Differential Binding of the NFE3 and CP1/NFY Transcription Factors to the Human \(\gamma\)- and \(\epsilon\)-Globin CCAAT Boxes*

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Naturally occurring nondeletional mutations affecting the distal CCAAT box of the human \(\gamma\)-globin gene promoter result in hereditary persistence of fetal hemoglobin in adult life. Although the distal CCAAT box is the target of several factors, including CP1/NFY, CDP, GATA-1, and NFE3, only NFE3 binding activity is consistently sensitive to well-characterized mutations in this region such as G\(-147\) → A, C\(-134\) → T, and \(\Delta 13\) hereditary persistence of fetal hemoglobin. We extensively characterized the binding specificities of NFE3 and demonstrated that NFE3 has unique properties with respect to other CCAAT box-binding proteins. Affinity-purified NFE3 from erythroid K562 cells binds the distal but not the proximal \(\gamma\)-globin CCAAT box, the single CCAAT box of the human \(\epsilon\)-globin promoter, and the proximal CCAAT box of the evolutionarily related Galago crassicaudatus \(\gamma\)-globin gene. Within the \(\epsilon\)-globin CCAAT box, NFE3 represents the major and almost exclusive binding activity. Disruption of such a binding site essentially inactivates the \(\epsilon\)-globin promoter, suggesting that NFE3 plays an important role in the embryonic expression of this gene.

In humans, different types of hemoglobins are produced during the embryonic, fetal, and adult stages (1). The first hemoglobin switch occurs early in gestation and involves the substitution of \(\alpha\) and \(\gamma\)-globin for \(\zeta\) and \(\epsilon\)-globin, respectively. At birth, \(\gamma\)-globin synthesis is almost completely switched off and is replaced by the synthesis of the major \(\beta\)-globin and the minor \(\delta\)-globin chains. Functional studies of the \(\beta\)-like globin cluster highlighted the role of the upstream locus control region in promoting high level erythroid-specific expression of the globin genes (2). However, temporal regulation of the expression of various globin genes, in particular \(\epsilon\) and \(\gamma\)-globin, appears to be largely dependent on sequences comprised within the genes themselves or in their immediate vicinity (3, 4).

Several different approaches have been taken to analyze functional elements in the promoters of the human globin genes. Evolutionarily conserved DNA motifs were identified and provided clues to the discovery of DNA binding motifs (5–7). Deletion and site-directed mutagenesis also identified potentially important motifs (8–10).

Finally, the existence of natural mutants within the human population provided in vivo evidence for the role of specific sequences (reviewed in Refs. 11 and 12). In particular, \(\beta\)-globin promoter mutations (\(\beta\)-thalassemia) demonstrated an important role of two conserved motifs, the TATA and the CACC box, in promoter function; surprisingly, no mutations have so far been identified in another highly conserved element, the CCAAT box. However, although these mutations significantly decrease \(\beta\)-globin expression, they do not appear to modify its temporal regulation.

On the other hand, mutations within the promoter of the fetal \(\gamma\)-globin gene do increase, often substantially, its postnatal and adult expression. Some of these mutations have been suggested to create better binding sites for transcription factors, such as GATA1 and Sp1 (13–18); others decrease or abolish the in vitro binding of nuclear proteins to the mutated \(\gamma\)-globin fragments and have been suggested to prevent the binding of negatively acting proteins that might repress transcription of the \(\gamma\)-globin gene postnatally (13, 19–21). The in vitro binding of one protein identified in these studies (NFE3) is consistently decreased with four different hereditary persistence of fetal hemoglobin (HPFH)\(^1\) mutations (Refs. 13 and 20 and present paper). We show that purified NFE3, in addition to binding to the distal \(\gamma\)-globin CCAAT box, also represents the major \(\epsilon\)-globin CCAAT box-binding protein.

MATERIALS AND METHODS

Cell Culture—The human erythroleukemic K562 cells were grown in a miniflask at a density of 1.5–1.8 \(\times\) 10\(^6\) cells/ml in RPMI 1640 medium supplemented with L-glutamine and 5% fetal calf serum.

Nuclear Extract Preparation and Protein Purification—K562 whole cell or nuclear extracts were prepared accordingly to reported methods (22, 23). For protein purification, the salt-precipitated proteins, obtained from 30 liters of cell culture, were dissolved in TM buffer (50 mM Tris, pH 7.9, 12.5 mM MgCl\(_2\), 5 mM EDTA, 10% glycerol) and loaded onto a heparin-Sepharose column. Bound proteins were step-eluted with TM buffer with 0.2, 0.4, 0.6, or 1 mM NaCl. The elution of NFE3 from the heparin and the subsequent columns was checked by gel mobility shift. The fractions eluted with 0.6 and 1 mM salt were pooled, and the proteins were precipitated with ammonium sulfate. The protein pellet was dissolved in TM buffer and loaded onto a Superdex-200 preparative grade column equilibrated with TM buffer with 0.1 mM NaCl. Fractions containing NFE3 were pooled and incubated with 500 \(\mu\)g of poly(dI-dC) and

\(^1\)The abbreviations used are: HPFH, hereditary persistence of fetal hemoglobin; DMS, dimethyl sulfate; CAT, chloramphenical acetyltransferase.
Nuclear or Whole Cell Extract

Heparin-Sepharose

CP1/NFY –
NFE3 –

Superdex-200

DNA-affinity

Ad D\textsubscript{H} ε NFE3-117

Fig. 1. Purification of NFE3. Gel mobility shift assays representing the degree of NFE3 fractionation are shown. In all cases the oligonucleotide probe was from the human ε-globin distal CCAAT box (D\textsubscript{H}). The fractions eluted at 0.4–2 M NaCl from the heparin column were also assayed by competition with unlabeled D\textsubscript{H}. CP1/NFY and NFE3 complexes are indicated. The asterisks indicate nonspecific complexes (see text). The protein eluted from a γ-affinity column was also tested by competition assays with CCAAT box region oligonucleotides (indicated above the figure) from the adenovirus type 2 origin of replication (Ad), the human γ-globin promoter distal CCAAT box (D\textsubscript{H}), the human ε-globin promoter CCAAT box (ε), a CP1/NFY canonical binding site from the human major histocompatibility complex gene promoter (NFY) and the –117 HPFH mutation (–117) of the γ-globin promoter (see Table I for nucleotide sequences).

125 μg of sonicated calf thymus DNA at 4°C for 15 min. The solution was cleared by centrifugation, and the supernatant was loaded onto a NHS-Sepharose column coupled to either the D\textsubscript{H} or the ε concatamerized oligonucleotides. The affinity chromatography was performed on a SMART system, and the bound proteins were eluted with a linear 0.1–0.8 M salt gradient.

In the supershift experiments shown in Figs. 2 and 7, the samples derived from extracts chromatographed onto a Sephacryl-300HR column. Fractions containing CP1/NFY or NFE3 activity were used.

Electrophoretic Mobility Shift Assay (24)—The standard binding reaction (20 μl) contained 0.1–0.2 ng of \textsuperscript{32}P-labeled oligonucleotide, 2–5 μg of crude nuclear protein, 1–3 μg of poly(dI-dC), and 2 μg of bovine serum albumin in a buffer consisting of 4 mM spermidine, 50 mM NaCl, 1 mM EDTA, 30 mM Tris-HCl, pH 7.9, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonil fluoride. Unlabeled competitor oligonucleotides were added to some reactions in a 100-fold excess relative to the labeled oligonucleotide. Affinity-purified fractions were used in all experiments, unless otherwise specified. When purified fractions were used, the poly(dI-dC) was added at a concentration of 2–10 ng/20-μl reaction mixture, and unlabeled competitors were in a 10-fold molar excess. The mixtures were kept on ice for 20 min. Gel electrophoresis was carried out in 5% acrylamide gels in 50 mM Tris borate, pH 8.2, at 20 °C or 4 °C (when purified fractions were used). The oligonucleotides used in this work are listed in Table I.

DNA affinity

RESULTS

Fig. 2. NFE3 is not an alternative form of CP1/NFY. Sephacryl-300HR CP1/NFY or NFE3 fractions were incubated with labeled D\textsubscript{H} in the presence of increasing amounts of antisera against the A \( (>\text{YA}) \) or the B \( (>\text{YB}) \) subunits of NFY or with two control antisera \( (>\text{GATA1} \) and \( >\text{Sp1}) \). CP1/NFY and NFE3 complexes are indicated with arrows. The asterisks indicate nonspecific complexes. For other abbreviations, see Fig. 1.

Purification of NFE3 from Human Erythroid K562 Cells—Experiments from the human erythroleukemic cell line K562 contain several factors that bind an oligonucleotide encompassing the human γ-globin distal CCAAT box from base –90 to –132 (long (L) D\textsubscript{H}); CP1/NFY, NFE3 and GATA-1 (13, 19–21). In particular, GATA-1 binds to a GATA-like motif (GACA) located between the two CCAAT boxes (19, 21). In order to simplify the pattern of complexes, we used in electrophoretic mobility shift assay (24) an oligonucleotide encompassing the human γ-globin distal CCAAT box from base –101 to –127 (D\textsubscript{H}) and lacking the GATA-1 binding site. Fig. 1 shows that D\textsubscript{H} is efficiently bound by CP1/NFY and NFE3 (input).

Nuclear or whole cell extracts from K562 cells were first processed by conventional chromatography to fractionate the NFE3 from CP1/NFY activity. This was achieved by salt step
elution of proteins bound to heparin-Sepharose. The NFE3 activity eluted in the 0.6 and 1 M salt fractions (Fig. 1). In the 1 M fraction a faster migrating complex was observed that is competed by D-H and is likely due to a proteolytic product of NFE3. Additional complexes, concomitantly with this band, are present in 0.4 and 2 M fractions. Because they are not competed by D-H, they represent nonspecific complexes (13).

CP1/NFY eluted in the 0.4 M fraction, though its binding activity could be restored only after dialysis against 0.1 M TM buffer (data not shown). The heparin-Sepharose 0.6 and 1 M fractions were further processed by Superdex 200 chromatography, and NFE3 was purified by affinity chromatography on a resin containing concatamerized D-H oligonucleotide (Fig. 1).

NFE3 Is Not an Alternative Form of CP1/NFY—

Affinity-purified NFE3 binds to the distal -globin CCAAT box (Ref. 13 and Fig. 1). In terms of binding specificities, CP1/NFY can be distinguished from NFE3 by its ability to bind to both the HPFH -117 mutant of the -globin distal CCAAT box region and the normal sequence, whereas NFE3 binds only to the latter (13, 20). In the active form, CP1/NFY consists of a heteromer composed of at least two subunits, A and B (29); it has been shown that alternative forms of CP1/NFY can originate from alternatively spliced NFY-A mRNA (30). Before proceeding to further purification, we tested whether NFE3 could be one alternative form or a degradation product of CP1/NFY. Antibody-mediated supershift assay was performed on Sephacyr-300HR fractionated CP1/NFY and NFE3. Two antisera raised against two invariant peptides of the NFYA (>YA) and the NFYB (>YB) subunits, respectively (31), were used. Neither antisera altered the NFE3-D-H complex even at concentrations capable of completely supershifting the NFY-D-H complex (Fig. 2).

NFE3 and CP1/NFY Bind to Overlapping but Distinct Sites

On the basis of binding specificity, NFE3 belongs to the heterogeneous class of CCAAT box-binding proteins. Because NFE3 and CP1/NFY complex with D-H (Fig. 3) shows that the two proteins contact the same bases in the core CCAAT motif (−115 and −114) and that both interact with base −117. However, NFE3 binding extends downstream to the core motif to bases −108 and −107. Although both proteins contact base −117, a G at this position seems critical only for NFE3 binding, as shown by experiments with the mutant G (−117) → A HPFH oligonucleotide (see below and Refs. 13 and 20). Indeed, the −117 HPFH oligonucleotide and the canonical CP1/NFY binding site from the mouse Eα gene promoter (Y box) have an A at the corresponding position with respect to the core motif (see Table I and “Discussion”).

NFE3 Does Not Bind −117, −114, and Δ13 HPFH Mutants and the Proximal CCAAT Box of the Human γ-Globin Promoter—

Affinity-purified NFE3 binds to the distal γ-globin CCAAT box but not to oligonucleotides carrying either the −117 and −114 HPFH point mutations (G → A and C → T, respectively) (32–34) or the 13-base pair HPFH deletion encompassing the distal CCAAT box (35), as demonstrated both by direct binding (Fig. 4A) and competition (Fig. 4B) experiments. These results are consistent with the criteria originally adopted to identify NFE3 activity in crude extracts (13, 20). An additional less characterized mutation (A (−110) → T) (36) detected in a low level HPFH, also abolishes the binding of NFE3 (data not shown). Moreover, NFE3 fails to bind to the proximal CCAAT box of the human γ-globin promoter (P-H) in spite of the high degree of homology between the two boxes (Fig. 4A and Table I). This finding, in agreement with the DMS interference data (Fig. 3), suggests that the two Cs at +3 and +4 downstream to the CCAAT core motif are critical for NFE3 binding.

NFE3 Binds to the Single CCAAT Box of the Human γ-Globin Promoter—

On the basis of binding specificity, NFE3 belongs to the heterogeneous class of CCAAT box-binding proteins. Be-

TABLE I

Sequences of oligonucleotides utilized in the present work

Nucleotide positions are relative to the CAP site. H, distal (D) or proximal (P) CCAAT boxes from the human γ-globin promoter; H and Hβ, human ε- or β-globin CCAAT boxes; Gγ, distal (D) or proximal (P) CCAAT boxes from the G. crassicaudatus γ-globin gene promoter; Eα, major histocompatibility complex II Eα gene promoter; Adori, adenovirus type 2 origin of replication.

| Name   | Position | Sequence                                              |
|--------|----------|-------------------------------------------------------|
| LD-H   | −132     | GGCACGCTTTGCCTGACAAAGGCAAACTT                        |
| D-H    | −127     | GGCACGCTTTGGACAAAGGCAAACTT                           |
| −117HPFH| −127     | GGCACGCTTTGGAAACAAAGGCAAACTT                         |
| −114HPFH| −132     | GGCACGCTTTGGACAAAGGCAAACTT                           |
| −110HPFH| −132     | GGCACGCTTTGGACAAAGGCAAACTT                           |
| Δ13HPFH| −132     | GGCACGCTTTGGACAAAGGCAAACTT                           |
| P-H    | −99      | GGCACGCTTTGGACAAAGGCAAACTT                           |
| Hβ     | −102     | GGCACGCTTTGGACAAAGGCAAACTT                           |
| Pγ     | −98      | GGCACGCTTTGGACAAAGGCAAACTT                           |
| Dγ     | −125     | GGCACGCTTTGGACAAAGGCAAACTT                           |
| Hα     | −89      | GGCACGCTTTGGACAAAGGCAAACTT                           |
| Eα     | −44      | GGCACGCTTTGGACAAAGGCAAACTT                           |
| Adori  | −22      | GGCACGCTTTGGACAAAGGCAAACTT                           |
NFE3 Binding to Globin Promoters

Fig. 4. Binding of affinity-purified NFE3 to wild type (γWT) and HPFH γ-globin CCAAT box regions. Labeled probes are indicated below the figure. −114, Δ13, and −117 indicate HPFH γ-globin mutated oligonucleotides (see Table I); for other abbreviations, see Fig. 1. A, direct binding. B, competition of the binding by the unlabeled oligonucleotides indicated on the top of the figure.

A

B

cause such an element is present in all globin gene promoters, we looked for NFE3 binding to other globin gene CCAAT boxes. Among the human globin gene CCAAT boxes, only the single ε-globin CCAAT box significantly bound NFE3 (Table I and Fig. 5A). When crude K562 cell extracts were incubated with the ε-globin CCAAT box, we observed a weak slow band (Fig. 5A, a) comigrating with the CP1/NFY complex obtained with the DγH oligonucleotide and a strong and broad band (Fig. 5A, b) partially overlapping with the position of the NFE3-DγH complex (Fig. 5A). An additional fast migrating band was observed, which by migration pattern and lack of specificity behaves as the nonspecific complex observed with D-γH. To test whether the protein(s) responsible for the band was NFE3, we processed whole cell extracts from K562 cells and purified the protein by affinity chromatography onto a resin coupled to the ε-globin oligonucleotide. NFE3 purified from either ε- or γ-resins yielded bands of similar mobility by electrophoretic mobility shift assay with DγH or ε probes, although the binding to the latter was much stronger (Fig. 5B), suggesting that the ε-globin CCAAT box is a better site for NFE3. The ε-globin-NFE3 complex was competed by an excess of unlabeled DγH or ε but not by −117 HPFH, NFY box or Ad2ori oligonucleotides (Fig. 5C), which contain CCAAT boxes capable of binding CP1/NFY but not NFE3.

DMS interference analysis shows that the interaction of NFE3 with the ε-globin CCAAT box is similar to that observed on the core DγH CCAAT box element (see Fig. 3) but extends an additional 7 bases upstream to the core motif (Fig. 6), suggesting that these bases might be responsible for the stronger binding to the ε-globin probe.

As shown in Fig. 5A the weak band a has a mobility similar to that of the CP1/NFY complex with DγH. However, using either a crude or a CP1/NFY Sephacryl-300HR fraction (Fig. 7A, lane 1), the complex (Fig. 5A, band a) was not significantly supershifted by either antiserum against CP1/NFY subunits A or B (Fig. 7, lanes 2–7) at concentrations that are able to completely supershift the CP1/NFY complex with DγH (compare with Fig. 2). Thus, NFE3 seems to be the major factor interacting with the ε-globin CCAAT box.

NFE3 Binds to the Proximal CCAAT Box of the Galago crassicaudatus γ-Globin Gene—The γ-globin genes of the prosimian G. crassicaudatus are known to be preferentially expressed during the embryonic rather than the fetal stage (5–7). The promoters of these genes contain a distal (DγG) and a proximal (PγG) CCAAT box. PγG shows a higher homology than DγG to the human ε-globin distal CCAAT box (Table I). It was interesting to observe that with crude extracts the PγG but not the DγG oligonucleotide generates a broad complex very similar to that observed with the human ε-globin oligonucleotide (Fig. 8A, lanes 1–3). Competition experiments show that the binding is consistent with all the criteria used to identify NFE3. In fact, the complex between NFE3 and the ε-globin oligonucleotide is competed by a low molar excess of PγG but not by DγG (Fig. 8A, lanes 6 and 8). Furthermore, the same results were obtained with affinity purified NFE3 (Fig. 8B). This finding, together with the embryonic expression of the prosimian γ-globin genes, suggests that NFE3 could play an important role in the expression of embryonic and fetal globin genes by binding to selective promoter elements.

NFE3 and CP1/NFY Binding Sites Are Functionally Interchangeable—As shown above, the CCAAT boxes of the embryonic ε- and fetal γ-globin genes strikingly differ in their relative abilities to bind NFE3 or CP1/NFY, respectively. To investigate the possibility that these binding differences have an effect on function, elements of the CCAAT boxes, necessary for NFE3 or CP1/NFY binding, respectively, were exchanged between the
promoters of the two genes and analyzed for their ability to drive CAT expression upon transfection in K562 cells.

We analyzed two types of constructs containing the human γ-globin promoter from −299 to +35 (HγCATwt) or the human ε-globin promoter from −220 to +18 (HεCATwt) linked to the CAT reporter gene (26). When K562 cells were electroporated with either HγCATwt or HεCATwt, the observed CAT expression levels were comparable. A CC → AA mutation in the single ε-globin CCAAT box (HεCATmut) that abolished the binding of NFE3 decreased CAT expression to the level of a control pro-

![Figure 5](image1.png)

**FIG. 5. Binding of NFE3 to ε-globin and DγH CCAAT boxes.** The labeled probes are indicated below the figure; unlabeled competitors are indicated above the figure. X, unrelated oligonucleotide. A, crude K562 nuclear extract. B, NFE3 fractions from the γ (lanes 1 and 2) and ε-affinity resin (lanes 3 and 4) tested by gel mobility shift assay with either DγH or ε probes. C, competition by unlabeled oligonucleotides of the binding of NFE3 eluted from ε-affinity resin to the ε probe. Ad, adenovirus.

![Figure 6](image2.png)

**FIG. 6. Methylation interference with binding to the ε-globin CCAAT box region.** Filled and open circles, strong and weak interference, respectively. A, top strand; B, bottom strand.
moterless plasmid. When the e-globin GAC triplet immediately downstream from the CCAAT box (positions -77 to -75) was replaced by an AGT triplet restoring the critical G (position -109) on the DγH region necessary for CP1/NFY binding (Fig. 3), the binding of NFE3 was abolished, as expected from the DMS interference data (Fig. 6), but was replaced by the binding of CP1/NFY (not shown). This mutant (HγCATmutAGT) was equally efficient as the wild type e-globin construct in K562 cells (Table II).

This result shows that either a NFE3 binding or a CP1/NFY binding site is sufficient (and necessary) for driving e-globin promoter activity. It should further be noticed that replacing the distal γ-globin CCAAT box within the γ-globin promoter with the e-globin CCAAT box had no effect (HγCATe) (Table II). These results have been confirmed using some of the same constructs linked to the strong 46-base pair erythroid enhancer (28) located in the human locus control region hypersensitive site II (Table II).

**DISCUSSION**

In the present work, we report a further characterization of the nuclear protein NFE3, a factor whose in vitro binding to the γ-globin distal CCAAT box was previously shown to be affected by HPFH mutations of this region (G\(\rightarrow\)A and -115 to -102 deletion, see Refs. 13 and 20). NFE3 appears to differ from CP1/NFY on the basis of both immunological and functional (in vitro DNA-protein interactions) criteria. In particular, antibodies directed against the A or B subunit of CP1/NFY do not affect the formation of the NF3-DNA complex, ruling out the possibility that NFE3 is a heterodimer of either CP1/NFY subunit with an unidentified additional protein (Fig. 2). In addition, DMS interference experiments show that NFE3 and CP1/NFY both interact with nucleotides -117, -115, and -114 of the γ-globin promoter, but only NFE3 extends its contacts to nucleotides -107 and -108 (Fig. 3). This result is in agreement with the inability of the -117 and -114 HPFH oligonucleotides to bind NFE3 (Fig. 4 and Ref. 13). Finally, the intensity of NFE3 binding to different oligonucleotides is inversely correlated to that of CP1/NFY (see Figs. 5A and 8A;
The interaction of NFE3 with bases downstream to the CCAAT box is likely to be important for sequence recognition. The most 5' C immediately downstream from the CCAAT box (position -108 in the human \( \gamma \)-globin promoter) is conserved in the sequences (P\( \gamma \)-G and e) able to bind NFE3 but not in those (D\( \gamma \)G and P\( \gamma \)H) unable to bind it (Table I); the G (antisense) residue at position -108 is critical in DMS interference experiments with D\( \gamma \)H, e (Figs. 3 and 6) and P\( \gamma \)G (not shown) oligonucleotides.

Other sequence differences, upstream to the CCAAT box, must be responsible for the stronger binding of NFE3 to the human \( \epsilon \)-globin and the G. crassicaudatus \( \gamma \)-globin CCAAT box regions, as indicated by DMS interference analysis of the \( \epsilon \)-globin-NFE3 complex (Fig. 6).

The strong binding of NFE3 to the human \( \epsilon \)-globin and G. crassicaudatus CCAAT box regions was unexpected. Previous binding studies (5-7, 37) have employed different gel shift patterns for the P\( \gamma \)-G and e human CCAAT box regions (but not for D\( \gamma \)H) that are similar to those reported by us to represent NFE3 binding and are likely to correspond to it. They also proposed a TGACCT motif as the element mediating the binding to P\( \gamma \)-G and e-globin oligonucleotides; however, this element (antisense -91-96) is only partially DMS-sensitive in the e-globin promoter (Fig. 6) and is modified to TGACCA and TGACCA in D\( \gamma \)H (Table I). A less extensive TGAC binding motif would, however, be fully consistent with the results of our DMS interference experiments.

The observation that both the fetal \( \gamma \)-globin gene and the embryonic human \( \epsilon \)-globin and G. crassicaudatus \( \gamma \)-globin genes have binding sites for NFE3 raises questions as to their role. The two embryonic genes have strong NFE3 binding sites, while showing little (G. crassicaudatus proximal CCAAT box) or no (human \( \epsilon \)-globin gene) CP1/NFY binding activities; in contrast, the fetal \( \gamma \)-globin gene has two very efficient CP1/NFY sites, and a low affinity NFE3 site. In agreement with these observations, genomic footprint analysis in K562 cells shows protection of nucleotides -117 (top strand) and -115 and -114 (bottom strand) in the distal CCAAT box of the human \( \gamma \)-globin gene and of the equivalent positions in the proximal CCAAT box (39, 40); these results are better explained by predominant occupancy of the distal CCAAT box by CP1/NFY rather than NFE3 (see also Fig. 3) in cells expressing the \( \gamma \)-globin gene. A mutation (H\( \gamma \)CATmut) in the e-globin CCAAT box that completely abolishes NFE3 binding results in the almost complete inactivation of the e-globin promoter in transfection experiments in K562 cells (Table II); however, a different mutation (H\( \gamma \)CATmut\( AGT \)) that also abolishes NFE3 binding but allows substantial CP1/NFY binding results in essentially normal activity of the promoter. Therefore these data suggest that, in the K562 environment, both NFE3 and CP1/NFY may act as positive transcription factors. The unique ability of the human \( \epsilon \)-globin and G. crassicaudatus embryonically expressed \( \gamma \)-globin genes to strongly bind NFE3, tempts one to speculate that NFE3 may be involved in the positive regulation of the embryonic globin genes. A similar idea was previously put forward by Gumucio et al. (6, 7), who observed the differential binding of unidentified proteins to the G. crassicaudatus versus the human proximal \( \gamma \)-globin CCAAT box (for further discussion, see also Ref. 38). A resolution of these issues must await transgenic assays and an accurate evaluation of the levels of NFE3 during development.

The present investigation was prompted by evidence provided by HPFH mutations in the CCAAT box region that the loss of NFE3 binding is a common factor in these conditions, suggesting that NFE3 may potentially act as a repressor of \( \gamma \)-globin gene activity. However, recent experiments\(^2\) show that specific disruption of the NFE3 site is not sufficient to cause adult overexpression (HPFH) of the mutated \( \gamma \)-globin gene in transgenic mice.

Our present evidence that NFE3 may be a positively acting factor for transcription (of the \( \epsilon \)-globin gene) is therefore not in contrast with the available information about its role in globin gene regulation, although alternative explanations for the HPFH phenotype caused by point mutations in the CCAAT box region must now be sought.

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