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Synthesis of a 600-nucleotide-long plus-strand DNA by virions of Moloney murine leukemia virus

(retroviruses/reverse transcription/plus-strong-stop DNA)

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ABSTRACT **A discrete, 600-nucleotide-long plus-strand DNA has been identified among the products of reverse transcription by virions of Moloney murine leukemia virus. Its polarity was shown by hybridization to minus-strand DNA. It appears to be copied from the right end of minus-strand DNA because (*i*) its restriction endonuclease cleavage pattern corresponds to the redundant 600-base segment found at either end of the ultimate double-stranded reverse transcription product, (*ii*) its synthesis is actinomycin D sensitive, and (*iii*) its synthesis begins during the first hour of a reverse transcription reaction when only the right-hand end of minus-strand DNA is available as template. We therefore call this DNA plus-strong-stop DNA by analogy with the minus-strong-stop DNA copied from the left end of the viral RNA. Both strong-stop DNAs are made early during *in vitro* reactions and decline in concentration later, consistent with postulated roles as initiators of long minus- and plus-strand DNA. Unlike minus-strong-stop DNA, plus-strong-stop DNA remains as a double-stranded nucleic acid after its synthesis, as shown by S1 nuclease resistance. A primer to initiate plus-strong-stop DNA synthesis has not been identified; the product found thus far has no detectable RNA attached to it.**

Although synthesis of minus-strand DNA during reverse transcription is at least partly understood, synthesis of plus-strand DNA is very poorly understood. Two clues exist. First, Varmus *et al.* (1) showed that during the synthesis of Rous sarcoma virus DNA, a short, discrete fragment of plus-strand DNA appears early after infection. Second, Hsu *et al.* (2), Shank *et al.* (3), and Gilboa *et al.* (4) have shown that the ultimate product of reverse transcription by Rous sarcoma virus and Moloney murine leukemia virus (M-MuLV) contains longer redundancies at its ends than are found in viral RNA. This second finding implies that there is reiterative copying of viral RNA during reverse transcription. Some of the reiteration can be explained by the jump of minus-strand strong-stop DNA, the initiator of minus-strand synthesis, from its place of synthesis at the 5′ end of the genome to the 3′ end of the genome (5, 6). This jump transfers about 100 nucleotides of DNA sequence from one end of the RNA genome to the other end of the reverse transcript, but the ultimate redundancy is longer and requires a second jump of sequence. The discrete plus strand found by Varmus *et al.* (1) could be responsible for a second jump [as both Baltimore *et al.* (7) and Shank *et al.* (3) have recently suggested].

For M-MuLV, a terminal redundancy of 600 bases exists in the ultimate 9-kilobase-pair (kbp) double-stranded reverse transcription product (4). Only 100 bases can arise from the first jump; about 50 redundant bases exist in the viral RNA (5) so that about 450 bases must come from another event. This reasoning led us to search among the products of endogenous reverse transcription by M-MuLV virions for a plus-strong-stop DNA. Its presence is reported here.

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Further properties of the molecules made in the endogenous reaction will be described elsewhere, and a precise model of reverse transcription will be presented there (8).

MATERIALS AND METHODS

Preparation of ^{32}P-Labeled *In Vitro* Reverse-Transcribed DNA. The preparation of *in vitro* reverse-transcribed DNA by use of detergent-disrupted M-MuLV virions has been described (4, 9). Briefly, virions adjusted to 4 mg of viral proteins per ml were incubated in the presence of 50 mM Tris (pH 8.3), 7 mM Mg acetate, 5 mM dithiothreitol, 0.01% Nonidet P-40 (Shell), 2 mM each of dATP, dCTP, dGTP, and dTTP, and 1 mCi of [α-^{32}P]dCTP per ml (>300 Ci/mmol, New England Nuclear; 1 Ci = 3.7×10^{10} becquerels) for varying lengths of time at 40°C. For isolation of the minus- and plus-strong-stop DNAs, 1–3 hr of incubation was found to be optimal. The reactions were terminated by adjusting the reaction mixture to 10 mM EDTA/0.25% sodium dodecyl sulfate and incubating with 2 mg of Pronase per ml for 30 min at 37°C. Nucleic acids were extracted twice with phenol/chloroform, 1:1 (vol/vol), precipitated with ethanol, subjected to Sephadex G-50 chromatography to remove salts and unincorporated triphosphates (4), and reprecipitated.

To make minus-strand DNA exclusively, we added 100 μg of actinomycin D (Act D) per ml (a kind gift from Merck, Sharp & Dohme) to the reaction mixture. The total DNA product made in the presence of Act D is referred to as (−)DNA.

Electrophoresis of Denatured DNA on Acrylamide Gels. Precipitated DNA samples were dissolved in 10 μl of doubly crystallized formamide to which 1 μl of a solution of 20 mM EDTA/0.1% bromophenol blue/0.1% xylene cyanol was added. The samples were denatured by boiling this mixture for 2 min in a water bath. They were then directly placed onto 5% polyacrylamide gels cast in buffer containing 90 mM Tris (pH 8.3), 90 mM boric acid, and 2.5 mM EDTA. The gels were subjected to electrophoresis in the same buffer at a constant 250 V until the bromophenol blue dye had reached the bottom (i.e., approximately 4 hr). Gel dimensions were $0.1 \times 10 \times 25$ cm.

Gels were either dried with Whatman paper as support and then autoradiographed or directly autoradiographed with Kodak RP-X-Omat film. For dried gels, images were enhanced with a lightning-plus Dupont Cronex intensifying screen. Simian virus 40 [^{32}P]DNA digested with the restriction enzyme *Hin*f was used as markers on the gel.

Elution of DNA from Acrylamide Gels. After the localization of DNA bands by autoradiography, the bands were sliced out and homogenized in 1.5-ml Eppendorf tubes with a glass rod drawn out to fit snugly into the tubes according to the procedure of Maxam and Gilbert (10). These were then incubated with 0.5 M ammonium acetate/10 mM Mg ace-

Abbreviations: M-MuLV, Moloney murine leukemia virus; Act D, actinomycin D; kbp, kilobase pair.

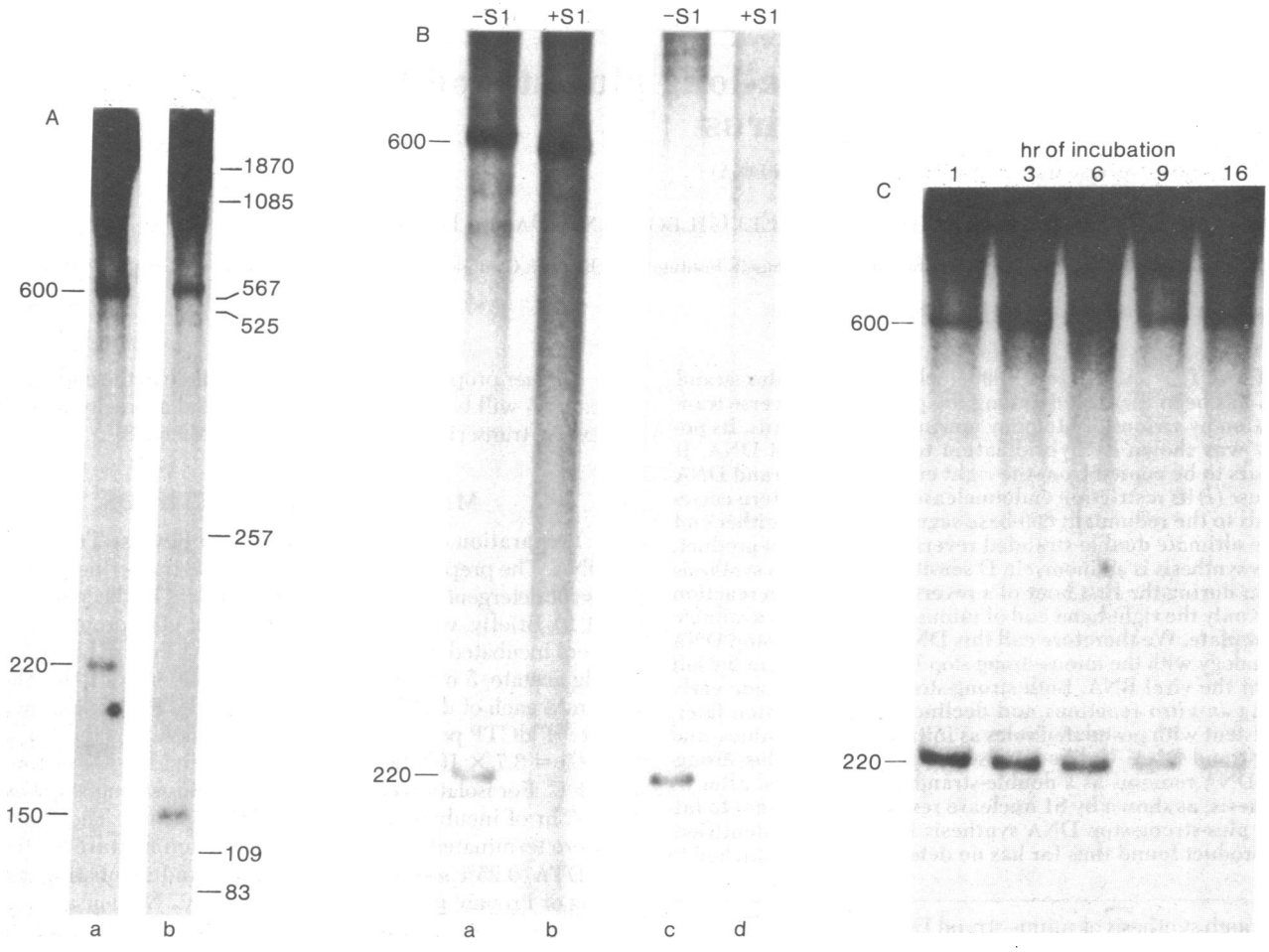

FIG. 1. Synthesis of 600-base DNA: effect of Act D, treatment with ribonuclease or S1 nuclease, and time course of synthesis. Number of bases is shown beside the lanes. (*A*) Total reverse transcription product was synthesized by incubation of virions at 40°C for 3 hr. Nucleic acids were prepared, and alcohol-precipitated samples were dissolved in 10 mM Tris, pH 7.5/0.1 mM EDTA. The samples were either untreated (lane a) or treated (lane b) with 0.02 mg of pancreatic RNase per ml (Worthington) at 37°C for 1 hr. Both samples were extracted with phenol/chloroform and denatured in 80% formamide prior to analysis by electrophoresis through a 5% polyacrylamide gel. (*B*) Endogenous [^{32}P]DNA was synthesized in the absence (lanes a and b) or presence (lanes c and d) of 100 μg of Act D per ml. Samples were processed and either left untreated (lanes a and c) or digested with S1 endonuclease (lanes b and d). DNA was then extracted with phenol/chloroform, denatured, and analyzed by electrophoresis through 5% polyacrylamide gel. (*C*) Samples of DNA from various times of reverse transcription were denatured and analyzed by electrophoresis. Endogenous reverse transcription was carried out in the presence of [α-^{32}P]dCTP for the indicated times and products were processed. The product from an equivalent volume of synthesis mixture was added for each lane.

tate/0.1% sodium dodecyl sulfate/0.1 mM EDTA at 37°C for 8–10 hr. The tubes were then centrifuged for 10 min at room temperature in a microfuge and the supernatants were collected. Incubations with buffer and centrifugations were repeated two to three more times until 90% of the label was extracted. Large pieces of gel were removed from the pooled supernatants by forcing the extract through a siliconized glass wool plug inside an Eppendorf tip. Unlabeled calf thymus DNA (4–8 μg) was added as carrier, extracted twice with phenol/chloroform, 1:1 (vol/vol), and then precipitated with alcohol.

CsCl/Guanidinium Chloride Centrifugation. To purify plus-strong-stop DNA, we isolated 5–10 ng of the 600-base DNA by elution from a gel and hybridized it to 2 μg of 70S viral RNA in the presence of 50% formamide/0.2 M 1,4-piperazinediethanesulfonic acid (Pipes), pH 6.4, for 2 hr at 56°C. Hybridization was terminated with the addition of 100 μl of 0.2 M Na acetate (pH 8.4), 20 mM Tris (pH 8.4), 5 mM EDTA, and 60 μg of yeast RNA per ml. The entire reaction mixture was added to a solution of 12.88 g of CsCl, 2.4 g of guanidinium chloride, and 10 ml of 10 mM Tris·HCl (pH 7.4) and centrifuged for 40 hr at 20°C in an SW50.1 rotor at 40,000 rpm (11). Fractions of 0.5 ml were collected, and radioactive DNA was located by its Cerenkov radiation.

Restriction Endonuclease Cleavage. The purified 600-base DNA was hybridized to 50 ng of (−)DNA in 50 μl of 1 M NaCl/20 mM Pipes, pH 6.4/1 mM EDTA at 68°C for 3 hr. Reactions were terminated by the addition of 1 ml of 0.2 M Na acetate, pH 8.4/20 mM Tris·HCl, pH 8.4/5 mM EDTA and precipitated by alcohol with 6 μg of yeast RNA as carrier. The precipitate was washed with 80% alcohol, dried, and dissolved in 20 μl of 10 mM Tris·HCl, pH 7.5/0.1 mM EDTA.

S1 Endonuclease Treatment. S1 endonuclease (Boehringer Mannheim) digestion was performed in the presence of 250 mM NaCl, 30 mM Na acetate (pH 4.5), 2 mM $ZnSO_4$, and 20 μg of denatured calf thymus DNA per ml at 45°C for 45 min. The amount of enzyme was selected by previous titration to ensure that over 90% of single-stranded DNA would be digested to acid solubility with virtually no loss of radioactivity from double-stranded DNA.

Reactions were terminated by the addition of 1 ml of 0.2 M Na acetate (pH 8.4), 20 mM Tris (pH 8.4), 5 mM EDTA, and 60 μg of yeast RNA per ml followed by alcohol precipitation.

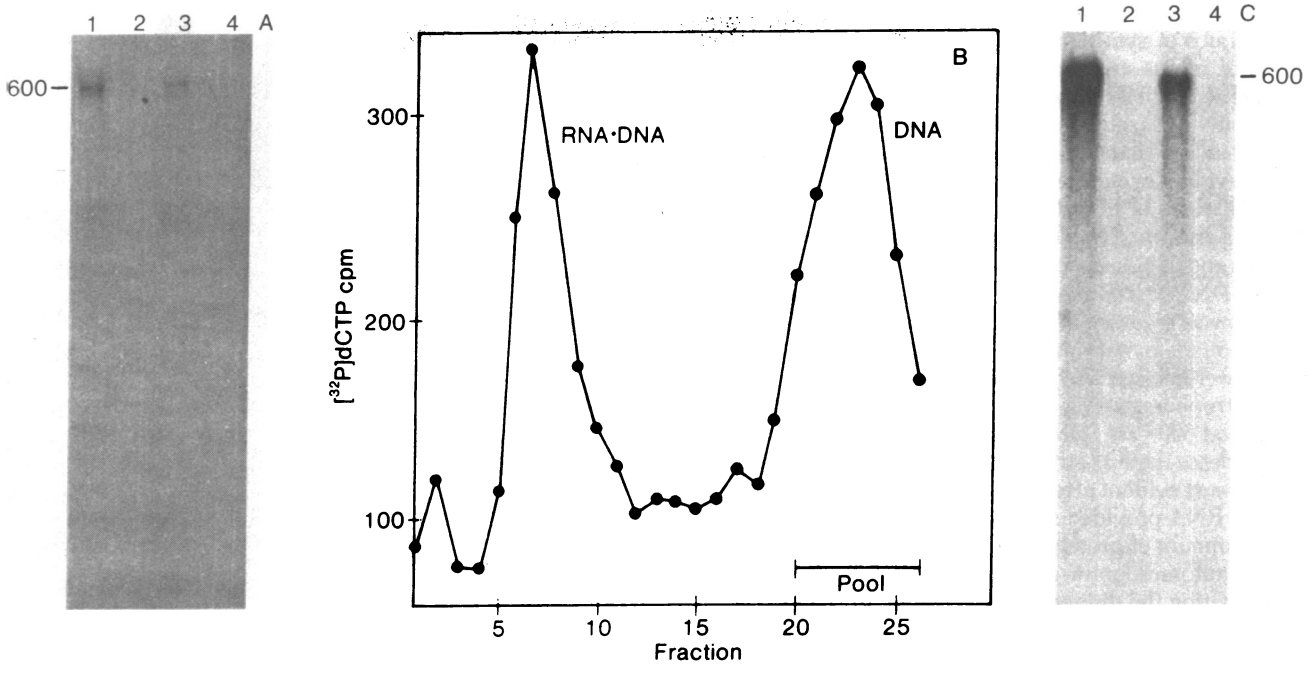

FIG. 2. Determination of polarity of 600-base DNA. (*A*) The 600-base DNA isolated from a polyacrylamide gel was hybridized to virion RNA (lanes 1 and 2), to (−)DNA (lane 3), or to itself (lane 4). After hybridization, samples were left untreated (lane 1) or treated with S1 nuclease (lanes 2–4) and subsequently processed and analyzed electrophoretically. (*B*) A hybridization mixture of 600-base [^{32}P]DNA and viral RNA was centrifuged to equilibrium in a guanidinium/CsCl gradient to separate the virion RNA·(−)DNA hybrids from the plus-strand DNA. The fractions at the top of the gradient, representing unhybridized plus-strand DNA, were pooled and precipitated with alcohol. (*C*) The pooled free DNA fraction from the guanidinium/CsCl gradient was hybridized and treated with S1 nuclease as described for *A*.

5′-End Labeling. The 9-kbp double-stranded DNA and total reverse-transcribed DNA were treated with calf intestinal phosphatase (Boehringer Mannheim) in the presence of 20 mM Tris, pH 8.6/10 mM $MgCl_2$ at 37°C for 30 min. The amount of calf intestinal phosphatase was chosen by prior titration to ensure complete removal of 5′-phosphate groups. Phosphatase-treated DNA was then incubated with [γ-^{32}P]ATP (3000 Ci/mmol) and 1 unit of polynucleotide kinase (New England Biolabs) in the presence of 50 mM Tris, pH 8.2/10 mM $MgCl_2$/5 mM dithiothreitol for 60 min at 37°C.

The 9-kbp DNA was cleaved with restriction endonuclease *Xho* I and the 2-kbp fragment was purified on agarose gels. This fragment represents the left end (4). Cleavage of 9-kbp DNA with restriction enzyme *Hpa* I and agarose gel purification of the 1050-base-pair-long fragment yielded the right end (4). Total reverse-transcribed, denatured, 5′-end-labeled DNA was electrophoresed on 5% polyacrylamide gels to yield the 600-base plus-strong-stop DNA.

RESULTS

Identification of a 600-Base DNA. To investigate whether a discrete, short, plus strand of DNA is made during *in vitro* reverse transcription, product DNA denatured in 80% formamide was analyzed by electrophoresis through a 5% polyacrylamide gel. Two discrete bands shorter than 1 kb were seen (Fig. 1*A*, lane a). One band was about 220 bases long and shifted to about 150 bases after digestion with pancreatic RNase (Fig. 1*A*, lane b). The 220-base DNA is known to be minus-strong-stop DNA attached to its tRNA primer, and ribonuclease will remove the tRNA (5). The second discrete DNA was 600 bases long and did not change mobility after RNase treatment.

To further examine synthesis of the 600-base DNA, we investigated the effects of Act D and S1 nuclease and the time course of its synthesis. Act D prevents DNA-dependent nucleic acid synthesis (12) and should therefore inhibit synthesis of the 600-base DNA if it represents plus-strand DNA copied from minus-strand DNA. When products of endogenous reactions with or without Act D were denatured and analyzed by electrophoresis, no 600-base DNA band was evident in the Act D-treated samples (Fig. 1*B*, lanes a and c). In both samples, however, minus-strong-stop DNA was evident. The Act D-treated sample also had a small amount of a band 500 bases long that is faintly evident in the absence of Act D and has not been investigated further.

To examine whether the 600-base DNA was found in a double-stranded form, we treated DNA made with or without Act D with S1 nuclease, denatured it, and analyzed it by electrophoresis. The Act D-treated sample again showed no 600-base DNA band (Fig. 1*B*, lane d), but the sample lacking the inhibitor contained such a band (Fig. 1*B*, lane b). The 600-base DNA is actually slightly smaller after S1 nuclease treatment; this effect has not been investigated further. Thus, the reverse transcription product had the majority of the 600-base DNA hydrogen-bonded to its complement, but the DNA made in Act D had no discrete DNA. The inability to detect the 600-base DNA in the presence of Act D suggests that it is made by DNA-dependent DNA synthesis and probably represents plus-strand DNA.

The S1 nuclease-treated DNA lacked any detectable minus-strong-stop DNA. Were this DNA hydrogen-bonded to its RNA template, it should have been protected. This result confirms an earlier study from this laboratory (unpublished data) in which it was shown that with or without phenol extraction, neither minus-strong-stop DNA nor longer minus-strand DNA made by reverse transcription was hydrogen-bonded to its RNA template. It appears that DNA copied from RNA does not remain hydrogen-bonded to its template although DNA copied from DNA does remain in a duplex configuration.

The time courses of synthesis of 600-base DNA and minus-

strong-stop DNA were examined by analyzing products after various times of synthesis (Fig. 1*C*). After 1 hr, a maximal amount of minus-strong-stop DNA synthesis was evident. It remained at a high level until 6 hr of reaction and then declined; by 16 hr it was barely evident. The 600-base DNA was more apparent at 3 hr than at 1 hr; it declined somewhat after 6 hr, but was evident even after 16 hr of reaction.

The 600-Base DNA Is Plus Strand. The polarity of 600-base DNA was examined by determining whether it hybridizes to minus-strand DNA or to (plus-strand) viral RNA. Labeled 600-base DNA band was excised from a polyacrylamide gel and the DNA was recovered. It was hybridized to viral RNA or to the DNA made by virions in the presence of Act D [(−)DNA]. Hybrids were detected by S1 nuclease treatment, denaturation, and electrophoresis (Fig. 2*A*). Self-annealed, 600-base DNA (lane 4) and 600-base DNA hybridized to RNA but not exposed to S1 nuclease (lane 1) served as controls. Protection against S1 nuclease was evident after hybridization to (−)DNA (lane 3), but viral RNA provided significantly less protection (lane 2). A small amount of protection by viral RNA was evident in the original autoradiogram and could represent some (−)DNA contaminating the discrete 600-base band; the band protected by viral RNA was wide and diffuse compared to the sharp band protected by (−)DNA. In an effort to eliminate this source of contamination, we hybridized DNA extracted from the 600-base band to 70S viral RNA. Unhybridized plus-strand DNA was separated from (−)DNA·RNA hybrids in a guanidinium/CsCl gradient (Fig. 2*B*) (11). Approximately 50% of the DNA banded at the density of DNA·RNA hybrids and presumably represented minus strands. The free DNA peak was pooled, precipitated, hybridized either to 35S RNA or to (−)DNA, digested with S1 nuclease, and analyzed by electrophoresis (Fig. 2*C*). The sample hybridized to (−)DNA (lane 3) showed extensive protection of a band the same size as the starting material (shown in lane 1). Viral RNA protected no detectable DNA (lane 2), and self-annealed material contained no protected DNA (lane 4). These experiments show that the 600-base discrete band of DNA represents plus-strand DNA. Hereafter we will therefore refer to it as plus-strong-stop DNA.

Location of Plus-Strong-Stop DNA on Viral Genome. Two approaches were adopted to determine what portion of (−)DNA is complementary to plus-strong-stop DNA. For the first analysis, we used the known restriction enzyme cleavage maps of M-MuLV DNA (4). Uniformly ^{32}P-labeled plus-strong-stop DNA was annealed to (−)DNA, digested with nucleases *Hae* III, *Pvu* II, or *Bam*HI, denatured, and analyzed by electrophoresis (Fig. 3). The digested fragments corresponded to the predicted sizes of fragments from either the right or left ends of the 9-kbp full-length reverse transcript of the M-MuLV genome (Fig. 3) (these ends have identical structures, ref. 4). *Bam*HI did not digest the DNA. *Pvu* II gave fragments of 115, 76, and 400 bases; the map (Fig. 3 *lower*) shows that a 115-base piece generated by *Pvu* II occurs at the left end of the reverse transcription product, a 400-base piece occurs at the right end, and 76-base pieces occur adjacent to both the left and right ends. *Hae* III gave fragments of 350 and 150 bases (smaller predicted *Hae* III fragments were not examined). The map in Fig. 4 shows that these *Hae* III fragments are the length of the *Hae* III left- and right-terminal fragments of the reverse transcription product. Thus, plus-strong-stop DNA has the general structure of the termini of the full-length reverse transcript.

To map plus-strong-stop DNA more precisely, we labeled its 5′ end with ^{32}P by using polynucleotide kinase and [γ-^{32}P]ATP. The single-stranded DNA was then partially hydrolyzed with *Hae* III for various times and the fragments were

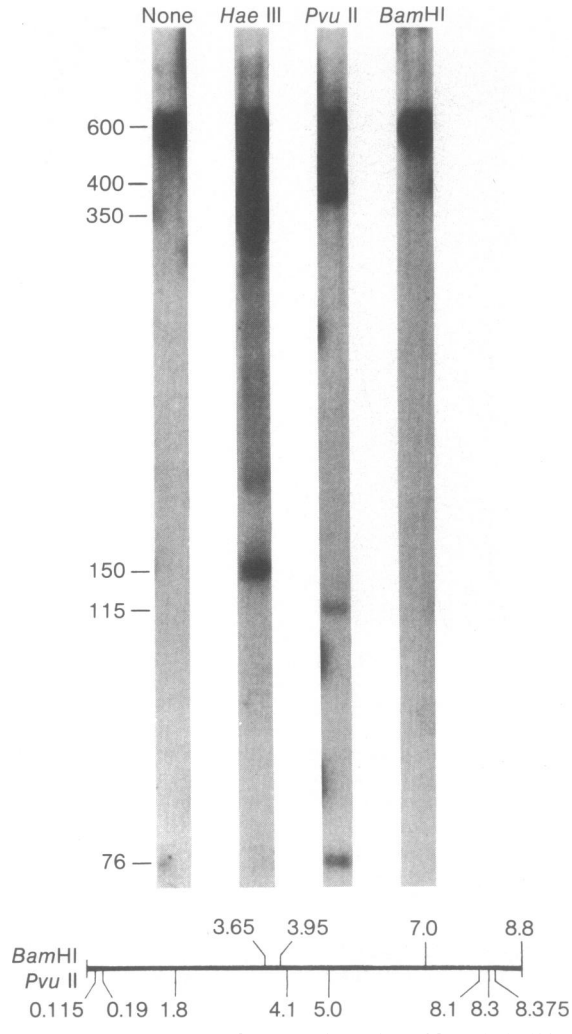

FIG. 3. Mapping of 600-base DNA on the 9-kbp DNA. (*Upper*) Uniformly ^{32}P-labeled 600-base DNA extracted from gels was made double stranded by hybridization to (−)DNA and digested with *Bam*HI, *Pvu* II, or *Hae* III or was left untreated. The cleavage products were analyzed electrophoretically after denaturation. (*Lower*) Cleavage sites of the full-length 8.8-kb DNA (4). The *Hae* III sites are shown in Fig. 4; 150 bases are to the left of the four sites and 350 bases are located to the right. The 600-base material left undigested presumably represents single-stranded minus-strand DNA contaminating the preparation.

separated by electrophoresis (Fig. 4*A*). For comparison, the 5′ termini at the left and right ends of the 9-kbp double-stranded product of reverse transcription (4) were labeled and partially digested as double-stranded DNAs with *Hae* III (Fig. 4 *B* and *C*). As shown in Fig. 4, the polarity of the DNA is such that the left end was labeled at its plus-strand end and the right end was labeled at its minus-strand end. The 600-base DNA gave three prominent labeled fragments, all of which were present in the *Hae* III digest of the left end; a minor contamination of right-end fragments was also present. The lack of one partial digest product from the left end (220 bases long) is a consequence of having digested single-stranded DNA; a digest of plus-strong-stop DNA hybridized to (−)DNA showed this band (and was also digested more completely than shown in Fig. 4*A*; unpublished results). These data imply that 600-base DNA has exactly the same structure as the left end of the 9-kbp DNA because the 150-base fragment representing this end is evident in both 600-base DNA and the left end of 9-kbp DNA.

Referring to the maps in Fig. 4, this result is consistent with

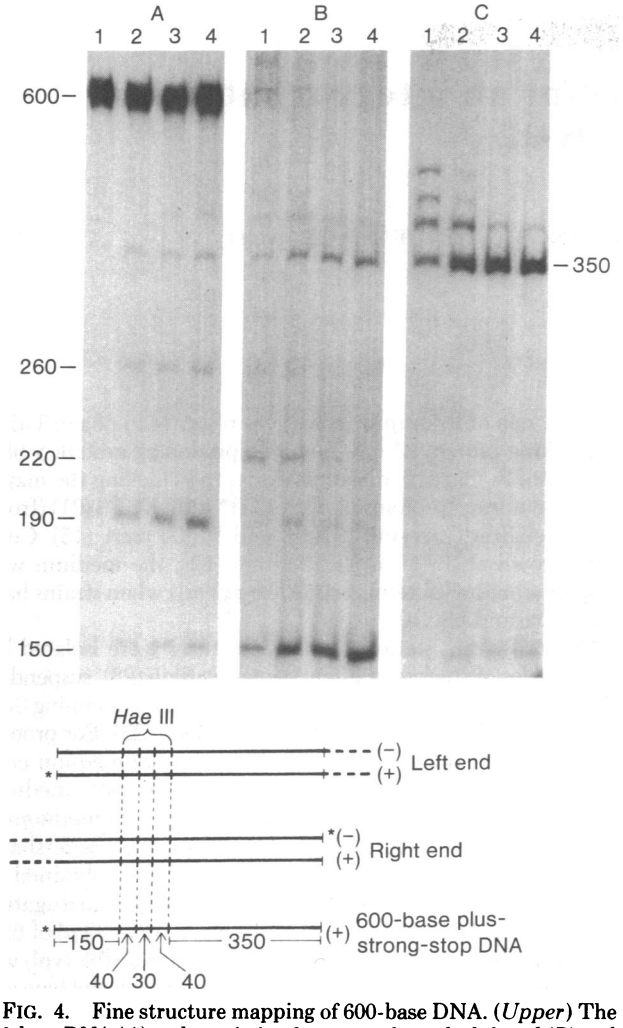

FIG. 4. Fine structure mapping of 600-base DNA. (*Upper*) The 600-base DNA (*A*) and restriction fragments from the left end (*B*) and right end (*C*) of 9-kpb DNA were labeled at the 5′ end with polynucleotide kinase and partially digested with *Hae* III endonuclease. Digestion was carried out at 25°C for 5 min (lanes 1), 20 min (lanes 2), and 1 hr (lanes 3) and at 37°C for 1 hr (lanes 4). (*Lower*) Comparison of the *Hae* III sites on the three substrates.

plus-strong-stop DNA arising from a start 600 bases from the right end. Because the two ends of 9-kbp DNA are identical, however, it could, in principle, arise from the left end of 9-kbp DNA. The short synthesis time used to make plus-strong-stop DNA (3 hr) argues that the DNA must come from the right end of the 9-kbp DNA because no left-end completion is evident after short times (4).

DISCUSSION

We have shown that among the products of endogenous reverse transcription is a 600-base DNA. This DNA is not made in the presence of Act D, hybridizes to (−)DNA but not to viral RNA, and maps at one end of 9-kbp ultimate product of reverse transcription. It is made within the first hour of *in vitro* synthesis, long before any full-length minus strands are evident. It must therefore be copied from nascent minus strands and must be copied from their right ends. It clearly deserves the name plus-strong-stop DNA by analogy to the minus-strong-stop DNA that is the initiator of minus-strand synthesis.

Varmus *et al.* (1) have identified a similar DNA made in cells recently infected by Rous sarcoma virus. It is made before completion of the minus strand and it represents a copy of the right end of the minus strand. Neither we nor Varmus *et al.* (1) have provided proof that plus-strong-stop DNA is actually the initiator of plus-strand DNA synthesis, but the fact that it is copied from the right end of minus-strand DNA but has the structure of the left end of 9-kbp DNA strongly argues that it is an initiator for plus-strand synthesis. As yet, 600-base DNA has not been sought in cells infected by M-MuLV.

An argument that both minus- and plus-strong-stop DNA are initiators can be made from the kinetics of their synthesis. Both appear early, reach a plateau of concentration, and then decline in concentration as long minus and plus strands accumulate. If the plateau and decline represent use of these DNAs as initiators of long DNA molecules, then minus-strong-stop DNA is used more efficiently than plus-strong-stop DNA. Their plateau concentrations are, however, approximately proportional to their molecular weights.

An important result of this work that was not made obvious during the presentation of the data is that 9-kbp DNA could be labeled by [γ-^{32}P]ATP at both ends (Fig. 4 *B* and *C*). This label was alkali resistant (unpublished results). It therefore appears that the 5′ ends in 9-kbp DNA are not blocked by anything but phosphate and are DNA ends. The tRNA primer that initiates minus-strong-stop DNA must therefore have been removed during reverse transcription. Recent experiments have directly demonstrated that this removal occurs shortly after plus-strong-stop DNA is completed (unpublished observations).

Elsewhere we will describe more details of the structure of plus-strong-stop DNA and we will propose how it might function as the initiator of synthesis of a full-length plus-strand DNA (8).

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