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LETTER

Structure of the Human α_2 Subunit Gene of the Glycine Receptor—Use of Vectorette and Alu–Exon PCR

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The α subunit of the glycine receptor is encoded by multiple genes that display developmental and tissue-specific expression. The α_1 subunit gene is expressed predominantly in the adult brain stem and spinal cord, whereas the α_2 subunit gene is expressed in fetal brain and spinal cord. We wished to determine the genomic organization of the human α_2 subunit gene as well as to define the 5′ ends of the α_1 and α_2 subunit genes. Gene structure can be defined rapidly from yeast artificial chromosome (YAC) DNA sources by the use of vectorette–exon polymerase chain reaction (PCR). However, YACs frequently contain small deletions that complicate the determination of the complete exon–intron structure of a gene, and this often necessitates the isolation of additional clones. In this study we have used vectorette–exon PCR from YAC DNA to define exons of the glycine receptor α_2 subunit gene. To define those exons that were absent in the isolated YACs, we used Alu–exon PCR on genomic DNA, using nested primers to obtain specificity in the PCR reactions. The α_2 subunit gene was found to contain nine exons varying in size from 68 bp (exons 3A and 3B) to 581 bp (exon 1). All of the intron–exon boundary sequences conform to consensus splice donor and acceptor sites. In addition, we have defined the 5′ end of this gene as well as that of the α_1 subunit gene by RACE–PCR. The structures of the α subunit glycine receptor genes in humans are very similar to each other and to the α subunit genes in mice.

[The sequence data described in this paper have been submitted to GenBank under accession no. U77724–U77732]

The advent of yeast artificial chromosomes (YACs) has allowed the rapid construction of physical maps of the mouse and human genomes. YAC contigs now exist for most of the human genome (Hudson et al. 1995; Dib et al. 1996). To define the structure of genes lying in these contigs, vectorette polymerase chain reaction (PCR) can be used to define the exon–intron boundaries without the need for additional cloning steps. This has the advantage of being simple and rapid and is especially useful in defining the organization of large genes such as dystrophin (Roberts et al. 1992). However, YACs often contain deletions and lack critical exons. It is therefore useful to have additional techniques to define exon–intron boundaries. In this paper we use vectorette–exon PCR on YAC DNA to identify the majority of the exons of the α_2 subunit gene of the glycine receptor and Alu–exon PCR on genomic DNA to define those exons that are not present in the YAC.

Glycine is a major inhibitory neurotransmitter in the central nervous system of vertebrates and invertebrates (Aprison and Daly 1978). It binds to a neurotransmitter receptor molecule called the glycine receptor that consists of a 48-kD α subunit, a 58-kD β subunit, and a 93-kD subunit called gephyrin (Pfieffer et al. 1982; Graham et al. 1985; Becker et al. 1986). The α subunit is encoded by multiple genes (Grenningloh et al. 1990; Kuhse et al. 1990; Matzenbach et al. 1994), which show specific expression patterns. The two most widely expressed α subunit genes are α_1 (the adult isoform) and α_2 (the fetal/neonatal form); α_1 replaces α_2 about two weeks after birth in mice (Becker et al. 1992). These two genes have been localized to 5q32 and Xp21.2–22.1 in humans and their syntenic groups on chromosome 11 and the X chromosome, respectively, in mice (Grenningloh et al. 1990; Warrington et al. 1992; Ryan et al. 1994). Mutations in the α_1 subunit

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result in the autosomal dominant condition hyperekplexia in humans (Shiang et al. 1993). Mice in which this gene is disrupted display the spasmodic (Ryan et al. 1994) and oscillator phenotypes (Buckwalter et al. 1994). Interestingly, the appearance of the mouse phenotypes coincides with the switch from α_2 to α_1. Two other α subunit genes, α_3 and α_4 provide additional heterogeneity to the glycine receptor. The four α subunit genes are distinct but display 80–85% sequence identity at the amino acid level. In particular, the extracellular region and the transmembrane segments display long stretches of amino acid identity. In the present study, we report the structure of the fetal form of the human α subunit, α_2, using vectorette PCR, Alu–exon PCR, and library screens. We have also identified an alternatively spliced transcript of the α_2 gene that we term α_2B and have defined the transcription initiation sites of both the α_2 and α_1 subunit genes.

RESULTS

Isolation and Characterization of Human α_2 GlyR genomic clones

Screening of the CEPH mega YAC library by PCR resulted in the isolation of two clones, 658b10 and 913a8. These candidate clones were characterized further by PCR using primers in the 5′ and 3′ ends of the α_2 gene. Only 913a8 was found to be positive with both sets of primers. It was therefore analyzed further by vectorette PCR to determine the exon–intron boundaries of the α_2 subunit gene.

The genomic structure of the human α_1 glycine receptor gene has been determined (Shiang et al. 1993). We hypothesized that the genomic structure of the α_2 glycine receptor gene would be very similar. By aligning the sequences of the α_1 and α_2 subunit genes we predicted the genomic structure of the α_2 gene. Synthetic oligonucleotides based on the α_2 cDNA sequence (Grenningloh et al. 1990) and predicted to lie in exons were then made and used to amplify vectorette libraries. In most cases, a single, distinct band was obtained with at least one of the three vectorette libraries used. In cases where more than one band was obtained by vectorette PCR, a nested reaction was carried out. In this manner, boundaries of exons 1, 2, 4, 8, and 9 were identified.

Attempts to isolate the remaining exons in this manner were unsuccessful as the YAC was found, based on PCR, to contain a deletion of these exons. We therefore used two alternative approaches. A human genomic library in phage was screened with two α_2 cDNA fragments corresponding to exons 2, 3, and 4 and exons 5, 6, and 7, respectively. This resulted in the isolation of exons 3 and 7 and the identification of their boundaries. Sequencing of clones containing exon 3 revealed the presence of a previously unreported exon separated from the third exon by 87 bp of intron sequence. Reverse-transcription PCR (RT–PCR) using a specific primer, EX3BRev, showed that this exon is utilized to give rise to an alternatively spliced α_2 transcript lacking exon 3 and instead containing the new exon. To identify the remaining exon boundaries, we used a novel procedure, which we term Alu–exon PCR. The Alu primer XBTC-65 (Nelson et al. 1989) along with a primer lying within the exon was used in an initial PCR reaction that resulted in a smear. A 1-μl aliquot was taken from this initial PCR reaction and was amplified with the Alu primer XBTC-65 and a new exon primer. This resulted in the amplification of a specific band. The resulting products were subcloned and sequenced.

Genomic Organization and DNA Sequencing of Exon–Intron Boundaries

The precise exon boundaries of the human α_2 glycine receptor gene were determined by comparing the genomic sequence with the cDNA sequence. As depicted in Table 1, the α_2 gene consists of nine exons varying in size from 68 bp (exons 3A and 3B) to 581 bp (exon 1). All of the intron–exon boundary sequences conform to consensus splice donor and acceptor sites (Shapiro and Senapathy 1987). Introns ended with the bases AG, which was preceded by a T/C-rich stretch of 8–12 bases, whereas introns started with the bases GT followed predominantly by A/GA/GG sequences. The size of the α_2 subunit gene has been difficult to determine because of deletions in YAC 913a8.

5′ Ends of α_2 and α_1 Glycine Receptor Genes

To determine the transcription initiation site of the α_2 subunit gene, 5′ RACE–PCR was carried out. Based on this experiment, a single transcription start site was found (Fig. 1). It is located 513 bp upstream of the translational start site or 127

Table 1. Nucleotide Sequences at Intron–Exon and Exon–Intron Junctions of the Human α_2 Glycine Receptor Gene

Exon	Amino acid no.	Nucleotide no.	5′ Intron	Exon sequence	3′ Intron	Exon size (bp)
1	1–23*	−191		TAGCCC---CTTCAG	gtaggtgaaa	581
2	23–68*	582	gagtgtttag	GACGGC---TTAAAG	gtaggttcca	134
3A	68–90	716	cattctgcag	GTCCTC---ACCATG	gtagtgctgc	68
3B	68–90	716	caacttgcag	GGCTCT---ACAATG	gtgagtggga	68
4	90–165*	784	atttctgtag	GACTAC---TATCAG	gtaagcctcc	224
5	165–193*	1008	gtttgctaag	CACCTT---AGAGTT	gtaagtcacc	83
6	193–240*	1091	tttacctcag	TTGGGT---ACACTG	gtaagtttct	138
7	240–312	1229	ttccttctag	GAAAGT---CCAAAG	gtaagaaatc	215
8	312–362	1444	tctctctcag	GTCTCC---AATAAG	gtatgattgc	150
9	362–454	1594	tccgtcagag	GAAGAC---		—

Indicated for each exon are the beginning and ending amino acid coded by the exon (asterisk indicates a split codon; numbered as in Grenningloh et al. 1990), the nucleotide sequences (numbered relative to the transcription start site) of the intron–exon boundaries, and the size of each exon (in base pairs). Intron sequences are in lowercase letters, exon sequences in uppercase letters.

bp upstream of the published nucleotide number 1 (Grenningloh et al. 1990). To confirm having isolated the ends of the α_2 gene, two primers, one designed to the 5′ end and the other lying immediately upstream of it, were used separately with a primer in exon 2 to carry out RT–PCR. In the first case the expected-size product was obtained; in the second, no amplification occurred. These results support the RACE–PCR data. Sequence analysis of a phage clone containing the 5′ genomic region has revealed the presence of a possible TATA box between −37 and −34 and multiple transcription factor binding sites. A similar analysis of the α_1 subunit gene upstream region based on a subclone of a cosmid containing exon 1 (Shiang et al. 1993) was carried out. Two consensus AP2 sites were found at −196 and −14, whereas a putative TATA box (TTTAAA) is located at −35 relative to the single transcription start site. The transcription start site of the α_1 gene was also determined by RACE–PCR and confirmed by RT–PCR (Fig. 1).

DISCUSSION

In this study, we have defined the genomic structure of the human α_2 subunit gene of the glycine receptor by vectorette and Alu–exon PCR. In addition, we have identified an alternatively spliced transcript of this gene. Finally, we have defined the transcriptional start site and sequenced the 5′ upstream region of this gene as well as that of the adult isoform, α_1. Vectorette PCR has already been shown to be a useful technique in determining exon–intron boundaries (Roberts et al. 1992). Here we show that Alu–exon PCR on total genomic DNA can be used to define the boundaries of exons that are lacking in the YACs. This, of course, depends on the presence of an Alu repeat sequence in the appropriate orientation within amplifiable distance of the exon. Absence of such a repeat, however, does not preclude determining gene structure by PCR. Vectorette PCR on genomic DNA in conjunction with nested reactions could be one approach used to circumvent this problem. These methods will be extremely useful in the rapid determination of gene structure, especially with the completion of a YAC contig of the human genome and the identification, sequencing, and positioning of expressed sequence tags (ESTs) on this contig.

The α_2 subunit gene consists of nine exons (Table 1). Like the α_1 subunit gene, exon 1 codes for the signal peptide, exon 4 for a major antigenic epitope common to the two subunits, and the last exon for the highly variable cytoplasmic loop and the fourth transmembrane domain (Matzenbach et al. 1994). Studies have also shown that the genomic organization of the α subunit genes within a species is highly conserved (Matzenbach et al. 1994). This extends across species as well, since the structure of the human α_2 subunit gene is very similar to the α subunit genes of the mouse. One notable differ-

A

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-191  ccccctttcagtatcccctcgctgaacgggcagtcgctctttgccttcgaa
-141  cagcaggcacgtcccctcttgaacttttcccgacccccgcccccaagcaac
 -91  tgaaaaaggaaacatgcgcacttcagagcttggagctgtccgagtgctga
 -41  aatttataaggctgggtaagatcacgtctccgtctcattctaTAGCCCTCA
  10  TTCCAGCACAGGATCTCAGCAATTTTTCCCTCTTCCCCCCACCCACTCCA
  60  GCGCGCAGAGTCCTTTCTTCTCTCTCATTTACAACTTCTTTTTAAAAGAA
 110  AACATTTTCTAGAAAAAGGGCTTTGCTAAACAGAAAAGATATAAAACAAA
 160  AGCCACAGCTATCTAGCATGGCATTGTCACCACTCCCTTTGCATGGTGAT
 210  GCGATTAAGGTAGCAGCATTTTTATTATTCAGGAAAAGCAGCTGGGGGAT
 260  TCATCAGTTCTGAGGCTTTGTCTTTCTGGGTTAACTGATGGTCCCAAGCC
 310  TCGGTTTGACCTGACCATGATGCCCAGGACTGGCACTTTTTCTTTTTTCT
 360  CAGCAAACTGTACAAAACCAAATCTCTTTTTGATTTTCAAGGAAACTAGG
 410  TTCCTGCCAAATTTTGATTGAATCTGGACAATAAACAGACACTTTGTCCT
 460  AGCATCTTTCTGGAATCATTTCGGGATATTTCCACAAGCAACACAGAAAC
 510  AGGAATGAACCGGCAGCTAGTGAACATTTTGACAGCCTTGTTTGCATTTT
 560  TCTTAGAGACAAACCACTTCAGgtaggtgaaacgactttgcatgttgata
 610  attaaattgtttaaaagagaatgtgtcatgtaattgtgtatttatctgtg
 660  ctctggactatattatttaattttata
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B

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-229  atttaactgaaggcagcgccgtcaaccgctttccccctcgccctaacccc
-179  ctcaggccggcccagagccataaagctctgttctctttacccaagatgct
-129  cgcccaatccaacggtccgaggccagcgctctgatctccacagaagattt
 -79  ttttccttgcagggagctggccgtttaaacagaaaaacagggcttgaaa
 -29  aaaaaaaaaaaaaaggaaatatacccaccCCCAAACGTGCCTCCCCAGCG
  22  CCGCAGGGAGCCAACAGACACGCTGGAGTTTAACAAACAGCAATACTCTT
  72  CGCGCTCCTGAAAAGCAGGTCTGGACGCTCTCCGTGGTGCCGAAACGCCT
 122  CGCAGCCGCCGCTGTCCGTGGTATCTACGACCCCCTCGCTCCAATTTCCC
 172  CTGGGGCTCTCCCTCCGCGCCCCTGTTCCCCGCCTCCCTTTAACATCTGG
 222  ATTATTTTTTGCAATAGCGCTTTCTGGTTTTGTAAGTGCCAATTTGAAAC
 272  ATTTTTTGCCCCCATAACTCGTGGACTACAAAGCACAAAGGACCTGAAAA
 322  ATGTACAGCTTCAATACTCTTCGACTCTACCTTTCGGGAGCCATTGTATT
 372  CTTCAGgtatgcaatttttctactgaccacatcgctctgatggaaatgggg
 422  ggaaagagtacctctgtccgtggtcccatcattttggggaagagaggggt
 472  gctgtattccactgcaatcgggtccttcca
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Figure 1 Nucleotide sequences of the 5′ ends of the α subunit genes of the glycine receptor. Sequences are based on genomic clones and 5′ RACE–PCR. Numbering in the case of both genes is relative to their transcription initiation sites. Uppercase letters denote exon sequence, lowercase letters, intron or 5′ genomic sequence. Indicated are putative transcription factor binding sites (underlined) and the TATA box (boxed) for each gene. Also indicated is the translational start site (in bold). (*A*) Sequence of the α_2 subunit gene; 1 μg of poly(A)$^+$ RNA from human fetal brain was reverse transcribed using primers shown in Table 2. RACE–PCR was carried out with the 5′ RACE kit (GIBCO-BRL) according to the manufacturer's instructions. (*B*) Sequence of the α_1 subunit gene. Template RNA was obtained from adult human spinal cord, and reverse transcription followed by PCR was carried out in a manner similar to that for the α_2 subunit gene.

ence between the genomic organization of human α_1 and α_2 glycine receptor genes is the presence of only eight exons in the former. However, exon 6 of the α_1 gene is equivalent to exons 5 and 6 of α_2. Kuhse et al. (1991) have shown that the rat α_2 subunit gene undergoes alternative splicing, the splice isoforms resulting from the use of exon 3A or 3B. Our study shows that a similar event occurs in humans. Phage 32A, positive for exon 3, was found to contain two exons displaying ~80% homology and separated by 87 bp of intron sequence. A primer specific for the new exon was used to selectively amplify and to provide evidence for the existence of a novel transcript containing this exon, which seems to be the equivalent of exon 3B of the rat α_2 gene. The functional significance of this alternative isoform, which we call human α_2B, is presently unclear.

Mutations in the glycine receptor result in the spastic, spasmodic, and oscillator phenotypes in mice. The spastic phenotype is attributable to a LINE-1 element insertion in the β subunit of the glycine receptor (Kingsmore et al. 1994; Mulhardt et al. 1994). The spasmodic and oscillator phenotypes are caused by mutations in the α_1 subunit. Interestingly, the symptoms that characterize these phenotypes become obvious only after the switch from the fetal α_2 to the adult α_1 subunit. This suggests that the α_2 subunit might be able to rescue the mutant phenotypes if expressed in a spatially and developmentally specific manner. The definition of the genomic structure of the α_2 subunit and the isolation of the 5′ upstream regions of this gene and the α_1 gene will help in understanding the elements that control the developmental switch from one subunit to the other. It will also be useful in any future experiments attempting to correct the mutant phenotypes by driving the α_2 gene under the control of the α_1 regulatory elements.

METHODS

Screening of YAC and Phage Libraries

The CEPH mega YAC library pools (Research Genetics, Huntsville, AL) were screened according to previously reported methods (Green and Olson 1990; Amemiya et al. 1992) using primers that lie in the 5′ and 3′ ends of the α_2 glycine receptor gene. A human genomic phage library was plated and grown on NZY–agar, replicated on nylon filters, and hybridized with two cDNA probes corresponding to exons 2, 3, and 4 and exons 5, 6, and 7, respectively, using standard methods (Sambrook et al. 1989). The probes were amplified by PCR, isolated in low-melt agarose, and labeled by random priming as described previously (Feinberg and Vogelstein 1983). Positive clones were digested with *Eco*RI and their inserts ligated into the Bluescript KS(+) vector for sequencing.

Vectorette and Alu–Exon PCR

Vectorette (Riley et al. 1990) and Alu–exon PCR on YAC and genomic DNA, respectively, were used to identify

most of the intron–exon boundaries of the α_2 glycine receptor gene. Low molecular weight DNA was prepared as described (Green and Olson 1990) except that Strataclean resin (Stratagene, La Jolla, CA) was used in place of phenol/chloroform/iso amyl alcohol extractions. Extracted DNA was digested separately with three different enzymes (*Rsa*I, *Eco*RV, and *Hinc*II) and vectorette libraries were constructed as described previously (Riley et al. 1990; Roberts et al. 1992; Carpten et al. 1994). PCR was carried out with the exon primers indicated in Table 2 and the vectorette primer. The PCR products were then either sequenced directly or subcloned and sequenced. For Alu–exon PCR, the Alu primer XBTC-65 (Nelson et al. 1989) was used with exon-specific primers. To obtain specific bands from Alu–exon PCR, it is necessary to perform a nested PCR reaction. The second PCR reaction used the same Alu primer but a new exon primer. In our experience there is little product resulting from inter-Alu PCR in these reactions, but a control PCR using just Alu primers is always included. The products were purified using Wizard PCR purification columns (Promega, Madison, WI) and cloned into the TA cloning vector (Invitrogen, San Diego, CA) for sequencing. The Alu–exon PCR was carried out under the following conditions: [94°C, 1 min; 58°C, 1 min; 72°C, 2 min] × 35 cycles. In each reaction, we used 1.5 units Taq polymerase, 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, and 25 ng of primer.

DNA Sequencing

Plasmid DNA from Bluescript/TA vector subclones or phage DNA purified through Wizard lambda prep columns

Table 2. Primers Used for Sequencing and PCR Amplification of the Human α_1 and α_2 Glycine Receptor Genes

Primer[a]	Application[b]	Position[c]	Sequence 5′ → 3′
$\alpha_2$5′	RT–PCR	1	TAGCCCTCATTCCAGCACA
$\alpha_2$ups	RT–PCR	–	TATAGGGCTGGGTAAGATCACGT
EX1F1	vPCR, SE, EE	165	AGCTATCTAGCATGGCATTG
EX1R1	vPCR, SE, rPCR, EE	343	CCAGTCCTGGGCATCATGG
EX2F1	RT	587	GCCGTCCTGAAGTGGTTTGTC
α_2GSP1	vPCR, SE	588	TTTCTGCAAAGACCATGACTC
EX2R1	vPCR, SE	608	GAGTCATGGTCTTTGCAGAAA
EX3F1	SE	716	GTCCTCCAGTAAACGTTAC
EX3BRev	RT–PCR	785	TCCATTGTAGTTTCTGCTATT
EX3R1	SE	786	GTCCATGGTCGTTTCTGTGA
EX4F1	vPCR, SE	789	CCGAGTGAATATTTTTCTGAG
EX4R1	vPCR, SE	965	AATTTGTTGTCAGTGGTGACA
EX5F1	aPCR	1009	CTCACCTTGACCTTATCCTG
EX5R2	aPCR, SE	1056	CATCGGAAAGTTCTTCAAGTC
EX5F2	aPCR, SE	1057	GATGTCCAGACCTGTACAAATG
EX5R1	aPCR	1088	CTCTCCAGCTGCATTGTACAGG
EX6F1	aPCR	1095	GTACACGATGAATGACCTGATAT
EX6R2	aPCR, SE	1114	TCAGGTCATTCATCGTGTACCCA
EX6F2	aPCR, SE	1124	GGTTAAGTGATGGTCCAGTGC
EX6R1	aPCR	1224	GTTGTAGTGCTTTGTACAGTAG
EX8F1	vPCR, SE	1445	TCTCCTATGTAAAAGCGATTGA
EX8R1	vPCR, SE	1561	GCAGGAACTCCTTGTGTTGC
EX9F1	EE	1723	GGAGATGCTATCAAGAAGAAG
EX9R1	vPCR, EE	1914	ACAACACTGAGGCAAGAAGG
α_1GSP1	RT	384	AGCAAGGCTGAAGTATACAATG
α_1GSP2	rPCR	148	TAGATACCACGGACAGCGGC
α_1GSP3	rPCR, SE	128	GGCTGCGAGGCGTTTCAGCA
$\alpha_1$5′	RT–PCR	4	AAACGTGCCTCCCCAGCCCGCA
$\alpha_1$ups	RT–PCR	–	TTACCCAAGATGCCGCCCAAT

All primers are specific to the α_2 subunit gene of the glycine receptor unless indicated by the letters α_1 before the name of the primer.

[a]Primer names refer to their exon specificity and their sense (F, forward) or antisense (R, reverse) orientation. GSP (gene specific primers) are all antisense.

[b]Applications of the primers: vPCR, vectorette PCR; aPCR, Alu–exon PCR; SE, sequencing of intron–exon boundaries; EE, exon–exon PCR; rPCR, 5′ RACE–PCR; RT, reverse transcription, RT–PCR, PCR carried out after reverse transcription.

[c]The position of the most 5′ nucleotide given. Sequences are numbered relative to the transcription start site.

(Promega, Madison, WI) was sequenced using vector-specific and exon-specific primers, respectively. Sequencing was performed with the Sequenase Version 2.0 sequencing kit according to the manufacturer's instructions (Amersham, Cleveland, OH).

5′ RACE and RT–PCR

The 5′ ends were amplified using a modified version of the RACE protocol (Frohman et al. 1988); 1 µg of poly(A)$^+$ RNA from human fetal brain was reverse transcribed at 45°C using the primer α_2GSP1 (Table 2). Excess primer was removed using a Sephacryl S-400 spun column and purified cDNA resuspended in 20 µl of water; 16 µl of cDNA was then tailed in a mix containing 2 µl 2 mM dCTP, 1 µl 10× tailing buffer and 10 units of terminal deoxynucleotidyl transferase (GIBCO-BRL, Gaithersburg, MD). After incubating at 37°C for 5 min, the reaction was stopped at 65°C and 2 µl amplified by PCR using the primer Ex1R1 and anchor primer (GIBCO-BRL). A nested reaction was carried out using Ex1R1 and the universal amplification primer (GIBCO-BRL). Cycling conditions were similar to those described earlier. The 5′ end of the α_1 gene was similarly obtained. RNA, however, was isolated from human spinal cord. Primers are indicated in Table 2. Amplification of exon 3B of the human α_2 subunit gene was carried out using primers EX3BRev and EX1F1. PCR reactions to confirm having isolated the transcriptional start site of the α_2 subunit gene were carried out using primers $\alpha_2$5′, $\alpha_2$ups, and EX2R1; the 5′ end of the α_1 gene was confirmed using primers $\alpha_1$5′, $\alpha_1$ups, and α_1GSP1. RT–PCR was carried out according to conditions described earlier, the only difference being that the template cDNA was obtained by random primed rather than gene-specific primed RNA.

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