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Functional expression of angiotensinogen depends on splicing enhancers in exon 2

Cibele C Cardoso^{1*}, Daniela A Cabrini^{2*}, Markus May^{1*}, Claudia S Bhagat¹, Nelida Eleno³, Cécile Cayla¹, Thomas Walther^{4,5}, Michael Bader¹

¹Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Str. 10, 13125 Berlin, Germany

²Department of Pharmacology, Polytechnique Center, Federal University of Paraná, PO Box 19031, 81530-900 Curitiba, Brazil

³Department of Physiology and Pharmacology, University of Salamanca, Av. Campo Charro s/n, 37007 Salamanca, Spain

⁴Department Experimental Cardiology, Excellence Cluster Cardio-Pulmonary System, Justus-Liebig-Universität Giessen, Giessen, Germany;

⁵Centre for Biomedical Research, Hull York Medical School, University of Hull, Hull HU6 7RX, UK.

Address correspondence to:

Prof. Dr. Michael Bader

Max-Delbrück Center for Molecular Medicine

Robert-Rössle-Str. 10

D-13125 Berlin-Buch, Germany

Email: mbader@mdc-berlin.de

Tel.: +49 30 9406 2193

Fax: +49 30 9406 2110

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* joint first authors

Abstract

Angiotensinogen belongs to the family of serpins and is the only precursor of the potent cardiovascular peptide, angiotensin II, the main effector of the renin-angiotensin system. The gene coding for this protein carries an internal exon (exon 2), the length of which (859 bp) by far exceeds the mean length of internal exons in vertebrates (<300bp). Here, we show that this essential exon is skipped in about 20% of all transcripts in liver, brain, and kidney of rats and mice. Deletion mutants of exon 2 revealed a 62 bp region located at its 5'-end which is important for its inclusion in the mature angiotensinogen mRNA in transfected COS7 cells. Using an artificial minigene, we defined sequences inside this region as exonic splicing enhancers. These data reveal a novel molecular mechanism important for the renin-angiotensin system with implications in the basic understanding and the therapeutically assessment of cardiovascular diseases.

Introduction

Splicing is an essential process in eukaryotic gene expression which starts in the cell nucleus with the transcription from DNA to an mRNA precursor (pre-mRNA). During transcription, introns are removed while exons are joined together (Matlin et al., 2010; Licatosi et al., 2010). For this process, consensus sequences at the 5' end (donor splice site, AG/GURAGU) and 3' end (acceptor splice site, CAG/G) of introns, as well as a preserved polypyrimidine tract and a degenerate branchpoint sequence located at about 20-50 nucleotides (nt) preceding the 3' splice site are identified by the spliceosome machinery composed of 5 small nuclear (sn)RNAs (U1, U2 and U4-U6) and a multitude of proteins. Alternative splicing of exons generates different mRNAs from the same pre-mRNA, consequently often also leading to multiple protein isoforms (Matlin et al., 2010). Regulatory elements for splicing which select exons for inclusion in the final mature mRNA can be secondary structures around splice sites, specific sequences so called exonic (ESE) or intronic (ISE) splicing enhancers and exon length (Cartegni et al., 2002; Singh et al., 2005). Serine/arginine-rich (SR) proteins bind to such elements to facilitate exon identification and promote splicing (Wahl et al., 2009).

Mammalian exons show a wide variety of lengths but while the first and ultimate exon can easily contain several kbs, internal exons are very limited in their maximal size (Robberson et al., 1990). Very few of the mammalian pre-mRNAs contain internal exons which exceed the length of 300 nt. Abnormal length of an exon can hamper its inclusion in the mature mRNA (Berget 1995; Humphrey et al., 1995).

One of the very few examples of an mRNA with a very long internal exon is the transcript coding for angiotensinogen. Angiotensinogen is a glycoprotein of the serpin family mainly produced in the liver. It is the natural substrate for renin required for the production of all active peptides of the renin-angiotensin system. The renin-angiotensin system is one of the most important cardiovascular hormone systems and a major target for drugs against cardiovascular diseases (Bader, 2010). Furthermore, it has been shown that the local production of angiotensins is involved in numerous

other physiological processes in tissues, such as the pancreas (Leung, 2007a; Leung, 2007b) and in brain (Bader, 2010).

The gene coding for angiotensinogen is composed of 5 exons and 4 introns (Fig. 1). Exon 2 is the longest exon (859 nt) and contains the translational start codon, the signal peptide and the angiotensin I coding sequences (Clouston et al., 1988). Notably, nearly all serpin genes carry internal exons, the length of which by far exceed the normal length of internal exons in other mammalian genes (50-300 bp) (Van Gent et al., 2003; Ragg et al., 2009).

Here, we show that a splice enhancer sequence in exon 2 of the angiotensinogen gene is important for its inclusion into mature mRNA.

Materials and Methods

Cloning of the mouse angiotensinogen gene

The whole mouse angiotensinogen gene was amplified by long range-polymerase chain reaction (LR-PCR) from mouse genomic DNA. The reaction was carried out using primers mapping to exon 1 (MAOGEx1, ATAGCTGTGCTTGTCTAGGTT) and exon 5 (MAOGEx5, CACCGGCCTTGTCTCCATGGC) in the presence of the thermostable Taq- and Pwo-polymerases and buffer 3 provided in the Expand Long Template PCR System kit (Roche Diagnostics, Mannheim, Germany). The PCR conditions were as previously described (Cayla et al., 2002) with an annealing temperature of 58°C. The fragment was cloned into an expression vector (pcDNA 3.1 (+), Invitrogen). The final construct was verified by sequencing and named pmAOG.

Generation of deletions in pmAOG

The construct pmAOG was linearized with Sfi I for 1 h at 50°C. Two micrograms of DNA were digested at 37°C for 10 min using the endo-/exonuclease Bal 31 (1 U/μl). After inactivation of the enzyme with EDTA (50 mM), the DNA was precipitated by sodium-acetate. The DNA endings were filled with T4 polymerase in the presence of dNTPs, creating blunt ends. By this method, it was possible to generate deletions of different size at the same site in exon 2 of the angiotensinogen gene. After re-ligation of the blunt ends, bacteria were transformed with the different size constructs;

colonies of bacteria were picked and grown in LB medium at 37°C for 12 h. DNA extraction from 2 µl of each bacterial colony was performed by adding TE buffer and incubating at 95°C for 5 min. The length of the deletions in exon 2 was analyzed by PCR with specific primers, (MMANG25, GGACACACAGAAGCAAATGC) and (MMANGI23, CCACTCCGTTTGTGAACCCA) and Taq polymerase (Invitrogen, Germany). The PCR reaction was performed with 36 cycles of denaturation at 95°C (45 sec), annealing at 55°C (30 sec) and extension at 72°C (1 min). The PCR products were visualized on a 1% agarose gel. After sequencing, 7 different clones with deletions between 56 and 677 bp in exon 2 were selected for further analysis.

Splicing assay

COS7 cells (ATCC, Manassas, VA, USA) were grown in Dulbecco's modified Eagle medium (DMEM) containing 1 mg/ml glucose (Invitrogen, Germany) supplemented with 10% fetal calf serum, 100 IU/ml penicillin/streptomycin in an incubator with constant temperature (37°C) and CO₂ level (5%). When the cells reached 80% confluence they were transfected with 20 µg of DNA using the calcium-phosphate co-precipitation method (Pesquero et al., 1994). 72 hours later, total RNA was extracted from the cells using TRIzol reagent (Invitrogen) followed by chloroform-isopropanol extraction as previously described (Pesquero et al., 1994). The samples were resuspended in RNase-free water and kept at -80°C until use.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

cDNA was generated using 2 µg of total RNA, random hexamer primers and the MMULV reverse transcriptase enzyme (Invitrogen) as described (Silva et al., 1999). The PCR reaction was performed using 10 µl of the RT reaction with Taq polymerase and the primers MAOGEx1 and MAOGEx4 (ACTCGGGGGTTGGTGTACC) for both, mouse and rat angiotensinogen mRNA.

Ribonuclease Protection Assay (RPA)

A mouse angiotensinogen cDNA probe containing 11 nt of exon 1, the whole exon 3 and two nucleotides of exon 4 was amplified by RT-PCR from mouse liver RNA resulting in a 281 bp cDNA

that was cloned into a pGEM-T vector (Promega). The plasmid was linearized with Spe I and transcribed in vitro with T7 polymerase in the presence of [α^{32} P]-UTP using a RNA-transcription kit (Stratagene, La Jolla, USA). The probe was used for RPA in an RPA II kit (Ambion, Austin, USA) as previously described (Schinke et al., 1999). Briefly, 20 μ g of total RNA from the transfected cells were hybridised with 40,000 cpm of the radiolabeled probe. Fragments protected from RNase A and T1 digestion were separated on a 5% acrylamide/8 M urea gel. The dried gel was exposed to a radiosensitive plate and analyzed using a Fujix BAS 2000 phosphoimager system (Fuji, Düsseldorf, Germany). The expression ratio between spliced and full-length protected fragments was calculated according to the band intensity values obtained by image analysis using the software program TINA version 2.08c.

Human β -globin minigene construct

Two fragments of the human β -globin gene were amplified by PCR in order to generate a minigene construct for in vivo splicing assays (Xu et al., 1993). The first fragment included a part of exon 1 (88 bp) and intron 1 (130 bp) and was generated with the primers, Ex1h β globin-fw (GAGAAGCTTGCCGTTACTGCCCTGTG, containing a Hind III restriction site) and In1h β globin-rev (ACCGGTACCCTAAGGGTGGGAAAATAGAC). The second fragment included intron 1 (130 bp) and a part of exon 2 (209 bp) and was amplified with the primers, In1h β globin-fw (TAAGGTACCAGGTTGGTATCAAGGTTACAAG) and Ex2h β globin-rev (TGCTCTAGAGGTTGTCCAGGTGAGCC, containing an Xba I restriction site). Both fragments were first cloned individually in pBluescript II SK (+) vector (Stratagene) and after restriction with Hind III and Kpn I for the first fragment and Kpn I and Xba I for the second, the fragments were subcloned into pcDNA3.1 (-) vector. In this way, a Kpn I restriction site was generated in a miniexon between the duplicated intron 1. Mouse angiotensinogen exon 2 DNA sequences (Sequences 1, 2, and 3) corresponding to the three regions predicted by the SELEX-database (Cartegni et al., 2003) to contain SR-protein binding sites, were cloned into the minigene construct after its linearization with Kpn I. For this purpose, forward and complementary reverse oligonucleotides (listed in Fig. 5B) were annealed to generate double stranded fragments having Kpn I sites on both ends. The primers used for

the splicing assay analysis map to exon 1 (MG-fw, ACGTGGATGAAGTTGGTGGT) and exon 2 (MG-rev, TTAGGGTTGCCATAACAGC) of the human β -globin minigene construct.

Electrophoretic mobility shift assay (EMSA)

HeLa cells (ATCC, Manassas, VA, USA) were grown in the same conditions described above for COS7 cells. After washing, the cells were scraped into 1 ml of ice-cold phosphate-buffered saline and nuclear extracts were prepared as described by Hattori et al. (Hattori et al., 1990). Synthetic complementary DNA oligonucleotides corresponding to the forward and reverse strand of Sequence 2 and Sequence 3 were annealed and cloned into pBluescript II SK(+) vector for generation of the probe as previously described (Pesquero et al., 1994). For EMSA we followed the protocol suggested by Black et al. (Black et al., 1998). Briefly, a RNA-protein binding reaction containing 30 μ g of HeLa cell nuclear extract, 3.2 mM MgAc, and 10 μ g unspecific RNA (yeast total RNA, Promega) was incubated at 30°C for 8 min. Then 1×10^6 cpm [α^{32} P]-UTP labelled RNAs of Sequence 2 or 3 were added to the reaction mixture and incubated at room temperature for 15 min. For competition studies, unlabeled RNA oligonucleotides corresponding to Sequence 2 or 3 (2* and 3*) were added at a 10-fold molar excess over the amount of radiolabeled probe. Protein-RNA complexes were resolved by electrophoresis in a 6% polyacrylamide non-denaturing gel run at 160 V for 2 h at 4°C. The gel was dried and exposed to X-ray film (Kodak, Rochester, NY, USA).

DNA sequence analysis

The prediction of SR protein binding sites was made using the SELEX-database (Cartegni et al., 2003).

Results

Expression analysis of angiotensinogen

We analyzed the expression of the angiotensinogen gene in different organs of NMRI mice and Sprague Dawley (SD) rats by RT-PCR using specific primers that map on exon 1 and exon 4 (Fig. 2A). The results revealed an expected band of 1295 bp corresponding to the amplification of the full-length angiotensinogen mRNA and to our surprise a second band of 438 bp that pointed to alternative splicing (Fig. 2B). Sequencing of this PCR product revealed that it is derived from an alternatively spliced mRNA lacking exon 2. In mouse brain and fat the full-length angiotensinogen mRNA is even not detectable (Fig. 2B, lower panel) and the short form is the predominant band. However, PCR favours the amplification of shorter fragments and can, thus, not be used for relative quantification.

Therefore, we analyzed the levels of expression of the angiotensinogen gene in the brain, kidney, and liver of NMRI mice additionally by ribonuclease protection assay (RPA; Fig. 2C). We used the lower cDNA band amplified by RT-PCR to generate a probe of 281 nt mapping to exons 1 and 3. Thus, the upper protected band in Fig. 2C corresponds to the spliced isoform of the mouse angiotensinogen gene lacking exon 2 and the lower band (270 nt) corresponds to the full-length angiotensinogen mRNA. Again the novel spliced isoform was detected in all tissues analyzed with a relative amount of about 20% of the full length angiotensinogen mRNA (Fig. 2C).

Splicing pattern of exon 2 deletion mutants

Exon 2 of the mouse angiotensinogen gene contains 859 nucleotides and is therefore of unusual length for an internal exon (Fig. 1). Thus, we hypothesized that it carries regulatory splicing elements, e.g. ESEs, that allows its integration in the full-length mRNA. To find such sequences, the whole mouse angiotensinogen gene, including all exons and introns, was cloned into an expression vector under the control of a viral promoter (CMV). In order to localize a putative ESE sequence, the construct was linearized with Sfi I, which cuts only once within exon 2 and nowhere else in the angiotensinogen gene, and deletions of different sizes were generated in exon 2 by Bal 31 digestion (Figs. 3 and 4B). COS7 cells were transfected with these deletion mutants and the splicing pattern was analyzed by RPA (Fig. 4A). Again the expression of both isoforms of the mouse angiotensinogen

mRNA was detected in all clones analyzed. Most clones expressed mainly the full-length mRNA. However, in cells transfected with clones M20 and BS, higher levels of the spliced isoform of the mRNA were detected, in which exon 2 is skipped (Fig. 4A). This is not depending on the length of the exons 2 in these two constructs, since M21 has about the same length and anyhow shows normal splicing. Since also clone M18 shows a normal splicing pattern, we localized possible ESEs in exon 2 in the 62 nucleotides between the Bst1107 I site and the 5'-end of the M18 deletion (Figs. 3 and 4B). M31 and M32 lack this putative ESE region but anyhow exhibit a normal splicing pattern. This can be explained by the fact that their length (235 and 182 nucleotides, respectively) has reached a number falling in the normal range of internal exons (Berget 1995).

Characterization of the ESE sequence in exon 2

The 62 nt long region identified by the *in vivo* splicing assay seems to contain ESE motifs that regulate alternative splicing of the mouse angiotensinogen gene. The SELEX-database (Cartegni et al., 2003) predicted three SR-protein binding sites in this region: one upstream region of 19 bp binding SRp55, SRp40 and SF2/ASF, an intermediate region of 14 bp binding SRp40, and a downstream region of 14 bp binding SRp55 and SF2/ASF (Fig. 3). Virtually, the same binding sites were predicted by the RESCUE-ESE database (Fairbrother et al., 2002).

In order to functionally dissect this putative ESE region, we cloned shorter sequences (Sequences 1, 2, and 3) of the 62 nt region into a minigene construct system that has been previously applied for the identification of ESEs (Xu et al., 1993). The minigene construct is composed of exons 1 and 2 of the human β -globin gene. Both exons are separated by a duplicated intron 1 of the same gene. Between the duplicated introns are an acceptor and a donor site for splicing defining a miniexon with a unique Kpn I restriction site (Fig. 5A). Different fragments of the angiotensinogen ESE region (Fig. 5B) were cloned in both orientations into this site for *in vivo* splicing analysis. Total RNA was extracted from COS7 cells three days after transfection with these constructs and RT-PCR analysis was performed (Fig. 5C). The sizes of the expected amplified products are listed in Fig. 5B. A longer product indicates the inclusion of the miniexon (insert and Kpn I site sequence) and therefore provides evidence for an ESE in the cloned sequence.

As shown in Fig. 5C, sequences 2 and 3f induced the inclusion of the miniexon into the mature mRNA, 1 and 3r did not. Sequence 2f showed the strongest splicing enhancer activity. Interestingly, it was even active in the reverse orientation (2r), which might be due to the possibility that a secondary structure important for ESE activity can be formed in both orientations.

Specific RNA-protein interaction within the mouse angiotensinogen ESE region

The functional analysis in *in vivo* splicing assays had revealed that Sequence 2 and 3 may carry ESEs. To analyze protein interactions in this region, RNAs corresponding to 2f, 2r, 3f, and 3r were labelled and used in electrophoretic mobility shift assays (EMSA) with nuclear extracts from HeLa cells (Fig. 6). EMSA revealed a strong formation of an RNA-protein complex with 3f. Although weak, such a complex formation was also observed with 2f, but not for 2r. A less efficient secondary structure formation of the short 2f and 2r oligonucleotides *in vitro* may explain these discrepant findings to the miniexon inclusion test (Fig. 5C), in which both sequences proved most efficient.

The specificity of complex formation was demonstrated by its inhibition by a 10-fold molar excess of unlabeled oligonucleotides (2f* or 3f*). The presence of 2f* in the initial reaction attenuated the formation of the complex for both 2f and 3f. An even stronger attenuation was observed when 3f* was added to the complex formed with 2f and 3f.

These data suggest that Sequence 2 contains a weak protein-binding site not able to compete for the proteins binding to 3f. Conversely, Sequence 3 seems to carry a stronger protein-binding site. Further protein interaction analysis will be applied for the identification of proteins binding specifically to the 2f and 3f regions to better understand the alternative splicing mechanism that allows the inclusion of exon 2 in the mature angiotensinogen gene.

Discussion

We have demonstrated the existence of a splicing variant isoform of the angiotensinogen mRNA in different mouse and rat tissues. In retrospect, this finding is not surprising since the angiotensinogen gene contains an internal exon (exon 2) with an unusual length of 859 bp exceeding the normal size (<300 bp). The same gene structure is shared by most serpins (Van Gent et al., 2003;

Ragg et al., 2009), such as antitrypsins and centerin, and comparable splicing mechanisms can be postulated for these proteins. For one serpin, C1 inhibitor, partial skipping of the long internal exon (in this case, exon 3) in human monocytes has already been described and discussed as a regulatory mechanism for its expression (Duponchel et al., 2006). We characterize sequences in the exon 2 of the angiotensinogen gene as ESEs since their deletion causes a partial skipping of this exon, their insertion in an artificial minixon promotes its inclusion in mature mRNA, and they specifically bind nuclear proteins. However, we can not exclude the existence of additional splicing enhancers elsewhere in the gene since deletion of these ESEs still allows the inclusion of exon 2 in part of the mature angiotensinogen mRNA (Fig. 4).

In theory, a second protein could be translated from the alternatively spliced angiotensinogen mRNA using an in-frame start codon in exon 3 and containing 199 amino acids. However, despite trying several antibodies we were not able to find this protein in Western blots or by immunoprecipitation. Since the residual protein after renin cleavage, des(AngI)angiotensinogen, has been described to have physiological functions, such as inhibition of angiogenesis (Corvol et al., 2003), also the 199 amino acid protein, if it exists, may have a biological role. This has to be clarified in future studies.

Taken together, the crucial precursor protein of the renin-angiotensin system, angiotensinogen, needs an enhanced splicing process to be functionally expressed. Notably, pharmacologically controlling this alternative splicing of angiotensinogen mRNA may be a novel strategy for the therapeutic interference with the renin-angiotensin system.

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Figure Legends

Fig. 1. Schematic drawing of the mouse angiotensinogen gene. Numbers denote the exons. The location of the start and stop codons as well as the signal peptide and the angiotensin I encoding sequences are indicated. Filled and open boxes refer to the coding and non-coding sequences, respectively. Thin broken lines between the boxes represent the intronic regions. Primers mapping to exon 1 (MAOGEx1, ATAGCTGTGCTTGTCTAGGTT) and exon 5 (MAOGEx5, CACCGGCCTTGTCTCCATGGC) were used to amplify the mouse angiotensinogen gene by long-range PCR.

Fig. 2. Angiotensinogen expression analysis in mouse and rat tissues. (A) Scheme of the mouse angiotensinogen cDNAs with and without exon 2. Arrow heads indicate sense and antisense primers used in the RT-PCR reaction. The probe of 281 nt for the ribonuclease protection assay (bar) maps to exons 1 and 3. (B) Reverse transcriptase-polymerase chain reaction on total RNA from different rat (upper panel) and mouse (lower panel) tissues. Marker (M, Φ X174/HaeIII). (C) Ribonuclease protection assay of total RNA from different mouse tissues (n = 5-6) with the 281 nt probe; Marker (M), brain (b), kidney (k), liver (l), fat (f).

Fig. 3. Sequence of the mouse angiotensinogen exon 2. The capital letters refer to the exonic sequence and the lower case letters refer to intronic sequences flanking exon 2 (based on ENSMUSG00000031980). The splice donor and acceptor sites around exon 2 are boxed. The start codon is overlined with a thin bar. The sequence coding for angiotensin I is overlined with a thick bar. The 5'-end (full forward arrow head) and the 3'-end (full reverse arrow head) of the different deletions generated by Bal 31 are shown. The 62 bp sequence between the Bst1107 I site and the 5'-end of M18 deletion that may carry ESEs is highlighted in bold. Thin underlines concern the three different regions of SR-protein binding sites predicted by the SELEX-database.

Fig. 4. In vitro splicing assay. (A) Ribonuclease protection assay (RPA) of total RNA extracted from COS7 cells after being transfected with angiotensinogen expression clones carrying different Bal 31 deletions of exon 2 (M5-M32) or a specific deletion between the Bst1107 I (B) and Sfi I (S) sites (BS). Cells were transfected with the full-length mouse angiotensinogen gene (mAOG) as positive control of the assay. COS7: total RNA from untransfected COS7 cells. (B) Schematic drawing of the exon 2 in the clones used for the splicing assay with the percentage of full-length mRNA in the total angiotensinogen mRNA detected by RPA. Means \pm SEM are shown. ESE, putative exonic splicing enhancer region.

Fig. 5. Minigene splicing assay. (A) Schematic drawing of the human β -globin minigene construct. Primers mapping to the human β -globin exon 1 (MG-fw, ACGTGGATGAAGTTGGTGGT) and exon 2 (MG-rev, TTAGGGTTGCCCAT AACAGC) were used to identify spliced and unspliced sequences generated with synthetic oligonucleotides cloned into the Kpn I site. (B) Table describing the sequence of the synthetic oligonucleotides and expected amplified DNA lengths of spliced and unspliced products from DNA sequences inserted into the minigene construct. (C) RT-PCR from total RNA isolated from COS7 cells transfected with the different minigene construct carrying the forward (1f, 2f, 3f) and reverse (2r, 3r) DNA sequences generated by synthetic oligonucleotides.

Fig. 6. Electrophoretic mobility shift assay (EMSA). Radiolabeled RNA generated by the transcription from synthetic oligonucleotides corresponding to Sequence 2 (reverse and forward) and Sequence 3 (reverse and forward) in the presence or absence (free RNA) of HeLa cell nuclear extract. For competition studies, unlabeled RNA oligonucleotides corresponding to Sequence 2 (2f*) or Sequence 3 (3f*) were added at a 10-fold molar excess over the amount of radiolabeled probe.

trip

Figure 1



trip

Figure 2

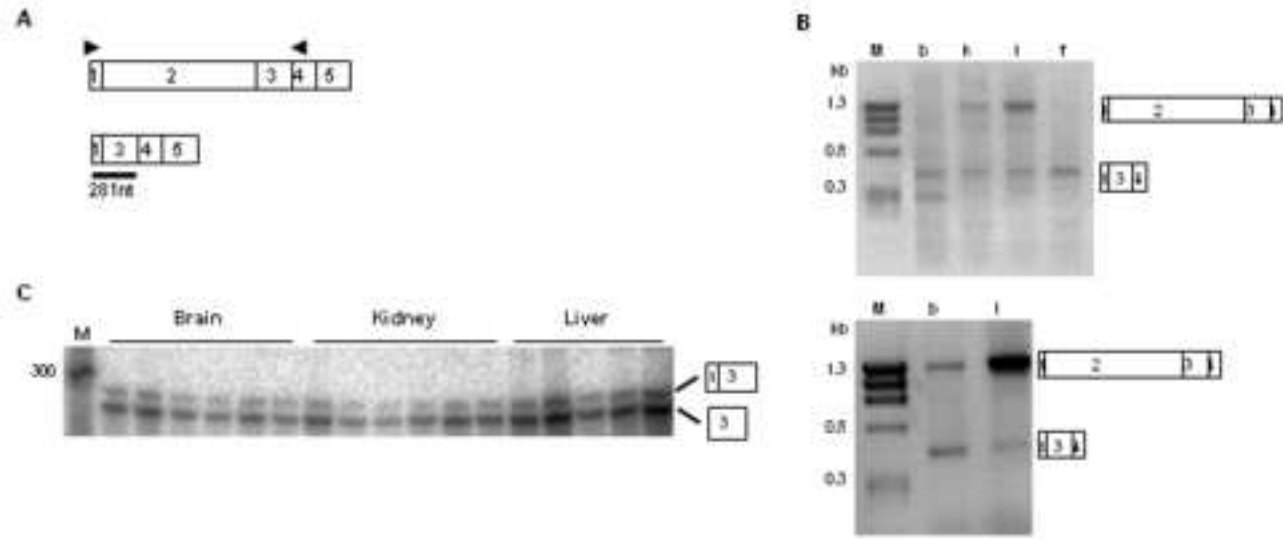




Figure 3



Figure 4

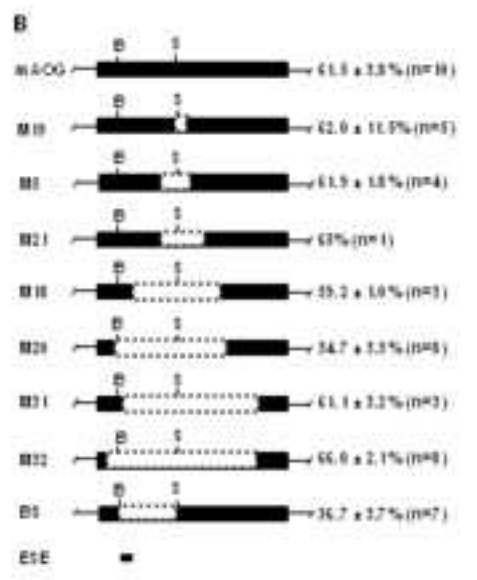
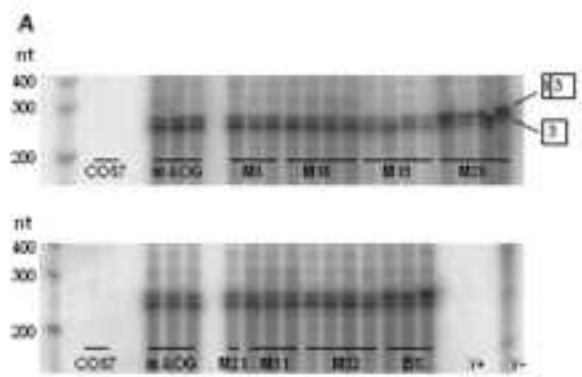


Figure 5

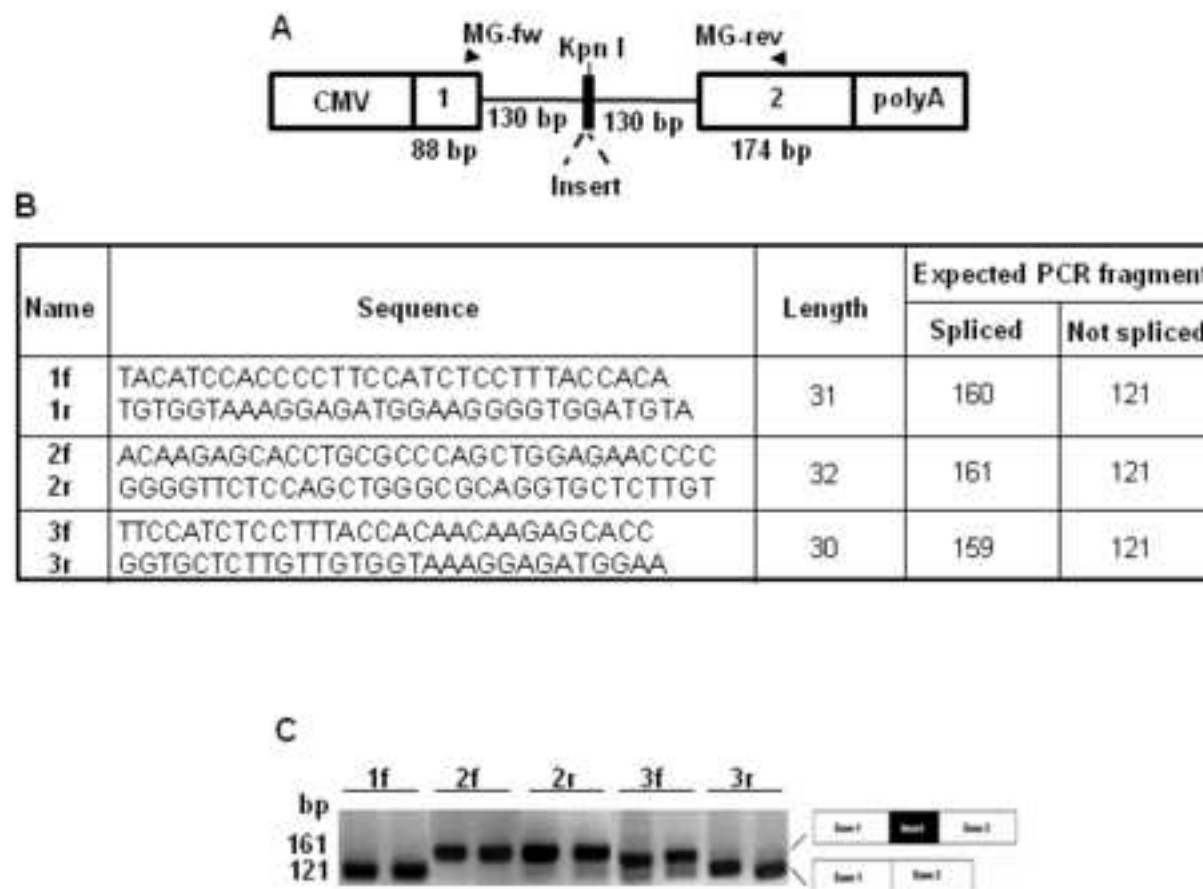


Figure 6

