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Filamentous Bacterial Viruses

VI. Role of fd Gene 2 in Deoxyribonucleic Acid Replication

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Functional gene 2 product was found to be necessary for fd deoxyribonucleic acid (DNA) synthesis throughout the life cycle of the virus. Bacteria which had been infected with a temperature-sensitive gene 2 mutant ceased to make virus-specific DNA when transferred to restrictive conditions at any time after infection, although current rounds of replication were completed.

The deoxyribonucleic acid (DNA) of the F-specific filamentous bacterial viruses (Ff) such as fd and M13 is a single-stranded circular molecule, weighing about 2×10^6 atomic mass units (13). After infection, a complementary strand is synthesized on the viral DNA to give a circular duplex form. This duplex multiplies under the control of viral gene 2, and later produces progeny single strands under the control of viral gene 5. The gene 2 product (2p) is a protein, since *amber* mutations in the gene have been identified. In the absence of 2p, the infecting viral DNA remains predominantly as a covalently closed duplex, form I (16). Gene 2 apparently controls the same function in Ff as gene A does in ϕX174 (6, 7, 9, 21). Synthesis of other viral proteins takes place in the absence of 2p (10, 22), indicating that the effect of 2p on DNA synthesis is specific, and not the consequence of a general effect of 2p on viral proteins.

To study the role of 2p in DNA replication, we have isolated a strain of fd which carries a temperature-sensitive mutation in gene 2. Using this strain, we show that gene 2 function is necessary throughout the life cycle of fd, but, in the absence of gene 2 function, replicating molecules collect as form I. Similar results have been obtained independently by N. Lin and D. Pratt (J. Mol. Biol., *in press*).

MATERIALS AND METHODS

Solutions and media. N1 solution contained 0.5 g of NaCl, 8.0 g of KCl, 0.1 g of $MgSO_4 \cdot 7H_2O$, 12.1 g of tris(hydroxymethyl)aminomethane (Tris), 1 liter of water, 0.087 g of KH_2PO_4, and 0.1 ml of 1 M $CaCl_2$; the *p*H was adjusted to 7.4 with HCl.

N7 medium contained 10 g of tryptone (Difco), 1 g of yeast extract, 8 g of NaCl, 1 g of glucose, 0.5 g of Tris, and 1 liter of water.

N11 medium was the same as N12 except that it contained only 0.023 g of KH_2PO_4 per liter.

N12 medium contained 1 ml of 1 M $CaCl_2$, 5 ml of 0.2 g/ml NH_4Cl, 1 ml of 1 μg/ml $FeCl_3$, 10 ml of 20% glucose, 10 ml of 5% Casamino Acids, and 5 ml of 2 mg/ml thiamine in 1 liter of N1.

N13 medium was the same as N12 but without Casamino Acids.

Tris-EDTA solution was 0.05 M Tris-0.003 M Na_2 ethylenediaminetetraacetate, adjusted to *p*H 8.1 with HCl.

SSC was 0.15 M NaCl-0.015 M sodium citrate, *p*H 7.0.

PM (preincubation medium) contained 0.2% Ficoll (molecular weight, 400,000), 0.02% polyvinylpyrrolidone (molecular weight, 3,600), 0.02% bovine serum albumin (BSA), and 0.05% sodium dodecyl sulfate (SDS).

Chemicals. Chloramphenicol was from Calbiochem, and was dissolved in N1 at a concentration of 2 mg/ml. Pronase was from Calbiochem, and was dissolved in 0.02 M NaH_2PO_4 at a concentration of 10 mg/ml. A precipitate which formed after storage at 0 C was removed by low-speed centrifugation. Mitomycin C was from Nutritional Biochemicals Corp.; lysozyme, BSA, and polyvinylpyrrolidine, from Sigma Chemical Co.; Ficoll, from Pharmacia; Sarkosyl NL97, from Geigy Chemicals; tryptone, yeast extract, and Casamino Acids (vitamin-free), from Difco; and thymine-methyl-^3H, thymidine-methyl-^3H, thymine-2-^{14}C, and ^{32}P-orthophosphoric acid (carrier-free), from New England Nuclear Corp.

Strains of Escherichia coli. Strain DM48 (Sup^-) is a λ^- derivative of strain S26, HfrC (11). Strain DM49 is a λ^- derivative of S26RIe (8), $Su\text{-}1^+$, obtained from R. C. Wilhelm. Strain DM130 is strain M93*A2(P)* (23), obtained via M. Oeschger; it has an *amber* mutation of the *trp* gene, and carries a temperature-sensitive $Su\text{-}3^+$ suppressor, so that it grows at 32 C but not at 42 C in the absence of tryptophan. Strain DM145 is an F'lac derivative of HF4704, an *E. coli* C strain which carries a mutation in the *uvrA* gene, and requires 2 μg of thymine/ml for growth. To obtain DM145, strain DM70 (11), which grows at 32 C in the absence of thymine, was further selected by use of trimethoprim (24). DM70 was streaked on N13 agar

plates containing 10 μg of trimethoprim/ml and 50 μg of thymine/ml, and was incubated at 32 C. A Thy$^-$ colony was selected and restreaked at 32 C on N12 plates containing 5 μg of thymine/ml. Colonies were then streaked on N12 plates containing 50 μg of thymidine/ml to select a *drm*$^-$ strain (2). This strain (DM145) requires thymine at both 32 and 42 C.

Strains of virus. Wild-type fd was used as type strain. The plating efficiency of fd was the same on DM48 and DM145, independent of which strain was used to prepare the virus stock, so there was no restriction between *E. coli* C and *E. coli* K-12.

Strain fd24 (13) carries an *amber* mutation in gene 5, and is referred to in this paper as *am5*.

Strain fd106 carries a temperature-sensitive mutation in gene 2, and is referred to in this paper as *ts2*. It is derived from strain fd18 (13), which carries an *amber* mutation in gene 2. To isolate this strain, a stock of fd18 was grown on a soft-agar layer in a suppressing host (DM49) at 32 C. This stock was then plated on DM48 (Sup$^-$) at 32 C, and single plaques were picked and tested for plaque-forming ability at 32 and 42 C. A clone with a reversion frequency (titer at 42 C to titer at 32 C) of 3×10^{-5} was selected and numbered fd106. The fact that the temperature-sensitive lesion is in gene 2 was confirmed by complementation tests (17).

Strain fd150 is a temperature-resistant derivative of fd18. The titer of fd18 is lower at 42 C than at 32 or 37 C (by 10^{-2} to 10^{-3}). Growth in liquid culture is also depressed at 42 C. Therefore, we selected a derivative (fd150) of fd18 which grows equally well at 42 and 32 C. Strain fd150 is referred to in this paper as *am2*.

Preparation of radioactive virus and DNA. Radioactive fd was prepared (26) by growing fd for 3 hr in N11 medium containing 0.08 mCi of ^{32}P-orthophosphate/ml, and purifying with a Sephadex G-100 column followed by equilibrium centrifugation. Marker viral DNA was extracted from ^{32}P-fd by phenol extraction (14).

Treatment of bacteria with mitomycin C. Strain DM145 was grown in medium N12 plus 5 μg of thymine/ml at 37 C to 2×10^8 colony formers/ml. The bacteria were centrifuged at room temperature in a Sorvall SS-1 centrifuge at 3,000 rev/min for 5 min, and gently resuspended in 0.2 volume of N12 containing 2 μg of thymine/ml. Mitomycin C was added to give 50 μg/ml, and the culture was incubated at 37 C without aeration or light for 10 min (12). The bacteria were washed twice by centrifugation with 1 volume of N1, resuspended in 1 volume of N12 plus 2 μg of thymine/ml, and incubated further at 32 C (unless otherwise indicated) for 2 min. The bacteria which had been treated with mitomycin C (MC-bacteria) were then infected with the appropriate fd strain at a multiplicity of 500 plaque-forming units (PFU)/bacterium. The time of addition of fd was zero on the time scale in all experiments. Incubation was continued at 32 C (the bacteria were grown at 37 C before infection to increase the fraction of infected bacteria).

In one experiment, MC-bacteria which were resuspended after mitomycin C treatment in the original growth medium were found to synthesize viral DNA at a rate only two-thirds that of a parallel culture which was resuspended in fresh N12 plus thymine.

E. coli cells which have not been treated with mitomycin C are referred to as "untreated bacteria."

MC-bacteria do not make appreciable amounts of DNA unless they are infected (11, 12, 16). In our hands, uninfected MC-bacteria synthesized an amount of DNA equivalent to less than five duplex molecules per bacterium over 60 min at 32 C. Increasing the incubation temperature to 42 C doubled this background rate of synthesis.

Several experiments indicated that viral DNA replication in MC-bacteria is not abnormal. After infection of MC-bacteria, viral DNA was synthesized in much the same pattern and amount as after infection of untreated bacteria (11). The eclipse period was also similar in treated and untreated bacteria, although the total yield of virions was reduced. Infected MC-bacteria continued to synthesize proteins up to 1 hr after infection. However, the addition of 180 μg of chloramphenicol/ml to the bacteria reduced the rate of protein synthesis to a few percent of the normal value, when chloramphenicol was added either at the time of infection or at some later time.

Changing temperature. When a change in the volume of the culture was undesirable, the change in temperature was effected by transferring the culture to a large flask equilibrated at the new temperature. Measurement of the change in temperature with a thermistor showed that the culture was within 0.5 C of the new temperature within 0.5 min after the transfer. Constant volume was less important when the DNA was to be analyzed by centrifugation of bacterial extracts. In such experiments, an increase in temperature was effected by diluting 1:5 into medium previously equilibrated at a temperature slightly higher than the desired final temperature, so that the final temperature was reached as fast as mixing took place.

Cultures were incubated in a gyratory water-bath shaker.

Measurement of DNA synthesis. Samples of 0.1 ml for measuring the amount of ^{3}H-thymine incorporation were collected on 2.4-cm glass-fiber filters (GF/A) which had been previously mounted on steel pins, soaked with about 0.1 ml of 1 N NaOH, and dried. Tests showed that incorporation of ^{3}H-thymine into DNA stopped at once on the filter. The filters were removed from the pins and spread between successive layers of Whatman no. 1 paper in a Büchner funnel. The filters were washed with 10% trichloroacetic acid followed by acetone, dried and placed in vials with 3 ml of toluene scintillator; radioactivity was measured in a scintillation counter.

The counting efficiency of ^{3}H on GF/A filters was 0.15 counts per min per disintegration per min under our conditions. There are about 3,800 thymine nucleotides per duplex fd DNA molecule (13). Therefore, at a specific activity of 10 μCi per 2 μg per ml, a bacterial concentration of 2×10^8/ml, and with 0.1-ml samples, there were 16 duplex replicative forms (RF) per bacterium for every 10^3 counts/min measured. This assumes that all bacteria are infected; if they are not, the number of RF per infected bac-

terium is higher. The eclipse period in MC-bacteria at 32 C was about 30 min. For experiments extending beyond this time, the number of "RF equivalents" included DNA incorporated into progeny intracellular viral DNA or released as free virions. Incorporation of radioactive label increased even when the label was added at the time of infection, so net synthesis and not turnover was being measured.

The rate of DNA synthesis was measured essentially as described previously (11). A 1-ml sample of bacteria was added to 2 μCi of ^{3}H-thymidine. After 1 min, 5 ml of iced 10% trichloroacetic acid was added; samples were collected on 2.4-cm Whatman GF/A filters, washed with trichloroacetic acid and acetone, dried, and counted.

Analysis by centrifugation. The incorporation of radioactive material was stopped by pipetting the culture into a large flask immersed in a dry ice-acetone mixture. The bacteria were thawed in the presence of 0.01 M KCN, centrifuged at 4 C in a Sorvall SS-34 centrifuge for 10 min at 5,000 rev/min, washed once with Tris-EDTA, and resuspended in Tris-EDTA at a concentration of no more than 5×10^9 bacteria/ml (in 0.2 ml for small gradients and 0.6 ml for large gradients). Then 0.1 volume of 0.1 M EDTA, *p*H 8.1, was added, and the sample was mixed with a Vortex mixer. Lysozyme solution was added to give 100 μg/ml and the mixture was incubated for 15 min at 0 C. Then enough 10% Sarkosyl NL97 was added to give 0.1%. Pronase solution was added to give a final concentration of 0.5 mg/ml. The sample was incubated for 2 hr at 37 C, and layered on the gradient.

Neutral high-salt gradients were 5 to 20% (w/v) linear sucrose gradients containing 0.5 M NaCl in Tris-EDTA. Alkaline high-salt gradients were 5 to 20% (w/v) linear sucrose gradients containing 0.3 N NaOH, 0.7 M NaCl, and 0.003 M EDTA. Since the sedimentation coefficient of single-stranded DNA is more sensitive to salt concentration (25) than to *p*H above 12.4 (27), we added the same amount of NaOH to the 20% sucrose solution as to the 5% sucrose solution, even though this gave a final *p*H of 12.8 for the 5% solution and 12.6 for the 20% solution. The 5 and 20% alkaline solutions were freshly mixed for each gradient from stocks of (i) 10 and 40% sucrose in 0.7 M NaCl-0.003 M EDTA, (ii) 3 N NaOH, and (iii) 0.7 M NaCl-0.003 M EDTA.

Gradients were centrifuged either in a Spinco SW 50.1 rotor, or in an SW 27 rotor when individual fractions were to be analyzed further. Samples were dropped from the bottom of the gradient, either directly onto GF/A filters, or into Analocups (Aloe Scientific) containing 50 μg of denatured salmon sperm DNA in 0.1 ml. In the latter case, 0.1 ml of each fraction was placed on GF/A filters. The filters were washed with 10% trichloroacetic acid and acetone, they were dried, and the radioactivity was measured.

Samples which were to be analyzed further by alakaline centrifugation were pooled from the Analocups, 0.1 volume of 3 M sodium acetate, *p*H 5, and 3 volumes of ethanol were added, and the sample was precipitated overnight at -20 C. The precipitate was collected by centrifugation at 15,000 rev/min for 30 min in a Sorvall SS-34 centrifuge at 4 C, and was resuspended in 0.2 ml of 0.3 N NaOH.

The position of DNA in the gradients was defined by the position of known markers, either internal or in a parallel gradient. Form I is the supercoiled viral duplex DNA with both strands covalently closed. Form II is the relaxed circular duplex, with at least one strand nicked; VS is the single-stranded viral DNA; and CS is the complementary strand. Radioactivity at "the position of form I," etc., refers to radioactivity which has the sedimentation coefficient of the marker in the experiment under discussion, but may not be identical to the marker when further characterized. In neutral high-salt velocity gradients, the order of sedimentation coefficients was VS > form I > form II. In alkaline high-salt velocity gradients, the order of sedimentation coefficients was form I > circular single-strand > linear single-strand.

Radioacitivity in dual-label experiments was corrected for spill from the second channel.

DNA-DNA hybridization. DNA-DNA hybridization on membrane filters was carried out by a modification of the Denhardt (4) procedure. Form I was isolated (20) and gamma-irradiated in a ^{60}Co source to create two to three single-strand nicks. Viral reference DNA was isolated by phenol extraction (14) from purified virions, and irradiated. Reference and input DNA were heated to 95 C for 10 min and then transferred to an ice bath to separate the strands of form II. The denatured reference DNA (5 μg of duplex DNA or 2.5 μg of viral DNA in 5 ml of 6 × SSC) was collected on 2.4-cm B-6 membrane filters (Schleicher & Schuell Co.), washed twice with 6 × SSC, and dried overnight in a desiccator followed by 6 hr at 80 C and 30 mm of Hg pressure in a vacuum oven. The dry reference filters of various types were stacked in 2.5-cm scintillation vials containing 0.7 ml of PM, and were incubated for 6 hr at 65 C. The input DNA, dissolved in 3 × SSC (irradiated and denatured) was added to the vials, and incubation was continued for a further 12 hr. The filters were washed on both sides with 6 × SSC and then with 0.001 M Tris, *p*H 9 (28). Less than 0.2% of the input radioactivity was found on a blank.

RESULTS

Requirement for 2p in DNA replication. To study the effect of inactivating gene 2 function on DNA replication, parallel cultures of MC-bacteria were infected with fd and with *ts2* (fd106) at 32 C in the presence of ^{3}H-thymine. At 20 min after infection, the cultures were shifted to 42 C. Incorporation of ^{3}H-thymine stopped immediately after the temperature of the *ts2* culture was raised, but incorporation was not affected by raising the temperature in the fd-infected culture (Fig. 1).

Incorporation of short pulses of ^{3}H-thymidine is a more sensitive measure of rate than is change in total incorporation. The amount of ^{3}H-thymidine incorporated during 1-min pulses at 32 C in MC-bacteria after infection with *ts2*

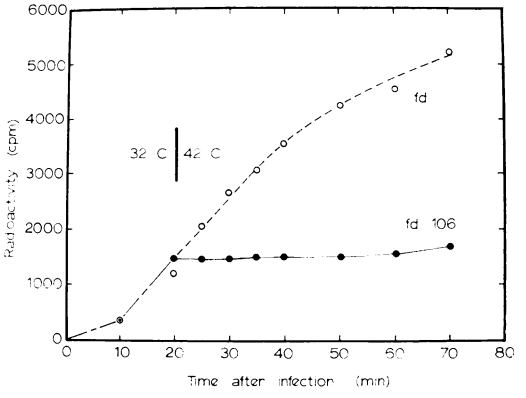

FIG. 1. *Effect of transfer to 42 C on synthesis of viral DNA. Two parallel cultures of mitomycin C-treated bacteria were infected with fd or ts2 (fd106) at 32 C. At the time of infection, 10 μCi per 2 μg of ^{3}H-thymine per ml was added. At 20 min after infection, the flasks were transferred to a water bath at 42 C and incubated further. Samples were taken to measure incorporation of radioactivity. Infected with fd, ○; infected with ts2, ●.*

(Fig. 2) followed the pattern previously observed for wild-type fd at 37 C (11): a rise in rate until about the end of the latent period, followed by a slow decrease in rate. When the temperature was raised to 42 C, incorporation of ^{3}H-thymidine decreased abruptly with a half-time of about 1 min. This was true both at a time after infection (12 min) when most DNA being synthesized was duplex DNA, and also at a time after infection (35 min) when most DNA being synthesized was single-stranded DNA. We conclude that 2p is required for synthesis of both duplex DNA and single-strand DNA.

Studies on the nature of the ts2 mutation. The temperature sensitivity of *ts2* could be due to a failure to make requisite new 2p at 42 C, or it could be due to an inactivation of existing 2p at 42 C. In the former case, inhibitors of protein synthesis such as chloramphenicol should also stop DNA synthesis. In fact, adding chloramphenicol at 32 C stopped DNA synthesis much more slowly than raising the temperature to 42 C (Fig. 3). Failure of chloramphenicol to inhibit Ff DNA synthesis after established infection has been observed by others (3). If possible secondary effects of chloramphenicol are discounted, this experiment suggests that the temperature sensitivity of *ts2* is due to inactivation of 2p at the non-permissive temperature, and not to failure to make new 2p.

We have also studied this question using a bacterial strain carrying a temperature-sensitive Su-3$^+$ suppressor, infected with *am2*. We expected that, although new 2p would not be made after the transfer to 42 C, preexisting 2p would be intact and would continue to promote DNA synthesis. Since no other proteins should be affected, in contrast to the chloramphenicol experiment of Fig. 3, we expected that new virions would continue to be made. In fact, transfer to 42 C stopped production of new *am2* virions at once, even though some new *ts2* virions were produced in a parallel culture (Fig. 4). The

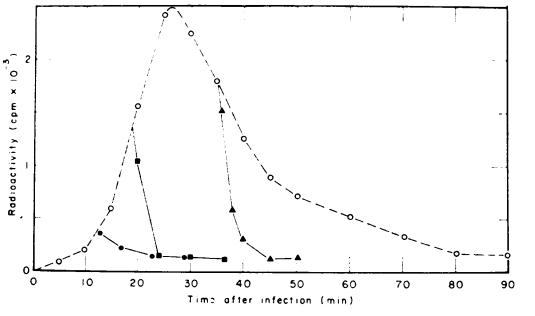

FIG. 2. *Synthesis of viral DNA after transfer to 42 C. Mitomycin C-treated bacteria were infected with ts2 at 32 C. At 12 min (●), 19 min (■), and 35 min (▲) after infection, portions of the culture were transferred to 42 C. Samples were taken at intervals to measure the amount of ^{3}H-thymidine incorporated in 1 min. Radioactivity at 32 C, open symbols; at 42 C, filled symbols.*

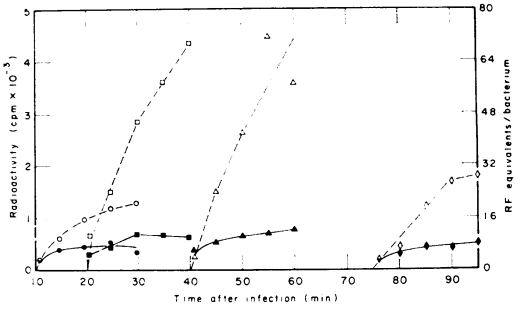

FIG. 3. *Synthesis of viral DNA after transfer to 42 C or to chloramphenicol at 32 C. Mitomycin C-treated bacteria were infected with fd106 at 32 C. At 10 min (circles), 20 min (squares), 40 min (triangles), and 75 min (diamonds), portions were either transferred to 42 C (filled symbols) or transferred into 180 μg of chloramphenicol/ml at 32 C (open symbols). At the time of transfer, 10 μCi per 2 μg of ^{3}H-thymine per ml was added, and samples were taken at successive times to measure incorporation of radioactivity. A portion of the same infected culture was incubated from the time of infection with ^{14}C-thymine at 32 C, and samples were taken at 10, 20, 40, and 75 min for analysis by alkaline velocity centrifugation. About half of the radioactivity had the sedimentation coefficient of form I, and the remainder had the sedimentation coefficient of viral DNA.*

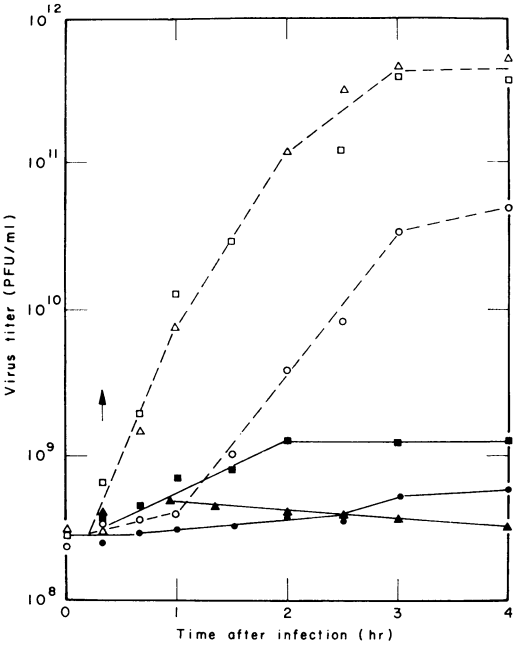

FIG. 4. *Growth of amber virus in a host carrying a temperature-sensitive suppressor. Strain DM130 was grown at 32 C in medium N7 supplemented with 20 μg of tryptophan/ml to 2 × 10⁸ colony formers/ml, and divided into three portions, which were infected with 2 × 10⁸ PFU/ml of am5 (circles), ts2 (squares), or am2 (triangles). After 20 min at 32 C, half of each culture was transferred to 42 C (filled symbols); the remainder was incubated further at 32 C (open symbols). Samples were taken at intervals to measure virus titer, with DM49 as indicator; the plates were incubated at 37 C for am2 and am5, and at 32 C for ts2. The arrow indicates the time of transfer to 42 C.*

difference between *am2* and *ts2* may reflect a reduced pool of 2p in the *am2* culture, due to poor suppression: in a similar experiment, in which growth of fd continued for 40 min after infection before transfer to 42 C, as many *am2* as *ts2* virions were released, but still fewer than 10 per bacterium.

This experiment is inconsistent with the simple interpretation of Fig. 3, that 2p is needed only for DNA synthesis, but new synthesis of 2p is not necessary for continued DNA synthesis. Controls, in which a temperature-insensitive Su-3⁺ strain of bacteria infected with *am2*, or temperature-sensitive bacteria infected with wild-type fd, were carried through the same temperature shift, gave normal virus yields. This indicates that the effect is directly due to inactivation of the suppressor function. Perhaps chloramphenicol has secondary effects on DNA synthesis (19), or perhaps 2p is needed for some viral function in addition to DNA synthesis.

Surprisingly, new synthesis of virions was also prevented by stopping new synthesis of gene 5 product (5p), about 10^4 to 10^5 molecules of which are present in the bacterium (1, 10, 15). Apparently there are limitations on the reuse of 5p at 42 C, perhaps related to its reduced ability to bind to DNA at this temperature (1).

Reversibility of the inactivation of 2p. To study the possibility that 2p synthesized at 42 C might recover its activity at 32 C, MC-bacteria were infected with *ts2* at 42 C for various lengths of time, and were then transferred to 32 C with or without chloramphenicol (Fig. 5). When the bacteria were transferred into chloramphenicol at 32 C at the time of infection, no DNA was made.

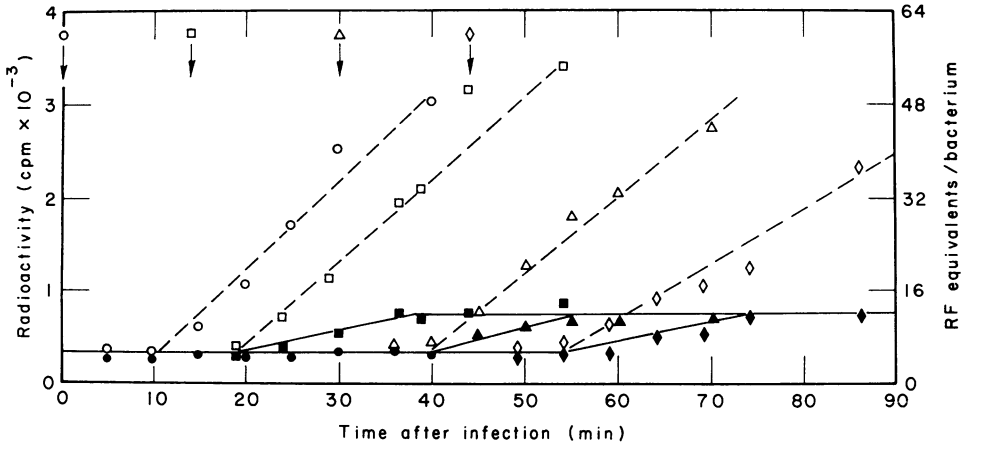

FIG. 5. *Synthesis of viral DNA after infection at 42 C. Mitomycin C-treated bacteria were infected with ts2 at 42 C. At 0 min (circles), 14 min (squares), 30 min (triangles), and 44 min (diamonds), portions were transferred to 32 C, either with (filled symbols) or without (open symbols) 180 μg of chloramphenicol/ml. At the time of transfer, 10 μCi per 2 μg of ³H-thymine per ml was added, and samples were taken to measure incorporation of radioactivity. The arrows indicate the times of transfer.*

However, if the bacteria were incubated at 42 C after infection, and then transferred into chloramphenicol at 32 C, about 5 to 10 duplex equivalents of ^{3}H-thymine were incorporated. This suggests that 2p which is synthesized at 42 C can become functional after transfer to 32 C. However, an increase in the length of the prior incubation at 42 C did not cause a corresponding increase in the amount of ^{3}H-thymine incorporated after transfer to chloramphenicol at 32 C. In contrast, an increase in the length of prior incubation at 32 C did cause a corresponding increase in the amount of ^{3}H-thymine incorporated after transfer to chloramphenicol (Fig. 3).

Fate of DNA after transfer to 42 C. The fate of viral DNA molecules which were synthesized before a transfer to 42 C was studied by adding radioactive DNA precursors to *ts2*-infected MC-bacteria, and examining extracts of bacteria after transfer to 42 C by velocity gradient centrifugation (Fig. 6). The fd DNA present in the bacteria at 60 min after infection, labeled by adding ^{14}C-thymine at the time of infection, corresponded to about 30 single-strand molecules and 100 duplex molecules, of which 15 were found at the position of form II and 85 at the position of form I (Fig. 6b, 0 min). By 10 min after a transfer to 42 C, the ^{14}C-thymine was virtually entirely at the position of form I (Fig. 6b, 10 min). Alkaline velocity gradient centrifugation analysis of the material at the position of form I confirmed that this was indeed form I (Fig. 6d, 10 min). At 10 min after infection, no clear peaks of ^{14}C radioactivity were seen in the gradients, indicating that there were fewer than five RF equivalents of viral DNA per bacterium.

The DNA molecules which incorporate a 1-min pulse of ^{3}H-thymidine are enriched for those which have either just replicated or are in the act of replication. These molecules were roughly equally distributed between the position of form I and the position of form II in the gradient before transfer to 42 C (Fig. 6a, 0 min; Fig. 6b, 0 min), but only 60% of the material at the position of form I in the neutral gradients was found still to sediment as form I in alkaline gradients (Fig. 6c, 0 min; Fig. 6d, 0 min). Therefore, only 25% of the material made during a 1-min pulse is form I, either at 10 min or at 60 min after infection. The material which sediments as form I in neutral but not in alkaline gradients may be in part rehlicative intermediates (26).

During the 10 min after transfer to 42 C, the DNA made just before transfer disappeared from the position of form II and collected at the position of form I. This was true for transfer to 42 C at either 10 or 60 min after infection (Fig. 6a and 6b). Although the radioactive thymidine remained in the culture after transfer to 42 C, no further incorporation into form II was found.

From analysis of the amount of ^{14}C-thymine incorporated after transfer to 42 C, we have estimated how many rounds of replication may take place after this transfer. During the 10 min after transfer to 42 C at 60 min after infection, about 10 single-strand equivalents per bacterium were synthesized. This is comparable to the 15 form II molecules per bacterium which are generating new single strands at this stage of infection (18). Therefore, we assume that each form II goes through one round of replication after the transfer to 42 C. This idea is supported by the fact that the increase in ^{3}H radioactivity between 0 and 10 min in Fig. 6b is comparable to the amount of ^{3}H radioactivity found at 0 min, at the end of the 1-min pulse. If the amount of ^{3}H-thymidine incorporated during 1 min is taken as a measure of the number of replicating molecules (26), then each molecule replicates once after transfer to 42 C.

Nature of the DNA synthesized after transfer to 42 C. The residual synthesis of DNA after transfer of *ts2* to 42 C was examined directly by adding ^{3}H-thymidine at 5 min after transfer. Most of the radioactivity was found in form I, for transfer at either 15 or 60 min after infection.

The radioactivity found in form I after a transfer to 42 C at 60 min after infection was approximately equally distributed between VS and CS (Table 1). The symmetrical labeling may reflect preferential depression of single-strand synthesis after transfer of *ts2* to 42 C. These experiments do not eliminate the possibility that a low level of residual synthesis of viral DNA continues indefinitely after a shift-up of *ts2*, but any such synthesis is less than 5% of the normal viral DNA synthesis.

DISCUSSION

Functional gene 2 protein is necessary for viral DNA replication throughout the life-cycle of fd, not only for duplex DNA synthesis, but also for progeny single-strand DNA synthesis. Therefore, the two types of DNA synthesis share some common mechanism, mediated by 2p.

When 2p function is inhibited, replicating DNA collects as form I, rather than as replicative intermediates or other open forms. This suggests that 2p may not be involved in the completion of partially replicated molecules, but instead is involved in initiation of new rounds of replication. However, even bacteria infected with wild-type fd are enriched in form I after a transfer to 42 C (N. Lin and D. Pratt, *personal communication*).

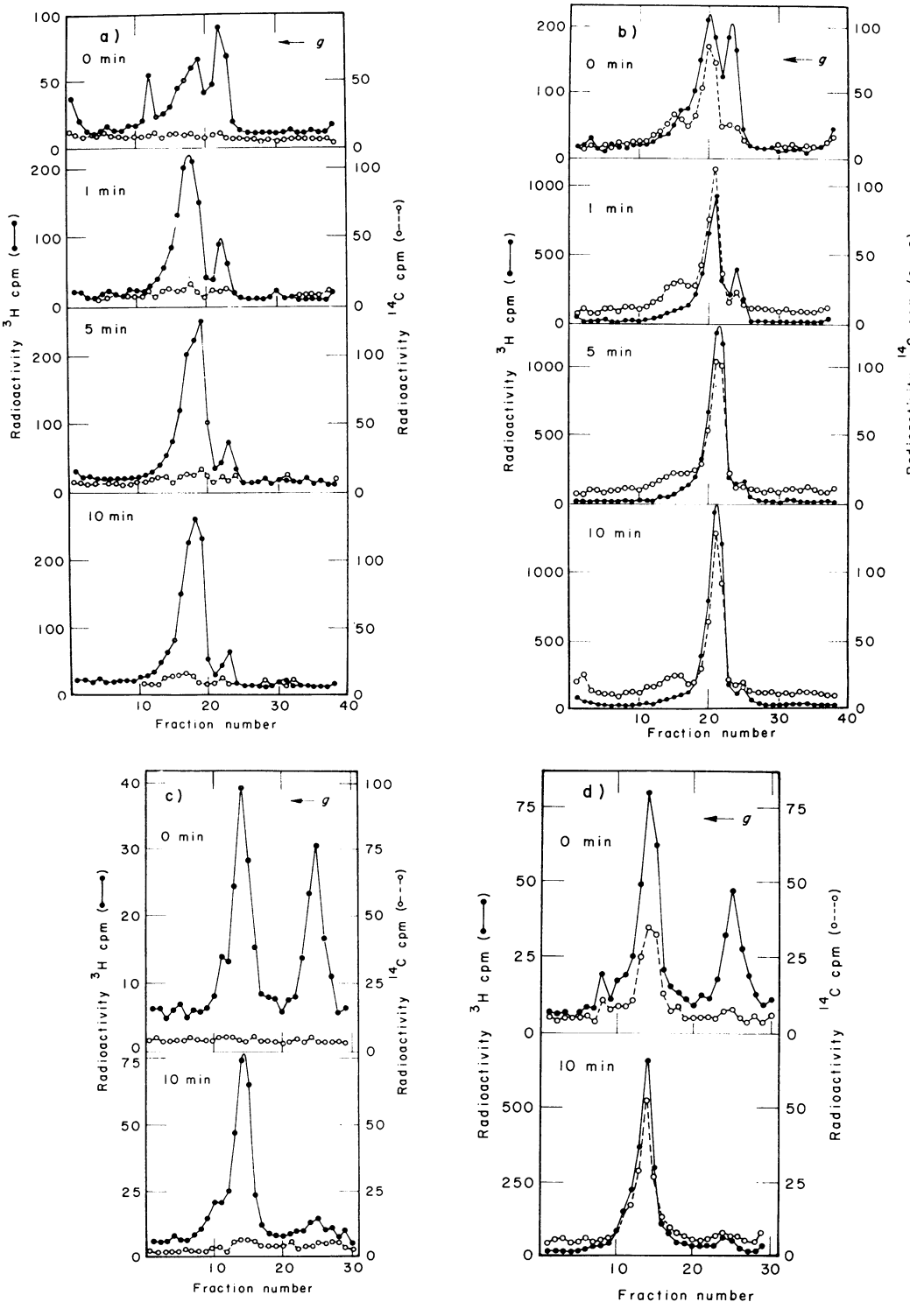

FIG. 6. *Analysis of the DNA synthesized before transfer to 42 C. Mitomycin C-treated bacteria were infected with ts2 at 32 C and labeled with 2 μCi per 2 μg of* ^{14}*C-thymine per ml at the time of infection. At 10 min (a) and 60 min (b) after infection, a portion of the culture was added to* ^{3}*H-thymidine (10 μCi/ml), and after 1 min was diluted fivefold into N12 at 42 C. Samples for neutral high-salt velocity centrifugation were taken at 0, 1, 5, and 10 min after transfer to 42 C. Incorporation was stopped with dry ice-acetone, and the bacteria were washed, lysed, and centrifuged at 23,000 rev/min for 15 hr at 4 C in a Spinco SW 27 rotor. Fractions of 1 ml were collected, and 0.1 ml of each fraction was dried onto a GF/A filter and counted. The form I region of each of these gradients was pooled and precipitated with sodium acetate and ethanol. The precipitate was collected by centrifugation, dissolved in 0.2 ml of 0.1 N NaOH, and analyzed by alkaline high-salt velocity centrifugation at 40,000 rev/min for 1 hr at 17 C in a Spinco SW 50.1 rotor. (c) Fractions 17 to 19 from gradient a, 0 min, and fractions 17 to 19 from gradient a, 10 min. (d) Fractions 19 to 21 from gradient b, 0 min, and fractions 20 to 22 from gradient b, 10 min.* ^{14}*C-thymine, ○;* ^{3}*H-thymidine, ●.*

TABLE 1. *Analysis of the viral DNA synthesized after shift-up*[a]

Reference DNA	Input DNA			
	Pulse		Continuous	
	Counts/min	Percent input	Counts/min	Percent input
Viral single strand	159	26	473	14
Duplex	240	40	753	23
Viral/duplex		0.66		0.63

[a] Mitomycin C-treated bacteria were infected with *ts2* at 32 C and labeled with 2 μCi per 2 μg of ^{14}C-thymine per ml at the time of infection. At 60 min after infection, the culture was transferred to 42 C, and 5 min later ^{3}H-thymidine (10 μCi/ml) was added. Incorporation was stopped after 5 min; the bacteria were washed, lysed, and centrifuged in a high-salt neutral gradient at 23,000 rev/min for 15 hr at 4 C in an SW 27 rotor. Most of the radioactivity, both ^{14}C and ^{3}H, sedimented at the position of form I. The fractions in this region were pooled, and the DNA was precipitated with ethanol and analyzed by DNA-DNA hybridizing. The percentage of the radioactivity (percent input) in the input DNA added to the incubation vials which were found attached to the filters after annealing and washing, as well as the absolute radioactivity, is shown.

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