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The effects of HPFH mutations in the human γ-globin promoter on binding of ubiquitous and erythroid specific nuclear factors

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ABSTRACT

Genetic evidence indicates that single point mutations in the γ-globin promoter may be the cause of high expression of the mutated gene in the adult period (Hereditary Persistence of Fetal Hemoglobin, HPFH). Here we show that one of these mutations characterized by a T ► C substitution at position -175 in a conserved octamer (ATGCAAAT) sequence, abolishes the ability of a ubiquitous octamer binding nuclear protein to bind a γ-globin promoter fragment containing the mutated sequence; however, the ability of two erythroid specific proteins to bind the same fragment is increased three to five fold. DMS interference and binding experiments with mutated fragments indicate that the ubiquitous protein recognizes the octamer sequence, while the erythroid specific proteins B2, B3 recognize flanking nucleotides. Competition experiments indicate that protein B2 corresponds to an erythroid-specific protein known to bind to a consensus GATAG sequence present at several locations in α, β and γ-globin genes. Although the distal CCAAT box region of the γ-globin gene shows a related sequence, an oligonucleotide including this sequence does not show any ability to bind the above mentioned erythroid protein; instead, it binds a different erythroid specific protein, in addition to a ubiquitous protein. The -117 G ► A mutation also known to cause HPFH, and mapping two nucleotides upstream from the CCAAT box, greatly decreases the binding of the erythroid-specific, but not that of the ubiquitous protein, to the CCAAT box region fragment.

INTRODUCTION

Several cases of Hereditary Persistence of Fetal Hemoglobin (HPFH) characterized by the selective overexpression, in adult age, of either the $^{A}\gamma$ or $^{G}\gamma$-globin gene, have been studied at the molecular level in recent years. Single point mutations in the promoter region of these genes have been consistently reported as the only detectable difference relative to normal genes (1-11); these mutations have never been observed in normal γ-globin genes (6,7,10). Moreover, identical promoter mutations have been detected in HPFH of different ethnic origins (2-5,9-13), the most notable cases being those of the -196 $^{A}\gamma$ C►T and of the -175 T►C mutations; the -196 mutation appears to be present both in Sardinian β-thalassemia and Italian HPFH cases, as well as in Chinese HPFH (2,5,9); in the Chinese case, the $^{A}\gamma$-globin gene carrying the mutation is clearly distinct on the basis of polymorphic

differences, from the two mediterranean cases, implying that identical mutations occurred independently. Moreover, the -175 mutation is detected both in $^{G}\gamma$-HPFH (in Sardinians and Americans of African descent) and in $^{A}\gamma$-HPFH (Huisman, personal communication) in the respective γ-globin alleles. These data make it extremely likely that the HPFH phenotypes in these patients are causally related precisely to the -196 and -175 mutations, rather than to any distant undetected mutation.

The molecular mechanisms generating HPFH have not yet been elucidated; however, it can be hypothesized that the mutations either decrease the affinity of a target DNA sequence for a repressor protein or increase the affinity for an activating factor.

Here, we report that the -175 T→C mutation detected in a $^{G}\gamma$-HPFH gene abolishes the binding of a ubiquitous "octamer-binding nuclear factor" (14) and increases the binding of two erythroid-specific factors (15) to a γ-globin DNA region comprising the conserved ATGCAAAT octamer. The erythroid specific protein is shown to be the same as that known to bind to other globin sequences in α, β and γ-globin genes, but is different from an erythroid-specific protein binding close to the distal CCAAT box of the γ-globin gene, and whose binding is also affected by another HPFH mutation, -117 G→A.

MATERIALS AND METHODS

Nuclear extracts

Nuclear extracts were prepared exactly according to ref. 16 from K562 (under different growth conditions), THP-1 and Raji (B-lymphoma) cells.

Oligonucleotides

Synthetic oligonucleotides corresponding to the normal, and -175 $^{G}\gamma$-HPFH sequence are shown in fig. 2. Oligonucleotides corresponding to the normal and -117 $^{A}\gamma$-HPFH genes have the sequence:

-117

Normal: Top 5' GCCTTGCCTTGACCAATAGCCTTGACA 3'

" : Bottom CGGAACGGAACTGGTTATCGGAACTGT

HPFH: A/T instead of G/C at position -117

The mouse α_1 promoter oligonucleotide (kindly provided by Giulio Superti-Furga) has the sequence

Top 5' CGGGCAACTGATAAGGATTCCCA 3'

Bottom GCCCGTTGACTATTCCTAAGGGT

Oligonucleotides were 5' labelled with ^{32}P-ATP and polynucleotide kinase and used for "gel shift assays" (14,15).

DMS interference

DMS interference analysis was performed according to refs. 14,17,18; either the top or bottom strand of the oligonucleotide (normal or HPFH) was 5' labelled with ^{32}-P, annealed with the corresponding complementary strand, DMS treated and incubated with nuclear extracts. Following a preparative "gel

shift assay", DNA was recovered from the various bands, piperidine treated (19) and analyzed on sequencing gels.

RESULTS

Previous experiments using nuclear extracts from the erythroleukemic cell line K562 showed that a labelled 45 nucleotides-long ApaI-BstNI fragment of the γ-globin promoter (from position -201 to -156 relative to the CAP site) is bound "in vitro" by a ubiquitous nuclear factor (B1, also known as octamer binding factor or OTF1), as well as by one or two erythroid specific factors (B2 and B3) which are particularly abundant in the erythroleukemic cell line K562 (15). To examine the effect of the -175 $^{G}\gamma$-HPFH mutation (11,13) on the binding of these proteins we used double-stranded oligonucleotides (normal and mutant versions) corresponding to the ApaI-BstNI fragment for "gel shift" experiments (14); with this assay, binding of factors to the labelled DNA generates three complexes (B1, ubiquitous factor; B2 and B3, erythroid factors) migrating more showly than the unbound DNA on acrylamide gels (15). Figure 1A shows that, using the mutant oligonucleotide, bands B2 and B3 are approximately three-fold stronger than using the normal oligonucleotide (band intensities can be compared directly as specific activities of the mutant and normal oligonucleotides were identical). As band B3 is apparent only in extracts from exponentially growing K562 cells (15), which may contain very low amounts of factor B1, binding to the latter protein was examined using extracts from the B -lymphoma Raji cell line. Figure 1B indicates that the -175 mutation abolishes the binding to the ubiquitous (20) octamer binding protein B1 (here labelled B1A), as well as the binding to the lymphocite specific factor (B1 B) (14,17,21). Overexposure of the gel in Figure 1A to visualize band B1, gives the same result (not shown).

The competition experiment in Figure 1C (using an extract from starved K562 cells (15) having abundant B1 and B2, but not B3) confirms these data; a 5-fold molar excess of unlabelled HPFH oligonucleotide, incubated with labelled normal oligonucleotide, almost completely suppresses band B2, while a 40-fold molar excess of the mutant is necessary to obtain a significant decrease of band B1; on the other hand, both bands are equally competed by the normal oligonucleotide, and a 20-40 fold molar excess of normal competitor is necessary to obtain the same degree of competition against B2 as that observed with a 5-fold excess of HPFH competitor. Using extracts from exponentially growing K562 cells, as in Figure 1A, B3 behaves as B2 (not shown).

Previous DNAse I protection experiments (15) only partially discriminated between the DNA regions interacting with factors responsible for bands B1 and B2, B3, respectively. In view of our present results, we wished to identify more precisely nucleotides involved in generating bands B1 and B2, B3, respectively. A "DMS interference" experiment (17) was carried out. Normal and HPFH oligonucleotides, 5' labelled at either the top (coding) or bottom (non-coding) strand were mildly treated with DMS to methylate a very low proportion of guanines; in a subsequent gel-shift experiment, only molecules in which guanines critical for DNA binding have not been methylated, are able to generate a shifted band. Piperidine treatment of the DNA from each shifted band is then used to

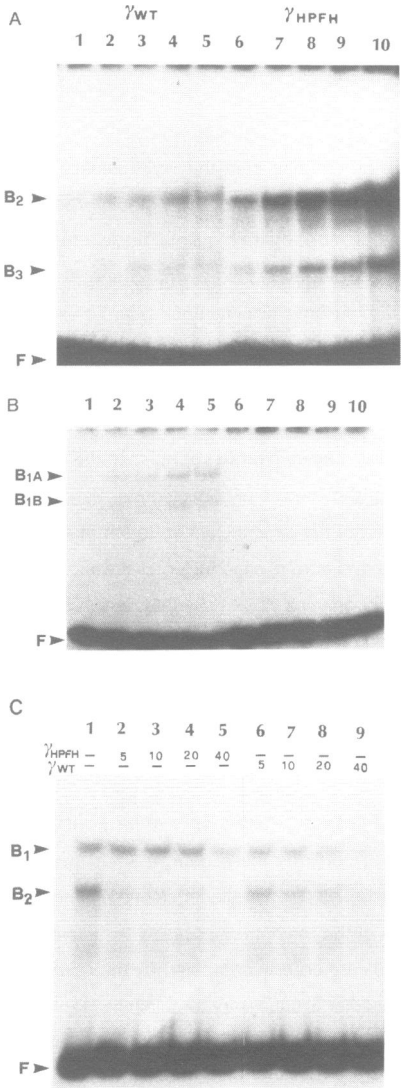

Figure 1.
Binding of wild type and -175 $^{G}\gamma$-HPFH oligonucleotides to nuclear factors.
A: lanes 1-5, normal (w.t.) oligonucleotide; lanes 6-10, HPFH oligonucleotide. Incubations were with 2,4,6,8 and 10 μgs of nuclear proteins from exponentially growing K562 cells.
B: same as in a; nuclear proteins from Raji cells.
C: labelled normal oligonucleotide incubated with nuclear extract from uninduced starved K562 in the presence of a 5-40 fold molar excess of unlabelled HPFH (lanes 2-5) or normal (lanes 6-9) oligonucleotide. Lane 1: no competitor. bands B1-B3 are indicated; B1 A. ubiquitous factor; B1 B: lymphoid specific octamer binding factor; F: unbound DNA.

cleave the molecule at the level of the reacted guanine, thus generating a band in a Maxam-Gilbert sequencing reaction (19). Thus, absence of a guanine band in a Maxam-Gilbert sequence indicates a particular guanine as essential for DNA-factor interaction. Figure 2 shows that on the top strand the guanine at position -180 of the normal sequence is essential for the binding to factor B1, but not B2 (similar data for B3, not shown). On the bottom strand, the guanine at position -179 is also important for B1, but not B2 binding. On the other hand, binding to B2 and B3 requires guanines at positions

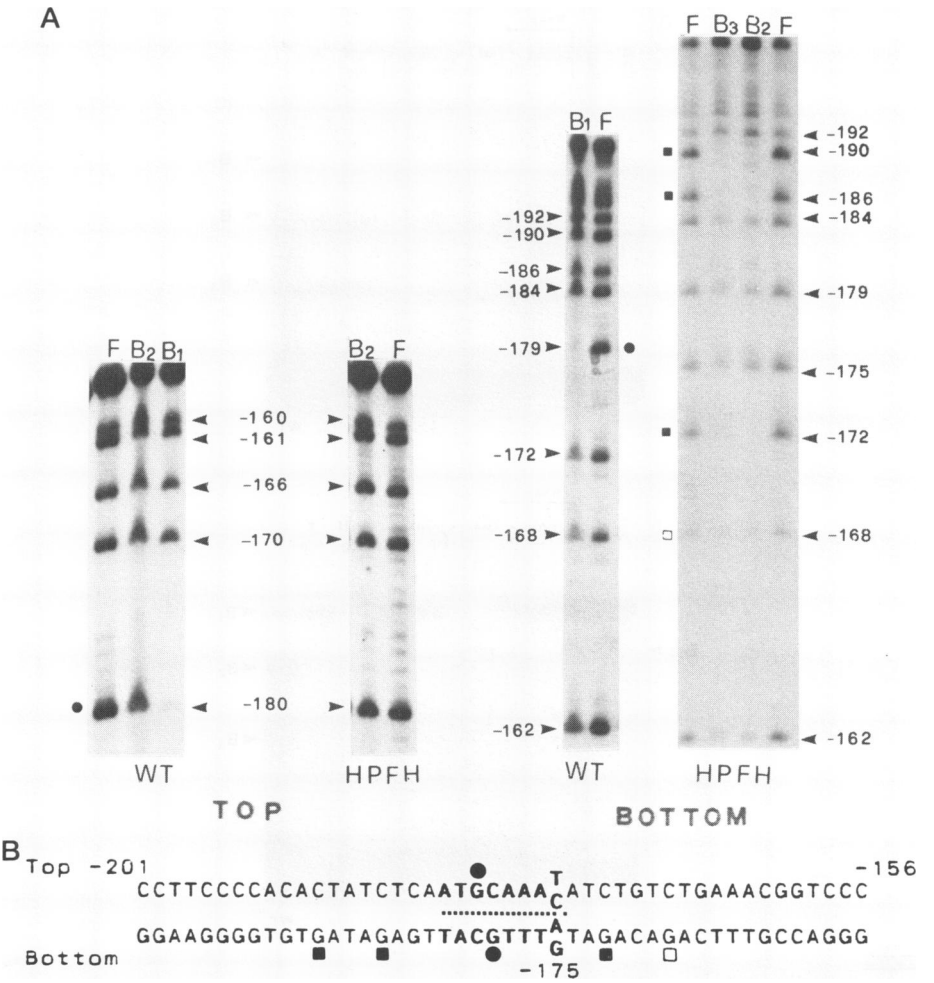

Figure 2.

DMS interference analysis of DNA-protein interaction using normal and HPFH oligonucleotides.
A: lanes B1, B2, B3 and F show analysis of piperidine cleavage products of DNA recovered from the respective bands from gel shift assays. An explanatory diagram of results shown in a, is given in B. Guanines whose methylation interferes with B1 (●) or B2, B3 (■) binding are shown; the octamer is indicated by a dotted line.

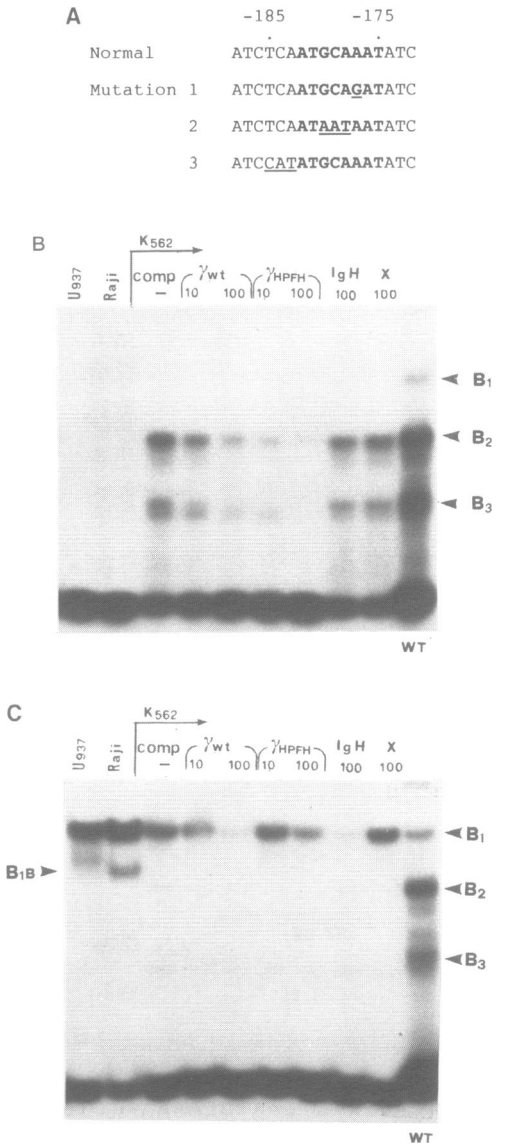

Figure 3.

Binding of wild type and mutated oligonucleotides to nuclear factors.

A: normal and mutated sequences (top strand).

B: binding of normal (wt) and mutated (mutation 1) oligonucleotides. Extracts used and unlabelled competitors are indicated at the top of the figure. Molar excesses of competitors are given. IgH: octamer containing immunoglobin heavy chain promoter ref. 14. X: an unrelated sequence from the c-fos promoter.

C: Binding of normal (wt) and mutated (mutation 3) oligonucleotides. Same as in B. B1B lymphoid specific octamer binding factor.

-190, -186 and -172 (outside the octamer); surprisingly the new guanine at position -175 (in the HPFH sequence) is apparently not directlyinvolved in the binding.

The above described binding data obtained with the -175 mutant and the DMS interference experiments suggest that nucleotides within the octamer may be necessary only for binding to the ubiquitous factor, while nucleotides at the border of the octamer may be important for binding to the erythroid-specific protein. This is confirmed by binding and competition experiments either using fragments mutated in the octamer at positions -177 (A►G) (fig. 3B) and -178 to -180 (ACG►TAA) (data not shown, same as for the previous mutation) or a fragment mutated immediately upstream to the octamer (-183 to -185, ACT►TAC) (fig. 3C).

Mutants in the octamer are unable to bind significantly either the ubiquitous protein (B1) present in U937, Raji and K562 cells or the B-lymphocite-specific protein of Raji cells, but are able to bind the erythroid specific proteins (B2, B3) of K562 cells (fig. 3B) at a normal level (data not shown). Conversely, the fragment mutated at positions 5' to the octamer binds normally to B1 proteins, but not to erythroid specific proteins B2, B3 (fig. 3C).

These data, taken together, strongly suggest that the erythroid specific factors B2, B3 may not be octamer binding proteins. This conclusion is supported by the inability of an antibody specific for the ubiquitous protein to affect band B2 (from K562 nuclear extracts), at concentrations that abolish band B1 from both U937 and K562 cell extracts (fig. 4). No effect of control anti-fos and anti-p53 antibodies was detected.

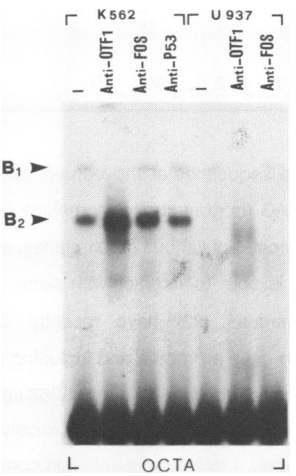

Figure 4.
Effect of anti-octamer binding protein (anti OTF1) antibody on proteins binding to the normal octamer oligonucleotide.
Nuclear extracts and antibodies used are indicated. Appropriate amounts of antibody were added to the incubation mixture ten minutes before the addition of the labelled oligonucleotide (last addition). Anti OTF1: a gift from Iain Matthaj (Heidelberg); anti-fos and anti p-53 from Oncor, Minneola, N.J..

TABLE I. Globin gene sequences containing the GATAG motif and known to bind to an erythroid specific protein (NFE); comparison with the CCAAT box region of the human γ-globin promoter

Human γ-globin octamer	Bottom	TGTGATAGAGTTACGTTTATAGACAGACT (-175)
Chicken β^A enhancer (24)	Bottom	CTTGATAGCAAAATGTTTATCTGCAA
	Top	GAACTATCGTTTTACAAATAGACGTT (+1897)
Chicken β^H promoter (23)	Bottom	AAAGATAGCAAATTTGGTAAATGTCCAA
	Top	TTTCTATCGTTTAAACCATGGACAGGTT (-61)
Chicken α^D promoter (25)	Top	TAAGATAAGGCCGGGGCGGGTGTACAGGGG
Mouse β-IVS2 (26)	Bottom	TCTGATAGGAA (+545)
Mouse α_1-promoter	Top	ACTGATAAGGA (-180)
Human β-globin enhancer	Bottom	TATGATAGGGT (+2302)
Human γ-globin CCAAT box (distal)	Top	TCCGATAACCAG (-117) (NORMAL)
		A (HPFH)
(proximal)	Top	TCTGATAACCAG (-92)
CONSENSUS		$T/_AGAT/_CAG/_APu$

On the other hand, inspection of the sequences at the borders of the γ-globin octamer shows the presence of motifs GATAG and GACAG (bottom strand) somehow related to that (Table I) present in sequences (promoters, introns, enhancers) of various globin genes; evidence has been presented that an erythroid-specific protein (NFE) is able to interact with some of these sequences (22-26). In particular, Superti-Furga and colleagues (22) have recently shown that an oligonucleotide corresponding to the mouse α_1-globin gene promoter, and including the sequence GATAAG, provides a high affinity site for an erythroid-specific protein (NFE) from K562 cells.

Fig. 5 shows that the α_1- and γ-octamer-oligonucleotides reciprocally compete and the mutant -175 γ-octamer-oligonucleotide is approximately 4 fold more efficient in competing protein binding to the α_1-sequence than the normal γ-oligonucleotide; the latter result parallels that obtained in competition experiments versus the homologous γ-globin sequence. Moreover, the band generated in gel shift experiments by the α_1-oligonucleotide comigrates with band B2 generated with the γ-oligonucleotide (fig. 6). Similar data (not shown) have also been obtained using an oligonucleotide corresponding to a GATAG-containing sequence of the human β-globin enhancer (see Table I). These results show that

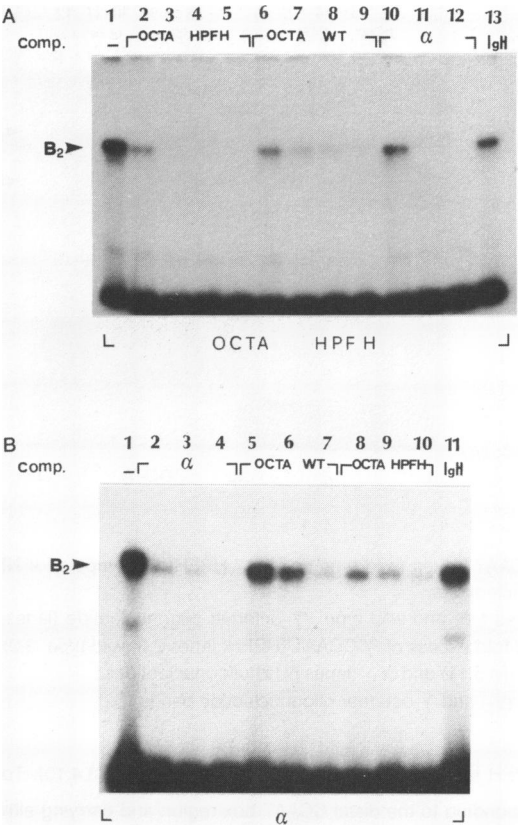

Figure 5.

Cross-competition for protein binding of γ-globin octamer (wild type and HPFH) and a mouse α_1-oligonucleotide containing a NFE-binding site (see Table I).

A: the -175 oligonucleotide was labelled and competed with unlabelled homologous (lanes 2-5), wild type γ-octamer - (lanes 6-9), α_1- (lanes 10-12) and IgH (lane 13) oligonucleotides. Molar excesses are 5-fold (lanes 2,6,10), 40-fold (lanes 3,7,11), 80-fold (lanes 4,8,12), 150-fold (lanes 5,9,13).

B: the α_1-oligonucleotide labelled and competed with unlabelled homologous α_1 (lanes 2-4), wild type γ-octamer - (lanes 5-7), HPFH γ-octamer - (lanes 8-10) and IgH octamer (lane 11) oligonucleotides. Molar excesses are 10-fold (lanes 2,5,8), 40-fold (lanes 3,6,9), 100-fold (lanes 4,7,10,11).

the erythroid-specific protein recognizing the region adjacent to the γ-globin octamer is the same as the protein recognizing other human and non human globin sequences.

Evidence independently obtained with an oligonucleotide corresponding to the γ-globin distal CCAAT box region indicates (see below) that an erythroid specific protein binds to this region; as a GATAA motif partially overlaps with the CCAAT box (see Table I), the possibility is raised that the GATAG protein might also be involved in binding to the distal CCAAT box region of the γ-globin promoter.

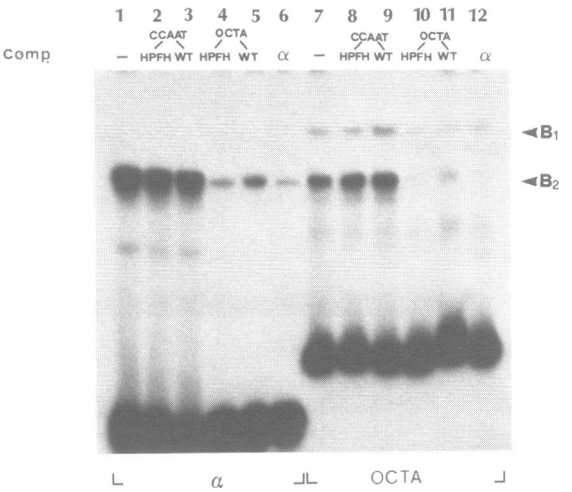

Figure 6.
Inability of γ-globin CCAAT boxes (wild type and -117 HPFH) to compete for NFE-protein versus γ-octamer and α_1-oligonucleotides.
α_1 oligonucleotide (lanes 1-6) and wild type γ-octamer oligonucleotide (lanes 7-12) were labelled and competed with a 80-fold excess of γ-CCAAT (HPFH: lanes 2,8; wild type: 3,9), γ-octamer (HPFH: lanes 4,10; wild type: lanes 5,11) and α_1- (lanes 6,12) oligonucleotides.
Note the comigration of α_1- and γ-octamer oligonucleotide bands (B2).

where an additional HPFH mutation (-117 G ► A) has been mapped (3,4,10). To test this possibility, oligonucleotides corresponding to the distal CCAAT box region and carrying either the normal or the mutated (HPFH) sequence, were used in competition experiments.

The CCAAT region γ-oligonucleotides fail to compete for the GATAG protein even at very high molar excesses with either the γ-octamer region or the α_1-oligonucleotide (fig. 6 lanes 2,3 and 8,9). These data, further strengthened by reciprocal competition experiments shown below, indicate that the oligonucleotide including the distal CCAAT box region does not contain a site with significant affinity for the GATAG protein.

Fig. 7 shows that the normal CCAAT region oligonucleotide generates, with uninduced K562 cell extracts, three bands: C1, C2 and C3. C1 and C3 are not erythroid specific; moreover, C3 is not significantly competed by excess unlabelled homologous oligonucleotide, indicating that it may be due to unspecific binding. On the other hand, C2 is erythroid-specific, being absent in extracts from Molt4, U937 and Raji cells. C2 is additionally well competed by the homologous unlabelled oligonucleotide; however, neither the unlabelled -175 (and normal) γ-octamer (fig. 7A) nor the α_1-oligonucletide compete for proteins generating bands C1,C2,C3 at molar excesses sufficient to abolish binding of labelled α_1- or -175 γ-oligonucleotides to the GATAG protein. In conclusion, these data indicate that the erythroid specific protein generating band B2 (GATAG protein, NFE) does not

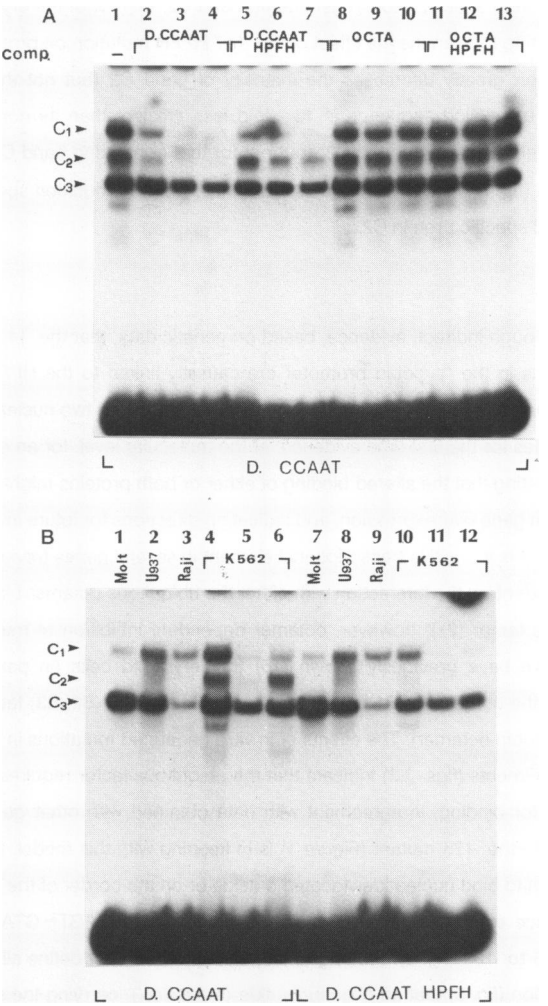

Figure 7.

Binding of wild type and -117 HPFH CCAAT boxes oligonucleotides to nuclear proteins from K562 or non-erythroid cells.

A: competition experiment with distal CCAAT oligonucleotides. Wild type CCAAT oligonucleotide was labelled and incubated with K562 extracts in the absence (lane 1) or presence of homologous CCAAT (lanes 2-4), HPFH CCAAT (lanes 5-7), wild type octamer (8-10) and HPFH octamer (11-13) oligonucleotides. Molar excesses are 20-fold (lane 2,5,8,11), 100-fold (lanes 3,6,9,12), 150-fold (lanes 4,7,10,13).

B: tissue specificity of band C2 and the effect of the-117 HPFH mutation on binding of distal CCAAT oligonucleotide.

Lanes 1-6: labelled wild type oligonucleotide; lanes 7-12: labelled HPFH oligonucleotide.

Lanes 1-3,7-9: non erythroid nuclear extracts. Lanes 4-6,10-12: K562 nuclear extracts.

Lanes 5 and 11: competition with a 100-fold molar excess of unlabelled wild type γ-CCAAT-oligonucleotide; lanes 6 and 12: competition with a 100 fold molar excess of HPFH γ-CCAAT oligonucleotide.

correspond to the erythroid specific protein generating band C2. In view of the erythroid specificity of band C2, it was interesting to examine the effect of the -117 HPFH mutation on protein binding. Fig. 7B shows that the mutation greatly decreases the intensity of band C2 (but not that of band C1); in addition, the mutated unlabelled sequence is 10 - fold less efficient than its normal counterpart in competing for the protein generating band C2, (but not for that generating band C1) with the labelled normal oligonucleotide (fig. 7A lanes 5-7; 7B lanes 5-6). Thus, the -117 mutation specifically affects the binding of the erythroid specific protein C2.

DISCUSSION

There is now strong, though indirect, evidence, based on genetic data, that the -117 and in particular -196 and -175 mutations in the γ-globin promoter are causally linked to the HPFH phenotype (see introduction); our observation that the -175 mutation alters the binding of two nuclear proteins to the γ-globin promoter provides for the first time evidence, at the molecular level, for an effect caused by an HPFH mutation, suggesting that the altered binding of either or both proteins might be the mechanism responsible for γ-globin gene overexpression, and indicating directions for future investigations.

The ATGCAAAT octamer is a positive transcriptional element in several genes (reviewed in refs. 20,21), its activity being dependent on the interaction with either the ubiquitous octamer-binding factor (20) or the lymphocite-specific factor (21); however, octamer dependent inhibition of transcription has also been reported (27). We have previously shown (15) that erythroid cells (in particular K562 cells) contain, in addition to the ubiquitous (B1) factor, two erythroid-specific (B2, B3) factors (binding to or in the vicinity of the γ-globin octamer). The effects of in vitro generated mutations in the octamer and of DMS interference experiments (figs. 2,3) indicate that the ubiquitous factor requires nucleotides within the γ-globin octamer for binding, in agreement with data obtained with other genes (17,28,29); the inability of B1 to bind to the -175 mutant (Figure 1) is in keeping with this model. On the other hand, factors B2 and B3 seem to bind nucleotides located outside or on the border of the octamer, as shown by the DMS interference experiment (fig. 2) and by the effect of the AGT►GTA mutation (bottom strand) at position -185 to -183 (fig. 3). Although these data alone do not define all the contact points precisely, the observation (fig. 5) that an oligonucleotide (mouse α_1) carrying the sequence GATAAG competes efficiently for binding versus the γ-globin oligonucleotide, carrying the related sequences GATAG (5') and GACAGATA (3') to the octamer and is itself well competed by the same γ-oligonucleotide (and particularly by the -175 mutant) strengthens the evidence implicating sequences at the 5' and 3' border of the octamer as responsible for binding to the erythroid specific factor; that the ubiquitous octamer protein is not present in complex B2, B3 is also shown by the failure of anti-octamer binding protein antibodies to affect these complexes (fig. 4). In conclusion, these data show that factors B1 and B2, B3 are unrelated proteins binding to different, but partially overlapping, sequences; B2 is due to the same protein (or a strictly related one) as the GATAG (NFE) protein binding to several globin sequences (Table I).

The effect of the -175 mutation on γ-globin expression in adult cells is formally consistent with two

different, although not mutually exclusive, possibilities: factor B1, whose binding is inhibited by the mutation, might act as a repressor, while factors B2, B3, whose binding is increased, might act as activators. Alternatively, B1 might be a weaker activator than B2, B3. Unfortunately, we do not know the relative proportions of these proteins in adult erythroid cells (where the HPFH phenotype is expressed); this evaluation is difficult even in K562 cells where factors B2, B3, after disappearing within two hours from hemin induction of differentiation (15), are again observed around 24 hours (in preparation), and slowly decrease to very low levels at 72-96 hours (15).

In transfection experiments in K562 cells the mutant -175 HPFH promoter is slightly (2 fold) more active than the normal promoter (in preparation). The small effect of -175 mutation in the γ-globin promoter on its expression upon transfection into K562 cells is not necessarily inconsistent with the suggested functional roles of B1 and B2, B3 in the pathogenesis of HPFH. In fact, in these cells the γ-globin promoter is almost completely derepressed; conversely, it is possible that full repression of γ-globin transcription, as observed in adult cells, requires cooperative interactions of several factors acting at multiple regions, as indicated by the existence of HPFH mutations in the distal CCAAT box (-117) (see below)and the -200 regions. In adult cells, disruption of repressor-DNA interaction at a single region, might then be sufficient to cause significant γ-globin gene overexpression. By the same reasoning, redundancy of active regions in the γ-globin promoter (for example octamer, CACCC and duplicated CCAAT boxes) and high levels of transacting factors might make negligible in K562 cells the effect of a mutation increasing the affinity of a target DNA sequence for its transactivating factor; in adult cells, in the presence of limiting factors, this effect might be significant.

A sequence (GATAA) partially matching that present in the high affinity site of the mouse α_1-oligonucleotide is present in both γ-globin CCAAT box regions; four nucleotides upstream of the end of this sequence in the distal CCAAT box maps the G►A mutation presumably responsible for -117 $^{A}\gamma$ HPFH (3,4,10), suggesting that the GATAG protein might also bind to this sequence and be involved in this type of HPFH. However, competition experiments with the α_1 and γ-octamer oligonucleotides clearly show that the oligonucleotide corresponding to the distal CCAAT box region does not contain any site capable of efficient binding of the GATAG protein (Figs. 6,7). This result is in agreement with data by Superti-Furga et al. (22) indicating that a binding site for this protein is present 3' to the distal CCAAT box but does not overlap with it; this site is not conserved in our oligonucleotide. On the other hand, an erythroid-specific band (C2 in figs. 6,7) is generated using the CCAAT box region oligonucleotide, that is not competed by either the octamer-region or α_1-oligonucleotide (Figs. 6,7). Significantly, the -117 HPFH mutation greatly decreases this band and the ability of the unlabelled oligonucleotide to compete versus the normal oligonucleotide for generating band C2 (Figs. 6,7). The binding and competition data discussed above, indicate that the erythroid-specific protein responsible for band C2 and whose binding is affected by the -117 HPFH mutation, is a new protein unrelated to the GATAG protein and recognizing an as yet undefined sequence. Using a different oligonucleotide Superti-Furga et al. (22) also demonstrated that the -117 mutation strongly decreases an erythroid specific band; the identity of the erythroid proteins responsible for his and our binding data is possible,

although not proven. We suggest that the erythroid specific protein responsible for band C2 might functionally act as a repressor in adult cells; by strongly decreasing this binding, the mutation might cause HPFH.

Thus, the data obtained with the -175 and -117 HPFH mutations suggest the very interesting possibility that two regions mediating repression may be located in the γ-globin promoter within a distance of 60-70 nucleotides, interspersed with sites capable of binding activating proteins. The binding site for the erythroid factor generating band C2 is being presently defined precisely to better understand these points.

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