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Identification of a transcriptional silencer in the 5′-flanking region of the human ε-globin gene

(ε-globin gene silencer/transcriptional regulation)

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ABSTRACT **We have studied the 5′-flanking sequences required for the transcriptional regulation of human ε-globin gene expression. A series of deletion mutants of the human ε-globin gene 5′-flanking sequences were constructed and linked to the bacterial chloramphenicol acetyltransferase gene. Expression of these constructs was tested in HeLa cells and the human erythroleukemia K-562 cells. By measuring chloramphenicol acetyltransferase activities and mRNA levels we found that the sequence between −177 and −392 base pairs (bp) relative to the mRNA initiation site exerts a negative effect on ε-globin promoter activity. This effect is more pronounced in HeLa cells compared with K-562 cells. To further characterize the negative control region we cloned the DNA sequence between −177 and −392 bp either 5′ or 3′ of the ε-globin promoter and in either orientation. Our data indicate that this negative control region inhibits the ε-globin promoter activity in a position- and orientation-independent manner, thus suggesting that it is a silencer. In addition, the silencer also inhibits the expression from the *Herpesvirus* thymidine kinase promoter. Sequence comparison reveals that there are three short regions within the silencer that share extensive homology with those found in other negative control DNA elements. Our results therefore indicate that an upstream silencer element is present in the ε-globin gene and that it may play an important role in the control of ε-globin gene expression during development.**

The human embryonic ε-globin gene is a member of the β-like globin gene family. This gene is expressed in a tissue-specific and developmental stage-specific manner (1). During early gestation the ε-globin gene is expressed in yolk sac islands in the embryo. At ≈5 weeks of gestation, the site of hematopoiesis shifts to the liver, the embryonic ε-globin gene is gradually turned off, and the fetal γ-globin gene is turned on. This transition is completed well before 10 weeks of gestation. Although there are cellular models to explain the regulation of developmental switching, the molecular mechanisms governing this switching are still not clear.

The ε-globin gene, like other globin genes, has been shown to contain several promoter elements within the first ≈100 base pairs (bp) 5′ of the RNA initiation site. Two typical higher eukaryotic promoter elements, the TATA box and the CCAAT box, are located at −28 bp and −82 bp, respectively (2). A third conserved sequence, the CCAAC box, which is common to the β-like globin genes (3), is found at −110 bp. Functional analysis by linker-scanning and point mutation revealed that the three conserved elements are necessary for promoter function in the β-like globin genes (3–7). Deletion analysis showed that the sequences upstream from the CACCC element are not required for constitutive promoter activity (5, 6, 8). While the known conserved promoter elements seem to play an essential role in promoting nonregulated or constitutive expression of the globin genes, several enhancer elements have been shown to play a role in tissue- or development-specific regulation. Hesse *et al.* (9) reported that the chicken adult β-globin gene contains such an enhancer in the 3′-flanking region. The human β-globin gene was also found to contain two tissue-specific enhancers located in the structural β-globin gene and 3′-flanking sequences, respectively (10, 11), and in the human γ-globin gene an enhancer with no apparent tissue specificity has been found (12).

Many other genes have been shown to contain distal promoter control elements extended further upstream of the canonical promoter region. In many cases these elements are involved in regulation of tissue-specific expression. Examples include the insulin (13), chymotrypsin (13), interferon β (14, 15), and albumin genes (16). Because the 5′-flanking sequences of the ε-globin gene have not been studied in detail, except for the known conserved promoter elements, we set out to investigate whether there are sequences upstream of the CACCC box that influence the promoter activity.

By introducing plasmids containing deletions of 5′-flanking regions into cultured cells, we have identified a DNA fragment between −392 and −177 bp relative to the cap site that negatively regulates ε-globin promoter activity. This negative control element acts both 5′ and 3′ of the transcription unit and in an orientation-independent manner, thus complying with the definition of a silencer (17). Comparison of the inhibitory effect in HeLa cells and K-562 cells showed that the negative effect of the silencer is stronger in the former. By sequence analysis we found that several short regions within the silencer element share extensive homology with sequences found in the negative regulatory elements of other genes. Our results suggest that this silencer element may play an important role in the regulation of ε-globin gene activity.

MATERIALS AND METHODS

Plasmid Constructs. The ε-globin deletion mutants were constructed by deleting part of the ε-globin 5′-flanking sequences from pεGLCAT (18) by using appropriate restriction enzyme sites. The restriction enzymes (Bethesda Research Laboratories) used were *Hin*cII and *Bam*HI for pεΔ392 and pεΔ177, respectively.

Tissue Culture, Transfection, and CAT Assay. K-562 cells (ATCC CCL243) were maintained in RPMI 1640 medium containing 10% fetal calf serum. HeLa cells (ATCC CCL2) were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. For transfection, cells were plated 24 hr before transfection at a density of 5×10^6 cells per

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Abbreviation: CAT, chloramphenicol acetyltransferase.

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100-mm dish and 1×10^6 cells per 100-mm dish for K-562 and HeLa cells, respectively. The method described by Wigler *et al.* (19) was used for transfection, except that the DNA precipitate was kept on the cells for 7–8 hr and the cells were harvested 48 hr after transfection. Under our standard conditions the transfection efficiencies within one experiment were very similar as determined by Southern blot and slot blot hybridizations after Hirt extraction (20). Chloramphenicol acetyltransferase (CAT) assays were performed by the methods of Gorman *et al.* (21), except that protein extracts were preheated at 65°C for 10 min to destroy interfering activity (18). Each CAT assay contained 1000 μg or 500 μg of protein and was incubated for 12 hr or 2 hr for K-562 cells or HeLa cells, respectively. CAT activities were determined under such conditions that the conversion of the standard plasmid pεΔ177 was at ≈10% for K-562 cells and ≈50% for HeLa cells. Under these conditions promoterless constructs (pSVOCAT) had <5% (usually 1–2%) of the activity of pεΔ177 [see figure 2 in Cao *et al.* (22)].

RNA Isolation and Analysis. Total RNA was isolated by using a modified procedure of Auffray and Rougeon (23). Transfected cells were washed once with cold phosphate-buffered saline. Cells were dissociated from dishes by either pipeting (K-562) or trypsin-EDTA (HeLa) treatment and washed twice with cold phosphate-buffered saline, and then lysed by adding 3 M LiCl/6 M urea and stirred vigorously in a Vortex mixer. The cell lysate was stored at −20°C. The RNA pellet was recovered by centrifugation and dissolved in urea/Tris solution (7 M urea/10 mm Tris·HCl, pH 8.0/200 mM NaCl/2% SDS), extracted twice with phenol/chloroform, and precipitated by ethanol. The RNA was then treated with DNase I (Promega Biotech) at a concentration of 30 units per 200 μg of RNA at 37°C for 20 min followed by phenol/chloroform extractions and ethanol precipitation. RNA analysis was performed using an RNase T1 system (Riboprobe, Promega Biotech) according to the manufacturer's recommended procedure (Promega Biotech). The RNA probe was synthesized from pGEMHE plasmid by using SP6 RNA polymerase as described (22). pGEMHE was constructed by inserting the *Eco*RV–*Eco*RI fragment spanning the ε-globin gene cap site (from −274 bp to +277 bp with respect to the cap site of pεGLCAT) into the *Eco*RI–*Hin*cII site of pGEM-4 in the antisense orientation.

RESULTS

The Sequence Between −177 bp and −392 bp Is a Negative Control Region. In a previous report we described the presence of a negative control element upstream of −274 bp of the ε-globin gene (22). In this report, we have further delineated this negative region. To encompass the probable full extent of the negative element, we have primarily compared our −177-bp deletion construct with a new deletion mutant at −392 bp. These ε-globin gene 5′ deletion mutants were fused to the bacterial CAT gene to generate plasmids pεΔ177 and pεΔ392 (Fig. 1*A*). These plasmids were transfected into either the human erythroleukemia K-562 cells or HeLa cells (nonerythroid cells), and CAT activities were determined. Analysis of the CAT activities revealed a similar expression pattern in both K-562 cells and HeLa cells: pεΔ177 showed a higher level of expression than pεΔ392 (Table 1). This difference suggested that the DNA sequence between −177 bp and −392 bp contains a negative control element to the ε-globin promoter. Further deletions 5′ of −392 bp did not show decreased expression compared with pεΔ392 (data not shown). To quantitate the difference in CAT activity between pεΔ177 and pεΔ392, we normalized the CAT level of pεΔ392 to pεΔ177, and the results are shown in Table 1. While the negative control element inhibits ε-globin promoter activity

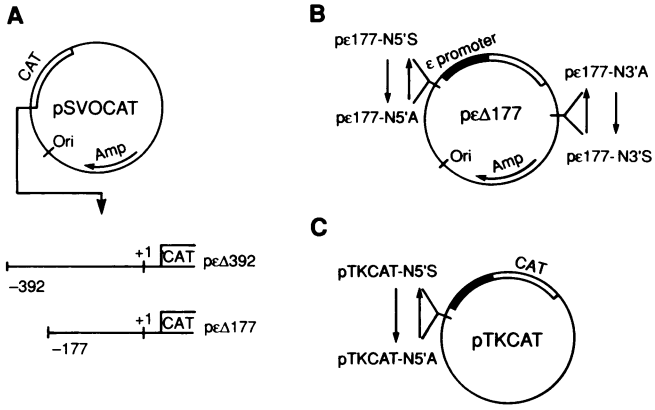


FIG. 1. Plasmid structures. (*A*) Plasmids pεΔ392 and pεΔ177. Numbers indicate the length of the 5′-flanking sequences relative to the ε-globin gene cap site. (*B*) Plasmids pε177-N5′S, pε177-N5′A, pε177-N3′S, and pε177-N3′A. The 215-bp negative control region was subcloned into pεΔ177 either 5′ or 3′ of the transcriptional unit in both sense and antisense orientations to generate the four plasmids. (*C*) Plasmids pTKCAT-N5′S and pTKCAT-N5′A. The 215-bp negative control region was inserted 5′ of the thymidine kinase promoter in either sense or antisense orientation.

in both HeLa cells and K-562 cells, the level of inhibition is higher in HeLa cells (10-fold) than in K-562 cells (3-fold).

To determine whether the levels of CAT activity reflected changes in CAT mRNA level, we measured the steady-state CAT mRNA concentration in HeLa cells transfected with pεΔ392 and pεΔ177 by RNase T1 analysis. The probe used in the RNA analysis was synthesized from pGEMHE. Correctly initiated CAT mRNA would protect a fragment of 277 nucleotides from RNase T1 digestion. As a control for transfection efficiency and RNA recovery, we included the plasmid pSV2neo in each transfection as an internal control. Fig. 2 shows that while levels of neo mRNA are comparable in cells transfected with pεΔ177 and pεΔ392, the CAT mRNA level was much higher in pεΔ177-transfected cells. This is consistent with the CAT assay results and indicates that the negative control element inhibits transcription. Note that there are also transcripts initiated from two minor upstream initiation sites, and their expression levels appear not to be influenced by the negative control region (Fig. 2).

The Negative Control Region Acts as a Silencer. To determine whether the negative control region can act as a silencer (17, 24, 25), i.e., in a position- and orientation-independent manner, we tested the effects of the fragment on the highest expression plasmid pεΔ177 when it is placed in different positions and orientations. The 215-bp negative control region was subcloned either 5′ or 3′ of the transcriptional unit and in either orientation. The resulting plasmids, pε177-N5′S, pε177-N5′A, pε177-N3′S, and pε177-N3′A represent the constructions with the negative control region inserted in pεΔ177 in 5′ sense, 5′ antisense, 3′ sense, and 3′ antisense configurations, respectively (Fig. 1*B*). These plasmids were transfected into HeLa cells and K-562 cells, and CAT activities were determined. The results are shown in Table 2.

Table 1. CAT activities of 5′ deletion mutants in HeLa cells and K-562 cells

	pεΔ177	pεΔ392
HeLa	1.00 ± 0.07	0.11 ± 0.02
K-562	1.00 ± 0.04	0.37 ± 0.03

HeLa cells and K-562 cells were transfected with either pεΔ177 or pεΔ392, and CAT activities were determined as described. For each cell line the CAT activity of pεΔ177 was set as 1 and that of pεΔ392 was normalized to pεΔ177. The values represent the average ± SD of three independent transfection experiments.

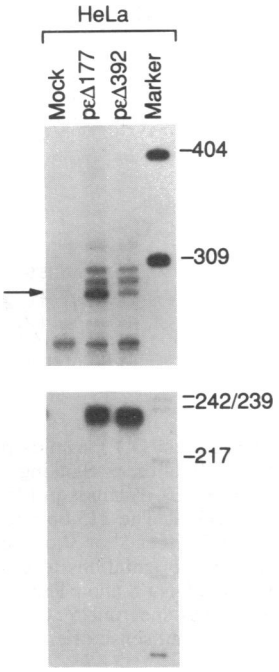


FIG. 2. RNA protection analysis of HeLa cells transfected with pεΔ177 and pεΔ392. HeLa cells were transfected with either pεΔ177 plus pSV2neo or pεΔ392 plus pSV2neo. Total RNA was isolated and analyzed by use of a Riboprobe in RNase T1 mapping as described by Promega Biotech; 20 μg of RNA was used in each analysis. (*Upper*) Arrow indicates the band protected by correctly initiated CAT mRNA. Exposure time was 25 hr. (*Lower*) Bands are internal control generated by neo mRNA. Exposure time was 4 hr.

Several conclusions can be drawn. (*i*) The negative control region inhibits ε-globin promoter activity in both HeLa cells and K-562 cells when this region is placed both 5′ and 3′ of the promoter and in both sense and antisense orientations; this fact indicates the negative control region is a silencer. (*ii*) The silencer has stronger inhibitory effect when it is 5′ of the promoter than when 3′ of the promoter. (*iii*) The inhibitory effect appears to be more prominent in HeLa cells than in K-562 cells. For example, when the negative control region was 5′ of the promoter, it produced 14-fold and 25-fold inhibition for sense and antisense orientation, respectively, in HeLa cells compared with 3-fold inhibition in K-562 cells.

The ε-Globin Gene Silencer Inhibits the Expression of pTKCAT. We next asked whether the ε-globin gene silencer acts on a heterologous promoter. To test this hypothesis the 215-bp fragment was cloned 5′ of pTKCAT, the herpes simplex virus thymidine kinase promoter–CAT plasmid (Fig. 1*C*). The resulting constructions, pTKCAT-N5′S and pTKCAT-N5′A as well as pTKCAT were introduced into HeLa cells, and CAT activity was determined. As shown in Table 3, CAT activities were strongly inhibited by the ε-globin gene silencer in either orientation. This result further confirms that the DNA sequence between −177 and −392 of the ε-globin gene is a silencer.

Table 3. Effects of the negative control region on the expression of pTKCAT

Relative CAT activity		
pTKCAT	pTKCAT-N5′S	pTKCAT-N5′A
1.00 ± 0.04	0.25 ± 0.12	0.10 ± 0.04

HeLa cells were transfected with plasmids as indicated. The CAT activity of pTKCAT was set to 1, and the others were normalized to pTKCAT. Each value represents the average ± SD of two independent transfection experiments.

DISCUSSION

By introducing recombinant plasmids into a transient expression system, we have identified a transcriptional silencer that negatively regulates expression from the ε-globin gene promoter. The silencer element is located between −177 and −392 bp relative to the cap site. This region has been shown to contain a DNase I-hypersensitive site at −200 bp in K-562 cells (26). Considering the close proximity of the silencer to the promoter elements (ATA, CCAAT, and CACCC), the silencer is likely to participate in the regulation of ε-globin gene activity.

Many findings suggest that sequences relevant to globin promoter function extend beyond the conserved promoter elements (8). For example, there are nuclease-hypersensitive sites 150–200 bp 5′ of the mRNA initiation site of the human ε-, γ-, and β-globin genes in cells or tissues where the genes are expressed (26–28). The chicken β-globin gene has a nuclease-hypersensitive region extending from −60 to −260 bp upstream from the cap site (29), and specific protein binding to 5′ portion of the region has been demonstrated (30). In the γ-globin gene, point mutations at −202 and −196 are associated with the syndrome of hereditary persistance of fetal hemoglobin (31, 32). Our findings provide further evidence supporting the functional importance of more distal sequences in the regulation of the β-like globin gene family. In the case of the ε-globin gene, while the conserved elements immediately upstream of the cap site produce constitutive promoter activity, the silencer can exert further negative regulation.

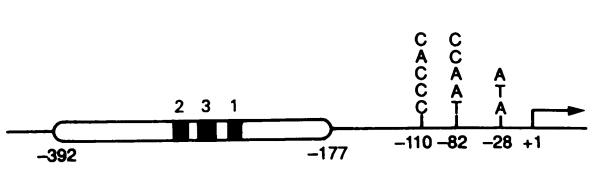
Gilmour *et al.* (33) have described the presence of a 1.11-kilobase negative regulatory sequence 5′ to the mouse β^{maj}-globin gene. Recently, Atweh *et al.* (34) reported a silencer from the human α-globin gene that inhibits simian virus 40 enhancer-dependent β-globin gene expression. The ε-globin gene silencer is distinct from the α-globin gene silencer in that it inhibits its own promoter at the natural position and, in this case, independent of an exogenous enhancer.

A comparison of sequences of the ε-globin gene silencer and other negative regulatory elements showed that there are three short regions that shared extensive homology among these genes (Fig. 3). There are two short regions from −244 to −252 and from −284 to −293 in the ε silencer that share 80–90% sequence homology with the box 1 and box 2 sequences found in the chicken lysozyme silencer and other negative regulatory elements (25), although in the opposite strand (Fig. 3 *B* and *C*). Furthermore, the two mismatched nucleotides in each homology region are all pyrimidine → pyrimidine substitutions. The

Table 2. Effects of the negative control region on the expression of pεΔ177

	Relative CAT activity				
Cells	pεΔ177	pε177-N5′S	pε177-N5′A	pε177-N3′S	pε177-N3′A
HeLa	1.00 ± 0.14	0.07 ± 0.02	0.04 ± 0.01	0.12 ± 0.04	0.27 ± 0.05
K-562	1.00 ± 0.08	0.35 ± 0.04	0.36 ± 0.06	0.43 ± 0.04	0.45 ± 0.11

HeLa cells and K-562 cells were transfected with plasmids as indicated, and CAT activities were determined. The CAT activity of pεΔ177 was set to 1, and the others were normalized to pεΔ177. Values show the average ± SD of three independent transfection experiments.



B Homology Region 1

ACCCTCTCT	Chicken Lysozyme Box 1
ACCCTCTtc	Human ε-Globin (-244 – -252)

C Homology Region 2

ATTCTCCTCC	Chicken Lysozyme Box 2
ATTCTCCTtt	Human ε-Globin (-284 – -293)

D Homology Region 3

TATCATTNNNNACGA	Yeast ABFI Binding Sequence
TATCATTTTGGAaGA	Human ε-Globin (-271 – -257)

FIG. 3. Comparison of the ε silencer and the negative consensus sequences. (*A*) Position of the ε-silencer in the ε-globin gene 5′-flanking region. The sequence between −392 and −177 is the silencer. The three black areas labeled 1, 2, and 3 represent the three homology regions shown in *B*, *C*, and *D*, respectively. Regions 1 and 2 are on the noncoding strand in the opposite orientation to that in the chicken lysozyme gene. (*B*) Homology region 1 and the box 1 sequence of the chicken lysozyme silencer. (*C*) Homology region 2 and the box 2 sequence of the chicken lysozyme silencer. (*D*) Homology region 3 and the consensus sequence of the ABFI protein binding site in a yeast silencer. N represents any nucleotide.

region between −271 and −257 shares more than 90% homology with the consensus sequence of a protein-binding site in a yeast silencer (35) (Fig. 3*D*).

The role of the silencer in regulation of the ε-globin gene activity during development awaits further study. At the present time we speculate that it could act either in a tissue-specific manner or in a developmental stage-specific manner. For example, the silencer could be active in nonerythroid tissues or at the end of embryonic stage of development, therefore down-regulating ε-globin gene expression. The fact that the ε silencer has a stronger effect in HeLa cells (nonerythroid cell line) than in K-562 cells (erythroid cell line) supports the idea that this silencer plays a role in conferring tissue-specific expression to the ε-globin gene. On the other hand, the fact that this silencer is partially active in K-562 cells [which seem to be arrested in an embryonic fetal stage (36, 37)] may indicate that it acts during the developmental switching process.

Note Added in Proof: Since submission of this manuscript, N. J. Proudfoot, P. Lamb, and P. Watt (Sir William Dunn School of Pathology, University of Oxford, Oxford, England) have communicated to us data showing evidence of negative regulation of the human ε-globin and ζ-globin genes.

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