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Deoxyribonucleic Acid Polymerase Associated with Avian Tumor Viruses: Secondary Structure of the Deoxyribonucleic Acid Product

LOIS FANSHIER, AXEL-CLAUDE GARAPIN, JEROME McDONNELL, ANTHONY FARAS, WARREN LEVINSON, AND J. MICHAEL BISHOP

Department of Microbiology, University of California, San Francisco, California 94122

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The products of the deoxyribonucleic acid (DNA) polymerase associated with Rous sarcoma virus and avian myeloblastosis virus were characterized by correlative analyses with equilibrium centrifugation and stepwise elution from hydroxyapatite. The initial enzymatic product consists of nascent DNA chains which are hydrogen-bonded to 70*S* viral ribonucleic acid (RNA), whereas the final enzymatic product is double-stranded DNA. Appreciable amounts of free single-stranded DNA were not detected at any point during the course of the enzymatic reaction, but the data in this regard are not decisive. The time course of synthesis of DNA:RNA hybrids and double-stranded DNA has been analyzed. It is concluded that the synthesis of double-stranded DNA is a sequel to and is probably dependent upon the synthesis of DNA:RNA hybrid.

A novel deoxyribonucleic acid (DNA) polymerase, which apparently transcribes DNA from a ribonucleic acid (RNA) template, has been discovered in association with the virions of RNA tumor viruses (1, 13). It has been suggested that the DNA synthesized by this enzyme serves as an intermediate in viral RNA replication, and that it is integrated into host DNA to provide for stable transformation (1, 12, 13). In view of these hypotheses, the precise nature of the enzymatic product is of substantial biological interest. Previous reports (6, 11) have established the fact that the enzyme initially synthesizes DNA:RNA hybrids. These are composed of nascent DNA chains hydrogen-bonded to molecules of their putative template, the 70*S* viral RNA. As the reaction progresses, DNA which is not associated with RNA also accumulates, eventually becoming the predominant product of enzymatic synthesis (6). On the presumption that this latter DNA may represent the biologically active form of enzymatic product, we have attempted to characterize its secondary structure by correlative analyses with equilibrium centrifugation and hydroxyapatite. We conclude that hybrid and nonhybrid DNA can be distinguished by use of a convenient batch-elution procedure with hydroxyapatite, and that the principal final product of the enzymatic reaction is double-stranded DNA. These studies have been performed with both the Schmidt-Ruppin strain of Rous sarcoma virus (RSV) and avian myeloblastosis virus, with essentially identical results. Unless otherwise stated, the illustrated results pertain to RSV.

MATERIALS AND METHODS

Reagents. ^{3}H-thymidine triphosphate (^{3}H-TTP), 10 to 12 Ci/mmole, was obtained from Schwarz BioResearch, Inc., or New England Nuclear Corp.

Pancreatic ribonuclease A was obtained from Worthington Biochemical Corp. Stock solutions were boiled for 10 min to inactivate contaminating deoxyribonuclease.

Pronase (B grade) and the deoxyribonucleoside triphosphates deoxyadenosine, deoxycytidine, and deoxyguanosine triphosphate (dATP, dCTP, and dGTP) were obtained from Calbiochem.

Phenol (reagent grade) was from Mallinckrodt Chemical Works; hydroxyapatite (Bio-Gel HT), from Bio-Rad Laboratories, Richmond, Calif.; Nonidet-P 40 (NP-40), from Shell Chemical Co.; and Cs_2SO_4 (optical grade), from Gallard-Schlesinger.

Sodium phosphate buffer was used at *p*H 7.8, and concentrations were standardized by use of refractive index.

Propagation and purification of virus. RSV, Schmidt-Ruppin strain, and avian myeloblastosis virus were prepared as described previously (4, 6). The protein content of purified virus (generally 1.0 to 1.5 mg/ml) was determined by the method of Lowry et al. (7).

Enzyme reaction. DNA polymerase associated with

RSV was assayed as described previously (6). Standard reaction mixtures contained the following: 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 8.1; 0.01 M $MgCl_2$; 100 to 500 μg of viral protein per ml; 6×10^{-5} M dATP, dGTP, and dCTP; 2% β-mercaptoethanol (v/v); 10^{-6} to 5×10^{-6} M ^{3}H-TTP; and an appropriate concentration of NP-40, determined individually for each preparation of purified virus. As noted previously (6), the optimal concentration of detergent is apparently a function of the concentration of viral protein in the reaction mixture.

Preparation of enzymatic product. Nucleic acids were routinely extracted from reaction mixtures by the addition of sodium dodecyl sulfate (0.5%, w/v) and self-digested Pronase (500 μg/ml), followed by incubation at 37 C for 45 min and two phenol extractions at room temperature. When necessary, the nucleic acids were precipitated with ethyl alcohol; unlabeled HeLa cell RNA was used as carrier (4). On occasion, the phenol extraction was omitted and the nucleic acids were analyzed directly on hydroxyapatite after dilution of the sodium dodecyl sulfate to 0.025%. This variation has no apparent effect on the adsorption and elution properties of the nucleic acids.

Analysis of nucleic acid secondary structure with hydroxyapatite. Chromatography on hydroxyapatite provides a convenient means by which to ascertain the secondary structure of DNA (2, 3, 9). The following batch procedure was employed to facilitate the processing of relatively large numbers of samples. For present purposes, it provided essentially the same information as would column chromatography, but allowed the analysis of as many as 15 samples in a period of 3 to 4 hr. Commercially available hydroxyapatite proved entirely satisfactory for the procedure. Slight variations in the elution properties of DNA were noted among different lots of hydroxyapatite and sodium phosphate buffers, but this is of little consequence because appropriate nucleic acid standards were included in every batch of analyses. The capacity of hydroxyapatite used in these experiments was approximately 1 mg of nucleic acid per ml of packed volume.

Nucleic acids (minimum of 2,000 counts/min) were adsorbed to 0.5 ml (packed volume) of hydroxyapatite by shaking or mechanical agitation in conical centrifuge tubes at room temperature for 5 min. When possible, adsorption was performed in 0.01 M sodium phosphate. However, the presence of sodium chloride (0.2 M) and ethylenediaminetetraacetate (EDTA, 2×10^{-3} M) had no effect on either the adsorption or subsequent elution of nucleic acids.

Nucleic acids were eluted from the hydroxyapatite by successive washes (each 2 ml) with sodium phosphate solutions of increasing concentrations. A full elution series utilized 0.05 M steps from 0.05 to 0.3 M, with two separate washes at each step. The 0.05 M washes were performed at room temperature for 5 min; all subsequent washes were at 55 C for 8 min (10). The hydroxyapatite was kept in suspension by intermittent agitation with a Pasteur pipette. At the conclusion of each wash, the hydroxyapatite was sedimented by centrifugation at 2,000 rev/min (Sorvall RC-3) for 1 min at room temperature. The clear supernatant was withdrawn, and the next sodium phosphate solution was added. Supernatants from each wash were analyzed for acid-precipitable radioactivity as described previously (4). The radioactivity eluted by the two washes at each concentration of phosphate was summed and expressed as the fraction of total radioactivity eluted by the entire series of washes. Recovery of nucleic acid (whether RNA, DNA, or DNA:RNA hybrid) was always greater than 90%.

Preparation of nucleic acid standards. The method used to isolate 70*S* RNA from purified RSV was described previously (4). DNA, labeled with ^{3}H-thymidine, was extracted from chick embryo fibroblasts according to the method of Martin (8), and was used without further fractionation. It contained double-stranded DNA with molecular weights of 10^7 to 3×10^7 daltons, as determined by the method of Burgi and Hershey (5); the native DNA (molecular weight, 2.8×10^7) of lambda phage was used as a sedimentation reference. ^{32}P-labeled lambda phage DNA and ^{3}H-labeled single-stranded DNA of fd phage (molecular weight, 2×10^6) were kindly provided by D. Roulland-Dussoix. The preparation of fd DNA used in these experiments had been largely converted from the native circular form to linear molecules by radioautolysis.

Analysis of nucleic acids by ultracentrifugation. Rate-zonal sedimentation through sucrose gradients and equilibrium centrifugation in Cs_2SO_4 have been described previously (6). Recoveries of nucleic acids from Cs_2SO_4 ranged between 50 and 90% provided that the gradients contained at least 0.001 M EDTA, that centrifugation was carried out in polyallomer tubes, and that optical-grade Cs_2SO_4 was used. We have observed no indication that the loss of nucleic acids in Cs_2SO_4 is in any manner selective with respect to DNA, RNA, and DNA:RNA hybrids.

RESULTS

Fractionation of single- and double-stranded DNA on hydroxyapatite: validation of the batch-elution procedure. The elution from hydroxyapatite of various single- and double-stranded DNA preparations is illustrated in Fig. 1. Native chick DNA eluted primarily in 0.25 M phosphate (Fig. 1a). If this eluate was recycled through a second series of adsorption and elutions, no DNA eluted prior to the 0.25 M phosphate wash. Thus, the small amount of DNA in the initial 0.1 to 0.15 M phosphate eluates (Fig. 1a) is probably single-stranded DNA rather than an artifact intrinsic to the adsorption-elution process. The effect of molecular weight on the elution of chick DNA was also examined. Native DNA, sheared to an average molecular weight of 10^6, eluted in a manner which is virtually identical to that of the unsheared DNA (Fig. 1b).

Denatured DNA of lambda phage (Fig. 1c) and chick fibroblasts (not illustrated) eluted

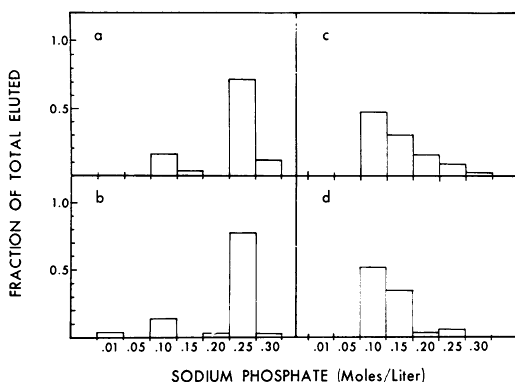

FIG. 1. *Fractionation of single- and double-stranded DNA on hydroxyapatite. DNA was prepared and analyzed on hydroxyapatite as described in Materials and Methods. (a) Native DNA from chick embryo fibroblasts, labeled with ^{3}H-thymidine. Molecular weight of this DNA was approximately 10^{7} to 3×10^{7} daltons. (b) Native DNA from chick fibroblasts as in (a), but sheared by sonic treatment to an average molecular weight of 10^{6} daltons. (c) ^{32}P-labeled DNA from lambda phage, denatured by boiling in 0.015 M NaCl-0.0015 M sodium citrate, pH 7, for 15 min, followed by quenching in an ice bath. (d) ^{3}H-labeled single-stranded DNA from fd phage.*

predominantly in 0.1 to 0.15 M phosphate. The small amounts of DNA eluting in 0.2 and 0.25 M phosphate probably represent partially renatured material. On recycling of the 0.1 and 0.15 M eluates, no nucleic acid was found in any of the washes containing more than 0.15 M phosphate. The single-stranded DNA of fd phage eluted almost entirely in 0.1 to 0.15 M phosphate (Fig. 1d). Consequently, it is a more convenient and predictable standard for the procedure than are the denatured DNA preparations. We have not examined the effect of molecular topology on elution; therefore, to avoid any uncertainty in this regard, we deliberately chose fd DNA which was primarily linear (see Materials and Methods).

The results illustrated in Fig. 1 have proven to be consistent throughout the course of our investigation. Only relative variations have been observed: if double-stranded DNA elutes primarily in 0.3 M phosphate (rather than in 0.25 M, as in Fig. 1), single-stranded DNA will inevitably elute predominantly in 0.15 M phosphate rather than in the manner illustrated in Fig. 1. No appreciable elution of single-stranded DNA in phosphate concentrations greater than 0.15 M has ever been observed. These relative variations necessitate the inclusion of both single- and double-stranded DNA standards in every batch of analyses, but do not otherwise impair the reliability of the procedure.

Elution of DNA:RNA hybrid from hydroxyapatite. The initial product of DNA synthesis by the virion-associated polymerase is a DNA:RNA hybrid (6, 11), consisting of short, nascent DNA chains, hydrogen-bonded to viral RNA (6, 11; *in preparation*). This material is first detected as DNA which co-sediments with 70*S* viral RNA during rate-zonal centrifugation (6; also, see Fig. 8), and which has a buoyant density identical to that of single-stranded RNA (6, 11). Because the 70*S* DNA:RNA complex is a major constituent of early enzymatic product (6), it was mandatory to examine the elution of this material from hydroxyapatite prior to applying the batch-elution procedure to unfractionated product. Purified complex elutes from hydroxyapatite in a manner quite similar to that of isolated 70*S* viral RNA (Fig. 2a and b). We attribute this observation to the fact that each molecule of 70*S* DNA:RNA hybrid apparently contains a large segment of RNA which is not associated with DNA (6). Consequently, the hybrid possesses physicochemical properties similar to those of 70*S* viral RNA, i.e., similar sedimentation coefficient and buoyant density (6, 11), and similar elution from hydroxyapatite.

Treatment of the 70*S* complex with ribonuclease in 0.3 M NaCl produces a DNA:RNA hybrid with a low sedimentation velocity (ca. 4*S*; *in preparation*) and approximately equimolar contents of DNA and RNA as judged by buoyant density (6). Presumably, only the RNA which is directly hydrogen-bonded to DNA escapes hydrolysis by the ribonuclease (14). Hybrid treated in this manner eluted from hydroxyapatite in a manner similar but not identical to that of single-stranded DNA (Fig. 2c); the single-stranded DNA control in this assay was identical to that illustrated in Fig. 1d. Ribonuclease treatment in 3 mM EDTA, a condition which would be expected to hydrolyze even the hydrogen-bonded RNA (14), resulted in only a slightly greater proportion of DNA eluting in 0.1 M phosphate (Fig. 2d). We cannot presently explain the absence of a more distinctive difference between the elutions of the materials treated with ribonuclease in high and low concentrations of salt, but the matter is of no major consequence to our present purpose. The important fact is that ribonuclease treatment effectively removes all hybrid DNA from the region of double-stranded DNA elution. Thus, ribonuclease treatment prior to the hydroxyapatite assay should allow an unambiguous assessment of the amount of double-stranded DNA present in enzymatic product. The utility of this maneuver will be further illustrated below.

Evolution of enzymatic product: correlative analysis with equilibrium centrifugation and

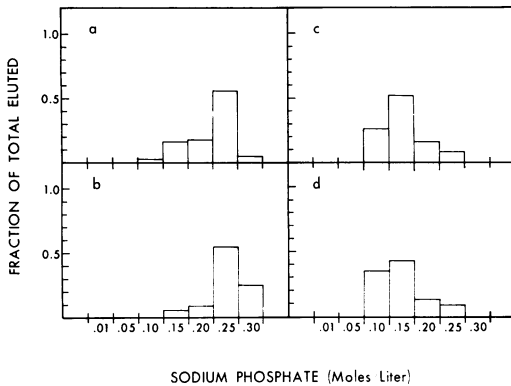

FIG. 2. *Elution of viral RNA and DNA:RNA hybrid from hydroxyapatite.* ^{32}*P-labeled 70S viral RNA was isolated from purified virus by phenol extraction and zonal centrifugation as described previously (4). DNA: RNA complex (70S) was prepared from a 1-hr enzymatic reaction. The product was extracted with sodium dodecyl sulfate-Pronase-phenol as described under Materials and Methods, and centrifuged through a 15 to 30% sucrose gradient containing 0.1 M NaCl-0.001 M EDTA-0.02 M Tris-hydrochloride, pH 7.4 (SW 65 rotor, 64,000 rev/min, 70 min, 4 C). The DNA co-sedimenting with 70S viral RNA was isolated from the gradient and stored frozen (−20 C) prior to analysis on hydroxyapatite. Adsorption to and elution from hydroxyapatite were not influenced by the presence of NaCl, EDTA, or sucrose. Appropriate control analyses were performed with single- and double-stranded DNA, but are not illustrated. The results were essentially as shown in Fig. 1. (a)* ^{3}*H-labeled 70S DNA:RNA complex, synthesized by RSV-associated DNA polymerase. (b)* ^{32}*P-labeled 70S viral RNA. (c) 70S DNA: RNA complex, treated with ribonuclease (0.1 μg/ml) in 0.3 M NaCl at room temperature for 30 min. After ribonuclease treatment, the sample was adsorbed directly onto hydroxyapatite. Adsorption under identical conditions had no effect on the subsequent elution of single- and double-stranded DNA controls. (d) 70S DNA:RNA complex, treated with ribonuclease (10 μg/ml) in 3 mM EDTA at 37 C for 1 hr. Thereafter, the sample was adsorbed directly to hydroxyapatite. These conditions had no effect on the adsorption and elution of appropriate DNA controls.*

hydroxyapatite. We previously reported that the early product of enzymatic synthesis consists primarily of DNA:RNA hybrid, whereas the late product contains DNA which is not associated with RNA (6). Figure 3 illustrates a more detailed analysis of the progress of the enzymatic reaction, obtained by use of equilibrium centrifugation in Cs_2SO_4. After 15 min of synthesis (Fig. 3a), the product consisted almost exclusively of DNA which had a buoyant density virtually identical to that of single-stranded RNA. This observation conforms to our previous report that the initial product is a 70*S* DNA:RNA complex with the same buoyant density as single-stranded RNA (6). The effect of ribonuclease treatment (in 0.3 M NaCl) on early (30 min) product is shown in Fig. 3b. The broad, symmetrical band with a mean density of 1.54 g/cc presumably represents the low-molecular-weight, equimolar hybrid of DNA:RNA obtained from the 70*S* complex as described above (Fig. 2). Hybrid material still predominated at 60 min (Fig. 3c), although there had been some reduction in the mean buoyant density. The latter change must reflect a decrease in the RNA-DNA ratio of the hybrid molecules, and could be due to lengthening of the nascent DNA chains or degradation by ribonuclease, or to a combination of these factors. We have data which indicate that there is considerable ribonuclease activity in the enzyme preparations under study in our laboratory (*in preparation*). Moreover, we presently have no evidence for the existence of other than short nascent DNA chains associated with viral RNA. Consequently, we must for the present ascribe all reductions in the buoyant density of hybrid product to degradative enzyme intrinsic to the reaction mixture.

After 4 hr of synthesis (Fig. 3d), the product consisted of two discrete populations, one banding in the DNA region of the density gradient (density ca. 1.45 g/cc) and the other corresponding to DNA:RNA hybrid (density ca. 1.54 g/cc). These data conform to our previous contention that at least two discrete enzymatic activities are operative (6, 7*a*), one producing the initial hybrid product and the other synthesizing nonhybrid DNA. However, the secondary structure of this latter DNA cannot be ascertained from its buoyant density, a fact which prompted the development and use of the hydroxyapatite assay.

Portions of the samples used in the equilibrium centrifugations illustrated by Fig. 3 were analyzed on hydroxyapatite (Fig. 4). Two primary conclusions can be drawn from these data. First, at no time did a significant proportion of the DNA elute in 0.1 M sodium phosphate. We therefore suggest that the product does not contain appreciable quantities of single-stranded DNA. The material which eluted in 0.15 M phosphate and which predominated early in the reaction must represent the hybrid molecules identified in equilibrium density gradients (Fig. 3a and c). The discrepancy between the elution pattern of this material and that of isolated 70*S* complex (Fig 2b) is probably due to partial degradation, as suggested by the results of equilibrium centrifugation (Fig. 3) and discussed above. Whatever the explanation of this discrepancy, it seems likely that the DNA eluting in 0.15 M phosphate represents (or has been derived from) hybrid structures, be-

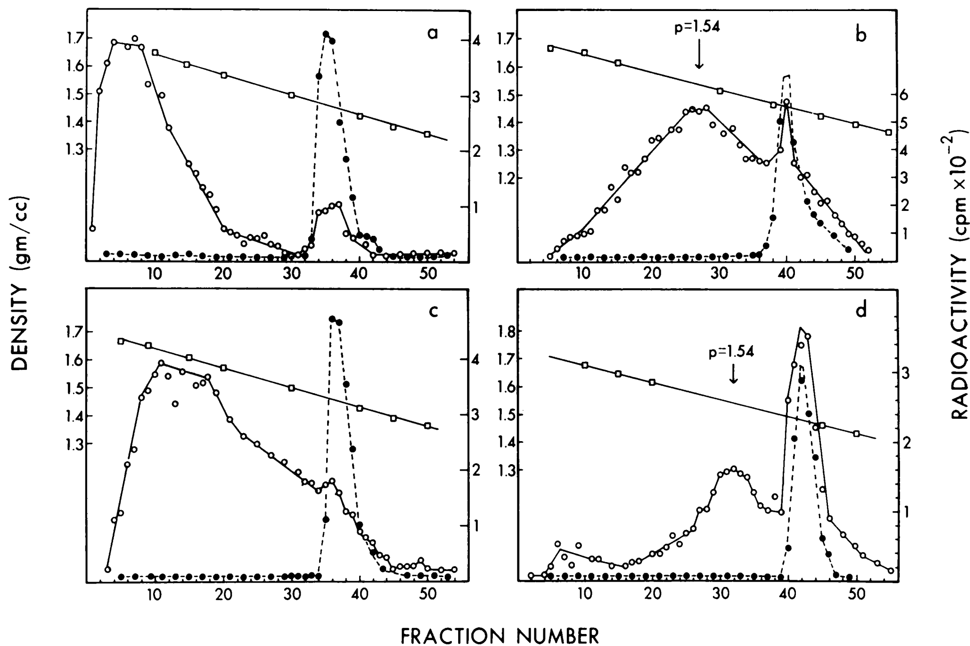

FIG. 3. *Equilibrium centrifugation of enzymatic product from various time points. A standard reaction mixture containing 500 μg of viral protein, was prepared as described in Materials and Methods and incubated at 37 C At the various times, samples were withdrawn for extraction with sodium dodecyl sulfate-Pronase-phenol. Residua phenol was removed by three extractions with ether, and portions of each sample were analyzed by equilibrium centrifugation in Cs_2SO_4. ^{32}P-labeled lambda phage DNA was used as a density marker.* (○) *^{3}H-labeled product;* (●) *^{32}P-labeled lambda DNA. (a) Fifteen-minute product. (b) Thirty-minute product, treated with ribonuclease (0.1 μg/ml) in 0.3 M NaCl at room temperature for 30 min. Prior to centrifugation, the sample was re-extracted twice with phenol and three times with ether. (c) One-hour product. (d) Four-hour product.*

cause no other form of DNA is apparent in equilibrium density gradients until after 60 min (Fig. 3). [The small amount of product DNA found in the region of lambda DNA at early time points (Fig. 3a and b) is an occasional finding of uncertain significance. Note that in the series of analyses illustrated by Fig. 3 virtually none of this material is visible at 1 hr (Fig. 3c). Both denatured lambda DNA and denatured enzymatic product band at approximately 1.48 g/cc in these gradients.] Second, appreciable quantities of double-stranded DNA (eluting in 0.25 M phosphate) began to appear at about 2 hr. At 4 hr, over 30% of the total product eluted in 0.25 M phosphate (Fig. 4f). By comparison with the results of equilibrium centrifugation (Fig. 3d), we conclude that the DNA banding at a density of approximately 1.45 g/cc consists mainly of double-stranded molecules. This issue will be examined further below.

Time course of accumulation of double-stranded DNA: use of a modified hydroxyapatite assay. After treatment with ribonuclease, the elution of the DNA:RNA hybrid enzymatic product from hydroxyapatite no longer overlapped that of double-stranded DNA (Fig. 2). This observation, and the fact that double-stranded DNA eluted almost entirely in 0.25 to 0.3 M phosphate, allowed a convenient abbreviation of the hydroxyapatite assay. Samples of nucleic acid were treated with ribonuclease in low concentrations of salt (usually 3 mM EDTA), and then adsorbed directly onto hydroxyapatite. Elutions were performed with 0.05, 0.20, and 0.30 M phosphate. Material eluting in 0.2 M phosphate was considered to be hybrid or single-stranded DNA, or both (Fig. 5a and c), whereas the 0.3 M phosphate eluate contained essentially all of the double-stranded DNA (Fig. 5b). The procedure does not discriminate between hybrid molecules and single-stranded DNA, but this is of no consequence because the product apparently contains insignificant amounts of single-stranded DNA (Fig. 4). Figures 5d-h illustrate the use of this procedure to analyze the accumulation of enzymatic products. Hybrid DNA (eluting in 0.2 M phosphate) was the exclusive product until approximately 2 hr, after which double-stranded DNA accumulated steadily. Late enzymatic

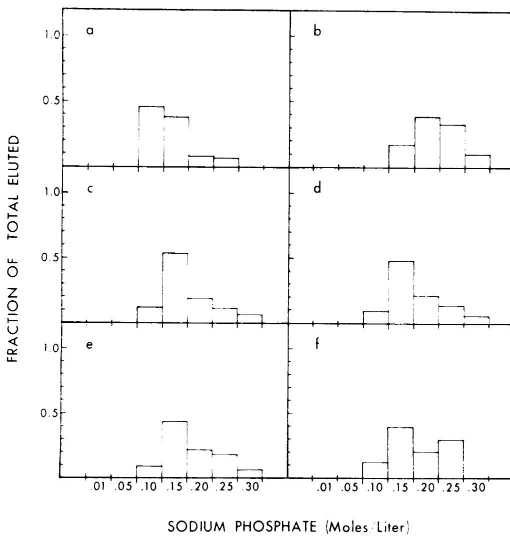

FIG. 4. *Analysis of enzymatic product on hydroxyapatite. Portions of the samples prepared as described in Fig. 3 were analyzed on hydroxyapatite. Controls included single-stranded fd DNA, the 70S DNA:RNA complex isolated as described for Fig. 2, and double-stranded DNA (the last is not illustrated; the results were identical to those shown in Fig. 1). None of the samples was treated with ribonuclease. (a) Single-stranded DNA of fd phage; (b) 70S DNA:RNA complex; (c) 30-min product; (d) 1-hr product; (e) -hr product; (f) 4-hr product.*

product was at least 70% double-stranded DNA (Fig. 5h), a more detailed analysis of which is presented below.

Final product of the enzymatic reaction is double-stranded DNA. After 12 hr of enzymatic synthesis, no hybrid molecules were detectable in the product by equilibrium centrifugation (Fig. 6a). Analysis of this late DNA on hydroxyapatite is illustrated in Fig. 7. The bulk (70%) of the material eluted in 0.25 M phosphate, in contrast to the double-stranded DNA control, a major portion of which eluted in 0.3 M phosphate in this particular assay (Fig. 7b). We attribute this difference to the extremely low molecular weight of the DNA product (6; also, see Fig. 8). Ribonuclease treatment had no effect on the elution pattern (Fig. 7d), a fact which indicates that hybrid material was not contributing to the nucleic acid eluted in 0.25 M phosphate. Denaturation of late product with either heat or alkali caused it to elute as single-stranded DNA (see Fig. 1d and 4a). We conclude that the final enzymatic product consists primarily of double-stranded DNA, the synthesis of which is initiated subsequent to the production of significant quantities of DNA:RNA hybrid.

Analysis of low-molecular-weight enzymatic product on hydroxyapatite. In addition to the 70S DNA:RNA complex described above, prolonged enzymatic reactions produced low-molecular-weight DNA with sedimentation coefficients of 4

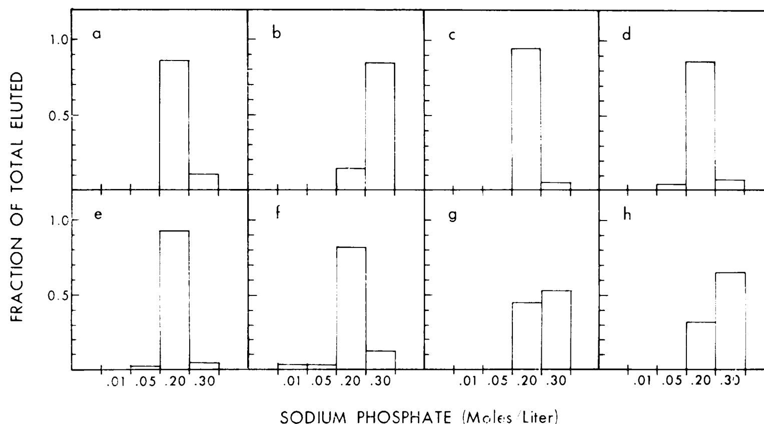

FIG. 5. *Abbreviated hydroxyapatite analysis of enzymatic product. Samples of a standard enzymatic reaction were extracted at various time points as described for Fig. 3. Small portions of these were diluted into 3 mM EDTA (maximum NaCl concentration, 0.01 M after dilution) and treated with ribonuclease (10 μg/ml) for 1 hr at 37 C prior to analysis on hydroxyapatite. All controls were treated with ribonuclease in the same manner. Nucleic acids were eluted from hydroxyapatite with successive washes of 0.05, 0.20, and 0.30 M sodium phosphate as described under Materials and Methods. (a) Single-stranded fd DNA; (b) double-stranded chick DNA; (c) 70S DNA:RNA complex, purified as described for Fig. 2; (d) 30-min product; (e) 1-hr product; (f) 2-hr product; (g) 4-hr product; (h) 12-hr product.*

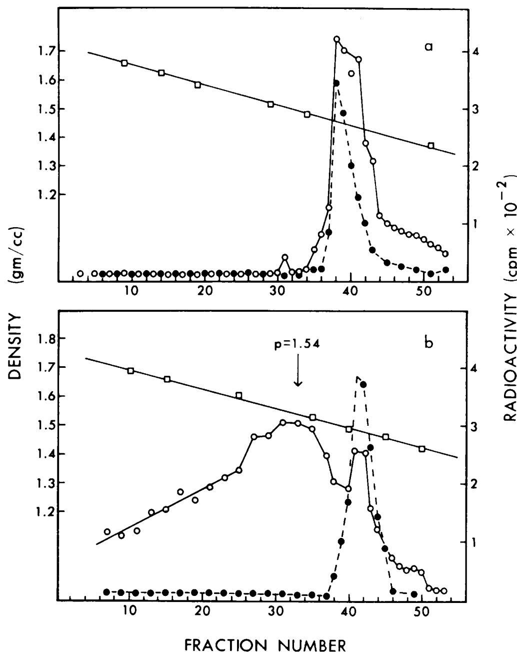

FIG. 6. *Analysis of enzymatic product by equilibrium centrifugation in* Cs_2SO_4. *(a) Avian myeloblastosis virus, 12-hr product. Nucleic acids were extracted from a standard reaction mixture [supplemented with phosphoenol pyruvate and pyruvate kinase (6)] after 12 hr of incubation. The energy-generating system has no effect on the nature of the product at any point in the reaction, but prolongs enzymatic activity by a factor of at least two (6). The nucleic acids were centrifuged to equilibrium in* Cs_2SO_4, *with* ^{32}P*-labeled lambda phage DNA as a density marker.* (○) ^3H*-labeled product;* (●) ^{32}P*-labeled lambda DNA. (b) Low-molecular-weight product at 1 hr. Nucleic acids were extracted from a standard reaction mixture at 1 hr, precipitated with ethyl alcohol, and separated into 70S complex and low-molecular-weight DNA by rate-zonal centrifugation through a 15 to 30% sucrose gradient (see Fig. 8). The DNA sedimenting at approximately 4 to 15S was recovered from the gradient and analyzed by equilibrium centrifugation in* Cs_2SO_4. ^{32}P*-labeled lambda phage DNA was used as a density marker.* (○) ^3H*-labeled product;* (●) ^{32}P*-labeled lambda DNA.*

to 15*S* (Fig. 8; also *in preparation*). As the reaction proceeded, the accumulation of low-molecular-weight DNA surpassed that of 70*S* complex, so that the former became the predominant enzymatic product by 2 hr (Fig. 8 and 9). Analyses with equilibrium centrifugation (Fig. 6b) and hydroxyapatite (Fig. 10 and 11) indicated that the low-molecular-weight material isolated by zonal centrifugation is composed of both double-stranded DNA and substantial amounts of hybrid. In fact, a sample taken at 30 min contained virtually no double-stranded DNA (Fig. 10a). As the reaction progressed, double-stranded DNA accumulated in a manner similar to that illustrated in Fig. 5. At early time points, the low-molecular-weight population geneıally contained proportionately more double-stranded DNA than did the unfractionated product (compare Fig. 10b and c to Fig. 5e and f) because of the absence of 70*S* hybrid from the isolated low-molecular-weight DNA. Nevertheless, DNA:RNA hybrids were present in the low-molecular-weight population, as indicated by the elution of radioactivity in 0.15 and 0.20 M phosphate, and by the fact that the elution profile changed after ribonuclease treatment (Fig. 11), with an increase in the DNA eluting in 0.10 to 0.15 M phosphate and a concomitant decrease in the amount of DNA in the 0.20 M eluate.

DISCUSSION

The exact function of the DNA polymerase associated with RNA tumor viruses has yet to be established, but it is generally assumed that some or all of the DNA synthesized by this enzyme becomes integrated into host DNA in order to provide for stable transformation (12). If this be the case, then the secondary structure of the final enzymatic product is of crucial importance because it would determine the mechanism by which integration is accomplished. For example,

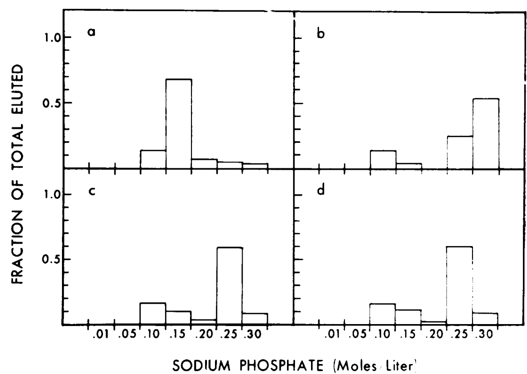

FIG. 7. *Analysis of 12-hr enzymatic product on hydroxyapatite. A portion of the 12-hr product (avian myeloblastosis virus) used for the equilibirum centrifugation illustrated in Fig. 6a was analyzed on hydroxyapatite. (a) Single-stranded fd phage DNA; (b) double-stranded chick DNA; (c) 12-hr enzymatic product; (d) 12-hr enzymatic product, treated with ribonuclease. A sample of the extracted product was diluted into 3 mM EDTA and treated with ribonuclease (10 μg/ml, 1 hr, 37 C) prior to analysis on hydroxyapatite.*

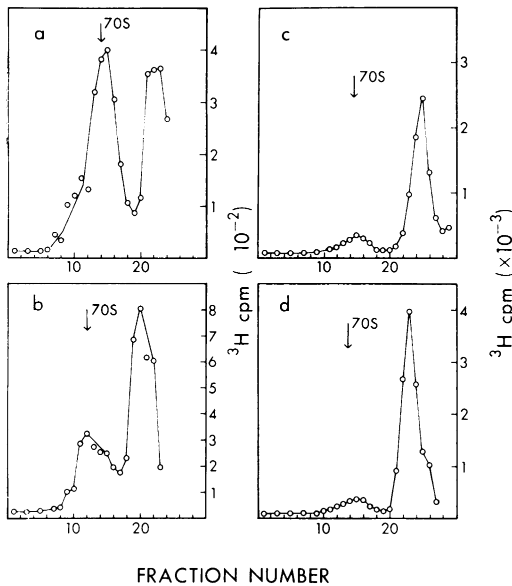

FIG. 8. *Zonal centrifugation of enzymatic product. Nucleic acids were extracted from a standard reaction mixture (containing 500 μg of viral protein) at various time points, precipitated with ethyl alcohol, and sedimented through gradients of 15 to 30% sucrose containing 0.1 M NaCl-0.001 M EDTA-0.02 M Tris-hydrochloride, pH 7.4 (SW 65 rotor, 60,000 rev/min, 70 min, 4 C). Gradient fractions were analyzed for acid precipitable radioactivity as described previously (6). The rapidly sedimenting DNA has previously been identified as a hydrogen-bonded complex of nascent DNA and viral RNA (6). The slowly sedimenting population (4 to 15S) was recovered from the gradients for further analysis (Fig. 6b and 10). ^{32}P-labeled 70S RSV RNA was used as a sedimentation marker. (a) Thirty-minute product. (b) One-hour product. (c) Two-hour product. (d) Four-hour product.*

a double-stranded product might require no further biochemical modification prior to integration by normal mechanisms of recombination. The potential biological significance of this issue prompted us to examine the secondary structure of polymerase product in some detail. Our data indicate that the principal final product of the enzymatic reaction (in the case of RSV and avian myeloblastosis virus) is double-stranded DNA. Appreciable amounts of single-stranded DNA could not be detected at any point during the course of the reaction, although the data in this regard are not decisive.

Synthesis of double-stranded DNA is preceded by the synthesis of DNA:RNA hybrids which presumably represent intermediates in the enzymatic reaction. The precise mechanism by which double-stranded DNA is synthesized has not yet been elucidated, but we have shown that the

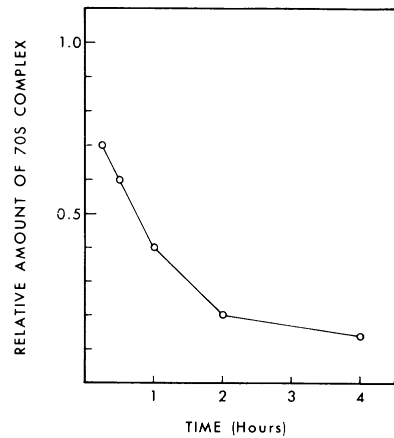

FIG. 9. *Proportions of 70S DNA:RNA complex and low-molecular-weight DNA at various time points in the polymerase reaction. Enzymatic product from various time points was analyzed by zonal centrifugation as in Fig. 8. The radioactivity contained in the 70S peak and that in the low-molecular-weight population were summed, and the ratio of the two was computed for each time point. The change in ratio as the reaction progresses is not due simply to cessation of the production of 70S complex, because there is an absolute increase in the latter for at least 2 to 3 hr (unpublished observations of the authors).*

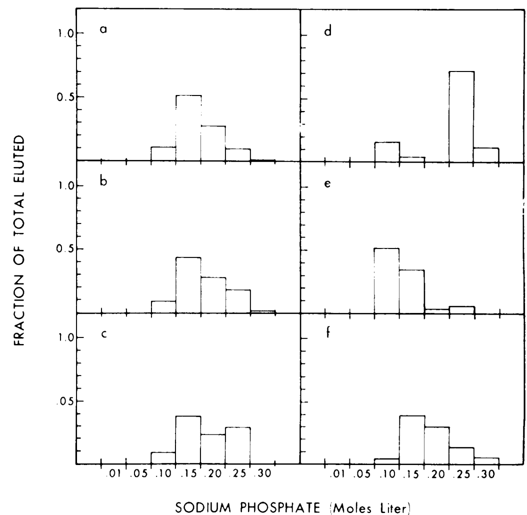

FIG. 10. *Analysis of low-molecular-weight product on hydroxyapatite. DNA sedimenting at approximately 4 to 15S was recovered from sucrose gradients similar to those illustrated in Fig. 8 and analyzed on hydroxyapatite. (a) Thirty-minute product. (b) One-hour product. (c) Two-hour product. (d) Double-stranded chick DNA. (e) Single-stranded fd DNA. (f) 70S DNA:RNA complex, prepared as described for Fig. 2.*

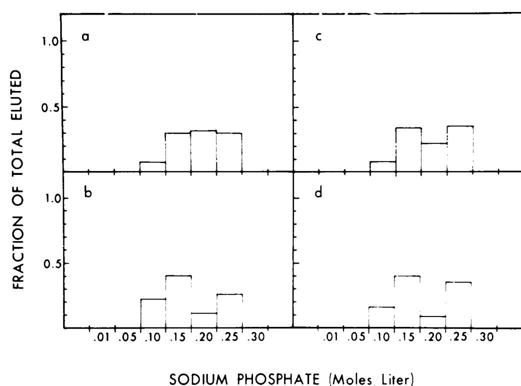

FIG. 11. *Analysis of ribonuclease-treated low-molecular-weight product on hydroxyapatite. DNA sedimenting at approximately 4 to 15S was recovered from sucrose gradients similar to those illustrated in Fig. 8, and was analyzed on hydroxyapatite before and after treatment with ribonuclease (10 μg/ml, 3 mM EDTA, 1 hr, 37 C). (a) Two-hour product, control. (b) Two-hour product, ribonuclease-treated. (c) Four-hour product, control. (d) Four-hour product, ribonuclease-treated.*

responsible enzyme can be inhibited by actinomycin D, and that it is capable of using exogenous DNA (either single- or double-stranded) as template (7*a*; *unpublished data*). In the absence of exogenous template, the synthesis of double-stranded DNA is totally dependent upon the synthesis of DNA:RNA hybrid. Thus, if synthesis of the latter is inhibited by treatment of the enzyme complex with ribonuclease prior to initiation of the reaction, the synthesis of double-stranded DNA is also inhibited (7*a*).

The initial product of the enzymatic reaction is a hydrogen-bonded complex of nascent DNA and viral RNA, with a sedimentation coefficient of approximately 70*S* (6). As the reaction progresses, substantial amounts of slowly sedimenting (4 to 15S) DNA:RNA hybrids accumulate (Fig. 10). This material contains only short (ca. 4*S*) chains of DNA (*in preparation*), and, for the present, we must assume that it is derived from the 70*S* hybrids by the degradative action of ribonuclease which is intrinsic to the virion-enzyme complex (*in preparation*). We have yet to determine whether or not these low-molecular-weight hybrids are active as templates for the synthesis of double-stranded DNA.

In its native state, the 70*S* hybrid cannot be reliably differentiated from double-stranded DNA by analysis on hydroxyapatite (Fig. 2). This observation is at variance with previous reports which described the successful resolution of DNA:RNA hybrids from both single- and double-stranded DNA by chromatography on hydroxyapatite (10). We ascribe this discrepancy to the fact that the 70*S* hybrid contains a very small proportion of DNA, and the nature of its elution is therefore determined by the RNA constituent. Consequently, the intact hybrid molecule elutes in a manner quite similar to that of isolated 70*S* viral RNA (Fig. 2). As expected, partial or complete degradation of the RNA constitutent substantially alters the elution of the hybrid (Fig. 2 and 10). This fact facilitates an unambiguous analysis of reaction product for the presence of double-stranded DNA, and should facilitate the use of the hydroxyapatite procedure in experiments designed to detect a precursor-product relationship between hybrid molecules and double-stranded DNA.

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