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A protein factor binding to an octamer motif in the γ-globin promoter disappears upon induction of differentiation and hemoglobin synthesis in K562 cells

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ABSTRACT

Using the electrophoretic mobility shift assay and the footprinting technique, we studied the binding of nuclear proteins from erythroid and non erythroid human cells to the promoter region of the human γ-globin gene. Two regions (A and B) of the promoter are bound by proteins present in uninduced K562 cells, but not in induced K562 cells nor in fetal liver erytroblasts; a protein binding to region A is also present in a variety of lymphoid and myeloid cells. Region B is centered on an octamer sequence identical to that present in immunoglobulin promoter and enhancers and other eukaryotic promoters; a B region binding protein common to K562 and other cells efficiently binds the octamer containing region of the histone H2B gene, while different B region proteins are more specific for uninduced K562 cells and the γ-globin octamer containing fragment. The possible role of these nuclear proteins in γ-globin gene regulation and/or cell differentiation is discussed.

INTRODUCTION

Gene regulation in animal cells is based on a variety of cis-acting DNA sequences, some common to many genes(1-4), some specific(4-7), which appear to interact with a variety of transacting factors regulating their activity. In a few cases, expression of a gene correlates, at least to some extent, with the presence of tissue-specific factor(s)(4,6) or its induction(5), suggesting that these factors may act as positive regulators. Human erythroleukemic cells K562 represent a useful model in the study of regulation of gene expression; these poorly differentiated cells can be induced to maturation along the erythroid pathway by a variety of chemical inducers, and rapidly accumulate relatively large amounts of embryonic and fetal hemoglobins. The latter phenomenon is largely mediated by transcriptional activation (8), but neither the factors related to cell differentiation nor those influencing globin gene expression, are known. Here we demonstrate the presence in uninduced K562 cells of protein(s) capable of binding to the human γ-globin promoter; hemin-induction of hemoglobin synthesis leads to the rapid disappearance of some of these proteins, generating a binding pattern very similar to that observed in fetal erythroblasts. One of the binding sites in the γ-globin promoter is centered

around an "octamer" motif common to immunoglobulin promoters and enhancers(3,4), histone H2B and U2 small nuclear RNA promoters(3).

MATERIAL AND METHODS

Cells

Human cell lines used were the following: K562 (erythroleukemia cells synthesizing embryonic and fetal hemoglobins), Molt 4 (T-lymphoma cells), U 937 and THP-1 (promonocytic leukemia cells); Raji (EBV-positive B-lymphoma cells) and HeLa (uterine carcinoma cells). K562 cells were induced to differentiation by addition of 60 μM bovine hemin for various times; U 937 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 90% pure) were obtained (9) from livers of fetuses (at the 16-18th week of gestation) legally aborted for social or medical reasons. Informed consent was obtained from the mothers.

DNA fragments

An Alu I fragment of the γ-globin promoter (from -299 to +36) was subcloned with Hind III linkers into the pSVO-plasmid (10); 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 Dignam et al.(11).

Electrophoretic mobility assay

The labelled fragments (0.1-0.5 ng), were incubated with nuclear extracts (5 μg of protein unless otherwise indicated) from various cell types according to Singh et al. (12), 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 (12).

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 min at 20°C; reactions were stopped with 5mM EDTA.
In experiments in which the concentration of the factor was sufficiently high to bind completely the DNA fragment, the incubation mixture was directly phenol extracted, ethanol precipitated, analyzed in denaturing sequencing gels and autoradiographed. In these experiments the DNAse I-protected fragment was compared with the corresponding fragment DNAse I-treated in the absence of nuclear extract. Alternately, after stopping the reaction, the incubation mixture was electrophoresed, the bands located by autoradiography, the fragments eluted from the excised bands, phenol extracted, ethanol precipitated and analyzed on sequencing gels.
Results obtained with either procedure were essentially identical.

RESULTS

To look for protein(s) binding to the γ-globin promoter, we used the electrophoretic mobility shift assay(12).A radiolabelled γ-globin fragment

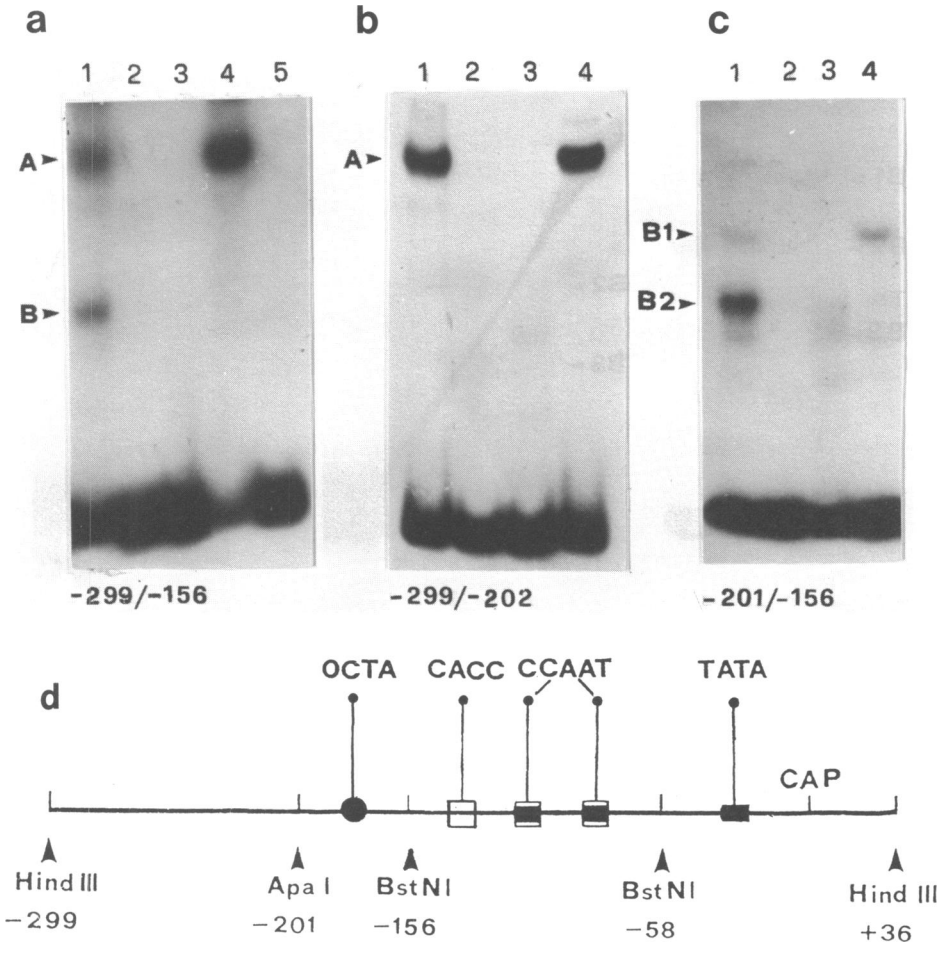

Figure 1.
Binding of nuclear factor(s) to human γ-globin promoter region: electrophoretic DNA binding assays.
a: Hind III-Bst NI; b: Hind III-Apa I; c: Apa I-Bst NI;
Lanes: 1: uninduced K562 nuclear extracts; 2: induced K562;
3: purified fetal liver erythroblasts (18th week of gestation);
4: Molt-4, 5: no extracts. Similar results as with Molt 4 (lane 4) are obtained with THP-1, U 937, Raji and HeLa cells.
d: restriction map of the γ-globin promoter region. The "OCTA", CACC and duplicated CCAAT elements start (5') at position -182, -152, -115, -88 respectively.

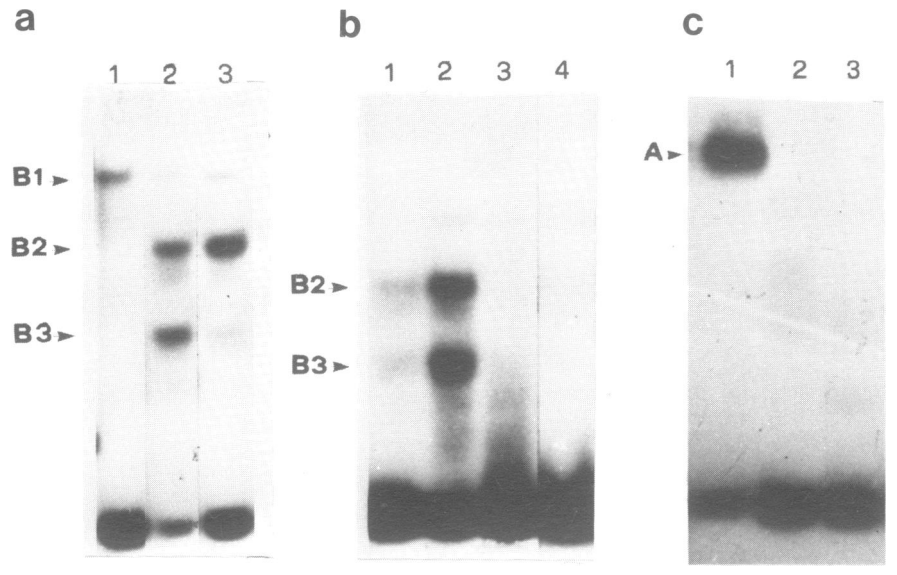

Figure 2.
Effects of growth conditions and cell differentiation on DNA binding-proteins. a: binding of the Apa I-Bst NI fragment. Lane 1: U 937 cells; lane 2: exponentially growing K562 cells ($0.5x10^6$ cells/ml); lane 3: K562 cells grown at high density ($1.5x10^6$ cells/ml);
b: binding of the Apa I-Bst NI fragment. Lane 1: fetal liver erythroblasts: lane 2: exponentially growing K562 cells; lane 3: hemin-induced K562 cells, 2hrs following hemin addition; lane 4: hemin-induced K562 cells, 4 days after induction. Benzidine positivity greater than 90%;
c: binding of the Hind III-Apa I fragment. Lane 1: exponentially growing K562 cells; lanes 2 and 3: hemin-induced K562 cells, 2hrs. and 4 days respectively following hemin addition.

spanning positions -299 to +36 relative to the CAP site was employed in these experiments; this fragment efficiently drives the activity of the CAT (chloramphenicol acetyl transferase) gene (10) in the absence of any enhancer when introduced into K562 cells and fetal liver erythroblasts (13). This fragment was incubated with nuclear extracts from uninduced and induced K562 cells, purified fetal liver erythroblasts, and non erythroid cells (T-lymphoma Molt 4, U 937, THP-1 and HeLa cells).
With all of these extracts, a complex pattern was obtained, consisting of at least three retarded bands (not shown). To better resolve this pattern, smaller fragments were used. Using a Hind III-Bst NI fragment (from -299 to -156), uninduced K562 extracts generate two different bands (A, slow and B2, fast) (fig. 1a); both bands are very faint in induced K562 cells as well as

in fetal liver erythroblasts. However, a slow band, with mobility similar to band A, is generated with extracts from Molt-4 cells (fig.1a), THP-1 and U 937 cells (see below). Cutting the fragment with Apa I, yields two fragments, Hind III-Apa I (-299 to -202) and Apa I-Bst NI (-201 to -156); the Hind III-Apa I fragment binds factor(s) present in uninduced K562 (fig. 1b) and in non-erythroid cells (data for Molt-4 in fig. 1b), while the Apa I-Bst NI fragment generates a major retarded band with extracts from uninduced K562 cells only (fig. 1c); remarkably, the relative mobilities of the two bands generated by the Hind III-Apa I and Apa I-Bst NI fragments are similar to those of the two bands generated with the intact Hind III-Bst NI fragment. A slow band labelled B1 in fig.1c is also present with uninduced K562 cells extracts; a stronger band of similar mobility is seen also with other nuclear extracts tested (fig. 1c), including THP-1, U 937, Raji and HeLa cells (not shown).

The data shown in fig.1c are from K562 cells grown at high densities; we therefore checked for the effect of different growth conditions of uninduced K562 cells to be used for the preparation of nuclear extracts. While K562 cells grown at high densities approaching saturation (1.0-1.5 x 10^6 cells/ml) show a pattern essentially identical to that in fig. 1c, exponentially growing K562 cells (0.4-0.6 x 10^6 cells/ml) show lower amounts of bands B1 and B2, but additionally exhibit a band of faster mobility (B3) (fig. 2a).

The concentration of the protein generating band A of fig. 1b is essentially unmodified in these extracts (not shown). Since we can obtain good induction of K562 cell differentiation and hemoglobinization, in the absence of significant toxicity, only starting with cells growing at low concentrations, the induced K562 binding pattern should be compared with that obtained using extracts from exponentially growing cells. A kinetic analysis of the hemin effect indicates that already within 1-2 hours from induction an almost complete disappearance of bands B2 and B3 can be observed (fig. 2b); the same is true also for band A (fig. 2c).

Taken together, these results suggest that one, or possibly more, nuclear proteins of uninduced K562 cells bind at two sites of the γ-globin promoter; these proteins rapidly disappear following induction of differentiation. Thus, the binding pattern of K562 cells at the onset and at the terminal stages of differentiation (fig. 2b,c) closely resembles that of erythroblasts directly obtained from fetal livers (fig. 1c).

As shown above (fig.1) extracts from non erythroid cells generate a band with mobility similar to A using the Hind III-Apa I fragment. Competition experiments using the unlabelled homologous Hind III-Apa I or non homologous fragments (for example from the ß-globin promoter, nucleotides -113 to -64) indicate that band A is due to a specific protein DNA interaction (fig. 3a). Similarly to what occurs with K562 cells, induction of differentiation of THP-1 and U 937 cells (to macrophages) by 12-O-Tetradecanoylphorbol-13-acetate (TPA) treatment, leads to the disappearance of the nuclear factor generating band A (fig.3b).

It should be noticed that the lack of binding observed with certain extracts cannot be due to protein degradation or other artefacts, as demonstrated by

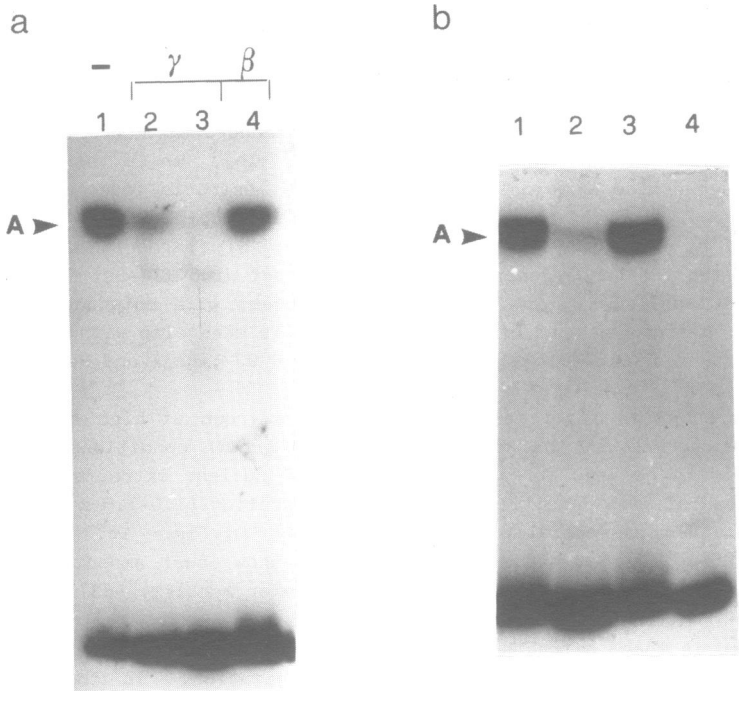

Figure 3.
Competition by homologous and non-homologous DNA fragments for the factor generating band A (a).
0.3 ngs of 5' labelled Hind III-Apa I fragment were mixed in the usual conditions with 0, 3, 12 ngs of the same unlabelled fragment (lanes 1-3), and with 10 ngs of a 50 nucleotide ß-globin fragment (60 fold molar excess) (lane 4) in uninduced K562 extract.
Effect of the induction of differentiation on binding to the Hind III-Apa I fragment (b).
Lane 1: uninduced THP-1 cells (0.5 x 10^6 cells/ml); lane 2: induced THP-1 cells; lane 3: uninduced U 937 cells (0.5 x 10^6 cells/ml); lane 4: induced U U937 cells.

the identical binding patterns obtained with all extracts using a Bst NI-Bst NI fragment (-155 -59) (fig.4). This fragment contains sequences previously known to be generally important for transcription like the duplicated CCATT boxes (1,14) and the CACC box common to both γ-and ß-globin genes (footprint experiments indicate that the major band is due to interaction with protein in the CACC box region; not shown).
To better define the site of interaction of proteins present in uninduced K562 cells (grown at high concentrations) with the γ-globin promoter, DNAse I footprint analysis (15,16) was employed; under these conditions, band B2 only

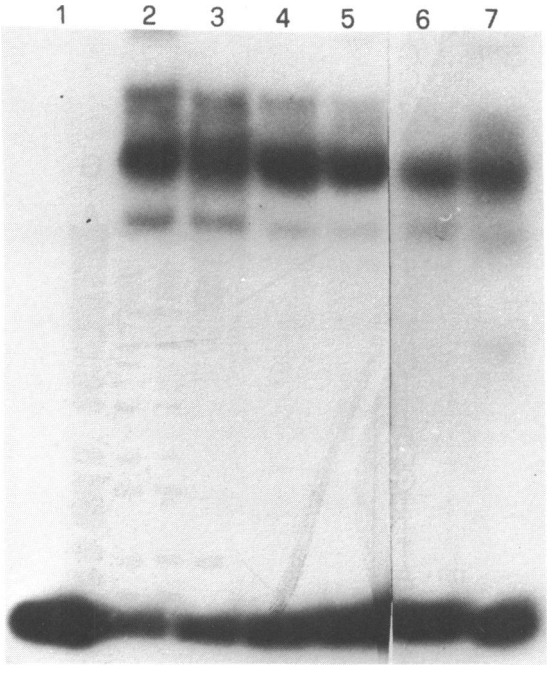

Figure 4.
DNA binding assays with fragment Bst NI-Bst NI.
Lane 1: no extract; lane 2: exponentially growing K562 cells; lane 3: 2 hrs induced K562 cells; lane 4: 4 days induced K 562 cells; lane 5: fetal liver erythroblasts; lane 6: uninduced THP-1 cells; lane 7: induced THP-1 cells.

is generated. The Apa I-Bst NI fragment showed (fig. 5) a protected region between positions -190 and -170; enhanced digestion was also demonstrated immediately upstream from the protected region (position -192). The protected region appears to be centered on an ATTTGCAT octamer, identical to that present in immunoglobulin, histone H2B and U2 snRNA genes (3,12); in our case, as in the V -immunoglobulin and U2 promoters (3,12), the octamer is in reverse orientation relative to the coding strand of the gene.
The presence in non erythroid cells of a slow band of mobility similar to B1 (of K562 cells) when using the Apa I- BstNI fragment suggests that a common motif (possibly the octamer itself) can be recognized by various factors present in different cells; indeed, a footprint of band B1 using K562 and Molt-4 extracts gives very similar protection patterns, both including the octamer. These patterns differ from those obtained with bands B2 (fig. 5) and B3 (fig.6) in that the B1 protected region extends more 3'and less 5' showing only two protected nucleotides upstream from the octamer; this contrasts with the symmetric protection around the octamer observed with both bands B2 and B3 (figs. 5 and 6). To test for the possibility that the octamer

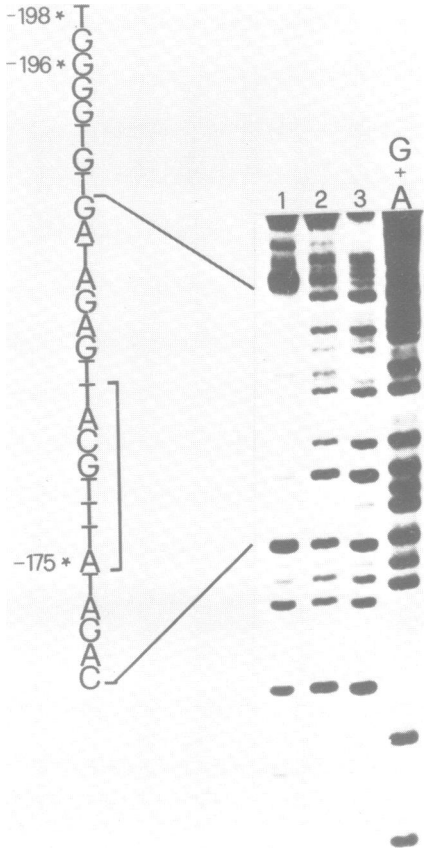

Figure 5.

DNAse I footprinting of factor-DNA complexes. The γ-globin Hind III fragment was 5'-end labelled at the Bst NI site and secondarily digested with Apa I; the purified Apa I-Bst NI fragment (1 ng, 20.000 cpm) was incubated in the absence (lane 3) or presence (7 and 14 μgs of protein) of extracts from uninduced K562 cells (lanes 2 and 1 respectively). At these protein concentrations, 50% and 100% of the fragment is bound, respectively. Following DNAse I digestion (2 μg DNAse/ml of reaction mixture) for 2 min at 20°C, the reactions were stopped with 5 mM EDTA, and the fragments were phenol extracted, ethanol precipitated, analyzed in a 20% denaturing sequencing gel and autoradiographed.

A+G chemical cleavage ladders were coelectrophoresed to map the binding domain. The protected region is shown. Asterisks indicate the positions of mutations correlated with γ-globin gene overexpression in adult life (see text).

A similar result is obtained by comparing band B2, excised from the gel, with the unbound band.

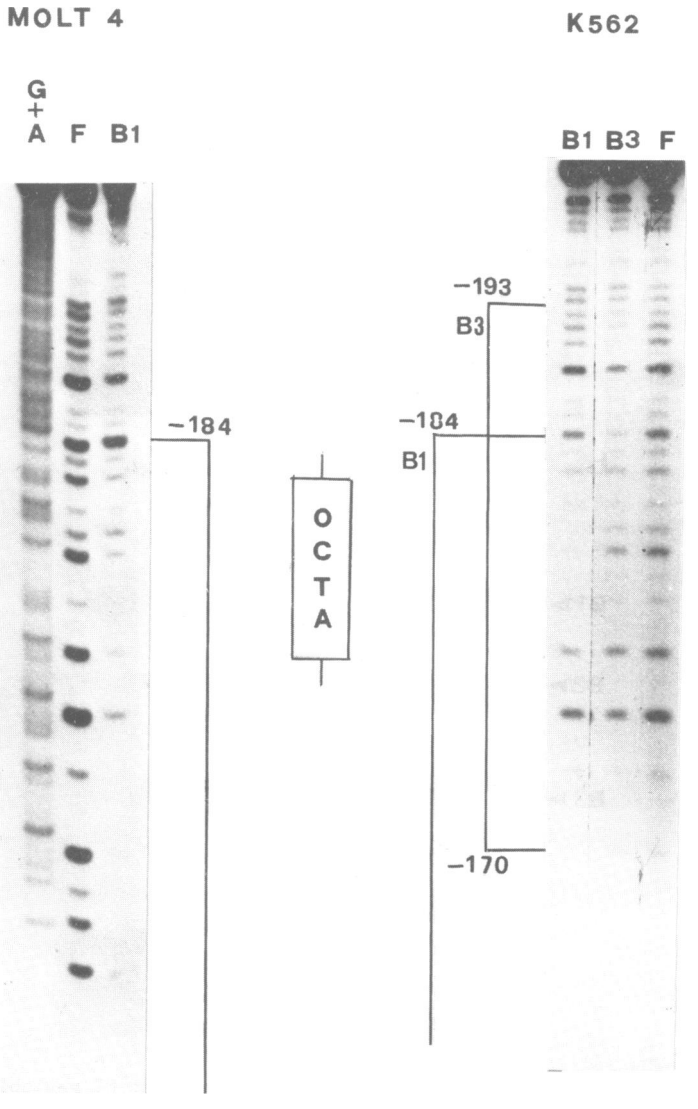

Figure 6.
DNAse I footprinting of band B1 from Molt 4 and K562 cells, and band B3 from uninduced K562 cells.
F: unbound fragment; B1 and B3: gel excised bands B1 and B3. The extent of protection is shown by the vertical bar; the endpoint of protection for both B1 bands is not visible in this gel, and was located at nucleotide - 164. For the sequence, see fig.5.

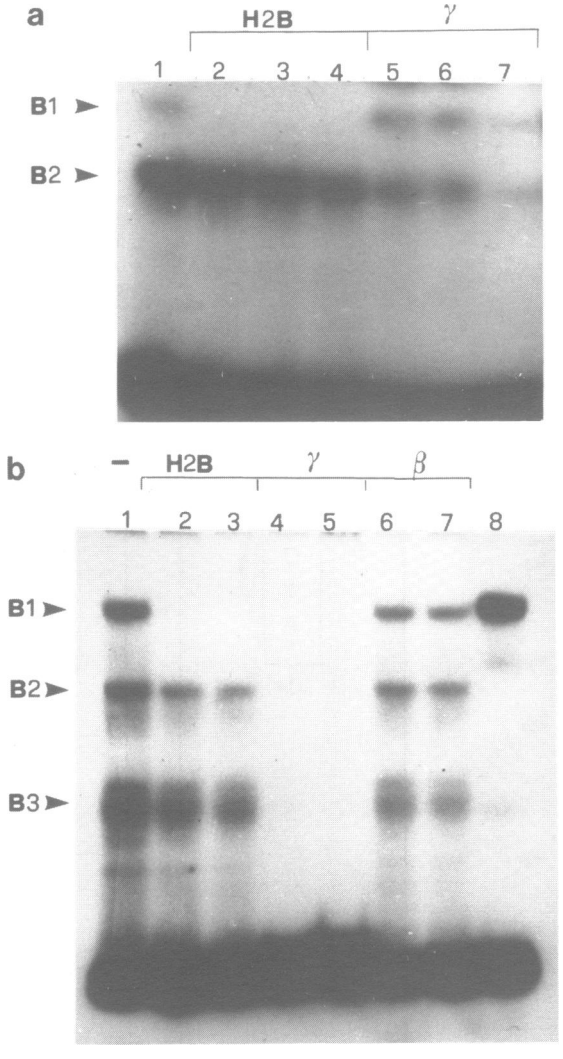

Figure 7.
Competition by the H2B oligonucleotide and the Apa I-Bst NI γ-globin fragment for factors generating bands B1 and B2 (a) and B3 (b).
a: 0.5 ngs of 5' labelled Apa I-Bst NI γ-globin fragment were mixed in the usual conditions with 0, 10, 40 and 100 fold molar excess of unlabelled H2B oligonucleotide or with 5, 10 and 20 fold molar excess of unlabelled Apa I - Bst NI fragment. Nuclear extract was from K562 cells grown at high densities.
b: 0.5 ngs of 5' labelled Apa I-BstNI γ-globin fragment were mixed with 0 (lane 1), or 100-200 fold molar excess of unlabelled H2B oligonucleotide (lanes 2-3), Apa I-BstNI fragment (lanes 4-5) and ß-globin fragment (lanes 6-7). Lane 8 shows the migration of the complex generated by a 5'-labelled H2B oligonucleotide. Extract used was from exponentially growing K562 cells.

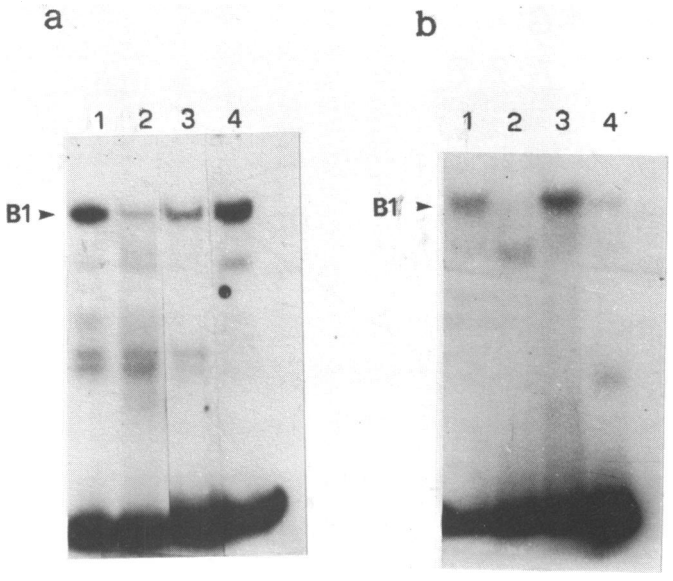

Figure 8.
DNA binding assay with an histone H2B octamer-containing oligonucleotide.
a, lane 1: exponentially growing K562 cells; lane 2: 2 hrs-induced K562 cells; lane 3: 4 days induced K562 cells; lane 4: exponentially growing Raji cells.
b, lane 1: uninduced THP-1 cells; lane 2: induced THP-1 cells; lane 3: uninduced U 937 cells; lane 4: induced U 937 cells.
The H2B oligonucleotide sequence is shown in fig.10.

is a commonly recognized element in bands B1, B2 and B3, a synthetic histone H2B oligonucleotide (45 mer) centered on the octamer sequence was used in binding and competition experiments; this sequence is known to bind a nuclear factor from HeLa cells (3). When an excess of unlabelled H2B oligonucleotide is added to labelled -globin Apa-BstNI fragment, the upper band B1, is completely competed out at a low molar excess (10 fold) of H2B oligonucleotide (fig. 7a, lanes 1 and 2). On the other hand, the intensities of bands B2 and B3 are slightly decreased by the H2B competitor only at the highest molar excesses tested (100-200 fold) (fig. 7a, lane 4 and fig. 7b, lanes 1-2). On the contrary, homologous competition with the Apa I-BstNI γ-promoter fragment eliminates all three bands already at a 20 fold excess (fig. 7a, lane 7 and fig. 7b, lanes 4-5). A 200 fold excess of unrelated competitor (ß-promoter nucleotides -113 to -64) has no significant effect (fig.7, lanes 6-7). Fig. 7, lane 8, also shows that, using K562 cell extracts, the electrophoretic mobility of band B1 obtained with labelled H2B is undistinguishable from that of band B1 obtained with the γ-globin fragment. In addition, all other nuclear extracts tested, generate with the H2B fragment a prominent band of mobility identical to B1 (fig.8).

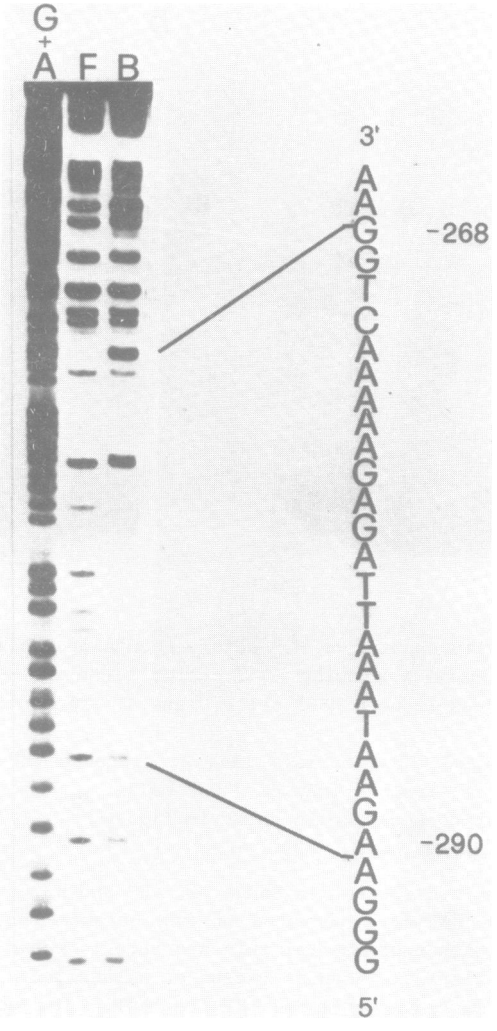

Figure 9.
DNAse I footprinting of the 5'labelled Hind III-Apa I fragment.
F: unbound fragment; B: bound fragment generating band A of figure 1b.

As in the case of bands A (fig. 1b),B2 and B3 (fig. 2), obtained with γ-globin probes, also the band generated with the H2B probe decreases following induction of K562 cells differentiation; however, this decrease is much less pronounced (five fold only) than in the former cases. Interestingly, induction of terminal differentiation of U 937 and THP-1 cells leads to the almost complete disappearance of the H2B generated band B1 (fig. 8b).
Similar binding data as with H2B were also obtained using a Bst NI-BstN1

H2B CODING STRAND -60 -50 -40 -30
gacttcaactcttcaccttATTTGCATaagcgattctatataaaag

γ-GLOBIN NON CODING STRAND -160 -170 -180 -190 -200
gggaccgtttcagacagatATTTGCATtgagatagtgtggggaagg

SV40 ENHANCER NON CODING STRAND -200 -232
cctggttgctgactaattgagatgcatgcTTTGCATacttctgcctgctggggag

Figure 10.
Comparison of octamer-containing histone H2B, γ-globin and SV40 enhancer fragments. Numbers refer to nucleotide positions relative to the CAP sites of H2B and γ-globin promoters; for SV40 enhancer, 1 refers to the first nucleotide of the early transcription unit.

fragment (55 nucleotides long) comprising a modified octamer from the SV40 enhancer; competition against complex B1 of the γ- fragment was also observed, although less efficiently than with the H2B oligonucleotide (data not shown).
The Hind III-Apa I fragment (generating band A of fig.1) also shows a protected region, (bordered and interrupted by several sites of enhanced cutting) (fig. 9), spanning from nucleotide -290 to -268; this region is unusually rich in A. The same region is also protected in uninduced THP-1 cells.

DISCUSSION

Using as a probe a DNA fragment including at least part of the γ-globin promoter, we have demonstrated the existence in nuclei of both erythroid and non-erythroid cells of proteins capable of binding to specific sequences in this promoter. Some of the binding proteins are much more abundant (or possibly exclusively present) in K562 than in non erythroid cells; in addition, significant variation has been observed in the levels of certain binding factors (but not of all factors), in relation to growth conditions and differentiation state of K562 cells.
The data shown in figures 1,2,5,6,7 indicate the existence in K562 cells of proteins capable of binding to a sequence containing an "octamer" identical to that present in several eukaryotic promoters and enhancers (3,17-19). It was previously shown (3,17,18) that two different octamer binding proteins exist, one ubiquitous (NF-A1), the other (NF-A2) restricted to lymphoid cells. Fig.10 indicates that there is no homology, except in the octamer, between the sequence of the H2B (and of the SV40 enhancer) and that of the γ-globin ApaI-BstNI fragment; the competition between these fragments for the factor responsible for band B1 therefore indicates that the octamer may be an element specifically required for the protein-DNA interaction. The slow band B1 observed upon incubation with K562 or other cells extracts of the octamer-containing γ-globin fragment is therefore likely to be due to interaction with the ubiquitous factor; on the contrary, the relatively faster bands B2 and B3 that are only slightly competed by the H2B fragment,

might represent either modified forms of the ubiquitous octamer binding factor or different types of octamer-binding proteins with high affinity for γ-globin sequences flanking the octamer, and specific (or selectively abundant) for uninduced K562 cells.

These data may have various functional implications; the evolutionary conservation of the octamer sequence as an important functional element of several eukaryotic and viral promoters and enhancers (3,17-19) is well known, and it is possible that the observed variation in the level of specific DNA binding factors may be related to the regulation of a class of genes containing the octamer, depending on growth conditions or cell cycle phases. In addition, the rapid disappearance of various proteins, including the octamer binding factor(s) responsible for bands B2 and B3 is, to the best of our knowledge, the earliest molecular event detectable following induction of K562 cell differentiation; this event might therefore lead to the coordinate inactivation during differentiation and erythroid maturation of a set of previously active genes requiring that protein for their expression. It is of interest that a population of maturing erythroblasts from fetal liver show absence of the protein generating band A and low levels of factors responsible for bands B2 and B3, although they maintain normal levels of the CACC binding protein (see figs. 1,2 and 4); thus, the disappearance of factors observed in K562 cells may also be relevant to the real "in vivo" differentiation of erythroblasts. It should also be noticed that the disappearance of proteins observed during K562 differentiation might, at least in part, be related to cell differentiation in general, rather than to specific erythroid differentiation; in fact, band A rapidly disappears in THP-I and U 937 cells following induction to terminal differentiation.

The additional question then arises whether the early disappearance of γ-globin promoter binding proteins(s) in the course of K562 differentiation (and the absence of this protein(s) in fetal liver erythroblasts) has any relationship with the significant transcriptional activation of the γ-globin genes during this process. No direct data are yet available on the effect of the octamer and of more upstream regions on γ-globin transcription, and expression experiments with appropriately mutagenized or deleted promoters are required to solve this problem. However, it is interesting to note that the octamer-containing binding site (responsible for bands B1, B2 and B3) overlaps, in part, with a region where several single point mutations occur, which are thought to be responsible for continued expression of high levels of either the $^{G}\gamma$-or $^{A}\gamma$-globin genes in adult life (20-24; for the -175 mutation, unpublished data of S.O; Schwartz, personal communication).

In addition, we have already mentioned the important functional role of the "octamer" in several eukaryotic genes: if the octamer binding protein(s), generating the uninduced K562-specific bands B2 and B3, has any role in γ-globin regulation, its early disappearance after K562 induction would suggest a negative regulatory effect, rather than the positive role suggested by studies with different promoters (25-27). This is intriguing, although it should be noticed that the effect of the interaction of the protein with the octamer might depend on the nature of flanking sequences and of additional proteins binding to that region; it is well known that a protein like E1A may

elicit inhibitory (28,29) or stimulatory (30) effects depending on the sequences and proteins with which it interacts; similarly, a CCAAT binding protein is known to support both DNA replication and transcription (14). Moreover, the presence of an octamer sequence in a viral thymidine kinase promoter has been shown to increase the transcriptional response of this promoter to a kappa-immunoglobulin enhancer, but to decrease that to a Moloney murine sarcoma virus enhancer (19). In addition, it has recently been shown (31) that a factor, binding to the CCAAT region of a testis H2B histone promoter, may prevent its interaction with a positively acting CCAAT-binding factor, thus mediating repression. Therefore, a possible negative role for the octamer binding protein (and the upstream binding factor) in γ-globin regulation is not necessarily incompatible with different effects of the ubiquitous octamer binding protein on other genes. Functional studies are in progress to answer these questions.

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