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An erythroid specific nuclear factor binding to the proximal CACCC box of the β-globin gene promoter

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ABSTRACT

We have used the gel retardation and DNAase I assays to investigate the binding of nuclear proteins to the human ß-globin promoter. Upon incubation with ß-globin promoter fragments containing the duplicated CACCC boxes, nuclear proteins from human erythroid cells generate complexes yielding four retarded bands in acrylamide gels; the three slowest bands are common to both erythroid and non erythroid cells. The fast band is present only in K562 erythroleukemic cells induced to differentiation and hemoglobin accumulation and in fetal and adult erythroblasts, but absent in uninduced K562 cells. Binding occurs on a short DNA region including the proximal CACCC box, and is not significantly competed by excess γ-globin fragments containing the CACCC box; the CACCC box appears to be essential for this binding, as shown by the failure of a fragment containing a natural ß-thalassemic mutation (-87, C ►G) to bind significantly to nuclear factors. These data suggest that the erythroid specific CACCC binding factor might play a role in the developmental activation of ß-globin transcription.

INTRODUCTION

The human ß- and γ-globin genes are differentially expressed during development; the ß-globin gene is transcribed at a low level (5-10% relative to total non α-globin RNA) already during fetal life (starting at the 5th week) and at the maximal level after birth, while the γ-globin genes are expressed almost exclusively during the embryonic and fetal periods. This transcriptional selectivity has been reproduced, in part, using cloned genes (or their fragments) transfected into cultured erythroid cells or inserted into the germ line of transgenic mice (1-21).

In addition, in vitro mutagenesis of the (murine) ß-globin promoter has precisely defined sequences (TATA, CCAAT and CACCC boxes) controlling transcription in non-erythroid cells (in the presence of an enhancer) as well as in erythroid cells (5,22). Some of these sequences correlate with those defined on the basis of the existence of natural ß-globin mutations (thalassemias), severely affecting ß-globin gene transcription (23-25).

In spite of this large body of information, little is known of the sequences determining erythroid- and developmentally-specific regulation, and of their interaction with controlling factors acting in trans. This in part stems from

the difficulties involved in transfecting with cloned genes the proper cells, human erythroblasts.
As an alternative approach to defining potentially important regulatory sequences, we have examined the in vitro binding to the ß-globin promoter of nuclear proteins from a variety of erythroid and non-erythroid human cells. Here we show that an erythroid specific factor binds to the normal, but not to a mutated, ß-globin CACCC box; this factor is absent in erythroid cells (K562) not producing ß-globin, but can be induced by agents causing the differentiation of these cells, and is additionally present in human fetal and adult erythroblasts properly synthesizing ß-globin.

MATERIAL AND METHODS

Cells

Human cell lines used were the following: K562 (erythroleukemia cells synthesizing embryonic and fetal hemoglobins), Molt 4 (T-lymphoma cells), U937 and THP-1 (promonocytic leukemia), Raji (EBV-positive B-lymphoma cells) and HeLa (uterine carcinoma cells). K562 were induced to differentiation by addition of 60 μM bovine hemin for various times; U937 and THP-1 cells were induced to differentiation to macrophages by addition of 12-O-Tetradecanoylphorbol-13-acetate (TPA) at a concentration of 50 ng/ml.
Fetal liver erythroblasts (approximately 95% pure) were obtained (26) from livers of fetuses (at the 16-18th week of gestation) legally aborted for social or medical reasons. Informed consent was obtained from mothers.
Adult marrow erythroblast were obtained from the bone marrow (BM) of patients with autoimmune haemolytic anaemia, whose smear revealed that more than 50-60% mononuclear cells were erythroblasts at different stages of differentiation. The BM aspirate was layered onto a 1077 Ficoll-Hypaque gradient. Cells at the interfacies were recovered, washed three times with sterile phosphate buffered saline (PBS) and incubated with HLe 1 monoclonal antibody (MoAb, Becton Dickinson, Mountain View, California, cat. N. 7460) which detects the CD45 antigen expressed on the surface of most lymphohaemopoietic cells with the notable exception of erythroid precursors. BM mononuclear cells resuspended in sterile PBS at the concentration of 30 x 10^6 cells/ml were incubated with HLe 1 purified ascitic protein (1 mg/ml at 1:1000 final dilution following dialysis and filtration) for 45'. at room temperature. After three further washings with PBS-BSA (Bovine Serum Albumin) 1%, the CD45+ cells were revealed by means of magnetic beads coated with Goat anti-mouse Ig (Dynabeads M-450, Dynal, Oslo, cat. N. 11006) and removed with the related magnetic particle concentrator (Dynal, Oslo, cat. N. 12002). The proportion of erythroblasts in the CD45-fraction was assessed morphologically on cytospins and proved to be 90%.

DNA fragments

A HinfI-NcoI fragment of the ß-globin promoter (from -280 to +49) was subcloned into the pGEM plasmid, with Hind III linkers; smaller fragments were generated by digestion with the indicated enzymes, and 5'-labelled with ^{32}P-ATP and T-4 Polynucleotide Kinase.

Nuclear extracts

Nuclear extracts were prepared exactly according to ref. 27.

Electrophoretic mobility assay

The labelled fragments (0.1-0.5 ng), were incubated (28) with nuclear extracts (5 μg of protein unless otherwise indicated) from various cell types, in the presence of optimal amounts (6 μg in a 20 μl reaction) of poly (dI-dC), electrophoresed in 50 mM Tris borate pH8.2 and autoradiographed (28).

DNAse I foot-printing

DNA fragments were 5' end labelled with polynucleotide kinase and ^{32}P-ATP and secondarily cut with the appropriate restriction enzyme to leave a single labelled 5' end. Fragments were incubated with nuclear extracts for 30' at 25°C; DNAse I (2 μg/ml of reaction mixture) was added, and the samples incubated for 2' at 20°C; reactions were stopped with 5mM EDTA, the incubation mixture was electrophoresed, tha bands located by autoradiography, the fragments eluted from the excised bands, phenol extracted, ethanol precipitated and analyzed on sequencing gels.

DMS interference (29)

A RsaI-BstNI fragment, 5'-end labelled at the BstNI site, was partially methylated with DMS (20°C, 11', 1 μl DMS/20 μl reaction); after stopping the reaction with 200 mM Tris Acetate, 0.2 M ß-mercaptoethanol, 0.2 mM EDTA, the fragment 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.

RESULTS

Tissue specific and ubiquitous nuclear factors binding to the ß-globin promoter.

To investigate the binding of nuclear proteins to the ß-globin promoter, the gel shift assay (28) was used. Using the full Hind III fragment of fig.1 (data not shown) or smaller fragments derived from it, a number of common bands are visible with all extracts tested (from erythroid and non-erythroid cells) (fig.1). However, an additional fast band (number 4, arrow) is clearly visible with extracts from induced K562 or fetal liver cells, but not from uninduced K562 or non erythroid cells (Molt 4); a slight, but reproducible difference in mobility between band 4 generated with K562 or fetal liver erythroblasts, respectively, is consistently observed; the reasons for the difference have not yet been investigated. The location of the protein binding site responsible for this band can be identified on the basis of the observation that the band appears with both RsaI-Hind III and DdeI-Hind III fragments (from -127 and -113 respectively to +49) (fig.1a and b) and with Hind III-BstNI fragment (-280 to -64) (fig.1c), suggesting region -113 to -64 as the relevant site; this is indeed the case (fig.1d). With this fragment, three common bands (1-3) are visible with all extracts, although their relative amounts are very variable between different cell types.

Competition experiments indicate that the tissue specific protein binds much more efficiently to the ß-than to the γ-globin promoter.

To demonstrate the specificity of binding to the ß-globin promoter, progressively increasing amounts of unlabelled Hind III-BstNI fragment were

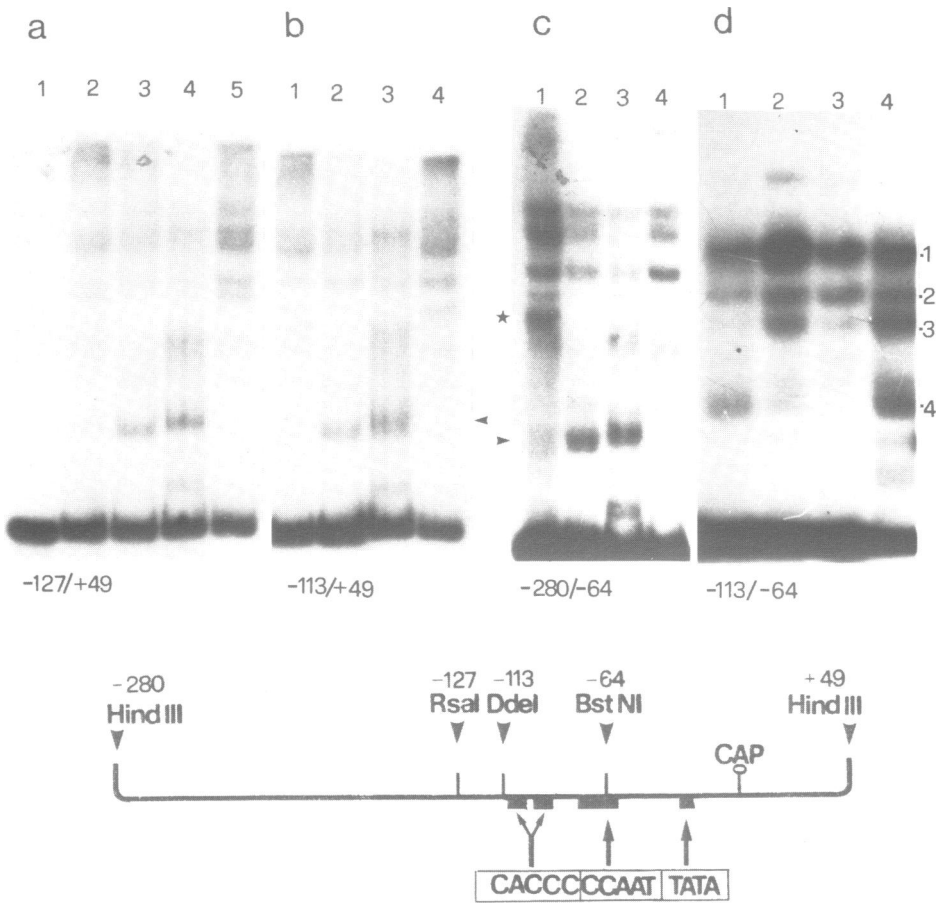

Figure 1.
DNA-protein complexes formed in vitro with the ß-globin promoter.
a: RsaI-Hind III fragment. Lanes: 1: no extract; 2: uninduced K562, 3: hemin-induced K562; 4: purified liver erythroblasts; 5: Molt 4.
b: DdeI-Hind III fragment. Lanes: 1: uninduced K562; 2: hemin-induced K562; 3: purified fetal liver erythroblasts; 4: Molt 4.
c: Hind III-BstNI fragment. Lanes: 1: uninduced K562; 2: hemin-induced K562; 3: purified fetal liver erythroblasts; 4: Molt 4.
d: DdeI-BstNI fragment. Lanes: 1: hemin-induced K562; 2 uninduced K562; 3: Molt 4; 4: purified fetal liver erythroblasts.
Asterisk indicates a complex that is formed with uninduced K562 extracts and is absent in fetal liver erythroblasts. It has been located on an upstream fragment and will be described in detail elsewhere.

added to the same labelled fragment in the presence of induced K562 cells extract (fig.2); all four bands are efficiently competed already at a 10-30 fold excess of competitor. On the contrary, a γ-globin promoter fragment (-299 to -53 relative to the CAP site), containing sequences homologous to the ß-globin promoter binding site (CACCC and CCAAT boxes) is unable, at the same molar excess, to compete significantly band 4; it should be noticed that the ubiquitous bands 1-3 are already greatly competed at a 10-30 fold excess.

Footprint and DMS interference experiments show protection of the proximal CACCC box of the ß-globin promoter.

The DdeI-BstNI fragment contains both the CACCC and CCAAT boxes, which are well known trascription activating elements (22). To investigate whether any

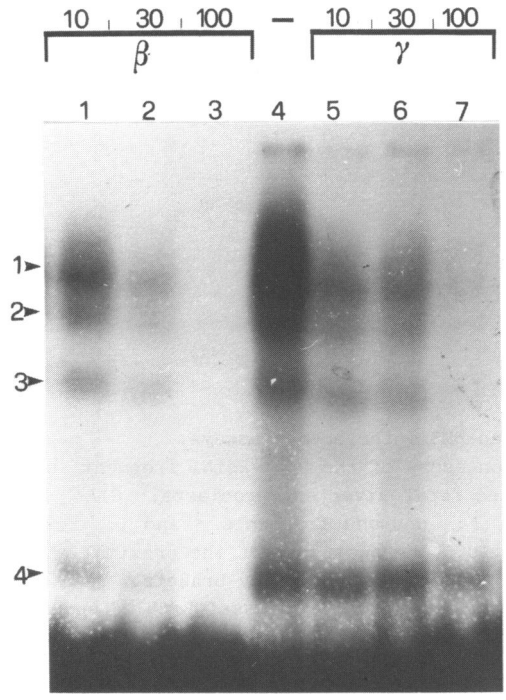

Figure 2.

Competition experiments using hemin induced K562 nuclear extract and labelled Hind III-BstNI fragment with homologous and non-homologous unlabelled competitors.

Lanes: 1-3: 10,30,100 fold molar excess of unlabelled ß-globin HindIII-BstNI fragment; 4: no specific competitor; 5-7: 10, 30, 100 fold molar excess of γ-globin promoter fragment containing CACCC and CCAAT boxes (-299 to -53 relative to the CAP site). Bands are labelled 1-4 as in fig. 1c, d.

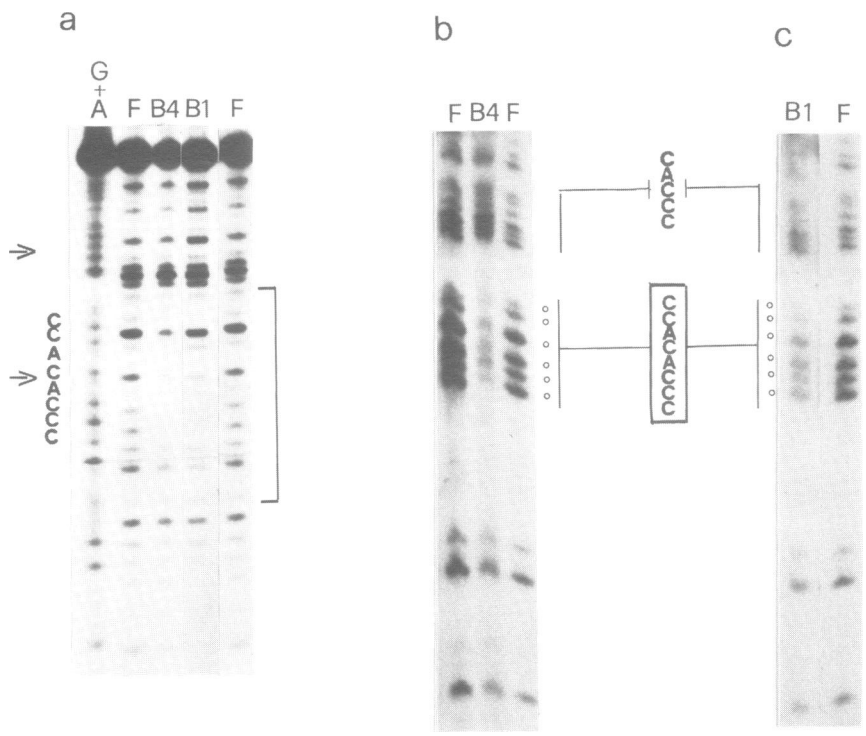

Figure 3.
DNAseI protection and DMS interference assays.
a: DNAse I protection assay of the DdeI-BstNI fragment incubated with nuclear extract from purified fetal liver erythroblasts. G+A: chemical degradation ladder of G and A; F: unbound fragments 4 and 1: retarded bands 4 and 1 respectively of Fig.1d. The positions of the proximal and distal CACCC boxes are indicated by arrows; the footprint by brakets.
b,c: DMS interference analysis of the RsaI-BstNI fragment. b: F: unbound fragment; B4: band B4. c: F: unbound fragment; B1: band B1.
Protected guanines are indicated by open circles.

of these elements is involved in the binding, bands 1 and 4 were footprinted with DNaseI (30). After DNaseI treatment, the incubation mixture was run on acrylamide gels, individual bands were recovered, denatured and further analysed on sequencing gels. With both bands protection was centered on the proximal CACCC box, extending approximately from nucleotide -95 to nucleotide -82 (fig.3a). The faint bands 2 and 3 gave essentially the same result (data not shown). To better define the nucleotides involved in the binding, DMS interference experiments (29) were performed. A RsaI-BstNI fragment (extending from -127 to -64), 5' labelled at the BstNI site (non coding strand), was partially methylated with DMS before the binding reaction. The bound and free fragments were separated and the purified bands analyzed,

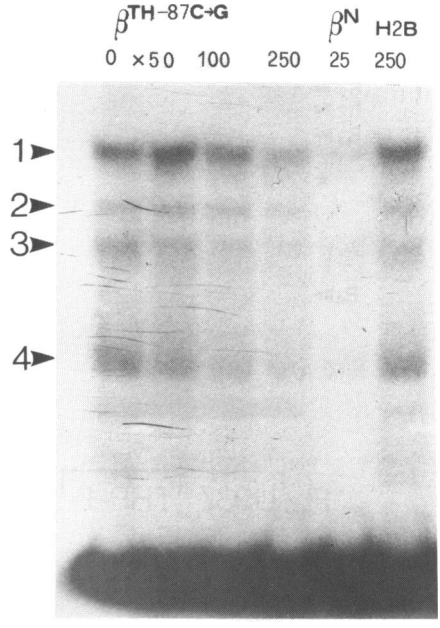

Figure 4.
Competition experiments using hemin-induced K562 extracts and 5'-labelled DdeI-BstNI fragment with unlabelled normal and ß-thalassemic (C ► G substitution, position -87) DdeI-BstNI synthetic oligonucleotides.
The relative molar excess of unlabelled competitor DNA is indicated for each lane. Competitions with a normal DdeI-BstNI fragment and an unrelated fragment H2B (a synthetic oligonucleotide containing sequences from histone H2B gene promoter) were included as controls.

after piperidine treatment, on sequencing gels. Fig.3b, c shows that methylation of the guanines at positions -86, -87, -88, -90, -92 and -93 strongly interferes with the ability of the fragment to bind to nuclear factors generating bands 1 and 4 (similar results were also obtained with bands B2 and B3, not shown). Interestingly, the guanines at positions -101, -102, -103, -105, -107 and -108 corresponding to the distal CACCC box, show similar intensities of reaction with both the free and the bound fragments, indicating that these nucleotides are not detectably involved in binding. This result is in agreement with the lack of DNAse I protection of the distal CACCC box shown in fig.3a.
Identical results were obtained with band 4 generated with either induced K562 cells or fetal liver erythroblasts (not shown).
A naturally occurring mutation in the CACCC box severely affects nuclear protein binding.
Some inherited defects of ß-globin gene expression are due to single point mutations in the CACCC box (25); we therefore asked whether any correlation

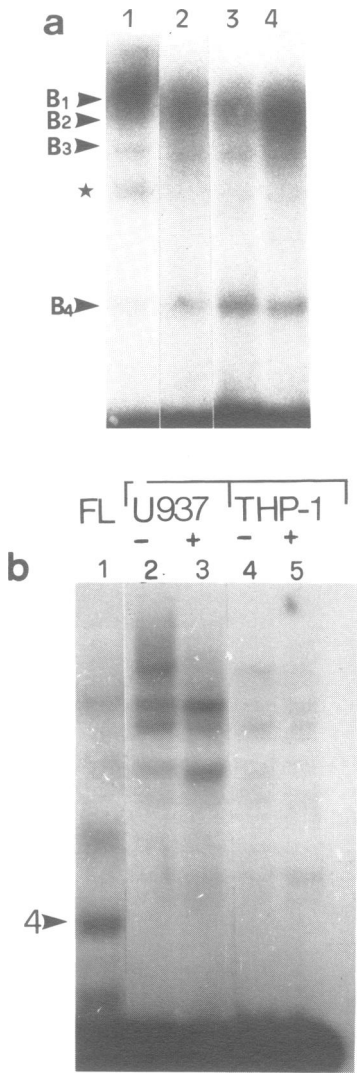

Figure 5.
Kinetics of complex 4 induction by hemin in K562 cells (a); binding patterns in other inducible cell lines (b).
a: lanes: 1: uninduced exponentially growing K562 cells; 2-3: hemin-induced K562 cells, 2 hours and 24 hours, after hemin addition. Benzidine positivity was greater than 90% at day 4. 4: 1-ß-D-Arabinofuranosylcytosine-induced K562 cells, 4 days after induction. Asterisk as in Figure 1 c.
b: lanes: 1: purified fetal liver erythroblasts; 2: uninduced U937 cells; 3: TPA-induced U937 cell; 4: uninduced THP-1 cells; 5: TPA-induced TPH-1 cells. The fragment used in these experiments was Hind III-BstNI.

existed between the occurrence of mutation in the CACCC box and defective binding of any of the factors generating bands 1-4. A synthetic double stranded oligonucleotide exactly corresponding to the DdeI-BstNI fragment, but carrying a C ►G substitution at position -87, was synthesized; this oligonucleotide generates extremely weak bands with either erythroid or non erythroid nuclear extracts (less than 5% relative to the normal fragment) (data not shown). To provide a better estimate of the relative affinities of the normal and mutated fragments, we performed competition experiments. While the normal unlabelled DdeI-BstNI fragment almost completely competes all four bands at a 25-fold molar excess, the mutated oligonucleotide gives about 50% competition at a 250-fold molar excess (fig.4). To ascertain that no aspecific competition occurs at high molar excesses, an unrelated oligonucleotide of a similar size (a fragment from the Histone H2B gene containing an "octamer" (32) sequence) was also used, showing no effect.

The protein generating band 4 is rapidly induced in K562 cells following erythroid differentiation and is not present in other hematopoietic cell lines.

Fig.5a shows that band 4 is already visible within two hours following hemin-induction of differentiation in K562 cells, and increases to a high level within 24 hours. In addition, the appearance of the new band is not strictly dependent on the nature of the chemical inducer, as the same band is

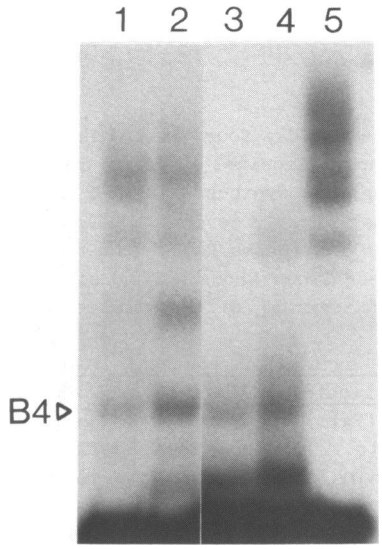

Figure 6.
DNA-protein complexes formed with adult erythroblasts extract.
Lanes: 1: hemin-induced K562, 3 μgr of protein; 2: fetal liver erythroblasts, 3 μgs of protein; 3 and 4: adult erythroblasts, 0.5 and 1 μgr of protein; 5: Raji, 3 μgs of protein. Fragment used was HindIII-BstNI.

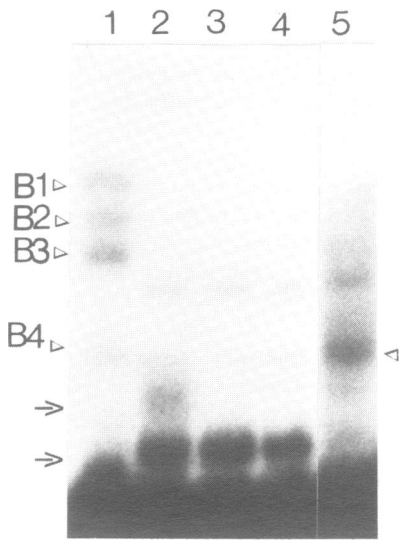

Figure 7.
Proteinase K digestion of uninduced K562 extract.
Lanes: 1: no proteinase K; 2-4: 1, 10, 100 ngr of proteinase K, 10' at 37°C; 5:induced K562, untreated. Arrows show shifted bands resulting froms digestion. Fragment used was RsaI-BstNI.

present at similar levels (at day four) following treatment with a cytostatic drug (1-ß-D-Arabinofuranosylcitosine, Ara-C) capable of inducing terminal differentiation and hemoglobin synthesis in K562 cells (33).
In addition to Molt 4 (see fig.1), several non erythroid cell lines were also examined (HeLa, the B-cell lymphoma Raji, the monocytic leukemia lines U937 and THP-1). None of these lines shows a band similar in mobility to band 4, even after induction of terminal differentiation with tetradecanoylphorbol acetate (TPA) (fig.5b).
The protein generating band 4 is present in adult human erythroblasts actively synthesizing ß-globin.
We have already shown (fig.1) that band 4 is generated using extracts from fetal liver erythroblasts. Although these cells synthesize ß-globin, the majority (90%) of non-α globin chains is represented by γ-globin. We therefore purified erythroblasts from the bone marrow of adult patients being examined for severe non thalassemic anemias, whose non-α globin synthesis is represented almost exclusively by ß-globin. Nuclear extracts from these cells yield a strong band 4, in addition to weaker bands 1-3 (fig. 6); based on the proportion of nuclear extract used in these binding experiments, we calculate that the protein responsible for band 4 is approximately two-three- fold more abundant in adult erythroblasts than in fetal liver or induced K562 cells.

Control experiments indicate that B4 is not generated by proteolysis acting on B1-B3.

Recent work (29) has shown that treatment of DNA binding proteins with a variety of proteases can release a protease-resistant DNA binding "core" domain in an active form; as band B4 is fastest migrating one, it might represent the product of protease degradation of bands B1-B3. We therefore treated extracts from uninduced or induced K562 cells with various amounts of proteinase K (29); fig.7 (using uninduced K562 cells extract) indicates that this treatment, though generating fast-migrating DNA-binding proteins, does not generate any band comparable to band B4. As the specificity of the exogenous protease used might differ from that of endogenous K562 proteases (if any were present) we preincubated at 37°C K562 extracts (induced and non induced) under variuos ionic conditions before the binding reactions. These experiments (data not shown) indicate a remarkable stability of the binding pattern for at least 30 minutes of preincubation and no generation of band B4 from the slower bands B1-B3. Similarly, mixing experiments between extracts generating and non-generating band B4, yield a band B4 of intermediate intensity, ruling out the possibility that the absence of band B4 in certain extracts results from protease activity. These data indicate that band B4 is unlikely to represent a protease digestion artefact.

DISCUSSION

Previous studies of the transcriptional regulation of the human ß-globin gene gave contrasting results as to the role of the ß-globin promoter. In transgenic mice, a ß-globin promoter containing 614 nucleotides flanking the CAP site is unable to drive transcription of a hybrid ß-globin or ß-globin metallothionein gene (21); in addition, a complete ß-globin gene containing only 48 nucleotides 5' to the CAP site is still transcribed specifically, at a low level, in erythroid cells (11), suggesting that the ß-globin promoter, per se, might be a relatively unimportant element for erythroid specific transcription of the ß-globin gene. In contrast, in mouse erythroleukemia cells the ß-globin promoter used in the present experiments (18) or a slightly longer one (20) is able to drive erythroid- and developmentally-specific expression of the chloramphenicol-acetyl-transferase and neo-resistance genes, respectively. In these cells, the ß-globin promoter can also be significantly activated following hexamethylenbisacetamide-induced cell differentiation (7). The latter results suggest that, in addition to other sequences, possibly represented by the recently described 3' enhancer (19), ß-globin promoter sequences may be involved in the erythroid- and developmentally-specific regulation of ß-globin gene expression. The question then arises whether promoter sequences (TATA, CCAAT and CACCC boxes) previously defined as being important in general for transcription (22) are involved in specific erythroid regulation. We have addressed this problem by looking at nuclear proteins capable of interacting *in vitro* with ß-globin promoter fragments. We show here that a nuclear protein present in erythroid cells, is able to interact with a short DNA region including the proximal CACCC box of the ß-globin promoter (fig.3). This nuclear protein is specific for erythroid cells, being observed in differentiating erythroleukemic K562

cells (but not in uninduced K562 cells), as well as in fresh erythroblasts purified either from human fetal livers or from adult bone marrows.
The binding to the CACCC box region requires the integrity of this element, as shown by the strong effect of a C ► G substitution at position -87; this mutation is known to greatly decrease ß-globin gene transcription in vivo, causing ß-thalassemia (23,24). Moreover, DMS interference experiments (fig.3b, c) show that all the guanines (in the non coding strand) corresponding to the CACCC box are important for protein binding. This is in agreement with the observation that all of the point mutations generated in this element in the mouse ß-globin promoter have a strong effect on transcription (22); in addition, ß-thalassemic mutations may also involve position -88 (25). Interestingly, neither footprinting nor DMS-interference experiments, show significant involvement of the upstream CACCC box in binding; this result indicates that nucleotide differences between the sequences flanking the two CACCC boxes may also significantly affect the ability of this element to bind nuclear proteins. The fact that mutations in the proximal CACCC box have a drastic effect on ß-globin transcription in spite of the existence, few nucleotides upstream, of a similar element, potentially able to compensate for the defective one, may well be explained by our data indicating that the upstream CACCC box is unable to bind significantly to factors B1-B4. This observation is in keeping with the notion that the CACCC box is duplicated in human, rabbit and goat ß-globin genes, but not in mouse and other species.
Although we can demonstrate only little difference in the binding patterns of factors B1-B3 and B4 (fig. 3 and data not shown), we believe B4 to be a genuinely different protein than B1-B3. In fact, B4 is present only in differentiated erythroid cells (induced K562 cells, fetal and adult erythroblasts). In addition, mixing experiments between extracts generating and non-generating band B4 indicate that the latters do not contain protease or other activities capable of preventing the appearance of band 4. On the other hand, protease treatment (fig.7) or preincubation of extracts under different conditions fail to generate band B4 (in extracts not exhibiting that band) or to increase its proportion relative to B1-B3 (in extracts already showing band B4); these data indicate that B4 is unlikely to represent a degradation product of B1-B3. Finally, B4 differs from B1-B3 as to the specificity of binding to different promoters; we have previously shown (ref. 34, fig.4) that a γ-globin fragment containing the CACCC box generates only three bands, comparable to B1-B3 (and due to interaction with the CACCC box, not shown) with all tested extracts, including induced K562 cells; no erythroid-specific band like B4 is seen with this fragment. This observation is confirmed by experiments (fig.2) showing that the same γ-globin fragment competes efficiently with the ß-globin fragment for generating bands B1-B3, but not for generating band B4. The erythroid specificity of the protein generating band 4, its apparent developmental regulation (as indicated by its absence in uninduced K562 cells and presence in fetal and adult erythroblasts), its preference for the ß-globin versus the γ-globin promoter, and its ability to recognize a motif of proven functional significance, suggest that the erythroid-specific nuclear factor generating

band 4 might be involved in the erythroid-specific regulation of the ß-globin promoter; this protein might be either a modified form of the ubiquitous protein(s) generating bands 1-3, or an altogether different factor. It is interesting in this context to recall a similar situation occurring in B-lymphocites; these cells appear to contain both a ubiquitous and a lymphocite-specific nuclear protein (28,30,32,35) capable of binding a conserved "octamer" sequence, present in immunoglobulin as well as in several non immunoglobulin genes. As the "octamer" alone is able to confer lymphocite-specific expression to non immunoglobulin genes, like globin (36) or renin (37), the lymphocite-specific factor may be involved in tissue specific regulation of immunoglobulin genes; a similar model may be proposed and is experimentally testable for ß-globin promoter regulation by the CACCC binding factor. An objection to the role of the erythroid specific factor in ß-globin regulation might be suggested by the fact that the endogenous ß-globin promoter is inactive in induced K562 cells (4), where the factor is present. These data indicate that the factor may not be sufficient, per se, but they do not rule out the possibility that the factor, in conjunction with other proteins, is involved in, or necessary for, ß-globin gene expression. K562 cells are "frozen" at a stage preceding, but close to, the fetal stage when ß-globin synthesis initiates, as indicated by the observation of δ-globin gene transcription in these cells (38), and by a report suggesting that 5'-azacytidine treatment may induce low level ß-globin transcription (39). Other events, like ß-globin gene demethylation (40) and 3' enhancer activation (19) might additionally be required, at more advanced stages, to promote ß-globin gene activity. Moreover, and more importantly, recent experiments (41; Antoniou and Grosveld, quoted in ref. 42) indicate that the lack of activity of the ß-globin promoter in K562 cells may be explained, at least in part, by negative regulation acting on specific sequences of the ß-globin promoter; in addition, induction of K562 cells by hemin is reported to allow expression of a transfected ß-globin promoter (41). These results are fully in keeping with our observation that hemin induces the protein generating band 4 in K562 cells (present paper), and causes the disappearance of two proteins, binding to the ß-globin promoter -253 to -230 and -207 to -128 regions (in preparation). These same two proteins are absent in fetal and adult erythroblasts.

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