CHARACTERIZATION OF TRANSCRIPTION FACTORS BINDING TO -120 GATA MOTIF OF RAT β\textsubscript{b\textsubscript{miny}} – GLOBIN PROMOTER

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(Received 25. April 2005)

The aim of this study was to elucidate the regulation of rat adult β\textsubscript{b\textsubscript{miny}}-globin gene transcription. We used DNaseI footprinting, gel mobility shift and supershift assays to characterize transcription factors involved in this regulation. In this study we analyzed GATA motif at – 120 bp in the distal promoter of β\textsubscript{b\textsubscript{miny}}-globin gene. Footprint analysis revealed the binding of nuclear factors from MEL cells to the GATA motif. By using gel mobility shift assay two protein complexes were detected. The faster migrating complex was erythroid-specific and more abundant in differentiating MEL cells. Competition experiments with GATA-1 oligonucleotides and GATA-1 protein antibodies confirmed binding of GATA-1 transcription factor to GATA motif at – 120 bp regulation of rat adult β\textsubscript{b\textsubscript{miny}}-globin gene.

Key words: rat β-globin genes, transcription regulation, GATA-1

INTRODUCTION

The developmental stage and tissue specific expression of mammalian - globin genes is controlled by complex interplay between cis-acting DNA elements and trans-acting regulatory factors. The murine erythroleukemia (MEL) cells represent a useful system for molecular identification of all factors involved in this process. (Friend et al., 1971). This cell line is blocked at the proerythroblast stage of erythroid development and can be induced by dimethylsulfoxide (DMSO) to terminally differentiate in the cell culture in a process that closely mimics the event of normal erythropoiesis. In vitro induction and differentiation by DMSO is demonstrated by a decrease in cell volume, reorganization of the cytoplasmatic membrane, an appearance of red and benzidine-positive cells and an induced expression of a variety of genes associated with more mature erythrocytes, most notably globins (Friend et al., 1971).

Perfect tuning of globin gene expression during erythroid cell differentiation is conducted by defined regulatory elements. Different regions within the promoter, the gene, and the locus control region (LCR) (Townes and Behringer, 1990), contribute to a variable extent to -globin transcriptional induction in a
context- and assay-dependent manner (Antoniou et al., 1988; Antoniou and Grosveld, 1990; Cowie and Mayers, 1988; de Boer et al., 1988; Schreck et al., 1990; Wright et al., 1984). Much of the complex regulation of the gene, however, appears to be mediated by the LCR with the promoter region. The promoter region spanning 100 bp 5' from the transcription initiation site (cap site) of -globin promoter is named the minimal promoter (de Boer et al., 1988). It consists of three conserved regions responsible for efficient transcription of the -globin gene: a TATA element at -30 bp, CAAT box at -70 bp and CACC element at -90 bp from the cap site (reviewed in reference Popović et al., 1993). Another cis-acting element found in the form of tandemly repeated decamer in the minimal promoters (-53 to -32 bp) of adult -globin genes is DRE (Stuve and Mayers, 1990).

Upstream distal -globin promoter region contains additional regulatory elements. Of major importance is T/A (GATA) A/G motif recognized by a highly conserved zinc finger domain of GATA-1 protein (Orkin, 1992; Wall et al., 1988). GATA binding sites are found in the promoters of erythroid-specific genes, as well as within - and - LCRs (Orkin, 1992).

GATA-1 protein is highly expressed in hematopoietic cells of the erythroid, megakaryocytes and mast cells lineages, and at low levels present in multipotent cells and Sertoli cells of testis (Elefanty et al., 1996). Besides, GATA-1 protein is monitored in studies on uninduced MEL cells and DMSO – induced and hydroxyurea – induced and uninduced HEL cells and assigned as a stimulator of the erythroid-differentiation (Aumont et al., 1993; Zhao et al., 1999). It is a major transcription factor of the erythroid lineage implicated in erythroid specific induction of -globin gene transcription (Wall et al., 1988).

In this paper we have shown that GATA-1 is a transcription factor that specifically binds to the GATA motif at -120 bp in rat \( b \text{miny-globin distal promoter.} \) Hence we have postulated its involvement in erythroid induction of transcription.

MATERIALS AND METHODS

Oligonucleotides

The following oligonucleotides were used in gel shift analysis:

1. 5'- TAGTTATGGC TATCATCTCT GAACCC -rat GATA. The oligonucleotide represents a part of rat \( b \text{miny-globin promoter located from -129 to -104 bp upstream of the cap site.} \)

2. 5'- TCAGGGCTTT GATAGCAGTC TCTGCAAGGC CAGGGCC - human GATA. The oligonucleotide represents the GATA-1 binding site from DNase I hypersensitive site 4 of the human LCR (Pruzina et al., 1991).

Cell culture

The MEL cell line C88 and HeLa cell line were maintained in a modification of Eagle’s minimal medium with 10% fetal calf serum. The medium was enriched with 0.1 mM nonessential amino acids (ala, asp, asn, glu, gly, pro, ser). MEL cells
were induced to differentiate by culturing for 4 days in the presence of 2% (v/v) DMSO.

**Preparation of nuclear extracts**

Nuclear extracts from MEL cells were prepared by the method described by Gorski *et al.* (1986). All the modifications introduced in Gorski’s method by deBoer *et al.* (1988) were also applied.

HeLa cell nuclear extracts were prepared by the standard method of Dignam *et al.* (1983).

All the final (NH₄)₂SO₄ nuclear pellets were dissolved in buffer D (20 mM Hepes, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM dithiothreitol, 0.5 mM EDTA, 0.5 mM phenylmethylsulphonyl-fluoride) in a final concentration of 1 mg of nuclear extracts in 1 ml of buffer D.

**DNasel footprinting assays**

DNasel footprinting assays were done as described by de Boer *et al.* (1988). A pUC18 vector containing the promoter and a part of the b₉₉ miny-globin gene was digested with Ncol. The linearized vector was labeled with (¹³⁵P) ATP using polynucleotide kinase. Secondary digestion was done with EcoRI. An 800 bp fragment containing the rat b₉₉ miny globin promoter was isolated.

Each 25 µl footprinting assay reaction contained 1 fmol of 32P-labeled DNA (~3000 cpm), 1 µg polydI:polydC and 10 or 50 µg of protein extracts. All other components were mixed on ice after which the extract was added. The assay mix was incubated at room temperature for 20 min. After cooling on ice for a few minutes 0.25 – 2.0 µg of DNasel was added and the reaction was incubated on ice for 90 s. The samples were run on 6% sequencing gels alongside G+A tracks of the same DNA (Maxam and Gilbert, 1980).

**Gel shift assays and competition studies**

Gel shift assays were performed as previously described (deBoer *et al.*, 1988). Briefly, each 20 µl gel shift assay reaction contained 0.1 ng of 32P-labeled oligonucleotide, 2 µg of poly(dI):poly(dC), 10 – 14 µg of protein extract and 2 µl of the binding buffer (50 mM Tris (pH 8.0), 5 mM DTT, 5 mM EDTA, 250 mM NaCl and 10 % glycerol). All other components were mixed on ice and then the extract was added. The assay mix was incubated at room temperature for 30 min. After the addition of 1 µl 10% glycerol containing 0.05% xylene cyanole and 0.05% bromophenol blue, the samples were run on 4% acrylamide: 0.13% methylene bis-acrylamide gel for 1 hr at 60 V/cm² in 1x TBE running buffer.

For competition experiments, the unlabeled oligonucleotides were added to the assay mixes before the addition of the extract. The competitor oligonucleotides were added in 100-fold molar excess.

Each assay was performed at least three times using nuclear protein extracts from different isolations.

Rat anti-mouse GATA-1 IgG mAb N6 was used in competition studies (supershift) (a 1:4 dilution of the growth medium of a confluent culture of the
myeloma cell line producing this antibody, source: J.D. Engel, Evanston, & P. Sjak, Rotterdam) (Wyatt et al., 1993). 1 μl of antibody was added in the above described gel shift assay reaction mixture.

RESULTS

DNaseI footprinting analysis of -120 GATA motif

To elucidate cis-acting regulatory elements essential for transcriptional regulation of rat \( b^{\text{bminy}} \)-globin gene, we have performed DNaseI footprint analysis (Figure 1). In this study, region adjacent to minimal promoter is evaluated. A footprint over the GATA motif at –120 bp is seen in uninduced MEL cells. It is more prominent in differentiating MEL cells and absent in nonerythroid HeLa cells.

![Figure 1. Footprinting analysis of the non-coding strand (from -90 to -150) of the rat \( b^{\text{bminy}} \)-globin promoter with 10 or 50 μg of nuclear extracts from MEL and HeLa cells. First 4 lanes represent four different concentrations of DNaseI digestion without extract. G+A tract is a Maxam-Gilbert depurination of the same fragment. GATA motif is indicated.](image)

**GATA motif at -120 bp binds erythroid-specific and differentiation – dependent protein complex**

Binding patterns of transcription factors to the GATA motif of rat \( b^{\text{bminy}} \)-globin promoter were examined with nuclear extracts from uninduced and DMSO-induced MEL cells (Figure 2). As a control, nuclear extract from HeLa cells was used (data not shown). GATA oligonucleotide used in gel mobility shift analysis encompasses GATA motif and surrounding sequences (from -129 to -104 bp upstream of the cap site of rat \( b^{\text{bminy}} \)-globin gene). GATA-1-binding
oligonucleotide from the human LCR served as cold competitor (Pruzina et al., 1991).

The fragment containing the GATA motif from rat b\textsuperscript{m\textsubscript{in\textsubscript{y}}}-globin gene promoter showed two major (c\textsubscript{1} and c\textsubscript{2}) bands after binding the nuclear extracts from both uninduced and DMSO-induced MEL cells (Figure 2). Both of the major bands were competed by oligonucleotide itself. The faster migrating c\textsubscript{1} complex was erythroid-specific and more abundant in differentiating MEL cells. Competition with GATA-1 oligonucleotide from the human LCR competed efficiently c\textsubscript{1} complex. We presumed that erythroid-specific c\textsubscript{1} complex is GATA-1 protein.

<table>
<thead>
<tr>
<th>probe</th>
<th>rat b\textsuperscript{m\textsubscript{in\textsubscript{y}}}-globin GATA motif</th>
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<tr>
<td>competitor</td>
<td>itself</td>
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<td>nuclear extract ((\mu)g)</td>
<td>MEL uninduced (14)</td>
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</table>

Figure 2. Gel mobility shift assay of the rat b\textsuperscript{m\textsubscript{in\textsubscript{y}}}-globin GATA motif with nuclear extracts from MEL cells (uninduced and DMSO-induced). The competitor oligonucleotides, the binding fragment itself and human LCR GATA-1 motif, are used in molar concentrations 100-fold over probe. The protein DNA complexes were separated on 4% polyacrilamide gel. Formed complexes are designated as c\textsubscript{1} and c\textsubscript{2}. 
**GATA motif at -120 bp binds GATA-1 protein**

The confirmation of specific GATA-1 factor binding to GATA motif at -120 bp is obtained by using GATA-1 antibodies in gel shift analysis. Formation of GATA-1 complex with oligonucleotide from rat β_{\text{miny}}-globin promoter was competed by GATA-1 antibodies in assays with extracts from uninduced MEL cells (Figure 3). The competition efficiency depends on the concentration of GATA-1 antibodies used.

From this set of experiments we concluded that GATA-1 transcription factor specifically binds to -120 GATA motif of rat β_{\text{miny}}-globin promoter.

![Figure 3. Supershift assay of the rat β_{\text{miny}}-globin GATA motif with nuclear extracts from MEL cells. The competitor, anti-GATA-1 antibody N6 was included in a different concentration. The protein DNA complexes were separated on 4% polyacrilamide gel. The free probe, the mobility of the bound GATA-1 complex and the mobility of the antibody-GATA-1-probe complex (supershift) are indicated.](image)
DISCUSSION

Among mammals studied so far, rats have the largest number of active adult β-globin genes. Therefore, it is interesting to find out if the transcriptional regulation of rat adult β-globin genes is different, compared to human and murine.

Besides the locus control region (LCR) the promoter is the key regulatory region for β-globin gene transcription. The adult β-globin-like promoters contain several elements whose sequence and location are evolutionally conserved. Comparison of the promoters of the human, mouse and rat β-globin genes reveals certain similarities, but also some differences that might be relevant to the regulation of these genes. Extensive studies of β-globin promoters have identified evolutionally conserved transcriptional regulatory elements in the minimal promoter, critical for a high-level of promoter function (TATA, CCAAT, -DRE, CACCC).

In previous studies a DNA-binding activity with specificity for the DRE from rat b^miny-globin promoter was identified and characterized (Pavlović et al., 1999). It was also confirmed that the DRE regulatory element contributed to the transcriptional inducibility of rat b^miny-globin gene in murine (MEL) and rat erythroleukemia cells (REL) (Pavlović et al., 2003). Mouse m^maj-globin DRE promoter element resembles the same regulatory element from the rat proximal promoter. It contributes significantly to erythroid-specific induction of transcription and DRE binding activity is detected (Stuve and Myers, 1993.) On the contrary, the human β-globin minimal promoter provides erythroid-specific induction of transcription only if combined with the LCR, and there was no evidence of DNA-binding activity with specificity for the DRE (Antoniou et al., 1995).

In this paper we have described additional specificity of the rat adult β-globin gene promoter regarding -120 GATA motif adjacent to rat b^miny-globin minimal promoter.

GATA-motif (T/A (GATA) A/G), except the one at -9 in human and -60 bp in mouse β-globin promoter, is common in distal β-globin promoters (Antoniou et al., 1995; Macleod et al., 1991). It is placed at -200 bp in human, -215 bp in mouse and -120 bp in rat β-globin promoters (deBoer et al., 1988; Macleod et al., 1991; Pavlović et al., 1999).

We have characterized the transcription factor that specifically binds to the -120 GATA motif of rat b^miny-globin gene promoter as GATA-1, by using gel mobility shift competition (both oligonucleotide and antibody) studies. The -120 region from the human β-globin promoter does not contain GATA consensus sequence, yet a weak binding activity of GATA-1 has been detected (de Boer et al., 1988). Both GATA-1 binding site and binding activity are absent at the position -120 of murine m^maj-globin promoter.

GATA-1 is the almost universal activator of β-globin genes, as shown for humans and mice (reviewed in Pavlović et al., 1996). It is a central transcription activator of erythroid differentiation (Sieweke and Graf, 1998). However, two exceptions where GATA-1 protein acts as a repressor have been noticed. Whyatt et al. showed that GATA-1 binding to (T/C)AAG motif in distal promoter region of
the human \(^{\alpha}\)-globin gene regulates the correct developmental suppression of \(^{\beta}\)-globin expression (Whyatt et al., 1993). Also, study of Ramchandran and colleagues demonstrated that GATA-1 bound to seven tandem GATA repeats in the 5’ boundary area of human \(^{\beta}\)-globin LCR, prevents HS2 enhancer activity (Ramchandran et al., 2000).

Taking into consideration the fact that the binding activity of GATA-1 to the -120 GATA motif of rat \(^{\beta}\)miny-globin gene is increased in differentiating MEL cells, we presume that it is involved in erythroid-specific induction of transcription, but it remains to be resolved by functional studies.

In previous studies GATA-1 was shown to physically interact and cooperate with the Krüppel family zinc finger proteins Sp1 and EKLF, which is required for the activation of the adult \(^{\beta}\)-globin gene in the final stages of definitive erythropoiesis (Merika et al., 1995). It is also confirmed that GATA-1 is associated with its cofactor FOG (Friend of GATA), essential for terminal erythroid maturation (Fox et al., 1998; Tsang et al., 1998).

Molecular identification of all other individual proteins in -120 GATA-motif complexes will elucidate the mechanisms of regulation of rat \(^{\beta}\)miny-globin gene transcription.

Moreover, further analysis of the interactions of LCR with the promoter elements that play different roles in different species will provide better insight into the regulation of transcription of \(^{\beta}\)-globin genes.

ACKNOWLEDGEMENT:
We would like to thank to Dr S.Philipsen for N6 GATA-1 IgG mAb. This work was supported by grant 1417 and 1438 from the Ministry of Science and Technology of Serbia.

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KARAKTERIZACIJA TRANSKRIPCIONIH FAKTORA KOJI SE VEZUJU ZA GATA MOTIV NA POZICIJI -120 U PROMOTORU \( \beta_{\text{miny}} \)-GLOBINSKOG GENA PACOVA

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SADRŽAJ

Cilj ovog rada je bio rasvetljavanje regulacije transkripcije adultnog \( \beta_{\text{miny}} \)-globinskog gena pacova. Da bi se okarakterisali transkripcioni faktori uključeni u ovu regulaciju, upotrebljena je metodologija koja je obuhvatila "footprint" (otisak proteina na DNK), "gel shift" (metoda usporene pokretljivosti u gelu) i "supershift" (metoda kompeticije antitelom u "gel shift"-u) eseje. U ovom radu je analiziran GATA motiv lociran na -120 bp distalnog promotora \( \beta_{\text{miny}} \)-globinskog gena. "Footprint" analiza je otkrila vezivanje nuklearnih faktora iz MEL \( \beta \)elija za GATA motiv. Uz pomoć "gel shift" eseja detektovana su dva proteinska kompleksa. Kompleks koji je brže migrirao u gelu je eritroidno-specifičan i zastupljeniji je u diferencirajućim MEL \( \beta \)elijama. Eksperimenti kompeticije sa GATA-1 oligonukleotidom i GATA-1 antitelim potvrdili su vezivanje GATA-1 transkripcionog faktora za GATA motiv na poziciji -120 adultnog \( \beta_{\text{miny}} \)-globinskog gena pacova.