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Covalent Linkage Between Ribonucleic Acid Primer and Deoxyribonucleic Acid Product of the Avian Myeloblastosis Virus Deoxyribonucleic Acid Polymerase

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Initiation of deoxyribonucleic acid (DNA) synthesis by the avian myeloblastosis virus DNA polymerase was previously suggested to involve a ribonucleic acid (RNA) primer, the initial product being a DNA molecule joined by a phosphodiester bond to the RNA primer. The existence and nature of such an RNA-DNA joint was investigated by assaying for transfer of a ^{32}P atom from an α-^{32}P-deoxyribonucleotide to a 2'(3')-ribonucleotide after alkaline hydrolysis of the polymerase product. Such a transfer was observed, but only from α-^{32}P-deoxyadenosine triphosphate and only to 2'(3')-adenosine monophosphate. This same transfer was observed in both the endogenous DNA polymerase reaction of purified virions and the reconstructed reaction of purified DNA polymerase plus purified 60 to 70*S* viral RNA. These results indicate a high level of specificity for the initiation process and support the idea of a low-molecular-weight initiator RNA as part of the 60 to 70*S* RNA complex.

Virions of ribonucleic acid (RNA) tumor viruses contain a deoxyribonucleic acid (DNA) polymerase which will synthesize a faithful copy of viral RNA (1, 6, 31). The enzyme is readily solubilized and purified from the virions and will utilize a wide variety of polymers as templates (3, 7, 12, 14, 16, 24, 27, 32, 34). Like other known DNA polymerases, the RNA tumor virus DNA polymerase is apparently unable to initiate polymerization of deoxyribonucleotides de novo on a single-stranded template but requires a primer containing a 3'-OH (3, 25). The primer is physically incorporated at the 5' end of the product (25). Properties of the virion DNA polymerase have been reviewed recently (Temin and Baltimore, Advan. Virus Res., *in press*).

With detergent-treated virions, the initial reaction product formed when the viral DNA polymerase copies the endogenous viral RNA is DNA attached to the 60 to 70*S* RNA (10, 20, 23, 26). The DNA product can be released from most of the viral RNA by methods which disrupt hydrogen bonds, but analysis of the released product in Cs_2SO_4 buoyant density gradients has indicated that it is a covalently linked RNA-DNA molecule (19, 32). This result suggests that the primer for endogenous DNA synthesis might be an RNA molecule. When purified DNA polymerase is used to copy 60 to 70*S* RNA in a "reconstructed system," an apparent covalent RNA-DNA molecule is also formed (32).

In the present study, we have examined the formation of RNA-DNA covalent joints by performing isotope transfer experiments (15). The rationale behind these experiments is described in Fig. 1. If there is an RNA primer to which the DNA is attached, the 3'-OH end of the RNA primer will enter into a phosphodiester bond with the first deoxyribonucleotide, and then further polymerization of deoxyribonucleotides will occur. If the α-phosphate of the deoxyribonucleotide triphosphate is labeled with ^{32}P, alkaline hydrolysis of the RNA-DNA joint should release one or more ^{32}P-containing 2'(3')-ribomononucleotides. Evidence for such a covalent bond has been obtained in these experiments, and its composition has been established to be $\cdots$pApdA$\cdots$ in both the endogenous reaction and a reconstructed system consisting of purified avian myeloblastosis virus (AMV) DNA polymerase and 60 to 70*S* AMV RNA. Flügel and Wells (9) recently published evidence for an RNA-DNA covalent joint, but they concluded that the major joint is $\cdots$pUpdC$\cdots$ and that the $\cdots$pApdA$\cdots$ is a minor component.

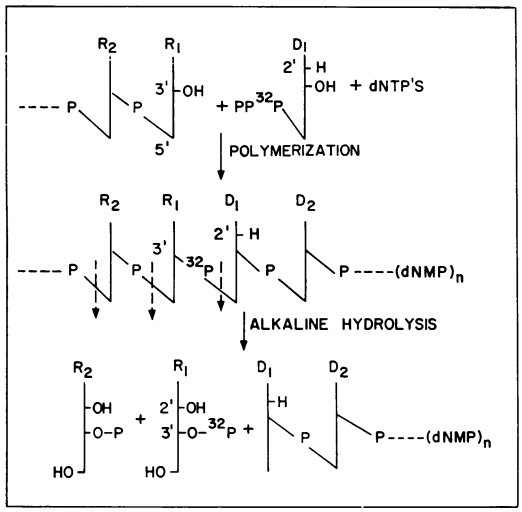

FIG. 1. *Schematic representation of the transfer of a ^{32}P atom from the α position of a deoxyribonucleotide to the 2'(3') position on a ribonucleotide. R_2 and R_1 represent penultimate and ultimate ribonucleotides of the presumed RNA primer. D_1 and D_2 represent the initial two deoxyribonucleotides incorporated. The final ribonucleotide products will be either 2' or 3', but only 3' is indicated.*

MATERIALS AND METHODS

Materials. α-^{32}P-deoxyribonucleoside triphosphates were purchased from International Chemical and Nuclear, Inc., Irvine, Calif. The α-^{32}P deoxyadenosine triphosphate (dATP) was 95 to 98% pure by electrophoretic analysis in our laboratory. The impurities appear as deoxyadenosine diphosphate and monophosphate (dADP and dAMP). ^{3}H-labeled deoxyribonucleoside triphosphates were purchased from New England Nuclear Corp., Boston, Mass. The AMV was generously supplied by J. Beard. Preparation of purified virions, AMV DNA polymerase, and 60 to 70S AMV RNA was carried out as described earlier (3, 32).

Preparation of products: (i) reconstructed system. The product was synthesized by use of purified 60 to 70S RNA and purified AMV DNA polymerase. A typical reaction mixture of 0.2 ml consisted of the following components: 50 mM tris(hydroxymethyl)-aminomethane-hydrochloride (*p*H 8.3), 10 mM dithiothreitol, 6 mM magnesium acetate, 60 mM sodium chloride, 1 mM unlabeled deoxyribonucleoside triphosphates, α-^{32}P-labeled deoxyribonucleoside triphosphates as specified in each experiment, 1 to 2 μg of 60 to 70S viral RNA, and 0.2–0.5 μg of AMV DNA polymerase.

(ii) **Endogenous reaction.** The same reaction mixture was used as for the reconstructed system except that 0.2% Nonidet P-40 was added and purified virions were used in place of the AMV polymerase and viral RNA. From 0.65 to 1.3 mg of viral protein was used per assay except for deoxycytidine triphosphate (dCTP) incorporation, in which case 65 μg was used because larger amounts were inhibitory to incorporation.

All reactions were carried out in a nitrogen atmosphere and incubated at 37 C for 60 min, unless otherwise specified. The reaction was stopped by adding sarkosyl to a final concentration of 1.5%. The reaction product was then separated from unincorporated substrates by chromatography on G-50 Sephadex. The peak fractions of excluded material were combined and lyophilized.

For the endogenous reaction, the product at the end of the reaction was extracted with a mixture of an equal volume of phenol and chloroform plus 1% isoamyl alcohol. After shaking for 5 min at room temperature, the mixture was centrifuged at 3,500 rev/min for 10 min in a PR-6 International centrifuge. The aqueous phase was reextracted with phenol-chloroform, and the product was then extracted twice with an equal volume of ether to remove the phenol. Ether was removed by bubbling nitrogen gas through the solution. The recovery of the product was over 50%. The product was then chromatographed on G-50 Sephadex and lyophilized.

Electrophoresis. Products were hydrolyzed with 0.3 N KOH by incubation at 37 C for 18 hr. They were neutralized with 20% perchloric acid, and the precipitate was discarded by centrifugation. The recovery was 70 to 90% of the radioactivity. The supernatant fluid was analyzed further by paper electrophoresis.

Samples of 50 to 100 μliters were spotted on a 56-cm long strip of Whatman no. 1 paper. To each sample was added a mixture (about 15 to 20 μg of each) of 2'(3')-adenosine, cytidine, uridine, and guanosine monophosphates (AMP, CMP, UMP, and GMP) as markers. Electrophoresis was carried out in precooled Savant tanks in pyridine-acetate buffer, *p*H 3.5 (30 ml of pyridine and 300 ml of glacial acetic acid made up to 6 liters with water), for 3 hr at 3,000 v. At the end of the electrophoresis, the paper was dried in a warm stream of air and then placed in a tank saturated with ammonia vapors to neutralize the pyridine in order to reduce its absorption of ultraviolet light. The paper was dried in air again; it was then scanned under ultraviolet light and the absorbing spots were marked. Strips of the paper 1 cm wide were cut and counted in scintillation fluid (4 g of Omnifluor/liter of toluene) in a Beckman scintillation counter. In a typical experiment, between 5,000 and 10,000 counts per min of product were analyzed. The background was 20 to 30 counts per min.

RESULTS

Incorporation of each deoxynucleotide. Four reaction mixtures of the reconstructed system containing all four deoxyribonucleoside triphosphates, with one labeled, were incubated for 90 min to investigate whether all of the four substrates were incorporated and whether they appeared in equimolar amounts. The results (Table 1) indicated incorporation of all four substrates and that the DNA contained an excess of deoxyguanosine monophosphate (dGMP) and only 14% dAMP.

α-^{32}P-nucleotide transfers. Each of the four α-^{32}P-deoxyribonucleoside triphosphates was incubated separately with both the reconstructed and the endogenous systems, and the products were purified. Alkaline hydrolysis of these products followed by electrophoresis yielded detectable radioactivity migrating from the origin of the electropherogram only if α-^{32}P-dATP was the labeled substrate (Table 2 and Fig. 2). About 11 to 15% of the total radioactivity in the product of the reconstructed system and 19 to 23% in the endogenous reaction appeared coincident with the marker spot of 2'(3')-AMP.

TABLE 1. *Extent of incorporation of the four deoxyribonucleotides*[a]

Labeled substrate	Deoxynucleotide monophosphate incorporation (pmoles)
dATP	14.3
dCTP	28.0
dTTP	16.0
dGTP	42.0

[a] Standard reaction mixtures contained (in 0.1 ml) 60 nmoles of each of three unlabeled deoxyribonucleoside triphosphates. ^{3}H-labeled deoxyribonucleoside triphosphates used were as follows: deoxyadenosine triphosphate (dATP), 0.23 nmoles (3,500 counts per min per pmole); deoxycytidine triphosphate (dCTP), 0.16 nmole (5,000 counts per min per pmole); deoxythymidine triphosphate (dTTP), 0.16 nmole (5,000 counts per min per pmole); deoxyguanosine triphosphate (dGTP), 0.2 nmole (4,000 counts per min per pmole). Avian myeloblastosis virus DNA polymerase used was 0.3 μg. The amount of 60 to 70*S* RNA was 2,000 pmoles of nucleotides. Incubation was carried out for 90 min at 37 C. The product was precipitated, collected on filters, and counted as described (2).

To investigate whether the radioactivity in the 2'(3')-AMP region arose from a ribonucleotide-deoxyribonucleotide joint in the product, two analyses were performed. First, unhydrolyzed α-^{32}P-dAMP-labeled product was submitted to electrophoresis. No radioactivity was observed to leave the origin. Second, the possibility of 5'-^{32}P-dAMP being carried over and released by hydrolysis was examined by extending the time of electrophoresis sufficiently to separate 5'-dAMP from 2'-AMP and 3'-AMP. Figure 3 shows that after extended electrophoresis the ^{32}P derived from the polymerase product was well separated from marker 5'-^{3}H-dAMP and was exactly coincident with marker 2'-AMP and 3'-AMP.

Kinetics of initiation. Figure 4 shows that, although incorporation of α-^{32}P-dAMP in the reconstructed system was continuous during 90 min of incubation, the percent transfer of radioactivity to 2'(3')-AMP remained constant after about 15 min of incubation. Similar results were obtained with the endogenous reaction. These results suggest that initiation of new chains occurs throughout the period of reaction. The ratio of total incorporation to radioactivity in 2'(3')-AMP suggests that the average size of the DNA chains synthesized is about 35 to 70 nucleotides (*see below*). Size determination of the product on neutral or alkaline sucrose gradients (not shown here) indicated that the majority of the product is never larger than 4*S* from 15 to 60 min of incubation.

Other transfers. The transfer experiment has been repeated numerous times with all four labeled deoxyribonucleoside triphosphates. The transfer from α-^{32}P-dATP to ^{32}P-2'(3')-AMP was always observed. Flügel and Wells (9) have reported transfer from α-^{32}P-dCTP to 2'(3')-UMP, and we have occasionally seen an apparent transfer from α-^{32}P-dCTP to ^{32}P-2'(3')-CMP and

TABLE 2. *Transfer of ^{32}P to ribonucleotides*[a]

Nature of reaction	Substrate	Percent recovered in			
		CMP	AMP	GMP	UMP
AMV virions (endogenous system)	α-^{32}P-dCTP	<1	<1	<1	<1
	α-^{32}P-dATP	<1	19–23	<1	<1
	α-^{32}P-dGTP	<1	<1	<1	<1
	α-^{32}P-dTTP	<1	<1	<1	<1
AMV polymerase plus 60 to 70*S* AMV RNA (reconstructed system)	α-^{32}P-dCTP	<2	<1	<1	<1
	α-^{32}P-dATP	<1	11–15	<1	<1
	α-^{32}P-dGTP	<1	<1	<1	<1
	α-^{32}P-dTTP	<1	<1	<1	<1

[a] Data are taken from a series of experiments like that depicted in Fig. 2, with the use of three different preparations of 70*S* RNA and DNA polymerase for the reconstructed system. All α-^{32}P-deoxynucleotide triphosphates were examined at least twice. The data for dCTP exclude two experiments where transfer to 2'(3')-CMP and 2'(3')-AMP was observed. This was not reproducible (see Fig. 5).

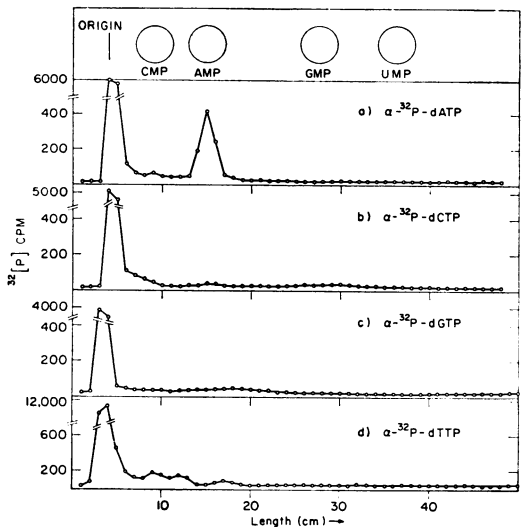

FIG. 2. *α-32P-transfer with individual deoxyribonucleoside triphosphates in the reconstructed system. Standard reaction mixtures in 0.2 ml containing the following amounts of individual α-32P-labeled deoxyribonucleoside triphosphates were used: (a) 0.7 nmole of α-32P-dATP (22,000 counts per min per pmole); approximately 100,000 counts per min were incorporated and about 10,000 to 15,000 counts per min of hydrolyzed sample were applied to the paper for electrophoresis; (b) 0.58 nmole of α-32P-dCTP (25,000 counts per min per pmole); about 180,000 counts per min were incorporated, and about 10,000 to 15,000 counts per min of hydrolyzed material were subjected to electrophoresis; (c) 1 nmole of α-32P-dGTP (15,000 counts per min per pmole); about 200,000 counts per min were incorporated and 10,000 to 15,000 counts per min of hydrolyzed material were analyzed by electrophoresis; (d) 0.54 nmole of α-32P-dTTP (28,000 counts per min per pmole); about 80,000 counts per min were incorporated and after hydrolysis about 20,000 to 30,000 counts per min were analyzed on paper electrophoresis.*

^{32}P-2′(3′)-AMP. To investigate whether such transfers from α-^{32}P-dCTP do occur, but that lability of the ribonucleotide deoxycytidine monophosphate (dCMP) bonds make them evanescent, we analyzed the product of a reaction containing α-^{32}P-dCTP after various times of synthesis. Figure 5 shows that, although incorporation of α-^{32}P-dCMP is linear until 90 min, there is no detectable transfer of radioactivity at any time to any of the possible ribonucleotides. We therefore conclude that the occasional transfer which we observed was probably an artifact, possibly due to contamination in the precursors. Our inability to reproduce the results of Flügel and Wells (9) could be due to slight differences in technique (see Discussion).

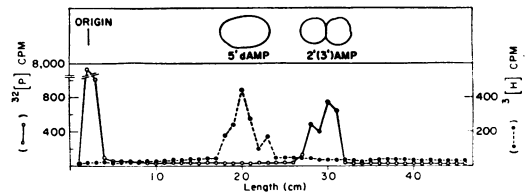

FIG. 3. *Co-electrophoresis of hydrolyzed α-32P-dATP product and 3H-dAMP. Approximately 15,000 counts per min of hydrolyzed α-32P-dATP-labeled product from Fig. 2a were mixed with 10,000 to 20,000 counts/min of 3H-labeled 5′-dAMP. Electrophoresis was carried out for 4.5 hr at 4,000 v. Symbols: ○, 32P-containing sample; ●, 3H-labeled sample.*

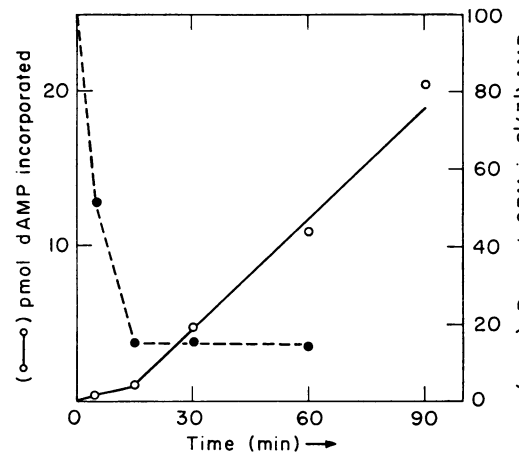

FIG. 4. *Kinetics of initiation and incorporation of α-32P-dATP in reconstructed system. Standard reaction mixture in 1.0 ml containing 4 nmoles of α-32P-dATP (20,000 counts per min per pmole) and 5,300 pmoles of nucleotides of 60 to 70S RNA was incubated at 37 C. Samples of 0.2 ml were withdrawn after various times, and the reaction was stopped by adding sarkosyl to a final concentration of 1%. Approximately 15,000 to 20,000 counts per min of each sample were subjected to electrophoresis. Symbols: ○, dAMP incorporation; ●, percent transfer of radioactivity to 2′(3′)-AMP.*

DISCUSSION

The recovery of a 2′(3′)-ribonucleotide after alkaline digestion of the DNA product labeled by an α-^{32}P-deoxyribonucleoside triphosphate confirms the idea that the initial product of the reaction in which AMV DNA polymerase copies 60 to 70S viral RNA is a covalently linked RNA-DNA molecule (9, 14, 32). The endogenous reaction and a reconstructed system consisting of purified DNA polymerase and 60 to 70S viral RNA both give the same result. The finding of a ^{32}P-transfer also shows that some and presumably all of the covalently linked RNA is at the 5′ end of the newly made DNA and therefore supports the

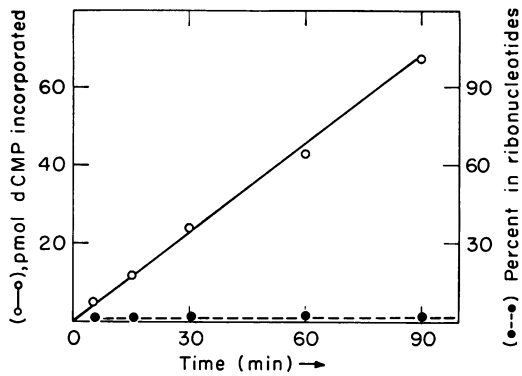

FIG. 5. *Kinetics of initiation and incorporation of α-^{32}P-dCTP in reconstructed system. Standard reaction mixture in 1.0 ml containing 1.75 nmoles of α-^{32}P-dCTP (20,000 counts per min per pmole) and 8,000 pmoles of nucleotides of 60 to 70S RNA were incubated at 37 C. Samples of 0.2 ml were withdrawn after various times. Approximately 30,000 to 40,000 counts per min of hydrolyzed product were analyzed by electrophoresis. Symbols:* ○, *^{32}P incorporation (counts per minute);* ●, *percent transfer of radioactivity to 2′(3′)-mononucleotides.*

concept that the role of the RNA is to act as a primer for DNA synthesis.

The existence of only 1 of the 16 possible ^{32}P-transfers argues that the initiation being observed is quite specific. It will be interesting to determine whether all tumor viruses form a ···pApdA··· joint. Since AMV is a mixture of viruses (22), it would appear that all of these viruses, at least, form the same bond. M. Bishop et al. (Proc. M. D. Anderson Symp., 1972, *in press*) have also found a ···pApdA··· joint with Rous sarcoma virus. The specificity of initiation in the tumor virus system can be compared to the results with M13 phage. Initiation of phage DNA synthesis also involves an RNA primer and pA-OH is also the predominant 3′ end of the primer, but significant ^{32}P-transfer was observed from all deoxyribonucleoside triphosphates (4, 35; Brutlug and Kornberg, *personal communication*).

We had earlier argued that the RNA primer in the 60 to 70S RNA must be much smaller than the 35S RNA subunits of the 60 to 70S RNA complex (32). Increasing evidence is accumulating in support of the idea of a primer of about 100 nucleotides in length (Faras et al., *manuscript submitted for publication*; Canaani and Duesberg, Proc. M. D. Anderson Symp., 1972, *in press*; R. Erikson, *personal communication*). The present results indicate that the primer has a 3′ end of pA-OH. Previous results had indicated that the 35S subunits of the 60 to 70S RNA had 3′ ends of pU-OH (8, 21). More recent evidence, however, suggests that the poly (A) in virion RNA (11, 13, 18) occurs at the 3′ end of the RNA (Datta and Baluda, *personal communication*; Erikson, *personal communication*), as it does in many other RNAs (5, 17; Darnell, *personal communication*; Yogo and Wimmer, *in press*). A pA-OH end for the 35S RNA has been very recently reported (28). If the 3′ end of the 35S RNA is pU-OH, our present results suggest that the primer is separate from the 35S RNA. If the 3′ end of the 35S RNA is poly (A), our earlier demonstration that the primer can be removed from the product by pancreatic ribonuclease in 0.3 M NaCl also argues that the primer is separate from the 35S RNA (32). We therefore conclude that the 60 to 70S RNA complex probably contains lower-molecular-weight primer RNAs. The pA-OH end of the primers is consistent with their being transfer RNA.

The occurrence of 10 to 20% of the α-^{32}P-dAMP radioactivity in 2′(3′)-^{32}P-AMP after alkaline hydrolysis suggests that the average chain lengths of the product must be very small. Since only 14% of the product is dAMP (see Table 1), the calculated chain lengths are about 35 to 70 rather than 20 to 40, which would be the case if all four deoxyribonucleotides occurred in equal amounts. Analysis of the product of both the reconstructed and endogenous systems on sucrose gradients confirms the short length. It is noteworthy that the AMV enzyme can make an 8S DNA when globin messenger RNA is the template and oligo(dT) is the primer (33). Such a DNA is about four times the size of the AMV RNA-stimulated product (29), suggesting that the small size of the product made on AMV RNA is due to a block of some sort in the AMV RNA rather than a limitation on the length of product which can be synthesized by the AMV DNA polymerase (19).

The finding of a ···pApdA··· joint in both the endogenous and reconstructed systems indicates the similarity of the two systems and that a true reconstruction of the endogenous reaction is possible with purified components. This supports the earlier conclusion of Taylor et al. (30), who showed that the DNAs formed in the two systems cross-hybridize.

Two other laboratories have reported transfer experiments of the type we have described here. Parsons, Bromley, Haroz, and Weissmann (*personal communication*) found transfer of about 0.3% of the incorporated label from α-^{32}P-thymidine triphosphate to predominantly 2′(3′)-AMP. We would not have detected such a low level of transfer, and Parsons et al. did not test α-^{32}P-dATP. Flügel and Wells (9), studying only the endogenous reaction activated by treatment of the

virus with ether, found transfer from α-^{32}P-dCTP to 2′(3′)-UMP and to a lesser extent the transfer from α-^{32}P-dATP to 2′(3′)-AMP which we have observed. We have not repeated their use of ether and cannot explain the discrepancy between our results.

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