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Alternative 5′-untranslated first exons of the mouse *Cyp19A1* (aromatase) gene

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ABSTRACT

The human aromatase gene (*CYP19A1*) has eleven tissue-specific untranslated first exons, while only three have been described in the mouse *Cyp19A1* namely brain-, ovary- and testis-specific exons 1. The present study aims to elucidate the complete structure of the mouse *Cyp19A1* gene. We detected aromatase transcripts in mouse bone, aorta, hypothalamus, adipose, gonads and placenta, but not nulliparous mammary fat pad. BestFit algorithm analysis against the human *CYP19A1* has identified ten putative first exons upstream of mouse *Cyp19A1*. Based on these putative sequences, we were able to design specific primers for RT-PCR and detected for the first time, the presence of exons I.4 and I.3 in murine fat and gonads, respectively. These are novel 5′UTRs of mouse *Cyp19A1*. Using RT-PCR and 5′ RACE, we confirmed the expression of exon 1f in the hypothalamus and proximal exon P2 in the ovary. The testis-specific exon 1 begins 217 bp further upstream than previously reported. Putative exons 2a, I.5, I.7, I.6 and I.2 were not detected in mouse tissues. Therefore, we showed that mouse *Cyp19A1* contains more tissue-specific first exons than previously thought and displays a similar genomic organization to human *CYP19A1*.

1. Introduction

The cytochrome P450 aromatase (aromatase) enzyme plays the key role in estrogen biosynthesis by aromatizing C19 androgens to estrogens. In non-pregnant premenopausal women, the ovary is the primary site of aromatase expression and hence is the systemic source of estrogen. Extragonadal tissues also express aromatase and have the ability to produce estrogen locally, and this has important intracrine or paracrine effects on homeostasis such as cardiovascular health, metabolism and behavior, especially in men and postmenopausal women [1].

Aromatase expression in premenopausal women is found in granulosa cells and the corpus luteum of the ovary [2]. It is also detected in human testicular leydig and sertoli cells [3], the epididymis [4], germ cells [5], syncytotrophoblasts of the placenta [6] and numerous fetal tissues [7,8]. Extragonadal tissues expressing aromatase included adipose mesenchymal tissue [9], skin fibroblasts [10], bone osteoblasts and osteoclasts [11], skeletal and smooth muscle [12], vascular endothelium [13] and the brain, markedly in the hypothalamus, frontal- and temporal-neocortices [14,15].

The human aromatase is encoded by the *CYP19A1* gene located at chromosome 15q21.1, which consists of nine coding exons (2–10) and a 5′ untranslated region (5′UTR), altogether spanning approximately 123 kb in length [16]. The coding exons occupy 30 kb and the remaining 93 kb contain alternative promoter and untranslated first exons. To date, eleven tissue-specific alternative promoters/first exons have been characterized: promoter/exon I.1 (placenta major), 2a (placenta minor), I.4 (adipose, skin), I.5 (fetal tissues), I.7 (endothelium), 1f (brain), I.2 (placenta minor), I.6 (bone), I.3 (adipose and breast cancer), promoter II/exon PII (gonads, adipose and breast cancer) and the newly discovered I.8 [17]. These promoters allow the regulation of *CYP19A1* expression in a tissue-specific manner. For example, promoter 2 drives aromatase expression in the ovary while promoter 1f is used to direct aromatase expression mainly in the brain, and promoter I.1 directs expression in the placenta. This is due to the presence of unique cell signaling pathways and transcription factors that exist in different tissues, to which different promoters are responsive [9]. Activation of tissue-specific promoters triggers the transcriptional splicing of their associated exons 1 to the coding exons at a common splice junction (*CSJ*) 38 bp upstream of the transcription start site, resulting in alternative aromatase transcripts with unique 5′ ends. However, the mechanisms regulating the use of tissue-specific promoters and the resulting tissue-specific alternative splicing are not completely understood.

Abbreviations: 5′ RACE, 5′ rapid amplification of cDNA ends; 5′UTR, 5′untranslated region; bp, base pair; *CSJ*, common splice junction; nt, nucleotide.

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Since 5′ ends are not translated, the resulting aromatase coding sequence and protein sequence are identical regardless of the promoter being used. Although alternative exons 1 are tissue-specific, they are by no means the only transcript variant present in one particular tissue. For instance, exons I.1, 2a and I.2 are all expressed in the placenta (although the latter two at very low levels), and adipose tissue contains transcript variants of exons I.4, I.3 and P2 [9].

The 5′UTR of human *CYP19A1* and that of some other species have been studied extensively. Most vertebrates produce estrogen principally in the brain and gonads, with a small subset of mammals also synthesizing estrogen in the placenta including human, horse, pig [18,19], cow, sheep [20], rabbit [21] and the hyena [19]. Species as ancient as the Atlantic stingray (fish) are found to express aromatase in ovarian follicles, brain, testis, pituitary and in the kidney [22]. It was first discovered in cattle that aromatase transcripts with different 5′-UTRs also exist in non-human species [23]. The bovine aromatase gene is regulated by up to six alternate promoters: I.1 (placenta), I.2a (placenta), I.2b (placenta), I.3 (placenta and testis), I.4 (brain) and promoter 2 (granulosa cells of ovary). In the rat, three alternative promoters have been discovered, namely promoter 1f (brain), promoter 2 and promoter PI.tr (gonads) [24]. Species such as pig and fish inherited another mechanism of tissue-specific expression of aromatase. For example, aromatase in teleost fish, is encoded by two loci *Cyp19A1a* and *Cyp19A1b* for ovary- and brain-specific expression, respectively [25,26]. The pig is unique in that it possesses at least three aromatase isozymes encoded by three separate genes: a gonad-, a placenta- and preimplanted blastocyte-specific aromatase [27,28]. Of the aromatase species sequenced so far, that of mouse *Cyp19A1* (chromosome 9) has the highest homology of 81% with human *CYP19A1*, followed by the equine, rat and chicken at 78%, 77% and 73%, respectively [29,30]. However the 5′UTR of the mouse as characterized so far is simpler than that of *CYP19A1.* To date, only three tissue-specific promoters/exons 1 have been described, namely the brain-, ovary- and the more recently discovered testis-specific exon 1 altogether spanning 31 kb upstream of the transcription start site [31,32].

Aromatase expression is detected in mouse ovary and brain, whereas transcript levels elsewhere in the mouse have been proven difficult to detect. Despite using sensitive methods such as fluorometrical quantitative polymerase chain reaction (PCR), Harada and Yamada [33] found no aromatase expression in mouse liver, thymus and spleen, also in the lung, kidney, adipose tissue, placenta and cerebellum and medulla of the brain, which do express aromatase in human [34,35]. However, earlier experiments have detected aromatase activity in the mouse testis, specifically in Leydig, Sertoli and germ cells [36,37]. Aromatase transcript and protein expression were also reported in mouse vascular smooth muscle cells [38] and vessel walls of the aorta [39] and possibly in preimplantation mouse embryos [40,41].

Mouse tissues have been demonstrated to express reporter genes through introduced human *CYP19A1* promoters, suggesting that equivalent promoters may also exist for the mouse *Cyp19A1*. For example, transgenic mice carrying the human placenta-specific promoter 1 driving growth hormone expression were found to direct growth hormone expression specifically in the placenta [42] although it is described that the mouse placenta does not express aromatase endogenously. A partial promoter I.4 construct was observed to direct reporter gene expression in transgenic mouse mammary gland and skin [43], and similarly, the human promoter 2 directed expression in mouse ovary [44]. The evolutionary conservation of regulatory machinery for aromatase expression suggests the conservation of alternative first exons and promoters.

This study aims to fully characterize the mouse *Cyp19A1* gene in order to determine if other alternative exons 1 and promoters exist, using alignment programs with the human *CYP19A1* as well as RT-PCR and 5′ RACE on various mouse tissues. If aromatase is regulated in a similar fashion in the mouse as in the human, we will be able to more effectively use the mouse as a model to understand the regulation of aromatase expression in the human, and especially to selectively inhibit or enhance aromatase expression in different tissues using the mouse model. This will facilitate the search for novel therapies for estrogen-related diseases.

2. Materials and methods

2.1. Genomic analysis

The sequences of ten first exons of human *CYP19A1* were obtained via literature search (GenBank assession nos.) exons I.1 (X55983), 2a (D14473), I.4 (L21982), I.5 (S71536), I.7 (AF419337), 1f (D29757), I.2 (S96437), I.6 [45], I.3 (D21241) and P2 (S52794).

The human sequences were individually aligned to the DNA sequence 5′ to the mouse *Cyp19A1* on chromosome 9, which was available publicly from NCBI (www.ncbi.nlm.nih.gov/mapview). The sequence extends from the 5′-end of exon 2 of *Cyp19A1* to the next gene immediately upstream of *Cyp19A1*, *Gliomedin, Gldn* (GenBank accession no. NM177350). Alignments were performed using the *BestFit Algorithm* available from WebANGIS (www.angis.org.au). The significance of sequence homology was assessed based on the length and the percentage identity with the matching human first exon. Homologous sequences of the mouse were used to design exon 1-specific forward primers for RT-PCR.

Transcription factor binding sites were investigated for the putative promoter I.4 of mouse *Cyp19A1*. Consensus sequences of the following *cis*-elements were obtained from the literature: Gamma activating site (GAS, 5′TTNCNNNAA) [46], glucocorticoid responsive element, GRE, full (5′ACANNNTGTNCT) and half (5′TGTTCN) sites [47], and the GC-rich Sp1 site (5′ACCCCGCCCA) [48]. These sequences along with 3.5 kb of genomic DNA upstream of the putative exon I.4 were put through an online program, MatInspector (Genomatix, http://www.genomatix.de/products/MatInspector/) to be compared. TATA boxes and Sp1 sites were identified using the same program by comparing the 3.5 kb gDNA to inbuilt libraries (general core promoter elements and vertebrate GC-box factors SP1/GC).

2.2. Tissue collection and RNA preparation

Gonadal fat, hypothalamus, testis, and visceral fat were collected from 6 month-old SV129J × C57BL WT male mice. Descending aorta, bone, mammary gland and ovary were collected from female littermates. Placentae were collected from 12 week-old, 2 week-pregnant female mice by cesarean section. Mice were killed by CO_2 asphyxiation. Tissues were harvested and immediately stored either in RNA*later*™ (Ambion Inc., TX, USA) or snap-frozen in liquid nitrogen, and stored at −80 °C until use. All procedures were carried out with approval by Monash Medical Centre Animals Ethics Committee B.

Total RNA was isolated from tissue samples using the single-step Ultraspec™ RNA isolation system (Biotecx, TX, USA) and DNAse-treated (Ambion, TX, USA). The quality of RNA samples was analyzed by gel electrophoresis (1% agarose, 1× TBE, 100V, 45 min) with ethidium bromide staining. Sample concentration was determined by UV absorbance at 260 nm wavelength with a spectrophotometer (Radiometer Pacific, Copenhagen).

2.3. 5′ RACE (5′ rapid amplification of cDNA ends)

In order to isolate aromatase transcripts from different mouse tissues and determine their 5′ ends, 5′ RACE was performed using the 5′/3′-RACE kit (Roche, Mannheim, Germany). Total RNA (1 μg)

Table 1
Comparison of first exons between human *CYP19A1* and mouse *Cyp19A1* by computer and experimental analyses.

Human first exon	Computer analysis Length of mouse DNA matched to human (%); Sequence homology with mouse (%)	RT-PCR (Mouse tissues)	5′ RACE (Mouse tissues)
1.1 (placenta)	31/103 bp (30%); 66%	–	–
2a (placenta)	33/109 bp (30%); 76%	–	–
1.4 (adipose, bone)	119/323 bp (37%); 75%	Gonadal fat	–
1.5 (fetal tissues)	55/95 bp (58%); 60%	–	–
1.7 (aorta)	49/101 bp (49%); 69%	–	–
1f (brain)	139/139 bp (100%); 94%	Hypothalamus	Hypothalamus Gonadal fat Placenta
1.2 (placenta)	411/971 bp (42%); 66%	–	–
1.6 (bone)	131/167 bp (78%); 63%	–	–
1.3 (ovary, adipose)	203/203 (100%); 88.5%	Ovary, testis	–
P2 (ovary, testis)	125/132 bp (95%); 64%	Ovary	Ovary Visceral fat
Mouse exon 1 E_{tes} (testis)	–	Testis	Testis

Computer (BestFit alignment) and experimental (5′ RACE) analyses of human *CYP19A1* first exons and the unique mouse testis-specific exon 1 in mouse tissues.

was reverse transcribed with an exon VI-specific primer (E6R, 5′TGGTTTGATCAGGAGAGCTTGC), in a 20 μl-reaction containing 1 μl transcriptor reverse transcriptase (Roche, Mannheim, Germany), 4 μl cDNA synthesis buffer, 2 μl dNTPs (10 mM) and 1.25 μl 10 μM E6R primer. The resultant cDNA (20 μl) was purified using High Pure PCR Product Purification Kit (Roche, Mannheim, Germany) as instructed in the 5′/3′-RACE protocol.

A homopolymeric dA-tail was appended to the purified cDNA using recombinant terminal transferase (Roche, Mannheim, Germany) in a 25 μl-reaction. Purified cDNA (19 μl) was first incubated with 2.5 μl 10× reaction buffer (Roche, Mannheim, Germany) and 2.5 μl dATP (2 mM), at 94 °C for 3 min then chilled on ice for 5 min. Terminal Transferase enzyme (1 μl 80 U/μl) (Roche, Mannheim, Germany) was then added to the reaction. After incubation at 37 °C for 25 min (20–30 min), the enzyme was heat inactivated at 70 °C for 10 min and the sample was kept on ice until amplification.

Tailed-cDNA was first amplified by Taq DNA polymerase (Promega, Madison, WI, USA) using an exon 3-specific primer (E3R3, 5′CCCAGACAGTAGCCAGGAC) and the 5′ Oligo dT-anchor primer provided (Roche, Mannheim, Germany). The PCR reaction (50 μl) contained 0.5 μl 5 U/μl Taq DNA polymerase (Promega, Madison, WI, USA), 5 μl 10× reaction buffer, 1 μl Oligo dT-anchor primer (Roche, Mannheim, Germany), 1.25 μl 10 μM E3R3 primer and 1 μl 10 mM dNTPs, carried out under the following conditions: 1 cycle of initial denaturation (94 °C for 2 min); 10 cycles of further denaturation (94 °C for 15 s), primer annealing (60 °C, specific to E3R3 for 30 s) and extension (72 °C for 40 s); 25 more cycles of previous 3 steps with extra 20 s of extension time added successively to each cycle; and 1 cycle of final extension (72 °C for 7 min).

A second "nested" PCR was performed using an exon 2-specific primer (E2R1, 5′CCAAATCAGGAGAAGGAGG) and the 5′ PCR anchor primer was supplied (Roche, Mannheim, Germany). Product from first amplification was diluted (1:20), of which 1 μl was amplified in a 50 μl-reaction described as above but using a different set of primers: PCR anchor primer, which complements the short oligo of Oligo-dT anchor primer (1 μl; Roche, Mannheim, Germany) and 1 μl 10 μM E2R1. The conditions for nested-PCR were exactly the same as those in the first amplification, with the exception of annealing temperature, which was 57 °C (optimized annealing temperature for E2R1).

Final RACE products were cloned into the pGEM-T vector (Promega, Madison, WI, USA) for sequencing using two vector-specific primers that complement either side of the 5′-end insert (RT7 5′TAATACGACTCACTATAGGG and Sp6 5′TTCTATAGTGTCACCTAAAT).

2.4. RT-PCR

RT-PCR was performed to investigate the expression of alternative first exons in various mouse tissues, using forward primers designed from mouse sequences homologous to the human first exon sequences (Table 1). Exon 1-specific primers were designed based on the putative mouse *Cyp19A1* first exons, or from 5′-RACE products. Total RNA (0.5 μg) was reverse transcribed amplified in a 50 μl reaction using SuperScript™ III RT/Platinum® *Taq* Mix (*Invitrogen, Carisbad, CA*) with the following conditions: 1 cycle of 60 °C for 30 min (cDNA synthesis), 1 cycle of 94 °C for 2 min; 40 cycles of 94 °C for 15 s, specific annealing temperature (Table 2) for 30 s, 68 °C for the duration (s) specific to the expected product (i.e. 1 min per 1000 bp; Table 2); and a final cycle of extension at 68 °C for 5 min. RT-PCR products were analyzed by gel electrophoresis and gel-purified (Qiagen, Hilden, Germany) for sequence analysis.

2.5. DNA sequencing

Sequencing was performed using an Applied Biosystems 3130xl genetic analyzer by the standard dideoxy method. Sequencing results were analyzed using BLASTn and BLAST alignment algorithms available from NCBI (www.ncbi.nlm.nih.gov/blast).

3. Results

3.1. Genomic analysis of the mouse Cyp19A1 5′UTR

The 5′UTR of the human aromatase gene (*CYP19A1*) consists of eleven alternative promoters and associated untranslated first exons spanning 93 kb. The known 5′UTR of the mouse aromatase gene (*Cyp19A1*) spans only 36 kb upstream of the transcription start site. However, our genomic analysis revealed that the next known gene 5′ to *Cyp19A1*, *Gldn,* is 93 kb away (data not shown) in chromosome 9, band 9A5.3. We hypothesized that this 93 kb of genomic DNA upstream of *Cyp19A1* may contain additional alternative first exons to the three known ones—a proximal ovary-specific, a testis-specific and distal brain-specific exon 1 located 120 bp, 8500 bp and 35,943 bp upstream of the transcription start site, respectively.

By comparing the 93 kb genomic DNA upstream of *Cyp19A1* against that of *CYP19A1* (BestFit Alignment program, ANGIS), we identified ten sequences located 5′ to the mouse *Cyp19A1* that were homologous to the human 5′-untranslated first exons (Fig. 1). The percentage identity between these human and mouse sequences reached 60% or above, and the length of matching exons 1 was

Table 2
RT-PCR oligo primers and specific PCR conditions.

Oligo primers	Sequence (5′ → 3′)	Anneal temperature (°C)	Extension time (s)	Expected product size (bp)
1.4F E3R1	AGTTGGAATTTGTGACCTCTA ATTCCCAGACAGTAGCCAG	65	12	204
1fF E3R3	GCATCATTAGCAAAACTCACC CCCAGACAGTAGCCAGGAC	55	16	262
P2F E3R3	GGAGCCACAGCCAAACC CCCAGACAGTAGCCAGGAC	56	16	260
T4F (E_{tes}) E2R2	CAAGTAGAAGCAAGAAACGGGTAT GGCCCATGATCAGCAGGAG	58	14	238
1.3F E2/3R	CGTCACTCTACTAACTCAAGGGC AGGACCTGGTATTGAAGACGA	54	22	372
E4F E6R	TGGAGAACAATTCGCCCTTTC TGGTTTGATCAGGAGAGCTTGC	62	14	230
2aF E3R1	GCAGAATTCAGAAATTATGTTC ATTCCCAGACAGTAGCCAG	–	–	183
1.7F E3R2	AT CCACCT CCAAGATAGAGACAG CAATTCCCAGACAGTAGCCAG	–	–	208
1.2F E2R1	CT GGAGCAAGT GAGAAAGAGA CCAAATCAGGAGAAGGAGG	–	–	296
1.6F E3R3	TCCCTGTAAGAGTTTTTGATAA CCCAGACAGTAGCCAGGAC	–	–	270
CycF CycR	CTTGGGCCGCGTCTCCTTC TGCCGCCAGTGCCATTAT	60	11	180

Designed according to putative first exons of mouse *Cyp19A1* obtained from BestFit analysis. T4F and E2R2 primers were designed from a testis RACE clone. E, exon; F, forward primer; R, reverse primer; e.g. E3R1, exon 3-specific reverse primer 1; E4F and E6R complement coding exons 4 and 6 of *Cyp19A1*, respectively; *Cyc*, *Cyclophilin* (housekeeping gene).

at least 30%, with the highest for human exons 1f, I.3 and P2 (more than 95%; Table 1). Moreover, these putative exons 1 of mouse *Cyp19A1* were distributed in almost the same order as those in human *CYP19A1*, except for exon I.1, which was found 15 kb upstream of transcription start site in the mouse *Cyp19A1* compared to being the furthest first exon (93 kb upstream) in human *CYP19A1* (Fig. 1).

In order to investigate whether these putative exons 1 are indeed expressed in the mouse, we designed oligo primers (Table 2) based on the putative *Cyp19A1* exons 1, and performed RT-PCR on various mouse tissues. Exon I.1- and exon I.5-specific primers were not used due to an overall low sequence homology between human and mouse, and the involvement of fetal tissues, respectively.

3.2. Aromatase expression in mouse tissues

Using RT-PCR, we first confirmed that aromatase transcripts were detectable in mouse bone, aorta, hypothalamus, ovary, placenta, testis, gonadal and visceral fat, but not in nulliparous female mouse mammary gland (Fig. 2). Sequence analyses confirmed that the sequences of all products were homologous to the expected

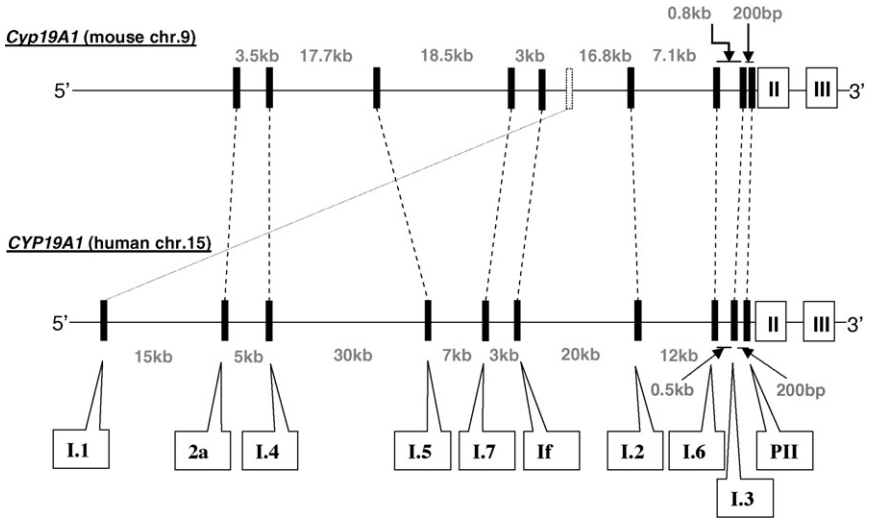

Fig. 1. Putative 5′-untranslated first exons of the mouse *Cyp19A1* gene and known human *CYP19A1* exons 1. Positions of potential mouse *Cyp19A1* first exons were predicted by aligning the known human *CYP19A1* 5′UTR to 93 kb genomic DNA upstream of the mouse *Cyp19A1* coding exons. Sequence alignment and homologies were obtained using an internet-based program BestFit analysis (WebANGIS: www.angis.org.au). The positions of predicted mouse exons 1 (except exon 1.1) are found in a similar order of that of known human exons 1.

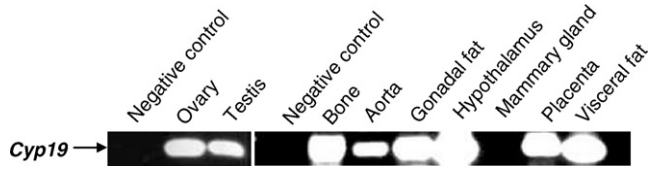

Fig. 2. Aromatase expression in mouse tissues. Aromatase transcripts were detected in mouse ovary, testis, bone, aorta, gonadal and visceral adipose tissues, hypothalamus and the placenta, using one step RT-PCR, 0.5 μg of total RNA, 40 cycles of PCR amplification for all transcripts in all tissues, and RT-PCR primers E4F and E6R, which crossed coding exons 4 and 6 of the mouse *Cyp19A1* (230 bp). No aromatase expression was detected in the mammary gland of a virgin female mouse (6 months). Identities of all PCR products have been confirmed by sequence analyses.

amplicon of coding exons 4–6 of *Cyp19A1*, with the intron spliced out at the predicted sites.

3.3. *Cyp19A1* 5′ ends discovered using 5′ RACE

5′ rapid amplification of cDNA ends (5′ RACE) was performed to characterize the 5′UTRs of aromatase transcripts from the mouse tissues that were expressing aromatase. Briefly, aromatase transcripts were first amplified using a specific, distal 3′ primer, followed by a nested-amplification using a more proximal 3′ primer, together with non-specific 5′ anchors that anneal to the 5′ end of specific transcripts at each round of amplification. Purified amplicons were then cloned into pGEMT vector for sequencing to identify the 5′ end sequence of the specific transcript. In total, 83 clones of RACE products were isolated and sequenced from mouse ovary, testis, hypothalamus, gonadal fat, visceral fat, bone and aorta.

3.3.1. *Testis*

Eight RACE clones were analyzed, all of which were identified as mouse aromatase identical to the published aromatase cDNA sequence (GenBank accession no. D00659). Five of the clones did not contain any 5′UTRs. The remaining 3 clones (coded T3, T4 and T7) each contained a novel 5′UTR identical to sequences within the 93 kb genomic DNA upstream of *Cyp19A1*.

Clone T4 contained a 272 bp-5′ end (Fig. 3) that was shown to be 8.5 kb upstream of the translation start site based on BLAST alignment analysis. In relation to other known *Cyp19A1* exons 1, the 5′ end of this clone was ~27 kb downstream of brain-specific first exon and ~8.5 kb upstream of ovary-specific first exon. The same splice junction, 15 bp upstream of the translation start, was identified in this RACE amplicon. This novel first exon matched to a previously reported sequence in the mouse testis denoted as putative promoter P_{tes}. This 272 bp-5′end showed 97% homology to a sequence previously published: *Mus musculus partial Cyp19A1 gene promoter and exon 1* by Vanselow and colleagues (GenBank accession no. AJ437576.1) are also known as P_{tes} [32], and our current sequence is 217 bp longer. We will refer to this novel exon 1 as E_{tes}, which was submitted to Genebank (GenBank accession no. FJ619058).

Clone T3 contained a 405 bp-5′ end, which consisted of 3 parts spliced together to the same junction 15 bp upstream of the translation start site, and the splice sites among the 3 parts confirmed the GT/AG rule for RNA splicing (supplementary Fig. 3Ai). The first part was 317 bp, the second part was 30 bp and the third part was 58 bp. BLAST search did not identify this amplicon with any expressed sequence previously published. Nevertheless, BLAST alignment indicated that parts 1, 2 and 3 were situated approximately 77 kb, 76.3 kb and 75 kb upstream of the translation start site (suppl. Fig. 3Aii).

Clone T7 revealed a short 5′ end of 57 bp (suppl. Fig. 3Bi), located 5.9 kb upstream of the translation start, as indicated by BLAST search (suppl. Fig. 3Bii). The common splice site was somewhat

Fig. 3. Mouse testis aromatase transcript variant 2 from RACE analysis. The sequence of 5′ RACE product (clone T4) from mouse testis. *Lower case*, vector sequence; *lower case-underlined*, partial PCR-anchor primer; the added polyA tail was totally missing; *Upper case bold*, the extended version of testis-specific 5′ end, with the new sequence *dot-underlined* and its specific forward primer, T4f. The remainder of the exon was identical to the testis-specific first exon of *Cyp19A1* previously published (GenBank accession no. AJ43757601); *CSJ*, common splice junction 15nt upstream of translation start; *Upper case not bold*, partial coding exon 2 with the translation start site ATG *underlined* and specific reverse primer, E2r2. The sequence of this clone has been confirmed by sequence analysis using both the forward T7 and reverse SP6 primers. GenBank accession no. FJ619058.

shorter than the previous two testicular clones, i.e. only 7 bp instead of 15nt upstream of translation start site. Although RACE revealed three unique 5′ ends, only that from clone T4 was confirmed to be expressed in the mouse testis by RT-PCR (E_{tes}; Fig. 4).

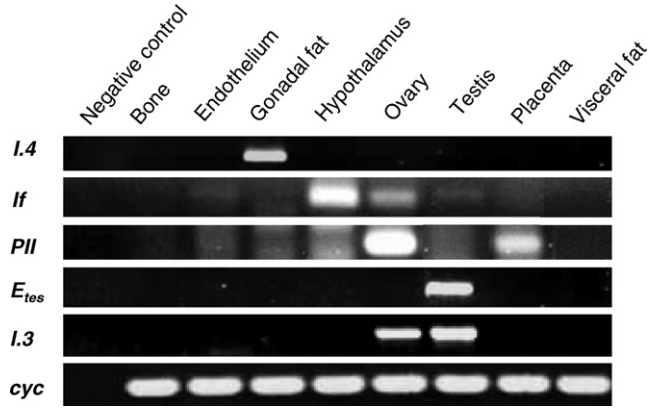

Fig. 4. Alternative 5′ ends of *Cyp19A1* in mouse tissues. RT-PCR (40 PCR cycles) was performed on 0.5 μg of total RNA from mouse tissues using oligo primers designed from putative first exon sequences. Exons 1f and P2 were detected from the hypothalamus and ovary, respectively, as previously reported. The adipose-specific exon 1.4 (GenBank accession no. FJ619059) was discovered in mouse gonadal adipose tissue, while exon 1.3 (GenBank accession no. FJ619060) was expressed in mouse ovary and testis. The expression of testis-specific exon 1 (E_{tes}) discovered through 5′ RACE was also confirmed here by RT-PCR. Housekeeping gene, *Cyclophilin* (*Cyc*), was amplified in all cDNA samples.

3.3.2. Ovary

Five clones of ovarian aromatase cDNA were isolated and analyzed. Four of these were successfully sequenced and contained the mouse aromatase sequence with 100% homology to the published sequence of *Mus musculus mRNA for aromatase* (GenBank accession no. NM007810.1). Sequencing of the fifth clone was not successful due to the presence of an extended poly-T stretch, which interfered with the sequencing reaction. Three of the four clones did not contain a 5′ end sequence. The last remaining clone (O4) contained a 120 bp 5′ end sequence upstream of the translation start site, which had a 100% homology with mouse *ovary-specific 5′ flanking region* (GenBank accession no. D67046; suppl. Fig. 3C). This region was 63% homologous to human exon P2 (GenBank accession no. D21241; data not shown) and this percentage identity was consistent with the prediction made using BestFit analysis (Table 2). RT-PCR using exon P2-specific primers confirmed the expression of first exon P2 in mouse ovary (Fig. 4).

3.3.3. Hypothalamus (brain)

Eight clones were selected for analysis, of which seven were identified as mouse aromatase according to the reported sequence of *Mus musculus mRNA for aromatase* (GenBank accession nos. NM007810.1 and D00659.1). Sequencing of the eighth clone was unsuccessful. Only three of the seven clones contained a 5′ end for analysis. Each of these three clones contained 5′ end of 17–19 bp, which was spliced to exon 2 at the common splice site 15 bp upstream of translation start (suppl. Fig. 3Di). These 5′ end sequences are 100% homologous to mouse brain-specific first exon (GenBank accession no. D67045.1; suppl. Fig. 3Dii), 86% homologous to human exon 1f despite their short lengths (GenBank accession no. D29757; data not shown). RT-PCR confirmed the expression of exon 1f in hypothalamus (Fig. 4).

3.3.4. Placenta

Eight RACE clones from mouse placenta were selected for analysis, all of which were identified as aromatase (GenBank accession no. NM007810.1; suppl. Fig. 3E), containing short 5′ ends ranging 16–18 bp long. The 5′ ends of these amplicons were 100% homologous to mouse brain-specific exon 1 (GenBank accession no. D67045.1; suppl. Fig. 3Eii), and were spliced to the common splice site 15 bp upstream of translation start site. However, RT-PCR did not detect exon 1f expression in mouse placenta (Fig. 4).

3.3.5. Gonadal fat

Eight RACE clones of gonadal fat were sequenced, all of which were identified as aromatase (suppl. Fig. 3F). Similar to data from mouse placenta, these clones contained a partial brain-specific exon 1f (GenBank accession no. D67045.1). Seven of the eight clones contained very short 5′ end sequences of 17–18 bp long. One clone, denoted G1, contained the longest 5′ end of 92 bp that showed 100% homology with the brain-specific exon 1f (suppl. Fig. 3Fii). This 5′ end was spliced to the common splice site 15 bp upstream of the translation start site. However, we failed to amplify using RT-PCR any aromatase cDNA containing brain-specific exon 1f from mouse gonadal fat (Fig. 4).

3.3.6. Visceral fat

Five clones were sequence-analyzed, and all of which were identified as mouse aromatase cDNA (GenBank accession nos. NM007810.1 and D00659.1; suppl. Fig. 3G). Each clone contained a 35 bp-5′ end that appeared to be a truncated version of the ovary-specific exon PII (suppl. Fig. 3Gii). Nevertheless, we failed to amplify P2-specific amplicons from visceral fat by RT-PCR (Fig. 4).

3.3.7. Bone

Two separate RACE experiments were carried out on mouse bone tissue and in total, 22 clones were sequenced. None of the nine clones from the first RACE experiment were identified as aromatase cDNA (data not shown). It was then identified that the nested primer E2R has a 100% homology to mouse *chondroadherin* mRNA (GenBank accession no. BC 012672.1), which led to the non-specific amplification instead of aromatase exon 2 (data not shown).

The second RACE was attempted to resolve the issue of non-specificity by using a new group of gene-specific primers: E10R (exon 10), E6R (exon 6) and P2R (exon 3) (RACE 1: E6R [exon 6], E3R4 [exon 3] and E2R [exon 2]). Thirteen clones were selected for sequence analysis. Eight of these clones contained primer dimers as inserts, and BLAST alignment revealed that no parts of the clone resembled the aromatase cDNA; two clones were homologous to *chondroadherin* and *SRC* gene (GenBank accession nos. BC 012672.1 and AJ313176.1, respectively); the remaining 3 clones shared homology with various *Mus musculus* BAC clones on chromosome 9, which did not resemble sequences within the 93 kb putative 5′UTR of *Cyp19A1*. Consequently, no 5′ end of bone aromatase transcript was identified.

3.3.8. Aorta

Similarly, two RACE experiments were performed on mouse aorta and 27 clones were selected and analyzed. The first nested-PCR produced amplicons too large to contain 5′ ends (400–700 bp; data not shown), since the coding exon 2 is only 120 bp long. Nonetheless, 16 clones were selected and analyzed and only one was identified as mouse aromatase, however it did not contain a 5′ end (data not shown). Seven of the 16 clones contained E2R/PCR anchor primer dimer as inserts. Sequencing failed for two other clones. The remaining six clones contained non-specific amplicons including mouse aldolase, mannosidase, mitochondrial DNA, gelsolin and ferritin heavy chain mRNA (GenBank accession nos. 31982505, 6754621, 8096291, 26354754 and 15126787, respectively).

The second RACE was performed in the same fashion as described for the second bone RACE. Eleven clones were analyzed, of which six contained E3R/PCR anchor primer dimer as inserts; three clones were homologous to sequence contigs from chromosome 10 to 14 (GenBank accession nos. 50199069 and 38708140, respectively); sequencing failed for one clone, and another had a sequence homologous to a *Mus musculus chromosome 9 clone RP23-38G1* (GenBank accession no. 46849832), but this sequence was not found in the 5′UTR to *Cyp19A1*. Overall, no 5′ end of the aromatase transcript was identified for mouse aorta.

3.4. Cyp19A1 5′ ends confirmed by RT-PCR

In order to investigate whether the putative first exons are indeed expressed in mouse tissues, we designed oligo primers based on the putative *Cyp19A1* exons 1 (Table 2), and performed RT-PCR on various mouse tissues. Of the nine primer pairs, only the primers for exon 1f, I.3, I.4 and P2 were successfully optimized. Primers for the novel E_{tes} discovered via 5′ RACE were also designed from RACE clone T4 and were optimized. Optimization of the remaining 4 primer pairs (for 2a, I.7, I.6. I.2) was unsuccessful. All PCR amplicons were sequence-analyzed.

Exon I.4 is the first exon expressed mainly in human adipose tissue. We detected exon I.4 expression in the mouse for the first time, and it was expressed specifically in mouse gonadal adipose and not detected in visceral adipose or any other tissues (Fig. 4). The sequence of this novel mouse *Cyp19A1* exon I.4 transcript has been submitted to Genebank (GenBank accession no. FJ619058).

Exon 1f was expressed mainly in the mouse hypothalamus (Fig. 4). The RT-PCR product detected in the mouse ovary was not

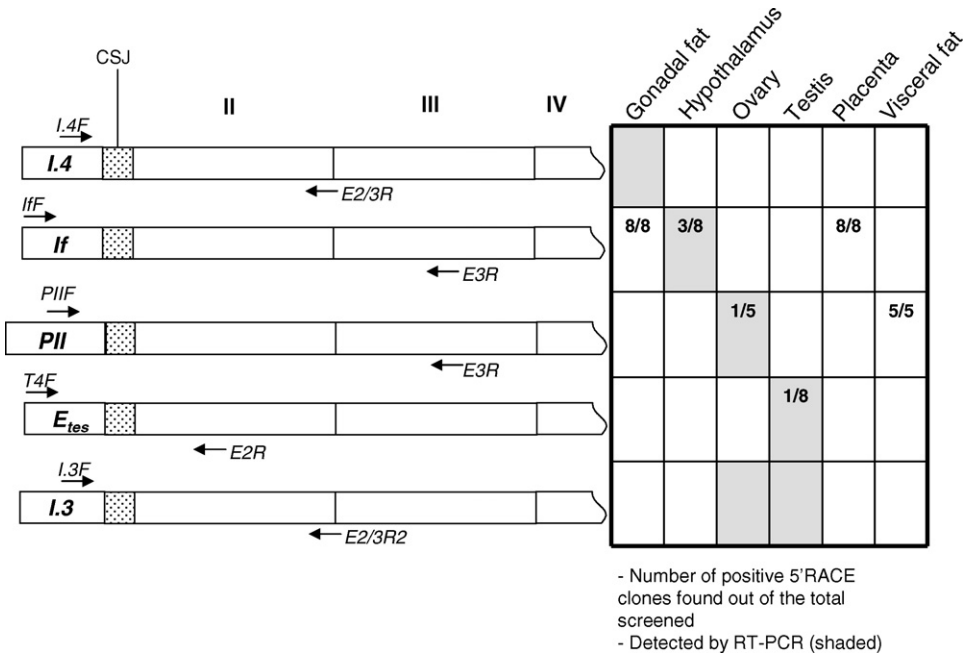

Fig. 5. Summary of RT-PCR and RACE results. The schematic presentation of tissue-specific aromatase transcripts with exons P2, If, E_{tes} as alternative first exons (left), which were detected by 5′ RACE in various mouse tissues, and 1.4 and 1.3 which were detected only by RT-PCR. The table (right) indicates the number of 5′ RACE clones bearing the corresponding specific first exons out of the total screened in each of the listed mouse tissues. Shaded cells (table) indicate that the expression of the corresponding tissue-specific aromatase transcript has been confirmed using RT-PCR primers (arrows).

a specific product according to sequence analysis. Exon 1f expression was not detectable in bone, visceral fat, placenta or gonadal fat, even though exon 1f was detected using RACE from gonadal fat and placenta.

Exon P2 expression was strongly evident in ovary (Fig. 4). Exon P2 was not detected in mouse bone, aorta, gonadal fat, hypothalamus, testis and visceral fat, despite the presence of some smearing (Fig. 4). The PCR product from placenta was a result of non-specific amplification as indicated by sequence analysis.

The testis-specific first exon, E_{tes}, was uniquely expressed in the mouse testis, and not in any other tissue investigated. Some PCR products were observed in aorta, gonadal fat, however, these were identified as non-specific amplicons. The *Cyp19A1* transcript containing the extended version of testis-specific exon 1 has been submitted to Genebank (GenBank accession no. FJ619058.

Exon I.3 is known to be expressed in the human ovary. Using the primers designed from the predicted mouse exon I.3, it was shown to be expressed in the mouse gonads (ovary and testis). The sequence of this novel mouse *Cyp19A1* exon I.3 transcript has been submitted to Genebank (GenBank accession no. FJ619060).

We were unable to detect the expression of exons I.6, I.2, 2a, or I.7 in any of mouse tissues tested, and it was difficult to optimize the primers when the tissue of expression is yet unknown in the mouse.

3.5. Genomic analysis of the putative promoter I.4 of Cyp19A1

The promoter region associated with the novel exon I.4 in mouse *Cyp19A1* was analyzed (Fig. 6). The distance between putative exon 2a and I.4 of *Cyp19A1* was approximately 3.5 kb, which was similar to that of human *CYP19A1*. The human *CYP19A1* promoter I.4 spans 1 kb, containing a silencer, gamma activating site (GAS), glucocorticoid responsive element (GRE), and a GC-rich Sp1 site within exon I.4 [49]. In the mouse *Cyp19A1* promoter I.4 region, three putative GAS *cis*-elements were found at −77,882, −77,527 and −77,990 upstreams of exon 2. Only two putative GRE half-sites were located at −76,388 and −76,341 (between the second and third GAS elements) and no full site was found. Four classical mouse TATA boxes were found at −77,970, −76,422, −75,882 and −75,137, flanking the GAS and GRE half-sites, and a GC-rich Sp1 site was located at −74,813 within exon I.4 as found in the human promoter I.4 (Fig. 6).

4. Discussion

The present study aimed to elucidate the 5′UTR of the mouse *Cyp19A1* transcripts. By identifying the locations of putative alternative exons 1, new promoter regions associated with these untranslated exons can be identified and subsequently characterized. Previous studies have identified three alternative 5′ ends expressed in ovary, brain and testis of mouse [31,32]. In this present study (Fig. 5), we have identified two additional untranslated exons 1: exon I.3 (gonads) and exon I.4 (gonadal adipose) of the mouse *Cyp19A1* and an extended version of the mouse testis-specific first exon (E_{tes}).

4.1. Ovary- and brain-specific aromatase expression in the mouse

Aromatase was expressed strongly in the mouse ovary and hypothalamus (brain). The transcription of a particular first exon implies the activation of its associated promoter. Aromatase expression in mouse ovary is driven by the proximal promoter 2, and in the brain by a more distal promoter 1f. The location and length of these exons 1 were consistent with previous findings, and indicate that use of these promoters is highly conserved between human and mouse. Our results from RT-PCR, but not RACE, amplified the full-length exon 1f from the mouse brain as previously reported [31], whereas both RT-PCR and RACE detected ovarian aromatase cDNA with full-length exon P2. Exon P2 was expressed exclusively in the ovary and exon 1f in the hypothalamus. This indicates that promoter 1f may not be present or play no role in aromatase expression in the ovary, and similarly with promoter 2 in the brain. In agreement with our data, Harada and colleagues also did not observe exon P2 in the mouse brain using RT-PCR [31]. However, exon P2-containing cDNA had been previously detected in brain [32] and in the testis

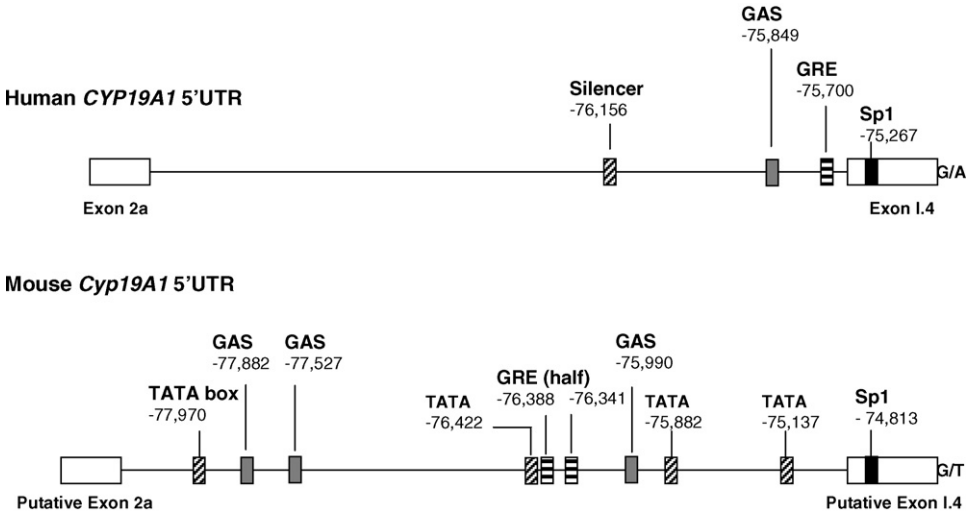

Fig. 6. The putative promoter 1.4 of mouse *Cyp19A1*. Comparison with the characterized human *CYP19A1* promoter 1.4 that contains a Silencer (TATTAAA; −76, 156), gamma Activating Site, *GAS* (TTCCTGTGAAA; −75, 849), Glucocorticoid receptor Responsive Element, *GRE* (AGAAGATTCTGTTCT; −75, 700) and Sp1 site (GGGCGGGG; −75, 267). Putative *cis*-elements were identified by comparing libraries of consensus sequences to genomic DNA 3.5 kb upstream of the putative exon 1.4 using the MatInspector program, Genomatix (http://www.genomatix.de/products/MatInspector/). Four putative TATA boxes (TATAAA; −77,970; −76,422; −75,882; −75,137), three GAS sites (TTACCCTAA; −77,882; −77,527; −75,990), two GRE half-sites (TGTTCT; −76,388; −76,341) and a Sp1 site (GAGCCCAGCCCCATG; −74,813) were located, shown with sequences and positions relative to the start of coding exon 2. Further studies are required to investigate their biological significance.

of adult mouse, as well as exon 1f in mouse gonads. [31,32], albeit at low levels of expression. The method employed was nested real-time PCR [32], which may have increased the specificity as well as maximized product amplification compared to the standard RT-PCR used in this current study.

4.2. Testis-specific aromatase expression in the mouse

Unlike the rat, aromatase expression in the mouse testis is not driven by promoter 2 but by a unique, yet to be characterized promoter (denoted as P_{tes}) that has no known equivalents in other species [32], including the male germ cell promoter, P1.tr, described in the rat testes by Silandre et al. [50]. The expression of this novel first exon (E_{tes}) was detectable in the adult mouse testis by both RACE and RT-PCR methods. Additionally, we discovered that E_{tes} begins 217 bp further upstream than previously reported. Our data predict that P_{tes} is located approximately 8.6 kb upstream of the translation start site in exon 2 and is spliced to exon 2 at the 15nt common splice junction. Further studies are required to characterize the 5′-flanking promoter region that controls the expression of this testis-specific exon. We confirmed that E_{tes} was only expressed in the mouse testis and not in any other tissues, which is in contrast to the findings of Golovine and colleagues, who detected expression of E_{tes} in ovary, brain and testis of adult mouse, when a proximal reverse primer (exon 3) was used but not with a distal one (exon X) [32].

Two minor aromatase cDNA variants were also discovered by RACE from mouse testis, which contained 5′ end sequences that were identified within the putative 5′UTR of *Cyp19A1*. Sequence analysis showed that these variants may encode functional aromatase enzymes in the testis, since the 5′ ends were indeed spliced onto exon 2.

However, RT-PCR failed to reproduce these products despite multiple attempts and therefore they may be considered as RACE artifacts. Minor aromatase transcript variants do exist and were first observed in human adipose tissue from RACE analysis by Mahendroo et al., where human truncated exon I.3, or exon I.4 along with exon I.2 were spliced to coding exon 2 [9]. The physiological significance of these transcripts resulting from 'infrequent splicing events' is uncertain. This speculation will require further experimentation

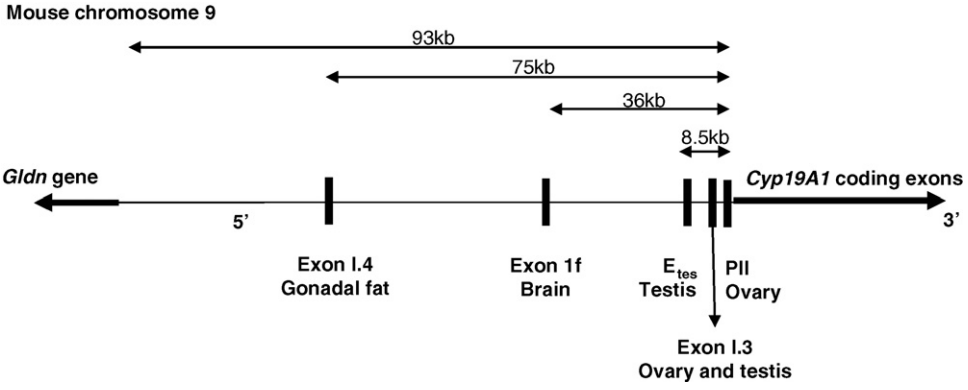

Fig. 7. The proposed mouse *Cyp19A1* gene. Based on computer analyses and experimental evidence, we propose that the mouse *Cyp19A1* gene also has an adipose-specific exon 1.4 that is approximately 75 kb upstream of the transcription start site; also present is a testis-specific exon 1 which was found by 5′ RACE and confirmed by RT-PCR to be 217 bp longer than previously described, located 8.5 kb upstream of the transcription start site; exon 1.3, approximately 200 bp upstream of the transcription start site, was detected in mouse gonads. Brain- and ovary-specific first exons were readily detected as reported previously.

and it will be useful to investigate the significance of multiple aromatase transcript variants in the testis as a future objective.

Our preliminary data also suggest that the *CYP19A1* promoter, I.3 has a role in aromatase expression in the mouse testis as exon I.3 expression was also detected by RT-PCR. Promoter I.3 is a cAMP-responsive promoter as is promoter 2, and cAMP has been shown to regulate aromatase expression in the testis [24].

4.3. Aromatase expression in mouse adipose tissues

The previous study using fluorometric PCR detected negligible aromatase transcript levels in mouse adipose tissue [33]. Moreover, RT-PCR did not detect ovary-specific exon P2 nor brain-specific 1f expression in mouse adipose tissue [31]. In this study, we detected by RT-PCR moderately high levels of aromatase expression in mouse gonadal and visceral fat, but not in the mammary fat pad.

The aromatase knockout (ArKO) mouse has shed light on the importance of local estrogen production in adipose tissue. This model is completely deficient in estrogen due to disruption of the *Cyp19A1* gene [51]. Male as well as female ArKO mice develop obesity, whereby the fat pads are disproportionately larger compared to wildtype (WT) counterparts [52]. This obese phenotype of the male ArKO mice implies a role for local aromatase expression in adipose tissues, since males do not have an ovarian source of circulatory estrogen as females do, as indicated by the very low serum estrogen levels in males. Thus normal fat metabolism would have to rely on local estrogen production in local tissues. It is unclear whether testicular estrogen contributes to the metabolism of the adjacent gonadal fat pads, but we speculate that aromatase expression is also tissue-specific regulated in gonadal fat.

Our RACE analysis detected brain-specific 5′ ends in mouse gonadal fat, and ovary-specific exon P2 in visceral fat. However, RT-PCR results did not confirm either of these findings. On the other hand, we detected exon I.4-specific transcripts in gonadal adipose tissue by RT-PCR. In human, exon I.4 was discovered in human adipose stromal cells obtained from women [9]. Aromatase expression was detected in mouse visceral fat however exon I.4 was not expressed in this adipose depot. Therefore a different regulatory mechanism may drive aromatase expression in visceral fat, which is yet to be determined. No aromatase expression was detected in nulliparous mouse mammary gland using RT-PCR, therefore exon 1-specific RT-PCR and RACE were not attempted on this tissue. A previous study has documented low aromatase activity in the mouse mammary gland, and this does not peak even during late pregnancy, as opposed to the goat mammary gland [53]. Future studies should include mammary fat pads from pregnant as well as lactating mice to further examine aromatase expression in this organ.

4.4. Aromatase expression in mouse placenta

A previous study had attempted to quantify aromatase mRNA level in the murine placenta using fluorometrical quantitative PCR, which involves product amplification with fluorescence-tagged primers. There was virtually no endogenous aromatase detected in the placenta of mouse [33], while aromatase expression in the human placenta is well established. Research suggests that the mouse placenta is not required to provide estrogen locally since the duration of pregnancy in rodents is short, and the ovarian supply of estrogen suffices [54]. Our present study did detect the presence of aromatase expression in the murine placenta by RACE analysis, suggesting the possibility of local estrogen production within the placenta. However, the source of aromatase is questionable. The aromatase transcripts may have originated from the rich vasculature of placenta and/or any fetal tissues attached to the placenta [35]. In human, aromatase expression occurs in the syncytiotrophoblast of placenta [6], a layer of tissue derived from the blastocyst as it implants into the endometrium, however in the mouse, preimplantation blastocysts do not express endogenous aromatase [41].

The expression of exon I.1 was not investigated further experimentally as we concluded that it had low homology with the human exon I.1 and was located in a different region of the 5′ end. RT-PCR on the putative exon 2a was attempted in mouse placenta but it was not successful. Our RACE data suggested that promoter 1f is involved in regulating aromatase expression in the murine placenta, since fragments of exon 1f were frequently obtained. However, our RT-PCR result did not support this observation. Previous studies have shown that the mouse placenta was able to drive reporter gene expression using human *CYP19A1* promoter I.1, which is a placenta-specific promoter [42]. This suggested that transcription factors required for promoter I.1 are either present or being recruited in order to activate human promoter I.1-directed reporter transcription in the mouse placenta. The issue of whether or not aromatase expression in murine placenta is significant remains to be decided.

4.5. A Comparison between mouse Cyp19A1 and human CYP19A1

Aromatase expression in human is regulated by the eleven alternative promoters of *CYP19A1* in a tissue-specific manner: I.1, 2a, I.8, I.4, I.5, I.7, If, I.2, I.6, I.3 and P2. Of these, I.1, I.4, If, I.3 and P2 are the major promoters whereas the others are of uncertain significance, although I.7 may be of importance in breast vascular endothelium [55]. These promoters, along with their specific first exon sequences are situated over 93 kb upstream of a 30 kb coding region consisted of nine coding exons. Similarly, the mouse *Cyp19A1* consists of nine coding exons spanning 27 kb, and a 5′UTR characterized to date to be 35 kb long, which includes three alternative exons 1 and their putative promoter regions—exon/promoter 1f, testis-specific first exon/P_{tes}, and exon P2/promoter 2 [32]. In this study, we found that the mouse *Cyp19A1* gene may also have a 90 kb 5′UTR upstream of its coding exons, which contains more alternative promoters and additional exons 1—exon I.3 and I.4. We were not able to demonstrate the presence of exons 2a and I.2 (placenta), I.7 (vascular endothelium), I.6 (bone) and I.5 (fetal tissues) in the mouse. Also, the issue of the role, if any, of the putative exon I.1 sequence in the mouse is unclear, because of its low homology to the human sequence and its altered position relative to the other first exons.

It was previously thought that a major difference between human and mouse in terms of aromatase expression, is that the human has evolved to regulate aromatase in a more differential fashion than the rodent. However it is apparent from the studies presented here that aromatase expression in the mouse is more similar to that of the human than previously thought. Consistent with this, both human *CYP19A1* and mouse *Cyp19A1* have retained promoter activity of 1f and 2, leading to estrogen production in the central nervous system and gonads, respectively. Consistent with the essential roles in sexual behavior and reproduction in both sexes [56,57], promoters: 1f and 2 are highly conserved among almost all animal species. Additionally we now find that aromatase transcripts containing exon I.4 are present in mouse gonadal fat (Fig. 6), and that the mouse genome contains a promoter I.4 sequence analogous to the human. As shown in Fig. 6, a comparison of human and mouse promoter I.4 5′-upstream sequences reveals the presence of GAS elements and an Sp1 element, and GRE half-sites in the mouse, similar to our previous findings in the human, suggesting a similar mechanism of regulation [49]. To date, there is no direct evidence that suggests that glucocorticoid or cytokines regulate aromatase expression in mouse adipose tissue. Further investigation is warranted to determine whether these putative sites are indeed functional as *cis*-elements. As now seen in the mouse, exon I.3 in human is also widely expressed, in adipose tissues of the thigh, abdomen, cancerous breast [9,58], vascular endothelium and

smooth muscle cells [13]. It was also found in skeletal muscle [12] and in bone as splicing variants with exon I.6 [45].

A key difference between the human and mouse aromatase genes is the regulatory mechanism for aromatase expression in the male gonad. Such difference may be due to different transcription factors or regulators present or being recruited in testis. A previous report indicates that aromatase expression in mouse testis is regulated by the nuclear receptor factor, LRH-1 via a cAMP-sensitive promoter [59,60]. It was shown by these studies that promoter 2 has no role in regulating aromatase expression in the mouse testis. Therefore, we speculate that the cAMP-sensitive promoter is promoter I.3 which has two putative c/EBP elements (c/EBP1 at −478 and c/EBP2 at −444) if aligned to human promoter I.3 (suppl. Fig. 3H),

Human breast cancer is associated with increased aromatase expression in the adipose tissue surrounding the tumour, which involves promoter switching from I.4 as the major driver of aromatase expression to the cAMP-driven promoter 2. However, we did not detect aromatase expression in nulliparous mouse mammary fat pad. Exon I.4 was indeed expressed in the mouse, however it was detected only in gonadal adipose tissue.

4.6. Comparison of the RACE and RT-PCR approach

In this study, standard 5′ RACE and RT-PCR procedures were used to detect the expression of 5′ ends of mouse aromatase cDNA. Both methods employ coding exon-specific 3′ primers. The 5′ primers for RT-PCR were exon 1-specific oligonucleotides designed from sequences homologous to human *CYP19A1* exons 1, whereas PCR in RACE utilizes 'anchor'-nucleotides that anneal to 5′ ends of any transcripts.

We amplified the E_{tes} by RACE, which was further confirmed by RT-PCR using primers designed from the resultant RACE sequence. By contrast, we detected the expression of exon I.3 (gonad-specific) and exon I.4 (gonadal fat-specific) by RT-PCR but not by RACE (Fig. 5).

This could be explained by the fact that RT-PCR involves specific forward and reverse primers, whereas the PCR in RACE uses only one specific reverse primer (which was different to those used for RT-PCR) and a general forward primer (oligo-dT anchor or PCR anchor primer). Therefore, RT-PCR allows targeted amplification, while RACE gathers a variety of cDNA species. Due to higher specificity, RT-PCR tends to be an 'all or nothing' approach in detecting expression, whereas RACE detects multiple species of cDNA simultaneously. The minor species may not be detected. Moreover, the specificity of RACE products depends on multiple factors, such as the due addition of polyA tail sequence to the 5′ end of cDNA, and the 'anchoring' onto this sequence by oligo-dT primer. It is also a long multi-step process compared to RT-PCR, which increases the probability of errors and uncertainties. Therefore any RACE result requires verification by RT-PCR, which was performed in this study.

Both RT-PCR and RACE revealed a common splice junction, GT/AG, in most aromatase cDNAs detected i.e. amplicons containing exon P2, 1f and E_{tes} from mouse ovary and brain (RT-PCR data) and placenta, gonadal fat and visceral fat (RACE data). This is consistent with the general GT/AG rule for RNA splicing which was previously observed in mouse ovary- and brain-specific aromatase cDNA generated by RACE [31]. In this study, the 5′ splicing donor (GT) was found 15 bases away from the start of translation in all aromatase cDNA sequences. Since the 5′-splice donor marks the 3′ end of the untranslated exons, we utilized this splicing donor to define the length of putative exon 1. We are confident that amplicons containing this common splice junction are generated from real aromatase transcripts in the mouse. In human, the equivalent junction was found 38 bp upstream of the translation start site [16]. The significance of such difference between species is uncertain.

This study provides a starting point for future studies investigating differential regulation of aromatase expression in the mouse. Having identified two alternative first exons (exons I.3 and I.4) present in the human genome but not previously described in the mouse, we are now in a position to characterize their 5′ flanking regulatory regions, and to better understand how aromatase expression is regulated in the mouse gonads and adipose tissue via these alternative promoters.

In conclusion, this study reveals novel alternative 5′ ends of mouse *Cyp19A1* transcripts—exon I.3 and I.4. Our results confirmed that there are now five alternative 5′ ends spanning approximately 75 kb upstream of the mouse *Cyp19A1* coding region: exon I.4 (gonadal fat; −75 kb), 1f (brain; −36 kb), E_{tes} (testis; −36 kb), exon I.3 (ovary and testis; −200 bp), and proximal exon PII (ovary) (Fig. 7). We were unable to detect the expression of human-equivalent exons I.1, 2a, I.5, I.7, I.2 and I.6, in the adult mouse.

Note-added-in-Proof

Zhao et al. describe a novel promoter which controls *Cyp19A1* gene expression in mouse adipose tissue (Reproductive Biology and Endocrinology 2009, 7:37; doi:10.1186/1477-7827-7-37). The sequence of their adipose specific mouse exon is identical to our mouse exon I.4, however they do not report homology with the human exon I.4, whereas in our hands the homology is 75% (Table 1, supplementary Fig. 3I).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2009.03.010.

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