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A DNA-binding protein factor in K562 nuclear extract interacts with positive control region (PCR) in the 5'-flanking sequence of human β-globin gene

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ABSTRACT

It has been known that there are at least three regulatory regions (NCR1, NCR2 and PCR) in the 5'-flanking sequence (from -610 bp to +1 bp) of human β-globin gene and that the function of PCR is unique to the human erythroleukemia (K562) cells. Here we have detected a DNA-binding protein factor (termed NFEa) in K562 cells, which can bind specifically to the PCR of human β-globin gene. The sequence of the binding site is 5' ACTGATG3' (between -222 bp and -216 bp). The NFEa is erythroid-specific and perhaps specific for K562 cells. It seemed that this factor differed from the erythroid-specific transcriptional factor (NFE-1) using competition assay. The presence of the NFEa further supported that the function of the cis-acting element PCR was specific for K562 cells, and helps us to understand the mechanism of the regulation of the expression of human β-globin gene in the human K562 cells.

Key words: *human β-globin gene, positive control region, erythroid-specific factor.*

INTRODUCTION

The regulation of transcription of eukaryotic genes has been shown to be controlled by the sequence-specific binding of trans-acting regulatory factors to cis-acting DNA regulatory elements (such as promoters, enhancers and silencers)[1,2]. The level of transcription is thought to be controlled by direct or indirect interaction of the regulatory protein factors binding to cis-acting regulatory elements[3,4]. The human β-globin gene family is a model system to study the regulation of eukaryotic

gene expression, since it represents a group of genes expressed in a tissue-specific manner and are switched on and off in suceession at specific developmental stages. The understanding this switch could provide insights leading to unique approaches to the treatment of certain human diseases involving hemoglobin, including sickle cell disease, β thalassemia and erythroleukemia. The developmental stage-specific and tissue-specific expression of hemoglobin genes are considered to be probably mediated by some sequence-specific DNA-binding factors[5].

The human β-globin gene was cloned and sequenced in 1983[6]. It has been identified that there are at least two negative control regions(NCR1 and NCR2) and one positive control region(PCR) in 5'-flanking sequence (from -610 bp to +1 bp) of the human β-globin gene[7,8]. NCR1 is located between -610 bp and -490 bp 5' to the cap site of human β-globin gene, NCR2 between -338 bp and -233 bp, and PCR between -233 bp and -185 bp. Both NCR1 and NCR2 also act as classical silencers. In addition, it has been demonstrated that two protein factors(BP1 and BP2) in the K562 nuclear extract could bind to the negative regulatory regions[2]. However, little has been known about the function of the PCR of human β-globin gene yet.

In previous studies[2,7,8], it was known that the NCR1 and NCR2 could function in all three cell lines (K562 cells, MEL cells and R1610 cells). However, the function of the PCR in those cell lines was quite different. So it seemed that the PCR function perhaps was specific for K562 cells, and the PCR might play a critical role in the expression of human β-globin gene in K562 cells. In addition, we know that K562 cells are human erythroleukemia cells which only express the human embryonic arid fetal globin genes(ε- and γ-globin genes) but not adult β-globin gene. MEL cells are mouse erythroleukemia cells which can express adult β-globin gene. R1610 cells are Chinese hamster cells(non-erythroid cells).

In this study, we attempt to examine why the function of the PCR is specific for K562 cells. We have detected one DNA-binding protein factor (NFEa) in the K562 nuclear extract, which could bind to the PCR of human β-globin gene. The binding sequence is 5'ACTGATG3' (between -222 bp and -216 bp).

MATERIALS AND METHODS

Preparation of nuclear extracts

Nuclear extracts were prepared from K562 cells, R1610 cells and mouse fetal liver (MFL, 10 and 19 days of gestation) by a modification of the methods of deBoer[9] and Gorski[10]. The protein concentration was determined using the Bio-Rad dye-binding assay[11].

Preparation of DNA fragments

The DNA fragment of PCR for the gel mobility shift assays was a 30 bp RsaI-DdeI fragment (-223 bp to -194 bp) from the pβGLCAT plasmid [2] and 5'-end-labelled with T_4 polynucleotide kinase and γ-^{32}P-ATP. The DNA fragment for DNase I footprinting assays was a 166 bp Hinf I-Hph I fragment (-279 bp to -114 bp) containing the partial NCR2, complete PCR and partial promotor region of human β-globin gene. The Hinf I-Hind III fragment (389 bp) was first obtained from the pβGLCAT plasmid and labeled with T_4 polynucleotide kinase and γ-^{32}p-ATP, and this

5'-end-labeled DNA fragment was further digested by Hph I and isolated by electrophoresis on 5% polyacrylamide gels.

Footprinting protection assays

DNase I footprinting assays were performed according to the method previously described[12]. A 166 bp end-labeled (at one terminus) Hinf I-Hph I fragment (-279 bp to -114 bp) was used as the footprinting protection probe.

Gel mobility shift assays

Gel electrophoresis mobility shift assays were done according to the method by Strauss and Varshavsky[13]. Approximately 5 fmol of end-labeled DNA fragment was incubated for 40 min on ice with 2 to 6 μg of nuclear extracts. KCl was added to a final concentration of 100 m*M*, and 1 μg of poly(dI·dC) was used as non-specific competitor in 25 μl of the reaction volume. The DNA-complexes were resolved by 4% non-denaturing polyacrylamide gel electrophoresis.

In competition assays, the unlabeled DNA fragment as competitor was added at the same time as the probe DNA.

RESULTS

Characterization of the protein factor binding to the PCR by mobility shift assays

In previous studies[2,7,8], it has been known that the function of the PCR of human β-globin gene in different cell lines(K562 cells, R1610 cells, and MEL cells) was quite different because the βGLCAT expression could be detected in MEL cells and R1610 cells, but not in K562 cells after the PCR was deleted. So it seemed that the PCR function was specific for K562 cells. But, what is the reason for this? We considered that some protein factors which could interact with the PCR might exist in K562 cells. In order to testify this assumption, we used the gel mobility shift assays. Nuclear extracts have been prepared from two cell lines (K562 cells and R1610 cells) and mouse fetal liver (10 and 19 days of gestation). We observed one major band in K562 cells and one weak band in MFL cells (19 days of gestation) but not any band in R1610 cells (Fig 1). So we considered that one protein factor which could bind to the PCR did exist in K562 cells.

Analysis of the protein factor binding to the PCR by competition

NFE-1[14] (also termed GF-1[15], Eryf1[16]) was considered to be the first erythroid-specific regulatory protein which has been extensively studied. The recognition sequence for NFE-1, 5'WGATWR3' (W=A or T, R=A or G), appeared in the promoters and enhancers of all chicken globin genes and probably for all human and mouse globin genes as well. Point mutations which eliminated the binding of NFE-1 to promoter or enhancer sites decreased the expression of the corresponding gene[17]. So NFE-1 appeared to be a very important transcriptional activator in the regulation of hemoglobin genes.

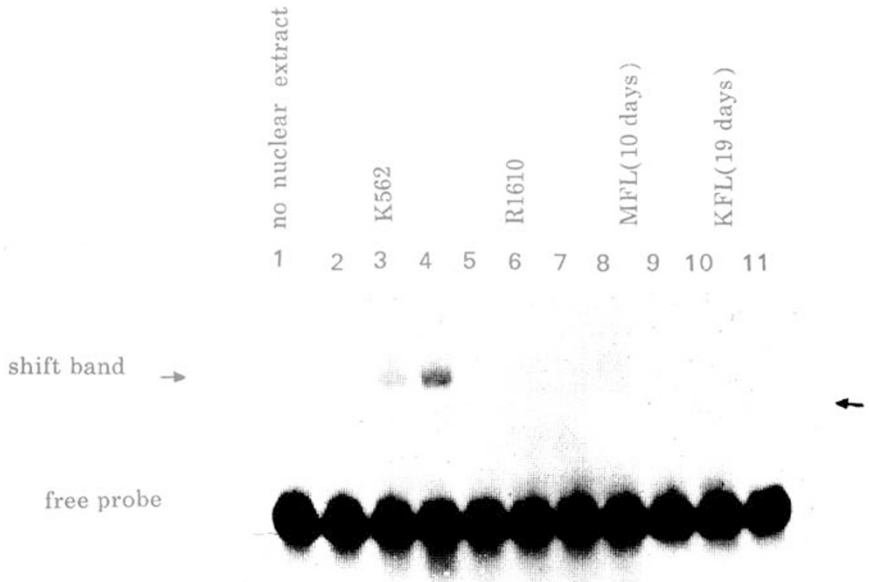

Fig 1. △ Gel mobility shift assays of the positive control region of human β-globin gene with different nuclear extracts(see Materials and Methods)
The probe (from -223 bp to -194 bp) was 5'-end labeled. Lane 1: no nuclear extract; lanes 2, 3 and 4: 2, 4, 6 μg of K562 nuclear extract, respectively; lanes 5, 6 and 7: 2, 4, 6 μg; of R1610 nuclear extract, respectively; lanes 8 and 9: 4, 6 μg of MFL nuclear extract 10 days of gestation), respectively; lanes 10 and 11: 4, 6 μg of MFL nuclear extract (19 d of gestation), respectively.

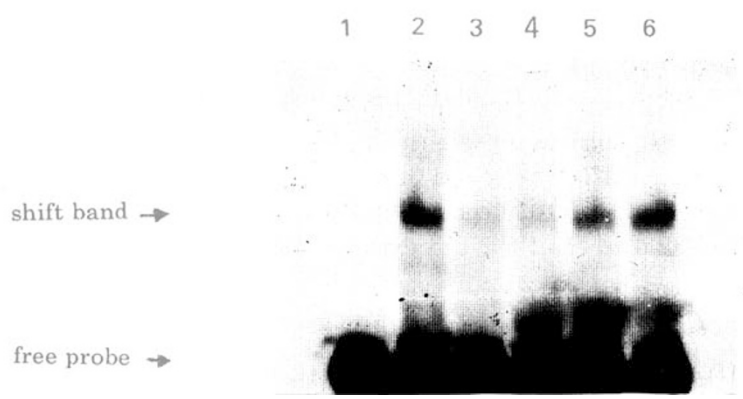

Fig 2. △ Gel mobility competition assays of the positive control region of human β-globin gene
The 5'-end labeled fragment was used, which was just same as the probe used in Fig 1. The sequence of a 30 bp PCR probe is 5'TACTGATGGTATGGGGCCAAGATATATC3', which contains one NFE-1 binding site. Competitor I is unlabeled 30 bp Rsa I-Dde I fragment (just same as the PCR probe). Competitor II is unlabeled 133 bp Dde I-Hind III fragment containing one NFE-1 binding site (5'CAATCT3') from -75 bp to -70 bp. Lane 1, no nuclear extract; lanes 2-6, 6 μg of K562 nuclear extract; lane 2, no competitor DNA; lanes 3 and 4, competitor I (added at 5 and 10 fold molar excess, respectively); lanes 5 and 6, competitor II (added at 50 and 100 fold molar excess, respectively).

Since the PCR of human β-globin gene also contains the binding site for NFE-1, we wondered if the protein factor was the same as the NFE-1. Therefore, we performed the gel mobility shift competition assay. Two unlabeled DNA fragments were used as competitors. Competitor II is a 133 bp Dde I-Hind III fragment (-113 bp to +20 bp) containing one binding site for NFE-1 (5'CAATCT3', -75 bp to -70 bp), and competitor I is a 30 bp Rsa I-Dde I fragment which is just the same as the PCR probe.

The results of competition assays showed that the mobility shift band of the protein factor binding to the PCR could not be competed by the unlabeled fragment (competitor II) at a 50-100 fold molar excess, but could be completely competed by the unlabeled fragment (competitor I) at a 5-10 fold molar excess (Fig 2). So we considered that this protein factor was quite different from the erythroid-specific transcriptional factor (NFE-1), and it was perhaps another erythroid-specific DNA-binding protein factor which may be specific for K562 cells. We designated this nuclear factor from the K562 erythroid cells NFEa.

Analysis of the PCR by DNase I footprinting

In order to determine the precise binding site of NFEa to the PCR of human β-globin gene, we performed DNase I footprinting protection assays using the 166 bp Hinf I-Hph I fragment containing the partial NCR2, complete PCR and partial promoter region of human β-globin gene as the footprinting probe.

In Fig 3(A, B), one protection region extending from -222 bp to -216 bp is shown only in K562 cells but not in R1610 cells or MFL cells (19 days of gestation). The DNA sequence of the protection region is 5'ACTGATG3'. In addition, we also observed another protection region located in the partial NCR2 in MFL cells (19 days of gestation).

The results of this footprinting assay further proved that NFEa can specifically bind to the PCR and the function of the PCR may be specific for K562 cells. Since the binding site of NFEa is located in the positive control region (PCR) of human β-globin gene, we considered that this NFEa was a transcriptional activator and it may play a critical role in the proximal regulation of human β-globin gene expression.

DISCUSSION

In previous studies, we observed that the function of the PCR in 5'-flanking sequence of human β-globin gene in three cell lines (K562 cells, MEL cells and R1610 cells) is quite different using the method of CAT assays[2,7,8]. In order to comprehend why the function of the PCR is specific for K562 cells, gel mobility shift and DNase I footprinting assays were performed using different nuclear extracts from two cell lines (K562 and R1610 cells) and mouse fetal liver. We have observed that one protein factor in the nuclear extract of human K562 cells, which we referred to as NFEa, can specifically bind to the PCR of human β-globin gene. So we considered

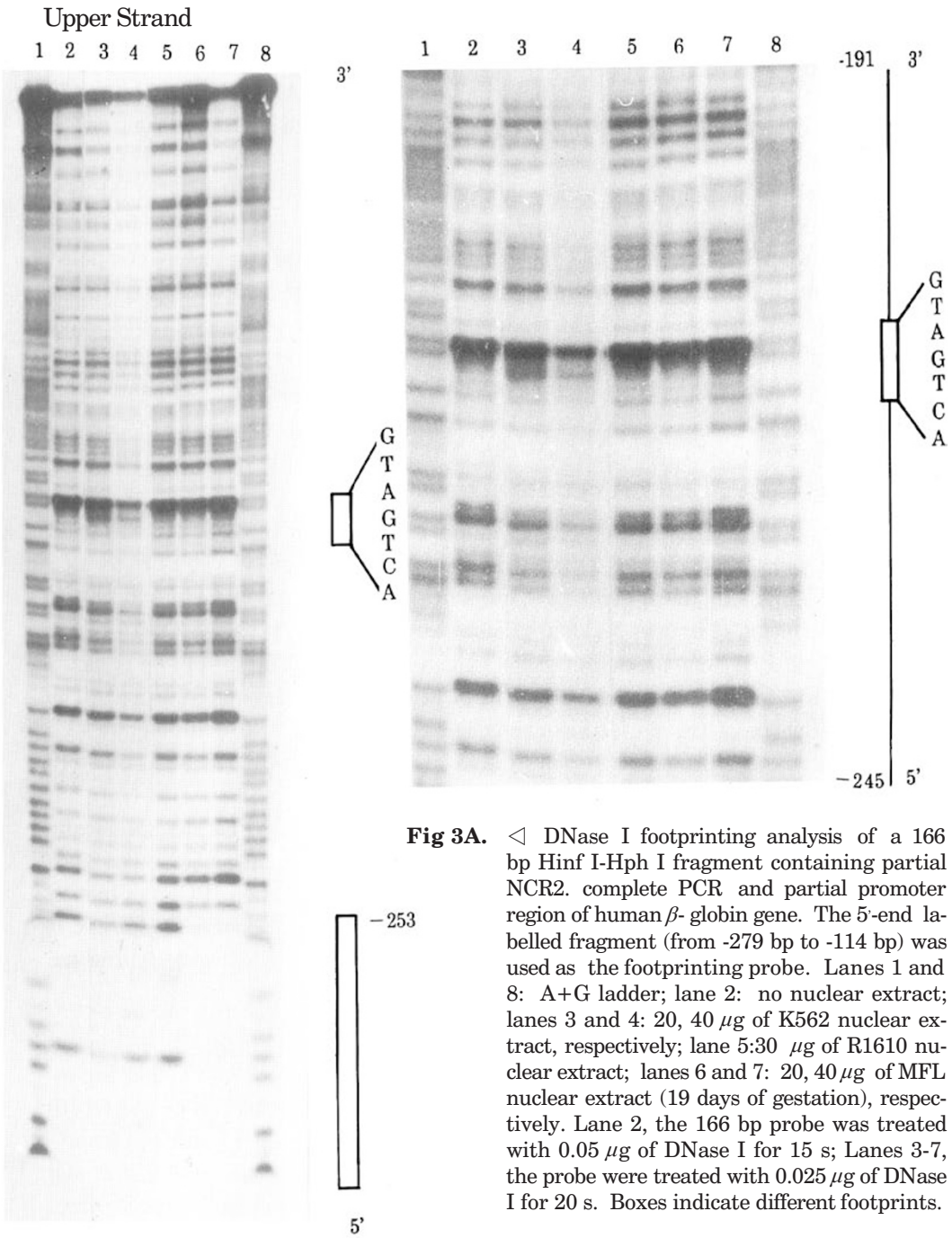

Fig 3A. ◁ DNase I footprinting analysis of a 166 bp Hinf I-Hph I fragment containing partial NCR2. complete PCR and partial promoter region of human β- globin gene. The 5'-end labelled fragment (from -279 bp to -114 bp) was used as the footprinting probe. Lanes 1 and 8: A+G ladder; lane 2: no nuclear extract; lanes 3 and 4: 20, 40 μg of K562 nuclear extract, respectively; lane 5:30 μg of R1610 nuclear extract; lanes 6 and 7: 20, 40 μg of MFL nuclear extract (19 days of gestation), respectively. Lane 2, the 166 bp probe was treated with 0.05 μg of DNase I for 15 s; Lanes 3-7, the probe were treated with 0.025 μg of DNase I for 20 s. Boxes indicate different footprints.

B. △ The magnified picture a part of the boxes shown in Fig 3A.

that the existence of this erythroid-specific DNA-binding factor(NFEa) only in K562 cells perhaps was the major reason why the function of the PCR is specific in K562 cells. Therefore, NFEa may play a critical role in the regulation (in proximal distance) of human β-globin gene in K562 cells, because the βGLCAT expression could be detected in MEL cells and R1610 cells while not in K562 cells if the PCR was deleted[7,8].

The competition assays have demonstrated that NFEa is distinct from the transcription activator, NFE-1. The recognition sequence (5'ACTGATG3') for NFEa is also different from that WGATWR (W=A or T, R=A or G) for NFE-1 using DNase I footprinting assays. Therefore, NFEa seemed to be another erythroid-specific nuclear transcriptional activator which can specifically bind to the PCR of human β-globin gene.

It is yet quite unclear why the human β-globin gene does not express in K562 cells. But it has been known that the lack of such an expression in K562 cells is not related to any major β-globin gene deletion or rearrangement, because normal K562 β-globin gene was demonstrated to be functional when used in a heterologous expression system such as COS cells or HeLa cells[5]. Therefore, the differential expression of β-globin genes in K562 cells appeared to be under the control of transacting factors[5]. From the result of our DNase I footprinting assays, we considered that NFE-1 was lower in K562 cells because we could not observe the protection region for NFE-1 using the K562 nuclear extract although the PCR of human β-globin gene contains the binding site for NFE-1. deBoer et al[9] also thought that the amount of NFE-1 was lower in K562 cells[9]. Since NFE-1 binding sites are present in a lot of regulatory regions (promoters and/or enhancers) for all chicken globin genes and probably for all human and mouse globin genes as well[18], this factor was thought to be a very potent transcriptional activator in erythroid cells. On the other hand, there are two negative regulatory proteins (BP1 and BP2) in K562 cells, which can bind to NCR1 and NCR2 in the 5'-flanking sequence of human β- globin gene[2], and these two negative control regions are located just adjacent to the positive control region(PCR). Because of the decrement of the very potent transcriptional activator(NFE-1) and the appearance of two negative regulatory factors, the β-globin gene could not be expressed under such condition, even though another erythroid-specific positive regulatory factor(NFEa) appeared in K562 cells. However, the β-globin gene could express in K562 cells only when one or two negative control regions were deleted. The molecular mechanism of the regulation of human β-globin gene expression in these cells still needs further exploration.

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REFERENCES

[1] Mitchell P, Tjian R. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science 1989; **245:**371-8.
[2] Berg PE, Williams DM, Qian RL, et al. A common protein binds to two silencers 5' to the human β-globin gene. Nucleic Acid Res 1989; **17:** 8833-52.
[3] Ptashne M. How eukaryotic transcriptional activators work. Nature 1988; 335: 683-89.
[4] Pugh BF, Tjian R. Mechanism of transcriptional activation by SP1 evidence for coactivators. Cell 1990;**61:** 1187-97.
[5] Wada Y, Noguchi TC. *In vitro* differential expression of human globin genes. J Biol Chem 1988; **263:** 12142-6.
[6] Poncz M, Schwartz E, Ballantine M, Surry S. Nucleotide sequence analysis of the $\delta\beta$-globin gene region in humans. J Biol Chem 1983; **258:**: 1599-609.
[7] Qian Ruo-Lan, Williams DM, Gong Y, et al. Regulation of β-globin gene expression by 5' DNA sequences. Blood 1986; **68** (Suppl 1): 77a.
[8] Qian RL, Williams DM, Cao SX, et al. Modulation of the β-globin gene promoter by 5' negative and positive control regions. Blood 1987; **70**(Suppl 1): 79a.
[9] deBoer E, Antoniou M, Mignotte V, Wall L, Grosveld F. The human β-globin promoter; nuclear factors and erythroid specific induction of transcription. EMBO J 1988; **7:** 4203-12.
[10] Gorski K, Carneiro M, Schibler U. Tissue-specific *in vitro* transcription from the mouse albumin promoter. Cell 1986; **47:** 767-76.
[11] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein- Dye binding. Anal Biochem 1976; **72:** 248-54.
[12] Qian RL, Chen YD, Song QB, Hu YL. The binding of HMG proteins to the 5' -flanking sequence of human β-globin gene. Science in China(Part B) 1992; **1:**62-8 (in Chinese).
[13] Strauss F, Varshavsky A. A protein binds to a satellite DNA repeat at three specific sites that would be brought into mutual proximity by DNA folding in the nucleosome. Cell 1984; **37:** 889-901.
[14] Wall L, deBoer E, Grosveld F. The human β-globin gene 3' enhancer contains multiple binding sites for an erythroid-specific protein. Genes & Dev 1988; **2:** 1089-100.
[15] Martin DIK, Tsai SF, Orkin SH. Increased γ-globin expression in a nondeletion HPFH mediated by an erythroid-specific DNA-binding factor. Nature 1989; **338:** 435-8.
[16] Evans T, Reitman M, Felsenfeld G. An erythroid-specific DNA- binding factor recognizes a regulatory sequence common to all chicken globin genes. Proc Natl Acad Sci USA 1988; **85:** 5976-80.
[17] Watt P, Lamb P, Squire L, Proudfoot NJ. A factor binding GATAAG confers tissue specificity on the promoter of the human ζ -globin gene. Nucleic Acids Res 1990; **18:** 1339-50.
[18] Evans T, Felsenfeld G, Reitman M. Control of globin gene transcription. Annu Rev Cell Biol 1990; **6:** 95-124.

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