

# Control of Splicing Efficiency by the Mouse Histone H2a Element in a Murine Leukemia Virus-based Retroviral Vector

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While using various human complementary DNA (cDNA) sequences in the context of the murine leukemia virus (MLV)-based retroviral vector, it was found that a retroviral vector containing some human cDNA sequences produces unusually low viral titer. One of those sequences is that for the human IL-1 receptor antagonist protein (IL1RN). The RNA analysis showed that a cryptic splice acceptor sequence is present in the middle of its coding region, resulting in the deletion of the packaging signal sequence and the removal of some coding sequences that lead to low viral titer and a low level of the transgene product. We tested whether the mouse Hist2h2aa1 element (mH2aE), previously shown to suppress the splicing function, could inhibit the cryptic splicing in the context of MLV-based retroviral vectors. It was found that the mH2aE could efficiently suppress such unwanted splicing event, thus increasing the amount of unspliced transcript, which eventually led to the increase in the level of IL1RN expression and viral titer. The mH2aE could also be used to control unusually high splicing activity. Our data suggested that the mH2aE could be used for the fine-tuning of the splicing process, thus improving the level of gene expression and viral titer in the context of retroviral vectors.

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## INTRODUCTION

The murine leukemia virus (MLV)-based retroviral vector is still one of the most widely used gene delivery vehicles employed in gene therapy clinical trials.<sup>1</sup> Despite this wide use, however, there are still many limiting factors that need to be improved upon in order for MLV-based vectors to become a therapeutically viable form of gene delivery system.<sup>2-7</sup> The two most prominent problems are the possibility of insertional mutagenesis, which has recently become a primary safety concern,<sup>8</sup> and the amount of the transgene product, which determines the actual therapeutic effects. The latter aspect is influenced largely by the

number of transduced cells and the level of gene expression in a given cell.

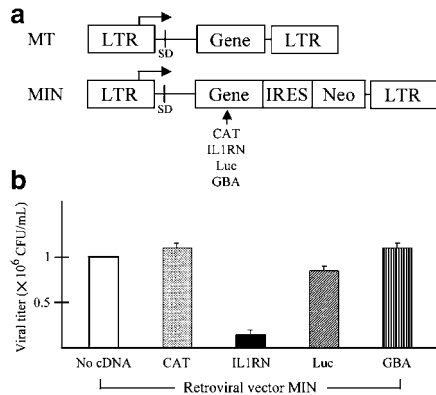
We previously reported a minimum-sized retroviral vector called MT, which does not contain any viral coding sequences.<sup>9</sup> Because of the lack of viral open-reading frames, the possibility of homologous recombination between the packaging genome and the vector sequences is virtually nil in this retroviral vector. A variety of human complementary DNA (cDNA) sequences have been cloned into MT or its derivative vectors in order to test its performance in terms of viral titer and the level of gene expression. During this study, it was found that some human cDNA sequences produced significantly lower viral titer than other human sequences. The subsequent analysis indicated that these human cDNA sequences contained a cryptic splice acceptor sequence that is efficiently used in the context of the retroviral vector, although it is not active in its original site. The presence of such cryptic splice site could greatly affect the performance of the retroviral vector because it influences the level of gene expression and viral titer both in the producer cell line and in the transduced target cell.

In this report, we investigated the possible use of the mouse Hist2h2aa1 element (mH2aE).<sup>10</sup> This sequence is present in the middle of the coding sequence of the mouse Hist2h2aa1 gene, and has previously been shown to inhibit the splicing function in the artificially arranged site as well as in its natural place. It was found that the insertion of the mH2aE could suppress the undesirable splicing activity in the context of the retroviral vector. Its activity was not restricted to one particular sequence. The suppressive effect of the mH2aE was enhanced with the increased copy number. Our data suggest that the mH2aE can be used to improve the performance of retroviral vectors by controlling the level of gene expression and viral titer.

## RESULTS AND DISCUSSION

MIN is a minimum-sized MLV-based retroviral vector derived from MT, containing an internal ribosomal entry site from encephalomyelitis virus and the bacterial *Neo* gene as a selectable marker (Figure 1a).<sup>11</sup> Several cDNA sequences, including those encoding chloramphenicol acetyl transferase (CAT), human

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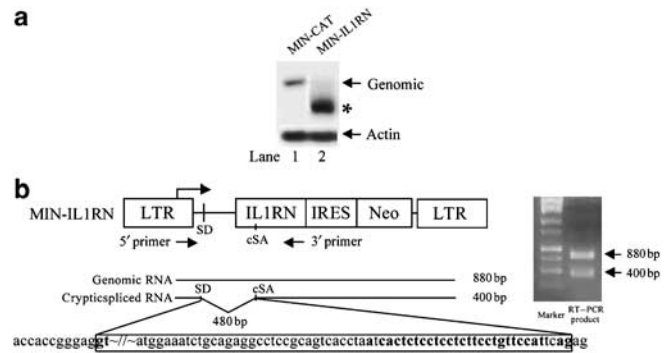
**Figure 1** Effect of various cDNA sequences on viral titer in the context of the MT retroviral vector. **(a)** Schematic diagram of a prototype retroviral vector used in the study. MT is a retroviral vector that does not contain any viral coding sequences.<sup>11</sup> MIN was derived from MT, and contained an internal ribosomal entry site from the encephalomyelitis virus and the bacterial *Neo* gene as a selectable marker.<sup>11</sup> The cDNA sequence encoding CAT, IL1RN, luciferase (Luc) and glucosylceramidase (GBA) was inserted into the *Bam*HI site of MIN. **(b)** Comparison of viral titers of retroviral vectors, containing different cDNA sequences.

interleukin 1 receptor antagonist (IL1RN), luciferase, and glucosylceramidase (GDA), were cloned into MIN (**Figure 1a**), and viral titers were compared. When the IL1RN cDNA was used, the viral titer was approximately 10-fold lower than MIN vectors containing other cDNA sequences (**Figure 1b**).

In an effort to understand the molecular basis of this result, the RNA content of respective retroviral vectors was studied. As a control, a retroviral vector harboring the bacterial CAT sequence was used. The sizes of CAT and IL1RN cDNA were supposed to be 660 and 534 bp, respectively. MIN-CAT produced a genomic transcript of expected size (**Figure 2a**, lane 1). However, MIN-IL1RN generated an RNA band that was unusually broad and appeared to contain more than one RNA species with a shorter size (**Figure 2a**, lane 2).

To identify what these were, total RNAs were prepared and subjected to reverse transcription–polymerase chain reaction (RT–PCR) using a pair of the primers as indicated in **Figure 2b**. Agarose gel electrophoresis of PCR products reproducibly showed two RNA bands (**Figure 2b**). One was an approximately 880-bp-long fragment, which was the expected size, whereas the smaller fragment was only 400 bp long. The nucleotide sequence analysis showed that a smaller band was an aberrant form of IL1RN cDNA resulting from the splicing between the splice donor in the retroviral vector and the cryptic splice acceptor present in the IL1RN coding region (**Figure 2b**). Therefore, the smaller RNA band is a truncated form of IL1RN containing a deletion of 480 bp, which harbors almost all the consensus *cis*-acting sequences needed for the splicing function. This result suggested that cryptic splicing decreased the amount of the genomic size transcript containing the packaging signal sequence, thereby decreasing viral titer as observed in **Figure 1**.

It appears that many human cDNA sequences contain the cryptic splice sequence, which may lead to a similar result. Therefore, we tested whether cryptic splicing could be inhibited in the context of the MLV-based retroviral vector by introducing



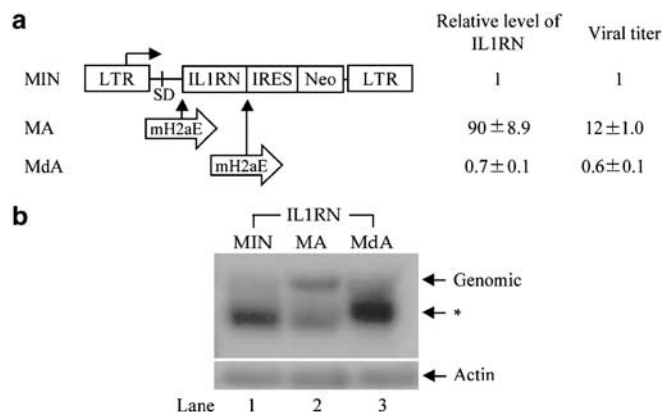
**Figure 2** Presence of cryptic splice site in the IL1RN cDNA sequence. **(a)** Northern blot analysis of a retroviral vector containing the IL1RN cDNA sequence. Total RNAs were prepared from 293T cells transfected with two retroviral vector DNAs, MIN-CAT and MIN-IL1RN, and followed by Northern blot analysis using the *Neo* sequence as a probe. Cellular  $\beta$ -actin RNA was used as a loading control. Genomic, unspliced genomic transcript; subgenomic, spliced RNA; asterisk (\*), aberrantly spliced RNA; Actin,  $\beta$ -actin RNA. **(b)** Determination of the cryptic splicing site in the IL1RN coding sequence. Total RNAs were subjected to RT–PCR, cloning, and nucleotide sequence analysis. The gel photo shows that the two RNA species were produced from the MIN-IL1RN retroviral vector. The precise location of cryptic splice acceptor site (cSA) is indicated. The region indicated as a box contains all the *cis*-elements needed for splicing. The arrows indicate the position of primers used for PCR.

a 100-bp sequence (mH2aE) from the mouse *Hist2h2aa1* gene, which has been reported to suppress cryptic splicing through a yet unknown mechanism(s).<sup>10,12</sup>

The mH2aE was inserted into two different sites of the MIN retroviral vector. MA- and Mda-IL1RN contain the mH2aE upstream and downstream from the IL1RN coding sequence, respectively (**Figure 3a**). 293T cells were transfected with MA- and Mda-IL1RN, and MIN-IL1RN as a control, together with amphotropic packaging constructs, pVM-GP and pVM-AE.<sup>9</sup> Total RNAs were analyzed by Northern blot using the IL1RN cDNA sequence as a probe (**Figure 3b**). The MIN-IL1RN vector reproducibly produced a large amount of aberrantly spliced RNAs (**Figure 3b**, lane 1), and RNA from Mda-IL1RN showed a pattern similar to that from MIN-IL1RN (**Figure 3b**, lane 3). However, MA-IL1RN produced an approximately 10-fold higher level of genomic transcript than MIN-IL1RN (**Figure 3b**, lane 2).

Cell-free viral supernatants from transfected 293T cells were used to transduce HT1080 and then compared for their viral titer and level of gene expression. MA-IL1RN produced a 90-fold higher level of IL1RN protein and 12-fold higher viral titer as compared with MIN-IL1RN. However, Mda-IL1RN gave results similar to those obtained with MIN-IL1RN (**Figure 3a**). These data suggested that the mH2aE introduced in the retroviral vector MIN-IL1RN effectively inhibited the cryptic splicing. This effect was highly position-dependent, because the same sequence inserted downstream from the cryptic splice acceptor could not suppress the abnormal splicing event.

The result from the RNA analysis of MA-IL1RN presented in **Figure 3b** indicated that the use of one mH2aE was still insufficient to suppress completely the cryptic splicing, as approximately half of the amount of RNA made was still an aberrant RNA species. To improve the performance of the

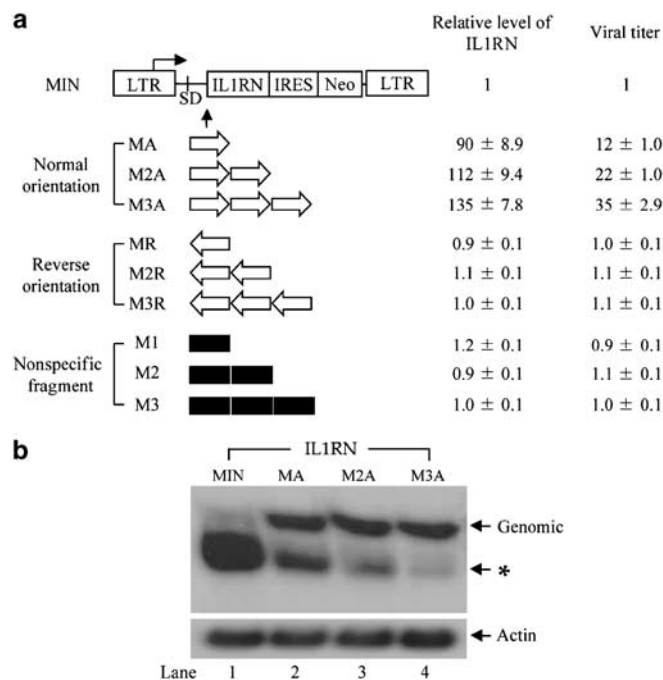


**Figure 3** Effect of mH2aE on splicing, viral titer, and the level of gene expression in the retroviral vector MIN-IL1RN. **(a)** Comparison of the level of gene expression and viral titer in transduced HT1080 cells. The level of IL1RN was determined by enzyme-linked immunosorbent assay using the commercially available kit from R&D Systems (Minneapolis, MN; DRA00). Cell-free viral supernatants were taken from 293T cells transfected with respective retroviral vectors, and used to transduce HT1080 cells to compare the level of gene expression and viral titer. Viral titer was determined by counting the number of G418-resistant colonies of transduced HT1080 cells. **(b)** Northern blot analysis of retroviral vectors containing the mH2aE. Total RNAs were prepared from 293T cells transfected with retroviral vector DNAs (MIN-, MA-, and MdA-IL1RN), followed by Northern blot analysis using IL1RN as a probe.

mH2aE, we tested whether multiple copies of the mH2aE could inhibit cryptic splicing more efficiently. We added one, two, and three copies of the mH2aE to MIN vector (**Figure 4a**) and compared them for RNA content. The ratio of genomic to spliced RNA increased proportionally with the increased number of mH2aE copies (**Figure 4b**). We also compared the retroviral vectors for their viral titer and the level of gene expression in transduced HT1080 cells. All mH2aE-inserted retroviral vectors gave higher levels of gene expression and viral titer than MIN-IL1RN. The viral titer and the level of gene expression increased with the increased number of copies (**Figure 4a**). These results suggested that the additional copy number of mH2aE more efficiently suppressed the aberrant splicing, thus raising the level of the genomic transcript.

To study the orientation effect, we inserted one, two, and three copies of mH2aE into the MIN IL1RN vector in an opposite direction, resulting in MR-, M2R-, and M3R-IL1RN. All these constructs gave results similar to MIN-IL1RN lacking the mH2aE sequence in transduced HT1080 cells (**Figure 4a**). Therefore, the histone element is not able to suppress the cryptic splicing event, when placed in an opposite direction, suggesting that it might work at the level of RNA.

To test whether the effects of mH2aE were not due merely to the presence of a nonspecific “spacer” sequence, we also used a similar-sized DNA fragment amplified from plasmid pUC18 by PCR. One, two, and three copies of the amplified fragment were inserted into the MIN IL1RN vector, resulting in M1-, M2-, and M3-IL1RN. The presence of this randomly selected DNA fragment did not have any significant effect on the performance of the retroviral vector (**Figure 4a**). These data suggested that the effects of the mH2aE were specific to this

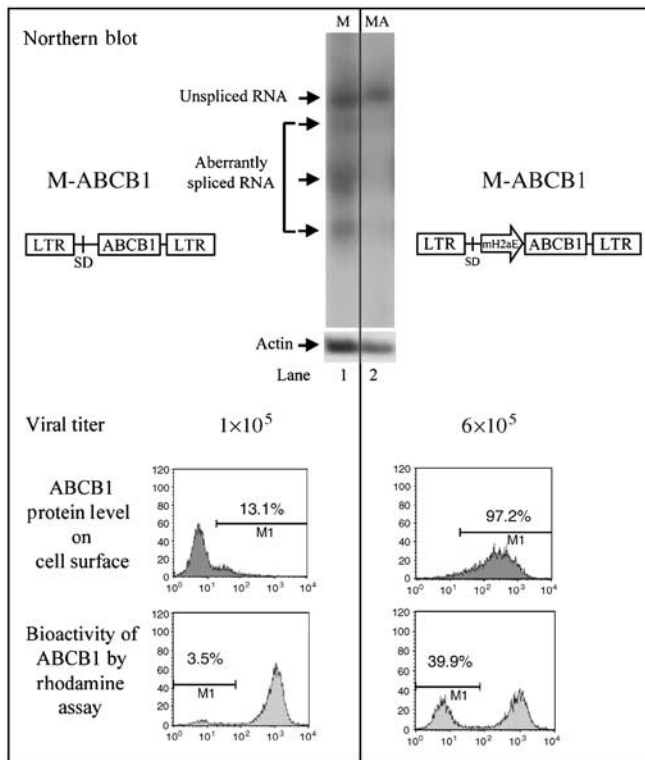


**Figure 4** Effect of copy number and orientation of mH2aE on splicing, the level of gene expression, and viral titer in a retroviral vector. **(a)** Comparison of the level of gene expression and viral titer in transduced HT1080 cells. The level of gene expression and viral titer were measured as described in **Figure 3**. An arrow indicates one copy of mH2aE and its orientation. A black box represents one copy of random DNA fragment. **(b)** Northern blot analysis of retroviral vectors containing a different copy number of the mH2aE. Total RNAs were prepared from 293T cells transfected with retroviral vector DNAs (MIN-, MA-, M2A-, and M3A-IL1RN), followed by Northern blot analysis using IL1RN as a probe.

particular sequence. The molecular mechanism for how the mH2aE works in the context of retroviral vectors remains to be elucidated.

To ensure that the above results are not restricted to one particular gene, we tested the effect of the mH2aE in the retroviral vector containing the ABCB1 gene (MDR1 or P-glycoprotein 1). Human ABCB1 cDNA has been recognized to produce many aberrant RNA species in the context of the retroviral vector, resulting from several cryptic splicing sites present in the middle of its coding region.<sup>13,14,15</sup> Indeed, when the human ABCB1 cDNA was cloned into MT (M-ABCB1, **Figure 5**), viral titer and the level of gene expression were decreased significantly, presumably owing to extensive aberrant splicing (**Figure 5**). However, the introduction of the mH2aE to M-ABCB1 improved the RNA expression profile, viral titer, and the level of protein level.

Northern blot analysis showed that M-ABCB1 produced four distinctive, but broad, RNA bands (**Figure 5**). The size of an unspliced RNA band is supposed to be 5,058 bp. All other smaller bands result from alternative splicing between the retroviral splice donor and cryptic splice sites present in ABCB1.<sup>13</sup> The broad RNA band probably indicates a mixture of different RNA species (**Figure 5**). When the mH2a element was present in the retroviral vector (MA-ABCB1), however, the only major RNA band was the size of genomic transcript,

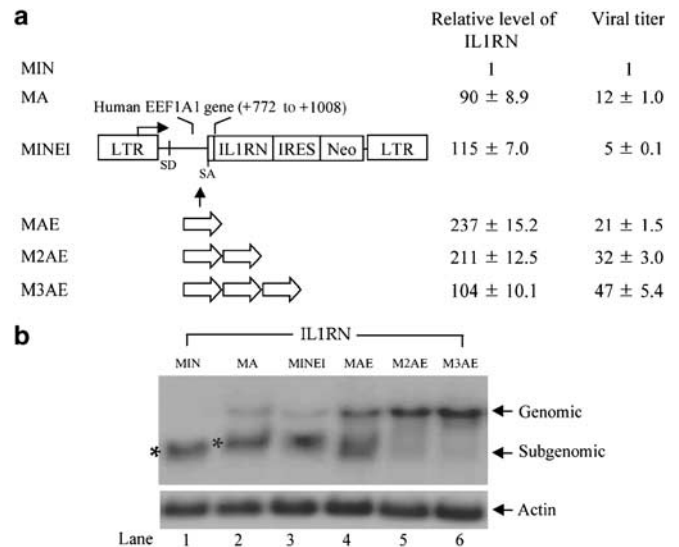


**Figure 5** Effect of mH2aE on splicing, viral titer, and the level of gene expression in a retroviral vector containing the human ABCB1 cDNA sequence coding for P-glycoprotein 1. Schematic diagram of retroviral vector containing the ABCB1 sequence. The ABCB1 cDNA sequence was cloned into MT, resulting in M-ABCB1. MA-ABCB1 was derived from M-ABCB1 by inserting one copy of the mH2aE upstream of the ABCB1 coding sequence. Viral titer was determined by counting the number of vincristine-resistant colonies of transduced HT1080 cells. Total RNAs were prepared from 293T cells transfected with retroviral vector DNAs (M- and MA-ABCB1), followed by Northern blot analysis using ABCB1 as a probe. The protein level of ABCB1 was measured by flow cytometry using anti-P-glycoprotein, MRK-16.<sup>16</sup> Comparison of the activity of ABCB1 by rhodamine 123 efflux assay in 293T cells transfected with respective retroviral vector DNAs.

suggesting the suppression of aberrant splicing by mH2aE (**Figure 5**, lane 2).

Cell-free viral supernatants were used to transduce HT1080 and then compared for their viral titer, as determined by vincristine-resistant colony-forming assay, and also the level of gene expression by flow cytometry using anti-ABCB1, MRK-16.<sup>16</sup> MA-ABCB1 produced 6-fold higher viral titer and a 7.4-fold higher level of ABCB1 than M-ABCB1 (**Figure 5**). To be certain, a functional assay was performed by using a fluorochrome rhodamine 123 (Rh123) efflux assay. In this assay, cells expressing no or low levels of ABCB1 produce stronger fluorescence, whereas those with a high-level expression of this efflux pump protein efficiently extrude the dye and produce lower fluorescence. As presented in **Figure 5**, retroviral vector MA-ABCB1 showed much higher expression of ABCB1 (39.92%) than control vector M-ABCB1 (3.48%). These results suggested that the effect of the mH2aE was not restricted to a specific reporter gene.

Next, we tested whether the mH2aE could also be used to control the unusually high splicing efficiency of the retroviral



**Figure 6** Effect of mH2aE in retroviral vectors containing the heterologous splice acceptor sequence from the human EEF1A1 gene. **(a)** Comparison of the level of gene expression and viral titer in transduced HT1080 cells. The levels of gene expression and viral titer were measured as described in **Figure 3**. An arrow indicates one copy of mH2aE and its orientation. **(b)** Total RNAs were prepared from 293T cells transfected with retroviral vector DNAs (MIN-, MA-, MINEI-, MAE-, M2AE-, and M3AE-IL1RN), followed by Northern blot analysis using IL1RN as a probe.

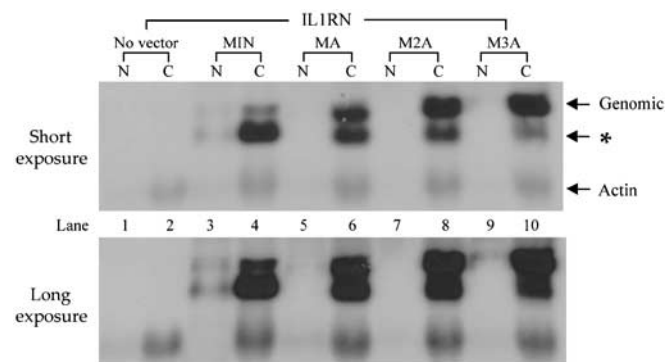
vector. We previously developed a retroviral vector, MINEI, that contains the splice acceptor sequence from the human elongation factor 1 alpha 1 (EEF1A1) gene.<sup>11</sup> MINEI drives a significantly higher level of gene expression. However, one disadvantage in using the vector is that the primary transcript of MINEI was too efficiently spliced to the subgenomic RNA, resulting in a low level of genomic RNA and thus leading to a low viral titer.<sup>11</sup>

We introduced one, two, or three copies of this histone sequence (mH2aE) upstream from the splice acceptor of MINEI-IL1RN, resulting in MAE-, M2AE-, and M3AE-IL1RN, respectively (**Figure 6a**). MIN- and MA-IL1RN, used as controls, demonstrated a similar pattern of RNA expression as revealed in **Figure 3b**. MINEI produced a large amount of spliced RNAs relative to the genomic RNA (**Figure 6b**, lane 3). Because most of the genomic RNAs made in this vector were spliced, the level of IL1RN protein was very high (**Figure 6a**). This vector also produced a higher level of genomic transcript, and thus a 5-fold higher viral titer as compared with MIN-IL1RN (**Figures 6a** and **b**). When the mH2aE was inserted, the ratio of genomic to subgenomic RNA increased with the increase in the number of copies of the splicing control sequence (**Figure 6b**, lanes 4-6). Consistent with this result, viral titer increased with the increased number of mH2aE sequence (**Figure 6a**). However, the level of gene expression was lowered somewhat when three copies were present, presumably because of the decreased amount of spliced RNA (**Figure 6a**). However, it is worth noting that M3AE still produced almost 10-fold higher viral titer than MINEI, whereas the level of gene expression was comparable. All these data indicated that the mH2aE could be

used to control the undesirable splicing event in the context of the retroviral vector.

The mH2aE is known to affect multiple steps of pre-mRNA processing.<sup>10</sup> We tested whether the mH2aE could facilitate the nuclear export of retroviral RNA transcripts. Cytoplasmic and nuclear RNAs were prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and analyzed by Northern blot hybridization using a <sup>32</sup>P-labeled IL1RN or  $\beta$ -actin probe. A significant amount of genomic and aberrantly spliced RNAs was observed in the nuclear fraction of MIN-IL1RN (Figure 7, lane 3). However, in retroviral vectors containing the mH2aE, virtually no RNA was detected in the nucleus, suggesting the efficient transport of RNAs from the nucleus to the cytoplasm (Figure 7, lanes 5–10). These results suggested that RNA containing the mH2aE element might promote nuclear export of RNAs in an MLV-based retroviral vector and interact with the general export proteins and also the different (or additional) types of SR proteins.

In order for the retroviral vector to be a viable form of gene delivery vehicle in actual clinical settings, many problems still need to be overcome, most notably safety and the level of gene expression, which are two of the most important factors determining the efficacy of gene therapy. Our data have significant implications for developing the improved version of retroviral vectors. There are a considerable number of human cDNA sequences that cause aberrant splicing in the context of the retroviral vector. Undesirable splicing can lead to lower viral titer owing to the removal of the packaging signal sequence as well as the lower level gene expression resulting from the truncation of the coding sequence. Thus far, it has been very laborious or impossible to correct this problem owing to the difficulty associated with the manipulation of nucleotide sequences in the coding region. Our results showed that the mH2aE could be used more or less universally to control the aberrant splicing, thus increasing the level of gene expression and viral titer.



**Figure 7** Effect of mH2aE on the nuclear export of RNAs in retroviral vectors. Nuclear and cytoplasmic RNAs were prepared from 293T cells transfected with retroviral vector DNAs (MIN-, MA-, M2A-, and M3A-IL1RN). Cytoplasmic RNA (10  $\mu$ g) and nuclear RNA (5  $\mu$ g) were subjected to Northern blot analysis. N, nuclear RNA; C, cytoplasmic RNA. Because nuclear RNA produced faint bands relative to cytoplasmic RNA, two different exposures of X-ray film are shown.

## MATERIALS AND METHODS

**Plasmids.** The structure of the MIN and MINEI vectors has been previously described.<sup>11</sup> MT was constructed by removing the IRES-Neo cassette from MIN. To construct a retroviral vector containing the mH2aE, the element was amplified using cDNA from mouse cells as a template. The nucleotide sequences of the primer used for PCR are as follows:

mH2aF: 5'-AGATCTGTCTGAGCTTTTGGAGTACGTCGTCCTTTAGGTT  
BglII

mH2aR: 5'-GGATCCGTTTAAACACCTGAAATGGAAGAAAAAACTTT  
BamHI

GAA

The amplified fragment was initially cloned into pGEM T easy (Promega, WI). The BglII–BamHI fragment was cloned into the BamHI site of MIN, resulting in MA. To construct a retroviral vector containing the nonspecific DNA, the 100 bp-long DNA fragment was amplified from the pUC18 plasmid (Promega, WI) as a template, using the following primer pair:

RDF: 5'-AGATCTTCCCCGAAAAGTGCCACCTGACGTTAA  
BglII

RDFR: 5'-GGATCCACGAAAGGGCCTCGTGATACGCCTATTTTT  
BamHI

The amplified fragment was initially inserted into pGEM T easy (Promega, WI). The BglII–BamHI fragment was then cloned into the BamHI site of MIN, resulting in M1. M2 and M3 were also prepared by cloning two and three copies of the same fragment, respectively. To construct the retroviral vectors expressing CAT, the BamHI CAT fragment from MIN-CAT<sup>11</sup> was inserted into the BamHI site of each MIN-derived vector, so that the CAT gene was linked with Neo through encephalomyelitis virus internal ribosomal entry site. To construct retroviral vectors expressing human IL1RN, IL1RN cDNA was cloned from the total RNA of human peripheral blood lymphocytes by RT-PCR. The nucleotide sequences of primers used in this step are as follows:

IRAPF: 5'-GGATCCATGGAAATCTGCAGAGGCCTCCGCAGTCAC  
BamHI

IRAPR: 5'-AGATCTCTACTCGTCCTCCTGGAAGTAGAATTTGGT  
BglII

The BamHI–BglII fragment of IL1RN cDNA was inserted into the BamHI site of the MIN-derived vector. To construct retroviral vectors expressing human P-glycoprotein 1 (ABCB1), cDNA was cloned from the total RNA of human peripheral blood lymphocytes by RT-PCR. The nucleotide sequences of primers used in this step are as follows:

MDRF: 5'-ATCGAT AGATCTATGGAGTTTTCAAGTCCTTCCAGA  
ClaI BglII

GAGGAATGTCC

MDRR: 5'-GTCGAC AGATCTCTACTGGCGACGCCA  
Sall BglII

The amplified fragment was cloned into pGEM T easy (Promega, WI) and its nucleotide sequence was confirmed. The BglII fragment was cloned into the BamHI site of the MIN-derived vector.

**Transfection and transduction.** The retroviral vectors were transfected into 293T (CRL1609) cells each with pVM-GP and pVM-AE plasmids expressing gag-pol and amphotropic env,<sup>9</sup> respectively, using the FuGene6 (Roche, Indianapolis, IN), according to the manufacturer's instructions. Supernatants from the transfected 293T cells were collected, usually 48 h after transfection, filtered through a 0.45- $\mu$ m filter, and used to transduce HT1080 (CCL-121) cells seeded at  $2 \times 10^5$  in a 60-mm dish on the previous day. One milliliter of the viral supernatant was added in the presence of 8  $\mu$ g/mL polybrene. Two days following transduction, cells and supernatants were harvested and assayed.

**Estimation of viral titer.** Cell-free viral supernatants obtained from 293T cells were used to transduce HT1080 cells plated at a cell number

of  $2.5 \times 10^5$  on a 60-mm dish the previous day. Serially diluted viral supernatants were added to HT1080 in the presence of 8  $\mu\text{g}/\text{mL}$  polybrene. The next day, transduced cells were transferred to 100-mm plates and selected in the presence of G418 or vincristine until visible colonies were formed. Viral titer was estimated by counting the number of drug-resistant colonies.

**RT-PCR.** cDNAs were synthesized from 1  $\mu\text{g}$  of RNA sample of MIN-IL1RN by reverse transcription. RT-PCR was performed with one primer specific to the 5'-LTR and the other primer specific for IL1RN cDNA. The sequences of primers are as follows:  
Forward: 5'-ATCGGGAGACCCCTGCCCA  
Reverse: 5'-CTACTCGTCTCTCTGGAAGTA

**Fluorescence-activated cell sorting analysis.** For the analysis of ABCB1 expression, 2 days after transduction with M- and MA-ABCB1,  $1 \times 10^6$  HT1080 cells were washed once with phosphate-buffered saline containing 0.1% sodium azide (fluorescence-activated cell sorting buffer). Cells were stained with saturating amounts of antibody MRK-16 (Kamiya Biomedical MC-012) for 30 min at 4°C.<sup>16</sup> Cells were then washed twice with fluorescence-activated cell sorting buffer and stained again with fluorescein isothiocyanate-conjugated anti-mouse antibody (F-2883, Sigma, St Louis, MO). Subsequently, cells were washed three times and suspended in 500  $\mu\text{L}$  of fluorescence-activated cell sorting buffer. Flow cytometry was performed on a FACSort (Becton Dickinson, San Jose, CA) with the aid of CellQuest (Becton Dickinson) data acquisition and analysis software.

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