Mutant murine leukemia virus Gag proteins lacking proline at the N-terminus of the capsid domain block infectivity in virions containing wild-type Gag

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Abstract

We have investigated the properties of murine leukemia virus Gag mutants in which the p12-CA cleavage site is altered. In one mutant, the cleavage is blocked; in the other, the conserved proline at the N-terminus of CA has been replaced with glycine. No infectivity was detected in either mutant. Mutant particles cannot synthesize full-length DNA upon infecting permissive cells. Particles composed of a mixture of wild-type and mutant proteins have severely impaired infectivity. These mixed particles are defective in their ability to synthesize DNA upon infection, but this defect is less severe than the loss of infectivity. Thus, proteins lacking the correct N-terminus of CA inhibit DNA synthesis and also interfere with formation or integration of a full-length, normal provirus. The results imply that CA proteins function as part of a large, highly organized structure in reverse transcription and apparently at a later step as well.

Keywords: Retroviruses; Capsid proteins; Infectivity; Reverse transcription; Dominant negative; Trans-dominant; Phenotypic mixing; Virus maturation

Introduction

The principal structural component of a retrovirus particle is the virus-coded Gag protein. After the particle is released from the virus-producing cell, Gag is cleaved by the viral protease (PR) into a series of cleavage products, always including (from N- to C-terminus) matrix (MA), capsid (CA), and nucleocapsid (NC). This series of cleavage events is termed “maturation” of the particle and is essential for infectivity (Swanstrom and Wills, 1997).

The structure of the CA protein of HIV-1 has been investigated in detail, using both nuclear magnetic resonance and X-ray crystallography. Retroviral CA proteins are composed of two domains connected by a flexible linker. Their N-terminal residue is always proline. Following the cleavage event that generates this N-terminus, the first ~50 amino acids of CA fold into a β-hairpin, and the proline forms a buried salt bridge with an internal aspartate residue (Gamble et al., 1996; Gitti et al., 1996; Tang et al., 2002).

Maturation entails a drastic change in the morphology of the virion. One of the hallmarks of this change is the appearance of the “mature core” of the particle, a densely staining body in the interior of the virion. Recent studies on structures formed in vitro by purified CA proteins of HIV-1 and murine leukemia virus (MLV) show that the mature core is composed of CA molecules. Within this core, CA proteins are evidently arranged in a lattice in which the N-terminal domains of groups of six CA molecules are in hexameric rings; each ring is joined to neighboring rings by dimeric contacts between C-terminal domains (Ganser et al., 2003; Li et al., 2000; Mortuza et al., 2004). The planar hexagonal lattice is closed by the presence of twelve pentameric “defects” (Ganser et al., 1999).

Several types of evidence suggest that the correct mature core structure is essential for infectivity, and that this structure depends in turn upon the presence of the proline at the N-
terminus of CA. Thus, mutants in which the proline is replaced by another amino acid (Fitzon et al., 2000) (see below) or in which the release of CA from Gag is inhibited by a change in the cleavage site (Gottlinger et al., 1989; Oshima et al., 2004) are not infectious. Similar results are also observed when the aspartate partner in the salt bridge in HIV-1 CA is replaced by alanine (Tang et al., 2003).

In the present work, we have analyzed the properties of MLV mutants at the N-terminus of CA. We have also studied MLV particles containing a mixture of mutant and wild-type proteins. The mutants investigated here included one in which maturation cleavage at the N-terminus of CA is blocked by a change in the residue preceding proline in Gag and one in which the N-terminal proline in CA was replaced by glycine. We found that both of these mutants potently inhibit the functions of the wild-type, reducing the specific infectivity of the mixed particles. The data indicate that these mutant proteins interfere with the ability of the particle to synthesize viral DNA upon entering the new host cell. The results support the hypothesis that an organized assemblage of CA molecules in the mature particle plays a crucial role in facilitating reverse transcription; this functional complex is evidently disrupted by the presence of mutant proteins in the particle, even when wild-type proteins are in excess. However, the inhibition of DNA synthesis did not appear to be sufficient to explain the loss of infectivity in the mixed particles. Thus, the data further suggest that the correct structure of the mature core is also required for the correct formation of the final DNA product or for a subsequent step, such as integration of the DNA into the chromosome of the host cell.

Results

Properties of “S2G”

The MLV Gag protein, Pr65, is cleaved into MA, p12, CA, and NC during virus maturation, as indicated in Fig. 1A. We have previously described mutants at cleavage site 2 (“S2”), the p12-CA cleavage site (Oshima et al., 2004). We found that replacement of the tyrosine at the C-terminus of p12 (the P1 position of S2) with a charged residue, such as aspartate, completely blocked cleavage at this site. This mutant was designated S2D. The location of this mutant in the Gag protein is illustrated schematically in Fig. 1A. We also observed that cleavage at the other two sites of maturation cleavage, particularly the CA-NC cleavage site, was somewhat inefficient in S2D particles (Oshima et al., 2004). In the present work, we have also investigated a mutant in which the proline at the N-terminus of CA (i.e., the P1’ position of site 2) was replaced by glycine; we refer to this mutant as “S2G” for convenience.

Fig. 1B shows an analysis of S2G particles, using immunoblotting with anti-CA antiserum. Also included in the figure, for comparison, are particles of S2D (in which there is no free CA, but only the 42-kDa p12-CA fusion protein (Oshima et al., 2004)), as well as wild-type and PR− particles. It can be seen that the S2G profile contains a substantial amount of CA protein. There is also a significant level of a protein migrating just slightly faster than the 42-kDa protein of S2D; this is p40, the CA-NC fusion protein, since it reacts with anti-NC as well as anti-CA antisera (data not shown). p40 was previously observed in S3R particles, in which cleavage between CA and NC was blocked (Oshima et al., 2004) (see Fig. 1A). Thus, despite the fact that this mutant is altered at the N-terminus of CA, it exhibits efficient cleavage at the N-terminus, but only partial cleavage at the C-terminus, of CA. Both S2G and S2D particles also contain two larger intermediates, presumably MA-p12-CA and p12-CA-NC.

The morphology of S2G particles was also examined. Released virions were collected for thin-section electron microscopy by immunoprecipitation with anti-SU antibody as described (Oshima et al., 2004). We found (Fig. 2) that they are similar to wild-type MLV particles, but that their structure is considerably more irregular. For example, many particles contain condensed material in their interiors but lack a clearly defined core. Other particles resemble immature particles, but

![Fig. 1](image-url) Cleavage of MLV Gag and CA-containing protein species in S2G virions. (A) (top row) Scheme showing normal cleavage of MLV Gag into MA, p12, CA, and NC; (middle row) production of p42 in mutants such as S2D which block cleavage at site 2 (between p12 and CA); (bottom row) production of p40 when cleavage at site 3 is blocked. (B) Particles of S2G (lane 1), S2D (lane 2), wild-type MLV (lane 3), and PR− MLV (lane 4) were collected from culture fluid and analyzed by immunoblotting with anti-CA antiserum. Mobilities of Pr65Gag, p30CA, and the p42 and p40 cleavage intermediates are indicated.
the ring of darkly staining material under the membrane of the particle is incomplete. Examples of these morphologies are in the field shown in Fig. 2B.

We also measured infectivity of S2G particles, using either a luciferase reporter or the green fluorescent protein (GFP)-based system described earlier (Oshima et al., 2004). No infectivity was detected in either of these assays, since the luciferase or GFP activity induced by the mutant particles was no higher than in cultures “mock-infected” using culture fluids from cells transfected with the empty vector pGCcos3neo; the luciferase data are shown in Table 1. The specific infectivity of these particles is at least several hundred-fold below that of wild-type particles.

We have previously analyzed the genomic RNA in S2D mutant particles (Oshima et al., 2004). These tests gave no suggestion that packaging of the RNA was reduced in the S2D particles. However, it was conceivable that packaging is defective in S2G particles. We therefore produced S2G particles, along with wild-type control particles, in 293T cells stably transfected with the MLV-derived vector pLXSH (Miller et al., 1993) (see below). We analyzed the particles for hygromycin phosphotransferase (hph) RNA by real-time RT-PCR. No significant differences between hph RNA contents of S2G and wild-type (or S2D) virions were observed (data not shown).

Properties of virions containing a mixture of wild-type and S2D or S2G Gag proteins

The focus of the present study was the biological properties of MLV particles composed of a mixture of wild-type and mutant Gag proteins. We measured infectivity by co-transfecting 293T cells with an MLV-based reporter vector along with the MLV proviral clones being analyzed.

A series of 293T cultures was transfected with constant amounts of reporter vectors and wild-type MLV plasmid together with graded amounts of S2D or S2G mutant plasmid. Virions in the culture fluid of the transfected cells were collected and assayed for their Gag protein profile and their infectivity. Immunoblots are shown in Fig. 3. The only proteins detected with anti-p30CA antiserum in the wild-type particles (lanes 1–4) are p30CA and a small amount of the uncleaved precursor, Pr65Gag. As noted above, however, the S2D clone (lane 10) gives rise to virions that contain p42 (the p12-CA fusion protein) and two larger intermediates in addition to Pr65Gag (Oshima et al., 2004). When increasing amounts of S2D plasmid are added to the wild-type plasmid (lanes 5–9), these same species, as well as free CA, are observed. A culture transfected with equal amounts of the two plasmids (lane 7) produces particles containing approximately equal amounts of CA and p42. Thus, neither of the MLV plasmids affects the contribution of the other plasmid to the virions produced by the co-transfected cells (It is interesting to note one deviation from this simple statement. There is more free CA in lane 9 than in lanes 5–8, despite the fact that CA is only produced from the wild-type plasmid, which is at a constant level in lanes 5–9. It seems likely that virus assembly is cooperative, so that the expression of S2D Gag increases virion production and, consequently, the export of wild-type Gag in virus particles.) Similar results were obtained in the cultures co-transfected with wild-type plasmid and increasing amounts of S2G (lanes 12–17).

Infectivity of mixed virus stocks like those shown in lanes 5–9 and 12–16 was assessed by infecting 293T cells expressing MCAT (Albritton et al., 1989) with them and assaying the infected cultures 48 h later for luciferase activity. Normalized results of these assays are shown in Fig. 4. It is evident that the infectivity values are substantially lower in the mixed particles than in the wild-type controls; for example, particles with equal amounts of wild-type and S2D protein have a titer several hundred-fold below that of the wild-type control. Similar results have also been obtained in assays on NIH3T3 cells (data not shown). (In the experiment presented here, S2D was more detrimental in its effects on infectivity than S2G. However, in some experiments, S2G has been fully as deleterious as S2D.

Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>LUC</th>
<th>Protein, µg/ml</th>
<th>Hph copies</th>
<th>CCR5 copies</th>
<th>Hph/CCR5</th>
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</thead>
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<tr>
<td>WT</td>
<td>30,876 ± 1344</td>
<td>370</td>
<td>16,472 ± 4664</td>
<td>36,641 ± 2289</td>
<td>0.449</td>
</tr>
<tr>
<td>S2D</td>
<td>37 ± 6</td>
<td>395</td>
<td>9.3 ± 1.8</td>
<td>73,530 ± 7035</td>
<td>0.00001</td>
</tr>
<tr>
<td>S2G</td>
<td>43 ± 12</td>
<td>483</td>
<td>4.6 ± 5.3</td>
<td>89,833 ± 13,016</td>
<td>0.00005</td>
</tr>
<tr>
<td>RT</td>
<td>N.D.</td>
<td>363</td>
<td>14.8 ± 9.8</td>
<td>80,719 ± 16,743</td>
<td>0.0002</td>
</tr>
<tr>
<td>pGCcos3neo</td>
<td>30 ± 0</td>
<td>341</td>
<td>5.8 ± 5.7</td>
<td>100,117 ± 13,844</td>
<td>0.00008</td>
</tr>
</tbody>
</table>

Virus particles were produced by transfecting pLXSH-containing cells with 4 µg of wild-type or mutant MLV plasmid. Each plate also received 4 µg of pBabe-Luc DNA. Infectivity was assessed by measuring luciferase activity in extracts of 293T/MCAT cells infected by the particles, while the amount of hph DNA in the infected cells was used to measure reverse transcription. The single-copy cellular gene CCR5 was used as a DNA recovery control. Luciferase activity ("LUC") is expressed as relative light units per 20 µl of cell extract. N.D., not determined.
We have been unable to determine the reason for this variation between experiments.

It seemed possible that these results are due to some peculiarity of the luciferase reporter system used here. To test this possibility, we included pLZRS-EGFP, as well as pBABE-Luc, in the co-transfections with mixtures of mutant and wild-type plasmids and assayed the culture fluids for the titer of GFP-inducing infectious units as well as for the infectivity of the luciferase vector. The relative titers obtained with the two reporter systems were virtually superimposable (data not shown). Thus, the reduced infectivity of mixed particles containing S2D or S2G protein is evidently independent of the assay used and presumably reflects an intrinsic property of these virions.

As noted above, S2G particles are inefficiently cleaved at the C-terminus of CA, so that they give rise to a significant amount of the p40 CA-NC fusion protein (Figs. 1B, 3). It seemed possible that this property was responsible for the ability of S2G Gag to reduce the infectivity of mixed particles (Fig. 4). To test this hypothesis, we also assessed the infectivity of particles produced following co-transfection of wild-type and S3R MLV; the leucine at the C-terminus of CA has been replaced in S3R with arginine, completely preventing cleavage between CA and NC (Oshima et al., 2004). The results of these tests are shown in Fig. 5. As can be seen in Fig. 5B, the admixture of S3R Gag protein (shown in Fig. 5A) had almost no effect on the infectivity of the particles. Thus, the inhibition of wild-type infectivity by the S2G Gag protein is probably not due to the inefficiency of cleavage at site 3.

DNA synthesis upon infection with particles containing both wild-type and mutant Gag proteins

It was of interest to determine whether the presence of the S2D or S2G proteins in the mixed particles interfered with the ability of the particles to synthesize viral DNA upon entry into susceptible cells. We therefore measured reverse transcription products, using real-time PCR, at 24 h after infection with these particles. One potential technical problem in these experiments is the presence in the virus preparations of plasmid DNA from the transient transfection used to produce the particles. To eliminate this background, we adopted the following strategy. 293T cells were first stably transfected with the MLV-derived vector pLXSH, which contains the coding sequence for hph (Miller et al., 1993). The wild-type and mutant MLV clones (together with pBABE-Luc, which enabled us to conveniently measure the infectivity of the progeny viruses) were then co-transfected into these hygromycin-resistant cells, and virus produced following this transient transfection was finally analyzed for its ability to synthesize hph DNA following infection of permissive cells as described in Materials and methods. Cells were lysed 24 h after infection and assayed for hph DNA by real-time PCR. It should be noted that hph DNA synthesis depends upon completion of the preceding steps in reverse transcription, i.e., (−) strand strong-stop DNA synthesis and the first strand transfer event. To control for possible variation in recovery of DNA from the infected cells, we also.
measured the amount of the single-copy gene CCR5 in our cell extracts. The variation among all samples with respect to CCR5 content was less than 3-fold (data not shown).

We found (Table 1) that particles containing only S2D or S2G mutant proteins were incapable of synthesizing hph DNA; that is, the corrected values of "hph DNA" in the infected cells were always at least 5000-fold lower than in the wild-type controls and were never significantly higher than those in mock-infected control cells. Thus, cleavage at the N-terminus of CA is apparently essential for reverse transcription of the full-length viral genome, and replacement of the conserved proline residue with glycine at this site is also incompatible with synthesis of the entire DNA product. We then measured hph DNA synthesized in cells infected with the virions containing mixtures of wild-type and S2D or S2G proteins. Results (following normalization to the wild-type control) are shown in the closed circles in Fig. 6. It is obvious that as the proportion of mutant protein in the virion increases, the particle becomes progressively impaired in its ability to perform reverse transcription. It is striking to note, however, that the inhibition of viral infectivity induced by the presence of the mutant proteins (dashed lines) is, in general, significantly greater than the inhibition of hph DNA synthesis (closed circles). In other words, many particles which synthesize DNA in newly infected cells still fail to establish infection.

In an effort to further define the block in infection imposed by the presence of Gag proteins with alterations at the N-terminus of CA, we also measured the level of hph DNA in cells that had been passaged for 11 days following infection. As shown in the open circles in Fig. 6, the relative levels of

Fig. 5. Properties of virions produced following co-transfection of wild-type and S3R plasmid DNA. 10-cm dishes of 293T cells were transfected with 4 μg of wild-type plasmid + 4 or 8 μg of S3R or S2G plasmid. Control cultures were transfected with 4 μg of wild-type or mutant plasmids alone. All cultures also received 4 μg of pBabe-Luc DNA. Total plasmid DNA in the transfections was kept constant by addition of pGCcos3neo DNA. (A) Protein composition of the virions from the transfected cells, as analyzed by immunoblotting with anti-CA antiserum; (B) infectivity of the virions, assayed by infecting NIH3T3 cells and measuring luciferase activity 48 h later. Luciferase values were divided by the activity obtained with 4 μg wild-type plasmid + 8 μg pGCcos3neo DNA. Closed circles: effect of S3R; open circles: effect of S2G.

Fig. 6. Effect of S2D (A) and S2G (B) on infectivity of and DNA synthesis by MLV particles. 293T cells expressing MCAT were infected with the virions analyzed in Fig. 3 and were lysed 24 h later for assay of hph DNA (closed circles) and for 2-LTR circular DNA (triangles). Replicate plates were lysed 48 h later for luciferase activity (dashed lines; data as in Fig. 4). Replicate cultures were also passaged for 11 days and then lysed and assayed for hph DNA (open circles). All DNA measurements were also corrected for differences in recovery of CCR5 DNA.
hph DNA at this time-point were virtually superimposable with the relative levels of infected cells (dashed lines). These results suggest that a uniform fraction – perhaps all or nearly all – of the hph DNA molecules that were stably associated with the infected cells were present in functional, integrated proviruses.

It seemed possible that the hph DNA molecules that are not stably associated with the cells could be blocked with respect to entry into the nucleus, prior to proviral integration. The presence of closed 2-LTR circular DNA is frequently taken as an indicator of entry of viral DNA into the nucleus (e.g., Yuan et al., 2002). We therefore measured the level of 2-LTR circles in the cells 24 h after infection. As shown in Fig. 6 (triangles), the relative levels of these circles are significantly higher than the levels of infection or of stable hph DNA and correspond closely with the level of hph DNA in the 24-h lysates. Thus, there is no indication that the nuclear entry of DNA molecules is blocked in the cells infected with the mixed virions.

**Discussion**

We have shown here (Fig. 4) that two MLV Gag proteins which lack the absolutely conserved proline residue at the N-terminus of the capsid domain are potent dominant-negative inhibitors of infectivity when present in virions together with the wild-type protein. In one of these, S2D, the change in the Gag coding sequence prevents cleavage at the p12-CA boundary, so that no free N-terminus of CA is formed during virus maturation. In contrast, the other mutant studied, S2G, undergoes efficient cleavage at this site, but the resulting N-terminal residue is glycine rather than proline. (Cleaveage at the C-terminus of CA is, however, incomplete in S2G mutant particles (Fig. 1B). It is somewhat surprising that the cleavage at site 3 is inhibited by the replacement of proline with glycine at site 2).

We attempted to identify the point in the viral replication cycle that is blocked in the particles containing both wild-type and mutant Gag proteins. We found (Fig. 6) that the proportion of mixed particles synthesizing late (i.e., hph) DNA products is reduced in the mixed particles but is still significantly higher than the proportion that successfully establishes infection. The presence of the mutant Gag proteins also has a less drastic effect on the synthesis of 2-LTR circular DNA than on infection or stable association of viral DNA with the cells, presumably in integrated proviruses. Taken together, these results imply that there is a fraction of the phenotypically mixed virions which synthesizes DNA, and that this DNA enters the nucleus but is not integrated into cellular DNA. One hypothesis that could explain these results is that these DNA molecules are unsuitable substrates for integrase: perhaps there are subtle defects in their ends, as has been previously described for mutants in NC (Gorelick et al., 1999) and, in HIV-1, in the RNase H domain of reverse transcriptase (Julias et al., 2002).

The ability of the mutant proteins to interfere with the function of the wild-type CA in the mixed particles implies that wild-type proteins do not function as monomers, but as part of a large, organized assemblage; this large functional unit is apparently disrupted and inactivated by the presence of mutant proteins within it. This structure is presumably the mature core of the virion. Mutants elsewhere within HIV-1 CA have previously been shown to interfere with infectivity in mixed particles (Furuta et al., 1997; Mammano et al., 1994; Trono et al., 1989). Interestingly, a recent study of poliovirus pointed out that genes in which mutations show trans-dominant effects can be particularly attractive targets for antiviral therapy; indeed, screening for trans-dominance can be used in the selection of drug targets (Crowder and Kirkegaard, 2005). Taken together, these observations highlight the potential utility of particle assembly and maturation as potential antiretroviral drug targets.

Unfortunately, the lack of basic molecular understanding and of convenient screens has made it impossible to exploit these targets to date.

The results presented here have, in part, been foreshadowed by previous reports using low doses of PR inhibitors on HIV-1. It was found in these studies that drug concentrations which only prevent cleavage in a small fraction of the Gag and Pol proteins lead to production of noninfectious particles (Kaplan et al., 1993; Krausslich, 1992). It was also reported that the loss of infectivity in these particles is more severe than the defect in reverse transcription (Kaplan et al., 1993). Thus, these results, like those in the present study, imply that a minority of uncleaved viral proteins (as in the S2D: wild-type mixed particles) can block infectivity without blocking DNA synthesis. However, they do not provide information as to which cleavage sites are critical for infection.

The present results also add to the growing body of evidence indicating that the mature core of a retrovirus particle plays a crucial role in reverse transcription. Thus, mutations in HIV-1 CA that alter the stability of the core in viral lysates affect the kinetics of DNA synthesis during infection (Forshey et al., 2002). In addition, mutations that replace either the N-terminal proline or its aspartate partner in the buried salt bridge in CA interfere with both formation of the normal core morphology and the initiation of viral DNA synthesis upon infection (Fitzon et al., 2000; Tang et al., 2003; von Schwedler et al., 1998). These studies all suggest that uncoating of the viral genome is a critical, tightly regulated step in infection which requires the correct conformation of the N-terminus of CA. In MLV also, mutants near the N-terminus of CA have been shown to be impaired in reverse transcription (Auerbach et al., 2003). However, we are not aware of prior reports implicating CA in the successful completion of integration-competent viral DNA copies, as suggested by the data in Fig. 6. In this connection, it is interesting to note that retroviral CA proteins are also the target of several cellular restriction systems, including Fv-1, Lv-1, and Ref-1 (Lee and KewalRamani, 2004). It appears that some of these systems interfere with infection before viral DNA synthesis (Stremlau et al., 2004; Towers et al., 2000), while others interfere with a subsequent event (Jolicoeur and Rassart, 1980; Yang et al., 1980); again, these observations would suggest that CA functions both in the initiation of reverse transcription and in its successful completion or in the integration of the DNA product.
Materials and methods

Cells and viruses

All experiments described here were performed with full-length Moloney MLV proviral clones ultimately derived from the infectious clone pRR88 (Fu and Rein, 1993). Virus particles were produced by transient transfection of 293T cells, using Transit 293 (Mirrus) in accord with the manufacturer’s instructions. When viruses were to be analyzed for infectivity, either pBABE-Luc, which contains the firefly luciferase gene from pGL3 (Promega) in pBabe Puro (Morgenstern and Land, 1990), or pLZRS-EGFP, an MLV-based vector encoding GFP (Dardalhon et al., 1999), was co-transfected with the MLV proviral constructs being analyzed. Infectivity was then measured by infecting either NIH3T3 cells or 293T cells expressing the ecotropic MLV receptor MCAT (Albritton et al., 1989) (a kind gift of J. Cunningham), and either enumerating GFP-positive cells as described (Oshima et al., 2004) or assaying cell extracts for luciferase activity using the Luciferase Assay System (Promega) 48 h after infection. The cell extracts were also assayed for total protein content using the BCA assay system (Pierce) according to the manufacturer’s instructions. MLV containing a mutation at the active site of reverse transcriptase, changing residues 223 and 224 from aspartate to lysine and leucine, respectively (a kind gift from Robert Gorelick, SAIC Frederick), was used as a negative control. Except where specified otherwise, all techniques were as described (Oshima et al., 2004).

Electron microscopy

Released virus was examined after it was collected by immunoprecipitation on protein G-Sepharose beads as previously described (Campbell et al., 2002).

Viral RNA

Virions were assayed for hph RNA by real-time RT-PCR as described (Hibbert et al., 2004).

Viral DNA synthesis

The ability of virus particles to perform DNA synthesis upon infecting new host cells was assayed as follows. Viruses were produced by transient transfection of 293T cells that had previously been stably transfected with pLXSH (Miller et al., 1993) and selected for hygromycin resistance. The virus particles obtained following the transient transfection were then used to infect 293T cells expressing MCAT. 24 h later, the cells were lysed by the QIAamp DNA Mini Kit (Qiagen, Valencia, CA), and the cell extracts were assayed for hygromycin phosphotransferase (hph) DNA by real-time PCR as described (Hibbert et al., 2004). Two-LTR circles were assayed by real-time PCR using 5′-GTC TCGCTTGGAGGTCT-3′ as the forward primer, 5′-CAT CTG TTC GTCCACC TTG ATC TGA-3′ as the reverse primer, and 5′-FAM-GGC GTT ACT TAA GCT AGC TTG CCA-3′ as the probe. These reagents were designed to anneal to sequences found in both MLV and pLXSH. The extracts were also assayed for the single-copy gene CCR5 by real-time PCR in order to control for differences in recovery of DNA from the extracts; these assays used 5′-CCA GAA GAG CTT AGA CAT CCG-3′ for forward primer, 5′-GCC AAG CAG CTT AGA GGT TAC T-3′ for reverse primer, and 5′-FAM-TCCCTACAAGAAAACCTCCCGG-TAMRA-3′ for probe. Plasmids used as standards in these two assays were a kind gift of Robert Gorelick.

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