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Deoxyribonucleic Acid Polymerase of Rous Sarcoma Virus: Studies on the Mechanism of Double-Stranded Deoxyribonucleic Acid Synthesis

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The deoxyribonucleic acid (DNA) polymerase of Rous sarcoma virus synthesizes both single- and double-stranded DNA, utilizing the ribonucleic acid (RNA) of the viral genome as the initial template. Results of pulse-chase experiments indicate that the single-stranded DNA serves as unconserved template and precursor for the synthesis of double-stranded DNA. The latter reaction is apparently initiated in association with the viral RNA and may involve a partially double-stranded intermediate form.

The virions of ribonucleic acid (RNA) tumor viruses contain at least two deoxyribonucleic acid (DNA) polymerase activities: (i) transcription of RNA into single-stranded DNA, using the RNA of the viral genome as template (1, 16), and (ii) the subsequent synthesis of double-stranded DNA (4, 5), utilizing product of the first reaction as template (6, 14). The RNA-dependent reaction results in the formation of DNA:RNA hybrids, which presumably represent intermediates in the synthesis of double-stranded DNA (7, 12, 13). However, the precise mechanism of double-stranded DNA synthesis has yet to be elucidated. The present study was undertaken to determine whether the single-stranded DNA constituent of the hybrid intermediate serves as a direct precursor of double-stranded DNA and to elucidate further the role of DNA:RNA hybrid in the enzymatic reaction.

MATERIALS AND METHODS

Reagents. ^{3}H-thymidine triphosphate (TTP), 10 to 15 Ci/mmole, was from the New England Nuclear Corp. Pancreatic ribonuclease A was from Worthington Biochemicals, Inc. Stock solutions were boiled for 10 min to inactivate any contaminating deoxyribonuclease. Pronase (B grade) was from Calbiochem. It was self-digested at 37 C for 2 hr before use. Deoxyribonucleoside triphosphates, deoxyadenosine triphosphate, deoxycytidine triphosphate, and deoxyguanosine triphosphate, were from Calbiochem. Phenol (reagent grade) was from Mallinckrodt. Hydroxyapatite (Biol-Gel HT) was from Bio-Rad, Richmond, Calif. Nonidet P40 (NP-40) was from the Shell Chemical Co. Actinomycin D was a gift from Merck, Sharp and Dohme, Inc.

Propagation and purification of virus. The Schmidt-Ruppin strain of Rous sarcoma virus was grown in chick embryo fibroblasts and purified as described previously (2).

Extraction and purification of viral RNA. Rous sarcoma virus RNA was extracted from purified virus with sodium dodecyl sulfate and phenol (2). The 70*S* RNA was isolated by zonal centrifugation through density gradients of sucrose (2, 7).

DNA polymerase reaction. Details of the enzyme reaction mixture have been reported (7). ^{3}H-TTP was used as labeled precursor. Enzymatic activity was elicited by treating virus suspensions with various concentrations of NP-40. The detergent was included in the reaction mixture, which was warmed to 37 C before addition of appropriate amounts of purified virus. Under these conditions, DNA synthesis began immediately. Determination of acid-precipitable radioactivity was accomplished as described previously (7).

Extraction and purification of enzymatic product. Reaction mixtures were treated with sodium dodecyl sulfate (0.5%, w/v) and Pronase (500 μg/ml) for 45 min at 37 C and then centrifuged through density gradients of 15 to 30% sucrose containing 0.1 M NaCl-0.001 M ethylenediaminetetraacetic acid (EDTA)-0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, *p*H 7.4. Centrifugation was carried out in a Spinco SW 41 rotor at 40,000 rev/min for 180 min at 4 C. Fractions (0.3 ml) were collected, and samples were taken for determination of acid-precipitable radioactivity (7). Appropriate regions of the gradient were pooled and stored at −20 C pending further analysis.

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Fractionation of DNA on hydroxyapatite. Single-stranded DNA and double-stranded DNA were separated by step-wise elution from hydroxyapatite as described previously (4). Nucleic acids were treated with ribonuclease (10 μg/ml, 3 mM EDTA, 37 C, 1 hr) to disrupt DNA:RNA hybrids. This procedure is necessitated by the fact that the elution of native hybrids from hydroxyapatite substantially overlaps that of double-stranded DNA (4). Consequently, analysis on hydroxyapatite cannot distinguish between free single-stranded DNA and that which was originally complexed to RNA.

Rate-zonal centrifugation. All analyses were carried out in density gradients of 15 to 30% sucrose containing 0.1 M NaCl-0.001 M EDTA-0.02 M Tris-hydrochloride, *p*H 7.4. Conditions of centrifugation are given for individual experiments.

RESULTS

Single-stranded DNA as precursor to double-stranded DNA: pulse-chase experiments with virion polymerase. The overall synthesis of DNA in vitro by Rous sarcoma virus polymerase is probably accomplished in several sequential steps (Fig. 1): (i) synthesis of a short piece of single-stranded DNA, utilizing 70*S* viral RNA as template and resulting in a DNA:RNA hybrid with a long tail of single-stranded RNA (7); and (ii) synthesis of double-stranded DNA (4), either directly from the hybrid or by way of an intermediate form of uncertain structure (*see below*). This model gives rise to a fundamental question regarding the mechanism of double-stranded DNA synthesis: does such synthesis proceed in a conservative or a semiconservative manner, i.e., does single-stranded DNA remain in the hybrid state and function simply as template (conservative mechanism) or is it actually a direct precursor of double-stranded DNA (semiconservative mechanism)? In an effort to answer this question, we have performed "pulse-chase" experiments in which a brief period of labeling with radioactive precursor is followed by periods of "chase" with an excess of unlabeled precursor. As a matter of convenience, we have utilized an enzymatic reaction in which the rate of DNA synthesis has been reduced well below the maximum (6). This reduction was accomplished by utilizing a concentration of nucleoside triphosphate precursor (8×10^{-7} M ^{3}H-TTP) which limits the reaction rate to 1% of the maximum (6) and by treating the virus suspensions with suboptimal amounts of nonionic detergent (7). The consequence of this latter maneuver is illustrated in Fig. 2, which contrasts the kinetics of DNA synthesis elicited by 0.005 and 0.01% NP-40. At the lower concentration of detergent, detectable DNA synthesis began only after an appreciable lag (ca. 15 min) and then proceeded at a rate which was somewhat lower than that observed with the higher concentration of detergent. When analyzed by rate-zonal centrifugation and elution from hydroxyapatite, the DNA synthesized by the detergent-limited reaction (i.e., 0.005% NP-40) proved to be qualitatively similar to that synthesized at higher concentrations of detergent, although the appearance of double-stranded DNA was appreciably delayed (6; *unpublished data*).

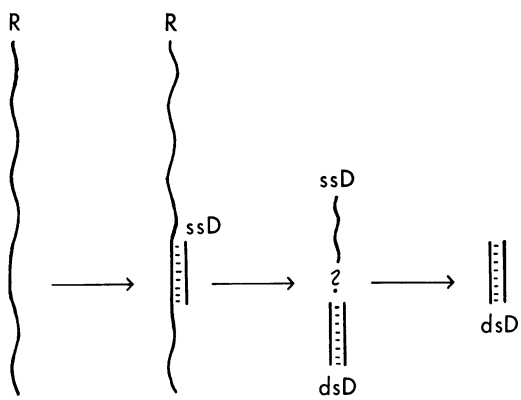

FIG. 1. *Hypothetical model for transcription of viral RNA. R, single-stranded viral RNA; ssD, single-stranded DNA; ds DNA, double-stranded DNA. This model incorporates the presently known features of the transcription of tumor virus RNA into DNA. (i) The initial reaction is RNA-dependent (1, 16), involving the synthesis of a short piece of single-stranded DNA utilizing 70S viral RNA as template and resulting in an RNA:DNA hybrid with a long tail of single-stranded RNA (7, 12); the transcribed region of viral RNA was chosen arbitrarily, and no effort has been made to portray the fact that the entire viral genome is ultimately transcribed (2a). (ii) The final reaction is probably DNA-dependent, in that it is actinomycin-sensitive (8) and capable of accepting exogenous double-stranded DNA as template (8, 9a, 13a). (iii) The final product appears to be double-stranded DNA (4, 5). (iv) Single-stranded DNA, free from viral RNA, may or may not be an intermediate in the synthesis of double-stranded DNA (9).*

The kinetics of representative "pulse-chase" experiments are illustrated in Fig. 3. Addition of unlabeled TTP 45 min after the onset of DNA synthesis sharply curtailed further incorporation of the radioactive precursor, although incorporation was not completely arrested until approximately 1 hr after the chase. By contrast, addition of actinomycin to the reaction mixture simultaneously with the chase resulted in the immediate cessation of incorporation. If actinomycin was present from the onset of the reaction, synthesis of DNA during the hour before the chase was reduced by a factor of two. This is a consequence

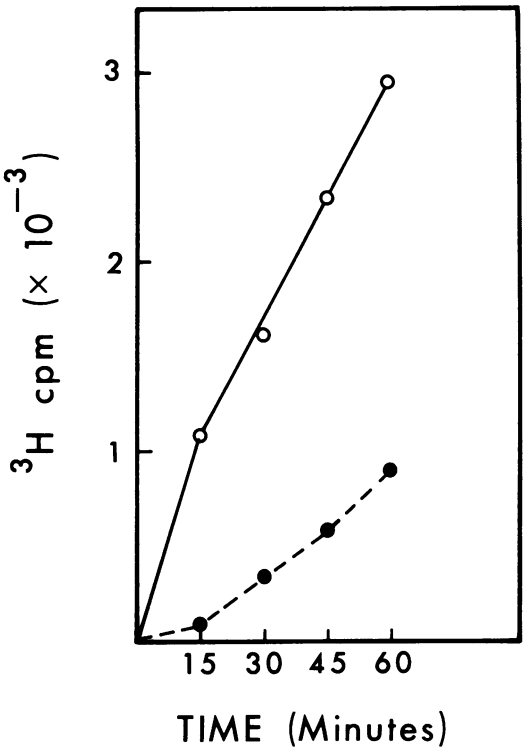

FIG. 2. *Effect of reduced detergent concentration on synthesis of DNA by Rous sarcoma virus polymerase. Standard reaction mixtures, containing 200 μg of virus protein per ml, 8 × 10^{-7} M ^{3}H-thymidine triphosphate, and either 0.005% (●) or 0.01% (○) Nonidet P40 were incubated at 37 C. Samples were withdrawn at the indicated time points for determination of acid-precipitable radioactivity.*

of the previously described inhibitory effect of actinomycin on the DNA-dependent portion of the reaction (8).

Fate of single-stranded DNA during a chase. The fate of the single-stranded DNA which is labeled during the course of a pulse was followed by analysis of enzymatic product on hydroxyapatite (Fig. 4). As noted above, the detergent-limited reaction synthesized double-stranded DNA very slowly (Fig. 4, left panel). After the addition of excess unlabeled precursor (TTP), however, a major portion (ca. 75%) of the labeled DNA was converted to a double-stranded form, with a concomitant decrease in labeled single-stranded DNA (Fig. 4, right panel). Addition of actinomycin at the time of the chase completely blocked this conversion (Fig. 4, right panel). These data indicate that most of the single-stranded DNA synthesized during the initial phase of the reaction is eventually incorporated into double-stranded DNA, presumably by serving as an unconserved template.

The conversion of single- to double-stranded DNA suggests that single-stranded DNA was continuously displaced from its initial hybrid state (Fig. 4), possibly by the ongoing synthesis of successive chains of DNA against the RNA template (*see below*; Fig. 9). It should be possible to detect this putative displacement by zonal centrifugation because the final double-stranded enzymatic product has a relatively low sedimentation velocity (7), whereas the DNA:RNA hybrid sediments rapidly (ca. 70*S* at early time points). A comparison by zonal centrifugation of control and "pulse-chase" reaction products is illustrated in Fig. 5 and 6. As expected, the early product (through 60 min) of the control reaction consisted primarily of the DNA:RNA hybrid which cosedimented precisely with 70*S* viral

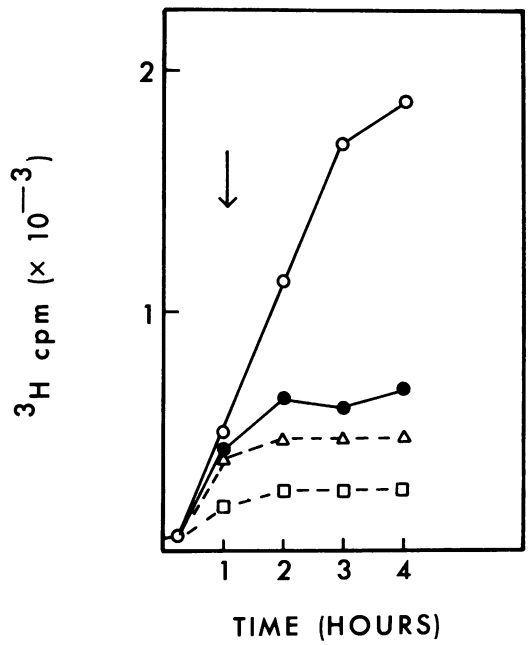

FIG. 3. *Pulse-chase experiments with virion polymerase. Replicate reaction mixtures were prepared with 200 μg of virus protein per ml, 8 × 10^{-7} M ^{3}H-thymidine triphosphate (TTP), and 0.005% Nonidet P40. Samples were withdrawn at the indicated time points for determination of acid-precipitable radioactivity. At 60 min (arrow), three of the four reaction mixtures received additions of unlabeled TTP in 100-fold excess of the ^{3}H-TTP. Actinomycin (50 μg/ml) was added to one of the chase reaction mixtures at the onset of the reaction and to another simultaneously with the chase of unlabeled TTP. Symbols: ○, control (not chased); ●, chase; □, chase, with actinomycin added at the onset of the reaction; △, chase, with simultaneous addition of unlabeled TTP and actinomycin at 60 min.*

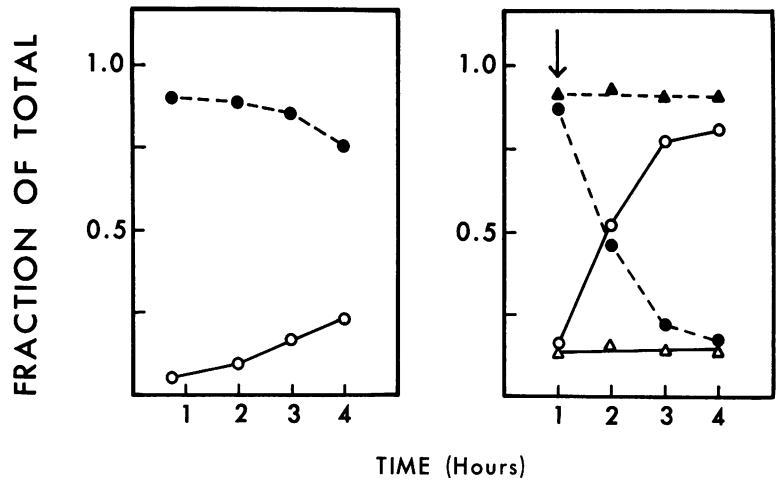

FIG. 4. *Fate of pulse-labeled DNA. Replicate reaction mixtures were prepared as for Fig. 3. Three of the four reactions were "chased" with unlabeled thymidine triphosphate at 60 min (arrow). Samples were taken at the indicated time points, extracted, and analyzed for relative content of radioactive single- and double-stranded DNA by elution from hydroxyapatite after treatment with ribonuclease (4). This procedure does not distinguish single-stranded DNA contained in hybrid structures from that which is not complexed with RNA (4). The left-hand panel illustrates the results of a control (not "chased") reaction. Symbols: ●, single-stranded DNA; ○, double-stranded DNA. The right-hand panel illustrates the results of the chased reactions. Symbols: ●, single-stranded DNA in chased reaction; ○, double-stranded DNA in chased reaction; ▲, single-stranded DNA in chased reaction containing actinomycin (50 μg/ml, added at the time of the chase); △, double-stranded DNA in the chased reaction containing actinomycin.*

RNA (Fig. 5a). By 2 hr, there was a slight but consistent discrepancy between the sedimentation velocity of 70S viral RNA and that of the hybrid enzymatic product (Fig. 5d). This discrepancy was even more apparent at 4 hr (Fig. 5e), although the bulk of the hybrid population was still coincident with the 70S viral RNA in the sucrose gradient.

These progressive changes in sedimentation velocity were accentuated by a chase (Fig. 6). At 4 hr, none of the labeled enzymatic product cosedimented with 70S viral RNA (Fig. 6e). Nevertheless, an appreciable portion of the product (ca. 40 to 50%) still sedimented more rapidly than the slowly sedimenting population which contains the final double-stranded product of the reaction (4).

Ribonuclease treatment of the rapidly sedimenting DNA isolated from control and pulse-chase reactions converted these DNA species to a low-molecular-weight form (Fig. 7). Thus, all of the rapidly sedimenting DNA present at 4 hr in both the control and the "pulse-chase" reactions consisted of low-molecular-weight DNA associated with high-molecular-weight single-stranded RNA. These observations do not conform to the expectation that the conversion of single- to double-stranded DNA is accompanied by displacement of the former from the DNA:RNA hybrid and prompted us to examine the secondary structure of the DNA in question by analysis on hydroxyapatite. In agreement with our previously published results (4), the rapidly sedimenting hybrid isolated from either a control or a pulse-chase reaction at an early time point contained only single-stranded DNA (Fig. 8c). At 4 hr, however, the hybrid from a control reaction contained an appreciable amount (ca. 25%) of double-stranded DNA (Fig. 8e), and the hybrid from a "pulse-chase" reaction contained primarily double-stranded DNA (ca. 70%; Fig. 8g). In both cases, treatment of the hybrid with alkali completely denatured the double-stranded DNA constituent (Fig. 8f and h). These observations indicate that conversion of single- to double-stranded DNA occurred in association with the initial template for DNA synthesis, the 70S viral RNA, and account in large measure for the fact that a chase did not completely displace radiolabel from the rapidly sedimenting form of enzymatic product.

Actinomycin, which completely inhibits the DNA-dependent portions of virion-associated DNA synthesis (8), blocked the conversion of pulse-labeled single-stranded DNA into double-stranded DNA (Fig. 4). Consequently, hybrid synthesized in the presence of actinomycin con-

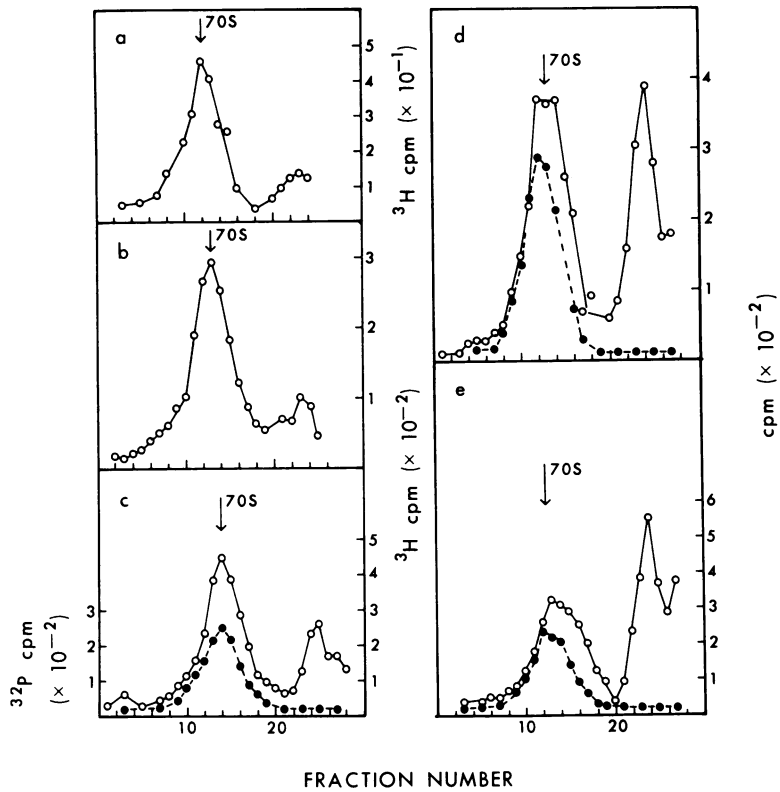

FIG. 5. *Rate-zonal centrifugation of enzymatic product from a control reaction. A standard reaction mixture was prepared with 300 μg of virus protein and 4 × 10^{-6}M ^{3}H-thymidine triphosphate (TTP). This concentration of ^{3}H-TTP was required to obtain sufficient enzymatic product to allow multiple centrifugal analyses. Samples were withdrawn at the indicated time points, extracted with sodium dodecyl sulfate and Pronase, and analyzed by centrifugation through density gradients of sucrose in an SW 41 rotor. ^{32}P-labeled 70S viral RNA was included as a sedimentation reference. (a) At 15 min. The 70S sedimentation marker is not illustrated but was coincident with the rapidly sedimenting enzymatic product. (b) At 30 min. The 70S sedimentation on marker is not illustrated but was coincident with the rapidly sedimenting enzymatic product. (c) At 60 min. (d) At 120 min. (e) At 240 min. Symbols: ○, ^{3}H-labeled enzymatic product; ●, ^{32}P-labeled 70S viral RNA.*

tained only single-stranded DNA at all time points during the course of both control and pulse-chase reactions (Table 1).

DISCUSSION

Hypothetical model for DNA synthesis. On the basis of the preceding data, we propose a preliminary model for the synthesis of DNA by the virion polymerases (Fig. 9). This model incorporates the four principle features of the reaction which have been elucidated to date. (i) The initial event is synthesis of short segments of single-stranded DNA, utilizing 70S RNA as template and resulting in the formation of a DNA:RNA hybrid. (ii) A portion of the DNA contained in this hybrid is accessible to digestion by the single strand-specific endonuclease of *Neurospora* (9), i.e., the hybrid apparently possesses branches of single-stranded DNA. (iii) Single-stranded DNA serves as template and precursor for the synthesis of double-stranded DNA. (iv) The synthesis of double-stranded DNA appears to occur, or at least to be initiated, in association with the viral RNA (Fig. 8 and reference 8).

We suggest that the branched hybrid results from displacement of a completed (or partially completed) DNA strand by the growth of a newly initiated strand. In this regard, the model is similar to that proposed by Manly et al. (9) and draws upon familiar hypothetical mechanisms for the synthesis of single-stranded viral RNA (15).

To accomodate our observation that the synthesis of double-stranded DNA occurs in association with the DNA:RNA hybrid (Fig.

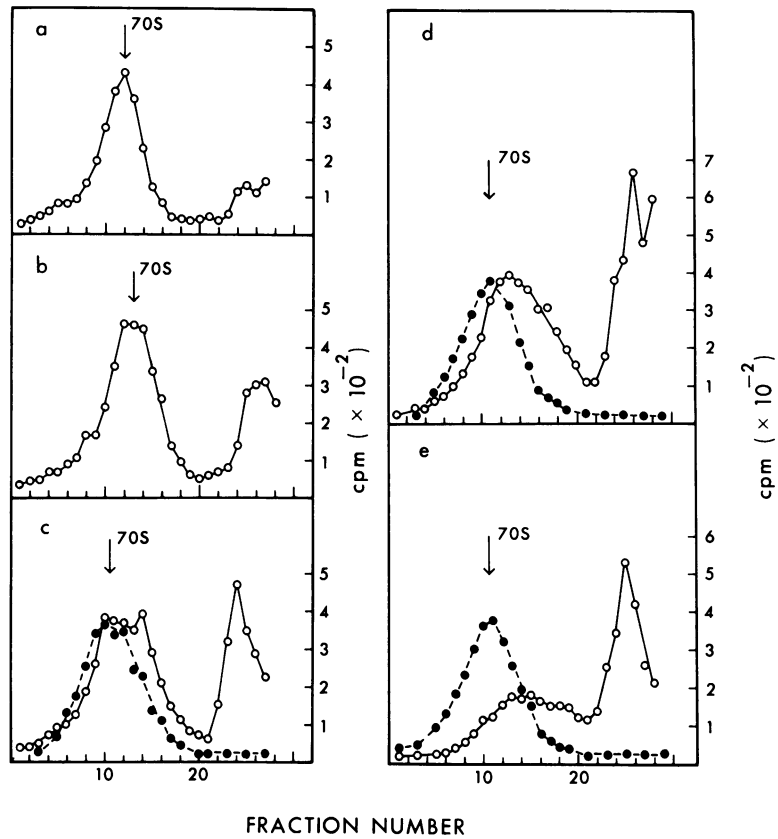

FIG. 6. *Rate-zonal centrifugation of enzymatic product from a chased reaction. A reaction mixture identical to that described for Fig. 5 was prepared. After 15 min of incubation at 37 C, unlabeled thymidine triphosphate (TTP) was added in 100-fold excess of the ^{3}H-TTP. Samples of the reaction mixture were extracted and analyzed by rate-zonal centrifugation as in Fig. 5. ^{32}P-labeled 70S viral RNA was included as a sedimentation reference. (a) At 15 min. Sample taken immediately before the chase. (b) At 30 min. (c) At 60 min. (d) At 120 min. (e) At 240 min. Symbols: ○, ^{3}H-labeled enzymatic product; ●, ^{32}P-labeled 70S viral RNA.*

6 and 8), we propose that DNA synthesis is initiated against the partially displaced single strands before they are released from the hybrid. We assume that, as in other instances (3), the synthesis of DNA is unidirectional (i.e., from the 5′ terminus towards the 3′ terminus). Consequently, a partially displaced strand can serve as template for further DNA synthesis only if there are repeated initiations in the manner suggested by Okazaki et al. for the replication of bacterial DNA (10). These initiations, in turn, would give rise to covalent gaps in the nascent DNA strand which would have to be closed by the action of a ligase enzyme (11). According to the model, double-stranded DNA could not be completed until after its release from the hybrid. Moreover, disruption of the hybrid by hydrolysis with ribonuclease in low concentrations of electrolytes would release incomplete (i.e., partially single-stranded) double-stranded DNA (Fig. 9). Attempts to identify and isolate DNA of this sort from disrupted hybrid are now in progress.

The foregoing proposals are clearly provisional and are subject to the criticism that the data were obtained with crude enzyme preparations. Such preparations may be contaminated with endonucleases and other possible sources of artifact. However, we have shown that, under the conditions used for these experiments, the Rous sarcoma virus virion is not disrupted, the viral genome (i.e., the primary template for DNA synthesis) remains intact, and no deoxyribonuclease active against double-stranded DNA is detectable in the reaction mixture (*in preparation*). Consequently, DNA synthesis is effected by an ostensibly undisturbed and coordinated series of enzymatic reactions which may well

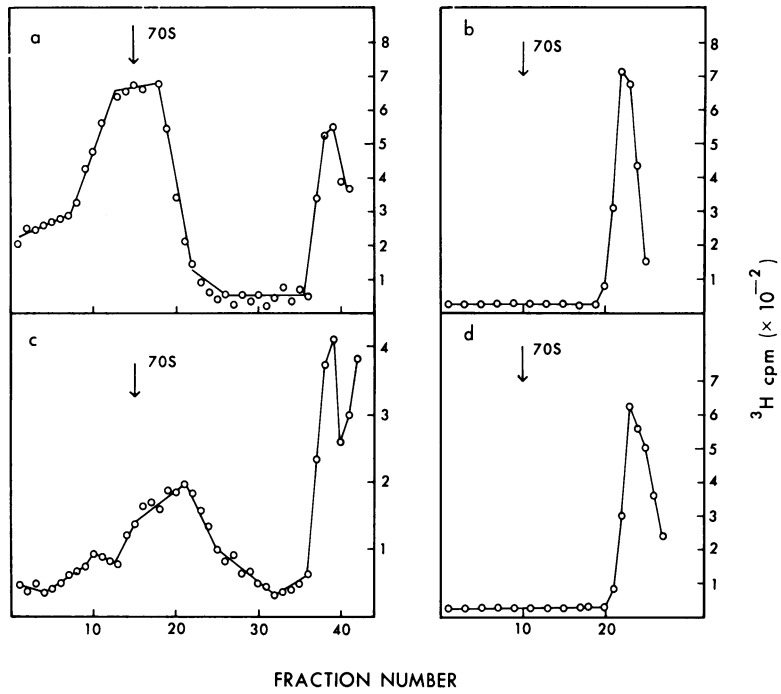

FIG. 7. *Effect of ribonuclease on rapidly sedimenting enzymatic product. Reaction mixtures similar to those described for Fig. 5 and 6 were prepared in duplicate. One reaction was chased with unlabeled TTP at 15 min. After 240 min of incubation, the entire reaction mixtures were extracted and centrifuged through sucrose density gradients. The rapidly sedimenting material was isolated from the gradients, treated with ribonuclease (10 μg/ml, 3 mM ethylenediamine tetraacetic acid), and analyzed by recentrifugation under the same conditions as before. ^{32}P-labeled 70S viral RNA was added directly to the samples before the initial centrifugation, but was centrifuged in a separate bucket for the second analysis to avoid degradation by the ribonuclease. (a) Control reaction. (b) Control reaction; 70S hybrid isolated from the gradient illustrated in (a), treated with ribonuclease, and recentrifuged. (c) Chase reaction. (d) Chase reaction; 70S hybrid isolated from the gradient illustrated in (c), treated with ribonuclease, and recentrifuged.*

approximate the circumstances that follow viral infection. The issue can only be resolved by comparisons between the present experimental system and purified, template-dependent enzymes (*in preparation*).

Sedimentation properties of the DNA:RNA hybrid. The initial product of enzymatic synthesis is a DNA:RNA hybrid which is composed of a small fragment of nascent DNA hydrogen-bonded to 70S viral RNA. The sedimentation velocity of this material is apparently determined solely by the RNA constituent (7). Consequently, the hybrid co-sediments with 70S viral RNA (Fig. 5). As the enzymatic reaction progresses, however, there is a detectable reduction in the sedimentation velocity of at least a portion of the hybrid population (Fig. 5d and e). Moreover, the hybrid which retains label after a prolonged chase has a considerably reduced sedimentation velocity (Fig. 6e). Much of the labeled DNA contained in the latter form of hybrid elutes from hydroxyapatite as if it were double-stranded (Fig. 8g), and we have suggested that this DNA represents branches of partially completed double-strands which are still associated with the hybrid intermediate (Fig. 9). It seems reasonable to expect that the addition of a double-stranded branch to the DNA:RNA hybrid in this manner would retard its sedimentation through sucrose and that the extent of this retardation would be determined by the length and number of double-stranded branches. After a chase period, any label still remaining associated with hybrid structures would be in the double-stranded branches nearest to completion, and this portion of the hybrid population would be most retarded in its sedimentation velocity (Fig. 6e).

Kinetics of DNA synthesis at 0.005% NP-40. RNA tumor viruses must be treated with a nonionic detergent to elicit DNA polymerase activity. The only possible exception to this is the

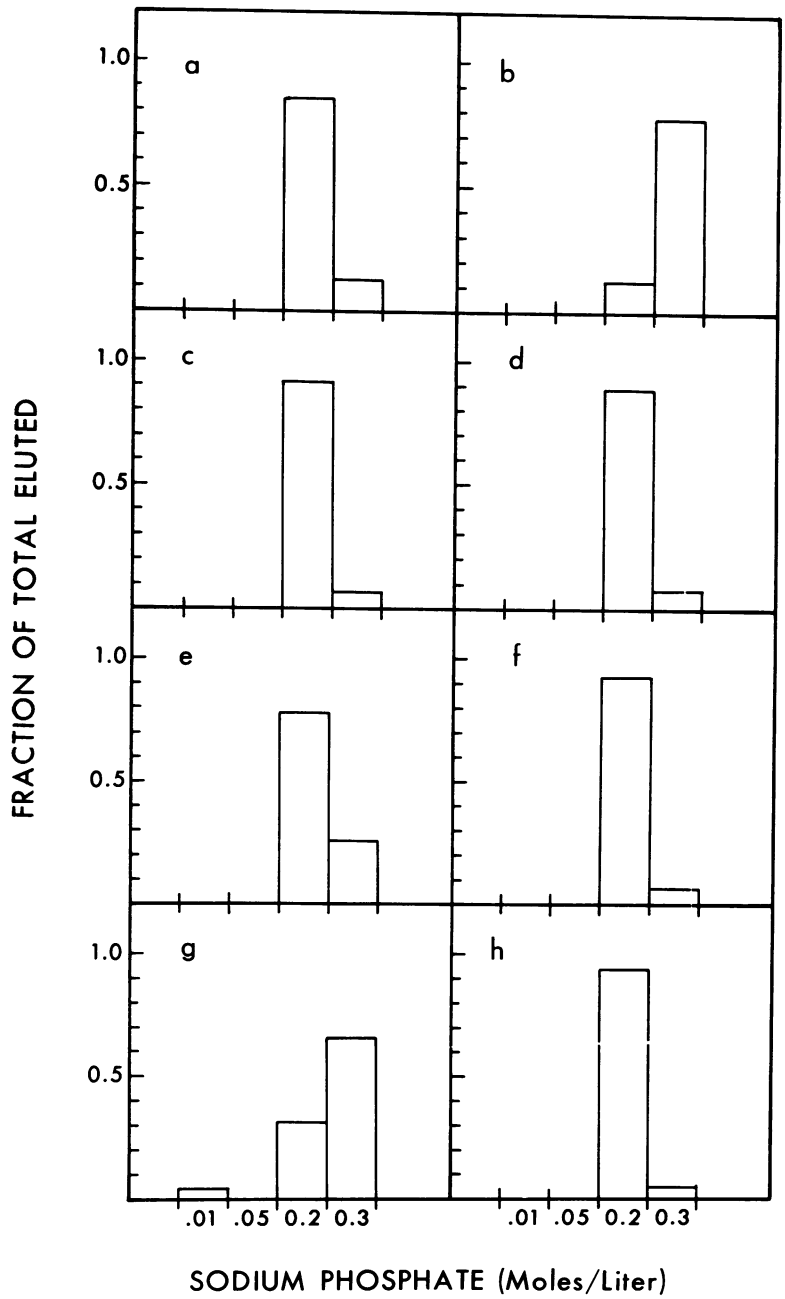

FIG. 8. *Analysis of rapidly sedimenting enzymatic product on hydroxyapatite. Control and chase reactions were performed as described for Fig. 5 and 6. Samples were withdrawn at 15 and 240 min for extraction and centrifugation through sucrose gradients. The results were essentially as illustrated in Fig. 5 and 6. Rapidly sedimenting product was isolated from the gradients, treated with ribonuclease (10 μg/ml, 3 mM ethylenediaminetetraacetic acid, 1 hr, 37 C) to release the DNA from RNA, and analyzed by elution from hydroxyapatite (4). Single-stranded DNA of fd phage and double-stranded avian DNA were included in the analyses as controls (4). (a) Single-stranded fd DNA. (b) Double-stranded DNA from chick fibroblasts. (c) At 15 min, rapidly sedimenting product from either control or chase reaction. The results were identical. (d) Same samples as c but treated with NaOH (0.6 N, 1 hr, 37 C). (e) At 240 min, rapidly sedimenting product from control reaction. (f) Same sample as e but treated with NaOH. (g) At 240 min, rapidly sedimenting product from chase reaction. (h) Same sample as g but treated with NaOH.*

case of virus which has been stored for an appreciable period of time (1). This requirement for detergent probably involves partial or complete disruption of the viral envelope, presumably to allow penetration of the nucleoside triphosphates to the interior of the virion. Consequently, extremely low concentrations of detergent elicit only limited amounts of enzymatic activity (7) and may also appreciably delay the onset of detectable DNA synthesis (Fig. 2). Whether this represents limitation of precursor access to the enzyme or some other structural constraint on enzymatic activity, or both, is presently indeterminate.

TABLE 1. *Nature of the DNA in rapidly sedimenting hybrid*

Determination	Single-stranded DNA (%)	Double-stranded DNA (%)
Control[a]	86	14
Chase[b]	34	66
Chase plus actinomycin[c]	83	17

[a] Rapidly sedimenting hybrid was isolated at 4 hr from a control reaction and analyzed on hydroxyapatite as described for Fig. 8.

[b] Reaction was chased at 15 min with unlabeled thymidine triphosphate (see Fig. 6). Rapidly sedimenting hybrid was isolated and analyzed as in footnote *a*.

[c] Reaction was carried out in the presence of actinomycin D (50 μg/ml) and chased at 15 min. Rapidly sedimenting hybrid was isolated and analyzed as in footnote *a*.

Characteristics of the chase reaction. Addition of large excesses of unlabeled TTP do not immediately arrest the incorporation of ^{3}H-TTP into an acid-precipitable state unless DNA-dependent synthesis is inhibited with a high concentration of actinomycin (Fig. 3). This observation suggests that the RNA-dependent reaction responds to a chase more quickly than does the DNA-dependent reaction, but no explanation for such differential response is apparent at present.

The transition of radioactive DNA from a single- to double-stranded form after a chase is never complete (Fig. 4). We cannot presently explain this observation. It could represent an artifact of the in vitro reaction or a biochemically significant restriction on the extent to which the single-stranded product is copied by viral DNA-dependent polymerase.

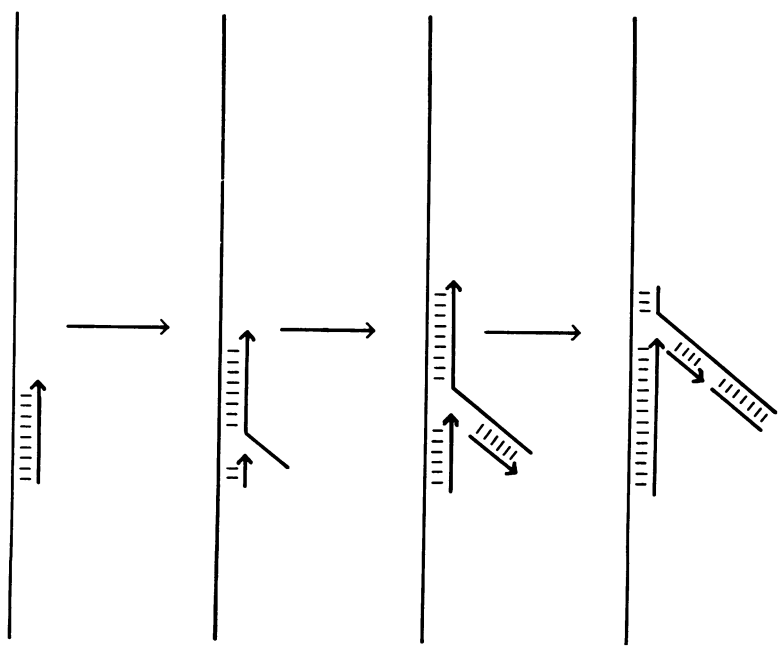

FIG. 9. *Hypothetical model for the synthesis of double-stranded DNA by virion polymerase. Horizontal bars represent hydrogen bonds. The growing termini of nascent DNA chains are indicated by arrowheads. The long bar represents single-stranded RNA template, the transcribed region of which was chosen arbitrarily. DNA synthesis was assumed to be unidirectional.*

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