

used as a vehicle control. Each assay was performed in duplicate and repeated at least three times.

RESULTS

Confirmation and Classification of Alternative Splicings of *ERRγ* Gene—To date, three independent investigations have revealed six alternative splicing sites for the human *ERRγ* mRNA gene and eight different *ERRγ* mRNA variants (13–15). However, there is no systematic study to unify these results, and thus it is unclear whether or not all of these variants are present simultaneously in one species, such as humans. Thus, we first attempted to confirm the full-length sequences of cDNAs derived from the eight *ERRγ* mRNA splicing variants.

To amplify each *ERRγ* mRNA isoform—*ERRγ1*, *ERRγ2*, *ERRγ2-gig* and *ERRγ3*—PCR was conducted successfully by using a series of forward sense and reverse antisense primer sets was designed for each isoform (Table 1) and commercially available pancreas and skeletal muscle cDNAs. As a result, seven of the eight splice variants reported were definitely identified, but we could not identify *ERRγ3* in this study. Although we carefully searched many other human tissues, the mRNA corresponding to *ERRγ3* was not detected. Instead, we identified another novel variant *ERRγ2-bcd* (accession number AB362218). It should be noted that these variants have variable nucleotide sequences of the 5'-UTR, with no structural changes of *ERRγ2* protein.

We now know nine splicing variants in total. It should be noted, however, that these variants afford or produce three distinctly different protein isoforms (Fig. 1), as the variants are classified into three mRNA isoforms: *ERRγ1*, *ERRγ2* and *ERRγ3*. Here, type-2 mRNA *ERRγ2* consists of seven subclasses of splicing variants: *ERRγ2-df*, *ERRγ2-def*, *ERRγ2-di*, *ERRγ2-d*, *ERRγ2-ad*, *ERRγ2-bcd* and *ERRγ2-gig* (Fig. 1C), although all of

these variants produce the same receptor protein molecule of *ERRγ2*. The variants are constituted from 15 distinct exons, A–O, coded in the human genomic DNA in the very broad region of chromosome 1 (about 1,000 kbp) (Fig. 1B). The heterogeneity at the 5'-UTR is due to the presence of alternative transcription start sites and alternative splicing sites.

The type-1 protein isoform *ERRγ1* has an additional 23-mer elongated N-terminal sequence. Type-2 mRNA genes, *ERRγ2* isoforms, include six variants containing the exon D-based fragment (designated *d*) in the 5'UTR. *ERRγ2-ad*, where *ad* indicates that the exons *a* and *d* are involved in this order, was first isolated from the human fetal brain library by Eudy *et al.* (13). On the other hand, *ERRγ2-gig* has been found only in the skeletal muscle cDNA library (14). The mRNA *ERRγ3-bcf* gene producing *ERRγ3* has recently been reported by Kojo *et al.* (15), although we could not identify this gene in the present study. The *ERRγ3* protein isoform has a deletion of 39-mer amino-acid residues in the DNA-binding domain of *ERRγ2*, resulting in an incomplete construction of the DNA binding site. In addition, it was found that *ERRγ* mRNAs each have two alternative polyadenylation isoforms (13).

Quantitative Analysis of *ERRγ* mRNA Genes as a Whole by Real-Time PCR—By means of real-time PCR, the total expression amount of the human *ERRγ* mRNA genes was estimated to amplify the region common to all the splicing variants. We designated the *hERRγ-whole* mRNA segment. We did confirm that there is no contamination of the genomic DNA in these RNA samples, since we could not amplify any cDNA when we used the primer sets directly for the samples.

To analyse the *hERRγ-whole* mRNA gene by real-time PCR, the primer set of sense *hERRγwholeF* and antisense *hERRγwholeR* was utilized (Table 2). For the quantification of each *ERRγ* splicing variant, real-time PCR was carried out by using a series of primer sets

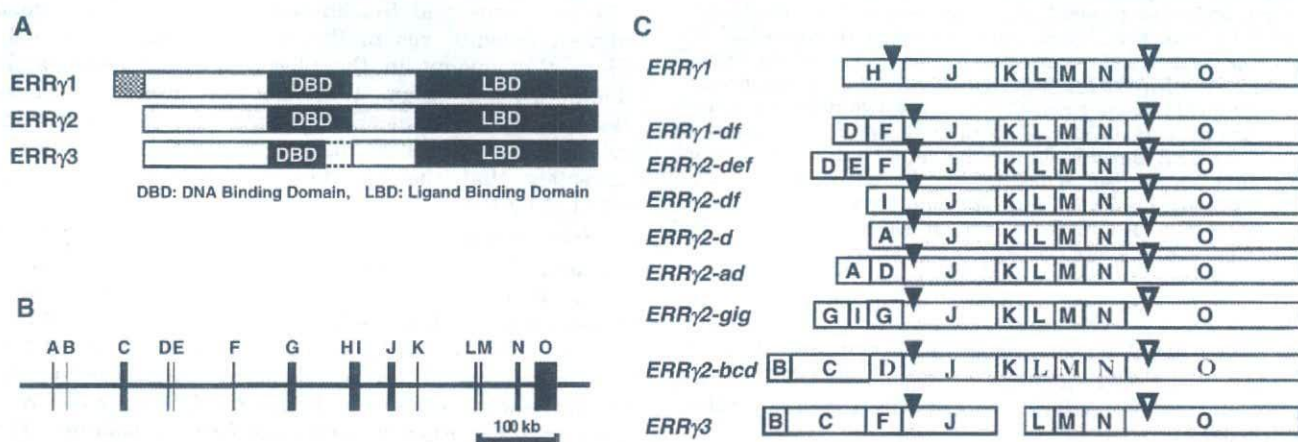


Fig. 1. Structural constitution of *ERRγ* mRNA isoforms and *ERRγ* protein isoforms. (A) Structure of *ERRγ* protein isoforms. *ERRγ1* has 23-mer amino-acid extension at the N-terminus of ordinary *ERRγ*, namely *ERRγ2*. *ERRγ3* has a 39-mer amino-acid deletion in the DNA binding domain. (B) Structural constitution of exon and intron of human

ERRγ genomic gene. Alphabetic letters A–O each indicate an independent exon. (C) Structural constitution of exons in nine *ERRγ* mRNA isoforms. Closed arrowheads indicate the position of an AUG initiation codon, and open arrowheads indicate the position of a termination codon in the *ERRγ* open reading frame.

Table 2. The nucleotide sequences of the primers used for the quantification of whole *ERR γ* mRNA and each *ERR γ* mRNA isoform.

Name of primers	Oligonucleotide sequences	Length of products (bp)
Primers for quantification of <i>ERRγ-whole</i> mRNA		
h <i>ERRγ</i> wholeF	5'-CAGAATGTCAAACAAAGATCGACAC-3'	148
h <i>ERRγ</i> wholeR ^a	5'-GGTTGAACTGTAGCTCCCCTG-3'	
Primers for quantification of <i>ERRγ1</i> mRNA		
h <i>ERRγ1</i> F	5'-GCACATGGATTCGGTAGAACTTTG-3'	215
h <i>ERRγ1</i> R ^a	5'-GGTTGAACTGTAGCTCCCCTG-3'	
Primers for quantification of <i>ERRγ2</i> mRNA		
h <i>ERRγ2</i> F	5'-TACGCTAACACTGTCGAGTTTG-3'	183, 300, 338, 359 ^b
h <i>ERRγ2</i> R ^a	5'-GGTTGAACTGTAGCTCCCCTG-3'	
Primers for quantification of <i>ERRγ2-gig</i> mRNA		
h <i>ERRγ2-gig</i> F	5'-GCCACCACATCTCGATTCAAAG-3'	339
h <i>ERRγ2-gig</i> R ^a	5'-GGTTGAACTGTAGCTCCCCTG-3'	
Primers for quantification of <i>ERRγ2-bcd</i> mRNA		
h <i>ERRγ2-bcd</i> F	5'-GATGTTGCTACACGGTCCCTTAC-3'	208
h <i>ERRγ2-bcd</i> R	5'-TGATCTTCTGCAAAGACCTACTTC-3'	
Primers for quantification of <i>gapdh</i> mRNA		
hg <i>apdh</i> F	5'-CAGCAAGAGCACAAGAGGAAGA-3'	107
hg <i>apdh</i> R	5'-GTCTACATGGCAACTGTGAGGAG-3'	

^aThese antisense primers have the same nucleotide sequence. ^bThe number of products depends on the number of alternative splicing sites in the particular region amplified.

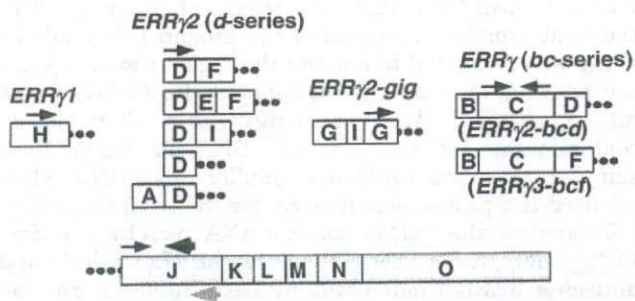


Fig. 2. Design strategy of forward sense and reverse antisense primers for real-time PCR quantification of *ERR γ* mRNA isoforms. Rightwards arrows indicate the forward sense primers, while leftwards arrows indicate the reverse antisense primers. Sense primers are specific to each mRNA isoform. For *ERR γ 2* containing exon D, *ERR γ 2-df*, *def*, *di*, *d*, *ad* and *bcd* the same sense primer was set in exon D (Table 2). For *ERR γ 2-bcd* isoforms, sense and antisense primers were set in the same exon, C. There are two different reverse antisense primers in exon J, besides the antisense primer in exon C for *ERR γ 2-bcd* isoforms. The large black leftward arrow is a universal antisense primer for the quantification of all isoforms except for *ERR γ 2-bcd*. The large grey leftward arrow is an *ERR γ* -specific antisense oligonucleotide for the reverse transcription to prepare cDNAs.

(Fig. 2, Table 2). These primers were designed not to amplify the *ERR γ* sequence on the genomic DNA, setting both sense and antisense primers in the independent exon region.

Real-time PCR was carried out at least three times for the cDNAs prepared from each total RNA sample of human adult kidney, placenta, ovary, uterus, prostate, testis and brain, as well as from the human fetal brain. We determined the number of molecules of the whole mRNA and respective mRNA isoforms, with

1×10^5 molecules of *gapdh* mRNA being the internal standard. For example, the molecular number of the *hERR γ -whole* mRNA of adult brain was 942 per 1×10^5 *gapdh* mRNA (Table 3). The fetal brain had 739 molecules (about 80% of the molecular number of adult brain).

In kidney, *hERR γ -whole* mRNA had a molecular number of 4,369, approximately 4.6-fold that of the adult brain (Table 3). Among the reproductive organ tissues, the placenta was found to express the highest number of *hERR γ -whole* mRNA molecule, 4,544, ~5% greater than that of kidney and thus about 5-fold that of the adult brain. This very high expression of *hERR γ* mRNA genes in the placenta was of course found to exceed those of other reproductive organs: 151 in ovary, 167 in uterus and 571 in testis (Fig. 3). The second highest amount was in the prostate (1,637) (Table 3). Thus, the amount in the placenta was approximately 3-fold greater than that in the prostate. These results suggest that *ERR γ* plays a very significant role in the placental functions. This may indicate adversely that the placenta is potentially the most affected by BPA.

Tissue Distribution Analysis of *ERR γ* mRNA Splicing Variants by Real-Time PCR—After real-time PCR to measure the *ERR γ -whole* mRNAs, we measured the ratio of each *ERR γ* mRNA isoform—*ERR γ 1*, *ERR γ 2*, *ERR γ 2-gig* and *ERR γ 3*—in the tissues. The amount of *ERR γ 2-gig* needs to be estimated separately, because the forward sense primer specific for *ERR γ 2-gig* is different from that for other *d*-containing *ERR γ 2* isoforms. The approximate ratio of each mRNA isoform was calculated against the total number of copies of all mRNA isoforms. As a result, we could calculate the ratio of each *ERR γ* mRNA isoform among the total amount of *ERR γ -whole* mRNA (Table 3 and Fig. 4). Detailed data for these analyses are shown in the Table 4, in which donor

Table 3. The results of real-time PCR quantification of *ERRγ*-whole mRNA and its subtypes in human tissues.

Tissues	<i>ERRγ</i> -whole mRNA ^a	<i>ERRγ</i> mRNA isoforms (%)			
		Type-1	Type-2		
			<i>d</i> -series	<i>gig</i>	<i>bcd</i> ^b
Brain (adult)	942 ± 24	26.1 ± 9.5	73.5 ± 9.8	0.3 ± 0.2	0.1 ± 0.1
Brain (fetal)	739 ± 195	30.2 ± 11.6	68.8 ± 11.1	0.8 ± 0.6	0.2 ± 0.2
Kidney	4369 ± 1276	11.1 ± 2.8	88.4 ± 3.1	0.3 ± 0.2	0.2 ± 0.1
Pancreas	2742 ± 798	4.0 ± 0.3	94.7 ± 1.2	0.7 ± 0.7	0.6 ± 0.5
Skeletal muscle	247 ± 48	45.2 ± 18.3	7.1 ± 1.0	47.7 ± 18.1	0.0 ± 0.0
Placenta	4544 ± 1572	98.9 ± 0.7	1.1 ± 0.7	0.0 ± 0.0	0.0 ± 0.0
Prostate	1637 ± 217	20.2 ± 2.7	78.2 ± 3.4	1.4 ± 0.6	0.2 ± 0.2
Testis	571 ± 93	23.6 ± 1.9	63.8 ± 1.6	11.0 ± 0.4	1.6 ± 1.1
Ovary	151 ± 50	18.0 ± 4.1	76.2 ± 8.1	3.5 ± 3.5	2.3 ± 2.2
Uterus	167 ± 127	4.3 ± 2.3	93.1 ± 3.7	2.6 ± 2.6	0.0 ± 0.0

^aThe amount of mRNA was calculated as the number of molecules per 1.0×10^5 *gapdh* mRNA molecules. ^bThe analysis with specific sense and antisense primers, both of which were set in the same exon C (Fig. 2) was originally designed to measure the total amount of *c*-containing *ERR* mRNAs including *ERRγ2-bcd* and *ERRγ3-bcf*. Since the amount of *ERRγ3-bcf* that gives *ERRγ3* was negligible, the measurement gave the amount of only *ERRγ2-bcd* that affords *ERRγ2*.

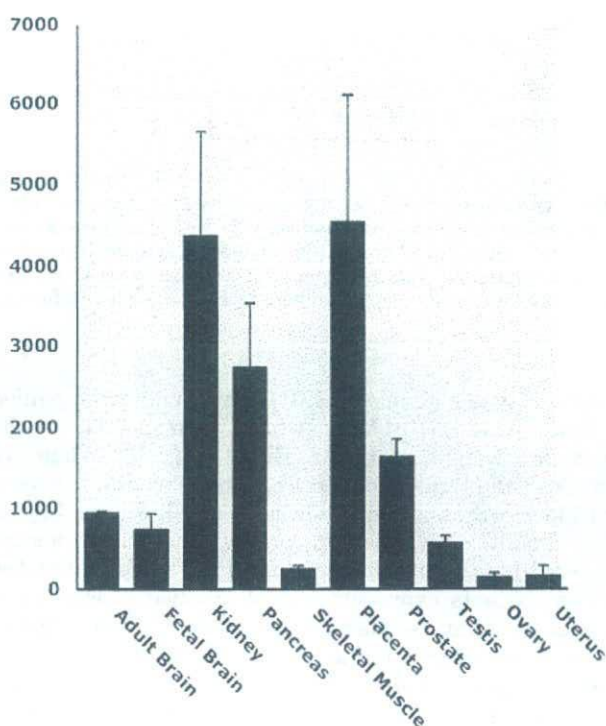


Fig. 3. Quantitative real-time PCR for estimation of *ERRγ* mRNA expression in human brains and reproductive organs. The *gapdh* mRNA gene was used as an internal control. The copy number per 1×10^5 *gapdh* mRNA was estimated for *ERRγ*-whole mRNA in each tissue. The error bars indicate SEM.

information such as age and sex is given for samples provided from each agent.

It should be noted that in the placenta, the *ERRγ1* mRNA isoform is accounted for 98.9% of the quantity of all mRNA isoforms (Table 3). This predominance of the type-1 mRNA isoform is very surprising and unique among the human tissues. All other human tissues

express *ERRγ2* as a major mRNA isoform. It is likely that *ERRγ1* produced from the *ERRγ1* mRNA isoform may play a crucial and central role in the placenta.

Reproductive tissues other than placenta expressed the *ERRγ2* mRNA isoform much more than *ERRγ1*. The content of *ERRγ2* mRNA isoform was estimated as 79.6% in prostate, and 81.9% and 95.7% in ovary and uterus, respectively (Table 3). Since the expression level of *ERRγ2-gig* mRNA was almost negligible in these tissues (Table 4), estimated *ERRγ2* mRNA isoform includes exclusively six subclasses of splicing variants (*d*-series that contain: *ERRγ2-df*, *ERRγ2-def*, *ERRγ2-di*, *ERRγ2-d*, *ERRγ2-ad* and *ERRγ2-bcd*) (Fig. 1C). As for the testis, however, a prominent inconsistency was found. It contained about 11% of the *ERRγ2-gig* mRNA isoform. Furthermore, a discrepancy between the sum of all mRNA isoforms and the *ERRγ*-whole mRNA—the sum of total numbers of *ERRγ* mRNA isoform molecules was clearly smaller (by about 10%) than the total molecular number of *ERRγ*-whole mRNA (data not shown)—strongly suggested the presence of mRNA isoform(s) other than those measured.

The adult and fetal brains were found to have almost the same isoform constitutions (Table 3). The expression ratios of *ERRγ1* and *ERRγ2* mRNAs respectively were 30.2% and 69.8% in the fetal brain and 26.1% and 73.9% in the adult brain. Thus, brain is the tissue in which type-2 *ERRγ* mRNA is expressed predominantly. In kidney, the expression levels of *ERRγ1* and *ERRγ2* mRNAs were estimated to be approximately 11.1% and 88.9%, respectively. Pancreas was also one of the tissues in which type-2 *ERRγ* mRNA was expressed predominantly, 4.0% *ERRγ1* and 96.0% *ERRγ2*. The sum of *ERRγ1* and *ERRγ2* mRNAs occupied approximately 100% in these tissues.

The constitutional ratio of *ERRγ1*, *ERRγ2(d-series)* and *ERRγ2-gig* mRNAs in skeletal muscle was unique (Table 3). Their expression ratios were 45.2%, 7.1% and 47.7%, respectively. Skeletal muscle was found to be the tissue in which type-2 *ERRγ2-gig* mRNA is expressed very highly.

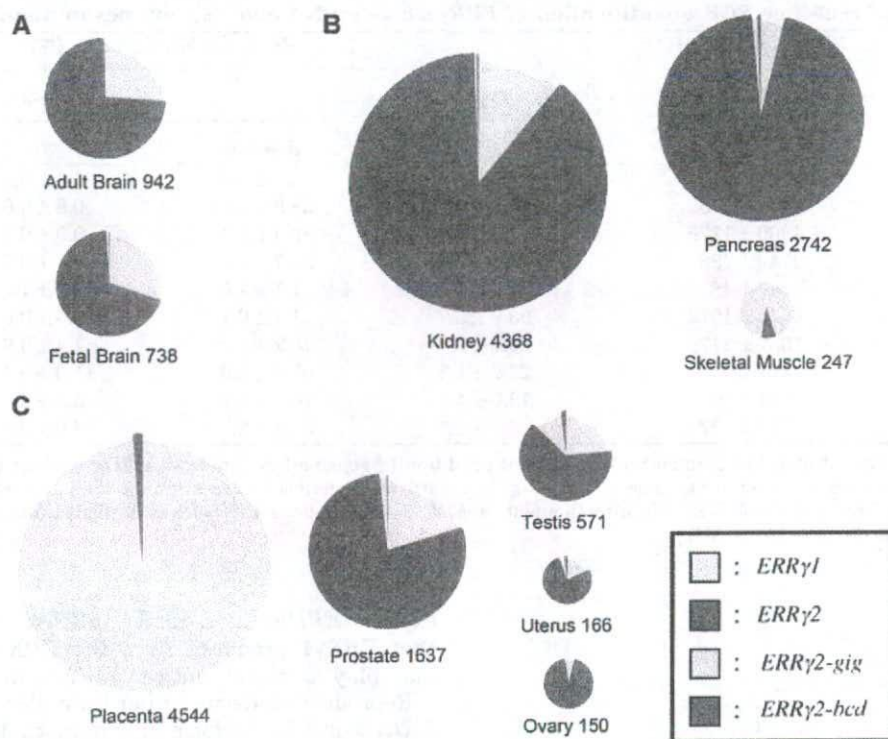


Fig. 4. Quantitative real-time PCR for estimation of *ERRγ*-whole mRNA expression and percentage of the constitutions of *ERRγ* mRNA isoforms in human brains (A), standard tissues (kidney, pancreas and skeletal muscle) (B) and reproductive organs (C). The circled area shows the total expression amount of *ERRγ*-whole mRNA in each

tissue. The area of each color region in the circle is proportional to the expression amount of the *ERRγ* mRNA isoforms. Blue, purple, yellow and red areas indicate *ERRγ1*, *ERRγ2*(*d*-series), *ERRγ2-gig* and *ERRγ2-bcd* mRNA isoforms, respectively. The expression rates of each *ERRγ* mRNA isoform were calculated against the sum of the copy numbers of *ERRγ* mRNA isoforms.

In the estimation of the tissue distributions of *ERRγ* mRNA splicing variants, another important issue is to calculate the actual amount of each mRNA isoform. As shown in Fig. 4, *ERRγ1* mRNA isoform was most abundant in placenta, the amount of this isoform ($4,544 \times 98.9\%$ as in Table 3 = 4,494 molecules) being exclusive and predominant. The kidney is the tissue in which the *ERRγ2* mRNA isoform is richest (3,884). Pancreas (2,632) and prostate (1,306) are also abounding in *ERRγ2*. As for the *ERRγ2-gig* mRNA isoform, skeletal muscle (118) and testis (63) contain it relatively highly.

Western Blotting Detection of *ERRγ* Protein Isoforms—To verify the amount of type-1 *ERRγ* protein isoform expressed in the placenta, Western blotting was carried out for the lysates of the human placenta and kidney. Kidney was selected as a reference because of its expected high expression (~90%) of the type-2 *ERRγ* protein isoform (see above). *ERRγ* protein isoforms were detected by using a monoclonal antibody specific for the N-terminal region (1–100) of *ERRγ2*, because this antibody can detect all three isoforms: *ERRγ1*, *ERRγ2* and *ERRγ3*. The calculated molecular weight (51,313) of type-1 *ERRγ* is larger than that of type-2 standard *ERRγ* by ~2,700, apparently due to the N-terminal addition of 23-mer amino-acid residues.

The lysate isolated from kidney exhibited an intense protein band of 49 kDa, corresponding to the molecular

weight of type-2 standard *ERRγ*. This band accompanied a faint band of 51 kDa, which corresponds to the molecular weight of type-1 *ERRγ* (Fig. 5). When we examined the lysate isolated from the placenta, the band of 51 kDa was markedly detected, as shown in Fig. 5. This result very clearly proves the consequences observed for the mRNA isoforms. It is evident that the mRNA variants expressed indeed produce a consequent protein and that the placenta expresses the type-1 *ERRγ* protein isoform predominantly and exclusively.

Transcription Activity of Type-1 and Type-2 *ERRγ* Isoforms in the Reporter Gene Assay—*ERRγ* is a constitutively active nuclear receptor that exhibits a high basal activity with no ligand. In the present study, we examined the reporter gene activity of type-1 and type-2 *ERRγ* isoforms by means of the luciferase reporter gene assay using HeLa cells. To normalize for transfection efficiency, we simultaneously carried out the SEAP assay (16). When the type-1 *ERRγ* isoform was compared with the type-2 standard *ERRγ* isoform, the constitutive activity level of type-1 *ERRγ* was found to be about 50% higher than that of type-2 *ERRγ*. As shown in Fig. 6, the type-2 *ERRγ* isoform exhibited significantly elevated constitutive activity (210% of the basal activity). Type-1 *ERRγ* isoform also exhibited considerably elevated constitutive activity (260%), a notably higher level than that of type-2. The results indicate that the N-terminal

Table 4. Donor information for quantification of $ERR\gamma$ -whole mRNA and its subtypes by quantitative real-time PCR.

Tissues	Agent sources ^a	Age	Sex ^b	Number of donors	$ERR\gamma$ -whole mRNA ^c	$ERR\gamma$ mRNA isoforms (%) ^d			
						Type-1	Type-2		
							<i>d</i> -series	<i>gig</i>	<i>bcd</i> ^e
Brain (adult)	C	47-55	M	2	933	45.0	54.0	0.7	0.3
	S	66	F	1	906	18.1	81.9	0.0	0.0
	B	30	M	1	988	15.2	84.7	0.1	0.0
Brain (fetal)	C	26-40 ^f	M/F	21	555	15.5	82.1	1.9	0.5
	S	19 ^f	M	1	1128	53.2	46.8	0.0	0.0
	B	28 ^f	F	1	533	21.9	77.4	0.6	0.1
Kidney	C	18-59	M/F	14	2540	16.5	82.4	0.7	0.4
	S	67	F	1	3742	7.2	92.7	0.0	0.1
	B	24	M	1	6824	9.7	90.2	0.0	0.1
Pancreas	C	35	M	1	4024	3.8	92.4	2.2	1.6
	S	72	M	1	1278	3.5	96.5	0.0	0.0
	B	44	M	1	2925	4.6	95.3	0.0	0.1
Skeletal muscle	C	20-68	M/F	7	152	18.1	8.7	73.1	0.1
	S	85	F	1	307	80.0	7.3	12.7	0.0
	B	87	F	1	283	37.3	5.4	57.3	0.0
Placenta	C	21-33	F	16	7538	99.6	0.3	0.1	0.0
	S	28	F	1	3880	99.5	0.5	0.0	0.0
	B	26	F	1	2214	97.6	2.4	0.0	0.0
Prostate	C	21-50	M	32	1420	22.8	74.8	2.0	0.4
	B	69	M	1	1854	17.5	81.6	0.9	0.0
Testis	C	24-64	M	39	693	21.7	62.9	11.7	3.7
	S	72	M	1	389	21.8	66.9	10.3	1.0
	B	27	M	1	632	27.3	61.7	11.0	0.0
Ovary	C	20-60	F	15	70	20.8	61.9	10.6	6.7
	S	49	F	1	139	10.0	90.0	0.0	0.0
	B	46	F	1	243	23.2	76.8	0.0	0.0
Uterus	C	40-61	F	3	70	4.9	87.3	7.8	0.0
	S	88	F	1	419	8.0	91.9	0.0	0.1
	B	49	F	1	11	0.0	100	0.0	0.0

^aThe samples of total RNA extracted from human tissues were purchased from Clontech (C), Stratagene (S) and BioChain (B). ^bM and F represent male and female, respectively. ^cThe amount of mRNA was calculated as the number of molecules per 1.0×10^5 *gapdh* mRNA molecules. ^dThe same antisense primer for *hERR\gamma*-whole mRNA was utilized for the quantification of *ERR\gamma1* and *ERR\gamma2* (Table 2). ^eThe real-time PCR quantification to measure *c*-containing *ERR* mRNAs was carried out. Since the amount of *ERR\gamma3-bcf* was negligible, this counting was found to be just for *ERR\gamma2-bcd* that affords *ERR\gamma2*. ^fThe age of fetal brain is shown by the number of weeks.



Fig. 5. Western blotting analyses of $ERR\gamma$ protein isoform expression levels. An intense protein band of 49 kDa in kidney lysate corresponds to the molecular weight of type-2 standard $ERR\gamma$. The accompanying faint band of 51 kDa corresponds to the molecular weight of type-1 $ERR\gamma$. A major strong band of 51 kDa in the placenta lysate corresponds also to this type-1 $ERR\gamma$. Other bands are supposed to be specific or non-specific protein bands derived from the first antibody.

23-mer elongation has a distinct effect on the reporter gene transactivation activity of $ERR\gamma$.

4-OHT deactivated the ordinary standard type-2 $ERR\gamma$, as reported (7, 17), diminishing the basal activity of $ERR\gamma$ by up to 60–80% at a concentration of $10 \mu\text{M}$ (Fig. 7A). These were exactly revealed for the type-1 $ERR\gamma$ isoform, $ERR\gamma1$. BPA, on the other hand, showed no effect on the basal constitutive activity of $ERR\gamma$ even at a concentration of $10 \mu\text{M}$, completely preserving $ERR\gamma$'s high constitutive activity (Fig. 7A). The inverse agonist activity of 4-OHT for $ERR\gamma1$ was reversed or inhibited by BPA in a dose-dependent manner (Fig 7B). This reversing activity of BPA, namely, inverse antagonist activity of BPA, was revealed originally for the type-2 $ERR\gamma$ isoform (7).

DISCUSSION

Extremely High Expression of $ERR\gamma$ mRNA in the Placenta—In the present study, using commercially available human gene samples of reproductive organ

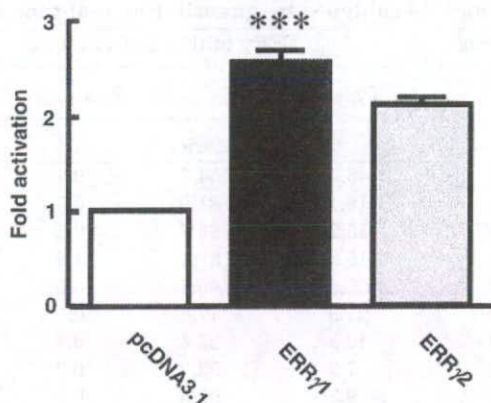


Fig. 6. Transcription activity of type-1 and type-2 $ERR\gamma$ isoforms in the reporter gene assay. The type-1 $ERR\gamma$ isoform is 23-mer larger than the type-2 ordinary $ERR\gamma$ isoform at the N-terminus. HeLa cells were transfected with the luciferase reporter gene ($3 \times ERRE$) and the expression plasmid of the wild-type of either type-1 or type-2 $ERR\gamma$. After 24 h, luciferase activity was measured. Cells treated with 1% BSA/PBS were used as a vehicle control. Each assay was performed in duplicate and repeated at least three times. To normalize the transfection efficiency, the SEAP assay, in which a second plasmid that constitutively expresses an activity that can be clearly differentiated from SEAP, was co-transfected simultaneously.

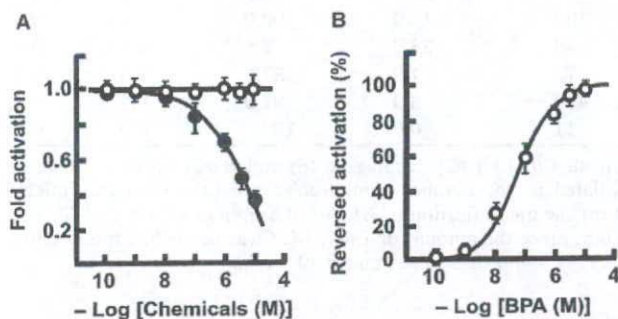


Fig. 7. The luciferase reporter gene assay of BPA and 4-hydroxytamoxifen (4-OHT) for type-1 $ERR\gamma$. (A) Deactivation of the fully activated human type-1 $ERR\gamma$ isoform by the inverse agonist 4-hydroxytamoxifen (4-OHT) (filled circle) and sustainment by the inverse antagonist BPA (open circle). (B) Inverse antagonist activity of BPA (open circle) against the inverse agonist activity of $1.0 \mu\text{M}$ 4-OHT in $ERR\gamma 1$. An amount of $1.0 \mu\text{M}$ 4-OHT exhibited ~ 0.4 -fold deactivation, and the inverse antagonist activities are shown by the percentage of relative activity to reverse this deactivation activity. The high basal constitutive activity of type-1 $ERR\gamma$ isoform was evaluated with the luciferase reporter plasmid ($pGL3/3 \times ERRE$), and the highest activity was estimated in a cell preparation of 1.0×10^6 HeLa cells/well.

tissues and brains, we succeeded in the tissue distribution analyses of the $ERR\gamma$ -whole mRNA gene and the constitutional ratios of a series of $ERR\gamma$ mRNA isoforms. Real-time PCR for whole- $ERR\gamma$ mRNA demonstrated an unexpectedly high expression of $ERR\gamma$ mRNA in the placenta (Fig. 3). Western blotting also confirmed the expression of $ERR\gamma$ protein (Fig. 5).

For accuracy in the quantification of $hERR\gamma$ -whole mRNA, we repeated the real-time PCR. By using a new set of sense and antisense primers set at the 3' terminal region, the results eventually obtained were almost the same as those of the first quantification shown in Fig. 3 (data not shown). For further confirmation, we tested internal controls other than *gapdh* mRNA. Those include the mRNA genes of human β -actin, ubiquitin C, *sdha* (succinate dehydrogenase complex, subunit A) and *hprt1* (hypoxanthine phosphoribosyl-transferase I). The results for the amount of $ERR\gamma$ mRNA were almost the same as those obtained by the quantification using *gapdh* mRNA (data not shown). These further evidenced that the $ERR\gamma$ mRNA expression in the human placenta is extreme, and the highest among the tissues examined.

Other reproductive organ tissues, such as ovary, uterus and testis, also express $ERR\gamma$ mRNA, but at very low levels: 3.3%, 3.7% and 12.6% that of the placenta, respectively. Compared to these tissues, the considerably high expression of $ERR\gamma$ mRNA in the prostate should be noted. The prostate had the second highest amount of $ERR\gamma$ mRNA, approximately 36% of that in the placenta.

Predominant Expression of Type-1 Isoform of $ERR\gamma$ mRNA in the Placenta—Nuclear receptors usually have several mRNA and protein isoforms by alternative splicing mechanisms, resulting in the exhibition of their functions in a tissue-specific or developmental stage-specific manner (10, 11). Unfortunately, there is little understanding not only of the physiological functions of splicing variants, especially *in vivo*, but also of their tissue distributions in the majority of tissues throughout the body.

All transcript $ERR\gamma$ mRNA variants consist of several distinct exons coded on human genomic DNA in the very broad region of chromosome 1 (about 1,000 kbp). As shown in Fig. 1B, the exons are thought to be distinguished between those in a variable region (A~I) and those in a consistent region (J~O). The latter includes almost all of the open reading frames of $ERR\gamma$, while 1–3 exons are selected from the variable region to form a 5'-UTR. Including alternative polyadenylation mechanisms, the gene expression of $ERR\gamma$ appears to be severely regulated in a post-transcriptional manner. The sequence difference of the 5'-UTR should bring about a different translational efficiency, depending on the stability of mRNA produced and the presence of some upstream open reading frames (uORF) (18).

Type-1 $ERR\gamma$ isoform ($ERR\gamma 1$) has an additional 23-mer amino-acid residue extension at the N-terminus, and exhibits about 50% increased basal constitutive activity relative to that of $ERR\gamma 2$ (Fig. 6). Although this isoform possesses exactly the same structure of its ligand-binding domain as other isoform types, its activation mechanism may differ from those of other isoforms by having this 23-mer N-terminal elongation. This might bring about unique tissue-specific function(s) in the placenta.

High Concentration of BPA in the Placenta due to High Expression of Type-1 $ERR\gamma$ —BPA is an industrial chemical, and exposure to it is now widespread (19, 20). We know now that BPA binds to $ERR\gamma$ about 100 times greater than to $ER\alpha$ and $ER\beta$, and that BPA exhibits a distinct inhibition activity against 4-OHT in $ERR\gamma$.

In the present study, we did know that there are three isoforms possessing precisely the same ligand-binding domain (LBD). It should be noted that BPA can bind to all these *ERR γ* isoforms in any tissue.

This strongly suggests that the concentration of BPA in human tissues is directly proportional to the amount of *ERR γ* proteins. In our estimation in the present study involving reproductive organ tissues, the expression of *ERR γ* mRNA is largest in the placenta and second largest in the prostate. The BPA concentration in the placenta has been reported to be approximately five times higher than that in maternal and fetal plasma (21). It is now quite reasonable to believe that the high concentration of BPA is due to the large amount of *ERR γ* protein isoforms, almost entirely type 1, in the human placenta.

The Effects of BPA Accumulation or Binding to Type-1 ERR γ on Placental Functions—The placenta receives nutrients, oxygen, antibodies and hormones from the mother's blood and removes waste. It forms the placental barrier, which filters out some substances that could harm the fetus. However, some substances, including BPA and viruses, are not filtered out, suggesting that the placenta does not act as a barrier against BPA (21, 22). In addition to transferring gases and nutrients, the placenta also has metabolic and endocrine activity. It produces estrogen, relaxin, and human chorionic gonadotropin, progesterone and somatomammotropin, all of which are important in maintaining pregnancy and the large amounts of glucose and lipids in the maternal blood. It is now evident that the placenta expresses BPA receptor *ERR γ* very highly. What would happen with the accumulation of BPA in the placenta?

Placentation in BPA-administered mice during pregnancy was reported to be abnormal (23), directly decreasing the number of embryos. In addition, almost all mouse neonates exposed to BPA were dead within 3 days after birth. Thus, BPA might disrupt the placental functions directly or indirectly, and might affect the mortality of neonates through indirect exposure of embryos. These are likely mediated through the BPA receptor *ERR γ* s, at least in part. BPA administration has also been reported to significantly increase the weight of the uterus and the number and fertilization quality of sperm (24). DNA microarray analysis has shown that BPA administration increases the mRNAs of some nuclear receptors in mouse placenta (25). These results suggest that BPA affects the transcriptional regulation in the placenta or other reproductive organs through certain particular transcription factors.

Based on the fact that BPA strongly binds to *ERR γ* , the abnormality and probable change of gene expression in the placenta are likely accompanied by BPA binding to *ERR γ* . In this study, we demonstrated that the *ERR γ 1* mRNA gene expresses almost fully in the placenta, and that the resulting type-1 *ERR γ* receptor is noticeably more potent than the resulting type-2 *ERR γ* receptor that expresses dominantly in other tissues. BPA would sustain unnecessarily this very high basal constitutional activity of type-1 *ERR γ* receptor in the placenta.

Other Human Tissues with High Expression of ERR γ mRNA—Adult and fetal brains, kidney and pancreas

were the tissues in which *ERR γ* expresses significantly and considerably highly. In these tissues, type-2 *ERR γ* mRNA is expressed predominantly, while the expression levels of *ERR γ 3* is almost negligible. The expression ratios of *ERR γ 1* and *ERR γ 2* mRNAs respectively varied 4–30% and 69–95%. As compared to the type-2 *ERR γ* isoform (*ERR γ 2*), type-1 isoform *ERR γ 1* has an additional 23-mer amino-acid elongation at the N-terminus. Although *ERR γ 1* exhibits about 50% increased basal constitutive activity relative to that of *ERR γ 2* (see above), physiological functions in these tissues have never been clarified nor analysed for both *ERR γ 1* and *ERR γ 2*.

As for the prostate, the mRNA gene was for the most part (~80%) *ERR γ 2*. Various effects of estrogenic chemicals including BPA have been reported for the prostate. For example, acceleration in the proliferation rate of prostate epithelium during fetal life was noted to disrupt permanently the cellular control systems and to predispose the prostate to disease in adulthood (26, 27). The effects of estrogens on the prostate, or the effects of their involvement in prostate cancer development and benign prostatic hyperplasia, are likely mediated through their ERs. In addition to the androgen receptor, which plays a central role in the normal development and neoplastic growth of the prostate gland, estrogens have long been suggested to play synergetic or distinct roles in the same processes. However, studies from ER knockout mouse models have shown neither ER α nor ER β affects the targeted disruption of prostatic phenotype and function (28). This strongly suggests the involvement of one or more nuclear receptors other than ER and that *ERR γ* is a probable candidate for involvement in prostatic growth and development. *ERR γ* might play regulatory roles in normal and neoplastic prostatic cells by sharing similar ER-mediated pathways or acting independently.

CONCLUSION

The present study provides a valuable blueprint of *ERR γ* mRNA expression and important clues to understanding BPA's low-dose effects in humans. For instance, although the issue of bioavailability of parent BPA in humans has been contentious, the present results strongly suggest that the BPA concentration is proportional to the expression amount of *ERR γ* . There are scientific debates over whether or not low doses of BPA can have developmental or reproductive effects in humans. Now, it is clear that *ERR γ* , the receptor of BPA, abounds in brains and reproductive tissues such as the placenta and prostate.

Strong expression of *ERR γ* as a possible receptor of BPA in both the placenta and the fetal brain could have important implications for newborns. *ERR γ* is also a probable candidate for involvement in prostatic growth and development. However, the physiological roles of *ERR γ* are poorly understood at the moment. It is thus important to clarify such physiological functions and characteristics of *ERR γ* . Moreover, it is crucial to examine the content and extent of which BPA may influence these roles.

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CONFLICT OF INTEREST

None declared.

REFERENCES

- Krishnan, A.V., Stathis, P., Permuth, S.F., Tokes, L., and Feldman, D. (1993) Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology* **132**, 2279–2286
- Olea, N., Pulgar, R., Perez, P., Olea-Serrano, F., Rivas, A., Novillo-Fertrell, A., Pedraza, V., Soto, A.M., and Sonnenschein, C. (1996) Estrogenicity of resin-based composites and sealants used in dentistry. *Environ. Health Perspect.* **104**, 298–305
- Sohoni, P. and Sumpter, J.P. (1998) Several environmental oestrogens are also anti-androgens. *J. Endocrinol.* **158**, 327–339
- Xu, L.C., Sun, H., Chen, J.F., Bian, Q., Qian, J., Song, L., and Wang, X.R. (2005) Evaluation of androgen receptor transcriptional activities of bisphenol A, octylphenol and nonylphenol *in vitro*. *Toxicology* **216**, 197–203
- Okada, H., Tokunaga, T., Liu, X., Takayanagi, S., Matsushima, A., and Shimohigashi, Y. (2008) Direct evidence revealing structural elements essential for the high binding ability of bisphenol A to human estrogen-related receptor- γ . *Environ. Health Perspect.* **116**, 32–38
- Liu, X., Matsushima, A., Okada, H., Tokunaga, T., Isozaki, K., and Shimohigashi, Y. (2007) Receptor binding characteristic of the endocrine disruptor bisphenol A for the human nuclear estrogen-related receptor γ . Chief and corroborative hydrogen bonds of the bisphenol A phenol-hydroxyl group with Arg316 and Glu275 residues. *FEBS J.* **274**, 6340–6351
- Takayanagi, S., Tokunaga, T., Liu, X., Okada, H., Matsushima, A., and Shimohigashi, Y. (2006) Endocrine disruptor bisphenol A strongly binds to human estrogen-related receptor γ (ERR γ) with high constitutive activity. *Toxicol. Lett.* **167**, 95–105
- Matsushima, A., Kakuta, Y., Teramoto, T., Koshiba, T., Liu, X., Okada, H., Tokunaga, T., Kawabata, S., Kimura, M., and Shimohigashi, Y. (2007) Structural evidence for endocrine disruptor bisphenol A binding to human nuclear receptor ERR γ . *J. Biochem.* **142**, 517–524
- Matsushima, A., Teramoto, T., Okada, H., Liu, X., Tokunaga, T., Kakuta, Y., and Shimohigashi, Y. (2008) ERR γ tethers strongly bisphenol A and 4- α -cumylphenol in an induced-fit manner. *Biochem. Biophys. Res. Commun.* **373**, 408–413
- Lu, N.Z. and Cidlowski, J.A. (2005) Translational regulatory mechanisms generate N-terminal glucocorticoid receptor isoforms with unique transcriptional target genes. *Mol. Cell* **18**, 331–342
- Wang, Z., Zhang, X., Shen, P., Loggie, B.W., Chang, Y., and Deuel, T.F. (2006) A variant of estrogen receptor- α , hER- α 36: Transduction of estrogen- and antiestrogen-dependent membrane-initiated mitogenic signaling. *Proc. Natl Acad. Sci. USA* **103**, 9063–9068
- NTP-CERHR Monograph on the potential human reproductive developmental effects of bisphenol A (NIH Publication No. 08–5994; September 2008).
- Eudy, J.D., Yao, S., Weston, M.D., Ma-Edmonds, M., Talmadge, C.B., Cheng, J.J., Kimberling, W.J., and Sumegi, J. (1998) Isolation of a gene encoding a novel member of the nuclear receptor superfamily from the critical region of Usher syndrom type IIa at 1q41. *Genomics* **50**, 382–384
- Heard, D.J., Norby, P.L., Holloway, J., and Vissing, H. (2000) Human ERR γ , a third member of the estrogen receptor-related receptor (ERR) subfamily of orphan nuclear receptors: tissue-specific isoforms are expressed during development and in the adult. *Mol. Endocrinol.* **14**, 382–392
- Kojo, H., Tajima, K., Fukagawa, M., Isogai, T., and Nishimura, S. (2006) A novel estrogen receptor-related protein γ splice variant lacking a DNA binding domain exon modulates transcriptional activity of a moderate range of nuclear receptors. *J. Steroid Biochem. Mol. Biol.* **98**, 181–192
- Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual* Cold Spring Harbor Laboratory Press, 3rd edn., Cold Spring Harbor, NY
- Coward, P., Lee, D., Hull, M.V., and Lehmann, J.M. (2001) 4-Hydroxytamoxifen binds to and deactivates the estrogen-related receptor gamma. *Proc. Natl Acad. Sci. USA* **98**, 8880–8884
- Meijer, H.A. and Thomas, A.A. (2002) Control of eukaryotic protein synthesis by upstream open reading frames in the 5'-untranslated region of an mRNA. *Biochem. J.* **367**, 1–11
- vom Saal, F.S. (2006) Bisphenol A eliminates brain and behavior sex dimorphisms in mice: how low can you go? *Endocrinology* **147**, 3679–3680
- Welshons, W.V., Nagel, S.C., and vom Saal, F.S. (2006) Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure. *Endocrinology* **147**, S56–S69
- Schonfelder, G., Wittfoht, W., Hopp, H., Talsness, C.E., Paul, M., and Chahoud, I. (2002) Parent bisphenol A accumulation in the human maternal-fetal-placental unit. *Environ. Health Perspect.* **110**, 703–707
- Takahashi, O. and Oishi, S. (2000) Disposition of orally administered 2,2-bis(4-hydroxyphenyl)propane (bisphenol A) in pregnant rats and the placental transfer to fetuses. *Environ. Health Perspect.* **108**, 931–935
- Tachibana, T., Wakimoto, Y., Nakamuta, N., Pichitraslip, T., Wakitani, S., Kusakabe, K., Hondo, E., and Kiso, Y. (2007) Effects of bisphenol A (BPA) on placentation and survival of the neonates in mice. *J. Reprod. Dev.* **53**, 509–514
- Welshons, W.V., Thayer, K.A., Judy, B.M., Taylor, J.A., Curran, E.M., and vom Saal, F.S. (2003) Large effects from small exposures. I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. *Environ. Health Perspect.* **111**, 994–1006
- Imanishi, S., Manabe, N., Nishizawa, H., Morita, M., Sugimoto, M., Iwahori, M., and Miyamoto, H. (2003) Effects of oral exposure of bisphenol A on mRNA expression of nuclear receptors in murine placental assessed by DNA microarray. *J. Reprod. Dev.* **49**, 329–336
- Richter, C.A., Taylor, J.A., Ruhlen, R.L., Welshons, W.V., and vom Saal, F.S. (2007) Estradiol and bisphenol A stimulate androgen receptor and estrogen receptor gene expression in fetal mouse prostate mesenchyme cells. *Environ. Health Perspect.* **115**, 902–908
- Timms, B.G., Howdeshell, K.L., Barton, L., Bradley, S., Richter, C.A., and vom Saal, F.S. (2005) Estrogenic chemicals in plastic and oral contraceptives disrupt development of the fetal mouse prostate and urethra. *Proc. Natl Acad. Sci. USA* **102**, 7014–7019
- Jarred, R.A., McPherson, S.J., Bianco, J.J., Couse, J.F., Korach, K.S., and Risbridger, G.P. (2002) Prostate phenotypes in estrogen modulated transgenic mice. *Trends Endocrinol. Metab.* **13**, 163–168