Increased Sp1 binding mediates erythroid-specific overexpression of a mutated (HPFH) γ-globulin promoter

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ABSTRACT

The −198 T→C mutation in the promoter of the $^{A}\gamma$-globin gene increases 20−30 fold the expression of this gene in adult erythroid cells of patients (Hereditary Persistence of Fetal Hemoglobin, HPFH). We show here that this mutation creates a strong binding site, resembling a CACCC box, for two ubiquitous nuclear proteins, one of which is Sp1. The mutated promoter is four to five-fold more efficient than a normal γ-globin promoter in driving expression of a CAT reporter plasmid when transfected into erythroid cells. The overexpression of the mutant is abolished by the introduction of an additional mutation disrupting the new binding site. No overexpression of the mutant is observed in non-erythroid cells, indicating that the ubiquitous factors bound on the mutated sequence must cooperate with erythroid specific factors.

INTRODUCTION

In man, γ-globin chains are almost completely replaced by β- (and δ-) globin chains at birth; the molecular mechanisms underlying this fetal to adult globin switch are not understood [1]. A number of point mutations in the promoter of either the $^{G}\gamma$ or the $^{A}\gamma$-globin gene, are consistently associated, in different populations, with greatly elevated expression in adult life of the mutated γ-globin gene (Hereditary Persistence of Fetal Hemoglobin, HPFH) [1,2]. To investigate the molecular basis of these disorders, we have characterized the binding of nuclear proteins from erythroleukemic cells to normal and HPFH γ-globin promoters. Here we report that the −198 T→C mutation (British HPFH) [3] generates a strong binding site for the ubiquitous factor Sp1 [4] and an additional uncharacterized protein; overactivity of the mutated promoter in erythroid, but not in non-erythroid cells, depends on the increased binding of these proteins. Thus, altered developmental regulation of a tissue-specific gene might be due to mutations causing better binding of either tissue-specific [5,6] or ubiquitous factors.

METHODS

Electrophoretic mobility assay

Nuclear extracts from exponentially growing cells were prepared exactly according to ref. 7, in the presence of leupeptin, aprotinin and pepstatin. 5'-end labelled oligonucleotides (top or bottom strand) were annealed with an excess of the complementary oligonucleotide [8] and repurified by polyacrylamide gel electrophoresis. *In vitro* binding of nuclear protein to DNA and electrophoretic runs were according to refs. 9,10; briefly, the standard assay contained (in a 20 μl reaction): 0.1−0.2 ngs of ^{32}P-labelled oligonucleotide, 1−5 μgs of nuclear protein, 3 μgs 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, 1 mM DTT,

0.5 mM PMSF. Unlabelled competitor oligonucleotides were as specified in figure legends. Incubation was at 20°C for 30'. Gel electrophoresis was in 50 mM Tris borate pH 8.2, 1 mM EDTA/5% polyacrylamide gels at 10 V per cm for 2−3 hours.

Oligonucleotides

The sequence of the −198 HPFH γ-globin oligonucleotide is given in Figure 2. The normal sequence has T instead of C at position −198. The −202 and −196 HPFH mutations have G and T, instead of C, at the corresponding positions. Other oligonucleotides are listed below (top strand sequence):

Sp1: 5'GCCCCTAACTCCGCCCAGTTC3' (ref. 11)
GT-I: 5'GACTTTCCACACCCTAACTGAC3' (ref. 11)
Sp1: 5'GATCCCCCGCCCC3'

human β-globin promoter:

5'TGTGAAGCCACACCCTAGGGTT3' (ref. 12)

human β-globin enhancer:

5'GTCTTATTACCCTATCATAGGCCCACCCCAAATGGAAGTCCCATTCTTCC3' (ref. 12)

Methylation interference [13,14]

The oligonucleotide, 5'-end labelled on either the top or bottom strand, was partially methylated by DMS treatment (1 μl DMS in a 20 μl reaction containing 50 mM Na Cacodilate pH 8, 10 mM $MgCl_2$, 0.1 mM EDTA, for 11 min. at 20°C); after stopping the reaction with 200 mM Tris Acetate, 0.2M β-mercaptoethanol, 0.2 mM EDTA, the DNA was ethanol precipitated, incubated with nuclear extracts and migrated on polyacrylamide gels. Bands were recovered, piperidine treated (90°C, 30', 1 M piperidine), extensively lyophilized and analyzed on sequencing gels.

Plasmids for CAT assays

An Alu I fragment (−299 to + 35) of the normal human γ-globin promoter was joined by linkers to the Hind III site of the plasmid pSVo CAT [15]. Other mutants were derived from this plasmid by site directed mutagenesis; 5' oligonucleotides carrying the required mutations were used in conjunction with a 3' oligonucleotide carrying a Hind III linker to amplify (using the normal pSVo-γ globin-CAT plasmid as a template) with Taq polymerase the γ-globin promoter fragment extending from −210 to + 35 (plus Hind III linker); after double digestion with Apa I and Hind III, the amplified (and mutated) fragment was inserted into the normal pSVo γ-CAT, replacing the corresponding normal Apa I-Hind III fragment.

Transfection and CAT assay

2×10^7 cells were transfected by electroporation exactly as described in ref. 6. Two days after transfection, cells were lysed; aliquots of lysates, containing equal amounts of protein, were assayed for CAT activity by measuring conversion of ^{14}C-chloramphenicol to acetylated forms (by thin layer chromatography) [15] and to the butyrylated form (by phase extraction) [16], as described in ref. 6.

RESULTS

The mutation responsible for British HPFH (−198 T→C) [3,17] lies in a GC-rich region of the $^{A}\gamma$-globin promoter, that has not been characterized so far for its ability to bind nuclear proteins *in vitro*. By using gel retardation assays, we investigated the binding of nuclear proteins to oligonucleotides encoding either the normal or the HPFH −200 region;

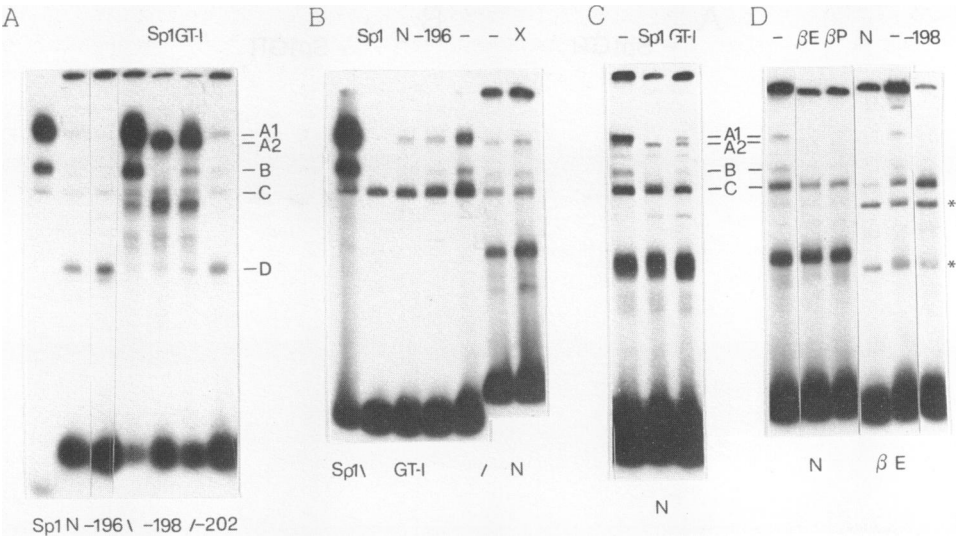


Figure 1. Binding of normal and HPFH oligonucleotides to nuclear proteins from K562 cells. Labelled oligonucleotides used for gel shift assays are indicated below the figure and unlabelled competitor oligonucleotides (in a 250-fold molar excess, when present) above the figure. (A) Binding of Sp1, normal −200 region oligonucleotide, −196, −198 and −202 HPFH oligonucleotides. Lanes 5 and 6: unlabelled competitor Sp1 and GT-I, respectively, were added. Data shown for the Sp1 oligonucleotide were obtained using the sequence from ref. 11; a different Sp1 oligonucleotide (see Methods) gave identical results. −202, −198 and −196 designate the respective HPFH mutations introduced into the γ-globin oligonucleotide. N: normal −200 region oligonucleotide; GT-I: see ref. 11. (B) Competition by unlabelled Sp1 and −200 region oligonucleotides of binding to GT-I oligonucleotide. Lane 1: binding of Sp1 oligonucleotide; lanes 2−4: binding of GT-I in the presence of competitor Sp1, normal −200 region, −196 HPFH oligonucleotides; lane 5: binding of GT-I, no competitor added. Lanes 6,7: binding of normal −200 region oligonucleotide, in the absence or presence of: X, unrelated oligonucleotide (normal γ-globin CCAAT region, ref. 25; similar results obtained with octamer-containing oligonucleotides from Immunoglobulin and Histone genes, ref. 30). (C) Competition by unlabelled Sp1 and GT-I oligonucleotides of binding to the normal −200 region oligonucleotide. Lane 1: no competitor; lanes 2,3: Sp1 and GT-I competitors, respectively. Note that the experiment in Fig. 1C is 15-fold overexposed relative to that in Fig. 1A. (D) Reciprocal competition between −200 region oligonucleotides and β-globin enhancer and promoter oligonucleotides. Lanes 1−3: binding of normal −200 region oligonucleotide; no competitor, β-globin enhancer (βE) and promoter (βP) oligonucleotides added, respectively. Lanes 4−6: binding of β-globin enhancer oligonucleotide; normal −200 region (lane 1) and -198 HPFH (lane 3) competitor oligonucleotides added. Asterisks indicate bands specific for the βE oligonucleotide.

three HPFH mutations (−202 C→G, −198 T→C, −196 C→T) [3,17−20] were tested.

Figure 1A shows that the −198 mutation dramatically enhances the intensity of the three slow bands A1, A2 and B, generated by the −200 region oligonucleotide (band A consists of a poorly resolved doublet); on the other hand, the −202 mutation slightly increases bands A1 and B, while the −196 mutation decreases all three bands. Two additional bands (C and D) are rather variable with different extracts and are not significantly affected by HPFH mutations (although apparently decreased by the −198 mutation under conditions of almost complete binding of the oligonucleotide, the intensity of band D is normal or slightly increased under conditions of vast oligonucleotide excess—not shown—. Band D did not give any clear DMS interference pattern (see below) and has not been further characterized).

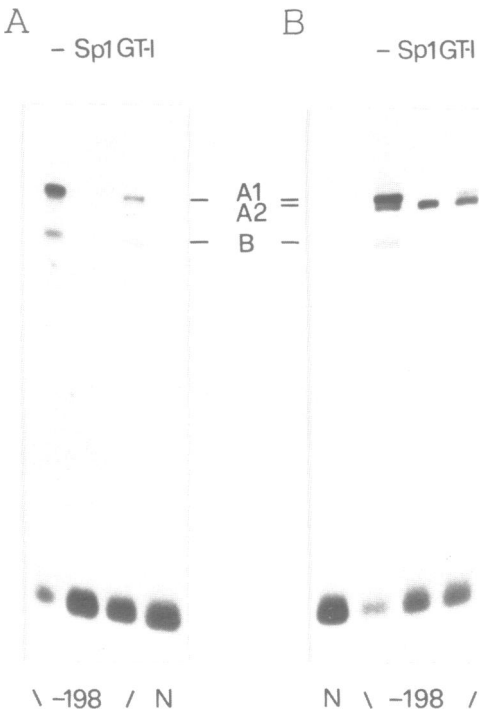


Figure 2. Binding of normal and −198 oligonucleotides to purified Sp1 (A) and K562 nuclear proteins (B).

Inspection of the sequence of the oligonucleotide identifies motifs resembling the Sp1 consensus [4,21] and the CACCC box; an oligonucleotide encoding the latter element (from the mouse β-globin promoter) is also known [11] to bind both Sp1 and the partially tissue-specific factor TEF-2 [22], a protein originally identified by its ability to bind the GT-I motif of the SV40 enhancer [11]. Indeed, (Fig. 1B and data not shown) two different Sp1 consensus oligonucleotides generate strong bands of mobilities corresponding to A1 and B, and a fainter band C, while a GT-I oligonucleotide yields the same bands, B being fainter and C stronger than with the Sp1 oligonucleotide; this pattern is similar to that described by Xiao et al. [11] with different extracts. In competition experiments, unlabelled Sp1 or GT-I oligonucleotides greatly decrease bands A1 and B (but not A2) obtained with the −198 (Fig. 1A; see also Fig. 2) and normal (Fig. 1C) oligonucleotides; reciprocally, bands A and B obtained with GT-I (Fig. 1B) or Sp1 (not shown) oligonucleotides are competed by −200 region oligonucleotides, though less efficiently than by Sp1 or GT-I. Band C is only partially competed by all oligonucleotides used, including the homologous one; band C is thus probably due, in part, to unspecific binding. No competition of bands A1 and B is observed with several unrelated oligonucleotides (Fig. 1B and data not shown).

DNA regions including CACCC box sequences from the mouse [11] and human [12,14] β-globin promoter, and the human β-globin enhancer [12] bind nuclear proteins generating a very similar pattern (Fig. 1D and ref. 12) to that obtained with the −200 region oligonucleotide. Unlabelled oligonucleotides encoding the human promoter and enhancer

CACCC box sequences compete for binding with the normal −200 oligonucleotide, abolishing bands A1, A2 and B, and slightly decreasing band C (Fig. 1D); when using the −198 mutant probe 20−30 fold higher levels of β-globin competitor are needed (not shown). However, an oligonucleotide containing a mutated β-globin promoter CACCC box, causing greatly decreased *in vivo* expression of β-globin (−87 C→G -thalassemia) [23] and unable to significantly bind nuclear proteins [14], is very inefficient in competing against the −200 region (not shown). Reciprocally, the normal and −198 oligonucleotides compete against the β-globin promoter and enhancer oligonucleotides (Fig. 1D). These data indicate that the −198 mutation strengthens a site in the γ-globin −200 region, capable of the same factor binding activity as a known β-globin transcriptional activator.

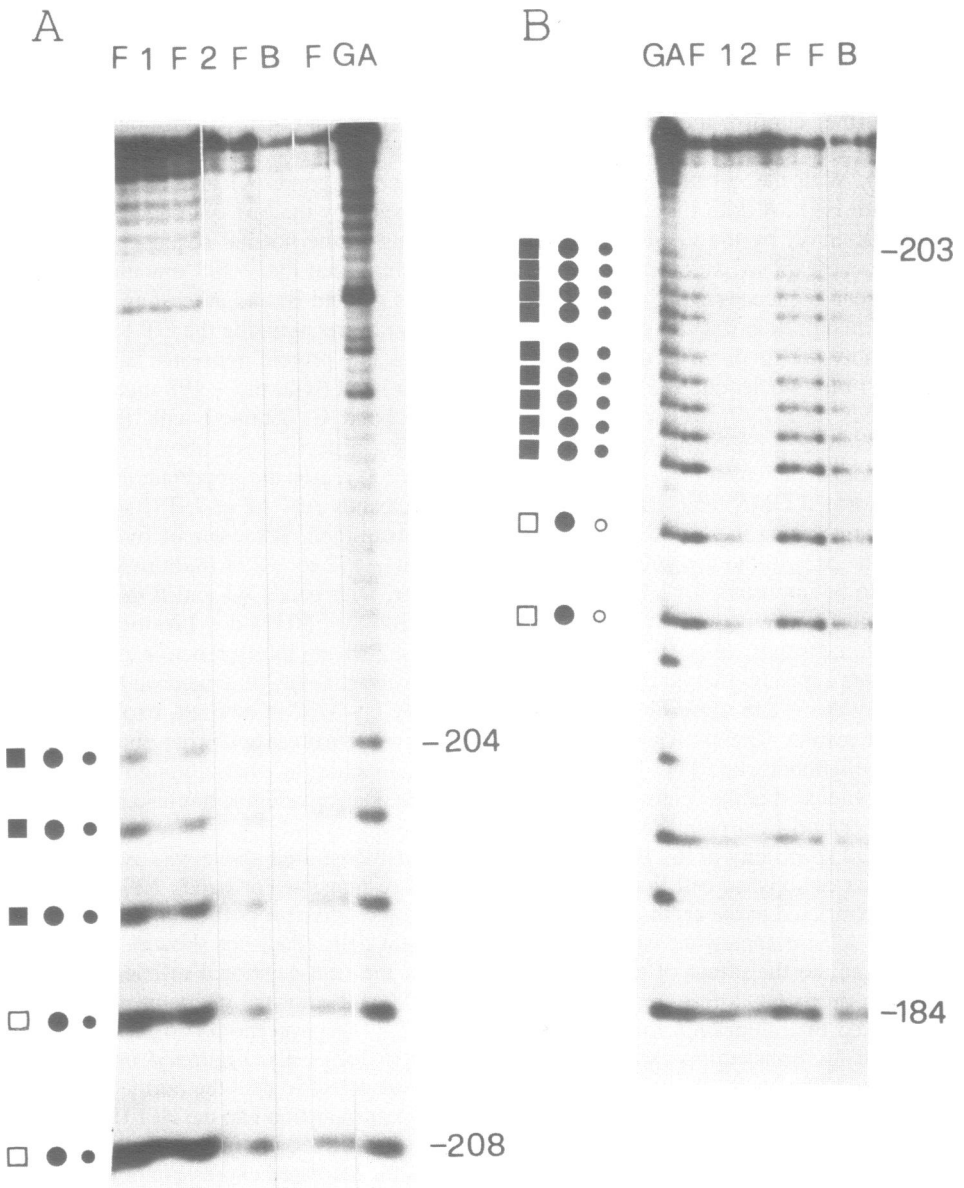
To further confirm the ability of the −200 region to bind Sp1, affinity-purified Sp1 [24] was used in gel-shift experiments with the −198 HPFH and normal oligonucleotides (Fig. 2). With the −198 oligonucleotides, Sp1 generates bands A1 and B (but not A2) (compare Fig. 2A with Fig. 2B) which can be competed by the Sp1 oligonucleotide and, less efficiently, by the GT-I oligonucleotide; binding to the normal sequence is visible only in overexposed autoradiographs. These data show that the −198 mutation greatly strengthens sequences capable of binding Sp1 (bands A1 and B) and at least an additional unidentified protein (band A2). The protein (TEF-2) [22] recognizing the GT-I motif [11] is probably not involved in the increased binding, as this protein generates band C with the GT-I oligonucleotide [11] and the corresponding band (with the −198 mutant) is not increased; TEF-2, however, might contribute to band C obtained with the normal oligonucleotide to a small extent (most binding being aspecific, see above).

To understand which nucleotides in the γ-globin −200 region are important for binding, we performed DMS interference experiments [13]; bands A1, A2 and B (from binding experiments using the −198 HPFH mutant) were examined. A cluster of five guanines (−208 to 203) in the top strand, and guanines from −192 to −203 (bottom strand) are shown to be important for binding (Fig. 3); however, with bands A1 and B the strongest DMS interference is on the three guanines from −206 to −203 and −196 to −198 (top and bottom strands, respectively), while with band A2 strong interference is also noticed with other adjacent guanines. For every band, the new guanine generated on the bottom strand by the −198 mutation, appears to be strongly involved in binding, explaining the gel shift results. The similarity of the −198 interference pattern with that obtained with an Sp1 oligonucleotide [11] is also apparent; note, in addition, that although a cytidine is usually located at the center of the consensus Sp1 sequence, adenine has also been found at this position [21] (see Fig. 3C).

The cell specificity of the DNA binding proteins demonstrated above was examined by looking at established cell lines in culture; proteins responsible for bands A1, A2 and B are not restricted to the erythroid lineage, although the relative proportions between bands A1 and A2 are somewhat variable between different cells (data not shown).

To examine the effect of the −198 mutation on the transcriptional efficiency of the γ-globin promoter, a fragment (from −299 to +35) of the normal or mutated promoter was linked to a chloramphenicolacetyltransferase (CAT)gene in the pSVo plasmid [15], and used for transfection experiments in erythroid (K562) cells. Figures 4 and 5 show that the −198 mutant promoter is four to five-fold more effective than the normal promoter in driving CAT expression in K562 cells; however, the introduction into the HPFH promoter of an additional mutation (CCC→GAA, −197 to −195) reversing (not shown) the effect on binding of the −198 T→C substitution, abrogates the increased expression of the HPFH

promoter. This result is consistent with the failure of the −196 HPFH mutant to overexpress the CAT gene. These experiments have been carried out using four independent preparations of the various plasmids, and yielded consistent results. Figure 5A summarizes the overall data in K562 cells; in transfection experiments with non erythroid cells (HL60, BJA/B) the expression of the γ-globin promoter was very low, similar to that of the promoter-less



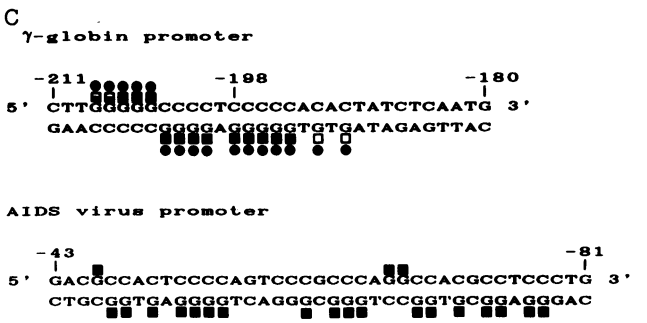


Figure 3. Methylation interference with binding of the −198 HPFH mutant. A: top strand. B: bottom strand. Comparable amounts of unbound DNA (F) and DNA obtained from bands A1, A2 and B (1,2 and B), respectively, were run on adjacent lanes. Reproducibly observed methylation interference is indicated by squares (band A1), large circles (A2) and small circles (B); filled symbols: strong methylation effect; open symbols: weak effect. C: interpretation of the experiments in A and B, and comparison of interference data on the −198 HPFH with those obtained with the AIDS virus promoter (ref. 21). Sp1: squares; A2: circles.

plasmid pSVo (as previously shown, ref. 6), and was not increased by the −198 mutation (Fig. 5B).

DISCUSSION

In this paper, we have shown that a mutation (−198 T→C) of the γ-globin promoter, causing HPFH, greatly increases the binding of two ubiquitous factors (Sp1 and protein A2). Sp1 binding is identified on the basis of similar migration of bands A1 and B obtained with γ-globin and consensus Sp1 oligonucleotides (Fig. 1A), competition experiments (Fig. 1) and identity of gel shift patterns demonstrated using purified Sp1 versus nuclear extracts (Fig. 2). As shown by methylation interference experiments (Fig. 3) the new guanine (bottom strand) introduced by the mutation is essential for increased binding of both Sp1 and protein A2; it creates a stretch of guanines, interrupted by an adenine, that closely resembles the Sp1 consensus. Although most Sp1 binding sites have cytidine as the central nucleotide between guanines [4], adenine is found in the AIDS virus promoter sites (Fig.

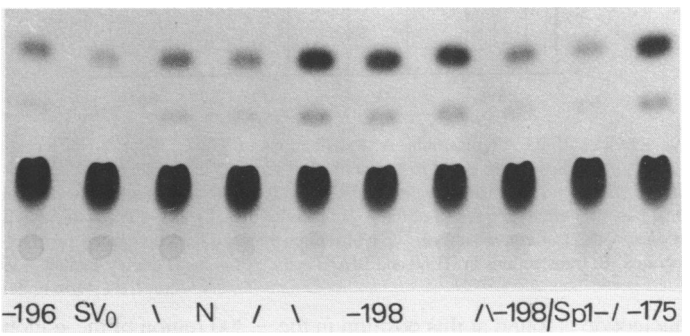


Figure 4. Transfection experiments with γ-CAT plasmids in K562 cells. SVo: pSVo [15]; −198/Sp1-: doubly mutant promoter containing the −198 HPFH sequence together with a CCC→GAA mutation (−197 to −195); N: normal.

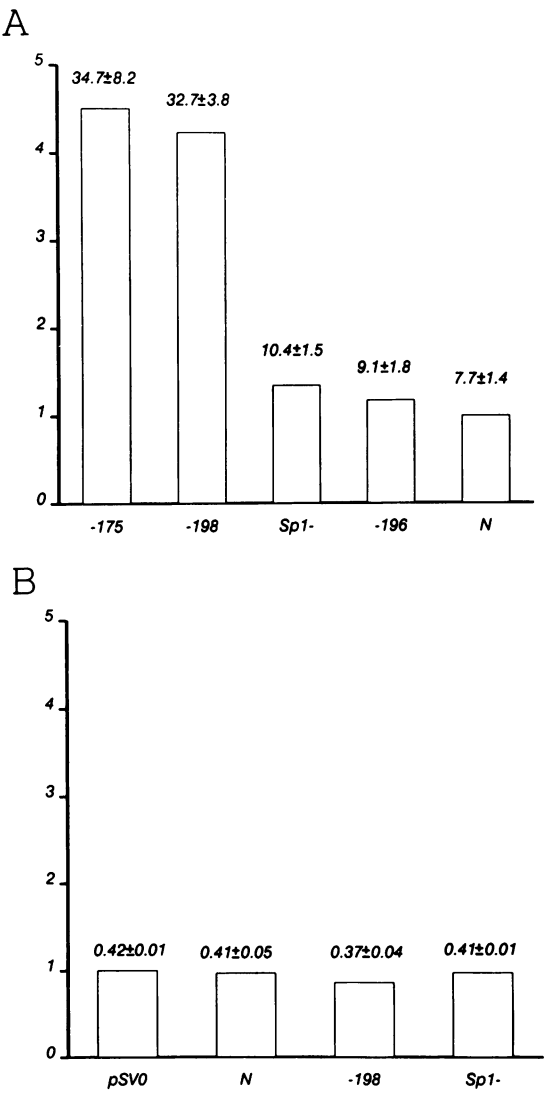


Figure 5. Relative activities of γ-CAT plasmids. A: transfections of K562 cells. Four different preparations of plasmids −198, N and −198/Sp1- were tested in a total of 21, 18 and 15 independent transfections. Two different preparations of the −196 and −175 plasmids were used (10 and 5 transfections). Activities are calculated relative to that of the normal plasmid, after subtraction of the pSVo activity (usually 25−40% of the activity of the normal plasmid). Proportion ($\times 10^{-3}$) of conversion of ^{14}C-Chloramphenicol to the butyrylated form (6,16) is indicated on top of the histograms. B: transfections in HL60 and BJA/B cells. Sp1− is the doubly-mutant −198-Sp1-plasmid.

3); the same nucleotide is found at this position in the −200 region of the γ-globin promoter, whereas thymine is present in the mouse and human β-globin promoter and enhancer sites binding the A1 and B proteins [11,12,14].

The complementary strand to the guanine stretch in these regions shows a typical (β-globin) or variant (γ-globin) CACCC box sequence; thus, CACCC boxes contain a subset of Sp1 binding sites [11] characterized by adenine or thymine, instead of cytosine, in the central part of the guanine stretch. We do not know yet the nature of band A2; although the protein responsible for it recognizes on the γ-globin and β-globin promoters (present paper and ref. 12) a sequence very similar to the Sp1 consensus, typical Sp1 oligonucleotides neither generate nor compete band A2 (Fig. 1C and Fig. 2). Thus, protein A2 might either be a different protein than Sp1, or a modified form of Sp1 with altered binding characteristics.

In adult individuals, γ-globin genes are almost completely silent; point mutations in the γ-globin promoter (HPFH) may increase the expression of the mutated gene to levels of 15−100% relative to the adjacent adult β-globin gene (whose activity is correspondingly decreased [19,20]; for a review, see ref. 2). Two hypotheses have been proposed to explain the increased activity of the HPFH γ-globin gene. First, the mutation might decrease the binding of tissue specific or ubiquitous negatively acting factors; examples of these possibilities are the −117 G→A and the 13 nucleotides (−117 to −105) deletion HPFH [8,10,25]; unfortunately, no functional effects of the mutation have been detected by transfection with the available experimental models. Second, the mutation might create or strengthen a binding site for a positively acting factor; the −175 HPFH mutation increases the activity of the γ-globin promoter in transfection experiments [5,6] by altering [5,25] the binding of the erythroid-specific factor NFE1-GF1 [5,8,25,26].

The −198 mutation greatly enhances Sp1 and A2 binding and causes a four to five-fold erythroid-specific overexpression of the promoter in transfection experiments; this increase compares with a 20 to 30 -fold *in vivo* overexpression of the mutated gene [3,27], a slight discrepancy that could depend on the embryonic-fetal (rather than adult [27]) type of the human cells available for transfections. For comparison, note that the −175 mutation increases γ-globin promoter activity in transfection assays by only three to five-fold [5,6; see also Fig. 4] versus a 50−200 fold *in vivo* increase [2]. Overexpression of the −198 plasmid is dependent on increased binding to the Sp1 motif, overlapping with a CACCC box-like sequence as shown by the reversal of the effect of the −198 mutation by disruption of the Sp1-CACCC site, and by the inability of the −196 mutation to increase both binding and expression.

The CACCC box is a well known functional element of several genes [28], including β-globin, and mutations inactivating this element greatly decrease *in vivo* β-globin gene transcription [23] and *in vitro* binding of Sp1 and protein A2 to it [12,14]. We therefore suggest that increased binding of Sp1 and/or A2 proteins is, at least in part, responsible for the HPFH phenotype observed *in vivo*. We can not exclude the possibility that additional proteins, in adult cells, might bind to the −200 region and be affected in their binding, in particular by the −202 and −196 HPFH mutations, whose *in vivo* effects remain unexplained so far; this hypothesis is also suggested by recent results demonstrating (in chicken erythroid cells) a developmentally regulated protein (PAL) capable of inhibiting β-globin transcription by binding to a G-rich region immediately upstream to a CACCC box [29], binding the CON activator.

How could increased binding of non tissue- and gene-specific factors, like Sp1 and A2, lead to specific erythroid overexpression (and abnormal developmental control) of a tissue specific gene, like γ-globin? The observation that the greatly increased binding of Sp1 and A2 is not sufficient to allow expression in non-erythroid tissues, but is highly effective

in erythroid cells, implies that these factors might either directly cooperate with erythroid specific factors or stimulate nonspecific factors already cooperating with erythroid specific proteins. A candidate erythroid specific factor is NFE1-GF1 [5,8,25,26,30], that has binding sites in the proximal [8] as well as in the distal part of the promoter [5,25,30,31]. Significantly, the distal binding site, extending from approximately −192 to −168 [5,25], is immediately downstream to the strong Sp1-A2 sites created by the −198 mutation and is the one site whose mutation (−175 HPFH, see above) leads to considerable erythroid-specific overexpression of the γ-globin gene; simultaneous binding *in vitro* of proteins A and B and NFE1-GF1 to this DNA region has been observed [32] in preliminary studies. Thus, it is possible that mutations 'activating' either the Sp1-A2 binding sites, or the adjacent NFE1-GF1 site, allow cooperation between these factors and increase promoter efficiency specifically in erythroid cells. Alternatively, factors bound at either of the distal 'activated' sites might cooperate with factors bound at the proximal CACCC or NFE1-GF1 boxes. Truncation [31] and site-directed mutagenesis experiments [5,6] (and our unpublished data) indicate that the proximal, but not the distal, binding sites of the γ-globin promoter, are most relevant for the activity of the normal γ-globin promoter in embryonic-fetal cells; consequently, the appearance of new strong factor binding sites in the distal region, might allow the γ-globin promoter to escape normal mechanisms inactivating, at birth, the proximal γ-globin region.

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