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EVIDENCE FOR A GLOBIN PROMOTER-SPECIFIC SILENCER ELEMENT LOCATED UPSTREAM OF THE HUMAN δ-GLOBIN GENE

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Summary: We describe the negative regulatory activity of a 1.7 kilobase (kb) region (*R*) in the human β-globin locus located between 4.0 and 2.3 kb upstream of the δ-globin gene capsite, using a transient assay with the chloramphenicol acetyltransferase (CAT) reporter gene in mouse erythroleukemia (MEL) cells. The *R* region is deleted in most cases of deletion hereditary persistence of fetal hemoglobin (HPFH), but is unaffected in most δβ°-thalassemias. However, no experiments addressing its function in globin gene expression have been reported to date. We show that *R* inhibits CAT expression of constructs containing a fetal (γ) or adult (β) globin gene promoter, but does not affect expression of similar constructs using a non-globin (SV40) promoter. The inhibitory effect on the β-globin promoter can be localized to a 651 bp sub-region of *R*. For the γ-globin promoter, no sub-region of *R* can reproduce the level of inhibition associated with the entire region.

The differences between deletion breakpoints found in cases of deletion hereditary persistence of fetal hemoglobin (HPFH) and δβ°-thalassemia have been studied for many years in attempts to explain the difference in the level of HbF between the two phenotypes (1). Evidence has been presented that enhancer sequences downstream of the 3' breakpoint, brought into the vicinity of the fetal (γ) genes by the deletion, might explain the elevated γ-globin expression in some instances of HPFH (2). On the other hand, the presence of negative regulatory elements affecting γ-globin expression in the region between the γ- and δ-globin genes, deleted in HPFH but not in δβ°-thalassemia, has also been postulated (3-6).

Here we examine the effect of a 1.7 Kb region (*R*), 4.0 to 2.3 kb upstream of the δ-globin gene capsite, on globin promoter function, since this region is more often deleted in HPFH than in

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The abbreviations used are: CAT, chloramphenicol acetyltransferase; kb, kilobase(s); bp, base pair(s); HPFH, hereditary persistence of fetal hemoglobin; MEL cells, mouse erythroleukemia cells.

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$\delta\beta^0$-thalassemia (1), and thus could constitute such a negative regulatory element. The region has a number of interesting features: (a) the presence of two *Alu* family repeats; (b) consensus binding sites for ubiquitous (Sp1) and erythroid-specific (GATA-1) transcription factors; (c) several direct and inverted repeats; and (d) sequences homologous to well-characterized silencer elements for the chicken lysozyme (7), yeast ABFI (8), and human ε-globin (9,10) genes (see fig. 1). To address the possible regulatory role of this region we subcloned it, in both orientations, downstream of a CAT gene driven by either the γ- or β-globin promoters or a non-globin (SV40) promoter, and transfected the resulting plasmids into mouse erythroleukemia (MEL) cells. The CAT activities for each promoter were compared in the presence and absence of *R*. The inclusion of *R* resulted in an approximately six-fold reduction in activity for both globin promoter-driven constructs, while no effect of *R* was evident for the SV40 promoter-driven construct. To characterize the minimal sequence (or sequences) responsible for the observed effect, we similarly analyzed five restriction fragments of *R* for the ability to affect globin promoter function. In the case of the β-promoter-containing construct, we found much of the inhibitory activity could be localized to a 651 bp sub-region which contains one of the *Alu* repeats. For the γ-promoter-containing construct, however, no sub-region could reproduce the inhibitory effect of *R*.

METHODS

Plasmids. Plasmids containing the CAT gene driven by γ- and β-globin promoters (pγCAT and pβCAT) were kindly provided by Dr. S. Ottolenghi (11, 12). The γ-globin promoter includes sequences from -299 to +35 relative to the capsite; the β-globin promoter from -276 to +49. Plasmid pSV2CAT, provided by Dr. S. Feo (University of Palermo), contains 323 bp of SV40 promoter sequence. The *R* region was obtained using the polymerase chain reaction with specific primers and analyzed by restriction mapping (see fig. 1). The *R* region was subcloned into the various CAT plasmids by insertion into a Bam HI site downstream of the CAT gene. All of the various sub-regions of *R* (see fig. 1) were obtained by restriction enzyme digestion and gel purification and were similarly cloned into pγCAT and pβCAT.

Transfections and CAT assays. Transfections were performed using the DOTAP liposomal transfection reagent (Boehringer Mannheim) with minor modifications to the manufacturer's instructions. For each transfection, 1 x 10^6 cells in 5 ml of tissue culture medium were plated at 7 to 8% CO_2. Five μg of plasmid DNA was dissolved in 150 μl of HBS (150 mM NaCl, 20 mM HEPES, pH 7.4). In a different tube, 40 μl of DOTAP was mixed with 110 μl of HBS and then added to the DNA solution. After incubation at room temperature for 10 minutes, the mixture was added to the cells. Twenty four hours later, 5 ml of fresh medium was added to each plate. The next day the same number of cells from each transfection was collected, and CAT assays were performed as described (13) using [^{14}C]-chloramphenicol.

To control for transfection efficiency, 10% of the cytoplasmic lysate was used for dot blot analysis as described (14), using [^{32}P]-labeled pSV2CAT plasmid as probe. Signals on dot blot (see fig. 2) were quantitated with a beta counter. Percent acetylated [^{14}C]-chloramphenicol for each transfected clone was determined by cutting out thin layer chromatography spots corresponding to acetylated and unacetylated chloramphenicol (see fig. 2) and counting in a scintillation counter. Total counts of acetylated chloramphenicol was divided by total counts of unacetylated plus acetylated chloramphenicol and multiplied by 100 to give the percent acetylated [^{14}C]-chloramphenicol. This value was then divided by the total counts obtained on dot blot analysis of the same clone to give the percent CAT activity corrected for transfection efficiency.

RESULTS

Effect of DNA region *R* on globin and non-globin promoter activity. For each plasmid analyzed, a minimum of six independent transfections were performed. Average

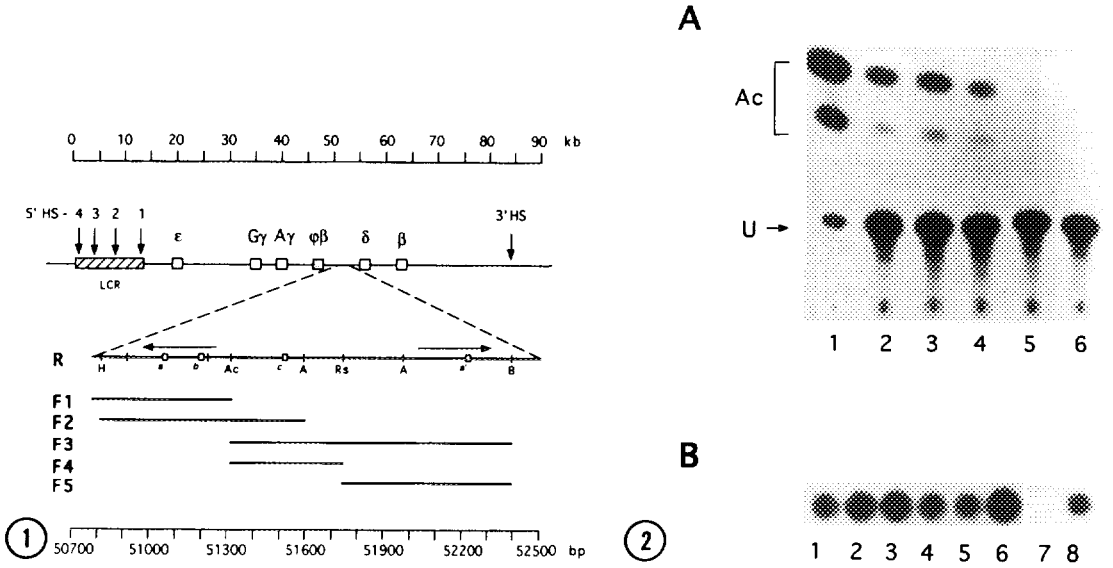

Figure 1. Diagram of the human β-globin gene cluster on chromosome 11, with an expanded view of region *R*. Horizontal arrows mark the position and orientation of *Alu* family sequences. Boxes *a*, *b* and *c* represent short sequences with homology to well-characterized silencer elements (*a*: ATTCTCCTNY in chicken lysozyme box 2 and human ε-globin; *b*: ACCCTCYYY in lysozyme box 1 and ε-globin; *c*: TATCATYNNNNA in yeast ABFI and ε-globin). Sequence a' in the 3' *Alu* repeat is almost identical to sequence a in the 5' *Alu* sequence. The restriction sites indicated (H=*Hgi* AI; A=*Ava* I; B=*Bgl* II; Ac=*Acc* I; unmarked and Rs=*Rsa* I) show the enzymes used to obtain the 5 sub-regions analyzed.

Figure 2. (A) Autoradiogram of a typical CAT assay thin layer chromatography plate. Lane 1, pβCAT; lane 2, pβCAT-R >; lane 3, pβCAT-R <; lane 4, pγCAT; lane 5, pγCAT -R >; lane 6, pγCAT-R < (> indicates the 3' insert in forward orientation, < indicates the insert in reverse orientation). Unacetylated (*U*) and acetylated (*Ac*) forms of [^{14}C]-chloramphenicol are indicated. (B) Dot blot assays of the same samples assayed in (A) using pSV2CAT as probe. Lanes 1 through 6 are the same as in (A). Lane 7, 200 ng of λ-DNA as negative control; lane 8, 100 ng of pSV2CAT as positive control. CAT activities were normalized for transfection efficiency as described in Methods.

CAT activity was compared for plasmids with and without region *R*, corrected for transfection efficiency as described in Methods. As seen in figure 3, in MEL cells the inclusion of *R* conferred an approximately 6-fold reduction in CAT activity to plasmids containing either γ- (pγCAT) or β- (pβCAT) globin promoters. This effect was independent of the orientation of the *R* insert. No such effect was observed with plasmids driven by the SV40 promoter (pSV2CAT, fig. 3c).

Effect of different sub-regions of *R* on the β- and γ-globin promoters. To define the DNA sequence (or sequences) responsible for the globin promoter-specific inhibitory activity of *R*, we analyzed five restriction fragments of the region (see fig.1) for their effect on pγCAT and pβCAT activity in MEL cells. Figure 3a shows that sub-region F5 comes very close to reproducing the effect of region *R* on β promoter activity, causing a 5-fold decrease in pβCAT activity in an orientation independent manner (figure 3a, pβCAT-F5). The plasmid with sub-region F3 in forward orientation (pβCAT-F3, black box) also shows a five-fold decrease in CAT

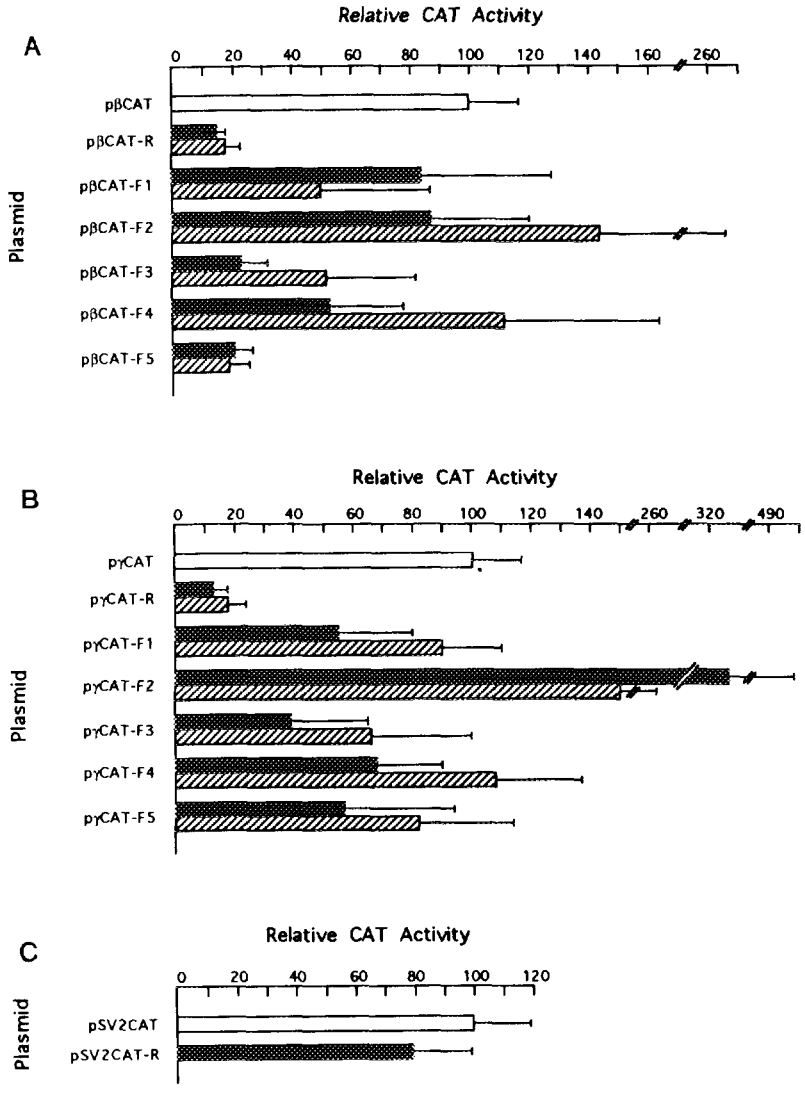

Fig. 3. (A) Relative CAT activities obtained with β-globin promoter-containing constructs. The basal activity of the β-globin promoter (pβCAT) is assigned a relative value of 100%. CAT activities represented by black boxes are for 3' inserts in forward orientation relative to the promoter; striped boxes represent activities for plasmids with the 3' insert in reverse orientation. The various sub-regions of *R* (F1 to F5) are the same as those shown in figure 1. (B) Relative CAT activities obtained with γ-globin promoter-containing constructs, with basal CAT activity of the γ-globin promoter (pγCAT) assigned a relative value of 100%. (C) CAT activities for the SV40 promoter with and without region *R* (in forward orientation only).

activity compared to pβCAT. In reverse orientation (pβCAT-F3, striped box), however, the reduction in CAT activity is to only approximately 50% of pβCAT. Sub-regions F1, F2 and F4 have no significant effect on βCAT activity. The inhibitory effect of *R* observed on the γ-globin promoter is not reproduced by any of the individual fragments from the region (fig. 3b). The plasmid containing sub-region F3 in the forward orientation (pγCAT-F3, black) shows a reduction

in CAT activity to approximately 40% of that of pγCAT, but in reverse orientation F3 reduces CAT activity to only about 65% of baseline. The results indicate that the six-fold reduction in CAT activity can only be accomplished with the γ promoter when the *R* region is intact.

DISCUSSION

On the basis of the difference in the 5' breakpoints among δβ°-thalassemia and HPFH phenotypes, it has been postulated that negative regulatory elements affecting γ-globin gene expression exist in the region between the γ- and δ-globin genes, since deletions removing this region result in the continued expression of γ-globin in adult life (1, 3-6). Recently a novel δβ°-thalassemia (Eastern-European) has been described (15) with a 3' breakpoint similar to HPFH-5 (16) but with a 5' breakpoint closer to the δ gene. The DNA region which is retained in the Eastern European δβ°-thalassemia and deleted in HPFH-5 (and thus may function in silencing the γ genes in adult cells) corresponds almost exactly to the *R* region used in our experiments.

We show that *R* has an inhibitory effect on globin promoters, but exerts no effect on a non-globin (SV40) promoter. It is perhaps unexpected that region *R* can inhibit the β-globin promoter as well as the γ-globin promoter. We do not know, however, how this region would interact with the globin promoters *in vivo*. It may be that other DNA sequences within the β-globin gene cluster are necessary in conjunction with *R* for it to exert its full negative effect on the γ promoter but not on the β promoter. For example, a region adjacent to *R*, one kb 5' to the δ gene, may also contain DNA sequences - and bind protein factors - which affect γ gene silencing (17). Such synergistic interactions among multiple elements upstream of a minimal promoter has been already reported for the human ε-globin gene (18). We do not know the mechanism of the inhibitory effect of region *R* (and sub-regions F3 and F5) on globin promoters, but preliminary evidence in our laboratory indicates that it may involve the interaction between these DNA sequences and developmental stage-specific DNA-binding factors (unpublished data).

It is not clear why we were unable to reproduce the inhibitory effect of *R* on the γ promoter with the various sub-regions of *R*, since we were able to do this with the β promoter. It may be that the entire region must remain intact for it to function in γ gene inhibition, or that we disrupted an important binding site for a protein factor when creating the sub-regions used in our experiments. It has been observed that some DNA-binding factors interact poorly with short DNA sequences, and need additional adjacent sequences for binding, possibly because of a particular DNA structure requirement (17).

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