Design of Retrovirus Vectors for Transfer and Expression of the Human β -Globin Gene

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Regulated expression of the human β -globin gene has been demonstrated in cultured murine erythroleukemia cells and in mice after retrovirus-mediated gene transfer. However, the low titer of recombinant viruses described to date results in relatively inefficient gene transfer, which limits their usefulness for animal studies and for potential gene therapy in humans for diseases involving defective β -globin genes. We found regions that interfered with virus production within intron 2 of the β -globin gene and on both sides of the gene. The flanking regions could be removed, but intron 2 was required for β -globin expression. Inclusion of β -globin introns necessitates an antisense orientation of the gene within the retrovirus vector. However, we found no effect of the antisense β -globin transcription on virus production. A region downstream of the β -globin gene that stimulates expression of the gene in transgenic mice was included in the viruses without detrimental effects on virus titer. Virus titers of over 10^6 CFU/ml were obtained with the final vector design, which retained the ability to direct regulated expression of human β -globin in murine erythroleukemia cells. The vector also allowed transfer and expression of the human β -globin gene in hematopoietic cells (CFU-S cells) in mice.

An understanding of the β -globin gene and its control and the existence of a variety of human diseases caused by defects in the β -globin gene have led to consideration of disease treatment by transfer of a normal gene into bone marrow of affected individuals. Retrovirus vectors currently offer the best vehicle for gene transfer into marrow cells, and vectors carrying the β -globin gene have been used to transfer the β -globin gene into murine erythroleukemia (MEL) cells, human erythroid progenitor cells (BFU-E cells), and bone marrow of mice. MEL cells are arrested at a relatively late stage of erythroid development, but can be induced to differentiate by using a variety of inducers (9, 18). During differentiation, a large increase in mRNA and protein production from the endogenous globin genes is observed. Following retrovirus-mediated transfer of the human β -globin gene into MEL cells, mRNA and protein production from the introduced human β -globin gene was inducible (4, 7, 13, 15) and in two studies was made at levels approaching that of the endogenous mouse genes on a per-gene-copy basis (4, 13). Transfer of the β -globin gene into human erythroid progenitor cells (BFU-E) resulted in production of RNA from the transferred human gene at 5% of the level of the endogenous gene (4). Retrovirus vectors have also been used to transfer the human β -globin gene into mouse bone marrow. Infusion of infected marrow into lethally irradiated mice resulted in tissue-specific expression of the gene in erythroid cells, albeit at levels 100-fold lower than those of the endogenous mouse genes (8).

One problem with current vectors which severely limits their usefulness is their relatively low titer, from 7×10^3 to 5×10^5 CFU/ml (4, 7, 8, 13, 15). The low frequency of gene transfer into bone marrow cells capable of reconstituting mice (18 DNA-positive animals of 104 tested) (8) is a possible consequence of this problem. Amphotropic retroviral vectors have been used to transfer genes into human hematopoietic progenitor cells, including erythroid progenitors, but

only the highest-titer vectors (10^6 to 10^7 CFU/ml) yield useful frequencies of transfer (11, 12). Indeed, we find very poor infection of human BFU-E cells by using our β -globin vector (4). Thus, development of higher-titer viruses carrying the β -globin gene is required to facilitate gene transfer experiments and for potential application of these techniques to gene therapy in humans.

In this study, we examined the factors leading to the low titer of β -globin viruses and made alterations in the virus which resulted in a substantial increase in titer without affecting β -globin gene expression. In addition, we incorporated into the viruses a region downstream of the human β -globin gene that is important for regulated human β -globin expression in transgenic mice (3, 14, 26). The new virus allowed efficient infection of murine hematopoietic cells (CFU-S cells) and human β -globin expression was detected in resultant hematopoietic colonies in mice.

MATERIALS AND METHODS

Cell culture. Cells were grown in Dulbecco modified Eagle medium with high glucose (4.5 g/liter) supplemented with 10% calf serum (ψ 2 cells) or 10% fetal bovine serum (all other cell lines). Concentrations of G418 are calculated by using the weight of dry powder, of which about 50% was active. Previously described cell lines include PA317 (19) (ATCC CRL 9078), adenine phosphoribosyltransferase-negative (APRT⁻) tetraploid MEL cells selected to be semiaherent (gift of P. Mellon, originally obtained from A. Deisseroth), thymidine kinase-negative (TK⁻) NIH 3T3 (20), and ψ 2 (17). PA317 retrovirus packaging cells used here were either from an early passage of the cell line or were reselected in hypoxanthine-aminopterin-thymidine medium as described previously (5). For induction of differentiation, MEL cells were seeded at 5×10^4 cells per ml in medium containing 3 mM *N,N'*-hexamethylene-bisacetamide (HMBA; Sigma Chemical Co., St. Louis, Mo.) on day 1. On day 4, medium was removed from the cells following low-speed centrifugation and the cells were resuspended in the same volume of

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FIG. 1. Human β -globin gene and surrounding sequences. Sequence numbers are given relative to the RNA cap site at +1. Boxes indicate exons, and hatched regions indicate the protein-coding region. A *Bgl*II site (AGATCT) was inserted between bases +40 and +41, as indicated, to mark the gene (4). Landmark restriction sites are given, and consensus poly(A) signals (AATAAA) and orientation of the signals are indicated by arrows. Inserts used in different vectors are diagrammed below the β -globin gene.

Retrovirus vectors. Retrovirus vectors expressing the neomycin phosphotransferase (*neo*) drug resistance gene were used to transfer β -globin genes. The prefix p indicates the plasmid form of the virus. Retrovirus sequence numbers are as described previously (27). The vector pLNL-XHC contains the *neo* gene inserted into a DNA clone of Moloney murine leukemia virus in place of Moloney murine leukemia virus sequences from 1039 to 7673 and contains *Xho*I, *Hind*III, *Cla*I restriction sites between the *neo* gene and the 3' long terminal repeat (LTR) for insertion of additional genes (5). The vector pLNSL7 contains (in the direction of transcription) the 5' LTR through base 544 from Moloney murine sarcoma virus, bases 569 to 1038 from Moloney murine leukemia virus, to *Bgl*II-to-*Sal*I fragment from transposon Tn5 containing the *neo* gene (2), a *Pvu*II-to-*Hind*III fragment from simian virus 40 containing the simian virus 40 early promoter, unique *Stu*I, *Avr*II, *Hind*III, and *Cla*I sites for inserting cDNAs, and bases 7764 through the 3' LTR from Moloney murine leukemia virus. In addition, the *gag* start codon in pLNSL7 was changed from ATG to TAG to prevent possible *gag* protein translation (5). Both LNL-XHC and LNSL7 were produced at equal titer by using retrovirus packaging cell lines.

β -Globin genes modified by insertion of a *Bgl*II site as a transcriptional marker (Fig. 1) (4) were inserted into pLNL-XHC between the *neo* gene and the 3' LTR (pLNB*HP, pLNB*HP MG⁺, and pLNB*HP MG⁻) and into pLNSL7 between the *neo* gene and the 3' LTR in place of the simian virus 40 promoter (all other β -globin vectors). Retrovirus vector names indicate the order of genes in the virus and important features of the vector. For example, LNB*HP indicates a retrovirus vector consisting of a viral LTR (L) driving *neo* (N) followed by a transcriptionally marked β -globin gene (B*) contained in an *Hpa*I-to-*Pst*I (HP) genomic fragment. The vectors LNB*MG⁺, LNB*MG⁻, and LNB*WT⁻ described in a previous publication (4) have been renamed LNB*HP MG⁺, LNB*HP MG⁻, and LNB*HP, respectively, to allow consistent nomenclature use in this report. To make LNB*SA RP (reversed promoter), the *Sna*BI (-265)-to-*Bgl*II (+43) fragment containing the β -globin promoter (Fig. 1) in LNB*SA was excised and reinserted in reverse orientation by using a *Bgl*II linker to adapt the blunt-end *Sna*BI sites to the *Bgl*II sites. The *Bgl*II site is not present in the normal β -globin gene but is present in the marked gene (Fig. 1). To make LNB*SA P⁻ (promoter minus), the same fragment was removed from LNB*SA entirely, again using a *Bgl*II linker to join the *Sna*BI and *Bgl*II sites.

Generation of virus from retrovirus vector constructs. Virus was generated from plasmid constructs as previously described (22). Briefly, plasmids containing the viral constructs were transfected into ψ 2 ecotropic retrovirus packaging cells, and after 2 days virus was harvested and used to infect PA317 amphotropic retrovirus packaging cells. The cells were then seeded into selective medium, and clonal cell lines containing single integrated proviruses were isolated. The structures of integrated β -globin viruses were analyzed by Southern analysis (16) with a β -globin minigene probe. By using restriction enzymes that cleave only in each LTR of the virus (*Kpn*I) and other enzymes that cut once within and at sites present at both ends of the β -globin insert (*Bam*HI and *Hind*III), we confirmed that the fragment sizes produced by the integrated provirus matched those of the original plasmid construct.

Virus assay. Virus was harvested from virus-producing cells by incubating confluent dishes of the cells with fresh medium for 16 h and then removing the medium and subjecting it to centrifugation at $3,000 \times g$ for 5 min to remove cells and debris. For assay of viruses carrying the *neo* gene, recipient cells were seeded at 5×10^5 per 60-mm dish on day 1. On day 2, the medium was changed to medium containing 4 μ g of Polybrene per ml and various amounts of test virus were added. On day 3, the cells were split 1:10 into medium containing 1 mg of G418 (about 50% active) per ml. Colonies were stained and counted on day 9. Amphotropic helper virus was measured by the S⁺L⁻ assay as previously described (20). The presence of helper virus in mice was monitored by using the XC assay (23) as previously described (20), except that recipient NIH 3T3 TK⁻ cells were cocultivated with 10 μ l of fresh blood instead of virus-containing medium for 16 h and blood cells were washed from the plate before the cells were trypsinized for the XC assay.

Infection of murine bone marrow cells and CFU-S assay. 5-Fluorouracil (150 mg/kg; LyphoMed Inc.) was administered intravenously to 6- to 8-week-old female C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine). Mice were sacrificed 48 h later by cervical dislocation, and marrow was flushed from femurs and tibias by using cocultivation medium (Iscoves medium with 10% heat-inactivated fetal bovine serum [Hyclone Laboratories, Logan, Utah], 10% WEHI-3 cell conditioned medium, 4 μ g of Polybrene per ml, penicillin, and streptomycin). Marrow was cocultivated on subconfluent monolayers of irradiated (2,100 rads) virus producer cells for 24 h. Hematopoietic cells were washed off the monolayer and cultured for 36 to 48 h in the presence of 1 mg of G418 per ml. Marrow was washed with and resuspended in Hanks buffered saline solution without calcium, magnesium, or phenol red and injected into female WBB6F₁/J-W/W^v mice (Jackson Laboratory) which had received either no irradiation or 400 rads. At 12 days postinjection, individual CFU-S colonies were dissected out and dissociated and total nucleic acid was isolated (4).

RESULTS

LNB*HP viruses: β -globin viruses containing the *Hpa*I-to-*Pst*I genomic fragment. Figure 1 depicts the human β -globin gene and surrounding sequences. Before insertion into viruses, the β -globin gene was modified by insertion of a 6-base-pair *Bgl*II site into the nontranslated region of exon 1 (Fig. 1). This was done to allow measurement of transcription from the gene in human cells already expressing the endogenous β -globin gene. When we began these experi-

TABLE 1. Characteristics of PA317 cells containing β -globin vectors^a

Vector	Correct structure?	Titer (CFU/ml) in:		β -Globin RNA expression in PA317 cells?
		PA317	ψ 2	
Parental				
<i>neo</i> -Virus	>90%	1×10^7	1×10^7	—
LNB*HP	3/20	5×10^4	5×10^5	+
LNB*HP MG ⁻	6/8	2×10^6	ND ^b	—
LNB*HP MG ⁺	7/8	4×10^6	ND	—
LNB*SX	2/13	2×10^4	4×10^5	+
LNB*SA	13/15	2×10^5	4×10^6	+
LNB*SA 1 ⁻ 2 ⁺	7/8	5×10^5	ND	+
LNB*SA 1 ⁺ 2 ⁻	7/8	2×10^6	ND	+/-
LNB*SA MG	4/4	4×10^6	ND	—
LNB*SA RP	7/10	3×10^5	ND	—

^a Clonal PA317 cell lines containing the indicated retrovirus vectors were generated as described in Materials and Methods. The number of clones with the correct proviral structure is indicated as the ratio of clones with the correct structure to the total analyzed. The highest titer obtained from PA317 cells containing an unrearranged virus is indicated for each vector. Synthesis of β -globin RNA in PA317 cells containing each vector is indicated, as determined by Northern analysis with a β -globin probe. Virus from PA317 cells was used to infect ψ 2 cells, infected clones were isolated, and the best titer obtained from ψ 2 cells containing an unrearranged provirus is indicated. At the time of assay, the ψ 2 cells were helper virus free (<10 PFU/ml), as measured by the XC assay (20, 23). After continued passage, helper virus was detected, as has previously been reported for the parental *neo*-virus (22).

^b ND, Not determined.

ments, the region from *Hpa*I (base -814) to *Pst*I (base +2163) appeared to be sufficient for developmentally regulated tissue-specific expression of the gene in transgenic mice, and we inserted this fragment into a selectable retrovirus in reverse orientation with respect to viral transcription. We also inserted the same fragment containing an intronless β -globin gene, or minigene, into the selectable retrovirus in both orientations. Virus generated by using PA317 retrovirus packaging cells (19) was made at high titer from vectors carrying the β -globin minigene in either the forward (LNB*HP MG⁺) or reverse (LNB*HP MG⁻) orientation, while the vector containing the β -globin gene in the reverse orientation (LNB*HP) yielded virus with a much lower titer (Table 1) (4). Conversely, transcription of β -globin was only detected in fibroblasts, MEL cells, or BFU-E colonies infected with the vector carrying the β -globin gene with introns and not in cells infected with either vector carrying a β -globin minigene (4). Thus, transfer of a transcriptionally active β -globin gene appeared to be incompatible with producing high-titer virus.

To understand how the different β -globin inserts affected the production of virus, we examined RNA from PA317 cells containing the different vectors and from virions produced by these cells (Fig. 2A). PA317 cells infected with the parental *neo*-virus contained two predominant viral RNAs (depicted in Fig. 2B), a full-length viral RNA which is packaged into virions and a spliced RNA which is not packaged because it lacks the signal required for packaging (1, 5). In contrast, PA317 cells infected with the β -globin viruses contained multiple viral RNAs, and virions produced by the cells contained at least two RNAs, of which only a minor portion was the full-length viral RNA (Fig. 2A). The effects on titer that we observed (Table 1) correlated with the proportion of full-length viral RNA observed in cells and in virions, which was reduced for the minigene viruses and was barely detectable for the LNB*HP virus (Fig. 2A). Thus, the low titer of these β -globin viruses was apparently due to the

low abundance of full-length viral RNA in virus-producing cells and in virions produced by the cells.

To understand the reasons for the low abundance of full-length viral RNA, we analyzed the transcription patterns of the vectors, and we propose the transcription patterns shown in Fig. 2B to explain the RNA data in Fig. 2A. The transcripts found in PA317 cells harboring the LNB*HP MG⁺ virus can be correlated with known transcriptional signals in the virus. There are two poly(A) signals in this virus, one in the 3' LTR and one at the end of the β -globin minigene where polyadenylation of β -globin transcripts normally occurs [signals labeled (A)_n, Fig. 2B]. In addition, splicing can occur between the 5' LTR and *neo* sequences in the parental *neo*-virus (1, 5). Thus, we expect four viral transcripts in cells containing LNB*HP MG⁺ (Fig. 2B). Indeed, three bands were detected in PA317 cells containing the LNB*HP MG⁺ virus (indicated by dots in Fig. 2A) corresponding in order of decreasing size to expected RNAs a, b plus c, and d (Fig. 2B), assuming that the abundant 28S rRNA (4.5 kilobases [kb]) caused bands b and c to comigrate. Analysis of cellular RNA with *neo* or β -globin probes revealed a similar pattern of bands. The sizes of the two RNA species in virions were consistent with expected packaging of unspliced RNAs a and c (Fig. 2B). Thus, viral RNA species found in cells containing LNB*HP MG⁺ and in virions produced by the cells match the predicted RNA species depicted in Fig. 2B. In addition, since RNA corresponding to the full-length viral transcript was observed in cells and in virions, this indicates that transcription can occur across the β -globin poly(A) signal present in the virus.

In the LNB*HP MG⁻ virus, the β -globin minigene is inserted in reverse orientation with respect to viral transcription. No known poly(A) or splicing signals are present in the inserted β -globin sequences, but there is one consensus poly(A) signal (AATAAA) in the β -globin gene and four such signals clustered at the 3' end of the insert (asterisks, Fig. 2B). β -Globin and *neo* probes revealed transcripts of similar size in LNB*HP MG⁻-infected cells (indicated by dots in Fig. 2A) which correspond in order of decreasing size to proposed transcripts a, c, b, and d (Fig. 2B), although as noted above the separation of bands b and c is complicated by their proximity to the 4.5-kb rRNA band. Two transcripts were detected in virions harvested from PA317 cells containing LNB*HP MG⁻ (Fig. 2A), and their sizes matched proposed transcripts a and c (Fig. 2B). Thus, RNA species found in PA317 cells containing LNB*HP MG⁻ and in virions produced by the cells can be accounted for by splicing known to occur in the parental *neo*-virus and the presence of an additional RNA termination signal in the β -globin insert, which coincides with several consensus poly(A) signals. Note that analysis of RNA in LNB*HP MG⁺ and LNB*HP MG⁻ virions reveals equivalent-size bands which correspond to the predicted full-length viral transcript, as expected since the viruses are the same size, and different-size smaller bands corresponding to early RNA termination at different positions in the two viruses.

Finally, one might expect that the pattern of RNA species observed for the LNB*HP virus would be similar to that of LNB*HP MG⁻, but shifted up 1 kb in size to reflect the addition of the β -globin introns. The observed pattern was more complex (Fig. 2A), but consistent with premature RNA termination within intron 2 of the β -globin gene (Fig. 2B). Proposed RNAs e and f (Fig. 2B) predominated in PA317 cells containing the virus (lower two dots, Fig. 2A), and RNA species e was the major RNA in virions produced from these cells (lower dot, Fig. 2A). Thus, it appears that

FIG. 2. Analysis of RNA from β -globin vectors containing the *HpaI*-to-*PstI* β -globin fragment. (A) RNAs from PA317 cells containing β -globin vectors and from virions produced by the cells were subjected to electrophoresis in agarose gels containing formaldehyde and probed by using an *HpaI*-to-*PstI* β -globin minigene probe or a *neo* probe by standard methods (16). The positions of the human β -globin mRNA ($h\beta^*$), the full-length RNAs from the minigene viruses (MG), and the full-length RNA from the intron-containing β -globin virus (HP) are indicated. Positions of the 28S (4.5 kb) and 18S (1.8 kb) rRNAs are indicated, and specific bands discussed in the text are indicated by dots to the left of each lane. (B) Proposed RNA species transcribed from retrovirus vectors. Approximate sizes of the RNAs are given in kilobases assuming a 200-base-pair poly(A) tail. Asterisks indicate consensus poly(A) signals (AATAAA) in the transcriptional orientation of the viral promoter, and arrows indicate promoters. (A)_n indicates known poly(A) sites in the orientation of the viral promoter, and arrows indicate promoters.

FIG. 3. Comparison of RNA in PA317 cells containing LNB*HP or LNB*SX. Northern (RNA) analysis was performed as described in the legend to Fig. 2A. One PA317 clonal cell line containing LNB*HP and three independent clones containing LNB*SX were analyzed. The sizes of the ribosomal markers and the position of the human β -globin ($h\beta^*$) RNA are indicated.

sequences in intron 2 in the reverse orientation β -globin gene act to reduce the amount of full-length viral RNA in PA317 cells containing the LNB*HP virus and in virions produced by these cells. RNA with the size expected from the β -globin gene was detected in cells containing this virus. This β -globin transcript was not detected in virions produced by these cells, even though it is transcribed in an antisense orientation with respect to the viral genome and thus could hybridize to viral RNA. In summary, the low titer of the β -globin viruses appears to be due to early termination of viral transcription, which reduces the amount of full-length viral RNA in virions.

LNB*SX: a β -globin virus containing the *SphI*-to-*XbaI* genomic fragment. In an attempt to improve the titer of virus containing the β -globin gene, we constructed a virus that did not contain the *HpaI* (–814)-to-*SphI* (–615) region of the gene which contained the four consensus poly(A) signals (Fig. 1), in which early termination of RNA transcription appeared to occur. In addition, evidence had accumulated suggesting involvement of sequences downstream of the gene in β -globin gene regulation (3, 14, 26). Thus, we included these sequences in the LNB*SX virus, which contains the region from *SphI* to *XbaI* inserted in reverse orientation into a *neo*-virus. PA317 cell lines containing this vector were generated and screened as for the LNB*HP virus. Unfortunately, we observed rates of rearrangement of the LNB*SX virus in PA317 cells that were as high as those seen with LNB*HP. In both cases, the rearranged viruses were smaller than the parental virus and had suffered deletions of β -globin sequences. The best titer from a PA317 cell clone containing an unrearranged LNB*SX provirus was 2×10^4 , even lower than that obtained with LNB*HP (Table 1).

Analysis of RNA in PA317 cells containing either the LNB*SX or LNB*HP virus (Fig. 3) provided an explanation for the low titer of the LNB*SX virus. Transcripts from the viral LTR through the β -globin gene were reduced in LNB*SX-infected cells in comparison with those in

LNB*HP-infected cells. In contrast, the amount of RNA transcribed from the β -globin gene was similar in cells infected with either virus. Since the probe used in these experiments was a β -globin minigene probe (positions –814 to +2163, Fig. 1), it is likely that an additional RNA termination signal in the *PstI*-to-*XbaI* fragment present in LNB*SX, possibly the poly(A) signal at position +2637 (Fig. 1), leads to termination before the region recognized by the probe. Apparently, the titer of the LNB*SX virus was not improved compared with that of LNB*HP because, while we may have removed one termination signal, we introduced another.

LNB*SA: a β -globin virus containing the *SphI*-to-*AvrII* genomic fragment. Assuming that the poly(A) signal at position +2637 (Fig. 1) was responsible for the low titer of the LNB*SX virus, we deleted this signal in our next β -globin virus, LNB*SA, which contains the *SphI* (–615)-to-*AvrII* (+2482) region containing the β -globin gene (Fig. 1) in reverse orientation. This virus still contains the region downstream of the β -globin gene involved in regulation of the gene, which has been localized between bases +2163 and +2422 (3). In contrast to results obtained with LNB*HP and LNB*SX, most of the PA317 cell lines infected with LNB*SA virus contained unrearranged proviruses, and the highest titer observed was 10-fold higher than that of LNB*SX and 4-fold higher than that of LNB*HP (Table 1). Therefore, we conclude that sequences in the regions flanking the β -globin gene from positions –814 to –615 and from positions +2482 to +3287 inhibit virus production when inserted into a retrovirus in reverse orientation. RNA analysis suggests that this result is due to premature termination of RNA transcription, which reduces the amount of full-length viral RNA and thus also reduces the titer of virus produced.

Role of β -globin introns in LNB*SA virus. The titer obtained from the LNB*SA virus (2×10^5 CFU/ml) was better than that of other viruses containing a β -globin gene with introns but was still far from the titer obtained with the minigene viruses (2×10^6 to 4×10^6 CFU/ml). To determine which intron was responsible for the decreased titer, we constructed three new viruses with structures identical to that of LNB*SA except that LNB*SA 1[–]2⁺ lacked intron 1, LNB*SA 1⁺2[–] lacked intron 2, and LNB*SA MG lacked both β -globin introns. Most of the PA317 lines generated from these viruses contained unrearranged proviruses, as we found for the parental LNB*SA virus (Table 1). The minigene virus LNB*SA MG (which does not contain β -globin introns) had the highest titer, 20-fold higher than LNB*SA, a result similar to that obtained previously with the LNB*HP viruses (Table 1). Virus titers from the LNB*SA 1[–]2⁺ and LNB*SA 1⁺2[–] viruses were intermediate to those of LNB*SA and LNB*SA MG, but clearly the presence of intron 2 best correlated with reduced virus titer (Table 1).

We next analyzed the RNA in the PA317 cells containing LNB*SA and its derivatives (Fig. 4). A transcript of the size expected from the β -globin gene was present in LNB*SA- and LNB*SA 1[–]2⁺-infected cells but not in LNB*SA 1⁺2[–]- or LNB*SA MG-infected PA317 cells. Quantitation of β -globin transcripts by an RNA protection assay revealed similar amounts in LNB*SA- and LNB*SA 1[–]2⁺-infected cells, 7% of this level in LNB*SA 1⁺2[–]-infected cells, and only 1% in LNB*SA MG-infected cells. Thus, transcription of the β -globin gene strongly depended on the presence of β -globin intron 2. Expected full-length proviral transcripts (indicated by dots in Fig. 4) were abundant in LNB*SA MG- and LNB*SA 1⁺2[–]-infected cells, were greatly reduced in

FIG. 4. Northern analysis of RNA in PA317 cells containing LNB*SA and its derivatives lacking one or more introns. RNA from independent PA317 clonal cell lines containing LNB*SA MG (1⁻2⁻), LNB*SA 1⁺2⁻ (1⁺2⁻), LNB*SA 1⁻2⁺ (1⁻2⁺), and LNB*SA (1⁺2⁺) was analyzed as described in the legend to Fig. 2A. The sizes of rRNAs and the position of the human β -globin mRNA ($h\beta^+$) are shown, and the full-length viral RNA species for each cell line is indicated by a dot to the left of each lane.

LNB*SA 1⁻2⁺-infected cells, and were barely detectable in LNB*SA-infected cells. As anticipated, the amount of full-length viral transcript present (Fig. 4) correlated with the titer of the virus (Table 1). Finally, the amount of full-length viral transcript, and thus the titer of virus, was inversely related to the amount of β -globin message present in the virus-infected cells.

Effect of β -globin gene transcription on virus production. The results presented above lead to the unfortunate conclusion that the titer of viruses containing the β -globin gene in reverse orientation is inversely related to the ability of the virus to make a β -globin mRNA. We considered three possible explanations for this effect. (i) The introns themselves, in particular intron 2, contain signals that inhibit production of the full-length viral RNA; (ii) the presence of an active promoter interfered with viral transcription; or (iii) the presence of the β -globin mRNA, which has an antisense orientation with respect to viral mRNA, caused rapid degradation of the viral RNA or in some other manner affected accumulation of viral RNA. To test the latter two possibilities, we altered LNB*SA to prevent production of antisense RNA. In LNB*SA RP the β -globin promoter was reversed, and in LNB*SA P⁻ the promoter was removed entirely.

PA317 cell lines containing LNB*SA RP were made and screened as described above. Both the percentage of clones containing an unrearranged virus and the titer of virus produced from the best clone were similar to results obtained with the parental LNB*SA virus (Table 1). As expected, RNA of the size expected from the β -globin gene was not detected in PA317 cells containing LNB*SA RP by using a β -globin probe (data not shown). Viral transcripts detected were similar in sizes and abundance to those of LNB*SA (data not shown). Thus, the low titer of the LNB*SA virus was not due to reverse orientation transcription (with respect to viral transcription) of the β -globin gene or to the presence of β -globin RNA in the virus-producing cells which has an antisense orientation with respect to viral transcripts.

Next, we tested the ability of pLNB*SA P⁻ to make high-titer virus. For this assay, we used a transient virus production assay in which retrovirus-packaging cells are transfected with the virus constructs, virus is harvested 2 days later, and the titer of the virus is measured. This assay is much faster than the assay used for the viruses described so far, and we found a good correlation between the results of the two assays with β -globin viruses. The results show that pLNB*SA, pLNB*SA RP, and pLNB*SA P⁻ all produce virus at a titer that is at least 20-fold lower than that of pLNB*SA MG or the parental *neo*-virus (Table 2). That the titer of LNB*SA P⁻ was not improved indicates that the presence of an active β -globin promoter in LNB*SA is not responsible for the low titer of this virus. Therefore, we conclude that the inverse correlation between β -globin expression and virus titer observed for the LNB*SA, LNB*SA 1⁺2⁻, LNB*SA 1⁻2⁺, and LNB*SA MG viruses does not reflect inhibition of virus production as a result of β -globin gene transcription. Instead, we conclude that β -globin intron 2 contains signals that inhibit transcription of the full-length viral RNA, which results in reduced virus titer.

β -Globin virus production from ψ 2 retrovirus-packaging cells. We tested the ability of another retrovirus-packaging cell line, ψ 2 (17), to produce several of the vectors. Interestingly, the titers of β -globin viruses LNB*HP, LNB*SX, and LNB*SA were 10- to 20-fold higher from ψ 2 cells than from PA317 cells, even though the highest titer of the parental *neo*-virus was similar in both cell lines (Table 1). The titer of LNB*SA was 10-fold higher than that of LNB*HP or LNB*SX, consistent with results found with PA317 cells. Helper virus was not detected in any of these clonal lines at the time of assay and thus cannot explain the high titer of the β -globin vectors.

Expression of β -globin gene in infected MEL cells. We infected MEL cells with several of the β -globin viruses and measured expression of the transferred β -globin gene in uninduced and induced MEL cells by using an RNA protection assay (Table 3). We found that all viruses containing the β -globin gene with both introns as well as the virus containing only intron 2 (LNB*SA 1⁻2⁺) directed similar amounts of correctly initiated β -globin RNA synthesis in induced cells. In contrast, β -globin RNA was reduced more than 10-fold in MEL cells infected with the virus containing intron 1 but not intron 2 (LNB*SA 1⁺2⁻) and was not detected in MEL cells infected with the minigene virus. These results parallel those obtained with fibroblasts (Fig. 4) and show that intron 2 is required for efficient β -globin expression. In addition, β -globin RNA was induced to similar extents in MEL cells containing all the viruses except for LNB*SA MG, in which β -globin RNA was not detected. Thus, either β -globin intron can be deleted without affecting the ability of

TABLE 2. Effect of β -globin promoter alterations on virus production^a

Vector	Transient titer (CFU/ml)
pLNB*SA	1 × 10 ⁴
pLNB*SA RP	6 × 10 ³
pLNB*SA P ⁻	1 × 10 ⁴
pLNB*SA MG	2 × 10 ⁵
<i>neo</i> -Virus	3 × 10 ⁵

^a Each of the indicated vectors (10 μ g) was transfected into ψ 2 cells plated the day before at 5 × 10⁵ per 6-cm dish by using calcium phosphate coprecipitation as described previously (21). The cells were fed 1 day after transfection, and after 2 days virus was harvested and quantitated.

TABLE 3. β -Globin expression in MEL cells^a

Vector	β -Globin RNA [% of total poly(A) ⁺ RNA]		Avg induced RNA level compared with LNB*SA (%)	Avg fold induction
	Uninduced	Induced		
LNB*HP	0.0073	0.066	110	9
LNB*SX	0.0031	0.039	70	13
LNB*SA	0.0044	0.058	100	13
LNB*SA 1 ⁻ 2 ⁺	0.0017	0.027	50	15
LNB*SA 1 ⁺ 2 ⁻	0.0003	0.0023	4	8
LNB*SA MG	<0.0002	<0.0002	<0.3	

^a β -Globin RNA production was measured in induced and uninduced MEL cells by an RNA protection assay as previously described (4). Results are averages of two experiments for each vector. Cells analyzed are pools of G418-resistant MEL cells resulting from infection with virus from PA317 cells containing the indicated vectors.

the gene to respond to induction. Finally, these results show that the presence of the additional region downstream of the β -globin gene implicated in control of gene expression (which is present in LNB*SX and LNB*SA but not in LNB*HP) had no significant effect on β -globin expression in MEL cells.

The presence of human β -globin polypeptide in infected MEL cells was determined by immunofluorescence with an antibody for human β -globin which does not recognize mouse β -globin (24). MEL cells infected with the LNB*HP and LNB*SA viruses displayed similar fluorescence, with 20 to 40% of the cells in these nonclonal populations showing bright fluorescence (data not shown). Thus, cells infected with the high-titer LNB*SA virus make human β -globin polypeptide in amounts similar to those demonstrated previously for the LNB*HP virus (4).

Expression of human β -globin in infected mouse hematopoietic cells. We used the high-titer virus from ψ 2 cells producing the LNB*SA virus to infect bone marrow from mice. Infected marrow was infused into W/W^v anemic mice, and after 12 days spleen colonies were isolated and analyzed for the presence of vector DNA and for human β -globin RNA expression. Twelve-day CFU-S colonies arise from primitive hematopoietic cells which give rise to colonies containing various hematopoietic lineages, including erythroid cells. In separate experiments, 6 of 9 and 10 of 10 CFU-S colonies contained a provirus of the expected structure (data not shown). Every infected CFU-S colony contained correctly initiated human β -globin mRNA, although the level of expression varied over 100-fold (Fig. 5). CFU-S colonies contain a variable mixture of erythroid and nonerythroid cells, which may account for part of the variation in expression. However, we have no evidence that human β -globin expression is indeed erythroid specific in these experiments. No helper virus was detected in similarly treated mice analyzed after 1 month, showing that infection was not the result of virus spread. Thus, LNB*SA was able to infect primitive hematopoietic cells and the transduced globin gene was expressed in the resulting colonies.

DISCUSSION

We made a variety of alterations to increase the titer of viruses containing the human β -globin gene. Several signals in both intron 2 and regions flanking the β -globin gene interfered with the retrovirus life cycle. We could eliminate the inhibitory flanking signals but not the signal(s) in intron 2, because the presence of this intron was required for

FIG. 5. Human β -globin RNA ($h\beta^*$) synthesis in infected CFU-S colonies. Bone marrow was cocultivated with ψ 2 cells producing LNB*SA, selected in G418, and infused into anemic W/W^v mice. Twelve-day CFU-S colonies were isolated, and total nucleic acid from provirus-containing CFU-S colonies was analyzed by an RNA protection assay (4). RNA from a MEL cell line infected with a β -globin gene-containing virus was used as a marker.

efficient β -globin expression. For the virus containing a forward orientation β -globin minigene (LNB*HP MG⁺), the interfering signal was apparently the normal poly(A) signal present at the end of the β -globin gene, which led to decreased full-length virus transcription. Less than full-length transcription in other β -globin viruses also correlated with the presence of consensus poly(A) signals (AATAAA) within the virus, but could be due to other signals for 3' RNA processing or for termination of transcription. In addition, not all consensus poly(A) signals are functional and not all functional poly(A) signals share the AATAAA consensus sequence (6); thus, the elimination of AATAAA signals might not be sufficient to increase full-length viral transcription.

We explored the effect on β -globin expression of a region downstream of the β -globin gene that has been implicated in β -globin regulation, but found no effect on expression in MEL cells or in fibroblasts. In addition, limited experiments suggest that this region has no effect on β -globin expression in animals reconstituted with infected marrow (8). However, inclusion of this region in the LNB*SA virus does not appreciably affect virus titer; therefore, we retained the sequence. We hope that regions flanking the globin locus which are responsible for position-independent high-level expression of β -globin in mice (10) can eventually be incorporated into these vectors. At present, however, the localization of these elements is not precise enough to allow insertion of the region without exceeding retrovirus size limits.

Problems that have been encountered when using retroviral vectors to transfer genes can be grouped into three categories: inappropriate splicing, early termination of viral

RNA, and competition of multiple promoters carried by the viruses (for a review, see reference 25). Since the β -globin introns were found to be necessary for β -globin expression, we were limited to insertion of the gene in reverse orientation to avoid removal of the introns by splicing during virus replication. Less than full-length viral transcription was encountered with reverse orientation inserts, which was reduced but not eliminated in redesigned viruses. We have direct evidence that possible competition between viral and β -globin promoters or possible effects of antisense RNA did not affect virus titer, since reversal or removal of the β -globin promoter had no effect on virus titer. The difficulties encountered with retrovirus-mediated transfer and expression of β -globin contrast with the ease with which another transcriptionally regulated gene, a rat growth hormone minigene, was transferred and expressed after insertion into a retrovirus vector (21). Despite these difficulties, systematic modification of viruses containing the β -globin gene led to a β -globin virus which can be made at a relatively high titer. Still higher titers might be achieved if the element(s) in intron 2 which interferes with transcription can be altered without affecting β -globin expression, for example, by site-specific mutagenesis of the presumed poly(A) signal.

In generating PA317 cell lines containing β -globin viruses, we found many lines containing rearranged proviruses (Table 1), especially with LNB*HP and LNB*SX. These viruses were in general shorter than the parental virus and had suffered various deletions (some complete) of the β -globin insert. Similar deletions were found in several independent clones, suggesting that either alternative splicing or recombination in regions of homology was responsible. The rearranged viruses were always able to replicate to a higher titer than the original virus. For a given vector there was a correlation between the transient titer of virus obtained from ψ 2 cells (e.g., Table 2), the highest titer that could be obtained from PA317 cells infected with the vector (Table 1), and the proportion of PA317 clones analyzed that contained unrearranged proviruses (Table 1). We interpret these results to mean that recombinant viruses are generated during transfection for all viruses, but only if the transfected virus replicates very poorly are the rearranged viruses detected at high frequency in the infected PA317 cells. Once packaging cell lines containing single unrearranged viruses were generated, little rearrangement was detected when virus from these cells was used to infect other cells.

The LNB*SA virus developed here can be obtained from ψ 2 cells at 4×10^6 CFU/ml, and this titer allowed transfer of the β -globin gene into murine CFU-S cells. Twelve-day CFU-S cells share several characteristics with more primitive hematopoietic stem cells. They are present in bone marrow at low abundance, they cycle slowly, and they give rise to cells of multiple lineages. We showed that up to 100% of 12-day CFU-S cells can be infected and that every infected CFU-S cell expresses correctly initiated human β -globin mRNA. The high titer and ability of this virus to express the β -globin gene in vivo will assist further studies of the long-term expression of the introduced β -globin gene in transplanted mice and in human hematopoietic cells.

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