JOURNAL OF VIROLOGY, Apr. 1991, p. 1952–1959
0022-538X/91/041952-08$02.00/0

Vol. 65, No. 4

Equine Infectious Anemia Virus and Human Immunodeficiency Virus DNA Synthesis In Vitro: Characterization of the Endogenous Reverse Transcriptase Reaction

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Received 26 October 1990/Accepted 31 December 1990

The endogenous reverse transcriptase reaction of equine infectious anemia virus (EIAV) has been studied, and conditions allowing synthesis of full-length minus-strand DNA have been determined. In contrast to results reported for other retroviruses, synthesis of EIAV full-length minus-strand DNA was not impaired by high concentrations of Nonidet P-40, a nonionic detergent used to make the virion envelope permeable. All components of the reaction were titrated for maximum synthesis of complete minus strands, and a time course under the standardized conditions was determined. Minor subgenomic bands were observed in some cases, and both the size and proportion varied with reaction conditions. Conditions established for full-length EIAV DNA synthesis also allowed full-genome-length human immunodeficiency virus type 1 DNA synthesis. The human immunodeficiency virus type 1 DNA product contained a greater proportion of reverse transcripts that were shorter than the complete virus genome. Also in contrast to EIAV, the endogenous synthesis of high-molecular-weight human immunodeficiency virus type 1 DNA was drastically reduced at Nonidet P-40 concentrations above 0.02%. These results indicated that a detergent-stable core is not a property shared by all lentiviruses. The EIAV virion synthetic machinery is unusually stable and provides a convenient system for further in vitro study of reverse transcription.

With the discovery of reverse transcriptase 20 years ago, RNA tumor viruses (retroviruses) were shown to be capable of synthesizing DNA in vitro when supplied with appropriate deoxynucleoside triphosphates (dNTPs), a divalent cation, a buffer, and a reagent to make the virion envelope permeable (3, 47). Under the appropriate conditions, reverse transcription of viral RNA within the core, termed the endogenous reverse transcriptase reaction, results in the synthesis of a genetically complete, infectious viral DNA (2, 5, 10, 18, 42). The endogenous reaction mimics early events in retrovirus-infected cells and has provided important insights into the mechanism of viral DNA synthesis (19). In general, the ability of permeabilized virions to synthesize a complete viral genome-length reverse transcript requires relatively high dNTP levels and careful titration of the reaction components, especially the detergent used to make the virion permeable (12, 25, 41, 49). Although there have been reports of full-length intact duplex DNA being made in vitro (7, 19), the vast majority of synthesized product contains an intact minus strand and a discontinuous positive strand (6, 10, 14, 29, 49), similar to the cytoplasmic forms detected early after infection (17, 22, 46, 48). Other DNA forms have been detected as well, including branched structures and noncovalently closed circles (15, 18, 23, 24). The discontinuous property of the positive strands presumably arises from initiation of positive-strand DNA synthesis on several RNA primers distributed over the minus-strand template (14, 46) in addition to the specific positive-strand strong stop primer (35, 36). The individual segments are not covalently linked, and therefore when denatured they migrate in electrophoretic gels as randomly sized molecules that are shorter than the full genome. For reasons that are not clear, segmented positive strands are more prominent with avian sarcoma/leukosis viruses DNA than with murine leukemia virus and mouse mammary tumor virus DNA (28).

It was reported more than 10 years ago that equine infectious anemia virus (EIAV), a lentivirus, was well suited for the study of the endogenous reverse transcriptase reaction (39). In particular, this virus rapidly synthesizes high-molecular-weight DNA in the presence of low dNTP concentrations; furthermore, the concentrations of other components do not appear to be critical. We wished to further characterize the products of the endogenous reaction with EIAV as a potential model for the analysis of the reverse transcriptase reaction of the related lentivirus, human immunodeficiency virus type 1 (HIV-1). The endogenous reaction has been studied to a limited extent in the early characterization of HIV-1 (38) and was used as a recombinant DNA cloning strategy for both HIV-1 and HIV-2 (1, 9). More recently, optimized conditions were reported for total DNA synthesis (51); however, the size and structure of the product were not fully characterized.

We report here that several parameters that have been considered to be critical for full-length DNA synthesis with other retroviruses were more widely tolerated in the EIAV endogenous reaction, most notably the detergent concentration. EIAV is therefore a very convenient experimental system with which to study retrovirus replication in vitro. The endogenous reaction was performed with HIV-1 under similar conditions, and full-length DNA was observed. The major discrete products of the HIV-1 reaction, however, were transcripts shorter than the complete viral genome. EIAV and HIV-1 differ markedly in their sensitivity to detergents, suggesting that a stable core DNA synthesis complex is not shared by all lentiviruses.

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MATERIALS AND METHODS

Virus and cell culture. Equine dermis cells chronically infected with the Malmquist strain of EIAV (34) (generously provided by L. Coggins and F. Fuller) were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Medium from roller-bottle cultures was harvested daily and clarified by centrifugation at 2,600 × *g* for 15 min. Virus was concentrated by centrifugation at 95,000 × *g* for 60 min; pellets were suspended in TE (10 mM Tris-HCl [pH 8.0]–1 mM EDTA, pooled, and centrifuged again at 151,000 × *g* for 60 min. The final 1,000-fold-concentrated virus in TE typically contained 10 mg of protein per ml as determined by a colorimetric protein assay reagent (Pierce). HIV-1 strain IIIB was purchased from Pan Data Systems, Rockville, Md. The virus was prepared by pressure filtration of low-serum-containing medium from infected H9 cells, followed by pelleting through a glycerol cushion and banding in a sucrose gradient.

Exogenous reverse transcriptase reaction. The standard exogenous reverse transcriptase assay consisted of mixing 10 μl of clarified cell culture medium with 20 μl of a cocktail to give final concentrations of 50 mM Tris-HCl (pH 7.9), 50 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.05% Nonidet P-40 (NP-40), 0.33 A_{260} units of poly(rA)-poly(dT)$_{12-18}$ per ml, and 0.5 μM [^{3}H]dTTP (ca. 67 Ci/mmol). The reaction was incubated for 90 min at 37°C, and 25 μl was spotted onto a 2-cm^2 square of DE81 chromatography paper (Whatman). Unincorporated [^{3}H]dTTP was removed by washing in 5% Na_2HPO_4. Filters were washed briefly in H_2O and finally in 95% ethanol before they were dried and counted in Ready-Safe (Beckman Instruments, Inc.) liquid scintillation cocktail.

Endogenous reverse transcriptase reaction. The standard endogenous reaction conditions consisted of 25 μl containing 1 mg of protein per ml, 100 mM Tris-HCl (pH 8.0), 15 mM NaCl, 0.8 mM magnesium acetate, 15 mM dithiothreitol, 0.1 mM each dNTP, [^{32}P]dCTP (12 Ci/mmol), and 0.02% NP-40. Reactions were incubated for 2 h at 41°C. Modifications to the standard reaction are indicated in each figure legend. The reaction was stopped by the addition of an equal volume of stop buffer (final concentrations of 0.5% sodium dodecyl sulfate, 25 mM EDTA, 100 mM NaCl) and extracted with phenol-chloroform-isoamyl alcohol (25:24:1). The DNA product was precipitated in ethanol, centrifuged, and dissolved in TE. Samples were electrophoresed in neutral 0.9% agarose in Tris-acetate running buffer, either native or after alkaline hydrolysis to remove viral RNA and denature the DNA product into single strands. Gels were dried and exposed to Kodak X-Omat AR film for autoradiography. In some cases DNA was synthesized without [^{32}P]dCTP and was transferred to nitrocellulose membranes for hybridization by the Southern procedure (45). NP-40, Triton X-100, and melittin were purchased from Sigma Chemical Co., St. Louis, Mo. Lipofectin was purchased from BRL, Gaithersburg, Md.

Hybridization. DNA bound to nitrocellulose membranes was hybridized to ^{32}P-labeled oligonucleotide probes in 6× SSC (1× is 0.15 M NaCl–0.015 M sodium citrate), 0.1% sodium dodecyl sulfate, and 0.1% sodium pyrophosphate overnight at 42°C. After hybridization, the membrane was washed briefly in 2× SSC at 42°C and then washed twice in 0.2× SSC–0.1% sodium dodecyl sulfate–0.1% sodium pyrophosphate at 50°C for 30 min each. The membrane was dried and exposed to X-Omat AR film for autoradiography. The following oligonucleotides were employed: 5′ GGGTCTGC GCCCCTCTCA 3′, which is complementary to the positive strand immediately 3′ to the primer binding site (nucleotides 343 through 360 [26]) and designated the positive-strand leader probe; and 5′ TGAGAGGGGCGCAGACCC 3′, which is complementary to the minus strand (nucleotide positions 343 through 360) and designated the minus-strand leader probe.

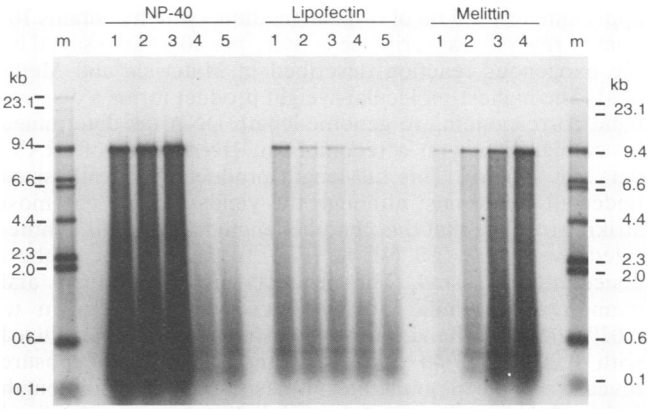

FIG. 1. Titration of permeabilizing agents. [^{32}P]DNA was synthesized by EIAV virions permeabilized by various concentrations of NP-40, Lipofectin, or melittin and electrophoresed in neutral agarose after NaOH treatment. Concentrations of NP-40 (lanes): 1, 2.0%; 2, 0.2%; 3, 0.02%; 4, 0.002%; 5, 0%. Concentrations of Lipofectin (lanes): 1, 200 μg/ml; 2, 100 μg/ml; 3, 50 μg/ml; 4, 25 μg/ml; 5, 0 μg/ml. Concentrations of melittin (lanes): 1, 200 μg/ml; 2, 100 μg/ml; 3, 50 μg/ml; 4, 25 μg/ml. Size markers (m) were *Hin*dIII fragments of bacteriophage lambda DNA. The single strands of several marker fragments appear as doublets in neutral agarose gels.

RESULTS AND DISCUSSION

Titration of reagents used to permeabilize EIAV virions for endogenous reverse transcription. The traditional method for making retroviral envelopes permeable to dNTPs is to treat with a low concentration of a nonionic detergent such as Triton X-100 or NP-40. It has long been known that the detergent concentration suitable for endogenous synthesis of full-length DNA has a narrow range (e.g., between 0.01 and 0.02% NP-40 or Triton X-100) (25). The optimum concentration usually has to be titrated for each virus preparation. Low concentrations are not adequate to permeabilize the virions, and higher-than-optimum concentrations result in the synthesis of predominately low-molecular-weight products. Some aspect of core architecture or the enzymatic machinery is sensitive to high detergent concentrations, resulting in indiscriminate template usage and synthesis of small DNA molecules (37).

As alternatives to nonionic detergents, melittin, a membrane-reactive peptide that has been shown to permeabilize retroviral envelopes (5), and a previously untested reagent, Lipofectin, were compared with NP-40 for their ability to activate the EIAV endogenous reaction. Lipofectin, a cationic liposome, was considered to be a candidate reagent to disrupt the viral envelope since it was known to have membrane-fusing properties (16). An autoradiograph of alkaline-denatured DNA synthesized in EIAV virions treated with these three reagents at various concentrations is shown in Fig. 1. The standard reaction contained 25 μg of protein derived from pelleted culture medium. This corresponds to

approximately 2.5 ml of original medium (which contains 10^5 dpm of reverse transcriptase activity per 10 μl as assayed by the exogenous reaction described in Materials and Methods). The highest-molecular-weight product forms a discrete band corresponding to genome-length DNA (as determined by comigration with a recombinant DNA clone of EIAV; data not shown). This full-length product was synthesized under all conditions, although the yields varied. The most striking result is that this genome-length transcript is synthesized even with 2% NP-40 (Fig. 1, lane 1), the highest concentration tested. The products are qualitatively and quantitatively similar at NP-40 concentrations down to 0.02% (lane 3). The virion was not sufficiently permeabilized with 0.002% NP-40 (lane 4), although a long exposure reveals a small amount of synthesis, including full-length DNA, at this concentration and in the nonpermeabilized control (lane 5). The methods used for virus preparation may have disrupted the envelope enough to account for this small amount of DNA synthesis in the absence of detergent treatment. Triton X-100 was also tested over the same concentration range and gave the same results (data not shown). As noted above, full-length DNA synthesis in high detergent concentrations was in contrast to what had been observed with other retroviruses and suggested that EIAV had a relatively stable core synthetic machinery. Recently reported biochemical characterization of EIAV cores prepared by banding in Ficoll gradients indicates that core structures are physically stable (40).

Lipofectin and melittin were both capable of activating the endogenous reaction, but, at the concentrations tested, neither resulted in yields as great as those with the NP-40-activated reaction. There was a clear dose-dependent yield of product in the Lipofectin-activated reaction. Concentrations higher than 200 μg/ml may have been optimum; however, achieving higher concentrations was limited by the 1-mg/ml commercially available stock of Lipofectin. Melittin exhibited an inverse dose response, with greater yields at lower concentrations, consistent with previous results with Rous-associated virus type 2 (5). Lower concentrations of melittin than those tested may have resulted in a yield comparable to that of NP-40. Melittin is an appropriate reagent to use with detergent-sensitive viruses; however, we suggest that NP-40 is the reagent of choice for permeabilizing EIAV because it has a very broad effective concentration range.

Divalent cation and dNTP concentrations. Earlier work characterizing the endogenous reverse transcriptase reaction with avian and murine retroviruses demonstrated that high-molecular-weight reverse transcripts could be produced when dNTP levels were high (0.2 to 5 mM for each dNTP) (12, 41). By contrast, it has been reported that EIAV does not require high concentrations of dNTPs to synthesize full-length DNA (39). When dNTP concentration requirements were examined under the standard EIAV reaction conditions, the synthesis of full-length DNA was not dependent upon high concentrations of dNTPs (data not shown). The concentration we adopted for our standard protocol was 0.1 mM for each dNTP.

It has been reported that although high dNTP concentrations favor synthesis of high-molecular-weight DNA, completely full-length Moloney murine leukemia virus DNA is produced only when Mg^{2+} is rate limiting (just below the total dNTP concentration) (43). The rate of DNA synthesis is stimulated when Mg^{2+} is in excess of the dNTP levels, but the products are predominately of low molecular weight. It was speculated that Mg^{2+} affects RNA stability and that template degradation may be responsible for the short transcripts. However, it should be noted that Mn^{2+} rather than Mg^{2+} is the preferred divalent cation for murine leukemia virus reverse transcriptase (20, 44). In contrast, DNA synthesis by avian sarcoma virus (10) and EIAV (39) has been reported to lack sensitivity to high concentrations of divalent cation.

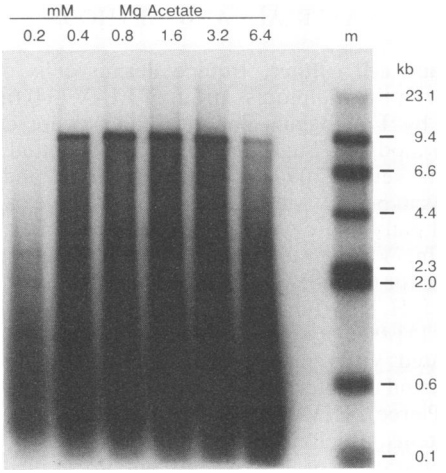

FIG. 2. Magnesium acetate titration. [^{32}P]DNA was synthesized by EIAV virions in a standard reaction with 0.1 mM each dNTP, except the concentration of magnesium acetate was varied from 6.4 to 0.2 mM in serial twofold dilutions. Products were extracted and subjected to electrophoresis as described in the legend to Fig. 1. m, Size markers.

We have characterized the EIAV endogenous reverse transcriptase reaction for its sensitivity to Mg^{2+} and dNTP levels. The effect on DNA synthesis of varying the Mg^{2+} concentration is shown in Fig. 2. Each of the four dNTPs was 0.1 mM, giving a combined dNTP concentration of 0.4 mM. The 0.2 mM magnesium acetate concentration is therefore 0.5 times the combined dNTP concentration (i.e., $[Mg^{2+}]/[dNTP] = 0.5$) and is not sufficient to stimulate the synthesis of genome-length product in the standard 2-h reaction. The 0.4 mM magnesium acetate concentration is equivalent to the combined dNTP concentration and is sufficient for synthesis of full-length product (lane 2). When the magnesium acetate concentration was 0.8, 1.6, or 3.2 mM (i.e., 2, 4, or 8 times the combined dNTP concentration), the synthesis of full-length DNA was slightly better than 0.4 mM. Even at 6.4 mM Mg^{2+} (i.e., 16 times the total dNTP level), full-length DNA was made; however, the yield was lower than that with $[Mg^{2+}]/[dNTP]$ ratios of 1 through 8. These results indicated that the synthesis of EIAV genome-length DNA did not require rate-limiting concentrations of Mg^{2+}.

The previous experiment demonstrated that Mg^{2+} at various concentrations in excess of the 0.4 mM combined dNTP concentration did not impair the ability of EIAV reverse transcriptase to completely copy the genome. However, as revealed by the following experiment, there is a relationship between the dNTP concentration and the Mg^{2+} concentration that affects DNA synthesis. The endogenous reaction was run at six different dNTP concentrations, beginning at 0.4 mM for each dNTP (Fig. 3, lane 1) and with twofold serial dilutions down to 0.0125 mM for each dNTP (lane 6). The magnesium acetate concentration was also serially diluted such that the ratio of Mg^{2+} to total dNTPs remained

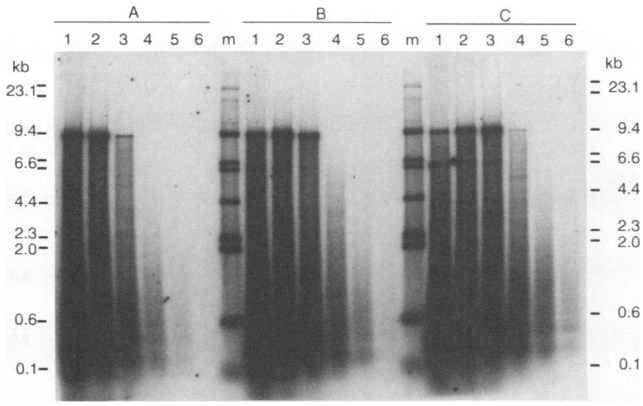

FIG. 3. dNTP titration at various magnesium acetate concentrations. [^{32}P]DNA was synthesized by EIAV virions in a standard reaction, except levels of each dNTP were varied as follows (lanes): 1, 0.4 mM; 2, 0.2 mM; 3, 0.1 mM; 4, 0.05 mM; 5, 0.025 mM; 6, 0.0125 mM. The magnesium acetate concentration was adjusted to be equal to the total dNTP concentration for each different sample in group A ([Mg^{2+}]/[dNTP] ratio of 1), twice the total dNTP in group B ([Mg^{2+}]/[dNTP] ratio of 2), and four times the total dNTP for each different sample in group C ([Mg^{2+}]/[dNTP] ratio of 4). Conditions of electrophoresis and markers are as in Fig. 1.

constant at 1, 2, or 4. Thus, in group A of Fig. 3, the [Mg^{2+}]/[dNTP] ratio was 1 and the Mg^{2+} concentration varied from 1.6 mM (lane 1) down to 0.05 mM (lane 6); in group B, the [Mg^{2+}]/[dNTP] ratio was 2 and the Mg^{2+} concentration varied from 3.2 mM (lane 1) down to 0.1 mM (lane 6); in group C, the [Mg^{2+}]/[dNTP] ratio was 4 and the Mg^{2+} concentration varied from 6.4 mM (lane 1) down to 0.2 mM (lane 6). The results from this experiment indicated that in the presence of 0.4 mM (lane 1) and 0.2 mM each dNTP (lane 2) genome-length DNA was synthesized at approximately the same yield under all three [Mg^{2+}]/[dNTP] ratios. Thus, at relatively high dNTP levels, the magnesium acetate does not have to be in excess to achieve complete reverse transcription of the RNA template. At 0.1 mM for each dNTP (lane 3), the Mg^{2+} was limiting with the [Mg^{2+}]/[dNTP] ratio of 1 (group A) and less DNA was made than at [Mg^{2+}]/[dNTP] ratios of 2 and 4 (groups B and C). This effect is even more pronounced as the dNTP concentration is further reduced. At 0.05 mM for each dNTP (lane 4), the Mg^{2+} at [Mg^{2+}]/[dNTP] ratios of 1 and 2 was severely limiting, and no detectable genome-length DNA was produced (groups A and B). A reduced but detectable amount of full-length DNA was synthesized at the ratio of 4 (group C). No full-length product was detected when the dNTP levels were 0.025 or 0.0125 mM each (lanes 5 and 6); however, there was a trend toward higher yield and higher-molecular-weight products with the higher [Mg^{2+}]/[dNTP] ratios. For example, the autoradiographic signal is undetectable in group A (Fig. 3A, lane 6) but detectable in group B (Fig. 3B, lane 6) and shows a stronger signal in group C (Fig. 3C, lane 6).

Our results indicate that when dNTP concentrations are relatively low (i.e., 0.1 mM or lower) it is important to use Mg^{2+} in excess of the dNTP concentration to synthesize a high yield of DNA. Another interesting observation from the experiment shown in Fig. 3 was that the relative proportion of discrete products that were shorter than the complete genome increased as the [Mg^{2+}]/[dNTP] ratio increased. In particular, a band at approximately 6.6 kb is most prominent in group C (ratio of 4), intermediate in group B (ratio of 2), and least prominent in group A (ratio of 1) (Fig. 3). Other subgenomic bands follow the same pattern. Although the DNA strand polarity has not been determined, these bands likely represented premature termination of the minus strand. Decreased processivity of reverse transcriptase or increased stability of template configurations that impeded transcription may have occurred under these conditions.

The remaining components of the reaction have been titrated for synthesis of full-length DNA and observed to have little effect (data not shown). NaCl and dithiothreitol may be omitted; however, varying the NaCl concentration did have subtle effects on the ratio of full-length DNA synthesis to total DNA synthesis and on the presence of minor subgenomic bands. Although the reaction works quite well in the absence of added NaCl or dithiothreitol, a pH buffer (50 to 100 mM Tris-HCl) is required. The endogenous reaction was run at 37, 39, and 41°C at pH 7.5, 8.0, and 8.3, and no significant differences were observed over this narrow range (data not shown). Based on all of these titrations, the standard protocol we adopted was to incubate the reaction at 41°C in 100 mM Tris-HCl (pH 8.0), 15 mM NaCl, 15 mM dithiothreitol, 0.1 mM each dNTP, 0.8 mM magnesium acetate, 0.02% NP-40, and 1 mg of protein per ml.

Time course. For detergent-treated avian and murine retroviruses, full-length DNA is often observed only after incubation for 3 to 6 h, and it is common to run reactions for 16 to 24 h (10, 15, 41). To determine the time course of EIAV DNA synthesis with our standard reaction conditions, samples were taken at various times and processed as described above. An autoradiograph of the alkaline-denatured [^{32}P]DNA electrophoresed in a neutral agarose gel is shown in Fig. 4A. By 30 min, the first time point, the product looks qualitatively like it does at later times (up to 4 h, the last time point), although the maximum yield is not reached until 90 min. This is similar to the time course of Rous-associated virus type 2 DNA synthesis in a melittin-activated endogenous reverse transcriptase reaction (6) and somewhat more rapid than previously reported for EIAV (39); this may simply be due to the use of more sensitive gel electrophoresis and autoradiography techniques. To determine whether the product contains sequences predicted to be near the 5′ end of genomic minus-strand DNA, DNA was synthesized without ^{32}P and transferred to a nitrocellulose membrane for Southern blot hybridization with a specific probe. An oligonucleotide of 18 bases complementary to the leader region of the minus-strand DNA (minus-strand leader probe as defined in Materials and Methods) was used; this oligonucleotide should hybridize only to minus-strand DNA, which is essentially full length. The leader region specificity was verified by hybridization to restriction enzyme fragments derived from an infectious molecularly cloned EIAV (50) (data not shown). The leader-specific probe hybridized to the high-molecular-weight band present at 30 min (Fig. 4B), supporting the conclusion that this band was a full-length minus strand. Unexpectedly, two additional minor minus-strand bands (one approximately 6.6 kb and one approximately 1.7 kb) were detected with the minus-strand leader probe. A close examination of Fig. 4A, and other figures where [^{32}P]DNA is shown, reveals additional subgenomic bands, suggesting that the two bands detected with the minus-strand leader probe are a specific subset. The origin of subgenomic reverse transcripts containing the leader sequence is unknown, but several possibilities could be considered. This hybridization pattern could have been produced by reverse transcription of spliced RNA or RNA from genomes con-

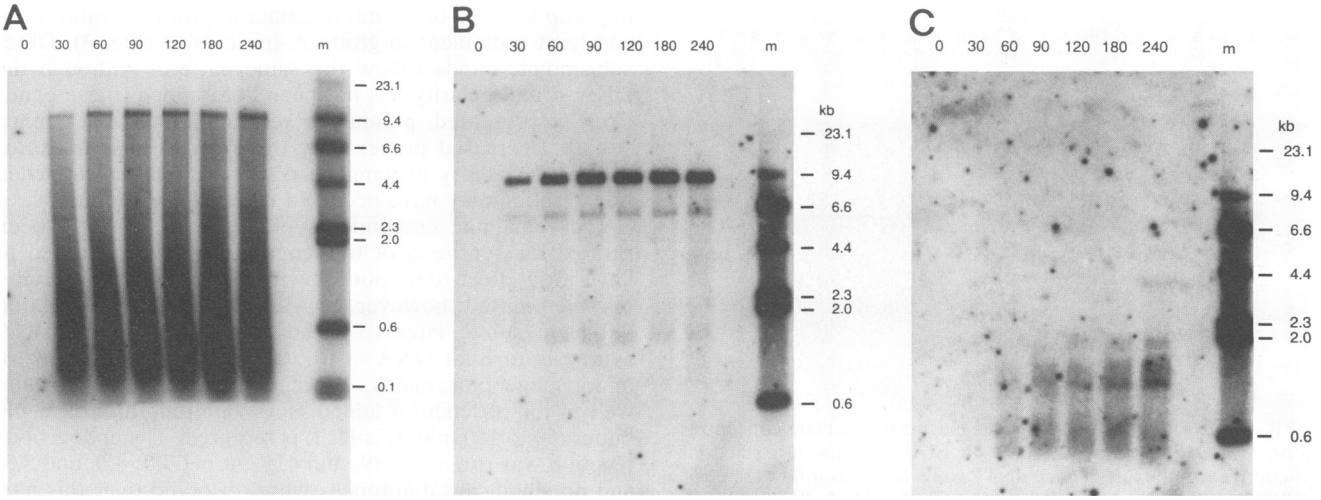

FIG. 4. Time course of DNA synthesis. DNA was synthesized by EIAV virions in a standard reaction, and time points were taken at 30- or 60-min intervals. Electrophoresis and autoradiography were as described in the legend to Fig. 1. (A) [^{32}P]DNA, (B) Southern transfer of unlabeled DNA hybridized to the minus-strand leader probe, (C) Southern transfer of unlabeled DNA hybridized to the plus-strand leader probe. m, Size markers.

taining specific deletions, or possibly by aberrant initiation or jumping (template switching) to specific internal sites. There is some precedent in the literature for jumping to internal sites (8, 13), although this has not been widely studied. Under conditions of high detergent concentration, presumably random jumping to alternate templates has been observed with avian type C retroviruses (37). Characterization of the subgenomic bands detected by the minus-strand leader probe is currently underway.

The complement of the minus-strand leader oligonucleotide probe was also hybridized to a Southern transfer of the time course experiment (Fig. 4C). This positive-strand leader probe detected only low-molecular-weight products, indicating that full-length positive strands are not synthesized. EIAV may be like the avian retroviruses, in which both in vitro- and in vivo-synthesized DNAs have discontinuous positive strands (6, 22, 28, 46, 48). Visna virus, a related lentivirus, has been shown to synthesize a linear DNA with an intact minus strand and a single gap in the positive strand, creating two approximately equal segments (21).

Products of the endogenous reaction were also subjected to electrophoresis without prior denaturation with NaOH. ^{32}P-labeled and unlabeled DNA hybridized with the positive-strand leader oligonucleotide probe exhibited two bands, one at the expected size for EIAV linear duplex DNA, and another that migrated more slowly (data not shown). The more slowly migrating form was not stable to NaOH or formamide denaturation and may have been noncovalently joined circles. Circles are predicted as an intermediate in the reverse transcription of the viral genome (19) and have been observed in other endogenous reverse transcriptase systems (4, 7, 15, 18, 24). Preliminary restriction mapping of the product was consistent with the slower-migrating form being circular. Additional characterization is currently underway.

HIV-1 endogenous reverse transcriptase reaction. DNA has been synthesized with HIV-1 with conditions that allow full-length DNA synthesis with EIAV. Shown in Fig. 5 is the result of titration with NP-40. Complete genome-length HIV-1 DNA was synthesized at NP-40 concentrations of 0.025% and lower, including the control without NP-40. At 0.05 and 0.1% NP-40 this product was not observed, and the amount of low-molecular-weight DNA increased. Thus, in contrast to EIAV, under these conditions the detergent concentration is critically important to yield a full-genome-length product for HIV-1. Surprisingly, the control reaction that was not treated with detergent synthesized a significant amount of DNA. The procedure used to prepare HIV-1, which differs form the procedure used to prepare EIAV (see Materials and Methods), possibly disrupted the viral envelope to a greater extent and made it permeable to the reaction components. We have observed variation among different HIV-1 preparations in the amount of reverse transcription that occurred in the absence of added detergent,

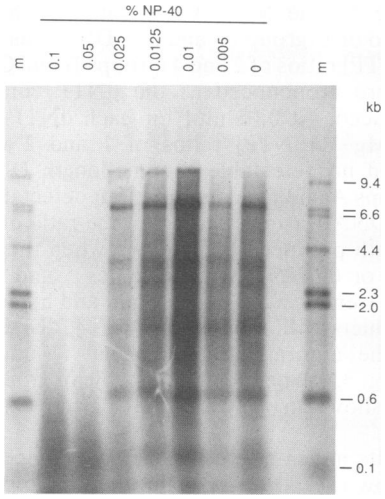

FIG. 5. NP-40 titration in the HIV-1 endogenous reaction. [^{32}P]DNA was synthesized by HIV-1 by a modified EIAV protocol. The reaction mixture was the same as the standard EIAV reaction, except that dNTPs were used at 0.2 mM each, magnesium acetate was 1.5 mM, and protein was 0.5 mg/ml. NP-40 concentrations are indicated. m, Size markers.

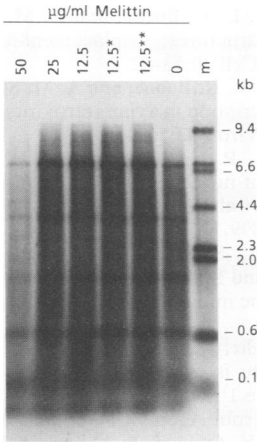

FIG. 6. Melittin titration in the HIV-1 endogenous reaction. [^{32}P]DNA was synthesized by HIV-1 with a modified EIAV protocol as described in the legend to Fig. 5. *, 50 μg of Lipofectin per ml in combination with 12.5 μg of melittin per ml; **, 0.01% NP40 in combination with 12.5 μg of melittin per ml; m, size markers.

but it was always greater than that observed with EIAV or type C retroviruses in the absence of detergent (data not shown).

Although full-length DNA was made, the most prominent band in the reactions conducted at 0.05% NP40 and below corresponded to an approximately 7-kb reverse transcript. Several other discrete bands were observed. Currently, these shorter reverse transcripts are being characterized to identify their structure and reveal their origin. Preliminary evidence (data not shown) indicates that the bands greater than 600 bp in length contain the minus-strand primer binding site and sequences in the leader region. As discussed previously in the case of EIAV, probes near to the 5′ end would be expected to only hybridize to minus strands that were completed. This suggests that some of the reverse transcripts that are shorter than the complete virus genome are not due to premature termination of reverse transcription. It will be important to determine whether these discrete size products are the result of an aberrant reverse transcription process or are due to accurate reverse transcription of spliced RNAs or RNAs transcribed from proviruses containing deletions.

There have been several reports in which the endogenous reverse transcriptase reaction has been performed with HIV-1, although the products have not been characterized by gel electrophoresis (27, 38, 51). With conditions optimized for total incorporation of dNTP, melittin has recently been shown to be more efficient than Triton X-100 in stimulating synthesis (51). We titrated melittin in the HIV endogenous reaction (Fig. 6). The untreated control was sufficiently permeable to allow synthesis in this experiment (Fig. 5). Consistent with the NP-40-treated virus, many discrete subgenomic bands were produced. DNA synthesis was stimulated at melittin concentrations of 25 and 12.5 μg/ml, and full-length DNA was barely detectable. A combination of 12.5 μg of melittin per ml plus 50 μg of lipofectin per ml or 12.5 μg of melittin per ml plus 0.01% NP-40 did not improve the yield. Melittin was inhibitory at 50 μg/ml. Given the sensitivity of HIV-1 to detergent concentration, melittin may be the reagent of choice for this virus. We have not consistently produced full-length DNA with melittin or NP-40; the conditions established for EIAV may not be optimum for HIV-1. Apparently, important differences exist between EIAV and HIV-1 in the core structure or synthetic machinery. It is possible that differences in the methods of virus preparation (see Materials and Methods) could account for some of the differences observed between the endogenous reverse transcription of EIAV and HIV-1. The reaction conditions established for EIAV provide a starting point for additional refinement in the HIV-1 system.

Reverse transcriptase is an important target for antiretroviral agents, and the exogenous reverse transcriptase reaction (i.e., synthetic template-primer) has been used as an assay to identify active compounds. Although the exogenous reaction is more easily and economically performed, the endogenous reaction may also be valuable in drug discovery and mechanism of action studies. Recent studies have demonstrated that the sensitivity to zidovudine (AZT) of mutant HIV-1 reverse transcriptase, as measured by the exogenous reaction, is not predictive of the sensitivity to AZT of HIV-1 in cell culture. Specifically, mutations in the reverse transcriptase gene which resulted in AZT-resistant enzyme produced by a bacterial expression vector conferred hypersensitivity to AZT when introduced into infectious virus and assayed by plaque reduction (32). In an enzyme assay, the virion associated reverse transcriptase exhibited the same resistance as the bacterially produced enzyme (33). Furthermore, HIV-1 reverse transcriptase mutants created in vitro to mimic clinical isolates that are AZT resistant (30) are indeed resistant in cell culture, but the reverse transcriptase does not exhibit an altered K_i when assayed by the exogenous reaction (31). Apparently, reverse transcriptase has different properties when assayed outside of the authentic viral core synthetic machinery. A case in point is the observation that the U5 leader stem secondary structure is important in the efficient initiation of reverse transcription both in vivo and in the endogenous reaction but not when reverse transcriptase is added to purified virion RNA (11). Perhaps an optimized endogenous reverse transcriptase reaction might more closely mimic what occurs in vivo and permit the study of reverse transcriptase-inhibitor interactions under more relevant conditions.

In summary, we have demonstrated a wide range of conditions that support the synthesis of EIAV full-length DNA in an endogenous reverse transcriptase reaction. Most notably, the detergent concentration is not a critical parameter, suggesting that the core or synthetic complex is a stable structure. HIV-1, however, is sensitive to detergent concentration like all other reported retroviruses. HIV-1 is capable of synthesizing full-genome-length DNA under conditions optimum for EIAV; however, the major products observed with the current virus preparation were less than full genome length. The mechanism responsible for the reverse transcription of a high proportion of discrete molecules shorter than the complete viral genome is currently under investigation.

ACKNOWLEDGMENTS

We thank Fred Fuller and LeRoy Coggins for the generous gift of EIAV-infected equine dermis cells, the molecular clone of EIAV designated pER, and advice on the EIAV system. We also thank Phil Furman and George Painter for many helpful discussions on the structure and function of reverse transcriptase and Marty St. Clair for assistance in working with HIV-1 in the BSL3 laboratory. We also appreciate the critical review of the manuscript by Fred Fuller, Bill Phelps, and Catherine Krebs Heitman.

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