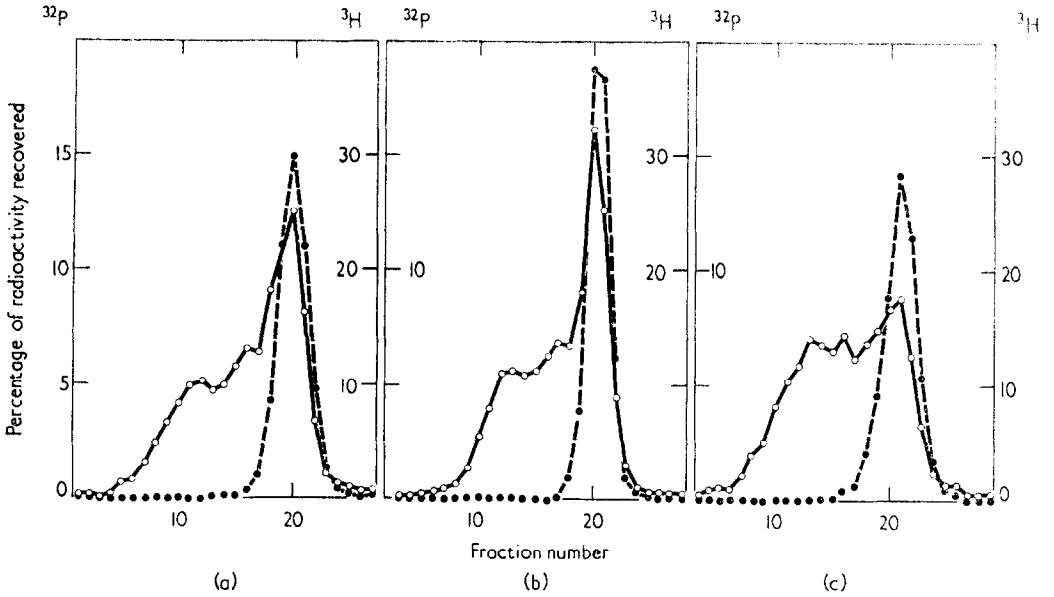

FIG. 5. CsCl density-gradient analysis of T4 *am* EB6 DNA (poly$^-$) after treatment with joining enzyme and DNA polymerase. DNA from *am* EB6-infected cells, corresponding to fraction 17 in Fig. 1 (a), was divided into three portions, then processed as described in the legend to Fig. 4.

(a) No enzyme added; (b) *E. coli* joining enzyme (2·5 units) was added; (c) *E. coli* joining enzyme (2·5 units) and DNA polymerase (5 units) were added at 0 min. —○—○—, ^{32}P; —●—●—, ^{3}H.

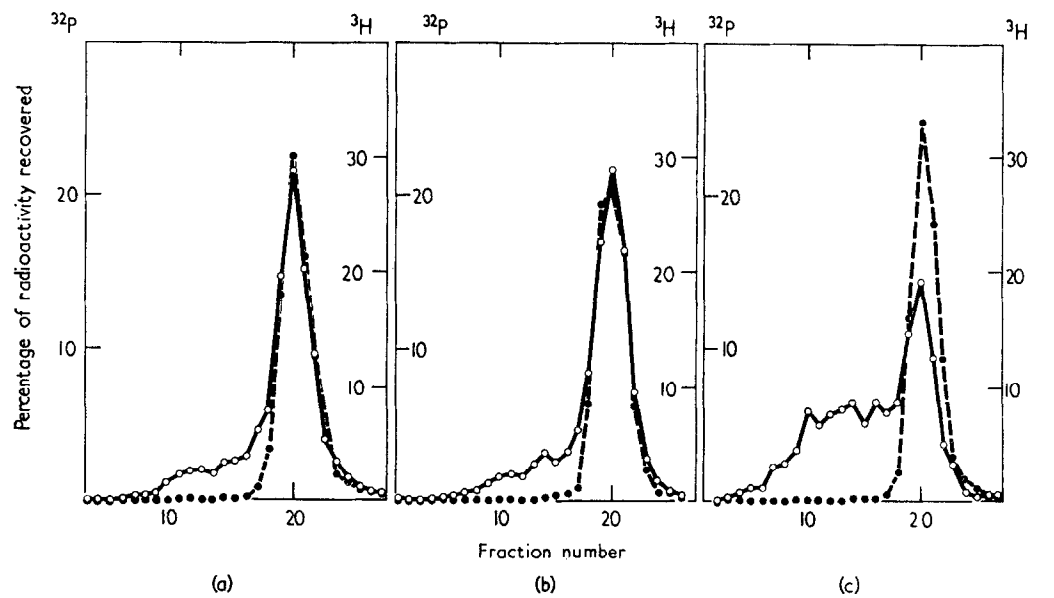

FIG. 6. CsCl density-gradient analysis of T4 *am* X5 DNA (genes 41 to 45) after treatment with joining enzyme and DNA polymerase. DNA from *am* X5-infected cells, corresponding to fraction 21 in Fig. 3 (a), was divided into three portions, then processed as described in the legend to Fig. 4.

(a) No enzyme added; (b) *E. coli* joining enzyme (2·5 units) was added to the reaction mixture at 0, 10 and 20 min; (c) *E. coli* joining enzyme was added to the reaction mixture as described in (b) and T4-induced DNA polymerase (3 units) was added at 0 min. —○—○—, ^{32}P; —●—●—, ^{3}H.

earlier by Tomizawa (1967) in which conversion *in vitro* of joint to recombinant molecules was observed after incubation of a preparation of joint T4 DNA molecules with crude T4-infected cell extracts in the presence of the four deoxynucleoside triphosphates. His failure to observe the conversion with purified T4 DNA polymerase may reasonably be attributed to the absence of ligase in these preparations.

In the experiments described above, using *am* EB6-605 DNA as substrate, 0·5 to 5 units of DNA polymerase (Richardson *et al.*, 1964) and 3 to 15 units of joining enzyme (Olivera & Lehman, 1967*a*) were used. The extent of conversion of joint to recombinant molecules observed was in the range of 30 to 60%. Higher concentrations of these enzymes or longer periods of incubation did not significantly increase the extent of conversion. A 3′-phosphoryl terminus would be expected to inhibit the action of both DNA polymerase and joining enzyme (Richardson, Schildkraut & Kornberg, 1963; Zimmerman, Little, Oshinsky& Gellert, 1967); the lack of a 5′-phosphoryl group would prevent the joining reaction (Zimmerman *et al.*, 1967). However, there was no effect of pre-treatment of the DNA with 0·004 unit of *E. coli* exonuclease III to remove possible 3′-phosphoryl termini (Richardson & Kornberg, 1964) or 0·1 unit of polynucleotide kinase and ATP to restore 5′-phosphoryl groups which may have been removed in the course of isolation of the DNA (Richardson, 1965; Novogrodsky, Tal, Traub & Hurwitz, 1966).

(c) *DNA joining enzyme and DNA polymerase activity in extracts of* E. coli *BB infected with T4 amber mutants*

The experiments described above imply that, although the *E. coli* DNA polymerase can replace the phage-induced enzyme in the conversion *in vivo* of joint to recombinant molecules, the *E. coli* joining enzyme is incapable of substituting for the corresponding phage-induced enzyme. The levels of *E. coli* joining enzyme, T4-induced ligase and DNA polymerase were therefore examined in cells infected with T4 mutants defective in the polymerase and ligase genes. As shown in Table 1 and Figure 7, a marked

TABLE 1

DNA joining enzyme activity in extracts of Escherichia coli *BB infected with T4 amber mutants*

	Extracts from cells infected with	Cofactor additions			
		None	NAD	ATP	NAD + ATP
		(units/mg protein)			
1.	(Uninfected cells)	0·40	2·7	0·47	2·7
2.	*am* EB6 (poly.$^-$)	0·04	1·4	5·2	6·8
3.	*am* EB6 + CM†	0·07	1·8	0·06	1·9
4.	*am* EB6-605 (poly$^-$, lig$^-$)	<0·01	0·37	<0·01	0·35
5.	*am* X5	0·05	1·8	5·3	8·4
	1 + 4‡	0·57	3·0	0·64	3·5
	2 + 4‡	0·09	1·8	7·0	8·4

Extracts were prepared at 60 min after infection. Assays of joining enzyme were carried out as described under Methods except that 10 mM-β-mercaptoethanol was added and incubation was for 15 min at 30°C.

† 30 μg of chloramphenicol/ml. (CM) was added 5 min before infection.

‡ Equal amounts of protein from the two extracts were mixed as indicated.

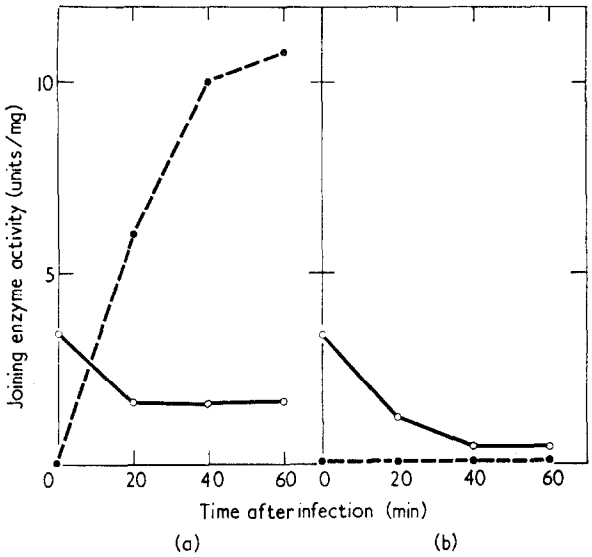

FIG. 7. Joining enzyme activity following infection of *E. coli* BB with (a) T4 *am* EB6 and (b) *am* EB6-605. Preparation of infected cell extracts and assay of joining enzyme activity were performed as described under Methods. --●--●--, + ATP; —○—○—, +NAD.

decrease (85%) in NAD-dependent joining enzyme activity did, in fact, occur in cells of *E. coli* BB infected with *am* EB6-605 (defective in both the polymerase and ligase). Infection with *am* EB6 (defective only in the polymerase) resulted in an approximately twofold decrease in activity. As expected, infection of *E. coli* with *am* EB6 or *am* X5 led to the induction of high levels of the ATP-dependent ligase, an increase which was blocked by infection in the presence of chloramphenicol. The decrease in activity observed in the case of the *am* EB6-605-infected cells did not appear to be due to the generation of a freely dissociable enzyme inhibitor since mixing of extracts obtained from uninfected and *am* EB6-605-infected cells produced the expected level of joining activity.

Assays of *E. coli* DNA polymerase activity in cells infected with *am* EB6 or *am* EB6-605 showed an apparent decrease of 40 to 50%.

5. Discussion

Infection of *E. coli* BB with an amber mutant defective in the T4 DNA polymerase (*am* EB6) leads to the generation of joint, as well as recombinant, DNA molecules; after infection with mutants defective in both the polymerase and ligase (*am* EB6-605), only joint molecules are formed. Thus, an active T4 ligase is necessary for the formation *in vivo* of recombinant DNA molecules from their hydrogen-bonded precursors. On the other hand, Richardson and his colleagues (Richardson *et al.*, 1968) have found that infection of the restrictive host with an amber mutant in the ligase structural gene (*am* H39X) (Fareed & Richardson, 1967), in the presence of 5-fluorodeoxyuridine to suppress DNA synthesis completely (Cohen, Flaks, Barner, Loeb & Lichtenstein, 1958), did result in the formation of recombinant molecules. Similarly, infection with mutants defective both in the ligase gene and gene 44 (required for phage DNA synthesis but whose product has not been identified) did not block completely the

formation of recombinant molecules. In another type of experiment, Kozinski & Kozinski (1969) have observed that parent to progeny recombination still occurred after infection of *E. coli* B with T4 *am* H39X. Barring a significant but as yet undetermined difference between *am* E605 and H39X†, it would appear that T4 mutants defective in either the polymerase or ligase alone produce covalently-linked recombinant molecules, but when both enzymes are defective, covalent linkage does not occur.

The conversion of joint DNA molecules to the covalently-linked recombinant forms *in vitro* requires the action of DNA polymerase in addition to ligase, suggesting that the joint DNA molecules, as isolated, contain nucleotide gaps which must be filled in before the ligase is able to catalyze the final covalent closure required to generate recombinant molecules. In these experiments we have used the T4 DNA polymerase in preference to the *E. coli* enzyme because of the absence of the 5′ to 3′ exonuclease component recently shown to be associated with the host enzyme (Klett, Cerami & Reich, 1968; Cozzarelli, Kelly & Kornberg, 1969).

Since a functional T4-induced DNA polymerase does not appear to be essential for the formation of recombinant molecules *in vivo*, it may be that the host DNA polymerase is capable of promoting the limited amount of DNA synthesis required to replace the missing nucleotide residues and fill in the gap. Assays of *E. coli* DNA polymerase activity in extracts obtained from cells infected with T4 amber mutants in the polymerase gene indicate that the apparent level of host DNA polymerase activity decreases by no more than a factor of two following infection; moreover, the addition of nucleotides at such a gap, using the apposing DNA strand as template, is a type of repair synthesis known to be catalyzed with high efficiency by the purified *E. coli* enzyme (Kornberg, 1969).

The presence of gaps in the isolated joint molecules, despite the apparent activity of the *E. coli* DNA polymerase *in vivo*, may be a reflection of the vulnerability to exonuclease action of nucleotide residues added to the gaps but not fully sealed in the absence of a functional ligase.

On the other hand, it is not clear why the host joining enzyme is unable to substitute for the phage-induced ligase in a comparable manner. It is obviously able to do so *in vitro*. Assays of extracts of *E. coli* BB infected with the mutant *am* EB6-605 have shown a significant drop in NAD-dependent joining enzyme activity which does not appear to be due to the generation of a freely dissociable enzyme inhibitor. A smaller (twofold) decrease was also observed after infection with *am* EB6, which possesses a functional ligase gene. It is not clear how much significance should be attached to these apparent decreases in *E. coli* joining enzyme activity following phage infection and to what extent, if any, they contribute to the seeming inability of the host enzyme to function in the formation of recombinant DNA molecules. As one explanation, the induction of high levels of endonuclease activity after T4 infection (Hurwitz, Becker, Gefter & Gold, 1967; Paoletti & Lehman, unpublished experiments) might lead to the introduction of large numbers of nicks in the host genome. In the absence of a functional phage-induced ligase, the *E. coli* enzyme may be rapidly saturated by

† The ligase activity after infection of the permissive host (CR63) with *am* H39X is approximately one-half that observed after infection with wild-type T4. On the other hand, *am* E605 induces the synthesis of a highly thermolabile ligase in CR63 which can only be detected by assay at 25°C and where activity is only 10% of that found in cells infected with the wild-type phage (Fareed & Richardson, 1967).

this nicked DNA and as a consequence be unavailable for the joining of the labeled substrate provided in the assay. Similarly, *in vivo*, in the absence of T4 ligase, the concentration of *E. coli* joining enzyme might be too low to meet the demands of the joining step of T4 recombination in the presence of *E. coli* DNA with a large excess of sites to which the host enzyme may be bound.

Since the host enzyme has a rather unusual and highly specific cofactor requirement, i.e. NAD, phage-induced alterations in the NADH/NAD ratio might be expected to affect the reactivity *in vivo* of the joining enzyme. However, this possibility seems unlikely for at least two reasons: first, energy metabolism is known not to be grossly affected as a result of phage infection (Cohen & Anderson, 1946); secondly, the K_m of the *E. coli* joining enzyme for NAD is exceedingly low (approximately 10^{-7} M; Olivera & Lehman, 1967*b*; Zimmerman, *et al.*, 1967); furthermore, NADH is not an inhibitor of the enzyme (Zimmerman *et al.*, 1967) so that were the level of NAD in the infected cell to fall precipitously, a sufficient concentration of this coenzyme would most likely still be available to serve the needs of the joining reaction.

It is worth noting that normal T4 DNA synthesis does not occur in cells infected with mutants defective in the ligase gene (Hosoda, 1967). It would therefore appear that the host polynucleotide joining enzyme is incapable of substituting for the phage-induced ligase in this respect as well. Finally, it is possible that the *E. coli* joining enzyme is, for reasons of physical inaccessibility, unable to act on T4 DNA *in vivo* despite its ability to do so *in vitro*. Further studies of the fate of the host polynucleotide joining enzyme are clearly required and may provide some insight into this problem, as well as the general question of the functionality or non-functionality of host enzymes in T4-infected cells.

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