Nucleic Acids Research, 1994, Vol. 22, No. 1 47–52

Mutagenicity and pausing of HIV reverse transcriptase during HIV plus-strand DNA synthesis

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Received October 6, 1993; Revised and Accepted November 30, 1993

ABSTRACT

The unusually high frequency of misincorporation by HIV-1 reverse transcriptase (HIV RT) is likely to be the major factor in the rapid accumulation of viral mutations in AIDS, especially in the *env* gene. To investigate the ability of HIV RT to copy the *env* gene, we subcloned an HIV *env* gene fragment into a single-stranded DNA vector and measured the progression of synthesis by HIV RT. We observed that HIV RT, but not RT from avian myeloblastosis virus, DNA polymerase-α or T7 DNA polymerase, pauses specifically at polydeoxyadenosine stretches within the *env* gene. The frequency of bypassing the polyadenosine stretches by HIV RT is enhanced by increasing the ratio of enzyme to template. We measured the fidelity of DNA synthesis within a segment of the hypervariable region 1 of the *env* gene (V-1) containing a polydeoxyadenosine sequence by repetitively copying the DNA by HIV RT, and then cloning and sequencing the copied fragments. We found that 27 % of the errors identified in V-1 sequence were frameshift mutations opposite the polyadenosine tract, a site where strong pausing was observed. Pausing of HIV RT at the polyadenosine tract could be enhanced by either distamycin A or netropsin, (A-T)-rich minor groove binding peptides. Moreover, netropsin increases the frequency of frameshift mutations in experiments in which HIV RT catalyzes gap filling synthesis within the *lacZ* gene in double-stranded circular M13mp2 DNA. These combined results suggest that the enhanced mutation frequency may be due to increased pausing at netropsin-modified polyadenosine tracts. Therefore, netropsin and related A-T binding chemicals may selectively enhance frameshift mutagenesis induced by HIV RT and yield predominantly non-viable virus.

INTRODUCTION

HIV-1 is a retrovirus with a 9.7 kilobase single-stranded RNA genome replicated by HIV-1 reverse transcriptase (HIV RT) (1). The high frequency of misincorporation by HIV RT is likely to be central to the high mutation rate exhibited by this virus (2, 3). All viral isolates constitute a mixed population of closely related yet genetically distinct genomes, referred to as quasispecies (4). This hypermutability is particularly evident in the *env* gene and could be a major impediment to the development of effective vaccines against the viral coat protein. The localization of most of the mutations within the *env* gene in five hypervariable regions (5, 6) could be the result of initial errors by the HIV RT followed by strong selection for the most viable mutants (7–9). Successful chemotherapeutic agents used in the treatment of AIDS are nucleoside analogs that interfere with HIV replication by specifically inhibiting incorporation by the viral reverse transcriptase. These include 3′-azido-3′-deoxythymidine (AZT), 2′ ,3′-dideoxyinosine (ddI) and 2′ ,3′-dideoxycytidine (ddC). However, the efficiency of these agents is limited because HIV strains resistant to AZT and other analogs appear rapidly during therapy (10–16); mutations in the reverse transcriptase gene have been documented in these resistant viral strains.

HIV RT not only copies the RNA viral strand into a minus-strand DNA, but also uses the resultant cDNA to synthesize a double-stranded DNA intermediate that is integrated into the host cell genome (1, 17). HIV RT is highly error prone in copying a DNA template (18–22). It has been shown that homopolymeric dT and dA runs in this template cause strong pauses during the copying of a DNA template by HIV RT *in vitro* (23–25). The effects of pausing during DNA polymerization on hypermutability are not fully understood (19, 26). The present results show that the HIV RT pauses strongly and specifically at polyadenosine runs during *env* gene plus-strand DNA synthesis. We show that the diminution in the rate of synthesis opposite polyadenosine runs is more extensive for HIV RT than for several other DNA

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polymerases. Using a cell-free assay for HIV RT fidelity that mimics the replicative steps in the viral life cycle, we have shown that a correlation exists between an HIV RT pause site on poly(A) tracts and a hot spot for frameshift mutation. We also present evidence that (A+T)-rich minor groove-binding peptides increase pausing and frameshift mutations by HIV RT.

MATERIALS AND METHODS

Bacteria

Escherichia coli MC1061 [*hsdR, mcrB, araD, 139D-(araABC-leu), 7679DlacX74, galU, galK, rpsL, thi*], the transformation host, and a F′ derivative of strain CSH50 [*D(pro-lac)thi, ara, strA/F′(proAB, Ia-cIa-zDM15)*], used as an indicator for the transfected *E.coli* MC1061, were provided by T.A.Kunkel (NIEHS, NC).

Chemicals and enzymes

HIV RT and calf thymus DNA polymerase α were purified as previously described (2, 18). T7 DNA polymerase, sequenase and avian myeloblastosis virus reverse transcriptase (AMV RT) were obtained from US Biochemical Corp. One unit of DNA polymerase α catalyzes the incorporation of 1 nmole of α-^{32}P-dTTP in 30 min at 37°C on activated calf thymus DNA (18). One unit of HIV RT, AMV RT or T7 DNA polymerase is the amount of enzyme required to incorporate 1.0 nmole of [^{3}H]TTP into an acid-insoluble product in 10 minutes at 37°C using polydA as a template (27). BamHI was from United States Biochemical Corp. T4 polynucleotide kinase and EcoRI were from New England BioLabs. Ligase was purchased from BRL and Taq DNA polymerase was from Perkin Elmer Cetus. [γ-^{32}P] ATP (3000 Ci/mmol) was from Amersham Corp. Netropsin was from Serva. Deoxynucleoside triphosphates and Distamycin A were purchased from Sigma.

DNA templates and primers

pME235, a pBluescript KS+ derivative plasmid containing the entire coding sequences of gp120 from an HIV-1 Bru isolate, was kindly provided by M.Emerman (FHCRC, WA). Single-stranded DNA containing the *env* gene was prepared upon transfection of VCS M13 helper phage purchased from Stratagene (27). The oligonucleotide primers used for extension reactions were synthesized by Operon Technologies, Inc. Gapped DNA was prepared by the method of Kunkel (28).

HIV RT-mediated PCR and mutation detection

Oligonucleotide primers used for amplification were 5′ end-labeled with [γ-^{32}P] ATP by T4 polynucleotide kinase as described (27). The two *env* primers used were Olig 001 with a BamHI site (ATG GGA TCC AAG CCT AAA GCC ATG TG) and Olig 002 with an EcoRI site (GGA ATT CTT TCT GCA CCT TAC CTC). The PCR reaction was conducted in 100 μl containing 20 ng template, 10 pmol primers, 60 mM Tris−HCl (pH 8.2), 1.0 mM dithiothreitol, 7 mM $MgCl_2$, 0.5 mM EDTA, 10 mM KCl and 500 μM each of the four dNTPs. DNA was amplified by 25 PCR cycles of 2 min at 95°C, 2 min at 25°C followed by 3 min at 37°C with 3 units HIV RT. The exponential DNA accumulation during amplification was monitored by agarose gel electrophoresis and scintillation counting.

Amplified *env* hypervariable region 1 (V-1) DNA and double-stranded M13mp18 vectors DNA were incubated in 50 μl reaction mix at 37°C for 60 min, containing 10 units each of EcoRI and BamHI, 50 mM Tris−HCl (pH 8.0), 10 mM $MgCl_2$ and 100 mM NaCl. The DNA was extracted with phenol/chloroform and ethanol precipitated. The residual oligonucleotide primers were removed by Centricon-30 filtration (Amicon). The *env* fragment was ligated into M13 vector DNA at a ratio of five to one in 10 μl at 16°C overnight, in a reaction mixture containing: 50 mM Tris−HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% polyethylene glycol-8000 and 2 units T4 ligase. The ligated products were transformed into *E.coli* MC1061 cells with Gene-pulser electroporator (Bio-Rad). The individual viral plaques were isolated and DNA was sequenced as described (18).

Primer extension experiments

The reaction conditions for primer extension were as described (18, 24). Briefly, in assays with HIV RT and AMV RT, the reaction mixture contained, in a final volume of 15 μl: 60 mM Tris−HCl buffer (pH 8.2), 7 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA, 10 mM KCl, 100 μM of dATP, dCTP, dGTP and dTTP, 60 to 100 ng of 5′-[γ-^{32}P]-labeled primer-template, and 0.05 to 10 units of RT. With calf thymus DNA polymerase α, the reaction mixture contained, in a final volume of 15 μl: 20 mM Hepes buffer (pH 7.8), 3 mM $MgCl_2$, 1 mM DTT, 20 to 500 μM of dATP, dCTP, dGTP and dTTP, 60 to 100 ng of 5′-[γ-^{32}P]-labeled primer-template, and 3 to 6 units of DNA polymerase α. For assays with bacteriophage T7 DNA polymerase the reaction mixture contained, in a final volume of 15 μl: 80 mM potassium phosphate buffer (pH 7.5), 6 mM $MgCl_2$, 5mM DTT, 25 mM NaCl, 500 μM of dATP, dCTP, dGTP, and dTTP, 60 to 100 ng of 5′-[γ-^{32}P]-labeled primer-template, and 0.1 to 10 units of T7 DNA polymerase. The extended DNA was further purified by centrifugation through Sephadex G-50 columns and the labeled products were analyzed on 8 % polyacrylamide/urea gel.

Analysis of the fidelity of HIV RT copying *lacZ* gene DNA

Double-stranded circular M13mp2 DNA (200 ng) containing a 361-nucleotide single-stranded gap within the *lacZ* gene was incubated for 60 min at 37°C in the HIV RT reaction buffer described above in the presence or absence of 10 mM netropsin. The gap was then filled by 0.4 units of HIV-RT for 60 min at 37°C in the presence of 0.5 units of ligase. *E.coli* MC1061 cells were electroporated in the presence of 2 μl of the ligation reaction and the transformed cells were plated with *E.coli* CSH50 cells (3, 21). Colorless plaques were picked, and the single-stranded DNA was isolated and sequenced.

RESULTS

Pause sites of HIV RT within the envelope gene

To study the progression of DNA synthesis by HIV RT, we first prepared single-stranded DNA containing the envelope gene, and annealed it with each of four different primers: the universal primer complementary to the vector sequence adjacent to the insert and three other primers complementary to nucleotides 161−189, 514−530 and 1106−1133 of the envelope gene. Each of the primed templates was incubated with 0.4 units of HIV RT for one hour at 37°C, the newly synthesized DNA products were fractionated by polyacrylamide gel electrophoresis and visualized by autoradiography. The reaction products migrated as a population of DNA fragments with a limited number of discrete sizes (Fig. 1), indicating pausing of DNA synthesis opposite specific nucleotide sequences within the envelope gene. All of

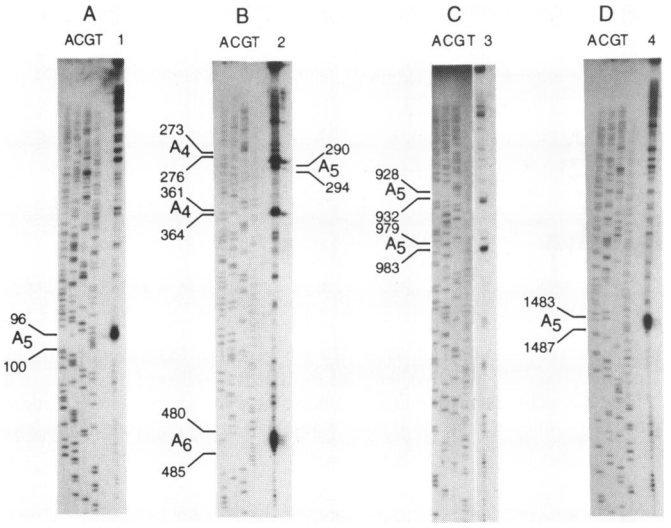

Figure 1. Pause sites of HIV RT along the envelope gene of HIV.100 ng of single-stranded pBluescript/*env* gene recombinant DNA was annealed with 5′-[γ-^{32}P]-labeled primers covering the nucleotides 161−189 (Panel **A**), nucleotides 514−530 (Panel **B**) or nucleotides 1106−1133 (Panel **C**) of the envelope gene or the sequence 3′ of the inserted envelope gene (Panel **D**). The annealed template were then extended in the presence of 0.4 units of HIV RT at 37°C for 60 min.. Lanes A, C, G, and T show the dideoxynucleotide sequencing reactions on the *env* gene using the corresponding primers.

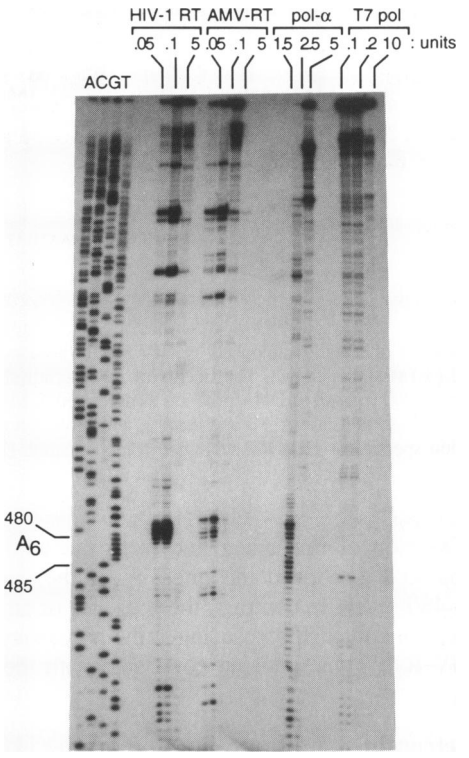

Figure 2. Primer extension and pausing of HIV RT, AMV RT, calf thymus DNA polymerase α, and T7 DNA polymerase along the *env* gene.DNA synthesis was conducted for 60 min. at 37°C with 100 ng of primed single-stranded pBluescript/*env* gene recombinant DNA and the three concentrations of enzymes as indicated. The primer used in this experiment covers the nucleotides 514−530 of *env* gene. Lanes A, C, G, and T show the result of sequencing reactions with the same primed template.

the clearly delineated pausing sites occured at positions opposite template sequences of 4−6 adjacent adenosines corresponding to nucleotides 96−100 (Fig 1A, lane 1), nucleotides 273−276, 290−294, 361−364 and 480−485 (Fig. 1B, lane 2), nucleotides 928−932 and 979−983 (Fig. 1C, lane 3), and nucleotides 1483−1487 (Fig. 1D, lane 4). In similar experiments carried out with a 12.5-fold higher ratio of HIV RT to DNA, each of these pause sites was bypassed , resulting in an increase in the length of the newly synthesized products (data not shown). To assess whether the pausing of HIV RT at polyadenosine tracts is specific to this enzyme, we analyzed the progression of three additional DNA polymerases along the same single-stranded DNA construct annealed to a primer complementary to nucleotides 514−530. The same prominent pause site opposite the polydeoxyadenosine tract at nucleotides 480−485 in the *env* gene was observed using small amounts of HIV RT (0.05 and 0.1 units) and was again no longer observed with 5 units, presumably as a result of bypass of this sequence as evidenced by the formation of longer DNA at the top of the gel (Fig. 2). Extension of the same template by low concentrations of the AMV RT (0.05 and 0.1 units) and the eucaryotic DNA polymerase-α (2.5 units) was interrupted at or in the vicinity of the polyadenosine tract, but the intensity of the pause was much lower compared to that observed for HIV RT. Like HIV RT, a higher concentration of AMV RT or DNA polymerase-α resulted in bypass of the polydeoxyadenosine stretch and an increase in the accumulation of high molecular weight products. In contrast, the progresion of DNA synthesis with T7 DNA polymerase was not diminished at the polydeoxyadenosine sequence with any of the three concentrations tested.

Specificity of mutagenesis by HIV RT in copying *env* hypervariable region 1 DNA

To analyze the type of mutations produced by HIV RT in copying DNA, we developed an assay based on an HIV RT-mediated polymerase chain reaction, utilizing a portion of the *env* gene (V-1) as a template. A 153-base segment of the V-1 fragment was repetitively copied by HIV RT using a PCR protocol in which fresh enzyme was added after each denaturation step. Because we used an excess of HIV RT in this reaction to ensure complete DNA synthesis, there was no detectable pausing for HIV RT during PCR. The HIV RT amplified product was cloned into double-stranded M13 DNA. Single-stranded recombinant DNA was isolated from individual clones and the nucleotide sequence of the V-1 region was determined. Mutations presumably arose during copying by HIV RT. There were a total of 64 mutations at 36 different positions within the V-1 region (Fig. 3). The most frequent mutations were single base substitutions (76%); 16 of 46 of the substitutions were GC → AT and 19 were AT → GC transtitions. Of the 64 mutations, 18 were frameshifts, 17 of which occurred opposite the stretch of six deoxyadenosines at positions 480−485, the strong pause site for HIV RT copying a single-stranded DNA template (see Fig. 1 B). Twelve of these frameshifts were +1 additions, three were +2 additions and two were −1 deletions.

We have used these different mutants with frameshifts as templates to examine the effect of length of the polydeoxyadenosine tract in determining pausing by HIV RT. In these experiments single-stranded DNA from the original clone containing the stretch of six deoxyadenosines (dA6), from the −1 deletion mutant (dA5), and from the +1 addition mutant (dA7) were used as templates in separate extension experiments

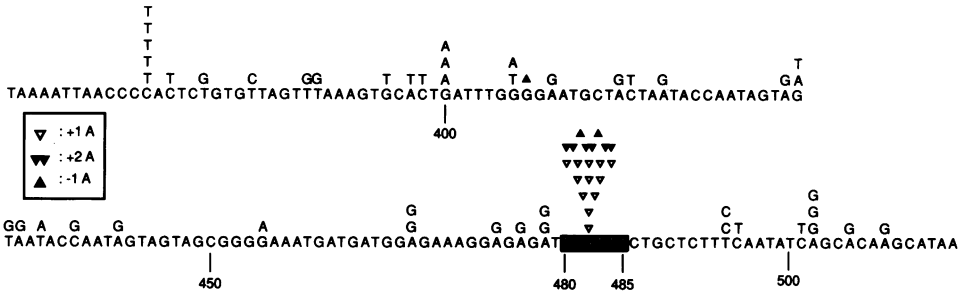

Figure 3. Spectrum of mutations induced by HIV RT during the DNA synthesis of the hypervariable region 1 (V-1) of HIV *env* gene.The 153-base target of the HIV *env* viral (+) strand is shown. The fidelity of DNA synthesis was measured by repetitively copying the DNA template by HIV RT, followed by cloning and sequencing the newly synthesized DNA products as described in Materials and Methods. Single-base substitutions are displayed above the wild type sequence. Deletions are shown as , and additions as for +1 and for +2, with a black box indicating the presumed origin of deleted or added nucleotides. Data in this experiment constitute part of mutation spectra for HIV RT submitted for publication (Ji & Loeb, 1993).

(Fig. 4). Deletion of one adenosine from the wild type V-1 sequence markedly reduced the intensity of the pause site at nucleotides 480−485. In contrast, the presence of an additional adjacent adenosine intensified pausing at the polydeoxyadenosine tract by HIV RT. Thus, pausing correlated with the length of the polydA.

Effect of netropsin and distamycin A on primer extension by HIV RT

The above studies on primer extension and RT fidelity identified the polydeoxyadenosine tracts in the envelope gene as major pause sites for HIV RT as well as hot spots for frameshift mutations. Sequences with polydeoxyadenosine tracts are known to be recognized by a class of (A+T)-rich minor groove-binding peptides. We hypothesized that such peptides, by binding preferentially to polyadenosine-rich tracts of the envelope gene, could enhance the termination of replication by HIV RT at these sites and also increase the frequency of mutations. We tested this hypothesis by assessing the influence of distamycin A and netropsin elongation and termination by HIV RT. Single-stranded pBluescript/*env* recombinant DNA was annealed to the 5′-[γ-^{32}P]-labeled 17-mer universal primer located upstream from the *env* gene. The annealed template was incubated with 50 or 100 mM of either distamycin A or netropsin and then copied with 10 units of HIV RT. With this high concentration of enzyme and in the absence of either peptide, the single-stranded *env* DNA extension product was of high molecular weight and there was a lack of pausing by HIV RT at the polydeoxyadenosine tract at position 1483−1487 (Fig. 5, lane C). However, in the presence of either 50 mM distamycin A or 50 mM netropsin there was a diminution in the total amount of DNA synthesis catalyzed by HIV RT and synthesis was terminated at the poly(A) tract (Fig. 5), mimicking the natural pause site found with small amounts of HIV RT.

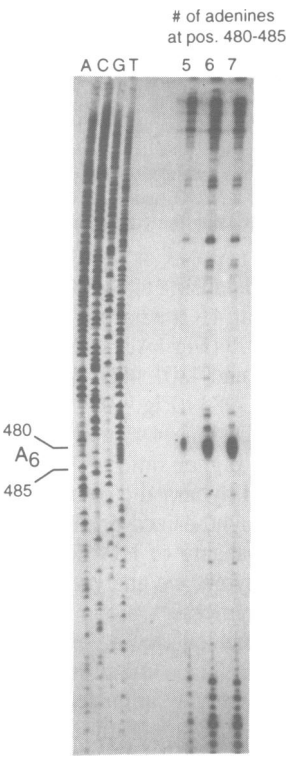

Figure 4. Pause sites of HIV RT on *env* V-1 DNA templates containing frameshift mutations at the deoxyadenosine tract. 5′-^{32}P-labeled 17-mer universal primer was annealed to 100 ng each of three different single-stranded M13/*env* gene recombinant DNAs. The wild type sequence contains 6 deoxyadenosines and the mutant sequences contain 5 or 7 deoxyadenosines after a −1 deletion or a +1 addition, respectively, within the deoxyadenosine tract at position 480−485. Reactions were incubated for 60 min. at 37°C in presence of 0.4 unit of HIV RT.

Enhancement of mutagenesis of HIV RT by netropsin

To examine the effect of (A+T)-rich minor groove-binding peptides on HIV RT mediated mutagenesis, we incubated an M13mp2 replicative form molecule, containing a 361-nucleotide single-stranded gap spanning the 5′ regulatory region and the first nucleotides of the coding region of the β-galactosidase gene, in the presence or absence of 10 mM netropsin. This concentration of netropsin strongly inhibited DNA synthesis by HIV RT *in vitro* at the polydeoxyadenosine tract (nucleotides 1483−1487 on the *env* gene) and these termination sites were bypassed only at low frequency as indicated by the appearance of a small amount of high molecular weight products (data not shown). We determined the frameshift mutation frequency from gap-filling synthesis by HIV RT in the presence or absence of the peptide by scoring the mutants due to the absence of α-complementation. Mutations that result in abolishment of β-galactosidase yield white plaques on X-gal plates. The results,

Table I. Effect of netropsin on fidelity of HIV RT copying *lacZ* gene DNA

		Total plaques	White plaques	Mutation frequency (%)
I	−Netropsin	669	29	4.3
	+Netropsin	818	162	19.8
II	−Netropsin	1090	64	5.9
	+Netropsin	601	64	10.6

Double-stranded circular M13mp2 DNA containing a single-stranded gap within the *lacZ* gene was incubated in the presence or absence of netropsin. The gap was then filled by HIV-RT and the DNA transfected into *E.coli*. M13 carrying mutations within the nonessential *lacZ* gene for inactivation of β-galactosidase were identified as white plaques due to an absence of α-complementation. The mutations were confirmed by DNA sequencing. The background frequency of white plaques in the absence of netropsin and HIV RT was 0.5 per cent.

presented in Table I, show that in two different experiments, HIV RT induced mutations with a frequency of 4−6 % when filling the *lacZ* gene gap. Pretreatment of the gapped DNA template with 10 mM netropsin caused a 2- to 5-fold increase in mutations by HIV RT (10−20%). Thirteen of these mutations were sequenced: 10 were frameshift mutations and 3 were single base substitutions. In contrast, the percentage of frameshift mutations by HIV RT along is about 20−30% (19−21). These data demonstrate the capacity of netropsin to increase HIV RT-induced frameshift mutations during the copying of the *lacZ* gene.

DISCUSSION

The fidelity of HIV RT was studied by measuring the progression of plus-strand DNA synthesis and the frequency of production of different types of mutations. A portion of the HIV envelope gene was used as a template for synthesis because this gene contains the most variable portions of the viral genome and this variability may be essential to viral evasion of host cell defenses (1). We found that HIV RT specifically paused at eight polydeoxyadenosine tracts along *env* gene, a finding in agreement with earlier reports (23−25). To explore the potential mutagenic consequences of these pauses, we developed a cell-free assay for HIV RT fidelity copying its own genome. Cloned vectors containing the *env* variable region 1 fragment (V-1) constituted a homogeneous template for analyzing the accuracy of catalysis by HIV RT *in vitro*. Mutations produced by HIV RT in the V-1 gene were detected after multiple rounds of replication carried out in the absence of any selection pressure. From these and other studies (unpublished results) we calculate that the overall error rate of HIV RT is about one error per 5400 nucleotides during copying DNA. This exceptionally high error rate could account for 3 to 4 mutations per HIV genome per replication cycle (1−3). The frequency of misincorporation using the *env* gene as a template appears to be similar to that obtained with other DNA templates indicating that envelope gene DNA sequence itself does not dictate the hypervariability exhibited by this region (9). While studies are needed on the production of errors in the envelope gene with an RNA template, it seems likely that the hypervariability observed after viral infections in humans result from the selection of more viable clones.

The mutagenic spectrum we obtained from copying of the envelope gene fragment by HIV RT identified one hot spot for frameshift mutations in V-1 region, corresponding to the polydeoxyadenosine pause site at positions 480−485. The association of pausing with misincorporation has also been demonstrated in studies with M13 DNA and DNA polymerase-α (26). With M13 DNA as a template, Kunkel's group observed

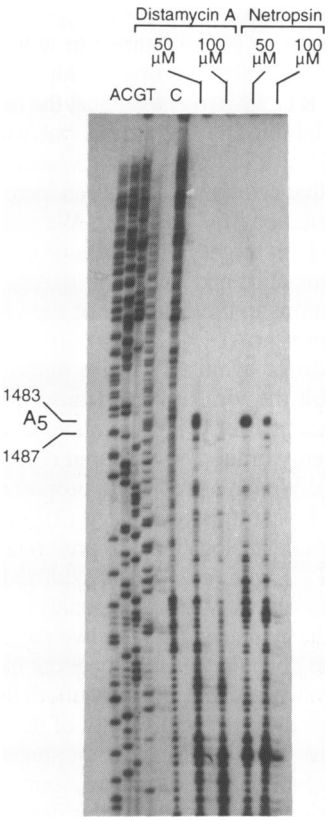

Figure 5. Effect of distamycin A and netropsin treatments on the replication of *env* gene DNA by excess amount of HIV RT. Primed, single-stranded pBluesript/*env* gene recombinant DNA was treated with the indicated concentrations of distamycin A and netropsin for 60 min. at 37°C. Extension was thereafter conducted for 60 min. at 37°C in presence of 10 units of HIV RT. Lane C indicates a control experiment without drug. The sequence of the *env* gene is shown in lanes A, C, G and T.

that HIV RT generates frameshift mutations at a high frequency, about 1 mutation per 4300 nucleotide incorporation (19). The percent of frameshift mutations produced by HIV RT was greater than that of any other DNA copying enzymes so far analyzed including those involved in eucaryotic DNA replication (18−20). Studying the fidelity and processivity of HIV RT copying along the M13 *lacZa* gene, Bebenek *et al.* observed that frameshift mutations were the only ones that correlated with diminished processivity (19). The correlation between pausing sites and frameshift hot spots at polydeoxyadenosine regions observed here

suggests that frameshifts at this site occur through a primer-template slippage mechanism (29).

We used distamycin A and netropsin, two antibiotics known to interact in the minor groove of DNA at sites consisting of at least four AT base pairs (30, 31), in an attempt to increase pausing and mutagenicity of HIV RT. Both drugs have been reported to inhibit the multiplication of DNA viruses as well as several retroviruses (32, 33). The most direct mechanism for the inhibition of viral replication would be based on their DNA binding properties. Our finding that distamycin A and netropsin enhance the pausing of HIV RT at polydA during *env* plus strand DNA synthesis suggests that the peptides bind within the oligo-deoxyadenosine tracts in a single-stranded DNA molecule, forming a complex which interrupts the progression of DNA synthesis by HIV RT.

Because the position of the pause site within the *env* gene at position 480–485 correlates strongly with a frameshift mutation hot spot for HIV RT, we hypothesize that the binding of netropsin would not only inhibit DNA synthesis but would also increase mutagenesis by HIV RT. Using an M13 *lacZα* complementation assay, we found that netropsin does indeed increase the production of frameshift mutations by HIV RT. We conclude that DNA binding agents which target polyadenosine clusters are likely to increase both termination and frameshift mutagenesis by HIV RT. Frameshift mutations in the *env* gene of the virus would lead to non-viable progeny.

Many of the drugs against HIV are nucleotide analogs that specifically inhibit the viral reverse transcriptase (1). Therapy with these analogs or even with non nucleoside analogs against HIV RT is markedly limited by the rapid emergence of resistant variants (11–16). We have previously proposed that the opposite approach should be carefully evaluated (2); that is to use highly mutagenic nucleoside analogs that are preferentially incorporated by the viral RT. Eigen *et al.* has explored this idea using mathematical models (4). From an analysis of viral isolates obtained during the course of AIDS in two patients, Nowak *et al.* concluded that the diversity of *env* sequences increased with time (34). Their data suggest that the immunodeficiency syndrome becomes manifested when the diversity of the *env* gene exceeds the host's immune capacity. Most of the mutations observed *in vivo* are base substitutions. However, our data suggest that frameshift mutations also might occur frequently during the course of expansion of viral variability but might not be observed within the viral isolates. Therefore, agents that increase the frequency of frameshift mutations by the RT may provide a rapid approach to enhance the production of non-viable virions during the course of viral infection. While netropsin and distamycin are toxic to host cells, a wide spectrum of analogs has been synthesized (32, 33) and can be evaluated for increasing the frequency of HIV mutagenesis. The high frequency of frameshift introduced by these analogs might be partially advantageous in biasing the viral spectrum toward the production of non viable progeny. This approach could greatly lessen the problem of rapid emergence of viral resistance (10–16).

ACKNOWLEDGEMENTS

We would like to thank Alan Herr, Brenda Donehower and Dominic Connolly for their excellent technical assistance and Kris Carroll for graphics work. This work was contributed equally by JJ and JSH and supported by an OIG from the National Institutes of Health Grant, 35-CA-39903.

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