Expression of the Human γ-Globin Gene after Retroviral Transfer to Transformed Erythroid Cells†

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ABSTRACT: Regulation of the human fetal (γ) globin gene and a series of mutant γ-globin genes was studied after retroviral transfer into erythroid cells with fetal or adult patterns of endogenous globin gene expression. Steady-state RNA from a virally transferred $^{A}\gamma$-globin gene with a normal promoter increased after induction of erythroid maturation of murine erythroleukemia cells and comprised from 2% to 23% of the mouse β^{maj}-globin RNA level. RNA expression from the virally transferred $^{A}\gamma$-globin gene comprised 23% of the endogenous $^{G}\gamma$- + $^{A}\gamma$-globin expression in K 562 cells after treatment with hemin. Expression from a virally transferred γ- or β-globin gene exceeded endogenous γ- or β-globin expression by a factor of 6 or more in the human erythroleukemia line KMOE, in which the endogenous globin genes are weakly inducible. In these experiments, no difference in expression was observed between the gene with the normal promoter and an $^{A}\gamma$-globin gene with a point mutation in its promoter (−196 C-to-T) that has been associated with hereditary persistence of fetal hemoglobin (HPFH). To test for cis-acting determinants located within the introns of the γ-globin gene, expression was measured from a set of γ-globin genes configured with either intron alone or with neither intron. In contrast to an intronless β-globin gene, which is not expressed in MEL cells, the intronless γ-globin gene was expressed in MEL cells at 24% of the level of an intron-containing gene. The data show that γ-globin genes can be expressed at substantial levels in transformed erythroid cell lines after retroviral transfer and that fundamental differences exist in the extent to which introns influence expression of γ- and β-globin genes.

The human fetal or γ-globin genes are active during the last trimester of fetal development and decline in activity during the perinatal period as expression from the β-globin gene rises (Stamatoyannopoulos & Nienhuis, 1987; Karlsson & Nienhuis, 1985). To facilitate the study of fetal globin gene sequences that mediate γ-globin activation and repression, we cloned the $^{A}\gamma$-globin gene with either a normal or an HPFH promoter (−196 C-to-T; Gelinas et al., 1986; Ottolenghi et al., 1985) and several other derivatives into a retroviral vector to take advantage of the efficiency of gene transfer, collinear integration of DNA sequences, and the wide host range afforded by these vectors (Miller, 1989). These vectors were used to transfer the γ-globin gene as a 3.3-kilobase (kb) *Hin*dIII fragment into murine and human erythroid cell lines having either an adult (MEL), embryonic plus fetal (K562), or fetal plus adult (KMOE) pattern of endogenous globin gene expression. As assayed in transgenic mice, this fragment results in tissue-specific, developmentally regulated γ-globin expression (Chada et al., 1986; Kollias et al., 1986). The γ-globin gene (Rixon & Gelinas, 1988) used in our studies was also "marked" to permit quantitation of its transcripts independently from RNA transcribed from the endogeneous γ-globin genes in the human K562 and KMOE cell lines.

Although the induction of γ-globin RNA expression after transfer to MEL cells has been well documented (Anagnou et al., 1986; Charnay & Henry, 1986; Chao, 1986; Stoeckert et al., 1987), other studies that used a variant of MEL cells deficient in thymidine kinase observed induction of β-globin but not γ-globin RNA in a majority of the clones studied (Wright et al., 1983, 1984). Thus, we studied the pattern of γ-globin expression in three MEL cell variants to determine if they might differ in their ability to express a transferred γ-globin gene. Identification of a MEL cell variant that might not express transferred γ-globin genes could lead to an assay for cis-acting determinants of γ-globin gene activation and repression, such as the determinants that mediate the nondeletion HPFH phenotype. A Greek HPFH (−117 G-to-A) $^{A}\gamma$-globin gene was reported to express more steady-state RNA than a normal γ-globin gene after stable transformation of MEL cells (Charnay & Henry, 1986), while no difference in expression was found in another study of the same gene that utilized retroviral vectors (Stoeckert et al., 1987).

We also studied the expression of normal or HPFH γ-globin genes in K562 (Lozzio & Lozzio, 1975) and KMOE (Kaku et al., 1984) cells, in which endogenous γ- or both γ- and β-globin genes, respectively, are active. Although expression of a hybrid γ/β-globin gene was reported after transfer to K562 cells (Lin et al., 1987; Donovan-Peluso et al., 1987), expression from a transferred γ-globin gene with its natural 5′ and 3′ flanking sequences has not been reported. KMOE cells were included in our study since it has been reported that, after retroviral transfer to these cells, the Greek HPFH γ-globin gene was more active than a normal $^{A}\gamma$-globin gene (Stoeckert et al., 1987).

Since high-titer viruses are the most useful in gene transfer studies, but previous work from our laboratory showed that the introns of the β-globin gene had a negative correlation with virus titers (Bender et al., 1988; Miller et al., 1988), we studied the influence of the introns of the γ-globin gene on virus titers and RNA expression. γ-Globin genes configured with no introns (but normal 5′ and 3′ flanking sequences) or with the first or the second intron alone revealed differences in expression compared to the corresponding β-globin gene con-

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structs. These data suggest that the introns of the γ- and β-globin genes contribute in different ways to gene expression. We also compared the γ-globin retroviruses to vectors that carry the human β-globin gene (Cone et al., 1987; Karlsson et al., 1987; Lerner et al., 1987; Bender et al., 1988; Miller et al., 1988) to evaluate their potential utility in gene therapy protocols.

Experimental Procedures

Cell Lines and DNA Constructs. Transformed human embryonic kidney cells (line 293; Graham et al., 1977), Hep G2 cells, PA317 cells (ATCC CRL 9078) (Miller & Buttimore, 1986), NIH 3T3 (*tk*⁻) cells (Wei et al., 1981), and strains 585, B10, and 179D of MEL cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. ψ-2 cells were grown in this medium with 10% calf serum (Mann et al., 1983). K562 and KMOE cells were grown in RPMI medium supplemented with 5% fetal calf serum. ^{A}γ-Globin genes with normal or HPFH (−196 C-to-T) promoters were subcloned on the 3.3-kb *Hin*dIII fragments derived from the cosmid clones as described (Gelinas et al., 1986; Rixon & Gelinas, 1988). These fragments contained 1345 bp of 5′ globin flanking sequence and 380 bp of 3′flanking sequence. Derivatives of the normal ^{A}γ-globin gene that contained either the first intron, the second intron, or neither intron were prepared by replacing a portion of the ^{A}γ-globin gene with the appropriate fragment from the γ-globin cDNA clone pJW151 (Wilson et al., 1978). To render transcripts from these genes distinguishable from endogenous γ-globin RNA in K562 and KMOE cells, each of these genes included a 6-bp deletion in the 5′ nontranslated region as previously described (Rixon & Gelinas, 1988). This short deletion, denoted with an asterisk (γ* RNA), had no effect on transcription in a transient assay system (Rixon & Gelinas, 1988). The replication-defective retroviral vector LNL-XHC has long terminal repeats derived from the Moloney murine leukemia virus and the gene for neomycin phosphotransferase (*neo*) (Bender et al., 1987). *Neo* is expressed from an RNA that originates in the 5′ long terminal repeat (LTR) of the vector. The vector LNβ*SA, which was used to transfer the human β-globin gene into KMOE-2/05 cells, has been described in detail (Miller et al., 1988).

Cell Infections. Preparation of retrovirus-producing cell lines with a transfection–infection protocol, titering of virus on NIH 3T3 (*tk*⁻) cells, and selection of infected adherent cell lines in G418 have been described (Miller & Buttimore, 1986; Miller et al., 1988). The XC assay was used to detect replication-competent helper virus as described (Miller et al., 1985). In most experiments, K562, KMOE, and MEL cells were infected by cocultivation. Briefly, 1×10^5 cells were overlaid overnight on PA317 γ-virus- (or β-virus-) producing cell lines that had been seeded 2 days earlier at 5×10^5 cells per 6-cm dish. After 16–20 h, the cells were diluted 10-fold into medium containing G418 at 1 mg of dry powder/mL. In some experiments, MEL cells were infected with virus supernatants as described (Bender et al., 1988). For induction, MEL cells at a density of 5×10^4 cells/mL were grown for 4 days in 3 mM *N,N′*-hexamethylenebis(acetamide) (HMBA; Sigma Chemical Co.). K562 cells in RPMI medium with 10% fetal calf serum were treated with 20 μM hemin for 4 days (Dean et al., 1981). KMOE-2/05 cells were maintained in RPMI medium with 5% fetal calf serum for 7 days and then switched to RPMI with 10% fetal calf serum, 50 μM hemin, and 0.05 μM arabinocytidine (araC) for 4 days. Concentrations of araC higher than 1 μM resulted in substantial cell death.

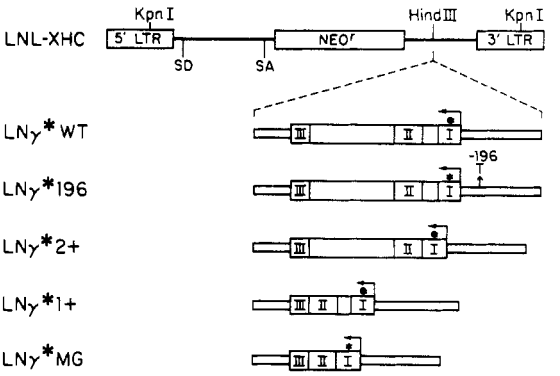

FIGURE 1: Virus structures. LTR, long terminal repeat of the Moloney murine leukemia virus; SD, splice donor; SA, splice acceptor; NEOʳ, bacterial neomycin phosphotransferase gene. Boxes labeled I, II, and III represent the three exons of the ^{A}γ-globin gene. An asterisk indicates a six-base-pair deltion in the 5′ untranslated region. The direction of globin transcription is indicated with an arrow.

RNA Measurements. Isolation of total cellular RNA and quantitation of RNA derived from endogenous or from virally transferred γ- or β-globin genes by solution hybridization with labeled RNA probes and RNase protection analysis have been described (Rixon & Gelinas, 1988). In some experiments, total cellular RNA was prepared by a simpler method (Chomczynski & Sacchi, 1987). The construction of the plasmids used as substrates for T3 or T7 polymerase transcription of ^{32}P-labeled probes specific for human β-globin, mouse β^{maj}-globin, and human γ-globin RNA has been described in detail (Bender et al., 1988; Rixon & Gelinas, 1988). Given the number of uridine residues in a given protected RNA fragment and the specific activity of the [α-^{32}P]UTP that was used to synthesize the probe as listed by the manufacturer, the specific activity of each preparation of probe was calculated. The mass of a specific mRNA present in a hybridization sample was then calculated by multiplying the probe's specific activity by the observed radioactivity in a protected mRNA fragment recovered from a gel. Here we report specific mRNA levels as a percentage of the total poly(A)+ RNA in the cell with the assumption that 1% of the total cellular RNA is poly(A)+.

Immunofluorescence Analysis. Human γ-globin protein was detected in MEL cells induced for 6 days with HMBA by using a monoclonal antibody specific for human γ-globin chains as previously described (Stamatoyannopoulos et al., 1983).

Results

Retroviral Vectors Carrying the Human ^{A}γ-Globin Gene.* The genomic 3.3-kilobase (kb) *Hin*dIII fragments carrying the normal or nondeletion (−196 C-to-T) HPFH ^{A}γ-globin genes were cloned into the *Hin*dIII site of the retroviral vector pLNL-XHC in the opposite transcriptional orientation with respect to the promoter in the 5′ LTR to form the retroviral derivatives LNγ*WT and LNγ*196 as shown in Figure 1. Vectors were also prepared for derivatives of LNγ*WT in which the first globin intron had been deleted (LNγ*2+), the second globin intron had been deleted (LNγ*1+), or both introns had been deleted to form an intronless gene or minigene (LNγ*MG). The latter three constructs had the same 5′ and 3′ flanking sequences and the mark in the 5′ untranslated region described for the ^{A}γ-globin gene in LNγ*WT.

Amphotropic packaging cell lines producing each of these viruses were prepared by transfection of ψ-2 (or PE501) cells followed by infection of PA317 cells, selection in G418, and expansion and characterization of the resulting clonal cell lines. Although only 1 in 16 G418-resistant PA317 cell lines proved

Table I: PA317 Cell Lines Producing Amphotropic Virus Containing Normal and HPFH $^{A}\gamma$-Globin

vector	max titer[a] (CFU/mL)	helper virus (FFU/mL)
LNL-XHC	1.4×10^7	<2
LNγ*wt	1.2×10^5	<2
LNγ*196	3.7×10^5	<2
LNγ*2+	1.0×10^5	nd
LNγ*1+	2.0×10^6	nd
LNγ*MG	6.0×10^6	nd
LNβ*WT-[b]	5.0×10^4	<2

[a] The highest titer on NIH 3T3 cells from the producer clone with the correct proviral structure; nd, not determined; CFU, colony-forming units; FFU, focus-forming units. [b] Previously reported in Bender et al. (1988).

to contain an unrearranged copy of the LNγ*WT provirus by *Kpn*I digestion of the DNA and Southern blot analysis (data not shown), this line produced virus at a titer of 1.2×10^5 CFU/mL (Table I). This titer was slightly higher than the titer we observed for the human β-globin gene cloned into the same vector (5.0×10^4 CFU/mL), and it proved acceptable for subsequent gene transfer studies. Virions from the PA317/LNγ*WT-producing line faithfully transmitted the proviral structure in subsequent studies. No replication-competent helper virus was detected in these virus-producing cell lines. Although the titer of the LNγ*2+ virus, which lacked the first globin intron, was comparable to the titer of the LNγ*WT virus, the titers of the LNγ*1+ and LNγ*MG viruses, which lacked the second intron or both introns, respectively, were 20–60 times higher than the titer of the LNγ*WT virus. We previously observed that the introns of the β-globin gene also decreased virus titers (Bender et al., 1988; Miller et al., 1988).

Expression of the Human γ-Globin Gene after Viral Transfer to MEL Cells. Three MEL cell lines were infected with the γ*-globin gene containing retroviruses to determine if they might differ in their ability to express a transferred γ-globin gene. Diploid MEL 585 cells (Deisseroth & Hendrick, 1978) and tetraploid 179D cells (Deisseroth & Hendrick, 1979) are adenine phosphoribosyltransferase deficient and are permissive for expression of transferred γ- or β-globin genes (Charnay & Henry, 1983; Anagnou et al., 1986; Chao, 1986; Karlsson et al., 1987; Bender et al., 1988; Cone et al., 1987; LaFlamme et al., 1987). The 707 B10/1 line of MEL cells (B10) is deficient in thymidine kinase (Spandidos & Paul, 1982), and transfected γ-globin genes were not inducible in a closely related thymidine kinase deficient MEL line F14-12B2 (Wright et al., 1983, 1984). Each of the three MEL cell lines was infected with the LNγ*WT and LNγ*196 viruses, and the steady-state levels of mouse β^{maj}-globin and human γ*-globin RNA were determined in G418-resistant populations before and after induction with HMBA for 4 days. Figure 2 shows the ribonuclease protection results for the MEL

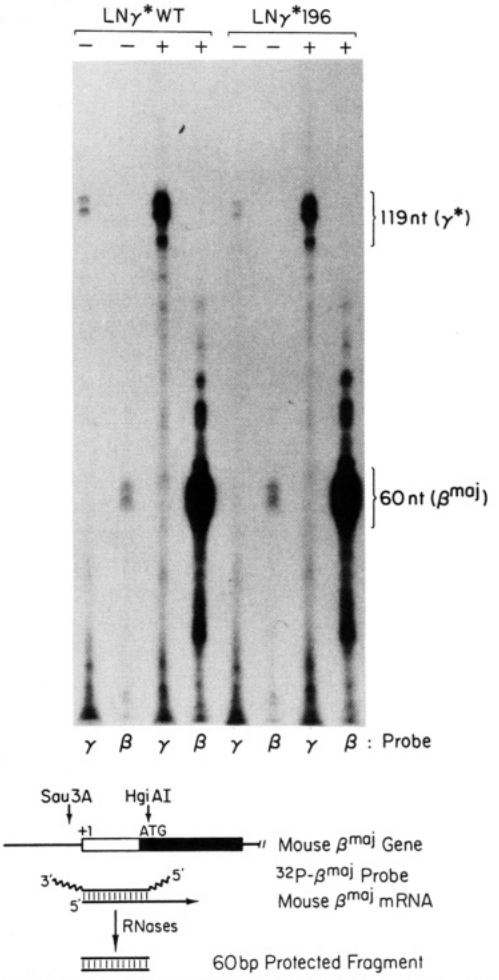

FIGURE 2: Ribonuclease protection of infected MEL cells. A quantity of 8 μg of RNA from MEL/179D cells infected with the LNγ*WT virus was analyzed by ribonuclease protection before induction (−), and 5.2 μg of RNA was analyzed after induction (+). A quantiy of 6.4 μg of RNA from these cells infected with the LNγ*196 virus was analyzed before induction, and 6.6 μg of RNA was analyzed after induction. γ* RNA was measured with the human γ probe; mouse β^{maj}-globin RNA was measured with the mouse β-globin probe. Control hybridizations (not shown) between the human γ probe and uninfected induced MEL cell RNA were negative.

179D cells, and quantitated RNA levels are shown in Table II for all three MEL cell lines. Low levels of steady-state RNA were observed for the mouse β^{maj}-globin gene and the human γ*-globin genes before induction. In all three lines, steady-state RNA levels increased substantially after induction with HMBA, indicating that the virally transferred human γ*-globin gene was regulated in a tissue-specific fashion. Induction of the γ*-globin genes was lower than that observed for the mouse β^{maj}-globin genes. The 585 and B10 strains of MEL cells showed higher RNA levels from the γ*-globin gene

Table II: Globin Expression as a Percentage of Poly(A)+ RNA in Infected MEL Cells

	m-β^{maj} RNA[a]			h-γ* RNA[a]			
MEL type/vector	U	I	ind	U	I	ind	ratio[b]
585/LNγ*WT	0.0008	1.2	1500	0.003	0.028	9.0	2
585/LNγ*196	<0.0003	1.0	>1000	0.016	0.041	2.6	4
B10/LNγ*WT	<0.0003	0.18	>600	0.0092	0.040	4.3	23
B10/LNγ*196	<0.0003	0.043	>140	0.030	0.14	4.7	337
179D/LNγ*WT	0.016	2.4	150	0.0047	0.14	29.0	6
179D/LNγ*196	0.022	1.9	86	0.0030	0.071	23.0	4

[a] The amount of mouse β^{maj}- or γ*-specific RNA in 10-μg samples of total cellular RNA, expressed as a percentage of the total poly(A)+ RNA, assuming that poly(A)+ RNA comprises 1% of the total; U, uninduced; I, induced; ind, induction ratio or I divided by U. [b] Ratio of h-γ* RNA to m-β^{maj} RNA in induced cells, expressed as a percentage.

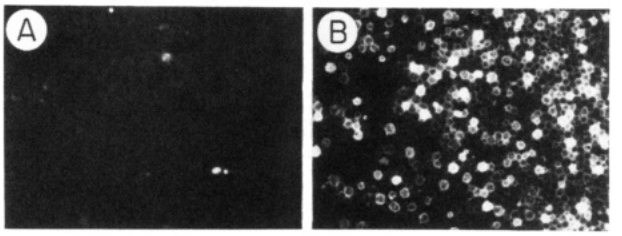

FIGURE 3: Immunofluorescence of infected MEL cells. Uninfected MEL B10 cells (A) or MEL B10 cells infected with LNγ*WT (B) were induced for 6 days with HMBA, fixed on slides, and stained with a monoclonal antibody specific for human γ-globin chains.

driven by the HPFH promoter compared to the γ*-globin gene with the normal promoter after HMBA induction. But in the 179D strain the opposite was the case, and more RNA was measured from the gene with the normal promoter as compared to that from the −196 HPFH promoter. In these experiments the MEL 585 and 179D lines reproducibly showed 5–10 times higher levels of β^{maj}-globin RNA after induction than the B10 line. γ* RNA represented from 0.03% to 0.14% of the poly(A)+ RNA in these MEL cell lines, which represents 2–6% of the mouse β^{maj}-globin RNA in the MEL 585 and 179D cells, with no correction for the number of endogenous mouse β^{maj}-globin genes. In the MEL B10 cells, the γ* RNA levels represent the same proportion of the total poly(A)+ RNA but represent a larger fraction of the β^{maj}-globin RNA because much lower levels of mouse β^{maj}-globin RNA were made.

Due to concerns that the γ* RNA might not be active in protein synthesis because of possible antisense effects arising from the two opposing promoters in the vectors or because the 6-bp deletion in the γ-globin 5′ nontranslated region might interfere with ribosome binding, we tested whether RNA derived from the virally transferred gene was active in protein synthesis. Using an indirect immunofluorescence assay with an antibody specific for human γ-globin chains, we found that about 25% of the induced MEL B10 cells infected with the LNγ*WT virus fluoresced brightly, whereas induced uninfected cells were negative (Figure 3). Studies on induced populations of MEL cells that had been infected with the LNβ*WT− retrovirus revealed a similar pattern of immunofluorescence with an antibody specific for human β-globin chains (Bender et al., 1988). Although no difference in RNA expression was detected between the marked γ-globin gene and a control γ-globin gene in a series of transient expression assays (Rixon & Gelinas, 1988), detection of γ-globin protein chains by immunofluorescence shows that the 6-bp deletion in the 5′ nontranslated region of the ^{A}γ-globin gene does not prevent translation of the γ* mRNA.

Expression of the Human γ-Globin Gene after Viral Transfer to K562 Cells. Expression of the normal and HPFH γ*-globin genes was studied after virial transfer to the human erythroleukemia cell line K562, in which embryonic and fetal globin genes are active (Rutherford et al., 1979; Benz et al., 1980; Dean et al., 1983). K562 cells were infected by cocultivation and selected in G418, and populations of G418-resistant cells were analyzed before and after treatment with hemin. RNA derived from endogenous γ-globin genes was detected in the same lane as RNA derived from the virally transferred γ*-globin gene (Figure 4); the quantitative results are given in Table III. RNA expressed from the proviral copy of the normal or HPFH γ-globin gene represented 31–37% of the endogenous levels before treatment with hemin. After growth in hemin for 4 days, endogenous γ-globin RNA levels increased slightly, but γ* RNA from the virally transferred

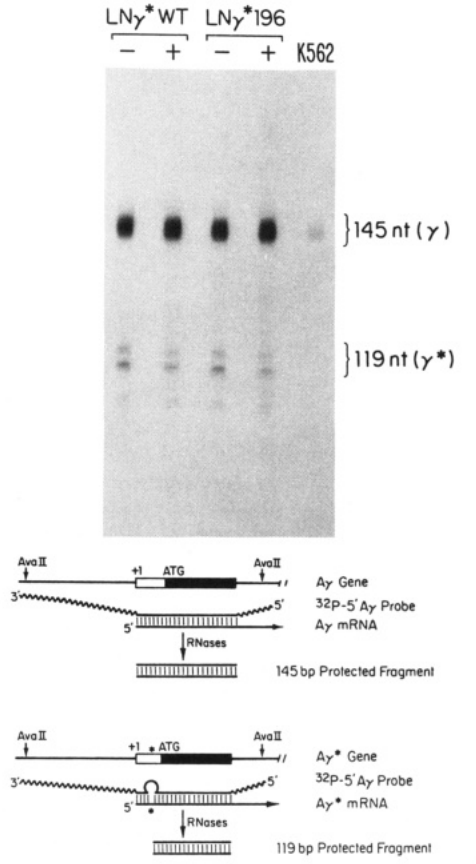

FIGURE 4: Ribonuclease protection of infected K562 cells. A quantity of 12.5 μg of total RNA from K562 cells infected with either the LNγ*WT or the LNγ*196 virus before induction (−) or after induction (+) was analyzed for virally transferred γ* RNA (γ*) and endogenous γ-globin RNA (γ) by ribonuclease protection analysis. The ribonuclease protection pattern of 6.6 μg of uninfected K562 RNA is shown as a control for endogenous γ-globin RNA.

Table III: Endogenous and Virally Transferred γ-Globin Expression in Infected K562 Cells as a Percentage of Poly(A)+ RNA

	^{G}γ + ^{A}γ RNA			h-γ* RNA			ratio[a]	
vector	U	I	ind	U	I	ind	U	I
LNγ*WT	0.15	0.17	1.1	0.056	0.041	0.73	37	23
LNγ*196	0.16	0.18	1.1	0.050	0.035	0.70	31	20

[a] Ratio of h-γ* RNA to total endogenous ^{G}γ + ^{A}γ RNA, expressed as a percentage.

γ*-globin genes decreased slightly. These results are not corrected for the number of endogenous γ-globin genes, which may be as high as six, since the K562 cell line was originally shown to have three copies of chromosome 11 (Lozzio & Lozzio, 1975). There was no significant difference in the expression of the wild-type and nondeletion −196 HFPH γ*-globin gene before or after treatment with hemin.

Expression of the Human γ-Globin Gene after Viral Transfer to KMOE Cells. We next studied expression of marked γ- and β-globin genes after retroviral transfer to KMOE cells. Although it has been reported that treatment of KMOE-2/05 cells with araC leads to the induction of α- and β-globin protein chains (Kaku et al., 1984; Takihara et al., 1987) or β-globin RNA with no detectable γ-globin expression (Stoeckert et al., 1987), we could detect no β-globin RNA after treatment with araC alone. But treatment of the cells with hemin and araC resulted in induction of γ- and β-globin RNA from very low preinduced levels (Figure 5 and Table IV). KMOE cells were first infected with a virus containing a marked human β-globin gene (LNβ*SA) and,

Table IV: Globin Expression as a Percentage of Poly(A)+ RNA in KMOE Cells Infected with LNγ*WT, LNγ*196, or LNβ*SA[a]

	$^{G}\gamma + ^{A}\gamma$			h-β			h-γ*			h-β*			
vector	U	I	ind	U	I	ind	U	I	ind	U	I	ind	ratio[b]
LNγ*WT[a]	0.36	9.3	26	0.014	0.88	63	23	67	3				700
LNγ*196[a]	0.46	2.8	6	0.004	0.51	127	47	43	0.9				1500
LNβ*SA[a]	0.40	11	27	0.09	1.2	13				6	7.4	1.2	600

[a] Values listed for percentages of uninduced (U) and induced (I) poly(A)+ RNA have been multiplied by 10^3 to simplify comparison. For absolute percentages, or before comparison to Tables II and III, divide the listed values by 10^3. [b] Ratio of h-γ* (or h-β*) RNA to the endogenous $^{G}\gamma + ^{A}\gamma$ RNA (or h-β RNA) in induced cells, expressed as a percentage.

Table V: γ-Globin RNA Expression as a Function of Intron Configuration in Nonerythroid PA317 Cells

vector	PA317 (cpm/μg) [mean]	% control
LNγ*WT	148	100
LNγ*2+[a]	285, 104, 63 [151]	102
LNγ*1+[a]	18, 74, 90 [61]	41
LNγ*MG[a]	7.1, 10.2, 28 [15]	10

[a] Data given for three independently derived cell lines.

in separate experiments, LNγ*WT and LNγ*196. Figure 5 shows the RNA levels before and after induction of the transferred β- or γ-globin genes along with endogenous globin expression. β*- and γ*-globin RNA expression attained 0.007% or 0.06% of the total poly(A)+ RNA, respectively, and thus exceeded expression from the endogenous globin genes in these cell populations. The γ* RNA levels in KMOE cells were comparable to the levels observed in MEL cells (Table II). Low endogenous globin RNA levels [less than 0.01% of the total poly(A)+ RNA] are consistent with the original observation that only a few percent of KMOE cells became benzidine positive after induction (Kaku et al., 1984). Although induced KMOE cells show both fetal and adult gene expression, no significant difference in steady-state RNA levels was observed for the γ-globin genes linked to the normal or the −196 HPFH promoters.

Expression of the Human γ-Globin Gene with Different Intron Configurations. Expression of the γ-globin gene with different configurations of introns was studied in nonerythroid PA317 cells and in G418-resistant populations of MEL 585 cells. The γ-globin minigene expressed 10–24% as well as the control gene with both introns in fibroblasts or in MEL cells after 6 days of induction (Tables V and VI). In contrast, the β-globin minigene was expressed at a level at least 100-fold lower than that of its control gene in MEL cells (Bender et al., 1988; Miller et al., 1988). Restoring the first intron (LNγ*1+) allowed expression to 41% or 58% of the control level. A β-globin gene containing its first intron was expressed at only 4% of the control level in MEL cells (Miller et al., 1988). The γ-globin gene with the second intron alone (LNγ*2+) was expressed at normal or near-normal levels. The β-globin gene configured with only its second intron is also expressed at normal or near-normal levels (Miller et al., 1988; R. Gelinas, unpublished observations).

Discussion

We studied the expression of a human γ-globin gene after retroviral transfer to several erythroid cell lines. Although expression from a γ-globin gene has previously been reported after transfer to nonerythroid (Busslinger et al., 1983; Anagnou et al., 1986; Rixon & Gelinas, 1988) or erythroid cell lines (Anagnou et al., 1986; Charnay & Henry, 1986; Wright et al., 1983, 1984), the amount of γ-globin RNA expression either in absolute terms or relative to an endogenous globin gene in the same cell was not always reported. Variations in the cell lines and in the induction protocols employed in these studies have also complicated interpretation of results. Often,

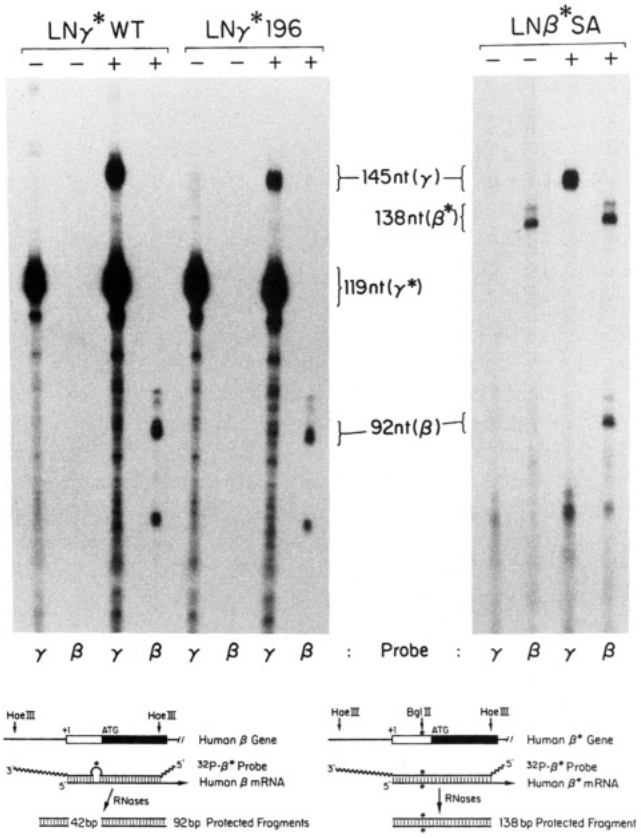

FIGURE 5: Ribonuclease protection of infected KMOE cells. A quantity of 10 μg of total RNA from KMOE-2/05 cells infected with either the LNγ*WT, LNγ*196, or LNβ*SA virus before induction (−) or after induction (+) was analyzed for virally transferred γ*- or β*-globin RNA or endogenous γ- or β-globin RNA by using ribonuclease protection analysis.

the levels of γ-globin RNA observed in these studies were quite low. Here we report expression from the virally transferred γ-globin gene as a percentage of the total poly(A)-containing RNA and relative to endogenous globin expression, based on a quantitative RNase protection assay.

Virally transferred γ-globin genes responded to chemical induction of erythroid differentiation in parallel to the endogenous mouse β^{maj}-globin gene in all three variants of the MEL cell line we tested. The mouse β^{maj}-globin gene typically showed 100–1000-fold induction in these experiments, but the human γ-globin gene induced only 3–29-fold, in part because its final steady-state RNA level was lower than the mouse β^{maj}-globin RNA level but also as a consequence of its expression before induction. After induction, the steady-state level of human γ-globin RNA was 2–23% of the level of the mouse β^{maj}-globin RNA, which is comparable to the level of human β-globin RNA accumulation reported after retroviral transfer into populations of MEL cells (Bender et al., 1988; Karlsson et al., 1987).

We found that the β^{maj}-globin gene itself induced less well in the MEL B10 line than in either of the other MEL cell lines.

The amount of γ-globin RNA actually exceeded endogenous mouse β^{maj}-globin expression in one induced MEL B10 population. The γ-globin expression before induction in the MEL cell lines [0.003–0.03% of the total poly(A)+ RNA] was comparable to the γ-globin expression we observed for virally transferred γ-globin genes into nonerythroid cells (data not shown) and is therefore the level of expression to be expected after selection for the phenotype of G418 resistance alone. Position effects that arise after selection for *neo* expression have been postulated in the past to help account for the expression of transfected β- (Bender et al., 1988; Antoniou et al., 1988) or γ/β-hybrid globin genes (Donovan-Peluso et al., 1987; Wright et al., 1984) prior to induction in erythroid cells. The γ* RNA level was higher from the γ-globin gene driven by the HPFH promoter than from the normal promoter in one MEL cell population, but it was lower in the two other MEL cell populations. These differences in expression are probably not significant.

The three different MEL cell line variants we studied showed no intrinsic differences in their ability to express transferred normal or HPFH γ-globin genes. In spite of the reports of Wright et al. (1983, 1984) which suggested that MEL lines are able to differentially regulate transferred β- and γ-globin genes, we have no evidence that these MEL cell lines vary in their ability to express transferred γ-globin genes. Differences in experimental protocols and the constructions employed may be more important in accounting for our results.

Expression from the virally transferred γ-globin gene attained 37% of the total endogenous $^{G}\gamma$- + $^{A}\gamma$-globin expression in K562 cells and declined slightly to 23% of the total after treatment with hemin. The transcriptional productivity of the virally transferred γ-globin gene thus compares favorably with the endogenous γ-globin genes, since genomic hybridizations showed that this K562 cell population contained only one copy per cell of the LNγ*WT provirus. The endogenous γ-globin genes in these virus-infected K562 cells did not induce appreciably after treatment with hemin. In tests prior to virus infection, we found that our sample of the K562 cell line showed a 4-fold induction of total γ-globin RNA (data not shown). Other investigators have shown that γ/β-globin fusion genes may not always respond to erythroid induction after stable transfection of K562 cells whose endogenous globin genes induce normally (Donovan-Peluso et al., 1987). Thus it would seem unlikely that effects of virus infection or transfection alone are responsible for the poor induction we observed with K562 cells, although these possibilities cannot be excluded. No significant difference in expression was observed for the genes driven by the normal or HPFH promoters. Assuming that the C-to-T substitution at position −196 is indeed the determinant for this form of HPFH, it could be argued that these fetal program cells might be unresponsive to a mutation that is normally expressed in adult erythroid cells.

We found that retrovirally transferred β- and γ-globin genes expressed more RNA than their endogenous counterparts in KMOE cells after induction with a combination of hemin and araC. We found that this combination of inducing agents resulted in a 6–27-fold increase in RNA levels from the endogenous γ-globin genes and a 13–127-fold increase in endogenous β-globin RNA levels. In spite of these induction ranges, steady-state RNA levels from the endogenous globin genes were exceeded by the RNA levels attained by the virally transferred globin genes. Expression from the virally transferred γ-globin gene in KMOE cell populations was comparable to its expression in MEL cell populations when viewed as a percentage of the total poly(A)-containing RNA. No significant difference in steady-state RNA level was observed for the γ-globin genes driven by the normal or HPFH promoter in any cell line tested. In another study that also used retroviruses as gene transfer vectors, a Greek HPFH gene was expressed at a slightly higher level than a control γ-globin gene in KMOE cells that had been induced with araC alone (Stoeckert et al., 1987).

As we had previously noted for β-retroviruses (Bender et al., 1988; Miller et al., 1988), γ-globin viruses with no introns or only the first intron were produced at titers only slightly inferior to the vector alone, with no globin gene. Addition of the second intron, or both introns, caused virus titers to fall 20–60-fold, which suggests that sequences in the γ-globin introns interfere in some way with retroviral replication. Expression of these genes revealed striking differences compared to the corresponding β-globin forms. The γ-globin minigene was expressed at 24% of the control level in MEL cells, whereas expression from a β-globin minigene was less than 0.3% of the control level after viral transfer to MEL cells (Miller et al., 1988) or after construction of transgenic mice (Brinster et al., 1988). Moreover, since the γ-globin minigene was expressed at moderate levels in nonerythroid and erythroid cells, splicing of a globin transcript is not a prerequisite for accumulation of steady-state RNA. Steady-state γ-globin RNA levels were 40–100% of control levels from the genes that contained either the first or the second intron, consistent with a general effect of splicing on message stability (Chung & Perry, 1989).

The ratio of γ* RNA to endogenous γ- or β^{maj}-globin RNA attained in K562 cells (20–23%) or MEL cells (2–23%) compares favorably to the levels of human β-globin RNA expression previously reported after retroviral transfer into populations of MEL cells, which was 10% in one study (Bender et al., 1988) and 7–40% in another (Karlsson et al., 1987). The β-globin retrovirus used in the former study (LNβ*WT−) was capable of infecting human BFU-E as shown by detection of h-β*-globin RNA in the nucleated erythroid progeny cells that comprised the bursts (Bender et al., 1988). Since the γ-globin retroviruses described here can be prepared at the same or slightly higher titers, studies of normal and HPFH γ-globin gene expression are now possible in normal adult erythroid cells to test if these cells are responsive to cis-acting determinants of γ-globin activation and repression presented on transferred genes. Addition of DNA sequences from the dominant control region (Grosveld et al., 1987) or the locus activation region (Forrester et al., 1987) of the β-like globin gene cluster to the vectors described here may raise the level of expression from a transferred globin gene. Derivatives of the locus activation region have been identified by gene transfer experiments with transgenic mice (Ryan et al., 1989) and MEL cells (van Assendelft et al., 1989; Forrester et al., 1989) that are small enough to be added to a retroviral vector and may result in improved expression of a transferred globin gene.

The ability to transfer a γ-globin gene into normal hematopoietic progenitor cells is also relevant to the question of the utility of γ-globin retroviruses for gene therapy. Using the vector LNβ*SA, we demonstrated transfer of the human β-globin gene into pluripotent hematopoietic stem cells of the mouse (Bender et al., 1989). But a γ-globin gene would offer no advantage over a β-globin gene in a gene addition therapy for thalassemia major and would require in addition that the natural tendency for fetal globin gene repression in adult erythroid cells be overcome. In view of the potent antisickling activity of γ-globin chains, which may surpass that of $^{A}\beta$-globin

Table VI: γ-Globin Expression as a Function of Intron Configuration in MEL Cells after Induction with HMBA

vector	expt	cpm[a]	% control
LNγ*WT	1	909	100
	2	3027	100
LNγ*2+	1	594	65
	2	2333	77
LNγ*1+	1	667	73
	2	1757	58
LNγ*MG	1	223	25
	2	736	24

[a] Ten micrograms of total cellular RNA was analyzed in each case; data are corrected for background.

chains (Noguchi et al., 1988), γ-globin chains might be more valuable than $^{A}\beta$-globin chains as part of a gene addition therapy for sickle cell disease.

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The Rate and Structural Consequences of Proline Cis–Trans Isomerization in Calbindin D_{9k}: NMR Studies of the Minor (*cis*-Pro43) Isoform and the Pro43Gly Mutant†

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ABSTRACT: The EF-hand calcium-binding protein, calbindin D_{9k}, exists in solution in the calcium-loaded state, as a 1:3 equilibrium mixture of two isoforms, the result of cis–trans isomerism at the Gly42–Pro43 peptide bond [Chazin et al. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 2195–2198]. Nuclear magnetic resonance (NMR) studies of the minor (*cis*-Pro43) isoform and the Pro43 → Gly mutant are reported here. The rate of cis → trans isomerization at the Pro43 peptide bond in the wild-type protein was determined by line-shape analysis at elevated temperatures, using a sample in which all amino acids, except Ser and Val, were deuterated. The cis → trans rate is calculated to be 0.2 s^{-1} at 25 °C, corresponding to a free energy of activation, $\Delta G^{\ddagger}$, of 77 kJ/mol. The complete sequence-specific ^1H NMR assignments of the *cis*-Pro43 isoform and the Pro43 → Gly mutant in the calcium-loaded state have been obtained by using standard methods combined with comparisons to the previously assigned major (*trans*-Pro43) isoform. This has permitted detailed comparative analysis of ^1H NMR chemical shifts, backbone scalar coupling constants, and nuclear Overhauser effects. The minor isoform has a global fold that is identical with that of the major isoform. Structural changes imposed by cis–trans isomerization at Pro43 are highly localized to the linker loop (containing Pro43) that joins the two EF hands. The Pro43 → Gly mutant has a global fold that is identical with the wild-type protein, but does not exhibit conformational heterogeneity. Only very limited structural differences are observed between mutant and wild-type protein, and these are also highly localized to the linker loop. The ion-binding properties of the mutant, as determined by ^{43}Ca and ^{113}Cd NMR, are found to be very similar to the wild-type protein. These results provide crucial evidence that justifies the calculation of high-resolution three-dimensional structures of the Pro43Gly mutant, rather than of the conformationally heterogeneous wild-type protein.

Ca^{2+} ions play an important role in the regulation of a variety of cellular functions. This role is mediated by a class of highly homologous Ca^{2+}-binding proteins, the calmodulin superfamily (Kretsinger, 1987), that respond rapidly to transient increases in intracellular Ca^{2+} concentration. The mechanism of action for these proteins is believed to be based on marked conformational changes that are induced by Ca^{2+} binding. While knowledge about the details of the molecular structure has been obtained from the highly refined X-ray diffraction studies of parvalbumin (Moews & Kretsinger, 1975), calbindin D_{9k}[1] (Szebenyi & Moffat, 1986), calmodulin (Babu et al., 1988), and troponin C (Herzberg & James, 1988; Satyshur et al., 1988), in no case have crystals suitable for X-ray structure determination been obtained for *both* calcium-free and calcium-bound states of one of these proteins. Solution structure determination utilizing two-dimensional ^1H NMR[2] [reviewed in Wüthrich (1989)] is uniquely suited to overcome this problem.

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[1] Formerly the 9-kDa intestinal calcium binding protein (ICaBP).
[2] Abbreviations: 1D, one-dimensional; 2D, two-dimensional; COSY, correlated spectroscopy; 2QF-COSY, two quantum filtered COSY; DR-COSY, double relayed COSY; FID, free induction decay; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, 2D NOE spectroscopy; Quin2, 2-[[2-[bis(carboxymethyl)amino]-5-methylphenoxy]methyl]-6-methoxy-8-[bis(carboxymethyl)amino]quinoline; r-calbindin, the minor A form of bovine calbindin D_{9k} with an additional methionine at the N-terminus, produced by recombinant DNA methodology; P43G, mutant of r-calbindin with Pro43 substituted by glycine; P20G, mutant with Pro20 substituted by glycine; N21Δ, mutant with Asn21 deleted.

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