

Review

Integration Site Selection by Retroviral Vectors: Molecular Mechanism and Clinical Consequences

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Abstract

Retroviral DNA integration into the host cell genome is an essential feature of the retroviral life cycle. The ability to integrate their DNA into the DNA of infected cells also makes retroviruses attractive vectors for delivery of therapeutic genes into the genome of cells carrying adverse mutations in their cellular DNA. Sequencing of the entire human genome has enabled identification of integration site preferences of both replication-competent retroviruses and retroviral vectors. These results, together with the unfortunate outcome of a gene therapy trial, in which integration of a retroviral vector in the vicinity of a protooncogene was associated with the development of leukemia, have stimulated efforts to elucidate the molecular mechanism underlying integration site selection by retroviral vectors, as well as the development of methods to direct integration to specific DNA sequences and chromosomal regions. This review outlines our current knowledge of the mechanism of integration site selection by retroviruses *in vitro*, in cultured cells, and *in vivo*; the outcome of several of the more recent gene therapy trials, which employed these vectors; and the efforts of several laboratories to develop vectors that integrate at predetermined sites in the human genome.

Introduction

EARLY STEPS of the retroviral life cycle include entry, reverse transcription, import of viral DNA and certain viral proteins into the cell nucleus, and finally, insertion of viral DNA into the host cell genome (Fig. 1; Flint *et al.*, 2004). The joining of viral to host cell DNA is termed *integration*, and like the other early steps, is shared by both retroviruses and retroviral vectors. The ability to catalyze integration makes retroviral vectors a valuable tool for gene therapy. The inserted DNA becomes a part of the cellular genome and is thus stably maintained in the infected cells.

The mechanism of integration (how viral DNA is joined to host cell DNA) has been thoroughly studied *in vitro* and the role of viral proteins in the process has been revealed to a large extent. In addition to viral proteins, integration also involves host cell proteins. Some of these have been identified. Their roles, as well as those of viral proteins, are discussed in the first section of this review. In contrast to the mechanism of integration, integration site selection, that is, where in the host cell genome integration occurs, and the process of selection of integration site(s), has been until re-

cently less well understood. The integration site selection process has been studied *in vitro*, as well as in cultured cells, and *in vivo*, in both human and murine models. Studies taking advantage of the human genome project have identified integration site preferences for several species of retroviruses and retroviral vectors. These results, as well as our current understanding of the underlying molecular mechanism of integration site selection, are summarized in the second part of this review. The next section discusses the role integration site selection plays in gene therapy in light of results that point to highly significant clinical consequences of the process in several gene therapy trials. The final section of this review describes attempts to control the process by genetic manipulation of the proteins that are involved in integration.

Integration

The retroviral enzyme integrase (IN) plays an essential role in integration. This enzyme is contained in the virion and later the preintegration complex (see Fig. 1). IN works as a multimer (probably a tetramer) and can catalyze integration reactions *in vitro*, even in the absence of other viral

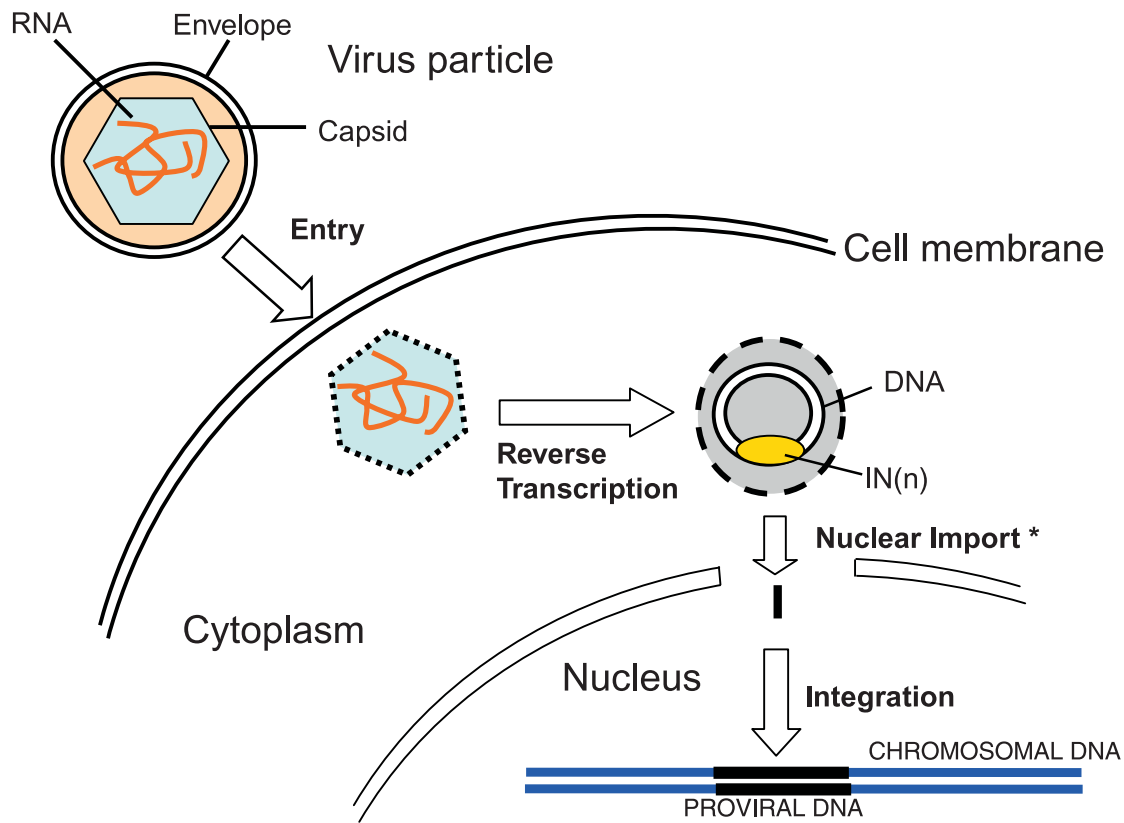


FIG. 1. Early steps of the retroviral life cycle. After entry, the RNA genome of the retrovirus or retroviral vector undergoes reverse transcription in the cytoplasm of infected cells. Viral DNA, integrase (IN), and certain viral and cellular proteins constitute the preintegration complex, which is imported into the nucleus (Flint *et al.*, 2004). IN then catalyzes the integration of viral DNA into the host cell DNA. As indicated by the asterisk (*), this process applies only to some retroviruses, such as human immunodeficiency virus (HIV)-1 and avian sarcoma virus (ASV; Flint *et al.*, 2004). Murine leukemia virus (MLV) and MLV-based vectors cannot pass readily through the nuclear pore and access the host cell DNA unless the nuclear envelope is dissolved during mitosis (Roe *et al.*, 1993). IN(n), integrase multimer.

or cellular proteins (Coffin *et al.*, 1997; Flint *et al.*, 2004). The integration reaction itself can be divided into two distinct steps. In the first step, IN removes two nucleotides from the 3' ends of the viral DNA, the synthesis of which was completed by the viral enzyme reverse transcriptase (Fig. 2; Coffin *et al.*, 1997; Flint *et al.*, 2004). This step of integration is termed *processing* (Katz and Skalka, 1994; Coffin *et al.*, 1997; Flint *et al.*, 2004). The second step of integration, called *joining*, occurs when the viral preintegration complex reaches the host cell chromatin (see Figs. 1 and 2) (Coffin *et al.*, 1997; Flint *et al.*, 2004). During joining, IN catalyzes a coupled cleavage-joining reaction, in which the 3' ends of viral DNA are joined to host cell DNA (Fig. 2). The product of the integration reaction is an intermediate, in which viral DNA is flanked by short single-stranded gaps in host DNA. The two steps of integration are then followed by postintegration repair, which includes trimming of the 5' ends of viral DNA, filling of the single-stranded gaps in host DNA, ligation of the host cell DNA sequences to the 5' ends of viral DNA, and, finally, reconstitution of appropriate chromatin structure at the integration site. Postintegration repair is not performed by IN, but requires host cell DNA repair proteins (Daniel *et al.*, 1999; Lau *et al.*, 2005; Skalka and Katz, 2005; Daniel, 2006; Smith and Daniel, 2006).

Integration can be reconstituted *in vitro* with purified IN

and model DNA substrates. Whereas most of these assays employ oligonucleotides, and only one end of a DNA substrate that represents viral DNA is joined to target DNA, some assays use miniviral DNA substrates and demonstrate concerted processing and joining of two viral ends (Flint *et al.*, 2004). IN is thus sufficient to perform both steps of the integration reaction *in vitro*. *In vivo*, however, integration is likely to depend on host cell proteins that may facilitate the reaction. Several cellular proteins have attracted considerable interest as potential cofactors of integration. A yeast two-hybrid screen identified a human immunodeficiency virus (HIV)-1 IN-binding protein termed INI1 (integrase interactor 1; Kalpana *et al.*, 1994). At first INI1 was shown to increase integration efficiency when added to the integration reaction *in vitro* (Kalpana *et al.*, 1994). Experiments with small interfering RNA (siRNA) targeting INI1 (Ariumi *et al.*, 2006) showed that HIV-1 replication was significantly reduced in cells with knocked-down INI1. However, integration was reported to occur in INI1-deficient cultured cells just as efficiently as when INI1 is present (Boese *et al.*, 2004). Thus, it is now believed that INI1 does not seem to affect integration per se. However, INI1 has been demonstrated to be involved in other steps of the retroviral life cycle (Ariumi *et al.*, 2006; Mahmoudi *et al.*, 2006; Treand *et al.*, 2006). Similar to INI1, a cellular high-mobility group protein-1 (HMG-1) was found

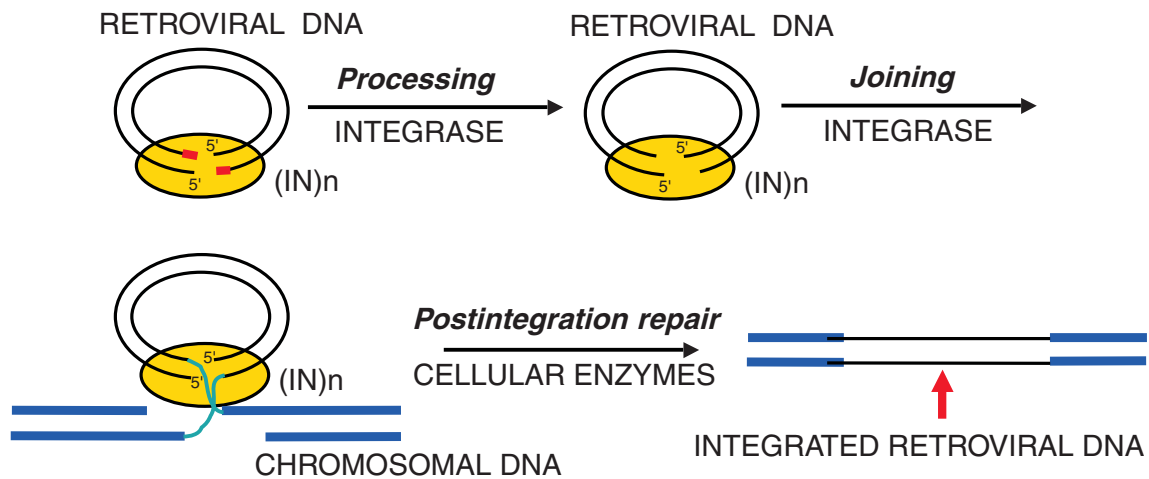


FIG. 2. Retroviral DNA integration. For details see text. IN(n), integrase multimer.

to enhance integration *in vitro* (Aiyar *et al.*, 1996). HMG proteins are nonhistone chromatin proteins and such enhancement could be due to their DNA-bending ability (Aiyar *et al.*, 1996; Flint *et al.*, 2004). A closely related protein, HMG-I(Y), was found in HIV-1 preintegration complexes isolated from infected cultured cells (Farnet and Bushman, 1997). As with HMG-1, HMG-I(Y), as well as HMG-2, enhance integration *in vitro* (Aiyar *et al.*, 1996; Farnet and Bushman, 1997; Hindmarsh *et al.*, 1999). However, experiments with cells lacking HMG-I(Y) failed to demonstrate a role for this protein in integration (Beitzel and Bushman, 2003).

The role of HMG proteins in integration thus remains unclear. The proper target for integration is host cell DNA, and integration into viral DNA itself, called *autointegration*, is expected to abort the retroviral life cycle. A biochemical analysis of murine leukemia virus (MLV) preintegration complexes identified the presence of a small protein (89 amino acids) that prevents autointegration of viral DNA, and was thus called the barrier-to-autointegration factor (BAF) (Lee and Craigie, 1998). BAF was also found in HIV-1 preintegration complexes, also blocking autointegration (Lin and Engelman, 2003). Finally, in 2003, use of the yeast two-hybrid system led to the isolation of a new HIV-1 IN-binding protein, a previously identified cellular protein termed LEDGF/p75 (lens epithelium-derived growth factor; Cherepanov *et al.*, 2003). Ironically, knockout experiments demonstrated that LEDGF/p75 is not a lens growth factor (Sutherland *et al.*, 2006). Instead, animals lacking the mouse LEDGF/p75 homolog, PSIP1 (PC4 and SFRS1-interacting protein-1), have skeletal abnormalities, indicating that this protein is involved in bone development (Sutherland *et al.*, 2006). However, suppression of LEDGF/p75 with siRNA, as well as experiments with primary LEDGF/p75 null cells from the LEDGF/p75 null transgenic animals, showed that integration of HIV-1-based vectors is reduced 89–96% in the absence of LEDGF/p75 (Llano *et al.*, 2006a; Shun *et al.*, 2007). Therefore, LEDGF/p75 is required for efficient integration of HIV-1 DNA. Interestingly, LEDGF/p75 does not bind to MLV IN, nor is it required for MLV integration (Llano *et al.*, 2004b; Busschots *et al.*, 2005; Shun *et al.*, 2007). LEDGF/p75 enhances integration *in vitro* and, in addition to this effect, plays a major role in integration site selection of HIV-1 and

HIV-1-based vectors, as discussed below (Ciuffi *et al.*, 2005, 2006; Llano *et al.*, 2006b; Shun *et al.*, 2007).

In summary, retroviral DNA integration is catalyzed by the viral protein integrase, but host cell proteins appear to play a significant role in enhancing the efficiency of the reaction, and in preventing autointegration.

Integration Site Preferences of Retroviruses and Retroviral Vectors

Integration can occur anywhere throughout the host cell genome and there are no strict host sequence requirements for site selection. However, integration is not random (Schroder *et al.*, 2002; Wu *et al.*, 2003; Mitchell *et al.*, 2004). *In vitro* systems, which employ purified IN and naked target DNA, demonstrated that certain DNA-binding proteins can prevent access of IN to target DNA and thus block integration at their binding sites (Pryciak and Varmus, 1992; Bushman, 1994). In contrast, bending or distortion of target DNA appears to stimulate integration (Pryciak and Varmus, 1992; Pryciak *et al.*, 1992a; Pruss *et al.*, 1994a,b; Katz *et al.*, 1998). When naked target DNA is replaced by DNA that is partly wrapped around nucleosomes, distortion of DNA promotes integration within the nucleosome-bound DNA (Pryciak and Varmus, 1992; Pryciak *et al.*, 1992b; Pruss *et al.*, 1994a). These systems thus reveal certain integration site preferences within model DNA substrates. However, as host DNA exists in a higher order chromatin structure, the results of these *in vitro* studies may not be relevant to events that take place in infected cells. To remedy this deficiency of *in vitro* systems, one system employed an extended 13-nucleosome array, which could be compacted into a higher order chromatin structure by addition of the histone H1 (Taganov *et al.*, 2004). It was observed that the chromatin structure affects integration site selection of HIV-1 and avian sarcoma virus (ASV) IN proteins in opposite ways. Specifically, HIV-1 IN-mediated integration was decreased after compaction of the target chromatin, whereas ASV IN-mediated integration was more efficient after compaction. These data suggested that a higher order chromatin structure may indeed affect site selection, and different retroviral species may exhibit different integration preferences.

There are approximately 25,000 genes in the human genome (International Human Genome Sequencing Consortium, 2004). Early studies suggested that retroviruses may integrate in or in the vicinity of transcription units (Mooslehner *et al.*, 1990; Scherdin *et al.*, 1990). However, these studies were hampered by a relatively low number of the identified transcription sites (Bushman *et al.*, 2005). Moreover, it was not clear what percentage of the genome contains these “favored” integration sites. The situation changed after the sequencing of the human genome was completed, which enabled a true statistical analysis of integration sites. Large-scale analyses of integration site selection of HIV-1 in human T cell lines demonstrated that approximately 70% of integration events occurred in genes (Schroder *et al.*, 2002; Bushman *et al.*, 2005). As about 30% should be expected if integration were random, this preference for genes is highly significant. In addition, some integration “hotspots” were found (11q13 chromosomal region). No preferences were detected within transcription units. Similar data were obtained with pseudotyped HIV-1-based vectors (Schroder *et al.*, 2002). Integration preferences of the related simian immunodeficiency virus (SIV), an SIV-based vector, and HIV-2 closely resemble that of HIV-1 (Hematti *et al.*, 2004; Crise *et al.*, 2005; MacNeil *et al.*, 2006). Integration preferences of the feline immunodeficiency virus (FIV) also resemble HIV-1 preferences (Kang *et al.*, 2006).

MLV shows different integration preferences compared with HIV-1. Approximately 20% of integration events occur in the vicinity of the 5' ends of transcription units (Wu *et al.*, 2003). The remaining integration sites are distributed in a somewhat random fashion. In addition, approximately 17% of MLV integration events occur in the vicinity of CpG islands (Mitchell *et al.*, 2004), and an increased frequency of integration was also noted in the vicinity of DNase I-hypersensitive sites (11%; Lewinski *et al.*, 2006). Similar data were obtained with MLV-based vectors (Wu *et al.*, 2003; Mitchell *et al.*, 2004). Finally, a preference, albeit a weak one, for integration near transcription start sites was shown by foamy viruses (FVs; Trobridge *et al.*, 2006; Beard *et al.*, 2007). FV also shows a preference for CpG islands that is similar to that of MLV (Trobridge *et al.*, 2006).

Avian retroviruses and vectors exhibit yet another integration preference: only a weak preference for genes was detected (about 40%) and no MLV-like preference for 5' ends of transcription units (Mitchell *et al.*, 2004; Narezkina *et al.*, 2004). Interestingly, high levels of transcription may even inhibit ASV integration in genes (Weidhaas *et al.*, 2000; Maxfield *et al.*, 2005). These preferences are consistent with the above-described data from the *in vitro* system, which used nucleosomal arrays (Taganov *et al.*, 2004). Interestingly, the human T-leukemia virus type 1 (HTLV-1) and mouse mammary tumor virus (MMTV), like avian retroviruses, do not specifically target genes and transcription start sites (Derse *et al.*, 2007; Faschinger *et al.*, 2008).

Finally, careful examination of a large number of integration sites from HIV-1, SIV, MLV, and avian sarcoma-leukosis viruses revealed symmetric base preferences surrounding integration sites (Holman and Coffin, 2005; Wu *et al.*, 2005). These weak consensus sequences are virus specific and possibly reflect the influence of IN on integration site selection (Holman and Coffin, 2005). This hypothesis is also supported by the symmetry of the target site sequence, because

IN likely functions as a tetramer (Coffin *et al.*, 1997; Flint *et al.*, 2004; Wu *et al.*, 2005; and see above).

In summary, integration preferences described in this section are distinct for different groups of retroviruses. The first group, exemplified by HIV-1 and including HIV-2, SIV, and FIV, preferentially integrates into genes. The second group consists of MLV and FV. These retroviruses show preferences for 5' ends of transcription units and CpG islands. Finally, the last group includes ASV, HTLV-1, and MMTV. Members of this group show weak or no preferences for genes or transcription start sites. In addition to these preferences, the specific DNA sequence appears to play a role in integration site selection, but the primary mechanism seems to be independent of the DNA sequence, and likely includes cellular cofactors and other cellular structures. The next section summarizes our current understanding of virus–cell interactions that partake in integration site selection.

Mechanism of Integration Site Selection

As noted previously, IN shows low specificity for binding to host cell DNA. Therefore, it would seem natural for host cell proteins to participate in the integration process. In 2003, Debyser and coworkers used the yeast two-hybrid system to identify a new HIV-1 IN-binding protein, termed LEDGF/p75 (Cherepanov *et al.*, 2003). As noted above, LEDGF/p75 is required for efficient integration of HIV-1 DNA. A molecular analysis showed that LEDGF/p75 is a transcription factor and has a C-terminal IN-binding domain and N-terminal chromatin-binding domain (Maertens *et al.*, 2003; Cherepanov *et al.*, 2004; Vanegas *et al.*, 2005; Llano *et al.*, 2006b; Turlure *et al.*, 2006). Chromatin binding is mediated by PWWP and AT-hook motifs in the N-terminal domain of LEDGF/p75 (Llano *et al.*, 2006b; Turlure *et al.*, 2006). In cultured cells, LEDGF/p75 was found associated with preintegration complexes of HIV-1 and FIV (Llano *et al.*, 2004b). In addition to stimulating IN activity *in vitro*, LEDGF/p75 appears to prevent degradation of ectopically expressed HIV-1 IN by the proteasome, and therefore might contribute to the stability of preintegration complexes during infection (Maertens *et al.*, 2003; Llano *et al.*, 2004a). In addition to these potential LEDGF/p75 effects on IN and integration, an analysis of the integration sites in LEDGF/p75 null cells showed that the residual integration in these cells no longer occurs preferentially in active genes (Shun *et al.*, 2007). Instead, integration occurred preferentially in the vicinity of promoters and CpG islands (Shun *et al.*, 2007). The symmetric base preferences surrounding the integration site remained preserved (Holman and Coffin, 2005; Shun *et al.*, 2007). Thus, in the absence of LEDGF/p75, HIV-1 integration site preferences resemble those of MLV (Shun *et al.*, 2007). Taken together, these results strongly support the hypothesis that LEDGF/p75 targets HIV-1 (and other lentiviral) integration into active genes by tethering the IN protein to chromatin (Fig. 3).

Although LEDGF/p75 appears to be a major HIV-1 IN-binding cellular protein, other factors are likely involved in integration site selection by HIV-1 and HIV-1-based vectors. Analysis of a large number of integration sites showed that favored integration sites occur in the vicinity of certain computer-predicted epigenetic marks, such as histone H3 K4 methylation, H4 acetylation, or H3 acetylation (Wang *et al.*,

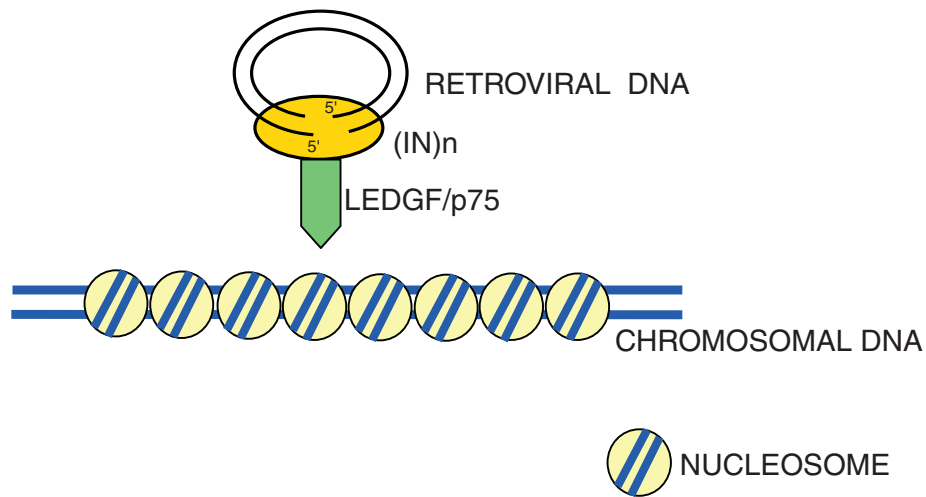


FIG. 3. LEDGF/p75 role in integration site selection. LEDGF/p75 binds to specific sites, thereby mediating contact between IN and host cell chromatin. This association tethers the HIV-1 preintegration complex near or in genes.

2007). Taken together, these data may suggest that the chromatin structure, including the histone code, may also affect integration site selection (Fig. 4). However, final proof that these marks play a role in integration site selection has yet to be shown. Additional factors that may affect integration site selection have been identified. Knockdown of the T-cell lineage-specific chromatin organizer, SATB1 (special AT-rich sequence-binding protein-1), reduces HIV-1 integration near SATB1-binding sites (Kumar *et al.*, 2007). SATB1 thus appears to be involved in integration site selection, by an un-

known mechanism. Finally, it has been suggested that the cellular protein Ku80, which is present in the preintegration complex, targets integration toward chromatin domains prone to silencing (Li *et al.*, 2001; Masson *et al.*, 2007).

In contrast to HIV-1, integration of MLV-based and ASV-based vectors does not seem to be influenced by LEDGF/p75 (see above; and see Mitchell *et al.*, 2004; Narezkina *et al.*, 2004). What determines ASV integration site selection is not known. In the case of MLV, a study of HIV chimeras with MLV genes showed that MLV IN seems to be the principal

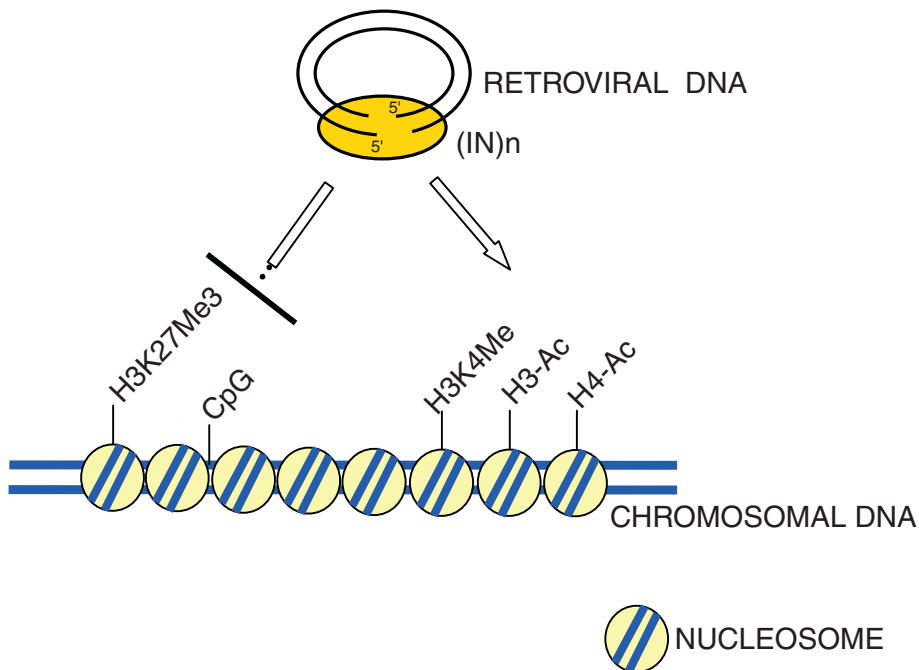


FIG. 4. A possible role for chromatin structure in HIV-1 DNA integration. HIV-1 DNA integration occurs preferentially in the vicinity of certain epigenetic modifications, such as H3 K4 methylation (H3K4Me) and H3 and H4 acetylation (H3-Ac and H4-Ac, respectively). Integration seems to be disfavored in the vicinity of H3K27 trimethylation (H3K27Me3) and CpG islands in the DNA (see text and Wang *et al.*, 2007).

determinant of integration site selection (Lewinski *et al.*, 2006). Interestingly, Gag-derived proteins play an auxiliary role in the process, as an HIV chimera containing MLV Gag displayed targeting preferences different from those of both HIV and MLV (Lewinski *et al.*, 2006). These results thus support a different mechanism of integration site selection for MLV versus HIV. Finally, in addition to Gag proteins, other viral proteins could play a role in integration site selection. However, examination of auxiliary proteins in SIV integration showed that Vif, Vpr, Vpx, Nef, Env, and promoter or enhancer regions are not required for preferential SIV integration into genes (Monse *et al.*, 2006).

In summary, more recent results have increased our understanding of the mechanism of retroviral integration site selection and show a major role of host cell proteins in the process. However, the process has yet to be completely understood and it is likely that new players in retroviral integration site selection will be revealed in future studies.

Integration Site Selection and Gene Therapy

Gene therapy trials, as well as gene therapy experiments in animal systems, commonly employ vectors that are based on either MLV or HIV-1. What could be the significance of the integration preferences of these vectors in gene therapy trials?

The tendency of MLV-based vectors to integrate at the 5' end of transcription units, and HIV-1 preferences for genes, could suggest increased danger of an adverse event during gene therapy, due to either activation or disruption of a cellular gene which could potentially stimulate tumorigenesis. Alternatively, it is possible that integration at an undesirable site occurs at such a low frequency that it may not affect a therapeutic outcome. In this context, it is important to note that carcinogenesis is a multistep process and even if integration occurs in a "wrong spot," it may not lead to tumor development (Hahn and Weinberg, 2002; Baum *et al.*, 2006).

Supporting evidence for the hypothesis that integration may have adverse consequences was provided by a human gene therapy trial involving children with X-linked severe combined immunodeficiency (SCID-X1) (Gunzburg, 2003; Hacein-Bey-Abina *et al.*, 2003; Alexander *et al.*, 2007; Bushman, 2007; Deichmann *et al.*, 2007). In this trial, which used an MLV-based vector, 4 of 11 patients developed T cell leukemia. In addition, it has been reported that a single patient (of 10 enrolled) developed leukemia in another SCID-X1 gene therapy trial (Alexander *et al.*, 2007; Schwarzwaelder *et al.*, 2007; Thrasher and Gaspar, 2007). A sequencing analysis of the T cells from two of the patients in the first trial, who developed leukemia first, demonstrated clonal expansion of T cell clones that contained an insertion of the vector in the vicinity of (and subsequent activation of) the Lin-1, Isl-1, Mec-3 (LIM) domain only-2 (LMO2) protooncogene by the long terminal repeat (LTR) enhancer of the vector (Hacein-Bey-Abina *et al.*, 2003). In addition, it appears that insertion in the vicinity of the LMO2 protooncogene also occurred in the patient from the second trial (Thrasher and Gaspar, 2007). These results strongly suggest that vector integration at a "dangerous" location in the human genome contributed to the development of leukemia in these patients. There could be other factors that played a role in the development of leukemia and have not yet been fully delineated. These may in-

clude expression of the transgene and a chromosomal rearrangement (Hacein-Bey-Abina *et al.*, 2003; Pike-Overzet *et al.*, 2006; Thrasher *et al.*, 2006; Woods *et al.*, 2006). Nevertheless, the association of leukemia with integration in the vicinity of a protooncogene suggests a high significance of integration site selection for the success or failure of gene therapy approaches using retroviral vectors.

A follow-up analysis of patients in these trials showed nonrandom distribution of integration sites *in vivo* (Deichmann *et al.*, 2007; Schwarzwaelder *et al.*, 2007). Integrations were found to occur preferentially near the 5' ends of genes and associated CpG islands (Bushman, 2007; Deichmann *et al.*, 2007; Schwarzwaelder *et al.*, 2007). This is consistent with results obtained with MLV in cultured cells (see above). Comparison of integration sites in vector-transduced cells before infusion into patients, with integration sites in cells that were recovered from patients after infusion, indicated that vector integration likely influences cell engraftment, survival, and proliferation *in vivo* (Deichmann *et al.*, 2007; Schwarzwaelder *et al.*, 2007). Likewise, clonal evolution was observed in an ADA-SCID gene therapy trial (Aiuti *et al.*, 2007). However, in this case, no adverse effects were associated with vector integration (Aiuti *et al.*, 2007). In a similar vein, integration was observed to deregulate gene expression, but did not lead to development of leukemias in other gene therapy trials (Ott *et al.*, 2006; Recchia *et al.*, 2006). In addition, the effect of integration sites on the outcome of gene therapy experiments in animal models generally resembled those observed in human gene therapy trials (Li *et al.*, 2002; Calmels *et al.*, 2005; Kustikova *et al.*, 2005; Modlich *et al.*, 2005; Montini *et al.*, 2006).

In summary, insertion of retroviral DNA into certain chromosomal locations was associated with the development of leukemias in both animal models and human gene therapy trials. However, these insertions may not be sufficient to induce malignant transformation and other events may contribute to the development of malignancies in these cases (Hacein-Bey-Abina *et al.*, 2003; Dave *et al.*, 2004). Nevertheless, these cases highlight a necessity for further improvements in the design of retroviral vectors so that they are less likely to integrate at undesirable sites in the human genome, thereby having an increased safety margin.

Efforts to Direct Integration to Predetermined DNA Sequences and Chromosomal Regions

Given the potential of retroviral vectors to integrate into undesirable sites in the human genome, it is not surprising that efforts in several laboratories have been directed toward the development of a vector that would integrate in a predetermined DNA sequence or chromosomal region. Because integration is catalyzed by IN, this protein became the focus of early efforts. Three laboratories (Bushman, 1994; Goulaouic and Chow, 1996; Katz *et al.*, 1996) initially used a strikingly similar approach to the problem. All three groups constructed fusion proteins, which consisted of IN protein either from HIV-1 or ASV, and a DNA-binding sequence from a cellular or bacterial protein. In two cases, the DNA-binding domain (DBD) was from the *Escherichia coli* LexA repressor (Goulaouic and Chow, 1996; Katz *et al.*, 1996), and in one case it was from the phage λ repressor (Bushman, 1994), fused to the N or C terminus of IN. The resulting fu-

sion proteins directed integration to their target sites in DNA *in vitro* (Bushman, 1994; Goulaouic and Chow, 1996; Katz *et al.*, 1996). Targeting integration in cultured cells, however, proved to be more difficult. The ASV IN–LexA fusion proved to be a target for the viral protease protein, which deleted a majority of the heterologous DBD (Katz *et al.*, 1996). We then attempted to mutate the protease recognition site between the DBD and the rest of the protein. The resulting mutant protein indeed proved to be resistant to viral protease *in vitro* and stable when the mutant gene was transfected together with the rest of viral DNA into chicken DF-1 cells; however, we did not obtain any viral particles containing the mutant protein, possibly because of a failure to incorporate into the virion (R. Daniel and A.M. Skalka, unpublished data). Similar problems were encountered when the LexA DBD was replaced with the DBD of the cellular GATA-6 protein (R. Daniel and A.M. Skalka, unpublished data). In another report, HIV-1 IN was fused to the zinc finger protein zif268 (Bushman and Miller, 1997). This fusion protein was incorporated into HIV-1 virions, but the virus lost its infectivity. However, viruses containing mixtures of wild-type IN and the fusion protein IN–zif268 were infective. Preintegration complexes of these viruses, when purified from cells, were able to target integration near zif268 recognition sites *in vitro* (Bushman and Miller, 1997). It is not clear whether these viruses exhibited the same properties in cultured cells.

Chow and coworkers demonstrated that an HIV-1 IN–LexA fusion protein can target integration to LexA-binding sequences *in vitro* (Goulaouic and Chow, 1996). Direct cloning in the HIV-1 IN gene region is difficult because of

an overlap with the open reading frame of *vif* and the presence of a splice acceptor site (Purcell and Martin, 1993). Thus, these investigators took advantage of the packaging properties of the HIV-1 Vpr protein, which enable other proteins to be incorporated into HIV-1 particles *in trans*, when they are fused to Vpr (Wu *et al.*, 1995). Vpr is incorporated into the particle through an interaction with the C terminus of the p6 protein in Gag (for review and summary articles on particle formation see Lu *et al.*, 1995; Kondo and Gottlinger, 1996; Flint *et al.*, 2004; Hill *et al.*, 2005). It should be noted that there are about 100–200 copies of Vpr and approximately 50–100 copies of IN per virion (Flint *et al.*, 2004). The wild-type IN protein in these viruses was inactivated by the D64V mutation in the IN catalytic site and the IN–LexA protein was then fused to Vpr so it would be incorporated into the virion. The viral particle carrying IN–LexA could infect and integrate its DNA in host cells, at an efficiency of 17 to 24% of the wild-type virus (Holmes-Son and Chow, 2000). To understand the mechanism of complementation, virus–host DNA junctions from cells infected with these viruses were then cloned and sequenced. Correct integration hallmarks were observed, including the duplication of host DNA sequence flanking the provirus, as well as 5′-TG/CA-3′ ends of viral DNA (Holmes-Son and Chow, 2000). LexA binds to its operator DNA sequence with a relatively low specificity (Lewis *et al.*, 1994). To increase specificity, IN was then fused to a designed polydactyl zinc finger protein E2C, which binds to a unique 18-bp sequence in the human genome (Beerli *et al.*, 1998, 2000; Tan *et al.*, 2004). These fusion proteins again targeted integration preferentially to the E2C-

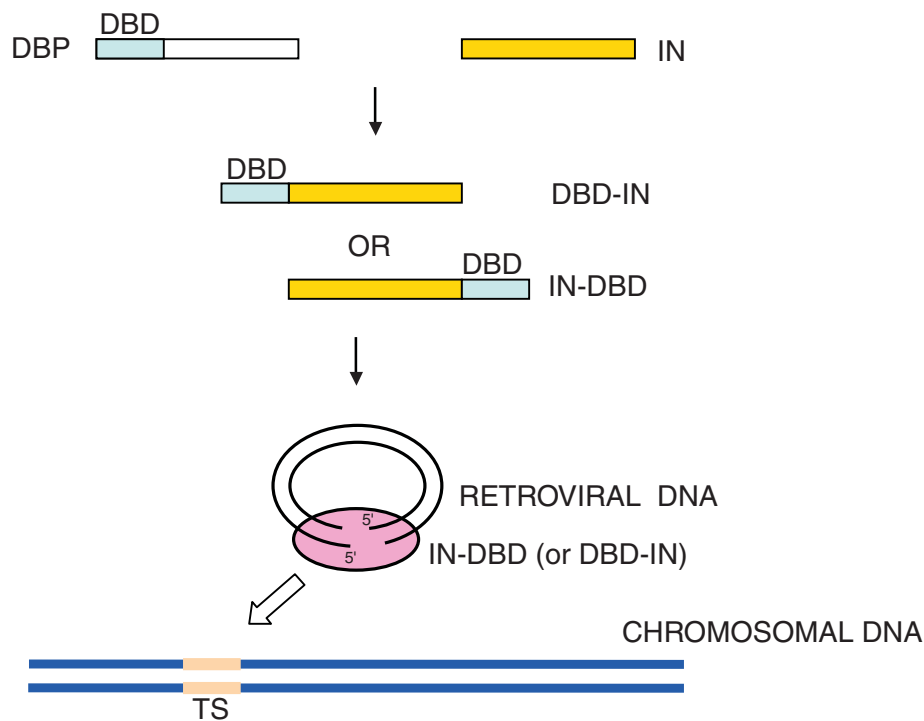


FIG. 5. A model for targeting integration by fusion proteins of integrase and a heterologous DNA-binding domain. A DNA-binding domain (DBD) of a cellular DNA-binding protein (DBP) can be fused to either the N terminus or C terminus of IN. The resulting fusion protein can be incorporated into the virion and the preintegration complex, and targets integration toward the DNA sequences recognized by the DBD, the target site (TS).

binding site *in vitro*. These proteins were then incorporated into virions *in trans*, as with IN–LexA fusion proteins (Tan *et al.*, 2006). Viruses containing E2C fused to the C terminus of IN had 7-fold higher preference for integrating near the E2C-binding site than viruses containing wild-type IN. Viruses containing E2C fused to the N terminus of IN had a 10-fold higher preference for the E2C-binding site when compared with wild-type IN. Therefore, these fusion proteins can affect integration site selection in cultured cells. However, it should be noted that the overall efficiency of retroviral DNA integration in this case was 4- to 100-fold lower than with wild-type IN, which is an undesirable side effect (Tan *et al.*, 2006). Moreover, integration in the vicinity of the E2C site is still relatively rare even when compared with the overall number of integration events in these cells (up to 1.5% of the total integration sites when the N-terminal fusion protein is used; Tan *et al.*, 2006). However, these experiments provide proof-of-principle evidence that it is possible to target integration in cultured cells by manipulation of the IN protein (Fig. 5).

As described above, LEDGF/p75 is a major cellular cofactor for integration of HIV-1 DNA. Therefore, hypothetically it should be possible to target integration by manipulation of this cellular protein. One approach is to use fusion proteins that contain the LEDGF/p75 IN-binding domain and a DNA-binding domain of a transcription factor of known specificity. Indeed, it had been shown that a fusion of LEDGF/p75 with the DNA-binding domain of phage λ repressor directs integration to λ R-binding sites *in vitro* (Ciuffi *et al.*, 2006). However, it remains to be seen whether these proteins can target integration in cells.

Finally, a new and innovative approach has been described that uses engineered zinc finger nucleases (ZFNs) and integrase-defective lentiviral vectors (IDLVs) to target gene addition to specific regions (Lombardo *et al.*, 2007). ZFNs are designed to induce a double-stranded DNA break in the chromosome of a target cell, at a predetermined location (Kim *et al.*, 1996). Two different ZFNs are required for a break to occur, because of a requirement for dimer formation (Bitinaite *et al.*, 1998). This break is then repaired by the cellular DNA repair system, which involves either nonhomologous end joining or homologous recombination. The latter repair system copies a homologous DNA sequence during the repair process (Cahill *et al.*, 2006). ZFNs were shown to mediate integration of extrachromosomal DNA into a specified location when expressed in the target cell. The extrachromosomal DNA, which was delivered by transfection, carried locus-specific homology arms, in addition to a gene of interest (Moehle *et al.*, 2007). To improve efficiency of the ZFN gene delivery system, the Naldini laboratory used the above-mentioned IDLVs, which carry an inactivating mutation in the IN gene (Lombardo *et al.*, 2007). IDLVs cannot integrate; however, they can still perform reverse transcription and a gene of interest can be expressed, albeit transiently, from unintegrated DNA. Two different ZFNs and a gene of interest were subcloned into two IDLVs, which were then used to cotransduce a variety of cell types. Between 5 and 50% of infected cells were stably transduced by this method, with gene addition occurring at the ZFN target site (Lombardo *et al.*, 2007). The specificity of this ZFN method thus compares favorably with the above-described method, which uses IN fusion proteins.

In summary, a persistent effort by several laboratories has led to the development of a variety of systems for potential targeting of integration to predetermined regions of the human genome. These encouraging results may lead to safer methods used to employ retroviral vectors in gene therapy applications. New therapeutic approaches that take advantage of the novel properties of these systems are promising, and are being explored.

Summary and Future Directions

Results from a variety of experiments have increased our understanding of the integration preferences of retroviruses and retroviral vectors, as well as of the molecular mechanism underlying integration site selection. Clinical evidence shows that integration at an undesirable site of the patient's genome can have negative consequences for the outcome of the gene therapy-based treatment. Therefore, new approaches are needed to increase the safety of retroviral vectors. Among the possibilities outlined in this review are a switch to retroviral vectors that are based on avian, foamy, and possibly HIV-1 retroviruses rather than MLV; genetic manipulation of the vector integrases and/or cellular factors that are involved in integration; and use of IDLV vectors that carry designed zinc finger nucleases in order to target integration to predetermined chromosomal regions. It is hoped that these research directions will result in new vectors, which may minimize the risk of adverse events in gene therapy applications.

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