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Research article

# Detection of novel transcripts and evaluation of expression levels of *Igf2* in mouse placenta

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## Abstract

The imprinted *Igf2* gene (Insulin-like growth factor 2) encodes a growth factor that plays an important role in the formation of the placenta and embryonic development. This study results showed that several new transcripts of the *Igf2* gene were detected in the placenta of mice, and the new promoter in the placenta P0L and PU2 were found. Expression levels of these promoters as well as of the *Igf2* gene were evaluated in the placentas of mice. The expression levels of these two promoters were investigated in different tissues; P0L is very well expressed in the brain and is clearly expressed in the placenta, tongue, and kidney; P0 is well expressed in the placenta, but also in the kidney and heart; PU2 is expressed in addition to the placenta, tongue, and muscles.

**Keywords:** Gene expression, Placenta, *Igf2*, Promoter

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# INTRODUCTION

Gene expression refers to the process that translates the genetic information of genes into mRNAs and proteins. A typical human cell expresses only approximately 20% of the total number of genes at a determined time, from which only 1.5% are coded for proteins (Biressi et al., 2007). The expression of each gene is controlled by various mechanisms, among them, contributrion by gene promoters is one of the most important mechanism.

Promoters can be approximately 100 - 1000 base-pairs long (Reeve et al., 1985). The expression of the Insulin-like growth factor 2 (*Igf2*) gene is quite complicated because it is carried out by some promoters and occurred only on paternally inherited allele. The expression levels of different promoters are completely different depending on the tissue and the mouse strain (Sambrook and Russell, 2001; Constância et al., 2002; Clermont et al., 2012). In mammals, *Igf2* controlled embryonic growth and development. This imprinted gene is located on the chromosome 7 in mice and the chromosome 11p15 in humans. It is paternally expressed both in humans and in mice. The regulation of *Igf2* gene expression is extremely complex. In adults, it is also involved in some pathways that lead to cancer (Nielsen et al., 1999; Sharan et al., 2007; Bhusari et al., 2011). A recent work has just suggested that *Igf2* promotes tumor progression by deactivating the P53 pathway (Clermont et al., 2012). Although *Igf2* is over-expressed in many cancers, its measurement has not yet made it possible to detect cancerous tumors very early. It should be noted, however, that the measurement of *Igf2* is included, along with 18 other factors, in a blood test for the evaluation of epithelial ovarian cancers. When the *Igf2* gene was inactivated in mouse embryos, the weight of the offspring was 40% lower than that of the normal offspring issued from the same litter (DeChiara et al., 1990; Ohta et al., 2008). Many subsequent studies have confirmed that the *Igf2* gene plays an important and direct role in the proliferation, differentiation and differentiation of cells in mammals (DeChiara et al., 1990). Previous studies showed that, there are 4 promoters that drive *Igf2* expression levels; among them, P1-P3 are transcribed in all tissues, while P0 is expressed specifically in the placenta (Meinsma et al., 1991; Clermont et al., 2012). There are some significant differences in the size of resulting transcripts (Leighton et al., 1995; Monk et al., 2006). Furthermore, this gene also plays an important role in the development and differentiation of the placenta. In mice knocked out of *Igf2*, the size of the placenta was reduced, resulting in a body weight at birth that was only 60% compared to wild-type mice (DeChiara et al., 1990; Hardouin et al., 2011). Therefore, identifying new transcrits as well as new promoters in the placenta is really important and necessary to accurately study the expression level of the *Igf2* gene in the mouse placenta. The role of this gene in the process of proliferation as well as the formation of the placenta and fetus.

# MATERIALS AND METHODS

## Generation of mouse crosses

*Mus musculus* were fed at the Institute of Molecular Genetics of Montpellier (France). Several crosses were generated. In the SD7/Dom and

Dom/SD7 crosses, the paternal chromosomes were either wild-type or issued from the *Mus spretus* strain (SD7). U2/Dom is a mutant mouse cross that removes the U2 exon from the maternal *Igf2* allele. The mouse used in this study has been approved by the inspector in charge of veterinary public health from the same office at the "He´rault pre´fecture" (Agreement Nu34-31). The primers used in the experiments are shown in Table 1.

**Table 1** Primer sequences.

| Amplicon names | Forward primer sequence | Reverse primer sequence |
|---|---|---|
| P0 mRNA | 5'-ATTGACCCAGCCAGCGGATC-3' | 5'-CTGTACTCTAGTCGCTTCGTAG-3' |
| P1 | 5'-CTCGTCACTTCTCCTACGGTG-3' | 5'-CCCAGTCGTTTTCCTGGACAC-3' |
| P2 | 5'-GTTCTGTCCCGTCGCACATT-3' | 5'-GGTATGCAAACCGAACAGCG-3' |
| P3 | 5'-CTGGACATTAGCTTCTCCTG-3' | 5'-CTGAAGTTGGGTAAGGAGGC-3 |
| I1bS | AACCCAGGGTTCTGAGTCTC | GAGACTCAGAACCCTGGGTT |
| U2S2 | GCTAGAGCATCCCGAGACTC | CATTCAGTTCTGGGAGCGTGG |
| Igf2 total* | 5'-CATCGTCCCCTGATCGTGTTAC-3' | 5'-GGAACTGTCCCTGCTCAAGA-3' |
| 5'Race | 5'-CGACTGGAGCACGAGGACACTGA-3' | |
| GeneRacer RNA oligo | 5'CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA3' | |

**This amplification allows quantification of Igf2 mRNAs issued from all known promoters*

## DNA extraction

Tissues were homogenized at room temperature in 2 mL of buffer. 40 µl proteinase (20 µg /ml) dissolved in pK 2X buffer was then added. After 4 hours of incubation at 50°C, the reaction was extracted with 4 ml of phenol/chloroform (1:1) and centrifuged for 30 minutes at 20°C at 6000 rpm. The supernatant was precipitated with 8 ml of EtOH and 300 µl of 5M NaCl at -20°C for 12 hours or overnight. The reaction mixture was centrifuged to collect the RNA pellet that was washed with 500 µl 70% EtOH. The DNA was then dried at room temperature.

## Extraction of RNA

RNA extraction was similar to DNA extraction with a few differences: after adding PK, we performed DNase I treatment to remove DNA. During extraction (by acidic phenol), centrifugation was carried out at a low temperature (4°C). After RNA was obtained, total RNA was extracted through a column containing oligo dT to separate the mRNA from the mixture. The mRNA was then recovered by elution.

The 5' RACE method: This method is used to screen new putative promoters. To 2 µg of DNase treated ARN, add 1 µl of Buffer CIP (Calf Instestine Alakine Phosphatase) 10 X, 1µl of RNAseOut (40U/µl), 1µl of CIP (10U/1µl) and 5µl of DEPC. Incubate the samples at 50°C for 1 hour, then add 90 µl of DEPC and 100 µl phenol:chloroform. Strongly vortex the samples during 30 seconds and centrifuge at 16000rpm for 5 minutes at room temperature. Collect the supernatant then add 2 µl of glycogen (10mg/ml), 10 µl of 3M sodium acetate (pH 5.2) and mix well. Add 220 µl of 95% ethanol, vortex, and put on ice for approximately 10 minutes. Centrifuge at 4°C for 20 minutes (16000rpm) and wash the pellet with 500 µl of 70% ethanol. Centrifuge, dry and add 7 µl of DEPC. Add 1 µl of TAP (Tobacco Acid Pyrophosphate)

buffer 10X, 1 µl of RNaseOut (40U/µl) and 1 µl of TAP (0.5 U/µl) (total volume is 10 µl). Gently shake and incubate the mixture at 37°C for 1 hour. Centrifuge to precipitate the RNA and dissolve it in 7 µl of DEPC. Next, add this reaction into a tube containing a 0.25 µg of GeneRacer RNA Oligo, incubating at 65°C for 5 minutes, cool the tube in ice for 2 minutes then add 1 µl of 10X ligase buffer, 1 µl of ATP (10 mM), 1 µl of RnaseOut (40 U/µl), and 1 µl of T4 RNA ligase (5 U/µl). Incubate the mixture at 37°C for 1 hour and then centrifuge it to collect RNA and add 10 µl of DEPC water. Store the reaction mixture at -20°C.

### PCR Quantifying

In this work, the Gapdh reference gene was used to calculate the relative quantities of mRNAs of the target gene (*Igf2*) expressed in the samples. The reaction mixture consisted of 1 µl of cDNA (5 ng/µl), 1 µl of qPCR mix (containing dNTP, MgCl2, qPCR 10X buffer), 0.5 µl of each primer (10 µM stock) and 7 µl of sterilized water. The positions of the primers used in the experiments are shown in Figure 2 (black arrowheads). PCR program: 95°C, 2 minutes and then 41 cycles (95°C/5s; 52°C/15s; 72°C/30s).

## RESULTS

Transcripts from the placenta: The demonstration of new transcripts strongly expressed in the placenta, other than those originating from the P0 promoter, led us to revisit the expression of the *Igf2* gene in the placenta. We proceeded in the same way as before, using the 5'RACE technique and RNA from the placenta at the e17 embryonic stage. To facilitate understanding of the results that follow, we subdivided intron 1 (I1a - I1c), located between exons U1 and U2 of the *Igf2* gene, into three parts, and exon U2 (U2a/U2b) into two parts (Figure 1).

Transcripts from the P0 promoter: Using nested PCR using an antisense primer located in exon U1, we were able to characterize a classic transcript similar to that from the P0 promoter (Figure 2).

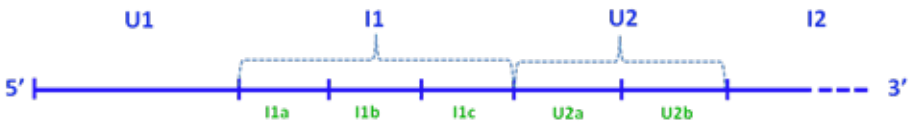



**Figure 1** Schematic of the first intron and the U2 exon of the *Igf2* gene. We have recut the first intron into 3 parts (I1a, I1b and I1c) and the U2 exon into two parts (U2a, U2b) in order to facilitate the understanding of the alternative splicings which will be described later.

Exon U1–new exon (I1b) splicing is classic, while exon U1b–U2b splicing could be explained by involving the minor snRNPs U11 and U12 (Figure 3) (Incorvaia and Padgett, 1998). Due to its larger size compared to the transcripts from the P0 promoter, we named this new transcript P0L ("long" P0, Figure 4).

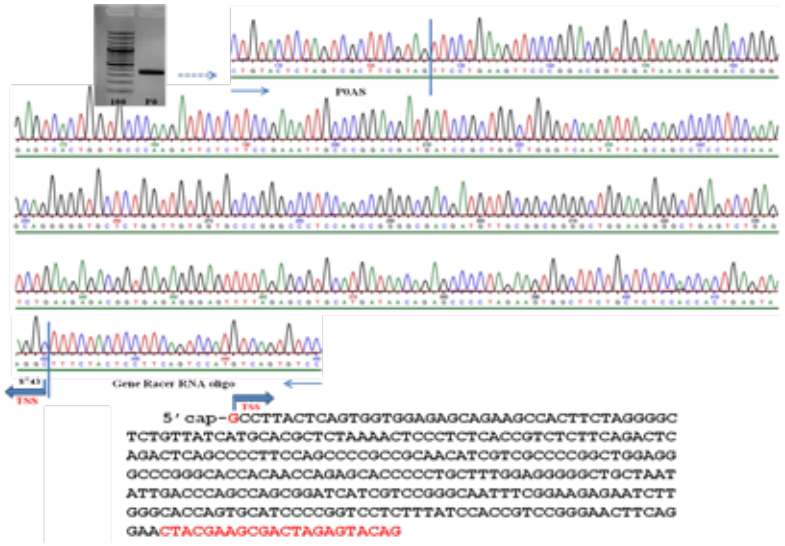
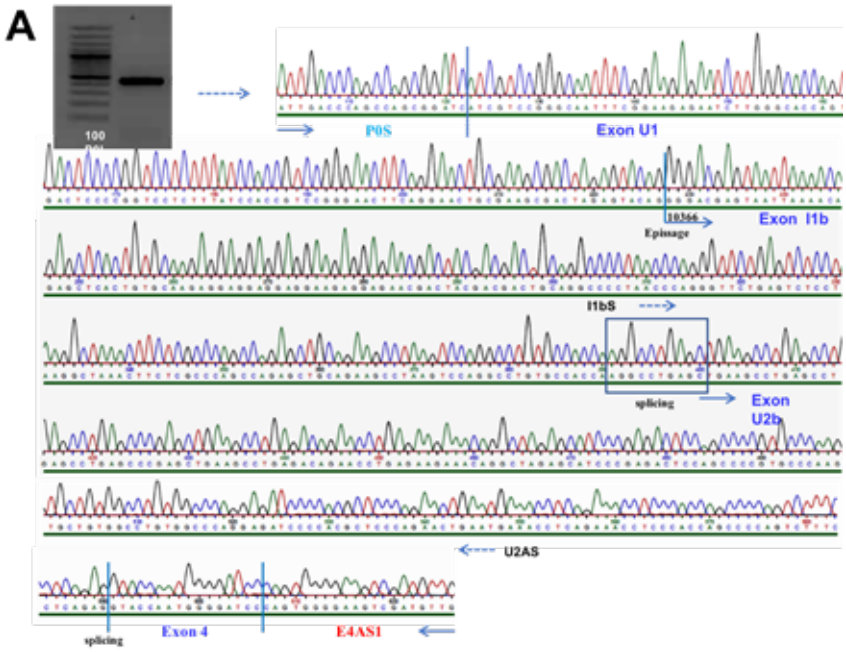



**Figure 2** Characterization of TSS of P0 promoter RNAs in the placenta E17 in mice. After ligation of the 5'RACE oligoribonucleotide, we performed a specific RT from exon 4 (E4-AS1 primer) before performing a nested PCR in exon U1 (P0AS) based on the results of the literature (Moore et al. 1997). The product of this PCR was analyzed on agarose gel and cloned for sequencing.

Using specific primers located in exons U1a, I1b, U2b and exon 4 of *Igf2*, we verified by RT–qPCR that this rare splicing indeed exists in the placenta and comes from the same TSS as that of the promoter classic P0 (Figure 3B).

It was therefore necessary to verify whether, in these mutant mice (ΔDMR1-U2), there were (or not) such transcripts, originating from the P0 promoter, since the latter was not removed in these mutants. Our results show without ambiguity that the transcripts from the P0 promoter are present in mice in the absence of the U2 exon (Figure 4A). Some of the transcripts from the P0 promoter therefore behave like the majority of other *Igf2* transcripts with the possibility of direct splicing of a first specific exon with exon 4 (common to all *Igf2* transcripts). This transcript is called P0c ("short" P0) (Figure 4B).



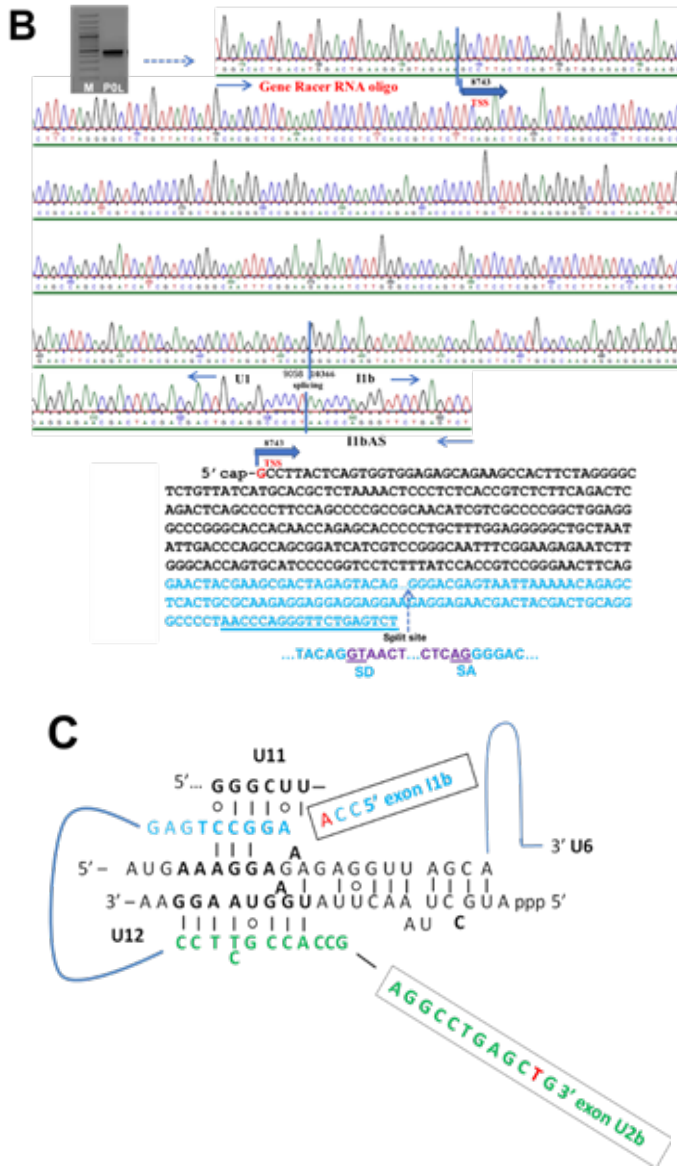



**Figure 3** Characterization of new transcripts from the P0 promoter of *Igf2* resulting from alternative splicing in the E17 placenta in mice. A. shows an amplification of RNAs originating from P0 (primer P0S) and spliced to exon 4 (primer E4AS1) whose size did not correspond to any of the known transcripts originating from the P0 promoter. For this reason, we sequenced it and discovered the new splice sites shown in the figure. The boxed region represents a new non-canonical splice site (with imprecision in the junction because the same nucleotides are present at the 3' end of I1b and the 5' end of U2b) which is detailed in C. B. characterization of the TSS of this new transcript. After ligation of the 5'RACE oligoribonucleotide and RT from exon 4, we performed a nested PCR with a primer in the new exon I1b (I1bAS primer). C. mechanistic hypothesis of non-canonical splicing involving U11-U12 snRNAs. The structure was established according to a model presented in the literature (Incorvaia and Padgett, 1998).

Transcripts initiated in exon U2: PU2 promoters: For several years, the U2 exon has been described as a specific exon of the "classic" placental P0 transcript. Using a primer that straddles the U2 - E4 exon splice sites and the 5'P

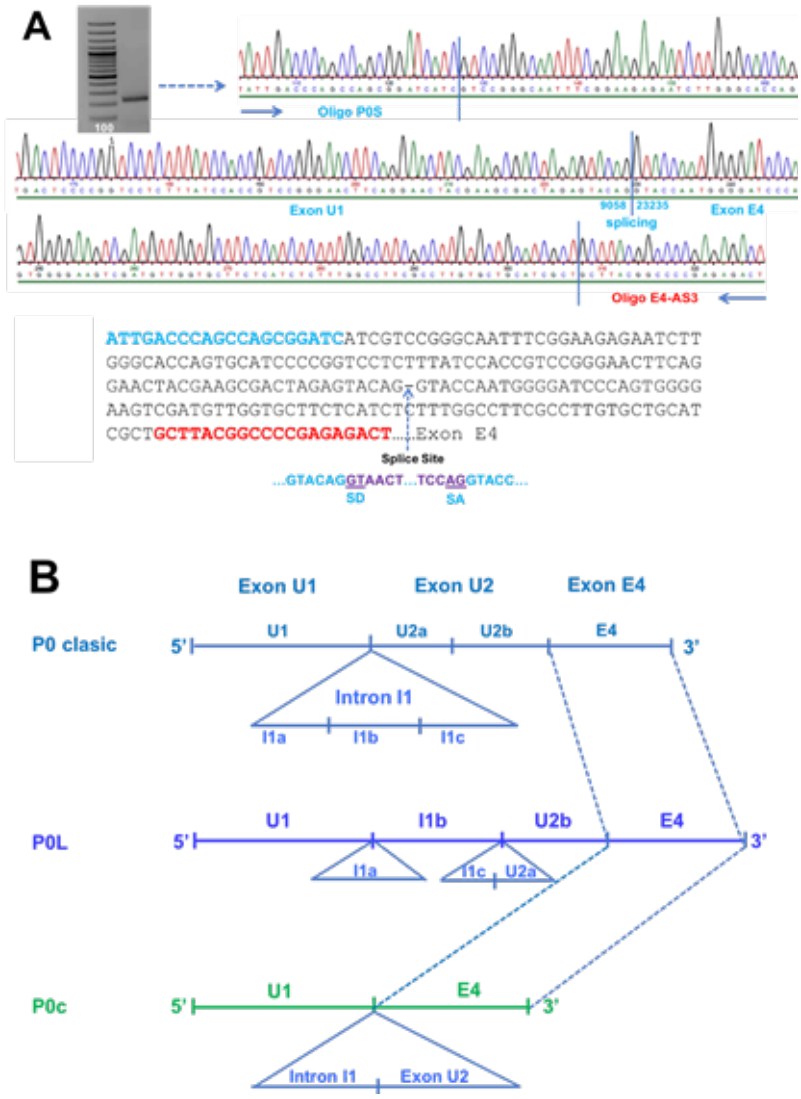



**Figure 4** Demonstration of direct splicing between exon U1 and exon 4 of the *Igf2* gene. A. Represents the result of an amplification with the P0S (blue) and E4AS3 (red) primers which unambiguously demonstrates the existence of splicing between the U1 and E4 exon in mice mutated on the paternal allele (dom/Δ DMR1-U2). B. Summary diagram of the 3 transcripts containing exon U1 and alternative splicings with exon 4.

RACE PCR primer that identifies the 5' end of the *Igf2* RNAs, one would expect to find transcripts from P0. Our surprise was to highlight new transcription initiation sites in the U2 sequence. Indeed, we observed the presence of 3 possible initiations in a sequence of 30 nucleotides located at the 5' end of exon U2. This observation led us to group these initiations under the term "transcripts from the PU2 promoter". Note that this new exon has an ATG in phase with that of the *Igf2* preprotein and that it would therefore lead to the translation of 16 additional amino acids at its 5' end (Figure 5). This observation is also valid when the U2 exon is transcribed from the P0 promoter.

Level of expression of placental promoters in mouse tissues: We have grouped under the generic term "placental transcripts" all RNAs originating from the P0 and PU2 promoters. They were quantified by RT–qPCR as before. However, the measurement of RNAs carried out with a specific primer of the

U2 exon includes both the transcripts from the PU2 promoters as well as part of those from the P0 promoter. On the other hand, thanks to a judicious choice of oligonucleotides, we were able to design PCRs specific for transcripts from the P0 and P0L promoters. The results (Figure 6A) show that placental transcripts from: P0L is very well expressed in the brain and is clearly expressed in the placenta, the tongue, and also in the kidney. Then, P0 is well expressed in the placenta but also in the kidney and the heart. The rest PU2 is expressed in addition to the placenta, tongue and muscles.

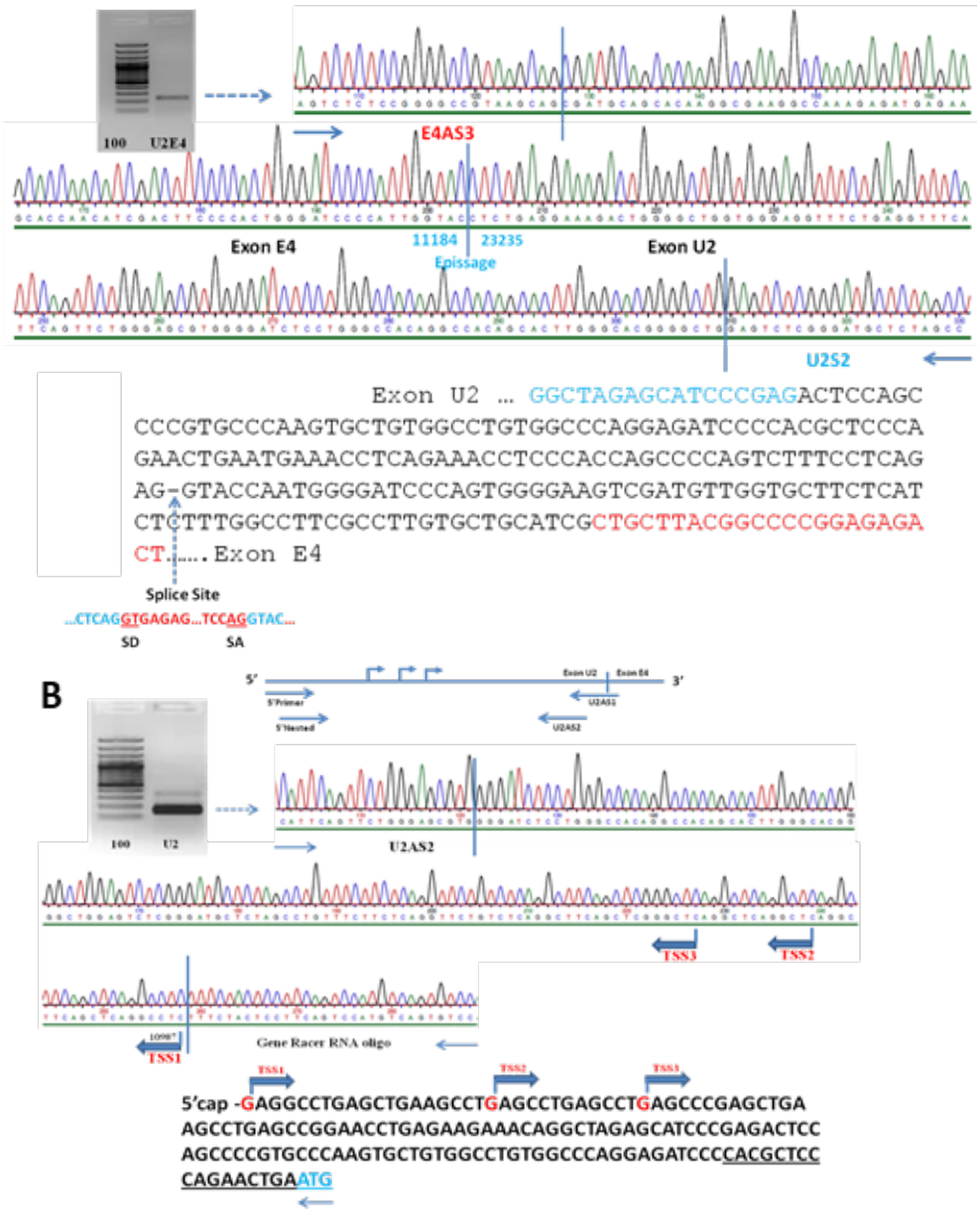



**Figure 5** Characterization of TSS of RNAs transcribed from exon U2 in the mouse placenta. A. Splice site between exon U2 and exon 4. B. The strategy is always the same except that the RT is performed using a primer on the junction exon 2-exon 4 (U2AS1). The three initiation sites are indicated in the figure along with a nested primer (U2AS2) which is underlined and contains an ATG in phase with that of the IGF2 pre-protein.

## DISCUSSION

One of the major aspects that emerges from reading this thesis undoubtedly lies in the measurement of the expression levels of *Igf2* RNA. Indeed, it is clear that, if we want to approach the regulation of the expression of the *Igf2* gene as a whole, it is imperative to carry out a minimum of 5' RT-PCR amplifications judiciously chosen in successive portions of the molecule of the *Igf2* RNA. All the new transcripts of the promoters in the placenta were found by 5'-RACE and RT-PCR. Firstly, It includes both exons U1 and U2. However, its initiation site is located in the DMR0 (Differentially Methylated Regions) a hundred nucleotides upstream of the one reported (Moore et al., 1997). The position of this new transcription initiation site is 142,669,936 on mouse

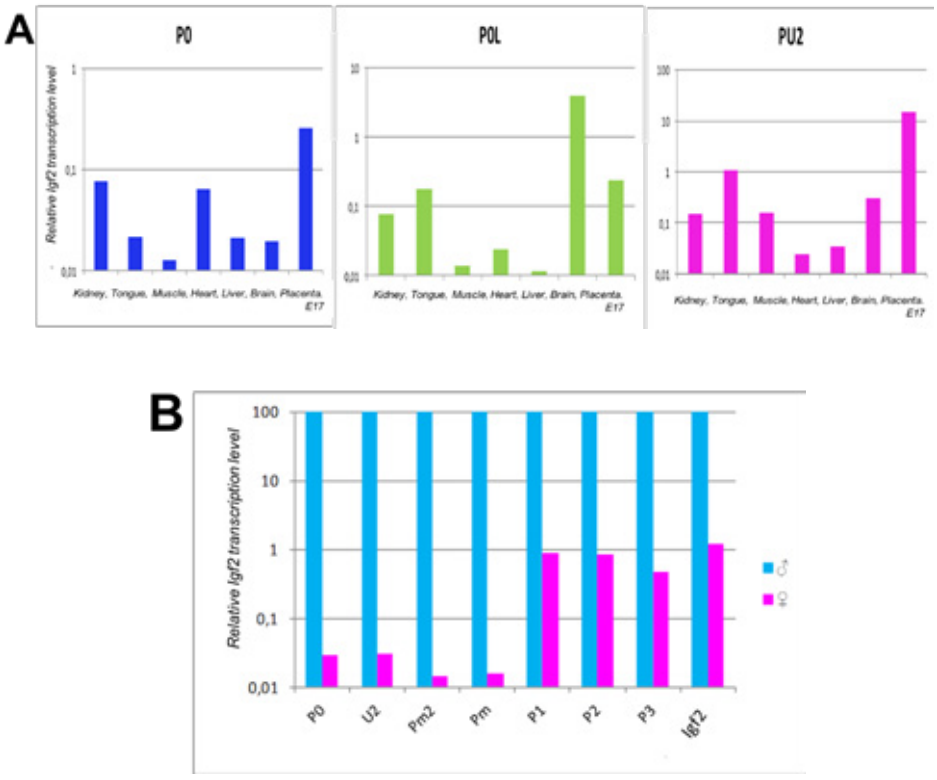



**Figure 6** Comparison of expression levels (A) and allelic transcription (B) of RNAs from different *Igf2* promoters in mice. A. represents the level of expression of RNAs from placental promoters in 7 tissues of dom/dom mice. B. represents the expression levels of each allele determined in the placenta (E15.5) of dom/SD7 mice as described for each promoter.

chromosome 7 (Dec. 2011 GRC m38/mm10) (Figure 2). Relative to the insulin polyadenylation site, this promoter is located 8743 nucleotides downstream. Among the RNAs from this P0 promoter, we have identified another transcript that presents an alternative splicing with splice sites that do not correspond to a classic 5' GT…AG 3' intron. This new *Igf2* transcript contains exon U1, a new exon (I1b) and the 3' part of exon U2 (U2b) (Figure 3A). Then, still using RT–PCR, we also found direct splicing between exon U1 and exon 4 of *Igf2*. This observation is important insofar as the deletion of exon U2 has been

attributed to the inactivation of RNAs from the P0 promoter (ΔDMR1-U2) (Constância et al., 2002). After discovering new promoters and new copies, we investigated their expression levels in the placenta and compared with other promoters. Allele-specific analysis of these expressions shows that these promoters are subject, like Pm and Pm2, to a very strong parental genomic imprint that is two orders of magnitude greater than that of the classic promoters in the placenta (Figure 6B). However, in pathological conditions in adults, the expression of *Igf2* from modest promoters that we have discovered is important. Indeed, the expression of the classic promoters is repressed in the adult, and therefore, the activation of one of these promoters (as we have seen in the *H19* KO -/- myoblasts transfected by the *H19* gene could lead to consequent re-expression of *Igf2*.

## CONCLUSIONS

From placental tissue, novel transcripts of *Igf2* were detected. Two new promoters were found and named P0L and PU2. The expression levels of these two promoters were evaluated and compared with other promoters of the *Igf2* gene. Among 2 news promotor in placenta, P0L is very well expressed in the brain and is clearly expressed in the placenta, the tongue, and also in the kidney. PU2 is expressed in addition to the placenta, tongue and muscles. With P0 is well expressed in the placenta but also in the kidney and the heart. Allele-specific analysis of these expressions shows that these promoters are subject, like Pm and Pm2, to a very strong parental genomic imprint that is two orders of magnitude greater than that of the classic promoters in the placenta

## ACKNOWLEDGEMENTS

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## AUTHOR CONTRIBUTIONS

**Van Giang Tran;** Contributed to sample preparation, counted meristic charaters and wrote the manuscript.
**Guy Cathala;** Gave the idea of study, planned the experiments, analyzed data and took the lead in writing the manuscript.

## CONFLICT OF INTEREST

We have no conflict of interest.

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