JOURNAL OF VIROLOGY, June 1977, p. 619-625

Vol. 22, No. 3
Printed in U.S.A.

# In Vitro System That Synthesizes Circular Viral DNA of Bacteriophage φX174

RYOZABURO MUKAI AND MASAKI HAYASHI*

*Department of Biology, University of California, San Diego, La Jolla, California 92093*

Received for publication 21 December 1976

An extract prepared from *Escherichia coli* cells infected with φX174 bacteriophage was capable of incorporating dTTP into phage-specific DNAs in vitro. The synthesized DNAs were associated with proteins and sedimented with *S* values of 20, 50, and 90 in a sucrose gradient sedimentation. DNA isolated from 20S material was open circular replicative form (RF), DNA in 50S material was replicative-form DNA with an extended single-stranded viral DNA that ranged up to one genome in length, and DNA in 90S material consisted of circular and linear single-stranded viral DNA of full genome length and single-stranded viral DNA shorter than full genome length. Pulse and pulse-chase experiments indicated that 90S material derived from 50S material.

Three different modes of phage-specific DNA synthesis have been identified in *Escherichia coli* cells infected with circular, single-stranded (SS) DNA bacteriophage φX174 (2, 14). Immediately after its penetration into the host cell, the circular SS DNA is converted to a double-stranded form (parental replicative form [RF]). The parental RF replicates semiconservatively to produce a number of progeny RF molecules. Finally, SS viral DNA is synthesized from RF molecules.

A particularly interesting feature of φX174 morphogenesis is that production of intact SS viral DNA depends upon the functions of seven phage-coded genes including those specifying virion structural proteins F, G, and H and nonvirion protein genes A, B, C, and D. The only known phage gene that is not involved in the maturation process is gene E (lysis).

An intermediate of viral DNA synthesis was described previously (4, 8, 10, 15). It consists of an RF molecule with an extended tail of SS DNA (rolling circle of sigma [σ] structure [3]). The length of viral DNA in the σ structure ranges from one to two genomes. Recently, Fujisawa and Hayashi found a DNA-protein complex that is a precursor for mature phage in cells infected with wild-type phage (5, 6). The complex sedimented with an *S* value of 50 in sucrose gradient centrifugation (50S complex) and contained the σ structure DNA and viral-coded proteins A, A* (smaller gene A protein [11, 12]), D, F, G, H, and J and several host proteins. They showed that (i) elongation of viral DNA occurred in the 50S complex, and (ii) cleavage of viral DNA into unit length from the σ structure occurred in the 50S complex with the action of the gene A protein.

We found that an extract prepared from infected cells was capable of synthesizing circular SS viral DNA in vitro. The system mimics the mode of SS DNA synthesis in vivo and would be a useful system for elucidation of the morphogenetic pathway of the phage.

## MATERIALS AND METHODS

**Preparation of cell extract.** *E. coli* H570-22 (a *thyA* derivative of H570 [F⁻ *tsx*ʳ λˢ φXˢ Strʳ *polA1 endA rna*] was grown at 37°C in tryptone-KCl medium (1% tryptone, 0.5% KCl) supplemented with 10 mM potassium phosphate buffer ($KPO_4$, pH 7.3), 0.5% glucose, and 5 μg of thymidine per ml to an absorbancy at 660 nm of 0.8. $MgCl_2$ and $CaCl_2$ were added to the culture at concentrations of 10 and 5 mM, respectively, and the culture was infected with N11 (a lysis gene E mutant of φX174) at a multiplicity of infection of 10 and kept at 37°C with vigorous aeration. The infected culture was quickly chilled in an ice bath 35 min after infection. Cells were harvested by centrifugation and washed twice with buffer A (50 mM Tris-hydrochloride, 0.1 mM EDTA [pH 7.3 at 25°C]) and twice with buffer A containing 10% sucrose. The washed cells were suspended to $1.25 \times 10^{11}$/ml in buffer A containing 10% sucrose, 100 mM KCl, 500 μg of bovine serum albumin (BSA) per ml, and 200 μg of *E. coli* tRNA per ml and incubated with 5 μg of T4 lysozyme per ml for 30 min at 0°C; then 1 mM of dithiothreitol was added to the incubation mixture. The mixture was brought to 30°C for 2 min, chilled at 0°C for 20 min, and centrifuged at 30,000 × *g* for 20 min at 0°C in a Spinco SW50.1 rotor. The supernatant fraction (cell extract) contained less than $10^6$ intact cells or spheroplasts per ml when counted under a light microscope.

**Reaction mixture.** A standard reaction mixture (0.25 ml) consisted of: cell extract, 0.15 ml; Tris-hydrochloride (pH 7.1 at 30°C), 50 mM; dATP, dGTP, dCTP, and [³H]dTTP, 0.1 mM each; ATP, 0.2

mM; $MgCl_2$, 15 mM; nicotinamide adenine dinucleotide, 2 mM; β-mercaptoethanol, 10 mM; and bovine serum albumin, 500 μg/ml. At the indicated times, 80 μl of the reaction mixture was taken and diluted with 200 μl of ice-cold buffer B (50 mM Tris-hydrochloride [pH 7.5 at 0°C], 100 mM KCl, 5 mM EDTA, and 500 μg of bovine serum albumin), and 100 μl of the diluted reaction mixture was centrifuged in a sucrose gradient (5 to 30% in 10 mM Tris-hydrochloride [pH 7.25]-100 mM NaCl-5 mM EDTA with a 0.15-ml cushion of 55% CsCl in 50% sucrose at the bottom of the gradient) at 49,000 rpm at 0°C in a Spinco SW50.1 rotor for 100 min. Fractions were collected onto Whatman 3 MM square filters, which were washed with ice-cold 6% trichloroacetic acid and then with ethanol, dried, and counted.

When the total incorporation of dTTP into DNA was measured, 15 μl of the reaction mixture diluted with buffer B was taken and mixed with 0.2 ml of 0.2 N NaOH-20 mM Na $PP_i$ and 200 μg of calf thymus DNA per ml and incubated at 90°C for 10 min. DNA was precipitated with 10% trichloroacetic acid at 0°C. The precipitate was collected on Whatman GF/C glass filters and counted.

**Isolation and characterization of product DNAs.** A complete reaction mixture was centrifuged in a sucrose gradient as described above. Fractions were collected in tubes containing sodium dodecyl sulfate, Sarkosyl, and EDTA to give final concentrations of 0.2%, 1%, and 10 mM, respectively, and incubated at 55°C for 20 min. Fractions in corresponding 20S, 50S, or 90S regions of the sucrose gradients were pooled. Each pooled fraction was incubated with 200 μg of RNase A per ml and 50 U of RNase T1 per ml for 50 min at 37°C. Pronase (500 μg/ml) was added to the RNase-treated samples, and the mixtures were dialyzed against a buffer containing 5 mM Tris-hydrochloride (pH 8.0), 5 mM EDTA, and 0.1% sodium dodecyl sulfate for 12 h at room temperature. DNA was purified from the Pronase-treated samples by the phenol method, precipitated with 2 volumes of ethanol, dissolved in a solution of 10 mM Tris-hydrochloride (pH 8.0), 10 mM NaCl, and 5 mM EDTA, and dialyzed against the same buffer overnight. The purified DNA was analyzed (i) in a neutral sucrose gradient of low ionic strength (5 to 20% sucrose gradient in 5 mM EDTA, pH 9.5) at 49,000 rpm for 150 min at 18°C in a Spinco SW50.1 rotor (13), or (ii) in an alkaline sucrose gradient (5 to 20% sucrose gradient in 5 mM EDTA-0.2 N NaOH) at 49,000 rpm for 250 min at 18°C, or (iii) in an alkaline CsCl density gradient.

**DNA-DNA duplex formation.** DNA samples purified as described above were mixed with formamide to a final concentration of 96% formamide and heated for 10 min at 95°C. Denatured DNA (2,000 cpm; ca. 0.1 μg) was mixed with (final volume, 0.2 ml) 35% formamide, 0.2 M sodium acetate (pH 5.0), and denatured DNAs (200 μg of *E. coli* DNA per ml, 5 μg of RF DNA per ml, or 5 μg of SS viral DNA per ml), and the mixture was incubated at 30°C for 14 h. A 0.8-ml volume of water, 2,000 U of S1 nuclease, and 0.5 mM $ZnCl_2$ were added to the mixture, which was then incubated for 60 min at 37°C. Ice-cold trichloroacetic acid (7%)-precipitable counts of the mixture were taken as hybridized counts.

## RESULTS AND DISCUSSION

**Characterization of the products in the in vitro system.** Cell extracts prepared as described above supported dTTP incorporation into acid-insoluble material for about 8 min at 30°C (Fig. 1c). When a complete reaction mixture was directly fractionated by sucrose gradient centrifugation, the incorporated counts were distributed into three fractions (90S, 50S, and 20S materials) as shown in Fig. 1a (*S* values of these fractions were estimated by using mature phage as a sedimentation marker [114S], Fig. 1b). Counts incorporated into 90S increased with time (Fig. 1a). Materials from

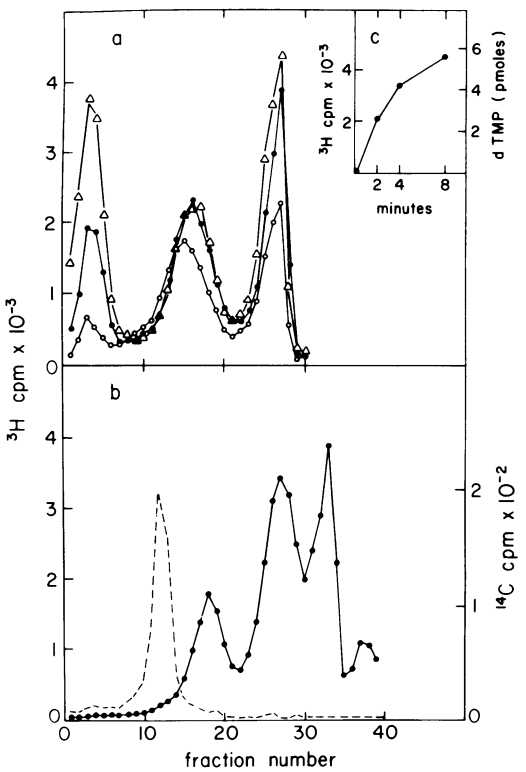


FIG. 1. *Kinetics of dTTP incorporation and product analysis of the in vitro system. (a) A complete reaction mixture ([$^3$H]dTTP, 880 cpm/pmol) was incubated at 30°C and, at 2* (○), *4* (●), *and 8* (△) *min, samples were taken and analyzed in sucrose gradients as detailed in the text. Centrifugation time was 100 min. Fractions 31 through 39 were discarded. (b) A portion of the reaction mixture of (a) incubated for 4 min was mixed with [$^{14}$C]thymine-labeled mature phage (114S) and analyzed in a sucrose gradient as described above, except that the centrifugation time was for 45 min.* (- - -) $^{14}$C; (●) $^3$H. *Sedimentation in (a) and (b) and in the subsequent sections was from right to left. (c) Kinetics of [$^3$H]dTTP incorporation into trichloroacetic acid-insoluble material. A 4.25-μl equivalent of the reaction mixture was measured as described in the text.*

each of the peak regions of Fig. 1b (90S, 50S, and 20S) were deproteinized by treatment with Pronase in the presence of 0.2% sodium dodecyl sulfate, and purified DNAs were hybridized to *E. coli* DNA, to RF DNA, or to viral DNA (Table 1). DNA from the 90S, 50S, or 20S region hybridized to RF DNA but not to viral DNA or to *E. coli* DNA, indicating that the product DNA in the reaction mixture contained sequences homologous to viral (plus-strand) DNA.

DNA from the 90S, 50S, or 20S region in Fig. 1a was analyzed by neutral or alkaline sucrose gradient sedimentation or alkaline CsCl density gradient centrifugations. (Fig. 2 and 3).

DNA from the 20S region of Fig. 1b sedimented at the position of the RFII marker in a neutral sucrose gradient (Fig. 2a) and at the position of DNA of linear unit length in an alkaline sucrose gradient (Fig. 2b). The DNA banded at the position of viral DNA (plus strand) in an alkaline CsCl density gradient (Fig. 2c).

DNA from the 50S region of Fig. 1b sedimented heterogeneously between RFI and RFII in a neutral sucrose gradient (Fig. 2d). In an alkaline sucrose gradient, the DNA from the 50S region sedimented heterogeneously between 18S (linear viral DNA two genomes in length) and 14S (linear viral DNA one genome in length), with a distinct peak at 14S (Fig. 2e). When the DNA from the 50S region was first treated with S1 nuclease (which is specific for SS DNA [1]) and then analyzed by alkaline sucrose gradient sedimentation, products from the digestion sedimented at the position of linear viral DNA one genome in length and digested material was recovered near the top of the gradient (Fig. 2f). In an alkali CsCl density gradient, the DNA from the 50S region banded at the position of viral DNA (data not shown).

DNA in the 90S region of Fig. 1b sedimented at the position of the viral strand, tailing toward the lower-molecular-weight region in a neutral sucrose gradient (Fig. 3a). In an alkaline sucrose gradient, the DNA in the 90S region sedimented at three positions corresponding to complete circular viral DNA, complete linear viral DNA, and smaller than full-genome-length DNA (Fig. 3b). DNA from the 90S region had the same density as that of viral DNA in an alkaline CsCl density gradient (Fig. 3c).

Ratios of circular and linear DNAs in the 90S region seemed to change with incubation time. If the incubation of the reaction mixture was limited to 2 min, the DNA isolated from the 90S region was circular and linear, one full genome in length (Fig. 3d, compare with 3b). When the DNA from the 90S region was treated with S1 nuclease first and then sedimented in an alkaline gradient, the circular and linear DNAs were digested completely with the nuclease, and the digested material was recovered near the top of the gradient (Fig. 3e). However, when DNA from the 90S region was treated with exonuclease I of *E. coli* and sedimented in an alkaline gradient (Fig. 3f), DNA sedimenting at the position of circular DNA was resistant to the nuclease, but the DNA originally sedimenting at the position of linear DNA was digested with exonuclease I.

These analyses of DNA in each region derived from the reaction mixture by sucrose density sedimentation indicates that the 20S material contains RFII, the 50S material consists of replicating SS DNA (the $\sigma$ structure), and the 90S material contains complete circular SS DNA and linear DNAs.

**Precursor and product relationship among 20S, 50S, and 90S materials.** A complete reaction mixture was incubated with labeled dTTP for 20 s, and then an excess amount of unlabeled dTTP was added. Samples were withdrawn at designated times and analyzed by sucrose density centrifugations. Results are shown in Fig. 4 (a to f), and the percentages of the total counts in 20S, 50S, and 90S material existing at the end of the pulse-chase periods are plotted in Fig. 4g.

At the end of the pulse period, radioactive counts were incorporated into 20S and 50S materials. Upon the chase, counts in the 20S material increased before the formation of radioactive 90S material (at 1 min). Counts in the 20S material then decreased and reached a plateau, while counts in 50S material decreased continuously with a concomitant increase of the

TABLE 1. *Hybridization test of DNAs from 20S, 50S, and 90S materials*

| Sample DNA | Input cpm | cpm hybridized to: | | | |
|---|---|---|---|---|---|
| | | SS DNA | RF DNA | *E. coli* DNA | No DNA[a] |
| From 20S | 2,010 | 80 | 1,834 | 64 | 82 |
| From 50S | 1,980 | 25 | 1,662 | 40 | 43 |
| From 90S | 2,100 | 58 | 1,921 | 46 | 46 |
| *E. coli*[b] | 1,500 | 80 | 76 | 860 | 78 |
| RF[c] | 3,040 | 1,021 | 2,672 | 46 | 120 |
| SS[d] | 2,820 | 29 | 2,520 | 21 | 38 |

[a] No DNA except sample DNA was added to the reaction mixture.

[b] Isolated from uninfected [$^3$H]thymidine-labeled HF4704 cells as described previously (13).

[c] [$^3$H]thymidine-labeled RF DNA isolated as detailed previously (9).

[d] Isolated from [$^3$H]thymidine-labeled φX174.

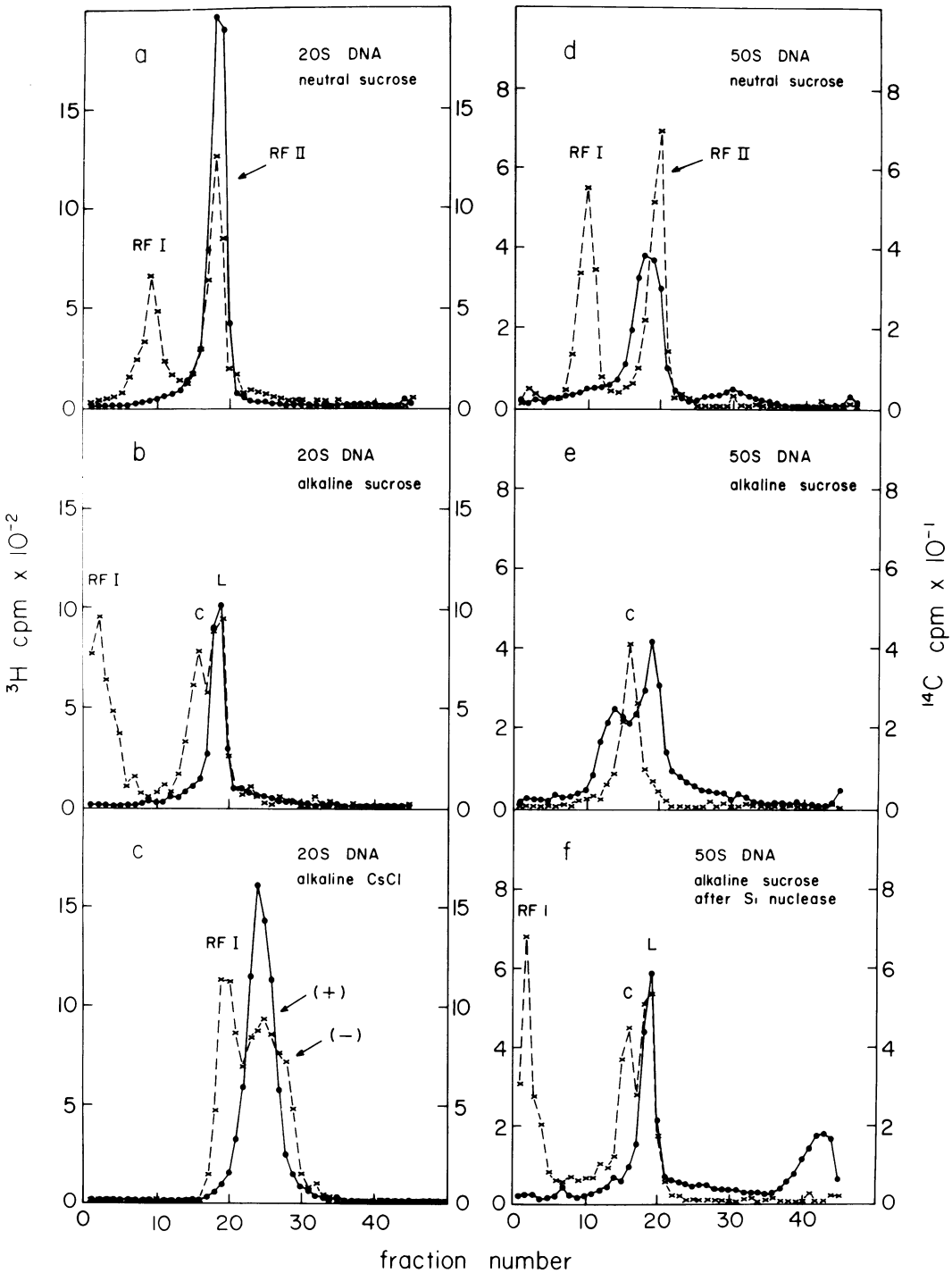


FIG. 2. *Characterization of DNAs in 20S and 50S regions. A standard reaction mixture ([$^{3}$H]TTP, 3,760 cpm/pmol) was incubated for 4 min at 30°C, and centrifuged through a sucrose gradient as described in the legend to Fig. 1. 20S or 50S regions were pooled, and DNAs were purified from the pooled fractions and analyzed by sucrose sedimentation or in CsCl density gradients as described in the text. (a) DNA from the 20S region; neutral sucrose gradient; markers, RFI and RFII. (b) DNA from the 20S region; alkaline sucrose gradient; markers, RFI and RFII (separated into circular [C] and linear [L] DNAs). (c) DNA from the 20S region; alkaline CsCl density gradient; markers, RFI (heaviest) and RFII (plus strand [+], heaver; minus strand [−], lighter). (d) DNA from the 50S region; neutral sucrose gradient; markers RFI and RFII. (e) DNA from the 50S region; alkaline sucrose gradient; marker, viral DNA. (f) DNA from the 50S region was incubated with 90 U of S1 nuclease per 0.15 ml in a buffer containing 50 mM acetate (pH 5.0) and 0.5 mM $ZnCl_2$ for 10 min at 37°C and was centrifuged in an alkaline sucrose gradient; markers, RFI and RFII. Marker RFI, RFII, and viral DNAs were labeled with [$^{14}$C]thymine.* (×) $^{14}$C *counts;* (●) $^{3}$H *counts.*

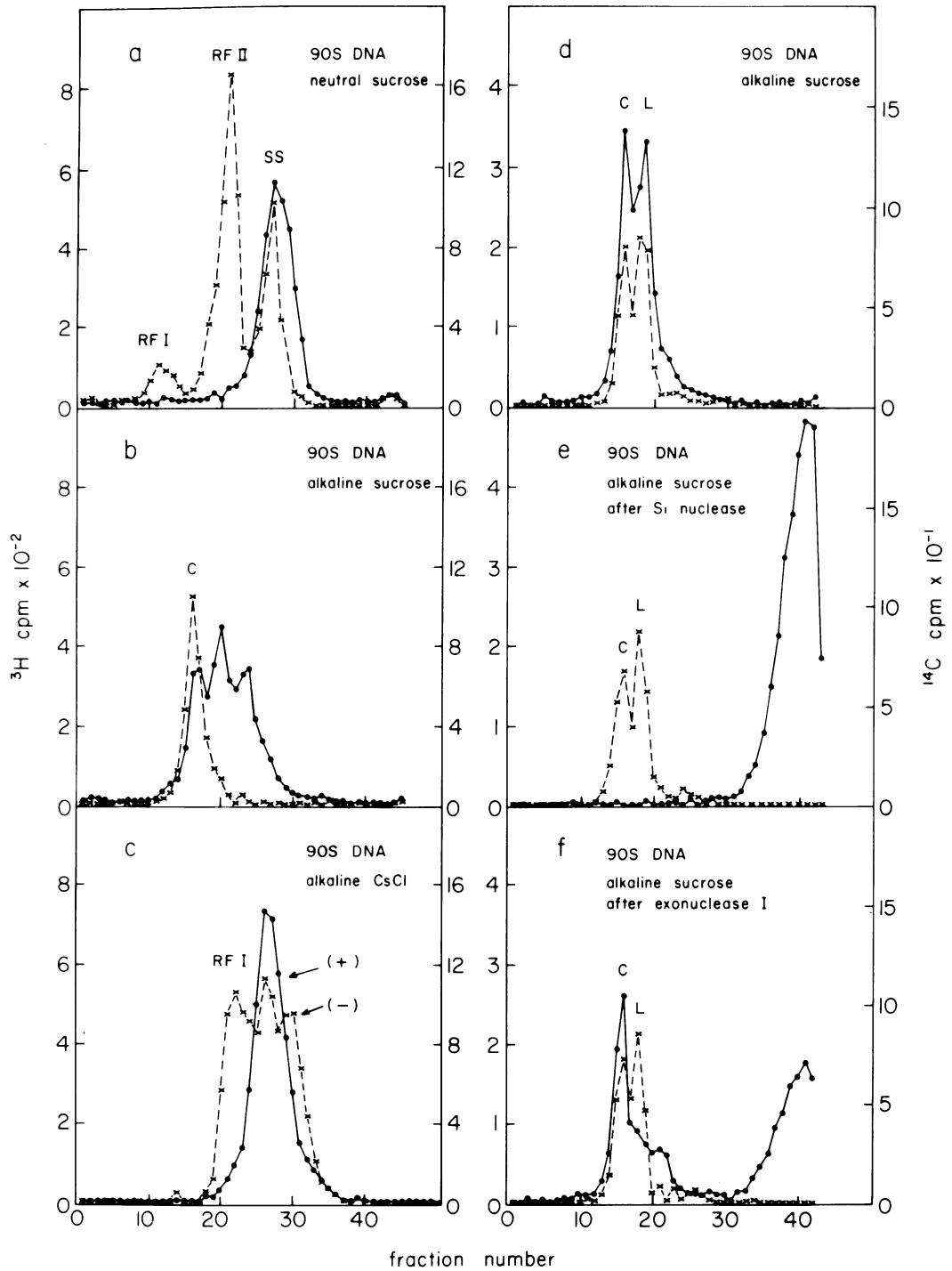


FIG. 3. *Characterization of DNA from the 90S region. (a, b, and c) DNA from the 90S region isolated from the reaction mixture used in Fig. 2 (4-min incubation) was analyzed. (d, e, and f) A standard reaction mixture was incubated for 2 min at 30°C, and DNA from the 90S region was isolated, as described in the legend to Fig. 2, and analyzed. (a) Neutral sucrose gradient; markers, RFI, RFII, and viral DNA (SS). (b) Alkaline sucrose gradient; marker, circular viral DNA. (c) Alkaline CsCl density gradient; markers, RFI and RFII. (d) Alkaline sucrose gradient; marker, RFII. (e) DNA of the 90S region was first treated with S1 nuclease as described in the legend of Fig. 2f and then analyzed in an alkaline sucrose gradient; marker, RFII. (f) DNA of the 90S region was treated with 4.5 U of E. coli exonuclease I per 0.15 ml in a buffer containing 67 mM glycine (pH 9.5), 6.7 mM $MgCl_2$, and 1 mM β-mercaptoethanol for 30 min at 37°C and was centrifuged in an alkaline sucrose gradient; marker, RFII. DNA from the 90S region ($^3$H, ●). Markers RFI, RFII, or viral DNA was labeled with [$^{14}$C]thymine (×).*

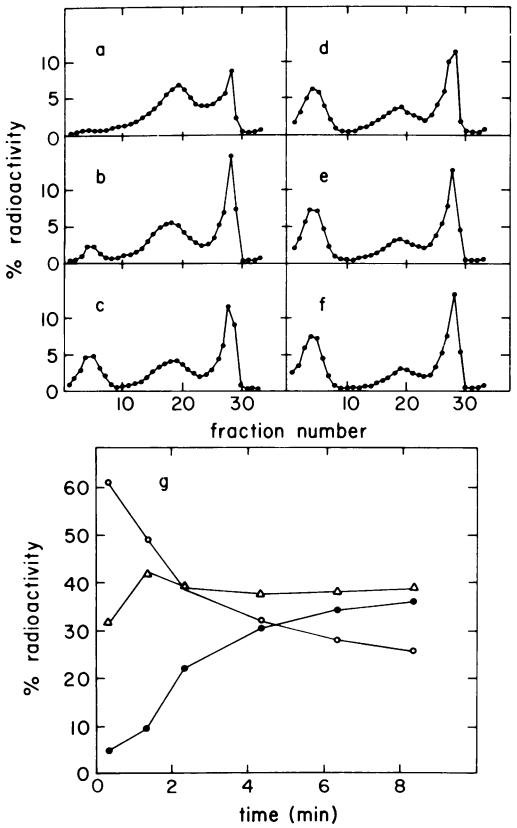


FIG. 4. *Pulse and pulse-chase labeling of the in vitro products. A standard reaction mixture with 5.4 μM α-[$^{32}$P]dTTP (22,400 cpm/pmol) was incubated for 20 s at 30°C, and then unlabeled dTTP was added to a final concentration of 1 mM. A 35-μl volume was taken at the end of the pulse and during the chase period and was mixed with 200 μl of buffer B. A 190-μl portion of the diluted reaction mixture was centrifuged as described in the legend to Fig. 1. (A gradual decrease of trichloroacetic acid-precipitable counts was observed during the chase period; 70% of the counts existing at the end of pulse period were recovered at the end of the 8-min chase.) (a) 20-s pulse; (b) 1-min chase; (c) 2-min chase; (d) 4-min chase; (e) 6-min chase; (f) 8-min chase. (g) Radioactive counts recovered in the 90S region (fractions 1 through 8) (●), the 50S region (fractions 9 through 23) (○), and the 20S region (fractions 24 through 30) (△) are expressed as percentages of the total counts.*

counts in the 90S material. If we assume that the 90S material is equivalent to phage (because the circular DNA in 90S is the end product of this in vitro system as far as the viral DNA synthesis is concerned), we can compare the labeling patterns in this pulse-chase experiment to those in vivo.

Previously, Fujisawa and Hayashi proposed a model of the in vivo precursor and product relationship among RFII, the 50S complex, and mature phage, based on the in vivo pulse and pulse-chase experiments (Fig. 5) (5–7). They observed that synthesis of SS viral DNA initiated at RFII. RFII with a short tail of SS DNA then associated with a capsomeric protein complex (omega complex) to form the 50S complex. Elongation of viral DNA continued in the 50S complex until one round of replication of viral DNA was completed. Cleavage on the viral strand by the action of the gene A product occurred to form an RFII molecule and a complex between linear DNA and the omega structure. This complex would be further processed to mature phage. The RFII released after the cleavage of the 50S complex would enter into the pool of RFII, and the formation of the 50S complex would be repeated.

Some of the features of the in vitro labeling pattern of 20S, 50S, and 90S material in Fig. 4g can be explained by this model. When the system was pulse-labeled with dTTP, radioactive counts were incorporated into endogenously existing RFII (20S) and the σ structure (in the 50S). During the pulse and subsequent chase period, 90S material that contained circular viral DNA was formed. However, radioactive counts would not be expected to appear in the 90S region until one round of viral DNA replication had been completed in 50S material. Before one round of viral DNA replication is completed, endogenously existing 50S material is converted to 90S material (non-radioactive) and RFII (radioactive), and de novo formation of 50S material should occur from the radioactive RFII and the omega complex. The 50S material would then produce radioactive 90S material upon completion of viral DNA replication.

The in vitro kinetics (Fig. 4g) indicate that much of the radioactive dTTP, initially incorporated in 50S material, is rapidly chased into RFII (20S) and, after a lag of about 1 min, into

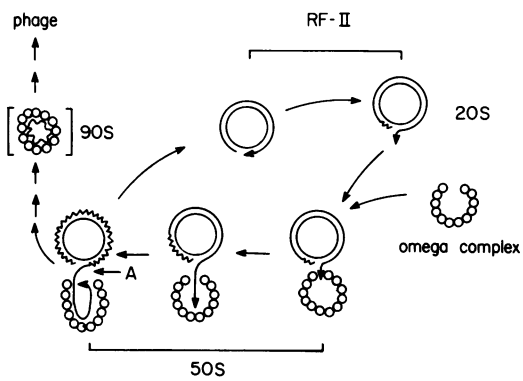


FIG. 5. *Model of the relationship among 20S, 50S, and 90S material. A detailed explanation of the model is given in the text.*

90S material. These features of the in vitro kinetics are consistent with the model previously proposed for in vivo replication of φX174 viral DNA.

Certain aspects of the kinetics shown in Fig. 4g apparently deviate from results that might be predicted by this model. These discrepancies can be explained in the following ways. The rapid decrease of 50S material may be due to a small pool size of the endogenous omega complex or to slow formation of the 50S material. During the chase period, no apparent decrease of radioactivity in RF (20S) was observed. It is possible that the asynchrony of formation of 50S and 90S materials in the present system led to a fortuitous balance between the decrease of labeled RFII due to formation of the 50S material and the increase of labeled RF due to its release from the 50S material. Because the rate of incorporation of dTTP into DNA decreased rapidly with time in the present system (Fig. 1), it is also possible that de novo formation of the 50S material took place only during early times of incubation. In separate experiments, we (manuscript in preparation) obtained evidence indicating de novo formation of 50S material from RFII and the omega complex in an in vitro system. The system consisted of extracts prepared (as described in this paper) from cells infected with different mutant phages. Mixing of appropriate extracts resulted in the formation of 50S and 90S materials (in vitro complementation).

The origin of DNAs in linear forms existing in the 90S material is not known. These DNAs may be derived from cleavage of circular DNA by nuclease(s) with some subsequent degradation, yielding the less-than-one-genome-length DNAs. Alternatively, full-length linear DNA may be a result of failure of ligation. It is possible that the DNA of smaller than unit length would be derived from premature termination of elongation in the 50S material. The premature termination would also be reflected in the rapid decrease of the 50S material in Fig. 4. The molecular structure of the 90S material is unknown. It has not been detected as a precursor for mature phage in vivo. If the 90S material is a precursor, our present system is deficient in protein(s) responsible for the conversion of the 90S material to mature phage. Alternatively, it is possible that the 90S material is an artificial aggregate between SS DNA and proteins.

Finally, if the in vitro synthesis of viral DNA in the present system proceeds by way of a sigma structure derived from RFII, the synthesis of radioactive, circular, full-genome-length viral DNA in the 90S material must be the result of de novo initiation, elongation, termination, and circularization.

ACKNOWLEDGMENTS

We are grateful to K. Spindler for invaluable help with the manuscript and to M. Sugiura and A. Tsugita for generous gifts of exonuclease I and T4 lysozyme, respectively.

This work was supported by grant BMS 74-10710 from the National Science Foundation and by Public Health Service grant GM-12934 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. Ando, T. 1966. A nuclease specific for heat-denatured DNA isolated from a product of Aspergillus oryzae. Biochim. Biophys. Acta 114:158–168.
2. Denhardt, D. T. 1975. The single-stranded DNA phages. CRC Crit. Rev. Microbiol. 4:161–222.
3. Dressler, D. 1970. The rolling circle for φX DNA replication. II. Synthesis of single-stranded DNA circles. Proc. Natl. Acad. Sci. U.S.A. 67:1934–1942.
4. Dressler, D. H., and D. T. Denhardt. 1968. On the mechanisms of φX single-stranded DNA synthesis. Nature (London) 219:346–351.
5. Fujisawa, H., and M. Hayashi. 1976. A viral DNA synthesizing intermediate complex isolated during assembly of bacteriophage φX174. J. Virol. 19:409–415.
6. Fujisawa, H., and M. Hayashi. 1976. Gene A product of φX174 is required for site-specific endonucleolytic cleavage during single-stranded DNA synthesis in vivo. J. Virol. 19:416–424.
7. Fujisawa, H., and M. Hayashi. 1977. Function of gene C and gene D products of bacteriophage φX174. J. Virol. 21:506–515.
8. Gilbert, W., and D. Dressler. 1968. DNA replication: the rolling circle model. Cold Spring Harbor Symp. Quant. Biol. 33:473–484.
9. Hayashi, Y., and M. Hayashi. 1971. Template activities of the φX-174 replicative allomorphic deoxyribonucleic acid. Biochemistry 10:4212–4218.
10. Knippers, R., A. Razin, R. Davis, and R. L. Sinsheimer. 1967. The process of infection with bacteriophage φX-174. XXIX. *In vivo* studies of the synthesis of the single-stranded DNA. J. Mol. Biol. 45:237–263.
11. Linney, E. A., and M. Hayashi. 1973. Two proteins of gene A of φX-174. Nature (London) New Biol. 248:6–8.
12. Linney, E. A., M. N. Hayashi, and M. Hayashi. 1972. Gene A of φX-174. I. Isolation and identification of its products. Virology 50:381–387.
13. Siegel, J. E. D., and M. Hayashi. 1969. φX-174 bacteriophage mutants which affect deoxyribonucleic acid synthesis. Virology 4:400–407.
14. Sinsheimer, R. L. 1968. Bacteriophage φX-174 and related viruses. Prog. Nucleic Acid Res. Mol. Biol. 8:115–168.
15. Sinsheimer, R. L., R. Knippers, and T. Komano. 1968. Stages in the replication of bacteriophage φX-174 DNA *in vivo*. Cold Spring Harbor Symp. Quant. Biol. 33:443–448.