

Activation of the δ -Globin Gene by the β -Globin Gene CACCC Motif

Submitted 02/22/99; revised 07/12/99

(communicated by George Stamatoyannopoulos, M.D., Dr. Sci., 07/13/99)

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ABSTRACT: The promoter region of adult β globin genes in humans and other mammals contains conserved regions of pivotal importance for their regulated tissue specific expression. These include the CACCC and CAAT motifs. The CACCC motif is duplicated in humans and others mammals. The human δ -globin gene lacks these conserved regions and its expression in normal individuals is about 3% that of the β globin gene. Previous studies have shown that the introduction of the β -globin CACCC or CAAT can activate the δ -globin gene promoter, but the effect of the distal CACCC element has not yet been tested. In the present study, using site-specific mutagenesis, we have introduced the consensus sequence for the distal and proximal CACCC motif and the CAAT box alone or in combination in the wild-type δ -globin gene promoter. The resulting mutants, as well as the wild type (wt) δ - and β -globin gene promoters, have been analyzed in a transient expression assay in Cos7, K562, and MEL cell lines. The results show that the CACCC boxes can increase the transcription efficiency of the δ -globin gene promoter in both erythroid and non-erythroid cell systems. The contribution of the two CACCC elements is almost equal in the non-erythroid (Cos7) and erythroid embryonic-fetal cell lines (K562), while the proximal CACCC element is more active in adult erythroid cells (MEL). Nonetheless, duplication of this element does not appear to affect the efficiency of the promoter synergistically. Furthermore, to assess the competitive ability of the δ globin promoter containing the proximal or distal CACCC consensus sequences over the wt β globin gene promoter, we have carried out transient expression experiments using DNA constructs in which the δ and β globin gene promoters are linked in cis and are sharing a single enhancer (competitive transient expression). The results show that both CACCC elements are able to activate the δ globin gene promoter in Cos7 and K562 cells, although to a different extent, whereas only the proximal CACCC element is effective in increasing the transcription efficiency in MEL cells. These findings are in agreement with the more severe clinical phenotype produced by the β -thalassemia mutations affecting the proximal CACCC box as compared with those within the distal CACCC box. The Erythroid Kruppel Like Factor (EKLF) is a nuclear protein restricted to erythroid cells which specifically bind the CACCC box sequence and activate the β -globin gene. In the present study we carried out transactivation experiments of the mutagenized δ -globin gene promoter by introducing an EKLF expressing construct in erythroid cells. Constructs containing the proximal but not those bearing the distal CACCC element are transactivated. Our results indicate that the proximal CACCC box and, to a lesser extent, also the distal box have a role in the regulated stage specific expression of a β -like globin gene, and show that the insertion of a single CACCC motif in the δ -globin gene promoter is sufficient to increase its activity. Nevertheless only the δ globin gene promoter containing the proximal CACCC element is able to compete with the wt β globin gene promoter in the adult erythroid environment. These findings have potential relevance for the future prospective treatment of inherited hemoglobinopathies based on the conversion of the low functioning δ -globin gene into a high functioning β -like globin gene.

Keywords: globin gene promoters, expression in non-erythroid and erythroid cells, CACCC and CAAT boxes

INTRODUCTION

The promoter region of the adult mammal β -globin genes contains evolutionary conserved sequences that are essential for its regulated tissue-specific expression (1-5). They include the TATA, CAAT, CACCC and GATA1 consensus sequences. These DNA motifs bind ubiquitous as

well as tissue specific transacting factors, of which the erythroid specific factor Erythroid Kruppel-like Factor (EKLF), a zinc fingers protein, which binds the CACCC motif, is essential for the activation of the β globin gene in definitive adult erythroid cells (6-9).

The CACCC box of the β -globin promoter is duplicated in humans, in a large proportion of the primates and in some other mammals. The

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relevance of this duplication for β -like promoter activity has not yet been clarified. Deletional and random point mutagenesis of the β -globin gene promoter has demonstrated the critical role of the proximal CACCC motif (from position –85 to –92 from the Cap site) in determining the overall expression of the gene but it has also indicated the existence of a not negligible effect of the distal element (from position –96 to –101 from the Cap site) (2-4). Comparative analyses of the phenotype of β -thalassemia mutations affecting either the proximal or the distal CACCC box have shown a relatively more severe phenotype from mutations affecting the proximal box as compared to those of the distal box, which when mutated usually result in the very mild β -thal silent phenotype (10-13).

In normal adults the δ -globin gene, which has a high homology with the β -globin gene, produces less than 3% of the total β -like globin chains. Instability of the δ -globin mRNA, a lack of intragenic enhancer and/or absence of consensus CAAT and CACCC boxes in the promoter are the molecular mechanisms proposed to explain its low expression (14-17). The insertion of the proximal CACCC motif and the replacement of the CCAAC motif, 60 bp upstream from the Cap site with a normal CCAAT box have recently been shown to activate the δ -globin gene promoter in HeLa, K562 and MEL cells (18,19). More recently activation of the δ -globin gene promoter by the insertion of the consensus sequence for the proximal CACCC box has also been obtained in adult primary erythroid cells (20). These studies, however, have not evaluated the role if any of the distal CACCC box and the potential synergistic effect of the combination of both CACCC boxes and CAAT box inserted at the location corresponding to their original position in the β -globin gene.

The aims of this study are: (a) to determine the feasibility of activating the δ -globin gene promoter by inserting the proximal and distal CACCC motifs as well as the CAAT box of the

wt β -globin gene alone or in combination; (b) to evaluate the activation of the mutagenized δ -globin gene promoter by EKLF; (c) to assess the competitive ability of the CACCC containing δ -globin gene promoter over the wt β -globin gene promoter. The results suggest that (a) in a transient expression assay both CACCC motifs are able to activate the δ -globin gene promoter; (b) in the adult erythroid environment (MEL cells) the proximal CACCC element is more active than the distal one in this effect; (c) EKLF binds and transactivates only the δ -globin gene promoter that contains the proximal CACCC motif; (d) the proximal but not the distal CACCC element activates the δ -globin gene promoter in competition with the wt β -globin gene promoter in adult erythroid environment (MEL cells).

These results have potential significance for future perspectives of activation of the δ chain production in the treatment of sickle cell anemia and β -thalassemia.

MATERIALS AND METHODS

Expression Vectors

Transfection studies have been performed using constructs generated by cloning the δ - (or β -) globin gene promoter into the expression vector pESL for erythroid cells (K562 and MEL cells) and into the pSVAL vector for non-erythroid cells (COS-7 cells). The resulting δ pESL and δ pSVAL constructs are schematically shown in Fig. 1A. The pESL is an eukaryotic expression vector based on the luciferase reporter gene, driven by an erythroid enhancer deriving from the Bal I/Ssp I fragment of the hypersensitive site 2 (5'-HS2) (21). The pSVAL is an SV40 based luciferase vector derived from the plasmid pSV2A1 Δ 5 (22); it maintains the SV40 enhancer but lacks the origin of replication and the early and late promoter regions of the virus. The δ -globin promoter was obtained by

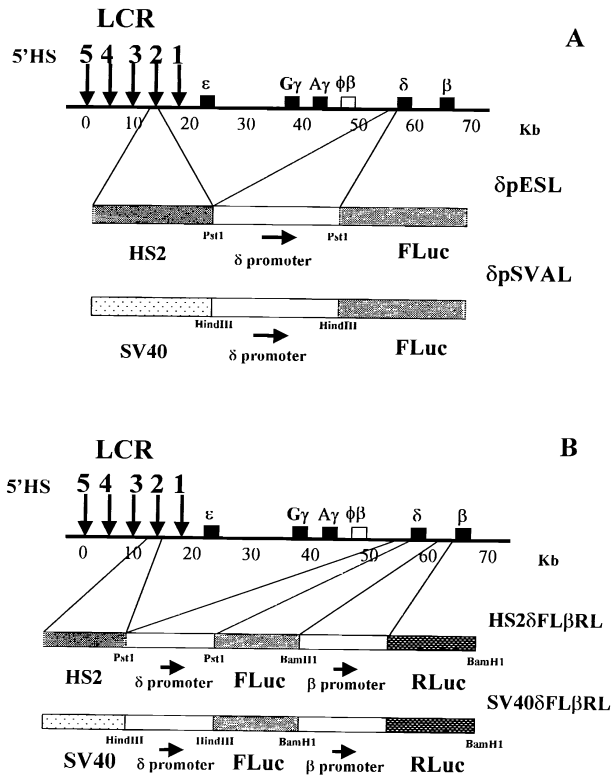


Figure 1. Schematic representation of the constructs used in the present study. FLuc: Firefly Luciferase cDNA; RLuc Renilla Luciferase cDNA. Arrows indicate the orientation of the δ promoter. Restriction enzymes site used in the cloning procedure are indicated. (A) Schematic representation of the constructs used in the non competitive transient assays: δ pESL and δ pSVAL. (B) Schematic representation of the constructs used in the competitive transient assays: HS2 δ FL β R β RL, SV40 δ FL β R β RL.

Identical constructs with the β globin gene promoter in place of the δ globin gene promoter have been produced for this study (β pESL, β pSVAL, HS2 β FL β R β RL, SV40 β FL β R β RL) and are described in Materials and Methods.

PCR amplifying a 464 bp fragment spanning the promoter regions from position -417 to +47 from the Cap site. Either oligonucleotides 1 and 2 in Fig. 2, which contain HindIII linkers, or 3 and 4 in the same figure, which contain PstI linkers were used as PCR primers. The resulting PCR product was cloned into the unique PstI or HindIII site of the pESL or pSVAL vectors, respectively. The β globin gene promoter was obtained by PCR using as primers oligonucleotide n.2 (Fig. 2) and oligonucleotide CGCTG-

CAGCATCTACATATCCCAAAA (which contains HindIII linkers) or oligonucleotide n.4 and oligonucleotide CCAAGCTTCATCTACATATCCCAAAA (which contain a PstI linker). Both couples of primers amplify a 448 bp fragment from position +47 to position -401 from the Cap site. The resulting PCR product was cloned into the expression vectors pESL and pSVAL as described before obtaining the β pESL and the β pSVAL constructs. Competitive transfection studies have been carried out using constructs containing a single HS2 or SV40 enhancer and the δ and β globin gene promoters driving the Firefly and Renilla luciferase, respectively (HS2 δ FL β R β RL and SV40 δ FL β R β RL in Fig. 1B). Constructs containing two identical β globin promoters (HS2 β FL β R β RL and SV40 β FL β R β RL) have been also produced and used as a standard control in transfection experiments. These constructs have been obtained by cloning a 1.8 Kb BamHI fragment from plasmid β Ren (kindly provided by Dr. P. Moi) into the β or δ pESL and the β or δ pSVAL constructs. The BamHI fragment contains the Renilla cDNA luciferase under the control of a 450 bp β globin gene promoter. For transactivation experiments we used the pcDNA1 vector (Promega) containing full length 1.6 kb human EKLf cDNA under the control of the CMV promoter, cloned into the EcoRI site (kindly provided by Dr P. Moi). The human EKLf cDNA has been obtained by screening of a λ GT11 human fetal liver cDNA library using a mouse EKLf cDNA as probe.

Site Specific Mutagenesis

The nucleotide sequence of the promoter mutants as well as the sequences and position of the oligonucleotides used in this study are summarized in Fig. 2A. The sequences of the mutated δ globin gene promoters are shown in Fig. 1B were obtained by *in vitro* site specific mutagenesis using the megaprimer method (23) as follows:

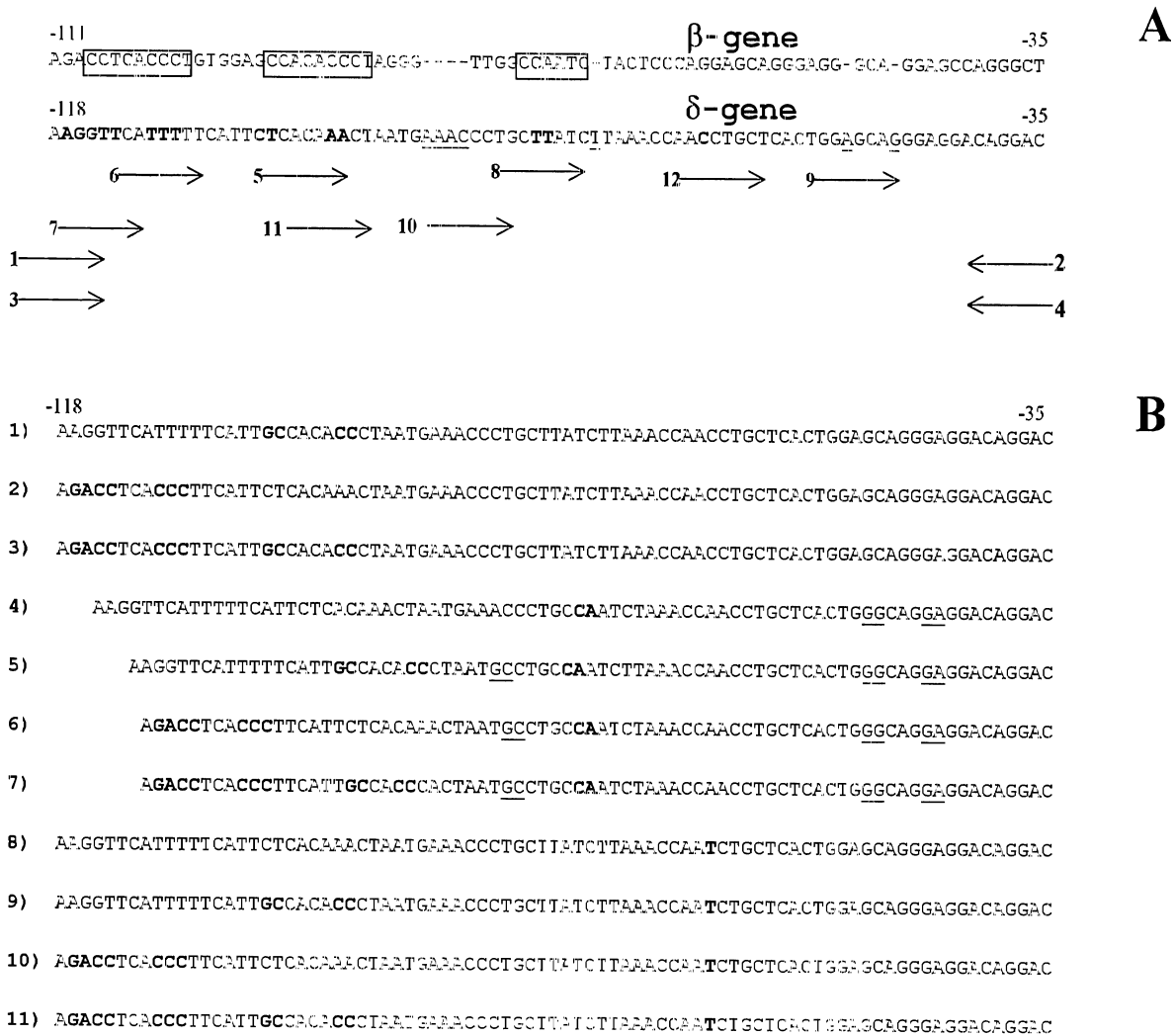


Figure 2. (A) Orientation and approximate position of the oligonucleotides used in the mutagenesis of the δ -globin promoter (only sequences from position -118 to -35 of the δ -globin gene and from -35 to -111 of the β -globin gene are shown). Nucleotide deletions are underlined and substitutions are in boldface. In the corresponding promoter sequence of the β -globin gene, the CACCC and CAAT consensus motifs are boxed and alignment gaps are represented with hyphens. The oligonucleotide sequences are as follows: (1) CCAAGCTTGAGGCAAAGAAGAAGT; (2) CCAAGCTTGTCTGTTTGAGGTTGCT; (3) CGCTGCAGGAGGCAAAGAAGAAGT; (4) CGCTGCAGGCTGTTTGAGGTTGCT; (5) TTCATTGCCACACCCTAATGAAA; (6) GAAGGTTACCCTTCATTCTC; (7) AGTGGATGAGACCTCACCTTCA; (8) AAACCCTGCCAATCTAAACCAACC; (9) TGCTCACTGGGCAGGAGGACAGG; (10) CTCACAACTAATGCCTGCCAATCTAAA; (11) TTCATTGCCACACCCTAATGCCT; (12) TTAACCAATCTGCTCACT. (B) Nucleotide sequences of the δ globin gene promoter mutant used in the present study (only sequences from position -118 to -35 of the δ -globin gene are shown). Position in which nucleotides have been deleted are underlined and nucleotides that have been substitutions are in boldface.

Mutants from 1 to 7 have been first created using oligonucleotides 1 and 2 in Fig. 2, which contain HindIII linkers, as 5' and 3' end primers. The resulting PCR products was restriction digested by HindIII and cloned into the pSVAL expression vector to produce the δ pSVAL

constructs used to transfect Cos7 cells. These mutants have then been used as template for PCR reactions with oligonucleotides 3 and 4 in Fig. 2A, which contain PstI linkers. The resulting PCR product has been PstI restriction digested and cloned into the pESL expression vector to

produce the δ pESL constructs used to transfect K562 and MEL cells. Mutants from 8 to 11 have been directly created using oligonucleotides 3 and 4 in Fig. 2A and cloned into pESL.

Mutant 1: proximal CACC containing δ globin gene promoter. The megaprimer has been obtained using the wt δ globin gene promoter as template and the oligonucleotide 5 in Fig. 2A.

Mutant 2: distal CACCC containing δ globin gene promoter. The distal CACCC box consensus sequence has been created in two steps. (A) oligonucleotide 6 in Fig. 2A was used for the production of the megaprimer. This oligonucleotide contains only the 3' half part of the distal CACCC consensus sequence; (B) using as a template the PCR product from step A oligonucleotides 7 in Fig. 2A was used to obtain the complete distal CACCC consensus sequence.

Mutant 3: proximal and distal CACCC containing δ globin gene promoter. This mutant was created using the distal CACCC containing promoter as a template and the procedure described for mutant 1.

Mutant 4: CAAT (at position -74) containing δ globin gene promoter. Since the distance between the CAAT consensus sequence and the TATA box is strictly evolutionary conserved to mimic the β globin spatial organization of these elements in the δ globin gene promoter we constructed mutant 4 in two steps: (A) Creation of the CAAT consensus sequence using oligonucleotide n. 8 in Fig. 2A and the wt δ globin gene promoter as a template. (B) Deletion of nt -47 and -51 using oligonucleotide n. 9 in Fig. 2A and mutant from step A as template.

Mutant 5: CAAT (at position -74) and proximal CACCC containing δ globin gene promoter. The distance between the CACCC and the CAAT boxes and that of these element from the TATA box are strictly evolutionary conserved. To mimic the β globin spatial organization of these elements in the δ globin gene promoter we constructed mutant n.5 in two steps using as starting template mutant n.4; (A)

deletion of the sequence AAAC from position -83 to -86 using oligonucleotide n.10 in Fig. 2A; (C) creation of the proximal CACCC consensus sequence using oligonucleotide n. 11 in Fig. 2A and the mutant obtained in step A as template.

Mutant 6: CAAT and distal CACCC box containing δ globin gene promoter. Mutant 6 have been created using as a template the δ globin gene promoter from step A of mutant 5 and the procedure described for mutant 2.

Mutant 7: CAAT (at position -74), proximal and distal CACCC containing δ globin gene promoter. This δ globin gene promoter mutant has been obtained creating the consensus sequence for the proximal CACCC box using as template mutant 6 and oligonucleotide 11 in Fig. 2A.

Mutant 8: CAAT (at position -64) containing δ globin gene promoter. This mutant has been created as using as a template the wt δ globin gene promoter and oligonucleotide n. 12 in Fig. 2A.

Mutant 9: CAAT (at position -64) and proximal CACCC containing δ globin gene promoter. This mutant has been created as mutant 1 using as a template the mutated δ globin gene promoter n8.

Mutant 10:CAAT (at position -64) and distal CACCC containing δ globin gene promoter. This mutant has been created as mutant 2 using as a template the mutated δ globin gene promoter n9.

Mutant 11: CAAT (at position -64), proximal and distal CACCC containing δ globin gene promoter. This mutant has been created as described for mutant 3 using as template the δ globin gene promoter mutant n. 10.

Cloning into the expression vector has been carried out as described before by standard techniques. The correct orientation of all the promoter inside the expression vectors has been verified by restriction mapping and the presence of the correct sequence has been verified by direct sequence of plasmid DNA by standard techniques.

Cell Lines

C88 Mouse Erythroleukemia (MEL) and K562 cell lines were grown in RPMI 1160 media supplemented with 10% fetal calf serum and antibiotics. Cos7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum and antibiotics.

DNA Transfection and Transient Assays

Plasmid DNA was prepared by alkaline lysis and PEG precipitation. Transfection was carried out with Lipofectin (Gibco BRL) following the manufacturer's instructions. Semiconfluent Cos7 cells were transfected using 5 μ g of the supercoiled plasmid DNA and harvested after 48 hrs. In each experiment 3 μ g of the plasmid pCH110 (Pharmacia), which contains a β -galactosidase gene, were co-transfected as an internal control of transfection efficiency. C88 MEL and K562 cells were transfected as described by Antoniou et al (24). In each experiment 3 μ g of supercoiled test plasmid DNA was used. 1 μ g of the pRL-CMV (Promega) Plasmid containing the Renilla Luciferase cDNA was cotransfected in each assay as a control for transfection efficiency. Since in the constructs used for the competitive transient assay the test (δ -globin gene promoter driving the Firefly Luciferase cDNA) and the control (β -globin gene promoter driving the Renilla Luciferase cDNA) are in the same construct we did not include transfection efficiency control in our experiments. The Renilla and Firefly luciferase activity were assayed, in the same test tube, using Dual-Luciferase Reporter Assay System (Promega) following the manufacturer condition. β -galactosidase activity was assayed by standard techniques. Protein extracts were obtained by freezing and thawing the cell pellets three times. Protein concentration was determined by the Bradford assay using a BioRad kit.

Transactivation experiments were carried out cotransfecting 1 μ g of human EKLF cDNA

cloned into PcDNA1(Promega). To assess whether the observed average value of the Relative Luciferase Activity (RLA) between the different constructs was significantly different we used the T test of Student.

RESULTS

Transient Expression

Expression studies in Cos7 cells. In Cos 7 cells the ratio between the δ and β mRNA for transiently expressed β and δ globin genes is close to that seen *in vivo* in erythroid cells from normal individuals (14). In the present study, the promoter region of the wt human δ -globin gene has been mutagenized at specific sites to reproduce the consensus sequences for the CACCC and CAAT boxes in the order and spatial organization that these elements have in the β -globin gene (Fig. 2). Each single and multiple promoter mutants as well as the normal β - and δ -globin gene promoter were transiently expressed in Cos7 cell lines using the firefly cDNA luciferase as reporter gene.

A schematic representation of the β - and δ -globin gene promoter constructs used in this study as well as their Relative Luciferase Activity (RLA) in Cos7 cells are shown in Fig. 3 as a mean of at least 3 separate sets of experiments. In each experiment the RLA of the β -globin promoter has been considered as 100% and all the other values have been calculated as a percentage of β -globin promoter activity.

The luciferase gene driven by the δ -globin gene promoter is expressed at a 4% (+/- 0.97) level compared to the β -globin gene. This ratio is in good agreement with the expression ratio of 1:40 seen in an earlier study (25). As shown in Fig. 3 the introduction of a single consensus sequence either for the proximal or for the distal CACCC box in an otherwise wt δ -globin gene promoter increases the RLA to a comparable extent, up to 80% (+/-14.11) for mutant

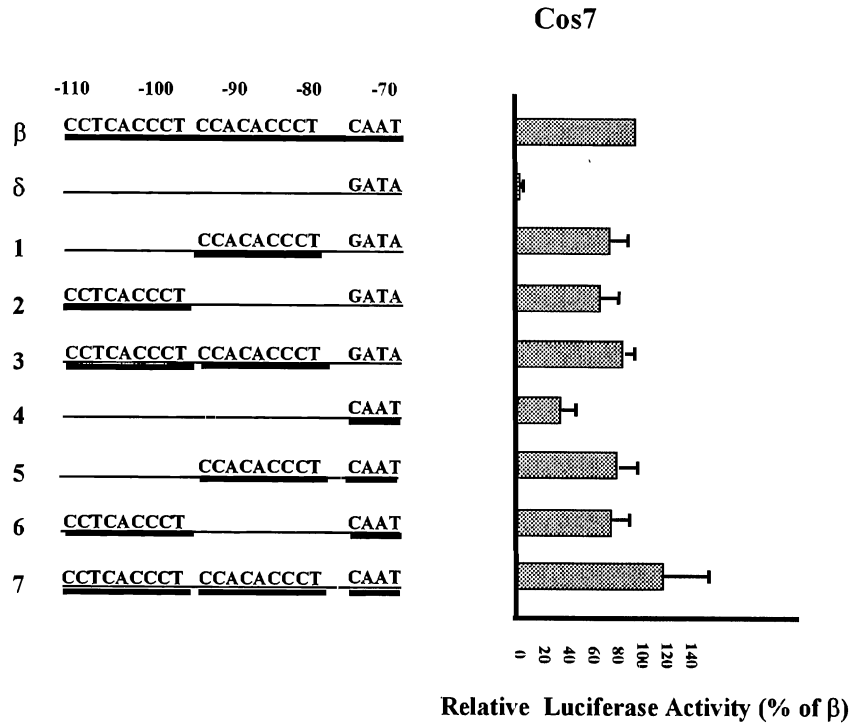


Figure 3. Relative expression of the different promoter mutants in COS7 cells. The wt β -, the δ -globin gene and the mutant promoters are represented schematically on the left. Sequences derived from the β - and δ - globin gene are indicated by thick and thin lines, respectively. On the right, aligned with the corresponding construct, is the relative luciferase activity (RLA), expressed as a percentage of activity of the wt β - globin promoter.

1 (proximal CACCC element) and up to 75% (+/- 13.65) for mutant 2 (distal CACCC element). The presence of both CACCC elements raises the RLA up to 86% (+/-6) (3 in Fig. 3). Thus in this assay the two CACCC boxes do not show any additive or synergic effect on the RLA. The δ -globin promoter mutant, in which the consensus sequence for the CAAT element has been created at position -74 bp from the Cap site, corresponding to its position in the β -globin gene (4 in Fig. 3), activates the δ -globin gene promoter up to 39% (+/-9) as compared to the β -globin gene promoter. In the promoter mutants 5 and 6 in Fig. 3, the consensus sequence either for the proximal (mut. 5) or the distal (mut. 6) CACCC element has been introduced in a promoter containing CAAT box. In these constructs the distance between these elements and the TATA box is the same as in the β -globin gene promoter (Fig. 3). The presence of the proximal or distal

CACCC element together with the CAAT consensus rise the RLA up to 84% (+/-13) and 80% (+/- 12.5), respectively, for the constructs containing the proximal and the distal element. When we assayed the mutant containing both the CACCC elements and the CAAT element in the same spatial organization as in the WT β -globin promoter the RLA increased to 127% (+/-40), (mut. 7 in Fig. 3). This average value is not significantly different from that obtained with the single proximal or distal CACCC element ($p=0.062$ and $p=0.129$, respectively). In conclusion all elements tested in this system can activate the δ -globin gene promoter, though none cooperates to increase the expression level.

Expression studies in K562 cells. K562 cells have an embryonic-fetal pattern of globin expression, but they have a detectable level of δ -globin production. These cells, however are able

to switch from γ to β chain expression (26). A schematic representation of the β - and δ -globin gene promoter constructs used in this study as well as their Relative Luciferase Activity (RLA) in K562 cells are shown in Fig. 4 as a mean of at least three separate sets of experiments.

In K562 cells, the wt δ -globin gene promoter is expressed at a considerably higher level than in non erythroid cells (65.4% +/-4.9), in agreement with the fact that in these cells the endogenous δ -globin gene is active. The presence of the proximal or distal CACCC consensus sequence in an otherwise wt δ -globin gene promoter increases the RLA with respect to the control (mutant 1 and 2 in Fig. 4). The increase in expression

between the constructs containing the proximal (182% +/-15.7) and those with the distal element is not significantly different (140% +/-27) (p=0.210)). Though further increasing the RLA (288% +/-58), the presence of both elements does not result in a cooperative activation of the δ promoter and shows less than an additive effect (mutant 3 in Fig. 4).

The introduction of a CAAT consensus sequence at position -74 nt from the δ Cap site into the δ -globin gene promoter at a position corresponding to the CAAT box of the β -globin gene considerably decreases the δ expression (10.7% +/- 2.5) (mutant 4 in Fig. 4). This effect is most likely explained by the removal of a

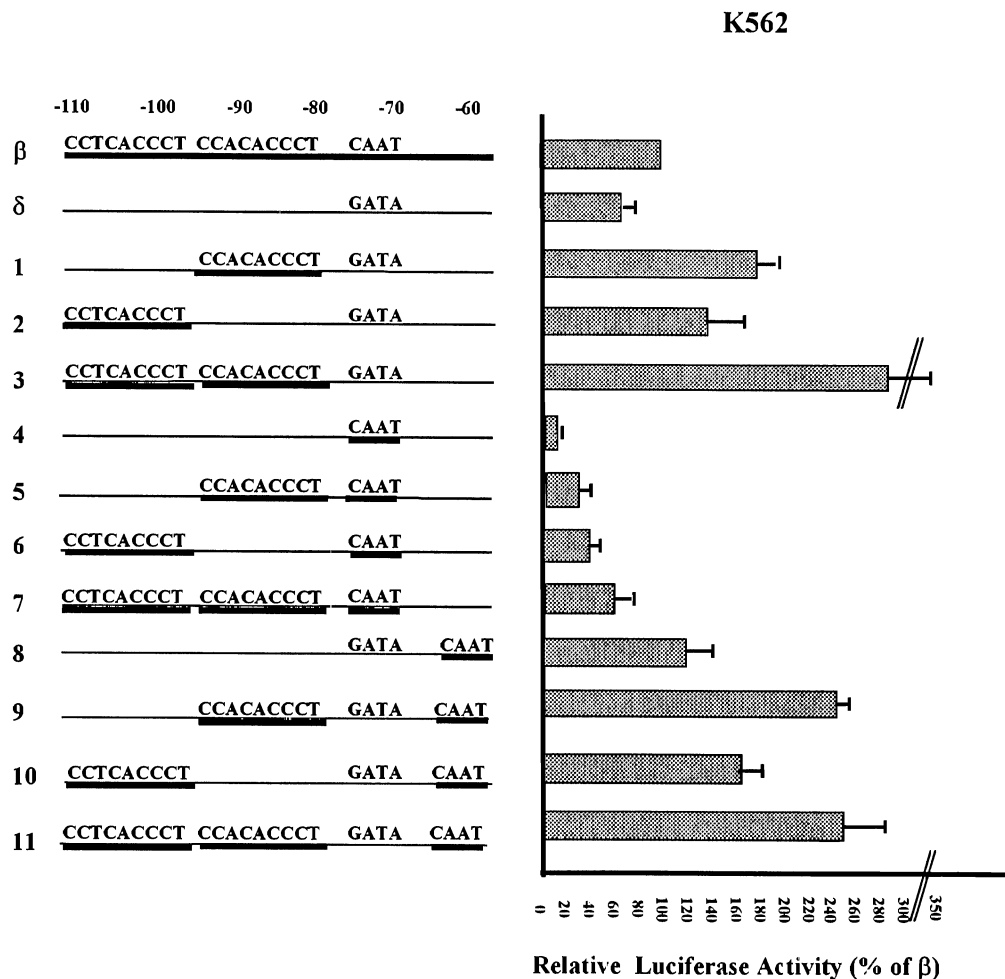


Figure 4. Relative expression of the different promoter mutants in K562 cells. The wt β -, the δ -globin gene and the mutant promoters are represented schematically on the left. Sequences derived from the β - and δ - globin gene are indicated by thick and thin lines, respectively. On the right, aligned with the corresponding construct, is the relative luciferase activity (RLA), expressed as a percentage of activity of the wt β - globin promoter.

coincident GATA1 binding site (26). Thus the presence of the CAAT consensus sequence does not compensate for the loss of the GATA1 binding site, even though the β -globin CAAT consensus sequence has been shown to bind GATA1 (5). The introduction of either one of the CACCC boxes into a δ -globin gene containing the β CAAT box does not compensate the loss of the GATA1 binding site (17.2% +/-3 and 26% +/- 6 for the constructs containing the proximal and distal elements respectively, mutants 5 and 6 in Fig. 4). These experiments indicate that in order to activate the δ -globin gene promoter in erythroid cells, the CACCC boxes need the

presence of a GATA1 site at position -74. The wt δ globin gene promoter contains, at position -64 from the Cap site, the nucleotide sequence CCAAC, which is very close to the consensus site for the CAAT box. An earlier study has shown that the substitution of the CCAAC with the CCAAT sequence activates the δ -globin gene promoter in erythroid and non erythroid cell lines. In our experiments, the construct containing the CCAAT motif at position -64 (mutant 8 in Fig. 4) increases the RLA up to 127% +/-22 (2X compared to the CAAT-less δ promoter). Nevertheless, the introduction of the CCAAT box at position -64 does not significantly increase the

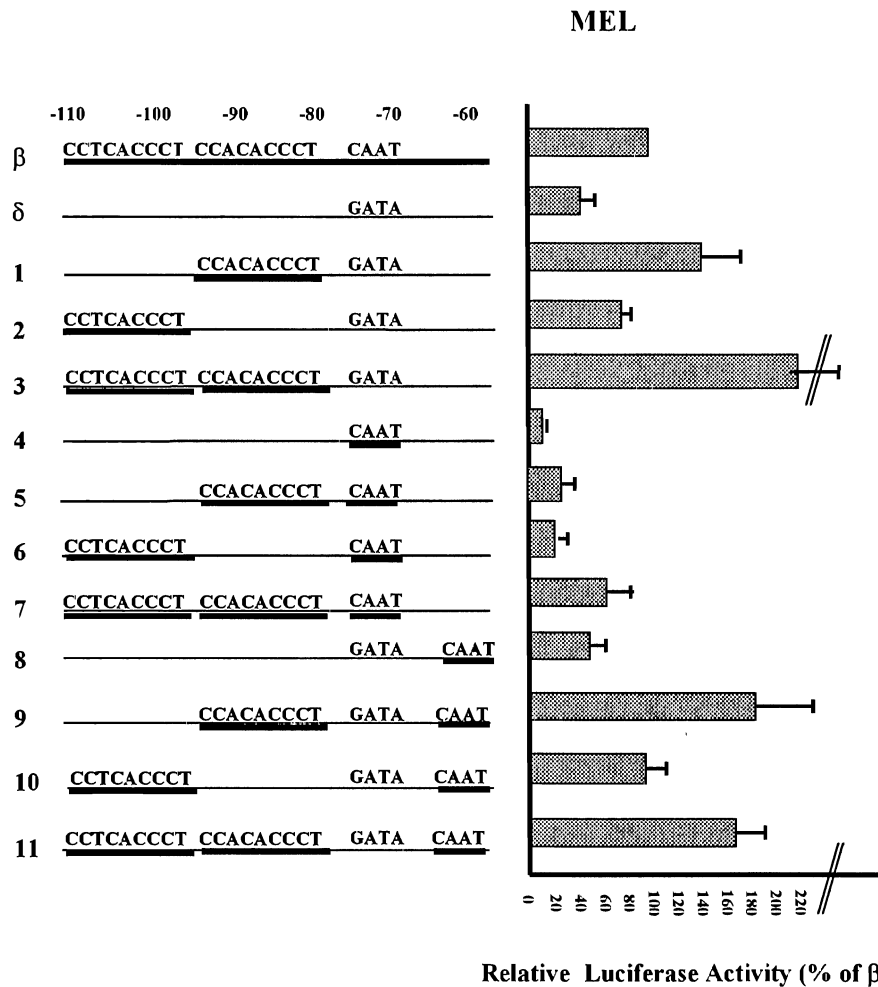


Figure 5. Relative expression of the different promoter mutants in MEL cells. The wt β -, the δ -globin gene and the mutant promoters are represented schematically on the left. Sequences derived from the β - and δ - globin gene are indicated by thick and thin lines, respectively. On the right, aligned with the corresponding construct, is the relative luciferase activity (RLA), expressed as a percentage of activity of the wt β - globin promoter.

expression of the δ -globin containing CACCC construct ($p=0,117$ and $p=0,486$ for the proximal and distal element respectively) (mutant 9 and 10 in Fig. 4). The δ -globin gene promoter containing both the CACCC elements and the CAAT box is indeed expressed at a 254% \pm 39 level, i.e., indicating absence of an additive effect from these motifs.

Expression studies in MEL cells. A schematic representation of the β - and δ -globin gene promoter constructs used in this study as well as their Relative Luciferase Activity (RLA) in MEL cells are shown in Fig. 5 as a mean of at least 3 separate sets of experiments.

In MEL cells the wt δ -globin gene promoter is expressed at the 38% (\pm 5) level with respect to the β -globin gene promoter. The presence of either the proximal or the distal CACCC consensus sequence in an otherwise wt δ -globin gene promoter increases the RLA with respect to the control. The increase in expression is higher in the construct containing the proximal motif (144% \pm 34, mutant 1 in Fig. 5) than in the one containing the distal motif (78% \pm 8,8, mutant 2 in Fig. 3) ($p=0.003$). The presence of both CACC motifs (mutant 3 in Fig. 5) increases the RLA further (216% \pm 94). As in K562 the removal of the GATA1 site at position -74 by introducing the β -like CAAT consensus sequence, abolishes the δ -promoter function (RLA 17% \pm 4.7, mutant 4 in Fig. 5). Similarly the introduction of the CACCC box in the CAAT-containing δ -promoter (at position -74) does not have a considerable effect on the RLA (mutants 5,6 and 7 in Fig. 5). This result indicates that in the adult erythroid environment this GATA1 site is strictly required for the normal δ -globin promoter to function and for the promoter to be activated by the CACCC box.

The introduction of a CAAT consensus sequence at position -64 has a poor effect on the RLA (41% \pm 7.5, mutant 8 in Fig. 5), which is not significantly different from that of the wt δ -

globin gene promoter ($p=0.413$). The insertion of either the proximal or the distal CACCC element activates the CAAT-containing δ -globin gene promoter, but the extent of activation is not significantly different from the corresponding CAAT-less δ -globin mutant promoter ($p=0.160$, $p=0.488$ and $p=0.259$, respectively). (184% \pm 47, 86% \pm 22.7, 165 \pm 38 for the proximal, distal and both elements, respectively, mutants line 9,10 and 11 in Fig. 5.)

Transactivation. We have carried out transactivation experiments in MEL and K562 cells. As shown in Fig. 6, in MEL cells, the wt β -globin and δ -globin gene promoter containing the proximal CACCC box show a marked increase in transcription following cotransfection with the EKLF construct. The increase in RLA is about 45X for the wt β -globin gene promoter and from 5 to 10X for the δ -globin promoter containing the proximal CACCC element (mutant n 1,3,5 and 7 in Fig. 6). EKLF, however, shows a very small transactivation effect (2X or less) on the CACCC distal δ -globin promoter (mutants 2 and 6 in Fig. 6) and no effect at all on the remaining constructs.

In K562 cells, EKLF has a small transactivation effect on the wt β -globin promoter and no effect at all on the δ -globin gene constructs (data not shown).

Competitive Transient Expression

In view of a possible activation of the δ globin gene to compensate the loss of function of the β globin gene in Thalassemia and Sickle Cell Anemia is relevant to evaluate the activation of the CACCC containing δ globin promoter in competition with the β globin gene promoter. To this aim we have carried out transient expression experiments using constructs in which the wt β globin gene promoter and the distal or proximal CACCC containing δ globin gene promoter (mutant 1 and 2 in Fig. 2B), as well as the wt δ

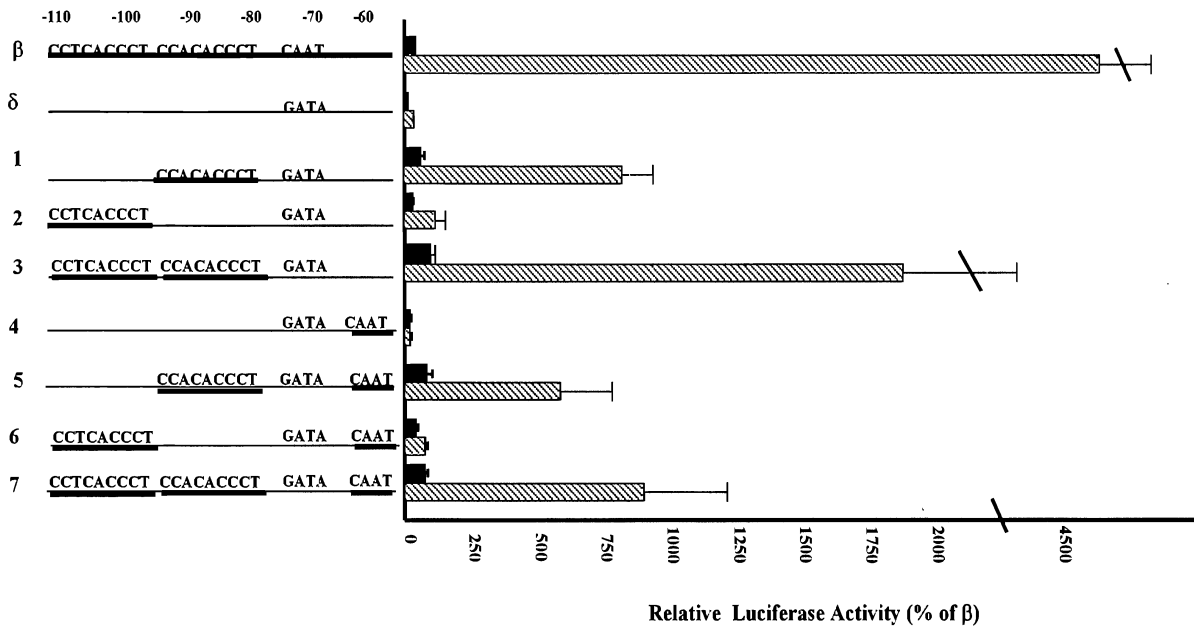


Figure 6. Relative expression of the different promoter mutants in MEL cells before (black bars) and after (stripped bars) transactivation by EKLf. Sequences derived from the β - and δ - globin gene are shown by thick and thin lines, respectively. On the right, aligned with the corresponding construct, is the relative luciferase activity (RLA), expressed as a percentage of activity of the non-transactivated wt β -globin promoter.

globin gene promoter, are linked in cis to a single enhancer (HS2 δ FL β R and SV40 δ FL β R in Fig. 1B). In these constructs the wt β -globin gene promoter drives the Renilla Luciferase cDNA while the δ -globin gene promoter drives the Firefly Luciferase cDNA. The Renilla Luciferase has an high sensitivity compared to the Firefly Luciferase. In order to assess the magnitude of these different activity in our assays we included in the transfections a construct containing two β -globin gene promoters, one of them driving the Firefly Luciferase and the other the Renilla Luciferase (HS2 β FL β R and SV40 β FL β R, see Materials and Methods). The ratio between the Firefly and the Renilla Luciferase obtained with these constructs has been considered as 100%. The ratio between the Firefly and the Renilla Luciferase obtained with the δ containing constructs has been expressed as relative percentage of the ratio obtained with the HS2 β FL β R and SV40 β FL β R constructs in the different cell lines.

Expression studies in Cos7 cells. As shown in Fig. 7A the wt δ globin gene promoter shows an RLA of 9.2% (+/-0.9) of the β globin gene promoter. The δ globin gene promoter containing the proximal CACCC consensus sequence raises the RLA up to 77% (+/-10) (mutant 1 in Fig. 7A), i.e., 8.3 times the wt δ globin gene promoter. The distal CACCC motif increases the promoter activity up to 27.5% (+/-5.8) (Mutant 2 in Fig. 7A), i.e., 3 times compared to the wt δ globin gene promoter.

Expression studies in K562 cells. As shown in Fig. 7B in agreement with the noncompetitive transient expression experiments the wt δ globin gene promoter shows an high basal activity (RLA= 81% +/- 6,2). The presence of the proximal or distal CACCC consensus sequence increases the activity of the δ globin gene promoter (mutant 1 and 2 in Fig. 7B). The activation obtained by the proximal CACCC element is significantly higher of the distal

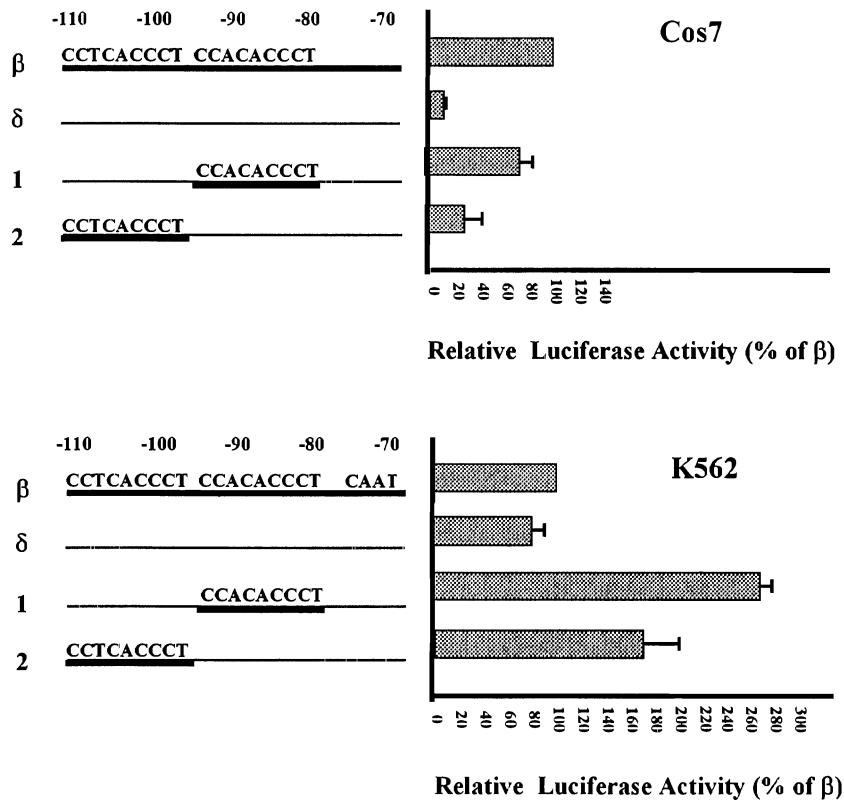


Figure 7. (A) Relative expression of the wt δ and CACCC containing promoter mutants in competitive transient assay in Cos7 cells. (B) Relative expression of the wt δ and CACCC containing promoter mutants in competitive transient assay in K562 cells. The wt β -, the δ -globin gene and the mutant promoters are represented schematically on the left. Sequences derived from the β - and δ -globin gene are indicated by thick and thin lines, respectively. On the right, aligned with the corresponding construct, is the relative luciferase activity (RLA), expressed as a percentage of activity of the wt β -globin promoter.

element (270% \pm 8.8 and 173 \pm 18.1), respectively.

Expression studies in MEL cells. As shown in Fig. 8 the wt δ globin gene promoter in this competition system shows a basal activity of 29% (\pm 9.3) of the β globin gene promoter. The proximal CACCC containing δ globin gene promoter increase the RLA up to 62% (\pm 21.6) (mutant 1 in Fig. 8A) while the distal element has no effect (RLA=19% \pm 6.2) (mutant 2 in Fig. 8A).

Transactivation. In transactivation experiments by hEKLf in MEL cells the wt δ globin gene promoter shows an RLA of 7% (\pm 7) compared to the β . This ratio is considerably

lower to that obtained without hEKLf cotransfection, in agreement with the lack of transactivation by EKLf of the wt δ globin gene promoter. The δ globin gene promoter containing the proximal CACCC box increased its expression up to 50% (\pm 11.4) (mutant 1 in Fig. 8B) while the distal has little effect (11.7% \pm 3.7) (mutant 2 in Fig. 8B).

DISCUSSION

The results produced in this study show that both the proximal and the distal CACCC motifs of the β -globin gene are able to activate the δ -globin gene promoter. However, in Cos7 and K562 cells the δ -globin gene promoter is

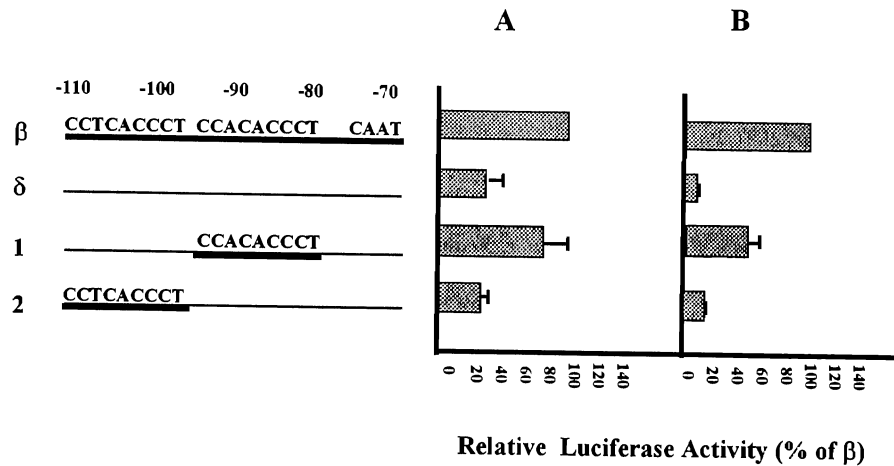


Figure 8. Relative expression of the wt δ and CACCC containing promoter mutants in competitive transient assay in MEL cells without (A) and with (B) EKLf transactivation. The wt β , the wt δ -globin and the mutant promoters are represented schematically on the left. Sequences derived from the β - and δ - globin gene are indicated by thick and thin lines, respectively. On the right, aligned with the corresponding construct, is the relative luciferase activity (RLA), expressed as a percentage of activity of the wt β - globin promoter.

activated to a similar extent by either motif, whereas in MEL cells the activation is more marked with the construct containing the proximal motif than with the one containing the distal motif. When in competition for a shared enhancer with a cis linked wt β globin gene promoter only the proximal CACCC element is able to activate the δ globin gene in adult erythroid cells while in non erythroid (Cos7) and embryonic fetal erythroid cells (K562) both elements are able to activate the promoter even though to a different extent. These findings indicate that in an adult erythroid environment such as the MEL cells, the proximal CACCC motif is more effective in activating the δ -globin gene promoter than the distal one. These data also suggest that the low *in vivo* expression of the δ -globin gene is most likely to be related to the lack of CACCC motifs. These experimental results agree with the different effect on the expression of the β -globin gene of β -thal mutations that affect the proximal or the distal CACCC box. Mutations of the proximal element indeed have a more severe phenotype than the mutation of the

distal element (10-13). Experiments with constructs containing the two CACCC motifs indicate that both in erythroid and non-erythroid cells the effect of these two elements together is less than additive, suggesting that the duplication of this CACCC box is not absolutely necessary for the high expression levels of a β -like globin gene. This conclusion is also supported by the observation that only one of the CACCC elements, i.e., either the proximal or the distal, is present in many mammals. The presence of two elements, however, may have some cooperative effect, as will be discussed later on.

In order to evaluate the effect of introducing a CAAT box in the δ -globin gene promoter, we carried out two sets of experiments, namely the introduction of the CAAT box at position -74 corresponding to the position of the CAAT box of the β -globin gene, and the creation of a CAAT sequence at position -64 in place of the CAAC sequence as already done by DC Tang et al. (19). The creation of a CAAT sequence in the δ -globin gene promoter at position -74 from the Cap site, which is the position corresponding to the CAAT

box of the β -globin gene, abolishes the promoter function in erythroid cells while activates the δ globin gene promoter in not erythroid cells. This effect is most likely related to the removal of a coincidental GATA-1 site (27) at position from -74 to -77 in this CAAT containing construct which may substitute the function of the CAAT sequence of the β -globin gene in the δ -globin gene promoter, and is therefore essential to promoter activity. The other two GATA1 binding sites in the δ -globin gene promoter at positions -250 and -350 (19) are indeed unable to compensate for the loss of the -74 site. On the other hand our study suggests defective GATA1 binding activity of the created CAAT box in the δ -globin gene framework, in contrast to the CAAT box of the β -globin gene which is indeed able to bind GATA1 (5). This postulated defective GATA-1 binding to the CAAT box containing δ -globin promoter construct probably also explains the lack of synergism between the CAAT and CACCC elements in conferring erythroid specific activity to the promoter (28,29).

Furthermore our experiments have shown that in erythroid cells the activation of the δ -globin gene promoter by the CACCC box absolutely requires the presence of GATA1 at position -74 . This finding is probably related to the fact that in order to have erythroid specific activity, a promoter requires the presence of a GATA1 and EKLF-Sp1 (CACCC box) consensus element that should be spatially organized to interact with each other (28,29).

The introduction of the CAAT box at position -64 determines the activation of the δ -globin gene promoter in K562 cells, but it has no effect in MEL cells. Previous studies have found activation of the δ -globin gene promoter in both K562 and MEL cells although in MEL cells the extent of activation was significantly less than in K562 cells (19). The discrepancy between our results and those of Tang et al. is most likely to be related to the different constructs used and especially to the fact that the SV40 enhancer was

used instead of the HS2 in our constructs. In our experiments in adult erythroid environment the lack of any effect in the introduction of the CAAT motif at position -64 in the δ -globin gene promoter may be related to its different position from the CAP site compared to the wt β -globin promoter.

Transactivation experiments with EKLF constructs in MEL cells have shown a markedly increased expression from the δ -globin gene promoter construct containing the proximal CACCC motif but a limited effect with the construct bearing the distal CACCC motif. In agreement with our results, a previous report shows that the transactivation by EKLF of the β globin gene promoter is not related to the presence of the distal CACCC box (30). EKLF is indispensable for the *in vivo* activation of the β globin gene in definitive erythroid cells as demonstrated by gene ablation experiments (8,9). It strongly binds to the consensus sequence for the proximal CACCC element as well as to the HS3 of the Locus Control Region (LCR) (31) but not to the distal CACCC element (32-34). It should be noted, however, that in respect to EKLF the distal CACCC box appear to act more efficiently in competition assay than in binding reaction (P. Moi submitted). In addition, the β -thal mutation C-T at position -101 in the distal CACCC box, which has the silent β -thal phenotype (13), has a suboptimal response to EKLF stimulation in the transactivation assay in MEL cells (P. Moi submitted). These findings together indicate that although weak, EKLF binding to the distal CACCC box is still necessary for full globin transactivation to take place and may explain the basal promoter activity of our construct containing the distal CACCC box in the adult erythroid environment. Furthermore evolutionary considerations indicate a role for the distal CACCC element in the overall expression of adult β -like globin genes. We should consider in fact that in some mammals like mice and rats the β -globin gene is duplicated. Of the two β -globin genes, the minor gene is expressed

prevalently in the fetal period and shows a single distal CACCC box in its promoter, whereas the major gene, which is, expressed mostly in the adulthood displays only the proximal CACCC element (35). Taken together these considerations suggest that the distal CACCC element may play a major role in the fetal-newborn period. It should be noted in this connection that β -thal mutation C-T substitution at position -101 inside the distal CACCC box has a more severe phenotype in the neonatal period than in adult life (36).

In K562 cells EKLF failed to transactivate any of the δ mutants and has a small effect on the wt β globin gene promoter (3X). H. Asano and G. Stamatoyannopoulos have reported a transactivation of the wt β globin gene promoter of about 8X in K562 cells (30), still markedly lower of the extent of transactivation level in MEL cells shown in the present report (45X). To this regard it is of interest to note that EKLF is present at all developmental stages in erythroid cells but exerts its action specifically only in adult definitive erythroid cells (37). The molecular mechanism by which EKLF is effective only on the adult β globin gene in definitive erythroid cells is still unclear. Different chromatin structures at different stages of erythroid development or stage specific post-transcriptional modifications may have a role (32,38,39). The high basal activity of the CACCC containing δ -globin promoter in K562 cells is most likely due to the effect of other CACCC binding transacting factors. Similarly the promoter activity of the proximal and distal CACCC motif containing δ -promoter in Cos7 cells may be related to the different cell environment and specifically to the difference in the ubiquitous transacting factors binding to the CACCC sequences as well as to the absence of EKLF. A similar explanation together with the lack of the GATA-1 factor may be assumed for the promoter activity of the -74 CAAT containing δ -promoter.

We have also carried out transient expression experiments in Cos7, K562 and MEL cells in order to evaluate the competitive ability of the

CACCC containing δ globin gene promoter toward the wt β globin gene promoter. In this competition assay the basal level of expression of the wt δ globin gene promoter is in agreement with the data obtained in the non-competitive assay. In all the cell types the presence of the consensus sequence for the proximal CACCC box increases the promoter activity in competition with the wt β globin gene promoter. The δ globin gene promoter containing the consensus sequence for the distal CACCC box shows an increase in the competitive ability only in the non erythroid (Cos7 cells) and embryonic fetal erythroid (K562 cells) environment.

In adult erythroid environment (MEL cells) the activation of the δ globin gene promoter by the proximal CACCC box is of only about 2 times compared to the wt δ globin gene promoter. However, because of the high basal activity of the δ globin gene promoter in erythroid cells in transient expression assay the final activity raises to an RLA of 77% of the β globin gene promoter. It is also noteworthy that in transactivation experiments by hEKLF the activity of the wt δ globin promoter is 7%, much closer to the *in vivo* normal expression level, and rises up to 50% introducing the proximal CACCC consensus sequence.

In conclusion our data show that the insertion of the consensus sequence for the proximal CACCC box by creating an EKLF binding site is sufficient to activate the δ -globin gene promoter in a transient expression assay. These findings are in agreement with a recent report, in which the creation of a proximal CACCC EKLF binding site on the δ -globin gene promoter was seen to activate the δ -globin gene in competition with an *in cis* β -globin gene in a stable transfection experiment (18).

All these findings raise the prospective of ameliorating homozygous β -thal or the sickle cell disorder by reactivation of the δ chain production. This optimistic view, however, is hampered by preliminary findings indicating that extremely high levels of HbA2 may be associated, in a transgenic

mouse model, with red blood cell abnormalities (40). These abnormalities, however, have been noted in a transgenic mouse that was also homozygous for the β -major gene deletion and had high levels of non assembled human α -globin chain which may explain the observed abnormalities. Further research is therefore needed to clarify whether the activation of the δ -chain production has a toxic effect on red blood cells, and to explore the usefulness of its activation in the treatment of β -thalassemia and sickle disorders. Experiments with transgenic mice are in progress to further assess the role *in vivo* of the proximal and distal CACCC elements and to estimate the level of activation achievable by the δ -globin gene *in vivo* and in competition with a β globin gene.

ACKNOWLEDGMENTS

This work was sponsored by the World Health Organization and supported by grants from Assessorato Igiene e Sanita' Regione Sardegna, "Patologia Molecolare, Genetica e Terapia Genetico-Somatica della β -thalassemia," L.R. 30.04.1990 No. 11 and Programma di Educazione Sanitaria "Prevenzione Malattie Genetiche nella Popolazione Sarda," DGR 1526, 1597 and 4842; National Research Council (CNR), Istituto di Ricerca sulle Talassemie ed Anemie Mediterranee, Cagliari; Target Project "Diagnosi Molecolare di Talassemie Intermedie," contract No. 95.00633.PF99 and Target Project "Diagnostica delle Talassemie," contract No. 95.04671.ST75; Finanziamenti Università Studi per la Ricerca Scientifica: quota 60-40% to A. Cao.

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