Human APOBEC3G (hA3G) has been identified as an anti-HIV-1 host factor (1). hA3G belongs to an APOBEC superfamily containing at least 10 members, which share a cytidine deaminase motif (a conserved His-X-Glu and Cys-X-Cys zinc coordination motif) (2). The APOBEC family in humans includes APOBEC1 (hA1), APOBEC2 (hA2), APOBEC3A-H (hA3A-H), and activation-induced cytidine deaminase. The APOBEC proteins are capable of inhibiting the replication of a wide variety of retroviruses and non-retroviruses, suggesting that these proteins represent a novel component of innate immunity to viral infection (for review, see Refs. 3 and 4). The virus counters hA3G’s anti-viral activity with the viral protein Vif (virion infectivity factor), which binds to hA3G, and targets hA3G for proteasomal degradation (5, 6). Vif is thus required for HIV-1 replication in cell types that constitutively express hA3G (termed “non-permissive” cells), such as primary T lymphocytes, macrophages, and T-cell lines such as H9. Vif is not required for viral replication in cells not expressing hA3G (“permissive” cells) such as SupT1, Jurkat, 293, HeLa, and CEM-SS lines (7–9).

HIV-1 containing hA3G shows a reduced ability to produce new viral DNA upon infecting cells (10–13). It has been suggested that this results from the degradation of newly synthesized viral DNA, rather than the inhibition of new DNA synthesis. Thus, because the small amount of minus strand cDNA that is made in newly infected cells (~5% of wild type) contains 1–2% of the cytosines deaminated by hA3G to form uracil (12, 14–16), it has been suggested that most newly synthesized viral DNA edited in this manner will be degraded by the DNA repair system. For example, DNA glycosylases such as Ung2, a uracil DNA glycosylase packaged into HIV-1 (17, 18), can recognize an altered base and remove the base by apurinic/apyrimidinic endonuclease 1, resulting in either a 5’-deoxyribose phosphate group that is a substrate for DNA repair enzymes or in the degradation of the DNA (19). However, a reduction in Ung2 incorporation into HIV-1 containing hA3G, using either an Ung2 inhibitor or virus-producing cells lacking endogenous Ung2 activity, had no effect on the antiviral capacity of hA3G, i.e. in the presence of hA3G, viral infectivity and resulting synthesis of viral DNA are reduced equally with or without viral Ung2 (20). Furthermore, several reports have shown that mutant hA3G (21–23) and mutant hA3F (24) that have lost their cytidine deaminase activity still show strong anti-HIV-1 activity and a reduction in viral DNA synthesis, and hA3F and/or hA3G can also inhibit hepatitis B virus replication with little or no editing (25, 26).

One should then consider the possibility that hA3G inhibits reverse transcription. We have recently reported that Vif-negative virions containing hA3G show a 55% reduction in the production of early viral DNA in newly infected cells and that this is correlated with a similar reduction in the initiation of reverse transcription (22). In this report, we provide further evidence that the greater (~95%) reduction in late DNA synthesis can be accounted for by the hA3G-induced inhibition of minus and plus strand transfer steps in reverse transcription.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction, Cell Transfections, and Virus Purification**—BH10 is a simian virus 40-based vector that contains full-length wild-type HIV-1 proviral DNA. The construction of BH10.Vif+, as well as wild-type and mutant forms of
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hA3G has been previously described (27). The culture and transfection of HEK-293T cells with these plasmids using Lipofectamine 2000 (Invitrogen), and the isolation of virions 48 h post-transfection from the cell supernatant, were done as previously described (22, 27). Unless stated otherwise, 293T cells were transfected with 2 μg of HIV-1 proviral DNA and 1 μg of plasmid coding for wild-type or mutant forms of hA3G. The total amount of plasmid DNA used for transfection was kept constant in controls by replacing plasmid coding for hA3G with the empty vector, pcDNA3.1. Viral p24 was measured with a commercial kit available for p24 antigen capture (Abbott Laboratories). Culture of SupT1 and its infection with the HIV-1 produced from 293T cells, were as previously described (22).

**Protein Analysis**—Cellular and viral proteins were extracted with radioummune precipitation assay buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40, 2 mg/ml aprotinin, 2 mg/ml leupeptin, 1 mg/ml pepstatin A, 100 mg/ml phenylmethylsulfonyl fluoride). The cell and viral lysates were analyzed by SDS-PAGE (10% acrylamide), followed by blotting onto nitrocellulose membranes (GE HealthCare). Western blots were probed with monoclonal antibodies that are specifically reactive with HIV-1 capsid (Zepto Metros Inc.), hA3G (NIH AIDS Research and Reference Reagent Program), hemagglutinin (HA, Santa Cruz Biotechnology, Inc.), and β-actin (Sigma), or with Vif-specific polyclonal antisemum to HIV-1 (NIH AIDS Research and Reference Reagent Program). Detection of proteins was performed by enhanced chemiluminescence (PerkinElmer Life Sciences), using as secondary antibodies anti-mouse (for hA3G, capsid, HA, and β-actin) and anti-rabbit (for Vif) antibodies, both obtained from GE HealthCare. Bands in Western blots were quantitated using the ImageJ 1.35s automated digitizing system (National Institutes of Health).

**Quantitation of Viral Genomic RNA in the Cell**—SupT1 cells were infected with equal amounts of wild-type or Vif-negative HIV-1, and then were collected at the different time point of post-infection, as above described. Total cellular RNA was extracted with TRIzol (Invitrogen), and β-actin mRNA was quantitated using real-time reverse transcription (RT)-PCR as described previously (28). Samples of total cellular RNA containing equal amounts of β-actin mRNA were used to generate cDNA containing the 5′-terminal repeat of the viral genome, using SuperScript II reverse transcriptase (Invitrogen) and the U5-R primer, 5′-GGGTCTGAGGATCTCAG-3′. The amount of cDNA containing the 5′-terminal repeat of the viral genome was determined using real-time fluorescence-monitored PCR with a pair of primers US-R forward and reverse, as described above.

**In Vitro Analysis of Minus Strand Transfer**—Using the MEGAscript kit (Ambion), an RNA template of 384 nt was synthesized from the linearized Tp-2 DNA (29). The RNA template contained both the 5′ and 3′ R regions and is competent to support strand transfer. An 18-nt DNA oligonucleotide (5′-GTCCTGTGTTGAGGGGCCCA, complimentary to the PBS, was used as primer in the reactions described below. The DNA oligomer, 5′-end-labeled using [γ-32P]ATP and T4 kinase, was annealed to the RNA template by heating at 85 °C for 5 min and gradually cooling to room temperature. Final reactions contained 2.25 μM NCP7, 50 nM RNA template, 100 nM primer DNA, 0.1 mM of each dNTP, 100 ng of RT, 50 mM Tris-Cl (pH 7.5), 75 mM KCl, 5 mM Mg-Cl, 1 mM dithiothreitol, and 10 units of RNase inhibitor (Ambion), in a volume of 20 μl. Various concentrations of purified hA3G or hA3G-containing cell lysate were added to the reaction mixtures to test their effects on minus strand transfer. Reactions were incubated at 37 °C for 40 min, followed by the addition of 1 μl of protease K, and further incubated at 65 °C for 20 min. After extraction by phenol/chloroform, the deproteinized reaction products were resolved by electrophoresis in a 5% denaturing polyacrylamide gel containing 7 M urea, and the different bands were quantitated by phosphorimaging (Molecular Dynamics). Minus strand transfer efficiency is determined by the ratio of T-DNA (transfer DNA, 296 nt): total DNA (minus strand strong stop (ss) DNA (200 nt) plus T-DNA).

Recombinant hA3G was produced in the Baculo Expression System as a T-tag fusion protein, and was provided by the NIH AIDS Research and Reference Reagent Program (Catalog number 10068). The 72-amino acid HIV-1 NCP7 peptide used in this analysis was prepared by solid-phase chemical synthesis as previously described (30). Recombinant HIV-1 RT (p66/51)
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In this work, we have investigated whether the reduction in the production of late DNA synthesis is due to the inhibition by hA3G of either DNA elongation, strand transfers, or of both these processes. Using real-time fluorescence-monitored PCR with equal amounts of cellular DNA, we monitored over a 24-h period post-infection the synthesis of viral cDNA intermediates in a permissive cell line, SupT1, that had been infected with Vif-negative HIV-1 containing or lacking hA3G. As shown in Fig. 1A, five pairs of primers were used to quantitate viral cDNA intermediates containing sequences for either R-U5, R-U3, pol, gag, or U5-gag, which represent, respectively, −sss DNA, post-minus strand transfer of early, middle, and late minus single strand DNA, and post-plus strand transfer late DNA. As shown in Fig. 1 (B–F), −sss DNA (R-U5) and late viral DNA production after the second strand transfer (U5-gag) reached maximum concentrations at 8 and 12 h post-infection, respectively, with intermediate DNA species reaching peak concentrations at intermediate times.

A comparison of the synthesis of each DNA region in the absence or presence of hA3G, is presented graphically in Fig. 1G. In agreement with our previous report (22), the presence of hA3G in Vif-negative HIV-1 caused a 48% reduction in −sss DNA and a 96% reduction in the full-length minus single strand DNA. A new observation is that the reduction in late DNA synthesis appears to reflect inhibition of the DNA strand transfer steps and not the rates of DNA elongation. Thus, the similar reductions in the three minus strand viral DNA intermediates produced after the first strand transfer (84%, 83%, and 85% for R-U3, pol, and gag, respectively) suggests that hA3G has no significant impact on the extension of minus strand viral DNA after the minus strand transfer. However, it can be seen that an additional drop in DNA production occurs after the second strand transfer (U5-gag). These results suggest that the reduction in late viral DNA synthesis by hA3G may be caused primarily by the inhibition of strand transfers during reverse transcription, and this is further examined below using an in vitro minus strand transfer system.

hA3G Inhibits the Minus Strand Transfer in Vitro—The ability of hA3G to inhibit minus strand transfer was measured using an in vitro assay that is described under "Experimental Procedures" and is depicted in Fig. 2A. A DNA primer, 5′-end-labeled with 32P, and complimentary to the PBS, was annealed to the PBS of a synthetic RNA template that contains, in sequence, R, U5, PBS, U3, and R, and serves as both the donor and acceptor RNA in the strand transfer reaction. The addition of HIV-1 RT produces a 200-nucleotide −sss DNA, whereas the addition of HIV-1 mature nucleocapsid (NCp7) is used to facilitate minus strand transfer and generate a 296-nucleotide transfer DNA product (T-DNA). Resolution of the radioactive...
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FIGURE 1. Effect of hA3G on production of HIV-1 DNA. Cellular DNA was extracted at different times post-infection from SupT1 cells infected with BH10Vif—lacking or containing hA3G. Viral DNA intermediates were monitored by real-time PCR, as described under “Experimental Procedures.” A, schematic diagram of reverse transcription. Small arrows indicate the PCR primers used to detect the following DNA intermediates: R-US (minus strand strong stop DNA intermediates in the absence (spots) or presence (lines) of hA3G. The bar graphs in panel G represent the means of results of experiments performed at least three times, and the error bars represent standard deviations.

reaction products by one-dimensional PAGE is shown in Fig. 2B. The synthesis of these two DNA species is seen in lane 3. The reaction products resolved in lanes 1 and 2 show no strand transfer and contain either RNaseH-negative RT (lane 1) or lack NCp7 (lane 2).

A recent study has revealed that ~7 (±4) molecules of hA3G are incorporated into virions produced from activated human peripheral blood mononuclear cells (36). This result suggests that, during reverse transcription, the molar ratio of hA3G to HIV-1 genomic RNA is ~3.5:1. To avoid a biologically irrelevant effect resulting from a high concentration of hA3G, we have tested low concentrations of purified hA3G, from 0.05 to 12.5 nM in the reactions, in which the molar ratio of hA3G to the 50 nM RNA template never exceeds 0.25. Similarly, estimates of NCp7 molecules/virion have ranged from 1400 (37) to 5000 (38). Thus the hA3G:NCp7 molar ratio in virions may be 0.001–0.005. The in vitro strand transfer reaction contains 2.25 μM NCp7, and thus never exceeds an hA3G:NCp7 molar ratio of 0.006.

As shown in Fig. 2B, with increasing concentrations of purified hA3G added to the strand transfer reaction, the synthesis of T-DNA is gradually reduced (lanes 4–8), and Fig. 2C plots the relative amounts of total DNA and DNA resulting from minus strand transfer (T-DNA/total DNA) obtained with increasing hA3G, whose concentrations are plotted on a logarithmic scale. Inhibition of minus strand transfer by hA3G increases from 11% to 76% as the hA3G concentrations increase from 0.2 nM to 3.2 nM. Experiments in which hA3G was replaced with bovine serum albumin did not result in any inhibition of minus strand transfer (data not shown). hA3G binds to both DNA and RNA (2, 39), but the replacement of hA3G with another RNA-binding protein, QKI-6 (lane 10) (40), has no effect upon strand transfer, making it less likely that hA3G inhibits strand transfer by sterically blocking the annealing of DNA to viral RNA.

Although hA3G strongly reduced
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This observation suggests that the reduction in strand transfer DNA product induced by hA3G did not result from a general inhibition of reverse transcription by hA3G. This is shown further in Fig. 2D, in which increasing concentrations of hA3G are added in the absence of NCp7, and no changes in the synthesis of −ssDNA are seen.

Inhibition of Minus Strand Transfer Occurs Independently of hA3G Editing—We next investigated whether the hA3G inhibition of minus strand transfer depends upon the editing activity of this enzyme. The editing activity of hA3G is specific for single-stranded DNA (41), and only the −ssDNA is a potential editing substrate for hA3G during minus strand transfer. As indicated in Fig. 1A, minus strand transfer is facilitated by annealing of sequences in the −ssDNA (R’) to the complementary R sequence at the 3’ terminus of viral RNA. C to U mutations introduced into R’ could hinder first strand transfer. We therefore cloned and sequenced the R-U5 region of −ssDNA produced from the in vitro strand transfer reaction. Ten independent nucleotide sequences were determined for −ssDNAs that had been produced in the absence or presence of hA3G, and no C to T (U) mutations were detected. This confirms previous observations that DNA mutations induced by hA3G were least frequent in the 5’-long terminal repeat, probably representing less than 3% of total cytidines within the R-U5 region (41).

We also tested the ability of mutant hA3G lacking editing activity to inhibit minus strand transfer in vivo, determined by ratios of the early DNA intermediate synthesized immediately after the minus strand transfer in vivo (U3-R) to −ssDNA, using real-time fluorescence-monitored PCR, as described above. The mutant forms of hA3G tested are shown in Fig. 3A. hA3G 1–156 and hA3G 105–384 contain the N- or C-terminal zinc coordination motifs, respectively, whereas hA3G 104–245 contains the linker sequences between these two motifs. We have previously reported that all three hA3G fragments were incorporated efficiently into HIV-1, but none were found to deaminate viral DNA in vivo (22, 27). 293T cells were transfected with plasmids coding for either wild-type or mutant forms of hA3G (Fig. 3A), and Western blots of viral lysates (Fig. 3B) show approximately equal viral content of these wild-type and mutant hA3G species. SupT1 cells were then infected with equal amounts of viruses containing similar amounts of wt or mutant hA3G species, and the effect of each mutant form of hA3G on minus strand transfer was quantitated. The results are graphically presented in Fig. 3B. Both hA3G 1–156 and hA3G 105–384, which show very little editing activity, inhibit the first strand transfer in vivo 60–70% as efficiently as wild-type hA3G. hA3G 104–245, missing both zinc coordination motifs, has almost no effect on the strand transfer. These results indicate that the inhibition of strand transfer can occur independently of the cytidine deamination editing activity of hA3G.

We have also analyzed the effect of the wild-type and mutant hA3G species upon minus strand transfer in vitro. Because we were unable to purify mutant hA3G fragments, we have tested the abilities of lysates of 293T cells expressing one or another of these species to inhibit minus strand transfer in vitro. An RNase inhibitor, SUPERaseIn™, was added during the preparation of cell lysates. This inhibitor is effective only against RNase A-type enzymes, but not RNaseH, and therefore does not interrupt the strand transfer assay. The Western blot in Fig. 4B shows the content of each hA3G species in the cell lysate per equal amounts of β-actin.

As shown in Fig. 4B, increasing amounts of 293T cell lysate (25–200 ng of cell protein) containing wild-type hA3G caused a gradual reduction in T-DNA products in a dose-dependent manner (lanes 3–6), whereas addition of cell lysate not expressing hA3G had no effect on minus strand transfer (lanes 7–10). In Fig. 4C, the ratios (T-DNA/total DNA) obtained relative to the ratio obtained for strand transfer in the absence of hA3G (panel A, lane 2) are plotted, and it can be seen that 1) minus strand transfer was inhibited from 12% to 74%, and 2) no significant reduction in total DNA synthesis was detected. These results demonstrate that, as with purified hA3G, hA3G present in cell lysates can be used to interrupt minus strand transfer in
The effect of wild-type and mutant hA3G upon minus strand transfer in vivo. A, wild-type and mutant hA3G. The filled rectangles represent the two zinc coordination units, and the numbers represent the amino acid positions. B, Western blots of lysates of Vif-negative HIV-1 produced from 293T cells expressing wild-type or mutant hA3G. Upper panel: blots probed with anti-HA. Lower panel: blots probed with anti-p24. C, comparison of abilities of viral mutant hA3G species to inhibit strand transfer in vivo and deaminate viral DNA. The in vivo inhibition of strand transfer was measured using real-time RT-PCR, as described in Fig. 1. The cytidine deaminating activity of wild-type and mutant hA3G species was determined by sequencing viral DNA with anti-HA.

We further investigated the effect of hA3G on the degradation of RNA template using the in vitro minus strand transfer assay described in Fig. 2, except that the synthetic RNA template was labeled by the incorporation of [α-32P]UTP, and an unlabeled DNA oligonucleotide primer was used. The full-length (384 nucleotides) labeled RNA was resolved by one-dimensional PAGE. Under reverse transcriptase conditions, but in the absence of added RT and hA3G, the full-length RNA remains intact (Fig. 5B, lane 7), whereas incubation with wild-type RT for 20 min generates one major 200 nucleotide cleavage product, accompanied by a >95% reduction in full-length RNA (Fig. 5B, lane 1). The addition of increased concentrations of hA3G (0.8, 3.2, and 12.5 nM) was accompanied by increased amounts of full-length RNA in a dose-dependent manner.
resulting in 11%, 24, and 37% of full-length RNA remaining (Fig. 5B, lanes 2–4). The use of RNaseH(–)/H11002 RT for reverse transcription resulted in no significant reduction in the RNA template, with or without the treatment of 12.5 nM hA3G (Fig. 5B, lanes 5 and 6), indicating that the decrease in RNA template observed did result from RNaseH-mediated RNA degradation, and that the retention of RNA template by hA3G reflects its ability to interrupt the degradation process.

HIV-1 NCp7 has been shown to facilitate the degradation of RNA template during DNA strand transfer (43). Because an interaction of hA3G with NCp7 may facilitate the incorporation of hA3G into virions, we investigated whether such an interaction might also be responsible for the inhibition of RNA template degradation, by performing the in vitro assay described above in the absence of NCp7. In Fig. 5C, we have plotted the amount of RNA template remaining during reverse transcription with or without NCp7 in the presence of increasing hA3G and compared this to a plot of the amount of first strand transfer occurring with increasing hA3G (obtained from Fig. 2C). Comparison of the three plots indicates that reduced RNA template degradation occurs independently of the presence of NCp7 and is correlated with the inhibitory effect of hA3G on the minus strand transfer.

**DISCUSSION**

In this report and elsewhere (22), we have presented evidence that a strong reduction in reverse transcription plays an important role in the inhibition of HIV-1 replication by hA3G. We have previously reported that the reduction in production of –sss DNA (~55%) is correlated with a similar reduction in the ability to initiate reverse transcription (22), whereas in this report, evidence is presented that the reduction in late DNA synthesis (~95%) is correlated with an hA3G-induced inhibition of minus and plus strand transfer. As shown in Fig. 1, hA3G inhibits the minus and plus strand transfers during reverse transcription in vivo. In the presence of viral hA3G, –sss DNA synthesis decreases ~2-fold. Synthesis of viral DNA immediately after the minus strand transfer (U3-R) decreases another 3- to 4-fold, without further reduction in the synthesis of later minus strand DNA sequences (pol and gag). However, synthesis of plus strand DNA after the plus strand transfer (U5-gag) undergoes another 3- to 4-fold decrease. To test our interpre-

**FIGURE 4.** The effect of wild-type and mutant hA3G upon minus strand transfer in vitro. A. Western blots of lysates of 293T cells expressing wild-type or mutant hA3G. Upper panel: blots probed with anti-HA. Lower panel: blots probed with anti-β-actin. B, resolution by one-dimensional-PAGE of –sss DNA and T-DNA, synthesized in the presence of increasing amounts (25 to 200 ng of cell protein) of cell lysates containing (lanes 3–6) or lacking (lanes 7–10) hA3G. Lanes 1 and 2, reactions contain no hA3G, and lack or contain NCp7, respectively. C, quantitation of panel B data. y axis: total DNA = –sss DNA plus T-DNA; strand transfer = T-DNA/total DNA. D, comparison of abilities of mutant hA3G variants (Fig. 3A) to inhibit strand transfer in vitro. The inhibition of strand transfer was measured as for experiments using wild-type hA3G described in Figs. 2. The bar graphs in D represent the means of results of experiments performed at least three times, and the error bars represent standard deviations.
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During minus strand transfer, the release of the annealed DNA from the 5’-region of template RNA requires the degradation of template RNA by the RNaseH activity associated with reverse transcriptase. Fig. 5 demonstrates that hA3G inhibits the degradation of the template RNA, both in vivo and in vitro. The mechanism for this remains unclear. Because hA3G has been reported to bind to RT (44), there might be a direct interaction between hA3G and RT that reduces RNaseH activity without affecting DNA elongation, because the polymerase activity of HIV-1 RT is not coupled to its RNaseH activity (45). Alternatively, because hA3G is able to bind efficiently to single-stranded DNA and RNA (39), the interaction of hA3G with the RNA/DNA hybrid may make the substrate less available to RNaseH.

hA3G may also disrupt NCp7 function in reverse transcription by binding to NCp7. hA3G inhibits both tRNA\textsuperscript{A\textsubscript{ly}3\textsubscript{ys}} priming (22) and DNA strand transfer, and both processes are facilitated by NCp7 (42), whose sequences in Gag are also required for the incorporation of hA3G into HIV-1 (27, 46–49). NCp7 is required for strand transfer, but the experiment shown in Fig. 5B is not a strand transfer reaction, i.e. inhibition of cleavage of RNA template by hA3G occurs independently of strand transfer, and of NCp7. So, although hA3G could inhibit strand trans-
fer by inhibiting template RNA cleavage, hA3G may alternatively, or in addition to inhibiting RNA cleavage, inhibit strand transfer through its interaction with NCp7. NCp7 is known to be important in facilitating strand transfer by both facilitating removal of cleaved RNA from the DNA template (which was not measured in the experiments in Fig. 5) and through facilitating annealing of the DNA to the RNA acceptor (42). Whether hA3G has an affect on either of these two steps in strand transfer is not known, but this remains a possibility because it has been reported that hA3G inhibits tRNA^Lys^ annealing to viral RNA through its interaction with NCp7 (50). Indeed, as shown in Fig. 5C, the addition of hA3G is able to inhibit ~80% of minus strand transfer in vitro but only 37% of RNA template degradation.

We have previously shown that, in HIV-1 produced from 293T cells transfected with 1 μg of hA3G plasmid, the viral content of hA3G is ~10 times greater than in virions produced from the naturally non-permissive H9 cell line (22). The question arises as to whether the inhibition of strand transfer during viral DNA synthesis that occurs after viral infection of SupT1 cells is an artifact of this increased content of viral hA3G. We suggest that this is not likely, based upon studies on the effect of cells is an artifact of this increased content of viral hA3G. Virions produced from 293T cells expressing hA3G also show a similar reduction in tRNA^Lys^ annealing to viral RNA through its interaction with NCp7 (50). Indeed, as shown in Fig. 5C, the addition of hA3G is able to inhibit ~80% of minus strand transfer in vitro but only 37% of RNA template degradation.

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