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Deoxyribonucleic Acid Polymerases of Rous Sarcoma Virus: Kinetics of Deoxyribonucleic Acid Synthesis and Specificity of the Products

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The deoxyribonucleic acid (DNA) polymerase(s) of Rous sarcoma virus synthesizes two principal products—single-stranded DNA in the form of a DNA:ribonucleic acid (RNA) hybrid and double-stranded DNA. All of the single-stranded product and 50% of the double-stranded product can be hybridized to 70*S* viral RNA. These results, in combination with data obtained by analysis of the kinetics of double-stranded DNA synthesis, indicate that the synthesis of double-stranded DNA is a sequel to the synthesis of single-stranded DNA and is dependent upon the latter for the provision of initial template.

The virions of ribonucleic acid (RNA) tumor viruses contain at least two deoxyribonucleic acid (DNA) polymerase activities: (i) synthesis of single-stranded DNA in association with the RNA genome of the virus (1, 7, 9, 10, 14), and (ii) synthesis of double-stranded DNA (5) by an enzyme which is capable of utilizing double-stranded DNA as template (8, 11). We have examined the interrelationship between these two reactions in the case of Rous sarcoma virus (RSV) by analyzing the kinetics of DNA synthesis at limiting substrate concentrations. The results suggest that the synthesis of double-stranded DNA is a sequel to the synthesis of single-stranded DNA, utilizing product of the latter reaction as template. This hypothesis was tested further by performing hybridization experiments with purified single- and double-stranded product. Both classes of product were found to contain nucleotide sequences which are complementary to the RNA of the viral genome. These data confirm previous reports that the virion-associated DNA polymerase(s) employs viral RNA as template (1, 7, 9, 10, 14) and demonstrate that both the single- and double-stranded products are virus specific.

MATERIALS AND METHODS

Reagents. ^{3}H-thymidine triphosphate (^{3}H-TTP), 10 to 15 Ci/mmoles, was obtained from New England Nuclear Corp., Boston, Mass.

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

Pronase (B grade, self-digested at 37 C for 2 hr prior to use) and the deoxyribonucleoside triphosphates deoxyadenosine, deoxycytidine, and deoxyguanosine triphosphate (dATP, dCTP, and dGTP) were obtained from Calbiochem, Los Angeles, Calif.

Phenol (reagent grade) was from Mallinckrodt Chemical Works, St. Louis, Mo.; hydroxyapatite (Bio-Gel HT), from Bio-Rad Laboratories, Richmond, Calif.; formamide (reagent grade), from J. T. Baker; and Cs_2SO_4 (optical grade), from Gallard Schlesinger.

Propagation and purification of virus. The Schmidt-Ruppin strain of RSV was grown in chick embryo fibroblasts and purified as described previously (3).

Extraction and purification of viral RNA. RSV RNA was extracted from purified virus with sodium dodecyl sulfate and phenol (3). When required, 70*S* RNA was isolated by zonal centrifugation through sucrose density gradients (3), followed by dialysis against 0.001 M ethylenediaminetetraacetate and concentration by flash evaporation. Poliovirus RNA was isolated from purified virus as described previously (2).

DNA polymerase reaction; extraction of enzymatic product. Details of the enzyme reaction mixture have been reported previously (7). ^{3}H-TTP was used as the labeled precursor. Enzymatic product was extracted by treating reaction mixtures with Pronase (500 μg/ml) and sodium dodecyl sulfate (0.5% w/v) for 45 min at 37 C, followed by two phenol extractions at room temperature and ethanol precipitation (7).

Rate-zonal and equilibrium density gradient centrifugation. The preparation, use, and fractionation of

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sucrose density gradients and equilibrium density gradients of Cs_2SO_4 have been described previously (5, 7). In the present experiments, the Cs_2SO_4 solutions were adjusted to a density of approximately 1.58 g/cc prior to centrifugation.

Fractionation of DNA on hydroxyapatite. Single- and double-stranded DNA were separated by step-wise elution from hydroxyapatite, after disruption of hybrid structures by treatment with ribonuclease (5). This latter maneuver is necessitated because the elution of native DNA:RNA hybrids from hydroxyapatite substantially overlaps that of double-stranded DNA (5). Single-stranded DNA, derived from the hybrids by ribonuclease treatment, elutes from hydroxyapatite in 0.2 M sodium phosphate, whereas double-stranded DNA elutes primarily in 0.3 M sodium phosphate (5).

Hybridization of DNA to RNA. Preparations of DNA were treated with 0.4 N NaOH for a minimum of 12 hr at 37 C in order to denature DNA and hydrolyze RNA. After neutralization, the DNA was mixed with viral RNA in a solution containing 0.1 M NaCl–0.02 M tris(hydroxymethyl)aminomethane-(Tris)-hydrochloride (*p*H 7.4)–50% (v/v) formamide. The amount of enzymatic product used was computed on the basis of the specific activity of the ^{3}H-TTP with corrections for relative counting efficiencies. The nucleic acid mixture was heated to 80 C for 2 min to denature any secondary structure of the RNA and was then quenched in an ice bath. Hybridization was carried out at 37 C for 20 to 24 hr. This procedure is similar to that described by Spiegelman et al. (10) and is designed to minimize thermal degradation of RNA.

RESULTS

Isolation of single- and double-stranded DNA; kinetics of DNA synthesis. The RSV-associated DNA polymerase synthesizes both single- and double-stranded DNA (5, 7). The single-stranded product appears first as a DNA:RNA hybrid in which the DNA is hydrogen-bonded to its putative template—70*S* viral RNA (10; A.-C. Garapin et al., *unpublished data*). However, some single-stranded chains of DNA may be released from the hybrid state prior to the synthesis of double-stranded DNA (K. Manly and D. Baltimore, *personal communication*). In the discussion that follows, all single-stranded product, whether in a hybrid or free state, will be referred to as single-stranded DNA.

We have prepared both single- and double-stranded product in purified form and have examined the specificity of each by hybridization with viral RNA. Double-stranded DNA was isolated by step-wise elution from hydroxyapatite (Fig. 1). Denaturation of the purified double-stranded product with 0.4 N NaOH quantitatively converts it to single-stranded DNA (Fig. 1d). This observation is in contrast to the report that a portion of the double-stranded DNA synthesized by the polymerase of avian myeloblastosis virus cannot be irreversibly denatured (6).

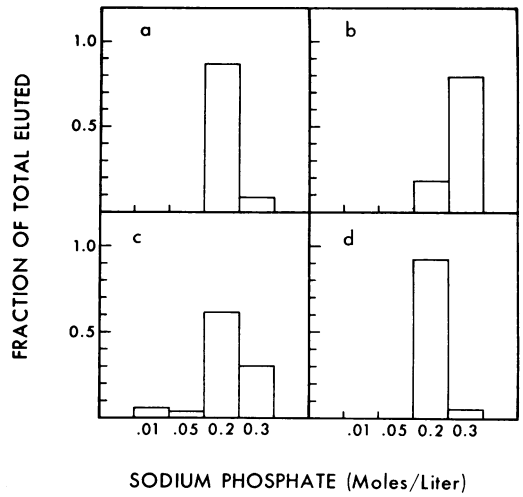

FIG. 1. *Fractionation of enzymatic product on hydroxyapatite. A standard reaction mixture containing 300 μg of viral protein, 5.5 × 10^{-5} M dATP, dCTP, and dGTP, and 8 × 10^{-7} M ^{3}H-TTP was incubated at 37 C for 4 hr, then extracted with sodium dodecyl sulfate-Pronase-phenol, and precipitated with ethanol as described under Materials and Methods. Approximately 0.07 μg (100,000 counts/min) of DNA was treated with ribonuclease (10 μg/ml for 1 hr at 37 C in 3 mM ethylenediaminetetraacetic acid, adsorbed to hydroxyapatite (1 ml packed volume), and eluted in a step-wise manner (5). The elution of denatured enzymatic product and of single- and double-stranded control DNA's, prepared as described previously (5), are also illustrated. (a) Single-stranded fd phage DNA (5,000 counts/min); (b) double-stranded chick fibroblast DNA (5,000 counts/min); (c) unfractionated enzymatic product (100,000 counts/min); (d) double-stranded enzymatic product (5,000 counts/min) denatured with alkali. A portion of the enzymatic product which elutes from hydroxyapatite in 0.3 M sodium phosphate (as in panel C) was denatured with alkali (0.6 N NaOH at 37 C for 1 hr) and reanalyzed on hydroxyapatite.*

Single-stranded DNA was prepared by exploiting the kinetics of the enzymatic reaction. The relative yields of single- and double-stranded product at a given time in the reaction can be regulated by varying the concentration of one of the triphosphates (TTP in this instance) while maintaining the other precursors in excess (Fig. 2). Moreover, at the lowest concentration (8 × 10^{-7} M) of TTP used in these experiments, there is an appreciable lag before double-stranded DNA can be detected in the reaction product (Fig. 3). A reaction of this sort, terminated after 1 hr of incubation, constitutes a convenient source of pure single-stranded DNA. The con-

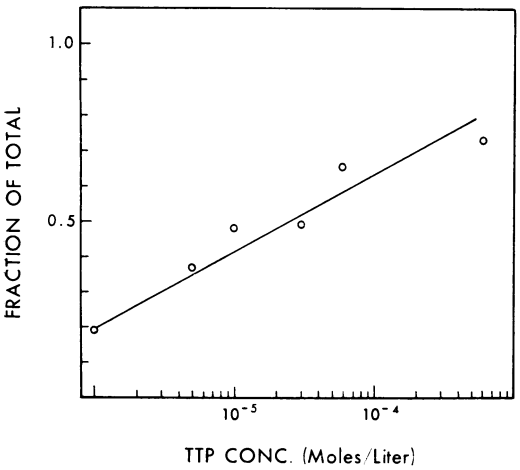

FIG. 2. *Effect of precursor concentration on proportional yield of double-stranded DNA. Standard enzymatic reaction mixtures were prepared with a constant amount of unlabeled dATP, dCTP, and dGTP (5×10^{-4} M) and varying amounts of ^{3}H-TTP. After 2 hr of incubation at 37 C, DNA was extracted by treatment with sodium dodecyl sulfate (0.5% w/v) and Pronase (500 μg/ml) for 45 min at 37 C. Portions of each extract were dialyzed against 1,000 volumes of 3 mM ethylenediaminetetraacetic acid, treated with ribonuclease (10 μg/ml at 37 C for 1 hr) to destroy all hybrid structures (see reference 5) and analyzed on hydroxyapatite as described previously (5). The proportion of enzymatic product which elutes as double-stranded DNA (i.e., in 0.3 M sodium phosphate) has been plotted as a function of the TTP concentration. Note that in every instance, the concentration of the three unlabeled nucleoside triphosphates was equivalent to or in excess of that of the TTP.*

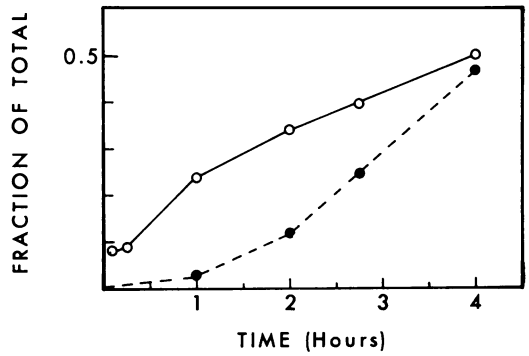

FIG. 3. *Effect of precursor concentration on the kinetics of double-stranded DNA synthesis. Reaction mixtures similar to those described for Fig. 2 were prepared with two concentrations of ^{3}H-TTP, 8×10^{-7} M and 4×10^{-6} M. At the indicated time points, samples were withdrawn from both reaction mixtures, extracted with sodium dodecyl sulfate and Pronase, dialyzed against 3 mM ethylenediaminetetraacetic acid, treated with ribonuclease (10 μg/ml at 37 C for 1 hr), and analyzed on hydroxyapatite as described for Fig. 2. The fraction of each sample which eluted in 0.3 M sodium phosphate (i.e., double-stranded DNA) has been plotted as a function of the time at which the sample was withdrawn from the reaction mixture. ○, 4×10^{-6} M TTP; ●, 8×10^{-7} M TTP.*

centration of precursor is critical. At higher levels of TTP (e.g., 4×10^{-6} M), double-stranded DNA appears quite early in the course of the reaction (Fig. 3). These observations reflect the fact that the concentrations of TTP in question limit the overall rate of the reaction. Figure 4 illustrates the relationship between TTP concentration (with the other nucleoside triphosphates in excess) and the rate of DNA synthesis as a whole. A maximum rate has not been achieved at the highest TTP concentration (5×10^{-4} M) tested. The reactions used to prepare single- and double-stranded DNA, as described for Fig. 1 and 5, were carried out at a TTP concentration of 8×10^{-7} M. This corresponds to a reaction rate of approximately 1 pmole of DNA per μg of viral protein per 120 min (Fig. 4), i.e., less than 1% of the maximum reaction velocity, achieved in this series of experiments.

Hybridization of product DNA to viral RNA. The nucleotide sequence of the single-stranded DNA which constitutes the initial enzymatic product should be entirely complementary to the putative template, 70S viral RNA. The experiments illustrated in Fig. 5a and 6a confirm this supposition. Virtually all of the early product can be annealed to 70S viral RNA when RNA is present in substantial excess of DNA (ca. 700:1). The resulting hybrids have a buoyant density identical to that of single-stranded RNA, indicating that only a small amount of DNA is hybridized to any given RNA molecule.

The specificity of the hybridization reaction is illustrated by the fact that enzymatic product cannot be annealed to poliovirus RNA (Fig. 5b). Furthermore, no hybrid structures are formed if enzymatic product is mixed with RSV RNA and analyzed in density gradients of Cs_2SO_4 without prior incubation at 37 C (not illustrated). Thus, the results illustrated in Fig. 5a and 6 cannot be ascribed to entrapment of DNA by coprecipitation with RNA in Cs_2SO_4 (12).

Approximately 50% of purified double-stranded product anneals to viral RNA (Fig. 6b). We conclude that the double-stranded DNA is a virus-specific product containing sequences which are complementary to viral RNA. The non-complementary sequences are presumably identical to a region (or regions) in the viral genome.

The final recovery of radioactivity from the

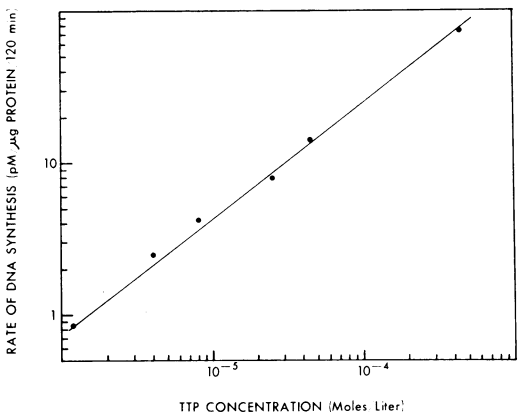

FIG. 4. *Relationship between TTP concentration and rate of DNA synthesis. Replicate reaction mixtures, containing 20 μg of viral protein and 5.5 × 10^{-4} M dATP, dCTP, and dGTP, were prepared with varying concentrations of ^{3}H-TTP. The amount of DNA synthesized by each mixture over a 2 hr period was determined (7) and plotted as a function of TTP concentration. In every instance, the incorporation of ^{3}H-TTP was linear for the duration of the reaction and the concentration of the unlabled nucleoside triphosphates was equivalent to or in excess of that of the ^{3}H-TTP.*

Cs_2SO_4 gradients was approximately 50% for both the single- and double-stranded enzymatic product (Fig. 6a and b; *see legends*). This result suggests that the losses during centrifugation are not selective, i.e., that the recoveries of hybridized and free DNA are approximately the same.

DISCUSSION

Two DNA polymerase activities associated with RSV can be readily distinguished by manipulation of reaction conditions (e.g., Fig. 3). At very low substrate concentrations, there is an appreciable period of time during which only the initial enzymatic product (single-stranded DNA, eigher free or complexed to viral RNA) is detectable. This lag in the appearance of the final enzymatic product (double-stranded DNA) suggests that the synthesis of single-stranded DNA is a necessary precursor to the synthesis of double-stranded DNA and that the latter reaction can initiate only after the synthesis of single-stranded DNA has progressed to a certain critical extent.

It should be emphasized that the analysis of reaction kinetics presented above is of relative value only. The enzyme preparation under study is not highly purified, and the reaction is quite complex with at least two enzymatic activities participating. Nevertheless, the data make it clear that our experiments have been carried out at limiting substrate concentrations, a circumstance which has allowed a partial exploration of the relationship between the two DNA polymerase activities.

Both the single- and double-stranded enzymatic products contain nucleotide sequences which are

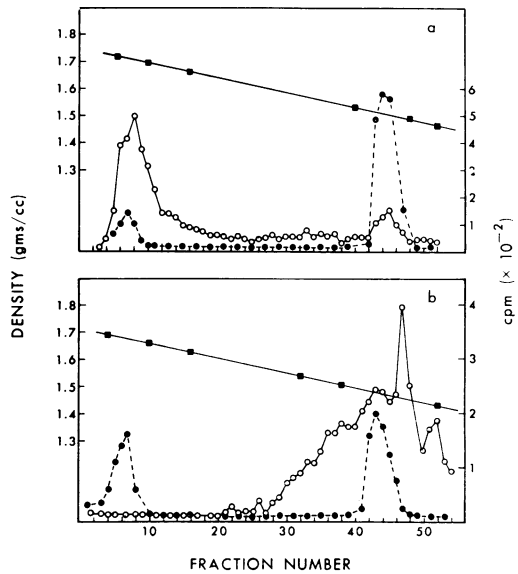

FIG. 5. *Hybridization of single-stranded product to viral RNA. A standard reaction mixture, containing 5 × 10^{-4} M dATP, dCTP, and dGTP and 8 × 10^{-7} M ^{3}H-TTP, was incubated at 37 C for 1 hr. Under these conditions, the enzymatic product consists almost entirely of single-stranded DNA (mainly in the form of DNA:RNA hybrids; see Fig. 3 and ref. 5). Nucleic acids were extracted as described under Materials and Methods, precipitated with ethanol, and treated with NaOH (0.4 N at 37 C for 16 hr) to completely hydrolyze RNA. After neutralization, ca. 0.007 μg (10,000 counts/min) of DNA was mixed with 5 μg of 70S viral RNA in 0.3 ml of 0.1 M NaCl–0.02 M Tris: hydrochloride (pH 7.4)–50% (v/v) formamide, and incubated at 37 C for 24 hr. The nucleic acids were then analyzed in equilibrium density gradients of Cs_2SO_4 as described under Materials and Methods. Lambda phage DNA and Escherichia coli RNA, both labeled with ^{32}P, were added to serve as density markers. The DNA banded at a density of ca. 1.50 g/cc, the RNA at 1.68 to 1.70 g/cc. Centrifugation was carried out in a Spinco SW-50 rotor at 33,000 rev/min at 4 C for 60 hr. ○, ^{3}H-labeled enzymatic product; ●, ^{32}P-labeled lambda phage DNA and E. coli RNA; ■, density (g/cc). (a) Hybridization with RSV RNA (5 μg). Approximately 5,000 counts/min were recovered from the Cs_2SO_4 gradient. (b) Hybridization with poliovirus RNA (5 μg). These data also represent the results obtained if a mixture of enzymatic product and RSV RNA (5 μg) is centrifuged without prior incubation at 37 C (i.e., without performing the annealing reaction). Approximately 5,000 counts/min were recovered.*

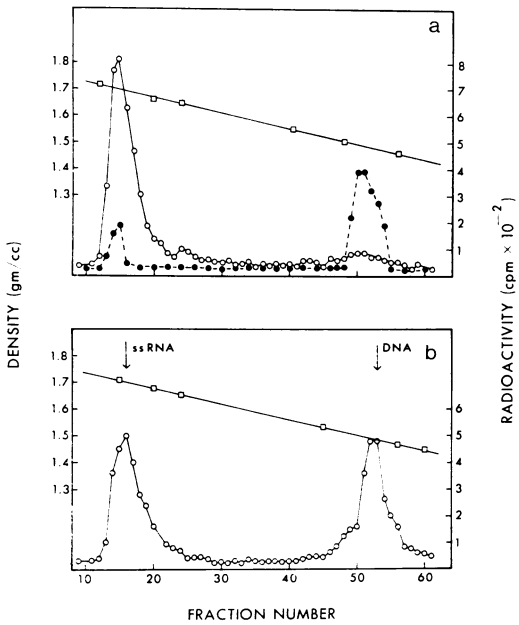

FIG. 6. *Hybridization of enzymatic product to viral RNA: comparison of single- and double-stranded DNA. Single-stranded enzymatic product was prepared as described for Fig. 5. Double-stranded product was isolated by elution from hydroxyapatite as in Fig. 1. Treatment with NaOH and hybridization with 5 μg of RSV RNA was carried out as described for Fig. 5. Equivalent amounts of single- and double-stranded product were used. Centrifugation in Cs_2SO_4 with ^{32}P-labeled DNA and RNA markers was carried out as described for Fig. 5.* ○, *^3H-labeled enzymatic product;* ●, *^{32}P-labeled lambda phage DNA and Escherichia coli RNA;* □, *density (g/cc). (a) Hybridization of single-stranded DNA (10,000 counts/min, ca. 0.007 μg) with 5 μg of RSV RNA. Approximately 4,900 counts/min of product were recovered in the hybrid band (at the density of single-stranded RNA) and 400 counts/min in the DNA band. This represents an overall recovery of 53%. (b) Hybridization of double-stranded DNA (10,000 counts/min, ca. 0.007 μg) with 5 μg of RSV RNA. Arrows indicate the positions of the RNA and DNA markers. Approximately 2,900 counts/min of product were recovered in the hybrid band (at the density of RNA) and 2,800 counts/min in the DNA band. This represents an overall recovery of 57%.*

complementary to regions in the 70S viral RNA. The extent of this complementarity, i.e., the fraction of the total viral genome which is actually represented among the sequences composing the enzymatic product, has yet to be determined. The present experiments do not provide any information in this regard. Hybridization was carried out with RNA in substantial excess of DNA. Consequently, only a small amount of DNA annealed to any given RNA molecule, and the resulting hybrids have a buoyant density virtually identical to that of single-stranded RNA (Fig. 5a and 6). Formation of this type of hybrid is facilitated by the extremely low molecular weight of the enzymatic product (9, 10), but it has no implication regarding what proportion of the viral genome is transcribed. This last issue will have to be examined with other procedures such as hybridization with DNA in excess and determination of the reassociation kinetics for the complementary strands of the DNA product (4).

The chronological relationship between the synthesis of single- and double-stranded DNA (Fig. 3 and reference 5) and the fact that both forms of enzymatic product contain virus-specific nucleotide sequences suggest the following sequence of events. Segments of the 70S viral RNA are transcribed into single-stranded DNA. After this reaction has progressed to a certain extent, the synthesis of double-stranded DNA is initiated, utilizing the primary enzymatic product as template in a manner which is presently indeterminate. The product of this second reaction could then serve as template for the virion-associated DNA-dependent polymerase (8, 11). This would provide for amplification of double-stranded product—an advantageous event if double-stranded DNA is, in fact, the biologically active product of the enzymatic reaction (1, 13, 14).

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