Structure of Murine Sarcoma Virus DNA Replicative Intermediates Synthesized In Vitro

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Moloney murine sarcoma virions synthesize discrete DNA products in vitro which closely resemble those found in vivo shortly after infection. These in vitro products have been isolated by electrophoresis and mapped with restriction endonucleases. In addition to the full-genome-length 6-kilobase pair linear DNA, a 5.4-kilobase pair circular DNA molecule, an incomplete linear DNA molecule, and a 600-base pair molecule were detected. The 6-kilobase pair DNA contained a 600-base pair direct terminal repeat which was missing from the circular form and was partially represented on the incomplete linear DNA molecule. The 600-base pair DNA contained sequences which were present in the 600-base pair direct repeat on the 6-kilobase pair DNA. The order of synthesis and the structure of these molecules detected in the in vitro reaction suggest that they are crucial intermediates in the formation of the final product of in vitro reverse transcription. A model which accounts for the synthesis of all of these molecules during the initial stages of viral replication is suggested.

The genetic information in retroviruses is contained in two apparently identical single-stranded RNA subunits; in the case of Moloney murine sarcoma virus (MSV), each is approximately 30S (12). The conversion of an RNA subunit into double-stranded DNA, which can integrate into the host genome and serve as a template for the synthesis of genomic and mRNA, is central to the life cycle of these viruses (see reference 3 for a review). Studies of viral DNA in infected cells of avian and murine systems have thus far revealed little about the initiation of the synthesis of either strand, but they have defined three major products of viral DNA synthesis: (i) a linear duplex containing (−) strands the length of an RNA subunit (5 to 10 kilobases [kb]) and (+) strands generally less than 2 kb in length (4, 16–18, 32, 38, 41, 47; unpublished data); (ii) covalently closed circular double-stranded DNAs of genome length and subgenomic length (20, 32, 37, 41, 49); and (iii) duplex DNA covalently integrated into the host genome (26, 32, 35). The unintegrated linear species appears first in the cytoplasm within a few hours after infection (46). It has been shown by pulse-chase experiments to be transported to the nucleus, where it is converted to closed circular DNA (39). The closed circular DNA is the putative precursor to the integrated form (20), but this relationship has not been established experimentally. In addition, during the synthesis of Rous sarcoma virus (RSV) DNA, a short discrete fragment of (+) strand DNA appears early after infection (47). The linear duplex DNA form in avian and murine systems contains a large terminal redundancy (300 to 600 base pairs [bp]) not found in viral RNA (2, 4, 25, 38).

Until recently, studies in vitro have succeeded principally in deciphering the initial events in the synthesis of the first, or (−), strand of viral DNA (complementary to the viral genome). Priming of the synthesis of this strand is performed by a tRNA species (tRNA^{Trp} in the case of avian viruses [21] or tRNA^{Pro} in the case of murine viruses [6, 23, 31]) which is hydrogen bonded to a subunit of the viral genome near its 5' terminus (see references 11, 42, 44, and 45 for a review). The polymerase reads to the end of the subunit, generating a relatively homogeneous class of short DNA transcripts called strong-stop DNAs (8, 14, 23) and then presumably continues synthesis on one of the two 3' termini present in each diploid viral genome. The observation that DNA synthesis is initiated near the 5' end of the genome means that some mechanism must exist for the elongation of this 5' transcript along the 3' end of the genome if the entire genome is to be copied. This mechanism presumably involves the short terminal redundancy found on the genomes of avian viruses (7, 8, 10, 24, 36, 40, 43) and murine viruses (6). Under appropriate conditions, full-length (−) strand DNA has been synthesized in vitro (27, 33, 34). Little is known about the details of the priming and synthesis of the second, or (+), strand of viral DNA.

Recently, Benz and Dina (2) and Gilboa et al. (19) have shown that purified detergent-disrupted virions from MSV and murine leukemia virus (MLV) supported the complete replication of genomic RNA to full-length double-stranded DNA. The major product was a 6-kilobase pair (kbp) MSV DNA (or an 8.8-kbp MLV DNA) that contained a 600-bp direct repeat at both ends of the genome. This repeat contained sequences copied from the 5' and 3' ends of the RNA genome. MSV DNA molecules synthesized in vitro were indistinguishable from those made in vivo shortly after infection. The presence of this repeat posed an additional constraint on retrovirus replication, implying that a mechanism must exist that allows reverse transcriptase to copy 5'- and 3'-terminal genomic sequences twice. In addition to the 6-kbp MSV DNA, we reported the synthesis of a circular form, a 600-bp fragment, and an incomplete linear double-stranded MSV DNA molecule during the course of the reaction. We report here a full characterization of the reaction products and a model for retrovirus replication in vitro that best accounts for their formation and order of appearance. The implications of this model and analogies to events occurring in vivo will be discussed.

MATERIALS AND METHODS

Growth of cells and virus stocks. The virus used in these studies was clone 124 of Moloney MSV propagated in TB cells (1, 12). The virus was grown in a Bellco autoharvester in HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid)–Dulbecco-modified Eagle medium supplemented with 10% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Medium was harvested every 6 h and pooled, and the virions were purified as described previously (12).

Synthesis of DNA in detergent-disrupted virions. Virions at a concentration of 5 mg of protein per ml were disrupted with Triton X-100 (final concentration, 0.028 to 0.030%) in a reaction mixture containing 50 mM Tris-hydrochloride (pH 7.5), 20 mM dithiothreitol, 2 mM MgCl$_2$, and the four deoxynucleoside triphosphates (dNTP's) at 1 mM each. The DNA was labeled with α-^{32}P-dNTP's (Amersham Corp. or New England Nuclear Corp.) at a specific activity of 5 to 50 cpm/pmol. Incubations were at 37°C for 16 to 24 h. These conditions were found to optimize the synthesis of full-length double-stranded products.

The DNA products were extracted with phenol saturated with 50 mM Tris (pH 7.5)–0.1 M NaCl–1 mM EDTA–0.1% sodium dodecyl sulfate and precipitated twice with 2.5 volumes of ethanol. This material was digested with an excess of S1 nuclease (5 U/μg of DNA; Sigma Chemical Co.) in 100 mM sodium acetate buffer (pH 4.5)–0.2 M NaCl–1 mM ZnCl$_2$ at 37°C for 30 min.

Isolation and purification of viral DNA. Double-stranded full-length viral DNA was isolated by electrophoresis on 1.5% neutral agarose (SeaKem,

HGT) horizontal slab gels (5 mm thick and 25 cm long) in 50 mM borate–1 mM EDTA buffer (pH 8) at 150 V for about 16 h. Restriction fragments of T7 DNA or phage α3 ^{32}P-labeled replicative-form II DNA served as molecular weight markers. The DNA was stained with ethidium bromide (0.1 μg/ml in 0.25 M ammonium acetate) for 30 min and visualized by UV illumination, and the bands were cut out. The DNA was eluted by crushing the agarose in 0.5 M ammonium acetate–10 mM EDTA–0.1% sodium dodecyl sulfate, incubating for 16 h at 37°C, and separating the agarose from the DNA by centrifuging the mixture over silanized glass wool in a micropipette tip. The DNA was precipitated twice with 2.5 volumes of ethanol at −70°C and collected by centrifugation. When necessary, DNA preparations were further purified by Bio-Gel A-5M chromatography in 4-ml Quik-Sep (Isolab) columns with 50 mM Tris–0.1 M NaCl–2.5 mM EDTA–0.1% sodium dodecyl sulfate–10% glycerol–0.01% diethylpyrocarbonate as the solvent, ethanol precipitated twice, and stored in 10 mM Tris (pH 7.5). The final yield was from 50 to 80% of the starting material.

Alkaline gel electrophoresis. Alkaline 1.5% agarose gels (either vertical [2 mm thick and 15 cm long] or horizontal [5 mm thick and 25 cm long]) in 30 mM NaOH–2 mM EDTA were run at 50 to 100 V for 8 to 30 h. Gels were stained with 0.1 μg of ethidium bromide per ml in 0.5 M ammonium acetate and exposed for autoradiography.

RESULTS

Since MSV is a replication-defective virus, stocks of MSV inevitably contain helper virus. To obtain preparations of in vitro-synthesized MSV DNA minimally contaminated with DNA transcribed from the helper virus genome, we utilized virus produced by the MSV 124 cell line (1), which produces a high ratio of MSV to helper Moloney MLV. This cell line has been found to produce the 9-kb helper virus genomic RNA in very small amounts (2%), whereas the remainder of the RNA molecules represent the 30S MSV genomic RNA of approximately 6 kb (12, 28, 29). Most of the viral DNA made by MSV 124 virions in vivo and in vitro has been shown to be MSV specific (2, 13).

Synthesis of (−) strand DNA in vitro. As observed with MLV, in conditions of low deoxynucleoside triphosphates (100 μM), 10 mM MgCl$_2$, and 40 μg of actinomycin D per ml, transcription of single-stranded genomic MSV RNA into DNA by the endogenous reverse transcriptase resulted in short DNA molecules complementary to the 5' end of the genome. These early transcripts (strong-stop DNAs) were the predominant early single-stranded DNA products of MSV transcription and were approximately 135, 75, and 50 bases in length (Fig. 1). The extension of (−) strand MSV DNA occurs under conditions of high dNTP concentration
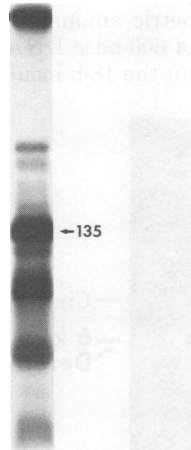
FIG. 1. *Early (−) strand DNA transcripts (strong-stop DNA). The reaction mixture contained 50 mM Tris-hydrochloride (pH 7.5), 10 mM MgCl₂, 100 μM dNTP's [α-³²P]dCTP, 20,000 cpm/pmol), 20 mM dithiothreitol, 40 μg of actinomycin D per ml, and 0.028 to 0.030% Triton X-100. The incubation was started with the addition of purified virions to a final concentration of 5 mg of protein per ml and lasted 60 min at 37°C. The reaction was extracted with an equal volume of saturated phenol (10 mM Tris [pH 7.5], 0.1 M NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate) three times, and the aqueous phase was precipitated with 2.5 volumes of ethanol at −70°C. The precipitate was washed with 70% ethanol, dried under vacuum, and suspended in 99% deionized formamide-0.1% xylene cyanol and bromophenol blue. The sample was electrophoresed at 10 W for 2.5 h on a 12% polyacrylamide-7 M urea gel in 90 mM Tris-borate (pH 8.3)-2 mM EDTA. The gel was exposed for autoradiography at −70°C with Kodak XR-5 film.*

(1 mM) and low divalent cation concentrations (Fig. 2A). Extension to a nearly full-genome-length product in the presence of actinomycin D required length (12 to 18 h) incubation at 37°C and resulted in little or no (+) strand synthesis. We detected no synthesis of the 9-kbp helper virus DNA. A comparison of MSV DNA synthesized in reaction mixtures with and without actinomycin D showed that the presence of actinomycin D resulted in a (−) strand DNA product 600 bases shorter than that synthesized when actinomycin D was absent. In addition, a 600-base DNA fragment synthesized in reactions not containing actinomycin D disappeared when actinomycin D was present (Fig. 2B). Similar results have been obtained for MLV (S. Mitra, personal communication).

Synthesis of double-stranded MSV DNA in vitro. Purified MSV virions synthesized S1 nuclease-resistant, genome-length, fully intact double-stranded DNA which contained direct repeated sequences derived from the 5′ and 3′
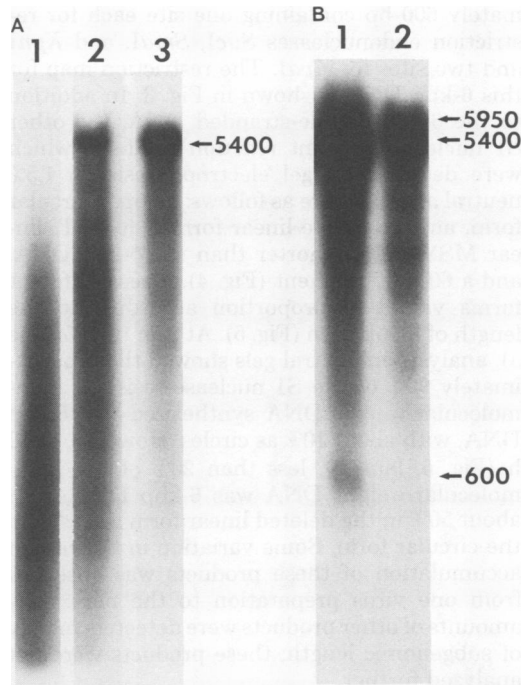

FIG. 2. *(A) Formation of high-molecular-weight DNA at different divalent cation concentrations. Reaction conditions were as described in the legend to Fig. 1 with the following exceptions: dNTP concentrations were 1 mM in each reaction, MgCl₂ was varied from 2 to 10 mM, and incubations were for 18 h at 37°C. The reaction mixtures were processed as described above but were fractionated on a vertical slab gel apparatus (1.5% agarose in 30 mM NaOH-2 mM EDTA; 15 cm long and 0.3 cm thick). Electrophoresis was at 50 V for 8 h, and autoradiography was performed as described above. (Lane 1) Products formed with 10 mM MgCl₂ and 1 mM dNTP's; (lane 2) products formed with 5 mM MgCl₂ and 1 mM dNTP's; (lane 3) products formed with 2 mM MgCl₂ and 1 mM dNTP's. (B) Effect of actinomycin D on product formation. Reaction mixtures were as described above with 2 mM MgCl₂ and 1 mM dNTP's. Incubations were for 18 h at 37°C, and products were extracted and fractionated on an alkaline gel as described in (A). (Lane 1) Reaction products made when actinomycin D was omitted; (lane 2) reaction products formed in the presence of actinomycin D.*

ends of the MSV RNA genome (2). This product, which represented 10 to 30% of the total DNA formed in the reaction, could not be distinguished from the linear double-stranded DNA which was extracted from cells shortly after infection with MSV. This synthesis represented replication of approximately 10% of the virion RNA present in the in vitro reaction. The repeat present on this 5,950-bp double-stranded DNA product (6-kbp DNA) consisted of approxi-

mately 600 bp containing one site each for restriction endonucleases SacI, SmaI, and KpnI and two sites for AvaI. The restriction map for this 6-kbp DNA is shown in Fig. 3. In addition to the 6-kbp double-stranded DNA, the other S1 nuclease-resistant reaction products which were detected by gel electrophoresis on 1.5% neutral agarose were as follows: an open circular form, an incomplete linear form ("deleted" linear MSV DNA) shorter than the 6-kbp DNA, and a 600-bp fragment (Fig. 4). These different forms varied in proportion according to the length of incubation (Fig. 5). At 24 h (Fig. 5, lane a), analysis on neutral gels showed that approximately 90% of the S1 nuclease-resistant high-molecular-weight DNA synthesized was 6-kbp DNA, with about 10% as circles. However, at 12 h (Fig. 5, lane b), less than 20% of the high-molecular-weight DNA was 6-kbp DNA, with about 50% in the deleted linear form and 30% in the circular form. Some variation in the rate of accumulation of these products was observed from one virus preparation to the next. Low amounts of other products were detected, mostly of subgenomic length; these products were not analyzed further.

Time course of appearance of different (+) strand DNA products. For determining the order of appearance of uniformly labeled (+) strand DNA products during the course of the reaction, samples of the reaction mixtures were withdrawn, processed as previously described, and analyzed by electrophoresis on denaturing alkaline agarose gels. On denaturing gels, open circular MSV DNA migrated as 5.4-kb linear DNA, shorter than the 6-kb DNA final product. This analysis (Fig. 6A and B) shows the results from time points at 0.5, 1, 2, 6, and 18 h and at an 18-h time point from a reaction mixture which included 40 µg actinomycin D per ml. It was apparent that at 1 h very little full-length (5,400- to 6,000-base) material had been synthesized (7% of total incorporation), but some 600-base DNA could be seen. The appearance of a barely visible 600-base band was significant since it would contain only 1/10 the radioactivity present in the full-length product if present in

stoichiometric amounts (Fig. 6A, lane 2). The amount of 600-base DNA appeared to increase throughout the 18-h incubation. At 2 h, a prom-
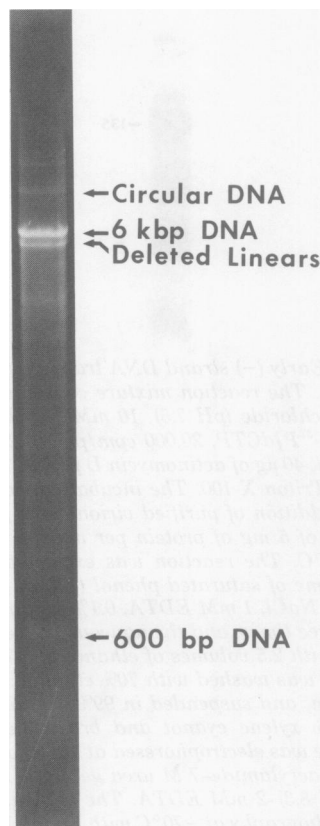

FIG. 4. *Analysis of in vitro-synthesized double-stranded DNA on a neutral agarose gel. The reaction mixture (3.0 ml) contained 50 mM Tris-hydrochloride (pH 7.5), 2 mM MgCl₂, 1 mM dNTP's ([α-³²P]dCTP, 40 cpm/pmol), 20 mM dithiothreitol, 0.030% Triton X-100, and 5 mg of viral protein per ml. Incubation was for 24 h at 37°C. The reaction mixture was phenol-chloroform extracted three times, ethanol precipitated, digested with S1 nuclease (5 U/µg of DNA), fractionated on a wickless horizontal "submarine" slab gel (1.5% agarose in 50 mM borate, pH 8.3), and stained with ethidium bromide as described previously (2).*
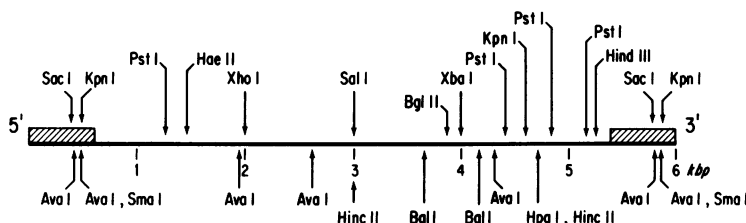

FIG. 3. *Restriction endonuclease map of MSV 6-kbp DNA. The data for this restriction map have been reported previously (2).*
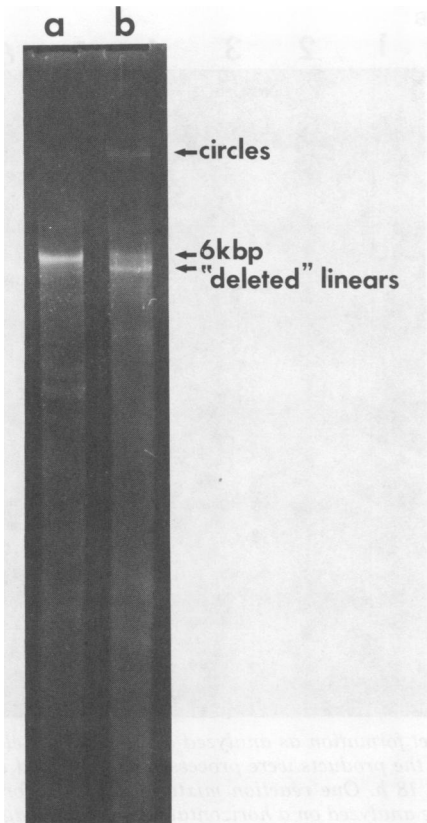
FIG. 5. *Formation of high-molecular-weight double-stranded DNA products in different proportions at 12 and 24 h of incubation. Reaction mixtures were as described in the legend to Fig. 4, and products were isolated and fractionated as described in the same legend, except this gel was electrophoresed on a Studier apparatus which had wicks (30). (Lane a) Incubation for 24 h; (lane b) incubation for 12 h.*

inent 5,400-base band was visible, whereas no 6-kb DNA had yet been synthesized (Fig. 6A and B, lane 3). At 6 h (Fig. 6A and B, lane 4), the 5,400-base band increased in amount, and for the first time 6-kb DNA appeared. By 18 h of incubation, both the 5,400-base DNA and the 6-kb DNA were present in roughly equivalent amounts.

This time course demonstrated two important points: first, the 600-base DNA appeared relatively early during (+) strand synthesis and was completely absent when (+) strand synthesis was inhibited by actinomycin D (Fig. 6A and B, lane 6); second, the formation of circular and deleted linear MSV DNA clearly preceded the formation of 6-kb MSV DNA. It should be noted that no detectable strong-stop DNA was observed at the earliest time points, i.e., 10 min (data not shown) and 0.5 h (Fig. 6A and B, lane

1). The formation of high-molecular-weight products is shown graphically in Fig. 7. It is apparent from this experiment and from the experiment shown in Fig. 5 that 6-kb DNA accumulated as the amount of 5.4-kb DNA (the deleted linear and circular forms of MSV DNA) decreased. From these experiments, several questions arose as to the nature of the products formed. (i) What was the polarity of the 600-base DNA which appeared early in the reaction? (ii) Did the 6-kb ultimate product contain both (+) and (−) strand DNA? The following experiments were designed to answer these questions. Additional experiments, to be described later, demonstrated the structure and the regions of the MSV genomes represented by these forms.

Polarity of the 600-base DNA and 6-kb DNA. To determine the polarity of the 600-base DNA fragment, we isolated this DNA from alkaline agarose gels. The purified fragment was then hybridized either to an excess of MSV viral RNA (+) strand or to an excess of (−) strand MSV DNA, which was prepared from reactions containing actinomycin D, and isolated from alkaline agarose gels. The 600-base DNA hybridized to (−) strand MSV DNA but not to MSV viral RNA, indicating that this molecule was of (+) strand polarity (Table 1). Similar analysis of the DNA eluted from the 6-kb band from alkaline gels demonstrated that both intact 6-kb (+) strand DNA and 6-kb (−) strand DNA were present in this product (Table 1).

Structure of circular forms of reaction products. Double-stranded open circular molecules of MSV DNA were generated when (+) strand synthesis was allowed to occur. These molecules were detected in neutral agarose gels and were shown to be noncovalently closed, as evidenced by their migration as nearly full-length (5.4-kb) MSV DNA when electrophoresed in alkaline gels. The finding that denatured circular molecules migrated as single-stranded DNA, 600 bases shorter than 6-kb DNA, raised the question as to whether these molecules represented circular 6-kbp DNA, from which the 600-bp repeat described above had been deleted. To test this hypothesis, we performed several experiments. Circular MSV DNA was eluted from neutral gels and digested with restriction enzymes that cleaved the 6-kbp DNA at only one site. The products of these digestions were run on neutral gels with 6-kbp DNA, incomplete linear DNA, and *HpaI*-cleaved T7 DNA as markers (Fig. 8A). The circular molecules cleaved at one site with *BglII* (Fig. 8A, lane 4) generated linear molecules about 600 bp shorter than the 6-kbp DNA. The presence of two minor bands corresponding to 4,000 and 2,000 bp probably reflected the slight contamination of the
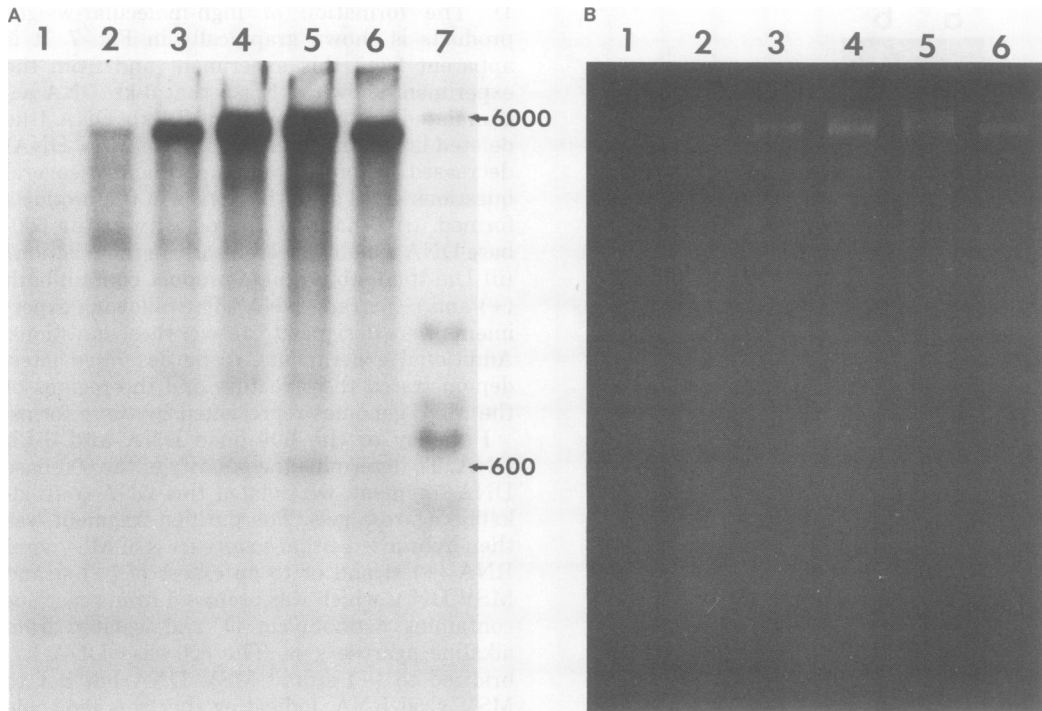
FIG. 6. *(A) Autoradiography of the time course of product formation as analyzed by alkaline gel electrophoresis. Reaction mixtures were as described above, and the products were processed as described in the legend to Fig. 4, except incubations were from 30 min to 18 h. One reaction mixture incubated for 18 h contained 40 μg of actinomycin D per ml. The products were analyzed on a horizontal slab gel (1.5% agarose in 30 mM NaOH–2 mM EDTA; 5 mm thick and 25 cm long) run for 30 h at 100 V. (Lane 1) DNA products formed in 30 min; (lane 2) DNA products formed in 1 h; (lane 3) DNA products formed in 2 h; (lane 4) DNA products formed in 6 h; (lane 5) DNA products formed in 18 h; (lane 6) products formed in an 18-h reaction containing actinomycin D; (lane 7) ^{32}P-labeled phage α3 replicative-form II DNA digested with HaeIII. (B) Ethidium bromide stain of the time course experiments. The same gel as shown in (A) was stained with ethidium bromide (0.1 μg/ml) in 0.5 M ammonium acetate buffer (pH 8). (Lane 1) DNA products formed in 30 min; (lane 2) DNA products formed in 1 h; (lane 3) DNA products formed in 2 h; (lane 4) DNA products formed in 6 h; (lane 5) DNA products formed in 18 h; (lane 6) products formed in an 18-h reaction containing actinomycin D.*

circular DNA with 6-kbp DNA. Circular MSV DNA digested with SacI (Fig. 8A, lane 5), which cleaves within the 600-bp repeat present on the 6-kbp DNA, generated a single 5,400-bp band. No 600-bp fragment was present. This suggested that the circular MSV DNA contained only one copy of the 600-bp repeat. As an additional test, circular DNA was cleaved with KpnI. According to the restriction map (Fig. 3), if the 600-bp repeat were deleted from one end, cleavage by KpnI should produce a 4,100-bp and a 1,300-bp fragment (Fig. 8A, lane 6). When the circular molecule was digested with a combination of enzymes which do not cleave within the repeat (XhoI and HindIII), a fragment (3,250 bp) extending in the 5′ to 3′ direction from the XhoI site to the HindIII site would be generated, regardless of the presence or absence of the repeat. In the 3′ to 5′ direction, a 2,750-bp fragment would result if the repeat were present, whereas this fragment would be shorter by the length of the deletion if the repeat were absent. The fragment shown in Fig. 8B, lane 1, is 2,150 bp, or 600 bp shorter. Similarly, when the circles are cut with PstI, a fragment of 2,150 bp would result if the 600-bp repeat were present. No fragment of this size was detected (Fig. 8B, lane 2). A 1,550-bp fragment was present instead, confirming the results obtained with HindIII and XhoI. All other fragments expected from cleavage with PstI were present. The presence in submolar amounts of additional bands corresponding to 750 and 800 bp (Fig. 8B, lanes 1 and 2) was probably the result of minor contamination of the circular DNA preparation with linear DNA. In all cases, no 600-bp fragment was gen-

erated from the cleavage of circular DNA by enzymes which cleaved within the repeated regions. From these data, we concluded that this product of the reaction was indeed circular MSV DNA, shorter by 600 bp than the 6-kbp DNA. This 600-bp deletion consisted of all, or nearly all, of the sequences comprising the direct terminal repeat present on the linear 6-kbp DNA molecules.

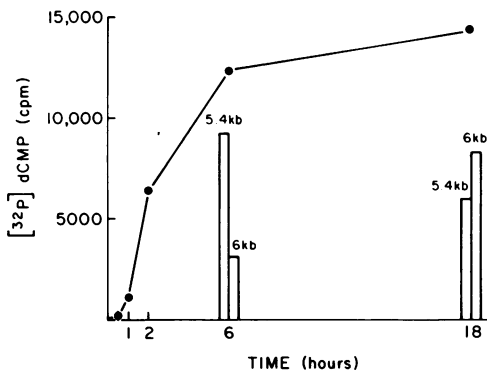## Structure of linear deleted double-

stranded MSV DNA. In addition to circular DNA, the 6-kbp DNA, and the 600-bp DNA, linear double-stranded molecules slightly shorter than 6-kbp DNA were detected (Fig. 4). The amounts of this product detected varied in inverse proportion to the amount of 6-kbp DNA formed (Fig. 5). Was this deletion similar to the deletion described above for the circular molecules? For testing this possibility, these molecules were fractionated and purified as described above for circular and 6-kbp DNA. Experiments were performed to determine the location and extent of the missing sequences.

In parallel digestions, completed 6-kbp DNA and deleted linear MSV DNA were digested with $XhoI$ and with the combinations of $XhoI$ plus $SacI$ and $XhoI$ plus $KpnI$. Digestion of 6-kbp DNA with $XhoI$ produced two cleavage products, a 2,000-bp 5' molecule and a 4,000-bp 3' molecule (Fig. 9, lane 3). Digestion of the deleted linear DNA produced the expected 4,000-bp 3' molecule, but the 5' cleavage product was heterogeneous and did not appear as a distinct band (Fig. 9, lane 4). Combined digestion with $KpnI$ plus $XhoI$ and with $SacI$ plus $XhoI$ demonstrated that both the $KpnI$ and $SacI$ sites were present on the deleted DNA since the internal fragments released were identical to those released from the completed 6-kbp DNA (Fig. 9, lanes 5 to 8). However, the short 5'-terminal fragments (480 and 400 bp) were only

FIG. 7. *Time course of incorporation of* ^{32}P *into high-molecular-weight products. High-molecular-weight products from reactions incubated for various times were processed and fractionated as described in the legend to Fig. 6. Bands were localized by ethidium bromide staining, cut out, and* ^{32}P *quantitated by Cerenkov radiation.*

TABLE 1. *Hybridization of 600-nucleotide-long complementary DNA to viral RNA and (−) strand DNA* [a]

Expt	DNA or RNA hybridized	Input cpm [b]	Cpm bound to hydroxylapatite (%) [c]
1. Self-annealing of 600-nucleotide DNA	600-nucleotide DNA	500	24.0
2. Annealing to (−) strand 5.4-kb DNA	600-nucleotide DNA plus (−) strand 5.4-kb DNA	500 plus 5,000	78.0
3. Annealing to viral RNA	600-nucleotide DNA plus viral RNA [d]	1,000 plus unlabeled RNA	12.0
4. Self-annealing of (−) strand 5.4-kb DNA [e]	(−) strand 5.4-kb DNA	5,000	13.5
5. Self-annealing of 6-kb DNA [f]	6-kb DNA	5,000	90.0

[a] All DNAs used in these experiments were eluted from the gel shown in Fig. 6. The 5.4-kb (−) strand DNA was made in the presence of actinomycin D. Annealing conditions for all of the experiments described above were as follows: 0.3 M sodium acetate (pH 6.1)–50% formamide at 42°C in a volume of 10 μl. All samples were annealed to a C_0t of 10^{-1} and assayed by hydroxylapatite chromatography.

[b] The specific activity of all DNA samples used in these experiments was 40 cpm/pmol of [α-^{32}P]dCMP. In experiment no. 2 the stoichiometry between 600-base DNA and (−) strand 5.4-kb DNA was 1:1.

[c] The amount of radioactivity bound to hydroxylapatite under the conditions used for this experiment does not measure the true extent of hybrid formation, but simply the presence of double-stranded regions on each molecule retained.

[d] The viral RNA input was 0.1 μg of unlabeled purified 70S MSV RNA.

[e] This value is a measure of intramolecular plus intermolecular annealing. The background binding of fully denatured (−) strand molecules at zero time was 11.5%.

[f] This value is a measure of intramolecular plus intermolecular annealing. The background binding of fully denatured 6-kb DNA molecules at zero time was 15.5%.
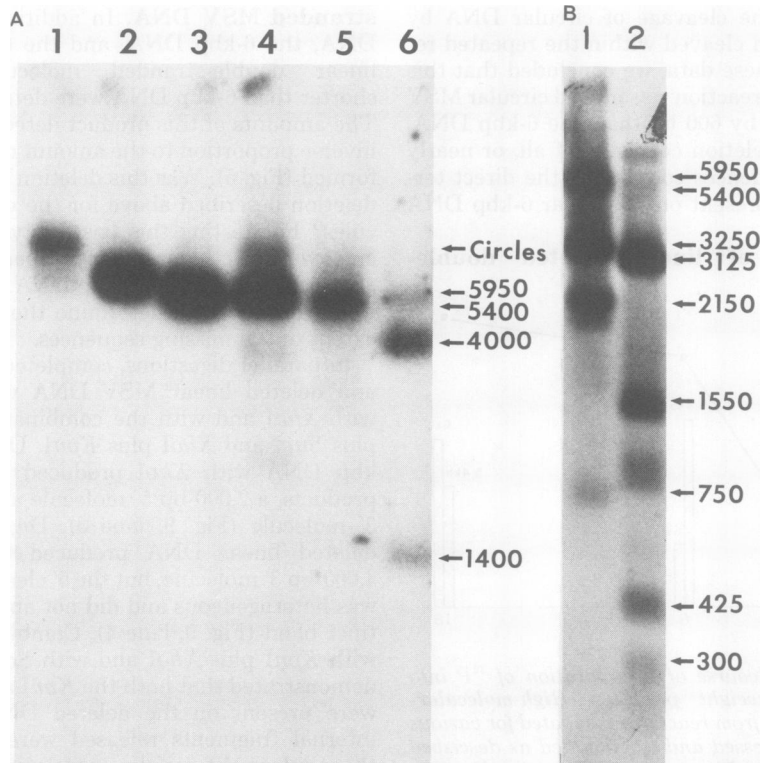
FIG. 8. (A) Analysis of circular MSV DNA. Reactions and processing of products were performed as described in the legend to Fig. 4. The band corresponding to circular DNA was cut out, eluted, and purified as described previously (2). Samples of this material were digested with restriction endonucleases. The cleavage products of these reactions were fractionated on horizontal 1.5% agarose submarine slab gels (in 50 mM borate [pH 8]–2 mM EDTA buffer; 5 mm thick and 25 cm long). (Lane 1) Circular MSV DNA, undigested; (lane 2) 6-kbp DNA, undigested; (lane 3) incomplete MSV DNA, undigested; (lane 4) circular MSV DNA digested with BglII; (lane 5) circular MSV DNA digested with SacI; (lane 6) circular MSV DNA digested with KpnI. (B) Analysis of 600-bp deletion in circular MSV DNA. Reactions, processing of DNA, and electrophoresis were performed as in (A). (Lane 1) Circular MSV DNA digested with XhoI and HindIII; (lane 2) circular MSV DNA digested with PstI.

present in digests of 6-kbp DNA (Fig. 9, lanes 5 and 7). The 5'-terminal fragments released from the deleted DNA did not appear on the autoradiogram as discrete bands. The 3' (right-hand) of the molecule appeared to be less heterogeneous since the 3' fragments released by these digestions formed distinct bands identical in size to those released from the completed 6-kbp DNA. We concluded from these experiments that the sequences missing from the deleted linear DNA derived primarily from the 5' (left-hand) end of the molecule and consisted of most, but not all, of the 600-bp repeat, since both SacI and KpnI sites were present on the molecule. This conclusion has an important consequence for the mechanism of MSV replication, which will be discussed below.

Structure of 600-bp DNA. The early ap-

pearance of a 600-bp DNA fragment detected apart from a 6-kbp DNA and 5,400-bp circular DNA (identical to the 6-kbp DNA but lacking a 600-bp terminal repeat) raised the intriguing question as to whether the 600-bp DNA consisted of sequences present in the repeat. To test this possibility, we isolated the 600-bp DNA and purified it from neutral agarose gels as described above. If the 600-bp DNA did represent the repeat, then digestions with restriction endonucleases SacI and AvaI should produce the cleavage products predicted from the restriction map of the repeat itself (Fig. 10). From the results of the experiments, we concluded that the 600-bp DNA did, in fact, consist of the repeated sequences found on the termini of the 6-kbp DNA. As predicted, SacI produced fragments of 400 and 200 bp, and AvaI produced fragments of 400
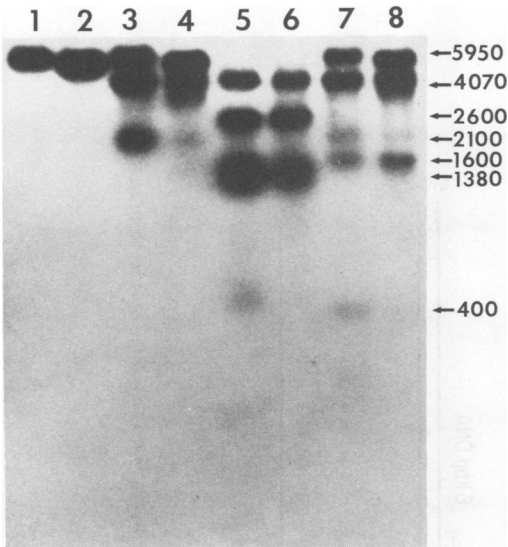
FIG. 9. Comparative analysis of incomplete linear and 6-kbp DNA. Incomplete linear MSV DNA and 6-kbp DNA were isolated, purified, and digested with enzymes to determine the location and extent of the sequences missing from the incomplete molecules. Gel electrophoresis was performed as described in the legend to Fig. 4. (Lane 1) 6-kbp linear MSV DNA, undigested; (lane 2) incomplete linear MSV DNA, undigested; (lane 3) 6-kbp DNA digested with XhoI; (lane 4) incomplete linear DNA digested with XhoI; (lane 5) 6-kbp DNA digested with XhoI and KpnI; (lane 6) incomplete linear DNA digested with XhoI and KpnI; (lane 7) 6-kbp DNA digested with XhoI and SacI; (lane 8) incomplete linear DNA digested with XhoI and SacI. T7 DNA cleaved with HpaI was run as a marker (data not shown).

and 125 bp. The shortest fragment produced by AvaI was not visible on the autoradiogram shown in Fig. 10.

The 600-bp fragment described here for MSV was very similar to the 600-bp repeat reported by Gilboa et al. (19) for MLV. However, the MSV 600-bp fragment lacked the XbaI site reported to be present on the MLV 600-bp repeat. We would also point out that a 600-base-long DNA was detected at low concentrations when isolated (−) strand DNA was used as a template for Escherichia coli DNA polymerase I. At present, the structure of this DNA has not been determined, and we do not know whether this 600-base DNA also represents the repeated sequence.

DISCUSSION

In this report, we have analyzed the intermediate DNA products generated in vitro by reverse transcription of MSV 124 RNA. The polarity and the physical location of these DNAs

with respect to the viral RNA genome have been established. Figure 11 outlines the proposed overall pathway of the synthetic process and depicts the main intermediate products that have been isolated and characterized. We now present a model which we believe accounts for all of the data.

(i) Initiation of (−) strand DNA synthesis: strong-stop DNA. The first discrete-sized DNA products of (−) strand polarity are a set of short DNAs about 50, 75, and 135 bases long (strong-stop DNAs) (Fig. 1 and reference 22). After reaching the 5′ end of the RNA template, DNA synthesis cannot continue without interruption on the same linear RNA molecule (Fig. 11a). It has been shown that further elongation of the (−) strand DNA chain depends on the removal of a short stretch of the 5′ end of the RNA template, presumably by RNase H activity (9, 15, 22).

(ii) Elongation of nascent (−) strand DNA by circularization via genomic terminal redundancy. The newly made strong-stop DNAs contain a short sequence (50 to 60 bases) at the
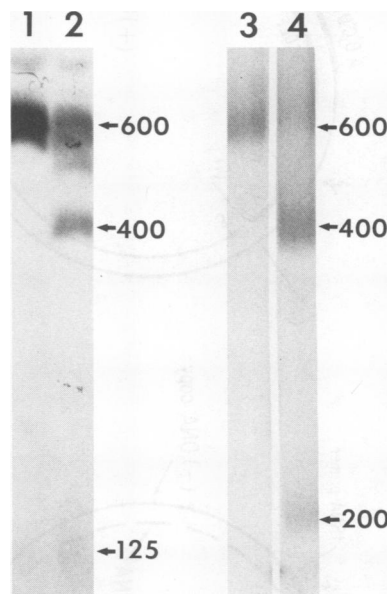

FIG. 10. Analysis of the 600-bp DNA. The 600-bp DNA was eluted from neutral agarose gels and purified. After restriction endonuclease cleavage, products were fractionated on a polyacrylamide-bisacrylamide gel (20:1, wt/wt) in 50 mM Tris-borate (pH 8.3)–1 mM EDTA (20 by 40 by 0.15 cm) at 35 W for 90 min. Autoradiography was done as described in the legend to Fig. 1. (Lane 1) 600-bp DNA, undigested; (lane 2) 600-bp DNA digested with AvaI; (lane 3) 600-bp DNA, undigested; (lane 4) 600-bp DNA digested with SacI.
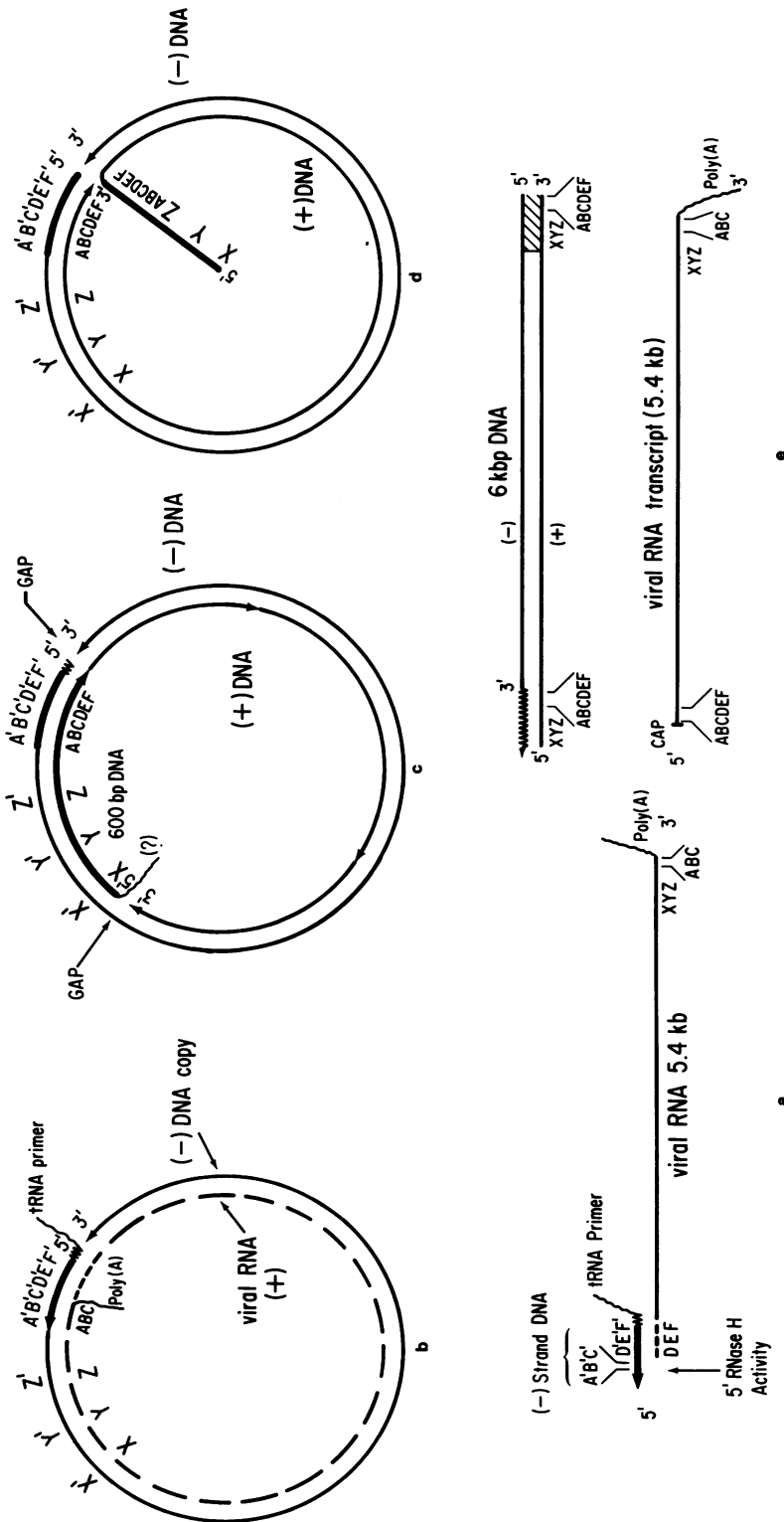
FIG. 11. *Proposed model for conversion of MSV viral RNA to double-stranded 6-kbp MSV DNA. Each step in this model is described in the Discussion. For clarity, the lengths of each intermediate are not drawn to scale.*

3' end which can anneal to a complementary RNA sequence located at the 3' end of the RNA genome, adjacent to the polyadenylic acid tail. Since these repeated sequences are annealed to the nascent (−) strand DNA they will be present only once in the newly made full-length DNA copy. DNA synthesis can now proceed in a 5' to 3' direction by elongating the strong-stop DNA back to the tRNA priming site (Fig. 11b). The product of this partial reaction is a 5.4-kb DNA molecule of (−) strand polarity. We have detected this molecule after as little as 1 h of incubation (Fig. 6A); it represents the main DNA product synthesized in the presence of actinomycin D (Fig. 2B).

(iii) Initiation of (+) strand DNA synthesis with the 600-base DNA and elongation of (+) strand DNA. We postulate that the priming of (+) strand DNA synthesis occurs at a specific site, leading to the formation of a 600-base molecule which extends as far as the 5' end of the nascent (−) strand DNA (Fig. 11c). The mechanism by which this strand is initiated is completely unknown. A 600-base molecule of (+) strand polarity has been found to accumulate in parallel with 5.4-kb (−) strand DNA (Fig. 6A). Moreover, we have isolated a double-stranded 600-bp molecule after S1 nuclease digestion of the total DNA made in vitro and have shown it to be a copy of the terminal repeat found on the 6-kbp DNA. Elongation of (+) strand DNA uses the (−) strand DNA as a template and proceeds up to the 5' end of the (+) strand DNA (Fig. 11c). The molecule formed in this process is a 5.4-kb open circle containing only one copy of the 600-bp repeat. The location of the gaps on the circular DNA is shown in Fig. 11. Circular molecules have been shown to constitute as much as 30% of the total high-molecular-weight double-stranded DNA made in vitro at 12 h (Fig. 5).

(iv and v) Displacement of (+) strand 600-bp DNA, regeneration of the short internal genomic redundancy, and completion of the 6-kbp DNA. Completion of the viral DNA to yield 6-kbp DNA could occur by displacement of either the (−) or the (+) strand 600-bp region. When the displacement is completed, the ends of the DNA are no longer base paired, and the circle will open. Reverse transcriptase can then fill in the remaining 600 bases on the single-stranded gap left by opening the circle (Fig. 11d and e). We suggest that this series of reactions begins by displacement of the (+) strand. In fact, when the (+) strand of the genome is displaced, a molecule with a complete right end (3' end of the viral RNA) repeat is formed, although the 5'

(left end) repeat has not yet been completed (Fig. 11e). We have found linear molecules that after digestion with S1 nuclease are shorter than the 6-kbp DNAs. These molecules have a copy of the 600-bp repeat at their 3' end (Fig. 9) and contain part, but not all, of the repeat at the 5' end. The displacement reaction is probably the rate-limiting step accounting for the relatively long lag between the accumulation of circular and incomplete linear forms and the appearance of the final product, the 6-kbp DNA. This incomplete linear molecule is the last proposed intermediate in the chain of events that leads to the formation of the 6-kbp MSV DNA.

Analogies with events occuring in vivo. We have previously shown that the DNA product of the in vitro reaction is indistinguishable from the 6-kbp DNA synthesized in vivo (2). Several other DNA intermediates have been described in other retrovirus systems in vivo which have been detected also in the course of the in vitro reaction. For example, in vivo observations by Varmus et al. (47) indicate that, as with MSV, (+) strand RSV DNA synthesis may depend on the early formation of a short (+) strand DNA fragment (300 bases long) which contains sequences from both the 3' and 5' ends of the RNA genome. This 300-base DNA presumably would represent a copy of the (+) strand RSV DNA repeat (38). Hsu et al. (25) and Shank et al. (38) have reported the presence of circular RSV DNA molecules with only one 300-bp repeat unit in RSV-infected fibroblasts. Yoshimura and Weinberg (49) have described similar circular molecules in MLV-infected cells. Although supercoiled circular DNA appears to be a common form of viral DNA in vivo (48), we have not found significant amounts of covalently closed full-sized circles synthesized in vitro. This could be explained by the fact that the in vitro reaction may lack some component indispensable for the enzymatic steps involved in the formation of supercoiled DNA molecules. Another discrepancy between the structures that we have detected in vitro and those detected in vivo is the appearance of circular DNA before the appearance of 6-kbp linear DNA. In vivo circular DNA has not been detected in the cytoplasm of infected cells, but has been detected in the nucleus after mature full-length viral DNA has already appeared in the cytoplasm. This could be explained by the fact that in vitro the reaction which leads to the formation of the 6-kbp DNA from the circular DNA requires displacement of the (+) strand DNA representing the 600-bp repeat. In vitro this reaction appears to be rate limiting in the formation of 6-kbp DNA, taking

4 to 12 h, which suggests that a critical component of this reaction is present in limiting amounts. In vivo this step may not be rate limiting, and circular DNA seen as a discrete intermediate in vitro may only be a transient intermediate in the cytoplasm.

Implications of the model. The proposed model has several implications, as follows.

(i) The mechanism of growth of the (−) strand viral DNA involves a base-pairing step that causes the specific loss of the short direct repeat present at the 5′ and 3′ termini of the viral RNA. Displacement and synthesis of the direct terminal repeat at the end of the replication cycle, a process which copies a region of the genome extending beyond the 5′ terminus, allow for the recovery of the sequences which would otherwise be lost.

(ii) The viral RNA transcript begins and ends within the short terminal repeated sequences shown as ABC in Fig. 11e. If viral RNA is derived from a larger nuclear precursor, these areas must contain processing sites. If viral RNA is the primary product of transcription, the 600-bp repeat must contain both promoter and terminator sequences.

(iii) The simplest model that accounts for the data reported here and by others (E. Gilboa, personal communication) need employ only one subunit to generate 6-kbp DNA. However, it is possible that the RNA subunit which is used as a template for (−) strand elongation is not the same subunit on which strong-stop DNA was synthesized. Additionally, the two subunits present in the virion may serve the function of preserving fidelity during transcription. As suggested by Coffin (5), alignment of the subunits during (−) strand synthesis would allow the polymerase to switch strands at a nick or gap in the original subunit and to continue transcription on the other subunit. Additionally, although Fig. 11 shows (+) strand synthesis beginning after the completion of (−) strand DNA synthesis, it is necessary for (−) strand synthesis to proceed only as far as X′, which represents the 5′ terminus of the 600-base early (+) strand DNA, for (+) strand synthesis to begin.

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