

Chromosomal integration of LTR-flanked DNA in yeast expressing HIV-1 integrase: down regulation by RAD51

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ABSTRACT

HIV-1 integrase (IN) is the key enzyme catalyzing the proviral DNA integration step. Although the enzyme catalyzes the integration step accurately *in vitro*, whether IN is sufficient for *in vivo* integration and how it interacts with the cellular machinery remains unclear. We set up a yeast cellular integration system where integrase was expressed as the sole HIV-1 protein and targeted the chromosomes. In this simple eukaryotic model, integrase is necessary and sufficient for the insertion of a DNA containing viral LTRs into the genome, thereby allowing the study of the isolated integration step independently of other viral mechanisms. Furthermore, the yeast system was used to identify cellular mechanisms involved in the integration step and allowed us to show the role of homologous recombination systems. We demonstrated physical interactions between HIV-1 IN and RAD51 protein and showed that HIV-1 integrase activity could be inhibited both in the cell and *in vitro* by RAD51 protein. Our data allowed the identification of RAD51 as a novel *in vitro* IN cofactor able to down regulate the activity of this retroviral enzyme, thereby acting as a potential cellular restriction factor to HIV infection.

INTRODUCTION

Retroviral integration is mediated by the preintegration complex (PIC) whose composition has not yet been completely elucidated. HIV-1 integrase (IN) is a major component of the PIC and is necessary and sufficient for the integration reaction *in vitro* (1, 2). Although recombinant IN can efficiently perform both 3'-processing and the

insertion of one viral extremity *in vitro*, some differences in the integration activity of the recombinant protein and its activity in the partially purified PIC have been described. Several cellular factors associated with the PIC, such as the high mobility group protein HMGA1, the transcription factor INI1 or LEDGF/p75, have been shown to stimulate *in vitro* integration reactions and/or play an important role in the HIV-1 infection cycle (3–7). The lack of these factors in the *in vitro* integration assays may account for the differences observed between these two systems. However, it has recently been reported that recombinant IN, purified under certain conditions, performed a concerted integration reaction which was almost as efficient as that catalyzed by the PIC (8). Thus we investigated herein whether IN might be the minimal viral protein required for integration in a cellular context. Previous attempts to evaluate the activity of HIV-1 IN over-expressed in eukaryotic cells failed to describe any specific integration (9). We have previously described a yeast eukaryotic model in which HIV-1 IN expressed as the sole viral protein produces a lethal effect linked to its previously described non-sequence-specific endonucleolytic activity (10–12). We, thus, established a system based on this model, thereby allowing the analysis of the integration step independently of other viral mechanisms.

Here we report for the first time that HIV-1 IN expressed as the sole retroviral protein in eukaryotic cells was sufficient to catalyze the complete integration of a DNA containing two viral LTRs into the nuclear genome. Using this model, we demonstrated the RAD51 dependent pathway of homologous recombination (HR) down regulates the integration activities catalyzed by IN both *in vitro* and in yeast.

MATERIALS AND METHODS

Yeast strains, culture media and growth conditions

Yeast strains The nomenclature used for the yeast strains was previously described (11). The following haploid yeast

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strains were used: W303.1A (h.RAD52⁺) (*MAT α* , *ura3-52*, *leu2-3,112*, *ade2-1*, *his3-11, 15*, *trp1-1*); W839-5C (h.rad52⁻) (isogenic to W303.1A except *rad52::TRP1*); BY4742 Δ rad18 (h.rad18⁻) (*MAT α* , *ura3 Δ 0*, *leu2 Δ 0*, *lys2 Δ 0*, *his3 Δ 1*, *rad18::kanMX4*); BY4742 Δ rad51 (h.rad51) (*MAT α* , *ura3 Δ 0*, *leu2 Δ 0*, *lys2 Δ 0*, *his3 Δ 1*, *rad51::kanMX4*).

Culture media Yeast selective media: YNB lacking uracil and leucine (0.67% yeast nitrogen base without amino acids, 0.1–8% glucose); YCAD lacking uracil (YNB 2% glucose supplemented with 0.5% casamino acids). Amino acids and bases (20–30 mg/l) were added as required. For the selection of zeocin-resistant cells, YCAD was supplemented with 400 μ g/ml zeocin (INVITROGEN).

Growth conditions Liquid cultures were performed in Erlenmeyer flasks filled to a fifth of their capacity and then shaken. Solid media were obtained by supplementing liquid media with 2% bacto-agar. Yeast strains were grown at 30°C.

DNA materials

All DNA vectors and PCR products were purified using DNA purification systems from PROMEGA (Wizard plus SV mini-prep and Wizard SV Gel kits). PCR amplifications were done under standard conditions using *Taq* polymerase (PROMEGA).

Expression vectors The HIV-1 IN gene was obtained from a cloned genomic provirus of a San Francisco isolate (SF2) (13). pHIV1SF2IN and pHIV1SF2IN-D116A were derived from the yeast/*Escherichia coli* shuttle plasmid pBS24.1 described previously (10).

DNA substrates of the yeast integration assay Various DNAs were used: (i) A 1212 bp double-stranded linear DNA flanked by the two sequences mimicking the HIV-1 U3 and U5 viral ends carries the zeocin-resistance encoding gene under the control of the yeast TEF1 promoter. This fragment was generated by PCR on pTEF1/Zeo vector (INVITROGEN) using primers 5'-U3-Zeo (5'-ACTGGAAGGGGCTAATTCACCCACACACCATAGCTTCAAATGTTTCTACTCC-3') and 3'-U5-Zeo (5'-ACTGCTAGAGATTTCCACAAGCTTGCAAATTAAGCCTTCGAGCGTCC-3'); (ii) a pre-processed substrate was prepared by PCR on pTEF1/Zeo with primers NdeI-5'-U3-Zeo (5'-GGAATTCATATGGAAGGGGCTAATTCACCCACACACCATAGCTTCAAATGTTTCTACTCC-3') and NdeI-3'-U5-Zeo (5'-GGAATTCATATGCTAGAGATTTCCACAAGCTTGCAAATTAAGCCTTCGAGCGTCCC-3'). The NdeI restriction site is underlined. The resulting PCR product was purified and used directly as the unprocessed integration substrate. The amplification product was also cloned into pGEM-T vector according to the manufacturer's recommendations (PROMEGA) and the pGEM-T-LTR-Zeo obtained vector was subjected to NdeI restriction cuts. The 1212 bp insert obtained displayed the 3'-OH recessed viral ends mimicking the 3'-processed LTR at each extremity; (iii) a 1169 bp DNA fragment with no viral LTRs was constructed by PCR amplification on pTEF1/Zeo using the following primers: 5'-Cont-Zeo (5'-CCCACACACCATAGCTTCAAATGTTTCTACTCC-3') and 3'-Cont-Zeo (5'-TTGCAAATTAAGCCTTCGAGCGTCCC-3'). Positions of the different primers are shown on Figure 1.

Yeast integration assay

The strategy used to follow integration of the DNA substrate containing the viral LTRs into yeast genome is presented in Figure 1. Yeast cells harboring either pHIV1SF2IN or pHIV1SF2IN-D116A vectors were grown for 72 h under maximal expression conditions (10 ml YNB containing 0.1% glucose and supplemented with required amino acids) until an OD_{600nm} of \sim 10 was attained (stationary phase). Proteins were then extracted and analyzed by SDS-PAGE in order to determine IN expression. Yeast aliquots were used for transformation with an excess of DNA substrate (7×10^{11} molecules) under conditions previously described (14). After transformation, 10^9 viable cells calculated by counting the number of cells recovered after plating on non-selective medium were cultured in YCAD liquid medium for 0–6 h and then plated on solid YCAD medium supplemented with zeocin (400 μ g/ml) to select the cells expressing the *Sh ble* gene from the DNA substrate. After five days of culture, resistant clones were recovered, stocked and analyzed further. Transformants were selected on medium lacking uracil and histidine.

PCR, Southern blot and sequencing analysis of the integration events

The chromosomal DNA from the selected zeocin-resistant clones was prepared as described previously (15) and then submitted to PCR amplification using 5'-pTEF1 (5'-TT CATTAGAAAGAAAGCATAGC-3') and 3'-U5-Zeo primers. Under positive integration conditions, an amplification product of 800 bp is expected. Southern blot analysis was performed on genomic DNA according to a previously described protocol (16) and using BamHI restriction enzyme (PROMEGA) and the ³²P-labeled ODN, 5'-³²P-U3-Zeo described above as probe. Sequencing was performed using chromosomal DNA digested by BamHI, religated with T4 DNA ligase (PROMEGA) and amplified using 5'-U3-junction (5'-GATGCGCGGAGTCCGA-3') and 3'-U5-junction (5'-AG ACGCGTGTACGCATGTA-3') primers. The amplification products were used in PCR-based sequencing (ABI Prism big dye terminator cycle sequencing ready reaction kit, Applied Biosystems) using the same primers.

HIV-1 IN purification, *in vitro* IN activities and ELISA assay

HIV-1 IN purification and *in vitro* assays were described previously (17). Human wild type RAD51 proteins were purified as reported before (18) and bacterial integrin class I recombinase IntI1 (kind gift from Pr C. Quentin) were added in the assay when mentioned. Integration products were quantified with ImageJ software.

ELISA experiments were done as follows. Plate wells were coated overnight at 4°C with 30 pmol of the recombination proteins diluted in a 0.1 M carbonate solution. Wells were washed with a solution of PBS containing 0.05% Tween (PBS 0.05% Tween), saturated for 1 h with BSA (10 mg/ml) and washed three times with PBS 0.05% Tween solution. IN (30 pmol) was added and incubated for 1 h at 37°C. After washing three times with PBS 0.05% Tween, anti-IN primary antibodies were incubated for 2 h at 37°C. After three washings, secondary antibodies were incubated for 1 h at 37°C and the interaction was revealed with

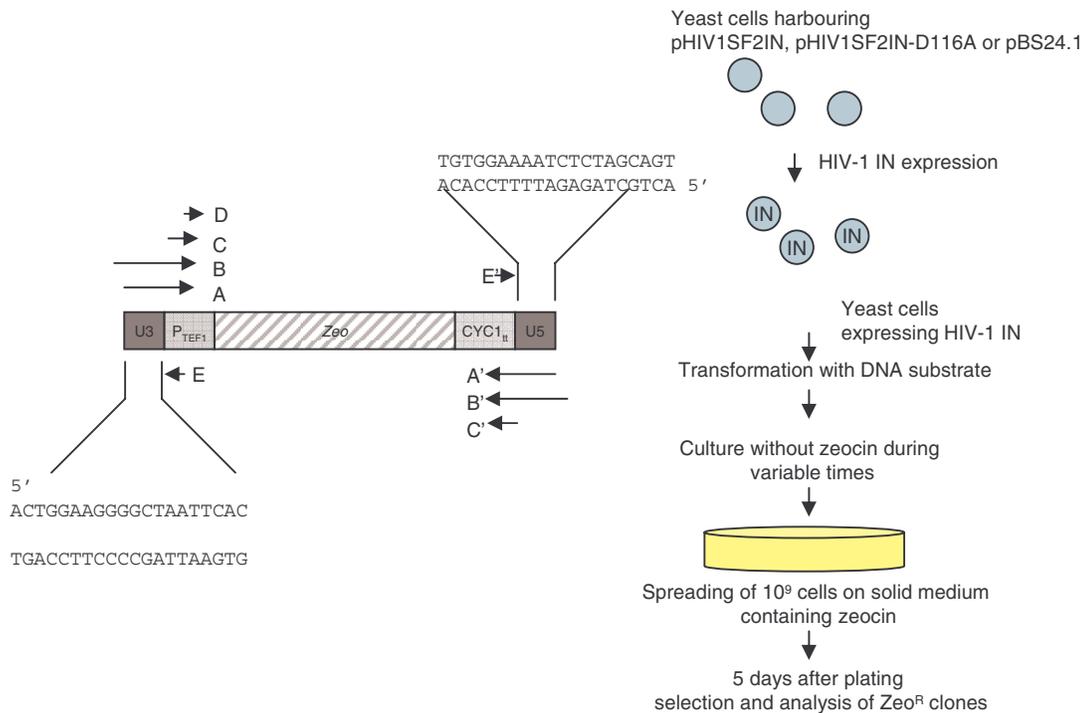


Figure 1. Yeast integration assay. Yeast cells (h.RAD52⁺) containing pHIV1SF2IN, pHIV1SF2IN-D116A or empty pBS24.1 were grown for 3 days under conditions of IN expression in a medium containing 0.1% glucose without leucine. Yeast cells were then transformed with the integration DNA substrate containing the *Sh Ble* ORF (*Zeo*) flanked by the LTR sequences. Cells were grown in the absence of zeocin for variable times and then plated on solid medium containing 400 μ g/ml zeocin. Resistant clones were selected five days after plating and analyzed. P_{TEF1}: TEF1 promoter, CYC1_{tt}: CYC1 transcription termination sequence. Arrows indicate primer positions used for the generation of different substrates, PCR and Southern blot analysis: 5'-U3-*Zeo* (A), 3'-U5-*Zeo* (A'), NdeI-5'-U3-*Zeo* (B), NdeI-3'-U5-*Zeo* (B'), 5'-Cont-*Zeo* (C), 3'-Cont-*Zeo* (C'), 5'-pTEF1 (D), 5'-U3-junction (E) and 3'-U5-junction (E').

Amplex-Red (Invitrogen) after washing with PBS. The absorbance was determined at 530/590 nm.

Pull down experiments

Purified hRAD51 and BSA were coupled to Affi-Gel 15 beads (Bio-Rad) to create matrices containing 3 mg/ml of RAD51 and 12 mg/ml BSA. To examine complex formation, HIV-1 IN (4 μ g) was mixed with 5 μ l Affi-RAD51 or Affi-BSA beads in 100 μ l buffer B (25 mM Tris at pH 7.4, 0.5 mM EDTA, 1 mM DTT, 0.01% Igepal CA-630, 75 mM NaCl and 10% glycerol) at 4°C for 60 min. The Affi-hRAD51 mixture also contained 2 μ g of BSA. The beads were washed twice with 100 μ l of the same buffer, before eluting the bound proteins with 30 μ l SDS-PAGE loading buffer. The supernatant (S), wash (W) and SDS eluate (E), 10 μ l each, were analyzed by 12% SDS-PAGE and Coomassie blue staining.

RESULTS

HIV-1 integrase integrates a DNA carrying two LTRs into the yeast genome

The h.RAD52⁺ yeast strain expressing HIV-1 IN was grown under optimal condition for the expression of an active enzyme (72 h, 0.1% glucose as determined previously (10), confirmed by both western blot and *in vitro* activities).

To detect the integration, we implemented the strategy shown in Figure 1. A DNA fragment containing the two HIV-1 U3 and U5 LTRs ends flanking the zeocin-resistance

encoding gene under the dependence of the yeast constitutive TEF1 promoter was introduced by electroporation into the cells after IN expression. Yeast cells were grown further for 1–5 h without selection to allow: (i) the interaction between IN and its substrate, (ii) nuclear import of both DNA and IN and (iii) integration of the DNA fragment. Approximately 10⁹ viable yeast cells were then plated on a solid medium containing 400 μ g/ml of zeocin and the zeocin-resistant clones were selected 5 days after plating. The first resistant clones were observed after 2 h of culture and the maximum number was attained after 4–5 h (Figure 2A). Zeocin-resistance analysis of the selected clones indicated a 100-fold increase compared to that of the initial yeast strain, thus confirming the acquisition of resistance.

Using yeast strains expressing the inactive mutant D116A IN or no IN only few clones were observed (Figure 2B and C), indicating that only a minor portion of the clones obtained with the cells expressing wild type IN (wt IN) was linked to an IN-independent process as the putative acquisition of mutational resistance.

Molecular analysis of the resistant clones

To confirm that the zeocin-resistant clones were representative of genuine integration events, several different molecular assays were performed. The expected 800 bp fragment was detected by PCR on total genomic DNA of the clones using internal primers for all the resistant clones obtained from h.RAD52⁺ (wt IN), whereas this product was

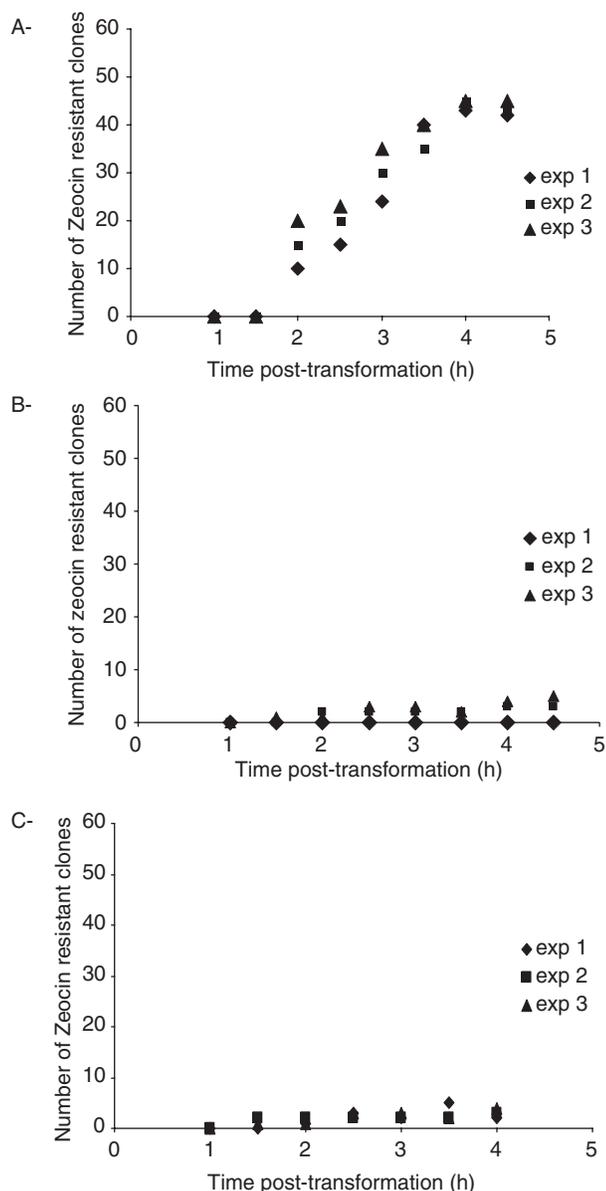


Figure 2. Selection of zeocin-resistant clones from yeast cells expressing wt IN (A), D116A IN (B) or no IN (C). Yeast cells (109) were grown from 0 to 5 h after transformation with the DNA integration substrate containing the two LTRs under non-selective conditions. Cells were plated on solid medium containing zeocin and after five days the zeocin-resistant clones were recovered. The results of three independent experiments are reported.

not detected with the total DNA from h.RAD52⁺ (D116A IN) clones or with the control DNA from non IN-expressing yeast (Figure 3A).

Southern blot analysis using 5'-end (³²P) radiolabeled U3-Zeo as probe revealed a band for h.RAD52⁺(wt IN) clones but not for h.RAD52⁺ expressing D116A IN or no IN (Figure 3B). The negative result obtained with h.RAD52⁺ (D116A) clones confirmed that the resistance observed in that case was not due to integration of the substrate into the genome but rather to another cellular mechanism.

To identify the integration loci and analyze the fidelity of integration events, total genomic DNA of 20 previously

selected clones were sequenced as described in the Materials and Methods section. For all of these clones, the integrated DNA was found disrupting a distinct open reading frame (ORF) (Figure 3C shows an e.g. of the genetic structure of the integrated fragments of the three clones analyzed in Figure 3A and B).

Since the sequence of the junctions between integrated LTRs and the target DNA constitutes a specific signature of the IN involved in this process, they were carefully analyzed. The 5 bp repeats characterizing HIV integration were recovered for nine clones, confirming that HIV-1 IN was responsible for the process (Figure 3D). In the remaining clones no repeat was found, whereas a deletion of the ORF sequence was observed (from 5 to 10 bp) and correlated well with the *in vitro* concerted integration data (Figure 3D). Likewise, nearly 50% of the *in vitro* integration products present under our conditions contained the specific 5 bp duplications.

IN can thus catalyze, viral DNA integration in yeast despite the lack of other viral factors thereby providing a way to study several parameters of the isolated retroviral integration step, such as the influence of viral DNA ends structure and the involvement of cellular machinery.

Influence of viral LTR structure on integration in yeast

Recently it has been shown that the processing of viral DNA ends by IN could channel their concerted integration (19). To determine the role of the viral ends structure and their integration in a cellular context, we followed IN activity using different DNA substrates. A DNA substrate that lacked LTR sequences was first used, in which case we recovered a number of resistant clones identical to the background (Figure 4) as observed in yeast cells expressing the inactive D116A IN (Figure 2B). PCR and Southern blot analysis of the corresponding genomic DNA also gave negative results indicating that, in contrast with the results obtained with the substrate containing the two HIV-1 LTRs, no IN-dependent integration was detected in the absence of viral sequences.

Since the DNA substrate used in our yeast integration assay carried the two non-processed LTR ends, it can be assumed from our results that HIV-1 IN could catalyze both 3'-processing and strand transfer in yeast cells. We thus sought to determine whether integration in yeast could be affected by pre-processing the DNA substrate as in *in vitro* assays. When the processed DNA was used in our system, a significantly higher number of resistant clones were obtained in comparison with the blunt-ended DNA (Figure 4). In addition, the maximum number of selected clones was attained earlier (~3 h), suggesting that the global rate of integration was improved. This result suggests that processing is a limiting step of the integration and can be dissociated in time from strand transfer IN activity.

We also conclude that (i) IN is the minimal retroviral protein needed for DNA integration with two LTRs into eukaryotic cellular DNA and (ii) some or all of the counterparts involved in HIV-1 integration in human infected cells are also present in yeast. These findings led us to further study the cellular mechanisms involved with the retroviral integration step.

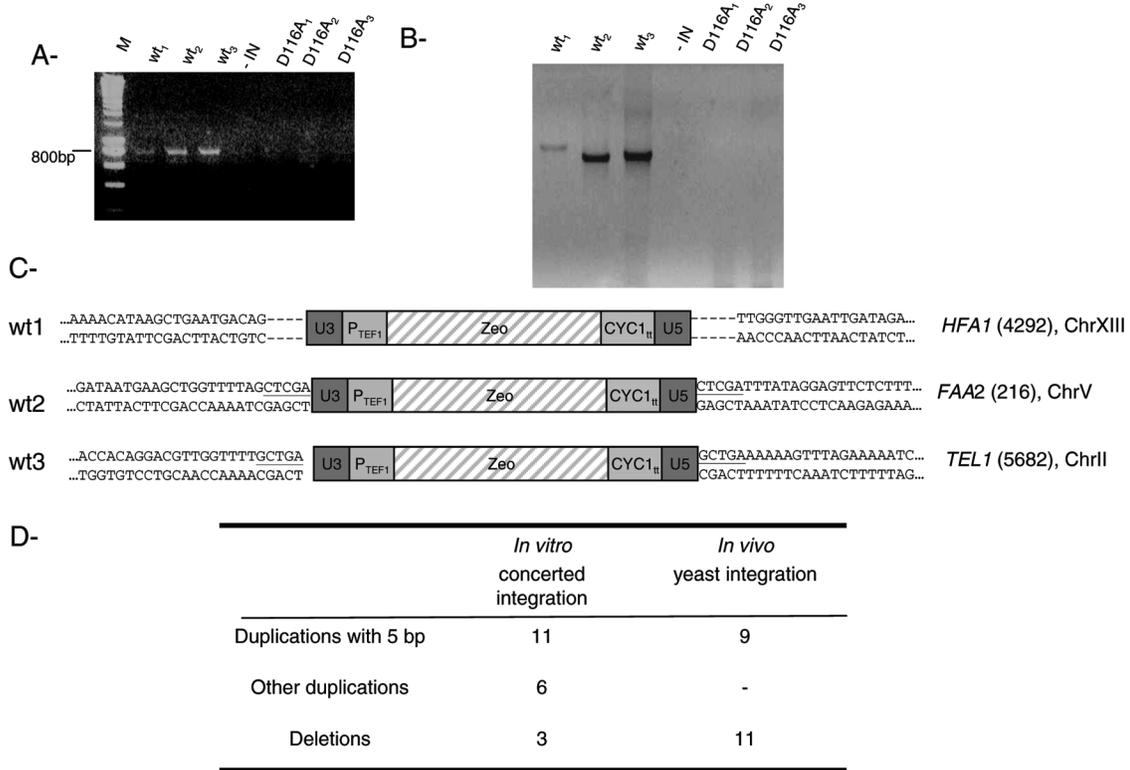


Figure 3. PCR (A), Southern blot (B) and sequence analysis (C) of the selected zeocin-resistant clones. (A). Aliquots from three different clones obtained from cells that expressed wt IN (wt₁₋₃), D116A IN (D116A₁₋₃), or no IN (-IN) were subjected to total genomic DNA extraction and PCR using 5'-U3-Zeo and 3'-U5-Zeo primers (B). Total genomic DNA was digested by BamHI restriction enzyme and submitted to Southern blot analysis with (5'-³²P) 5'-U3-Zeo as a probe. M: DNA size marker (bp). (C) Total genomic DNA was cut by BamHI restriction enzyme, ligated, amplified using 5'-U3-junction and 3'-U5-junction primers and sequenced using the same primers. The name of the disrupted ORF is mentioned as well as the integration site inside the gene. The 5 bp repeats are underlined, dotted lines indicate base deletions at the integration site. (D) The sequences of the integrated products obtained either in an *in vitro* concerted integration assay [data obtained from Ref. (17)] or from the *in vivo* yeast integration system were compared. The number of duplications and deletions in both systems are reported. 'Other duplications' indicate the number of cases that exhibited duplications comprising sizes from 3 to 6 bp.

Viral DNA integration in yeast is down regulated by RAD51

The last step of proviral integration is the repair of the DNA gaps flanking the integrated product. Several viral or cellular proteins have been proposed to be involved in this mechanism. New data reported here suggest that, in addition to cellular factors, no viral proteins other than IN play a major role in this repair activity.

Cellular repair systems have been previously proposed to be important for retroviral infection, such as the proteins belonging to the RAD52 epistasis group (20), but no direct evidence of their role in the integration step have been shown. Some of the proteins belonging to the RAD repair system, as RAD18 or RAD51, have been proposed to function either in the retroviral integration step in human cells (RAD18), or in the integration of retrovirus-like elements in yeast (RAD51) (21,22). In contrast to the case in human cells, the deletion of RAD51 and RAD18 in yeast does not lead to deleterious effects. We thus took into advantage the possibility of using our yeast system to study the role of those factors specifically on the isolated integration step by using the corresponding DNA repair-deficient mutant yeast strains.

The previously described lethal phenotype observed in a *rad52*⁻ deficient yeast strain (10, 11) further complicated the analysis of the role of RAD52 in the cellular integration

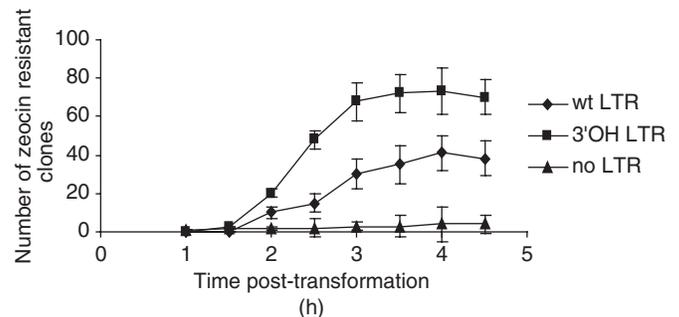


Figure 4. Effect of LTRs on integration catalyzed by IN in yeast. Experiments similar to those described in Figure 3 were performed using either the DNA substrate containing the complete LTR sequences (wt LTR), the 3'-processed LTRs (3'-OH LTR) or no LTR.

mechanism but, as an alternative, we performed the integration assay in haploid yeast strains deficient for either yeast RAD18 or RAD51 encoding genes (respectively *h.rad51*⁻ and *h.rad18*⁻). No difference was observed in the number of zeocin-resistant clones obtained with *h.RAD52*⁺ or *h.rad18*⁻ cells but, in contrast, a significantly higher number of zeocin-resistant clones were obtained in *h.rad51*⁻ in an extremely reproducible way (*P*-value = 0.03 from five independent experiments, Figure 5). In addition, no such increase was observed in the yeast cells expressing either the D116A

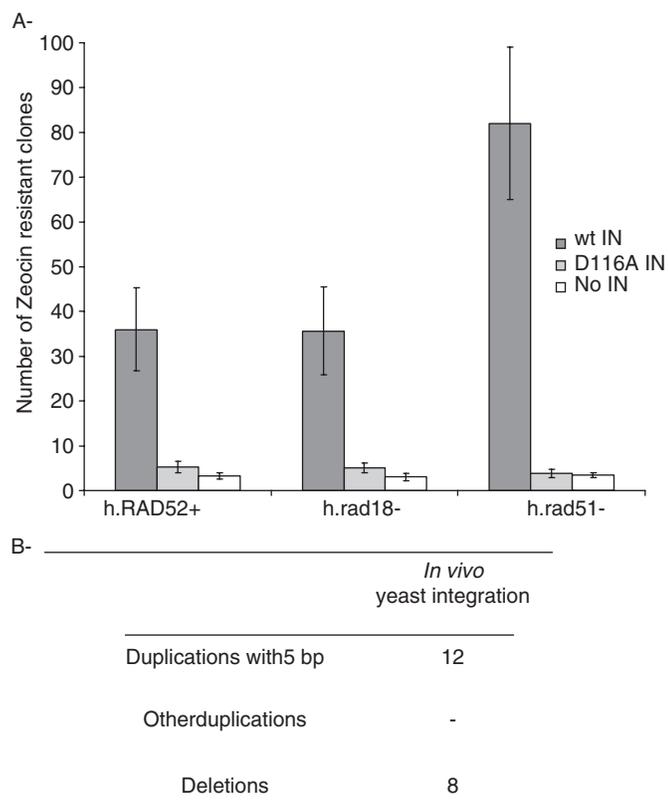


Figure 5. Effect of recombination factors on yeast integration catalyzed by IN. The integration assay described in Figure 2 was performed using the h.RAD52⁺ yeast strain (h.RAD52⁺), or yeast cells disrupted for RAD18 (h.rad18⁻) and RAD51 (h.rad51⁻) (A). The number of clones recovered 5 h post-transformation is reported. Values are the mean \pm SD (error bars) of five independent experiments. Twenty clones selected in h.rad51⁻ yeast strain were analyzed for the structure of their integration loci (B). The number of duplications and deletions are reported.

mutant or no IN, indicating that the increase of the integration events observed in the presence of IN was probably not due to a global stimulation of DNA mobility.

PCR and Southern blot analysis of the genomic DNA of the clones confirmed that the higher number of clones obtained with h.rad51⁻ was due to an increase in integration events catalyzed by IN (data not shown). Sequencing of several selected clones in the rad51 deleted strain revealed a similar ratio of loci presenting the 5 bp duplication compared to the results obtained with the wild type yeast strain (see Figures 5B and 3D). This indicates that the absence of RAD51 increased the number of integration events catalyzed by IN without significantly affecting the quality of the integration reaction products. Taken together, these results suggest a possible negative regulation of integration by the RAD51 DNA repair system in yeast. To determine whether this effect was due to a direct inhibition of IN activity by RAD51 we further analyzed the effect of the human RAD51 on *in vitro* integration activity catalyzed by IN.

Human Rad51 interacts with IN and inhibits its *in vitro* concerted integration activity

Physical hRAD51-IN interaction was first checked by ELISA experiments using highly purified enzymes. As reported

in Figure 6A IN was found to bind hRAD51 with notable efficiency (close to the auto-association level of the enzyme). The interaction was also specific since no binding of IN was observed with non coated wells or with the bacterial class 1 integrase recombinase. Nevertheless, as HIV-1 IN is known for its low solubility, the fixed format of ELISA could lead to biased results. In addition, we performed *in vitro* pull down assays using hRAD51 protein which had been previously coupled to Affi-gel beads. As shown in Figure 6B HIV-1 IN was found to associate with Affi-hRAD51 beads specifically, since no co-elution was observed in presence of Affi-BSA beads. It is noteworthy that the Affi-hRAD51 does not bind all the free IN protein. This could possibly be due to the fact that Affi-beads contain RAD51 ring structures in which not all the surfaces are accessible for additional interactions.

Our results indicate that IN and hRAD51 interact *in vitro*. We wondered whether this interaction could be responsible of the IN activity inhibition observed in yeast cells. For this purpose we analyzed the effects of hRAD51 on *in vitro* IN integration activity. Increasing amounts of hRAD51 were added in a standard concerted integration assay. Under our conditions hRAD51 caused a strong inhibition of the IN activity (Figure 7A). Quantification of the inhibitory effect of hRAD51 allowed us to determine an IC₅₀ of 0.5 μ M (Figure 7B). hRAD51 is known to interact with DNA in presence of ATP (18). Since some ATP may still be present in the integration labeling reaction it remains to determine the part of inhibition due to the DNA binding property of RAD51 and/or to its the direct interaction with IN.

DISCUSSION

It has recently been shown that IN exhibits an ability to complete a concerted integration reaction *in vitro* similar to that of purified PICs from virus-infected cells (8) suggesting that IN is the minimal requirement to accomplish the whole nuclear integration process in the cell.

A previous study reported the *in vivo* activity of avian IN in eukaryotic cells, indicating that the enzyme was able to enhance the integration of a transgene by specific retroviral integration (23). However, all previous attempts to evaluate the *in vivo* activity of HIV-1 IN expressed as the sole viral protein in eukaryotic cells were unsuccessful in detecting specific integration (9). In order to determine whether HIV-1 IN alone is able to catalyze viral DNA integration, we chose a simpler model to set up a eukaryotic integration system based on the use of yeast cells.

Given the strong conservation of genomics and functional processes between *Saccharomyces cerevisiae* and human cells, this yeast has been used to study several steps of the retroviral life (24–28). In our laboratory, yeast has been also used as a tool and a model in HIV-1 integration studies to search for the eukaryotic partners of IN (10,11,15,29–32).

Integration of a two LTR retroviral-like DNA in yeast

Using DNA containing two HIV-1 LTRs and yeast cells expressing wt HIV-1 IN, we observed that IN alone could integrate the proviral DNA-like substrate into the nuclear genome. Several controls such as the use of an inactive

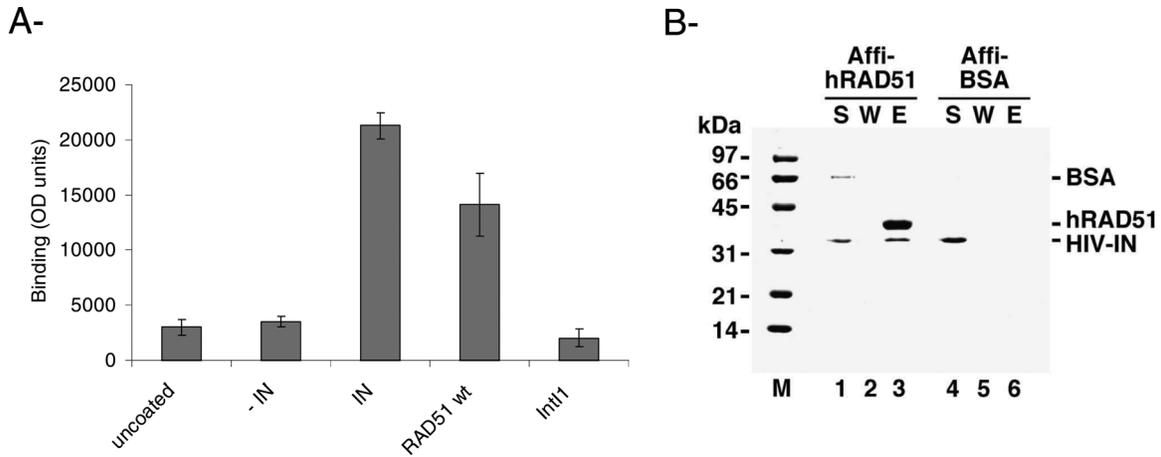


Figure 6. *In vitro* interaction between human RAD51 and HIV-1 IN (A) ELISA assay. Experiments were performed with a plate either uncoated or coated with the reported protein (30 pmol) and revealed with anti-IN antibodies after IN loading (30 pmol of proteins). The signal observed with anti-IN serum on RAD51 coated plate was also reported (-IN) (B) Pull down assay. HIV-IN was subject to affinity pull-down with Affi-beads conjugated to hRAD51 (Affi-RAD51; lanes 1-3) or BSA (Affi-BSA; lanes 4-6). The supernatant (S), wash (W) and SDS eluate (E) from the above reactions were analyzed by SDS-PAGE and Coomassie blue staining.

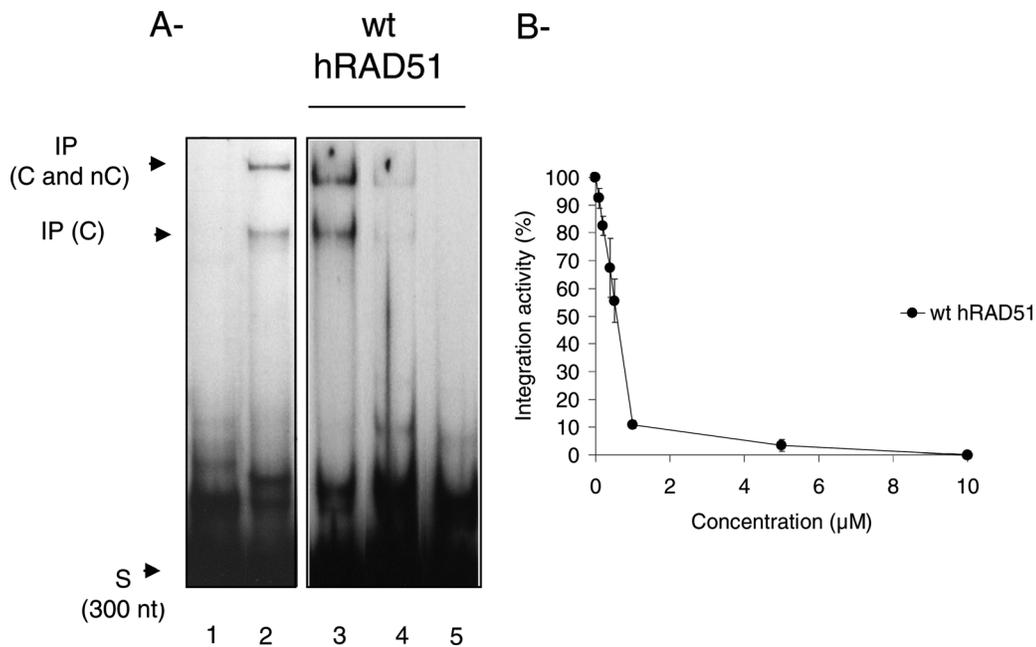


Figure 7. *In vitro* effect of human RAD51 proteins on integration catalyzed by IN (A) Concerted integration assays performed with 250 nM of IN, 100 ng of receptor plasmid and 10 ng of donor DNA in absence (lane 2) or in presence of 0.1, 1 or 10 μM of purified human wild type RAD51 (lanes 3, 4 and 5). Lane 1: substrate alone; S: substrate, IP: integration product, C: concerted integration product, nC: non-concerted integration product. (B) Quantification of the integration product obtained in presence of increasing amounts of wild type RAD51 proteins. Values are the mean ± SD (error bars) of three independent experiments.

mutated D116A IN or substrates lacking the LTRs, in addition to PCR and Southern blot analysis, strongly supported integration of the DNA substrate into the yeast nuclear genome catalyzed by HIV-1 IN. Furthermore, the sequencing of the chromosomal integration locus from the selected clones displayed the HIV-1 specific 5 bp repeats at the junction between LTRs and host DNA, demonstrating that IN catalyzes integration with the same specificity in both yeast and human infected cells. However, deletions were also

observed suggesting that defective integration can also occur in yeast. We previously demonstrated *in vitro* that concerted integration was ascribed to the oligomeric state of the enzyme since the tetrameric form of IN specifically catalyzed the HIV-1 specific integration leading to 5 bp duplication (17). This suggests that the defective integration observed in yeast cells might be due to non-tetrameric or poorly folded tetramers of IN. In addition, defective integration could be due to the absence or low levels of specific

factors in yeast cells that are normally present in infected cells. In mammalian cells, PIC could act by preventing those deleterious events and/or optimizing the basal IN activity in order to allow highly effective and specific integration. Interestingly, no yeast orthologs of human LEDGF/p75, a recently described IN cofactor, have been found. Since LEDGF/p75 has been described to stabilize tetramers of IN (33), the lack of this factor in yeast could lead to a low proportion of tetrameric IN and thus to the partial defective integration observed.

Taken together our data suggests that in the absence of other HIV-1 viral factors, IN carries out all the reactions required for proviral integration in a eukaryotic cell, including LTR interaction, nuclear migration (after or before IN-DNA complex formation), chromosomal targeting, LTR 3'-processing and integration.

The stimulation of the integration rate observed when a pre-processed substrate was used (Figure 4) suggests that 3'-processing is a limiting step of integration in our system. This result indicates that IN recognize a pre-processed substrate before the integration step. Thus, the intracellular processing and integration steps can be chronologically distinguished.

Involvement of DNA repair systems in the retroviral integration step?

As previously described, in addition to viral factors other cellular proteins (InI1, LEDGF/P75, BAF...) are probably involved in the integration process (4, 6, 33–36). These factors may or may not be associated with the PIC and could be involved in several steps of the integration process such as nuclear entry and host DNA targeting. However, one step of the retroviral integration process that still remains obscure is the repair of the gaps generated during the insertion of viral DNA into the host cell chromosomes. Full integration of a viral DNA in the presence of IN as the sole viral protein reported here indicates that the cellular DNA repair step is essentially performed by the nuclear machinery, as suggested by others (37, 38), without ruling out the possible involvement of others viral proteins in the PIC context favoring this crucial mechanism. Previously, it has been proposed that the cellular DNA repair systems play a role in this mechanism (39–41). Recently it has been shown that HR systems of DNA repair, such as RAD52, can modulate retroviral infection probably by acting at the integration level (20). To determine the role of HR systems in the yeast integration model, we used mutants deficient in proteins of the RAD52 epistasis group. Lethality induced by HIV-1 IN expression in *rad52*⁻ deficient cells strongly suggests, as reported before (11), that the double strand breaks taking place during defective or extensive integration events could be repaired early by this factor explaining why increased retroviral integration is the first major phenotype described for a RAD52 deficiency in mammalian cells (20). Other proteins of the HR pathway have been previously proposed to play a role in the infection. We thus studied RAD51, another factor belonging to the same epistasis group as RAD52, and also potentially involved in integration. In addition we analyzed the potential involvement of RAD18 in integration step as previously proposed (21).

The RAD51 protein down-regulates the integration catalyzed by HIV-1 IN both *in vitro* and in yeast expressing the retroviral enzyme

The lethality induced by the inhibition of RAD51 in mammalian cells complicated previous studies of the potential role of this factor in retroviral integration (20). In contrast, RAD51 and RAD18 can be deleted in yeast without affecting the life of the cell allowing us to specifically study their involvement in the cellular activity of IN. For this purpose, we used two yeast strains deficient for both RAD18 and RAD51. While no effect on integration was observed when the RAD18 gene was deleted, RAD51 deletion induced a significant increase in integration by IN (Figure 5). This result is consistent with data reported by Lau *et al.* (20) indicating that deletion of proteins of the repair system close to RAD52 led to an increase in cellular transduction by HIV-1 and suppression of viral infection.

The *in vitro* inhibition of concerted integration catalyzed by recombinant IN by the human RAD51, in addition to the *in vitro* binding of this factor with IN, strongly suggests a negative regulation of retroviral integration by RAD51 probably via a direct allosteric effect. hRAD51 could act by a dual mechanism involving (i) a direct effect on IN protein and (ii) a competitive effect with IN for its substrates by DNA binding on cellular or viral integration intermediates. The detailed biochemical and molecular mechanism involved in this inhibitory effect remains to be studied.

The differential effect observed with RAD52, RAD51 and RAD18 repair proteins suggests that each one could play a different function in proviral integration. The increased integration observed in mammalian cells deficient for RAD52 indicates that this factor modulates the number of integration events probably by repairing the DNA cuts early before completion of the integration and/or as proposed before by capping the retroviral DNA ends (20). According to our results, RAD51 also performs a negative control of the integration but the mechanism leading to the inhibition is different than for RAD52 and may involve a direct interaction of IN with RAD51. According to this model, retroviral integration may be considered as a mutagenic event by the cell whose answer is to modulate the viral mechanism by acting on two integration partners: IN and DNA in a competitive 'struggle for life'. The important link between retroviral integration and cellular DNA repair processes deserves further studies in order to establish the biological implication of the IN-RAD51 interaction in HIV-1 infected cells.

In addition to highlighting several interactions between HIV-1 integration and cellular mechanisms, our data underscore the potential use of the yeast *S.cerevisiae* as a tool of choice for studying the retroviral integration isolated from other steps of the infection. The demonstration of IN activity in yeast indicates that the viral protein may interact with conserved orthologous factors in human HIV-1 infected cells as previously described (15, 32). A further advantage of the yeast model with its highly characterized and accessible genetics is that it is now possible to dissect the retroviral cycle by focusing on a unique viral protein, the HIV-1 IN. Studies of the integration loci reported in our work also resulted in the possibility to use the yeast system for extended

analysis of the host DNA targeting by IN and the role of additional cellular factors in this mechanism.

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