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Analysis of avian myeloblastosis viral RNA and *in vitro* synthesis of proviral DNA

(translation *in vitro*/transcription *in vitro*/cDNA)

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ABSTRACT **Two virus-specific RNA species of 7.5 and 7.0 kilobases have been identified in avian myeloblastosis virus (AMV) by denaturing gel electrophoresis and blot hybridization analysis, and they were found to be in a 10:1 ratio. The individual RNAs directed the cell-free synthesis of the 76,000-dalton "gag" protein and the 180,000-dalton "gag-pol" protein, thereby demonstrating 5′ sequence homology of approximately 4.9 kilobases between the two species. Synthesis of these two precursor proteins by the AMV genome indicates structural differences between AMV and other avian acute leukemia viruses. The two viral RNAs were transcribed into complete cDNA copies with AMV DNA polymerase. Linear proviruses were found to be 90–100% resistant to S1 nuclease. Analysis of single-stranded transcripts demonstrated two distinct species of 2.6 and 2.3 × 10^6 daltons, and analysis of duplexes formed from the single-stranded transcripts demonstrated species of 5.2 and 4.0 × 10^6 daltons.**

Acute avian leukemia viruses are defective and can replicate only in the presence of helper viruses. Avian erythroblastosis virus (AEV), avian myelocytomatosis virus (MC29), and avian myeloblastosis virus (AMV) can induce primarily erythroblastic leukemia, myelocytomatosis carcinomas of the liver or kidney, and acute myeloblastic leukemia, respectively. Additionally, AMV with its myeloblastosis-associated virus (MAV) will produce nephroblastoma, lymphoid leukosis, and osteopetrosis in chickens and will transform hematopoietic cells *in vitro* (1). Infection of cells by RNA tumor viruses is a prerequisite for viral transformation and depends on the conversion of the RNA genome into proviral DNA. Two forms of viral DNA have been detected in infected cells: linear molecules and covalently closed circular molecules. Both forms have been shown to be infectious (2, 3). A detailed model of reverse transcription in proviral synthesis has been recently proposed; in this model minus and plus DNA strands are elongated in a continuous fashion, yielding a double-stranded molecule containing 600-nucleotide direct repeats at both ends (4). These terminal repeat sequences appear to function in provirus integration and in the virus life cycle (5), and they may be necessary for efficient transformation of cells by viral transforming regions (6).

In this paper we present evidence that AMV contains two distinct RNA genomic species, presumably corresponding to AMV-associated helpers and the defective leukemogenic virus. In addition, we report conditions for the synthesis of double-stranded DNA complementary to the AMV viral RNAs. A preliminary report of this work has been made (7).

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MATERIALS AND METHODS

Materials. Freshly pelleted AMV and purified AMV DNA polymerase (reverse transcriptase) were provided through the Virus Cancer Program of the National Cancer Institute under contract N01 CP 33291. Rous sarcoma virus (RSV) was obtained from Electro-Nucleonics Laboratories (Bethesda, MD); methylmercury hydroxide, from Alfa Chemicals (Danvers, MA); poly(rU)-Sepharose, from Collaborative Research (Waltham, MA); endonuclease *Eco*RI-digested λ DNA and RNA blot transfer system, from Bethesda Research Laboratories (Rockville, MD); hexadecyltrimethylammonium bromide, from Sigma; unlabeled deoxynucleoside triphosphates, from P-L Biochemicals; [^{3}H]deoxycytidine triphosphates (15–30 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels), [^{3}H]deoxyguanosine triphosphate (5–15 Ci/mmol), reticulocyte lysate translation kit, En^{3}Hance, and Aquasol, from New England Nuclear; and deoxycytidine [^{32}P]triphosphate (350 Ci/mmol) and deoxyguanosine [^{32}P]triphosphate (350 Ci/mmol), from Amersham.

Extraction of Viral RNAs. RNAs were extracted from fresh viral pellets as described (8). Viral RNA was fractionated on sucrose gradients, and 70S RNA was precipitated with ethanol. Pellets were resuspended and heated to 90°C for 3 min, and the RNA was refractionated on sucrose gradients. The 35S RNA peak was precipitated and stored in ethanol until use. Individual RNA species were isolated from denaturing acid/urea/agarose gels by melting the agarose and extracting the RNAs with a quaternary ammonium detergent (9).

Agarose Gel Electrophoresis and Northern Hybridization Analysis. Methylmercury hydroxide and acid/urea/agarose gel electrophoresis were carried out as described (10), with the horizontal gel system of McDonnell *et al.* (11). Transfer of RNA to diazobenzyloxymethyl-paper and hybridization analysis were carried out as described by Alwine *et al.* (12). Nondenaturing gels and formaldehyde/agarose gels were run in a water-jacketed horizontal system obtained from Bethesda Research Laboratories. Gels containing ^{32}P-labeled DNAs were soaked in methanol for 2 hr, dried under reduced pressure, and subjected to autoradiography.

Cell-Free Protein Synthesis and Analysis by Polyacrylamide Gel Electrophoresis. One microgram of 35S RNA isolated from AMV and 1 μg of each of the individual RNAs extracted from acid/urea gels were used to direct protein synthesis in a rabbit reticulocyte lysate cell-free translation system (13).

Abbreviations: AMV, avian myeloblastosis virus; MAV, myeloblastosis-associated virus; RSV, Rous sarcoma virus; kb, kilobase(s); sDNA, second strand DNA (the complement of cDNA).

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[^{35}S]Methionine-labeled protein products were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis in the discontinuous buffer system described by Laemmli (14). Gels were fixed in 25% 2-propanol/10% acetic acid (vol/vol) solution, soaked in En^{3}Hance and water for 1 hr, dried under reduced pressure, and subjected to fluorography.

cDNA Preparation. The standard reaction mixture was prepared at 4°C and contained 50 mM Tris·HCl (pH 8.3)/10 mM $MgCl_2$/30 mM 2-mercaptoethanol/70 mM KCl, the four deoxynucleoside triphosphates (including the labeled species) at 500 μM $(dT)_{10}$ at 100 μg/ml, 4 mM sodium pyrophosphate, 35S RNA at 10 μg/ml and AMV DNA polymerase at 20 units/μg of RNA (7). The 35S RNA template was heated at 60°C for 2 min, quickly chilled, and then added to the mixture. After incubation at 42°C for 1 hr, the reaction was terminated by adding EDTA to 10 mM and sodium lauroyl sarcosinate to 1% (wt/vol), and the mixture was extracted with phenol/chloroform/isoamyl alcohol. cDNA products were fractionated on 10–30% linear sucrose gradient containing 0.1 M NaOH, 0.7 M NaCl, and 10 mM EDTA. Centrifugation was carried out at 40,000 rpm for 20 hr at 10°C in a Beckman SW 41 rotor. The gradient was fractionated, and peak fractions containing cDNA were pooled, neutralized with Tris·HCl (pH 7.5) to 50 mM and 5 M HCl to 0.1 M and precipitated with ethanol.

Duplex DNA Preparation. The reaction was carried out in a 100-μl volume and contained 50 mM Tris/acetate (pH 8.3), 10 mM $MgCl_2$, 30 mM 2-mercaptoethanol, 70 mM KCl, 500 μM deoxynucleotide triphosphates (including ^{32}P-labeled compounds), 4 mM sodium pyrophosphate, full-length [^{3}H]cDNA at 0.5–5 μg/ml, and AMV DNA polymerase at 20–30 units/μg. The reaction mixture was incubated at 42°C for 1 hr. The double-strandedness of the DNA was measured by S1 nuclease treatment as described (15). After phenol/chloroform/isoamyl alcohol extraction, the material was fractionated on neutral sucrose gradients under conditions used for 35S RNA preparation. The peak DNA fractions obtained from these procedures were precipitated with ethanol and stored at −20°C until further use.

Electron Microscopic Analysis of DNAs. Grids were prepared and DNA was deposited on grids by a modification of the method described by Westphal and Lai (16). DNA was brought into hyperphase solution [55% (vol/vol) formamide/2.7 M urea/9 mM EDTA/90 mM N-[tris(hydroxymethyl)methyl]glycine (Tricine)/90 mM NaCl], and the mixture was heated at 53°C for 30 s. It was immediately placed on ice. The mixture was then spread in a Kleinschmidt monolayer on deionized water (hypophase) (17). Collodion-coated grids treated with cytochrome *c* were used for picking up DNA by touching the spread hyperphase. After a washing with acetone, the grids were dried on filter paper. For content enhancement, the grids were rotary shadowed with platinum/palladium evaporated from an electron wire. Measurements were assigned by comparison with simian virus 40 DNA.

RESULTS

Analysis of RNAs from AMV on Denaturing Gels. Total RNA from freshly pelleted AMV was extracted and analyzed on denaturing agarose gels containing urea (Fig. 1). Three RNA species were detected by ethidium bromide staining from the total AMV RNA preparation (lane 1). A faster-migrating band was removed upon passage through a poly(rU)-Sepharose column that comigrated with the cellular 18S RNA. Quantitation of the relative amounts of the poly(rA)-containing RNA bands by densitometric analysis showed the slower-migrating species to be present in approximately 10-fold excess of the faster-migrating RNA. These two RNA species contain 7500 and 7000 ribonucleotides, respectively, as estimated on the basis of migration relative to RSV genomic RNA and rRNA markers.

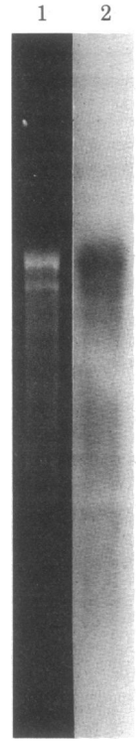

FIG. 1. Analysis of total AMV RNA on acid/urea denaturing 0.8% agarose gels. Lane 1, ethidium bromide-stained gel; lane 2, blot hybridization analysis of virus-specific RNA, utilizing a full-length cDNA transcript of poly(A)-containing RNA.

Blot Hybridization Analysis of Viral RNAs. The RNAs were further analyzed by the hybridization technique of Alwine *et al.* (12), utilizing two different [^{32}P]cDNA probes (Fig. 1). A full-length oligo(dT)-primed cDNA transcript of 35S RNA from AMV (prepared as described below) hybridized specifically to the two major RNAs isolated from AMV (lane 2). The two major RNA bands were obtained with cDNAs whose synthesis was primed by calf thymus DNA. Thus, because the 7.5- and 7.0-kilobase bands are of viral origin and exist in a ratio of 10:1, it is reasonable to assign the larger species to be the helper virus

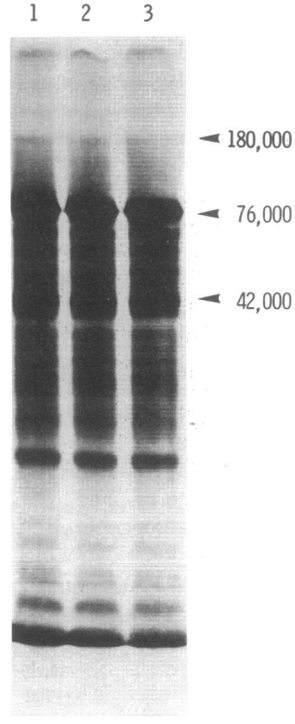

FIG. 2. Polypeptides obtained from *in vitro* translation of RNA isolated from AMV as displayed on $NaDodSO_4$/polyacrylamide gels. Lane 1, polypeptides obtained from the translation of total poly(rA)-containing RNA; lane 2, polypeptides obtained from 7.5-kb RNA; lane 3, polypeptides obtained from 7.0-kb RNA. In addition to the 42,000-dalton endogenous polypeptide product, the 180,000- and 76,000-dalton peptides could be detected in all three cases.

genome and the smaller RNA to be the defective AMV genome (18).

Purification and Cell-Free Translation of the Two Major Viral RNAs. The two viral RNA bands were individually extracted from acid/urea gels by partitioning the molecules into 1-butanol as their quaternary ammonium salts followed by recovery from aqueous solution as their sodium salts. Their intactness was confirmed by reelectrophoresis in a denaturing methylmercury hydroxide gel. The RNA species are nearly homogenous and about 90% intact. The purified RNAs as well as the mixture were translated in a rabbit reticulocyte lysate cell-free translation system (13). Analysis of [^{35}S]methionine-labeled translation products on NaDodSO$_4$/polyacrylamide gels showed prominent protein bands of 76,000 and 180,000 daltons, in all cases, which correspond to the "gag" and "gag-pol" polypeptides of the virus (Fig. 2). Thus the two RNA species appear to contain identical 5′ sequences of 4.9 kb.

Synthesis of Full-Length cDNA. The procedure used was a modification of the methods of Myers *et al.* (19). For the transcription of complete cDNA copies, the isolation of intact RNA template and the use of a concentrated AMV DNA polymerase preparations were important. Particular attention was paid to eliminate nuclease activity associated with the virus. As previously reported (20), the addition of 4 mM pyrophosphate causes a large shift in the size distribution, with 80% of the transcripts sedimenting at the size of the complete DNA copy (Fig. 3). The peak material was neutralized, precipitated with ethanol, and examined on denaturing gels. Two distinct species of DNA could be detected (Fig. 3 *Inset*); the larger species of 2.6×10^6 daltons represents a complete copy of the viral genome (19), and the smaller species is $2.0–2.3 \times 10^6$ daltons. Reactions in the absence of pyrophosphate result in low yields of complete cDNA transcripts (Fig. 3).

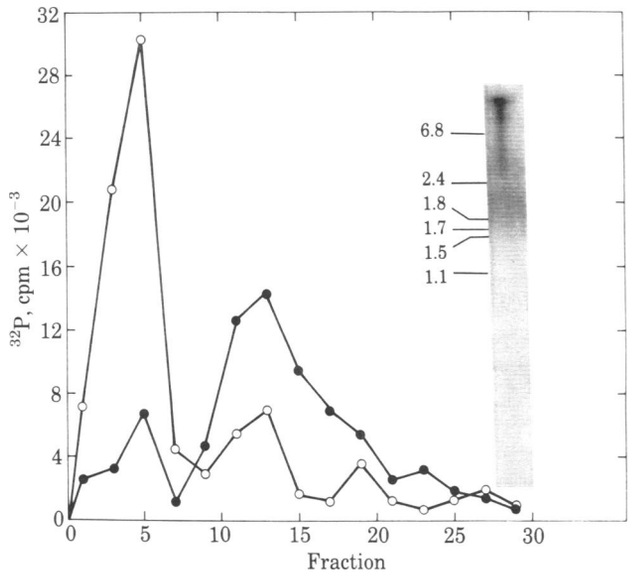

FIG. 3. Analysis of AMV cDNA products by alkaline sucrose gradient centrifugation and denaturing 1.4% agarose/4.4 M formaldehyde gel electrophoresis. Standard reverse transcriptase reaction mixtures containing (○) or lacking (●) 4 mM sodium pyrophosphate were prepared as described in the text. The reaction was terminated after 1 hr by adding EDTA to 10 mM and sodium lauroylsarcosinate to 1% (wt/vol). The aqueous layer was adjusted to 0.1 M NaOH, 0.7 M NaCl, and 10 mM EDTA. The samples were layered on an 11.2-ml 10–30% linear sucrose gradient containing 0.1 M NaOH, 0.7 M NaCl, and 10 mM EDTA. Centrifugation was for 20 hr at 10°C and 40,000 rpm in a Beckman SW 41 rotor. (*Inset*) Denaturing gel electrophoresis pattern of ^{32}P-labeled cDNA synthesized by using standard reaction conditions with 4 mM pyrophosphate and [^{32}P]dCTP (2.0 Ci/mmol) as the labeled compound. The product was treated and centrifuged as described above. Pooled fractions 2–7 were analyzed by electrophoresis for 2 hr at 100 V and bands were detected by autoradiography. An *Eco*RI λ phage DNA digest was used as markers and molecular weights × 10^{-6} are shown.

Synthesis of Linear Duplex DNAs. Second strand DNA (sDNA) synthesis of transcripts of several eukaryotic messages has been accomplished with *Escherichia coli* polymerase I or AMV DNA polymerase (21). With the optimal reaction conditions determined for the first strand synthesis, we examined cDNA-dependent kinetics of second strand synthesis by AMV DNA polymerase in the absence of oligo(dT) (Fig. 4). Samples removed at the times indicated were examined for DNA synthesis, as well as resistance to S1 nuclease digestion. There is an increase in DNA synthesis up to 30 min of incubation, beyond which no further synthesis occurs. After 30 min, almost 100% of the template is protected from S1 nuclease digestion. Inasmuch as sDNA synthesis occurs in the absence of exogenous primer, it strongly suggests the presence of a hairpin structure at the 3′ end of the cDNA. To verify the hairpin structure, we subjected cDNA to S1 nuclease digestion under nondenaturing and denaturing conditions (heating at 100°C for 1 min and then quickly chilling). Under nondenaturing conditions, 11% of the cDNA template behaves as though it is double stranded, and after heat denaturation half that amount appears to have regained its double-stranded structure (Table 1). DNA labeled with [^{3}H]dCTP in the first strand and [^{32}P]dGTP in the second was treated with S1 nuclease. Second strand synthesis increases the S1 nuclease resistance of the [^{3}H]cDNA, whereas the sDNA is totally resistant to digestion. Heat denaturation increases the susceptibility of the two strands to nuclease attack. However, more than half the double-stranded material regains resistance during the initial minutes of the reaction. This further suggests the presence of the hairpin structure, which facilitates rapid renaturation of the first and second strands. The double-stranded DNA synthesized by the second AMV DNA polymerase reaction was fractionated on a neutral sucrose gradient to determine the size distribution of the DNA chains. ^{3}H-Labeled first strand and ^{32}P-labeled second strand cosedimented

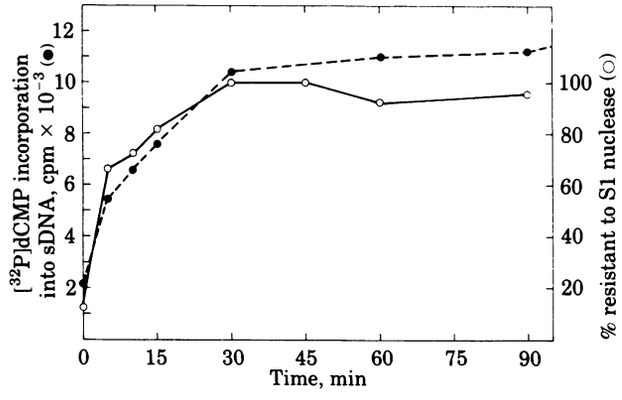

FIG. 4. Time course of AMV sDNA synthesis. [^{3}H]cDNA (66 ng) was incubated in a 100-μl reaction mixture with [^{32}P]dCTP as the labeled triphosphate (2.4 Ci/mmol). Three 2-μl aliquots were removed at the times indicated; one of these was precipitated with trichloroacetic acid to monitor incorporation of [^{32}P]dCMP into the second strand (●). The other two were used to determine the kinetics of acquisition of S1 nuclease resistance of the first strand (○). One aliquot was added to the standard S1 nuclease reaction mix containing enzyme, while the other was used in a control reaction lacking enzyme. Values represent the percent of acid-precipitable material remaining compared to the control reaction lacking enzyme.

Table 1. S1 nuclease analysis of cDNA and cDNA·sDNA

DNA sample	Conditions		Resistance, %	
	S1 nuclease	Denaturation	First strand	Second strand
[^{3}H]cDNA	−	−	100	NA
	+	−	11.2	NA
	+	+	5.3	NA
[^{3}H]cDNA·[^{32}P]sDNA	−	−	100	100
	+	−	91.9	100
	+	+	53.0	57.8

Under standard reaction conditions, 63 ng of [^{3}H]cDNA and 52 ng of [^{3}H]cDNA·[^{32}P]sDNA were subjected to S1 nuclease digestion. Samples were denatured by heating at 100°C for 1 min and then quickly chilling in ice. Values represent means from triplicate measurements of trichloroacetic acid-precipitable material. NA, not applicable.

as a sharp peak (Fig. 5*A*). Peak fractions were precipitated with ethanol and subjected to electrophoresis on nondenaturing agarose gels. Two distinct major species with molecular weights of 5.2 and 4.0 × 10^6 can be detected (Fig. 5*A Inset*). Electron microscopic analysis of DNA isolated from the neutral gradient showed linear duplex DNA molecules with a molecular weight of 5.2 × 10^6 (Fig. 5*B*). These species made up 20% of the double-stranded DNA duplexes synthesized.

DISCUSSION

AMV is a mixture of at least three viral species (22); AMV-associated helpers MAV-1 and MAV-2 and the defective AMV that is responsible for the leukemogenic process on specific myeloblast target cells.

In this report, we present evidence of two distinct RNA species from AMV. These differ in size by at least 500 bases and are present in viral preparations at a ratio of 10:1 (Fig. 1, lane 1). By analogy to related defective avian virus systems, the 7.5-kb RNA would represent the helper virus, while the 7.0-kb RNA should be the defective AMV genome (1). It is interesting to note that both genomic species have the identical 5′-end 4.9 kb, because both RNAs are translated into the "gag-pol" 180,000-dalton polypeptide (Fig. 2) (13). This would suggest that MAV and AMV genome organization are closer to that of the transformation-defective RSV genome and significantly different from the avian erythroblastosis virus and avian myelocytomatosis virus systems, in which an aberrant "gag-x" transforming polypeptide is encoded by the viral genome (23). Our data do not explain, however, the defectiveness or transforming nature of AMV, but they do predict that these functions are located within the 3′ 2 kb of the genome. The presence of the polymerase sequence in the AMV genome is reflected in the size of the AMV RNA (7.0 kb), which is larger than the genomes of other known acute viruses (5–6 kb). The specificity of the AMV to transform only hematopoietic cells could be embodied in the structural differences that exist between AMV and other acute leukemia viruses.

We have also established the conditions that allow the synthesis of the complete AMV linear duplex DNA with AMV DNA polymerase. Attempts to prepare cDNA from AMV RNA with reported conditions (19) resulted in low yields. Thus, we found it necessary to optimize the pertinent characteristics by using highly purified enzyme and intact RNA. AMV 35S RNA was isolated from freshly pelleted virus to maximize the yields of intact templates. Addition of pyrophosphate to reaction mixtures was necessary to suppress the residual nucleolytic activity expressed under transcription conditions and was accompanied by a striking increase in the average transcript size (Fig. 3).

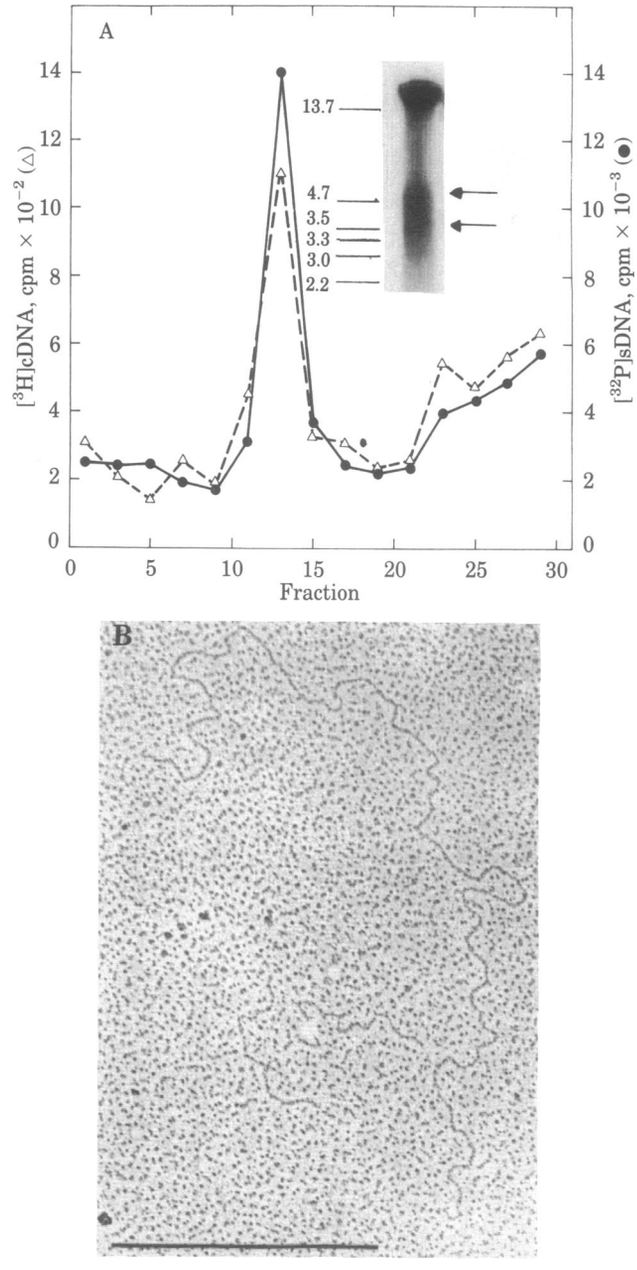

FIG. 5. Duplex DNA. (*A*) Gradient and electrophoresis. [^{32}P]sDNA was synthesized in a standard reaction mixture containing [^{32}P]dGTP (2.2 Ci/mmol) as the labeled compound and 71 ng of [^{3}H]cDNA as the template-primer. The recovered DNA samples were resuspended in 500 μl of gradient buffer and fractionated on 5–30% linear sucrose gradients. Samples (20 μl) were precipitated with trichloroacetic acid and filtered. Precipitates on the filters were monitored for ^{3}H and ^{32}P radioactivity. Peak fractions 10–15 were pooled from the gradient and precipitated with ethanol. The DNA precipitate was electrophoresed on a nondenaturing 1.4% agarose gel for 2 hr at 100 V. (*Inset*) Autoradiogram of the peak product, with positions of *Eco*RI-digested λ phage DNA markers indicated. Arrows on the right point to labeled DNAs with molecular weights of 5.2 and 4.0 × 10^6. (*B*) Electron microscopy. The DNA was viewed by using a Siemens electron microscope with a magnification of ×10,000. Size measurements were obtained by comparison to simian virus 40 DNA. The inserted bar represents 1 μm, which corresponds to 1 × 10^6 daltons under the conditions used.

Variation in the enzyme-to-RNA ratio also had a dramatic effect on synthesis of complete transcripts. Large copies are produced only at ratios of 20:1 or higher. Excess enzyme in reactions allowed for "read-through" of the entire RNA molecule. This may be due to enhanced binding of the template for the duration of

the polymerization process and transcription of regions with high secondary structure. Monitored characteristics showed that the reaction was rapid and independent of incubation temperature and deoxynucleoside triphosphate concentration.

For the synthesis of double-stranded cDNAs, published reports have described the self-priming ability of the cDNAs to synthesize the second DNA strand. AMV cDNA treated with S1 nuclease under nondenaturing and denaturing conditions contained double-stranded regions (Table 1). This suggested that the cDNA might be able to self-prime for second strand synthesis. AMV DNA polymerase readily catalyzed this reaction without the addition of exogenous primer, as monitored by the increased incorporation of ^{32}P-labeled deoxynucleotides and increased resistance of the cDNA template to single-strand specific S1 nuclease (Fig. 4 and Table 1). One product of this two-step reaction is a full-length duplex DNA (Fig. 5) that is 90–100% resistant to S1 nuclease. The 5.2×10^6 dalton species is, as far as we know, the largest DNA molecule yet synthesized in a reconstructed enzymatic system *in vitro*. Analysis of the viral RNA template (Fig. 1, lane 1), genomic length cDNA (Fig. 3), and linear duplex (Fig. 5) on agarose gels reproducibly demonstrates two distinct species of each. cDNA transcripts with molecular weights of 2.6 and 2.3×10^6 correspond closely in size to possible RNA templates of 7.5 and 7.0 kb, respectively. Likewise, duplex DNAs of 5.2 and 4.0×10^6 daltons would be predicted with the two cDNAs as templates.

All strains of *in vitro* transforming acute leukemia viruses tested so far are defective for replication and require a helper virus of the nondefective lymphatic-leukemia type (1, 23, 24). The AMV BAI-A strain is probably coharvested with a helper MAV; preparations of 35S RNA may therefore contain both AMV and MAV genomes. These could act independently as templates in the synthesis of cDNA transcripts, with the ultimate formation of two duplex DNAs. Similar findings have not been observed in earlier reports on the synthesis of cDNA of AMV (19). The two duplexes represent linear proviruses synthesized *in vitro*. Although these molecules lack the terminally repeated 600-base-pair sequences, the molecular cloning of the DNAs would allow for production of the total information encoded in the RNA genomes. Also, one can predict the presence of at least two proviral DNA species in AMV-infected cells of equal or larger size, depending on the number and size of the terminally repeated sequences present. Preliminary restriction enzyme analysis of the two duplex DNAs suggests that they are different in some portion of the molecule. The differences appear most certainly to exist at the 3′ portion of the genome due to the 5′ sequence homology shown by the translation of the RNAs.

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