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A Novel Nuclear Protein Which Binds to Gγ and Aγ Globin Promoters and Modulates Hemoglobin Synthesis in K_{562} Cells

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Abstract Nuclear extract of human erythroleukemic cell line K_{562} contains a 70 kDa protein which is gradually reduced when cells are induced to express globin genes by 25 μM hemin. When globin synthesis was inhibited by cycloheximide (100 μg/ml) or Actinomycin D (1 μg/ml), the disappearance of this protein was prevented. The 70 kDa nuclear protein exhibited strong binding to Gγ and Aγ globin promoters but not to β-globin promoter. This suggests that this 70 kDa nuclear protein may be involved in the regulation of fetal globin gene expression.

Key words: nuclear protein, globin promoters, K_{562} cells, hemoglobin, hemin

Increased level of fetal hemoglobin is known to ameliorate the severity of many forms of β-globin disorders [Charache et al., 1991; Perrine et al., 1989; Veith et al., 1975]. The molecular mechanism of fetal hemoglobin gene expression in erythroid cells, however, is not understood. The human erythroleukemic cell line K_{562} (upon induction by hemin) expresses embryonic (ε) and fetal (γ) globins but not adult globin (β) [Lozzio et al., 1975; Thompson et al., 1983; Charnay and Maniatis, 1983; Dean et al., 1983]. K_{562} cells are often used for understanding the mechanism of fetal hemoglobin gene expression [Mitchell et al., 1989; Evans et al., 1988]. In order to understand the molecular mechanisms accounting for tissue specific developmental regulation of the globin gene families, many investigators have focussed their attention on identifying cis-elements and trans-regulatory protein factors involved in mediating globin gene transcription [deBoer et al., 1988; Gumuchio et al., 1988; Mantovani et al., 1988; Catala et al., 1989].

In this report, nuclear extracts isolated from hemin induced and uninduced K_{562} cells were analyzed for their binding to regulatory sequences of different globin genes. When using "gel retardation" assays, we have found 3 major nuclear proteins from K_{562} extracts binding with oligonucleotide containing AP-1 and NFE-2 sites. Incubating nuclear proteins from K_{562} extracts with β-globin, Gγ and Aγ promoters, only one 70 kDa protein bound specifically to Gγ and Aγ promoters but not to β globin promoter. This protein is associated with the repressed state of γ-globin gene and is shown to decrease when fetal hemoglobin is expressed.

MATERIALS AND METHODS

K_{562} cells and the β-promoter and 1st and 2nd exons cloned into pBR328 were purchased from ATCC, Maryland. Cells were grown in suspension culture in RPMI-1640 medium supplemented with glutamine (2 mM), HEPES (25 mM), Penicillin (50 IU/ml), Streptomycin (50 ug/ml) and 10% FCS at 37°C. Differentiation was induced by seeding exponentially growing cells into complete medium supplemented with 20 um hemin for at least 48 h.

Preparation of Nuclear Extracts

K_{562} cells (10×10^6 cells) from exponentially grown culture were collected by centrifugation and washed twice with cold phosphate buffer solution (PBS). The final pellet was suspended in cold lysing buffer (10 mM Tris, HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$ and 0.5% NP-40). After 5 min incubation on ice, the nuclei were collected by centrifugation at 500 x g for 10 min. The nuclei were washed once with lysis buffer.

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Under phase contrast microscopy, the nuclei appeared intact and devoid of attached cytoplasm or cell debris. The nuclear extract was prepared as described by Dignam et al., 1983. The nuclei were suspended in buffer containing 20 mM HEPES pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT, homogenized and then centrifuged for 30 min at 25,000 x g. The supernatant was dialyzed against same buffer overnight, centrifuged and stored in aliquots at $-70°$.

SDS-Polyacrylamide Gel Electrophoresis

Electrophoretic separation of the nuclear proteins was performed in 12% polyacrylamide gel containing 0.1% sodium dodecyl sulphate as described by Laemmli, 1970. Gels were run at constant current at 40 mAmp for approximately 120 minutes using Bio-Rad Mini II apparatus. After electrophoresis, gels were fixed and stained with silver as described by Wray et al. 1981.

Mobility Shift Electrophoresis

This was performed essentially as described [Singh et al., 1986]. The [^{32}P]-labeled oligonucleotide containing AP-1 and NFE-2 (see Scheme 1) binding sites (48 mer oligonucleotide construct, approximately 19,000 CPM/ng) was incubated in a total volume of 20 μl containing 10 mM Tris-HCl (pH 7.5) 1 mM dithiothreitol, 1 mM EDTA, 40 mM KCl and 10% glycerol. After 20 min incubation at 25°C, an aliquot of this reaction mixture was mixed with sample buffer and immediately loaded onto a 4% polyacrylamide gel and electrophoresed at 150v for 2 h with a Tris-borate-EDTA buffer (TBE). After electrophoresis, the gels were fixed, dried and autoradiographed.

Promoter Binding Assay

We have developed an alternative to the gel shift assay. The gamma promoters (Aγ, Gγ), and β-globin promoter were cloned into a bluescript and pBR322 vectors respectively. Following purification, 1–5 μg of each plasmid was incubated with 5 μl of glass milk component of the GENE CLEAN system, from BI0101 Corp., LaJolla, CA. The binding of DNA to the matrix was tested, then each matrix with its bound DNA was used with K_{562} nuclear extract. Five micrograms of pBR322 bound to the glass milk was used in every experiment as the negative control.

Each reaction was carried out in a 25 μl reaction volume containing binding buffer, 1–2 μg nuclear protein, 1 ug of poly d(I)-d(C) and 1 μg of matrix bound promoter. At the end of incubation, the reaction mix was cooled on ice, centrifuged and the supernatant was analyzed on SDS-gel electrophoresis as described above.

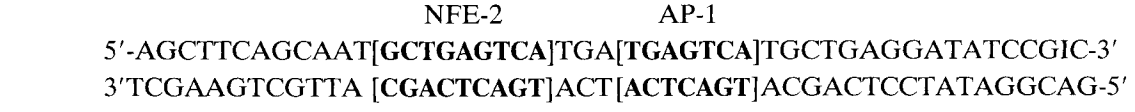
Hemoglobin Determination

Hemoglobin containing cells were scored as benzidine positive cells. Benzidine stain was freshly prepared before use.

RESULTS

The induction of fetal hemoglobin gene expression during erythroid differentiation of K_{562} cells provides a well defined model for the investigation of specific nuclear factors involved in globin gene expression. Fetal hemoglobin gene expression increases 20 to 30 fold in K_{562} cells within 48 h of incubation in the presence of 25 μM hemin [Charnay and Maniatis, 1983; Dean et al., 1983]. Though the mechanism of activation of globin synthesis by hemin is not understood, genetic elements and protein factors are thought to be involved in this activation process. To test this possibility, we examined nuclear proteins isolated from induced and non-induced K_{562} cells that selectively bind to known DNA sequences in the 5′ end of the enhancer region (HS-II) and the promoter region of the γ-globin.

An in vitro gel mobility shift assay was used to test for binding activities of nuclear protein extracts from hemin-induced and non-induced K_{562} cells. A [^{32}P]-labelled oligonucleotide construct (ds 48 mer) similar to the 5′ end of the β globin enhancer region (HS-II) of β globin cluster was used as a probe in this assay. This oligonucleotide contruct contains two transcription activating factors AP-1 and NFE-2 binding



Scheme 1.

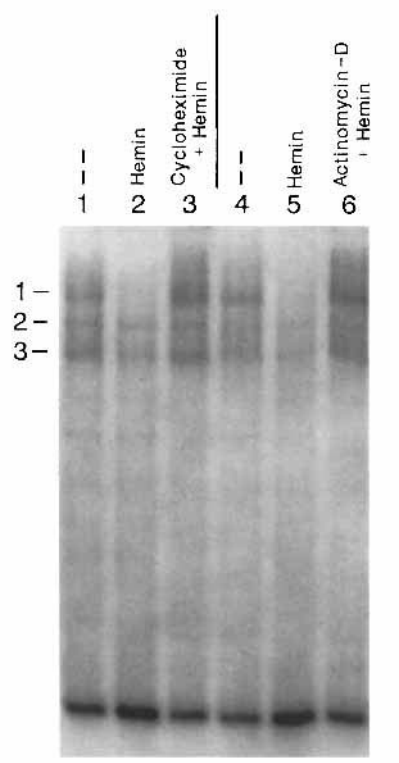


Fig. 1. Gel shift assay. Gel shift electrophoresis assays were carried out using 48 mer oligonucleotide (32,000 CPM) containing one copy of each AP-1 and NFE-2 factor binding sites.

NFE-2 AP-1
5'-AGCTTCAGCAAT[**GCTGAGTCA**]TGA[**TGAGTCA**]TGCTGAGGATATCCGTC-3'
3'TCGAAGTCGTTA [**CGACTCAGT**]ACT[**ACTCAGT**]ACGACTCCTATAGGCAG-5'

Assay contained either 2.5 ug of nuclear protein extract from unstimulated K_{562} cells. The optimum binding was determined by using varying amounts of nuclear protein and oligonucleotide. No protein/oligo only control was run each time (not shown in figure).

sites. As shown in Figure 1, three protein-DNA complexes (band shifts 1, 2 and 3) were evident with the uninduced nuclear extract (lane 1) indicating either the presence of three proteins interacting with oligonuleotide or the probe has multiple binding sites for one or more proteins. One of the bands (band shift 1) was reduced when incubated with K_{562} cells extract from hemin treated cells. However, band 1 was not reduced in the presence of 100 ug/ml cycloheximide or 1 ug/ml Actinomycin D indicating that disappearence of band 1 may be coordinated with RNA and protein synthesis. These results suggest that the presence of hemin regulated HS-II enhancer binding protein in K_{562} extract.

In order to explore nuclear proteins involved in binding to globin promoters, uninduced K_{562} nuclear extracts was incubated with Gγ, Aγ, and

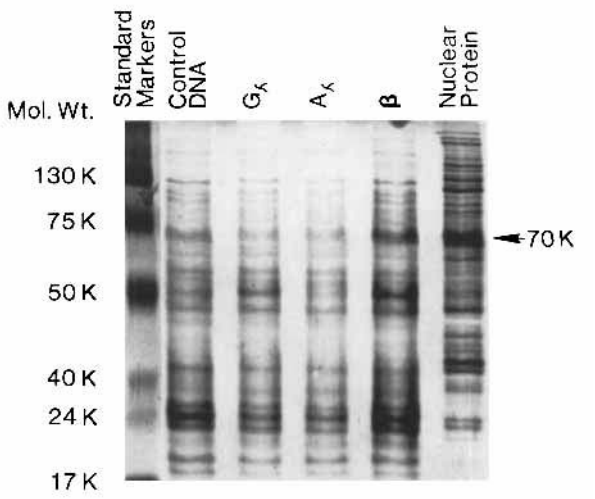


Fig. 2. SDS-electrophoresis analysis of K_{562} nuclear proteins. Nuclear proteins isolated from uninduced K_{562} cells were incubated with β, Gγ and Aγ promoters in the presence of 1 μg of poly d(I)-d(C) for 20 min at 25° as described in Materials and Methods. At the end of incubations, the reaction mixture was centrifuged and the supernatant was analyzed on 12% SDS-polyacrylamide gel electrophoresis.

β globin promoters and Control DNA (plasmid pBR322) for 10 min at 25°C in the presence of poly d(I)-d(C). At the end of incubation time, the reaction mixture was centrifuged and an aliquot of the supernatant was analyzed on SDS-PAGE. A 70 kd protein (as shown by arrow) was reduced in Gγ and Aγ promoter treated extracts as compared to the extracts treated with β globin promoter or control DNA (pBR322) (Figure 2). The binding was concentration dependent and reproducible.

To determine the possible role of 70 kDa protein in fetal globin gene expression, K_{562} cells were incubated at different time intervals in the presence of 25 μM hemin. At the end of incubation, nuclear protein extracts were isolated and analyzed on SDS gel electrophoresis. The nuclear protein extracts from hemin induced K_{562} cells exhibit a time dependent and gradual decrease in 137 kDa and 70 kDa proteins (Figure 3). However, these two proteins remained constant in un-induced K_{562} nuclear extracts. When globin gene expression in hemin induced K_{562} cells was inhibited by addition of cycloheximide (100 μg/ml) or Actinomycin D (1 μg/ml) the degradation of 70 kDa protein was prevented (Figure 4). The 70 kDa protein was rather stabilized in the presence of these inhibitors. These results suggest that the reduction of the 70 kDa protein due to hemin induction parallels the increase in γ-globin gene expression.

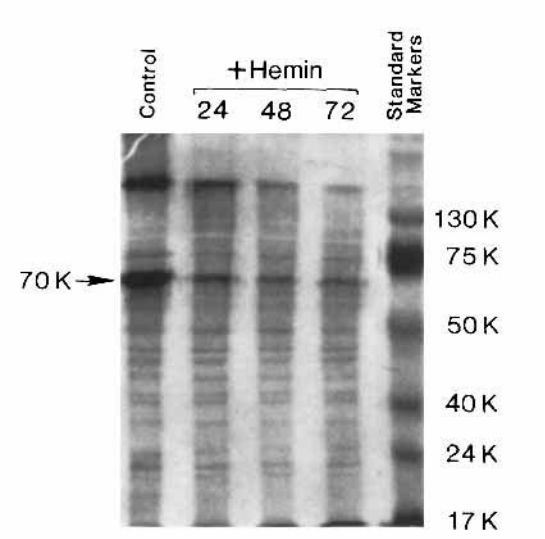


Fig. 3. Electrophoretic analysis of nuclear proteins isolated from K_{562} cells incubated at 24, 48, 72 and 96 h in the presence of 25 μM hemin. At the end of incubations, cells were centrifuged, washed and suspended in gel buffer and analyzed on 12% SDS-polyacrylamide gel as described in Materials and Methods.

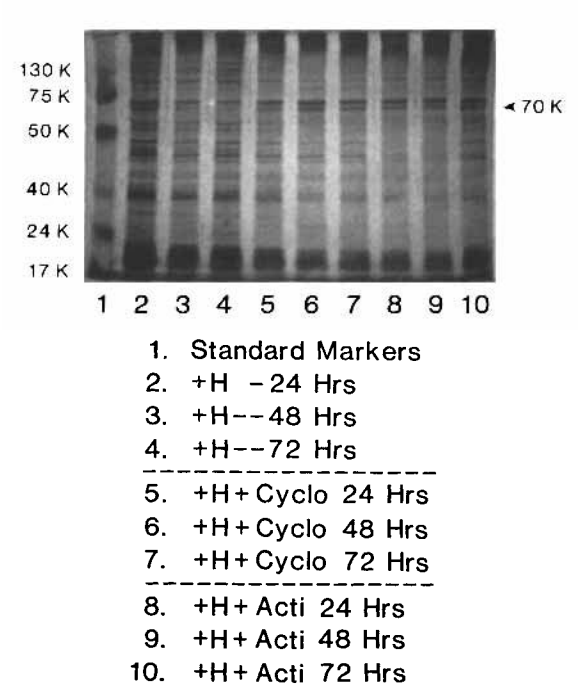


Fig. 4. SDS-polyacrylamide gel electrophoretic analysis of nuclear proteins isolated from hemin induced (25 μM) K_{562} cells incubated at 24, 48, 72 and 96 hrs in the presence of cycloheximide (100 μg/ml) and Actinomycin-D (1 μg/ml).

DISCUSSION

K_{562} cells seem to contain several DNA binding proteins; some are binding specifically to enhancer region and some are promoter-region specific. Three nuclear proteins are binding to AP-1 and NFE-2 binding sites of HS-2 enhancer region. One of the proteins was significantly reduced in hemin treated extract and this disappearance was sensitive to both Actinomycin-D and cycloheximide. These results indicate the involvement of this protein in hemin induced globin gene expression. Isolation and characterization of this protein is in progress. The SDS-gel electrophoretic analysis of the K_{562} nuclear extracts treated with Gγ, Aγ and β globin promoters followed by removal of promoter-protein complexes by centrifugation showed that a protein with approximately 70 kDa molecular weight diminishes in Gγ and Aγ but not β globin promoter treated K_{562} cells. These results indicate that 70 kDa protein specifically bind to Gγ and Aγ promoters but not to the β promoter. When ^{32}P-labelled 300 base pair Gγ promoter sequence was incubated with K_{562} nuclear extract, we observed one band shift, whose intensity is increased with increasing K_{562} nuclear extract [unpublished results]. It is possible that a single band shift observed on gel shift assay may be due to at least a 70 kDa protein. Additional experiments such as South Western assay and DNAase-I footprint studies may be required before 70 kDa protein can be conclusively implicated in globin gene promoter binding. Furthermore, the nuclear extracts from hemin induced K_{562} cells exhibited a time dependent decrease in 137 kDa and 70 kDa proteins. When protein or RNA synthesis are inhibited by cycloheximide and Actinomycin-D, respectively, the disappearance of the 70 kDa protein is prevented indicating the association of 70 kd protein with the repressed state of γ-globin gene expression. At this time we do not know the nature of the protein which binds to HS-II enhancer. It is possible that both 70 kDa protein and HS-II enhancer binding protein may be involved in fetal hemoglobin gene expression.

These results suggest that hemin induction of fetal globin gene expression requires the removal of a 70 kDa protein bound to γ-globin promoters and is thus indicative of presence of a negative control mechanism in γ-globin gene expression. The role of hemin in the negative regulatory control mechanism of 70 kDa protein in globin gene expression requires further investigation. Since no binding of the 70 kDa protein to comparable region of β globin promoter is found, this discrimination suggests that the 70 kDa protein could play a role in the differential

expression of γ and β globin genes. We hope this information will provide a more detailed understanding of gene expression in mammalian cells and in developing ways to maintain production of hemoglobin F in individuals who have altered or deficient β-globin chain production.

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