Journal of Cellular Biochemistry 76:376–385 (2000)

ARTICLES

Both Locus Control Region and Proximal Regulatory Elements Direct the Developmental Regulation of β-Globin Gene Cluster

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Abstract Using ligation-mediated PCR and in vivo footprinting methods to study the status of DNA-protein interaction at hypersensitive site 2 of locus control region and β^{maj} promoter of erythroid cells of fetal liver and adult bone marrow, we found that during different developmental periods, the status of DNA-protein interaction at both hypersensitive site 2 and β^{maj} promoter changed significantly, and indicated that locus control region might function through a looping mechanism to regulate the expression of downstream genes, and that distal regulatory elements (locus control region, hypersensitive sites) as well as proximal regulatory elements (promoter, enhancer) of β-globin gene cluster participate in the regulation of developmental specificity. J. Cell. Biochem. 76:376–385, 2000.

Key words: β-globin gene; HS; LM-PCR; in vivo footprinting; developmental regulation; gene expression

β-globin gene cluster has been one of the favorite model systems for analyzing the control of gene expression, particularly developmental regulation. The β-like globin genes are expressed sequentially from 5′ to 3′ by the genome orientation in an erythroid-specific and developmental stage-specific manner during the erythroid development. Although clustered in distinct chromosomal loci, the expression of α-like and β-like globin chains is balanced. A region of four DNase I hypersensitive sites (HS) upstream of β-globin family has been extensively studied. This region can confer copy number-dependent, integration-independent, high level expression of linked globin genes in cell lines and transgenic mice, so it has been designated the locus control region (LCR) [Grosveld et al., 1987].

It is now known that individual HS activity of LCR is defined to its core sequences, and the HS core sequences of the LCR and the promoter of individual globin genes have quite a few binding sites for erythroid-specific as well as ubiquitous proteins. The involvement of specific DNA-protein interaction in the formation of HSs, in the assembly of basal transcription apparatus, and in the contact between LCR and downstream gene promoter is suggested by a large body of experimental evidence [Fraser et al., 1993].

Homologues to the human LCR have been examined in other species. HS1–4 has been isolated and sequenced from mouse genome. The mouse LCR has a function similar to human LCR's [Moon et al., 1990].

Several models have been proposed to explain the LCR activity. Among them, the looping proposal is the most convincing [Baron, 1997], although short of direct experimental evidence. The study of DNA-protein interaction is important and significant to elucidate the mechanism of LCR action and globin gene switching.

In this article, we followed the principle of "design at molecule, observe at whole animal," and used in vivo footprinting and ligation-mediated PCR (LM-PCR) to study DNA-protein interaction at HS2 of LCR and β-globin gene promoter of erythroid cells in different developmental periods to obtain information on the regulation of β-globin gene cluster.

Grant sponsor: National Natural Science Foundation of China; Grant numbers: 39470390 and 39893320; Grant sponsor: Science Foundation for Chinese Outstanding Youth; Grant number: 3952006.

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Received 29 June 1999; Accepted 29 July 1999

Print compilation © 2000 Wiley-Liss, Inc.
This article published online in Wiley InterScience, January 2000.

MATERIALS AND METHODS

Animal

Male Kunming adult mice were used to prepare bone marrow. Normal and healthy mice were mated to produce embryos. Then the pregnant mice were killed and the fetal livers prepared at 14 days post-coitus (dpc).

Preparation of Cell Suspension and Separation of Nucleated Erythroid Cells

Nucleated erythroid cells of fetal liver and bone marrow were obtained from 40%–70% percoll gradient separation. The purified erythroid cells were resuspended in DMEM media without serum, and the cell concentration was adjusted to 5×10^7/ml [Zhang et al., 1988].

In Vivo Methylation

The in vivo methylation of suspension cells was carried out according to the method introduced by Mueller and Wold [1995]. The final concentration of dimethyl sulfate (DMS) was 0.1%, incubated at 37°C for 2 min.

Extraction of DNA and In Vitro Methylation

Isolation of genomic DNA and in vitro methylation on control protein-free DNA were performed according to Mueller and Wold [1995].

Base-Specific DNA Cleavage

Guanine-specific piperidine cleavage of in vivo and in vitro methylated DNA was done according to molecular cloning [Sambrook et al., 1989].

LM-PCR

LM-PCR genomic footprinting was performed essentially as described by Garrity and Wold [1992]; the primers used were the same as those used by Reddy and Shen [1993].

Labeling Reaction and Sequencing PAGE

Finally, the PCR products were used as substrate in two cycles of labeling reaction. After extraction with phenol-chloroform and precipitation with ethanol, the labeled products were resuspended in 15 μl of sequencing formamide dye, applying 3.5 μl samples to 8 M urea-6% PAGE sequencing gels. Gels were dried and exposed to X-ray film at −70°C for 2–3 days [Garrity et al., 1992].

Statistics

After sequencing PAGE, the qualified gels were exposed to phosphate screen and scanned by PhosphorImager (Molecular Dynamics). The percent protection or hypersensitivity was determined by comparing the reactivity of individual G residues with the reactivity of corresponding G residues on the naked DNA lane (in vitro methylation control). Using several G residues nearby as a control that has no reactivity, the reactivity of control G residues was defined as 100%. G residues that were protected or hypersensitive 10%–40% were classified as weak hypersensitivity or weak protection. G residues having 40% or more reactivity were classified as strong hypersensitivity or strong protection. In order to verify the reproducibility of in vivo footprints, every set of reactions, sequencing PAGE, and PhosphorImager scanning was repeated at least twice under similar conditions.

RESULTS

We used LM-PCR and in vivo footprinting to study the status of DNA-protein interaction at both HS2 of LCR and β^{maj} promoter. The structure of mouse β-globin gene cluster, the arrays of cis-acting elements at HS2, and β^{maj} promoter are presented in Figure 1.

In Vivo Footprints of HS2

The mouse HS2, just like the human's, consists of CACC/GT, NFE2/AP1, and GATA elements which are the binding sites of Sp1 (or EKLF, BKLF/TEF2) [Asano et al., 1998; Kadonaga et al., 1987; Xiao et al., 1987], NFE2 (or AP1, Nrf2, LCRF1/Nrf1) [Andrews et al., 1993; Kataoka et al., 1995; Oyake et al., 1996], and GATA-1 [Trainor et al., 1996], respectively. The interaction of these cis-acting elements and relevant trans-acting factor is required for LCR formation and LCR function. In order to facilitate the description, "G" combining with number up (coding strand) or down (non-coding strand) was used to refer to individual "G" residue. The calculated volumes of individual residues are summarized in Table I.

CACC/GT Element

The sequence of this element and flanking region is as follows:

```
                  12   131415
TCTCTACTCCCCACCCTGTGGGTGTGTTCA
AGAGATGAGGGGTGGGACACCCACACAAGT
 1 2  3 4567 8910          11
```

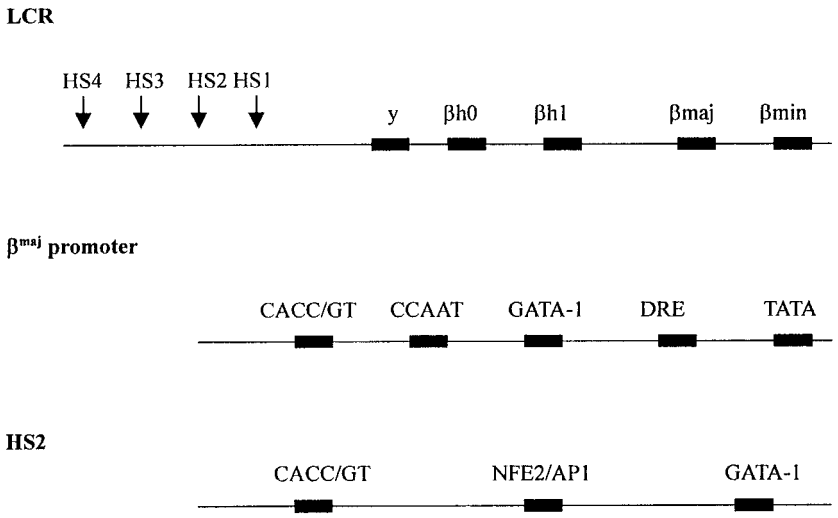

Fig. 1. The structure of mouse β globin gene family, cis-acting elements at HS2 and β globin gene promoter.

This CACC/GT motif, located upstream of HS2, was footprinted in both tissues, but the patterns of footprints were different. Results of in vivo footprints of the coding strand of this element are illustrated in Figure 2A. Compared to in vitro control, the reactivity of G residues was the same in both tissues, that is to say, G12 was hypersensitive, and G13, G14, and G15 were protected from methylation. On the other hand, in vivo footprinting pattern of the non-coding strand exhibited differences between these two tissues, G4, G5, G7, G8, G9, and G11 were protected in fetal liver. In bone marrow, however, only G4 and G11 were protected, whereas G3 was also strongly hypersensitive (Fig. 2B).

Although the reactivity of G residues of the coding strand was similar in both fetal liver and bone marrow, the results of the non-coding strand indicated that DNA-protein interaction at this element was different.

NFE2/AP1 Element

DNA sequence corresponding to this element is as follows:

```
9   10  1112 1314  15 1617   18 19
GCACAGCAGTGCTGAGTCATGCTGAGTCATGCTG
CGTGTCGTCACGACTCAGTACGACTCAGTACGAC
 1 2  3    4     5   6     7   8
```

The results of in vivo footprints at this site indicated that DNA-protein interaction mode at this element has changed in these two tissues. As shown in Figure 2, the reactivities of G residues in both fetal liver and bone marrow exhibited significant differences both from coding and non-coding strand. Results of coding strand showed that, in fetal liver, G11 was hypersensitive, while G12, G14, G15, and G17 were protected. However, in bone marrow, G11, G14, and G17 were hypersensitive, whereas G10, G16, G18, and G19 were protected (Fig. 2A).

The results of non-coding strand are shown in Figure 2B. In fetal liver, G1, G3, G5, and G7 were protected. G4 and G6 were strongly hypersensitive. In bone marrow, however, G1, G3, and G7 were protected, and G6 was strongly hypersensitive as in fetal liver. G4 and G5 did not exhibit any reactivities, and G2 was strongly hypersensitive only in bone marrow.

GATA Element

Located downstream of HS2 is the following sequence of GATA element:

```
TAGTCACGATAGACCCAGA (non-coding strand)
  1    2   3      4
```

This element was footprinted in both fetal and bone marrow, and demonstrated similar reactivity, that is to say, G1, G2, and G3 were protected in these two tissues (Fig. 2B). In vivo footprints of HS2 are summarized in Figure 3.

In Vivo Footprints of β^{maj} Promoter

The mouse β^{maj} promoter consists of five cis-acting elements: CACC/GT, CCAAT, GATA,

TABLE I. Quantitation of the Reactivity of G Residues[a]

	HS2			β-Globin gene promoter	
	Fetal liver	Bone marrow		Fetal liver	Bone marrow
CACC/GT			CACC/GT		
G3	100	200	G1	150	150
G4	60	70	G2	60	150
G5	70	100	G3	100	160
G7	70	100	G5	80	130
G8	50	100	G6	80	150
G9	70	100	G7	50	100
G10	90	100	G8	50	100
G11	60	60	G9	50	120
G12	150	150	G10	70	130
G13	80	80	CCAAT		
G14	80	80	G1	130	120
G15	70	60	G2	120	120
NFE2/AP1			G4	140	100
G1	70	80	G6	100	120
G2	100	170	G7	70	70
G3	80	80	GATA-1-DRE		
G4	200	100	G3	30	60
G5	70	100	G4	40	100
G6	230	160	G5	70	100
G7	40	80	G6	20	100
G8	100	100	G7	20	100
G10	100	50	G8	30	80
G11	130	120	G9	70	70
G12	50	100	G10	50	100
G14	40	130	G11	100	130
G15	30	90	G12	40	50
G16	100	80	G13	20	40
G17	60	120	G15	80	100
G18	100	60	G16	80	100
G19	90	50	G17	100	80
GATA-1			G18	120	120
G1	70	80	G19	80	120
G2	70	80	G20	70	100
G3	50	50			

[a]After sequencing PAGE, the qualified gels were exposed to phosphate screen and scanned by PhosphorImager (Molecular Dynamics). The protection or hypersensitivity percentage was determined by comparing the reactivity of individual G residues with the reactivity of corresponding G residues on the naked DNA lane (the reactivity of control G residues was defined as 100%).

DRE, and TATA. These elements are necessary for its appropriate transcription activation. CACC/GT, CCAAT, GATA, and TATA are recognized by Sp1 (or EKLF, BKLF/TEF2) [Asano et al., 1998; Kadonaga et al., 1987; Xiao et al.,1987], NFE3 or other factors [Ronchi et al., 1995; Evans et al., 1990], GATA-1 [Trainor et al., 1996], and TFIID [Evans et al., 1990], respectively. The two DRE elements are very crucial to the induction of globin gene expression, but the factor which recognizes and binds to this element is not yet identified [Stuve et al., 1990].

CACC/GT Element

This element,

```
  1 2         34
TAGAGCCACACCCTGGT
ATCTCGGTGTGGGACCA
    56 7 8910
```

located at the most 5′ end of the promoter, was important for cell-type specificity. G1, G2, and G3 were hypersensitive in bone marrow, G1

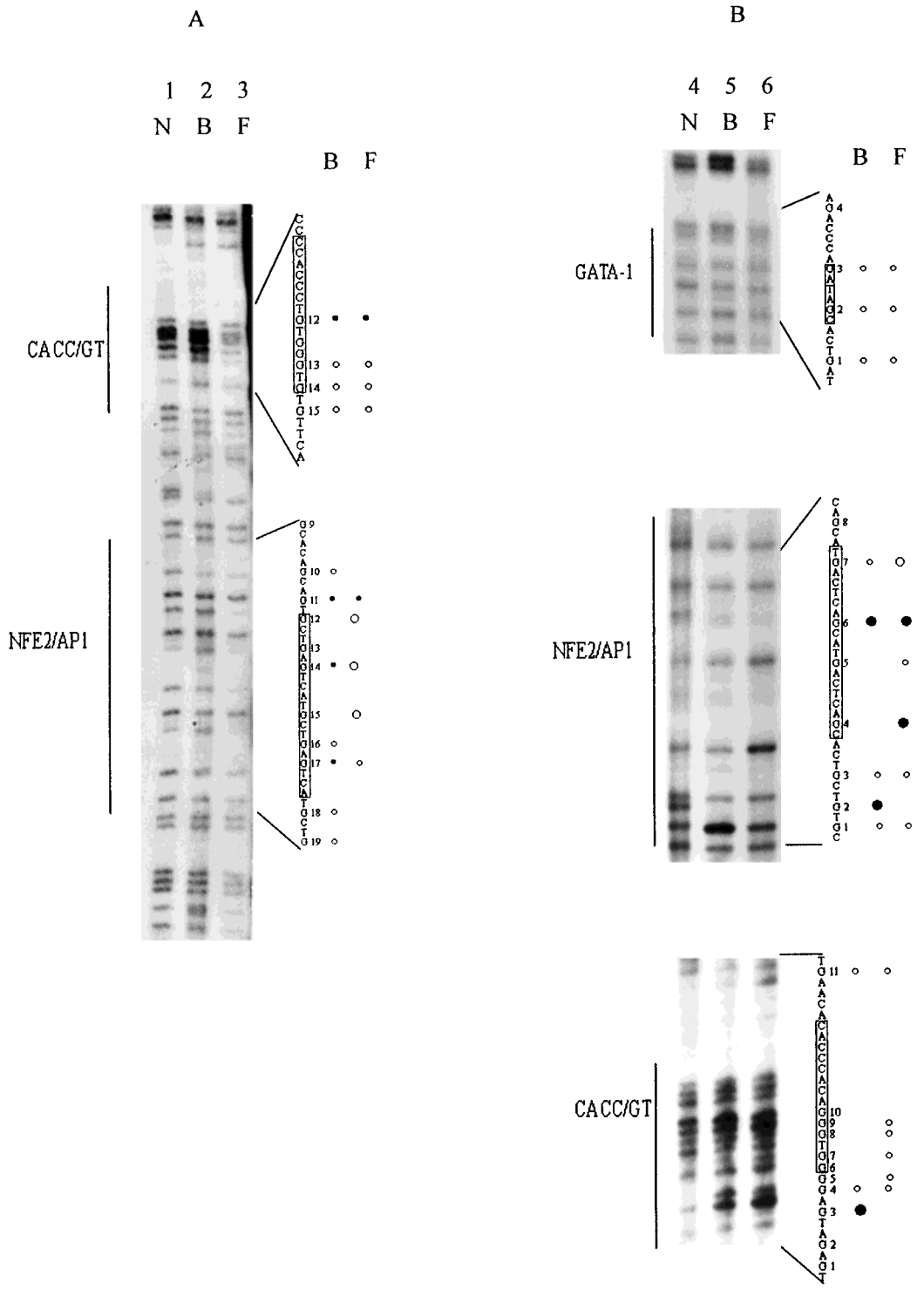

Fig. 2. Autoradiographs of in vivo footprints of LCR-HS2 of β globin gene cluster of mouse bone marrow and fetal liver. **A**: Coding strand. **B**: Non-coding strand. The vertical lines on the left represent different motifs, the corresponding DNA sequence is on the right. Open and solid circles denote protection and hypersensitivity, respectively. Sizes of the circles represent the relative extents of G reactivity. **Lanes 1,4**: Control sample of in vitro methylation (N). **Lanes 2,5**: Bone marrow sample of in vivo methylation (B). **Lanes 3,6**: Fetal liver sample of in vivo methylation (F).

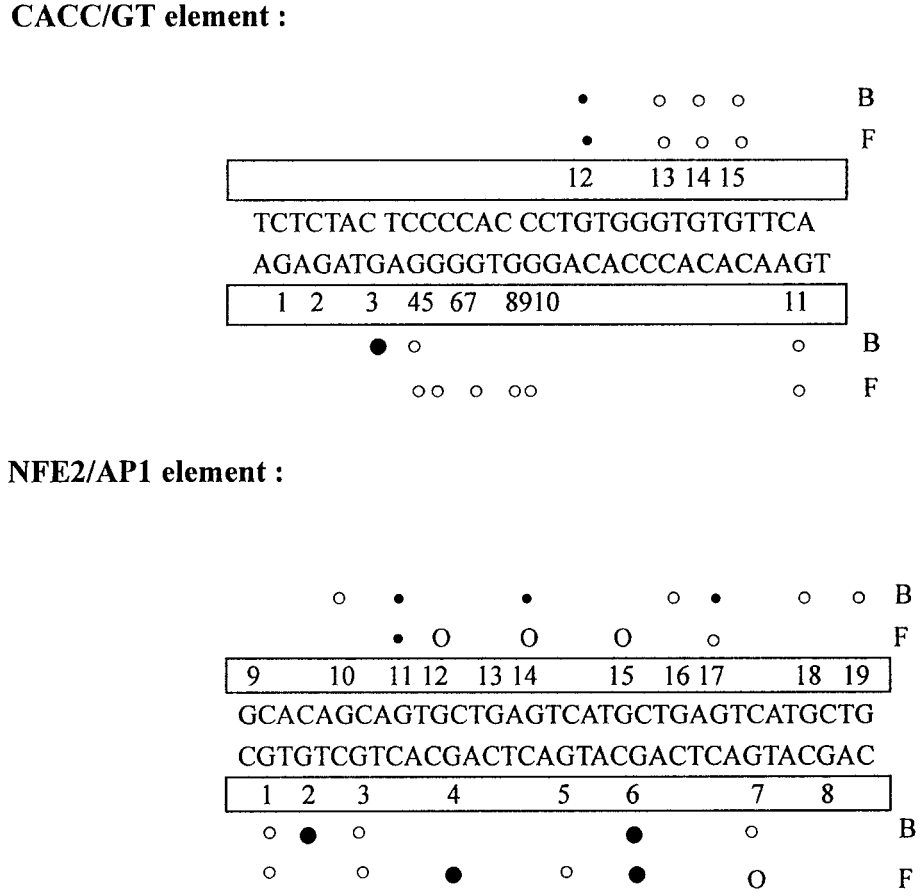

Fig. 3. Summary of in vivo footprinting of HS2 of LCR of bone marrow and fetal liver. B, bone marrow; F, fetal liver; ○, weak protection; ●, weak hypersensitivity. ○, strong protection; ●, strong hypersensitivity.

was also hypersensitive in fetal liver, and G2 was protected (Fig. 4A). The results of the non-coding strand also showed the difference of in vivo footprints of fetal liver vs. bone marrow. G5, G6, G9, and G10 were hypersensitive in bone marrow, while G5, G6, G7, G8, G9, and G10 were all protected in fetal liver (Fig 4B).

The difference between the G reactivity of the coding strand and that of the non-coding strand implied that different DNA-protein interactions formed in these two tissues.

CCAAT Element

The DNA sequence of this element and its flanking region is as follows:

```
  123       4
AAGGGCCAATCTGC
TTCCCGGTTAGACG
   56    7
```

As shown in Figure 4A, G1, G2, and G4 were hypersensitive in fetal liver, while only G1 and

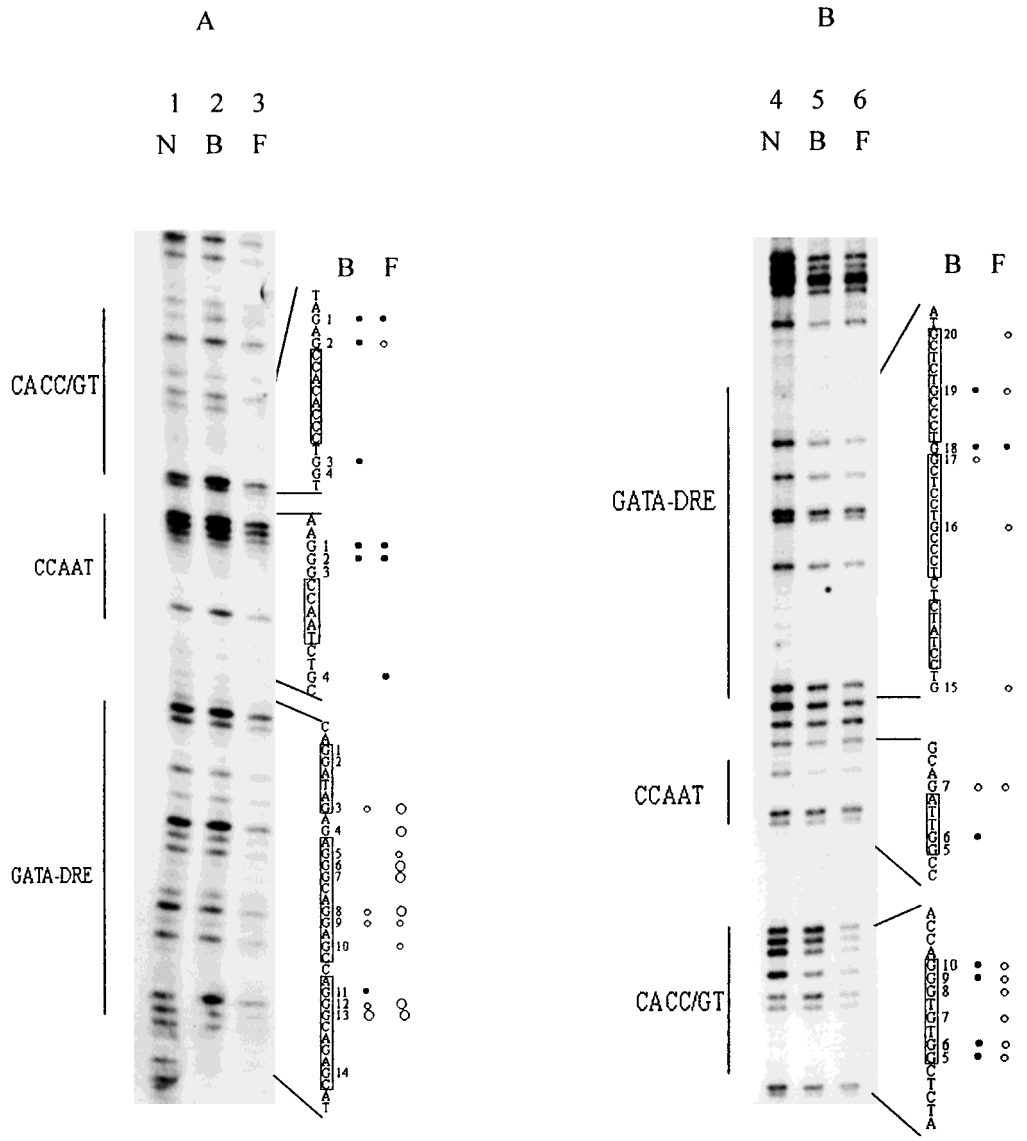

Fig. 4. Autoradiographs of in vivo footprints of β globin gene promoter of mouse bone marrow and fetal liver. **A**: Coding strand. **B**: Non-coding strand. The vertical lines on the left represent different motifs, the corresponding DNA sequence is on the right. Open and solid circles denote protection and hypersensitivity, respectively. Sizes of the circles represent the relative extent of G reactivity. **Lanes 1,4**: Control sample of in vitro methylation (N). **Lanes 2,5**: Bone marrow sample of in vivo methylation (B). **Lanes 3,6**: Fetal liver sample of in vivo methylation (F).

G2 were hypersensitive in bone marrow. The in vivo footprints of the non-coding strand showed that G7 was protected in both fetal liver and bone marrow, and an additional hypersensitivity of G6 was found only in bone marrow (Fig. 4B).

GATA-DRE Elements

```
  12   3 4 567  89 10 111213  14
CAGGATAGAGAGGGCAGGAGCCAGGGCAGAGCAT
GTCCTATCTCTCCCGTCCTCGGTCCCGTCTCGTA
15            16    1718  19   20
```

The significant difference of G reactivity was seen in this region. In vivo footprints of coding strand was shown in Figure 4. G3, G4, G5, G6, G7, G8, G9, G10, G12, and G13 were protected in fetal liver, however, only G3, G8, G9, G12, and G13 were protected in bone marrow. Additionally, G11 was hypersensitive in bone marrow. The results of the non-coding strand also exhibited the same trend. In fetal liver, G15, G16, G19, and G20 were protected and G18 was hypersensitive. G20 was protected and G18 was hypersensitive in bone marrow. In addition, an extra hypersensitivity (G19) was found in bone marrow.

The reactivity of G residues at these elements was different in these two tissues. On the

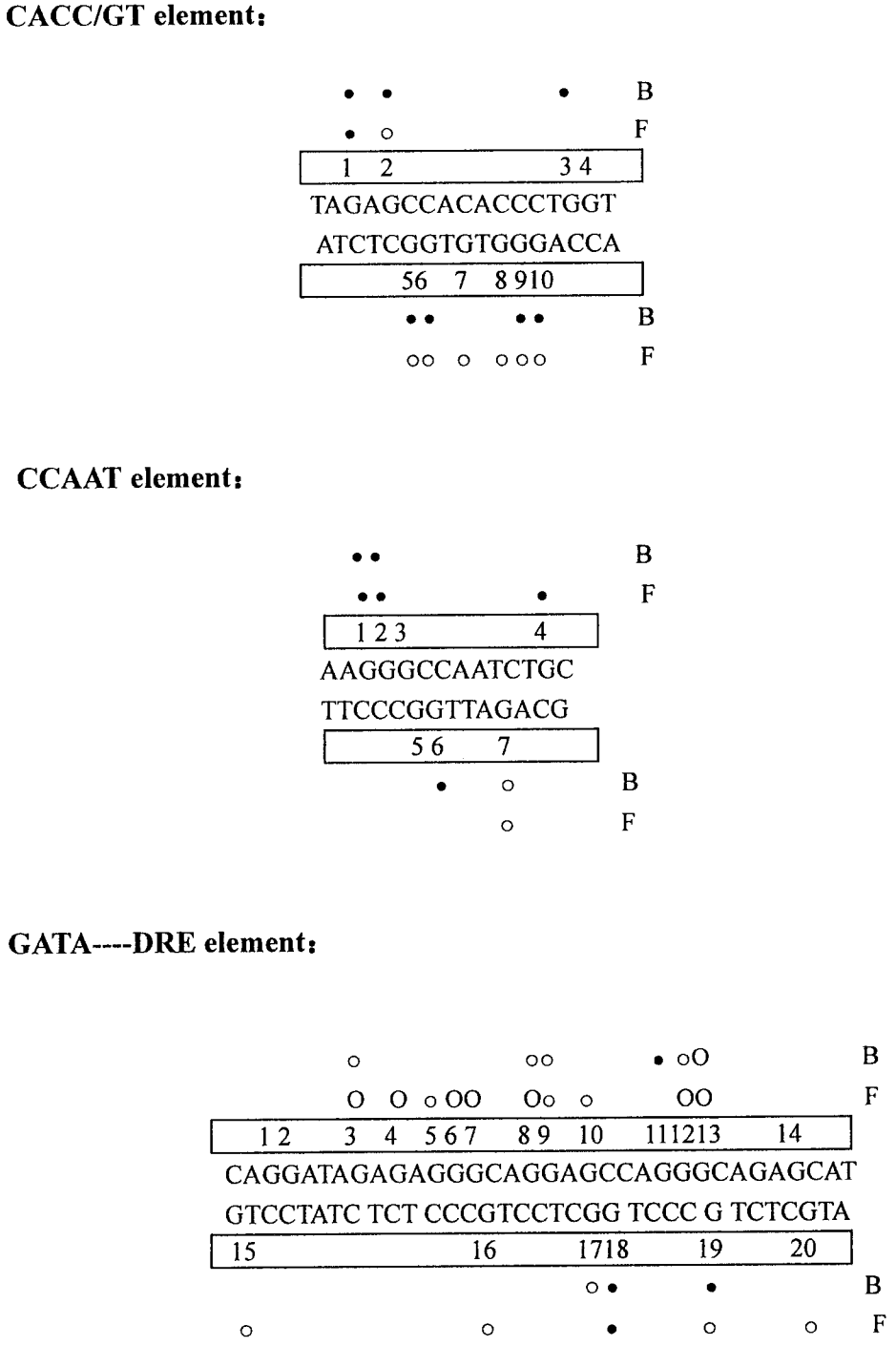

Fig. 5. Summary of in vivo footprinting of β-globin gene promoter of bone marrow and fetal liver. B, bone marrow; F, fetal liver; ○, weak protection; ●, weak hypersensitivity. ○, strong protection; ●, strong hypersensitivity.

coding strand, extensive protection occurred in fetal liver. Aside from protection, a significant difference was that G11 was hypersensitive in bone marrow. On the non-coding strand, some different G residues were protected in these two sites. It is interesting that the reactivity of G19 was protected in fetal liver, but hypersensitive in bone marrow.

In vivo footprints of β^{maj} promoter are summarized in Figure 5.

DISCUSSION

DNA-protein interaction in vivo can be revealed by in vivo footprinting study. The invention and its application in in vivo footprinting

study of LM-PCR have greatly improved the execution of DNA-protein interaction study in vivo [Mueller and Wold, 1989].

Mouse erythroid formation and differentiation begin in the blood island of the embryonic yolk sac at 7 dpc. The primitive nucleated erythroid cells appear in these blood islands and express embryonic globin genes. The site of erythropoiesis begins to shift to the fetal liver at 10–11 dpc, then to bone marrow, and adult-type globin genes begin to be expressed.

In present study, we used in vivo footprinting and LM-PCR to study the DNA-protein interaction mode at HS2 of LCR and β^{maj} promoter of mouse erythroid cells of different developmental periods (fetal liver and bone marrow). Percoll gradient separation was used to obtain purified nucleated erythroid cells to satisfy the need of this experiment. Our results showed that DNA-protein interaction mode at HS2 and β^{maj} promoter was different in these two tissues.

It is generally thought that fetal liver and bone marrow are two sites of erythropoiesis in adult mice. But the pattern of globin gene expression is significantly different in these two periods. β^{minor} gene expression is gradually down-regulated during ontogeny, while β^{maj} globin gene expression is up-regulated and dominates in late period of erythropoiesis. All these episodes must be accompanied and related to corresponding regulation process happening in cells. Now, it is widely accepted that the difference of trans-acting factor environment at different developmental stages results in the change of DNA-protein (cis-acting element—trans-acting factor) interaction, and affects the transcription activation of relevant globin genes at last.

The results described here demonstrated that the mode of DNA-protein interaction at both HS2 and β^{maj} promoter of erythroid cells of fetal liver and bone marrow was significantly different, implying that different DNA-protein interaction regulates the expression of relevant genes at these two periods. The difference of DNA-protein interaction may be quality-related or quantity-related or both.

Several models have been proposed to clarify the mechanism of LCR activity. At present, it is generally agreed that individual HS elements interact with one another to form a functional unit (holocomplex) which flip-flops via looping among globin genes, although no direct experimental evidence supports this view [Wijgerde et al., 1995]. At present, however, there is no universal agreement as to the role of LCR in the regulation of the globin gene switching during ontogenesis. According to one hypothesis, tissue- and stage-specificity may be conferred only by gene-proximal sequence (promoter and enhancer) of individual genes. The other hypothesis states that the switching process is determined by the combinatory interaction of gene-proximal regulatory elements and LCR [Baron, 1997; Orkin, 1995].

Our results demonstrated that DNA-protein interaction modes at regulatory elements of HS2 (CACC/GT, NFE2/AP1, GATA) and β^{maj} promoter (CACC/GT, CCAAT, GATA, DRE, TATA) of erythroid cells of both fetal liver and bone marrow were significantly different, and implied that LCR may function through a looping mechanism. DNA-Protein complex can form at HS2 and β^{maj} promoter at any given instance, and a looping may form between these two complexes mediated by protein-protein interaction. The change of trans-acting factor environment during development will necessarily result in the change of DNA-protein interaction at HSs or at promoter (or both). Because of the alternate influence between the two DNA-protein complexes, we can infer that the status of in vivo footprints at any regulatory element will also change. Our results are consistent with this view.

The expression of β-globin gene in transgenic mice with LCR-β-globin gene construct loses developmental specificity, individual HSs within the β-LCR contributes preferentially to the developmental regulation of specific globin genes [Behringer et al., 1990; Orkin et al., 1981; Wood, 1996]. The disruption of HS3 in YAC-transgenic mice differentially influences the expression of γ-globin gene during development, it means that the interaction mode between LCR (or individual HSs) and downstream genes is different at different developmental stage [Navas et al., 1998]. Human HS3 does not exhibit in vivo footprints in K562 cells [Ikuta et al.,1991. Strauss et al., 1992], it is footprinted in HU11 cells only after being induced with DMSO, so it seems that HS3 is developmentally regulated [Ikuta et al., 1996].

Although human HS2 has in vivo footprints throughout ontogenesis (K562 cells and human erythroblast) [Ikuta et al., 1991, 1996; Reddy et al., 1991, 1994], the status of in vivo footprints in K562 cells is different before and after induction [Ikuta et al., 1991]. The in vivo footprints of CACC/GT element of HSs in HU11 cell line which expresses γ globin gene are different

from that in HU11 cell line which expresses β globin gene [Ikuta et al., 1996]. Our results, combined with other experimental evidence described above, support the view that LCR participates in developmental regulation of β-globin gene cluster.

ACKNOWLEDGMENT

The authors thank Professor Jing-bo Zhang for his valuable suggestion on the preparation of this paper.

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