GENES: STRUCTURE AND REGULATION:

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Cooperation and Competition between the Binding of COUP-TFII and NF-Y on Human ϵ- and γ-Globin Gene Promoters*

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The nuclear receptor COUP-TFII was recently shown to bind to the promoter of the ϵ- and γ-globin genes and was identified as the nuclear factor NF-E3. Transgenic experiments and genetic evidence from humans affected with hereditary persistence of fetal hemoglobin suggest that NF-E3 may be a repressor of adult ϵ and γ expression. We show that, on the ϵ-promoter, recombinant COUP-TFII binds to two sites, the more downstream of which overlaps with an NF-Y binding CCAAT box. Binding occurs efficiently to either the 5′ or the 3′ COUP-TFII site but not to both sites simultaneously. However, adding recombinant NF-Y induces the formation of a stable COUP-TFII·NF-Y-promoter complex at concentrations of COUP-TFII that would not give significant binding in the absence of NF-Y. Mutations of the promoter indicate that COUP-TFII cooperates with NF-Y when bound to the 5′ site, whereas binding at the 3′ site is mutually exclusive. Likewise, in the γ-promoter, COUP-TFII binds to a site overlapping the distal member of a duplicated CCAAT box, competing with NF-Y binding. Transfections in K562 cells show that both the mutation of the 5′ COUP-TFII or of the NF-Y site on the ϵ-promoter decrease the activity of a luciferase reporter; the mutation of the 3′ COUP-TFII site has little effect. These results, together with transgenic experiments suggesting a repressive activity of COUP-TFII on the ϵ-promoter and the observation that, on the 3′ site, COUP-TFII and NF-Y binding is mutually exclusive, suggest that COUP-TFII may exert different effects on ϵ transcription depending on whether it binds to the 5′ or to the 3′ site. At the 5′ site, COUP-TFII might cooperate with NF-Y, forming a stable complex, and stimulate transcription; at the 3′ site, COUP-TFII might compete for binding with NF-Y and, directly or indirectly, decrease gene activity.

The non-α-globin genes are clustered in several species within a relatively small chromosomal region. The expression of these genes is precisely regulated both spatially and quantitatively during embryonic, fetal, and postnatal development to match the expression of α-globin genes, resulting in a perfectly balanced α/non-α synthetic ratio (1–3).

In man, the predominant non-α-globin chain during the embryonic period is ϵ-globin, which around the third month of gestation (embryonic-fetal switch) is replaced by γ-globin (encoded by two non-allelic genes, ^{G}γ- and ^{A}γ-globin) and finally, around birth, by β-globin (fetal-adult switch). DNA sequences regulating globin gene expression have been extensively investigated; in addition to the upstream locus control region, essential for the correct activity of all the genes in the cluster (4–8), several promoter, enhancer, and other non-conventional elements have been described (9, 10).

Despite the detailed knowledge of the DNA elements regulating globin expression, the mechanisms underlying the transition from embryonic (ϵ) to fetal (γ) and then to adult (β) gene expression remain largely unclarified. In particular, no transcription factor has been detected whose activity during development varies in a way consistent with the changes observed in globin gene expression at the various stages; even erythroid Krüppel-like protein, although necessary for β-globin (11–14) but not ϵ- and γ-globin expression, is present and active during the embryonic and early fetal stages, when β-globin is not yet expressed (13).

Some clues to the nature of the DNA sequences controlling the temporal expression of γ-globin genes have come from inherited conditions usually observed in heterozygous individuals and known as hereditary persistence of fetal hemoglobin (HPFH)[1] (1–3). Such individuals present, postnatally, moderate or high levels of fetal hemoglobin ($\alpha_2\gamma_2$). Some HPFHs are caused by point mutations in either the ^{G}γ or ^{A}γ-globin gene; the mutated gene is selectively overexpressed in adults. Six different mutations causing HPFH cluster around the double CCAAT box region and affect the binding of several proteins (3, 15–24). Among them, all of the four different HPFH mutations studied so far greatly diminish or abolish the binding of a protein called NF-E3 (23).

These results suggested that NF-E3 might be a γ-globin repressor and that the inability of the γ-globin promoter to bind it might be the underlying cause of the HPFH phenotype. Given the large number of proteins binding to this region, it has not been possible to design an artificial mutation that fully reproduces the binding abnormalities observed with spontaneous HPFH mutations and also causes HPFH in transgenic models; however, two mutations causing HPFH in humans have the same effect in transgenic mice (24, 25). Additional evidence that NF-E3 may be a repressor was also provided by more recent experiments showing that an extensive mutation of both the distal and the proximal NF-E3 binding sites on the

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[1] The abbreviations used are: HPFH, hereditary persistence of fetal hemoglobin; EMSA, electrophoresis mobility shift assay; GST, glutathione *S*-transferase.

ϵ-globin promoter causes persistent expression of an ϵ-globin transgene in adult erythroid cells (26). In these studies it was shown that COUP-TFII, an orphan nuclear receptor, is either NF-E3 or, more likely, a part of an NF-E3 complex, whose composition varies during mouse development.

In both the ϵ- and γ-globin promoters, the NF-E3 binding sites partially overlap an NF-Y binding site. NF-Y is a trimeric transcription factor composed of three subunits (A, B, C) that binds to the CCAAT box motif (27). Some observations suggest the relevance of NF-Y for γ-globin promoter activity in HPFH. (i) A mutation of the proximal γ-globin CCAAT box within a transgenic γ-globin HPFH construct carrying the −117 HPFH G → A mutation, adjacent but not overlapping the distal CCAAT box, almost completely suppresses the overexpression of γ-globin in adult cells, which is normally caused by the HPFH mutation (25). Note that the mutation of both CCAAT boxes has no effect on the embryonic expression of a normal γ-globin gene, indicating that at this stage factors other than NF-Y are responsible for the high level of γ-globin activity. (ii) HPFH point mutations, which mutate the CCAAT box, thus decreasing NF-Y binding, induce much lower levels of fetal hemoglobin expression in adults than the −117 HPFH G → A mutation, which does not affect the CCAAT box.

In this paper, we have investigated by *in vitro* electrophoresis mobility shift assay (EMSA) the simultaneous binding of recombinant COUP-TFII·NF-E3 and NF-Y to normal and mutated ϵ- and γ-globin promoters. We show that COUP-TFII and NF-Y binding can be either mutually exclusive or cooperative, depending on the particular binding site used. On the basis of these observations and transfection experiments, we propose that COUP-TFII may act as a modulator of ϵ-globin transcription by cooperating and/or interfering with NF-Y binding.

EXPERIMENTAL PROCEDURES

Recombinant Proteins Preparation—NF-YA and NF-YB cDNA fragments were cloned into the *Escherichia coli* expression vector pET3b (29); NF-YC was polymerase chain reaction-cloned into the PET32b vector (30). Expression and purification of the recombinant proteins were as described in Mantovani *et al.* (29) and Bellorini *et al.* (30), in particular, the three subunits were purified on a Ni^{2+}-agarose column by means of the His tag, either from soluble BL21 LysS bacterial extracts or from renatured inclusion bodies.

Native NF-Y protein was purified from CH27 cells according to Kadonaga and Tjian (31). The COUP-TFII-pGEX-3X expression plasmid was kindly provided by Dr. Paulweber (32).

To produce glutathione *S*-transferase (GST)-fused COUP-TFII recombinant protein, BL21 LysS bacteria were transformed, grown in LB at mid-logarithmic phase (0.7–0.8 *A* at 600 nm) and induced with isopropyl-1-thio-β-D-galactopyranoside (1 mM) for 3 h at 37 °C. Cells were pelleted and frozen at −80 °C; upon thawing, they were resuspended in BC300 (300 mM KCl, 20 mM Hepes pH 7.9, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride), sonicated over a total of 5 min with 30-s pulses, and centrifuged. The supernatant was then processed for purification; soluble bacterial extracts were loaded on a glutathione-Sepharose 4B (Amersham Pharmacia Biotech) column, and then GST-COUP-TFII was eluted at 30 mM glutathione according to standard protocols. For control experiments, GST was removed by proteolytic cleavage with FactorXa (Amersham Pharmacia Biotech). Extracts from COS cells transfected with a COUP-TFII expression vector were provided by Dr. B. Paulweber.

EMSA—Nuclear extract preparation, *in vitro* incubation of labeled oligonucleotides with nuclear proteins, and electrophoretic analysis (EMSA) were performed according to standard protocols, as previously described in Refs. 23, 25, and 28. ^{32}P-Labeled oligonucleotides (0.1–0.5 ng) were incubated for 20 min at 4 °C with the recombinant proteins in a buffer containing 5% glycerol, 50 mM NaCl, 20 mM Tris, pH 7.9, 0.5 mM EDTA, 5 mM $MgCl_2$, and 1 mM dithiothreitol. The reaction mix was then run into a 5% polyacrylamide gel (acrylamide/bisacrylamide ratio of 29:1) at 4 °C. The sequence of the oligonucleotides used is described in Table I.

Competition experiments were performed using 20–50-fold molar excess of unlabeled oligonucleotides. The anti NF-Y B subunit antibody was generated by R. Mantovani as described in Ref. 29. The anti-ARP-1 T-19 (COUP-TFII) antibody was purchased from Santa Cruz Biotechnology (sc-6578)

Plasmid Construction—The ϵ-globin promoter spanning from nucleotide −220 to nucleotide +18 (23) was joined by linkers to the *Hin*dIII site in the pGL2basic (Promega) luciferase reporter vector. A 46-base pair oligonucleotide corresponding to the erythroid-specific enhancer derived from the human locus control region hypersensitive site II (33) was inserted in the pGL2 *Bgl*II site upstream to the promoter. All the mutant constructs tested were produced by polymerase chain reaction techniques and entirely sequenced.

Transfection Experiments—K562 cells were grown in RPMI 1640 medium supplemented with L-glutamine and 5% fetal calf serum. 10^7 exponentially growing K562 cells were electroporated at 400 V, 960 microfarads with a Bio-Rad apparatus in 0.8 ml of phosphate-buffered saline with 10 μg of plasmid according to Ronchi *et al.* (23). To normalize for transfection efficiency, 700 ng of pRL-TK plasmid (Promega, dual luciferase reporter system) were cotransfected in each sample. After 48 h, extracts were prepared, and the double luciferase activity was tested according to the Promega protocol. All experiments were repeated in triplicate with at least three independent plasmid preparations.

RESULTS

COUP-TFII and NF-Y Interact upon Binding to the ϵ-Globin Promoter in Vitro—Previous experiments (23, 26, 28) showed that both NF-E3 and (weakly) NF-Y bind to the ϵ-globin promoter in the CCAAT box region; in addition, the same two factors bind to the CCAAT box region of the γ-globin promoter, although in this case NF-Y binding is much stronger, and NF-E3 binding is weaker with respect to the ϵ-globin promoter (23, 28).

Mignotte and co-workers (26) recently proposed that COUP-TFII, an orphan nuclear receptor, is part of the NF-E3 complex (26). With K562 extracts, NF-E3 runs as a poorly resolved doublet; Fig. 1, *lane 2*, shows that an antibody against COUP-TFII almost completely supershifts the NF-E3 complex formed with an ϵ-globin promoter oligonucleotide (ϵ2DR, Table I).

In addition, recombinant COUP-TFII obtained by transfection of an expression vector in COS cells, generates on the same oligonucleotide a band that migrates as the slower portion of the NF-E3 band (Fig. 1*B*). These data confirm that COUP-TFII is at least a component of NF-E3.

There are two putative NF-E3/COUP-TFII binding sites on the human ϵ-globin promoter (26); the 3′ site overlaps the NF-Y binding CCAAT box (Fig. 2). To characterize the binding of COUP-TFII to this region, we analyzed by EMSA the interaction of synthetic oligonucleotides with recombinant COUP-TFII obtained as a bacterially produced protein fused to GST at its NH_2 terminus. Using the ϵ2DR oligonucleotide, a single band is present at low concentrations of COUP-TFII; at the highest COUP-TFII concentration (*lane 4*), a smear of slower mobility is observed, suggesting that, if a complex with two COUP-TFII molecules is formed, it is very unstable.

NF-Y was previously shown to bind, albeit weakly, to the ϵ-globin promoter (23, 28). We incubated increasing amounts of the three recombinant A, B, C subunits of NF-Y with the same ϵ-globin oligonucleotide; at an intermediate concentration of protein, a strong NF-Y band was formed of a mobility slower than the COUP-TFII complex (Fig. 2*A*) as expected (23). To test whether COUP-TFII and NF-Y can bind simultaneously to the same DNA molecule, we then incubated (Fig. 2*A*, *lanes 8–11*) a fixed amount of NF-Y (corresponding to that used in Fig. 2, *lane 6*) together with increasing amounts of COUP-TFII (*i.e.* the same amounts as used in *lanes 1–4* in the absence of NF-Y). Fig. 2*A* shows that, at the lowest COUP-TFII concentration, which gives essentially no binding by itself (*lane1*), most of the NF-Y complex is shifted up to a slower mobility (*lane 8*), suggesting the simultaneous binding of both proteins on the same DNA molecule; no COUP-TFII band is present, in agreement

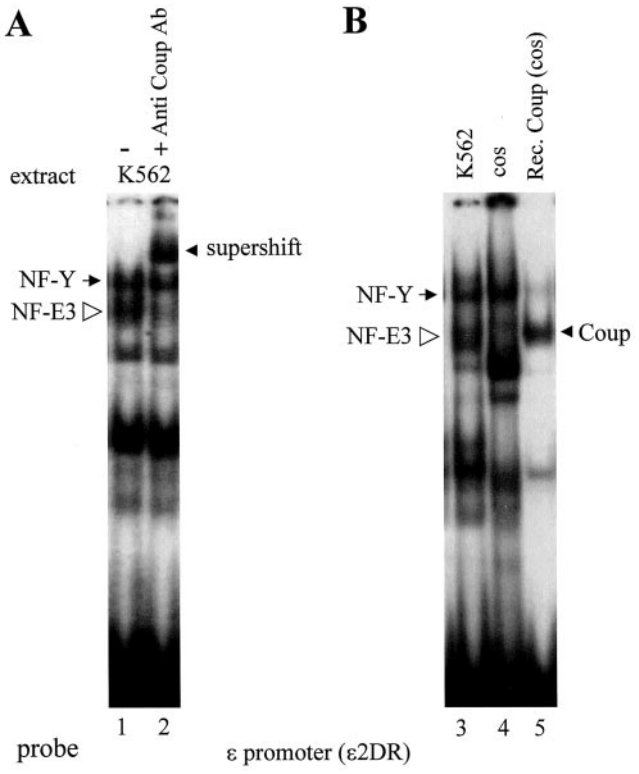

FIG. 1. **EMSA analysis of the ϵ-promoter region encompassing the CCAAT box.** *A*, 4 μg of K562 nuclear extracts were incubated with the ^{32}P-labeled oligonucleotide ϵ2DR (Table I, oligonucleotide 1). In *lane 2* the complex corresponding to NF-E3 (23, 26) is supershifted by an anti-COUP-TFII antibody. *B*, 0.5 μg of nuclear extract from COS cells transfected with a COUP-TFII expression vector (*lane 5*) generates a band of similar mobility as NF-E3 (compare *lanes 3* and *5*). This band is absent in the extract from non-transfected COS cells even at a much higher protein concentration (6 μg, *lane 4*).

TABLE I
Wild type and mutated transcription factor binding sites

All the oligonucleotides used for gel shift experiments are listed (upper strand only). The CCAAT box is underlined; mutations are in bold lowercase letters. MHC, major histocompatibility complex.

1. CCCTGAGGGACACAGGTCAGCCTTGACCAATGACTTTTAAGTA (ϵ2DR)
2. CCCTGAG-G**gac**CAGC**gac**GCCTcGACCAATGA**ggg**TTAAGTA (ϵ2DRmut)
3. CCCTGAG-G**gac**CAGG**gac**GCCTTGACCAATGACTTTTAAGTA (ϵ2DR5′mut)
4. CCCTGAGGGACACAGGTCAGCCT**c**GACCAATGA**ggg**TTAAGTA (ϵ2DR3′mut)
5. CCCTGAGGGACACAGGTCAGCCTTGACCA**g**TGACTTTTAAGTA (ϵ2DR NF-Ymut)
6. ACTGAACCCTTGACCCCTGCCCT (human apoAI promoter, COUP-TFII consensus oligo)
7. ATTTTTCTGATTGGTTAAAAGT (MHC Ea NF-Y consensus oligo)
8. CTAGGCCTTGCCTTGACCAATAGCCTTGACAAGGCAAACTTGACCAATAGTCTTAGAG (γ promoter, wild type)
9. GCCTTGCCTTGACCAATAGCCTTGACA (γ promoter, distal CCAAT box)
10. GCCTTGCCTT**a**ACCAATAGCCTTGACA (γ promoter distal CCAAT box, −117 HPFH)
11. GCCTTGCCTTGACCAAT**a**CCTTGACA (γ promoter, distal CCAAT box, mutant −109)
12. GCCTTGCCTTGACCAATAG**tt**TTGACA (γ promoter, distal CCAAT box, double mutant −107/−108)

with the expected result (compare with *lane 1*). When higher concentrations of COUP-TFII are used, the NF-Y band is progressively shifted to the position of the slower complex (*lanes 8–11*), and the COUP-TFII single band appears. These results indicate that COUP-TFII, although unable by itself to bind to the ϵ-globin promoter at low concentrations, readily does so in the presence of NF-Y.

A number of control experiments (Fig. 2*B*) were also carried out to verify the specificity of the observed bands. In particular, the binding of recombinant COUP-TFII to the ϵ-globin promoter is competed by an oligonucleotide carrying the human apoAI COUP-TFII binding site (Table I, Refs. 26 and 32, Fig. 2*B*, *lane 16*, and data not shown) but not by an unrelated oligonucleotide (not shown). In addition, an antibody against the YB subunit of NF-Y supershifts both the NF-Y band and the slower complex formed by the addition of an intermediate concentration of COUP-TFII (Fig. 2*B*, *lanes 13* and *18*), confirming the presence of NF-Y in the latter band. Furthermore, both the upper and lower band are competed by an excess of unlabeled NF-Y binding oligonucleotide (major histocompatibility complex Ea NF-Y consensus oligo (Table I, Refs. 23 and 29, and Fig. 2*B*, *lane 19*), but only the upper band is competed by an unlabeled COUP-TFII binding site (*lane 20*). Taken together, these experiments indicate that the slowest band observed in the presence of both COUP-TFII and NF-Y is a complex containing both proteins.

The upper complex observed in Fig. 2 might either be because of the binding of COUP-TFII and NF-Y to the same DNA molecule or to protein-protein interaction between COUP-TFII and NF-Y before binding. An artifactual effect due to the fused moieties of recombinant COUP-TFII and NF-Y (see "Experimental Procedures" and Refs. 29, 30, and 32) was excluded by adding Factor Xa-treated COUP-TFII to a K562 nuclear extract (Fig. 2*C*); the NF-Y band (*lane 21*) was quantitatively shifted to the upper position (*lane 22*). As a control, COUP-TFII added to a mutant oligonucleotide lacking COUP-TFII sites (ϵ2DRmut) (see below, Fig. 4) failed to shift the NF-Y band (*lane 23*). The same results were obtained with native NF-Y purified from CH27 cells (not shown).

In addition, we ruled out the possibility of a stable COUP-TFII-NF-Y interaction in the absence of DNA; recombinant GST-COUP-TFII·NF-Y were mixed together and passed onto a Sepharose column carrying immobilized anti-NF-YA antibody. Whereas both COUP-TFII and NF-Y were present in the un-

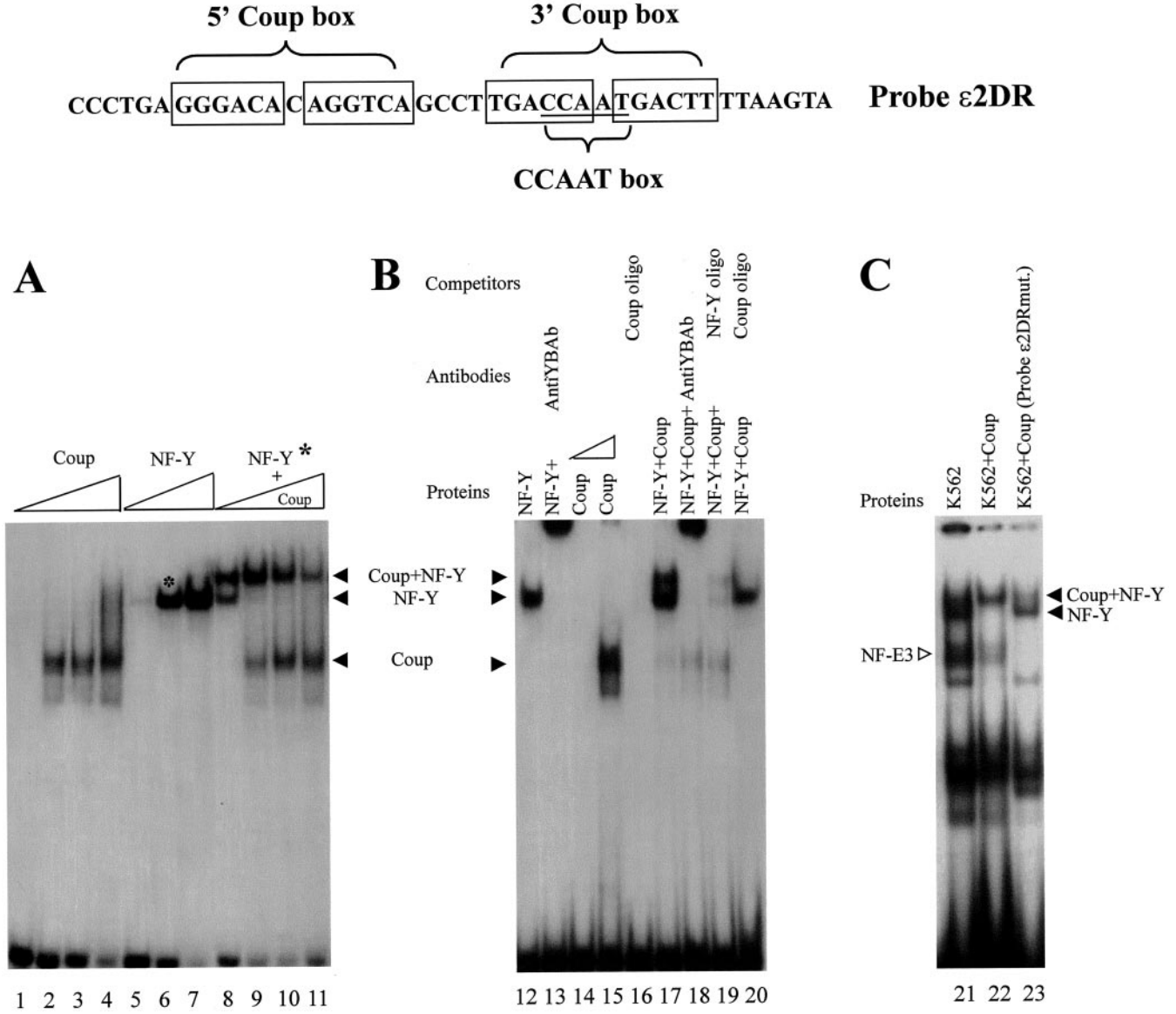

FIG. 2. **EMSA analysis of COUP-TFII and NF-Y binding on the ϵ-globin promoter probe.** Recombinant GST-COUP-TFII and NF-Y subunits A, B, and C were incubated with a ^{32}P-labeled ϵ-globin promoter oligonucleotide, and their interaction was analyzed by gel shift. *A*, *lanes 1–4*; *lane 1* corresponds to the minimal level of recombinant COUP-TFII protein. The following lanes (*lanes 2–4*) correspond to a 3-fold increase each of recombinant GST-COUP-TFII over the previous lane. *Lanes 5–7*, binding with an increasing amount (3-fold increase per *lane*) of recombinant NF-Y. *Lanes 8–11*, a fixed amount of NF-Y (see *asterisk*, *lane 6*) is added to the same amount of COUP-TFII as used in *lanes 1* to *4*. *B*, control competitions and supershifts. The band generated by NF-Y (*lane 12*) is supershifted by an antibody raised against the YB subunit of NF-Y (*lane 13*) (29). The COUP-TFII single band (*lanes 14–15*, see also *lanes 1–2*, *panel A*) is competed by a 20-fold excess of unlabeled apolipoprotein AI COUP-TFII consensus oligonucleotide (32) (*lane 16*). *Lane 17*, the slowest band (*arrow*, COUP+NF-Y), formed by mixing together COUP-TFII and NF-Y, is either supershifted by the anti-YB antibody (*lane 18*) or competed by a 20-fold excess of either the unlabeled major histocompatibility complex class II Ea Y-box consensus oligo for NF-Y (28) or the apoA COUP-TFII oligonucleotide (*lanes 19* and *20*, respectively). Recombinant proteins and unlabeled competitors or antibodies are indicated on the top of the figure. *C*, the addition of bacterial COUP-TFII cleaved with Factor Xa to K562 nuclear extracts (4 μg) quantitatively supershifts the NF-Y band to form a slower complex (*lane 22*). This upper band is not formed when a mutant probe (ϵ2DRmut, Table I) lacking both COUP-TFII sites is used (*lane 23*).

bound fraction (as assayed by Western blot with anti-NF-YB, anti-COUP-TFII, and anti-GST antibodies), only NF-Y was retained by the column (data not shown).

NF-Y Bound to the ϵ-Globin Promoter Favors COUP-TFII Binding—The experiment shown in Fig. 2*A* indicates that COUP-TFII is able to bind to NF-Y-bound ϵ-globin DNA at concentrations that would not allow significant binding to DNA alone. To provide a quantitative evaluation of this phenomenon, we incubated COUP-TFII at a wide range of concentrations with a fixed amount of DNA in the presence or absence of a given amount of NF-Y. As shown in Fig. 3*A*, ≈50% of the NF-Y-bound DNA was shifted to the upper band at a concentration of 9 COUP-TFII arbitrary units. In contrast, a similar amount of COUP-TFII band was formed at a much higher COUP-TFII concentration (Fig. 3*A*, 60 units, *lane 8*). This experiment likely underestimates the real difference; in fact, the concentration of the NF-Y-bound DNA is approximately 5-fold lower than that of the total probe. As shown in Fig. 3*B*, diminishing the target DNA dramatically decreases binding. For this reason, the COUP-TFII binding experiment was also carried out at lower DNA concentrations (0.3× and 0.15× and data not shown); under these conditions, a 50% shift of the probe because of COUP-TFII binding is observed between 120 and 240 units of protein (Fig. 3*A*, *right*), *i.e.* at a protein concentration that is 12–25-fold higher than that needed to shift 50% of the NF-Y-bound probe.

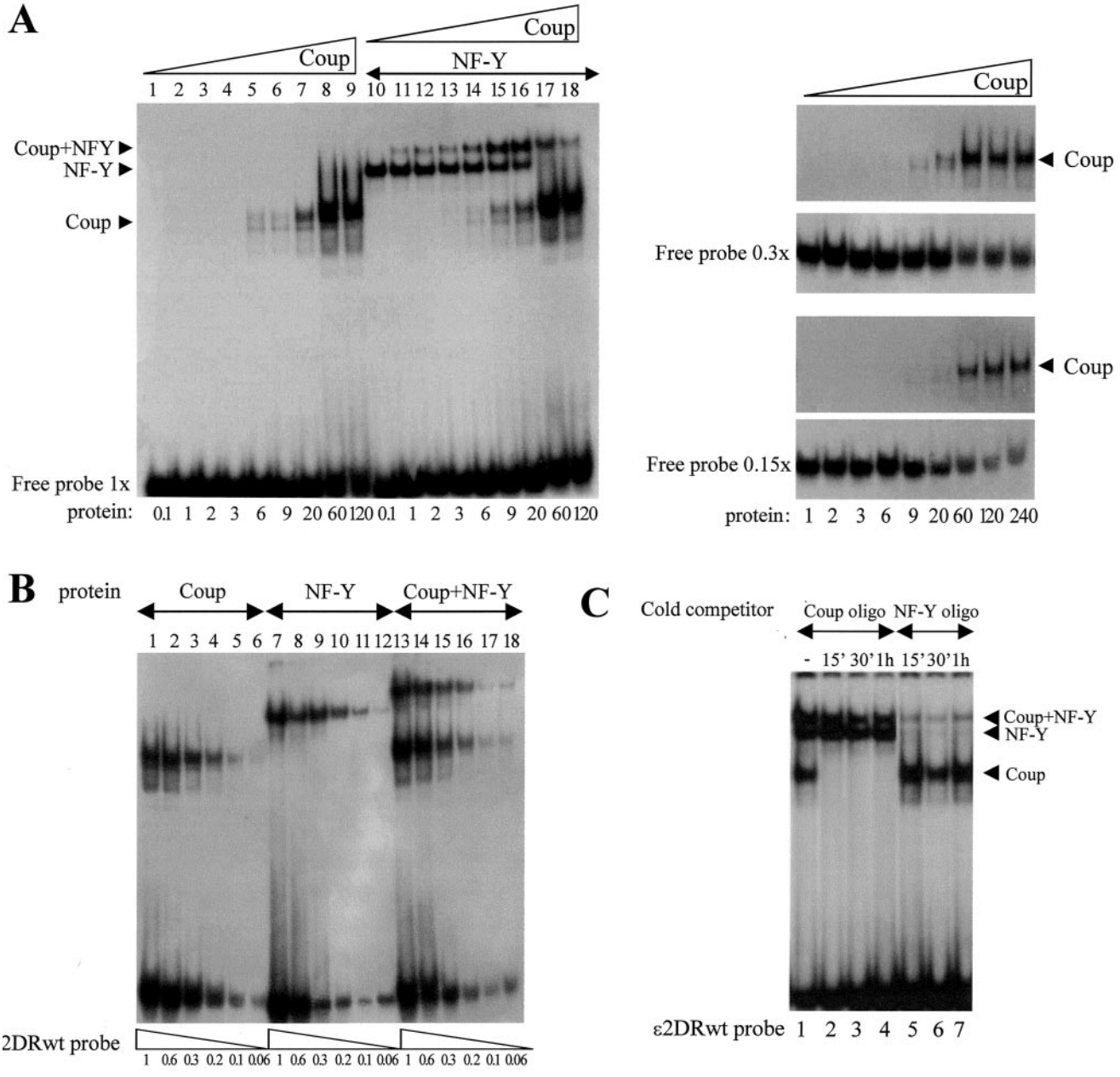

FIG. 3. **Kinetic analysis of the formation of the COUP-TFII·NF-Y·ϵ-promoter complex.** *A*, increasing amounts of recombinant COUP-TFII were added to ϵ2DR probe in the absence (*lanes 1–9*) or in the presence (*lanes 10–18*) of a given amount of NF-Y. The quantity of COUP-TFII, given in arbitrary units on the basis of appropriate dilutions of the recombinant protein, is indicated at the bottom of the gel. In the *right panels*, the same experiment has been carried out using a 0.3× and a 0.1× concentration of the labeled probe, respectively. *B*, constant amounts of COUP-TFII (*lanes 1–6*), NF-Y (*lanes 7–12*), or both (*lanes 13–18*) were incubated with decreasing amounts of probe (see the bottom of the gel for dilutions). *C*, incubation of appropriate amounts of NF-Y and COUP-TFII with ϵ2DR probe for 20′ at 4 °C was followed by competition with a 20-fold excess of unlabeled oligonucleotides containing the binding sites for either COUP-TFII (COUP oligo, *lanes 2–4*) or NF-Y (NF-Y oligo, *lanes 5–8*). Unlabeled competitors were added at 4 °C for the time indicated on the top of the figure before loading the reaction mixture onto the gel. *wt*, wild type.

Furthermore, we assayed the stability of the complexes by incubating the intact ϵ-globin oligonucleotide with appropriate amounts of COUP-TFII and NF-Y, allowing for the formation of all three complexes. We then added cold competitor oligonucleotides carrying either the COUP-TFII or NF-Y consensus (see Table I). The mixture was kept for various times at 4 °C before loading onto the gel. Fig. 3*C* shows that the unlabeled COUP-TFII competitor oligonucleotide readily abolishes (within 15 min of incubation) the single COUP-TFII band (*lane 2*) but only slowly decreases the upper COUP-TFII·NF-Y·DNA complex upon incubation for 60 min, as indicated by the ratio of the upper COUP-TFII·NF-Y *versus* the intermediate NF-Y band (*lanes 2–4*). In contrast, in a similar experiment, unlabeled NF-Y binding oligonucleotide almost fully competes the NF-Y intermediate band and significantly reduces the proportion of the upper complex within 15 min (Fig. 3*C*, *lanes 5–7*). These results show that NF-Y, bound to the ϵ-globin promoter, somehow helps COUP-TFII form a relatively stable complex.

The Formation of the COUP-TFII·NF-Y-ϵ-Globin Promoter Complex Requires the 5′ COUP-TFII Binding Site—In the previous section, we showed that a complex including both COUP-TFII and NF-Y is formed on a single ϵ-globin DNA promoter oligonucleotide. Are both COUP-TFII binding sites required for this effect?

To answer this question, we first introduced into the ϵ-globin oligonucleotide a mutation known to abrogate both the NF-E3/

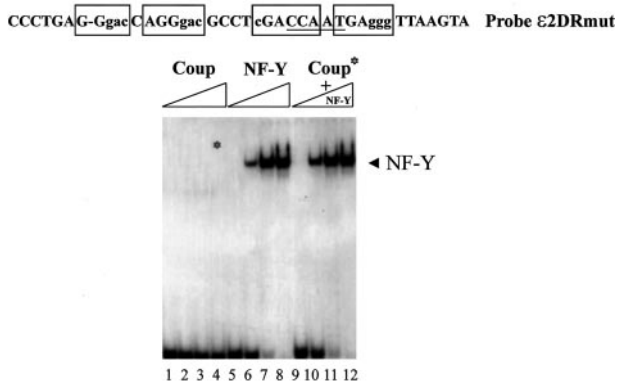

FIG. 4. **EMSA analysis of the double COUP-TFII mutant ϵ-globin oligonucleotide.** *Lane 1–4*, no COUP-TFII binding is observed in the presence of a mutation abolishing both the COUP-TFII sites on the ϵ-promoter. The amounts of added COUP-TFII are the same as in Fig. 2*A*, *lanes 1–4*. *Lanes 5–9*, increasing amounts of NF-Y bind the mutated probe. In *lanes 5–7* the amounts of NF-Y correspond to those in Fig. 2*A*, *lanes 5–7*. Each lane is a further 3-fold increase. When the same amounts of NF-Y are added to a fixed amount of recombinant COUP-TFII (as in *lane 4*, see the *asterisk*), no upper band corresponding to the COUP-TFII·NF-Y·ϵ-promoter complex is observed (*lanes 9–12*).

COUP-TFII consensus motifs (Table I, ϵ2DRmut oligonucleotide) (26). Fig. 4 shows that this mutation does not prevent the binding of NF-Y (*lanes 6–8*) but abrogates the formation of the COUP-TFII band (*lanes 1–4*) and of the upper COUP-TFII·NF-Y band (*lanes 9–12*); thus, COUP-TFII must bind DNA to allow the formation of the complex.

Then, we mutated either the 5′ or the 3′ COUP-TFII binding sites; on the 5′-mutated probe, as shown in Fig. 5, *lanes 1–3*, recombinant COUP-TFII is still able to generate a complex of the usual mobility, but the addition of increasing amounts of NF-Y fails to generate the COUP-TFII·NF-Y slow complex (*lanes 7–9*). Thus, the 3′ COUP-TFII site alone is not able to induce the formation of the upper complex.

We repeated the same experiment using the ϵ-oligonucleotide mutated in the 3′ COUP-TFII site. At an intermediate COUP-TFII concentration, a low level of NF-Y is already able to form both the NF-Y-ϵ-oligonucleotide and the COUP-TFII·NF-Y·ϵ-oligonucleotide complexes; at higher NF-Y levels, a strong progressive increase of the upper band together with a decrease of the COUP-TFII single band is observed (Fig. 5, *lanes 16–18*). These results indicate that the formation of the upper complex requires COUP-TFII bound at the 5′ site and a NF-Y molecule bound on the CCAAT box overlapping the 3′ COUP-TFII site.

A Mutation of the COUP-TFII Binding Site in the ϵ-Globin Promoter Causing Persistent Expression of the ϵ-Globin Gene in Transgenic Mice Also Increases NF-Y Binding in Vitro—It was previously reported (26) that an extensive mutation of the COUP-TFII binding motifs in the ϵ-globin promoter of an ϵ-globin transgene greatly increases its activity postnatally, a situation recalling the effect observed for the γ-globin gene in some HPFHs. Because these mutations include nucleotides immediately flanking the core of the NF-Y binding motif (*i.e.* the CCAAT box), we tested the ability of the mutant *versus* the normal sequence to bind NF-Y. Fig. 6 shows that the mutant oligonucleotide binds recombinant NF-Y significantly better than the normal one.

In the γ-Globin Promoter, Either COUP-TFII or NF-Y Binds Their Overlapping Binding Sites—In the human γ-globin promoter the CCAAT box is duplicated, and a single COUP-TFII/NF-E3 site overlaps the distal (5′) CCAAT box. The existence of an overlapping NF-Y·COUP-TFII site is reminiscent of the ϵ-globin promoter, where the 3′ COUP-TFII site and the CCAAT box overlap.

We therefore tested the binding of COUP-TFII and NF-Y on the γ-globin promoter region encompassing these sites. Fig. 7*A* shows that both COUP-TFII (*lanes 1–3*) and NF-Y (*lanes 4–6*) bind separately to the γ-globin probe, giving bands of relative mobility similar to those generated on the ϵ-globin promoter (not shown). When increasing amounts of COUP-TFII are added to a fixed amount of NF-Y, a strong increase of the COUP-TFII band is observed (together with a modest decrease of the NF-Y band), but no upper band is observed that might suggest the formation of a COUP-TFII·NF-Y·γ-globin complex (*lanes 7–9*); the same result is obtained when increasing amounts of NF-Y are added to a fixed amount of COUP-TFII (*lanes 10–12*). Thus, either COUP-TFII or NF-Y, but not both proteins simultaneously, can bind to the distal CCAAT box region on the γ-globin promoter. This is consistent with the failure to observe any double NF-E3·NF-Y complex with the γ-globin promoter in previous studies (23, 26).

COUP-TFII binding to the γ-globin oligonucleotide is similar to NF-E3 binding in other respects as well; Fig. 7*B* shows that the G → A −117 HPFH mutation, which strongly impairs NF-E3 binding (23), also greatly decreases COUP-TFII binding (*lanes 28–30 versus lanes 19–21*). Similarly, two artificial (*i.e.* not observed in patients) mutations, which greatly decrease or increase NF-E3 binding, respectively (23, 25, 58), have the same effect on recombinant COUP-TFII binding (*lanes 22* and *27*).

COUP-TFII·NF-E3 Binding to the 5′ and 3′ Sites of the ϵ-Globin Promoter May Have Different Functional Effects—To evaluate the functional role of the 5′ and 3′ COUP-TFII binding sites on the ϵ-globin promoter, we linked a 220-nucleotide fragment of the promoter to the strong HSII locus control region enhancer to drive the expression of a luciferase reporter gene. The same mutations as assayed in gel shifts were introduced in the 5′ or 3′ COUP-TFII or in the NF-Y binding sites. The erythroid leukemia cell line K562, which expresses high levels of both COUP-TFII and NF-E3 and also expresses ϵ-globin mRNA, was used for transient transfection experiments. All experiments were normalized for transfection efficiency by evaluating the activity of a cotransfected *Renilla* luciferase plasmid.

As shown in Fig. 8, the mutation of the 5′ COUP-TFII binding site leads to a significant decrease in promoter activity (60%) relative to the control. On the other hand, the mutation of the 3′ site gives a marginal increase in comparison to the wild type control promoter. However, when compared with the 5′ COUP-TFII site mutation, the activity of the 3′ mutation is clearly increased (more than 3-fold). When both mutations (5′ and 3′) are combined, the activity of the promoter is intermediate between that of the two individual mutants and only slightly lower than that of the intact promoter.

Overall, these data suggest that although the binding of COUP-TFII to the 5′ site may stimulate ϵ-globin promoter activity, the binding on the 3′ site has little (if any) activating effect or even a moderate repressive effect (compare wild type *versus* 3′ mutant and double mutant *versus* 5′ mutant). Thus, the two COUP-TFII sites have clearly different if not opposite functional roles. Finally, the mutation of the NF-Y binding site causes a 70% decrease of the ϵ-globin promoter activity as previously shown (23).

DISCUSSION

The recent proposal that COUP-TFII is a component of the nuclear factor NF-E3 has provided a clue to the further elucidation of molecular mechanisms controlling the transition from embryonic to fetal (ϵ- to γ-globin) and fetal to adult (γ- to β-globin) expression. NF-E3/COUP-TFII has been suggested to represent a repressor of both γ- and ϵ-globin genes (26), but in

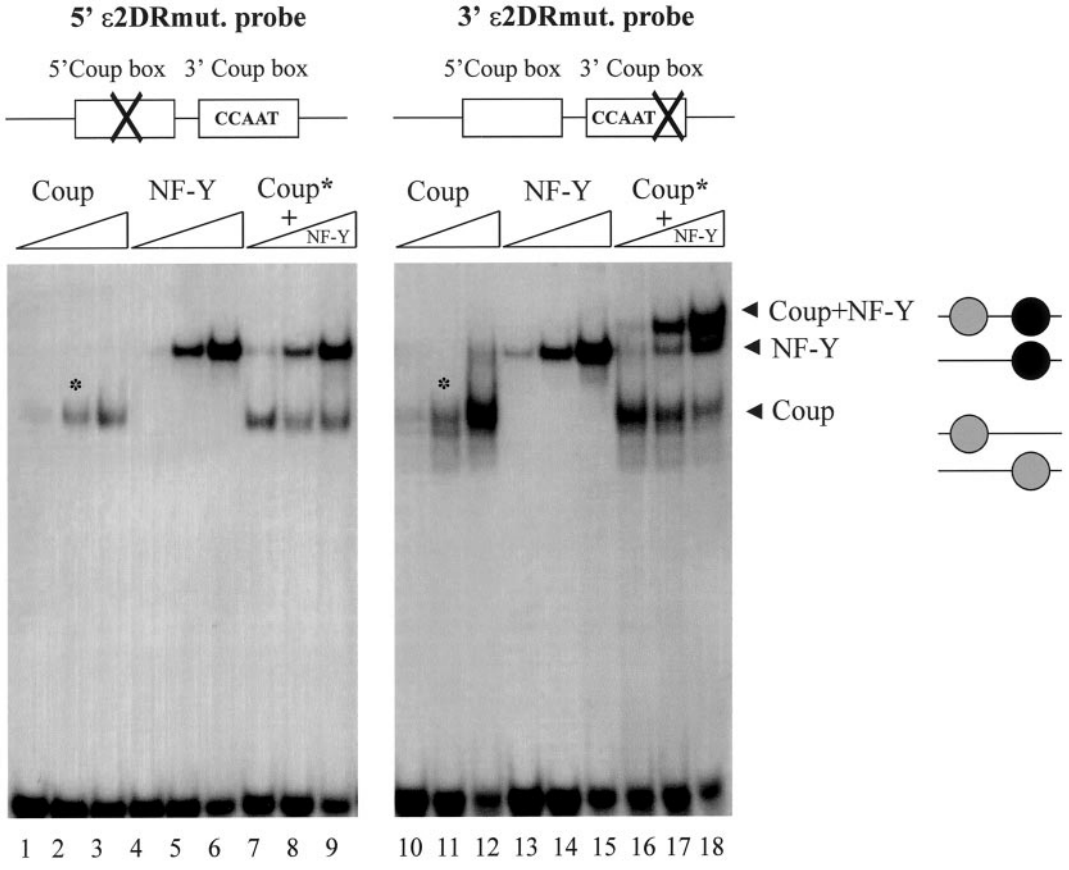

FIG. 5. **EMSA analysis of the single 5′ or 3′ COUP-TFII binding sites mutants.** COUP-TFII (*lanes 1–3* and *10–12*), NF-Y (*lanes 4–6* and *13–15*), or both recombinant proteins (at the same concentrations as in Fig. 2*A*) (*lanes 7–9* and *16–18*) were incubated with a labeled probe mutated in either the 5′ or the 3′ COUP-TFII binding site, respectively (see Table I for the sequence of the mutated oligonucleotides 3 and 4, respectively).

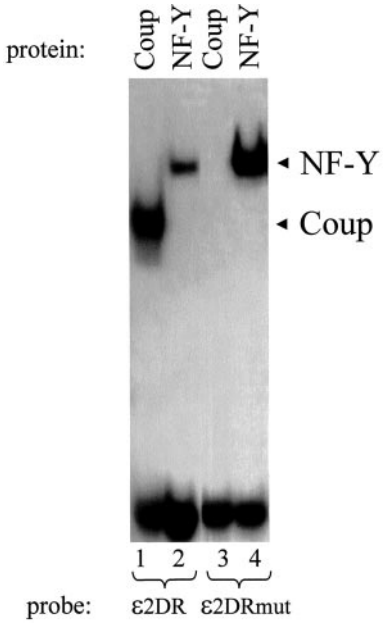

FIG. 6. **A mutation of the COUPTF-II sites in the ϵ-globin promoter causing adult overexpression in transgenic mice (26) also increases NF-Y binding, as measured by EMSA.** The same amounts of COUP-TFII and NF-Y were incubated with either the wild type (*lanes 1–2*) or the mutated (*lanes 3–4*) ϵ-globin promoter (see Table I, oligonucleotides 1 and 2). Recombinant proteins used are indicated on the *top* of the figure; probes are listed at the *bottom*.

the absence of a well defined molecule, it has not been possible so far to design molecular investigations of its binding to the globin promoter and its interactions with other transcription factors. Here we report that COUP-TFII binds to two different sites (5′ and 3′) in the ϵ-globin promoter and that NF-Y bound at the CCAAT box strongly synergizes with COUP-TFII binding at its 5′ site to form a stable complex. The functional implications of these findings are discussed below.

NF-E3 and COUP-TFII—Mignotte and co-workers (26) suggest that COUP-TFII is related to NF-E3. We showed that an antibody against COUP-TFII supershifts the ϵ-globin-NF-E3 complex (Fig. 1*A*). In addition, EMSA experiments with normal and mutant ϵ- and γ-globin oligonucleotides show that COUP-TFII has similar binding specificity as NF-E3 (Fig. 7; see also Refs. 23, 25, and 58).

Although these experiments confirm that COUP-TFII is related to NF-E3, it is not yet clear whether COUP-TFII and NF-E3 are exactly the same molecule; in this regard, recombinant COUP-TFII from COS cells forms a complex with the ϵ-globin promoter that runs as the slower component of the NF-E3 band (Fig. 1*B*). This difference might simply be because of different post-translational modifications in COS *versus* K562 cells; alternatively, NF-E3 might represent either a COUP-TFII homodimer or a heterodimer with a different partner or both. Further evidence that its properties vary during development emphasizes the complexity of this molecule (26).

COUP-TFII and NF-Y Synergize to Form a Stable Complex on the ϵ-Globin Promoter—There are two potential COUP-TFII binding sites on the ϵ-globin promoter (26); by mutating either the 5′ or the 3′ site, we have shown (Fig. 5) that each site is able to bind a COUP-TFII molecule. It is unclear whether a complex with two COUP-TFII molecules is formed on the normal oligonucleotide; if it is formed, it is not stable enough to survive the gel fractionation step, as suggested by the reproducible detection of a smear of higher molecular weight than the single

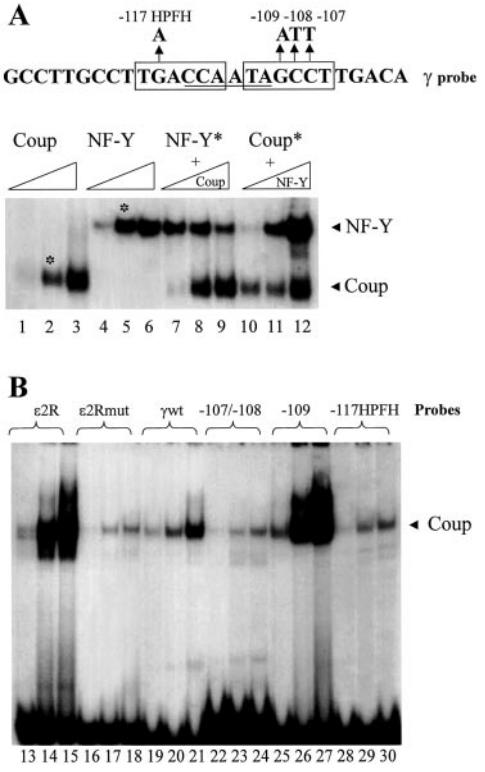

FIG. 7. **COUP-TFII and NF-Y binding on the human γ-globin wild type and mutated promoter.** A)Increasing amounts of recombinant COUP-TFII (*lanes 1–3*) or NF-Y (*lanes 4–6*) were incubated with a γ-globin-labeled probe encompassing the distal CCAAT box region (Table I, oligonucleotide 8). In *lanes 7–9*, increasing COUP-TFII protein was mixed with the fixed NF-Y amount used in *lane 5* (see the *asterisk*). In *lanes 10–12*, COUP-TFII was kept at the concentration used in *lane 2* (*asterisk*), and NF-Y was progressively increased, as in *lanes 4–6*. *B*, different amounts of COUP-TFII were incubated with the wild type (*wt*) γ-probe (*lanes 13–15*) and with the different γ-promoter mutants indicated on the top of the figure and listed in Table I.

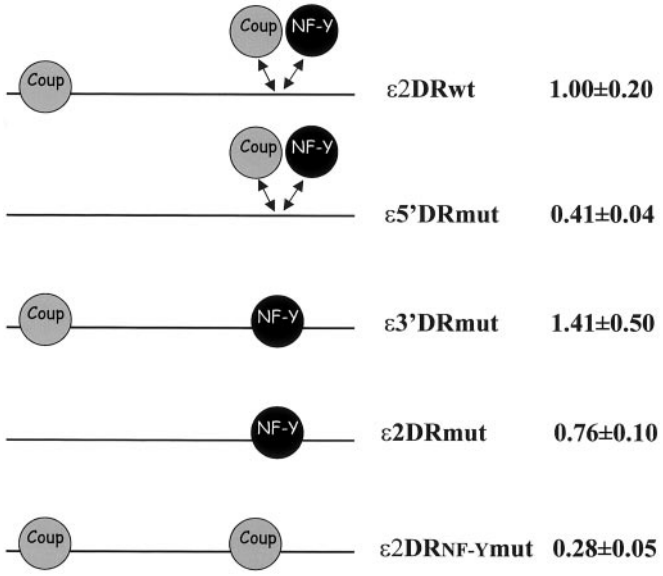

FIG. 8. **Effect of different mutations of either COUP-TFII or NF-Y binding sites on the ϵ-promoter activity in transient transfection experiments in K562 cells.** The same mutations tested in gel shift analysis were introduced in a 220-nucleotide ϵ-promoter fragment driving the expression of a luciferase reporter gene (Promega). To enhance the activity of these constructs, a core locus control region HSII 46-nucleotide fragment (33) was also inserted upstream to the promoter. Transfection efficiency was normalized using a thymidine kinase *Renilla* luciferase control vector (Promega). All the experiments were repeated in triplicate with at least three independent plasmid preparations. *wt*, wild type.

COUP-TFII band (Fig. 2*A*, *lane 4*, and Fig. 3*A*, *lanes 8–9*). The inability to form a stable complex might be due to several reasons, such as steric hindrance, the need for additional molecules that would stabilize the complex, or for posttranslational modifications such as phosphorylation or acetylation.

In the presence of NF-Y, COUP-TFII readily binds (Fig. 2*A*, *lanes 8–11*) to the ϵ-globin promoter, even at concentrations that would not allow binding in the absence of NF-Y (Fig. 2, *lanes 1* and *8*). Binding must occur at the 5′-COUP-TFII site and not at the 3′ site, as shown by the effect of mutations of either sites (Fig. 5). The kinetic analysis shown in Fig. 3 shows that COUP-TFII has higher affinity for ϵ-globin DNA in the presence than in the absence of NF-Y. Once formed, the COUP-TFII·NF-Y-ϵ-promoter complex is competed more efficiently by unlabeled NF-Y oligonucleotide than by the same molar excess of a COUP-TFII consensus oligonucleotide (Fig. 3*C*). Taken together these results suggest that NF-Y, bound to the CCAAT box overlapping the 3′ COUP-TFII site, helps recruit a COUP-TFII molecule on the 5′ COUP-TFII site.

What is the mechanism of the facilitating effect of bound NF-Y on COUP-TFII binding at the 5′ COUP-TFII site? (Fig. 2, *lane 8* and Fig. 3). It is possible to envisage that COUP-TFII and NF-Y might interact before binding, forming a complex with higher affinity for the ϵ-globin promoter than either factor alone. However, we failed to demonstrate any stable interaction between COUP-TFII and NF-Y onto an anti-YA-conjugated Sepharose column (not shown). In addition, the formation of the COUP-TFII·NF-Y-ϵ-promoter complex requires direct DNA binding of COUP-TFII specifically at the 5′ site (Fig. 5); when both COUP-TFII sites are mutated, the residual NF-Y site is not able to load the putative COUP-TFII·NF-Y complex onto the promoter (Fig. 4). Similarly, the single 3′ COUP-TFII site, in the absence of the 5′ COUP-TFII site, is not sufficient to form the COUP-TFII·NF-Y-ϵ-promoter complex (Fig. 5).

An alternative explanation is that the observed "cooperation" between COUP-TFII and NF-Y reflects protein-protein interaction that occurs between DNA-bound molecules, leading to stabilization of the complex, as indeed is shown in Fig. 3*C*. Presently, we have no direct evidence for such protein-protein interaction; it is interesting, however, that we were able to reproduce (data not shown) the same synergism between COUP-TFII and NF-Y (as shown in Figs. 2 and 3) using a "mini" NF-Y molecule composed of the YA9, YB4, and YC5 subunit mutants previously described (34); these mutants retain only the domains for subunit-subunit interactions and DNA binding. Finally, an additional explanation not mutually exclusive with the previous one is suggested by our observation that NF-Y strongly bends DNA upon binding (28) and significantly reorientates it in space. When two molecules of NF-Y are bound simultaneously to appropriately spaced NF-Y binding sites, the resulting complex is more stable than the complex formed by a single NF-Y molecule (28). Thus, NF-Y-induced bending on the ϵ-globin promoter might reorient it in such a way as to favor high affinity binding to the DNA and to promote protein-protein interactions.

The Binding of NF-Y and COUP-TFII Can Be Mutually Exclusive—When the 5′ COUP-TFII binding site in the ϵ-globin promoter is mutated, the simultaneous binding of NF-Y to the CCAAT box and of COUP-TFII to the 3′ site is never observed, even at high concentrations of proteins, as indicated by the absence of any band slower than those generated by the single NF-Y and COUP-TFII bands (Fig. 5 and data not shown). Similarly, using the γ-globin promoter, either the NF-Y- or the COUP-TFII-band, but never a band containing both proteins, is

observed (Fig. 7*A*). Because the CCAAT box overlaps with the 3′ COUP-TFII site in the ϵ-globin and with the single COUP-TFII site in the γ-globin promoter, the implication of this finding is that the binding of either NF-Y or COUP-TFII factor to its cognate site is mutually exclusive.

Different Functional Roles of the Two COUP-TFII Binding Sites—We tested the activity of the normal and mutated ϵ-globin promoter by transient transfection into the erythroid cell line K562, which contains relatively high levels of both NF-Y and NF-E3/COUP-TFII and expresses ϵ- and γ-globin RNAs.

The effects of mutations of the 5′ COUP-TFII site and of the NF-Y binding site (Fig. 8) suggest that both proteins can behave as positively acting factors when bound to the ϵ-promoter. In fact, abolishing the binding of either COUPTF-II at the 5′ site or of NF-Y (to the CCAAT box) leads to a moderate but significant decrease of the activity of the promoter. However, the binding of COUP-TFII *per se* does not necessarily lead to activation; indeed, the ϵ-globin promoter mutated in the 3′ COUP-TFII site is as active and possibly more active as the normal ϵ-globin promoter and clearly much more active than the 5′ COUP-TFII mutated promoter; this suggests that COUP-TFII binding at the 3′ site is either functionally irrelevant or, if anything, negatively acting. The interpretation of these findings must take into account the experiments of Mignotte and co-workers (26), who reported that the mutation of both COUP-TFII binding sites (corresponding to construct ϵ2DRmut in Fig. 7) leads to significant expression of the ϵ-globin gene in adult erythroid cells of transgenic mice, indicating that COUP-TFII bound at either the 5′, the 3′, or at both its binding sites may be a repressor. With the caveat that our data were obtained in a different system (*i.e.* in a fetal-embryonic cell environment), our results would suggest that the repressor activity ascribed to COUP-TFII by Mignotte and co-workers (26) is because of its binding at the 3′ but not at the 5′ site.

There are several possible explanations for these findings. (i) Depending on binding to the 5′ or 3′ site, COUP-TFII, an orphan receptor, might interact with different factors and/or recruit additional proteins (transcription factors, acetylases, deacetylases) to the promoter, thus behaving either as an activator or a repressor (35–37). Steroid receptors are well known to either repress or stimulate transcription depending on the binding site and on recruitment of corepressors and coactivators (38). COUP-TFII was indeed previously shown to contain repressor domains (39–42) capable of forming inactive heterodimers with receptors such as RXR (39). Interactions with corepressors such as N-COR (43–44) and inhibition of gene expression dependent on other receptors (vitamin D3, thyroid hormone, retinoic acid) was also demonstrated (39). (ii) COUP-TFII, when bound to the 5′ site, might synergize with NF-Y bound to the CCAAT box (Fig. 2) and form a more stable complex (Fig. 3), thus providing greater transcriptional activity; however, at the 3′ COUP-TFII site overlapping to the CCAAT box, NF-Y and COUP-TFII might compete for binding (Fig. 5, *lanes 7–9*). Thus, even if endowed with positive transcriptional activity, COUP-TFII bound to the 3′ site might fail to increase (or might even repress) the activity of the promoter by preventing the binding and, thus, the activity of the strong activator NF-Y. (iii) More complex interpretations are also possible based on the notion that, although NF-Y generally has a positive role on promoter activity, it may also play a negative role in some instances. Although in most cases NF-Y cooperates with transcription factors binding to nearby sites in establishing a stable DNA-protein complex that leads to the recruitment of additional factors and activation (45–49), in other cases, as in the albumin promoter, the cooperative binding of NF-Y and a different factor leads to functional impairment (50). Moreover, the NF-Y site may be the focus of positive as well as negative regulation by proteins, including nuclear receptors, that do not contact DNA directly (51–56).

As mentioned above, a mutation (identical to that used in the present work) of both COUP-TFII sites of the ϵ-globin promoter resulted in a substantial level of adult activity of a transgenic ϵ-globin gene (26). The effect of this mutation would have been to abolish both the 5′ COUP-TFII site-dependent positive activity and the 3′ site-dependent activity, thus indirectly favoring NF-Y binding. Moreover, the 3′ mutation, although not affecting the core NF-Y binding motif (*i.e.* the CCAAT box), creates a better NF-Y consensus in the flanking region (27), that is expected to strengthen the normally weak NF-Y binding activity (28); indeed, as shown in Fig. 6, the mutant oligonucleotide binds NF-Y significantly better than the normal sequence. Thus, the mutation might favor NF-Y binding by two independent mechanisms, *i.e.* lack of competition for binding between COUP-TFII and NF-Y and the higher affinity for NF-Y.

Recently, Tanimoto *et al.* (57) showed that the two direct repeat sequences in the ϵ-globin gene that bind COUP-TFII also recognize a protein, direct repeat crythroid-definitive protein, that is enriched in mouse erythroleukemia cell extracts. Mutation of either one or both repeats leads to significant activation of the ϵ-globin gene in definitive erythropoiesis. These authors suggest that DRED might be a definitive stage-specific suppressor of ϵ-globin expression. Because the mutations of the COUP-TF-II binding sites that we introduced would likely affect DRED binding, the question arises of whether the observed functional effects (Fig. 8) depend on interference with DRED binding. We think that this is not the case for the following reasons. (i) DRED appears to be abundant in definitive-type mouse erythroleukemia cells but not in primitive-type K562 cells (that we used for transfection experiments) and is predominantly active in adult stage cells (57). (ii) The significant decrease of ϵ-globin expression that we observe with the 5′ COUP-TFII site mutation is inconsistent with the postulated inhibitory role of DRED binding on gene activity. Thus, we conclude that the functional effects of the ϵ-globin promoter mutations we observe in K562 cells are unlikely to depend on interference with DRED binding.

Implications for HPFH—Although the known HPFH mutations (16–22) in the γ-globin COUP-TFII·CCAAT region differently affect the *in vitro* DNA binding of several nuclear proteins, the binding of NF-E3 is greatly decreased by all of the four mutations tested so far (23), and this is the only consistent effect of the HPFH mutations. On this basis, it was suggested that NF-E3 might be a repressor of γ-globin expression in adult life (23).

Two of the HPFH mutations (−117 G → A and −114 C → T) have been tested in transgenic mice and show an HPFH phenotype (24, 25). However, the loss of binding of NF-E3 *per se* is not likely to be sufficient to cause HPFH, because a mutation (−107–108 CC → TT) that greatly decreases NF-E3 binding (Ref. 25 and Fig. 7) does not cause HPFH in transgenic mice (25). It is likely that the binding of other proteins in addition to NF-E3 has to be affected to generate an HPFH phenotype. One important factor affecting the level of γ-globin expression may be the binding of NF-Y itself; the disruption of the proximal CCAAT box within a γ-globin promoter carrying the −117 HPFH mutation (upstream to the distal CCAAT box) almost completely abolishes the HPFH phenotype, although the embryonic expression of a normal (*i.e.* non-HPFH) γ-globin gene is not affected by the loss of both the CCAAT boxes (25). Thus, a different combination of transcription factors may be required for adult expression of the HPFH γ-globin gene as opposed to

the embryonic expression, and NF-Y may be one of the critical players. We have shown that once NF-Y is bound to the proximal CCAAT box, it is able to "cooperate" with NF-Y bound to the distal CCAAT box, generating a stable NF-Y/NF-Y/γ-globin promoter complex (28), an effect that is reminiscent of the formation of the NF-Y/COUP-TFII-ϵ-promoter complex observed in this paper.

In Fig. 7*B* we have shown that recombinant COUP-TFII behaves similarly to NF-E3 in EMSA experiments; both bind much better to the ϵ-globin promoter than to the γ-promoter (*lanes 13–15* and *19–21*; see also Ref. 23). In addition, two mutations strongly reducing NF-E3 binding (−117 G → A HPFH and −107–108 CC → TT) also do so with COUP-TFII, and a mutation strongly increasing NF-E3 binding (−109 G → A; Ref. 58) correspondingly increases COUP-TFII binding. These data are in agreement (Ref. 26 and the present paper, Fig. 1) with the conclusion that COUP-TFII and NF-E3 are closely related molecules. Significantly, the binding of COUP-TFII and NF-Y to the γ-globin promoter is mutually exclusive (Fig. 7*A*) as on the 3′ COUP-TFII and NF-Y sites in the ϵ-promoter.

Our present results are consistent with two possible hypotheses on the mechanism of HPFH. (i) COUP-TFII, by binding to its site, might hinder NF-Y binding to the overlapping distal CCAAT box. This might, in turn, prevent its cooperation with NF-Y bound to the proximal CCAAT box (28). We thus suggest that the loss of NF-E3/COUP-TFII binding might contribute to the HPFH phenotype, at least in part by favoring NF-Y binding and NF-Y-dependent activation of the γ-globin promoter. (ii) COUP-TFII binding to the distal CCAAT box region might directly lead to γ-globin inhibition through its repressor domains, as also postulated for COUP-TFII binding on the ϵ-globin gene (see preceding paragraph).

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