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Effects of a second intron on recombinant MFG retroviral vector

Brief Report

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Summary. The retroviral vectors based on an MFG-type backbone have superior expression characteristics, in part, due to the presence of the retroviral chimeric intron (MFG intron). We tested the hypothesis that inclusion of a second intron in MFG vectors may influence packaging and/or LTR-driven transgene expression. We constructed two MFG retroviral vectors, MFG/hFIXc and MFG/hFIXm2, containing human factor IX (hFIX) cDNA without and with a 0.3-kb hFIX intron, respectively. When tested with primary mouse myoblasts or HepG2 cells in culture for transient expression activity, pMFG/hFIXm2 plasmid produced two-to-three fold higher hFIX than pMFG/hFIXc. These vectors produced equivalent retroviral titers from packaging cells. In transduced cells, the splicing of the MFG intron in the retroviral transcripts occured at a similar efficiency; however, MFG/hFIXc virus gave two-fold higher hFIX expression than that of the MFG/hFIXm2 viral infection. Analyses of MFG/hFIXm2 virion RNA and transduced cell genomic DNA suggested that, although the hFIX intron containing viral RNA are packaged, these viruses fail to integrate their transgenes into the genome of transduced cells, suggesting a block at the reverse transcription and/or integration steps. Similar results were also obtained with the prototype vectors, LIXcSN and LIXm2SN, lacking the MFG intron. Together, these results suggest that a hFIX cDNA sequence in the retroviral vectors performs better over hFIX intron-containing minigene.

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Recombinant retroviral vectors, derived from Moloney murine leukemia virus (MoMLV), contain the viral Long Terminal Repeat (LTR) promoter driven trans-

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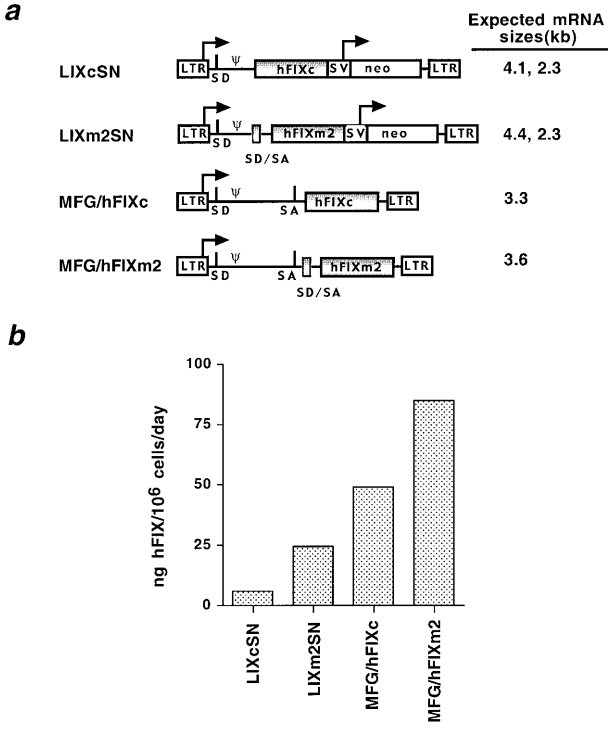
gene placed downstream of the packaging signal, Ψ, replacing the viral *gag* (3′ portion), *pol* and *env* regions [18]. The packaging signal, flanked by viral splicing donor (SD) site and the transgene, has a strong secondary structure and can interfere with the translation of the following gene. In the absence of an authentic splicing acceptor (SA) site preceding the transgene in vectors often referred to as LN-type (as LIXcSN), a cryptic SA is used to ensure that the Ψ sequence is spliced out for efficient translation of the transgene insert sequence [2]. LIXcSN contains human Factor IX (hFIX) cDNA under the LTR promoter and also contains an SV40 promoter-neomycin-resistance gene expression unit (Fig. 1a) [30]. MFG retroviral vector contains a segment comprising of an authentic viral SA and 5′-untranslated region of the viral *env* gene preceding the transgene insert and thus recreates the presence of a viral intron [21]. The first methionine codon of the transgene coincides to the viral *env* start codon in these vectors. Expression of a transgene from the MFG vectors can be 7- to 50-fold higher than that from LN vectors [4, 13]. The ratio of spliced to unspliced vector RNA is increased by up to four-fold for MFG vectors in transduced cells, which in part can explain higher expression from MFG vectors [4, 11, 13]. However, the mechanism(s) responsible for higher expression from MFG vectors is not fully understood. Other mechanisms include a possible presence of internal ribosome entry segment (IRES) in *env* leader [7]. It is also possible that, the chimeric viral intron allows efficient spliceosome assembly with viral RNA in packaging cells, thus resulting in higher levels of viral RNA for packaging and producing higher titers than the LN-type vectors [17].



Similar as in wild-type retrovirus, the chimeric intron in MFG vector is also partially spliced in the packaging cells and since only the RNAs that retain the intron are packaged, all vectors in target cells must have this intron. On the other hand, retention of heterologous intron(s) within the transgene and placed in the same orientation as retroviral intron have shown mixed results. Early experiments suggested that heterologous introns included in retroviral vectors are inefficiently (∼10%) spliced prior to packaging and may require 7–9 passages for complete

Fig. 1. a Construction of retroviral vectors. Vectors used in the present studies contain 0.59-kb LTR promoter, Ψ packaging signal containing region and 1.4-kb hFIXc or hFIXm2 containing a 0.3-kb internally truncated first intron. LN-type vectors (LIXcSN and LIXm2SN) or MFG vectors (MFG/hFIXc and MFG/hFIXm2) have 0.4-kb partial and 1.27-kb chimeric viral introns, respectively. The 3′-untranslated region of vectors between transgene and LTR is 0.14-kb in all vectors. The 1.7 kb SV-neo represents SV40 promoter and neomycin resistance gene, respectively. SD and SA refer to splice donor and splice acceptor, respectively. The expected RNA transcript sizes are shown on the right. Plasmid vectors, pMFG/hFIXc and pMFG/hFIXm2 were constructed starting with p-416hFIXc and p-416hFIXm2, which were previously constructed and contain 416-bp hFIX promoter and hFIX cDNA or hFIX cDNA with a 0.3-kb intron I, respectively [14]. An *Nco* I linker (5′-AGCCATGGCT) was introduced between hFIX 3′-end and poly (A) signal sequence of hFIX plasmids at the *Bam* HI site. Similarly, a *Bsm* BI adapter designed to give an *Nco* I compatible sticky end (5′-CTAGCGTCTCACATGCAG-3′ and 5′-CGCTGCATGTGAGACG-3′) was introduced at the 5′-end of hFIX gene. pMFG/hFIXc and pMFG/hFIXm2 expression vectors were obtained by ligating hFIXc or hFIXm2 genes at the *Nco* I site of pMFG [21]. Use of the *Bsm* BI adapter allowed that the translational start codon corresponded to that of the viral *env* without altering the second codon of hFIX. pLIXm2SN was constructed by replacing the hFIXc of pLIXcSN with hFIXm2 after digesting with *Bam* HI. Retroviral vectors were prepared using the Phoenix-A cell line [9, 12] which was grown in Dulbecco's modified essential medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and penicillin-streptomycin at 37 °C. Cells at 60–80% confluency in 9-cm diameter dishes were transfected overnight with 24 μg of retroviral plasmid DNAs. Next morning, medium was replaced with 4 ml of fresh medium, and plates were incubated in humidified incubator at 32 °C. After 48 hours, recombinant retroviruses (culture supernatants) were harvested, filtered through 0.22 μ filters, aliquoted and stored at −70 °C until use. **b** Transient expression of hFIX by myoblasts transfected with expression plasmid vector DNAs. Mouse myoblasts (∼2.5×10^5 cells) were plated on 6-well plates one day prior to transfections and grown in DMEM supplemented with 20% FBS and 0.5% chicken embryo extracts (CEE) [28]. Cells were transfected next day with 1 μg of plasmid DNA mixed with FuGENE6 (Roche Biochemicals) (transfection efficiency of 5–10%) [26]. The transfection medium was replaced with fresh medium supplemented with Vitamin K_1 (10 μg/ml) and $BaSO_4$-treated FBS [27] on the following day, harvested 48 h thereafter and used for measuring hFIX by ELISA [14] as previously described. At the end of experiment, the total myoblast cell number was ∼4×10^6 per well. Results shown from three independent experiments normalized to 10^6 cells and 24 h are shown (standard deviation, <5%). Similarly, HepG2 cells (3×10^5 cells plated on 6-cm dishes one day prior to transfection) were transfected with 8 μg of plasmid DNA using FuGENE6 [15]. Cell number at the end of experiment was ∼5×10^6. The results normalized to 10^6 cells per 24 h are described in the text

removal [24]. Overall, splicing of heterologous introns occurs with varying efficiency and in some cases introns were completely retained in retroviral vectors ([20] and references therein). However, these experiments were performed using the LN-type or similar vectors. In order to force package the heterologous introns, the intron-containing genes were also placed by other groups in a reverse orientation relative to that of viral intron and LTR promoter [10, 23]. This reverse orientation required transgene expression to be driven by heterologous promoters in these vectors. However, cryptic poly(A) or splicing signals in the complementary strand of heterologous intron or transgene in these situations may interfere with retroviral packaging or transduction [26].

Expression of transgenes from the LTR promoter in MFG vector has some merit as shown by the use of this backbone for the first-ever report of a successful gene therapy trial in humans [5]. Furthermore, as discussed above the packaging of heterologous introns in retroviral RNA is possible. Therefore, we tested the possibility of enhancing the performance of MFG LTR driven vectors by inclusion of transgene contained intron as described below.

We previously observed that an internally truncated version of human coagulation factor IX (hFIX) intron I increased hFIX expression by 10–12 fold and does not contain poly(A) or cryptic splicing signals [14, 26]. Therefore, we hypothesized that in the MFG context it may be possible to achieve packaging of virions with the viral genomes containing this hFIX intron in the same orientation as the viral intron, thus further enhancing transgene expression driven by the LTR promoter. We tested this by MFG vectors with hFIX (cDNA with and without an intron) as a reporter gene (Fig. 1a). MFG retroviral vectors constructed, MFG/hFIXc and MFG/hFIXm2, contained hFIX cDNA or hFIX minigene (cDNA with an intron), respectively, and then hFIX expression levels were analyzed in comparison to those of LN-type vectors, LIXcSN or LIXm2SN (Fig. 1a).

Myoblasts [28] transiently transfected with hFIX minigene vectors, MFG/hFIXm2 or LIXm2SN, produced 2- or 4-fold higher hFIX than those transfected with hFIX cDNA containing vectors, MFG/hFIXc or LIXcSN, respectively (Fig. 1b). Similarly, levels of hFIX produced by HepG2 cells transfected with LIXcSN, LIXm2SN, MFG/hFIXc and MFG/hFIXm2 were 16 ± 9, 35 ± 12, 30 ± 14 and 98 ± 37 ng/10^6 cells/24 hours, respectively (Mean $\pm$ SD, $n = 6$) (see legend to Fig. 1b). Enhanced hFIX expression from hFIX minigene containing retroviral plasmids over those with hFIX cDNA is consistent with previous results obtained with non-retroviral plasmid vectors [14, 26].

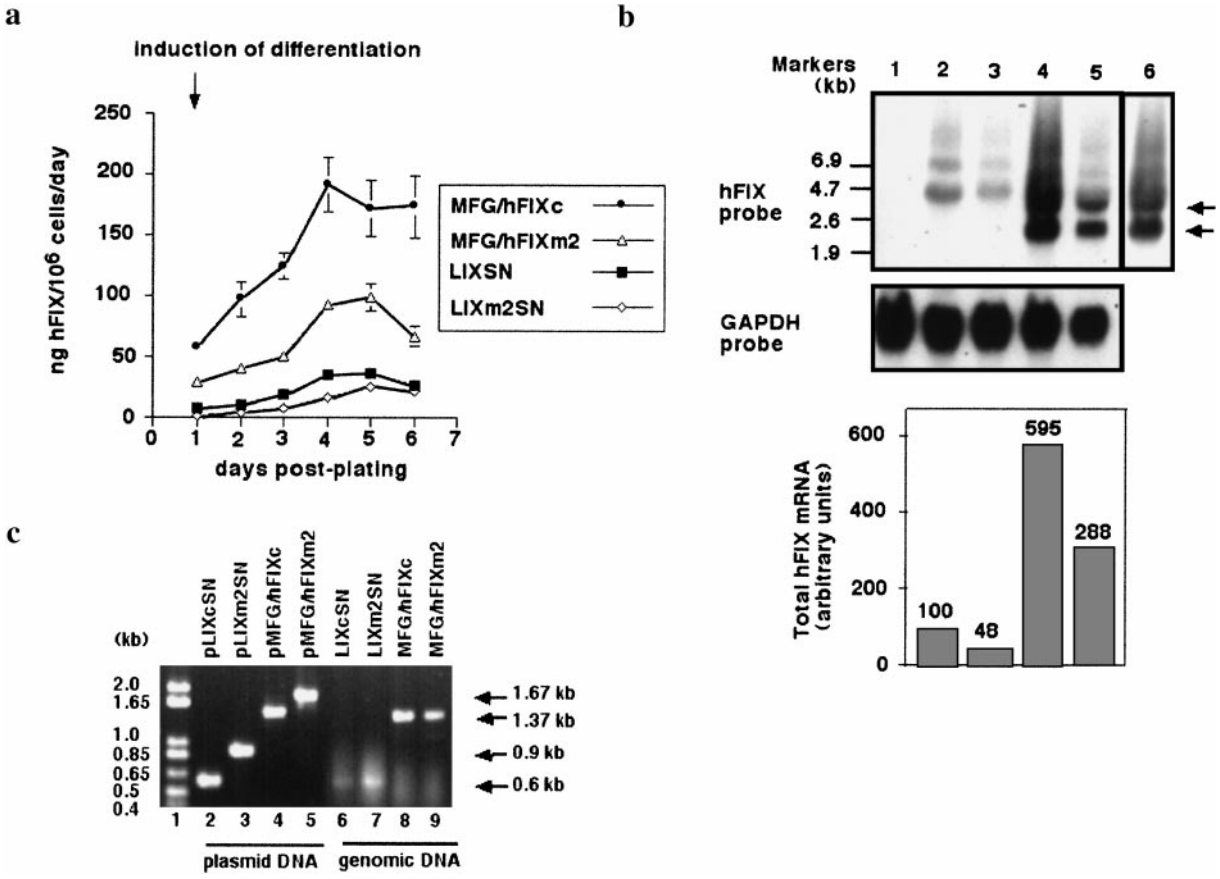
Effects of the hFIX minigene intron on retroviral transduction were then examined by using retroviral vectors prepared by transient transfection of Phoenix A retroviral packaging cell line [9, 12]. Relative titers of recombinant viruses were determined by RNA slot-blot hybridization [19] using hFIX DNA probe. Viral titers relative to LIXcSN (defined 100) were 102 ± 30, 129 ± 46 and 117 ± 28 (Mean $\pm$ SEM, n=5) for LIXm2SN, MFG/hFIXc and MFG/hFIXm2, respectively. These results suggested that the hFIX intron does not confer any significant increase in titer, and all vectors are packaged with near equal efficiency. Primary mouse myoblasts were then transduced with retroviral supernatant and hFIX ex-

pression was determined by Enzyme Linked Immunosorbant Assay (ELISA [14]). Both LIXcSN and LIXm2SN produced hFIX at low levels, agreeing with the previous results (Fig. 2a) [8, 26, 29]. Surprisingly, LIXcSN expressed up to two-fold higher hFIX than LIXm2SN (Fig. 2a). Myotubes transduced with MFG/hFIXc also expressed two-fold higher levels of hFIX compared to MFG/hFIXm2 transduced myotubes (172–191 versus 67–99 ng hFIX/10^6 cells/24 h) (Fig. 2a). Similar results were obtained in transduced HepG2 cells, though the hFIX levels produced were lower than those by myoblasts. HepG2 cells transduced with MFG/hFIXc expressed hFIX at ~2-fold higher levels than with MFG/hFIXm2 (21 versus 10 ng/10^6 cells/24 h; n=4). Together, these results suggested that the presence of hFIX intron in MFG vectors neither contributes to enhancement of viral titer nor hFIX transgene expression.

In transduced myoblasts, hFIX mRNA levels were in order of MFG/hFIXc> MFG/hFIXm2>LIXcSN>LIXm2SN (Fig. 2b), which is consistent with the hFIX protein expression levels (Fig. 2a). The lower hFIX mRNA levels of LIXcSN or LIXm2SN may be due to the non-MFG retroviral backbone in these constructs and also in part to the presence of SV-neo (Fig. 1a and 2b, lanes 2 and 3). Importantly, the ratio of unspliced and spliced transcripts was very similar for MFG/hFIXc or MFG/hFIXm2, 1.13 and 1.19, respectively (Fig. 2b, lanes 4–6). Thus, splicing ratios alone cannot explain the differences in hFIX expression level between MFG/hFIXc and MFG/hFIXm2 vectors.

Since viral RNA is reverse transcribed and integrated into genomic DNA prior to transgene expression, the state of integrated vector in genomic DNA of the transduced cells should reflect the status of viral genomic RNA in virions. When genomic DNA isolated from the transduced myoblasts was subjected to PCR analysis using primers specific to the U3 region in retroviral LTR and the second exon of hFIX, MFG/hFIXc transduced myoblasts gave a 1.37-kb band, as expected (Fig. 2c, lane 8). However, MFG/hFIXm2 transduced myoblasts did not give the expected 1.67-kb band which represents the intron-retained form, but instead, gave the 1.37-kb product corresponding to intron-spliced form (Fig. 2c, lane 9). Nucleotide sequencing of the junction region of hFIX exon 1 and 2 in 1.37-kb PCR products from MFG/hFIXm2 transduced cells indicated that the splicing of hFIX intron was complete and not aberrant (data not shown). Similarly, genomic DNA from LIXm2SN transduced myoblasts did not have the hFIX intron sequence (Fig. 2c, lanes 6 and 7). These results suggested that hFIX intron was not present in the genomic RNA of MFG/hFIXm2 or LIXm2SN viruses that were used for transduction. However, Northern blot analysis of genomic RNA prepared from virions concentrated from the retroviral supernatant according to the method described by Cepko [6] showed reactivity to hFIX intron fragment hybridization probe (data not shown), Together, these analyses suggested that the viral RNA with or without the hFIX intron sequence are packaged, but the hFIX intron containing viral RNA fails to integrate into the genome of the transduced cells. This suggested that presence of the hFIX intron sequence may possibly suppress reverse transcription and/or integration steps in the target cells, and only a small population of MFG/hFIXm2 or LIXm2SN viral RNA, can get integrated,

Fig. 2. a Expression of hFIX from myoblasts and myotubes transduced with recombinant retroviruses. Transduction of mouse myoblasts was performed by plating the cells at 10^5 cells/well (6-well plate) to obtain 30% confluency on the next day. Cells were then infected with 2 ml of viral supernatant in the presence of 8 μg/ml polybrene with centrifugation at 1180×g for 30 min at 32 °C using a microplate carrier. The first infection was followed by additional infections at 10 and 24 h later to obtain virtually 100% transduction. For quantitative determination of hFIX production, 5×10^5 transduced myoblasts were plated per well of 6-well plates in 2 ml of growth medium (DMEM supplemented with Vitamin K_1 (10 μg/ml), 20% $BaSO_4$-treated FBS and 0.5% CEE) for 1 day, followed by changing to differentiation medium (DMEM/Vitamin K_1/2.5% $BaSO_4$-treated FBS) and incubation for 3 additional days. The medium was then switched to growth medium and cells were incubated for 2 more days. The medium was collected and replenished every 24 h and assayed for hFIX by ELISA. Myoblasts were >90% confluent at the start of differentiation. On day 2, cell numbers were counted in two wells and used for normalizing results to per 10^6 cells. Decline in expression of hFIX from MFG/hFIXm2 transduced cells on day 6 is due to cell release from the plates as previously observed [26]. **b** Northern blot analysis. Northern blot analysis was done as previously described [14] using 10 μg total RNA prepared from transduced myoblasts and 1% agarose/formaldehyde gel. The ^{32}P labeled hFIX minigene DNA was used as the hybridization probe. As an internal control, 1.1-kb *Eco* RI-*Hind* III cDNA fragment of cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (IMAGE. consortium clone ID 360840; American Type Culture Collection) was used. Relative ratios of GAPDH mRNA bands in this experiment were 2.11, 1.85, 1.84 and 1.0 for LIXcSN, LIXm2SN, MFG/hFIXc and MFG/hFIXm2 transduced myoblasts, respectively. Arrows on the right indicate possible spliced and unspliced transcripts from myoblasts transduced with MFG/hFIXc and MFG/hFIXm2 viruses (*4–6*) as judged from expected sizes (shown in Fig. 1a) and were used for calculating unspliced/spliced RNA ratios (see text). This ratio could not be determined for LIXcSN and LIXm2SN since the spliced transcripts were not clearly visible (*2* and *3*). Higher molecular size RNA bands, also seen previously [26], in all lanes are also possibly full-length transcripts that are migrating anamolously, due to unknown mechanisms. *1* RNA from uninfected myoblasts. *2–5* RNA from myoblasts infected with LIXcSN, LIXm2SN, MFG/hFIXc, and MFG/hFIXm2, respectively. *6* is a shorter exposure of *4*. The histogram underneath represents total hFIX RNA amounts for *2–5*, expressed relative to LIXcSN, which were quantified with the STORM phosphorimager (Molecular Dynamics). The arbitrary RNA units in the histogram have been corrected for total RNA loaded per lane based on the measured values of GAPDH mRNA. **c** PCR analysis of genomic DNA isolated from transduced myoblasts. Aliquots of genomic DNA (5 μg) were subjected to PCR using 0.4 μM each of forward primer in retroviral promoter region (5′-TCTGTGTGATTGACTACCCG-3′) and reverse primer in hFIX exon 2 (5′-GGCCGATTCAGAATTTTGTTG-3′) in the presence of 0.2 mM each of dATP, dCTP and dTTP, and 0.15 mM of dGTP, 0.05 mM of 7-deaza-dGTP, 2.5% (v/v) formamide and AmpliTaq DNA polymerase (Roche). PCR was composed of an initial 3 min denaturation at 94 °C, followed by 25 cycles of 1 min denaturation at 94 °C, 1 min annealing at 52 °C and extension at 72 °C for 1 min. One-tenth of the reaction products were resolved on 0.7% agarose gel and stained with ethidium bromide. *1* 1 Kb Plus DNA Ladder (Gibco-BRL). *2–5* PCR products of plasmid DNAs as labeled. *6–9* PCR products of genomic DNAs isolated from myoblasts transduced with viruses as indicated. Arrows indicate the expected products, in sizes shown



contributing towards transgene expression. This proposed mechanism may also explain why the overall levels of hFIX mRNA are significantly higher in MFG/hFIXc or LIXcSN transduced cells than MFG/hFIXm2 or LIXm2SN transduced cells, respectively.

As shown for factor VIII, some coding sequence of the transgene can grossly alter retroviral titer and transgene expression [16]. Furthermore, the neomycin resistance gene has been shown to exert negative effects on expression of the other transgene in the same retroviral vector in a multi-cystronic organization [3]. Unlike factor VIII, we do not observe any reduction in the titer of MFG/hFIXm2 vectors, suggesting a possibility that the hFIX intron sequence may exert a negative control at a post-packaging, but pre-integration step(s) in target cells [25]. A short polypurine tract near 3′-end of a retroviral genome is involved in plus-strand DNA synthesis during retroviral replication. Although BLAST [1] search of the 0.3-kb hFIX intron did not reveal any homology to retroviral sequences, the hFIX intron sequence has several stretches of A and G nucleotides, suggesting a possibility that the hFIX intron may interfere in a dominant negative fashion with the wild-type polypurine tract during reverse transcription of the vector RNA prior to integration in transduced cells [22]. This possibility will be further tested.

In summary, our results indicate that in the MFG retroviral context, the hFIX minigene intron has a negative influence on transgene expression, presumably due to the interference effects of the second intron on retroviral generation and transduction processes.

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