JOURNAL OF VIROLOGY, Dec. 1973, p. 1491-1502
Vol. 12, No. 3

Printed in U.S.A.

RNA-DNA Covalent Bonds Between the RNA Primers and the DNA Products Formed by RNA Tumor Virus DNA Polymerase

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Received for publication 2 August 1973

Initiation of DNA synthesis by endogenous RNA primer molecules was studied with three different RNA tumor viruses. The influence of the method of virus disruption on the observed RNA-DNA bonds was ascertained. Ether disrupted virions of both murine leukemia virus (MuLV) and the B77 strain of avian sarcoma virus (B77 virus) have rC-dC and rA-dA covalent linkages between RNA primers and newly synthesized DNA. None of the 14 other possible bonds were formed. Ether-disrupted virions of avian myeloblastosis virus (AMV) have rU-dC and rA-dA linkages. In contrast, work reported herein and from other laboratories shows that Nonidet P-40 (NP-40)-disrupted virions of all three viruses have only the rA-dA junction. Studies with virus particles which were first disrupted with ether and then treated with NP-40 indicated that the detergent treatment disallowed the formation of the ribopyrimidine-dC internucleotide bond. The same transfers are found with AMV in the presence or absence of actinomycin D, where only single-stranded DNA is formed. This finding is consistent with the notion that virtually all of the significant primers have been recognized. In contrast to mature virions, transfer experiments with ether-disrupted early harvest (5 min) MuLV showed only the rC-dC bond; the rA-dA bond was absent. The short-time harvest contains a significantly higher proportion of infectious virions than 24-h harvests. Also, since the RNA from early harvest virus is appreciably more homogenous than the RNA of mature MuLV, it is concluded that the ribopyrimidine-dC linkage is the more significant initiation event from a biochemical standpoint.

RNA tumor viruses contain a DNA polymerase which can utilize high molecular weight viral RNA as a template for DNA synthesis (27). Several reports (2, 16, 29) have indicated that RNA tumor viruses also contain an RNA primer. This was conclusively demonstrated by analysis of the RNA-DNA convalent bond formed between the primer RNA and the newly synthesized DNA molecule (8, 31). Previous work from this laboratory (8) showed that the RNA-DNA joining reaction was specific since only -rU-dC- and -rA-dA- bonds were observed out of the 16 possible linkages. These studies, with ether-disrupted avian myeloblastosis virus (AMV), suggested that at least two RNA molecules could serve as primers.

Later work with either Nonidet P-40 (NP-40)-disrupted virions of AMV or with purified high molecular weight RNA and purified DNA polymerase from AMV (28) verified the -rA-dA- linkage. However, the -rU-dC- bond was not detected in these studies, suggesting that a factor, possibly an enzyme or an RNA molecule (primer?), was lost or destroyed during the purification processes. In addition, studies with other enzymatic activities clearly demonstrated marked differences between NP-40-disrupted and ether-disrupted virus preparations (7). Hence, it is understandable that somewhat different results were found as a function of the technique used for virus activation. Recent studies with other viruses confirmed the presence of the -rA-dA- bond. These are studies with NP-40-disrupted virions of Schmidt-Ruppin strain of Rous sarcoma virus (RSV) and with purified 70S RNA and the DNA polymerase from this virus (25) and studies with NP-40-disrupted virions of Rauscher murine leukemia

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virus (MuLV) (21). In none of these studies were other linkages found. Thus, the -rU-dC- linkage was observed only in ether-disrupted virions of AMV.

In an effort to resolve the apparent discrepancies between the types of transfers found under the different conditions, we performed further studies with different RNA tumor viruses. The following questions were studied. (i) Are similar types of transfers found with other viruses? This answer should imply if the primers for initiation of DNA synthesis are similar or if they must be different. (ii) Is the -rU-dC- type of transfer unique to ether-disrupted AMV or are similar bonds formed with other viruses which are ether-disrupted? (iii) Why is a difference observed in the types of transfers found with ether-disrupted and with NP-40-disrupted virions? (iv) Does the presence of actinomycin D influence the types of transfers observed under conditions where the formation of double-stranded DNA is suppressed (10, 23, 30)? (v) What types of RNA-DNA bonds are formed by immature particles of RLV?

The general scheme of this study is outlined in Fig. 1. A purified virus preparation (either AMV or MuLV or B77 virus) was activated by either ether extraction or by treatment with the nonionic detergent NP-40. A DNA polymerase reaction was then performed by using this preparation as a source of the enzymatic activity, RNA primer and template. Deoxyribonucleoside triphosphates (dNTP's), one of which contained an α-^{32}P-label, were added (endogenous reaction). After a suitable reaction time, the ^{32}P-polynucleotide product was purified from other polymer material which was also radioactively labeled. The RNA-DNA product was then subjected to an alkaline hydrolysis step (31) to provide a specific and sensitive assay for the phosphodiester junction between an RNA primer and a DNA chain grafted onto its 3'-end. Paper chromatography or electrophoresis in several different systems served as a suitable means of ascertaining the composition of the ribomononucleotides.

MATERIALS AND METHODS

Materials. α-^{32}P-dNTP, 10 to 50 Ci/mmol were obtained from I.C.N. and N.E.N. and were routinely checked for purity in solvent systems A and B (described below); only products which were >85% pure were used. ^{3}H-uridine was purchased from N.E.N. All other materials were as described previously (8, 31).

Viruses. B77 virus stock was obtained from H. Temin. The virus was grown on primary cultures of fibroblasts, prepared from 12 day old white Leghorn chicken embryos (Sunnyside Hatchery Co., Oregon, Wis.). Cells were grown in medium 199 (Gibco) containing 20% tryptose phosphate broth, 10% calf serum, and 5% chicken serum. Four hours after seeding into roller culture bottles (1,410 cm^2; rotated at 0.2 rpm), the cells were infected with a multiplicity of 0.5 focus-forming units per cell. Five to 7 days after infection, the culture fluid was harvested once daily for several days. In the case of nonlabeled virus, plastic strips were added to the roller bottle to increase the total surface area (14).

MuLV was purified from culture fluid of RRTC cells. This cell line was established from a Rauscher virus-induced rat leukemia, grown in MEM (Gibco) containing 10% calf serum, 5% fetal calf serum, and supplemented with L-glutamine and nonessential amino acids (Gibco).

Labeling and purification of virus. Virus was labeled in complete medium containing 20 μCi/ml of uridine-5-^3H (12 Ci/mmol). Routinely, virus was harvested 24 h after the addition of labeled uridine. For the early harvest experiment, virus was harvested 24 h after the addition of ^{3}H-uridine (10 mCi/roller bottle containing 30 ml); virus was harvested at 5-min (or 3-h) intervals. Tissue culture fluid was centrifuged at 10,000 rpm for 10 min to remove whole cells and debris. Virus was concentrated from the supernatant by centrifugation at 25,000 rpm for 60 min in an SW27 rotor onto a 3-ml cushion of 65% sucrose in TNE buffer (0.01 M Tris-hydrochloride, 0.15 M NaCl, 0.001 M EDTA, pH 7.2). Virus was collected in 1 ml, diluted to 6 ml with TNE buffer and then was subjected to equilibrium density gradient centrifugation in 15 to 60% sucrose (wt/vol in TNE buffer) gradients (25,000 rpm for 3 h in an SW27 rotor). The banded virus from density gradient fractions was concentrated by sedimentation (25,000 rpm, 60 min in an SW27 rotor).

Early harvest virus was obtained essentially by the procedure of Cheung et al. (4). Because of the large volumes of tissue culture fluid, the early harvest virus was concentrated first by precipitation with ammonium sulfate (final concentration 30% wt/vol) and then purified as described above. Due to scarcity of material, protein concentration was not determined.

AMV was purified as described previously (8, 31).

Viral assays. B77 virus was titered by focus formation on chicken cells (26). MuLV infectivity was determined by the UV-XC test as described by Rowe (22); III6A cells (kindly provided by W. Rowe, TR810/58159 F 1701 III6AP$_{105}$) were used as indicator cells. A modification in this test was the use of polybrene (20 mg/ml) rather than DEAE-dextrane (20 mg/ml). Furthermore, the embryo cells were overlaid with XC cells 4 days after injection, rather than after 7 days (as described by Rowe).

For the determination of infectivity of early harvest virus, the test was modified as follows: after a 45-min adsorption period at 4 C, the cells were shifted to 37 C for 2 min and then washed two times with cold 0.25% trypsin solution. Complete medium at 37 C was then added. In the control, the wash was done before the temperature shift.

In order to determine the relative infectivity of virions from harvests at different time intervals, virus was grown in the presence of ^{3}H-uridine. The virions

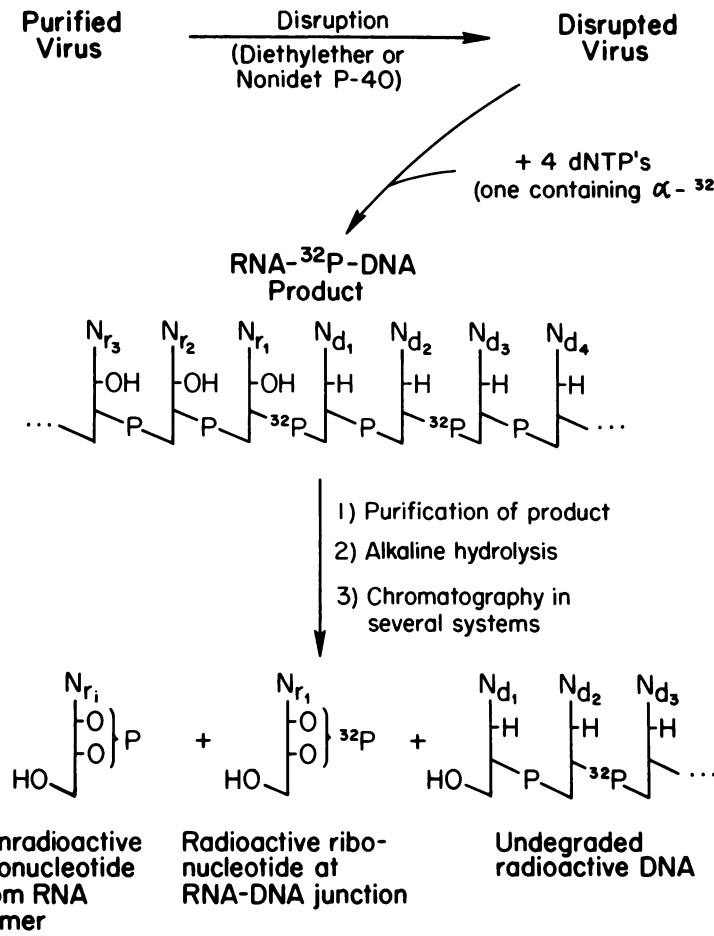

FIG. 1. *Outline of method used to elucidate the nucleotide sequence at the RNA-DNA covalent bond. Template strand is not shown.*

were purified by equilibrium density centrifugation; radioactivity in the peak area served as reference for the number of virions in the infectivity assay (Table 6).

Endogenous DNA polymerase reaction. All assays for AMV and B77 virus DNA polymerase were carried out as described before (8). MuLV DNA polymerase was assayed with the following reaction mixture (1.0-ml volume): Tris-hydrochloride (pH 8.3), 50 mM; $MgCl_2$, 5 mM; KCl, 45 mM; dithiothreitol DTT, 30 mM; $MnCl_2$, 2 mM; three nonradioactive dNTP's each at 10 to 20 μM and one α-^{32}P-dNTP (10^7 to 10×10^8 counts per min per reaction; 0.1 μM). Ether- or NP-40 disrupted virus (30 μg of protein/ml) was added, and the mixture was incubated at 35 C. Reactions were 10 min unless indicated otherwise.

Virus disruption. Ether disruption was carried out by adding an equal volume of diethylether to purified virus and the mixture was agitated on a Vortex mixer for 2 min at room temperature. The ether phase and gel-like interface was discarded. NP-40 was used at a final concentration of 0.1%.

Analysis of RNA-DNA products. The published procedure (8, 31) was used with the following modifications. After dialysis, the product was further purified from unpolymerized monomer by column chromatography on Sephadex G-75. The ^{32}P-polymer products were excluded from the column and checked for trichloroacetic acid-insolubility. At least 95% of the isotope was found to be insoluble; a control chromatogram to which was applied 10^4 to 10.0×10^4 counts/min was run routinely in system B (described below). In experiments in which α-^{32}P-dCTP or α-^{32}P-dGTP was the labeled substrate, a small amount of a fast moving lipid-nucleotide-protein complex appeared (9). In these cases, the labeled polynucleotide was purified from the complex by preparative paper chromatography in system B. The polynucleotide at the origin was eluted with a 50% ethanol-1% ammonium hydroxide solution; recovery was quantitative (>90%). The polymer material was subsequently treated with alkali (0.3 N NaOH) and analyzed as described previously (8). As an additional control, a sample of the polymer material, prior to alkaline

hydrolysis, was treated with pancreatic DNase (100 μg/ml) and venom phosphodiesterase (50 μg/ml). The polymer was totally (>99.95%) converted to mononucleotides in 75 min at 37 C by this treatment.

It is not possible to deduce the relative amounts of the transfers shown in Tables 1 to 5 since no effort was made to use labeled dNTP's at the same specific activity. To optimize the reliability of comparative data, reactions and product workups should be performed in parallel, as discussed previously (8). In addition, only radioactive dNTP's which have had their specific activity carefully determined can be used and internal standards should be employed, since these systems are known to contain nucleotides, phosphatases, nucleoside diphosphokinase, and other interfering factors (8). These considerations were not emphasized in these studies. Instead, various preparations of three different viruses were studied over a period of 18 months. In addition, efforts were made to definitively identify the ribomonophosphates formed after alkaline hydrolysis by employing several chromatographic systems which were chosen because of their ability to resolve the four rNp's and to resolve the appropriate deoxynucleotides from ribonucleotides.

The number average molecular weight of the DNA product which can be deduced from the nucleotide incorporation data and the amount of RNA-DNA junction is in agreement with our previous determination (8).

Chromatography. Descending paper chromatography was performed on Whatman no. 1 paper in the following systems: A: 0.1 M sodium phosphate (pH 6.8) (100 ml)-ammonium sulfate (60 g)-*n*-propanol (2 ml); B: isobutyric acid-concentrated NH_4OH-H_2O (66:1:33); C: *n*-propanol-concentrated HCl-H_2O (85:20:20). Electrophoresis was performed on Whatman no. 1 paper in the pyridine-acetic acid system at 3,000 V for 90 min (system D). Chromatograms were routinely developed for 15 to 24 h except for System C which was run for 45 to 48 h.

The relative mobilities of the mononucleotides of interest in the different systems are the following (increasing R_f values): system A, 3'-Ap, 2'-Ap, Gp, Up, Cp; system B, Gp, Up, Cp, Ap; system C, d-pA, Gp, Ap, Cp, d-pC, Up; system D, Cp, Ap, Gp, Up. Systems A, C, and D were used routinely and C was especially useful for separating deoxy- from ribomonophosphates. (The R_f values in A and C are shown in Fig. 3, 4 and 7.) System D mobilities were as described (30). System B was especially useful for separating Gp from Ap.

Between 800 and 10,000 counts/min were routinely applied to the chromatograms (or electropherograms). Since a charcoal adsorption step (8) was included in the workup procedure, most of this radioactivity was in mononucleotides and only a small amount was in polymer due to irreversible adsorption of the polymer to the charcoal (8). Recovery of mononucleotides was quantitative (>90%) in all cases.

The background levels of radioactivity presented in Tables 1 to 5 varied from 50 to 200 counts/min, depending on the amount of radioactivity applied to the chromatogram. The apparent high backgrounds shown on some tables are accentuated by the method of data presentation. Since chromatograms were cut into strips and counted by liquid scintillation, at times a flat tracing on the radiochromatogram scanner showed some counts per minute by the former method. Only well-distinguished peaks of isotope in more than one chromatography system are acknowledged as genuine transfers in Results.

Sucrose gradient centrifugation of viral RNA. Virus was purified as described above. Yeast RNA was added to a concentration of 125 μg/ml and SDS was added to a final concentration of 1%. The mixture was kept at 22 C for 30 min. The solution was then layered onto a 5 to 20% sucrose gradient (made up in TNE containing 0.2% SDS) and the gradient was centrifuged at 65,000 rpm in an SW65 rotor for 50 min at 15 C. Fractions were collected and radioactivity was determined as previously described (8).

RESULTS

Kinetics of DNA synthesis and RNA degradation. Before transfer experiments were performed with any virus preparation, pilot experiments were performed to determine the kinetics of DNA synthesis. A typical experiment is shown in Fig. 2 with B77 virus and similar results were found with MuLV and AMV preparations. DNA synthesis proceeded throughout the 60-min time course; each of the four nucleotides was incorporated as shown previously (8).

In the same time period, more than 75% of the ^{3}H-viral RNA was degraded. Although the assay for acid-insolubility which was used would not detect a small number of endonucleolytic cuts, no major breakdown of viral RNA was observed within the first 10 min under these conditions. Since this pattern of degradation was observed for all viral preparations tested, reactions were routinely stopped after 10 min in order to minimize the breakdown of viral RNA. Previous studies (7) showed that the presence of RNase or RNase H could influence the types of transfers observed if overt measures were taken to express these activities. However, under normal reaction conditions, such as reported herein, it was concluded that these potential interfering factors were latent. The high degree of specificity for the transfers supports this contention.

RNA-DNA junctions in MuLV. After we reported (8) the formation of the rU-dC and the rA-dA linkages in ether-disrupted AMV, several reports appeared (10, 21, 28) indicating that only an rA-dA bond was formed by NP-40-disrupted preparations of Schmidt-Ruppin strain of RSV, MuLV or AMV. To attempt to resolve this discrepancy, we performed the transfer study on other viruses which were disrupted with ether. Ether seems to be a suitable disrupting agent (13) since it allows

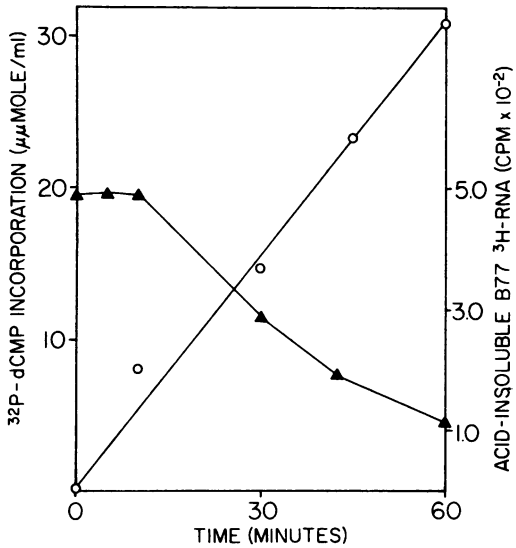

FIG. 2. *Kinetics of nucleotide incorporation and ^{3}H-RNA breakdown for endogenous reaction by disrupted B77 virions. Symbols:* O, *α-^{32}P-dCTP as substrate for DNA polymerase;* ▲, *^{3}H-uridine-labeled B77 virus RNA as substrate for degradative activities. The latter activity was assayed as described previously (19).*

expression of at least as much DNA polymerase activity as NP-40 disruption (7).

MuLV was freshly prepared, purified by ultracentrifugation and disrupted with ether. A complete transfer study was then performed as outlined in Fig. 1. When the α-^{32}P-dNTP substrate was dCTP, virtually all of the radioactivity was transferred to the 2′ and 3′ isomers of rCMP (Fig. 3). That this labeled product was not rUMP or dCMP was conclusively shown by chromatography of other samples in system D (Table 1) and system C. dCMP and rCMP were well separated (results not shown) in the latter system; hence this "transfer" cannot be due to trace contamination of the reaction product with dCMP. Thus, for ether-disrupted MuLV, an rC-dC linkage was formed instead of the rU-dC linkage found in ether-disrupted AMV (8).

When the label was in α-^{32}P-dATP initially, a double peak of radioactivity is observed on the paper chromatogram (Fig. 4) which comigrates with the 2′- and 3′-rAMP markers. Moreover, the ^{32}P activity was also found to comigrate with 2′ (3′) rAMP in an electropherogram (Table 1). No transfers to any ribomonophosphates were found with either α-^{32}P-dGTP or with α-^{32}P-dTTP although extensive incorporation of ^{32}P activity into newly synthesized DNA was observed (results not shown). We conclude therefore that during the endogenous reaction of mature MuLV (disrupted with ether), an rA-dA bond also is formed. This result can be compared with the report by Gilden et al. (21) of an rA-dA linkage in NP-40-disrupted RLV particles. Hence, the MuLV system is similar to the AMV system since both ribopyrimidine-dC and rA-dA bonds are found after ether disruption and only the rA-dA bond is found after NP-40 disruption.

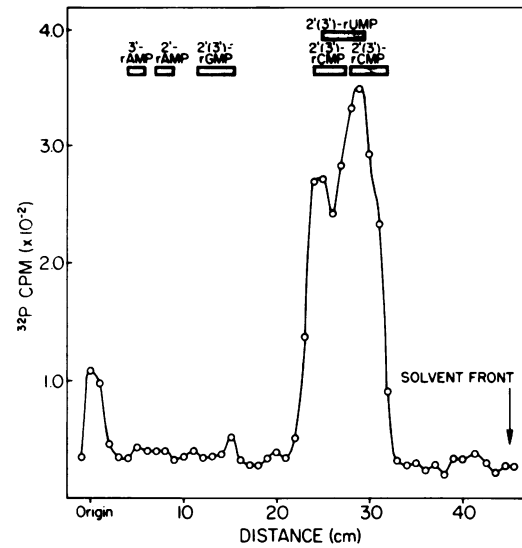

FIG. 3. *Radiochromatogram of alkaline hydrolysate of reaction product formed with α-^{32}P-dCTP substrate in ether-disrupted MuLV. Chromatography was in system A.*

TABLE 1. *Distribution of radioactivity after alkaline hydrolysis of products of MuLV DNA polymerase endogenous reaction*[a]

α-^{32}P-dNTP substrate	Counts per min in 2′ (3′)-ribomonophosphates				Chromatography system
	rAMP	rGMP	rUMP	rCMP	
dCTP[b]	60	30	80	1,710	D
dATP	5,820	70	60	50	D
dGTP	10	30	50	70	A
dTTP	20	40	30	50	D

[a] Purified virus was disrupted with ether for 5 min. A 10-min endogenous reaction was performed at 35 C. Product workup included a charcoal adsorption step. Other details are in Materials and Methods.

[b] The data shown for transfers when dCTP was the labeled substrate are typical of three separate determinations performed at different times over a 5-month period. However, in a recent single experiment, small amounts of transfers from dCMP to rUp and rAp (17 and 8%, respectively, of dC→rC transfer) were found.

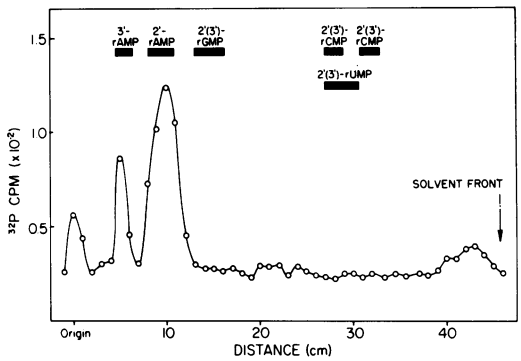

FIG. 4. *Radiochromatogram of alkaline hydrolysate of reaction product formed with α-³²P-dATP substrate in ether-disrupted MuLV. Chromatography was in system A. The small amount of radioactivity near the solvent front is Pi which is generated occasionally during analyses as found previously (9).*

RNA-DNA junctions in B77 virus. B77 virus was also studied as outlined in Fig. 1 to determine if the RNA-DNA bonds formed were influenced by the method of disruption, as for RLV and for AMV. When the radioactive dNTP was dCTP, the ^{32}P was transferred to rCp (Table 2) when the virus was disrupted with ether. This same result was found when another sample was analyzed in system A (results not shown). However, when the particles were disrupted with NP-40, no appreciable transfer was observed from dCTP to any ribonucleotide (Table 2) even though α-^{32}P-dCMP was readily incorporated into acid-insoluble polymeric product.

When α-^{32}P-dATP was the sole radioactive substrate, an rA-dA covalent bond was formed by ether disrupted virions of B77 virus (Table 2); this result was verified by electrophoretic analysis in system D. Neither the dC → rC nor the dA → rA transfer can be attributed to trace contamination with the deoxyribonucleotide since chromatography systems were used which resolved these molecules. No transfer occurred after alkaline hydrolysis from either α-^{32}P-dGTP, and in a separate experiment, from α-^{32}P-dTTP (Table 2).

We conclude, therefore, that only rA-dA and rC-dC bonds are formed during the endogenous reaction of ether-disrupted virions of B77 virus. However, the transfer from dCMP to rCp is not found when NP-40 is the disrupting agent but only the dAMP to rAp transfer is observed (also found by M. Bishop, personal communication). Thus, for three different viruses, rC-dC (or rU-dC) and rA-dA bonds are found for ether-disrupted virions, whereas only rA-dA bonds are found for NP-40-disrupted virions.

Core particles of B77 virus were prepared and transfer experiments were performed to ascertain if the RNA molecules that function as primers with disrupted virions in fact reside in the viral nucleoid. The cores (1) had the expected density (1.25 to 1.26 g/cm³) in a sucrose gradient and they were highly active in incorporating dNTP's into DNA (15 pmol of ^{3}H-dAMP per ml per 30 min). The transfer experiments proved that only dA → rA transfer was observed. No more, or fewer, transfers were found. Since cores were produced by the combined treatment with ether and NP-40 (1), it was not possible to assay for the rC-dC bond. Hence, the RNA primer for the rA-dA bond is an integral part (5) of the B77 viral nucleoid.

Effect of disruption technique on RNA-DNA bonds. The studies described above show that the types of RNA primers which are utilized by the DNA polymerases are affected by the method of virus disruption (ether versus NP-40). To clarify these differences, AMV was extracted first with ether, and then NP-40 (to 0.1%) was added to the aqueous layer. The endogenous reaction was then performed with this "doubly disrupted" preparation and a transfer experiment was performed on the product of this reaction (Table 3). Two radioactive nucleoside triphosphates (α-^{32}P-dATP and α-^{32}P-dCTP) were present simultaneously in this experiment; the other triphosphates were added but were nonradioactive.

A transfer to rAp (presumably an rA-dA bond) is still formed as expected. However, only negligible amounts of ^{32}P activity are found in the rUMP and rCMP regions (definite peaks of radioactivity were not observed). Another sample was analyzed in system A (results not shown) where virtually all (>80%) of the iso-

TABLE 2. *Distribution of radioactivity after alkaline hydrolysis of products of B77 virus DNA polymerase endogenous reaction*[a]

α-³²P-dNTP substrate	Disruption method	Counts per min in 2' (3')-ribomonophosphates				Chromatography system
		rAMP	rGMP	rUMP	rCMP	
dCTP	ether	120	40	70	790	D
dCTP	NP-40	90	20	120	90	C
dATP	ether	730	30	140	30	A
dGTP	ether	10	20	30	60	A
dTTP	ether	70	20	40	40	A

[a] Purified virus was disrupted with ether for 1 min at room temperature or with NP-40 for 15 min at 0 C. An endogenous reaction was then carried out for 10 min at 37 C. Other details are in Materials and Methods.

TABLE 3. *Distribution of radioactivity after alkaline hydrolysis of products of AMV DNA polymerase endogenous reaction: ether and NP-40 disruption*[a]

α-^{32}P-dNTP substrate	Counts per min in 2′ (3′)-ribomonophosphates				Chromatography system
	rAMP	rGMP	rUMP	rCMP	
dATP + dCTP	890	20	100	130	C

[a] Purified virus was disrupted with ether for 1 min at room temperature; the interface and ether layer were discarded and residual ether was removed from the aqueous phase by bubbling N_2 through the solution. The solution was then made 0.3% in NP-40 and kept at 0 C for 15 min. A 10-min endogenous reaction was then performed at 37 C. Other details are in Materials and Methods. All samples were desalted with charcoal prior to chromatography.

tope was found in rAp. Previous studies (8) have clearly demonstrated the presence of the dC → rU transfer in AMV which is disrupted with only ether. In fact, the dC → rU transfer was the predominant RNA-DNA linkage. Thus, the transfer from dC to rUp is abolished by the addition of the detergent.

Hence, this study buttresses the contention (7) that either NP-40 treatment causes inactivation of the primer with the 3′-ribopyrimidine or it activates some agent (such as an enzyme) which is deleterious to the dC → ribopyrimidine transfer.

Possible effect of actinomycin D on formation of RNA-DNA bonds. The presence of actinomycin D (AM) in either an endogenous reaction or in a purified system allows the formation of only single-stranded DNA; only a small amount of double-stranded DNA product is synthesized (10, 23, 30). In addition, the DNA product formed in the presence of AM is a relatively complete copy of the viral RNA (10). In contrast, the DNA product formed when AM is not added to the endogenous reaction is a preferential copy of a limited region of the viral genome. The single-stranded DNA which is produced in the presence of the antibiotic apparently is still of low molecular weight.

Since a larger amount of the RNA template is transcribed in the presence of AM but the DNA product is the same size in its presence or absence, it was significant to determine if new RNA-DNA bonds were found in the presence of AM. Preliminary kinetic experiments in the presence of varying amounts of AM (from 78 to 312 μg/ml) showed extents of inhibition similar to that reported previously (23). The reaction products of the ether-disrupted AMV in the presence of 119 μg of AM per ml were purified and analyzed as described above. α-^{32}P-dATP and α-^{32}P-dCTP were the labeled substrates tested (in separate experiments). As observed in the absence of AM, an rA-dA and an rU-dC bond were found in the presence of the antibiotic (Table 4). Thus, no new RNA-DNA junctions were observed; likewise, neither of the RNA-DNA linkages were lost.

RNA-DNA bonds formed by early harvest virus. It was reported recently (3, 4, 6) that the RNA from rapid harvest (3 to 30 min) RNA tumor viruses is somewhat smaller than the RNA from mature virions. Also, the RNA is appreciably more homogeneous than the RNA isolated from mature virions. We performed isotope transfer experiments with early harvest MuLV to determine if the RNA-DNA linkages formed in their endogenous reaction differ from those observed in mature virus.

RNA was extracted from ^{3}H-uridine-labeled early harvest virus and banded on a sucrose gradient (Fig. 5). The homogeneity of this RNA, compared to the RNA from 24-h virions, is apparent and the slightly smaller size of the early harvest RNA was also observed, as reported previously. Thus it is clear that these virions are similar to those described previously.

Figure 6 shows the kinetics of incorporation of dNMP into DNA during the endogenous reaction in early harvest virions. All four nucleotides are incorporated readily; reasons for the variable extents of incorporation were discussed previously (8).

Table 5 shows the results of the transfer experiments. As shown above (Table 1) with mature virions of MuLV, a transfer from dCMP to rCp is observed. Figure 7a shows a typical chromatographic analysis in another solvent system. The 450 counts/min in Table 5, line 1, suggesting a small amount of rA-dC bond, is not

TABLE 4. *Distribution of radioactivity after alkaline hydrolysis of products of AMV DNA polymerase endogenous reaction in presence of actinomycin D*[a]

α-^{32}P-dNTP substrate	Counts per min in 2′ (3′)-ribomonophosphates				Chromatography system
	rAMP	rGMP	rUMP	rCMP	
dCTP	90	30	380	30	D
dATP	1,290	80	130	120	A

[a] AMV was disrupted with ether for 1 min; 119 μg of actinomycin D per ml were added to the incubation mixture. The endogenous reaction was for 10 min at 37 C. Product workup included a charcoal adsorption step. Other details are as described in Materials and Methods. dGTP and dTTP were not tested as labeled substrates.

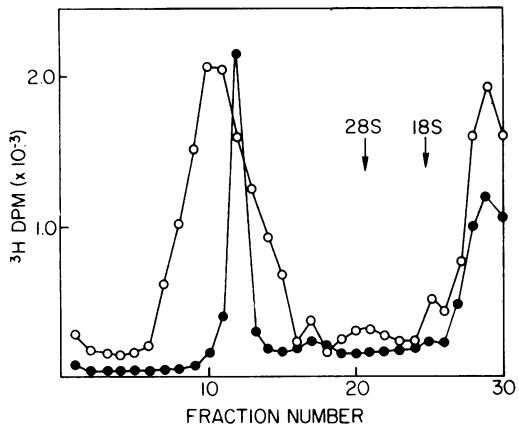

FIG. 5. *Analysis of viral RNA by sucrose gradient centrifugation. ^{3}H-labeled virus was disrupted and analyzed as described in Materials and Methods. Symbols: O, 24-h harvest; ●, 5-min harvest.*

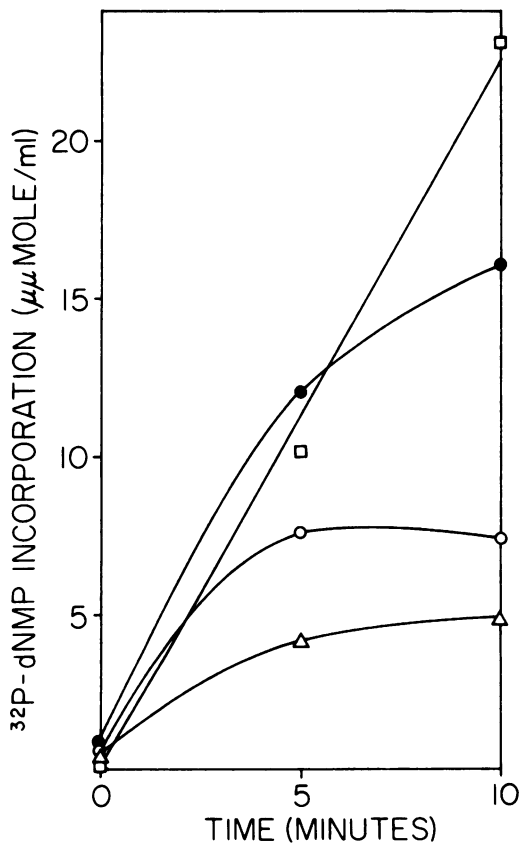

FIG. 6. *Kinetics of nucleotide incorporation for endogenous reaction by early harvest (5 min) MuLV. All four dNTP's were present in all reactions. Virions were ether-extracted for 5 min. Symbols: △, α-^{32}P-dATP; O, α-^{32}P-dTTP; ●, α-^{32}P-dCTP; □, α-^{32}P-dGTP.*

considered significant since this transfer was not found in two other experiments.

However, the results found when α-^{32}P-dATP was the substrate for the early harvest virions contrast strikingly with the results with mature virions. Table 5 and Fig. 7b show that the dA → rA transfer found with mature virions (Table 1) is absent for the early harvest virus. This result was found for determinations with three different preparations of early harvest MuLV and in all chromatography systems employed. Any small amounts of apparent transfers shown in Table 5 or Fig. 7b are not significant since they were not observed when identical samples were analyzed in other systems. Analyses were also performed with dGTP and with dTTP as labeled substrates; as with mature virions no transfers were found.

Infectivity of early harvest MuLV. The relative infectivity of particles harvested at different times after infection was determined. Table 6 shows that the 5-min virions are appreciably more infectious than the 24-h virions. The relative infectivity of 3-h virions was approximately the same (1.3×10^3) as found for the 5-min virus. Hence, to perform studies on virus populations with a maximally high percentage of biologically active particles, early harvest virions must be employed. These early harvest particles apparently are not "immature" with respect to infectivity.

DISCUSSION

Summary of transfers. These studies were aimed at providing biochemical information on the biologically significant RNA primer(s) for DNA synthesis. Since the initiation of DNA synthesis is probably a key event in the oncogenic capacity of RNA tumor viruses, studies

TABLE 5. *Distribution of radioactivity after alkaline hydrolysis of products of early harvest (5 min) MuLV DNA polymerase endogenous reaction*[a]

α-^{32}P-dNTP substrate	Counts per min in 2′ (3′)-ribomonophosphates				Chromatography system
	rAMP	rGMP	rUMP	rCMP	
dCTP	450	150	110	5,870	D
dATP	50	50	230	120	C
dGTP	60	110	80	70	A
dTTP	60	40	60	90	A

[a] Immature MuLV particles were disrupted with ether for 5 min at room temperature and the endogenous DNA polymerase reaction was carried out at 35 C for 10 min. Other details are in Materials and Methods. The workup of all products after alkaline hydrolysis included a charcoal adsorption step.

were focused on factors which could influence the RNA-DNA linkages between RNA primers and nascent DNA chains.

Table 7 presents the available information on RNA-DNA junctions formed by RNA tumor virus DNA polymerases. Both ribopyrimidine-dC and rA-dA linkages are formed by ether-disrupted virions, whereas only rA-dA bonds are formed by NP-40 disrupted virions or purified systems. These results suggest that the same type of RNA molecules serve as primers for all four viruses and indicates a high degree of molecular homogeneity of the primers, at least as regards the 3'-ends. AMV is a mixture of viruses (20); however, it is apparent that this does not endow it with more or fewer initiation events than found for the purer viruses.

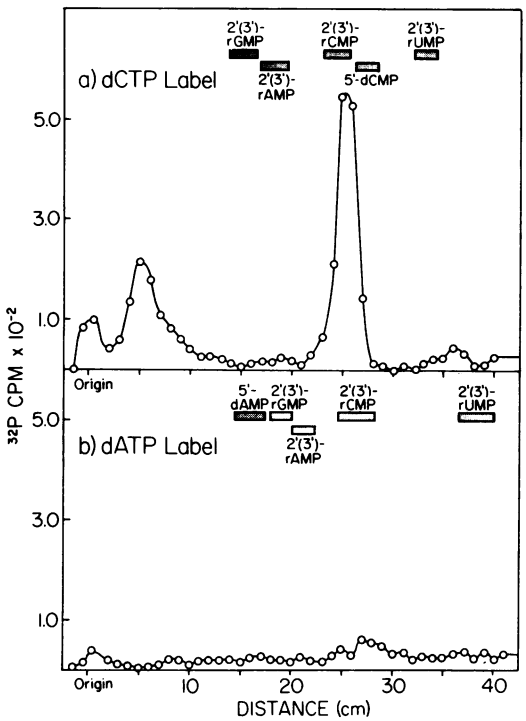

FIG. 7. *Radiochromatograms of alkaline hydrolysates of reaction products formed with early harvest (5 min) MuLV. Chromatography was in system C. The small peak of radioactivity close to the origin in panel a presumably consists of deoxyribooligonucleotides since this system is run for 48 h. Since less nucleotide incorporation is found in immature virions, it is possible that shorter DNAs are formed. Panel a: α-32P-dCTP substrate, panel b: α-32P-dATP substrate.*

Transfers with early harvest virions. Since we consider the endogenous system to be capable of providing biologically useful information for these types of studies (see below), variations of this system were investigated in which it was anticipated that the viral RNA would be in its most native and least degraded state. Studies were performed with early harvest particles of MuLV since their RNA is more homogeneous (3, 4) than for mature virus. Unexpectedly, only the rC-dC linkage was found in immature MuLV; the rA-dA bond was not observed. This observation is important from a biochemical standpoint, since the early harvest virus RNA is much less heterogeneous in size than the mature virus RNA and hence is apparently less degraded. A highly nicked (or gapped) molecule is a superior template-primer to a more intact molecule (11, 12, 31), since there are more potential primer sites. Hence, fewer transfers would be expected in the more homogeneous system (early harvest particles).

Since this more homogeneous system does not form the rA-dA bond, and since a higher percentage of rapid harvest virions are infectious, it can be concluded that the rC-dC bond is the

TABLE 6. *Relative infectivity of MuLV harvested after different time intervals*

Time of harvest	PFU/10³ dpm
5 min[a]	4.8 × 10³
24 h	2.4 × 10²

[a] Control: wash before temperature shift; 56 PFU/10³ dpm.

TABLE 7. *Summary of RNA-DNA covalent linkages*

Virus	Endogenous system		Purified system
	Disruption method		70S RNA + purified DNA polymerase
	Ether	NP-40	
Avian myeloblastosis virus	rU-dC and rA-dA (9)	rA-dA (30, 8)	rA-dA (30)
Rous sarcoma virus (Schmidt-Ruppin)	ND[a]	rA-dA (27)	rA-dA (27)
B77 virus	rC-dC and rA-dA (RH)	rA-dA (RH)	ND
Rauscher leukemia virus	rC-dC and rA-dA (RH)	rA-dA (22)	ND

[a] ND, not determined; RH, reported herein; references are in brackets.

more important initiation event. However, it is conceivable that the RNA with the 3'-adenosine is "developed" during the conversion of homogeneous to heterogeneous subunit RNA (4) and that this primer is of no less importance from a biological standpoint. It will be of interest to understand the initiation event with NP-40-disrupted early harvest virus.

These data do not prove that either of these types of transfers reflect the biologically significant initiation event. Even for the rapid-harvest virions, only a small proportion of all virus particles are infectious. The noninfectious particles may also contain competent template-primers for DNA synthesis. Further work is necessary to elucidate the biological relevance of these findings.

This change in the RNA primer is the third difference noted between early harvest and mature virus. The other two are size and homogeneity of RNA (3, 4, 6) and polypeptide constitution (4). The apparent differences (4) in templating activity between 30 to 40*S* RNA from immature and mature RSV are, no doubt, due to the integrity of the RNA from the early-harvest virions. The less nicked molecule is an inferior template-primer to the more degraded molecule (11, 12, 31). The same explanation probably holds for the differences observed for 5-min and 24-h virus when exogenous DNA was added as a template-primer (4). Since the immature RNA is in a more native form (less nicked), addition of a nicked exogenous molecule causes a marked stimulation (4) in the observed DNA polymerase reaction.

Influence of disruption method. Pronounced differences in initiation events are found (Table 7) as a function of the method of virion disruption. Specifically, both rpyr-dC and rA-dA bonds are found when ether extraction is the disrupting agent, whereas only the rA-dA bond is formed when NP-40 is used instead of ether. Studies with virions which were treated first with ether and then NP-40 show that the detergent disallows the rpyr-dC bond by either inactivating the primer, perhaps by denaturing it from its template (a recognized effect of detergents), or by activating some deleterious agent. Previous studies (7) have shown that ether is an appropriate agent for expressing DNA metabolizing enzymes since virions disrupted by the detergent, but not by ether, contain a potent phosphatase which can destroy dNTP's. A number of other significant differences between these two systems may exist and are currently under study.

Transfers in presence of AM. It is believed that most or all of the significant RNA-DNA linkages have been recognized (Table 7) even though only a small portion of the viral genome is replicated into DNA product (10). The reason for this belief is that the same bonds are observed in the presence and absence of actinomycin D, an agent that promotes the synthesis of single-stranded DNA which is almost a complete copy of viral RNA (10). The molecular basis for this effect by AM is obscure (reviewed in 32).

General comments on systems. An ideal system for identifying the primer for DNA synthesis is difficult to devise. Utilization of a highly purified DNA polymerase and 70*S* viral RNA might seem a logical choice. However, it is likely that proteins (enzymes) other than the DNA polymerase are involved in DNA synthesis in vivo; examples are DNA ligase (19), a stimulatory protein (15), and other activities (7, 18). If only the DNA polymerase is added to the reaction, only a partial answer may be obtained. Also, utilization of only the 70*S* component as template and primer means that an appreciable fraction of the total viral RNA is not analyzed for its priming ability. On the other hand, if disrupted virus preparations (endogenous reaction) are employed, these problems are circumvented and it is conceivable that this in situ system may maintain the in vivo significant primer-template relationship. However, this system also has certain inherent disadvantages such as the potential presence of deleterious factors such as nucleases. Nevertheless, we have decided to focus on the endogenous system instead of the purified system, and to attempt to assay for the presence or effect of possible interfering factors such as RNases or RNase H (7). It was concluded that these activities did not influence the types of RNA-DNA bonds formed under normal reaction conditions. This entire study presupposes that the biologically significant primer for DNA synthesis is contained in the virus, which may be unwarranted.

In summary, neither system is ideal. However, it is apparent that a biased answer may be obtained if a highly purified system is used, whereas it is possible that an unbiased answer can be found with the less pure system which more closely mimics the in vivo situation. That a less than completely purified system is necessary to obtain biologically meaningful information has been recognized and widely exploited by laboratories studying bacterial DNA replication (R. D. Wells and R. B. Inman, in press).

Comparison with other transfer studies. It may, or may not, be significant that similar types of RNA-DNA linkages have been found in other systems. Sugino and Okazaki (24 and

personal communication) have shown recently in permeabilized *E. coli* that discontinuous DNA fragments are linked to RNA through both rU-dC or rC-dC linkages and that the rC-dC bond is preferred in intact *E. coli*. Moreover, Reichard and co-workers (17), studying nuclei of polyoma infected cells, have shown that newly synthesized DNA is joined to RNA fragments through an rX-dC linkage where X is any of the four ribonucleotides. Thus, on the basis of these studies (one in a bacterial system and one in a DNA virus-infected animal system) and the results with four different RNA tumor viruses (Table 7), it is tempting to speculate on the universality of the initiation event.

ACKNOWLEDGMENTS

This investigation was supported by funds from the National Cancer Institute (NO 1-CM-12275) and the National Science Foundation (GB-30528X) to R. D. W. and a N.I.H. Research Grant (CA-07175) to McArdle Laboratory, University of Wisconsin-Madison.

We thank R. C. Nowinski for support and advice, J. and D. Beard for generously providing AMV, D. Bolognesi for help in preparing labeled RNA, P. F. Schendel for helpful suggestions, and J. Dahlberg, R. Nowinski, and H. Temin for useful criticism of the manuscript.

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