

Proteins binding to the 5'-flanking regulatory elements of the human β -globin gene¹

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ABSTRACT

The binding of nuclear proteins prepared from mouse erythroid tissue in different developmental stages to the 5'-flanking regulatory elements of human β -globin gene, two negative control regions(NCR1, -610 to -490 bp; NCR2, -338 to -233bp), was identified. Two stage specific protein factors corresponding to embryonic and fetal stages were found to be capable of binding to NCR2. These data provided evidence that the cis acting elements of the 5'-flanking region might be involved in the developmental control of β globin gene and NCR2 might be responsible in part for the silence of β -globin gene in the embryonic and fetal stages.

Key words: *human β -globin gene, negative control region, stage specific factor.*

INTRODUCTION

The human β -like globin genes are located in the short arm of chromosome 11 in a cluster of 5 genes, ϵ - γ^G - γ^A - δ - β in a 5'-3' order that reflects their developmental expression[1]. These genes are expressed specifically in erythroid tissue at high levels and differentially expressed in a precise temporal order during development. The ϵ gene is expressed in the embryonic yolk sac, the γ gene in fetal liver and the δ and β genes mainly in adult bone marrow. The globin gene family has served as a model for the study of developmental stage and tissue specific gene control. Hemoglobin

1. Project was supported by grants from Shanghai Joint Laboratory of Life Sciences, Academia Sinica, and the National Natural Sciences Foundation.

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switching is also of medical interest because the reactivation of fetal hemoglobin in patients with sickle cell disease or β thalassemia ameliorates their diseases.

How the human globin gene expression is developmentally switched has been studied for years. Recently, the discovery of the locus control region (LCR) and experiments in transgenic mice have provided insights on the molecular control of globin gene switching. The LCR, lying 6 to 20 kb upstream of the human ϵ gene, consists of a series of deoxyribonuclease (DNase) I hypersensitive sites that are specific for the cells of the erythroid lineage and are developmentally stable[2]. The entire set of β -like globin genes is controlled by the LCR[3, 4]. Experiments in transgenic mice have shown that human γ and β -globin genes expressed sequentially during development but at a lower level in the absence of LCR [5,6,7]. The linkage of LCR to γ or β -globin gene confers high level expression and position independence on human globin transgene in mice[4,8]. Initially, Behringer et al[5] and Enver et al[7] reported that linkage of γ -globin gene alone to LCR resulted in γ globin gene expression throughout the developmental stages, while linkage of both γ and β -globin genes to LCR conferred a correct stage developmental expression, which led to a proposition that γ to β -globin switching is regulated by reciprocal competition. However, Dellon and Grosveld[9] reinvestigated the problem and found that γ -globin gene was silenced at adult stages and the silencing was independent of the presence of the β -globin gene. Similar results were obtained with the ϵ -globin gene[10,11]. These results supported that even in the presence of LCR, the ϵ -and γ -globin gene were suppressed at later stages of development in the absence of competition from other genes and indicated the importance of silencing elements during development. A competition study of α , γ and β globin genes combination has shown that the pattern of developmental expression of the γ and β -globin genes is affected by their positions relative to LCR[12]. This has led to a general nonreciprocal competition model for the developmental regulation of the globin genes. This model proposed that the competition is influenced by the sequences immediately flanking the genes besides the positions of the genes relative to LCR.

Previous studies [13,14] have identified that there are at least three regulatory sequences 5' to the human β -globin gene, i.e., two negative control regions (NCR1, -610 to -490bp; NCR2, -338 to -233bp) and one positive control region (PCR, -233, to -185 bp). NCR1 and NCR2 acted as classical silencers and they decreased the expression of a heterologous gene in a position and orientation independent manner. PCR is specific for K562 cells and has no effect on MEL (murine erythroleukemia) cells. In this paper, we used gel electrophoresis mobility shift assay to detect proteins capable of binding to the two silencers, NCR1 and NCR2, to see if the 5'-flanking element would possibly be involved in the control of β -globin gene expression during development. As a result, we propose that the negative regulation of β -globin gene, like that of ϵ -globin gene, seems to be an important component of tissue-and developmental stage-specific globin gene expression.

MATERIALS AND METHODS

1. Mouse matings and dissection

Mouse matings and dissection were performed by the method described by Whitelaw et al[15]. Mouse matings were assumed to occur at the midpoint of dark cycle (midnight). The females in which a plug was identified were collected. Pregnant females were sacrificed by cervical dislocation in the morning on d 10.5, d 13.5 and d 17.5. Embryos were dissected free of decidua and the placenta was removed. Yolk sac or fetal livers were dissected from the embryos and washed in phosphate buffered saline. From baby mice of 1 d old, liver and spleen were dissected out. Brain was also collected from maternal tissue as nonerythroid control.

2. Protein extract preparation

Nuclear protein extracts were prepared according to the method described by Gorski et al[16] with a little modification. All manipulations were performed in cold and all solutions, tubes, and centrifuges were chilled to 4°C. Mouse tissues (about 1 g of liver, spleen or brain) were put into 2.5 ml homogenization buffer (10 mM Tris-HCl, pH 7.5, 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 M sucrose, 10% glycerol) separately and homogenized by a 10 ml glass homogenizer, striking for 4-5 times. The homogenates were diluted to 8.0 ml with a homogenization buffer, layered over 1.5 ml cushion of the same buffer, and centrifuged at 35,000 rpm for 40 min in a RP-65T rotor. The nuclear pellets were resuspended in 8 ml of 9:1 (V/V) mixture of homogenization buffer and glycerol. The homogenates were again layered over 1.5 ml cushion as described above and recentrifuged at 38,000 rpm for 30 min.

The pelleted nuclei were resuspended with pipette in 3 ml of nuclear lysis buffer (10 mM Tris-HCl, pH 7.5, 100 mM KCl, 3 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol). 0.3 ml of 4 M (NH₄)₂SO₄, pH 7.9 was added drop by drop and the mixture was gently shaken at 0°C for 1 h. The viscous lysates were then centrifuged at 100,000 g for 1 h to pellet chromatin. Solid (NH₄)₂SO₄ (0.3 g/ml) was added to the supernatants over 10 min. After being kept at 0°C for 30 min, the precipitated proteins were sedimented by centrifugation at 100,000 g for 30 min. The protein pellets were dissolved in 0.5 ml dialyzation buffer (25 mM Tris-HCl, pH 7.5, 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10 % glycerol) and dialyzed against the dialyzation buffer twice at 4°C overnight. After removal of the precipitate formed during dialyzation by centrifugation, the protein extracts were stored at -20°C for using. An aliquot was taken and the protein concentration was determined according to Bradford's method[17].

3. Gel Electrophoresis mobility shift assays

Double stranded DNA fragments were prepared by restriction enzyme reactions from the pGLCAT plasmid[14] containing NCR1, NCR2, and PCR. The DNA fragment for NCR1 was a 270 bp HindIII-AecI fragment (-643 to -373 bp), for NCR2 148 bp AccI-RsaI fragment (-372 to -224 bp), and for PCR 30 bp RsaI-DdeI fragment (-223 to -194 bp). The DNA fragments were 5'-end labeled with (γ -³²P) ATP by T4 polynucleotide kinase.

Gel electrophoresis mobility shift assays were performed according to the method described by Strauss and Varshavsky[18]. The binding reaction (25 μ l) contained approximately 5 fM of end-labeled DNA fragmenL 0-2 μ g of nuclear protein extract, and 1 μ g nonspecific competitor poly d(I-C) in a reaction buffer consisting of 10 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM MgCl₂, 1 mM EDTA, 10 mM DTT, 4% glycerol, and 0.1% Triton X-100. The reaction mixtures were incubated for 1 h at 0°C and were analyzed on a 4% non-denaturing polyacrylamide gel. Gel was dried and autoradiographed at -20°C.

RESULTS

Gel electrophoresis mobility shift assays were used to detect proteins capable of

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binding to the 5'-flanking two silencers, NCR1 and NCR2, of human β -globin gene with nuclear extracts of mouse embryonic tissues at different developmental stages. The pattern of expression of the β -like globin genes in mouse is quite analogous to that of human and involves two developmental switches (β H1 to $\epsilon\gamma$ 2 and $\epsilon\gamma$ 2 to β)[15]. The β H1 globin gene is expressed in the largest amount on d 10.5 in yolk sac during mouse gestation[15]. $\epsilon\gamma$ 2 has its peak on d 13.5 in fetal liver and β -globin becomes predominant on d 15.5. Two major changes were involved here, the first occurred with the major reduction in β H1-globin at d 12.5 and the second occurred with the switch from $\epsilon\gamma$ 2 to adult β -globin at d 15.5. According to these considerations we chose female mice of d 10.5, d 13.5, and d 17.5 in gestation as well as baby mice of 1 d old as materials representing expression stages of ϵ , γ and β globin genes respectively.

The first silencer, NCR1, was analysed with a 5'-end-labeled 270 bp fragment (-643 to -373). When this fragment was incubated with the nuclear extracts prepared from gestation mice of d 10.5, d 13.5, d 17.5, or 1 d old mice, 2 bands with decreased mobility were observed, indicated by the arrows in Fig 1. The band A1 appeared in all erythroid samples except that of embryonic d 10.5. On the other hand, A2, was detected in the samples of adult stage, d 17.5 mouse liver and 1 d old mouse

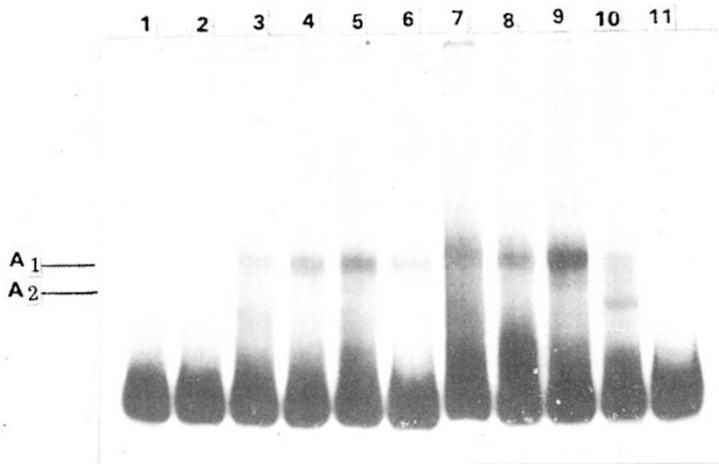


Fig 1. Gel electrophoresis mobility shift assay of the 5'-flanking negative control region I(NCR1) of human β -globin gene against the nuclear protein extracts of mouse erythroid tissues of different developmental stages (see Materials and Methods). The DNA fragment (-643 to -373 bp) for NCR1 were 5'-end labelled. Lane 1, no nuclear protein; Lane 2, d 10.5 yolk sac (0.3 μ g); Lanes 3 and 7; d 13.5 liver (0.3 and 0.6 μ g); Lanes 4 and 8, d 17.5 liver (0.6 and 1.2 μ g); Lanes 5 and 9, spleen of one day old mice (0.3 and 0.6 μ g); Lane 6, liver of one day old mice (0.3 μ g); Lane 10, K562 cells (1 μ g); Lane 11, brain of one day old mice (0.3 μ g).

spleen (Fig 1, Lanes, 4, 8, 5, and 9). Nothing were detected for d 10.5 (Fig 1, Lane 2). The binding of proteins to the NCR1 indicated the presence of possible repressors to β -globin gene during development. The shift bands of K562 cells (Fig 1, Lane 10) were different from those of mouse erythroid tissues. Both bands A1 and A2 were not observed in the nuclear extract of brain (Fig 1, Lane 11), indicating that both shift bands were erythroid specific.

When the second silencer was analyzed with a 5'-end-labeled 148 bp fragment (-372 to -224 bp), 2 decreased mobility bands as indicated by B1 and B2 in Fig 2B were observed in the sample of d 10.5 (Fig 2B Lanes 2 and 3). The B1 band of d 10.5 was not observed in the sample of d 13.5 (Fig 2A, Lanes 3 and 7, Fig 2B, Lanes 4 and 5), while the band B2 became more predominant on d 13.5 (Fig 2B, Lanes 4 and 5, Fig 2A, Lanes 3 and 7). Both band B1 and B2 were not observed in the samples of adult stage, d 17.5 liver and the spleen and the liver of 1 d old mice (Fig 2B, Lanes 6 and 7, Fig 2A, Lanes 4, 5, 9 and 6). Therefore, both bands B1 and B2 were considered to be stage-specific binding proteins corresponding to the embryonic and fetal stages respectively. The B1 and B2 bands appeared in the stages when β -globin gene was not expressed but disappeared when β -globin

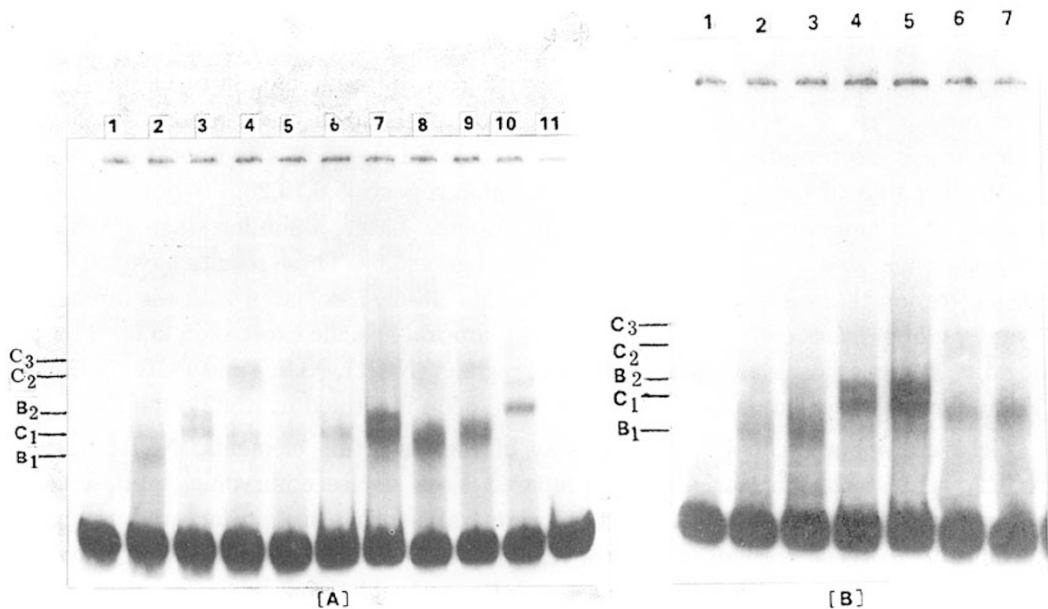


Fig 2. Gel electrophoresis mobility shift assay of the 5'-flanking negative control region 2 (NCR2) of human β -globin gene. The DNA fragment (-372 to -224 bp) for NCR2 were 5'-end labelled. [A], Lane 1, no nuclear protein; Lane 2, d 10.5 yolk sac (0.3 μ g); Lanes 3 and 7, d 13.5 liver (0.3 and 0.6 μ g); Lanes 4 and 8, d 17.5 liver (0.6 and 1.2 μ g); Lanes 5 and 9, spleen of one day old mice (0.3 and 0.6 μ g); Lane 6, liver of one day old mice (0.3 μ g); Lane 10, K562 cells (1 μ g); Lane 11, brain of one day old mice (0.3 μ g). [B], Lane 1, no nuclear protein; Lanes 2 and 3, d 10.5 yolk sac (0.3 and 0.6 μ g); Lanes 4 and 5, d 13.5 liver (0.3 and 0.6 μ g); Lanes 6 and 7, d 17.5 liver (0.6 and 1.2 μ g).

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gene was expressed. Thus, the presence of band B1 and B2 might indicate that there were trans-acting protein factors interacting with the second silencer of β -globin gene during the embryonic and fetal stages in the gestation of mice to cause silencing of β -globin gene.

There was another main decreased mobility band, C1, observed in the sample of d 13.5 (Fig 2A, Lane 3). The band C1 was also observed in the samples of adult stage, d 17.5 liver and spleen and liver of 1 d old mice (Fig 2A, Lanes 4, 5, and 6). There were 2 other main decreased mobility bands, C2 and C3, in the samples of d 17.5 liver and spleen of 1 d old mice (Fig 2A, Lanes 4, 8, Fig 2B, Lanes 6, 7, Fig 2A, Lanes 5, 9). The band C3 could be detected in the samples of d 13.5 in the high protein concentration (Fig 2B, Lanes 4 and 5, Fig 2A, Lane 7). These results showed that C1, C2, and C3 bands had no obvious stage-specificity. The mobility shift bands of nuclear extract from K562 cells were different from those of erythroid tissue of mice (Fig 2A, Lane 10) and no shift bands were observed for the nuclear protein of brain (Fig 2A, Lane 11).

DISCUSSION

Human β -like globin genes express in a developmental stage-specific manner from embryonic (ϵ) in the yolk sac, to fetal (γ) during intrauterine life, and to adult (β) after birth. Previous studies in transgenic mice have shown that human β -globin gene alone is expressed specifically in adult erythroid tissue in a temporal pattern paralleling that of the endogenous mouse β -globin genes[5,6,19,20]. β -globin gene is silenced in embryonic and fetal erythroid tissue. Later, a similar stage specific expression has also been obtained with γ -globin gene[5,6]. These results have led to the suggestion that cis-acting regulatory elements closely associated with the human β and γ globin genes could confer tissue-and temporal-specific expression [5,6]. Two negative and one positive regulatory control regions (NCR1, NCR2 and PCR) in the 5'-flanking region of human β -globin gene have been identified in K562 cells [13,14].

Gel electrophoresis mobility shift assay of the cis-acting elements, NCR1 and NCR2, against the nuclear proteins prepared from mouse embryonic, fetal, and adult erythroid tissue showed that there were trans-acting protein factors binding specifically to these regulatory control regions. These results together with those of specific expression of β -globin gene in adult erythroid tissue in transgenic mice suggested that the immediate 5'-flanking cis-acting elements of β -globin gene were probably involved in the stage specific developmental regulation. Deletion analysis for induced K562 cells showed a 3.1-fold increase in CAT gene expression upon deletion of NCR1, but a 138-fold increase upon deletion of NCR2[14]. This result indicated that the NCR2 was more powerful and important than NCR1 to the regulation of β -globin gene expression. Gel mobility shift assays showed that 2 stage-specific nuclear proteins of erythroid tissue on d 10.5 and d 13.5 in mouse gestation could bind to NCR2. The gene expression on d 10.5 and d 13.5 is β H1

and $\epsilon\gamma 2$ in mice corresponding respectively to the stages of human embryonic ϵ and fetal γ globin gene expression [15]. The detection of stage specific binding factors to NCR2 together with its predominant silencing role to the β -globin gene as shown in transfected K562 cells suggested that the silencing of β -globin gene was not simply passive due to its inferiority to ϵ or γ globin gene in competition for LCR[12], but was probably caused actively by the stage specific repressors in the embryonic and fetal stages. The interaction of NCR2 with the trans-acting protein factors may be responsible in part for the stage silencing. Support for this conclusion also came from preliminary results of the transient expression of β -globin gene in murine erythroleukemia (MEL) cells (which expressed the endogenous adult murine globin gene) but not in the K562 cells (which expressed embryonic and fetal globin genes) [21].

Recently, the cis-acting silencing elements which are involved in the stage developmental control have been demonstrated with ϵ -globin gene in transgenic mice [10]. Deletion of the ϵ -globin silencer region (-467 to-180 bp) in a construct containing the LCR and human ϵ -globin gene resulted in some loss of developmental specificity giving rise to detectable expression of the human ϵ -globin transgene in adult mice. The sequences immediately flanking the ϵ , γ and β globin genes mediated the silencing and activation of the genes which had been incorporated into a general model proposed for the developmental expression of human β -like globin genes based on the distal relationships of the globin genes in the human β -globin cluster and their interaction with LCR[9,12].

The NCRI of β -globin gene did not show obvious stage-specific shift bands in gel mobility shift assays. One band, A1, was common to all stages except that of d 10.5. The PCR of β -globin gene had a protein binding pattern similar to that of NCR1 (data not shown). Meanwhile, we also have identified a stage specific trans-acting factor binding to the promoter region of human β -globin gene. The appearance of which is concomitant with the β -globin gene expression[22]. Taken together, these data indicated that the stage-specific cis-elements and trans-acting factors may be responsible for the control of the expression of β -globin gene.

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Received 16-4-1993. Revised 6-8-1993. Accepted 14-8-1993