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Construction of Human Factor IX Expression Vectors in Retroviral Vector Frames Optimized for Muscle Cells

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ABSTRACT

Development of a highly refined human factor IX (hFIX) expression vector system is critical for establishing a durable hemophilia B gene therapy. Here we report construction of a series of retroviral vectors and identification of an optimal basic structure and components for expressing hFIX in skeletal muscle cells. These vectors, which are derived from Moloney murine leukemia virus (MoMLV) with its enhancer sequence in the 3′ long terminal repeat (LTR) deleted, contained internal hFIX expression units inserted in forward configuration without or with a viral vector intron sequence (pdL or pdLIn vector frame, respectively) or in inverted configuration without a viral vector intron sequence (pdLi frame). Internal expression units contained a hFIX cDNA or hFIX minigene (hIXm1 or hIXm2) derived from the hFIX cDNA by insertion of a shortened first intron sequence of the hFIX gene. Regardless of the promoter and vector frame used, both hIXm1 and hIXm2 gave 10- to 14-fold higher hFIX expression compared to those with hFIX cDNA. Internal hFIX transcriptional control units of these vectors were composed of various promoters linked with or without the muscle creatine kinase enhancer (Me) sequence. Promoters tested included those of α-actin (αA775), β-actin (βA280), cytochrome oxidase (CO1250 and CO650), myogenin (Mg1031 and Mg353), and Rous sarcoma virus (RSV). βA200, which was derived from βA280 by eliminating potential polyadenylation sites, was also tested. As extensively examined with the myogenin promoter, presence of one or multiple copies of Me in the vectors elevated the expression activity in myotubes by 4.5- to 19-fold over those without Me, but not significantly in myoblasts. Similar enhancements in expression activity with Me were also observed with other promoters, except those of RSV and CO. The latter two showed only modest enhancements in the presence of Me. As assayed with myotubes in culture, the general order of hFIX expression activity of various promoters with four copies of Me in the three different vector frames was βA280 $\approx$ βA200 $>$ Mg353 $>$ Mg1031 $\approx$ RSV $\approx$ CO650 $\approx$ αA775 $>$ CO1250. One exception was that CO650 showed significantly less activity in pdLi-type vectors than in the pdLIn vectors. Based on the systematic analyses of various structural components, a group of pdLi vectors consisting of βA200, two to four copies of Me, and hIXm2 was identified to have the optimal basic vector structure to be used in retrovirus for hFIX expression in differentiated skeletal muscle cells. The present studies provide the critical first step for establishing a highly refined hemophilia B gene therapy based on skeletal muscle-targeted hFIX gene transfer.

OVERVIEW SUMMARY

Skeletal muscle cells may serve as an efficient medium for systemic production of transgene products in a gene therapy. Retroviral vector constructs were prepared using human factor IX minigenes, various muscle-specific or nonspecific promoters, and muscle creatine kinase enhancer. These vectors were tested systematically to determine an optimal basic structure for producing factor IX in mouse skeletal muscle cells. The basic retroviral vector structure

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for an optimal expression of factor IX in skeletal muscle cells may contain an internal factor IX expression cassette composed of a β-actin promoter, muscle creatine kinase enhancer, and human factor IX minigene in a reverse orientation relative to the 5′ long terminal repeat.

INTRODUCTION

SKELETAL MUSCLES comprise a large portion of human and animal bodies, and are easily accessible for various manipulations. Skeletal myoblasts can be prepared from muscle tissue biopsy samples and expanded in culture to large numbers (Webster *et al.*, 1988; Rando and Blau, 1994). Intramuscularly implanted myoblasts genetically modified in culture not only fuse with the existing host myofiber cells or among themselves, generating new muscle cells, but also survive as muscle precursor cells (satellite cells) (Yao and Kurachi, 1993). Skeletal muscle has also been shown to have high systemic delivery efficiency of various transgene products (Yao and Kurachi, 1992). Accordingly, muscle-targeted gene transfer has been intensively studied for its potential application for establishing gene therapies for various disorders (Barr and Leiden, 1991; Yao and Kurachi, 1992; Bansal *et al.*, 1994; Baque *et al.*, 1994; Hamamori *et al.*, 1995). The potential of myoblast-mediated gene transfer to be developed into a durable gene delivery method has been extensively tested for various genes such as factor IX (Dai *et al.*, 1992; Yao and Kurachi, 1992; Yao *et al.*, 1994), growth hormone (Dhawan *et al.*, 1991; Dahler *et al.*, 1994), erythropoietin (Hamamori *et al.*, 1995), glucocerebrosidase (Bansal *et al.*, 1994), and other proteins (Jiao *et al.*, 1993). Muscle-targeted gene transfer may be achieved by various methods, such as *in vivo* direct gene transfer using viral vectors (Ragot *et al.*, 1993; Vincent *et al.*, 1993; Acsadi *et al.*, 1995; Dai *et al.*, 1995) and naked vector DNAs (Davis *et al.*, 1993a,b; Manthorpe *et al.*, 1993) or *ex vivo* gene transfer (myoblast-mediated gene transfer) (Barr and Leiden, 1991; Dhawan *et al.*, 1991; Yao and Kurachi, 1992, 1994; Salvatori *et al.*, 1993; Vitadello *et al.*, 1994; Hamamori *et al.*, 1995; Trivedi and Dickson, 1995).

For establishment of a durable gene therapy method, development of a highly refined gene delivery system has become a crucial issue (Miller, 1992a,b). Retroviral vector systems have been extensively used to introduce foreign genes into various target cells including skeletal muscle cells (Dhawan *et al.*, 1991; Yao and Kurachi, 1992; Yao *et al.*, 1991, 1994; Miller, 1992b). Most retroviral vectors used to date are derivatives of Moloney murine leukemia virus (MoMLV) (Miller, 1992a,b). Although its long terminal repeat (LTR) promoter is generally strong in various cell types and also can direct stable transgene expression in skeletal muscle cells (Yao *et al.*, 1994), the LTR promoter is not differentiated muscle cell specific. Expression of foreign genes under its control is not elevated much upon myoblast differentiation. Transcriptional control units with various cell type-specific promoters and enhancers in retroviral vectors can confer cell-specific and persistent expression of the transgenes (Petropoulos *et al.*, 1992; Anson and Occhiodoro, 1994; Dahler *et al.*, 1994).

Recently, we reported that primary mouse skeletal myoblasts transduced with recombinant retroviral vectors containing a human factor IX (hFIX) cDNA under control of the LTR or chicken β-actin promoter (βA) with murine muscle creatine kinase (MCK) enhancer (Me) can express hFIX at a high level, equivalent to that observed for C2C12 cells (established murine myoblast line) (Yao and Kurachi, 1992; Yao *et al.*, 1994). The *in vivo* hFIX expression levels of the primary myoblasts were, however, significantly lower than those observed with C2C12 cells in a similar experiment (Yao *et al.*, 1994). It was concluded that the observed difference between the primary myoblasts and C2C12 cells is likely due to their cellular properties, such as fusion efficiency and *in vivo* stability, rather than due to a possible promoter inactivation as reported by others (Palmer *et al.*, 1991; Scharfmann *et al.*, 1991; Dai *et al.*, 1992). These observations strongly suggested the necessity for extensive refinement of two aspects of the muscle-targeted gene delivery system, the hFIX expression vector structure and the cellular procedures involved.

The present paper deals with the first phase of our intensive studies on the hFIX expression vector, defining its structural components and organization required for an optimal production of hFIX in differentiated muscle cells (myotubes). We have constructed a series of hFIX expression vectors in three different vector frames derived from MoMLV, with a battery of different muscle-specific and nonspecific promoters combined with Me and hFIX minigenes. They were constructed so that the hFIX expression cassette can be easily transferred into various other viral and nonviral vector systems for further testing. These vectors were subjected to systematic expression analyses with primary mouse skeletal myoblasts and myotubes, thus establishing the basic structure in retroviral vector frame for the optimal hFIX expression in muscle cells.

MATERIALS AND METHODS

Construction of hFIX expression vectors

Promoter Sequences: Muscle-specific and nonspecific promoters were prepared by excising from the previously prepared vectors, or by polymerase chain reactions (PCR) using 5′ and 3′ primers with *Xho* I and *Bam* HI linker sequences, respectively, and genomic DNA or cloned DNA fragments as the templates (Table 1). PCR was carried out as previously described (Yao *et al.*, 1994), and resulting DNA fragments containing various promoter sequences were digested with *Xho* I and *Bam* HI, purified by agarose gel electrophoresis, and inserted into various expression vectors at their *Xho* I/*Bam* HI sites. Specific procedures for preparation of each promoter sequence are described below.

A long myogenin promoter, Mg1031, which contains the sequence-spanning nucleotide −1,013 to +18 of the myogenin gene, was derived by PCR with a pair of primers and pMyo1565CAT as the template (Edmondson *et al.*, 1992) (Table 1). The PCR product was digested with *Xho* I and *Bam* HI, generating Mg1031 with *Xho* I and *Bam* HI sites at its 5′ and 3′ ends, respectively. A short myogenin promoter sequence fragment, Mg353, containing the region spanning nucleotides −335 to +18 of the myogenin gene, was prepared by removing the 5′ half of Mg1031 (nucleotides −1,031 through −334) by cleaving with *Sma* I at nucleotide −334. A *Xho* I linker was then ligated at the *Sma* I site followed by *Xho* I digestion.

TABLE 1. PCR PRIMERS FOR PREPARATION OF PROMOTER SEQUENCES

Promoter	Primer Sequence*	Nucleotide Position	PCR Template	Reference
Mg1031 (Mouse)	5' forward: 5'-CAT CTC GAG TAC AGG GGA ACG CCA GGG C-3'	nt -1013 to -993	pMyo1565CAT	Edmondson et al., 1992
	3' reverse: 5'-TAT GGA TCC CCC AAG CTC CCG CAG CCC C-3'	nt +18 to -3		
CO1251	5' forward: 5'-CCG CTC GAG GTC CCT AGA CTG AGG CAC-3'	nt-1189 to -1170	pCOX8HCAT	Lomax et al., 1995
CO650 (Calf)	5' forward: 5'-CCG CTC GAG CGA CCT CCC GGG GCC AGC-3'	nt-588 to -569		
	3' reverse: 5'-CGC GGA TCC AGT TGT AGG GCT GAG CTC CT-3'	nt +62 to +43		
αA775 (Human)	5' forward: 5'-ACT GCT CGA GGT GGC CCT CTG TGC GGT GG-3'	nt -689 to -672	Human genomic DNA	Taylor et al., 1988
	3' reverse: 5'-CAC TGG ATC CGG GTA GCT ACA ACT GCT ACT-3'	nt +86 to +66		
βA200 (Chick)	5' forward: 5'-CCT CCT CGA GGG CCC TT*T* *T*TT *T*TT TTG TGC AGC GAT GGG GG-3'	nt -1199 to -1174	pdLMe2βA280 IX	Kost et al., 1983; Yao et al., 1994
	3' reverse: 5'-CAC GGA TCC TAG GTC CCG CCC GCC GCG CGC TT-3'	nt -1000 to -1018		

*The underlined sequences indicate restriction sites built into the PCR primers. The italic Ts in βA200 5' primer sequence indicate they are different from the original gene sequence with adenine residues.

Bovine cytochrome oxidase (CO8H) promoter sequences were prepared by PCR using pCOX8HCAT containing bovine CO8H 5'-flanking sequence as the template (Table 1) (Lomax *et al.*, 1995). Long (1,251 bp) and short (650 bp) forms of the CO promoter, CO1251 and CO650, were prepared by using either one of the two 5' forward primers (corresponding at nucleotides −1,189 to −1,170 and nucleotides −588 to −569) and a common 3' primer (nucleotides +62 to +43) (Table 1).

Human α-actin promoter (αA775) was prepared by PCR using human genomic DNA as the template (Taylor *et al.*, 1988) (Table 1). Because of the presence of an *Xho* I site at nucleotide −86 within the αA promoter sequence, the PCR product was first digested with *Bam* HI and then subjected to partial digestion with *Xho* I, thus obtaining αA775.

A Rous sarcoma virus (RSV) promoter fragment was obtained from pERT9 (Invitrogen) as follows. A unique *Xho* I site present in the RSV promoter sequence contained in pERT9 was eliminated by *Xho* I digestion followed by blunt-ending and religation. A *Sal* I–*Bam* HI fragment (~600 bp) containing the RSV promoter sequence was isolated from the resulting vector and inserted into pBluescript vector (Stratagene) at *Sal* I/*Bam* HI sites, generating pBluescriptRSV. The *Xho* I/*Bam* HI fragment containing RSV promoter sequence was then generated by digestion of pBluescriptRSV with *Xho* I and *Bam* HI.

Chicken β-actin promoter (βA280, containing a region spanning nucleotides −1,279 through −1,000) (Kost *et al.*, 1983) was derived from a hFIX expression vector, pdLMe2βA280hIX (previously named pdLMMBAIX), constructed in our previous study (Yao *et al.*, 1994). A short form of the βA promoter sequence (βA200) containing the region nucleotides −1,199 through −1,000 was prepared by PCR using this vector as the template and primers listed in Table 1. The 5' primer contained thymine residues replacing the adenine residues present in the original gene sequence at nucleotides −1,197, −1,196, and −1,193 (italics in Table 1) to disrupt potential polyadenylation site sequences. The 5' forward primer contained *Xho* I and *Apa* I linker sequences in its 5' end region, while the 3' reverse primer contained *Avr* II and *Bam* HI sequences. These sites were included to make the fragment versatile in transferring into other vectors.

MCK Enhancer (Me): The region (hereafter referred to as Me) spanning nucleotides −1,351 to −1,050 of the 5'-flanking sequence of mouse MCK gene contains a strong muscle-specific enhancer activity (Sternberg *et al.*, 1988). A fragment containing two Me copies (Me2) was constructed by inserting two 301-bp *Xho* I fragments (a single copy of Me), previously prepared (Yao *et al.*, 1994), into pBluescript vector at *Xho* I site in a tandem orientation. The *Xho* I site in between the two Me sequences was eliminated by blunt-ending. Fragments containing four copies of Me (Me4) in a tandem orientation was constructed in a similar manner by using Me2 as the starting fragment.

Vector Frames: Three different vector frames, pdL, pdLIn, and pdLi, were used in constructing the hFIX expression vectors (Fig. 1A).

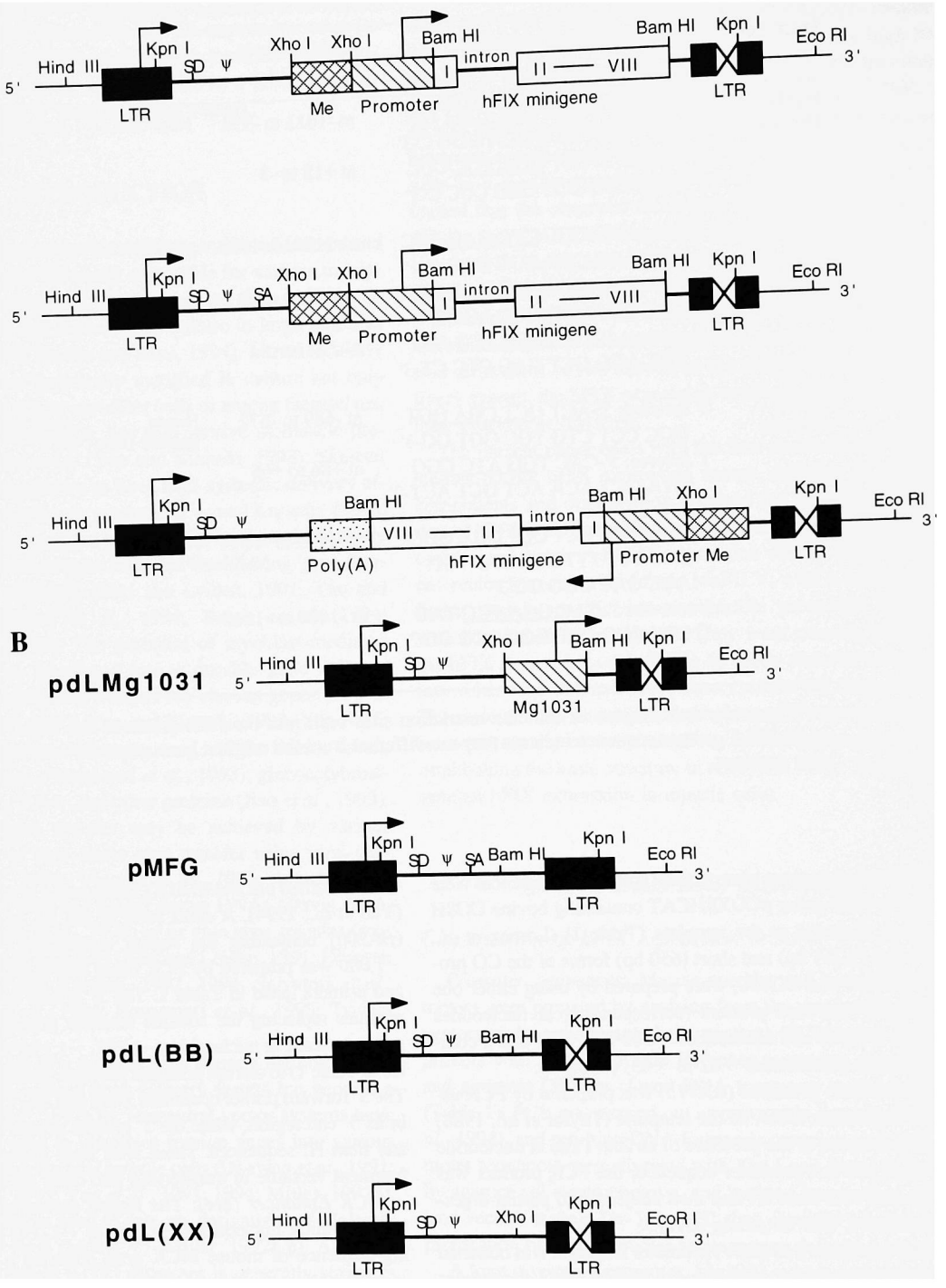

FIG. 1. A. Three basic retroviral vector frames used to construct new hFIX expression vectors. All vectors are derivatives of MoMLV. pdL indicates a vector frame with the 3′ LTR with its enhancer region deleted. pdLIn has the same structure as pdL, except that it is inserted with the MFG-derived vector intron sequence. pdLi has the same vector frame as pdL, except that the internal FIX expression unit is inserted in an inverted orientation with regard to the 5′ LTR. B. Intermediate constructs used for preparing pdLIn- and pdLi-type vectors. The unique *Bam* HI site in pMFG was converted to *Xho* I, and a *Hin*d III–*Xho* I fragment derived from pMFG was inserted into the *Hin*d III–*Xho* I sites of pdLMg1031, replacing the original *Hin*d III–*Xho* I portion, generating pdLInMg1031. pdL(XX) and pdL(BB) were derived from pdLMg1031 by converting its *Bam* HI site to *Xho* I and the *Xho* I site to *Bam* HI. They were used to generate pdLiMg1031 (see text). LTR, Long terminal repeat; SD, splicing donor site; SA, splicing acceptor site; *ψ*, viral packaging signal; Me, MCK enhancer; intron, shortened first intron of hFIX gene in its natural position; I–VIII, exons of the FIX gene. Arrows show the sites and the direction of transcription. The X in the 3′ LTR indicates the deleted enhancer region. Relevant restriction sites are shown with short vertical bars. The constructs shown are inserted in the pBR322 vector at the *Hin*d III and *Eco* RI sites.

pdL vector frame was derived from pdLMe2βA280hIX (Yao *et al.*, 1994) by deleting its Me2βA280hIX portion with *Xho* I/*Bam* HI digestion. This vector has its U3 enhancer region (178 bp) in the 3′ LTR deleted (Wilson *et al.*, 1988). pdLMe2-βA280hIXm1 was constructed by replacing the hFIX cDNA in pdLMe2βA280hIX with a hFIX minigene, hIXm1, which contains the first intron sequence of the hFIX gene with its middle 4.8-kb sequence truncated out of the original 6.2 kb (Kurachi *et al.*, 1995). The Mg1031 fragment prepared as described above was inserted into the pdL vector frame, thus generating pdLMg1031 (Fig. 1B). hIXm1 was then inserted into this intermediate vector at the unique *Bam* HI site, generating pdLMg1031hIXm1. Four copies of Me (Me4) were then inserted into this vector at the *Xho* I site, producing pdLMe4Mg1031hIXm1. The Mg1031 promoter sequence in these vectors was then replaced by various other promoter sequences (Mg353, βA280), thus generating a series of pdL-type vectors.

pdLIn vector frame was derived from plasmid pdLMg1031 and pMFG (Dranoff *et al.*, 1993) (Fig. 1B). pMFG contained an intron sequence (In) in the retroviral vector frame that was derived from the splicing donor sequence (SD) located 5′ to the packaging sequence region (ψ), and a splicing acceptor sequence (SA) derived from the envelope gene sequence. A unique *Bam* HI site located downstream of the viral intron sequence of pMFG was converted to an *Xho* I site by ligating an *Xho* I linker. The plasmid DNA was then cleaved with *Xho* I and *Hind* III (Fig. 1B), generating a 2.2-kb *Hind* III–*Xho* I fragment containing the 5′ LTR, packaging signal sequence, and complete splicing sequence. This fragment was then inserted into pdLMg1031 at *Hind* III/*Xho* I sites, replacing the corresponding *Hind* III–*Xho* I fragment (2.4 kb), thus generating pdLInMg1031. hIXm1 and Me (1, 2, and 4 copies) were then inserted into these vectors at the unique *Bam* HI and *Xho* I sites in a similar manner as described for pdL vectors, generating the pdLIn vectors with Mg1031 and hIXm1. The Mg1031 promoter sequence was then replaced with various other promoter sequences (Mg353, βA280, CO1250, CO650, and RSV), thus resulting in a series of pdLIn-type vectors.

The pdLi vector frame, which contains an internal hFIX expression unit in an inverted orientation relative to the 5′ LTR, was derived from the pdL vector frame. The unique *Xho* I and *Bam* HI sites in the pdL vector frame were converted to *Bam* HI and *Xho* I, respectively. The resulting two intermediate constructs, pdL(BB) and pdL(XX), which have a unique *Bam* HI or *Xho* I site, respectively (Fig. 1B), were then digested with a combination of *Hind* III and *Bam* HI or *Hind* III and *Xho* I, generating a 2.4-kb *Hind* III–*Bam* HI fragment containing 5′ LTR, SD, and ψ signal of pdL, or 5.6-kb *Xho* I–*Hind* III fragment containing 3′ LTR and pBR322 backbone sequence. These two fragments and Mg1031 fragment with *Xho* I/*Bam* HI ends were ligated, thus producing pdLiMg1031 with its Mg1031 sequence in an inverted orientation relative to the 5′ LTR. A *Bam* HI–*Bgl* II fragment (0.38 kb) containing the 3′ untranslated region of the hFIX gene with its poly(A) signal sequence was prepared from a hFIX expression vector, p-416hIXm1 (Kurachi *et al.*, 1995). This fragment was inserted into pdLiMg1031 at the *Bam* HI site in the same direction as the Mg1031 promoter, generating pdLi(pA)Mg1031. hIXm1 and Me2 or Me4 were then inserted into the vectors at *Bam* HI and *Xho* I sites, respectively, generating pdLihIXm1Mg-1031, pdLihIXm1Mg1031Me2, and pdLihIXm1Mg1031Me4. Mg1031 in these vectors was then replaced by various other promoters (Mg353, βA280, βA200, αA775, and CO650), and hIXm1 in some constructs was replaced with hIXm2, another hFIX minigene, thus generating a series of pdLi-type vectors (Table 2). hIXm2 was derived from hIXm1 by further shortening the intron sequence by 1.1 kb leaving only a 0.3 kb intron sequence of the hFIX gene (Kurachi *et al.*, 1995).

All PCR-derived DNA fragments and ligation sites of the essential components in the newly constructed vectors were sequenced to confirm their correct structures. The vector plasmid DNAs used for transfection experiments were purified using a Qiagen plasmid kit or two cycles of ultracentrifugation banding through CsCl gradient.

Cells and culture conditions

Primary skeletal myoblasts were isolated from SCID mouse hind leg muscles as previously described (Yao and Kurachi, 1993) and used in the present expression assays. Cells were grown in Dulbecco's modified essential medium (DMEM) (GIBCO/BRL) supplemented with 20% fetal calf serum (FCS), 0.5% chicken embryo extract (CEE) (GIBCO/BRL), and streptomycin and penicillin. All cell culture dishes were kept at 37°C in humidified incubators with 5% CO_2.

Enzyme-linked immunosorbent assay of hFIX

hFIX produced was quantified by hFIX-specific enzyme-linked immunosorbent assay (ELISA) using a murine anti-hFIX monoclonal antibody (AHIX-5041, Haematologic Technologies Inc.) as the catching antibody and rabbit polyclonal anti-hFIX as the detecting antibody (Yao *et al.*, 1991; Kurachi *et al.*, 1995). A microtiter plate reader (Bio-Tek model EL312e) was used for quantitation. This ELISA detected neither bovine nor murine factor IX at any significant level, permitting a precise determination of hFIX produced at sub-nanogram levels in the culture medium.

Transient expression of hFIX in culture

For assaying the transient expression activities of the vectors, LIPOFECTAMINE-mediated cell transfection was used according to the manufacturer's instruction (GIBCO/BRL). Twenty-four hours prior to transfection, primary myoblasts were plated at a density of 2×10^5/well in six-well cell culture plates (Corning). Expression vector DNA (2 μg) and pCH110 DNA (β-Gal expression vector, 0.2 μg) (Herbomel *et al.*, 1984) were mixed with LIPOFECTAMINE (30 μg) in Opti-MEM (GIBCO/BRL; total volume 100 μl) and incubated at room temperature for 15 min. Aliquots of Opti-MEM (900 μl) were then added and the DNA–LIPOFECTAMINE complexes formed were added to each well with cells, which were in advance rinsed once with Opti-MEM. Cells were then incubated at 37°C under 5% CO_2 for 6 hr, followed by addition of 1-ml aliquots of the myoblast culture medium. Twenty hours later, the DNA–LIPOFECTAMINE transfection medium was removed, and the cells were washed twice with phosphate-buffered saline. Two milliliters of hFIX assay medium (DMEM, 20% $BaSO_4$-treated FCS, 10 μg/ml Vitamin K1, and 0.5% CEE) were then

TABLE 2. HUMAN FACTOR IX EXPRESSION VECTORS

Vector Type*		Vector Name	Promoter (Size in bp)	Me (Copy No.)	hFIX Gene	hFIX (ng/ml)**
pdL	1	pdLMg1031hIXm1	Murine Myogenin (1031)	0	m1	16.6±2.3
	2	pdLMe4Mg1031hIXm1	Murine Myogenin (1031)	4	m1	78.4±7.9
	3	pdLMg353hIXm1	Murine Myogenin (353)	0	m1	25.5±4.4
	4	pdLMe4Mg353hIXm1	Murine Myogenin (353)	4	m1	98.9±11.5
	5	pdLMe2βA280hIX	Chicken β-Actin (280)	2	cDNA	5.3±2.3
	6	pdLMe2βA280hIXm1	Chicken β-Actin (280)	2	m1	126.7±10.7
	7	pdLMe4βA280hIXm1	Chicken β-Actin (280)	4	m1	161.4±16.1
pdLIn	8	pdLInMg1031hIX	Murine Myogenin (1031)	0	cDNA	1.5±0.5
	9	pdLInMg1031hIXm1	Murine Myogenin (1031)	0	m1	21.0±7.1
	10	pdLInMe1Mg1031hIXm1	Murine Myogenin (1031)	1	m1	40.6±2.7
	11	pdLInMe2Mg1031hIXm1	Murine Myogenin (1031)	2	m1	71.8±11.2
	12	pdLInMe4Mg1031hIXm1	Murine Myogenin (1031)	4	m1	87.1±8.8
	13	pdLInMg353hIXm1	Murine Myogenin (353)	0	m1	33.1±6.2
	14	pdLInMe4Mg353hIXm1	Murine Myogenin (353)	4	m1	106.2±12.6
	15	pdLInβA280hIXm1	Chicken β-Actin (280)	0	m1	54.2±3.4
	16	pdLInMe4βA280hIXm1	Chicken β-Actin (280)	4	m1	160.7±13.2
	17	pdLInCO650hIXm1	Calf Cyto Oxidase (650)	0	m1	53.9±9.7
	18	pdLInMe4CO650hIXm1	Calf Cyto Oxidase (650)	4	m1	81.6±8.0
	19	pdLInCO1250hIXm1	Calf Cyto Oxidase (1250)	0	m1	8.8±3.4
	20	pdLInRSVhIXm1	Rous Sarcoma Virus	0	m1	49.2±5.6
	21	pdLInMe4RSVhIXm1	Rous Sarcoma Virus	4	m1	86.6±7.9
pdLi	22	pdLihIXm1Mg1031	Murine Myogenin (1031)	0	m1	4.4±0.6
	23	pdLihIXm1Mg1031Me2	Murine Myogenin (1031)	2	m1	46.4±2.1
	24	pdLihIXm1Mg1031Me4	Murine Myogenin (1031)	4	m1	85.9±12.4
	25	pdLihIXm1Mg353Me4	Murine Myogenin (353)	4	m1	118.5±14.6
	26	pdLihIXm2Mg353Me4	Murine Myogenin (353)	4	m2	91.3±12.8
	27	pdLihIXm1βA280Me4	Chicken β-Actin (280)	4	m1	153.5±16.8
	28	pdLihIXm2βA280Me4	Chicken β-Actin (280)	4	m2	146.9±16.4
	29	pdLihIXm1βA200	Chicken β-Actin (200)	0	m1	30.7±4.0
	30	pdLihIXm2βA200	Chicken β-Actin (200)	0	m2	28.3±3.1
	31	pdLihIXm2βA200Me4	Chicken β-Actin (200)	4	m2	141.5±5.9
	32	pdLihIXm1CO650Me4	Calf Cyto Oxidase (650)	4	m1	44.4±5.1
	33	pdLihIXm1αA775Me4	Human α-actin (775)	4	m1	80.3±8.6
LIXSN	34	LIXSN	MoMLV LTR	0	cDNA	10.2±0.7

*pdL, Retroviral vector frame with its 3′ LTR enhancer region (178 bp) deleted; pdLIn, vector frame with a splicing unit derived from the MFG vector and the 3′ LTR enhancer region deleted as in pdL; pdLi, vector frame with the same vector backbone as pdL, except the inverted orientation of the internal hFIX expression unit in relation to 5′ LTR.

**hFIX produced into the culture medium of myotubes on day 7 shown in ng/ml, which is equivalent to those in ng/10^6 cells/24 hr. Average hFIX production levels with SD (n = 4–6 from two to four independent experiments) are shown. The expression levels of different vectors were normalized to the level of one to two vectors commonly tested in different sets of experiments.

added. The medium was collected every 24 hr and the same volume of fresh medium was added. After 48 hr, myoblast differentiation medium containing 2% $BaSO_4$-treated horse serum (HS) was added to two out of four wells for each construct. This was replaced with fresh medium every 24 hr up to day 6 to induce myoblast differentiation into myotubes. Cell numbers at the time of switching to the differentiation medium was about 2×10^6/well at 80–90% confluency. Under these conditions, differentiation was completed in 2.5–3 days. At the end of day 6, the medium was replaced with the hFIX assay medium and incubated for additional 24 hr. Amounts of hFIX protein produced into the medium were quantified by ELISA and normalized to the β-Gal activity (internal control for transfection efficiency). Myoblast cell numbers, β-Gal activities, and total cellular protein amounts were determined using the remaining two wells at the end of day 2. β-Gal activity was determined according to Eustice *et al.* (1991) with minor modifications. The total protein concentration of cell extracts was measured

by the Bio-Rad protein assay kit (Bio-Rad Laboratories) according to the manufacturer's instruction.

pLIXSN and pdLMe2βAhIX, which we previously reported (Yao *et al.*, 1991, 1994), were used as reference expression vectors in assaying the newly constructed vectors for their transient expression activities.

Northern blot analysis of muscle cells transfected with hFIX expression vectors

Polyadenylated RNAs were prepared using the QuickPrep *Micro* mRNA purification kit (Pharmacia Biotech) from cells on day 2 (myoblasts) and day 5 (myotubes) after transfection, and subjected to Northern blot analysis as previously described (Kurachi *et al.*, 1995). hFIX cDNA labeled with ^{32}P to a specific activity of $\sim 1 \times 10^9$ cpm/μg was used as the hybridization probe for blotted filter membranes. After stripping off the hFIX probe, the membrane was rehybridized with the ^{32}P-labeled human 18S ribosome RNA cDNA (American Type Culture Collection) as controls for equal loading of mRNA samples. RNA levels were quantified by a PhosphorImager (Molecular Dynamics) and hFIX mRNA levels were normalized to those observed for 18S ribosome RNA.

RESULTS

Construction of hFIX expression vectors and their expression activities

hFIX expression vectors and transient expression activities assayed with myotubes are summarized in Table 2. All the vectors contained an internal hFIX expression cassette inserted in one of the three MoMLV-derived vector frames (Fig. 1A). Internal hFIX expression cassettes consisted of various combinations of a promoter (either muscle-specific, viral, or housekeeping gene promoters), muscle creatine kinase enhancer (Me) in different copy numbers, and a hFIX minigene in two different forms. Promoters tested included those of βA in two lengths (βA280 and βA200, the latter with all potential polyadenylation sites in the complementary strand eliminated), Mg with two different lengths (Mg1031 and Mg353), muscle-specific CO with two different lengths (CO1250 and CO650), RSV, and αA (αA775). hFIX sequences used were cDNA (1.4 kb) and two minigenes, hIXm1 (2.9 kg in length) and hIXm2 (1.7 kb), containing truncated first-intron sequences. These vectors were systematically tested in a transient expression assay system with skeletal myoblasts and thereof derived myotubes to determine the relationship between various structural components and hFIX expression activity. Efficiency of myoblast transfection with LIPOFECTAMINE in the present study was approximately 20%.

Effects of minigenes

As shown in Fig. 2, pdLInMg1031hIXm1 showed 10- to 14-fold higher expression level in myotubes over that of pdLInMg1031hIX, indicating gross elevations in hFIX expression in the presence of hIXm1 over that with hFIX cDNA. Similar elevation due to the use of hIXm1 was also observed with βA280 promoter (Table 2). The expression activity of

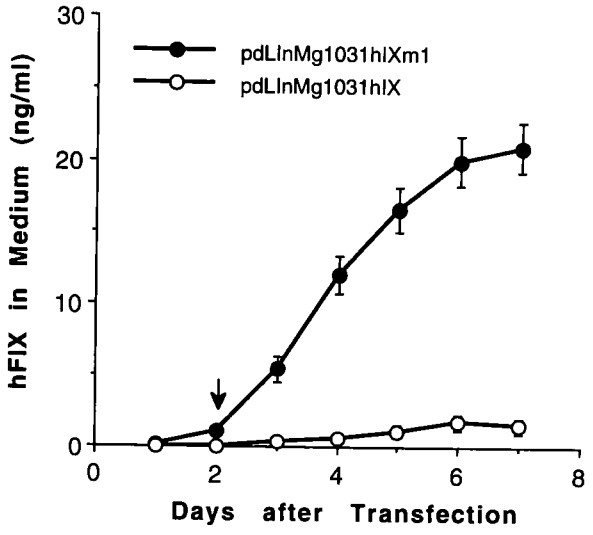

FIG. 2. Transient expression of hFIX by pdLInMg1031hIXm1 (●) and pdLInMg1031hIX (○) from skeletal muscle cells. Details of experimental conditions are described in Materials and Methods. Murine myoblasts (2×10^5/well) were plated in a six-well plate 1 day prior to transfection. Cells were transfected with hFIX expression vector DNAs and pCH110 plasmid DNA (internal transfection efficiency control) using lipofectamine-mediated transfection. Twenty hours later, the transfection medium was replaced with 2 ml of fresh complete growth medium (day 1). The medium was collected for ELISA every 24 hr and was replaced with the fresh medium. Differentiation medium with 2% horse serum was added to cells beginning on day 3 through day 6. Then the fresh growth medium was added to the cells for an additional 24 hr. Cell number at the beginning of differentiation was about 2×10^6/well. Expression levels shown in ng/ml are equivalent to those in ng/1×10^6 cells per 24 hr. Vertical bars indicate standard deviations ($n = 4$). Arrow indicates the time point of switching to the differentiation medium.

hIXm2 was only slightly lower than that of hIXm1, as shown by comparison between pdLihIXm1Mg353Me4 and pdLihIXm2Mg353Me4, pdLihIXm1βA280Me4 and pdLihIXm2βA280Me4, or pdLihIXm1βA200 and pdLihIXm2βA200 (Table 2). These results were in accord with our previous observations with the hFIX promoter (Kurachi *et al.*, 1995). Therefore, hFIX minigenes were included in the newly constructed hFIX expression vectors in this study.

Effects of different vector frames

Three different retroviral vector frames, pdL, pdLIn, and pdLi, designed in the present study, permitted us to test several critical aspects of the basic structures of FIX expression vectors.

pdLIn vectors without Me (pdLInMg353hIXm1 and pdLInMg1031hIXm1) showed only marginally (20–30%, $p = 0.052$) higher expression activities than pdL vectors (pdLMg353hIXm1 and pdLMg1031hIXm1) (Table 2), suggesting only a minor contribution, if any, of the MFG vector intron sequence with the 5′ LTR promoter to the overall expression levels. As shown for pdLMe4Mg353hIXm1 and pdLInMe4Mg353hIXm1, as well as for pdLMe4Mg1031hIXm1 and pdLInMe4Mg1031hIXm1, presence of Me greatly reduced the difference,

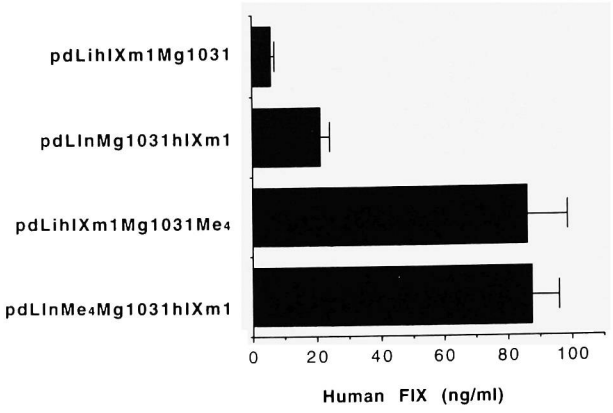

FIG. 3. Comparison of transient expression activities among the representative pdLIn and pdLi vectors with the Mg1031 promoter in the absence and presence of Me4. Experimental conditions are similar as described in Fig. 2. Transient expression activities of the vectors from myotubes were analyzed on day 7. Thin lines with vertical bars indicate standard deviations of three independent experiments.

and both pdL- and pdLIn-type vectors expressed hFIX equally well. Similarly, in the presence of Me, βA280 could drive hFIX expression equally well for both pdL and pdLIn-type vectors.

pdLi vectors were designed to test the hFIX expression unit in the inverted orientation. Such vector structure was considered to be important in avoiding splicing of the intron sequence contained in the hFIX minigenes during retroviral RNA genome production in the packaging cells (Cone *et al.*, 1987; Miller *et al.*, 1988). pdLihIXm1Mg1031, which contained no Me, showed only 27% and 21% expression levels of those of pdLMg1031hIXm1 and pdLInMg1031hIXm1, respectively. In the presence of Me, however, pdLihIXm1Mg1031Me4 expressed hFIX at the levels equivalent to the counterpart constructs of pdL and pdLIn-type vectors, pdLMe4Mg1031hIXm1 and pdLInMe4Mg1031hIXm1 (Fig. 3, Table 2).

Effects of MCK enhancers

As typically shown for a series of pdLInMg1031hIXm1 vectors with one, two, and four copies of Me (vectors 10–13 in Table 2), the overall hFIX expression level was elevated 2.0-, 3.7-, and 4.5-fold over that with no Me (pdLInMg1031hIXm1) (Fig. 4). The first one and two copies of Me gave the most drastic increases in expression, while the additional two copies present in Me4 gave less enhancing effects per Me copy. pdLInMg353hIXm1 series vectors, which contained the short Mg promoter also showed similar enhancements with increasing number of Me copy (Table 2). Similar general effects of Me were also observed with both pdL- and pdLi-type vectors. Among the three types of vectors, however, enhancing effects of Me were most dramatic with pdLi-type vectors as typically shown for pdLihIXm1Mg1031 series of vectors (Table 2, Fig. 5). In comparison to pdLihIXm1Mg1031, which expressed 4.4 ng/ml of hFIX on day 7, vectors with two and four Me copies (pdLihIXm1Mg1031Me2 and pdLihIXm1Mg1031Me4, respectively) expressed 46.4 and 85.9 ng/ml (10- and 19.5-fold enhancements), respectively (Fig. 5). The substantial differences in the enhancement rate between pdLi and the other two vector types (pdL and pdLIn) are due to the lower basal activity of pdLi vectors in the absence of Me than those of pdL and pdLIn vectors (Table 2). As mentioned above, the hFIX expression levels of pdLi, pdL, and pdLIn vectors with four Me copies (Me4) are not significantly different.

Effects of different promoters

Different promoters tested in the hFIX expression vectors showed substantially varied hFIX expression activities and muscle cell specificity. Vectors with Mg1031 promoter showed marked preference for myotubes, as expected, from the previous report (Edmondson *et al.*, 1992) (Figs. 2 and 6). When the culture medium was switched to the differentiation medium on day 2 (end of 48 hr after transfection), hFIX expression of pdLInMg1031hIXm1 rose from 1.6 ng/ml medium with myoblasts to 21.0 ng/ml on day 7 with myotubes, a 13.1-fold increase (Fig. 6). This large increase was not due to a modest increase in myoblast cell number (<20% at most) observed after switching the medium to a differentiation medium. The observed myotube-specific increase in expression was also observed with the short form of myogenin promoter (Mg353). pdLInMg353hIXm1 expressed 33.1 ng/ml on day 7, a 13.2-fold increase over that on day 2 (2.5 ng/ml medium). Vectors with Mg353 showed about 20–60% higher expression levels than with Mg1031 ($p = 0.010$) (Figs. 6 and 7). On day 7, pdLInMg1031hIXm1 expressed 21.0 ng/ml medium of hFIX (average of four independent experiments), whereas the pdLInMg353hIXm1 expressed 33.1 ng/ml medium (Table 2). Higher expression levels were also observed for Mg353 over Mg1031 in the presence of Me, regardless of different vector frames (pdL or pdLi).

CO650 and CO1250 showed similar differentiated muscle cell-specific expression (data not shown). CO650 also had a significantly higher basal activity than CO1250. pdLInCO650-hIXm1 expressed 53.9 ng/ml medium of hFIX (average of three independent assays), whereas pdLInCO1250hIXm1 gave only 8.8 ng/ml (Table 2).

CO650, RSV, and βA280 promoters had similar basal promoter activities, which were substantially higher than those of

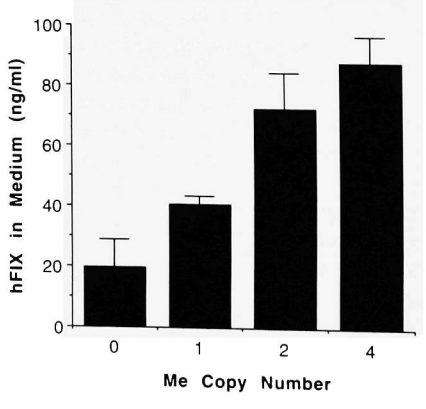

FIG. 4. Effects of increasing copy numbers of MCK enhancer (Me) on transient expression of hFIX by pdLInMg1031IXm1. Averages of hFIX amounts produced from myotubes (day 7) in two experiments are shown with the observed expression ranges (vertical bars).

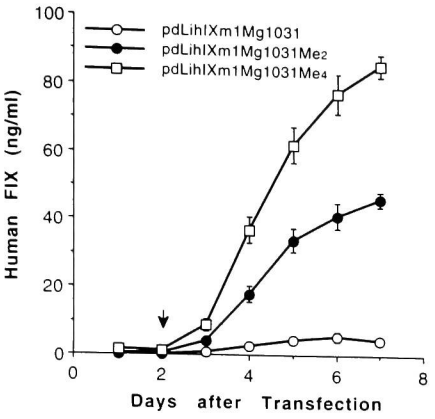

FIG. 5. Expression of hFIX in myoblasts and myotubes by pdLi vectors with Mg1031 promoter and MCK enhancer (none, two, and four). Expression conditions were similar to those described in Fig. 2. (○) pdLihIXm1Mg1031; (●) pdLhIXm1-Mg1031Me2; (□) pdLihIXm1Mg1031Me4. Arrow indicates the medium switch for cell differentiation. Vertical bars are standard deviations of triplicated assays.

Mg1031 and Mg353 (Table 2, Fig. 7a). In the presence of Me4, however, Mg353 gave a 30% and 23% higher activities than those of CO650 and RSV, whereas βA280 gave the highest activity level of all vectors tested, which was 51% higher than that of Mg353.

Regardless of the vector frames used, βA promoter with Me consistently showed the highest expression activity in myotubes among the promoters tested. Me4 could enhance the activity of RSV and CO650 in pdLIn vectors by 1.5- and 1.8-fold, respectively, over those without Me. The enhanced expression levels of these vectors, however, were equivalent to the level only half of that of βA construct (pdLInMe4βA280hIXm1) (Fig. 7A). αA775, another differentiated muscle cell specific promoter, gave a promoter activity similar to that of Mg1031

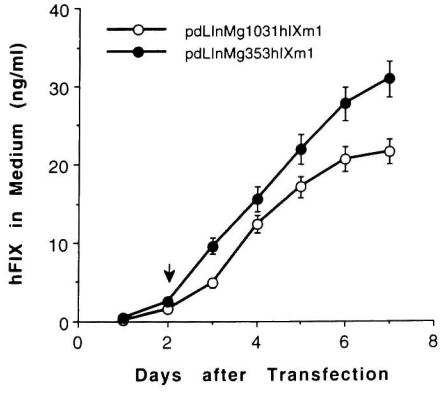

FIG. 6. hFIX expression activities of myogenin promoters Mg1031 and Mg353 in skeletal muscle cells. Assay conditions were similar to those in Fig. 2. The results from two constructs, pdLInMg1031hIXm1 (○) and pdLInMg353hIXm1 (●) are shown. The arrow indicates medium switch for cell differentiation. Results of a set of representative experiment with four duplicates are shown with standard deviations (vertical bars).

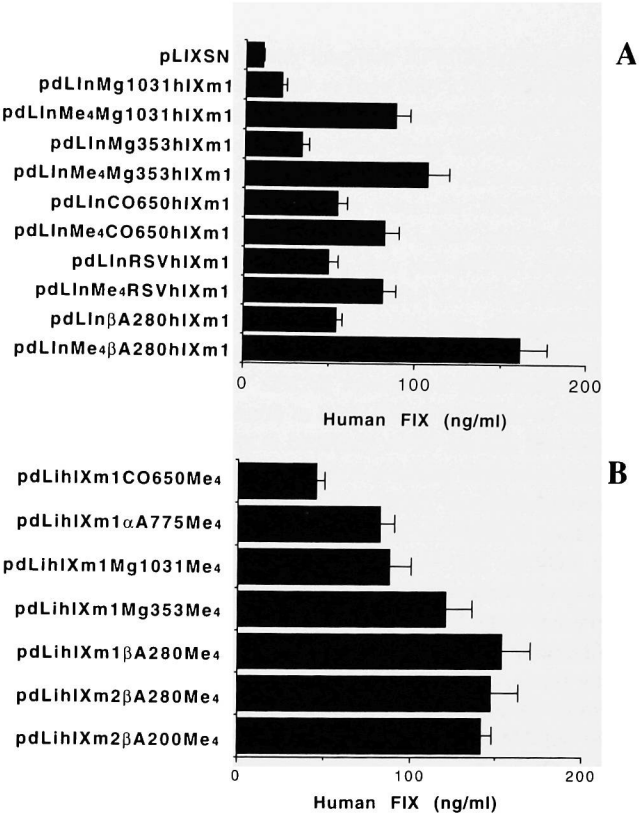

FIG. 7. Comparison of hFIX expression activities by vectors with different promoters with or without Me4. Experimental conditions were similar to those in Fig. 2. A. pdLIn vectors with or without Me4. pLIXSN is included as a reference vector. Thin lines with vertical bars indicate standard deviations of four duplicated assays. Results shown are from the day 7 data of a representative experiment. B. pdLi vectors with Me4. hFIX expressed on day 7 of two experiments is shown with the observed ranges.

as shown for pdLihIXm1αA775Me4 and pdLihIXm1Mg-1031Me4 (Fig. 7B).

The order of the promoter strength in pdL-type and pdLIn-type vectors with Me4 was determined with myotubes as, βA280 > Mg353 > Mg1031 ≈ RSV ≈ CO650 > CO1250 (Fig. 7A, Table 2). Various promoters with Me4 in the pdLi frame also gave a similar order in their strength, except that CO650 activity was only 52% of Mg1031. hFIX expression levels of pdLInMe4βA280hIXm1 and pdLihIXm1βA280Me4 in myotubes (160.7 ± 12.6 and 153.5 ± 16.8 ng/ml, respectively) were 15- to 16-fold and 30-fold higher than those of our previous vectors, pLIXSN and pdLMe2βAhIX, respectively (Yao *et al.*, 1994). In the present transient assay system, the latter two vectors expressed 10.2 ± 0.7 and 5.3 ± 2.3 ng/ml of hFIX, respectively (Table 2).

Structure of highly refined expression vector

On the basis of the results summarized above, the optimal basic structure of the hFIX expression vector to be used for production of hFIX by muscle-targeted gene transfer should contain βA promoter, Me2 or Me4, and the hFIX minigene. Among the three vector frames used, only the pdLi-type frame should be able to prevent a loss of the expression-augmenting effects

of the intron of hFIX minigene due to its splicing in the packaging cells. pdLi-type vectors with Me4 expressed hFIX at the levels equivalent to the counterpart pdL and pdLIn vectors, indicating that the basic pdLi vector structure functions well.

To construct a further refined vector with the pdLi vector frame, βA280 promoter was modified by truncating its 5′ 80-bp sequence, where two potential polyadenylation signal sequences (AATAAA) were present in the complementary strand (Kost *et al.*, 1993; Miller *et al.*, 1988). In addition, several base substitutions were also made to the remaining promoter sequence to eliminate all other potential polyadenylation sites, thus generating a promoter βA200. These modifications were done to eliminate possibilities of premature polyadenylation of the viral genome RNA produced in the packaging cells. All of the removed or modified regions contain no known specific enhancer elements. βA200 still retained a promoter activity very similar to βA280 as shown with both hIXm1 and hIXm2 (Table 2 and Fig. 7B). Because hIXm2 was also devoid of potential polyadenylation sites in the complementary strands, which are present in the hIXm1 in its intron sequence region, we utilized hIXm2 in a highly optimized vector system with the pdLi vector frame. pdLihIXm2βA200Me4 expressed hFIX at a level of 146.9 ng/ml medium from myotubes, an equivalent level of that of pdLihIXm1β A280Me4, indicating no significant detrimental effects of these modifications of the βA promoter and the hFIX minigene (Fig. 7B).

Northern blot analysis of muscle cells transfected with hFIX expression vectors

As shown in Fig. 8, pdLMe4βA280hIXm1, pdLInMe4βA-280hIXm1, pdLihIXm1βA280Me4, and pdLihIXm2βA-200Me4 produced approximately equal amounts of the major hFIX mRNA bands (~1.9 kb in lanes 7 and 8; ~1.6 kb in lanes 5 and 6), which were produced by the internal promoter, in myotubes on day 5 (third day after the initiation of differentiation). In myotubes, pdLihIXm2βA200Me4 (lane 5) and pdLihIXm1-βA280Me4 (lane 6) produced approximately four- to five-fold more mRNA than pdLihIXm1βA200 (lane 4). pdLihIXm1β-A280Me4 produced a significantly less amount of mRNA in myoblasts (day 2) (lanes 2) in comparison to that in myotubes (day 5) (lanes 6). These are consistent with the relative hFIX expression levels of these vectors observed in myotubes and myoblasts. Interestingly, pdLihIXm1βA200 also consistently gave a higher hFIX mRNA level in myotubes on day 5 than in myoblasts on day 2.

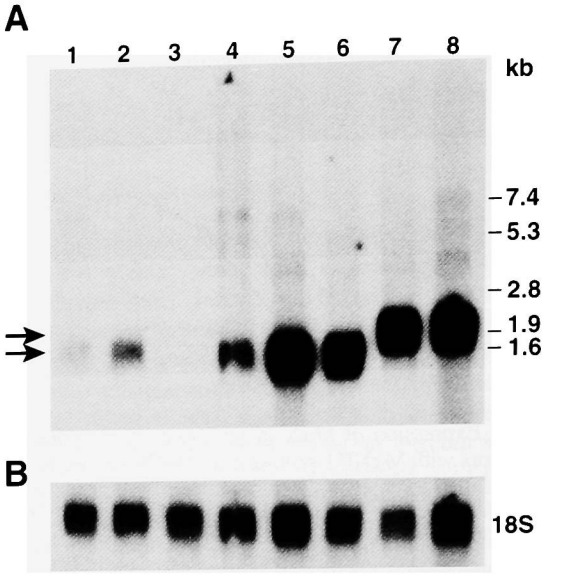

FIG. 8. Northern blot analysis of muscle cells transfected with hFIX expression vectors. A. Lanes 1 and 2 contain poly(A)$^+$RNA samples prepared from the myoblasts on day 2 post transfection with pdLihIXm1βA200 and pdLihIXm-1βA280Me4, respectively. Lanes 4–8 contain poly(A)$^+$RNA samples prepared from the myotubes derived from myoblasts on day 5 post transfection with pdLihIXm1βA200, pdLihIXm-2βA200Me4, pdLihIXm1βA280Me4, pdLInMe4βA280hIX-m1, and pdLMe4βA280hIXm1, respectively. Lane 3 contains poly(A)$^+$RNA prepared from the mock-transfected muscle cells. Numbers on the right side indicate RNA molecular weight marker. Arrows on the left side indicate the position of hFIX mRNA bands of ~1.9 kb in lanes 7 and 8 and ~1.6 kb in lanes 1, 2, 4, 5, and 6, respectively. B. The presence of approximately equivalent RNAs in all lanes is shown by the presence of 18S ribosomal RNA. See the text for the experimental conditions.

DISCUSSION

Skeletal muscle cells can be relatively easily targeted for *in vivo* and *ex vivo* gene transfers and their great potential to serve as an efficient and safe medium for producing various recombinant proteins has been demonstrated (Barr and Leiden, 1991; Dai *et al.*, 1992; Yao and Kurachi, 1992; Bansal *et al.*, 1994; Baque *et al.*, 1994; Hamamori *et al.*, 1995). To establish a durable muscle-targeted gene therapy, however, development of a highly refined gene expression vector system has become one of the most critical issues. In the present study, we constructed a series of new hFIX expression vectors in three different retroviral vector frames and systematically analyzed the relationship among vector structure, expression components, and hFIX expression activity.

Effects of different vector frames

Three basic retroviral vector frames, pdL, pdLIn, and pdLi (Fig. 1), provided us with an excellent model system to examine several critical issues in developing an optimal hFIX retroviral vector structure. These issues include: (i) how high an expression level can be achieved by improving pdL-type vectors; (ii) how much effect does the MFG-type viral intron sequence contained in pdLIn-type vectors have on the internal transcriptional control unit; (iii) what is the relative strength of the 5′ LTR promoter in the different vector frames; (iv) what is the transcriptional activity level that can be achieved by the internal hFIX expression unit with pdLi in relation to pdL and pdLIn vectors; and (v) what is the basic vector frame to be included in an optimal hFIX retroviral vector.

pdL-type vectors, which are the simplest of the three vector frames tested in the present study, were previously used in human gene therapy of hypercholesterolemia (Grossman *et al.*, 1994). After a series of testings with the hFIX minigene, different promoters and additional muscle-specific enhancers

(Me), a specific combination of these components as represented in pdLMe4βA280hIXm1, gave the highest hFIX expression levels among the pdL vectors (Table 2).

The pdLIn-type vectors, which have a MFG-derived vector intron sequence, were of particular interest because of their potential for an elevated transgene expression. In recent years, the MFG retroviral vector has been reported for its capability to express transgenes at substantial levels in various cell types such as hematopoietic cells (Ohashi *et al.*, 1992; Dranoff *et al.*, 1993). More recently, Dwarki *et al.* (1995) reported a successful expression of B-domain-deleted factor VIII in fibroblasts and endothelial cells, as well as myoblasts, by using a MFG retroviral vector. In the present study, pdLIn vectors without any Me gave only slightly better expression levels (20–30%) than their counterpart pdL vectors (Table 2). The differences were statistically not significant. hFIX expression levels of pdLMe4βA280hIXm1 and pdLInMe4βA280hIXm1, which have strong internal hFIX expression activities, are very similar to each other (Table 2).

These results indicate that: (i) the 5′ LTR without or with the MFG-type viral intron sequence in the pdL and pdLIn vectors does contribute to the overall hFIX expression, but not significantly enough to affect the hFIX expression when the internal hFIX expression unit is very strong; and (ii) neither a cryptic splicing acceptor site nor the splicing acceptor site of the hIXm1 internal intron is likely to be used in combination with the splicing donor site in pdL-type vectors. The MFG-derived intron is only partially spliced both in the packaging cells and transduced target cells (Dwarki *et al.*, 1995). The viral packaging sequence is contained in the MFG-derived intron sequence and, therefore, the viral RNAs of all recombinant MFG viruses must contain the intron sequence. In the targeted cells, the intron is again partially spliced (Dwarki *et al.*, 1995). In the transient assays, the MFG-derived intron of pdLIn vectors may be partially spliced, resulting in some minor contribution to the hFIX production, particularly in the present of a strong internal hFIX transcriptional unit, which has a much higher expression activity than the LTR in myotubes.

When the MFG vector is used in a virus form, its transgene expression in the transduced target cells is likely elevated due to the positive effects of splicing of the intron sequence, thus resulting in an increased mRNA level for the transgene. As shown in Fig. 7 and Table 2, pdLihIXm1βA280Me4 has an expression activity equivalent to pdLMe4βA280hIXm1 and pdLInMe4βA280hIXm1, supporting that, with a strong internal promoter, neither the 5′ LTR promoter nor the MFG-derived intron significantly affects the overall hFIX expression activity of these vectors. This was consistent with the mRNA levels (Fig. 8). This situation is much different from the original MFG vector, which uses its LTR promoter to drive expression of the inserted gene (Dranoff *et al.*, 1993; Dwarki *et al.*, 1995). Presence of possible cryptic elements, which may function as ATG site(s) or premature polyadenylation sites in the internal enhancer and promoter regions of the pdLIn vectors, may also minimize the effective use of mRNAs generated by the 5′ LTR promoter in producing hFIX. Such possibilities, however, may be minimal, because, in the absence of Me, pdLIn vectors appear to express marginally higher levels of hFIX than those of pdL vectors with various promoters (Table 2).

Unless the intron sequence of hIXm1 is retained in the genomic RNA of recombinant hFIX viruses, no grossly elevated production of the hFIX mRNA due to the positive effects of such an intron can be expected in the target cells (Kurachi et al., 1995). The pdLi series of vectors was designed to test the basic vector structure that can be used to prepare a retroviral vector capable of preventing such a loss of the internal intron due to splicing in the viral packaging cells (Cone *et al.*, 1987; Miller *et al.*, 1988). pdLihIXm1Mg1031, with no Me, can only express hFIX at a level 21% and 27% of those of pdLInMg1031hIXm1 and pdLMg1031hIXm1, respectively. These results indicate some contribution of the 5′ LTR promoter activity with or without the MFG-derived intron in pdLIn and pdL vectors. Alternatively, transcripts generated by the 5′ LTR may complex with the transcripts produced by the internal promoter in the reverse orientation, thus reducing the effective mRNA amount for producing factor IX. With a strong internal hFIX transcriptional unit as in pdLihIXm1Mg353Me4 or pdLihIXm1βA280Me4, the overall expression activities as well as mRNA levels of the pdLi-type vectors becomes indifferent from the counterpart constructs of pdLIn and pdL, indicating that the modest contribution of the 5′ LTR is easily overcome (Figs. 3, 7, and 8).

These results indicate that the basic structure of the pdLi-type vectors should permit us to take a full advantage of the hFIX minigene. It is possible that LTR-driven transcription for producing the virus genomic RNA might be affected due to the strong internal promoter activity. Promoters with Me, a differentiated muscle cell-specific enhancer, however, may function at the minimal efficiency in the fibroblast-derived packaging cells. Other possibilities, such as the presence of potential cryptic splicing donor and acceptor sites in the pdLi-type vectors cannot be ruled out (Jonsson *et al.*, 1995). These possibilities are currently being tested in our laboratory.

Effects of additional muscle specific enhancer elements

A region spanning nucleotides −1,351 to −1,050 of the 5′-flanking sequence of the mouse MCK gene (designated Me in the present study) contains one CArG-box and two E boxes and shows a strong differentiated muscle cell-specific enhancer activity (Sternberg *et al.*, 1988). This region not only enhances the MCK promoter (Sternberg *et al.*, 1988) but also confers enhanced muscle-specific expression of heterologous gene promoters such as simian virus 40, CMV, and c-*fos* (Sternberg *et al.*, 1988; Dai *et al.*, 1992; Martin *et al.*, 1994). We also reported an augmentation of βA promoter activity by Me (Yao *et al.*, 1994). In the present study, a systematic analysis of the enhancer effects of Me was carried out. With increasing copy numbers of Me, hFIX expression by pdL, pdLIn, and pdLi vectors with not only muscle-specific promoters such as Mg, CO650, and αA775, but also with non-muscle-specific promoters such as RSV and βA, was greatly increased (Fig. 7). In general, Me affects more strongly the promoters with relatively weak basal activities, such as Mg1031, than those with strong activities, such as RSV and CO (Figs. 6 and 7). This, however, appears to be very much dependent with the promoter sequence, because βA280, which has as strong a basal activity as RSV and CO650, is also enhanced as much as Mg353. Expression of pdLi-type vectors is more affected by Me than those of pdL- or pdLIn-type vectors, suggesting that within the retroviral vec-

tor frame Me primarily affects the closely linked internal promoter; the 5′ LTR located at a distant position is less affected.

Expression activities of different promoters

Myogenin is one of the well-defined muscle-specific transcriptional factors (Edmondson *et al.*, 1992; Schwarz *et al.*, 1992). The Mg promoter has a strong specificity for differentiated muscle cells and was used in the present study as a model promoter for testing various conditions. As shown with pdLInMg1031hIXm1, a long form of myogenin promoter, Mg1031, shows approximately 10-fold higher level of hFIX expression in myotubes than in myoblasts in good agreement with the previous observations (Edmondson *et al.*, 1992). In the present study, the short form of the myogenin promoter Mg353 not only retained the differentiated muscle cell specific activity, but its expression activity was also higher by about 1.6-fold than that of Mg1031 (Figs. 6 and 7). These observations suggest that the region spanning nucleotides −353 to −1,031, which is present only in Mg1031, and contains additional E boxes, contributes rather negatively to the transcriptional activity. This difference between Mg1031 and Mg353 was observed regardless of the vector type (pdL, pdLIn, or pdLi) and presence or absence of Me (see also Figs. 6 and 7). Interestingly, a short form of the calf CO promoter, CO650, also gave a substantially higher expression activity than that of the long form, CO1250 (Table 2). The proximal regions up to nucleotide −353 in the Mg promoter and up to nucleotide −588 in the CO promoter contain multiple muscle-specific enhancer elements (Edmondson *et al.*, 1992; Lomax *et al.*, 1995). It is not known at the present time why the longer forms, which contain additional enhancer elements, produce lower activities than the short form. The longer forms may also contain hitherto unidentified silencer elements. Four Me copies increased the expression activity of CO650 by only 1.5-fold as observed for pdLInCO650hIXm1 and pdLInMe4CO650hIXm1 (Fig. 7A, Table 2). A similar, rather poor enhancement of the RSV promoter (1.6-fold) with Me was also observed. βA280 has a basal activity equivalent to RSV and CO650 promoters, but its activity was enhanced three-fold with Me4, equivalent to that of Mg353 (3.2-fold) (Fig. 7A, Table 2). These results suggest that the enhancer activity of Me is dependent on the promoter sequence in addition to the vector frame context as discussed above. αA, another muscle-specific promoter, is as strong as Mg1031 in the presence of Me4.

Among promoters tested, both βA280 and βA200 with Me4 consistently produce hFIX in muscle cells at the highest level in all three vector frames. It is important to find that βA200, which is modified from βA280, still has an expression activity comparable to βA280. Presence of an increasing copy number of Me in the vector results in a higher hFIX expression. However, to minimize potential rearrangements of the vector DNA in the process of gene transfer and chromosomal integration, as small as two Me copies, and not more than four copies, may be preferred. These multiple Me copies insure a substantial increase in hFIX production in skeletal muscle cells.

Optimal basic structure of hFIX retroviral vector for skeletal muscle cells

Through the systematic analyses carried out and as discussed above, we conclude that the basic structure of a retroviral vector for achieving an optimal hFIX expression in differentiated muscle cells should have a pdLi vector frame with an internal hFIX expression cassette composed of two (and no more than four) Me copies, βA200 and hIXm2. pdLihIXm2βA200Me2 and pdLihIXm2βA200Me4 satisfy all of the conditions considered for maximizing hFIX expression. In transient assays, pdLihIXm2βA200Me4 expressed hFIX at levels 15- or 30-fold higher than those of the previous-generation retroviral vectors, LIXSN or pdLMe2βA280hIX, respectively (Table 2). These refinements made to the basic structure of the vector insure not only the enhanced muscle-specific, high-level expression of hFIX in the target cells, but also possible preparation of recombinant virus at a high yield in the packaging cells. The 3′ LTR with its enhancer deleted should assure a minimal interference of hFIX expression directed by the reversed internal expression unit in the target cells. Preparation of recombinant viruses of pdLihIXm2βA200Me2 and pdLihIXm2βA200Me4 are in progress.

The internal hFIX expression unit as well as each structural component such as enhancer, promoter, and hFIX minigene of the vectors described in this study have been constructed as versatile cassettes. They can be easily transferred into various different types of vector systems such as adenovirus, AAV, or nonviral vector systems, and their enhancer, promoter, or gene can also be easily exchanged with others to target not only the FIX gene but also various other genes of interest into different cell types and tissues. For example, proteins were produced at levels as high as hFIX by replacing the hIXm1 sequence in pdLihIXm1βA280Me4 with those of protein C or hirudin cDNA (unpublished data).

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