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A Fetal Globin Gene Mutation in $^{A}\gamma$ Nondeletion Hereditary Persistence of Fetal Hemoglobin Increases Promoter Strength in a Nonerythroid Cell

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Single base substitutions have been identified in the promoter regions of $^{A}\gamma$-globin genes from individuals with certain types of nondeletion $^{A}\gamma$ hereditary persistence of fetal hemoglobin (HPFH). The presence of these mutations is closely associated with the $^{A}\gamma$ HPFH phenotype, but proof that they are the nondeletion HPFH determinants is lacking. To test directly whether these base substitutions can result in an increase in $^{A}\gamma$-globin gene transcription, we studied cosmid clones containing the $^{G}\gamma$- through β-globin gene regions from individuals with Greek-type (G-to-A base substitution at −117) and Chinese-type (C-to-T base substitution at −196) $^{A}\gamma$ HPFH in a transient expression assay. When tested as part of a cosmid clone, the Greek HPFH $^{A}\gamma$-globin gene consistently produced about 1.4 times as much RNA as the wild-type $^{A}\gamma$-globin gene when standardized against RNA transcribed from the $^{G}\gamma$ genes in *cis*. The relative strengths of the normal and HPFH $^{A}\gamma$-globin gene promoters were also compared in transient expression assays with plasmids containing the $^{A}\gamma$-globin genes. Pseudo-wild-type $^{A}\gamma$-globin genes containing a short, transcriptionally neutral deletion were used so that two $^{A}\gamma$-globin genes that differed in their promoter sequences could be compared in the same transfection. The plasmid transient expression results indicated a 1.3- to 1.4-fold increase in steady-state RNA levels from the Greek-type $^{A}\gamma$ HPFH promoter compared with the wild-type $^{A}\gamma$ promoter, while no difference was documented between the Chinese-type $^{A}\gamma$ HPFH promoter and the wild-type $^{A}\gamma$ promoter.

During normal development, expression within the human β-globin gene cluster undergoes a sequential change, switching from the embryonic (ε) to the fetal ($^{G}\gamma$, $^{A}\gamma$) to the adult (δ, β) genes (22, 23). The mechanisms underlying these switches remain to be elucidated. Analysis of naturally occurring mutants with altered developmental expression may provide information on the switching process. Two such classes of mutations have been described, termed deletion and nondeletion hereditary persistence of fetal hemoglobin (HPFH), that result in continued expression of the fetal γ-globin genes into adulthood (34). In contrast to the deletion HPFH forms, the nondeletion HPFH phenotypes have been shown to be associated with single base changes in the immediate 5′-flanking sequences of the fetal γ-globin genes (9, 10, 14–16). The remaining structural sequences appear to be identical to wild type (28).

In normal adults, the circulating level of fetal hemoglobin is less than 1% of the total (22) with a $^{G}\gamma$-to-$^{A}\gamma$ polypeptide ratio of about 2/3 (27). In the Greek- and Chinese-type $^{A}\gamma$ nondeletion HPFH, the level of fetal hemoglobin is 10- to 20-fold higher than normal and the fetal globin polypeptide is predominantly $^{A}\gamma$ (22). Although concordance studies have shown a correlation between the presence of these single base substitutions and the nondeletion HPFH phenotype (8, 32), no in vitro system exists that establishes a causative relationship between the mutations and the phenotype of $^{A}\gamma$ nondeletion HPFH. Nucleated erythroblasts, in which globin genes are normally transcribed and thus in which the nondeletion HPFH phenotype is generated, are largely confined to the bone marrow and at present are not easily amenable to gene transfer studies. For these reasons, we initially studied nondeletion HPFH promoter function by using a transient expression assay in a nonerythroid cell line.

Transient expression experiments have previously been used to analyze differences in promoter activities. Two examples of single base mutations in the human β-globin gene promoter that result in β-thalassemia owing to decreased RNA levels have been described. A β-globin gene containing a point mutation at position −87 was shown to transcribe at only 10% of normal levels (31), while a mutation in the TATA region (position −29) also resulted in lower levels of transcription (26) in transient expression assays in nonerythroid cells. Myers and co-workers (25) systematically mutagenized the mouse β^{maj}-globin gene promoter and assayed the effects on relative transcription levels by transient expression in HeLa cells. Similar expression assays have also been done to analyze the differential expression of γ-globin gene promoter deletions relative to cotransfected β-globin genes in nonerythroid cells (1).

We investigated the mechanism of nondeletion $^{A}\gamma$ HPFH by testing two different nondeletion $^{A}\gamma$ HPFH mutant alleles, Greek type with a G-to-A base substitution at −117 (−117 HPFH) and Chinese type with a C-to-T base substitution at −196 (−196 HPFH), in a transient expression assay utilizing 293 cells. 293 cells are human embryonic kidney cells transformed with adenovirus type 5 DNA (17). In transient expression assays, these cells are able to transcribe the human β-globin gene without an additional viral enhancer element in *cis* (30). In addition, when a simian virus 40 enhancer was linked to a β-globin gene, no augmentation in transcription was observed (30). We therefore reasoned that by using 293 cells, differences in γ-globin promoter strength might be detected, without an overriding or interfering influence of a linked enhancer. Although 293 cells are nonerythroid, fundamental functions of promoter activity may be revealed by transcriptional assays in these cells. Our

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studies showed that the −117 HPFH promoter is 1.4 times stronger than the wild-type promoter, while no consistent difference was observed for the −196 HPFH gene. Although the 1.4-fold increase seen in 293 cells with the Greek-type $^{A}\gamma$-globin gene is too small to fully account for the steady-state level of $^{A}\gamma$ polypeptide in vivo, the observed promoter strength difference is attributable solely to the −117 G-to-A transition. This suggests that this genotypic difference leads to the phenotypic difference observed in vivo.

MATERIALS AND METHODS

Cosmid and plasmid cloning. Cosmid clones cCH1, cCH2, and cES2 have been described previously (14, 15). They consist of the 39-kilobase-pair (kb) *Kpn*I fragment from the human β-globin locus cloned into a derivative of the cosmid vector pHC79 (19) which accepts *Kpn*I target fragments (15). Plasmid DNAs were generated by subcloning the 3.3-kb *Hin*dIII fragment encompassing the $^{A}\gamma$-globin gene into pUC19 (35). Plasmid nomenclature is as follows: p$^{A}\gamma$wt, p$^{A}\gamma$117, and p$^{A}\gamma$196 refer to the wild-type, −117 HPFH, and −196 HPFH genes, respectively.

Transient expression. 293 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. DNA was introduced into the cells by standard calcium phosphate precipitation methods (33). In all transfections, the DNA was added to cells at 40% confluency. After 12 to 16 h, the medium was changed, and RNA was harvested after 48 h. In the cosmid transient expression experiments, 10 μg of DNA was used and RNA was prepared by lysing the cells in the tissue culture dish with guanidine isothiocyanate (6) followed by an overnight 147,000 × *g* spin through a 5.7 M CsCl cushion. The RNA pellet was suspended, digested with proteinase K (1 mg/ml), and stored under ethanol until ready for use. For the transient expression assays with plasmid DNA, RNA was prepared by guanidine monothiocyanate lysis and selective LiCl precipitation as described previously (4).

Primer extension. Primer extension was performed by the method of Eisenberg et al. (12). Oligonucleotides specific for the RNA to be analyzed were end labeled with [γ-^{32}P]ATP (3,000 Ci/mmol; New England Nuclear Corp., Boston, Mass.) and T4 polynucleotide kinase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) (24). The sequence of the γ-specific oligonucleotide is 5′-CGTCTGGACTAG GAGCTTATTGA-3′ and that of the β-specific oligonucleotide is 5′-CCACAGGGCAGTAACGGCAGA-3′. The labeled oligonucleotides were recovered by purification on a 12% polyacrylamide gel and eluted overnight in 0.5 M ammonium acetate. The labeled oligonucleotides were annealed to total RNA prepared from each transfection and extended with avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.). Excess RNA was removed by RNase A digestion, and the extended DNA products were fractionated on an 8% polyacrylamide–7 M urea gel and compared with a DNA sequence ladder for size determination.

Site-directed mutagenesis. The method of Zoller and Smith (36) was used to introduce a 6-base-pair deletion in the 5′ nontranslated region of the $^{A}\gamma$-globin gene. Bases +21 to +26 relative to the cap site were removed by using an oligonucleotide with the sequence 5′-CTTATTGATA GACGTTCC-3′. The mutagenesis was done on the *Stu*I (−385)-to-*Bst*EII (+155) fragment of the $^{A}\gamma$-globin gene cloned into M13mp19 (35). After sequence confirmation, the mutagenized region was reintroduced as the *Apa*I (−206)-to-*Bgl*I (+94) fragment into plasmids containing the 3.3-kb *Hin*dIII fragment encompassing the $^{A}\gamma$ gene. The mutagenized γ genes are denoted throughout with an asterisk (*).

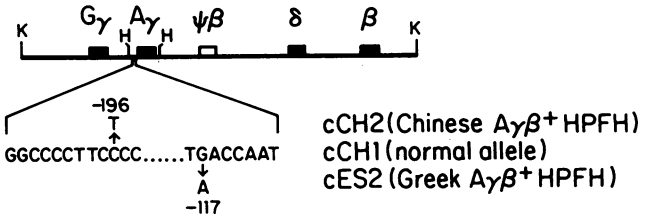

FIG. 1. Cosmid clones from the β-globin locus of two individuals with nondeletion $^{A}\gamma$ HPFH. One individual had heterozygous Chinese $^{A}\gamma$ nondeletion HPFH (CH), while the other had heterozygous Greek $^{A}\gamma$ nondeletion HPFH (ES). The sequence changes observed to be associated with the nondeletion HPFH phenotype are indicated. Each $^{A}\gamma$ gene was also subcloned as the 3.3-kb *Hin*dIII fragment into pUC19. K, *Kpn*I; H, *Hin*dIII.

RNase protection. RNase protection was performed as described by Bender et al. (submitted for publication). The RNA probe used to measure γ and γ* RNA was generated by cloning the 340-base-pair *Ava*II fragment from $^{A}\gamma$ gene positions −160 to +182 into pVZ1, a derivative of Bluescript-plus (Stratagene, Inc., San Diego, Calif.) which contains a larger polycloning segment (S. Henikoff, unpublished data). The RNA probe used to measure $^{A}\gamma$ and $^{G}\gamma$ RNA was generated from the *Pst*I (+1456)-to-*Sph*I (+1672) fragment from the 3′ end of the $^{A}\gamma$ gene which spans the poly(A) addition site. This is the area of greatest divergence between $^{A}\gamma$ and $^{G}\gamma$ RNA where four consecutive base differences occur three nucleotides 3′ to the termination codon. Preliminary hybridization tests with human fetal liver RNA and K562 cellular RNA demonstrated the ability of the probe to discriminate between $^{G}\gamma$ and $^{A}\gamma$ RNA sequences (data not shown) (see Fig. 3). Both the 5′ $^{A}\gamma$ and 3′ $^{A}\gamma$ RNA probes were synthesized with T3 RNA polymerase (Bethesda Research Laboratories). Protected fragments were fractionated on a 5% polyacrylamide–7 M urea gel and cut out, and disintegrations per minute were quantitated by scintillation counting.

RNA quantitation. Moles of RNA were calculated from the number of disintegrations per minute recovered after counting (assuming a counting efficiency of 100%), the specific activity of the RNA probe, and the number of moles of incorporated [α-^{32}P]UTP per protected fragment.

RESULTS

Transient expression of cosmid DNA. A schematic representation of the three cosmid clones used for transient expression experiments in 293 cells is indicated in Fig. 1. Cosmid cCH1 contains the 39-kb *Kpn*I fragment of the β-globin locus isolated from an individual with Chinese $^{A}\gamma$ HPFH. This cosmid clone contains normal γ- and γ-globin genes. The 39-kb *Kpn*I fragment from the other allele of this individual is cloned in the cosmid cCH2. DNA sequencing of the $^{A}\gamma$ gene from this cosmid indicated a C-to-T transition at position −196 (14). A cosmid clone containing the 39-kb *Kpn*I fragment from an individual with Greek $^{A}\gamma$ HPFH was also used. This cosmid, cES2, contains an $^{A}\gamma$ gene with the −117 G-to-A mutation (15).

To test whether the single base substitutions in the nondeletion HPFH $^{A}\gamma$-globin gene promoters have an influence

on promoter function in a transient expression assay, we introduced the three cosmids as supercoiled molecules into 293 cells by calcium phosphate-mediated DNA transfection (33). Total RNA was isolated, and the presence of correctly initiated γ- and β-globin RNA was assayed by primer extension. The γ oligonucleotide will anneal with its most 5′ base at positions +51 of γ-globin RNA and will yield a γ-globin RNA-specific primer extension product of 51 nucleotides. The γ oligonucleotide will anneal to both $^{A}\gamma$ and $^{G}\gamma$ RNA since they are identical throughout this region. The β oligonucleotide will anneal specifically to β-globin RNA with its most 5′ base at position 98.

Analysis of γ- and β-globin RNA transcribed during transient expression of cosmid DNA. Figure 2 shows the reverse transcriptase extension products of RNA from each cosmid from a transient expression experiment. The extension products with the β-specific primer correspond to the expected length of 98 bases. K562 cells express high levels of fetal hemoglobin (3) and therefore serve as a positive control for γ-globin RNA. Primer extension products having the expected length of 51 nucleotides are seen with K562 RNA. However, the primer extension signal from the γ-specific oligonucleotide shows five distinct bands. This is not an artifact of our transient expression system since not only are the same five bands visible from primer extension of K562 cellular RNA, but of fetal liver RNA (see Fig. 4) and human bone marrow RNA (not shown) as well. This characteristic pattern of γ-globin RNA 5′ termini could be the result of microheterogeneity in transcription initiation or inability of the reverse transcriptase to accurately read the 5′ end of the message. This analysis demonstrates that the γ-globin genes on each cosmid transcribe RNA which is correctly initiated compared with a K562 RNA standard. It is also clear that these are the major transcripts from the γ- and β-globin genes, and no aberrantly initiated transcripts are detected. Differences in signal strength between the lanes in Fig. 2 reflect differences in RNA yields which arise from minor variations in transfection efficiency. Such differences did not affect the quantitative results since the essential data were ratio comparisons of steady-state RNA levels from separate transfections (see below).

To assess potential differences in promoter activity between the wild-type and nondeletion HPFH $^{A}\gamma$-globin genes, we compared the amounts of RNA transcribed from the γ-globin genes on each cosmid. β-Globin RNA transcribed from the β gene on each cosmid was used as a reference. RNA probes were used to quantitate the γ and β RNA levels as described in Materials and Methods with the following assumption. Since the γ- and β-globin gene structural sequences are the same in each cosmid (13–15) all the γ and all the β transcripts will be identical and the stability of the RNA transcribed from each cosmid will be the same. Therefore, differences in accumulated steady-state levels of a given RNA should reflect differences in transcription initiation rates or promoter strength. However, measurements of the γ-to-β molar RNA ratios indicated no increase in total γ RNA from the cosmids containing the nondeletion $^{A}\gamma$ HPFH alleles (data not shown). A ratio of γ-to-β RNA of approximately 2 was observed from each transient expression, regardless of the cosmid used. This could be explained if there was either little or no increase in $^{A}\gamma$-globin gene transcription from the HPFH $^{A}\gamma$ genes or if the total γ-specific transcription was held constant by some mechanism. To measure $^{A}\gamma$ RNA independently of $^{G}\gamma$ RNA, we designed an RNA probe which discriminates between the two. This probe allowed us to compare $^{A}\gamma$-globin RNA

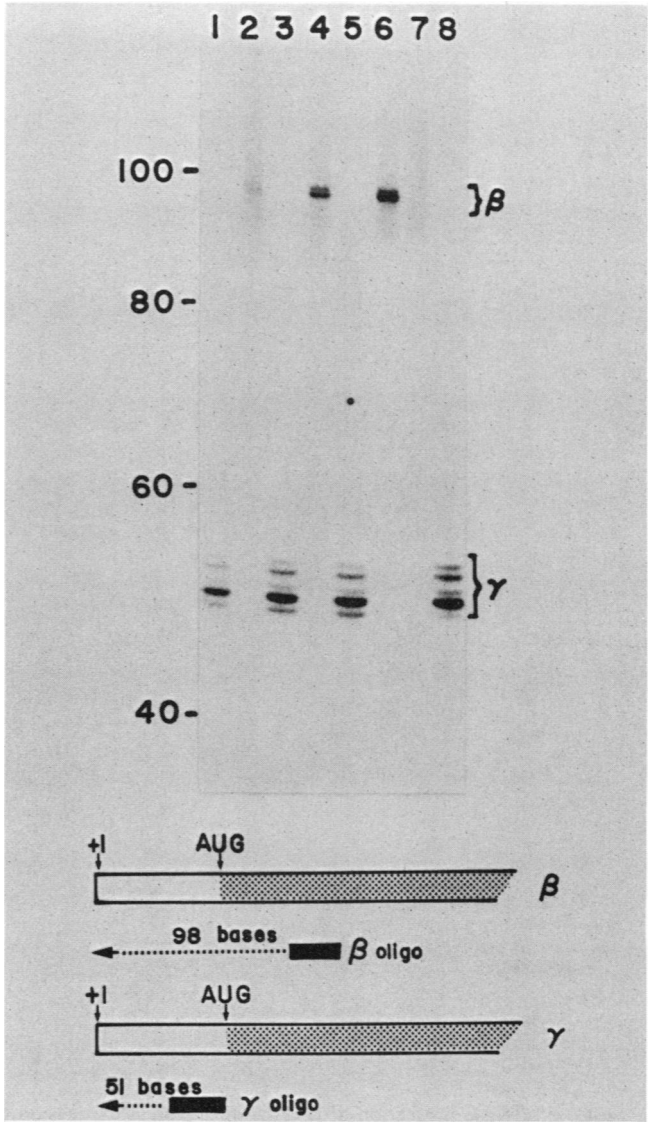

FIG. 2. Primer extension analysis of RNA isolated from 293 cells after transient expression of cosmid DNA. Shown are extension products of RNA from cES2 (lanes 1 and 2), cCH1 (lanes 3 and 4), and cCH2 (lanes 5 and 6). A 60-μg portion of RNA was used for extension with the β-oligonucleotide primer, while 30 μg of RNA was used for extension with the γ-oligonucleotide primer. Lane 7 contains extension products of no RNA, while lane 8 contains extension products from 5 μg of K562 cellular RNA. Lanes 1, 3, 5, and 8 are RNA samples extended with the γ-specific oligonucleotide. Lanes 2, 4, 6, and 7 are RNA samples extended with the β-specific oligonucleotide. The γ RNAs should yield an extension product of 51 nucleotides, while the β RNAs should yield an extension product of 98 nucleotides as indicated in the diagram. Numbers on left show size in nucleotides.

levels transcribed from the different promoters on each cosmid, using RNA derived from the linked $^{G}\gamma$ gene as a reference.

Independent quantitation of $^{A}\gamma$- and $^{G}\gamma$-globin RNA. Figure 3 shows the RNase-protected fragments from the 3′ $^{A}\gamma$ probe after annealing with RNA from the cosmid transfections. The expected $^{A}\gamma$ and $^{G}\gamma$ protection patterns are observed from K562 cellular RNA, and the specificity of the probe is demonstrated by the protected $^{A}\gamma$ signal from the RNA sample containing only $^{A}\gamma$ RNA. The $^{A}\gamma$-specific RNA

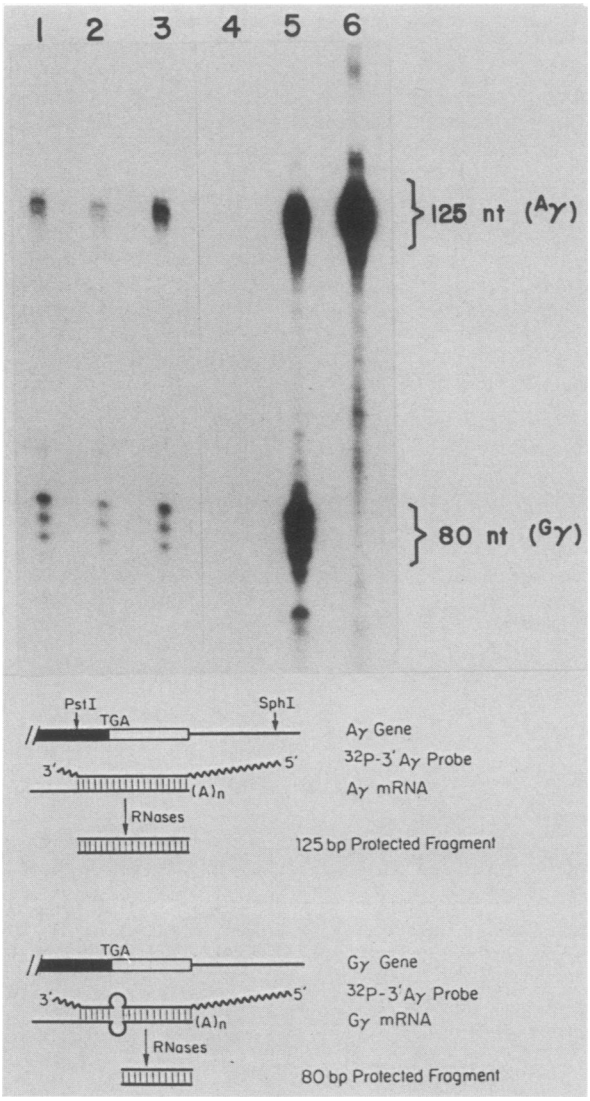

FIG. 3. RNase protection of RNA from transient expression of cosmid DNA in 293 cells, using the 3' $^{A}\gamma$ probe to measure $^{A}\gamma$ and $^{G}\gamma$ RNA. Diagrams indicating the 3' $^{A}\gamma$ probe and expected RNase-protected fragments from either $^{A}\gamma$ or $^{G}\gamma$ RNA are shown. Lanes: 1, 14 μg of RNA from cCH1 transfection; 2, 20 μg of RNA from cCH2 transfection; 3, 10 μg of RNA from cES2 transfection; 4, 6 μg of 293 cellular RNA; 5, 2 μg of K562 cellular RNA; 6, 4.5 μg of RNA from transfection of a plasmid containing only an $^{A}\gamma$ gene. nt, Nucleotides.

was generated by transient transfection in 293 cells of a plasmid containing an intact $^{A}\gamma$-globin gene. Both $^{A}\gamma$ and $^{G}\gamma$ RNA protected fragments are visible in the lanes from the cosmid transfection RNA. Table 1 shows the quantitated levels of $^{A}\gamma$ and $^{G}\gamma$ RNA and the molar ratios of $^{A}\gamma$ RNA to $^{G}\gamma$ RNA from each cosmid transfection. In three separate transfection experiments, cosmid cES2 (which contains the $^{A}\gamma$ gene with the −117 base substitution and a wild-type $^{G}\gamma$ gene) had a higher $^{A}\gamma$ RNA-to-$^{G}\gamma$ RNA ratio than cosmid cCH1 (which contains wild-type $^{A}\gamma$ and $^{G}\gamma$ genes). The difference in steady-state RNA levels was 1.3- to 1.5-fold. Cosmid cCH2 (which contains the $^{A}\gamma$ gene with the −196 base substitution and a wild-type $^{G}\gamma$ gene) had a $^{A}\gamma$ RNA-to-$^{G}\gamma$ RNA ratio similar to that of cosmid cCH1. However, the difference in $^{A}\gamma$ RNA levels between the −196 HPFH gene and the wild-type gene measured from repetitive cosmid transfections varied from 1.0- to 1.3-fold.

Transcriptional marking of γ-globin gene. In the transient expression experiments with the cosmid DNA, the $^{A}\gamma$ genes of interest and the genes used for reference were present on the same 45-kb circular DNA molecule. An abnormally expressed HPFH or reference gene arising from transcription interference or polarity mechanisms could potentially bias the calculated ratios. To see whether the relative promoter activities observed with the cosmid transfection would be maintained on smaller DNA fragments, we performed transient expression assays in 293 cells on plasmid forms of the wild-type and nondeletion HPFH $^{A}\gamma$ genes. Separate plasmid constructs containing the $^{A}\gamma$ 3.3-kb *Hin*dIII frament (Fig. 1) were generated with the wild-type promoter and the nondeletion HPFH promoter mutations. The plasmids were identical except for the presence of the single base substitutions that are associated with the nondeletion HPFH phenotype. In addition, a second set of plasmids was constructed with the three promoters adjacent to a pseudo-wild-type or marked $^{A}\gamma$ gene which has a 6-base-pair deletion in its 5' nontranslated region. RNA transcribed from these two reporter genes, the normal and marked $^{A}\gamma$ genes, in the same cell could be distinguished by primer extension or RNA protection assays. The site of the deletion did not interfere with transcription as demonstrated by introducing an intact $^{A}\gamma$-globin gene and marked $^{A}\gamma$-globin gene into 293 cells for transient expression. The presence of correctly initiated γ RNA was assayed by primer extension with the γ-globin-specific oligonucleotide. Figure 4 shows the primer extension results of RNA from the wild-type gene with and without the 5' transcriptional mark. No difference in transcription initiation was observed between the two genes except that the marked gene gave rise to RNA that was 6 bases shorter. In addition, the marked RNA was translated. Mouse erythroleukemia cells transfected with the marked $^{A}\gamma$ gene produce $^{A}\gamma$-globin chains which can be detected by an immunofluorescence assay specific for human γ chains (T. Enver, M. Rixon, and T. Papayannopoulou, unpublished observation).

Transient expression of plasmid DNA. Using the normal and marked $^{A}\gamma$-globin structural sequences as reporter genes, we made a direct comparison of steady-state RNA levels transcribed from the wild-type and nondeletion HPFH $^{A}\gamma$-globin promoters in the same transient expression assay. A 1-to-1 molar ratio of input plasmid DNA, with 10 μg of each, suggested that the −117 HPFH promoter transcribed between 1.5- and 2.0-fold more RNA than the wild-type $^{A}\gamma$ gene, whereas results with the −196 HPFH promoter were more ambiguous (data not shown). To avoid potential problems owing to DNA toxicity effects at this level of input plasmid DNA or possible effects on the final steady-state RNA levels arising from the two reporter genes which differ slightly in sequence and to see whether the promoter strength differences previously observed by transfection of

TABLE 1. Steady-state $^{A}\gamma$- and $^{G}\gamma$-globin RNA levels in 293 cells transfected with cosmid DNA

Cosmid	$^{A}\gamma$ RNA (10^{-17} mol)[a]	$^{G}\gamma$ RNA (10^{-17} mol)[a]	Molar RNA ratio ($^{A}\gamma/^{G}\gamma$)
cCH1	1.39	1.54	0.90
cCH2	0.76	0.84	0.90
cES2	1.88	1.44	1.30

[a] RNA calculated from counts per minute protected.

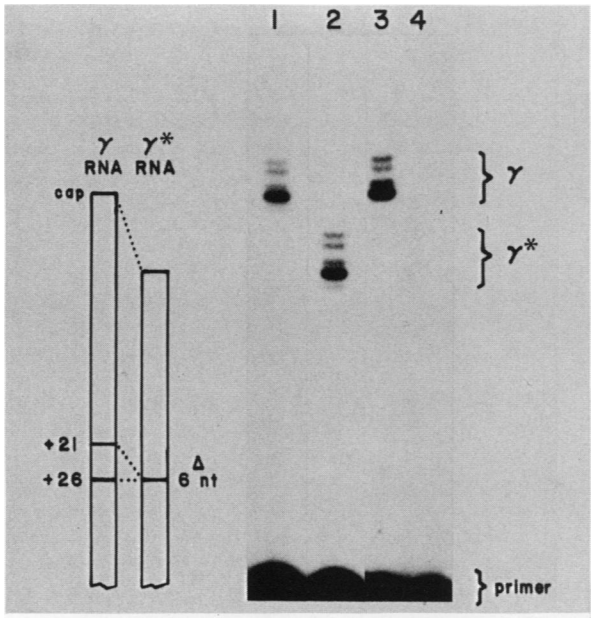

FIG. 4. Primer extension of the γ-specific oligonucleotide on full-length and 6-base deletion-marked $^{A}\gamma$-globin RNA. RNA was generated by transient expression in 293 cells of plasmids containing full-length and marked $^{A}\gamma$-globin genes. Lanes: 1, 17 μg of total cellular RNA containing full-length $^{A}\gamma$-globin RNA; 2, 17 μg of total cellular RNA containing marked $^{A}\gamma$-globin RNA; 3, 300 ng of human fetal liver RNA; 4, no RNA control. A diagram indicating the difference in the RNAs is shown on the left. nt, Nucleotides.

the cosmids would be maintained even at low DNA concentrations with a reference gene in *trans*, we compared transient expression over a wide range of input plasmid DNA ratios. A saturation assay and self-comparison experiment were used to validate the suitability of this cotransfection strategy. First, a saturation assay was performed to determine the DNA toxicity limit for calcium phosphate-mediated transfection of 293 cells. A linear increase in γ-globin-specific RNA was detected when up to 15 μg of input plasmid DNA was used (data not shown). For this reason, approximately 15 μg of total plasmid DNA per transfection was used in the comparison assays to give a wide range as possible in molar DNA ratios. Next, self-comparisons were done in which the wild-type promoter (or a nondeletion HPFH promoter) upstream of the marked and unmarked $^{A}\gamma$-globin genes was tested in a cotransfection. The amount of each plasmid used varied from 0.25 to 15 μg, which gave input DNA ratio limits of 1/60 and 60/1. The data shown in Table 2 and Fig. 6A show that neither promoter competed with itself when upstream of the two reporter genes.

−117 HPFH promoter comparison. For the direct comparison, the wild-type promoter linked to a marked $^{A}\gamma$ gene was cotransfected with the −117 HPFH promoter linked to an unmarked $^{A}\gamma$ gene. The reciprocal combination of constructs was also tested to control for any possible differences in the synthesis or analysis of γ* RNA. The amounts of unmarked and marked γ-globin RNA produced during the transient transfection were determined by RNase protection. Figure 5 shows the RNA protection results of one of the transient expression comparison assays. In this case, an unmarked gene with the −117 HPFH promoter is compared with a marked gene with a wild-type promoter. The signal from the −117 HPFH promoter increased from lane 3 to 13 and the signal from the wild-type promoter decreased as the ratio of input plasmid DNA was varied while the total amount of transfected DNA remained constant. The quantitation of the protected unmarked and marked RNAs from the self-comparisons of each promoter on both reporter genes and the comparisons between the wild-type and the −117 HPFH promoters is shown in Table 2. The molar ratios of RNA were calculated for comparison with the molar ratios of input DNA. Input molar DNA ratios below 1/7.5 and above 7.5/1 are not included since the calculated RNA ratios were no longer responding linearly owing to the overwhelming signal from the transfected plasmid at the higher mass. The total sum of the moles of γ and γ* RNA was not the same from different transfections. This was probably due to intrinsic variability in the transient expression protocol. However, the molar ratios of γ-to-γ* RNA were unaffected by separate transfections (wild-type promoter comparison, see Tables 2 and 4) or separate RNase protections on the same RNA sample (data not shown).

Figure 6A plots the data of Table 2 as the logarithm of the molar ratio of steady-state RNA as a function of the logarithm of the molar ratio of input plasmid DNA from the comparisons of the wild-type and −117 HPFH $^{A}\gamma$ promoters. The self-comparisons of a given promoter linked to both of the reporter genes demonstrated that there is no overt influence of the marked gene on promoter activity. This is evident at the point at which equal amounts of input plasmid DNA were used and molar RNA ratios of 1.1 were observed. In contrast, the plots from the direct comparisons between the wild-type and the −117 HPFH promoters were shifted away from the self-comparison lines in the direction of the −117 component over a wide range of input plasmid DNA. Regardless of which reporter gene was used, the observed shift in the lines corresponded to a difference in steady-state RNA levels which would result if the −117 HPFH promoter was about 1.3- to 1.4-fold stronger than the wild-type promoter. This is easily observed by comparing the RNA ratio

TABLE 2. Steady-state γ and γ* RNA levels from plasmid transient expression in 293 cells comparing the wild-type and −117 HPFH promoters

Plasmid DNA	Plasmid mass ratio	γ RNA (10^{-17} mol)[a]	γ* RNA (10^{-17} mol)[a]	Molar RNA ratio (γ/γ*)
$p^{A}\gamma wt$ +	1/7.5	16.6	63.8	0.26
$p^{A}\gamma wt^{*}$	1/3	21.8	45.5	0.48
	1/1	27.9	25.4	1.1
	3/1	22.5	9.6	2.3
	7.5/1	26.8	6.1	4.4
$p^{A}\gamma 117$ +	1/7.5	8.6	39.7	0.22
$p^{A}\gamma 117^{*}$	1/3	12.4	29.2	0.42
	1/1	27.7	26.0	1.1
	3/1	52.2	19.9	2.6
	7.5/1	67.2	14.4	4.7
$p^{A}\gamma 117$ +	1/7.5	22.5	67.3	0.33
$p^{A}\gamma wt^{*}$	1/3	31.1	51.8	0.60
	1/1	40.1	27.2	1.5
	3/1	61.7	19.3	3.2
	7.5/1	63.7	12.1	5.3
$p^{A}\gamma wt$ +	1/7.5	6.7	32.1	0.21
$p^{A}\gamma 117^{*}$	1/3	13.3	38.8	0.34
	1/1	30.4	37.7	0.81
	3/1	29.5	15.0	2.0
	7.5/1	39.9	10.6	3.8

[a] RNA calculated from counts per minute protected.

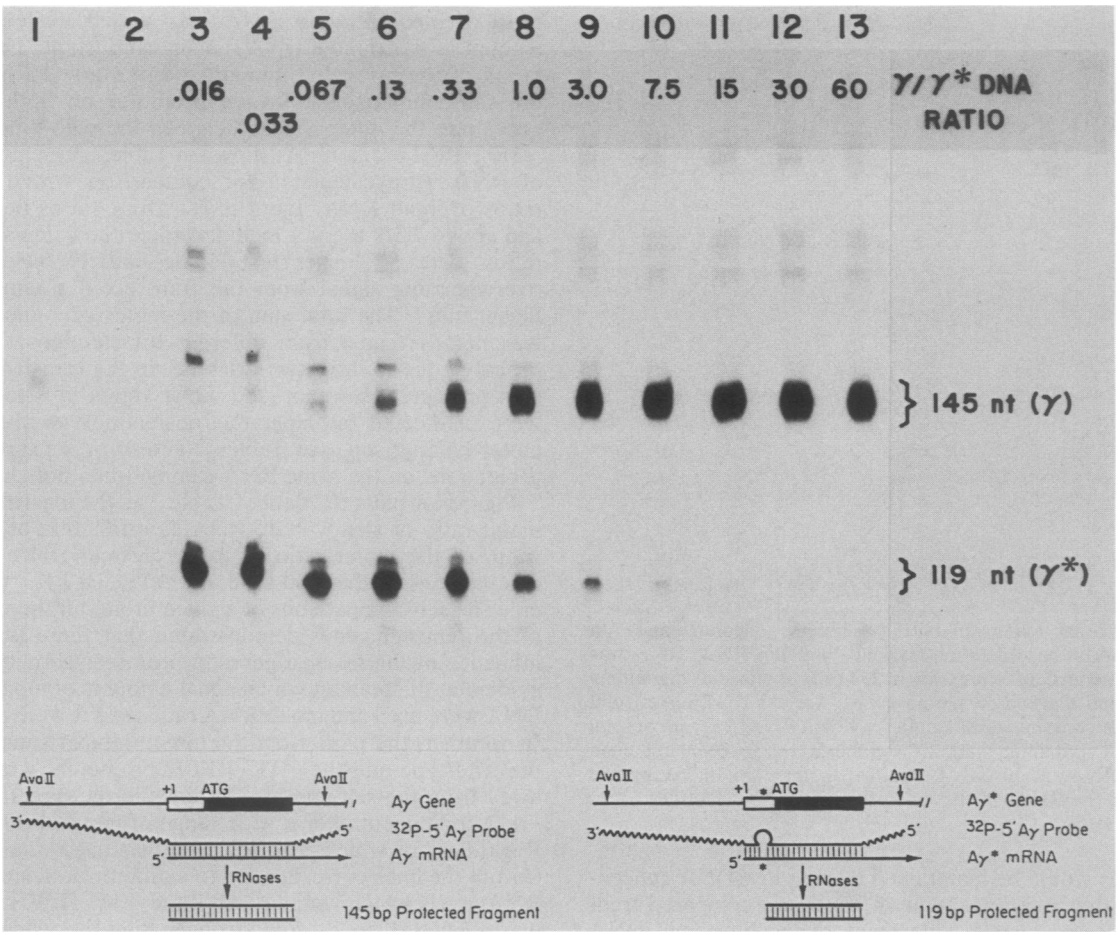

FIG. 5. RNase protection of transient expression RNA from a plasmid comparison series of $p^{A}\gamma 117$ and $p^{A}\gamma wt^{*}$ with the 5' $^{A}\gamma$ RNA probe. A diagram below the figure indicates how γ- and γ*-specific protected fragments are generated from the 5' $^{A}\gamma$ probe. Lane 1, 25 ng of human fetal liver RNA; lane 2, 6 μg of 293 cellular RNA; lanes 3 to 13, 5 μg of RNA from the transfection comparison of $p^{A}\gamma 117$ and $p^{A}\gamma wt^{*}$. The mass ratio of $p^{A}\gamma 117$ to $p^{A}\gamma wt^{*}$ DNA tested in each transfection is given below the lane number (γ/γ* DNA ratio). For the transfection comparison series, $p^{A}\gamma 117$ input DNA mass increased from 0.25, 0.5, 1, 2, 4, 8, 12, 15, 15, and 15 to 15 μg, while $p^{A}\gamma wt^{*}$ DNA mass decreased from 15, 15, 15, 15, 12, 8, 4, 2, 1, and 0.5 to 0.25 μg. nt, Nucleotides.

values from Table 2 in which equimolar amounts of input plasmid DNA were used. Both comparisons between the wild-type and −117 HPFH promoters give the same result, whether or not the unmarked gene (line shifts up) or marked gene (line shifts down) is used with the −117 HPFH promoter. These results are very similar to the results seen with the $^{A}\gamma$ RNA-to-$^{G}\gamma$ RNA ratios calculated from the cosmid DNA transfections (Table 1). Assuming no difference in RNA stability, we interpret these steady-state RNA values to indicate that the promoter containing the −117 base substitution is transcribed at a rate approximately 1.4 times greater than the wild-type $^{A}\gamma$-globin gene.

In a separate set of transfections, the mass of $p^{A}\gamma 117$ and $p^{A}\gamma wt^{*}$ was varied while maintaining a constant 1-to-1 molar ratio of input plasmid DNA. Table 3 shows that the ratio of RNA transcribed from the −117 HPFH promoter compared with RNA transcribed from the wild-type promoters was consistently 1.5. Therefore, even at lower amounts of total transfected plasmid DNA, the transcriptional difference is still observed. Since this ratio was constant when a total of 2, 4, 8, or 16 μg of input DNA was used, the transcriptional factors involved in discriminating between the two promoters are not limiting.

−196 HPFH promoter comparison. Table 4 and Fig. 6B show the calculated ratios and plots from the comparison series between the wild-type promoter and the −196 HPFH promoter over input plasmid DNA ratios of 3/1 to 1/3. In this case, no difference is evident to suggest that either of these two genes has a transcriptional advantage over the other when tested in *trans*.

DISCUSSION

Transient expression assays in 293 cells tested whether the single base substitutions found at positions −117 and −196 in $^{A}\gamma$-globin genes from individuals with nondeletion HPFH lead to elevated expression of $^{A}\gamma$ RNA. With reporter genes either in *cis* or in *trans*, these assays demonstrated that the −117 HPFH promoter is 1.4 times stronger than the wild-type $^{A}\gamma$-globin promoter. This promoter strength difference is solely attributable to the G-to-A transition mutation at position −117 and thus establishes a biochemical phenotype for this mutation in a transient assay which explains in part the Greek-type $^{A}\gamma$ HPFH phenotype in vivo. Results with the −196 HPFH promoter suggested no transcriptional difference compared with the wild-type promoter.

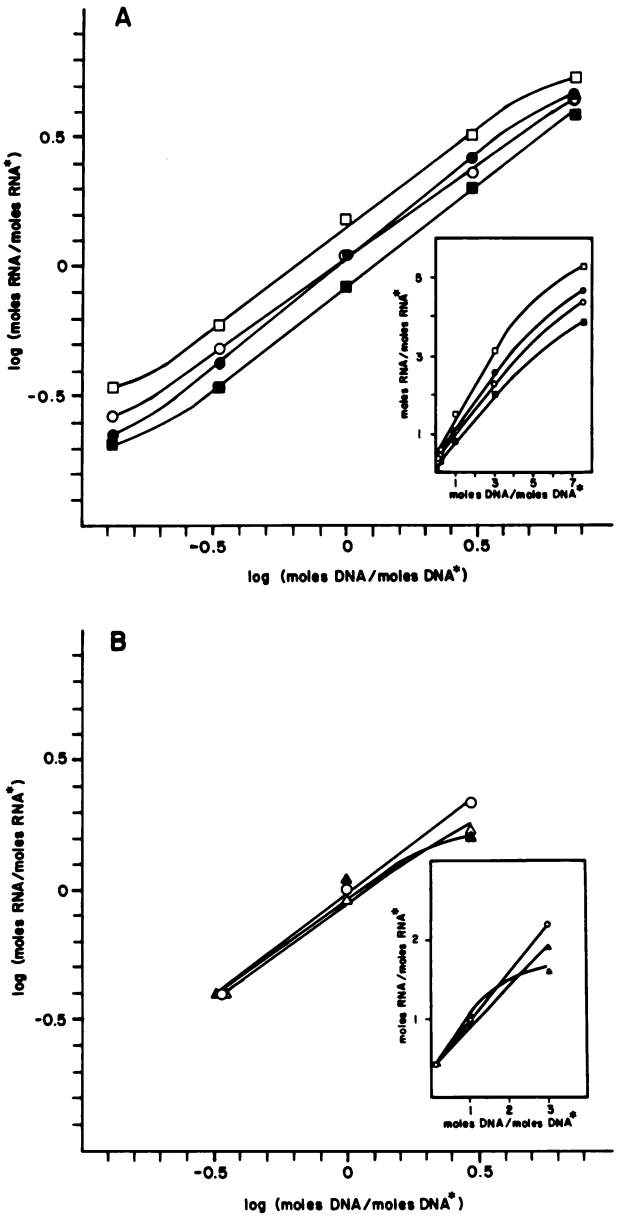

FIG. 6. (A) Data from Table 2 plotted as the logarithm of the molar RNA ratios as a function of the logarithm of the molar input plasmid DNA ratios. Inset shows the same data plotted on a linear scale. Symbols: ○, $p^{A}\gamma wt + p^{A}\gamma wt^{*}$; ●, $p^{A}\gamma 117 + p^{A}\gamma 117^{*}$; □, $p^{A}\gamma 117 + p^{A}\gamma wt^{*}$; ■, $p^{A}\gamma wt + p^{A}\gamma 117^{*}$. (B) Data from Table 3 plotted in the same manner as in panel A. Symbols: ○, $p^{A}\gamma wt + p^{A}\gamma wt^{*}$; △, $p^{A}\gamma 196 + p^{A}\gamma wt^{*}$; ▲, $p^{A}\gamma wt + p^{A}\gamma 196^{*}$. Assuming RNA is transcribed from a gene promoter (DNA) at a given rate k, then the transcriptional efficiency can be described by RNA = (DNA)k. Comparing the RNA transcribed from one promoter (DNA) with another (DNA*), yields RNA/RNA* = (DNA/DNA*) (k/k^{*}). Taking the logarithm of this equation gives log RNA/RNA* = log DNA/DNA* + log k/k^{*}. Plotting log RNA/RNA* as a function of log DNA/DNA* results in a line described by the constant, log k/k^{*}. Differences in k or k^{*} will manifest themselves by a shift in the line owing to a change in the value of log k/k^{*}.

Since differences in promoter activities have been demonstrated by transient expression, it seemed plausible that the genes encoding the polypeptides that are expressed at elevated levels in individuals with nondeletion HPFH could also be analyzed by this method. Although previous studies have demonstrated that transient expression of globin genes with linked enhancers in HeLa cells can be used to differentiate between promoter strengths (1, 25), 293 cells were chosen for these experiments on the basis of their ability to transcribe exogenous β-globin genes without the presence of linked enhancers. It is possible, however, that the transcriptional differences we observed in 293 cells would also be present in HeLa or 293 cells even if an enhancer element had been included in *cis*.

TABLE 3. Comparison of steady-state RNA levels after transient expression of equimolar masses of $p^{A}\gamma 117$ and $p^{A}\gamma wt^{*}$

Mass of plasmids (μg)	γ RNA (10^{-17} mol)[a]	γ* RNA (10^{-17} mol)[a]	Molar RNA ratio (γ/γ*)
1	0.17	0.11	1.5
2	1.17	0.74	1.5
4	5.7	3.7	1.5

[a] RNA calculated from counts per minute protected.

Cosmids containing the 39-kb *Kpn*I fragment from the human β-globin locus were introduced into 293 cells by calcium phosphate-mediated DNA transfection. Three separate cosmids were tested, one containing normal $^{G}\gamma$-, $^{A}\gamma$-, and β-globin genes and two with different nondeletion HPFH $^{A}\gamma$ genes (Fig. 1). Normal RNA transcripts could be detected by primer extension from the linked γ- and β-globin genes encoded on the cosmids (Fig. 2). Independent quantitation of $^{A}\gamma$ and $^{G}\gamma$ RNA levels was performed with an RNA probe that can discriminate between the two. Comparison of $^{A}\gamma$ RNA-to-$^{G}\gamma$ RNA ratios indicated that the −117 HPFH promoter was approximately 1.4 times more active than the wild-type gene. Normal and HPFH promoters were also compared in *trans* by transient expression assays in 293 cells with normal and marked $^{A}\gamma$-globin reporter genes, and a similar increase in transcription was observed between the wild-type and −117 HPFH $^{A}\gamma$-globin promoters. Two other reports have also shown an increase in RNA levels transcribed from the promoter containing the −117 base substitution after stable transformation of erythroid cells (5, 29) and when quantitiated in induced KMOE cells, the difference, on average, was twofold higher than wild type (29).

Position −117 is within the conserved CCAAT box region found in embryonic and fetal β-like globin genes (11) 2 bases 5′ to the distal CCAAT sequence. A mutation 2 bases 5′ to the CCAAT box sequence that results in increased transcription is not unprecedented. Myers and co-workers (25) created two changes in the mouse β^{maj}-globin gene promoter

TABLE 4. Steady-state γ and γ* RNA levels from plasmid transient expression in 293 cells comparing the wild-type and −196 HPFH promoters

Plasmid DNA	Plasmid mass ratio	γ RNA (10^{-17} mol)[a]	γ* RNA (10^{-17} mol)[a]	Molar RNA ratio (γ/γ*)
$p^{A}\gamma wt$ +	1/3	7.7	19.4	0.4
$p^{A}\gamma wt^{*}$	1/1	11.9	11.6	1.0
	3/1	11.1	6.4	2.2
$p^{A}\gamma 196$ +	1/3	5.4	13.6	0.40
$p^{A}\gamma wt^{*}$	1/1	2.2	2.4	0.92
	3/1	9.5	4.9	1.9
$p^{A}\gamma wt$ +	1/3	5.0	12.6	0.40
$p^{A}\gamma 196^{*}$	1/1	13.2	12.4	1.1
	3/1	10.1	6.4	1.6

[a] RNA calculated from counts per minute protected.

that increased transcription over threefold compared with that of wild type when assayed by transient expression in HeLa cells, a nonerythroid cell line. These changes were at positions 1 and 2 bases 5′ to the CCAAT sequence. Changes elsewhere in the mouse β^{maj}-globin gene promoter had either no effect or resulted in a decrease in RNA levels. In addition, Graves and co-workers (18) using an in vitro transcription assay demonstrated a 1.5-fold increase in transcription over that of wild type when the nucleotide 2 bases 5′ to the CCAAT sequence from the herpes simplex virus thymidine kinase gene was mutated.

Nuclear proteins that bind to CCAAT boxes are widely distributed and have been reported in nuclear extracts from rat liver (18), HeLa cells (21), and MEL cells (7). Although some data suggest that at least one of these factors can have a positive effect on transcription in vitro (21), a consistent effect on transcription, either positive or negative, has not been convincingly demonstrated for the others, which in some cases may bind to non-CCAAT sequences as well (20).

The results presented here suggest that 293 cells, even though they are nonerythroid in origin, contain a transcriptional regulatory factor, or factors, that differentially recognizes the promoter of the $^{A}\gamma$-globin gene depending on the presence of a single base change at position −117. If this factor has a positive effect on transcription, the mutation at position −117 may lead to higher affinities through stronger binding. If the factor has a negative effect on transcription, the −117 mutation may lead to weaker affinity, allowing higher transcription. A CCAAT displacement protein from sea urchin, which may play a negative regulatory role on histone H2b gene expression, has been described which would fit the negative regulatory factor model (2).

In contrast to the −117 HPFH promoter mutation, results in 293 cells with the −196 HPFH promoter mutation indicated no difference in promoter strength compared with the wild-type promoter. In erythroid cells, nucleotide positions −117 and −196 of the $^{A}\gamma$ globin gene may be within regions that are recognized by a combination of general and erythroid-specific *trans*-acting factors that regulate fetal gene expression. It is likely that 293 cells would lack erythroid developmental specific factors if they exist, but they may contain some general transcription factors that are similar to those found in nucleated erythrocytes. Thus, the −117 HPFH promoter may show only a portion of the promoter strength difference it demonstrates in vivo, and the −196 HPFH promoter may show no difference compared to a normal $^{A}\gamma$ promoter in the 293 cell assay.

In vivo, the difference in accumulated fetal hemoglobin between normal and HPFH individuals is 10- to 20-fold. These differences would not result from the 1.4-fold difference in steady-state RNA levels we observed in these 293 cell transfections with the −117 HPFH promoter. Extension of the promoter comparison assay with the marked $^{A}\gamma$ reporter gene in normal human nucleated erythroblasts may reveal a greater difference in transcription between the wild-type and nondeletion HPFH γ-globin genes and differences in transcription between the wild-type and −196 HPFH gene. The creation of the transcriptionally marked $^{A}\gamma$ gene will make it possible to study the expression of $^{A}\gamma$ genes in these human cells containing endogenous γ-globin transcripts when gene transfer systems become more efficient.

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