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Pseudodiploid Genome Organization Aids Full-Length Human Immunodeficiency Virus Type 1 DNA Synthesis▽

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Template switching between copackaged human immunodeficiency virus type 1 (HIV-1) genomic RNAs is genetically silent when identical RNAs are copackaged but yields recombinants when virions contain two distinct RNAs. Sequencing has revealed that errors at retroviral recombination junctions are infrequent, suggesting that template switching is not intrinsically mutagenic. Here, we tested the hypothesis that template switching may instead contribute to replication fidelity. This hypothesis predicts that reverse transcription of a single-copy gene will be more error prone than replication in the presence of a second copy. To test this, HIV-1-based vectors containing both *lacZ* and the puromycin resistance marker were expressed either alone or with an excess of an "empty" vector lacking *lacZ* and *puro*. This resulted in virions with either RNA homodimers or haploid genomes with only a single *lacZ-puro* RNA. In untreated cells, *lacZ* inactivation rates suggested that haploid vector reverse transcription was slightly more error prone than that of homodimerized pseudodiploid vectors. Haploid reverse transcription was at least threefold more error prone than pseudodiploid-templated synthesis when slowed by hydroxyurea treatment or stopped prematurely with zidovudine. Individual products of one- and two-copy genes revealed both nucleotide substitutions and deletions, with deletions more frequent than point mutations among haploid genome products. Similar spectra of defective products were observed at early reverse transcription time points and among products of haploid virions. These results indicate that faithful, full-length reverse transcription products were underrepresented in the absence of a reserve of genetic information and suggest that template switching contributes to HIV-1 genomic integrity.

Retroviral recombination results from reverse transcriptase template switching between copackaged RNAs during viral DNA synthesis. When two different viruses infect a single cell, a portion of that cell's viral progeny will contain one each of the two parental viral genomes. During a subsequent round of viral replication, template switching between these copackaged RNAs can result in genetic recombination, which is an important source of virus variability (1, 17, 21, 31). Because at most one provirus is generated per virion and thus only one copy of information from each locus is transmitted to progeny, this two-copy genome organization, which distinguishes retroviruses from all other kinds of viruses, is best described as "pseudodiploid."

Whereas recombinogenic template switching occurs during the synthesis of essentially every retroviral DNA, marker segregation can result only for virions in which two different RNAs were copackaged. Although there is some evidence that individual cell coinfection is common in human immunodeficiency virus (HIV)-infected individuals (16), it is likely that many retroviruses are the products of singly infected cells and therefore contain two RNAs that, barring errors introduced by host RNA polymerase II, are identical. Thus, recombinogenic template switching is often genetically silent. Retroviruses presumably gain a selective advantage by copackaging two copies of their genome and using both as templates during reverse transcription. However, the nature of this advantage is uncertain.

Based primarily on the results of cell-free reverse transcriptase reactions, it was suggested previously that template switching might be error prone and thus an important source of genetic variation (27). However, studies examining crossover junctions generated during retroviral replication appear to refute this speculation. Although most such studies have been performed with simple retroviruses and thus may not pertain to HIV type 1 (HIV-1), these analyses suggest that whereas high-frequency errors do occur at the required first-strand transfer site (19), recombinogenic template switching is not particularly error prone (4, 29, 36, 42, 44).

Another possible advantage conferred by template switching is that it might serve as a fidelity mechanism and promote genomic stability. The "forced copy choice" model for retroviral recombination, which suggests that reverse transcriptase template switching results when reverse transcriptase encounters a template RNA break, was conceived to explain how retroviruses could replicate broken RNA genomes (7). Template breakage is likely not required for recombinogenic template switching, since parameters that should not affect RNA integrity, such as mutations in reverse transcriptase (5, 14) or limiting deoxynucleotide concentrations (15, 23, 30), appear capable of influencing recombination rates at least as much as RNA damage (13). Nonetheless, template switching may contribute to replication error avoidance by serving as a salvage

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pathway for reverse transcription elongation complexes that are halted by conditions such as template damage, substrate limitation, or enzymatic defects.

A prediction of forced copy choice recombination models is that retroviral replication errors should increase under conditions where template switching is not possible, as would be the case if a reverse transcription complex contained only a single RNA template of a particular genome segment instead of the normal homdimeric RNA genome. The experiments presented here tested this prediction and showed that preventing homologous template switching by reducing template copy numbers correlated with higher gene inactivation rates and lower frequencies of intact genome synthesis.

MATERIALS AND METHODS

Cells. 293T is a human embryonic kidney cell line that expresses adenoviral E1A and simian virus 40 (SV40) large T antigen. 293T cells were grown from a master cell bank (40) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. A 293T cell clone containing a single integrated retroviral DNA was produced by transducing 293T cells with the retroviral vector described below, selecting clones in puromycin, and screening colonies for high *lac*Z expression.

Plasmids. pHEF-VSVG (6) is a vesicular stomatitis virus (VSV) G protein expression construct that was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Lung-Ji Chang. pCMVΔR8.2 (22) encodes all HIV-1 proteins except the envelope glycoproteins and Vpu.

$p_{CMV}Lac_{SV40}Puro$, which encodes an HIV-1-based vector that expresses *lacZ* from the cytomegalovirus (CMV) immediate-early promoter and puromycin resistance from an SV40 promoter, was previously called pHIVlac (1). The SV40 promoter in $p_{CMV}Lac_{SV40}Puro$ was replaced with the Rous sarcoma virus (RSV) promoter (nucleotides 483 to 806 from pREP8; Invitrogen) to produce $p_{CMV}Lac_{RSV}Puro$. To generate $p_{CMV\ S/D-}Lac_{RSV}Puro$, the CMV promoter in $p_{CMV}Lac_{RSV}Puro$ was altered by site-directed mutagenesis so that the putative splice donor site (ACGGTAAAT) was changed to TTCCGTCTC.

A minimal HIV-based "empty" vector, pEmpty, was produced from $p_{CMV}Lac_{SV40}Puro$ by replacing the internal promoters and genes plus downstream long terminal repeat (LTR) with an altered 3′-LTR. The 3′-LTR fragment was produced by overlapping PCR using the primers 5′-CCATCGATAAGACAAGATATCCTTGATCTGTGGA-3′, 5′-GCGGAAAGTCCCTTGTAGCA-3′, 5′-AGGGACTTTCCGCACTAGTTCTGGTTAGACCAGATCTGAG-3′, and 5′-CTGCAGACTTGAAGCACTCAAGGCAAGCT-3′. The resulting PCR fragment was digested with ClaI plus PstI and used to replace the corresponding fragment in $p_{CMV}Lac_{SV40}Puro$. As a result, the 3′ end of the RNA encoded by pEmpty lacked the polypurine tract and most of the 3′ LTR, including R sequences required to accept minus-strand transfer products. An additional retroviral vector, called $p_{CMV}Lac_{SV40}$, was produced from $p_{CMV}Lac_{SV40}Puro$ by replacing the puromycin resistance gene and downstream LTR with the altered 3′-LTR described above.

Virus production and infection. 293T cells were transfected with expression clones by calcium phosphate precipitation (40) to produce replication-defective HIV particles bearing VSV G envelope protein. Virus-containing medium was harvested from 293T cells at 48 and 72 hours posttransfection, passed through 0.2-μm filters, and stored at −70°C.

Fresh 293T cells were infected with retroviral vectors and selected in 1 μg/ml puromycin at 48 hours postinfection. Twelve days later, puromycin-resistant colonies were fixed and stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for β-galactosidase activity as described previously (3). Viral vectors were produced for serial passage by transfecting pools of puromycin-resistant clones with helper plasmids (pHEF-VSVG and pCMVΔR8.2), and the resulting virions were harvested for further infections at 2 days posttransfection. To infect hydroxyurea (HU)-treated cells, 293T cells were pretreated with the indicated concentrations of HU (Sigma-Aldrich, St. Louis, MO) for 2 hours and the medium was replaced with medium containing fresh HU and retroviral particles. At 12 hours postinfection, the medium was replaced with medium containing 200 μM zidovudine (AZT), and colonies were selected in puromycin and stained with X-Gal as described above. Where indicated, cells were exposed to 200 μM AZT at 1.5 h postinfection to limit the time available to complete reverse transcription.

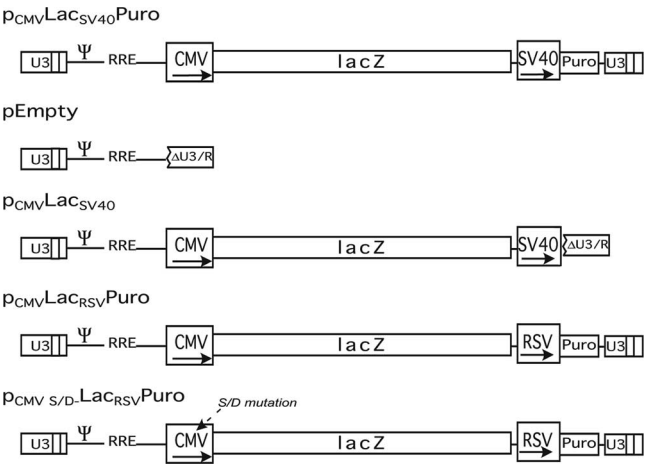

FIG. 1. Plasmids used to express HIV-based retroviral vectors, as described in the text. CMV, SV40, and RSV indicate promoters from the respective viruses. CMV S/D− indicates a variant CMV promoter cassette in which a putative 5′ splice site was mutationally inactivated. The diagrams are not precisely to scale.

Integrated provirus structure analysis. Individual puromycin-resistant colonies were expanded and divided to generate duplicate samples of cloned cells for X-Gal staining and for DNA preparation. Total cell DNA was prepared using the DNeasy tissue kit (Qiagen, Valencia, CA). Integrated HIV vectors were amplified by PCR using the following primer pairs (numbering refers to coordinates of the $_{CMV}Lac_{SV40}Puro$ proviral sequence and F or R to forward or reverse primers, respectively): 205F, CGACAGGCCCGAAGGAATA; 955R, GTAAAACGACGGGATCTAGCATG; 931F, TCCATGCTAGATCCCGTCG; 1683R, TAGGTAGTCACGCAACTCGCC; 1563F, GCTGCATAAACCGACTACACAAA; 2313R, GTGGCCTGATTCATTCCCC; 2259F, CGATCGTAATCACCCGAGTGT; 3014R, ACTGTGAGCCAGAGTTGCCC; 2784F, ACACCAGCAGCAGTTTTTCCA; 3539R, GCCACTTCAACATCAACGGTAAT; 5221F, GGATTGATGGTAGTGGTCAAATG; 6075R, CCTCACTACTTCTGGAATAGCTCAG; 6348F, GGAGAGCGTCGAAGCG; and 6623R, TAGAAGGGGAGGTTGCGG. Primers were removed from the amplified fragments using the QIAquick PCR purification kit (Qiagen, Valencia, CA) or by agarose gel electrophoresis and purification using the QIAquick gel extraction kit (Qiagen, Valencia, CA). All puromycin-resistant cell clones yielded *puro*-specific PCR products. When lack of another PCR product indicated a proviral deletion, alternate primer pairs were used to generate deletion junction-containing fragments. The nucleotide sequences of PCR fragments were determined by the University of Michigan DNA Sequencing Core Facility.

RESULTS

Establishing baseline replication error rates with *lacZ-puro* vectors. The HIV-1-based vectors used for this study were expressed from the plasmid $p_{CMV}Lac_{SV40}Puro$, which contains *lacZ* driven by a CMV promoter and the puromycin resistance gene expressed from an SV40 promoter, or from its derivatives (Fig. 1). Coexpression of $p_{CMV}Lac_{SV40}Puro$ with HIV-1 helper function plasmids in 293T cells produced viral particles that each contained two copies of the $_{CMV}Lac_{SV40}Puro$ vector RNA. These particles were used to infect fresh 293T cells, the infected cells resulting from a single round of reverse transcription and integration were selected in medium containing puromycin, and proviral *lacZ* mutation frequencies (measured by the loss of functional β-galactosidase) were determined by white-per-total (blue plus white) puromycin-resistant colony counts observed upon X-Gal staining.

Such screens for loss of phenotype are commonly used to monitor retroviral mutation frequencies. However, nonstain-

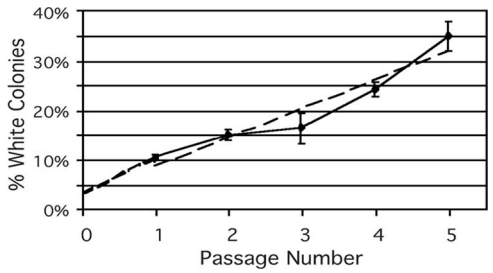

FIG. 2. Accumulation of *lacZ* mutations during serial passage of $_{CMV}Lac_{SV40}$Puro. Viral particles containing two $_{CMV}Lac_{SV40}$Puro genomes were serially passaged on 293T cells, and mutations in the *lacZ* indicator gene were monitored by determining the number of white colonies following staining with X-Gal. The solid line connects the mean data from three experiments, and the error bars represent standard deviations of the means. The dotted line represents the least-squares fit for a line representing the data by the equation $y = 5.8x + 2.7$ ($r^2 = 0.92$).

ing white provirus-containing colonies could result either from *lacZ* inactivating mutations or from other factors. For example, because gene expression levels vary by integration site, some white colonies may result if integration site position effects limit *lacZ* expression to below levels detectable by X-Gal staining.

To calibrate the assay used here and address the relative contributions of *lacZ* inactivation and of positional effects to total white-colony formation, a population of $_{CMV}Lac_{SV40}$Puro vectors was passaged serially for five sequential single rounds of replication in 293T cells (Fig. 2). This was achieved as follows. After one round of vector reverse transcription and integration, the population of infected cells was selected in puromycin. One plate of puromycin-resistant cells was X-Gal stained to generate the white-colony percentage data presented in Fig. 2, while the colonies on a duplicate plate were pooled. The integrated viral vectors in this infected cell pool were then remobilized by transfection with helper function plasmids. To perform a subsequent round of reverse transcription, the resulting viral particles were used to infect duplicate plates of fresh cells: one to score for X-Gal staining and the other as a source of integrated vectors for a subsequent round of remobilization.

The percentage of colonies that were white due to position effects was then estimated by plotting white-colony proportions over sequential viral generations. Total white-colony frequencies were assumed to reflect the sum of white colonies due to integration position effects plus those due to mutations arising in *lacZ* during viral replication. The percentage that are white due to position effects should remain fairly constant from one viral generation to the next, while those that are white due to *lacZ* mutation should increase with each round of reverse transcription. Thus, the percentage of white colonies not accounted for by *lacZ* mutations can be predicted by extrapolating the data to the *y* intercept to represent the number of white colonies present before viral replication (zero passages).

The results showed that serial passage of $_{CMV}Lac_{SV40}$Puro resulted in a nearly linear increase in white colonies over sequential rounds of viral replication (Fig. 2). Approximately 10% of all puromycin-resistant colonies showed no detectable β-galactosidase activity after one round of infection, with this number increasing to 35% white colonies following five rounds of reverse transcription. These data suggest that $_{CMV}Lac_{SV40}$Puro vectors accumulated *lacZ*-inactivating mutations at a rate of approximately 6% per replication cycle (linear slope), with a frequency of white colonies due to positional effects of about 3% (*y* intercept). Note that relatively few products of reverse transcription conditions where this background appears significant (e.g., pseudodiploid genomes from untreated cells and single replication cycles) were characterized in this study, and whether or not detected point mutations inactivated *lacZ* was not rigorously explored. Thus, the sequencing analyses described below lack the power to confirm this deduced position effect value.

Comparing defective product frequencies with one or two copies of *lacZ* template RNA. To test the hypothesis that template switching contributes to replication fidelity, a skewed vector expression approach was used to produce viral particles containing predominantly single copies of *lac*Z (26). When two different HIV-1-based RNAs are coexpressed in individual virus-producing cells, the vector RNAs segregate into virions at random, generating encapsidated RNA homo- and heterodimers in proportions predictable by the Hardy-Weinberg equation (10). Thus, experimentally, RNA dimer proportions in HIV-1 populations can be controlled by altering vector coexpression ratios.

Here, generating haploid virions containing a single RNA copy of *lacZ* involved coexpressing $p_{CMV}Lac_{SV40}$Puro with a molar excess of an additional vector plasmid called pEmpty (Fig. 1). pEmpty was produced by deleting *lacZ*, the puromycin resistance gene, and their respective promoters from $p_{CMV}Lac_{SV40}$Puro, as well as ablating sequences needed for reverse transcription from the downstream LTR. As a result, pEmpty generated a short RNA vector that could heterodimerize with $_{CMV}Lac_{SV40}$Puro RNA to facilitate its packaging but that could not contribute, either directly or via homologous recombination, to the production of puromycin resistance-conferring proviruses. RNase protection assays showed that vectors produced by transfection with a 20-fold molar excess of pEmpty compared to $p_{CMV}Lac_{SV40}$Puro contained an average of 25-fold more Empty than $_{CMV}Lac_{SV40}$Puro RNA copies, thus confirming that pEmpty was efficiently expressed and encapsidated (data not shown). In addition, the molecular analysis of $_{CMV}Lac_{SV40}$Puro proviral products of heterozygous virions confirmed that recombination with Empty RNAs was not detected in puromycin-resistant proviruses (see below).

In the skewed vector approach used here, producer cells were transfected with a 20-fold molar excess of pEmpty over $p_{CMV}Lac_{SV40}$Puro, the resulting virions were harvested, and fresh cells were infected and selected in puromycin. The puromycin resistance colony-forming titer that was observed under these conditions was reduced 100-fold relative to that for virions produced without pEmpty cotransfection. From a 20:1 cotransfection ratio, virions containing $_{CMV}Lac_{SV40}$Puro homodimers, $_{CMV}Lac_{SV40}$Puro/Empty heterodimers, or Empty RNA homodimers are predicted to arise at 0.2%, 9.1%, and 90.7%, respectively, according to the Hardy-Weinberg equation. Thus, much but not all of the observed titer reduction could be explained by predicted proportions of Empty vector homodimers. Of the predicted 9.3% of all particles that should

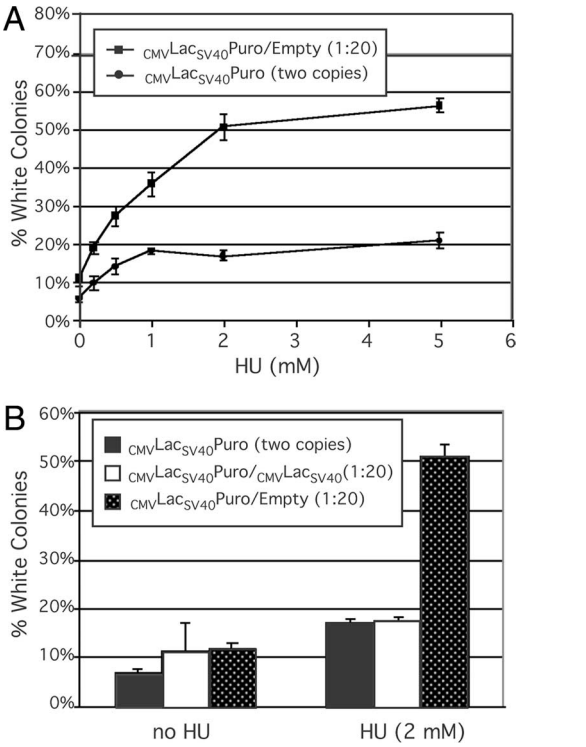

FIG. 3. *lacZ* inactivation frequencies for haploid and pseudodiploid genome virions. (A) Effects of template copy number on mutation frequency. Vectors containing either two copies or primarily a single copy of *lacZ* were used to infect 293T cells in a single-round infection assay. Each point represents the mean from four experiments, with error bars showing the standard errors. (B) Error rates for heterozygous virions with one or with primarily two *lacZ* gene copies. Bars represent the means from two experiments, with error bars showing the standard deviations of the means.

be capable of producing puromycin resistance-conferring proviruses from a 20:1 transfection, 98% should contain a single copy of *lacZ* RNA.

Using this approach, the frequencies of *lacZ*-inactivating mutations arising during reverse transcription of virions with RNA genome populations comprised either of principally a single *lacZ* template or of two gene copies were compared (Fig. 3A). Initial results comparing white-colony titers for single-copy *lacZ* vectors to those for two *lacZ* templates revealed a modest, albeit consistent, twofold increase in apparent *lacZ* inactivation rates in the absence of a second *lacZ* gene copy (Fig. 3A [data from experiments with 0 mM HU]).

The defects associated with haploid genome reverse transcription were subsequently magnified by carrying out reverse transcription under mutagenic conditions. The above findings (Fig. 2) showing that integration position can contribute to white-colony proportions suggested that the apparent magnitude of haploid genome-associated defects might be masked by the presence of position effect products and thus would become more evident under conditions where this background was reduced relative to the total number of white colonies. This was achieved by increasing viral mutation rates by infecting cells that had been treated with the concentrations of HU indicated in Fig. 3A. HU inhibits ribonucleotide reductase and rapidly depletes intracellular deoxynucleoside triphosphate (dNTP) pools, resulting in increased retroviral mutation rates (30). Because HU treatment raises retroviral mutation rates but should not affect integration site-dependent white-colony background levels, we anticipated that HU treatment would increase the "signal-to-noise" ratio and allow a clearer assessment of effects on reverse transcription. Results from infections of HU-treated cells revealed that reverse transcription products of virions containing two copies of *lacZ* contained significantly fewer *lacZ*-inactivating mutations than those of virions bearing only a single *lacZ* template (Fig. 3A). Factoring in 3% as the integration site-dependent background white-colony frequency, *lacZ* inactivation was more than threefold more frequent when a single copy of *lacZ* was reverse transcribed than when proviruses were templated by homodimeric genomes.

Confirming that *lacZ* gene copy number, and not RNA heterodimerization, affects white-colony product frequency. An additional test was performed to ensure that the skewed RNA expression approach used to generate vectors with a single *lacZ* template did not cause the observed high *lacZ* inactivation frequency. For this approach, a new vector called p_{CMV}-Lac_{SV40} was created, in which the intact *lacZ* gene was retained but from which the puromycin resistance coding sequences of $p_{CMV}Lac_{SV40}$Puro were deleted (Fig. 1). Most virions capable of conferring puromycin resistance that were generated in the presence of excess $_{CMV}Lac_{SV40}$ RNA (which replaces Empty RNA in the experiments here) and limiting $_{CMV}Lac_{SV40}$Puro RNA would be predicted to contain RNA heterodimers with two copies of *lacZ* but only one *puro* gene copy. Fresh cells infected with virions produced under these conditions displayed low puromycin resistance titers similar to those of $_{CMV}Lac_{SV40}$Puro/Empty heterodimers. However, as shown in Fig. 3B, in either the presence or absence of HU, the *lacZ* inactivation rate among products of $_{CMV}Lac_{SV40}$Puro/$_{CMV}Lac_{SV40}$ heterodimeric virions was indistinguishable from rates with $_{CMV}Lac_{SV40}$Puro homodimers. These results contrasted with the marked increase in white colonies observed for $_{CMV}Lac_{SV40}$Puro/Empty heterodimer products and support the notion that the higher white colony frequency observed during infection with single-*lacZ*-copy virions was primarily dependent on the *lacZ* copy number.

Addressing contributions of packaged subgenomic RNAs to haploid genome-associated replication defects. Extracting proviral sequences by PCR from infected cell clones revealed that one 4-kilobase deletion arose at a high frequency (Fig. 4A). This common deletion, which was observed in nearly 60% of all analyzed $_{CMV}Lac_{SV40}$Puro white proviruses, was generated by haploid and by pseudodiploid RNA genomes and whether or not cells were treated with HU.

Analysis of this product's deletion endpoints revealed strong splice site consensus sequence similarity (37), suggesting that the use of cryptic splice sites had generated spliced RNAs that were encapsidated within the virion population and served as templates for these deletion products (Fig. 4A). To provide confirmatory evidence for the likelihood that the putative splice donor and acceptor sites were involved in deletion product formation, additional vectors, called $p_{CMV}Lac_{RSV}$Puro or $p_{CMV\ S/D-}$ Lac_{RSV}Puro, were designed that lacked one or both of the splice junctions, respectively (Fig. 1). The potential 3′

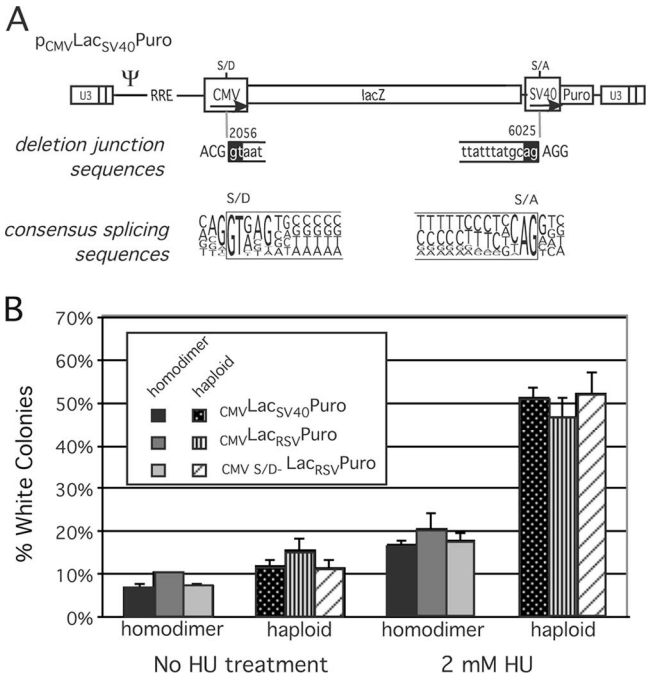

FIG. 4. Effects of eliminating potential subgenomic spliced product. (A) Schematic illustration of $p_{CMV}Lac_{SV40}Puro$, indicating the positions and sequences of potential splice donor and acceptor sites. Numbers 2056 and 6025 indicate the vector map positions that comprise the common 4-kb deletion's junctions; the residues indicated in uppercase were retained in the deletion product, whereas nucleotides indicated in lowercase were deleted. The major class splicing consensus sequence is from reference 37. (B) *lacZ* inactivation frequencies for haploid and pseudodiploid genome virions. A comparison of observed values for vectors that possessed the major putative splice sites ($_{CMV}Lac_{RSV}Puro$), for one that lacked the putative splice acceptor ($_{CMV}Lac_{SV40}Puro$), and for one that lacked both putative splice donor and splice acceptor ($_{CMV\ S/D-}Lac_{RSV}Puro$) is shown. Mutations in the *lac*Z indicator gene were measured as the number of white colonies following staining with X-Gal. Bars show the means from two experiments, with error bars representing the standard deviations of the means.

splice signal (designated S/A for "splice acceptor" in Fig. 4A) was removed by replacing the SV40 promoter with the RSV promoter to generate $p_{CMV}Lac_{RSV}Puro$, and the 5′ splice site (S/D in Fig. 4A) was altered by site-directed mutagenesis, yielding $p_{CMV\ S/D-}\ Lac_{RSV}Puro$.

These new vectors were then tested to address whether or not mutating these putative splice junctions would eliminate haploid genome-associated increases in *lacZ* inactivation rates. Each of the three *lacZ-puro* vectors was expressed either alone or in the presence of excess pEmpty, and the resulting virions were used to direct one round of replication in either untreated or HU-treated cells (Fig. 4B). The results revealed that all three *lacZ-puro* vectors, when reverse transcribed from haploid genomes produced by transfection with an excess of pEmpty, exhibited higher white-colony frequencies than the corresponding products from homodimeric virions (Fig. 4B). As had been observed for $_{CMV}Lac_{SV40}Puro$, increases in white-colony formation were more pronounced in the presence of HU for both the vector derivatives (right half of Fig. 4B). These results confirmed the correlation between *lacZ* RNA template copy number and proviral error rates and indicated that the high-frequency putative spliced RNA products of $_{CMV}Lac_{SV40}Puro$ were not solely responsible for the higher white-colony rates observed with single-copy *lacZ* vectors. Thus, the property of enhanced defective genome synthesis correlated with imbalanced gene copy number, even in the absence of the previous major product.

Assessing the spectra of *lacZ*-inactivating mutations. The *lacZ* genes from many individual β-galactosidase-negative proviruses (each templated by one of the three *lacZ-puro* vectors, expressed either alone or with excess pEmpty, and reverse transcribed in the presence or absence of HU) were then isolated, mapped, and sequenced (Table 1). Of the total of 82 such white-colony provirus structures summarized in Table 1, 53 contained large deletions in *lacZ* and 29 differed from the parental sequence only by the possession of one or more small mutations, including point mutations and frameshifts or small deletions revealed only by DNA sequencing.

As introduced above, initial work with $p_{CMV}Lac_{SV40}Puro$ products revealed that 23 out of 26 large deletions shared the same 5′ and 3′ deletion junctions (S/D1 and S/A5 in Fig. 5A). As predicted by the assumption that these common deletions were products of spliced subgenomic RNAs, analysis of $p_{CMV}Lac_{RSV}Puro$ products (which were designed to lack S/A5 [Fig. 5A]) revealed the use of the same 5′ junction that was observed for the original $_{CMV}Lac_{SV40}Puro$ vector (S/D1) but joining to different cryptic 3′ splice sites (S/A1 to -4). In contrast, all these putative spliced RNA products were absent among proviruses generated by $p_{CMV\ S/D-}\ Lac_{RSV}Puro$ (Table 1), which lacked both S/D1 and S/A5. However, a second putative splice donor (S/D2 at vector map position 4673) was revealed by the structures of two additional proviral clones derived from $p_{CMV}\ Lac_{RSV}Puro$. Both of these deletion products resulted from use of the same potential splice acceptor site (S/A4 in Fig. 5A).

The deletion junctions of three deletion-containing products

TABLE 1. Distribution of *lacZ* mutations[a]

Vector	No. of *lacZ* copies per virion	HU (mM)	No. of clones with:	
			Deletions[b]	Point mutations
$_{CMV}Lac_{SV40}Puro$	2	0	1 (0, 1)	3
	2	2	2 (2, 0)	2
	1	0	5 (3, 2)	1
	1	2	18 (18, 0)	2
$_{CMV}Lac_{RSV}Puro$	2	0	2 (2, 0)	3
	2	2	4 (3, 1)	3
	1	0	7 (2, 5)	0
	1	2	10 (3, 7)	8
$_{CMV\ S/D-}\ Lac_{RSV}Puro$	2	0	1 (1, 0)	2
	2	2	0	3
	1	0	1 (0, 1)	2
	1	2	3 (0, 3)	0

[a] Reverse transcription was terminated at 12 h postinfection by the addition of AZT.

[b] The data are presented as the total number of clones with large deletions, followed in parentheses by the number that were likely generated from spliced RNA templates (Fig. 5A) and the number that arose by such means as nonhomologous recombination (Fig. 5B), respectively.

FIG. 5. Observed *lacZ-puro* vector deletion products. (A) Deletions likely generated by spliced templates. The structure expected from an intact $_{CMV}Lac_{SV40}$Puro provirus is shown together with putative 5′ (S/D1 and S/D2) and 3′ (S/A1 to -5) deletion junctions revealed by molecular analysis of individual reverse transcription products (note that the S/D and S/A presented in Fig. 4 are the same as S/D1 and S/A5 here). Numbers indicate map positions of the deletion junctions relative to the intact provirus, where 1 is the first nucleotide in the 5′ LTR. Deletion junction sequences that remained in deleted replication products are shown in uppercase, while deleted sequences are in lowercase. (B) Deletion junctions generated by nonhomologous recombination. Examples of deletions that lack putative splicing signals and were likely generated by nonhomologous recombination are shown. The clone number is indicated on the left. Numbers above and below each deletion junction indicate the nucleotide positions relative to the intact provirus, where 1 is the first nucleotide in the 5′ LTR. Sequence homologies at deletion junctions are boxed. Clones 3, 107, and 417 were generated by infection with $_{CMV}Lac_{SV40}$Puro, while clones 502 and 546 were produced using $_{CMV}Lac_{RSV}$Puro. Clone 546 contained an 11-nucleotide insertion that, when used in a BLAST query with its flanking viral sequences, revealed 19 nucleotides that matched sequences located on human chromosome 20 (GenBank accession no. AL359828), possibly indicating that it contained a transductive insertion (9). Clones 910, 927, and 963 were generated during shortened periods of reverse transcription (see Fig. 6) by $_{CMV}Lac_{SV40}$Puro, $_{CMV}Lac_{RSV}$Puro, or $_{CMVS/D-}$ Lac_{RSV}Puro, respectively.

of $p_{CMV}Lac_{SV40}$Puro, about half of those from $p_{CMV}Lac_{RSV}$Puro, all characterized clones with deletions generated from $p_{CMV\ S/D-}Lac_{RSV}$Puro, and many early-time-point products (described below) did not display features suggestive of cryptic RNA splicing but instead displayed structures typical of retroviral nonhomologous recombination-generated deletions (Fig. 5B). Most of these products contained 1 to 11 nucleotides of microhomology at the deletion junctions, one clone showed no deletion junction-associated homology (clone 963), and one product contained an 11-base insertion between the deletion junctions (clone 546). Junctions with microhomology, no homology, and insertions within deletions are all common nonhomologous recombination products (9).

Taken together, these data indicate that most if not all of the deletions observed in *lacZ-puro* vector products can be explained either by the ability of reverse transcriptase to use spliced subgenomic RNA templates or by viral nonhomologous recombination.

In contrast to the deletion derivatives characterized above, 29 of the 82 "white-colony" proviruses did not contain length variation but instead were revealed by sequencing to differ from the parental sequence by point mutations in *lacZ*. Although point mutations appeared to contribute to *lacZ* inactivation less frequently than deletions, some clones contained multiple changes, with as many as 10 point mutations in a single mutant *lacZ* gene (data not shown). Interestingly, 27 of the total of 51 observed nucleotide substitutions were G-to-A transitions.

Although the numbers of sequences considered here limit the statistical power of these interpretations, when all products of the three vectors were considered together, the following trends emerged. Of 12 clones generated by pseudodiploid virions in the absence of HU, 8 were inactivated by point mutations (Table 1, rows 1, 5, and 9), while 4 contained deletions. Among 14 proviruses templated by homodimerized RNAs in cells treated with HU, 8 contained nucleotide substitutions and 6 contained deletions (Table 1, rows 2, 6, and 10). Thus, the defective reverse transcription products generated by vectors containing two *lacZ* templates included approximately equal proportions of alleles with *lacZ*-inactivating point mutations and with deletions.

In contrast, clones resulting from infection with single-gene-copy *lacZ* vectors predominantly contained deletions (Table 1, rows 3, 4, 7, 8, 11, and 12). Thirteen analyzed clones generated by haploid vectors in untreated cells contained deletions, while only three were disrupted by point mutations. Among 41 analyzed products of haploid vectors reverse transcribed in HU-treated cells, 31 contained deletions and only 10 contained point mutations (Table 1, rows 4, 8, and 12).

Assessing underrepresentation of intact genome products at early time points during reverse transcription. Cataloging of defective reverse transcription products (Table 1 and discussed above) showed that the spliced-RNA-templated 4-kb $_{CMV}Lac_{SV40}$Puro deletion was observed among products generated with or without HU treatment and among products of both haploid and pseudodiploid homozygous virions. This suggested that the spliced RNA that templated the deletion product was constitutively present in virion RNA populations. These observations also raised the possibility that the reason the spliced RNA's proviral form was overrepresented among

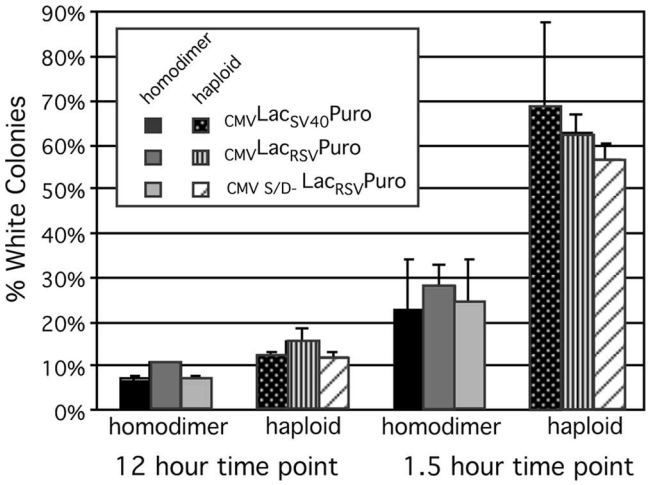

FIG. 6. *lacZ* inactivation rates for products of limited-time reverse transcription. AZT was added at either 12 h or 1.5 h postinfection to stop reverse transcription. Mutations in the *lacZ* indicator gene were measured as the number of white colonies following staining with X-Gal. Bars show the means from two experiments, with error bars representing the standard deviations of the means.

haploid genome products was due to disadvantages of full-length genome synthesis in the absence of a second RNA gene copy.

Some of the increased proportions of *lacZ* inactivation observed in the presence of HU (Fig. 3) may reflect a selective advantage of deletion products, which could be completed faster than full-length *lacZ* vectors. The half-time to completion of HIV DNA synthesis is about 4 h under optimal intracellular conditions (24), and reverse transcription in the presence of HU takes even longer due to limiting substrate availability (15, 23, 30). Here, single-copy *lacZ* gene vectors were more prone to generating *lacZ* inactivation products than were homozygous genomes, and many defective products contained deletions. Thus, we hypothesized that part of the reason that virions containing a single copy of the assayed *lacZ* gene might be less capable of generating full-length DNAs than were homozygous genomes was that reverse transcriptase elongation for single-gene-copy genomes proceeded less efficiently than when a second copy was present.

To test this hypothesis, proviruses generated during limited durations of DNA synthesis were examined. This approach addressed whether or not the same spectra of deletion products that were enriched by skewed vector ratios also were overrepresented under conditions known to disfavor full-length provirus synthesis. Reverse transcription was disrupted prematurely by treating cells with inhibitory concentrations of AZT at 1.5 h after initiation of infection (Fig. 6). This resulted in roughly 70-fold decreases in proviral product yields, as determined by puromycin resistance titers (not shown). Note that no HU was used in these experiments.

Consistent with the HU treatment results, haploid vectors had higher *lacZ* inactivation rates than pseudodiploid vectors when the time of reverse transcription was limited (right half of Fig. 6). Structural analysis of defective products of pseudodiploid particles revealed that the same common 5′ junction observed above among skewed vector products was also predominant among clones derived from limited reverse transcription duration (1.5 h), representing 9 of 10 deletions and 2 of 6 deletions for $p_{CMV}Lac_{SV40}Puro$ and $p_{CMV}Lac_{RSV}Puro$, respectively (Table 2). Thus, the same spliced-RNA-templated deletion products became overrepresented both by limiting durations of reverse transcription and by infection with haploid virions. These observations demonstrate that products of late-time-point synthesis for haploid vectors resemble those of much earlier time points for pseudodiploid genomes, thus suggesting that the presence of two intact viral genomes is more favorable for full-length viral DNA synthesis.

DISCUSSION

Retroviruses such as HIV-1 normally package two complete copies of their RNA genome, and both RNA templates participate in the generation of a single copy of viral DNA (2). The work here compared the frequency of defective provirus formation for HIV-1 reverse transcription complexes containing haploid templates to those with normal homodimeric RNA pseudodiploid genomes, to address whether or not possessing two viral RNA templates provides retroviruses with a means for overcoming reverse transcription impediments. Monitoring of *lacZ* inactivation rates revealed that defective *lacZ* DNA copy formation occurred up to threefold more frequently when replication was limited to a single template than for the normal homozygous genome.

Molecular analysis of individual defective replication products revealed that gene-inactivating mutations were fairly equally divided between point mutations and sequence deletions when virions contained homodimeric, pseudodiploid genomes. Among the point mutations observed, most were G-to-A substitutions, which is suggestive of the activity of the APOBEC3 family of cellular deaminases. These substitutions occurred even though the helper plasmid used encoded Vif (22) and virions were produced in 293T cells, which have little deaminase activity and are permissive for HIV *vif* mutants (34). Eighteen of 23 observed G-to-A mutations were in the context of GA dinucleotides, which is more consistent with APOBEC3F than 3G activity (20, 35, 39).

In contrast to the error distribution among products of two-gene-copy virions, most defective products generated from single-gene-copy virions contained deletions. Many deletions appeared to be templated by spliced subgenomic RNAs. The spliced RNAs revealed by these reverse transcription products

TABLE 2. Distribution of *lacZ* mutations in clones generated after limited reverse transcription[a]

Vector	No. of *lacZ* copies per virion	HU (mM)	No. of clones with:	
			Deletions[b]	Point mutations
$_{CMV}Lac_{SV40}Puro$	2	0	11 (9, 2)	0
$_{CMV}Lac_{RSV}Puro$	2	0	6 (2, 4)	3
$_{CMV\ S/D-}\ Lac_{RSV}Puro$	2	0	6 (0, 6)	3

[a] Reverse transcription was terminated at 1.5 h postinfection by the addition of AZT.

[b] The data are presented as the total number of clones with large deletions, followed in parentheses by the number that were likely generated from spliced RNA templates (Fig. 5A) and the number that arose by such means as nonhomologous recombination (Fig. 5B), respectively.

were generated using cryptic splice donors and acceptors that were far downstream of 5′ packaging signals, and thus splicing did not interfere with RNA packaging. These spliced RNAs were likely constitutive low-level components of the encapsidated virion RNA population. Although the splice signals observed here resided in nonviral vector sequences, some instances of packaging and reverse transcription of virus-derived subgenomic RNAs have been reported (11, 12). Many deletions that appeared to be mediated by nonhomologous recombination were detected among the defective reverse transcription products studied here as well.

The results reported here suggest that like limiting the time available for reverse transcription by AZT treatment (Fig. 6), reverse transcription using a single template provides conditions that disfavor intact genome synthesis. Under conditions that disfavored full-length genome production, shorter products (templated by spliced RNAs or generated by nonhomologous recombination-mediated deletion) became overrepresented in the proviral population. Note that much of the template copy number-associated difference observed here was evident only when magnified by HU treatment. Additionally, many observed defective products were templated by short, aberrantly spliced RNAs. Thus, although preventing the formation of the predominant spliced product did not relieve template copy number-associated replication defects, as revealed by screening for gene inactivation, the limited numbers of individual products characterized prevent an assessment of whether single-template replication increases defective products solely by disfavoring lengthy product formation or whether there is some influence on nonhomologous template switching or other error rates as well.

Advantages of RNA copackaging for intact genome synthesis, as indicated by the results here, may be important to viral replication in vivo. Some target cells for HIV infection, especially macrophages and resting T cells, are predominantly nondividing cells containing low concentrations of dNTPs (8, 18, 43). The fact that HIV replication is challenged in these cells is supported by the analysis of HIV proviruses from primary patient cells. Many of these proviruses are defective, and significant deletions of viral sequences are common (33). Thus, the virus's pseudodiploid genome organization may be particularly beneficial to successful HIV replication under conditions that increase the time required to complete viral DNA synthesis, such as low dNTP concentrations.

Based on the observations reported here and the remarkable frequency of template switching observed during HIV DNA synthesis (roughly one crossover per kilobase [25, 32, 44]), we propose a model wherein the second copackaged RNA genome is recruited to and serves as part of the reverse transcriptase elongation complex. This model suggests that the presence of the acceptor template may aid processivity by facilitating recombinogenic acceptor template recruitment when the donor template is damaged or other impediments are encountered, and/or it may prevent the formation of secondary structures in the nascent DNA that could impede reverse transcriptase elongation. Evidence for some aspects of the model that secondary RNAs may aid reverse transcriptase processivity, such as the recruitment of acceptor templates into elongating reverse transcription complexes prior to template switching, have been provided previously (28). When templates are broken or elongation is otherwise prevented, the only recourse with a single-copy RNA would be abortive synthesis or deletion, and since nonhomologous recombination occurs only 0.1 to 1% as frequently as homologous recombination, this would decrease proviral yield as well as quality (41). Speculatively, observed titer decreases in excess of those predicted by RNA ratios, as well as the observation of increased frequency of deletions among products templated by single-copy genes, may suggest that the pseudodiploid nature of retroviruses also contributes to the lower frequency of deletion-containing defective-interfering particles observed in retroviral populations than is seen for many other RNA viruses (38).

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