

Molecular analysis of the human fetal-to-adult globin switching : the effects of the HPFH mutations in the γ -globin promoters

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GLOBIN SWITCHING: THE EFFECTS OF THE HPFH
MUTATIONS IN THE γ -GLOBIN PROMOTERS

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**MOLECULAR ANALYSIS OF THE HUMAN FETAL-TO-ADULT
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MUTATIONS IN THE γ -GLOBIN PROMOTERS**

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ABSTRACT

The human fetal-to-adult hemoglobin switch, which normally occurs at birth, is characterized by reduced γ -globin gene expression and increased activity of the adult β -globin gene. The mechanism controlling this developmental switch has remained elusive. Hereditary persistence of fetal hemoglobin (HPFH) is a condition characterized by continued expression of the γ -globin gene in adult life. Analysis of a Japanese HPFH family had revealed that the C-T transition at position -114 within the distal CCAAT box of the γ -globin gene associated with the HPFH allele. In the vicinity of the distal CCAAT box, other two mutations (-117 C-T, 13 bp del) had been identified in individuals with a HPFH phenotype. Functional analysis of these mutant promoters in erythroid cell lines suggested that the distal CCAAT box works positively in fetus but negatively in adult on the expression of the γ -globin gene. Further study on transgenic mice showed that the -114 mutation was responsible for the elevated expression of the γ -globin gene in the adult. To elucidate the molecular mechanism underlying the persistent expression of the γ -globin genes associated with the HPFH mutations, interaction of the mutant promoters with nuclear factors was analyzed. Relevance of the nuclear factor, NFE3, to the γ -globin regulation was suggested by the affected binding of NFE3 to the altered distal CCAAT boxes with HPFH mutations (-117, -114, 13 bp del). In addition, an uncharacterized factor "X", which generates a low mobility complex with the -114 mutant CCAAT box on gel retardation assay, may be involved in elevation of the γ -globin expression in an individual with the -114 mutation.

INTRODUCTION

The human β -like globin genes are distributed over 65 kb of DNA on the short arm of chromosome 11 (Fig. 1). Five functional genes contained within this region are regulated in a tissue- and developmental stage-specific manner (reviewed in 1, 2). The ϵ -globin gene is active primarily in the primitive erythroid cells that develop in the blood islands of yolk sac. Between the 5th and 10th weeks of gestation, there is a shift to "definitive" fetal liver erythropoiesis, and during this time the ϵ gene is switched off and the two γ -globin genes begin to be expressed. The γ -globin genes are maximally active through most of fetal life and are switched off around the time of birth. In contrast, the "adult" genes, δ and β , are active at only a low level in the fetal liver erythroid cells and are turned on fully only after birth, as the site of erythropoiesis shifts from liver to bone marrow. The expression of these genes is regulated, predominantly at the transcriptional level. Because of its own intrinsic interest as well as its importance in approaches to the therapy of the hemoglobinopathies, understanding regulation of the human β -like globin genes has been the focus of much research. The mechanism of this regulation, however, is still poorly understood.

Hereditary persistence of fetal hemoglobin (HPFH) is a clinically benign condition manifested by the continued expression of one or both γ -globin genes in the adults (3). This results in increased production of fetal hemoglobin (HbF; $\alpha_2\gamma_2$) which normally occupies less than 1% of total hemoglobin in adults. Different molecular lesions are associated with this phenotype and can be classified into two forms, the deletion and nondeletion types. Various deletional forms in which the δ - and β -globin genes are missing have been characterized (1). In the nondeletional form, the entire globin genes in the β -globin gene cluster are intact. In this latter type, several point mutations have been identified in the promoter region of the overexpressed γ -globin gene. The concordance of the mutations with a HPFH phenotype suggests that they mark

important regulatory sequences in the γ -globin genes. Trans-acting factors which interact with these sequences may be integral parts in the puzzle of γ -globin gene regulation. The regions in which their mutations are associated with overexpression of the gene include the distal CCAAT box (positions -111 to -115), the octamer sequence; ATGCAAAT (positions -182 to -175) and the sequence between 196 and 202 bp 5' to the cap site of the γ -globin gene (Fig. 1). Several nuclear factors binding to these parts of regions have been found.

Recently, a C-T substitution within the distal CCAAT box (at position -114) in the G γ -globin promoter of the Japanese HPFH family had been identified at our laboratory (4, 5). In the vicinity of the distal CCAAT box, other two forms of mutations associated with the HPFH phenotypes had been reported (6-8). The mutations are a G-A substitution at 2 bp upstream of the distal CCAAT box (position -117) and a 13 bp deletion including the distal CCAAT box. In this report I have pursued a detailed analysis of the functional role of the distal CCAAT box in the γ -globin regulation and of trans-acting factors binding to this region. Observations on the transient expression in K562 and KU812 demonstrated that the distal CCAAT box had a negative role in adulthood. Collinear with this, the presence of the -114 mutation resulted in persistence of γ -globin expression in adult mice. In vitro DNA-protein interaction assays showed the loss of binding of the transcription factor NFE3 (9, 10) to the HPFH mutant γ -globin promoters, suggesting that it may act as an negative regulator of the γ -globin gene in adult. In addition, the -114 C-T mutation which alters the CCAAT motif to the sequence, CTAAT, increased the ability of binding to an uncharacterized nuclear factor. This factor may be a positive regulator and involved in the persistent expression of the γ -globin gene with the -114 mutation.

Fig. 1

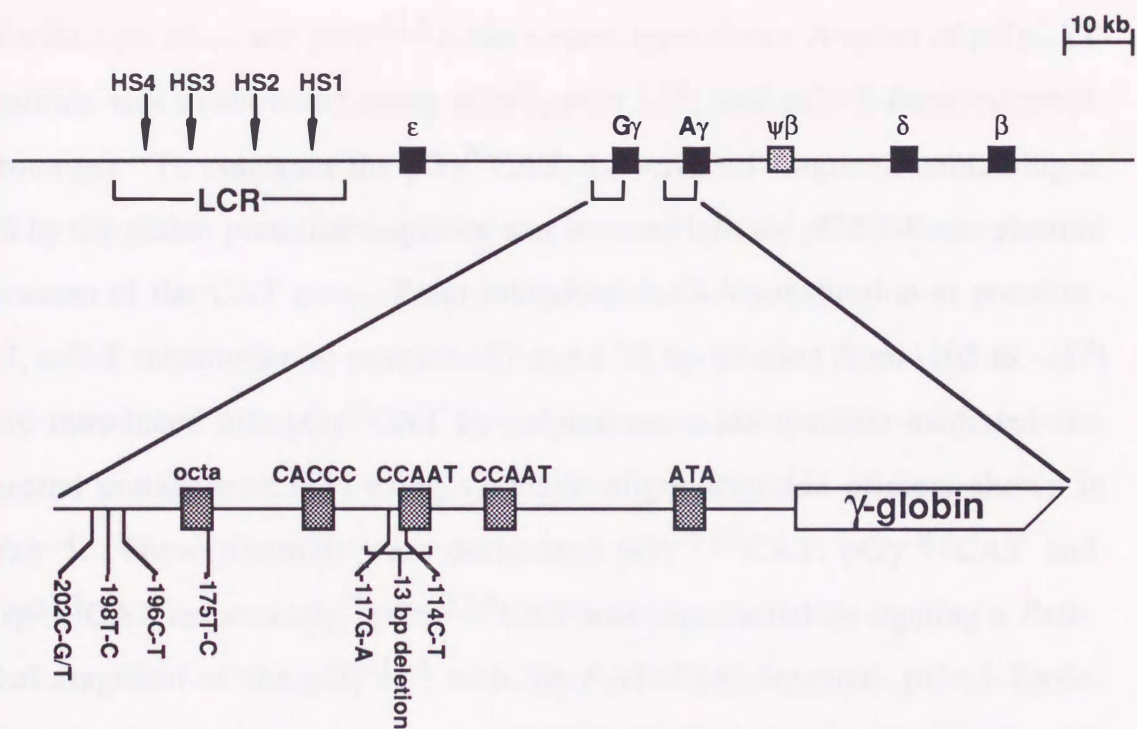


Fig. 1. Organization of the human β -like globin genes (top) and the 5' region of the γ -globin gene (bottom). The ATA box, the duplicated CCAAT boxes, the CACCC box and the octamer sequence are shown as shaded rectangles. Point mutations associated with HPFH are also indicated.

MATERIALS AND METHODS

Plasmid constructions. pG γ^N and pG γ^{114} (4) were made by inserting the 578 bp fragment amplified by polymerase chain reaction using Japanese HPFH patient's genomic DNA heterozygous for a C-T transition at position -114 into *HincII* site of pUC13 vectors. The 578 bp fragment spans from the position -564 to +14 to the cap site of the G γ -globin gene. pG γ^N is a clone of the wild type allele and pG γ^{114} is the mutant type allele. A series of pG γ CAT plasmids was constructed using pG γ^N , pG γ^{114} , and pCAT-Basic plasmid (Promega). To construct the pG γ^N CAT, a *SmaI-PstI* fragment containing a 576 bp G γ -globin promoter sequence was inserted into the pCAT-Basic plasmid upstream of the CAT gene. Point mutations (a G-A substitution at position -117, a C-T substitution at position -87 and a 13 bp deletion from -105 to -117) were introduced into pG γ^N CAT by polymerase chain reaction mediated site directed mutagenesis (11) using synthetic oligonucleotide primers shown in Table 1. These plasmids were designated pG γ^{117} CAT, pG γ^{87} CAT and pG $\gamma^{\Delta 13}$ CAT respectively. pG γ^{114} CAT was constructed by ligating a *PstI-XbaI* fragment of the pG γ^{114} with the *PstI-XbaI*-digested pCAT-Basic plasmid. To construct a series of p μ LCRG γ CAT plasmids, *HindIII-BamHI* fragments containing the 578 bp G γ -globin promoter and the CAT gene derived from pG γ CAT plasmids were ligated to the *HindIII-XhoI*-digested p μ LCR (12, 13). pG γ^N BN and pG γ^{114} BN were generated by inserting a 78 bp *BalI-NaeI* fragment spanning from the position -131 to -53 to the G γ -globin gene cap site into *HincII* site of the pBluescript SK+ plasmid vector.

Cell lines and preparation of nuclear extracts. Four nonerythroid cell lines, HeLa cell, RPMI human B-cell, JEG3 human chorionic cell, F9 mouse teratocarcinoma cell lines and three erythroid cell lines; human leukemia cell lines that have erythroid properties, K562, KU812 and a mouse erythroleukemia cell line, MEL were used in this study. HeLa, JEG3 and F9

cells were grown in Dulbecco's modified Eagle medium (Nissui Pharmaceutical Co.) containing 10% fetal calf serum. MEL cells were maintained in modified Eagle's medium (Nissui Pharmaceutical Co.) supplemented with 10% fetal calf serum. RPMI, K562 and KU812 cells were cultured in RPMI 1640 medium (Gibco Laboratories) supplemented with 10% fetal calf serum. In some experiments, K562 cells were induced to differentiate by addition of 50 μ M hemin to the medium for 2 to 4 days. For MEL cells, however, induction for differentiation was performed by growing the cultures for 4 days in the presence of 2% dimethylsulfoxide (Sigma). Nuclear extracts were prepared by the methods of Dignam et al. (14) with slight modification. In addition to the PMSF protease inhibitor, 2 mg/ml each of antipain, leupeptin and pepustatin A were also included in the buffers and NaCl in buffer B was replaced with KCl. For DNase I footprinting assay, nuclear extracts were prepared according to Shapiro et al. (15). The amount of protein in the extracts were determined by Bradford's dye binding assay using a Bio-Rad reagent (Bio-Rad Laboratories).

Transient expression studies. MEL, K562 and KU812 cells were transfected by electroporation. DNA was purified by the alkaline lysis method followed by banding in cesium chloride density gradients. Each pG γ CAT plasmid DNA (20 μ g), together with 2 μ g of an RSV promoter-luciferase gene fusion plasmid, pRSVLUC (16), was mixed with $1-3 \times 10^7$ cells in 0.8 ml of ice-cold phosphate-buffered saline. A single pulse at 400 V and 960 μ F was delivered by GENE PULSER (Bio Rad). The cells were then kept on ice for 10 min and incubated at 37°C for 48 hrs in appropriate media. In the case of MEL and K562, the cells were induced to differentiate by adding dimethylsulfoxide or hemin after transfection. The chloramphenicol acetyltransferase assay was performed as described (17). The luciferase activity was measured (16) on Lumat LB9501 luminometer (Berthold).

Production of transgenic mice. The construction of pA γ / β was described previously (18). pG γ^{114} / β was created by replacing a 3.3 kb *Hind*III fragment of the A γ -globin gene in pA γ / β with a 3.3 kb fragment containing the entire G γ -globin gene associated with the C-T substitution at nucleotide position -114. This G γ -globin fragment was the product of polymerase chain reaction using Japanese HPFH patient's genomic DNA. Two oligonucleotide primers with the sequence 5'-GGTCTTTTAGCCGCCTAACA-3' and 5'-TTTCTTCGTTGAGCCCCCTTC-3' were used for amplification. A 8.9 kb *Cla*I-*Sal*I fragments (Fig. 3) of pA γ / β and pG γ^{114} / β were purified by agarose gel electrophoresis from vector sequences. After further purification by banding in cesium chloride and dialysis, the DNA was microinjected into male pronuclei F2 hybrid eggs obtained by mating C57BL/6J X DBA/2 parents as described (19, 20). Transgenic mice were identified by Southern blot analysis (21) of the DNA obtained from tail biopsies (22). The transgene copy numbers were determined by dot blot hybridization using human genomic DNA as a standard.

Analysis of mRNA in transgenic mice tissues. Total RNA was prepared using the guanidine thiocyanate procedure (23) from 10.5-day yolk sac, 13.5 and 16.5-day fetal liver, and adult spleen (about two months old). The levels of β -, γ -, β_{maj} -, and β_{h1} -globin mRNAs were determined using quantitative ribonuclease protection assay (24) and intensities of protected bands were quantitated with Fuji Bio Image analyzer BAS2000 (Fuji Film Co, Japan). The following globin-specific probes were used: (i) for human β -globin mRNA, pBH β 2, a 655 bp *Acc*I fragment linearized with *Pst*I to give a 143 bp protected fragment; (ii) for human γ -globin mRNA, pBH γ 2, a 342 bp *Ava*II fragment linearized with *Pst*I to give a 145 bp protected fragment; (iii) for mouse β_{maj} -globin mRNA, pBM β_{maj} , a 441 bp *Hinc*II-*Bam*HI fragment linearized with *Ava*II to give a 189 bp protected fragment; (iv) for mouse β_{h1} -

globin mRNA, pBM β h1, a 423 bp *Xba*I-*Bam*HI fragment linearized with *Xba*I to give a 143 bp protected fragment.

Gel retardation assay. Synthetic oligonucleotides, listed in Table 1, were labeled at their 5'-end using T4-kinase and annealed to the 5-fold excess of the unlabeled complementary counterparts in annealing buffer (100 mM NaCl, 100 mM Tris-HCl pH7.8 and 1 mM EDTA) by heating to 85°C for 2-3 min followed by slowly lowering the temperature to 4°C. These annealed oligonucleotides were diluted to an appropriate concentration. The standard binding reaction mixture (20 μ l) contained 5-10 μ g nuclear extract protein in 10 mM HEPES pH 7.6, 50 mM KCl, 0.25 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, 10% glycerol and 1.25 mg poly(dI-dC)poly(dI-dC) (Pharmacia). The mixture was left on ice for 20 min prior to the addition of the 32 P-labeled probe (10-20 fmol) or the probe-competitor mixture. The reaction mixture was then left on ice for another 20 min. Electrophoresis was carried out at 4°C on a 4% native polyacrylamide gel which had been pre-run (at 20 mA for 1 hr) in 0.4 X TBE. Electrophoresis of the sample was continued for 1-1.5 hrs and the gel was then dried and autoradiographed.

DNaseI footprinting assay. The 99 bp *Hind*III-*Xho*I fragments from the plasmids pG γ ^NBN and pG γ ¹¹⁴BN, α - 32 P dCTP labeled at the 3' end of the *Xho*I site (for coding strand) or the *Hind*III site (for noncoding strand), were used as probes. The reaction mixture (50 μ l) contained about 50 μ g nuclear extract protein, 2.5 μ g poly(dI-dC)poly(dI-dC) (Pharmacia) and 5 fmol of a singly end labeled probe in DNase reaction buffer containing 10 mM HEPES pH 7.6, 50 mM KCl, 0.25 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF and 10% glycerol. After standing on ice for 30 min, an equal volume of DNase reaction buffer was added (37°C) and incubated at 25°C for 1 min followed by digestion with 3 μ l of DNaseI (Takara Shuzo) diluted in 20 mM Tris-HCl pH7.5, 5 mM CaCl₂, 10 mM MgCl₂ and 50% glycerol at 25°C for 1 min. The reaction was

terminated by adding 25 ml of stop solution (1.5 M sodium acetate pH5.2, 20mM EDTA and 100 mg/ml tRNA). The partially digested DNA was recovered by phenol/chloroform extraction and ethanol precipitation. The pelleted DNA was dissolved in formamide dye and analyzed on an 8% sequencing gel. An appropriate amount of the probe was also subjected to chemical cleavage at G and A bases, according to Maxam and Gilbert (25), for use as a marker.

Methylation interference assay. Synthetic double-stranded oligonucleotide probes were end labeled with γ - ^{32}P ATP and T4-kinase either in the sense or the antisense strand, partially methylated at G residues by dimethyl sulfate according to Maxam and Gilbert (25). The methylated probe was incubated with the nuclear extract and then electrophoresed as described above. Free and bound DNA was eluted from the acrylamide gel, purified on DEAE 81 cellulose and then cleaved at methylated G residues in 100 μl of 1 M piperidine at 95°C for 30 min prior to analysis on a 20% denaturing sequencing gel.

Table 1. The sequences of the top strand oligonucleotides used in binding assays.

Specific oligonucleotides	Sequence (5' to 3')
Normal γ -distal CCAAT (D/CCAAT)	GCCAGCCTTGCCTTGAC <u>CCAAT</u> AGCCTTGAC
-114 C-T γ -distal CCAAT (D/-114 C-T)	GCCAGCCTTGCCTTGAC <u>CTAAT</u> AGCCTTGAC
-117 G-A γ -distal CCAAT (D/-117 G-A)	GCCAGCCTTGCCTTAAC <u>CCAAT</u> AGCCTTGAC
13 bp del γ -distal CCAAT (D/ Δ 13)	TGGCCAGCCTTGCCTTGACAAGGCAAACCTTGA
-109 G-A γ -distal CCAAT (D/-109 G-A)	GCCAGCCTTGCCTTGAC <u>CCAATA</u> aCCTTGAC
Normal γ -proximal CCAAT (P/CCAAT)	AGGCAAACCTTGAC <u>CCAAT</u> AGTCTTAGAGTAT
-87 G-A γ -proximal CCAAT (P/-87 C-T)	AGGCAAACCTTGAC <u>CTAAT</u> AGTCTTAGAGTAT
Normal β -globin CCAAT (β /CCAAT)	TAGGGTTGG <u>CCAAT</u> CTACTCCCAGGAGCAG

The CCAAT motif and its mutant derivatives are underlined. Abbreviations for each oligonucleotide are indicated in parentheses.

RESULTS

A) Functional analysis of the γ -globin promoters

Activity of the mutant γ -globin promoters in erythroid cells. To evaluate the effects of the HPFH mutations in the vicinity of the distal CCAAT box on the γ -globin gene expression, a series of the G γ -globin promoter-CAT fusion genes were analyzed in transient expression studies using K562 and KU812 cell lines. These cell lines are derived from human leukemia and have erythroid properties. K562 cells express embryonic (ϵ) and fetal (γ) globin chains (27, 28), while KU812 cells express fetal and adult (β) globin chains (28, 29). The G γ CAT constructs used are shown in Fig. 2. In addition to the HPFH mutations (-117 G-A, -114 C-T, 13 bp del), a C-T substitution at position -87 which alters the same position of the proximal CCAAT motif to CTAAT as the -114 mutation in the distal one was tested. Relative activities of the mutant promoters to the normal one are summarized in Table 2. In an embryonic-fetal erythroid back ground (K562), the HPFH mutations, as well as the -87 C-T substitution decreased the promoter activity. On the other hand, the HPFH mutant promoters represented equal or slightly higher activity to the normal one in a fetal-adult erythroid back ground (KU812). The -87 C-T mutation, however, decreased promoter activity also in KU812.

The activity of the stage-specific promoter elements within the β -globin gene cluster is significantly influenced by the distant regulatory element, the locus control region (LCR) (30). The LCR is situated between 10 and 30 kbp upstream from the ϵ -globin gene and consists of four erythroid-specific DNaseI hypersensitive sites. High level, stage-specific expression of the β -like globin genes represents an integration of the activity of the LCR. To evaluate effects of the LCR to the HPFH mutant promoters, G γ CAT constructs linked to the μ LCR (12, 13) were also tested in transient expression assays. Activity of the normal G γ -globin promoter was enhanced about 70 fold by the μ LCR in K562

(compare the values of $G\gamma$ NCAT and μ LCR- $G\gamma$ NCAT in the lower column in Table 2). Similar enhancement was observed on the mutant promoters (compare the values of the μ LCR-less constructs in the upper column standardized to $G\gamma$ NCAT with the values of the μ LCR-linked constructs in lower column standardized to μ LCR- $G\gamma$ NCAT). These results indicate that the transcriptional enhancement by the LCR is equally significant in normal and mutant γ -globin promoters and would be in agreement with the finding that the LCR can invoke induced expression of unrelated transgenes in erythroid cell (31-33).

Fig. 2

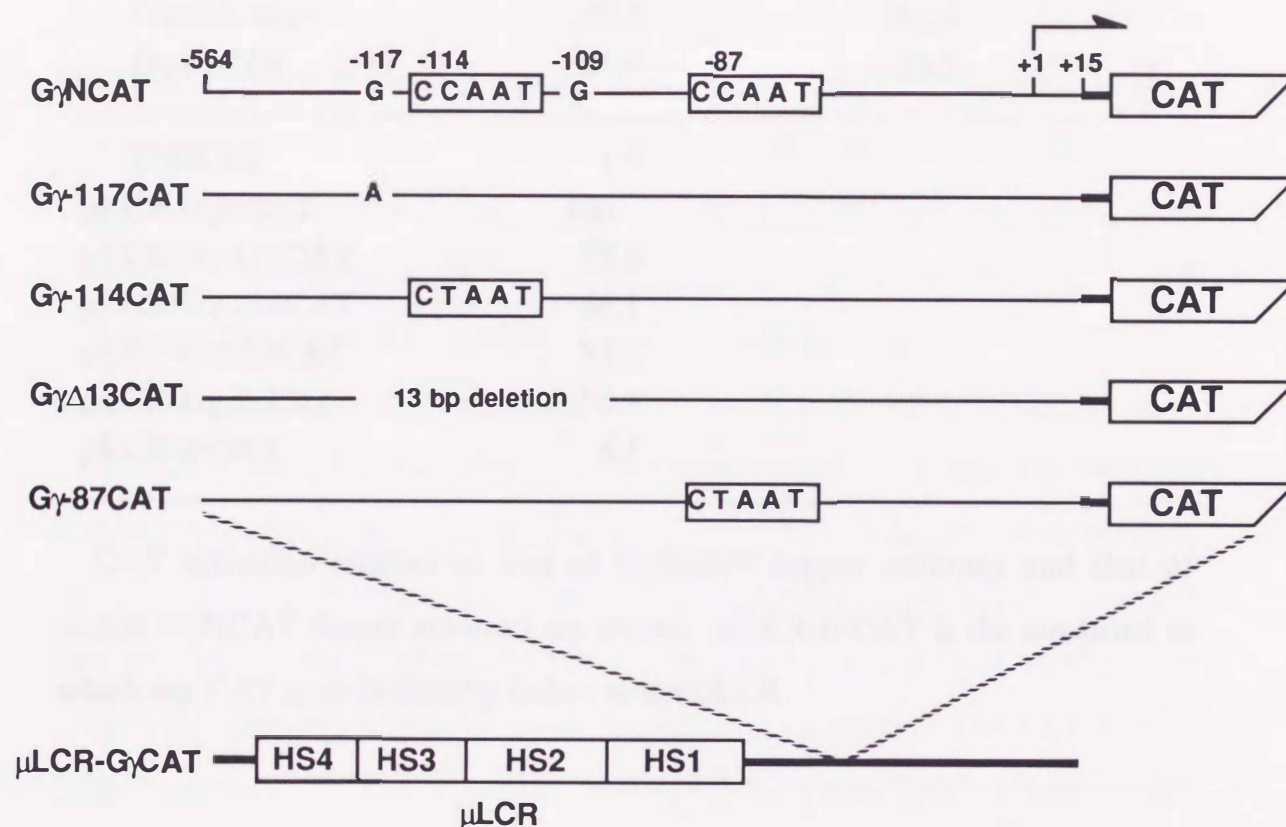


Fig. 2. Structures of CAT plasmids. The $G\gamma$ -globin promoter region corresponding to -564 to +5 was linked to the CAT gene. Duplicated CCAAT boxes are indicated by open boxes. A series of μ LCR- $G\gamma$ CAT plasmids were constructed by linking each $G\gamma$ CAT fusion gene to downstream of the μ LCR. Thick bars show the sequences derived from plasmid vectors.

Table 2. Activities of mutant $\text{G}\gamma$ -globin promoters in K562 and KU812

CAT construct	K562	KU812
$\text{G}\gamma\text{NCAT}$	100	100
$\text{G}\gamma\text{-117CAT}$	73.5	158.9
$\text{G}\gamma\text{-114CAT}$	26.0	102.7
$\text{G}\gamma\Delta\text{13CAT}$	45.7	101.4
$\text{G}\gamma\text{-87CAT}$	21.1	56.5
$\text{G}\gamma\text{NCAT}$	1.5	
$\mu\text{LCR-G}\gamma\text{NCAT}$	100	
$\mu\text{LCR-G}\gamma\text{-117CAT}$	67.4	
$\mu\text{LCR-G}\gamma\text{-114CAT}$	66.1	
$\mu\text{LCR-G}\gamma\Delta\text{13CAT}$	53.6	
$\mu\text{LCR-G}\gamma\text{-87CAT}$	54.9	
$\mu\text{LCR-0-CAT}$	6.1	

CAT activities relative to that of $\text{G}\gamma\text{NCAT}$ (upper column) and that of $\mu\text{LCR-G}\gamma\text{NCAT}$ (lower column) are shown. $\mu\text{LCR-0-CAT}$ is the construct in which the CAT gene is directly linked to the μLCR .

Human γ -to- β globin gene switching in transgenic mice. The cell systems simulate in vivo erythropoiesis only in part. They, therefore have certain disadvantages, in particular, for studying the switch from embryonic to fetal to adult globin gene expression. Indeed, there is no human cell lines manifesting complete adult erythroid properties. A more powerful approach to the study of switching mechanisms is the development of transgenic mice in which expression of the introduced human globin genes is regulated as their endogenous counterparts. To establish such transgenic mice, the $A\gamma/\beta$ construct, in which the human γ - and β -globin genes were juxtaposed and oriented in the same directions (Fig. 3), was introduced into mice and expression profiles of the transgenes were determined.

Five lines bearing various numbers of the $A\gamma/\beta$ -globin gene construct were obtained (Table 3) (18). Southern blot analysis showed that the injected fragments appeared to be arranged in the head-to-tail configuration in all lines (data not shown). Expression of the introduced β -globin gene in adult spleen of transgenic mice was measured by ribonuclease protection assay. Two lines, 3-4 and 11-5 expressed detectable amounts of human β -globin mRNA (Table 3). Further analysis for these two lines revealed that human β -globin mRNA was not detected in liver, brain and skeletal muscle of the adult mice. The expression of the β -globin gene was limited to the erythroid tissues. To investigate the expression pattern of the introduced human globin genes throughout development, total RNAs from 10.5-day yolk sac, 13.5 and 16.5-day fetal liver, and adult spleen of lines 3-4 and 11-5 were prepared. The relative abundance of human and mouse globin mRNAs were determined by ribonuclease protection assay using an appropriate amount of total RNA samples. Fig. 4 illustrates a representative result obtained from line 3-4. Although intensity of the protected bands could not be compared directly between different developmental stages because of different RNA sources, a significant increase of β -globin expression was observed between 13.5 and 16.5-days. On the other hand, a striking change was not observed in γ -globin

gene expression. Table 4 shows relative expression levels of human and mouse globin mRNAs represented as a percentage of the level of mouse β_{maj} -globin mRNA expressed at adult spleen. In 10.5-day yolk sac of line 3-4, the β -globin expression level was higher than that of the γ -globin gene. As described previously (34, 35), the β_{h1} -globin gene (mouse embryonic type) was predominantly expressed, but the β_{maj} -globin gene (mouse adult type) was barely expressed in 10.5-day yolk sac of the mouse. It is likely that the presence of β -globin mRNA in embryos is due to contamination by maternal erythroid tissues because β_{maj} -globin mRNA was detected in 10.5-day yolk sac (Table 3). Contamination by maternal RNA seems to be more significant in line 3-4 because exogenous β -globin expression in adult of line 3-4 was much higher (34.5 % of endogenous β_{maj}) than that in line 11-5. Since the amounts of β - and γ -globin mRNA derived from maternal tissues in embryonic samples should correlate with the relative level of mouse β_{maj} -globin mRNA in 10.5-day yolk sac, the amounts of contaminated messages during embryo preparation of line 3-4 appear to be 1.33 and 0.05 as β - and γ -globin messages, respectively. After compensation for contamination by maternal RNAs, relative expression levels of β - and γ -globin mRNAs turn to be 0 and 0.05, respectively in 10.5-day yolk sac of line 3-4 (Table 4 parenthesized). Fig. 5 shows the ratio of the γ -globin mRNA expression to the total exogenous mRNA ($\gamma/\beta+\gamma$) at four developmental stages of transgenic mice. The switch from γ - to β -globin expression was observed around 13.5 day of gestation of transgenic mice.

Using this transgenic mice system, effects of the -114 HPFH mutation of γ -globin regulation was analyzed. The expression pattern of the $G\gamma^{114}/\beta$ -globin construct, in which the $A\gamma$ -globin gene in the $A\gamma/\beta$ construct was replaced with the $G\gamma$ -globin gene with the -114 C-T mutation, was analyzed by RNase protection assay throughout mouse development (Table 4). Fig. 5 shows the $\gamma/\beta+\gamma$ ratio during development. The expression level of the γ -globin gene was not reduced in fetal and adult stages and remained equivalent to that of the β -

globin gene. The persistent expression of the γ -globin gene associated with the -114 C-T substitution in mice demonstrates that this mutation in the distal CCAAT box is the cause of the raised γ -globin level in the adults of the HPFH patients and is not just a linked polymorphism.

Fig. 3

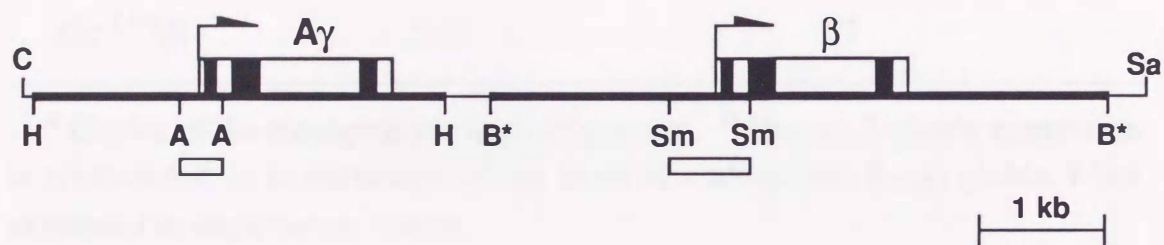


Fig. 3. The A γ / β -globin gene construct introduced into mice. The human A γ -globin *Hind*III fragment and the human β -globin *Bgl*III fragment (thick bar) were juxtaposed and oriented in the same direction. Thin bars show the sequences derived from plasmid (pBR322). Arrows indicate the transcription start sites. The coding regions of each globin gene are shown by solid boxes. Open boxes under the diagram indicate the locations of the probes used for ribonuclease protection assay. Abbreviations are: A, *Acc*I; B*, *Bgl*III (these sites are lost); C, *Cl*aI; H, *Hind*III; Sa, *Sal*I; Sm, *Sma*I.

Table 3. Copy number of transgenic mice and relative expression of the human β - to mouse β_{maj} - globin gene in adult mouse spleen

Lines	Copy number ^a	human β /mouse β_{maj} (%) ^b
A γ / β 3-4	80-150	34.5
11-5	33-42	6.9
5-3	6-13	0
4-5	50	0
8-2	3	0
G γ ¹¹⁴ / β	5-10	1.7

^a Copies of the transgene per haploid genome. ^b Human β -globin expression is represented as a percentage of the level of endogenous β_{maj} -globin RNA expressed in adult mouse spleen.

Fig. 4

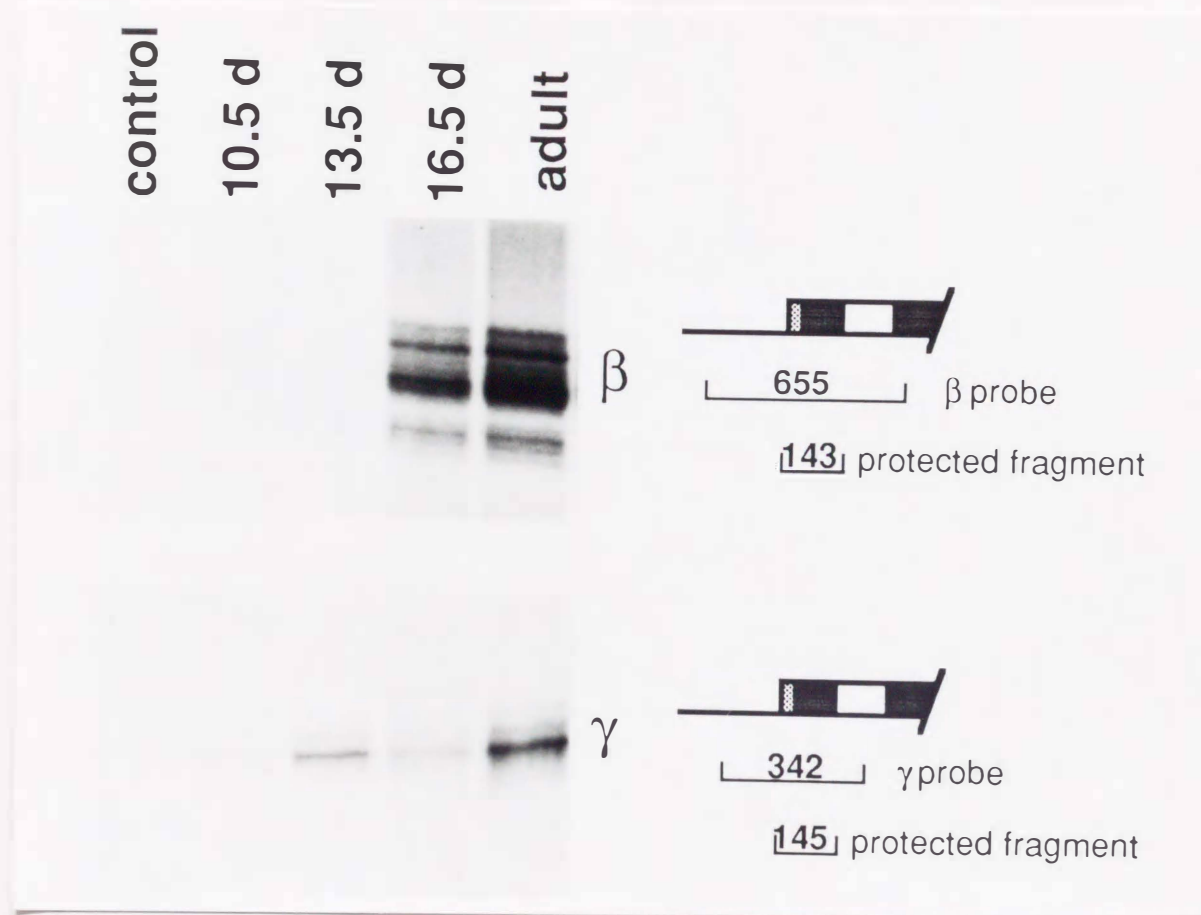


Fig. 4. RNase protection analysis against human γ - and β -globin mRNAs in transgenic mice (line 3-4) during development. Five μ g or 10 μ g total RNA prepared from erythroid tissues at indicated days of gestation were hybridized to human β - or γ -globin specific RNA probes and RNase resistant fragments were analyzed by electrophoresis on a 6% polyacrylamide sequence gel. RNase protected fragments are indicated at the right. Negative control consisted of 20 μ g tRNA. Probes are diagramed at the right.

Table 4. Relative abundance of human and mouse globin mRNAs per microgram total RNA prepared from transgenic mice.

Lines	mRNA	10.5 day	13.5 day	16.5 day	Adult
A γ / β 3-4	human β	0.42(0)	0.95*	13.3	34.5
	human γ	0.10(0.05)	0.41	0.28	1.19
	mouse β_{maj}	3.87	63.6	168.2	100
	mouse β_{h1}	9.75	0.83	0	0
A γ / β 11-5	human β	0	1.11*	2.42	6.50
	human γ	0.05	0.09	0.07	0.24
	mouse β_{maj}	2.05*	155.3*	219.9	100
	mouse β_{h1}	3.80	0.06*	0*	0*
G γ ¹¹⁴ / β	human β	0	7.65*	15.4	1.7
	human γ	0.24	9.68	8.29	1.15
	mouse β_{maj}	1.17	258.8	351.2	100
	mouse β_{h1}	9.85*	0.24*	0*	0*

Human and mouse globin expression levels in 1 μ g total RNA prepared at indicated time points are represented as a percentage of the mouse β_{maj} -globin mRNA level in 1 μ g total RNA of adult mice spleen. Expression values shown are averages from duplicated or triplicated analyses except those marked by asterisks. Compensated values for maternal RNA contamination are parenthesized.

Fig. 5

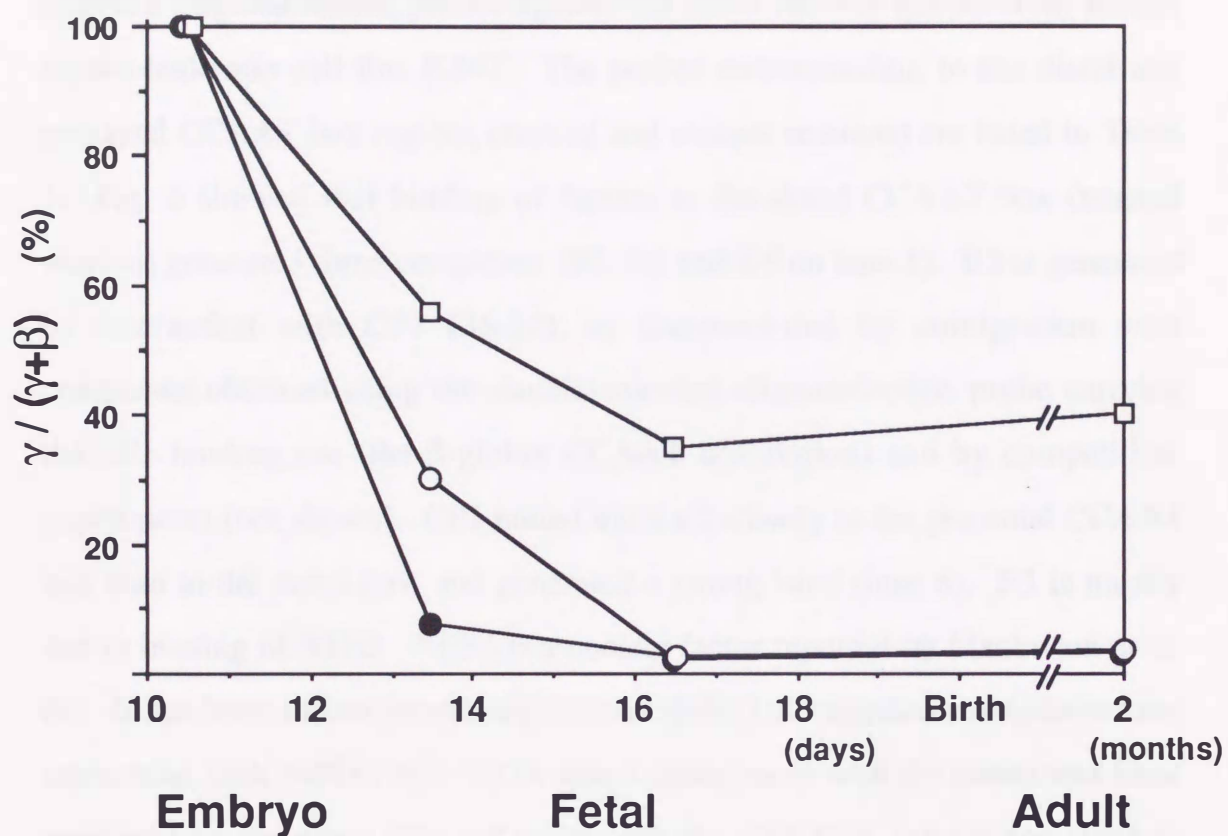


Fig. 5. Relative changes in the γ - and β -globin gene expression during ontogeny of transgenic mice. The relative γ -globin mRNA levels were plotted as a fraction of total exogenous human globin ($\gamma + \beta$) mRNA. Filled circle, $A\gamma/\beta(3-4)$; open circle, $A\gamma/\beta(11-5)$; open rectangle, $G\gamma^{114}/\beta(3-4)$.

B) Interaction of trans-acting factors with the γ -globin promoters

Effects of the HPFH mutations on the binding of trans-acting factors. To elucidate the effects of the HPFH mutations on the binding of trans-acting factors, gel mobility shift assays were performed using double-stranded oligonucleotide probes against the crude nuclear extract from human erythroleukemic cell line K562. The probes corresponding to the distal and proximal CCAAT box regions (normal and mutant versions) are listed in Table 1. Fig. 6 showed that binding of factors to the distal CCAAT box (normal version) generated three complexes (B2, B3 and B4 on lane 1). B2 is generated by interaction with CP1 (36-38), as demonstrated by comigration with complexes obtained using the double-stranded oligonucleotide probe carrying the CP1 binding site (the β -globin CCAAT box region) and by competition experiments (not shown). CP1 bound more effectively to the proximal CCAAT box than to the distal box, and generated a strong band (lane 6). B3 is mostly due to binding of NFE3. NFE3 is a nuclear factor reported by Mantovani et al. (9). It has been shown previously that the -109 G-A mutation strengthened the interaction with NFE3 (10). B3 in lane 1 comigrated with the prominent band generated by the distal CCAAT probe with the -109 G-A substitution (lane 5) and was competed out effectively by the unlabeled -109 G-A mutant probe (not shown). B4 was observed on all the probes. The binding of B4, however, could not be suppressed on self competition experiments. It could therefore be considered as a nonspecific binding. The -117 HPFH mutant probe gave B2 (CP1) with almost the same intensity to the normal distal CCAAT probe but the -114 C-T and the 13 bp deletion HPFH mutant probes did not generate B2 (lanes 2, 3 and 4). This is consistent with previous results (9, 10, 39, 40). The -117 G-A substitution diminished the binding property of NFE3 and only the faint band corresponding to B3 was observed in lane 2. The -114 C-T probe gave a faint smear band nearly the same position as B3 (lane 3). With the 13 bp deletion probe, a complex with almost the same but slightly high mobility to the

B3 was also detected (lane 4). Specificity of the bands generated by the -114 and the 13 bp deletion mutant probes was confirmed by self competition analyses (not shown). The inability of the unlabeled -109 mutant probe, which has high affinity with NFE3, to compete out these two bands reveals that these are not due to binding of NFE3. It should be noted that the -114 C-T mutant probe generated a strong band with low mobility (B1 in lane 3). Although, faint bands corresponding to B1 were observed on the -117 and the 13 bp deletion mutant probes, these are likely to be nonspecific binding since unlabeled homologous oligonucleotide can not compete significantly (not shown). Interestingly, the -87 C-T mutant probe, in which the proximal CCAAT motif is altered to CTAAT, also generated a strong band with the same mobility as B1 (lane 7). Reciprocal competition experiments between the -114 and the -87 mutant probes demonstrated that the factors comprising these bands are identical. This factor, refer to "X" in this issue, is likely to bind to the CTAAT motif.

Decreased binding of NFE3 to the HPFH mutant promoters was investigated further by a competition experiment. To directly quantitate the relative affinity of each of the normal and the HPFH mutant γ sequences for NFE3, ability of the corresponding oligonucleotides to compete NFE3 (B3) from the high-affinity NFE3 probe, the -109 G-A, was examined. For these experiments, a series of parallel binding reactions containing the K562 extract and the labeled -109 G-A mutant probe were incubated with the increasing amount of unlabeled probes (Fig. 7). The normal sequence prevented formation of NFE3 complex 28% at 5 fold and 72% at 50 fold excess concentration (lanes 2 and 3). A slight inhibition of NFE3 binding, 41% by the 50 fold excess competitor, was detected on the -117 G-A mutant version (lane 5). A significant competition was not observed on the -114 C-T (lanes 6 and 7) and the 13 bp deletion (lanes 8 and 9) mutant versions. Decreased binding of NFE3 is the common effect of the -117 G-A, -114 C-T and 13 bp deletion HPFH mutations and likely to be the

integral part of the continuous expression of the γ -globin gene in adulthood of the HPFH patients.

Fig. 6

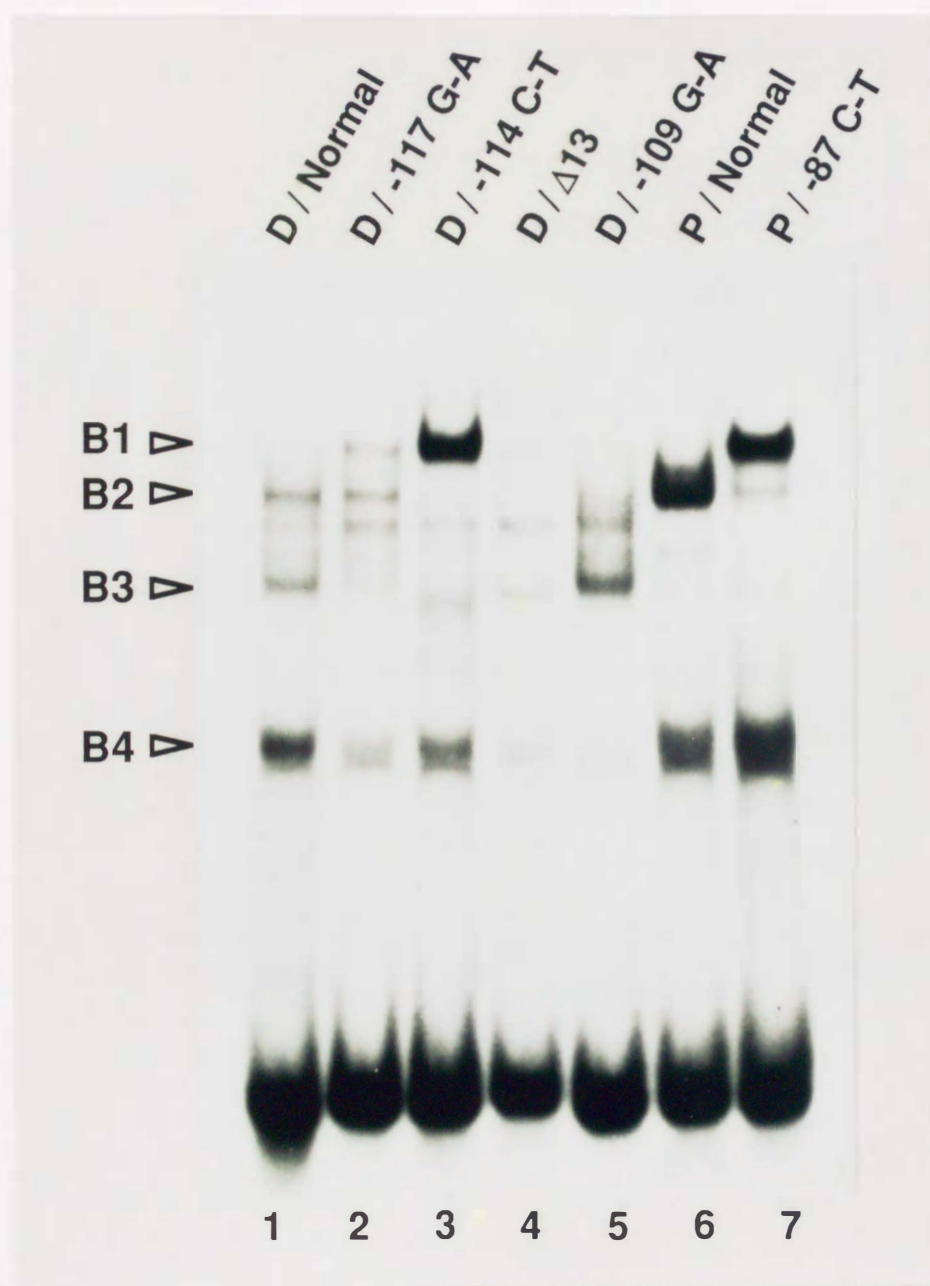


Fig. 6. Gel mobility shift assay of the normal and mutant oligonucleotide probes corresponding to the distal or proximal CCAAT box region using the nuclear extract from K562. The probes used are shown at the top and their sequences are listed in Table 1.

Fig. 7

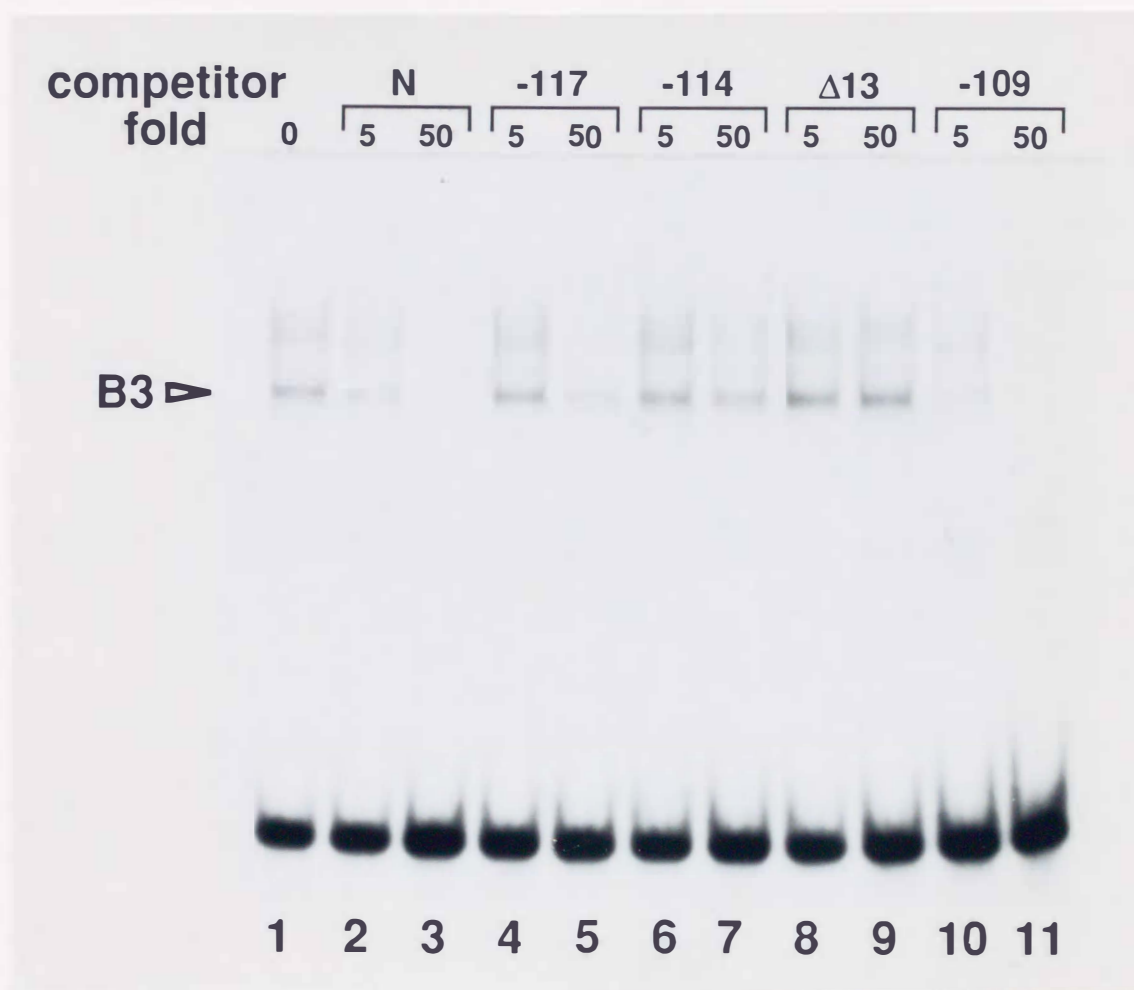


Fig. 7. Quantitation of the relative affinity of the distal CCAAT box bearing the HPFH mutations to NFE3. The labeled -109 G-A mutant probe, which binds efficiently to NFE3, was incubated in K562 extracts in the presence of 5-50 fold unlabeled competitors (as indicated at the top of the figure). Lane 1 is the binding pattern without competitors. Competitors used are indicated at the top.

Tissue specificity of NFE3 and the factor "X". Tissue specificity of NFE3 and the factor "X" was further analyzed using nuclear extracts from various cell lines. Fig. 8 shows a gel mobility shift experiment of the distal CCAAT and -109 G-A (lane 2) probes. The NFE3 (B3) complex with the distal CCAAT probe was formed in nuclear extracts from human nonerythroid cell lines HeLa and JEG3 but not in mouse nonerythroid cell line F9 (lanes 4, 5 and 6). With the nuclear extract from the human B-cell line RPMI, the distal CCAAT probe generated faint smear at the position of B3 (lane 3). This smear band was strengthened when the -109 G-A mutant probe was used (lane 2) and effectively competed out by the unlabeled -109 probe (not shown). These lines of evidence suggest that this faint smear was the complex formed by partially degraded NFE3. NFE3 was originally reported as a erythroid specific factor because of the absence in extracts from three human nonerythroid blood cell lines; Molt4, U937 and Raji cells (9). My results, however, demonstrate the existence of NFE3 in variety types of human cells. NFE3 may be unstable especially in nonerythroid blood cells and hardly to be detected by in vitro binding assay just as the case of RPMI. Fig. 9 demonstrates a gel mobility shift experiment of the -114 C-T probe using variety types of nuclear extracts. The nuclear factor "X" must be a ubiquitous factor, being present in all extracts tested.

Fig. 8

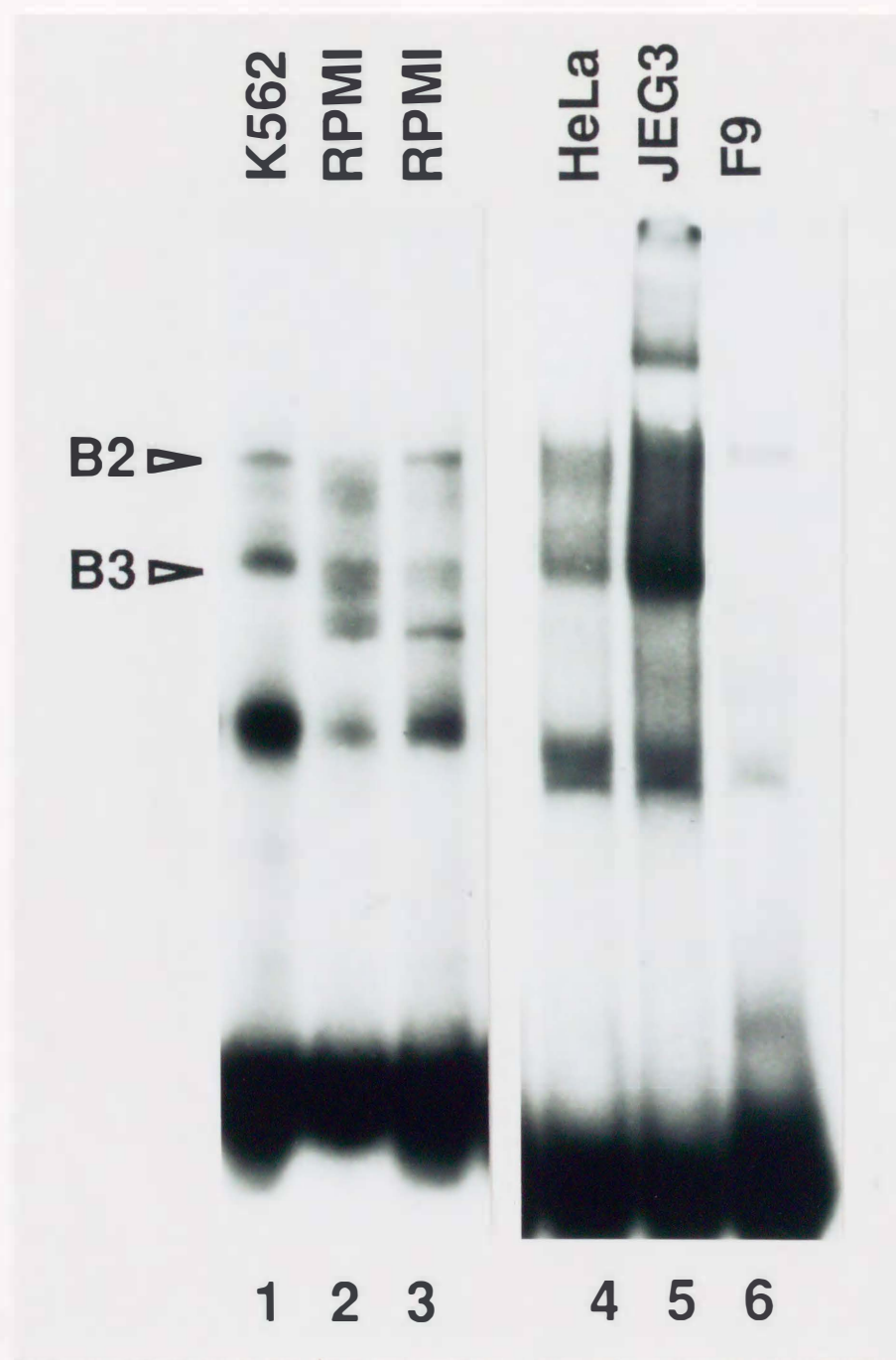


Fig 8. Tissue specificity of band B3 (NFE3). Gel mobility shift assay using nuclear extracts from various nonerythroid cell lines. Lane 1 is a binding pattern of the normal distal CCAAT probe with the K562 extract as a reference. Lanes 2 and 3 are a binding of the -109 mutant and normal distal probe with the RPMI extract, respectively. Lanes 4 to 6 are binding of the normal distal probe with nuclear extracts from HeLa, JEG3 and F9.

Fig. 9

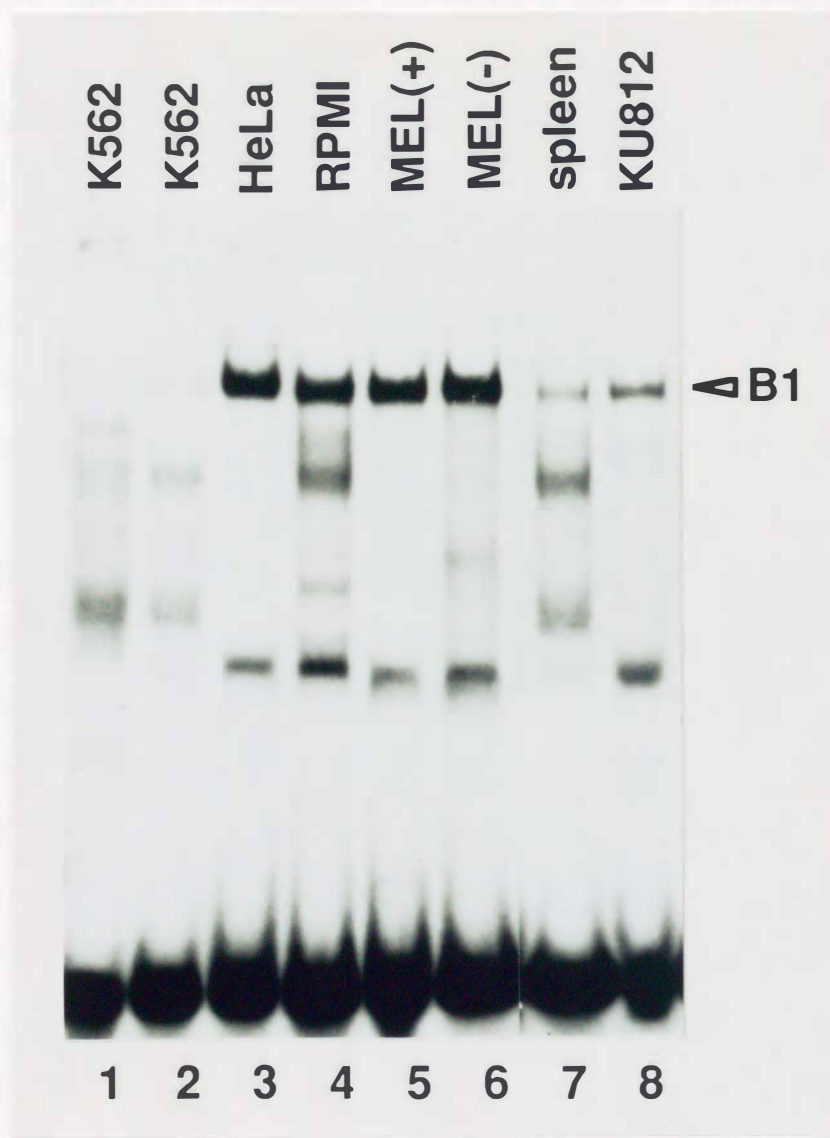


Fig. 9. Tissue specificity of band B1. Gel mobility shift assay of the -114 HPFH mutant probe using nuclear extracts from various erythroid and nonerythroid cells (as indicated at the top of the figure). Lane 1 is a binding pattern of the normal distal CCAAT probe with the K562 extract as a reference. MEL(+) and MEL(-) represent nuclear extracts from differentiated MEL (induced by DMSO) and un-induced MEL, respectively. Lane 7 is a binding with the nuclear extract from a spleen of a anemic mouse by the phenylhydrazine treatment (41).

Interaction of the distal CCAAT box with CP1 and NFE3. Though CP1 and NFE3 interact with the same distal CCAAT probe (normal version), it is not known whether the interactions of CP1 and of NFE3 are mutually exclusive. To address this question, a competition experiment in the gel mobility shift assay was performed under a condition using excess amounts of the nuclear extract. If CP1 and NFE3 bind to the distal CCAAT probe simultaneously, a supershifting band due to a complex containing these two factors could be observed. On the contrary, if the interactions are mutually exclusive, removal of CP1 with an oligonucleotide that specifically binds to this factor but not to NFE3, would enhance the formation of the complex containing NFE3, and vice versa. The unlabeled normal proximal CCAAT probe, which interacts efficiently with CP1 but not with NFE3 (lane 6 in Fig. 6), and the -109 G-A distal CCAAT probe, which interacts efficiently with NFE3 but inefficiently with CP1 (lane 5 in Fig. 6), were used as competitors against the normal distal CCAAT probe on the mobility shift assay (Fig. 10). On the absence of the competitors, there was no supershifting band (lane 4). Generation of the bands B2 and B3 were inhibited with the 5 to 100 fold excess amounts of the normal proximal CCAAT (lanes 5, 6 and 7) and -109 G-A distal CCAAT probes (lanes 3, 2 and 1), respectively. Enhancement of the bands B2 by the NFE3 specific competitor or B3 by the CP1 specific competitor was not detected and cross competition was observed by the 100 fold excess competitors (lanes 1 and 7). Absence of the supershifting band suggest that binding of CP1 and NFE3 to the distal CCAAT region may be mutually exclusive, although the elimination of the one factor didn't enhance the binding of the other factor. Because of the low affinity of these factors to the distal CCAAT probe in this condition, complex formation of the one factor might not be enhanced in the absence of the other factor. Indeed, affinity of CP1 and NFE3 to the distal CCAAT probe is so weak that 60 % of the probe exists in non-binding fraction on the gel mobility shift analyses using excess amounts of the nuclear extract.

Fig. 10

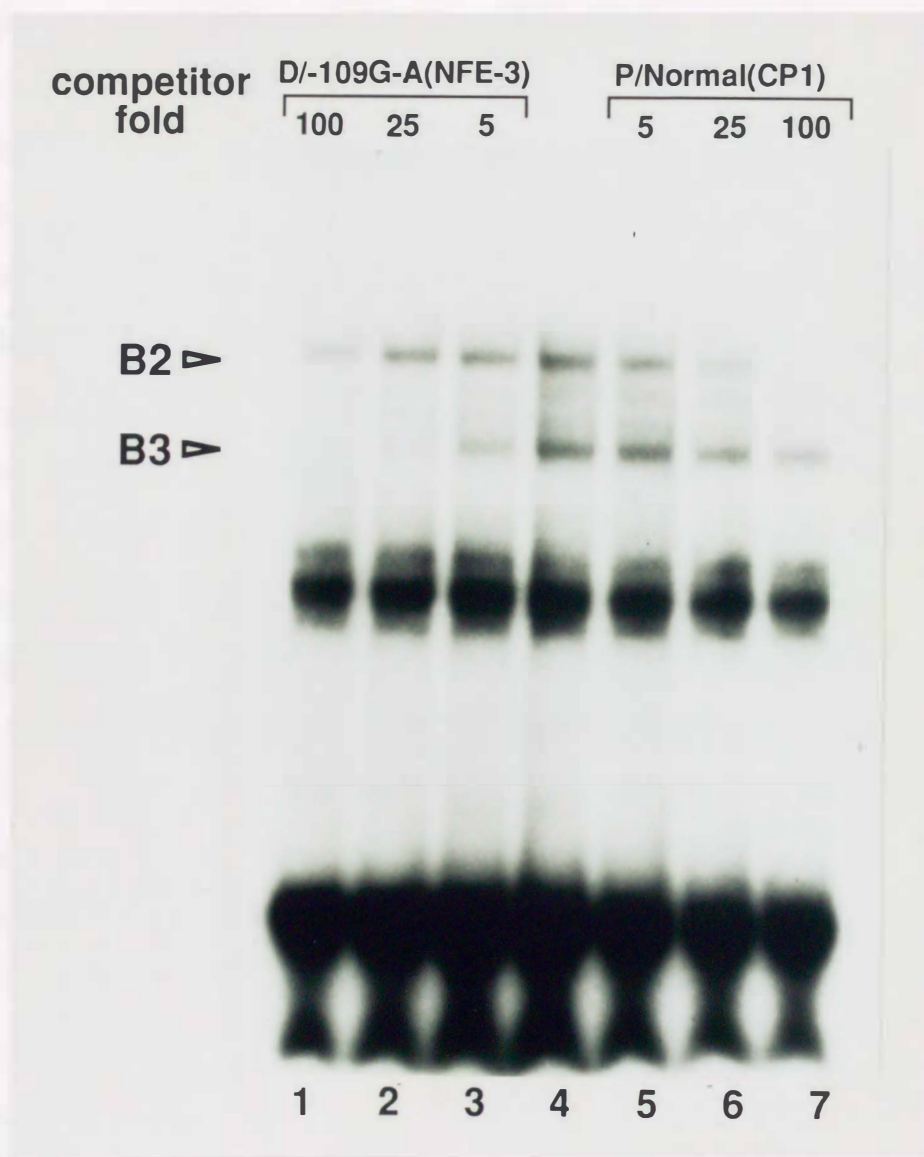


Fig. 10. Competitive binding assay for CP1 and NFE3 using excess amounts of the nuclear extract. The labeled normal distal CCAAT probe was incubated with the K562 extract under the condition described in Materials and Methods except that 30 μ g of the nuclear extract was used. Lanes 1 to 3 and 5 to 7 include the unlabeled -109 G-A or the normal proximal CCAAT competitors (5-100 fold) respectively.

Determination of the binding sites of the trans-acting factors. To localize the binding sites of the factors, DNaseI footprinting experiments were performed. A *BalI-NaeI* fragment containing the two CCAAT boxes of the γ -globin promoter was used as a probe (see materials and methods). Both of the normal and the -114 C-T HPFH mutant sequences were analyzed on footprinting experiments using the K562 nuclear extract. Two regions protected from DNaseI digestion were appeared on the normal probe (Fig. 11). The first footprint spanned sequences from -122 to -105 relative to the cap site and included the distal CCAAT box. The second footprint covered bases from -95 to -78 and included the proximal CCAAT box. On the mutant probe, two footprints were also observed. The protected region around the proximal CCAAT box was just the same with that of the normal probe. This footprint on the proximal CCAAT motif is likely due to the binding of CP1. The footprint covering the -114 mutant distal box was slightly different from that of the normal one. The upstream border was a little diminished and the downstream border was slightly expanded on both strands. This difference is consistent with the results of the gel mobility shift assay (lanes 1 and 3 in Fig. 6). The footprint on the normal distal box is likely to be a binding of CP1 and/or NFE3. That on the -114 mutant distal box is likely to be a binding of the factor "X".

The contact sites of CP1, NFE3 and the factor "X" on the distal CCAAT region were further determined by the method of methylation interference (42). The normal and the -114 C-T distal CCAAT probes used in gel mobility shift assays were partially methylated at G residues by dimethyl sulfate and then used in gel mobility shift experiments with K562 extracts. Bound and free DNA was eluted from the gel, cleaved by piperidine and analyzed on a 20% denaturing sequencing gel. Methylated G residues that interfere with protein binding are under-represented in the G-ladder of the bound DNA. The interference patterns of CP1 (B2), NFE3 (B3) and the factor "X" (B1) are shown in Fig. 12. In lanes F, B1, B2 and B3, about a half nucleotide shifted ladders were observed,

although those were absent in lane G (chemical cleavage at G bases by Maxam-Gilbert method). These are likely to be products incompletely cleaved by piperidine. The two G residues in the complementary (lower) strand of the distal CCAAT box (at position -115 and -114) were essential for the binding to CP1 (left panel in Fig. 12). This is consistent with the previous results showing that CP1 contacts with the G residues in the homologous position of the proximal CCAAT box (39). On the lower strand, obvious interference of NFE3 binding was not observed (left panel). Binding of the factor "X" was strongly interfered by a methylation of a G residue at position -115 in the complementary strand of the CTAAT motif (middle panel). The G residues at position -117 and -109 in the sense strand also interfered the factor "X" weakly (right panel). Methylated G residues that interfere with binding of the factors are summarized in the bottom of Fig. 12.

Fig. 11

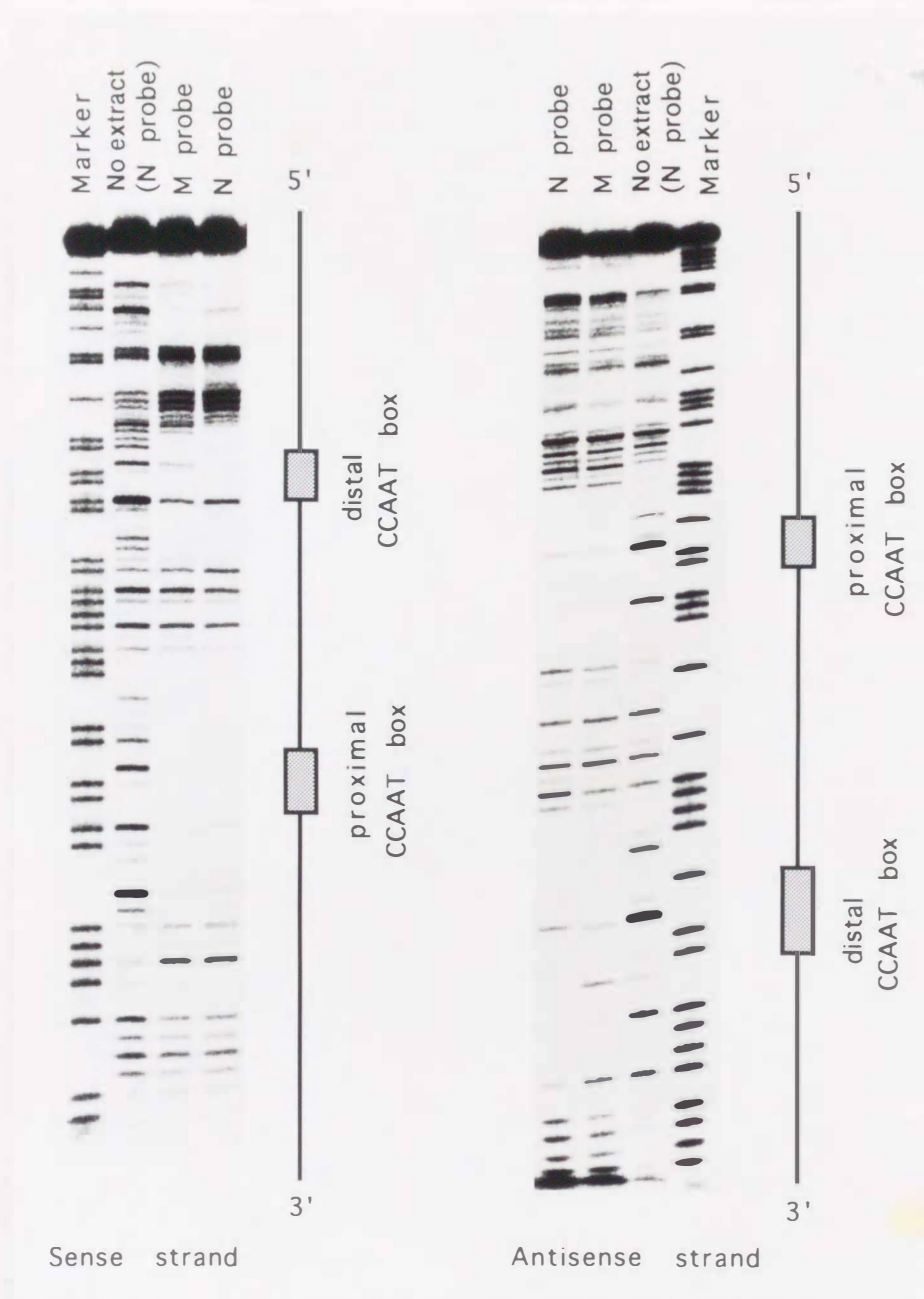
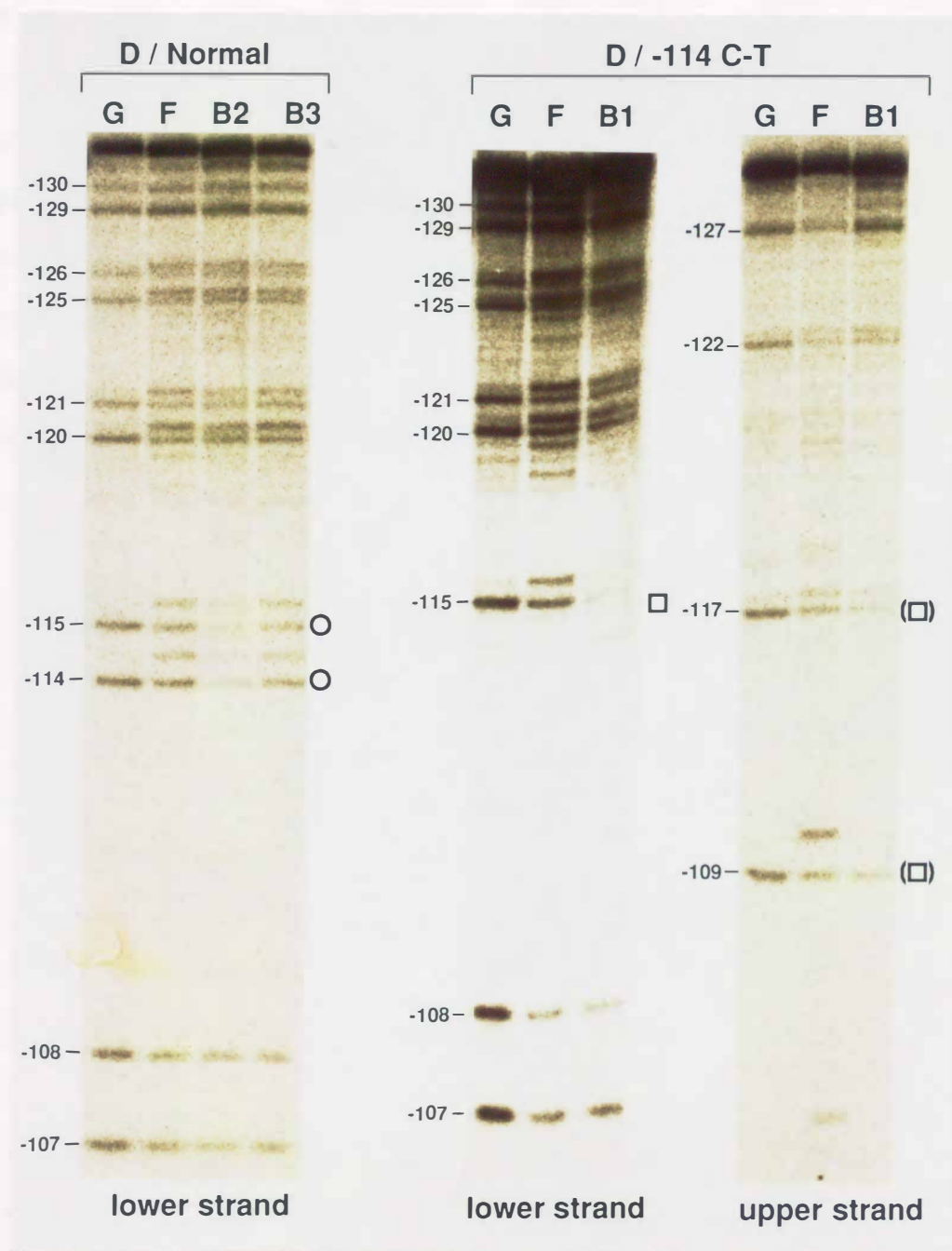


Fig. 11 DNaseI footprinting of the *GyBallI-NaeI* fragment containing the two CCAAT boxes in the K562 nuclear extract. The normal (N probe) and the -114 mutant (M probe) DNA probes were bound to the K562 nuclear extract, digested with DNaseI and analyzed on an 8% sequencing gel (lanes labeled "N probe" or "M probe" at the top). The normal probes digested with DNaseI without the nuclear extract or chemically cleaved at G and A residues were also analyzed for a references (lanes labeled "No extract" and "Marker" respectively).

Fig. 12



(□) (□)
 GCCAGCCTTGCCTTGACCAATAGCCTTGAC
 CGGTCGGAACGGAAGTGGTTATCGGAAGT
 (□) (□)
 (□) (□)

Fig. 12. Methylation interference analysis of CP1, NFE3 and the factor "X" to the distal CCAAT box region. The normal distal CCAAT and the -114 C-T mutant oligonucleotide (30 bp) used in gel mobility shift assays were end labeled in the coding or noncoding strand and utilized for methylation interference analysis. Lanes B1, B2, B3 and F (unbound DNA) show analysis of piperidine cleavage products of DNA recovered from the respective bands from the gel mobility shift assay. Lane G represents the ladders obtained from chemical cleavage at G bases by Maxam-Gilbert method. G residues whose methylation interferes with B1 ($\square$) or B2 ($\circ$) binding are shown. The distal CCAAT motif is indicated by the underline.

DISCUSSION

A great variety of molecular lesions associated with abnormal expression of the γ -globin genes indicates that multiple sequence elements both 5' and 3' of these genes are involved in their developmental regulation. One of the key elements controlling this process appears to be the duplicated CCAAT region around which three HPFH mutations are clustered.

To verify the role of this region in γ -globin regulation, functional analyses were undertaken using human erythroid cell lines. The HPFH mutant promoters were not upregulated in a embryonic-fetal erythroid environment (K562). This is in agreement with the results of Ulrich et al. (43) and Mantovani et al. (10) in which the -117 G-A and the 13 bp deletion HPFH mutant promoters were not expressed higher level than the wild-type promoter in K562. In contrast, the -117 G-A mutant promoter was overexpressed (1.6 fold) in KU812 representing a fetal-adult erythroid property. Stoeckert et al. (44) had also shown that the A γ -globin gene containing the -117 G-A HPFH mutation was more inducible than a normal one in cytosine arabinoside (Ara-C) treated KMOE cells. KMOE is a human erythroleukemia cell line and has the ability to selectively increase β -globin levels after treatment with Ara-C. Similarly, the -114 C-T and the 13 bp deletion HPFH mutant promoters were downregulated in K562 but were as active as to the normal promoter in KU812. On the other hand disruption of the proximal CCAAT motif by the -87 C-T substitution resulted in diminished strength of the promoter in both cell lines. These results suggest that the proximal CCAAT box may have a positive effect on the γ -globin transcription in both fetus and adult. The regulation of the γ -globin expression by the distal CCAAT box is complicated. The decreased activity of the HPFH mutant promoters in K562 cells suggests that the distal CCAAT box has a positive role in fetus. The equal or rather increased activity in KU812 leads to a supposition that the distal CCAAT box has a negative role in adult. Since KU812 expresses the γ - and β -globin genes and has both fetal and adult properties, the activity of

the mutant promoters in this cell line might reflect a sum of the effects of the mutations in fetal and in adult erythroid cells. A recent study by Stamatoyannopoulos et al. (45) using transgenic mice carrying various γ gene promoter truncations indicates that elements involved in the γ gene silencing exist downstream from -141 relative to the cap site. Such elements may include the distal CCAAT box.

Unfortunately, due to the lack of proper human adult erythroid cell lines, obvious overexpression of the γ -globin gene caused by the HPFH mutations could not be observed. A clue to the functional analyses of the globin regulation comes from experiments using transgenic mice. As reported previously (34, 46, 47), the transgenic mice bearing the $A\gamma/\beta$ -globin construct schematically shown in Fig. 3 expressed the human β -globin gene as the adult mouse globin gene and the human γ -globin gene as the mouse embryonic globin gene. The human γ -to- β switch was observed around 13.5 day in gestation of the transgenic mice. In this system the $G\gamma$ -globin gene associated with the -114 C-T HPFH mutation represented persistence of the γ -globin expression at an equivalent level of human β -globin expression in fetal and adult mice (Fig. 5). It was previously shown that the $A\gamma$ -globin gene associated with the -117 G-A substitution also overrides the repression in adult mice (40).

Because expression of introduced genes depends on the integration loci, it is difficult to analyze the effect of modification on the introduced gene construct. To overcome position-dependent expression of the globin gene in transgenic mice, the globin gene constructs with the locus control region (LCR) were used in several studies (13, 32, 48-53). Although the LCR conferred high-level position independent erythroid-specific expression, the γ - or β -globin gene attached individually to the LCR was expressed throughout mouse development (13, 48, 49, 53). The LCR-linked gene with heterologous promoters such as that of the thymidine kinase gene, was also expressed in an erythroid-specific manner (31-33), indicating that the effect is not promoter specific. These observations suggest that each globin gene should escape the effect of the LCR

at specific stages of development to be expressed in a developmentally regulated manner. Recently it was revealed that the relative order of the globin genes and distances from the LCR are critical for temporally-specific interaction between the LCR and the globin genes (40, 54). Therefore, the globin gene constructs, whose expression was strictly regulated in transgenic mice, were mostly large (40, 48, 49, 52) and its manipulations were laborious. In addition, it is difficult to obtain adult lines that pass on the transgenes because of severe globin chain imbalance due to overexpression of the exogenous gene driven by the LCR (48, 50). The constructs used in this study contain two genes. The one gene can be considered as a reference for the another and both genes are regulated individually without the LCR through mouse development. This transgenic mouse system enables us to analyze the expression of the test gene by regarding the expression of the β -globin gene linked to it as the erythroid-specific reference gene.

To elucidate the molecular mechanism underlying the persistent expression of the γ -globin genes associated with the HPFH mutations, interactions of the mutant promoters with nuclear factors were analyzed. At least four different nuclear proteins had been known to bind to the γ -globin promoter CCAAT box region in vitro (4, 9, 10, 39, 55, 56). Among them one is the vertebrate homologue of the sea-urchin CCAAT displacement protein (CDP) (57) which binds to the entire duplicated CCAAT region. CP1 (36, 37), a potential activator of the γ -globin promoter, is able to bind to the CCAAT motifs. GATA1 (58), a erythroid specific factor, binds to the degraded GATA motif that exist in the middle of the two CCAAT boxes. NFE3, originally reported as a erythroid specific factor (9), binds to the proximity of the distal CCAAT box. Previous studies had demonstrated that effects of the HPFH mutations in the vicinity of the distal CCAAT box (-117 G-A, -114 C-T, 13 bp del) on binding to CDP, CP1 and GATA1 are various (9, 10, 39). For instance, binding of CP1 to the distal CCAAT box is slightly increased by the -117 mutation but reduced by the -114 and the 13 bp deletion mutations. In contrast, the -117 and

the 13 bp deletion mutations commonly reduce the affinity to NFE3. In gel mobility shift assay, the band B3, deduced to be the NFE3 complex, appeared to have a defect on binding to the -114 as well as the -117 and 13 bp deletion HPFH mutant distal CCAAT regions. This common effect of these mutations can apply to the common mechanism of the elevated expression in the individuals with these mutations. Difference of the sequence specificity of CP1 and NFE3 was confirmed by gel mobility shift analysis and methylation interference assay. However, simultaneous binding of CP1 and NFE3 was not observed on gel mobility shift analysis under a nuclear extract excess condition. Interactions of these factors and the distal CCAAT region may be mutually exclusive. NFE3 therefore, could act as suppressor by inhibiting interaction of CP1 with the distal CCAAT box in adulthood. Mantovani et al. (9) found that NFE3 is absent in extracts from human nonerythroid blood cell lines; Molt4, U937 and Raji cells. However, the obvious B3 (probably NFE3) complex was observed in human nonerythroid cell lines, HeLa and JEG3. I can not ruled out a possibility that NFE3 described by Mantovani et al. is a different factor from B3 represented in this study, however, the binding profile of B3 to the probes on gel mobility shift assays strongly suggest the identity of B3 and NFE3. Further characterization of NFE3 is required to address this possibility.

The nuclear factor "X" (B1), found in both erythroid and non-erythroid cells, appeared to bind effectively to the -114 C-T HPFH mutant distal CCAAT box. This factor is likely to recognize the "CTAAT" motif and can also bind to the -87 C-T mutant probe in which the proximal CCAAT motif is altered to CTAAT. The factor "X" may be an activator and accidental binding of this factor to the -114 HPFH mutant distal CCAAT box could cause to activate the γ -globin gene in adults of the individuals with this mutation.

Fig. 13 represents the hypothetical mechanisms underlying overexpression of the γ -globin gene associated with the -114 C-T HPFH mutation in adulthood. In a normal situation, NFE3 binds to the distal CCAAT box and inhibits CP1 mediated upregulation (middle panel). In the HPFH condition, however, the -

114 C-T mutation eliminates the binding of NFE3 as well as CP1. The nuclear factor "X" binds to this mutant distal CCAAT motif, "CTAAT", and activate the γ gene (lower panel). Alternatively, NFE3 may have effects on interaction of upstream and/or downstream regulatory regions with trans acting factors by mediating protein-protein interactions or by altering DNA topology to repress the γ -globin gene. Elimination of NFE3 binding by the HPFH mutations may be sufficient to override the γ -globin suppression in adulthood.

Fig. 13

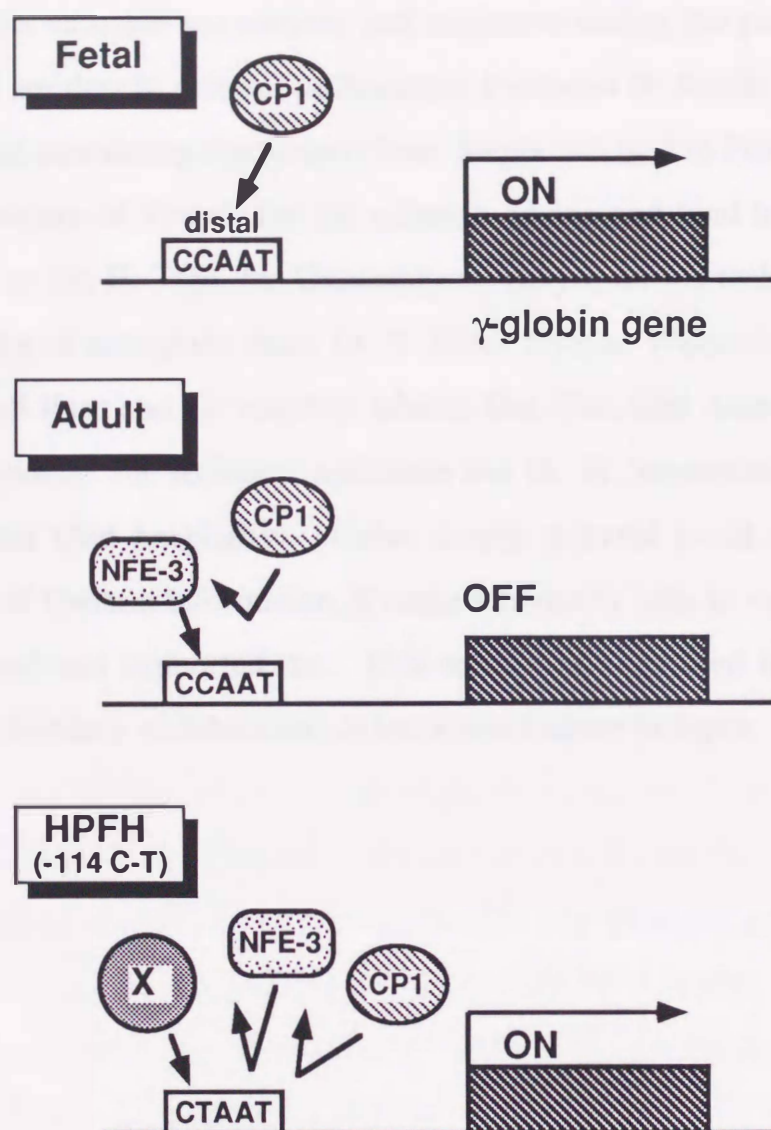


Fig. 13. Schematic representation of the hypothetical mechanisms involving the loss of NFE3 binding to the distal CCAAT motif of the γ -globin gene in overexpression of the gene in HPFH. Binding of the factor "X" to the -114 mutant distal "CTAAT" motif is also involved.

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