Incorporation of Uracil into Minus Strand DNA Affects the Specificity of Plus Strand Synthesis Initiation during Lentiviral Reverse Transcription*

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Many retroviruses either encode dUTP pyrophosphatase (dUTPase) or package host-derived uracil DNA glycosylase as a means to limit the accumulation of uracil in DNA strands, suggesting that uracil is detrimental to one or more steps in the viral life cycle. In the present study, the effects of DNA uracilation on (−) strand DNA synthesis, RNase H activity, and (+) strand DNA synthesis were investigated in a cell-free system. This system uses the activities of purified human immunodeficiency virus type 1 (HIV-1) reverse transcriptase to convert single-stranded RNA to double-stranded DNA in a single reaction mixture. Substitution of dUTP for dTTP had no effect on (−) strand synthesis but significantly decreased yields of (+) strand DNA. Mapping of nascent (+) strand 5′ ends revealed that this was due to decreased initiation from polypurine tracts with a concomitant increase in initiation at non-polypurine tract sites. Aberrant initiation correlated with a change in RNase H cleavage specificity when assayed on preformed RNA-DNA duplexes containing uracilated DNA, suggesting that appropriate "selection" of the (+) strand primer is affected. Collectively, these data suggest that accumulation of uracil in retroviral DNA may disrupt the viral life cycle by altering the specificity of (+) strand DNA synthesis initiation during reverse transcription.

Human immunodeficiency virus type 1 (HIV-1), like other retroviruses, is a plus-stranded RNA virus that replicates through a DNA intermediate. Viral reverse transcriptase (RT), a DNA polymerase that uses RNA and DNA templates, converts the viral RNA to double-stranded DNA after host cell infection (Fig. 1). This DNA is then integrated into a host cell chromosome, where it serves as a template for synthesis of new viral RNA. Full-length viral RNA and some viral gene products are subsequently packaged into progeny virions (reviewed in Refs. 1 and 2).

In HIV-1, both minus (−) and plus (+) strand DNA synthesis initiate from RNA primers (Fig. 1). The former requires a cellular tRNA annealed near the 5′ end of the genomic RNA (Fig. 1, step 1), whereas primers for the latter are generated by specific RNase H-mediated cleavage of viral RNA (indicated in Fig. 1 with scissors). Following (−) strand DNA synthesis, selective degradation of viral RNA primers leaves short (+) strand RNA fragments annealed to the (−) strand DNA (3). One of these fragments, a highly conserved, purine-rich sequence referred to as the 3′ polypurine tract (3′-PPT), is located near the 3′ end of the genome and is used as an obligatory primer for (+) strand synthesis in all retroviruses (Fig. 1, step 4). Precise cleavage at (Fig. 1, black scissors) and initiation from the 3′-PPT is required to define the 5′ end of the "left" long terminal repeat and maintain the viral integrase (IN) attachment site (att; (2)) (Fig. 1, gray oval). A second copy of the PPT is located near the center of the genome (cPPT) and is also used to prime (+) strand DNA synthesis in HIV-1 and other lentiviruses (4–7). The pol gene in all retroviruses universally encodes RT and IN. However, in some non-primate lentiviruses such as feline immunodeficiency virus (FIV) and equine infectious anemia virus (EIAV), the pol gene also encodes a dUTP pyrophosphatase (dUTPase) (8). This enzyme hydrolyzes dUTP to dUMP and pyrophosphate and thus provides dUMP as a precursor for de novo synthesis of dTTP in host cells (9). dUTPase also ensures that the dUTP/dTTP ratio is low to minimize uracil incorporation into DNA (9). Previous studies revealed that retroviruses encoding defective dUTPase exhibit delayed replication kinetics in macrophages (10–13) and accumulate G-to-A base substitutions (14, 15). Delayed replication in macrophages resulted from a block in the replication cycle at one or more steps after viral DNA synthesis (10). Thus, it was proposed that dUTPase functions primarily to increase replication efficiency and fidelity. It was further speculated the absence of dUTPase in some retroviruses may contribute to genetic diversity (15).

Interestingly, although primate lentiviruses lack dUTPase, they recruit and package cellular uracil DNA glycosylase (UDG) in the virion through a Vpr- or IN-dependent mechanism (16, 17). UDG removes uracil bases from DNA as part of the base excision repair pathway (9). Although UDG and dUTPase are mechanistically different enzymes, they both function to exclude uracil from DNA strands. This further suggests that
PBS, PPT, and U3 regions are indicated, with lowercase described in the legend for Fig. 2 are shown in the central PPT is omitted. Steps recapitulated in the cell-free assay arrowheads accepted model of retroviral DNA synthesis (1). shown by the 3′/H11032 indicated by the 3′/H11032 strand DNA product of end in U3. This approximates the positions of primers HIV 9091 and viral DNA. ruses have mechanisms to avoid uracil incorporation into RNA-DNA hybrid. A model is proposed to explain why retroviral DNA synthesis, RNase H activity, and (+) strand DNA synthesis initiation were evaluated in a cell-free system. This system uses purified RT and its associated RNase H to convert regions of single-strand RNA to double-strand DNA in a single incubation (18). In dUTP-containing reactions, DNA synthesis catalyzed by HIV-1 RT proceeded normally, but the creation and extension of PPT primers were significantly compromised. Failure to correctly “select” and extend the PPT primers was largely explained by additional experiments in which the effects of uracil on RNase H cleavage specificity were evaluated. The results showed that cleavage specificity is altered by the presence of uracil in the DNA strand of a PPT-containing RNA-DNA hybrid. A model is proposed to explain why retroviruses have mechanisms to avoid uracil incorporation into viral DNA.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following reagents were from Amersham Biosciences: Hybond N+ nylon membrane, Sephadex G-50, RNaseT1, RNaseU2, Ultrapure dNTPs, [γ-32P]dATP (3000 Ci/mmol), [α-32P]dCTP (6000 Ci/mmol), Redivue sequencing terminator mix [α-32P]dNTPs (1500 Ci/mmol), and thermosteadamidase radiolabeled terminator cycle sequencing kit. T7 RNA polymerase in vitro transcription kits, rNTPs, RNasin, and pGEM-3Zf (+) were from Promega. T4 polynucleotide kinase and shrimp alkaline phosphatase were purchased from U. S. Biochemical Corp. Quick Spin Sephadex G-25 columns were from Roche Molecular Biochemicals. Plasmids pHIV-pol and pHIV-nef and oligonucleotide 9091 were described previously (18, 19). Oligonucleotide FIV 9133 is complementary to nucleotides (nt) 9133–9152 of the FIV molecular clone 34TF10 (20). Oligonucleotides were synthesized by Operon Technologies. Restriction enzymes and general reagents were from Invitrogen or Fisher.

Recombinant wild-type and RNase H-minus (D44S/N) HIV-1 RTs were prepared according to published procedures (18, 19) and were >95% homogeneous as determined from Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis. The wild-type RT had a specific activity of 2.5 units/μg on poly(rC)/oligo(dG) (units defined in Ref. 19) and was >33% active as determined by titration with 5′/H11032 U5 long terminal repeat DNA-primer-template (21, 22). FIV RT (1 unit/μl) was purified from the Petaluma virus strain using the procedure and unit definition of North et al. (23). All RT preparations were stored in multiple aliquots at −70 °C. Shortly before conducting experiments, their activities were reassessed in dilution and time course assays on each of the substrates described below. RT concentrations and incubation times were chosen that spanned the linear range of product formation in each experiment. Thus, all experiments were conducted under “steady-state” conditions of synthesis in which the products assayed arose in direct proportion to RT concentration and time.

**Preparation of RNA Templates—**pFIV-PPT was created by subcloning a NheI/SpeI fragment of p34TFT10 (nt 8287–9229) into the XbaI site of pGEM-Zf(+) (20). FIV 3′-PPT RNA (1 kb) was generated by in vitro transcription of HindIII-linearized pFIV-PPT as recommended by Promega. HIV-1 nef RNA (1.5 kb), containing the 3′-PPT, was described previously (18). The 73-nt HIV-1 3′-PPT and 61-nt cPPT RNAs were generated by in vitro transcription of PCR products. The 5′ and 3′ primers for the cPPT PCR product were complementary to nt 4772–4789 and 4807–4826, respectively (based on NL4–3 HIV-1 sequence). The 5′ and 3′ primers for the 3′-PPT PCR product were complementary to nt 9042–9059 and 9091–9110, respectively. PCR conditions and product purification were as described (24), except the annealing temperature was 55 °C. The PCR primers added a T7 RNA polymerase promoter sequence (5′-TCTAATACGACTCACTATAAGGGAGA-3′) to the 5′ end of each product amplified from pHIV-pol or pHIV-nef. In some cases, the 73-nt 3′-PPT and 61-nt cPPT RNAs were precipitated with ethanol and 10 μM MgCl2 (25), dephosphorylated with alkaline phosphatase, and 5′-end-labeled with [γ-32P]dATP as described (26), and free nt were removed by G-25 spin columns. In all cases, RNAs were purified by 7 μl urea/8% PAGE and eluted by the crush and soak method (25).

**Cell-free DNA Synthesis Reactions—**Annealing and extension steps for processivity reactions were performed as described (18, 19). See the figure legends for details. The assay for (+) strand synthesis initiation is described in detail elsewhere (18). Reaction products were treated with 0.3 n NaOH, neutralized and passed through Sephadex G-50 spin columns (18), or treated with 45 mM EDTA followed by G-50 spin columns. Products were resolved by 7 M urea/8% PAGE and transferred to Hybond N+ nylon membranes. Nascent (+) strand RNAs were visualized by PhosphorImager analysis after hybridization to a radioactively labeled (+) strand specific probe. The probe was the same oligonucleotide used as primer for initial (−) strand synthesis. Thus, the probe will only detect (+) strand DNAs that have been initiated by RT to the 5′ end of the (−) strand DNA initiated from the same primer-probe sequence. Plus strand DNA 5′ ends are identified by their electrophoretic mobility relative to a sequencing ladder created with the same primer. See the figure legends for specific conditions.

**HIV-1 RT/RNase H Cleavage Assays—**5′-end-labeled (+) strand RNA (40 μM) was hybridized to a 35-mer (−) strand DNA oligonucleotide (400 nM) in 50 mM potassium acetate, 10 mM Tris-HCl, pH 8.0, at 65 °C for 10 min, 37 °C for 15 min, 25 °C for 15 min, and 4 °C for 15 min. RNA-DNA duplexes were diluted 5-fold and incubated at 37 °C with 0.7 units of HIV-1 RT/μl in 10-μl reactions containing 25 μM Tris-HCl, pH 8.0, 30 mM KCl, 10 mM MgCl2, 0, or 100 μM each of dNTP, RNasin (1 unit/μl), and 2 μM dithiothreitol. Reactions were held by the addition of an equal volume of 90% formamide, 10 mM EDTA, pH 8.0, 0.1% bromphenol blue, and 0.1% xylene cyanol. Samples were loaded on prerun 7 μl urea/8% PAGE and eluted by the crush and soak method (25).

Fig. 1. RNase H activity is required for retroviral DNA synthesis. RNA (gray lines) is converted to DNA (black lines) by RT in the accepted model of retroviral DNA synthesis (1). Arrowheads indicate the 3′ direction of DNA polymerization. RNase H cleavage events are indicated by the scissors. The precise cleavage event required to liberate the 3′ end of the 3′-PPT to serve as a (+) strand synthesis primer is shown by the black scissors. DNA synthesis from the ppt and the subsequent ppt primer removal must be precise to faithfully generate the U3 end of the 5′ long terminal repeat (gray ovals). Genomic R, U5, PBS, PPT, and U3 regions are indicated, with lowercase letters corresponding to (+) and (−) strand, respectively. For simplicity, the central ppt is omitted. Steps recapitulated in the cell-free assay described in the legend for Fig. 2 are shown in the gray box. The (−) strand DNA product of step 2 is shown as an intermediate having a 3′ end in U3. This approximates the positions of primers HIV 9091 and FIV 9133 used in the cell-free assay (Fig. 2).

uracil accumulation in retroviral DNA is detrimental to some critical step or steps in the viral life cycle.

In this work, the effects of uracilated DNA on (−) strand DNA synthesis, RNase H activity, and (+) strand DNA synthesis initiation were evaluated in a cell-free system. This system uses purified RT and its associated RNase H to convert regions of single-strand RNA to double-strand DNA in a single incubation (18). In dUTP-containing reactions, DNA synthesis catalyzed by HIV-1 RT proceeded normally, but the creation and extension of PPT primers were significantly compromised. Failure to correctly “select” and extend the PPT primers was largely explained by additional experiments in which the effects of uracil on RNase H cleavage specificity were evaluated. The results showed that cleavage specificity is altered by the presence of uracil in the DNA strand of a PPT-containing RNA-DNA hybrid. A model is proposed to explain why retroviruses have mechanisms to avoid uracil incorporation into viral DNA.

![Image](https://via.placeholder.com/150)
dUTP Minimally Affects (−) Strand DNA Synthesis—To determine how dUMP incorporation affects reverse transcription, (−) strand DNA synthesis reactions were examined in a cell-free system (Fig. 2, step 1). A 1.5-kb RNA derived from the 3′ end of the HIV-1 genome was hybridized to a [5′-32P]-labeled 20-mer DNA primer and incubated for varying lengths of time with a fixed amount of purified, recombinant HIV-1 RT (Fig. 3). Two different reaction sets were performed: one containing all four normal dNTPs and (−) strand RNA-DNA hybrids were identified after urea-PAGE separation by a blotting assay that uses a strand-specific 32P-labeled oligonucleotide probe identical to the original primer used for (−) strand polymerization. The PPT sequence is indicated by the filled rectangle, and expected (−) strand products are indicated as P1, P2, and P3. See “Experimental Procedures” and Ref. 18 for details. 

Resolution of primer extension products by sequencing gels revealed that primer extension terminated at distinct sites called “pause sites,” where RT frequently dissociates from the primer-template (19). The pausing pattern in each reaction set remained essentially unchanged as the incubation time increased, and total extension products increased linearly with time up to about 15 min (Fig. 3). Thus, RT and unextended primer-template are in the steady state for −15 min, and the products result from multiple turnovers initiated at the excess of primer-template. The HIV-1 RT pause pattern was largely unaffected by dUMP incorporation with the exception of a single site where pausing was significantly reduced −90 nt from the primer terminus (indicated by the filled circle). In addition, the steady-state rates of primer extension (1−3 nt/sec) were comparable in the dTTP and dUTP reaction sets. Similar results were obtained with FIV RT (data not shown). Taken together, these data show that dUMP is incorporated efficiently in place of dTMP by HIV-1 and FIV RTs. 

dUMP Decreases Initiation of (+) Strand DNA Synthesis from PPTs—The sequence of the central and 3′-PPTs in HIV-1 (+) strand RNA is 5′-AAAAAGAAAAAGGGGGG-3′ (27). This A-rich sequence is a good template for dUMP incorporation by RT (Fig. 3), and the uracil in the complementary (−) strand DNA may alter the utilization of the PPT for (+) strand DNA synthesis. This was tested using a cell-free assay that converts regions of single-strand RNA to double-strand DNA in a single incubation (Fig. 2, steps 1−3). This RT-dependent system recapitulates several of the required intermediate steps of retroviral DNA synthesis: RNA-templated (−) strand DNA synthesis, RNase H cleavage and preferential (+) strand initiation at PPTs, and subsequent DNA-templated (+) strand DNA synthesis (18). Briefly, HIV-1 or FIV RNAs containing PPTs are hybridized to oligonucleotide primers and incubated with RT in the presence of either a dTTP- or a dUTP-containing dNTP set. More RT is required in these reactions to increase yields of (+) strand DNA intermediates and in turn achieve steady-state accumulation of nascent (+) strand DNA. Half of the products of each reaction are subjected to alkaline hydrolysis to degrade the RNA, and all reaction products are resolved by denaturing PAGE and transferred to a nylon membrane. Nascent (+) strand DNA products are detected by probing with 32P-labeled strand-specific oligonucleotides. The 5′ ends of nascent DNA or RNA-DNA chimeras (nascent DNAs still attached to RNA primers) are identified by mapping against sequence ladders. 

Formation of (+) strand DNA requires (−) strand DNA synthesis and yields three groups of reaction products (Fig. 2, step 3 products, and Fig. 4, P1, P2, and P3). P1 bands are nascent (+) strand DNAs liberated from the PPT by RNase H. P2 bands are (+) strand DNAs covalently linked to the PPT RNA primer. The bands labeled P3 reflect various (+) strand DNA synthesis products.
dUTP alters the specificity of (+) strand DNA synthesis initiation. Cell-free DNA synthesis reactions (Fig. 2) were carried out at 37 °C as described in “Experimental Procedures” and Ref. 18 using 30 nM primer-template and matched activities of either HIV-1 or FIV RT (~17 units/ml). Reaction products were separated by urea-PAGE, transferred to nylon membranes, and probed with 32P-labeled 9091 (−) strand DNA oligonucleotide (A) or FIV 9133 (−) strand oligonucleotide (B). The (+) strand sequences shown in the center of each figure were generated with the same (−) strand primers used for probing. The primers used for sequencing were phosphorylated, and the reactions contained [α-32P]dNTPs. The locations of the HIV-1 3′-PPT and FIV 3′-PPT are indicated by the black bars on the side of the sequence lanes of A and B, respectively, and products P1, P2, and P3 and residual (+) strand RNA fragments are indicated on the left of each panel. Reaction products that were analyzed directly are indicated as −NaOH, whereas products that were incubated with alkali prior to electrophoresis are labeled +NaOH. Incubations containing dCTP, dATP, dGTP, and dTTP are labeled dUTP: −, and reactions where dUTP was substituted for dTTP are indicated as dUTP: +. The 5′ end of P1 is identified by comparing its mobility with the sequencing ladder. The 5′ end of the P2 cluster cannot be identified precisely because P2 is an RNA-DNA hybrid, and RNA imparts a reduction in PAGE mobility by ~10% (28). A, HIV-1 nef RNA/9091 (−) strand initiation products produced by HIV-1 RT. Reaction times were 10 (−NaOH only), 20, 30, 45, 60, and 90 min. The (−) lane is a 60-min incubation lacking dNTPs. The inset shows an enlarged and darker view of the P3 products. B, FIV 3′-PPT RNA/9133 (+) strand initiation products. Incubation times were 15, 30, 45, 60, and 90 min. The RT used for catalysis is indicated on the top of each panel. Reaction products shown were analyzed directly on the gel without alkali treatment. The (−) lanes are 60-min reactions lacking RT.
DNAs initiated from non-PPT primers upstream (5') of the PPT. The P2 bands and several of the P3 bands disappeared and/or showed an increased gel mobility after NaOH treatment (Fig. 4A, +NaOH lanes), demonstrating that RNA primers were still linked to the nascent (+) strand DNAs and not yet removed by RT/RNase H. Specific (+) strand DNA initiation products were not detected in reactions where an RNase H-minus HIV-1 RT mutant was used (D443N; (18)) or in reactions lacking the (−) strand DNA primer (data not shown).

Initiation of (+) strand DNA synthesis from the HIV-1 3'-PPT required RT and dNTPs (Fig. 4A, −NaOH lanes and data not shown). In reactions containing dTTP in the absence of dUTP, two PPT initiation products of similar intensity were detected with 5' ends mapping to the last G residue and the adjacent A residue at the 3' end of the PPT (Fig. 4A, −NaOH lanes, bands labeled P1). NaOH treatment yielded two additional P1 bands (Fig. 4A, +NaOH lanes, dTTP-containing reactions), revealing the four-band pattern observed previously (18). Substitution of dUTP for dTTP in these reactions significantly decreased the amount of P1 and caused a slight increase in the mobility of the P1 bands, presumably due to the absence of the C5 methyl group in uracil (Fig. 4A, −NaOH lanes, dUTP reactions). Reactions containing dUTP yielded shorter P2 products (Fig. 4A). In addition, the intensity of non-PPT initiation products (P3) increased in reactions containing dUTP relative to the dTTP-containing reactions (Fig. 4A, right inset).

Using an FIV RNA-DNA substrate, HIV-1 and FIV RTs recognized and initiated (+) strand DNA synthesis from the FIV PPT (Fig. 4B). The amount of PPT initiation decreased in dUTP-containing reactions, and a decrease in heterogeneity at the PPT 5' end was observed with HIV-1 RT (Fig. 4B, bands labeled P1 and P2, respectively). These data demonstrate that the PPT itself, not the specific RT or flanking sequences (HIV-1 versus FIV), directs (+) strand synthesis initiation in these experiments (see also Refs. 18 and 28–30). Thus, the effect of dUMP on primer formation and recognition by RT is directed predominately by the double-strand PPT region.

PPT-initiated (+) strand DNAs (P1) were detected as early as 10 min (Figs. 4A and 5A) and accumulated linearly with time for up to 60 min, whereas the amount of P2 was essentially constant over the 90-min incubation (Fig. 5A and data not shown). Presumably P2 never accumulated due to rapid removal of the PPT by RNase H (31). The rate of P1 accumulation decreased ∼3-fold in the dUTP reaction set (Fig. 5A). This decrease must result from an effect on either RNase H cleavage and/or subsequent PPT primer selection and extension because (−) strand synthesis is unaffected by uracil (Fig. 3). Interestingly, the quantity of non-PPT initiated products increased significantly when dUTP was included in the reactions, and their rate of formation increased 3-fold (Fig. 5B). Taken together, the data in Fig. 4 show that uracil-DNA diminished specific PPT initiation and concomitantly increased the rate of non-PPT initiation.

dUMP in (−) Strand DNA Decreases RNase H Cutting at the PPT—The change in (+) strand DNA initiation specificity imparted by dUMP incorporation may be the result of altered RNase H activity. An RT/RNase H activity assay was used to directly investigate the influence of dUMP on RNase H cleavage of the HIV-1 PPTs (Fig. 6). [5'-32P]-HIV-1 RNAs (61-mer containing the ePPT or 73-mer containing the 3'-PPT) were hybridized to 35-mer (−) strand DNAs containing either dTMP or dUTP (Fig. 6A). These substrates position the polymerase active site at the 3' end of the (−) strand DNA and the RNase H active site 18 nt behind (i.e., toward the 5' end of the (−) strand) to allow precise RNA cleavage between the last G residue of the PPT and the adjacent A residue. The substrate design suppresses cleavage directed by the 5' end of RNA (26). RNA-DNA hybrids are incubated with RT and dNTPs for various time points, and the resultant RNase H cleavage products are resolved by urea-PAGE and visualized by PhosphorImager. Unlike the concerted assay (Fig. 4), HIV-1 RT is present in limiting quantities for this experiment to study RNase H activity under steady-state conditions. Thus, the observed cleavage products result primarily from hydrolysis of the initial substrate.

The RNA-DNA duplex containing dTMP was cleaved by RT/RNase H between the A and G residue precisely at the 3' end of both the 3' and ePPTs, similar to the positive control lane (+) (Fig. 6, B and C, reaction set labeled DNA/T and indicated with an arrow). Note that RNA fragments generated by RNase H activity have a 3'-hydroxyl, causing them to migrate −1 nt slower than the corresponding fragments of the RNA sequence marker that have 3'-phosphates left by RNases U2 and T1 (32). Cleavage also occurred 1 nt inside the 3' end of each PPT. These two RNA fragments are consistent with the two major (−) strand synthesis initiation sites observed in Fig. 4. The remainder of the PPT sequence was resistant to RNase H in these reaction conditions. RT extended the (−) strand DNA oligonucleotide, resulting in significant RNA cleavage 14–18 nt from the 5' end of each RNA substrate (Fig. 6, B and C, bottom left). The amount of each of these cleavage products increased with time on the 3' end of the cPPT substrates. No cleavage products were detected in the absence of RT (Fig. 6, −lanes) nor in control reactions incubated with HIV-1 D443N RT lacking
FIG. 6. HIV-1 RT RNase H cleavage specificity at the PPT is altered by dUMP in (−) strand DNA. In A, the sequences of the RNA/DNA duplex substrates used for the cleavage assay are shown. The RNAs were annealed separately to each 35-mer (−) strand DNA oligonucleotide. RNAs were in vitro transcribed from PCR products and 32P-labeled at the 5′ end as detailed under “Experimental Procedures.” The sequence GGGCGA at the 5′ end of each RNA is derived from the 3′ end of the T7 RNA polymerase promoter. The central and 3′-PPT sequences are highlighted with the gray box. RNA/DNA duplexes were incubated with HIV-1 RT as described under “Experimental Procedures” for 1, 2, 5, 10, 20, or 40 min and resolved by urea-PAGE. B, 3′-PPT. C, cPPT. PPT duplexes formed with the dTMP-containing 35-mer are indicated as DNA/T, whereas duplexes formed with the dUMP-containing 35-mer are indicated as DNA/U. Uncleaved RNA appears at the top of the gel panel, whereas specific cleavage products are labeled on the left. Lanes labeled (+) are reactions on the DNA/T duplex where the concentration of MgCl2 was lowered to 300 μM. Our unpublished observations revealed that concentrations of MgCl2 below 400 μM result in precise PPT cleavage by RT/RNase H on this template. (−) indicates 20-min incubations in the absence of RT. The PPT is indicated on the right of the panel adjacent to the RNA sequence derived from RNaseT1 (cleaves 3′ of G residues) and RNaseU2 (cleaves 3′ of A residues). The RNA substrate was subjected to alkali treatment to generate a ladder (L).
RNase H activity (data not shown).

The amount of precise PPT cleavage product decreased on duplexes containing dUMP in place of dTMP (Fig. 6, reaction sets labeled DNA/U). Analysis of the data indicates that the precise PPT cut represents only 17 ± 6% of the total cleavage products observed at and within the 3′' and cPPTs. In contrast, on the DNA/T substrate, the precise cut accounted for 44 ± 8% of the cleavage products at the PPT (10-min time points). The cleavage pattern 14–18 nt from the 5′ end of the RNA was unaffected by uracil in the (−) strand DNA. Interestingly, the total amount of RNA-DNA/U duplex that was cleaved by RNase H was equal to or slightly increased relative to the RNA-DNA/T duplex. Thus, RNase H activity is specifically decreased only at the 3′ end of the PPT in the presence of dUMP and not at other sites on the RNA/DNA duplex. Additional imprecise RNA cleavage occurred within the PPT (Fig. 6, B and C) as observed in Fig. 4A (−NaOH reaction set). However, these shorter PPT RNA fragments were incapable of serving as (+) strand primers based on the absence of (+) strand DNA with 5′ ends mapping to this sequence (Fig. 4A, +NaOH reactions). In the absence of DNA synthesis, the total RNase H activity on all PPT substrates increased (data not shown). In addition, the cleavage specificity decreased, and the PPT sequence itself was significantly more susceptible to cleavage by RNase H when the (−) strand DNA contained uracil. In summary, uracil DNA causes a decrease in precise PPT cleavage while increasing nonspecific cleavage within the PPT, demonstrating that the activity of RNase H is greatly affected by uracil in the DNA/RNA duplex.

**DISCUSSION**

Lentiviral virions contain enzymes that are predicted to limit the amount of uracil present in viral DNA, suggesting that incorporated dUMP may disrupt an essential step in the viral life cycle. In this work, we investigated whether incorporated dUMP alters primer-template recognition by RT in biochemical assays of (−) strand DNA synthesis, RNase H activity, and (+) strand DNA synthesis initiation.

RTs from both HIV-1 and FIV efficiently utilized dUTP to synthesize (−) strand DNA (Fig. 3 and data not shown); neither the steady-state polymerization rates nor the processivity were altered. Likewise, the 3′-5′ RNase H activity of HIV-1 RT (33) was similar on substrates containing either thymine or uracil bases (data not shown). Thus, dUMP residues in the primer strand do not impede movement of RT along the nucleic acid substrate. Polymerization of dUMP is not unique to RT as other DNA polymerases also readily incorporate dUMP (9). Therefore, efficient incorporation and subsequent extension of dUMP are general properties of DNA polymerases.

The effect of uracil-mediated aberrant initiation of (+) strand synthesis would be decreased viral DNA integration frequency. Plus-strand DNA originating downstream of the 3′-PPT (i.e. within u3, r, u5, or pbs; Fig. 1, steps 4 and 5) would compromise and/or eradicate the critical att site, thereby preventing integration (43). In addition, IN binds less efficiently to viral DNAs with minor or major groove perturbations imparted by base analogs (44). Thus, dUMP incorporation is deleterious for retroviruses because it affects specific protein/DNA interactions and consequently alters critical protein functions. This hypothesis is supported by the biochemical data presented in Figs. 4–6. Furthermore, the observations in macrophages that uracilmodified EIAV DNA forms proviruses 2.5 times less efficiently than viral DNA lacking uracil, and the subsequent decreased transcription of uracilmodified EIAV DNA, provide additional evidence for this hypothesis (10). Moreover, interactions of several other viral and cellular proteins with DNA are also altered by the presence of uracil (45–47). Thus, we propose that retroviruses require dUTPase and/or UDG to exclude dUMP from viral DNA, ensuring that critical protein/DNA interactions occur with the highest possible efficiency and fidelity, thereby enhancing viral fitness.

The substrates used in our studies contained exclusively dTMP or dUMP to maximize the effects in the cell-free system. The replicating virus has access to small amounts of dUTP (48); therefore, the effects on initiation or integration may be less pronounced, particularly if, as expected, the effects are proportional to the amount of uracil in the DNA. Nevertheless, even
subtle impairment of integration efficiency and the resultant decreased viral fitness will have significant effects on overall virus replication after many replication cycles in a selective environment (49, 50).

A previous study concluded that the function of dUTPase is to maintain genetic stability by preventing the misincorporation of dUMP opposite template G residues (15). The work reported here suggests an alternative reason for the necessity of dUMP opposite template G residues (15). The fact that several virus families have enzymatic means to minimize uracilated DNA sites formed by UDG (51). The presence of UDG in HIV-1 virions (16, 17) suggests that retroviral DNA requires HIV-1 DNA has not been directly tested. The presence of UDG reported here suggests an alternative reason for the necessity of virus replication after many replication cycles in a selective environment (49, 50).

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Incorporation of Uracil into Minus Strand DNA Affects the Specificity of Plus Strand Synthesis Initiation during Lentiviral Reverse Transcription

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