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Development of a human immunodeficiency virus-1 in vitro DNA synthesis system to study reverse transcriptase inhibitors

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Summary

A Human immunodeficiency virus type-1 endogenous reverse transcriptase reaction was developed as an in vitro assay to study the inhibition of reverse transcription by antiviral compounds. Conditions were established for producing genomic length (−) strand DNA in high yields and measuring the inhibition of this transcript as the assay endpoint. In addition to genomic length (−) strand DNA, a novel segmented (−) strand product composed of a 6.0 kb reverse transcript of the 5′ 2/3 of the viral RNA genome and a 3.5 kb reverse transcript of the 3′ 1/3 was observed. The most prominent (+) strand product was the size expected for plus-strong stop DNA. Additional minor (+) strand species were also observed. The triphosphate form of the nucleoside analog inhibitor 3′-azido-3′deoxythymidine (RETROVIR, Zidovudine, AZT) and BI-RG-587 (nevirapine), a non nucleoside inhibitor, were used to demonstrate the utility of the endogenous system for the analysis of reverse transcriptase inhibitors. In a standard reaction, synthesis of genomic length DNA was 50% inhibited by 0.1 μM AZTTP and 0.1 μM nevirapine.

HIV-1; Endogenous reverse transcription; AZTTP; nevirapine

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Introduction

Human immunodeficiency virus type-1 (HIV-1), like all other replication competent retroviruses, encodes a reverse transcriptase (RT) that is essential for viral replication. Following virus attachment and penetration of susceptible cells (Coffin, 1990; Varmus, 1988), RT converts genomic viral RNA into double-stranded DNA within the viral core. The endogenous reverse transcriptase reaction is an in vitro system which mimics these early events of retroviral replication. The genomic RNA is reverse transcribed in vitro when virions are supplied with the appropriate divalent cations, deoxynucleoside 5′ triphosphates (dNTPs), buffer, and a reagent to permeabilize the envelope (Baltimore, 1970; Temin and Mizutani, 1970). The endogenous reaction with C-type retroviruses has provided important insights into the mechanism of retroviral minus (−) and plus (+) strand synthesis. In general, the products synthesized in the reaction include genomic length (−) strand and discontinuous (+) strand DNA (Junghans et al., 1975; Rothenberg and Baltimore, 1976; Verma, 1978). These products are similar to the viral DNA forms detected in the cytoplasm early after infection (Gianni and Weinberg, 1975; Hsu and Taylor, 1982; Varmus et al., 1976). Synthesis of genetically complete, infectious viral DNA in the endogenous reaction has been reported for several retroviral systems (Boone and Skalka, 1981; Gilboa et al., 1979a; Rothenberg and Baltimore, 1976).

More recently, the endogenous reaction has been used to synthesize HIV-1 DNA in vitro (Borroto-Esoda and Boone, 1991; Yong et al., 1990) and to study the inhibition of HIV-1 RT by compounds that inhibit viral replication (Debyser et al., 1992; Lacey et al., 1992). The endogenous reaction has several advantages over the more common exogenous RT assay that relies on exogenously added homopolymeric template-primer for the analysis of RT inhibitors. Because virion RNA is the template, analogs of all four of the natural deoxynucleoside 5′ triphosphates can be analyzed as inhibitors. The reaction presumably occurs within the viral core where other virion proteins may influence the complex steps that occur during synthesis of viral DNA. Finally, compounds that inhibit the RNA-dependent DNA synthesis, DNA-dependent DNA synthesis, and RNase H activity of RT can each be examined in this system.

We report here conditions that have been optimized for synthesis of genomic length minus strand DNA in the HIV-1 endogenous reverse transcriptase reaction and characterize several prominent subgenomic products. The utility of measuring the in vitro synthesis of full length HIV-1 DNA as an assay for inhibitors of RT is demonstrated with the antiviral compounds AZTTP and nevirapine (Merluzzi et al., 1990).

Materials and Methods

Endogenous reverse transcriptase reaction

HIV-1 strain IIIB was obtained from Universal Biotechnology Inc., Rockville, MD. The virus was prepared by pressure filtration of low-serum-containing media (< 1% fetal bovine serum) collected from infected H9 cells, followed by centrifugation through a glycerol cushion and banding in a sucrose gradient. The final viral preparation had a protein concentration of 1 mg per ml and contained 10^5 infectious units per ml as determined by p24 antigen capture. Typical endogenous reaction conditions consisted of 30 μl containing 0.33 mg of protein per ml, 100 mM Tris-HCl (pH 8.1), 15 mM NaCl, 3 mM $MgCl_2$, 0.5 mM each dNTP, 1 mM EGTA and 0.1% NP-40. Reactions were incubated for 2 h at 39°C and then stopped by the addition of an equal volume of stop buffer (final concentration of 0.5% SDS, 25 mM EDTA, 100 mM NaCl). The samples were then treated with 1 μl of a 20 mg/ml solution of Proteinase K, incubated for 1 h at 55°C and extracted with phenol/chloroform/isoamyl alcohol (25:24:1). The DNA product was precipitated in ethanol, centrifuged and resuspended in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Samples were denatured in 0.3 M NaOH and subjected to electrophoresis in 1% agarose (30 mM NaCl, 2 mM EDTA) in alkaline running buffer consisting of 30 mM NaOH, 2 mM EDTA. Following electrophoresis, DNA products were transferred to nylon reinforced nitrocellulose (Schleicher & Schuell) by the method of Southern (Southern, 1975).

Hybridization

Membrane bound DNA was hybridized to ^{32}P-labeled RNA or DNA probes in 50% formamide, 5 × SSC (1 × = 0.15 M NaCl, 0.015 M sodium citrate), 10 × Denhardt's solution (1 × = 0.02% ficoll, 0.02% polyvinyl pyrrolidone, 0.02% BSA), 50 mM Tris-HCl (pH 7.5), 0.5% SDS, 5 mM EDTA, 1 mM EGTA, 0.1% sodium pyrophosphate, and 100 μg/ml herring sperm DNA overnight at 42°C. Following hybridization, the membrane was washed in 2 × SSC at 42°C for 60 min then twice in 0.2 × SSC 0.1% SDS, 0.1% sodium pyrophosphate at 68°C for 20 min each.

Hybridization probes

Fig. 1 illustrates the regions of the HIV-1 genome that were used as probes to detect the products of the endogenous reverse transcriptase reaction. The plasmid pBKBH10S, an 8.9 kb fragment of the HTLV-III_B plasmid clone pBH10 (Hahn et al., 1984) inserted into the *Sst*I site of pBluescript, was used to generate the following probes:

3′(+). RNA probe specific to 3′(+) strand DNA sequences was generated from the T3 RNA polymerase promoter following digestion of the plasmid with *Nco*I (nucleotide (nt) No. 5670).

5′(−). A subclone of plasmid pBKBH10S from the 5′ *Sst*I site to a *Pst*I site at nt 736 was used to generate a RNA probe specific to the (−) cDNA strand at

the 5′ end of the genome from the T7 RNA polymerase promoter. RNA transcripts initiated from the T3 RNA polymerase promoter of this clone were used to probe for (+) strand DNA sequences at the 5′ end.

The infectious molecular clone HXB2 (Fisher et al., 1985) was used as the template for PCR (Saiki et al., 1988) amplification of a 500 bp fragment specific to the 3′ end of the envelope coding region **(env)**. The DNA was generated with the primer pairs 5′ccgagatcttcagacctggagg3′ (nt No. 7617) and 5′ctctgtcccactc-catccaggtc3′ (nt No. 8123). The fragment was labeled by the random primer method (Feinberg and Vogelstein, 1983).

Results

Optimization of the HIV-1 endogenous reverse transcriptase reaction for synthesis of genomic length DNA

The endogenous reverse transcriptase system is not a steady-state enzyme assay from which kinetic measurements are easily made. The most conveniently measured end point of the system is complete reverse transcription of the viral RNA template. This may also be the most relevant endpoint as an in vitro surrogate for the inhibition of viral replication. With HIV-1, as with many retroviruses, full length DNA has not been reported to be an abundant product of the endogenous RT reaction. Also, HIV-1 has been reported to synthesize DNA only in a narrow detergent concentration range (Borroto-Esoda and Boone, 1991; Yong et al., 1990), and therefore requires a careful titration for each virus lot as has been standard practice in the retrovirus field for many years (Collett and Faras, 1976). We recently reported a study (Borroto-Esoda and Boone, 1991) in which Equine infectious anemia virus (EIAV) appeared to be unique in its ability to synthesize DNA over a wide range of detergent concentrations presumably because of an unusually stable core synthetic machinery. In that study, HIV-1 appeared to be typical in having a narrow concentration range. The results of a titration of NP-40 as the detergent used to permeabilize sucrose gradient banded HIV-1 are shown in Fig. 2. The reaction

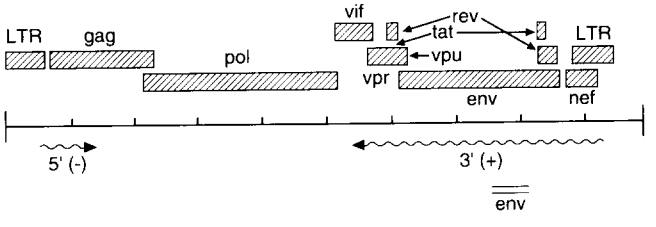

Fig. 1. Probes used in the analysis of the HIV-1 DNA synthesized in the endogenous reverse transcriptase system. Probes are depicted below the genome. Wavy lines represent RNA probes generated from the HIV-1 clone pBKBH10S and double lines represent double-stranded DNA probes derived from the HIV-1 clone HXB2 as described in Materials and Methods.

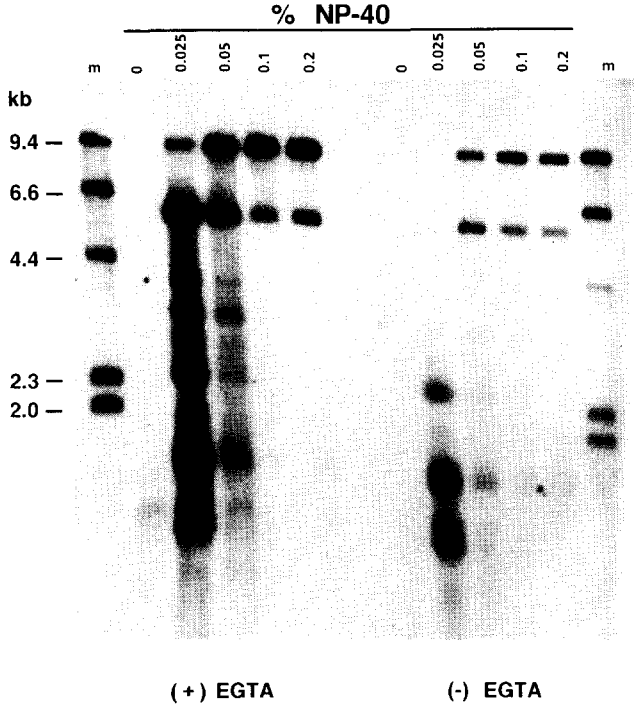

Fig. 2. Titration of NP-40 and EGTA in the HIV-1 endogenous reverse transcriptase system. DNA was synthesized by HIV-1 virions permeabilized by various concentrations of NP-40 in the presence or absence of 1 mM EGTA. The DNA was electrophoresed in alkaline agarose gels, transferred to nitrocellulose and hybridized with the RNA probe **5′(−)** as described in Materials and Methods. Size markers (m) were *Hin*dIII fragments of bacteriophage lambda DNA.

conditions were as described in Materials and Methods with the exception that a comparison was made of the products synthesized in the presence or absence of 1 mM ethylene glycol-bis(*B*-aminoethyl ether)*N,N,N′*,N′-tetraacetic acid (EGTA), a Ca^{2+} chelator. The products were analyzed by hybridization with a probe specific for the (−) strand near the 5′ end of the RNA genome, **p5′(−)**. Although the total yield of products detected by this probe, composed primarily of subgenomic species, exhibited the expected relatively narrow detergent concentration range, the synthesis of genomic length HIV-1 DNA did not. The maximum yield of genomic length DNA was achieved only at NP-40 concentrations higher than optimum for total product yield and higher than typically used in endogenous reactions. Inclusion of EGTA improved the yield of HIV-1 DNA at all NP-40 concentrations tested, but the most dramatic effect was at 0.025% NP-40. Genomic length DNA was detected only in the presence of EGTA at 0.025% NP-40, which is a typically used concentration for activating endogenous reverse transcription. The highest molecular weight

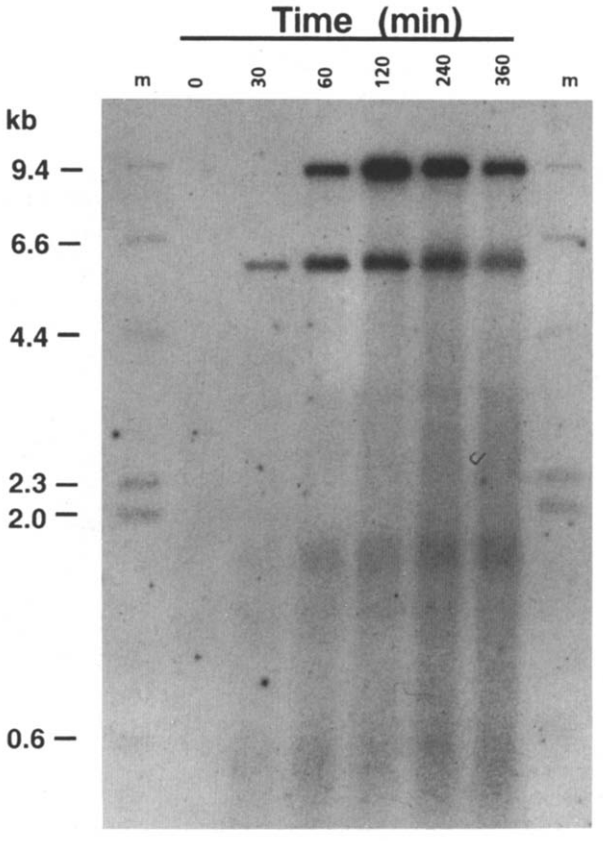

Fig. 3. Time course of DNA synthesis in the HIV-1 endogenous reverse transcriptase system. DNA was synthesized by HIV-1 virions as described in Materials and Methods and time points were taken at 30′, 60′, 120′, 240′, and 360′. Electrophoresis and hybridization with the RNA probe 5′(−) are as described in the legend to Fig. 2.

product corresponded to genomic length HIV-1 DNA containing one LTR (approximately 9.2 kb) as determined by comigration of a molecular HIV-1 clone (data not shown). Interestingly, a prominent 6 kb subgenomic (−) strand product did persist at high NP-40 concentrations. This product will be discussed further in connection with a more detailed analysis of the reaction products in a later section.

To determine the time course of HIV-1 DNA synthesis, samples were taken from an endogenous reaction at varying intervals and the products analyzed by hybridization with a probe specific for (−) strand DNA (Fig. 3). Genomic length HIV-1 DNA was first observed after 1 h whereas the prominent 6 kb transcript was synthesized by 30 min. Assuming initiation at the tRNA primer, this corresponds to polymerization of more than 3 nucleotides per second, a

rate that is approximately 3 times faster than that previously reported for synthesis of HIV-1 DNA in an endogenous reaction (Debyser et al., 1992). Incubation up to 6 h showed a similar pattern of transcripts although the intensity of the genomic length product was less than that observed after 2 h of synthesis. This decrease in genomic length DNA was accompanied by a slight increase in the amount of lower molecular weight transcripts and may be due to the activity of nucleases present in the preparation. A 2 h incubation time was selected for the standard protocol.

The HIV-1 endogenous reaction was also optimized for the concentration of dNTPs, Mg^{2+}, salt, and pH. As has been reported with avian and murine retroviruses (Collett and Faras, 1976; Rothenberg and Baltimore, 1976) and in contrast to what has been observed with EIAV (Borroto-Esoda and Boone, 1991), HIV-1 required relatively high concentrations of dNTPs for the synthesis of genomic length DNA. The concentration adopted for standard reactions was 500 μM. Genomic length HIV-1 DNA was not efficiently synthesized at concentrations below 250 μM each dNTP (data not shown).

In contrast to a previous report that demonstrates a higher HIV-1 RT activity on poly(rA)-oligo(dT) templates with K^+ vs. Na^+ (Hoffman et al., 1985), our experiments showed greater synthesis of genomic length DNA in the presence of NaCl (data not shown). Concentrations of NaCl ranging from 5 to 75 mM resulted in similar yields of genomic length HIV-1 DNA (data not shown). As with EIAV, the HIV-1 standard reaction utilizes 15 mM NaCl. Mg^{2+} has been shown to be the preferred divalent cation of HIV-1 RT (Hoffman et al., 1985). Synthesis of genomic length HIV-1 DNA in the endogenous reaction occurred at concentrations of $MgCl_2$ ranging from 1 to 6 mM. However, at the higher $MgCl_2$ concentrations, there was a marked decrease in the total synthesis of HIV-1 DNA (data not shown). The Mg^{2+} concentration selected for routine use was 3 mM.

We investigated the pH optimum of HIV-1 RT in the endogenous reaction by changing the pH of the Tris-HCl buffer (Fig. 4). Synthesis of genomic length HIV-1 was optimal between pH 8.0 and 9.0 and was almost completely inhibited at pH 7.5 and below. Previous results have demonstrated the activity of HIV-RT over a broad pH range, 6.6–9.0, with peak activity occurring at pH 8.0 (Hoffman et al., 1985; Yong et al., 1990). However, it is important to note that while the isolated enzyme is reported to be active, synthesis of genomic length HIV-1 DNA in the endogenous reaction is inhibited at pH 7.5 and below. Standard reactions were buffered at pH 8.1.

Characterization of the HIV-1 DNA products synthesized in the endogenous reverse transcriptase reaction

The HIV-1 endogenous reverse transcriptase reaction was conducted using optimized conditions and [^{32}P]dCTP (12 Ci/mmol) was included to radiolabel all products. The DNA products were subjected to electrophoresis under denaturing conditions and the gel was dried and exposed for autoradiography rather than transferred by the Southern procedure (Fig. 5A). Three prominent

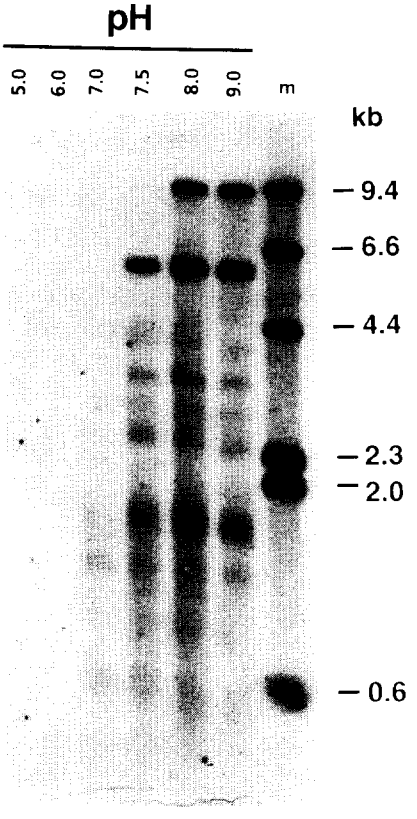

Fig. 4. Effect of pH on DNA synthesis in the HIV-1 endogenous reverse transcriptase system. DNA was synthesized by HIV-1 virions in a standard reaction as described in Materials and Methods while varying the pH of the Tris-HCl buffer. Electrophoresis and hybridization with the RNA probe **5′(−)** are as described in the legend to Fig. 2.

bands were apparent at 9.2, 6, and 3.5 kb, as well as less resolved bands at approximately 0.7 and 0.4 kb, the sizes expected for (+) and (−) strong stop DNAs, respectively. A similar pattern was observed when the products of an unlabeled reaction were hybridized with a probe representative of the entire HIV-1 genome (data not shown).

The products synthesized in a non radiolabeled standard reaction were further characterized by hybridization with specific in vitro RNA transcripts and DNA fragment probes. Using the **p5′(−)** probe (Fig. 5B) two prominent products were detected: genomic length DNA and a 6 kb reverse transcript. A probe specific to a region of the envelope gene at the 3′ end of the genomic RNA (**env**) was used to analyze the HIV-1 DNA products (Fig. 5C). This probe did not detect the 6 kb transcript but instead hybridized with a 3.5 kb product

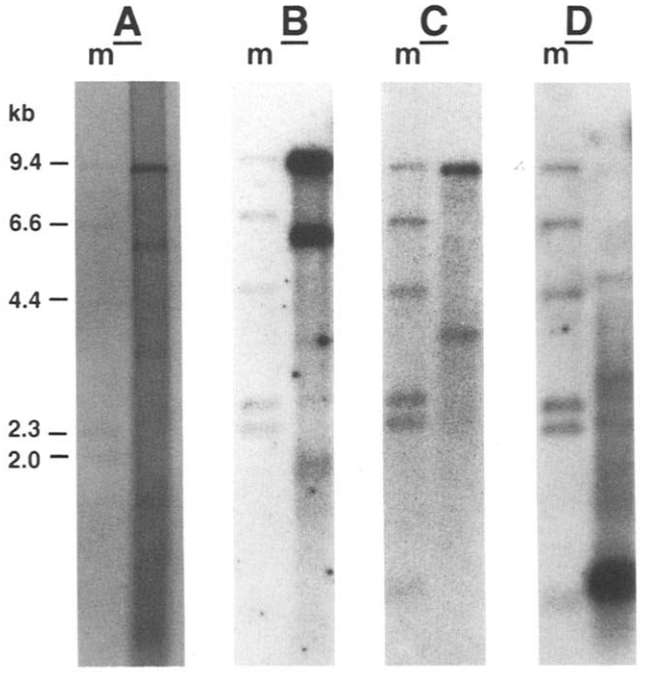

Fig. 5. Characterization of HIV-1 DNA products synthesized in the endogenous reverse transcriptase system. HIV-1 DNA was synthesized and subjected to electrophoresis and blotting as described in the legend to Fig. 2. (A) HIV-1 DNA synthesized with the addition of [^{32}P]dCTP. (B) Hybridization with a RNA probe specific for (−) cDNA strand 5′ sequences, **5′**(−). (C) Hybridization to a DNA probe specific for 3′ sequences, **env**. (D) Hybridization with a RNA probe specific for (+) cDNA strand 3′ sequences, **3′**(+).

and with full length genomic transcripts.

Synthesis of (+) strand HIV-1 DNA in the endogenous reaction was analyzed by hybridization with a probe, **3′**(+), representing the 3′ half of the HIV-1 genome and specific for (+) strand DNA (Fig. 5D). This probe detected a major band at 0.7 kb, most likely (+) strong stop DNA (Mitra et al., 1979), as well as other sub-genomic length transcripts. This probe also detected a (+) strand transcript of 4.9 kb which is consistent with the size expected for (+) strand transcripts initiated from the central polypurine tract (Charneau and Clavel, 1991; Charneau et al., 1992). Genomic length (+) strand HIV-1 DNA was not detected with this probe or with a probe specific to (+) strand at the 5′ end of the viral genome (data not shown).

Inhibition of DNA synthesis in the HIV-1 endogenous reverse transcriptase reaction

Inhibition of viral DNA synthesis in the endogenous HIV-1 reaction by the antiviral compound AZTTP is demonstrated in Fig. 6. In this assay, the

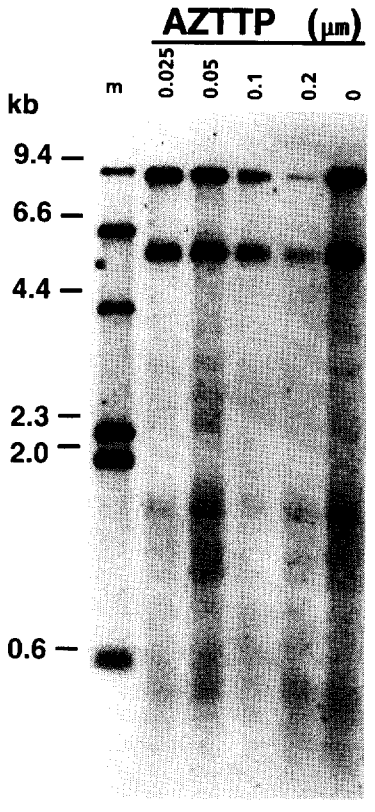

Fig. 6. Inhibition of DNA synthesis in the HIV-1 endogenous reverse transcriptase system by AZTTP. DNA was synthesized by HIV-1 virions as described in Materials and Methods with the addition of increasing concentrations of AZTTP. Electrophoresis and hybridization with the RNA probe 5′(−) were as described in the legend to Fig. 2.

concentration of each of the four natural dNTPs was 500 μM. The autoradiograph shown in Fig. 6 demonstrates the decrease in genomic length DNA synthesis as the AZTTP concentration increases. This same filter was analyzed by scanning with a Molecular Dynamics PhosphorImager followed by quantitation of the signal observed for genomic length HIV-1 DNA at each concentration of inhibitor, Fig. 7. Inhibition of DNA synthesis is expressed as a percentage of the maximum signal obtained from reactions conducted in the absence of inhibitor. The presence of 0.025 μM AZTTP was not sufficient for inhibition of genomic length DNA synthesis. However, when the concentration of AZTTP reached 0.2 μM, synthesis of genomic length DNA was inhibited by greater than 80% with respect to the control. An AZTTP concentration of 0.1 μM consistently resulted in 50% inhibition of full length (−) strand DNA. Fig. 7 also illustrates the inhibition of full length (−) strand synthesis by the non

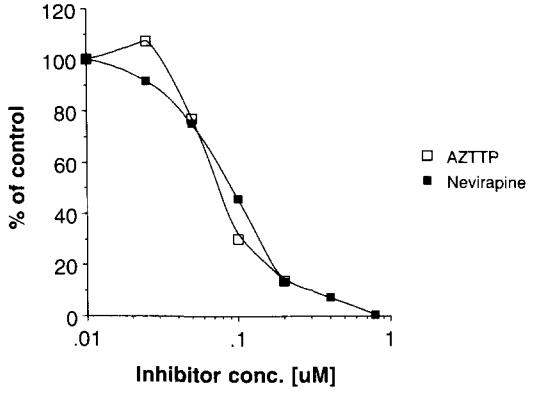

Fig. 7. Inhibition of DNA synthesis in the HIV-1 endogenous reverse transcriptase system by AZTTP and nevirapine. Synthesis of HIV-1 DNA in the presence of increasing concentrations of AZTTP was analyzed by scanning the filter from Fig. 6 with a Molecular Dynamics PhosphorImager followed by quantitation of the signal observed for full length HIV-1 DNA at each inhibitor concentration. A similar analysis was conducted on a filter (not shown) which contained the products of an endogenous reaction carried out in the presence of nevirapine. Inhibition of DNA synthesis is expressed as a percentage of the maximum signal obtained from reactions run in the absence of inhibitor.

nucleoside reverse transcriptase inhibitor nevirapine in the endogenous reaction. The concentration of nevirapine required for 50% inhibition of full length (−) strand synthesis was 0.1 μM.

Discussion

The present studies of HIV-1 endogenous reverse transcription have revealed that the optimum detergent concentration for synthesis of genomic length DNA is higher and has a broader range than that which allows synthesis of other products of the reaction. The general observation of reduced endogenous reverse transcription product formation at high detergent concentrations, including the literature on C-type retroviruses, may be more related to the presence of nucleases and the lack of detailed characterization of the products than to disruption of a critical structural complex within the viral core required for synthesis. EGTA, a Ca^{2+} chelator, is a standard component of some RT assay protocols (Hafkemeyer et al., 1991; Hoffman et al., 1985; Schwartz et al., 1988), and has recently been shown to prevent Ca^{2+}-dependent nuclease degradation of template and products in HIV-1 RT assays (Buckheit and Swanstrom, 1991; Recker et al., 1991). The importance of this reagent has also recently been demonstrated in an HIV-1 endogenous reaction (Debyser et al., 1992). Inclusion of EGTA in our reactions improved the yield of total as well as genomic length products. These observations illustrate the importance of

measuring genomic length DNA rather than total DNA synthesis as the end point for optimization of reaction conditions.

Approximately 6 discrete subgenomic reverse transcripts were detected at the lower NP-40 concentrations. Premature termination of reverse transcription would create subgenomic size (−) strand DNA, but these DNAs would not be detected by the probe used in Fig. 2, **p5′**(−). Subgenomic DNAs detected by this probe are therefore not due to premature termination of reverse transcription. Two plausible explanations for subgenomic (−) strand species containing the 5′ end sequences are: within the population, initiation of minus-strand DNA occurred at alternate sites along the RNA templates, or, initiation occurred normally at the tRNA primer, but (−) strong stop jumped to an alternate internal site in the RNA template rather than to the normally utilized R region at the 3′ end (Gilboa et al., 1979b). Additional primers which would be required for (−) strand initiation at internal sites have not been reported. Also, no internal sequences have been identified by homology to the R region that would be likely candidates for alternate jump sites. Although a mechanism which could direct the jump to a specific alternate site is not known, the sensitivity of subgenomic DNA synthesis to detergent concentration suggests that core structure might be important. Perhaps jumping to the authentic R region at the 3′ end requires a more open core structure which is only achieved in vitro at high detergent concentrations. The discrete banding pattern of subgenomic products indicates a high degree of specificity in the mechanism.

The major 9.2 and 6 kb products observed by direct labeling (Fig. 5A) correspond to the same two major products that were detected by the **p5′**(−) probe (Fig. 2–4). The 3.5 kb product detected by the **env** probe was most likely the same prominent 3.5 kb product observed by direct incorporation of radiolabel during synthesis (Fig. 5A). The failure of the **env** probe to detect the 6 kb product is consistent with either of the two synthetic mechanisms previously mentioned: a novel (−) strand initiation site, or, aberrant jumping to an internal site. Attempts to analyze the 6 kb product for evidence that this DNA initiated at the tRNA and jumped to an internal site in the genome have been inconclusive. We speculate that reverse transcription generating the 3.5 kb product terminated specifically at the point where the downstream portion of the template was removed by RNase H during synthesis of the 6 kb product.

The concentration of AZTTP required to inhibit the synthesis of genomic length HIV-1 DNA is dependent on the concentration of the natural nucleoside 5′ triphosphate with which it competes. In the endogenous reaction, a dTTP to AZTTP ratio of 2500/1 (500 μM dTTP/0.2 μM AZTTP) inhibited the synthesis of genomic length DNA by greater than 80%. Inhibition of genomic length product synthesis by 50% was calculated to be achieved with 0.1 μM AZTTP, which corresponds to a dTTP/AZTTP ratio of 5000/1. Lower molecular weight products detected with other probes (not shown) were not efficiently inhibited at the concentrations of AZTTP tested, consistent with the dependence of product length on the probability of incorporating a chain-terminating nucleotide (Reardon, 1992). Inhibition of reverse transcriptase by non

nucleoside compounds can also be analyzed in the endogenous reaction as illustrated with nevirapine. The concentration of nevirapine required for inhibition of 50% of the genomic length DNA was 0.1 μM.

In summary, we have further refined the HIV-1 endogenous reverse transcriptase reaction and have characterized the prominent subgenomic bands synthesized. Inhibition of genomic length DNA synthesis was used as an in vitro end point to assay the inhibition of viral replication by the nucleoside 5′ triphosphate analog AZTTP. A ratio of dTTP/AZTTP of 5000/1 was sufficient for 50% inhibition of genomic length HIV-1 DNA. Inhibition of genomic length DNA by various candidate antiviral compounds has been studied (Wilson et al., 1993) and a manuscript describing endogenous reaction studies involving HIV-1 strains resistant to antiviral compounds is in preparation.

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