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Cis- and trans-acting elements involved in the regulation of the erythroid promoter of the human porphobilinogen deaminase gene

(tissue-specific transcription/erythroid cells/NF-E1/NF-E2/AP1)

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ABSTRACT **Two cis-acting sequences, recognized by two erythroid-specific trans-acting factors, are involved in the regulation of the erythroid promoter of the human gene coding for porphobilinogen deaminase (PBGD). The first region, located at −70, binds the erythroid factor NF-E1, and point mutations within this region abolish the induction of transcription of this promoter during murine erythroleukemia (MEL) cell differentiation. The second region, located at −160, binds the erythroid-specific factor NF-E2 and the ubiquitous factor AP1. Using UV cross-linking, we show that NF-E2 has a higher molecular weight than AP1, demonstrating that NF-E2 is not an erythroid-specific degradation product of AP1. By point mutagenesis of the NF-E2/AP1 binding site, we define mutations that abolish binding of either NF-E2 alone or AP1 and NF-E2 together. Regulation of transcription of the PBGD erythroid promoter is abolished by those mutations, suggesting that NF-E2 but not AP1 is necessary for correct regulation of this promoter in erythroid cells.**

The coordinated appearance of specific gene products during cellular differentiation is partly regulated at the gene transcription level. Therefore, the knowledge of the mechanisms that control the transcription of genes activated during a particular differentiation is essential. The erythroid differentiation results in the production of massive amounts of hemoglobin, which requires the coordinated production of globin chains and heme molecules. The synthesis of globin chains primarily results from transcriptional activation of the globin genes (1, 2), and the enhanced heme production is ensured by the activation of the enzymes involved in its biosynthesis (3). For at least three of these enzymes, this activation occurs at the gene transcription level (4–6).

The human β-globin gene activation is regulated by at least four cis-acting regions. The first one, located at the 5′end of the β-globin gene cluster, seems to regulate the whole cluster (7). The other three are located in the immediate vicinity of the β-globin transcription unit and consist of a promoter and two downstream enhancers (8). Alone or in combination, these three elements give rise to regulated erythroid expression (8, 9). The characterization of trans-acting factors that bind those different regulatory regions has shown that, in addition to several ubiquitous proteins, an erythroid-specific factor, called NF-E1, binds the 3′ enhancer and the promoter of the human β-globin gene (10). A similar situation is found in chicken, where an erythrocyte-specific DNA binding factor recognizes a regulatory sequence present in all chicken globin genes (11). Indeed, those two factors recognize the same sequence ($\overset{A}{C}$-Y-$\overset{T}{A}$-A-T-C-$\overset{A}{T}$-Y, where Y is an unspecified pyrimidine nucleoside), which is also present in the promoter of other erythroid-specific genes (12).

We have focused our work on the regulation of the expression of the human gene coding for porphobilinogen deaminase (PBGD; EC 4.3.1.8), the third enzyme of the heme biosynthetic pathway. This gene has two overlapping transcription units, each with its own promoter. The upstream promoter is active in all cell types, whereas the downstream one is erythroid specific. After differential splicing, two mRNAs are produced having a specific first exon and 13 common exons (13). The erythroid promoter (−714 to +78) fused to the herpes simplex virus (HSV) thymidine kinase coding sequence (*tk*) is correcetly expressed and regulated during murine erythroleukemia (MEL) cell differentiation (14). This same construct was inactive in nonerythroid cells and only weakly stimulated by a heterologous non-tissue-specific enhancer. These results suggest that structural features within the *PBGD* erythroid promoter are responsible for its strict erythroid-specific expression. Using DNase cleavage inhibition and gel retardation techniques, we have shown that two erythroid-specific factors recognize sequences in the *PBGD* erythroid promoter (15). One of these factors is NF-E1 and binds three sites located at −180, −70, and +45. The second erythroid-specific factor, which we called NF-E2, recognizes an AP1 consensus sequence located at −160. Furthermore, mammalian NF-E2 binds region II of the chicken β-globin enhancer *in vitro* (15), a region that mediates erythroid-positive-acting activity of this enhancer *in vivo* (16).

In this paper we describe expression studies designed to determine the contribution of NF-E1 and NF-E2 binding to the erythroid specificity of the *PBGD* erythroid promoter, and we further characterize the erythroid-specific complex NF-E2. The results suggest that two binding sites, namely the NF-E2 site and the −70 NF-E1 site, are required for transcriptional regulation of the *PBGD* erythroid promoter during MEL cell differentiation.

MATERIALS AND METHODS

Oligonucleotides. The oligonucleotides used for gel retardation and site-directed mutagenesis of the −160 region contained the following sequences (coding strand): Wild-type (oligonucleotide wt) 5′-CCTCCAGTGACTCAGCACAGGTTCCCCAG-3′; oligonucleotide A (−162 mutant), 5′-CCTCCAGTTACTCAGCACAGGTTCCCCAG-3′; and oligonucleotide B (−155 mutant), 5′-CCTCCAGTGACTCAGAACAGGTTCCCCAG-3′. The oligonucleotides used for site-directed mutagenesis of the NF-E1 binding sites are: −180, 5′-GTCTGTTGTCACGCATGAATCTAT-3′; −70, 5′-CTGATGGGCCGGTGACAGTTACCCACCT-3′; +45, 5′-GCAGGTCCTAAGCGTGCCTCCCTC-3′.

Hybrid Gene Constructs. PBGD-CAT was the parent vector used. It is a pBL-CAT3 (17)-based construct with the human *PBGD* erythroid promoter (−714 to +78) fused to the

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Abbreviations: MEL, murine erythroleukemia; PBGD, porphobilinogen deaminase; CAT, chloramphenicol acetyltransferase.

chloramphenicol acetyltransferase (CAT) bacterial gene and followed by the *t* intron and polyadenylylation signals from simian virus 40. For deletions, various restriction enzymes that cut the −714 to +78 region of the *PBGD* erythroid promoter were used, and each deleted promoter was fused to the CAT gene. For site-directed mutagenesis, the −714 to +78 region was subcloned into phage M13mp18 and used as template for mutagenesis by using the Amersham "oligonucleotide-directed *in vitro* mutagenesis system."

Protein–DNA Interactions. Gel retardation assays were performed as described by de Boer *et al.* (9) with K562 nuclear extract. UV cross-linking experiments were carried out as described (18).

Transfections into MEL cells. Protoplast fusions were carried out as described (1).

Assay of CAT Activity. CAT assays were done as described by Gorman *et al.* (19).

Mapping of the Initiation Site of CAT Fusion mRNAs by Polymerase Chain Reaction. In order to check the initiation site, three oligonucleotides were used: oligonucleotide 1 (25 nucleotides) is complementary to a specific sequence of the PBGD-CAT mRNA and is located 128 base pairs (bp) downstream from the correct *PBGD* initiation site; oligonucleotides 2 and 3 contain the sequence of the fusion gene from +1 to +25 and from −25 to −1, respectively. Total RNA from transfected cells (10 μg) was denatured at 70°C and then incubated with 100 mM Tris·HCl, pH 8.3/140 mM KCl/10 mM $MgCl_2$/20 mM 2-mercaptoethanol/1 mM each dNTP containing 100 ng of oligonucleotide 1, 40 units of RNasin, and 10 units of avian myeloblastosis virus reverse transcriptase in a final volume of 50 μl for 40 min at 42°C. RNA was hydrolyzed with NaOH at 65°C. After precipitation, one-fourth of the cDNA sample was subjected to 20–30 cycles of 1 min at 94°C/1 min at 55°C/1 min at 72°C in 10 mM Tris·HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/0.01% gelatin/0.2 mM each dNTP containing 70 ng of oligonucleotide 1, 70 ng of oligonucleotide 2 or 3 (in two separate reactions), and 5 units of *Thermus aquaticus* polymerase (Perkin–Elmer/Cetus) in a final volume of 0.1 ml. Twenty-five microliters was loaded on an 8% nondenaturing acrylamide gel, and DNA was visualized by ethidium bromide staining and UV transillumination. The sizes expected are: oligos 1 + 2, 128 bp; oligos 1 + 3, 153 bp.

RESULTS

Deletion Analysis of the *PBGD* Erythroid Promoter Defined Two Regions Involved in Its Erythroid Expression. We have shown (14) that a hybrid gene containing the *PBGD* erythroid promoter [nucleotides −714 to +78] fused to the HSV *tk* coding sequence was correctly expressed and regulated when introduced into MEL cells. To delimit the sequences involved in the regulation of the *PBGD* erythroid promoter, we performed sequential 5′ deletions of this promoter and linked the remaining sequences to the CAT coding sequence. These constructs were then inserted into a plasmid containing the hamster gene for adenine phosphoribosyltransferase (APRT) and transformed into *Echerichia coli*. After protoplast fusion with MEL $APRT^-$ cells, several pools of >100 independent $APRT^+$ clones were obtained, and CAT activity was measured before and after MEL cell differentiation. CAT fusion mRNAs are often unstable in eukaryotic cells, which makes primer extension analysis of low-expression constructs difficult. Therefore, we developed a modification of the polymerase chain reaction method to check the initiation of transcription of the transfected genes (see *Materials and Methods*). We were unable to detect any band with oligonucleotides 1+3 (compare lane 1+2 with lane 1+3 of Fig. 1*A*) and this demonstrated that the measured CAT activity reflected the transcriptional activity of the *PBGD* erythroid promoter. Two regions seemed to be involved in the erythroid expression of the PBGD erythroid promoter (Fig. 1*B*). One, located between −243 and −112, was involved in the inducibility of this promoter during MEL cell differentiation, and the other one, located between −80 and −50, was involved in the correct initiation of transcription in erythroid cells.

Effects of Point Mutations at the NF-E1 Binding Sites. We have shown (15) that the −243 to +78 region of the *PBGD* erythroid promoter has three binding sites for the erythroid-specific factor NF-E1: a weak binding site at +45 and two strong ones at −70 and −180. As all of those sites lie inside the −243 to +78 region that is sufficient for the erythroid regulation of the *PBGD* erythroid promoter, we performed site-directed mutagenesis on each of them and analyzed the regulation of the mutated promoters during MEL cell differentiation. All the mutations abolished binding of NF-E1 *in vitro* (data not shown). The mutated promoters were then fused to the CAT coding sequence and transfected into MEL cells. CAT activity was measured before addition of hexamethylenebisacetamide and after 80 hr of its presence. The −70 NF-E1 binding site was necessary for correct regulation of the *PBGD* erythroid promoter during MEL cell differentiation, whereas mutations within the other two NF-E1

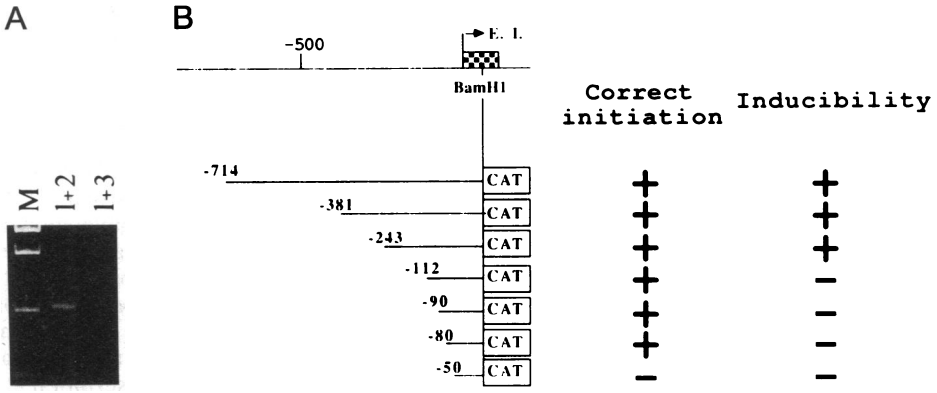

FIG. 1. Effect of 5′ deletions on the expression of the *PBGD* erythroid promoter during MEL cell differentiation. (*A*) Result of a typical polymerase chain reaction experiment performed with RNA isolated from MEL cells transfected with the human *PBGD* erythroid promoter fused to the CAT coding sequence. Lanes M: *Hae* III digest of OX174 replicative form (fragments from 72 to 234 bp); 1+2, product of amplification using oligonucleotides 1 and 2 (correct and upstream initiations); 1+3, product of amplification using oligonucleotides 1 and 3 (upstream initiations). (*B*) Expression of mutated *PBGD* erythroid promoters. The various constructs shown (*Left*) were introduced into MEL cells by protoplast fusion and pools of >100 independent clones were analyzed before (*Center*) and after (*Right*) induction in 5 mM hexamethylenebisacetamide. E. I. indicates the PBGD erythroid initiation site.

binding sites had no effect on the induction of transcription of the *PBGD* erythroid promoter (Fig. 2).

Characterization of the Erythoid Factor NF-E2. Then we focused our attention on the second erythroid-specific factor that binds to this promoter—i.e., NF-E2. A powerful tool for studying the structure of a protein–DNA complex is cross-linking analysis. We have used UV to cross-link the NF-E2–oligonucleotide wt and the AP1–oligonucleotide wt complexes separated in a preparative gel retardation experiment. The gel was then sliced, and the slices containing each of the AP1 and NF-E2 complexes were put on the top of a sodium dodecyl sulfate/polyacrylamide gel together with protein size markers. After migration and staining, the complexes were revealed by autoradiography. Fig. 3 illustrates a typical experiment. The cross-linked AP1–oligonucleotide wt complex migrated at a position indicative of a molecular mass of 55 kDa, whereas the cross-linked NF-E2–oligonucleotide wt complex migrated at a position indicative of a molecular mass of 60 kDa. Knowing that oligonucleotide wt has a molecular mass of 16 kDa and assuming that the contributions of the protein and DNA to the molecular weight observed are additive (18), we found the respective molecular weight of AP1 and NF-E2 to be 39 kDa and 44 kDa.

Effect of Point Mutations in the NF-E2 Binding Site. To characterize further the function of this AP1/NF-E2 binding site, we had to find sequence motifs with altered binding affinities for these complexes. We have previously shown that binding of the erythroid specific factor NF-E2 to the TGACTCA AP1 consensus sequence was more sensitive to methylation of a guanine at position −155 (15). We designed two oligonucleotides, one (oligonucleotide A) with a mutation within the AP1 consensus sequence [TGACTCA → TTACTCA] and one (oligonucleotide B) with a mutation at the 3′ end of the AP1/NF-E2 consensus sequence [TGACTCAGC → TGACTCAGA]. A gel retardation experiment, shown in Fig. 4*A*, indicated that oligonucleotide A did not bind AP1 or NF-E2, whereas oligonucleotide B still bound AP1 but not NF-E2. Unfortunately, we were unable to find an oligonucleotide that bound NF-E2 but not AP1. Using oligonucleotides A and B, we performed site-directed mutagenesis on the promoter and made two mutants, one with no AP1/NF-E2 binding site and the other with a sequence recognized by AP1 only. Both mutations abolished correct regulation of the *PBGD* erythroid promoter during MEL cell differentiation (Fig. 4*B*), suggesting that NF-E2 but not AP1 was necessary for correct regulation of this promoter in erythroid cells.

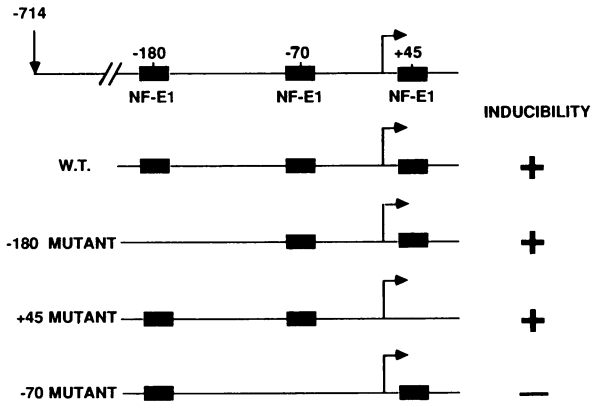

FIG. 2. Effect of point mutations at the NF-E1 binding sites. Using site-directed mutagenesis, the −180, −70, or +45 NF-E1 binding site of the human *PBGD* erythroid promoter was destroyed. Each mutated promoter was then fused to the CAT gene and introduced into MEL cells. Its regulation was studied by measuring CAT activity before and after hexamethylenebisacetamide-induced MEL cell differentiation.

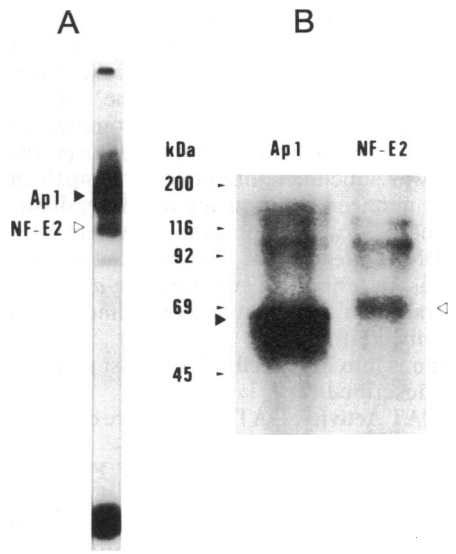

FIG. 3. Native and UV-cross-linked AP1 and NF-E2 complexes. (*A*) Native complexes. Gel retardation was set up by using crude K562 nuclear extract, and labeled oligonucleotide wt. Symbols show the AP1 complex (▶) and the NF-E2 complex (▷). (*B*) Cross-linked complexes. A preparative gel retardation was done with labeled oligonucleotide wt containing BrdUrd in the noncoding strand and was UV-illuminated. The AP1 and NF-E2 bands were excised, put on the top of a sodium dodecyl sulfate/polyacrylamide gel along with protein molecular mass markers, and electrophoresed.

DISCUSSION

Transcription regulation of tissue-specific genes could be achieved either by combinatorial use of ubiquitous trans-acting factors (20) or by cooperation between tissue-specific and ubiquitous DNA-binding factors. This latter phenomenon occurs in B cells where the immunoglobulin promoters bind a tissue-specific factor and various ubiquitous factors (21) and in liver cells where many hepatocyte-specific promoters interact with hepatocyte nuclear factor 1 (22) and ubiquitous factors (23).

In this paper, we have shown that two cis-acting sequences that bind the erythroid-specific DNA binding factors NF-E1 and NF-E2 *in vitro* are functionally necessary for correct expression and regulation of the human *PBGD* erythroid promoter during MEL cell differentiation.

The first region we have identified is located between positions −50 and −80 and binds the erythroid-specific factor NF-E1. This region is necessary for correct initiation in erythroid cells; thus, NF-E1 might be involved in the formation of an active initiation complex. This, indeed, could explain part of the strict tissue specificity of the human *PBGD* erythroid promoter as NF-E1 is present in erythroid cells only (10). Interestingly, 20 bases upstream from this NF-E1 binding site lies a sequence, CCCCACCC, which matches the consensus CAC box found upstream of the β-globin gene cap site in multiple species. We have shown that this CAC box forms the same complexes as observed with the human β-globin promoter (15) and that its deletion greatly reduces the expression of the *PBGD* erythroid promoter transiently introduced into nonerythroid cells (manuscript in preparation). Thus, the CAC-NF-E1 association might allow erythroid-specific initiation of transcription at a reasonable level. This same association is found in erythroid-specific promoters of several genes (Fig. 5). Most of these genes lack the consensus TATA box, have one or very few initiation sites (13, 24), and are transcribed at a low level when compared to the β-globin gene. Interestingly, the spacing between the CAC box and the NF-E1 binding site is always an approximate multiple of 10 bp, suggesting that stereospecific align-

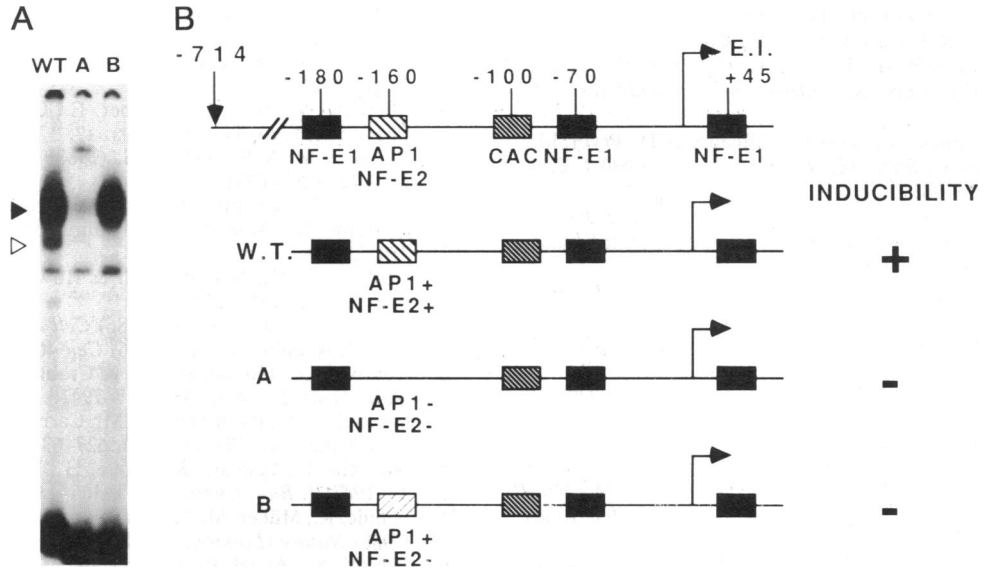

FIG. 4. Effect of point mutations in the NF-E2 binding site. (*A*) Native AP1 and NF-E2 complexes obtained with oligonucleotide wt, oligonucleotide A, and oligonucleotide B. The symbols are the same as in Fig. 3. (*B*) Effect of site-directed mutagenesis on the AP1 consensus sequence of the *PBGD* erythroid promoter. Two mutated promoters were obtained by mutagenesis with oligonucleotide A or B. Each mutated promoter was fused to the CAT gene and introduced into MEL cells. CAT activity was measured before and after 80 hr of MEL cell differentiation. E. I. indicates the *PBGD* erythroid initiation site.

ment of these two elements is necessary for correct expression of those erythroid-specific promoters. This situation is similar to that observed in the rat tryptophane oxygenase gene, where cooperativity between the glucocorticoid receptor and a CAC box binding factor has been demonstrated (25).

The second region we have identified is located between positions −112 and −243 and binds the erythroid factors NF-E1 and NF-E2 in addition to several ubiquitous complexes (15). In the *PBGD* erythroid promoter, this region is necessary for correct regulation during MEL cell differentiation; thus, we focused our work on the erythroid-specific trans-acting factors. Site-directed mutagenesis of the −180 NF-E1 binding site did not affect the regulation of the PBGD erythroid promoter during MEL cell differentiation, suggesting that this binding site is not necessary for transcriptional induction. However, as previously observed for β-globin constructs stably introduced into MEL cells (8), we found position-dependent expression levels before MEL cell differentiation. Thus, we cannot determine the contribution of each of the elements of the *PBGD* erythroid promoter to its efficient transcription in erythroid cells, and the −180 NF-E1 binding site might be quantitatively but not qualitatively involved in the transcriptional activation of the *PBGD* erythroid promoter. The second erythroid-specific factor that binds to this −112 to −243 region has been called NF-E2. This factor competed with the ubiquitous AP1 factor for binding to the TGACTCA AP1 consensus sequence. As the NF-E2–oligonucleotide complex migrated faster than the AP1–oligonucleotide complex on nondenaturing gels, NF-E2 might be a degradation product of AP1 generated in an erythroid-specific fashion. Using UV cross-linking, we have shown that NF-E2 has a higher molecular weight than AP1 and, thus, is not an erythroid-specific degradation product of AP1. In addition, NF-E2 cannot be derived from c-*jun* by alternative splicing, as it has been shown that the human gene encoding c-*jun* has no intron (26). Finally, NF-E2 might be an erythroid-specific posttranscriptional modification of AP1. However, we do not favor this explanation since glycosylations and phosphorylations do not seem to alter the binding specificity of a number of trans-acting factors (27) and since AP1 and NF-E2 displayed distinct patterns of contact sites on the AP1 consensus sequence. Thus, NF-E2 and AP1 are probably different factors that bind the same sequence. We also have shown that NF-E2 but not AP1 is necessary for correct regulation of the *PBGD* erythroid promoter during MEL cell differentiation, demonstrating a biological function of this erythroid-specific factor. These results have to be compared to the study of Reitman and Felsenfeld (16) who found that mutations within the AP1 consensus sequence of the chicken β-globin enhancer dramatically reduced its efficiency in erythroid cells. Thus, as for NF-E1, NF-E2 might be involved in the coregulated transcriptional activation of erythroid-specific genes.

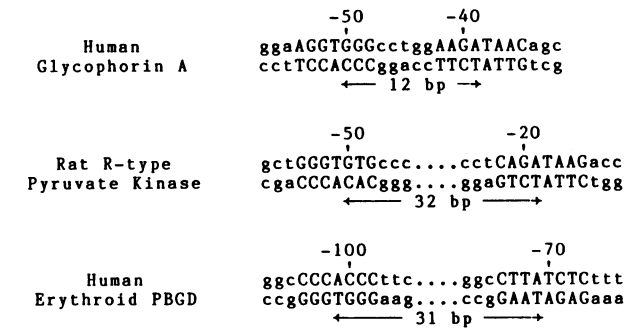

FIG. 5. Modular structure of various erythroid-specific promoters. The first nucleotide of each promoter region is numbered −1. The CAC and NF-E1 motifs are printed in capital letters.

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