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Genome Organization of RNA Tumor Viruses

I. In Vitro Synthesis of Full-Genome-Length Single-Stranded and Double-Stranded Viral DNA Transcripts

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Genome-length complementary DNA (cDNA) transcripts were synthesized in vitro by using purified virions of avian myeloblastosis virus, Moloney murine leukemia virus, and clone 124 mouse sarcoma virus. The size of the genome-length cDNA transcripts was measured on either alkaline sucrose gradients or alkaline agarose gels. The longest cDNA transcripts synthesized by using avian myeloblastosis virus, Moloney murine leukemia virus, and clone 124 mouse sarcoma virus were 7, 9, and 6 kilobases (kb), respectively. The in vitro system used was capable of synthesizing double-stranded DNA, but the plus strands (same polarity as the viral RNA) were only 0.5 to 1.2 kb long. Lone Moloney murine leukemia virus cDNA transcripts were used as templates to synthesize the second plus strand. Essentially two strategies were employed as follows. (i) The 3′ ends of the cDNA transcripts were extended by addition of 50 to 100 dAMP residues by terminal deoxynucleotidyl transferase. The $(dA)_n$-tailed cDNA transcripts were used as templates along with an oligomer of dT as primer and *Escherichia coli* DNA polymerase to synthesize the plus strands. (ii) DNase-digested calf thymus DNA was used to prime the synthesis of plus strands on long cDNA with *E. coli* DNA polymerase I. In both cases, the synthesis of the plus strands was monitored by increased resistance of the cDNA templates to single-strand-specific S_1 nuclease. The double-stranded DNA was fractionated on neutral sucrose gradients. Analysis of the double-stranded DNA synthesized by using oligo(dT) primer showed the plus strands to be about 5 to 6 kb long, whereas the plus strands synthesized by using DNase-digested calf thymus DNA primers were only 0.3 to 0.5 kb long. Double-stranded DNA synthesized by either method has an average size of 6×10^6 daltons. Double-stranded DNA was also synthesized by using cDNA transcripts as templates without the addition of any primers. In this case, the plus strands were covalently linked to the template strand and were not representative of the whole parent strand.

Infection by RNA tumor viruses requires the conversion of their genetic material into DNA (2, 43-45, 50, 57), which can be found in the cell after infection (13, 48, 59). The viral RNA is first transcribed into complementary DNA (cDNA), which in turn acts as a template to synthesize the second strand (17; H. E. Varmus, S. Heasley, H.-J. Kung, H. Opperman, V. C. Smith, J. M. Bishop, and P. R. Shank, J. Mol. Biol., in press). In cells infected with either avian or murine retroviruses, the supercoiled, circular, and linear forms of viral DNA have been identified (13, 15, 16, 30). The linear form of the viral DNA, however, represents a majority of the viral DNA identified in the infected cell (13, 15, 38, 48, 59). The negative strand (DNA strand complementary to the viral RNA) of the linear viral DNA appears to be of full genome length, whereas the positive strand (same polarity as the viral RNA) consists of small DNA transcripts (13, 38, 47, 48, 59). The conversion of the viral genetic material into cDNA is carried out by reverse transcriptase, which is encoded by the viral RNA (50, 57). Viral mutants defective in this function are unable to establish infection (24, 46, 49, 53, 56, 57). When purified virions are lysed with nonionic detergent and incubated with substrates, DNA is synthesized that has the nucleic acid sequence complexity of the entire viral genome (8, 11). However, the average size of the cDNA transcripts on alkaline sucrose gradients is about 4 to 5S (ca. 500 nucleotides) (11, 44). Several research groups have recently been able to synthesize small amounts of near-genome-length cDNA transcripts. Collett and Faras (6) observed that at a concentration of 200 μm of each

of the precursors, cDNA transcripts larger than 16S (5 kilobases [kb]) can be synthesized by using purified virions of Rous sarcoma virus. Junghans et al. (21), however, showed that the synthesis of long DNA transcripts complementary to Rous sarcoma virus can be synthesized if optimal detergent concentrations are used. Rothenberg and Baltimore (32, 33) have demonstrated that at high deoxynucleoside triphosphate precursor concentrations near full-length cDNA transcripts to murine leukemia virus (MLV) RNA can be synthesized. Meyers et al. (29) have reported the synthesis of large cDNA transcripts by using the reconstructed system of avian myeloblastosis virus (AMV) RNA as template, oligo(dT) as primer, and purified reverse transcriptase. Recently, Rothenberg et al. (34) have reported that the in vitro-synthesized cDNA using MLV is infectious.

Over the last years, we have been interested in studying the organization of the genomes of avian and murine RNA tumor viruses. We have taken the following two approaches: (i) to synthesize in vitro viral DNA and employ the use of restriction endonucleases to construct a physical map of the genome, and (ii) to form heteroduplexes between the full-length cDNA transcripts of a given virus and RNA from another virus and analyze them under an electron microscope. Both of these approaches require the availability of relatively large amounts of full-genome-length cDNA transcripts. In this manuscript, we describe the conditions of synthesis of large cDNA transcripts from AMV, a cloned isolate of Moloney MLV (M-MLV) and Moloney murine sarcoma virus (M-MSV), clone 124. The in vitro system described here is capable of synthesizing both strands of DNA; however, the second strand (plus strand) is synthesized in small fragments. Furthermore, long cDNA transcripts appear to be entirely single stranded. Our efforts to cleave full-genome-length cDNA transcripts with restriction endonucleases like *Hae* III or *Hpa* II, known to cleave single-stranded DNA (3, 18), were not reproducible. To overcome this problem, we decided to synthesize the second strand of DNA, using the cDNA strand as template. We have used two approaches to synthesize double-stranded viral DNA as follows. (i) The 3′ end of the minus strand is extended by 50 to 100 dAMP residues by employing the use of terminal deoxynucleotidyl transferase (terminal transferase [14, 20, 25, 35]). The dA-tailed cDNA transcripts are selected by binding to oligo(dT)-cellulose column and used as template, with an oligomer of dT as primer, and *Escherichia coli* DNA polymerase I (23). The reaction is monitored by determining the increased resistance of the cDNA strand to single-strand-specific nuclease S_1 (40, 58). The reaction is stopped when the template is over 70% S_1-resistant, and double-stranded DNA is isolated from the unreacted DNA by sedimentation on neutral sucrose gradients. (ii) The second approach is essentially that described by Taylor et al. (41, 42) to utilize DNase-digested calf thymus DNA as primers. The minus strand is incubated with calf thymus DNA primers, and *E. coli* DNA polymerase I and the reaction are monitored as described above. The double-stranded DNA is then isolated on neutral sucrose gradients. The advantage of the first procedure is that the plus strand is nearly the length of the template strand, whereas the plus strands synthesized by using calf thymus DNA primers are only 0.5 to 1.0 kb long. However, the second approach is more efficient and involves less manipulations. The results obtained by these two approaches and the usefulness of the double-stranded DNA in constructing physical maps by bacterial restriction endonucleases (31) are discussed.

MATERIALS AND METHODS

Viruses. AMV was obtained from Life Sciences Inc. through the courtesy of the Office of Program Resources and Logistics of the National Cancer Institute. Cloned isolates of M-MLV clone 1 obtained from Hung Fan and stocks of clone 124 MSV obtained from Karen Beemon were grown as described previously (12). The medium was harvested between 24 and 72 h and, after a low-speed spin (2,000 rpm in ICE), was stored at −70°C. Virions were purified as described before (51). The viral protein concentration was determined as described before (51), and reverse transcriptase activity was assayed by employing exogenous template as described (51). Purified virions from clone 1 M-MLV often showed two bands in discontinuous sucrose gradients. Both bands had reverse transcriptase activity. In general, viruses that showed less than 1,000 units of polymerase activity (1 unit defined in reference 52) per mg of viral protein were not used to make cDNA.

Unlabeled deoxyribonucleoside triphosphates were obtained from P. L. Biochemicals; ^{3}H-labeled dTTP was obtained from Schwarz/Mann; and ^{32}P-labeled deoxyribonucleoside triphosphates were obtained from ICN, Irvine, Calif. Oligo(dT)-cellulose (type 3) was obtained from Collaborative Research, Inc. Terminal transferase was a gift of John Abelson and for later experiments was obtained from Boehringer Mannheim Corp. Chymotrypsin-treated *E. coli* DNA polymerase I was also obtained from Boehringer Mannheim. Calf thymus DNA was obtained from Miles Laboratories, Inc.

Synthesis of cDNA. A typical reaction mixture contained 50 mM Tris-hydrochloride (pH 8.3), 10 mM dithiothreitol, 6 mM magnesium acetate, 60 mM NaCl, 2 mM each of dATP, dCTP, dGTP, dTTP, 0.02 to 0.2% Nonidet P-40 for avian viruses and 0.01% for murine viruses, and 2 to 3 mg of viral protein per 1.0

ml of the reaction mixture. Reactions were carried out in glass tubes and were flushed with N_2 to prevent oxidation of the reducing agent. At the end of the reaction, portions were withdrawn and the amount of acid-precipitable material was determined as described. If the reaction volume was less than 2.0 ml, it was chromatographed as described. If the reaction volume was less than 2.0 ml, it was chromatographed on a G-75 Sephadex column to separate unincorporated radioactivity and substrates. The column buffer contained 0.2 M LiCl, 0.01 M Tris-hydrochloride (pH 7.5), and 0.005 M EDTA. Portions from the effluent were withdrawn, and peak samples with radioactivity were combined and adjusted to a final concentration of 0.4 M NaCl, 1% sodium dodecyl sulfate, and 0.01 M EDTA. Equal volumes of phenol saturated with Tris-hydrochloride buffer, pH 8.0, were added to it and shaken vigorously. One volume of chloroform-isoamyl-alcohol was added, and the mixture was shaken and centrifuged in a bench top clinical centrifuge. The aqueous layer was reextracted with phenol and chloroform-isoamylalcohol, followed by extraction twice with chloroform-isoamylalcohol. The aqueous phase was withdrawn, adjusted to 40 μg of yeast RNA as carrier per ml, and nucleic acids were precipitated with 2.5 volumes of chilled ethanol. Nucleic acids were recovered by centrifugation in 15-ml Corex tubes at 15,000 × *g* for 20 min. The pellets were resuspended in standard buffer (10^{-2} M Tris and 10^{-3} M EDTA, pH 7.5). If the reaction volume was larger than 2.0 ml, then the nucleic acids were extracted with phenol-chloroform as described above and precipitated with ethanol. The precipitate was dissolved in small amounts of standard buffer and chromatographed on G-75 Sephadex column. Restriction endonuclease, *Bam* I-treated ^{14}C-labeled polyoma DNA, and *Hae* III-digested single-stranded phage φX174 DNA fragments did not undergo any change in size during the incubation period for cDNA synthesis, thus suggesting that the in vitro system is free of nucleases.

Addition of $(dA)_n$ residues. The reaction mixture in 0.05 ml contained 0.2 M potassium cacodylate buffer (pH 7.4), 1.6 mM β-mercaptoethanol, 2 mM cobalt chloride, 0.08 mM ^3H- or ^{32}P-labeled dATP, and cDNA transcript. The mixture was incubated at 37°C for 5 min, and 1 μl of sample was withdrawn to determine zero time incorporation. About 8 to 10 units of enzyme were used if less than 0.2 μg of cDNA transcripts was added in the reaction mixture. (The operational definition of a unit of terminal transferase obtained from the lab of John Abelson was the amount of enzyme required to add 50 dA or dT residues per μg of *Eco* RI-digested lambda DNA fragment [about 0.4 pmol of ends] in 5 to 15 min at 37°C.) The reaction mixture was incubated at 37°C for periods of time, depending on the amount of cDNA transcript added to the reaction. The number of nucleotides added was determined by the following formula provided by J. Beckman of the University of California at San Diego: number of nucleotides = $[c/(\text{number of ends} \times d)] \times [b/(a \times 10^{-6}\ \text{g})]$ where a = micrograms of DNA sample, b = molecular weight of the fragment, c = counts per minute incorporation, d = specific activity in counts per minute per picomole, and number of ends = 1 (as our long cDNA transcripts are single-stranded). The reaction was terminated either by addition of 4 μl of 0.5 M EDTA or 0.25 ml of binding buffer (see below).

Oligo(dT)-cellulose chromatography. This was performed as follows. About 100 mg of oligo$(dT)_{12-18}$-cellulose (type 3) was equilibrated in binding buffer containing 0.01 M Tris-hydrochloride (pH 7.4), 0.4 M NaCl (chelated), 0.001 M EDTA, and 0.5% sodium dodecyl sulfate. About 50 μg of yRNA was adsorbed to the column to prevent any nonspecific binding. The column was washed with 5 volumes of binding buffer, and the sample was loaded and allowed to adsorb for 30 min. The column was washed with 5 column volumes of binding buffer and eluted with elution buffer containing 0.01 M Tris-hydrochloride (pH 7.4) and 0.5% sodium dodecyl sulfate buffer. The material eluting with elution buffer was brought to a salt concentration of 0.2 M LiCl, carrier yRNA was added to a final concentration of 40 μg/ml, and nucleic acids were precipitated with ethanol.

***E. coli* DNA polymerase I reaction.** The reaction mixture in 0.1 ml contained 20 mM Tris-hydrochloride (pH 7.5), 4 mM dithiothreitol, 10 mM magnesium acetate, 60 mM NaCl, 1 mM each of the four deoxyribonucleoside triphosphates, and desired amounts of labeled precursor and template. About 200 pmol of $(dT)_{10}$ or $(dT)_{12-18}$ was added as primer when up to 400 pmol of $(dA)_n$-tailed cDNA transcripts was used as template. About 200 μg of DNase-digested calf thymus DNA primer was added to a reaction containing up to 400 pmol of cDNA transcripts. The DNase-digested calf thymus DNA primers were made as described (41, 42). The reaction was carried out at 22 to 23°C, and incorporation was measured by determining trichloroacetic acid-precipitable radioactivity. The double-strandedness of the material was measured by S_1 nuclease as described in the hybridization section. The reaction was stopped by addition of 4 μl of 0.5 M EDTA, and the unincorporated radioactivity was removed by chromatography on G-75 Sephadex columns as described above. If no radioactive precursor was added, the reaction was directly layered on a gradient.

Alkaline sucrose gradients. The DNA sample was adjusted to 0.3 M KOH and centrifuged in 5 to 20% alkaline sucrose gradients prepared in buffer containing 0.7 M LiCl, 0.3 M KOH, and 0.005 M EDTA, pH 12.4. After centrifugation for appropriate lengths of time, the gradients were collected by puncturing the bottom of the tube. Polyallomer tubes treated with a solution containing 3 μg of bovine serum albumin per ml, 10 mM Tris-hydrochloride buffer (pH 7.4), and 1 mM EDTA to reduce nonspecific sticking of single-stranded cDNA transcripts were used for centrifugation. If the cDNA synthesized was labeled with ^{32}P, the amount of radioactivity in each sample was determined by counting Cerenkov radiations. For ^3H-labeled DNA samples, portions were withdrawn and counted in scintillation fluid as described (54). Labeled form II of polyoma DNA (a gift from M. Vogt) was used routinely as the standard for estimating the size of the cDNA transcripts. The open circles of polyoma DNA sedimented at 16S, corresponding to an average size of 5 kb, as determined by the equation of Studier (39). The closed circles sedimented at 18S, representing the size of approximately 6.5 kb. All conversions of

S values to molecular weights were done by using the equation of Studier (39). Standards were either included in the gradient containing the sample or run in parallel gradients. The samples of interest were neutralized with acetic acid and precipitated with ethanol. Both LiCl and potassium acetate are highly soluble in ethanol.

Neutral sucrose gradients. Sucrose gradients (5 to 20%) were prepared in buffers containing 50 mM Tris-hydrochloride (pH 7.4), 1 M LiCl, and 5 mM EDTA. The samples were layered in a volume of 200 μl, and centrifugation was carried out in polyallomer tubes in the SW50.1 rotor for the desired amount of time. Forms I and II polyoma DNA (gift of M. Vogt) sedimenting at 20 and 16S (45) and full-genome-length cDNA transcript sedimenting at 38S were included as standards in parallel gradients. The gradient was fractionated by puncturing the bottom of the tube, and the distribution of DNA was determined by measuring radioactivity in various fractions. Fractions with peak radioactivity sedimenting between 18 to 21S (or lower S values if smaller cDNA transcripts were used as templates) were combined and precipitated with ethanol.

Agarose gel electrophoresis. (i) Alkaline gels. The horizontal gel system described by McDonnell et al. (26) was used with few modifications. The gel was made in buffer containing 30 mM NaCl and 2 mM EDTA, and the sample was loaded in the same buffer. The running buffer contained 30 mM NaOH and 2 mM EDTA, pH 12.6. The electrophoresis was carried out in a cold room (4°C) at 2 V/cm for 19 h. The buffer in the two chambers was circulated during the run to keep constant pH. At the end of electrophoresis, the marker was visualized with staining with ethidium bromide (100 μg/ml), and then the gel was dried under vacuum and autoradiographed. *Bam* HI-treated polyoma DNA and *Hind* III-digested lambda DNA were boiled in 0.3 N NaOH for 10 min and used as standards.

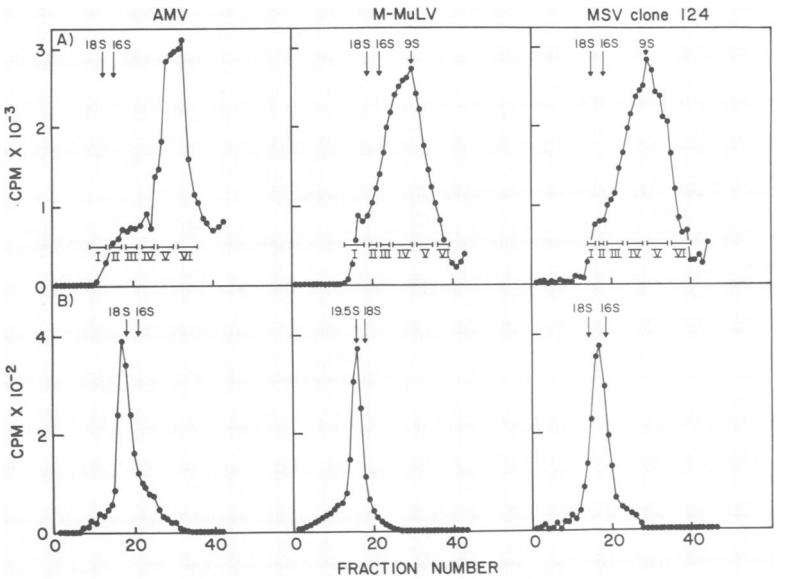
(ii) Neutral gels. Samples were subjected to electrophoresis in 1.2% agarose gels made in Tris-borate buffer, pH 8.0, as described (36). A horizontal gel apparatus was used, and the gel was 43 cm long and 4 mM thick. Samples were loaded in 25 μl of buffer containing bromophenol blue as dye marker. Lambda DNA digested with *Hind* III and adeno-2 DNA digested with *Sma* I were used as standards for determining molecular weights. The gel was dried under vacuum and subjected to autoradiography. The standards were visualized by staining the gel with ethidium bromide. Details of the gel electrophoresis procedure will be published in the accompanying paper (52).

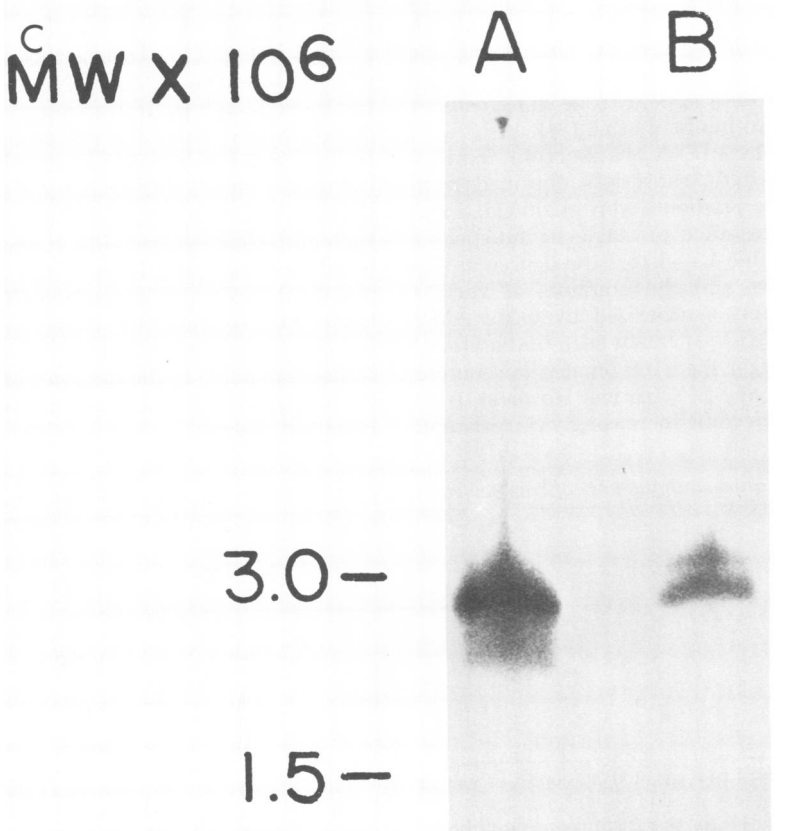
Hybridization. Hybridizations were carried out in buffer containing 0.5 M NaCl, 0.01 M TES buffer, pH 7.4, 0.001 M EDTA, and 0.1% sodium dodecyl sulfate. All solutions were chelated by chromatography through a column containing Chelex resin. Incubations were carried out in 5- to 20-μl samples at 68°C. The samples were layered with paraffin oil to prevent loss of material. After incubations for appropriate lengths of time, the samples were withdrawn and added to a 10-volume excess of buffer containing 0.25 M potassium acetate (pH 4.5), 0.01 M $ZnSO_4$, 20 μg of denatured calf thymus DNA per ml, and 5 μg of native calf thymus DNA per ml. The samples were divided into two parts. To one half was added 2 μl of S_1 nuclease (1 unit), and it was incubated for 90 min at 45°C. The other half was incubated at 45°C without enzyme. At the end of the incubations, residual acid-precipitable radioactivity was determined. To determine the degree of hairpins formed, samples were incubated for 1 to 2 min at 45°C and then immediately assayed for sensitivity to S_1 nuclease.

RESULTS

Size of the cDNA transcripts. Figure 1A shows the sedimentation profiles in alkaline sucrose gradients of the cDNA synthesized at optimum conditions by using purified virions of AMV, clone 1 MLV, and clone 124 MSV. The majority of cDNA transcripts sediment at about 8 to 10S. The largest fraction (fraction I) of the total cDNA transcripts synthesized by using AMV or clone 1 MLV (shown in Fig. 1a) were resedimented on alkaline sucrose gradients, and their profiles are shown in Fig. 1B. Fraction I of AMV cDNA sediments slightly ahead of 18S marker, representing a size of about 7 kb, which

FIG. 1. *Size analysis of cDNA transcripts on alkaline sucrose gradients and alkaline agarose gels. (A) Analysis of total cDNA. The reaction conditions described in the text were employed to synthesize cDNA to AMV, clone 1 M-MLV, and clone 124 MSV. Either [^{3}H]dTTP or [^{32}P]dTTP was included as the labeled precursor (specific activity 200 to 2,000 cpm/pmol), and the reaction was incubated for 8 to 16 h at 37°C. The cDNA was processed as described in the text and analyzed on alkaline sucrose gradients. Small portions from each gradient fraction were counted to determine radioactivity. Labeled polyoma DNA form II was included as a size marker. (B) Resedimentation of long cDNA transcripts. The regions of the gradient marked as fraction I in (A) were pooled, neutralized, and precipitated with ethanol. In the panel showing MSV cDNA, region II of the gradient was pooled. The samples were resuspended in alkaline buffer and centrifuged on 5 to 20% alkaline sucrose gradients in SW41 rotor for 15 h at 32,000 rpm at 22°C. The gradients were collected and assayed as described before (50). Labeled polyoma DNA form II was included as a size marker. (C) Electrophoresis of fraction I of clone 1 M-MLV cDNA on alkaline agarose gels. ^{32}P-labeled fraction I (about 800 to 1,000 cpm) obtained from alkaline sucrose gradients was neutralized, and the sample was taken in 20 μl of buffer containing 30 mM NaCl and 2 mM EDTA. Panels (A) and (B) represent fraction I from two different experiments. The molecular weights are determined by using phage λ DNA digested with restriction endonuclease Hind III and polyoma DNA digested with Bam I. A molecular weight of 3×10^6 shown in the figure is denatured Hind IIIB fragment of phage DNA, and 1.5×10^6 molecular weight is denatured linear form of polyoma DNA.*


AMV
M-MuLV
MSV clone 124
A)
B)
18S 16S
18S 16S 9S
19.5S 18S
CPM X 10⁻³
CPM X 10⁻²
I II III IV V VI
FRACTION NUMBER
0 20 40


C
MW X 10⁶
A
B
3.0
1.5

is very close to the 7.5-kb subunit size of AMV RNA (9). On the other hand, fraction I of clone 1 MLV cDNA sediments at about 19.5S, representing a size of about 9 kb (19), which is in agreement with the molecular weight of clone 1 MLV RNA of about 3×10^6. The average size of the genomic RNA of clone 124 MSV is 6 kb (7, 19). Resedimentation of fraction II of cDNA to clone 124 MSV (Fig. 1B) appears to have an average size greater than 16S (>5 kb). The virions of clone 124 MSV have been shown to contain 1 to 7% of the genome of helper MLV (7). It can be observed from the profile of total cDNA synthesized from clone 124 MSV that there is some material sedimenting ahead of 18S marker (6.5 kb).

Figure 1C shows the size of fraction I of clone 1 MLV cDNA made from two different preparations (A and B) on alkaline agarose gels. Fraction I appears to have two distinct bands of average molecular weights of 2.8×10^6 to 2.9×10^6 and 3.0×10^6 to 3.2×10^6. The ratios of the two bands differ in different experiments. Rothenberg et al. (34) have also recently reported that on alkaline agarose gels full-length cDNA transcripts of M-MLV have two size classes, one of which is about 600 nucleotides shorter than the other. Our data appear to be in agreement with those of Rothenberg et al. (34).

Nature of the cDNA transcripts. Various fractions of cDNA transcripts obtained from alkaline sucrose gradients were reannealed and assayed for resistance to single-strand-specific nuclease S_1. Table 1 registers the degree of single-strandedness and the amounts of various cDNA transcripts synthesized by using AMV and clone 1 M-MLV. It can be seen that material sedimenting larger than 16S on alkaline sucrose gradients is largely S_1 sensitive. However, fractions III to VI become increasingly S_1 resistant. Table 1 also shows the amounts of various fractions of cDNA transcripts synthesized by AMV and clone 1 M-MLV. Similar data were obtained from cDNA transcripts synthesized using purified virions of MSV (data not shown). The amount of full-genome-length cDNA transcript varies from experiment to experiment, but it is always greater than 5% of the total cDNA synthesized.

It has been reported that large amounts of in vivo-synthesized proviral DNA are linear (59). The negative strand of the linear proviral DNA appears to be of genomic length, whereas the positive strands (having the polarity of the genome) have an average size of 0.8 to 1.2 kb (59; Varmus et al., J. Mol. Biol., in press). We were interested in determining whether the second strand on DNA can be synthesized in vitro. The long cDNA transcripts appear to be entirely single stranded; however, the smaller cDNA transcripts appear to be quite S_1 resistant (Table 1). If the entire second strand of DNA was synthesized but had an average length of 0.5 to 1.2 kb, then on alkaline sucrose gradients most of the second strand will sediment in fractions IV to VI (Fig. 1A). To test this possibility, we decided to anneal AMV cDNA fraction V with fractions I and II. If fractions I and II were transcribed to make second strands of DNA and the transcripts were small in size, then annealing fractions I and II with fraction V should render them S_1 resistant. Table 2 shows the results of such annealing experiments. It can be seen that both fractions I and II have now become completely S_1 resistant, suggesting that they were transcribed to make the second strand of DNA, but the second strand was small in size. cDNA transcripts made in the presence of actinomycin D (100 μg/ml) are essentially all single stranded (data not shown).

TABLE 1. *Nature and amounts of various fractions of AMV and clone 1 M-MLV DNA transcripts*[a]

Fraction of cDNA	S	kb	Total S_1 resistance		% of total cDNA	
			AMV	Clone 1 M-MLV	AMV	Clone 1 M-MLV
I	>18	7–9	0	<2	5.5	6
II	>16	5–7	1	<2	6.5	8
III	>14	4–5	16	12	10	13
IV	>11	2–4	19	30	22	25
V	>7	0.75–2	80	19	31	30
VI	>3	0.25–0.75	60	15	36	18

[a] Approximately 2 to 3 ng (400–500 cpm) of various fractions indicated in Fig. 1A were pooled, neutralized with acetic acid, and annealed to a C_0t of 2.4×10^{-2} mol·s/liter under the conditions described in the text. Samples were assayed for S_1 resistance as described. The degree of hairpins formed was scored and was found to be less than 2 to 5%. The values shown in the table are given after subtracting the values of the hairpins formed. The percentage of each fraction present in the total cDNA was determined by dividing the amount of DNA in various fractions marked in Fig. 1A with total cDNA present in the gradient. The values are an average of at least 10 different experiments.

Addition of $(dA)_n$ residues at the 3′ end of long cDNA transcripts by terminal transferase. Figure 2 shows the kinetics of incorporation of [^{32}P]dAMP molecules at the 3′ end of ^{3}H-labeled full-genome-length cDNA transcripts synthesized by using purified virions of clone 1 M-MLV. It can be seen that the incorporation is linear with time and after incubation for 15 min an average of 50 to 60 nucleotides are incorporated. Unincorporated substrate was removed by chromatography on a G-75 Sephadex column, and the material eluting in the void volume was precipitated with ethanol. The [^{32}P]dA-tailed [^{3}H]cDNA transcripts were selected from the unreacted molecules or those [^{3}H]cDNA transcripts having less than 20 to 30 $(dA)_n$ residues by chromatography on an oligo(dT)-cellulose column. Table 3 shows that over 90% of the ^{3}H- and ^{32}P-labeled material was bound to the column, suggesting that a majority of cDNA transcripts contained at least 30 or more dAMP residues. The parent template does not bind to oligo(dT)-cellulose column, suggesting that it has no stretches of dA residues greater than 30.

TABLE 2. *Reannealing of ^{32}P-labeled AMV DNA in fraction V to ^{3}H-labeled AMV DNA in fractions I and II*[a]

Fraction no.	% S_1 resistance of ^{3}H-labeled cDNA
I	1
I + V	100
II	2
II + V	98

[a] ^{3}H-labeled fractions I and II (0.6 ng, 1,200 cpm) were annealed with 15 ng of fraction V (low specific activity, ^{32}P-labeled, about 200 cpm) in the buffer described in the text at 68°C to a C_0t of 7.2×10^{-2} mol·s/liter. The percent annealing was assessed by analyzing with S_1 nuclease. Fractions I and II, upon self-annealing, showed only 1 to 2% S_1 resistance.

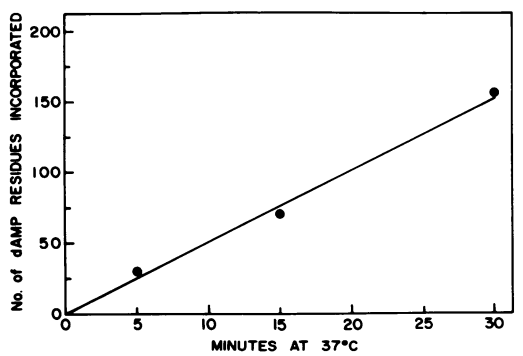


FIG. 2. *Kinetics of incorporation of [α-^{32}P]dATP by terminal deoxynucleotidyl transferase. The reaction mixture described in the text was used. About 0.2 μg of the genome-length clone 1 M-MLV cDNA transcripts obtained from alkaline sucrose gradients (Fig. 1B) was used as substrate, and 5-μl portions were withdrawn at various time intervals to determine trichloroacetic acid-insoluble radioactivity.*

TABLE 3. *Selection of $(dA)_n$-tailed cDNA transcripts on oligo(dT)-cellulose column*[a]

Sample	% Bound to oligo(dT)-cellulose column	
	^{3}H	^{32}P
[^{3}H]cDNA transcript (>7.5 kb)	0	
[^{32}P]$(dA)_{50-60}$ [^{3}H]cDNA transcripts	>90	>90

[a] ^{3}H-labeled cDNA transcripts were bound to oligo(dT)-cellulose (type 3) column before and after addition of $(dA)_n$ residues described in the text. The bound material was eluted with low-salt buffer as described. The amount of the material bound to the column remained constant even after two cycles of binding and elution from the column.

Figures 3a and b show the alkaline sucrose gradient profiles of the [^{3}H]cDNA transcripts before and after addition of ^{32}P-labeled $(dA)_n$ residues by terminal transferase. Because the ^{3}H-labeled parent strand before and after addition of ^{32}P-labeled $(dA)_n$ residues cosediments, we conclude that the [^{32}P]dAMP residues are covalently linked to the [^{3}H]cDNA transcript. Furthermore, the data indicate that the integrity of the template molecules remains unaffected during the reaction.

Synthesis of second strand of DNA by using $(dA)_n$-tailed cDNA transcripts as templates. The second strand of DNA is synthesized by using ^{3}H-labeled cDNA transcripts containing an average of 50 to 60 $(dA)_n$ residues at their 3′ end as templates, oligo$(dT)_{12-18}$ as primer, deoxyribonucleoside triphosphates including a labeled precursor, and *E. coli* DNA polymerase I. Figure 4 shows the rate of incorporation and the percent S_1 resistance of template at various time intervals during the reaction. It appears that maximum levels of incorporation are reached after 12 h of incubation at room temperature (23°C). At this point, the template is over 70% S_1 resistant. The ^{32}P-labeled plus strand of DNA is over 95% S_1 resistant, suggesting that the parent and the daughter strands remain hydrogen bonded. Both the amount of incorporation and the S_1 resistance of the template do not change significantly for the next 10 to 11 h of incubation. Addition of enzyme or substrates after 23 h of incubation stimulates some more incorporation. At completion of the reaction, the template is over 80% S_1 resistant, and the plus strand is about 85% S_1 resistant. Upon lone periods of incubation, the plus strand

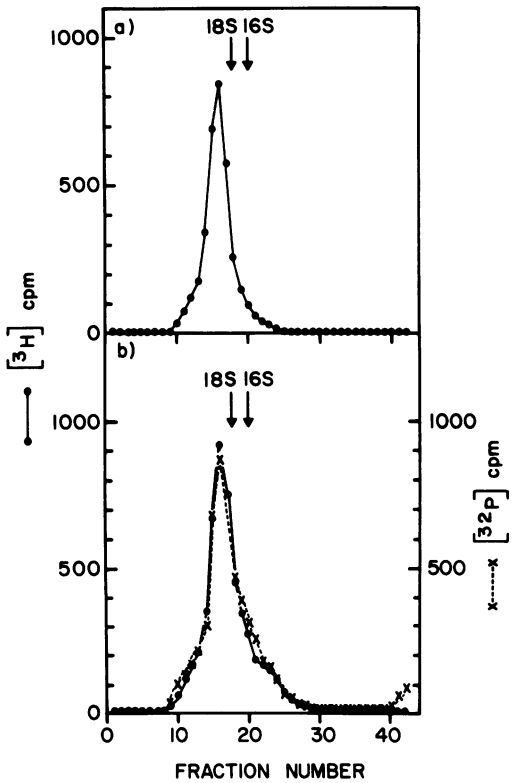


FIG. 3. *Size analysis of cDNA transcripts before and after the addition of $(dA)_n$ residues. A small fraction of ^3H-labeled M-MLV cDNA transcripts before and after the addition of ^{32}P-labeled $(dA)_n$ residues was analyzed by centrifugation on alkaline sucrose gradients. ^3H-labeled form II of polyoma DNA was used as marker in parallel gradients.*

appears to fall off the parent strand. This is not due to the 5'-3' exonuclease activity associated with *E. coli* DNA polymerase, as protease-treated *E. coli* DNA pol I (4, 22, 23) gives essentially similar data (data not shown). The net amount of plus strand of DNA synthesized under these conditions is about 80% of the input amount of the cDNA template.

Synthesis of the second strand of DNA by utilizing DNase-digested calf thymus DNA primers. Figure 5 shows the kinetics of incorporation and S_1 resistance of the template by using calf thymus DNA primers and cDNA transcript as template. The incorporation is considerably faster than that observed in the previous case, and the reaction essentially reaches saturation in 4 to 5 h. The reaction is monitored by increased S_1 resistance of the template and is terminated when the template is over 95% S_1 resistant. If the reaction is not terminated, incorporation continues, and the template appears to become less S_1 resistant.

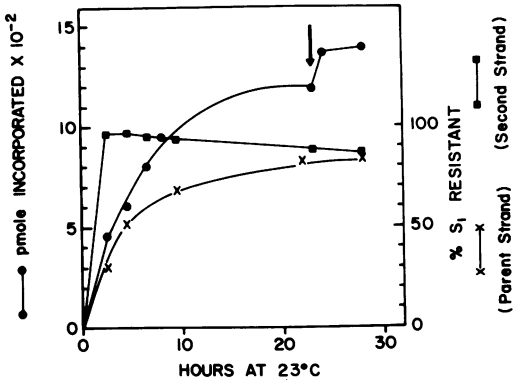


FIG. 4. *Kinetics of synthesis of plus strand of DNA by using $(dA)_n$ cDNA and oligo(dT) as template-primer. The reaction conditions described in the text were employed. The parent strand was ^{32}P-labeled, and ^3H-labeled dTTP was included in the reaction to monitor its progress. Portions were withdrawn at various time intervals and one-third of the portions was directly assayed for trichloroacetic acid-precipitable radioactivity, and the other two-thirds of the material was assayed for S_1 nuclease resistance of the parent strand and the second strand of DNA. The arrow indicates where additional E. coli DNA polymerase I was added.*

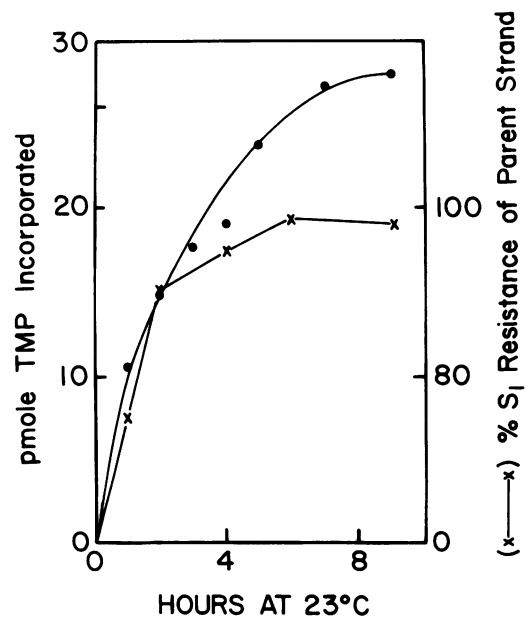


FIG. 5. *Kinetics of synthesis of plus strand of DNA by using DNase-digested calf thymus DNA primers. ^{32}P-labeled cDNA transcripts were used as template in the reaction mixture described in the text, along with [^3H]dTTP as the labeled precursor and DNase-digested calf thymus DNA primers. Portions were withdrawn at various time intervals and assayed for incorporation and resistance to S_1 nuclease.*

Isolation and characterization of double-stranded DNA. It has been reported that linear double-stranded DNA synthesized in vivo sediments between 18 and 21S on neutral sucrose gradients, whereas the parent strand sediments at 36 to 38S (13, 59). The double-stranded DNA synthesized by either of the two above methods was fractionated on neutral sucrose gradients, and the material sedimenting between 18 and 21S was isolated. Figure 6a shows the profile of double-stranded DNA synthesized by oligo(dT) primer and Fig. 6b displays the profile of double-stranded DNA synthesized by calf thymus DNA primers. It can be seen that a majority of double-stranded DNA synthesized by calf thymus primers sediments as a sharp peak, whereas the oligo(dT)-primed double-stranded DNA has a broader peak. The template DNA is included in the gradient shown in Fig. 6a and sediments at predicted values of 38S (13).

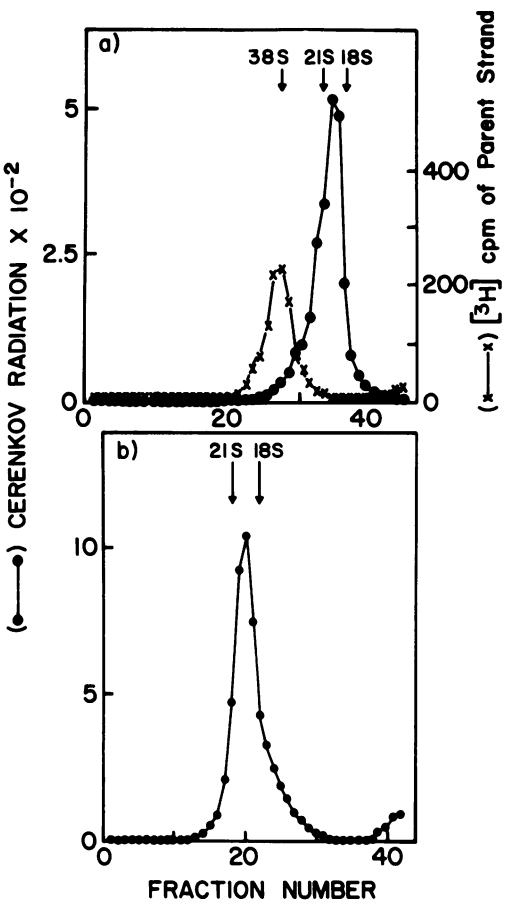


FIG. 6. *Fractionation of double-stranded DNA on neutral sucrose gradients. Upon completion of E. coli DNA polymerase I reaction, the samples were fractionated on 5 to 20% neutral sucrose gradients as described in the text. In parallel gradients, unreacted parent cDNA transcripts and forms I and II of polyoma DNA sedimenting at 38S (13), 20, and 16S (45), respectively, were used as size markers. The values of 21 and 18S shown in the figure were obtained by using 20 and 16S polyoma DNA markers as standards. (a) Double-stranded DNA synthesized by using oligo(dT) as primer. (b) Double-stranded DNA synthesized by using DNase-digested calf thymus DNA primers.*

Analysis of the material sedimenting faster than the 18 to 21S region of the gradient in Fig. 6a shows that the parent strand is full genomic length but the second strand is smaller in size. The larger the S value of the fraction, the smaller the second strand (data not shown).

Figure 7 shows the analysis of the size of the double-stranded DNA synthesized by either method on agarose gels. Figure 7a and b show the size of the full-genome-length parent strand before and after the synthesis of the second strand by using calf thymus DNA primers. The double-stranded DNA was completely resistant to S_1 nuclease. It has been shown before (Varmus et al., J. Mol. Biol., in press) that single-stranded DNA migrates faster on neutral agarose gels than double-stranded DNA. Double-stranded DNA synthesized by utilizing either calf thymus DNA primers (Fig. 7b) or by oligo(dT) priming (data not shown) has a molecular weight of 5.9×10^6 to 6.1×10^6, which is the observed size of the in vivo-isolated linear DNA. Figure 7c and d, show the electrophoretic profiles of fractional-length cDNA transcripts (molecular weight about 3×10^6 to 3.6×10^6) before and after the synthesis of the second strand. The distribution of DNA in Fig. 7d is quite broad because a broad size class of the parent DNA was used as template (Fig. 7c). Thus, it appears that regardless of the size of the parent cDNA transcripts, both methods are capable of synthesizing double-stranded viral DNA.

The peak fractions of double-stranded DNA isolated from neutral sucrose gradients appear to be over 95% S_1 resistant. Although the double-stranded DNA appears to sediment at predicted values and has an average molecular weight of 6×10^6, the data obtained by using either neutral sucrose gradients or agarose gels do not show that both strands are of full genomic length. To answer this question, we have analyzed the double-stranded DNA on alkaline sucrose gradients. Figures 8a and b show the profiles of DNA synthesized either by oligo(dT) primer or by DNase-digested calf thymus DNA primers. It can be seen that the template DNA (Fig. 8a) sediments at about 19S, whereas the plus strand of DNA synthesized by oligo(dT) primer sediments heterogeneously with an average size of 5 to 6 kb. Some fraction of the plus strands also

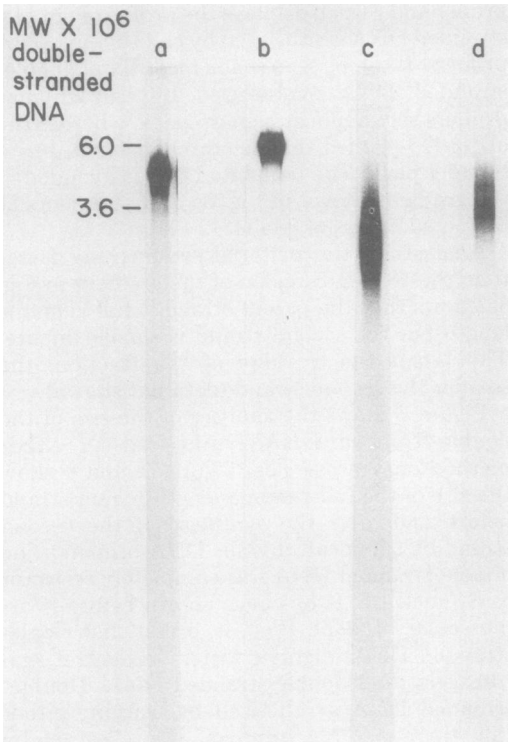


FIG. 7. *Size analysis of DNA by electrophoresis on agarose gels. The parent strand obtained from alkaline sucrose gradients was made double-stranded by using DNase-digested calf thymus DNA primers. It was fractionated on neutral sucrose gradients (see Fig. 6) and analyzed on 1.2% agarose gels. Electrophoresis was carried out at 2.5 V/cm for 16 h. Panels a and b show full-genome-length clone 1 M-MLV cDNA transcripts before (a) and after (b) being converted to double-stranded form. Panels c and d represent 3 × 10^6 to 3.5 × 10^6 long cDNA transcript of clone 1 M-MLV before (c) and after (d) the synthesis of the second strand. The single-stranded cDNA transcripts migrated ahead of double-stranded DNA on these gels. Autoradiographs of the dried gels are shown here. The two molecular weight markers shown are fragment B of phage lambda DNA digested with HindIII and HindIII-digested fragment A of M-MLV DNA.*

sediments along with the template strand. In Fig. 8b, the template cDNA transcript used was about 3.5 × 10^6 daltons. It seems to maintain its integrity during the reaction. However, the plus strands synthesized by calf thymus DNA primer appear to be no larger than 5S (0.3 to 0.4 kb).

Synthesis of double-stranded DNA without primer. It has been shown that various DNAs can form a hairpin and their 3′ ends can then serve as a primer to initiate DNA synthesis (10, 23). In the case of globin cDNA, both the reverse transcriptase from AMV and *E. coli* DNA polymerase I can make second strands of DNA, using the 3′ end of the hairpin as primer (28, 55). We were interested in determining whether the long cDNA transcripts of MLV can also be self-primed to make plus strands of DNA. Figure 9a shows the kinetics of incorporation and S_1 resistance of large single-stranded cDNA transcripts used as template along with *E. coli* DNA polymerase I. Maximum levels of incorporation are achieved after 8 to 10 h of incubation, and little incorporation is observed if the incubations are carried out for another 10 h. After 8 h of incubation, over 50% of the template becomes S_1 resistant. Further incubation with

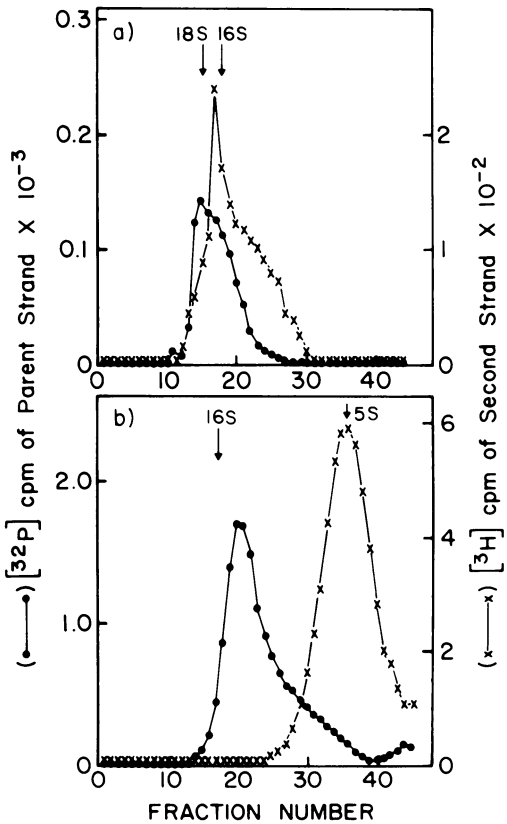


FIG. 8. *Analysis of double-stranded DNA on alkaline sucrose gradients. Fractions of double-stranded DNA obtained from neutral sucrose gradients were analyzed on alkaline sucrose gradients. (a) Double-stranded DNA made by using genome length $(dA)_n$-tailed ^{32}P-labeled cDNA transcripts and oligo$(dT)_{12-18}$ as primer. (b) Double-stranded DNA synthesized by using 4- to 5-kb-long ^{32}P-labeled cDNA transcripts as template and DNase-digested calf thymus DNA as primers. Labeled form II of polyoma DNA sedimenting at 18 and 16S was centrifuged in parallel gradients.*

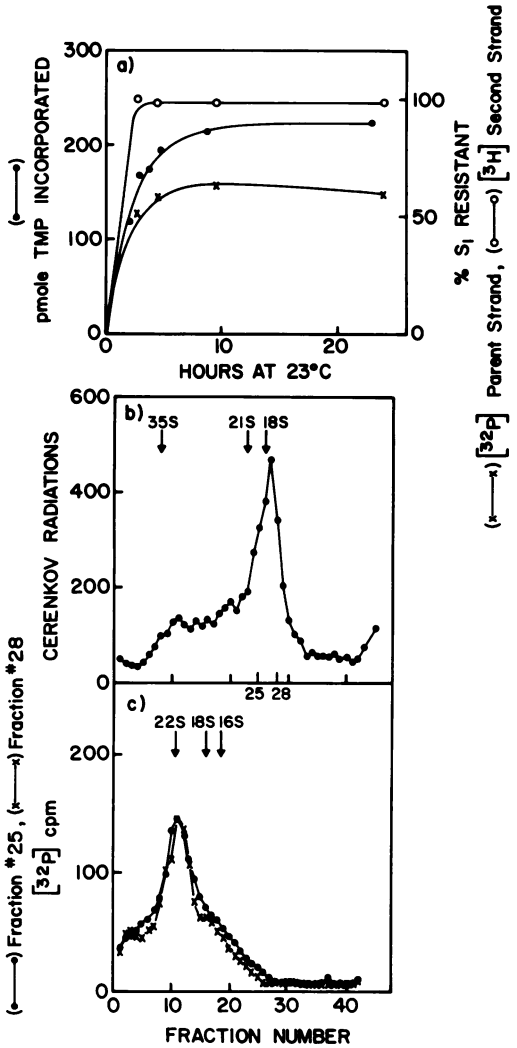


FIG. 9. *Kinetics of synthesis and analysis of double-stranded DNA synthesized without exogenously added primers. (a) Rate of reaction. The reaction conditions described in the text were used except that no primer was added. The cDNA transcripts used were ^{32}P-labeled, and [^{3}H]dTTP was included as labeled precursor to monitor the reaction. Portions were withdrawn at various time intervals, and a portion was assayed for trichloroacetic acid-precipitable radioactivity to determine the amount of incorporation. The other portions of the samples were assayed for resistance to S_1 nuclease as described in the text and the legend to Fig. 4. (b) Fractionation of the double-stranded DNA on neutral sucrose gradients. As described in the text and the legend to Fig. 6. Labeled forms I and II of polyoma DNA and 8-kb-long cDNA transcripts were used as standard markers for size analysis. (c) Analysis of double-stranded DNA on alkaline sucrose gradients. Fractions 25 and 28 from neutral sucrose gradients (b) were analyzed on alkaline sucrose gradients. Labeled polyoma DNA form II was included as size marker in a parallel gradient.*

more enzyme or substrate did not enhance S_1 resistance. The plus strand remains completely S_1 resistant during the reaction. If, however, cDNA transcripts of an average length of 5 kb are used as template, over 70 to 80% of the resulting hybrid appears to be double stranded (data not shown). Figure 9b shows the neutral sucrose gradient profiles of double-stranded DNA synthesized by utilizing approximately 7-kb-long cDNA transcripts as template primer and *E. coli* DNA polymerase I. At completion of the reaction, the parent strand was over 50% S_1 resistant. Compared with two previous methods of synthesizing the second strand of DNA, the double-stranded DNA sediments with a more heterogeneous profile. Over 20 to 30% of DNA sediments ahead of 21S, and some material sediments at a density of parent single-stranded DNA transcript. A large proportion of the material sedimented between 14 and 21S. Figure 9c shows the alkaline sucrose gradient profiles of materials sedimenting slightly behind (fraction 28) and slightly ahead of 18S (fraction 25) marker. The data indicate that in both fractions some of the DNA sediments at a position where the parent cDNA transcript sediments (ca. 19S). However, about 30 to 40% of the material sedimented ahead of 19S, with a leading edge at about 22S. Thus, it appears that the plus strand of DNA is covalently linked to the parent strand and, thus, sediments faster than parent cDNA on density gradients. Furthermore, this double-stranded DNA reanneals with zero order kinetics, suggesting that the two strands form hairpins. We have, however, so far never been able to make double-stranded DNA of an average size of 18 kb by this procedure. The largest double-stranded DNA sediments on alkaline sucrose gradients at about 22S (11 to 12 kb). Upon analysis on agarose gels, this double-stranded DNA migrates as a broad band with an average molecular weight of 4.5×10^6 to 5×10^6 (data not shown). If this double-stranded DNA is used as substrate for restriction endonucleases, the pattern of fragments generated is quite different from that obtained by using double-stranded DNA made by the other two methods. Some fragments, however, do appear to have the same molecular weights as those obtained from double-stranded DNA made by other methods (unpublished data).

DISCUSSION

Genome-length cDNA transcripts were synthesized in vitro by using purified virion of AMV, M-MLV and clone 124 MSV. The long M-MLV cDNA transcripts were then used as templates to synthesize the second plus strand of the viral DNA. This paper describes the characterization

and conditions for the synthesis of double-stranded viral DNA transcripts.

Synthesis of genomic-length cDNA transcripts. It appears that a small but reproducible amount of genome-length cDNA transcripts can be synthesized in vitro from various avian and murine RNA tumor viruses. The most crucial parameters required for the synthesis of long cDNA transcripts appear to be very high concentrations of precursors and an optimal concentration of divalent ion. In addition, it is important to determine the optimal detergent concentration for different viruses. Junghans et al. (21) have shown that full-length cDNA transcripts can be synthesized from Rous sarcoma virus at a low substrate concentration if low levels of detergent are used to lyse virions. We are unable to synthesize full-length cDNA transcripts at a concentration lower than 1 mM each of the four precursors. The amount of full-length cDNA transcripts synthesized in a reaction reflects the amount of intact full-length viral RNA molecules present in the virion. The data shown here are obtained from viruses in which the media were stored at −70°C before purifying the virions. Once the virions were purified, however, they were not frozen. In a parallel experiment, virion 70S RNA was isolated and heat denatured to generate 35S subunits. Less than 10% of the viral RNA sedimented at regions greater than 35S. Thus, it appears that the efficiency of transcription of genome-length cDNA transcripts depends on the state of genomic RNA.

The in vitro system is capable of synthesizing both strands of the viral DNA. However, the plus strand appears to be synthesized in small fragments. These results can be explained if one assumes that as the complementary cDNA minus strand elongates, the viral RNA is digested by RNase H associated with reverse transcriptase. This RNase H digestion leaves 3′-OH ends on the template, which can now serve as primer to initiate DNA synthesis. The viral reverse transcriptase-associated RNase H is an exonuclease and generates products which have 3′-OH and 5′-phosphate ends (50). This kind of mechanism will also suggest that the synthesis of the plus strand of the DNA will be observed only after the synthesis of the minus strand of DNA has been initiated. Furthermore, the synthesis of plus strands will be initiated before the synthesis of the minus strand is completed. We have isolated DNA at various periods after incubation and determined its degree of double strandedness by annealing and assaying for S_1 resistance. Our data suggest that the second strand of DNA does not initiate until the minus strand of cDNA is at least 1 to 1.5 kb in length (Fung and Verma, unpublished data). Varmus et al. (J. Mol. Biol., in press) have shown that in quail tumor cells acutely infected with avian sarcoma virus, plus strands were detected 45 min after infection. At this stage, the minus strand is only 1 to 2 kb long. Thus, contrary to the model presented by Haseltine and Baltimore (17), it is not necessary to complete the synthesis of the minus strand before initiating the synthesis of plus strands. Furthermore, the largest plus strands observed by Varmus et al. (J. Mol. Biol., in press) were about 0.5 to 1.3 kb long, similar to those synthesized in vitro. Thus, the in vitro data of synthesis of double-stranded DNA are very similar to those reported for viral DNA isolated in vivo from either avian sarcoma virus-infected or MLV-infected cells.

Synthesis of double-stranded viral DNA. Essentially two approaches were employed as described below.

(i) Extending the 3′ end of cDNA transcript with dAMP. The cDNA transcript was extended by addition of an average of 40 to 60 dAMP residues at the 3′-OH end with terminal transferase (14). The reaction is quite efficient; over 90% of the substrate gets adenylated with at least 30 dAMP residues as judged by its binding to oligo(dT)-cellulose columns (Table 3). In some experiments, we have also extended the 3′ end by addition of dTMP residues. Although cobalt is used as the divalent ion, it can be substituted by Mg^{2+}. The reaction with terminal transferase appears to be much less efficient if large amounts of carrier are present. Commercially available terminal transferase is generally very dilute, requires long periods of incubation, and, thus, enhances the chances of nicking of parent cDNA molecules.

The $(dA)_n$-tailed cDNA transcript can be transcribed into complementary strand by using an oligomer of dT as primer and *E. coli* DNA polymerase I. The primer can be as small as $(dT)_4$, and the largest primer we have used is $(dT)_{12\text{-}18}$. In some experiments we have used purified AMV reverse transcriptase to replace *E. coli* DNA polymerase I. However, our preparation of purified AMV DNA polymerase introduced at least one or two nicks per 10,000 nucleotides. Furthermore, for optimal DNA synthesis, using purified AMV reverse transcriptase, high substrate concentrations are required. One of the purposes of making a second strand was to make it of high specific activity so that we can use it for constructing physical maps of the genome. The requirement of high concentrations of precursors made the use of AMV reverse transcriptase less attractive. However, the reaction with AMV reverse transcriptase can be

carried out at 37°C and is essentially completed in 4 to 5 h. The AMV reverse transcriptase, in addition, is devoid of 3'-5' or 5'-3' exonucleolytic activities and, thus, will not digest full-length double-stranded DNA (50). In the experiments described here, the concentration of precursors used for *E. coli* DNA polymerase was quite high (1 mM each of four dNTP's). However, this is not necessary, and the reaction can be carried out at a concentration of 10 to 20 μM of each of the four precursors. We have recently synthesized double-stranded AKR viral DNA, where the plus strand was labeled with high-specific-activity ^{32}P-labeled dTTP using 15 μM of the labeled substrate. The reaction with *E. coli* DNA polymerase I is carried out at room temperature (22°C) to avoid formation of triplex structures. Because *E. coli* DNA polymerase does contain very potent 5'-3' exonuclease activity (23), which utilizes double-stranded DNA as substrate, we used chymotrypsin-treated *E. coli* DNA polymerase (4, 22, 23), which is devoid of 5'-3' exonuclease activity. No significant difference in the data was found, except that with protease-digested *E. coli* DNA polymerase I the rate of reaction was slower (data not shown).

(ii) Use of DNase-digested calf thymus DNA primer. This method has successfully been used to synthesize representative cDNA probes from avian and murine RNA tumor viruses (41). The DNase-digested autoclaved calf thymus DNA is about 4 to 8 nucleotides long (41). The most likely mode of synthesis of second strand of DNA is by displacement of newly synthesized strands. If the reaction is allowed to proceed past where the single-stranded template strand has become S_1 resistant, the amount of incorporation continues and a yield of second strand greater than that of input DNA is obtained. Although we have not tested it directly, the displaced strands can also act as templates. Recently Varmus et al. (J. Mol. Biol., in press) have also successfully used this approach to synthesize double-stranded DNA by utilizing genome-length minus strand isolated in vivo, after infecting quail tumor cells with avian sarcoma viruses as template.

(iii) Relative advantages of these two methods. The major advantage of the $(dA)_n$-tailing method is that very long transcripts of plus strand of DNA can be synthesized. This is so far the only method to obtain biochemically usable quantities of linear double-stranded viral DNA where both strands are of near genome length. The other available method is to convert in vivo-isolated circular viral DNA into linear form by restriction endonucleases, which have one cleavage site in the proviral DNA (60). Furthermore, this is a very useful method for us, as we would like to use this double-stranded DNA to generate deletion mutants by employing the methodology described for papova viruses (45). Another advantage of this method turned out to be the ease with which the 5'-terminal restriction fragment of in vitro-synthesized viral DNA could be located. If ^{32}P-labeled cDNA transcript was extended with [^{3}H]dATP, the fragment containing both ^{3}H and ^{32}P radioactivities must come from the 5' end of the viral genome.

The advantage of DNase-digested calf thymus DNA as primers is the efficiency of the reaction. The single-stranded cDNA transcript can be directly converted to double-stranded DNA without going through the manipulation of addition of $(dA)_n$ residues. The recoveries are generally higher, and the reaction reaches completion much faster. However, this method suffers from the fact that the second strand consists of small DNA fragments. It is perhaps possible to make long second strand by using DNase ligase (5, 37) to join the fragments.

Self-priming of cDNA transcripts. All DNA polymerases require primers to initiate DNA synthesis (1, 23, 44). It was first shown that T4 DNA can form hairpins and serve as a primer to synthesize the complementary strand (10). Subsequently, it has been shown that many in vitro-synthesized cDNA's can self-prime to make double-stranded DNA (27, 28, 55). Apparently the 3' end of the DNA folds back on itself to reanneal to some complementary part and provides a free 3'-OH end to form a phosphodiester bond with the incoming deoxynucleoside triphosphate. The hairpin loop remains, however, single-stranded. It appears that the size of the hairpin loop in case of full-length cDNA transcript as template is rather large. This may explain why the maximum S_1 resistance seen with over 7-kb cDNA transcripts is not more than 50%. Furthermore, the analysis of this covalently attached double-stranded DNA shows that the largest cDNA transcript is only 1.5 times the size of the parent molecule. If this double-stranded DNA is treated with S_1, the parent strand sediments at approximately half its size (unpublished data). Finally, if the self-primed double-stranded DNA is used as substrate for digestion with restriction endonuclease, *Bam* HI, a discrete band of approximate molecular weight of 1.25×10^6 is observed (data not shown). This fragment corresponds in size to the *Bam* IC fragment (52) which represents the 3' end of the M-MLV genomic RNA. Thus, it will appear that once the synthesis of the plus strand initiates, it will continue to elongate until completion. When cDNA transcripts of an av-

erage length of 4 to 5 kb are used as templates, over 75% of the resulting hybrid is S_1 resistant and upon analysis of alkaline sucrose gradient has an average size of 4 to 8 kb. We are now examining the size of the loop by electron microscopy and by restriction endonucleases. These studies, however, will not distinguish whether the in vivo mode of synthesis of second strand of proviral DNA is by self-priming of the minus strand or by use of template RNA as primer.

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