The $^A\gamma$-195 (C→G) mutation in hereditary persistence of fetal hemoglobin is not associated with activation of a reporter gene *in vitro*

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Abstract

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Hereditary persistence of fetal hemoglobin is an uncommon, benign disorder in which the expression of γ-globin genes persists into adult life. Several point mutations have been associated with the increased γ-globin gene promoter activity. We evaluated the -195 (C→G) mutation by a functional *in vitro* assay based on the luciferase reporter gene system. The results indicated that the increased promoter activity observed *in vivo* could not be reproduced *in vitro* under the conditions employed, suggesting that other factors may be involved in the over-expression of the γ-globin gene containing the -195 (C→G) mutation. Furthermore, this is the first time that the -195 (C→G) mutation of the $^A\gamma$-globin gene has been evaluated by *in vitro* gene expression.

Nondeletional hereditary persistence of fetal hemoglobin (HPFH) is a condition characterized by a continuous expression of γ-globin genes during adult life. This increase is associated with specific single-base mutations in the promoter region of either the $^G\gamma$- or $^A\gamma$-globin gene (1). These point mutations are clustered in three distinct regions of the 5'-flanking DNA of the affected γ-globin genes (2).

The complex mechanism of γ-globin gene control regulation is thought to be a consequence of the binding modification of a number of different *trans*-acting factors to critical regions of the γ-globin gene promoters, the *cis*-elements. The mutations may prevent the binding of negative regulatory factors or enhance the binding of positive regulatory factors, thus interfering with gene expression. The mechanism by which the mutations cause up-regulation of the γ-globin gene promoter might be of considerable practical value, as it could lead to the development of methods to increase the levels of functional fetal hemoglobin in patients with hemoglobinopathies.

A C→G substitution was identified in a Brazilian individual (3) and, more recently, another six individuals (three unrelated and three members of a family) were identified in which the presence of a -195 (C→G) mutation was closely associated with the high fetal hemoglobin phenotype (4). The effect of this mutation has not yet been assessed in functional assays and this is the first time that this mutation has been evaluated by *in vitro* gene expression. In the present study we used the reporter gene approach based on the

luciferase gene to assess the effect of a -195 (C→G) mutation in the $^A\gamma$-globin gene promoter.

The results indicated that the observed *in vivo* phenotype of high fetal hemoglobin levels cannot be translated into increased *in vitro* promoter function when compared to the wild-type promoter under the experimental conditions described, suggesting that in addition to the mutation other factors are involved in controlling the promoter function.

A blood sample was collected in EDTA and DNA was isolated by the method of Poncz et al. (5). The $^A\gamma$ promoter of 675 bp was prepared by polymerase chain reaction (PCR) amplification from a control and a patient with C→G mutation at position -195 using genomic DNA as template and the primers P 133 and P 134 (Table 1), which were engineered to contain an *Xho*I (forward primer) or a *Hin*dIII site (reverse primer) at their 5' ends (Table 1).

PCR mutagenesis was used to generate a 377-bp fragment of the PCR products containing the T→C mutation at position -175 in three stages as described by Motum et al. (6). Stage 3 was modified to include primers P 6 and P 134 (Table 1), engineered to contain an *Xho*I (forward primer) or a *Hin*dIII site (reverse primer) at their 5' ends.

All samples were denatured at 95°C for 10 min before adding the *Taq* DNA polymerase enzyme (Gibco, Grand Island, NY, USA) and then amplified for 30 cycles with denaturation

at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min in an automated PTC 200 thermal cycler (M.J. Research Inc., Watertown, MA, USA), followed by a final extension at 72°C for 10 min.

PCR products were double-digested with *Xho*I and *Hin*dIII. The purified products were identified by 1% agarose gel electrophoresis with TAE buffer and stained with the fluorescent dye ethidium bromide (7). The promoters prepared as described above were inserted into the *Xho*I-*Hin*dIII sites of the pGL2-Basic (Promega, Madison, WI, USA), a promoterless luciferase reporter gene vector.

Supercoiled plasmid DNA prepared for transfection was grown in *Escherichia coli* strain DH5α (Bethesda Research Laboratories, Bethesda, MD, USA) and purified with a cesium chloride density gradient (7). At least two different plasmid preparations of each construct were used for transfection. For all experiments, the integrity and concentrations of all plasmids to be transfected were verified by 1% agarose gel electrophoresis and the concentration and purity was evaluated by spectrophotometry at 260/280 nm.

All the γ-globin gene promoter constructs were sequenced using the Sequenase kit (United States Biochemical Corporation, Cleveland, OH, USA) and primers GL1 and GL2 of the pGL2 expression vector (Promega) to verify the correctness of the amplified promoter segment and its appropriate insertion into the pGL2-Basic vector, and correct insertion and sequence of the $^A\gamma$-globin gene promoter construct were confirmed by sequencing.

K562 cells (8) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco), at 37°C and 5% CO_2. The cells were transfected with 20 μg of γ-globin gene promoter luciferase constructs and 5 μg of SV40/ß-galactosidase expression vector (Promega), which was used as an internal control for transfection efficiency, by electroporation in a Gene Pulser (Bio-

Table 1 - $^A\gamma$-Globin oligonucleotide primers for PCR.

[1]Underlined bases added to produce an XhoI site (sense primer). [2]Underlined bases added to produce a HindIII site (antisense primer). *Mutated base to produce -175 (T→C) base substitution. S, Sense; AS, antisense.

Primer	Location	Sequence (5'→3')
P 133	-622 to -591	ATCTCGAGTGAAACTGTGGTCTTTATGA[1]
P 6	-324 to -305	ATCTCGAGCTATGATGGGAGAAGGAAAC[1]
P 134	+33 to +53	CTAAGCTTTCTGGACTAGGAGCTTATTG[2]
-175 S	-186 to -165	CTCAATGCAAAC*ATCTGTCTG
-175 AS	-186 to -165	CAGACAGATG*TTTGCATTGAG

Rad Laboratories, Richmond, CA, USA) as described by Motum et al. (6). Luciferase assays were performed using the Luciferase Assay System (Promega) as recommended by the manufacturer and light emission was measured with a luminometer monolight 2010 (Analytical Luminescence Laboratory, San Diego, CA, USA). The luciferase activity associated with each construct was corrected for differences in transfection efficiency based on the results of the ß-galactosidase assay (9). All transfections were performed at least in triplicate and repeated several times.

Data are reported as mean ± SD. Groups were compared by standard analysis of variance (ANOVA).

The results of the transfection studies are summarized in Table 2. As expected, the -175 (T→C) site-directed mutation increased the expression of the $^A\gamma$-globin gene promoter construct by 2.2-fold compared to the wild-type sequence (activity 1.0) in K562. The construct containing the -195 (C→G) mutation was compared with the wild-type $^A\gamma$-globin gene promoter construct (activity 1.0) and was not significantly different in K562 (Table 2). The promoterless luciferase (pGL2) activity was 30-fold less than in other constructs (data not shown).

HPFH is a condition characterized by the continuous expression of fetal hemoglobin during adult life. Several mutations in the γ-globin promoter have been described. However, their contribution to the phenotype remains poorly understood. In the present study, we used a luciferase reporter gene approach to determine the effect of the -195 (C→G) mutation in the $^A\gamma$-globin promoter on the K562 cell line, using the -175 (T→C) mutation in the $^A\gamma$-globin promoter as a positive control.

Several studies have demonstrated up-regulation of the -175 (T→C) mutation (6). We used this mutation as a positive control in order to determine the ability of this system to reproduce the up-regulation of a $^A\gamma$-globin gene promoter luciferase construct *in vitro*. Our analysis showed that this construct containing the -175 site-directed mutation is able to increase luciferase expression, with a two-fold higher expression than the wild-type sequence. This mutation was in a conserved "octamer" position in the γ-globin gene promoter and was recognized by the ubiquitously distributed octamer-binding protein (OBP). Ottolenghi et al. (10) showed that the -175 (T→C) mutation abolishes binding of the OBP and therefore, in its absence, the ability of erythroid-specific GATA proteins to bind adjacent sites on both sides of the octamer is increased. In our studies, we observed that this mutation led to a two-fold higher promoter function. These results were consistent with previous data, which reported an increase of 2.5- to 5-fold (11-13). The increment in promoter strength observed in our studies and others was much less than the *in vivo* 50 to 100 up-regulation of the mutated HPFH gene (11).

Our analysis of the HPFH-associated γ-globin gene promoter containing the -195 (C→G) mutation was not translated into increased promoter function in transient expression assays. The failure to detect up-regulation by this mutation, however, did not preclude the potential role of this mutation in altering γ-globin gene expression, since several other nondeletional HPFH mutations, including the -202 and -196, were not overexpressed in the transient expression system (14,15).

Table 2 - Ratio of luciferase/ß-galactosidase activity (relative to wild type) of $^A\gamma$-globin with the -195 (C→G) and -175 (T→C) mutation in the K562 cell line.

The estimate is reported as mean ± SD. CI: Confidence interval; N: number of assays performed. *P<0.05 compared to wild type (Student t-test).

Construct	Estimate	95% CI	N
Wild type	1.0		64
-175 (T→C)	2.19 ± 0.26*	1.90-2.46	25
-195 (C→G)	1.06 ± 0.05	0.98-1.14	39

Ulrich et al. (16) suggested that point mutations at positions -202, -196 and -195 reduce triplex stability by disrupting critical Hoogsteen base pairs and the -198 substitution is the only HPFH mutation in the γ-200 region that does not destabilize the secondary DNA structure. The -198 (C→T) mutation increases γ-globin promoter strength in transiently transfected fetal erythroid cells or an *in vitro* transcription assay, while the -202 and -196 substitutions do not.

These results suggest that the phenotype observed *in vivo* is not only dependent on the *trans*-acting factor/*cis*-acting element alteration but is also dependent on DNA structure modifications, which may be difficult to reproduce *in vitro*. Another factor that may affect γ-globin expression *in vitro* and was not used in our experiments is the locus control region (LCR). Over the last decade, the importance of the LCR has been well documented (17). Recently, Langdon and Kaufman (18) studied the elements required for interaction of the γ-globin promoter with 5'HS2 in the K562 cell line. They demonstrated that the HPFH γ-globin promoter linked to 5'HS2 showed a two- to three-fold higher expression than the wild-type promoter.

In this paper, we report that the *in vitro* functional activity of the ^Aγ-globin promoter containing the -195 (C→G) point mutation is not different from the wild-type promoter and that other *in vitro* and *in vivo* studies including this mutation and the sequences of the LCR would clarify the effect of the HPFH point mutation and this control region on the expression of the γ-globin genes.

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