SHORT COMMUNICATION

Identification of α NF- κ B site in the negative regulatory element(ϵ -NRAII) of human ϵ -globin gene and its binding protein NF- κ B p50 in the nuclei of K562 cells

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ABSTRACT

The developmental control of the human e-globin gene expression is mediated by transcription regulatory elements in the 5' flanking DNA of this gene. Sequence analysis has revealed a DNA motif (GGGGAATTTGCT) similar to NF- κ B consensus sequence resides in the negative regulatory element (-3028bp ~ -2902bp, termed ϵ -NRAII) 5' to the cap site of this gene. NRF DNA fragment (-3010bp ~ -2986bp) containing the NF- κ B motif similar sequence was synthesized and used in electrophoresis mobility shift assay (EMSA) and competitive analysis. Data showed that a protein factor from nuclear extracts of K562 cells specifically interacted with NRF DNA fragment. The synthetic NF DNA fragment (containing NF- κ B consensus sequence) could compete for the protein binding, but MNF DNA fragment (mutated NF- κ B motif) could not, suggesting that the binding protein is a member of NF- κ B/Rel family. Western blot assay demonstrated that the molecular weight of NF- κ B protein in the nuclei of K562 cells is 50ku. We suggested that NF- κ B p50 may play an important role in the regulation of human e-globin gene expression.

Key words: *Human e-globin gene, negative regulatory element, NF- κ B p50.*

INTRODUCTION

The human b-like globin genes are arranged as a cluster of five genes(ϵ , γ , δ and β) in the order of their temporal expression. The human embryonic ϵ -globin gene is expressed in the blood island of the embryonic yolk sac and is silenced completely at 6 ~ 8 w of gestation in the fetal liver [1]. Studies on transgenic mice suggested that the regulation of ϵ -globin gene expression is autonomous. The activating and silencing of e-globin gene expression rely on distal locus control region (LCR) and proximal regulatory elements[2, 3].

The NF- κ B /Rel family is composed of five distinct DNA-binding subunits (p50, p52, p65 (RelA), c-Rel, and Rel-B[4-6]). The different members of NF- κ B /Rel family can be associated into various homo- or heterodimers through a highly conserved N-terminal 300-aa region known as the rel homology domain. In monocytes, NF- κ B family has been shown as important transcription factor in the expression of cytokine genes, of cell-surface receptors and in the expression of human immunodeficiency virus. In those cells, DNA binding activity of NF- κ B can be detected without intentional stimulation[7].

Li et al identified a strong negative regulatory element ϵ -NRAII (-3028 ~ -2902bp) around -3.0kb 5' to the cap site of human e-globin gene and specu-

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lated that this element might play an important role in the regulation of this gene[8]. Using Sequence analysis of ε -NRAII, we show here that a DNA motif (-3004bp ~ -2993bp) similar to NF- κ B consensus sequence exists in the NRF DNA fragment (-3010bp ~ -2986bp). EMSA and competitive analysis confirmed that a member of NF- κ B protein in nuclear extracts of K562 cells could bind to this motif in the NRF DNA fragment. Western blot assay showed that the member of NF- κ B/Rel family in the nuclei of K562 cells is p50.

MATERIALS AND METHODS

Sequence analysis of protein binding sites in ε -NRAII

The sequence of ε -NRAII was put into the library of TRANSFAC4.0[9], in which proteins related to transcriptional regulation were collected, DNA sequence could be scanned and binding sites for transcription factors could be outlined.

Cell culture and preparation of nuclear extracts

K562 cells were cultured in RPMI 1640 medium (GIBCO-BRL) supplemented with 10% newborn calf serum, streptomycin (100 mg/ml), penicillin (100 μ g/ml) and glutamin (2 μ M)[10]. Nuclear extracts were prepared as described by Dignam etc[11]. The concentrations of nuclear extracts were determined according to Bradford method[12].

Electrophoresis mobility shift assay and competition analysis

The sequence of NRF DNA fragment was synthesized:

5' ATCTGTAGCAAATTCCTGAAAA 3'

3' TAGACATCGTTTAAGGGGACTTTT 5'

The sequences of competitors were synthesized as follows:

1, NF DNA fragment containing the NF- κ B consensus sequence[13]:

5' AGT TGA GGG GAC TTT CCC AGG C 3'
3' TCA ACT CCC CTG AAA GGG TCC G 5'

2, MNF DNA fragment (bases mutated were indicated in small letter):

5' TGA Gaa tgc TTc ata AGG 3'
3' ACT Ctt acG AAg tat TCC 5'

NRF and NF DNA fragments were 5' end labeled with [γ - 32 P]-ATP. EMSA was performed as described by Strauss etc[14]. 5' end labeled NRF or NF fragments (~ 5 fmol) were incubated with 2 ~ 4 μ g nuclear extracts in 25ul reaction solution at 0 $^{\circ}$ C for 45 min with enough nonspecific competitor polydI-polydC. DNA-protein complexes were resolved by 6% non-denaturing polyacrylamide gel electrophoresis. For competition analysis, unlabeled

competitors at various amounts were preincubated with nuclear extracts in reaction mixture on ice for 10 min prior to the addition of the labeled probe.

Western blot assay[15]

Nuclear proteins (100 μ g) isolated from K562 cells were separated by 12% SDS-PAGE and transferred to nitro-cellulose membrane as recommended by Bio-Rad Company. The membranes were blocked for 2 h in TBST buffer (10 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1% Tween-20) with 5% nonfat milk, and were washed with TBST buffer for 3 times. Then they were incubated successively with anti- NF- κ B/p50 monoclonal antibody and HRP labeled secondary antibody for 2 h respectively at room temperature. Finally the membrane was detected with ECL (BBST).

RESULTS

Computer sequence analysis of ε -NRAII

Li et al previously revealed a GATA-1 binding site in the ε -NRAII and identified that GATA factor in K562 cells could specifically bind to the GATA-1 consensus motif in this regulatory element. They demonstrated that the GATA-1 transcription factor might play a negative regulatory role through its binding to ε -NRAII[8]. Using computer sequence analysis, a DNA sequence (-3004bp ~ -2993bp, Fig 1) similar to NF- κ B consensus binding motif was found within ε -NRAII.

5' ATCTGT AGCAAATTCCTG CTGAAAA 3'
3' TAGACA TCGTTTAAGGGG GACTTTT 5'

Fig 1. Sequence boxed in ε -NRAII is similar to NF- κ B consensus binding sequence.

The consensus motif of NF- κ B family is NGGGA MTTYCCN (M=A, C; Y=C, T), while the sequence in NRF DNA fragment is GGGGAATTTGCT. Members of NF- κ B family are very important regulatory factors in transcriptional control of multiple genes related to immunity, apoptosis, cell proliferation and so on[16]. To elucidate whether there are NF- κ B family members interacting with ε -NRAII in K562 cells, NRF DNA fragments were synthesized and used in EMSA.

NF- κ B protein binding to NRF DNA fragment

NF- κ B existing in the nuclei of K562 cells has been reported[17], [18]. To identify NRF DNA frag-

ment binding protein, EMSA and competition analysis were carried out. EMSA with NF DNA fragment as a probe showed that one shift band (NF- κ B) could

Fig 2. EMSA analysis of nuclear extracts isolated from K562 cells with NF DNA fragments as a probe. 1, labeled NF fragments without nuclear extract; 2, labeled NF fragment with 4 μ g of nuclear extracts from K562 cells; 3, 4 and 5 labeled NF fragment with 4 μ g of nuclear extracts from K562 cells, unlabeled NF, NRF and MNF DNA fragments were added as competitors at 100 fold molar excess respectively.

Fig 3. EMSA analysis of nuclear extracts isolated from K562 cells with NRF DNA fragments as a probe. 1, labeled NRF fragments without nuclear extract; 2, labeled NRF fragments with 4 μ g of nuclear extracts from K562 cells; 3-5, labeled NRF fragments with 4 mg of nuclear extracts from K562 cells, unlabeled NRF DNA fragments were added as competitor at 10, 50 and 100 molar excess respectively; 6-8, labeled NRF fragments with 4 μ g of nuclear extracts from K562 cells, unlabeled NF DNA fragments were added as competitor at 10, 50 and 100 molar excess respectively; 9-10 labeled NRF fragments with 4 μ g of nuclear extracts from K562 cells, unlabeled MNF DNA fragments were added as competitor at 50 and 100 molar excess respectively.

be detected with nuclear extracts from K562 cells (Fig 2, lane 2). Both NF and NRF DNA fragments were able to compete for this band (Fig 2, lane 3 and 4), whereas MNF DNA fragment was unable to compete for it (Fig 2, lane 5). Furthermore, EMSA with NRF DNA fragment was also performed and one shift band (band A) was detectable (Fig 3, lane 2). Both NRF and NF DNA fragments were capable of competing for band A (Fig 3, lanes 3-5 and lanes 6-8), while, MNF DNA fragment was also unable to compete for it (Fig 3, lane 9-10). Those results indicate that the protein factor in K562 cells, which can bind to both NF and NRF DNA fragments, belongs to NF- κ B family, and also confirmed that e-NRAII contains a NF- κ B binding site.

The member of NF- κ B family in K562 is p50

Using Western blot assay, we have further revealed that at least one member of NF- κ B family exists in the nuclei of K562 cells without stimulation and the molecular weight of it is 50ku compared with protein markers (Fig 4).

Fig 4. Western blot assay. Monoclonal Ab anti-NF- κ B p50 was used. M, Standard protein markers; 1-4, 15, 30, 45 and 60 μ g of nuclear extracts from K562 cells respectively.

DISCUSSION

Many regulatory elements have been identified in the DNA 5' to the cap site of human e-globin gene and they are located in four regions [around -3.0kb relative to the cap site[8], between -2.0kb and 460bp[19], between -460bp and -180bp[8], [19-22] and in the proximal promoter (-177bp ~ +1bp)[19]. eNRA and ePRA located around -3.0kb are much conservative in human, orangutan and galago[8].

NF- κ B can upregulate the expression of human b-globin gene in interferon induced K562 cells[23].

Sequence analysis of ϵ -NRAII (-3028bp to -2902bp) revealed that a motif similar to NF-kB consensus sequence exists in this DNA fragment except a previously confirmed GATA-1 binding site[8]. Results presented here demonstrate for the first time that NF-kB binding site exists in the regulatory elements (ϵ -NRAII) in the 5' DNA sequence of ϵ -globin gene. Data also confirm that at least one member of NF-kB/Rel family active in the nuclei of K562 cells is p50. We suggest that NF- κ B may play an important role in the regulation of ϵ -globin gene. And whether NF- κ B p50 directly regulates the expression of ϵ -globin gene through the interaction with the negative regulatory element ϵ -NRAII is now under further investigation.

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REFERENCES

- [1] Karlsson S, Nienhuis AW. Developmental regulation of human globin gene. *Ann Rev Biochem* 1985; **54**:1071-108.
- [2] Raich N, Enver T, Nakamoto B et al. Autonomous development control of human embryonic globin gene switching in transgenic mice. *Science* 1990; **250**:1147-9.
- [3] Shih DM, Wall RJ, Shapiro SG. Developmentally regulated and erythroid-specific expression of the human embryonic-globin gene in transgenic mice. *Nucleic Acids Res* 1990; **18**:5465-72.
- [4] Bauerle PA, T Henkel. Function and activation of NF-kB in the immune system. *Annu Rev Immunol* 1994; **12**: 141-79.
- [5] Jr Baldwin AS. The NF- κ B and I-kB proteins: new discoveries and insights. *Annu Rev Immunol* 1996; **14**:649-83.
- [6] Ghosh S, MJ May, EB Kopp. NF-kB and rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* 1998; **16**:225-60.
- [7] Frankenberger M, Pforte A, Sternsdorf T, Passlick B, Baeuerle PA, Ziegler-Heitbrock HW. Constitutive nuclear NF-kappa B in cells of the monocyte lineage. *Biochem J* 1994; **15**; **304** (Pt 1):87-94.
- [8] Li J, Noguchi CT, Miller W, Hardson R, Schechter AN. Multiple regulatory elements in the 5'-flanking sequence of the human epsilon-globin gene. *J Biol Chem* 1998; **273**: 10202-9.
- [9] Heinemeyer T et al. Expanding the TRANSFAC database towards an expert system of regulatory molecular mechanisms. *Nucleic Acids Res* 1999; **7**:318-22.
- [10] Yan ZJ, Jiang C, Qian RL. Trans-acting factors from the human fetal liver binding to the human epsilon-globin gene silencer. *Cell Res* 1997; **7**(2):151-9
- [11] Dignam JD, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* 1983; **11**(5):1475-89.
- [12] Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**(1):248-54.
- [13] Zabel U, Schreck R, Baeuerle PA. DNA binding of purified transcription factor NF-kappaB. *J Biol Chem* 1991; **266**:252-60.
- [14] 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 nucleosomes. *Cell* 1984; **37**:889-901.
- [15] Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning A laboratory manual*, 2nd ed, 1989; 888-98.
- [16] Perkins ND. Achieving transcriptional specificity with NF-kappa B. *Int J Biochem Cell Biol* 1997; **29**(12):1433-48.
- [17] Lavrovsky Y, Schwartzman ML, Levere RD, Kappas A, Abraham NG. Identification of binding sites for transcription factors NF-kB and AP-2 in the promoter region of the human heme oxygenase 1 gene. *Proc Natl Acad Sci USA* 1994; **21**; **91**(13):5987-91.
- [18] Sater RA. Basal expression of the human macrophage colony-stimulating factor (M-CSF) gene in K562 cells. *Leuk Res* 1994; **18**(2):133-43.
- [19] Li Q, Blau CA, Clegg CH et al. Multiple epsilon-promoter elements participate in the developmental control of epsilon-globin genes in transgenic mice. *J Biol Chem* 1998; **273**:17361-67.
- [20] Raich N, Papayannopoulou T, Stamatoyannopoulos G et al. Demonstration of a human epsilon-globin gene silencer with studies in transgenic mice. *Blood* 1992; **79**: 861-4.
- [21] Raich N, Clegg CH, Grofti J et al. GATA1 and YY1 are developmental repressor of the human epsilon-globin gene. *EMBO J* 1995; **14**:801-9.
- [22] Peters B, Merezinskaya N, Diffley JF et al. Protein-DNA interactions in the epsilon-globin gene silencer. *J Biol Chem*, 1993; **268**:3430-7.
- [23] Moitreyee CK, Suraksha A, Swarup AS. Potential role of NF-kB and RXR beta like proteins in interferon induced HLA class I and beta globin gene transcription in K562 erythroleukaemia cells. *Mol Cell Biochem* 1998; **178**(1-2):103-12.