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Tissue-specific expression of the human aromatase cytochrome P-450 gene by alternative use of multiple exons 1 and promoters, and switching of tissue-specific exons 1 in carcinogenesis

(alternative splicing/estrogen synthetase/tissue-specific promoter)

NOBUHIRO HARADA*†, TOSHIAKI UTSUMI‡, AND YASUYUKI TAKAGI*

*Division of Molecular Genetics, Institute for Comprehensive Medical Science, and ‡Department of Surgery, Fujita Health University, School of Medicine Toyoake, Aichi 470-11, Japan

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ABSTRACT **Extensive screening of aromatase cDNA was carried out in cDNA libraries from various human tissues. The DNA sequences of all the isolated cDNA clones were identical in the region encoded by exons 2–10 of the aromatase gene. However, tissue-specific sequences, which were classified into four groups, were observed in the 5′ portions of the clones corresponding to the region encoded by exon 1. All of them were also found in clones isolated from a human genomic library and mapped between exons 1 and 2 of the human aromatase gene reported previously, suggesting the presence of multiple exons 1 and promoters in the gene. Reverse transcription–PCR analyses of aromatase mRNAs in various tissues revealed that aromatase transcripts are tissue-specifically spliced by alternative use of multiple exons 1, although minor forms of the transcripts were also present in each tissue. Aromatase mRNA is spliced from 10 exons in most tissues, but from 9 exons in the prostate and from 10 or 11 exons in the placenta. This suggests that tissue-specific regulation of the aromatase gene in various tissues may be explained by alternative use of multiple exons 1 flanked with tissue-specific promoters. The alternative use of multiple exons 1 for liver transcripts was found to change developmentally. Furthermore, switch from an adipose-specific exon 1 to another type of exon 1 was observed in aromatase transcripts of adipose tissues of three of five breast cancer patients.**

Aromatase, also called cytochrome P-450$_{AROM}$ or estrogen synthetase, is a unique member of the cytochrome P-450 superfamily. It is localized mainly in the ovary and placenta and catalyzes the conversion of androgen to estrogen, which is a rate-limiting step in estrogen biosynthesis. This enzyme activity has been found in various extragonadal tissues such as the brain (1), liver (2), skin fibroblasts (3), and adipose tissue (4) as well as in gonadal tissues. It is important that the tissue-specific expression of aromatase is strictly regulated, because estrogen has been suggested to be involved in various physiological functions through not only endocrine but also autocrine or paracrine actions and to function as a growth or differentiation factor. Indeed, the expression of aromatase has been reported to be regulated tissue specifically by many kinds of factors, including cAMP, phorbol esters, glucocorticoid, insulin, androgen, epidermal growth factor, fibroblast growth factor, and transforming growth factor. Interestingly, some of these factors have different effects on the expressions of aromatase in different tissues (5–7).

The human aromatase gene has been isolated (8–10), using human aromatase cDNA as a probe. The gene is larger than other members of the cytochrome P-450 superfamily, consisting of 10 exons and spanning at least 70 kb (9). Furthermore, exon 1, encoding the only 5′ untranslated region, is separated from exon 2 by an intron of more than 35 kb. Recent studies demonstrated the presence of multiple exons 1 in several human genes such as aldolase A (11), insulin-like growth factor-II (12), glucokinase (13), parathyroid hormone-like peptide (14), prolactin (15), gonadotropin-releasing hormone (16), and glucocorticoid receptor (17). As analysis of genomic clones of human aromatase suggested that the aromatase gene exists in the human haploid genome as a single copy (9), the tissue-specific expression of this gene by alternative utilization of multiple exons 1 and promoters is an attractive idea to explain its complex regulation by various factors.

Recently, the aromatase mRNAs in the ovary and adipose stromal cells were shown to be transcribed from 79 and 84 bp upstream, respectively, of the exon 2 identified in placenta (18, 19). This finding indicates that the aromatase genes in the ovary and adipose stromal cells utilize a new exon 1 containing the placental exon 2 instead of the placental exon 1 and that their expressions are regulated by a new promoter, explaining in part the tissue-specific regulation of the human aromatase gene. Furthermore, a unique form of aromatase mRNA has been found in human skin fibroblasts and shown to be formed by alternative use of a different exon 1 from tissue-specific exons 1 (20).

In this study, to determine how many tissue-specific exons 1 of the human aromatase gene there are and how aromatase transcripts are spliced in various tissues, we isolated aromatase cDNAs from all available cDNA libraries of various human tissues in which the expression of aromatase has been reported.§ The results suggested that the human aromatase gene is expressed tissue specifically by alternative splicing of four major types of exons 1. We also detected tissue-specific expression of several minor forms of aromatase mRNA by alternative splicing, and we observed switching of a tissue-specific exon 1 in the adipose tissue of breast cancer patients.

MATERIALS AND METHODS

Materials. The cDNA libraries of human fetal liver (tissue from a 26-week-old male fetus), adult liver (from a normal woman), testis (from a healthy 50-year-old man), ovary (a pool of normal whole ovaries of four normal Caucasian women 15, 42, 42, and 46 years old), prostate (from a 25-year-old Caucasian), and brain amygdala (tissue from a disease-free 20-year-old Black man) and poly(A)$^+$ RNAs from human fetal liver (a 21-week-old fetus), adult liver (from an adult autopsied woman), ovary (a pool from four Cauca-

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†To whom reprint requests should be addressed.

§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. D21240 and D21241).

sian women), fetal brain (a pool of 20- to 26-week-old fetal brains), and adult brain (from a 15-year-old Caucasian woman) were purchased from Clontech. Human skin fibroblasts (primary culture from skin of the arms of four healthy Japanese men), prostate (the peripheral tissue of two Japanese prostate cancers), placenta (from four healthy Japanese women), and adipose tissue (from the abdomen or breast of Japanese) were obtained with informed consent. Poly(A)$^+$ RNAs from these tissues were prepared as described by Chirgwin *et al.* (21). The human placental aromatase cDNA, hA-24, was used as a probe for screening cDNA libraries. A Charon 4A genomic DNA library was kindly provided by C. Setoyama and K. Shimada (Kumamoto University). The oligonucleotide primers 1a (5′-CTGGAGGGCTGAACACGTGG-3′), 1a′ (5′-CCGAGCACAGGACCTTCCGTCC-3′), 1b (5′-GACCAACTGGAGCCTGACAG-3′), 1c (5′-CCTTGTTTTGACTTGTAACCA-3′), 1c′ (5′-TGTTTTATAAATGTGATCAGACATT-3′), 1d (5′-AACAGGAGCTATAGATGAAC-3′), 1d′ (5′-CTGTGGAAATCAAAGGGACA-3′), 2a (5′-GGTGTCAGAAACCCTGTG-3′), 2a′ (5′-GGATTTCCAAATGTTGCTTTG-3′), 2b (5′-CCTCTGAGGTCAAGGAACAC-3′), 2c (5′-GTGGCAGCAGGCATGGCTTC-3′), and 2d (5′-GTGCCCTCATAATTCCACAC-3′), and a hybridization probe F (5′-TACAGTACAGATTCACTTAC-3′) were synthesized (see Fig. 3). Probe F, specific for the region between exons 1c and 1d, was labeled with digoxigenin-ddUTP (dd = dideoxy) by terminal deoxynucleotidyltransferase (Boehringer Mannheim). Probes A, G, C, and D, specific for exons 1a, 2a, 1c, and 1d, were prepared by PCR amplification of isolated DNA fragments of the aromatase gene with primers specific for each exon, 1a and 1a′, 2a and 2a′, 1c and 1c′, and 1d and 1d′, respectively, in the presence of digoxigenin-dUTP.

Isolation of Aromatase cDNAs from Various Human Tissues. The cDNA libraries from various human tissues were screened with the *Eco*RI fragment (0.38 kb) of placental aromatase cDNA as a probe (22). The aromatase cDNAs from human skin fibroblasts and adipose tissue were isolated by the RACE (rapid amplification of the cDNA end) method as described (20). The DNA sequences of the isolated clones were determined by the dideoxynucleotide chain-termination method (23) with *Taq* DNA polymerase.

Isolation of Multiple Exons 1 of the Aromatase Gene from a Human Genomic Library. Multiple exons 1 of the aromatase gene were isolated from a human genomic library by using as probes DNA fragments corresponding to exon 1, which were amplified from the isolated clones by PCR. The isolated genomic clones were mapped on the aromatase gene by the method of Rackwitz *et al.* (24) and sequenced.

Analysis of Alternative Usage of Exons 1 in Aromatase mRNA from Various Human Tissues. The mRNA fractions from various human tissues were reverse-transcribed with reverse transcriptase (Rous-associated virus 2; Takara Shuzo, Kyoto) and a specific antisense primer 2d. The resulting cDNAs were amplified by PCR using primer 2d with primer 1a, 1b, 1c, or 1d for 20 cycles. After agarose gel electrophoresis, Southern blotting analysis of the PCR products was carried out by hybridization with a digoxigenin-labeled DNA fragment, which was prepared from aromatase cDNA by PCR amplification using primers 2b and 2c in the presence of digoxigenin-dUTP. Hybridized bands were visualized with anti-digoxigenin antibody–alkaline phosphatase conjugate and a luminescent reagent, 3-(2′-spiroadamantane)-4-methoxy-4-(3′′-phosphoryloxy)phenyl-1,2-dioxetan (AMPPD; Boehringer Mannheim), according to the manufacturer's instructions.

RESULTS

Isolation of Aromatase cDNAs with Unique DNA Sequences on the 5′ Side from Various Human Tissues. Extensive screening of cDNA libraries from various tissues revealed the presence of four kinds of unique DNA sequences on the 5′ side of aromatase cDNAs, as shown in Fig. 1. All the isolated clones had the same DNA sequences in the region encoded by exons 2–10 of the aromatase gene, but had one of four kinds of unique DNA sequence on the 5′-side region corresponding to exon 1. Furthermore, the sequence observed in the clones depended on the tissue source of the library, indicating tissue-specific expression of the four kinds of aromatase mRNA. Thus, the aromatase mRNAs in various tissues were tentatively classified into four types: prostate-specific mRNA, ovary-specific mRNA, fetal liver/skin fibroblast-specific mRNA, and placenta-specific mRNA. According to this classification, the brain amygdala and adipose tissue mainly express low levels of the fetal liver/skin fibroblast-specific aromatase mRNA species and the ovary expresses the prostate-specific mRNA as well as the ovary-specific mRNA. The placenta also expresses a minor species of aromatase mRNA similar in structure to the placenta-specific mRNA but with an insertion of 109 bases.

Isolation of Alternative Exons 1 from a Human Genomic Library. A human genomic library was screened for the unique sequences found on the 5′ side of aromatase cDNA. As shown in Fig. 2, all the isolated genomic clones were mapped upstream of exon 2 of the aromatase gene, indicating that the unique sequences found in the aromatase cDNAs were due to alternative splicing of the multiple exons 1. The placenta-specific exon 1 (exon 1a) and the fetal liver/skin fibroblast-specific exon 1 (exon 1b) were located more than 35 and 17 kb, respectively, upstream of placental exon 2, whereas the ovary-specific exon 1 (exon 1c) was located within 305 bp upstream of the placental exon 2. Exon 1c was separated by 23 bp from exon 1d and by 102 bp of an intron from placental exon 2 (Fig. 2). Prostate-specific exon 1 (exon 1d) was formed by transcription initiated 79 bp upstream of

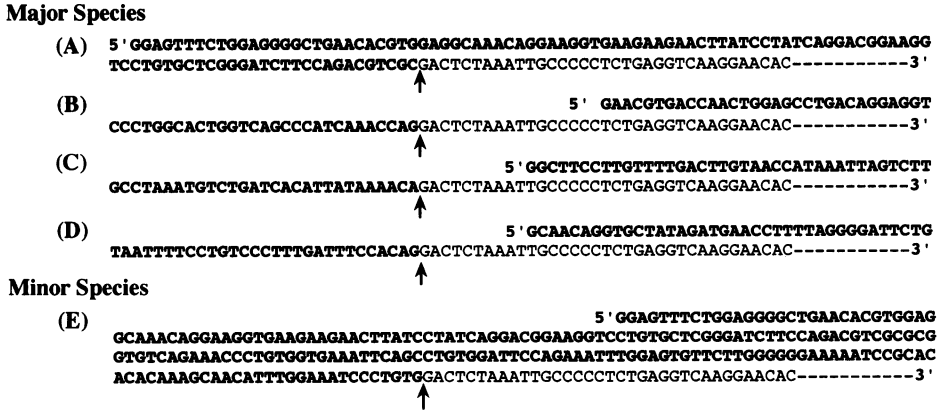

FIG. 1. DNA sequences in the 5′ regions of aromatase cDNA isolated from various human tissues. Four major species (A–D) and one minor species (E) of aromatase cDNAs were isolated from the placenta (A and E), fetal liver (B), skin fibroblasts (B), brain amygdala (B), ovary (C), prostate (D), and testis (D). Arrows indicate 5′ boundaries of regions encoded by exon 2 of the gene. Boldface letters show unique DNA sequences in the regions encoded by exon 1.

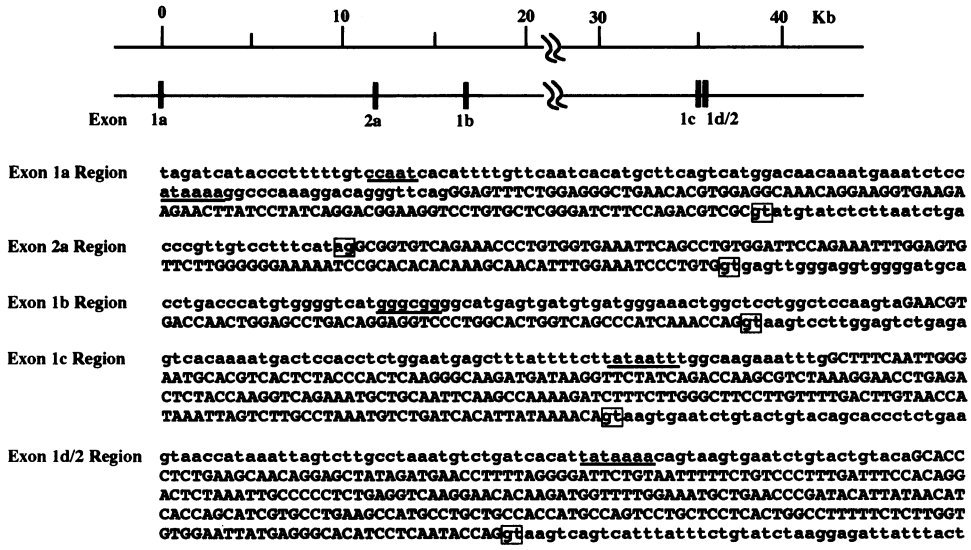

FIG. 2. Mapping of exons 1 or 2 of the aromatase gene containing unique sequences found in cDNAs and DNA sequences around the exons. Four exons 1 and an exon 2 were mapped. Capital letters in the DNA sequences show exons 1a, 1b, 1c, 1d, and 2a containing unique sequences in species A, B, C, D, and E, respectively, of aromatase cDNAs. Lowercase letters in the DNA sequences indicate regions of promoters or introns. As exon 1d contains exon 2 for exons 1a, 1b, and 1c, it is shown as exon 1d/2. Typical TATAA, CAAT, and GC boxes in the promoter regions are underlined. The GT/AG consensus sequences for splice junctions are boxed.

the placental exon 2 to the 3′ end of exon 2 without splicing at the junction of the placental intron 1 and exon 2. So, in the prostate, aromatase mRNA is encoded by 9 exons of the aromatase gene. On the other hand, the 109-bp sequence observed in the minor species of placental aromatase mRNA was located 23 kb upstream of placental exon 2 and the adjacent sequences at the two ends were AG at the 5′ end and GT at the 3′ end, corresponding to splicing consensus sequences. These results indicate that this region functions as a new exon 2 for the minor species of placental aromatase mRNA and, consequently, that the mRNA is formed from 11 exons of the aromatase gene. Each exon 1 found was associated with the splicing donor sequence GT at the 3′ end according to the canonical GT/AG rule and with promoter motifs in the upstream region. Exons 1a, 1c, and 1d, specific for the placenta, ovary, and prostate, each have a typical TATA box in their 20- to 30-bp upstream region, whereas exon 1b, specific for fetal liver/skin fibroblasts, has a typical GC box in the upstream region.

Analysis of Alternative Usage of Multiple Exons 1 in Aromatase Transcripts of Various Tissues. For determination of whether aromatase transcripts are tissue-specifically spliced by alternative usage of multiple exons 1, RNA fractions from various tissues were reverse-transcribed and analyzed by PCR using one of the exon 1-specific primers, 1a–1d, and an exon 2-specific antisense primer, 2d. The major PCR products were formed tissue specifically as shown in Fig. 3*A*, suggesting alternative usage of multiple exons 1 in various

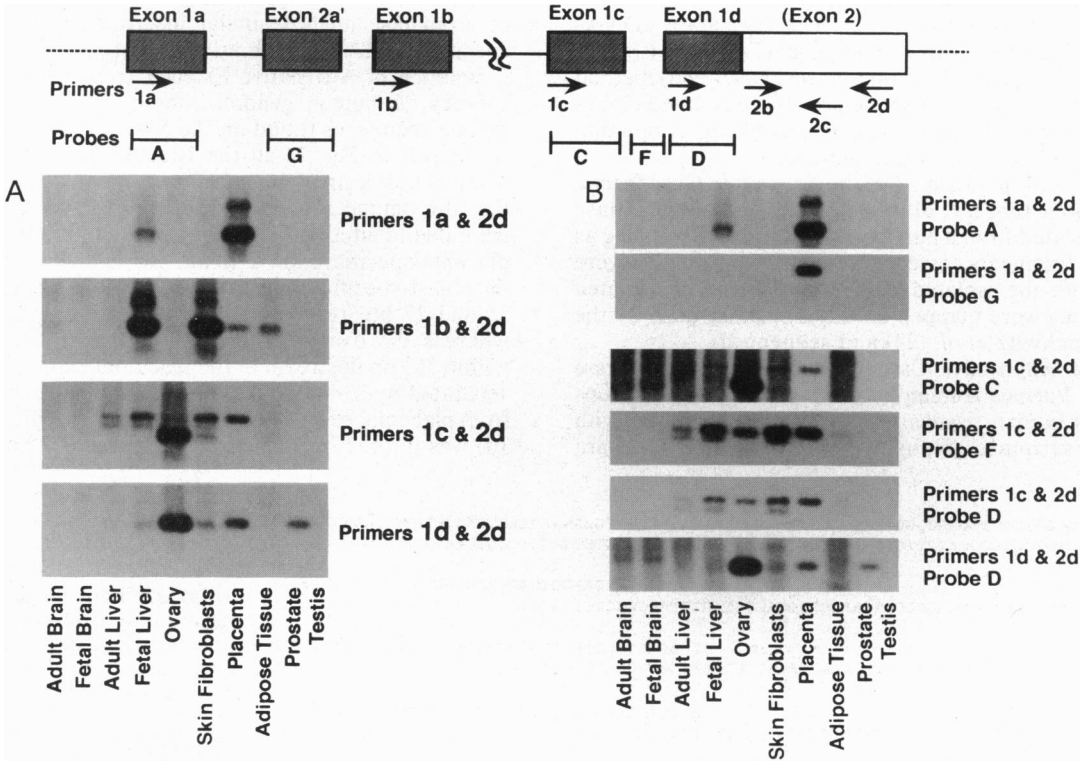

FIG. 3. Southern analysis of tissue-specific use of alternative exons 1 in various human tissues. Poly(A)$^+$ RNA fractions (3 ng of placental RNA for PCR using primers 1a and 2d, and 3 μg in other cases) from various tissues were subjected to reverse transcription and PCR using primer 2d and 1a, 1b, 1c, or 1d. (*A*) The PCR products were analyzed by hybridization with a digoxigenin-labeled DNA fragment which had been prepared from exon 2 by PCR using primers 2b and 2c in the presence of digoxigenin-dUTP. (*B*) Digoxigenin-labeled probes A, G, C, F, and D were used for Southern analysis of the PCR products.

tissues and tissue-specific splicing. Apparently, exons 1a, 1b, 1c, and 1d were mainly used for the aromatase mRNAs of placenta, skin fibroblasts and fetal liver, ovary, and ovary and prostate, respectively. Aromatase mRNAs in adult brain and adipose tissue were transcribed from exon 1b, although their expression levels were low. A major transcript using exon 1b and a minor transcript using exon 1c were observed in fetal liver, whereas in adult liver, the major transcript using exon 1b had completely disappeared and only the minor transcript using exon 1c was observed. The transcript in the ovary seems to use exons 1c and 1d equally for alternative splicing. Leaky levels of minor transcripts were also found in fetal liver, skin fibroblasts, and placenta. These results are summarized in Table 1.

Interestingly, the PCR product derived from RNA in the ovary was smaller than PCR products derived from other tissues. Further characterization was carried out with three specific probes, C, F, and D, as shown in Fig. 3*B*. The PCR using primers 1c and 2d gave an intense band for the ovary and weak bands for other tissues on Southern blotting analysis with probe C, specific for exon 1c. Furthermore, the sizes of the PCR products of the ovary and other tissues were distinctly different. However, the same PCR products gave bands of almost identical sizes on analysis with probes F and D, which are specific for the region between exons 1c and 1d and exon 1d, respectively, and the intensity of the band from the ovary was greatly weakened to a level comparable to bands from other tissues. On the other hand, the PCR using primers 1d and 2d gave intense bands for the ovary on analysis with probe D, whereas the PCR using primers 1c and 2d gave weaker bands. These results strongly suggest that aromatase mRNA in the ovary is present as two major species, one being spliced alternatively between exons 1c and 1d and the other being transcribed from exon 1d. Probably, the transcriptions of aromatase mRNA in other tissues as well as the minor species in the ovary are initiated from exon 1c to form a new long exon 1 containing exons 1c, 1d, and 2, without splicing of exon 1c or 1d. The aromatase transcripts in the prostate were amplified by PCR using only primers 1d and 2d with probe D for detection. These results indicated that transcription of aromatase in the prostate is initiated from exon 1d. Primer extension analysis was performed to confirm transcriptional initiation from exons 1c and 1d in the ovary (data not shown). At least three kinds of aromatase mRNAs were found in the ovary, their transcriptions being initiated from positions 79 and 261 bp upstream from the 3′ end of placental exon 2.

Two products of different sizes were obtained when placental RNA was amplified by the PCR using primers 1a and 2d, as shown in Fig. 3. Both products were detected by hybridization with probe A specific for exon 1a, whereas only the larger product was detected with probe G, specific for exon 2a, as shown in Fig. 3*B*. These results indicate that the larger product corresponds to the smaller one with an extra exon 2a insert and is the same as the minor species of placental aromatase mRNA (Fig. 1).

Table 1. Tissue-specific utilization of alternative exons 1 of the aromatase gene

Tissue	Relative utilization			
	Exon 1a	Exon 1b	Exon 1c	Exon 1d
Brain	−	+	−	−
Fetal liver	+	+++	+	+
Adult liver	−	−	+	±
Ovary	−	−	+++	+++
Skin fibroblasts	−	+++	+	+
Placenta	++++	+	+	+
Adipose tissue	−	+	±	−
Prostate	−	−	−	+
Testis	−	−	−	±

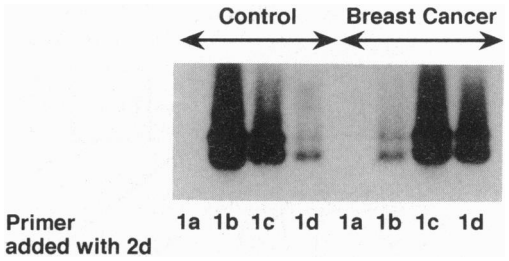

FIG. 4. Southern analysis of switching of tissue-specific exons 1 in breast adipose tissue. RNA fractions from adipose tissues of one control and one breast cancer patient were analyzed by the same procedures as for Fig. 3*A*.

Switching of an Adipose-Specific Exon 1b to Exons 1c and 1d in Adipose Tissues from Breast Cancer Patients. Breast adipose tissues in breast cancer patients often show elevated expressions of aromatase. Therefore we examined the alternative use of exons 1 for aromatase transcripts in adipose tissues from healthy controls and breast cancer patients with increased aromatase mRNA (Fig. 4). Aromatase transcripts from healthy controls showed tissue-specific use of exon 1b, as seen in Figs. 3 and 4, whereas those from three of five breast cancer patients examined showed a switch from exon 1b to exons 1c and 1d.

DISCUSSION

Aromatase is known to be widely distributed in extragonadal as well as gonadal tissues and to take part in various important functions through estrogen biosynthesis. For its special roles in various tissues, its tissue-specific expression is regulated by various factors. Recently, aromatase was suggested to have multiple promoters attached to multiple exons 1 and to use tissue-specific exons 1 and promoters by alternative splicing in the placenta, ovary, and adipose tissues (19, 25). In the present study, we isolated four major species of aromatase cDNA from cDNA libraries of various human tissues and found that their mRNAs are expressed tissue specifically and differ only in the portions encoded by exon 1 of the gene. Furthermore, the DNA sequences of these portions were found in the upstream region of exon 2 of the aromatase gene. These results suggest that aromatase mRNA is expressed by alternative splicing using a tissue-specific exon 1. Similar alternative splicing from multiple exons 1 and promoters has been shown for the transcripts of several genes (11–17), and so this tissue-specific expression by alternative use of multiple exons 1 and promoters is not unique to the aromatase gene.

Since the original isolation of an exon 1 used for placental mRNA (8–10), three other exons 1 have been obtained from mRNAs of placenta (26), adipose stromal cells (18), ovary (19), and skin fibroblasts (20). The exon 1a in this study is identical in its sequence and transcriptional start site to placental exon 1 (9, 10) or exon I.1 (8) isolated previously. Although another exon 1, exon I.2, used for placental mRNA was found in a minor species of aromatase mRNA (26), we could not isolate it in the present study. Thus, at least five different exons 1 of the human aromatase gene are now known, as shown in Fig. 5. Ovary and adipose stromal cells are reported to utilize an exon (18, 19) consisting of placental exon 2 and its 79- or 84-bp upstream region, as a new exon 1. This exon utilized in the ovary (18) has the same sequence and transcriptional initiation site as exon 1d, which was confirmed to initiate the transcription 79 bp upstream from placental exon 2 by primer extension experiments (data not shown). However, we could not identify exon 1d transcribed from 84 bp upstream of placental exon 2 in the ovary,

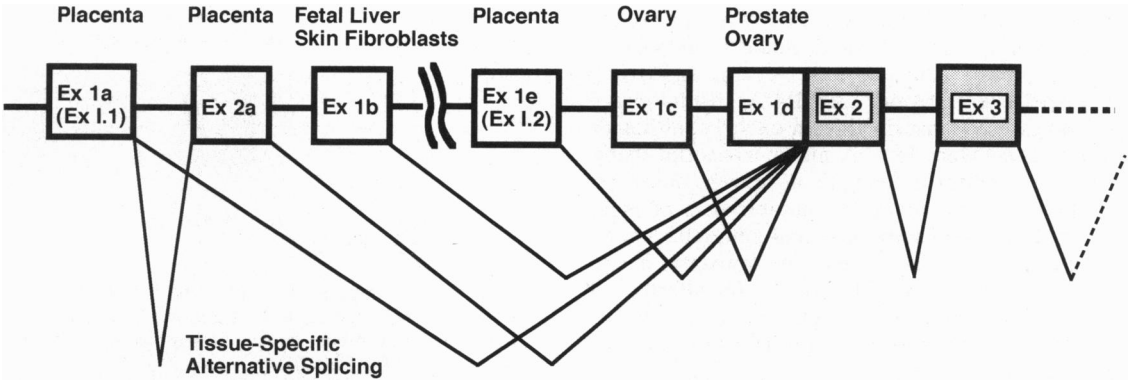

FIG. 5. Schema of alternative utilization of tissue-specific exons 1 and promoters of the human aromatase gene. Exon I.1 and exon I.2 in the figure are those of Simpson *et al.* (25).

prostate, or testis as in adipose stromal cells. Previous findings are inconsistent with the present finding of use of both exons 1c and 1d in the ovary and exon 1b in adipose tissue. As aromatase in the ovary is mainly localized in granulosa cells before ovulation and in lutein cells after ovulation, and its responses to various regulatory factors seem to change during cell differentiation in the ovarian sex cycle (7), exon 1 of the aromatase gene used may change from one type to another during the ovarian sex cycle. In this study we used pooled aromatase mRNA from the ovaries of four women, and so our results may indicate the overall usage of exons 1 in different stages. Similarly, differences between growth conditions or cell types of adipose stromal cells in primary culture and adipose tissue *in vivo* may result in switch from one type of exon 1 to another. In fact, we observed developmental change of alternative exons 1 in the liver, and a similar switch in adipose tissues from healthy women to that in three of five patients with breast cancer showing increased aromatase mRNA. Aromatase is an important etiological factor in some human breast cancers by inducing overproduction of estrogens as growth factors, resulting in proliferation of hormone-dependent cancer cells. In menopausal women with breast cancer, breast adipose tissue is a major source of estrogens, and aromatase in breast adipose tissue seems to have a significant association with the presence of tumors (27–29). From this finding, we postulate that the regulation of aromatase in breast adipose tissue becomes abnormal during carcinogenesis due to switching of exons 1 and promoters, resulting in overproduction of estrogens under control of a new promoter. For confirmation of this hypothesis of switching of exons 1 in breast cancer, it is essential to examine more clinical cases of breast cancer and analyze the mechanisms regulating transcription from each of the five exons 1.

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