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Effect of the Internal Promoter on Insertional Gene Activation by Lentiviral Vectors with an Intact HIV Long Terminal Repeat[▽]

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Insertional mutagenesis by viral vectors is a problem in gene therapy. We recently reported that lentiviral vectors with an intact HIV long terminal repeat (LTR) caused insertional gene activation by transcripts from the 5′ LTR splicing to an adjacent gene. Here we demonstrate that the level of transcription from the 5′ LTR, and also insertional gene activation, is dependent on the internal promoter in the vector. We also show that there are more transcripts originating from the 5′ LTR than from, or reading through, the 3′ LTR. This study will allow the design of safer lentiviral vectors for applications in which an intact HIV LTR is required.

Insertional mutagenesis (IM) by gammaretroviral vectors caused leukemias in 5 out of 20 patients treated for X-linked severe combined immune deficiency in Paris and London (6, 7). This has created a need for assays to assess vector safety more thoroughly (10, 11, 14). Previously, we developed an assay to assess IM based on the cell line Bcl-15, which is dependent on interleukin-3 (IL-3) for survival (3, 4). Gammaretroviral and lentiviral vectors were tested and found to generate IL-3-independent mutants at similar rates, but the mechanisms differed. Gammaretroviral vectors caused mutagenesis through insertion in the IL-3 gene or genes listed as common insertion sites in the Retroviral Tagged Cancer Gene Database (1). Lentiviral vectors induced mutagenesis through insertion into the growth hormone receptor (*Ghr*) gene. Our extensive RNA analyses revealed that transcription of a fusion mRNA transcript initiated from the HIV 5′ long terminal repeat (LTR) and that the mRNA was spliced from the HIV splice donor site to exon 2 of *Ghr* (the first coding exon), leading to the expression of a full-length GHR protein (3). In the work presented here, we further analyzed the mechanism of mutagenesis in lentiviral vectors, testing the contribution of internal vector elements in pHV. We have also extended our analysis to a clinical vector with intact HIV LTRs which has been used for the treatment of HIV infection.

Two derivatives of the pHV lentiviral vector were tested in our IM assay (3) to determine which parts of the vector contributed to the activation of *Ghr* expression. pUBIQ contains the human ubiquitin promoter in place of spleen focus-forming virus (SFFV), and pΔWPRE has the woodchuck posttranscriptional regulatory element (WPRE) deleted (Fig. 1). Three separate IM assays were carried out with pHV, pUBIQ, and pΔWPRE. Briefly, Bcl-15 cells were infected and 48 h later plated in 24-well plates at a density of 10^6 per well without IL-3 but with 1 μg/ml bovine growth hormone to select for mutants with insertions in *Ghr* (3). The number of mutant wells was then scored. Table 1 shows that removal of the WPRE element did not affect the number of mutant wells, whereas substitution with the ubiquitin promoter significantly decreased the frequency. We also analyzed a derivative of the vector VRX496 used in gene therapy for HIV infection. This vector has an intact HIV LTR and is designed to express an HIV antisense sequence when cells carrying the vector become infected with HIV and express the viral transcriptional activator Tat; the phase I trial showed immune function improvement in some patients (9). VRX494 (8) encodes the HIV antisense sequence and also green fluorescent protein (GFP) under the control of the 5′ LTR and does not contain an internal promoter (Fig. 1). Table 1 shows that this vector did not generate any mutant wells in the Bcl-15 cell assay.

We analyzed the number of independent mutants by performing a Southern blot assay with a GFP probe and integration site cloning using inverse PCR on the mutants obtained in experiment 1 (see http://www.ac.uk/infection-immunity/themes

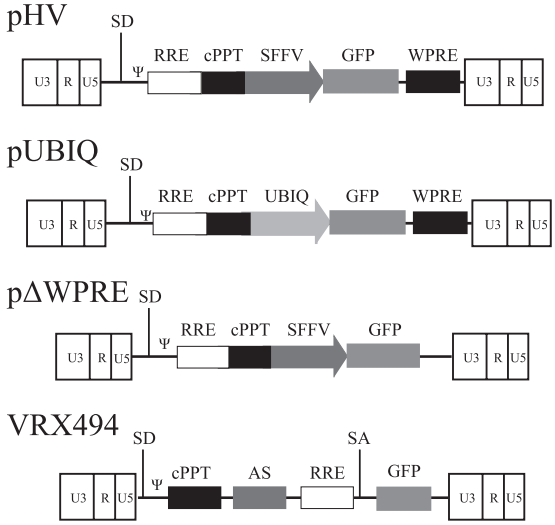

FIG. 1. Lentiviral vectors containing wild-type HIV LTRs in this study. SFFV, SFFV promoter; cPPT, central polypurine tract; RRE, Rev-responsive element; AS, antisense sequence to *env*; SD, slice donor; SA, slice acceptor.

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TABLE 1. Mutagenesis frequency[a]

Vector and expt no.	No. of cells screened	No. of integrants screened	No. of mutant wells	Cell frequency	Integrant frequency
pHV					
1	3.6×10^7	2.9×10^8	14	3.89×10^{-7}	4.83×10^{-8}
2	3.6×10^7	3.5×10^8	17	4.72×10^{-7}	4.86×10^{-8}
3	3.6×10^7	8.6×10^7	19	5.2×10^{-7}	2.2×10^{-7}
4	3.6×10^7	2.8×10^8	12	3.33×10^{-7}	4.25×10^{-8}
pUBIQ					
1	7.2×10^7	3.2×10^8	1	1.39×10^{-8}	3.13×10^{-9}
2	2.16×10^8	1.77×10^8	0	$<4.63 \times 10^{-9}$	$<5.69 \times 10^{-9}$
3	3.6×10^7	4.7×10^8	0	$<2.78 \times 10^{-8}$	$<2.13 \times 10^{-9}$
pΔWPRE					
1	3.6×10^7	3.2×10^8	7	1.94×10^{-7}	2.19×10^{-8}
2	3.6×10^7	1.03×10^8	7	1.94×10^{-7}	6.8×10^{-8}
3	3.6×10^7	3.9×10^8	17	4.72×10^{-7}	4.36×10^{-8}
VRX494					
1	7.2×10^7	9.9×10^7	0	$<1.4 \times 10^{-8}$	$<1.0 \times 10^{-8}$
2	3.6×10^7	2.94×10^8	0	$<2.78 \times 10^{-8}$	$<3.4 \times 10^{-9}$
3	3.6×10^7	2.91×10^8	0	$<2.78 \times 10^{-8}$	$<3.43 \times 10^{-9}$
Mock					
1	3.6×10^7		0	$<2.78 \times 10^{-8}$	
2	3.6×10^7		0	$<2.78 \times 10^{-8}$	
3	3.6×10^7		3	8.33×10^{-8}	
4	3.6×10^7		0	$<2.78 \times 10^{-8}$	
5	3.6×10^7		0	$<2.78 \times 10^{-8}$	

[a] For each vector, three or four experiments were conducted. The cell frequency was calculated by dividing the number of mutant wells by the total number of target cells in each experiment. The number-of-integrants calculation was based on the average number of vector copies per cell determined by qPCR at 72 h posttransduction. The integrant frequency was calculated as number of mutant wells/total number of integrants in each experiment. The frequencies of mutants obtained with pHV and pΔWPRE were not significantly different ($P = 0.4$), pHV was significantly more mutagenic than both pUBIQ ($P = 0.0286$) and VRX494 ($P = 0.0286$). pUBIQ and VRX494 were not significantly different from mock treatment ($P = 0.5$ and $P = 0.7143$). *P* values were calculated by using the Mann-Whitney U test.

/tak_supp). As we previously reported (3), the majority of mutant wells contained independent mutants. Interestingly, the UBIQ mutant was a background mutant having no vector insertion (see http://www.ac.uk/infection-immunity/themes/tak_supp). All of the other mutants analyzed had insertions in the first intron of *Ghr* in the same orientation as the gene (see http://www.ac.uk/infection-immunity/themes/tak_supp), and reverse transcription (RT)-PCR analysis revealed that all of the mutants expressed the HIV-*Ghr* fusion mRNA transcript (data not shown).

The fact that no mutants were obtained with pUBIQ showed that the SFFV LTR was necessary for mutagenesis observed with the pHV and pΔWPRE vectors. We reasoned that expression from the HIV LTR might be increased by the presence of the SFFV LTR enhancer but to a lesser extent by that of the weaker ubiquitin enhancer; this could then provide a sufficient level of HIV-*Ghr* fusion mRNA to allow a growth hormone response. To test this, we isolated single-cell clones from populations of cells infected with pHV or pUBIQ; the mean copy numbers of vector insertions in the sets of clones were 0.91 and 1.03, respectively (for the measurement of the vector copy number and expression for each clone, see http://www.ac.uk/infection-immunity/themes/tak_supp). Quantitative RT-PCR (qRT-PCR) for the HIV leader sequence showed that pHV clones collectively had significantly higher levels of HIV LTR-driven expression than pUBIQ clones (Fig. 2A). The SFFV promoter also gave a higher level of GFP expression in Bcl-15 cells than the ubiquitin promoter, as demonstrated by fluorescence-activated cell sorter analysis of bulk-infected populations of cells (Fig. 2B). We conclude that the stronger SFFV LTR enhancer/promoter increases the level of expression from the 5′ LTR; in the case of integrants in *Ghr*, this presumably allows selection of growth hormone-responsive cells.

We have identified expression from the 5′ LTR, followed by splicing to an adjacent gene, as the main mechanism of insertional gene activation by lentiviral vectors in the Bcl-15 cell assay. However, transcription from the 3′ LTR and readthrough of transcription through the 3′ LTR have also been proposed as mechanisms of IM (15). Indeed, we have detected low expression of RNA corresponding to downstream, but not upstream, regions of the vector integration in five HV mutants (Fig. 3A and B). We therefore decided to measure the quantities of all of the known vector mRNA transcripts initially in a Bcl-15-derived cell line with a single integrant in *Ghr*. Primers were designed to measure the unspliced transcript (RRE), the fusion transcript (HIV Gh), and two transcripts resulting from splicing to cryptic acceptor sites (Cryp1 and Cryp2, identified by sequencing of RT-PCR products; see http://www.ac.uk/infection-immunity/themes/tak_supp). Transcription at the 3′ LTR was assessed by using two primers sets; one detects readthrough transcription initiated upstream

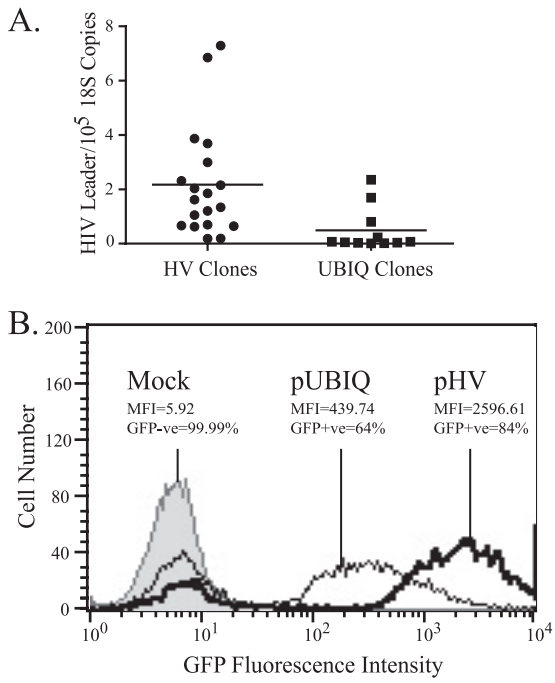

FIG. 2. HIV promoter activity in pHV and pUBIQ IL-3-dependent clones. (A) HIV leader expression is shown for 19 HV and 11 UBIQ clones. Each data point represents the expression of a single clone. The mean is shown by a black line. For the PCR primers used in qRT-PCR assays, see http://www.ac.uk/infection-immunity/themes/tak_supp. The difference between HV and UBIQ is significant ($P = 0.0141$) by the unpaired *t* test. (B) FACS plot showing difference in enhanced GFP (eGFP) expression between cells transduced with pHV and pUBIQ. The MFI (mean fluorescence intensity) for eGFP-positive (GFP+ve) populations is shown for pHV and pUBIQ. GFP−ve, eGFP negative.

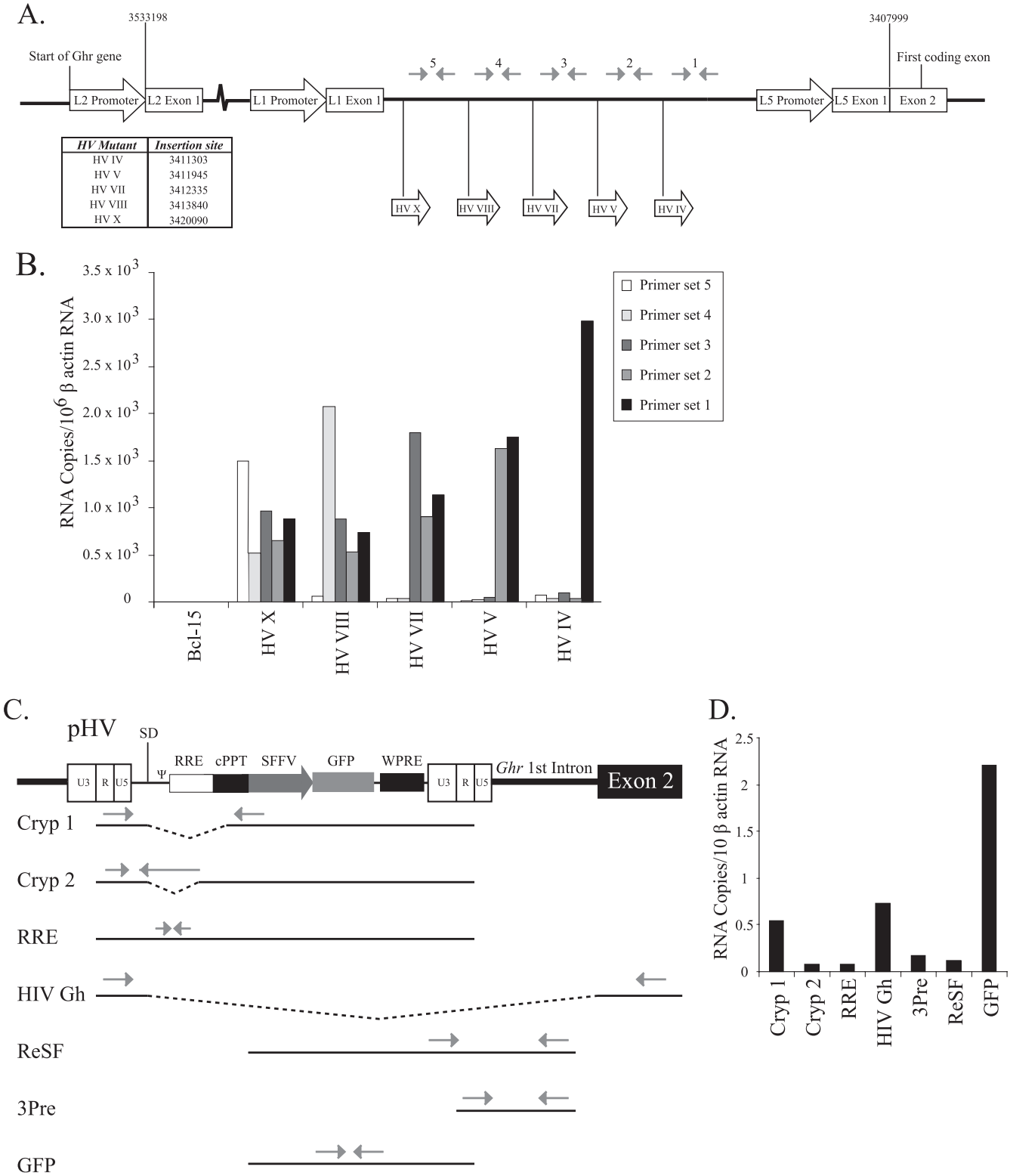

FIG. 3. Quantities of mRNA transcripts initiated within an integrated HV vector in *Ghr*. (A) Schematic of the *Ghr* first intron. Integration sites from five mutants are marked (HV IV, V, VII, VIII, and X). Converging arrows illustrate the primers used, and primer sets are labeled 1 to 5. (B) qRT-PCR of the *Ghr* first intron using each primer set on each mutant. The number of *Ghr* mRNA transcripts was normalized to the number of β-actin mRNA transcripts. (C) Schematic showing possible mRNA transcripts initiated within an HV vector and the primers used in the qRT-PCR to determine their quantities. (D) Results of the qRT-PCR on the HV V mutant. Absolute numbers of transcripts were normalized to the number of β-actin transcripts.

of the 3′ LTR (ReSF), and the other detects the sum of this and 3′ LTR-initiated transcription (3Pre) (Fig. 3C). qRT-PCR was conducted, and all transcripts were normalized to β-actin mRNA copy numbers. Transcripts initiated from the 5′ LTR were expressed at a much higher level than those initiated from the 3′ LTR (Fig. 3D). In this clone, the transcript spliced to *Ghr* was second in abundance to the SFFV-driven GFP mRNA, and the total numbers of cryptically spliced transcripts were also higher than the unspliced transcript numbers. For an analysis of vector transcription in further HV single-cell clones or mutants with insertions in *Ghr*, together with a Northern blot analysis, see http://www.ac.uk/infection-immunity/themes/tak_supp. In each case, the total level of spliced transcripts is higher than that of unspliced transcripts, in agreement with the predominance of a transcript of about 3 kb, the approximate size of the cryptic spliced RNAs. Thus, we conclude that most transcripts initiating at the 5′ LTR splice either within the vector or to an adjacent gene. Furthermore, readthrough transcription and 3′ LTR activity pose a limited risk to vector safety.

Previous work in our lab established an *in vitro* insertional gene activation assay and found that lentiviral vectors caused IM through insertion in the *Ghr* locus and activity of the HIV LTR (3). A similar mechanism of mutagenesis was recently reported in the tumor-prone mouse model (13). The work presented here addressed the role of vector design in this insertional gene activation. The SFFV element increases the level of transcripts initiating in the 5′ LTR. We propose that there is a threshold of *Ghr* fusion transcripts required for response to growth hormone and that SFFV enhances transcription to this level but the ubiquitin promoter does not. In our assay, the WPRE element does not cause an increase in the frequency of mutagenesis. In agreement with our data, recent studies using the bone marrow replating assay have also shown that removing the WPRE element from vectors does not change the frequency of mutants (12). The limitations of these data should be noted; the Bcl-15 assay detects splicing to an adjacent gene, and the bone marrow replating assay detects enhancer insertion, mainly in the Evi1 locus. The WPRE element could still cause IM by other mechanisms.

Deletion of the U3 region of the LTR of pHV, to generate the self-inactivating (SIN) lentiviral vector pCSGW (5), prevented insertional activation of *Ghr* in the Bcl-15 assay (3). For many gene therapy applications, SIN vectors will be appropriate. Such vectors have been shown to cause insertional Evi1 activation (12) or perturb adjacent gene expression (2, 10). It remains to be determined whether the internal promoter is acting as a classical enhancer in all of these studies, and careful promoter choice will reduce such activity. However, in a clinical trial for the treatment of HIV infection, the vector VRX496 with intact HIV LTRs has been used (9). This approach allows the expression of proteins or RNAs that specifically inhibit HIV replication to be induced in HIV-infected cells by the viral transcription activator Tat. We tested this type of vector and did not obtain any mutants. This could be due to low expression from the HIV LTR in the absence of an internal promoter or to the inclusion of a splice acceptor sequence, reducing the chances of aberrant splicing outside the vector.

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ERRATUM

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Volume 84, no. 9, p. 4856–4859, 2010. Throughout, the URL for the supplemental material should read "http://www.ucl.ac.uk /infection-immunity/themes/tak_supp".

Page 4856, legend to Fig. 1, lines 3 and 4: "slice" should read "splice".