Frequency of Direct Repeat Deletion in a Human Immunodeficiency Virus Type 1 Vector during Reverse Transcription in Human Cells

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Retroviral genetic rearrangements can result from reverse transcriptase template switching. Most published data suggest that errors such as base misincorporation occur at similar frequencies for HIV-1 and for simple retroviruses such as spleen necrosis virus (SNV) and murine leukemia virus (MuLV). However, previous reports have suggested that template switch-mediated recombination is much more frequent for HIV-1 than for simple retroviruses. In this report, direct repeat deletion vectors similar to those previously used for measuring template switching events for SNV and MuLV were developed for HIV-1. Forward mutation rates and the frequency of template switching during a single cycle of HIV-1 replication were determined. The frequency of HIV-1-mediated repeat deletion was measured for three separate internal repeats in lacZ and was compared to rates observed with identical repeats for MuLV. The results indicated that the error rate and the frequency of repeat deletion of HIV-1 were similar to those of MuLV.

Key Words: genetic recombination; template switching; HIV-1 replication.

INTRODUCTION

Cumulative evidence suggests that, similar to other members of the Retroviridae family, HIV-1 reverse transcription is prone to both nucleotide misincorporation (mutations) and genetic recombination. Although the steady state composition of retroviral populations is determined more by selection than by error frequency, the high rate of errors associated with HIV-1 replication is an important factor in HIV-1 disease because it provides the genetic diversity from which variants can be selected.

Many studies with purified enzymes have demonstrated that HIV-1 reverse transcriptase base misincorporation rates are high and that this kind of mutation also arises frequently during viral replication (Preston and Dougherty, 1996). Although the rate of misincorporation during HIV-1 replication is much higher than that of the cellular replication machinery, it is about 20-fold lower than values obtained for purified HIV-1 RT (Mansky and Temin, 1995). Most studies suggest that misincorporation rates during lentivirus replication are similar to or somewhat higher than those for simple retroviruses (Pathak and Temin, 1990a,b; Mansky, 2000; Mansky and Temin, 1994). For example, in a study that used lacZα as a reporter, a forward mutation rate of 3.4 × 10⁻¹⁰ mutations per base per HIV-1 replication cycle was observed (Mansky and Temin, 1995). This is twofold higher than values obtained during spleen necrosis virus (SNV) replication (Pathak and Temin, 1990a,b).

Some studies suggest that genetic recombination may be a more significant source of retroviral phenotypic variation than base misincorporation, especially for HIV-1 (Mansky, 2000; Parthasarathi et al., 1995). Retroviral genetic recombination results from template switching between copackaged RNAs (Hu and Temin, 1990; Hunter, 1978; Stuhlmann and Berg, 1992). One approach to the study of retroviral recombination is to introduce genetic markers into two different retroviral vectors and to examine the progeny proviral DNA from virions containing these coexpressed RNAs (Yu et al., 1997). By this approach, the recombination frequency of HIV-1 was determined to be two to three recombination events per genome per replication cycle (Jetzt et al., 2000). This is roughly 10-fold higher than rates determined using similar approaches for SNV (Hu and Temin, 1990) and murine leukemia virus (MuLV) (Anderson et al., 1998).

Another approach for studying retroviral recombination-related properties is to measure direct repeat deletion. Repeated sequences are deleted frequently during retroviral replication (Rhode et al., 1987), and deletion rates have been measured previously for SNV and MuLV (Delviks et al., 1997; Delviks and Pathak, 1999; Julias et al., 1995; Pathak and Temin, 1990a; Pfeiffer et al., 1999). Though not an absolute rule, MuLV and SNV deletion frequencies correlate roughly with repeat length (Delviks et al., 1997; Delviks and Pathak, 1999; Julias et al., 1995; Pfeiffer et al., 1999; Zhang and Temin, 1994). Repeat deletion rates have not been reported for HIV-1.

Here, we established a system for analyzing HIV-1 recombination-related properties.
mutation and direct repeat deletion rates during single cycles of viral replication. In contrast to reported large differences in intermolecular recombination frequencies between HIV-1 and certain simple retroviruses, we found that mutation rates and direct repeat deletion rates for HIV-1 and MuLV were very similar.

RESULTS
Vector and cell system to study HIV-1 direct repeat deletion during a single cycle of replication

An assay was established to examine direct repeat deletion rates during single cycles of HIV-1 replication. A single cycle is defined as starting with transcription and virus production in the producer cell and then continuing through infection, reverse transcription, and proviral DNA integration into the target cell. In our system, replication was limited to a single cycle because vector virus was pseudotyped with ecotropic MuLV envelope that was constitutively expressed by the producer cells (Pfeiffer et al., 1999), and 293-derived cells that express ecotropic receptor were used as the human target cell line (mATRC1/293 cells) (Malhotra et al., 1996). Vector virus generated in the producer cells could not infect producer cells because they lack ecotropic receptor, and no infectious virus was produced by infected target cells since the target cells were devoid of helper function. The titer of vector virus was typically $10^4$ transducing units per milliliter, which is about one order of magnitude lower than those reported for similar HIV-1 vectors pseudotyped with amphotropic envelope or VSV-G (Naldini et al., 1996b).

For the assays employed here, the lentivirus helper construct, pCMVΔR8.2 (Naldini et al., 1996a), provided all HIV-1 proteins except envelope in trans for HIV-1-based transducing vectors (Fig. 1A). The wild-type transducing vector, pHIVlac, was a derivative of pH'R'CMVlacZ (Naldini et al., 1996b), engineered to include a puromycin resistance gene driven by the SV40 early promoter. pHIVlac117, pHIVlac284, and pHIVlac971 differed from pHIVlac in that each contained a 117-, 284-, or 971-bp direct repeat within lacZ, respectively.

To produce viral particles, individual transducing vector and helper constructs were cotransfected into producer cells, and virus was harvested and used to infect susceptible human cells (Malhotra et al., 1996) (Fig. 1B). After 2 weeks of selection, puromycin-resistant colonies were X-gal stained. If precise repeat deletion occurred during reverse transcription, cells should stain blue; if the repeats were maintained, colonies would not stain. Deletion rates were calculated as ratios of blue colonies to the total number of colonies. For each transducing vector, virus was prepared from three separate cotransfections, and two independent infections were performed in triplicate on each virus stock to assess deletion rates during single replication cycles.

lacZ inactivation during HIV-1 replication

Due to HIV-1’s high mutation rate, the reconstituted lacZ gene in some proviral DNAs may be inactivated even if precise deletion takes place. To determine the inactivation rate of lacZ in our system, pHIVlac (which contains uninterrupted lacZ) was cotransfected into producer cells with pCMVΔR8.2, target cells were infected, and puromycin-resistant colonies were counted after X-gal staining. The inactivation rate of lacZ was calculated as the ratio of white colonies to total colonies (Table 1). The results suggested that lacZ was inactivated at a rate of 6.4% for HIV-1 during single replication cycles, which is comparable to frequencies measured for MuLV (Julias et al., 1997; Julias and Pathak, 1998; Pfeiffer et al., 1999). The roughly 1.6-fold difference between our measurement and previously reported HIV-1 error frequencies (Mansky and Temin, 1995) may not be significant or it may reflect the different approaches used.

Deletion rates for different length direct repeats

To determine repeat deletion values for HIV-1, the cotransfection, infection, selection, and staining protocol outlined above was repeated using pHIVlac117, pHIVlac284, and pHIVlac971 as transducing vectors and pCMVΔR8.2 as helper (Table 2). The deletion rates measured by blue/total colony ratios for 117, 284, and 971 base direct repeats were 6.4, 18.6, and 81.4%, respectively. Factoring in the 6.4% lacZ inactivation rate determined above, calculated deletion frequencies were 6.8, 19.9, and 87%, respectively.

mATRC1/293 cells were also infected with MuLV-based vectors that contained the same lacZ repeats to compare deletion frequencies for the MuLV and HIV-1 replication machinery. The values determined here for MuLV infection of mATRC1/293 cells are shown in Table 3. In our previous work, murine NIH-3T3 cells were infected with these same vectors, and MuLV repeat deletion values ranged from $4.8 \times 10^{-2}$ per base per cycle of replication (5.6% deletion of a 117 base repeat) to $10.6 \times 10^{-2}$ per base per cycle (30% deletion of a 284 base repeat), with an intermediate value of $6.8 \times 10^{-2}$ per base per cycle (66% deletion of a 971 base repeat) for the longest repeat studied (Pfeiffer et al., 1999). As indicated in Fig. 2, the rates of repeat deletion for MuLV vectors reverse-transcribed in human mATRC1/293 cells were very similar to the MuLV deletion frequencies previously determined for products generated in murine NIH-3T3 cells.

Effect of HU and AZT treatment on lacZ inactivation and repeat deletion rates

HU inhibits cellular ribonucleotide reductase and can induce dNTP pool imbalance. HU treatment has been shown to modestly increase direct repeat deletion rates
for MuLV and lacZ inactivation rates in SNV and MuLV (Julias and Pathak, 1998; Pfeiffer et al., 1999; Svarovskaia et al., 2000). AZT, the RT nucleoside inhibitor widely used in clinical therapy of HIV disease, also affects mutation rates (Julias et al., 1997; Mansky and Bernard, 2000).

To address effects of HU and AZT treatment on lacZ inactivation in our system, mATRC1/293 cells were infected with HIVlac vector virus and incubated in culture media supplemented with 0.2 mM HU or 1.0 μM AZT, with untreated cells used as a control (Table 1). The HU treatment resulted in 8.5% lacZ inactivation, or a 1.3-fold increase compared to untreated cells. This slight in-
crease for HIV-1 is lower than the reported 1.8- to 2.7-fold increases for MuLV and a 2.1-fold increase reported for SNV undergoing reverse transcription in HU-treated cells (Julias and Pathak, 1998; Pfeiffer et al., 1999). When target cells were infected in the presence of AZT, the lacZ inactivation rate was 7.3%, or 1.1-fold higher than in untreated cells. This magnitude of effect is lower than those reported in other studies, where treatment with a similar concentration of AZT increased the MuLV lacZ inactivation rate threefold (Julias et al., 1997). Even lower concentrations of AZT were found to increase lacZ inactivation for SNV by 7- to 10-fold (Julias et al., 1997) and to increase forward mutation of lacZa 7-fold for HIV-1 (Mansky and Bernard, 2000). The cause of the differences in magnitudes of AZT effects between values determined here and those reported previously was not determined, but differences may reflect cell-line specific differences in AZT processing or investigator-specific differences in methodology.

To study the effect of HU and AZT treatment on HIV-1 direct repeat deletion, target cells were infected with HIVlac117 virus and incubated in media containing 0.2 mM HU or 1.0 mM AZT. Deletion rates were determined as above (Table 4). When treated with 0.2 mM HU, the deletion rate increased to 11.4%. This 1.8-fold increase upon HU treatment is similar in magnitude to that which we observed for the identical lacZ repeat in MuLV vectors (Pfeiffer et al., 1999) and is very similar to the 1.7-fold increase observed by another group that subsequently measured HU effects on MuLV repeat deletion (Svarovskaia et al., 2000). When treated with 1.0 mM AZT, the deletion rate increased to 10.3%, a 1.6-fold increase over the deletion rate in untreated target cells.

**DISCUSSION**

We established a system for examining HIV-1 direct repeat deletion rates during single cycles of viral repli-

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**TABLE 1**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>No. of blue colonies</th>
<th>No. of white colonies</th>
<th>Inactivation rate (%)(^a)</th>
<th>No. of blue colonies</th>
<th>No. of white colonies</th>
<th>Inactivation rate (%)(^a)</th>
<th>No. of blue colonies</th>
<th>No. of white colonies</th>
<th>Inactivation rate (%)(^a)</th>
</tr>
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<tr>
<td>1.1</td>
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<td>17</td>
<td>8.0</td>
</tr>
<tr>
<td>1.2</td>
<td>3313</td>
<td>240</td>
<td>6.8</td>
<td>2207</td>
<td>258</td>
<td>10.5</td>
<td>2104</td>
<td>213</td>
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<tr>
<td>2.1</td>
<td>726</td>
<td>36</td>
<td>4.7</td>
<td>194</td>
<td>15</td>
<td>7.2</td>
<td>256</td>
<td>20</td>
<td>7.2</td>
</tr>
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<td>3034</td>
<td>218</td>
<td>6.7</td>
<td>1621</td>
<td>144</td>
<td>8.2</td>
<td>602</td>
<td>39</td>
<td>6.1</td>
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<td>1637</td>
<td>85</td>
<td>4.9</td>
<td>682</td>
<td>64</td>
<td>8.6</td>
<td>628</td>
<td>42</td>
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<td>3240</td>
<td>173</td>
<td>5.1</td>
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<td>87</td>
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<td></td>
<td></td>
<td>8.5</td>
<td></td>
<td></td>
<td>7.3</td>
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<td></td>
</tr>
<tr>
<td>SD</td>
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<td></td>
<td></td>
<td>1.4</td>
<td></td>
<td></td>
<td>1.2</td>
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<td></td>
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</tbody>
</table>

\(^a\) The first number indicates separate cotransfections from which viruses were produced, and the second number indicates separate infections performed in triplicate. Data provided are sums of values from the three dishes.

\(^\) The lacZ inactivation rate was calculated as the ratio of the number of white colonies to the total number of colonies.

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**TABLE 2**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>117-base repeat</th>
<th>284-base repeat</th>
<th>971-base repeat</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>No. of blue colonies</td>
<td>No. of white colonies</td>
<td>Deletion rate (%)(^a)</td>
</tr>
<tr>
<td>1.1</td>
<td>99</td>
<td>1437</td>
<td>6.4</td>
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<td>1.2</td>
<td>500</td>
<td>5546</td>
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<tr>
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<td>30</td>
<td>620</td>
<td>4.6</td>
</tr>
<tr>
<td>Avg</td>
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<tr>
<td>SD</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) The first number indicates separate cotransfections from which viruses were produced, and the second number indicates separate infections performed in triplicate. Data provided are sums of values from the three dishes.

\(^\) The deletion rate was calculated as the ratio of the number of blue colonies to the total number of colonies.
cation. Direct repeat deletion, measured by the frequency of marker gene reconstitution, has been used as a measure of homologous recombination frequency during both cellular and viral DNA synthesis (Bierne et al., 1997; Delviks et al., 1997; Delviks and Pathak, 1999; Julias et al., 1995; Pfeiffer et al., 2000; Pfeiffer et al., 1999; Svarovskaia et al., 2000; Xu and Boeke, 1987; Zhang and Sapp, 1999). An important feature of this new HIV-1-based assay was that the lacZ expression unit and the repeats within lacZ used to measure deletion frequencies were identical to those previously used to assess MuLV template switching (Pfeiffer et al., 1999). This was important for allowing a comparison of deletion frequencies by MuLV and HIV-1 because reverse transcriptase errors do not occur at uniform frequencies and are highly context-dependent (Anderson et al., 1998; Ricchetti and Buc, 1990; Wooley et al., 1998). For example, sequences that potentially form secondary structures have been shown to be hot spots for both mutation and recombination (Jetzt et al., 2000; Jones et al., 1994; Pathak and Temin, 1992). These and other poorly understood template features that affect error frequencies are not present at uniform frequencies throughout templates. The system used here eliminated template structure and sequence context as variables and allowed direct comparison of MuLV replication properties in murine cells to those of HIV-1 in human cells.

Findings reported here demonstrated that direct repeat deletion frequencies of MuLV and HIV-1, when both viruses were tested with identical repeats in the same human cell type, were very similar. The measured values agreed well with deletion rates previously determined by others for MuLV in murine cells and SNV in canine cells (Delviks et al., 1997; Delviks and Pathak, 1999; Julias et al., 1995; Pfeiffer et al., 1999; Svarovskaia et al., 2000). Thus, we conclude that HIV-1 deletes repeats at a fre-

**TABLE 3**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Wild type</th>
<th>117-base repeat</th>
<th>284-base repeat</th>
<th>971-base repeat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of blue colonies</td>
<td>No. of white colonies</td>
<td>Inactivation rate (%)</td>
<td>No. of blue colonies</td>
</tr>
<tr>
<td>1</td>
<td>3639</td>
<td>337</td>
<td>8.5</td>
<td>2947</td>
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<td>2</td>
<td>1908</td>
<td>168</td>
<td>8.1</td>
<td>1909</td>
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<tr>
<td>Avg</td>
<td>8.3</td>
<td>3.1</td>
<td></td>
<td>3.1</td>
</tr>
<tr>
<td>SD</td>
<td>0.3</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Each experiment involves independent infection of six dishes. Data provided are sums of values from the six dishes.
* The lacZ inactivation rate was calculated as the ratio of the number of white colonies to the total number of colonies.
* The deletion rate was calculated as the ratio of the number of blue colonies to the total number of colonies.

**FIG. 2.** lacZ inactivation and repeat deletion frequencies for HIV-1 and MuLV. Comparison of lacZ inactivation rates is shown in the insert and repeat deletion frequencies are indicated in the main figure. Values for HIV-1 vectors assayed in mATRC1/293 cells (white bars), MuLV in mATRC1/293 cells (gray bars), and MuLV assayed in NIH-3T3 cells (black bars; from Pfeiffer et al., 1999). Repeat deletion values were corrected to account for lacZ inactivation frequencies.
quency similar to simple retroviruses such as to MuLV and SNV. This finding was unexpected because previous measurements of intermolecular template switching rates between copackaged RNAs have suggested that HIV-1 template switching is about 10-fold more frequent than that of SNV or MuLV (Hu and Temin, 1990; Anderson et al., 1998; Jetzt et al., 2000).

Possible explanations for this new finding of similar repeat deletion rates for HIV-1 and MuLV—despite vast reported recombination differences—range from technical differences of no biological importance to potentially significant differences in replication properties. For example, whereas HIV-1 intermolecular switching in a single-cycle assay occurred two or three times per cycle (Jetzt et al., 2000), data with replication-competent virus from a different research group suggests only one crossover occurs per replication cycle (St Louis et al., 1998). It is similarly possible that if another research group using different approaches addressed simple retrovirus recombination rates, they might be revised upward. Thus, technical differences between labs might erode the reported 10-fold difference between HIV-1 and simple retrovirus recombination frequencies.

Another possibility is that although the highly mosaic nature of natural HIV-1 recombinants appears consistent with high recombination frequencies (Quinones-Mateu and Arts, 1999), these mosaic genomes may have resulted from negative interference, which occurs during the replication of simple retroviruses (Hu et al., 1997). Negative interference is the phenomenon in which retroviral genomes that show any evidence of genetic recombination tend to display more crossovers than would be predicted based on calculated average recombination rates. If previously described HIV-1 recombinants are not typical HIV-1 reverse transcription products but instead products of a rare “recombining population” (Anderson et al., 1998), then HIV-1 and simple retrovirus recombination rates may be indistinguishable over all.

A third possibility is that direct repeat deletion and intermolecular recombination may differ mechanistically, so one cannot be used as a predictor for the other. A possible precedent for this notion lies in the observation that direct repeat deletion in Escherichia coli is independent of the recombination protein RecA, whereas other forms of homologous recombination are RecA-dependent (Lovett et al., 1993).

A fourth possibility is that template switching frequencies may be the same for the studied retroviruses but that their genome organizations may differ, thus leading to the appearance of differences where none exist. Retroviral recombination is detectable only when it occurs between genetically distinct templates (Hu et al., 1993). Although the possibility that RNA copackaging may not be random has been discussed (Hu et al., 1997), a general assumption has been that when two similar retroviral RNAs are coexpressed in single cells, they will dimerize and thus copackage randomly, and hence numbers of “heterozygous” particles can be calculated from the total amount of each RNA in the population. However, how RNAs are recruited for packaging is not well understood, and there is some evidence that these processes may differ among retroviruses (Kaye and Lever, 1999). If MuLV RNA homodimers were packaged preferentially but dimerization and copackaging were random for HIV-1, HIV-1 recombination would appear to be more frequent than that of MuLV even if template switching rates were indistinguishable.

**MATERIALS AND METHODS**

**Plasmids and cells**

The HIV-1 transducing vector, pHIVlac, was a derivative of pHRCMVlacZ (Naldini et al., 1996b), which con-
tains cis-acting sequences necessary for reverse transcription, packaging, and integration. To construct pHIVlac, the NheI plasmid backbone of pHIVlac was replaced with an EcoRI-XbaI fragment from pUC18. Then a 1-kb Asp718-SalI fragment containing an SV40 early promoter and a puromycin-resistance gene from an MuLV-based vector (Kulpa et al., 1997) was inserted downstream of lacZ. pHIVlac117, pHIVlac284, and pHIVlac971 were constructed by replacing the FspI–EcoRI lacZ fragment of pHIVlac with corresponding fragments from MuLV-based vectors, pLaac-117, pLaac-284, and pLaac-971 (Pfeiffer et al., 1999). The helper function plasmid used, pCMVΔR8.2 (Naldini et al., 1996a), encodes all HIV-1 trans-acting proteins including Vpu but not envelope, driven by a CMV immediate early promoter.

ET cells are a 293T derivative that constitutively expresses ecotropic envelope (Pfeiffer et al., 1999). mATRC1/293 cells are derived from the human 293 cell line and stably express the receptor for ecotropic Env (Malhotra et al., 1998). Both of these cell lines and their derivatives were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gemini).

Transfection and virus harvesting

HIVlac and HIVlac vector viruses were generated by transiently cotransfecting ET cells with pCMVΔR8.2 and the corresponding transducing vector plasmid by calcium phosphate precipitation (Miller et al., 1993). Virus was harvested by replacing culture medium 16–24 h after transfection and collecting medium 36–48 h posttransfection. Residual cells were removed from the medium by 4°C low-speed centrifugation for 5 min at 3000 g. Virus was stored at −70°C prior to use. For each HIV-1 transducing vector, three viral stocks were prepared from separate cotransfections.

MuLV vector viruses were produced using the same approaches, except that ET cells were cotransfected with the MuLV helper construct, pMLVΨ+, and the MuLV transducing constructs, pLaac-117, pLaac-284, and pLaac-971 (Pfeiffer et al., 1999), respectively. Virus stocks were prepared and stored as described above and used to infect mATRC1/293 cells as described below for the HIV-1 based vectors.

Infection and functional assay for LacZ activity

mATRC1/293 cells in 6-cm-diameter dishes were infected 1 h in the presence of 8 μg/ml of hexadimethrine bromide (Polybrene; Sigma). Puromycin (Sigma) selection, cell fixation, and 5-bromo-4-chloro-3′-galactopyranoside (X-gal; Sigma) staining were performed as previously reported (Pfeiffer et al., 1999) except that infected cells in each 6-cm-diameter dish were subcultured into one or two 10-cm-diameter dishes before puromycin selection. Infection with each virus stock was performed in parallel in three or six dishes as indicated. The numbers of blue and white colonies were summed for each infection experiment and were tabulated in Tables 1–4.

To determine lacZ inactivation rate, ET cells were cotransfected with pHIVlac and pCMVΔR8.2 helper, virus was harvested, and mATRC1/293 cells were infected, selected in puromycin, and stained as above. The inactivation rate was calculated as the ratio of white colonies per total colonies. To determine repeat deletion rates, pHIVlac117, pHIVlac284, or pHIVlac971 was used in cotransfections with helper. Where indicated, infected cells were maintained in media supplemented with 0.2 mM hydroxyurea (HU; Sigma) or 1.0 μM 3′-azido-3′-deoxythymidine (AZT; Sigma) until puromycin selection was initiated. Results reported in Tables 1 and 4 are from experiments in which HU or AZT treatment was started at the end of the 1-h infection period. Experimental repetitions were also performed in which HU or AZT treatment was initiated 4 h before infection and continued through the infection period. Under the latter condition, HU and AZT treatment resulted in increases in lacZ inactivation and deletion rates that were within the range of 1.3- to 1.8-fold, values which are similar to those obtained under conditions used to generate the data reported in the tables (data not shown).

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