JOURNAL OF VIROLOGY, July 1984, p. 26–33
0022-538X/84/070026-08$02.00/0
Copyright © 1984, American Society for Microbiology
Vol. 51, No. 1

RNA-Primed Initiation of Moloney Murine Leukemia Virus Plus Strands by Reverse Transcriptase In Vitro

WENDI I. FINSTON AND JAMES J. CHAMPOUX*

Department of Microbiology and Immunology, School of Medicine, University of Washington, Seattle, Washington 98195

Received 16 January 1984/Accepted 29 March 1984

A 190-base-pair DNA-RNA hybrid containing the Moloney murine leukemia virus origin of plus-strand DNA synthesis was constructed and used as a source of template-primer for the reverse transcriptase in vitro. Synthesis was shown to initiate precisely at the known plus-strand origin. The observation that some of the origin fragments retained ribonucleotide residues on their 5′ ends suggests that the primer for chain initiation is an RNA molecule left behind by RNase H during the degradation of the RNA moiety of the DNA-RNA hybrid. If the RNase H is responsible for creating the correct primer terminus, then it must possess a specific endonucleolytic activity capable of recognizing the sequence in the RNA where plus strands are initiated. The 16-base RNase A-resistant fragment which spans the plus-strand origin can also serve as a source of the specific plus-strand primer RNA. Evidence is presented that some of the plus-strand origin fragments synthesized in the endogenous reaction contain 5′ ribonucleotides, suggesting that specific RNA primers for plus-strand initiation may be generated during reverse transcription in vivo as well.

Replication of retrovirus genome RNA is accomplished by reverse transcription of the RNA into double-stranded DNA, integration of the DNA into the host chromosome, and transcription of the integrated proviral DNA into RNA. The enzyme reverse transcriptase, which is responsible for copying the RNA into DNA early after infection, is carried within virus particles (1, 32). The process of reverse transcription has been studied in vitro with detergent-treated virions, and detailed models have been presented for the various steps in the overall process (11, 12, 31, 33).

Like all other known DNA polymerases, reverse transcriptase has an absolute requirement for a primer to initiate new chains (2). The synthesis of the minus-strand DNA is primed by a cellular tRNA molecule which is packaged along with the genome RNA in virions (36). After the genome RNA has been used as a template for minus-strand synthesis, the RNA component of the resulting DNA-RNA hybrid is degraded, presumably by the RNase H activity which has been found to be associated with all known reverse transcriptases (3, 7–10, 14, 21).

The mechanism by which plus-strand DNA synthesis is primed is unknown. Although multiple priming events might be involved in the synthesis of the complete plus-strand DNA (24, 34), studies on the nature of the first plus strand to be synthesized (called plus strong-stop DNA) indicate that the origin for plus-strand initiation is located near the 5′ end of the newly synthesized minus-strand DNA (15, 16, 20). Moreover, for Moloney murine leukemia virus (M-MuLV), the site at which plus strong-stop DNA initiates has been found to be unique and to begin with the sequence 5′-AATGAAAGA (19) (Fig. 1). This sequence is preceded by a string of 13 purine residues in the RNA, which has been suspected to be important for the specific priming of plus strands (30). The precise initiation of plus strands is important for the generation of the left end of the left long terminal repeat, which in turn is probably important, in conjunction with the right long terminal repeat sequence, for integration (26, 28).

It has been suggested that the degradation of the genome RNA in the vicinity of the plus-strand origin by RNase H might leave a specific RNA fragment which primes the synthesis of plus strands (22–24, 37). To test this hypothesis, we initiated experiments designed to reconstruct the process of plus-strand initiation in vitro. A DNA-RNA hybrid, 190 base pairs in length containing the origin for M-MuLV plus-strand synthesis, was tested as a source of primer-template for DNA synthesis by M-MuLV reverse transcriptase. We show here that synthesis can initiate precisely at the known plus-strand origin, suggesting that the RNase H activity is capable of cleaving the RNA strands of the DNA-RNA hybrids to generate the correct primer RNA. After this work was submitted, a paper by Smith et al. (29) appeared which described an in vitro system for the priming of Rous sarcoma virus plus strands which is dependent on added RNA.

MATERIALS AND METHODS

Cloning of M-MuLV plus-strand origin in M13mp7. The *Hin*dIII-*Sac*I M-MuLV proviral DNA fragment (27), corresponding to the right third of the genome, was cloned in pBR322 by using *Eco*RI linkers. The 190-base *Pvu*II fragment containing the plus-strand origin (27) was subcloned from this plasmid by insertion into the *Hin*cII site of M13mp7 by blunt-end ligation by standard recombinant DNA techniques (18). Recombinant phage isolates containing inserts of opposite polarity were identified by their ability to hybridize as described by Messing (18). The recombinant phage containing the minus strand was identified by hybridization to an M13mp2 recombinant phage DNA previously shown to contain an insert of the same polarity as the viral M-MuLV genome RNA.

Excision of a single-stranded insert from M13mp7 recombinant phage DNA. The presence of a region of self-complementarity within the multicloning site of M13mp7 DNA allows one to use certain restriction enzymes to excise an insert directly from the single-stranded DNA (4). The method for the isolation of single-stranded M13 phage DNA has been described previously (4). Before excision of the insert with *Eco*RI, the phage DNA was heated to 100°C in 0.2 M NaCl for 2 min. The reaction mixture for excision (final volume, 0.8 ml) contained 100 mM Tris-hydrochloride (pH 7.5), 5 mM $MgCl_2$, 50 mM NaCl, 100 μg of DNA per ml, and

* Corresponding author.

400 U of *Eco*RI (New England Biolabs). After the reaction was incubated at 10°C for 18 h, the 190-base excised fragment was separated from the larger vector DNA by sedimentation in a 5 to 20% alkaline sucrose gradient in a SW56 rotor at 50,000 rpm and 20°C for 4 h. For some experiments the isolated fragment was 3′ end labeled with ^{32}P by fill-in with the large fragment of *E. coli* DNA polymerase I as previously described (4). The structure of the isolated minus-strand fragment, referred to as P190 DNA, was verified by the sequencing method of Maxam and Gilbert (17).

Isolation of M-MuLV RNA. Clone 1 M-MuLV was grown and purified by the MIT Cell Culture Center as described previously (11). Approximately 7 mg of virus was suspended in 20 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA, and sodium dodecyl sulfate was added to a final concentration of 0.5%. The lysed virions were extracted twice with phenol-chloroform (1:1) and twice with chloroform-isoamyl alcohol (24:1) and precipitated with 2 volumes of ethanol. RNA concentration was determined from the absorbance at 260 nm, taking an absorbance value of 1.0 as 40 μg/ml.

Preparation of DNA-RNA hybrids. The hybridization reaction (volume, 50 μl) contained 35 ng of isolated P190 fragment DNA and 6.7 μg of M-MuLV RNA in 0.2 M NaCl. After incubation for 2 h at 65°C, NaCl was added to 0.5 M, and the complexes were treated with a combination of RNase A (20 μg/ml) and T1 RNase (1,000 U) for 30 min at 37°C to remove the RNA tails and degrade any excess unhybridized RNA. T1 RNase was included in the reaction to degrade any of the polypurine origin fragments which might have been produced by the action of RNase A on any unhybridized M-MuLV RNA. Sodium dodecyl sulfate was added to 0.1%, and the products were extracted twice with phenol and twice with chloroform-isoamyl alcohol (24:1). The samples were diluted to 0.3 M NaCl and precipitated by the addition of 2 volumes of ethanol, followed by ethanol precipitations in the presence of 2 M ammonium acetate and 0.3 M sodium acetate.

Purification of M-MuLV reverse transcriptase. The DNA-polymerizing activity of the reverse transcriptase was assayed by the incorporation of ^{32}P-labeled dATP into acid-insoluble material by using single-stranded DNA as a template and oligonucleotides generated by extensive pancreatic DNase digestion of calf thymus DNA as primers (13). The reaction mixture (final volume, 10 μl) contained 50 mM Tris-hydrochloride (pH 8.0), 10 mM $MgCl_2$, 50 mM KCl, 0.4 mM dithiothreitol, 10 μg of M13mp9 single-stranded DNA per ml, 1 μl of oligonucleotide primers, 0.2 mM dCTP, 0.2 mM dTTP, 0.2 mM dGTP, and 0.1 mM [α-^{32}P]dATP (1 to 2 Ci/mmol). After incubation at 37°C for 20 min, an 8-μl portion was spotted onto a glass fiber filter, and acid-precipitable radioactivity was determined as described previously (6). One unit of enzyme activity was defined as the amount of enzyme which resulted in the incorporation of 1 pmol of dATP in 20 min under these reaction conditions.

The reverse transcriptase was purified by DEAE and phosphocellulose chromatography of the proteins from 33 mg of detergent-treated M-MuLV virions by the method of Verma and Baltimore (35) with the modifications described by Gerard (10). After concentration by dialysis against 30% polyethylene glycol, the enzyme was stored at −80°C at a concentration of 20,000 U/ml.

Reverse transcriptase reaction conditions. Reaction mixtures (final volume, 20 to 50 μl) contained 50 mM Tris-hydrochloride (pH 8.0), 10 mM $MgCl_2$, 50 mM KCl, 0.4 mM dithiothreitol, 0.2 mM dCTP, 0.2 mM dTTP, 0.2 mM dGTP, 25 μM [α-^{32}P]dATP (40 Ci/mmol), 6.0 to 10 ng of P190 DNA-RNA hybrids, and 2,000 U of M-MuLV reverse transcriptase per ml. The reactions were incubated at 37°C for 60 min and terminated by the addition of excess EDTA. The products were ethanol precipitated in the presence of 2 M ammonium acetate to remove unincorporatd deoxynucleotides and, where indicated, cut with a restriction enzyme under the conditions recommended by the supplier.

M-MuLV endogenous reaction. The endogenous reverse transcription reaction conditions were described previously (19). Briefly, the reaction contained, in addition to the standard salts mixture described above, 1 mM each of the deoxyribonucleoside triphosphates and 0.01% Nonidet P-40. In some reactions the products were uniformly labeled by the addition of ^{32}P-labeled dATP to a final specific activity of 1.0 Ci/mmol. After incubation at 40°C for 3 to 15 h, the reaction was terminated by the addition of excess EDTA and sodium dodecyl sulfate to 0.25%. The lysed virions were treated with proteinase K at 100 μg/ml, extracted once with phenol and twice with chloroform-isoamyl alcohol (24:1), and ethanol precipitated in the presence of 2 M ammonium acetate. The products were cut with *Pvu*II (New England Biolabs) and extracted with phenol and chloroform-isoamyl alcohol, and the 122-base plus-strand origin fragment was selected out of the mixture by hybridization to the M13mp7 recombinant phage containing the P190 M-MuLV minus-strand fragment. The hybrids were isolated by sedimentation in a 5 to 20% neutral sucrose gradient in the SW56 rotor at 50,000 rpm and 20°C for 2 h. For use as a marker in the sequencing gel analyses, the hybrids were cut with *Dde*I to generate the specific 77-base origin fragment.

Other procedures. Alkaline hydrolysis of RNA was accomplished by the addition of NaOH to 0.33 M and incubation of the mixture at 65°C for 20 min. The sample was neutralized with acetic acid and precipitated with 2 volumes of ethanol. The reaction conditions (final volume, 10 μl) for labeling 5′-hydroxyl termini in DNA fragments after alkaline hydrolysis were 70 mM Tris-hydrochloride (pH 7.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 to 1.0 μM [γ-^{32}P]ATP (2,900 Ci/mmol), and 4 U of polynucleotide kinase (New England Biolabs). After incubation for 6 h at 0°C, the unincorporated ATP was removed by ethanol precipitation in the presence of 2 M ammonium acetate. Nondenaturing polyacrylamide gel electrophoresis was carried out in a Tris-borate-EDTA (pH 8.3) buffer as described previously (20). Before the gel analysis, the samples were ethanol precipitated, dissolved in 80% formamide containing 5 mM EDTA, and denatured by heating to 100°C for 2 min. Simian virus 40 *Hin*fI fragments which had been 5′ end labeled with polynucleotide kinase were used as markers. The gels were dried onto Whatman 3MM paper, and autoradiograms were obtained by exposure at −80°C. The procedure for DNA sequence determination by base-specific chemical cleavages and analysis by polyacrylamide-urea gel electrophoresis was that described by Maxam and Gilbert (17).

RESULTS

DNA-RNA hybrids containing M-MuLV plus-strand origin. The minus strand of the 190-base M-MuLV *Pvu*II fragment containing the origin for plus-strand synthesis was cloned into the *Hin*cII site of M13mp7 (18). Because of the self-complementarity of the sequences within the polycloning region of the single-stranded vector DNA, the recombinant phage DNA can base pair over a short region that flanks the inserted sequences. The resulting duplex region contains the recognition sequences for several restriction enzymes including *Eco*RI. Excision of the single-stranded insert from

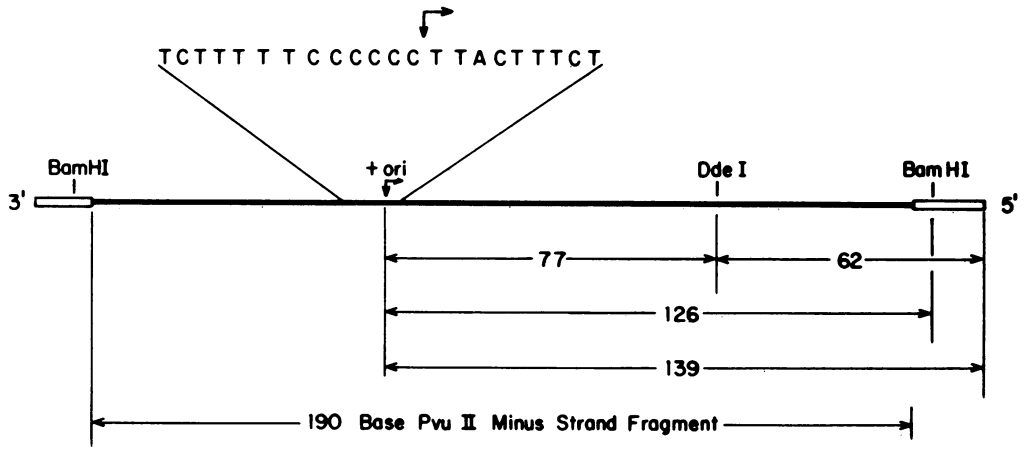

FIG. 1. Structure of the M-MuLV plus-strand origin-containing fragment. The minus strand of the M-MuLV 190-base *Pvu*II fragment was cloned in M13mp7 and excised with *Eco*RI to give the structure shown (P190 DNA). The M-MuLV *Pvu*II fragment sequences are indicated by the solid line. The open boxes at both ends of the solid line indicate the sequences contributed by the M13mp7 vector to the excised fragment. These sequences are complementary, enabling the two ends of the fragment to base pair to form a panhandle-type structure (not shown for simplicity). The location of the plus-strand origin in the *Pvu*II fragment and also within the DNA sequence around the origin is indicated by the two perpendicular arrows. The distances from the origin to the restriction enzyme sites and to the right end of the fragment are given in nucleotides.

the phage DNA with *Eco*RI (4) gives a fragment with the structure shown in Fig. 1 (P190 DNA).

M-MuLV RNA was hybridized to the isolated single-stranded P190 fragment, and the unpaired RNA tails were removed by treatment with pancreatic RNase A. The resulting structures were characterized to confirm that DNA-RNA hybrids had been formed and that they were relatively homogeneous in structure. The products of the hybridization reaction, labeled with ^{32}P at the 3′ ends of the DNA strands, were analyzed by electrophoresis through a 5% polyacrylamide gel adjacent to both unhybridized DNA and the duplexes formed when opposite polarities of the DNA fragment were annealed. When the P190 fragment was annealed to a complementary strand of the same size, the mobility of the resulting duplex DNA was greater than either one of the single-stranded fragments (Fig. 2, lanes 1, 2, and 3). Thus, under these conditions of gel electrophoresis, the duplex molecules migrated faster than the corresponding single-stranded fragments of the same length, suggesting that mobility through the gel was influenced by the secondary and probably tertiary structure of the molecule. The molecules which formed when the M-MuLV RNA was annealed to the P190 fragment and the resulting complexes were treated with RNase A should have contained 190 base pairs of hybrid structure flanked by short regions of single-stranded DNA at each end (see Fig. 1). The terminal single-stranded regions were complementary and would be expected to base pair to generate a circular molecule with a panhandle. It is difficult to predict the behavior of such structures in a nondenaturing 5% polyacrylamide gel, but the slower mobility of the hybrids in relation to the single-stranded DNAs (Fig. 2, lane 5) was at least consistent with the observation that fractionation under these conditions was quite sensitive to secondary and tertiary structure of the molecules. The presence of a single discrete species suggested that most of the structures were probably full-length DNA-RNA hybrids. As expected, the DNA-RNA hybrids, when analyzed before the RNase A digestion, migrated with a broad range of mobilities (Fig. 2, lane 4) due to variability in the lengths of the RNA tails.

DNA synthesis by M-MuLV reverse transcriptase with DNA-RNA hybrids as a source of primer-template. To test the hypothesis that RNase H is capable of generating the correct primer for plus-strand initiation, the DNA-RNA hybrids described above were incubated with M-MuLV reverse transcriptase in the presence of ^{32}P-labeled dATP. The products were cut with the restriction enzyme *Dde*I, denatured, and analyzed by electrophoresis through a 5% polyacrylamide gel (Fig. 3). Synthesis, which initiated from primers located at the plus-strand origin and continued to the end of the template DNA, would be predicted to yield fragments 77 and 62 bases in length after *Dde*I cutting (Fig. 1). The results (Fig. 3, lane 1) indicated that the two major DNA fragments produced under these conditions migrated at positions corresponding to these lengths. In other experiments in which uncut products were subjected to a similar analysis (data not shown), the major fragment produced had a length of 139 bases, and in addition, a fragment with an apparent length of 119 bases was occasionally observed. The 119-base fragment appears to result from the termination of synthesis at the duplex region which forms when the two complementary ends of the template DNA base pair. Two bands corresponding to these lengths are also visible in Fig. 3 (lane 1) and apparently resulted from incomplete cutting by the *Dde*I. The slowest labeled species (which comigrated with the 237-base marker fragment) was the template DNA itself (224 bases in length), which was 3′ end labeled by fill-in synthesis of the *Eco*RI site at the end of the panhandle by reverse transcriptase.

Two results indicated that synthesis of the 77-base fragment required the presence of RNA. First, incubation of the template DNA with reverse transcriptase in the absence of added RNA resulted in extensive end labeling of the template and the labeling of a few other DNA fragments, but none of the products had lengths near 77 nucleotides (Fig. 3, lane 4). The origin of the three fragments with lengths in the range from 150 to 160 bases in this experiment is unknown. Second, pretreatment of the M-MuLV with RNase T1, which cleaves the RNA within the G-rich primer region, eliminated the synthesis of the 77-base fragment (Fig. 3, lane 3).

However, treatment of the RNA with RNase A before

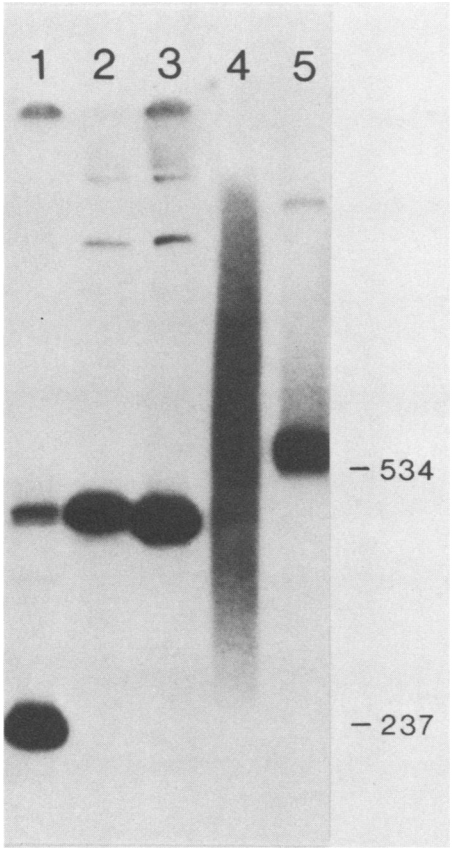

FIG. 2. Characterization of the hybrids formed between the P190 fragment and M-MuLV RNA by electrophoresis in a neutral polyacrylamide gel. Single-stranded P190 fragment DNA (3′ end labeled with ^{32}P) was hybridized with the complementary DNA fragment of the same size and subjected to electrophoresis through a 5% polyacrylamide gel for 2.5 h at 8 V/cm (lane 1) alongside the P190 fragment DNA alone (lane 2) and the complementary DNA strand alone (lane 3). M-MuLV RNA was hybridized to the P190 fragment DNA as described in the text and similarly analyzed before (lane 4) and after (lane 5) RNase A treatment in the presence of 0.5 M NaCl. The numbers along the right side indicate the positions of double-stranded simian virus 40 *Hin*fI markers run on the same gel.

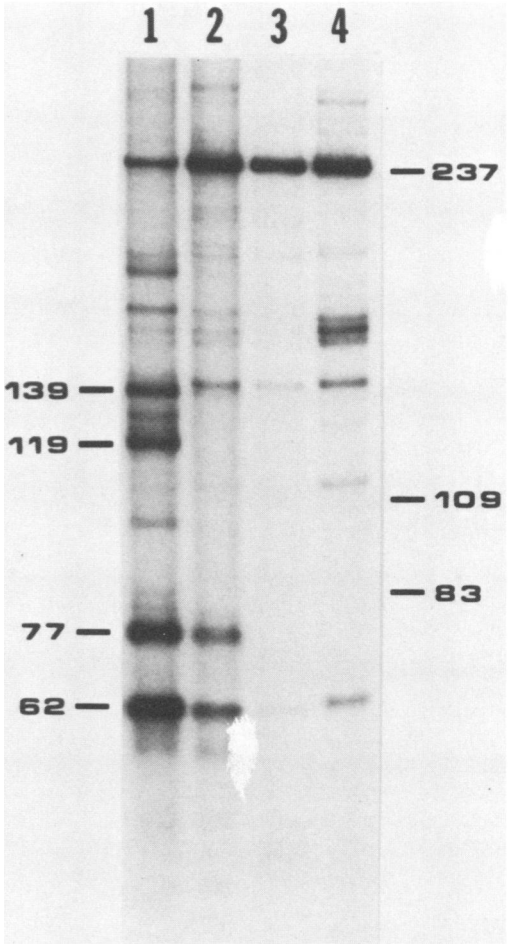

FIG. 3. Electrophoretic analysis of reverse transcriptase reaction products cut with restriction enzyme *Dde*I. Each reaction contained, in addition to the standard salts mixture, [α-^{32}P]dATP, 2,000 U of M-MuLV reverse transcriptase per ml and, as sources of template and potential primers, P190 fragment DNA hybridized to M-MuLV RNA (lane 1), P190 fragment DNA plus RNase A-treated M-MuLV RNA (lane 2), and P190 fragment DNA plus RNase T1-treated M-MuLV RNA (lane 3). Lane 4 contained only P190 fragment DNA. The products of the reactions were cut with *Dde*I, denatured, and subjected to electrophoresis through a 5% polyacrylamide gel for 2 h at 8 V/cm. The DNA bands were detected by autoradiography. The numbers along the right side indicate the positions of the single-stranded simian virus 40 *Hin*fI marker fragments run on the same gel. The numbers on the left indicate the sizes of the major reaction products.

allowing it to hybridize to the template DNA resulted in synthesis that appeared to initiate correctly from the plus-strand origin (Fig. 3, lane 2). This result suggested that the RNase A-resistant polypurine segment derived from the origin region of the RNA was sufficient as a precursor for the formation of the plus-strand primer (see below). It should be noted that the synthesis observed with the DNA-RNA hybrids could not have been due to the presence of the RNase A-resistant polypurine fragments contaminating the hybrid preparations since T1 RNase (which cuts 3′ to G residues), in addition to RNase A, was used in preparing the DNA-RNA hybrids.

Mapping the 5′ end of the in vitro-synthesized fragment. The major DNA fragment synthesized by the reverse transcriptase under the direction of the DNA-RNA hybrids had a length of ca. 139 bases as determined by mobility in a polyacrylamide gel containing 8 M urea (Fig. 4, lane 1), consistent with initiation having occurred at the plus-strand origin. After treatment with the restriction enzymes *Bam*HI and *Dde*I, the mobility of the major band shifted to the expected positions for fragments with lengths of 126 and 77 bases, respectively (Fig. 4, lanes 2 and 3). As will be shown below, the multiple species which have mobilities slightly slower than the major fragments with lengths of 139, 126, and 77 bases probably contain residual RNA.

It has been shown previously that M-MuLV plus strands are initiated in the endogenous reaction with the DNA sequence 5′-AATG and that after cutting the products of the reaction with *Pvu*II, 122-base fragments can be isolated which possess this sequence at their 5′ ends (19). Precise mapping of the 5′ start point for the in vitro-synthesized DNA was accomplished by comparing the mobility of the *Dde*I fragment produced in the reconstruction experiments with the mobility of the 122-base origin fragment which had been synthesized in the endogenous reaction and recut with *Dde*I (Fig. 4, lanes 3 and 4). The identical mobilities of the

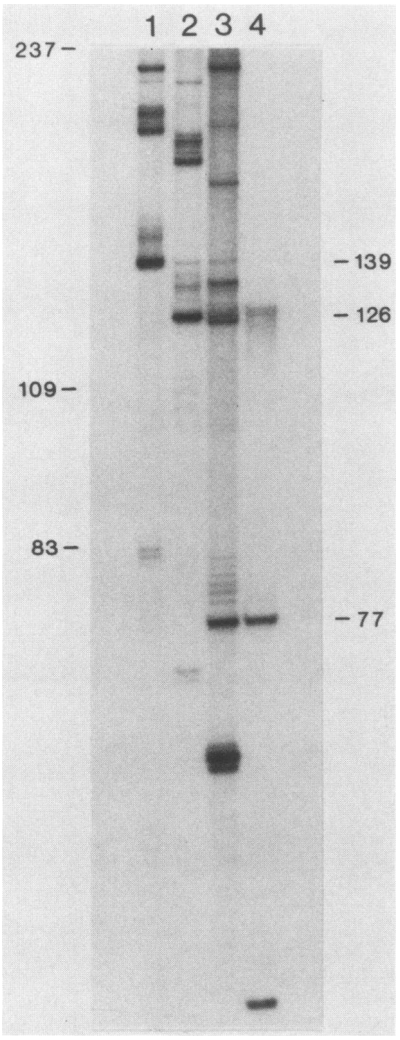

FIG. 4. Size determination of M-MuLV reverse transcriptase products using P190 fragment DNA hybridized to M-MuLV RNA as a template. Comparison with DNA synthesized in the endogenous reaction. P190 fragment DNA-RNA hybrids were incubated with reverse transcriptase under standard reaction conditions in the presence of [α-^{32}P]dATP, and the reaction was split into three portions. One portion was denatured and analyzed directly on an 8% polyacrylamide gel containing 8 M urea at 29 V/cm for 2.5 h (lane 1). The other two portions were treated with either *Bam*HI (lane 2) or *Dde*I (lane 3) before denaturation and electrophoretic analysis. Lane 4 contained plus strong-stop DNA (122 nucleotide-long *Pvu*II fragment) isolated from the endogenous reaction and cut with *Dde*I to give a fragment 77 nucleotides in length. The numbers along the left side show the positions of the single-stranded simian virus 40 *Hin*fI marker fragments run through the same gel. The numbers along the right side indicate the sizes of the major reaction products.

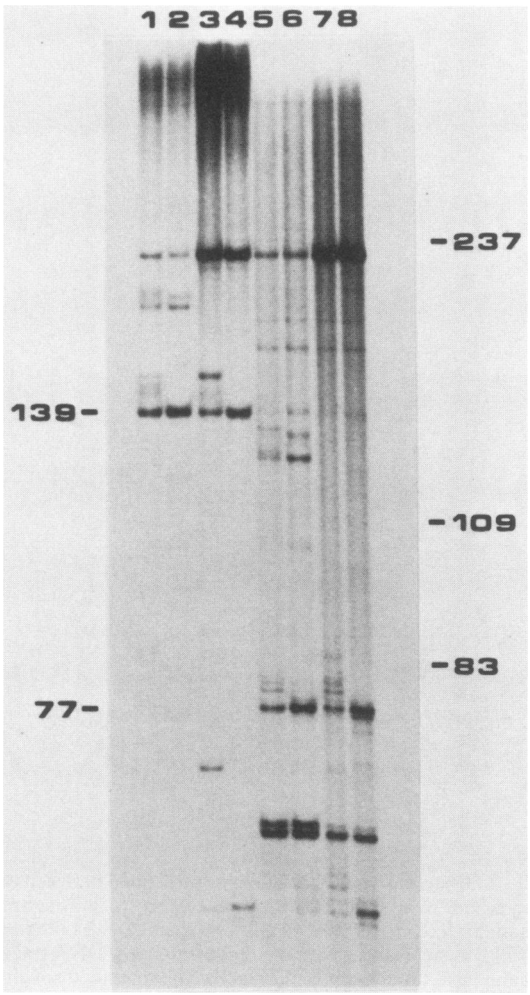

FIG. 5. Electrophoretic analysis of reverse transcriptase reaction products before and after treatment with alkali. P190 fragment DNA was the template for the M-MuLV reverse transcriptase, and the source of primers was either M-MuLV RNA which had been hybridized to the fragment as described in the legend to Fig. 4 (lanes 1, 2, 5, and 6) or RNase A-treated M-MuLV RNA (lanes 3, 4, 7, and 8). The products were either analyzed directly (lanes 1 through 4) or cut with *Dde*I before analysis (lanes 5 through 8). The samples run in the even-numbered lanes were treated with alkali before the electrophoresis. Electrophoresis was as described in the legend to Fig. 4. The numbers along the right side indicate the positions of the single-stranded simian virus 40 *Hin*fI marker fragments. The numbers along the left side mark the positions of the major reactions products.

two fragments in a sequencing gel indicated that the reverse transcriptase was initiating plus strands properly in vitro when presented with the DNA-RNA hybrids containing the plus-strand origin sequence.

The significance of the slower-migrating species seen near the top of Fig. 4, lane 1, is unknown. The length of the most prominent species was ca. 190 bases, placing its initiation point ca. 50 bases upstream of the normal plus-strand origin or only about 14 bases away from the duplex panhandle structure formed when the two ends of the template base pair. Whether this initiation site is normally used in the endogenous reaction or is an artifact of the in vitro system is unknown.

DNA strands initiated with RNA primers in vitro. From the results of the experiments shown in Fig. 3, it appeared that pretreatment of M-MuLV RNA with RNase A did not prevent the synthesis of the 77-base origin fragment by reverse transcriptase. To further investigate the size requirement of the RNA for priming, experiments were carried out with intact and RNase A-treated M-MuLV RNA as a source of primers. To explore the possibility that some of the DNA chains synthesized in these reactions might retain ribonucleotides on their 5′ ends, portions of the products were treated

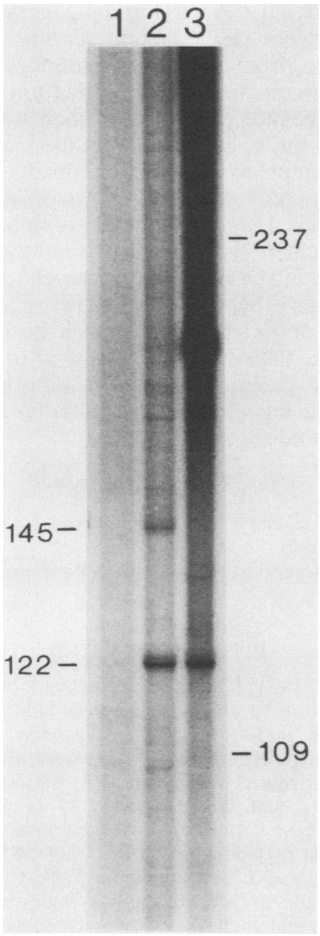

FIG. 6. Detection of RNA-primed plus-strand origin fragments from the endogenous reaction. The DNA which had been synthesized in the endogenous reaction was treated with alkali to remove any ribonucleotides from 5′ ends, and any resulting 5′-hydroxyl groups were labeled with ^{32}P by using polynucleotide kinase. The products of the kinase reaction were hybridized to M13mp7 containing the P190 minus-strain DNA insert, and the hybrids were isolated by sucrose gradient sedimentation. The isolated hybrids were denatured and subjected to electrophoresis in an 8% polyacrylamide gel containing 8 M urea for 2.5 h at 29 V/cm (lane 2). Lane 1 contained a control sample which had been treated in the same fashion except the alkaline hydrolysis step was omitted. Lane 3 contained uniformly labeled 122-base *Pvu*II origin fragment isolated from an endogenous reaction run in the presence of ^{32}P-labeled dATP. The numbers along the right indicate the positions of the simian virus 40 *Hin*fI marker fragments run in the same gel. The numbers along the left side indicate the sizes of the two major products labeled by the above protocol.

with alkali to remove any ribonucleotide residues before the electrophoretic analysis. Two conclusions can be drawn from the results of this experiment (Fig. 5). First, the synthesis of the 77-base fragment in the reaction containing RNase A-treated RNA indicated that the polypurine region near the origin could substitute for the intact RNA as a source of RNA primers for the precise initiation of DNA strands at the plus-strand origin (Fig. 5, lanes 5 through 8). Second, treatment of the products with alkali before the gel analysis (Fig. 5, even-numbered lanes) eliminated a set of minor bands which migrated slower than the 139-base or the 77-base major bands. Moreover, the alkali treatment appeared to shift radioactivity from the minor bands to the positions of the major bands, indicating that the retention of ribonucleotides by some of the origin fragments synthesized in vitro was responsible for the slower mobility of the DNA.

Evidence for RNA priming of plus strands in the endogenous reaction. Plus-strand origin fragments isolated from the M-MuLV endogenous reaction have previously been shown to be resistant to phosphorylation by polynucleotide kinase unless the DNA had been subjected to prior treatment with a phosphatase (20). Therefore, most of the origin fragments contained 5′ phosphate groups. Any strands containing 5′ ribonucleotide residues as remnants of RNA primers would similarly be resistant to phosphorylation by the kinase. However, treatment of such molecules with alkali should leave a 5′-hydroxyl terminus, which would be a substrate for polynucleotide kinase. These facts served as the basis for experiments designed to detect even trace amounts of RNA-primed plus strands which retained 5′ ribonucleotides.

DNA synthesis in Nonidet P-40-treated M-MuLV virions was allowed to proceed for 15 h at 40°C in the presence of unlabeled deoxynucleoside triphosphates. The virions were lysed with detergent, and the products were extracted as described above. After cutting with the restriction enzyme *Pvu*II, half of the material was subjected to alkaline hydrolysis to remove any ribonucleotides from nascent chains. Both the control sample and the alkali-treated sample were treated with polynucleotide kinase in the presence of γ-^{32}P-labeled ATP to label any free 5′ hydroxyl groups. The products of the kinase reaction were hybridized to the M13mp7 recombinant phage DNA containing the minus-strand sequences of the plus-strand origin region (P190 fragment) and the hybrids isolated by sedimentation in neutral sucrose. The labeled DNA was then subjected to electrophoresis in a polyacrylamide-urea gel with uniformly labeled 122-base *Pvu*II origin fragment from the endogenous reaction as a size marker. In the control sample, which had been treated with polynucleotide kinase without prior treatment with alkali, no specific DNA bands were labeled (Fig. 6, lane 1). However, prior treatment with alkali resulted in the specific labeling of a DNA fragment which comigrated with the 122-base origin fragment isolated from the endogenous reaction (Fig. 6, lanes 2 and 3). Although any alkali-sensitive blocking group on the 5′ ends of the nascent strands could have been responsible for this observation, the most likely interpretation of this result is that the ends of some of the strands were indeed blocked by the ribonucleotide residues which had originally been responsible for priming their synthesis. The size of the labeled DNA fragments after *Pvu*II cutting and the fact that they hybridized to the P190 minus-strand DNA indicated that these ends corresponded to the 5′ termini of plus strands initiated at the plus-strand origin.

In the same experiment it was noted that a small amount of a fragment 145 bases in length was also labeled by the combined alkali and kinase treatments. Although experiments were not undertaken to positively identify the origin of this fragment, it is possible that it corresponds to the minus strong-stop DNA (15), which by virtue of the attached tRNA primer would be labeled by the kinase only after treatment with alkali. Presumably the minus strong-stop DNA is capable of hyridizing weakly to the minus strands containing the plus-strand origin because the first 13 bases are the same on the plus and minus strands at the two corresponding origins (27). Consistent with this suggestion it was observed that the hybrids involving the putative minus strong-stop DNA were melted by heating to 60°C in 10 mM Tris-hydrochloride–1 mM EDTA (data not shown).

DISCUSSION

By using a 190-base-pair DNA-RNA hybrid that contained the M-MuLV plus-strand origin sequence as a source of template-primers for the reverse transcriptase, we showed that some activity in the reverse transcriptase preparation is capable of generating the correct RNA primer for the initiation of plus-strand DNA synthesis. The conclusion that initiation occurs precisely at the correct site is based on the observation that when the 3′ ends of the fragments are fixed by *Dde*I cutting, the length of the fragment produced in the in vitro reconstruction experiment (measured by a method which resolves polynucleotide chains differing in length by a single nucleotide) is exactly the same as the length of the origin fragment produced in the endogenous reaction. From the observation that some of the origin fragments synthesized in the in vitro reaction as well as in the endogenous reaction retain 5′ ribonucleotides, we infer that probably all of the chains are initiated with RNA primers which are subsequently removed by RNase H.

The generation of the correct RNA primer terminus requires a specific endonucleolytic scission between the G and the A residues, indicated by the arrow in the RNA sequence 5′....GGGGGG↓AAUG....3′, when the RNA is base paired to the DNA at the origin. The oligoribonucleotides downstream of the break point must become displaced from the template DNA, whereas the upstream purine-rich oligonucleotide must remain hydrogen-bonded to the DNA. The fact that the 3′-terminal six residues form rG · dC base pairs may be important in stabilizing the base pairing of the primer to the template DNA. From the nature of the reaction we presume that the enzyme activity associated with reverse transcriptase which catalyzes this breakage is RNase H. If this is true, then RNase H must possess a specificity for RNA sequence not heretofore demonstrated.

There is another situation in which RNase H appears to cut a polynucleotide chain bearing this or a similar sequence. Omer and Faras (25) have shown that the avian myeloblastosis virus (AMV) RNase H can remove the tRNA primer from minus strong-stop DNA strands by a single cleavage at the junction between the RNA and the DNA chains. Similar studies with M-MuLV plus strands which had been synthesized in vitro and which retained an RNA primer revealed that the AMV RNase H removes the priming RNA fragment intact by cleaving at the junction between the RNA and the DNA (5). In both of these situations the basis for the recognition of the cleavage sequence may simply be the site at which the polynucleotide chain switches from ribonucleotides to deoxyribonucleotides. However, in both of these cases as well as in the situation where the enzyme generates the primer for plus-strand synthesis, the sequence at the new 5′ end created by the cleavage is 5′-AATGAAA. Therefore, the sequence at the site of cleavage may play some role in determining the specificity of the enzyme in all three situations. The nucleotides at the 3′ end of the broken strand must, however, be ribonucleotides since no cleavage was observed when duplex DNA containing these sequences was tested as a substrate for the enzyme (data not shown).

The results presented here provide some insight as to the limits of the DNA-RNA recognition region which is required for the correct processing of the RNA to yield the plus-strand primer. Based on the specificity of RNase A for pyrimidines, the RNase A-resistant fragment, which when hybridized to the template DNA serves as well as the complete DNA-RNA hybrid as a substrate for the production of the correct primer, should have the sequence 5′-AGAAAAAGGGGGGAAU-3′. The generation of the plus-strand primer from this fragment requires the removal of three nucleotides from the 3′ end. Therefore, the recognition region which directs the enzyme in the formation of the plus-strand primer cannot extend more than three bases in the RNA strand in the 3′ direction from the cleavage site.

Previous attempts to detect any remnants of RNA primers on the 5′ ends of plus strands synthesized in the endogenous reaction have failed (20), presumably because primer removal is highly efficient. By labeling with polynucleotide kinase after treatment of the products of the endogenous reaction with alkali, it has proven possible to identify a small fraction of plus-strand origin fragments which probably contain 5′ ribonucleotides. This result lends support to the idea that the mechanism for generating plus-strand primers which we observed in the in vitro-reconstructed reaction may also operate in the endogenous reaction.

ACKNOWLEDGMENTS

We thank Michael Been and Louise Schibig for helpful comments during the preparation of the manuscript.

This work was supported by grant NP-400 from the American Cancer Society.

LITERATURE CITED

1. **Baltimore, D.** 1970. RNA-dependent DNA polymerase in virions of RNA tumour viruses. Nature (London) **226**:1209–1211.
2. **Baltimore, D., and D. Smoler.** 1971. Primer requirement and template specificity of the DNA polymerase of RNA tumor viruses. Proc. Nat. Acad. Sci. U.S.A. **68**:1507–1511.
3. **Baltimore, D., and D. F. Smoler.** 1972. Association of an endoribonuclease with the avian myeloblastosis virus deoxyribonucleic acid polymerase. J. Biol. Chem. **247**:7282–7287.
4. **Been, M. D., and J. J. Champoux.** 1983. Cutting of M13mp7 phage DNA and excision of cloned single-stranded sequences by restriction endonucleases. Methods Enzymol. **101**:90–98.
5. **Champoux, J. J., E. Gilboa, and D. Baltimore.** 1984. Mechanism of RNA primer removal by the RNase H activity of avian myeloblastosis virus reverse transcriptase. J. Virol. **49**:686–691.
6. **Champoux, J. J., and B. L. McConaughy.** 1975. Priming of superhelical SV40 DNA by *Escherichia coli* RNA polymerase for in vitro DNA synthesis. Biochemistry **14**:307–316.
7. **Collett, M. S., P. Dierks, J. T. Parsons, and A. J. Faras.** 1978. RNase H hydrolysis of the 5′ terminus of the avian sarcoma virus genome during reverse transcription. Nature (London) **272**:181–183.
8. **Darlix, J.-L., P. A. Bromley, and P.-F. Spahr.** 1977. Extensive in vitro transcription of Rous sarcoma virus RNA by avian myeloblastosis virus DNA polymerase and concurrent activation of the associated RNase H. J. Virol. **23**:659–668.
9. **Friedrich, R., and K. Moelling.** 1979. Effect of viral RNase H on the avian sarcoma viral genome during early transcription in vitro. J. Virol. **31**:630–638.
10. **Gerard, G. F.** 1981. Mechanism of action of Moloney murine leukemia virus RNA-directed DNA polymerase associated RNase H (RNase H I). Biochemistry **20**:256–265.
11. **Gilboa, E., S. Goff, A. Shields, F. Yoshimura, S. Mitra, and D. Baltimore.** 1979. In vitro synthesis of a 9 kbp terminally redundant DNA carrying the infectivity of Moloney murine leukemia virus. Cell **16**:863–874.
12. **Gilboa, E., S. W. Mitra, S. Goff, and D. Baltimore.** 1980. A detailed model of reverse transcription and tests of crucial aspects. Cell **18**:93–100.
13. **Goulian, M., S. H. Goulian, E. E. Codd, and A. Z. Blumenfield.** 1973. Properties of oligonucleotides that determine priming activity with *Escherichia coli* deoxyribonucleic acid polymerase I. Biochemistry **12**:2893–2901.
14. **Grandgenett, D. P., G. F. Gerard, and M. Green.** 1972. Ribonuclease H: a ubiquitous activity in virions of ribonucleic acid tumor viruses. J. Virol. **10**:1136–1142.

15. **Haseltine, W. A., D. G. Kleid, A. Panet, E. Rothenberg, and D. Baltimore.** 1976. Ordered transcription of RNA tumor virus genomes. J. Mol. Biol. **106**:109–131.
16. **Kung, H. J., Y. K. Fung, J. E. Majors, J. M. Bishop, and H. E. Varmus.** 1981. Synthesis of plus strands of retroviral DNA in cells infected with avian sarcoma virus and mouse mammary tumor virus. J. Virol. **37**:127–138.
17. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
18. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–78.
19. **Mitra, S. W., M. Chow, J. Champoux, and D. Baltimore.** 1982. Synthesis of murine leukemia virus plus strong stop DNA initiates at a unique site. J. Biol. Chem. **257**:5983–5986.
20. **Mitra, S. W., S. Goff, E. Gilboa, and D. Baltimore.** 1979. Synthesis of a 600-nucleotide-long plus-strand DNA by virions of Moloney murine leukemia virus. Proc. Nat. Acad. Sci. U.S.A. **76**:4355–4359.
21. **Moelling, K., D. P. Bolognesi, H. Bauer, W. Busen, H. W. Plassmann, and P. Hausen.** 1971. Association of viral reverse transcriptase with an enzyme degrading the RNA moiety of RNA-DNA hybrids. Nature (London) New Biol. **234**:240–243.
22. **Myers, J. C., C. Dobkin, and S. Spiegelman.** 1980. RNA primer used in synthesis of anticomplementary DNA by reverse transcriptase of avian myeloblastosis virus. Proc. Nat. Acad. Sci. U.S.A. **77**:1316–1320.
23. **Olsen, J. C., and K. F. Watson.** 1980. Avian retrovirus RNA-directed DNA synthesis by purified reverse transcriptase. Covalent linkage of RNA to plus strand DNA. Biochem. Biophys. Res. Commun. **97**:1376–1383.
24. **Olsen, J. C., and K. F. Watson.** 1982. Reverse transcription of avian myeloblastosis virus 35S RNA. Early synthesis of plus strand DNA of discrete size in reconstructed reactions. Nucleic Acids Res. **10**:1009–1027.
25. **Omer, C. A., and A. J. Faras.** 1982. Mechanism of release of the avian retrovirus $tRNA^{Trp}$ primer molecule from viral DNA by ribonuclease H during reverse transcription. Cell **30**:797–805.
26. **Panganiban, A. T., and H. M. Temin.** 1983. Nature (London) **306**:155–160.
27. **Shinnick, T. M., R. A. Lerner, and J. G. Sutcliffe.** 1981. Nucleotide sequence of Moloney murine leukaemia virus. Nature (London) **293**:543–548.
28. **Shoemaker, C. S., S. Goff, E. Gilboa, M. Paskind, S. W. Mitra, and D. Baltimore.** 1980. Structure of a cloned circular Moloney murine leukemia virus DNA molecule containing an inverted segment: implications for retrovirus integration. Proc. Nat. Acad. Sci. U.S.A. **77**:3932–3936.
29. **Smith, J. K., A. Cywinski, and J. M. Taylor.** 1984. Initiation of plus-strand DNA synthesis during reverse transcription of an avian retrovirus genome. J. Virol. **49**:200–204.
30. **Sorge, J., and S. Hughes.** 1982. Polypurine tract adjacent to the U_3 region of the Rous sarcoma virus genome provides a *cis*-acting function. J. Virol. **43**:482–488.
31. **Swanstrom, R., H. E. Varmus, and J. M. Bishop.** 1981. The terminal redundancy of the retrovirus genome facilitates chain elongation by reverse transcriptase. J. Biol. Chem. **256**:1115–1121.
32. **Temin, H. M., and S. Mizutani.** 1970. RNA-directed DNA polymerase in virions of Rous sarcoma virus. Nature (London) **226**:1211–1213.
33. **Varmus, H. E.** 1982. Form and function of retroviral proviruses. Science **216**:812–820.
34. **Varmus, H. E., S. Heasley, H.-J. Kung, H. Oppermann, V. C. Smith, J. M. Bishop, and P. R. Shank.** 1978. Kinetics of synthesis, structure and purification of avian sarcoma virus-specific DNA made in the cytoplasm of acutely infected cells. J. Mol. Biol. **120**:55–82.
35. **Verma, I. M., and D. Baltimore.** 1973. Purification of the RNA-directed DNA polymerase from avian myeloblastosis virus and its assay with polynucleotide templates. Methods Enzymol. **29**:125–143.
36. **Verma, I. M., N. L. Meuth, and D. Baltimore.** 1972. Covalent linkage between ribonucleic acid primer and deoxyribonucleic acid product of the avian myeloblastosis virus deoxyribonucleic acid polymerase. J. Virol. **10**:622–627.
37. **Watson, K. F., P. L. Schendel, M. J. Rosok, and L. R. Ramsey.** 1979. Model RNA-directed DNA synthesis by avian myeloblastosis virus DNA polymerase and its associated RNase H. Biochemistry **18**:3210–3219.