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# The deletion of the distal CCAAT box region of the $^{A}\gamma$-globin gene in black HPFH abolishes the binding of the erythroid specific protein NFE3 and of the CCAAT displacement protein

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ABSTRACT

Non-deletion Hereditary Persistence of Fetal Hemoglobin (HPFH) is characterized by great elevation of the synthesis, in adult age, of fetal hemoglobin (HbF), of either the $^{A}\gamma$ or $^{G}\gamma$ type. Strong genetic evidence indicates point mutations in the $^{G}\gamma$ - or $^{A}\gamma$ -globin promoter as responsible for overexpression of the mutated gene. Here we report that a 13 nucleotides deletion in the CCAAT box region of the $^{A}\gamma$ -globin promoter, associated with greater than 100 fold overexpression of the gene, abolishes the in vitro binding of the ubiquitous factors CP1 and CDP (CCAAT displacement protein) and of the erythroid specific protein NFE3. Loss of NFE3 binding is consistent with a similar effect of the -117 G►A HPFH mutation, suggesting a possible role of NFE3 as a negatively acting factor. In addition, loss of CDP binding indicates that this alteration might also contribute to the HPFH phenotype in this particular case, suggesting possible heterogeneity of the mechanisms causing HPFH.

## INTRODUCTION

Hereditary Persistence of Fetal Hemoglobin (HPFH) is a clinically benign condition characterized by the continued expression in adult life of one, or both, of the duplicated $\gamma$ -globin genes which are normally expressed at significant levels in the fetal period only (1,2). Large deletions involving the adult $\delta$ - and $\beta$ -globin genes (sometimes together with the fetal $^{A}\gamma$ -globin gene) are responsible for a subclass of HPFH, in which both $\gamma$ -globin genes in cis to the deletion are usually kept active ($^{G}\gamma$ $^{A}\gamma$ -HPFH, $^{G}\gamma$ $^{A}\gamma$-thalassemia; $^{G}\gamma$ -$\delta\beta$thalassemia in those cases in which the $^{A}\gamma$ -gene is included in the deletion); on the other hand, point mutations in the promoter of the singly overexpressed $^{A}\gamma$ - or $^{G}\gamma$ -globin gene have been consistently reported in a subclass of HPFH not detectable associated with deletions (3-15). A causal relationship of these mutations to the HPFH phenotype is strongly, though indirectly, supported by two types of genetic evidence: - the same mutation is present in HPFH $\gamma$ -globin genes of patients of different ethnic origins (reviewed in refs. 12,14) indicating independent occurrence of the mutation; this point is strengthened by haplotype analysis and by the observation of the same mutation (-175 T►C) in either the $^{G}\gamma$ - or $^{A}\gamma$ -globin gene in different HPFH patients (12,13-15); - the mutation thought to be responsible for HPFH has never been detected, in population analyses, in normal individuals (4,10-11).

The molecular mechanisms underlying the greatly elevated expression of $\gamma$ -globin genes in HPFH are unknown. Recently, the analysis of the effects of two HPFH mutations (-175 T►C and -117 G►A) on the ability of nuclear erythroid proteins to bind to $\gamma$ -globin promoter fragments has indicated that

several proteins may be affected simultaneously, and suggested that the HPFH phenotype might be the result of increased binding of activator proteins as well as of loss of binding of inhibitor proteins (16-20). However, the complex effect of the mutation raises the question which of the observed alterations in binding is responsible for HPFH, and by which mechanism (loss of inhibition rather than activation). In order to get further insight into this problem, we have now studied a newly discovered $^{A}\gamma$-HPFH (21), characterized by great elevation of HbF (~30% in heterozygotes), that is associated with a 13 nucleotides deletion in the CCAAT box region of the $^{A}\gamma$-globin promoter.

Here we show that loss of binding of the erythroid factor NFE3 is the only common effect of the -117 G→A and 13 nt. deletion HPFH's; in addition, we demonstrate complete loss of binding of the ubiquitous protein CDP (22). The possible significance of altered binding of NFE3, CDP and of the erythroid specific protein NFE1 (16,17) in this and other types of HPFH is discussed.

## MATERIALS AND METHODS

### Preparation of nuclear extracts

Nuclear extracts were prepared exactly as described by Dignam et al. (23) from exponentially growing K562 cells (24). Some extracts were prepared with the modifications (ammonium sulphate precipitation followed by dialysis) described by Superti-Furga et al. (17); the latter extracts were used for studying CDP binding (Figure 5).

### Oligonucleotides

Oligonucleotides were prepared and annealed according to ref. 17. The sequences of these oligonucleotides are shown in Figure 2 or indicated in the papers quoted in the text.

The human -globin enhancer oligonucleotide (see Figure 3) has the sequence (top strand only is indicated):

5' GTCTTATTACCCTATCATAGGCCCACCCCAAATGGAAGTCCCATTCTTCC 3'; this oligonucleotide contains an NFE1 binding (underlined) site (16,25) and at least one CP1 binding site (25; R. M. and S. O.; unpublished data).

The human β-globin enhancer oligonucleotide (see Figure 5) has the sequence 5' CAGGGACATGATAAGGGAGCCAA 3'.

### Electrophoretic mobility shift assay (26)

Binding reactions and electrophoretic runs were carried out essentially according to ref. 24; briefly, the standard assay contained (in a 20 µl reaction): 0.1-0.2 ngs of $^{32}$P-labelled oligonucleotide, 3-9 µgs of nuclear protein, 3µgs of poly (dI-dC), 2 µgs of bovine serum albumin in a buffer consisting of 4 mM spermidine, 50 mM NaCl, 1 mM EDTA, 10 mM Tris HCl pH 7.9, 1mM DDT, 0.5 mM PMSF. Unlabelled competitor DNAs were as specified in Legend to Figures. For optimal detection of NFE3, the higher amounts (6-9 µgs) of nuclear proteins were used. Incubation was at 20°C for 30'. Gel electrophoresis was in 5% acrylamide gels in 50 mM Tris borate pH 8.2. Experiments shown in Figure 5 were performed according to conditions specially modified to optimize detection of both NFE1 and CDP (17).

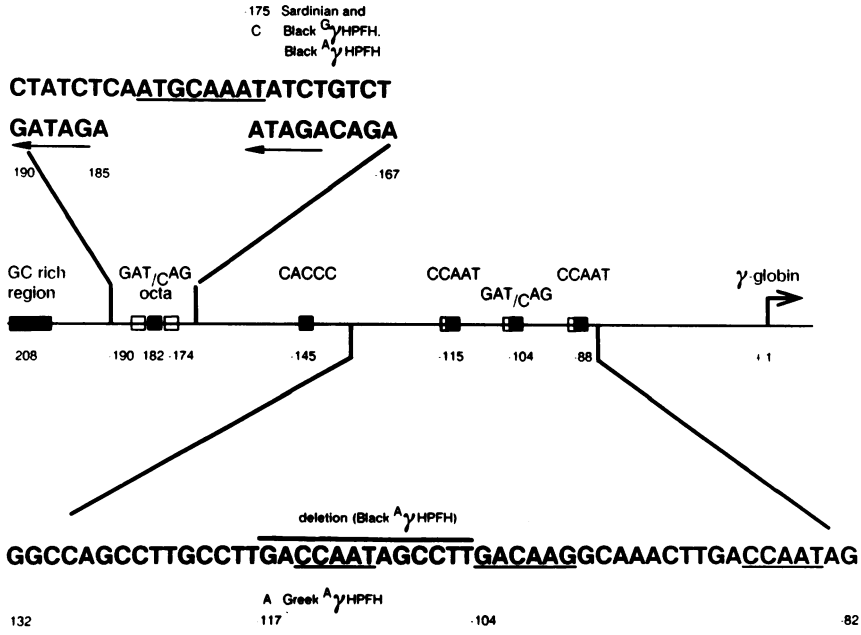



Figure 1
Promoter structure of the γ-globin genes and HPFH mutations in this region. Boxes indicate various nuclear protein binding sites; the TGA motifs (open half boxes) and the CCAAT box are essential for CDP binding. CP1 binds to the sequence CCAAT, NFE1 to the consensus GAT/CAG, present at two sites, proximal and distal.

## Transfection

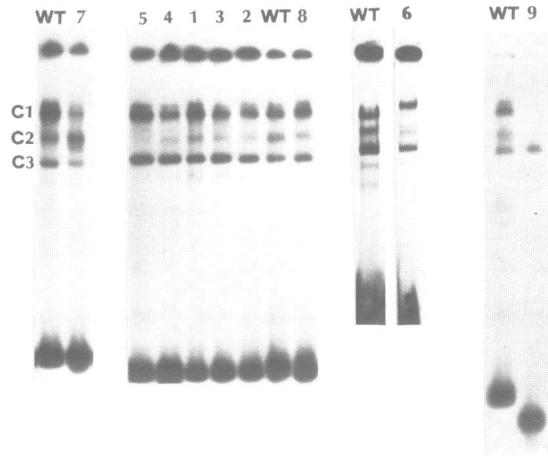
$2x10^7$ cells in 0.8 ml of phosphate buffered saline were transfected by electroporation at 400 Volts, 400 μF, in the presence of 30 μgs of CAT reporter plasmids (27) driven by normal or mutant γ-globin promoters (28). Cells were recovered two days after transfection and lysed; CAT activity was assayed according to refs. 27,28.

## RESULTS

### The binding site of NFE-3 overlaps with the distal CCAAT box

At least four different proteins bind in vitro to the human γ-globin promoter CCAAT box region (Figure 1). Two of these proteins are ubiquitously present in animal cells: CP1, a transcriptional activator (29) and CDP, a putative repressor (22). The other two proteins are erythroid specific and will be referred to as NFE1 (17) and NFE3 (see Figure 7 in ref. 16) respectively (NFE3 was referred to as NFE2 in ref. 19; it is called here NFE3 to avoid confusion with the different factor NFE2 described by Mignotte et al. (30).

Previous experiments (16) had shown that the -117 G→A substitution causing Greek HPFH almost completely abolishes NFE3 binding; to better locate the binding site for the this protein, we tested




-132 -122 -117 -109 -91

GGCCAGCCTTGCCTTGACCAATAGCCTTGACAAGGCAAACTT

| | |
|---|---|
| wt 42 mer | ---------- |
| wt 27 mer | ---------- |
| 1 | ----------A---------- |
| 2 | ----------C---------- |
| 3 | ----------TT---------- |
| 4 | ----------CC---------- |
| 5 | ----------A---------- |
| 6 | ----------C---------- |
| 7 | ----------A---------- |
| 8 | ---------- |
| 9 | ---------- |
| 10 | ---------- |
| 11 | ---------- 13 nt. HPFH deletion ---------- |

Figure 2
Sequence requirements for binding of NFE3 to the $\gamma$ -globin CCAAT region. Oligonucleotides containing the mutations indicated in the lower part of the figure were annealed and used for electrophoretic mobility shift analysis. Oligonucleotides used are indicated by their respective numbers on the top of the figure. The effect of the mutations on NFE3 binding was evaluated densitometrically by reference to bands C1 and/or C3. For oligonucleotide 2 the decrease was 40-60%, for oligonucleotides 3 and 4 50-80%, for oligonucleotides 5 and 6~90 and~80% (4 experiments).
Lower part: the distal CCAAT box and the NFE1 binding sequence are underlined; GCCTTG motifs and their polarity are indicated by arrows.

mutant oligonucleotides for NFE-3 binding by gel shift assays (16,24,26). With the normal 27 mer used (Figure 2) three bands are detected: C1, C2 and C3 (16). C1 is generated by interaction with a CCAAT binding protein (CP1) and C2 by interaction with NFE3; C3 is known to be due to unspecific binding. With the exception of the G►A change at position -122, all point mutations from positions -122 to -116 significantly (50-80%) decrease band C2 (Figure 2). These data identify the GCCTTG motif (-122 to -117) as an important part of the recognition element for NFE3; this motif is repeated twice 5' and twice 3' to the CCAAT box (the most 3' repeat is in reverse orientation relative to the other ones).

The comparison of the binding of oligonucleotides of different lengths indicates that the two GCCTTG motifs immediately flanking the CCAAT box are important for NFE-3 binding (see lack of binding of oligonucleotides 9 and 10 and the ~3-fold increased binding of oligonucleotide 7, -109 G►A); on the other hand, the first and fourth GCCTTG motifs are not essential as oligonucleotide 8, lacking the first motif, and the normal 27 mer, lacking the fourth one, bind as well as the normal 42 mer (Figure 2 and data not shown). Of the sequences comprised between the second and fourth motif, only the A at position -116 appears to be important, as shown by the greatly decreased (~80%) binding of oligonucleotide 6; C►T substitutions at positions -115, 114 have only a slight effect (not shown).

Finally, although the NFE-1 core recognition element at position -104 to -98 (GACAAGG) is not necessary for NFE-3 binding (see normal 27 mer), the observation that the -117 G►A substitution affects both NFE-3 and NFE-1 binding (16,17) implies that the respective binding sites of these two proteins also somewhat overlap.

The HPFH- 13 nucleotide deletion in the $^{A}\gamma$ -globin promoter abolishes the binding of CP1 and NFE3

To evaluate the effect of the 13 nucleotide deletion associated with HPFH on binding of nuclear proteins, we used a 42 nt. oligomer and its 29 nt. corresponding mutated version (Figure 2, oligonucleotide 11) for gel shift assays (Figures 3A and B). This 42-mer contains the distal CCAAT box sequence and the NFE1 and NFE3 binding sites; it generates with nuclear extracts from the erythroid cell line K562 three different bands, migrating exactly as those generated by the normal 27 mer and labelled in the same way. C1 corresponds to the band generated by a CCAAT box binding protein, presumably CP1 (17), C2 is generated by interaction with NFE3 and C3 is mostly due to binding of NFE1, as demonstrated by comigration (not shown) with complexes obtained using oligonucleotides carrying the appropriate binding sites and by competition experiments (Figure 2, see legend for details). Note, in addition, that the NFE1 band is somewhat contaminated by a band due to the same unspecific binding as observed with the 27-mer (16).

The effects of the HPFH 13-nt deletion are seen both by direct binding and by competition experiments; the deletion abolishes both CP1 and NFE3, but not NFE1 binding (Figure 3). Figure 3A, lane 1 indicates that the HPFH 29-mer does not generate C1 and C2 while band C3 is essentially normal. The inability of the HPFH oligomer to bind CP1 and NFE3 is confirmed by competition experiments, showing that the unlabelled oligomer is unable to decrease bands C1 and C2 generated with labelled 42-mer (Figure 3B, lane 3); a 20-mer (Figure 2, oligonucleotide 9) lacking both the CP1 and NFE-3 sites is equally unable to compete (Figure 3B, lane 4); under the same conditions, a control 27 mer known to

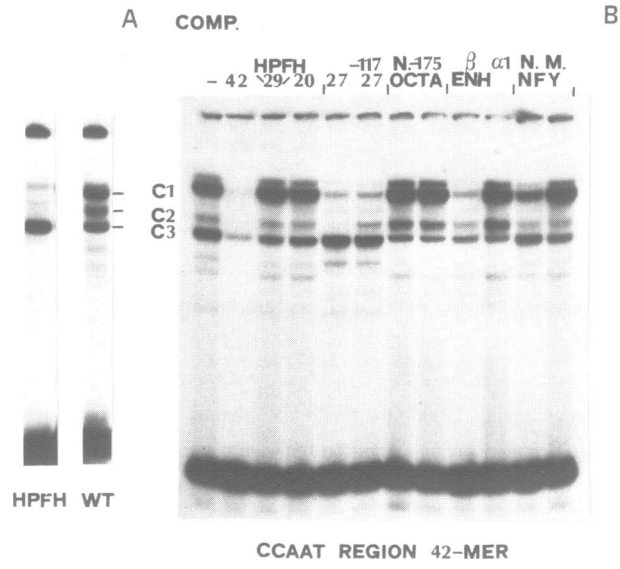



Figure 3
Binding of K562 nuclear proteins to normal and 13 nt. HPFH deletion oligonucleotides. (A) Labelled normal 42 mer and 13 nt. HPFH 29 mer were used for electrophoretic mobility shift analysis; lane 1 HPFH, lane 2 normal. (B) Labelled normal 42 mer was incubated in K562 cell extracts in the presence of excess (50-200 fold) unlabelled competitor (as indicated on the top of the figure) and analysed as above. Competitors are wt. 42 mer, HPFH 29 mer, deleted oligonucleotide 9, wild type and -117 HPFH 27 mer (see Figure 2), normal and -175 HPFH $\gamma$-globin octamer region, human NFE1 binding site from $\beta$-globin enhancer and mouse $\alpha_1$ globin oligonucleotides (16), normal and mutated 27 mer MHC-E$_\alpha$ CCAAT box oligonucleotides (31). Bands C1, C2 and C3 are assigned to CP1, NFE3 and NFE1, respectively, on the basis of comigration with bands generated by appropriate marker oligonucleotides (not shown) and competition experiments. Briefly, band C1 is competed specifically by oligonucleotides containing a CCAAT box capable of binding CP1 or its mouse homolog NFY (31), i.e. wild type and -117 HPFH 27 mer (16,17) (lanes 5 and 6), human $\beta$-globin enhancer (25) (lane 9) and normal (lane 11), but not mutated (lane 12) MHC-E$_\alpha$ oligonucleotides (31); note that 90-100% competition can be obtained (not shown) with higher levels (200-fold instead of 50 fold) of competitor MHC-E$_\alpha$. Band C2 is competed only by normal, not by -117 HPFH 27 mer (16) (lanes 5 and 6). Band C3 is competed (partially, due to comigration with an unspecific band, see lane 2) by HPFH 29 mer normal and -175 HPFH $\gamma$-globin octamer region (16), human $\beta$-globin enhancer (16,25) and mouse $\alpha_1$-globin (16,34) oligonucleotides, all containing a NFE-1 binding site (lanes 3,7-10).

bind both CP1 and NFE-3 greatly decreases (Figure 3, lane 5) bands C1 and C2, while the -117 HPFH 27-mer (unable to bind NFE3, ref. 16) competes only against band C1, but not C2 (Figure 3, lane 6).

The HPFH deletion does not significantly affect NFE1 binding

The presence of an unspecific component in band C3 prevents a conclusive direct analysis of NFE-1 binding. To circumvent this problem, we used unlabelled normal 42-mer and HPFH 29-mer in competition experiments against labelled distal $\gamma$-globin NFE-1 binding site (-175 HPFH version, 16). Figure 4 indicates a very similar competition; the normal sequence appears to be at most twice as

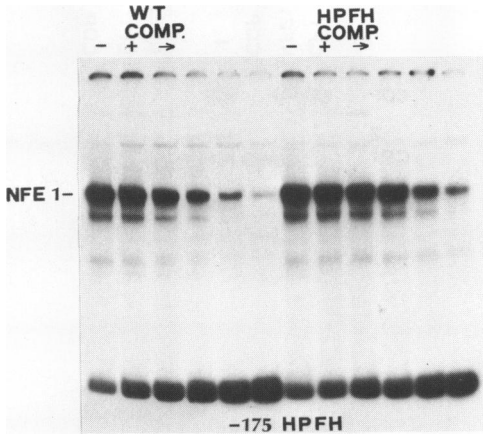



Figure 4
Competition of unlabelled wild type 42 mer and HPFH 29 mer for the NFE1 binding site of labelled -175 HPFH oligonucleotide. Labelled -175 HPFH oligonucleotide (16) (0.2-0.4 nanograms) was incubated with 5,10,20,40,80 nanograms (arrow) of unlabelled competitor in K562 cell extracts and analyzed by elctrophoretic mobility shift experiments.

efficient as the HPFH-sequence in preventing binding to the -175 HPFH NFE-1 site. In parallel competition experiments an unlabelled oligonucleotide corresponding to the distal NFE1 binding site (positions -190 to -168, ref. 16) competes against the labelled oligonucleotide approximately four fold better than normal 42-mer and HPFH 29-mer (not shown).

The HPFH deletion abolishes CDP binding

To study CDP binding, a labelled BalI-HpaII fragment (17) from the normal and HPFH $\gamma$-globin promoters was directly used in gel shift experiments; nuclear extract preparation and binding conditions are optimized for detecting CDP binding according to Superti-Furga et al. (17), and differ from those used in Figures 2-4 to detect NFE1, NFE3 and CP1 (NFE3 is not detected under these conditions). Figure 5 shows that the CDP band (clearly identified by its disappearance upon competition with a CDP-binding site from the histone sperm $H_2B$ gene, see ref. 22) is completely suppressed by the HPFH deletion (compare lanes 6-8 with lane 5).

In addition, the experiments in fig. 5 confirm that the HPFH mutation leaves NFE-1 binding essentially intact (compare lane 6 with lane 5); under these conditions, the unspecific band comigrating with C3 and not self -competed (Figure 3) is not seen (see lanes 3,4,7 and 9), allowing direct evaluation of NFE-1 binding. As the fragment used in this experiment contains the proximal CCAAT box, spared by the deletion, CP1 binding is apparently not decreased by the HPFH deletion; actually, while the zed band (NFE-1 plus CP1 bound to the same fragment) is very similar using the normal and mutated oligomers, the CP1 band is somewhat increased. This effect might be a secondary consequence of the loss of the CDP binding site, allowing increased reaction between CCAAT box and CP1.

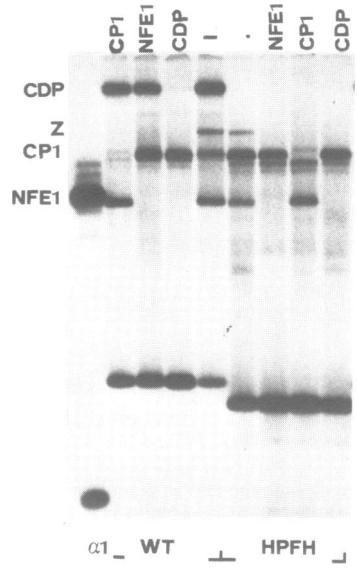



Figure 5
Electrophoretic mobility shift analysis of CDP and NFE1 binding to normal and -13 nt. deletion HPFH. A BalI-HpaII fragment (17) from the normal or HPFH DNA was 5' terminally labelled with polynucleotide kynase, incubated in K562 cell extract with excess (300 fold) competitor under conditions optimized for CDP assays (17) and used for mobility shift analysis. Competitors are indicated on the top of the figure as follows: CP1: CCAAT box of mouse $\alpha_1$ globin (see ref. 17, Table I); NFE1: NFE1 binding site GATAAG from the human $\beta$-globin enhancer (25; see the sequence in Materials and Methods); CDP: a PVUII-FokI fragment from the histone sperm $H_2B$ promoter, containing a CDP binding site and mutated in the CCAAT box (see ref. 22, figure 5). This oligonucleotide happens to contain some NFE1 binding sequences, and it is thus also a competitor for NFE1. In the first lane, labelled $\alpha_1$-mouse oligonucleotide, containing a NFE1-binding site (16,34) was used as a control. Bands are indicated: for complex Z (CP1 + NFE1) see ref. 17.

Functional analysis

A fragment from the promoter of the 13 nucleotide deletion HPFH (from position -299 to +35) was joined by linkers to the Hind III site of the CAT-reporter plasmid pSVo (27,28). This, and similarly constructed plasmids carrying the normal or the -175 HPFH promoter sequences, were transfected by electroporation into K562 cells; after two days of culture, chloramphenicol acetyltransferase activity was determined (27). In agreement with previous results (28,33) the -175 HPFH plasmid gave an approximately four-fold stimulation relative to the normal control; however, the 13 nucleotides deletion mutant was as active as the normal promoter. Thus, the loss of the binding sites for CP1, CDP and NFE3 is not reflected, in K562 cells, in increased activity of the promoter, contrary to the *in vivo* data. Similarly negative results have been reported, in K562 and MEL cells for the -202 and -196 HPFH mutations (33); this probably reflects the fact that these cells allow expression of endogenous or transfected $\gamma$-globin genes, while the HPFH phenotype is observed, *in vivo*, in cells in which normal $\gamma$-globin genes are almost completely repressed.

## DISCUSSION

The black HPFH 13 nucleotides deletion removes the distal CCAAT box and one of the TGA repeats, thought to be essential for CDP binding (17), and the third GCCTTG repeat, leading to complete loss of binding of CP1 (to the distal CCAAT box), CDP and NFE-3.

The binding of NFE3 can now be clearly discriminated from that of either CP1 or NFE1. In fact, partial or total competition for CP1 by unlabelled nucleotides (Figure 3B, lanes 9 and 11) does not decrease NFE3 binding; on the other hand, normal or increased binding of CP1 to the -117 HPFH oligonucleotide (16,17,32; see also Figure 2) is associated with almost complete disappearance of NFE3 binding (16). Similarly, competition for NFE1 does not decrease NFE3 binding (16; see also Figure 3, lanes 7-10); moreover, the wild type 27 mer binds NFE3, but not NFE1 (16), while che HPFH 29 mer binds NFE1, but not NFE3 (Figure 4).

Recent attempts to understand the HPFH phenotype have focused on alterations in binding of nuclear proteins to the mutated promoter (16,17,32,33). The -175 T► C HPFH mutation increases (four-fold) the activity of the $\gamma$-globin promoter in transfection assays in K562 cells (28,33), by increasing (16) or "altering" (33) the binding of NFE1 to a distal site in the -195 to -170 region; it is unclear whether this change is relevant to the *in vivo* HPFH phenotype (28), but the data do suggest that NFE1 binding to the distal site has a positive effect on $\gamma$-globin promoter activity. Unfortunately, due to the lack of proper human adult erythroid cell lines the -175 HPFH promoter is unique in showing a functional effect in transfection assays (33 and present paper); at present, clues to the molecular mechanisms of HPFH can only be obtained by comparison of binding alterations with the *in vivo* phenotype.

Two HPFH mutations affect the duplicated CCAAT box region: the -117 and the 13 nucleotides deletion HPFH's. In the -117 HPFH the binding of both NFE1 and NFE3 is greatly decreased (16,17) while the binding of CP1 and CDP is slightly increased (17,32); in the 13 nucleotides deletion HPFH, the binding of CP1, CDP and NFE3, but not NFE1, is lost. Though the two mutations are located in the same region, it is possible that the molecular mechanisms underlying the HPFH phenotype are heterogeneous; if this is the case, any one of the proteins (CDP,CP1,NFE3) whose binding is affected by the 13 nucleotides deletion might behave as a negatively acting factor for $\gamma$-globin expression. CDP was postulated to be such a factor on the basis of its dominant binding (versus CCAAT box binding factors) to the sea urchin $H_2B$ histone or human $\gamma$-globin promoters (18,22). On the other hand, if similar mechanisms are responsible for the two types of HPFH, the loss of NFE3 binding is the only common alteration in both the -117 and 13 nucleotides deletion HPFH, suggesting a negatively acting role for this protein. Continuous search for new HPFH mutations and the development of functional assays in adult-type erythroid cells are necessary to understand the role of these proteins in HPFH.

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