Processing of an HIV Replication Intermediate by the Human DNA Replication Enzyme FEN1*

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Jeffrey A. Rumbaugh‡, Gloria M. Fuentes, and Robert A. Bambara‡‡

From the Departments of Biochemistry and Microbiology and the Cancer Center, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

The role of human FEN1 (flap endonuclease-1), an RTH1 (RAD two homolog-1) class nuclease, in the replication of human immunodeficiency virus (HIV) type 1 has been examined using model substrates. FEN1 is able to endonucleolytically cleave a primer annealed to a template, but with a 5'-unannealed tail. The HIV (+)-strand is synthesized as two discontinuous segments, with the upstream segment displacing the downstream segment to form a central (+)-strand overlap. Given a substrate with the exact HIV nucleotide sequence, FEN1 was able to remove the overlap. After extension of the upstream primer with DNA polymerase ε, human DNA ligase I was able to complete the continuous double strand as would occur for an integrated provirus. FEN1 may represent a target for new therapeutic interventions.

The multifunctional, virally encoded reverse transcriptase can perform all the DNA synthesis steps required in the general model of retroviral replication (1). In this model, (+)-strand synthesis is initiated from a single RNA polypurine tract primer derived from a location near the 3'-end of the viral genome (3'-PPT). However, in many retroviruses including human immunodeficiency virus (HIV), the causative agent of AIDS, there is also a perfect copy of the polypurine tract sequence present in a central position (cPPT) (2–4). The polypurine tract is believed to resist reverse transcriptase-directed RNase H action and to carry out a priming function because its sequence imparts a structure more like a DNA-DNA helix than an RNA-DNA helix (5). Having the same sequence as the 3'-PPT, the cPPT should be a second site for (+)-strand initiation (6, 7). After a step called the second strand transfer, the 3'-PPT-initiated segment is extended from upstream of the cPPT segment. This upstream segment displaces the downstream strand 88–98 nucleotides and then terminates, with reverse transcriptase dissociation mediated via a cis-acting central termination sequence (CTS) (8–11). Viral DNA with this overlap structure is capable of integration, but it is not found following transport to the nucleus, suggesting that it is rapidly repaired by normal cellular mechanisms (12). Mutation of either the cPPT or the CTS (8–10) profoundly impairs HIV replication and infectivity, even when the overlaid amino acid sequence is preserved. Thus, central initiation and termination, and preservation of the overlap, are critical steps in the HIV life cycle, yet reverse transcriptase lacks the DNA cleavage activity necessary to perform this repair (1). As expected, we failed to observe specific reverse transcriptase-directed cleavage of the overlap substrate (data not shown).

The retroviral integrase, a component of the preintegration complex, is another virus-encoded protein that might repair the overlap. It has been shown to cleave single stranded flap structures (13, 14). The enzymatic activity is mediated through a one-step transesterification reaction and is referred to as a “DNA splicing” activity because of its similarity to RNA splicing. Although integrase activity has been observed with single-stranded flaps that mimic the terminal HIV-1 LTR sequences, there are no reported data that this enzyme can cleave the flaps generated during the central termination process.

Host enzymes have been shown to play a role in HIV infection; but often these factors have not been specifically identified, and none have been shown to participate in the replication process (15–17). For example, cellular RNase H cannot substitute for reverse transcriptase-associated RNase H (18). Identification of the cellular factors involved in central (+)-strand processing might guide new therapeutic efforts. FEN1 (flap endonuclease-1) is a likely candidate to process the overlap region because of its substrate specificity. As reviewed by Bambara et al. (19), FEN1 removes initiator RNA from Okazaki fragments during lagging strand DNA synthesis in human cells. It may also participate in DNA repair because it can traverse and remove adducts and abasic sites (20–22). FEN1 has both exonucleolytic and endonucleolytic activities and can cleave both RNA and DNA (23–28). To make an endonucleolytic cut, it must recognize the 5'-end of an unannealed tail, apparently sliding along the single strand to the point of cleavage, near the position of annealing (20). The enzyme also often requires an upstream primer immediately adjacent to the site of cleavage (23). The HIV overlap region has all of these structural characteristics. Since FEN1 is essential (29, 30), it will be difficult to study the effect of mutations or knockouts on HIV replication due to an overlying effect on cell viability. We therefore chose to first study the role of FEN1 in HIV replication in vitro, with the expectation that the resulting information would indicate the potential value of further analysis in vivo.

Although the HIV overlap has many of the structural characteristics required by FEN1, its sequence suggests the presence of at least two stem loops. It was unclear whether FEN1 could traverse these stem loops because it is inhibited by primers that anneal to form double-stranded regions on the tail flap (20). Additionally, the region clearly has an atypical structure since it promotes dissociation of the reverse transcriptase at
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The CTS after the limited displacement synthesis that creates the overlap. The overlap region contains several oligo(dA) tracts, which, along with the stem-loop structures, could create bends in the DNA, resulting in a unique structure (11). This structure might generally exclude protein-DNA interactions, including binding of FEN1. In this study, the ability of FEN1 to process the HIV overlap was examined using a substrate that models the replication intermediate that would occur following central termination. We provide biochemical evidence that the human DNA replication enzyme FEN1 removes the overlap created by synthesis from both the 3'-PPT and the cPPT.

EXPERIMENTAL PROCEDURES

Materials—Unlabeled nucleotides were purchased from Amersham Pharmacia Biotech, and radiolabeled nucleotides (3000 mCi/mmol) were from NEN Life Science Products. Oligonucleotides were synthesized by Genosys Biotechnologies, Inc. (The Woodlands, TX). T4 polynucleotide kinase and Sequenase (Version 2.0) were obtained from U. S. Biochemical Corp. T4 DNA ligase, calf intestinal phosphatase, RNase inhibitor, and snake venom phosphodiesterase were from Boehringer Mannheim. All other reagents were from Sigma.

Enzyme Purification—Recombinant human FEN1 was purified as described (31, 32). The final preparation was eluted by SDS-polyacrylamide gel electrophoresis and silver staining. The enzyme was greater than 70 °C. Recombinant human replication protein A (RPA) was purified as described (32). DNA polymerase ε (33) and recombinant human DNA ligase 1 (34) were purified as described. The ligase preparation had a specific activity of 5,000,000 units/mg, with 1 unit defined as the amount of protein that ligates 20 fmol of linear nicked oligonucleotide substrate in 5 min at 37 °C.

Substrates—The HIV overlap substrate was made from the following oligomers: template 1, 3'-AGCGGTCCAAGAGCACGTCTGGCC-CAATAAATGTTCCGTGCTTGGTTAGA; upstream primer 1a, 5'-TCCG-CCAGGGTTTCTCCGTCAGGAC; downstream primer 1a, 5'-ATTGGGGGTACAGTGCAGGGGAAAGAATAGTAGACATAATAGCAACAG-AATTGG-9; downstream primer 1b, 5'-TTGAAAATTTTCCGCTTATACAGGACAGCAGAATCC. Downstream primers 1a and 1b were 5'-phosphorylated using T4 polynucleotide kinase and ATP. Downstream primer 1b was radiolabeled by addition of a single nucleotide at the 3' end. upstream primer 1b, producing downstream primer 1, which is the (+)-strand sequence from nucleotides 4380 to 4507 of the HIV provirus (2). Bulk ligation was done by T4 DNA ligase using linker template 2 (3'-AATGGTTTTATGTTTTAAGTTTTAAAAGCCC-AAAAAATA) in 60 mM Bis-Tris (pH 7.0), 0.5% glycerol, 0.1 mg/ml bovine serum albumin, 5 mM β-mercaptoethanol, 10 mM MgCl2, 10 mM ATP, 20 pmol of each oligomer, and 10 units of enzyme in a final volume of 250 μl at 37 °C overnight. Ligated downstream primer 1 was isolated via 5% denaturing gel. The complete overlapping HIV substrate was constructed by annealing upstream primer 1 and downstream primer 1 to template 1. Upstream primer 1 fully anneals to template 1 and forms a nick with the first annealed nucleotide of downstream primer 1. The underlined sequence of downstream primer 1 above represents the region annealed to template 1, whereas the remainder forms a 5'-unannealed tail. Boldface nucleotides signify areas in the tail that could anneal to each other to form stable secondary structure.

The unannealed flap is 98 nucleotides long, representing the distance from the cPPT to the major site of termination in the center of the genome. Pause sites prior to the 98-nucleotide site, particularly one at 88 nucleotides, are relatively easily overrun, suggesting that they simply reduce enzyme efficiency as it approaches the final stop site (8–11). In the event reverse transcriptase ever did dissociate after forming a shorter tail, which would have even less secondary structure, FEN1 could still act since it can navigate the entire 98-nucleotide tail. Following DNA initiation, the cPPT is expected to be removed quickly by RNase H action (1), so we have not included it in our substrate. However, since FEN1 can traverse RNA (28), its presence should not affect cleavage.

Enzyme Assays—Assays of cleavage by FEN1 were performed under the same conditions as described for bulk ligation except with no ATP, using 10 fmol of substrate in a volume of 25 μl/reaction. Reactions were initiated by addition of 15 ng of FEN1/reaction and stopped at the appropriate times with an equal volume of 98% formamide, 10 mM EDTA (pH 8.0), and 0.01% (w/v) each xylene cyanol and bromphenol blue and heating at 95 °C for 5 min. Zero time controls were removed from the mixture before addition of enzyme. Products were separated by 7 μm, 5% polyacrylamide gel (denaturing) gel electrophoresis and visualized by autoradiography. Blocking, upstream, and other primers were annealed as appropriate. Adjustments are as noted in the figure legends. Quantification was done using a Molecular Dynamics PhosphorImager and ImageQuant software. Snake venom phosphodiesterase ladders of unrelated DNA and cationization with related and unrelated DNAs of known size were used as size markers.

RESULTS

FEN1 Can Cleave an HIV (+)-Strand DNA Overlap Substrate Despite the Secondary Structure of the Overlap—Since the structure of the HIV central overlap is similar to the preferred substrates of FEN1, we hypothesized that this cellular DNA replication enzyme might function in viral overlap processing. However, we wondered whether FEN1 action might be inhibited since the enzyme had not previously been observed to traverse such a long tail containing stem loops and other secondary structure. Surprisingly, incubation of the HIV substrate with FEN1 produced highly efficient cleavage at the expected location near the junction of the single-stranded overlap with the template (Fig. 1A). Although cleavage rates vary substantially depending on the particular substrate used, the cleavage rate of the HIV substrate was similar to that of other non-HIV substrate sequence endonucleolytic substrates (data not shown). As with other endonucleolytic substrates, the overlap was removed as an intact segment. No intermediate products were observed between starting material and final product at any time point, including points under a minute (lanes 2–5). Lane M is shown here to illustrate that the main cleavage product of FEN1 on this substrate comigrates with a chemically synthesized 29-mer, representing the 3′-most 29 annealed nucleotides of the downstream primer. Annealing of a complementary primer to the HIV tail inhibited cleavage, whereas addition of a noncomplementary primer did not (data not shown). Evidently, the natural foldback is not a sufficiently stable double-stranded region to inhibit cleavage.

Since FEN1 had not previously been shown to cleave substrates with stem loops, we wished to verify that significant secondary structure is indeed present in the HIV overlap. To do so, we first analyzed the effect of increasing salt concentrations on cleavage activity. As salt is increased, annealed regions should hybridize more tightly, making it more difficult for FEN1 to access the cleavage site. Fig. 1B shows that increasing salt concentrations progressively inhibit cleavage. Increased salt will, however, inhibit RNA directly by disrupting catalysis. With the HIV substrate, the observed inhibition is therefore likely due to a combination of effects on the enzyme directly and on the stems.

To provide more direct evidence for flap secondary structure, we added oligomer a to the HIV substrate in order to favor FEN1 cleavage at one of the anticipated flap stem loops. The
FIG. 1. FEN1 cleaves an HIV overlap substrate despite secondary structure. A, time course for endonucleolytic removal of an overlap having the exact HIV nucleotide sequence that is displaced during HIV (+)-strand DNA synthesis. Incubation was for 0, 5, 15, 30, and 45 s and 1, 5, 15, 30, 45, and 60 min in lanes 1–11, respectively. The upper band represents starting substrate, and the lower bands represent 3'-labeled fragments remaining after cleavage. Lane M is a marker lane containing a 29-mer representing the 3'-most 29 annealed nucleotides of the downstream primer. B, inhibition of cleavage by increasing salt concentrations. Incubation was for 60 min. Lanes 3–7 contained 20, 40, 60, and 80 mM NaCl, respectively; lanes 2 and 8 had 100 mM NaCl; and lanes 1 and 2 had no enzyme. C, upstream primer-dependent cleavage occurs at the base of a stem loop present within the overlap. Oligomer a is present only in lanes 4–9. Structures to the right depict how addition of oligomer a can promote the observed internal cleavages at the sites located by the arrows. Oligomer a is 3’-TGTITTTAAGTTTTAAAA. Lanes 1 and 4 had no enzyme and no incubation. The incubation time for lanes 2 and 5 was 1 min; for lanes 3 and 6, 5 min; and for lanes 7–9, 15, 30, and 60 min, respectively. D, FEN1 can cleave a substrate with a 10-nucleotide stem in the tail. Downstream primer 2 is 5’-GGGGGTACAAATTTAATTTTTTGTGCAGGGGACGGTTTATTACGGGACACGGAGAAA. The underlined sequence anneals to template 1; boldface nucleotides anneal to each other. The substrate was formed by annealing downstream primer 2 and upstream primer 1 to template 1. Downstream primer
substrate depiction shown to the right of the 64-nucleotide band in Fig. 1C demonstrates how a stem-loop structure can form the downstream double-stranded region of a flap substrate. For cleavage, FEN1 often requires an upstream primer, directly adjacent to the flap. We hypothesized that if a stem loop were present, adding the appropriate oligonucleotide would promote upstream primer-dependent cleavage. If no stem loop were present, the primer might act as a block for cleavage at the original flap annealing point, but it would be unlikely to produce cleavage on the single strand. Fig. 1C shows that adding the appropriate primer does promote cleavage at the expected location at the base of the proposed stem loop. Under the conditions used in this experiment, the primer did not entirely prevent cleavage at the original flap. It did, however, significantly inhibit this cleavage compared with that seen in the same experiment with no oligomer added (compare lanes 2 and 3 with lanes 5 and 6). While serving as an upstream primer for one of the natural stems, oligomer a anneals over the other stem. This result is significant because it shows that FEN1 can be inhibited by a double strand on the flap, but the natural HIV double strand in the same location is not sufficient for extensive inhibition. In this experiment, cleavage occurred at an unexpected site near the 5′-end of the tail. Presumably, an internal sequence of the primer, probably one of the dT runs, can transiently anneal to a complementary dA tract at this location on the overlap. Such annealing could create another flap structure, as depicted. Evidently allowing upstream primer-independent cleavage, this structure produced an additional unanticipated product.

The observation that FEN1 can bypass stem loops has implications for its role in Okazaki fragment processing and base excision repair. We wanted to know whether it could cleave a substrate with a stem loop both different and more stable than that in the natural HIV substrate. Fig. 1D demonstrates that FEN1 can cleave a substrate in which the HIV overlap downstream primer has been replaced by a downstream primer with a 10-nucleotide fully complementary stem in the tail. This result supports the general ability of FEN1 to cleave substrates with flap foldbacks, but clearly much more work would be required to determine how the size, sequence, and flap location of such structures might affect cleavage. Our intention here is to focus on the suitability of the HIV substrate.

NC and RPA Have Little Effect on FEN1 Cleavage of the HIV Overlap—We decided to explore the influence of other factors that might affect FEN1 cleavage of the HIV overlap. We hypothesized that human RPA and viral NC play a role. Both proteins have single-stranded binding and strand melting activities (35, 36). By binding the overlap, they might interfere with movement of the nuclease to the cleavage point. Alternatively, they might melt any existing secondary structure or otherwise promote cleavage. Fig. 2A illustrates the influence of NC. PhosphorImager quantitation suggested that NC is neutral to slightly stimulatory up to 50% coating of substrate (lane 5), but very inhibitory by 100% coverage (lane 6). Results of three to six experiments indicated that percent cleavage was 31.7 ± 4.5% with no NC (lane 2) and 34.7 ± 2.2% at 25% coating (lane 4). By 100% coating (lane 6), cleavage fell to 13.2 ± 2.6%. Not surprisingly, NC is neutral or may even stimulate cleavage when present at low levels that can melt secondary structure. The inhibition observed at high levels is also not surprising. Protein-DNA aggregation at high concentrations has been previously observed for Escherichia coli single-strand binding protein (35) and could explain the inhibition of cleavage.

RPA behaved similarly, being neutral to slightly stimulatory at low concentrations, but inhibitory at higher concentrations (Fig. 2B). Percent cleavage was 31.7 ± 4.5% with no RPA (lane 2) and 43.4 ± 4.3% at 0.78% coverage (lane 4) and declined from there. At high concentrations, RPA destroyed the endonucleolytic substrate by completely unannealing the downstream primer from the template, a phenomenon that we have previously observed by native polyacrylamide gel electrophoresis using oligomer substrates (data not shown). Unannealing should not be a problem in vivo because the full-length provirus or integrated viral DNA would be too long for RPA to dissociate. Moreover, the normal cellular activities of FEN1 are carried out in the presence of RPA, implying that these molecules are adapted to each other. Overall, there is no evidence that either binding protein would
have a major effect on the cleavage reaction in vivo.

**FEN1, DNA Polymerase, and DNA Ligase Can Complete Overlap Repair, Forming Fully Double-stranded DNA**—To demonstrate that FEN1 can participate in HIV metabolism, we reconstituted overlap repair to make completely double-stranded DNA. After cleavage to remove a tail, FEN1 typically leaves a nick or gap between remaining upstream and downstream primers (20). During normal Okazaki fragment processing or DNA repair, a polymerase would be expected to fill the gap, and a ligase would seal the primers together (28). We surmised that completion of HIV replication would occur in the same way. In Fig. 3A, we used mammalian DNA polymerase ε and human DNA ligase I to perform these functions because both participate in DNA replication and cooperate with FEN1 (19, 37, 38). We note, however, that any one or more of at least three human polymerases are involved as well. Since the central processing steps of HIV replication are critical in the viral life cycle (8–10) and ligase are involved as well. Since the central processing steps of HIV replication are critical in the viral life cycle (8–10) but often overlooked, we feel that more focused exploration could produce new therapeutic possibilities.

**DISCUSSION**

Generally, the structure of the HIV overlap flap is consistent with the preferred FEN1 substrate. The sequence of the tested substrate was identical to that of HIV-1, except that we changed the sequence of the upstream primer-template so that the template was not complementary to the flap. In vivo, annealing of the upstream primer and the overlapping downstream flap would be in equilibrium. We have shown previously that FEN1 can cleave 5'-flaps equilibrating in this manner because the upstream primer also promotes displacement of the downstream flap (27, 28). In fact, the strand displacement process may promote 5'-tail formation over a 3'-tail because the polymerase binds the 3'-end of the upstream primer. The presence or lack of upstream template complementarity should not influence cleavage at the base of the tail (28).

Many host proteins have been shown previously to play a role in HIV infection (15–17), but this is the first time a cellular enzyme has been identified with a substrate specificity appropriate for direct participation in HIV replication. In Fig. 3B, we present a model of the steps of HIV replication likely to involve
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human enzymes. The presence of two (+)-strand initiation sites is believed to increase replication efficiency by allowing simultaneous synthesis of two segments, each approximately half the total (+)-strand length (6, 7). It is unclear whether the overlap serves any other function, but the strong replication advantage shown with the wild-type cPT and CTS suggests that it may (8–10). Since the overlap is removed as an intact segment by FEN1 (Fig. 1A), it may survive to perform other functions. For example, it may promote (+)-strand recombination by a mechanism similar to that described previously (39, 40). Alternatively, the released overlap would simply be degraded. We note that FEN1 is a member of a family of enzymes, with other members that might be able to remove the overlap. In particular, XPG, a nucleotide excision repair nuclease, may substitute for FEN1, a possibility requiring further study.

FEN1-directed cleavage is more likely since XPG, although capable of cleaving flaps, acts in concert with the nucleotide excision repair complex of proteins. It cleaves on the 3′-side of a damaged nucleotide where the single-stranded portion of a bubble structure reanneals (41).

The possibility that retroviral integrase can cleave central termination region flaps also remains to be investigated. Previous examination of the disintegration reaction catalyzed by the HIV-1 integrase has shown that single-stranded tail structures with the HIV terminal LTR sequence at the annealing point can serve as substrates for flap cleavage (13, 14). However, integration intermediates with single-stranded flaps are cleaved at a lower efficiency than the corresponding native double-stranded structure. Additionally, alteration of the conserved 3′-CA nucleotides at the base of the flap lowered cleavage efficiency even further. At this point, the high activity of FEN1 in the nucleus, its structure specificity, and its low sequence specificity make it a very strong candidate for this reaction.

For the gap-filling step, we propose a human DNA polymerase, rather than reverse transcriptase itself, because overlap repair would presumably occur after integration, certainly after nuclear entry, as part of normal chromosomal repair mechanisms. Additionally, reverse transcriptase dissociates at the CTS (8–11). We have chosen to use polymerase β, in particular, might be ideally suited to the role since it appears designed to fill very short gaps (42). Although our results suggest that polymerase ε can perform the function, we have not ruled out the possibility that any of the other human polymerases might participate in vivo.

Even though there clearly is secondary structure in the overlap flap (Fig. 1, B–D), it does not interfere with FEN1-directed cleavage (Fig. 1A). Although previous results have shown that primers annealed to a flap are inhibitory (20), FEN1 may more readily traverse a double-stranded region if it is in the form of a foldback. Alternatively, the stability of the HIV foldbacks may simply not have been sufficiently great to interfere with tracking, binding, and cleavage by FEN1.

Deletion of the yeast homolog of FEN1 caused an increase in expansion frequency of trinucleotide repeats, the hallmark of several human genetic diseases, including myotonic dystrophy, Huntington’s disease, several ataxias, and fragile X syndrome (43). FEN1 mutation may also correlate with increased risk for colorectal cancer (44–46). This study suggests a mechanism by which FEN1 can play a role in another human disease, AIDS. Knowledge of this mechanism may eventually provide a new target for antiviral therapy. Such therapy would be complicated because FEN1 is essential for normal cellular DNA replication. Since the cPT-initiated overlap is very specifically defined, starting and ending at precise genomic locations, we envision approaching the problem with antisense technology. Targeting oligonucleotides to certain regions on or near the overlap might prevent binding or cleaving of the HIV substrate, without significantly inhibiting FEN1 action on normal cellular substrates.

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