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Organ-Specific and Age-Dependent Expression of Insulin-like Growth Factor-I (IGF-I) mRNA Variants: IGF-IA and IB mRNAs in the Mouse

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ABSTRACT—Insulin-like growth factor-I (IGF-I) gene generates several IGF-I mRNA variants by alternative splicing. Two promoters are present in mouse IGF-I gene. Each promoter encodes two IGF-I mRNA variants (IGF-IA and IGF-IB mRNAs). Variants differ by the presence (IGF-IB) or absence (IGF-IA) of a 52-bp insert in the E domain-coding region. Functional differences among IGF-I mRNAs, and regulatory mechanisms for alternative splicing of IGF-I mRNA are not yet known. We analyzed the expression of mouse IGF-IA and IGF-IB mRNAs using SYBR Green real-time RT-PCR. In the liver, IGF-I mRNA expression increased from 10 days of age to 45 days. In the uterus and ovary, IGF-I mRNA expression increased from 21 days of age, and then decreased at 45 days. In the kidney, IGF-I mRNA expression decreased from 10 days of age. IGF-IA mRNA levels were higher than IGF-IB mRNA levels in all organs examined. Estradiol-17β (E2) treatment in ovariectomized mice increased uterine IGF-IA and IGF-IB mRNA levels from 3 hr after injection, and highest levels for both mRNAs were detected at 6 hr, and relative increase was greater for IGF-IB mRNA than for IGF-IA mRNA. These results suggest that expression of IGF-I mRNA variants is regulated in organ-specific and age-dependent manners, and estrogen is involved in the change of IGF-I mRNA variant expression.

Key words: insulin like growth factor-I (IGF-I), uterus, estradiol, mouse

INTRODUCTION

Insulin-like growth factor-I (IGF-I) is composed of 70 amino acids, and a single-chain polypeptide regulating proliferation, apoptosis and differentiation of various cells (LeRoith *et al*., 1995). IGF-I genes have been characterized in humans, rats, mice and chicken (Rotwein *et al*., 1986; Shimatsu and Rotwein, 1987a; Kajimoto and Rotwein, 1989; Kamai *et al*., 1996). Rat and mouse IGF-I gene contains six exons spanning almost 100 kb of genomic DNA (Shimatsu and Rotwein, 1987b; Kamai *et al*., 1996). Exons 1 and 2 are leader exons, and encode distinct 5'-untranslated regions and signal peptide sequences. From the two leader exons, two types of IGF-I transcripts (class 1 and class 2) are encoded (Hall *et al*., 1992; Shemer *et al*., 1992; Pell *et al*., 1993; Yang *et al*., 1995; Kamai *et al*., 1996). Exon 3 encodes the remainder of the signal peptide and the first part of the B domain of IGF-I peptide. Exon 4 encodes the remainder of the B, C, and D domains, as well as the first part of the E peptide moiety of prohormone. Exon 5 is an alternatively spliced cassette exon of 52 base pairs (bp). Alternative splicing of IGF-I mRNA results in the synthesis of various types of IGF-I mRNA variants in humans (Tan *et al*., 2002), rats (Daughaday and Rotwein, 1989), and mice (Klotz *et al*., 2000). Inclusion of exon 5 changes the reading frame of mRNAs, and results in the difference in the size and sequence of the carboxyl terminal peptide of pro-IGF-I. These two mRNAs differ by the presence or absence of

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exon 5 (Bell *et al*., 1986; Kamai *et al*., 1996). IGF-IA is encoded by mRNAs derived by splicing of exon 4 and exon 6, and IGF-IB is encoded by mRNAs including exons 4, 5 and 6.

Alternative splicing is a widespread process used in higher eukaryotes to regulate gene expression and functional diversification of proteins. Regulatory mechanisms of alternative splicing and functional roles of IGF-I mRNA variants are not clear. Therefore, the present study aimed to establish a reliable method for measuring expression levels of splice variants. We also investigated expression profiles of the two splice variants of IGF-I mRNA: IGF-IA and IGF-IB mRNAs to address changes in the expression of the splice variants, since precise functional differences between IGF-IA and IGF-IB still remain unknown.

Estrogen stimulates IGF-I mRNA expression in the uterus (Murphy *et al*., 1987; Kapur *et al*., 1992; Inoue *et al*., 2005). IGF-I is a mediator of estrogen-induced growth in the uterine epithelium (Beck and Garner, 1992; Baker *et al*., 1996; Shiraga *et al*., 1997; Sato *et al*., 2002; Inoue *et al*., 2005). Therefore, we also studied effect of estradiol-17β (E2) on IGF-I mRNA isoforms in the mouse uterus.

It is important to choose internal control genes suitable for analysis of gene expression, since the expression of some of housekeeping genes, which are often used as internal controls, changes under experimental conditions. Therefore, we compared expression of β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), elongation factor-I α (EF-Iα), hypoxanthine-guanine phosphoribosyltransferase (HPRT) and histone H2A.X genes. Expression of β-actin, GAPDH, EF-Iα, HPRT mRNAs varied widely after estrogen treatment, but H2A.X mRNA levels were constant throughout estrogen treatment. Based on these findings we used H2A.X gene as an internal control gene in the present study.

MATERIALS AND METHODS

Animals

Male and female ICR mice (CLEA Japan Inc., Osaka, Japan) were used in the present study. They were kept in a temperature–controlled animal room with free access to CE-7 commercial diet (CLEA Japan) and tap water *ad libitum*. All animal care and experiments were performed in accordance with the Guidelines for Animal Experimentation of Okayama University, Japan.

Ovariectomy and estrogen treatment

Adult female mice were ovariectomized at 8 weeks of age under light ether anesthesia. Seven days later, they were subcutaneously injected with estradiol-17β (E2, Sigma-Aldrich, St. Louis, MO, USA) at doses of 25 and 250 ng/mouse (0.1 ml), or the same volume of sesame oil. E2 was dissolved in sesame oil. At 0, 3, 6, 12, 24, 48, 72 and 96 hr after E2 treatment uteri were collected for RNA extraction.

RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted using the method of Chomczynski and Sacchi (1987). Total RNA (2 μg) in a final volume of 20 μl was subjected to reverse transcription (RT) reaction using Superscript Preamplification System for First Strand cDNA Systems (Gibco BRL, Gaithersburg, MD, USA) with oligo-dT primer according to the manufacturer's instructions.

PCR was performed using specific primer pairs for class 1-IGF-I cDNA and class 2-IGF-I cDNA. Forward primer for class 1-IGF-I cDNA (5'- ATGGGGAAAATCAGCAGTC-3') located in exon 1 and forward primer for class 2-IGF-I-cDNA (5'-CTGCCTGTGTAAACGACCCGG-3') located in exon 2, reverse primer (5'-TGTTTTGCAGGTTGCTCAAGCA-3') located in exon 6 (Kamai *et al*., 1996; Weihua *et al*., 2000). Expected sizes of amplified products for class 1-IGF-IA, class 1-IGF-IB, class 2-IGF-IA and class 2-IGF-IB cDNAs are 534-bp, 586-bp, 514-bp and 566-bp, respectively. PCR was performed using Takara Taq (Takara, Otsu, Japan) and a thermal cycler (Gene Amp PCR System 9700, Applied Biosystems, Branchburg, NJ). Conditions for PCR were as follows: initial activation of DNA polymerase by a 20-sec incubation at 90°C: 30 cycles of reactions including denaturation for 1 min at 95°C, annealing for 1min at 55°C and extension for 2 min at 72°C; and additional extension of 10 min at 60°C.

Ten micro liter aliquots of PCR product of each reaction were electrophoresed on 2% agarose gel, stained with ethidium bromide, and photographed under ultraviolet rays.

Real-time PCR

Real-time PCR was conducted with an initial denaturing at 95°C for 15 min, and then involved 40 cycles of 94°C, 30 sec; 60°C, 45 sec; 72°C, 30 sec using a Quantitect SYBR Green PCR kit (Qiagen, Hilden, Germany) and a LightCycler (Roche Diagnostics, F. Hoffmann-La Roche Ltd, Basel, Switzerland). A melting-curve analysis was performed to confirm the absence of primer dimers. Quantification was made by using the fit point method described in the LightCycler Software. To validate the present real-time PCR protocol, gene-specific standard curves for H2A.X, IGF-IA, IGF-IB, estrogen receptor α (ERα) and IGF-I receptor (IGF-IR) cDNAs were generated from serial 10 fold dilutions of the RT-product mixture. Linearity of amplification was observed in the range from 10-fold dilution to 1000-fold dilution in all cDNA samples.

To verify the specificity of each PCR quantification method, PCR products were sequenced. For each target gene, PCR in a 20-μl reaction volume was performed to obtain sufficient amounts of PCR products. Five micro liter PCR product aliquots were applied on a 3% agarose gel to check the presence of single bands. Remainings of PCR products were purified with PCR Purification Systems (Marligen Biosciences Inc. Ijamsville, MD, USA), and directly sequenced with BigDye Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems, Foster City, CA, USA) using the appropriate PCR primers. Following purification of sequenced products by ethanol precipitation, samples were run on an ABI310 Genetic Analyzer (Applied Biosystems).

Primer design for Real-time PCR

IGF-I cDNA variant-specific primers IGF-IA and IGF-IB cDNA-specific primer sets consisted of a common upstream forward primer (5'-TCGTCTTCACACCTCTTCTACCTG-3') located in exon 3, an IGF-IA cDNA-specific reverse primer (5'-CAAATGTACTTCCTTCTGAGTCTTG-3'; 333-bp amplified product), and an IGF-IB cDNA-specific reverse primer (5'-TAGGGACGGGATTCTGAGTCT-3'; 333-bp amplified product). The IGF-IA cDNA-specific reverse primer spans the boundaries of exon 4 and 6, and the IGF-IB cDNA-specific reverse primer spans the boundaries of exon 4 and 5 (Fig. 1).

Other primers Other primers were as follows. For IGF-I-F1: forward, 5'-GATACACATCATGTCGTCTTCACAC-3' and reverse, 5'-TGTTTTGCAGGTTGCTCAAGCA-3' which should result in a 467 bp product with IGF-IA cDNA and in a 519 bp product with IGF-IB cDNA. For β-actin: forward, 5'-TGCGTGACATCAAAGAGAAG-3' and reverse, 5'- GATGCCACAGGATTCCATAC-3' which should give rise to a 197 bp product (Steuerwald *et al*., 1999). For GAPDH:

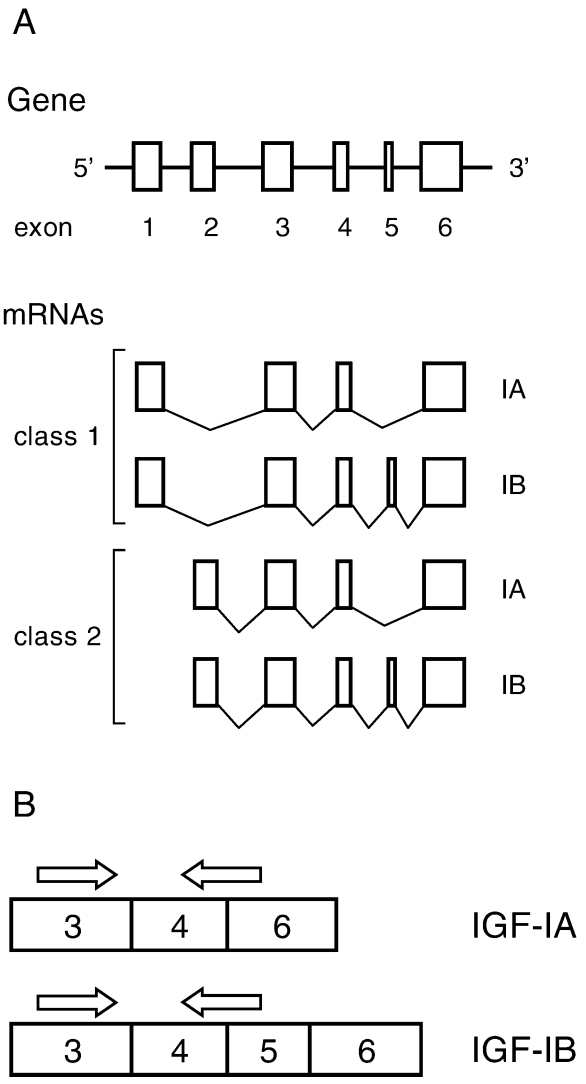

Fig. 1. Structure and expression of mouse IGF-I gene (A) and location of IGF-IA cDNA and IGF-IB cDNA specific primers (B). In A, an overview of the mouse IGF-I gene and its mRNA variants are presented. Boxes indicate exons, and lines indicate introns and flanking regions. In B, arrows above the exons indicate the positions and orientations of primers. IGF-IA cDNA- and IGF-IB cDNA-specific primer sets were composed of the common forward primer and IGF-IA cDNA- and IGF-IB cDNA-specific reverse primers. IGF-IA cDNA-specific reverse primer is located at the exon 4-6 junction and the IGF-IB cDNA-specific reverse primer is located at the exon 4-5 junction. The exon size is not drawn to the scale.

forward, 5'-TGTCAGCAATGCATCCTGCA-3' and reverse, 5'-CCGTTCAGCTCTGGGATGAC-3' which should give rise to a 239 bp product (Lekanne Deprez *et al*., 2002). For EF-Iα: forward, 5'-AAAGCTCAGAAGGCTAAATG-3' and reverse, 5'- AAGGTTTTACGATGCATTG-3' which should give rise to a 156-bp product (Lekanne Deprez *et al*., 2002). For H2A.X: forward, 5'-GTCGTGGCAAGCAAGGAG-3' and reverse, 5'- GATCTCGGCCGTTAGGTACTC-3' which should give rise to a 182 bp product (Robert *et al*., 2002). For HPRT: forward, 5'-GCTGGTGAAAAGGACCTC-3' and reverse, 5'- CACAGGACTAGAACACCTGC-3' should give rise to a 248 bp product (Lekanne Deprez *et al*., 2002). For ERα: forward, 5'-AATTCTGACAATCGACGCCAG-3' and reverse, 5'- GTGCTTCAACATTCTCCCTCCTC-3' should give rise to a 346 bp product (Weihua *et al*., 2000). For IGF-IR: forward, 5'-CAATCTATTCACAAGCCTCC-3' and reverse, 5'- GGAAAAAGAGAGGAGACGGA-3' which should give rise to a 250 bp product (Ostlund *et al*., 2001). All these primers used in the present study were synthesized by Gibco BRL Custom Primers (Life technologies Asia Pacific, Yokohama, Japan).

Real-time PCR linearity with IGF-IA and IGF-IB cDNA

The linearity of real-time PCR was determined using IGF-IA and IGF-IB cDNAs (10^2, 10^3, 10^5 and 10^7 copies/reaction) as standard samples. Under the present conditions for real-time PCR of IGF-IA and IGF-IB cDNAs, the minimum detection levels were 10 copies/reaction (data not shown). Amplification of both IGF-IA and IGF-IB cDNA templates at different concentrations showed linearity in a range from 10^2 copies to 10^7 copies.

Determination of amplification specificity of specific primers for IGF-IA and IGF-IB cDNAs

IGF-IA and IGF-IB cDNA fragments were amplified by RT-PCR with the primer set IGF-I-F1, and the PCR products were electrophoresed on agarose gels. After ethidium bromide staining, IGF-IA cDNA (467 bp) and IGF-IB cDNA (519 bp) fragments were excised from the gel and subcloned in pCR4-TOPO (Invitrogen, Carsbad, CA, USA). After the sequence determination, EcoRI-digested fragments were electrophoresed, and the fragment was isolated, and quantified by the measurement of optical density at 260 nm. The copy numbers for each cDNA were calculated using the following equation: 1 μg of 1000 bp DNA=9.1×10^{11} molecules (Overbergh *et al*., 1999). Serially diluted cDNA templates (IGF-IA cDNA and IGF-IB cDNA fragments) were amplified by real-time

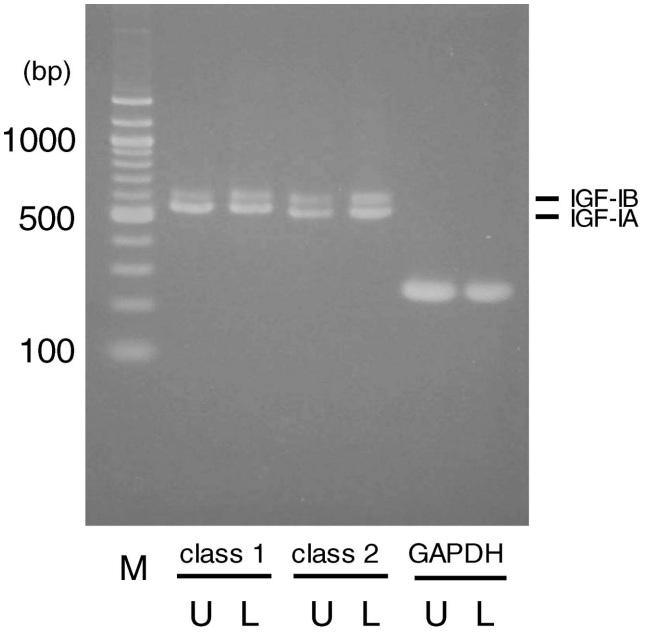

Fig. 2. RT-PCR analysis of the expression of IGF-I mRNA variants in the mouse uterus (U) and liver (L) using exon 1- and exon 2-specific forward primer and exon 6 complementary strand specific reverse primer. Class 1 variants were generated with a pair of exon 1-specific forward primer and exon 6 complementary strand specific primer, and class 2 variants with a pair of exon 2-specific forward primer and exon 6 complementary strand specific primer. Lower bands correspond to IGF-IA cDNAs, and higher bands correspond to IGF-IB cDNAs. GAPDH cDNA was amplified as an internal control. A 100-bp ladder was used as a molecular size marker (M). The number on the left depicts the size of products (bp).

PCR and the standard curves were constructed.

Statistics

Data were presented as the mean ± standard error of means (SEM), and analyzed by analysis of variance, followed by Bonferroni/Dunn test.

RESULTS

Expression of each class of IGF-I mRNA

Class 1-IGF-I mRNA (derived from exon 1) and class 2-IGF-I mRNA (derived from exon 2) were detected in liver

Table 1. Amplification specificities of IGF-I cDNA variant specific primers

Primer	Template	Template concentration ($\times 10^7$ copies/ml)	IGF-I cDNA variant concentration ($\times 10^3$ copies/ml)	Amplification specificity (%)
IGF-IA	IGF-IB	1	3.5±0.2	0.04
IGF-IB	IGF-IA	1	5.3±0.2	0.05

The standard curve was generated by serial 10-fold dilutions. Linearity of each primer ranged from 10^2 (copies/ml) to 10^7 (copies/ml). IGF-IA cDNA was quantified in the IGF-IB cDNA-specific PCR analysis, and conversely IGF-IB cDNA was quantified in the IGF-IA cDNA-specific PCR analysis. The assay was conducted in duplicate. Data are expressed as the mean±SEM (n=3).

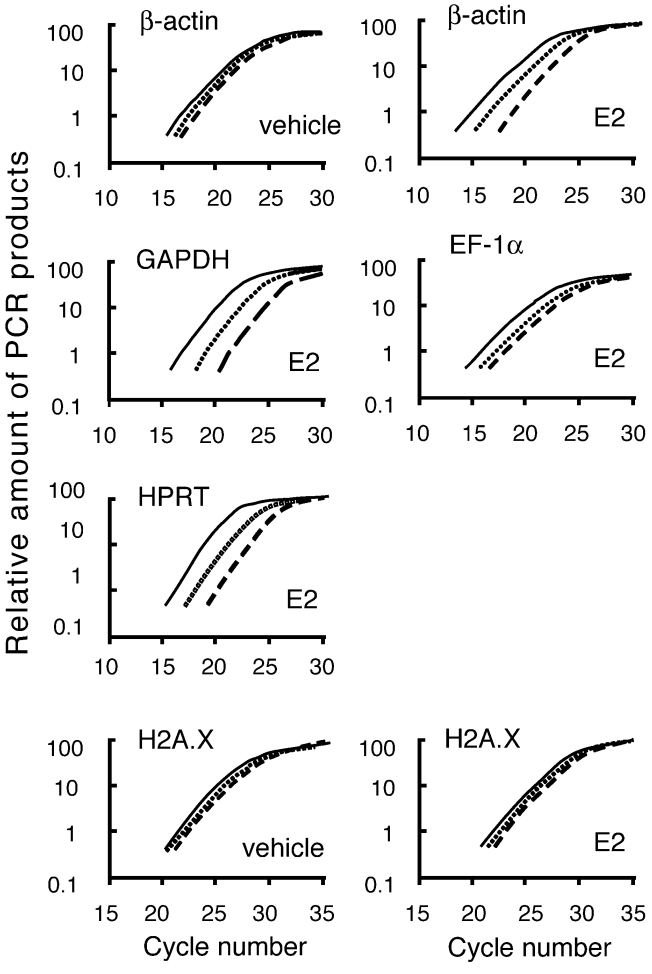

Fig. 3. Real-time PCR analysis of mRNA levels of 5 housekeeping genes, β-actin, GAPDH, EF-Iα, HPRT and H2A.X in the uterus of ovariectomized mice given E2 injection. Samples were obtained at 0 hr (broken line), 3 hr (dotted line) and 6 hr (continuous line) from ovariectomized mice after a single E2 injection (250 ng/mouse) or the vehicle (sesame oil) injection. Real-time PCR amplification was performed in duplicate, and three mice were used for each group. Typical amplification patterns were shown.

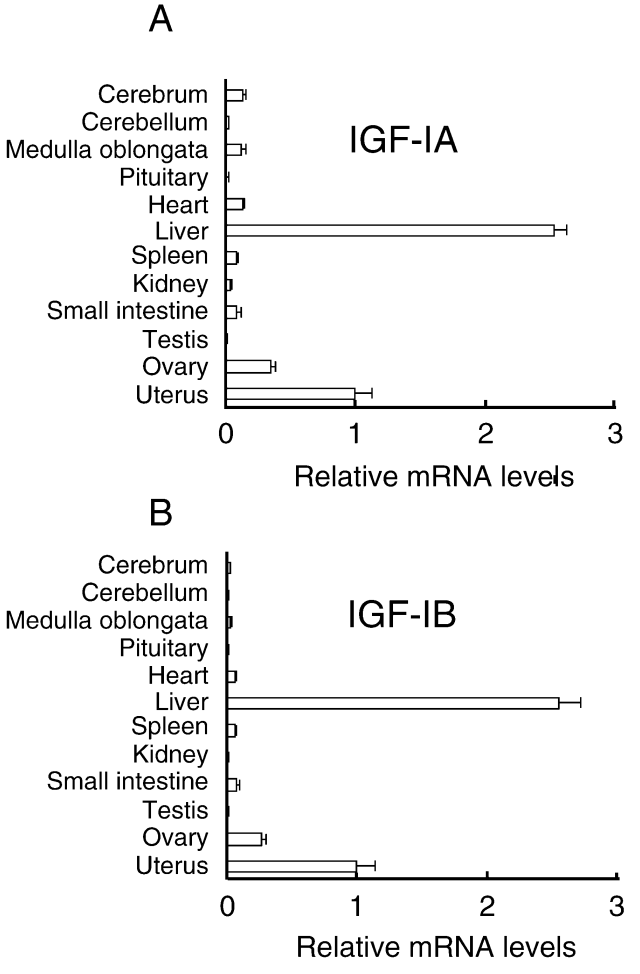

Fig. 4. Expression of IGF-IA (A) and IGF-IB (B) mRNAs in various mouse organs. Total RNA was prepared from the cerebrum, cerebellum, medulla oblongata, pituitary, heart, liver, spleen, kidney, small intestine, testis, ovary and uterus of five mice at random stages of the estrous cycle. IGF-IA and IGF-IB mRNA levels were quantified by the real-time PCR method. The value of uterine mRNA expression was defined as 1. Variant mRNA levels were normalized relative to the H2A.X mRNA levels. Data are expressed as the mean±SEM (n=5).

and uterus (Fig. 2). In both tissues, four types of IGF-I mRNAs were detected: class 1-IGF-IA, class 1-IGF-IB, class 2-IGF-IA and class 2-IGF-IB. Each lane contains two bands, a band with a low mobility corresponds to IGF-IB mRNA containing a 52 bp insertion, and a band with a high mobility corresponds to IGF-IA mRNA.

Amplification specificity of IGF-I mRNA variant specific primers

To assess the amplification specificity of each primer set, IGF-IA cDNA fragment (10^7 copies/μl) was quantified using the IGF-IB primer set, and the copy number was found to be 3.5×10^3 copies/μl (Table 1). In contrast, when the IGF-IB cDNA fragment (10^7 copies/μl) was quantified using the IGF-IA primer set, the copy number was found to be 5.3×10^3 copies/μl. These results indicate that the amplification efficiency of the IGF-IA cDNA-specific primer set using IGF-IA cDNA as a template was 2.8×10^3 times as high as the amplification efficiency using IGF-IB cDNA using as a template. On the other hand, the amplification efficiency of IGF-IB cDNA-specific primer set using IGF-IB cDNA as a template was 1.9×10^3 times as high as the amplification efficiency using IGF-IA cDNA as a template.

Determination of internal standard genes suitable for normalizing estrogen-induced mRNA expression

Five housekeeping genes, β-actin, GAPDH, EF-Iα, HPRT and H2A.X are often employed as internal controls for the analysis of mRNA expression. We analyzed changes in mRNA levels of these genes after estrogen treatment by real-time PCR, to study the validity of the use of these genes as an internal control for mRNA analysis. RNA samples were obtained from the uterus of ovariectomized mice at 0, 3 and 6 hr after E2 injection (250 ng/mouse). Fig. 3 shows typical examples of amplification of the PCR products. In each graph, the dotted, broken and solid lines indicate amplification of cDNAs obtained from mice at 0, 3 and 6 hr after E2 injection, respectively. Plots of β-actin and H2A.X expressions for 0, 3 and 6 hr in the vehicle-treated mice overlapped, indicating that β-actin and H2A.X mRNA levels did not change within 6 hr. On the other hand, plots of GAPDH, EF-Iα, HPRT and β-actin expressions at 3 and 6 hr after E2 injection shifted to the left side from the plots at 0 hr, but plots of H2A.X mRNA levels at 3 and 6 hr did not. These results indicate that E2 treatment increased GAPDH, EF-Iα, HPRT and β-actin mRNA levels, indicating that E2 stimulated transcription of GAPDH, EF-Iα, HPRT and β-actin genes. From these results we concluded that H2A.X gene was appropriate as the internal control in the present study. In the following experiments, we used H2A.X gene as the internal control of mRNA levels for the normalization of mRNA levels.

Real-time PCR analysis of expression patterns of IGF-IA and IGF-IB mRNAs in various mouse organs

IGF-IA and IGF-IB mRNA expression levels in several organs were determined by real-time PCR (Fig. 4). IGF-IA and IGF-IB mRNAs were detected in the uterus, cerebrum,

Table 2. Real-time PCR analysis of IGF-IA and IGF-IA-IB mRNA levels in various mouse organs

	Relative mRNA level (arbitrary unit)			Percentage of IGF-IB mRNA
	IGF-IA (A)	IGF-IB (B)	Total IGF-I mRNA (A + B)	(B/A+B) (%)
Cerebrum	1.10±0.20	0.02±0.01	1.12±0.24	1.8
Cerebellum	0.23±0.02	0.01±0.00	0.23±0.04	4.3
Medulla oblongata	1.03±0.33	0.03±0.01	1.06±0.35	2.8
Pituitary	0.14±0.02	0.01±0.00	0.14±0.03	7.1
Heart	1.13±0.13	0.06±0.01	1.20±0.23	5.0
Liver	21.71±0.79	2.55±0.18	24.26±4.73	10.5
Spleen	0.69±0.10	0.06±0.01	0.74±0.14	8.1
Kidney	0.35±0.07	0.02±0.00	0.36±0.07	5.6
Small intestine	0.77±0.25	0.08±0.02	0.84±0.27	9.5
Testis	0.10±0.02	0.01±0.00	0.11±0.02	9.1
Ovary	2.99±0.27	0.26±0.04	3.25±0.61	8.0
Uterus	8.56±1.11	1.00±0.14	9.56±1.68	10.5

RNA samples from the uterus, cerebrum, cerebellum, medulla oblongata, pituitary, heart, liver, spleen, kidney, small intestine, testis and ovary of five 45-day-old mice at random stages of estrous cycles were quantified by IGF-IA and IGF-IB cDNA specific PCR. The arbitrary unit value (A.U) of IGF-IA and IGF-IB cDNA was determined as follows; the IGF-IA cDNA to IGF-IB cDNA expression ratio was determined by using concentrations determined from IGF-IA cDNA fragments and IGF-IB cDNA fragments. As a result, the cDNA expression ratio was estimated to be about 8.6: 1. The value of uterine IGF-IB cDNA level was defined as 1.0. The IGF-IA cDNA level was defined as the IGF-IB cDNA level multiplied by 8.6. Data are the mean±SEM of five independent experiments.

cerebellum, medulla oblongata, pituitary, heart, liver, spleen, kidney, small intestine, testes and ovaries of 45-day-old mice. The estimated amount of IGF-I mRNAs of both isoforms was highest in the liver.

Relative proportions of IGF-IA mRNA and IGF-IB mRNA in various organs

Relative proportions of IGF-IA and IGF-IB mRNA levels in various organs were determined as follows: RNA samples obtained from various organs were pooled, and the pooled sample was used as the standard sample for measurements of IGF-IA and IGF-IB mRNAs. Copy numbers of both IGF-IA mRNA and IGF-IB mRNA in the pooled samples were determined using known copies of IGF-IA cDNA and IGF-IB cDNA fragments as quantification standards, respectively. Thus, the relative proportion of IGF-IA and IGF-IB mRNA levels in the pooled sample was 8.6:1. The arbitrary unit value (A.U.) of IGF-IA and IGF-IB mRNA levels was determined. The relative proportions of IGF-IA and IGF-IB mRNAs differed among various organs (Table 2). The liver contained the highest amount of IGF-I mRNAs of both variants, and in the uterus, IGF-I mRNA levels were approximately half of the hepatic mRNA levels. The ovaries contained a relatively high amount of IGF-I mRNA when compared to the other remaining organs. The liver contained the highest IGF-I mRNA level, and the uterus and ovary contained high IGF-I mRNA levels compared with the other organs. IGF-IB mRNA level was lower than IGF-IA mRNA level in all organs studied. The relative proportions of IGF-IB mRNAs ranged from 10.5% of liver and uterus to 1.8% of cerebrum.

Postnatal changes in IGF-IA and IGF-IB mRNA expression

IGF-IA and IGF-IB mRNA expressions in the uterus, ovary, liver and kidney were studied in 10-, 20- and 45-day-old female mice (Fig. 5). IGF-IA and IGF-IB mRNA levels in 10-day-old mice were defined as 1. In the ovaries, IGF-IA mRNA and IGF-IB mRNA levels were relatively constant. In the uteri, IGF-IA mRNA and IGF-IB mRNA increased until 21 days, and then decreased from 45 days. In the liver, IGF-IA mRNA and IGF-IB mRNA levels increased during the observation period studied, in contrast, in the kidneys, IGF-I mRNA levels decreased.

Effect of E2 on IGF-IA and IGF-IB mRNA expressions in the uterus of ovariectomized mice

The time course of E2 effect on uterine IGF-IA and IGF-IB mRNA levels was studied by real-time PCR (Fig. 6). E2 treatment (25 and 250 ng) increased IGF-IA and IGF-IB mRNA levels from 3 hr after E2 injection. The highest levels of IGF-IA and IGF-IB mRNAs were detected at 6 hr irrespec-

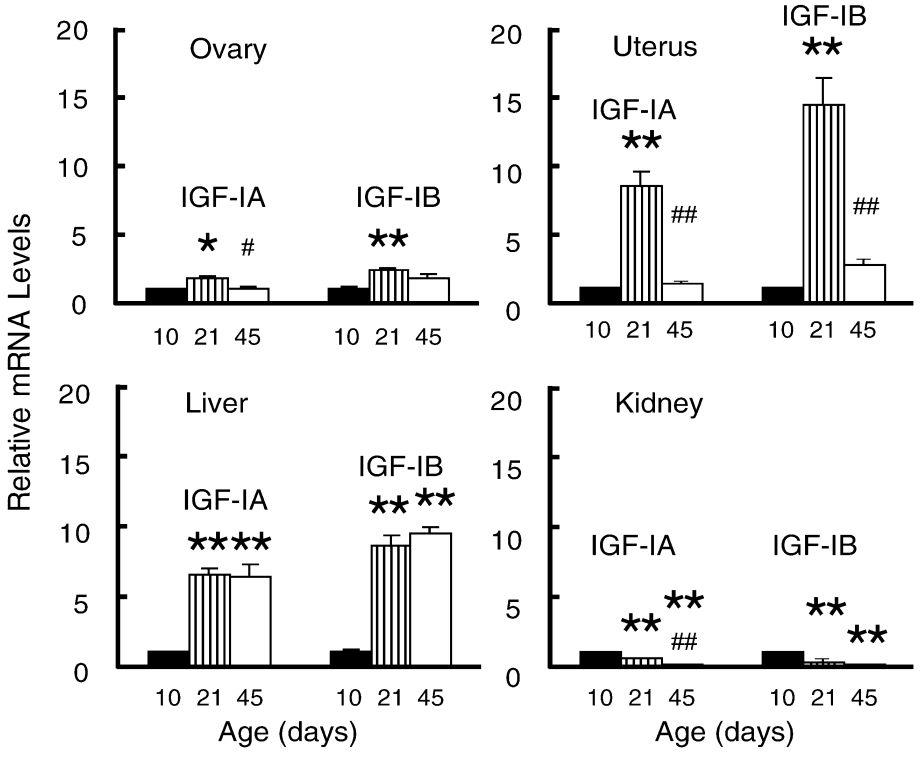

Fig. 5. Postnatal changes in IGF-IA and IGF-IB mRNA expressions. IGF-IA and IGF-IB mRNA levels were analyzed by real-time PCR in the ovary, uterus, liver and kidney obtained at 10 days of age (closed column), 21 days (striped column) and 45 days at random stages of the estrous cycle (open column). Variant mRNA levels were normalized relative to the H2A.X mRNA levels. The mRNA levels at 10 days of age were defined as 1. Data are expressed as the mean±SEM (n=5). * $P<0.05$, ** $P<0.01$, significantly different from the level at 10 days of age. # $P<0.05$, ## $P<0.01$, significantly different from the level at 21 days of age.

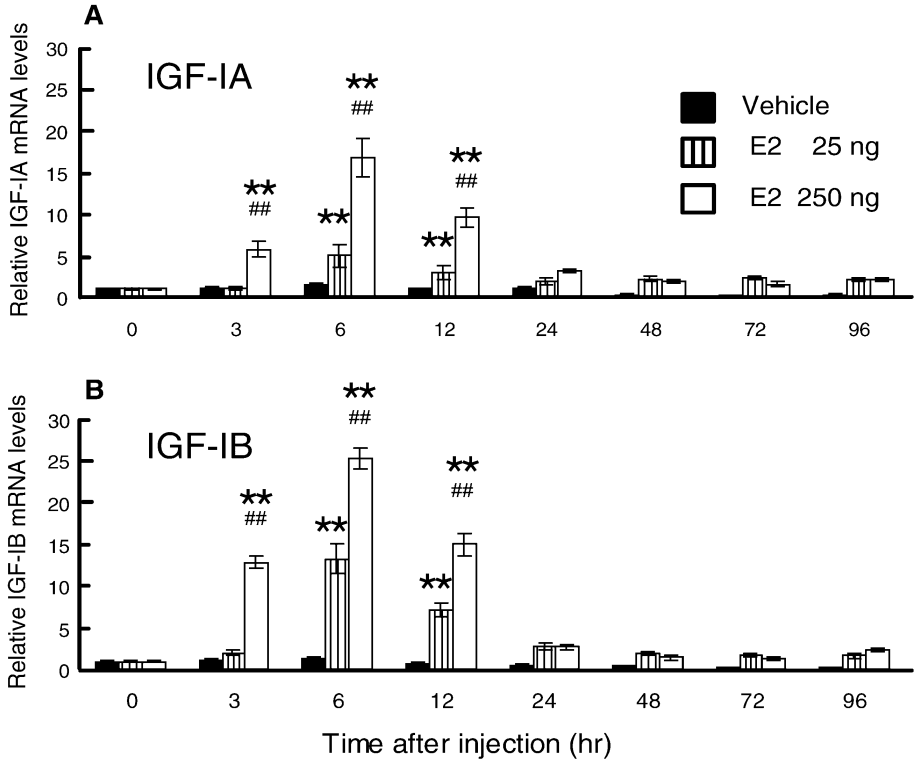

Fig. 6. Effect of E2 on IGF-IA mRNA (A) and IGF-IB mRNA (B) expressions in the uteri of ovariectomized mice. Ovariectomized mice were given a single injection of E2 (25 ng/mouse, striped column; 250 ng/mouse, open column) or sesame oil (closed column). IGF-I mRNA levels were determined by real time-PCR. Variant mRNA levels were normalized relative to the H2A.X mRNA levels. IGF-I mRNA levels were normalized relative to H2A.X mRNA levels. The mRNA level at 0 hr was defined as 1. Data are expressed as the mean±SEM (n=3). * $P<0.05$, ** $P<0.01$, significantly different from the vehicle-treated control. # $P<0.05$, ## $P<0.01$, significantly different from the E2 (25 ng)-treated group.

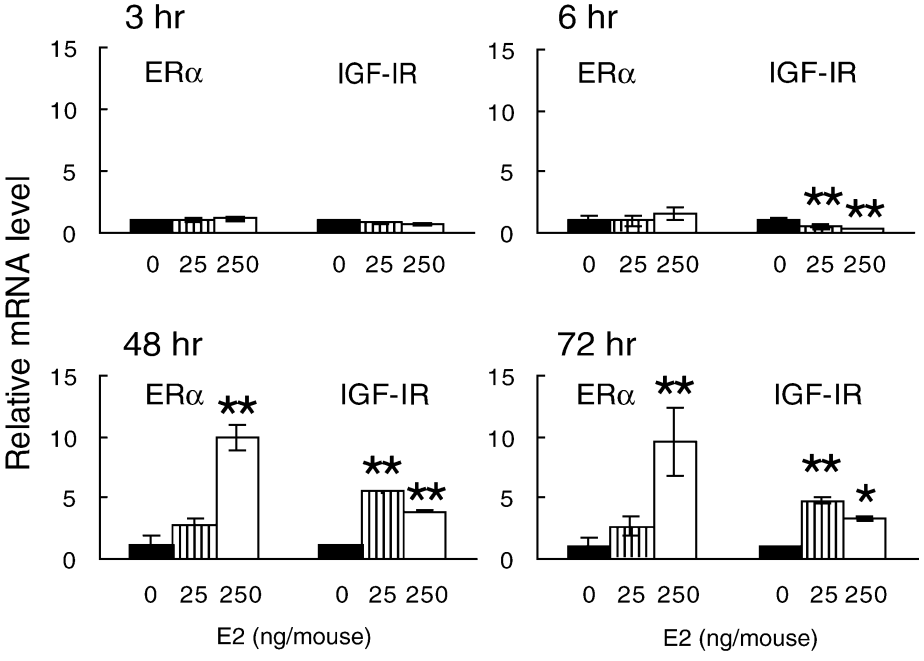

Fig. 7. Effect of E2 on uterine ERα and IGF-IR mRNA levels in ovariectomized mice. Ovariectomized mice were given a single injection of E2 (25 ng/mouse, striped column; 250 ng/mouse, open column) or sesame oil (closed column). The mRNA levels at 3, 6, 48 and 72 hr after E2 injection were determined by real time-PCR. ERα and IGF-IR mRNA levels were normalized relative to the H2A.X mRNA levels. Each mRNA level of the vehicle-treated uterus was defined as 1. Data are expressed as the mean±SEM (n=3). ** $P<0.01$, significantly different from the vehicle-treated control.

tive of the doses used. At 6 hr, a 25-fold increase in IGF-IB mRNA levels was detected in response to E2 treatment (250 ng), while a 18-fold increase in IGF-IA mRNA levels was induced by the same dose of E2. Similarly, a 15-fold increase in IGF-IB mRNA levels in response to E2 treatment (25 ng) was detected, and a 6-fold increase in IGF-IA mRNA levels was detected. From 3 to 12 hr after E2 treatment, both IGF-IA and IGF-IB mRNA levels in the uterus of E2 (250 ng)-treated mice were higher than those of E2 (25ng)-treated mice. At 24 hr, IGF-IA mRNA levels returned to control levels.

Effect of E2 on ERα and IGF-IR mRNA expression in the uteri of ovariectomized mice

Changes in uterine ERα and IGF-IR mRNA levels induced by E2 treatment in ovariectomized mice were studied by real-time PCR (Fig. 7). ERα mRNA levels were not influenced by E2 treatment at 6 hr, but were elevated at 48 and 72 hr (E2, 250 ng). IGF-IR mRNA levels were decreased at 6 hr, and were elevated at 48 and 72 hr after E2 treatment (25 and 250 ng).

DISCUSSION

The present study was aimed at establishing a method for measuring expression levels of IGF-I mRNA splice variants for better understanding their expression pattern. We successfully measured IGF-IA and IGF-IB mRNA levels with primer sets specific to the respective splice variants. The primer encompassing the variant specific exon boundary can distinguish IGF-I mRNA variants in which there is no difference except for the presence of insertion. Specificity of each primer for PCR amplification was confirmed using IGF-IA and IGF-IB cDNAs as templates. The amplification efficiency of the IGF-IA cDNA-specific primer set using IGF-IA cDNA as templates was 10^3 times as high as the efficiency using IGF-IB cDNA as templates. Conversely, the amplification efficiency of the IGF-IB cDNA-specific primer set using IGF-IB cDNA was also at least 10^3 times more efficient than when IGF-IA cDNA was used as templates. Therefore, we concluded that the specificity of each primer is sufficient to measure the target splice variant in the presence of the other splice variant, although both the variant specific primer sets are able to detect the other splice variant. The method established in the present study for analyzing IGF-I mRNA variants is useful in future studies of the splice variants expressed in cultured cells or small tissue fragments. The present finding will provide a basis for the study of tissue-specific regulatory mechanisms of IGF-I gene expression, in particular usage of the IGF-I gene promoter and regulation of alternative splicing.

Using the present method we for the first time demonstrated that IGF-IA mRNA was more abundant than IGF-IB mRNA in organs studied in the mouse and that the relative amount of these two IGF-I mRNA variants differed among the organs and temporally changed in several organs. These findings indicate that IGF-IA and IGF-IB mRNA expression in the mouse organs is organ-specifically and temporally regulated.

Liver is a major source of systemic IGF-I (Isaksson *et al*., 1987). We found that liver contained the highest amount of IGF-I mRNAs of both variants, and in the uterus and ovary. In the human liver, IGF-IA mRNA is more expressed in IGF-IB mRNA (Nagaoka *et al*., 1991), which is in agreement with the present study. IGF-IA and IGF-IB mRNA expressions varied among various organs and appear to be organ-specifically regulated. In addition, the present study showed postnatal changes of IGF-I mRNA expression and their pattern differed among organs studied. In the liver, IGF-I mRNA expression increased at 21 days of age and did not change at 45 days. In the ovary and uterus, IGF-I mRNA expression increased at 21 days of age and decreased at 45 days. In the kidney, IGF-I mRNA expression was low and decreased in tissue development. Thus, expression of each IGF-I mRNA splice variant was temporally regulated in organ-specific manners.

Hepatic IGF-I expression was mainly controlled by growth hormone (GH) (Bichell *et al*., 1992). The Janus kinase 2-signal transducer and activator of transcription 5b (JAK2-STAT5b) pathway are activated by GH in the mouse liver (Yoshizato *et al*., 2004). Estrogen treatment does not affect hepatic IGF-I expression, while it stimulates uterine IGF-I expression (Murphy and Friesen, 1988). The AP-1 motif located in the chicken IGF-I gene promoter is associated with estrogen-induced expression in IGF-I mRNA (Umayahara *et al*., 1994). On the other hand, estrogen may decrease IGF-I gene expression through a cAMP pathway in rat osteoblasts (McCarthy *et al*., 1997). Thus, IGF-I gene expression seems to be controlled organ-specifically.

IGF-I expression in the ovary is dependent on the cell type and functional status of the ovarian follicles. IGF-I mRNA is expressed in the granulosa cells of growing antral follicles in several species (Oliver *et al*., 1989; Samaras *et al*., 1993). Maximal expression of the IGF-I gene was observed on 20 days of age in the rat ovary (Levy *et al*., 1992), and this coincides with early development of the follicles destined to be the products of first ovulation (Ojeda *et al*., 1980). Since serum estrogen increases at 10 days of age and reaches maximum levels at 17 days of age, but gradually then decreases and remains at low levels after 30 days of age (Döhler and Wuttke, 1975), the high IGF-I mRNA expression correlates with and may be induced by high estrogen levels. Similarly, high uterine IGF-I expression may be due to the local high estrogen levels (Ghahary *et al*., 1990).

Both IGF-IA and IGF-IB mRNA levels in ovariectomized mouse uteri were elevated by E2 treatment, which is in agreement with a previous study carried out in the rat uterus (Klotz *et al*., 2000), and importantly, the relative increase in IGF-IB mRNA levels was higher than that in IGF-IA mRNA levels. Therefore, estrogen is considered to be associated with changes of IGF-I mRNA variant expression. Estrogen

may stimulate the alternative splicing that generates the IGF-IB mRNA variant. Interestingly, Smith *et al.* (2002) found that an exonic splicing enhancer is located in human alternative exon 5 of the IGF-I gene, and that the serine-arginine protein splicing factor-2/alternative splicing factor promotes the splicing of human IGF-I mRNA. If the similar system operates in the mouse uterus, it is probable that estrogen activates this system in the mouse uterus. On the other hand, estrogen-induced changes in expression of uterine IGF-I mRNA variants may be due to changes in uterine cell populations in estrogen-treated mice, estrogen stimulates the proliferation of luminal and glandular epithelial cells in the uterus (Huet-Hudson *et al*., 1989). Future analysis of IGF-I mRNA variant expression using culture systems of isolated endometrial epithelial and stromal cells may reveal the role of estrogen on the regulation of alternative splicing of IGF-I transcripts.

Both class 1- and class 2-IGF-I mRNAs were expressed in liver and uterus, each class was composed of IGF-IA and IGF-IB mRNAs. IGF-IA mRNA levels were higher than IGF-IB mRNA levels in both class 1- and class 2-IGF-I mRNAs in liver and uterus. Fasting specifically decreased IGF-IB mRNA synthesis in rat liver (Zhang *et al*., 1997). In the mammalian IGF-I gene, exon 1 and 2 are differently spliced to exon 3, producing alternative class 1 and class 2 transcripts. In the rat liver, transcription initiation at exon 2 was reduced in diabetes and fasting. Class 1 transcripts are ubiquitous and predominant in all tissues, and Class 2 transcripts are detected in liver (Hoyt *et al*., 1988). O'Sullivan *et al*. (2002) studied regulation of class 1 and class 2-IGF-I mRNAs in lamb liver, and showed that GH may stabilize mature class 2-IGF-I mRNA or destabilize mature class 1-IGF-I mRNA and the class 2-IGF-I mRNA may have a greater overall translational potential. These findings suggest that the transcription start site and alternative splicing of IGF-I mRNA are regulated by hormonal factors, nutrients and other factors.

In human IGF-I mRNA variants, IGF-IA and IGF-IB precursor have different nuclear localization (Tan *et al*., 2002). The nuclear localization signal situated in the C-terminal part of the exon 5-encoded domain is similar to signal sequence in several other growth factors. The last four amino acid residues of the rat and mouse IGF-IB exon 5 domain (E domain) match the motif R/K-R/K-X-R/K that is found in several nuclear localization signals (Tan *et al*., 2002). Human Eb domain, which corresponds to rodent E domain, is not cleaved. We propose that pro-IGF-I and the IGF-IB have some biological activities in the nucleus or after proteolytic processing.

Expression of uterine ERα and IGF-IR mRNA induced by E2 treatment in the uterus of ovariectomized mice was studied. ERα mRNA levels were elevated from 48 hr and still remained high at 72 hr. In the rat uterus, ERα mRNA expression was high at 24 hr in the uterine epithelial cells (Nephew *et al*., 2000). These results indicate that uterine ERα expression was up-regulated by estrogen, which agrees with a previous report (Bergman *et al*., 1992). ERα expression is decreased by IGF-I in the human breast cancer cell line MCF-7 (Stoica *et al*., 2000). IGF-IR is constitutively expressed in the mouse uterus throughout the estrous cycle (Kapur *et al*., 1992; Henemyre and Markoff, 1999). In the present study, IGF-IR mRNA was expressed and its expression was suppressed up to 24 hr after E2 injection, but remarkably elevated at 48 hr, and remained high at 72 hr. In terms of IGF-IR mRNA expression, biphasic regulatory mechanism may be operated in the mouse uterus. Since estrogen stimulates IGF-I production in the uterus as shown in the present study and previous studies (Murphy *et al*., 1987; Klotz *et al*., 2000; Inoue *et al*., 2005), estrogen-associated regulation of ERα and IGF-IR expression may have complicated systems under the presence of uterine IGF-I system governed by estrogen.

In conclusion, variant specific real-time PCR study of mouse IGF-I mRNA production revealed that the expression of IGF-I mRNA splice variants changed in organ-specific and age-dependent manners in the uterus, ovary, liver and kidney. Estrogen treatment stimulated the production of uterine IGF-I mRNA, and influenced the proportion of IGF-I mRNA splice variants.

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