Table 1
Effect of organotin and related chemicals on HAT activity

Chemical	Abbreviation	Relative HAT activity[a]	CAS no.	Purity (%)	Source
Triphenyltin chloride	TPT	$2.03 \pm 0.13^{**}$	639-58-7	>95	Aldrich Chemicals
Diphenyltin dichloride	DPT	$1.63 \pm 0.061^{**}$	1135-99-5	>96	Aldrich Chemicals
Monohenyltin trichloride	MPT	0.97 ± 0.026	1124-19-2	>98	Aldrich Chemicals
Tributyltin chloride	TBT	$2.22 \pm 0.13^{**}$	1461-22-9	>95	Tokyo Kasei Kogyo
Dibutyltin dichloride	DBT	$1.81 \pm 0.045^{**}$	683-18-1	>97	Tokyo Kasei Kogyo
Monobutyltin trichloride	MBT	1.01 ± 0.037	1118-46-3	>95	Aldrich Chemicals
Tin chloride	$SnCl_4$	0.91 ± 0.025	10025-69-1	>97	Nacalai tesque
Tetrabutyltin	TetBT	1.05 ± 0.042	1461-25-2	>93	Aldrich Chemicals
Trimethyltin chloride	TMT	0.95 ± 0.011	1066-45-1	>98	Aldrich Chemicals
Triethyltin bromide	TET	$1.27 \pm 0.034^{*}$	2767-54-6	>97	Aldrich Chemicals
Tripropyltin chloride	TPrT	$3.09 \pm 0.080^{**}$	2279-76-7	>98	Merck Schuchardt
Triphenylsilanol	TPSiOH	1.14 ± 0.23	791-31-1	>95	Merck Schuchardt
Triphenylmethane	TPM	0.95 ± 0.12	519-73-3	>98	Kanto Chemical
Triphenylethylene	TPE	0.91 ± 0.12	58-72-0	>98	Kanto Chemical

[a] Core histones and 10 μM of chemicals were used for the HAT assay. Relative HAT activity shows mean HAT activities (±S.D.) relative to a control treated without chemical (three independent experiments). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to control.

1% protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan)), and 3 M KCl was added to a final concentration of 0.55 M. Nuclear lysate was gently mixed on ice for 30 min and centrifuged at 40,000 rpm in a Beckman 50.2 Ti rotor (Beckman, Fullerton, CA, USA) for 40 min at 4 °C. The RLNE was diluted by adding the same volume of nuclear lysis buffer without KCl and $MgCl_2$ and then incubating with preequilibrated Ni^{2+}-NTA agarose overnight at 4 °C on a rotating wheel. This suspension was poured into a column and the flowthrough was collected. The column was then washed with five column volumes of the nuclear lysis buffer without KCl and $MgCl_2$, and the retained proteins were eluted with a solution consisting of 20 mM imidazole (pH 7.5), 100 mM KCl, 10% glycerol, 5 mM 2-mercaptoethanol, and 1% protease inhibitor cocktail.

2.3. Western blotting and antibodies

Ten microliters of each fraction of column eluate was electrophoresed on an SDS-polyacrylamide gel, transferred to nitrocellulose, and detected with an ECL Western blotting analysis detection system (Amersham Biosciences, Piscataway, NJ, USA). Antibodies against P300, CBP, and GCN5 were obtained from Santa Cruz Biotech (Santa Cruz, CA, USA). Anti-PCAF antibody was kindly provided by Dr. Yoshihiro Nakatani (Harvard Medical School, Boston, MA, USA).

2.4. HAT assay

HAT assays were performed as follows: 1.5 μg core histones or nucleosome histones was incubated together with 5 μl of eluate from the Ni^{2+}-NTA agarose column, ^{3}H-labeled acetyl-CoA (0.25 μCi), and 1.2 μl of the test compound in 30 μl of HAT buffer (50 mM Tris–HCl, pH 8.0, 50 mM KCl, 5% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium butyrate) at 30 °C for 30 min. Histones were prepared as described previously (Owen-Hughes et al., 1999). After incubation, 15 μl of reaction mixture was transferred to a P81 phosphocellulose filter (Whatman, Brentford, UK) and allowed to air-dry. Filters were washed three times in wash buffer (50 mM $NaHCO_3$-$NaCO_3$, pH 9.2) and air-dried. The samples were counted in a scintillation counter (Beckman) for 10 min. The remaining 15 μl was subjected to SDS-polyacrylamide gel electrophoresis (PAGE). All gels were stained with Coomassie Brilliant blue to ensure loading of equivalent amounts of histone in each lane, then destained and flourographed.

2.5. Statistics

All results are expressed as means ± standard deviations (S.D.). Statistical analysis was performed by Dunnett's method.

3. Results

3.1. Partial purification of HAT complex from RLNE

To observe the effects of suspected EDCs on HAT activity, we used partly purified HAT complex from RLNE. Many HATs function as catalytic subunits in HAT complexes, and the specificity and the activity of HAT complexes are different from those of recombinant HATs. For example, recombinant GCN5 can acetylate core histones well, but it exhibits poor nucleosomal HAT activity (Balasubramanian et al., 2002; Grant et al., 1997). Recombinant SAS2 does not show HAT activity, but a complex including SAS2 can acetylate histones (Sutton et al., 2003). Grant and coworkers reported that some native HAT complexes in yeast bind to Ni^{2+}-NTA agarose (Grant et al., 1997). We wondered whether native mammalian HAT complexes bind to Ni^{2+}-NTA agarose. RLNE was incubated with Ni^{2+}-NTA agarose, and bound proteins were eluted with a buffer containing imidazole. Bound fraction included at least four HATs: GCN5, PCAF, P300, and CBP (Fig. 1).

3.2. Effect of TBT and TPT on core HAT activity

Nickel and curcumin (diferuloylmethane) were found to inhibit the acetylation of histones in vitro using recombinant histone acetyltransferase (Balasubramanyam et al., 2004; Broday et al., 2000), indicating that heavy metals and low molecular compounds may affect HAT activity. Suspected EDCs were screened for inhibition and activation of the HAT activity of partly purified HATs by the standard HAT assay procedure using core histone as substrate (Eberharter et al., 1998) and 20 chemicals in the list of chemicals suspected of having endocrine disrupting effects, as published by the Strategic Programs on Environmental Endocrine Disrupters '98, the Japan Environmental Agency (www.env.go.jp/en/pol/speed98/sp98.pdf). Tested 20 chemicals (benzophenone, octachlorostyrene, diethyl phthalate, butyl benzyl phthalate, diethylhexyl adipate, TPT, diethylhexyl phthalate, dicyclohexyl phthalate, di-*n*-butyl phthalate, TBT, 4-nonylphenol, *p*-octylphenol, bisphenol A, 2,4-dichlorophenol, 4-nitrotoluene, di-*n*-pentyl phthalate, dipropyl phthalate, pentachlorophenol, amitrole, and dihexyl phthalate) did not inhibit HAT activity. Interestingly TBT and TPT enhanced HAT activity but other chemicals did not (Fig. 2A and data not shown). HAT activity in the presence of various concentrations of TBT and TPT was assayed, and both chemicals demonstrated dose-dependent enhancement of HAT activity (Fig. 2A). TBT and TPT at both 1 and 10 μM promoted HAT activity but at 0.1 μM had little, if any, effect. Fluorography indicated that partly purified HATs from RLNE acetylated histones H2A, H3 and H4 and that these histones were more effectively acetylated in the presence of TBT or TPT (Fig. 2A, middle panel). A partly purified HAT fraction includes several kinds of HATs and HDACs, but TBT and TPT did not show HDAC inhibitory activity (data not shown). HDACs are classified into three groups, class I, II, and III. Assay reaction mixtures include butyrate, an inhibitor for class I and II HDACs (Ajamian et al., 2004). Further, TBT and TPT enhanced HAT activity in the presence of nicotinamide, an inhibitor for class III HDACs (Bitterman et al., 2002) (data not shown). These results also indicate that TBT and TPT do not inhibit HDAC activity.

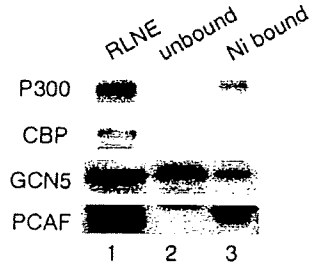

Fig. 1. Partial purification of HATs from RLNE. Western blots were performed with RLNE (lane 1), flow-through from a Ni^{2+}-NTA agarose column (unbound fraction, lane 2), and eluate from a Ni^{2+}-NTA agarose column (Ni-bound, lane 3) using antibodies against P300, CBP, GCN5, and PCAF.

We investigated the effects of organotin and related chemicals on HAT activity. TBT is metabolized to dibutyltin (DBT), monobutyltin (MBT), and inorganic tin; and TPT is metabolized to diphenyltin (DPT), monophenyltin (MPT), and inorganic tin (Horiguchi et al., 1997). The effects of these metabolites of the organotin compounds on HAT activity were also analyzed (Table 1). DBT and DPT enhanced HAT activity, but MBT, MPT, and $SnCl_4$ had no effect. DBT and DPT showed less enhancement of HAT activity than TPT and TBT, but tetrabutyltin did not affect HAT activity (Table 1). These results indicate that trialkyltin com-

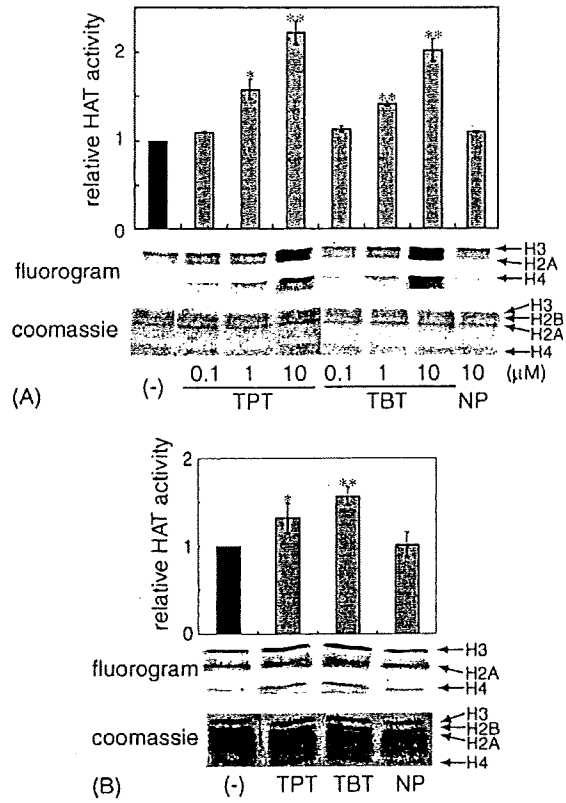

Fig. 2. Effect of TPT and TBT on HAT activity. (A) HAT assays with core histones as substrates and partly purified HAT fraction from RLNE as enzyme. The bar graph shows mean HAT activities (±S.D.) relative to a control treated without chemical (three independent experiments). 4-nonylphenol (NP) was used as a negative control. $^{*}P<0.05$ and $^{**}P<0.01$ compared to control. Products of HAT assays were separated by SDS-PAGE on 18% polyacrylamide gels to resolve the histones. The gels were stained with Coomassie Brilliant blue to visualize proteins (lower panel) and were then dried and visualized by fluorography (middle panel). (B) HAT assays in the absence or presence of chemicals (10 μM), TPT, TBT, and NP, using nucleosome histones as substrates were performed in triplicate as described in the legend to (A). A typical fluorogram from three independent experiments that showed similar results is shown.

pounds are more effective enhancers than other alkyltin compounds.

Next, we focused on the number of carbon alkyl chain in the trialkyltin compounds. Trimethyltin (TMT) did not affect HAT activity, and triethyltin (TET) was a poor promoter of HAT activity. Interestingly, tripropyltin was a more effective enhancer than the compounds containing one or more butyl or phenyl groups. The compounds containing carbon or silicon in place of tin were used to determine whether tin is essential for the promotion of HAT activity by organotin compounds. However, triphenylsilanol, triphenylmethane, and triphenylethylene did not affect HAT activity (Table 1).

3.3. Effect of TBT and TPT on nucleosomal HAT activity

Several transcription co-activators possess HAT activity, and the acetylation of nucleosomes associated with the promoter is correlated with transcriptional activation (Ikeda et al., 1999; Sterner and Berger, 2000; Utley et al., 1998). To investigate the effect of TPT and TBT on the nucleosomal HAT activity, we used nucleosomal histones as substrates instead of core histones (Fig. 2B). The partly purified HAT fraction from RLNE includes HAT activity for nucleosome histones, which was promoted by adding TPT and TBT to the reaction. This means that these compounds enhance the core HAT activity as well as the nucleosomal HAT activity.

4. Discussion

We demonstrated that HAT activity is enhanced by certain organotin compounds, including TPT and TBT. Here we found that: (1) trialkyltin compounds are more effective enhancers of HAT activity than mono- and dialkyltin compounds; (2) tin compounds with short alkyl chains showed no effect; and (3) the tin atom is important for the enhancement of HAT activity. On the basis of these results, it is likely that the acetyl CoA binding pocket (active site) or the substrate-binding site of HATs can tolerate a small compound, such as TMT or TET. These results suggest that both a carbon chain of a suitable length and a charge of tin are required for the regulating HAT activity. The crystal structure of HATs with acetyl CoA and/or histone has been determined (Dutnall et al., 1998; Rojas et al., 1999; Yan et al., 2000). Therefore, this information might be useful for analyzing the molecular mechanism of the enhancement of HAT activity by organotin compounds. However, we cannot rule out an alternative possibility that the organotin compounds affects histones. For example, organotin compounds may release histones from a inhibitor of acetyltransferase complex, which binds to histones and masks them from being HAT substrates

(Seo et al., 2001), or change the structure of the histone tails and making them better substrates. Zoroddu et al. propose that the binding of Ni (II) can produce a secondary structure with organized side-chain orientation in the amino terminal tail of histone H4 (Zoroddu et al., 2002). Some compounds including heavy metal(s) may affect histones. We used a partly purified HAT fraction so that the HAT complexes would be in their native form. To clarify the mechanism of HAT activity enhancement by organotin compounds, we are proceeding with identification of the specific HAT whose activity is activated by organotin compounds.

A cDNA microarray analysis revealed that expression of about 130 genes was induced by treatment of the ascidian *Ciona intestinalis* with TBT (Azumi et al., 2004). We previously reported that the level of the mRNA for aromatase/CYP19, which is essential for converting androgenic to estrogenic steroids, was increased by treatment of human choriocarcinoma JAR cells with TBT (Nakanishi et al., 2002). The mechanism of the induction of these mRNAs by TBT has not been elucidated yet. Aberrant HAT activity induced by TBT treatment might cause unusual expression of these genes.

HAT activity is required for the regulation of gene expression and histone acetylation has a fundamental biological role. Organotin compounds have various influences on physical function including the hormone and immune systems, embryogenesis, development, etc. In previous studies, reasonable butyltin concentrations were detected in human liver and blood (Kannan et al., 1999; Lo et al., 2003). Aberrant HAT activity in vivo induced by organotin compounds may cause abnormal development in human and wildlife. Our data indicate that the organotin compounds have unique effects on HATs independent of their EDC activities and suggest that the varied toxicities of the organotin compounds may be caused by aberrant gene expression following altered histone acetylation.

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Rapid Communication

Cloning and Characterization of a cDNA Encoding the Histone Acetyltransferase MOZ (Monocytic Leukemia Zinc Finger Protein) in the Rat

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Many DNA-binding transcription factors require coactivators for their function. Some of these coactivators have histone acetyltransferase (HAT) activity, which is important for transcription from chromatin template. We cloned a cDNA encoding the rat homolog of monocytic leukemia zinc finger protein (MOZ), a member of the MYST (MOZ, Ybf2/Sas3, Sas2, and Tip60) acetyltransferase family. Rat MOZ (rnMOZ) encoded 1998 amino acids and was composed of 16 exons. Comparison of the rnMOZ and human MOZ amino acid sequences revealed 89% identity over the whole sequence and 100% identity in the MYST region, which is essential for HAT activity. Further, we identified physical interaction between rnMOZ and basic leucine zipper (bZIP)-type DNA-binding proteins, including c-Jun and CCAAT/enhancer binding proteins. This finding suggests that MOZ may function in multiple cellular processes through various bZIP-type transcription factors.

Key words: histone acetyltransferase, MOZ, transcription, c-Jun, C/EBP, bZIP family

INTRODUCTION

Transcription of eukaryotic genes is controlled by various regulatory elements, termed promoters, enhancers, and silencers. These elements are recognized by sequence-specific DNA-binding proteins.[1] A virtual explosion of information in the field of eukaryotic gene regulation has revealed that many DNA-binding transcription factors require coactivators that have histone acetyltransferase (HAT) activity.[2] Active chromatin has been associated with hyperacetylation of histones, binding of transcriptional regulators, and active transcription.[3]

Some HATs in multiple-protein complexes are recruited by DNA-binding transcription factors to chromatin, whereas others are physically associated with DNA-binding proteins. MOZ is a member of the MYST (MOZ, Ybf2/SAS3, SAS2, and TIP60) family of HATs, which play key roles in various nuclear functions[2] and frequently are rearranged in leukemia.[2,4] Only human MOZ (hsMOZ) cDNA had been cloned previously.[5] Although the MOZ complex has been identified,[4] physical interaction between MOZ and DNA-binding transcription factors has not yet been reported. Candidates for associating factors include the basic region leucine zipper (bZIP) proteins, which are a large class of transcription factors including c-Jun, c-Fos, and CCAAT/enhancer binding proteins (C/EBPs) that are crucial for cell proliferation, cell differentiation, and cancer development.[6]

We cloned rat MOZ (rnMOZ) cDNA and showed that rnMOZ interacted with various bZIP-type transcription factors.

MATERIALS AND METHODS

Cloning of rnMOZ cDNA and Plasmid Construction—All animal care and handling procedures were approved by the animal care and use committee of Osaka University. Total RNA was prepared from Wister rat liver by using TriZol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. The open reading frame (ORF) of rnMOZ was

amplified by reverse transcriptase – polymerase chain reaction (RT-PCR); PCR conditions are found in Table 1. The seven fragments we obtained were subcloned into pBluescript KS (Stratagene, La Jolla, CA) and sequenced. To subclone the full-length rnMOZ ORF into the *Eco*RI–*Not*I site of the mammalian expression vector pCI (Promega, Madison, WI), cDNA fragments were assembled using suitable internal restriction sites. Glutathione *S*-transferase (GST)-fused rat c-Jun constructs were generated by PCR from pRJ101[7] (kindly provided Dr. Masayoshi Imagawa of Nagoya City University). GST-c-Jun (FL) (contains full-length Jun), GST-c-Jun (AD) (contains the Jun transactivation domain), and GST-c-Jun (DBD) (includes the DNA-binding domain of Jun) were constructed by inserting fragments corresponding to amino acid residues 1–334, 1–146, and 257–334, respectively, into the *Bam*HI–*Xho*I site of pGEX4T-1 (Amersham Biosciences, Piscataway, NJ). All fragments amplified by PCR were verified by DNA sequencing. GST-fused C/EBPα and C/EBPβ expression plasmids[8] were kindly provided by Dr. Robert Hache of Ottawa University.

GST Pull-down Assay—GST fusion proteins were expressed in *Escherichia coli* as described by the manufacturer (Amersham Biosciences) and cross-linked to glutathione–Sepharose 4B with dimethylpimelimidate. [^{35}S]-labeled MOZ protein was produced from pCI-MOZ by *in vitro* transcription–translation with the TNT T7-coupled reticulocyte lysate system (Promega). A 5 μl aliquot of the reticulocyte lysate reaction containing [^{35}S]-labeled MOZ protein was incubated for 3 h at 4 °C with GST fusion proteins in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM KCl, 10% glycerol, 0.1% Tween 20, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. After extensive washes, bound proteins were separated by SDS – polyacrylamide gel electrophoresis and detected by autoradiography.

RESULTS AND DISCUSSION

Molecular Cloning of cDNA Encoding rnMOZ

To generate PCR primers for identification of rnMOZ cDNA by RT-PCR, we compared the sequence of human MOZ (hsMOZ, GenBank accession no. U47742) with mouse expressed sequence tags (accession nos. AK028058, BC024786, and AK054354) and designed our primers on the basis of sequences identical between human and mouse. Seven fragments amplified from rat total RNA were sequenced and covered the entire ORF. The predicted primer-generated sequences, except for primers including the translation start and stop codons, were confirmed by sequencing of the amplified regions. The rnMOZ ORF (accession no. AB195309) included 5994 nucleotides and encoded 1998 amino acids (Fig. 1A).

Comparison of rnMOZ cDNA with Rat Genome Sequence

The genome sequence of the Brown Norway rat, recently determined by the Rat Genome Sequencing Project Consortium, is available in the international sequence databases.[9] Comparison of rnMOZ with the genomic sequence revealed that the *rnMOZ* gene was located at 16q12.5 and comprised 16 exons spanning >75 kb (Fig. 1B); all of the exon–intron boundaries were consistent with the GT/AG rule (data not shown). The sequence of rnMOZ cDNA was the same as the genomic sequence, except that the C, T, and C at nucleotides 3699, 4329, and 5058 of the cDNA were T, G, and T, respectively, in the genome. These differences may be due to differences in the stem lines. The predicted sequences of primers including the translation start and stop codons were also identical to the genome sequences, except that A at position 9 of cDNA was G in the genome sequence; however the predicted amino acids of rnMOZ were completely identical with those from the genomic sequence.

Comparison of rnMOZ with hsMOZ

Functional domains of hsMOZ have been identified.[10] Comparison of rnMOZ with hsMOZ revealed 89% identity over the whole sequence, and the percentage of identity at each domain is shown in Fig. 1C. All domains were highly conserved. In particular, the sequences of the MYST, PHD, and Met-rich domains, which are important for HAT activity and transcriptional regulation, were nearly identical between the orthologs.

Association of rnMOZ with bZIP-type Transcription Factors

Recruitment of HAT activity by DNA-binding transcription factors is a trigger for gene expression from chromatin. We observed the *in vitro* interaction between rnMOZ and various DNA-binding proteins by using the GST pull-down assay. c-Jun is a bZIP-type DNA-binding transcription factor,[6] and the GST pull-down assay revealed that [^{35}S]-labeled MOZ was retained on beads cross-linked with GST-c-Jun (FL) but not with GST alone. This finding suggests that rnMOZ is physically associated with c-Jun. The transactivation domain (AD) and the DNA-binding domain (DBD) of c-Jun have been determined.[11] We tried to identify the region in c-Jun that is required for interaction with rnMOZ and found that DBD, but not AD, of c-Jun was necessary for interaction with rnMOZ (Fig. 2). Further, we showed that both C/EBPα and C/EBPβ—other bZIP family members—also physically associate with rnMOZ (Fig. 2). These findings are the first evidence that MOZ interacts with DNA-binding transcription factors, and they suggest that MOZ may influence multiple cellular functions through bZIP-type transcription factors.

Acknowledgements This research was supported in part by grants from the Japanese Ministry of Education, Science, Sports, and Culture and by the Sasakawa Scientific Research Grant from the Japan Science Society. We also thank the staff of the Radioisotope Research Center, Osaka University.

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Table 1. PCR Condition

Amplifed Position	Primer Sequence	Denature	Annealing	Extension	Cycles
1- 993	5'-ATAGAATTCATGGTAAAACTCGCTAACC-3' 5'-AAGCGGCCGCGTTAATAGCGCCGTTTTATC-3'	94 °C, 1 min	54°C, 1 min	72°C, 1 min	36
804-2336	5'-TCGCGATCAAGGCAAAAACG-3' 5'-ACTATGACTGGAGTCCAGCG-3'	94 °C, 1 min	54°C, 1 min	72°C, 2 min	43
1969-3524	5'-GGCAGGTTTCTCATCGATTTCA-3' 5'-TTAAATCCTGGTTTCCGTCCAGG-3'	94 °C, 1 min	54°C, 1 min	72°C, 2 min	40
3271-4722	5'-AAGACATCCTTAGGTGTCAGGCTT-3' 5'-GTTATTCCCACAAATACTGCTG-3'	94 °C, 1 min	56°C, 1 min	72°C, 1.5 min	40
4576-5386	5'-AAATGGATGTGCCTTCCGTATC-3' 5'-AACGGCTAAGGGATGAGATGGA-3'	94 °C, 1 min	56°C, 1 min	72°C, 2 min	40
4576-5997	5'-AAATGGATGTGCCTTCCGTATC-3' 5'-TTTGCGGCCGCATCATCTTCTCATGTAAGG-3'	94 °C, 1 min	54°C, 1 min	72°C, 2 min	40
5100-5997	5'-ATTGAATTCATGAACAACAGCTTCACTGC-3' 5'-TTTGCGGCCGCATCATCTTCTCATGTAAGG-3'	94 °C, 1 min	56°C, 1 min	72°C, 1 min	36

(A)

(B)

(C)

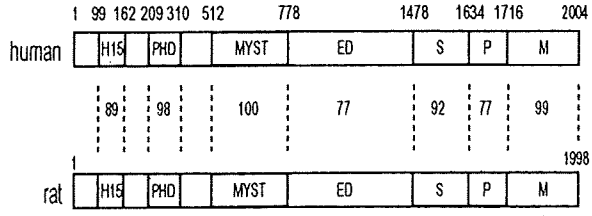

Fig. 1. Molecular Cloning of cDNA Encoding rnMOZ

(A) Deduced amino-acids sequence of rnMOZ. The numbers on the left refer to the corresponding to amino acid positions. (B) Schematic representation of the rnMOZ gene structure. The boxes indicate the relative sizes and positions of exons. (C) Comparison of the functional domains of orthologous MOZs. H15, histone H1- and H5-like motif; PHD, plant homeodomain zinc fingers; MYST, MYST acetyltransferase domain; ED, S, P, and M are Glu/Asp-, Ser-, Pro-, and Met-rich regions, respectively.

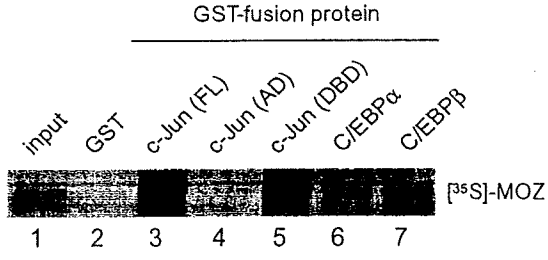

Fig. 2. Association of rnMOZ with bZIP-type transcription Factors

[^{35}S]-MOZ was incubated with GST (lane 2) or GST-fused c-Jun (FL), c-Jun (AD), c-Jun (DBD), C/EBPα, and C/EBPβ (lanes 3-7). MOZ protein retained on the GST-conjugated beads after extensive washes was analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The amount of input (lane 1) was equivalent to 10 % of the reaction in the assay.

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

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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

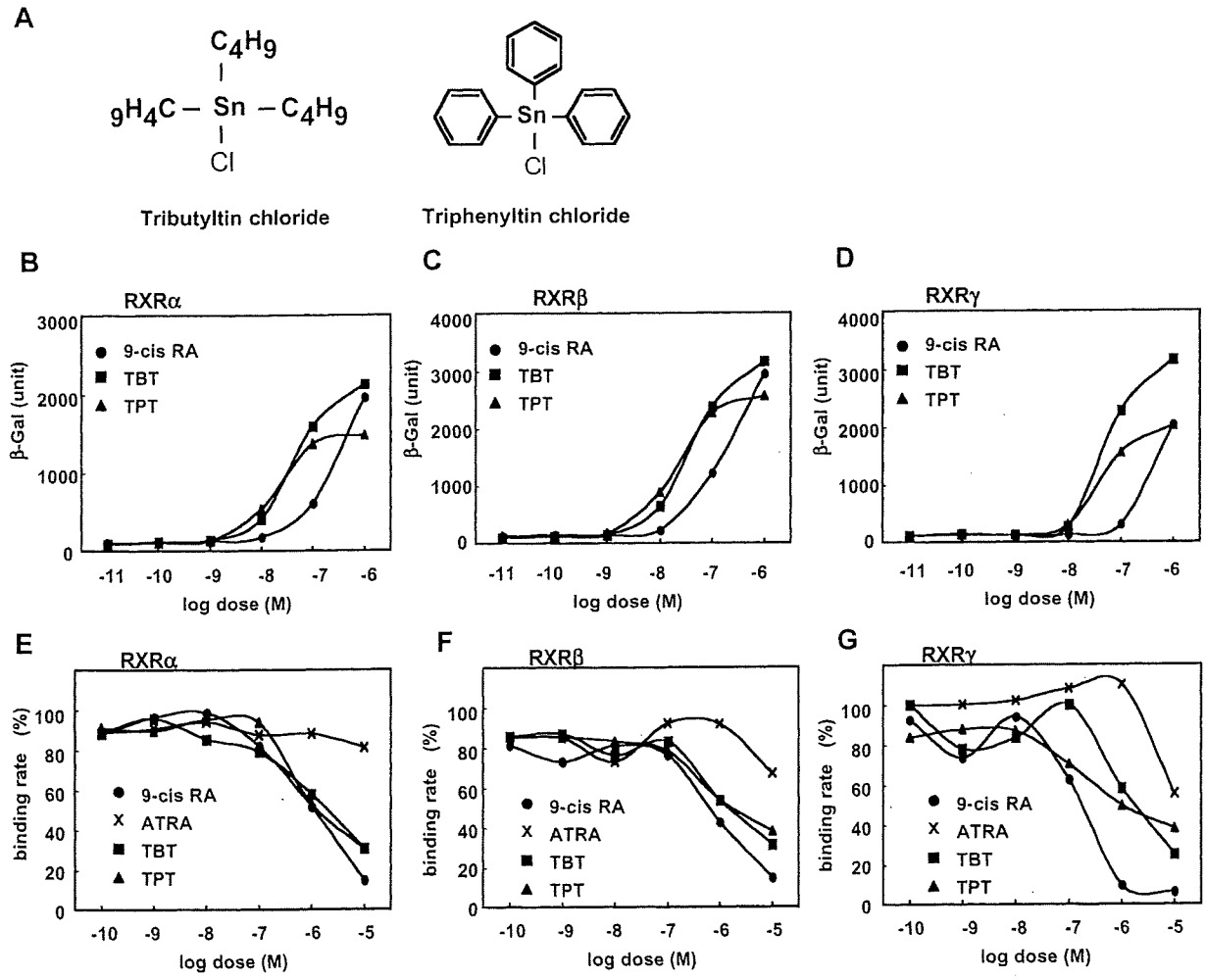

FIGURE 1. RXR activation by TBT and TPT. Structures of organotin compounds are shown in panel A. Yeast strain Y190 was transformed with GAL4AD fused to TIF2 and GAL4DBD fused to LBD of human RXRα (B), RXRβ (C), or RXRγ (D). Chemicals were added to yeast cultures in doses ranging from 10^{-11} to 10^{-6} 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.

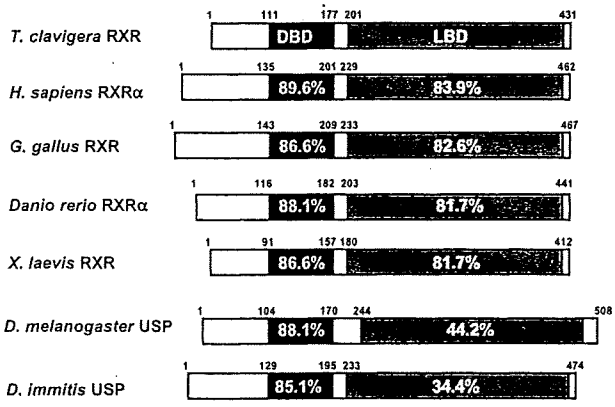

FIGURE 2. Comparison of the deduced amino acid sequences of rock shell RXR with related nuclear receptors. The similarity in the DBD and LBD between rock shell RXR and related nuclear receptors is indicated as percentage amino acid identity. The database accession numbers for the sequences are as follows: *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

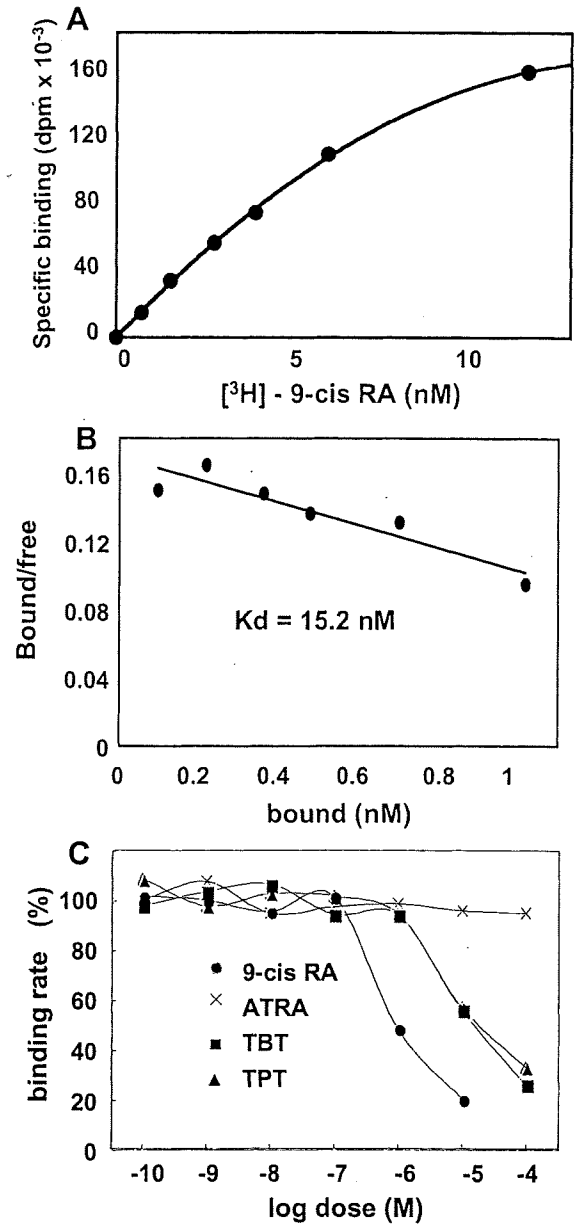

FIGURE 3. 9-cis RA and organotin compounds bind rock shell RXR in vitro. (A) The LBD of rock shell RXR expressed in *E. coli* was incubated with increasing concentrations of ^{3}H-labeled 9-cis RA in the absence (total binding) or presence of 400-fold nonlabeled 9-cis RA (nonspecific binding). Nonspecific 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

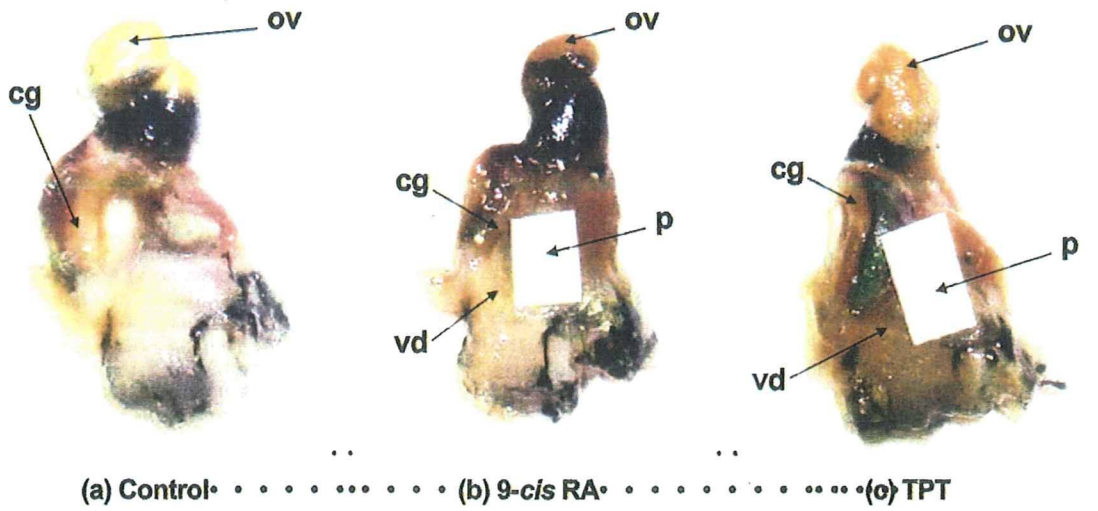

FIGURE 4. Substantial penis growth observed in the female rock shells after 1 month of 9-cis RA injections: cg, capsule gland; ov, ovary; p, penis; vd, vas deferens. (A) Neither penis nor vas deferens was observed in the control female (after shell removal). (B) Substantial penis growth as well as vas 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.

Acknowledgments

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Development of Standardized *in Vitro* Assay System for Estrogen Receptors and Species Specificity of Binding Ability of 4-Nonylphenol and *p*-Octylphenol

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The *in vitro* binding assay seems to be a useful first screening method for endocrine disrupting chemicals. The various methods have been developed and applied to the testing of chemicals. Although these assays should be applied to estrogen receptors (ER) of not only humans but also wildlife, a standardized system is yet to be established. Furthermore, a method for *Xenopus* ER is not yet developed. We previously expressed the ligand-binding domain (LBD) of quail ERα and ERβ as a fusion protein with glutathione S-transferase, and developed a competitive enzyme immunoassay for detecting the capacity of chemicals to bind ERs. It seems that this system is a powerful tool, since it needs no special equipment. In this report, we first produced ER-LBD protein of human, *Xenopus* and medaka as well as quail. Then, we established a competitive enzyme immunoassay for these ERs as a standardized method, and compared the species specificity of the ability of 4-nonylphenol and *p*-octylphenol to bind ERs. Although a significant difference was not detected among ERβ of human, quail and medaka, 4-nonylphenol and *p*-octylphenol exhibited the higher affinity for the medaka ERα than human ERα. These results indicate the species specificity of the capacity of chemicals to bind ERs.

Key words —— endocrine disrupting chemicals, estrogen receptor, enzyme immunoassay, endocrine disruptor, *in vitro* binding assay, species specificity

INTRODUCTION

Endocrine disrupting chemicals (EDCs) also called endocrine disruptors (EDs) seem to be substances affecting reproductive functions through the estrogen-estrogen receptor (ER) signaling pathway.[1,2] Although the mechanisms of disruption are not fully understood, screening methods are definitely needed. Indeed, *in vivo* approaches including a one-generation study and uterotrophic assay, and *in vitro* techniques including a receptor binding assay and reporter gene assay have been developed.[1–4] The Ministry of the Environment of Japan (MoE) released a document entitled "Strategic Programs on Environmental Endocrine Disrupters '98 (SPEED'98)" in 1998, and selected 65 substances as high-priority chemicals to be tested. For initial screening *in vitro*, the receptor binding assay is often utilized. Using the fluorescence polalization method, the capacity of chemicals to bind human ERα and ERβ was evaluated. The Japanese medaka (*Oryzias latipes*) ERα and ERβ were tested using a radio-competitive assay (Ministry of Environment, Japan; http://www.env.go.jp/en/topic/edcs.html). We previously established the competitive enzyme immunoassay (EIA) for analysis of the capacity to bind quail ERα and ERβ, and then tested the chemicals selected by MoE.[5]

Recently, it has been proposed that the effects of EDCs are considered not only in humans but also in wildlife, such as birds, frogs and fish, and indeed adverse effects on human and wildlife were reported. However, a standardized method is not yet established. Moreover, a method for use with frog ER

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has not been developed. The Organization for Economic Cooperation and Development (OECD) and MoE have started the development of various test methods for humans, fish, reptiles, amphibians, and birds (Ministry of Environment, Japan; http://www.env.go.jp/en/topic/edcs.html, OECD; http://www.oecd.org/home/).[6] For this purpose, a cheap and easy screening method was required. However, the competitive assay system using radiolabeled estrogen needs special equipment and regulation for handling. Although a kit for the fluorescence polarization method for human ERα and ERβ is available, this system also needs special equipment. The yeast two-hybrid system is a superior method in terms of cost and handling.[7,8] However, since a cofactor is needed for each species, it is not suitable for an evaluation of the binding to many species of ERs.

The competitive EIA for detecting the ability of EDCs to bind ERs has several advantages. First, it needs no special equipment and no special techniques. Second, it is less expensive than other methods. Therefore, once the recombinant ERs are ready, the assay system can be established quickly for ERs in all species.

In this report, we first established a competitive EIA system for *Xenopus laevis* ER. Furthermore, we developed similar assay systems for ERs in human and medaka as well as quail. Using these standardized assay systems, we evaluated the species specificity of the binding of 4-nonylphenol and *p*-octylphenol, which are known as EDCs, to various ERs.

MATERIALS AND METHODS

Chemicals —— 4-Nonylphenol and *p*-octylphenol were supplied by Dr. Kawashima at JAPAN NUS Co., Ltd. (Tokyo, Japan), where large amounts of chemicals of reagent grade have been stocked for various tests at the request of MoE. These chemicals were dissolved in dimethyl sulfoxide (DMSO). All other chemicals are of reagent grade.

Plasmid Construction —— A DNA fragment containing the ligand-binding domain (LBD) of *Xenopus* ER (721–1758 bp and 241–586 aa in open reading frame) was subcloned into the *Eco*RI-*Sal*I sites in pGEX-4T-1, coding glutathione S-transferase (GST) (Amersham Biosciences Corp., U.S.A.). Finally, the recombinant plasmid was introduced into *Escherichia coli* (*E. coli*), BL21 (DE3) (Novagen, EMD Biosciences Inc., Germany). The construction of pGEX-4T-1-ERα- and ERβ-LBD for human and quail was described previously.[5,9] pGEX-4T-1-ERα- and ERβ-LBDs for medaka were a gift from Dr. Nakai at Chemicals Evaluation and Research Institute, Japan. Thus, pGEX-4T-1 was used for all constructs for the expression of ER-LBDs.

Expression and Purification of GST-ER Fusion Protein —— BL21 harboring pGEX-4T-1-ERα- or ERβ-LBD was cultured in LB medium at 30°C. At OD_{600} = 0.4, isopropyl-1-thio-β-D-galacto pyranoside (IPTG) was added at a final concentration of 0.5 mM. After incubation for 2 hr, the cells were harvested, suspended in 14.4 ml of B-0.1 [20 mM Tris (pH 7.5), 10% glycerol, 0.1 M KCl, 5 mM $MgCl_2$, and 1 mM DTT]. 1.6 ml of 1 mg/ml lysozyme in B-1 [20 mM Tris (pH 7.5), 10% glycerol, 1 M KCl, 5 mM $MgCl_2$, and 1 mM DTT] was added and the cells were disrupted by sonication. This suspension was mixed with 16 ml of B-1, and further stirred gently for 0.5 hr at 4°C. By centrifugation at 12000 rpm for 15 min at 4°C, the soluble fraction including GST-ERs was obtained.

For the purification of GST-ER-LBDs, 0.42 ml of GSH-Sepharose 4B (Amersham Biosciences Corp., U.S.A.) was added to 32 ml of the soluble fraction, and stirred gently for 0.5 hr at 4°C, and then the mixture was packed into the column. After washing thoroughly, the bound GST-ERs were eluted with 1 ml of 20 mM GSH in B-1.

Competitive Enzyme Immunoassay —— The principle of *in vitro* binding assay for ERs is based on the enzyme-linked competitive immunoassay. This assay was performed using the kit, Ligand Screening System-ERα or ERβ, (TOYOBO Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. The only change was the replacement of the human ER in the kit with GST-ERα-LBD or ERβ-LBD of human, quail, *Xenopus* and medaka. 17β-Estradiol (E2) and diethylstilbestrol (DES) were diluted with DMSO at a concentration of $8/3 \times 10^{-4}$–$8/3 \times 10^{-8}$ M, and further diluted with the dilution buffer attached in the kit at a final concentration of $8/3 \times 10^{-6}$–$8/3 \times 10^{-10}$ M (final DMSO concentration, 1%). The test chemicals were diluted in the same way at a final concentration of $8/3 \times 10^{-4}$–$8/3 \times 10^{-8}$ M.

The assay was done according to the manufacturer's instructions. The protocol consists of three steps as follows. Step 1: receptor-ligand binding; 20 μl (0.45–12.6 pmol; see RESULTS in detail) of GST-ER in B-1 buffer including 20 mM GSH described above, 30 μl of various amounts of test

chemicals or standard DES in the dilution buffer attached in the kit, and 30 μl of E2 (8/3 × 4 nM) in the dilution buffer attached in the kit were mixed and incubated for 1 hr on ice. Step 2: antigen-antibody reaction; after the incubation, 50 μl out of 80 μl of the mixture was transferred to an E2-coated plate, and 50 μl of E2-horse radish peroxidase (HRP) solution was added. The mixture was incubated for 1 hr on ice. Step 3: enzyme reaction; the plate was washed and the enzyme reaction was done at 37°C for 20 min. The absorbance at 450 nm was measured with a 1420 ARVO Multilabel Counter (Wallac, Gaithersburg, U.S.A.).

The % inhibition of binding was calculated as follows: $(A_{DMSO\ control}-A_{test\ sample})/(A_{DMSO\ control}-A_{DES\ at\ 112\ nM}) \times 100$. IC_{50} was obtained from the concentration giving 50% inhibition when the inhibition by 112 nM DES is 100%. Relative binding affinity (RBA) was obtained by dividing the IC_{50} of DES by the IC_{50} of the test sample.

RESULTS

Comparison of Amino Acid Sequences of ER-LBDs in Various Species

In the nuclear hormone receptors, DNA binding domains (DBDs) were found in the N-terminal half, and LBD were located in the C-terminal half. The nucleotide and amino acid sequences of ER-DBDs were well conserved among human, quail, *Xenopus*, and medaka. The similarity of amino acid sequences is more than 95%. On the other hand, the similarity of amino acid sequences of ER-LBDs was slightly lower among the four species. As shown in Fig. 1, both ERα and ERβ of medaka revealed less similarity than the other species, suggesting that the characteristics of ligand binding might be different. Only one form of *Xenopus* ER was identified. Although the amino acid sequences of *Xenopus* ER show 99% similarity to DBDs of ERα and ERβ in human and quail, these show 85 and 87% similarity to LBDs of ERα in human and quail, respectively (Fig. 1). On the other hand, it shows only 59 and 60% similarity to LBDs of ERβ in human and quail, respectively. Therefore, *Xenopus* ER seems to be ERα.

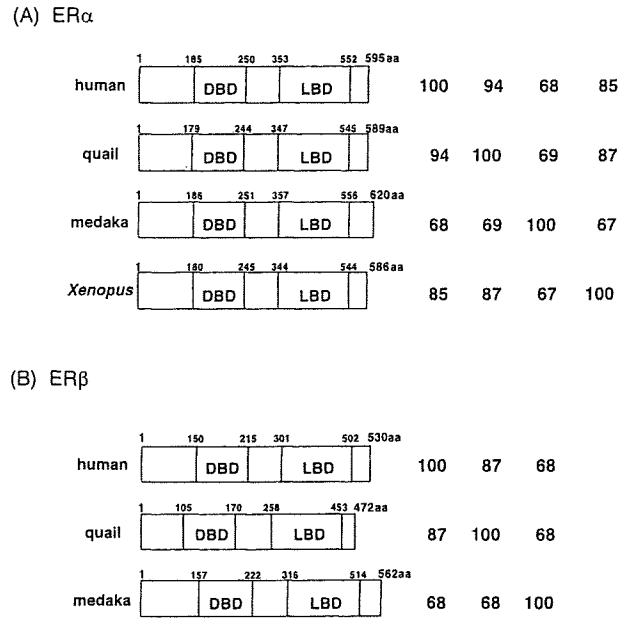

Fig. 1. Similarity of Amino Acids in the Ligand Binding Domain of ERα and ERβ in Various Species

(A); ERα, (B); ERβ. The left panels show a schematic representation of DBD and LBD in ERs. The number of amino acids is shown. The right panels show the % similarity of amino acids in LBD in various species.

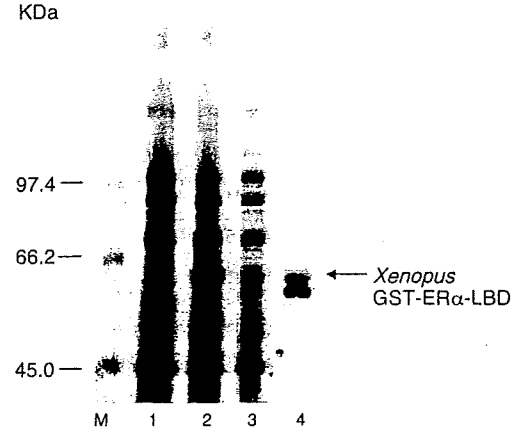

Fig. 2. Expression and Purification of *Xenopus* GST-ERα-LBD

The *Xenopus* GST-ERα-LBD fusion protein was expressed in *E. coli* BL21 (DE3), and then purified using GSH-Sepharose. The samples were loaded on 8% SDS-polyacrylamide gel, and stained with coomassie brilliant blue. Lane M: size marker; Lanes 1: whole protein solubilized from untreated cells; Lanes 2: whole protein solubilized from IPTG-treated cells; Lanes 3: soluble fraction from IPTG-treated cells; Lanes 4: purified GST-ERα-LBD from soluble fraction. The arrow shows a band for *Xenopus* GST-ERα-LBD. The band below the GST-ERα-LBD is a nonspecific band.

Expression and Purification of GST-ER Fusion Protein

For the development of an *in vitro* binding assay system for *Xenopus* ERα, we first expressed the LBD of *Xenopus* ERα as a GST fusion protein in *E. coli*. With the addition of IPTG, GST-ERα-LBD was induced and recovered partly in the soluble fraction (Fig. 2). This soluble fraction was applied to a GSH-Sepharose column and eluted with GSH. The purity

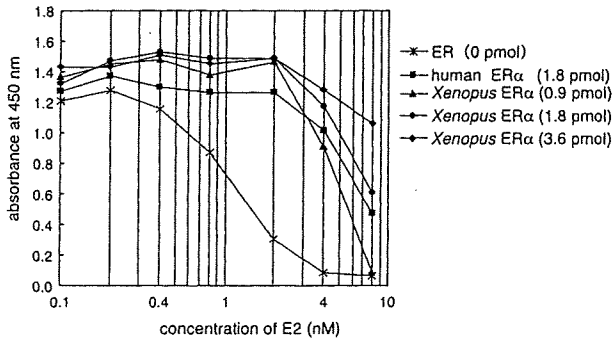

Fig. 3. Measurement of the Amount of Free Ligand in the Presence of *Xenopus* GST-ERα-LBD

For determination of the most suitable amount of GST-ERα-LBD used, the free ligand test was performed using various amounts of GST-ERα-LBD. As a control, human ERα was used.

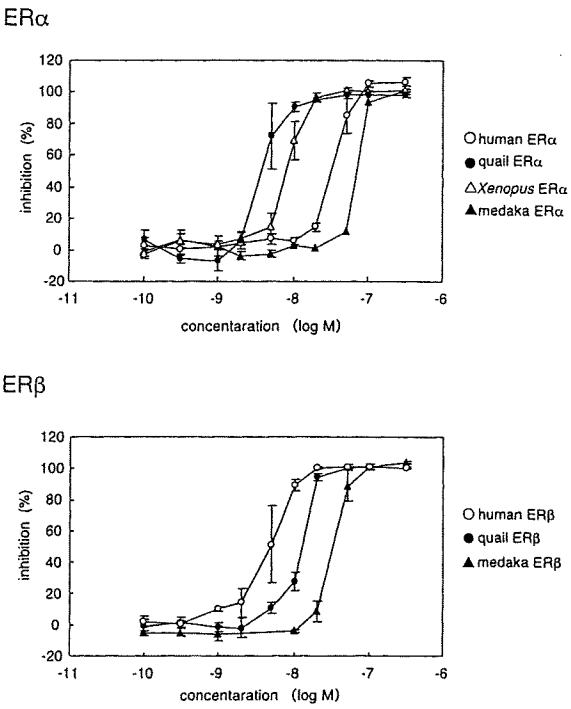

Fig. 4. Standard Curves of DES Obtained by Competitive Enzyme Immunoassay

The inhibition curves against DES for ERα (A) and ERβ (B) are shown. Values are the mean and standard deviation (n = 3–4).

and amount of the resultant purified GST-*Xenopus* ERα-LBD (MW = 63.8 kDa) was checked by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). Although the purified GST-*Xenopus* ERα-LBD was identified by the position of induced proteins by IPTG and the molecular mass and also the purification fold, the nonspecific protein was also co-purified. This nonspecific protein was always recovered with the purification of GST-ERs for other species, and found at the same position in SDS-PAGE when GSH-Sepharose column was used for purification. It seems that this protein does not have any effect on the competitive enzyme immunoassay.[5] GST-ER-LBDs for other species were expressed and purified in the same way.

Development of Competitive Enzyme Immunoassay for *Xenopus* ERα and ERs in Other Species

For the development of a competitive EIA for *Xenopus* ERα, the optimum amount of GST-ERα-LBD used should be determined. Using various amounts of E2 and *Xenopus* GST-ERα-LBD, the amount of free ligand which does not make a complex with GST-ERα-LBD was determined (Fig. 3). Since the final concentration of E2 used in the kit is 4 nM, it is required that up to 4 nM of E2 be trapped with GST-ERα-LBD, whereas the E2 above 4 nM exists free. As shown in Fig. 3, when used 1.8 pmol/well, 2 nM of E2 was completely trapped, while small amounts of E2 exists free in 4 nM of E2. In this regard, the optimum amount of *Xenopus* ERα used was determined as 1.8 pmol/well. In the same way, the optimum amounts of ERs in other species were also determined. The amounts determined were 0.45, 3.6, 12.6, 3.6, 3.6 and 7.2 pmol/well for human ERα and ERβ, quail ERα and ERβ and medaka ERα and ERβ, respectively.

Next, we developed the competitive EIA using these amounts of recombinant ERs. The standard curve for *Xenopus* ERα was made as percent inhibition when that by 112 nM DES was 100%. Those for other ERs were made in the same way. In Fig. 4, the standard curves of ERα in four different species and of ERβ in three different species are shown. IC_{50}s of DES for various ERs are listed in Table 1. The quail ERα and medaka ERα showed the highest and lowest affinities, 3.8×10^{-9} M and 6.5×10^{-8} M, respectively. Thus, IC_{50} values among the four species and two types of ER revealed differences of one order of magnitude.

Comparison of Binding Capacity of 4-Nonylphenol and *p*-Octylphenol for ERs in Four Species

In the previous report, 4-nonylphenol and *p*-octylphenol revealed relatively strong binding to both quail ERα and ERβ.[5] Using this competitive enzyme immunoassay system, we next tested the capacity of 4-nonylphenol and *p*-octylphenol to bind 7 kinds of ERs. The results are shown in Figs. 5 and