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RNA primer used in synthesis of anticomplementary DNA by reverse transcriptase of avian myeloblastosis virus

(hybrid nuclease/RNA·cDNA hybrid/phage T4 RNA ligase/two-stage reaction/DNA nucleotidyltransferase)

JEANNE C. MYERS*, CARL DOBKIN†, AND S. SPIEGELMAN‡

Institute of Cancer Research and Department of Human Genetics and Development, College of Physicians and Surgeons, Columbia University, New York, New York 10032

Contributed by Sol Spiegelman, December 4, 1979

ABSTRACT When either the homologous RNA (avian myeloblastosis virus RNA) or a heterologous RNA (poliovirus RNA) was used as a template, the anticomplementary DNA synthesized *in vitro* by avian myeloblastosis virus reverse transcriptase (RNA-directed DNA nucleotidyltransferase, EC 2.7.7.7) was primed by fragments of the original RNA template that usually had adenosine at their 3′ ends. When we used phage T4 RNA ligase (EC 6.5.1.3) to label the 3′ end of the RNA template fragments contained in the RNA·cDNA hybrid intermediate, adenosine was found to be the principal nucleoside carrying the label. We infer from these results that the ribonuclease H (hybrid nuclease) activity of the reverse transcriptase creates fragments of the original RNA template with adenosine as the principal 3′ terminus and that these fragments serve as primers for the synthesis of anticomplementary DNA.

Avian myeloblastosis virus (AMV) reverse transcriptase is an RNA- (or DNA-) directed DNA polymerase (DNA nucleotidyltransferase, EC 2.7.7.7) that is responsible for the conversion of the single-stranded viral RNA into double-stranded DNA that can be integrated into the host genome (1–3). In addition to catalyzing the polymerization of DNA, the enzyme has an integral ribonuclease H activity that degrades the RNA in an RNA·DNA hybrid (4–7). The enzyme synthesizes complementary DNA (cDNA), using the viral RNA as template and a tRNA primer (8), degrades the RNA with its ribonuclease H activity, and synthesizes DNA identical to the RNA (anticomplementary DNA), using the cDNA as template.

The isolated enzyme exhibits these activities clearly *in vitro*. Highly purified AMV reverse transcriptase will extend an oligo(dT) primer and polymerize full-length cDNA from the natural AMV RNA template (9) or from a heterologous polyadenylylated template such as poliovirus RNA (10). Anticomplementary DNA is synthesized *in vitro* in 5–8S segments (11) analogous to the segmented anticomplementary DNA that has been observed during viral infection (12–16).

The presence of 4 mM pyrophosphate (PP_i) or high concentrations of ribo- or deoxyribonucleoside triphosphates in the reaction mixture inhibits the synthesis of anticomplementary DNA and increases the amount of full-length cDNA that is synthesized (9, 10). The product of a reverse transcriptase reaction containing one of these inhibitors is a hybrid of cDNA and nicked template RNA (17). When this RNA·cDNA hybrid intermediate is isolated and then used as template in a reaction in the absence of an inhibitor, the reverse transcriptase degrades the RNA in the RNA·cDNA hybrid and replaces it with anticomplementary DNA (17). The synthesis of complementary and anticomplementary DNA can therefore be dissociated *in vitro*. For example, by including 4 mM PP_i in the reaction, synthesis is restricted to the completion of the RNA·cDNA hybrid; when the PP_i is reduced to 2 mM, the RNA in the hybrid is replaced with anticomplementary DNA.

The primers used for the synthesis of anticomplementary DNA have not been identified unambiguously. It has been suggested (3, 4, 11, 18, 19) that the ribonuclease H activity of this enzyme degrades the RNA in the RNA·cDNA hybrid, and in so doing, creates the primers for the segments of anticomplementary DNA observed both *in vitro* and *in vivo*. The results of the experiments described here indicate that the fragments of the original RNA template do in fact serve as primers for anticomplementary DNA synthesis *in vitro*. In addition, we find that most of the primers used for anticomplementary DNA synthesis possess a 3′-terminal adenosine, and that most of the RNA fragments in the RNA·cDNA hybrid intermediate also terminate with adenosine. The same results are obtained when either the homologous or a heterologous RNA serves as the template.

MATERIALS AND METHODS

Reverse Transcriptase. RNA-directed nucleotidyltransferase was isolated from AMV by DEAE-cellulose (Whatman) and CM-Sepharose (Pharmacia) column chromatography and concentrated using a collodion bag (20). Units are defined in ref. 20.

RNA and RNA·cDNA Hybrid Templates. Poliovirus RNA and AMV RNA were isolated as described (9, 10). A hybrid of poliovirus RNA and its full-length [^{3}H]cDNA was isolated from a reverse transcriptase reaction mixture containing 4 mM PP_i and fractionated on a sodium dodecyl sulfate/sucrose gradient (17). A hybrid of AMV RNA and its full-length [^{3}H]cDNA was synthesized (9) and then isolated by the same procedure.

Synthesis of Anticomplementary DNA from Poliovirus RNA·cDNA Hybrid. The four 25-μl reaction mixtures, which differed only in the labeled deoxyribonucleotide that was present, contained 50 mM Tris·HCl (pH 8.3), 8 mM $MgCl_2$, 40 mM KCl, 0.4 mM dithiothreitol, 0.0125 mM (each) deoxyribonucleoside 5′-triphosphate [one of which was α-^{32}P-labeled (New England Nuclear) at 10 Ci/mmol (1 Ci = 3.7×10^{10} becquerels)], poliovirus [^{3}H]cDNA (in the form of an RNA·cDNA hybrid) at 20 μg/ml, and AMV reverse transcriptase at 50 units/ml.

Synthesis of Anticomplementary DNA from Poliovirus RNA and AMV RNA in a Two-Stage Reaction. There were four separate synthetic reactions for each type of RNA and each reaction was completed in two stages. In the first stage, in which

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Abbreviation: AMV, avian myeloblastosis virus.

* Present address: Department of Biochemistry, College of Medicine and Dentistry of New Jersey, Rutgers Medical School, Piscataway, NJ 08854.
† Present address: Institut für Molekularbiologie I, Universitat Zürich, Hönggerberg, CH-8093 Zürich, Switzerland.
‡ To whom correspondence should be addressed.

the [^{3}H]cDNA was synthesized, each 50-μl reaction mixture contained 50 mM Tris·HCl (pH 8.3), 8 mM $MgCl_2$, 40 mM KCl, 4 mM $NaPP_i$, 0.4 mM dithiothreitol, 0.2 mM (each) deoxyribonucleoside triphosphate (including [^{3}H]dCTP (New England Nuclear) at 5 Ci/mmol), and $(dT)_{12-18}$ (Collaborative Research) at 5 μg/ml. The reaction mixtures with poliovirus RNA template contained RNA at 20 μg/ml and reverse transcriptase at 80 units/ml. The reaction mixtures with AMV template RNA contained RNA at 40 μg/ml and reverse transcriptase at 200 units/ml. The mixtures were incubated for 45 min at 37°C.

In the second stage, each reaction mixture was increased to 100 μl to reduce the PP_i concentration to 2 mM, thus allowing anticomplementary DNA synthesis to occur. The original concentrations of Tris·HCl, $MgCl_2$, KCl, and dithiothreitol were maintained. Each second-stage reaction mixture contained one α-^{32}P-labeled deoxyribonucleoside triphosphate at 0.1 mM and 10 Ci/mmol, and the three other triphosphates at 0.2 mM. Five units of reverse transcriptase was added to each poliovirus RNA reaction mixture and 15 units to each AMV RNA reaction mixture. Incubation was continued for 20 min at 37°C.

Gradient Analyses. The density of nucleic acid hybrid structures present in the reverse transcriptase reaction mixtures was determined by cesium sulfate equilibrium density gradient centrifugation as described (17). The densities of denatured reaction products were determined by diluting 1 μl of the reaction mixture in 300 μl of 1 mM EDTA and incubating it at 100°C for 5 min before centrifugation in cesium sulfate.

The size of ^{3}H-labeled complementary DNA and ^{32}P-labeled anticomplementary DNA present in the reverse transcriptase reaction mixtures was determined by centrifugation through gradients of alkaline sucrose (9, 10) with phage f1 [^{3}H]DNA present as a marker in parallel gradients.

Isolation of Reaction Products. The RNA and DNA present at termination of each reverse transcriptase reaction were isolated by phenol/cresol/chloroform extraction and Sephadex G-50 (Pharmacia) column chromatography (10). Twenty-five μg of yeast RNA (P-L Biochemicals) was added to the excluded fraction and the nucleic acid mixture was precipitated with 2 vol of ethanol overnight at −20°C. The precipitate was then collected by centrifugation and washed with 70% ethanol (17).

Alkaline Hydrolysis of Reaction Products and Thin-Layer Chromatography. Nucleic acids isolated from each reaction mixture (containing 25 μg yeast RNA) were suspended in 8 μl of 0.5 mM EDTA. Two microliters of 0.5 M NaOH was then added and the mixture was sealed in a 50-μl capillary tube and hydrolyzed by incubation at 100°C for 20 min (21). The hydrolysate was neutralized with 1 M $HClO_4$ and the resulting $KClO_4$ precipitate was collected at one end of a sealed 25-μl capillary tube by centrifugation at 4°C and then discarded.

Twenty-five nanomoles of each ribo- and deoxyribonucleoside 3′(2′)-monophosphate (P-L Biochemicals) was added to the hydrolysate. This mixture (containing 1–5 × 10^5 cpm) was then applied to a polyethyleneimine-cellulose thin-layer plate (Machery-Nagel, West Germany) and chromatographed in two dimensions to separate all ribo- and deoxyribonucleotides from each other and from polymerized DNA (22). The chromatograms were autoradiographed overnight at −70°C with Kodak X-Omat R film and a Cronex Lightning-plus intensifier screen (Du Pont). The positions of the (unlabeled) ribo- and deoxyribonucleotides were determined by visualization under short-wave ultraviolet light. The portion of the chromatograph containing each ribonucleotide spot was cut out and the amount of radioactivity it contained was determined by liquid scintillation counting. Between 0.1 and 0.9% of the radioactivity on the chromatogram was present in the ribonucleotide spots; the remainder was present as polymerized DNA.

Ribonucleotide Analysis. It was sometimes necessary to further identify the radioactivity in parts of the chromatogram. To this end, the radioactive material was eluted from the polyethyleneimine-cellulose with triethylammonium bicarbonate (23). To separate orthophosphate from guanosine monophosphate, or to define any incompletely separated ribonucleotides, the eluted material was applied to 3MM paper (Whatman) and analyzed by electrophoresis at pH 3.5 (24). To determine whether any of the labeled adenosine in the chromatogram was N^6-methylated (25), this material was eluted, mixed with 25 nmol of unlabeled *N*^{6}-methyladenosine 3′(2′)-monophosphate [prepared from 1-methyladenosine 3′(2′)-monophosphate (P-L Biochemicals)] and chromatographed in isopropyl alcohol/1% ammonium sulfate, 3:2 vol/vol (26).

Analysis of the RNA Component of the RNA·cDNA Hybrid. RNA·cDNA hybrids resulting from reverse transcription of poliovirus RNA and of AMV RNA in the presence of PP_i were isolated as described above and their RNA component was 3′-end labeled by phage T4 RNA ligase (EC 6.5.1.3) essentially under the conditions described by England and Uhlenbeck (27). Four-tenths microgram of the RNA (in the form of a RNA·cDNA hybrid) was suspended in 11 μl of H_2O and denatured by incubation at 100°C for 1 min while sealed in a 50-μl capillary tube. At 0°C the solution was brought to 20 μl containing 50 mM Tris·HCl (pH 7.6), 12 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 10% (vol/vol) dimethyl sulfoxide, 0.01 mg of bovine serum albumin per ml, 600 pM [5′-^{32}P]pCp (at 450 Ci/mmol, New England Nuclear), and T4 RNA ligase (P-L Biochemicals) at 350 units/ml (which also contributed ATP at 5 μM final concentration). After incubation at 4°C for 25 hr, 6 × 10^5 cpm was incorporated. End-labeled RNA was isolated by phenol/cresol/chloroform extraction and Sephadex G-50 chromatography, and collected as described above. Ten percent of this material was mixed with 25 μg of yeast RNA, hydrolyzed, and analyzed by thin-layer chromatography as described above.

The amount of labeled poly(A) in the RNA from each RNA·cDNA hybrid was determined by suspending 20% of the end-labeled RNA in a 1-ml solution of 1 mM EDTA containing 0.6 μg of [^{3}H]poly(A) (3 × 10^5 cpm/μg) and incubating it at 100°C for 3 min. The mixture was brought to 0.5 M NaCl and applied to a 1 × 0.5 cm column of oligo(dT)-cellulose equilibrated in 0.5 M NaCl. Less than 2% of the ^{32}P-end-labeled material was retained, while more than 95% of the [^{3}H]poly(A) was bound to the column.

T4 RNA Ligase Control. Randomly fragmented poliovirus RNA (1.5 μg) (approximately 3500 nucleotides average length) was treated with 0.14 unit of bacterial alkaline phosphatase (EC 3.1.3.1; Worthington) for 15 min at 37°C in a 50-μl volume of 50 mM Tris·HCl (pH 8.0). The RNA was isolated, precipitated, and collected as described above. This material was dissolved in 11 μl of H_2O and incubated with T4 ligase and [5′-^{32}P]pCp as described above, resulting in the incorporation of 6 × 10^4 cpm of ^{32}P. The labeled material was then applied to oligo(dT)-cellulose as described above. The [^{32}P]RNA (48%) that did not bind to the oligo(dT)-cellulose because it lacked the 3′-poly(A) segment was precipitated with 2 vol of ethanol, hydrolyzed, and analyzed by thin-layer chromatography.

RESULTS

Experimental Design. The ribonuclease activity of AMV reverse transcriptase degrades the RNA strand of an RNA·cDNA hybrid into oligonucleotides that have a hydroxyl group at their 3′ end (4–7). If these fragments serve as primers for the synthesis of anticomplementary DNA in the reverse transcriptase reaction, phosphodiester bonds will be formed between their 3′-terminal ribonucleotides and adjacent deoxyribonucleotides.

To detect these linkages we analyzed anticomplementary

DNA synthesized *in vitro* with α-^{32}P-labeled deoxynucleoside triphosphates, using a separate reaction for each labeled nucleotide. Two types of reverse transcriptase reaction conditions were employed. In one, each differently labeled reaction mixture contained an RNA·(full-length)cDNA hybrid as the template and no PP_i. In the other, each reaction was templated with RNA and was completed in two stages. The first stage contained no ^{32}P-labeled triphosphates and included 4 mM PP_i, which limited synthesis to the completion of the cDNA; in the second stage, one α-^{32}P-labeled deoxyribonucleoside triphosphate was added to each reaction mixture and the concentration of PP_i was reduced to allow synthesis of anticomplementary DNA. By employing an RNA·(full-length)cDNA hybrid as the reverse-transcriptase-reaction template, or by allowing the cDNA to be completed in the first stage of an RNA-templated reaction, we ensured that the action of ribonuclease H would yield representative RNA fragments that could serve as primers for anticomplementary DNA synthesis. The labeled anticomplementary DNA was treated with alkali, and the resulting hydrolysate was analyzed by two-dimensional thin-layer chromatography to separate the individual ribonucleoside monophosphates from polymerized DNA and from any deoxyribonucleotides (22). If RNA fragments serve as primers for anticomplementary DNA synthesis, some of their 3′-ribonucleotides will have been linked to an α-^{32}P-labeled deoxyribonucleotide. Alkaline hydrolysis of this link should then yield a ^{32}P-labeled ribonucleotide (28) that can be detected in the chromatogram.

Anticomplementary DNA from Poliovirus RNA·(Full-Length)cDNA Template. We first analyzed the anticomplementary DNA synthesized in reverse transcriptase reactions templated with a hybrid of poliovirus RNA and full-length [^{3}H]cDNA isolated from a reverse transcriptase reaction mixture containing 4 mM PP_i (10, 11, 17). As shown in Fig. 1, ^{32}P-labeled deoxyribonucleotides were incorporated into 5–8S segments of anticomplementary DNA that replaced the RNA in the hybrid during the reaction. When this material was hydrolyzed and analyzed by thin-layer chromatography, 0.1–0.4% of the incorporated ^{32}P from each reaction was found in the ribonucleotide spots in the chromatogram. Thus, the anticomplementary DNA contained linkages between ribo- and deoxyribonucleotides, indicating that fragments of RNA in the RNA·cDNA hybrid served as primers for the synthesis of anticomplementary DNA.

These analyses also showed that adenosine was the principal base labeled in all four differently labeled reactions (Table 1). (The predominance of labeled adenosine ribonucleotide in the hydrolysates of all four reactions could not be due to the presence of the 3′-poly(A) region of the RNA, because primer fragments from this region can be labeled only by [α-^{32}P]-deoxyadenosine.)

Anticomplementary DNA from Homologous and Heterologous RNA Templates. To investigate further the primers arising from the RNA template *in vitro*, we compared the anticomplementary DNA from reverse transcriptase reactions templated with poliovirus RNA and with AMV RNA. Each reaction was completed in two stages, and the reaction intermediates present at each stage were analyzed by molecular hybridization and by velocity and isopycnic gradient centrifugation. The first-stage mixture (45 min) contained ^{3}H-labeled deoxyribonucleoside triphosphates and 4 mM PP_i, which restricted synthesis to the completion of complementary DNA. In the second-stage mixture (20 min), the PP_i concentration was reduced to 2 mM and one α-^{32}P-labeled deoxyribonucleoside triphosphate was added and incorporated into anticomplementary DNA (see Fig. 2). When the [^{32}P]DNA synthesized in the second stage of the reaction was fractionated and analyzed

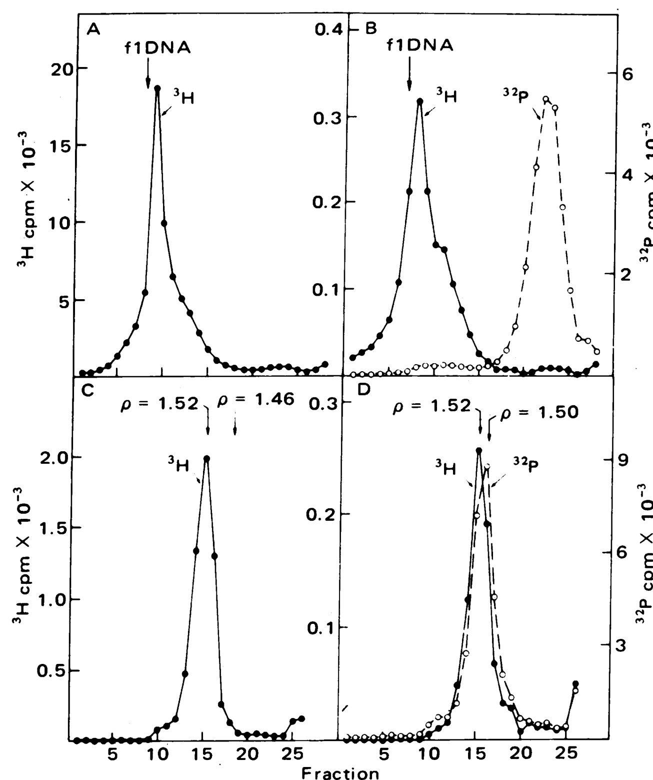

FIG. 1. Gradient analyses of reverse transcriptase reactions in which poliovirus RNA·[^{3}H]cDNA hybrid templated synthesis of ^{32}P-labeled anticomplementary DNA. (*A*) Alkaline sucrose gradient of [^{3}H]cDNA present in the hybrid. (*B*) Alkaline sucrose gradient of ^{3}H- and ^{32}P-labeled anticomplementary DNA at termination of the 20-min reaction. (*C*) Cesium sulfate density gradient of poliovirus RNA·[^{3}H]cDNA hybrid template. (*D*) Cesium sulfate density gradient of ^{3}H- and ^{32}P-labeled anticomplementary DNA at termination of the reaction. Densities ρ are given in g/ml.

by hybridization, more than 90% of it was found to be identical to the RNA template (see Table 2).

Gradient analysis of the reaction products (see Fig. 3) showed that the [^{3}H]cDNA completed during the first stage of the reaction was incorporated into an RNA·cDNA hybrid. In the second stage the RNA component of the hybrid was degraded and replaced by anticomplementary [^{32}P]DNA and the density of the hybrid shifted toward that of DNA. When the hybrid present at the end of the reaction was dissociated and then analyzed on a density gradient, the [^{3}H]cDNA banded sharply in

Table 1. Ribonucleotides labeled by deoxyribonucleoside α-^{32}P-triphosphates incorporated into anticomplementary DNA

Template	α-^{32}P-Labeled deoxynucleotide	Labeled ribonucleotides in hydrolyzed reaction products, %			
		AMP	CMP	GMP	UMP
Poliovirus RNA·cDNA hybrid	dATP	79	3	8	9
	dCTP	67	18	4	11
	dGTP	77	1	5	17
	dTTP	77	5	11	7
Poliovirus RNA (two-stage reaction)	dATP	67	6	13	13
	dCTP	65	10	8	17
	dGTP	62	3	13	22
	dTTP	69	6	13	12
AMV RNA (two-stage reaction)	dATP	63	8	12	17
	dCTP	64	8	12	15
	dGTP	63	4	13	20
	dTTP	67	13	6	14

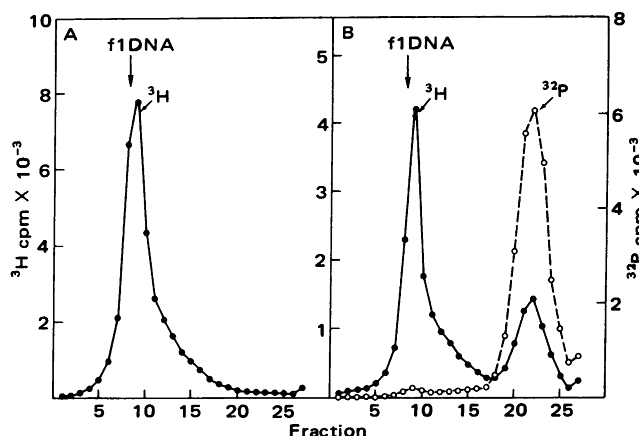

FIG. 2. Alkaline sucrose gradient analyses of poliovirus cDNA synthesized in a two-stage reverse transcriptase reaction. (*A*) Poliovirus [^{3}H]cDNA synthesized in the presence of 4 mM PP_i during the first stage incubation (45 min). (*B*) Anticomplementary [^{32}P]DNA synthesized in the presence of 2 mM PP_i during the second stage incubation (20 min).

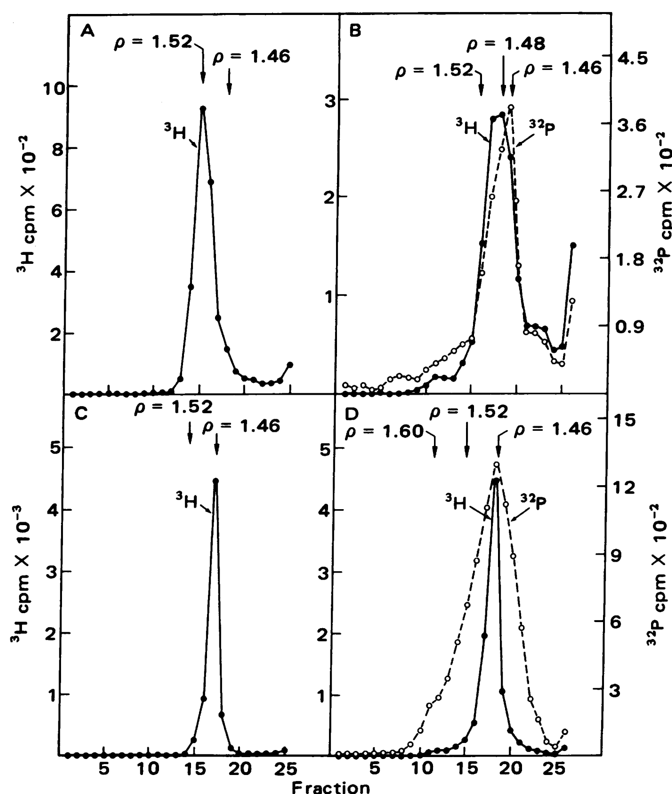

FIG. 3. Cesium sulfate density gradient analyses of hybrid structures in the two-stage reaction. (*A*) Poliovirus RNA·[^{3}H]cDNA hybrid formed in the presence of 4 mM PP_i during the first stage incubation (45 min). (*B*) Hybrid structures resulting from synthesis that occurred in the second stage incubation (20 min) in the presence of 2 mM PP_i and ^{32}P-labeled deoxyribonucleoside triphosphates. (*C*) [^{3}H]cDNA shown in *A* after it was melted. (*D*) [^{3}H]cDNA and anticomplementary [^{32}P]DNA shown in *B* after it was melted.

the DNA region, whereas the behavior of the anticomplementary [^{32}P]DNA suggested that it is covalently linked to some RNA.

AMV RNA and poliovirus RNA were used as templates under the same two-stage reaction conditions. The reaction products were isolated and hydrolyzed, and the hydrolysates were analyzed by thin-layer chromatography as before (see Fig. 4). The analyses of the reactions with homologous RNA and with heterologous RNA as template yielded essentially identical results. More than 60% of the template RNA fragments that served as primers for anticomplementary DNA synthesis had a 3′-terminal adenosine (Table 1).

RNA Component of the RNA·cDNA Hybrid Intermediate. If most of the segments of anticomplementary DNA are linked to an adenosine because the available RNA fragments that could serve as primers usually end in adenosine, then most of the fragments of template RNA in the RNA·cDNA hybrid intermediate should terminate with this ribonucleoside. We therefore isolated AMV RNA·cDNA and poliovirus RNA·cDNA hybrids and labeled the 3′ ends of their RNA component by using T4 RNA ligase and [5′-^{32}P]pCp (27). Oligo(dT)-cellulose chromatography of the RNA isolated from the reaction mixture showed that it contained no labeled poly(A). This material was treated with base and analyzed on thin-layer plates as before (see Fig. 5). In a control reaction, randomly fragmented poliovirus RNA was incubated with T4 RNA ligase and [5′-^{32}P]pCp. The polyadenylylated 3′ end of the intact RNA was removed by oligo(dT)-cellulose chromatography, and the poliovirus RNA fragments that had been 3′-end-labeled were hydrolyzed and analyzed on a thin-layer plate.

Table 2. Hybridization analysis of AMV [^{32}P]DNA synthesized in the two-stage reaction

Nucleic acid	% micrococcal nuclease resistance after hybridization with AMV [^{3}H]cDNA	AMV [^{32}P]DNA
AMV RNA	99.6	8.7
Escherichia coli RNA	6.2	8.2
AMV [^{32}P]DNA	89.4	—

The conditions for hybridization and micrococcal nuclease assays have been described (10, 11). Two-tenths microgram of RNA and 1.5–3.0 ng of DNA were present in each 20-μl reaction mixture except in the DNA·DNA hybridization, for which 20 ng of cDNA was added. All reaction mixtures were incubated at 68°C to attain $C_{RNA\ or\ RNA}\cdot t$ = 0.1 mol of nucleotide·liter^{-1}·sec. AMV [^{3}H]cDNA was isolated from a synthetic reaction mixture containing 4 mM PP_i (9). [^{32}P]DNA synthesized in a two-stage reaction was fractionated on an alkaline sucrose gradient (17).

The results are shown in Table 3. The hydrolysate of the control reaction mixture contained approximately equal amounts of all four ^{32}P-labeled ribonucleotides, indicating that all fragments in the ligase reaction were labeled indiscriminately. In contrast, the hydrolysates of the 3′-end-labeled RNA from AMV and poliovirus RNA·cDNA hybrids revealed that adenylate was the principally labeled ribonucleotide.

We infer from these and the previous results that fragments of the original RNA template that are present in the RNA·cDNA hybrid intermediate usually terminate with an adenosine at their 3′-end and that these fragments serve as primers for the synthesis of anticomplementary DNA.

DISCUSSION

When we examined the anticomplementary DNA synthesized by AMV reverse transcriptase *in vitro*, we found that it was covalently linked to fragments of the original RNA template, and that the linkage usually occurred at an adenosine ribonucleotide. Similar observations have recently been made by J. Olsen and K. Watson (ref. 19 and personal communication), who used AMV RNA as template with different reaction conditions and analytical techniques. By end-labeling the RNA fragments present in the RNA·cDNA hybrid intermediate, we found that most of the potential primers for anticomplementary DNA have a 3′-terminal adenosine. These results imply that once the RNA has served as template and has been incorporated into the growing RNA·cDNA hybrid, it is cleaved endonucleolytically, and the cleavages occur preferentially at an

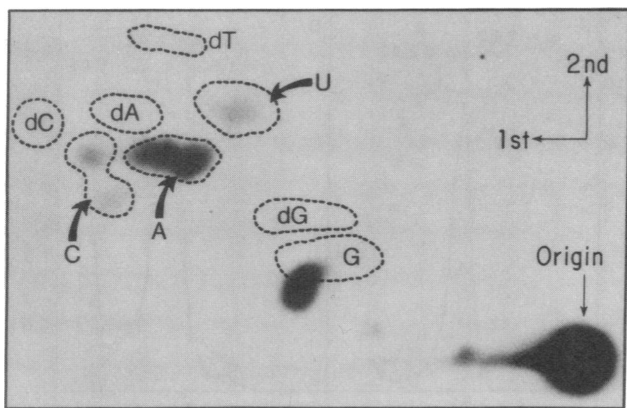

FIG. 4. Two-dimensional thin-layer chromatography of hydrolyzed AMV anticomplementary DNA labeled with [α-^{32}P]dTTP. The hydrolyzed material from a reverse transcriptase reaction was mixed with unlabeled marker ribo- and deoxyribonucleotides and applied to a polyethyleneimine-cellulose thin-layer plate at the origin. The plate was developed in two dimensions, the marker mononucleotides were located with ultraviolet illumination, and the plate was autoradiographed to visualize the radioactive material. Broken lines indicate the positions of all eight marker ribo- and deoxyribonucleoside 3′-monophosphates on this autoradiograph. Analysis of the radioactive material overlapping the G spot showed that it was orthophosphate. Analysis of the labeled adenosine from a thin-layer plate indicated that no N^6-methyladenosine had been linked to DNA.

adenosine. The resulting RNA fragments then serve as primers for the synthesis of anticomplementary DNA. Because adenosine was found predominantly as the 3′ terminus with the homologous RNA template and with a heterologous one, it seems likely that the linkage site is determined by the enzyme rather than the template.

We have incorporated these conclusions into the following model of the synthetic and degradative activities of AMV reverse transcriptase *in vitro*. In the presence of 4 mM PP_i, the reverse transcriptase extends the oligo(dT) primer to synthesize a strand of full-length complementary DNA and the RNA template in the growing RNA·cDNA hybrid is usually nicked at an adenosine. The PP_i apparently prevents further degradation of the RNA and consequently inhibits the synthesis of anticomplementary DNA (11, 17). When the PP_i is removed, the enzyme's ribonuclease H activity degrades the RNA fragments in the hybrid and the reverse transcriptase replaces them with segments of anticomplementary DNA for which they serve as primers. If no inhibitor such as PP_i is present, the reverse transcriptase begins to synthesize anticomplementary DNA as soon as an RNA primer becomes available.

Although we detected no specificity for the first deoxyribonucleotide incorporated into the anticomplementary DNA

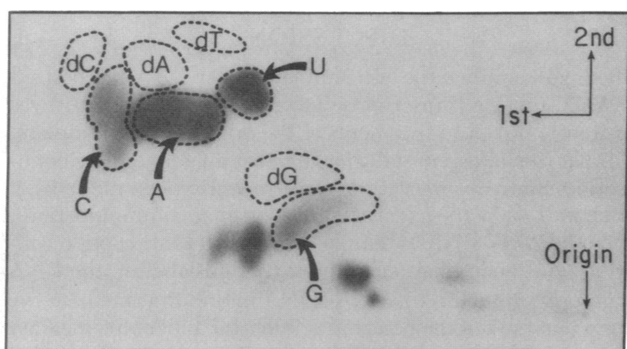

FIG. 5. Two-dimensional thin-layer chromatography of hydrolyzed 3′-end-labeled RNA from an AMV RNA·cDNA hybrid intermediate. Chromatography and autoradiography were performed as described for Fig. 4.

Table 3. Ribonucleotides labeled with T4 RNA ligase and [5′-^{32}P]pCp

RNA source	Labeled ribonucleotides, %			
	AMP	CMP	GMP	UMP
Poliovirus RNA·cDNA hybrid	68	11	6	16
AMV RNA·cDNA hybrid	53	18	8	21
Fragmented poliovirus RNA	20	28	22	30

segments, it is possible that the endonucleolytic activity that creates the primers requires more than a single adenosine to recognize a cleavage site. It is possible to investigate the activity of AMV reverse transcriptase *in vitro* by using MDV-1 RNA, a small completely sequenced molecule (29) as a template. Analysis of the RNA component of an MDV-1 RNA·cDNA hybrid intermediate would reveal the common features of the RNA fragments that serve as primers for anticomplementary DNA.

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