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A stage-specific protein factor binding to a CACCC motif in both human β-globin gene promoter and 5'-HS2 region[1]

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ABSTRACT

The DNaseI hypersensitive site 2 (HS2) of human β-globin locus control region (LCR) is required for the high level expression of human β-globin genes. In the present study, a stage-specific protein factor (LPF-β) was identified in the nuclear extract prepared from mouse fetal liver at d 18 of gestation, which could bind to the HS2 region of human β-globin LCR. We also found that the shift band of LPF-β factor could be competed by human β-globin promoter. However, it couldn't be competed by human ε-globin promoter or by human ^{A}-γ-globin promoter. Furthermore, our data demonstrated that the binding-sequence of LPF-β factor is 5'CACACCCTA 3', which is located at the HS2 region of β-LCR (from -10845 to -10853 bp) and human β-globin promoter (from -92 to -84 bp). We speculated that these regions containing the CACCC box in both the human β-globin promoter and HS2 might function as stage selector elements in the regulation of human β-globin switching and the LPF-β factor might be a stage-specific protein factor involved in the regulation of human β-globin gene expression.

Key words: *β-globin promoter, HS2 of β-globin LCR, trans-acting factor.*

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INTRODUCTION

The human β-globin gene cluster spans a region of 70 kb on chromosome 11 and contains five developmentally regulated genes in the order 5'-ε, ^{G}γ, ^{A}γ, δ, β-3' (Fig 1). In the early stage of human development, the embryonic yolk sac is the haematopoietic tissue and expresses the ε-globin gene. This is followed by a first switch to the γ-globin genes in the fetal liver and a second switch to the β-globin gene in adult bone marrow[1].

Four developmentally stable DNase I hypersensitive sites are located 6 to 20 kb upstream of human ε-globin gene (Fig 1). These sequences are critical for high-level expression of human ε, γ and β-globin genes and, therefore, have been designated the locus control region (LCR) [2-4]. Naturally occuring deletions of LCR sequences render intact human β-globin gene inactive in a subset of individuals with β-thalassemia (-low or absent β-globin production)[5-7].

Introduction of human γ- or β-globin gene, including proximal flanking sequences, into transgenic mice and erythroid cells in culture led to the tissue-specific and developmentally stage-specific expression, but the level of expression was much lower than that of the endogenous globin genes[8-9]. These results suggested that although there were many important cis-regulatory elements in the proximal flanking sequences (promoter, positive and negative control region, etc), some crucial elements for physiological expression were omitted, If the LCR was present along with the γ- or β-globin gene, it enhanced human γ- and β-globin gene expression 300-fold, The expression of the γ-globin genes was developmentally correct. However, the β-globin gene was expressed at all stages of development[3, 10]. If the LCR was linked to the γ- and β-globin genes in tandem, stage-specific expression was restored. Human γ-globin genes were expressed in the fetal stage, and human β-globin gene in adult stage only[11]. These results demonstrated that promoter competition might play a major role in globin gene switching. The outcome of this competition probably depended on some stage-specific factors which cause the LCR to favor one promoter over the other at different stages.

In this paper, we attempt to examine how the distal regulatory element, HS2, cooperates with the proximal regulatory element (such as human β, γ and ε-globin promoters) in developmental regulation. Using gel mobility shift assays, we detected at least a stage-specific protein factor, termed LPF-β, from the nuclear extract of mouse fetal liver at d 18 of gestation. LPF- β could bind to the HS2 region of human β-globin LCR. Furthermore, We observed that the shift band of LPF-β could be competed by human β-globin promoter. However, it couldn't be competed by human ε - or ^{A}γ-globin promoter. The binding-motif of LPF-β factor is 5' CACACCCTA 3', which is located at HS2 of β-LCR (from -10845 to -10853 bp) and human β-globin promoter (from -92 to -84 bp). Therefore, we speculated that both CACCC elements in the β-globin promoter and HS2 might be involved in the developmental regulation of human β-globin gene expression.

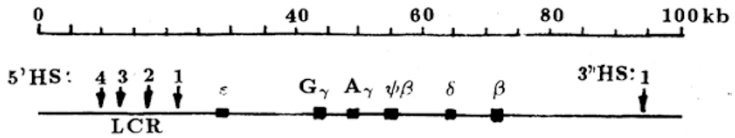

Fig 1. The human β-globin gene cluster. $\psi\beta$ is a pseudogene.

MATERIALS AND METHODS

Preparation of nuclear extracts

Nuclear extracts were prepared from mouse haematopoietic tissues at d 10, d 14 and d 18 of gestation according to the methods of Gorski[12]. The concentration of protein was determined using Bio-Rad dye-binding assay[13].

Preparation of DNA fragment

The DNA fragment of HS2 for the gel mobility shift assays was a 285 bp HaeIII-XbaI fragment (from -10965 to -10681 bp), and it was 5'-end-labelled with (γ-P^{32})ATP (3000 Ci/mmol) using T4 polynucleotide kinase. Five DNA competitive fragments were prepared: human β-globin promoter (from -112 to +20 bp); human $^{A}\gamma$-globin promoter (from -399 to +34bp); human ε-globin promoter (from -177 to +30 bp); competitor I (synthetic oligonucleotides corresponding to the β-globin promoter from -92 to-84 bp, 5'CACACACCCTAA 3') and competitor II (synthetic oligonucleotides containing the binding-motif of GATA-1,5'GTTGCAGATAGACATT 3').

Gel mobility shift assay

Gel mobility shift assays were carried out as described by Strauss and Varshavsky[14]. A 285 bp HaeIII-XbaI fragment of HS2 was used as a probe. Approximately 5 fmol of end-labeled DNA were incubated for 30 min on ice with 2 to 6 μg of nuclear extracts. Poly dI.dC (1 μg) was added as a non-specific competitor. DNA-protein complexes were resolved by 4 % non-denaturing polyacrylamide gel electrophoresis:

DNase I footprinting assay

A DNA probe (from -113 to +20 bp) was labeled at 5'-end with T4 polynucleotide kinase. The DNase I footprinting assay was performed according to the conditions described by Berg et al[15].

RESULTS

A stage-specific protein factor identified in the nuclear extract of mouse fetal liver at d 18 of gestation

The pattern of expression of mouse β-like globin genes is quite analogous to that of human's. Experiments with transgenic mice showed that human ε-globin gene was expressed in the mouse yolk sac. Human γ-globin genes were expressed in the mouse yolk sac and early fetal liver, and were silenced after d 16 of gestation. As the mouse adult β^{maj}-globin gene, human β-globin gene was expressed in the mouse fetal liver after d 11 of gestation and in adult bone marrow. Later it rose rapidly to maximum level by d 16 of gestation, and remained in the adult stage[16]. According

to these data, mouse haematopoietic tissues at d 10, d 14 and d 18 of gestation represent embryonic, fetal and adult haematopoietic tissues. Baby mouse liver and adult mouse brain represent non-erythroid tissues.

In gel mobility shift assay, we found that the patterns of shift band at different developmental stages were different. One major band (band A) in the nuclear extract from mouse fetal liver at d 18 of gestation was detected (Fig 2, lane 3). However, it couldn't be detected in the nuclear extract from mouse haematopoietic tissues at d 10 and d 14 of gestation (Fig 2, lanes 1-2), and in the nuclear extract from baby mouse liver and adult mouse brain (Fig 2, lanes 4-5). These data demonstrated that this protein factor (band A), termed LPF-β, might be a tissue-specific and adult stage-specific protein factor, which could bind to the HS2 region of β-globin LCR.

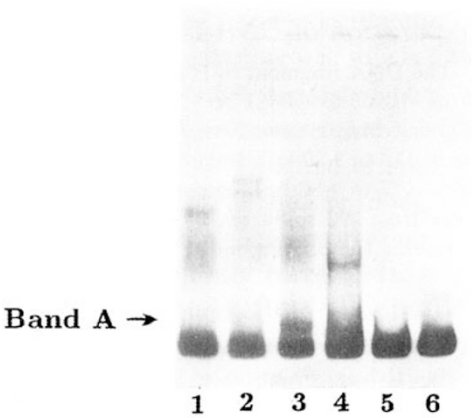

Fig 2. Gel mobility shift assays of the HS2 region of human β-LCR with the nuclear extracts from different mouse tissues
A 285 bp HaeIII-XbaI HS2 fragment was 5'-end labeled as a probe. Lanes 1-3: labeled fragment with 2 μg of the nuclear extract from mouse fetal liver at d 10, d 14 and d 18 of gestation. Lane 4: labeled fragment with 2 μg of the nuclear extract from baby mouse liver. Lane 5: labeled fragment with 2μg of the nuclear extract from adult mouse brain. Lane 6: labeled fragment without nuclear extract.

Analysis of trans–acting factor binding to HS2 by competition assays

For investigating the interaction between HS2 of β-LCR and the β-globin promoters, competitive gel mobility shift assays were performed with the nuclear extract of mouse fetal liver at d 18 of gestation. The Hae III-XbaI fragment of the HS2 region was used as a probe. Unlabeled competitors, human ε, $^{A}\gamma$, β-globin promoter fragments, were added at 100 fold molar excess. We observed that band A could be competed by addition of unlabeled human β-globin promoter fragment (Fig 3, Lane 3), but not by addition of unlabeled human $^{A}\gamma$ or ε-globin promoter fragments (Fig 3, lanes 4-5 respectively). Since human β-globin gene could be expressed in transgenic mouse fetal liver at d 18 of gestation, and LPF- β could interact with HS2 and adult β-globin promoter, but not interact with embryonic ε-globin promoter or fetal $^{A}\gamma$-globin promoter, we speculated that the stage-specific protein factor LPF- β might play an important role in human β-globin gene expression.

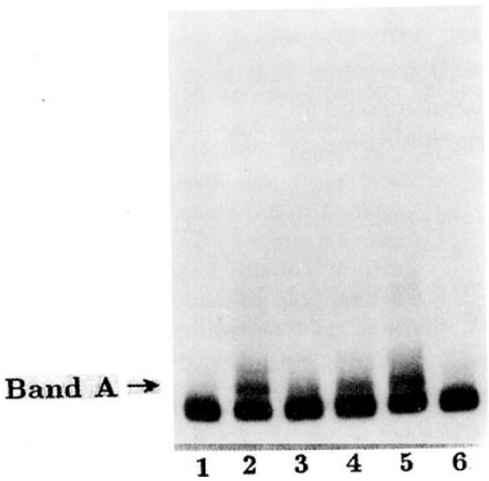

Fig 3. Gel mobility shift assays of LPF-β factor binding to the HS2 region, competed by various promoters of β-like globin genes A 285 bp HaeIII-XbaI HS2 fragment was 5'-end labeled as a probe. Lanes 1 and 6: labeled fragment without nuclear extract. Lane 2: labeled fragment with 2μg of nuclear extract. Lane 3: labeled fragment with 2μg of nuclear extract. Unlabeled human β-globin promoter fragment (from -112 to +20 bp) was added as a competitor at 100 fold molar excess. Lane 4: labeled fragment with 2μg of nuclear extract. Unlabeled human $^{A}\gamma$-globin promoter fragment (from -399 to +34 bp) was added as a competitor at 100 fold molar excess. Lane 5: labeled fragment with 2μg of nuclear extract. Unlabeled human ε-globin promoter fragment (from -177 to +30 bp) was added as a competitor at 100 fold molar excess.

The precise binding site of the protein factor LPF-β

In order to determine the precise binding sites of the protein factor LPF-β in human β-globin promoter and the HS2 region, DNase I footprinting protection assays were performed. In Fig 4, One strong protection region (from -90 to -66 bp) was only detected in the nuclear extract at d 18 of gestation (Fig 4, Lanes 6-7). These data demonstrated that a stage-specific factor in the nuclear extract from mouse fetal liver at d 18 of gestation could also specifically bind to the region of the β-globin promoter (from -90 to -66 bp). Meanwhile, we observed that both proximal CACCC box and CCAAT box were included in this protected region.

Comparing this protected region with the sequence of HS2, we found that a sequence (5' CACACCCTA 3', from-92 to -84 bp) of human β-globin promoter was also present in HS2 (from -10845 to -10853 bp). In order to determine the binding-site of LPF-β factor in the HS2 region, the competitive gel mobility shift assays were carried out with two synthetic oligonucleotides. Competitor I was 5' CACACACCCTAA 3', contained in both human β-globin promoter (from -92 to -66 bp) and the HS2 region (from -10845 to -10853 bp). Competitor II was 5' GTTGCAGATAGACATT 3', which contained GATA-1 binding-motif. We observed that the shift band A was competed by competitor I (Fig 5, Lanes 3-4), not by competitor II (Fig 5, Lane 2).

The results of the footprinting assays and competitive gel shift assays demonstrated that the stage-specific factor LPF-β bound to the common sequences in both human β-globin promoter and HS2. The common sequences were 5' CACACCCTA 3'. Therefore, we suggested that the interaction between human β-globin promoter and HS2 of β-LCR through this stage-specific factor LPF-β might partly

account for the transcription switching from fetal (γ) to adult (β) globin gene.

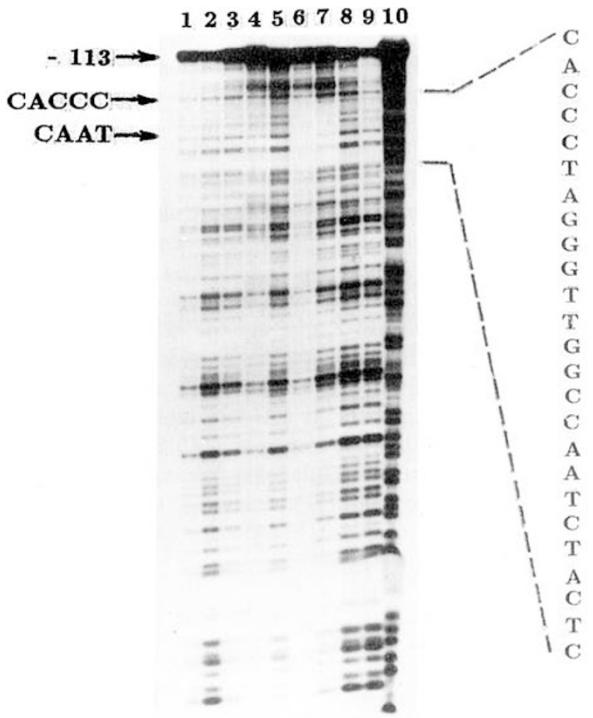

Fig 4. DNaseI footprinting analysis of the human β-globin promoter The probe (from -112 bp to +20 bp) was 5'-end labeled. Lanes 1 to 3: Labeled fragment without nuclear extract. Lanes 4 to 5: Labeled fragment with 8 and 12μg of nuclear extract from mouse fetal liver at d 10 of gestation respectively. Lanes 6 to 7: Labeled fragment with 10 and 20μg of nuclear extract from mouse fetal liver at d 18 of gestation respectively. Lanes 8 to 9: Labeled fragment with 7 and 14μg of nuclear extract from baby mouse liver respectively. Lane 10: A+G ladder.

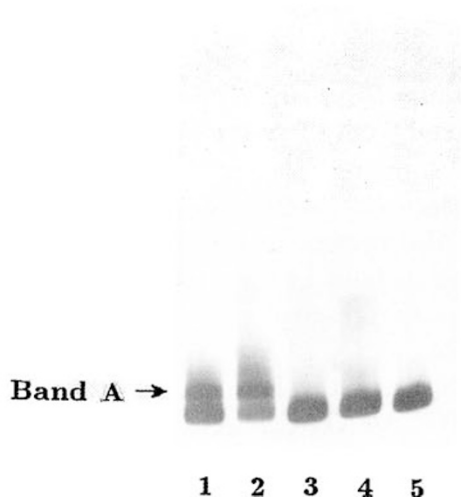

Fig 5. Gel mobility shift assays of LPF-β binding to the HS2 region, compet oligonucleotides containing CACC and GATA-1 binding motif
A 285 bp HaeIII-XbaI HS2 fragmer 5'-end labeled as a probe. Lanes 1: l fragment with 2μg of nuclear extract 2: labeled fragment with 2μg of nucle tract. Unlabeled competitor II was at a 100 fold molar excess. Lanes 3 beled fragment with 2μg of nuclear e Unlabeled competitor I was added a 50 fold molar excess respectively. L labeled fragment without nuclear e

DISCUSSION

Four erythroid-specific DNase I hypersensitive sites are located 6 to 20 kb upstream of the ε-globin gene in the human β-globin gene family. These sequences are critical for high-level expression of human ε, γ and β-globin genes[2-4]. They function as powerful enhancers that stimulate globin gene expression. However, how LCR interacts with promoters in the developmental regulation of globin gene

expression is still unclear.

Here we identified an erythroid-specific and stage-specific protein factor (LPF-β) from the nuclear extract of mouse fetal liver at d 18 of gestation. LPF-β factor could bind to the HS2 region, and it could be competed by human β-globin promoter, not by human ε- and $^{A}\gamma$-globin promoters. Furthermore, our data demonstrated that the binding-motif of LPF-β factor is 5'CACACCCTA 3', which is located in both the HS2 region (from -10845 to -10853 bp) and human β-globin promoter (from -92 to -84 bp).

For several reasons, we suggested that LPF-β factor might play an important role in the regulation of human adult β-globin gene switching. First, LPF-β is a stage-specific protein factor detected in the nuclear extract from mouse fetal liver at d 18 of gestation. Experiments with transgenic mouse showed that human β-globin gene could be expressed in mouse fetal liver at this stage. Second, the binding-motif of LPF-β is located in both human LCR and β - globin promoter. Finally, the binding-motif of LPF-β factor in human β-globin promoter is very crucial. Naturally occurring point mutations (C→T, -88 bp; C→G, -87 bp) give rise to β-thalassemia[17-18].

Human β-globin promoter has two CACCC elements, one at -90, the other at -105, but other globin genes have only one. Three CACCC binding proteins have been identified (Sp1, B4 and EKLF) at the proximal CACCC box of human β-globin promoter. Sp1 is an ubiquitous regulatory protein factor. It has been shown to be capable of looping DNA[19-20]. B4 seems to be a fetal and adult erythroid regulatory protein, not an adult stage-specific protein factor like LPF-β[21]. In addition, the Erythroid Kruppel-like factor (EKLF) is a recently found erythroid-specific transcription factor that bind to the proximal CACCC box of human β-globin promoter. The binding-motif of EKLF is very similar to that of LPF-β factor[22-23]. However, LPF-β factor in the nuclear extract prepared from mouse fetal liver at d 18 of gestation is a tissue-specific and stage-specific factor, and it could only be competed by human β-globin promoter, not by human ε- or $^{A}\gamma$-promoter. These data suggested that LPF-β could specifically bind to the CACCC box both in human β -globin promoter and in the HS2 region of β-LCR. For these reasons, we suggested that LPF-β factor might differ from EKLF.

In the HS2 region, multiple regulatory elements were identified to be required for maximal enhancer activity. Such elements included Sp1, NF-E2, GATA-1 and USF binding-sites[24]. Deletion of each of these binding-sites significantly decreased human β-globin gene expression in transgenic mice. But all of these trans-acting factors were not stage-specific. According to our data, we speculate that when human β-globin gene is going to be expressed, the adult stage-specific protein factor LPF -β comes into play. It can bind to the common sequences within both the distal regulatory element (HS2 of β-LCR) and the proximal regulatory element (β-globin promoter). So we suggest that LPF-β factor may self-associate, bringing together distant DNA segments to loop DNA. The formation of such DNA loop structure

may increase the concentration of activators (such as GATA-1, NF-E2, Sp1, USF and LPF- β itself) at human β-globin promoter, and then may activate and maintain the expression of human β-globin gene.

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