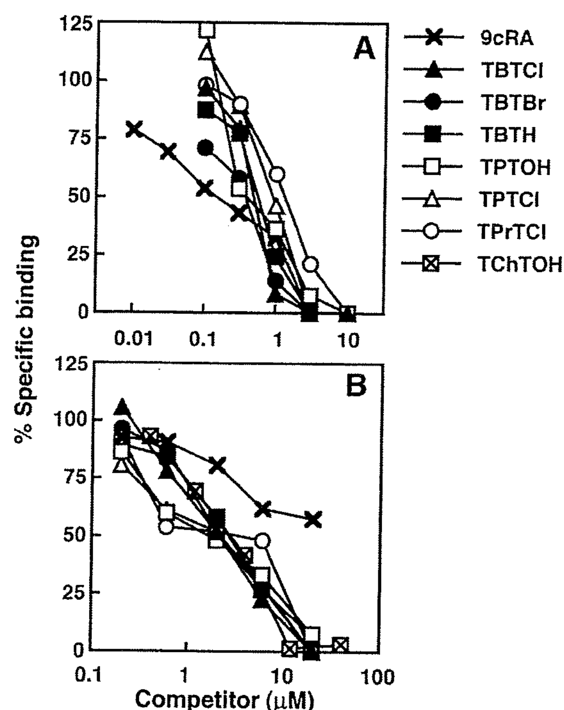

Fig. 8. Competition by 9cRA and Alkyltin Compounds with [^{3}H]9cRA (A) and [^{14}C]TPTOH (B) for Binding to the LBD of hRXRα

The LBD of hRXRα protein was incubated with increasing concentrations of unlabeled 9cRA or alkyltin compounds as competitors in the presence of [^{3}H]9cRA or [^{14}C]TPTOH as ligand. Results are expressed as percent specific binding. Each experiment was performed at least twice, and representative curves are shown.

Table 1. IC_{50} of Tin Compounds for Competition of [^{3}H]9cRA and [^{14}C]TPTOH Binding to hRXRα, and EC_{50} of Tin Compounds for GAL-RXR Reporter Assay

Compound	IC_{50} of Competition Assay (μM)		EC_{50} of GAL-RXR Activity (nM)
	[^{3}H]9cRA	[^{14}C]TPTOH	
9cRA	0.107	26.9	10.2
TBTCl	0.468	2.26	14.4
TBTH	0.475	2.37	12.3
TBTBr	0.265	2.56	10.4
TPTOH	0.527	1.82	13.9
TPTCl	0.792	1.63	13.4
TPrTCl	0.921	2.11	9.0
TChTOH	>10	2.68	17.3
TeBT	>10	23.7	559
TBVT	>10	>200	2960
TETBr	6.76	80.1	N.D.[a]
TMTCl	>10	>200	N.D.
TOTH	>10	>200	N.D.
$SnCl_4$	>10	>200	N.D.
$MBTCl_3$	>10	>200	N.D.
$DBTCl_2$	>10	>200	N.D.
$MPTCl_3$	>10	>200	N.D.
$DPTCl_2$	>10	>200	N.D.

[a] Not detectable.

a dose-dependent manner to levels approaching those induced by 9cRA and LG100268.

Because RAR agonists induced mRNA expression of aromatase (Fig. 4B), we examined the effectiveness of cotransfection with a human RARβ expression plasmid in stimulating the LUC activity by these alkyltin compounds to identify whether RAR is involved in alkyltin-induced aromatase expression (Fig. 9B). In the presence of the natural RAR ligands, atRA or 9cRA, RAR statistically significantly increased the expression of LUC ($P < 0.05$ or $P < 0.01$), whereas RAR had no effect on the LUC activity induced by LG100268, TBTCl, or TPTOH (Fig. 9B). These results suggest that the expression of human placental aromatase induced by these alkyltin compounds is involved in a ligand-dependent signaling pathway of RXRs but not in transactivation of RXR-RAR.

DISCUSSION

Although our previous study of human placental cells showed that TBTCl and TPTOH enhance hCG secretion and aromatase activity with an accompanying increase in the mRNA expression of each factor (27), the underlying molecular mechanism had remained unclear. In our previous study, we examined the effect of TBTCl and TPTOH on cAMP concentrations in human choriocarcinoma cells, because hCG production and aromatase activity in the human placenta are both well known to be controlled by cAMP-dependent intracellular signal pathways (16, 17, 45, 46). However, neither of these trialkyltin compounds altered cAMP production (27). We then speculated that activation of RXRs is a common signaling pathway of alkyltin-stimulated hCG production and aromatase activity, because both of these events are induced by specific ligands of RXRs (Fig. 4 and Refs. 9, 10, 13, and 14). In our present study, we provide evidence that trialkyltin compounds stimulate the transcription of RXRs because of a high-affinity interaction with the LBD of the receptor. In addition, trialkyltin compounds stimulate the expression of an LUC reporter construct containing the human placental promoter I.1 sequence of aromatase via a ligand-dependent signaling pathway of RXR.

In humans, the tissue-specific expression of aromatase is strictly regulated. Human aromatase is a single-copy gene composed of 10 exons: exons II through X encode the aromatase protein as well as the 3′-untranslated region of mRNA common to all estrogen-producing tissues (16). There are a number of variants of exon I. These encode the 5′-untranslated regions of various aromatase mRNAs, which are selectively expressed in some tissues by alternative splicing (16, 43, 44). The tissue-specific expression of aromatase in humans appears to be mediated by tis-

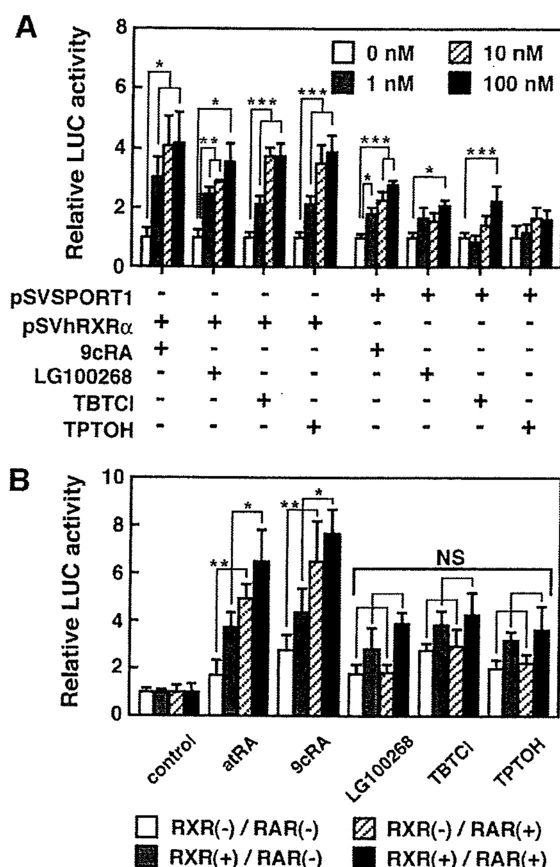

Fig. 9. Ability of TBTCl and TPTOH to Increase Transcription of an LUC Reporter Gene Containing the Human Placental Promoter I.1 Sequence of Aromatase via the Activation of RXR but not RAR

A, JEG-3 cells were cotransfected with 10 ng of either pSVhRXRα or pSVSPORT1 in addition to 50 ng PGVArom and then treated with various concentrations of 9cRA, LG100268, TBTCl, or TPTOH. B, JEG-3 cells were cotransfected with 10 ng pSVhRXRα or pSV40hRARβ, or both, in addition to 50 ng PGVArom, and then treated with 100 nM of atRA, 9cRA, LG100268, TBTCl, or TPTOH. pRL-TK (2 ng) was cotransfected as the control for normalization (see *Materials and Methods*). The results were expressed as average fold activation ± 1 SD after normalization to *Renilla* LUC activity. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.005$. NS, Not significant ($P \geq 0.05$).

sue-specific promoters lying upstream of the respective exon I sequences and by the binding of transcription factors to specific regions of each promoter. In the placenta, aromatase is driven by the placental major promoter (I.1), and the transcript contains exon I.1. In contrast to our results, Saitoh *et al.* (47) recently reported that TBT inhibits aromatase activity and decreases mRNA levels in ovarian granulosa cells. They also suggested that TBT-induced suppression of aromatase in the cells is partly regulated at the transcriptional level because of association with the cAMP-protein kinase A pathway or regulation by the steroidogenic tissue-specific transcriptional factor adrenal 4 binding protein/steroidogenic factor 1. In contrast to those in the placenta, ovarian transcripts of aromatase contain a 5′ sequence immediately upstream of the translation start site, because expression of the gene in the ovary uses a proximal promoter (II) that is strongly regulated by adrenal 4 binding protein/steroidogenic factor 1 and cAMP. In addition, RXR and PPARγ ligands suppress the mRNA expression of aromatase in ovarian granulosa cells (48, 49). Therefore, in light of our findings, TBT-induced suppression of aromatase in ovarian granulosa cells may involve RXR activation.

The RXRs stand out as unique members of the type II nuclear receptor subfamily and play a dual role in nuclear receptor signaling. On one hand, they can bind to their own response element (DR1) as a homodimer and activate transcription in response to their ligands, and, on the other hand, they serve as partners for other nuclear receptors (1–3). Trialkyltin compounds bind to RXRs to induce their transcription. In turn, the expression of hCG and aromatase induced by these compounds may involve either RXR-homodimer or -heterodimer, or both. The existence of three types of heterodimers—nonpermissive, conditionally permissive, and fully permissive—has been described. Nonpermissive heterodimers include RXR-TR and RXR-VDR, which cannot be activated by RXR agonist regardless of the presence (or absence) of the agonist of its partner receptor; formation of the heterodimer is thought to preclude the binding of ligand to RXR (50, 51). RXR-ligand-dependent transcription in promoter I.1 of human aromatase is reported to be regulated by RXR-VDR heterodimers, owing to binding to the imperfect palindromic sequence located from −183 to −172 bp upstream of the transcriptional start site (13). However, within these complexes, RXR acts as a silent partner, as described earlier (50). In addition, we used a GAL-VDR chimeric receptor to confirm that TBT and TPT could not activate transcription of VDR (data not shown). Consequently, both alkyltin-induced aromatase activity and mRNA expression may not involve the association of RXR-VDR. Although RXR-TR heterodimer generally is believed to be nonpermissive (51), Castillo *et al.* (52) recently demonstrated that RXR-TR heterodimer can function as a permissive heterodimer to allow 9cRA-induced stimulation of prolactin gene transcription in rat pituitary cells. Accordingly, we used a synthetic DR4 reporter gene to examine whether TBTCl and TPTOH stimulate transcription of RXR-TR heterodimer. However, these trialkyltin compounds had no effect on transactivation of these complexes in the presence or absence of T_3. We then used a GAL-TR chimeric receptor to confirm that TBT and TPT could not activate transcription of TR (data not shown). These results suggest that these trialkyltin-induced transcriptional activities also do not involve the association of RXR-TR.

As an example of the second type of heterodimer, the RXR-RAR heterodimer exhibits conditional permissivity because full response to RXR agonist occurs only in the presence of an RAR agonist (38, 41). Our

results showed TBT and TPT function as RXR agonists but not RAR agonist. Although RXR-RAR heterodimer generally is believed to be nonpermissive in the absence of RAR agonist, LG100754, which binds to RXRs but not RARs, strongly transactivates this heterodimer pair (53). In addition, the mRNA expression of both hCG and aromatase is also induced by RAR-specific agonist (Fig. 4), and it is possible that in light of the result in Fig. 9B, the transcription from promoter I.1 of human aromatase may be regulated by RXR-RAR heterodimers. However, TBTCl and TPTOH failed to transactivate RXR-RAR heterodimer on either RAR response element (DR2 and DR5) reporter elements or promoter I.1 elements of human aromatase (Figs. 5B and 9B). These results suggest that, unlike the effect of LG100754, these trialkyltin-induced transcriptional activities do not involve the association of RXR-RAR.

The third type is the permissive heterodimers, such as PPAR-RXR and FXR-RXR, which exhibit dual ligand permissivity, because they can be activated by the agonists of either RXR or its partner receptor, or both, in a more-than-additive fashion (35–40). The PPAR ligand 15dPGJ$_2$, PPARγ-specific ligand rosiglitazone, and FXR ligand CDCA all failed to increase mRNA expression of aromatase in Jar cells (Fig. 4B), suggesting that neither PPAR-RXR nor FXR-RXR heterodimers are involved in organotin-induced aromatase expression in the human placenta and that RXR homodimer may be required for organotin-induced aromatase expression. By contrast, PPAR agonists, in addition to RXR and RAR agonists, stimulate mRNA expression of hCGβ, as previously described (Fig. 4 and Refs. 9, 10, 12, and 14). These findings indicate that organotin-induced hCGβ expression might involve either PPAR-RXR heterodimers or RXR homodimer.

To address these possibilities, we constructed a LUC reporter plasmid containing the promoter sequence (−455 to +365 bp) of hCGβ5, which is the predominant hCGβ subunit expressed in the human placenta, and assessed the effectiveness of TBTCl and TPTOH in stimulating LUC activity by using JEG-3 cells cotransfected with a human RXRα expression plasmid and the hCGβ5-LUC reporter plasmid. However, trialkyltin compounds, RXR, and PPAR ligands failed to stimulate LUC expression (data not shown), whereas cAMP analogs stimulated gene expression, as previously described (45). Furthermore, Tarrade *et al.* (14) reported that ligand-dependent mRNA expression of hCGβ is transcriptionally controlled by PPARγ-RXR heterodimers, which bind to DR1 as well as does RXR homodimer. However, like us, they failed to detect expression of reporter gene constructs containing imperfect DR1 motifs in the regulatory region of the hCGβ gene. Transcriptional regulation in promoter I.1 of human aromatase and the hCGβ promoter is not yet fully understood, and neither the PPAR response element nor the RXRE involved in both promoter activation by RXR ligands has been identified. Further studies are needed to clarify the precise mechanism of action of RXRs in the expression of human placental aromatase and hCG, because the ligand-dependent signaling pathways of RXRs appear intricate.

We assayed 15 tin compounds, in addition to TBTCl and TPTOH, for their ability not only to induce hCG production, aromatase activity, and mRNA expression of both factors but also to activate RXR through binding to the LBD of the receptor. hCG production and aromatase activity did not differ significantly among the TBT and TPT derivatives. In addition, the abilities to bind to the LBD of RXR and activate the receptor were similar among these compounds, because they all competed with both [^{3}H]9cRA and [^{14}C]TPTOH for binding to RXR approximately as well as did TBTCl and TPTOH. These results suggest that the exact identity of the ligand on the trialkyltin (as long as it is not another alkyl group) is relatively unimportant for binding to RXR.

By contrast, approximately 50- to 100-fold higher concentrations of tetraalkyltin compounds such as TeBT and TBVT were needed to elicit a response, compared with those of the TBT and TPT derivatives. In addition, although the tetraalkyltin compounds stimulated transcription through RXR, they hardly competed with [^{3}H]9cRA for binding to the LBD of RXR. This observation may indicate that the tetraalkyltin compounds were metabolically converted to the active form in the cells. This hypothesis is supported by the general trend of the previous results showing that organotin compounds undergo dealkylation by the microsomal monooxygenase system, which is dependent on cytochrome P450 in the liver and other organs (54–56). The presence of a fourth alkyl group on the tin atom may interfere with the binding of alkyltin compounds to RXR, and activation of the receptor by these tetraalkyltin compounds may be the result of their metabolic conversion in cells to the active dealkylated form (*e.g.* TBT). Such events are reminiscent of an early observation that atRA could activate RXR in cells because of its metabolic conversion to the high-affinity ligand 9cRA (57). Although the dialkyltin compounds neither bind to nor activate RXR, DBTCl$_2$ and DPTCl$_2$ induced expression of the mRNA of aromatase and hCGβ, respectively. It remains unclear why these dialkyltin compounds induced expression of the mRNA of aromatase or hCGβ, but the induction appears to be caused by a mechanism other than activation of RXRs.

Among the trialkyltin compounds other than TBT and TPT derivatives, TPrTCl was most active. TPrTCl activated transcription of RXR as well as did 9cRA and, like TBTCl and TPTOH, completely out competed both [^{3}H]9cRA and [^{14}C]TPTOH for binding to RXR. TETBr bound weakly to RXR, but we were unable to detect TETBr-induced transcription of RXR and mRNA expression of hCGβ and aromatase. The fact that TETBr is cytotoxic at concentrations greater than 300 nM, according to the result of the [^{3}H]thymidine uptake assay (data not shown), may render TETBr-stimulated RXR activation undetectable. TChTOH, which activated transcription of RXR, completely out competes [^{14}C]TPTOH for binding to the LBD of RXR, whereas it

cannot out compete [^{3}H]9cRA at all. This difference in the ability of TChTOH to compete with [^{3}H]9cRA and [^{14}C]TPTOH may be caused by differences in their ligand-protein contacts (see following paragraph). These results suggest that the affinity of tin compounds to RXR is related to both the numbers and lengths of their alkyl groups.

RXR has been characterized as a nuclear receptor that demonstrates a highly restricted substrate specificity. Until recently, 9cRA was defined as the most potent RXR activator. However, various fatty acids, such as docosahexaenoic acid (DHA) and phytanic acid, and methoprene acid, a synthetic juvenile hormone analog used as an insect regulator, have been identified as RXR ligands (58–62). In addition, several retinoids with RXR selectivity have been developed (63), because retinoids are important therapeutic agents in the treatment of cancer and proliferative diseases of the skin. Although fatty acid- or methoprene acid-induced RXR activation requires greater than 1000-fold higher concentrations than that induced by 9cRA (58–62), the protein-ligand interactions of almost all RXR ligands share several common characteristics. Recently, Egea *et al.* (64) have obtained some interesting findings through analyzing the crystal structures of RXR with DHA, the synthetic ligand BMS649, and 9cRA. For example, RXR ligands contain a carboxylate group, which is important in their ability to be buried stably in the predominantly hydrophobic pocket. This functional group is involved in an ionic interaction with the strictly conserved basic residue R316 of helix H5 and forms a hydrogen bond with the backbone carbonyl amide group of the β-turn residue A327. Furthermore, ligand atoms C14–C22 of DHA and the tetrahydrotetramethylnaptho group of BMS649 occupy the same hydrophobic cavity (delineated by helices H3, H7, and H11), in which the β-ionone ring of 9cRA is buried stably. However, although trialkyltin compounds bind RXR with high specificity and induce RXR activation at doses similar to those of 9cRA, they lack a carboxylate group. Further, except for the TPT derivatives and TChTOH, RXR-stimulating trialkyltin compounds also lack sufficiently long fatty acid and cyclic functional groups, both of which might be buried in the β-ionone binding subpocket. In the competitive ligand-binding assay, RXR-stimulating trialkyltin compounds, except for TChTOH, completely out competed both [^{3}H]9cRA and [^{14}C]TPTOH, whereas 9cRA could not completely out-compete [^{14}C]TPTOH for binding to RXR. In addition, TChTOH out competed [^{14}C]TPTOH but not [^{3}H]9cRA. Together, these results suggest that the protein-ligand interaction of trialkyltin compounds and RXR is very different from those seen with other RXR ligands. Indeed, previous studies have reported that, despite the overall similarity of protein-ligand interactions, RXR ligands differ, especially within the L-shaped binding pocket. Although further studies are necessary to clarify which amino acid of RXR is important to the binding of trialkyltin compounds to the ligand-binding pocket, the ligand-protein contacts of these trialkyltin compounds are probably unique to them.

To our knowledge, ours is the first study to clarify the molecular mechanism of trialkyltin-induced endocrine-disrupting effects in the human placenta. Through RXR activation, trialkyltin compounds may be potent endocrine disruptors of other human tissues, because these compounds alter the endocrine functions, differentiation, and other processes of several human cell types (21, 47, 65–67). Furthermore, we demonstrated that trialkyltin compounds function as RXR ligands with novel structures, which bind to the LBD of RXR with high affinity and stimulate transcription of the receptor. We believe that our results provide information useful in the design of novel RXR ligands.

MATERIALS AND METHODS

Chemicals and Cell Culture

TBTCl, TPTOH, TOTH, and $DBTCl_2$ were obtained from Tokyo Kasei Kougyo (Tokyo, Japan). $SnCl_4$, TMTCl, TETBr, TChTOH, $MBTCl_3$, $MPTCl_3$, $DPTCl_2$, TPTCl, TBTH, TBTBr, TeBT, and TBVT were obtained from Aldrich Chemicals (Milwaukee, WI). 9cRA, atRA, AM580, and CDCA were obtained from Sigma Chemical Co. (St. Louis, MO). $15dPGJ_2$ and rosiglitazone were obtained from Cayman Chemical (Ann Arbor, MI). TPrTCl was obtained from Merck (Darmstadt, Germany). LG100268 (>95% pure) was synthesized in the Medical Chemistry Laboratories of Fujisawa Pharmaceutical (Osaka, Japan). Human choriocarcinoma cell lines Jar and JEG-3 were obtained from American Type Culture Collection (ATCC; Manassas, VA). Jar cells (ATCC No. HTB-144) were cultured in RPMI 1640 medium with 2 mM L-glutamine, 1 mM pyruvate, 4.5 g/liter glucose, and 10% fetal calf serum (FCS). JEG-3 cells (ATCC no. HTB-36) were cultured in MEM with 2 mM L-glutamine, 0.1 mM MEM nonessential amino acid solution (Invitrogen, Carlsbad, CA), and 10% FCS. To determine the effect of tin compounds on hCG secretion, aromatase activity, and mRNA expression of Jar cells, the cells were seeded and precultured for 24 h and then treated with either various concentrations of tin compounds in 0.1% dimethyl sulfoxide (DMSO) or vehicle alone (0.1% DMSO) for an additional 24 or 48 h. In control experiments, 0.1% DMSO did not alter hCG secretion, aromatase activity, mRNA expression of hCGβ and aromatase, or the results of reporter gene assays in any of the cell lines examined.

Determination of hCG Production in Culture Media

hCG production was assessed as previously described (27). Jar cells were seeded in 48-well plates (4×10^4 cells per well) in regular culture medium supplemented with 5% charcoal-stripped FCS instead of 10% normal FCS. After 24 h, cells were treated with various tin compounds for 48 h. To determine hCG production, the cells were then washed and cultured in fresh medium for another 24 h. Culture supernatant was collected, and hCG concentration was determined by ELISA. Microtiter ELISA plates were coated with 5 μg/ml rabbit polyclonal antibody against intact hCG in 0.05 M sodium bicarbonate, 0.02 M sodium carbonate buffer (pH 9.6) overnight at 4 C. They were blocked for 2 h at room temperature with 1% (wt/vol) gelatin in PBS, washed with 0.05% (vol/vol) Tween 20 in PBS (TPBS), and incubated for 2 h at 37 C with 50 μl collected test samples. After being washed three times with TPBS, the plates then were incubated for 2 h at 37

C with 1:1000 mouse monoclonal antibody against the β-subunit of hCG. After being washed with TPBS, the plates were incubated for an additional 2 h at 37 C with 1:1000 rabbit antimouse IgG1 antibody conjugated with horseradish peroxidase (Zymed Laboratories, Inc., South San Francisco, CA). The plates then were washed with TPBS and developed using 2.5 mM 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Sigma) in 0.1 M citrate buffered solution containing 0.015% H_2O_2. The reaction was stopped by the addition of 0.1% NaN_3, after which the plates were read at a wavelength of 415 nm in a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA). The level of hCG in the culture supernatant was calculated from a standard curve by using Microplate Manager III software (Bio-Rad). The standardized hCG was a kind gift from Teikoku Hormone Manufacturing (Tokyo, Japan).

Aromatase Assay

Aromatase activity was determined as previously described (27). Jar cells were seeded in 12-well plates (1.5×10^5 cells per well) in regular culture medium supplemented with 5% charcoal-stripped FCS instead of 10% normal FCS. After 24 h, cells were treated with various tin compounds for 48 h. At the end of each treatment, cells were washed three times with PBS. Aromatase activity then was determined by tritium release assay. This method measures production of ^3H_2O, which forms as a result of aromatization of the substrate [1β-^{3}H]androst-4-ene-3,17-dione (New England Nuclear, Boston, MA). Serum-free medium containing [1β-^{3}H]androst-4-ene-3,17-dione solution (54 nM) was prepared, and 0.5 ml of this solution was added to each well. In addition, wells containing media alone were tested to provide control values for aromatase activity. After incubation for 1 h, each plate was placed on ice, and 200 μl of culture medium was withdrawn from each well. The medium was extracted with 500 μl chloroform, vortexed, and then centrifuged for 1 min at 9000 × *g*. A 100-μl aliquot of the aqueous phase was mixed with 100 μl of a 5% wt/vol charcoal 0.5% wt/vol dextran T-70 suspension, vortexed, and then incubated for 10 min. After centrifugation of the solution for 5 min at 9000 × *g*, a 150-μl aliquot was removed to measure the level of radioactivity by liquid scintillation.

Quantitative RT-PCR

Jar cells were seeded in 100-mm tissue culture plates (1×10^6 cells) and treated with various compounds in regular culture medium supplemented with 5% charcoal-stripped FCS instead of 10% normal FCS, after which total RNA was extracted from them using TRIzol reagent (Invitrogen). mRNA expression of hCGβ and aromatase in Jar cells was determined by quantitative RT-PCR, as previously described (27). Total RNA (5 μg) extracted from Jar cells was reverse transcribed using SuperScript II reagent (Invitrogen) and oligo-(dT) as primer. The reaction was performed by incubation for 1 h at 42 C. After termination of cDNA synthesis, reaction mixtures were diluted with 4 volumes of Tris-EDTA. Aliquots (2 μl) of diluted reverse transcribed products were amplified in a reaction mixture containing 2× buffer from the QuantiTect SYBR Green PCR kit (QIAGEN, Valencia, CA) and 0.5 μM of each primer by using LightCycler (Roche Diagnostics, Mannheim, Germany). After preincubation at 95 C for 15 min, PCR was performed with 35–40 cycles of denaturation at 95 C for 15 sec, annealing at 65 C for 30 sec, and elongation at 72 C for 10 sec. Primers used were as follows: human aromatase, 5′-CCGGCCTTGTTCGTATGGTCA-3′ and 5′-CAACACGTCCACATAGCCCGA-3′; hCGβ, 5′-CCGTGTGCATCACCGTCAACA-3′ and 5′-GTTGCACACCACCTGAGGCAG-3′; and human β-actin, 5′-CTACGAGCTGCCTGACGGC-3′ and 5′-GCCACAGGACTCCATGCCC-3′.

Plasmid Construction

Human RXRα and RARβ cDNAs were amplified by RT-PCR using total RNA from JEG-3 cells. The amplified RXRα fragment was cloned into pSVSPORT1 (Invitrogen), whereas the RARβ fragment was cloned into a simian virus 40 promotor-containing expression vector. The resulting RXRα and RARβ expression vectors were termed pSVhRXRα and pSV40hRARβ, respectively. A 2.4-kb promoter I.1 sequence of the human aromatase gene (−2295 to +107 bp) was PCR amplified from JEG-3 genomic DNA. *Kpn*I and *Sma*I sites were introduced into the 5′- and 3′-termini, respectively, of the amplified fragment, which then was subcloned into the *Kpn*I–*Sma*I site of PGVB2 (Nippon Gene, Tokyo, Japan); the resulting reporter construct was termed PGVArom.

To construct a reporter plasmid containing an RXRE and RAR response element, response elements were cloned into the *Sma*I site of pTAL-Luc (CLONTECH Laboratories, Inc., Palo Alto, CA); response elements with the *underlined* consensus hexanucleotide sequence were as follows: DR1 × 2 (5′-<u>AGGTCA</u> a <u>AGGTCA</u> a <u>AGGTCA</u> a <u>AGGTCA</u>-3′); DR2 × 2 (5′- aa <u>AGGTCA</u> aa <u>AGGTCA</u> ccatcccgggaaa <u>AGGTCA</u> aa <u>AGGTCA</u> cc-3′); DR5 × 2 (5′-aa <u>AGGTCA</u> ccgaa <u>AGGTCA</u> ccatcccgggaaa <u>AGGTCA</u> ccgaa <u>AGGTCA</u> cc-3′); the resulting reporter constructs were termed pTALDR1, pTALDR2, and pTALDR5, respectively. The LBDs of hRXRα (codons 201–693), hRXRβ (codons 275–534), and hRXRγ (codons 172–455) were amplified by RT-PCR using mRNA from human liver and kidney and subcloned into pGEX-4T (Amersham Biosciences, Piscataway, NJ). These constructs were used for generation of glutathione *S*-transferase (GST)-hRXR fusion proteins. For chimeric receptor assay, the LBD of hRXRα was fused to the C-terminal end of GAL4-DNA binding domain (amino acids 1–147) in the pBK-CMV expression vector (Stratagene, La Jolla, CA) to yield pBK-CMV-GAL4-hRXRα. All sequences synthesized by PCR were confirmed by DNA sequencing. The plasmid p4×UAS-tk-luc, a LUC reporter construct containing four copies of the GAL4 binding site [upstream activating sequence (UAS) of GAL1] followed by a thymidine kinase promoter, was a kind gift from Dr. Y. Kamei (National Institute of Health and Nutrition, Japan).

Transient Transfection Assay

Transfection was performed with Lipofectamine regent (Invitrogen) in accordance with the manufacturer's instructions. JEG-3 cells (3×10^4 cells) were seeded in 24-well plates 24 h before transfection with the optimal dose of each DNA construct. At 18 h after transfection, various compounds were added to the transfected cells, which were then cultured in regular culture medium supplemented with 1% charcoal-stripped FCS instead of 10% normal FCS. The cells were harvested 30 h later, and extracts were prepared and assayed for LUC activity by using the dual-LUC reporter assay system (Promega Corp., Madison, WI) in accordance with the manufacturer's instructions. To normalize LUC activity for transfection and harvesting efficiency, the *Renilla* LUC control reporter construct pRL-TK (Promega) was cotransfected as an internal standard in all reporter experiments. The results are expressed as the average relative LUC activity of at least quadruplicate samples.

Ligand Binding Assay

The GST-RXR fusions were expressed in *Escherichia coli* DH5α cells and purified according to the manufacturer's (Amersham Biosciences) instructions. The purified proteins (30 μg/ml) were incubated with increasing concentrations of either [^{3}H]9cRA (1.63 tBq/mmol, Amersham Biosciences) or [^{14}C]TPTOH (2.04 gBq/mmol, Amersham Biosciences) with or without a 100-fold molar excess of each unlabeled compound. After incubation at 4 C for 1 h, specific binding was

determined by hydroxyapatite binding assay as described elsewhere (68). Binding in the presence of a 100-fold molar excess of unlabeled ligand was defined as nonspecific binding; specific binding was defined as total binding minus nonspecific binding. Similarly, tin compounds were used to compete for [^{3}H]9cRA and [^{14}C]TPTOH in this assay to determine the binding preferences of RXRs.

Statistics

Data were analyzed by the two-tailed unpaired Student's *t* test by using SPSS software (SPSS, Inc., Chicago, IL). Control and treatment group data were always obtained from equal numbers of replicate experiments. Values with $P < 0.05$ were considered statistically significant.

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BIODEGRADATION OF BISPHENOL A AND DISAPPEARANCE OF ITS ESTROGENIC ACTIVITY BY THE GREEN ALGA *CHLORELLA FUSCA* VAR. *VACUOLATA*

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Abstract—Bisphenol A (BPA) is known as an endocrine disruptor and often is found in landfill leachates. Removal of BPA by green alga, *Chlorella fusca*, was characterized, because we previously found that various phenols were well removed by this strain, including BPA. *Chlorella fusca* was able to remove almost all BPA in the concentration range from 10 to 80 μM for 168 h under continuous illumination at 18 W/m². At the low light intensity of 2 W/m², 82% of 40 μM BPA was removed, and only 27% was removed in the dark. Moreover, *C. fusca* could remove 90% of 40 μM BPA under the 8:16-h light:dark condition, which was almost as high as that under the continuous-light condition. The amount of BPA contained in the cells was less than the amount of BPA removed from the medium. Monohydroxybisphenol A was detected as an intermediate of BPA degradation. Moreover, estrogenic activity that originated from BPA in the culture medium also completely disappeared. Based on these results, BPA was finally degraded to compounds having nonestrogenic activity. Therefore, *C. fusca* can be considered a useful organism to remove BPA from landfill leachates.

Keywords—Bisphenol A *Chlorella fusca* Estrogenic activity Landfill leachate Monohydroxybisphenol A

INTRODUCTION

Microalgae frequently have been used in processes for removing pollutants, such as nitrogen and phosphorus, from wastewaters [1]. Biosorption of heavy metals and biodegradation of hazardous organic compounds by microalgae also have been reported [2,3]. In a previous study, we found that the green alga, *Chlorella fusca* var. *vacuolata* IAM C-28, which was obtained from the Institute of Applied Microbiology Culture Collection (Tokyo, Japan), could remove various kinds of phenols, such as chlorophenols, nitrophenols, and bisphenol A (BPA; 2,2-bis(4-hydroxyphenyl)propane), from medium [4].

Recently, BPA has often been detected in aquatic and soil environments [5,6]. Bisphenol A has an acute toxicity to aquatic organisms. It was reported that a median lethal concentration for fish is 3 to 15 mg/L and that a median effective concentration for green algae is 1 to 3 mg/L [6]. Because BPA also has endocrine-disrupting activity, it is hazardous to animals even at low concentrations [7,8]. Bisphenol A is widely used as a material to produce polycarbonate, epoxy resins, flame retardants, and other products. These plastics are finally disposed of into landfills, and BPA often is detected in landfill leachates at higher concentrations than those in environmental water [9,10]. Therefore, leachates are thought to be significant sources of BPA in aquatic environments. In Japan, landfill sites are categorized into three types depending on waste contents. Waste plastics, rubber, glasses, ceramics, nontoxic metals, and construction scrap materials are disposed of into landfill sites for stable industrial wastes. Industrial wastes containing toxic substances at high level are disposed of into strictly controlled landfills for industrial wastes. The other industrial and domestic wastes are disposed of into leachate-controlled landfills equipped with a leachate-treatment system. Urase et al. [11] and Yamada et al. [12] reported that more than 90% of the BPA contained in leachates from controlled-type landfill sites was removed by treatment processes consisting of activated sludge method, coagulation, sedimentation, sand filtration, and activated carbon adsorption. However, BPA also is detected in leachate from landfill sites for stable industrial wastes, which are not equipped with a leachate-treatment system [11,12]. Because the cost of the usual leachate-treatment system is high, a low-cost treatment system is required to remove BPA in leachates from open-landfill sites.

The present report focuses on the utilization of microalgae for removing BPA from landfill leachates. We evaluated the ability of *C. fusca* to remove BPA in batch experiments under light, dark, and light-dark cycle conditions. We also investigated the degradation of BPA by the cells, and then we identified an intermediate of BPA degradation. The disappearance of estrogenic activity originated from BPA in the culture medium also was analyzed by the yeast two-hybrid assay [13].

MATERIALS AND METHODS

Microorganism and culture condition

Chlorella fusca was precultivated in a modified Bristol medium at 27.5°C under continuous illumination with white fluorescent light at an intensity of 18 W/m² and aerated with air containing 1% CO_2. After 7 d, cells were harvested and inoculated into a 100-ml glass test tube as described previously [4]. Algal cells were cultivated under the same conditions as used for precultivation with 40 μM BPA as basal condition for BPA removal. To evaluate the ability of *C. fusca* for BPA

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removal, the BPA concentration was varied from 10 to 160 μM. To examine the effect of illuminating conditions on BPA removal, the light intensity was changed from 0 to 36 W/m^2 at the surface of the glass test tube, and the effect of a light-dark cycle (8:16-h light:dark photoperiod; light intensity, 18 W/m^2) also was investigated. The concentration of BPA and the cell density were measured every 24 h after addition of BPA. All the batch experiments were undertaken in triplicate.

Analytical methods

The cell growth was evaluated by the optical density at 680 nm and measured by a spectrophotometer (U-2000; Hitachi, Tokyo, Japan). The concentration of BPA was measured using a high-performance liquid chromatography (HPLC) system (D-7000 series; Hitachi) with a diode-array detector (L-4500; Hitachi) at 280 nm. The pretreatment method for the HPLC sample was as follows: The culture (2 ml) was centrifuged (11,000 *g*, 4°C, 5 min), 200 μl of the supernatant were mixed with 20 μl of 6 N HCl to remove proteins, and a second centrifugation was performed (17,360 *g*, 4°C, 11 min). The supernatant (40 μl) was then injected into the HPLC system, which was equipped with a reversed-phase column (250 × 4.6 mm I. D., 5 mm; Mightysil RP-18; Kanto Chemical, Tokyo, Japan). Acetonitrile/50 mM potassium dihydrogenphosphate buffer (pH 2.5; 50/50 [v/v]) was used as the mobile phase at a flow rate of 0.7 ml/min. Degradation intermediates of BPA were identified using the HPLC–mass spectrometry (LC-MS) system (LCQ Advantage; Thermo Finnigan, San Jose, CA, USA) with an electrospray ionization interface. The HPLC sample (50 μl) was injected into the LC-MS system equipped with a reversed-phase column (250 × 4.6 mm I. D., 5 mm; L-column; Chemical Evaluation and Research Institute, Tokyo, Japan). Water/acetonitrile (60/40 [v/v]) was used as the mobile phase at a flow rate of 1.0 ml/min. The electrospray ionization interface was operated in a negative-ion mode.

Extraction of BPA from algal cells

Chlorella fusca was cultivated in the presence of 40 μM BPA in the light at 18 W/m^2. Algal cells were harvested from 5 ml of culture by centrifugation (845 *g*, 5°C, 5 min) and washed three times with distilled water. Cell pellets were resuspended in 5 ml of distilled water. After ultrasonic disruption (Sonifier 450; Branson, Danbury, CT, USA) of the cells, 12.5 ml of methanol and 6.25 ml of chloroform were added. This solution was shaken for 1 h. Next, 6.25 ml of distilled water and 6.25 ml of chloroform were added to the solution, and the organic phase was collected by centrifugation (845 *g*, 5°C, 5 min). This treatment was repeated four times. Chloroform was evaporated completely from the organic phase by a rotary evaporator. The residual matter was dissolved in 10 ml of methanol and then analyzed by HPLC.

Chemical oxidation of BPA by Fremy's salt

Bisphenol A was oxidized by potassium nitrosodisulfonate (Fremy's salt; Sigma-Aldrich, St. Louis, MO, USA) to prepare monohydroxybisphenol A according to a previously reported method [14]. Fremy's salt (0.1 g) was added to 66 ml of 25 mM sodium dihydrogenphosphate buffer solution containing 13 mM BPA in a 200-ml Erlenmeyer flask. The mixture was shaken for 20 min at 25°C and then extracted with chloroform. After the organic phase was collected and chloroform evaporated, the residual matter containing red crystals was dissolved in 10 ml of ethanol. The solution was analyzed by LC-MS.

Estrogenic activity of the culture supernatant

The estrogenic activity of the culture supernatant was estimated by a two-hybrid yeast assay using the recombinant yeast *Saccharomyces cerevisiae* Y190 with pGBT9-ER LBD and pGAD424-TIF2 as described by Nishikawa et al. [13] with some modifications. Then, 250 μl of the supernatant of the *C. fusca* culture containing BPA or of that containing ethanol as a negative control were mixed with 200 μl of synthetic dextrose medium. The yeast, which had been precultured for 48 h, was added to the mixture at a volume of 50 μl and then cultivated for 4 h at 30°C in a culture-tube rotator (LD-76; Labinco B.V., Breda, The Netherlands) at 30 rpm. After the optical density at 595 nm (OD_{595}) of the culture was measured, the yeast cells were collected by centrifugation (1,100 *g*, 4°C, 5 min) from 350 μl of the culture and then resuspended in 200 μl of Z buffer (pH 7.0) containing 21.49 g of $Na_2HPO_4 \cdot 12H_2O$, 6.22 g of $NaH_2PO_4 \cdot 2H_2O$, 0.75 g of KCl, 0.246 g of $MgSO_4 \cdot 7H_2O$, 0.27 ml of β-mercaptoethanol, and 1 g of Zymolyase 20T (Seikagaku, Tokyo, Japan) in 1 L. Samples were incubated for 15 min at 37°C, and then enzymatic reaction was started by adding 40 μl of 4 g/L *o*-nitrophenyl-β-D-galactopyranoside. The mixtures were incubated for 90 min at 30°C, and 100 μl of 1 M Na_2CO_3 were added to stop the reaction. After centrifugation (17,360 *g*, 4°C, 5 min), the absorbance of the supernatant at 415 and 550 nm (A_{415} and A_{550}, respectively) was measured. Estrogenic activity was indicated as β-galactosidase activity (U) calculated by the following equation:

$$U = (A_{415} - (1.75 \cdot A_{550})) \times 10^3/(t \cdot v \cdot OD_{595})$$

where t is the time of reaction (90 min) and v is the volume of mixture used in the assay (0.05 ml).

RESULTS

Effects of initial BPA concentration on its removal and algal growth

The effect of initial BPA concentration on its removal was investigated. The concentration of BPA in uninoculated media was found not to decrease (data not shown). *Chlorella fusca* was cultivated with BPA in the concentration range from 10 to 160 μM for 168 h under the continuous-light condition (18 W/m^2). Figure 1 shows the BPA removal and cell growth. More than 95% of BPA was removed at the concentration between 10 and 80 μM, although only 70% of 160 μM BPA was removed. Therefore, it can be assumed that BPA was removed by the algal cells. This strain grew well in the BPA concentration range from 10 to 40 μM. Because the concentration of BPA in Japanese landfill leachates generally was lower than 40 μM [9–12], the initial BPA concentration was set at 40 μM for subsequent experiments.

Effects of light condition on BPA removal

To investigate the effects of light intensity on BPA removal, *C. fusca* was cultivated in the light-intensity range from 0 to 36 W/m^2 for 168 h (Fig. 2). The growth rates of this strain decreased with decreasing light intensity, and growth did not occur in the dark. The BPA removal was 98% at 36 W/m^2 and 82% at 2 W/m^2, although the removal decreased to 27% in the dark. Therefore, light illumination was an important parameter for achieving a high ability to remove BPA.

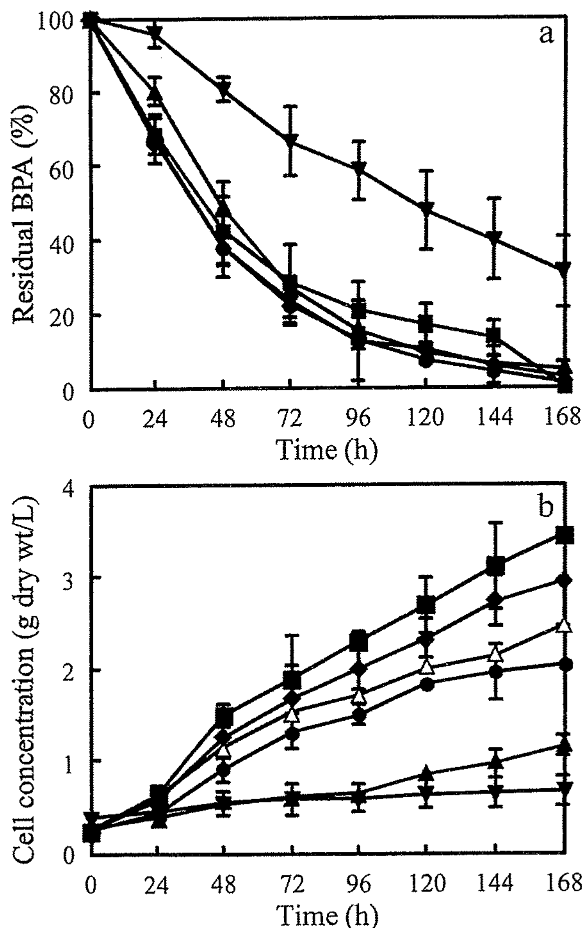

Fig. 1. Effect of initial bisphenol A (BPA) concentrations on its removal (a) and growth (b). The initial BPA concentrations were 0 μM (△), 10 μM (■), 20 μM (◆), 40 μM (●), 80 μM (▲), and 180 μM (▼). The light intensity was 18 W/m². Values were the averages of triplicate determinations. Error bars indicate standard deviations.

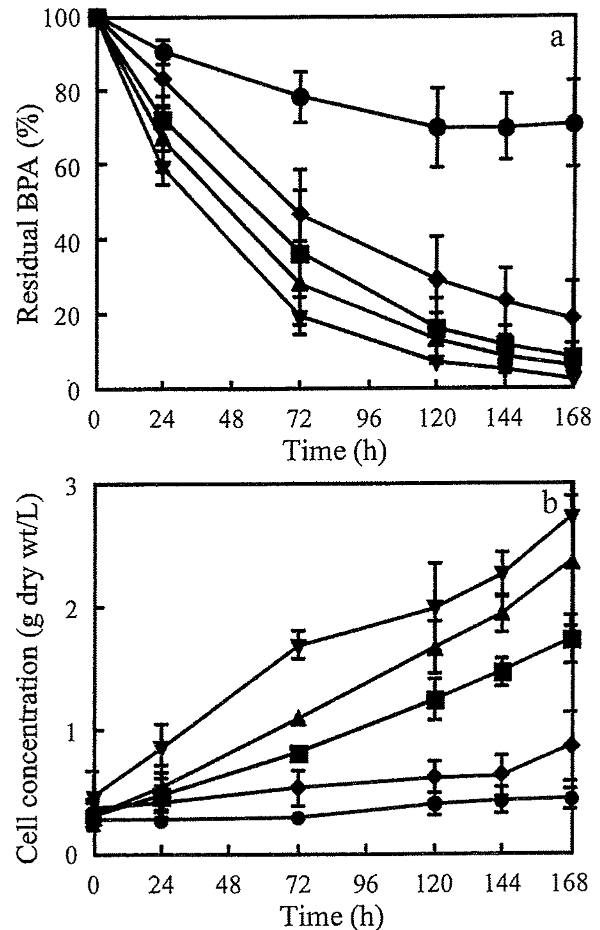

Fig. 2. Effect of light intensity on bisphenol A (BPA) removal (a) and growth (b). Light intensities tested were 0 W/m² (●), 2 W/m² (◆), 9 W/m² (■), 18 W/m² (▲), and 36 W/m² (▼). Values were the averages of triplicate determinations. Error bars indicate standard deviations.

Removal of BPA under the 8:16-h light:dark photoperiod (light intensity, 18 W/m²) also was investigated (Fig. 3). *Chlorella fusca* grew well and removed 90% of BPA, a result not very different from that obtained under continuous light (18 W/m²) for 168 h.

Biodegradation of BPA by C. fusca

To determine whether BPA was degraded by the cells or simply accumulated in the cells, the amount of BPA in the cells cultivated at 18 W/m² was analyzed (Fig. 4). The amount of BPA contained in the cells was significantly less than the amount of BPA removed from the medium. At the end of the cultivation time, BPA in the cells finally was decreased below the detection limit of HPLC analysis. This result indicates that BPA removal by *C. fusca* was caused by biodegradation by the cells rather than by simple accumulation in the cells.

On the HPLC chromatogram, an unknown peak at 8.2 min was observed in the culture medium in the light. A typical chromatogram is shown in Figure 5a. At 2 W/m², this peak area increased with decreasing BPA until 72 h and then decreased (data not shown). This peak was analyzed by LC-MS, and the mass spectrum of it revealed a parent-ion peak $[M-1]^-$ at *m/z* 243.11 (Fig. 6a). Because the molecular weight of BPA is 228.29, it was suggested that hydroxylation occurred in BPA. Based on these LC-MS results, this compound was thought to be monohydroxybisphenol A, an intermediate of BPA degradation by *C. fusca*. Atkinson and Roy [14] reported that monoquinone and monohydroxy derivatives of BPA were produced by BPA oxidation using Fremy's salt, strong oxidizing agent, and treatment with 1 N HCl. Therefore, monohydroxybisphenol A was produced according to their method and compared with the intermediate of BPA degraded by *C. fusca* using HPLC and LC-MS. The BPA degradation intermediate showed the same retention time (8.2 min) and mass spectrum as those of monohydroxybisphenol A (Figs. 5b and 6b).

Disappearance of estrogenic activity in the culture

To determine if estrogenic activity originated from BPA in the culture medium of *C. fusca* was decreased on BPA removal, the change of this activity was analyzed by yeast two-hybrid assay (Fig. 7). The activity decreased with decreasing BPA concentration and then completely disappeared.

DISCUSSION

In the present study, the green alga *C. fusca* well degraded BPA in the concentration range from 10 to 80 μM in the light (Fig. 1a). Yamamoto et al. [9] reported that the highest BPA concentration detected in landfill leachates was 75 μM, indicating that it is possible for *C. fusca* to remove BPA at this

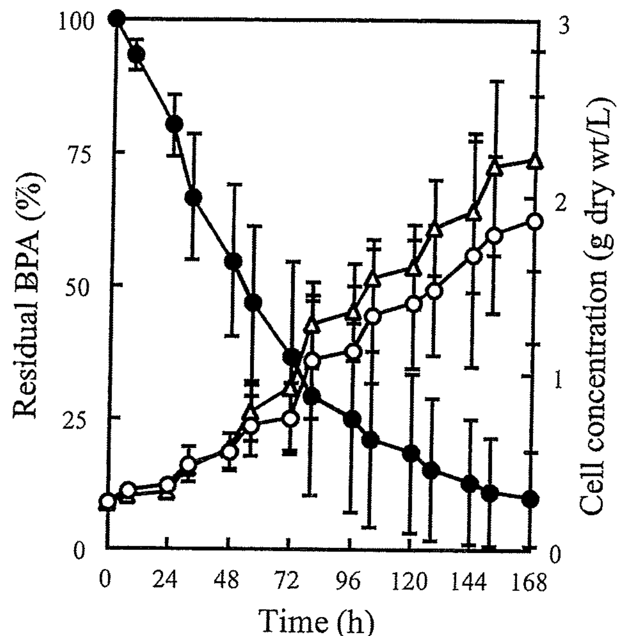

Fig. 3. Removal of bisphenol A (BPA) and growth under light-dark cycle condition. The BPA residual was shown (●). Algal cell concentration measured in media containing BPA (○) and not containing BPA (△), both incubated under 8:16-h light:dark photoperiod. Values were the averages of triplicate determinations. Error bars indicate standard deviations.

concentration. Several reports have appeared regarding the removal of BPA by bacteria. The gram-negative bacterium strain MV-1, isolated from the sludge of a wastewater treatment plant at a plastic manufacturing facility, was able to use BPA as a sole carbon and energy source. This bacterium removed 1.1 mM BPA completely from culture for 4 h [15]. Ronen and Abeliovich [16] reported that the gram-negative bacterium

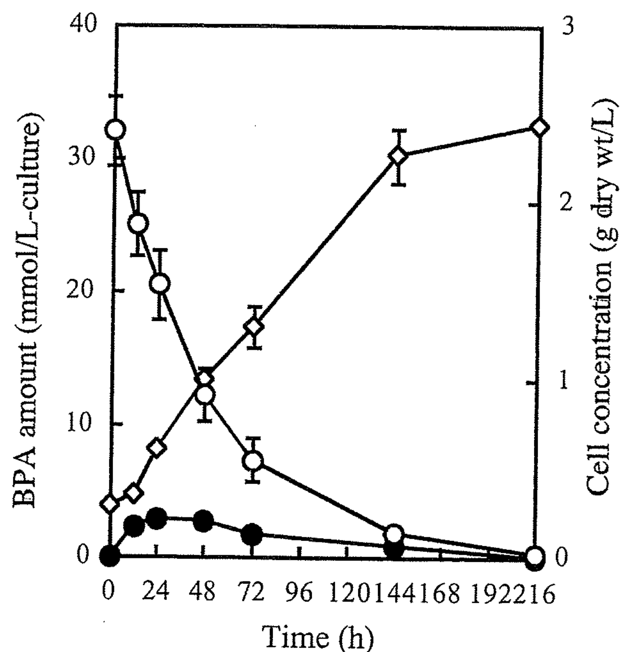

Fig. 4. Removal of bisphenol A (BPA) from the culture medium and change of its amount in the cells. Error bars indicate standard deviations. ○ = BPA amount in the culture medium; ● = BPA amount in cells; ◇ = cell amount.

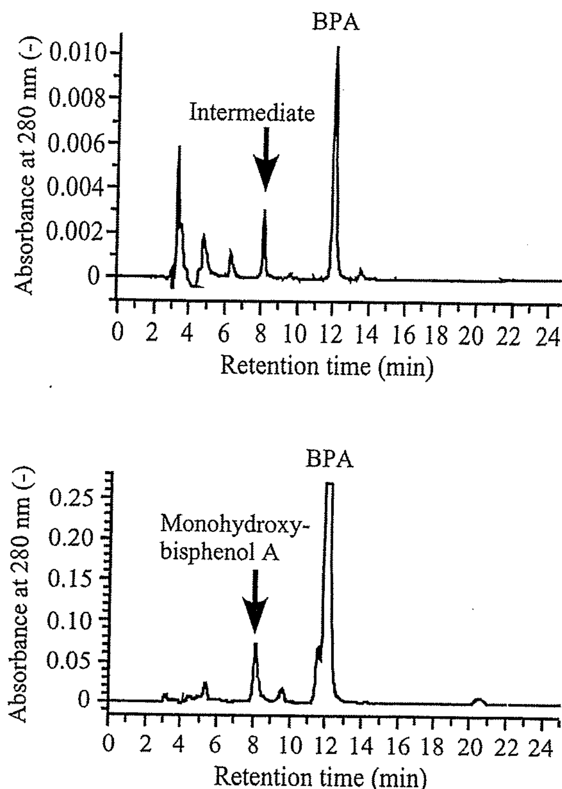

Fig. 5. High-performance liquid chromatograms of oxidized and degraded intermediate of bisphenol A (BPA) by *Chlorella fusca* (**top**) and Fremy's salt (**bottom**).

WH1, isolated from BPA-contaminated soil, removed 900 μM BPA completely from culture for 96 h. Kang and Kondo [17] reported that two *Pseudomonas* strains isolated from a river-water sample in Japan removed approximately 90% of 4 μM BPA for 240 h. However, these heterotrophic bacteria sometimes are difficult to use directly for treatment of BPA in landfill leachates, because the level of biologically available organic carbon sources usually is low. Therefore, it is necessary to add an external organic carbon source to maintain the biomass and the ability to remove BPA. On the other hand, microalgae can grow by using CO_2 as a carbon source. In this case, no need exists to supply any organic carbon sources. Therefore, *C. fusca* is thought to be useful to remove BPA contained in landfill leachates.

Chlorella fusca showed a high ability to remove BPA in the light-intensity range of 2 to 36 W/m^2, although this ability for removal decreased in the dark (Fig. 2). These results suggest that the light-illumination during cultivation is an important factor for the treatment of BPA by *C. fusca*. Bisphenol A also was removed in the light-dark cycle (Fig. 3), indicating that *C. fusca* would be a useful organism for BPA removal in the day-and-night cycle at most temperate or tropical outdoor sites.

As shown in Figure 4, the taken-up BPA was degraded in the cells. The degradation intermediate of BPA by *C. fusca* was identified as monohydroxybisphenol A, and this compound disappeared in the subsequent cultivation. Therefore, monohydroxybisphenol A would be a primary intermediate of BPA degradation in the light. In the case of bacteria, several intermediates of BPA degradation have been reported. The MV-1 strain has two pathways for BPA degradation [15,18]. The major pathway produces two primary intermediates, 4-

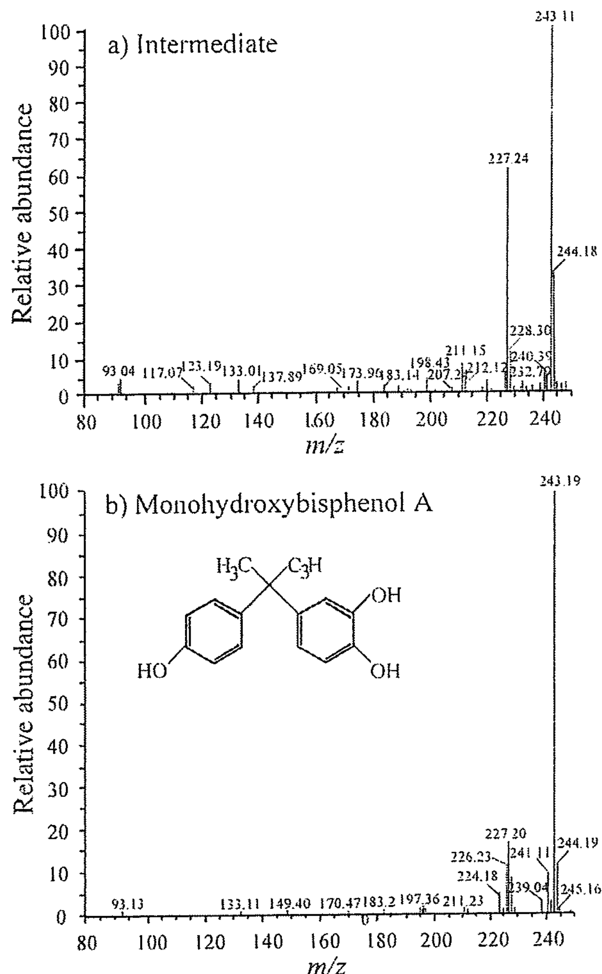

Fig. 6. Liquid chromatography–mass spectra of degraded intermediate of bisphenol A (BPA) by *Chlorella fusca* (a) and monohydroxybisphenol A (b).

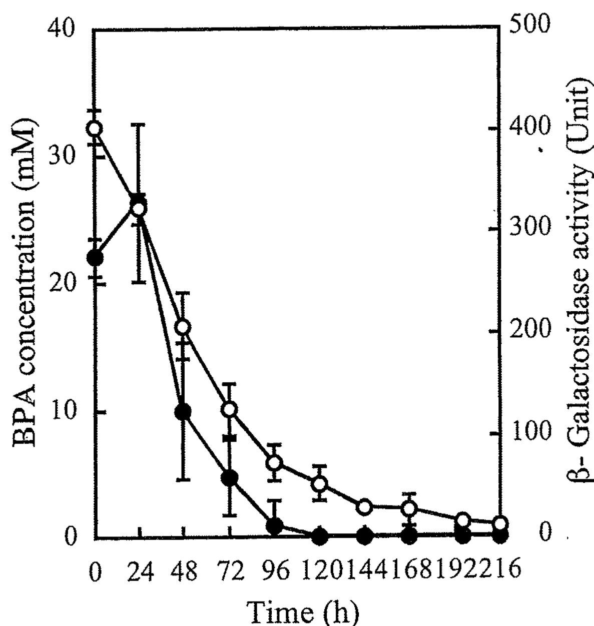

Fig. 7. Change of bisphenol A (BPA) concentration and estrogenic activity in the culture medium. Error bars indicate standard deviations. ○ = residual of BPA; ● = estrogenic activity in the culture medium.

hydroxybenzoic acid and 4-hydroxyacetophenone, and these intermediates were not detected in the case of *C. fusca*. On the other hand, monohydroxybisphenol A was reported as an intermediate of BPA degradation by potato (*Solanum tuberosum*) [19]. The BPA degradation pathway of *C. fusca* is similar to that in higher plants and not bacteria.

The reported degradation intermediates of BPA by heterotrophic bacteria have lower estrogenic activity compared with BPA [20]. The estrogenic activity also disappeared with the degradation of BPA by *C. fusca* (Fig. 7), indicating that *C. fusca* degrades BPA to intermediates with no estrogenic activity, including monohydroxybisphenol A. Therefore, it is possible to decrease the environmental effect of BPA using *C. fusca*.

The present study is, to our knowledge, the first to show that microalga *C. fusca* has the ability to degrade BPA. Microalgae have been employed to remove nitrogen and phosphorus in domestic wastewater by using a large-scale pond system. A similar system would be possible to remove BPA from landfill leachates, although further detailed studies will be required to achieve practical outdoor cultivation of *C. fusca* that maintain a high ability for BPA removal.

Acknowledgement—The present study was partly supported by a grant from the Heiwa Nakajima Foundation.

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Involvement of the Retinoid X Receptor in the Development of Imposex Caused by Organotins in Gastropods

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Organotin compounds released from antifouling paints, such as tributyltin (TBT) and triphenyltin (TPT), are potent inducers of imposex (a superimposition of male genital tracts, such as penis and vas deferens, on females) in marine gastropods. Little is known about the induction mechanism of gastropod imposex. Here, we show that organotins bind the human retinoid X receptors (hRXRs) with high affinity and that injection of 9-cis retinoic acid (RA), the natural ligand of hRXRs, into females of the rock shell (*Thais clavigera*) induces the development of imposex. Cloning of the RXR homologue from *T. clavigera* revealed that the ligand-binding domain of rock shell RXR was very similar to vertebrate RXR and bound to both 9-cis RA and to organotins. These suggest that RXR plays an important role in inducing the development of imposex, namely, the differentiation and growth of male genital tracts in female gastropods.

Introduction

Organotin compounds, such as tributyltin (TBT) and triphenyltin (TPT), have been used worldwide in antifouling paints for ships and fishing nets since the mid-1960s and released into the marine environment resulting in a worldwide pollution (*1*). TBT and TPT are very toxic to organisms, including marine species (*2–5*). One of the most interesting toxic effects of TBT and TPT to marine organisms is the induction of the development of imposex in gastropods (*6*). Imposex (as an abbreviation of imposed sexual organs) is defined to be an irreversible syndrome imposing male genital tracts, such as penis and vas deferens, upon female gastropods (*7*). Gastropod imposex is known to be typically induced by very low concentrations of TBT and/or TPT (*7–18*). Reproductive failure is involved at severely affected stages of imposex, due to either oviduct blockage by vas deferens formation or ovarian spermatogenesis, resulting in population declines and/or mass extinction (*6, 19, 20*). Approximately 150 species of gastropods including the rock shell (*Thais clavigera*) have been observed to be affected by imposex in the world (*6*). Gastropod imposex is thought to be one manifestation of endocrine disruption in wildlife (*6*). Despite several hypotheses about imposex induction mechanisms, such as those involving aromatase inhibition, testosterone excretion–inhibition, functional disorder of female cerebropleural ganglia, and involvement of a neuropeptide–APGWamide (*21–25*), the exact physiological/biochemical pathway is still unclear.

The occurrence of reproductive abnormalities in wildlife may be associated with exposure to environmental pollutants capable of mimicking the action of natural hormones (*26*). The nuclear receptors of intrinsic hormone systems are likely to be targets of industrial chemicals because they are originally mediators for fat-soluble, low molecular weight agents such as steroid hormones, thyroid hormones, fat-soluble vitamins, and fatty acids. Forty-eight members of the nuclear receptor family have been shown to exist in the human genome (*27*). Information on the ability of chemicals to bind nuclear receptor family members is therefore important for environmental risk assessment.

To determine if environmental pollutants can bind to members of the nuclear receptor family, we constructed assay systems for human nuclear receptors including ERα, ERβ, AR, PR, GR, MR, RARα, RARβ, RARγ, TRα, TRβ, VDR, RXRα, RXRβ, RXRγ, CAR, and SXR based on a yeast two-hybrid system (*28*). In the course of the study on suspected endocrine disrupters, we found that TBT and TPT strongly enhanced the protein–protein interaction between human RXRs (hRXRs) and coactivator TIF2 to a somewhat greater extent than 9-cis retinoic acid (RA), the natural ligand of RXR.

Here, we will show the results of interaction between organotin compounds, such as TBT and TPT, and hRXR. We will also report the results of cloning of the RXR homologue from the rock shell (*T. clavigera*), its binding characteristics to both 9-cis RA and organotins, and results of the in vivo injection experiment of 9-cis RA using the rock shell. On the basis of these results, we will discuss involvement of the RXR in the development of imposex caused by organotins in gastropods.

Experimental Methods

Yeast Two-Hybrid Assay. We cloned the ligand-binding domain of nuclear receptors including ERα, ERβ, AR, PR, GR, MR, RARα, RARβ, RARγ, TRα, TRβ, VDR, RXRα, RXRβ, RXRγ, CAR, and SXR by RT-PCR from human mRNA (Origin Technologies, Inc.). All sequences were confirmed to be identical to the database by sequencing. These genes were subcloned into pGBT9 (Clontech, Palo Alto, CA) so that they were in the same translational reading frame as the vector's GAL4 DNA binding domain. pGBT9-NRs and pGAD424-TIF2 were introduced into *Saccharomyces cervisiae* Y190. Transformed yeasts were incubated with test chemicals for 4 h at 30 °C, and then β-galacosidase activity was measured as described in Nishikawa et al. (*28*).

Ligand Binding Assay. The LBD of hRXRα (codons 201–693), hRXRβ (codons 275–534), hRXRγ (codons 172–455), and the rock shell RXR (sRXR) (codons 177–431) were subcloned into pGEX-4T (Pharmacia, Uppsala, Sweden). GST-RXRs fusions were expressed in *Escherichia coli* BL21 and purified according to the standard procedure (Pharmacia, Uppsala, Sweden). The purified proteins (30 μg/mL) were incubated with increasing concentrations of 9-cis-[20-methyl-^{3}H]retinoic acid (69.4 Ci/mmol, NEN Life Science Products, Inc.) with or without a 400-fold molar excess of

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TABLE 1. Body Size of Female Rock Shells (*Thais clavigera*) Used in the Injection Experiment (February 14–March 14, 2003)[a]

	control	RA	TPT
shell height (mm)	21.6 ± 1.8	21.5 ± 1.5	20.3 ± 1.4
shell width (mm)	14.5 ± 1.2	14.4 ± 1.2	14.2 ± 0.5
shell weight (g)	2.0 ± 0.5	1.9 ± 0.5	1.7 ± 0.3

[a] Mean ± standard deviation.

TABLE 2. Quality of Artificial Seawater during the Experimental Period (February 14–March 14, 2003)[a]

	control	RA	TPT
water temp (°C)	18.1 ± 0.1	18.2 ± 0.1	18.2 ± 0.1
pH	8.28 ± 0.02	8.31 ± 0.02	8.31 ± 0.04
salinity (‰)	33.5 ± 1.0	33.5 ± 0.9	33.4 ± 1.0

[a] Mean ± standard deviation.

unlabeled 9-cis RA. After incubation at 4 °C for 1 h, specific binding was determined by hydroxyapatite binding assay (*29*). Similarly, organotin compounds were used to compete for 9-cis RA in this assay to determine the binding preference for RXRs.

DNA Cloning. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using total RNA derived from male *T. clavigera*. Degenerate primers used for amplification of RXR were synthesized as follows: F-primer, 5′-TGYGARGGNTGYAARGGNTTYTTYAARMG-3′; R-primer, 5′-RAAGTGNGGVABNMKYTTVGCCCAYTC-3′. A single 390-bp fragment was obtained and sequenced. The fragment was used as a probe for screening in a cDNA library made with λ-ZAP II phagemid vector (Stratagen, Kirkland, WA). The 5′ end of the cDNA was cloned using 5′-Full RACE Core Set (Takara Bio, Shiga, Japan). The amplified products were analyzed by agarose gel electrophoresis, isolated from the gel, cloned into a pBluescript. Five independent clones were sequenced.

In Vivo Injection Experiment. The rock shell specimens were collected at Hiraiso in Ibaraki Prefecture, Japan, in December 2002 for experiments to investigate the effect of 9-cis RA. The rock shells were reared in a laboratory aquarium for approximately 2 months in artificial seawater (Senju Pharmaceutical Co. Ltd., Japan) with live mussels (*Septifer virgatus*) collected at Hiraiso as feed. Before the experiments, the rock shells were narcotized by exposure to a 72 g/L solution of magnesium chloride hexahydrate to allow the selection of females. As a male rock shell has a large penis behind the right tentacle, female shells were easily recognized by its absence (*16, 18*). The female rock shells were divided

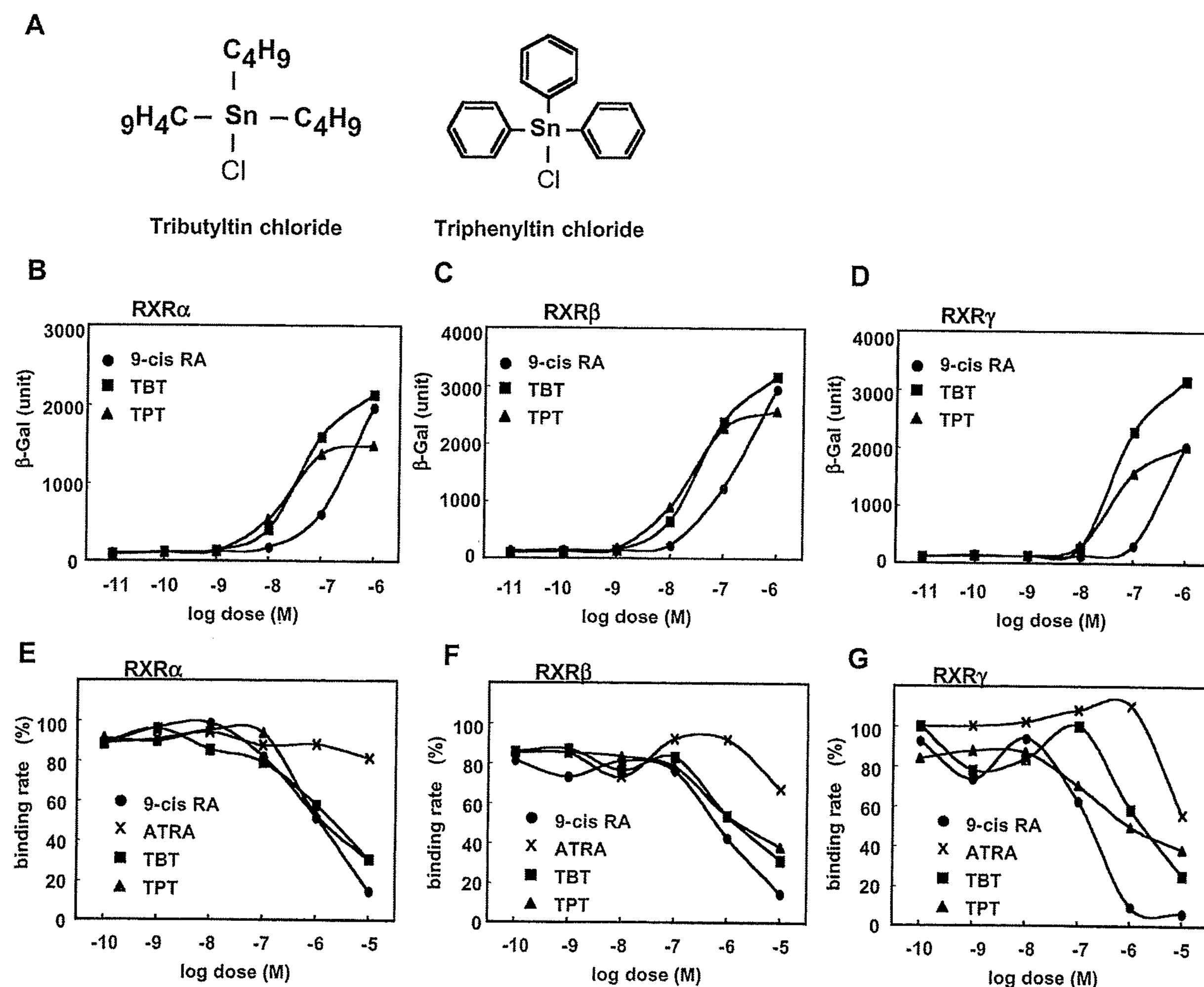

FIGURE 1. RXR activation by TBT and TPT. Structures of organotin compounds are shown in panel A. Yeast strain Y190 was transformed with GAL4AD fused to TIF2 and GAL4DBD fused to LBD of human RXRα (B), RXRβ (C), or RXRγ (D). Chemicals were added to yeast cultures in doses ranging from 10^{-11} to 10^{-6} M. Following 4 h incubation, yeasts were disrupted and assayed for β-galactosidase activity. Data points are means of three independent experiments. For in vitro binding assay, LBDs of RXRα (E), RXRβ (F), or RXRγ (G) were expressed in *E. coli* as fusion proteins with GST. Increasing amounts of chemicals were added to RXRs with 9-*cis*-[20-methyl-^{3}H]retinoic acid for competitive binding assays.

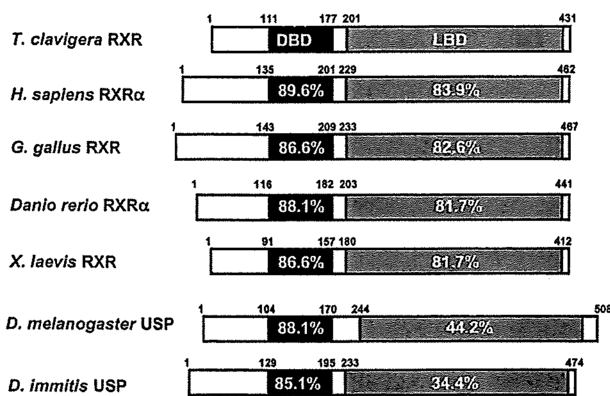

FIGURE 2. Comparison of the deduced amino acid sequences of rock shell RXR with related nuclear receptors. The similarity in the DBD and LBD between rock shell RXR and related nuclear receptors is indicated as percentage amino acid identity. The database accession numbers for the sequences are as follows: *T. clavigera*, AY704160; *H. sapiens*, NM 002957; *G. gallus*, X58997; *Danio rerio*, U29940; *X. laevis*, X87366; *D. melanogaster*, NM 057433; *D. immitis*, AF438230.

into three experimental groups of 20 animals each: for 9-cis RA injection, for triphenyltin (TPT) injection, and for control. 9-cis RA (Wako Pure Chemicals Industries, Ltd., Japan) was prepared in a fetal bovine serum (FBS; Flow Laboratories Inc.) and was injected into the foot at an application rate of approximately 1 μg/g wet wt of soft tissue of the rock shell. FBS was injected to the control animals. Triphenyltin chloride (TPTCl; Tokyo Kasei Kogyo Co., Japan, 98% pure) was used as a positive control agent and was also injected at a rate of approximately 1 μg/g wet wt of soft tissue of the rock shell. The body size of the female rock shells used in the injection experiment is shown in Table 1. After the injection of each test solution, the rock shells were kept in 2 L glass beakers in separate groups, in flow-through systems of artificial seawater saturated with oxygen (10 L/d), with live mussels as feed, for 1 month. Temperature of experimental seawater was maintained to be 18 ± 1 °C. The quality of artificial seawater during the experimental period (February 14–March 14, 2003) is summarized in Table 2. After this time, animals were removed for imposex examination (*16*). Parameters concerning gastropod imposex [the incidence of imposex (percentage occurrence of imposex individuals among females used in the experimental group), mean values of penis length (measured by automatic/digital caliper), and the vas deferens sequence (VDS) index (an index for the degree of development of vas deferens in the imposex-exhibiting female; the VDS index for the rock shell is similar to that for the dog-whelk reported by Gibbs et al.; *12*)] were calculated for each experimental group (*12, 16*), and the statistical significance of any difference to the control group was tested. The statistical significance of the incidence of imposex was determined using Fisher's *t*-test, and an analysis of variance (ANOVA) was carried out for penis length and VDS index (*24*).

Results and Discussion

Interaction between Organotin Compounds (TBT and TPT) and hRXR. We found that TBT and TPT (Figure 1A) strongly enhanced the protein–protein interaction between hRXRs and coactivator TIF2 (Figure 1B–D) to a somewhat greater extent than 9-cis RA, the natural ligand of RXR (*30, 31*). Because the interaction of nuclear receptor with coactivator correlated with transcriptional activity (*32*), organotin compounds, such as TBT and TPT, were thought to be agonists for hRXRs. However, TBT and TPT showed no activity to other nuclear receptors including retinoic acid receptors

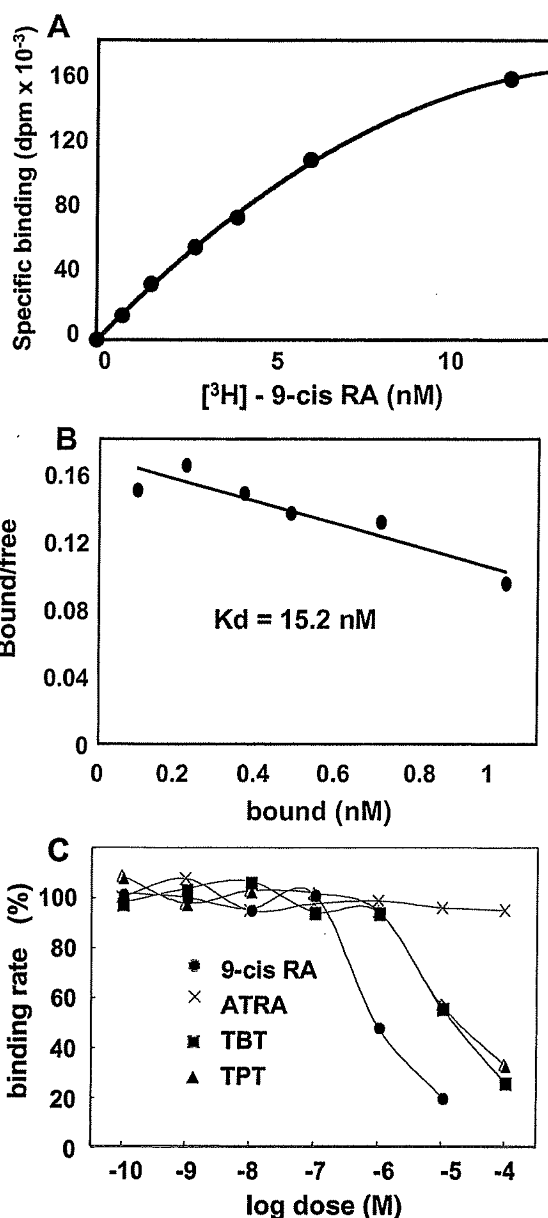

FIGURE 3. 9-cis RA and organotin compounds bind rock shell RXR in vitro. (A) The LBD of rock shell RXR expressed in *E. coli* was incubated with increasing concentrations of ^{3}H-labeled 9-cis RA in the absence (total binding) or presence of 400-fold nonlabeled 9-cis RA (nonspecific binding). Nonspecific binding was subtracted from total binding and plotted as specific binding. (B) Scatchard analysis. Specific 9-cis RA binding to rock shell RXR was transformed by Scatchard analysis and plotted. Linear regression yielded K_d = 15.2 nM. (C) Competition assay. The LBD of rock shell RXR was incubated with increasing concentrations of nonlabeled 9-cis RA, ATRA, TBT, or TPT in the presence of ^{3}H-labeled 9-cis RA.

(RARs) (*33*). While 9-cis RA is known to be a ligand for RARs as well as RXRs (*30*), organotin compounds are specific for RXRs. To confirm the binding of organotin compounds to hRXRs, we carried out an in vitro competition assay against ^{3}H-labeled 9-cis RA and found that TBT and TPT bound to RXRs as well as 9-cis RA (Figure 1E–G). The observation that TBT and TPT could act as agonists for hRXRs led us to investigate the involvement of RXR in the development of imposex in gastropods.

Cloned Rock Shell RXR (sRXR). We tried to clone the RXR cDNA from *T. clavigera*. Comparison of the RXR protein sequences in various species revealed significant similarities in the P-box in the DNA binding domain (DBD) and

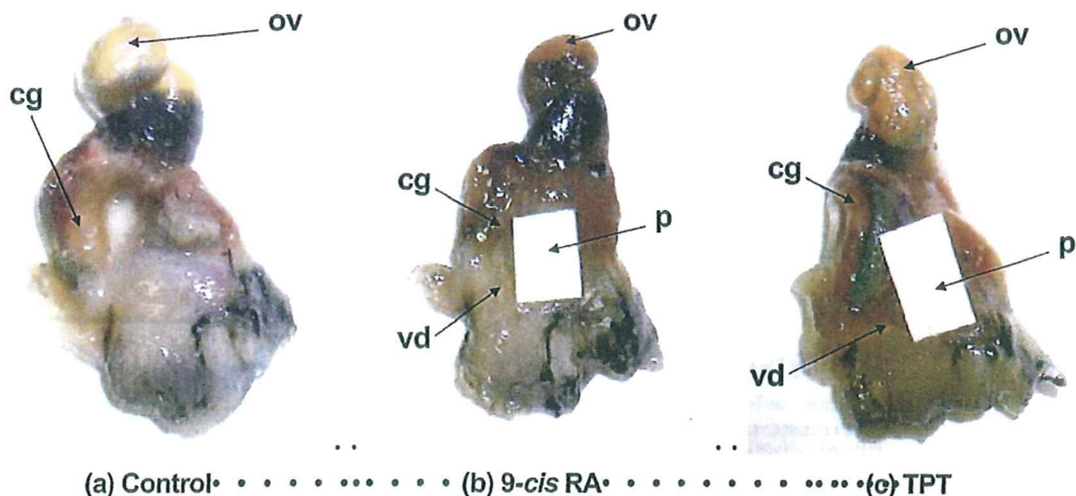

FIGURE 4. Substantial penis growth observed in the female rock shells after 1 month of 9-cis RA injections: cg, capsule gland; ov, ovary; p, penis; vd, vas deferens. (A) Neither penis nor vas deferens was observed in the control female (after shell removal). (B) Substantial penis growth as well as vas deferens development was observed in the female which received 9-cis RA injection (after shell removal; penis length: 6.06 mm). (C) Substantial penis growth as well as vas deferens development was also observed in the positive control female that received TPT injection (after shell removal; penis length: 6.50 mm). Imposex symptoms based on penis length and vas deferens sequence (VDS) index of the females that received 9-cis RA injections were clearly promoted, similar to those of females receiving TPT injections.

helix 4 in the ligand-binding domain (LBD) (*34, 35*). By using degenerate primers deduced from these peptide sequences, we obtained a segment of *T. clavigera* RXR. Next, the *T. clavigera* cDNA library was screened to high precision using the RT-PCR product as a probe. Given that the cDNA isolated by screening was truncated, the 5′ end was amplified by RACE. Comparison of the rock shell RXR (sRXR) protein sequence with the Genebank database revealed that sRXR is closely related to vertebrate RXRs and invertebrate homologues (Figure 2). The highest homology with other species is in the DBD where 85−90% of the amino acids residues are identical (Figure 2). The LBD of sRXR also shows considerable homology with vertebrate RXRs but much less homology to ultraspiracle (USP), the RXR homologue found in *Drosophila*.

Ligand Binding Assay. Vertebrate RXRs bind to 9-cis RA, but insect USP does not (*30, 31, 36*). The LBD of sRXR protein, expressed after fusion with GST in bacteria, bound to 9-cis RA with $K_d = 15.2$ nM (Figure 3A,B), similar to values reported for vertebrate RXRs (*30*). These data implied that *T. clavigera* RXR could bind to 9-cis RA, even though *T. clavigera* is a gastropod mollusk. The sRXR fusion protein also bound to organotin compounds, such as TBT or TPT (Figure 3C). On the other hand, sRXR did not bind to all-trans RA (ATRA) in contrast to human RXRs that bind to ATRA even with low affinity (*30*) (Figure 3C; Figure 1E−G). The jellyfish RXR has also been reported to bind 9-cis RA with high affinity but not to ATRA (*37*).

In Vivo Injection Experiment To Examine the Involvement of RXR in the Development of Imposex in *Thais clavigera*: Effect of 9-cis RA Inducing and/or Promoting the Development of Imposex. To further verify the involvement of RXR in the development of imposex in gastropods, live female rock shells (*T. clavigera*) collected at Hiraiso in Ibaraki Prefecture, Japan (an area of low organotin contamination: see Horiguchi et al.; *18*) were injected with 9-cis RA. Results of these experiments are shown in Table 3 as well as Figure 4. Imposex was significantly induced in female *T. clavigera*, which received the injection of 9-cis RA ($p < 0.01$; Table 3), and substantial penis growth was observed in them after 1 month of 9-cis RA injections (Table 3; Figure 4). Their increased penis length and VDS index were significant when compared with controls ($p < 0.01$ and $p < 0.001$, respectively; Table 3).

TABLE 3. Incidence of Imposex (IOI), Penis Length (PL), and Vas Deferens Sequence Index (VDS) in Female Rock Shells (*Thais clavigera*) after 1 Month of Injections[a]

	control	RA	TPT
IOI (%)	10	50**	80**
PL (mm)	0.04 ± 0.13	2.87 ± 2.39**	3.77 ± 2.16***
VDS	0.20 ± 0.63	3.80 ± 0.42***	3.63 ± 0.74***

[a] Mean ± standard deviation. **, $p < 0.01$. ***, $p < 0.001$.

These results suggest that much 9-cis RA could bring about induction and/or promotion of the development of imposex in *T. clavigera* through its binding to RXR. Relatively large variance for the penis length in females that received injections of 9-cis RA may have resulted from differences in the rate of metabolism of 9-cis RA among female rock shells used in the experiment, although it is not known if *T. clavigera* inherently has a biosynthetic system for RA.

9-cis RA is the first substance, except for certain organotin compounds, that has been confirmed to induce and/or promote the development of imposex in gastopods, especially in terms of penis growth in females. As both TBT and TPT were observed to have agonistic activity to the RXR, it is strongly suggested that gastoropod imposex could be mediated by RXR.

Mode of Action of Organotins on the Development of Imposex in Gastropods. Several hypotheses have been proposed concerning the imposex induction mechanism, and they can be summarized as (i) increased androgen levels, such as testosterone, due to aromatase inhibition by TBT (*21*); (ii) inhibition by TBT of the excretion of sulfate conjugates of androgens (*22*); (iii) disturbance by TBT of penis morphogenic/retrogressive factor released from pedal/cerebropleural ganglia (*23*); and (iv) increase in a neuropeptide, APGWamide, level caused by TBT (*24, 25*). Experimental evidence, however, is weak for these four hypotheses. There is a lack of correlation between the time course of the increase in testosterone titers and penis growth in females in the aromatase inhibition hypothesis (*21*), and there is a possibility that the results given in support of the testosterone excretion−inhibition hypothesis (*22*) may reflect a phenomenon that is at least partly short-term and/or associated with acutely

toxic TBT concentrations (*20*). The effect of APGWamide to induce and/or promote the development of imposex also appears weak based on experimental results of incidence of imposex and penis growth (*24*, *25*).

In addition, it should be noted that substantial penis length has been observed in natural populations of imposex-exhibiting females distributed in coastal areas severely contaminated with TBT and/or TPT, as well as in females that received injections of or were exposed to TBT or TPT in the laboratory (*8*–*12*, *16*, *18*), and that little is known about basic endocrinology in invertebrates including mollusks (*38*). The penis length in female gastropods observed in the experiments given in support of the aromatase inhibition hypothesis, and the APGWamide involvement hypothesis was small (*21*, *24*, *25*). This contradiction concerning imposex development, especially penis length in imposex-exhibiting females, strongly suggested that gastropod imposex could be primarily induced and promoted by a factor other than increased androgen levels caused by aromatase inhibition or the neuropeptide, APGWamide. Moreover, there has not been any experimental evidence on purified aromatase protein itself (or aromatase at the protein level) in invertebrates, but only reports on aromatase-like activity in invertebrates including mollusks (*39*–*41*). The role of steroid sex hormones, similar to those of vertebrates, are still uncertain in invertebrates, because certain peptides have been reported to act as sex hormones in invertebrates such as *Aplysia californica* (Mollusca: Opisthobranchia), *Lymnaea stagnalis* (Mollusca: Plumonata), and *Armadillidium vulgare* (Arthropoda: Malacostraca) (*42*–*44*). In contrast, RXR is rather well-conserved from invertebrates to vertebrates (Figure 2).

In this paper, we have shown that TBT and TPT are high affinity ligands for RXR and that the natural ligand of RXR significantly caused the development of imposex in female rock shells. These results imply that RXR plays an important role in the induction/differentiation and growth of male genital tracts in female gastropods. Further studies on a heterodimer partner, coupling factors, and target genes of sRXR with molecular biological and immunohistochemical techniques are necessary to clarify the entire mode of action of TBT and/or TPT on the development of imposex in gastropods.

RXRs are key factors involved in the mediation of several hormone response systems via their association with other nuclear receptors as heterodimer partner (*45*). The knockouts of RXRs in the mouse have provided important information in the physiological functions of these receptors. RXRα null mice died in utero and exhibited a hypoplasic ventricular myocardium and ocular abnormalities (*46*, *47*). Approximately 50% of RXRβ null mice died before or at birth, and males of the remaining null mutants were sterile, owing to the aberrant lipid metabolism in Sertoli cells (*48*). On the other hand, 9-cis RA is difficult to detect in vivo, and its action is remained to be obscure (*49*). Our result that injection of 9-cis RA into female gastropods induced and/or promoted the development of imposex may provide some insight into the physiological function of 9-cis RA.

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