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Nonreplicated DNA and DNA Fragments in T4 r⁻ Bacteriophage Particles: Phenotypic Mixing of a Phage Protein

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"Conservative phage" containing a genome derived from an infecting phage particle which has not undergone replication in the cell but nevertheless has become encapsulated and released in a normal phage particle, are found after infection of *Escherichia coli* with rII⁻ or rI⁻ mutants under conditions which result in rapid lysis. If such conservative phage are derived from a mixed infection with v^+ and v_i phage, they display phenotypic mixing of the v gene product (an endonuclease carried in the phage particle). Populations of rI and rII mutant phage grown under conditions of rapid lysis include particles containing short DNA fragments. It is suggested that a "maturation defect", common to rI and rII mutants, but absent in rIII mutants, may account for the encapsulation of nonreplicated DNA as well as that of the DNA fragments.

Mutations in the rII gene of bacteriophage T4 have pleiotropic effects. Mutant phage are restricted in lambda-lysogenic K-12 strains, and display rapid lysis in *Escherichia coli* B. After infection of either host there is increased leakage of some cellular metabolites as compared to what is found after infection with wild-type cells (5, 10, 29), and in *E. coli* B also an increase in phospholipid degradation (2). Mutations in rII partially suppress mutations in genes 30 (polynucleotide ligase; 3, 7, 14), t (cell lysis; 13) and z (tail fibers and adsorption; 28) after infection of *E. coli* B. In this host, net parent-to-progeny transfer of labeled DNA is low, and very heterogeneous contributions of parental DNA, including entire nonreplicated parental genomes, are found in progeny phages (6).

The products of the rIIA and rIIB cistrons appear to be proteins which become associated with the cell membrane during development (9, 11, 25, 33), but the mechanisms underlying the effects described above are still basically unknown. Some of these characteristics are shared by rapid lysis mutants in genes other than rII, others are not.

The experiments described in this communication were undertaken in order to characterize phage produced in cells infected with rII mutants as compared to phage mutant in other rapid lysis genes or wild-type phage. In the first section the nature of "conservative phage" (progeny phage containing nonreplicated DNA) is further explored. One possible explanation of their appearance is that the infection results in a compartmentation of the cell where only genomes located in "replication regions" can replicate, although both replicated and nonreplicated DNA matures. If this compartmentation reflects a physical seclusion, nonreplicated DNA would be expected to be inaccessible to all enzymes and proteins coded by the replicative DNA, and thus not display phenotypic mixing. Alternatively, "conservative transfer" may reflect a "nonreplicative state" of the DNA, without its physical isolation from replicating genomes. This alternative should allow phenotypic mixing in a mixed infection.

The product of the v gene of T4 is an endonuclease carried in the phage particle which will produce breaks in UV-damaged DNA in vivo (31) as well as in vitro (C. J. Castillo, Ph.D. thesis, Univ. of Pennsylvania, 1973; R. B. Shames, personal communication). The v_i mutation results in the production of an altered protein lacking this enzymatic activity. Probably as a result of this, v_i phage are more sensitive to UV light than are wild-type T4 phage (12).

Progeny phage from a mixed infection with v^+ and v_i phage behave phenotypically as if they contain both normal (v^+) and mutant (v_i) enzyme molecules. When assayed for DNA breakage after single infection in the presence of chloramphenicol, all DNA from such mixed

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progeny is nicked to an extent that appears proportional to the ratio of v^+ to v_i parental phages (31). In addition, UV irradiation of progeny phage resulting from such a mixed infection does not result in differential inactivation of the two phage types, but v^+ and v_i genotypes are present in equal proportion before irradiation and among survivors after a UV dose sufficient to kill 50% of v^+ and 95% of v_i phage (R. B. Shames, personal communication). These observations suggested to us a system where phenotypic mixing in conservative phage could be tested, and it will be demonstrated that conservative phage display phenotypic mixing of this protein.

During the course of this investigation it became apparent that purified rII⁻ phage differed in several respects from wild-type O_1^r phage. These differences were outlined in a previous communication (K. C., Fed. Proc., **29**:864, 1970) and are described in the second part of the paper. In the third part it is shown that these characteristics of rII⁻ phage are seen also in rI⁻, but not in rIII⁻, mutants.

MATERIALS AND METHODS

All experiments were performed in TCG medium (18) (except the experiment illustrated in Fig. 11, where TAG medium [22] was used for the preparation of the parental phage) at 37 C, and all manipulations involving 5-bromodeoxyuridine (BUdR)-labeled cells or phage were carried out in dim light. Standard plating methods were used to estimate bacteria and phage titres (1).

Strains. The *E. coli* strains B23 and CR63 were used in the experiments described here. The bacteriophage strains were T4B O_1^r (osmotic shock resistant, "wild type" in this study), T4D r59 (rIIA⁻; missense mutation), T4D r59 v_i (constructed from r59 and v_i single mutants; rIIA⁻ and UV sensitive), T4D r48 (rI⁻) and T4D r67 (rIII⁻). The rI and rII mutants were obtained from H. Berger, the rIII mutant was from S. Weintraub, and the v_i mutant was from W. Harm.

Preparation of phage. (i) Randomly isotope-labeled phage ("parental phage") were prepared essentially as previously described (15) by a single cycle of growth in [^{32}P]P_i- (P, 5 μg/ml, specific activity 0.1 to 5 mCi/mg), [^{3}H]adenine- (0.2 μg/ml, specific activity 0.2 mCi/mg), or [^{3}H]thymidine- (5 μg/ml, specific activity, 0.1 to 20 mCi/mg; 5 μg of 5-fluorodeoxyuridine [FUdR] per ml and 20 μg of uracil per ml) containing TCG medium. Viability of ^{32}P-labeled phage was calculated as PFU per particle, the latter estimated from the ^{32}P content of the phage (1 μg of P corresponds to 5×10^{10} T4-equivalent units of DNA). Unless otherwise stated, parental phage were prepared in *E. coli* CR63. This yields a uniform population of parental rII⁻ phage, since in this host strain the "maturation defect" (see Results and Discussion) of rII mutants is suppressed.

(ii) Progeny phage were prepared essentially as previously described (6, 15). Bacteria were pelleted from a fresh broth culture and grown for one to two generations to 3×10^8 cells/ml in BUdR-containing ("heavy"; 200 μg of BUdR per ml, 5 μg of FUdR per ml, 20 μg of uracil per ml) TCG. The culture was infected with either ^{32}P-labeled ("light"; non-BUdR-labeled) phage, or with a mixture of ^{32}P-labeled light and cold heavy phage. The MOI was calculated from the total phage particles (from the ^{32}P-content or DNA content of the preparations) added per viable cell. Five minutes after infection, the cells were sedimented and resuspended in fresh heavy medium in order to remove unadsorbed parental phage. Approximately 70 to 90% of the parental radioactivity was recovered in the cell pellets. The residual level of contaminating viable parental phage after resuspension (PFU resistant to chloroform treatment of the resuspended infected cells) was $\leq 10^{-3}$ phage per infected cell, whereas the average burst of r⁻ phage was 20 to 40 viable particles per infected cell. Lysis occurred 25 to 40 min after infection with r⁻ mutants, and was induced with $CHCl_3$ 90 min after infection under conditions of lysis inhibition. The lysate was treated with DNase (pancreatic endonuclease I) and progeny phage were purified as described previously (6, 15). The final phage pellet was resuspended in 0.3 ml of 0.15 M NaCl-0.01 M Tris-hydrochloride, pH 7.4, and banded in CsCl. Conservative transfer typically resulted in a bimodal distribution of parental label similar to that illustrated in Fig. 10 A, B, and D. Fractions corresponding to the light peak, operationally defined as "conservative phage" were pooled (the slight displacement of this light peak from the peak of reference light phage is due to a host-dependent modification of the phage; see Fig. 9 and 12). The isolated light phage fractions were dialyzed for 4 h against 0.15 M NaCl-0.01 M Tris-hydrochloride, pH 7.4, at room temperature. Although the T4D strains employed are not resistant to osmotic shock, control experiments showed that, by dialyzing the pooled fractions rather than diluting them in order to remove CsCl, full viability of the phage was preserved.

Irradiation. Phage were suspended in 0.15 M NaCl-0.01 M Tris-hydrochloride, pH 7.4, during irradiation.

(i) UV irradiation (260 nm) was performed with a GE germicidal lamp without a filter. Phage samples were gently agitated during irradiation.

(ii) Black light irradiation was performed with a lamp giving monochromatic light at 340 nm. Phage suspensions were chilled with circulating ice water during irradiation.

Gradient centrifugations. Phage and DNA were prepared for CsCl and sucrose gradient analysis as previously described (16, 23).

Phage CsCl gradients were prepared in broth containing 0.2 M $MgCl_2$, and had an average density of 1.5 g/cm^3. The gradients were centrifuged 18 h at 26,500 rpm in a Spinco SW50 rotor. Increasing the duration of the spin to 48 h somewhat sharpened the bands of osmotic-shock-sensitive phage, but did not alter their relative position. Analysis of phage DNA was performed in CsCl of average density 1.75 g/cm^3

in 0.15 M NaCl-0.015 M sodium citrate. These gradients were centrifuged for 72 h at 29,600 rpm in a Spinco SW50 rotor.

Sucrose gradients contained 5 to 20% sucrose in 1 M NaCl (neutral gradients) or 1 M NaCl-0.2 M NaOH (alkaline gradients). They were centrifuged at 26,700 rpm for 3 h in a Spinco SW50 rotor.

All gradients were collected from the bottom, and radioactivity in each fraction was estimated in a Packard Tri-Carb scintillation spectrometer by using a toluene-based scintillation fluid.

With the exception for CsCl gradients of some r⁻ phage, all added label was quantitatively recovered in the collected fractions.

RESULTS

Fate of the v gene product in phage containing nonreplicated parental DNA. The success of an experiment measuring the extent of phenotypic mixing among conservative rII^-v_i phage resulting from a mixed infection with rII^-v_i and rII^-v^+ phage depends critically upon the quantitative removal of all progeny phage containing newly synthesized DNA as well as that of conservative progeny from the co-infecting v^+ phage. The isolation procedure, described in Materials and Methods, utilized BUdR labeling and preparative density gradients followed by inactivation of BUdR-labeled phage in the pooled fractions with black light irradiation. Some preliminary experiments were required to test this system.

(i) Inactivation of phage by irradiation with UV or black light. The presence of BUdR in T4 phage DNA results in increased radiation sensitivity (32). Our results with respect to near visible and UV light are illustrated in Fig. 1 and 2.

Black light ("BL." 340 nm; Fig. 1) caused an exponential inactivation of heavy phage (with BUdR-substituted DNA) to about 0.02% survivors while not affecting the light phage (containing non-BUdR-labeled DNA) significantly. Deficiency in the v gene does not result in sensitivity to this type of irradiation.

Heavy phage are also more sensitive to UV irradiation 260 nm, Fig. 2) (32) than wild-type light phage, and displayed kinetics of inactivation very similar to those of light v_i phage. Moreover, v_i phage were not further sensitized to UV irradiation by substitution with BUdR. This observation made it necessary to ascertain whether sufficient quantities of functional v protein is produced in BUdR medium to permit phenotypic mixing in a mixed infection.

(ii) Sympathetic nicking with BUdR-labeled rescuer phage. Although DNA from UV-irradiated v_i phage remains intact upon

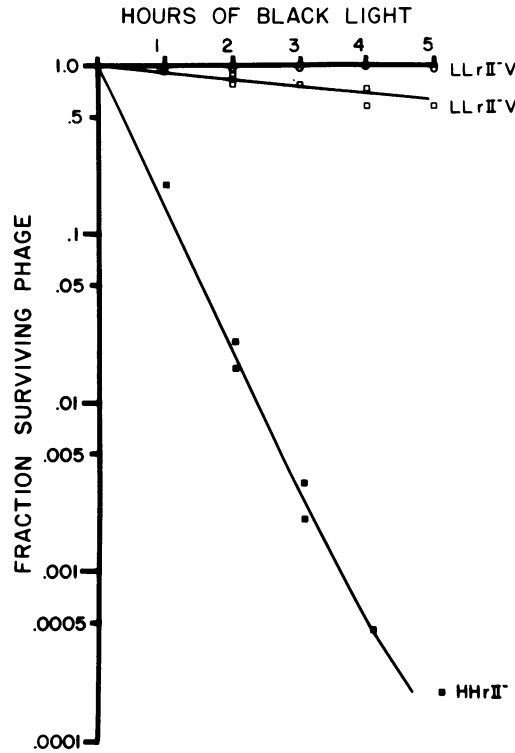


FIG. 1. *Inactivation of different phage types by black light. Heavy (containing BUdR-substituted DNA) rII^-v^+ phage (closed squares) were prepared as described in Materials and Methods ("preparation of progeny phages"). (The experiment illustrated in Fig. 5 and 6 was performed with another portion of these BUdR-labeled host cells.) Light (containing DNA which is not BUdR-substituted) rII^-v_i (open circles) and rII^-v^+ (open squares) phage were prepared in the same way, by using regular TCG instead of BUdR medium. The phage preparations were then exposed to irradiation at 340 nm for increasing lengths of time, and the fraction surviving phage was plotted as a function of time of irradiation.*

infection in the presence of a protein-synthesis-inhibitor, co-infection with v^+ phage results in breakage of the irradiated v_i DNA ("sympathetic nicking") (31). The ability of heavy phage to induce sympathetic nicking of UV-irradiated v_i phage DNA was tested as follows. Light bacteria were infected (in the presence of chloramphenicol) with light ^{32}P-labeled UV-irradiated rII^-v_i phage (A), the same UV-irradiated phage together with BUdR-labeled cold rII^-v^+ phage (B) or ^{32}P-labeled UV-irradiated light rII^-v^+ phage (C). This preparation of heavy phage was exponentially inactivated by black light irradiation to 0.05% survivors in 5 h (cf Fig. 1), demonstrating homoge-

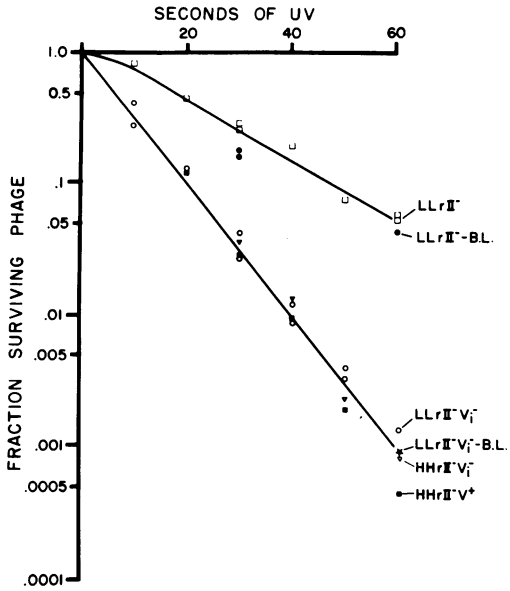


FIG. 2. *UV-inactivation of light and heavy v_i and v^+ phage. Phage preparations as specified in the figure were exposed to irradiation at 260 nm for varying lengths of time. B.L. after phage designation refers to a sample that was exposed to black light for 5 h prior to UV irradiation. This had no effect on the kinetics of UV inactivation.*

neous BUdR substitution (data not shown). Samples of intracellular phage DNA taken 3 min after infection were analyzed by alkaline sucrose gradient sedimentation. The results, illustrated in Fig. 3, are very similar to what was found by Shames et al. (31) by using only light phage: DNA from UV-irradiated v_i phage remains intact after infection (panel A), whereas DNA from v^+ phage is rapidly broken to an extent corresponding to the UV dose (panel C). Mixed infection with BUdR-labeled phage allows a corresponding extent of breakage of the v_i DNA (panel B).

This experiment shows that BUdR-labeled phage contain a normal amount of functional v protein. Therefore, sufficient quantities of v^+ protein should be produced in BUdR-containing medium to make the phenotypic mixing experiment feasible.

(iii) Phenotypic mixing in conservative phage. The design of the phenotypic mixing experiment is outlined in Fig. 4. Heavy *E. coli* B23 was infected with light ^{32}P-labeled rII^-v_i phage, either alone (MOI = 6, part A) or together with heavy rII^-v^+ cold phage (MOI = 3 for each phage type, part B). After lysis of the cultures, progeny phage were purified and banded in CsCl. In this progeny all phage containing newly synthesized DNA, as well as the conservative rescuer in part B, will contain BUdR-substituted DNA, and the only light phage will be the conservative rII^-v_i phage. These phage were isolated from the CsCl gradients. The pooled light fractions were then exposed to black light. Figure 5 shows that there was a measurable contamination of the light peak with heavy phage, but the phage surviving the black light illumination were as resistant to black light as the parental light phage. The relatively high contamination of the light peak was not unexpected. The gradients were collected from the bottom, which in our experience results in about 1% of the heavy phage band being recovered in the area of light phage. Irradiation of the unfractionated progeny failed to reveal biphasic inactivation kinetics (data not shown); thus, although a significant fraction of parental label is recovered in conservative phage, these phage form a very small minority in the entire burst. The inactivation plateau, which was reached at 0.5 to 1% survivors, cannot be accounted for by inefficient BUdR uptake during growth. The heavy phage used to obtain the graph in Fig. 1 was prepared from another portion of the heavy cells used in this mixed infection experiment, proving a very homogeneous BUdR-substitution. It was concluded, therefore, that the plateau observed in Fig. 5 represented the conservative light phages, which are insensitive to black light.

The genotype of the black-light-resistant phages was tested by picking several plaques, resuspending the phage in a small volume of buffer, and irradiating these suspensions with UV light. The results are shown in Table 1. It is demonstrated that all phage obtained are UV-sensitive, and thus only v_i genotypes are present in the viable phage from either preparation of conservative phage.

To see whether there was any phenotypic resistance to UV light among conservative rII^-v_i phage from the mixed infection, the rate of UV inactivation of conservative phage from both parts of the experiment was tested. Figure 6 shows the results of the experiment. The conservative rII^-v_i phage derived from the infection without rescuer (part A) were as sensitive to UV as the parental rII^-v_i phage. On the other hand, the conservative rII^-v_i phage from the mixed infection (part B) were as resistant to UV as rII^-v^+ phage. (In another repetition of this experiment conservative progeny from the mixed infection were slightly less resistant than

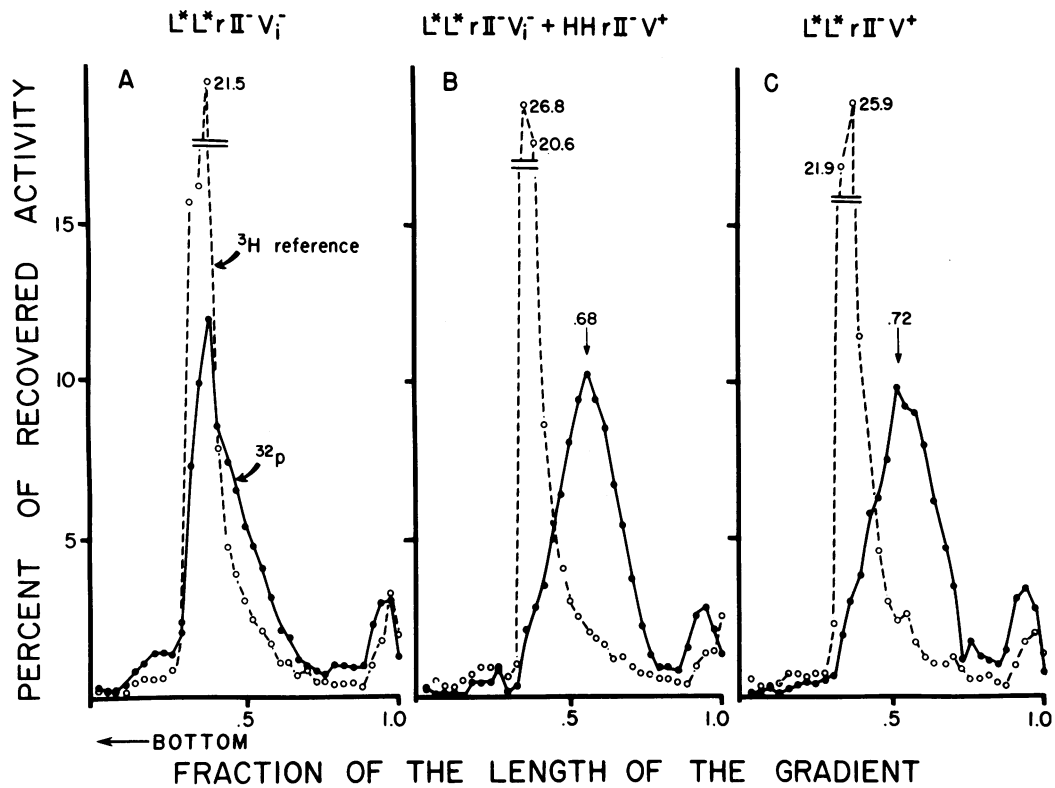


FIG. 3. *Sympathetic nicking of DNA from v_i phage by co-infecting heavy v^+ phage. Light ^{32}P-labeled phage (rII^-v_i or rII^-v^+) were irradiated with UV (six lethal hits, calibrated with v^+ phage) and used to infect light bacteria, either alone (MOI = 5, panels A and C, respectively), or together with cold heavy rII^-v^+ phage (MOI = 5 for each phage, panel (B). Chloramphenicol (100 μg/ml) was added to the bacteria just prior to infection. Three minutes after infection, samples of the infected cells were withdrawn, chilled in two volumes of 0.05 M NaCl-0.02 M EDTA-0.05 M Tris-hydrochloride, pH 7.5, sedimented, and resuspended in half the original volume of the same EDTA-salt buffer. The samples were supplemented with ^{3}H-labeled phage to serve as a sedimentation marker, lysed with lysozyme and Triton X-100 (17) followed by KOH to a final concentration of 0.2 M, incubated at 37 C for 30 min, and then analyzed in alkaline sucrose gradients. In this and following graphs of gradients, ^{3}H is represented by broken lines and ^{32}P by solid lines. Arrows with numbers in this and following graphs of sucrose gradients indicate the distance from the top of the gradient relative to the distance travelled by intact T4 DNA (D_2/D_1) (20).*

the rII^-v^+ phage.) It is obvious that phenotypic mixing has taken place in the mixedly infected cells. The results of this experiment provide additional evidence that viable conservative phage are true progeny phage containing DNA that has entered the cell, not participated in replication, but has been encapsulated again and released as a mature particle. In addition, this experiment shows that in encapsulation a random assortment of intracellular phage v protein was utilized.

The unusual parent-to-progeny transfer pattern observed in cells infected with rII mutants suggested that phage particles of an abnormal nature were produced. A more detailed characterization of phage particles and their DNA content was therefore undertaken.

Host-dependent characteristics of rII^- phage particles. $T4rII^-$ phages labeled either in those DNA portions transferred from a labeled parental phage by recombination during growth ("parental label") (6) or labeled randomly by growing the phage in radioactive medium ("progeny label") differ from wild-type T4B O_1^r phage after conventional purification of the phage. Figure 7 shows that only a fraction of the DNA label, whether "parental" or "progeny", in rII^-·B phage is recovered in a phage band upon centrifugation in CsCl. Residual label can be recovered from a pellet which forms on top of the gradient during centrifugation and which sticks to the walls of the tube upon

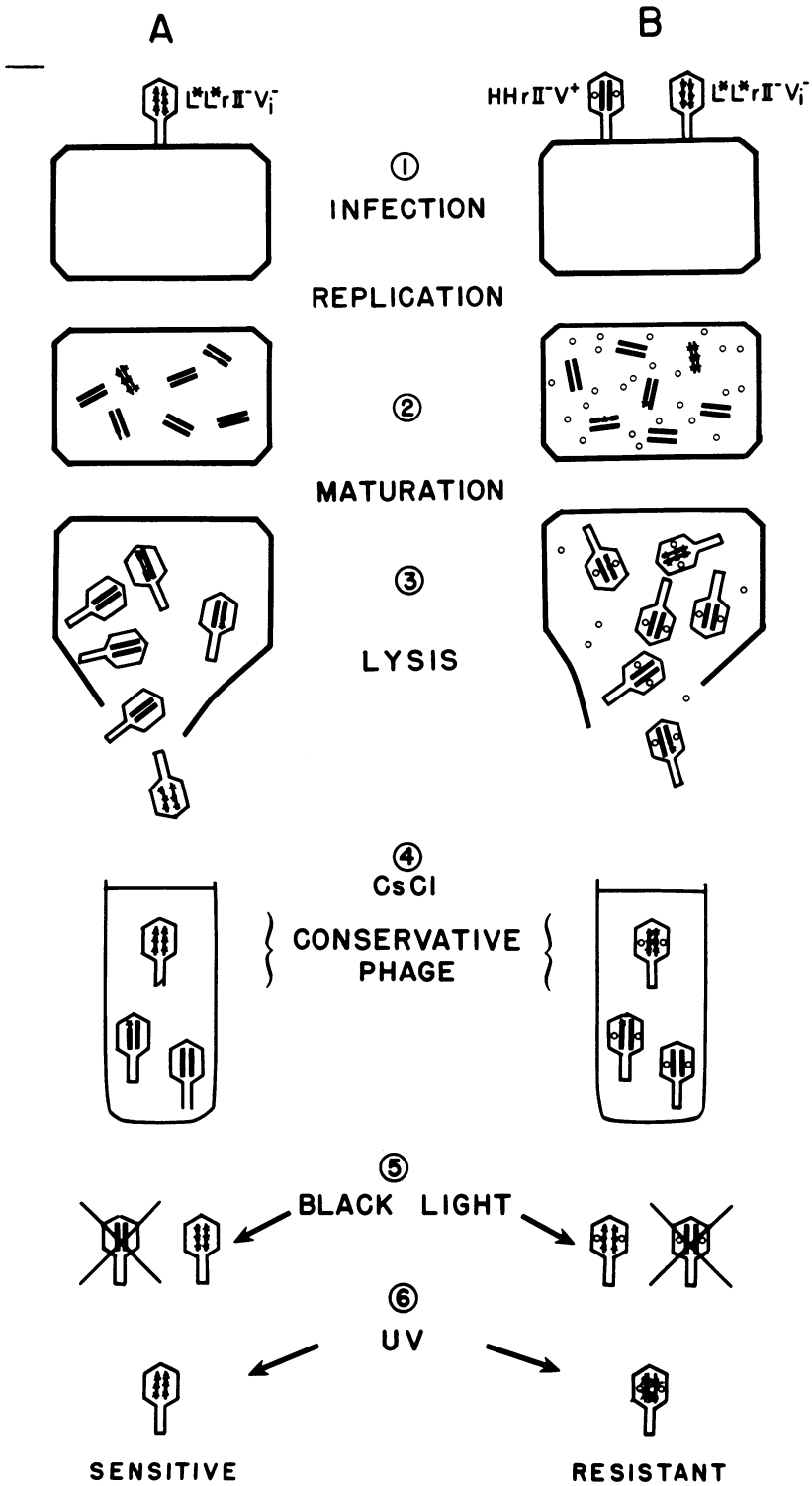


FIG. 4. *Scheme of experiment demonstrating phenotypic mixing of the v gene product in conservative rII⁻v, phage. The wild-type v gene product is represented as circles in the right column (part B). The scheme is not intended as an accurate model of the vegetative cycle of phage development, nor of the actual configuration of DNA and v protein in the mature particles. For more detailed description of the figure, see text.*

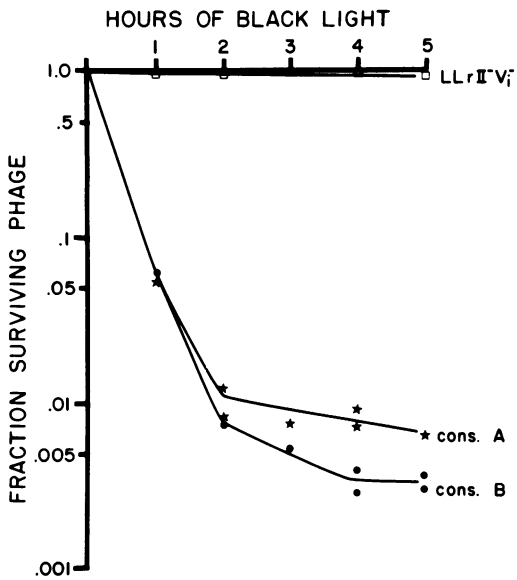


FIG. 5. *Black light irradiation of conservative rII⁻v_i phage. Conservative phage derived from an infection of heavy E. coli B23 with light ^{32}P-labeled rII⁻v_i phage (MOI = 6, part A) or the same light ^{32}P-labeled phage together with heavy rII⁻v^+ phage (MOI = 3 for each phage type, part B) were irradiated with black light, and the fraction surviving phage plotted was as a function of irradiation time. (The inactivation kinetics for heavy rII⁻v^+ phage grown in another portion of these host cells is illustrated in Fig. 1.) The light rII⁻v_i parental phage used in the experiment were irradiated at the same time as a control.*

TABLE 1. *UV sensitivity of phage progeny from conservative rII⁻ phage*

Plaque	Percent bacteriophage surviving after 60 s of irradiation[a]			
	Concn A (no rescuer)	Concn B (with rescuer)	rII⁻v^+ Stock	rII⁻v_i Stock
1	0.077	0.52	3.4	0.13
2	0.018	0.11	4.7	0.058
3	0.031	0.061	5.1	0.096
4	0.20	0.10	3.2	
5	0.12	0.097		
6		0.061		
7		0.52		
8		0.086		

[a] Phage samples from the experiment outlined in Fig. 4 were irradiated with black light for 5 h and plated. From these plates plaques were picked, resuspended in 1 ml of 0.15 M NaCl-0.01 M Tris-hydrochloride, pH 7.4, and saturated with $CHCl_3$. The suspensions were irradiated with UV, and the fraction surviving phage was calculated. Each entry in the table represents phage from one plaque.

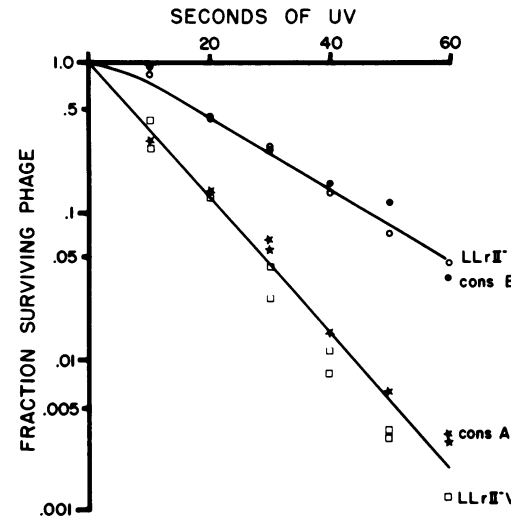


FIG. 6. *UV irradiation of conservative rII⁻v_i phage. Conservative phage obtained in the experiment described in the legend to Fig. 5 were exposed to 5 h of black light irradiation (Fig. 5), and subsequently irradiated with UV light. The graph shows UV inactivation curves for conservative phage from cells infected with rII⁻v_i phage alone (part A) as well as from cells mixedly infected with rII⁻v_i and heavy rII⁻v^+ phage (part B). At the same time inactivation curves for the rII⁻v_i parental phage used in this experiment, and for the rII⁻v^+ light phage stock used to prepare the heavy co-infecting phage were obtained.*

collection. The "parental label" associated with this floating fraction was previously analyzed and found to be mainly in conservative phage DNA (6). The relative proportion between banding and floating phage varies considerably from preparation to preparation, although the distribution illustrated in Fig. 7 is rather typical. In contrast to this, r^+ phage always band quantitatively in this type of gradient.

The right panel in Fig. 7 illustrates the sedimentation pattern of DNA from the same phage in an alkaline sucrose gradient. Native DNA showed essentially similar migration patterns (a gradient of this type is illustrated in Fig. 13). Note the distinctly bimodal sedimentation pattern. The recovery of added label in this gradient is quantitative, i.e., the pattern observed represents DNA from "CsCl banding phage" as well as from the "CsCl floating fraction."

rII⁻ phages prepared in *E. coli* B display lower viability (PFU per phage-equivalent unit of ^{32}P-DNA) than either rII⁻·CR phage or wild-type phage (6). To test the possibility that defective phage were present, some physical

properties of rII⁻·B phage were investigated. ^{32}P-labeled rII⁻·B phage were mixed with ^{3}H-labeled O_1^r phage, and the mixtures were subjected to various treatments. The ratio of ^{32}P to ^{3}H was not reduced by trichloroacetic acid precipitation, precipitation with anti-T4 antiserum, or DNase treatment followed by trichloroacetic acid precipitation ($^{32}P/^{3}H$ = 1.1, 1.02, and 1.2, respectively, where the ratio in the original mixture was 1.0). However, when the mixture was allowed to adsorb to bacteria, the $^{32}P/^{3}H$ ratio in the cell pellet was reduced to 0.72. It is likely that the nonadsorbing moiety forms the "CsCl floating fraction." The rII⁻·B phage preparation used for these assays contained 72% of its DNA in "banding phage," and alkaline and neutral sucrose gradient sedimentation of its DNA revealed a peak at D_2/D_1 = 0.6 containing approximately 20% of the labeled DNA.

Neither rII⁻·B nor rII⁻·CR phage coband with the wild-type reference phage in CsCl

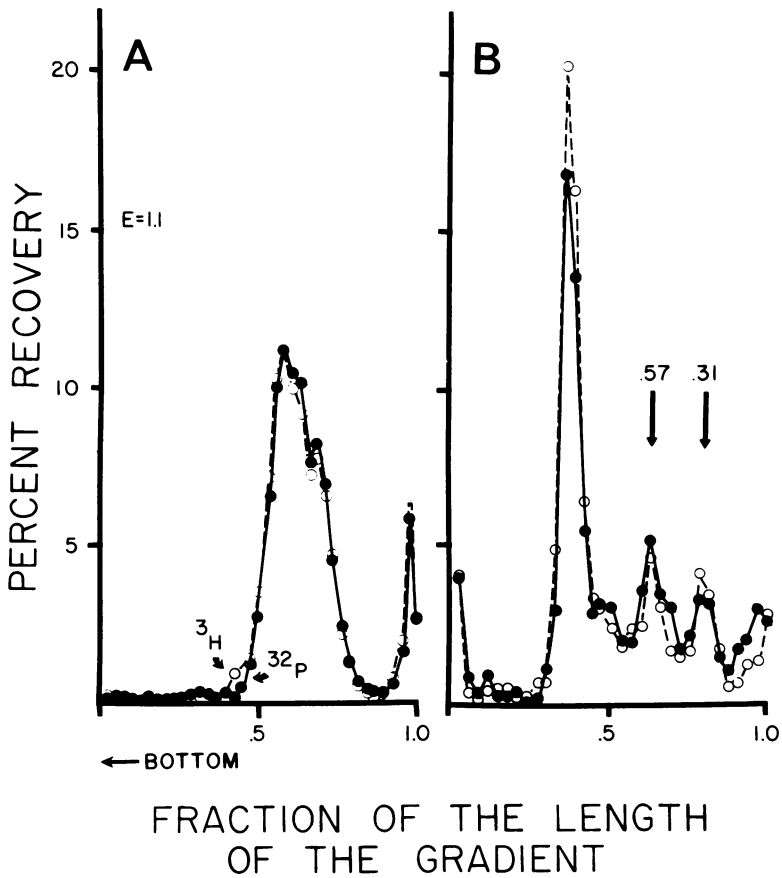


FIG. 7. *Identical fate of parental and newly synthesized DNA in rII⁻ phage. E. coli B23 was grown for two generations in light TCG medium (no BUdR) and infected with ^{32}P-labeled (specific activity 2 mCi/mg of P) light rII⁻ phage (MOI = 7). Five minutes after infection the cells were sedimented and resuspended in an equal volume of fresh light TCG containing [^{3}H] TdR (5 μg/ml, specific activity 1 mCi/mg), FUdR (5 μg/ml), and uracil (20 μg/ml). Forty-five minutes after infection (40 min after resuspension) $CHCl_3$ was added to complete lysis, and the phage was purified from the lysate. The left panel shows a CsCl gradient of the phage. The recovery of isotopes in the collected fractions was 62.6% for ^{3}H and 66.4% for ^{32}P. Residual counts could be extracted from a pellet forming on top of the gradient which remained in the tube upon fractionation. The efficiency of recovery (E) in this and following graphs of CsCl gradients is calculated as $^{32}P/^{3}H$ recovered in the collected fractions divided by $^{32}P/^{3}H$ in the input to the gradient. The right panel shows an alkaline sucrose gradient of the phage DNA. The phage was mixed with an equal volume of 0.5 M KOH-0.15 M NaCl-0.001 M EDTA and gently layered on top of the gradient which was then centrifuged, as described in Materials and Methods. The peak at D_2/D_1 = 0.6 is always found in preparations of rII⁻·B phage, whereas the peak at D_2/D_1 = 0.3 is not always present.*

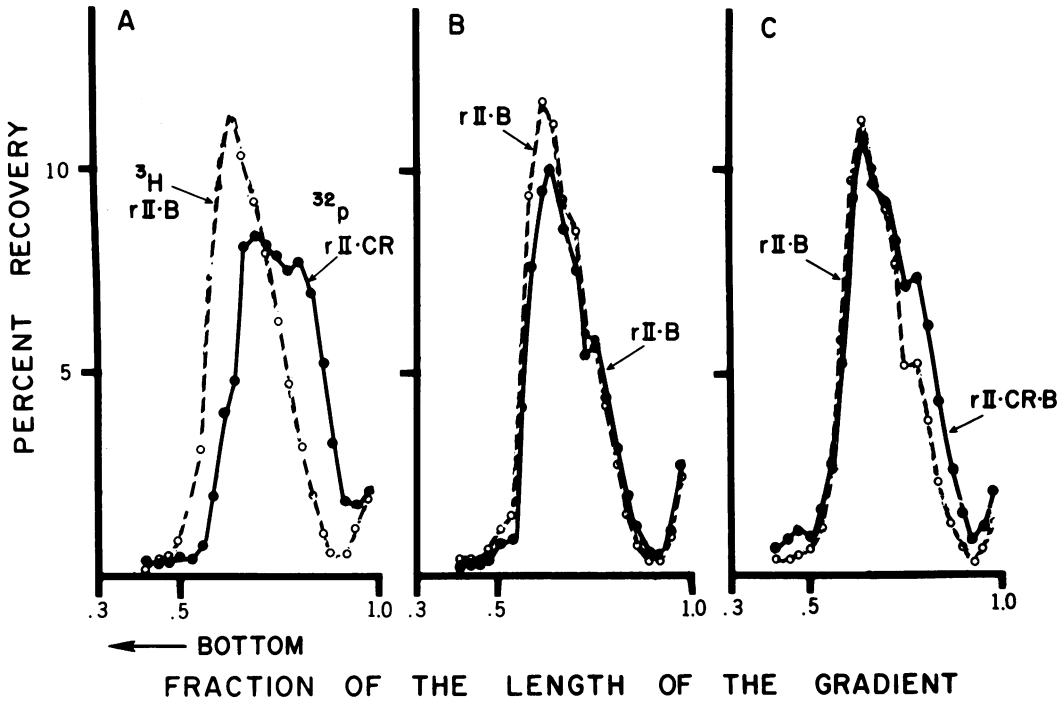


FIG. 8. *Buoyant density of rII$^-$·B and rII$^-$·CR phage. Light ^{3}H-labeled and ^{32}P-labeled rII$^-$ phage were prepared in E. coli B23 or CR63 (Materials and Methods: preparation of parental phage) and analyzed in CsCl (panels A and B). In addition, the ^{32}P-labeled rII$^-$·CR phage was used to infect E. coli B23, and progeny phage from this infection (designated rII$^-$·CR·B) was analyzed together with ^{3}H-labeled rII$^-$·B phage (panel C). No density label, such as BUdR, was present during the preparation of any of these phages.*

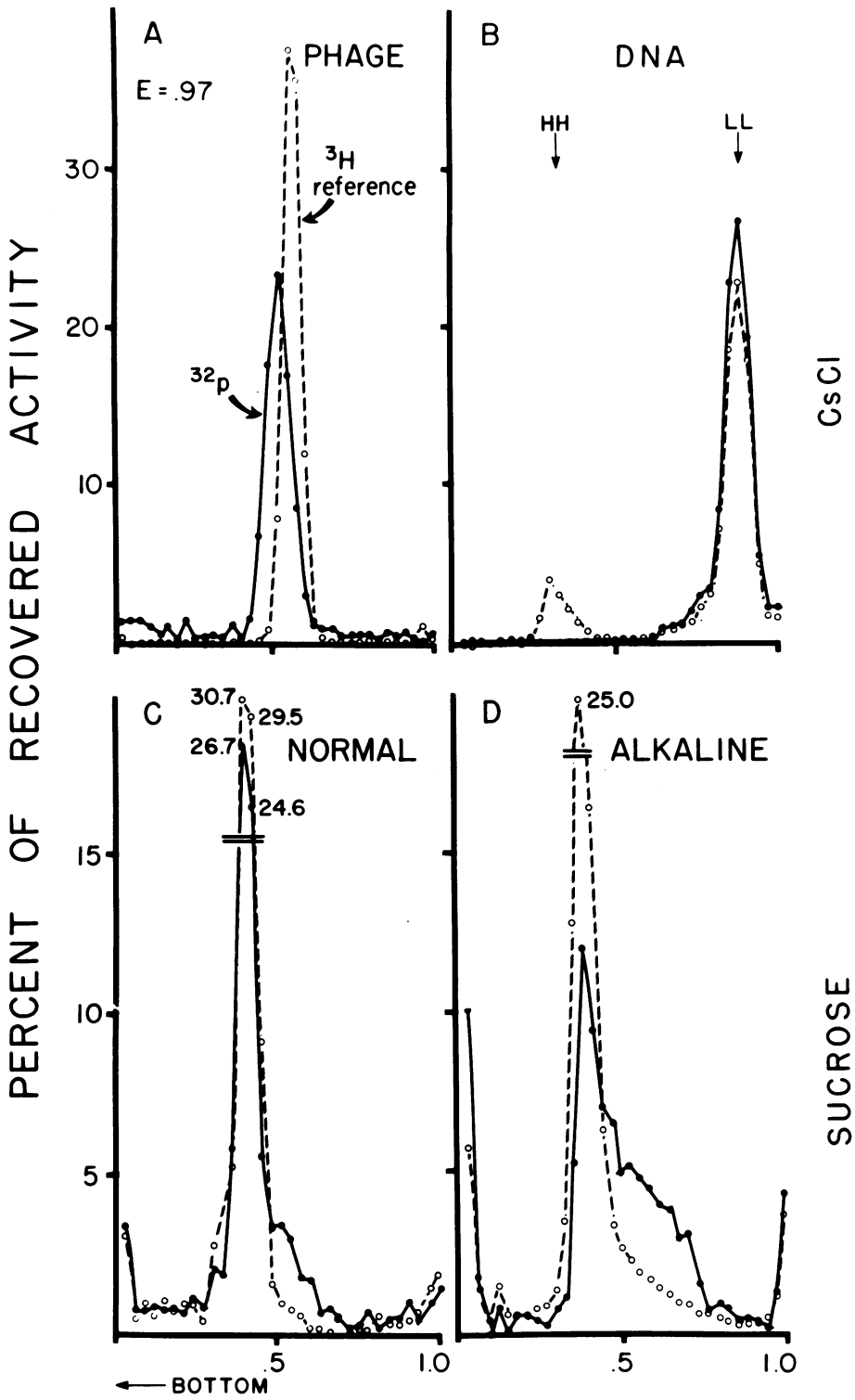
gradients. Figure 8 demonstrates that the relative increase in density of rII$^-$ phage depends on the host in which the particles were encapsulated: rII$^-$·B phage are slightly denser than rII$^-$·CR phage (which in turn are denser than reference phage, Fig. 12) regardless of whether the phage DNA is randomly labeled with ^{32}P or ^{3}H (panels A and B) or labeled only in those portions of the DNA transferred from the parental rII$^-$·CR phage (panel C).

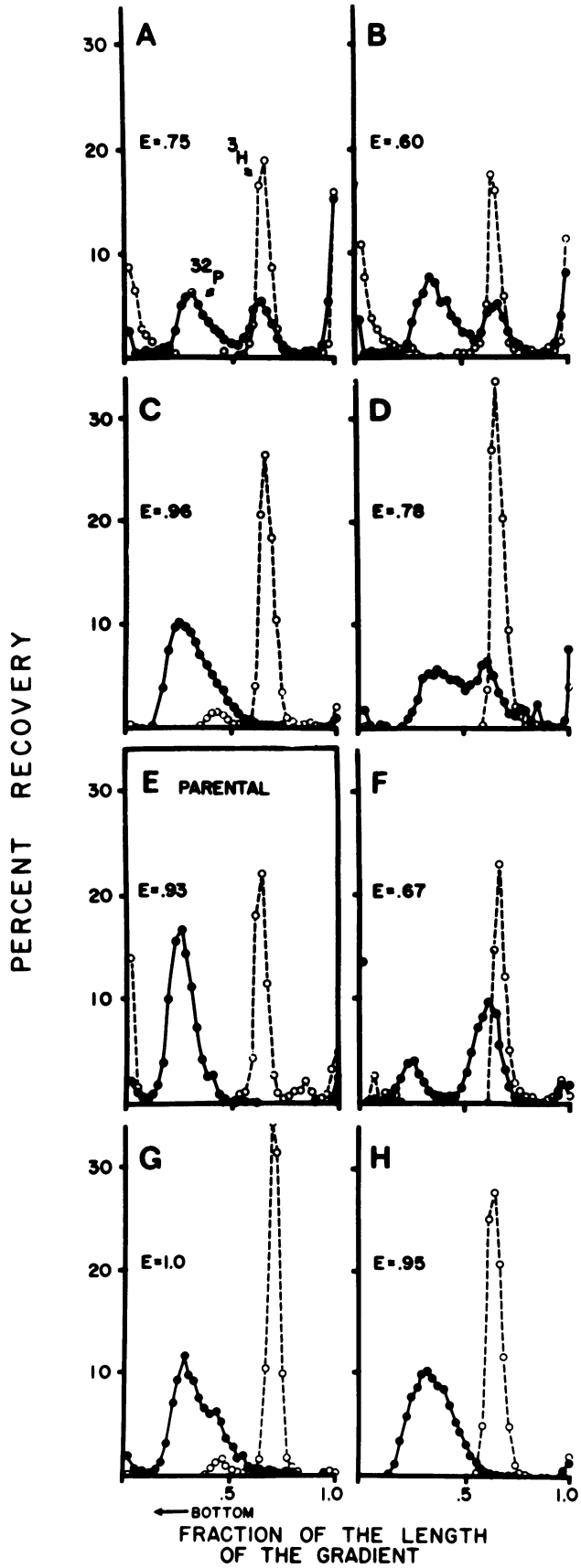
The relative density increment does not reflect an increase in the DNA density, nor in the quantity of DNA per phage. Reanalysis of phage from a CsCl band, and its DNA, is shown in Fig. 9. Phage isolated from CsCl reband in the original location (panel A), whereas DNA isolated from such phage cobands with reference DNA in CsCl (panel B) and comigrates with intact reference DNA in both normal (panel C) and alkaline (panel D) sucrose gradients, although the phage itself retains its increased buoyant density.

It should be noted that, although a shoulder on the alkaline sucrose gradient peak of phage DNA (Fig. 9) indicates some fragmentation of the DNA, the distinct peak at $D_2/D_1 = 0.6$, which was present in the sucrose gradients in Fig. 7 and also seen in Fig. 13, is absent in the gradients illustrated in Fig. 9. The most likely explanation for this difference is that the DNA fragments are found in the "CsCl floating fraction" (cf. efficiencies of recovery in CsCl in Fig. 7 and 9). The low buoyant density of the phage in this fraction suggests that only one or a few fragments are contained in one band.

We do not know the nature of this phage

FIG. 9. *Reanalysis of rII$^-$·B phage and its DNA. Light ^{32}P-labeled rII$^-$·B phage was banded in CsCl. Fractions corresponding to the phage peak were pooled, dialyzed, and reanalyzed together with ^{3}H-labeled references. Part was rebanded in CsCl (panel A). Another part was extracted with two volumes of phenol followed by four ether-washes of the aqueous phase, and the DNA was analyzed in CsCl (panel B) as well as in a neutral sucrose gradient (panel C). A third portion of the phage was mixed with an equal volume of 0.5 M KOH-0.15 M NaCl-0.001 M EDTA and analyzed in an alkaline sucrose gradient (panel D). Both isotopes were quantitatively recovered in these gradients.*


A
PHAGE
E = .97
^{3}H reference
^{32}P
B
DNA
HH
LL
CsCl
C
30.7
29.5
26.7
NORMAL
24.6
D
25.0
ALKALINE
SUCROSE
PERCENT OF RECOVERED ACTIVITY
30
20
10
15
10
5
.5
1.0
BOTTOM
FRACTION OF THE LENGTH OF THE GRADIENT


A
E=.75
$^{3}H_s$
$^{32}P_s$
B
E=.60
C
E=.96
D
E=.78
E PARENTAL
E=.93
F
E=.67
G
E=1.0
H
E=.95
30
20
10
.5
1.0
BOTTOM
PERCENT RECOVERY
FRACTION OF THE LENGTH OF THE GRADIENT

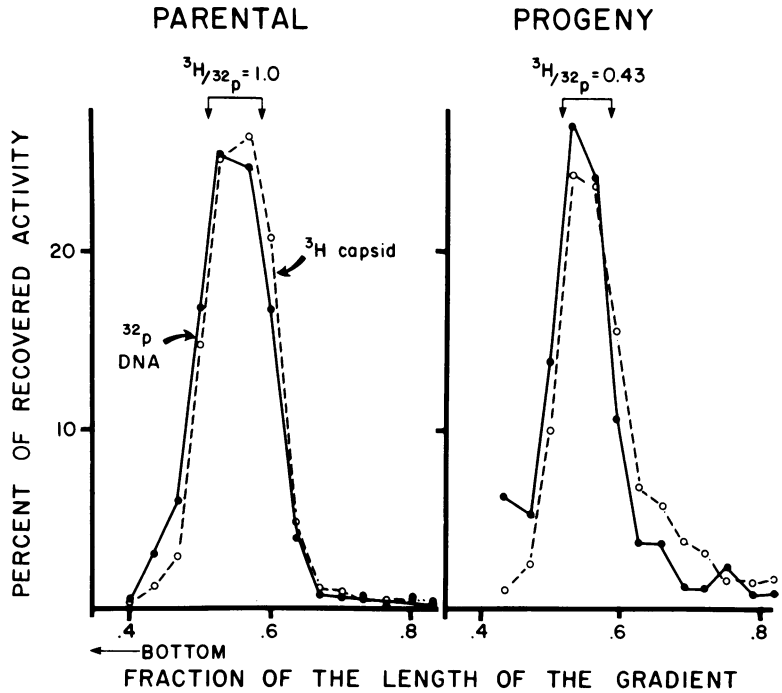
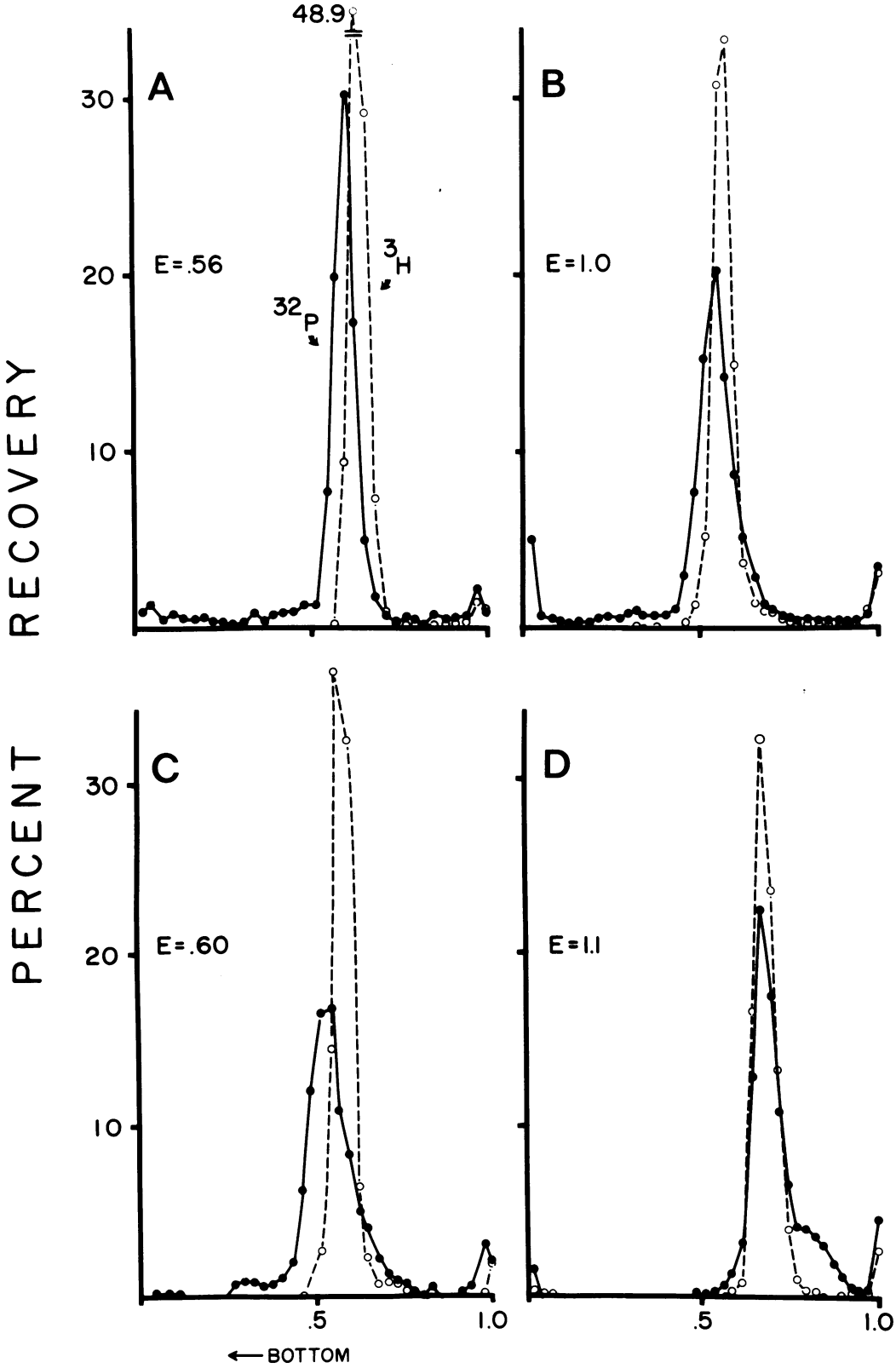


FIG. 11. *Conservative transfer after infection of heavy E. coli B23 with light rI⁻ phage labeled with [³H]leucine and [³²P]P_i. The parental phage was grown in CR63 in TAG medium (22) as described previously (6) ([³H]leucine, 0.4 μg/ml, specific activity 300 mCi/mg; P 5 μg/ml, specific activity 10 mCi/mg). The viability of this phage was 80%, which is unusually high for rI⁻ phage. Progeny of this phage were prepared as described in Materials and Methods by using an MOI of 0.4 particles per cell. (Another portion of the culture was infected with an MOI of 5 particles per cell. Analysis of progeny from this culture gave results very similar to those shown here for the singly infected cells.) Both parental and progeny phage were analyzed in CsCl. No references were added to these gradients. The amounts of radioactivity recovered between 40 and 80% of the length of the gradients, within which region the light parental phage banded, were summed and considered as 100% of recovered activities in constructing the graphs. The average specific activity of ³H/³²P in the peak was obtained by summing the recovered activities in the fractions between the arrows and is expressed by the numbers over these arrows.*

density marker. It provides a useful tool, however, in discriminating between phages prepared in one or the other host, which was utilized in the previous study of parent-to-progeny transfer (6). Also the rI mutant (Fig. 12) and an amber phage (17) are denser than O_1^r phage, and a spontaneous revertant of the r59 mutation, isolated as a plaque-former on K-12(λ) which displays neither rapid lysis nor conservative transfer, retains the increased density (unpublished observations). The increased density is not a characteristic of D strains as opposed to B strains: T4DrIII67 co-bands with T4BO_1^r (Fig. 12D).

Parent-to-progeny transfer, phage density, and DNA fragments in other rapid-lysis mutants of T4. The occurrence of conservative phage among progeny from different r mutants was analyzed in a manner analogous to that described by Kozinski (15) and utilized in the preceding study (6). The parent-to-progeny transfer was studied in both *E. coli* B and in *E. coli* K12 in order to possibly correlate an occurrence of conservative transfer to rapid lysis,

FIG. 10. *Parent-to-progeny transfer of DNA from different r mutants in E. coli CR63 or B23. Cultures of E. coli B23 or CR63 grown in heavy medium were divided each in three portions. Each portion was infected with a ³²P-labeled light r⁻ parental phage (MOI = 8), and the resulting progeny were isolated. Progeny phage were similarly prepared from light E. coli B23 infected with heavy ³²P-labeled rII⁻ phage. The purified progeny phage and the heavy parental phage were banded in CsCl together with ³H-labeled light O_1^r phage. Left panels, transfer in CR63, right panels, transfer in B23. Top two rows and bottom row, transfer of light parental DNA in heavy cells. A, rI⁻ in CR63; B, rI⁻ in B23; C, rII⁻ in CR63; D, rII⁻ in B23; G, rIII⁻ in CR63; H, rIII⁻ in B23. The third row shows transfer with reversed density labels (heavy parental DNA in light cells). E, Parental heavy rII⁻ phage prepared in CR63; F, transfer of DNA from this phage in light B23.*


48.9
A
B
30
20
10
E=.56
E=1.0
^{3}H
^{32}P
RECOVERY
C
D
E=.60
E=1.1
PERCENT
.5
1.0
BOTTOM
FRACTION OF THE LENGTH
OF THE GRADIENT

since mutants in the rI gene display rapid lysis in both hosts, whereas mutants in rII or rIII display rapid lysis only in *E. coli* B and are lysis inhibited in other strains.

Figure 10 shows that, in the case of rI (panels A and B) and rII (panels D and F) mutants, a sizable portion of the parental label transferred to the progeny appears at the parental density location. This type of transfer is found in both host strains when infected with the rI mutant, but only in *E. coli* B when infected with the rII mutant, which correlates occurrence of rapid lysis and conservative transfer in rI⁻ or rII⁻ infected cells. In the case of the rIII mutant no such correlation is found.

To verify that the appearance of parental label at parental density in the CsCl gradient of rI progeny represented true conservative transfer, the transfer of parental DNA from a phage with labeled capsid was studied. This experiment was patterned on one previously described using an rII⁻ parental phage (6) where the specific activity of capsid label over DNA label was compared for parental and conservative progeny phage. The results, illustrated in Fig. 11, were essentially identical to what was found with rII⁻ phage. Also in this case conservative phage from singly infected cells displayed a considerably lower capsid label to DNA label ratio, indicating that a large proportion of these phage contained parental DNA in an unlabeled capsid. Note, however, that there is no difference in density between parental and conservative progeny phage, in contrast to what was found with rII⁻ phage (6). This agrees with our (unpublished) observation that rI⁻·B and rI⁻·CR phage have the same buoyant density in CsCl.

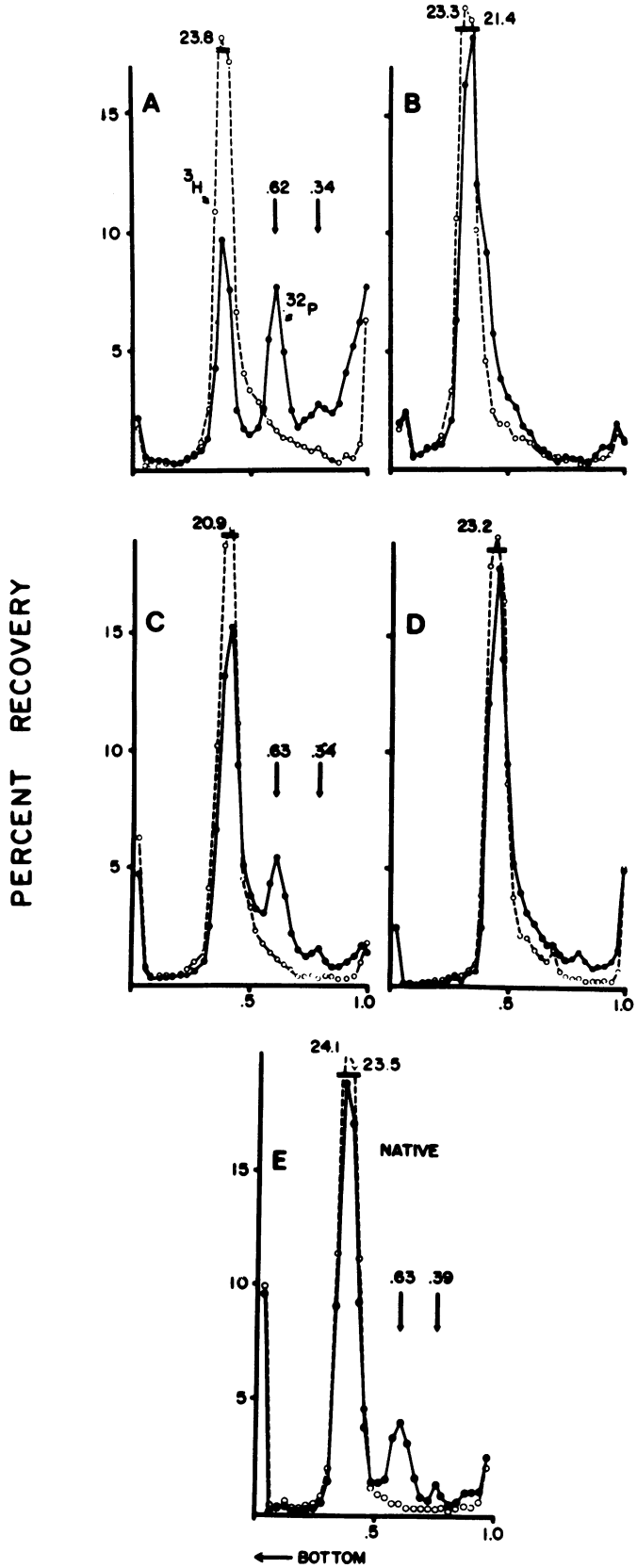
These experiments demonstrated that rI and rII mutants both display conservative transfer, but the rIII mutant does not. When the studies described earlier were extended to include other rapid lysis mutants of T4, what has been described here for rII⁻·B phage was found to apply also to rI⁻ phage (Fig. 12 and 13 and unpublished observations): phage have low "viability," purified phage preparations include a sizable fraction of particles with much lower buoyant density than normal phage, and there are two size classes of DNA associated with the phage. The rI mutant displays these properties in all host strains that have been tested, but rII mutants do so only when the phage is prepared in *E. coli* B (K. Carlson, Ph.D. thesis, Univ. of Pennsylvania, 1971). rIII mutants do not display any of these characteristics.

DISCUSSION

The mode of transfer of parental DNA to progeny rII⁻ (or rI⁻) phage is quite heterogeneous, with contributions of parental DNA ranging from short, semiconservatively replicated pieces to fully conservative, nonreplicated molecules (6). Three lines of evidence support the conclusion that those phages containing an entire nonreplicated parental chromosome ("conservative phage") are indeed newly formed progeny particles and not contaminating parental phage. (i) Conservative phage derived from an infection with parental phage labeled with [^{3}H]leucine as well as [^{32}P]phosphate contained significantly less ^{3}H than did the parental phage (ref. 6 and our Fig. 11). (ii) The displacement between ^{3}H (representing contaminating parental phage) and ^{32}P (representing mostly conservative phage) in the CsCl gradient of this conservative rII⁻ progeny phage shows that the conservative phage have acquired the density characteristic for phage grown in the second host (6). (iii) Conservative phage derived from a mixed infection display phenotypic mixing of a phage protein, the v gene product (Fig. 9).

It should perhaps be emphasized here that the failure of a portion of the injected parental DNA to replicate is not unique to r mutants, but rather common under the experimental conditions employed here. The intracellular development of rII⁻ and wild-type phage in *E. coli* B, as measured by the rate and extent of DNA replication and molecular recombination, is indistinguishable (K. Carlson, Ph.D. thesis, Univ. of Pennsylvania, 1971). Unique to cells infected with rII⁻ (or rI⁻) phage is that the nonreplicated DNA is repackaged and released in a newly formed phage particle. We previously interpreted the unusual parent-to-progeny transfer patterns as indicating reduced molecular recombination in this system. However, the results can also be interpreted in terms of defects in maturation and DNA packaging. Since intracellular molecular recombination appears normal (l.c.), this view may be more valid. This might suggest a defect common to rII and rI mutants which allows the encapsulation of various DNA structures that would normally be unacceptable to the phage-assembly apparatus, such as nonreplicated parental

FIG. 12. *Buoyant densities of r⁻ phages. Light ^{32}P-labeled phage (specific activity 1 mCi/mg of P) were prepared in E. coli B23 or CR63 and banded in CsCl together with ^{3}H-labeled light O_1^r phage. A, rI⁻·B; B, rII⁻·CR; rII⁻·B; D, rIII⁻·B.*


23.8
23.3
21.4
A
B
15
10
5
^{3}H
.62
.34
^{32}P
20.9
23.2
C
D
.63
.54
.5
1.0
24.1
23.5
E
NATIVE
.63
.39
BOTTOM
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OF THE GRADIENT

DNA and also DNA fragments. Several fragments may be present in a particle, totalling an approximately normal amount of DNA (this was suggested by the observation [6] of a class of "hybrid" phage resulting from infecting heavy cells with light parental phage, which proved to contain parental DNA in conservative, not hybrid, form), or they may total much less than the normal amount of DNA yielding particles with abnormally low buoyant density (the "CsCl pellet fraction"). The fact that this phenotype is seen only in *E. coli* B with rII mutants, but also in *E. coli* K-12 with the rI mutant (as is rapid lysis with these mutants) suggests that host factors are involved in the expression of the phenotype.

Conservative transfer involves about 10 to 30% of the DNA transferred from the parental phage in cells infected singly or multiply with rI or rII mutants (Fig. 10 and 11; ref. 6). Thus, not only does nonreplicated DNA mature, but it must also code for its own maturation functions. This implies late transcription without concomitant DNA replication, which is not thought to be normal in T4 infection (4, 19, 24, 26, 30; review in 21) although kinetics of RNA synthesis upon shift from nonreplicative to replicative states (using temperature-sensitive mutations in DNA polymerase, for example) (8, 24) suggests that late mRNA may be transcribed from a nonreplicated parental genome.

The reasons for the normally observed requirement for DNA replication during late transcription are not well understood. The two phenomena may be "uncoupled" in mutants deficient in genes 30 (ligase) and 46 (exonuclease?) (27). Deficiency in the rII (or rI) gene may similarly allow an "uncoupling" of replication and late transcription, if for instance the DNA from the infecting phage is inaccessible to the DNA replication enzyme(s), yet accessible to enzymes involved in the transcription and maturation. This inaccessibility could reflect a compartmentation of the cell excluding nonreplicative DNA from enzymes and other proteins coded from replicative DNA. The observation of phenotypic mixing, however, suggests that there is no physical compartmentation in the cells, so if this explanation is correct, "inaccessibility" should reflect conditions of the DNA itself or its immediate surroundings (e.g., the replicative complex). Although the fairly constant ratio of conservative to replicative transfer independent of the multiplicity of infection suggests that the modifications may be permanently "engraved" upon a DNA molecule, predestinating a certain proportion of infecting genomes for conservative transfer, the fact that conservative phage are viable (plaque forming) nevertheless indicates that conservative transfer may affect a random 10 to 30% of the injected molecules.

A different explanation for the observed "uncoupling" is that in normal T4 infection there are low levels of late transcription in the absence of DNA replication, which may not be adequate to be detected in hybridization-competition experiments (4, 19, 26, 30) but sufficient to allow the encapsulation of one or a few phage particles.

A choice between the two alternatives outlined above is not possible on the basis of existing data, but will have to await a better understanding of the controls of transcription and maturation in T4.

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FIG. 13. *Sucrose gradients of DNA from r phages. DNA from the phage preparations illustrated in Fig. 12 was analyzed in alkaline sucrose gradients as described in legend to Fig. 7 together with intact ^{3}H-labeled reference DNA from O_1^r phage. A, rI ·B; B, rII$^-$·CR; C, rII$^-$·B; D, rIII$^-$·B. DNA extracted from the rII$^-$·B phage as described in legend to Fig. 9 was also analyzed in a normal sucrose gradient (panel E). The analyses documented in this and the preceding figure were repeated with different rII mutants (missense and deletions in either or both cistrons), and in the case of the rI mutant several different host strains were used, all with essentially the same results.*

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