The EMBO Journal Vol.16 No.16 pp.5123–5138, 1997

Complementation of integrase function in HIV-1 virions

Thomas M.Fletcher III[1], Marcelo A.Soares[1], Silvia McPhearson[2], Huxiong Hui[1], MaryAnn Wiskerchen[3], Mark A.Muesing[4], George M.Shaw[1,2,5], Andrew D.Leavitt[6], Jef D.Boeke[7] and Beatrice H.Hahn[1,2,8]

[1]Department of Medicine, [2]Department of Microbiology and [5]Howard Hughes Medical Institute, University of Alabama at Birmingham, 701 S.19th Street, LHRB 613, Birmingham, AL 35294, [3]Promega, Inc., Madison, WI 53711, [4]Aaron Diamond AIDS Research Center, New York, NY 10016, [6]Departments of Laboratory Medicine and Internal Medicine, University of California at San Francisco, San Francisco, CA 94143-0100 and [7]Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

[8]Corresponding author
e-mail: bhahn@shaw-hahn.dom.uab.edu

Proviral integration is essential for HIV-1 replication and represents an important potential target for antiviral drug design. Although much is known about the integration process from studies of purified integrase (IN) protein and synthetic target DNA, provirus formation in virally infected cells remains incompletely understood since reconstituted *in vitro* assays do not fully reproduce *in vivo* integration events. We have developed a novel experimental system in which IN-mutant HIV-1 molecular clones are complemented *in trans* by Vpr–IN fusion proteins, thereby enabling the study of IN function in replicating viruses. Using this approach we found that (i) Vpr-linked IN is efficiently packaged into virions independent of the Gag–Pol polyprotein, (ii) fusion proteins containing a natural RT/IN processing site are cleaved by the viral protease and (iii) only the cleaved IN protein complements IN-defective HIV-1 efficiently. Vpr-mediated packaging restored IN function to a wide variety of IN-deficient HIV-1 strains including zinc finger, catalytic core and C-terminal domain mutants as well as viruses from which IN was completely deleted. Furthermore, *trans* complemented IN protein mediated a bona fide integration reaction, as demonstrated by the precise processing of proviral ends (5′-TG...CA-3′) and the generation of an HIV-1-specific (5 bp) duplication of adjoining host sequences. Intragenic complementation between IN mutants defective in different protein domains was also observed, thereby providing the first evidence for IN multimerization *in vivo*.

Keywords: functional IN subdomains/HIV-1 integrase/IN multimerization/*trans* complementation/Vpr

Introduction

Efficient replication of retroviruses (including HIV-1) requires the insertion of a DNA copy of the viral genome into the chromosome of infected host cells (Goff, 1992; Kulkosky and Skalka, 1994; Farnet and Bushman, 1996). This integration process generates the proviral template for subsequent viral gene expression and is mediated by the viral integrase (IN) protein. IN is one of three enzymes encoded by the viral *pol* gene, and is expressed and incorporated into virions as part of a Gag–Pol polyprotein. After budding and release of virus particles from infected cells, this polyprotein is cleaved by the viral protease into individual components, a process essential for virus replication. Upon infection of new target cells, IN remains associated with a large nucleoprotein (preintegration) complex which contains the newly synthesized viral DNA as well as Gag and Pol proteins (Bowerman *et al.*, 1989) and, in the case of HIV/SIV, the accessory proteins Vpr and Vpx (Heinzinger *et al.*, 1994; Fletcher *et al.*, 1996) as well as at least one cellular protein (Farnet and Bushman, 1997). The preintegration complex migrates to the nucleus where proviral integration takes place.

Most of the mechanistic details of the integration reaction are derived from *in vitro* studies of purified IN protein acting on short oligonucleotides that mimic viral ends and also serve as target DNA (Bushman and Craigie, 1991; Engelman *et al.*, 1991; Kulkosky and Skalka, 1994). From these and other analyses it has become apparent that integration proceeds as a two step process. In the cytoplasm, IN mediates an endonucleolytic reaction that generally removes two nucleotides from the 3′ ends of the newly synthesized (blunt ended) linear viral DNA in a 3′ processing reaction. After transport to the nucleus, the recessed 3′ ends of the viral DNA are joined to host chromosomal DNA in a concerted strand transfer reaction. The two ends of viral DNA join the target DNA in a staggered fashion, which results in the duplication of host cell sequences immediately flanking the inserted provirus. The length of this duplication is virus-specific and, in the case of HIV-1, comprises a 5 bp direct repeat (Muesing *et al.*, 1985; Bushman *et al.*, 1990; Vink *et al.*, 1990). Purified IN protein can also catalyze the reverse strand transfer reaction, termed ‘disintegration’, when supplied with a synthetic gapped intermediate substrate (Chow *et al.*, 1992).

Phylogenetic comparisons, mutational analyses, partial protease cleavage and structural studies have all shown that the HIV-1 IN protein (like that of other retroviruses) consists of functionally distinct subdomains (see Kulkosky and Skalka, 1994; Plasterk, 1995; Farnet and Bushman, 1996). The N-terminal region (located between residues 1 and 50) contains a highly conserved ‘HHCC’ motif, which resembles the zinc finger domains of some transcription factors (Burke *et al.*, 1992; McEuen *et al.*, 1992; Bushman *et al.*, 1993). The exact contribution of this zinc finger domain to IN catalytic activity remains unclear, because mutational analyses have produced varying

results, ranging from minor effects to complete abrogation of function (Drehlich *et al.*, 1992; Engelman and Craigie, 1992; Schauer and Billich, 1992; Bushman *et al.*, 1993; Leavitt *et al.*, 1993; Vincent *et al.*, 1993; Vink *et al.*, 1993). Nevertheless, there is evidence suggesting that the HHCC domain plays a role in the formation of stable complexes between integrase and viral DNA (Hazuda *et al.*, 1994; Ellison and Brown, 1994; Ellison *et al.*, 1995). The central region (located between residues 50 and 212) contains a triad of three invariant acidic residues (Asp64, Asp116 and Glu152), commonly called the D,D-35-E domain, which are evolutionarily highly conserved among retroviral IN proteins as well as various eukaryotic and prokaryotic transposases (Kulkosky *et al.*, 1992; Doak *et al.*, 1994; Rice and Mizuuchi, 1995). Replacement of any of these acidic residues results in the loss of all enzymatic activities including the disintegration reaction, indicating that this domain constitutes the catalytic core of the enzyme (Engelman and Craigie, 1992; van Gent *et al.*, 1992; Leavitt *et al.*, 1993). Finally, a less conserved C-terminal domain is also required for 3′ processing and forward reactions; although its boundaries are not clearly defined, most investigators place it between residues 212 and 288. This domain contains extensive positively charged surfaces and is believed to have non-specific DNA binding activity (Vink *et al.*, 1993; Woerner and Marcus-Sekura, 1993; Engelman *et al.*, 1994). The precise function of the C-terminus of IN remains unknown.

Functional subdomains of integrase have also been defined by *in vitro* complementation studies (Engelman *et al.*, 1993; van Gent *et al.*, 1993) which demonstrated that certain combinations of enzymatically inactive IN mutants efficiently catalyze 3′ processing and strand transfer reactions when assayed as mixed multimers. For example, zinc finger and catalytic domain mutants complemented each other *in trans*, i.e. they could be supplied on two different monomers, while the C-terminal region of integrase could function both *in trans* and *in cis* relative to the catalytic core. By contrast, no complementation was observed between proteins with mutations in the same functional domain (e.g. different active site mutations). IN can thus form functional multimers *in vitro* and domains critical for integration can be supplied by different subunits in an oligomeric complex (Engelman *et al.*, 1993; van Gent *et al.*, 1993). Based on these *in vitro* experiments, it has been proposed that provirus formation *in vivo* (i.e. in virally infected cells) is also mediated by active IN multimers (Plasterk, 1995). However, direct evidence for this is lacking, since *in vitro* integration assays generally examine only 'half reactions', i.e. insertion of a single viral DNA end into a single strand of target DNA, and thus do not fully reproduce the integration events that occur *in vivo*.

To further define the functions of integrase during *in vivo* integration, several groups of investigators have begun to analyze IN mutants in the context of infectious molecular clones of HIV-1. Interestingly, these studies have provided evidence for additional roles of IN in HIV-1 replication (Engelman *et al.*, 1995; Masuda *et al.*, 1995; Wiskerchen and Muesing, 1995; Cannon *et al.*, 1996; Leavitt *et al.*, 1996; Taddeo *et al.*, 1996). For example, mutations in the C-terminal domain of integrase, which have little to no effect on *in vitro* IN enzymatic activity (Leavitt *et al.*, 1993), abrogate proviral integration when introduced into an infectious molecular clone (Cannon *et al.*, 1996; Leavitt *et al.*, 1996). Careful analysis of particle morphology, protein composition, viral DNA synthesis and nuclear import revealed no differences between the C-terminal integrase mutants and wild-type HIV-1, suggesting an altered interaction of integrase with the target cell DNA (Cannon *et al.*, 1996; Leavitt *et al.*, 1996). Another interesting phenotype resulted from analyses of viral constructs with substitutions in the conserved His or Cys residues of the N-terminal HHCC domain (Masuda *et al.*, 1995; Leavitt *et al.*, 1996). Upon infection of new cells, these HHCC mutants were severely impaired in their ability to synthesize viral DNA, although they contained a fully functional reverse transcriptase enzyme and wild-type levels of packaged viral RNA (Masuda *et al.*, 1995; Leavitt *et al.*, 1996). These data thus indicated an effect of integrase on reverse transcription, possibly through alteration of the preintegration complex.

Given the complexity of IN activities *in vivo*, we wished to develop a *trans*-complementation system that would allow us to probe integrase function in the context of replicating virions. To mediate virion incorporation in the absence of genomic expression, we fused IN to Vpr, an HIV-1 accessory protein which is present in virions in equimolar quantities to the viral Gag proteins (Lu *et al.*, 1993; Paxton *et al.*, 1993), represents a known component of the viral preintegration complex (Heinzinger *et al.*, 1994) and has previously been shown to have the capacity to target heterologous fusion proteins to the HIV-1 particle (Fletcher *et al.*, 1995; Wu *et al.*, 1995). Coexpressing Vpr–IN fusion constructs with IN-mutant HIV-1 molecular clones, we found that IN can be efficiently packaged by this novel route and that *trans* complemented IN protein can restore provirus formation to IN-defective virions. We also found that proteolytic cleavage of IN from its Vpr fusion partner is required for efficient complementation. Finally, we demonstrated that intragenic complementation between IN mutants defective in different protein domains is possible, thus providing the first evidence for IN multimerization *in vivo*.

Results

Integrase is efficiently packaged into HIV-1 virions as a Vpr fusion protein

We have previously shown that virion-associated accessory proteins of HIV (i.e. Vpr, Vpx and Vif) can be utilized to target foreign proteins to the HIV particle (Fletcher *et al.*, 1995; Wu *et al.*, 1995). To investigate whether this same strategy could be used to complement functionally impaired virion components, e.g. a defective IN protein, we prepared Vpr-integrase gene fusions and control constructs (Figure 1A, left panel) and tested their ability to express proteins with virion targeting capabilities. R–IN was generated by ligating the 3′ end of *vpr* in-frame to the 5′ end of integrase, while R–PC–IN was engineered to contain an additional 45 bp of *pol* sequences upstream of IN conserving the natural RT/IN protease cleavage site (PC). Control constructs contained either *vpr* alone (R) or *vpr* fused to PC sequences (R–PC). These gene fusions were cloned into an HIV-2 LTR/rev responsive element (RRE) regulated vector (pLR2P) known to mediate high

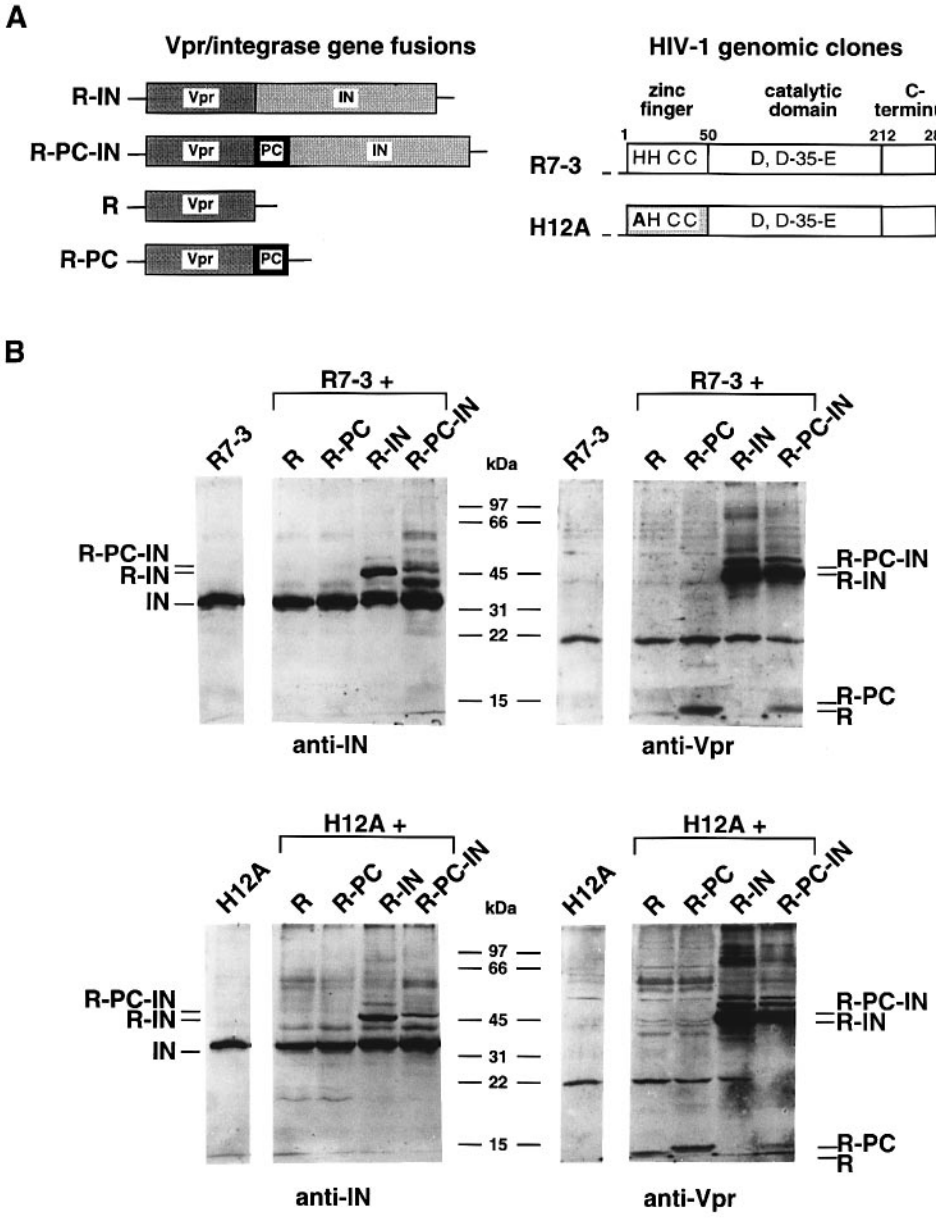

Fig. 1. Efficient packaging of Vpr–IN fusion proteins into HIV-1 virions. (**A**) Schematic representation of Vpr–integrase fusion constructs (R–IN; R–PC–IN), control constructs (R; R–PC), and HIV-1 molecular clones containing wild-type (R7-3) or mutant (H12A) integrase genes (IN domains and their boundaries are indicated). PC comprises 45 bp of *pol* sequence immediately upstream of the natural RT/IN cleavage site (also see Figure 3). R7-3 and H12A are isogenic, except for a single amino acid substitution in the zinc finger domain (highlighted). (**B**) Western blots of transfection-derived virion preparations (200 ng of p24 per lane) probed with anti-IN and anti-Vpr antibodies. R7-3 and H12A molecular clones were transfected alone (left lanes of each panel) or in combination with R, R–PC, R–IN and R–PC–IN constructs. Bands corresponding to the various fusion proteins as well as wild-type integrase (IN) are indicated. An additional IN-reactive protein of 38 kDa in R–PC–IN complemented virions likely represents a cleavage product processed at a non-natural site (non-specific processing is known to occur in the context of Vpr fusion proteins; Wu *et al.*, 1995). The same protein is also apparent in R–PC–IN containing virion preparations shown in Figure 3B (middle panel) and Figure 5A (right upper panel). Neither the R7-3 nor the H12A molecular clones encode a functional Vpr protein (the 22 kDa band present in all virion preparations probed with the anti-Vpr antiserum is an a non-specific reaction product).

level expression (Wu *et al.*, 1995) and cotransfected with HIV-1 molecular clones containing either wild-type (R7-3) or mutant (H12A) integrase coding regions (Figure 1A, right panel).

To assess packaging of the Vpr fusion proteins, transfection-derived virions were pelleted through 20% sucrose and their protein profiles were examined by Western blot analysis. As shown in Figure 1B, R–IN and R–PC–IN fusion proteins were readily detectable in R7-3 as well as H12A derived virions. As expected, R–PC–IN was slightly larger than R–IN. However, the intensity of the R–PC–IN band was diminished relative to that of R–IN, while the intensity of the corresponding wild-type integrase band (IN) was increased, suggesting partial cleavage by the viral protease. This was confirmed by blots probed with anti-Vpr antibodies which showed that only virions containing the R–PC–IN (but not the R–IN) fusion protein exhibited a 13 kDa Vpr-reactive protein. Since both R7-3 and H12A encode a prematurely truncated (and thus unstable) Vpr protein, which is undetectable on immuno-

blots (Wiskerchen and Muesing, 1995), this small Vpr-reactive protein most likely represents a protease cleavage product. Moreover, comigration with the R–PC translation product (which contains 15 amino acids of PC sequence in addition to Vpr), rather than the slightly smaller R translation product (which resembles the native Vpr protein), suggests that protease processing had occurred at the intended (i.e. natural) site. These results indicate that Vpr–integrase fusion proteins are efficiently packaged into HIV-1 virions and accessible to processing by the viral protease at the RT/IN cleavage site.

Vpr-mediated packaging of integrase restores the biological activity of a zinc finger mutant HIV-1 molecular clone

To investigate whether Vpr-mediated packaging supplied a functional integrase protein, we cotransfected an IN-defective HIV-1 molecular clone (H12A) with R–PC–IN and R–IN, and tested the resulting virions for biological activity in the MAGI cell assay (wild-type R7-3 HIV-1 was analyzed in parallel for control). MAGI (HeLa-CD4-LTR-β-gal) cells contain a β-galactosidase gene (β-gal) stably integrated under the control of an HIV-1 LTR (Kimpton and Emerman, 1992). Since the β-gal gene also encodes a nuclear localization signal (NLS), infection with wild-type HIV-1 results in the formation of blue nuclei. Viruses with defective integrase genes, including H12A, score negative in this assay, because the induction of blue nuclei requires *tat* gene expression from an integrated provirus to activate the LTR-β-gal construct. The MAGI cell assay has thus been widely used to characterize the biological activity of IN-mutant molecular clones of HIV-1, except for catalytic triad mutants which are believed to express Tat from unintegrated viral DNA and generate blue nuclei even in the absence of viral integration (Engelman *et al.*, 1995; Wiskerchen and Muesing, 1995).

Transfection-derived virion preparations were normalized for p24 content and used to infect MAGI cells. As expected, wild-type HIV-1 (R7-3) yielded large numbers of blue nuclei when transfected alone (~1×10^4 per 10 ng of p24) or in combination with R–IN, R–PC–IN, R and R–PC constructs (0.3–0.5×10^4 per 10 ng of p24; Figure 2A depicts results for cotransfection with R–PC–IN). By contrast, virions derived from the H12A molecular clone produced no blue nuclei (Figure 2B), consistent with previous reports of a severe DNA synthesis defect associated with mutations of the HHCC domain (Engelman *et al.*, 1995; Masuda *et al.*, 1995; Wiskerchen and Muesing, 1995; Leavitt *et al.*, 1996). There were also no blue nuclei detectable in cultures infected with virions derived from H12A/R–IN cotransfections (Figure 2C), despite efficient packaging of the R–IN fusion protein (see Figure 1B). However, virions derived from H12A/R–PC–IN cotransfections yielded considerable numbers of blue nuclei (Figure 2D). Since equivalent amounts of virions (based on p24 content) were used for all MAGI cell infections, the biological activity of wild-type and complemented IN-mutant HIV-1 could be compared. Counting several different fields from two independent experiments, we estimated that R–PC–IN restored the IN defect of H12A-derived virions to ~20% of wild-type activity. Importantly, complementation efficiency appeared to depend on the amount of R–PC–IN fusion protein packaged into virions. As shown in Figure 2E, maximal numbers of blue nuclei were generated when H12A and R–PC–IN constructs were cotransfected in ratios (wt/wt) of 1:4–1:8 (a ratio of 1:5 was used for all subsequent experiments). These results thus indicate that the R–PC–IN fusion protein is functionally active and can restore IN function to a zinc finger mutant HIV-1 molecular clone.

Proteolytic cleavage of integrase is required for efficient in vivo complementation

The fact that R–IN-complemented H12A virions failed to produce blue nuclei in the MAGI cell assay indicated that virion incorporation of the fusion protein alone was not sufficient for restoration of integrase function. To investigate directly whether cleavage of the fusion protein was required, we mutated the RT/IN cleavage site in R–PC–IN (Figure 3A) by substituting a single nucleotide (CTA→ATA) in the codon immediately 5′ of the N-terminus of IN (P1 position of the cleavage site), thus generating R–PC^M–IN. Based on previous analyses of HIV-1 protease processing sites (Pettit *et al.*, 1991), we expected the resulting amino acid substitution (Leu to Ile) to abrogate, or at least greatly diminish, protease processing. Sequence analysis of the entire R–PC^M–IN construct confirmed the C to A substitution and excluded inadvertent PCR-induced mutations.

Figure 3B depicts the protein profiles of sucrose pelleted virions derived from cotransfections of H12A with R, R–PC, R–IN and R–PC–IN as well as the newly generated R–PC^M–IN construct. The results show that the introduced amino acid substitution indeed inhibited (or at least greatly reduced) protease cleavage of the R–PC^M–IN fusion protein. No Vpr-reactive cleavage product was detectable on blots probed with an anti-Vpr antibody (upper panel), and there was no decrease in the intensity of the full length R–PC^M–IN fusion protein relative to R–IN (middle panel). Analysis of the same virion preparations in the MAGI cell assay (Figure 3C) documented that cleavage was essential for *trans*-complementation. H12A cotransfected with R–PC–IN yielded the expected number of blue nuclei (see Figure 2). However, the same clone cotransfected with R–PC^M–IN produced virtually no blue nuclei (there were only one or two per plate). These data thus indicate that cleavage of IN from its Vpr fusion partner is required for complementation of an HIV-1 molecular clone defective in its HHCC domain.

Defects in all three functional domains of IN can be complemented by packaging integrase in trans

To examine whether R–PC–IN could complement a broader spectrum of integrase mutations and to prove that this complementation indeed resulted in bona fide provirus formation, we characterized additional HIV-1 molecular clones with point or deletion mutations in their IN coding region (Figure 4A). These included a catalytic core mutant (D116A), a combined central region/C-terminus mutant which lacked 68 amino acid residues between positions 181 and 249 and contained two amino acid substitutions (E85A/E87A) in the central domain (M2) and a mutant in which integrase expression was abrogated due to stop codons at the RT/IN junction (ΔIN). These clones were selected because they contained mutations in all three functional domains of integrase and were known to be

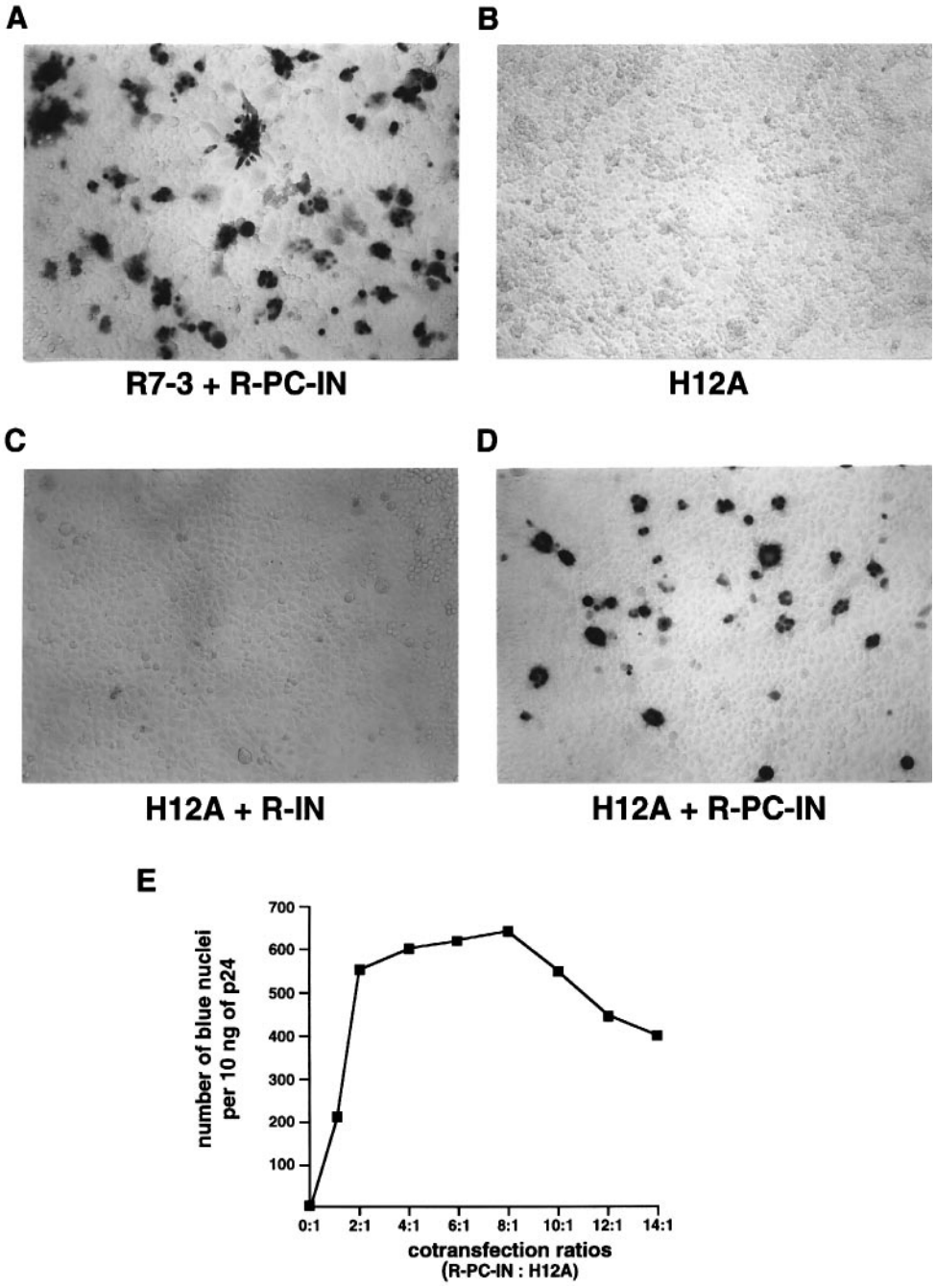

Fig. 2. Vpr-mediated packaging of integrase restores IN function to an IN-defective (H12A) HIV-1 molecular clone. (**A**)–(**D**) Analysis of fusion protein containing wild-type (R7-3) and IN-mutant (H12A) HIV-1 virions in the MAGI cell assay. Blue nuclei indicate single cell infections with viruses containing a functional IN protein (see text for details of the experiment). (**E**) Determination of optimal cotransfection ratios for efficient Vpr-mediated IN complementation. Maximal numbers of blue nuclei were generated when H12A and R–PC–IN were cotransfected in ratios (wt/wt) of 4:1 to 8:1.

integration defective (Wiskerchen and Muesing, 1995). They were also available as proviral constructs containing a selectable marker cassette (SV40 *gpt*) in place of their *env* coding region (Figure 4B), allowing characterization in a single round integration assay (Wiskerchen and Muesing, 1995).

Viral stocks were prepared by transfecting the various *gpt* constructs with and without R–PC–IN. All constructs were also pseudotyped with MuLV *env* to provide a functional envelope glycoprotein. Virus stocks were pelleted through a sucrose cushion, normalized for p24 content, and examined by Western blot analysis. This was done to ensure efficient packaging and cleavage of the R–PC–IN fusion protein, and to confirm the authenticity of the proviral *gpt* constructs used for transfection (Figure 5A). For example, ΔIN *gpt*-derived virions exhibited no reactivity with anti-IN antibodies, confirming the inability of the ΔIN construct to express integrase. After transfection of ΔIN *gpt* with R–PC–IN, however, wild-type IN (32 kDa) and R–PC (13 kDa) bands were apparent, indicating efficient packaging and cleavage of the fusion protein. Virus stocks were then used to infect susceptible target

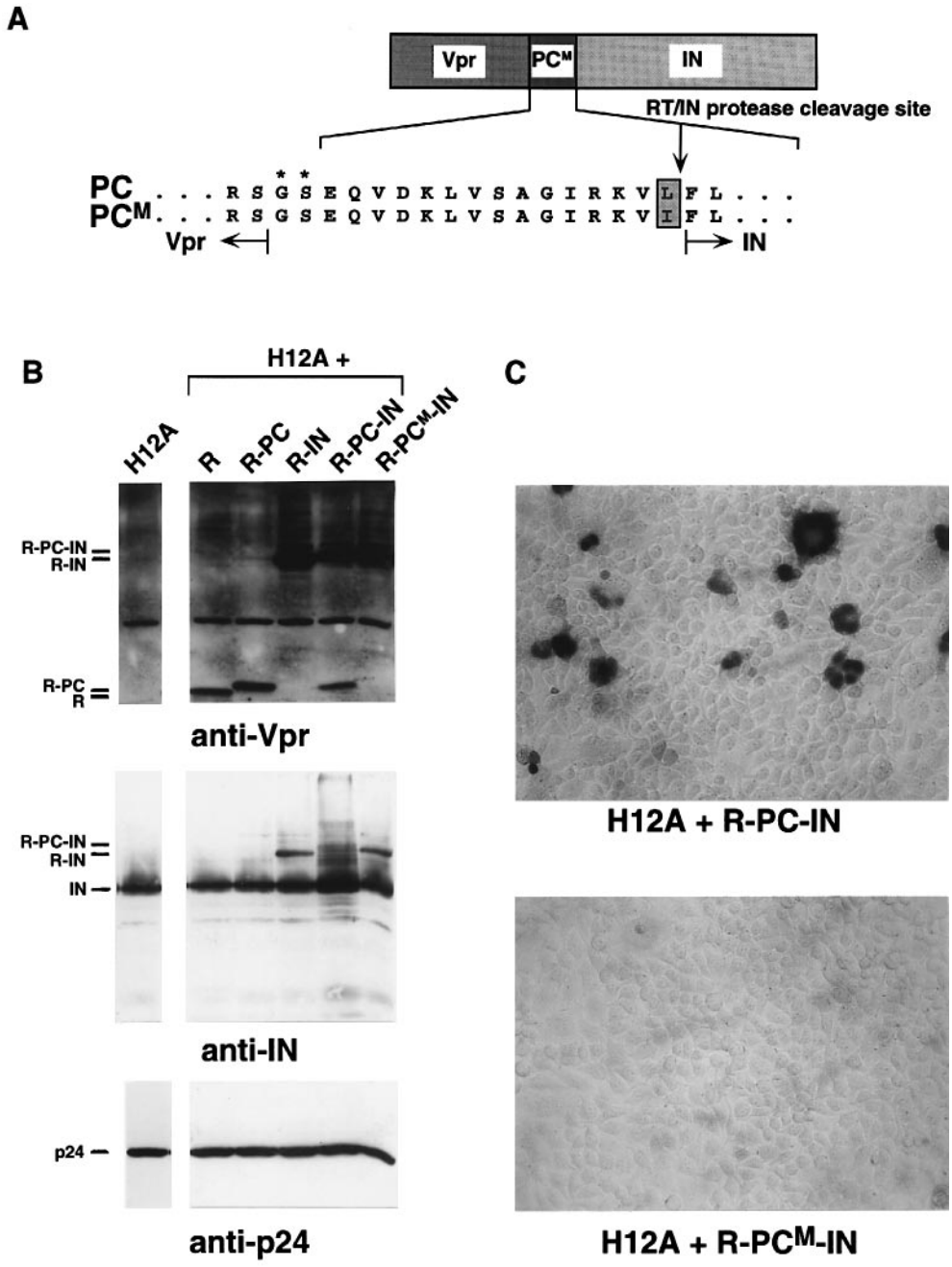

Fig. 3. Proteolytic cleavage of IN from its Vpr fusion partner is required for efficient *in vivo* complementation. (**A**) Schematic representation of wild-type (PC) and mutated (PC^M) RT/IN protease cleavage sites. The single Leu to Ile substitution is highlighted. Two amino acids generated by the introduction of a *Bam*HI restriction site used for fusion gene construction are denoted by asterisks (*). (**B**) Protein profiles of H12A-derived virions containing R, R–PC, R–IN, R–PC–IN and R–PC^M–IN fusion proteins. The absence of an R–PC band in virions containing the R–PC^M–IN fusion protein indicates that proteolytic cleavage at the RT/IN site did not occur (the blot is overexposed to rule out partial cleavage). (**C**) R–PC^M–IN-containing H12A virions lack biological activity in the MAGI cell assay.

cells and infected cell clones were selected as described (Landau *et al.*, 1991). Because the *gpt* constructs are defective in their *env* gene (compare Figure 4B), all progeny virions produced after the first round of infection are replication incompetent. Moreover, each mycophenolic acid resistant colony reflects an individual integration event because sustained *gpt* expression requires integration. The number of resistant colonies observed for any virus preparation thus provides a direct measure of the number of integrated proviruses (Wiskerchen and Muesing, 1995).

Table I summarizes the number of resistant colonies for each HIV-1 construct in the presence and absence of R–PC–IN complementation, as well as integration frequencies relative to R–PC–IN-complemented wild-type virus (the number of colonies obtained for R7-3 *gpt*/MuLV *env*/R–PC–IN derived virions was arbitrarily assigned a value of 100; all other values are relative to this number). In agreement with previous results (Wiskerchen and Muesing, 1995), none of the IN mutants transfected with MuLV *env* alone resulted in appreciable numbers of resistant colonies (Figure 5B, upper panel), indicating that their genomes were unable to integrate into the target cell

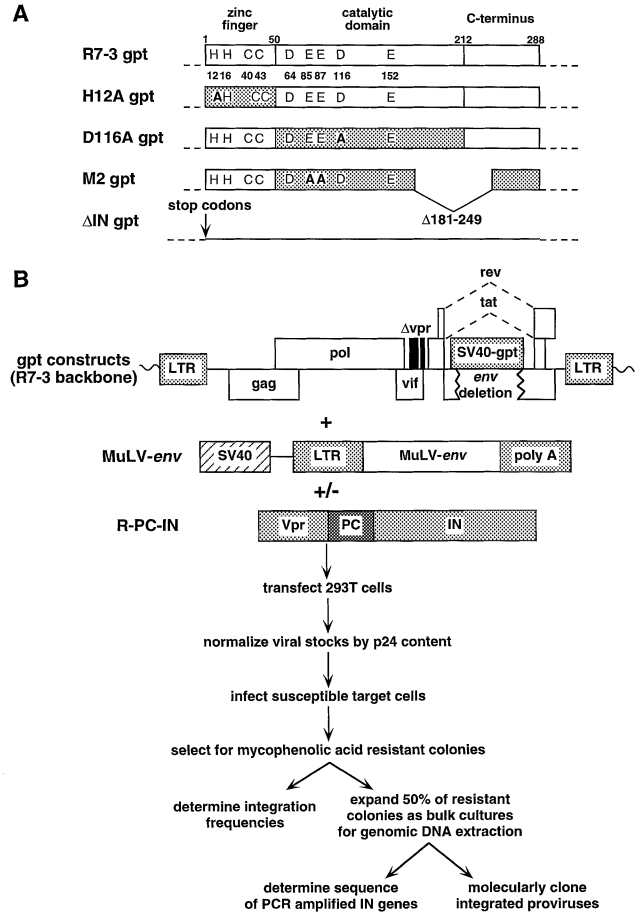

Fig. 4. Generation of an expanded set of IN-mutant molecular clones. (**A**) Schematic representation of wild-type (R7-3 *gpt*) and IN-mutant HIV-1 molecular clones with defects in three functional domains (highlighted). Amino acid substitutions and deletions are indicated. ΔIN contains two in-frame stop codons at the RT/IN junction. (**B**) Experimental outline of the single round integration assay (see text for details).

genome. By contrast, the same mutants complemented with R–PC–IN produced considerable quantities of resistant colonies (Figure 5B; lower panel), although not all IN defects were restored to the same extent. R–PC–IN-complemented zinc finger (H12A *gpt*) and catalytic domain (D116A *gpt*) mutants yielded on average 16% and 21% of wild-type activity, respectively, whereas M2 *gpt* and ΔIN *gpt* yielded only 5.3% and 1.7%. Since R–PC–IN packaging and processing were equivalent in all four virion preparations (Figure 5A), the considerably lower complementation efficiencies for M2 *gpt* and ΔIN *gpt* are probably the result of pleiotropic effects of their extensive IN mutations (i.e. removal of major parts of the IN coding region may impair the function of neighboring Pol domains).

Uncleaved R–IN fusion protein retains some in vivo complementation activity

The availability of a larger panel of IN-mutant molecular clones prompted us to re-examine the requirement for proteolytic cleavage of integrase from its fusion partner for *in vivo* activity in the single round integration assay (Table II). Testing R–IN and R–PC–IN in parallel, we found no restoration of integrase function in R–IN complemented H12A *gpt*, M2 *gpt* or ΔIN *gpt* virion preparations. By contrast, viral stocks derived by cotransfection of D116A *gpt* with R–IN reproducibly yielded ~10-fold more resistant colonies than D116A *gpt* alone, although this level of complementation was 100-fold lower than that of R–PC–IN. The R–IN fusion protein therefore retains some complementing activity in the context of replicating virus, but only when combined with certain IN mutations, such as D116A. Restoration of integrase function in all other mutant viruses required cleavage of wild-type IN from its Vpr fusion partner.

Restoration of IN function is due to complementation and not recombination

Packaging of R–PC–IN fusion proteins requires co-expression of R–PC–IN and IN-mutant proviral constructs in the same cell. To examine whether the observed restoration of integrase function was due to recombination between the cotransfected plasmids, we generated bulk cultures from mycophenolic acid resistant colonies of R–PC–IN-complemented H12A *gpt*, D116A *gpt*, M2 *gpt* and ΔIN *gpt* molecular clones and examined their integrated proviruses for the presence of wild-type IN sequences. This was done by combining half of all colonies obtained from each complementation experiment, expanding them *in vitro* and amplifying their IN coding region by single round PCR for population sequence analysis. Using this approach, we confirmed all expected integrase mutations, including the single nucleotide substitutions in H12A *gpt* and D116A *gpt*, the internal deletion in M2 *gpt* and the translational stop codons in ΔIN *gpt* (data not shown). Importantly, there was no evidence for wild-type integrase sequences or sequence mixtures in any of the amplification products, indicating that 90% (or more) of the pooled colonies harbored the expected IN-defective provirus (population sequence analysis detects point mutants comprising as little as 10% of an overall virus population; Wei *et al.*, 1995). These data thus indicate that the vast majority of R–PC–IN-mediated restoration of integrase function is due to complementation and not recombination.

Independent evidence for complementation also derives from the results shown in Table II. Using constructs which are virtually identical at the DNA level, i.e. R–IN and R–PC–IN, we observed efficient complementation of D116A *gpt* only with the construct that contained the intact protease cleavage site (R–PC–IN). If this restoration of IN function was due to recombination of cotransfected plasmids in the producer cell, then both R–IN and R–PC–IN should have generated similar numbers of drug resistant colonies. Instead, there is a >75-fold difference in the extent of proviral integration. These results (along with our intragenic complementation studies described below) argue strongly against recombination as a major contributor to the R–PC–IN-mediated restoration of integrase function.

Trans-complemented integrase mediates a bona fide integration reaction

Although the data in Figure 5B and Table I strongly suggest that packaging of R–PC–IN into IN-mutant HIV-1 mediates provirus formation, they do not prove that this integration event is specific. For example, one could speculate that virion association of the R–PC–IN fusion

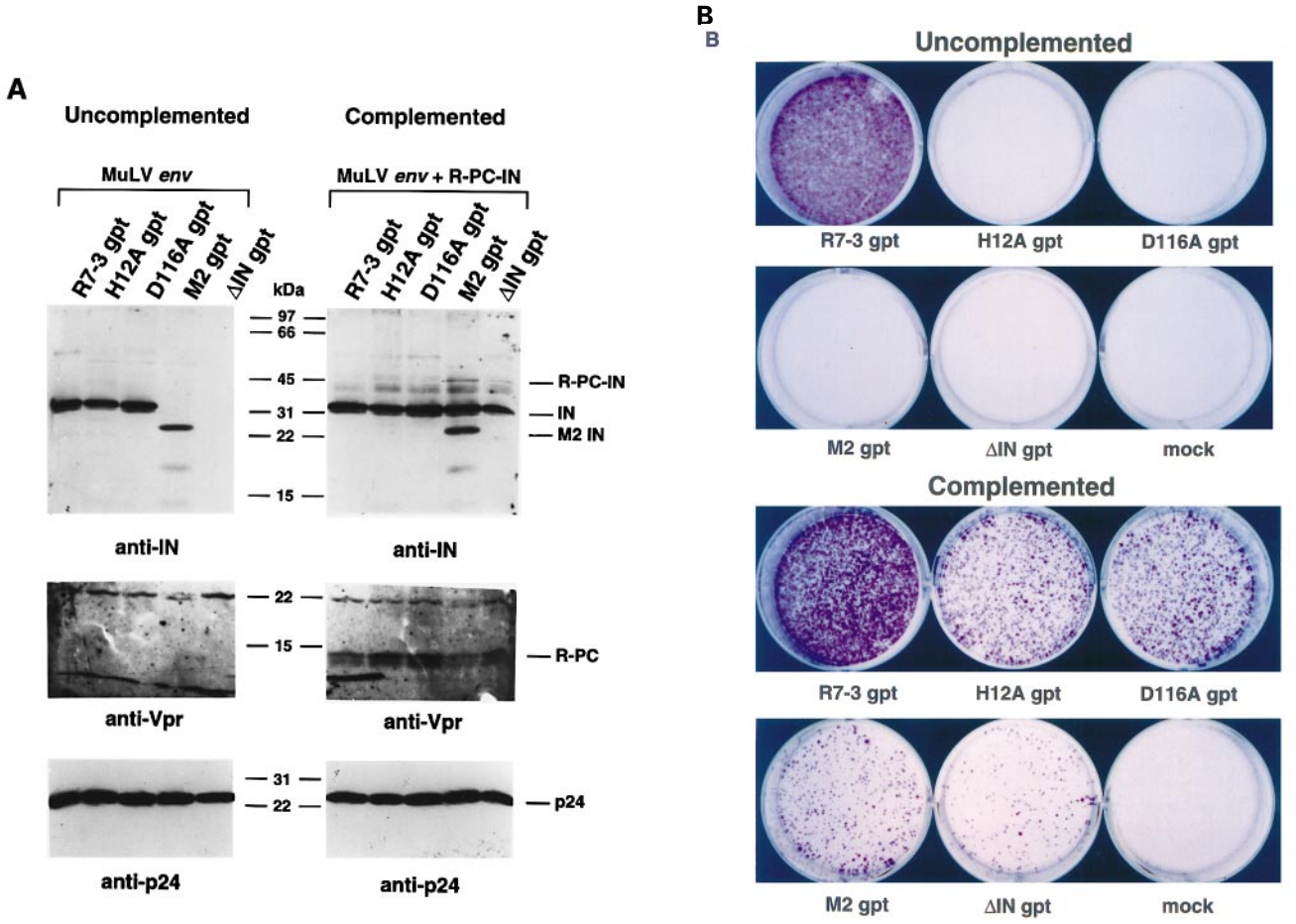

Fig. 5. *Trans* complementation of HIV-1 molecular clones with defects in multiple integrase domains. (**A**) Western blots of uncomplemented (left panels) and R–PC–IN-complemented (right panels) virion preparations probed with anti-IN, anti-Vpr and anti-Gag p24 antibodies. The presence of an R–PC protein in all complemented virion preparations indicates efficient packaging and cleavage of the R–PC–IN fusion protein. (**B**) Colony formation of uncomplemented and R–PC–IN-complemented virion preparations. Target cells were infected with uncomplemented and complemented wild-type (R7-3) and IN-mutant viruses, plated in six-well plates at various dilutions (a dilution of 1:20 is shown), and grown in selection medium for 8–9 days. Mycophenolic acid resistant colonies (purple dots) represent individual integration events.

Table I. Integration frequencies of uncomplemented and R–PC–IN-complemented IN–mutant HIV-1 molecular clones

Constructs	MuLV *env*		MuLV *env* + R–PC–IN	
	Resistant colonies per 20 ng of p24[a]	Relative frequency[b]	Resistant colonies per 20 ng of p24[a]	Relative frequency[b]
R7-3 (wt) *gpt*	2.8×10^5	149	1.9×10^5	100
H12A *gpt*	<1[c]	<0.001	3.0×10^4	16
D116A *gpt*[d]	85[d]	0.045[d]	4.0×10^4	21
M2 *gpt*	<1[c]	<0.001	1.0×10^4	5.3
ΔIN *gpt*	<1[c]	<0.001	3.2×10^3	1.7

[a]Data are averaged from two independent experiments, each plated in quadruplicate.
[b]The value obtained for R7-3 wt gpt + MuLV *env* + R–PC–IN is arbitrarily set to 100. All other values are relative to this number.
[c]<1 indicates that no colonies were observed in a 1:2 dilution (see Materials and methods).
[d]As reported previously, uncomplemented D116A *gpt* yielded a small number of mycophenolic acid resistant colonies (Wiskerchen and Muesing, 1995).

protein promotes genome insertion by an IN-independent mechanism. To investigate this possibility, we constructed genomic libraries (in lambda phage) from R–PC–IN-complemented H12A *gpt* and M2 *gpt* bulk cultures and cloned several integrated proviruses. Four hybridization positive lambda phage clones were identified (three for M2 *gpt* and one for H12A *gpt*), all of which were mapped with appropriate restriction enzymes and subjected to sequence analysis (Figure 6A).

To characterize their integration sites, all proviral clones were sequenced across their 5′ and 3′ LTR junctions. This analysis revealed a 5 bp direct repeat immediately adjacent to all four proviral insertion sites as well as intact proviral termini ending with the highly conserved TG (5′) and CA

Table II. Cleavage requirement of integrase for the complementation of HIV-1 clones with defects in different IN domains

Fusion constructs	Genomic clones							
	H12A *gpt*		D116A *gpt*		M2 *gpt*		ΔIN *gpt*	
	RC/20 ng p24[a]	RF[b]	RC/20 ng p24[a]	RF[b]	RC/20 ng p24[a]	RF[b]	RC/20 ng p24[a]	RF[b]
None	<1[c]	<0.002	44	0.14	<1[c]	<0.01	<1[c]	<0.03
R–IN	<1[c]	<0.002	420	1.3	<1[c]	<0.01	<1[c]	<0.03
R–PC–IN	3.9×10^4	100	3.2×10^4	100	7.6×10^3	100	3.0×10^3	100

RC/20 ng p24 = resistant colonies per 20 ng of p24. RF = relative frequency.
[a]Data were combined from different experiments (the low level complementation of D116A *gpt* by R–IN was confirmed in three independent experiments).
[b]The value of R–PC–IN complementation for each IN-mutant molecular clone is arbitrarily set to 100. The other two values are relative to this number.
[c]<1 indicates that no colonies were observed in a 1:2 dilution.

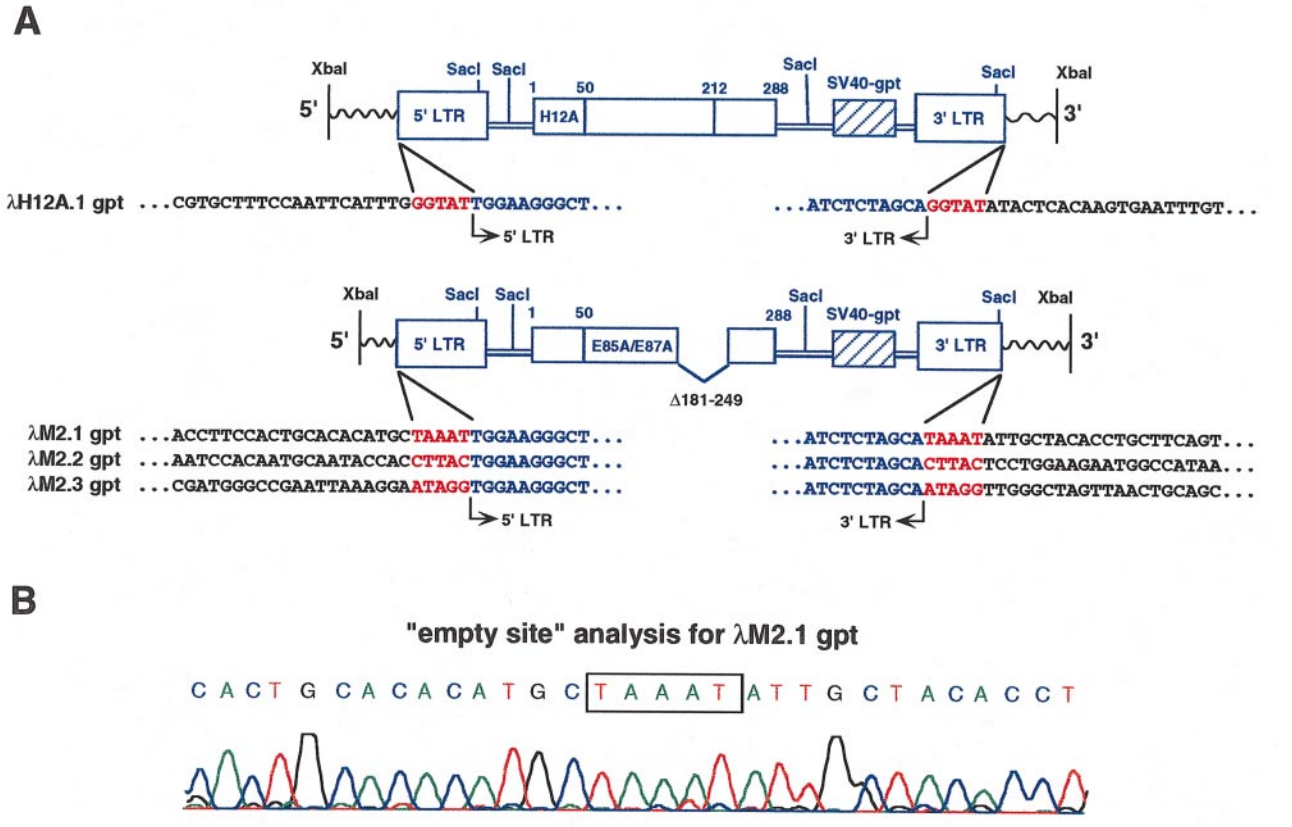

Fig. 6. *Trans*-complemented IN protein mediates a bona fide integration reaction. (**A**) Lambda phage cloning of integrated proviruses from expanded (R–PC–IN-complemented) H12A *gpt* and M2 *gpt* bulk cultures. Four positive lambda phage clones were identified and sequenced across their 5′ and 3′ LTR junctions. 5′ and 3′ proviral termini (5′-TG...CA-3′), duplicated host sequences (5 bp direct repeat), and flanking cellular sequences are shown in blue, red and black, respectively. (**B**) Analysis of an 'empty' integration site. Uninfected target cell DNA was amplified with primers flanking the λM2.1 *gpt* integration site and sequenced without interim cloning. The TAAAT motif found duplicated after M2.1 *gpt* integration is boxed.

(3′) dinucleotides. This precise processing and insertion of the viral DNA ends, along with the (HIV-1-specific) 5 bp duplication of adjoining host sequences, indicates that the H12A *gpt* and M2 *gpt* proviruses were indeed the result of an IN-mediated retroviral integration reaction. This was further confirmed by sequence analysis of one of the integration sites prior to proviral insertion (Figure 6B). Genomic sequences amplified from uninfected cellular DNA with primers flanking the λM2.1 *gpt* insertion site contained only a single copy of the TAAAT motif found duplicated after λM2.1 *gpt* integration. In addition, there was no evidence for sequence rearrangements or deletions of cellular sequences flanking this motif. The retention of the respective IN mutation was also confirmed for each clone by sequence analysis, again ruling out recombination or inadvertent contamination with wild-type virus as the reason for complementation (data not shown). Taken together, these results demonstrate that provirus formation mediated by a *trans* complemented integrase is mechanistically indistinguishable from that of

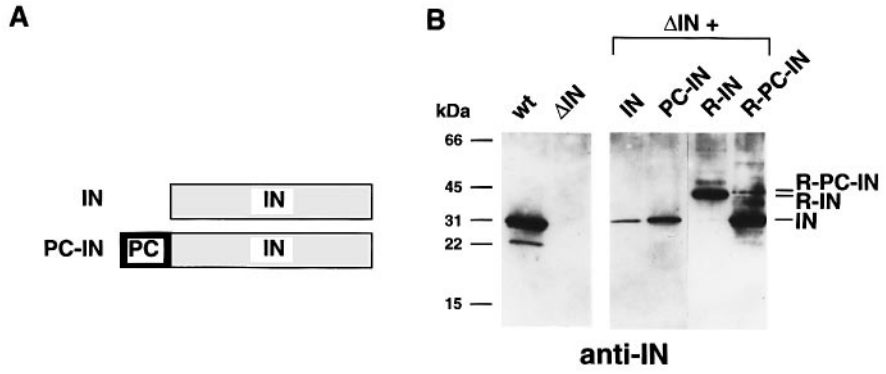

Fig. 7. Packaging of small quantities of IN in the absence of a Vpr fusion partner. (**A**) Schematic representation of IN and PC–IN constructs. (**B**) Western blots of virion preparations derived from cotransfections of ΔIN with IN, PC–IN, R–IN and R–PC–IN (probed with an anti-IN antibody). Wild-type IN bands in lanes 3 and 4 indicate that IN and PC–IN proteins are packaged in small quantities (comigration of IN and PC–IN suggests processing of PC–IN by the viral protease).

a provirally encoded enzyme packaged as part of the Gag–Pol precursor.

Vpr is required for efficient packaging of integrase into HIV-1 particles

Because Vpr is an integral component of the HIV-1 preintegration complex (Trono, 1995; Emerman, 1996), we examined whether this protein influences the complementation process in addition to targeting the fusion protein to the virion. For this purpose, we constructed the expression plasmids IN and PC–IN (Figure 7A), and transfected them (along with control constructs) with the ΔIN proviral clone. The protein profiles of the resulting virions are shown in Figure 7B (ΔIN does not express an integrase protein and thus allows assessment of IN and PC–IN packaging). This analysis revealed that integrase could indeed be packaged in the absence of the Vpr fusion partner. However, the amount of virion incorporation was approximately one to two orders of magnitude lower than when mediated by the Vpr fusion protein (similar results were obtained when IN and PC–IN were cotransfected with the M2 genomic clone; data not shown).

To investigate whether integrase packaged in the absence of Vpr was also functional, we determined the integration frequencies of H12A *gpt*-derived virions complemented with either IN or PC–IN. This experiment indicated that both constructs mediated very low level complementation, with IN yielding 22 and PC–IN 38 resistant colonies per 20 ng of p24, respectively. In comparison, R–PC–IN produced 1.1×10^4 resistant colonies in the same experiment (data not shown). The extent of IN- and PC–IN-mediated complementation thus amounted to only 0.2% and 0.4% of R–PC–IN activity, respectively. Given the low level of IN and PC–IN packaging (Figure 7B), these results again point to a relationship between the amount of incorporated integrase and its biological activity (note that the size of PC–IN in Figure 7B suggests that its PC segment is removed by the viral protease). The data also indicate that the primary (and probably only) function of Vpr in the complementation process is to mediate efficient targeting of integrase to the budding virus particle. Nevertheless, Vpr is not absolutely necessary for virion incorporation since small amounts of IN and PC–IN were found in virus particles even in the absence of this fusion partner. One explanation is that the transient co-transfection approach mediates protein expression at such high levels that packaging occurs by a non-specific mechanism. A more intriguing possibility is that IN and PC–IN interact directly (albeit weakly) with the Gag–Pol polyprotein during viral assembly. Further studies will be necessary to examine this possibility.

Intragenic complementation of mutant integrase proteins in vivo

Having confirmed the specificity of Vpr-mediated IN complementation, we utilized this experimental approach to examine whether integrase forms biologically active multimers *in vivo*. For this purpose, we prepared a panel of R–PC–IN^M constructs containing point mutations in zinc finger (H12A, H16A), catalytic core (D64A, D116A) and the central region (R199A) of IN and determined their ability to complement IN-mutant HIV-1 molecular clones (H12A *gpt*, D116A *gpt*) in a single round integration assay (Table III). Importantly, all of these mutants were previously tested in the context of molecular clones of HIV-1 and were known to render the virus integration defective (Leavitt *et al.*, 1995; Wiskerchen and Muesing, 1995). Viral stocks were prepared by transfection of *gpt*-containing genomic clones, MuLV *env* for pseudotyping and R–PC–IN^M constructs, and efficient packaging and cleavage of the Vpr based fusion proteins were confirmed by Western blot analysis (data not shown). Genomic clones were also transfected with R–PC–IN to assess the efficiency of intragenic complementation relative to complementation with wild-type integrase. Finally, wild-type HIV-1 (R7-3 *gpt*) was transfected with R–PC–IN^M constructs to examine whether any of the IN mutants under study had a dominant-negative phenotype.

Table III summarizes the results of these experiments and demonstrates successful complementation between some but not all IN mutants analyzed. For example, H12A *gpt* yielded virtually no colonies when combined with itself (H12A) or a second zinc finger mutant (H16A), but produced 180 and 190 colonies (per 20 ng of p24) when combined with the R–PC–IN^M constructs D64A and D116A, respectively. Similarly, D116A *gpt* yielded only a very small number of background colonies when complemented with itself or D64A, but produced 340 and 250 colonies when combined with H12A and H16A,

Table III. Intragenic complementation between integrase mutants

Fusion constructs	Genomic clones				
	H12A *gpt*		D116 *gpt*		R7-3 (wt) *gpt*
	RC/20 ng p24[a]	Comp. rel. to R–PC–IN[b]	RC/20 ng p24[a]	Comp. rel. to R–PC–IN[b]	RC/20 ng p24[a]
None	<1[c]	<0.002	44[d]	0.14	3.0×10^5
R–PC–IN	3.9×10^4	100	3.2×10^4	100	2.2×10^5
R–PC–IN^M (H12A)	<1[c]	<0.002	340	1.1	3.3×10^5
R–PC–IN^M (H16A)	5	0.01	250	0.8	2.7×10^5
R–PC–IN^M (D64A)	180	0.5	58	0.18	2.9×10^5
R–PC–IN^M (D116A)	190	0.5	15	0.05	3.0×10^5
R–PC–IN^M (R199A)	111	0.3	1.4×10^4	44	3.0×10^5

RC/20 ng p24 = resistant colonies per 20 ng of p24. Comp. rel. to R–PC–IN = Complementation relative to R–PC–IN.
[a]Data are reported for one of three representative experiments (plated in duplicate).
[b]Complementation frequencies of R–PC–IN^M constructs are expressed relative to R–PC–IN values, which are arbitrarily set to 100 for each genomic construct.
[c]<1 indicates that no colonies were observed in a 1:2 dilution.
[d]As reported previously, uncomplemented D116A *gpt* yielded a small number of resistant colonies (Wiskerchen and Muesing, 1995)

respectively. Although intragenic complementation mediated by the various R–PC–IN^M constructs was considerably less efficient than complementation mediated by wild-type integrase (R–PC–IN), the number of resistant colonies was reproducibly one to two orders of magnitude above background. In agreement with previous *in vitro* studies (Engelman *et al.*, 1993; van Gent *et al.*, 1993), these results thus demonstrate that zinc finger and catalytic triad mutants can restore each other's defect *in vivo*, at least partially. Interestingly, much higher levels of complementation were observed between D116A *gpt* and the R–PC–IN^M (R199A) construct. In three independent experiments, complementation efficiencies ranged between 28% and 44% of R–PC–IN activity (data from one representative experiment are shown in Table III). At the same time, only poor *in vivo* complementation was seen between H12A *gpt* and R–PC–IN^M (R199A) (0.3% of R–PC–IN activity), despite the fact that these two mutants map to IN regions previously reported to *trans* complement each other *in vitro* (Engelman *et al.*, 1993; van Gent *et al.*, 1993). These results thus indicate that efficient *in vivo* complementation of IN mutants is possible and that particle associated IN is comprised of subdomains which function in a truly independent manner.

In this context, it should again be emphasized that the results in Table III argue for intragenic complementation, and not for recombination, as the reason for restoration of IN function. If recombination events between the various R–PC–IN^M constructs and the respective genomic clones were responsible for the observed drug resistant colonies, then the extent of viral integration should correlate with the distance between the two mutations in the integrase gene. This is clearly not the case; for example, D116A is separated from H12A and R199A by a similar distance (104 and 83 codons, respectively), yet cotransfection of D116A *gpt* with R–PC–IN (R199A) resulted in 1.4×10^4 colonies, while cotransfection of H12A *gpt* with R–PC–IN (D116A) resulted in only 190 colonies. Also, as expected from complementation, pairwise combinations of mutations in the same functional domains did not restore function whereas those in separate domains did. Thus, like in the case of R–PC–IN, the pattern of restoration of IN function among the various domain mutants is consistent with complementation and not with homologous recombination.

Discussion

We describe a novel *trans*-complementation assay which can be used to restore integrase function to IN-mutant HIV-1 virions. Traditional *in vivo* complementation approaches, e.g. coinfection of cells with mutant and helper (wild-type) virus or expression of the wild-type protein in cells infected with mutant virus, are not applicable to HIV-1 integrase, because this enzyme (as well as other *pol*-encoded proteins) is packaged into virus particles as part of the Gag–Pol polyprotein. Moreover, after infection of new target cells, IN remains associated with a nucleoprotein complex (the preintegration complex) which transports the viral DNA to the nucleus. Thus, unless properly assembled into this preintegration complex, IN provided *in trans* is unlikely to access its substrates, the viral and cellular DNA. By co-expressing an IN-mutant HIV-1 provirus with a *trans*-complementation plasmid that expresses IN in the form of a Vpr–IN fusion protein, we are circumventing the requirement of a polyprotein for integrase packaging, yet mediating virion incorporation in such a way that natural interactions of IN with other virion or cellular components during assembly, maturation and early infection steps are preserved. As shown in Figure 6, this approach promotes a bona fide complementation reaction and not an illegitimate bypass of the integration mechanism, because the resultant proviruses are indistinguishable from proviruses established by genomically encoded IN.

The availability of this novel complementation assay has allowed us to address a number of questions concerning integrase biology which have previously been experimentally inaccessible. These include (i) the conditions under which IN must be delivered to the virus particle in order to be biologically active, (ii) the role of IN proteolytic cleavage for zinc finger and catalytic domain function and (iii) the composition of functional IN multimers in replicating virions.

IN must be proteolytically cleaved from its Vpr fusion partner to be active in vivo

Vpr-packaged IN does not complement efficiently unless it is cleaved from its fusion partner by the viral protease. Figure 2 and Table II show that only the R–PC–IN construct in which IN and Vpr sequences are separated by the native RT/IN protease cleavage site, but not the R–IN construct, restores provirus formation of IN-mutant HIV-1 to significant levels. Moreover, introduction of a single amino acid substitution into the PC segment that blocks cleavage also blocks *in vivo* complementation (Figure 3). This dependence on the presence of a protease cleavage site could be explained in one of three ways: (i) IN requires a very specific N-terminal sequence or structure that is generated by cleavage of the viral protease, (ii) Vpr-tethered IN is targeted to an unfavorable location within the virion and/or newly infected cells such that it cannot access its DNA substrates or (iii) IN enzymatic activity is impaired in the context of an N-terminal fusion protein. Since several N- and C-terminal IN fusion proteins have previously been shown to efficiently mediate *in vitro* 3′ processing and joining reactions (Bushman, 1994; Miller *et al.*, 1995; Katz *et al.*, 1996), the latter possibility seems unlikely. Instead, we favor the second hypothesis because it is consistent with current knowledge of HIV-1 virion architecture and early infection events. Nevertheless, our data also suggest that complementation of the zinc finger function may require an intact (natural) N-terminus.

Although the intravirion distribution of Vpr is still a subject of investigation, circumstantial evidence suggests that only a small portion of this protein locates inside the viral core (Yu *et al.*, 1993; Kewalramani and Emerman, 1996; Sato *et al.*, 1996). Assuming that Vpr and Vpr-linked fusion proteins are targeted to similar sites in the virus particle, it seems likely that the bulk of Vpr-tethered IN locates to a virion compartment, such as the outer core surface or the space between the core and the membrane, in which it cannot access viral DNA. Moreover, by analogy with the matrix protein (Gallay *et al.*, 1995b; Bukrinskaya *et al.*, 1996), only a small fraction of virion-associated Vpr (and thus Vpr-linked integrase) is believed to be incorporated into the preintegration complex. We thus hypothesize that most of the Vpr-tethered IN protein is inaccessible for complementation because it is probably targeted to an unfavorable location within the virion as well as in infected cells. By contrast, when cleaved from Vpr by the viral protease—a process that presumably takes place during assembly when all other viral proteolytic cleavages occur—IN appears to be free to associate with other core components similar to the virally encoded integrase, resulting in a more physiological intra-virion distribution.

Nevertheless, a portion of Vpr is known to associate with the HIV-1 preintegration complex and to mediate—together with the matrix protein—its transport to the nucleus in non-dividing cells (Bukrinsky *et al.*, 1993a,b; Heinzinger *et al.*, 1994; von Schwedler *et al.*, 1994; Gallay *et al.*, 1995a; Emerman, 1996). A fraction of Vpr-tethered integrase may therefore also be incorporated into this complex and could retain at least some *in vivo* complementation activity, unless its function was obstructed by other components of the nuclear transport machinery. Interestingly, Table II provides evidence for such R–IN-mediated complementation, but only in the context of the D116A *gpt* molecular clone. Transfection of this catalytic domain mutant with R–IN yielded one order of magnitude more resistant colonies than uncomplemented D116A *gpt*. By contrast, no colonies were observed with identically generated (R–IN-complemented) H12A *gpt*, M2 *gpt* and ΔIN *gpt* virion preparations. These results thus indicate that R–IN can, in fact, restore the integration defect of certain IN-mutants, albeit only at a very low level (1.3% of R–PC–IN activity). However, its complete failure to complement H12A *gpt*, M2 *gpt* and ΔIN *gpt* constructs, which either contain (H12A, ΔIN) or are likely to contain (M2) defective HHCC domains, suggests that restoration of zinc finger function requires both 'untethering' of IN from Vpr and that the natural IN N-terminus. We are currently testing this hypothesis by engineering alternative PR processing sites into R–PC–IN that will mediate proteolytic processing (and thus removal) of Vpr, but which also alter IN by leaving 10–15 additional (heterologous) amino acids at its N-terminus.

Intragenic complementation of IN mutants in vivo

Complementation analysis of a limited set of IN mutants revealed that some of them were capable of mediating provirus formation when assayed as mixed multimers (Table III). The most efficient complementation was observed between the D116A *gpt* genomic clone and the R–PC–IN^{M} (R199A) construct, while zinc finger and catalytic core mutants complemented each other only modestly. This documentation of provirus formation by pairwise combinations of IN mutants, which by themselves render HIV-1 molecular clones integration defective, provides the best evidence yet that IN functions in the form of multimeric complexes *in vivo*. Zinc finger and catalytic domain mutants have previously been shown to complement each other in *in vitro* integration assays which has led to their classification as functionally independent subdomains (Engelman *et al.*, 1993; van Gent *et al.*, 1993). However, the level of complementation in these experiments was considerably higher (20–40% of wild-type activity) than the level of complementation observed in our *in vivo* analyses (~1% of wild-type activity). Since our results were reproducible, not due to dominant-negative effects of either IN mutation and 'symmetrical' (i.e. equivalent regardless of whether virion incorporation was mediated by proviral clones or R–PC–IN^{M} constructs), we conclude that provirus formation depends on the functional integrity of zinc finger and catalytic core domains to a much larger extent than suggested by *in vitro* assays. This may be because both domains could actually be required for catalyzing the proviral integration reaction, e.g. both may be necessary for 3′ processing. Alternatively, zinc finger mutations may be less readily complemented by other IN mutants because they are defective in integrative as well as non-integrative (e.g. viral DNA synthesis) functions. Quantitative analyses of viral DNA synthesis and provirus formation in cells infected with virions containing pairwise combinations of zinc finger/catalytic domain mutant IN proteins may help to distinguish between these possibilities.

The complementation matrix in Table III also provided new insights into whether virion-associated integrase multimers consist of truly independent functional sub-

domains. For example, R–PC–IN^{M} (R199A) complemented the D116A *gpt* genomic clone very efficiently (44% of wild-type activity), while the same mutant had only very modest effects when co-expressed with the H12A *gpt* zinc finger mutant (0.3% of wild-type activity). Amino acid residues 187–234 have previously been reported to function only *in cis* relative to the catalytic core (Engelman *et al.*, 1993), yet we show here that R199A complements a catalytic core mutant efficiently *in trans*. One explanation is that R199A may affect an *in vivo*-specific 'C-terminal function' rather than a 'core function' that is required *in vitro*, in which case some of the boundaries of currently established integrase subdomains may require revision. Another possibility is that the R199A mutation efficiently complements D116A *gpt* because it affects only a non-catalytic integrase function, such as target recognition. A mutation similar to R199A (R199C) has previously been shown to retain near wild-type *in vitro* 3′ processing, strand transfer and disintegration activities (Leavitt *et al.*, 1993), while totally abrogating proviral integration in the context of an HIV-1 genomic clone (Leavitt *et al.*, 1996). Assuming that R199A has the same *in vitro* phenotype, the complementation results suggest that R199A supplies the catalytic activity, while D116A *gpt* provides the non-catalytic function required for provirus formation. This would indicate that IN can function very efficiently when composed of mixtures of catalytic and non-catalytic domain mutants which, in turn, would suggest that the active IN multimer *in vivo* may consist of individual subunits with either catalytic or non-catalytic functions. We are currently testing this hypothesis by analyzing additional IN mutants with presumed non-catalytic IN defects (e.g. W235E; Leavitt *et al.*, 1996).

Identification of pleiotropic integrase mutants

In vivo analyses of IN mutants are complicated by the fact that IN is normally expressed and packaged as part of a large polyprotein. Although some IN mutants made in this context affect only single biochemical functions, there are examples of mutations that affect the function of integrase as well as that of neighboring Gag–Pol regions. For example, certain IN mutations have been reported to affect virion morphology, particle production, as well as levels of virion-associated IN and RT proteins (Cannon *et al.*, 1994; Engelman *et al.*, 1995; Bukovsky and Goettlinger, 1996). Since our complementation strategy bypasses the Gag–Pol assembly mechanism, such pleiotropic mutants should behave quite differently in our system. Mutants with an effect strictly limited to IN itself would be defective whether they are packaged as part of Gag–Pol or as a R–PC–IN fusion protein. By contrast, pleiotropic mutants would be expected to exhibit a much more severe defect when expressed in the context of a molecular clone, and would thus be more resistant to complementation by R–PC–IN. For example, our ability to complement H12A *gpt* and D116A *gpt* mutants more efficiently then M2 *gpt* and ΔIN *gpt* could be due to the fact that the latter two have multiple effects on viral replication. Removal of all (ΔIN) or parts (M2) of integrase may alter the function of neighboring Gag–Pol regions which would not be expected to be complemented by only packaging an alternative IN protein. Analysis of a larger panel of well characterized IN mutants should allow us to determine whether the R–PC–IN complementation assay is generally useful for the characterization of pleiotropic IN mutations.

A safer HIV-1-based vector system

Because of HIV's unique ability to replicate in non-dividing cells, efforts are underway in several laboratories to develop HIV based retroviral delivery vectors for eventual gene therapy applications (Parolin *et al.*, 1994; Corbeau *et al.*, 1996; Naldini *et al.*, 1996). A problem with this approach is that such vector systems must be safe, i.e. they must be designed in such a way that reversion to wild-type virus is not possible. Thus, cell lines or plasmids producing structural proteins cannot express viral RNA that is packagable into the resulting particles. While this problem has generally been approached by mutation and/or deletion of the packaging signal, instances have been described in which wild-type virus emerged as a result of homologous recombination between helper and vector genomes (for review see Vile and Russell, 1995). The experiments described here show that IN can be produced *in trans* from an R–PC–IN fusion construct and can complement a virus from which IN has been fully deleted. Similarly, Wu *et al.* (1997) have shown that Vpr–RT fusions can complement HIV-1 genomes defective in their RT domain. Thus, it is theoretically possible to generate HIV packaging constructs or cell lines that express Env, Gag, as well as portions of Pol separately. Such a strategy would greatly decrease the likelihood of recombination or reversion of a mutated or deleted packaging signal, and thus the generation of infectious virus.

A strategy for delivering modified IN proteins

A second gene therapy application that has received considerable attention is the possibility of directing retroviral IN proteins to certain sites in the genome by fusing their coding regions to those of sequence-specific DNA binding proteins (DBPs) (Bushman, 1994, 1995; Miller *et al.*, 1995; Goulaouic and Chow, 1996; Katz *et al.*, 1996). While this approach has worked well *in vitro*, attempts to use these fusion proteins to replace the native IN gene *in vivo* have met with only partial success. Propagation of IN gene fusion containing retroviral genomes in tissue culture has revealed that they are often replication attenuated and/or genetically unstable, i.e. they lose the introduced fusion gene after only a limited number of replication cycles. In addition, their construction is difficult because of overlapping regulatory and coding regions and because the packaging capacity of the parental retrovirus limits the size of the gene that can be expressed. Using the approach described here, it should be relatively straightforward to provide R–PC–IN–DBP fusions *in trans* and to use these to complement IN-deleted viruses. Such complemented viruses could then be analyzed for targeted integration. Given the sensitivity of this system, several DBP fusion candidates could be screened rapidly in the context of a single ΔIN virus construct without the interference of any wild-type IN protein. Studies are underway to investigate these possibilities.

Materials and methods

Construction of Vpr–IN gene fusions

Vpr and IN coding regions were amplified from HIV-1/YU-2 (Li *et al.*, 1991) and HIV-1/R7-3 (Feinberg *et al.*, 1991), respectively, using primers R1 (5′-TGAGccatggAACAAGCCCCAGAAGACCAA-3′) and R2 (5′-GCGggatccGGATCTACTGGCTCCATTTCT-3′), as well as IN1 (5′-CGCggatccTTTTTAGATGGAATAGATAAG-3′) and IN3 (5′-GGCctcgagCTAATCCTCATCCTGTCTACT-3′; lower case letters indicate restriction sites used for fusion gene construction). Integrase was also amplified using primers IN2 (5′-CGCggatccGAACAAGTAGATAAATTAGTC-3′) and IN3 to derive an IN fragment that contained the natural RT/IN protease cleavage site (PC). Amplification products were subcloned into pLR2P (Wu *et al.*, 1995) previously shown to mediate high level expression of Vpr-based fusion proteins. The resulting constructs R–IN and R–PC–IN (Figure 1) were then used as templates for the amplification of additional constructs, including R using primers R1 and R3 (5′-GGctcgagCTAGGATCTACTGGCTCCATTTCT-3′); R–PC using R1 and R4 (5′-GGCctcgagCTATAGTACTTTCCTGATTCC-3′); IN using IN4 (5′-TGAGccatggGATTTTTAGATGGAATAGAT-3′) and IN2; PC–IN using IN5 (5′-TGAGccatggAACAAGTAGATAAATTAGTC-3′) and IN2; and PC^{M}–IN using IN6 (5′-TCCggatccGAACAAGTAGATAAATTAGTCAGTGCTGGAATCAGGAAAGTAATATTTTTA-3′) and IN2. Finally, all R–PC–IN^{M} constructs listed in Table III were generated by replacing the wild-type integrase coding region of R–PC–IN with mutant integrase genes amplified (using primers IN2 and IN3) from IN-mutant HIV-1 proviral clones (Wiskerchen and Muesing, 1995). All constructs were sequenced in their entirety to exclude amino acid substitutions due to PCR misincorporations. Their sequences (in pLR2P) as well as specific amplification conditions are available upon request.

Construction of IN-mutant HIV-1 genomic clones

The HIV-1 molecular clones R7-3 (Feinberg *et al.*, 1991) and R7-3 *gpt* as well as their IN-mutant derivatives (except ΔIN) have been described previously (Wiskerchen and Muesing, 1995). ΔIN was generated by introducing two in-frame stop codons at the RT/IN junction using PCR mutagenesis. Two internal fragments of R7-3 were amplified using primers *Eco*RV (5′-GGATTAgatatcAGTACAATGTGCTTCCAC-3′) and *Eco*47IIIa (5′-CTATTagcgctCATTATAGTACTTTCCTGA-3′), as well as *Eco*47IIIb (5′-TTTTagcgctAATAGATAAGGCCCAAGATG-3′) and *Nde*I (5′-AACATAcatatgGTGTTTTACTAAACTTTT-3′), ligated at the *Eco*47IIIb site and recloned into the R7-3 backbone (the introduced nucleotide substitutions are underlined). The *gpt* containing derivative of ΔIN was then generated by replacing the *Sal*I–*Bam*HI *env*-containing fragment with the corresponding *gpt*-containing fragment from HIV *gpt* (Wiskerchen and Muesing, 1995). Importantly, all IN-mutant HIV-1 molecular clones used in this study are isogeneic with R7-3 or R7-3 *gpt*. Consequently, they all contain a defective *vpr* gene and lack the *vpu* initiation codon, but encode a functional *nef* gene (Miller *et al.*, 1994).

Preparation and characterization of viral stocks

All viral stocks were generated by transfection of 293T cells using a commercially available calcium phosphate/DNA precipitation kit (Stratagene, La Jolla, CA) according to manufacturer's recommendations. For analysis in the MAGI cell assay, HIV-1 molecular clones (wild-type or IN-mutant) were transfected alone (5 μg), or in combination with Vpr–integrase fusion and control constructs (25 μg). For the single round integration assay, HIV-1 *gpt* constructs (5 μg) were either transfected with SV-A-MLV *env* (5 μg) alone (Wiskerchen and Muesing, 1995), or with SV-A-MLV *env* and Vpr–IN fusion constructs (25 μg). Transfections were performed in 100 mm Petri dishes at 30–50% confluency and virus-containing supernatants were harvested either 24 (MAGI) or 48 h (integration assay) post transfection. Supernatants were normalized for p24 content (Coulter Diagnostics, Hialeah, FL) and stored at –70°C prior to analysis in either the MAGI or the single round integration assays. Aliquots were also pelleted through a 20% sucrose cushion and analyzed for protein content and processing on immunoblots (Fletcher *et al.*, 1996) using anti-IN (Grandgenett and Goodarzi, 1994), anti-Vpr (Wu *et al.*, 1995) and anti-Gag p24 (Minassian *et al.*, 1988) antibodies.

MAGI cell assay

8×10^{4} MAGI (HeLa-CD4-LTR-β-gal) cells were plated (per well) in a 12-well format and infected in duplicate with wild-type or IN-mutant HIV-1 virions (complemented or uncomplemented with R–PC–IN) using the equivalent of 50, 5 and 0.5 ng of p24 in the presence of 20 μg/ml DEAE dextran. 40 to 48 h post infection, cells were fixed, stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as previously described (Kimpton and Emerman, 1992) and blue nuclei were counted.

Single round integration assay

Complementation of IN-mutant HIV-1 molecular clones was quantified in a single round integration assay (Wiskerchen and Muesing, 1995). Briefly, viral stocks derived from double (proviral *gpt* constructs + SV-A-MLV-*env*) or triple (proviral *gpt* constructs + SV-A-MLV-*env* + R–PC–IN/IN^{M}) transfections were normalized for p24 content (20 ng/ml) and used to infect MAGI cells at 30% confluency in six-well plates in the presence of polybrene (8 μg/ml). 4 h post-infection, virus-containing supernatant was removed and cells were washed twice with serum-free medium. Cells were then grown in non-selective medium for an additional 40 h, trypsinized, resuspended in 6 ml of selection medium containing mycophenolic acid (50 μg/ml) and plated in 100 mm dishes (1:2 dilution) as well as six-well plates (1:20, 1:200 and 1:2000 dilutions). The selection medium was changed every other day, and colonies were stained (0.2% crystal violet, 25% isopropanol and 5% acetic acid) and counted 8–9 days after infection. Counts were multiplied by the dilution factor and expressed as numbers of resistant colonies per 20 ng of p24.

PCR amplification of mutant integrase genes from expanded gpt cultures

Bulk cultures of mycophenolic acid resistant colonies (derived from R–PC–IN complementation of H12A *gpt*, D116A *gpt*, M2 *gpt* and ΔIN *gpt* molecular clones) were expanded and genomic DNA was extracted for single round PCR amplification of IN coding regions using primers IN2 and IN3. Importantly, each bulk culture was generated by combining all colonies from the 1:2 dilution plate, and thus represented half of all integration events resulting from that complementation experiment. Amplification products were purified by agarose electrophoresis and sequenced directly (without interim cloning) using cycle sequencing and dye terminator methodologies on an automated DNA sequencer (model 373A; Applied Biosystems, Inc.). Sequences were analyzed using the program Sequencher (Gene Codes Corp., Ann Arbor, MI).

Lambda phage cloning

Genomic lambda phage libraries were constructed and screened as previously described (Maniatis *et al.*, 1982; Li *et al.*, 1991). Briefly, high molecular weight DNA from expanded (R–PC–IN-complemented) M2 *gpt* and H12A *gpt* bulk cultures were digested with *Xba*I (an enzyme known not to cut the R7-3 *gpt* genome), fractionated by sucrose gradient centrifugation (10–40%) to enrich for fragments 12–20 kb in length, and ligated into the purified arms of λDASHII (Stratagene, La Jolla, CA). Ligation products were packaged *in vitro* (Gigapack II Gold, Stratagene, La Jolla, CA), titered and plated on LE392 cells. Recombinant phage clones (20 000 plaques per plate)were screened with a full length HIV-1 probe (BH10; Hahn *et al.*, 1984). Positive phage recombinants were plaque purified and their restriction maps determined by multiple enzyme digestions. Phage clones containing full length integrated HIV-1 genomes were digested with *Xba*I and *Sac*I (which cleaves the LTR of R7-3) and the restriction fragments containing 5′ and 3′ flanking cellular sequences as well as the integrase coding region were sequenced directly.

'Empty integration site' analysis

Genomic DNA was extracted from uninfected MAGI cells and subjected to single round PCR amplification using primer pairs that flanked the proviral insertion site of λM2.1 *gpt* (forward primer 5′-GGTGAGGTTAGGGCCGGG-3′; reverse primer 5′-GCATACACACACACACATTGTGAAATG-3′). The resulting 330 bp fragment was purified by agarose gel electrophoresis and sequenced without interim cloning.

Acknowledgements

We thank J.Kappes and X.Wu for the Vpr antiserum and pLR2P expression plasmid; J.Conway for p24 antigen determinations; S.A.Chow, R.Swanstrom, M.Emerman, M.Stevenson and C.D.Morrow for helpful discussions and J.Wilson for artwork and preparation of the manuscript. The anti-IN antiserum was obtained through the AIDS Research and Reagent Program (Cat. #757), Division of AIDS, NIAID, NIH (originally contributed by D.Grandgenett). This work was supported by grants from the National Institutes of Health (U01 AI35282, R01 AI34748, R01 AI35467), by shared facilities of the UAB Center for AIDS Research (P30 AI27767) and by the Birmingham Veterans Administration Medical

Center. T.M.F. was supported by an NIH training grant (T32 AI07150) and M.A.S. by the Brazilian National Council of Scientific and Technological Development (Brazil-260024/94-5).

References

Bowerman,B., Brown,P.O., Bishop,J.M. and Varmus,H.E. (1989) A nucleoprotein complex mediates the integration of retroviral DNA. *Genes Dev.*, **3**, 469–478.

Bukovsky,A. and Göttlinger,H. (1996) Lack of integrase can markedly affect human immunodeficiency virus type 1 particle production in the presence of an active viral protease. *J. Virol.*, **70**, 6820–6825.

Bukrinskaya,A.G., Ghorpade,A., Heinzinger,N.K., Smithgall,T.E., Lewis,R.E. and Stevenson,M. (1996) Phosphorylation-dependent human immunodeficiency virus type 1 infection and nuclear targeting of viral DNA. *Proc. Natl Acad. Sci. USA*, **93**, 367–371.

Bukrinsky,M.I., Haggerty,S., Dempsey,M.P., Sharova,N., Adzhubei,A., Spitz,L., Lewis,P., Goldfarb,D., Emerman,M. and Stevenson,M. (1993a) A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. *Nature*, **365**, 666–669.

Bukrinsky,M.I., Sharova,N., McDonald,T.L., Pushkarskaya,T., Tarpley, W.G. and Stevenson,M. (1993b) Association of integrase, matrix and reverse transcriptase antigens of human immunodeficiency virus type 1 with viral nucleic acids following acute infection. *Proc. Natl Acad. Sci. USA*, **90**, 6125–6129.

Burke,C. J., Sanyal,G., Bruner,M.W., Ryan,J.A., LaFemina,R.L., Robbins,H.L., Zeft,A.S., Middaugh,C.R. and Cordingley,M.G. (1992) Structural implications of spectroscopic characterization of a putative zinc-finger peptide from HIV-1 integrase. *J. Biol. Chem.*, **267**, 9639–9644.

Bushman,F.D. (1994) Tethering human immunodeficiency virus 1 integrase to a DNA site directs integration to nearby sequences. *Proc. Natl Acad. Sci. USA*, **91**, 9233–9237.

Bushman,F.D. (1995) Targeting retroviral integration. *Science*, **267**, 1443–1444.

Bushman,F.D. and Craigie,R. (1991) Activities of human immunodeficiency virus (HIV) integration protein *in vitro*: specific cleavage and integration of HIV DNA. *Proc. Natl Acad. Sci. USA*, **88**, 1339–1343.

Bushman,F.D., Fujiwara,T. and Craigie,R. (1990) Retroviral DNA integration directed by HIV integration protein *in vitro*. *Science*, **249**, 1555–1558.

Bushman,F.D., Engelman,A., Palmer,I., Wingfield,P. and Craigie,R. (1993) Domains of the integrase protein of human immunodeficiency virus type 1 responsible for polynucleotidyl transfer and zinc binding. *Proc. Natl Acad. Sci. USA*, **90**, 3428–3432.

Cannon,P.M., Wilson,W., Byles,E., Kingsman,S.M. and Kingsman,A.J. (1994) Human immunodeficiency virus type 1 integrase: effect on viral replication of mutations at highly conserved residues. *J. Virol.*, **68**, 4768–4775.

Cannon,P.M., Byles,E.D., Kingsman,S.M. and Kingsman,A.J. (1996) Conserved sequences in the carboxyl terminus of integrase that are essential for human immunodeficiency virus type 1 replication. *J. Virol.*, **70**, 651–657.

Chow,S.A., Vincent,K.A., Ellison,V. and Brown,P.O. (1992) Reversal of integration and DNA splicing mediated by integrase of human immunodeficiency virus. *Science*, **255**, 723–726.

Corbeau,P., Kraus,G. and Wong-Staal,F. (1996) Efficient gene transfer by a human immunodeficiency virus type 1 (HIV-1) derived vector utilizing a stable HIV packaging cell line. *Proc. Natl Acad. Sci. USA*, **93**, 14070–14075.

Doak,T.G., Doerder,F.P., Jahn,C.L. and Herrick,G. (1994) A proposed super-family of transposase genes: transposon-like elements in ciliated protozoa and a common 'D35E' motif. *Proc. Natl Acad. Sci. USA*, **91**, 942–946.

Drelich,M., Wilheim,R. and Mous,J. (1992) Identification of amino acid residues critical for endonuclease and integration activities of HIV-1 IN protein *in vitro*. *Virology*, **188**, 459–468.

Ellison,V. and Brown,P.O. (1994) A stable complex between integrase and viral DNA ends mediates human immunodeficiency virus integration *in vitro*. *Proc. Natl Acad. Sci. USA*, **91**, 7316–7320.

Ellison,V., Gerton,J., Vincent,K.A. and Brown,P.O. (1995) An essential interaction between distinct domains of HIV-1 integrase mediates assembly of the active multimer. *J. Biol. Chem.*, **270**, 3320–3326.

Emerman,M. (1996) HIV-1, Vpr and the cell cycle. *Curr. Biol.*, **6**, 1096–1103.

Engelman,A. and Craigie,R. (1992) Identification of conserved amino acid residues critical for human immunodeficiency virus type 1 integrase function *in vitro*. *J. Virol.*, **66**, 6361–6369.

Engelman,A., Mizuuchi,K. and Craigie,R. (1991) HIV-1 DNA integration: mechanism of viral DNA cleavage and DNA strand transfer. *Cell*, **67**, 1211–1221.

Engelman,A., Bushman,F.D. and Craigie, R. (1993) Identification of discrete functional domains of HIV-1 integrase and their organization within an active multimeric complex. *EMBO J.*, **12**, 3269–3275.

Engelman,A., Hickman,A.B. and Craigie,R. (1994) The core and carboxyl-terminal domains of the integrase protein of human immunodeficiency virus type 1 each contribute to nonspecific DNA binding. *J. Virol.*, **68**, 5911–5917.

Engelman,A., Englund,G., Orenstein,J.M., Martin,M.A. and Craigie,R. (1995) Multiple effects of mutations in human immunodeficiency virus type 1 integrase on viral replication. *J. Virol.*, **69**, 2729–2736.

Farnet,C.M. and Bushman,F.D. (1996) HIV cDNA integration: molecular biology and inhibitor development. *AIDS*, **10**, S3-S11.

Farnet,C.M. and Bushman,F.D. (1997) HIV-1 cDNA integration: requirement of HMG I(Y) protein for function of preintegration complexes *in vitro*. *Cell*, **88**, 483–492.

Feinberg,M.B., Baltimore,D. and Frankel,A.D. (1991) The role of Tat in the HIV life cycle indicates a primary effect on transcriptional elongation. *Proc. Natl Acad. Sci. USA*, **88**, 4045–4050.

Fletcher,T.M.,III, Wu,X., Liu,S., McPherson,S., Shaw,G.M., Boeke,J.D., Muesing,M.A., Kappes,J.C., Stevenson,M. and Hahn,B.H. (1995) Virion associated accessory proteins: new targets for antiviral therapy. In Girard,M. and Dodet,B. (eds), *Retroviruses of Human AIDS and Related Animal Diseases.* Dixeme Colloque des Cent Gardes, pp. 99–106

Fletcher,T.M.,III, Brichacek,B., Sharova,M., Newman,M.A., Stivatis,G., Sharp,P.M., Emerman,M., Hahn,B.H. and Stevenson,M. (1996) Nuclear import and cell cycle arrest functions of the HIV-1 Vpr protein are encoded by two separate genes in HIV-1/SIVsm. *EMBO J.*, **15**, 6155–6165.

Gallay,P., Swingler,S., Aiken,C. and Trono,D. (1995a) HIV-1 infection of nondividing cells: C-terminal tyrosine phosphorylation of the viral matrix protein is a key regulator. *Cell*, **80**, 379–388.

Gallay,P., Swingler,S., Song,J., Bushman,F.D. and Trono,D. (1995b) HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase. *Cell*, **83**, 569–576.

Goff,S.P. (1992) Genetics of retroviral integration. *Annu. Rev. Genet.*, **26**, 527–544.

Goulaouic,H. and Chow,S.A. (1996) Directed integration of viral DNA mediate by fusion proteins consisting of human immunodeficiency virus type 1 integrase and *Escherichia coli* LexA protein. *J. Virol.*, **70**, 37–46.

Grandgenett,D.P. and Goodarzi,G. (1994) Folding of the multidomain human immunodeficiency virus type I integrase. *Protein Sci.*, **3**, 888–897.

Hahn,B.H., Shaw,G.M., Arya,S.K., Popovic,M., Gallo,R.C. and Wong-Staal,F. (1984) Molecular cloning and characterization of the HTLV-III virus associated with AIDS. *Nature*, **312**, 166–169.

Hazuda,D.J., Wolfe,A.L., Hastings,J.C., Robbins,H.L., Graham,P.L., LaFemina,R.L. and Emini,E.A. (1994) Viral long terminal repeat substrate binding characteristics of the human immunodeficiency virus type 1 integrase. *J. Biol. Chem.*, **269**, 3999–4004.

Heinzinger,N.K., Bukrinsky,M.I., Haggerty,S.A., Ragland,A.M., Kewalramani,V., Lee,M.-A., Gendelman,H.E., Ratner,L., Stevenson,M. and Emerman,M. (1994) The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. *Proc. Natl Acad. Sci. USA*, **91**, 7311–7315.

Katz,R.A., Merkel,G. and Skalka,A.M. (1996) Targeting of retroviral integrase by fusion to a heterologous DNA binding domain: *In vitro* activities and incorporation of a fusion protein into viral particles. *Virology*, **217**, 178–190.

Kewalramani,V.N. and Emerman,M. (1996) Vpx association with mature core structures of HIV-2. *Virology*, **218**, 159–168.

Kimpton,J. and Emerman,M. (1992) Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated b-galactosidase gene. *J. Virol.*, **66**, 2232–2239.

Kulkosky,J., Jones,K.S., Katz,R.A., Mack,J.P.G. and Skalka,A.M. (1992) Residues critical for retroviral integrative recombination in a region that is highly conserved among retroviral/retrotransposon integrases and bacterial insertion sequence transposases. *Mol. Cell. Biol.*, **12**, 2331–2338.

Kulkosky,J. and Skalka,A.M. (1994) Molecular mechanism of retroviral DNA integration. *Pharmac. Ther.*, **61**, 185–203.

Landau,N.R., Page,K.A. and Littman,D.R. (1991) Pseudotyping with human T-cell leukemia virus type 1 broadens the human immunodeficiency virus host range. *J. Virol.*, **65**, 162–169.

Leavitt,A.D., Shiue,L. and Varmus,H.E. (1993) Site-directed mutagenesis of HIV-1 integrase demonstrates differential effects on integrase function *in vitro*. *J. Biol. Chem.*, **268**, 2113–2119.

Leavitt,A.D., Robles,G., Alesandro,N. and Varmus,H.E. (1996) Human immunodeficiency virus type 1 integrase mutants retain *in vitro* integrase activity yet fail to integrate viral DNA efficiently during infection. *J. Virol.*, **70**, 721–728.

Li,Y., Kappes,J.C., Conway,J.A., Price,R.W., Shaw,G.M. and Hahn,B.H. (1991) Molecular characterization of human immunodeficiency virus type 1 cloned directly from uncultured human brain tissue: identification of replication-competent and -defective viral genomes. *J. Virol.*, **65**, 3973–3985.

Lu,Y.-L., Spearman,P. and Ratner,L. (1993) Human immunodeficiency virus type 1 viral protein R localization in infected cells and virions. *J. Virol.*, **67**, 6542–6550.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 269–295.

Masuda,T., Planelles,V., Krogstad,P. and Chen,I.S.Y. (1995) Genetic analysis of human immunodeficiency virus type 1 integrase and the U3 att site: unusual phenotype of mutants in the Zinc finger-like domain. *J. Virol.*, **69**, 6687–6696.

McEuen,A.R., Edwards,B., Koepke,K.A., Ball,A.E., Jennings,B.A., Wolstenholme,A.J., Danson,M.J. and Hough,D.W. (1992) Zinc binding by retroviral integrase. *Biochem. Biophys. Res. Commun.*, **189**, 813–818.

Miller,M.D., Warmerdam,M.T., Gaston,I., Greene,W.C. and Feinberg, M.B. (1994) The human immunodeficiency virus-1 nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages. *J. Exp. Med.*, **179**, 101–113.

Miller,M.D., Bor,Y.-C. and Bushman,F.D. (1995) Target DNA capture by HIV-1 integration complexes. *Curr. Biol.*, **5**, 1047–1056.

Minassian,A.A., Kalyanaraman,V.S., Gallo,R.C. and Popovic,M. (1988) Monoclonal antibodies against human immunodeficiency virus (HIV) type 2 core proteins: Cross-reactivity with HIV type 1 and simian immunodeficiency virus. *Proc. Natl Acad. Sci. USA*, **85**, 6939–6943.

Muesing,M. A., Smith,D.H., Cabradilla,C.D., Benton,C.V., Lasky,L.A. and Capon,D.J. (1985) Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus. *Nature*, **313**, 450–458.

Naldini,L., Blomer,U., Gallay,P., Ory,D., Mulligan,R., Gage,F.H., Verma,I.M. and Trono,D. (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science*, **272**, 263–267.

Parolin,C., Dorfman,T., Palu,G., Göttlinger,H. and Sodroski,J. (1994) Analysis in human immunodeficiency virus type 1 vectors of cis-acting sequences that affect gene transfer into human lymphocytes. *J. Virol.*, **68**, 3888–3895.

Paxton,W., Connor,R.I. and Landau,N.R. (1993) Incorporation of Vpr into human immunodeficiency virus type 1 virions: requirement for the p6 region of gag and mutational analysis. *J. Virol.*, **67**, 7229–7237.

Pettit,S.C., Simsic,J., Loeb,D.D., Everitt,L., Hutchison,C.A.,III and Swanstrom,R. (1991) Analysis of retroviral protease cleavage sites reveals two types of cleavage sites and the structural requirements of the P1 amino acid. *J. Biol. Chem.*, **266**, 14539–14547.

Plasterk,R.H.A. (1995) The HIV integrase catalytic core. *Struct. Biol.*, **2**, 87–90.

Rice,P. and Mizuuchi,K. (1995) Structure of the bacteriophage Mu transposase core: a common structural motif for DNA transposition and retroviral integration. *Cell*, **82**, 209–220.

Sato,A., Yoshimoto,J., Isaka,Y., Miki,S., Suyama,A., Adachi,A., Hayami,M., Fujiwara,T. and Yoshie,O. (1996) Evidence for direct association of Vpr and matrix protein p17 within the HIV-1 virion. *Virology*, **220**, 208–212.

Schauer,M. and Billich,A. (1992) The N-terminal region of HIV-1 integrase is required for integration activity, but not for DNA-binding. *Biochem. Biophys. Res. Commun.*, **185**, 874–880.

Taddeo,B., Carlini,F., Verani,P. and Engelman,A. (1996) Reversion of a human immunodeficiency virus type 1 integrase mutant at a second site restores enzyme function and virus infectivity. *J. Virol.*, **70**, 8277–8284.

Trono,D. (1995) HIV accessory proteins: Leading roles for the supporting cast. *Cell*, **82**, 189–192.

van Gent,D.C., Oude Groeneger,A.A.M and Plasterk,R.H.A. (1992) Mutational analysis of the integrase protein of human immunodeficiency virus type 2. *Proc. Natl Acad. Sci. USA*, **89**, 9598–9602.

van Gent,D.C., Vink,C., Oude Groeneger,A.A.M. and Plasterk,R.H.A. (1993) Complementation between HIV integrase proteins mutated in different domains. *EMBO J.*, **12**, 3261–3267.

von Schwedler,U., Kornbluth,R.S. and Trono,D. (1994) The nuclear localization signal of the matrix protein of human immunodeficiency virus type 1 allows the establishment of infection in macrophages and quiescent T lymphocytes. *Proc. Natl Acad. Sci. USA*, **91**, 6992–6996.

Vile,R.G. and Russell,S.J. (1995) Retroviruses as vectors. *Br. Med. Bull.*, **51**, 12–30.

Vincent,K.A., Ellison,V., Chow,S.A. and Brown,P.O. (1993) Characterization of human immunodeficiency virus type 1 integrase expressed in *Escherichia coli* and analysis of variants with amino-terminal mutations. *J. Virol.*, **67**, 425–437.

Vink,C., Groenink,M., Elgersma,Y., Fouchier,R.A.M., Tersmette,M. and Plasterk,R.H.A. (1990) Analysis of the junctions between human immunodeficiency virus type 1 proviral DNA and human DNA. *J. Virol.*, **64**, 5626–5627.

Vink,C., Oude Groeneger,A.A.M. and Plasterk,R.H.A. (1993) Identification of the catalytic and DNA-binding region of the human immunodeficiency virus type 1 integrase protein. *Nucleic Acids Res.*, **21**, 1419–1425.

Wiskerchen,M. and Muesing,M.A. (1995) Human immunodeficiency virus type 1 integrase: effects of mutations on viral ability to integrate, direct viral gene expression from unintegrated viral DNA templates, and sustain viral propagation in primary cells. *J. Virol.*, **69**, 376–386.

Wei,X., Ghosh,S.K., Taylor,M.E., Johnson,V.A., Emini,E.A., Deutsch,P., Lifson,J.D., Bonhoeffer,S., Nowak,M.A., Hahn,B.H., Saag,M.S. and Shaw,G.M. (1995) Viral dynamics in human immunodeficiency virus type 1 infection. *Nature*, **373**, 117–122.

Woerner,A.M. and Marcus-Sekura,C.J. (1993) Characterization of a DNA binding domain in the C-terminus of HIV-1 integrase by deletion mutagenesis. *Nucleic Acids Res.*, **21**, 3507–3511.

Wu,X., Liu,H., Xiao,H., Kim,J., Seshaiah,P., Natsoulis,G., Boeke,J.D., Hahn,B.H. and Kappes,J.C. (1995) Targeting foreign proteins to human immunodeficiency virus particles via fusion with Vpr and Vpx. *J. Virol.*, **69**, 3389–98.

Wu,X., Liu,H.M. and Kappes,J.C. (1997) Virion incorporation of Vpr-RT fusion protein rescues replication of RT-defective HIV-1. 4th Conference on Retroviruses and Opportunistic Infections, Washington, D.C.

Yu,X., Matsuda,F.M., Yu,Q.-C., Lee,T.-H. and Essex,M. (1993) Vpx of Simian Immunodeficiency virus is located primarily outside the virus core in mature virions. *J. Virol.*, **67**, 4386–4390.

Received on April 1, 1997; revised on May 25, 1997