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Biochemical and Biophysical Research Communications 327 (2005) 261–267
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Evaluation of the activity of HIV-1 integrase over-expressed in eukaryotic cells

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Received 18 November 2004
Available online 13 December 2004

Abstract

Since the integration of viral DNA in the host genome is an essential step in the replication cycle of HIV-1, an active search for inhibitors of the integration step is ongoing. Our laboratory has been working on the development of a cellular integration system. Such a system would be helpful in the study of the HIV-1 integration process and, eventually, could be used in the search for new inhibitors that selectively interfere with HIV integration. We have previously selected stable cell lines (293T-IN^S) that constitutively express high levels of HIV-1 integrase (IN) from a synthetic gene [FASEB J. 14 (2000) 1389]. We have now constructed linear DNA substrates containing the terminal HIV LTR sequences (so called 'mini-HIV') and EGFP as reporter gene to evaluate whether IN can improve the integration of transfected linear DNA. After electroporation of this mini-HIV we observed a 2- to 3-fold increase in EGFP expression in IN expressing cell lines relative to control cells. The increase in EGFP expression was still evident after passaging of the cells. The effect was observed with linear DNA but not with circular DNA, thus excluding an effect on DNA uptake. The increase was the highest in the 293T-IN^S(D64V) cell line due to an increase in the amount of total mini-HIV DNA and 2-LTR circles as quantified by Q-PCR. Our data suggest that IN over-expressed in our cell lines interacts with the incoming DNA, protects it from nuclease degradation but does not catalyze the integration as such.

Keywords: HIV-1; Integrase; Mini-HIV; 293T-IN^S

Integration of the linear viral DNA into the cellular genome is an obligatory step in the replication cycle of the human immunodeficiency virus type 1 (HIV-1) [2,3]. After viral entry reverse transcriptase (RT) copies the viral genomic RNA into a linear double-stranded cDNA, that is subsequently inserted into the host cell genome by the viral enzyme integrase (IN). IN is localized in a cytoplasmic nucleoprotein complex, termed the pre-integration complex (PIC), which is composed of viral proteins, the double-stranded viral cDNA, and some cellular proteins (for a review see [3]). In this PIC the integrase first catalyzes the removal of the pGT dinucleotide at the 3′ ends of the long terminal repeats (LTR), a reaction referred to as 3′ processing. Following nuclear entry, IN mediates the strand transfer of the processed 3′ ends to the 5′ phosphates of a double-stranded staggered cut in the cellular DNA. Repair of the remaining gaps between the unjoined viral 5′ ends and the chromosome most probably involves cellular enzymes. The transport of the PIC through the nuclear pores is mediated by an active, energy-dependent process [4] and requires both viral and cellular proteins. The precise contribution of nuclear localization signals in matrix, Vpr, and integrase remains controversial (reviewed by [5]). Since integration is an essential and specific step for HIV-1 replication, integration is an attractive target for antiviral therapy [6,7]. While an intensive search for HIV integrase inhibitors is ongoing, a detailed study of the integrase enzyme in vitro as well as in vivo is necessary.

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.11.157

The biochemistry of retroviral integration has been elucidated from in vitro studies using recombinant IN protein and a synthetic oligonucleotide DNA substrate that mimics one of the viral LTRs [8,9]. Alternatively, long double-stranded DNA molecules flanked by both LTR ends, the so-called mini-HIV DNA substrates, have been used; mini-HIV DNA supports efficient but non-concerted integration in vitro [10]. Another approach to study integration in vitro consists of isolating subviral particles, that contain integrase and viral cDNA, from acutely infected cells. When providing these pre-integration complexes (PICs) with double-stranded DNA, both ends of the viral cDNA will be integrated in a concerted fashion [2]. These in vitro assays have contributed much toward the currently accepted mechanism of retroviral integration [3]. The HIV integration in the context of viral replication has been studied by introducing mutations in IN followed by the analysis of the replication cycle of the mutant virus. Mutations in HIV-1 IN affect steps distinct from integration, including uncoating [11,12], reverse transcription [13], and viral particle formation [14]. These pleiotropic effects suggest that IN is implicated in various replication steps. Apart from neglecting the interaction with other viral proteins, the in vitro integrase assays do not account for the cellular co-factors that participate in the integration process in vivo. The host factors HMG-1a [15,16], integrase interactor 1 (Ini1) [17], and barrier to autointegration factor (BAF) [18] have been put forward as co-factors of HIV integration. More recently, a new cellular IN co-factor was identified, namely lens-epithelium derived growth factor (LEDGF)/p75, that stimulates the strand transfer activity in vitro [19] and appears to be required for the nuclear and chromosomal targeting of HIV-1 IN in human cells [20].

So far, the cell biology of HIV integration has been studied in the context of viral infection or using the PIC assay. To investigate the integration reactions in the eukaryotic cell, we attempted to over-express HIV integrase in human cells to catalyze the integration of mini-HIV DNA in the chromosome in the absence of other viral factors. This system could also facilitate integrase inhibitor development. We have previously reported on a human 293T cell line that stably expresses HIV-1 integrase, encoded by a synthetic gene [1]. We proved the enzymatic activity of the integrase by complementation of IN-defective HIV-1-derived vector particles. Lentiviral vectors carrying catalytically inactive IN(D64V) were capable of stably transducing 293T cells when complemented in the producer cells with integrase expressed from the synthetic gene. Moreover, when the cell line that stably expresses integrase was transduced with the defective lentiviral vector particles, complementation of integrase activity was detected as well [1]. In the present study, we evaluated whether HIV-1 integrase stably expressed in the human 293T cells was per se able to stimulate the integration of mini-HIV DNA substrates in the absence of the viral particle. Hereto we designed mini-HIV DNA substrates containing the EGFP reporter gene, facilitating the analysis of the transfection efficiency by FACS.

Materials and methods

Cell culture. 293T cells, E1A transformed human embryonic kidney cells expressing SV40 large T antigen, were obtained from Dr. O. Danos (Généthon, Evry, France). 293T cells were grown in Dulbecco's modified Eagle's medium with glutamax (DMEM, Invitrogen) supplemented with 10% FCS (Biochrom AG) and 20 μg/ml gentamicin (Invitrogen) at 37 °C in 5% CO_2 humidified atmosphere. The stable cell lines, transfected with pCEP (Invitrogen) [293T-pCEP], pCEP-IN^S [293T-IN^S] or pCEP-IN^S(D64V) [293T-IN^S(D64V)], were cultured in the presence of 200 μg/ml hygromycin B (Invitrogen).

Western blotting. For Western blotting we used rabbit polyclonal antibodies raised, in-house, against recombinant HIV-1 integrase. The detection of tubulin was performed by using a commercially available antibody (Sigma). Western blotting was performed using the ECL+ chemiluminescent detection system (Amersham–Pharmacia Biotech) with biotinylated goat anti-rabbit antibodies and streptavidin-horseradish peroxidase (Amersham–Pharmacia Biotech).

The plasmid constructs. pUC-19 was digested with *Sca*I (Gibco-BRL). The product was ligated with Ready-to-Go T4 DNA ligase (Amersham–Pharmacia) to the kanamycin resistance gene, isolated from pCP15 (obtained from P. Cherepanov) by restriction with *Xba*I (Gibco-BRL), and the ends were blunted using T4 DNA polymerase (Gibco-BRL). The resulting plasmid, pUC-Km, was used as backbone for further constructions. The pUC-CMV-EGFP plasmid was obtained by subcloning the 1.6 kb *Ase*I/*Afl*II fragment of pEGFP-N1 (Clontech) in the *Sma*I site of pUC-Km. The *Ase*I and *Afl*II ends were filled in using T4 DNA polymerase. The LTR ends, present in the pU3U5 plasmid [10], were subcloned in pUC-Km as a *Eco*RI/*Kpn*I insert, resulting in the plasmid pLTR-Km. The *EGFP* gene was subcloned in the same way as the former construct to result in the pLTR-CMV-EGFP plasmid. Before electroporation the plasmids were linearized by *Sca*I digestion, followed by phenol–chloroform extraction and ethanol precipitation.

Transfection procedure. Cells were passaged the day before transfection to obtain 80–90% confluency 24 h later. Before transfection, cells were trypsinized and counted. Cells were diluted to a concentration of 8×10^5 cells/ml. Five micrograms of DNA was added to 500 μl cell suspension in an electroporation cuvette (Eurogentec). Electroporation was done at 250 V and 1050 μF in an Easyjet One electroporator (Eurogentec). After electroporation cells were grown in a 24-well plate. The day after transfection the medium was changed. Part of the transfected cells were passaged (10-fold dilution) after 72 h and analyzed after 120 h.

FACS analysis of reporter gene expression. The EGFP expression was analyzed 72 and 120 h postelectroporation. Cells were fixed in 2% paraformaldehyde prior to FACS analysis, using a FACS-calibur flow cytometer (Becton–Dickinson). The data were analyzed using the CellQuest software package provided with the instrument.

Quantification of circular DNA. Cellular DNA was extracted, 72 h (i.e., before passaging) and 120 h (i.e., after passaging) after electroporation, by phenol–chloroform extraction and ethanol precipitation. For the quantification of the total amount of transfected DNA a real-time quantitative PCR (Q-PCR) was performed using the following primers and probe: 5′-TGAGGGATCTCTAGTTACCAGAGTCA-3′ as forward primer; 5′-CCTGGGAGCTCTCTGGCTAA-3′ as reverse primer; and 5′-FAM-ACAACAGACGGGCACACACTACTTGAAGC-TAMRA-3′ as probe. U3–U5 circles were quantified by Q-PCR—the same one as that used for quantification of 2-LTR circles—using the following primers and probe: 5′-GTGCCCGTCTGTTGTGTGA

CT-3′ as forward primer; 5′-CTTGTCTTCTTTGGGAGTGAATT AGC-3′ as reverse primer; and 5′-FAM-TCCACACTGACTAAA AGGGTCTGAGGGATCTCT-TAMRA-3′ as probe. Reactions were done in 50 μl containing 1× TaqMan universal master mix (Applied Biosystems, Lennik, Belgium), 400 ng DNA, 300 nM primers, and 150 nM probe. A standard curve was run in parallel, using dilutions of the pU3U5 plasmid [10]. After initial incubations at 50 °C for 2 min and at 95 °C for 10 min, 40 cycles of amplification were carried out at 95 °C for 15 s, followed by 1 min at 60 °C. Reactions were analyzed using the ABI Prism model 7700 sequence detection system (Applied Biosystems).

Results

Over-expression of HIV-1 IN increases transfection of mini-HIV DNA

To verify whether the presence of IN in eukaryotic cells could stimulate the integration of linear DNA, we constructed the following DNA substrates (Fig. 1). We cloned the *EGFP* gene downstream of a CMV promoter in a plasmid containing the U3–U5 sequence of HIV-1, pU3U5 [10]. The resulting plasmid was named pLTR-CMV-GFP. To create the mini-HIV DNA substrate the plasmid was linearized by *Sca*I digestion, resulting in a linear DNA strand flanked by U3 and U5. As control for the LTR sequence, we also constructed the plasmid, pUC-CMV-GFP, containing the CMV-*EGFP* gene without the U3–U5 sequence. Following cell lines were used for transfection: (1) 293T-pCEP, serving as a control cell line, contains the backbone plasmid pCEP4; (2) 293T-IN^S, stably expresses wild type IN; and (3) 293T-IN^S(D64V), stably expresses the catalytically inactive mutant IN^S(D64V). The expression of IN and IN(D64V) was verified by Western blotting (Fig. 2).

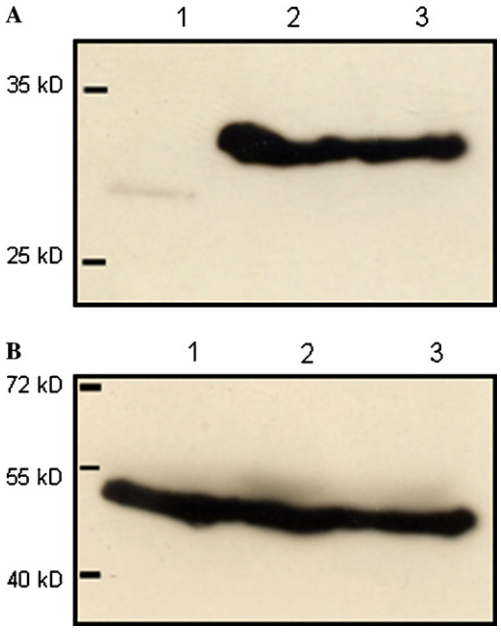

Fig. 2. Western blot analysis of stable expression of HIV-1 IN in 293T cells. 293T cells (lane 1) were transfected with pCEP-IN^S (lane 2) and pCEP-IN^S(D64V) (lane 3), and selected with 200 μg/ml hygromycin B. Extracts of the selected stable cell lines, representing 25 μg of total protein, were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto PVDF membranes. (A) Integrase was detected by Western blotting using polyclonal antibodies against HIV-1 integrase and the ECL+ detection system. (B) To check for equal loading, tubulin levels were visualized in the same samples using specific antibodies. At the left side, molecular weights are indicated, based on the protein standard.

The linearized plasmids pUC-CMV-EGFP and pLTR-CMV-EGFP were electroporated in the three cell lines. FACS analysis was used to measure EGFP expression

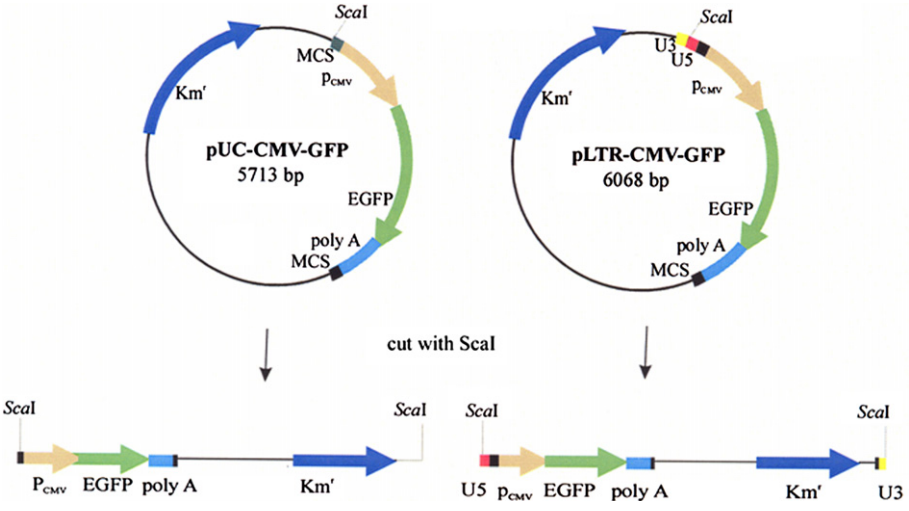

Fig. 1. Plasmid maps of the DNA substrates. The 1.6 kb *Ase*I/*Afl*II fragment from EGFP-N1, containing CMV-EGFP, was cloned in the *Sma*I site of pUC-Km and pLTR-Km, resulting in pUC-CMV-EGFP and pLTR-CMV-EGFP, respectively. To obtain a linear mini-HIV DNA substrate we linearized the plasmids by *Sca*I digestion, followed by phenol–chloroform extraction and ethanol precipitation. KmR, kanamycin resistance gene; MCS, multiple cloning site; and P_{CMV}, CMV-promoter.

after 72 h. The percentage of cells expressing EGFP above cut-off was used as a measure of transient transfection efficiency. As shown in Fig. 3 and Table 1, the EGFP reporter gene signal was 2- to 3-fold higher with linearized pLTR-CMV-EGFP in cells over-expressing IN, compared to the control cell line. With the linearized pUC-CMV-EGFP, that does not contain the LTR ends, the effect in the 293T-IN^S cells was less pronounced. Remarkably, the measured EGFP signal was highest in the cells stably expressing mutant IN [IN(D64V)] using DNA substrates with or without LTR.

Table 1
Effect of over-expression of HIV-1 IN on the transient transfection efficiency of DNA in 293T cells

	Relative transfection efficiency (Transient)[a]	
	293T-IN^S	293T-IN^S(D64V)
Linear pLTR-CMV-EGFP	2.22 ± 0.86	2.92 ± 0.36
Linear pUC-CMV-EGFP	1.36 ± 0.13	2.71 ± 0.31
Circular pLTR-CMV-EGFP	1.02 ± 0.1	1.34 ± 0.55
Circular pUC-CMV-EGFP	0.96 ± 0.18	1.2 ± 0.42

[a] Transfection efficiency 72 h after electroporation, as measured by FACS analysis, relative to the transfection efficiency obtained in the 293T-pCEP cell line. Data represent average values ± SD from three independent experiments.

Effect of IN on the transfection efficiency of circular DNA

Since the stable expression of IN seemed to have a stimulatory effect on the transfection efficiency of linear DNA, we verified if the presence of IN also improved the transfection efficiency of circular plasmid DNA. Hereto the three cell lines were electroporated in parallel with circular pLTR-CMV-EGFP and pUC-CMV-EGFP. Neither before nor after passaging of transfected cells a significant difference between EGFP reporter gene signal could be observed between the cells expressing wild type IN and the control cell line (Table 1 and Fig. 4). In cells expressing mutant IN a small increase in transfection efficiency was seen.

Stable increase in EGFP expression in cells over-expressing IN

The linear DNA substrates used in the assay contain a cytomegalovirus (CMV) promoter upstream of the *EGFP* reporter gene, causing transient expression from non-integrated DNA substrates. To eliminate EGFP expression from non-integrated linear DNA, cells were passaged once or twice (10-fold dilution). Although there was a clear drop of about 60% in the level of EGFP expression in the different cell lines after passaging, the 2- to 3-fold increase in EGFP signal from the linearized pLTR-CMV-EGFP could still be observed in the cells expressing IN or IN(D64V), as shown in Fig. 4.

IN(D64V) stimulates circularization of the DNA substrates

Since the observed increase in stable EGFP signal was always highest in the 293T-IN^S(D64V) cells, catalytic activity of IN was apparently not required for the effect. Possibly, by interacting with the linear DNA, integrase protects it against nuclease degradation in

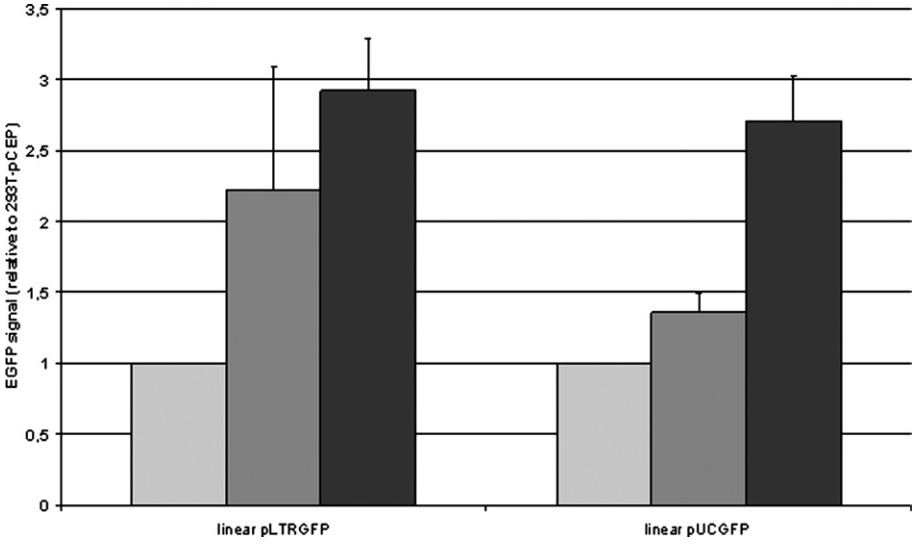

Fig. 3. Integrase increases the transient transfection efficiency of linear DNA. Five micrograms of linearized pLTR-CMV-EGFP or pUC-CMV-EGFP were electroporated in 4×10^5 293T-pCEP cells (light gray), 293T-IN^{S}cells (dark gray), and 293T-IN^S(D64V) cells (black). The EGFP expression was analyzed 72 h postelectroporation by FACS analysis. The percentage of EGFP positive cells, relative to this percentage in the 293T-pCEP control cells, is given and the values represent the average ± SD from three independent experiments.

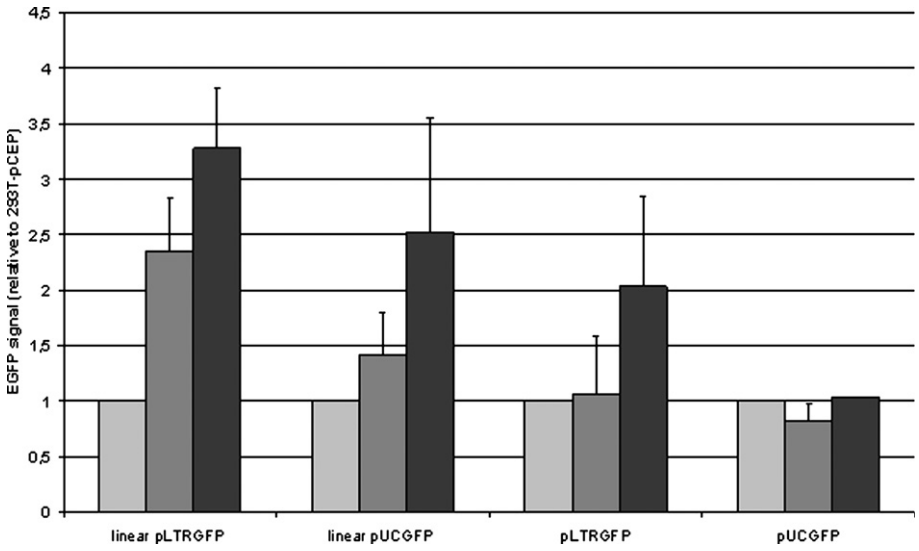

Fig. 4. The increase in transfection efficiency of linear DNA substrates upon over-expression of IN is retained after passaging. Five micrograms of linearized pLTR-CMV-EGFP, pUC-CMV-EGFP, circular pLTR-CMV-EGFP or pUC-CMV-EGFP were electroporated in 4×10^5 293T-pCEP cells (light gray), 293T-IN^S cells (dark gray), and 293T-IN^S(D64V) cells (black). FACS analysis was performed 120 h postelectroporation. The percentage of EGFP positive cells, relative to this percentage in the 293T-pCEP control cells, is given and the values represent the average ± SD from three independent experiments.

the cytoplasm. Alternatively, the nucleophilic integrase may facilitate the nuclear import of mini-HIV DNA. During HIV replication, the 2-LTR circles are used as a marker for nuclear import. Therefore, we electroporated the 293T-pCEP and 293T-IN^S(D64V) cell lines, extracted cellular DNA before and after passaging of the cells, and performed quantitative PCR analysis, measuring specifically the amount of total DNA substrate and U3–U5 circles. In 293T-IN^S(D64V) cells, the amounts of total DNA substrate (Fig. 5A) and U3–U5 circles (Fig. 5B) were 2-fold higher than in the control cells, 72 h after electroporation. After passaging the difference in the amount of U3–U5 circles was lost (Fig. 5B). Although the total DNA remained 1.6-fold higher in the 293T-IN^S(D64V) cells (Fig. 5A) after dilution, most of the probably unintegrated linear DNA was lost. With circular plasmid DNA comparable amounts of total DNA were detected in both cell lines 72 h after electroporation. This rules out better cellular uptake of DNA in integrase expressing cells.

Discussion

The replication cycle of HIV-1 requires a functional viral integrase for a productive infection. Integrase is necessary and sufficient to catalyze the 3′ processing and strand transfer of retroviral integration in in vitro assays [21,22]. In the present study we investigated whether the presence of the viral integrase in human cells is, by itself, sufficient to catalyze the integration of naked mini-HIV DNA in these cells. Our data suggest that the stable over-expression of HIV-1 IN in 293T cells can stimulate the transfection efficiency of linear mini-HIV DNA, transiently as well as stably. Transfection efficiency could easily be measured by FACS analysis since we used mini-HIV DNA carrying the *EGFP* gene. Although we observed a 2- to 3-fold increase in EGFP expression after electroporation of linear DNA in the cells over-expressing IN, we cannot attribute this result only to the catalytic activity of IN, since the highest increase was always seen in cells over-expressing the catalytically inactive D64V mutant. This could suggest that the aspecific DNA binding activity of IN is able to protect the naked DNA from nuclease digestion within the cytoplasm and the nucleus. Alternatively, the karyophilic properties of HIV integrase may be responsible for facilitating the nuclear import of mini-HIV DNA. Possibly, both mechanisms are dependent on each other: by binding to linear DNA and by increasing nuclear import integrase will protect the DNA from degradation in the cytoplasm. To corroborate this hypothesis we quantified the amount of total transfected DNA and U3–U5 circles in the 293T cells that express mutant integrase and support the highest transfection efficiency. During HIV replication 2-LTR circles are known to be formed in the nucleus by cellular enzymes [23] and they are used as marker for nuclear import of the viral genome. Since we used a mini-HIV DNA substrate, we took the number of U3–U5 circles as a measure for the efficiency of nuclear import of the transfected DNA. In the cells over-expressing IN(D64V) an increase of total mini-HIV DNA substrate and of U3–U5 circles was seen 72 h after transfection. Since it is known that 2-LTR cir-

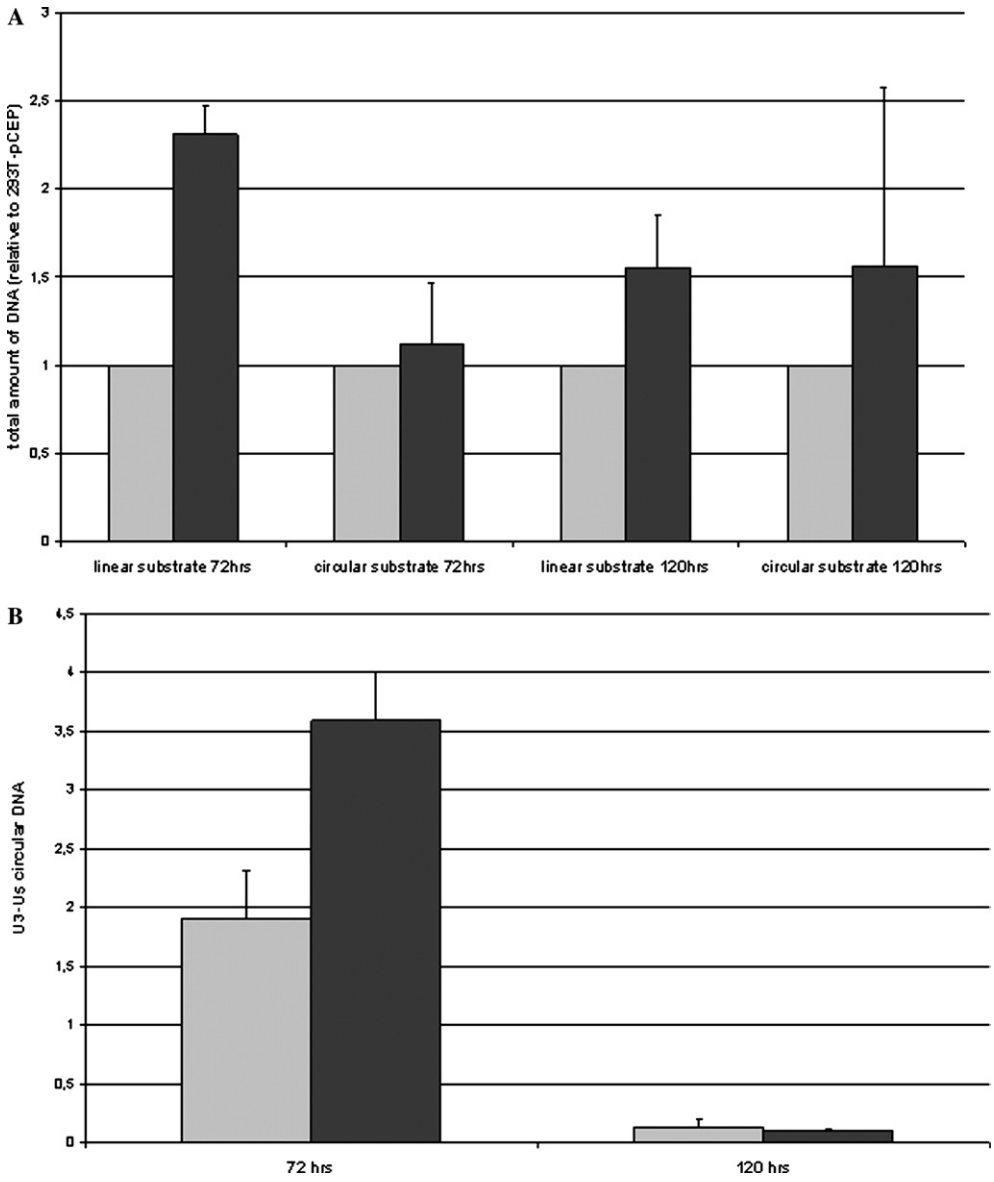

Fig. 5. The presence of IN(D64V) in 293T cells protects linear DNA ends from degradation, resulting in a higher amount of totally transfected DNA and an increase in U3–U5 circles. 293T-pCEP (light gray) and 293T-IN^{S}(D64V) (black) cells were electroporated with linearized and circular pLTR-CMV-EGFP. Cellular DNA was extracted, 72 h (before passaging) and 120 h (after passaging) after electroporation, by phenol–chloroform extraction and ethanol precipitation. Q-PCR analysis was performed to quantify the total amount of transfected DNA (A) and the amount of recircularized linear DNA, the so-called U3–U5 circles (B). Values represent the average ± SD from duplicate experiments.

cles are diluted upon cell division [24,25], it is not surprising that the increase in U3–U5 circles was lost upon passaging of the cells. Still, the transient increase in U3–U5 circles in cells expressing mutant integrase corroborates our hypothesis that IN mediates the presence of increased amounts of mini-HIV DNA substrate in the nucleus. The higher amount of total mini-HIV DNA in cells over-expressing mutant integrase that is partially resistant to dilution points to a protection of the incoming naked linear DNA by interacting with IN and an increased insertion of this DNA in the chromosome possibly through the process of non-homologous recombination. A direct effect of IN over-expression on the uptake of the DNA in the cells was excluded since the effect was not seen with circular plasmid DNA.

An indirect effect due to the selection procedure of the cells is possible, although we obtained our data relative to a control cell line, 293T-pCEP, selected the same way. Moreover, all the experiments were done in polyclonal cell lines, excluding artifacts linked to clonal differences. Alternatively, stable over-expression of IN in the cells may have altered expression of other proteins that affect DNA stability.

Recently, Yao et al. [26] demonstrated a 4-fold increase in efficiency of integration of a selectable transgene after microinjecting avian myeloblastosis virus

(AMV) IN–DNA complexes in mammalian cells. The increase in resistant colonies was dependent on both *att* sites of the DNA. By sequencing three integration events Yao et al. [26] concluded that the microinjection of the AMV IN–DNA complex resulted in an authentic integration of the U5–U3 DNA with a 6-bp duplication. Although this does not prove that the overall increase in transfection efficiency was due to authentic integration (and perhaps is also partially due to non-homologous recombination), integration seems to occur in their system. Their approach was different from ours since they microinjected a pre-assembled IN–DNA complex in the cells and used a selectable marker, thereby selecting for stable integration. Moreover, AMV integrase is known to have distinct biochemical features, especially with regard to concerted integration of both LTR ends [27,28]. When we cloned and sequenced mini-HIV DNA stably inserted in the chromosome, we could not retrieve the hallmark of HIV-1 integration, a preferential terminal 2-bp deletion (data not shown). Instead, a range of terminal deletions were observed. The requirement for a pre-assembled complex of AMV-IN and DNA to obtain authentic concerted integration points to another difference with our system. In our cells stably over-expressing HIV-1 integrase, most of the enzyme is present in the nucleus. We do not know whether nuclear HIV integrase is capable of forming the appropriate IN–DNA complex required for concerted integration. We attributed the observed 2- to 3-fold difference in reporter gene expression to a protection from nuclease degradation in the nucleus. It may well be that the integrase present in the nucleus and attached to the chromosomes is in an inactivated form, explaining why mutant integrase could also enhance the transfection of linear DNA. Despite the fact that Yao et al. [26] obtained some authentic integration in the absence of any other viral co-factor, viral co-factors or cellular co-factors activated upon HIV infection may be required for efficient HIV integration.

Our data are suggestive for an interaction of linear DNA substrates with HIV-1 integrase in the cell. We now plan to use this system to further investigate this interaction in the individual cell using fluorescently labelled DNA substrates and to examine the role of viral and cellular co-factors.

Acknowledgments

We thank Dr. Peter Cherepanov for the design and construction of the synthetic IN^S gene and the sequencing of inserted mini-HIV DNA. We thank Dr. Rik Gijsbers and Jan De Rijck for a critical reading of the manuscript. B.V.M. is funded by a grant from the Flemish Institute supporting Scientific-Technological Research in Industry (IWT). This work was supported by the European Commission (LSHB-CT-2003-503480).

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