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Expression of the Affected Aγ Globin Gene Associated with Greek Nondeletion Hereditary Persistence of Fetal Hemoglobin

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The overexpressed Aγ globin gene in the Greek type of nondeletion hereditary persistence of fetal hemoglobin has a unique single-base substitution located at position −117 relative to the site of transcription initiation. This gene and its normal counterpart were transferred into cultured cell lines by using a retroviral vector. The only difference in expression between the transferred normal and mutant γ genes was observed in the human erythroleukemia cell line KMOE after exposure of the cells to cytosine arabinoside, a condition that resulted in an adult pattern of endogenous globin gene expression by the cells and was associated with increased expression of the mutant gene.

Molecular analyses of nondeletion forms of hereditary persistence of fetal hemoglobin (HPFH) have provided important information regarding specific DNA sequences that are involved in the developmental regulation of globin gene expression. HPFH is a heterogeneous set of clinically benign disorders with the phenotype of elevated γ globin chain production in adults. In a Greek form of nondeletion HPFH it is the fetal Aγ gene that is overexpressed. A base substitution has been reported 117 base pairs 5′ to the site of transcription initiation (position −117) of the affected Aγ globin gene in this disorder (6, 8). The position −117 HPFH mutation borders a known transcriptional regulatory element, the CCAAT box. The mutation changes the distal duplicated CCAAT box of the Aγ globin gene from TTGAC CAATA to TTAACCAATA. This change is the first known naturally occurring mutation at this site and is analogous to an in vitro-generated mutation found to increase expression of a mouse-human β globin hybrid gene in HeLa cells (15).

We report here studies of the expression of the mutant nondeletion HPFH Aγ gene and its normal counterpart upon transfer into a variety of tissue culture cell lines by means of the retroviral vector pZipNeoSV(X)1 (3) (hereafter called SVX).

The 3.3-kilobase (kb) *Hin*dIII fragment, containing either the normal or Greek HPFH Aγ globin gene isolated from plasmid subclones, was cloned (using *Bgl*II linkers) into the *Bam*HI site of the vector between the 5′ long terminal repeat and the neomycin resistance (*neo*) gene (see Fig. 4b). Transcription from viral and globin promoters is in opposite directions as indicated by arrows. SVX constructs containing either the normal Aγ globin gene or the Greek HPFH Aγ globin gene were transfected into the amphotropic viral packaging cell line ψ-AM (7), using calcium phosphate-mediated gene transfer. Isolation of ψ-AM cell clones containing the viral constructs and infection of 3T3 cells were done as previously described (7). Viral titers, assayed using 3T3 cells, typically ranged from 10^2 to 10^3 G418-resistant 3T3 colonies per ml of ψ-AM medium.

For infection of erythroleukemia cells, 10^5 cells were added to a near-confluent 100-mm plate culture of a ψ-AM virus producer cell clone in 10 ml of Dulbecco minimal essential medium containing 10% fetal calf serum and 8 μg of Polybrene (Aldrich) per ml. After 4 h of cocultivation at

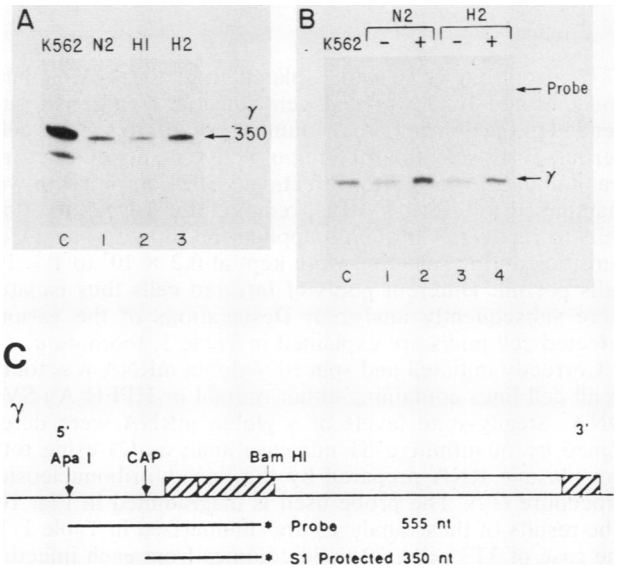

FIG. 1. Analysis of transferred Aγ globin gene expression in 3T3 and MEL cells. (A) S1 protection analyses of γ mRNA (γ 350) isolated from normal (N2) and HPFH (H1 and H2) Aγ-SVX-infected 3T3 cells. A 20-μg sample of total cytoplasmic RNA was used for each S1 analysis. Hemin-induced cytoplasmic K562 RNA was used as a positive control. (B) S1 protection analyses of γ mRNA isolated from normal (N2) and HPFH (H2) Aγ-SVX-infected MEL cells. A 20-μg sample of total cytoplasmic RNA from untreated (−) and DMSO-induced (+) MEL cells was used for each S1 analysis. K562 RNA was used as a positive control as for panel A. (C) The *Apa*I-*Bam*HI fragment from the γ cDNA-gene hybrid clone pγΔ IVS1 was used as the probe. This probe lacks the first intron and results in a fragment of 350 base pairs, protected by normally initiated and spliced γ mRNA, which extends from within the second exon to the site of transcription initiation.

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TABLE 1. Levels of γ globin mRNA in transfected cell lines[a]

Cell line	Normal Aγ cells				HPFH Aγ cells			
	Sample	Treatment[b]	γ mRNA copies per cell	Relative induction	Sample	Treatment[b]	γ mRNA copies per cell	Relative induction
3T3	N1	None	5		H1	None	11	
	N2	None	17		H2	None	18	
MEL	N1	None	16		H1	None	55	
		DMSO	28			DMSO	99	
	N2	None	48		H2	None	44	
		DMSO	106			DMSO	70	
KMOE[c]	N1	None	65	0.97	H1	None	21	6.00
		AraC	63			AraC	126	
	N2	None	47	0.34	H2	None	57	4.77
		AraC	16			AraC	272	
	N3	None	44	1.27	H3	None	59	3.42
		AraC	56			AraC	202	
	N4	None	54	3.31	H4	None	11	4.91
		AraC	179			AraC	54	
	N5	None	58	1.55	H5	None	68	1.12
		AraC	90			AraC	76	
					H6	None	18	3.33
						AraC	60	
					H7	None	47	6.72
						AraC	316	

[a] Values given are γ mRNA copies per cell based on densitometric tracings of autoradiographic films from S1 protection experiments (see Fig. 1 and 3). Cytoplasmic RNA from hemin-induced K562 cells calibrated against purified 10S γ mRNA was used as the quantitation standard. 3T3 and MEL values represent single S1 experiments. KMOE values are the average of two S1 analyses of a single induction experiment. Before averaging, KMOE γ values were normalized using actin levels as an internal reference. Relative induction is the ratio of araC-treated to untreated KMOE γ mRNA. 3T3, MEL, and KMOE cells labeled N1, N2, and N3 or H1, H2, and H4 are from independent infections using normal Aγ-SVX or HPFH Aγ-SVX producing ψ-AM clones, respectively.

[b] None, Untreated cells.

[c] KMOE cells labeled N4 and N5 are from independent infections using ψ-AM clone N1. Similarly, KMOE cell line H3 is from an independent infection using ψ-AM clone H1; H5 is from ψ-AM clone H4; H6 and H7 are from ψ-AM clone H2. Average values (mRNA copies per cell) for normal cells: untreated, 53.6; treated, 80.8. Average values (mRNA copies per cell) for HPFH cells: untreated, 40.1; treated, 158.0

37°C, erythroid cells were isolated from the ψ-AM plate, concentrated by low-speed centrifugation, and then suspended in the appropriate medium at 2×10^4 to 5×10^4 cells per ml. G418 selection (0.5 mg of active compound per ml) was initiated 2 days after infection. Selection pressure was maintained for 10 to 14 days, removed for 1 week to allow cells to recover, and then reapplied for 10 to 14 days. Cell densities during selection were kept at 0.2×10^5 to 1×10^5 cells per ml. Different pools of infected cells thus isolated were subsequently analyzed. Designations of the various infected cell pools are explained in Table 1, footnote *a*.

Correctly initiated and spliced γ globin mRNA was found in all cell lines containing either normal or HPFH Aγ-SVX DNA. Steady-state levels of γ globin mRNA were determined by quantitative S1 nuclease analysis (2) using total cytoplasmic RNA prepared by the vanadyl-ribonucleoside procedure (12). The probe used is diagrammed in Fig. 1C. The results of these analyses are summarized in Table 1. In the case of 3T3 cells, 20 to 30 colonies from each infection were pooled for RNA analysis. There was no significant difference between the mRNA levels derived from the normal and Greek HPFH Aγ globin genes in 3T3 cells (Fig. 1A, Table 1).

Expression of transferred genes in mouse erythroleukemia cells. Both the normal and the Greek HPFH Aγ globin genes produced higher levels of γ mRNA in MEL cells (16 to 55 copies of γ mRNA per cell) than in 3T3 cells (5 to 18 copies per cell). DNA blot-hybridization (16) analysis of the infected MEL cell pools indicated that there was approximately one copy of Aγ-SVX proviral DNA per cell (data not shown). MEL cells produce very low levels of endogenous globin unless induced to differentiate with compounds such as dimethyl sulfoxide (DMSO) (13). Four-day exposure to 1.8% DMSO of MEL cells containing the introduced γ genes resulted in approximately a twofold increase in γ mRNA (Fig. 1B). Spliced retroviral transcripts were also induced by DMSO (data not shown). DMSO induction of human γ

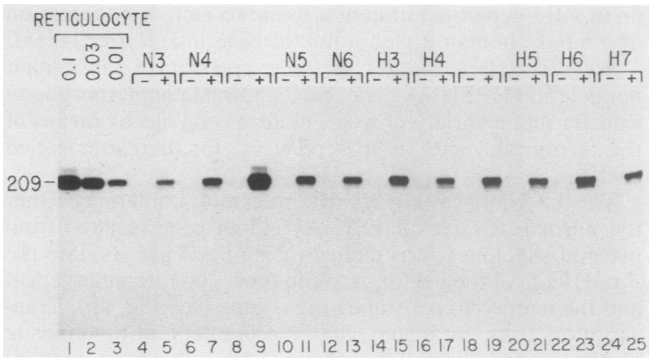

FIG. 2. Induction of endogenous β globin expression in KMOE cells by araC. Steady-state levels of β globin mRNA in uninduced (−) and araC-induced (+) KMOE cells were determined by quantitative S1 nuclease analysis. A 5′-end-labeled 1.9-kb *Bam*HI fragment isolated from the human β globin gene was used as a probe. This fragment extends from the *Bam*HI site within exon 2 to a position 5′ to the site of transcription initiation and results in the protection of a 209-base-pair fragment corresponding to the 5′ end of exon 2. A variety of cell lines are shown, including cell lines infected with the normal (N3 to N6) and the HPFH (H3 to H7) Aγ-SVX constructs as well as cell lines which had not been successfully infected (unlabeled) as determined by the lack of retroviral sequences in DNA blot-hybridization analyses (data not shown). Lanes 1 through 3 show dilutions of reticulocyte RNA; the number of micrograms of RNA used is shown above each lane.

globin genes in MEL cells has been previously reported (1, 4); however, lack of induction has also been reported (17, 18). Nevertheless, there was no apparent effect of the position −117 HPFH mutation on the level of γ mRNA in either uninduced or DMSO-induced MEL cells (Fig. 1B and Table 1). In contrast, Charnay and Henry (4) observed higher levels of γ mRNA expression from the −117 γ gene than from the normal γ gene in both uninduced and induced MEL cells after DNA-mediated gene transfer.

Transfer of genes into the human erythroleukemia cell line KMOE. The human erythroleukemia cell line KMOE-2/05 provides an interesting system for studying differences between β and γ globin gene regulation, since KMOE cells have the ability to selectively increase β globin levels after treatment with cytosine arabinoside (araC) (11). KMOE cells were treated with araC in the following manner. Cells were first grown in 5% fetal calf serum for 6 days, followed by 2 days in 15% fetal calf serum, and then diluted to 5×10^4 cells per ml. After 2 days, araC was added to 10^{-5} M, and RNA was prepared 4 days later.

We found that the levels of γ and β mRNA were very low and approximately equal in untreated cells, and while araC treatment consistently resulted in a substantial increase in endogenous β mRNA levels (Fig. 2), it did not change endogenous γ mRNA levels (Fig. 3, lanes 9 and 10). This finding is in agreement with earlier reports (11). The level of endogenous γ globin gene expression in KMOE cells is sufficiently low so as not to interfere with the analysis of the introduced Aγ genes.

Approximately one copy of Aγ-SVX DNA per cell was found in most KMOE cell pools as determined by densitometric scanning of autoradiograms from Southern blot analysis (Fig. 4). A 10-μg sample of high-molecular-weight DNA from KMOE cells was digested to completion with *Bam*HI and *Bgl*II and probed with the 3-kb *Stu*I fragment shown in Fig. 4 by the method of Meinkoth and Wahl (14). Although 10^5 KMOE cells were treated with 10^3 to 10^4 3T3-transforming virus particles, five of nine cell pools examined (N1, N3, N4, H5, and H7) contained one predominant junction fragment (indicated by * in Fig. 4), suggesting a single insertional event. This lack of pool complexity could be the result of a lower efficiency of infectivity of KMOE cells relative to 3T3 cells. Alternatively, single clonal cell populations may have overgrown and dominated in these pools during growth in G418. Some cell pools (H2, H4) were found to contain many insertion sites as demonstrated by the lack of detectable junction fragments (Fig. 4).

Expression of transferred genes in KMOE cells. KMOE cells containing the normal Aγ provirus had γ globin mRNA levels of approximately 50 copies per cell, ranging from 44 to 65 copies per cell (Fig. 3, Table 1). This level is assumed to be nearly entirely due to the introduced Aγ globin gene since the γ globin mRNA level in uninfected cells is one to five copies per cell (Fig. 3; data not shown). KMOE cells containing the HPFH Aγ globin gene had an average of 40 copies per cell, with a range of 11 to 68 copies per cell (Table 1). The difference between the mRNA levels of the normal Aγ gene (50 copies per cell) and the HPFH Aγ gene (40 copies per cell) is not statistically significant. Therefore, no difference is apparent between the steady-state mRNA levels of the normal and HPFH Aγ genes in untreated KMOE cells.

AraC treatment increased γ globin mRNA levels 3.3- to 6.7-fold in six of the seven KMOE cell lines carrying the HPFH Aγ globin gene (Table 1 and Fig. 5). In contrast, only one of five KMOE cell lines carrying the transferred normal

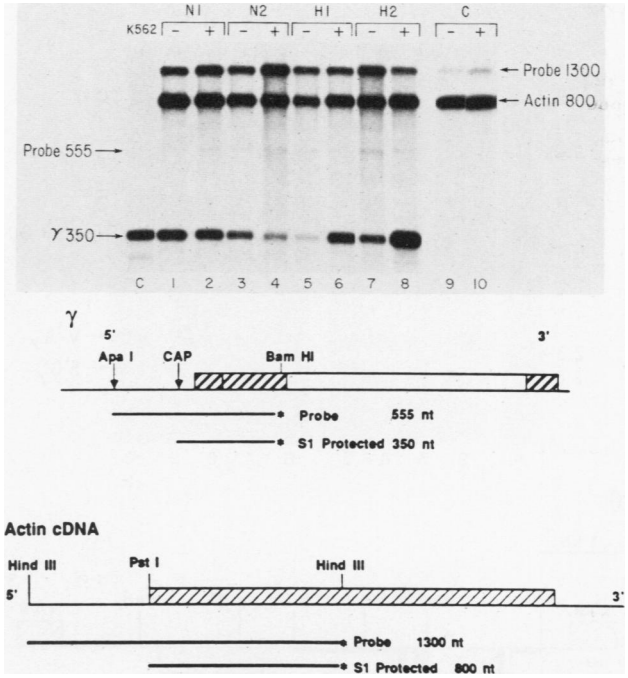

FIG. 3. Analysis of γ globin gene expression in Aγ-SVX-infected KMOE cells. (Top) S1 protection analysis of γ (γ 350) and actin (actin 800) mRNA isolated from normal (N1 and N2) and HPFH (H1 and H2) Aγ-SVX-infected KMOE cells and uninfected KMOE control (c). A 20-μg sample of total cytoplasmic RNA from untreated (−) and araC-induced (+) KMOE cells was used for each S1 analysis. The K562 lane represents approximately 100 γ mRNA copies per cell per 20 μg of sample cytoplasmic RNA (see Table 1). Shown below are the γ and actin probes prepared from the γ cDNA-gene hybrid clone pγΔIVS1 and from pHF1 (9a), respectively.

Aγ gene increased γ mRNA levels appreciably (N4: 3.3-fold) (Table 1 and Fig. 5). AraC induction of γ mRNA levels in all other KMOE lines carrying the normal γ gene was 1.6-fold or less. The anomalous behavior of KMOE cell lines H5 (did not induce) and N4 (did induce) may be attributable to chromosomal sequences flanking the inserted Aγ-SVX provirus. Southern blot analysis of H5 and N4 indicated that both contain predominantly one insertion site (Fig. 4). Endogenous β globin transcripts were inducible in all cell lines tested (Fig. 2). Thus, the araC induction pattern of the transferred HPFH Aγ globin gene in KMOE cells is similar to that of the endogenous β globin gene, while the induction pattern of the transferred normal Aγ gene is similar to that of the endogenous γ globin genes.

Possible role of the −117 mutation in control of gene expression. Our analyses of transferred normal and Greek HPFH Aγ globin genes in a number of cell lines demonstrated only one situation in which the transferred HPFH Aγ gene was expressed differently from the normal gene. This difference was in the human erythroleukemic cell line KMOE, in which the transferred HPFH Aγ gene was responsive to araC while the transferred normal Aγ gene was not (Fig. 3 and Table 1). In most cell lines or pools, a three- to fourfold "induction" of the HPFH Aγ gene relative to the transferred normal Aγ was observed in araC-treated KMOE cells (Table 1). This level is much smaller than the 20- to 40-fold overexpression, compared to normal, of fetal hemoglobin observed in vivo in adult individuals with Greek

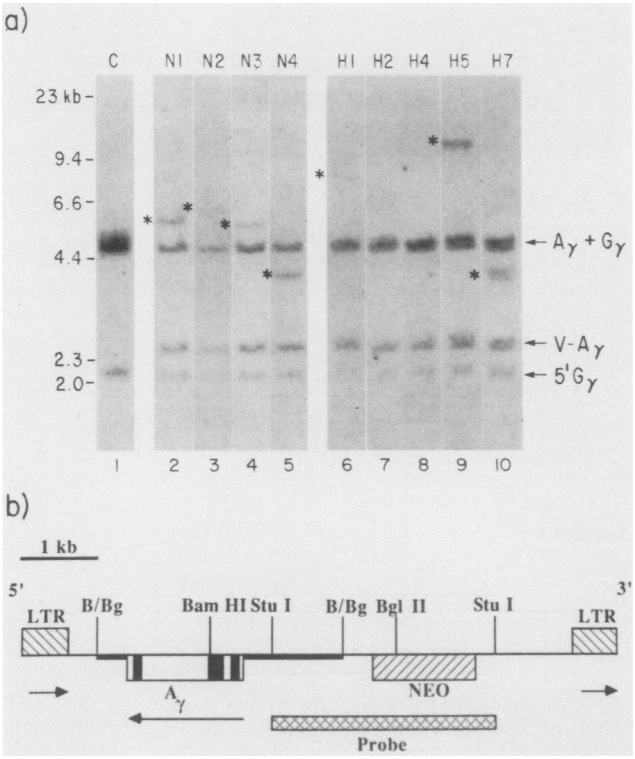

FIG. 4. Southern blot analysis of uninfected KMOE control (c) and KMOE cell lines infected with normal (N1 through N4) or HPFH (H1, H2, H4, H5, H7) Aγ-SVX virus. Bands observed due to endogenous γ globin genes are the 2.1-kb *Bgl*II-*Bam*HI fragment from the Gγ gene (5′ Gγ) and the 4.9-kb *Bam*HI fragment containing the 3′ Gγ and 5′ Aγ sequences (Aγ + Gγ). Infected KMOE cells also show a band corresponding to the internal 2.5-kb *Bam*HI-*Bgl*II fragment of the Aγ-SVX provirus (V-Aγ) plus additional bands (*) corresponding to junction fragments containing 3′ sequences of the SVX provirus and variable lengths of contiguous chromosomal DNA.

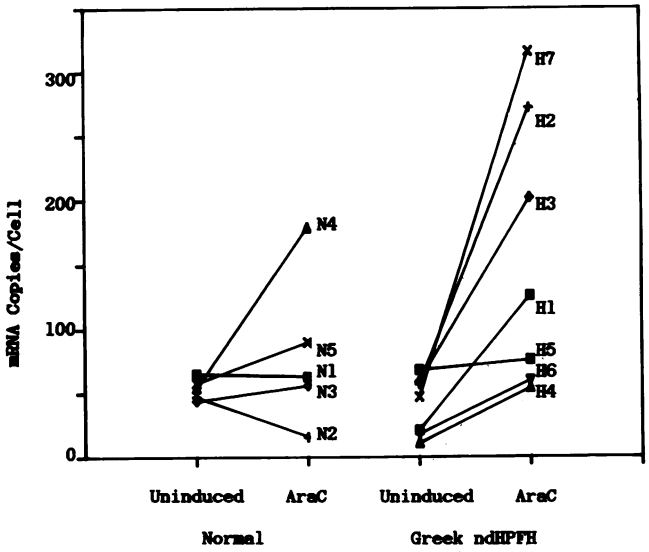

FIG. 5. Induction of γ globin mRNA levels by treatment with araC in normal and HPFH Aγ-SVX-infected KMOE cells. γ mRNA levels in infected cells are given in copies per cell and are from values listed in Table 1.

HPFH. The discrepancy between the in vivo and the tissue culture results may be due to the limited ability of KMOE cells to express globin genes. It is also possible that sequences normally flanking the Aγ gene which were not included in the Aγ-SVX construct are necessary for obtaining the full magnitude of HPFH γ gene overexpression.

The position −117 mutation, located near the distal CCAAT box of the affected Aγ gene, is the only HPFH-specific mutation associated with the transferred Greek HPFH Aγ globin gene (8; unpublished data), indicating that this mutation is very likely to be responsible for the increased γ mRNA levels in araC-induced KMOE cells. CCAAT-box binding proteins for the mouse γ globin gene as well as for viral thymidine kinase genes have been described (5, 9, 10). If the position −117 mutation affected the binding of a general CCAAT-box binding protein, differences in expression levels between normal and HPFH Aγ globin genes might be expected in all cell systems studied. Collins et al. have found that the chloramphenicol acetyltransferase (CAT) gene driven by the position −117 HPFH Aγ promoter overexpresses threefold in mouse fibroblasts and MEL cells relative to CAT genes driven by the normal γ promoter (F. S. Collins, D. M. Bodine, W. K. Lockwood, J. L. Cole, L. Mickley, and T. Ley, Clin. Res. **34**:454a, 1986). However, we found no clear effect of this mutation on the expression of the Aγ globin gene in 3T3 cells, MEL cells, DMSO-treated MEL cells, and untreated KMOE cells (Table 1). The experiments of Collins et al. differ from ours in two important respects. One is that γ-CAT hybrid genes were studied in a transient expression system, whereas the Aγ-SVX genes were stably integrated into the genome. The other difference is that our experiments utilized the entire γ globin gene, including 5′ and 3′ flanking sequences, whereas the γ-CAT experiments utilized only a short segment of the γ gene promoter. In nonerythroid or mouse cell lines, the rate-limiting steps of γ gene transcription may involve DNA sequences that are not present in the γ-CAT constructs, but are present on the Aγ-SVX constructs. Although the position −117 mutation may influence a general mechanism of transcriptional control, this effect may only be observed when other rate-limiting steps of transcription are overcome. If the position −117 mutation does influence a general mechanism of transcriptional control, as suggested by the γ-CAT hybrid gene experiments, the results presented here may indicate that this mechanism of control is rate limiting only in a human adult erythroid cellular environment.

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