

Primary T-lymphocytes rescue the replication of HIV-1 DIS RNA mutants in part by facilitating reverse transcription

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ABSTRACT

The dimerization initiation site (DIS) stem-loop within the HIV-1 RNA genome is vital for the production of infectious virions in T-cell lines but not in primary cells. In comparison to peripheral blood mononuclear cells (PBMCs), which can support the replication of both wild type and HIV-1 DIS RNA mutants, we have found that DIS RNA mutants are up to 100 000-fold less infectious than wild-type HIV-1 in T-cell lines. We have also found that the cell-type-dependent replication of HIV-1 DIS RNA mutants is largely producer cell-dependent, with mutants displaying a greater defect in viral cDNA synthesis when viruses were not derived from PBMCs. While many examples exist of host–pathogen interplays that are mediated via proteins, analogous examples which rely on nucleic acid triggers are limited. Our data provide evidence to illustrate that primary T-lymphocytes rescue, in part, the replication of HIV-1 DIS RNA mutants through mediating the reverse transcription process in a cell-type-dependent manner. Our data also suggest the presence of a host cell factor that acts within the virus producer cells. In addition to providing an example of an RNA-mediated cell-type-dependent block to viral replication, our data also provides evidence which help to resolve the dilemma of how HIV-1 genomes with mismatched DIS sequences can recombine to generate chimeric viral RNA genomes.

INTRODUCTION

All retroviruses, including human immunodeficiency virus type 1 (HIV-1), contain two copies of genomic RNA that are noncovalently linked near their 5' end to form dimers (1,2). The formation of a dimeric RNA genome is critical for HIV-1 viral replication and HIV-1 genomic RNA

dimerization is believed to facilitate reverse transcription (3,4), to increase the rate of genetic recombination (5–7) and to contribute to overall genetic diversity (8,9).

Formation of HIV-1 genomic RNA dimers is initiated by a region within the 5' untranslated region (5'UTR) of the HIV-1 RNA genome known as the dimer initiation site (DIS) stem-loop (10–13). The HIV-1 5'-UTR is one of the most conserved parts of the HIV-1 RNA genome, and contains extensive secondary and tertiary structures that form function domains which regulate key steps in the viral replication cycle (14). These domains include signals for transcriptional transactivation (the TAR domain), polyadenylation [the Poly(A) signal], reverse transcription (the primer binding site; PBS), genomic RNA dimerization (the DIS/SL1), splicing (the splice donor site; SD/SL2), genomic RNA encapsidation (the Ψ /SL3) and translation (the gag start codon; AUG/SL4) (14). The HIV-1 DIS stem-loop consists of a short 35 base sequence that is located between the primer binding site and the major splice donor site, which folds into a hairpin structure with an exposed palindromic sequence flanked by 5' and 3' purines within its loop (15). This highly conserved palindrome sequence, which consists of either a 5'-GCGCGC-3', 5'-GTGCAC-3' or 5'-GTGCGC-3' (16), within the DIS stem-loop is important for the formation of viral RNA dimers *in vitro* (10,11). According to the proposed model for dimer formation, contact between two DIS hairpins is initiated by base pairing of the self-complementary palindrome sequences to form what is known as the kissing-loop complex (17–19). In a structural rearrangement activated by the HIV-1 nucleocapsid protein, this initial kissing-loop complex is then transformed into a more stable dimer with extended interstrand base pairing known as the extended duplex (20–22). This rearrangement is thought to be associated with viral particle maturation, but experimental evidence illustrating that virion genomic RNA undergoes the same reorganization *in vivo* is currently lacking.

Studies using cell-free RNA binding assays have shown that both the palindrome sequence and the stem-loop of

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the HIV-1 DIS are essential for the dimerization of short sequences of viral RNA *in vitro* (10,11). However, significant amounts of dimerized viral genomic RNA can be found in full length mutant viruses that contain severe disruptions of the DIS structure (23,24); although these RNA dimers appear to have an abnormal conformation, failing to form discrete dimers. These data imply the involvement of other viral RNA sequences in the dimerization process. In support of this, cell-free assays with synthetic RNA molecules showed that antisense nucleotide oligomers that bind to a separate stem-loop structure in the HIV-1 5' UTR (SL3) and a downstream GA-rich RNA region were able to inhibit RNA dimerization (25). In the context of full length HIV-1, DIS stem-loop mutations lead to decreased genomic RNA dimerization (24,26), genomic RNA packaging (3,23,26–29) and virus infectivity (3,26,27,30).

Deletion of the complete DIS stem-loop has been shown to strongly decrease HIV-1 infectious titres; however, we have previously demonstrated that the requirement for the DIS RNA stem in the replication of HIV-1 is cell-type dependent (23), with HIV-1 mutants lacking the DIS stem being replication competent in primary peripheral blood mononuclear cells (PBMCs) but not in a SupT1 T-cell line. Long-term passage of DIS RNA mutants in PBMCs did not restore the DIS stem or lead to the detection of compensatory mutations (23). The ability of HIV-1 DIS RNA mutants to replicate in PBMCs has been validated independently using a primary isolate from a patient sample (31). However, in contrast to these studies, it has been reported that a Δ DIS HIV-1 mutant was not replication competent in cord blood mononuclear cells (CBMCs) (32). The biological distinctions between PBMCs and CBMCs are currently unclear and it is unknown whether these contrasting results are due to the differential proteinomic profiles of these two distinct primary cell populations.

While numerous protein-mediated cell-type-dependent restrictions have been reported (33–35), examples of RNA-mediated cell-type-dependent restriction are few and far between. Recently, the role of RNA in cell biology has been reassessed, revealing that RNA sequences may have a direct role in regulating biological processes (36–38). It is unclear whether the observed RNA-mediated restriction of HIV-1 DIS mutants is limited to SupT1 cells or if it is a widespread phenomenon across other T-cell lines. It is also unknown what the RNA structural requirements that govern this restriction process are or at what stage of the viral replication cycle this cell-type-dependent restriction acts.

The dimerization of HIV-1 genomic DNA is also thought to play a role in the creation of intersubtype recombinant viruses. It is believed that the formation and packaging of heterologous genomic RNA dimers consisting of RNA genomes from two different HIV-1 subtypes relies on the ability of the two complementary viral DIS sequences to mediate RNA dimerization (39). To date, three different HIV-1 DIS sequences have been identified and different HIV-1 subtypes utilize different DIS sequences (5'-GCG CGC-3' subtype B and D, 5'-GTGCAC-3' subtype A, C, E, F and G and 5'-GTGCGC-3' subtype D and G) (16).

Mismatching DIS sequences are thought to reduce genomic RNA dimerization (40) and a recent study has suggested that mismatching DIS sequences between HIV-1 subtypes is a major restriction in HIV-1 intersubtype recombination (39). Yet, it is clear that intersubtype recombination between viruses that contain mismatching DIS sequences does occur *in vivo* (41,42), but the precise mechanisms that mediate this biological process remain undetermined.

In this study, we have investigated the replication of HIV-1 DIS RNA mutants in both primary T-lymphocytes and laboratory adapted T-cell lines. Multiple round replication assays revealed that all HIV-1 DIS mutants tested were replication competent in PBMCs and monocyte-derived macrophages (MDMs), with a maximum reduction in infectivity of ~10-fold compared to wild-type (WT). Whereas, a panel of human T-cell lines displayed a significantly reduced capacity to support the replication of DIS defective HIV-1 with up to a 100 000-fold decrease in infectivity compared to WT HIV-1. In a single round luciferase reporter virus assay, the replication of HIV-1 DIS mutants was impaired at an early step in the viral life cycle. Examination of HIV-1 reverse transcription products demonstrated that HIV-1 DIS mutants were defective in the synthesis of viral cDNA in a producer cell-type-dependent manner; with the synthesis of viral cDNA significantly increased when input viruses were produced from PBMCs compared to virus derived from 293T cells. Our data provide evidence to illustrate that primary T-lymphocytes rescue, in part, the replication of HIV-1 DIS RNA mutants through mediating the reverse transcription process in a cell-type-dependent manner. This mechanism may help to explain the dilemma of how different HIV-1 subtypes with mismatched DIS sequences (such as B and C) are able to recombine to yield HIV-1 intersubtype recombinants.

MATERIALS AND METHODS

Construction of plasmid DNAs

WT HIV-1 and a panel of five DIS RNA mutants (NL_{ACGCGT}, NL_{GCGCGC}, NL_{Palindrome neg}, NL_{Ext Stem} and NL_{Ext Loop}) were used in this study to examine the RNA structural requirements, which govern the cell-type-dependent replication of HIV-1 DIS RNA mutants. Two types of mutants were used, those with deletions in the DIS stem-loop, which were designed to abolish the stem-loop structure (NL_{Palindrome neg}, NL_{ACGCGT}, NL_{GCGCGC}), and those with insertions in the DIS stem-loop, which aimed to maintain a stem-loop structure but alter either the length of the stem (NL_{Ext Stem}) or the loop (NL_{Ext Loop}) (Figure 1). The WT HIV-1 proviral DNA NL4.3 was obtained from the NIH AIDS Reagents Program from Dr Malcolm Martin (43). The numbering of RNA sequences in Figure 1 is based on the RNA genome of NL4.3. NL_{ACGCGT} and NL_{GCGCGC} have been previously published (23). NL_{GCGCGC}, which contains the WT DIS palindrome sequence, was generated by replacement of the 35-nucleotide (nt) sequence encompassing the DIS stem-loop (HIV-1 RNA residues 242–276) within the NL4.3 proviral DNA with the 10 nt palindrome sequence

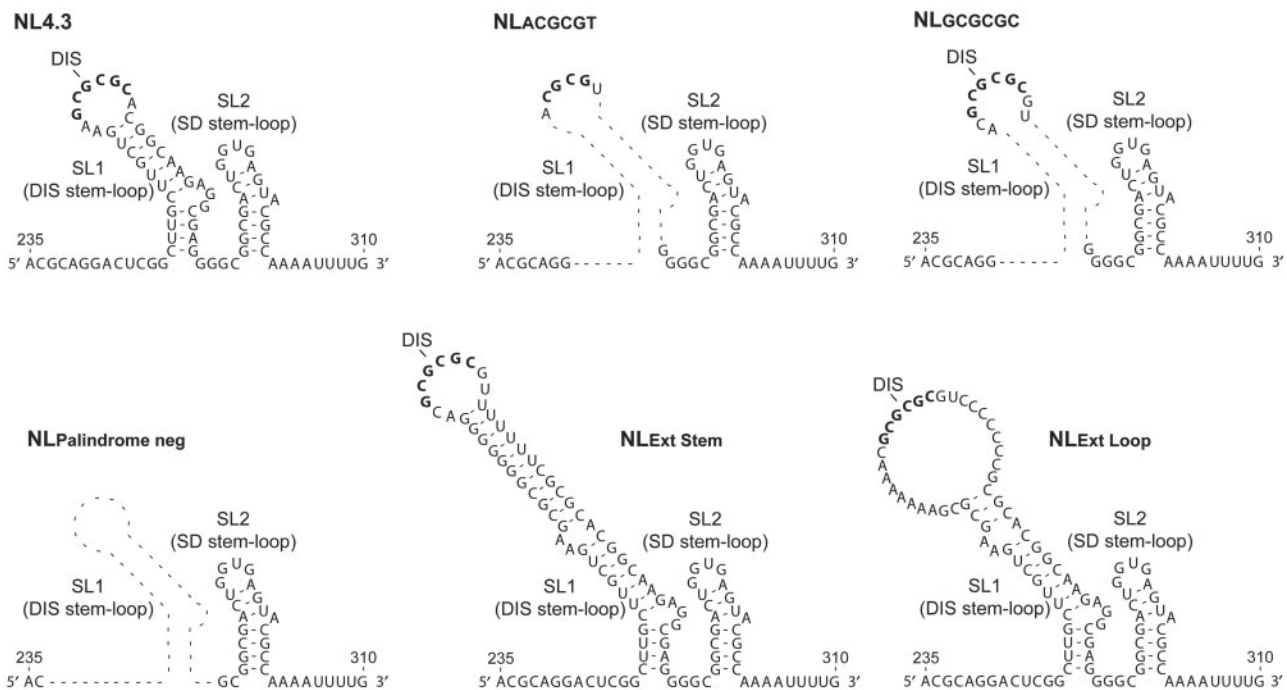


Figure 1. Schematic representation of the WT and mutant DIS stem-loop sequences in the HIV-1 RNA genome. The numerical values are based on the RNA nucleotide position of NL4.3. The deletion sequences are indicated as dotted lines. The bold font highlights the palindromic sequences within the stem-loop.

(5' ACGCGCGCGT 3'). Similarly, NL_{ACGCGT} was constructed by replacing the same 35 nt sequence (HIV-1 RNA residues 242–276) within the NL4.3 proviral DNA with an arbitrarily chosen 6 nt palindromic sequence (5' ACGCGT 3'). NL_{Palindrome neg} was created by deletion of a 43 nt sequence encompassing the DIS stem-loop (HIV-1 RNA residues 237–279). NL_{Ext Stem} and NL_{Ext Loop} were constructed via the insertion of a 26 nt sequence (5' CGCGGGGGGACGCGCGCGTTTTTTT 3' and 5'CGCGAAAAAACGCGCGTCCCCC 3', respectively) into the BshII site of NL4.3. All mutants were constructed via site-directed mutagenesis using specific PCR primers. DNA sequencing was performed to confirm the presence of the desired mutations and the absence of spontaneous mutations via PCR mutagenesis.

Macrophage tropic HIV-1 constructs were generated by substitution of the EcoRI/BamHI fragment of the NL4.3-based plasmids with the EcoRI/BamHI fragment from pNL(AD8). The pNL(AD8) HIV-1 AD8 Macrophage-Tropic R5 clone was obtained through the AIDS Research and Reference Reagent Program, from Dr Eric O. Freed (44). HIV-1 luciferase reporter virus constructs were used to identify the stage at which the replication of the DIS mutants was blocked. The HIV-1 luciferase reporter virus constructs, which contain the firefly luciferase gene inserted into the HIV-1 *nef* gene, were generated by substitution of the XhoI/BamHI fragment of the NL4.3-based plasmids with the XhoI/BamHI fragment from pNL4.3.Luc.R-E-. The pNL4.3.Luc.R-E- clone was obtained through the AIDS Research and Reference Reagent Program, from Dr Nathaniel Landau (45,46). The HIV-1 luciferase reporter virus constructs also contained a stop codon and

frameshift mutation in the second exon of the *rev* gene, which was introduced via a 22 bp insertion (5' GATC CAATAGACGCGTCTATGG 3') into the BamHI site of the NL4.3 and NL(AD8)-based plasmids.

The vesicular stomatitis virus envelope glycoprotein expression plasmid pHCMV-G was generously provided by Dr Jane Burns (University of California, San Diego). The pNLA1 and pNLA1(AD8) are HIV-1 Env and accessory protein expression vectors (a kind gift from Dr Damian Purcell, University of Melbourne) that express all HIV-1 proteins with the exception of Gag and GagPol under the HIV-1 LTR-control.

Virus production

Mutant and WT HIV-1 particles were produced either by poly(ethylenimine) (PEI; Polysciences Inc., Warrington, PA, USA) transfection of 293T cells or by infection of phytohemagglutinin (PHA) stimulated PBMCs. 293T cells were maintained in Dulbecco's modified Eagle medium/high modified (with 4500 mg/l dextrose and 4 mM L-glutamine) (DMEM; SAFC Biosciences, Lenexa, KS, USA), supplemented with 10% vol/vol heat-inactivated cosmic calf serum (CCS; Hyclone, Tauranga, New Zealand), 100 U/ml of penicillin and 100 µg/ml of streptomycin (Invitrogen, Mount Waverley, Victoria, Australia). VSV-G pseudotyped HIV-1 was produced by co-transfection of the NL4.3 and NL(AD8) based plasmids with pHCMV-G at a 1:0.6 molar ratio. HIV-1 luciferase reporter viruses were produced by co-transfection of the envelope, *nef* and *rev* defective NL4.3 and NL(AD8)-based luciferase reporter plasmids with pNLA1 or pNLA1(AD8) at a 1:1 molar ratio, which provided the

HIV-1 envelope, nef and rev proteins *in trans*. Supernatants were collected 36 h posttransfection.

PBMCs were isolated from buffy packs (supplied by the Red Cross Blood Bank, Melbourne) as described previously (23). PBMCs were then stimulated with PHA (10 µg/ml; Remel, Lenexa, KS, USA) for 3 days and maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% vol/vol heat-inactivated CCS, 2 mM L-glutamine (Invitrogen), 50 µg/µl gentamycin (Pfizer, Bentley, WA, Australia) and 10 units of human interleukin 2 (IL-2; Roche, Mannheim, Germany) per milliliter. Equivalent amounts of VSV-G pseudotyped virus input, as determined by a HIV-1 antigen (p24 CA) Micro ELISA assay (Vironostika; Biomerieux, Boxtel, The Netherlands) were used to infect PHA stimulated PBMCs for 2 h at 37°C. Cells were then pelleted at 130g for 10 min and washed twice with PBS. Cell pellets were resuspended in fresh media and cells were cultured in 6-well plates. Supernatants were collected at 7 days postinfection.

Viral particles from the transfection or infection supernatant were purified and concentrated by filtration and ultracentrifugation through a 20% sucrose cushion using an L-90 ultracentrifuge (SW 41 rotor; Beckman, Fullerton, CA, USA) at 100 000g for 1 h at 4°C. Virus pellets were then resuspended and quantified using a HIV-1 antigen (p24 CA) Micro ELISA assay.

Replication kinetics and TCID₅₀

Freshly isolated PBMCs were either stimulated or used for monocyte and PBL isolation. Monocytes and PBLs were isolated by plastic adherence as previously described (47). Monocytes were cultured in Iscoves modified Dulbecco's media (Invitrogen) supplemented with 10% vol/vol heat-inactivated human serum (CELLect pooled human serum, MP Biomedicals, Solon, OH, USA), 2 mM L-glutamine and 50 µg/µl gentamycin adherent to plastic. Monocytes were differentiated into MDMs for 5–7 days before infection. PBMCs and PBLs were stimulated with PHA (10 µg/ml) for 3 days and maintained in RPMI 1640 medium supplemented with 10% vol/vol heat-inactivated CCS, 2 mM L-glutamine, 50 µg/µl gentamycin and 10 units of IL-2 per milliliter. Human T-cell lines (H9, A3.01, CEM-SS, CEM-T4 and SupT1) were maintained in RPMI 1640 medium supplemented with 10% vol/vol heat-inactivated CCS, 2 mM L-glutamine and 50 µg/µl gentamycin.

Sample virus stocks with equivalent levels of RT activity, as determined by a micro-RT assay (48), were added to 1×10^5 cells in 96-well tissue culture plates. Eight 10-fold serial dilutions of each virus were tested in triplicate. Supernatants were collected on days 3, 7, 10 and 14 postinfection (supernatants were also collected at Day 21 for MDM infections) and subsequently stored at -80°C. Viral infectivity was measured by monitoring the production of viral RT activity by using a micro-RT assay (48). The infectivities of WT and mutant viral particles were quantified by using a 50% tissue culture infective dose (TCID₅₀) method as previously described (49).

Luciferase reporter virus assay

A single round luciferase reporter virus assay was used to assess the impact of HIV-1 DIS RNA mutants on the early stages of viral replication. Infectivity was tested on virus stocks obtained from transfection of 293T cells. Equivalent amounts of virus input, as determined by a HIV-1 antigen (p24 CA) Micro ELISA assay were used to infect 5×10^5 cells in 48-well plates. The success of infection was determined by the level of luciferase activity in the cells, which was measured using a Luciferase Assay System (Promega, Madison, WI, USA). Forty-eight hours after infection, cells were harvested and lysed in luciferase lysis buffer [cell culture lysis buffer (CCLR)] at $\sim 2 \times 10^4$ cells/µl. Ten microliters of cell lysate was added to 50 µl of substrate buffer and luciferase activity was read using a FLUOstar optima microplate reader (BMG Labtech, Offenburg, Germany).

Real-time PCR

Quantification of HIV-1 reverse transcription products and standardization of cell numbers was performed using real-time PCR. PBMCs or SupT1 cells were infected with equivalent amounts of virus as determined by a HIV-1 antigen (p24 CA) Micro ELISA assay. Concentrated virus stocks were treated with 50 U/ml of DNase (Roche) for 15 min at 37°C before infection to remove any contaminating plasmid DNA from the transfection procedure. A heat-inactivated virus control (2 h at 56°C) was used to confirm the level of any residual plasmid DNA for each sample. Twenty-four hours postinfection cells were pelleted and lysed in PCR lysis buffer containing 1× PCR buffer (Roche) with 0.5% vol/vol Triton-X100, 0.5% vol/vol NP-40 and 75 µg/ml proteinase K (Roche). Samples were incubated at 56°C for 1 h before proteinase K was inactivated at 95°C for 10 min, samples were then stored at -20°C. Real-time PCR was performed on an iCycler (BioRad, Hercules, CA, USA). Each PCR reaction contained 1× SYBR Green I Master mix (BioRad), 400 nM each primer and 2.5 µl cell lysate in a 25 µl reaction volume. The HIV-1 specific primers M661/M667 (50) were used to detect complete HIV cDNA. HIV-1 PCR conditions were an initial denaturation at 95°C for 10 min followed by 40 rounds of cycling at 95°C for 15 s, then 61°C for 30 s. Cell numbers were standardized for the human CCR5 gene using the primers LK46 (sense; 5'-GCTGTGTTTGCCTCTCTCCCAGGA-3') and LK47 (antisense; 5'-CTCACAGCCCTGTGCCTCTTCTTC-3'). CCR5 PCR conditions were an initial denaturation at 95°C for 10 min followed by 45 rounds of cycling at 94°C for 20 s, 58.3°C for 30 s and 72° for 30 s.

RESULTS

Primary T-lymphocytes and macrophages are unique in their ability to rescue the replication of DIS-defective HIV-1 RNA mutants

Previous work has shown that the DIS stem-loop is critical for HIV-1 replication in T-cell lines (3,24,26–28). Subsequently, we have made a novel observation that

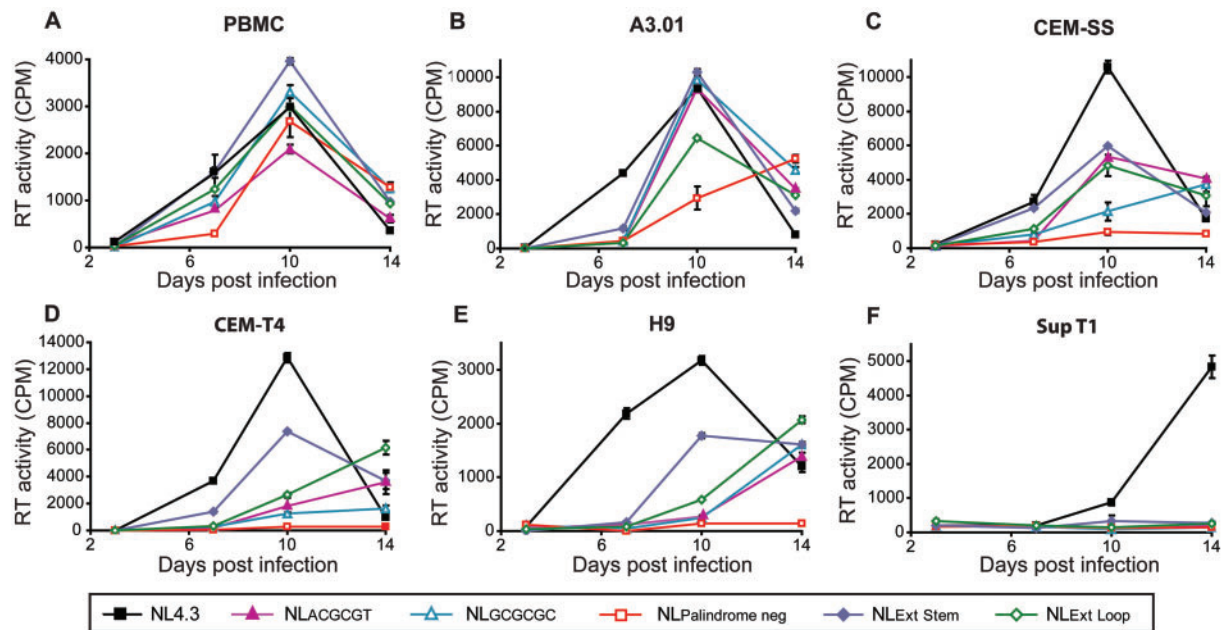


Figure 2. PBMCs but not T-cell lines can support the replication of HIV-1 DIS RNA mutants. WT HIV-1 and five HIV-1 DIS mutants (NL_{ACGCGT}, NL_{GCGCGC}, NL_{Palindrome neg}, NL_{Ext Stem} and NL_{Ext Loop}) were normalized for viral RT activity and used to infect PHA-stimulated freshly isolated PBMC (A) and a number of T-cell lines (B–F). Supernatants were collected 3, 7, 10 and 14 days postinfection and RT activity in each sample was measured. Results represent means and standard deviations of triplicate samples and are representative of six different PBMC donors and either four or six sets of independent experiments for the T-cells lines.

although the WT HIV-1 DIS is required for HIV-1 replication in the T-cell line, SupT1, the DIS stem-loop is dispensable for HIV-1 replication in PBMCs (23). In order to characterize the RNA structural requirements, which govern this cell-type-dependent replication of HIV-1 DIS mutants a panel of five HIV-1 RNA mutants were used in this study (NL_{ACGCGT}, NL_{GCGCGC}, NL_{Palindrome neg}, NL_{Ext Stem} and NL_{Ext Loop}) (Figure 1). Two types of mutants were used, those with deletions in the DIS stem-loop, which were designed to abolish the stem-loop structure and either remove (NL_{Palindrome neg}) or alter the palindrome sequence (NL_{ACGCGT}, NL_{GCGCGC}); and those with insertions in the DIS stem-loop, which aimed to maintain a stem-loop structure but alter either the length of the stem (NL_{Ext Stem}) or the loop (NL_{Ext Loop}). Replication kinetics of these HIV-1 DIS mutants were examined in both primary cells and a number of T-cell lines. Mutant and WT HIV-1 were generated by transfecting the indicated proviral DNA into 293T cells. In order to examine whether the observed RNA-mediated restriction of HIV-1 DIS mutants is limited to SupT1 cells or if it is a widespread phenomenon across other T-cell lines parallel infections were carried out using a panel of T-cell lines and PHA-stimulated PBMCs (Figure 2). The differential capacities of primary T-lymphocytes and SupT1 cells to support DIS RNA mutants resembles the ability of these cell types to support the replication of HIV-1 Vif mutants. The cell lines used in this study were chosen to evaluate whether the requirement for the DIS RNA mutants to replicate is Vif/APOBEC related. The T-cell lines used included Δ Vif permissive cell lines (CEM-SS and SupT1), nonpermissive cell lines

(CEM-T4, H9) and a T-cell line, which is semi-permissive (A3.01) (33).

All HIV-1 DIS mutants were replication defective in SupT1 cells (Figure 2F), whereas the HIV-1 DIS mutants were all replication competent in PBMCs (Figure 2A), which is consistent with our previously reported data using the NL_{ACGCGT} and NL_{GCGCGC} DIS mutants (23). In contrast to the SupT1 cells, the other T-cell lines tested showed differing capacities to support the replication of DIS defective HIV-1 (Figure 2B–E). In comparison to primary PBMCs, all T-cell types tested demonstrated a reduced capacity to support the replication of DIS defective HIV-1 (from high to low replication capacity: A3.01, CEM-SS, CEM-T4, H9 and SupT1).

The TCID₅₀ values of each virus were also measured to assess the relative infectivity among WT HIV-1 (NL4.3) and the DIS mutants in both PBMCs and the panel of T-cell lines (Table 1). The relative difference in TCID₅₀ value from the best performing virus (NL4.3) to the worst performing virus (NL_{Palindrome neg}) varied between the cell types, ranging from an \sim 10-fold difference in PBMCs to a $>100\,000$ -fold difference in SupT1, H9 and CEM-T4 cells (Table 1). The TCID₅₀ values obtained for WT HIV-1 were consistent between the different T-cell types (<2 -fold difference; Table 1), whereas the TCID₅₀ values obtained for the worst performing virus (NL_{Palindrome neg}) varied by up to >5000 -fold, demonstrating that the observed cell-type-dependent replication is specific for the DIS defective HIV-1 RNA mutants.

A consistent pattern in the ability of the different DIS RNA mutants to replicate within each cell type was also observed. Mutants that contained deletions in the DIS

Table 1. Virion infectivity (TCID₅₀) of WT and DIS mutant HIV-1 in PBMCs and T-cell lines

	Infectious particles/ml					
	PBMC	A3.01	CEM-SS	CEM-T4	H9	SupT1
NL4.3	3.16×10^7	3.16×10^7	3.16×10^7	1.78×10^7	3.16×10^7	3.16×10^7
NL _{ACGCGT}	1.78×10^6	3.16×10^6	1.78×10^5	3.16×10^3	3.16×10^3	3.16×10^2
NL _{GCGCGC}	3.16×10^6	3.16×10^5	3.16×10^5	3.16×10^3	3.16×10^3	3.16×10^2
NL _{Palindrome neg}	1.78×10^6	1.78×10^4	3.16×10^3	3.16×10^2	3.16×10^2	3.16×10^2
NL _{Ext Stem}	1.78×10^7	3.16×10^7	3.16×10^5	3.16×10^5	3.16×10^5	3.16×10^3
NL _{Ext Loop}	3.16×10^6	1.78×10^7	3.16×10^6	1.78×10^4	3.16×10^4	5.62×10^2

Results are representative of six different PBMC donors and either four or six sets of independent experiments for the T-cells lines.

stem-loop showed the largest reduction in infectivity with the most severely affected mutant containing the largest deletion covering both the palindromic DIS sequence and the stem-loop (NL_{Palindrome neg}). The best performing of the DIS mutants in all cell types were consistently the NL_{Ext Stem} and NL_{Ext Loop}, which both contain the WT palindrome sequence and represent the most subtle of the mutations tested.

In addition to examining the replication of HIV-1 DIS mutants in T-cell lines, the ability of our panel of HIV-1 DIS mutants to infect two natural target cells of HIV-1, peripheral blood lymphocytes (PBLs) and MDMs was also examined (Figure 3). The HIV-1 DIS mutants were all replication competent in both PBLs (Figure 3A) and MDMs (Figure 3B), with one of the mutants consistently achieving TCID₅₀ values similar to WT HIV-1 in both cell types (NL_{Ext Stem}) (Table 2). The other DIS mutants showed slightly reduced replication (~2- to 18-fold reduction in TCID₅₀ values) and slightly delayed replication for all the DIS mutants was observed in some donors (Figure 3A). Although overall the TCID₅₀ values for MDMs were lower than those for the PBL infections, the fold difference between the best performing virus (NL4.3) to the worst performing virus (NL_{Palindrome neg}) was the same for both primary cell subtypes (~6-fold; Table 2). Together these data show that, for equivalent input levels of virus, HIV-1 DIS RNA mutants are replication competent in a cell-type-dependent manner and that primary T-lymphocytes and macrophages are unique in their ability to rescue the replication of DIS-defective HIV-1 RNA mutants.

DIS defective HIV-1 is blocked at an early step of HIV-1 replication in both laboratory T-cell lines and the natural target cells of HIV-1

In order to identify whether the replication of HIV-1 DIS mutants is blocked at early or late stages of the viral replication cycle, single round luciferase reporter virus constructs were used to infect both primary cells and the T-cell line SupT1. Envelope, nef and rev defective luciferase reporter virus constructs, which contain the luciferase gene inserted into the HIV-1 *nef* coding region, were produced by cotransfection with HIV-1 envelope (either T-cell tropic or macrophage tropic), rev and nef proteins supplemented *in trans*. In this system, the luciferase reporter serves as a surrogate for the expression

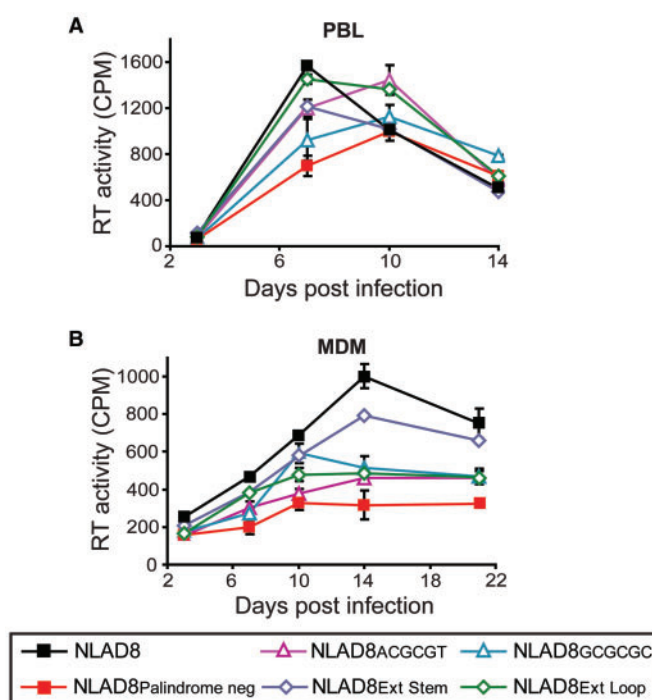


Figure 3. PBLs and MDMs possess unique capacities to support the replication of HIV-1 DIS RNA mutants. WT HIV-1 and five HIV-1 DIS mutants (NL_{ACGCGT}, NL_{GCGCGC}, NL_{Palindrome neg}, NL_{Ext Stem} and NL_{Ext Loop}) were normalized for viral RT activity and used to infect PHA-stimulated freshly isolated PBLs (A) or MDMs (B). Supernatants were collected 3, 7, 10, 14 and 21 days postinfection and RT activity in each sample was measured. Results represent means and standard deviations of triplicate samples and are representative of four different PBL donors and six different MDM donors.

of early proviral genes, with luciferase expression requiring only completion of virus entry, reverse transcription, nuclear translocation, proviral integration and transcription of the integrated provirus.

HIV-1 luciferase reporter viruses were produced by co-transfection of 293T cells and equivalent amounts of input virus, as measured by HIV-1 p24 CA protein, were used to carry out parallel infections of either, PBMCs and SupT1 cells (Figure 4A), or PBLs and MDMs (Figure 4B). All DIS mutants showed severely reduced luciferase activity compared to WT HIV-1 (10–30%) in all cell types tested demonstrating that the HIV-1 DIS mutants

Table 2. Virion infectivity (TCID₅₀) of WT and DIS mutant HIV-1 in PBLs and MDMs

	Infectious particles/ml	
	PBL	MDM
NL4.3	3.16×10^7	1.78×10^4
NL _{ACGCGT}	3.16×10^7	3.16×10^3
NL _{GCGCGC}	1.78×10^7	3.16×10^3
NL _{Palindrome neg}	5.62×10^6	3.16×10^3
NL _{Ext Stem}	3.16×10^7	1.78×10^4
NL _{Ext Loop}	3.16×10^7	5.62×10^3

Results are representative of four different PBL donors and six different MDM donors.

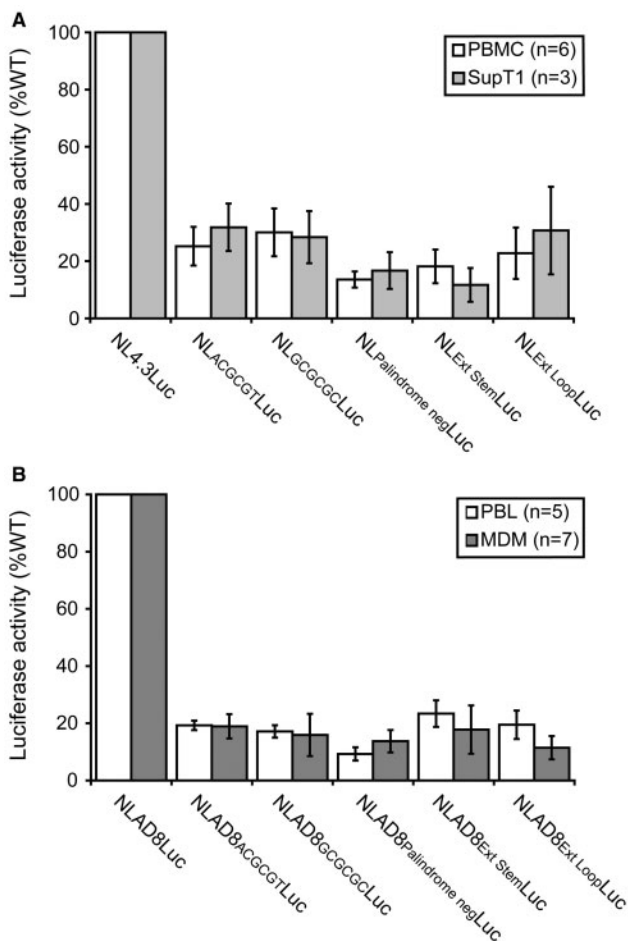


Figure 4. 293T derived HIV-1 DIS mutant viruses are unable to establish effective infection in PBMCs and SupT1 cells using a single round reporter virus system. WT HIV-1 and five HIV-1 DIS mutants (NL_{ACGCGT}, NL_{GCGCGC}, NL_{Palindrome neg}, NL_{Ext Stem} and NL_{Ext Loop}) containing the luciferase reporter gene were normalized for p24 capsid protein content and used to infect PBMC and SupT1 cells (A) or PBL and MDM (B). The level of luciferase activity was used as an indicator of infectivity, which was reported as a percentage (mean \pm SE) of the WT control.

were much less efficient at completing the early steps of viral replication. Surprisingly, the HIV-1 DIS mutants showed significantly reduced luciferase activity in both PBMCs and SupT1 cells, which does not directly reflect

the infectivity data generated using a multiple round infection system, in which all HIV-1 DIS mutants were replication competent in PBMCs but not in SupT1 cells (Figure 2). Furthermore, the HIV-1 DIS mutant viruses also showed a significantly reduced luciferase activity in both PBLs and MDMs, which again does not reflect the infectivity data generated using a multiple round infection system (Figure 3). These data suggest that DIS defective HIV-1 produced from 293T cells is blocked at an early step in the viral replication cycle, at some point before the expression of early proviral genes and protein production.

HIV-1 DIS mutants are defective in the synthesis of viral cDNA in a producer cell-type-dependent manner

Interestingly, we have demonstrated that our panel of DIS mutants display a cell-type-dependent ability to replicate using a multiple round infection assay (Figure 2), whereas using a single round reporter virus assay, all HIV-1 DIS mutants show a severe and consistent defect in infectivity in all cell types tested (Figure 4). One of the major differences that might contribute to this disparity is the identity of the virus producer cell. During multiple rounds of viral replication, the target cells of infection also become the virus producer cell from second round of infection onwards, consequently, viruses derived from 293T cells might not readily recapture such defects in single round infection assay. Therefore, whether the identity of the virus producer cell affected the ability of HIV-1 DIS mutant viruses to replicate was investigated.

Previous studies suggest that mutations in the HIV-1 DIS stem-loop lead to a defect in the early steps of viral replication, with a specific impairment during the 2nd template switch stage of reverse transcription (3,4), leading to a reduction in the synthesis of the viral cDNA. Results produced using a single round luciferase reporter virus assay (Figure 4) demonstrate that the panel of HIV-1 DIS mutants used in this study are unable to complete the early stages of the viral replication cycle with replication being blocked at some point before protein production. In order to pinpoint the exact stage at which the block to viral replication occurs, and to assess whether the cell-type-dependent replication of our HIV-1 DIS mutants represents a differing ability of the various cell types to support the reverse transcription of DIS defective HIV-1, real-time PCR analysis of HIV-1 reverse transcription products was used.

As SupT1 cells and other T-cell lines are unable to produce sufficient quantities of DIS mutant HIV-1 viruses for analysis, WT and DIS mutant HIV-1 viruses were produced by either transfection of 293T cells or infection of PBMCs. 293T cells were used as a surrogate for virus production in SupT1 cells. It is expected that if the virus producer cell is the major determinant of this cell-type-dependent replication of HIV-1 DIS-RNA mutants, HIV-1 DIS mutants generated from primary cells will have an enhanced capacity to synthesize viral cDNA than HIV-1 DIS mutants produced from laboratory adapted cells, such as 293T and SupT1 cells. Equivalent amounts of virus, as measured by HIV-1 p24 CA protein, were used to infect either SupT1 cells or PBMCs. Twenty-four hours

after infection cells were lysed and subjected to quantitative real-time PCR amplification to detect HIV-1 reverse transcription products. Full length viral DNA was detected by PCR using M661/M667 (LTR/gag) primers (50), which flank the PBS of the HIV-1 genome and represent the last region of the minus strand of viral DNA that is synthesized. Parallel infections using a heat inactivated virus control (2h at 56°C) were carried out for each virus to assess the level of any contaminating plasmid DNA. Average background levels were <5% of WT virus for all samples (Supplementary Figure S1).

Quantification of late reverse transcription products (complete cDNA) in HIV-1 infected cells showed that all 293T cell derived HIV-1 DIS RNA mutants were impaired in the production of viral DNA compared to WT HIV-1 (5–30%). This defect is found in both SupT1 cells and PBMCs when the virus was derived from 293T cells (Figure 5A and B). No significant difference in the levels of reverse transcription between the different DIS mutants was observed. In contrast, when virus derived from PBMCs was used to infect SupT1 cells and PBMCs, an increase in the amount of reverse transcription products was observed for all of the HIV-1 DIS mutants, except *NL_{Palindrome neg}*, in both cell types (Figure 5A and B). Importantly, the differences in the levels of reverse transcription between the different DIS mutants in PBMCs mirror the variability in TCID₅₀ values seen in PBMCs (Table 1). In contrast to the other HIV-1 DIS mutants tested, the ability of the *NL_{Palindrome neg}* mutant virus to complete reverse transcription was not producer cell dependent. Consequently, the differences in infectivity of the *NL_{Palindrome neg}* mutant in primary cells versus T-cells line cannot be explained by our reverse transcription data alone. This observation indicates that the *NL_{Palindrome neg}* mutant suffers additional defects at other stages in the viral life cycle.

In summary, our data show that HIV-1 DIS mutants are defective in the synthesis of viral cDNA in a producer cell-type-dependent manner, demonstrating that the ability of DIS defective HIV-1 to replicate is associated with the identity of the virus producing cell, not the target cell. These observations imply the presence of a cellular factor within the virus producing cell, which can regulate the ability of DIS defective HIV-1 to replicate.

DISCUSSION

In this study, we provide evidence that mutations in a noncoding region of the HIV-1 RNA genome affect the ability of the virus to synthesize viral cDNA in a cell-type-dependent manner, illustrating the importance of virus–host cell interplays via an RNA-trigger. Using a panel of five DIS mutants, we have demonstrated that both the DIS stem-loop structure and the presence of a palindromic sequence are not required for viral replication in the natural target cells of HIV-1 and that laboratory T-cell lines display varying abilities to support the replication of DIS defective HIV-1. We have also demonstrated that this cell-type-dependent replication is determined by the identity of the virus producing cell with primary

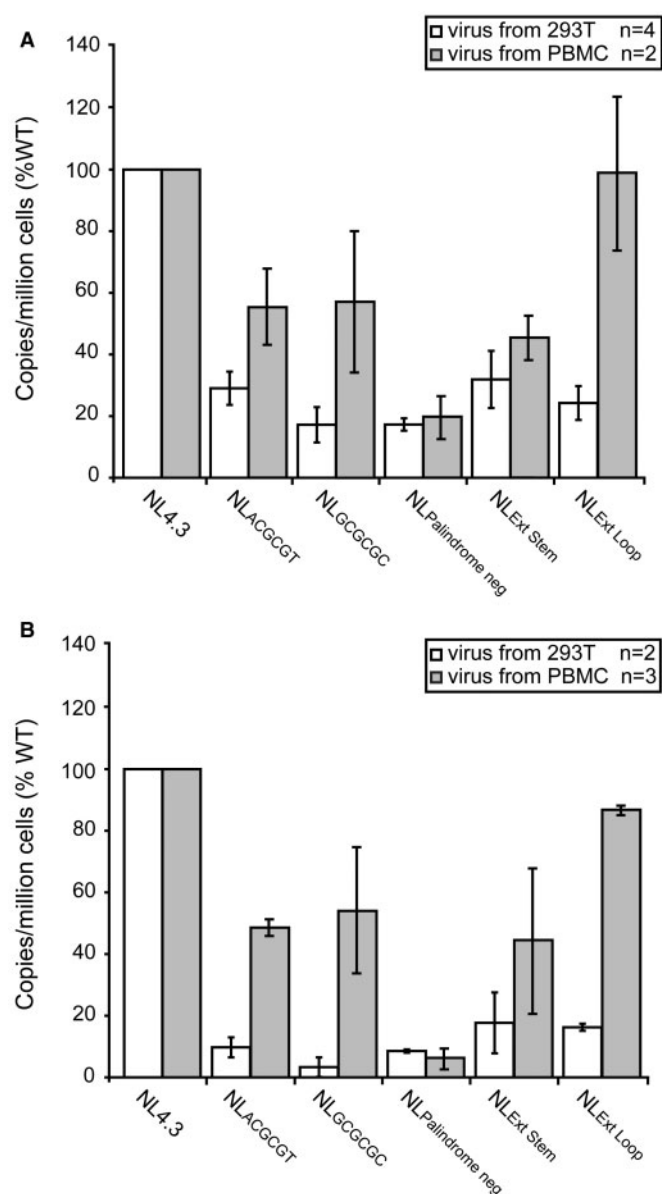


Figure 5. Production of HIV-1 DIS RNA mutants from PBMCs partially rescues defects in viral cDNA synthesis. WT and DIS mutant HIV-1 viruses were produced either by transfection of 293T cells or infection of PHA-stimulated freshly isolated PBMCs. Equivalent amounts of DNase treated virus were used to infect either SupT1 cells (A) or PBMCs (B). Cells were lysed 24h postinfection and HIV-1 cDNA was measured by real-time PCR. HIV-1 DNA copies were normalized by CCR5. Results are reported as a percentage of the WT control and represent means (\pm SE) of separate experiments.

T-lymphocytes rescuing the replication of HIV-1 DIS mutants by facilitating reverse transcription.

The dimerization of retroviral RNA genomes is a conserved phenomenon that is critical for viral replication. The process of HIV-1 RNA dimerization is thought to be largely regulated by the DIS, which is found near the 5' end of the HIV-1 RNA genome. We have previously observed that requirement of the HIV-1 DIS RNA sequence in viral replication is cell-type dependent (23). In this study, a panel of five DIS mutants were utilized to

examine the mechanism and the RNA structural requirements of the cell-type-dependent replication of DIS defective HIV-1. Infection of primary cells with these mutants revealed that all DIS mutants were able to replicate in both primary T-lymphocytes and MDMs, demonstrating the both the DIS stem-loop and the presence of a palindromic sequence are not required for viral replication in the natural target cells of HIV-1. In contrast, none of the DIS mutants tested were able to replicate in the laboratory adapted T-cell line SupT1, suggesting that the integrity of the entire native DIS stem-loop structure is absolutely required for HIV-1 replication in this T-cell line. Further analysis of these HIV-1 DIS mutants in additional T-cell lines demonstrated that primary cells are unique in their abilities to rescue the replication of HIV-1 DIS RNA mutants.

Infections using single round HIV-1 reporter virus constructs containing the same panel of DIS mutants demonstrated that these mutants were impaired at an early stage of the viral replication cycle, which is consistent with previously published work showing that mutations in the HIV-1 DIS can affect the reverse transcription process (4,51). Interestingly, despite being able to demonstrate a cell type dependent replication of HIV-1 DIS mutants in multiple round infectivity assays (Figures 2 and 3), this cell-type-dependent response was not evident in the single round HIV-1 reporter virus assay when viruses were derived from laboratory adapted cell lines, in which both PBMCs and SupT1 were used as target cells for analysis (Figure 4). These data suggest that it is not the virus target cell which determines the ability to support the replication of DIS defective HIV-1. Real-time PCR for HIV-1 reverse transcription products demonstrated that viral cDNA levels were significantly increased when the infection input virus was derived from PBMCs compared to virus derived from 293T cells with the exception of the NL_{Palindrome^{neg}} mutant (Figure 5). This observation may indicate that the NL_{Palindrome^{neg}} mutant suffers additional defects at other stages in the viral life cycle, which may not be present in the other DIS mutants tested. Thus, the cell-type-dependent replication of HIV-1 DIS mutants is largely, but not exclusively, associated with the identity of virus producer cell and not the target cell.

Our observations of the producer cell-dependent replication of HIV-1 DIS mutants imply the presence of a cellular factor (or group of cellular factors) within the virus producing cell, which can compensate for the requirement of a fully functional dimerization initiation site during subsequent rounds of HIV-1 replication. This observation that different cell types display varying DIS stem-loop mutant 'permissiveness' is reminiscent of the well-described interplay between HIV-1 Vif and the host cell APOBEC proteins (52–55), which is another cell-type-dependent phenomenon and is only evident during infection with a mutated HIV-1 virus. While our observations show some similarities to the effects of Vif/APOBEC on HIV-1 biology, we did not find any correlation between Δ Vif permissiveness and DIS mutant permissiveness.

Another difference between our observations and some of the better known host-cell restriction is that previous

studies provide examples of protein-mediated viral restrictions (33–35), while our work describes an unusual RNA-triggered viral replication defect. Our observed effect could be mediated either through direct packaging of a host cell factor(s) into HIV-1 virions or via an indirect effect during viral assembly. Although our observation suggests the presence of a cellular factor, our data cannot distinguish between the existence of a positive factor present in primary cells or a negative factor present in laboratory adapted T-cell lines. The discrepancy in the propagation of DIS stem-loop mutants in primary cells compared with T-cell lines could suggest the loss of specific host-cell factors during T-cell transformation, a process which is known to alter cellular gene expression (56). Such cellular factors might facilitate reverse transcription through direct or indirect interaction with the DIS stem during virion uncoating, perhaps by participating in template switching or recombination during the synthesis of viral cDNA. Heterokaryon formation has been classically used to examine cell-type-dependent responses to viral infection, but unfortunately, heterokaryon cell fusion experiments between primary T-lymphocytes and laboratory adapted T-cell lines have proven to be impossible due to the poor fusion potential of primary cells. A systematic array analysis of the mRNA profiles amongst the DIS RNA mutant permissive and nonpermissive cells, following by corresponding gene knock-down or knock-in experiments will be needed to identify this host-cell factor(s).

Our observed RNA-mediated cell-type-dependent replication defect of HIV-1 is unusual, but not impossible. Recent investigations have enriched our appreciation of the role of RNA in the regulation of a number of biological processes. It is worth noting that only 2% of the human genome is devoted to the synthesis of proteins (57) and the vast majority of RNA transcripts are nonprotein coding (noncoding) sequences without ascribed functions (58,59). There is growing evidence to suggest that these noncoding RNAs can function as 'signaling' molecules, which serve to regulate many cellular functions (60,61). As viruses have evolved to mimic host-cell machinery to support their own propagation, it is not surprising that HIV-1 has also evolved to utilize a noncoding RNA-mediated strategy. In support of this view, it has recently been shown that well-known members of the noncoding RNA family, the microRNAs (miRNAs), are manipulated by HIV-1 during viral replication (62).

In addition to describing an unusual RNA mediated cell-type-dependent block to HIV-1 replication, our data also help to reconcile the discrepancy between the importance of the DIS in genomic RNA selection (39,40) and the existence of recombinant HIV-1 forms that originate from HIV-1 subtypes containing mismatched DIS sequences (63–65). Previous work has suggested that mismatching DIS sequences are a severe restriction to the formation of heterodimeric RNA genomes and intersubtype recombination (39). However, HIV-1 genomes with mismatching DIS sequences must be capable of forming dimers, being packaged and facilitating reverse transcription as intersubtype recombinant viruses, such as HIV-1 B/C recombinants, exist in nature (63–65).

Our observations demonstrate that the natural target cells of HIV-1 are able to facilitate the reverse transcription process in the absence of a fully functional DIS, suggesting the DIS is not the only determining factor for recombination and helps to explain the occurrence of HIV-1 intersubtype recombinant forms between subtypes with mismatching DIS sequences *in vivo*. Our study illustrates the ability of primary T-lymphocytes and macrophages to rescue the replication of HIV-1 RNA mutants and implies a role for host-cell factors in maintaining the viral diversity of HIV-1. Delineating the mechanistic contributions of these host-cell factors in HIV-1 biology will ultimately reduce the capacity of mutant HIV-1 to be rescued *in vivo* and decrease the opportunity for these viruses to pass on beneficial mutations to progeny virions through retroviral recombination.

SUPPLEMENTARY DATA

Supplementary data are available at NAR Online.

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