Frequent Incorporation of Ribonucleotides during HIV-1 Reverse Transcription and Their Attenuated Repair in Macrophages*§

Received for publication, February 2, 2012, and in revised form, February 29, 2012. Published, JBC Papers in Press, March 1, 2012, DOI 10.1074/jbc.M112.348482

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Background: dNTPs are exceptionally low in nondividing macrophages, which promotes incorporation of rNMPs into HIV-1 DNA.

Results: We quantify the frequency of rNMP incorporation into viral DNA and their repair in macrophages and CD4+ T cells.

Conclusion: Abundant monoribonucleotides are incorporated into HIV-1 DNA in macrophages, wherein repair is attenuated.

Significance: Monoribonucleotides in DNA have implications for HIV-1 mutagenesis and therapy in macrophages.

Macrophages are well known long-lived reservoirs of HIV-1. Unlike activated CD4+ T cells, this nondividing HIV-1 target cell type contains a very low level of the deoxynucleoside triphosphates (dNTPs) required for proviral DNA synthesis whereas the ribonucleoside triphosphate (rNTP) levels remain in the millimolar range, resulting in an extremely low dNTP/rNTP ratio. Biochemical simulations demonstrate that HIV-1 reverse transcriptase (RT) efficiently incorporates ribonucleoside monophosphates (rNMPs) during DNA synthesis at this ratio, predicting frequent rNMP incorporation by the virus specifically in macrophages. Indeed, HIV-1 RT incorporates rNMPs at a remarkable rate of 1/146 nucleotides during macrophage infection. This greatly exceeds known rates for cellular replicative polymerases. In contrast, little or no rNTP incorporation is detected in CD4+ T cells. Repair of these rNMP lesions is also substantially delayed in macrophages compared with CD4+ T cells. Single rNMPs embedded in a DNA template are known to induce cellular DNA polymerase pausing, which mechanistically contributes to mutation synthesis. Indeed, we also observed that embedded rNMPs in a dsDNA template also induce HIV-1 RT DNA synthesis pausing. Moreover, unrepaired rNMPs incorporated into the provirus during HIV-1 reverse transcription would be generally mutagenic as was shown in Saccharomyces cerevisiae. Most importantly, the frequent incorporation of rNMPs makes them an ideal candidate for development of a new class of HIV RT inhibitors.

Lentiviruses such as human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) and simian immunodeficiency viruses uniquely replicate in both nondividing and dividing cells, whereas other retroviruses such as oncoretroviruses replicate exclusively in the latter (1, 2). Specifically, HIV-1 primarily infects activated CD4+ T cells and to a lesser extent, monocyte-derived macrophages. HIV-1 infection of terminally differentiated/nondividing cells such as macrophages and microglia directly impact viral pathogenesis. These cells are long-lived upon infection (3), in contrast to activated CD4+ T cells (4), and produce both virus and proinflammatory factors known to compound HIV-1-associated neurocognitive disorder (5–7). Macrophages also serve as a cellular reservoir of HIV-1 within the host and are compartmentalized in several tissues, especially in the brain, where elimination is challenging but essential for virus eradication (8–10).

Activated CD4+ T cells and macrophages differ enormously in cell cycle status and thus expression of ribonucleotide reductase R2 subunit, which determines the levels of the principal substrates, 2′-deoxynucleoside triphosphates (dNTPs), required for viral DNA synthesis mediated by HIV-1 reverse transcriptase (RT) (11). Moreover, a substantial challenge that lentiviruses encounter during infection of macrophages is the poor cellular dNTP availability (12, 13). This low intracellular dNTP concentration causes substrate binding to RT to be the rate-limiting step in proviral DNA synthesis in human primary macrophages (20–40 mM dNTP concentration), which harbor ~50–200 times lower dNTPs than activated CD4+ T cells (1–5 μM dNTP concentration) (12, 14).

It is increasingly apparent that rNTPs, which serve as the substrates of cellular RNA polymerases, cellular kinases, and are metabolic energy carriers, can be incorporated into DNA by cellular DNA polymerases during chromosomal DNA replication (15, 16). These events are normally infrequent because DNA polymerases, including HIV-1 RT, rigorously discriminate against nucleotides containing a 2′ OH such as rNTPs (17–20). However, high cellular concentrations of rNTPs (millimolar range) relative to dNTPs (nanomolar range) can promote rNMP incorporation during DNA replication. Therefore,

* This work was supported, in whole or in part, by National Institutes of Health Grants AI077401 (to B. K.), AI049781 (to B. K.), and GM049576 (to R. A. B.).
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‡ This article contains supplemental Figs. S1–S4.
§1 The abbreviations used are: dNTP, 2′-deoxynucleoside triphosphate; FEN1, flap endonuclease 1; qRT-PCR, quantitative real time PCR; rNMP, ribonucleoside monophosphate; rNTP, ribonucleoside triphosphate; 2LTR, two long terminal repeats; RNase Hα, ribonuclease Hα.

14280 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 287 • NUMBER 17 • APRIL 20, 2012

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 17, pp. 14280–14288, April 20, 2012
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when HIV-1 infects macrophages, HIV-1 RT faces the substrate specificity challenge during proviral DNA synthesis in the presence of limited dNTPs, which kinetically promotes rNMP incorporation. Here, we demonstrate that HIV-1 RT frequently incorporates rNMPs during proviral DNA synthesis in macrophages and that their capacity for repair of the resulting rNMP lesions is greatly reduced relative to activated CD4+ T cells.

EXPERIMENTAL PROCEDURES

Coupled Primer Extension and Alkaline Hydrolysis Assay—All DNA and RNA primers used in this study were purchased from Integrated DNA Technologies and Dharmacon Research, respectively. Assay mixtures (20 μl) contained 10 nM Template/primer, the RT protein concentrations specified in the individual figure legends, 4 dNTPs (Amersham Biosciences) at concentrations indicated in the figure legends, and 1× reaction buffer (50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM MgCl2, 0.5 μM (dT)20). Reactions were initiated by adding the RT proteins and incubated at 37 °C for the defined times. Reactions were terminated with 10 μl of 40 mM EDTA, 99% formamide. Reaction products were immediately denatured by incubating at 95 °C for 5 min, and 4 μl of each 30-μl final reaction mixture was quantitated by PhosphorImager analysis (PerkinElmer Life Sciences) of 14% polyacrylamide-urea denaturing gels (Sequagel; National Diagnostics, GA).

For the primer reaction described in Fig. 1A, a 5′-32P-labeled 17-mer DNA primer (5′-CGCGCCGAATTCCCGCT-3′) annealed to a 40-mer DNA (5′-AACCUUGGCUCAGGAUAUUUGCUCAGGGAUUUCGCGCG-3′; template/primer, 2.5/1) was used for various times under the reaction conditions described above using the nucleotide pools specified in the figure legends. These were then subjected to KOH or water, neutralized in the case of KOH, and separated by urea-PAGE as described previously.

Preparation of Nuclear Extracts—Nuclear extracts from 293T cells, Jurkats, primary CD4+ T cells, and macrophages were prepared by an adaptation of the protocol by Abmayr et al. (21). All buffers in this protocol contained 1× Roche Complete instead of 0.2 mM PMSF. Cell pellets were washed in PBS and resuspended in hypotonic buffer. They were then incubated on ice for 15 min and Dounce-homogenized with 3–22 strokes using pestle B. Suspension cells were more easily lysed and were homogenized with fewer strokes than adherent cells. Lysed cells were centrifuged at 3,300 × g for 15 min to separate the nuclei from the supernatant cytoplasmic fraction. Nuclei were extracted in an equal mixture of a low salt buffer (0.02 M KCl) and high salt buffer (1.6 M KCl) and were mixed on ice every 5 min for 30 min. Extracts were centrifuged at 22,065 × g for 30 min, and supernatants were removed and saved as nuclear extracts. Extracts were dialyzed overnight in the described dialysis buffer (21), but without 0.5 M DTT.

Quantitative (q)RT-PCR rNMP Assay—2LTR circles were isolated from transduced (D3) primary human macrophages or PHA and IL-2-activated CD4+ T cells and subsequently treated with Jurkat cell extract at 1/20 dilution or nuclear extract dialysis buffer. Dilution titrations were performed on Jurkat extract to minimize nulease activity and in conjunction with the oligo(dT) trap added to these reactions this activity was negligible (supplemental Fig. S2). We also confirmed that this activity is not trapped by residual genomic DNA present in 2LTR circle preparations and that rNMPs and not abasic sites, are stable for the 15 min 95 °C preincubation step in our standard qRT-PCR protocol described previously (22).

To calculate the overall frequency of ribonucleotide incorporation we utilized a Poisson calculation based upon the mean amount of 2LTR circles that were not degraded from four donors. We assumed these had 0 rNMP incorporated and then utilized the Poisson relationship to determine how many had at least 1 rNMP and with the known nucleotide length domain between the two qRT-PCR primers we calculated the probability of a single rNMP incorporation per number of base pairs.

RNase H2 Activity Assay—To determine RNase H2 activity in nuclear extracts, a 5′-32P-labeled dsDNA substrate containing a single rAMP (5′-GCAGAATA TTGCTGCGGGAATTCCCGCT-3′) was annealed to its DNA complement and used in a 20 μl reaction containing 10 nM substrate, 20 μM oligo(dT)20, 2 mM ATP, and reaction buffer. Nuclear extracts from macrophages and CD4+ T cells were normalized by total protein, determined by a Bradford assay, and then serially diluted 1/1, 1/4, 1/80, 1/160, 1/320, and 1/640 for each cell type unless otherwise described. Reactions were initiated with the addition of 4 μl of diluted extract or 4 μl of 300 mM KOH as a positive control and incubated at 37 °C for 30 min. Reactions were quenched with 10 μl of 40 mM EDTA, 99% formamide, and immediately denatured by incubating at 95 °C for 5 min. To analyze product formation, 4 μl of the 30-μl final reaction was separated by 16% urea-PAGE as described previously.

Flap Endonuclease 1 (FEN1) Activity Assay—To determine FEN1 activity in nuclear extracts, reactions containing a 5′-32P-labeled 10-nucleotide flap-containing substrate annealed to a 49-mer template along with an upstream primer (upstream primer, 5′-CGCCAGGTTTTTCAGCTACGACCA-3′; downstream primer, 5′-CAGTGGCGCTGCTTCAACAGG-ACGTTAGCTGCGG-3′; template, 5′-GCCACGTACGTGCTGTTGTAACCGGTTCTGACTGGGAAACCCCGTGGGG-3′) was used to observe FEN1 cleavage. Macrophage and CD4+ T cell nuclear extracts were normalized by total protein, determined by a Bradford assay, and then serially diluted at 1/40, 1/80, 1/160, 1/320, and 1/640 for each cell type unless otherwise described. Each reaction (15 μl) contained 10 nM substrate, 75 μM (dT)20, 2 mM ATP, and reaction buffer. Purified FEN1 (1.3 nM), a gift from Dr. Robert Bambara, was used in place of nuclear extract to act as a positive control. Reactions were initiated by addition of 4 μl of diluted extract and incubated at 37 °C for 30 min. Reactions were terminated and analyzed as described previously.

Western Blotting—The antibodies used in the Western blots were α-tubulin (Santa Cruz Biotechnology), FEN1 (Cell Signaling), RNase H2A (Abcam), RNase H2B (Abcam), and ECL anti-rabbit IgG or anti-mouse IgG (GE Healthcare). Whole cell lysates were collected in radioimmunoprecipitation assay buffer (10× PBS, 5% sodium deoxycholate, 10% SDS, Nonidet P-40) and then sonicated. Nuclear extracts were prepared as described above. All extracts were normalized by total protein concentration and diluted to the appropriate volumes.
Quantification of rNMPs Incorporated into HIV-1 DNA

FIGURE 1. Biochemical simulation of rNMP incorporation by HIV-1 RT in nucleotide pools found in human primary macrophages and CD4+ T cells. A–C. 5'-32P-17-mer DNA primer (10 nM) annealed to a 40-mer RNA template (A) was extended by HIV-1 RT (20 nM) in the presence of all four dNTPs and rNTPs found at physiological concentrations in human primary macrophages (B) or activated PBMC cells (C) (14) for 0, 2, 4, 6, 8, or 20 min. B and D, reaction products were then split and treated with 300 mM KOH or water and subjected to urea-PAGE. The KOH hydrolysis products (+PO4) at the three C template sites (1C, 2C, and 3C) are illustrated by the arrows in B, and the first rCMP incorporation site (1C) is magnified in D. E, quantification of the KOH hydrolysis products in D is shown as the percent of its dNMP extension product counterpart for both macrophage and peripheral blood mononuclear cell concentrations at sites 1C and 2C in A. These reactions were repeated with a second template/primer set encoding different sequences with identical results in supplemental Fig. S1, and assessment of the electrophoretic mobility of the alkaline hydrolysis products resulting from monoribonucleotides in DNA is also included in supplemental Fig. S1C.
proteins were quantified and stored in 10% glycerol dialysis buffer as described previously (24, 25).

**RNase H2A and FEN1 Depletion Assays**—To verify further that the cleavage products observed in the RNase H2 and FEN1 activity assays were not due to other enzymes in the extracts, nuclear extracts from macrophages and CD4\(^+\)/H11001 T cells were depleted of RNase H2A or FEN1 using a RNase H2A- or FEN1-specific antibody purchased from Abcam. To deplete the extracts, 10 \(\mu\)l of protein A/G plus-agarose beads from Santa Cruz Biotechnology was equilibrated with dialysis buffer, and 30 \(\mu\)l of diluted nuclear extract was added to the beads along with 3 \(\mu\)l of antibody. For a positive control, 3 \(\mu\)l of tubulin antibody purchased from Santa Cruz Biotechnology was added to an identical reaction. Reactions were mixed on a spinner at 4 °C overnight, and the following day they were centrifuged for 1 min at 10,000 \(\times\) g. For the RNase H2A depletion reaction, extracts were removed from the beads and added to 10 \(\mu\)l of newly equilibrated beads and mixed at 4 °C for 2 hr. Reactions were again centrifuged for 1 min at 10,000 \(\times\) g, and the extracts were removed from the beads and immediately assayed for RNase H2 or FEN1 activity using identical reaction conditions as described above.

**RESULTS**

HIV-1 RT Incorporates GMP Most Frequently during Proviral DNA Synthesis in Macrophage Nucleotide Pools—To monitor rNMP incorporation, we biochemically simulated DNA synthesis by HIV-1 RT using the dNTP/rNTP concentrations found in macrophages (Fig. 1B) and activated CD4\(^+\)/H11001 T cells (Fig. 1C). A 5' \(^{32}\)P-labeled DNA primer annealed to an RNA template was extended by a fixed amount of HIV-1 RT at either macrophage or T cell nucleotide concentrations, generating a 38-nucleotide product (F). Incorporated rNMPs were then detected by treating the products with potassium hydroxide (KOH), which hydrolyzes the 3' ends of rNMPs embedded in DNA (26). KOH hydrolysis products (see arrows in Fig. 1B) were detected in the macrophage simulations, but not in the T cell simulations (Fig. 1C), using two different primer extension assays (see supplemental Fig. S1). These results indicate that HIV-1 RT can efficiently incorporate rNMPs during DNA synthesis in an environment simulating macrophage dNTP/rNTP concentrations, but not at those found in activated CD4\(^+\) T cells. The visible rNMP incorporation sites in Fig. 1, B and C, correspond to GMP incorporation (quantified in Fig. 1E).
Quantification of rNMPs Incorporated into HIV-1 DNA

rNMPs Are Incorporated into HIV-1 Proviral DNA—Next, we tested whether HIV-1 incorporates rNMPs during reverse transcription in human primary macrophages and activated CD4⁺ T cells. For this virological test, we used RNase H2, which cleaves at the 5’ side of rNMPs in dsDNA (27). To do this, we first prepared a nuclear extract from Jurkat cells (21) containing substantial RNase H2 activity, which was confirmed biochemically with an RNase H2-specific cleavage assay (Fig. 2A). A radiolabeled 30-bp dsDNA containing a single rAMP in one strand (Fig. 2A) was treated with the nuclear extract, producing a 13-nucleotide cleavage product. To validate further that the cleavage was due to RNase H2 activity and not from other nucleases in the extract, RNase H2A was depleted from the extract using an anti-RNase H2A antibody (supplemental Fig. S3). We then isolated HIV-1 2LTR circle DNAs (22) from human primary macrophages and activated CD4⁺ T cells infected with a single round VSV-G pseudotyped D3HIV-GFP vector, that expresses all HIV-1 genes except env and nef, which is replaced with eGFP. Isolated 2LTR circle DNAs were incubated with the Jurkat nuclear extract containing RNase H2 activity or with buffer alone. In this reaction, any 2LTR circle DNAs containing rNMPs will be degraded by the RNase H2 activity in the extract. Next, we quantified the copy number of 2LTR circles in the macrophage and T cell DNA samples using a qPCR assay. As shown in Fig. 2, B and C, DNA samples from CD4⁺ T cells (four independent donors) displayed no significant reduction in 2LTR circle copy number after exposure to RNase H2-containing extract (Fig. 2C, left). In contrast, DNA samples from infected primary macrophages (four independent donors) showed a 48% reduction in the 2LTR circle DNA copy number (Fig. 2C, right) after RNase H2 treatment, suggesting that 48% of the 60-bp 2LTR circle fragments contain at least 1 rNMP (Fig. 2D). These data allowed us to estimate (Poisson calculation) the minimal incorporation rate of rNMPs during reverse transcription in macrophages to be about 1/146 nucleotides incorporated. Strikingly, this rate is 27 times higher than the enzymatic misincorporation rate of HIV-1 RT (28).

The rNMP incorporation rate determined in Fig. 2D used DNA samples collected at 24 h after infection to minimize cell-directed rNMP repair of the HIV 2LTR circle DNAs. However, when the experiment was repeated with a sample collected at 48 hr after infection (Fig. 2C), essentially identical results were obtained (Fig. 2C). This suggests that repair of incorporated rNMPs in proviral DNA is significantly impaired or delayed in macrophages.

RNase H2 and FEN1 Repair Activity Are Attenuated in Macrophages—First, we sought to test whether components of the human RNase H2 complex were expressed in HIV-1 target cells (supplemental Fig. S4). We found by Western blot analysis that they were expressed in both cell types, and we also determined that the RNase H2 complex was indeed active in these cells. To test this, we isolated native nuclear extracts (21) from activated CD4⁺ T cells and macrophages. As shown in Fig. 2A, Jurkat cells exhibit robust RNase H2 activity; however, it is unclear whether this complex would be active in terminally differentiated cells. Following extensive dialysis of these extracts to remove cellular metabolites and detection of RNase H2 by Western blotting (Fig. 3A), we treated a 5’ 32P-labeled dsDNA substrate containing a single rNMP (Fig. 3B) with nuclear extracts normalized by total protein from two blood donors of activated CD4⁺ T cells and monocyte-derived macrophages. We observed a specific product with slightly higher electrophoretic mobility than a KOH-treated sample, known to hydrolyze the 3’ side of an rNMP (Fig. 3C). This product is likely a consequence of RNase H2 cleavage due to the lack of any other known human enzyme with similar activity; however, we further confirmed that it was RNase H2 cleavage by immunoprecipitation depletion coupled with the RNase H2

![FIGURE 3. Human primary macrophage nuclear extracts exhibit reduced RNase H2 and FEN1 activity compared with CD4⁺ T cells. A, nuclear extracts from 293 FT cells, Jurkats, two donors of activated CD4⁺ T cells, and primary macrophages were normalized by total protein, and expression of RNase H2A and FEN1 were detected as described. B, depiction of RNase H2- and FEN1-specific substrates. C, nuclear extracts were diluted 1/1, 1/4, 1/80, 1/160, 1/320, and 1/640 RNase H2 activity assessed as described previously. D, nuclear extracts were diluted 1/10, 1/40, 1/80, 1/160, 1/320, and 1/640 and FEN1 activity assessed as described under “Experimental Procedures.”](image-url)
cleavage assay (supplemental Fig. S3B). This activity was observed in both donors, with a <1-fold difference between them.

Subsequent to RNase H2 cleavage 5’ of an rNMP in DNA, gap filling and strand displacement occur by a cellular repair or replicative polymerase, followed by FEN1 cleavage. We again used nuclear extracts obtained from primary cells, which were shown by Western blotting to contain FEN1 (Fig. 3A), in a FEN1-specific cleavage assay. When these nuclear extracts were titrated upon a 5’ 32P-labeled 10-nucleotide flap-containing substrate, we observed cleavage products identical to those described in C, except reactions contained either 100, 10, 1, 0.1, 0.01, or 0.001 mM dNTPs, with or without 2 mM ATP. 

Complete rNMP Repair Process Is Attenuated in Macrophages—We next investigated the ability of macrophages and activated CD4+ T cells to repair rNMPs embedded in dsDNA. This pathway is initiated by cleavage 5’ of a monoribonucleotide in DNA by RNase H2, which has been recently shown in a high throughput screen to be important for HIV-1 infection (29). This is followed by DNA gap filling, FEN1 cleavage, and ligation (27). This DNA repair system uses lagging strand DNA replication components required for Okazaki fragment maturation. However, there is no DNA replication in postmitotic macrophages therefore, we anticipated that macrophages would be impaired in their repair capacity compared with activated CD4+ T cells. Although both macrophages and CD4+ T cells express active RNase H2 (supplemental Fig. S4), the actual enzymatic activity of RNase H2 in macrophages was approximately 3-fold lower than that in activated CD4+ T cells (Fig. 4A). In addition, FEN1 activity (which was measured using a DNA substrate containing a FEN1-specific flap) was also approximately 3-fold lower than in activated CD4+ T cells (Fig. 4B). Finally, we analyzed the entire repair processing capacity after RNase H2 cleavage using the substrate illustrated in Fig. 4C. This DNA substrate self-anneals to form a partially dsDNA with a terminal fold-back loop containing an rCMP at its 5’ end.
The single-stranded region is annealed to a radiolabeled ssDNA leaving a nick 5’ of the rCMP. Completion of the repair process will generate a long DNA product that joins both of the DNA strands (**). To test the dNTP concentration dependence of rNMP repair we employed Jurkat nuclear extract and a titration of dNTPs. At low dNTP concentrations (100 and 10 nM) complete repair of this substrate was greatly reduced (Fig. 4D). Subsequently, we wanted to test extracts from macrophages and activated CD4+ T cells with their respective dNTP concentrations directly (as well as higher dNTP concentrations to assess maximal repair activity) using this assay. The T cell nuclear extract produced a much higher level of completely repaired product compared with the macrophage extract (Fig. 4E). This shows that macrophages have greatly reduced rNMP repair capacity compared with activated CD4+ T cells. Collectively, because every rNMP repair step in macrophages was less efficient than in activated CD4+ T cells, the overall rNMP repair process in macrophages is substantially delayed. This further supports the observation in Fig. 2C that no significant recovery of rNMP-free 2LTR circle DNAs were detected even at later time points after infection.

DISCUSSION

There is considerable evidence that cellular DNA polymerases do not always properly use their canonical dNTP substrates, such that in the context of an entire genome, rNMPs are commonly incorporated (15, 16). Yeast DNA polymerase ε incorporates rNMPs during chromosomal DNA replication at a rate of 1 in 1,250 nucleotides (16). Results indicate that HIV-1 incorporates rNMPs during proviral DNA synthesis in macrophages at the rate of 1 in 460 nucleotides. Interestingly, this noncanonical rNMP incorporation rate is 20–30 times higher than the incorrect dNMP incorporation rate of HIV-1 RT (28). This suggests that the initial rNMP incorporation during HIV-1 proviral DNA synthesis is frequent, and reverse transcription in macrophages results in 130 rNMP molecules incorporated/HIV-1 genome during infection. Frequent rNMP incorporation during proviral DNA synthesis in macrophages was logically predicted by our previous biochemical observation about the larger concentration disparity between dNTPs and rNTPs in macrophages compared with activated CD4+ T cells. Considering that cellular DNA polymerases also frequently incorporate noncanonical rNMPs and that cells are well equipped with a repair system for removing rNMPs embedded in dsDNA, it is a reasonable assumption that rNMPs incorporated during DNA synthesis must be detrimental to the cells. Indeed, defects...
in RNase H2, which is a key initial repair enzyme that specifically recognizes rNMPs embedded in dsDNA, cause the severe genetic disorder Aicardi-Goutières syndrome (33). Unlike DNA, RNA is labile to both heat and alkaline hydrolysis (26). Therefore, after cellular polymerases misincorporate rNMPs in DNA, they are quickly repaired by enzymes of the RNase H2 family found in prokaryota, eukaryota, and archaea. RNase H2 specifically nicks 5’ of an rNMP, or RNA oligomer, in DNA. Our data demonstrate that macromolecules express RNase H2, but it appears that the RNase H2 activity in macromolecules is lower than that in CD4+ T cells. Interestingly, different forms of the RNase H2 subunit (Fig. 3A and supplemental Fig. S4B) appear to exist in different cell types, supporting the idea that their activities may be differentially regulated. In addition, after RNase H2 cleavage of the 5’ side of embedded rNMPs, the highly coordinated actions of multiple enzymes are required to complete the repair process. One major kinetic block of the RNMP repair process that specifically exists in macromolecules is DNA gap repair, which requires cellular dNTPs. Considering the high Km values of host cellular DNA polymerases for dNTPs (micromolar range) and the extremely poor availability of cellular dNTPs in macromolecules (20 – 40 mM), it is a logical assumption that the gap repair process after RNase H2 removal is kinetically impaired in macrophages (Fig. 4), compared with activated CD4+ T cells.

Our observations detailed herein are directly relevant to other recent descriptions of the cellular incorporation and repair of rNMPs in chromosomal DNA. First, recent work with DNA polymerase ε revealed (16) that the replication of an RNMP-containing DNA template induces pausing of the DNA polymerase, which is one of the known elements that induces mutation synthesis (32). In this report, we have clearly demonstrated that HIV-1 RT recapitulates these results (Fig. 5). Second, work addressing the mutagenic impact of rNMPs in DNA has shown that a specific 2-bp deletion is present in the context of elevated ribonucleotide incorporation and in the absence of repair (15). It was further shown in yeast that DNA topoisomerase I, in conjunction with an rNMP, is responsible for this distinct mutational spectrum. Additionally, we have found 45 of the (AT)2 and three (TC)3 topoisomerase I sites described previously (34) in the NL4–3 HIV-1 genome. Therefore, it is a distinct possibility that mutagenesis impacts HIV-1 replication; however, it is unclear at this time whether topoisomerase I is expressed and active in macrophages. Third, the rNMPs embedded in proviral DNA, more specifically in the minus strand, may also affect host RNA polymerase II (viral transcription). Indeed, the effect of rNMPs on the process of transcription has not been reported, and this issue requires further investigation. Finally, because DNA repair systems change with cell cycle and DNA replication during S phase, most of the DNA repair pathways were extensively studied in dividing cells. The most studied DNA repair system in nondividing cells is transcription-coupled nucleotide excision repair (TC-NER), which is activated by stalled RNA polymerases at DNA damage sites. However, whether TC-NER can recognize the rNMPs embedded in DNA as it does with other well-known DNA lesions induced by UV and chemical modifications is still unclear. In summary, the virological impacts of rNMPs embedded in HIV-1 proviral DNA require further investigation.

This study reveals that HIV-1 frequently incorporates rNMPs during proviral DNA synthesis in macrophages compared with activated CD4+ T cells. This unexpectedly frequent rNMP incorporation by HIV-1 RT was predicted by the uniquely large concentration disparity between dNTPs and rNTPs in macrophages observed previously (14). Furthermore, the frequent rNMP incorporation by HIV-1 RT validates 3’ deoxyribonucleosides, termed ribonucleoside RT inhibitors, as a new class of anti-HIV therapeutic agents that specifically target HIV-1 reverse transcription in nondividing macrophages.

Acknowledgments—We thank Dr. Stephen Dewhurst and Erin Noble for critically reading this manuscript.

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