Ribonucleic Acid Directed Deoxyribonucleic Acid Synthesis by the Purified Deoxyribonucleic Acid Polymerase of Rous Sarcoma Virus. Characterization of the Enzymatic Product†

J. M. Taylor, A. J. Faras, H. E. Varmus, W. E. Levinson, and J. M. Bishop

ABSTRACT: The purified DNA polymerase of Rous sarcoma virus synthesizes both single- and double-stranded DNA with 70S viral RNA as template. Secondary structure of the enzymatic product was evaluated by both fractionation on hydroxylapatite and treatment with a single strand-specific nuclease. The two procedures gave virtually identical results. A variable portion of double-stranded product cannot be irreversibly denatured with heat unless first "nicked" by limited hydrolysis with DNase. The nearest-neighbor nucleotide composition of this nondenaturable DNA is not significantly different from that of unfractionated enzymatic product. Both single- and double-stranded enzymatic product contain nucleotide sequences complementary to the RNA template, and DNAs transcribed from different avian tumor virus RNAs share extensive sequence homology. Nascent DNA is hydrogen bonded to the high molecular weight template RNA and covalently linked to a low molecular weight polyribonucleotide. No DNA is released from template molecules without concomitant degradation of the RNA. The length of DNA chains synthesized by purified polymerase is identical with that of DNA synthesized by detergent-activated virions. Both virions and purified enzyme preferentially transcribe limited and homologous regions of viral RNA into double-stranded DNA.

The DNA polymerases associated with virions of RNA tumor viruses (Baltimore, 1970; Temin and Mizutani, 1970) are thought to play a central role in both viral replication and virus-induced cellular transformation (Temin, 1971). Confirmation of this hypothesis will require extensive characterization of enzymatic products and elucidation of the mechanisms of DNA synthesis. To this end, we have purified the DNA polymerase of Rous sarcoma virus (RSV),[1] and have concluded that the enzyme is a single protein (or protein aggregate) capable of responding to a large variety of nucleic acid templates (Faras *et al.*, 1972). The present communication describes the nature of the DNA synthesized by the purified polymerase under the direction of 70S RSV RNA template. We find that purified enzyme possesses the multiple synthetic capacities associated with intact virions, although little if any of the enzymatic product is released from its association with template RNA without concomitant degradation of the RNA. Despite the absence of appreciable quantities of nuclease, the purified enzyme retains two presently unexplained properties of crude enzyme preparations: (1) the individual chains of DNA product have relatively low molecular weights, and (2) a limited portion (*ca.* 5%) of the total template nucleotide sequence is preferentially transcribed into double-stranded DNA.

Materials and Methods

Reagents. Sources and preparation of most of the pertinent materials have been described in previous communications (Faras *et al.*, 1972; Garapin *et al.*, 1970; Fanshier *et al.*, 1971). Sephadex G-50 (coarse) was from Pharmacia, micrococcal nuclease and spleen phosphodiesterase were from Worthington, conidia of *Neurospora crassa* from Miles Laboratories, Inc., [^{3}H]TTP (15–20 Ci/mmole) from Schwarz BioResearch, [α-^{32}P]TTP (5 Ci/mmole) and [α-^{32}P]dCTP (5 Ci/mmole) from International Chemical and Nuclear Corp. The specific activity of 70S viral [^{32}P]RNA was generally 50,000–100,000 dpm/μg.

Preparation and Assay of Viral DNA Polymerase. The growth and purification of the Schmidt–Ruppin strain of RSV have been described (Bishop *et al.*, 1970), as have the purification of RNA-dependent DNA polymerase and conditions for enzymatic synthesis of DNA with various templates (Faras *et al.*, 1972).

Fractionation of Nucleic Acids. We have previously described our procedures for rate-zonal centrifugation in density gradients of sucrose (Bishop *et al.*, 1970), fractionation on hydroxylapatite (Fanshier *et al.*, 1971), and electrophoresis in ethylene diacrylate cross-linked gels of polyacrylamide (Bishop *et al.*, 1970).

Nucleic Acid Hybridization. Enzymatically synthesized DNA (7500 cpm of [^{3}H]TMP/ng) was treated with 0.6 N NaOH for 1 hr at 37°, then incubated with an excess (usually 1000:1) of RNA in 0.3 M NaCl–0.001 M EDTA–0.02 M Tris·HCl (pH 7.4) at 68° for 2–4 hr. Hybridization was detected by equilibrium centrifugation in Cs_2SO_4 (Garapin *et al.*, 1971a) or by fractionation on hydroxylapatite. The latter procedure has been described previously (Garapin *et al.*, 1971b), and is further validated in a separate communication (Leong *et al.*, 1972). It is based on the observation that the bulk of single-stranded DNA elutes from hydroxylapatite in 0.16 M sodium phosphate, whereas hybrids of DNA and RNA are quantitatively retained on hydroxylapatite at 0.16 M sodium phosphate and can be eluted with 0.4 M sodium phosphate (Garapin *et al.*, 1971b).

Complexity of Double-Stranded DNA. The details of this procedure have been reported (Varmus *et al.*, 1971, 1972).

† From the Department of Microbiology, University of California, San Francisco, California 94122. *Received January 31, 1972.* A preliminary report of this work was presented before the American Society of Biological Chemists, June 17, 1971. This investigation was supported by U. S. Public Health Service Grants AI 08864, CA 12380, CA 12705, AI 06862, and AI 00299 and Contract 71-2147 within the Special Virus-Cancer Program of the National Cancer Institute, National Institutes of Health, and the Public Health Service.

[1] Abbreviations used are: RSV, Rous sarcoma virus; AMV, avian myeloblastosis virus; C_0t, concentration × time, expressed as (mole sec)/l.; RNase, pancreatic ribonuclease.

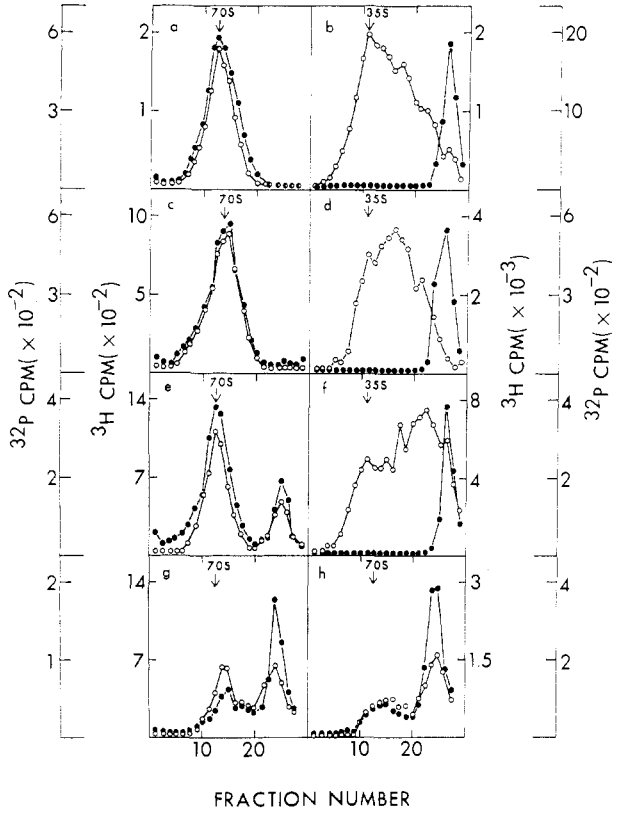

FIGURE 1: Rate-zonal centrifugation of enzymatic product. A standard reaction mixture (2.0 ml), containing 0.4 unit of purified RSV polymerase, ^{32}P-labeled 70S RSV RNA (2 μg/ml), and [^{3}H]TTP (50 μCi/ml) as the labeled precursor, was incubated at 37°. At the indicated time points, samples were withdrawn, treated with sodium dodecyl sulfate, 0.1%, pronase, 100 μg/ml, and passed through a 0.9 × 40 cm column of G-50 (coarse) Sephadex. The nucleic acids in the excluded volume were recovered by ethanol precipitation and analyzed by centrifugation in density gradients of sucrose, using an SW 50.1 rotor at 4°. (○) ^{32}P cpm; (●) ^{3}H cpm. Arrows indicate the positions of 70S RSV RNA and 35S poliovirus RNA, centrifuged in separate buckets. (a) Native product from 5 min reaction. Centrifugation: 50,000 rpm, 1 hr 40 min. (b) Denatured product from 5-min reaction. Nucleic acids were treated with dimethyl sulfoxide prior to centrifugation at 50,000 rpm for 3 hr 45 min. (c) Native product from 30-min reaction, centrifuged as in step a. (d) Denatured product from 30-min reaction. Denaturation and centrifugation as in step b. (e) Native product from 1-hr reaction, centrifuged as in step a. (f) Denatured product from 1-hr reaction. Denaturation and centrifugation as in step b. (g) Native product from 4-hr reaction, centrifuged as in step a. (h) Native product from 8-hr reaction, centrifuged as in step a.

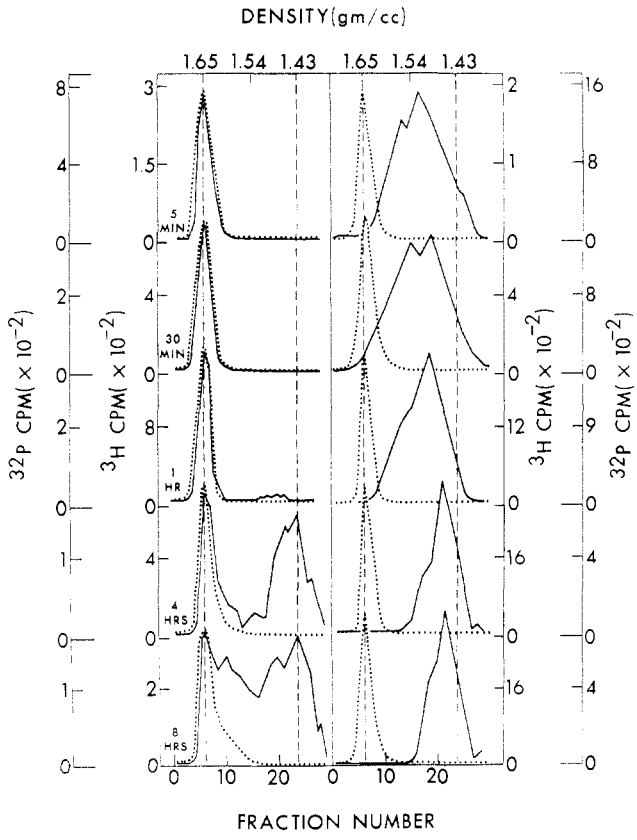

FIGURE 2: Equilibrium centrifugation of reaction product. Reaction products were prepared as described for Figure 1 and analyzed by centrifugation in Cs_2SO_4 (SW 50 rotor, 33,000 rpm, 66 hr, 4°). Markers indicate the positions of density references centrifuged in a separate tube: RNA, 70S RSV [^{32}P]RNA; DNA, denatured [^{32}P]-λ DNA. On occasion, the denatured λ DNA was included as an internal reference which could be readily distinguished by its density from the RSV [^{32}P]RNA used in the reaction. (· · · · ·) [^{32}P]-RNA; (—) [^{3}H]DNA (enzymatic product). Durations of individual reactions are indicated in the figure. Left column, native product. Right column, product denatured with dimethyl sulfoxide.

Double-stranded DNA was isolated by fractionation of enzymatic product on hydroxylapatite following treatment with RNase in 3 mM EDTA in order to disrupt RNA–DNA hybrids (Fanshier *et al.*, 1971). Double-stranded enzymatic product prepared in this manner is resistant to hydrolysis by the single strand-specific nuclease prepared from conidia of *Neurospora crassa* (see Table II). The DNA was denatured by boiling in 3 mM EDTA, sodium phosphate added to 0.4 M and the rate of reassociation determined at 68° (Varmus *et al.*, 1971, 1972; Britten and Kohne, 1968). Results were corrected to standard concentrations of salt and expressed in terms of the convention C_0t (concentration × time) (Britten and Kohne, 1968). The C_0t required to reassociate 50% of the total DNA was then used to compute complexity of nucleotide sequence (Varmus *et al.*, 1972; Britten and Kohne, 1968).

Purification of a Single Strand-Specific Nuclease from Conidia of N. crassa. Conidia were disrupted by sonication and the released nuclease was purified as described by Rabin *et al.* (1971), omitting the gel filtration and preparative electrophoresis. We have documented the properties and requirements of our preparations in a separate communication (Leong *et al.*, 1972). The enzyme is highly specific for single-stranded nucleic acids (DNA and RNA), and possesses both endo- and exonuclease activities as noted previously (Rabin *et al.*, 1971). Under the conditions used here, the circular single-stranded DNA genome of fd bacteriophage is degraded as rapidly as denatured λ phage DNA. The standard reaction mixture for the nuclease was as follows: 0.1 ml of 0.01 M $MgCl_2$–0.1 M Tris·HCl (pH 7.4), containing trace amounts of radioactive test DNA and 10 μg/ml of unlabeled, denatured calf thymus DNA. Extent of substrate hydrolysis was determined by acid precipitation (5% trichloroacetic acid) in the presence of 80 μg of carrier DNA (calf thymus). Results are expressed as the proportion of total acid-precipitable radioactivity resistant to hydrolysis under standard conditions.

Nuclease Test of Secondary Structure. This procedure is patterned after that reported by Manly *et al.* (1971). All operations were performed in duplicate. Separate samples of radiolabeled test DNA (1000–2000 cpm), contained in 0.1 ml, were

tested as follows: (a) precipitated directly as controls, (b) treated with *Neurospora* nuclease under standard conditions, (c) treated with RNase (10 μg/ml, 1 hr, 37°) in 0.02 M Tris·HCl (pH 7.4), followed by *Neurospora* nuclease under standard conditions, and (d) treated with RNase in 0.02 M Tris·HCl, then boiled for 10 min, and tested with the *Neurospora* nuclease. Typical results are illustrated in Table III. Pilot experiments demonstrated that the conditions of RNase digestion used here completely disrupt the DNA–RNA hybrids synthesized by RSV DNA polymerase (Garapin *et al.*, 1970; Spiegelman *et al.*, 1970; Rokutanda *et al.*, 1970).

Nearest-Neighbor Nucleotide Frequencies. DNA was synthesized with purified polymerase, using [α-^{32}P]TTP (or dCTP) as the radioactive substrate. The DNA was extracted as before (Garapin *et al.*, 1970), precipitated with ethanol, and passed through a 0.9 × 40 cm column of Sephadex G-50 (coarse) in order to eliminate unincorporated isotope and other low molecular weight materials. The DNA in the excluded volume was precipitated with ethanol, dissolved in 100 μl of water, evaporated to dryness on a siliconized plastic sheet, and dissolved in 10 μl of 0.01 M $CaCl_2$–0.1 M Tris (pH 7.5) containing micrococcal nuclease (150 U), splenic phosphodiesterase (0.10 U), and adenosine monophosphate (10 mM). After incubation for 2 hr at 37°, the samples were applied to Whatman 540 paper and subjected to electrophoresis in pyridine–acetate buffer (pH 3.5) (Sebring and Salzman, 1964). Electrophoresis was at 4500 V for 40 min at room temperature. The separated nucleotides were located by autoradiography, cut out, and counted by liquid scintillation.

Denaturation of Nucleic Acids with Dimethyl Sulfoxide. Nucleic acids were dissolved in 3 mM EDTA (pH 7), adjusted to 95% (v/v) dimethyl sulfoxide, incubated at 37° for 30 min, then precipitated with ethanol and recovered by centrifugation. The pellets were dissolved in 0.01 M EDTA–0.02 M Tris·HCl (pH 7.4) and the samples heated at 80° for 2 min prior to further analysis. The heat treatment is necessary to disrupt aggregates of nucleic acids which form during ethanol precipitation from dimethyl sulfoxide (Best *et al.*, 1972).

Results

Analysis of Enzymatic Product by Ultracentrifugation. The evolution of DNA synthesis by purified polymerase, using 70S RSV RNA as template, is illustrated in Figures 1 and 2. The initial enzymatic product both cosediments with template RNA (Figure 1a) and bands with RNA in equilibrium density gradients of Cs_2SO_4 (Figure 2). Treatment with dimethyl sulfoxide to disrupt hydrogen bonds dissociates the 70S RNA into its constituent subunits (Duesberg, 1968; Erikson, 1969; and Figure 1b) and frees the product DNA of its association with high molecular weight viral RNA (Figure 1b). The released DNA has a buoyant density intermediate between that of RNA and DNA (Figure 2). Treatment of the denatured product with either RNase (in 0.3 M NaCl) or alkali (pH 12) converts its density to that of free DNA (unpublished data). These observations conform to those of Verma *et al.* (1971), and support their conclusion that the initial product of the enzymatic reaction is covalently linked to an RNA primer molecule. The amount of materials used in the experiment of Figure 2 precludes direct identification of this RNA in the Cs_2SO_4 gradient.

DNA free of template is not detected until 1–4 hr of enzymatic synthesis (Figure 2), at which time there is manifest degradation of the template (Figure 1f,g). Concomitantly, the buoyant density of product DNA has shifted close to that of a DNA marker (Figure 2). These observations contrast sharply with the results using detergent-activated virions (Verma *et al.*, 1971; Fanshier *et al.*, 1971; and unpublished observations), where "free" DNA is observed as early as 15 min after the onset of DNA synthesis with similar precursor concentrations and at similar rates of DNA synthesis.

Secondary Structure of Product DNA. The secondary structure of enzymatic product has been assessed in two ways: (1) fractionation on hydroxylapatite, following digestion with RNase to disrupt DNA–RNA hybrids (Fanshier *et al.*, 1971), and (2) treatment with a single strand-specific nuclease of *N. crassa* in conjunction with RNase digestion at low concen-

TABLE I: Evaluation of Fractionation with Hydroxylapatite.

DNA[a]	Concentration of Sodium Phosphate	
	0.18 M (%)	0.4 M (%)
30-min reaction	0.55 (8)	0.45 (75)
18-hr reaction	0.20 (38)	0.80 (100)
Native λ	0.05 (5)	0.95 (100)
Denatured λ	0.95 (5)	0.05 (100)

[a] DNA was extracted from 30-min and 18-hr standard reactions (using purified polymerase and 70S RSV [^{32}P]RNA template as in Figure 1), then treated with RNase (100 μg/ml, 1 hr, 37°) in 3 mM EDTA and fractionated by elution from hydroxylapatite. The results of the hydroxylapatite fractionation are expressed as the proportion of total radioactivity ([^{3}H]DNA) recovered in each eluate. Numbers in parentheses indicate the extent of resistance of each eluate to digestion by *Neurospora* nuclease under standard conditions.

TABLE II: Synthesis of Double-Stranded DNA by Crude and Purified Polymerase.

DNA[a]	Hydroxylapatite (%)		Neurospora Nuclease (%)	
	Single Strand	Double Strand	Single Strand	Double Strand
Detergent-activated virions (30 min)	73	27	73	27
Detergent-activated virions (18 hr)	25	75	21	79
Purified polymerase (30 min)	55	45	54	46
Purified polymerase (18 hr)	22	78	24	76

[a] DNA was extracted from standard reaction mixtures at the indicated times, treated with RNase (100 μg/ml, 1 hr, 37°) in 3 mM EDTA, and analyzed by either fractionation on hydroxylapatite or exposure to *Neurospora* nuclease. The 70S [^{32}P]RNA used as template for purified enzyme was entirely acid soluble after the RNase digestion. Results are expressed as the percentage of total radioactivity ([^{3}H]DNA) classified as single and double stranded by each of the analytical procedures.

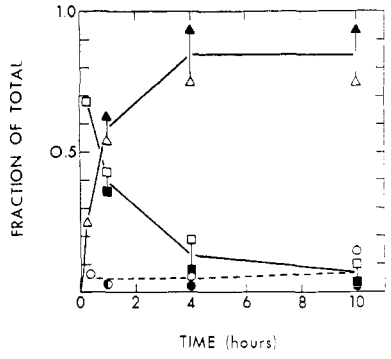

FIGURE 3: Synthesis of DNA by purified polymerase. A standard reaction mixture was prepared as in Figure 1; samples were taken at the indicated time points for testing with the *Neurospora* nuclease. Representative data are illustrated in Table III. The figure presents the results of two separate experiments, using different batches of enzyme and 70S RNA template. (○,●) DNA sensitive to nuclease hydrolysis in the native state, or single-stranded; (□,■) DNA sensitive to hydrolysis only after RNase treatment, or hybrid; (△,▲) DNA resistant to hydrolysis after RNase treatment, or double stranded.

trations of salt, as described previously by Manly *et al.* (1971). The hydroxylapatite procedure provides an estimate of the relative amounts of single- and double-stranded DNA but does not distinguish between free single-stranded DNA and single-stranded DNA contained in DNA–RNA hybrids (Fanshier *et al.*, 1971). By contrast, use of the *Neurospora* nuclease permits the differentiation of free single-stranded DNA, single-stranded DNA in hybrid form (*i.e.*, DNA sensitive to *Neurospora* nuclease only after prior treatment with RNase in low salt), and double-stranded DNA. We consider the latter procedure to be the more definitive of the two, and have therefore used it to evaluate the limitations of the hydroxylapatite assay.

Fractions of DNA eluting from hydroxylapatite as single- and double-stranded molecules were exposed to *Neurospora* nuclease, using native and denatured λ DNA as standards (Table I). "Double-stranded" DNA isolated from an early reaction product is partially susceptible (*ca.* 25%) to hydrolysis by *Neurospora* nuclease, implying that it includes single-stranded regions accessible to the enzyme. By contrast, the "double-stranded" DNA obtained from a prolonged reaction is completely resistant to the nuclease, and must therefore consist almost entirely of intact double helices.

DNA defined as single stranded by its elution from hydroxylapatite contains some nuclease-resistant material when isolated from a prolonged reaction (Table I). This can be largely accounted for by the small "spill" of double-stranded DNA into the 0.18 M sodium phosphate eluate.[2]

The product of enzymatic synthesis by both virions and purified enzyme consists of single- and double-stranded DNA when tested with either hydroxyapatite or *Neurospora* nuclease (Table II). The two procedures give virtually identical results, although this must to some extent be fortuitous in view of the data illustrated in Table I. The proportion of double-stranded DNA varies as a function of the reaction time (Table II) and precursor concentration (Garapin *et al.*, 1971).

[2] This statement is based on the following computation: (0.38) × (0.20) × (100) = 7.6% of total reaction product incorrectly classified as "single strand," *i.e.*, little more than the 5% of total double-stranded λ DNA which elutes in 0.18 M sodium phosphate

TABLE III: Test of Secondary Structure with *Neurospora* Nuclease.

DNA[a]	% Resistance		
	Native	RNase Treated	Boiled
Detergent-activated virions (30 min)	89	27	4
Purified enzyme (60 min)	97	64	15
70S Hybrid	83	29	5
Native λ	100	100	5
Denatured λ	5	7	4

[a] Products of 30- or 60-min reactions with virions and purified polymerase were extracted and tested without further fractionation. DNA–RNA hybrid (70S) was purified from a 2-hr reaction with detergent-activated virions as described previously (Faras *et al.*, 1971). It consists of 70S RNA hydrogen bonded to short chains of nascent DNA. The results are expressed as percentage of input radioactivity resistant to hydrolysis by *Neurospora* nuclease, and are interpreted as follows: DNA hydrolyzed in the native state represents free single strands, DNA hydrolyzed only after RNase treatment represents single strands contained in DNA–RNA hybrids, and DNA resistant to hydrolysis following RNase treatment is double stranded.

A complete analysis of secondary structure using *Neurospora* nuclease and DNA from an intermediate reaction time is illustrated in Table III. The results with DNA–RNA hybrid conform to those reported previously by Manly *et al.* (1971), and indicate that the hybrid intermediate contains single-stranded tails, single-stranded DNA hydrogen bonded to RNA, and double-stranded DNA. The progress of DNA synthesis by purified enzyme, monitored with the *Neurospora* nuclease test, is illustrated in Figure 3. Single-stranded DNA in hybrid form is the principal initial product. Subsequently, double-stranded DNA accumulates. Single-stranded DNA not in hybrid form is barely detectable until very late in the reaction, and has never constituted more than 10–15% of the total reaction product.

Molecular Weight of Product DNA. Single- and double-stranded DNA synthesized by purified polymerase with 70S RNA as template were analyzed by rate-zonal centrifugation (Figure 4a). Both populations of DNA are composed principally of low molecular weight forms. Comparisons of the DNA synthesized by the crude and purified enzyme are illustrated in Figures 4b and 5. Unfractionated enzymatic products were denatured with sodium hydroxide, neutralized, and analyzed by rate-zonal centrifugation (Figure 4b) and electrophoresis in polyacrylamide gels[3] (Figure 5). The crude and

[3] The electrophoresis of DNA in polyacrylamide gels is not a conventional procedure, and there is evidence that high molecular weight double-helical DNAs behave aberrantly during such analysis (Fisher and Dingman, 1971). However, extensive work in this laboratory has shown that both single- and double-stranded DNAs can be reliably analyzed by electrophoresis if they have molecular weights in the range encountered here (*i.e.*, $<1 \times 10^6$). Under these circumstances, electrophoretic mobilities are chiefly a function of molecular weight in much the same manner as described for RNA (Bishop *et al.*, 1967). These conclusions are substantiated by the comparative results of zonal centrifugation and electrophoresis illustrated in the present manuscript.

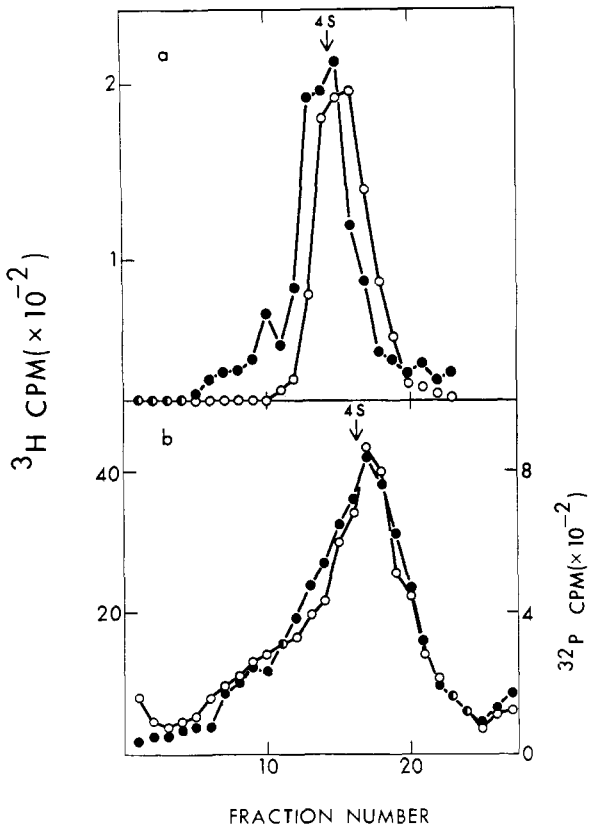

FIGURE 4: Velocity sedimentation of product DNA. (a) Single- and double-stranded products (^{3}H labeled) of purified polymerase. A reaction product (18 hr) was prepared with 70S RNA template as in Figure 1. Single- and double-stranded DNA were isolated by fractionation on hydroxylapatite, then centrifuged in 15–30% gradients of sucrose (0.1 M NaCl–0.001 M EDTA–0.02 M Tris·HCl, pH 7.4) with 4S [^{32}P]RNA as a sedimentation reference. SW 65 rotor, 60,000 rpm, 13 hr, 4°. The results of the two analyses have been superimposed. (●) Single-stranded DNA; (○) double-stranded DNA. Arrow denotes the position of 4S RNA. (b) Comparison of denatured products synthesized by crude and purified polymerase. DNA was prepared with an 18-hr reaction, using either detergent-activated virions ([^{3}H]TTP) or purified polymerase with 70S RNA as template ([α-^{32}P]TTP). The extracted products were mixed, treated with alkali (0.2 N NaOH for 10 min, 100°), neutralized, and sedimented in sucrose gradients as described in part a. (○) ^{3}H cpm (product of crude enzyme); (●) ^{32}P cpm (product of purified enzyme). The arrow denotes the position of 4S RNA, centrifuged in a separate bucket.

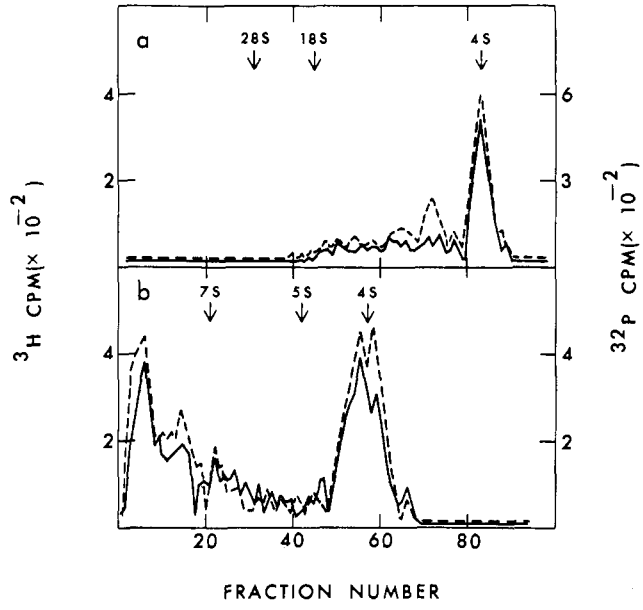

FIGURE 5: Electrophoresis of product DNA in polyacrylamide gels. DNA was prepared with detergent-activated virions and purified polymerase as described for Figure 4, mixed, treated with alkali (0.2 N NaOH, for 10 min, 100°), neutralized, and subjected to electrophoresis in polyacrylamide gels. (a) Electrophoresis in 2.25% polyacrylamide, 5 mA/gel, 3 hr, room temperature. Arrows denote the positions of 28S and 18S rRNAs, and 4S RNA, electrophoresed either in a separate gel, or in the presence of one or the other products. (b) Electrophoresis in 10% polyacrylamide, 5 mA/gel, 4 hr, 30 min, room temperature. Arrows denote the positions of 4S, 5S, and 7S RNAs electrophoresed as in part a. (—) ^{3}H cpm (product of crude enzyme); (– – –) ^{32}P cpm (product of purified enzyme).

purified polymerases synthesize DNA of essentially identical chain length. The results of velocity sedimentation and electrophoresis give similar estimates of molecular weight (50–100 nucleotides for the major forms). Small fractions of the denatured products appear as heterogeneous, relatively high molecular weight forms. The yield and size distribution of these products have been inconsistent.

Nearest-Neighbor Nucleotide Composition of Enzymatic Product. The nearest-neighbor nucleotides of TMP and dCMP in enzymatic product are illustrated in Table IV. These data indicate that the enzymatic product consists mainly of heteropolymer. There is no appreciable change in the nearest-neighbor frequencies as the reaction progresses.

Nondenaturable Double-Stranded Product. A varying proportion of double-stranded enzymatic product cannot be denatured by boiling (Table V) or treatment with alkali (data not shown). The amount of nondenaturable double-stranded DNA has generally been greater in products of prolonged reactions, and in products of purified polymerase as compared to crude enzyme. We have purified this material and find that its nearest-neighbor nucleotide composition is not remarkably different from that of unfractionated enzymatic product (Table VI). Thus, the reversible denaturation cannot be explained by the presence of rapidly reassociating polymers such as rA·dT. The preparation of *Neurospora* nuclease which we are using contains a limited amount of endonuclease active on double-stranded DNA, as manifested by the conversion of phage fd RF I DNA (supercoiled circles) to RF II (relaxed circles) without further hydrolysis. We have exploited this property of the nuclease preparation in order to introduce a limited number of single-chain scissions into double-stranded DNA. If double-stranded enzymatic product is treated with *Neurospora* nuclease under standard conditions prior to denaturation, the nondenaturable fraction is largely eliminated (Table V).

The presence of nondenaturable double-stranded DNA complicates the study of nucleotide sequences by hybridization and measurement of reassociation kinetics. Consequently, preparations of double-stranded enzymatic product containing relatively small amounts of nondenaturable material were used in the experiments which follow.

Complementarity between Product DNA and Template RNA. The template function of viral RNA was confirmed by nucleic acid hybridization. Product DNA, prepared with 70S RNA as template, was fractionated into single- and double-stranded forms by elution from hydroxylapatite and annealed with large excesses of purified 70S RNA. Hybridization was detected by centrifugation in Cs_2SO_4 (Garapin *et al.*, 1971a; Spiegelman *et al.*, 1970; Rokutanda *et al.*, 1970) and by fractionation on

TABLE IV: Nearest-Neighbor Nucleotide Composition of Enzymatic Product.

Precursor[a]	Reaction Time (min)	Nucleotide			
		dCMP	dAMP	dGMP	TMP
[^{32}P]TTP	5	0.25	0.29	0.26	0.21
	15	0.24	0.25	0.33	0.19
	60	0.23 (0.01)	0.25 (0.01)	0.29 (0.01)	0.24 (0.01)
[^{32}P]CTP	5	0.22	0.17	0.31	0.31
	15	0.23	0.18	0.27	0.33
	60	0.28 (0.01)	0.20 (0.02)	0.23 (0.03)	0.28 (0.03)

[a] DNA was synthesized with purified RSV polymerase, using 70S RSV RNA as template and either [α-^{32}P]TTP (140 μCi/ml) or [α-^{32}P]dCTP (140 μCi/ml) as the radioactive precursor. Enzymatic product was extracted at the indicated time points and analyzed for nearest-neighbor nucleotide composition as described in Materials and Methods. Results are expressed as the proportion of total radioactivity contained in the individual nucleotides following hydrolysis and electrophoretic separation. The data for 5 and 15 min represent a single determination, those for 60 min are the means of three separate measurements with standard error shown in parentheses.

hydroxylapatite (Garapin *et al.*, 1971b). Both procedures gave the same results. The bulk of the single-stranded product can be annealed to viral RNA (Figure 6a and Table VII). The resulting hybrids contain a great excess of RNA as compared to DNA, and therefore band at or near the buoyant density of single- and double-stranded RNA (Garapin *et al.*, 1971a,b; Spiegelman *et al.*, 1970; Rokutanda *et al.*, 1970). Products annealed in the absence of viral RNA band in the region of a reference DNA (Figure 6a,b). Using Cs_2SO_4 centrifugation it was found that approximately 50% of the double-stranded DNA annealed with viral RNA. This experiment could not be repeated with the hydroxylapatite procedure because double-stranded DNA and DNA–RNA hybrids cannot be adequately separated by fractionation on hydroxylapatite.

We have also compared the homology between the DNAs synthesized with template RNAs from two different avian tumor viruses (RSV and AMV). Hybridization of single-stranded products to homologous and heterologous RNA was measured by fractionation on hydroxylapatite (Table VII). The results indicate that DNA synthesized with either form of 70S RNA as template hybridizes equally well with both forms of RNA. The enzyme must therefore be copying sequences shared by the RSV and AMV RNAs. Complete hybridization with RSV RNA has been achieved repeatedly, but the amount of AMV RNA available to us was inadequate to permit further analysis.

TABLE V: Denaturation of Double-Stranded Product.

DNA[a]	% Resistance to *Neurospora* nuclease	
	Native	Denatured
Detergent-activated virions		
30 min	70	5–15
18 hr	100	5–10
Purified polymerase		
30 min	75	15–30
18 hr	100	20–65
18 hr nicked	100	5–10
λ	100	3–8

[a] Double-stranded DNA was prepared from enzymatic products by fractionation on hydroxylapatite as described in Table II. Portions were denatured by boiling in 0.02 M Tris·HCl (pH 7.4) for 10 min. Native and denatured samples were then tested for resistance to hydrolysis by *Neurospora* nuclease. In each instance, the range of results obtained with three separate preparations of enzymatic product are illustrated. Similar results were obtained with hydroxylapatite, and with alkali treatment for denaturation. A portion of the double-stranded DNA synthesized with purified polymerase in an 18-hr reaction was treated with *Neurospora* nuclease under standard conditions prior to denaturation. This sample is denoted "18 hr nicked" in the table.

TABLE VI: Nearest-Neighbor Nucleotide Composition of Nondenaturable Double-Stranded DNA.[a]

Nondenaturable DNA	Nucleotide			
	dCMP	dAMP	dGMP	TMP
Before nuclease treatment	0.24	0.25	0.25	0.26
After first cycle	0.25	0.25	0.22	0.28
After second cycle	0.31	0.22	0.19	0.28

[a] DNA was synthesized with purified polymerase, using 70S RNA as template and [α-^{32}P]TTP as the labeled precursor in an 18-hr reaction. Double-stranded DNA was isolated by fractionation on hydroxylapatite and boiled for 10 min in 3 mM EDTA. The resulting single-stranded DNA was exhaustively hydrolyzed with *Neurospora* nuclease, and the residual core of DNA (approximately 50% of the total DNA, now completely resistant to *Neurospora* nuclease) was purified by filtration through G-50 (coarse) Sephadex. A portion of the core fraction was recycled through the denaturation and nuclease treatments. Both preparations of nondenaturable DNA were then analyzed for the nearest-neighbor nucleotides of TMP. Results are expressed as in Table IV.

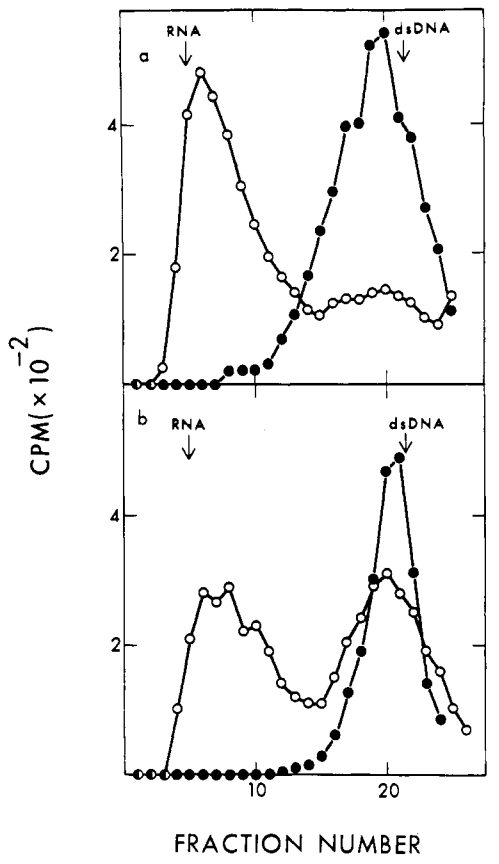

FIGURE 6: Complementarity between enzymatic product and template. [^{3}H]DNA, prepared with 70S RSV RNA as in Figure 1 and using a 2-hr reaction, was fractionated into single- and double-stranded forms by elution from hydroxylapatite and annealed with purified unlabeled 70S RSV RNA. The annealed nucleic acids were centrifuged to equilibrium in Cs_2SO_4 (SW 50 rotor, 33,000 rpm, 60 hr, 4°). Viral RNA and native λ DNA were centrifuged in a separate tube as density references, and ^{32}P-labeled λ DNA was used as an internal density marker. As controls, fractionated enzymatic product was mixed with viral RNA and centrifuged without performing the annealing reaction. Results similar to the controls were also obtained when enzymatic product was annealed with poliovirus RNA. (a) Single-stranded enzymatic product. The results with control and annealed samples are superimposed. (●) Unannealed product; (○) annealed product. Overall recovery of radioactivity was *ca.* 40% with 3209 cpm in the hybrid region (fractions 1–15) and 1340 cpm in the DNA region (fractions 16–25). (b) Double-stranded enzymatic product. The results with control and annealed samples are superimposed. (●) Unannealed product; (○) annealed product. Overall recovery of radioactivity was 46%, with 2164 cpm in the hybrid region (fractions 1–14) and 2463 cpm in the DNA region (fractions 15–26).

Complexity of the DNA Synthesized by Purified Polymerase. We and others have previously reported that detergent-activated virions of RNA tumor viruses preferentially transcribe a very limited portion (*ca.* 1–5%) of the viral genome into double-stranded DNA (Varmus *et al.*, 1971; Gelb *et al.*, 1971). This conclusion is based on measurements of the reassociation kinetics of double-stranded enzymatic product, performed and interpreted as described by Britten and Kohne (1968). Figure 7 illustrates a similar analysis of double-stranded DNA synthesized by purified enzyme with 70S RSV RNA as template. The results with the products of crude and purified RSV polymerase are quite similar. By reference to the reassociation kinetics of two standard forms of double-stranded DNA (replicative form of fd and genome of λ phage), we compute that the principal constituent of double-

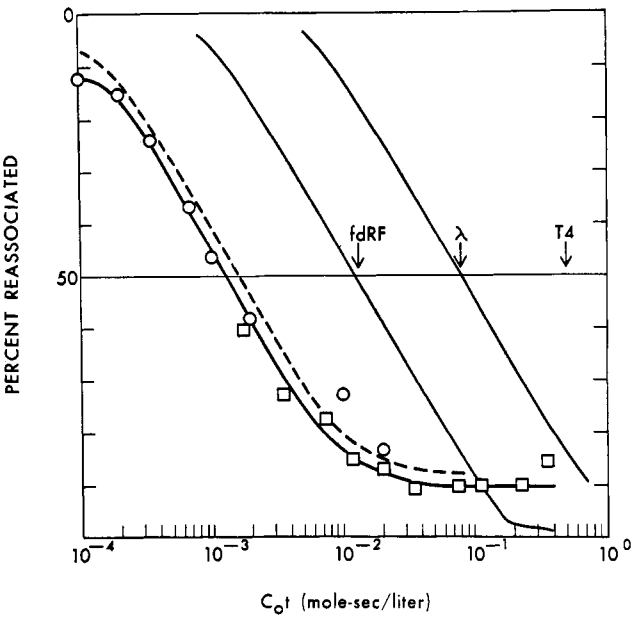

FIGURE 7: Complexity of double-stranded enzymatic product. Double-stranded DNA was isolated from 18-hr reactions with either detergent-activated RSV virions or purified RSV polymerase (70S RSV RNA as template). Reassociation kinetics of denatured DNAs were determined as described in Materials and Methods. The results with replicative form DNA of fd phage (mol wt 4.4 × 10^6) and λ phage DNA (mol wt 27 × 10^6) are also illustrated (Varmus *et al.*, 1971). (○,□) Product of purified enzyme; (– – –) product of crude enzyme; (—) fd and λ DNAs. The half C_0t for T4 phage DNA was taken from Britten and Kohne (1968).

stranded enzymatic product corresponds to unique nucleotide sequences with molecular weights of approximately 1 × 10^6 (Varmus *et al.*, 1971).

In our previous report, we were able to define a second minor constituent of double-stranded DNA synthesized by crude enzyme preparations (Varmus *et al.*, 1971). This DNA has a sequence complexity of 3–5 × 10^6, and represents 25–50% of the viral genome (Varmus *et al.*, 1971, 1972). To date, we have been unable to demonstrate DNA of this complexity in the product of purified enzyme because of the limited amounts of available material. However, the failure

TABLE VII: Hybridization of Enzymatic Product with Homologous and Heterologous Templates.[a]

Template	Test RNA (%)	
	RSV	AMV
RSV 70 S	97	80
AMV 70 S	87	76

[a] Enzymatic product was prepared with purified RSV polymerase and either RSV or AMV 70S RNA as template. After fractionation on hydroxylapatite, the single-stranded forms (*ca.* 1000 cpm/sample) were annealed with homologous and heterologous viral RNA. Hybridization was measured by elution from hydroxylapatite (see Methods section). The results are expressed as proportion of total DNA eluting from hydroxylapatite as hybrid. No hybrids are formed if the various DNAs are incubated with poliovirus RNA.

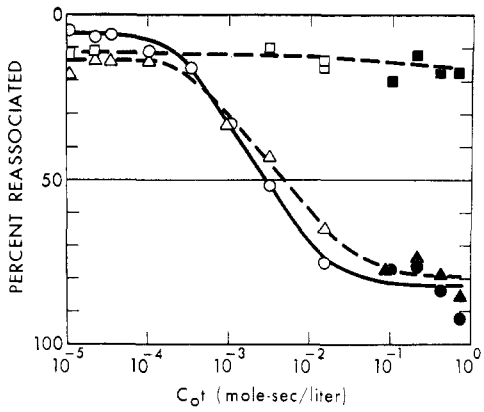

FIGURE 8: Homology of nucleotide sequence between products of crude and purified RSV polymerase. DNA was synthesized both with the purified polymerase ([^{3}H]TTP) and detergent-activated virions ([α-^{32}P]TTP). In each case, the double-stranded DNA was isolated by hydroxylapatite fraction and denatured by boiling and the kinetics of reassociation measured as described in Materials and Methods. The [^{3}H]DNA was allowed to reassociate either in the presence (△,▲) or absence (□,■) of a 1000-fold excess of [^{32}P]DNA (○,●). The data are plotted at the C_0t values of the [^{32}P]DNA. The open and closed symbols refer to two separate experiments.

to achieve complete reassociation under the conditions illustrated in Figure 7 suggests the presence of more complex sequences in the population of double-stranded DNA (Varmus *et al.*, 1971).

The double-stranded DNAs synthesized by detergent-activated virions and purified enzyme contain similar or identical nucleotide sequences, as judged by the results of reassociation experiments (Figure 8). ^{3}H-Labeled double-stranded product of purified enzyme was denatured, adjusted to a concentration too low to allow self-annealing, and incubated either with or without a large excess of denatured, ^{32}P-labeled product of detergent-activated virions. The [^{3}H]DNA incubated alone reassociates to only a limited extent (10–15%), whereas that incubated with the excess of [^{32}P]DNA reassociates extensively and with kinetics similar to those of the [^{32}P]DNA. We conclude that crude and purified enzyme preparations preferentially transcribe either identical or extensively homologous regions of the viral RNA template.

Discussion

Elution from Hydroxylapatite as a Criterion for Secondary Structure. This study and several previous reports (Fanshier *et al.*, 1971; Varmus *et al.*, 1971; Gelb *et al.*, 1971; Fujinaga *et al.*, 1970) have relied heavily upon hydroxylapatite for both analytical and preparative purposes. We therefore attempted to validate these procedures, utilizing resistance to hydrolysis by single strand-specific nuclease as the best criterion for double-helical structure. From the results of comparative analyses with hydroxylapatite and *Neurospora* nuclease (see Tables I and II), we conclude that estimates of secondary structure made with hydroxylapatite are subject to only minor artifacts under the present circumstances. However, we do not discount the probability that, in other situations, major artifacts may be introduced by the unpredictable behavior of partially double-helical molecules. Challenge with a single- or double strand-specific nuclease would appear to be the best available criterion for secondary structure of DNA. For present purposes, it is important to note that "double-stranded" DNA prepared from prolonged reactions with hydroxylapatite is entirely double helical, and therefore suitable for study of reassociation kinetics. Early reaction products contain DNA which elutes from hydroxylapatite in 0.4 M sodium phosphate, but which is hydrolyzed by *Neurospora* nuclease (Table II). This observation conforms to the previous suggestion that partially completed double-helical molecules constitute an intermediate form in the synthesis of double-stranded DNA (Faras *et al.*, 1971).

Synthesis of DNA by Detergent-Activated Virions and Purified Polymerase. The purified DNA polymerase of RSV apparently retains on a single protein molecule or complex all of the polymerase activities associated with intact virions. In response to 70S RNA template, the purified enzyme syntnesizes both single- and double-stranded DNA. However, no DNA is released from template molecules without concomitant degradation of the template. These observations are similar to those reported by Leis and Hurwitz for the DNA polymerases of AMV and Rauscher leukemia virus (Leis and Hurwitz, 1972). By contrast, detergent-activated virions rapidly synthesize DNA free of template. This discrepancy may indicate the presence of a "factor" within the virions which facilitates displacement of nascent DNA from template RNA (Leis and Hurwitz, 1972). For example, Mölling *et al.* have described a virion-associated hydrolytic enzyme specific for RNA–DNA hybrids (Mölling *et al.*, 1971). Alternatively, the synthesis of "free" DNA by virions may represent a trivial artifact due to other nucleases known to be present in purified preparations of virus (Mizutani *et al.*, 1970, 1971; Quintrell *et al.*, 1971). Whatever its explanation, the failure of purified "reverse transcriptase" to effectively synthesize DNA free of intact template raises serious conceptual problems with respect to the enzyme's putative function in viral replication and cellular transformation (Temin, 1971) which cannot presently be resolved.

A portion of the double-stranded DNA synthesized by purified polymerase is nondenaturable unless first nicked with DNase. These observations are consistent with the existence of "hairpin" forms in the population of double-stranded DNA, although the mechanism of their formation is unknown. Enzyme-active RSV virions generally do not synthesize an appreciable amount of nondenaturable double-stranded DNA. This could be due to the presence of DNase in the virions (Mizutani *et al.*, 1970, 1971), although we have been unable to detect significant amounts of DNase in our virus preparations (Quintrell *et al.*, 1971). The products of both crude (Fujinaga *et al.*, 1970) and purified (Leis and Hurwitz, 1972) preparations of AMV DNA polymerase contain significant amounts of nondenaturable double-stranded DNA, the nature of which has yet to be elucidated.

Initiation of DNA Synthesis on an RNA Primer Molecule. Previous reports have indicated that the synthesis of DNA by oncornavirus DNA polymerase is initiated on the 3′ hydroxyl of a polyribonucleotide (Verma *et al.*, 1971; Leis and Hurwitz, 1972). Our results confirm these observations, and further indicate that much of the DNA product remains covalently linked to RNA as late as 1 hr in the enzymatic reaction. This situation is substantially different from that with crude enzyme preparations, where DNA entirely free of RNA appears within 30 min (Verma *et al.*, 1971; and unpublished observation), and probably reflects the almost complete absence of nucleases from our enzyme preparations (Faras *et al.*, 1972). Given the lack of artifact due to nuclease, the results illustrated in Figure 1b indicate that the putative primer molecule must be relatively small because the entire covalently linked RNA–DNA complex has a sedimentation coefficient of less

than 10 S. The results of extensive analyses which substantiate this view will be reported subsequently (manuscript in preparation).

Molecular Weight of the Enzymatic Product. Extensive purification of the RSV polymerase, and in particular the complete elimination of nucleases, has had no effect on the molecular weight of the enzymatic product. In fact, the DNA chains synthesized by crude and purified enzyme are remarkably similar in length (see Figures 4 and 5). These observations conform to those made with purified polymerases from AMV (Leis and Hurwitz, 1972), and leave unreconciled the nature of the DNA synthesized *in vitro* and its putative role *in vivo* (Temin, 1971).

Extent of Genome Transcription by the Purified Enzyme. Detergent-activated virions of RSV transcribe at least a major portion of the viral genome into DNA. This statement is based on the results of Duesberg and Canaani (1970), who demonstrated that hybridization of viral RNA with large excesses of enzymatic product renders the bulk of the viral RNA resistant to digestion by RNase. We have performed similar experiments with crude preparations of RSV polymerase, and have confirmed these findings (H. E. Varmus, A. C. Garapin, and J. M. Bishop, unpublished observations). Experiments of this sort are not presently feasible with purified polymerase because of the inadequate amounts of materials available. However, by analyzing the reassociation kinetics of double-stranded DNA, we have obtained evidence for the preferential transcription of a very limited portion of the viral genome by purified polymerase. These findings conform to observations made with crude enzyme preparations (Varmus *et al.*, 1971; Gelb *et al.*, 1971). Moreover, the preferential transcription by crude and purified enzyme apparently involves the same or substantially homologous nucleotide sequences (Figure 8). The significance of this preferential transcription remains obscure. These results do not rule against the possible transcription of the entire viral genome. For example, the results from different laboratories with crude enzyme preparations can be reconciled by assuming that the bulk of the genome is represented infrequently among the enzymatic transcripts. This would explain both the requirement for very large excesses of DNA in the experiments of Duesberg and Canaani (1970) and our inability to detect double-stranded DNA sufficiently complex to represent the entire genome (Varmus *et al.*, 1971).

Acknowledgments

We thank H. Goodman for assistance in performing the nearest-neighbor analyses, L. Levintow for advice and support, J. Hurwitz and R. Leis for communication of data prior to publication, and L. Fanshier, N. Quintrell, K. Smith, and J. Jackson for technical assistance.

References

Baltimore, D. (1970), *Nature (London) 226*, 1209.
Best, M., Evans, B., and Bishop, J. M. (1972), *Virology* (in press).
Bishop, D. H. L., Claybrook, J. R., and Spiegelman, S. (1967), *J. Mol. Biol. 26*, 373.
Bishop, J. M., Levinson, W. E., Quintrell, N., Sullivan, D., Fanshier, L., and Jackson, J. (1970), *Virology 42*, 182.
Britten, R. J., and Kohne, D. E. (1968), *Science 161*, 529.
Duesberg, P. H. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 1511.
Duesberg, P. H., and Canaani, E. (1970), *Virology 42*, 783.
Erikson, R. L. (1969), *Virology 37*, 124.
Fanshier, L., Garapin, A. C., McDonnell, J. P., Faras, A., Levinson, W., and Bishop, J. M. (1971), *J. Virol. 7*, 77.
Faras, A., Fanshier, L., Garapin, A. C., Levinson, W., and Bishop, J. M. (1971), *J. Virol. 7*, 539.
Faras, A. J., Taylor, J. M., McDonnell, J. P., Levinson, W. E., and Bishop, J. M. (1972), *Biochemistry 11*, 2334.
Fisher, M. P., and Dingman, C. W. (1971), *Biochemistry 10*, 1895.
Fujinaga, K., Parsons, J. T., Beard, J. W., Beard, D., and Green, M. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1432.
Garapin, A. C., Fanshier, L., Leong, J., Jackson, J., Levinson, W., and Bishop, J. M. (1971a), *J. Virol. 7*, 227.
Garapin, A. C., Leong, J., Fanshier, L., Levinson, W. E., and Bishop, J. M. (1971b), *Biochem. Biophys. Res. Commun. 42*, 919.
Garapin, A. C., McDonnell, J., Levinson, W., Quintrell, N., Fanshier, L., and Bishop, J. M. (1970), *J. Virol. 6*, 589.
Gelb, L., Aaronson, S. A., and Martin, M. (1971), *Science 172*, 1353.
Leis, J. P., and Hurwitz, J. (1972), *J. Virol. 9*, 130.
Leong, J., *et al.* (1972), *J. Virol.* (in press).
Manly, K. F., Smoler, D. F., Bromfield, E., and Baltimore, D. (1971), *J. Virol. 7*, 106.
Mizutani, S., Boettiger, D., and Temin, H. M. (1970), *Nature (London) 228*, 424.
Mizutani, S., Temin, H. W., Kodama, M., and Wells, R. T. (1971), *Nature (London) 230*, 232.
Mölling, K., Bolognesi, D. P., Bauer, H., Büsen, W., Plassmann, H. W., and Hausen, P. (1971), *Nature (London), New Biol. 234*, 246.
Quintrell, N., Fanshier, L., Evans, B., Levinson, W. E., and Bishop, J. M. (1971), *J. Virol. 8*, 17.
Rabin, E. F., Preiss, B., and Fraser, M. J. (1971), *Prep. Biochem. 1*, 283.
Rokutanda, M., Rokutanda, H., Green, M., Fujinaga, K., Ray, R. K., and Gurgo, C. (1970), *Nature (London) 227*, 1026.
Sebring, E. D., and Salzman, N. P. (1964), *Anal. Biochem. 8*, 126.
Spiegelman, S., Burny, A., Das, M. R., Keydar, J., Schlom, J., Travnicek, M., and Watson, K. (1970), *Nature (London) 227*, 563.
Temin, H. M. (1971), *Annu. Rev. Microbiol. 25*, 609.
Temin, H. M., and Mizutani, S. (1970), *Nature (London) 226*, 1211.
Varmus, H. E., Levinson, W. E., and Bishop, J. M. (1971), *Nature (London), New Biol. 233*, 19.
Varmus, H. E., Weiss, R. A., Friis, R. R., Levinson, W. E., and Bishop, J. M. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 20.
Verma, I. M., Bromfeld, E., Manly, K. F., and Baltimore, D. (1971), *Nature (London), New Biol. 233*, 131.