Mechanisms That Prevent Template Inactivation by HIV-1 Reverse Transcriptase RNase H Cleavages*§

Vandana Purohit†, Bernard P. Roques*, Baek Kim*, and Robert A. Bambara†

From the Departments of †Biochemistry and Biophysics and *Microbiology and Immunology, University of Rochester, Rochester, New York 14642 and the §Unite de Pharmacochimie Moleculaire et Structurale, INSERM U266, CNRS UMR 8600, UFR des Sciences Pharmaceutiques et Biologiques, Universite Rene Descartes, 75270 Cedex 06, Paris, France

The RNase H activity of human immunodeficiency virus, type 1 (HIV-1) reverse transcriptase (RT) cleaves the viral genome concomitant with minus strand synthesis. We previously analyzed RT-mediated pausing and RNase H cleavage on a hairpin-containing RNA template system and reported that RT generated 3’ end-directed primary and secondary cuts while paused at the base of the hairpin during synthesis. Here, we report that all of the prominent cleavage products observed during primer extension on this template correlated with pause induced cuts. Products that persisted throughout the reaction corresponded to secondary cuts, about eight nucleotides in from the DNA primer terminus. This distance allows little overlap of intact template with the primer terminus. We considered whether secondary cuts could inactivate further synthesis by promoting dissociation of the primer from the template. As anticipated, 3’ end-directed secondary cuts decreased primer extendibility. This provides a plausible mechanism to explain the persistence of secondary cut products in our hairpin template system. Improving the efficiency of synthesis by increasing the concentration of dNTPs or addition of nucleocapsid protein (NC) reduced pausing and the generation of pause-related secondary cuts on this template. Further studies reveal that 3’ end-directed primary and secondary cleavages were also generated when synthesis was stalled by the presence of 3’-azido-3’-deoxythymidine at the primer terminus, possibly contributing to 3’-azido-3’-deoxythymidine inhibition. Considered together, the data reveal a role for NC and other factors that enhance DNA synthesis in the prevention of RNase H cleavages that could be detrimental to viral replication.

Human immunodeficiency virus, type 1 (HIV-1) reverse transcriptase (RT) is the virally encoded enzyme responsible for conversion of the single-stranded RNA genome of HIV-1 to a double-stranded DNA form that can be integrated into the host genome (1). RT can utilize both RNA and DNA as a template for DNA synthesis. In addition, RT possesses RNase H activity, meaning that it can cleave RNA within a RNA:DNA hybrid. Both the polymerase and RNase H activities of RT are essential for the reverse transcription of the viral genome.

RT is a p66/p51 heterodimer with both the polymerase and RNase H activities residing in the p66 subunit. When RT binds the primer terminus within the polymerase active site, the RNase H active site is positioned 18 nt upstream of the primer terminus (2–5). Concomitant with synthesis of the first, or minus DNA strand, the polymerizing RT generates cuts on the RNA template ~18 nt upstream, designated “polymerase-dependent” cuts. However, the polymerase and RNase H activities of RT are uncoupled and cuts occur less frequently than nucleotide incorporation (6). Studies in vitro indicate that RNase H cleavage by the polymerizing RT is not sufficient to fully degrade the RNA template and some fragments of RNA are left bound to the cDNA during minus strand synthesis (7). It is believed that these fragments are degraded by excess RT in the virion through a “polymerase independent” mechanism. In support, RT has been shown to have alternate modes of nucleic acid binding that do not require a 3’ primer terminus. For example, RT preferentially cleaves ~18 and ~8 nt from the 5’ end of an RNA fragment recessed on a longer DNA substrate (8, 9). Designated as 5’ end-directed primary and secondary cuts, respectively, these ~18 and ~8 nt cuts are created independently of each other and at distinctly different rates (10). In addition, the RT has been shown to make internal cuts that are not directed by primer termini (11). These internal and 5’ end-directed RNase H cleavages by RT are believed to contribute to clearing away the RNA template during minus strand synthesis.

Because of its critical role in the viral life cycle, RT is a drug target for treatment of HIV infection. Administration of nucleoside analogs, which are incorporated onto the elongating primer during viral replication but lack a 3’ hydroxyl group essential for further chain elongation, provides an efficient strategy to inhibit RT (12). A widely used drug that employs this strategy is 3’-azido-3’-deoxythymidine (AZT). Following prolonged administration of AZT to patients, mutations that confer resistance arise (6, 13). Several studies point to a resistance mechanism involving RT-mediated removal of AZT-terminated primer (14–16). RT was shown to excise an AZT residue from the primer terminus in the presence of an acceptor such as pyrophosphate (PP) or ATP (14, 17).
wild type RT, AZT-resistant RT is more efficient at ATP-mediated excision (16).

In vivo, reverse transcription takes place in the presence of virally encoded nucleocapsid protein (NC). NC has been shown to facilitate reverse transcription at multiple steps, including tRNA primer binding and removal, and minus and plus strand transfer (reviewed in Ref. 18). NC is a chaperone protein that promotes the folding of nucleic acids into more stable conformations and can enhance strand annealing (19–21). This activity has also been shown to alleviate pausing during synthesis by RT (22–25). Pause sites are positions where RT synthesis is stalled, often by the presence of a secondary structure such as a hairpin. NC has also been suggested to influence the RNase H activity of RT. In several studies, NC was observed to increase overall RNase H cleavage during primer extension (26, 27) and suppress the RNase H cleavage defect of an RT mutant (28). Moreover, NC has been shown to enhance secondary cleavages on blunt end substrates (29–31).

RT can switch templates during DNA synthesis. Two of these strand transfer events are required at the ends of the genome for the completion of viral replication (1). In addition, RT has been shown to mediate strand transfer within internal regions of the genome (32–34). These events can produce recombinant progeny virus if the template regions involved in switching are not identical. By modeling template switching during minus strand synthesis in vitro, several mechanisms that promote strand transfer have been elucidated (reviewed in Ref. 35). RNase H activity of RT is required for strand transfer in vitro (36, 37). In addition, several groups have reported enhancement of strand transfer upon the addition of NC (29, 38–40). Furthermore, studies in vitro have suggested that proper template structure (41) and RT pausing (26, 42, 43) enhance strand transfer. In support, Lancialut and Champoux (44) recently reported a correlation between enhanced pausing in vitro and higher recombination in vivo. However, templates with relatively less stable structures and less pausing also allow efficient strand transfer (45, 46), suggesting multiple mechanisms of strand transfer.

A series of studies from our group have defined elements of the mechanism of HIV-1 RT-mediated strand transfer in a template system containing a major hairpin sequence derived from the primer-binding site of the equine infectious anemia virus (EIAV) (27, 42). In this system, pausing at the base of the hairpin on the first, or donor, template enhances RNase H cleavage by the RT. This locally clears away the donor RNA and allows the second, or acceptor, template to interact with the cDNA before the hairpin base. The cDNA/acceptor hybrid propagates toward the cDNA primer 3’ terminus. Strand transfer is complete once the hybrid reaches the primer terminus and the acceptor serves as the template for DNA synthesis. Analysis of several other template systems provided further evidence that this multistep strand transfer mechanism that involves initial acceptor invasion upstream of the point of terminus transfer is a major pathway for strand transfer in HIV-1 (26, 46–48).

In a previous study examining RNase H mutants and strand transfer using the EIAV template, we identified prominent cleavages at the hairpin base of the EIAV RNA template generated during primer extension (49). We presented evidence indicating that they were created by a DNA 3’ end-directed primary and secondary cleavage mechanism mediated by RT while paused during synthesis. Because of their prominence in the degradation pattern of the donor template and their location at the hairpin base, we suggested that these 3’ end-directed primary and secondary cuts helped to create an acceptor invasion site for strand transfer. The generation of primary cuts at a pause site during synthesis was in agreement with the findings of Suo and Johnson (50). Other studies also identified 3’ end-directed secondary cuts in the absence of synthesis (31, 51–55).

In the current study we further examined cleavages during primer extension on the EIAV template. We specifically addressed whether secondary cleavages made during synthesis lead to template disruption, and, if so, how the virus suppresses disruption to prevent inactivation of synthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—RT was purified as described (27, 56) except that p66 and p51 subunits were purified separately and mixed in equal amounts prior to dialysis. DNA oligonucleotides and RNA templates T2 and T3 were obtained from Integrated DNA Technologies (Coralville, IA). AZTTP was purchased from Moravek Biochemicals (Brea, CA). Poly(rA)-oligo(dT) was obtained from Amersham Biosciences. For 5’ end labeling, [γ-32P]ATP was purchased from PerkinElmer Life Sciences. NotI, HindIII, and Escherichia coli RNase H were purchased from Invitrogen. All other enzymes and dNTP solutions were obtained from Roche Applied Science.

**Preparation of Substrates**—RNA template T1 (DI from pEIAV-Donor) was generated and purified as previously described (42, 49). To prepare RNA templates for 5’ end labeling, RNA was first treated with calf alkaline phosphatase to remove the 5’ phosphate. RNA and DNA were radiolabeled using polynucleotide kinase and [γ-32P]ATP. The labeled samples were purified using a Bio-Rad P-30 microspin column to remove unincorporated ATP. To anneal substrates for reactions, the templates were mixed in the appropriate ratio, heated for 5 min at 95 °C, and then cooled slowly to room temperature.

**Primer Extension and Template Degradation with RNA T1**—Reactions were performed as previously described (42, 49). To examine primer extension, a labeled DNA primer was used. A labeled RNA template was used in reactions to monitor template degradation. For both types of reactions the substrates were annealed as described above in a ratio of 2:1 primer to template. The substrates were incubated with RT for 5 min at 37 °C prior to initiation of the reaction by the addition of MgCl2 and dNTPs. Reactions were incubated at 37 °C and were terminated at appropriate times by the addition of 2X termination dye (20 mM EDTA (pH 8.0), 90% formamide, 0.1% each of bromphenol blue and xylene cyanole). Unless otherwise specified, final reaction conditions were 4 mM primer, 2 mM template, 32 mM RT, 6 mM MgCl2, 50 μM dNTPs, 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM dithiothreitol, and 1 mM EDTA. For primer extension control reactions, dCTP was replaced with ddCTP. To assess the amount of substrate available for RNase H cleavage in degradation assays, the substrate was incubated with 2 units of E. coli RNase H for 10 min. For reactions containing NC, 800% NC coating (expecting that 1 NC covers 7 nt) was added at 37 °C, 5 min prior to the addition of RT. The
Extendibility

strate was incubated with RT for 5 min at 37 °C and then MgCl₂ 5
ACACTATAGAATATGCATCACTAGTAAGCTTCAGG-
AGCTCGAAT-3’; O-C, 5’-CCCTGTTCGGGCGCCCAAACGTGTA-3’, O-E, 5’-GGATCTGAACAGACAAACTAGAGACA-3’.

Assay to Determine the Effect of RNase H Cleavage on Primer Extendibility—DNA primer P2 and RNA template T2, both radiolabeled, were annealed together in a 1:1 ratio. The substrates were incubated with RT for 5 min at 37 °C and then MgCl₂ was added to initiate RT cleavage of the template. Reactions were incubated at 37 °C and two aliquots were taken at appropriate times. To the aliquot identified as “quench” termination dye was added. To the other aliquot, identified as “extend,” dNTPs were added for an additional 5 min before addition of termination dye. The final reaction conditions were 4 mM primer, 4 mM template, 32 mM RT, 6 mM MgCl₂, 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA. The extend reactions contained 50 μM dNTPs. The reaction products were separated by PAGE and quantitated as described below.

The primer extension control was performed under the same conditions as the other extend reactions, except without preincubation with MgCl₂. The cleavage control was performed by the addition of 2 units of HIV-1 RT RNase H for 10 min. These controls were used to determine the maximum amount of substrate available for extension or cleavage. The percent of substrate that was extended or cleaved at each time point of the experiment was normalized to these control values. The substrates used were: P2, 5’-CTAGAGGATCCCCGGTGACCCGACGCTCAAT-3’ and T2, 5’-GGGGGAUUGCCAGGCUCGGUAACCCGGGAUCCUCUGAGCUG-3’.

Assay to Determine the Ability of RT to Generate 3’ End-directed RNase H Cuts on an AZT-terminated Substrate—5’ End-labeled DNA primers P3 and P4 were annealed to 5’ end-labeled RNA template T3 at a ratio of 1 to 1. The substrates were incubated with RT for 5 min at 37 °C before initiation. Following initiation, the reactions were incubated at 37 °C and terminated at appropriate times by the addition of 2X termination dye. The final reaction conditions were 4 mM primer, 4 mM template, 32 mM RT, 6 mM MgCl₂, 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA. Certain reactions contained 50 μM dNTPs, 3 mM ATP, and 100 μM PP₃, as specified. For reactions with 3 mM ATP, an additional 3 mM MgCl₂ was added. The reaction products were separated by PAGE and visualized and quantitated by Storm PhosphorImager and ImageQuant software version 1.2 (GE Healthcare). The primers used were: P1, 5’-TACGATTTAGGATGACACTATAG-3’; O-A, 5’-ACACTATAGAATATGCATCACTAGTAAGCTTCAGG-TGTTGCTCGC-3’, O-B, 5’-GGATGATGTCTGCAGGCGCTCT-CTA-3’; O-C, 5’-CCCTGTTCGGGCGCCCAAACGTGTA-3’, O-D, 5’-CCCTGTTCGGGCGCCCAAACGTGTA-3’, O-E, 5’-GGATCTGAACAGACAAACTAGAGACA-3’.

RESULTS

In a previous study we showed that during primer extension the RT created a series of prominent cleavages on the EIAV RNA template T1 before the base of the major hairpin in this substrate. The positioning of these cuts correlated with cuts produced by the RT in the absence of synthesis (no dNTPs) using a primer positioned at the base of the hairpin. Considered together, the data indicated that the RT generated 3’ end-directed primary and secondary cuts at this major pause site during primer extension. This study further defines the generation and consequences of the prominent cleavages created during primer extension on this template.

Paused Intermediates of Primer Extension That Persisted Throughout the Reaction Correlated with Secondary Cuts at the Sites of Pausing—We wanted to determine whether all the prominent template cleavage bands observed during primer extension on the EIAV template T1 would correspond to pause related cuts. Fig. 1A shows the extension profile observed when synthesis on this template was monitored by use of a 5’ end-labeled primer. Extension of the P1 primer (22 nt) produced two completed products: extension product (EP) and self-priming product (SP). The extension product is 136 nt long and is generated by extension of the P1 primer to the end of the T1 template. The self-priming product is 194 nt long and is generated from a completed extension product that folds back onto itself and is extended an additional 58 nt using the remaining 5’ region of cDNA as a template for further synthesis (see schematic in Fig. 1A). After 1 min of extension, stalling of the RT at the base of the major hairpin produced a pause product at +58 (identified as A in the figure). At subsequent time points pausing at +68, +79, +93, and +120, identified as B, C, D, and E, respectively, were observed.

Use of a 5’ end-labeled template instead of a labeled primer allowed for the monitoring of template cleavage during primer extension (see lanes labeled P1 in Fig. 1B). As the primer was extended, regions closer to the 5’ label became hybrid and available for RT-mediated RNase H cleavage. This explains the template cleavage pattern that was observed: cleavage fragments shifted from long products at early time points to shorter products at later time points. The prominent cleavage bands created during primer extension (positioned at +93, +83, +80, +75, +68, +60, +50, and +27) are labeled and indicated by circles to the left of the bands. In addition to these cleavage bands, prominent cleavage bands representing products less than 20 nt long were observed. Because these bands correspond to cuts at the 5’ terminus, they were not considered in our analysis of cuts during extension.

To correlate pausing and cleavage, we designed a series of short DNA oligonucleotides (O-A, O-B, O-C, O-D, and O-E) complementary to the T1 RNA template with 3’ terminated positioned at pause sites A, B, C, D, and E, respectively (+58, +68, +79, +93, and +120). When these oligonucleotides are annealed to the T1 RNA template, the resulting substrates model extension intermediates stalled at pause sites during synthesis. Incubation of RT with these substrates in the absence of
HIV-1 RT RNase H Cleavage Mechanism

dNTPs allowed for the detection of 3′ end-directed primary and secondary cuts generated at the pause sites. The RNA T1 cleavage fragments generated using these pause site oligonucleotides in the absence of synthesis were compared with T1 cleavage bands observed during synthesis using the P1 primer. On the right side of the gel, cleavage fragments generated by RT in the absence of synthesis using the pause site oligonucleotides, O-A, O-B, O-C, O-D, and O-E, are shown. As reported in the previous study (49), primary and secondary cleavage directed by O-A generated bands that correlated with the major cleavage bands observed during P1 primer extension at +93 and +83, respectively. Similarly, cleavage fragments generated using the other pause site oligonucleotides in the absence of synthesis also corresponded to prominent cleavage bands generated during synthesis with primer P1. We designated the longest cleaved segment as the primary cleavage product. The shortest fragments from 3′ end-directed cuts were designated as secondary cleavage products. Using these criteria, pause site oligonucleotides O-B, O-C, and O-D generated 3′ end-directed primary and secondary cuts (O-B, +83 and +80; O-C, +75 and +68; O-D, +60 and +50) that could readily be correlated to prominent cleavage bands created during synthesis with P1. For O-E, the secondary cleavage band at +27 corresponded to a prominent fragment generated during primer extension, however, other products generated with O-E were not clearly discernable during primer extension.

From this analysis it became apparent that all the major cleavage bands (+93, +83, +80, +75, +68, +60, +50, and +27) observed during synthesis with the P1 primer correlated with cuts generated in the absence of synthesis at positions of pausing (using O-A, O-B, O-C, O-D, and O-E). The cleavage pattern of the template during synthesis with P1 changed as the reaction progressed. The intensity of some of the bands (+83, +68, +50, and +27) did not change with time, whereas others (+93, +80, +75, and +60) faded after the first time points. The fading of cleavage bands is expected, because as the primer is further extended, regions of RNA closer to the 5′ end become susceptible for RNase H cleavage by RT.

As an RNase H control reaction for the T1 template degradation analysis, after 10 min of synthesis using primer P1, the pause site oligonucleotide O-E was added. This addition chased the +83, +68, and +50 template cleavage fragments to shorter

FIGURE 1. RT-mediated primer extension and template degradation on the EIAV RNA template, T1. The reactions were sampled at the times indicated above the lanes. Lane L, 10-bp DNA ladder. A, primer extension using 5′ end-labeled P1 primer. Positions of pausing at +58, +68, +79, +93, and +120 nt are marked A, B, C, D, and E to the right of the gel. The starting primer (+22) and the completed synthesis products of extension (+136) and self-priming (+194) are indicated by P1, EP, and SP, respectively. B, degradation of T1 (radiolabeled at the 5′ end) during primer extension compared with 3′ end-directed cleavage at pause sites. Lanes marked P1 show the template T1 cleavage fragments generated by RT during primer extension using primer P1 (in the presence of dNTPs). Prominent cleavage bands at +93, +83, +80, +75, +68, +60, +50, and +27 are indicated to the left of the gel. Lanes marked O-A, O-B, O-C, O-D, and O-E show 3′ end-directed cuts generated by RT in the absence of synthesis (no dNTPs) using primers whose 3′ termini are positioned at pause sites A, B, C, D, and E, respectively. Open circles to the left of the gel (+93, +75, and +60) indicate bands observed during primer extension that correlate with primary cuts at pause sites. Closed circles (+83, +68, +50, +27) indicate bands generated during synthesis that correlate with secondary cuts at pause sites. The cleavage bands that persisted throughout the primer extension reaction that correlate with secondary cuts at pause sites A, C, D, and E are marked by a, c, d, and e, respectively, to the right of the gel. Lane C, control reaction containing all the components except RT incubated for 30 min. The position of pausing and corresponding 3′ end-directed primary and secondary cleavages are summarized in Fig. S1, included as supplemental data.
products (data not shown). This control reaction indicated that the RT-mediated RNase H activity in the reaction was sufficient to fully degrade any hybridized template. Evidently, the prominent cleavage bands that persisted throughout the reaction were indicative of fragments not available for further RNase H cleavage, presumably because the primers were not further extended.

3' End-directed Secondary Cuts Could Dissociate the Primer from the Template—Significantly, all of the major cleavage products that persisted throughout the reaction (+83, +68, +50, and +27, labeled a, c, d, and e to the right of the gel, Fig. 1B) corresponded to secondary cuts at pause sites (A, C, D, and E). Because these segments were not further degraded, we considered the possibility that they derived from cuts that led to the termination of synthesis. As shown in Fig. 2, the mechanism by which these cuts are generated could create a situation in which the RT cleavage inactivates further primer extension. Generation of the primary cut creates an ~18-bp hybrid region at the primer terminus, which is probably an adequate length to remain annealed at 37 °C. However, 3' end-directed secondary cuts further cleave the ~18-nt RNA fragment to generate an RNA segment with as little as a 5–10-nt overlap with the primer terminus. Given the positioning of the cuts we considered it likely that generation of 3' end-directed secondary cleavages could inactivate the primer for further extension and explain why those cleavage bands persisted until the end of the reaction.

3' End-directed Secondary Cuts Decreased Primer Extend-ability—Next, we designed an assay to determine whether generation of 3' end-directed cuts could inactivate the primer-template for further extension. To do this, we examined the relationship between cleavage of the RNA template and extension of a DNA primer. We constructed a short template system in which both the primer and template were 5' end-labeled as shown in Fig. 3A. We incubated the substrate with RT in the presence of Mg$^{2+}$ for increasing times. At each time point, two aliquots were taken; the reaction was quenched by the addition of termination dye in one aliquot and in the other aliquot dNTPs were added to allow the RT to extend the primer for an additional 5 min before addition of termination dye. The quenched reactions monitor cleavage of the RNA template over time. The extension reactions determine the amount of primer that can still be extended by RT. The extension reaction time of 5 min was chosen because it was sufficient to produce the maximal amount of extended primers in control reactions.

The quenched reactions that were used to monitor the extent of RNA cleavage are labeled quench and shown on the left of the gel in Fig. 3A. After 1 min of incubation with RT and Mg$^{2+}$ most of the RNA template was cleaved to make the primary cleavage product (+28 to +25), indicated as T-1° in the figure. With increasing times, the amount

FIGURE 2. Synthesis inactivation by RNase H 3' end-directed primary and secondary cleavages. 3' end-directed primary cuts generate an RNA template fragment with ~18-nt overlap with the primer terminus. 3' End-directed secondary cuts further cleave the RNA template at the terminus to generate RNA fragments with as little as ~8 nt overlap with the primer terminus. The positioning of these cuts lead to dissociation of the template from the primer terminus and inactivation of subsequent synthesis.

FIGURE 3. Examination of relationship between RNase H cleavage of the template and primer extend-ability. A, 30-nt DNA primer P2 and 41-nt RNA template T2, both radiolabeled at the 5' end (as indicated by a star), were annealed together to form the substrate for the assay as shown above. The substrate was incubated with RT and Mg$^{2+}$ and at 1, 3, 6, 10, and 15 min, as indicated above the lanes, two aliquots were taken. To the aliquot labeled quench, termination dye was added. To the other aliquot, labeled extend, dNTPs were added for 5 min before being quenched by addition of termination dye. Positions of primary (+28 to +25) and secondary (+21 to +19) cleavage directed from the unextended primer are indicated by T-1° and T-2°, respectively. Extension of the primer (P) to the end of the template created a product 36-nt long, labeled P$_{ext}$. Lane TC, template control reaction in which 2 units of E. coli RNase H was added to the substrate. Lane C, starting material of the reaction. Primer extension reactions without preincubation with RT and Mg$^{2+}$, labeled Control Ext., are shown on the right on the gel. The times labeled above the control extension lanes indicate the duration of the primer extension reactions. Lane L, 10-bp DNA ladder. Lane numbers are marked below the gel. B, quantitation of the species present in the gel from A with: T, uncut template (filled diamonds), T-1°, primary cut (filled circles), T-2°, secondary cut (filled triangles), P$_{ext}$, extended primer (open circles), and P, unextended primer (open squares). Cleavage bands were quantitated from quench reactions and extension bands were quantitated from extend reactions. Values were corrected for the maximum amount of substrate that could be cleaved or extended in control reactions as described under "Experimental Procedures." C, the relationship between secondary cleavages and unextended primer as determined by an average of three independent experiments. T-2°, secondary cut (filled triangles), and P, unextended primer (open squares).
of uncut RNA template slightly decreased, but the major change in the cleavage pattern came from processing of the primary cleavage product to the secondary cleavage product (+21 to +19), indicated as T-2' in the figure.

Reactions that were used to monitor primer extension are labeled extend and shown to the right of the quench reactions in Fig. 3A. Extension of the 30-nt starting primer (P) to the end of the template resulted in a synthesis product of 36 nt (P$_{ex}$). The amount of primer that was extended after 1 min of preincubation with RT and Mg$^{2+}$ was slightly reduced compared with the control sample with no preincubation step (compare lane 8 to lane 14). With increasing time of preincubation with RT and Mg$^{2+}$, the amount of P$_{ex}$ decreased (lanes 8–12). When a DNA template was substituted for the RNA template, preincubation with RT and Mg$^{2+}$ did not reduce the amount of P$_{ex}$ (data not shown). This control reaction confirmed that the polymerase activity of RT.

Quantitation of the data obtained from Fig. 3A is shown in Fig. 3B. Although equimolar amounts of the primer and template were added, there was excess primer and template that did not anneal to each other. To correct for the amount of annealed substrate, the amounts of cleavage and extension were normalized as described under “Experimental Procedures.” Fig. 3B shows the distribution of primer and template species with increasing duration of preincubation with RT and Mg$^{2+}$ (as plotted on the x axis). The graph indicates that as the duration of the preincubation with RT and Mg$^{2+}$ was lengthened, the amount of primer that could be extended decreased. Fig. 3C highlights the relationship between the generation of the secondary cleavage product and the amount of unextended primer. These results show that as secondary cuts were generated, primer extendibility decreased proportionally. This experiment provides evidence that indeed 3' end-directed secondary cuts can inactivate further primer extension, presumably by dissociation of the primer from the template.

It seemed unlikely that the virus would have evolved to have such a high level of synthesis-related inactivation as observed in our primer extension reactions with T1. We considered the possibility that synthesis in vivo is more efficient than our in vitro reactions. We decided to try to improve the efficiency of our primer extension reactions in vitro to examine the effect such improvement would have on secondary cuts.

Increasing the Concentration of dNTPs Reduced Pausing and Pause Related Secondary Cuts—To facilitate more efficient synthesis on the EIAV template, we increased the concentration of dNTPs. Fig. 4A shows primer extension reactions with 10, 50, 150, 300, and 500 µM dNTPs. Pausing at A, B, C, D, and E is indicated to the left of the gel. As the concentration of dNTPs was increased from 10 to 500 µM, the amount of pausing at the base of the major hairpin (A) decreased substantially. Moreover, the intensity of pause sites B, C, D, and E also decreased, especially when compared with the amount of completed products generated. The ratio of primers stalled at pause site A compared with completed products (EP + SP) decreased nearly 30-fold compared with 10 to 500 µM reactions. Significantly, primers that stalled at pause sites using standard reaction conditions (50 µM dNTPs) were effectively chased to longer products at 500 µM dNTPs.

Next, we examined the RNase H cleavage pattern of the EIAV template during primer extension at increasing concentrations of dNTPs (Fig. 4B). At 10 and 50 µM dNTPs, prominent cleavage bands that persisted throughout the reaction were observed. These bands, positioned at +83, +68, +50, and +27 (marked a, c, d, and e to the right of the gel in Fig. 4B), correlated with secondary cleavages from pause sites at A, C, D, and E, as previously shown in Fig. 1. However, when dNTP concentrations were increased the intensity of these cleavage products diminished, especially when compared with the terminal cleavage fragments of less than 20 nt. For example, the ratio of cleavage fragment “a” to terminal cleavages decreased over 2-fold when comparing reactions at 50 and 500 µM dNTPs. These data indicated that as the efficiency of synthesis through pause sites improved, less secondary cuts were produced. Combined with the disappearance of synthesis intermediates, these results suggested that a consequence of more efficient synthesis was less primer-template dissociation at pause sites.

NC Reduced Pausing and Pause Related Secondary Cuts—Synthesis is likely to be more efficient in the cellular environ-
HIV-1 RT RNase H Cleavage Mechanism

To obtain additional information on the effects of NC, we examined primer extension during a single RT binding event by the addition of an RT trap (Fig. 5B). Under these conditions NC enhanced synthesis through pause sites. This was most evident when the intensity of completed products and paused products at the major pause sites (A, B, C, D, and E) were compared. Addition of NC reduced the ratio of pause product A to completed product (EP) 5-fold. A parallel effect was also observed at pause sites B, C, D, and E. Similar to what was observed in the absence of trap, NC increased the number of primers that initiated synthesis (≈2-fold) as indicated by control reactions shown on the right of the gel.

Next, we examined the effect of NC on RNase H cleavage of the template during primer extension (Fig. 5C). Control reactions in which E. coli RNase H was incubated with untreated and NC-treated substrates are shown on the right of the gel. In these control reactions, the amount of template that remained uncleaved was much less in NC-treated substrates compared with untreated substrates. This indicated that NC enhanced annealing of the primer and template, in agreement with the primer extension controls shown in A and B of this figure.

Time course reactions examining template cleavage during synthesis in the absence and presence of NC are shown on the left of the gel (Fig. 5C). In agreement with control reactions, which indicated that addition of NC improved annealing of the primer to the template, the amount of template that remained uncleaved in the time course extension reactions was greatly

\[ \text{FIGURE 5. Effect of NC on primer extension and template degradation using RNA template T1 and P1 primer.} \]  

The reactions were performed in the presence and absence of NC and sampled at 1, 3, 5, 10, 20, and 30 min, as indicated above the lanes. Lane L, 10-bp DNA ladder. A, primer extension by RT. Positions of pausing at +58, +68, +79, +93, and +120 nt, marked A, B, C, D, and E, respectively, to the left of the gel. The starting primer (+22) and the completed synthesis products of extension (+136) and self-priming (+194) are indicated by P1, EP, and SP, respectively. Extension controls, with ddCTP substituted for dCTP, are marked –C and +C, for reactions without and with NC, respectively. B, primer extension by RT during a single binding event. Extension controls –C and +C, are the same as described in A, except in the presence of trap. The trap control reaction, indicated by TC, was carried out with the same conditions as the extension reactions except that trap was added prior to the addition of RT. C, degradation of the RNA template T1 during primer extension. Persistent cleavage bands observed during primer extension (+83, +68, +50, and +27) that correlate with secondary cleavage bands from pause sites A, C, D, and E are indicated to the left of the gel by a, c, d, and e, respectively. Substrates treated with 2 units of E. coli RNase H for 10 min in the absence and presence of NC are labeled –C and +C, respectively. Lane C, starting material of the reaction.
HIV-1 RT RNase H Cleavage Mechanism

FIGURE 6. Examination of 3' end-directed primary and secondary cleavage using primers with dT (P3, indicated by arrowhead) or AZT (P4, indicated by a circle) at the 3' terminus. DNA primers P3 and P4 and RNA template T3 were 5' end-labeled (indicated by a star) and P3/T3 and P4/T3 templates were annealed. The reaction was sampled at 1, 3, 6, 10, and 16 min as indicated above the lanes. Control lanes, labeled C in the figure, show the starting material. The position of the primer and extended primer are indicated by P and P<sub>ext</sub>, respectively, to the left of the gel. The position of the uncut template and products of primary and secondary cuts directed from the unextended primer are indicated by T, T-1°, and T-2°, respectively. Template cleavages directed by the extended primer are bracketed and labeled, as well. A, P3/T3, with 50 μM dNTP and 6 mM MgCl<sub>2</sub>; B, P3/T3, with 6 mM MgCl<sub>2</sub>; C, P4/T3, with 6 mM MgCl<sub>2</sub>; D, P4/T3, with 50 μM dNTPs, 6 mM MgCl<sub>2</sub>; E, P4/T3 with 3 mM ATP; F, P4/T3, with 100 μM PP<sub>i</sub>, 50 μM dNTPs, 6 mM MgCl<sub>2</sub>

reduced in the reactions with NC. Accordingly, in extension reactions the RT-mediated RNase H cleavage was much greater in NC-treated samples compared with untreated samples. This is reflected not only in the disappearance of starting material, but also in the intensity of the terminal cleavage bands (marked to the left of the gel).

In addition to the effect of increasing the amount of overall template degradation, NC decreased the generation and persistence of secondary cuts at pause sites. In the reaction without NC, the persistent secondary cuts (labeled as a, c, d, and e to the left of the gel) were observed. These secondary cleavage bands were much weaker in the reactions with NC. NC effects on secondary cuts were even more pronounced when considered relative to the total amount of degraded products. For example, upon addition of NC, the ratio of secondary cleavage fragment a (+83) compared with terminal cleavage fragments decreased over 5-fold. NC had similar effects on the ratio of other persistent secondary cleavage fragments to terminal cleavage fragments: c (+68) decreased 6-fold; d (+60) decreased 5-fold; e (+27) decreased 2-fold. Considered together, the NC experiments indicated that by improving synthesis through pause sites, NC decreased pause mediated secondary cleavage during synthesis.

RT Generated 3' End-directed Primary and Secondary Cuts with an AZT-terminated Primer—We also considered another situation in which RNase H-induced template inactivation would take place: the halting of synthesis by incorporation of a nucleoside analogue. Unlike the short pauses anticipated during synthesis in vivo, the pause associated with chain termination may be long enough for secondary cuts to occur at significant frequencies. To determine whether RT could make primary and secondary cuts at an AZT-terminated primer, we compared 3' end-directed cleavages on two substrates; one with a normal dT and one with an AZT group at the 3' terminus. These experiments used a substrate design similar to those shown in Fig. 3, in which both the primer and the template were radiolabeled at the 5' end. As shown in Fig. 6B, when the primer with a dT at the 3' terminus (P3) was incubated with RT and Mg<sup>2+</sup>, a series of 3' end-directed primary and secondary cleavages positioned around +37 and +27 were observed (labeled as T-1° and T-2°, respectively). A similar set of cuts was observed when an AZT group was positioned at the 3' terminus (P4) (Fig. 6C). These data indicated that the presence of an AZT group at the terminus did not inhibit RT-mediated primary and secondary cleavage in the presence of Mg<sup>2+</sup>.

Next, we examined these same cleavages under conditions that allowed the RT to utilize its polymerase active site for synthesis. Fig. 6A shows incubation of the dT-terminated substrate with RT in the presence of Mg<sup>2+</sup> and dNTPs. The starting primer was readily extended 19 nt to the end of the template generating P<sub>ext</sub> as indicated to the left of the gel. We detected only very faint template cleavage bands between +37 and +27 from primary and secondary cleavages directed by the unextended primer. Instead, a template cleavage band around +18 was observed that correlated with RT cleavage directed by the extended primer, P<sub>ext</sub>. Even at 1 min, when the +18 band was very faint, indicating that some primers were partly extended, bands at +37 and +27 were only barely detectable. This indicated that with dT at the terminus, the RT incorporated nucleotides onto the primer before allowing significant cleavage directed by the unextended primer to take place.

We then measured cleavage with the AZT-terminated primer (P4) under conditions that allowed excision and synthe-
**HIV-1 RT RNase H Cleavage Mechanism**

In the presence of Mg$^{2+}$ and dNTPs, the RT was not expected to efficiently excise the terminal AZT. As anticipated, the RT generated 3' end-directed primary and secondary cuts in a manner similar to conditions with only Mg$^{2+}$ (compare C and D in Fig. 6). The RT has been shown to excise the chain-terminating nucleotide and then extend the primer under conditions that allow for ATP or PP$_i$-mediated phosphorolysis (14, 17). In agreement with the literature findings, RT was much more efficient at PP$_i$-mediated, compared with ATP-mediated excision (compare P$_{ext}$ in Fig. 6, E and F). Surprisingly, in the presence of either ATP or PP$_i$, template cleavage bands between +37 and +27 generated from 3' end-directed cleavage at the AZT-terminated primer were observed. Apparently, in the presence of either ATP or PP$_i$, some RTs cleaved the template before excising the AZT at the terminus.

**DISCUSSION**

In this study we examined the relationship between HIV-1 RT pausing during synthesis and template cleavage using an RNA template containing a large, stable hairpin sequence derived from the primer-binding site of the EIAV genome (T1). To correlate pausing and cleavage, the template cleavage pattern produced during synthesis with P1 was compared with cleavage fragments generated by RT in the absence of synthesis using oligonucleotides O-A, O-B, O-C, O-D, and O-E, whose termini were positioned at pause sites A, B, C, D, and E, respectively (Fig. 1). From this analysis several intriguing features of the cleavage pattern emerged. First, all the prominent cleavage fragments observed during primer extension correlated with cleavage products produced at pause sites. This finding implies that pausing during synthesis plays a dominant role in the positioning of cuts during synthesis. Moreover, although the RT was unable to extend the primer, presumably because of the delaying effect of the hairpin structure, it could advance on this structure to make secondary cuts. This behavior resulted in the formation of primary and secondary cuts at the same positions that would occur if the RT could not progress with synthesis.

Second, the prominent cleavage fragments that were observed in these reactions produced bands that appeared early in the reaction and persisted throughout the remainder of the reaction. The persistence of any cleavage fragments is unexpected, because as the primer is elongated, regions closer to the 5' end label become hybrid and therefore susceptible to RNase H cleavage. Significantly, the cleavage fragments that persisted throughout the measured period of primer extension all correlated with RT-mediated secondary cuts at pause sites.

To establish the generality of our observations regarding pausing and cleavage on the T1 substrate, we re-examined cleavage on two other template systems. Previously, we described synthesis-related cleavages at the base of TAR in an HIV-1 template designated D199 (26) and pause associated cleavage on a non-viral RNA template called D1 (41). Using primers that were positioned at these pause sites, we found that HIV-1 RT generated primary and secondary cleavages in the absence of synthesis that correlated with the prominent cleavages observed during synthesis (data not shown). Moreover, the cuts that were identified as pause associated secondary cuts were not chased to smaller fragments, in agreement with our observations using T1. These findings indicate that the cleavage characteristics at pause sites seen with T1 apply more broadly to RNA template systems.

Consideration of the positioning of 3' end-directed cuts indicated a possible mechanism for synthesis inactivation by dissociation of the primer from the template (Fig. 2). This possibility was evaluated using the P2/T2 template system (Fig. 3). The experiment revealed that RT cleavage could inactivate the template for further extension. In fact, the generation of secondary cuts was almost exactly proportional to the decrease in primer extension (Fig. 3C). The striking proportionality of this relationship indicates that, within the limits of the experiment, creation of a secondary cut immediately inactivated the primer. The same relationship was not observed for the primary cut: 50% of the template was cleaved to form primary cut fragments within 1 min, and yet almost 90% of the primer could be extended (Fig. 3B).

Presumably, the correlation between generation of secondary cleavages and template inactivation is a consequence of primer-template dissociation. This explanation is consistent with the persistence of secondary cleavage bands to the end of the reaction in the experiments with T1, because termination of synthesis on templates with secondary cuts would not allow additional cleavages between the secondary cut site and the labeled template 5' terminus. The data also suggest that the presence of the bound RT did not stabilize the primer-template structure after the secondary cut was made. Moreover, the primer did not transiently equilibrate with unbound, cleaved template strands producing primer-template structures that could be captured by the RT for synthesis.

Whereas a significant portion of the templates appeared to be inactivated by this mechanism in our EIAV system, it seemed unlikely that the virus would have evolved to have such a high level of synthesis-related inactivation. The divergence of strand transfer distribution profiles measured in vitro and ex vivo led Galetto et al. (58) to speculate that reverse transcription in vivo is more processive than in vitro. We considered whether more efficient primer extension in vivo is the basis of synthesis without significant primer inactivation. To address this issue, we improved the efficiency of synthesis in vitro by increasing the concentration of dNTPs. High concentrations of dNTPs produced less pausing and pause related cuts (Fig. 4). In particular, there was a weakening of the persistent secondary cut bands that we attributed to reduced pausing resulting from improved strand displacement synthesis. These high dNTP concentrations were well above the estimated physiological concentration range of 50 nM to 5 μM determined for macrophage and T cells, respectively (59), but we speculate that they provided a means of simulating the natural synthetic rate of the RT.

Several studies have shown that NC reduces pausing and improves the efficiency of synthesis by RT (22–25, 60). In agreement with these studies, we found that addition of NC reduced pausing in our template system (Fig. 5A). To better examine the effects of NC on synthesis, we repeated the primer extension reactions in the presence of trap (Fig. 5B), which sequestered any RT molecules that dissociated from the primer-template. These experiments allowed two effects of NC to be recognized. First, because the ratio of the EP product to the product at pause...
site A increased substantially with NC, it is clear that NC improved the amount of synthesis that the RT could carry out through the pause site without dissociating from the substrate. Second, in the trapped NC reactions, a considerable number of primers were still stalled at pause site A. In the NC-treated untrapped reactions these primers were chased to longer products. This difference indicates that NC also stimulated synthesis through the pause site upon RT re-binding to the hairpin base.

The effects of NC on RNase H activity were found to be complex. We showed that addition of NC increased the amount of overall template cleavage (Fig. 5C). This was most clearly observed when comparing the amount of uncleaved template in reactions in the presence and absence of NC. However, control reactions in which the NC-treated and untreated substrates were incubated with E. coli RNase H indicated that NC improved annealing of the primer to the template and created more templates that were available for RNase H cleavage. This finding was in agreement with extension control reactions that also showed that NC increased the amount of primers that were extended (Fig. 5, A and B). Together, these control reactions indicated that the greater RNase H cleavage observed upon addition of NC could be attributed almost entirely to improved annealing of substrates, rather than enhancement of RT RNase H activity, per se. Other studies from our laboratory have demonstrated similar enhancements of template cleavage with the addition of NC (27, 31), however, without the extension and cleavage controls employed here it was difficult to distinguish between the two possible scenarios that could explain these effects. Given the data presented here, we suggest that much of the improved RNase H cleavage observed with NC in vitro is the result of improved annealing rather than a direct enhancement of RT RNase H activity by NC. Based on our current results, we suggest that NC stimulation of RNase H activity be re-examined in the future using RNA templates with stably bound primers.

Beyond the effects of NC on overall template cleavage, with NC we observed substantially less of the persistent cleavage products a, c, d, and e that corresponded to secondary cleavage fragments generated from pause sites A, C, D, and E, respectively (Fig. 5C). We interpret this effect of NC to be indirect in that the reduction of secondary cleavage products was likely the result of reduced pausing rather than a direct effect of NC inhibition of 3′-end-directed secondary cuts. Several lines of evidence support these conclusions. First, the primer extension reactions indicated that NC enhanced synthesis through pause sites. Second, because the rate of secondary cleavage is much slower than primary cleavage and likely requires a reorientation of the RT on the primer terminus, the shortening of pauses is predicted to affect secondary cuts preferentially. Third, 3′-end-directed secondary cuts in the absence of synthesis were not diminished by the addition of NC (data not shown). Considered together, the results presented here suggest a novel role for NC in viral replication. The ability of NC to improve RT synthesis through structured regions reduces pausing and prevents the generation of 3′-end-directed secondary cuts that could be detrimental to viral replication by causing primer-template dissociation. This role complements other roles for NC in the enhancement of viral replication (reviewed in Refs. 18 and 61).

The role for NC in the prevention of 3′-end-directed secondary cuts at pause sites reported here seemingly contrasts with previous studies showing that addition of NC improved secondary cuts on substrates with blunt ends (26, 29 –31). However, blunt ends form a unique substrate in which the roles of the DNA 3′-end and the RNA 5′-end in orienting the RT have not been defined. Moreover, the influences of NC on RT binding at the end of an RNA-DNA hybrid have not been explored. NC effects on hybrid annealing might alter RT affinity for and movement on the hybrid. Alternatively, NC may stabilize RT binding for secondary cuts at a blunt end by a direct interaction. In support of the latter, several groups have reported evidence for such an interaction between NC and RT (28, 29, 62, 63). These phenomena are likely to be unrelated to the acceleration of strand displacement synthesis by NC, a process that leaves less time for pause related secondary cuts.

Our studies suggested that RNase H cleavage-mediated template inactivation is suppressed in vivo by NC. We wondered whether there are other conditions in vivo in which cleavage-mediated template inactivation plays an important role. We considered the situation when synthesis is stalled by the presence of a nucleotide analog at the primer terminus. We report here that RT generated 3′-end-directed cleavages with an AZT-terminated primer in the presence of Mg2+ (Fig. 6). The presence of the AZT group did not influence the positioning of the cuts as the pattern produced was the same as when a primer with dT at the terminus was used. Furthermore, when PPi or ATP was added to the reaction, some RTs cleaved the template rather than excised and extended the terminus. Because these 3′-end-directed primary and secondary cleavages can inactivate primer extension by a dissociation mechanism, the observation that RT makes these cleavages is consistent with the idea that there is a balance between RNase H cleavage and AZT incorporation, excision, and resumption of DNA synthesis as suggested by Nikolenko et al. (64). The potential contribution of RNase H cleavage to the therapeutic effects of AZT is not yet clear and further studies are needed to evaluate it.

Previously, we examined strand transfer on the template T1 used in this study (27, 42, 49). Our studies revealed a multistep mechanism for transfer in which the initial interaction of the acceptor with the cDNA occurred at the base of the major hairpin (pause site A) and transfer of most primer termini from the donor to the acceptor occurred in the stem-loop region of the hairpin (between pause sites C and E) (42). More recently, we investigated the RNase H cleavages responsible for creation of the invasion site, the region of cDNA accessible for acceptor interaction due to local clearing of the donor template. We identified cleavages of the donor template that were generated by 3′-end-directed primary and secondary cuts mediated by RT, while stalled at the base of the major hairpin and suggested that they were responsible for creating the invasion site for transfer (49). Our current findings indicate that 3′-end-directed secondary cuts promote dissociation of the primer from the template. If secondary cuts at the hairpin base mediated transfers, then the majority of primers would begin acceptor-template synthesis at the beginning of the hairpin. However, transfer distri-
HIV-1 RT RNase H Cleavage Mechanism

FIGURE 7. RT positioning for DNA 3' and RNA 5’ end-directed primary and secondary RNase H cuts. RT is illustrated as a rectangle with the polymerase (pol) and RNase H active sites labeled.

FIGURE 7.

DNA 3’ End-Directed  RNA 5’ End-Directed
Primary cleavage  
Secondary cleavage  
RNA 5’ End-Directed  RNA 3’ End-Directed
Primary cleavage  
Secondary cleavage

FIGURE 7.

HIV-1 RT RNase H Cleavage Mechanism

...ments small enough to allow rapid dissociation from the cDNA. This indicates a need for polymerization-independent cuts (not directed by the DNA 3’ end) to complete RNA template removal in preparation for plus strand synthesis. Second, the 5’ end-directed primary cut creates a template fragment with ~18 nt of overlap with the cDNA, a length probably insufficient to dissociate the fragment from the cDNA. This indicates a need for the 5’ end-directed secondary cut to further degrade the template to create fragments small enough to allow rapid dissociation from the cDNA. Considering these points, we think it likely that the ability of RT to generate both primary and secondary cleavages directed by the RNA 5’ end is important for clearing the cDNA for plus strand synthesis.

To generate RNA 5’ end-directed secondary cuts the RNase H active site of RT must be positioned ~8 nt from the 5’ end of the template. This would require RT to bind in an orientation ~10 nt closer to the 5’ end of the template relative to the positioning for the primary cut. A similar binding orientation is required for DNA 3’ end-directed secondary cuts (Fig. 7). For both 3’ and 5’ end-directed secondary cuts, the RNase H active site contacts the DNA and RNA strands in the same manner. The only difference is that the linear anchoring of the polymerase active site of RT is done by a DNA 3’ end in one case and an RNA 5’ end in the other. We propose that both secondary cuts are manifestations of the same mechanism and that the detrimental 3’ end-directed secondary cuts arise from the requirement for 5’ end-directed secondary cuts. If this interpretation is correct, the ability of NC to enhance synthesis through pause sites, the rapid primer extension rate proposed to occur in vivo, and other factors in the replication environment in vivo are critical. These characteristics of the replication system would have evolved to suppress generation of cuts that would otherwise be detrimental to viral replication.

Acknowledgments—We thank Drs. Mark Hanson and Mini Balakrishnan for helpful discussions and Min Song, Lu Gao, and Sean Rigby for critical reading of the manuscript.

REFERENCES

1. Telesnitsky, A., and Goff, S. P. (1997) in Retroviruses (Coffin, J. M., Hughes, S. H., and Varmus, H. E., eds) pp. 121–160, Cold Spring Harbor Press, Plainview, NY.
2. Furfine, E. S., and Reardon, J. E. (1991) J. Biol. Chem. 266, 406–412.
3. Gopalakrishnan, V., Peliska, J. A., and Benkovic, S. J. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 10763–10767.
4. Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1992) Science 256, 1763–1790.
5. Sarafianos, S. G., Das, K. Tanillo, C., Clark, A. D., Jr., Ding, J., Whitcomb, J. M., Boyer, P. L., Hughes, S. H., and Arnold, E. (2001) EMBO J. 20, 1449–1461.
6. Kati, V. M., Johnson, K. A., Jerva, L. F., and Anderson, K. S. (1992) J. Biol. Chem. 267, 25988–25997.
7. DeSantos, J. J., Biuser, R. G., Mallaber, L. M., Myers, T. W., Bambara, R. A., and Fay, P. J. (1991) J. Biol. Chem. 266, 7423–7431.
8. DeSantos, J. J., Mallabar, L. M., Fay, P. J., and Bambara, R. A. (1993) Nucleic Acids Res. 21, 4330–4338.
9. Palaniappan, C., Fuentes, G. M., Rodriguez-Rodriguez, L., Fay, P. J., and Bambara, R. A. (1996) J. Biol. Chem. 271, 2063–2070.
10. Wisniewski, M., Balakrishnan, M., Palaniappan, C., Fay, P. J., and Bambara, R. A. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 11978–11983.
11. Schultz, S. J., Zhang, M., and Champoux, J. J. (2004) J. Mol. Biol. 344, 635–652.
12. Eminni, E. A., and Fan, H. Y. (1997) in Retroviruses, (Coffin, J. M., Hughes, S. H., and Varmus, H. E., eds) pp. 637–708, Cold Spring Harbor Laboratory Press, Plainview, NY.
13. Larder, B. A., and Kemp, S. D. (1989) Science 246, 1155–1158.
14. Arion, D., Kaushik, N., McCormick, S., Borkow, G., and Parniak, M. A. (1998) Biochemistry 37, 15908–15917.
15. Boyer, P. L., Sarafianos, S. G., Arnold, E., and Hughes, S. H. (2001) J. Virol. 75, 4832–4842.
16. Meyer, P. R., Matsuura, S. E., Schinazi, R. F., So, A. G., and Scott, W. A. (2000) Antimicrob. Agents Chemother. 44, 3465–3472.
17. Meyer, P. R., Matsuura, S. E., So, A. G., and Scott, W. A. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 13471–13476.
18. Levin, J. G., Guo, J., Rouzina, I., and Musier-Forsyth, K. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 217–286.
19. Rein, A., Henderson, L. E., and Levin, J. G. (1998) Trends Biochem. Sci. 23, 297–301.
20. Tsuchihashi, Z., and Brown, P. O. (1994) J. Virol. 68, 5863–5870.
21. You, J. C., and McHenry, C. S. (1994) J. Biol. Chem. 269, 31491–31495.
22. Drummond, J. E., Mounts, P., Gorelick, R. J., Cassas-Finet, J. R., Bosche, W. J., Henderson, L. E., Waters, D. J., and Arthur, L. O. (1997) AIDS Res. Hum. Retroviruses 13, 533–543.
23. Li, X., Klarman, G. J., and Preston, B. D. (1996) Biochemistry 35, 132–143.
24. Klages, B. I., Huthoff, H. T., Das, A. T., Jeeninga, R. E., and Berkhout, B. (1999) Biochim. Biophys. Acta 1444, 355–370.
25. Wu, W., Henderson, L. E., Copeland, T. D., Gorelick, R. J., Bosche, W. J., Rein, A., and Levin, J. G. (1996) J. Virol. 70, 7132–7142.
26. Chen, Y., Balakrishnan, M., Roques, B. P., and Bambara, R. A. (2003) J. Biol. Chem. 278, 38368–38375.
27. Roda, R. H., Balakrishnan, M., Hanson, M. N., Wohlr, B. M., Le Grice, S. F., Roques, B. P., Gorelick, R. J., and Bambara, R. A. (2003) J. Biol. Chem. 278, 31536–31546.
28. Cameron, C. E., Ghosh, M., Le Grice, S. F., and Benkovic, S. J. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 6700–6705.
29. Peliska, J. A., Balasubramanian, S., Giedroc, D. P., and Benkovic, S. J. (1994)
Biochemistry 33, 13817–13823
30. Chen, Y., Balakrishnan, M., Roques, B. P., Fay, P. J., and Bambara, R. A. (2003) J. Biol. Chem. 278, 8006–8017
31. Wisniewski, M., Chen, Y., Balakrishnan, M., Palaniappan, C., Roques, B. P., Fay, P. J., and Bambara, R. A. (2002) J. Biol. Chem. 277, 28400–28410
32. Clavel, F., Hoggan, M. D., Willey, R. L., Strebelt, K., Martin, M. A., and Repaske, R. (1989) J. Virol. 63, 1455–1459
33. Goodrich, D. W., and Duesberg, P. H. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2052–2056
34. Hu, W. S., and Temin, H. M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1556–1560
35. Negroni, M., and Buc, H. (2001) Annu. Rev. Genet 35, 275–302
36. DeStefano, J. J., Mallaber, L. M., Rodriguez-Rodriguez, L., Fay, P. J., and Bambara, R. A. (1992) J. Virol. 66, 6370–6378
37. Smith, C. M., Smith, J. S., and Roth, M. J. (1999) J. Virol. 73, 6573–6581
38. DeStefano, J. J. (1995) Arch. Virol. 140, 1775–1789
39. Negroni, M., and Buc, H. (1999) J. Mol. Biol. 286, 15–31
40. Rodriguez-Rodriguez, L., Tsuchihashi, Z., Fuentes, G. M., Bambara, R. A., and Fay, P. J. (1995) J. Biol. Chem. 270, 15005–15011
41. Hanson, M. N., Balakrishnan, M., Roques, B. P., and Bambara, R. A. (2005) J. Mol. Biol. 353, 772–787
42. Roda, R. H., Balakrishnan, M., Kim, J. K., Roques, B. P., Fay, P. J., and Bambara, R. A. (2005) J. Biol. Chem. 277, 46900–46911
43. Wu, W., Blumberg, B. M., Fay, P. J., and Bambara, R. A. (1995) J. Biol. Chem. 270, 325–332
44. Lanciault, C., and Champoux, J. J. (2006) J. Virol. 80, 2483–2494
45. Balakrishnan, M., Fay, P. J., and Bambara, R. A. (2001) J. Biol. Chem. 276, 36482–36492
46. Derebail, S. S., and DeStefano, J. J. (2004) J. Biol. Chem. 279, 47446–47454
47. Balakrishnan, M., Roques, B. P., Fay, P. J., and Bambara, R. A. (2003) J. Virol. 77, 4710–4721
48. Negroni, M., and Buc, H. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6385–6390
49. Purohit, V., Balakrishnan, M., Kim, B., and Bambara, R. A. (2005) J. Biol. Chem. 280, 40534–40543
50. Suo, Z., and Johnson, K. A. (1997) Biochemistry 36, 12468–12476
51. Archer, R. H., Wisniewski, M., Chen, Y., Balakrishnan, M., Palaniappan, C., Roques, B. P., Fay, P. J., and Bambara, R. A. (2002) J. Biol. Chem. 277, 8390–8401
52. Archer, R. H., Wisniewski, M., Bambara, R. A., and Demeter, L. M. (2000) J. Biol. Chem. 274, 4087–4095
53. Ghosh, M., Howard, K. J., Cameron, C. E., Benkovic, S. J., Hughes, S. H., and Le Grice, S. F. (1995) J. Biol. Chem. 270, 801–818
54. Galetto, R., Moulen, A., Giacomoni, V., Veron, M., Charneau, P., and Negroni, M. (2004) EMBO J. 9, 1171–1176
55. Gotte, M., Arion, D., Parniak, M. A., and Wainberg, M. A. (2000) J. Virol. 74, 3579–3585
56. Druillennec, S., Caneparo, A., de Rocquigny, H., and Roques, B. P. (1999) J. Biol. Chem. 274, 11283–11288
57. Lener, D., Tanchou, V., Roques, B. P., Le Grice, S. F., and Darlix, J. L. (1998) J. Biol. Chem. 273, 33781–33786
58. Nikolenko, G. N., Palmer, S., Malardelli, F., Mellors, J. W., Coffin, J. M., and Pathak, V. K. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 2093–2098