Transcription of Preintegrated HIV-1 cDNA Modulates Cell Surface Expression of Major Histocompatibility Complex Class I via Nef

Richard D. Sloan, Björn D. Kuhl, Daniel A. Donahue, André Roland, Tamara Bar-Magen, and Mark A. Wainberg

McGill University AIDS Centre, Lady Davis Institute, Jewish General Hospital, Montréal, Québec, Canada; Department of Experimental Medicine, McGill University, Montréal, Québec, Canada; and Department of Microbiology and Immunology, McGill University, Montréal, Québec, Canada

Received 1 September 2010/Accepted 23 December 2010

Although transcription from unintegrated human immunodeficiency virus type 1 (HIV-1) DNA can occur inside infected cells, yielding all classes of viral mRNA transcripts, the translation of viral proteins is very limited. One of the proteins made is Nef, but it is unclear whether Nef produced in this way is able to play a role in immune evasion as occurs with integrated virus. We therefore asked whether transcription from preintegrated HIV-1 cDNAs could result in Nef-mediated modulation of cell surface major histocompatibility complex class I (MHC-I) expression. We infected a Rev-CEM green fluorescent protein (GFP) reporter cell line with virus and blocked integration though use of either an inactive integrase or the integrase inhibitor raltegravir. Infected cells were assayed by flow cytometry for cell surface expression of the HLA-A, HLA-B, and HLA-C allotypes (HLA-ABC), HLA-A31, and HLA-E. Viral RNA and DNA products were assayed via quantitative PCR (qPCR). The prevention of integration had no effect, relative to productively infected cells, on levels of expression of multiply spliced viral mRNA transcripts and Nef protein. Downregulation of HLA-ABC and HLA-A31 also occurred at levels similar to those seen in cells in which integration had occurred. Parallel experiments assaying cell surface HLA-ABC expression in infected activated primary CD4+ T cells produced a similar pattern of results. Hence, the capacity of HIV-1 to modulate MHC-I is not linked to its ability to integrate. Thus, Nef-mediated evasion of host immune responsiveness might be attributable, in part at least, to transcription from unintegrated viral DNA.

Retroviruses are defined by the integration of their reverse-transcribed genome into host cell chromatin. This process enables transcription and translation of viral genes by host cells, ultimately resulting in new viral progeny. However, human immunodeficiency virus type 1 (HIV-1) gene transcription and translation can also occur prior to, or even in the absence of, viral integration (8, 53, 57), since unintegrated, reverse-transcribed viral cDNAs can also serve as a template for transcription (22). Three species of unintegrated HIV cDNAs are found in natural infections; these are linear reverse-transcribed cDNA, which is the template for integration, and 1-long terminal repeat (LTR) and 2-LTR circular forms, which are the products of autointegration or nonhomologous recombination and nonhomologous end-joining events of linear cDNAs, respectively (15, 26, 37). The circular cDNAs were long considered to be “dead end” products, which cannot serve as templates for integration, though it is now understood that unintegrated cDNA can be complemented by superinfecting virus to yield productive infection (17, 39, 53).

Transcription of preintegrated HIV-1 cDNA can yield all classes of viral RNA transcripts (25, 37, 52); however, only the accessory and regulatory proteins Nef (18, 58), Tat (2, 14, 46), and Rev (22, 29) are translated in readily detectable amounts, and the full extent of the function of these proteins needs to be further characterized. Differences in transcription between integrated and unintegrated HIV-1 may be due to the fact that unintegrated HIV-1 cDNA is organized into chromatin structures, with histone modifications typical of silenced chromatin (23). Additionally, the low levels of Rev synthesized prior to integration may also limit the translation of unspliced viral RNA transcripts and ultimate expression of late gene products (58).

Studies of integrase-defective HIV-1 mutants that bear mutations in the catalytic D(64)D(116)E(152) triad of integrase have been particularly useful in the study of preintegration transcription (18, 36). Indeed, patterns of transcription and translation arising from unintegrated DNA following infection with D116N mutated HIV-1 are identical to those observed from preintegrated viral DNA and in infections of T-cell lines, activated CD4+ T cells, resting T cells, and macrophages (25, 56, 58). Transcription and translation from unintegrated cDNA following use of integrase strand transfer inhibitors (INSTIs) are also indistinguishable from those seen with preintegrated virus or integrase-defective virus (21, 58).

2-LTR circles were previously proposed as a likely transcriptional template, as their levels were found to be elevated when viral integration was inhibited (14, 21). Moreover, a novel viral transcript spanning the LTR-LTR junction was detected, demonstrating that 2-LTR circles can act as a transcriptional template (7). In contrast, a recent study calculated that there were insufficient levels of 2-LTR circles to account for the numbers...
of cells bearing transcriptionally active preintegrated virus (22, 54, 55).

Translation of nef, tat, and rev from preintegrated templates has been linked to a number of cellular effects which aid viral infection. For example, preintegration translation of tat and nef has been shown to increase the activation state of resting T cells, making them more amenable to productive infection (58). Preintegration translation of nef has been linked to reduced cell surface expression of CD4 in primary T cells and T cell lines (18, 36), and we have recently demonstrated that the CXCR4 and CCR5 coreceptors are also affected in this manner (45). In a study of macrophages, transcription of preintegrated HIV-1 cDNA was also linked to altered patterns of cytokine expression (25).

CD8+ cytotoxic T-lymphocytes (CTLs) play a central role in the adaptive immune response to control HIV-1, as they recognize viral antigens presented through major histocompatibility complex class I (MHC-I) on infected cells and can limit infection either by direct lysis (5) or through release of inhibitory factors, such as RANTES, MIP-1 alpha, or MIP-1 beta (9). Therefore, modulation of cell surface expression of MHC-I is a common viral immune evasion strategy and avoids recognition by CTLs, thereby preventing lysis of the infected cell (20). In the case of HIV-1, the virus-encoded protein Nef performs this function (11, 44). Nef downregulates MHC-I by forming a complex with the cytoplasmic tail of MHC-I and the clathrin adaptor AP-1 in the trans-Golgi network (TGN). This allows it to divert normal migration of newly synthesized MHC-I to the cell surface and instead targets it for endosomal degradation (28, 41, 47, 51). However, there is also some evidence to suggest that Nef may mediate accelerated endocytosis of MHC-I from the plasma membrane (28), and it has been suggested that cell type differences might also be important (24).

HIV-1 Nef can mediate downregulation of the MHC-I/human leukocyte antigen (HLA) HLA-A and HLA-B allotypes, which are recognized by CTLs. In contrast, HIV-1 does not downregulate HLA-C and HLA-E, which is advantageous since a reduction in cell surface expression of these allotypes would lead to natural killer (NK) cell-mediated lysis, as NK cells respond to reduced MHC-I levels (10). We therefore hypothesized that the preintegration translation of nef has the capacity to modulate cell surface MHC-I expression.

Here we show that preintegration transcription and translation of nef can modulate cell surface MHC-I in the same manner as when integration occurs. This suggests that transcription from preintegrated viral DNA can influence viral immune evasion even prior to viral integration into the host genome.

MATERIALS AND METHODS

Plasmids and cloning. The HIV-1 molecular clone pNL4-3 was altered through site-directed mutagenesis (Stratagene) to introduce termination codons in the first and third amino acids of the env gene (constructed termed pNL4-3-ΔEnv). Further modifications by mutagenesis included the substitution D116N in the integrase coding sequence of the pol gene (constructed termed pNL4-3-D116N-ΔEnv) and the introduction of termination codons into the first and third codons of the nef gene (constructed termed pNL4-3-ΔEnv-Nef and pNL4-3-D116N-ΔEnv-ΔNef). A pNL4-3-ΔNef construct with an intact env was also prepared in a similar manner.

Virus production. For infections of T-cell lines, pseudovirus was produced by cotransfection of 7 × 10⁴ 293T cells with 4 μg pVPlac-VSV-G (Stratagene), a vesicular stomatitis virus G protein (VSV-G) envelope-encoding construct, in combination with 12 μg of a pNL4-3 derivative (pNL4-3-ΔEnv, pNL4-3-D116N-ΔEnv, pNL4-3-ΔEnv-ΔNef, or pNL4-3-D116N-ΔEnv-ΔNef) via Lipofectamine (Invitrogen). For infections of primary CD4⁺ T cells, virus was prepared by the same method utilizing pNL4-3 or pNL4-3-ΔNef.

All transfection supernatants were harvested at 72 h posttransfection (p.i.), clarified by centrifugation for 5 min at 470 × g, and passed through a 0.45-μm filter. Virus was treated with 50 U/ml benzonase (Sigma) at 37°C for 20 min to digest contaminating plasmid DNA (40) and then stored at −80°C until use.

Cell culture and viral infections. Rev-CEM cells were chosen for our infections and were derived by transduction of the CEM-SS cell line (54). CEM-SS cells are a clone of virus “syncytium-sensitive” cells derived from CEM cells, also known as the CEM-CRF cell line (33). The CEM cell line was itself derived from a patient with acute leukemia (16); as the HLA serological profile of the original CEM line is known, so too is that of the Rev-CEM cell line (IMGT/HLA database [www.ebi.ac.uk]), which expresses the HLA-A31 haplotype, recognized by CTLs.

Rev-CEM cells (54), obtained through the NIH AIDS Research and Reference Program (courtesy of Yuntao Wu and Jon Marsh), were maintained in RPMI 1640 medium (Invitrogen), and 293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen), each supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin. Rev-CEM cells (2.5 × 10⁵) were infected with 250 ng p24 of virus in 24-well plates by spinoculation at 1,200 × g at 37°C for 2 h, followed by 1 h at 37°C, after which medium was replaced, resulting in a multiplicity of infection (MOI) of 0.1 for the wild-type (wt) virus as determined by green fluorescent protein (GFP) expression. Cells were infected with wt or Δnef virus, which was integrase competent or contained a defective D116N mutated integrase, respectively. In some cases, media were pretreated with 1 μM raltegravir (a gift of Merck Canada, Inc.) for 1 h prior to infection; after spinoculation, raltegravir-containing media were again used at a concentration of 1 μM.

For infections of primary CD4⁺ T cells, fresh peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of individual donors by using the Ficoll-Hypaque method. PBMCs were cultured in RPMI 1640 and stimulated with 10 μg/ml phytohemagglutinin A (PHA) and 20 U/ml human interleukin-2 (IL-2) for 72 h. CD4⁺ T cells were then purified by negative selection using an Invitrogen CD3 “CD4⁺ Untouched” kit.

Activated CD4⁺ T cells were pooled (2 × 10⁶) and infected with 80 ng p24 of either NL4-3 or NL4-3-ΔNeF virus via spinoculation at 1,500 × g at 37°C for 2 h in the presence of 8 μg/ml Polybrene, followed by incubation for 1 h at 37°C. Thereafter, cells were cultured with RPMI 1640 supplemented with 20 U/ml human IL-2. Some infections were incubated with 2 μM raltegravir for 1 h prior to infection, during infection, and after infection.

Total viral cDNA qPCR. For both integrated viral DNA and total viral cDNA quantitative PCR (qPCR) assays, cellular DNA was extracted with a DNeasy blood and tissue kit (Qiagen) and resuspended in 10 ng template diluted with undiluted samples (100 ng template) and 1:10 dilutions of each sample (10 ng template diluted with undiluted DNA; 100 ng DNA total) in the presence of 2 mM MgCl₂ and 200 μM deoxyribonucleoside triphosphates (dNTPs). A portion (9 μl) of the resulting first-round product was used as the template for the second-round nested reaction in the presence of 5 mM MgCl₂ (final concentration including MgCl₂, carryover from the first round) and 200 μM dNTPs, using the “wild-type” probe only. Second-round cycling conditions were 90°C for 2 min, 95°C for 1 min, and 45 cycles of 95°C for 15 s and 60°C for 30 s. Dually labeled probes were obtained from Biosearch Technologies (Novato, CA). To generate a standard curve for relative quantification of integrated DNA, Alu-gag PCR was first performed on a dilution series of DNA from infected Rev-CEM cells (diluted with DNA from uninfected cells).

Multiply spiked viral RNA transcript qRT-PCR. Total cellular RNA was extracted from infected cells 72 h p.i. with a Rentasy kit (Qiagen). Quantitative reverse transcription-PCR (qRT-PCR) was performed using one-step SuperScript III Platinum Taq kit (Invitrogen) with primers MS3Jr, 5' -CAGACTCAC CAGCTCTGTATCA-3' (nucleotides [nt] 6016 to 6041 of NL4-3), and MS3Jr, 5' -TTATTCCTTGGGGCGGTC-3' (nt 8368 to 8390 of NL4-3), in conjunction with the dually labeled probe 5'-FAM-AAAAACACTCCATTCAATT CGAGG-BHQ1-3' (where FAM is 6-carboxyfluorescein and BHQ-1 is black hole quencher 1) (nt 8396 to 8415 of NL4-3) (Biosearch Technologies). A PCR

Downloaded from http://jvi.asm.org on June 20, 2017 by guest
amplicon was produced with the primers MSJ3F and MSJ3R but incorporated a 5’ overhang containing the T7 promoter on the forward primer. An in vitro RNA standard was transcribed from the PCR amplicon using a MegaScript kit (Ambion), and serial dilutions of the standard were prepared in uninfected-cell total RNA extract. The amount of RNA amplified per reaction condition was 250 ng, with 150 nm probe, 0.2 μM primers, and 2.0 mM MgSO4. Cycling conditions were 50°C for 15 min, 95°C for 8 min, and then cycling of 95°C for 15 s and 60°C for 30 s. Cellular GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA was additionally amplified as a loading control using the same reaction and cycling conditions, with the primers GAPDHF, 5’-AGGTCGGAGTCAACGG and GAPDHR, 5’-GATGGCAACAATATCCACTTACCA-3’, in conjunction with the GAPDH probe, 5’-FAM-TCTATTGGCGCGGCTCGGT CAC-BHQ-1-3’.

**Western blot analysis.** Cells were collected at 72 h p.i. and pelleted by low-speed centrifugation at 470 g. The pellet was resuspended in RIPA buffer (0.15 M NaCl, 20 mM Tris, pH 7.4, 2 mM EDTA, 1% Triton X-100, and 1% deoxycholate). Cell lysates were normalized by the Bradford assay (Pierce) to 1 mg/ml total protein and resolved in a 12% SDS-polyacrylamide gel. Cell lysates were incubated for 60 min with 1:4,000 polyclonal anti-HIV-1 Nef antibody obtained from the NIH AIDS Research and Reference Reagent Program (catalog number 2949) and anti-rabbit IgG–alkaline phosphotase conjugate (secondary antibody) (1:10,000). The chemiluminescent reagent West-Pico (Pierce) was used to develop the blots.

**Cells surface HLA-ABC, HLA-A31, and HLA-E staining.** Cells that had been infected with virus or pseudovirus were stained at 72 h p.i. in phosphate-buffered saline (PBS) containing 3% fetal bovine serum and 0.1% sodium azide (FACS buffer) for 30 min at 4°C with one of the following mouse monoclonal antibodies (MAbs): phycoerythrin (PE)-conjugated anti-human HLA-A, HLA-B, and HLA-C allotype (HLA-ABC) Mab (clone DX17; BD Biosciences), unconjugated anti-human HLA-A31/30 (clone 4i103; Abcam), or PE-conjugated anti-human HLA-E (clone D3D12; eBioscience). Cells stained with unconjugated antibodies were washed twice in FACs buffer and then resuspended in FACs buffer containing PE-Cy5-conjugated monoclonal rat anti-mouse IgM (clone II/41; eBioscience). Cells were then fixed in a final concentration of 1% paraformaldehyde and resuspended in FACs buffer. A total of 10,000 to 20,000 cell events were assayed on a FACScalibur instrument (BD PharMingen); analysis was performed with BD CellQuest Pro 4.0.2 (BD PharMingen) and FCS Express 3 (DeNovo) software. Levels of receptors were quantified relative to those found after infection by Δ nef virus. These studies were controlled by subtracting background isotype fluorescence or secondary antibody fluorescence values from antibody receptor fluorescence measurements.

**Statistical analysis.** All statistical analyses were performed with GraphPad Prism 4.0 software. To test for statistically significant differences between pairs, unpaired two-tailed t tests were performed, with confidence intervals set at 95%. To test for significant differences between treatment groups, one-way analysis of variance (ANOVA) was performed, with confidence intervals set at 95%.

**RESULTS**

**Nef is expressed in the absence of integration.** We first sought to confirm that Nef is expressed from unintegrated DNA. Rev-CEM cells were infected with VSV-G-pseudotyped NL4-3 virus bearing either wt integrase or the integrase-defective mutation D116N, in conjunction with either wt nef or a Δ nef mutation. For some infections with wt integrase coding sequences, 1 μM integrase inhibitor raltegravir was added.

Analysis of late reverse transcripts (total viral cDNA) by qPCR revealed that there were no significant differences between the capacities of any of the infecting viruses to reverse transcribe the genome, regardless of ability to integrate (P = 0.38) (Fig. 1a), in accord with previous findings (4, 21).

Integration was then assayed with an Alu-HIV qPCR for integrated provirus. This assay confirmed that virus bearing the integrase mutation D116N did not integrate, as previously described (14). Nor was integration detectable with wt-integrase-bearing virus in the presence of 1 μM raltegravir. The presence or absence of an intact Nef coding sequence had no influence on the extent of integration observed (Fig. 1b).

We next used qRT-PCR to assay the capacity of our wt or...
nef-deleted integrating and nonintegrating viruses to produce multiply spliced viral RNA transcripts. The design of our primers should allow amplification of all classes of multiply spliced viral RNA transcripts, including those which code for Nef (38). Our analysis found that there were no significant differences between any of the viruses ($P \approx 0.43$) (Fig. 1c). Thus, the levels of multiply spliced transcripts produced in the absence of integration are generally equivalent to those produced following integration.

Western blot analysis next confirmed that the introduction of stop codons into the first and third codons of nef was sufficient to knock out Nef expression. For both the D116N mutated integrase virus and the wt integrase virus, in the presence of inhibitory concentrations of raltegravir, Nef expression was readily detectable despite an absence of integrase as detected by Alu-HIV qPCR (Fig. 1d). However, the GFP coding sequence is retained in the presence of both Tat and Rev, and GFP is expressed in the cell. In our experiments, infected Rev-CEM cells were detectable as GFP positive by flow cytometry, even when integration was not detectable by qPCR. This was because there was sufficient translation of Tat and Rev to induce GFP expression. The GFP-positive infected cells were stained with an antibody capable of detecting the HLA-A, HLA-B, and HLA-C allotypes (i.e., HLA-ABC).

For wt integrating virus, Nef mediated downregulation of HLA-ABC to 27% of the levels seen with Δ nef virus ($P < 0.0001$) (Fig. 2a and d). When integration was blocked either through the integrase D116N mutation or through the use of raltegravir, the extents of Nef-mediated downregulation were similar, i.e., to 27% and 34%, respectively ($P < 0.0001$ and $P = 0.0002$) (Fig. 2b, c, and d). An analysis of the viruses bearing wild-type nef genes found no significant differences between them ($P = 0.16$); hence, the ability of Nef to downregulate
HLA-ABC was not mediated by the capacity of the virus to integrate (Fig. 2d). We were also able to demonstrate that raltegravir itself did not affect expression of the epitopes recognized by the HLA-ABC antibody, as both untreated and treated cells had similar HLA-ABC expression levels ($P > 0.5$) (Fig. 2d).

**HLA-A is downregulated by Nef in the absence of integration.** In productive HIV-1 infections, Nef selectively downregulates the HLA class I allotypes recognized by cytotoxic T cells (HLA-A and HLA-B), while leaving those recognized by NK cells (HLA-C and HLA-E) unperturbed on the cell surface (10). Thus, our data for HLA-ABC were a compound result of Nef-mediated reductions of cell surface HLA-A and HLA-B, but with unaffected levels of HLA-C.

To more specifically measure Nef-mediated downregulation of HLA allotypes recognized by CTLs, we assayed HLA-A31 cell surface expression levels in Rev-CEM cells following infection with integrating and nonintegrating pseudovirus. With integrating infections, we observed that Nef mediated a reduction in levels of HLA-A31 to about 12% of the usual cell surface expression levels ($P < 0.0001$), relative to those seen with Δ nef virus (Fig. 3a and d). When integration was prevented, either through the integrase D116N mutation or through the use of raltegravir, the same degree of downregulation was observed, i.e., cell surface expression of HLA-A31 was reduced to 9% ($P < 0.0001$) or 17% ($P < 0.0001$), respectively (Fig. 3b, c, and d). We then compared levels of HLA-A31 following infection by all wt viruses and were unable to show any significant differences among them ($P > 0.5$). Thus, the capacity to reduce cell surface HLA-A31 via Nef is not linked to the ability of the infecting virus to integrate.

As with HLA-ABC, we saw no evidence of altered HLA-A31 expression levels due to the influence of raltegravir ($P > 0.5$) (Fig. 3d).

**HLA-E is unaffected by Nef irrespective of viral integration.** We next examined the specificity of our findings by assaying cell surface HLA-E levels, which can affect NK cell recognition of target cells (10, 46). HLA-E is ubiquitously expressed on B cells and T cells, as well as placental cells and trophoblasts, at low levels (48), and we were also able to identify it at low levels in the Rev-CEM cell line.

We found that neither integrating infections with wt virus nor nonintegrating infections that had been blocked by defective integrase or raltegravir resulted in any measurable Nef-mediated downregulation of cell surface HLA-E levels in Rev-CEM cells ($P > 0.5$) (Fig. 4). Following infection by all viruses studied, HLA-E levels were commonly around 2.25-fold higher than the untreated controls.
than in uninfected controls, irrespective of the status of nef or integrase or the use of raltegravir (Fig. 4). Neither wt virus nor Δ nef virus downregulates HLA-E. The results are overlaid in the histogram; wt virus is displayed in white and Δ nef virus in shaded gray. The same pattern of results is seen for virus bearing the integrase-deficient D116N mutation (b) or with virus bearing wt integrase but in the presence of inhibitory concentrations of raltegravir (c). The bar chart shows combined results of 3 independent experiments with duplicate infections with wt or defective D116N integrase (IN) with raltegravir (RAL) (d). Geometric means of fluorescence for each receptor are expressed relative to the level after infection by the Δ nef virus (100%). Error bars indicate standard deviations (SD).

FIG. 4. Lack of Nef-mediated downregulation of HLA-E by both integrating and nonintegrating virus. Analysis of infected GFP-positive cells (gate R2) for cell surface HLA-E expression. Representative results for wt-integrase-bearing virus (a). Neither wt virus nor Δ nef virus downregulates HLA-E. The results are overlaid in the histogram; wt virus is displayed in white and Δ nef virus in shaded gray. The same pattern of results is seen for virus bearing the integrase-deficient D116N mutation (b) or with virus bearing wt integrase but in the presence of inhibitory concentrations of raltegravir (c). The bar chart shows combined results of 3 independent experiments with duplicate infections with wt or defective D116N integrase (IN) with raltegravir (RAL) (d). Geometric means of fluorescence for each receptor are expressed relative to the level after infection by the Δ nef virus (100%). Error bars indicate standard deviations (SD).

**HLA-ABC is downregulated by Nef in primary CD4+ T cells in the absence of integration.** Given our findings with the Rev-CEM GFP cell line, we next wished to examine the effect of Nef on total MHC-I (HLA-ABC) cell surface expression in the absence of integration in a natural infection model using primary CD4+ T cells infected with replication-competent NL4-3 virus.

Our initial attempts to use a pNL4-3-derived GFP reporter virus to specifically measure HLA-ABC expression on infected cells were unsuccessful (data not shown). Infections with pNL4-3-Δ nef-eGFP-derived virus (61), which encodes enhanced GFP (eGFP) in place of Env, and infections using virus derived from the pBRNL4-3-Δ nef-IRES-eGFP construct (43), which encodes eGFP under an internal ribosome entry site (IRES) downstream from nef, both resulted in cells infected with non-integrating virus being nondetectable by eGFP, despite the fact that integrating virus commonly leads to detection levels of about 12% in our system.

We therefore performed our analysis using nonfluorescent wt NL4-3 virus and analyzed HLA-ABC expression on the total cell population rather than only on infected cells. We controlled the capacity of the virus to integrate through the use of 2 μM raltegravir, a concentration that we showed can block integration to below levels of detection by Alu-LTR qPCR. Thus, we were able to compare the capacities of Nef to mediate downregulation of cell surface HLA-ABC both in the presence and in the absence of integration.

We found that the nef-bearing wt virus could mediate an 18% reduction in HLA-ABC expression relative to the level for the Δ nef virus (P = 0.0086) (Fig. 5). In parallel infections in which integration was blocked with raltegravir, we again found evidence of cell surface HLA-ABC downregulation; a reduction in expression of 26% for wt virus relative to that for Δ nef virus (P = 0.0113) was observed (Fig. 5).

As these findings are based on HLA-ABC analysis of the bulk population, they cannot be directly compared to our findings in the Rev-CEM cell line. Additionally, infection with Δ nef virus typically results in infection levels that are reduced compared to those for wt virus (19). This was also observed when we infected cells with either wt or Δ nef pBRNL4-3-Δ nef-IRES-eGFP-derived virus, as we detected infection levels of 12% and 5%, respectively, when assaying for eGFP expression. Given these expected differences in infection rates, the 18% and 26% downregulation levels that we observed for integrating and nonintegrating virus, based on a comparison of wt nef to Δ nef virus, are likely an underestimate. Our results also demon-
strate that Nef-mediated HLA-ABC downregulation by nonintegrating virus in primary CD4$^+$ T cells is readily detectable and occurs to a degree similar to that for integrated virus.

**DISCUSSION**

It is evident that transcription and translation of HIV-1 genes prior to integration are common features of viral replication (8, 57). Studies of transcription prior to integration in resting T cells demonstrated an advantage to the virus, as nef and tat translation increased the activation state of the infected cell, which was thereby more likely to yield a productive infection (58). Equally, translation of nef prior to integration is particularly apparent in macrophages (25). Studies of transcription from unintegrated cDNA can be modeled by blocking integration, either through the use of defective integrase or by integrase strand transfer inhibitors, as the patterns of transcription and translation observed in these contexts are similar to those seen in studies of transcription prior to integration (21, 56, 58). Such studies demonstrated that preintegration translation of nef variously downregulates CD4, CXCR4, and CCR5 (18, 45), which may serve to limit signal transduction through these receptors (59) and possibly limit viral superinfection (31, 45, 49, 50). Similar studies have also demonstrated that preintegration translation of rev might limit superinfection of cells at the level of integration (29, 30). We therefore suspected that transcription of preintegrated cDNA could also result in modulation of cell surface MHC-I expression by Nef.

The experiments presented herein confirm our hypothesis and demonstrate that transcription from unintegrated HIV-1 can selectively downregulate HLA-ABC and HLA-A31 in the absence of any effect on HLA-E expression. In every instance, both for the Rev-CEM cell line and for primary CD4$^+$ T cells, the extent of downregulation observed was independent of the capacity of the virus to integrate its genome into the host cell. Surprisingly, we found that in Rev-CEM cells the levels of multiply spliced mRNA transcripts were equivalent either with or without integration. Though the transcription levels described are supportive of our findings for MHC-I expression, we were surprised that our attempts to analyze cells expressing eGFP via unintegrated eGFP reporter virus BRNL4-3-nef-IRES-eGFP or NL4-3-ΔE-eGFP (both for primary cells and for cell lines) were unsuccessful. Although levels of viral multiply spliced RNA transcripts were equivalent for both integrated and nonintegrated virus, it is clear, given the paucity of eGFP expression from the reporter viruses, that there are fundamental differences in transcription from these reporter viruses in the presence and absence of integration.

Our data for HLA-A31 downregulation in Rev-CEM cells are consistent with levels of downregulation mediated by Nef for the HLA-A allotype in T cell lines, though more extensive downregulation is typically seen in primary CD4$^+$ T cells (24). The results demonstrate that transcription from unintegrated virus both in T cell lines and in activated primary CD4$^+$ T cells yields fully functional levels of Nef that are capable of mediating MHC-I modulation. Given that the pattern of MHC-I modulation seen with integrating virus is already known to protect from CTLs (11) and that the same pattern is produced with nonintegrating virus, we conjecture that cells bearing unintegrated cDNA should be equally protected from CTL responses. We propose that synthesis of Nef prior to integration leads to comprehensive and selective HLA-A and HLA-B downregulation and subsequent CD8$^+$ CTL evasion, without affecting NK cell-mediated lysis, since levels of HLA-C and HLA-E cell surface expression are not affected. The fact that HLA-E levels were elevated after infection, independent of Nef, is consistent with observations that an epitope in p24 can lead to an upregulation of HLA-E cell surface levels (34).

During acute infection, the viral load usually diminishes, concomitant with the emergence of HIV-specific CTLs (6). Typically, CTL levels remain high throughout untreated HIV infection (1) and yet can decrease following administration of therapy that leads to a reduced viral load (35). Downmodulation of MHC-I is thought to be an important feature of CTL evasion by HIV, as individuals infected with Nef-defective HIV-1 show remarkably potent CTL responses (13). Equally, work with macaques infected by simian immunodeficiency virus SIVmac showed that introduction of a point mutation in Nef that disrupted MHC-I downmodulation quickly reverted within 4 weeks (32). These findings suggest that there is a strong selective pressure for HIV to initiate Nef-mediated immune evasion. We now show that this can occur even prior to attainment of complete HIV integration.

For activated CD4$^+$ T cells, MHC-I downregulation as a result of preintegration transcription might occur several hours prior to integration, giving the virus added time to evade immune responsiveness. For infections of resting T cells, which have slower viral replication kinetics than activated T cells,
there might be a delay of 2 to 3 days prior to integration during which preintegration transcription can occur, representing a further advantage for the virus (58). An even longer delay could apply to macrophages, e.g., up to 30 days (25). However, this depends on the same pattern of MHC-I modulation occurring in both resting T cells and macrophages, as we have shown here for activated primary CD4+ T cells.

Nef produced from preintegrated DNA seems to be fully functional in terms of MHC-I modulation, and it is likely that other aspects of Nef functions are also intact. Previous studies showed that preintegration synthesis of Nef can modulate CD4, CXCR4, and CCR5 expression as well as T-cell activation (8, 45, 58). In SIV, downregulation of the T-cell receptors (TCR) CD3 and CD28 is considered a critical function for T-cell viability. Anal. Biochem. 348:316–321. Given the clear benefit of avoiding CTL- and NK cell-mediated lysis, transcription of nef from preintegrated cDNA might be a key mechanism in immune evasion following HIV infection of a host cell.

ACKNOWLEDGMENTS

This work was supported by grants from the Canadian Institutes of Health Research (CIHR) and Merck Canada, Inc. R.D.S is the recipient of a postdoctoral fellowship jointly funded by the CIHR Canadian AIDS Research (CANFAR). D.A.D. is the recipient of a predoctoral fellowship from CIHR.

We thank Daria Hazuda of Merck, Inc., for helpful comments. We also thank Yuntao Wu and Jon Marsh for provision of the Rev-CEM cell line, Robert Siliciano for provision of the pNL4-3-Δε-E-GFP construct, and Jan Münch, Michael Schindler, and Frank Kirchhoff for provision of the pBRNL4-3-nef-IRES-E-GFP construct, all via the NIH AIDS Research and Reference Reagent Program. We also thank Carlos Collazo, Susan Colby-Germainio, and Maureen Oliviera of the McGill AIDS Centre and Christian Young of the Lady Davis Institute flow cytometry core facilities for valuable technical assistance.

REFERENCES