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Conformational Changes in the 5′ End of the HIV-1 Genome Dependent on the Debranching Enzyme DBR1 during Early Stages of Infection

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ABSTRACT Previous studies in our laboratory showed that the RNA debranching enzyme (DBR1) is not required for early steps in HIV cDNA formation but is necessary for synthesis of intermediate and late cDNA products. To further characterize this effect, we evaluated the topology of the 5′ end of the HIV-1 RNA genome during early infection with and without inhibition of DBR1 synthesis. Cells were transfected with DBR1 short hairpin RNA (shRNA) followed 48 h later by infection with an HIV-1-derived vector containing an RNase H-deficient reverse transcriptase (RT). RNA was isolated at several times postinfection and treated with various RNA-modifying enzymes prior to rapid amplification of 5′ cDNA ends (5′ RACE) for HIV-1 RNA and quantitative reverse transcriptase PCR (qRT-PCR). In infected cells, DBR1 knockdown inhibited detection of free HIV-1 RNA 5′ ends at all time points. The difference in detection of free HIV-1 RNA 5′ ends in infected DBR1 knockdown versus control cells was eliminated by *in vitro* incubation of infected cell RNAs with yeast or human DBR1 enzyme prior to 5′ RACE and qRT-PCR. This was dependent on the 2′-5′ phosphatase activity of DBR1, since it did not occur when we used the catalytically inactive DBR1(N85A) mutant. Finally, HIV-1 RNA from infected DBR1 knockdown cells was resistant to RNase R that degrades linear RNAs but not RNAs in circular or lariat-like conformations. These results provide evidence for formation of a lariat-like structure involving the 5′ end of HIV-1 RNA during an early step in infection and the involvement of DBR1 in resolving it.

IMPORTANCE Our findings support a new view of the early steps in HIV genome replication. We show that the HIV genomic RNA is rapidly decapped and forms a lariat-like structure after entering a cell. The lariat-like structure is subsequently resolved by the cellular enzyme DBR1, leaving a 5′ phosphate. This pathway is similar to the formation and resolution of pre-mRNA intron lariats and therefore suggests that similar mechanisms may be used by HIV. Our work therefore opens a new area of investigation in HIV replication and may ultimately uncover new targets for inhibiting HIV replication and for preventing the development of AIDS.

KEYWORDS DBR1, human immunodeficiency virus, reverse transcription

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During mRNA splicing, excision of group II introns from pre-mRNA by the spliceosome results in formation of intron lariats. In lariat RNAs, a 2′ hydroxyl group of an internal guanosine or adenosine nucleotide is linked to the 5′ phosphate of the excised intron (1–3). The unique 2′-5′ phosphodiester bonds in intron lariat RNAs are cleaved by a specific debranching enzyme, DBR1, resulting in a linear RNA with 5′ phosphate that is amenable to ribonucleotide recycling (4). Formation of a lariat form of the

genomic RNA also has been proposed to be an intermediate in the replication of the yeast retrotransposon Ty1 (5).

Ty1 is among the best characterized of the long terminal repeat (LTR) retrotransposons of the yeast *Saccharomyces cerevisiae* (5–8). Retrotransposons are inherited genetic elements that resemble integrated retroviruses, and they contain LTRs. When induced, they copy their RNAs into DNAs by reverse transcription, which are then reintegrated into new chromosomal sites (retrotransposition). Using a genetic screen aimed at identifying cellular factors involved in Ty1 retrotransposition, Chapman and Boeke found that DBR1 plays a role in Ty1 retrotransposition, although its function is not essential for cell viability (9). DBR1 mutant yeast strains produce mature cellular mRNAs, but accumulate intron lariats, and they are defective for both Ty1 transposition and cDNA formation (5, 6). Menees and colleagues demonstrated that the 2′-5′ phosphodiesterase activity of DBR1 is required for Ty1 retrotransposition (8). Furthermore, Cheng and Menees, reported that during retrotransposition, the Ty1 RNA forms a 2′-5′ branch characteristic of an RNA lariat, between the 5′ end of the RNA and the last nucleotide of the U3 region (5). The formation of a lariat-like structure was proposed to assist in the first strand transfer during reverse transcription. The involvement of a putative lariat in Ty1 retrotransposition, however, remains controversial. Coombes and Boeke found that despite using a number of lariat detection methods, they were unable to detect lariats from *in vivo* Ty1 RNA preparations (10). Thus, the mechanism of DBR1's role in Ty1 retrotransposition remains unclear.

The similarity of Ty1 to vertebrate retroviruses suggested that DBR1 might be important for cDNA synthesis in retroviruses, including the complex retrovirus human immunodeficiency virus type 1 (HIV-1). We previously reported a role for DBR1 in HIV-1 cDNA synthesis (11), and DBR1 has also been identified as a cellular gene important for HIV-1 replication in a genome-wide small interfering RNA (siRNA) screen (12). Using short hairpin RNA (shRNA)-mediated knockdown of DBR1, we inhibited the detection of late reverse transcription products and HIV capsid protein p24 at 24 h postinfection, but there was little effect on production of the early cDNA product known as minus-strand strong-stop DNA. This effect could be reversed if cells were cotransfected with a DBR1 expression plasmid (11). More recently, we confirmed that DBR1 shRNA inhibits HIV-1 reverse transcription during or after the minus-strand template switch (13). Moreover, we found that initiation of reverse transcription takes place in the cytoplasm, but completion of reverse transcription largely takes place in the nucleus or perinuclear region. Since DBR1 is a nuclear protein, this would be consistent with it playing a role in completion of reverse transcription in the nucleus.

Here, we further investigated the role of DBR1 in HIV-1 replication by examining the topology of the 5′ end of an HIV-1-based vector RNA genome during early infection with or without DBR1 knockdown. We utilized an HIV-1 vector preparation containing a mutant reverse transcriptase, RT(E478Q), that is active in DNA synthesis but defective for RNase H activity (14), to prevent degradation of the RNA genome during reverse transcription. We analyzed the configuration of the 5′ end of viral genomic RNA in infected cells by digestion *in vitro* with several RNA-modifying enzymes followed by 5′ rapid amplification of cDNA ends (RACE) and quantitative reverse transcriptase PCR (qRT-PCR). This approach allowed us to identify and quantify different forms of the infecting viral genome at different times early after infection. The results provided evidence for formation of a lariat-like structure of HIV-1 genomic RNA in cells early after infection that required DBR1 for resolution. The temporal formation and disappearance of this structure would be consistent with its involvement in reverse transcription at or near the minus-strand transfer step.

RESULTS

Detection of HIV-1 RNA in infected cells by 5′ RACE. In previous studies, we determined that DBR1 plays a role in HIV reverse transcription (11, 13). Here we tested if DBR1 might be resolving a lariat-like intermediate of the template viral RNA formed during minus-strand transfer, as proposed for Ty1. We hypothesized that there are

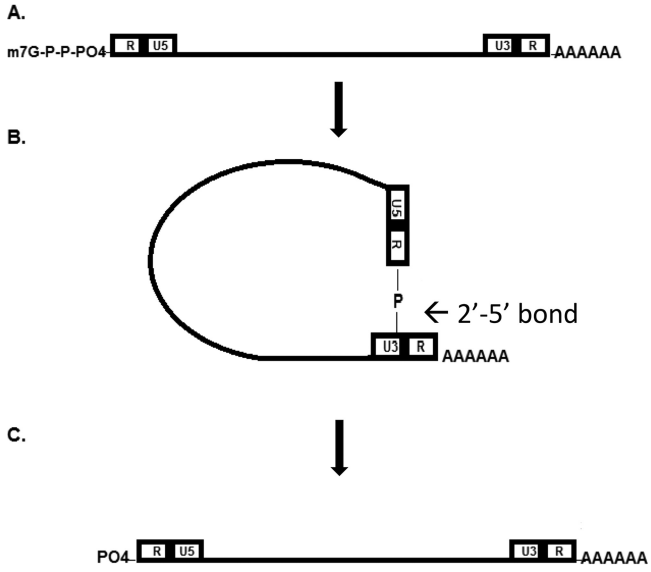
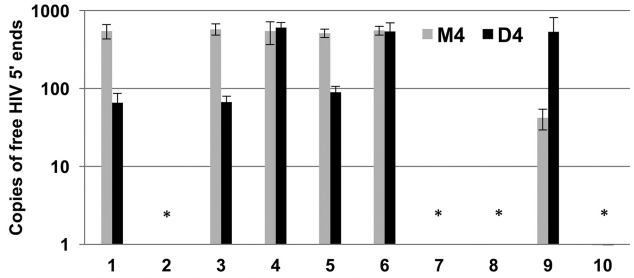


FIG 1 Potential HIV-1 RNA genome intermediate structures. (A) Capped single-strand HIV-1 RNA genome. (B) Lariat formed by a 2′-5′ phosphodiester bond between the 5′ nucleotide and the last nucleotide of the U3 region. (C) Uncapped single-strand RNA genome.

three HIV RNA genome structures at different stages of early infection and reverse transcription: capped linear viral RNA (the genomic form in the virion), a lariat intermediate, and uncapped linear RNA (Fig. 1), which occur in that temporal order. To study the state of the RNA genome during infection, we first transfected GHOST-R5X4 cells with the DBR1 shRNA D4 to knock down DBR1 expression or with a control shRNA with three mismatches in the core binding sequence, M4. We previously showed that the D4 shRNA efficiently reduces DBR1 RNA levels in transient transfections (13). After 48 h, the cells were infected with the HIV-1 vector HR-E pseudotyped with vesicular stomatitis virus G protein (VSV-G) at a multiplicity of infection (MOI) of 0.2. To prevent degradation of the vector RNA by the RNase H activity of reverse transcriptase during reverse transcription, the vector was packaged with the RT(E478Q) mutant. The E478Q mutation renders the RNase H subunit catalytically inactive but allows reverse transcription up to the point of the first strand transfer (14). Cells were harvested 12 h postinfection, and phosphorylated 5′ ends of viral vector RNA were detected by 5′ RACE and qRT-PCR with or without previous *in vitro* digestion with one or two relevant enzymes. In the 5′ RACE, an RNA oligonucleotide was ligated to RNAs containing free 5′ phosphate ends with RNA ligase, followed by qRT-PCR for HIV sequences with an HIV primer near the 5′ end (13). Copies of HIV-1 free 5′ end cDNA detected were normalized against copies of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA detected from the same cells to obtain the number of HIV-1 free 5′ end RNA copies per 2.5 μg of cellular RNA.

Preliminary analysis of HIV-1 RNA in infected cells. Analyses of RNA from vector-infected cells at 12 h postinfection are shown in Fig. 2. Phosphorylated uncapped 5′ ends were detected in the infected cells, and inhibition of DBR1 (D4) decreased the levels by 8.6-fold compared to cells treated with the control plasmid (M4) (treatment 1). This suggested that DBR1 activity is necessary for appearance of the phosphorylated uncapped (free) 5′ ends. As expected, treatment of the infected cell RNA with calf intestinal alkaline phosphatase (CIP [which removes free 5′ phosphates from RNA]) abolished detection of viral RNA (treatment 2); removal of the 5′ phosphate would prevent ligation of the RNA oligonucleotide necessary for 5′ RACE. Treatment of the RNA prior to 5′ RACE with tobacco acid pyrophosphatase (TAP [which removes 5′ cap structures and leaves a 5′ phosphate]) did not increase the amount of detectable free 5′ ends (treatment 3). This indicated that at 12 h postinfection, there was little capped



Treatment										
CIP	-	+	-	-	-	-	-	+ (1)	-	-
TAP	-	-	+	-	-		-	+	-	-
Yeast DBR1	-	-	-	+		-	-	-	+	-
Yeast DBR1 N85A	-	-	-	-	+	-	-	-	-	+
Human DBR1	-	-	-	-	-	+	-	-	-	-
RNAse R	-	-	-	-	-		+	-	+ (1)	+ (1)

FIG 2 Detection of free HIV-1 5′ ends following various RNA-modifying enzyme treatments. GHOST-R5X4 cells were transfected with DBR1 shRNA pHyper-D4 (D4) or DBR1 shRNA triple-mismatch pHyper-M4 (M4) for 48 h, followed by infection with DNase-treated HIV-1 vector (HR-E) packaged with the catalytically inactive E478Q RNase H mutant. Twelve hours later, the cells were lysed and RNA was isolated. Aliquots of 2.5 μg of RNA were used per enzyme treatment (denoted as "+"). Where noted, in two enzyme treatments "(1)" refers to the first enzyme used. Following enzymatic treatment, the RNA samples underwent 5′ RACE and then qRT-PCR to quantitate the copies of free 5′ ends in HIV-1 RNA. Copies of HIV-1 free 5′ ends were normalized to copies of GAPDH mRNA. The data represent three independent experiments processed for qRT-PCR in duplicate. Error bars denote standard deviations from the means. The *x* axis denotes each enzymatic treatment with a number, and the identity of the enzyme or enzymes used is shown below. Asterisks indicate values below a detectable threshold.

viral RNA. Indeed, when the RNA was first treated with CIP followed by TAP, there were no detectable ligatable HIV 5′ ends (treatment 8). The difference in detection of free 5′ ends of HIV-1 RNA in the D4- versus M4-transfected cells (treatment 1) suggested that there might be viral RNA in unresolved lariat-like forms in the D4-treated cells. When RNA from the D4-treated cells was digested *in vitro* with human or yeast DBR1 enzyme prior to 5′ RACE (treatments 4 and 6), the number of detectable 5′ HIV-1 RNA ends increased to the levels detected in the control, M4-treated, cells. Furthermore, this effect required DBR1 enzymatic activity since the yeast DBR1(N85A) mutant that lacks enzymatic activity (15) did not increase detectable free 5′ ends in RNA from D4-treated cells (treatment 5). These data supported the hypothesis that DBR1 treatment *in vitro* resolved a lariat-like structure involving the 5′ end of the HIV-1 vector genome in infected cells.

To further test this hypothesis, we treated RNA from infected cells with RNase R prior to *in vitro* DBR1 incubation. RNase R is a 3′-5′ exoribonuclease that degrades linear RNAs, while the loop portions of lariat and circular RNAs remain intact (16). Prior digestion of RNA from D4-transfected cells with RNase R had no effect on the increase in ligatable 5′ HIV-1 RNA ends after *in vitro* DBR-1 treatment (compare treatments 9 and 4), indicating that the great majority of HIV-1 RNA 5′ ends were in a lariat-like configuration under conditions of DBR1 knockdown, not linear. In contrast, prior RNase R digestion of RNA from M4-transfected cells reduced the detectable ligatable 5′ ends substantially (ca. 15-fold), although some positive signal was still detected (treatment 9 versus 4). Therefore, when DBR-1 was active in infected cells, at 12 h postinfection, the HIV-1 RNA was predominantly linear and uncapped, with a minor fraction in a lariat-like configuration.

Temporal effects of DBR1 shRNA knockdown on detection of free HIV 5′ ends in infected cells. To more precisely determine the effect of DBR1 shRNA inhibition on the detection of free 5′ends, we carried out infections as described in Fig. 2, the cells were harvested 2 to 24 h postinfection, and RNA was isolated. For comparison, the input vector preparation was also analyzed. RNAs were analyzed for free phosphory-

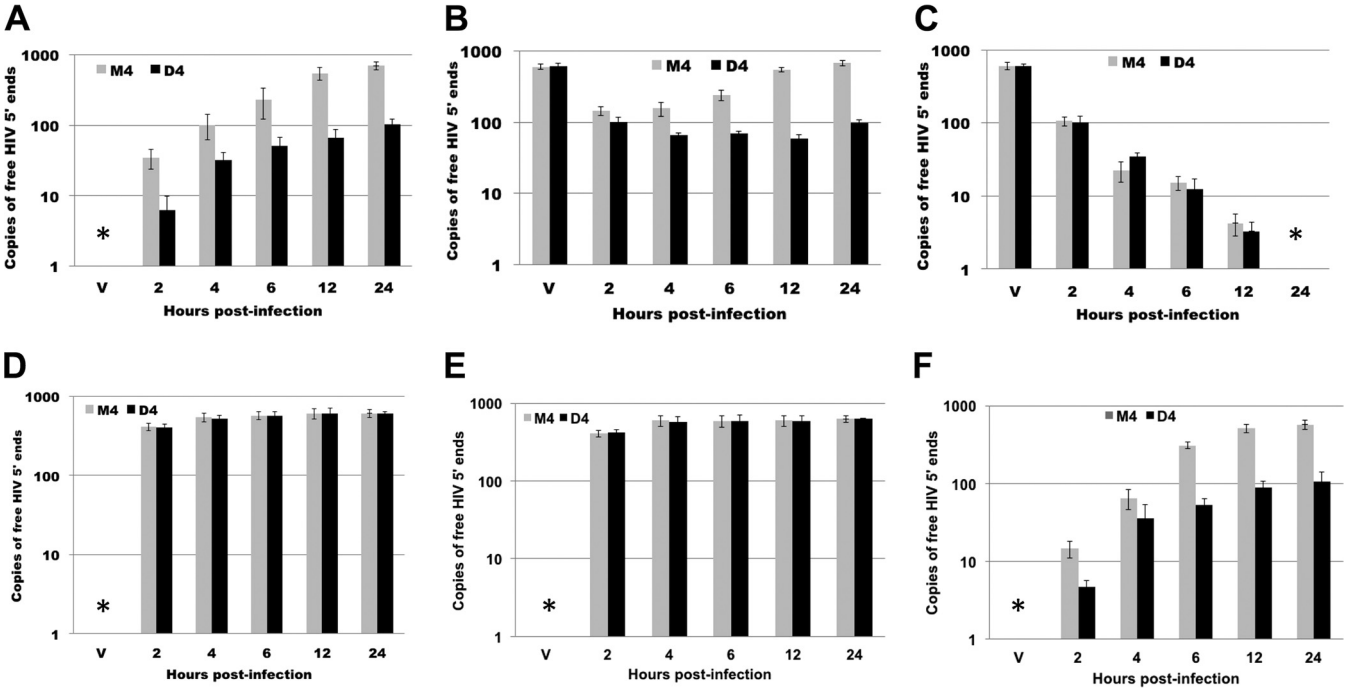


FIG 3 (A to F) Analysis of HIV-1 5′ ends from 2 to 24 h postinfection. Aliquots of 2.5 μg of RNA were used per 5′ RACE reaction, and qRT-PCR was used to quantitate the free 5′ ends of HIV-1 vector RNA. Copies of HIV-1 free 5′ ends were normalized to copies of GAPDH mRNA. Data represent three independent experiments processed for qRT-PCR in duplicate. Error bars denote standard deviations from the means. Asterisks indicate values below the threshold of detection. An equivalent amount of input HR-E vector (V) RNA was analyzed in parallel. (B) Aliquots of RNA were treated with TAP prior to the 5′ RACE and qRT-PCR for quantitation of free 5′ ends in HIV-1 RNA. (C) Aliquots of RNA were treated with CIP followed by TAP prior to 5′ RACE and qRT-PCR for quantitation of free 5′ ends in HIV-1 RNA. (D) Aliquots of RNA were treated with yeast DBR1 prior to 5′ RACE and qRT-PCR for quantitation of free 5′ ends in HIV-1 vector genomic RNA. (E) Aliquots of RNA were treated with human DBR1 prior to 5′ RACE and qRT-PCR for quantitation of free 5′ ends in HIV-1 vector genomic RNA. (F) RNA aliquots were treated with yeast DBR1(N85A) mutant enzyme prior to 5′ RACE and qRT-PCR for quantitation of free 5′ ends in HIV-1 RNA.

lated 5′ HIV-1 ends and normalized against copies of GAPDH mRNA as described above. When HIV-1 free 5′ ends were analyzed over time (Fig. 3A), the concentrations in both the control, M4-treated, cells and D4-treated cells increased more than 24-fold between 2 and 24 h postinfection. Moreover, detection of free HIV-1 vector 5′ ends was significantly lower at all time points when DBR1 was knocked down (maximal difference 6.7-fold; $P < 2.7 \times 10^{-5}$). As will be shown below, essentially all of the detectable viral RNA entered the cells by 2 h postinfection. Thus, the increase in detectable RNA in Fig. 3A reflected conversion of the 5′ end of vector RNA to the linear uncapped form as shown in Fig. 1C, and the conversion proceeded through an intermediate that required DBR1 for resolution.

It has been reported that the HIV-1 genomic RNA packaged in the virion contains a 5′ cap (17). Indeed, when the input vector RNA was analyzed by 5′ RACE (Fig. 3A), the free 5′ end signal was below the threshold of detection. As expected, *in vitro* treatment of RNA from input virions with TAP resulted in a strong 5′ RACE signal (Fig. 3B); moreover, treatment by CIP before TAP did not affect the strength of the 5′ RACE detection (Fig. 3C), indicating the absence of appreciable uncapped and phosphorylated 5′ ends in the virion genomic RNA.

DBR1 does not affect HIV-1 genomic RNA decapping during infection. In the 5′ RACE results shown in Fig. 3A, it was possible that DBR1 was inhibiting decapping of the HIV-1 genome, thereby leading to a decrease in the detection of free 5′ ends. To test this possibility, RNA aliquots from the M4 and D4 time course experiments shown in Fig. 3A were treated with TAP (Fig. 3B) or with CIP followed by TAP (Fig. 3C) prior to 5′ RACE. (Treatment of the RNA with CIP alone resulted in undetectable levels of viral RNA 5′ ends at all time points, as expected [data not shown].) In Fig. 3B, TAP treatment would give 5′ RACE signals from both capped viral RNA and linear uncapped RNA with a 5′ phosphate. Comparison of Fig. 3A and B shows that TAP increased the ligatable

HIV-1 5′ RNA ends at 2 h postinfection in both the M4- and D4-treated cells, but at other times, the differences were not statistically significant. These data suggest that at 2 h postinfection, capped viral RNA was still present, but at later times, the amounts of capped viral RNA decreased markedly.

To measure only capped viral RNA in the infected cells, we used treatment of RNAs with CIP (which dephosphorylates existing uncapped linear viral RNA) followed by TAP (which removes caps, leaving a ligatable 5′ end) before 5′ RACE and qRT-PCR (Fig. 3C). The amounts of capped HIV-1 RNA were highest at 2 h postinfection, and they rapidly decreased to very low levels by 6 h postinfection (note the log scale of the graphs in Fig. 3)—consistent with early decapping of viral genomes during infection. Moreover, equal amounts of capped HIV-1 RNA were detectable with or without DBR1 knockdown (D4 versus M4) at all time points. These data indicated that DBR1 is not required for decapping of HIV-1 genomes early during infection.

DBR1 treatment *in vitro* restores detection of free 5′ ends in RNA from DBR1 shRNA-treated cells. To further investigate the nature of the RNAs in the infected cells, we digested the RNAs from Fig. 3A with purified yeast (Fig. 3D) or human (Fig. 3E) DBR1 enzyme prior to the 5′ RACE. *In vitro* incubation with yeast DBR1 enhanced detectable free 5′ ends of HIV-1 RNA in the D4-treated cells to the levels detected in the control, M4-treated, cells at each of the time points (Fig. 3D). In contrast, DBR1 treatment had no effect on detection of 5′ ends in the input virion RNA. These results indicated that the amounts of viral RNA in the infected D4- and M4-treated cells were similar, but that in the absence of DBR1 viral RNA accumulated in a form that could not be detected by 5′ RACE. Similar results were obtained when the infected cell RNAs were digested *in vitro* with human DBR1 (Fig. 3E).

To test whether the results of the DBR1 incubation were dependent on the 2′-5′ phosphodiesterase activity of DBR1, we treated the same RNAs with yeast DBR1(N85A), which is catalytically inactive (15). We observed no significant difference between the DBR1(N85A)-treated and untreated RNAs (compare Fig. 3A and F), indicating that the catalytic activity of DBR1 is necessary for unmasking 5′ ends of HIV-1 genomic RNAs for 5′ RACE in the DBR1 knockdown cells.

Treatment of infected cell RNAs with DBR1 before 5′ RACE would allow detection of either RNAs that were in lariat-like structures or linear uncapped RNAs (Fig. 1). Thus, the total viral RNAs in infected cells could be determined from the 5′ RACE signals of Fig. 3D (or E) combined with those of Fig. 3C that quantified capped viral RNA. It was apparent that essentially the same amounts of total viral RNA were present at all time points and without regard to DBR1 knockdown (note the logarithmic scales). Therefore, under these conditions of infection, uptake of virus was complete by 2 h postinfection, and the changes in concentrations of the different 5′ end forms represent conversions between the different species.

DBR1 knockdown protects HIV-1 RNA from RNase R digestion. To determine whether any HIV-1 vector RNA genome in infected cells is in a circular or lariat-like form, we treated RNAs from the time course experiments in Fig. 3 with RNase R, which degrades linear RNAs, but not loop portions of lariat RNAs or circular RNAs (15). RNase R treatment was followed by treatment with wild-type (WT) yeast DBR1, followed by 5′ RACE (Fig. 4). For the input virion RNA, this combination of treatments did not lead to a detectable 5′ RACE signal, consistent with the absence of lariat-like structures. Moreover, treatment of virion RNA with TAP followed by RNase R gave no signal, indicating that the virion RNA is linear, as expected (data not shown). In contrast, by 2 h postinfection, the 5′ ends of the HIV-1 RNA were in RNase R-resistant forms that could be detected by 5′ RACE after digestion with DBR1, for both M4- and D4-treated cells. This indicated conversion of the capped linear viral RNA in the virion to a lariat-like form that could be resolved by *in vitro* incubation with DBR1. Furthermore, as infection progressed in the control, M4-treated, cells, the 5′ end of the viral genome rapidly became RNase R sensitive. By 6 h postinfection, there was a more than 7-fold reduction in the amount of RNase R-resistant viral genome, consistent with resolution of the

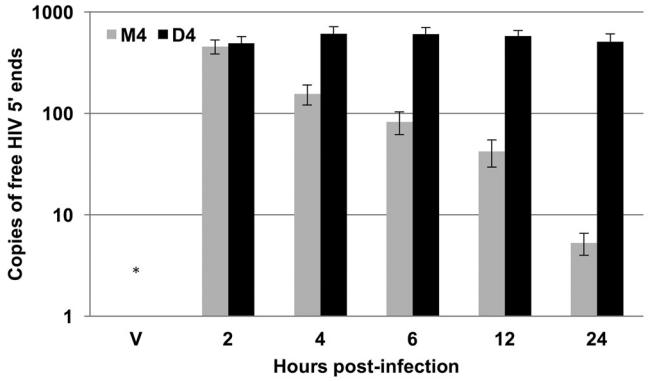


FIG 4 DBR1 knockdown in infected cells protects viral RNA from *in vitro* RNase R digestion. The same RNA samples used in Fig. 3 were treated with the RNase R followed by yeast DBR1 prior to 5′ RACE and qRT-PCR for quantitation of free 5′ ends in HIV-1 RNA. The data represent three independent experiments processed in duplicate. Error bars denote the standard deviations from the means. The asterisk indicates values below a detectable threshold.

lariat-like structures into linear uncapped RNA. In our previous studies of early HIV-1 infection, completion of strong-stop cDNA synthesis and minus-strand transfer occurred at 4 to 6 h after the initiation of infection (11, 12). In contrast, in the D4 DBR1 knockdown cells, the viral genome remained resistant to RNase R degradation but resolvable by DBR1 *in vitro*. This result indicates accumulation of the lariat-like viral RNA structure in these cells. Finally, regardless of DBR1 inhibition, we did not detect free HIV-1 vector genome 5′ ends when the RNA was treated with RNase R followed by DBR1(N85A) (data not shown).

Accumulation of a cellular intron after DBR1 knockdown. We also used the infected cell RNA samples to examine the effects of DBR1 knockdown on a cellular mRNA intron—intron 1 of GAPDH RNA. The same RNAs were analyzed for the presence of phosphorylated GAPDH intron 1 (presumably excised and linearized) by 5′ RACE and qRT-PCR (Fig. 5A). Inhibition of DBR1 (D4 treatment) led to a significant decrease (1.5- to 3.5-fold; $P < 0.01$) in the detection of excised GAPDH intron 1 RNA; the levels did not vary significantly from 2 to 24 h postinfection, which was expected since knockdown was initiated 2 days before. This reduction was likely due to the accumulation of unresolved GAPDH intron 1 lariats that were undetectable in the 5′ RACE assay. The fold difference between D4- and M4-transfected cells was relatively small—perhaps because once a lariat was resolved by DBR1 in M4 cells, the linearized intron RNA was relatively rapidly degraded through the nucleotide recycling pathway.

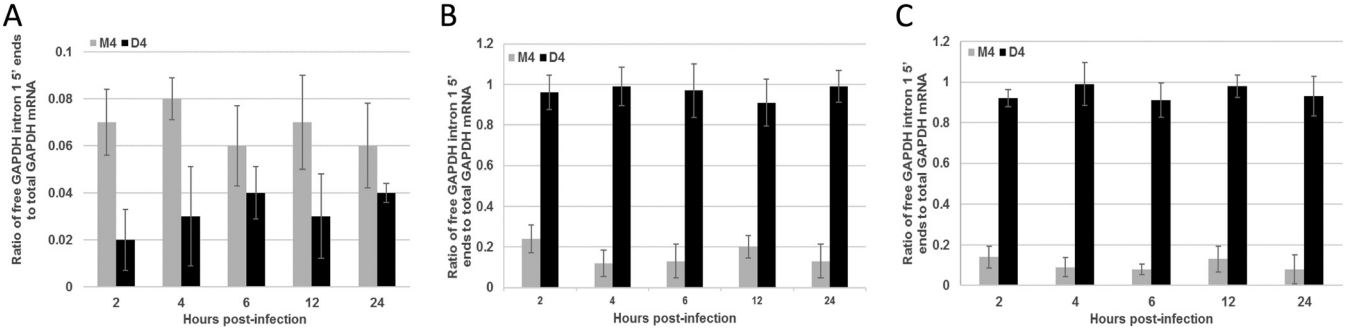


FIG 5 DBR1 knockdown affects GAPDH intron 1 accumulation. The same RNA samples used in Fig. 3 were treated as follows. In each case, the data represent three independent experiments processed in duplicate. Standard deviations from the means are denoted by whiskers. (A) RNA underwent the 5′ RACE reaction, and qRT-PCR was used to evaluate the ratio of free GAPDH intron 1 5′ ends to total GAPDH mRNA. (B) Aliquots of RNA were treated with yeast DBR1 prior to 5′ RACE and qRT-PCR for free 5′ ends in GAPDH intron 1 RNA. (C) RNA was treated *in vitro* with RNase R followed by yeast DBR1 prior to 5′ RACE and qRT-PCR for detection of free 5′ ends in GAPDH intron 1 RNA normalized to total GAPDH mRNA.

Treatment of the RNA from the D4-treated cells with yeast DBR1 before 5′ RACE resulted in a substantial increase (~30-fold) in detection of the GAPDH intron 1 (Fig. 5B), consistent with the great majority (>95%) being "trapped" in a lariat structure that could be resolved by DBR1 *in vitro* and detected by 5′ RACE. In contrast, in M4-treated cells, nearly 5-fold-lower levels of intron 1 RNAs were detected after DBR1 treatment, indicating that DBR1 knockdown led to accumulation of GAPDH intron 1 in lariats. As expected, treatment of the RNAs with the catalytically inactive DBR1(N85A) mutant, produced no change in the detection of GAPDH intron 1 free 5′ ends compared to the untreated samples (not shown).

Finally, the RNAs were first treated with RNase R followed by DBR1 and 5′ RACE. The majority of the GAPDH intron 1 RNA in the D4-treated cells was protected from RNase R digestion and could be detected by 5′ RACE after treatment with active DBR1 enzyme (Fig. 5C). In contrast, no GAPDH intron 1 5′ ends were detected when the RNAs were treated with RNase R followed by DBR1(N85A) (not shown). Thus, knockdown of DBR1 caused an accumulation of GAPDH intron 1 RNA in an RNase R-resistant form, consistent with a lariat. For the control, M4-treated, cells (no DBR knockdown) there was a small but significant difference ($P < 0.01$) in the detected GAPDH intron 1 RNA between the samples treated or not treated with RNase R prior to DBR1 incubation and 5′ RACE (Fig. 5B versus C). This would be consistent with the presence of some resolved and linearized introns in these cells, along with introns in lariat forms.

DISCUSSION

In this report we investigated the mechanism by which DBR1 mediates HIV-1 replication. We previously showed that knockdown of DBR1 by an shRNA decreased HIV-1 cDNA synthesis and replication (11, 13). The studies described here investigated the nature of the viral genome during the first 24 h of infection by focusing on the state of the 5′ end of the viral RNA. Features of these experiments included comparison of cells with and without DBR1 knockdown and use of a viral vector preparation containing RT with the E478Q mutation that allowed analysis of the genomic RNA without the complication of RT-mediated degradation of the template RNA. Our studies employed 5′ RACE to detect free (uncapped and phosphorylated) 5′ ends of genomic RNAs, and qRT-PCR of the products allowed improved analysis. In addition, *in vitro* digestion of RNAs from infected cells with different RNA-modifying enzymes prior to the 5′ RACE allowed us to distinguish capped linear viral RNA, viral RNA in a circular or lariat-like configuration, and uncapped linear viral RNA. Input viral vector RNA was capped and linear, as expected, but in infected cells, the viral RNA was converted rapidly (beginning by 2 h postinfection) into other forms. In control cells, the temporal order of these forms was (i) capped linear, (ii) lariat-like, and (iii) uncapped linear, and the timing was consistent with resolution of the lariat-like form concomitant with minus-strand transfer during cDNA synthesis. In DBR1 knockdown cells, there was an accumulation of lariat-like structures as well as a decrease in uncapped linear viral RNA, consistent with a requirement for DBR1 to resolve the lariats into linear viral RNA. In support of this, *in vitro* incubation of RNA from infected DBR1 knockdown cells with purified DBR1 enzyme increased the amount of ligatable 5′ viral RNA ends. Moreover, this was dependent on the catalytic activity of DBR1. The accumulation of lariat-like viral RNAs in infected DBR1 knockdown cells was also consistent with the persistence of the 5′ end of viral RNA in a form that was resistant to RNase R. The results reported here are consistent with formation and resolution of a lariat-like structure involving the 5′ end of HIV-1 RNA during reverse transcription in infected cells, as previously proposed for yeast Ty1 retrotransposition.

Previous studies suggesting an RNA lariat during yeast Ty1 retrotransposition have been controversial. The original study by Cheng and Menees indicated that the 5′ end structures of the Ty1 RNA in wild-type and *dbr1* mutant yeast strains were differentially accessible by 5′ RACE (5). In their study, they pretreated the RNAs with phosphatases and pyrophosphatases prior to annealing the RNA oligonucleotide to enable amplification of only capped RNAs; they compared the results with reactions where there was

no pretreatment, which would detect all uncapped RNAs. They found that in *dbr1* mutants, there was little or no detection of free 5′ ends regardless of the treatments, while in wild-type controls, 5′ ends were detectable in both the capped and uncapped forms (5). However, the results were not reproduced by Coombes and Boeke (10), who used the same 5′ RACE strategy and a semiquantitative RT-PCR analysis as well as sequencing of the amplified product. The latter investigators reported that there was no difference in the sequenced amplified products and no obvious difference in the amplification of 5′ ends between wild-type and *dbr1* mutant samples. These differing results prompted development of our strategy for analysis of the 5′ ends of HIV-1 RNA in infected cells, which employed 5′ RACE and qRT-PCR and which allowed identification of differences that may not have been evident in standard or semiquantitative RT-PCR. Moreover, *in vitro* digestion of the infected cell RNAs with different RNA-modifying enzymes prior to 5′ RACE allowed us to distinguish capped, lariat-like, and linear uncapped RNAs; the results with the different enzymes were internally consistent.

We found a significant difference in the detection of 5′ HIV-1 RNA ends between DBR1 shRNA-treated cells and controls (Fig. 3A), although it was not an absolute difference as was reported by Cheng and Menees (5) for yeast Ty1 retrotransposition. Analogous to the results of Coombes and Boeke (10), we could detect both capped and uncapped 5′ ends regardless of DBR1 inhibition, but in our studies, we found quantitative differences in the signals from control and DBR1 knockdown cells. This could be due to the fact that we employed a more sensitive qRT-PCR assay for quantification or that our studies were on HIV-1 employing shRNA knockdown versus yeast Ty1 retrotransposition in WT and *dbr1* mutant strains of *S. cerevisiae*.

In our previous study (13), we found that DBR1 was necessary for HIV-1 cDNA synthesis in infected cells under our standard conditions. However, if nuclear import of viral particles was blocked by the presence of a truncated form of the nuclear import factor CPSF-6, cDNA synthesis still occurred, but in this case it was cytoplasmic. Under these conditions, cytoplasmic cDNA synthesis was not DBR1 dependent. Thus, there appear to be both DBR1-dependent and -independent mechanisms of reverse transcription, although under standard conditions the DBR1-dependent mechanism seems to predominate. The existence of DBR1-independent reverse transcription could also be related to the fact that complete reverse transcription of viral RNA into double-stranded DNA can occur during *in vitro* incubation of isolated retroviral virions in the absence of added cellular factors, although it is inefficient (18).

Taken together, these results support a model in which lariat formation of viral genomic RNA is important for minus-strand transfer, with DBR1 being required to resolve the lariat to allow reverse transcription to proceed. Future experiments will focus on further elucidating the lariat-like structure, identifying cellular factors involved in its formation, and testing the overall model.

MATERIALS AND METHODS

Construction of siRNA expression vectors. The shRNA expression vector pHyper was a gift of Vicente Planelles, and we have described it previously (13). Briefly, the vector expresses a 23-nucleotide hairpin-type shRNA with a 6-nucleotide loop. The 19-nucleotide DBR1 sequence for D4, CTTGCTGTGCT GCGGCGAC, was used to create the DBR1 shRNA plasmid, designated pHyper-D4. The 19-nucleotide DBR1 triple-mismatch sequence for M4, CTTCCTGTACTGCAGCGAC, was used to create the DBR1 shRNA plasmid designated pHyper-M4.

Preparation and titration of HIV vector stocks. The pCMVΔR8.2-E478Q plasmid was derived from pCMVΔR8.2 (19, 20); both plasmids encode HIV-1 structural proteins and only differ in that pCMVΔR8.2 E478Q encodes the RNase H catalytically inactive reverse transcriptase mutant RT(E478Q) (14). The pMDG plasmid expressed the vesicular stomatitis virus G protein (VSV-G) (19, 20). The pHR-E vector plasmid contains a truncated HIV-1 *env* gene and the HIV-1-derived *cis*-acting sequences necessary for packaging, reverse transcription, and integration. Viral vector stocks were prepared by triple transfecting 293T cells (3.0×10^7 cells in 500-cm^2 plates) with 75 μg each of the pHR-E, pCMVΔR8.2-E478Q, and pMDG plasmids coprecipitated with calcium phosphate. Two days posttransfection, culture supernatant was collected and frozen at −80°C until needed. VSV-G-pseudotyped HIV-1 vector virions in the supernatant were quantified using quantitative real-time PCR (qPCR).

Cell culture, DNA transfection, and infection. GHOST-R5X4 cells have been extensively described previously (21). GHOST-R5X4 cells were cultured in Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal bovine serum (FBS), 50 μg/ml gentamicin, and 500 μg/ml G418. Human embryonic

kidney 293T cells were cultured in IMDM supplemented with 10% FBS and 50 μg/ml gentamicin. GHOST-R5X4 cells were seeded overnight at 10^6 cells per well in a six-well plate prior to transfection. Cells were transfected using Lipofectamine (Invitrogen) according to the manufacturer's instructions at a ratio of 1 μl of Lipofectamine per μg of plasmid DNA. Forty-eight hours after transfection, cells were infected with DNase I-treated viral stocks of VSV-G-pseudotyped HR-E containing the RNase H-deficient RT(E478Q) mutant. Cells were harvested for RNA isolation using TRIzol (Invitrogen) according to the manufacturer's instructions.

5′ RACE and RNA enzyme treatments. RNA was quantified by measuring the absorbance at 260 nm. RNA (2.5 μg) was treated with 2 U of DNase I (Fermentas) in 10 μl to remove contaminating DNA followed by heat inactivation of the enzyme (37°C for 20 min, 60°C for 5 min). RNA was then phenol-chloroform extracted and ethanol precipitated prior to subsequent treatments. RNA was treated when noted, with RNase R (Epicentre) according to the manufacturer's instructions. Two units of enzyme was used to treat the RNA at 37°C for 1 h. RNA was then phenol-chloroform extracted and ethanol precipitated prior to subsequent treatments. When noted, the RNA underwent debranching by incubation with DBR1. Yeast DBR1 and DBR1(N85A) were a gift from Beate Schwer (Weill Cornell Medical College, New York) (15). Human DRB1 was a gift from Eric Montemayor (University of Texas Health Sciences Center, San Antonio). Incubation with DBR1 was carried out as described previously (15) in 20 μl of 50 mM Tris-HCl (pH 7.0), 4 mM $MnCl_2$, 2.5 mM dithiothreitol (DTT), 25 mM NaCl, 0.01% Triton X-100, 0.1 mM EDTA, and 0.15% glycerol at 30°C for 90 min. RNA was then phenol-chloroform extracted and ethanol precipitated prior to subsequent treatments. Activity of the debranching enzymes was assayed by qRT-PCR using primers for the 5′ end RNA oligonucleotide and GAPDH intron. RNA was treated when noted with calf intestinal alkaline phosphatase (CIP [New England BioLabs]) in 10 μl of 50 mM Tris-HCl (pH 7.9), 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT at 25°C for 1 h. RNA was then phenol-chloroform extracted and ethanol precipitated prior to subsequent treatments. The RNA was treated when noted with tobacco acid pyrophosphatase (TAP [Epicentre]) in 10 μl of 50 mM sodium acetate (pH 6.0), 1 mM EDTA, 0.1% β-mercaptoethanol, and 0.01% Triton X-100 at 37°C for 1 h. RNA was then phenol-chloroform extracted and ethanol precipitated prior to ligation. All RNA samples underwent an RNA ligation reaction utilizing T4 RNA ligase I (New England BioLabs) according to the manufacturer's instructions. The RNA oligonucleotide utilized was G5-RNA (CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA). The reaction was carried out in 20 μl with 2 U of enzyme at 37°C for 1 h. RNA was then phenol-chloroform extracted and ethanol precipitated for qRT-PCR.

Real-time qRT-PCR. RNA was reverse transcribed using a first-strand cDNA synthesis kit (Fermentas) following the manufacturer's instructions. The total cDNA volume of 20 μl was frozen until real-time qPCR was performed. After thawing for PCR experiments, the cDNA was diluted 1:10 in distilled water (dH_2O), and 2 μl of diluted cDNA was used for each PCR. Each PCR mixture contained 7.5 μl SYBR green PCR master mix (Fermentas), 2 μl of template, and 1 μM each primer in a 15-μl reaction mixture. The RNA oligonucleotide-specific primer GeneRacer 5O (CGACTGGAGCACGAGGACACTGACAT [nucleotides 1 to 26]) and HIV-1-specific primer AA55 (CTGCTAGAGATTTTCCACACTGAC [nucleotides 635 to 612]) were used to detect free HIV-1 5′ ends. The RNA oligonucleotide primer G5O-15F (CGACTGGAGCACGAG [nucleotides 1 to 15]) and the GAPDH intron 1-specific primer GAPDH intron-1-93 (GGTGGGAACAGGTC CCCT [nucleotides 93 to 76]) were used to detect free GAPDH intron 1 5′ ends. The forward and reverse primer sequences for amplifying GAPDH were CAAATTCCATGGCACCGTCAA (nucleotides 264 to 284) and GTTGCTGTAGCCAAATTCGTTGT (nucleotides 333 to 314). Relative HIV-1 copy number was calculated by dividing the HIV-1 copy number (determined from the appropriate standard curve) by the GAPDH copy number.

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