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Influence of the polymorphisms of the α-major regulatory element HS-40 on *in vitro* gene expression

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The α-MRE is the major regulatory element responsible for the expression of human α-like globin genes. It is genetically polymorphic, and six different haplotypes, named A to F, have been identified in some population groups from Europe, Africa and Asia and in native Indians from two Brazilian Indian tribes. Most of the mutations that constitute the α-MRE haplotypes are located in flanking sequences of binding sites for nuclear factors. To our knowledge, there are no experimental studies evaluating whether such variability may influence the α-MRE enhancer activity. We analyzed and compared the expression of luciferase of nine constructs containing different α-MRE elements as enhancers. Genomic DNA samples from controls with A (wild-type α-MRE) and B haplotypes were used to generate C-F haplotypes by site-directed mutagenesis. In addition, three other elements containing only the G→A polymorphism at positions +130, +199, and +209, separately, were also tested. The different α-MRE elements were amplified and cloned into a plasmid containing the luciferase reporter gene and the SV40 promoter and used to transiently transfect K562 cells. A noticeable reduction in luciferase expression was observed with all constructs compared with the A haplotype. The greatest reductions occurred with the F haplotype (+96, C→A) and the isolated polymorphism +209, both located near the SP1 protein-binding sites believed not to be active *in vivo*. These are the first analyses of α-MRE polymorphisms on gene expression and demonstrate that these single nucleotide polymorphisms, although outside the binding sites for nuclear factors, are able to influence *in vitro* gene expression.

Key words: Alpha-major regulatory element; HS-40; Alpha-globin genes; Genetic polymorphisms; Gene expression

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Introduction

The erythroid lineage-specific and developmental stage-specific expression of the human α-like globin genes is controlled by an element located 40 kb upstream of the ζ-globin gene on chromosome 16p13.3, known as HS-40 or α-major regulatory element (α-MRE) (1,2). It has been demonstrated that the α-MRE confers high levels of expression to the α-like globin genes and behaves as an enhancer for the promoter activity of these genes (2-9).

The α-MRE is genetically polymorphic and its variability has been studied by Harteveld et al. (10) in seven population groups from Africa (Bantu-speaking and Pygmies), Europe (Dutch and Italians) and Asia (Chinese, East Indians, and Indonesians), and by Ribeiro et al. (11) in two Brazilian Indian tribes. Six haplotypes, denoted A to F, were detected. Only the A and B haplotypes are present in all groups analyzed. The other haplotypes (C to F) are present in low frequencies and in specific populations (10,11).

Most α-MRE single nucleotide polymorphisms (SNPs) are located either between binding sites for transcriptional factors or in a site considered not to be active *in vivo* (3,8,10,12). There are no studies evaluating whether these

natural mutations influence gene expression or not. Since clinical studies associating the α-MRE haplotypes with α-gene expression levels and hematological features are difficult to carry out due to the low frequencies of most haplotypes, the aim of the present study was to compare the *in vitro* gene expression of constructs having all six different α-MRE haplotypes as enhancers, in addition to the SV40 promoter and to the luciferase reporter gene. These constructs were used to transiently transfect K562 cells.

Material and Methods

Plasmid constructs

The α-MRE core fragment (350 bp) was first amplified by the polymerase chain reaction (PCR) (10) using genomic DNA from two controls: one with the wild-type α-MRE (A haplotype) and the other with the B haplotype. These fragments were used to generate the other haplotypes (C-F) and three other elements containing the +130, +199, and +209 polymorphisms separately by site-directed mutagenesis in a two-step PCR. In the first step, primer pairs A/D and B/C were used to amplify overlapping halves of the mutant segment; the two PCR products were combined and the full-length mutant sequence was amplified with primer pair A/B (oligonucleotide sequences in Table 1). The products were sequenced to determine if any additional mutations had been introduced during amplification.

The different amplified elements were cloned into a pGL2-promoter plasmid (Promega, USA) consisting of an SV40 promoter linked to the firefly luciferase reporter gene. The enhancer activity of the α-MRE on the SV40 promoter is similar to its activity on α-globin gene promoters (4).

Table 1. Primer sequences for site-directed mutagenesis.

Primers	Sequences
A: HM13-αMRE	5'-GCC GGG AGG CTC TCA GGA ACA AGA-3'
B: HBIO-αMRE	5'-CCG CAG CCT GGC TGT GAA CAC TTT-3'
C: P96s	5'-AAG GGT GGA GG**A** ATG CAG CTG TGG-3'
D: P96as	5'-CCA CAG CTG CAT **T**CC ACC CTT-3'
C: P130s	5'-AAA CAC TTG AG**A** GAG CAG ATA ACT-3'
D: P130as	5'-AGT TAT CTG CTC **T**CT CAA GTG TTT-3'
C: P199s	5'-AGT CAT CCT GT**A** GGG GTG GAG GTG-3'
D: P199as	5'-CAC CTC CAC CCC **T**AC AGG ATG ACT-3'
C: P209s	5'-TGG GGG TGG AG**A** TGG GAC AAG GGA-3'
D: P209as	5'-TCC CTT GTC CCA **T**CT CCA CCC CCA-3'
C: P212s	5'-GGG TGG AGG TG**C** GAC AAG GGA AAG-3'
D: P212as	5'-CTT TCC CTT GTC **G**CA CCT CCA CCC-3'

A and C indicate the forward primers (Ref. 10); B and D indicate the reverse primers; bold letters indicate the nucleotide substitutions in relation to the wild-type sequence.

Plasmid DNA was grown in DH5α bacteria and purified (Qiagen Plasmid Maxiprep, Germany). The correct sequences of the constructs were confirmed by sequencing. Only constructs containing the α-MRE in the same genomic orientation as the SV40 promoter were used in the transfection assays.

Nine constructs were obtained: one with the A haplotype, five with the other haplotypes (B-F) and three with the +130, +199 and +209 polymorphisms separately.

Cell culture and DNA transfection assay

Cells from the human K562 erythroleukemia cell line were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin-streptomycin (GIBCO, USA) (37°C, 5% CO_2) at a density of 1 x 10^6 cells per mL (13). Approximately 8 x 10^6 cells were transfected in suspension by electroporation (250 volts, 975 µF, 9 ms) with 20 µg of each construct and 1 µg of pRL-TK plasmid (Promega). The vector pRL-TK, which expresses *Renilla* luciferase, was used as an internal control for electroporation.

Luciferase assays

The cells were harvested 24 h post-transfection. The firefly and *Renilla* luciferase activities were measured in the cell lysates using a luminometer (Turner Designs, USA) and the dual luciferase reporter assay (Promega). The light output was measured for 15 s. The vectors pGL2-Basic and pGL2-Control (Promega) were used as negative and positive expression controls, respectively.

The firefly luciferase activity expressed by the constructs was normalized against the *Renilla* luciferase activity, expressed from the co-transfected pRL-TK vector. Electroporations were performed in triplicate, each experiment was repeated three times and the average expression level for each construct is reported in relative luminescence units.

Results and Discussion

The results of the transient transfection experiments are summarized in Figure 1. Analysis of variance (ANOVA) detected a significant decrease (P = 0.0025) with all the enhancers compared with the A haplotype. Interestingly, the greatest reductions of α-MRE function in K562 cells were observed with the constructs containing the isolated polymorphisms +96 and +209, both located near the SP1 protein-binding sites believed not to be active *in vivo*. These mutations were able to repress the α-MRE enhancer activity almost completely.

Previous studies using site-directed mutagenesis in the nuclear protein-binding sites and transient expression

assays demonstrated that the GATA-1 sites (B, C and D), both NF-E2 sites (5' and 3') and the CACC box (II) positively regulate the α-MRE function in α-like globin genes expression in K562 cells (14,15). Mutations in each of the six protein-binding sites reduced the gene expression by 50 to 90% (13).

Recently, Vernimmen et al. (16) proposed a model to explain the mouse α-globin locus activation during the erythroid differentiation process. According to it, active transcription first requires the recruitment of regulatory proteins and RNA polymerase II to the remote regulatory sequences; only late in the differentiation process is the transcriptional machinery recruited to the α-globin promoters with looping occurring between the regulatory elements and the α-globin genes. De Gobbi et al. (17) investigated gene activation in human erythropoiesis. All known cis-acting regulatory elements in the human α-cluster were identified and no additional erythroid-specific regulatory elements were found (reviewed in Ref. 18).

We analyzed the effects of the SNPs located in flanking sequences of α-MRE binding sites on the luciferase reporter gene expression in K562 cells. This kind of study allowed the simultaneous comparison of all base substitutions described in the α-MRE so far. In the transient expression assay employed here, they substantially reduced gene expression.

Since this type of assay reflects exactly the structure-function relationship during the final stage of transcriptional activation, when promoters and regulatory elements are already physically associated (15), one possible explanation for the present results is that the α-MRE polymorphisms may be causing a reduction of the binding affinity of regulatory proteins to DNA, consequently altering the configuration and function of the regulatory element and thus modifying the luciferase gene expression.

The greatest reductions of luciferase gene expression were observed with the constructs containing polymorphisms located near the SP1 protein-binding sites considered not to be active *in vivo*. These sites, however, have been well-conserved during evolution (2). Since an enhancer can sometimes function as a silencer (19,20), the drastic negative regulatory effect of the polymorphisms next to the CACC boxes suggests that these sites were occupied by factors other than SP1 involved in the positive or negative regulation of the α-MRE function in K562 cells.

Our results represent the first analysis of the effects of α-MRE polymorphisms on gene expression. They indicate that these SNPs, although outside the binding sites for nuclear factors, are able to influence the enhancer activity of the α-MRE. They may be related, *in vivo*, to variable levels of expression of the α-globin genes and α-thalassemia, especially in regions under malarial selective pressure. The hematological phenotype, however, will depend on the final genotype of each individual and compensatory mechanisms and epigenetic regulations occurring in complex organisms. Further experiments identifying DNA-protein interactions at these polymorphic sites should help to understand how these polymorphisms influence gene regulation.

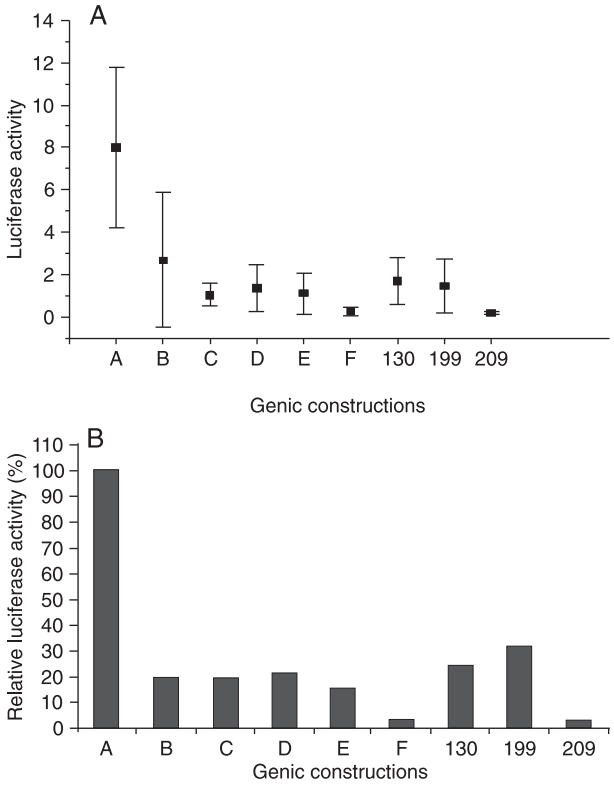

Figure 1. Effect of polymorphisms of the α-major regulatory element (α-MRE) HS-40 on *in vitro* gene expression. *A*, Luciferase activity of the different gene constructions in K562 cells. Data are reported as means ± SD relative luminescence units. *B*, Relative luciferase activities. The medians are shown as a percentage of the activity of the construction containing the wild-type α-MRE (A haplotype), defined as 100%. The gene constructions correspond to haplotypes A (wild-type) to F and to 3 isolated polymorphisms (G→A at positions 130, 199 and 209) of the cloned α-MRE fragment.

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