Deoxyribonucleic Acid Polymerase of Rous Sarcoma Virus: Reaction Conditions and Analysis of the Reaction Product Nucleic Acids

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Reaction conditions for Rous sarcoma virus ribonucleic acid (RNA)-instructed deoxyribonucleic acid (DNA) polymerase activity are described whereby the viral RNA is relatively protected from endogenous or added nuclease activity. Three analyses of reaction product nucleic acids (^3H -RNA, ^{32}P -DNA) were compared, namely, gel electrophoresis, Cs_2SO_4 gradient centrifugation, and hydroxyapatite column chromatography. It was found that hydroxyapatite analysis could be misleading unless the state of the template RNA was monitored concomitantly with the DNA analysis. Gel electrophoresis and Cs_2SO_4 gradient centrifugation gave comparable results. It was concluded that analyses of the product of reverse transcriptase reactions should not only refer to the template RNA and product DNA species, but also be performed with virus or viral RNA which do not have or obtain nicks in the 60S RNA. Otherwise, interpretation of the results would have the ambiguity of potential artifacts caused by those degraded RNA molecules.

Rous sarcoma virus (RSV) and other oncogenic viruses have been shown to possess a ribonucleic acid (RNA)-instructed deoxyribonucleic acid (DNA) polymerase in the virion particles (3, 37). It has been demonstrated by velocity gradient or Cs_2SO_4 equilibrium gradient centrifugation and hydroxyapatite analyses that reaction product DNA is initially associated with the template RNA but subsequently is recovered free (22-26, 29, 30, 32-35).

Our ultimate research objective with RSV is to determine whether the DNA transcribed from the viral RNA is eventually integrated into the host genome. One approach to this problem would be to pulse-label the product DNA in vitro and attempt to introduce the subviral particles (containing labeled DNA product) into susceptible cells where its subsequent fate could be monitored by following the labeled DNA component. Evidently it is critical in this type of investigation that the viral RNA retain its genome competence during the in vitro synthesis. If contaminating endonuclease activity resulted in degradation of the viral RNA before or during the pulse synthesis of DNA, then one could not meaningfully interpret the results obtained.

The purpose of the present study was to investigate concurrently the state and fate of the

viral RNA, together with the kinetics and properties of product DNA synthesis during the time-course of an in vitro reaction, in order to delineate reaction conditions in which the viral RNA remains undegraded. To do this, we employed ^3H -uridine-labeled RSV and ^{32}P - α -thymidine triphosphate (TTP) to label product DNA. Gel electrophoresis, hydroxyapatite analyses, and Cs_2SO_4 equilibrium gradient centrifugation were used to resolve reaction product nucleic acids. We were able to demonstrate that detergent concentration is a critical determinant of the fate of the viral RNA.

MATERIALS AND METHODS

Purification of virus. The Schmidt-Ruppin strain of RSV (RAV-free) was grown by using a modification of the procedure described by Hanafusa (27) and by employing secondary or tertiary chick embryo fibroblast (CEF) monolayer cultures derived from 12-day-old chick embryos (COFAL-negative, specific pathogen free eggs, SPAFAS, Norwich, Conn.). The CEF cultures were grown to confluency in 90-mm petri plates under 15 ml of growth medium containing Eagle's minimal essential medium (Auto-POW MEM, Flow Laboratories) supplemented with 10% Tryptose phosphate broth (Difco), and 5% calf serum. The cells were removed with 0.025% trypsin and 0.02% ethylenediaminetetraacetate (EDTA; pH 7; 3 ml/

plate) and concentrated by centrifugation. The cell pellet was suspended in a stock preparation of RSV to give a cell concentration of 6×10^6 cells per ml and a multiplicity of infection of about 10 focus-forming units per cell. The suspension also contained diethylaminoethyl-Dextran (56 $\mu\text{g}/\text{ml}$), and this mixture was incubated with occasional shaking at 37 C for 60 min. The cells were recovered by centrifugation and resuspended in fresh growth medium, and 10-ml volumes were dispensed into 90-mm petri plates (6×10^6 cells per plate). The plates were incubated at 39 C in a humidified atmosphere of 8 to 10% CO_2 in air until at least 90% of the cells had become transformed. The medium was then discarded and replaced with 10 ml of fresh medium containing 10 μCi of ^3H -uridine per ml. At 12-hr intervals, the medium was replaced with fresh growth medium containing ^3H -uridine, and harvested supernatant fluid was stored at 4 C. Four or six successive 12-hr harvests were pooled and stored at 4 C. In some experiments, unlabeled virus was prepared by omitting ^3H -uridine from the media.

Crude virus was initially clarified by centrifugation at 4 C and $10,000 \times g$ for 10 min. Virus was recovered from each liter of supernatant fluid by slow addition, with stirring, of 23 g of NaCl followed by 70 g of polyethylene glycol (Carbowax 6000; Union Carbide). After storage at 4 C for 2 hr (or more), the precipitated proteins and virus were recovered by centrifugation at $4000 \times g$ for 30 min at 4 C. The pellet was suspended in MEM (25 ml) and layered over a double cushion of sucrose in an SW27 Spinco centrifuge tube. The double cushion consisted of 3 ml of 80% (w/v) sucrose and 8 ml of 25% (w/v) sucrose, both in 0.15 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.5). The tube was centrifuged at 25,000 rev/min and 4 C for 180 min, and the band of material between the 25 and 80% sucrose layers was collected by pipette after removing and discarding the supernatant fluids. The band contained most of the virus and was diluted with 1 ml of 0.15 M NaCl, 0.01 M Tris-hydrochloride buffer (pH 7.5), immediately layered over a 60 to 30% (w/v) linear gradient of sucrose dissolved in 0.15 M NaCl, 0.01 M Tris-hydrochloride buffer (pH 7.5), and centrifuged to equilibrium at 25,000 rev/min in the Spinco SW27 rotor for 18 hr at 4 C. The visible virus band was collected by pipette, diluted threefold with 0.15 M NaCl, 0.01 M Tris-hydrochloride buffer (pH 7.5), and concentrated by centrifugation at 25,000 rev/min and 4 C in the SW27 rotor by using a 1-ml cushion of 80% (w/v) sucrose to collect the virus. After collection with a pipette, the virus was diluted with an equal volume of 100% glycerol and stored at 4 C or -20 C without loss of enzyme activity.

Preparation of ^{32}P - α -TTP. We recently described a procedure (6) based on methods described by Symons (36) for the large-scale preparation of ^{32}P - α -ribonucleoside triphosphates of high specific activity. The method involves the condensation of ^{32}P -phosphoric acid onto the free 5' hydroxyl of a protected precursor nucleoside, removal of the protecting group, and the enzymatic conversion of the 5' nucleotide monophosphate to its corresponding triphosphate. The procedure is also applicable to the preparation of deoxy-

ribonucleoside triphosphates (36). However we have found that when using the 3'-O-acetyl protected deoxyribonucleoside, the removal of the protecting group (36) results in some concomitant loss of the nucleoside, with a subsequent lower yield. Consequently we have used unprotected thymidine as the precursor for the initial condensation with ^{32}P -phosphoric acid. This resulted, as judged by paper electrophoresis, in the conversion of about 50% of the phosphoric acid to thymidine-5'-monophosphate, 20% to thymidine-3'-monophosphate and 10% to thymidine-3',5'-diphosphate. Uridine-5'-monophosphate kinase (6) was used to convert selectively the thymidine-5'-monophosphate to corresponding TTP and leave the thymidine-3'-monophosphate and thymidine-3',5'-diphosphate untouched. The triphosphate was recovered by Dowex-1- Cl^- chromatography as described for the preparation of ^{32}P - α -uridine triphosphate (6) and thereby freed from contaminating ^{32}P -thymidine-3'-monophosphate as well as free ^{32}P -phosphate and unconverted ^{32}P -thymidine-5'-monophosphate. The thymidine-3',5'-diphosphate, having the same charge as TTP, was eluted with the triphosphate from the Dowex-1- Cl^- column.

The procedure for preparing ^{32}P - α -TTP was therefore identical to the method we described before (6), except that thymidine was used as a precursor, and the procedures involved in removing the protecting group were omitted. The specific activity of the ^{32}P - α -TTP obtained was 5 mCi/ μmole , and the triphosphate preparation contained about 20% of contaminating thymidine-3',5'-diphosphate as judged by paper electrophoresis. Because the diphosphate did not interfere with DNA synthesis and its 3'-phosphate was not incorporated into product DNA (as judged by the fact that digestion of product with venom phosphodiesterase resulted in the recovery of only thymidine-5'-monophosphate), it was not removed.

Reaction conditions. The reaction mixture contained, per 125 μliters : 0.1 μmole each of deoxyadenosine triphosphate, deoxycytidine triphosphate, and deoxyguanosine triphosphate; 0.01 μmole of ^{32}P - α -TTP; 1 μmole of MgCl_2 ; 0.1 μmole of MnCl_2 ; 10 μmoles of NaCl; 0.2 μmole of dithiothreitol; 8 μmoles of Tris-hydrochloride buffer (pH 8.1); 10 μg of Triton N101; and 10 or 20 μg of RSV viral protein. These conditions were chosen for both optimal rate and overall sustained DNA synthesis. The optimal temperature of incubation was found to be 37 C, not 31 C as found for vesicular stomatitis, Kern Canyon, or influenza virion RNA transcriptases (1, 4, 6). The synthesis of DNA was linear for 8 hr under the conditions described here. The optimal pH of the reaction was 8.1, similar to the results obtained with three other virion transcriptases (1, 6). As with influenza transcriptase, there was a stimulatory effect on the enzyme rate by the inclusion of both manganese and magnesium ions (6).

Purification of reaction products for analysis by gel electrophoresis. Reaction products were purified by phenol-sodium dodecyl sulfate (SDS) extraction and Sephadex chromatography as described for the reaction products of vesicular stomatitis transcriptase products (8). Nucleic acid samples were subjected to polyacrylamide gel electrophoresis for 2 hr at 50 v and

10 ma/gel using unswollen bisacrylamide cross-linked, 1.8% polyacrylamide gels prepared as described previously (5). Because of the low concentration of acrylamide, the gels were difficult to handle before freezing. It was found that after extrusion from the Plexiglas electrophoresis tube, the gels had to be floated in water to regain their natural length before being carefully frozen over dry ice in a 1 by 1 by 15 cm trough. After freezing, the gels were sliced into 1-mm sections with a CO₂-freezing microtome as described previously (8), dissolved in 0.5 ml of 30% hydrogen peroxide at 50 C for 4 hr, and counted in a liquid scintillation counter by using Aquasol (New England Nuclear Corp., Boston, Mass.).

Melting of the reaction product nucleic acid. Nucleic acid samples (50 μ liters), suspended in 0.01 M sodium phosphate buffer, 0.005 M EDTA (pH 7), were heated at 100 C for 20 sec and fast-cooled in a dry ice-ethanol bath before electrophoresis.

Equilibrium Cs₂SO₄ gradient centrifugation. Nucleic acid samples containing 100 μ g of *Escherichia coli* DNA and 100 μ g of *E. coli* RNA were mixed with 1.8 ml of saturated Cs₂SO₄ and adjusted with 0.01 M NaCl, 0.01 M Tris-hydrochloride buffer (pH 7) to give a final volume of 3.6 ml and a refractive index of 1.360. The mixtures were centrifuged for 60 hr at 15 C and 31,000 rev/min in a Spinco SW56 rotor. By using an 18-gauge needle, 10-drop fractions were collected, and the optical density at 260 nm (OD₂₆₀) and refractive index of each sample were determined, as well as the distribution of acid-insoluble radioactivity.

Hydroxyapatite column chromatography (29). A 1.5-ml column of hydroxyapatite (Biogel HTP, Bio-Rad, Richmond, Calif.) was washed with five 1.5-ml volumes of 0.01 M phosphate buffer (pH 6.8) and 0.20 M NaCl at 60 C by using a water-jacketed column and preheated solutions. After the sample was loaded in low-phosphate buffer, the column was washed five times at 60 C with 1.5-ml volumes of 0.01 M phosphate buffer and 0.20 M NaCl. The column was then successively eluted at 60 C with 0.14, 0.16, 0.18, 0.20, 0.22, 0.24, 0.40, and 0.50 M phosphate buffer (pH 6.8) in 0.20 M NaCl. Eluants were monitored for content of acid-insoluble radioactivity or OD₂₆₀. The total content of label or OD₂₆₀ in the five washes is given. Usually all the material that would elute did so in the first three washes. Between 90 and 100% of the applied nucleic acids (RNA and DNA) was recovered.

RESULTS

Effect of Triton on RSV reaction conditions. In determining the optimal detergent concentration for the RNA-instructed DNA polymerase reaction (Table 1), we found that at supraoptimal detergent concentrations the reaction rate became progressively depressed. To investigate this further, the effect of Triton treatment on ³H-uridine-labeled virus was investigated by velocity centrifugation (Fig. 1). It was found that, at the lower Triton concentrations, viral RNA was associated with structures which sedimented only slightly more slowly than untreated virions. Virus treated with higher Triton concentrations were

TABLE 1. Effect of Triton N101 concentration on RSV DNA polymerase^a

Triton concn ^b	Product DNA TMP incorporated ^c
None	18
1	19
5	230
10	245
50	102
100	66
400	65
1,000	48

^a Reaction mixtures containing 10 μ g of RSV protein per 125 μ liters, and other constituents as indicated in Materials and Methods, were incubated at 37 C through 2 hr with various concentrations of Triton N101. Samples were removed at intervals to determine the amount of acid-insoluble ³²P-thymidine monophosphate (TMP) incorporated and the rate of incorporation.

^b Expressed as micrograms per 125 μ liters.

^c Expressed as picomoles of TMP incorporated per hour per milligram of protein.

FIG. 1. Sucrose gradient velocity centrifugation of ³H-RSV treated with low or high Triton N101 concentrations. Samples of ³H-uridine-labeled RSV in 0.10 M NaCl, 0.01 M Tris-hydrochloride buffer (pH 7.4) were incubated at 37 C for 3 min with Triton N101 at a concentration of 10 μ g (low Triton) or 400 μ g (high Triton) per 125 μ liters and centrifuged in 10 to 30% gradients of sucrose containing 0.15 M NaCl, 0.01 M Tris-hydrochloride buffer (pH 7.4), 0.0002 M dithiothreitol for 60 min at 3 C and 25,000 rev/min in a Spinco SW41 rotor. The distribution of acid-insoluble ³H radioactivity was determined and compared to an untreated control.

FIG. 2. Effect of ribonuclease on RSV DNA polymerase activity at two different reaction concentrations of Triton N101. Reaction mixtures containing 10 μ g of RSV protein and either 10 μ g of Triton N101 (A) or 400 μ g Triton N101 (B) per 125- μ liter reaction volume were incubated at 37 C. At indicated intervals (solid arrows), samples were withdrawn, and 5 μ g of ribonuclease A was added and the incubations were continued. The acid-insoluble radioactivity was determined on 50- μ liter volumes as shown. Pretreatment of 10 μ g of virus in 10- μ liter volume with 5 μ g of ribonuclease A for 10 min at 4 C (open arrows) before addition to the reaction mixture is also shown (–10 min, open arrow).

dissociated into smaller components (about 100S). When the integrity of the RNA was examined by gel electrophoresis immediately after treatment with high or low Triton, no difference in the resulting patterns of RNA was observed in comparison to untreated virus (see Fig. 3). However, when reaction mixtures were incubated at these Triton concentrations, it was discovered that in low Triton there was little degradation of the viral RNA (see Fig. 4), but, in the presence of high Triton, 70% of the 60S viral RNA was degraded by 1 hr (*data not shown*; see reference 32).

To test whether high Triton concentrations render the RNA-instructed DNA polymerase sensitive to exogenous ribonuclease, the effect of added nuclease was examined by using reaction mixtures containing either high or low Triton concentrations. The results (Fig. 2) confirm the suggestion that the enzyme reaction was less susceptible to inhibition by ribonuclease at low Triton concentrations.

Similar conclusions on the susceptibility of the RSV RNA-instructed DNA polymerase activity to endogenous ribonuclease at high detergent concentrations were recently obtained by Quintrell et al. (32).

Gel electrophoresis of the RNA species of RSV. A preparation of 3 H-uridine-labeled RSV was mixed with 32 P ribosomal RNA (28S and 18S species), treated with SDS to dissociate the virion RNA from other viral components (6), and then subjected to electrophoresis in a 1.8% polyacrylamide gel (Fig. 3). The profile of RNA species obtained was identical to that obtained by phenol extraction of 3 H-uridine-labeled virion particles (*see below*). By reference to the 32 P-

labeled ribosomal species, it was calculated that the apparent molecular weight of the principal viral RNA species was 8.5×10^6 daltons (60S). Five classes of RNA were obtained from the virion particles: 60S RNA, 28S RNA, 18S RNA, 7S RNA, and 4S RNA species. By dividing the radioactivity present in each peak by its apparent molecular weight (calculated by reference to the 32 P ribosomal markers), a molar ratio of 1:0.4:1:5:200 was obtained for the five RNA species. Although such an estimation is limited by the effect of base ratio and content of uridine, it shows that many different RNA molecules are associated with the virus preparation. We have not been successful in endeavors to dissociate the smaller RNA species from the virus preparation. In attempts to remove material which may be chelated or hydrogen-bonded to virions, we have tried preincubating virus with 0.025 M EDTA or 1 M NaCl followed by sucrose gradient equilibrium centrifugation (containing 0.025 M EDTA or 1 M NaCl) as well as subsequent velocity gradient centrifugation. After all of these treatments, the virus preparation still contained all five RNA species.

Kinetic analysis of the product synthesized by RSV. A kinetic analysis was undertaken by using 3 H-uridine-labeled RSV and 32 P- α -TTP to label the product species. The reaction products were extracted with phenol-cresol, purified from triphosphates by Sephadex chromatography (8), and resolved by gel electrophoresis (5). The results are shown in Fig. 4. The zero time sample (not shown) was similar to the 3 H profile shown in Fig. 3. The linear synthesis of product DNA, overall conservation of acid-insoluble 3 H label throughout the incubation, per cent distribution

FIG. 3. Polyacrylamide gel electrophoresis of ^3H -labeled RSV. A sample of ^3H -labeled RSV was mixed with sodium dodecyl sulfate (1% final concentration), glycerol (30% final concentration), and 10 μl of ^{32}P -labeled baby hamster kidney (BHK) single-stranded cellular RNA (containing mostly 28 and 18S RNA). The mixture was loaded directly on a bisacrylamide cross-linked, 1.8% polyacrylamide gel and subjected to electrophoresis for 120 min at 10 ma and 50 v. After electrophoresis the gel was frozen, sliced, and dissolved, and the radioactivity was determined with a liquid scintillation counter. The molecular weights of the ^{32}P -BHK 28S and 18S ribosomal RNA (7, 8) were used to calibrate the gel (upper scale) and to estimate the apparent molecular weights of the viral species (see text).

of ^3H between the five classes of virion RNA, and the per cent ^{32}P associated with viral 60S RNA species are all shown in Table 2.

The viral RNA was not substantially degraded during the first hour of reaction (Table 2). At early times at least, there was no evidence for product associated with any of the RNA species other than the high molecular weight 60S viral RNA. The amount of DNA associated with the 60S RNA increased throughout the 3 hr of incubation, it did not plateau, and its rate of increase was about half that of the rest of the DNA ("free" DNA; see below). The 2- and 3-hr samples contained RNA-DNA complexes which moved slightly more slowly on electrophoresis than the 60S viral RNA. Unfortunately, the difference in mobility was not sufficient to determine the

number of viral RNA species participating in DNA synthesis. Moreover, without a greater difference in mobility, the extent of transcription could not be determined from the relative specific activities of the viral RNA and product DNA as was determined with the RNA complexes formed by vesicular stomatitis virus transcriptase (4).

It was apparent that, between 60 and 180 min of incubation, a considerable amount of product DNA was synthesized having an electrophoretic mobility greater than the 60S viral RNA, but equal to or slower than the 4S RNA species. It was not possible to determine from the gel profiles whether these DNA species were free single- or double-stranded DNA or RNA-DNA hybrids. Samples of the reaction products were therefore subjected to Cs_2SO_4 centrifugation to separate the nucleic acids according to density (29, 34). This method permits differentiation of RNA from "free" DNA and from RNA-DNA hybrids (where the proportion of both is a substantial amount of the total hybrid). After centrifugation, it was found that the proportion of ^{32}P product DNA present in the RNA region of the Cs_2SO_4 gradient (Fig. 5) agrees well with the proportion of product DNA associated with the 60S viral RNA identified by gel electrophoresis (Table 2). The fact that ^{32}P product DNA was present in the RNA region indicates that its mass is very small relative to its associated RNA. Secondly, the fact that the majority of the residual product DNA was coincident with the *E. coli* DNA marker suggests, as pointed out by Spiegelman et al. and Manly et al. (29, 34), that it consists of free single- or double-stranded DNA, or both, or has very little associated RNA. The relative ^{32}P -to- ^3H radioactivity in the DNA region was very much higher than that in the RNA region of the Cs_2SO_4 gradients.

It is reasonable to suggest from these two different analyses of the same reaction product nucleic acids that product DNA both is associated with the 60S viral RNA and occurs free as single- or double-stranded DNA (or both) species having a wide assortment of electrophoretic mobilities in polyacrylamide gels. Similar conclusions have been drawn from analyses of the in vitro reaction product performed by velocity centrifugation, equilibrium Cs_2SO_4 centrifugation, specific enzymatic digestions, and hydroxyapatite analyses (22-26, 29, 30, 32-35).

Hydroxyapatite column chromatography of RSV reaction product nucleic acids. Two procedures have been used to determine the relative content of single- and double-stranded DNA among reaction product nucleic acids. Manly et al. (29), by using specific nuclease digestions

FIG. 4. Kinetic analysis of RSV reaction products by gel electrophoresis. A 30-fold reaction containing 20 μg of ^3H -uridine-labeled RSV protein per reaction mixture and ^{32}P - α -TTP to label the DNA product species was incubated at 37 C. At intervals, samples (0.7 ml) were removed, mixed with sodium dodecyl sulfate (SDS) (1% final concentration), 100 μg of *Escherichia coli* RNA, and extracted with an equal volume of phenol-cresol as described previously (8). After separation from triphosphates by Sephadex column chromatography (8), the acid-insoluble radioactivity was determined (Table 2), and the nucleic acids were recovered by alcohol precipitation from 0.4 M LiCl. The nucleic acids were freed from traces of SDS by a second 70% alcohol precipitation from 0.4 M LiCl, dried in vacuo, and dissolved in 200 μl of 0.01 M phosphate buffer, 0.005 M EDTA, pH 7. A portion from each sample was mixed with SDS (1% final concentration), glycerol (30% final concentration), and resolved by polyacrylamide gel electrophoresis as in Fig. 3.

TABLE 2. Recovery and distribution of ^3H -viral RNA and ^{32}P -product DNA from a kinetic analysis of an RSV DNA polymerase reaction^a

Time (min)	Total ^3H (counts/min)	Total ^{32}P (counts/min)	Viral RNA (%)					Product DNA (%)	
			60S	28S	18S	7S	4S	60S	Other
0	217,400	800	57	3	4	1	20	—	—
15	229,400	57,200	58	4	4	1	21	92	8
30	183,000	98,000	57	3	3	1	22	73	27
60	198,400	249,200	54	3	3	2	19	59	41
120	213,600	594,000	44	3	3	3	27	41	59
180	180,000	862,200	45	3	3	3	29	34	66

^a Recovery of ^3H -viral RNA and ^{32}P -product DNA in the kinetic analysis described in Fig. 4 are given. The per cent of the total ^3H label recovered after electrophoresis in five major fractions—60S, 28S, 18S, 7S, and 4S species—is shown. The unaccounted per cent of the ^3H label showed no particular distribution (see Fig. 3 and 4) and probably represented degradation species of RNA. Degradation of the viral RNA (e.g., 12% of the 60S species in 3 hr of incubation) presumably accounted for the increase in the 7S and 4S viral RNA through the kinetic analysis. The per cent recovery of ^{32}P product in association with the 60S RNA is given. It is noteworthy that the amount of 28S or 18S RNA through the reaction did not change substantially (see Fig. 4). These results suggest that the 28S and 18S RNA species, like the 60S RNA, are sequestered from endonuclease activity.

FIG. 5. Cs_2SO_4 equilibrium gradient centrifugation of the 60-min and 180-min RSV reaction product nucleic acids. Samples of the 60-min (A) and 180-min (B) RSV reaction product nucleic acids were mixed with *Escherichia coli* RNA and DNA and Cs_2SO_4 centrifuged to equilibrium, and fractionated as described in text. The refractive index (η), optical density (OD_{260}), and distribution of acid-insoluble radioactivity were used to determine the positions of carrier RNA and DNA and reaction product nucleic acids. The per cent of the total ^{32}P present in the "DNA" region of the two gradients (fractions 18 to top) were 46% and 71%, respectively (compare Table 2).

on reaction products resolved by Cs_2SO_4 gradient centrifugation, demonstrated that at early reaction times there were equal amounts of RNA-DNA hybrids and free single-stranded DNA, but little double-stranded DNA. At later reaction times, they obtained equal amounts of free single- or double-stranded DNA but little RNA-DNA hybrid. In contrast to these results, by using hydroxyapatite column chromatography, Fanshler et al. (22) were unable to detect free single-

stranded DNA at any stage of their reactions. In an attempt to reconcile these conflicting reports, we used hydroxyapatite to resolve the double-labeled reaction product nucleic acids obtained in the kinetic analysis described above (Fig. 4, Table 2). The procedure employed involved stepwise elution of hydroxyapatite columns with solutions of sodium phosphate buffer in 0.2 M NaCl at 60 C. The total reaction product nucleic acids were either resolved directly or pretreated

with ribonuclease (A and T_1) in 0.4 M NaCl before chromatography (Table 3).

The results obtained from hydroxyapatite analysis of RSV reaction nucleic acids (Table 3) seriously bring into question the validity of using this method of analysis on unresolved reaction products. The reaction products contained five RNA species (Fig. 3); therefore, the distribution of 3H label was difficult to interpret. The recovery

of ^{32}P -DNA could not be easily correlated to the analyses described in Fig. 4 and 5 and Table 2.

RSV RNA (0-min sample) eluted (56%) in the 0.18 to 0.24 M phosphate eluants but was also recovered in the 0.40 to 0.50 M eluants where free double-stranded DNA can be eluted. Consequently, it was not too surprising that we obtained DNA in all the higher phosphate eluants. The 15-min product had 92% of its DNA associated

TABLE 3. Fractionation of RSV reaction product nucleic acids by hydroxyapatite column chromatography^a

Eluant	0 min		15 min				30 min			
	Native		Native		Ribonuclease		Native		Ribonuclease	
	3H	^{32}P	3H	^{32}P	3H	^{32}P	3H	^{32}P	3H	^{32}P
0.01	0	0.3	0	0	0.3	0	0	0	0.2	0.2
0.14	110	0.8	138	0.8	1.7	4.7	98	1.0	2.5	6.4
0.16	29	0.6	46	0.3	0.4	0.6	61	1.9	0.3	0.6
0.18	62	0.2	92	1.0	0.3	0.6	79	1.7	0.5	1.2
0.20	135	0.2	138	0.9	0.4	0.2	116	2.2	0.8	1.1
0.22	94	0.6	61	0.6	0.4	0.2	37	1.5	0.8	0.5
0.24	101	1.2	77	1.0	1.0	0.4	30	0.9	1.0	0.9
0.40	150	7.8	161	2.3	5.2	0.8	92	3.6	3.2	1.8
0.50	22	0.2	54	0.5	0.2	0	98	1.1	0.2	0

Eluant	60 min				120 min				180 min			
	Native		Ribonuclease		Native		Ribonuclease		Native		Ribonuclease	
	3H	^{32}P	3H	^{32}P	3H	^{32}P	3H	^{32}P	3H	^{32}P	3H	^{32}P
0.01	0	0	0.3	0	0	0	0.4	0	0	0	0.6	0
0.14	109	6.3	1.6	11.7	118	22.4	2.2	29.3	151	26.3	2.2	27.3
0.16	91	1.3	0.3	2.6	111	4.8	0.4	4.6	52	6.3	0.5	9.0
0.18	73	3.0	0.4	3.6	104	8.0	0.5	6.9	58	8.8	0.5	10.4
0.20	91	3.3	0.5	2.9	156	8.8	0.6	7.7	70	12.5	0.8	13.4
0.22	36	3.0	0.7	2.9	325	8.0	1.0	8.5	41	12.5	0.7	16.4
0.24	30	5.0	1.2	4.5	59	13.6	2.0	13.9	35	23.8	1.7	32.8
0.40	48	8.9	4.4	4.5	52	15.2	4.5	12.4	99	32.5	4.5	21.3
0.50	121	2.3	0.4	0	33	0	0.2	0	64	2.5	0.3	0

^a Analyses of samples of the native products or products after ribonuclease digestion are shown (10 μ g of ribonuclease A per ml; 10 μ g of ribonuclease T_1 per ml in 0.4 M NaCl, 0.01 M Tris-hydrochloride buffer, pH 7.5; incubation at 37 C for 30 min). Nanogram recoveries of RNA and DNA were calculated for the total sample. Nanogram amounts were calculated from the specific activity of the 3H -viral RNA (3×10^5 counts per min per μ g) and the ^{32}P - α -thymidine triphosphate (DNA = 7.5×10^6 counts per min per μ g, calculated by assuming a 25 mole per cent of thymidine monophosphate in the product DNA). By using known nucleic acids and identical chromatography procedures, we determined that transfer RNA species elute (94%) between 0.14 M and 0.16 M phosphate buffer; 28S and 18S ribosomal RNA elute (88%) between 0.18 M and 0.24 M phosphate buffer; *Escherichia coli* native double-stranded DNA elutes (93%) at 0.40 M phosphate buffer; *E. coli* denatured single-stranded DNA elutes (88%) between 0.14 M and 0.16 M phosphate buffer; vesicular stomatitis virus single-stranded RNA elutes (79%) between 0.18 M and 0.24 M phosphate buffer; and deoxythymidine:riboadenosine (equimolar polymer) elutes (92%) between 0.22 M and 0.24 M phosphate buffer. Alkali digestion of the reaction product nucleic acids solubilized 99.99% of the 3H label, indicating that that much of the 3H label was present as RNA.

with 60S viral RNA (Fig. 4, Table 2). After hydroxyapatite chromatography, ^{32}P was recovered throughout the chromatogram. A similar wide distribution of reaction product DNA was obtained by Fanshier et al. (22). In contrast to their results, though, the amount and per cent of ^{32}P which we recovered in the low-salt eluants increased throughout the kinetic analysis. Because at any of the intervals examined there was much more RNA than DNA present in these low-salt eluants, we cannot prove that the ^{32}P is equivalent to free single-stranded DNA. A similar reason prohibits deduction that the observed increase in per cent and amount of product through the kinetic analysis in the 0.40 and 0.50 M eluants represents free double-stranded DNA. Evidently, hydroxyapatite analysis of reaction product nucleic acids previously resolved by equilibrium or velocity centrifugation or gel electrophoresis would be more useful in determining the presence of free single- or double-stranded DNA (29).

The ribonuclease-treated samples were more informative. Ribonuclease digestion was performed in 0.4 M NaCl to conserve RNA-DNA hybrids and resulted in 96% or greater digestion of the viral RNA (Table 3). After digestion of the 15-min sample, 70% of the product DNA was recovered in the 0.14 and 0.16 M phosphate eluants. The fact that there was less RNA than DNA in these fractions indicates that the DNA was *principally* single-stranded.

The per cent and amount of DNA recovered in the double-stranded DNA eluants (0.40 and 0.50 M phosphate) after ribonuclease digestion increased throughout the time-course. At later time points there was more DNA than RNA in these high-salt eluants; therefore, this DNA was principally in the form of double- or multi-stranded DNA. At the earlier time points, there was more RNA than DNA in these fractions, so we cannot definitively conclude that this ^{32}P product was in the form of double-stranded DNA.

DNA was also recovered post-ribonuclease in the fractions where we would expect to recover RNA-DNA hybrids (as defined by the elution pattern of deoxythymidine:riboadenosine). However, at the later time points these fractions contained substantially more DNA than RNA, which indicates that this DNA was not in equimolar RNA-DNA hybrids.

In conclusion, though hydroxyapatite is a valuable tool for DNA analyses, when RNA is present we would caution its use unless the state, amount, and relationship of the RNA is concomitantly monitored.

Melting the reaction product nucleic acids. To determine whether the product DNA is hydrogen-bonded or covalently linked to the template virion RNA, a melting experiment was performed. In contrast to the virion RNA of Newcastle disease virus (16) or vesicular stomatitis virus (9), the 60S RSV viral RNA can be dissociated by mild heat or dimethyl sulfoxide treatment and thereby shown to be composed of subunits (2, 12, 16, 17, 19–21, 31). Consequently, from the melting experiment, we expected to obtain the viral RNA in its dissociated subunit state, after resolution by gel electrophoresis. The conditions employed were identical to those we have used for similar analyses of influenza (7) and vesicular stomatitis transcription products (9). We would not expect from our previous experience to get breakage of covalently joined nucleic acid using this procedure.

The results of melting the 60-min reaction products (Fig. 6) indicated that the viral RNA was recovered in the heterogeneous mixture of sizes having molecular weights between 3×10^6 and 0.8×10^6 daltons. The ^3H profiles obtained from the other samples, including the zero time sample, were essentially identical, except that the 2-hr and 3-hr samples had more faster-moving species, presumably representing degradation products of the 60S viral RNA. It is noteworthy that there was little recovery of the ^3H label in the 8×10^5 to 5×10^4 dalton molecular weight region of the melted 60-min sample.

These results suggest that the 60S RNA, even though it appeared reasonably homogeneous before melting (Fig. 4), in fact consisted of many

FIG. 6. Melting the reaction product nucleic acids. Reaction product nucleic acids were melted by heat treatment and resolved by gel electrophoresis as described in Text. The 60-min sample is shown.

distinct RNA molecules. Because other results (2, 21) have suggested that the 60S RNA of RSV and other viruses is composed principally of several 35S subunits, we can only conclude that our preparation of viral RNA contained breaks in the RNA phosphodiester backbone ("nicks")—unexpressed in the 60S species because of hydrogen bonding. From the relative distribution of the ^3H label (assuming that the 12% of the total ^3H label present in the 35S species represents unnicked RNA subunits and that they are segregated together in the 60S species) we can calculate that the maximum percentage of original 60S species containing unnicked subunits is about 20% of the total 60S molecules (viz. 12/57, see Table 2). As this presumption that the unnicked subunits are segregated together in the same 60S species has no basis, this figure is an upper limit, and the true number of 60S species with unnicked subunits is probably very low.

We have also used 90% dimethyl sulfoxide treatment and heating at 60 C in 67% formamide to dissociate the viral RNA from product DNA and obtained identical results to those described above.

It can be seen from the results presented in Fig. 6 that the ^{32}P -DNA species did not show the same electrophoretic distribution as the principal viral RNA species, suggesting that they were not covalently joined to the larger template molecules.

DISCUSSION

RNA content of RSV. Many analyses of the RNA content of RSV and related viruses have shown that oncogenic virus preparations contain a 60S high-molecular-weight RNA and frequently also have 28S, 18S, 7S, and 4S RNA species (10–17, 19–21, 38–40).

Our estimate of the molecular weight for the RSV genome of 8.5×10^6 daltons is significantly lower than that of others (14–16), who have derived values of between 9.5×10^6 and 12×10^6 daltons based on sedimentation of the viral RNA or gross chemical analyses of the virion particles. Clearly, where an RNA exists as a conglomerate of several molecules, the derivation of molecular weight from sedimentation or electrophoretic criteria requires supporting chemical or physical evidence.

Our attempts to dissociate the other RNA species from the viral particles were negative. Although we cannot exclude the possibility that all five species of RNA are integral parts of the virion structure, a negative result does not prove relevance of these RNA species to the virions.

It has been shown that the viral 7S and 4S

RNA do not hybridize to the 60S viral RNA (10, 11, 40) and are therefore not complementary to the 60S genome. It is not known whether the 7S RNA is identical in sequence to part of the 60S (or other) viral RNA. The function of the 7S species is unknown; it does not contain methylated bases (11). The 4S RNA species, however, have been shown to contain methylated bases and to have sequences common to the host cell DNA, and at least some of them have amino acid acceptor (tRNA) capabilities (10, 14, 38–40). The molecular ratio of the 7S and tRNA species in the RSV preparation we used indicates that there are, on the average, around 5 and 200 molecules, respectively, per molecule of virion 60S RNA. Although the presence of the 28S and 18S RNA species was reported earlier (10, 11, 14), it is still not known whether they are involved in a ribosome structure. Their presence could possibly indicate that either the viral preparation contains membrane contaminants derived from the host cell or that virions have translational capabilities. It should be mentioned that influenza virus grown and purified from similar, albeit untransformed, CEF cells does not contain 28S, 18S, 7S, and 4S RNA species (7). An investigation into the association and location of the 28S and 18S RNA species is being undertaken to elucidate these possibilities.

Bader and Steck (2) pulse-labeled infected mouse splenic cells (JLSV₃) and harvested mouse leukemic virus (MLV) at 1-hr intervals thereafter. They demonstrated that the only labeled RNA in their preparation was the 60S viral RNA species. This suggests that the other RNA species present in our RSV preparations are probably present in contaminating membranes which cannot be removed by EDTA and high-salt treatment.

Subunits of RSV genome. RSV and other oncogenic viruses contain a 60S genome which can be dissociated by heat, dimethyl sulfoxide, urea, or formaldehyde treatment (2, 12, 16, 17, 19–21, 32). In the RSV preparations used in this study, the dissociated RNA showed considerable heterogeneity. Although the frequent occurrence of such heterogeneity is well documented (16, 17, 19–21), Erikson (21) showed that considerably less heterogeneity is obtained when virus is harvested at 3-hr intervals. Similarly, Bader and Steck (2) used MLV harvested at 1-hr intervals and showed that 90% of the 60S viral RNA can be converted into a 35S to 45S (4×10^6 daltons) RNA species. Although these harvesting procedures pose a logistics problem for enzyme analyses, they suggest that the heterogeneity we have observed may be the result of endonuclease activity before or during virus purification and

also suggest that the resulting degradation of the 60S RNA was unexpressed because of hydrogen bonding. Thus, determination of the number of subunits, an accurate molecular weight estimate of the 60S conglomerate as well as the 35S subunits, and a convincing demonstration that subunits can be resolved by gel electrophoresis, will have to await chemical and physical analyses carried out with preparations containing viral RNA lacking intramolecular nicks.

DNA polymerase enzyme of RSV. It was the purpose of this study to delineate reaction conditions whereby, during RSV RNA-instructed DNA synthesis, the viral RNA would remain intact. We have found, by using limited Triton concentrations, that the viral RNA can be conserved. The recovery of 60S viral RNA, as resolved by gel electrophoresis, was essentially unchanged throughout 1 hr of reaction incubation and showed a 20% loss through the total 3-hr incubation. After melting of the reaction product nucleic acids, the patterns of heterogeneous RNA species were also essentially similar throughout the reaction time-course. Reactions run in high Triton concentrations showed considerable degradation of the 60S viral RNA and were therefore deemed unsuitable for product analysis. The fact that the virions were substantially dissociated in high Triton (Fig. 1) suggests that contaminating endonuclease was the cause of the viral RNA degradation.

Product analysis of the RSV DNA polymerase reaction. The basic questions posed by oncogenic viral DNA polymerase reactions center around the following issues. (i) How is DNA synthesis initiated and accomplished? (ii) What are the intermediates in product synthesis? (iii) What are the physical states of the product species? (iv) How complete is the transcription process? (v) What happens to the product DNA in an infected cell? Although preliminary answers to several of these questions have been obtained (18, 22-26, 29, 30, 32, 35), we would question their relevance to the polymerase reaction as no concurrent template analyses were performed, and, therefore, we do not know if the DNA analyzed represented artifacts generated by degraded templates.

In the analyses described here, we found that the template RNA is mostly nicked. Although we have not determined whether DNA synthesis is affected on the 60S RNA templates possessing these nicks, the conclusion to be drawn from these experiments is clear. Not only should the state of the 60S template RNA species be monitored throughout a reaction time-course (as in Fig. 4), but also the subunit structure of the viral RNA should be examined before and after a reaction to

determine the true integrity of the viral RNA. Product analyses should only be performed with DNA associated with intact templates. In experiments with either detergent-dissociated virions or purified enzymes and viral RNA, if the viral RNA has nicks, then interpretation of the results will always have the ambiguity of potential artifacts. What happens when an enzyme is transcribing a template molecule and comes to a break in the template? Does it traverse the break, does it reverse and transcribe the DNA forming double-stranded DNA, or are it and its product prematurely displaced by succeeding enzymes from the template molecule?

Bearing these problems in mind, it is still of interest to note that an early RSV reaction product contained 92% of its DNA associated with the 60S RNA. However, after ribonuclease digestion, the DNA was recovered as single-stranded species with little associated RNA, suggesting that the original RNA-DNA complexes were multi-stranded with respect to product DNA strands. Thus it can be suggested that the initially synthesized DNA strand is displaced by succeeding strands of DNA synthesized on the same template RNA (29). The proportion of any of the initially synthesized DNA strands which remains hydrogen-bonded to the RNA might be so small that after ribonuclease digestion these DNA strands are released as if they were single-stranded molecules. This result should not be too surprising if reaction products contain free single-stranded DNA (29) and the virions possess several enzyme molecules (28).

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