Purification and Characterization of the Deoxyribonucleic Acid Polymerase Associated with Rous Sarcoma Virus†

A. J. Faras, J. M. Taylor, J. P. McDonnell, W. E. Levinson, and J. M. Bishop*

ABSTRACT: The DNA polymerase associated with the Schmidt–Ruppin strain of Rous sarcoma virus has been purified a minimum of 500-fold. The purified enzyme is completely template dependent and free of appreciable amounts of nuclease. Responses to various RNA and DNA templates apparently reside on a single protein or protein complex. The 70S RNAs of RNA tumor viruses are more efficient templates for the Rous sarcoma virus polymerase than any of the other natural RNAs tested. Under optimal conditions, the amount of DNA synthesized with 70S viral RNA as template is almost identical with that with synthetic polynucleotides. However, synthesis of DNA in excess of the amount of input template has not been observed under any circumstance. Preparations of Rous sarcoma virus contain a maximum of 2.5–5 polymerase molecules/virion.

Purified preparations of RNA tumor viruses contain two distinct DNA polymerase activities: RNA-dependent synthesis of single-stranded DNA (Baltimore, 1970; Temin and Mizutani, 1970; Manly *et al.*, 1971; Fanshier *et al.*, 1971), and DNA-dependent synthesis of double-stranded DNA (Manly *et al.*, 1971; McDonnell *et al.*, 1970; Spiegelman *et al.*, 1970b). Genome RNA of the virus serves as template for the synthesis of single-stranded DNA (Spiegelman *et al.*, 1970a; Rokutanda *et al.*, 1970; Garapin *et al.*, 1971), initiated on the 3′ terminus of a polyribonucleotide (Verma *et al.*, 1971; Leis and Hurwitz, 1972). The single-stranded DNA product in turn serves as template precursor for the synthesis of double-stranded DNA (Garapin *et al.*, 1971; Faras *et al.*, 1971). These results have been obtained mainly with crude enzyme preparations consisting of virions disrupted with nonionic detergents. Further study of the mechanism of enzymatic synthesis, detailed characterization of the synthetic capabilities of the virion enzyme(s), and identification of the protein molecule(s) responsible for these capabilities will all require purified preparations of the enzyme or enzymes in question.

Extensive purification of representative RNA-dependent DNA polymerases has now been achieved in several laboratories (Kacian *et al.*, 1971; Hurwitz and Leis, 1972), and we report here the purification of DNA polymerase from virions of Rous sarcoma virus (RSV).[1] We document the extent of purification and the removal of all but trace amounts of RNase, define conditions for optimum DNA synthesis, and present a preliminary characterization of the relative efficiency of various nucleic acids as templates for the purified enzyme. A subsequent report describes the nature of the DNA synthesized in response to viral RNA templates (Taylor *et al.*, 1972).

Materials and Methods

Reagents and Solutions. RNase A was from Worthington Biochemical Corp. Stock solutions were boiled for 10 min to inactivate contaminating DNase. The following lists the materials and the company where they were purchased: Nonidet-P40 (NP-40), Shell Chemical Co.; Triton X-100, Packard Instrument Co.; Poly(rA)·oligo(dT) and poly(rA)·poly(rU), Collaborative Research Inc.; Sephadex G-100, Pharmacia; phosphocellulose—Whatman P-11, prepared by successive washes with 0.1 N HCl in 50% (v/v) ethanol, 0.1 N NaOH, and 0.1 M EDTA; DEAE-cellulose—Whatman DE-11, washed sequentially with 0.1 N NaOH and 0.1 N HCl; deoxynucleoside triphosphates (dGTP, dATP, dCTP), Calbiochem; [^{3}H]TTP (15–20 Ci/mmole), Schwarz BioResearch, Inc.; [α-^{32}P]TTP (1–5 Ci/mmole), International Chemical and Nuclear Corp.; DEAE-cellulose buffer: 0.02 M Tris·HCl (pH 7.2)–0.2% (v/v) β-mercaptoethanol–0.2% NP-40–30% glycerol; phosphocellulose buffer—0.01 M sodium phosphate (pH 6.8)–0.001 M EDTA–0.2% β-mercaptoethanol–0.2% NP-40–30% glycerol; Sephadex buffer—0.4 M NaCl–0.01 M sodium phosphate (pH 6.8)–0.2% β-mercaptoethanol–0.2% NP-40–30% glycerol; pancreatic DNase, electrophoretically purified, Worthington Biochemical Corp. DNA polymerase I (fraction VII) of *E. coli*, purified according to Jovin *et al.* (1969), was a gift from Dr. J. Huberman.

Cells and Virus. The propagation of the Schmidt–Ruppin strain of Rous sarcoma virus (RSV) in chick embryo fibroblasts and purification of the virus have been described previously (Bishop *et al.*, 1970). Purified virus was stored at 4° in 0.01 M NaCl–0.001 M EDTA–0.02 M Tris·HCl (pH 7.4)–45% sucrose for up to 2 months without appreciable loss of enzyme activity. Purified poliovirus was prepared as described previously (Bishop *et al.*, 1969). R-17 bacteriophage was prepared according to Roblin (1968).

Enzyme Reactions. DNA polymerase was assayed by determining the incorporation of radiolabeled precursor into an acid-insoluble state (Garapin *et al.*, 1970). Enzyme samples (usually at concentrations of 0.2 enzyme unit and 2 μg of enzyme protein/ml) were incubated at 37° in the following reaction mixture: 0.1 M Tris·HCl (pH 8.1),[2] 0.01 M $MgCl_2$,

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[1] Abbreviations used are: RSV, Rous sarcoma virus; NP-40, Nonidet-P40; AMV, avian myeloblastosis virus; rA·dT, poly(rA)·oligo(dT).

[2] Previous determinations of the optimal pH for enzyme reactions carried out with disrupted virions led to the routine use of pH 8.1

2% β-mercaptoethanol, 5×10^{-5} M unlabeled deoxynucleoside triphosphates (dGTP, dATP, and dCTP), [^{3}H]TTP (generally 5×10^{-6} M), and template nucleic acid at specified concentrations. For assay of column and gradient fractions, templates were used at concentrations which provide maximum activities under standard conditions (see Figure 8). One unit of polymerase is defined as that amount of activity which catalyzes the incorporation of 1 nmole of [^{3}H]TTP in 120 min with calf thymus DNA (200 μg/ml) as template. This proved to be a consistent and reproducible value when independently standardized with another template, rA·dT. The inclusion of bovine serum albumin (200 μg/ml) in the reaction mixture stimulated DNA synthesis twofold without any other effect on the kinetics of synthesis. This maneuver was not used in the studies to be reported here unless otherwise noted. DNA-dependent RNA polymerase of *E. coli*, purified and assayed as described by Burgess (1969), was a gift from H. Eisen.

Measurement of Viral and Enzyme Protein. Protein was measured with the method of Lowry *et al.* (1951). The presence of glycerol and nonionic detergent in buffers interfered with the routine determination of protein in enzyme fractions, as did the limited amounts of protein available in the purified enzyme fractions. The procedure was therefore standardized twice with virus labeled with tritiated amino acids, and once by devoting an entire enzyme preparation to protein determinations. The specific activities (polymerase units/μg of protein) obtained on these three occasions did not vary by more than a factor of 2. Experimental results have been routinely expressed in terms of enzyme units rather than enzyme protein.

Assay of Alkaline Phosphatase. Alkaline phosphatase activity was assayed with two procedures: hydrolysis of *p*-nitrophenyl phosphate (Nisman, 1968) and release of the γ-phosphate from [γ-^{32}P]ATP. These assays were performed in collaboration with H. Boyer and J. Hedgpeth, who provided the [γ-^{32}P]ATP.

Solubilization of Enzyme. Unless otherwise stated, these and all subsequent manipulations were carried out at 4°. Purified virus was adjusted to a final protein concentration of 1–2 mg/ml in the following solution: 0.2 M sodium phosphate (pH 6.8), 0.01 M EDTA, 0.2% (v/v) β-mercaptoethanol, and 1.0% NP-40. The mixture was incubated for 30 min at 4° followed by 30 min at 37°. At the conclusion, enzyme activity with exogenous DNA template remained at maximum levels. Endogenous activity (without added template) was always reduced to less than 25% of the original level because of degradation of resident RNA template by the RNase present in all preparations of purified virus (Quintrell *et al.*, 1971). A minimum of 75% of all enzyme activity remained in the supernatant following centrifugation at 10,000 rpm for 15 min at 4° in a type 40 rotor.

Chromatography on DEAE-cellulose. The solubilized enzyme (10–20 mg of viral protein) was diluted tenfold in DEAE buffer and applied to a 0.9 × 20 cm column of DEAE-cellulose. All enzyme activity detectable with DNA and rA·dT as templates adsorbed to the column. Following a wash with two column volumes of DEAE buffer, the column was eluted with 0.4 M KCl in DEAE buffer. Recovery of enzyme activity ranged from 80 to 150%. In the absence of nonionic detergent, recovery dropped to 10–20%.

Chromatography on Phosphocellulose. The eluate from DEAE-cellulose was diluted tenfold in phosphocellulose buffer and applied to a 0.9 × 10 cm column. All detectable enzyme activity adsorbed to the column. Following a wash with two column volumes of phosphocellulose buffer, the column was eluted (10 ml/hr for 8 hr) with a gradient of 0–0.6 M NaCl in phosphocellulose buffer. Fractions of 2 ml were collected and aliquots assayed for enzyme activity with rA·dT and calf thymus DNA as templates. Recovery of enzyme activity ranged from 80 to 100% in the presence of nonionic detergent, 10–20% in the absence of detergent.

Concentration of Phosphocellulose Eluate. The fractions containing enzyme activity were pooled, diluted fourfold in phosphocellulose buffer, and passed through a 0.9 × 1 cm phosphocellulose column. All detectable enzyme activity adsorbed to the column, and could be eluted with 0.6 M NaCl–phosphocellulose buffer in a volume no greater than 2 ml. Recovery of enzyme activity was 100%.

Gel Filtration. The concentrated phosphocellulose eluate was passed through a 2.5 × 65 cm column of G-100 Sephadex, previously equilibrated with Sephadex buffer. Flow rate was maintained at 3–5 ml/hr. Fractions of 3 ml were collected and assayed for enzyme activity with 70S viral RNA, rA·dT, and calf thymus DNA. Recovery of enzyme activity was 100%.

Rate Zonal Centrifugation. Enzyme preparations were centrifuged in gradients of 20–40% glycerol containing 0.4 M NaCl–0.001 M EDTA–0.01 M sodium phosphate (pH 6.8)–0.2% NP-40–0.2% β-mercaptoethanol. Nucleic acids were analyzed in gradients of 15–30% sucrose containing 0.1 M NaCl–0.001 M EDTA–0.02 M Tris·HCl (pH 7.4). Conditions of centrifugation are given in the legends for individual figures.

Electrophoresis in Polyacrylamide Gels. Electrophoresis of RNA in ethylene diacrylate cross-linked gels of 2.25% polyacrylamide was performed as described previously (Bishop *et al.*, 1970).

Preparation of RNAs. The 70S RNA of RSV was extracted from purified virus with sodium dodecyl sulfate–phenol and isolated by rate zonal centrifugation (Bishop *et al.*, 1970). Purified 70S RNA of avian myeloblastosis virus (AMV) was a gift from Dr. R. Erikson. Poliovirus RNA was extracted from purified virus with phenol at 37° (Bishop *et al.*, 1969). r- and tRNAs were prepared from HeLa cells by phenol extraction at 60° and fractionation with 1 M NaCl (Bishop and Koch, 1967). The tRNA was further treated with DNase (10 μg/ml, 1 hr, 37°) prior to use as enzyme template. This was necessary to eliminate low levels of template activity due to residual DNA. RNA was extracted from R-17 bacteriophage with phenol, then further purified by rate-zonal centrifugation in sucrose gradients and ethanol precipitation of the 27S RNA.

Preparation of Iodinated Serum Albumin. Bovine serum albumin (twice crystallized, Nutritional Biochemicals) was labeled with ^{131}I, using the procedure of McConahey and Dixon (1966). When acid precipitated and trapped on glass fiber filters (Reeve-Angel), this material could be counted in the ^{32}P window of a Beckman LS-200B scintillation spectrometer with 10% spill into the ^{3}H channel.

DNA Templates. Calf thymus DNA, purchased from Sigma, was dissolved in 0.1 M Tris·HCl (pH 8.1) at a concentration of 2.0 mg/ml, and sonicated (in 20-ml volumes) for 2 min with a Branson sonifier in order to reduce viscosity. DNA extracted from purified λ bacteriophage was a gift from Dr. H. Boyer. DNA was prepared from suspension cultures of HeLa cells and from 11-day chicken embryos as described

(Garapin *et al.*, 1970). For the sake of consistency, this convention has been maintained in the work presented here, although subsequent determinations have shown that the purified enzyme is slightly more active (*ca.* 5–8%) at pH 7.5 (see Figure 6c).

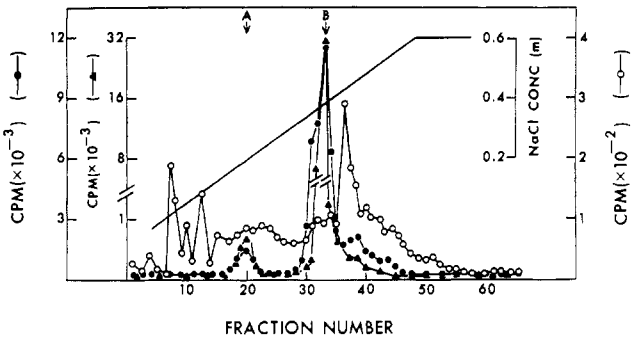

FIGURE 1: Chromatography of DNA polymerase on phosphocellulose. Purified RSV, labeled with [^{3}H]amino acids, was disrupted with NP-40 and chromatographed on DEAE-cellulose as described in the text. The eluate from DEAE-cellulose was then adsorbed to phosphocellulose and elution performed with a gradient of 0–0.6 M NaCl. Samples (20 μl) were taken for assay of enzyme activity with DNA (●) and rA·dT (▲) templates, using [α-^{32}P]TTP as the labeled precursor. Acid-precipitable cpm of [^{3}H]amino acids (○) was determined on separate samples (0.2 ml). (—) NaCl gradient.

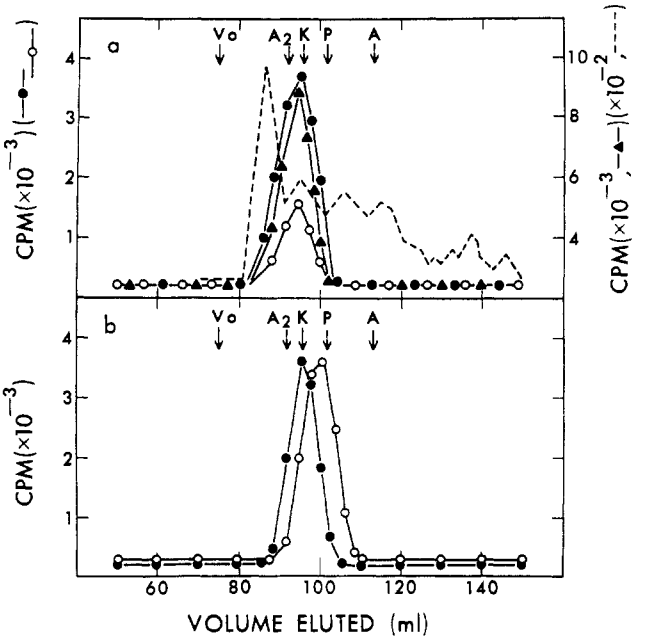

FIGURE 2: Gel filtration in G-100 Sephadex. (a) Template responses of form B. The second peak of enzymatic activity eluted from phosphocellulose (form B) was concentrated as described in Materials and Methods and filtered through a column of G-100 Sephadex in the presence of ^{131}I-labeled albumin. Samples (20 μl) were taken for assay of polymerase activity with 70S RSV RNA (○), rA·dT (▲), and calf thymus DNA (●), and for measurement of acid-precipitable [^{131}I]albumin and [^{3}H]amino acids (– – –). The column was also calibrated separately by simultaneous filtration of [^{131}I]-albumin, alkaline phosphatase (P) and *E. coli* DNA polymerase I (K). The albumin preparation contained monomeric (A) and dimeric (A_2) molecules. *E. coli* polymerase was assayed in the same manner as viral polymerase, using DNA template. V_0, exclusion volume. (b) Comparison of RSV polymerase forms A and B. Forms A and B eluted from phosphocellulose were analyzed separately, using [^{131}I]albumin as internal standard and the same column as in (a). Polymerase activity was assayed with DNA template. (○) Form A; (●) form B; A, monomer albumin; A_2, dimer albumin; P, alkaline phosphatase; K, *E. coli* DNA polymerase I; V_0, exclusion volume.

previously (Fanshier *et al.*, 1971). All DNAs were in 0.1 M Tris·HCl (pH 8.1) when used in enzyme reactions.

Results

Purification of the Enzyme. Enzyme activity was routinely followed through the course of purification with exogenous DNA template. The validity of this procedure will be discussed below. Table I summarizes the scheme which provides a minimum purification of 500-fold. Recoveries ranged from 60 to 100% throughout, provided that nonionic detergent (NP-40) was present. In its absence, recoveries from the DEAE-cellulose and phosphocellulose columns, and from the glycerol gradient, were substantially lower (10–20%). We do not understand the mechanism of this effect, but the results of reconstruction experiments indicate that the detergent has no perceptible effect on either the behavior of the enzyme *per se* or the general resolution achieved in these procedures. Triton X-100 has also been used with similar results.

All detectable polymerase activity in the crude preparation of disrupted virus adsorbs to DEAE-cellulose. When eluted,

TABLE I: Purification of Rous Sarcoma Virus DNA Polymerase.[a]

	Cumulative Recovery	Sp Act.	Rel Purity
Virus	1.0	0.012	1
DEAE-cellulose	0.8	0.05	4.2
Phosphocellulose	0.8	0.55	46
Sephadex G-100	0.7	5.6	470
Glycerol gradient	0.7	11.2	940

[a] Purified RSV, labeled with [^{3}H]amino acids, was used to prepare DNA polymerase as described in the text. Relative purity at each step was computed on the basis of enzymatic specific activity (*i.e.*, pmoles of DNA synthesized per 2 hr per cpm of acid-precipitable [^{3}H]amino acids). Similar results were obtained in a separate experiment, using unlabeled virus and chemical determination of protein concentrations. On both occasions, the final specific enzymatic activity was approximately 1 unit of enzyme/10 μg of protein. The total yields of enzyme protein have been in the range of 10–50 μg, starting with 10–50 mg of viral protein.

TABLE II: Responses of Polymerase Forms A and B to DNA and 70S RNA.

	Polymerase[a]	
Template	Form A	Form B
DNA	0.8	1.0
70S RNA	0.1	0.13
DNA–RNA	8	8

[a] Polymerase forms A and B, eluted from phosphocellulose, were used without further purification. Equal amounts of enzyme activity (0.01 unit) were assayed with calf thymus DNA (3 μg/ml) and 70S RNA (3 μg/ml) as templates. Results are expressed as pmoles of DNA synthesized in a 30-min period. Assays were performed in duplicate, and a background of 0.02 pmole has been subtracted from all samples.

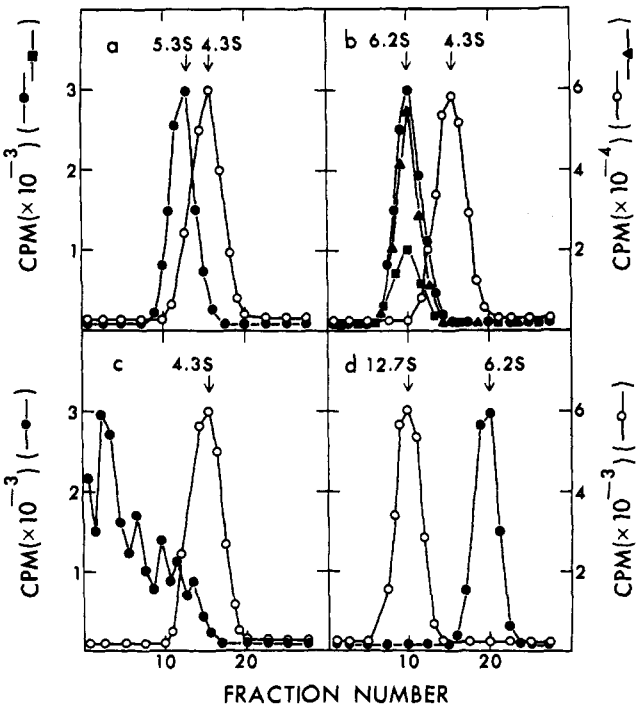

FIGURE 3: Rate-zonal centrifugation of polymerase forms A and B. Polymerase forms A and B as eluted from phosphocellulose were centrifuged in density gradients of 20–40% glycerol. [^{131}I]Albumin served as a sedimentation reference. Centrifugation in panels A, B, and C was carried out in an SW-65 rotor at 45,000 rpm for 16 hr. Centrifugation in panel D was at 40,000 rpm for 20 hr in an SW-41 rotor. All analyses were carried out at 4°. (a) Polymerase form A sedimented in 0.4 M NaCl: (●) [^{3}H]TMP (DNA as template); (○) ^{131}I cpm (albumin monomer). (b) Polymerase form B sedimented in 0.4 M NaCl: (●) [^{3}H]TMP (DNA template); (▲) [^{3}H]TMP (rA·dT template); (■) [^{3}H]TMP (70S RNA as template); (○) ^{131}I cpm (albumin). (c) Polymerase form B sedimented in 0.05 M NaCl: (●) [^{3}H]TMP (DNA template); (○) ^{131}I cpm (albumin). (d) Polymerase form B sedimented in 0.4 M NaCl with *E. coli* DNA-dependent RNA polymerase: (●) cpm [^{3}H]TMP (DNA polymerase); (○) cpm of [^{3}H]rGMP (RNA polymerase).

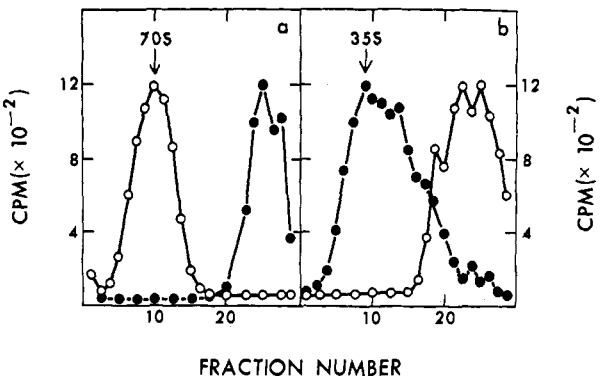

FIGURE 4: Analysis of polymerase fractions for RNase activity. 70S RSV [^{32}P]RNA was exposed to samples of enzyme fractions under standard conditions for the polymerase assay. Incubation was at 37° for 30 min, after which the reaction mixtures were extracted with sodium dodecyl sulfate–Pronase (Garapin *et al.*, 1970) and analyzed by centrifugation through gradients of 15–30% sucrose. Where indicated, the extracted reaction mixture was adjusted to 0.01 M EDTA and heated at 80° for 2 min prior to centrifugation. This procedure dissociates the 70S RNA into its constituent subunits without the introduction of appreciable numbers of heat-induced covalent breaks. ^{3}H-Labeled RNAs of RSV (70 S) or poliovirus (35 S) served as sedimentation references. (a) Centrifugation of native 70S RSV RNA after exposure to various polymerase fractions. The results of two separate centrifugations are superimposed. An internal sedimentation reference (70S RSV RNA) was present in each case. Centrifugation was carried out in an SW-50.1 rotor at 50,000 rpm for 1 hr, 45 min at 4°. (●) 70S RNA exposed to crude disrupted virions; (○) 70S RNA exposed to polymerase form B eluted from phosphocellulose. (b) Centrifugation of RSV RNA, denatured with heat following exposure to polymerase fractions. The results of two separate centrifugations are superimposed. An internal sedimentation reference (35S poliovirus RNA) was present in each case. Centrifugation was carried out in an SW-50.1 rotor at 50,000 rpm for 3 hr, 45 min at 4°. (●) RSV RNA denatured after exposure to reaction mixture lacking enzyme; (○) RSV RNA denatured after exposure to polymerase form B eluted from phosphocellulose.

it is totally dependent upon exogenous template. Chromatography on phosphocellulose yields two fractions of enzymatic activity (Figure 1). One (form A) constitutes approximately 10% of the total recovered activity and elutes in 0.2 M NaCl, the other (form B) represents the bulk (*ca.* 90%) of the activity and elutes in 0.4 M NaCl. This pattern is also observed if chromatography on DEAE-cellulose is omitted and the disrupted virus applied directly to phosphocellulose. If form B is diluted in buffer and rechromatographed on phosphocellulose, forms A and B are again obtained in the same ratio as above. The two enzyme fractions are therefore related, but probably differ in size and/or conformation, as will be discussed below. Only form B has been obtained in quantities sufficient to allow detailed characterization of template responses, but the proportional responses to DNA and 70S RNA are identical with forms A and B (Table II).

Gel filtration of form B in G-100 Sephadex is illustrated in Figure 2a. The position of the enzyme in relation to DNA polymerase I of *E. coli*, alkaline phosphatase, and serum albumin indicates a relative molecular weight of approximately 105,000.

Form A appears to have a slightly lower molecular weight (*ca.* 96,000) than form B when separately analyzed in the same G-100 column with the same markers (Figure 2b). The observed differences are small but consistent. If disrupted virus is run directly through the Sephadex column (omitting the preceding chromatographies), only form B can be delineated. We do not presently know whether this is due to failure to generate form A under these conditions, or to inadequate resolution in the column.

The centrifugation of purified enzyme with iodinated albumin in density gradients of glycerol is illustrated in Figure 3a,b. Form A sediments at 5.3 S, form B at 6.2 S. At NaCl concentrations below 0.2 M, form B aggregates extensively, leading to poor recoveries and erratic sedimentation behavior. An example is illustrated in Figure 3c. Because of this, 0.4 M NaCl was used routinely in both gel filtration and rate-zonal centrifugation. Sedimentation of enzyme activity directly from disrupted virus gives results identical with those obtained with form B. All of the ensuing studies were performed with polymerase form B.

Elimination of RNase. Purified preparations of RSV contain extensive amounts of RNase (Quintrell *et al.*, 1971), elimination of which was mandatory if the purified DNA polymerase was to be used with RNA templates. RNase activity was monitored by exposing 70S RSV RNA to the enzyme under conditions for the enzymatic reaction, then denaturing the RNA into its constituent subunits (Duesberg, 1968; Erikson, 1969) and analyzing it with either rate-zonal centrifugation or electrophoresis in polyacrylamide gels. This procedure provides an assay of maximum sensitivity, capable of detecting a limited number of chain scissions which might otherwise be

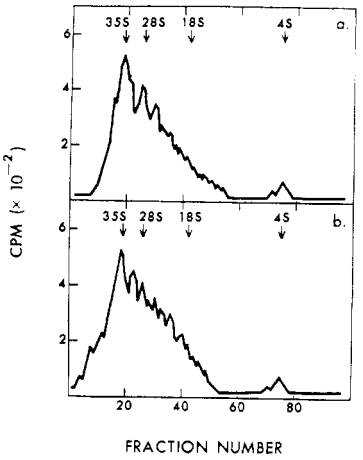

FIGURE 5. Analysis of RSV RNA in polyacrylamide gels following exposure to purified DNA polymerase. Polymerase form B was used after gel filtration in G-100 Sephadex. Exposure and denaturation of 70S RSV [^{32}P]RNA were carried out as described in Figure 4. Electrophoresis was performed with 6 × 100 mm gels of 2.25% polyacrylamide, 5 mA/gel, 3 hr at room temperature. 35S poliovirus [^{3}H]RNA and ^{3}H-labeled chick embryo fibroblast RNA were used as markers. (a) Control: 70S RSV [^{32}P]RNA was exposed to polymerase reaction mixture lacking enzyme, denatured, and electrophoresed; (b) enzyme-treated: 70S RSV [^{32}P]RNA was exposed to purified DNA polymerase (G-100 Sephadex fraction), denatured, and electrophoresed.

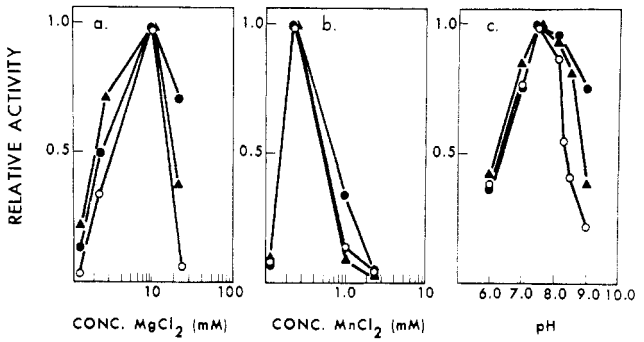

FIGURE 6: Determination of optimal reaction conditions. Purified RSV DNA polymerase (G-100 Sephadex fraction) was assayed under standard conditions, using a 2-hr incubation, and varying the indicated parameters. Assays were performed with 70S RSV RNA (○), rA·dT (▲), and calf thymus DNA (●), all at concentrations providing maximum activities (see Figure 8). (a) Optimal concentration of $MgCl_2$: no other divalent cation was added. (b) Optimal concentration of $MnCl_2$: no other divalent cation was added. (c) Optimal pH: $MgCl_2$ (0.01 M) was the divalent cation.

obscured by secondary structure. Typical results are given in Figure 4. Crude preparations of disrupted virus quickly degrade RSV RNA to small fragments detectable even without denaturation (Figure 4a). Partially purified enzyme preparations (phosphocellulose fractions or enzyme centrifuged directly into glycerol gradients) contain limited amounts of RNase, the effects of which are apparent only if the substrate RNA is denatured (Figure 4a,b). Only gel filtration in Sephadex reduces contamination with RNase to the point where few or no chain scissions are introduced during limited incubations (up to 30 min). In this instance the melted RNA was evaluated by electrophoresis in polyacrylamide gels in order to obtain maximum resolution (Figure 5). The profiles of high molecular weight RNA in these gels are generally comparable to those published by Duesberg and Vogt (1970), and there is no appreciable difference between the control and the enzyme-treated RNA. Two low molecular weight RNAs are also apparent after denaturation: a 4S form as previously described by Erikson and Erikson (1971), and a slightly larger form which comigrates with cellular 5S RNA in 10% polyacrylamide gels (A. J. Faras, unpublished observation). To date, we have been unable to prepare enzyme fractions which do not eventually degrade RNA, the first chain breaks becoming apparent after 1- to 2-hr incubations.

Coincidence of Template Responses. Crude preparations of disrupted RSV can utilize a variety of templates for the synthesis of DNA. The most important of these are: (1) the endogenous viral RNA (Baltimore, 1970; Temin and Mizutani, 1970); (2) various synthetic polynucleotides, particularly polyribonucleotides hydrogen bonded to oligomers of deoxyribonucleotides (Spiegelman *et al.*, 1970c; Baltimore and Smoler, 1971); and (3) exogenous DNA (McDonnell *et al.*, 1970; Spiegelman *et al.*, 1970b; Mizutani *et al.*, 1970). In our experience, these various template responses are coincident at all stages of enzyme purification where they can be examined, *i.e.*, whatever the template, the activity apparently resides on the same physical entity. Responses to exogenous DNA and rA·dT are coincident throughout the purification (Figures 1, 2, and 3). Examination of the response to exogenous viral RNA is more difficult, because it cannot be elicited until the bulk of the RNase has been eliminated. In our standard purification sequence, a significant and reliable response to 70S viral RNA is first observed following gel filtration (Figure 2), at which point it is coincident with the other template responses. However, a separate RNA-dependent activity could have escaped detection and been discarded earlier in the purification. We therefore prepared enzyme by either rate-zonal centrifugation or gel filtration directly after disruption of purified virus. Both procedures sufficiently reduce RNase contamination to allow the use of exogenous RNA template, and in both instances, the only response to such template is that which is coincident with the response to rA·dT and DNA. These experiments included centrifugations brief enough to detect much larger enzyme molecules using DNA-dependent RNA polymerase of *E. coli* as a sedimentation marker (Figure 3d). Molecules (or aggregates) even larger than this should not have escaped detection in the gel filtrations.

Properties of the Purified Enzyme

Determination of Optimal Reaction Conditions. Determinations of optimal concentrations of hydrogen ion, Mg^{2+}, and Mn^{2+} for each of three classes of template are illustrated in Figure 6. In each instance, the requirements are similar or identical for all three templates. Moreover, there is no significant difference between the maximum activities obtained with Mg^{2+} and those with Mn^{2+} (Table III), although the optimal concentrations of these cations are appreciably different. A previous report indicated that Mn^{2+} was far superior to Mg^{2+} for the transcription of duplex synthetic polynucleotides (Scolnick *et al.*, 1970), but the conditions of enzymatic synthesis and the nature of the templates (principally poly(A)·poly(U)) were appreciably different from those employed here. Magnesium was adopted as the standard divalent cation for the studies which follow. In our experience, Mg^{2+} and Mn^{2+} used together are not synergistic (Table III). We have not examined the effect of the combined cations on template specificity.

Stability of the Enzyme. The enzyme in undisrupted virions (both endogenous RNA-dependent activity and activity with

TABLE III: Synthesis of DNA with $MgCl_2$ and $MnCl_2$.

Template	Cation[a]		
	Mg^{2+}	Mn^{2+}	$Mg^{2+} + Mn^{2+}$
70S RNA	2.22	2.60	2.33
rA·dT	9.54	9.40	0.53
DNA	8.23	3.77	6.83

[a] Assays (0.04 ml) were carried out at optimal concentrations of divalent cation using the G-100 Sephadex fraction of purified polymerase at 0.2 unit/ml and all templates at 2 μg/ml. Results are expressed as pmoles of DNA synthesized in 1 hr at 37°.

the exogenous templates DNA and rA·dT) is completely stable for a minimum of 1 month when stored at 4°. By contrast, the various template responses of purified enzyme, stored at 4° in 30% glycerol–0.4 M NaCl, display differential labilities. The half-life of DNA-dependent activity is approximately 8 weeks, that of viral RNA-dependent activity 4 weeks. A precise determination of the half-life for the response to rA·dT has not been made, but it appears to be intermediate between the responses to DNA and viral RNA. All template responses are *less* stable at lower temperatures (−10 to −70°), even in the presence of 50% (v/v) glycerol.

Effect of Monovalent Cation. Monovalent cations inhibit the response of the enzyme to both RNA and DNA templates (Figure 7), although higher concentrations of cation are required to inhibit completely the response to DNA. The three cations tested (Na^+, K^+, and NH_4^+) have essentially identical effects at equivalent concentrations. Enzyme incubated in the presence of template and inhibiting concentrations of cation can be restored to full activity by dilution of the reaction mixture. By contrast, addition of more template does not reverse cation inhibition, even at template concentrations well below saturating levels (see below).

The preceding results have also been obtained with crude virions. In this case, endogenous polymerase activity is taken to represent response to RNA template. DNA-dependent activity is elicited by addition of exogenous DNA following treatment of the detergent-disrupted virions with RNase to eliminate the endogenous reaction (McDonnell *et al.*, 1970; Spiegelman *et al.*, 1970b).

Saturation of Enzyme with Template. Figure 8a illustrates the amounts of RNA and rA·dT required to saturate a given amount of purified enzyme. Saturation with DNA has not been achieved at the highest concentration tested (300 μg/ml of reaction mixture). On most occasions, the quantity of enzyme available for these studies was too small to allow routine estimation of protein concentration. Consequently, a standard volume of enzyme was used in each reaction, and every preparation of enzyme was independently calibrated in the manner illustrated in Figure 8. In every instance, the relative amount of each template required to obtain maximum response was constant.

The saturation of a given amount of template RNA by increasing amounts of enzyme is illustrated in Figure 8b. Below a critical concentration, the enzyme loses all perceptible activity. This observation suggests that the enzyme may be inactivated by dissociation into constituent subunits. Our

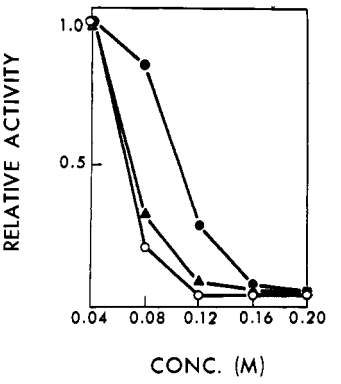

FIGURE 7: Effect of monovalent cations on DNA synthesis. Assays (0.04 ml) were carried out using the G-100 Sephadex fraction of purified polymerase at 0.2 unit/ml under standard conditions except for the addition of varying amounts of monovalent cation. NaCl, KCl, and NH_4Cl were tested independently, and gave essentially identical results. Templates were used at concentrations which give maximum activity (see Figure 8). Incubations were for 2 hr at 37°: (○) 70S RSV RNA template; (▲) rA·dT template; (●) calf thymus DNA template.

standard reactions have all been carried out with enzyme concentrations (0.1–0.2 U/ml) well in excess of the critical level.

Kinetics of DNA synthesis with viral RNA as template are illustrated in Figure 9a. The initial rate of the reaction is a function of precursor concentration (Figure 9b), but all reactions cease significant DNA synthesis after 1.5- to 4-hr incubation. Addition of either fresh enzyme or fresh template at this point has no effect, but DNA synthesis is renewed if *both* reagents are added. Thus, all components of the reaction mixture are stable except enzyme and template, but the nature of the events which cause enzyme inactivation is presently unknown. From data similar to that illustrated in Figure 9b, but plotted according to the method of Lineweaver and Burk, we have computed a K_m for TTP of 2×10^{-5} M for the initial

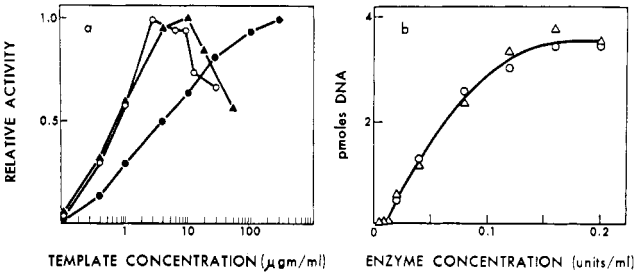

FIGURE 8: Template responses of purified polymerase. (a) Saturation of enzyme with template. Assays (0.1 ml) were carried out with the G-100 Sephadex fraction of purified polymerase (0.2 unit/ml; 2 μg of protein/ml) in the presence of varying concentrations of templates. [^{3}H]TTP was used at 2.3×10^{-6} mole/l. This is far below the saturating concentration of TTP (see Figure 9b), but represents the standard concentration used in characterizing the enzyme and its products. Preliminary data indicate that template saturation does not vary as a function of precursor concentration within the limits tested to date. (○) 70S RSV RNA template; (▲) rA·dT template; (●) calf thymus DNA template. Results have been normalized to maximum observed response during 1 hr at 37°. (b) Saturation of template with enzyme. Varying amounts of purified polymerase (G-100 Sephadex fraction) were assayed with 70S RNA (0.2 μg/ml) as template in a standard reaction mixture. Incubations were for 180 min at 37°. Results are expressed in terms of pmoles of DNA synthesized at different concentrations of enzyme.

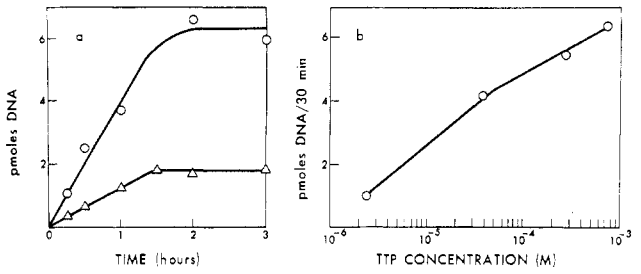

FIGURE 9: Kinetics of DNA synthesis and dependence on precursor concentration. All assays were carried out with the G-100 Sephadex fraction of purified polymerase (at 0.2 unit/ml). The template was 70S RSV RNA at 0.6 μg/ml. (a) Kinetics of DNA synthesis with viral RNA template and two concentrations of TTP. (△) [^{3}H]TTP at 2.4×10^{-6} mole/l.; (○) [^{3}H]TTP at 3.8×10^{-5} mole/l. Samples (50 μl) were taken at the indicated time points for acid precipitation. (b) Effect of precursor concentration on DNA synthesis with viral RNA template. The concentration of TTP was varied as indicated. Unlabeled precursors were always 2.2×10^{-3} mole/l. Results are expressed as pmoles of DNA synthesized per 30 min.

linear phase of DNA synthesis with viral RNA as template. This value is very similar to that obtained for the polymerase of Rauscher leukemia virus, using d(A,T) copolymer as template (Hurwitz and Leis, 1972).

Synthesis of DNA with rA·dT and DNA templates also ceases after approximately 4 hr (unpublished data). We have yet to determine the cause of this cessation.

The quantities of DNA synthesized in response to saturating amounts of the three classes of template and relatively high precursor concentration (see Figure 9b) are compared in Table IV. As reported previously (Kacian *et al.*, 1971; Spiegelman *et al.*, 1970c), rA·dT is the most efficient template on a weight basis. Extensive reactions (4 or more hr) synthesize quantities of DNA equivalent to 25–100% of the amount of poly(A) added as template. In the case of viral RNA, DNA synthesis has not exceeded 25% of the input template, either at or below saturating levels of template. Extent of DNA synthesis with DNA template has varied from one DNA preparation to another (maximum of 8%), but again, net synthesis has not been observed. These observations all conform to the view that the enzyme carries out only repair synthesis with all three classes of template (Leis and Hurwitz, 1972; Hurwitz and Leis, 1972). Net synthesis of DNA would require release of nascent DNA from template as DNA synthesis proceeds, something which has yet to be observed with the purified enzyme (Leis and Hurwitz, 1972) except under extreme circumstances (Taylor *et al.*, 1972).

TABLE IV: Maximum Synthesis of DNA with Various Templates.

	Template[a]		
	70S RNA	rA·dT	DNA
Yield of DNA	36	76	8.5
Proportion of input template (%)	21	45	5

[a] Assays (0.25 ml) were carried out using the G-100 Sephadex fraction of purified polymerase at 0.2 unit/ml and all templates at 0.6 μg/ml. [^{3}H]TTP diluted with unlabeled TTP was used at 3.6×10^{-3} mole/l., unlabeled triphosphates at 2.2×10^{-3} mole/l. Bovine serum albumin was present at 200 μg/ml. Results are expressed as pmoles of DNA synthesized per 15 hr at 37°.

TABLE V: Template Activities of Natural RNAs.[a]

Nucleic Acid	Rel Incorp (%)
RSV 70S RNA	100
RSV 70S RNA (pretreated with ribonuclease)	0.3
RSV 70S RNA (preheated 2 min at 80°)	1.3
AMV 70S RNA	138
Polio 35S RNA	6.2
R17 27S RNA	3.0
Total HeLa cell RNA (predominantly ribosomal)	5.8
HeLa tRNA	0.9
Calf thymus DNA	249
Poly(rA)·oligo(dT)	418
Poly (rA)·poly(rU)	3.5

[a] Standard assays (0.1 ml) were carried out with the G-100 Sephadex fraction of purified polymerase (at 0.2 unit/ml) and all templates at 2 μg/ml. Relative activities were computed from the amounts of DNA synthesized in 2 hr at 37°. RNase treatment (50 μg/ml, 60 min, 37°) was carried out prior to addition of polymerase. Controls were incubated in the same manner without RNase. Viral RNA was denatured by heating at 80° for 2 min in 0.01 M EDTA–0.02 M Tris-HCl (pH 7.4). This treatment dissociates the RNA into its constituent subunits (Duesberg, 1968; Erikson, 1969).

Comparative Template Response. Preparations of enzyme, standardized as illustrated in Figure 8, were used to compare the responses to a number of RNA templates (Table V). These determinations were performed at concentrations of RNA below saturation so that enzyme was in apparent excess. Under such conditions, 70S oncornavirus RNA is a more efficient template than any of the other natural RNAs tested. The response to RNA template can be eliminated by treatment of the reaction mixture with RNase. Treatment with DNase has no effect if the DNase is inactivated prior to initiating DNA synthesis (data not illustrated). The responses of the RSV enzyme to homologous (RSV) and heterologous (AMV) avian oncornavirus RNA are identical. Dissociation of 70S RNA into its constituent subunits (Duesberg, 1968; Erikson, 1969) by heat eradicates template activity. The reduction in activity obtained here is greater than that reported by Leis and Hurwitz (1972), but similar to that reported by Duesberg *et al.* (1971b).

Response to DNA Templates. Sonicated commercial preparations of calf thymus DNA are routinely effective as template without further activation. By contrast, the native DNAs of λ bacteriophage, HeLa cells, and avian embryos (all prepared in this laboratory) have little or no template activity (Table VI). Denaturation fails to activate these DNAs, but partial hydrolysis with DNase results in appreciable template activity in every instance (Table VI). More extensive hydrolysis abolishes template activity.

Absence of Other Enzyme Activities. Purified preparations of RSV DNA polymerase were tested for DNase activity with the following substrates: RFI of fd phage, and native and denatured DNA of λ phage. Tests were carried out at pH 8.1 and 5.0. After exposure to enzyme mixtures, the substrate DNAs were analyzed by centrifugation in neutral (RFI) or alkaline (native and denatured λ DNA) sucrose gradients as described previously (Quintrell *et al.*, 1971). None of these tests detected any DNase activity associated with the purified polymerase.

Tests for phosphatase activity (at pH 8.1) were also carried out, using two procedures: hydrolysis of *p*-nitrophenyl phosphate, and release of γ-^{32}P from [γ-^{32}P]ATP. Results were negative in both instances.

TABLE VI: Template Activities of Various DNAs.

Source of DNA	Nature of Template[a]				
	Native	Denatured	DNase (μg/ml) 0.1	1.0	10
Calf thymus	1.0		1.9	0.1	0.03
λ phage	0.12	0.12	2.9	0.18	0.09
HeLa cells	0.04	0.05	1.1	0.04	0.01
Chick embryos	0.11	0.15	1.1	0.02	0.01

[a] Standard reactions were carried out with the G-100 Sephadex fraction of purified polymerase for 2 hr. Templates were used at 10 μg/ml, *i.e.*, well below maximum levels (Figure 8). DNAs were denatured by boiling (10 min) at 100 μg/ml in 0.01 M Tris·HCl (pH 7.8). DNase treatment was carried out for 30 min at 37° with the DNA at 100 μg/ml in 0.01 M $MgCl_2$–0.02 M Tris·HCl (pH 7.4). The mixture was then heated at 60° for 30 min to inactivate DNase prior to carrying out the polymerase reaction. Results are expressed as amount of DNA synthesis relative to that obtained with native calf thymus DNA, the standard DNA template. All DNA preparations were sonicated as described in Materials and Methods. This procedure has no effect on the relative template activity of native or denatured materials.

Discussion

Molecular Locus of Template Responses. Previous reports have indicated that the multiple template responses characteristic of the DNA polymerase(s) associated with RNA tumor viruses reside on a single protein or protein complex (Kacian *et al.*, 1971; Hurwitz and Leis, 1972; Duesberg *et al.*, 1971a). Our data support this conclusion. Both single-step fractionation (rate-zonal centrifugation or gel filtration) and extensive purification yield one major population of enzyme activity, responsive to all the appropriate templates. Nevertheless, we cannot exclude the possibility that the presence of a separate, strictly RNA-dependent enzyme has been obscured by contamination with RNase. Our unpublished data indicate that the RNase associated with RSV virions is relatively small. It contaminates the upper third of the glycerol gradients illustrated above (Figure 3), and emerges from the G-100 Sephadex column subsequent to alkaline phosphatase.

Polymerase Forms A and B. Chromatography on phosphocellulose resolves two forms of DNA polymerase. These have different sedimentation coefficients and are differentially retarded in G-100 Sephadex (although the differences are small). Moreover, form A can be repeatedly generated from form B by rechromatographing the latter on phosphocellulose. The mechanism and significance of this transition are unknown, but the results of velocity sedimentation and gel filtration suggest that a change in either molecular weight or configuration takes place during the course of the chromatography. We have been unable to reproduce this phenomenon under any other circumstances (*e.g.*, centrifugation in low concentrations of electrolytes).

Our estimate of the molecular weight for RSV polymerase B (105,000, based on gel filtration) conforms to that computed for RSV polymerase from sedimentation data (Duesberg *et al.*, 1971a). To date, we have not obtained sufficient enzyme protein to permit conclusive electrophoretic analysis of polypeptide constitution. However, we have reached the provisional conclusion that our preparations contain a minimum of two polypeptides, with molecular weights of 65,000 and 105,000 measured by electrophoresis in gels of 10% polyacrylamide containing 0.1% sodium dodecyl sulfate. We have yet to determine the relationship between these polypeptides and enzymatic activity. The DNA polymerase of AMV has been reported to consist of two polypeptide subunits with molecular weights of 110,000 and 69,000 when analyzed in the presence of sodium dodecyl sulfate (Kacian *et al.*, 1971).

We have achieved a 500- to 1000-fold purification of active RSV polymerase with a molecular weight of approximately 105,000. Given a molecular weight of 2.7×10^8 for total virion protein (Vogt, 1965), we estimate that there are no more than 2.5–5 enzyme molecules/virus particle. Kacian *et al.* (1971) computed 5–17 molecules/virion of AMV.

Relative Efficiencies of RNA Templates. The 70S RNAs of oncornaviruses are more efficient templates for the RSV polymerase than any of the other natural RNAs tested. We cannot presently exclude the possibility that this is a trivial observation due to either some special feature of the reaction conditions or relative quality of the templates, but our results conform to those from other laboratories (Leis and Hurwitz, 1972; Kacian *et al.*, 1971; Duesberg *et al.*, 1971a).

The RNA-dependent RNA polymerase of Qβ bacteriophage displays a high order of template specificity (Haruna and Spiegelman, 1965) which apparently resides in the enzyme molecule. Template preference on the part of oncornavirus DNA polymerase could have a different locus. Transcription of DNA from viral RNA template initiates on the 3′ terminus of a polyribonucleotide (Verma *et al.*, 1971; Leis and Hurwitz, 1972), and the 70S RNA of oncornaviruses may have special structural features which facilitate initiation of this sort (Erikson and Erikson, 1971).

The extent of DNA synthesis with viral RNA template reported here (maximum of 25% of input template) conforms to or exceeds that observed in other laboratories (Leis and Hurwitz, 1972; Kacian *et al.*, 1971). There are two likely explanations for this failure to obtain more extensive DNA synthesis: (1) only a limited number of the input RNA molecules are competent to serve as templates, and (2) transcription by the enzyme is restricted to a limited portion of any given template molecule. For the present, it is impossible to distinguish between these two possibilities, although the extent of genome transcription with purified enzyme is quite similar to that with crude enzyme preparations when assessed in terms of the complexity of nucleotide sequences transcribed into double-stranded DNA (Taylor *et al.*, 1972; Varmus *et al.*, 1971).

Exogenous DNA as Template. It is now well established that

the initiation of DNA synthesis by RNA tumor virus polymerase requires a primer molecule with a 3′-hydroxyl terminus irrespective of whether the template is RNA or DNA (Verma *et al.*, 1971; Leis and Hurwitz, 1972; Hurwitz and Leis, 1972; Baltimore and Smoler, 1971; Smoler *et al.*, 1972). This fact explains the failure of carefully prepared native or denatured DNA to serve as template as well as the template activation of double-helical DNA by partial hydrolysis with DNase (Table VI). Commercially available DNA is apparently sufficiently degraded so as to require no further activation. In view of these conclusions the significance of previous reports that the principle product of synthesis on an exogenous DNA template is not covalently linked to the template (Spiegelman *et al.*, 1970b) and that denatured DNA is an efficient template for partially purified polymerase (Duesberg *et al.*, 1971a) is open to some question.

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