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RESEARCH ARTICLE

Igf2 Gene Expression Levels in Wild-Type and Mutant Mice

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Abstract:

Genomic imprinting occurs where only one allele of a gene is expressed depending on its parental origin. The imprinted Igf2 gene (Insulin-like growth factor 2) is encoding a growth factor, which play an important role in embryonic development and formation of the placenta. The regulation and expression of Igf2 is carried out by different promoters. Promoter expression is extremely complex in wild-type mice during development and is altered in several mutant mice bearing deletions at the Igf2/H19 locus. In this work, we analyzed the Igf2 RNA expression of the placenta-specific P0 promoter in placental tissue (embryonic day 17) of both wild-type and mutant mice. For all the other promoters, we used RNA extracted from liver tissues (postnatal day 7.5). All these RNAs were reverse transcribed to cDNA before quantifying expression levels of the promoters by quantitative PCR (qPCR).Our results show that transcriptions of Igf2 P2 and P3 promoters are the highest in all mice analyzed, except in ΔU2/Dom mutant mice where P0 and P1 promoters were highly expressed, while they display low expression in all the other mice strains analyzed. Furthermore, all promoters were stably expressed at high levels in wild-type and ΔU2/Dom mutation, but at a low level in Δ3/Dom

Keywords: Social Gene expression, Genomic imprinting, Igf2, Mutant strains, Promoter

Introduction

A typical human cell expresses only about 20% of the total number genes at a determined time, from all of them, only 1.5% are coding for proteins [2]. Gene expression refers to the processes that translates the genetic information of the genes to mRNAs and proteins. The expression of each gene is controlled and tightly regulated by various mechanisms within the cell. The main elements controlling the expression process are gene promoters. A promoter is a region of DNA that initiates transcription of a particular gene. Promoters are located around transcription start sites (TSS) of genes, on the same strand and upstream on the coding DNA sequence. Promoters can be about 100 -1000 base-pair long [17]. A gene can be controlled by one or several promoters. Researches on the mechanism of expression and regulation of gene expression in eukaryotes, and more particularly in mice, are particularly complex, especially for imprinting genes [3, 7]. The expression of the Insulin-Like Growth Factor 2 (Igf2) gene is quite complex because it is carried out by several promoters and only on the paternally inherited allele. The expression level of the different promoters is completely different depending on the tissue and the mouse strain [4, 5, 19]. In mammals, embryonic growth and development are controlled by a number of genes, among them is Igf2. This imprinted gene is located on the chromosome 11p15 in humans and the chromosome 7 in mice. It is paternally expressed both in humans and in mice. The regulation of Igf2 gene expression is extremely complex. The abnormal expression, loss imprinting or hypermethylation of this gene lead to the Beckwith-Wiedemann syndrome, the Silver-Russell syndrome or Wilms' tumors in children. In the adult, it is also involved in some pathways leading to cancers [1, 14, 20]. When the Igf2 gene was inactivated in mouse embryos, the weight of the offspring was 40% lower than the normal offspring issued from the same litter [6, 15]. Knockout of the endodermic specific enhancers, located at the Igf2/H19 locus, results is fetuses lacking Igf2 expression in the endoderm [9, 18]. Many subsequent studies have confirmed that Igf2 gene plays an important and direct role in the proliferation, differentiation and differentiation of cells in mammals [6]. There are 4 promoters that are driving Igf2 expression levels, among them P1-P3 are transcribed in all tissues, while P0 is expressed specifically in the placenta [4, 11, 12]. There are some significant differences in the size of the resulting transcripts [10, 13]. Furthermore, this gene also plays an important role in the development and differentiation of the placenta. In the Igf2 knockout mice, the size of the placenta was reduced, resulting in body weight at birth that is only 60% compared to wild mice wild [6, 8]. However, the tissue-specific expression level of the different promoters in wild and mutant mice has not yet been much explored.

Materials and Methods

Generation of mouse crosses

Mus musculus mice was 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 are either wild type or issued from the Mus spretus strain (SD7). The H19Δ3 mutant strain were provided by Luisa Dandolo's laboratory (INSERM Paris, France) [15]. Δ3/Dom is a cross where the maternal allele of the H19 gene is removed. H19 is an imprinted gene, repressed on the paternal allele and located in the same locus as Igf2. It is encoding a non-coding RNA that participates in the Igf2 gene regulation (Figure 1). U2/Dom is a mutant mouse cross that removes the U2 exon on the maternal Igf2 allele.

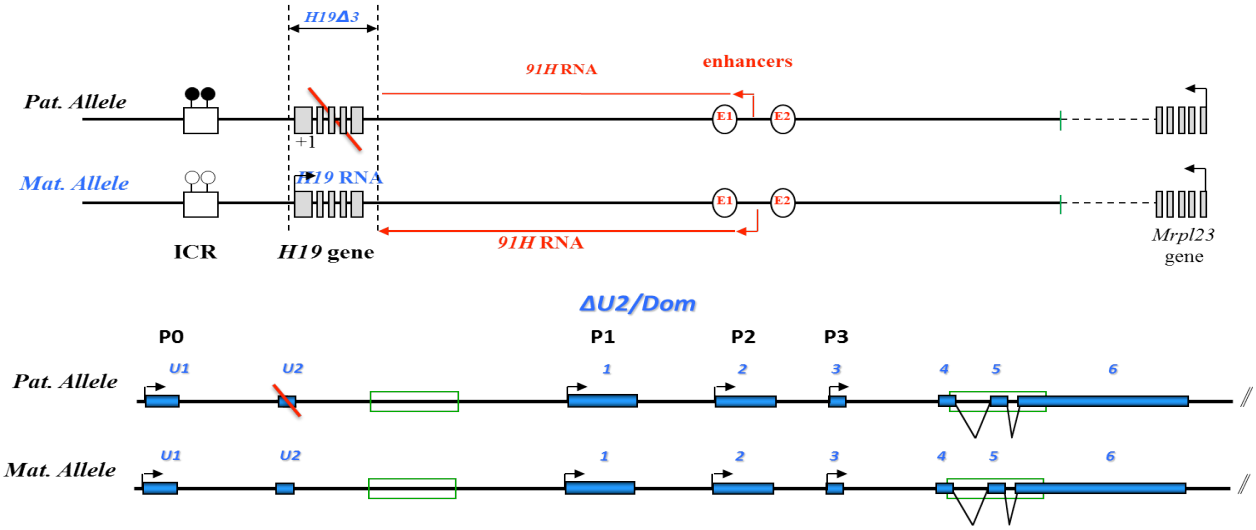

Figure 1. Genomic map of mutant mouse Δ3/Dom and ΔU2/Dom

Primers are 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'*
*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

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

Extraction of total RNA

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

Preparation of cDNA

To 2 µg of DNase treated RNA, add 1 µl of random primer or specific primer and 10 µl of sterile H2O. Incubate at 70°C for 10 minutes and then place in the ice for 5 minutes. Add 4 µl of buffer 5X (FS), 2 µl of DTT 0.1 M, 1 µl of dNTP 2.5 mM, 0.7 µl of reverse transcriptase. Stabilize the mixture for about 10 minutes at room temperature and incubate at 42°C for 1 hour. The cDNA is purified by glass balls and washing solution. Dilute cDNA 10 times for quantitative PCR or storage at -20°C.

Quantitative PCR

In this work, the Gapdh reference gene is used to calculate the relative quantities of mRNAs of the target gene (Igf2) expressed in the samples. The reaction mixture consists of 1 µl of cDNA (5 ng/µl), 1 µl of qPCR mix (containing dNTP, MgCl2, qPCR 10X buffer), 0.5 µl of each primers (10 µM stocks) 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/5 s; 52°C/15 s; 72°C/30 s).

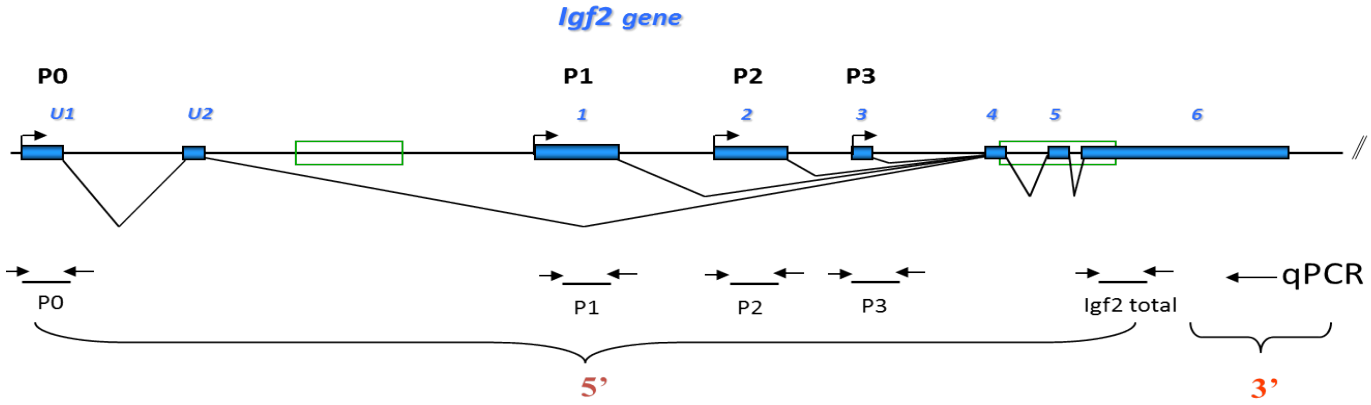

Figure 2. Map showing Igf2 promoters and the positions of the primers used in the experiments. The "Igf2 total" amplicon was designed to target exon 6 which is common to all known Igf2 mRNAs (issued from all promoters).

The 5'RACE method

This method is used to screen new putative promoters. To 2µg 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 at 50°C for 1 hour, then add 90 µl of DEPC and 100 µl phenol:chloroform. Vortex strongly during 30 seconds. Centrifuge at 16000rpm for 5 minutes at room temperature. Collect the supernatant and 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, put on ice for about 10 minutes. Centrifuge at 4°C for 20 minutes (16000rpm), 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), 1 µl of TAP (0.5 U/µl) (total volume is 10 µl). Gently shake and incubate at 37°C for 1 hour. Then centrifuge to precipitate the RNA and dissolve in 7 µl of DEPC. Next, add this reaction into a tube containing the 0.25 µg of GeneRacer RNA Oligo, incubating at 65°C for 5 minutes, cool the tube in ice for 2 minutes and add 1 µl of 10X ligase buffer, 1 µl of ATP (10 mM), 1 µl of RnaseOut (40 U/µl), 1 µl of T4 RNA ligase(5 U/µl). Incubate at 37°C for 1 hour and then centrifuge to collect RNA and add 10 µl of DEPC water. Store this reaction at -20°C.

Results

Amplification sizes of Igf2 transcripts

Total DNA and RNA was extracted from 7.5 day-old mouse livers according to the method described above. DNA was tested on agarose gel (Figure 3A). The image of DNA shows that the total DNA was not broken and relatively clean, and thus of good quality. The RNA was tested on agarose gel (Figure 3B) before quantification by spectrophotometer at 260 nm. The concentration were

determined to be 25 μg/ml. This concentration was sufficient to carry out qPCR quantifications.

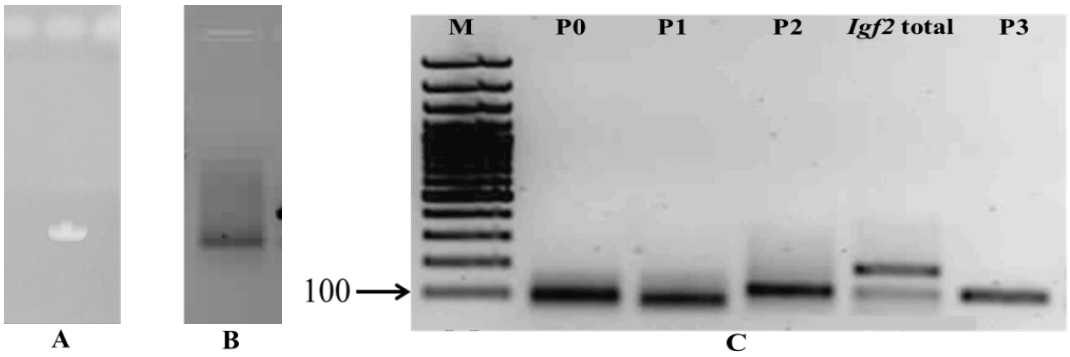

Figure 3. Image of total DNA (A) RNA (B) extracted from in wild mouse liver (d.7.5). The size of the amplicons obtained from the different Igf2 specific promoters were check in an agarose gel (C). M (100bp marker).

The Igf2 gene is located on mouse chromosome 7 and on human chromosome 11. In the mouse, this gene has a complex structure with 8 exons (U1, U2, 1,2,3,4,5,6) and four promoters (P0, P1, P2, P3). The size of the Igf2 mRNAs varies according to the length of each promoters. There are 3 exons (exons 4, 5, 6) are common to all transcripts and exon 4 contains the start codon.

Using cDNA from the Dom/SD7 cross (wild mouse) and promoter-specific primers, we quantify Igf2 gene expression from each promoters. The sizes of the resulting amplicons were as expected from the reference sequence of the mouse genome (P0: 120 bp, P1: 98 bp, P2: 135 bp, Igf2total: 201 bp, P3: 132 bp) (Figure 3C). It means that these primers are specifically designed for promoters and cDNA. These primers continue to be used to quantify the expression levels of different promoters in different mice.

Identification of a new placental transcript

Using the 5'RACE technique, we have identified a new Igf2 transcript in the mouse placenta. The transcription start site of this novel transcript is located within the U2 exon of the Igf2 gene. The new exon is 136 nucleotides in size and is named PU2 (Figure 4).

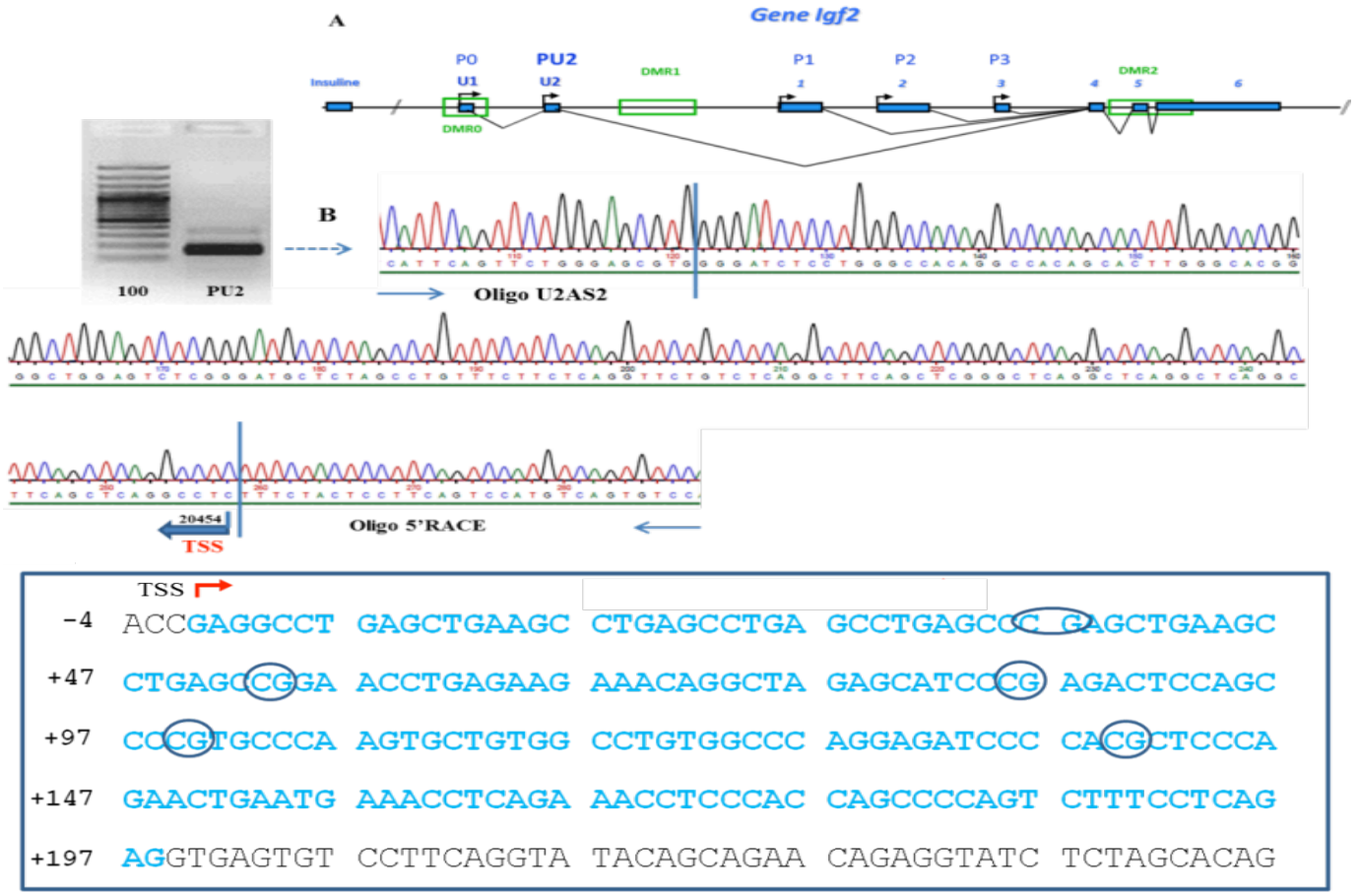

Figure 4. PU2 transcript position is depicted relative to the other Igf2 promoters (A). The sequence of the new PU2 promoter is shown (B, C).

Expression levels of specific Igf2 promoters

RNA from placental tissue (e17) was extracted from wild-type and mutant mouse strains and used for quantifications of the P0 promoter [16], while RNA from 7.5 days-old mouse livers was used to quantify all other Igf2 promoters. The level of expression of each promoters was compared to the expression level of the total Igf2 mRNA (100%). The highest expression levels were observed for the P3 promoter (80%), to P2 (35%), followed by P1 (20%) and the lowest is P0 (7%) (Figure 5).

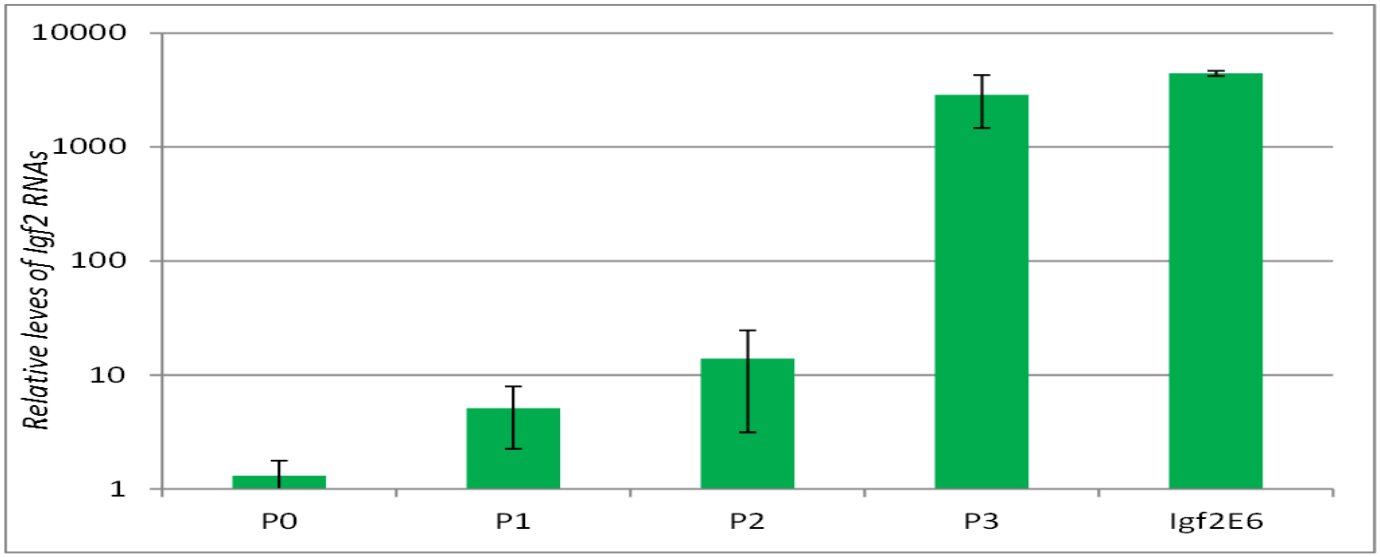

Figure 5. Levels of promoter expression (P0, P1, P2, P3), in wild mice in correlation with total Igf2 expression

Igf2 is a maternally imprinted gene, therefore its expressed arises only from alleles derived from the paternal chromosome. Thus, the elimination of the U2 exon on the maternal alleles in the ΔU2/Dom cross has virtually no effect on the expression level of this gene.

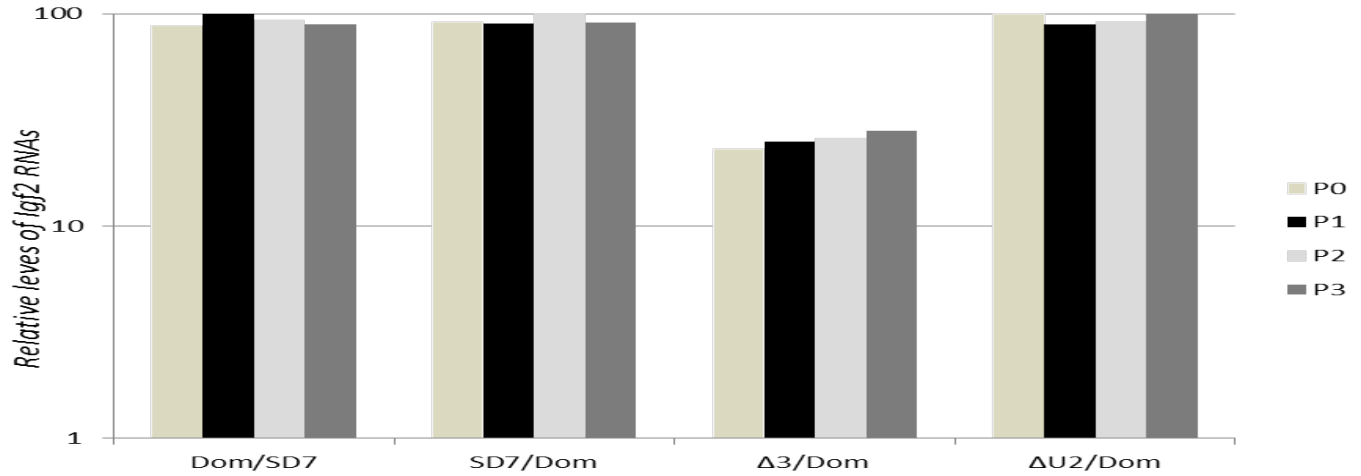

Figure 6. Levels of promoter expression (P0, P1, P2, P3) in wild mouse and mutant mouse cloning (using the Gapdh reference gene).

In contrast, in the two wild-type crosses (SD7/Dom and Dom/SD7), P0 and P1 exhibited stable but low levels (less than 50%) (Figure 6). We can see that the all promoters exhibited stable expression in the two wild-type crosses, but the expression decreased sharply when the H19Δ3 mutation occurred on the maternal allele (Δ3/Dom cross).

Discussion

Studies of RNA expression levels are often using the Northern-blot method. Northern-blots are usefull to study the size of full lenght mRNA molecules, but they often provide little knowledge on the identity of the mRNA variants that are vizualised. That is why, in this work, we prefered to use RT-qPCR and 5'RACE methods.

Our work shows that, when studying the expression of a mRNA, it is not only required to study different tissues at different developmental stages, but it is also necessary to check the expression levels throughout the entire mRNA molecule. This is particularly true for the Igf2 mRNAs for which the expression levels were here investigated at only four different promoters and two tissues (liver and placenta). Indeed, the Igf2 gene can be divided into three main areas that provide different mRNA molecules displaying different expression levels. The first area is encompassing the region of the promoters, the second area is containing the common exons (4,5,6) and the third area is the 3' region that contains the polyA tail signal (Figure 6).

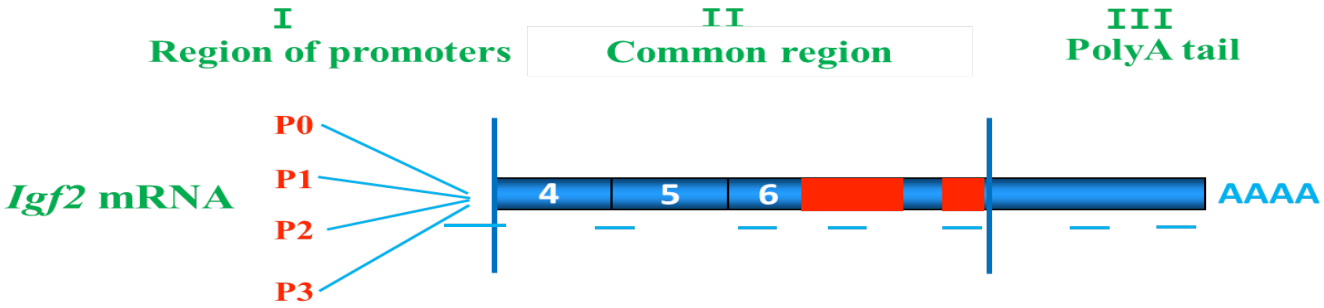

Figure 6. Main areas for study of Igf2 RNA expression

Wolfgang et al. (2011), among promoters of Igf2 in humans, P3 and P4 (equivalent to P2 and P3 in mice) contributed significantly to the total number of copies of Igf2, meaning that they were most strongly expressed in correlates with other promoters. Explaining this phenomenon, he said that, as the enhancer activated the differential methylated DMR2 region, it was itself considered to be a activative factor for two adjacent promoters [21].

When quantifying the expression levels of promoters in different mice, the results showed that all promoters exhibited low levels in the Δ3/Dom mutation, because of deletion of the H19 gene. This gene regulated the expression of Igf2 via a methylation-sensitive mechanism that is no longer active in the Δ3/Dom mutant, thus reducing the level of transcription of Igf2. In other mutants (ΔU2/Dom) as well as wild type, the expression levels of the promoters were quite high compared to the Δ3/Dom, especially P2 and P3, and were quite stable. About P0 and P1 promoters, only high expression in mutant ΔU2/Dom.

Conclusion

Specific primers for each promoter are designed and amplified precisely the size of each promoter from the cDNA (P0: 120 bp, P1: 98 bp, P2: 135 bp, P3: 132 bp, total Igf2: 201 bp). We have identified a new transcript in the mouse placenta, located on the U2 exon of the Igf2 gene. This new transcript is 136 nucleotides and is named PU2. In wild type mouse, the P3 promoter exhibited the highest (80%), followed by P2 (35%), and the remaining 2 promoters were lower than the total Igf2, P1 and P0 (less than 20%). There are different expressions of promoters in mutant and wild type mouse. The promoters exhibited low levels in the mutant Δ3/Dom. P2 and P3 are highly expressed and stable in both wild and mutant ΔU2/Dom. P0 and P1 are highly expressed only in the mutation ΔU2/Dom.

Acknowledgments

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