Volume 17 Number 11 1989 Nucleic Acids Research

Mutations in two regions upstream of the $^{A}\gamma$ globin gene canonical promoter affect gene expression

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Received February 8, 1989; Revised and Accepted April 17, 1989

ABSTRACT

Two regions upstream of the human fetal ($^{A}\gamma$) globin gene, which interact with protein factors from K562 and HeLa nuclear extracts, have functional significance in gene expression. One binding site (site I) is at a position -290 to -267 bp upstream of the transcription initiation site, the other (site II) is at -182 to -168 bp. Site II includes the octamer sequence (ATGCAAAT) found in an immunoglobulin enhancer and the histone H2b gene promoter. A point mutation (T→C) at -175, within the octamer sequence, is characteristic of a naturally occurring HPFH (hereditary persistence of fetal hemoglobin), and decreases factor binding to an oligonucleotide containing the octamer motif. Expression assays using a $^{A}\gamma$ globin promoter-CAT (chloramphenicol acetyl transferase) fusion gene show that the point mutation at -175 increases expression in erythroid, but not non-erythroid cells when compared to a wild-type construct. This correlates with the actual effect of the HPFH mutation in humans. This higher expression may result from a mechanism more complex than reduced binding of a negative regulator. A site I clustered-base substitution gives γ-CAT activity well below wild-type, suggesting that this factor is a positive regulator.

INTRODUCTION

The globin genes in vertebrates are regulated in both a developmental and tissue specific manner. In humans, embryonic (ε), fetal (γ) and adult (β) globin genes are differentially expressed in their respective developmental compartments (1). Recent studies have implicated both 5' and 3' flanking sequences, as well as internal regions (2,3), in the control of globin gene expression. Functional enhancer sequences have been identified in the 3' flanking region of the chicken β globin gene (4-6) and the human γ and β globin genes. The element 3' of the human β globin gene has been identified using both DNA mediated transfection (7,8) and transgenic mouse assays (2,3,9). This sequence is located approximately between 500 and 800 nucleotides downstream from the β globin gene. It is capable of promoting the expression of the normally silent fetal $^{A}\gamma$ globin gene in the adult erythroid compartment of transgenic mice when placed upstream of this gene (2). The β globin gene enhancer contains binding sites for an erythroid-specific protein (10). The γ globin gene enhancer is located in a fragment 400 to 1150 nucleotides downstream from the polyadenylation site. This fragment has been shown

to enhance expression when placed in a construct including the γ globin gene promoter, or other gene promoters, fused to the chloramphenicol transacetylase gene (11).

Sequences downstream of position -48 in the human β globin gene promoter are sufficient for tissue-specific gene expression (12). However, nucleotide sequences located at -259 to -137 of the human fetal ($^{A}\gamma$) globin gene are involved in developmental control since they are capable of activating a β globin promoter when assayed in stably transformed K562 cells, a cell line normally expressing fetal, but not adult, globin genes (13). Additionally, naturally occurring point mutations at -117, -175, -196, -198 and -202 from the γ globin gene are associated with hereditary persistence of fetal hemoglobin (HPFH), a condition in which the gene is expressed even into adult life (14-21). Taken together these observations strongly suggest a regulatory role for a DNA sequence element or elements upstream of the canonical promoter associated motifs such as the CCAAT and TATA boxes in γ globin gene regulation.

We and others have recently reported the binding and footprinting of factors to two 5' flanking regions of the human γ globin gene, upstream of the canonical promoter (22-26). One of these regions (site II) contains the octanucleotide motif (ATGCAAAT) found to be important in the expression of a variety of genes, including an immunoglobulin promoter and enhancer (27), U2 small nuclear RNA (28) and histone H2b promoter (29). More than one octamer-binding protein is known to exist, a lymphoid - specific (Oct-2) and a ubiquitous (Oct-1) factor have been identified and purified (30,31). Significant similarities between the Oct-1 and Oct-2 proteins are evident, and both proteins are similar to the Drosophila homeobox domain (32-34). The other region (site I), about 100 bp further upstream, does not appear to contain a binding site for a previously described DNA sequence specific binding protein. In this report, we use a transient DNA-mediated transfection assay to demonstrate the functional importance of these two sequences in the 5' flanking region of the human $^{A}\gamma$ globin gene.

MATERIALS AND METHODS

Plasmids and Probes. DNA probes, competitors, and constructs were prepared from pIBI30 and pUC plasmids containing cloned fragments of the Aγ globin gene: pIBI30- γH3.3 (Hind III, -1348 to +1950) and pTL (AluI, -300 to +36, kindly provided by T. Ley). The -300/-140 (AluI/NcoI), -256/-140 (HinfI/NcoI), -201/-140 (ApaI/NcoI) and -300/-206 (AluI/ApaI) probes were made from a derivative of pTL, and were end-labeled at the 3' end by fill-in using the Klenow fragment of DNA pol I and [α-^{32}P] dNTPs. The specific competitor in the binding assays in Fig. 1 was the -300/-140 fragment, the non-specific competitor was a fragment of a similar size from the β globin gene first intron.

In competition and binding assays involving the use of oligonucleotides, both the

coding and non-coding DNA strands were synthesized, and were annealed prior to use as competitor or 5' end-labeling with polynucleotide kinase. After annealing, the oligonucleotides were electrophoresed to insure that there was no single-stranded oligonucleotide detectable.

Clustered-base substitutions and point mutations were generated by oligonucleotide-directed mutagenesis using uracil - containing templates (35,36) and synthetic oligonucleotides with base mismatches.

pIBI30-γAdCAT was constructed by fusing the human Aγ globin gene at a SalI site clustered-base substitution of the ATAAA box, to the -40 region of the Ad2 major late promoter and in turn to a HindIII-BamHI fragment containing the chloramphenicol acetyl transferase gene. The Ad2 MLP sequence includes the tripartite leader sequence, TATAA box and transcription initiation site (37). In addition, t splice and polyA addition signals from SV40 are included in the γAdCAT construct.

Preparation of Nuclear Extracts and DNA Binding Assays. Nuclear extracts were prepared from K562 and HeLa cell lines essentially by the method of Dignam et al. (38), and binding reactions were as described by Singh et al. (39). Nuclear extracts were the last addition to the binding reactions. DNA-protein complexes were resolved by electrophoresis on 4% polyacrylamide gels run in 0.25X TBE (22mm Tris, 22mm Borate, 0.5mm EDTA) at 10V/cm.

Transfections and CAT assays. Transfections of the γAdCAT constructs were performed by electroporation into 293, HeLa, K562 and HEL (human erythroleukemic) cells. Cells were grown in DMEM (293 and HeLa) or RPMI media, supplemented with 10% fetal calf serum. Induced K562 and HEL cells were treated with 30 μM hemin one day before transfection, and for the 48 hour period prior to harvesting. CAT assays were performed using a standard protocol (37). At least two different plasmid preparations of each construct were used for transfections in all cases. The integrity of the DNA preparations was checked by agarose gel electrophoresis prior to transfection. The electroporation experiments were repeated several times, usually each construct was transfected in duplicate. If the results of these duplicate transfections varied greatly, both were disregarded. Most of the CAT assay trials were normalized for the amount of protein per reaction.

RESULTS

The 5' flanking sequence of the human γ globin gene has been implicated in its developmental and tissue-specific regulation. Using band retardation assays we and others (22-24,26) have identified DNA fragments upstream of the γ globin gene which bind factors present in nuclear extracts from an erythroleukemic cell line expressing the

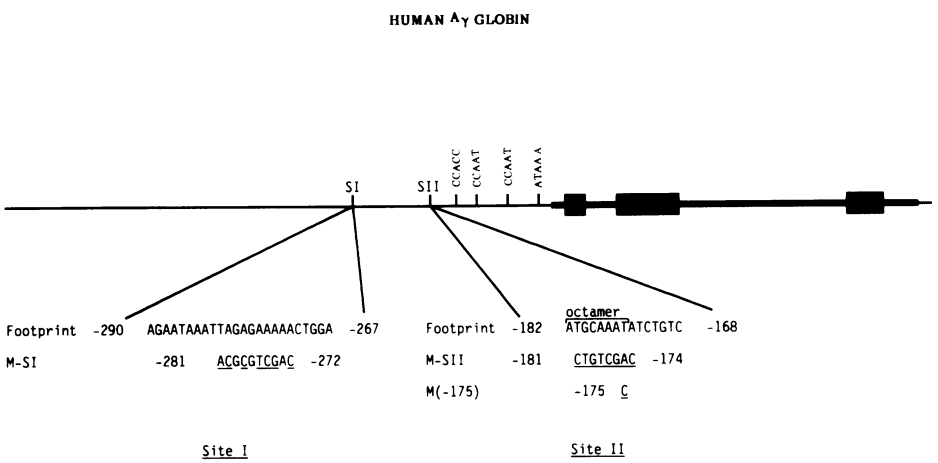

Fig. 1. Footprints of sites I and II, and mutations made to study γ globin expression. The bases included in the footprints of the major retarded bands for sites I and II in Fig. 2 are shown (unpublished results, 23, 24). Three mutations were created to determine their effect on γ globin expression. Underlined bases are mutated, all other bases in the footprinted area remain unchanged. The M-SI substitution is a replacement of the bases from -280 to -272 as indicated. An M-SII substitution in site II changes 7 of the 8 bases in the octamer sequence. -175 (T→C) correlates with a naturally occurring HPFH mutation.

gene (K562). Figure 1 depicts the sequence protected in footprint experiments of these two regions, designated sites I and II. The footprint assays were performed on both strands using K562 extracts and Cu-OP (40) or DNase I, through our own work (unpublished data) and that of others (23,24). The site I protected region extends from -290 to -267 bp. It does not contain a sequence recognized by any known binding protein. The sequence is A-T rich in composition. The site II protected region includes the octamer sequence and extends from -182 to -168 bp upstream of the γ globin gene.

We wished to study the requirements for factor interaction, and functional significance, of these two binding regions. To do this, clustered-base substitutions were introduced in site I and site II by *in vitro* mutagenesis (35,36), and the effect these exhibit on binding and expression was investigated. The bases which were mutated are depicted below the wild-type sequence and marked M-SI and M-SII (Fig. 1). All of the other bases in the footrpinted sequence remain the same as wild-type. The substitution in site I was from -281 to -272. In site II, seven of the eight bases of the octamer, and an additional base within the protected region were replaced (-181 to -174). A T→C point mutation at -175, M(-175), which correlates with a naturally occurring HPFH, was also made to test in functional assays.

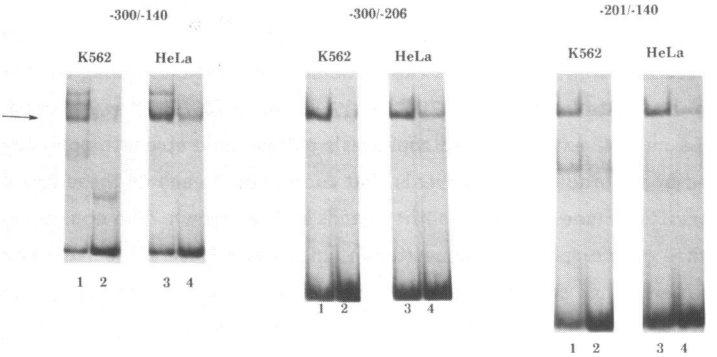

Fig. 2. DNA sequence-specific binding in two regions upstream from the $^{A}\gamma$ globin gene canonical promoter. 20 µl binding reactions were performed using about 1.5 ng of the indicated end-labeled restriction endonuclease fragments, 5 µg of poly-dI/dC as non-specific DNA competitor and 3 µl of K562 or HeLa nuclear extract (38). The restriction fragments used as probes and competitor DNAs employed are described in Materials and Methods. The non-specific competitor DNA used in lanes 1 and 3 is 150 ng of a conveniently available 180 bp fragment (from a subclone of the human β globin first intron). The specific competitor DNA in lanes 2 and 4 is 150 ng of a similar size fragment derived from the γ globin gene subclone pTL (see Material and Methods). The arrows indicate the major retarded band, the sequence of its footprint is shown in Figure 1.

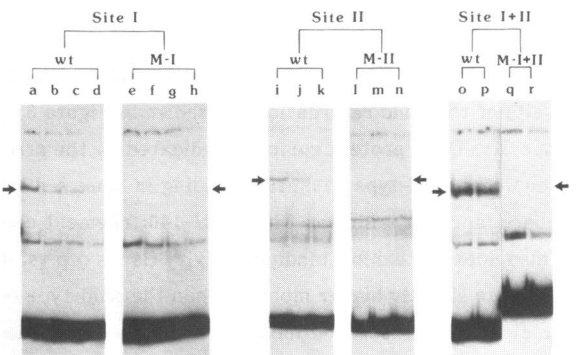

Fig. 3. Sites I and II are required for binding. A cluster of the site I and site II bases protected by nuclear factors in footprint assays were substituted by *in vitro* mutagenesis. The fragments used for binding are either the wild-type or clustered-base substitution version of site I (AluI/ApaI, -300/-206), site II (ApaI/NcoI, -201/-140) or site I plus site II (AluI/NcoI, -300/-140). Each probe was incubated with equal amounts of K562 nuclear extract, 5 µg of poly dI/dC and increasing concentrations of non-specific plasmid competitor (lanes a, b, e, f, i-r) or specific competitor pTL (lanes c, d, g and h). Lanes a-d have the same amount of competitor DNA added as e-h, i-k match l-n, and o-p are the same as q-r. The arrow indicates the position or expected position of the low mobility complex; it is absent with the M-I, M-II, and M-I+II mutations.

Clustered-base substitution of site I or II eliminates binding - Representative binding assays with normal DNA fragments containing site I and/or site II from the γ globin gene are shown in Figure 2. A radiolabeled fragment containing both site I and site II (-300/-140), site I only (-300/-206) or site II only (-201/-140) was incubated with K562 or HeLa nuclear extract. Electrophoresis on low ionic strength polyacrylamide gels followed by autoradiography reveals that factors bind each of these fragments with sequence specificity (see the low mobility bands at the arrow). The complexes formed in the presence of non-specific competitor in lanes 1 and 3 are effectively competed with the DNA sequence specific fragment used in lanes 2 and 4. The low mobility complexes resulting from binding of the three fragments are probably not due to an erythroid specific factor(s), since complexes of similar mobilities are observed using either K562 or HeLa cell nuclear extracts. The pattern of band retardation with these fragments as probes was identical whether K562 or MEL (mouse erythroleukemia cell) nuclear extracts were used (unpublished data). Higher mobility bands are often observed in binding assays with the -300/-140 and -201/-140 probes and K562 or MEL cell nuclear extracts (Fig. 2). These were not apparent with nuclear extracts from HeLa cells (see Discussion).

To provide evidence that the DNA sequences within the footprinted regions are indeed involved in the specific DNA protein interactions observed in gel binding assays, fragments with these sites altered, as indicated in Figure 1, were examined for binding activity. The -300/-140, -300/-206 and -201/-140 DNA fragments and their mutated counterparts (M-I, M-II and M-I + II) were incubated with K562 nuclear extract in binding assays. The results of the band retardation are shown in Figure 3; when site I or site II are altered, the specific DNA-protein complex (indicated by the arrow) is not observed. For site I, compare wild-type to mutant binding in lanes a-d vs. e-h, respectively. For site II, compare lanes i-k with l-n. The -300/-140 fragment containing both mutated binding sites also does not exhibit binding activity (lanes o-p vs. q-r). The significance of a faint band with slightly higher mobility than the wild-type complex in the M-I and M-II lanes is unclear. The results obtained with the clustered-base substitutions correlate with footprint data indicating the positions of two factor binding sites upstream of the human γ globin gene.

We found that the octamer sequence is not only necessary, but sufficient, for protein binding in an experiment using a synthetic DNA fragment as competitor against a labeled fragment containing site II (-256/-140). The 20 bp DNA fragment (GGATCCA-TGCAAATAAGCTT) was used as competitor. This includes the octamer sequence plus 6 bases of random flanking sequence 5' and 3'. This fragment competed for binding of the γ globin octamer binding factor (unpublished data). This indicates that the eight base pair motif, flanked by random sequence, is sufficient for binding.

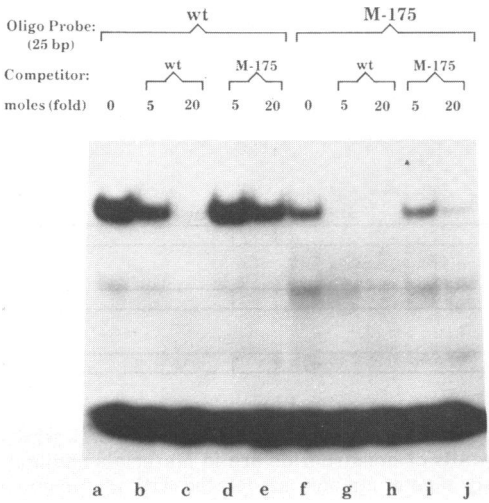

Fig. 4. An HPFH point mutation (-175 T→C) reduces factor binding. Two 25 bp oligonucleotides were constructed, and complementary strands annealed. One contains the octamer binding sequence surrounded by 8 or 9 bases of normal flanking sequence on either side (-190 CTATCTCAATGCAAATATCTGTCTG -166). The other is the same but for a T→C change at -175, within the octamer. The radiolabeled wild-type oligonucleotide is used as a probe in lanes a-e, a probe of the same specific activity from the -175 T→C oligonucleotide is in lanes f-j. The unlabeled wt oligonucleotide was used as competitor in lanes b, c, g and h; the -175 T→C oligonucleotide in lanes d,e,i and j. Each binding reaction contains 5 μg of poly dI/dC and an equal amount of K562 nuclear extract. The amount of competitor used in each lane is as shown.

Binding activities of oligonucleotides with or without an HPFH mutation - A mutation at position -175 (T→C) in the human γ globin gene is correlated with hereditary persistence of fetal hemoglobin, an asymptomatic condition in which levels of fetal hemoglobin are higher than normal in adults (1). Since the -175 mutation falls within site II, and indeed within the octanucleotide motif (ATGCAAAT), we wished to determine whether this single base change has an effect on nuclear factor binding. A synthetic 25 bp DNA fragment containing the octamer sequence plus 8 and 9 bases on either side of normal flanking sequence (-190 to -166), as well as a mutant DNA fragment, identical except for a single base (T→C, -175), were tested for their protein binding affinities in competition assays using K562 nuclear extract. The results are shown in Figure 4. The wild-type probe was used in lanes a-e and the mutant probe in lanes f-j. Radiolabeled mutant oligonucleotide (M-175) did not bind protein as well as a wild-type probe of the same specific activity. This is evident in a comparison of the assays in lanes a and f, which contain wild type and mutant probes, respectively, and no competitor DNA. Binding can be competed from either probe, however the unlabeled wild-type DNA fragment competes more effectively than unlabeled M(-175) (compare lanes b and c vs. d

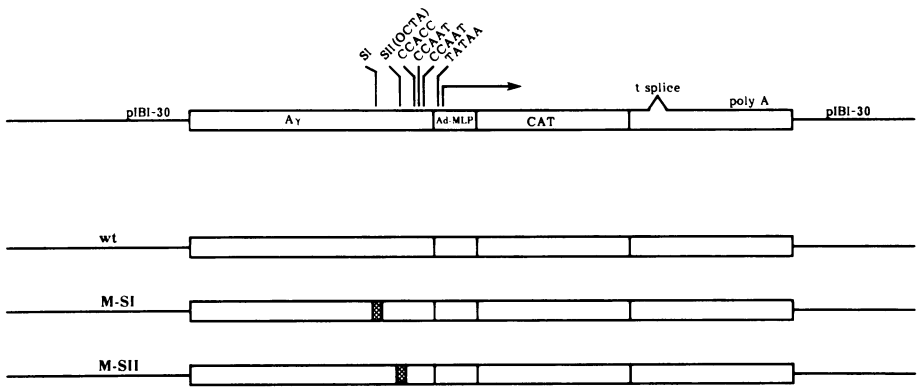

Fig. 5. The γAdCAT constructs. A schematic of the γAdCAT wild-type and mutant constructs is shown, details of construction are in Materials and Methods. CCACC represents a promoter sequence commonly associated with globin genes. It is located between 141 and 148 bp upstream from the start site of transcription (43). M-SI and M-SII contain the same clustered mutations as described in Fig. 1. Not shown here is the γAdCAT -M(-175) construct, which has the -175 T→C point mutation.

and e, g and h vs. i and j). These results show that, though the M(-175) mutant sequence can bind protein, it has a much lower binding affinity than the wild-type sequence. These *in vitro* results indicate that the mutant octanucleotide motif, upstream of the γ globin gene in erythroid cells with the HPFH mutation at -175, might not bind an octamer factor as readily as in normal cells. This suggests that the γ globin octamer binding factor may have a negative regulatory control on γ globin gene expression in normal adult erythroid cells, which is alleviated in the -175 T→C mutant.

Expression Assays - To investigate the effects of the clustered-base substitutions and the point mutation in the γ globin promoter region on gene regulation, mutant and wild-type γ globin promoters (-1350 to -32) were introduced into a construct containing the tripartite leader, TATAA box and transcription initiation site of the Adenovirus 2 MLP fused to the chloramphenicol acetyl transferase gene (Figure 5). The Ad2-MLP/CAT construct has previously been shown to express a high level of CAT activity (37), as well as accurate initiation of the Ad2/MLP (41).

Two of the mutations in the A_γ globin promoter investigated are M-SI and M-SII, the clustered-base substitutions in sites I and II. The substituted bases are identical to those depicted in Figure 1. These constructs are shown in Figure 5. A third mutant has a single change of T→C at -175 (M-175) analogous to the naturally occurring HPFH mutation. The parental and three mutant constructs were transfected by electroporation

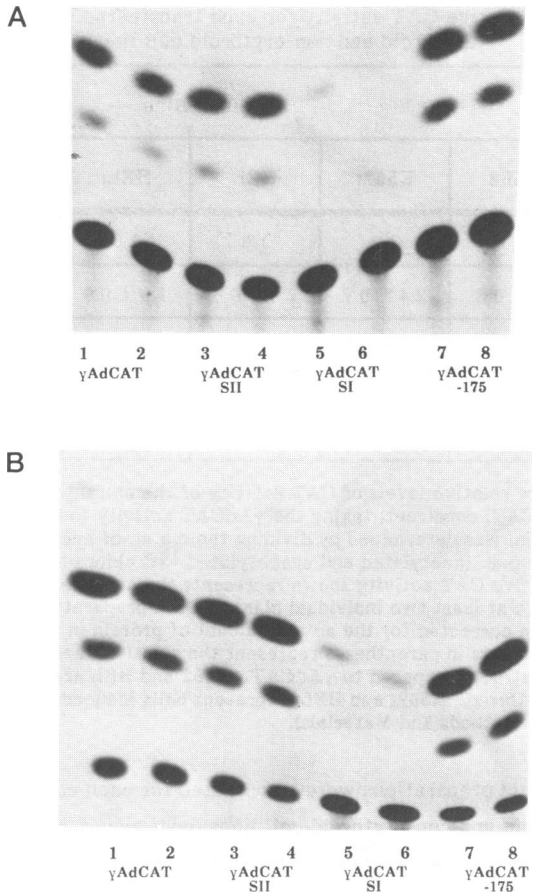

Fig. 6. Results from a representative CAT assay of transfected erythroid and nonerythroid cells. The constructs used in the transfection are as described in Figure 5. Lanes 1 and 2, two individual plasmid preparations of the wild-type γAdCAT construct; lane 3 and 4, γAdCAT-M-SII; lanes 5 and 6, γAdCAT-M-SI; lanes 7 and 8, γAdCAT-M (-175). A) CAT assay of transfected K562 erythroleukemic cells. B) CAT assay of transfected 293 cells (adenovirus transformed kidney cells).

into induced and uninduced K562 and HEL (human erythroleukemic) cells, as well as non-erythroid HeLa and 293 (human embryonic kidney) cells. The CAT activities relative to the wild-type were determined in transient expression assays. Representative autoradiograms of CAT assays with erythroid (K562) and non-erythroid (293) cell lines are shown in Figure 6. The results of at least six repeated sets of transfection experiments with each construct in each cell line are summarized in the Table. At least two

Table. Relative CAT activity for each transfected construct in erythroid and non-erythroid cell lines

Construct	Cell Line					
	K562	$K562_I$	HEL	HEL_I	293	HeLa
γAdCAT	1.0	1.0	1.0	1.0	1.0	1.0
γAdCAT-M (-175)	3.0±0.8	2.4 ± 0.7	2.5 ± 0.6	1.9 ± 0.6	0.8 ± 0.2	0.9 ± 0.1
γAdCAT-M-SII	1.2 ± 0.2	1.0 ± 0.2	1.8 ± 0.5	1.2 ± 0.4	1.0 ± 0.2	1.5 ± 0.4
γAdCAT-M-SI	0.03 ± 0.01 (-33X)	0.04 ± 0.02 (-25X)	0.05 ± 0.03 (-20X)	0.03 ± 0.01 (-33X)	0.02 ± 0.01 (-50X)	0.04 ± 0.02 (-25X)

Results shown are relative levels of CAT activity of the mutant constructs as compared to the wild-type (γAdCAT) construct, taking the γAdCAT activity to be 1.0 (or 100%). The % conversion for each trial was determined by dividing the c.p.m. of acetylated ^{14}C chloramphenicol by the total c.p.m. (acetylated and unacetylated ^{14}C chloramphenicol), and multiplying by 100. Each relative CAT activity shown represents the average of at least six independent transfections, with at least two individual plasmid DNA preparations. Most individual CAT assay results were corrected for the actual amount of protein in the extracts used for the CAT reaction. The numbers in parentheses represent the relative decrease in CAT activity of the γAdCAT-M-SI construct compared to γAdCAT. K562 and HEL are erythroid cells, 293 and HeLa cells are non-erythroid. $K562_I$ and HEL_I represent cells induced with hemin before and after transfection (see Methods and Materials).

individual DNA plasmid preparations were transfected for each construct. The use of two different erythroid and non-erythroid cell lines lends even further credence to the experiments, since the results are basically the same for K562 and HEL, or for HeLa and 293. In addition to the repeats within each cell line, since both induced and uninduced K562 and HEL cells were tested, a total of at least twenty-four transfections of each construct were done in erythroid cells.

The SI mutation results in 20-50X lower than wild-type CAT activity. This may indicate that the factor normally binding here is a positive regulator. This decrease in expression relative to wild-type is evident in all cell lines, erythroid and non-erythroid. In erythroid cells (K562 and HEL), the γAdCAT-M (-175) shows significantly increased expression (2-3X) over the wild-type $A\gamma$ globin promoter, this correlates with the observed situation in the naturally occurring -175 (T→C) HPFH and is not dependent upon induction. In contrast, the γAdCAT-M (-175) construct does not exhibit expression higher than γAdCAT wild-type when transfected into non-erythroid 293 or HeLa cells (0.8X and 0.9X, respectively). The CAT activity of the construct containing the clustered-base substitution in the octamer region, M-SII, is not different from wild-type

in erythroid or non-erythroid cells. The SII mutation includes a T→A change at -175, but additionally has mutations at all the other bases from -181 to -174.

Two other naturally occurring HPFH mutations, C→T at -196 and G→C at -202, were also generated in the γAdCAT construct and were examined in transient expression assays. We did not observe a significant increase in CAT expression (unpublished results).

DISCUSSION

Two regions upstream of the human A_γ globin gene, one from -168 to -182 (site II), and the other from -267 to -290 (site I), bind protein factors from human erythroid K562 and HeLa cell nuclear extracts (unpublished results,22-26). Site II encompasses an octamer sequence. A change of one base (-175, T→C) in the octamer region is correlated with hereditary persistence of fetal hemoglobin in human adults (42). Mutations in each of these regions decrease factor binding *in vitro*. Mutations in site I and at -175 affect the expression of a A_γ globin promoter - CAT fusion gene. A clustered-base substitution at site I (M-SI) decreases the level of CAT activity in both erythroid and non-erythroid cells (K562, HEL, 293 and HeLa). Expression of γ-AdCAT is increased in erythroid cells when the -175 T→C point mutation in site II is introduced, this is evident in both induced and uninduced K562 and HEL cells. However, the transcriptional activity of the construct with the -175 T→C mutation is the same as wild-type in non-erythroid 293 or HeLa cells. When every base from -174 to -181 is substituted in the γAdCAT-M-SII construct, little effect on the level of CAT activity is evident compared to wild-type.

The Adenovirus 2 major late promoter (Ad2MLP) was included in the CAT constructs in order to increase CAT assay sensitivity. This increased sensitivity does not appear to mask the effects of the mutations studied, since the -175 and SI γ globin promoter mutations result in changes in expression. Indeed, even the tissue-specific nature of the effect of the -175 mutation is evident in expression assays with a construct including the Ad2MLP. The γAdCAT constructs with the C→T -196 and G→C -202 HPFH mutations showed no increased expression over wild-type in our transient assays. The same result has been independently obtained with another γ globin construct containing these mutations (44).

When the distal binding sequence (site I) is mutated in γAdCAT-M-SI, expression is significantly less than wild-type (at least 20X), suggesting that the factor normally binding here has a positive regulatory effect. This decrease is evident in both erythroid and non-erythroid cells, suggesting that the factor binding at site I does not confer tissue specificity in γ-globin gene expression. Tne site I sequence (Fig. 1) is A-T rich. It

does not appear to include the recognition sequence for a known binding protein. The dramatic effect exerted by a clustered-base substitution of ten bases from this region suggests that it encompasses an important regulatory element worthy of further investigation.

The simplest explanation for the increase in expression observed in the human -175 (T→C) HPFH condition would be that a binding site for a repressor near -175 is eliminated due to the base change. When the -175 mutant is tested in *in vitro* binding assays with either HeLa or erythroid nuclear extracts, binding to the octamer region is decreased (Fig. 4, unpublished results). However, it seems unlikely that the octamer binding factor acts simply as a repressor, since the -175 (T→C) mutation results in increased expression of a γAdCAT fusion gene only in erythroid cells (see Table). This suggests that limiting the binding of the octamer factor is not solely responsible for the observed increase in γAdCAT expression. Also, the SII clustered-base substitution, which decreases octamer factor binding (Fig. 2), does not produce the increased γAdCAT expression evident with the -175 (T→C) mutation. This larger clustered-base substitution, from -181 to -174, might affect the function of a different binding factor(s), resulting in no enhancement of expression. This factor may be erythroid specific, since the -175 effect is only evident in erythroid cells.

In fact, methylation interference footprinting studies have revealed that an erythroid specific factor (EFγa or GF-1) binds at positions in the γ globin promoter bordering the octamer sequence both 5' and 3' (24,25,44). The fainter, faster migrating band present in the -201/-140 K562 nuclear extract binding assay in Fig. 2, and absent with the HeLa nuclear extract, likely corresponds to EFγa binding. Our SII mutation (-174 to -181) affects the octamer binding sequence and also overlaps bases in the EFγa binding site proximal to the γ globin transcription start site (25,44). Recently, Martin et al. (44) tested expression of a γ globin promoter-growth hormone fusion gene with mutations at both -175 and -186. These mutations affect both the octamer and distal EFγa binding sites. Their construct showed a wild-type rather than an enhanced level of expression in transient assays, similar to our results with the γAdCAT-M-SII construct. Martin et al. (44) concluded that EFγa must bind to the distal site for the T→C mutation at -175 to produce enhanced expression. Our results indicate that additional sequence, between -174 and -181, is also essential for this enhancement. To determine the nature of the involvement of the octamer binding factor and EFγa in both wild-type and -175 (T→C) γ globin gene expression, it will probably be necessary to purify the binding factors and study their function in subsequent *in vitro* transcription assays.

ACKNOWLEDGEMENTS

We appreciate the outstanding technical assistance of Michelle Hare, Kathy Frondorf, Mary Eileen Rath and Bonnie Richmond. We thank Elvira Ponce for critically reading the manuscript. We thank Jennifer Schroeder for preparation of the manuscript.

This study was supported by NIH grant DK 39585. J.A.L. is a recipient of NIH postdoctoral fellowship 1GM12817-01 F32. R.F.L. was a recipient of NIH postdoctoral fellowship 12331.

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