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Synthesis of Plus Strands of Retroviral DNA in Cells Infected with Avian Sarcoma Virus and Mouse Mammary Tumor Virus†

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The vast majority of plus strands synthesized in quail cells acutely infected with avian sarcoma virus were subgenomic in size, generally less than 3 kilobases (kb). A series of discrete species could be identified after agarose gel electrophoresis by annealing with various complementary DNAs, indicating specificity in the initiation and termination of plus strands. The first plus strand to appear (within 2 h postinfection) was similar in length to the long redundancy at the ends of linear DNA (0.35 kb), and it annealed with complementary DNAs specific for the 3′ and 5′ termini of viral RNA (Varmus et al., J. Mol. Biol. **120**:50–82, 1978). Several subgenomic plus-strand fragments (0.94, 1.38, 2.3, and 3.4 kb) annealed with these reagents. At least the 0.94- and 1.38-kb strands were located at the same end of linear DNA as the 0.35-kb strand, indicating that multiple specific sites for initiation were employed to generate strands which overlapped on the structural map. We were unable to detect RNA linked to plus strands isolated as early as 2.5 h postinfection; thus, the primers must be short (fewer than 50 to 100 nucleotides), rapidly removed, or not composed of RNA. To determine whether multiple priming events are a general property of retroviral DNA synthesis in vivo, we also examined plus strands of mouse mammary tumor virus DNA in chronically infected rat cells after induction of RNA and subsequent DNA synthesis with dexamethasone. In this case, multiple, discrete subgenomic DNA plus strands were not found when the same methods applied to avian sarcoma virus DNA were used; instead, the plus strands present in the linear DNA of mouse mammary tumor virus fell mainly into two classes: (i) strands of ca. 1.3 kb which appeared early in synthesis and were similar in size and genetic content to the terminally repeated sequence in linear DNA; and (ii) plus strands of the same length as linear DNA. A heterogeneous population of other strands diminished with time, was not found in completed molecules, and was probably composed of strands undergoing elongation. These two retroviruses thus appear to differ with respect to both the number of priming sites used for the synthesis of plus strands and the abundance of full-length plus strands. On the other hand, the major subgenomic plus strand of mouse mammary tumor virus DNA (1.3 kb) is probably the functional homolog of a major subgenomic plus strand of avian sarcoma virus DNA (0.35 kb). The significance of this plus strand species is discussed in the context of current models which hold that it is used as a template for the completion of the minus strand, thereby generating the long terminal redundancy.

The first extensively studied step in the replication of retroviruses is the synthesis of linear double-stranded DNA from a template of viral RNA by the virus-coded, RNA-directed DNA polymerase (11, 23, 35). The completed duplexes are composed of strands complementary to the viral genome (minus strands) and strands with the same chemical polarity as viral RNA (plus strands). Linear DNA is slightly longer than a subunit of viral RNA by virtue of terminal redundancies containing sequences present uniquely at the 3′ and 5′ ends of viral RNA (11, 23).

Priming of the minus strand is generally agreed to occur at a site near the 5′ end of viral RNA, where a cellular tRNA, base paired with the genome, initiates DNA synthesis (29). The minus strand then appears to be elongated as a continuous polynucleotide with a final length of up to 10 kilobases (kb); however, the rate of

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elongation is relatively slow (30), and the completion of the strand, with its terminal redundancies, requires two transfers between templates (11, 23).

In contrast, little is known about the priming of plus strands. Based upon early analyses of plus strands synthesized in infected cells (6, 31) or in vitro (8), plus strands appeared to constitute a rather heterogeneous collection of relatively short products, without evidence for specific initiation or termination sites. However, more recent use of fractionation methods with high resolution, hybridization reagents specific for defined portions of viral genomes, and favorable conditions for DNA synthesis in vitro has revealed that plus strands are synthesized by mechanisms with considerable precision. The first evidence for this view was the finding that the first plus strand to appear after infection by avian sarcoma virus (ASV) was homogeneous in size (ca. 300 nucleotides) and composed of sequences mapped to the 5′ end of minus-strand DNA (and to both ends of viral RNA) (30). The possible significance of this strand was appreciated when structural studies of completed linear ASV DNA revealed terminal repeat units of ca. 300 nucleotides composed of sequences from both ends of viral RNA (11, 23): the early plus strand might serve as the template for the synthesis of the 3′ end of the minus strand, thereby producing the terminal redundancy (1, 23, 32). This notion has gained considerable support from the finding of plus strands equal in size and genetic content to the terminal redundancy of murine leukemia virus (MuLV) linear DNA after synthesis of MuLV DNA in detergent-disrupted virions (4, 7, 15).

Despite the agreement about the requirement for a precisely initiated plus strand of this type (now referred to as the "plus-strand strong stop" [7]), there is confusion about the structure of other plus strands in linear DNA. We have reported previously that a heterogeneous collection of plus strands, generally fewer than 2 to 3 kb in length, was present in ASV DNA (30), although the resolving power of the methods for size fractionation were inadequate for distinctions among similar discrete species of plus strands. In contrast, plus strands in completed MuLV DNA synthesized in vitro are frequently full length, indicating that they may have been synthesized continuously and primed by the plus-strand strong stop (7, 15).

To examine the origins and implications of these discrepancies, we analyzed plus strands synthesized in cells infected by two retroviruses, ASV and mouse mammary tumor virus (MMTV), using high-resolution gel electrophoresis, the DNA transfer method of Southern (24), and annealing with complementary DNAs (cDNA's) specific for various regions of viral genomes. Our results indicated that retroviruses differ with respect to plus-strand structure: ASV plus strands are principally short (fewer than 3 kb), whereas MMTV plus strands are commonly full length (9 kb), suggesting that these viruses use different numbers of sites for priming of plus strands. Moreover, the multiple subgenomic plus strands of ASV fall into several discrete size classes, indicating that a high degree of specificity is involved in the initiation or termination or both of synthesis. We also found that the predicted plus-strand strong stop for MMTV is similar in size (1.3 kb) to the terminal redundancy in the DNA of this virus.

MATERIALS AND METHODS

Cells, viruses, and biochemical agents. QT-6 cells, derived from a fibrosarcoma induced in Japanese quail with methylcholanthrene (16), were used throughout the studies for ASV infections. Nondefective (nd) B77 and Prague subgroup A (PrA) viruses were recently cloned and kindly provided by P. Vogt, University of Southern California. The infecting stocks used in the current study were all transforming components, as documented by a single sharp RNA peak of 3.3×10^6 in a methylmercury hydroxide gel; no smaller RNA subunit could be detected. Transformation-defective (td) B77 and PrA were stocks obtained by long-term passaging of B77 and PrA viruses in quail cells. The resulting stocks were ≥95% td components as judged by the patterns of intact viral RNA, DNA, and *Eco*RI-digested viral DNA (H. J. Kung and P. Shank, unpublished data). No detectable *src* gene fragment could be identified in the latter assay. M1.54 cells, a line of cultured rat hepatoma cells infected with the GR strain of MMTV, were used for the MMTV analysis (21).

*Hae*III-digested ^{32}P-labeled M13 phage DNA, prepared by terminal labeling with polynucleotide kinase, was generously provided by R. Swanstrom, University of California, San Francisco. *Hae*III-digested φX174 replicative form I DNA and the restriction endonuclease *Bam*HI were purchased from New England BioLabs. The glyoxal solution was purchased from Aldrich Chemical Co., and [α-^{32}P]dCTP (400 Ci/mmol) was purchased from Amersham Corp.

Infection and isolation of cytoplasmic DNA. Infection and isolation of cytoplasmic DNA followed previously published procedures (23), except, in some experiments, the pancreatic RNase digestion and the subsequent pronase and phenol treatments were omitted to preserve the RNA molecules. Briefly, infected cells were lysed with 1% Nonidet P-40 and centrifuged to separate the nuclei from the cytoplasm. The cytoplasmic DNAs were then purified by pronase digestion and phenol and chloroform extractions and concentrated by ethanol precipitation. MMTV RNA synthesis (and subsequent DNA synthesis) was induced by the addition of 10^{-6} M dexamethasone.

Agarose gel electrophoresis and Southern transfer. (i) Neutral agarose gel. A 0.8% agarose gel was prepared in TEA buffer (50 mM Tris [pH 8.05], 20 mM sodium acetate, 2 mM EDTA, 18 mM sodium chloride [10]) and cast in a horizontal slab gel apparatus. After electrophoresis, the DNA samples were denatured in situ, neutralized, and transferred to membrane filter paper (Millipore Corp.) as previously described (23).

(ii) Alkaline agarose gel. A 1 or 1.4% agarose gel was prepared in 30 mM NaOH-2 mM EDTA (14). Agarose was dissolved in 2 mM EDTA (pH 7.5) by heating in an autoclave. After cooling to 50°C, the molten agarose solution was adjusted to 30 mM NaOH and immediately poured into the gel apparatus. Viral DNAs were loaded in the same buffer, using bromocresol blue as the marking dye. Either ^{32}P-labeled *Hae*III-digested M13 DNA or *Hae*III-digested φX174 DNA was included as the internal molecular size marker. After the run, the gel was neutralized for 2 h in 0.5 M Tris (pH 7.0)-3 M NaCl and stained at the same time with 1 μg of ethidium bromide per ml. The pattern of the molecular size marker was visualized and photographed under short-wavelength UV light. The DNAs were then ready to be transferred to the Millipore filter paper.

(iii) Glyoxal-phosphate agarose gel. A glyoxal-phosphate gel was used in this study to analyze the putative RNA primer molecule covalently linked to the plus-strand DNA. Before glyoxalation, the viral DNA samples were treated and dissociated into single-stranded molecules in one of the following ways: (a) heating at 100°C for 5 min in TE (5 mM Tris [pH 7.5], 0.1 mM EDTA) and quenching on ice, followed by ethanol precipitation; (b) heating as described in (a), followed by pancreatic RNase digestion (100 μg/ml, 37°C, 1 h), pronase digestion (250 μg/ml, 37°C, 1 h), phenol-chloroform extraction, and ethanol precipitation; or (c) treatment with 0.3 N NaOH at 37°C for 12 h, followed by neutralization with HCl and ethanol precipitation.

After such treatments, viral DNA samples were suspended in 10 mM sodium phosphate (pH 7.0) and incubated with 1 M glyoxal-50% dimethyl sulfoxide at 50°C for 1 h. The glyoxal was deionized through an I-300 (Fisher Scientific Co.) column just before use. The molecular size marker *Hae*III-digested φX174 DNA was denatured and treated with glyoxal in the same manner. The glyoxalated DNA samples were electrophoresed in a 1.4% agarose gel containing 10 mM sodium phosphate, pH 7.5. The buffer was continuously recirculated during the run. After the run, for the visualization of φX174 DNA, the gel was soaked in 50 mM NaOH for 1 h. This treatment, which removes the majority of glyoxal from nucleic acids, allows subsequent reformation of the secondary structure needed for ethidium bromide intercalations. The gel was then neutralized, stained with ethidium bromide, and transferred as before.

Filter hybridization and hybridization reagents. (i) [^{32}P]cDNA. The detailed procedures for the synthesis of the specific cDNA's were described previously (22, 27). The radiolabeled nucleotide in all of these reactions was [α-^{32}P]dCTP, and the specific activity of the cDNA was 2×10^8 cpm/μg.

(a) cDNA$_{rep}$. cDNA$_{rep}$, which represents 70% of the viral genomic sequences, was synthesized by using oligomers of calf thymus DNA to prime the synthesis on subunits of PrC RNA or C3H MMTV RNA by avian myeloblastosis virus polymerase as described previously (22).

(b) cDNA$_{3'}$. cDNA$_{3'}$, which carries the 3'-terminal sequences (200 to 300 nucleotides) of the viral genome, was synthesized by avian myeloblastosis virus polymerase on either 10S polyadenylic acid-containing RNA or the 35S subunit by using oligo(dT)$_{12-18}$ (P-L Biochemicals, Inc.) as the primer. Oligo(dT) primer cDNA$_{3'}$ was then purified by chromatography twice on oligo(dT)-cellulose after annealing to polyadenylic acid as described by Tal et al. (27) and Shank et al. (23).

(c) cDNA$_{5'}$. cDNA$_{5'}$, which represents the 5'-terminal 101 nucleotides of the ASV genome, was synthesized by detergent-activated B77 ASV as previously described (5). cDNA$_{5'}$ was purified twice by isolation from a 10% polyacrylamide gel (5, 23). cDNA$_{5'}$ was kindly provided by S. Hughes, University of California, San Francisco.

(d) cDNA$_{gp}$. cDNA$_{gp}$, which carries the envelope gene (ca. 2 kb) sequences, was selected from cDNA$_{rep}$ by annealing with RNA from a replication-defective mutant of ASV bearing a 21% deletion involving most or all of the *env* gene as described previously (23). cDNA$_{gp}$ was kindly provided by N. Quintrell, University of California, San Francisco.

(ii) ^{32}P-labeled 70S RNA. ^{32}P-labeled 70S RNA (specific activity, 10^6 cpm/μg) was extracted according to a previously published procedure (19) from B77 ASV harvested from cells labeled for 1 to 3 days with $^{32}PO_4$ at 2 mCi/ml.

(iii) Filter hybridization. Hybridizations with cDNA were conducted in a solution containing 50% formamide, 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 20 μg of yeast RNA per ml, 20 μg of salmon sperm DNA per ml, and 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.0) supplemented with Denhardt solution (3) at 41°C for 60 h. The filter was then washed once with 2× SSC at room temperature and twice with 0.1× SSC-0.1% sodium dodecyl sulfate at 50°C and rinsed with 0.1× SSC. The filter was then dried and exposed to X-ray film at −70°C in the presence of a Lightning-Plus intensifying screen. The procedure for hybridizations with [^{32}P]RNA was the same, but the washing was performed differently. The filter was first washed once with 2× SSC at room temperature and once with 6× SSC at 68°C. It was then subjected to pancreatic and T_1 RNase treatments (50 μg and 5 U, respectively, per ml) at 37°C for 30 min. Finally, the filter was rinsed with 2× SSC, dried, and exposed to film at −70°C.

(iv) Removal of radioactivity from filter for rehybridization. The filter was soaked in 80% formamide in TE in a tightly sealed plastic bag which was immersed in an 80°C water bath for 20 min. To insure that no residual radioactivity remained, the background for the filter was prechecked by autoradiography before the second hybridization.

Digestion of viral DNA with endo R·*Bam*HI. Samples were incubated in a solution containing 50

mM NaCl, 6 mM Tris (pH 7.4), 6 mM $MgCl_2$, 6 mM β-mercaptoethanol, and 100 mg of bovine serum albumin per ml with 1 U of enzyme per μg of DNA at 37°C for 1 h. The completeness of digestion was monitored by the inclusion of form I plasmid pBR313 DNA in the reaction mixture. A small sample was removed and analyzed in a gel for a demonstration of conversion from the circular to the linear form.

RESULTS

Multiple, discrete plus strands in ASV DNA. We previously assessed the size and genetic composition of plus strands of ASV DNA at various times after infection with hybridization probes specific for various regions of the viral genome to detect plus strands in fractions from rate-zonal sucrose gradients or in slices from polyacrylamide gels (30). In that study, we identified a dominant, homogeneous class of short plus strands (ca. 0.3 kb in length) containing sequences unique to both the 3′ and the 5′ ends of viral RNA. However, the remainder of the plus strands appeared to be heterogeneous in size and we found no evidence for full-length plus strands.

The DNA transfer method of Southern (24) permits a higher degree of resolution and sensitivity in the analysis of small amounts of plus-strand DNA present in infected cells than do methods that we previously employed. Using the transfer method, we identified a large number of discrete ASV plus-strand segments, in addition to the previously identified 0.3-kb strand.

A sampling of these additional subgenomic plus strands is shown in Fig. 1. In this experiment, DNA was extracted from the cytoplasm of QT-6 cells after infection for 24 h with either B77 or PrA ASV, subjected to electrophoresis under alkaline denaturing conditions, transferred to nitrocellulose sheets, and detected by hybridization with [^{32}P]$cDNA_{rep}$. (The annealing probe in this case scores only the plus strands but represents the entire ASV genome [see above].) Bands of DNA that were 1.3, 1.4, 2.4, and 2.6 kb in length could be identified in both the B77 and the PrA ASV samples; the 2.4- and 2.6-kb species are faint in lane A but can be readily observed in the original autoradiogram. In addition, several other bands can also be seen, particularly in lane B. The nonrandom size distribution of the plus-strand segments suggests that they are initiated and terminated with specificity and that the two strains of ASV share common mechanisms for plus-strand synthesis.

Although the experiment shown in Fig. 1 identified several plus-strand species, it seemed likely that we could detect a larger proportion of fragments with less complexity by sequential

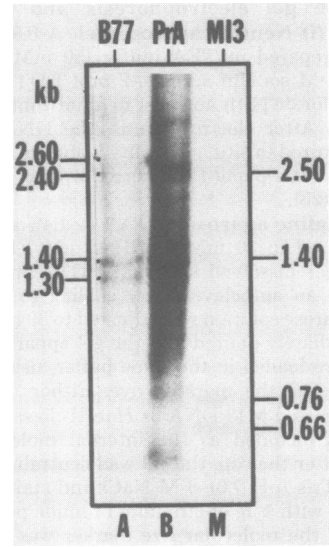

FIG. 1. *Plus strands of nd B77 and nd PrA viral DNAs as detected by $cDNA_{rep}$. Cytoplasmic DNA samples, isolated at 24 h after infection of QT-6 cells with nd viruses, were analyzed in a 1.4% agarose gel under alkaline conditions (30 mM NaOH, 2 mM EDTA). The DNA samples were then transferred to a Millipore filter and hybridized with $cDNA_{rep}$. (A) nd B77; (B) nd PrA; (M) HaeIII-digested ^{32}P-labeled M13 phage DNA which was used as a molecular size marker.*

annealings with cDNA's specific for various regions of the ASV genome. In the following sections, we describe results of tests for plus strands, using $cDNA_{3'}$, $cDNA_{5'}$, and $cDNA_{gp}$, representing the 3′ terminus, the 5′ terminus, and the *env* region of viral RNA, respectively.

(i) ASV plus strands annealing with $cDNA_{3'}$ and $cDNA_{5'}$. One of the plus strands which we failed to detect with $cDNA_{rep}$ (Fig. 1) is the dominant 0.3-kb strand found in our earlier study (30). Reexamination of the same samples of cytoplasmic DNA, using reagents specific for the terminal sequences of viral RNA, displayed the 0.3-kb species as a dominant band at the position expected for a single strand of ca. 0.35 kb (Fig. 2, lanes A to D). This species was found in the DNA of nd and td examples of PrA and B77 ASV sequences (Fig. 2, lanes H and I). It was about equally detectable with $cDNA_{3'}$ and $cDNA_{5'}$, as might be expected from the linkage of these sequences at both ends of viral DNA (see Fig. 3). In addition, several other well-defined plus strands could be detected with both reagents; in the case of the nd strains, the more abundant or major strands had lengths of 0.94, 1.38, 2.3, and 3.4 kb, but other minor strands with lengths of 0.52, 0.62, 0.74 kb, etc., were also

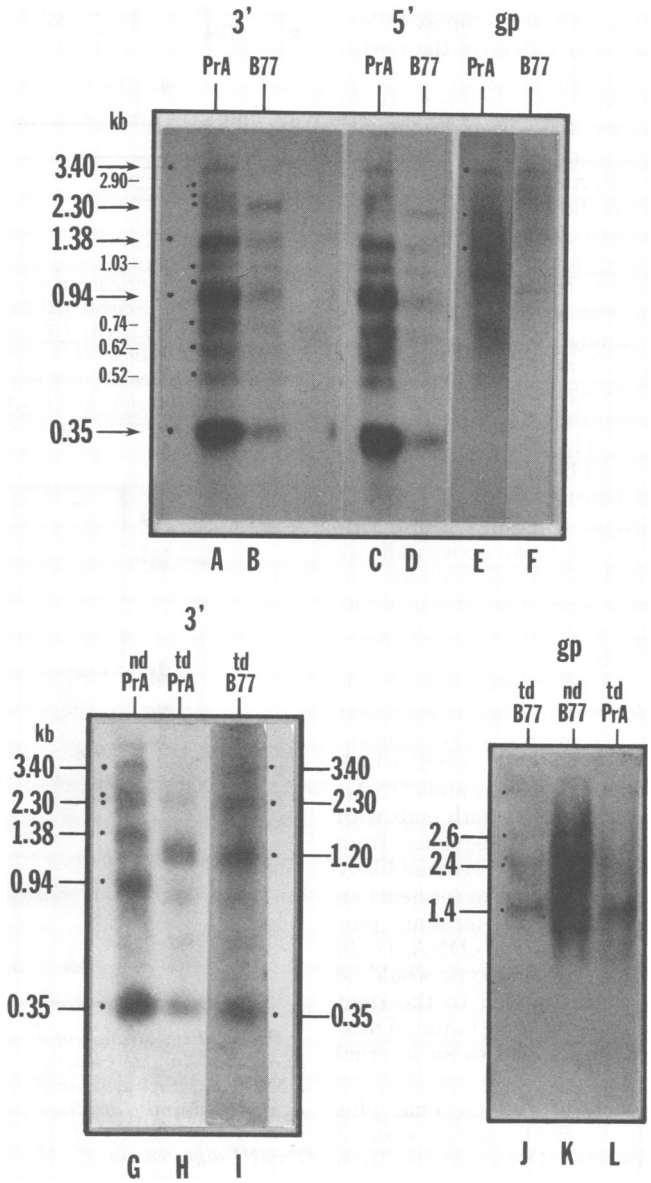

FIG. 2. *Plus strands of B77 and PrA viral DNAs as detected by $cDNA_{3'}$, $cDNA_{5'}$, or $cDNA_{gp}$. The 24-h cytoplasmic DNA samples were subjected to 1.4% (lanes A to I) or 1% (lanes J to L) alkaline agarose gel electrophoresis and hybridized with various cDNA's. The molecular sizes of the segments shown were calculated by using HaeIII-digested M13 DNA fragments run in parallel as a calibration. (A) nd PrA, $cDNA_{3'}$; (B) nd B77, $cDNA_{3'}$; (C) nd PrA, $cDNA_{5'}$; (D) nd B77, $cDNA_{5'}$; (E) nd PrA, $cDNA_{gp}$; (F) nd B77, $cDNA_{gp}$; (G) nd PrA, $cDNA_{3'}$; (H) td PrA, $cDNA_{3'}$; (I) td B77, $cDNA_{3'}$; (J) td B77, $cDNA_{gp}$; (K) nd B77, $cDNA_{gp}$; and (L) td PrA, $cDNA_{gp}$.*

discernible. The pattern was significantly altered with DNA from td strains; most obviously, the major strands of 1.38 and 0.94 kb were replaced by a strand of 1.2 kb (Fig. 2, lanes G to I). The position of the td deletion at ca. 0.5 kb from the 3' end of ASV RNA suggested that the altered plus strands were derived from the right-hand end of linear DNA (i.e., the first end to be synthesized [see Fig. 3]), in the manner used for the synthesis of the 0.35-kb strand. Further evidence for this view will be presented below.

(ii) ASV plus strands annealing with

$cDNA_{gp}$. With $cDNA_{gp}$, a more complex collection of plus strands was detected than with $cDNA_{3'}$ and $cDNA_{5'}$, as judged from the relatively diffuse distribution of radioactivity in lanes E and F. This may have been due in part to the greater genetic complexity of the probe; $cDNA_{gp}$ represents approximately 20% of the ASV genome, whereas the cDNA's for the termini represent only 1 to 3%. A greater number of initiation or termination or both sites for plus strands may also exist in the *env* portion of the genome. Electrophoresis in 1.0% agarose was more effective in defining discrete species (1.4, 2.4, and 2.6 kb [Fig. 2, lanes J to L]) than was electrophoresis in 1.4% agarose (Fig. 2, lanes E to F). The experiments shown in lanes E and F confirm the specificity of hybridization reagents, since the same filter had been tested previously with $cDNA_{3'}$ (Fig. 2, lanes A and B).

Physical mapping of the early major initiation sites. The results shown above indicate that many of the plus-strand segments are initiated at specific sites along the viral genome. For an understanding of the nature of the specificity in the initiations, it is necessary to map the initiation sites. As a first step toward this end, we mapped three major initiation sites near the right end of the cytoplasmic DNA, concentrating upon the distinctive plus strands which anneal efficiently with both $cDNA_{3'}$ and $cDNA_{5'}$. Since linear DNA terminates at both ends with a repeat unit composed of linked sequences from the 3′ and 5′ ends of viral RNA, it seemed likely that these plus strands extended to (or near) an end of linear DNA. Thus, the terminal plus strands detected with $cDNA_{3'}$ and $cDNA_{5'}$ could be of two types (Fig. 3): the first type would be initiated internally and extended to the right end, and the second type would be initiated near or at the left end and extended to an internal termination point.

To determine which of the terminal plus strands derive from the right and left ends of linear DNA, we subjected DNA isolated from the cytoplasm at 24 h after infection to digestion with the restriction endonuclease *Bam*HI, followed by electrophoresis in alkaline agarose gels and hybridization with $cDNA_{3'}$ (Fig. 4). *Bam*HI cleaved PrA ASV DNA asymmetrically at sites 0.6 kb from the left end and 8 kb from the right end of linear DNA (Fig. 3B). Plus strands derived from the right end and smaller than 8 kb thus will not be cleaved by *Bam*HI; conversely, plus strands derived from the left end and longer than 0.6 kb will be cleaved to produce a strand of 0.6 kb which will anneal with $cDNA_{3'}$. In this manner, we were able to determine the map positions of some of the prominent terminal plus strands without physically separating the

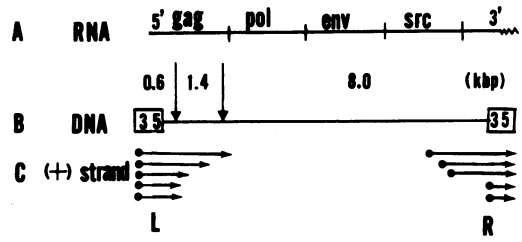

FIG. 3. *(A) Genetic map of the ASV genome: gag, group-specific antigen; pol, polymerase; env, envelope; and src, sarcoma. (B) BamHI cleavage map of PrA cytoplasmic DNA: 35, large terminal repeating sequences (350 nucleotides) which carry both the 3′ and the 5′ sequences (not to scale). (C) Plus-strand segments detectable by $cDNA_{3'}$ or $cDNA_{5'}$: arrows indicate the direction of plus-strand synthesis.*

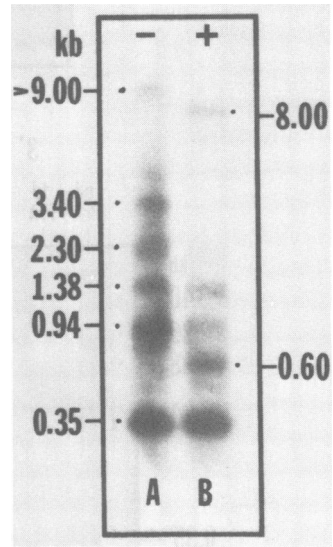

FIG. 4. *Plus strands of nd PrA viral DNA before and after BamHI cleavage. The 24-h cytoplasmic DNA of nd PrA was analyzed in a 1.4% alkaline agarose gel and hybridized with $cDNA_{3'}$. (A) Before BamHI digestion; (B) after BamHI digestion. For the BamHI-digested sample, the completeness of digestion was monitored by analyzing a sample of the reaction mixture in a neutral agarose gel; it displayed the expected 3.0-, 1.4-, and 0.6-kb fragments, and no partially digested fragments could be detected.*

*Bam*HI fragments from the right and left ends (Fig. 4).

The control (undigested) sample (Fig. 4, lane A) was very similar to the sample analyzed in Fig. 2, lane A, with prominent plus strands of 0.35, 0.94, 1.38, 2.3, and 3.4 kb when hybridized with $cDNA_{3'}$. In addition, we observed a faint band at the position expected for full-length plus strands (ca. 9 kb). After digestion with *Bam*HI, we observed the expected new fragment of 0.6

kb; this could be generated by cleavage of the subunit-sized strand (now reduced to 8 kb) or by cleavage of the 3.4- and 2.3-kb fragments, both of which were markedly reduced in intensity. More importantly, little or none of the 1.38- and 0.94-kb DNA has been cleaved by *Bam*HI, indicating that these two species, as well as the previously mapped 0.35-kb strand, are located at the right-hand end of linear DNA. These assignments were confirmed by the absence of the 1.38- and 0.94-kb strands in the DNA of td mutants of ASV (Fig. 2, lanes G and H). The predicted initiation sites for the 1.38- and 0.94-kb strands are located within the *src* gene of ASV (2a) and within the region deleted in the generation of td mutants (12). Possible signals for the initiation of plus strands at these sites will be considered below.

Kinetics of appearance of plus and minus strands of ASV. We took advantage of the simplicity and high resolution of the DNA transfer procedure to reexamine the kinetics of appearance of completed duplex DNA and its single-stranded components (Fig. 5). In accord with our previous report (30), 4 h is required for the synthesis of a full-length minus strand, detected by annealing with ^{32}P-labeled 70S RNA (lanes E to G); much greater amounts of a completed minus strand are found at 8 h postinfection. The delay presumably reflects a relatively slow rate of elongation of this continuously synthesized strand. Similar results were obtained in tests for completed duplex DNA in nondenaturing gels with $cDNA_{rep}$ (lanes A to D). At 2 h after infection, we found a small amount of low-molecular-weight DNA (observable only after prolonged autoradiographic exposure in lane A'). A faint band representing full-sized DNA was perceptible in the sample harvested at 4 h postinfection (lane B), but the bulk of the DNA migrated with the heterogeneity expected of incomplete molecules. With time, the ratio of completed to incompleted molecules increased markedly; at 24 h after infection, little, if any, incompleted DNA was present (lane D). Using $cDNA_{rep}$ to detect plus strands in a denaturing gel, we observed only a very faint 0.35-kb species in the sample harvested at 2 h postinfection, as expected from prior studies (30); this species was examined more thoroughly with $cDNA_{3'}$ and will be discussed below. At 4 and 8 h after infection (lanes I and J), we observed increasing amounts of a heterogeneous collection of plus strands, similar to that observed at 24 h postinfection (Fig. 1), with prominent bands representing strands of 1.3, 1.4, 1.7, and 2.5 kb. We have not attempted to map the position of these strands on the ASV genome; however, we have shown previously that most, if not all, portions of the genome are represented in plus strands of fewer than 3 kb in length (30). These findings, in conjunction with our inability to detect more than low levels of full-length plus strands (Fig. 4), suggest that the majority of completed ASV linear DNA is composed of a full-length minus strand and several subgenomic plus strands.

Kinetics of synthesis of terminal plus strands: lack of evidence for a large RNA primer. We examined samples isolated from the cytoplasm of QT-6 cells at 2.5, 4.5, and 24 h

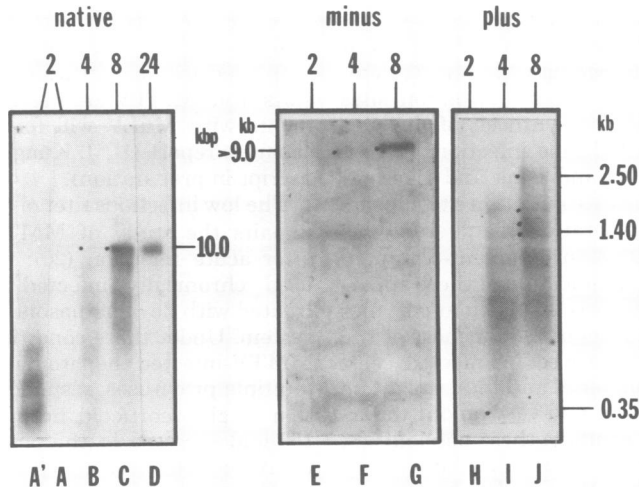

FIG. 5. *Kinetics of synthesis of nd PrA viral cytoplasmic DNA. The cytoplasmic DNAs of nd PrA virus were isolated at different intervals after infection. They were analyzed for the duplex structure (lanes A to D) in a 0.8% neutral agarose gel or for the minus-strand (lanes E to G) and plus-strand (lanes H to J) structures in a 1.4% alkaline agarose gel. The viral DNA samples were detected by radioactive $cDNA_{rep}$ or 70S RNA.*

postinfection with PrA ASV to follow the appearance of plus strands from the terminal regions of linear DNA and to determine whether appreciable amounts of RNA might remain linked to these plus strands, as suggested by a recent report (9). DNA was extracted without the use of RNases and then studied with and without treatment with alkali or with pancreatic RNase (Fig. 6). To measure the lengths of plus strands without further hydrolysis of ribonucleotides, the samples were denatured with heat, treated with glyoxal, and analyzed in neutral agarose gels (see above).

Again, only the plus strand of 0.35 kb was observed at 2.5 h postinfection; the terminal plus strand of 0.94 kb from the right-hand terminus appeared at 4.5 h postinfection, and all of the plus strands previously found at 24 h postinfection (Fig. 2 and 4) were again seen under these alternative conditions of electrophoresis. In no case, did we find convincing evidence for the sensitivity of these strands to alkaline hydrolysis or digestion with RNases. Thus, large tracts of RNA (i.e., primers) were not covalently linked to the plus strands. Figure 6 also shows that few, if any, ribonucleotides were incorporated into these strands, as has been described recently for the DNA of reticuloendotheliosis virus (2). Analysis of the 0.35-kb strand was particularly telling in this regard: in none of the three samples could we detect an alteration in mobility, even though the resolution of the gel was adequate to detect a loss of 50 to 100 nucleotides from molecules of 0.35 kb. Thus, if the plus strands are primed by RNA (see below), the primers are either too short to be detected in this analysis or removed soon after synthesis.

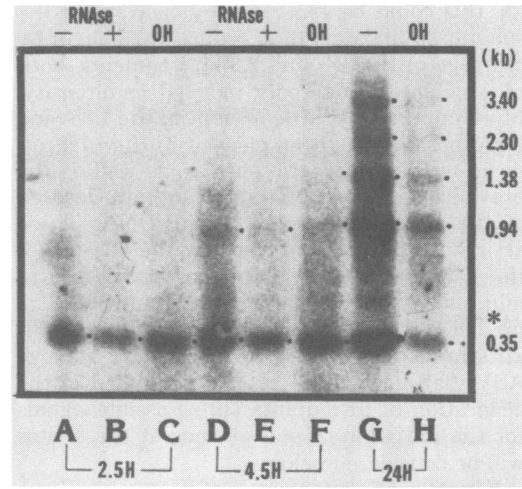

FIG. 6. *Kinetics of synthesis and structure of the terminal segments of nd PrA viral DNA. The cytoplasmic DNAs of nd PrA virus were isolated at different times after infection without prior RNase A treatment. These DNA samples were either heat denatured at 100°C for 5 min (lanes A, D, and G), heat denatured at 100°C for 5 min plus pancreatic RNase treatment (lanes B and E), or treated with 0.3 N NaOH (lanes C, F, and H). After glyoxalation, the samples were analyzed in a 1.4% agarose gel containing 10 mM sodium phosphate, pH 7.5. HaeIII-digested φX174 replicative form II DNA which underwent identical denaturation and glyoxalation was included in the viral DNA sample to serve as molecular size marker and identified by staining with ethidium bromide. *, Position at which a single-stranded nucleic acid of 450 nucleotides in size should migrate.*

Plus strands synthesized in cells infected with MMTV. The foregoing experiments demonstrate that multiple specific initiation sites must be used during the synthesis of the plus strands of ASV DNA during infection. In contrast, Gilboa et al. (7) and Dina and Benz (4) have reported that a single initiation site appears to be used for the generation of full-sized plus strands of MuLV DNA in detergent-disrupted virions. One possible implication of these apparently conflicting findings is that cellular enzymes or other factors are responsible for most of the multiple initiations observed in infected cells; alternatively, the number of initiations might be determined by the virus and vary among different retroviruses. To examine these possibilities, we analyzed plus strands synthesized in vivo by two retroviruses other than ASV—MuLV and MMTV. In both cases, we found evidence for only two major species of plus strand, one the length of the terminal redundancy and one full length, suggesting that these mammalian viruses differ from ASV in the number of initiation sites employed in the synthesis of plus strands. Results from MMTV are presented below; experiments with MuLV will form the subject of a separate report (H. J. Kung and S. Dube, manuscript in preparation).

The low infectious titer of most MMTV stocks impairs the study of MMTV DNA synthesis after acute infection (20); for this reason, we used chronically infected cell lines acutely treated with dexamethasone as an experimental system. Under these conditions, M1.54 cells, an MMTV-infected hepatoma cell line carrying multiple proviruses, respond to the administration of glucocorticoid hormones with a 50- to 150-fold increase in the concentration of viral RNA; the induction is followed by the relatively synchronous appearance of a few copies of viral DNA transcribed from newly synthesized RNA (20). By all available criteria (the kinetics of synthesis and structural forms), MMTV DNA appearing after induction is similar to other

kinds of retroviral DNA synthesized after acute infection (22).

Viral DNA is found in useful amounts at about 10 h after the addition of dexamethasone (i.e., at about 5 h after the maximal induction of viral RNA); during the first 48 h after induction, there is an increase in the proportion of mature forms of unintegrated DNA (completed linear and closed circular forms), as well as in the overall concentration of viral DNA. To examine the composition of plus strands in linear MMTV at different stages of synthesis, we prepared DNA from the cytoplasmic fraction of M1.54 cells at 10, 24, and 48 h after induction. In each case, the DNA was analyzed by gel electrophoresis in both native and denatured states, followed by hybridization with a $cDNA_{rep}$ probe which was made in the presence of actinomycin D and therefore detects primarily viral plus-strand material. In addition, we examined the plus strands in separated halves (*Eco*RI fragments) to locate any subgenomic fragments in finished portions of the linear DNA.

At 10 h after induction, most of the free MMTV DNA was incomplete, migrating heterogeneously in its native state in agarose gels (Fig. 7A). When these molecules were denatured for the analysis of plus strands, a major discrete single-stranded component of ca. 1.3 kb was detected by annealing with $cDNA_{rep}$ (Fig. 7A), in addition to a complex assortment of fragments of various lengths. These latter fragments were not present in isolated restriction fragments presumably derived from completed duplex DNA (Fig. 7C, D, and F to I). Thus, we suspect that they are intermediates in plus-strand synthesis, perhaps reflecting sites at which the polymerase pauses, and we have not used region-specific probes other than $cDNA_{3'}$ to analyze them further. However, there may also be components representing both full-length strands from defective molecules and subgenomic strands arising occasionally from secondary initiation sites. The 1.3-kb plus strand, on the other hand, is similar, if not identical, in size to the sequence which is terminally redundant in linear DNA (22; J. E. Majors and H. E. Varmus, Nature [London], in press); moreover, it anneals with $cDNA_{3'}$ (Fig. 7B) and thus must be positioned near or at one of the ends of linear DNA. This strand can be recovered from only the smaller of the two *Eco*RI fragments from linear DNA harvested at 10 or 24 h after induction (Fig. 7C and F versus D and G); since *Eco*RI cleaves 3.6 kb from the right-hand terminus of MMTV DNA (22), the 1.3-kb strand must be positioned at the right end of linear DNA. This is the location of structurally analogous plus strands found in ASV and MuLV DNAs in vivo and in vitro (4, 7).

Full-sized duplex DNA was recovered from the cytoplasmic fractions as the predominant form at 24 h after induction (Fig. 7E). Denaturation of the unfractionated mass revealed that an increasing proportion of the plus strands was also full sized, with a corresponding reduction in the 1.3-kb fragment and in the heterogeneous collection of plus strands. Furthermore, denaturation of the separated *Eco*RI fragments generated from linear DNA isolated at either 24 or 48 h after induction showed that the plus strands were almost entirely full length, with only a small subgenomic component (Fig. 7F to I). The time-dependent disappearance of these subgenomic species and their absence in the completed linear form and its *Eco*RI products suggested (but did not prove) that they are the

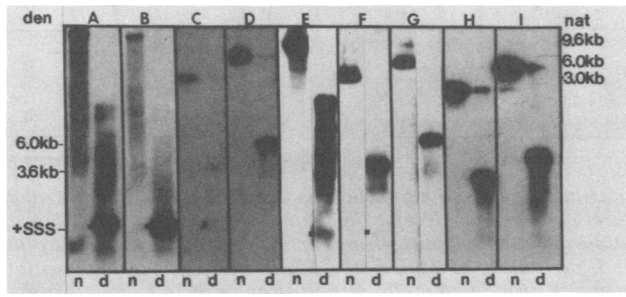

FIG. 7. *Structure of MMTV plus strands. MMTV cytoplasmic DNA was isolated after induction with 10^{-6} M dexamethasone. Each sample is shown as native (n) and denatured (d) after electrophoresis through a 0.8% neutral agarose gel. Except for that in (B), each sample was hybridized with MMTV $cDNA_{rep}$. (A) Unfractionated DNA at 10 h after induction; (B) filter from (A) hybridized with MMTV $cDNA_{3'}$; (E) unfractionated DNA at 24 h after induction; (C, F, and H) 3.6-kb EcoRI fragment from 10, 24, and 48 h after induction; (D, G, and I) 6-kb EcoRI fragment from 10, 24, and 48 h after induction. The size markers on the left refer to the positions of the indicated fragments in the denatured (den) lanes. +SSS refers to the 1.3-kb plus-strand strong-stop fragment. Those +SSS fragments referred to in the text are marked with a ■. The size markers on the right refer to the positions of the indicated fragments in the native (nat) lanes.*

precursors to the full-length plus strands, as suggested in the models proposed by Gilboa et al. (7) and Dina and Benz for MuLV DNA (4).

DISCUSSION

Sizes of plus strands of linear retroviral DNA. Using methods with an increased power of resolution, we confirmed our earlier findings that the plus strands in the linear unintegrated form of ASV DNA are mainly or exclusively subgenomic, that they include most or all of the viral sequences, and that they are initiated at specific sites. This report demonstrates that a large number of specific initiation sites must exist in several regions of the genome, since hybridization reagents which detect different portions of the genome can identify several different plus-strand species in completed linear duplexes (Fig. 2, 5, and 6).

This situation is clearly different from that observed with several other retroviruses. Most plus strands are full length when isolated from completed linear DNA made in vivo by MMTV (Fig. 7), MuLV (Kung and Dube, in preparation), or reticuloendotheliosis virus (2) or made in vitro by MuLV (4, 7). However, results with MMTV, MuLV, and ASV DNAs are similar in that in each case a dominant species of subgenomic plus strand is synthesized early in the production of linear DNA; this species always has the size and genetic composition of the sequences present at both ends of linear and proviral DNAs. This plus strand, which has been termed the plus-strand strong stop (7), appears from recent sequencing studies to be initiated at the end of a purine-rich sequence highly conserved among retroviruses and located at the boundary of the 3′ region of the RNA which is terminally repeated in viral DNA (23a, 24a; Majors and Varmus, in press; R. Swanstrom, W. J. Delorbe, J. M. Bishop, and H. E. Varmus, Proc. Natl. Acad. Sci., U.S.A., in press). The plus strand is synthesized by copying all of the 5′ end of the nascent minus strand, including sequences copied from the 3′ and 5′ ends of viral RNA (Fig. 8); it is probably extended further by copying the first 18 (or so) nucleotides of the tRNA primer for the minus strand, until synthesis halts when the polymerase encounters the first methylated base in the tRNA (7, 28; R. Swanstrom, unpublished data).

We have been unable, however, to document the existence of RNA covalently linked to segments of plus-strand DNA, even in intermediates isolated early after infection (Fig. 6; O. Richards, P. Shank, and authors, unpublished data), although covalent hybrids have been reported by others (9, 13, 25, 26). Chen and Temin (2) have recently described infrequent ribonucleotides in strands of spleen necrosis virus DNA; however, the presence of the ribonucleotides in both plus and minus strands of spleen necrosis virus DNA and their absence from ASV DNA (Fig. 6 and reference 2) suggest that they reflect the misincorporation of ribonucleotides by the spleen necrosis virus DNA polymerase,

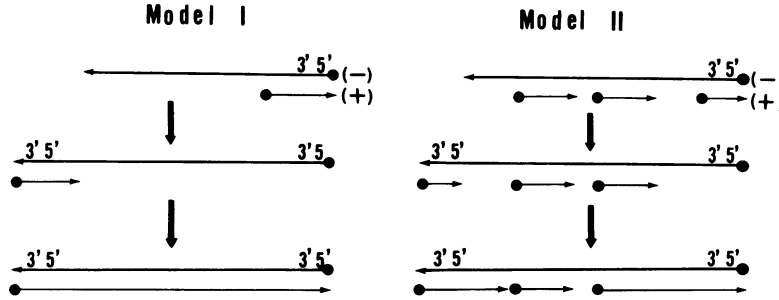

FIG. 8. *Effect of the number of plus-strand initiations upon the sizes of plus strands in completed linear DNA. The relevant features of current models for the synthesis of viral DNA (7, 32) are shown to indicate the effects of single and multiple initiations of plus strands upon the ultimate lengths of plus strands in completed linear DNAs. In model I, pertaining to MuLV and MMTV DNAs, the intermediate structure (top line) contains only a growing minus strand and a plus-strand strong stop (see text); transfer of the nascent minus strand from an RNA template to a template of the plus-strand strong stop allows the formation of the terminal redundancy (consisting of sequences from the 3′ and 5′ ends of viral RNA (3′5′; middle line); the plus strand can then be extended the full length of the minus strand (bottom line). In model II, pertaining to ASV DNA, multiple plus strands have been initiated, in addition to a plus-strand strong stop; as a consequence, the completed linear DNA may have plus strands of various sizes in all regions, including the right end, but few molecules, if any, will contain full-length plus strands. In this simplified diagram, we have omitted RNA templates and primers and have made no effort to consider the use of one or two subunits of viral RNA during DNA synthesis. (The use of two subunits could, for example, permit the retention of the plus-strand strong stop at the right end of completed linear DNA, as suggested by results shown in Fig. 4.) Arrows indicate the direction of synthesis, closed circles indicate 5′ termini of plus and minus strands.*

rather than a residue of RNA primers.

It is not yet apparent how the enzymes involved in the initiation of plus strands preserve the specificity that we have observed for several priming sites. Sequencing results alluded to earlier have revealed that the priming site for the plus-strand strong stop is purine-rich on the plus (RNA) strand. The relevant region in the ASV genome reads AAAAAGGGGGAAGT; the final 10 bases of this sequence are identical in the genome of MMTV (Majors and Varmus, in press) and very similar in MuLV DNA (3a, 23a, 24a). Other purine-rich regions, with short runs of oligo(A) and oligo(G), have been found at several points on the 5′ side of the plus-strand strong-stop priming site (2a). These might represent some of the other priming sites that we have mapped at approximately 0.94 and 1.38 kb from the right end of linear DNA, but more rigorous approaches to mapping are now required.

Our results indicate that multiple specific priming sites must exist for ASV plus strands, but they do not cast much light upon the priming mechanism per se. Studies of plus-strand initiation in vitro indicate that oligoribonucleotides of unknown length are likely to constitute the primers for plus strands and that RNase H activity is probably essential for the generation of primers (17, 34). Hence, it is attractive to assume that viral RNase H generates oligonucleotides suitable as primers from genomic RNA already copied into minus-strand DNA, but such oligonucleotides have yet to be identified and the implied specificity of RNase H has yet to be demonstrated.

Since we have only measured unlabeled species of viral DNA in our experiments, we have not examined the possibility that plus strands (and even minus strands) are synthesized in a highly discontinuous fashion as short strands of less than 0.3 kb in the manner described by Okazaki et al. (18), followed by partial or complete ligation to generate subgenomic plus and full-length minus strands. In this case, which we consider unlikely, the apparent preferred initiation sites would instead represent priming sites at which ligation was unlikely to occur. Assays for pulse-labeled plus strands will be required to deal effectively with this possibility.

Subgenomic plus strands have been reported in some cases to be the dominant species synthesized by MuLV in vivo (6) or in vitro (33). It seems likely, in retrospect, that the short strands observed in vivo were mainly the plus-strand strong stop, since the native molecules were found to be almost entirely single stranded (6).

Effect of plus-strand structure upon models for DNA synthesis. The discovery of a short ASV plus strand bearing sequences from both termini of viral RNA (30) prompted the subsequent suggestions that the terminal redundancy in linear DNA was generated by using this plus strand as a template to complete the synthesis of the minus strand (1, 23, 32). Strong evidence favoring this possibility and a detailed model for the synthesis of viral DNA which incorporates this step have been published recently by Gilboa et al. (7). One of the predictions of the model is that the plus-strand strong stop will be extended after it has base paired with the 3′ terminus of the newly completed minus strand (Fig. 8). In their report, Gilboa et al. predict that full-length plus strands will result from the extension, and their finding of genome-sized plus strands is interpreted as a validation of the model. However, it is apparent from the diagrams shown in Fig. 8 that the prediction of full-length plus strands is not central to the important features of the model. Thus, if only a single initiation site exists for plus strands, as appears to be the case for MuLV, MMTV, and perhaps reticuloendotheliosis virus full-length plus strands will result; but if multiple initiation sites exist, as we have found for ASV plus strands, then only a limited extension of the plus-strand strong stop will occur, unless there is considerable synthesis accompanied by strand displacement. However, the essential features of the synthetic process could be the same in the two types; the most significant difference might lie in the greater number of ligations required to form closed circular DNA from ASV linear DNA.

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