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Research Article

The rat β_b^{miny}-globin promoter: nuclear protein factors and erythroid-specific induction of transcription

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Abstract. We show that the rat adult β_b^{miny}-globin gene is transcriptionally active. The − 100-bp promoter region contains control elements that are important for the induction of transcription in rat erythroleukemia (REL) cells. By using DNaseI footprinting and gel mobility shift assays, we have shown that the CCAAT box, a regulatory element from an analyzed promoter region, binds NF-Y and GATA-1 transcription factors. Another regulatory element from this region, βDRE, binds erythroid-specific and ubiquitous factors from REL cells. Although both the CCAAT box region and βDRE element bind the same protein factors before and after induction of REL cells, we show by South-Western blot analysis that the concentrations of 150-kDa, 70-kDa and 60-kDa factors binding to the βDRE are increased in DMSO-induced REL cells.

Key words. Rat β-globin genes; transcription regulation; MEL cells; REL cells; induction of erythropoiesis.

The rat β-globin gene locus is unique for its organization. There are three rat adult β-globin gene haplotypes containing either five (haplotype b i c) or three genes (haplotype a) [1]. Three embryonic β-like globin genes are identified in the rat. Their structure and organization are very similar to those of mouse embryonic β-like globin genes [M. Stevanović, personal communication]. Detailed sequence analysis and comparison of adult rat β-globin genes with mouse β-globin genes reveal that the leftmost gene is the major-like gene and that at the opposite end downstream lies the minor-like gene (fig. 1). All of the genes lying between them are minor-major hybrids, indicating their origin by unequal crossing over [2]. For all internal genes the 5′ part is minor, whereas the 3′ untranslated and flanking regions are of the major type, with a breakpoint occurring somewhere before the 3′ untranslated regions, meaning that all the promoters of internal genes belong to the minor type β-globin promoters [2].

Haplotype b contains 5 genes: β_b^{maj}, $\beta_b^{minx'}$, β_b^{miny}, $\beta_{b/c}^{minx''}$, $\beta_{b/c}^{minz}$. Structurally, they all appear capable of activity. The β_b^{maj} gene shows in conceptual translation complete identity with the amino acid sequence of the most abundant rat adult β-globin chain [3]. Wong et al. showed that the $\beta_{b/c}^{minz}$ is active [4]. Sequence analysis of the β_b^{miny}-globin gene and its promoter region indicates that it might also be active [5].

The mechanisms of developmental-stage and tissue-specific regulation of transcription of β-globin genes have been extensively studied in different species. An important step in understanding this regulation is identification of the cis-acting regulatory sequences that are required for expression of globin genes in erythroid cells, as well as trans-acting protein factors which bind at different regulatory regions located through the gene cluster.

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The key regulatory element of the entire β-globin cluster, the locus control region or LCR, was discovered upstream of the β-like globin genes [6–8]. It has strong enhancer-like and chromatin-organizing properties [9–11]. Furthermore, the human β-globin gene and its immediate flanking regions contain at least three regulatory elements: the promoter and two enhancers [12].

Functional DNA sequences characterized in the proximal β-globin promoters up to 100 bp upstream from the cap site of different species are the TATA box (-30), βDRE motif (-53 to -32), CCAAT element (-75) and CACCC box (-90) [13–21]. DNA-protein interactions studies in vitro revealed several transcription factors binding at these regulatory elements [22–25].

We used murine erythroleukemia (MEL) and rat erythroleukemia (REL) cell lines for our investigations.

MEL cells are a widely used tissue-culture model for the study of β-globin gene expression. Due to the lack of appropriate homologous model systems, human β-globin gene expression has also been extensively studied in MEL cells. MEL cells represent adult erythroid development [26]. They are arrested at the proerythroblast stage and can be induced to terminally differentiate in vitro in a process that closely mimics the events of normal erythropoiesis. MEL cell differentiation is characterized by a large increase (10–50-fold) in the steady-state level of β-globin messenger RNA (mRNA) [27].

REL cells represent a permanent cell line derived from transplantable tumors from 7,12 dimethylbenz (α) anthracene-induced erythroleukemia in the Long-Evans rat [28]. This cell line maintains its erythroid nature. Erythroid differentiation can be induced by DMSO, as demonstrated by a decrease in cell size, the appearance of red and benzidine-positive cells and increased synthesis of adult globin chains [29].

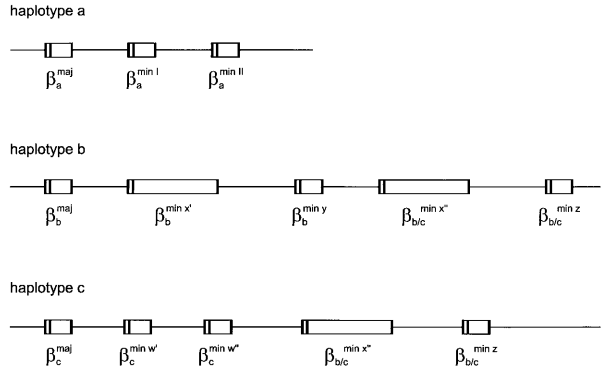

Figure 1. Schematic map of three haplotypes of the rat β–globin gene family. The positions of the genes are shown by the boxes, with the filled parts denoting exons.

In this paper, we show that the rat adult β_b^{miny}-globin gene is active, and we have investigated aspects of the transcriptional regulation of this globin gene. We have studied cis-acting elements from the proximal promoter of rat β_b^{miny}-globin gene and analyzed the binding of specific transcription factors in erythroid induction of transcription.

Materials and methods

Animals. Laboratory rats of the *Wistar* strain (3 months old) were used. Reticulocytosis was induced in rats by three daily injections of 1.25% (v/v) phenylhydrazine (PHZ) in isotonic saline (150 mM NaCl) (50 μg/g of body weight). Rats were bled by heart puncture on the fifth day, when reticulocyte counts varied from 40 to 55% [30].

Experiments on rats were done in line with the 'Ethical principles and guidelines for scientific experiments on animals' of the Swiss Academy of Medical Sciences.

Primers. The oligonucleotide primer used in primer extension analysis is 5′-GCT TCC TTGCT CCT GAAAATCTCGT. The 5′ end of the primer corresponds to coordinate 2311 of the pCAT-basic vector (Promega).

The oligonucleotide primers used in reverse transcription-polymerase chain reaction (RT-PCR) analysis are designed as follows: forward primer -5′-GGACACCC-CTTTCCACTTAC, corresponding to position 42–61 of rat β_b^{miny}-globin gene complementary DNA (cDNA); reverse primer -5′-CATATTGCCCAGGGAGCCTG, corresponding to position 330–312 of all rat β–globin genes cDNA-sequenced so far.

Oligonucleotides. The following oligonucleotides were used as probes in gel shift assays:

1. 5′-ACCCTCACAT TGCCCAATCT GCTCA-CACA — rat CCAAT. The probe represents a part of the promoter of the rat β_b^{miny}-globin gene located -89 to -61 bp upstream of the *cap* site.
2. 5′-CTCACACAGG ACAGAGTGAT CAGGG-GCCAG AATTT — rat βDRE. The probe represents a part of the promoter of the rat β_b^{miny}-globin gene located -68 to -34 bp upstream of the *cap* site.

The following oligonucleotides were used as competitors in gel shift assays:

1. 5′-TCAGGGCTTT GATAGCACTAT CTGCA-GAGC CAGGGCC — human GATA. The probe represents the GATA-1 binding site from the DNaseI hypersensitive site 4 of human LCR [31].
2. 5′-CGACCTAGGG TTGGCCAATC ACTCCAAG — human CCAAT. The probe represents a part of the human β-globin gene promoter located -57 to -86 bp upstream of the *cap* site [22].

DNA constructs. The hybrid rat β_b^{miny}-globin/*cat* gene was used as a reporter system for the functional analysis

of the promoter in these studies, pCATβ_b^{miny}-globin plasmid.

It consists of 122-bp fragment of the β_b^{miny}-globin promoter ($+27$ to -95) cloned into the polycloning site of the pCAT-Basic vector (Promega).

The parent vector used in the constructions was derived from a λ11 phage clone [1] by excision of the *Bam*HI fragment containing the 5′ region and the part of β_b^{miny}-globin gene, and its splicing into the *Bam*HI site in the polycloning site of the pUC18 vector. The next step was the insertion of a 1357-bp *Pst*I fragment containing 900 bp of promoter and the part of the β_b^{miny}-globin gene into the polycloning site of the pCAT-Basic vector. The removal of the β_b^{miny}-globin gene was achieved by *Hinc*II digestion. Finally, after the excision of distal parts of the β_b^{miny}-globin gene promoter by *Pst*I digestion, the construct used in this paper was generated.

PCR amplification of β_b^{miny}-globin RNA from reticulocytes. Rat reticulocyte RNA was prepared from phenylhydrazine-treated adult rat by the method of Berger et al. [32]. The quality of the RNA was assessed by electrophoresis in a 1% formaldehyde agarose gel.

One microgram of total cellular RNAs from rat reticulocytes was used as the template in RT reactions. RT was performed with the reverse primer listed below and AMV reverse transcriptase according to the instructions of the manufacturer (Pharmacia Biotech). Incubation for the RT step was at 42 °C for 60 min The polymerase chain reaction (PCR) conditions were 94 °C for 3 min followed by 35 cycles of 94 °C for 1 min, 68 °C for 1 min and 72 °C for 1 min, and a final cycle of 72 °C for 3 min. $MgCl_2$ (2 mM) was present in both the RT and PCR steps. PCR samples were analyzed in 4% agarose gels. The 5′ → 3′ sequences of the primers were CATATTGCCCAGGAGCCTG (reverse primer) and CATTCACCTTTCCCCACAGG (forward primer).

Cell culture and cell transfections. The MEL cell line C88, REL and the HeLa cell line were maintained in a modification of Eagle's minimal medium with 10% fetal calf serum. The medium was enriched with nonessential amino acids (Ala, Asp, Asn, Glu, Gly, Pro, Ser) all in final concentrations of 0.1 mM. pCATβ_b^{miny}-globin plasmid (75 μg) was linearized at the *Hin*dIII site and cotransfected with GSE 1417 plasmid [33] containing *tk-neo*^{R} (25 μg) into MEL and REL cells by electroporation, as follows: 3×10^7 cells in rapid, log phase were washed in PBS and resuspended in 1 ml of electro-shock buffer (ESB) (140 mM NaCl, 25 mM HEPES, pH 7.15, 0.75 mM Na_2HPO_4). The cells were electroporated in a 0.4-cm cuvette in a BioRad Gene Pulser apparatus set to deliver a 250 V, 960 μF. The transfection was divided into three independent populations and selected in 800 μg/ml of G418. Populations of transfected cells were ready for further use 2 weeks after drug selection. At this stage, each population was further subdivided, with one half maintained in normal selection medium. The other half was induced to differentiate by culturing for 4 days in the presence of 2% (v/v) DMSO.

Primer extension analysis. Total RNAs from MEL and REL cell lines transformed with pCATβ_b^{miny}-globin plasmid were isolated by acid guanidinium thiocyanate-phenol-chloroform extraction [34]. Fifty micrograms of total RNA and 8 pmol of *cat* primer (5′-GCTTCCT-TAGCTCCTGAAAATCTCG) were used for the primer extension analysis as described previously [35]. The products of the reactions were electrophoresed next to the DNA sequencing ladder [36] of the promoter DNA fragment generated by the same primer oligonucleotide.

CAT assays. Uninduced and induced REL cells transformed with pCATβ_b^{miny}-globin plasmid were pelleted and washed in PBS. The cells were resuspended in 200 μl of 0.25 M Tris buffer (pH 7.8) and subjected to three cycles of freezing and thawing. Extracts were incubated at 65 °C for 10 min to inactivate endogenous deacetylases. Cell extracts containing equal amounts of proteins (400 μg) [37] were incubated with [^{14}C]chloramphenicol (0.125 μCi) and unlabeled acetyl coenzyme A (30 mM) for 16 h at 37 °C. CAT activity was measured by thin-layer chromatography (TLC) [38].

Preparation of nuclear extracts. Nuclear extracts from MEL cells were prepared by the method described by Gorski et al. [39]. All the modifications introduced Gorski's method by deBoer et al. [22] were also applied. HeLa cell nuclear extracts were prepared by the standard method of Dignam et al. [40].

The preparation of nuclear extracts from REL cells was done by a modification of the method used for MEL cells [41].

All the final $(NH_4)_2SO_4$ 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.

DNaseI footprinting assays. DNaseI footprinting assays were done as described by de Boer et al. [22]. A pUC18 vector containing the promoter and the part of β_b^{miny}-globin gene was digested with *Nco*I. The linearized vector was labeled with (γ-^{32}P) ATP using polynucleotide kinase. The secondary digestion was done with EcoRI. An 800-bp fragment containing the rat β_b^{miny}-globin promoter was isolated.

Each 25-μl footprinting assay contained 1 fmol of ^{32}P-labeled DNA ($\sim$ 3000 cpm),

1 μg of polydI:polydC and 10 or 50 μg of protein extracts. All the other components were mixed on ice, and 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 DNaseI 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 [42].

Gel shift assays and competition studies. Gel shift assays were performed as previously described [22]. Briefly, each 20-μl gel shift assay contained 0.1 ng of ^{32}P-labeled oligonucleotide and 0.4 ng of the complementary unlabeled 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 the extract was added and the assay mix incubated at room temperature for 30 min. After the addition of 1 μl of 10% glycerol containing 0.05% xylene cyanole and 0.05% bromophenol blue, the samples were run on 4% acrylamide:0.13% methylene bisacrylamide gel for 1 h at 60 V/cm^2 in 1 × TBE running buffer.

For competition experiments, the unlabeled oligonucleotides in 1 μl of annealing buffer 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.

South-Western blot analysis. South-Western blot analysis was done as described by Michael et al. [43]. Briefly, nuclear proteins extracted from uninduced and induced REL cells (15 μg each) were electrophoretically separated on an 8% SDS-polyacrylamide gel and electrically transferred to a nitrocellulose membrane (Hybond C). Hybridization buffer contained 300,000 cpm/ml of ^{32}P-labeled probe (rat βDRE oligonucleotide) and 20 μg/ml of polydI:polydC. The incubation was at room temperature overnight. Signals were visualized by autoradiography.

Results

Rat β_b^{miny}-globin gene is transcriptionally active. RT-PCR was used to detect the β_b^{miny}-globin gene transcripts in RNAs isolated from reticulocytes of phenylhydrazine-treated adult rat. Due to the extreme homology of rat β-globin genes, the choice of primers specific for β_b^{miny}-globin gene was made after comparison of all rat β-globin gene sequences known so far [T. Okazaki, unpublished data, 4, 5, 44]. We used two primers: the first one was specific for the β_b^{miny}-globin gene (encompassing the sequence of the second and third exons), and the second was common to all the adult rat β-globin genes. The RT-PCR reaction yielded an expected product of 288 bp (fig. 2). Thus we demonstrated the transcriptional activity of the β_b^{miny}-globin gene in adult rat reticulocytes.

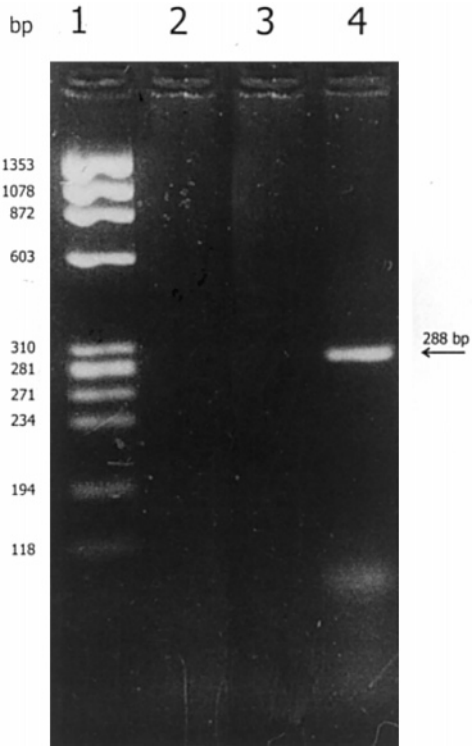

Figure 2. Detection of β_b^{miny}-globin RNA in rat reticulocytes by PCR amplification. Lanes 1, φX174/*Hae*III DNA marker; 2, control PCR amplification done in the absence of the template (water control); 3, control PCR amplification done in the absence of RT; 4, enzymatic amplification of β_b^{miny}-globin cDNA products (288 bp) (indicated by arrow).

Determination of the *cap* site of rat β_b^{miny}-globin gene. Primer extension analysis was performed to determine the start site of transcription of the β_b^{miny}-globin gene in MEL cells. The 5′ part of the β_b^{miny}-globin gene is extremely homologous to the same regions of murine and rat β-globins, and for that reason we were not able to perform primer extension using the oligonucleotide primer which encompassed the sequence of β_b^{miny}-globin gene. Instead, we fused β_b^{miny}-globin promoter to the *cat* reporter gene and used the oligonucleotide primer which corresponded to the *cat* gene to perform primer extension (see above). Figure 3 shows initiation of transcription at coordinate 53 upstream of the ATG codon in transformed MEL cells.

The start site of transcription is indicated as base + 1, and this is reflected in all the positions indicated in this paper (fig. 4).

Transcription of the rat β_b^{miny}-globin gene proximal promoter. In order to determine the functional role of the proximal rat β_b^{miny}-globin promoter, we tested whether the region from + 27 to −95 bp conferred transcrip-

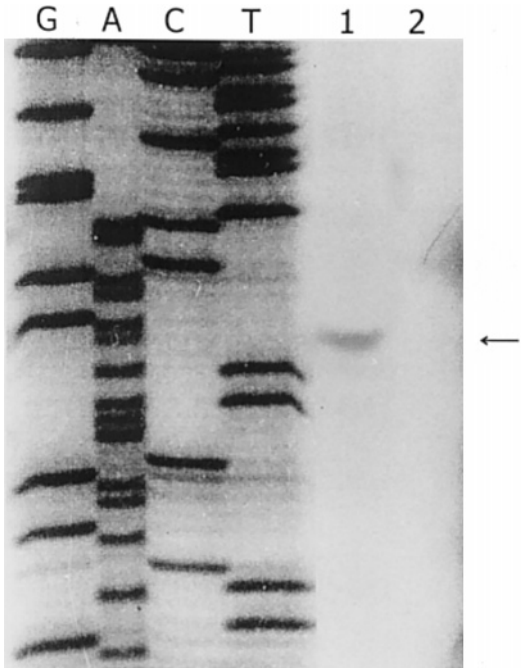

Figure 3. Primer-extension analysis of *cat* transcripts in MEL cells transformed with pCATβ_b^{miny}-globin plasmid. The autoradiogram represents data from primer extension with total RNA from MEL cells transformed with pCATβ_b^{miny}-globin plasmid (lane 1), and mock transfected cells (lane 2). The corresponding pCATβ_b^{miny}-globin (antisense) sequencing ladders are obtained by the same primer, specific for the *cat* reporter gene. The arrow indicates the position of the +1 nucleotide. Sequencing ladders represent the promoter fragment from 34 to 75 bp upstream of the ATG codon.

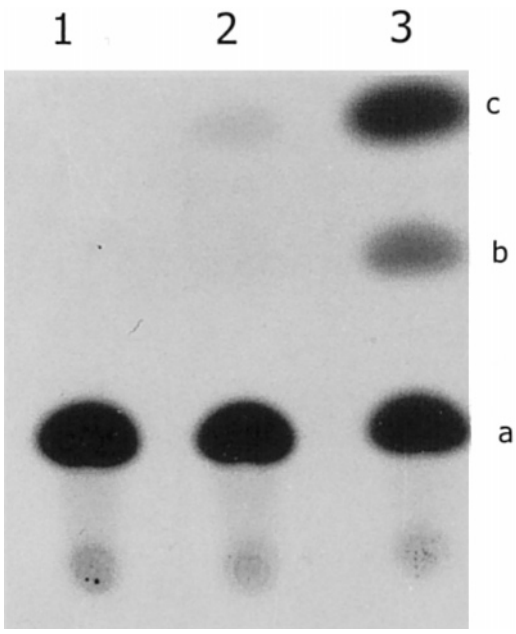

Figure 5. CAT enzymatic activities of recombinant CAT plasmids containing 122 bp (from +27 to −95) of the rat β_b^{miny}-promoter in REL cells.The levels of CAT activity in mock transfected REL cells (lane 1); REL cells transformed with pCAT β_b^{miny}-globin plasmid before (lane 2) and after the induction to differentiation with DMSO (lane 3) were determined by TLC assay. Unacetylated chloramphenicol spots in the chromatograph are marked by 'a', and acetylated chloramphenicol spots are marked by 'b' and 'c'.

tional inducibility in REL cells. This promoter fragment was cloned in the CAT reporter vector and introduced into REL cells by electroporation. For selection of transformants, the plasmid GSE 1417 [33], containing a thymidine kinase promoter-driven G418 resistance marker, was cotransfected. Three independent stable populations were established by selection in G418. Each population was subdivided, and one half was induced to differentiate for 4 days after addition of 2% DMSO. The levels of CAT activity before and after DMSO-

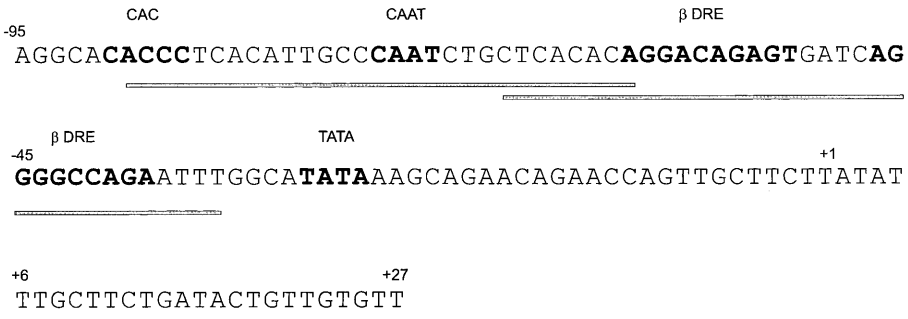

Figure 4. Sequence (+27 to −95) of the rat β_b^{miny}-globin promoter. The *cap* site determined in MEL cells is indicated as +1. The conserved consensus sequences are bolded. Bars indicate the oligonucleotides used in gel mobility shift assays.

induced differentiation were measured by CAT assay. The results of CAT assay analysis demonstrate that the proximal promoter has barely detectable activity in uninduced REL cells. Interestingly, we observed significant activation of the *cat* reporter gene after DMSO induction (fig. 5). Similar results were obtained with MEL cells (data not shown).

Analysis of the β_b^{miny}-globin gene promoter region by DNaseI footprinting. Next, we analyzed the cis-acting elements from the proximal promoter of the rat β_b^{miny}-globin gene by DNaseI footprint analysis. Since the proximal promoters of adult β-globin genes from different species contain common cis-acting elements that have been characterized using MEL cell nuclear extracts, we analyzed the promoter of the rat β_b^{miny}-globin gene for the binding of nuclear factors from MEL cells (fig. 6). Starting from the transcription initiation site, four footprinted regions are detected in the β_b^{miny}-globin gene proximal promoter: the TATA box region, the βDRE, the CCAAT box region and the CACCC box. There is also a big footprint over the *cap* site region. The region of the βDRE element (-38 to -61) does not show a footprint in HeLa cells (fig. 6).

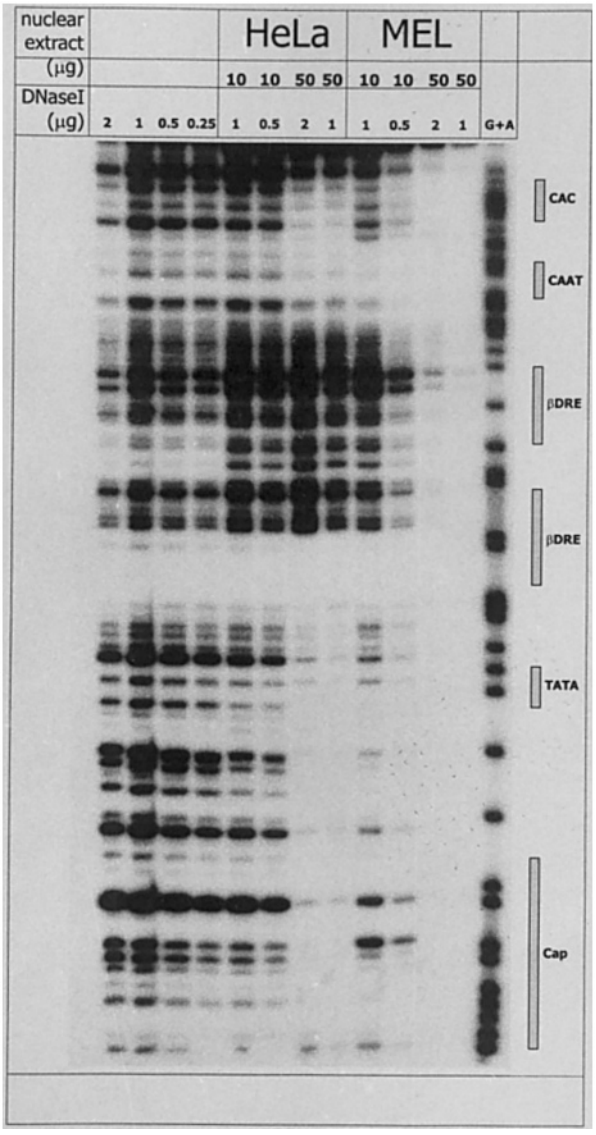

Figure 6. Footprinting analysis of the noncoding strand (from $+1$ to -95) of the rat β_b^{miny}-globin promoter with 10 or 50 μg of nuclear extracts from MEL and HeLa cells. Lanes 1–4 are four different concentrations of DNaseI digestion without extract. The G + A tract is a Maxam-Gilbert depurination of the same fragment. Bars indicate the different footprints.

Characterization of the protein factors by mobility shift assays. In order to characterize the interactions of trans-acting factors with each of the binding sites, we used two oligonucleotides in gel mobility shift assays for our further analysis. The first encompasses the CCAAT box and the other the βDRE element from the β_b^{miny}-globin proximal promoter (fig. 4).

These were characterized by using two oligonucleotides as cold competitors in the gel mobility shift assays: a GATA-1-binding oligonucleotide from human LCR [31] and a CCAAT box oligonucleotide from the human β-globin gene promoter [22]. This oligonucleotide binds NF-Y (formerly designated CP1) GATA-1 and NF-E6 [45].

We used nuclear proteins from REL and MEL cells in order to detect possible species and/or cell-type differences. The fragment containing the CCAAT box from rat β_b^{miny}-globin gene shows two major (a_1 and a_2) and several minor bands after binding the nuclear extracts from both uninduced and DMSO-induced REL cells. These complexes were competed by the human CCAAT box oligonucleotide. The faster migrating major complex was also competed by the human GATA-1 oligonucleotide (data not shown). The same complex was not observed in the HeLa extract, confirming its erythroid-specific nature (fig. 7). All the factors detected occur in both uninduced and DMSO-induced REL cells, although quantitative differences are observed. The same complexes were detected after binding of nuclear protein factors from MEL cells (data not shown).

The fragment containing the βDRE element consists of two βDRE repeats [15]. It shows four distinct complexes on gel with nuclear extracts from REL cells. The bands are specific because they are competed by the oligonucleotide itself. Gel shift assays with HeLa extracts suggest that three of them are erythroid-specific (fig. 8). After DMSO induction quantitative differences are observed in all complexes, indicating that factors binding at the βDRE element are increased in differentiating REL cells.

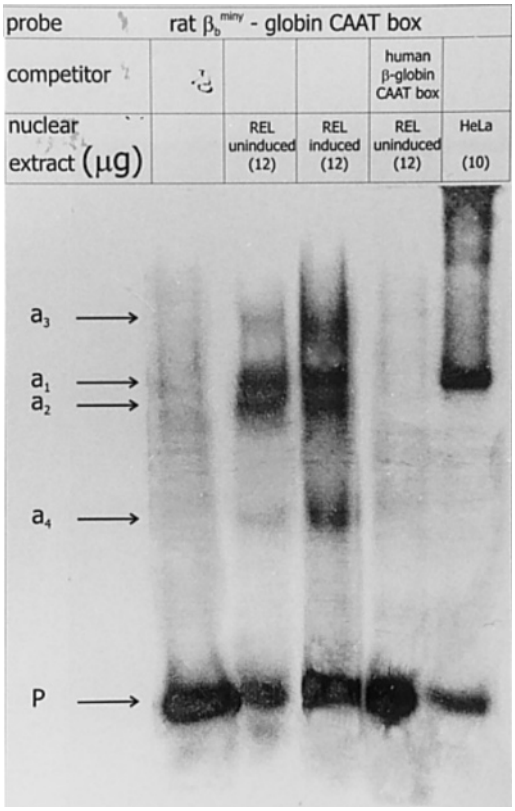

Figure 7. Gel mobility shift assay of the rat β_b^{miny}-globin CCAAT box with nuclear extracts from REL cells (uninduced and DMSO-induced) and HeLa cells. The competitor oligonucleotide, the human β-CAAT box is used in molar concentration 100-fold over probe. P, free unbound probe; a_1, NF-Y complexes; a_2, GATA-1 complexes; a_3, a_4, different CCAAT box complexes.

The same complexes are detected after binding of nuclear protein factors from MEL cells. After DMSO induction of MEL cells, quantitative differences were observed in three of them. The fastest migrating complex, clearly increased in DMSO-induced REL cells, is barely visible in both uninduced and induced MEL cells (data not shown). This is the only difference we have detected using nuclear protein factors from MEL and REL cells.

South-Western blot analysis of βDRE element from β_b^{miny}-globin promoter. In order to confirm the prominent increase in the amounts of βDRE-binding proteins in DMSO-induced REL cells and to characterize them better, we used South-Western blotting. Fifteen micrograms of nuclear proteins from uninduced and DMSO-induced REL cells were size-fractionated on SDS-polyacrylamide gels and immobilized on nitrocellulose sheets. These sheets were hybridized to a DNA fragment containing the rat β_b^{miny}-globin βDRE element. The results are shown in figure 9. We observe several proteins that bind to the βDRE probe. Three of these proteins, 150 kDa, 70 kDa and 60 kDa, are significantly increased in nuclear extract from DMSO-induced REL cells. South-Western blot analysis performed using nuclear extract from HeLa cells revealed that the 70-kDa protein is a ubiquitous transcription factor and that the 150-kDa and 60-kDa proteins are exclusively present in the REL cell nuclear extract (data not shown). Thus, we postulate that these erythroid-specific proteins are involved in erythroid-specific induction of transcription of the proximal rat β_b^{miny}-globin promoter.

Discussion

In this paper we have shown that the rat β_b^{miny}-globin gene is transcriptionally active. It has previously been shown that β_b^{maj} and $\beta_{b/c}^{minz}$ are active genes. Whether the $\beta_b^{minx'}$ and $\beta_{b/c}^{minx''}$ genes from haplotype b are active remains to be determined, but it is already certain that,

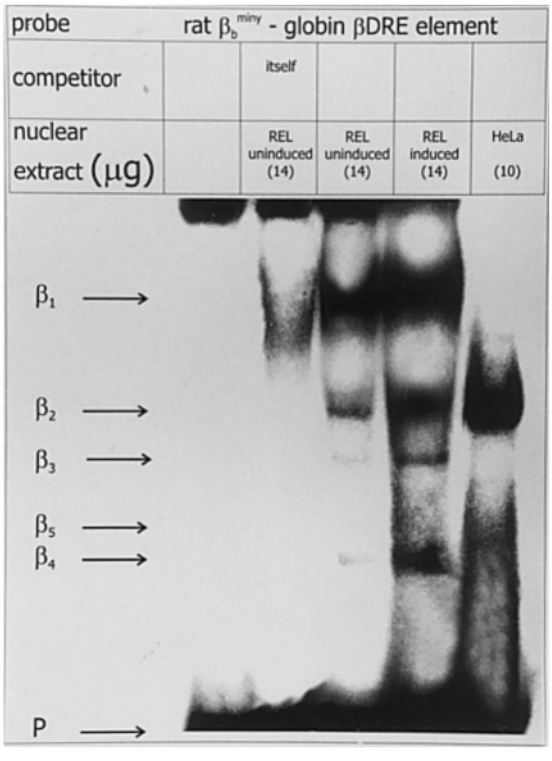

Figure 8. Gel mobility shift assay of the rat β_b^{miny}-globin βDRE element with nuclear extracts from REL cells (uninduced and DMSO-induced) and HeLa cells. The competitor oligonucleotide, the binding fragment itself, is used in molar concentration 100-fold over probe. P, free unbound probe; β_1, β_2, β_3, β_4, different βDRE complexes in REL cells, β_5, exclusive βDRE complex in HeLa cells.

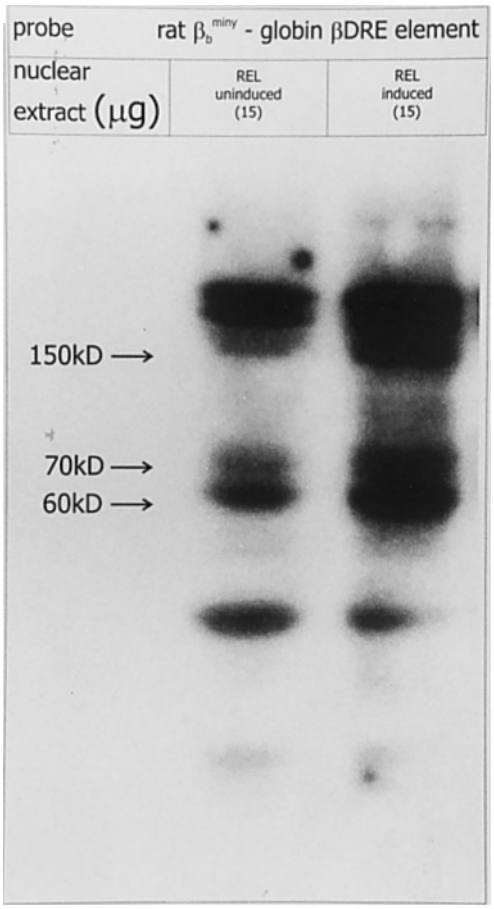

Figure 9. South-Western blot analysis of the interactions between a βDRE oligonucleotide probe and nuclear proteins from uninduced and DMSO-induced REL cells (15 μg each). The nuclear proteins of 150 kDa, 70 kDa and 60 kDa are indicated by arrows.

among mammals studied so far, rats have the largest number of active adult β-globin genes.

We have shown that transcription of the rat β_b^{miny}-globin gene initiates at coordinate 53 upstream of the ATG codon. The position of the *cap* site of the β_b^{miny}–globin gene determined in MEL cells does not coincide with the cap sites of human adult β–globin gene and the murine β^{maj}–globin gene, both determined in MEL cells: it is located at thymine, 3 bp further upstream [46].

In this paper we have demonstrated that the -95-bp fragment of the β_b^{miny}-globin promoter directs transcription of mRNA in stable transfection assays, and more interestingly, this promoter region is transcriptionally induced after MEL and REL cell differentiation. The promoter region spanning 100 bp 5′ from the transcription initiation site of the adult human β–globin gene was coined the minimal promoter [22]. In contrast to our results, deletion experiments of this promoter region demonstrated that the mutants containing the same conserved regulatory elements as our -95 rat β_b^{miny}-globin promoter (TATA box, βDRE, CCAAT box and proximal CACCC box) is not inducible during MEL cell differentiation [12]. It was shown that human β-globin gene promoter regulatory elements located between positions -160 and -138 are necessary for increased transcription upon differentiation. However, if combined with β-LCR, the human minimal β-globin promoter does provide erythroid-specific induction of transcription [47], as does the -95 rat β_b^{miny}-globin promoter in the absence of the LCR. 5′ deletion mutants of the murine β^{maj}-globin promoter indicate that 106 bp of the β-globin 5′-flanking sequences, containing at least four regulatory elements:—TATA box, βDRE, CCAAT box and CACCC box—is transcriptionally induced after MEL cell differentiation [14, 15, 19]. When both the CACCC and CCAAT elements are deleted in the -60 promoter, a small drop in inducibility of the β^{maj}-globin promoter is observed. An additional drop in inducibility occurs when the βDRE is removed, indicating that this element contributes significantly to induction [15]. Therefore, our rat β_b^{miny}-globin proximal promoter resembles the murine β^{maj}-globin promoter. Human β–globin minimal promoter appears to be differentially regulated during DMSO-induced erythroid differentiation.

Sequence and footprint analysis of the -95 β_b^{miny}-globin promoter region reveals four evolutionarily conserved transcriptional regulatory elements: the TATA element, βDRE, CCAAT box and CACCC box. In compliance with βDRE nomenclature [25], the promoter region from -38 to -58 of the rat β_b^{miny}-globin gene can be defined as a βDRE homology motif (βDREh), for its sequence shows 7/10 matches to the mouse and human βDRE repeat motifs. Similar to the human -90 CACCC box region, the rat β_b^{miny}-globin proximal promoter contains proximal and distal CACCC boxes. The -95 promoter region analyzed in this paper contains only the proximal CACCC box. We find that it shows a clear footprint.

Since we found that the -95 β_b^{miny}-globin promoter region is inducible upon MEL and REL cell differentiation, we analyzed the transcription factors binding at this promoter region in erythroid extracts. We used two oligonucleotides encompassing regulatory elements which are the candidates for inducibility of this promoter region, the CCAAT box and the βDRE, and performed gel shift analysis with MEL and REL cell nuclear extracts. The oligonucleotide containing the CCAAT box showed no qualitative difference in factors binding from uninduced and DMSO-induced REL cells. With both extracts two major complexes were observed. Based on competition analysis, we infer that

the two major complexes represent NF-Y [48, 49] and GATA-1 [22]. Further confirmation will be achieved by using antibodies and supershift analysis.

No differences were observed when REL and MEL cell nuclear protein factors were compared. The human β–globin -70 CCAAT box that we used binds NF-Y, GATA-1 and NF-E6 [45], whereas the mouse β^{maj}-globin promoter CCAAT box binds only NF-Y [50]. Thus, the rat β_b^{miny}-globin promoter CCAAT box resembles the human β-globin CCAAT box in this respect.

Antoniou et al. found no evidence for βDRE binding activity in the promoter of the human β-globin gene [23]. In contrast, Stuve and Myers have identified DNA binding activity with specificity for the βDRE from the promoter of the mouse β^{maj}-globin gene [25]. This binding activity, termed βDRf, for β-globin direct repeat factor, was detected in fractionated nuclear extracts from MEL cells, and has been partially purified from undifferentiated cells. We have found several complexes binding at the βDRE element from the rat β_b^{miny}-globin promoter in MEL and REL cell nuclear extracts.

We presume that the lack of inducibility of -100 human β–globin promoter in MEL cells could be explained by the fact that the βDRE element does not take part in the transcriptional regulation of this gene, whereas it has an active role in the inducibility of the rat β_b^{miny}-globin as well as the murine β^{maj}-globin proximal promoters.

The same complexes were found after binding of uninduced and DMSO-induced REL cell extracts, but quantitative differences are observed in all complexes. This indicates that the concentration of transcription factors binding to the βDRE element from the β_b^{miny}-globin gene is increased in DMSO-induced REL cells. South-Western blot analysis confirmed that there is, indeed, a significant increase in the amounts of two erythroid-specific transcription factors (150 kDa and 60 kDa) in nuclear extract from DMSO-induced REL cells. We presume that these factors are involved in erythroid-specific induction of transcription of the rat β_b^{miny}-globin.

Antoniou et al. have found that several transcription factors bind around the TATA box of the human β-globin gene [23]. One of them is erythroid factor GATA-1. The experiments of Matsuda et al. suggest that rat GATA-1 transcription factor is approximately 60 kDa in size [51]. We presume that the 60-kDa transcription factor which binds to the βDRE from the rat β_b^{miny}-globin gene is GATA-1. The confirmation will be achieved by using antibodies and supershift analysis.

In *Drosophila* and human cells, the TATA binding protein (TBP) of the transcription factor IID (TFIID) complex is tightly associated with multiple subunits termed TBP-associated factors (TAFs) that are essential for mediating regulation of RNA polymerase II transcription. TAFs are found to be essential for mediating activation by sequence-specific transcription factors. Surprisingly, DNA-binding studies indicated that $TAF_{II}150$ (150–kDa TAF) bound specifically to a DNA sequence overlapping an extended region outside of the TATA box. It binds to the promoter region independently of TBP [52]. The 150-kDa protein which binds to the βDRE element from rat β_b^{miny}-globin gene is possibly $TAF_{II}150$. Why is it bound to the βDRE element only in erythroid cells? One possible explanation is that $TAF_{II}150$ is a coactivator of GATA-1 transcription factor, and its coactivator function is realized through direct binding to promoter DNA. $TAF_{II}150$ could mediate GATA-1-dependent transcriptional activation. If GATA-1 and $TAF_{II}150$ are an interacting pair, $TAF_{II}150$ does not bind to the βDRE when GATA-1 is absent (HeLa extract). Similarly, there is evidence for a coactivator function of TAF in the human transcription factor Sp1 and its target $TAF_{II}110$ [53].

Our data show that all the detected factors binding to the proximal promoter of the β_b^{miny}-globin gene occur in uninduced and DMSO-induced REL cells, although there are quantitative differences. This is in agreement with what deBoer et al. have described for the transcription factors involved in the induction of transcription of the human β-globin gene during erythroid differentiation [22].

Antoniou et al. emphasized the importance of using a homologous model system regarding tissue specificity [23]. Our results for the βDRE element refer to another issue regarding the model system used in studies of globin gene expression, namely species specificity. In fact, the fastest complex, which is prominent in DMSO-induced REL cells, is barely visible in DMSO-induced MEL cells, similar to both uninduced REL and uninduced MEL cells. These results suggest that there might be differences in regulation of globin gene expression in systems originating from different species, but it remains to be confirmed in future studies.

We have used REL cells to study the regulation of transcription of globin genes, providing an additional tissue culture model that can be used for the analysis of gene expression and differentiation of erythroid cells. Although the rat is a commonly used laboratory animal, surprisingly little is known about globin gene regulation and erythropoiesis in this species. This is the first report on rat β-globin regulation of transcription. Similarity score comparison of the -95 promoter region of adult rat β-globin genes from haplotype b reveals extreme homology (98–100%) for four of them ($\beta_b^{minx'}$, β_b^{minx}, $\beta_{b/c}^{minx''}$ and $\beta_{b/c}^{minz}$). All of these promoters belong to the minor type β–globin promoters. In contrast, rat β_b^{maj}-globin promoter, the only major-type promoter in

haplotype b, does not show a high homology score (less than 50%) with the β_b^{miny}-globin promoter. We presume that our results of interactions among cis-acting elements and transcription factors characterized in this paper could be extrapolated to the other rat adult minor-type promoters, but this question will be addressed in future studies in our laboratory.

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