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Cloning and functional characterization of the rat α2B-adrenergic receptor gene promoter region: Evidence for binding sites for erythropoiesis-related transcription factors GATA1 and NF-E2

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Abstract

In the rat, the α2B-adrenergic receptor (α2B-AR) is encoded by the rat non-glycosylated (RNG) gene and is primarily expressed in the kidney, brain and liver of adult animals. High levels of α2B-AR are also found during fetal life in the placenta, liver and blood, where it is borne by cells of the erythropoietic lineage. As a first step to define the mechanisms responsible for the spatio-temporal pattern of α2B-AR expression, a genomic fragment containing 2.8 kb of the 5′-flanking region, the ORF and approximately 20 kb of the 3′-flanking region of the RNG gene was isolated. RNase protection assays performed on RNA from placenta or kidney using a series of riboprobes permitted to locate the transcription start site 372 bases upstream from the start codon. Transient transfection of various cells, including rat proximal tubule in primary culture, with constructs containing luciferase as a reporter gene demonstrated that: (i) the 5′-flanking region exhibited a strong and sense-dependent transcriptional activity and (ii) the 332 bp fragment (−732/−401 relative to the start codon), which lacks a TATA box but contains Sp1 sites, is sufficient to drive expression. Analysis of chromatin susceptibility to DNaseI digestion identified two hypersensitive sites (HS1 and HS2) located 1.7 and 1.0 kb, respectively, upstream from ATG and containing recognition sequences for erythroid transcription factors. EMSA showed specific binding of GATA1 and NF-E2 to these elements. Taken together, the results suggest that the chromatin environment in the vicinity of these boxes plays a critical role for α2B-AR expression during fetal life.

Keywords: α2b-adrenergic receptor; RNG; Promoter; Transcription factors; GATA1; NF-E2

1. Introduction

The α2-adrenergic receptors (α2-AR) are seven-transmembrane-domain receptors primarily coupled to heterotrimeric G proteins of the Gi/Go family, which affect cellular functions by changing the activity of a broad panel of effectors including adenylyl cyclase, phospholipases C, D and A_2, inwardly rectifying K^+-channels, voltage-gated Ca^{2+}-channels and mitogen-activated protein kinases (Erk1/2). α2-AR are widely distributed in the organism. Notably, they play a prominent role in the regulation of sympathetic nervous system tone, with a resultant decrease in heart rate and blood pressure, and are also involved in the control of a large number of other physiological functions such as lipolysis, platelet aggregation and insulin secretion. Pharmacological and molecular studies have demonstrated that the α2-AR family is heterogeneous and comprises three subtypes (α2A, α2B and α2C) encoded by distinct intronless genes [1]. The specific role of each subtype is not fully apprehended yet, but the generation of genetically engineered mice has recently allowed significant progress in the definition of their respective contributions to the regulation of the sympathetic nervous system and cardiovascular system. Examination of the phenotype of mice with inactivation of α2A-AR or/and α2C-AR demonstrated that both subtypes act as autoreceptors and thereby participate in presynaptic inhibition of noradrenaline release. The α2A-AR appears, however, to play a crucial role in the

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central action of α2-agonists, since most of the classical effects of these drugs, including hypotension, sedation and analgesia, are no longer observed in mice lacking this subtype (reviewed in [2]). On the contrary, administration of α2-agonist to α2B-AR knockout mice caused sustained hypotension but failed to elicit the initial transient increase in blood pressure observed in wild-type animals, indicating that this subtype is involved in peripheral vasoconstriction [3]. Moreover, because heterozygous mice lacking one allele of the α2B-AR gene do not display increased blood pressure in response to subtotal nephrectomy and dietary salt-loading, it is also believed that this subtype plays a crucial role in salt-induced hypertension [4].

The tissue distribution of the α2B-AR is still a matter of debate in humans, but has been rather well established in the rat. Its expression in adult rats is primarily restricted to kidney, liver and brain areas, including the thalamus and the nucleus tractus solitarii [5–8]. Remarkably high levels of α2B-AR are found during rat fetal life in the placenta, liver and blood, where it is borne by the hematopoietic cells of the erythrocyte lineage [9]. Although its function is still enigmatic in these cells, it was recently demonstrated that placental α2B-AR plays a major role in the development of the labyrinth vascular system and is crucial for mouse fetus survival [10]. Changes in the level of α2B-AR expression in brain and kidney were also found to be associated with pathological states. In particular, rat models for genetic or salt-induced hypertension exhibit an increased density of α2B-AR in their renal proximal tubule [11–15]. Since the receptor enhances Na^+-reabsorption via activation of NHE3 in this segment of the nephron [16] and because receptor up-regulation has been shown to precede the rise in blood pressure [17], it was hypothesized that this change may participate in the establishment and/or the maintenance of hypertension. Modifications in the level of α2B-AR expression were generally correlated with alterations in the amounts of its mRNA, but the mechanisms responsible for tissue-specific expression and transcriptional regulation of this subtype remain unclear.

The organization of the gene encoding the rat α2B-AR is completely unknown, in contrast to the α2A- and α2C-AR subtypes [18,19]. In an attempt to clarify the regulatory mechanisms of α2B-AR expression in rat tissues, we cloned and sequenced the promoter region of the rat non-glycosylated (RNG) gene. The functional characteristics of this region were investigated in rat proximal tubule cells in primary culture and in various established cell lines. DNaseI sensitivity experiments on rat fetal liver nuclei allowed us to map two hypersensitive sites, termed HS1 and HS2. Gel retardation assays demonstrated that HS1 contains functional recognition sequences for the erythroid-specific transcription factors, GATA1 and nuclear factor erythroid 2 (NF-E2), which may play a critical role for α2B-AR expression during fetal life.

2. Materials and methods

2.1. Screening the rat genomic library

The rat genomic library in the pWE15 cosmid vector (cat no. RL1032m, Clontech, Palo Alto, CA) was first screened using a [^{32}P]-labeled probe prepared from a PstI–PstI restriction fragment corresponding to nucleotides +629/+898 of the RNG coding region. Hybridization and washing were carried out as recommended by the manufacturer. Secondary screening was performed by PCR on bacterial colonies, using the sense 5′-AGCGCACTCCGCGCCGCATCA-3′ and antisense 5′-TTAGCTGAGGTTGGCACTCC-3′ primers which correspond to nucleotides +383/+403 and +694/+713 of the coding region, respectively. The isolated cosmid was characterized by Southern blot analysis. Restriction fragments were subcloned in either pBluescript® II KS$^+$ (pKS, Stratagene, La Jolla, CA) or pSP72 (Promega, Madison, WI) and sequenced by the dideoxy chain termination method using the ABI Prism DNA sequencing kit (Applied Biosystems, Foster City, CA). The nucleotide sequence has been deposited in GenBank under accession number AF366899 and was analyzed using the computer programs (TSSG, TSSW, TESS and POLYAH) available on-line at the Baylor College of Medicine (BCM, Houston, TX). Throughout the manuscript, nucleotides were numbered according to their position relative to the translation start site of the gene.

2.2. Generation of luciferase constructs

All the reporter gene constructs are numbered according to their positions relative to the translation initiation site and were generated from the promoterless luciferase vector pGL3-Basic (Promega). The NcoI–NcoI restriction fragment corresponding to bases −1877/+19 was inserted into the NcoI site of pGL3-Basic. Both orientations were obtained leading to the sense and anti-sense constructs, which were, respectively, termed pGL3-RNG −1877/+19 and pGL3-RNG +19/−1877. Deletion of the pGL3-RNG −1877/+19 with either BglII or NheI generated, respectively, pGL3-RNG −732/+19 and pGL3-RNG −406/+19. The pGL3-RNG −732/−401 construct was obtained by ligating the BglII-NheI fragment of the RNG gene into the same sites of pGL3-Basic. Finally, the pGL3-RNG −2779/+19 was constructed as follows: the BamHI–BamHI fragment (nucleotides −2779/−1175) was subcloned into the BamHI site of pKS, the XbaI-EcoRV fragment was excised and ligated with the 3.7 kb EcoRV-SalI fragment of pGL3-RNG −1877/+19 and the 2.8 kb SalI-NheI fragment of pGL3-Basic.

2.3. Proximal tubule cell isolation, cell culture and transfection

All experiments were conducted in accordance with the guiding principles for the care and use of animals. Prox-

imal tubule cells were isolated and cultured as previously described [20]. Briefly, Sprague-Dawley rats (4 weeks of age) were anesthetized by intra-peritoneal injection of sodium pentobarbital. Kidneys were rapidly excised, renal cortex was dissected and sliced in ice-cold Hank's-balanced saline solution (HBSS) buffered with 10 mM Hepes (pH 7.4). Slices were then incubated 30 min at 37 °C in 10 ml of HBSS containing 1 mg/ml BSA and 0.3 mg/ml collagenase. After two washes in HBSS, the preparation was deposited onto a self-forming Percoll gradient and centrifuged 30 min (20,000 g at 4 °C). The proximal tubule enriched fraction was collected, washed twice in DMEM/HAMF12 medium (1/1) and finally seeded on collagen-coated 6-well plates in the same medium supplemented with 5% FCS and containing 10 μg/ml insulin, 10 ng/ml EGF, 5 μM T3, 5 μg/ml transferrin and 100 nM dexamethasone. Two days post-seeding, cells were placed in FCS-free, but hormone-containing, DMEM/HAMF12. They were transfected the following day using the calcium phosphate method as described below for established cell lines.

The following established cell lines: MDCK (canine kidney), OK (opossum kidney), BHK21 (hamster kidney), NIH-3T3 (mouse fibroblasts) and HeLa (human cervix) were maintained in DMEM containing 10% FCS, 2 mM glutamine, 100 μg/ml streptomycin and 100 IU/ml penicillin. Cells were seeded at the appropriate density in 6-well plates and transfected by the calcium phosphate method using 4.5 μg of luciferase DNA and 0.5 μg of pCMV-βGal per dish. MEL (mouse erythroleukemia) cells were maintained in RPMI supplemented with 10% FCS, 2 mM glutamine, 100 μg/ml streptomycin and 100 IU/ml penicillin and transfected with lipofectin reagent (Invitrogen, Cergy Pontoise, France). Two days post-transfection, cells were rinsed in PBS and lysed in passive lysis buffer (Promega). The β-galactosidase activity was measured spectrophotometrically using *o*-nitrophenyl β-D-galactopyranoside as substrate and luciferase was assayed using the luciferase assay reagent (Promega). Luciferase activity was normalized to β-galactosidase and expressed as fold increase with regards to the promoterless vector pGL3-Basic.

2.4. Construction of the riboprobes

The 424 bp NheI-HindIII fragment of the RNG gene was subcloned into the SpeI and HindIII sites of pKS to generate pKS-RNG −405/+19. Subcloning of the BglII-AvrII fragment into pKS digested with BamHI and XbaI led to the pKS-RNG −731/−133 construct. The SpeI-SmaI restriction fragment ligated into the SpeI and EcoRV sites of pKS generated pKS-RNG −1085/−540. Linearization of pKS-RNG −405/+19 and pKS-RNG −1085/−540 with XbaI generated templates for probes 1 and 5, respectively. Templates for probes 2, 3 and 4 were obtained by cutting pKS-RNG −731/−133 with PvuII, NheI and EcoRI, respectively. Templates 1–5 served to generate the corresponding anti-sense labeled probes using [α^{32}P]UTP and T3 or T7 RNA polymerase (Promega). Positive controls correspond to cold sense RNA transcribed from pKS-RNG −405/+19 linearized with HindIII for probes 1 to 4 or from pKS-RNG −1085/−540 linearized with EcoRI for probe 5.

2.5. RNA extraction and RNase protection assay (RPA)

Tissues were rapidly frozen and pulverized in liquid nitrogen. Total RNA was extracted using the guanidinium thiocyanate/phenol/chloroform method [21]. RPA was performed on 80 μg of RNA as previously described [22], but with slight modifications. Lyophilized RNA was dissolved in 30 μl of hybridization buffer (80% formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM pipes, pH = 6.7) containing an excess of the [^{32}P]-labeled riboprobe. Samples were denatured 5 min at 95 °C and hybridized 14 h at 55 °C. Free probe was then digested with 5 units of RNase ONE (Promega) in 300 μl of TEN buffer (10 mM Tris/HCl, 5 mM EDTA, 300 mM NaCl, pH = 7.5). After 1 h at 37 °C, the reaction was stopped with 25 μl of 2% SDS containing 10 μg of carrier tRNA and protected hybrids were precipitated by the addition of 1 ml ethanol. RNA pellets were rinsed with 70% ethanol, dried, dissolved in 10 μl of sample buffer (97% formamide, 0.1% SDS, 0.05% bromophenol blue, 0.05% xylene cyanol, 10 mM Tris/HCl, pH = 7.0) and loaded on a 5% acrylamide/7 M urea gel. The gels were dried and exposed to X-ray film (Biomax, Kodak, Rochester, NY) with an intensifying screen or analyzed using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

2.6. DNaseI hypersensitivity assay

Pregnant Sprague-Dawley rats, at day 20 of gestation, were anesthetized by intra-peritoneal injection of sodium pentobarbital. Placenta and fetal liver were dissected and spleen was collected from the mother. Fetal liver and spleen were rapidly rinsed in ice-cold PBS, weighed and immediately homogenized in 10 volumes of ice-cold lysis buffer (10 mM Hepes pH = 7.9, 1% NP-40, 3 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF) using a Dounce homogenizer. After verification for integrity by light microscopy using trypan blue, nuclei were collected by gentle centrifugation (500 g, 10 min, 4 °C) and washed twice in lysis buffer. The nuclei were then loaded on a 10% sucrose cushion in lysis buffer and centrifuged 10 min at 1000 g. The final pellet was suspended in lysis buffer, the DNA content was estimated at 260 nm and adjusted to 0.1 μg/μl with 10 mM Hepes (pH 7.9). Aliquots of nuclei were treated with bovine pancreatic DNaseI at concentrations ranging from 0.1 to 0.8 μg/mg of DNA. After 10 min at 37 °C, the reaction was stopped by addition of EDTA. The nuclei were then successively treated by RNase A

(30 μg/ml, 15 min at 37 °C) and proteinase K (100 μg/ml, 5 h at 55 °C in 2% final SDS). DNA was extracted with phenol and chloroform, precipitated with isopropanol, rinsed in 70% ethanol and dissolved in water. After complete digestion with the appropriate restriction enzyme, samples were run on a 1.5% agarose 0.5× TBE gel (Genetical Tested Grade, Quantum Biotechnologies, Illkirch, France), treated 15 min with 0.25 M HCl, neutralized with 0.4 M NaOH and transferred onto a positively-charged nylon membrane (Hybond-N+, Amersham, Little Chalfont, UK) using 0.4 M NaOH. The membranes were prehybridized 4 h at 42 °C in 5× SSPE, 5× Denhardt's, containing 0.1% SDS, 50% formamide and 100 μg/ml salmon sperm DNA. They were then hybridized 16 h at 42 °C in the same buffer containing 10^6 cpm/ml of a specific probe corresponding to the region −406/+19 of the RNG gene labeled with [α^{32}P]dCTP using the Ready-To-Go DNA Labeling Beads (Amersham). Membrane was rinsed in 2× SSC, 0.1% SDS (10 min, twice) and in 0.1× SSC, 0.1% SDS (15 min, twice) and autoradiographied for 2–6 h.

2.7. Electrophoretic mobility shift assay (EMSA)

The oligonucleotides used as probes in EMSA were the following: RNG-GATA1a, 5′-TTGTCAGATATCCTGGGATTAG-3′ (nt −1679/−1658); RNG-GATA1b, 5′-TTGTTTAAGATTTATCATTTTATGTGT-3′ (nt −1776/−1750) and RNG-NFE2, 5′-CTTAAACTGCTGAGTCATCGCTCAG-3′ (nt −1607/−1583).

The oligonucleotides used in competition experiments corresponded to the GATA1 and NFE2 sites located at positions −83/−60 and −171/−142, respectively, of porphobilinogen deaminase (PBGD) gene and to the NFE2 site located at position −89/−69 of thromboxane synthase (TXS) gene: PBGD-GATA1, 5′-CTGATGGGCCTTATCTCTTTACCC-3′; PBGD-NFE2, 5′-TCCTCCAGTGACTCAGCACAGGTTCCCCAG-3′ and TXS-NFE2, 5′-AAAGTTGCTGATTCATTCCTT-3′.Probes were prepared as follows. Single-stranded oligonucleotides were labeled using [γ^{32}P]dATP and T4 polynucleotide kinase, purified on a Sephadex G-50 column, mixed with a four-fold excess of cold anti-complementary oligonucleotide, heated 2 min at 90 °C and allowed to cool slowly to room temperature. Unlabeled competitors were annealed in a similar way. Nuclear proteins were extracted from human erythroleukemia K562 cells according to the method developed by Dignam et al. [23]. As previously described [24], EMSA were performed in a 10 μl final volume of Tris-buffer (5 mM, pH = 8.0) containing 0.2 μg/μl poly(dI:dC), 0.5 mM DTT, 0.5 mM EDTA, 25 mM NaCl, 1% Ficoll, 50 pg/μl of the mentioned [^{32}P]-labeled probe and 10 μg of nuclear proteins. When specified, either a 100-fold excess of double-stranded cold competitor or an antibody directed against the NF-E2 p45 subunit was added. After a 30 min incubation period at room temperature, the reaction was stopped and samples loaded onto a 4% polyacrylamide, 0.25 × TBE gel.

3. Results

3.1. Cloning and sequence analysis of the genomic region surrounding the RNG gene

The screening of a rat genomic library allowed us to isolate a cosmid containing a 25 kb fragment of the RNG gene, which consisted in 2.8 kb of the 5′-flanking region, the ORF and approximately 20 kb of the 3′-flanking region. The first 6.3 kb of this fragment were subcloned and sequenced. This genomic region has an overall composition of 54% GC and its restriction map is depicted in Fig. 1. Search for RNA polymerase-II recognition sequences was performed using the promotor.dat (TSSG software) or the

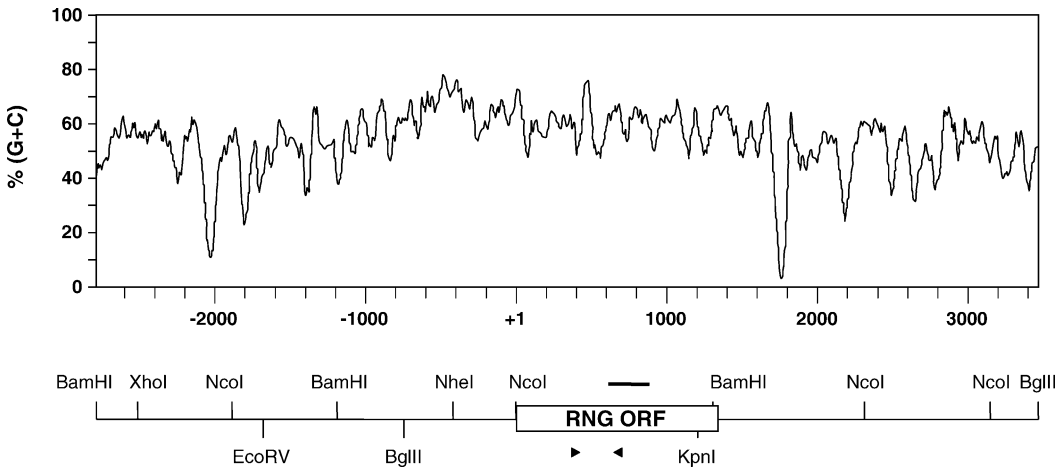

Fig. 1. Restriction map and GC content of the genomic region encompassing the RNG gene. The location of the probe (line) and primers (arrowheads) used to screen the rat genomic library is indicated. The isolated cosmid contains 2.8 kb of 5′-flanking region, the ORF and approximately 20 kb of 3′-flanking region. The restriction map and GC content of the BamHI/BglII fragment of the RNG gene (nt −2779/+3489 relative to ATG) are presented.

TRANSFAC (TSSW software) databases. Both programs predicted a unique promoter, located 372 (TSSG) or 358 bases (TSSW) upstream from ATG. On the other hand, the search for the mRNA 3′ end indicated the presence of a single poly-adenylation signal (AATAAA) located 1766–1771 bp downstream of the stop codon. The functionality of this signal was suggested by the existence of two expressed sequence tags (GenBank accession numbers AI511478 and AI549156), that both perfectly match our sequencing data and end 1848 bases downstream of the stop codon. The 3′-untranslated region also contains two T-rich regions, located 344–438 and 805–842 bp downstream of the ORF (77 and 79% of T, respectively), which might be involved in the regulation of mRNA stability. Analysis of the 5′-flanking region revealed that it lacks a conventional TATA or CCAAT box and does not contain a pyrimidine-rich sequence resembling an initiator [25]. By contrast, several putative binding sites for Sp1 (position −922/−913, −873/−864, −466/−455 and −440/−429) and C/EBPα (position −1165/−1158, −451/−444 and −421/−414) are present. The 5′-flanking region also contains potential binding sites for tissue-specific factors or transcription factors related to embryonic development, including Olf-1 (−90/−80), HNF-3 (−1574/−1565), HFH-2 (−1988/−1977) and Pax5 (−2625/−2612). A (GT)-repeat (i.e. $AG(GT)_{24}T$), which represents a putative binding site for the transcription factor WT1, was present at position −2070/−2020. Recognition sequences for transcription factors expressed by hematopoietic and erythroid cells were also predicted, including STAT6 (−623/−615), Ets-1 (−1501/−1492), GATA1 (−1768/−1758, −1674/−1665, −996/−987 and −822/−815) and NF-E2 (−1600/−1590). Finally, the 5′ non-coding region contains some special features, including an AT-rich region in position −2009/−1949 (89% AT) and an in-frame micro-ORF in position −165/−133, whose ATG codon is, however, not in a favorable context to initiate translation [26]. The nucleotide sequence of the 5′-flanking region is shown in Fig. 2.

3.2. Localization of the transcription start site

In order to identify the transcription initiation site of the RNG gene, a series of overlapping anti-sense riboprobes

Fig. 2. Sequence of the RNG gene promoter region. The sequence of the RNG gene spanning from BamHI to NcoI restriction sites (nt −2779/+21 relative to ATG) is presented. Putative binding sites for transcription factors are indicated as follows: Stat6, in bold; C/EBPα, underlined; Sp1, bold and underlined; Olf-1, underlined with open circles; HNF-3, underlined with open triangles; HFH-2, underlined with solid triangles; Pax-5, underlined with solid circles; Ets1, in bold and doubly underlined. Sequences for GATA1 are boxed and that for NF-E2 is in bold and boxed. The (GT)-repeat/WT1 site is in italics and within brackets; the AT-rich region is in lower case and within brackets; and the micro-ORF (−165/−133) is underlined with a dotted line and within brackets. The position of the transcription initiation site is indicated by an arrow.

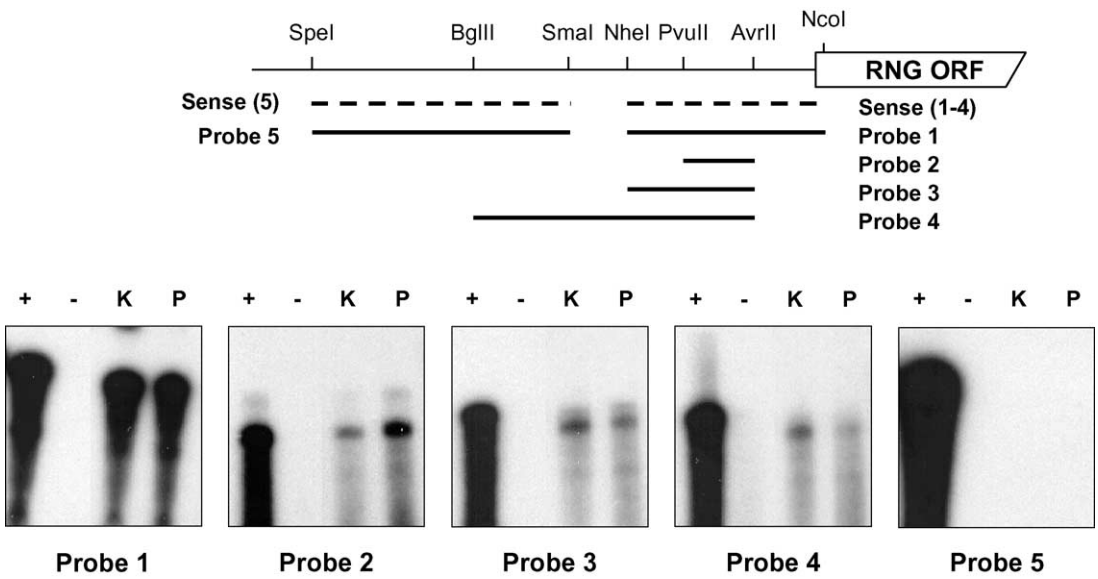

Fig. 3. Determination of the transcription start site of the RNG gene. The position of probes 1–5 is given. RPA was performed on RNA extracted from rat kidney (K) or placenta (P). Lanes (−) correspond to yeast tRNA. Lanes (+) are positive controls made with in vitro transcribed sense RNA corresponding to the NheI-NcoI fragment when probes 1–4 were used or to the SpeI-SmaI fragment when probe 5 was used.

was used in RPA on RNA extracted from kidney and placenta. The size and location of each probe are summarized in Fig. 3. Probe 1 corresponds to the NheI-NcoI restriction fragment (nt −405/+19), probe 2 to PvuII-AvrII (−287/−133), probe 3 to NheI-AvrII (−405/−133), probe 4 to BglII-AvrII (−731/−133) and probe 5 to SpeI-SmaI (−1085/−541). RPA performed with probe 1 revealed a strong signal from a hybrid, whose size was slightly shorter than that of the positive control obtained with the corresponding cold sense RNA transcribed in vitro. This result, which suggests that, in both kidney and placenta, transcription is initiated downstream of the NheI restriction site, was confirmed by the use of the other probes. Indeed, RPA performed with probe 5 gave no specific signal, whereas experiments with probe 2 yielded fully protected hybrids. Furthermore, probes 3 and 4, which share a common 5′ extremity but which then span to NheI or upstream from the NheI site, respectively, were merely partially protected. Strictly similar results were obtained when RPA was performed on fetal liver RNA. Analysis of the size of bands protected by probes 1, 3 and 4 indicated that transcription is initiated 372 ± 5 bases upstream from the ATG. This result is in good agreement with the computer prediction, as well as with the data obtained by Zeng et al. [27]. Indeed, when these authors isolated the RNG cDNA, the clone that extended furthest towards the 5′ terminal ended at position −368. Assuming that transcription of the RNG gene is initiated approximately 370 bases upstream from the ATG and that the poly-adenylation site is located 1848 bases downstream of the stop codon, the size of RNG mRNA is expected to be 3580 bases, not including the poly-A tail. This size is compatible with the result from Northern blot experiments with rat kidney poly-A+ RNA, indicating a signal of about 4 kb [27].

3.3. Functional characterization of the promoter in transfected cells

The transcriptional activity of the 5′-region of the RNG gene was first examined by transfecting rat proximal tubule cells in primary culture with luciferase constructs. These cells were chosen as hosts because they spontaneously express the α2B-AR [20]. The construct containing the entire 5′-flanking region (pGL3-RNG −2779/+19) exhibited a luciferase activity comparable to that observed with the SV40 early promoter (Fig. 4). The activity of pGL3-RNG −1877/+19 was similarly high; nevertheless, the promoter activity of this region was sense dependent as the construct containing the same fragment but in reverse orientation (pGL3-RNG +19/−1877) was devoid of luciferase activity. Further 5′-deletions indicated that the −732/+19 fragment still displayed activity, whereas the −406/+19 did not, suggesting that the region between nucleotides −732 and −406 is crucial for transcriptional activity. This view is also supported by results from transfection with pGL3-RNG −732/−401. This 332 bp fragment was indeed sufficient to promote luciferase activity, indicating that it contains the *cis*-elements required for optimal transcription.

To examine the RNG promoter transcription activity in different cellular contexts, all above-mentioned constructs were also assayed in cell lines of various origins, including MDCK, MEL, OK, NIH3T3, BHK21 and HeLa (Table 1). Although none of these cells naturally express the α2B-AR, the profile of activity for the different constructs was the same as in rat proximal tubule cells. Thus, in transient transfection experiments, the cloned fragment of the RNG promoter failed to display any cell specificity. This clearly contrasts with the restricted pattern of RNG

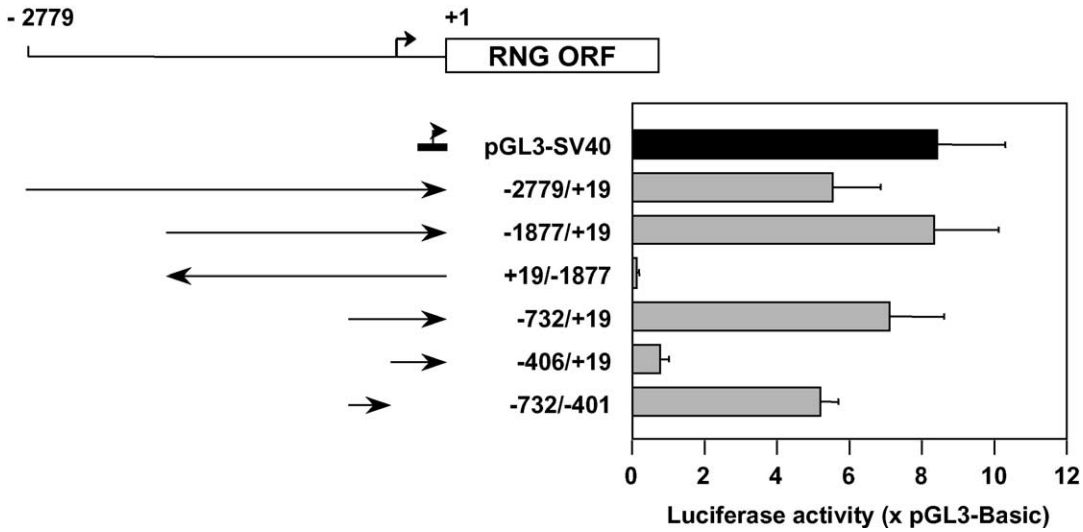

Fig. 4. Promoter activity of the RNG gene 5′-region in primary culture of proximal tubule cells. Rat proximal tubule cells were cultured and transfected with the indicated luciferase constructs and pCMV-βGal vector as described in the Section 2. Luciferase activity was measured 48 h post-transfection and normalized to β-galactosidase. Results are expressed as fold increase with regards to the promoterless vector pGL3-Basic. Reported values are means ± S.E.M. of 3–6 independent experiments, each performed in duplicate.

gene expression in vivo. Consequently, a different approach was considered in the attempt to identify the regions possibly involved in the targeted expression of this gene.

3.4. Identification of two DNaseI hypersensitivity sites in the RNG gene promoter

DNaseI hypersensitivity assays were performed in order to map regions of the RNG promoter that may be important for transcription. These experiments were carried out on nuclei prepared from two rat tissues, fetal liver and adult spleen. Whereas the former contains large amounts of α2B-AR, the latter does not and expresses the α2A-AR subtype only [9]. As shown in Fig. 5, in the absence of DNaseI treatment and after digestion with KpnI, the RNG probe detected a single band of about 20 kb in both tissues. The treatment of adult spleen nuclei with increasing concentrations of DNaseI resulted in a progressive decrease in the intensity of the 20 kb band without appearance of any other signal, indicating that this KpnI/KpnI genomic region was cut randomly. Very different were the data when fetal liver was considered. Indeed, two major bands (about 3 and 2 kb in size) were progressively generated by DNaseI treatment, revealing the presence of two hypersensitive sites (HS1 and HS2) in the KpnI/KpnI fragment. Southern blotting using NcoI as restriction enzyme allowed us to localize HS1 and HS2 at 1.70 ± 0.1 and 1.05 ± 0.1 kb, respectively, upstream from ATG. Interestingly, these two regions contain potential binding sites for transcription factors expressed in hematopoietic cells. Indeed, HS2 contains a putative binding site for GATA1 (nucleotides −996/−987), while HS1 covers two potential binding sites for GATA1 (nucleotides −1768/−1758 and −1672/−1665) and one for NF-E2 (nucleotides −1600/−1590). EMSA was therefore carried out in order to determine whether these sequences are functional.

3.5. NF-E2 and GATA1 transcription factors bind to the RNG gene promoter

The ability of NF-E2 and GATA1 to interact with their putative target sequences present in HS1 was examined by EMSA (Fig. 6). To this end, radiolabeled double-stranded

Table 1
Activity of the RNG promoter in various established cell lines Cells were transfected and assayed for luciferase and β-galactosidase activities as described in the Section 2

	MDCK	MEL	OK	NIH3T3	BHK21	HeLa
pGL3-SV40	16.2 ± 0.9	16.4 ± 2.9	15.7 ± 1.2	26.0 ± 5.4	40.3 ± 9.2	38.7 ± 13.5
pGL3-RNG −2779/+19	7.7 ± 2.1	4.9 ± 0.6	4.0 ± 0.3	7.9 ± 0.7	11.4 ± 3.5	18.8 ± 1.1
pGL3-RNG −1877/+19	6.5 ± 0.7	5.5 ± 1.2	5.0 ± 0.3	9.1 ± 0.8	12.1 ± 2.1	32.3 ± 6.6
pGL3-RNG +19/−1877	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
pGL3-RNG −732/+19	7.6 ± 1.4	6.9 ± 0.9	6.5 ± 1.0	9.0 ± 1.7	10.0 ± 1.5	36.2 ± 8.6
pGL3-RNG −406/+19	3.1 ± 0.3	2.1 ± 0.3	2.9 ± 0.5	1.7 ± 0.2	1.8 ± 0.4	6.5 ± 3.1
pGL3-RNG −732/−401	9.3 ± 1.6	5.1 ± 0.7	5.9 ± 1.1	5.1 ± 0.6	6.9 ± 0.9	14.9 ± 1.4

Results are expressed as fold increase regarding to the promoterless vector pGL3-Basic. Reported values are means ± S.E.M. of 3–9 independent experiments, each performed in duplicate.

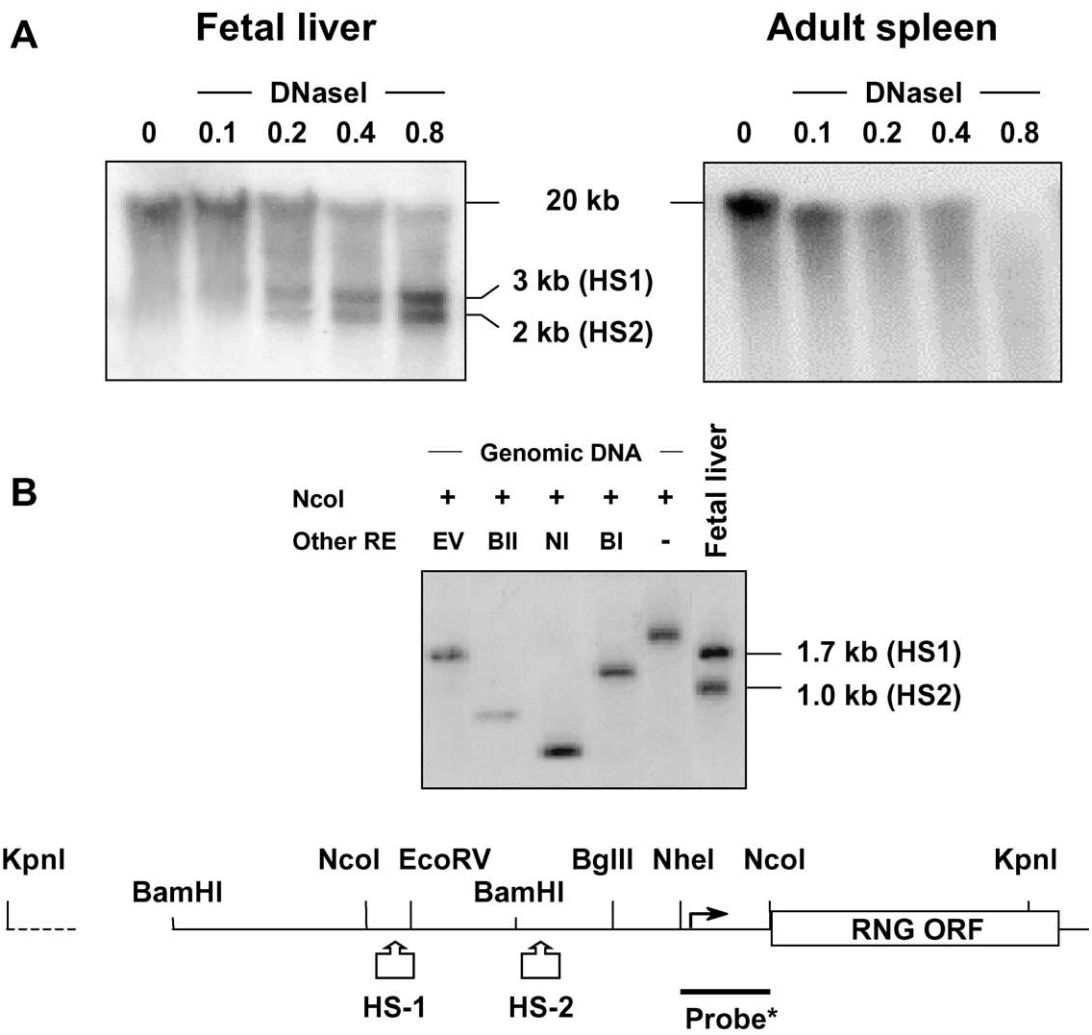

Fig. 5. Identification of DNaseI hypersensitive sites. Panel A: Nuclei from fetal liver and adult spleen were prepared as described in Section 2. After digestion by increasing amounts of DNaseI, genomic DNA was extracted, digested to completion with KpnI and submitted to a Southern blotting. Blots were hybridized with a [^{32}P]-labeled DNA probe corresponding to the NheI-NcoI restriction fragment. Panel B: DNA extracted from DNaseI-treated (0.8 μg) fetal liver nuclei was digested to completion with NcoI (fetal liver). DNase-untreated genomic DNA was also extracted and digested with NcoI alone, or in combination with EcoRV (EV), BglII (BII), NheI (NI) or BamHI (BI). Samples were submitted to Southern blotting using a [^{32}P]-labeled DNA probe corresponding to the NheI-NcoI restriction fragment.

oligonucleotides were incubated with nuclear extracts prepared from K562 cells. Incubation of nuclear extract with RNG-GATA1a probe (nucleotides −1679/−1658 of the RNG gene promoter) yielded a single retarded signal (Fig. 6, panel A) which was strongly attenuated by the addition of an excess of cold RNG-GATA1a or RNG-GATA1b probes. Moreover, it was completely blunted by the addition of a competitor corresponding to the region −83/−60 of the promoter of PBGD (PBGD-GATA1), which was previously reported to bind GATA1 [28]. Thus, RNG-GATA1a specifically binds GATA1 transcription factor. EMSA performed with RNG-GATA1b probe indicated that several factors are able to interact with the region of the promoter spanning nucleotides −1776/−1750 (Fig. 6, panel B). The retarded band with the highest mobility appears to be artifactual as it was not affected by the addition of cold RNG-GATA1b. Of the four other retarded bands, the one with highest mobility was strongly inhibited by an excess of cold RNG-GATA1a or RNG-GATA1b and totally disappeared in the presence of PBGD-GATA1, indicating that it corresponds to GATA1 binding. The three other bands with lowest mobility are specific to RNG-GATA1b as they disappeared with an excess of the corresponding cold probe, but they do not reflect GATA1 binding as they were not affected by RNG-GATA1a or PBGD-GATA1. The nature of the other protein factors interacting with the RNG-GATA1b sequence therefore remains to be established. Finally, the functionality of the NF-E2 site was examined using the RNG-NFE2 probe which spans nucleotides −1607/−1583 (Fig. 6, panel C). Two specific bands, eliminated in the presence of cold RNG-NFE2, were retarded in such experiments. They correspond to the binding of AP1 and NF-E2 transcription factors as they disappeared in the presence of competitors (PBGD-NFE2, TXS-NFE2) corresponding to the well characterized NF-E2 sites from the PBGD and TXS gene

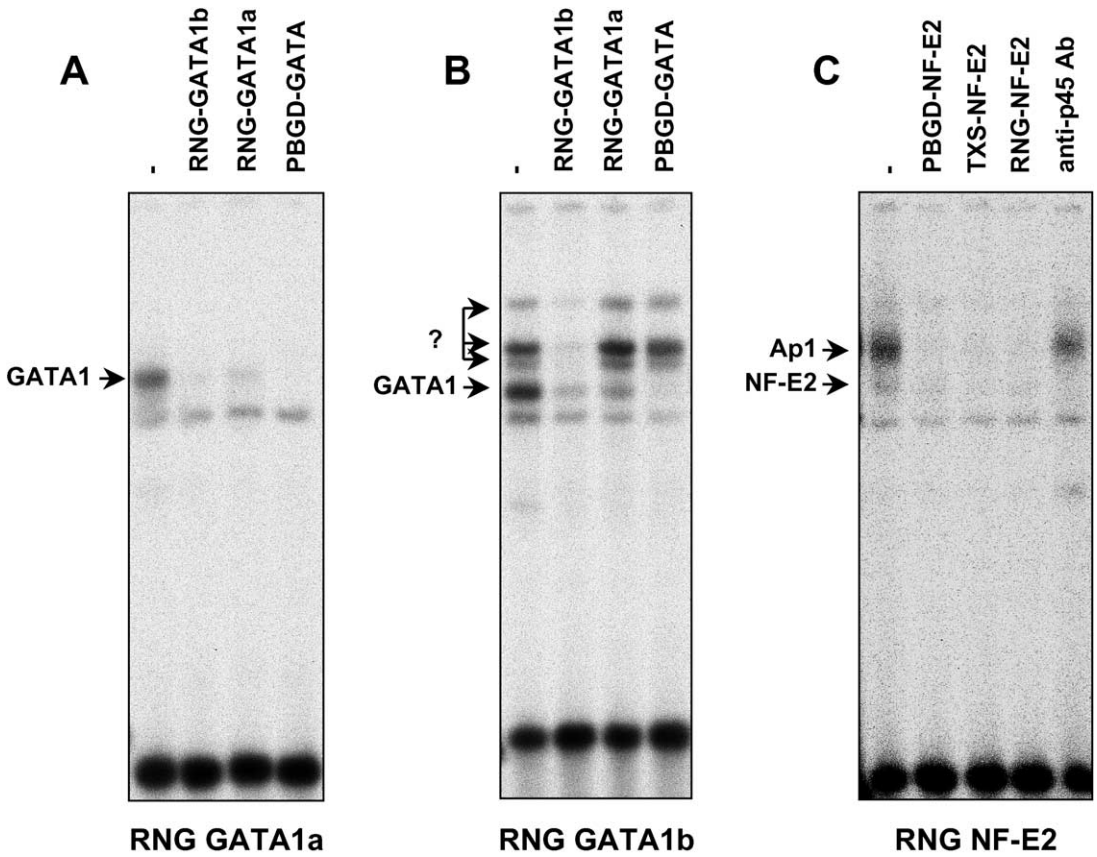

Fig. 6. Functional study of GATA1 and NF-E2 binding sites. Double-stranded oligonucleotides corresponding to nt −1679/−1658 (RNG-GATA1a) nt −1776/−1750 (RNG-GATA1b) and nt −1607/−1583 (RNG-NF-E2) were labeled, incubated with nuclear extracts from K562 cells and submitted to EMSA as described in Section 2. Panel A: RNG-GATA1a labeled probe was incubated in the absence (−) or in the presence of the indicated cold competitors (RNG-GATA1b, RNG-GATA1a or PBGD-GATA1). Panel B: RNG-GATA1b probe was incubated in the absence (−) or in the presence of the indicated cold competitors (RNG-GATA1b, RNG-GATA1a or PBGD-GATA1). Panel C: RNG-NF-E2 probe was incubated in the absence (−) or presence of the following competitors (PBGD-NF-E2, TXS-NF-E2 or RNG-NF-E2 itself); or in the presence of an antibody directed against the p45 subunit of NF-E2 (anti-p45 Ab).

promoters. Moreover, the band with higher mobility represents binding of NF-E2 as it is eliminated by the addition of an antibody directed against the p45 subunit of this transcription factor. Conversely, the cold RNG-NFE2 was able to disrupt the interaction of both AP1 and NF-E2 with their respective target sequences present in the PBGD and TXS promoters (not shown). Overall, these data demonstrated that functional NF-E2 and GATA1 binding sites are present in HS1, suggesting that these erythroid-specific transcription factors are involved in RNG gene expression in the liver during fetal life.

4. Discussion

Although several studies in the rat have demonstrated that α2B-AR expression is restricted to certain tissues and that receptor density undergoes important changes during development, the flanking regions of the gene encoding this subtype have so far remained unexplored. The present work was thus carried out as an initial approach to characterize the promoter region of the RNG gene and to define the elements that may account for its pattern of expression and regulation. The screening of a rat genomic library allowed us to isolate a fragment containing 2.8 kb of 5′-flanking region, the ORF and approximately 20 kb of 3′-flanking region. Alignment of the sequence of the RNG gene with those of mouse (GenBank accession no. M94583) and human (GenBank accession no. AF005900) indicated that whereas the coding blocks are nearly identical, the flanking regions largely diverge between rodents and man (Fig. 7). The 5′NC-region of rat shares 83% identity with mouse, but is very divergent from human with the exception of two short regions. The first (54 bp in length, 83% identity) contains the two SP1 sites located just upstream from the transcriptional start of the rat and human genes. The second (122 bp, 81% identity) contains a micro-ORF, a feature which is also present in both species. In the same way, rat and human 3′-flanks exhibit identity (82%) only within a 333 bp region which contains three AAAAA motifs in man, but only one in rat. Finally, rat and mouse contain a strictly identical T-rich region (i.e. TTTTTTGTTTGTTTGTTTGTTTGTTT) located, respectively, 805 and 2028 bp downstream of the stop codon. Among α2-AR genes, divergence between rodents and human seems to be a singularity of the α2B-AR, since the genomic regions surrounding α2A- and α2C-AR genes are

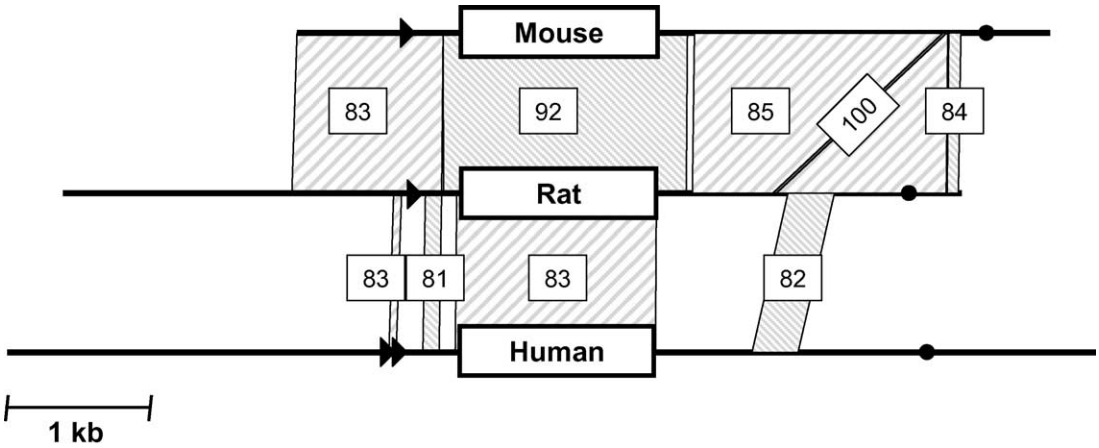

Fig. 7. Comparison of the sequences surrounding the rat, mouse and human α2B-AR encoding genes. The genomic sequence of the RNG gene (6268 bp) was aligned with the mouse (5265 bp) or the human (7605 bp) α2B-AR gene using pairwise blast at the NCBI. Open reading frames, transcription start sites and polyadenylation signals are indicated by rectangles, arrowheads and circles, respectively. Shaded regions indicate the percentage of identity to the rat sequence.

rather well conserved between the three species (>75% identity). As previously noted [29], this may explain the difference in the patterns of α2B-AR distribution observed between man and rat [30].

According to RPA performed on RNA extracted from kidney, placenta and fetal liver, the transcription start site is located approximately 370 bases upstream from ATG indicating that the α2B-AR expression is driven by the same promoter in adult and fetal tissues. Consistent with this result, assessment of promoter activity in transfected cells using luciferase as reporter revealed that the region located between nucleotides −732/−401 exhibited high transcriptional activity and thus corresponds to the minimal promoter. This region lacks the conventional TATA-box but contains two putative binding sites for each of the ubiquitous transcription factors, Sp1 and C/EBPα. The transcriptional activity of the 5′-flanking region was dependent on orientation, but not on the cellular context. This latter result was unexpected regarding the restricted expression pattern of the RNG gene in vivo. The transfection approach being inappropriate to identify promoter regions responsible for tissue-specific expression, DNaseI sensitivity assays were performed on nuclei prepared from two rat tissues (fetal liver, adult spleen) strongly expressing α2B-AR or not. Two hypersensitive sites, termed HS1 and HS2 and located, respectively, 1.70 and 1.05 kb upstream from ATG, were found in fetal liver, but not in spleen. As the HS1 region contains putative binding sites for transcription factors involved in erythroid-specific gene expression, the functionality of these sequences was explored. EMSA clearly demonstrated the binding of several transcription factors to sequences located in HS1. More precisely, the probe corresponding to nt −1607/−1583 bound NF-E2 as well as the ubiquitous factor Ap1, while the baits corresponding to nt −1679/−1658 and −1776/−1750 were recognized by GATA1. Interestingly, whereas the proximal GATA-box bound this transcription factor only, the distal box also bound other factors which remain to be identified. While the RNG promoter contains sequences which effectively bind erythroid-related transcription factors, transfection experiments indicated no consequence of their deletion on the promoter activity in MEL cells. This lack of effect may be due to the fact that transient transfection assays do not generally allow evaluation of HS activity because transfected DNA is not in a chromatin environment. Assessment of HS1 and/or HS2 function will thus require testing luciferase constructs in stable transfectants. The RNG promoter region contains additional transcription factor binding sites which may also participate in gene expression in hematopoietic cells and in the liver. They include one binding site for Ets-1 and two for GATA1, one of which is located within HS2, and two binding sites for members of the HNF3 family (HNF3 and HFN3/Forkhead Homologue 2, HFH2). Ets-1 and GATA1 were for example demonstrated to act additively to promote the expression of human glycoprotein IIB in megakaryocytes [31]. In HepG2 cells, HNF3γ was shown to regulate the constitutive expression of CYP3A4 in cooperation with C/EBPα [32]. The functionality of these elements was not investigated in the current work. Further studies, including DNaseI sensitivity assays on other tissues, will be also necessary to investigate whether these elements are involved in RNG gene expression.

NF-E2 is a member of the Cap‘n’Collar subfamily of basic region-leucine zipper transcription factors, which binds the consensus sequence TGCTGA(G/C)TCA. Such Ap1-extended motifs are found in regulatory regions of several erythroid- and megakaryocyte-specific genes, including α- and β-globin, TXS and heme biosynthetic enzymes (PBGD, ferrochelatase) [33–35]. NF-E2 is a heterodimer consisting of a large p45 subunit and a small p18 partner, also known as Maf-K. Whereas, expression of p18 is ubiquitous, that of p45 is restricted to cells from the

erythroid/megakaryocytic lineage. The p45 subunit lacks DNA-binding properties but has a transactivation domain and is able to interact with TAFII130 [36] and CBP [37,38]. Conversely, p18 confers to NF-E2 its DNA binding specificity. In addition to heterodimerizing with p45, Maf-K is able to homodimerize or to form dimers with other members of the Maf family (Maf-F and Maf-G), which are all devoid of transactivating activity. Consequently, according to the cell context and to the expression level of these different partners, the NF-E2 sequence can behave either as an enhancer or as a repressor [39]. Genetic disruption of p45 results in a high perinatal mortality (90%) due to a severe deficit in megakaryocyte maturation and platelet production [40]. Surprisingly, with regard to high expression in erythroid cells, surviving $p45^{-/-}$ mice only suffer from mild globin gene expression defect, suggesting that other factors can compensate for the lack of NF-E2 [41]. Redundancy was confirmed by invalidation of Maf genes. Indeed, $MafK^{-/-}$ and $MafG^{-/-}$ mice exhibit a normal phenotype [42] and mild thrombocytopenia weakly phenocopying $p45^{-/-}$ mice [43]. By contrast, disruption of both genes causes severe anemia, thrombocytopenia and red cell cytoskeletal defects resulting in perinatal lethality [44].

The zinc-finger transcription factor GATA1 binds a (A/T)GATA(A/G) motif and is also critical for expression of almost all erythroid-specific genes [45,46]. The GATA1 gene, which is located on the X chromosome, has been invalidated in male-derived ES cells. When injected into blastocysts, these $GATA^{-/0}$ cells were never seen to participate in erythrotopoiesis of the resulting chimeric mice [47]. They also failed to undergo erythroid differentiation in vitro [48]. Further evidence for the vital role of this transcription factor was provided by knockout mice. Whereas, heterozygous females ($GATA^{-/+}$) are viable, all hemizygous males ($GATA^{-/0}$) die at E11 from severe anemia [49] so homozygous female ($GATA^{-/-}$) cannot exist.

In conclusion, the present study permitted the transcription start site of the RNG gene to be located and a 332 bp DNA fragment to be identified as corresponding to the minimal promoter. Two DNaseI hypersensitive sites (HS1 and HS2) were found in nuclei from rat fetal liver. HS1 contains recognition sequences for transcription factors NF-E2 and GATA1 suggesting that it may act as an enhancer of RNG gene transcription in fetus erythropoietic tissues, but this hypothesis awaits experimental demonstration.

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