Reverse transcription of avian myeloblastosis virus 35S RNA. Early synthesis of plus strand DNA of discrete size in reconstructed reactions

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ABSTRACT

The early DNA products of reverse transcription have been analyzed from reconstructed reactions containing avian myeloblastosis virus 35S RNA·tRNA^{trp} complex and highly purified reverse transcriptase. We describe conditions for the synthesis of genome-length complementary DNA and two discrete species of plus strand DNA (the same chemical polarity as the viral RNA genome) about 300 and 400 nucleotides in length. Plus DNA₄₀₀ and plus DNA₃₀₀ were detected by molecular hybridization with DNA probes complementary to sequences from both the 3'- and 5'-ends of the viral RNA. Both species appear to be copied from the 5'-end of minus strand DNA by their hybridization properties and their early synthesis when only the 5'-end of minus strand DNA is available as template. Restriction endonuclease mapping of plus DNA₄₀₀ and plus DNA₃₀₀ rules out a precursor-product relationship between the two. Rather the results suggest a unique initiation site for both species, with plus DNA₄₀₀ containing internal sequences not present in plus DNA₃₀₀. Plus DNA₄₀₀ and plus DNA₃₀₀ appear to be analogous to early plus DNA species detected in cells early after retrovirus infection. Thus, purified reverse transcriptase appears to be enzymatically sufficient for synthesis of genome-length complementary DNA and initiation and synthesis of early plus strand DNA as observed in infected cells.

INTRODUCTION

A central event in the life-cycle of retroviruses is the copying of the single-stranded RNA genome into double-stranded DNA. This process known as reverse transcription is dependent upon an RNA-directed DNA polymerase residing in the virion (1,2) and encoded within the viral 35S RNA subunit (3). In reconstructed reactions containing purified reverse transcriptase, the bulk of the DNA complementary to the RNA [(-)DNA] is initiated on a tRNA specie [tRNA^{trp} for avian viruses (4)] which is hydrogen bonded close to the 5'-terminus of the viral RNA subunit (5,6). The reverse transcriptase reads to the 5'-end of the RNA producing a homogeneous class of short "strong stop" (-)DNA (cdNA_{5'}¹⁰⁰) and continues elongation at a 3'-terminal end of the RNA via a "jump" mechanism facilitated by the direct terminal redundancy present in

retrovirus genomes (7-9). The end products of this process in infected cells or in detergent-disrupted virus systems include complete double-stranded DNA copies of the viral RNA genome (6-10 kb) plus a reiterated region of 0.3-1.3 kb not present in the viral RNA and called the long terminal repeat (10-13). In infected cells (14,15) or in cell-free systems using either detergent-disrupted (16,17) or melittin-permeabilized virions (18), initiation of the second strand of DNA [(+)DNA] occurs prior to the completion of (-)DNA synthesis. Varmus *et al.* (14) detected a (+)DNA fragment about 300 bases long appearing in cells shortly after infection by avian sarcoma virus. This (+)DNA hybridized to radiolabeled DNA probes specific for both 3'- and 5'-terminal sequences of the viral RNA. More recent studies reveal that analogous (+)DNA molecules [referred to as plus strong stop DNA (17)] are synthesized in mouse mammary tumor virus-infected cells (15) and in various virus-disrupted systems *in vitro* (16-18).

Events leading to and including the initiation of the plus strand strong stop DNA are not clear. To fully understand the details of this synthetic process and to define the role of reverse transcriptase and its associated RNase H, we have initiated studies to characterize the synthesis of viral RNA-directed DNA including (+)DNA in reconstructed reactions containing only purified avian myeloblastosis virus (AMV) 35S viral RNA•tRNA^{trp} template-primer and reverse transcriptase. In this communication we describe the first evidence for synthesis of genome-length (-)DNA in such a system, and initiation of early (+)DNA by what may be the same mechanism operating *in vivo*.

MATERIALS AND METHODS

Virus. AMV(BAI strain A) was purified as described (19,20) from frozen plasma of leukemic chicks supplied through the Office of Program Resources and Logistics, Virus Cancer Program, National Cancer Institute, Bethesda, Maryland.

Purification of AMV DNA polymerase. AMV DNA polymerase was solubilized from purified AMV essentially as described (19) in a medium containing 6.7% (v/v) Nonidet P-40 (Particle Data Laboratories Ltd.), 0.8 M KCl, 10 mM KPO₄ (pH 8.0), and 5 mM dithiothreitol. The mixture was kept on ice for 45 min and then centrifuged at 20,000 x g for 15 min at 1°C. The pellet from this step contains all of the viral 60S RNA and about 30% of the free viral 4S RNA fraction (unpublished data of the authors), and was used as the source of RNA for these studies (see below). The supernatant was diluted 10-fold with 10 mM KPO₄ (pH 7.2), 5 mM DTT, and 5% (v/v) glycerol, and was applied to a DEAE-cellulose column (2 x 15 cm) (19,20). The enzyme was further purified to apparent homogeneity as determined by SDS-PAGE using CM-Sephadex chromatography and glycerol gradient centrifugation (19), and had no contaminating endo- or exonucleolytic activity.

RNA. 60S and 4S RNA fractions were purified from the 20,000 x g pellet

obtained from detergent-disrupted virions as described above by SDS-phenol extraction (21) and centrifugation through 10-30% (v/v) glycerol gradients containing 10 mM Tris-HCl (pH 7.4), 40 mM LiCl, 5 mM EDTA, and 0.2% (w/v) SDS for 3.75 hr at 21°C and 26,000 RPM using a SW 27 rotor. The 60S RNA was dissociated into its subunits without dissociation of tRNA^{trp} primer (22) by heating at 60-63°C for 3 min in 20 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 0.2% (w/v) SDS followed by oligo(dT)-cellulose chromatography (23). The poly(A)⁺ fraction was reheated and cycled 2-3x through oligo(dT)-cellulose. 35S RNA·tRNA^{trp} was obtained by 2 cycles of glycerol gradient centrifugation in a SW 41 rotor at 40,000 RPM for 5 hr as described above. Greater than 90% of the low molecular weight nucleic acid associated with 35S RNA prepared in this manner is tRNA^{trp} as determined by denaturing and nondenaturing polyacrylamide gel electrophoresis (24,25) of 5'-end [³²P]-labeled RNA complex. The 35S RNA devoid of tRNA^{trp} was obtained by velocity gradient centrifugation of 35S RNA·tRNA^{trp} complex through DMSO-sucrose gradients (26). tRNA^{trp} was purified from free viral 4S RNA essentially as described (27). The reconstituted 35S RNA·tRNA^{trp} complex was prepared by annealing DMSO-sucrose gradient purified 35S RNA with tRNA^{trp} as described (28) followed by ethanol precipitation and recentrifugation in glycerol gradients after reheating at 63°C to dissociate aggregates.

Synthesis of 35S viral RNA-directed DNA. Our standard reaction mixture has been formulated to give the maximum yield of large minus strand DNA. Reaction mixtures contained 50 mM Tris-HCl (pH 8.1), 5 mM MgCl₂, 10 mM dithiothreitol, 94 mM NaCl, 25 µg/ml 35S RNA·tRNA^{trp}, 0.2 mM each of dATP, dCTP, dGTP and dTTP (P-L Biochemicals), 50-500 µCi/ml [³H]dCTP (20-30 Ci/mmol, New England Nuclear) and saturating amounts of AMV DNA polymerase. The enzyme storage buffer contributed 30 mM KPO₄ (pH 8.0) and 12% glycerol to the reaction mixture. Following incubation at 39°C for various times, reactions were terminated by the addition of SDS and EDTA to 1% and 10 mM, respectively, and phenol extracted. Following G-50 Sephadex chromatography, [³H]DNA was stored at -20°C in 75% (v/v) ethanol containing 0.4 M NaCl.

Preparation of molecular hybridization probes. (a) cDNA₃¹⁰ and cDNA₃⁹. [³H]DNA (1-2 x 10⁷ cpm/µg) specific for the 5'-end of viral RNA was synthesized essentially as described by Friedrich et al. (29) in reaction mixtures containing 100 µg/ml actinomycin D (Sigma, Grade I), 100 µg/ml fragmented poly(A)⁻ RNA (>28S) isolated as described above from AMV 60S RNA, and saturating amounts of AMV DNA polymerase. The use of poly(A)⁻ fragments of viral RNA greatly suppresses the elongation of cDNA₃¹⁰ (unpublished data of the authors). Reactions were incubated at 41°C for 60-75 min and the DNA was purified as described above. The DNA was then fractionated by electrophoresis in gels of 10% polyacrylamide containing 98% formamide (25). The gels were sliced and the nucleic acids were eluted overnight at room temperature with 10 mM Tris-HCl (pH 7.7), 150 mM LiCl, 2 mM EDTA and 0.2% (w/v) SDS. Discrete species of DNA 175 bases long (cDNA₃¹⁰ + tRNA^{trp}) and about 115 bases long (cDNA₃⁹ + tRNA^{trp}) were pooled separately and the tRNA^{trp} primer removed by alkaline hydrolysis (0.3 N NaOH, 1 mM EDTA, 18-24 hr, 37°C). Each DNA specie was then neutralized, chromatographed on G-50 Sephadex, ethanol precipitated, and refractionated on 10% gels. (b) cDNA₃¹. cDNA₃¹ was synthesized as described (30) using 8-10S poly(A)⁺ fragments of AMV RNA as template and oligo(dT)₁₂₋₁₈ (P-L Biochemicals) as primer. Alkali-treated cDNA₃¹ was further purified by chromatography on oligo(dT)-cellulose after annealing with poly(A) as described (30). (c) 35S RNA. 35S RNA was purified by velocity centrifugation of 35S RNA·tRNA^{trp} complex through DMSO-sucrose gradients as described above and concentrated by ethanol precipitation. Prior to 5'-end labeling, the 5'-terminal cap was chemically removed as described (31).

5'-end labeling. Dephosphorylation of nucleic acids by *E. coli* alkaline phosphatase (P-L Biochemicals-minimal nuclease) and 5'-end labeling with γ-

[32 P]ATP (1,000-2,000 Ci/mmol, New England Nuclear) and T₄ polynucleotide kinase (P-L Biochemicals) was performed essentially as described (32).

Gel electrophoresis. Denaturing polyacrylamide gel electrophoresis was performed in 98% formamide or 7 M urea at room temperature as described by Maniatis *et al.* (25), except that 4.8% polyacrylamide-7 M urea gels were run at 12 mA constant current for 2.8 hr. Under these conditions, all *Hind* III restriction fragments of ϕ x174 RF DNA (72-1,353 nucleotides) remain denatured throughout the electrophoretic run. Samples were heated in 95% formamide, 1 mM NaPO₄ (pH 7.5), 0.01% bromophenol blue and 0.01% xylene cyanole FF for 90 sec at 97°C and then cooled in a saline-ice bath just prior to loading on the gel. Electrophoresis in alkaline agarose gels was performed as described (33).

Fluorography. Fluorography of agarose gels was performed as described by Chamberlain (34). Fluorography of polyacrylamide gels (>4%) was performed by the following modifications of the procedure of Bonner and Laskey (35). After electrophoresis, nucleic acids were fixed by soaking the gel in 5 volumes of 1% lanthanum acetate/1% acetic acid for 20 min. The gel was then dehydrated by careful washing in 20 volumes of glacial acetic acid for 10 min. Then the gel was soaked in 4 volumes of a 22% (w/v) solution of 2,5 diphenyl-oxazole (PPO) in glacial acetic acid for 30 min (longer times may result in deterioration of the gel). The PPO-impregnated gel was then immersed in excess water for 20-30 min to remove most of the acetic acid and to precipitate the PPO. Finally, the gel was dried and exposed to hypersensitized Kodak XR film at -70°C.

Transfer of DNA from agarose gels to diazobenzylloxymethyl (DBM)-paper and hybridization. DBM-paper was prepared using Schleicher and Schuell 589 paper and N-(3-nitrobenzylloxymethyl)-pyridinium chloride (Sigma Chemical Co.) according to Alwine *et al.* (36) as modified by Wahl *et al.* (37). Transfer of DNA from agarose gels (3 mm thick) to DBM-paper was according to Wahl *et al.* (37) except that the transfer was carried out at 4°C rather than at room temperature, and the partial depurination step was omitted. After the transfer step, the DNA-paper was pretreated and hybridized to 5'-[32 P]DNA in the presence of 10% dextran sulfate (37). When 5'-[32 P]RNA was used as probe, it was necessary to include 15 μ g/ml yeast tRNA and 25 μ g/ml poly(A) in both the pretreatment solution and hybridization buffer to minimize high backgrounds. Following hybridization at 42°C for 18-24 hr, the DNA-paper was washed at 42°C exactly as described (37) and then air dried on paper towels. The DNA-paper was exposed at -70°C to Kodak XR film sandwiched between intensifying screens.

Purification of (+)DNA₄₀₀ and (+)DNA₃₀₀. Purified DNA product (4-20 μ g) was dissolved in 0.18-0.45 ml of solution containing 80% formamide, 40 mM Pipes-NaOH (pH 6.4), 1 mM EDTA and a 30-40 fold mass excess of AMV 60S RNA. The solution was heated at 85°C for 3 min, and then chilled on ice. Then 4 M NaCl was added to a final concentration of 0.4 M NaCl and the nucleic acids were hybridized at 52°C for 60 min (uncorrected Cr_t > 0.2 mol $\cdot$ sec $\cdot$ L⁻¹). Following hybridization, the solution was cooled on ice and then mixed with 4.5-4.7 ml of a solution of 8.59 gm CsCl, 1.60 g guanidinium chloride, and 6.7 ml of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA (17,38). The nucleic acids were then centrifuged in a SW 50.1 rotor at 36K RPM and 20°C for 40 hr. Under these conditions, the RNA-(-)DNA hybrid pellets or is near the bottom of the tube while the unhybridized single-stranded (+)DNA is near the top of the gradient. Appropriate pools were made and the nucleic acids were concentrated by ethanol precipitation. Following alkaline hydrolysis to remove traces of RNA, the (+)DNA was fractionated by electrophoresis in cylindrical 5% polyacrylamide-98% formamide gels as described above. The gels were sliced and the nucleic acids were eluted as described above. (+)DNA₄₀₀ and (+)DNA₃₀₀ were located by hybridizing eluants with 1,000-2,000 cpm of radiolabeled cDNA₃ or cDNA₅ probes in a volume of 100 μ l containing 50% formamide, 10 mM Tris-HCl (pH 7.4), 0.6 M LiCl for 18 hr at 37°C. Following hybridization, reactions were

diluted with 1 ml of 0.1 M sodium acetate (pH 4.4), 0.2 M NaCl, 5 mM ZnSO₄, 10 µg of calf thymus DNA, and 5 units of S₁ nuclease which was prepared as described through the DEAE-cellulose step (39). Digestion with S₁ nuclease was carried out at 42°C for 90 min and 5% trichloroacetic acid-insoluble material was collected on nitrocellulose filters and counted in a liquid scintillation counter. Pools of (+)DNA₄₀₀ and (+)DNA₃₀₀ were made and further purified by hybridization to cDNA_{3'}-cellulose (10 µg cDNA_{3'}/10 mg ABM-cellulose) which had been prepared according to Noyes and Stark (40).

Restriction endonuclease cleavage. Purified, uniformly labeled [³²P]- or 5'-[³²P](+)DNA₄₀₀ or (+)DNA₃₀₀ (1-5 ng) was hybridized with 200-400 ng of unlabeled (-)DNA, isolated from either the guanidinium/CsCl gradient (above) or synthesized in the presence of 100 µg/ml actinomycin D as described by Taylor (41), for 3 hr at 69°C in 50 µl 20 mM Pipes-NaOH (pH 6.4), 0.5 M NaCl, and 1 mM EDTA. Hybridization was terminated by ethanol precipitation. *Hae* III or *Hind* III restriction endonuclease (Worthington) digestion was carried out for 2 hr at 37°C using conditions suggested by New England Biolabs, Inc.

RESULTS

Early DNA synthesis in reconstructed reverse transcription. Viral RNA-directed DNA synthesis was performed in reconstructed reactions containing only purified AMV 35S RNA•tRNA^{trp} complex and reverse transcriptase. DNA product was isolated at various times, denatured in formamide, and analyzed by electrophoresis in 4.8% polyacrylamide gels containing 7 M urea. As shown in Fig. 1, lane b, the predominant component observed after 5 min is a small DNA molecule about 175 bases long. Upon alkali treatment of the DNA product, the 175 base-long DNA disappears with the subsequent appearance of a DNA specie about 100 bases in length (cDNA_{5'}¹⁰⁰), reflecting the loss of the tRNA^{trp} primer (Fig. 1, lane c). The predominance of cDNA_{5'}¹⁰⁰ among the early reaction products in this reconstructed reaction is consistent with earlier studies demonstrating that most if not all of the (-) strand DNA is initiated on the tRNA^{trp} primer bound 100 nucleotides from the 5'-terminus of the viral 35S RNA (42). Other minor DNA products were also observed after 5 min of reverse transcription and have not been characterized. The small specie (about 150 bases long) and several larger species (500-1,000 bases long) are presumed to be incomplete cDNA_{5'}¹⁰⁰ and elongated products of cDNA_{5'}¹⁰⁰, respectively, since their lengths are decreased about 75 bases (length of tRNA^{trp}) by alkali treatment (Fig. 1, lane c, and data not shown).

Analysis of reaction products after 20 min and 60 min indicates a significant increase in the amount of larger DNA product (greater than 1,000 nucleotides) which is beyond the resolution of the 4.8% polyacrylamide gel (Fig. 1, lanes d-g). Accompanying the appearance of the larger DNA is a concomitant decrease in the amount of cDNA_{5'}¹⁰⁰. This arises from the elongation of cDNA_{5'}¹⁰⁰, which is presumably facilitated by its interaction with the direct terminal redundancy adjacent to the 3'-poly(A) tract in the viral RNA (7-9). In exper-

Figure 1. Analysis of early DNA products from reconstructed reactions. [^3H]DNA products taken at various times from 35S RNA $\cdot$ tRNA $^{\text{trp}}$ -directed reverse transcription reactions were denatured in formamide, fractionated by electrophoresis in a 4.8% polyacrylamide gel containing 7 M urea, and analyzed by fluorography. Samples in lanes c,e,g and i were alkali-treated prior to electrophoresis. (a) *Hae* III-digested ϕ x174 DNA size markers; (b and c) 5 min reaction; (d and e) 20 min reaction; (f and g) 60 min reaction; (h and i) 60 min reaction with 100 $\mu\text{g/ml}$ actinomycin D. Marker DNA lengths in nucleotides are indicated on the left. The positions of the cDNA $_{5'}$ 100 and hairpin DNA (hp) species prior to or after removal of the tRNA $^{\text{trp}}$ primer by alkali treatment and of the xDNA species referred to in the text are indicated to the right of the figure. The identities of these DNA bands were determined in separate experiments (not shown) in which DNAs were eluted from preparative gels and characterized further.

iments not shown using 5'-[^{32}P]-labeled tRNA $^{\text{trp}}$, we observe the covalent linkage of this larger DNA to tRNA $^{\text{trp}}$ and the decrease in cDNA $_{5'}$ 100 -tRNA $^{\text{trp}}$ with time, confirming the elongation of cDNA $_{5'}$ 100 . Also observed after 20 min are (i) a new species (designated hp-tRNA $^{\text{trp}}$ in Fig. 1) migrating slightly faster than the incomplete cDNA $_{5'}$ 100 species (Fig. 1, lane d); (ii) a species (designated x in Fig. 1) migrating rapidly (about 90 bases long); and (iii) 2-3 discrete species in the range of 300-400 bases long (Fig. 1, lanes d and e). HpDNA has an apparent length of 135-140 bases under our electrophoretic conditions (Fig. 1, lanes d and f) and is alkali-sensitive, shifting to a position of about 80 bases in length (Fig. 1, lanes e and g). It has been isolated from polyacrylamide gels (not shown) and appears to be structurally iden-

tical to hairpin DNA as previously described by Collett and Faras (43) and more recently by Swanstrom *et al.* (53).

The very small DNA specie (xDNA) and the DNA species in the range of 300-400 bases long are not appreciably sensitive to alkali (Fig. 1, lanes d and e), suggesting they may not be precursors to or elongation products of $\text{cDNA}_{5'}^{100}$. To examine this possibility further, we investigated the effects of actinomycin D on their synthesis. Actinomycin D inhibits the synthesis of DNA-directed DNA synthesis by reverse transcriptase (44,45) and has been used by others to preliminarily identify the synthesis of (+)DNA copied from newly synthesized (-)DNA (17,18). When products were analyzed after 60 min of DNA synthesis in the presence of actinomycin D, the appearance of the alkali-resistant DNA species was inhibited (Fig. 1, lane h). Unfortunately, we found in confirmation of earlier reports (46,47), that the elongation of $\text{cDNA}_{5'}^{100}$ was also impaired (compare Fig. 1, lanes g and i), making tenuous the preliminary assignment of the polarity of these actinomycin D-sensitive DNA species as (+)DNA species.

Identification of discrete (+)DNA molecules with (-)DNA probes. Initially, DNA synthesized in reconstructed reverse transcription reactions was isolated, alkali-treated, and subjected to electrophoresis under denaturing conditions in a 5% polyacrylamide gel containing 98% formamide. After slicing the gel, the DNA was eluted and hybridized to various (-) strand [^3H]DNA probes: (i) cDNA_3 , which is complementary to sequences 200-300 bases adjacent to the poly(A) tract in 35S RNA; (ii) $\text{cDNA}_{5'}^{100}$, which is initiated on tRNA^{trp} near the 5'-end of the template and complementary to 100 bases at the 5'-end of the RNA penultimate to the cap; and (iii) $\text{cDNA}_{5'}^{40}$, which is an incomplete transcript of $\text{cDNA}_{5'}^{100}$. The results indicate that all three [^3H]DNA probes hybridized to two major species of (+)DNA about 400 and 300 nucleotides long (Fig. 2, A-C), with the 400 base-long molecule representing 60-75% of the total in several experiments. We also performed hybridization experiments after transfer of the DNA from slab gels to DBM-paper (36). Again, the 400- and 300-base DNA molecules were observed, as well as another DNA about 170-175 bases long (Fig. 3, lane b). All three species of DNA were synthesized in reactions containing 60S RNA as template-primer or 35S RNA + tRNA^{trp} obtained either by partial heat denaturation of 60S RNA or by annealing purified 35S RNA to purified tRNA^{trp} (Fig. 3, lanes a-c, respectively). No DNA was detected by the (-)DNA probes in reactions containing purified 35S RNA devoid of tRNA^{trp} primer (Fig. 3, lane d).

DNA product from endogenous reactions of detergent-disrupted virions was

Figure 2. Detection of (+)DNA synthesis by hybridization with specific (-)DNA probes. Unlabeled, alkali-treated DNA product from a 2 hr reconstructed reaction (1 ml) containing 35S RNA•tRNA^{trp} template•primer complex was fractionated in a cylindrical 5% polyacrylamide gel containing 98% formamide, sliced, and eluted as described in Methods. One-twelfth of the eluted DNA from each sliced fraction was annealed to (A) [³H]cDNA₃₁ (1,060 cpm); (B) [³H]cDNA₅₁₀₀ (2,250 cpm); and (C) [³H]cDNA₃₁₀ (450 cpm). Positions and base lengths of denatured Hae III-digested ϕ x174 DNA marker fragments are indicated in panel A.

also analyzed. In contrast to the results just described for reconstructed reactions, the 400-base DNA molecule was by far the most predominant comprising more than 80% of the total DNA detected by the cDNA₅₁₀₀¹⁰⁰ probe (Fig. 3, lane e) or the cDNA₃₁ probe (data not shown). In addition, several minor species of DNA approximately 525, 700, 950 and 1,800 bases long were detected with the cDNA₅₁₀₀¹⁰⁰ probe (Fig. 3, lane e) or the cDNA₃₁ probe (not shown). These minor species were readily visible in the DNA product from an endogenous reaction, but were only very minor components in the DNA product from the reconstructed reaction. At present we cannot account for the differential synthesis in the endogenous and reconstructed reactions. The addition of detergent to the reconstructed reaction mixture did not alter the results qualitatively or quantitatively (data not shown).

The 90 base-long, alkali-resistant DNA specie (xDNA) which was observed

Figure 3. Detection of (+)DNA synthesis using various AMV RNA template·primers. Alkali-treated DNA product from 2½ hr reactions (20 µl) was fractionated by electrophoresis in an alkaline 1.2% agarose gel at 60 V for 6 hr at room temperature, transferred to DBM-paper and hybridized with [³²P]cDNA₅¹⁰⁰. The template·primer in each reaction was (a) 60S RNA; (b) 35S RNA·tRNA^{trp} purified from 63°C-heated 60S RNA; (c) 35S RNA·tRNA^{trp} obtained by annealing purified tRNA^{trp} to DMSO-sucrose gradient-purified 35S RNA; (d) 35S RNA obtained after DMSO-sucrose gradient; and (e) endogenous 60S RNA in 0.018% Triton X-100-disrupted AMV (540 µg/ml viral protein).

in the experiment described in Figure 1 was not detected by either hybridization method with the three (-)DNA probes used (Figs. 2 and 3) and has not been characterized further.

Detection of hairpin DNA bound to DBM-paper. The detection of a strongly hybridizing 175-nucleotide DNA in the DNA product transferred from agarose gels to DBM-paper (Fig. 3, lane b) was surprising since this had not been detected in the experiment described in Figure 2 in which radiolabeled cDNA₅¹⁰⁰ had been hybridized to DNA eluted from gel slices. We have positively identified this 175-nucleotide DNA as hairpin DNA (hpDNA). First, it was detected by the blot-hybridization procedure from reactions using highly purified cDNA₅¹⁰⁰ as template·primer in a self-priming reaction (43). The hpDNA was also identified by its apparently anomalous electrophoretic behavior in partially denaturing polyacrylamide gel electrophoresis containing 7 M urea and by its double-stranded properties on hydroxylapatite after heat denaturation followed by quick cooling. To explain its appearance in the blot hybridization experiment (Fig. 3), we suggest that this DNA specie was not fully renatured during the transfer from agarose gels to DBM-paper. Consequently, it was covalently attached to the paper in an extended configuration and subsequently detected by the cDNA₅¹⁰⁰ probe.

The polarity of DNA₄₀₀ and DNA₃₀₀. The results of hybridization studies

shown in Figures 2 and 3 strongly suggest that the DNA₄₀₀ and DNA₃₀₀ species synthesized in reconstructed reverse transcription are plus strand DNAs. To confirm this, DNA₄₀₀ and DNA₃₀₀ were shown to be of (+) strand polarity by complete fractionation from (-) strand DNA. Total DNA product was hybridized to 60S viral RNA and the (-)DNA-RNA hybrids were separated from (+)DNA by equilibrium density centrifugation in a guanidinium/CsCl gradient. Following alkaline hydrolysis, a portion of each of the purified (+) and (-) strand DNAs was subjected to electrophoresis in alkaline agarose gels, transferred to DBM-paper, and hybridized with either [³²P]cDNA_{5'}¹⁰⁰ or viral [³²P]35S RNA. It was found that only the (-) strand fraction contains DNA which hybridizes with 35S viral RNA (Fig. 4A, lane b) and no discrete DNA species appear in the region of 300-400 bases in length. In contrast, the (-) strand DNA probe (cDNA_{5'}¹⁰⁰) detected both DNA₄₀₀ and DNA₃₀₀ as well as hpDNA and an unidentified specie appearing between the DNA₃₀₀ and hpDNA (Fig. 4B, lane c). The only DNA in the (-)DNA fraction that was detected by cDNA_{5'}¹⁰⁰ was hpDNA (Fig. 4b, lane b). These results demonstrate that both DNA₄₀₀ and DNA₃₀₀ species can be com-

Figure 4. Polarity of DNA₄₀₀ and DNA₃₀₀. Four μ g of alkali-treated DNA product from a 35S RNA-tRNA^{trp}-directed reverse transcriptase reaction was fractionated into (+) strand and (-) strand DNA pools as described in Methods. 30% of the total DNA product was recovered as (+)DNA. 26 ng of the alkali-treated (-)DNA pool (lane b) and 31 ng of the alkali-treated (+)DNA pool (lane c) were fractionated by electrophoresis in alkaline 2.5% agarose gels, transferred to DBM-paper, and hybridized to (A) 5' [³²P]35S RNA. After autoradiography, the RNA probe was removed by treating the DNA-paper with 100 ml 0.3N NaOH for 30 min. Following autoradiography to confirm the removal of the [³²P] RNA, the DNA paper was rehybridized to (B) 5' [³²P]cDNA_{5'}¹⁰⁰. Lane a shows marker 5' [³²P] fragments of Hae III-digested ϕ X174 DNA whose lengths in nucleotides are indicated on the left.

pletely fractionated from (-)DNA and, therefore, represent true (+)DNA species. Hereafter they will be referred to as (+)DNA₄₀₀ and (+)DNA₃₀₀.

Kinetics of (+)DNA₄₀₀ and (+)DNA₃₀₀ synthesis. [³H]DNA product was purified from standard reconstructed reactions at various times, subjected to electrophoresis in alkaline agarose gels, and analyzed either by fluorography or by hybridization to [³²P]cDNA₅¹⁰⁰ after transfer to DBM-paper. In this experiment, the synthesis of total DNA was linear for 90 min, and then leveled off over the next 20 hr (data not shown). DNA product comparable in length to the viral 35S RNA genome (8.0 kilobases in length) required 1-2 hr for completion (Fig. 5A). In contrast, hybridization analysis with the cDNA₅¹⁰⁰ probe revealed that (+)DNA₄₀₀ and (+)DNA₃₀₀ were detectable after 5 min of synthesis

Figure 5. Kinetics of 35S RNA•tRNA^{trp}-directed DNA synthesis. [³H]DNA product was isolated at various times after initiation of reconstructed reverse transcription and fractionated on alkaline 1.2% agarose gels. (A) Fluorography of the fractionated DNA from various timed reactions. (B) Autoradiography of hybridization pattern with 5' [³²P]cDNA₅¹⁰⁰ after fractionation on agarose gel and transfer to DBM-paper. Positions and lengths of Hind III-digested λ DNA marker fragments are indicated.

(Fig. 5B). Thus, we conclude that these molecules are synthesized prior to completion of limit length DNA. Also, in spite of the synthesis of large DNA product (Fig. 5A), only very minor quantities of (+)DNA species larger than 400 bases long were detected with the cDNA_{5'}¹⁰⁰ probe even after 24 hr of synthesis. (Fig. 5B).

Relationship of (+)DNA₄₀₀ to (+)DNA₃₀₀. Our hybridization experiments indicate that (+)DNA₄₀₀ and (+)DNA₃₀₀ contain sequences representative of both the 5'- and 3'-termini of the viral RNA. In order to learn more about the structural relationship between these two (+)DNA species, each was purified as described in Methods, and restriction endonuclease maps were prepared. We chose to use endonuclease Hae III since results of sequencing cDNA_{5'}¹⁰⁰ from AMV had indicated a Hae III cleavage site 56 nucleotides from the initiation site of (-)DNA synthesis (8). Endonuclease Hind III was also chosen because it has been reported to cleave AMV unintegrated linear DNA within its long terminal repeat (48). Digestion of uniformly labeled (+)DNA₄₀₀ with Hind III produced four fragments approximately 270, 140, 130 and 120 nucleotides in length (Fig. 6A, lane c). To determine which fragment contained the 5'-terminal sequences, (+)DNA₄₀₀ was 5'-end labeled with ³²P₀₄ using γ-[³²P]ATP and polynucleotide kinase prior to analysis. It was found that in partial (Fig. 6B, lane b) or complete digests (not shown) only the 270 nucleotide fragment contained the label indicating that it was derived from the 5'-end. The absence of fragments other than the 270 nucleotide long fragment in partial digests of 5'-[³²P](+)DNA₄₀₀ suggests the presence of only one Hind III site in (+)DNA₄₀₀. Thus, the 140, 130, and 120 nucleotide long fragments are derived from the 3'-end of the molecule.

Uniformly labeled (+)DNA₃₀₀ had a similar Hind III digestion pattern (Fig. 6A, lane e) producing fragments approximately 170, 140, 130, and 120 nucleotides long. Again only one fragment, the 170 nucleotide fragment, contained the 5' end sequences (Fig. 6B, lane e). The set of fragments 120-140 nucleotides long comigrated with the 3'-fragments of the Hind III-digested (+)DNA₄₀₀ (Figure 6A, lanes c and e). The heterogeneity in the length of the 3'-end Hind III fragments may be due to variation in termination; for example, the 20 nucleotide difference between the 120 and 140 nucleotide fragments is close to that expected for copying the 3'-end of the tRNA^{trp} primer (51).

Partial Hae III digestion of (+)DNA₄₀₀ yielded fragments about 350, 300 and 110 nucleotides in length (Fig. 6B, lane c). A similar digestion of (+)DNA₃₀₀ gave products about 250, 200 and 110 nucleotides long (Fig. 6B, lane f).

Figure 6. Restriction endonuclease digests of purified $(+)\text{DNA}_{400}$ and $(+)\text{DNA}_{300}$. Uniformly $[^{32}\text{P}]$ -labeled or 5'- $[^{32}\text{P}]$ -labeled $(+)\text{DNA}_{400}$ and $(+)\text{DNA}_{300}$ were isolated from 60 min reconstructed reactions and hybridized to unlabeled $(-)\text{DNA}$ as described in Methods. The DNA was digested with either *Hind* III or *Hae* III, denatured in formamide, subjected to electrophoresis in a 4.8% polyacrylamide gel containing 7 M urea and analyzed by autoradiography. The positions of marker $[^{32}\text{P}]$ -labeled *Hae* III digests of ϕx174 DNA are indicated to the sides.

(A) *Hind* III analysis of uniformly $[^{32}\text{P}]$ -labeled DNA: (a) ϕx174 DNA markers; (b) undigested $(+)\text{DNA}_{400}$; (c) *Hind* III digest of $(+)\text{DNA}_{400}$; (d) undigested $(+)\text{DNA}_{300}$; (e) *Hind* III digest of $(+)\text{DNA}_{300}$.

(B) *Hae* III and *Hind* III analysis of 5'- $[^{32}\text{P}]$ -labeled DNA: (a) undigested $(+)\text{DNA}_{400}$; (b) *Hind* III digest of $(+)\text{DNA}_{400}$; (c) *Hae* III digest of $(+)\text{DNA}_{400}$; (d) undigested $(+)\text{DNA}_{300}$; (e) *Hind* III digest of $(+)\text{DNA}_{300}$; (f) *Hae* III digest of $(+)\text{DNA}_{300}$.

The resulting *Hae* III and *Hind* III maps of $(+)\text{DNA}_{400}$ and $(+)\text{DNA}_{300}$ (Fig. 7) indicate that each molecule contains homologous 3'-halves. However, both species also contain a *Hae* III site about 110 nucleotides from the 5'-end (Fig. 6B, lanes c and f). This suggests that $(+)\text{DNA}_{300}$ is missing about 100 nucleotides approximately 110-170 bases from the 5'-end that are present in $(+)\text{DNA}_{400}$.

DISCUSSION

The molecular events of retrovirus RNA-directed double-stranded DNA synthesis and the specific roles of reverse transcriptase, its associated RNase H, and other viral and cellular components in this process are presently not well understood. To elucidate the specific events of this mechanism, we have attempted to reconstruct the synthetic process using purified reaction components. In initial experiments, we employed a model ribohomopolymer template.

Figure 7. Summary of Hae III and Hind III cleavage sites in (+)DNA₄₀₀ and (+)DNA₃₀₀

primer and demonstrated the efficient synthesis of double-stranded DNA by reverse transcriptase (49). Also in that study, we documented the role of reverse transcriptase-associated RNase H in the generation of RNA primers for initiation of synthesis of the second strand of DNA. In similar experiments using viral RNA template-primer, we have shown that (+)DNA has RNA covalently linked at its 5'-terminus (50). In the present study, we have established reconstructed reaction conditions for synthesis of genome-length (-)DNA and have examined the early products of viral RNA-directed DNA synthesis. In confirmation of earlier reports, we observe the synthesis of the minus strand strong stop DNA (cDNA_{5'}¹⁰⁰) (42) and hairpin DNA (hpDNA) (43,53). To our surprise, hpDNA could be detected by hybridization to cDNA_{5'}¹⁰⁰ after its transfer to DBM-paper. Although we suspect that hpDNA may be an artifactual end product of the reconstructed reaction, its detection by blot-hybridization provides an assay for its presence in infected cells. Interestingly, hpDNA was not readily detectable in DNA product from the endogenous reaction of detergent-disrupted AMV. This result confirms the earlier results of Novak *et al.* (46) who reported a decrease in rapidly reannealing DNA using carefully optimized reaction conditions and the more recent results of Swanstrom *et al.* (53). These results may suggest that in the endogenous reaction under defined conditions, either the synthesis of hpDNA is suppressed and/or the correct elongation of cDNA_{5'}¹⁰⁰ is more efficient. In contrast, we have been unable to find conditions to significantly reduce the amount of hpDNA in the reconstructed reaction except by suppressing (+)DNA synthesis in general with the use of additives such as actinomycin D (Fig. 1, lane i).

As early as 10-20 min after initiation of viral RNA-directed DNA synthesis, two additional DNA species of discrete size (400 and 300 nucleotides in length) were detected. That they were not synthesized in the presence of

actinomycin D suggested their (+) strand polarity. This was confirmed in hybridization experiments with viral RNA and (-)DNA probes. Viral 35S RNA template without tRNA^{trp} primer was unable to direct the synthesis of any (+)DNA species detectable with either cDNA₃ or cDNA₅ probes. In contrast, reconstructed 35S RNA•tRNA^{trp} template-primer from the purified RNA components directed the synthesis of both (+)DNA₄₀₀ and (+)DNA₃₀₀. Therefore, no contaminating primer activity was associated with the 35S viral RNA, and tRNA^{trp} was acting as the initial primer for the (-)DNA template required for synthesis of these two (+)DNA species.

Both discrete (+)DNA species contain sequences of the 5' and 3' terminal regions of the viral RNA and appear early in the synthetic reaction (5-10 min) long before (-) strand synthesis is completed. They must, therefore, be copied from the 5'-region of newly synthesized (-) strands, and would appear to be analogous in size and sequence to a 350 base (+)DNA that has been detected in cells shortly after infection by avian sarcoma virus (14, 15). Further mapping evidence comes from our restriction endonuclease digestion data which are in general agreement with those of Bergmann et al. (48). They found Hind III cleavage sites within terminal redundancies of approximately 0.3×10^6 daltons in AMV-associated MAV-1 and MAV-2 unintegrated viral DNAs. Our interpretation of these results is that the reconstructed system containing only purified reverse transcriptase and viral 35S RNA•tRNA^{trp} template-primer is capable of synthesizing (+)DNA species similar to those observed *in vivo*. A formal proof of this must await analysis of small (+)DNAs found in infected cells. We are currently studying (+)DNA synthesis in AMV-infected cells.

Several possibilities exist regarding the relationship between (+)DNA₃₀₀ and (+)DNA₄₀₀. First, (+)DNA₃₀₀ could be a precursor of (+)DNA₄₀₀. This would assume that a single, relatively specific initiation site for (+)DNA synthesis exists on (-)DNA. Elongation of (+)DNA would proceed, copying (-)DNA back to its 5'-linkage with tRNA^{trp} and perhaps continue along part of the tRNA primer itself (51). If (+)DNA₃₀₀ were an incomplete transcript, it should be complementary to the cDNA₃ probe and possibly part of the cDNA₅¹⁰⁰ probe; but it would not be complementary to the cDNA₅⁴⁰ probe. Our results indicate that cDNA₅⁴⁰ does hybridize to (+)DNA₃₀₀ (Fig. 2C). Therefore, both (+)DNA species contain similar 3'-sequences, and a precursor-product relationship would tend to be excluded. Another possibility is that the two (+)DNA species are initiated at two different sites on (-)DNA about 100 bases apart. In this case, (+)DNA₃₀₀ and (+)DNA₄₀₀ would anneal to all three cDNA probes as observed (Fig. 2). However, this possibility appears to be ex-

cluded from the restriction enzyme cleavage data, which shows that both species contain a Hae III cleavage site about 110 bases from their 5' end (Figs. 6 and 7). This finding supports a third possibility that both (+)DNA₃₀₀ and (+)DNA₄₀₀ share a common initiation site, and the difference between the two is due to internally deleted sequences. We suggest that these sequences are to the 5'-side of the Hind III site and to the 3'-side of the left hand Hae III site.

Why are there (+)DNAs of different sizes that map in the same region? Since the standard AMV complex from chicken plasma is made up of at least three virus strains (48), the difference could be due to heterogeneity; e.g., deletions near the 3'-end of the viral RNA. This, however, seems unlikely in view of recent evidence suggesting that the viral components in AMV share highly conserved 3' sequences (i.e. the c-region) (54). Another possibility is that deletions occur during the reverse transcription process itself, either during the copying of the viral RNA or (-)DNA. At present, we cannot distinguish between these or other possibilities. Recently, Boone and Skalka (18) reported the presence of two (+)DNA species about 350 and 270 bases in length appearing early in the endogenous reaction of melittin-disrupted RAV-2. Unfortunately, these two molecules were not studied further and at present any analogies between RAV-2 and AMV cannot be made.

Larger, minor (+)DNA species (525-1,800 nucleotides) detected by the cDNA_{5'}¹⁰⁰ probe in the product from detergent-disrupted virions (Fig. 3, lane e) were, at best, only faintly detected in the DNA product from reconstructed reactions (Fig. 3, lane b and Fig. 5B). Such (+)DNAs, complementary to cDNA_{5'}¹⁰⁰, could arise by at least four mechanisms not all of which are mutually exclusive: (i) ligation of adjacent (+)DNAs to (+)DNA₄₀₀ or (+)DNA₃₀₀. This requires multiple initiations and a mechanism (e.g. a DNA ligase) for covalent joining of (+)DNA; (ii) strand displacement [as suggested recently by Boone and Skalka (52)] of (+)DNA₄₀₀ [(+)DNA₃₀₀] by (+)DNA initiated on (-)DNA downstream from the initiation point of (+)DNA₄₀₀ [(+)DNA₃₀₀]; (iii) elongation of (+)DNA₄₀₀ [(+)DNA₃₀₀] following the second transcriptional jump by (-)DNA (13,15); and (iv) failure to initiate (+)DNA synthesis at the (+)DNA₄₀₀ [(+)DNA₃₀₀] initiation site followed by a subsequent successful initiation at another (+) initiation site and read through. While we have not yet studied the synthesis or the structure of the larger (+)DNA species in detail, the results presented in this report suggest that factors other than reverse transcriptase are present in the virion but not present in the reconstructed reaction containing only purified reverse transcriptase that influence either

the initiation or maturation of (+)DNA. It should also be emphasized that the probes used in this study were specific for the ends of the viral RNA and represent only a minor fraction (<5%) of the total genome. Thus, our present results do not rule out the possible initiation of (+)DNA synthesis from other more internal regions. Indeed, our unpublished data with a cDNA probe representative of the entire genome suggest that (+)DNA synthesis from other regions is quite extensive. This is in agreement with the results obtained with other avian retrovirus systems (15,16,18). We are presently preparing well defined cDNA probes to further map (+)DNA synthesized from more internal regions.

What are the events leading to and including the initiation of (+)DNA? Recently, we have found that reverse transcriptase-associated RNase H is responsible for cleaving the 3'-poly(A) tract of 35S RNA shortly after the transcriptional "jump" from the 5'-end of the RNA to the direct terminal repeat adjacent to the poly(A) (Olson, J. C. and Watson, K. F., manuscript in preparation). It is then possible that RNase H hydrolyzes the RNA moiety of the RNA-DNA hybrid being formed by the elongation of (-)DNA. RNA removal would facilitate the synthesis of (+)DNA synthesis in the opposite direction, possibly primed by RNase H-degradation products. However, it remains to be demonstrated that viral RNA primer fragments are responsible for initiation of (+)DNA₄₀₀ and (+)DNA₃₀₀ synthesis. Whatever the mechanism, our present observation of synthesis of discrete (+)DNA species in this region of the viral genome indicates that reverse transcriptase is somewhat discriminatory in its choice of potential initiation sites. Thus, we suggest that further studies of the reconstructed reaction will provide considerable insight into the specific events of retrovirus RNA-directed (+)DNA synthesis.

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