Transcriptional and translational mechanisms of cytochrome *b*₅ reductase isoenzyme generation in humans

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Cytochrome *b*₅ reductase (*b*₅R) is an essential enzyme that exists in soluble and membrane-bound isoforms, each with specific functions. In the rat, the two forms are generated from alternative transcripts differing in the first exons. In contrast, the biogenesis of *b*₅R isoforms in the human is not yet well understood. In the present study we have detected three novel alternative exons, designated 1S, S' and 1B, located between the first alternative exon 1M and the common second exon in the human *b*₅R gene. Accordingly, multiple M-type, S-type and SS'-type and B-type transcripts are generated. All types of human *b*₅R transcript are expressed ubiquitously. An analysis of *in vitro* translation

products demonstrated an alternative use of different AUG initiators resulting in the production of various human *b*₅R protein isoforms. Our results indicate that the organization of the 5' region of the *b*₅R gene is not conserved between rodents and humans. Insertion of Alu elements into the human *b*₅R gene, in particular just upstream of the S/S' region, could be responsible for dynamic events of gene rearrangement during evolution.

Key words: alternative splicing, Alu sequences, erythroid promoter, gene evolution.

INTRODUCTION

NADH:cytochrome *b*₅ reductase (*b*₅R; EC 1.6.2.2) is a flavo-protein that catalyses the transfer of electrons from NADH to cytochrome *b*₅ within an NADH/cytochrome *b*₅ reductase/cytochrome *b*₅ complex.

Two forms of *b*₅R are known, a membrane-bound form and a soluble one. The membrane-bound form is present mainly in the endoplasmic reticulum (ER), mitochondria, nuclei, peroxisomes and plasma membranes of somatic cells [1–4]. The soluble form of *b*₅R is found mainly in erythrocytes of different species including humans [5], but has also been isolated from rat and rabbit livers and human placenta [6,7].

The two forms of *b*₅R, localized in different cellular compartments, are involved in various metabolic pathways. In erythrocytes, the NADH/cytochrome *b*₅ reductase/cytochrome *b*₅ complex present in the soluble form is responsible for the reduction of methaemoglobin [8]. In non-erythroid tissues the soluble *b*₅R isoform is a minor form. Nevertheless, a soluble electron transport system consisting of cytosolic *b*₅R and cytochrome *b*₅, promoting the hydroxylation of CMP-*N*-acetylneuraminic acid to *N*-glycolylneuraminic acid, has been described in the liver and other tissues from various species [9,10]. In contrast, the effective involvement of the cytosolic *b*₅R isoform in the bioreductive activation of mitomycins has been reported in Chinese hamster ovary cells [11]. The membrane-bound *b*₅R isoform transfers electrons from NADH to membrane-bound cytochrome *b*₅, which then transfers its electrons to different electron acceptors of the cells. In ER membranes, the NADH/cytochrome *b*₅ reductase/cytochrome *b*₅ complex is involved in fatty acid

desaturation [12], transferring the electrons to a non-haem-iron-containing stearyl-CoA desaturase enzyme (Δ -9 desaturase) or to other desaturases (Δ -5, Δ -6, Δ -1' desaturases) present in the ER. In human erythrocyte membranes, the membrane-bound *b*₅R isoform has been described to participate in vitamin E recycling [13], whereas its role in ascorbate regeneration was reported in both mitochondrial [14] and plasma membranes [4].

Both forms of *b*₅R protein have an identical hydrophilic catalytic domain and differ only in their N-termini. The major membrane-bound *b*₅R isoform has 25 additional hydrophobic amino acid residues, with an N-terminal myristoylation sequence. The erythrocytic membrane-bound *b*₅R isoform found in rat [15] contains at its N-terminus a non-myristoylated stretch of 29 hydrophobic amino acid residues. Both hydrophobic sequences are lacking in the soluble form of the protein.

The biosynthesis of different *b*₅R isoforms, known to be encoded by a single gene [16], was first elucidated in the rat [15,17]. Three types of *b*₅R mRNA have been found in addition to the major M-mRNA, which codes for the membrane-bound *b*₅R isoform. One is the erythroid-specific R-type mRNA and the other two are the X- and Y-type mRNA species expressed ubiquitously at a relatively low level [17]. These four types of rat mRNA differ in their first alternative and mutually exclusive exons, and also in the use of AUG initiation codons, leading to the synthesis of proteins bearing different N-termini: ubiquitous membrane-bound, erythroid membrane-bound, erythroid soluble and ubiquitous soluble proteins.

Here we report the expression of the human *b*₅R gene and especially the biogenesis of the human soluble *b*₅R isoform. In contrast with another paper [18], we found that in humans,

Abbreviations used: *b*₅R, cytochrome *b*₅ reductase; ER, endoplasmic reticulum; ORF, open reading frame; RT-PCR, reverse-transcriptase-mediated PCR; RACE, rapid amplification of cDNA ends.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers AF162683 and AF162684.

Figure 1 Nucleotide sequence of the human *b₅R* gene fragment encompassing exons 1S and S'

Exons are indicated by capital letters, introns by lower-case letters. Consensus donor and acceptor splicing sites surrounding exons 1S, S' and 2 are underlined with a double line. Primers in exon 2 used for 5' RACE PCR and analysis by RT-PCR are underlined with dotted arrows. The asterisk indicates the radiolabelled oligoprobe used in both experiments. The additional fragment bounded by two repeats (TGGGATCC) shown by bold square brackets is underlined by a single thin line. The plain arrows indicate primers used in the genomic DNA amplification of the new DNA sequence identified. The broken arrows indicate the upstream primers in 1S and S' exons used in the RT-PCR analysis of the expression profile. ORF1 starts with an ATG initiator codon (▼) and ends with a TGA stop codon (●) within exon 1S. ORF2 in exon 2 possesses two putative initiator codons (▼), both in phase with ORF1. Putative *cis*-acting elements discussed in the text are boxed upstream of exon 1S. The reverse-oriented Alu element class II detected approx. 190 bp upstream of exon 1S is underlined with a broken line.

in contrast with rats, no erythroid-specific transcript can be detected. Instead, *b₅R* activity in red cells seems to result from the translation of various types of ubiquitous transcript generated by the use of alternative promoters and splicing patterns. The evolutionary diversification of *b₅R* gene organization and expression between rodents and humans is discussed.

EXPERIMENTAL

Reticulocytes

Heparinized blood, obtained from either a sickle-cell-anaemia patient with 6% reticulocytes or from umbilical cord during delivery, was further treated by differential centrifugation to remove leucocytes [19] and to increase the reticulocyte proportion to approx. 25% for the two samples, as determined by staining with Brilliant Cresyl Blue.

General procedures

Southern blotting of PCR products subjected to PAGE was performed as described previously [20]. Blots were hybridized with oligonucleotides labelled at the 5'-OH group with [³²P]ATP (Amersham) by using T4 polynucleotide kinase. Sequencing of

Figure 2 Nucleotide sequence of exon 1B and flanking region in the human *b₅R* gene

Exons, introns and consensus splicing sites are represented as in Figure 1. The upstream primer used in the amplification of B-specific cDNA fragments is represented by the broken arrow at position nt 5229. The overlapping downstream primers used in the 5' RACE PCR characterization of exon B are represented by the dotted arrows at positions nt 5472–5497. The asterisk indicates the oligonucleotide probe. Two ATG codons at positions nt 5380 and 5407 in exon 1B are represented by open triangles (▼). The two AUG initiator codons in exon 2 are shown as in Figure 1. The upstream functionally active ATG used as an initiator in the B-type transcript is shown in bold letters and is marked by plain triangle (▼). Putative *cis*-acting elements located upstream of exon B are boxed.

recombinant plasmids was performed with a Sequenase kit (version 2.0; United States Biochemicals) in accordance with the instructions of the manufacturers. Total RNA was prepared from different human tissues, cultured cells and umbilical cord blood, and from the reticulocyte-enriched red blood cell preparations by using RNA-NOW single reagent (Biogentex). Total RNA from human fetal brain and liver was obtained from Clontech.

PCR

PCR experiments were performed in a DNA thermal cycler (Perkin-Elmer Cetus) with *Taq* DNA polymerase (Gibco BRL). The PCR conditions and buffer composition were as described previously [17]. Genomic DNA fragments were amplified from 500 ng of human DNA heated at 95 °C for 10 min in the reaction mixture before the addition of *Taq* DNA polymerase. The oligonucleotide primers were obtained from Genset (Paris, France).

Reverse-transcriptase-mediated PCR (RT-PCR)

cDNA synthesis was performed as described previously [17] with 10 µg of total RNA in 80 µl of buffer supplied in the Reverse Transcriptase kit from Gibco BRL. PCR amplification was performed with 5 µl of reverse transcriptase reaction. Detection of the M-mRNA by RT-PCR was performed with an upstream primer, 5'-ACGGTCTCGGCGGCGGC-3', located at nt -53 to -38 of the published sequence [21]. The nucleotide sequences of upstream primers used in RT-PCR analysis of the expression of S and B mRNA species are indicated in Figures 1 and 2.

5' Rapid amplification of cDNA ends (RACE) PCR cloning of human *b₅R* RNA extremities

The first strand (–) of *b₅R* cDNA was obtained from 2.5 µg of total human RNA species by extension of the appropriate sequence-specific primers with a recombinant MMLV (Moloney-

Figure 3 Translation of the human *b₅R* mRNA isoforms *in vitro*

(A) Schematic representation of the different 5' ends and predicted translation products for each of the truncated M, B, S and SS' cDNA constructs. The ATG codons marked ▼ were actually used as initiators in each transcript, including the ORF1-ATG within the exon 1S. The functional TGA stop codon in the exon 1S is marked ●; in ORF1-ATG deleted ΔS and ΔSS' cDNA constructs it is marked ○. In the M-cDNA construct, the boxed amino acid sequence corresponds to a myristoylation consensus. In the B, S and SS' transcripts, the hatched region within the common exon 2 represents the N-terminus of 13 uncharged residues whose sequence is underlined. (B) Autoradiograph of the translation products analysed by SDS/PAGE. The pH 5 fractions prepared from 50 μl of protein-synthesizing reticulocyte lysate for each construct were analysed by SDS/PAGE [12% (w/v) gel]. Lane 1, size markers; lanes 2, 3, 4 and 5 correspond to M, B, S and SS' cDNA constructs respectively; lane 6, control assay in which the non-recombinant vector was used in the transcription–translation analysis. The sizes of translated products are indicated at the right; the polypeptide corresponding to the ORF1 located in the exon S is boxed. (C) A section of the autoradiograph of the translation products obtained and analysed as described above. Lane 1, positions of molecular size markers; lanes 2 and 4, S and SS' cDNA constructs respectively; lanes 3 and 5, ORF1-ATG deleted (A) ΔS and ΔSS' cDNA constructs respectively. In (B) and (C) the positions of molecular mass markers (lane MW) are indicated at the left.

murine-leukaemia virus) reverse transcriptase kit (Gibco BRL). The excess of free nucleotides and primers was removed with a Centricon 100 spin filter (Amicon) in accordance with the instructions of the manufacturers. The cDNA species were extended with dGTP by terminal deoxynucleotidyltransferase (Boehringer Mannheim), and amplified with a nested specific downstream primer and the upstream anchor primer 5'-CCCTGCAGGAT(C)₁₄-3', in accordance with the modified RACE PCR method described by Frohman et al. [22]. Amplification was performed for 40 cycles; amplified cDNA species were analysed by Southern blotting and hybridized with the appropriate ³²P-labelled internal oligoprobes. The amplified cDNA species were cloned directly into a pGEM-T Easy vector system (Promega). The clones containing the 5' extremities of M cDNA were identified by hybridization with a 1M-specific exon oligoprobe, 5'-CCATGGGGGCCAGCTCAGC-3', corresponding to the coding sequence of the alternative 1M exon.

In vitro coupled transcription–translation analysis

Truncated cDNA species were amplified from human lymphoblasts and fetal liver by a RT-PCR technique with specific upstream primers (see Figures 1 and 2) and a common downstream primer corresponding to nt 21370–21387 [21] in exon 6 (5'-GGACTTTTGTGTCAGGTCGG-3'). To introduce an arti-

ficial stop codon, consecutive semi-nested amplification was performed with identical upper primers as above and a common lower primer located within the junction between exons 5 and 6 at nt 20872 at its 5' end and 21362 at its 3' end respectively [21]: 5'-CTACCCTTAGCCCTGCTAGACC-3'. Underlined nucleotides correspond to modifications of the natural sequence designed to create in-frame TAG, TAA and TAG stop codons respectively. Truncated cDNA species were subcloned into the pGEM-T Easy vector system (Promega). To obtain splicing variants for both 1S- and 1SS'-type S-mRNA species, the clones were first screened by plaque hybridization with an S'-exon-specific oligoprobe (Figure 1). The truncated S and SS' cDNA fragments were further used to prepare ORF1-ATG (in which ORF stands for open reading frame) deleted constructs ΔS and ΔSS' (Figure 3A) by excision of a 121 bp fragment between the *Sal*I site in the pGEM-T Easy vector polylinker and the *Bam*HI site located within the sequence of exon 1S, followed by filling and religation. A 1 μg sample of each circular plasmid DNA was used in a transcription–translation-coupled reticulocyte lysate system *in vitro* (TnT-SP6; Promega). The translation reaction was performed for 120 min at 30 °C in a 50 μl reaction mixture containing SP6 RNA polymerase and [³⁵S]methionine (Amersham), in accordance with the manufacturer's protocol. The pH 5 fraction from the protein-synthesizing reticulocyte lysate was prepared as described by Leroux and London [23]. The

aliquots (10–15 μ l) of reticulocyte lysate or pH 5 fraction corresponding to 50 μ l of *in vitro* translation mixture were analysed by SDS/PAGE [12 % (w/v) gel] and autoradiography. The size of the translated products was estimated in comparison with the Rainbow 14 C-methylated protein molecular mass markers (Amersham).

RESULTS

Cross-hybridization study of the upstream *b₅R* gene region in different species

Both rat (17 kb) and human (31 kb) *b₅R* genes were described [21,24] as possessing nine coding exons (1M–9), the sequences of which share 95 % nucleotide identity and 87 % amino acid identity. In rat, additional first alternative exons 1R, 1X and 1Y were described previously [15,17] and located in this order in the rat *b₅R* genomic sequence, between the first alternative exon 1M and the common second exon (Figure 4A). We first searched for the presence in the human *b₅R* gene of homologues of the 1R, 1X and 1Y exons. Fragments specific to these exons were radio-labelled and hybridized separately to a Zoo blot (Clontech) containing *Eco*RI-digested genomic DNA from nine different eukaryotic species. All three probes hybridized strongly to rat genomic DNA, moderately to mouse DNA and not at all to DNA from other species, including humans (results not shown). This suggests that the alternative 1R, 1X and 1Y exons described

in the rat are conserved only in mouse and that different 5′-*b₅R* gene structures occurred during evolution.

Characterization by 5′ RACE PCR of various *b₅R* transcripts in human fetal liver, reticulocytes, umbilical cord blood and lymphoblastoid cells

To identify a putative human erythroid-specific exon, the 5′ RACE PCR technique was used to characterize 5′ ends of the *b₅R* mRNA species prepared from three human erythroid tissues: fetal liver (from 22- and 40-week-old fetuses), reticulocytes and umbilical cord blood; and from one non-erythroid tissue (lymphoblasts). The first primer (5′-CCATCACCTCGAGAGGCC-3′) used contained nucleotides within the common second exon (Figure 1). After extension with terminal deoxynucleotidyl-transferase, cDNA was amplified with the upstream anchored primer (see the Experimental section) and an exon-2-specific nested downstream primer (5′-GCGCTCCACGCCAGCC-3′; Figure 1). Approximately 500 clones for each tissue were first screened by plaque hybridization with an oligoprobe corresponding to a sequence located within the common second exon (Figure 1). Positive clones were further hybridized with a human 1M-exon-specific oligoprobe (see the Experimental section and Figure 4B), allowing us to eliminate approx. 80 % of the clones corresponding to the known major M-mRNA transcript. Sequencing of remaining cDNA clones revealed three types of

Figure 4 Scheme of the 5′ *b₅R* gene organization and alternative promoters, transcripts and isoforms in rat and in human

(A) The rat *b₅R* gene possesses three first alternative exons 1R, 1X and 1Y located within a 6 kb DNA fragment separating the first alternative 1M exon and common exon 2. (B) In the human *b₅R* gene approx. 11.5 kb separate the first alternative 1M exon from exon 2. This fragment contains three exons: 1S, S′ and 1B. Exons 1S and 1B are the first alternative exons of the *b₅R* S-type and B-type transcripts respectively. The different *b₅R* transcripts resulting from the splicing of several alternative first exons to the common exon 2 are represented with positions of the functionally active translation start codons (▼). The non-conserved upstream AUG initiator located within human exon 2 is illustrated by a filled triangle under a star. The arrows in human *b₅R* gene correspond to complete sequences of direct (→) or indirect (←) oriented Alu sequences [21].

novel sequence located 5' to the second common exon. In all investigated tissue, the majority of the 5'-end *b₅R* cDNA clones contained the 1S exon partly described by Du et al. [25], for which the 3' border corresponds to position nt 2852 in the human gene [21]. However, most of the 1S-type transcripts were extended by a 5' 161 bp novel fragment, bounded by TGGGATCC repeats (Figure 1). Therefore the sequence in the described genomic region was renumbered; the total sequence of the 1S exon is given in Figure 1. A small percentage (approx. 20%) of the 5' cDNA clones were characterized by a 66 bp insert located between the 1S exon and the second common exon (Figure 1). This insert, corresponding to the nt 3193–3258 genomic fragment, was referred to as exon S', bounded by the consensus acceptor (agttAG) and donor (GTAage) splice sites. A new 5' RACE PCR experiment performed with 3' primers specific to the S' exon, using both human erythroid (fetal liver) and non-erythroid (lymphoblasts) mRNA species, confirmed that the S' exon was always associated with the 1S exon and was never detected in the M-type transcripts. This result favours the existence of two S-mRNA splicing variants in which the first alternative 1S exon is either directly spliced to the common exon 2 or to an alternative S' exon inserted between the 1S exon and the second common exon (Figures 1 and 4B).

Finally, a third rare species (approx. 3%) was detected in human fetal liver and lymphoblast mRNA species; it was devoid of both 1S and S' exons, containing instead a 26 bp fragment whose 3' bound corresponded to position nt 5497 according to our numbering and was followed by a consensus AGgtgagt donor splice site (Figure 2). This new sequence, referred to as exon 1B (Figures 3 and 4B), was further characterized by a new 5' RACE PCR experiment performed with fetal liver and lymphoblast RNA species primed by an oligonucleotide spanning nt 5481–5497 (Figure 2). The largest transcribed 1B sequence obtained from lymphoblast-derived cDNA clones encompassed 286 bp (Figure 2). The existence of transcripts including this 1B sequence was verified by RT-PCR with a 3' oligonucleotide in the common second exon and 5' oligonucleotide in exon 1B (nt 5229–5248; Figure 2) as primers: the expected sequence was easily amplified from lymphoblast and fetal liver RNA species (results not shown). In brief, 5' RACE PCR analysis of *b₅R* transcripts unable to hybridize with an M-specific probe in human erythroid and non-erythroid tissues permitted the detection of three species of transcripts whose 5' exons were of type 1S, 1SS' or 1B.

Expression profile of the different *b₅R* transcript species

Expression of the various *b₅R* mRNA species in different human tissues was studied by RT-PCR with primers specific for the common second exon (3' primer) and for each of the alternative first exons (1M, 1S and 1B). 1SS' species were specifically amplified with a 5' S'-specific primer (Figures 1 and 2). All types of transcript were expressed ubiquitously in all tissues investigated (heart, fetal brain, adult and fetal liver, reticulocytes and testis) and cell lines (lymphoblasts, non-induced fetal/erythroid K562 and pluripotent UT7), with the exception of the B-type transcript which was not detected in reticulocytes, K562 cells or testis (results not shown). In any case, no species seems to be specific for erythroid tissues.

Differential translation initiation of the multiple human *b₅R* transcripts

The ubiquitous expression of different human *b₅R* mRNA species suggests that the various *b₅R* isoforms in human tissues and cells

are generated mainly by alternative translation mechanisms. The putative translation initiator codons and open reading frames present in the different types of transcript were explored first.

Exon 1S possesses a 54-codon ORF, ORF1, starting with a putative in-frame AUG initiator codon at position nt 2849 and ending with a TGA stop codon at position nt 3013 at the 3' end of exon 1S (Figures 1 and 3A). The alternative S' exon does not contain a AUG codon. The common exon 2 contains two in-frame putative initiator codons (upstream CATAUGG and downstream CTCAUGA), the former unique to the human *b₅R* gene and the latter differing by only one mismatch from its rat counterpart (TTCAUGA) (Figure 3A) [15,17].

Finally, exon 1B contains a GGAAUGA sequence (position nt 5380) and, in phase, a downstream TGCAUGC element (position nt 5407), both putatively in frame with the coding sequence starting in exon 2 (Figures 2 and 3A).

Products translated *in vitro* from each construct were analysed by SDS/PAGE. The technique of precipitation of the protein-synthesizing reticulocyte lysate at pH 5 [23] permits the detection of non-haemoglobin proteins and increases the sensitivity of detection of minor polypeptides translated *in vitro*. Translation of the M-template produced a single major polypeptide of molecular mass 17.3 kDa (Figure 3B), reflecting a high efficiency of the AUG initiator codon within exon 1M whose flanking ACCAUGA sequence matches Kozak's consensus A/GCCAUGG sequence perfectly [26]. In the presence of this strong upstream initiator codon, the two downstream non-consensus translation initiators in the second exon are not used.

Translation of both 1S-type and 1SS'-type templates yielded three polypeptides of molecular masses 6.5, 14.6 and 16.2 kDa. The major 6.5 kDa polypeptide (54 residues) corresponds to ORF1 starting from the favourable initiator CCCAUGG sequence at nt 2848 and ending at the TGA stop codon nt 3010 (Figures 1 and 3A). Two minor 16.2 and 14.6 kDa polypeptides, probably resulting from the use of both upstream and downstream exon 2 AUGs, were detected only in pH5 concentrated fractions of lysates (Figures 3B and 3C). We speculate that the translation of ORF1 in exon 1S, stopping 12 bp upstream of the 5' AUG initiator of exon 2 (78 bp for the 1SS'-type transcripts), interferes with reinitiation at this AUG codon and might also affect the reinitiation at the downstream AUG in exon 2. To verify this hypothesis we created two constructs, ΔS and ΔSS', from which the ORF1-ATG initiator located in exon 1S had been deleted (see the Experimental section and Figure 3A). The translation of both Δ1S-type and Δ1SS'-type templates allowed us to express the two polypeptides observed with S and SS' templates; the 16.2 kDa species was largely predominant over the 14.6 kDa one (Figure 3C). This confirms that the translation of ORF1 interferes with use of the upstream AUG initiator in exon 2. The deletion of ORF1 therefore permits a more efficient initiation at this upstream AUG and the production of a 16.2 kDa polypeptide (Figure 3C). In contrast, in the presence of ORF1 the upstream AUG is repressed, favouring reinitiation at the downstream initiator codon in spite of its semi-favourable CTCAUGA context, resulting in synthesis of the 14.6 kDa polypeptide.

Finally, translation of the B-mRNA-type template yielded a single 16.2 kDa product, as expected for the upstream AUG initiator of exon 2 being used. Neither the downstream AUG initiator in exon 2 nor two weak putative initiator codons present in exon 1B (GGAAUGA and TGCAUGC) were used. These AUGs in exon 1B, if used, would have resulted in larger products: 21.6 and 20.0 kDa polypeptides respectively (Figure 3B). Such a result favours the existence of a novel *b₅R* isoform translated from S, SS' and B mRNA species, possessing

at its N-terminus a stretch of 12 hydrophobic uncharged residues devoid of myristoylation consensus. It is likely that this novel b_5R isoform is a membrane-bound enzyme different from the M-type isoform. The physiological role and cellular localization of this new isoenzyme remain to be determined.

DISCUSSION

The system of b_5R represents a model in which a single gene is able to generate several transcripts resulting from the use of alternative promoters. The different transcripts are translated in various isoenzymes with different subcellular localizations and biological activities. The biosynthesis of the soluble and membrane-bound b_5R isoforms was first elucidated in the rat [15,17]. We describe here the organization of the 5' alternative exons of the human b_5R gene. Using the anchored PCR technique applied to RNA species from erythroid and non-erythroid tissues, we cloned, sequenced and mapped three first alternative exons designated 1M, 1S, and 1B. In addition to the corresponding 1M, 1S and 1B mRNA species, a fourth species was characterized by the presence of an additional S' exon downstream of exon 1S (1SS' mRNA). The alternative exons 1M, 1S, S' and 1B are arranged in this order in the human b_5R genomic sequence (approx. 11.5 kb) between the first alternative exon 1M and the common second exon (Figure 4B).

During evolution, only the first alternative exon 1M was strongly conserved in both species, with 100% similarity in its 21 bp sequence [21,24]. Exon 1M is the first alternative exon of the major M-type transcript, coding for the membrane-bound myristoylated b_5R isoform mainly detected in the ER.

In contrast, the mechanisms involved in the production of the soluble b_5R in erythrocytes differ strikingly between rats and humans. The rat b_5R gene can be transcribed from an erythroid-specific promoter, resulting in the synthesis of a unique R-type mRNA whose 5' extremity is encoded by exon 1R [15,17]. The erythroid-specific promoter contains putative binding sites for the erythroid transcription factors NF-E2 and GATA-1 [27] and is sufficient to direct a tissue-specific expression in transgenic mice (A. Leroux, unpublished work). In humans, erythroid tissues do not synthesize this specific transcript but instead the ubiquitous 1S- and 1SS'-type mRNA species, which coexist with the 1M-type mRNA. These results disagree with those of Bulbarelli et al. [18], describing S-type transcripts specific for erythroid tissues, as judged from a RNase protection assay. However, although detected in terminally differentiated erythroblast culture and in reticulocytes, S-specific protected fragments were absent from haemin-treated K562 cells, a well-documented model of erythroid differentiation [28].

The differences between these results and our laboratory might be due to the different sensitivities of the methods used. The RNase protection assay is more quantitative but less sensitive than the RT-PCR that we used. This observation suggests that the human 1S/1SS'-type mRNA is a minor ubiquitously expressed b_5R transcript, probably present in larger amounts in terminal erythroblast culture and in reticulocytes. Actually, the erythroid tissues could be characterized only by an increased ratio of 1S/1SS'-type transcript to 1M-type in comparison with non-erythroid tissues.

The ubiquitous expression of the 1S/1SS'-type transcript is consistent with the analysis of the 5' flanking region of exon 1S devoid of consensus sequences for erythroid transcription factors NF-E2 and GATA-1 [27]. In addition to one GT box (GGTGGGTGG) already mentioned and two overlapping GC boxes (CCGCCC) [25], we observed the presence of a putative binding site for nuclear factor κB (GGGGATTACC) (Figure 1)

and, 190 nt upstream of exon 1S, a complete 258 bp Alu repeated sequence in the opposite orientation to that of the b_5R gene. This Alu element shows 86% identity with the consensus sequence of the class II Alu subfamily, the most represented class (more than 400 000 copies per genome), thought to have been inserted into the primate genome 32–57 million years ago [29–31].

These insertion events could have had a major role in the evolution of the 5' region of the b_5R gene. We hypothesize that rearrangements associated with Alu insertion disrupted the R-erythroid-specific regulatory region, leading to functional inactivation and the accumulation of subsequent mutations in this human genomic region. The occurrence of a new transcription pattern has possibly been influenced by *cis*-acting effects of the upstream Alu element, as reported for several human genes [32–34]. The complex system of multiple transcripts generated by the human b_5R gene was extended further by the finding of a novel 280 bp first alternative exon, referred to as 1B, associated with a B-type mRNA. Although the exon 1B 5' flanking sequence has features of a housekeeping-type promoter (the absence of TATA and CAAT boxes, putative binding sites for Sp1, AP-1, AP-4 and basic helix–loop–helix factors), 1B-type mRNA species are not equally distributed in the different tissues and cell lines analysed.

1M-, 1S- and 1B-type b_5R mRNA species are translated into different soluble and membrane-bound isoforms

Translation experiments performed *in vitro* for all types of b_5R mRNA species (1M, 1S, 1SS' and 1B type) showed an alternative use of different AUGs initiators resulting in the production of various protein isoforms: membrane-bound located in ER, soluble and a novel minor isoform probably located in membranes. Nevertheless, the mechanisms generating the soluble b_5R isoenzyme are clearly different in rodents and humans. Soluble erythrocytic b_5R isoenzyme is involved in the well-established reduction of methaemoglobin, whereas the biological significance of the soluble non-erythrocytic b_5R isoenzyme is not as clear. In the rat, the ubiquitous soluble b_5R isoform is produced by minor transcripts X and Y mRNA species possessing two alternative exons 1X and 1Y respectively (Figure 4A) [17]. These exons have no human counterparts; the human S and SS' mRNA species therefore produce the soluble b_5R isoform found in both erythroid and non-erythroid tissues.

In conclusion, our results demonstrate that the organization of the 5' b_5R gene region is not conserved between rodents and humans, which are thought to have diverged from a common ancestry approx. 95 million years ago [35]. Insertion of Alu elements into the human b_5R gene could have resulted in a profound transcriptional modification and genomic reorganization between the first alternative exon 1M and the second common exon, which are both well conserved in rodents and humans. Indeed, in addition to the Alu element inserted immediately upstream of the exon 1S, eight similar complete elements have previously been described in the genomic region [21] corresponding to the fragment between the exon 1B characterized in this study and the second common exon (Figure 4B). Novel mechanisms would then have been selected by evolution to direct the synthesis of the functionally essential erythrocyte-type soluble isoenzyme in primates, with the recruitment of new regulatory regions and alternative first exons permitting differential regulation of the use of the AUG initiators present in exon 2.

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