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Mapping of the Bovine Immunodeficiency Virus Packaging Signal and RRE and Incorporation into a Minimal Gene Transfer Vector

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Gene transfer systems based on lentiviruses have emerged as promising gene delivery vehicles for human gene therapy due to their ability to efficiently transduce nondividing target cells. Both primate and nonprimate lentiviruses have been used for construction of lentiviral vectors. An early generation of gene transfer system based on bovine immunodeficiency virus (BIV) has been developed (R. D. Berkowitz, H. Ilves, W. Y. Lin, K. Eckert, A. Coward, S. Tamaki, G. Veres, and I. Plavec, 2001, *J. Virol.* 75, 3371–3382). In this study, we mapped the BIV Rev response element (RRE) to 312 bp of the Env coding region. Furthermore, we compared transduction efficiencies of vectors containing different portions of the BIV Gag coding region and found that the first 104 bp of gag contains a functional part of the BIV packaging signal. These findings enabled the generation of a minimal BIV-based lentiviral vector. The minimal transfer vector construct consists of a self-inactivating long terminal repeats (LTR), minimal packaging sequence, putative central polypurine tract, minimal RRE, an internal promoter driving the gene of interest, and a woodchuck hepatitis posttranscriptional regulatory element. In addition, we constructed a BIV packaging construct containing *gag/pol,* minimal Rev/RRE, and the accessory gene *vpy.* The regulatory gene *tat* and the accessory genes *vif* and *vpw* have been inactivated or truncated. The current system has significantly reduced regions of homologies between the transfer vector and the packaging constructs. The vectors generated from this system achieved a titer of greater than 1×10^6 transducing units per milliliter and are fully functional as indicated by their ability to efficiently transduce both dividing and nondividing cells. These modifications should provide improved safety features for the BIV-based gene transfer system.

Key Words: lentiviral vector; bovine immunodeficiency virus; packaging signal; Rev response element.

INTRODUCTION

Various viral vectors have been explored as vehicles to deliver therapeutic genes for human gene therapy. Murine leukemia virus (MLV), adenovirus, and adenoassociated virus (AAV) based vectors have been widely used for such purposes. All these vector systems have their strengths and weaknesses. MLV-based vectors have been proven safe for human gene therapy yet suffer from an inability to transduce nondividing cells, which are often the therapeutic targets *in vivo* (Miller *et al.,* 1990; Roe *et al.,* 1993; Lewis and Emerman, 1994; Liu *et al.,* 1996; Uchida *et al.,* 1998). Human adenovirus based vectors are capable of efficiently transducing a variety of nondividing target cells. However, a strong host immune reaction and transient gene expression have limited the applications of the most commonly used vectors. AAV-based vectors have shown promise in preclinical and clinical trials; however, packaging capacity is a limitation.

Lentivirus-based gene transfer systems represent a promising gene delivery technology due to their ability to efficiently transduce a variety of nondividing target cells *in vitro* and *in vivo* (Poznansky *et al.,* 1991; Lewis *et al.,* 1992; Naldini *et al.,* 1996a,b; Blomer *et al.,* 1997; Kafri *et al.,* 1997; Miyoshi *et al.,* 1997; Gallichan *et al.,* 1998; Douglas *et al.,* 1999; Galileo *et al.,* 1999). In addition, lentiviral vector-mediated gene expression does not require *de novo* synthesis of viral proteins, reducing the potential elimination of target cells by the host immune system. The vector system has shown utility in preclinical animal models of Parkinson's disease and ocular diseases (Takahashi *et al.,* 1999; Deglon *et al.,* 2000; Kordower *et al.,* 2000). However, the lentiviral systems with the most promising results have been those based on human immunodeficiency virus (HIV), the causative agent of AIDS. Several animal lentivirus-based gene transfer systems have been developed, providing alternatives to HIV-based vectors (Olsen, 1998; Poeschla *et al.,* 1998; Mitrophanous *et al.,* 1999; Curran *et al.,* 2000; Mangeot *et al.,* 2000; Berkowitz *et al.,* 2001a,b).

Bovine immunodeficiency virus (BIV) is a lentivirus that infects a large number of cattle (Garvey *et al.,* 1990; Gonda *et al.,* 1994; St. Cyrcoats *et al.,* 1994). However, the significance of natural BIV infections to the health of herds has not been clearly established (Gonda *et al.,* 1994). Interestingly, BIV causes acquired immune dys-

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function in experimentally infected rabbits (Kalvatchev *et al.,* 1995; Walder *et al.,* 2001). In addition to its three structural genes (*gag, pol,* and *env*), BIV has two regulatory genes (*tat, rev*) and three accessory genes (*vif, vpw,* and *vpy*) in the central region. Also, it has been suggested that an additional open reading frame may exist at the end of *env,* which encodes a fourth accessory gene, *tmx,* in the same frame as *env* (Garvey *et al.,* 1990; Oberste *et al.,* 1993). Unlike HIV, BIV has not been intensively studied. The biological functions of the BIV accessory genes are unknown. Furthermore, locations of some important BIV elements such as the packaging signal sequence and Rev response element (RRE) have not been identified. There is no significant sequence homology between BIV and other lentiviruses except for the reverse transcriptase coding sequence. Therefore, it presents a significant challenge to derive an advanced gene transfer system from this virus. An early generation gene transfer system based on bovine immunodeficiency virus has been developed (Berkowitz *et al.,* 2001b). In this study, we mapped the location of the BIV packaging signal and RRE and generated a minimal transfer vector construct. We also modified the early generation packaging construct pBH2 (Berkowitz *et al.,* 2001b) by including the identified RRE and blocking expression of the accessory genes *vif, vpw,* and the regulatory gene *tat.* The BIV vectors produced with this system achieve a titer greater than 1×10^6 transducing units per milliliter and are fully functional as indicated by their efficient transduction of both dividing and nondividing cell lines, as well as human primary cells.

RESULTS

Minimal requirement of BIV *gag* sequence for packaging

It has been shown in HIV that approximately 50 nucleotides between the major splice donor site (MSD) and the *gag* initiation codon as well as the first 40 nucleotides of *gag* are critical for efficient RNA packaging (Lever *et al.,* 1989; Aldovini and Young, 1990; Clavel and Orenstein, 1990; Luban and Goff, 1994). The location of the BIV packaging sequence has not been identified. The early generation BIV vector pBS4MGppt had 509 bp of *gag* sequence remaining, which contained the putative packaging sequence (Berkowitz *et al.,* 2001b). To reduce the sequence homology between the transfer vector and our Gag/Pol expression packaging construct, we determined the minimal amounts of *gag* sequence required to preserve the packaging function. We made a series of deletions to the Gag-coding sequence from the 3′ end of the 509-bp *gag* sequence in pBS4MGppt, generating four constructs with 212 bp *gag* (pBIVminivec), 28 bp *gag* (pBv28), 54 bp *gag* (pBv54), and 104 bp *gag* (pBv104). Lentiviral vectors were generated from each of these four vector constructs by cotransfection with packaging and VSV-G expression constructs and compared with the parental vector pBSV4MGppt (Berkowitz *et al.,* 2001b). The number of vector particles were normalized by reverse transcriptase (RT) activity and equal amounts of RT-containing vector supernatants were used to transduce Cf2Th cells. As shown in Fig. 1, the vectors with either 212 or 104 bp of *gag* sequence achieved transduction efficiencies equivalent to the parental construct containing 509 bp of Gag-coding sequence. Thus the 405-bp gag deletion did not affect the putative packaging sequence (compare Figs. 1C and 1F to 1B). However, fur-

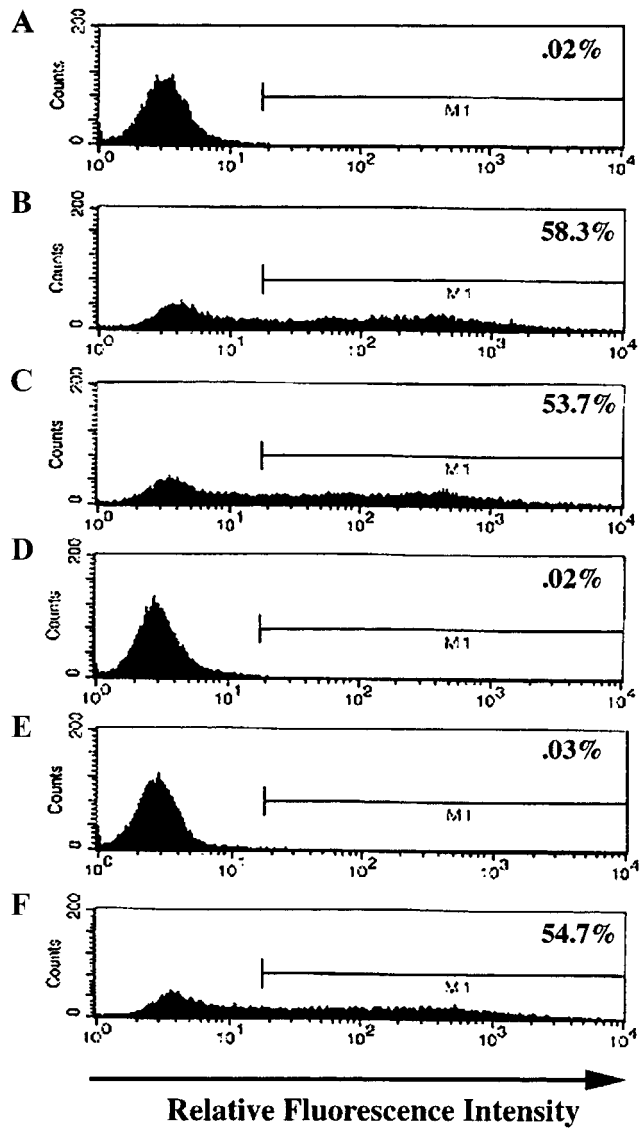

FIG. 1. The first 104 bp of gag sequence is important for BIV vector packaging. The same number of Cf2Th cells were transduced with Mock (A); or equal amounts of RT containing vectors pBS4MGppt (containing 509-bp gag, B); pBIVminivec (containing 212-bp gag, C), pBv28 (containing 28-bp gag, D), pBv54 (containing 54-bp gag, D), pBv104 (containing 104-bp gag, E). eGFP expression was measured by flow cytometry analysis 48 h posttransduction. The number in the panels represents the percentage of GFP-positive cells. The experiment was repeated three times with similar results.

TABLE 1

Mapping of BIV RRE

Construct	Base pairs	Primer sequence	Transduction efficiency
Positive control pBvΔG	6783–7561	n/a	100%
1. pBvRRE1	6783–7030	5′ ggcgaattcgatctaggaaaaaattttccg-3′ 3′ ggaagatctccacaaacccatagctgg-5′	1%
2. pBvRRE2	7005–7290	5′ cccgaattcaaaggtccccagc-3′ 3′ ggaagatctctctatggtgtaggac-5′	65%
3. pBVRRE3	7288–7561	5′ ccggaattcgagtttcatacttggag-3′ 3′ ggaagatcttgcactaaatggtc-5′	9%
4. pBvRRE4	6908–7181	5′ ccggaattccctaatactatgcc-3′ 3′ ggaagatctcttagccgtcgtgtgc-5′	38%
5. pBvRRE5	7192–7431	5′ ggcgaattcgggttgtgcaaaatgtg-3′ 3′ cctagatctcattccaagttttgct-5′	3%
6. pBvRRE6	6992–7303	5′ ccggaattcgtggattcttgtaaagg-3′ 3′ ggaagatctctccaagtatgaaactc-5′	106%
7. pBvRRE7	7048–7345	5′ ccagaattccaccaccatccctcc-3′ 3′ ggaagatctcaaccaaagaatact-5′	98%

Note. Seven different constructs that incorporated different regions of the putative RRE. All seven constructs were individually transfected into 293T cells together with the BIV packaging construct, pBH2M2, and the VSV-G expression plasmid. Forty-eight hours posttransfection viral supernatant was harvested. These supernatants were normalized by the amount of reverse transcriptase (RT) activity and the same amount of RT-containing vector supernatant was used to transduce 293T cells. Of the seven constructs created, only vectors generated from pBvRRE6 and pBvRRE7 (containing RRE6 and RRE7) transduced cells as efficiently as vectors produced by the parental construct containing the full 778-bp RRE. The bp numbers for each RRE correspond to the nucleotide position in BIV genomic RNA sequence isolate 127 (Garvey *et al.,* 1990).

ther deletion of the gag sequence resulted in defective vector particles, as indicated by the lack of transduction with vectors containing either 28 or 54 bp of *gag* (Figs. 1D and 1E). While the precise location and the secondary structure of the packaging signal sequence within the BIV vectors still remains unclear, the results suggest that the 5′ untranslated region (UTR) and the first 104 bp of Gag coding sequence are sufficient for packaging BIV vector RNA.

Identification of the BIV Rev response element

In HIV and other lentiviruses, Rev is responsible for nuclear export of unspliced viral genomic RNA and singly spliced mRNA by binding to the Rev response element. The BIV RRE sequence had not been identified. The early generation BIV vector pBC4MGppt contained the putative RRE sequence in a 778-bp envelope coding region (Berkowitz *et al.,* 2001b). Exclusion of this 778-bp putative RRE sequence resulted in defective vector particles (data not shown). To reduce the sequence homology between the transfer vector and the Gag/Pol packaging construct, we accurately mapped the location of the RRE within these 778 bp. We created seven different constructs that incorporated different regions of the putative RRE (Table 1). Each of the seven constructs was transfected into 293T cells together with the BIV packaging construct pBH2M2 (see Materials and Methods) and the VSV-G expression plasmid. Forty-eight hours posttransfection, viral supernatants were harvested. Each supernatant was normalized by the amount of reverse transcriptase activity, and the same amount of RT-containing vector supernatant was used to transduce 293T cells. Of the seven constructs created, only vectors generated from pBvRRE6 and pBvRRE7 (containing RRE6 and RRE7) transduced cells as efficiently as vectors produced by the parental construct containing the full 778-bp RRE sequence (Table 1). These data suggest that the sequence containing the RRE can be narrowed down to approximately 300 bp (Fig. 2A). RRE function is known to be orientation specific. To further verify that the 300-bp sequence was functioning as an RRE, we inserted it into the vector in reverse orientation. As expected (Fig. 2B), in reverse orientation, sequence was nonfunctional. Finally, vector particle production is Rev-dependent; removal of *rev* from the packaging construct diminished vector particle yield as determined by RT levels (data not shown).

With the mapping of packaging sequence and RRE, a minimal BIV transfer vector construct was generated (Fig. 3). Starting from the 5′ end, the minimal transfer vector construct contains the following: 5′ long terminal repeats (LTR) with a CMV immediate-early enhancer-promoter replacing U3, an intact UTR between LTR and *gag* coding region, 212- or 104-bp *gag* coding region, RRE6, an internal promoter driving a transgene, 3′ polypurine tract, 3′ LTR with a 332-bp deletion in U3, and a 40-bp SV40 late polyadenylation signal upstream enhancer element inserted in the place of the U3 deletion (Carswell and Alwine, 1989; Berkowitz *et al.,* 2001b). In addition, the woodchuck hepatitis virus posttranscriptional regulatory element (PRE) was incorporated into the

A

GTGGATTCTTGTAAAGGTCCCCAGCTATGGGTTTGTGGTAGTAAATGAC
ACAGATACACCACCATCCCTCCGCATCCGAAAGCCTCGAGCAGTCGGAC
TAGCAATATTCCTGCTTGTGCTGGCTATCATGGCCATCACATCCTCCTTG
GTGGCAGCTACAACGCTCGTGAACCAGCACACGACGGCTAAGGTTGTGG
AGAGGGTTGTGCAAAATGTGTCATATATTGCTCAAACCCAGGACCAATT
CACCCACCTGTTCAGGAATATAAACAACAGATTAAATGTCCTACACCATA
GAGTTTCATACTTGGA

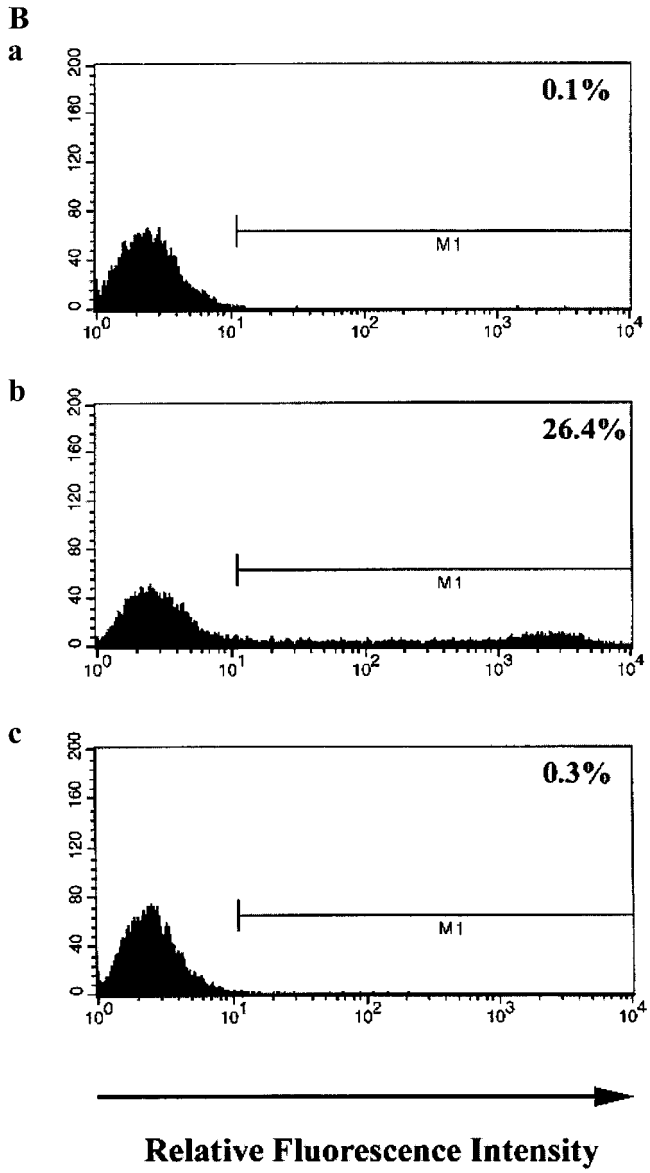

FIG. 2. (A) The 312-bp minimal BIV RRE sequence located within the envelope gene. This sequence is required and sufficient to support nuclear export. (B) Effect of orientation on RRE function. 293T cells were transduced with Mock (a), equal amounts of RT containing vectors generated by either pBv104 (b) or pBv104ERR (with RRE at reverse orientation) (c). Forty-eight hours posttransduction, the transduced cells were analyzed for eGFP expression by flow cytometry. The number in the panels represents the percentage of eGFP-positive cells. The experiment was repeated three times with similar results.

BIV transfer vector immediately downstream eGFP coding sequence. The PRE has been shown to be able to enhance transgene expression in the context of retroviral and lentiviral vectors (Zufferey *et al.,* 1999). The PRE element increased eGFP expression by three- to fivefold in the context of BIV vectors (data not shown). A putative BIV central purine tract was also included in the vector (Berkowitz *et al.,* 2001b) (Fig. 3).

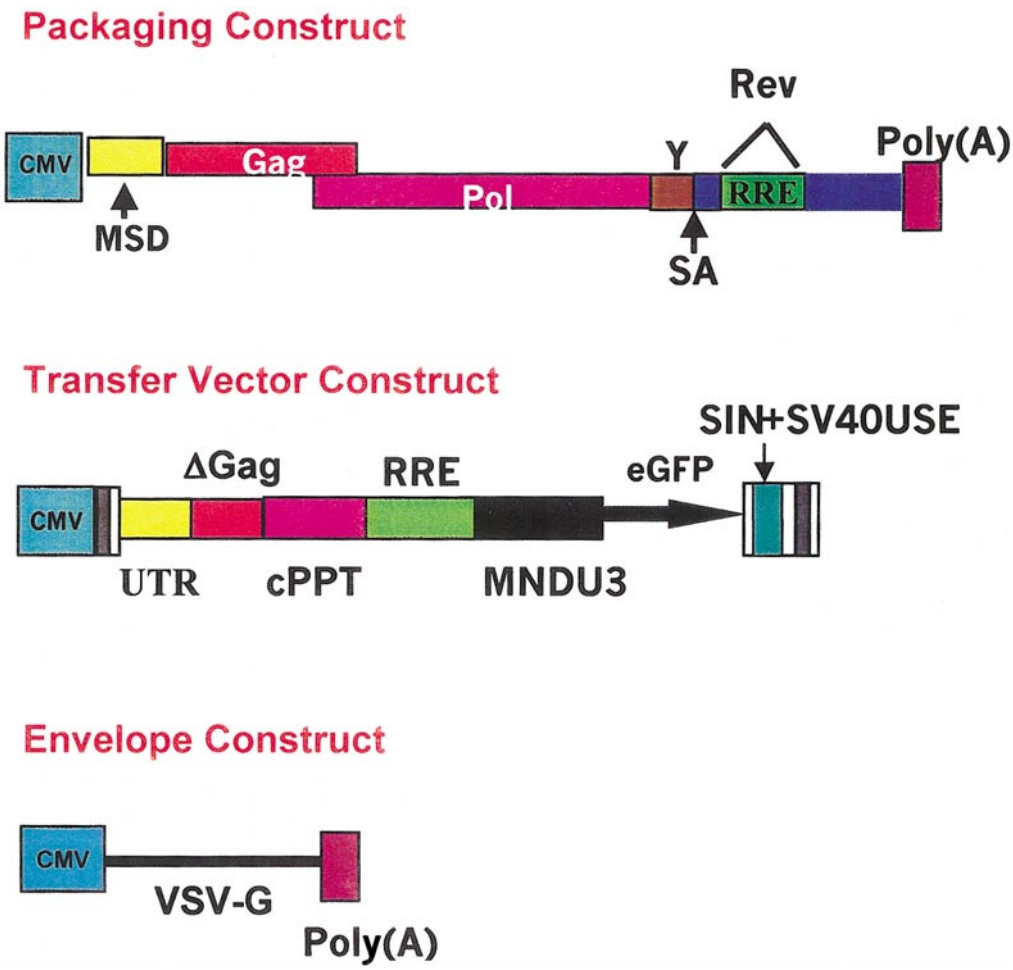

FIG. 3. Schematic representation of BIV-based gene transfer system. The BIV three plasmid gene transfer system contains the packaging construct, the transfer vector construct, and the envelope expression construct. CMV, CMV immediate early promoter; Poly(A), SV40 polyadenylation signal; MSD, major splice donor site; SA, splice acceptor site; Rev, BIV Rev; RRE, rev response element; UTR, untranslated region; ΔGAG, deletion in gag coding sequence; cPPT, central polypurine tract; MNDU3, a modified version of the myeloid proliferative sarcoma virus long terminal repeat U3 region serving as an internal promoter driving eGFP; SIN, self-inactivating; SV40USE, SV40 polyadenylation signal upstream enhancer element; VSV-G, vesicular stomatitis virus envelope glycoprotein G.

Modification of the BIV packaging construct

The packaging construct was derived from pBH2, which has previously been described (Berkowitz *et al.,* 2001b). In addition to *gag, pol,* and *env,* BIV has two regulatory genes, *tat* and *rev,* and four predicted accessory genes, *vif, vpw, vpy,* and *tmx.* HIV has four accessory genes, *vif, vpr, vpu,* and *nef,* some of which play important roles for viral pathogenesis (Kestler *et al.,* 1991; Trono, 1995) but are dispensable for the HIV vector-mediated gene transfer into nondividing cells (Parolin *et al.,* 1994; Reiser *et al.,* 1996; Zufferey *et al.,* 1997). We generated a packaging construct, pBH2M2RRE6, which has the *tat* ATG start site mutated to a stop codon, and the *vif* and *vpw* open reading frames disrupted by stop codons. Moreover, we also deleted 50 bp of untranslated leader sequence upstream of the major splice donor site (Fig. 3). These mutations were confirmed by direct DNA sequencing. In addition, the minimal RRE6 was introduced into the construct between the two exons coding for Rev.

Titer of minimal BIV vectors

We examined the titer of the BIV vectors produced from 293T cells cotransfected with the transfer vector, pBIVminvec, the packaging construct, pB2M2RRE6, and the VSV-G expression construct. Cf2Th cells (4×10^5) were transduced with different amounts of vectors encoding eGFP. Forty-eight hours posttransduction, 18.5% of cells scored positive for eGFP expression in the sample containing 50 μl of vector supernatant giving a relative vector titer of 1.5×10^6 transducing units (T.U.) per milliliter (Fig. 4). It should be noted that the titer scored by eGFP expression is not absolutely linear with the vector input.

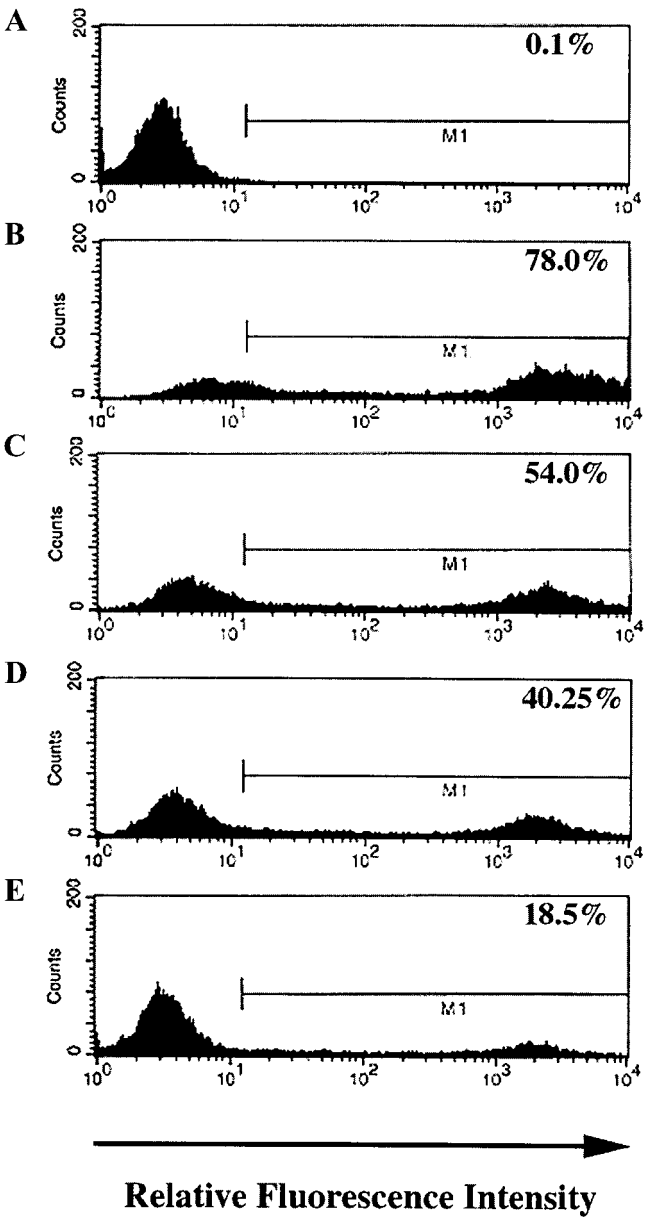

FIG. 4. Titration of BIV nonconcentrated vectors encoding eGFP. Cf2Th cells (4×10^5/per well) were transduced with 2 ml medium containing Mock (A); 500 μl of vectors (B); 250 μl of vectors (C); 100 μl of vectors (D); and 50 μl of vectors (E). Forty-eight hours posttransduction, the transduced cells were analyzed for eGFP expression by flow cytometry. The number in the panels represents the percentage of eGFP-positive cells. The experiment was repeated three times with similar results.

Transduction of dividing and nondividing cells

We evaluated the capacity of the BIV vectors encoding GFP to transduce dividing and nondividing cells. Vector supernatants were assayed for RT activity. Equal amounts of RT were used to transduce both dividing and nondividing HeLa and Neuro-2A cells (Figs. 5 and 6). To suppress cell division, cells were exposed to aphidicolin. VSV-G pseudotyped MLV vector served as a control to confirm the status of cell division. As expected, VSV-G pseudotyped MLV vector efficiently transduced both dividing HeLa and Neuro-2A cells (Figs. 5B and 6B), but not the aphidicolin-treated cells (Figs. 5E and 6E). In contrast, BIV vector efficiently transduced both nondividing HeLa and Neuro-2A cells with the minimal BIV vector at relatively high efficiencies (Figs. 5F and 6F). Therefore the vectors generated from the minimal transfer vector and the minimized packaging constructs were fully competent to mediate transgene expression in both dividing and nondividing cells.

To ensure that the modifications made to the packaging construct did not compromise the vector transduction efficiency, we did a side-by-side comparison of the vectors generated either by pBH2 or by pBH2M2RRE6. We found that pBH2 and pBH2M2RRE6 produced equivalent amounts of vector particles as indicated by RT assay (data not shown). Further, the same amount of RT-containing vectors generated from these two packaging constructs transduced both dividing and nondividing HeLa cells at equivalent efficiency (Fig. 7), suggesting that the accessory genes *vif* and *vpw* are dispensable for BIV vector-mediated gene expression *in vitro.*

Transduction of human primary cells

To examine the ability of the minimal BIV vector to transduce primary cells, we transduced human primary skeletal muscle cells treated with and without aphidicolin. MLV vector was used to confirm the status of cell division. As expected, MLV efficiently transduced the dividing primary cells (Fig. 8B), but did not score any significant transduction in the nondividing cells (Fig. 8E). In contrast, the BIV vector efficiently transduced both dividing and nondividing primary cells (Figs. 8C and 8F), confirming that the significant modification to the BIV transfer vector and packaging constructs did not affect the ability of the BIV vector to transduce both dividing and nondividing human primary cells. Together, these data suggest that accessory genes *vif* and *vpw* are dispensable for BIV vector-mediated gene expression *in vitro.* These data also confirmed that RRE6 is sufficient for BIV RRE-mediated nuclear export of viral BIV RNA.

DISCUSSION

A lentiviral vector system based on BIV has been reported (Berkowitz *et al.,* 2001b). Initial testing suggested that these vectors were able to transduce both dividing and nondividing cells *in vitro.* Moreover, BIV vectors efficiently transduced mouse retinal cells *in vivo* and expressed GFP for the 5-month duration of the study (Takahashi *et al.,* 2002). However, limited knowledge of BIV has made it difficult to develop an advanced version of a BIV-based gene transfer system. Significant homology between the BIV packaging and transfer vector constructs existed in the previous system (Berkowitz *et al.,*

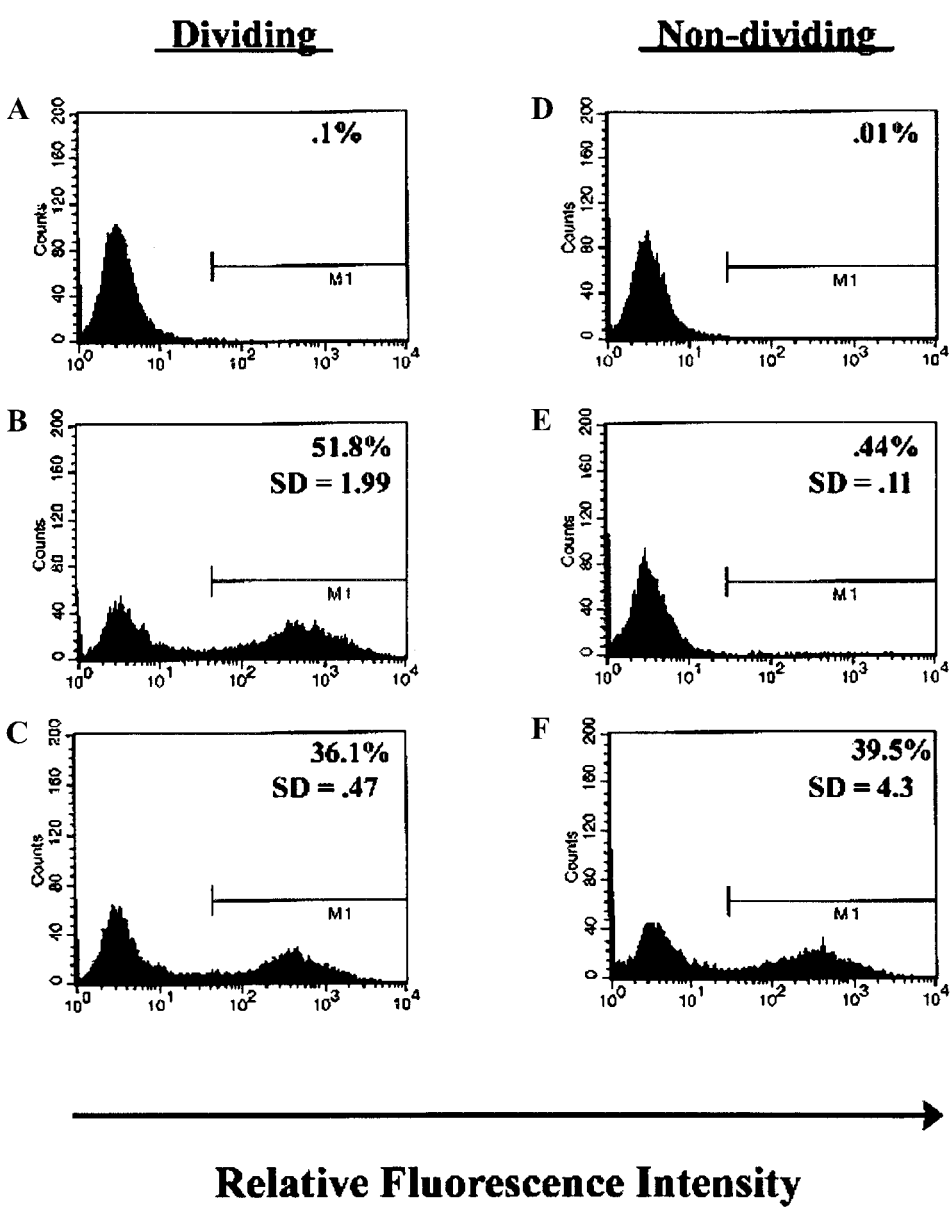

FIG. 5. Flow cytometry analysis of BIV vector-mediated eGFP expression in dividing and nondividing HeLa cells. (A, B, and C) Dividing cells. (D, E, and F) Aphidicolin-treated cells. Cells were then transduced with either Mock (A and D), MLV vector (B and E), or BIV vector (C and F), respectively. The number in the panels represents the percentage of eGFP-positive cells and is the mean of triplicate samples. SD refers to the standard deviation. The experiment was performed three times with similar results.

2001b). This was due in part to the fact that BIV packaging signal sequence and the BIV RRE had not been identified. Large portions of BIV *gag* and *env* coding sequence remained in the transfer vector and a large portion of *env* sequence remained in the packaging construct. Lentiviral packaging signal sequences usually extend into the *gag* coding region, although to different degrees depending upon the lentivirus (Lever *et al.,* 1989; Aldovini and Young, 1990; Clavel and Orenstein, 1990; Luban and Goff, 1994; Johnston *et al.,* 1999). The early generation BIV transfer vector was designed to contain 509 bp of *gag* sequence and 778 bp of *env* sequence to ensure incorporation of the packaging signal and RRE, respectively (Berkowitz *et al.,* 2001b). In this study, we were able to reduce the *gag* sequence to 104 bp without affecting transduction efficiency of the vector (Fig. 3).

Lentiviruses contain instability sequences (INS) found in *gag, pol, env,* and RRE (Luciw, 1996). To overcome this disadvantage, lentiviruses have evolved a unique mechanism that utilizes a Rev protein that binds to an RRE sequence to efficiently export viral genomic RNA and singly spliced mRNA out of the nucleus (Schwartz *et al.,* 1992; Luciw, 1996; Cullen, 1998). Although BIV Rev coding regions (two exons) have been identified (Oberste *et al.,* 1993), the precise location of BIV RRE had not been mapped. In the current study, we identified a 312-bp

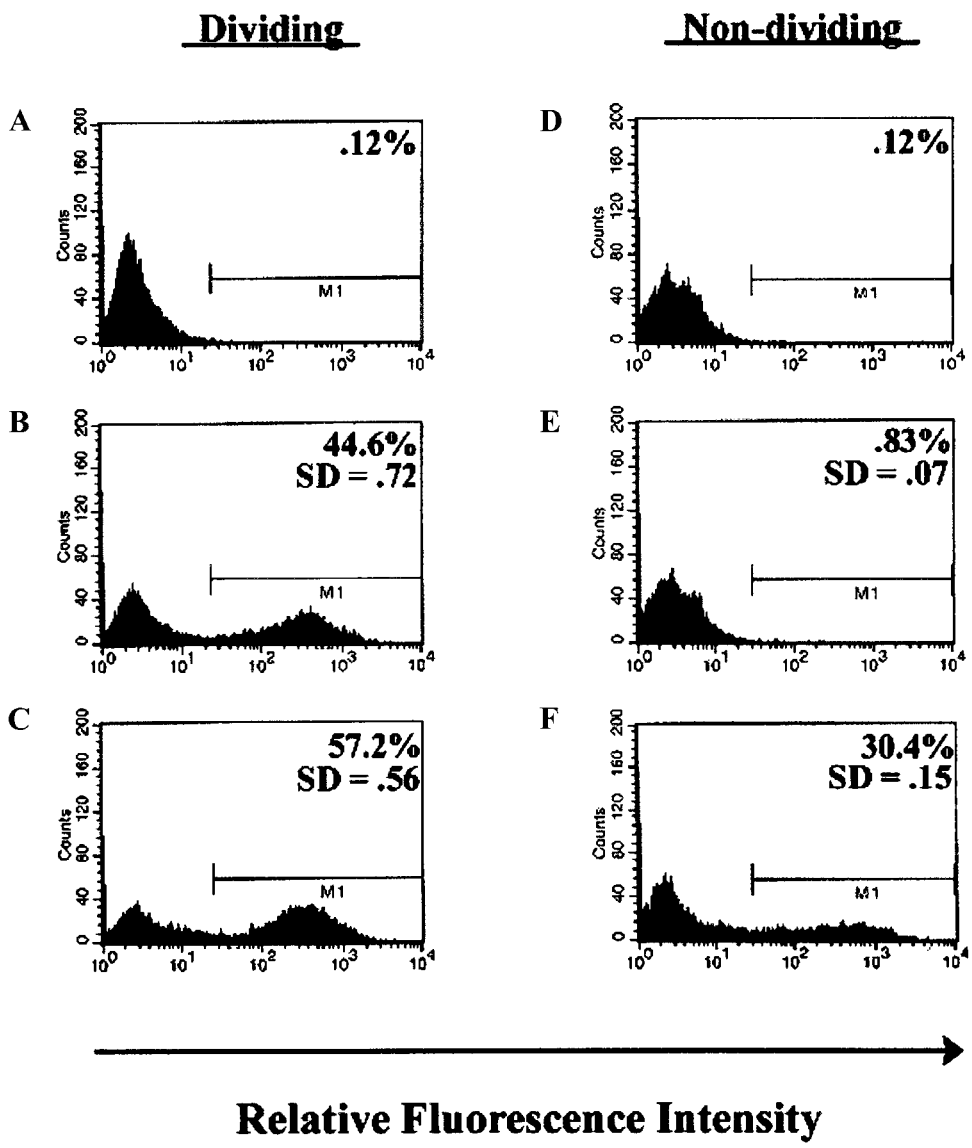

FIG. 6. Flow cytometry analysis of BIV vector mediated eGFP expression in dividing and nondividing Neuro2A cells. (A, B, and C) Dividing cells. (D, E, and F) Aphidicolin-treated cells. Cells were then transduced with either Mock (A and D), MLV vector (B and E), or BIV vector (C and F), respectively. The number in the panels represents the percentage of eGFP-positive cells and is the mean of triplicate samples. SD refers to the standard deviation. The experiment was performed three times with similar results.

sequence which contains the RRE (Fig. 2). Removal of this 312-bp sequence from the transfer vector rendered the vector particles defective, as indicated by their inability to transduce cells (data not shown).

By shortening the packaging signal sequence in the vector construct and the RRE in the vector and packaging constructs, we effectively reduced homologies between these two constructs. Furthermore, we have inactivated the regulatory gene *tat* in the packaging construct, as it is not needed for the self-inactivating vector (Zufferey *et al.,* 1998; Deglon *et al.,* 2000; Berkowitz *et al.,* 2001b).

BIV has four predicted accessory genes, *vif, vpw, vpy,* and *tmx.* The biological functions of these accessory genes in the BIV lifecycle and pathogenesis have not yet been delineated. Moreover, these accessory genes share no significant homology with the accessory genes found in other lentiviruses (Garvey *et al.,* 1990; Gonda *et al.,* 1994). We truncated *vif* and *vpw* in the packaging construct without affecting packaging function, suggesting they are dispensable for gene transfer. The BIV vectors generated from our minimized constructs are fully functional, suggesting the modifications made to the transfer vector construct, as well as the modifications made to the packaging construct, did not affect the performance of the vectors.

One of the major concerns with clinical applications of lentivirus-based gene transfer systems is the potential risk of generating replication competent lentiviruses (RCL). It has been suggested that the potential risk exists within HIV as well as other lentivirus-based systems (Wu

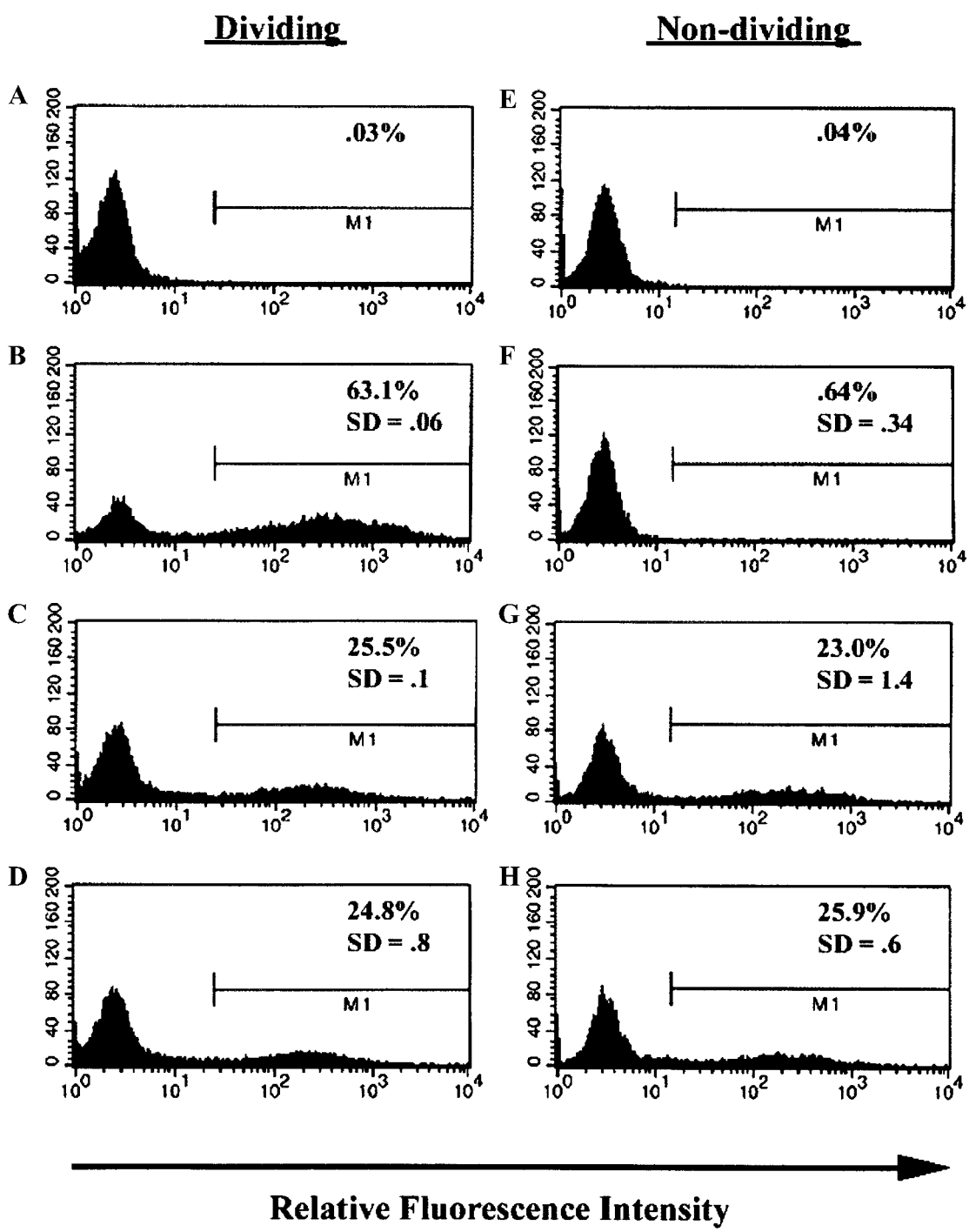

FIG. 7. Flow cytometry analysis of BIV vector-mediated eGFP expression in dividing and nondividing HeLa cells. Dividing cells (A, B, C, and D) and nondividing cells (E, F, G and H) transduced with either Mock (A and E), MLV vector (B and F), BIV vector generated from the parental packaging construct pBH2 (C and G), or BIV vector generated from the minimized packaging construct pBH2M2RRE6 (D and H). The number in the panels represents the percentage of eGFP-positive cells and is the mean of triplicate samples. SD refers to the standard deviation. The experiment was performed three times with similar results.

et al., 1997; Buchschacher and Wong-Staal, 2000; Trono, 2000). Several strategies are being employed to minimize the risk of generating RCL. The strategies utilized in these studies include separating the vector system components into multiple plasmids and minimizing the sequence homologies among the plasmids. The three-plasmid system described here could also be separated into four plasmids by expressing Rev in a different construct (Dull *et al.,* 1998). Alternatively, dividing the *gag/pol* coding sequence into two plasmids should presumably provide a safer lentivirus-based gene transfer system (Wu *et al.,* 1997, 2000). Finally, recoding *gag/pol* in the

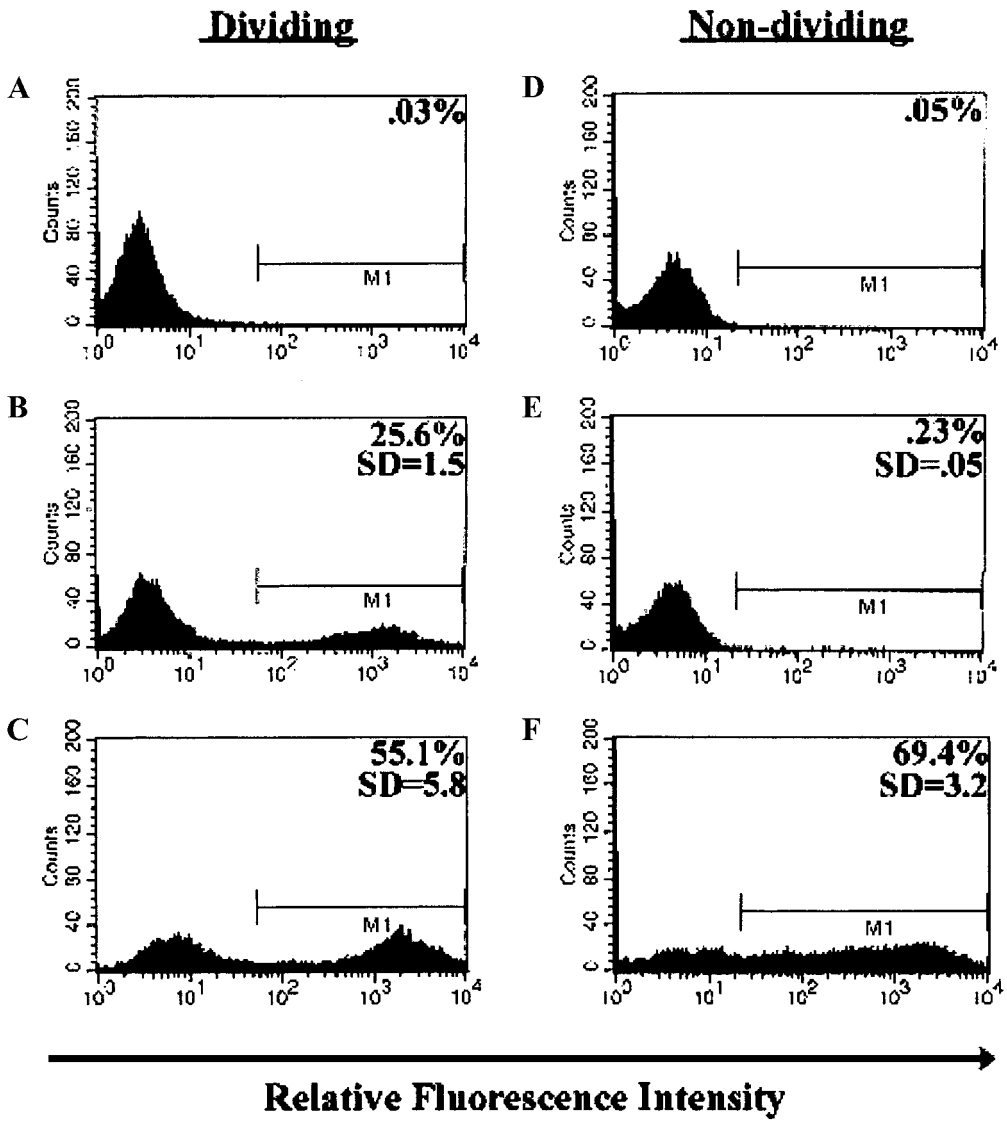

FIG. 8. Flow cytometry analysis of BIV vector-mediated eGFP expression in dividing and nondividing primary human skeletal muscle cells. (A, B, and C) Dividing cells. (D, E, and F) Aphidicolin-treated cells. Cells were transduced with either Mock (A and D), MLV vector (B and E), or BIV vector (C and F), respectively. The number in the panels represents the percentage of eGFP-positive cells and is the mean of triplicate samples. SD refers to the standard deviation. The experiment was performed three times with similar results.

packaging construct significantly reduces the homology between the packaging and the transfer vector constructs (Kotsopoulou *et al.,* 2000; Wagner *et al.,* 2000). Although homologies still exist in the BIV-based system, the identification of BIV packaging sequence and RRE and modifications we made to the BIV-based gene transfer system in this article should serve as a first step to the generation of an efficient and safer BIV-based vector system for human gene therapy.

MATERIALS AND METHODS

Plasmid construction

The transfer vector pBIVminivec was derived from pBC4MGppt, which has previously been described (Berkowitz *et al.,* 2001b). To facilitate the cloning, the entire BIV transfer vector coding sequence was cloned into the expression plasmid pBS II KS$^+$ (Stratagene, La Jolla, CA) by digesting pBC4MGppt with *Bsp*MI and ligating to pBS II KS$^+$ previously digested with *Hinc*II as a blunt-end ligation to create the plasmid pBv. The plasmid pBv was digested with *Bgl*II and *Eco*NI, Klenow treated, and re-ligated to remove a 297-bp fragment of the *gag* gene to create the plasmid pBvΔG, which contained 212-bp Gag coding sequence. Due to the lack of unique and convenient restriction sites immediately following the eGFP reporter gene, a unique *Pst*I site was incorporated using the primers WPRE5 (5′GAGCTGTACAAGTAAAGCGGCCAACCCTCCTGCAGAAACTCCTTTGGG-3′) and WPRE3 (5′GGAACAAAAGCTGGGTACCGGGCCC-

CCCC-3′) to create the plasmid pBvΔGPstI. The woodchuck hepatitis posttranscriptional regulatory element was then cloned into the backbone, pBvΔPstI, which was previously digested with *Pst*1, treated with Klenow, and ligated to the PRE fragment to create the plasmid pBvΔGPRE. The plasmid pBvΔGPRE was further modified by removing all of the putative RRE, which is about 778 bp in the original BIV transfer vector (Berkowitz *et al.,* 2001b), and replacing it with a 312-bp fragment of the RRE. First, the plasmid pBvΔGPRE was digested with *Kas*I and *Bbv*CI. This region was then PCR amplified with the primers RRE1 (5′GTTGGCGCCCAACGTGGGGCTCGAGTAAGAGAG-3′), RRE2 (5′AGATCTGAATTCTAAGTGACCTATTTC-3′), RRE3 (5′GAATTCAGATCTTATGGGAATGAAAGACC-3′), and RRE4 (5′AACTGCTGAGGGCGGGACCGCATCTGG-3′). RRE1 and RRE2 amplified upstream of the putative RRE and primers RRE3 and RRE4 amplified downstream of the putative RRE. The products were then mixed in equal molar ratios and amplified with primers RRE1 and RRE4. The final product incorporated the *Kas*I and *Bbv*CI sites, deleting the entire putative RRE. Furthermore, there were unique *Eco*RI and *Bgl*II sites incorporated to create junction sites between primers RRE2 and RRE3 for annealing of the final product, but primarily for subsequent cloning of various regions of the RRE. This PCR strategy created the plasmid pBvΔRRE. The putative RRE was then PCR amplified with seven sets of primers in various regions all encoding a 5′ *Eco*RI site and a 3′ *Bgl*II site to be cloned into the backbone, pBvΔRRE. Once created, each fragment was digested with *Eco*RI and *Bgl*II and cloned as described above (Table 1). The final construct created was pBIVminivec containing RRE6. The entire pBIVminivec was then subjected to DNA sequencing to confirm the integrity of the construct. The pBIVminivec was used for all experiments in this article unless indicated otherwise.

To further delete the remaining 212-bp Gag coding sequence in the pBIVminivec, the construct pBvΔRRE was used as the template to make deletions in the *gag* sequence to determine the location of the packaging signal. The construct pBvΔRRE, which was described above, was used to delete 184 bp of GAG by digesting with *Cla*I and *Hin*dIII, treating with Klenow, and then re-ligating. This cloning strategy resulted in a construct containing 28 bp of Gag coding sequence, creating the plasmid pBv28ΔRRE. Next we created a vector construct containing 54 bp of *gag* sequence. First, the template pBvΔRRE was digested with *Kas*I and *Eco*RI and alkaline phosphatase treated. Gag coding region was amplified using the primers NRS1 (5′AACAGTTGGCGCCCAACGTGGGGCTC-3′), NRS2 (5′ATGCATCACGTGGGGTGTCACCCTAACCTTACGAA-3′), NRS3 (5′CACGTGATGCATCGATCTAAAAGACAGATTGGC-3′), and NRS4 (5′CATAAGATCTGAATTCAATGATCTAAGTG-3′). NRS1 and NRS2 were used to amplify the 5′ region of the Gag start codon (ATG) to base-pair 54 of Gag. NRS3 and NRS4 amplified 3′ of the stop codon of Gag through the BIV cPPT. The products were then mixed in equal molar ratios and amplified with primers NRS1 and NRS4. The final product incorporated the *Kas*I and *Eco*RI sites, deleting the last 158 bp of Gag within the template, resulting in a construct containing 54 bp of Gag coding sequence. Furthermore, there were unique *Nsi*I and *Pml*I sites incorporated to create junction sites between primers NRS2 and NRS3 for annealing and screening of the final product. This PCR strategy created the plasmid pBv54ΔRRE. The same PCR strategy was implemented to create the construct pBv104ΔRRE. NRS1 and NRS4 were used as external primers and the new internal primers are NRS32 (5′ATGCATCACGTGATTCTAATGGCCCATTGAAGATTC-3′) and NRS33 (5′CACGTGATGCATCGATCTAAAAGACAGATTGGC-3′). NRS1 and NRS32 were used to amplify the 5′ region of the Gag start codon (ATG) to base-pair 104 of Gag. NRS33 and NRS4 amplified 3′ of the stop codon of Gag through the BIV cPPT. The products were then mixed in equal molar ratios and amplified with NRS1 and NRS4. The final product incorporated the *Kas*I and *Eco*RI sites, deleting the last 108 bp of Gag within the template pBvΔ104RRE. As described above, there were unique *Nsi*I and *Pml*I sites incorporated to create junction sites between NRS32 and NRS33 for annealing and screening of the final product. Finally, all three constructs, pBv28ΔRRE, pBv54ΔRRE, and pBv104ΔRRE, were digested with *Eco*RI and *Bgl*II, alkaline phosphatase treated, and then the minimal RRE6, as described above, was cloned in as an *Eco*RI and *Bgl*II fragment to create the plasmids pBv28, pBv54, and pBv104, respectively. All these transfer vector constructs were subjected to DNA sequencing to confirm the integrity of the construct. To generate the transfer vector construct with RRE at the reverse orientation, pBv104 was digested with *Eco*RI and *Bql*II to cut out the RRE6. Both fragments were then blunt-ended and the RRE6 was cloned back at the reverse orientation by ligating the two fragments, creating pBv104ERR.

The packaging construct pBH2M2RRE6 was derived from the previously described construct pBH2 (Berkowitz *et al.,* 2001b). First, primers were constructed to delete some of the 5′ untranslated leader sequence without affecting the MSD site. This region was PCR amplified with primers pBH2delpac2F (5′CATGTTGCATTG-3′) and pBH2delpac2R (5′GGTTCTCCGGAGTTCGAGACTGTTG-3′), using pBH2 as the template. The pBH2 plasmid and the PCR product were digested with *Spe*I and *Bsp*EI and then ligated to create pBH2ΔUTR. The next step was to inactivate the *tat* gene by mutating the start codon of exon 1 to a stop codon. The *Sal*I fragment from pBH2ΔUTR encoding the first exon of *tat* was subcloned into pUC19 (New England BioLabs, Beverly, MA). The subclone was then amplified with primers pBH2deltatF (5′CCACCCAAGGTCCGGGCTAATTGCCACAATCTATG-3′) and pBH2deltatR (5′CCACCCAAGGTCCGGGCTAATT-

GCCACAATCTATG-3′) using the Quickchange Site Directed Mutagenesis kit (Stratagene) to create the plasmid pUC19Δtat. pUC19Δtat was then digested with *Sal*I and cloned back into pBH2ΔUTRSalI to create the plasmid pBH2ΔUTRΔtat. pBH2ΔUTRΔtat was then digested with *Eco*RV and *Not*I and subcloned into pBSII KS$^+$ (Stratagene) to truncate *vif* and *vpw* by inserting in-frame stop codons 3′ to the end of the *pol* reading frame. The resulting plasmid created was pBSII KS$^+$pol. The subclone was then amplified using the primers StopWF (5′GAGTTCGTGAGGCAGACTGAATAGAGCATGACCGCGTGCG-3′) and StopWR (5′CGCACGCGGTCATGCTCTATTCAGTCTGCCTCACGAACTC-3′) using the Quickchange Site Directed Mutagenesis kit to create the plasmid pBSII KS$^+$STOPW. PBSII KS$^+$STOPW was then digested with *Eco*RV and *Not*I and cloned back into pBH2ΔUTRΔtat to create the plasmid pBH2M2. pBH2M2 was PCR amplified with the primers pBH2RRE5 (5′ACGGCTAGCGCGCGACCATTTAGTGC-3′) and pBH2RRE3 (5′CATGTCTGCTCG-3′) to remove all of the putative RRE. The pBH2M2 backbone and the PCR product were digested with *Nhe*I and *Xba*I and ligated to create the plasmid pBH2M2ΔRRE. Last, pBH2M2ΔRRE was digested with *Xba*I and Klenow treated. The RRE6 fragment containing the mapped RRE6, as described above, was digested with *Eco*RI and *Bgl*II, Klenow treated, and ligated into the pBH2M2ΔRRE to create the final construct pBH2M2RRE6. The packaging construct was then subjected to DNA sequencing to confirm the integrity of the construct.

The pVSV-G plasmid contains the vesicular stomatitis virus glycoprotein and has previously been described (Burns *et al.,* 1993).

Cell lines and culture conditions

293T cells and HeLa cells were cultured in Dulbecco's modified Eagle's medium (BRL Life Technology, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Salt Lake City, UT), 50 IU penicillin/ml, 50 μg streptomycin/ml, and 2 mm L-glutamine (Complete DMEM). Cf2Th cells, a cell line derived from canine thymus, was obtained from American Type Tissue Collection (ATCC) (Manassas, VA). Cf2Th cells were cultured in the same medium as that for 293T except with 20% FBS. Mouse neuronal and amoeboid stem cells (Neuro-2A) were also obtained from ATCC. Neuro-2A cells were cultured in minimal essential medium (BRL Life Technology) supplemented with 10% heat-inactivated fetal bovine serum, 50 IU penicillin/ml, 50 μg streptomycin/ml, 1.0 mM sodium pyruvate, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 0.1 mM nonessential amino acids. Human primary skeletal muscle cells (SkMC) were obtained from Clonetics (San Diego, CA). SkMC cells were cultured in SkBM basal media with the SkGM bullet kit containing 0.1% human epidermal growth factor, 1% insulin, 5% BSA, 5% fetuin, 1% gentamicin-amphotericin B, and 0.5 μM dexamethasone. Cell lines were maintained at 37°C in a humidified incubator with 5% CO_2.

Viral vector production

In this article, all the vectors (unless indicated otherwise) were generated with cotransfection of 293T cells with pBH2M2RRE6 (packaging construct), BIV transfer vector construct, and VSV-G expression construct. 293T cells were seeded at a density of 10×10^6 into 150-mm dishes overnight. The following day the medium was aspirated and replaced with fresh complete DMEM. The 293T cells were transfected 4 h later using the Profection mammalian transfection system calcium phosphate coprecipitation method (Promega, Madison, WI). Typically, 45 μg of the transfer vector, 45 μg of the packaging construct, and 13.5 μg of the VSV-G plasmid were used for each dish. After 24 h, the medium was aspirated and replaced with fresh complete DMEM. Viral supernatant was harvested at 48 h posttransfection, centrifuged at 2000 rpm for 10 min to clear cell debris, and stored frozen in aliquots at −80°C. Generation of VSV-G pseudotyped MLV vector encoding eGFP was described previously (Roe *et al.,* 1993; Liu *et al.,* 1996; Gallardo *et al.,* 1997). To measure the amount of vector particles shed into the medium, the medium is cleared of cellular debris by low-speed centrifugation; then 10 μl is lysed and analyzed for RT activity using a commercial kit (Roche Molecular Biochemicals, Indianapolis, IN).

Transduction

To transduce dividing cells, 1×10^5 cells were seeded per well into six-well dishes. After 24 h, the medium was aspirated and 2 ml of viral supernatant containing medium was added to the cells. Protamine sulfate (Sigma, St. Louis, MO) was then added to the wells at a final concentration of 8 μg/ml. Cells were then maintained at 37°C in a humidified incubator with 5% CO_2 for 3 h. After 3 h, viral supernatant was aspirated and replaced with fresh medium and incubated for 48 h at 37°C in a humidified incubator with 5% CO_2. To transduce nondividing cells, 1×10^5 cells were seeded per well into six-well dishes. After 24 h, aphidicolin was then added to a final concentration of 4 μg/ml (except for the human primary cells where the concentration was reduced to 2 μg/ml aphidicolin due to the toxicity). Sixteen hours posttreatment with aphidicolin, the medium was aspirated and 2 ml of viral supernatant containing medium was added to the cells in the presence of aphidicolin. Protamine sulfate was then added to the wells at a final concentration of 8 μg/ml. Cells were maintained at 37°C in a humidified incubator with 5% CO_2 for 3 h. Viral supernatant was then aspirated and replaced with fresh medium containing aphidocolin at a final concentration

of 2 μg/ml (except for the human primary cells where the concentration was reduced to 1 μg/ml aphidicolin) and then incubated for 48 h at 37°C in a humidified incubator with 5% CO_2.

Flow cytometry analysis

For analysis of eGFP expression, the medium was aspirated from the wells. The cells were then rinsed with 2 ml phosphate-buffered saline (PBS). The PBS was then aspirated and the cells were trypsinized, washed, and resuspended in PBS containing 5% heat-inactivated fetal bovine serum. The cells were analyzed for eGFP expression on a FACS Calibur (Becton–Dickinson Biosciences).

Vector titer

To determine the vector titer, Cf2Th cells (4×10^5 cells/per well) were transduced with 2 ml medium containing different amounts of viral vectors in six-well dishes in the presence of protamine sulfate. After 3 h, viral supernatant was aspirated and replaced with fresh medium and incubated for 48 h at 37°C in a humidified incubator with 5% CO_2. EGFP expression was then analyzed by flow cytometry analysis. The vector titer was calculated as follows: titer (transducing units/ml) = percentage of the positive cells $\times$ 4×10^5 cells $\times$ dilution factor.

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