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Synthesis of Replicative Form Deoxyribonucleic Acid and Messenger Ribonucleic Acid by Gene IV Mutants of Bacteriophage S13

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Gene IV mutants of bacteriophage S13 are known to be blocked in infectious replicative form (RF) DNA synthesis, producing only a small fraction of the RF formed by wild-type phage. This investigation shows that gene IV mutants form only parental RF and are blocked in the synthesis of any progeny RF, either infectious or noninfectious. This was determined by density labeling of RF in cells treated with mitomycin C to suppress host deoxyribonucleic acid (DNA) synthesis. RF synthesis was also studied in untreated cells, using methylated albumin columns to separate RF from host DNA. In this case it was also found that synthesis of progeny RF by gene IV mutants is negligible. It has been found by DNA-ribonucleic acid (RNA) hybridization experiments that gene IV mutants form at least as much or more messenger RNA than wild-type phage. Therefore, parental RF alone can form messenger RNA in appreciable amounts.

The two closely related single-stranded deoxyribonucleic (DNA) phages ϕX174 and S13 are known to form double-stranded replicative form DNA during phage development (13, 15). Of the seven known genes of phage S13 (14, 15) one, gene IV, has been shown to be involved in the synthesis of infectious double-stranded DNA (15). When suppressible mutants of gene IV are used to infect the nonpermissive host *Escherichia coli* C, synthesis of infectious replicative form (RF) proceeds for about 4 min and then ceases. The amount of RF synthesized by a gene IV mutant is only a small fraction of the total RF made in wild-type infected cells. A block in RF synthesis similar to that found for gene IV mutants can be produced if infection is carried out in the presence of chloramphenicol (100 to 150 μg/ml). It was concluded that the RF made by a gene IV mutant or by wild-type phage in 100 to 150 μg of chloramphenicol/ml is formed by a preexisting host enzyme, whereas the formation of all later RF involves the action of a phage-specified enzyme (15). These experiments measured only infectious RF. The purpose of our experiments was to determine whether the RF formed by gene IV mutants is exclusively parental, or whether any noninfectious progeny RF is formed. A finding of an appreciable amount of noninfectious progeny RF would suggest that the function of the gene IV product is the conversion of noninfectious RF to an infectious state.

This question has recently been investigated by Levine and Sinsheimer (8) for mutants of the homologous cistron VI of phage ϕX174.

In order to study the role of parental and progeny RF in messenger ribonucleic acid (mRNA) synthesis, the amounts of phage mRNA formed after infection by gene IV mutant phage and by wild-type phage were determined by DNA-RNA hybridization experiments.

MATERIALS AND METHODS

Media and reagents. Minimal "light" medium contained per liter: 5.8 gm of Na_2HPO_4, 3.0 gm of KH_2PO_4, 0.5 gm of NaCl, 100 mg of niacin, 1 ml of 1 M $MgSO_4$, 0.1 ml of 1 M $CaCl_2$, 0.1 ml of 10^{-2} M $FeCl_3$, 1.0 gm of glucose, and 1.1 gm of NH_4Cl.

"Heavy" medium is the same as minimal "light" medium except that it contains, per liter, 1.0 gm of ^{13}C-glucose (49.6% isotopic purity; Merck, Sharp and Dohme of Canada, Ltd.) and 1.1 gm of $^{15}NH_4Cl$ (99% purity, Biorad Laboratories) instead of the normal compounds.

Modified M9 medium and tris(hydroxymethyl)-aminomethane (Tris)-glycerol (TG) medium are described by Shleser et al. (12).

Lysing buffer is 0.1 M Tris-hydrochloride, 0.01 M ethylenediaminetetraacetate (*p*H 8.0).

Standard saline-citrate (SSC) is 0.15 M NaCl and 0.015 M sodium citrate.

Bacteria. *E. coli* C, the nonpermissive strain for

S13 *amber* mutants, *E. coli* C AP1, a host cell reactivation-negative derivative of *E. coli* C, and *Shigella dysenteriae* Y6R, a permissive strain for *amber* mutants were used.

Bacteriophage. The gene IV *amber* mutants used were *su*100 and *su*16. The gene V mutant used was *su*N15. Gene V mutants lack the ability to lyse the host but are like wild-type in ability to produce phage. The IV-V double mutant used was *su*100-V.

Preparation of ^{14}C-labeled phage. The permissive host *S. dysenteriae* Y6R was used for the gene IV-V mutant lysate; the nonpermissive host *E. coli* C was used for the gene V mutant lysate. Cells were grown to 5×10^8 per ml in modified M9 medium; deoxyadenosine was then added to a concentration of 150 μg/ml to facilitate incorporation of thymidine into a non-thymidine-requiring strain (1) and ^{14}C-thymidine (Schwarz Bio Research Inc., Orangeburg, N.Y.; specific activity, 53.6 mc/mmole) was added to a concentration of 0.2 μc/ml. $MgSO_4$ was added to a concentration of 10^{-2} M and the phage was added at a multiplicity of infection (MOI) of 10. For the gene IV-V mutant lysate, aeration was continued until foaming had stopped; the lysate was stored overnight in the cold and bacterial debris was collected by centrifuging at 15,000 $\times$ *g* for 15 min at 0 C. The supernatant fluid was discarded. The pellet was suspended in lysing buffer and then lysed with lysozyme at 150 μg/ml by freeze-thawing. Ribonuclease and deoxyribonuclease were each added to a concentration of 50 μg/ml, and the lysed pellet was incubated for 1 hr at room temperature. Residual bacterial debris was removed by centrifugation at 15,000 $\times$ *g* for 15 min, and the supernatant fluid was dialyzed against 0.02 M ammonium acetate (*p*H 7.4). The lysate was again centrifuged at 15,000 $\times$ *g* and the pellet was discarded. For the gene V mutant lysate, aeration was continued for 2 hr and the infected cells were collected by centrifugation. The rest of the procedure was the same as for the gene IV-V mutant lysate.

Mitomycin C treatment. Lindqvist and Sinsheimer (10) have shown that mitomycin C treatment inhibits cellular DNA synthesis while allowing normal phage particle formation if an HCR-negative cell strain is used. This treatment was carried out by us by growing preadapted *E. coli* C AP1 in heavy medium and then adding mitomycin C (Nutritional Biochemicals Corp., Cleveland, Ohio) to a concentration of 50 μg/ml and incubating for 10 min at 37 C. The cells were then harvested by filtration and suspended in heavy medium before infection.

Density labeling of RF. The procedure used for distinguishing parental RF from progeny RF was to infect *E. coli* C AP1 grown in heavy medium with light ^{14}C-thymidine phage in the presence of ^{3}H-thymidine. Under these conditions, parental RF should be of hybrid density and progeny RF should be of heavy density. To the mitomycin C-treated cells in heavy medium, ^{3}H-thymidine (New England Nuclear Corp., Boston, Mass.; specific activity, 11 c/mmole) was added to a final concentration of 3 μc/ml; deoxyadenosine was added to a concentration of 125 μg/ml, $MgSO_4$ to 10^{-2} M. Light, ^{14}C-thymidine-labeled phage was then added; an MOI of 10 was used for the gene IV-V mutant, whereas an MOI of 20 was used for the gene V mutant phage because the radioactivity per infectious particle of the latter lysate was rather low. [It has been found that MOI has no effect on the amount of progeny RF formed up to an MOI of 300 (E. S. Tessman, *unpublished data*).] The cells were incubated at 37 C for 8 min; at this time chloramphenicol (30 μg/ml) was added to the gene V mutant-infected culture but not to the gene IV-V mutant-infected culture. This concentration of chloramphenicol added at 8 min has been shown to stop single-stranded DNA synthesis, but not RF synthesis. At 30 min after infection, both cultures were chilled and were harvested by centrifugation. Any noninjected phage was removed by washing three times in 0.05 M sodium borate-0.003 M ethylenediaminetetraacetate, *p*H 9.5 (7). Lysis was accomplished by freezing and thawing three times in lysing buffer to which lysozyme (100 μg/ml) and ribonuclease (50 μg/ml in SSC, *p*H 5.0, heated for 10 min at 80 C) were added. Most of the remaining protein was removed by digesting for 7 hr at room temperature with 200 μg/ml of self-digested Pronase, followed by three successive phenol extractions. The DNA solution was adjusted to 0.3 M in NaCl and precipitated with 2.5 volumes of 95% ethyl alcohol. The precipitate was collected by centrifugation at 6,000 $\times$ *g* for 15 min in the cold, after which the ethyl alcohol was decanted and the DNA was dried in vacuo. The dried precipitate was then suspended in CsCl solution which was buffered with 0.1 $\times$ SSC.

Ultracentrifugation and fractionation of density-labeled RF. The DNA-CsCl solutions, made up to a density of 1.725 gm/cm^3, were transferred to cellulose nitrate tubes and centrifuged in the SW 39L rotor of the Spinco model L ultracentrifuge at 35,000 rev/min for 60 hr. Four-drop fractions were collected from the bottom of the tubes by using a no. 23 syringe needle. To each fraction, 0.5 ml of 0.05 M Tris-hydrochloride-0.05 M NaCl (pH 8.1) was added, and then 0.1 ml was removed for infectivity determinations. The remaining 0.4 ml was precipitated with 2 ml of 10% cold trichloroacetic acid, filtered on B4 membrane filters (Millipore Corp., Bedford, Mass.), dried, and counted by using a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

Preparation of RF for methylated albumin Celite-kieselguhr (MACK) chromatography. *E. coli* C was grown in TG medium to a concentration of 5×10^8 cells per ml and then infected either with wild-type S13 or with the gene IV mutants *su*16 or *su*100 at an MOI of 5 in the presence of 10^{-2} M $MgCl_2$. Labeling was carried out from 7 to 18 min after infection at 37 C by using either ^{32}P- or ^{3}H-thymidine. The cultures were chilled and the DNA was extracted as described by Shleser et al. (12) and chromatographed three times on MACK by the method of Hayashi et al. (4) as modified by Shleser et al. (12).

DNA-RNA hybridization. DNA for the hybridization experiments was obtained as follows. *E. coli* DNA was isolated by the method of Marmur (11). RF DNA was isolated from cells infected with wild-type S13 by methylated albumin-kieselguhr by the method of Hayashi et al. (4); single-stranded DNA was prepared

from purified wild-type S13 by the method of Eigner et al. (2). All three types of DNA were boiled for 10 min in 0.1 × SSC before hybridization.

Labeled RNA for the hybridization experiments was prepared as follows. *E. coli* C was grown in modified M9 medium to a concentration of 5×10^8 cells per ml and infected with phage at an MOI of 10; ^{3}H-uridine was added from 6 to 11 min after infection at 37 C. The cells were then poured over an equal volume of ice and centrifuged at 8,000 × *g* for 10 min. The pellet was suspended in Tris-Mg buffer (3×10^{-2} M Tris-hydrochloride, *p*H 7.3; 5×10^{-3} M $MgCl_2$) and washed; lysozyme (200 μg/ml) and deoxyribonuclease (30 μg/ml; Worthington Biochemical Corp., Freehold, N.J., electrophoretically purified) were then added. The cells were lysed by freeze-thawing, after which sodium lauryl sulfate was added to a final concentration of 0.2%. The mixture was extracted three times with water-saturated phenol, and then the RNA was precipitated by making the solution 0.3 M in sodium acetate, adding two volumes of ethyl alcohol and allowing it to stand overnight in the cold.

The precipitated RNA was collected by centrifugation and suspended in 2 × SSC. A constant amount of RNA (measured by optical density) was added to a number of DNA samples, each containing a different amount of RF DNA, cell DNA, or SS DNA.

Hybridization was carried out by the method of Hayshi and Hayashi (3). After ribonuclease treatment the hybrid was precipitated with 5% trichloroacetic acid and the amount of ^{3}H-uridine remaining on filters was determined by counting in a scintillation counter.

RESULTS

RF synthesis by gene IV mutants in cells treated with mitomycin C. To determine whether gene IV mutants are blocked in the conversion of noninfectious progeny RF to infectious progeny RF or instead are totally blocked in formation of any progeny RF, it was desirable to suppress synthesis of *E. coli* DNA. This was because infection does not shut off host DNA synthesis until some time after infection (6, 9) so that *E. coli* DNA, as well as any phage-specified DNA, would be labeled during this period of time. *E. coli* DNA synthesis was largely eliminated by treating cells with mitomycin C by the method of Lindqvist and Sinsheimer (10). Mitomycin C treatment of the HCR-negative strain *E. coli* C AP1 severely reduces host DNA synthesis (Fig. 1). However, when treated cells are infected with the gene V mutant, *su*N15 (normal except for ability to lyse cells), total DNA synthesis is approximately the same as for untreated infected cells (latter curve not shown). Figure 1 also shows that total DNA synthesis by treated cells infected with a gene IV mutant is very small, being approximately the same as for uninfected treated cells. However, the measurement of incorporation of radioisotope into total DNA does not permit a comparison of amounts of RF made by the gene IV and the gene V mutants.

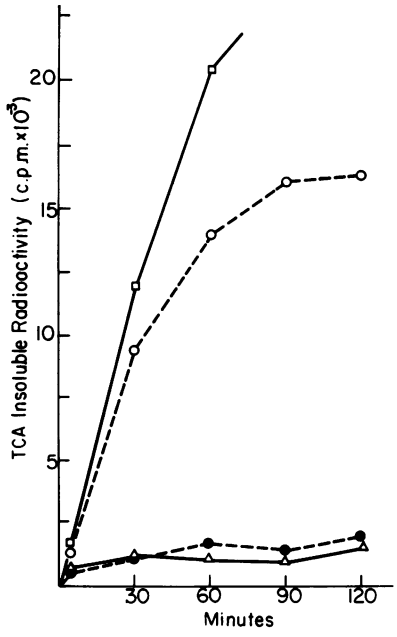

FIG. 1. *Effect of mitomycin C on DNA synthesis in uninfected or S13-infected E. coli C strain AP1. Cells were treated with mitomycin C as described in Material and Methods, harvested, and suspended in minimal medium to which 3 μc of ^{3}H-thymidine per ml (specific activity, 11.2 c/mmole), 2 μg of unlabeled thymidine per ml, and 125 μg of deoxyadenosine per ml were added. Samples were removed at the indicated times, precipitated in cold 10% trichloroacetic acid, and counted. Symbols: △, mitomycin C-treated, uninfected cells; □, untreated, uninfected cells; ○, mytomycin C-treated, gene V-infected cells; ●, mitomycin C-treated, gene IV-infected cells.*

A density-labeling experiment was performed to determine whether a gene IV mutant forms progeny RF. Mitomycin C-treated cells of *E. coli* C suspended in heavy (^{13}C, ^{15}N) ^{3}H-thymidine-labeled medium were infected with light phage labeled with ^{14}C-thymidine. Figure 2 shows the results of equilibrium banding in a CsCl density gradient of DNA extracted from a culture infected with a gene V mutant (Fig. 6a) and one infected with a gene IV-V mutant (Fig. 6b). In the gradient containing the DNA from the cells infected with the gene IV-V mutant, essentially only one species of RF is observed, this being DNA of the intermediate density predicted for a molecule composed of one heavy and one light strand. In contrast, in cells infected with the gene V mutant, the predominant RF has the density expected when both strands of the molecule have incorporated the heavy isotope. The ^{14}C radioactivity marks the region of the hybrid density

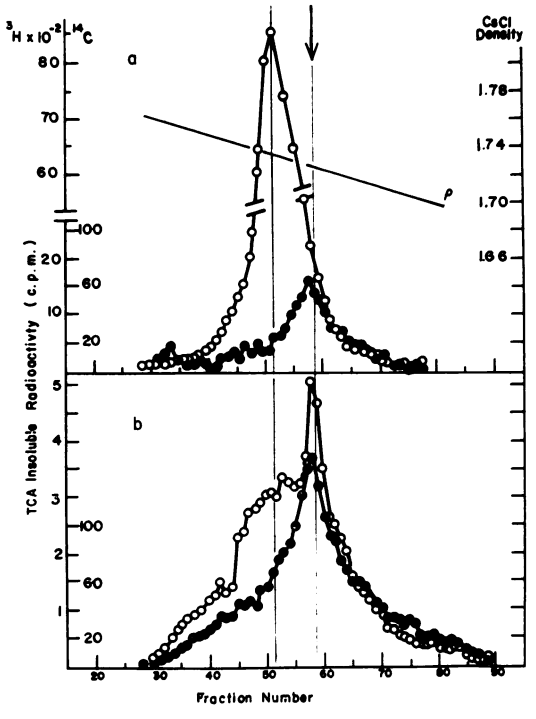

FIG. 2. *E. coli C in heavy medium containing ^{3}H-thymidine was infected with light, ^{14}C-thymidine-labeled phage at an MOI of 10 for the gene IV-V mutant and 20 for the gene V mutant. At 8 min after infection, chloramphenicol was added to a concentration of 30 μg/ml to prevent SS synthesis in the gene V mutant-infected culture; both cultures were then chilled 30 min after infection. Total DNA was extracted and centrifuged to equilibrium in a CsCl density gradient. (a) Infection with the gene V lysis mutant suN15. (b) Infection with the gene IV-V mutant su100. The arrow marks the position of S13 SS marker DNA. Symbols: ●, ^{14}C-thymidine; ○, ^{3}H-thymidine.*

parental RF molecules. The ^{3}H radioactivity marks the region of heavy progeny RF. The maximal amount of progeny RF formed by the gene IV-V mutant is about 0.16 RF molecules for each infecting phage particle. This value is an upper limit, since *E. coli* DNA has the same density as progeny RF (13).

RF synthesis by gene IV mutants in cells not treated with mitomycin C. To determine whether gene IV mutants form progeny RF in cells not treated with mitomycin C, MACK column chromatography was used to separate RF from *E. coli* DNA. This method has the limitation that only RF molecules having the same chromatographic properties as infectious RF would be observed. DNA from cells infected with wild-type S13 in the presence of ^{3}H-thymidine and from cells infected with a gene IV mutant (*su*100) in the presence of ^{32}P were mixed and chromatographed through a series of three MACK columns. Labeling was from 7 to 18 min after infection. This time period was chosen because in cells infected with a gene IV mutant, infectious RF synthesis is completed, under the conditions used, by 7 min. After each passage through a column, the RF-containing fractions were pooled and used for the next chromatography step. The RF region from each column was detected by measurement of infectivity and optical density. The results of the third chromatography step are shown in Figure 3a. The wild-type infected cell forms very much more RF than the gene IV-infected cell. Figure 3b demonstrates the same result for a second gene IV mutant, *su*16, when the isotopes were reversed. Furthermore, if the gene IV mutant DNA curves in Fig. 3a and 3b are corrected for *E. coli* DNA remaining in the RF region after three MACK chromatography steps, the amount of gene IV DNA be-

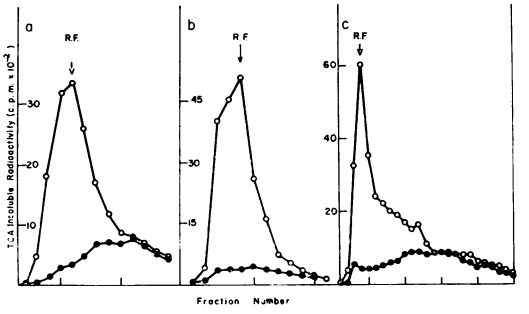

FIG. 3. *MACK chromatography of replicative form DNA from E. coli C infected with wild-type S13 or with gene IV mutant phage. (a) A culture of E. coli C in TG medium was divided in two parts. One part was infected with wild-type S13 at an MOI of 5 and then ^{32}P was added from 7 to 18 min at 37 C. The other part was infected with the gene IV mutant, su100, at the same MOI and labeled with ^{3}H-thymidine for the same time period. The cultures were chilled and mixed; total DNA was extracted, dialyzed, and chromatographed three times on MACK columns by the method of Hayashi et al. (4) as modified by Shleser et al. (12). Results of the last chromatography step are shown. Arrows show the positions of maximal optical density for purified carrier RF. (b) Conditions were the same as for (a), except that the gene IV mutant su16 was used instead of su100, and the isotopes were reversed in the two cultures. (c) Conditions were the same as for (a), except that one culture was left uninfected instead of being infected with gene IV mutant phage. In all cases, ^{3}H and ^{32}P counts were normalized at the region of the E. coli DNA maximum. Symbols: (a) ○, ^{32}P-labeled DNA from wild-type infected cells; ●, ^{3}H-thymidine-labeled DNA from cells infected with the gene IV mutant. (b) ○, ^{3}H-thymidine-labeled DNA from wild-type infected cells; ●, ^{32}P-labeled DNA from cells infected with the gene IV mutant. (c) ○, ^{3}H-thymidine-labeled DNA from wild-type injected cells; ●, ^{32}P-labeled DNA from uninfected cells.*

comes negligible. The extent of the correction can be seen in Fig. 3c, which shows the results of an experiment in which ^{3}H-thymidine-labeled DNA from cells infected with wild-type DNA was extracted and co-chromatographed three times with ^{32}P-labeled, uninfected *E. coli* C.

Synthesis of mRNA. The demonstration that gene IV mutants form no progeny RF suggested that these mutants might make only a small amount of mRNA compared with wild-type-infected cells which contain many times the number of RF templates. To test whether this is true, cells of *E. coli* C were infected with a gene IV mutant and pulsed with ^{3}H-uridine from 6 to 11 min after infection. The RNA made during this period would probably include mRNA of both the virus and the host. The total RNA made in wild-type- or gene IV mutant-infected cells was extracted and added to increasing amounts of three different kinds of DNA. Figure 4 indicates the degree to which the labeled RNA from cells infected with gene IV mutants or with wild-type phage hybridizes with *E. coli* DNA, with RF DNA, or with S13 single-stranded DNA. RNA made in infected cells hybridizes with purified RF as well as with *E. coli* DNA; it does not, however, hybridize with the single-stranded DNA of the virus particle. It is clear from Fig. 4 that cells infected with gene IV mutants form phage-specified mRNA and that the amount formed is greater than that for cells infected with wild-type phage.

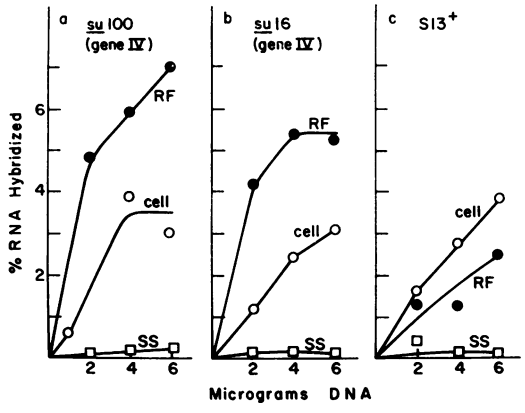

FIG. 4. *Hybridization of labeled RNA from cells infected (a) with the gene IV mutant, su100; (b) with the gene IV mutant, su16; or (c) wild-type S13, as a function of increasing total amount of E. coli DNA, RF DNA, or SS DNA. RNA extraction is described in Materials and Methods; hybridization was by the procedure of Hayashi and Hayashi* (3). *[The ratio of radioactivity to optical density of the purified RNA was determined prior to hybridization. The values of counts per minute per optical density unit were for (a), 400,000; for (b), 550,000; for (c), 210,000.] Each point shown in the figure is the average value from duplicate cultures. Symbols: ●, percentages of counts hybridized to RF DNA; ○, percentage of counts hybridized to E. coli DNA; □, percentage of counts hybridized to viral SS DNA.*

DISCUSSION

We conclude from the results of the density-labeling experiment that a gene IV mutant of phage S13 synthesizes no progeny RF. This experiment required mitomycin C treatment of the host cells to suppress host DNA synthesis, a treatment which permits extensive replication of progeny RF by a gene V mutant.

RF synthesis was also studied in cells that had not been treated with mitomycin C. This was done by using MACK column chromatography, which separates RF from host DNA. This method showed that also in the case of untreated cells there is negligible synthesis of any progeny RF by gene IV mutants. Levine and Sinsheimer (8), using the density-labeling method and mitomycin C-treated cells, recently found a similar result for a mutant of cistron VI of the closely related phage ϕX174.

Thus, the hypothesis is eliminated that the gene IV product might confer infectivity on a species of late RF which is noninfectious.

Gene IV must function to synthesize progeny RF from the template provided by the parental RF molecule. It is not known whether gene IV functions as a DNA polymerase or whether it converts the parental replicative form to a condition in which it may be replicated by another enzyme.

The DNA-RNA hybridization experiments represented in Fig. 4 show that parental RF alone can produce greater than normal amounts of mRNA. This result might be explained if parental RF normally serves as the main template for RNA synthesis. In the case of wild-type infection, there would be a competition between DNA- and RNA-synthesizing enzymes for the parental RF template. In the case of infection with gene IV mutants, the parental RF would be exclusively available as a template for RNA synthesis; therefore, more RNA synthesis might be expected.

These experiments do not determine whether all mRNA species can be made by parental RF. A study of the proteins formed after infection with a gene IV mutant showed that most, and possibly all, of the phage proteins are formed (12*a*). Therefore, most species of mRNA can be formed by parental RF alone.

The lack of hybridization of phage-specified RNA with SS shows further that for S13, just as for ϕX174 (5), most of the phage-specified RNA is synthesized by using the complementary

strand of DNA as a template. This observation verifies for S13 that synthesis of parental RF is obligatory for mRNA synthesis.

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