sists of two each of the core histones H2A, H2B, H3, and H4, all of which have a basic, unstructured amino terminal tail. These basic proteins are susceptible to a variety of posttranslational modifications, e.g., acetylation (Howe et al., 1999; Wu et al., 1986). One of the well-characterized modifications is acetylation of specific lysine residues, which is reversibly catalyzed by histone acetyltransferase (HAT) and histone deacetylase (HDAC).

The GCN5-related *N*-acetyltransferase family includes GCN5 and PCAF, which share a remarkable degree of homology throughout their sequences and are present in a multisubunit complex consisting of more than 20 distinct polypeptides (Ogryzko et al., 1998). GCN5 and PCAF are transcriptional coactivators with intrinsic HAT activity; they contribute to transcriptional activation by acetylating chromatin (Sterner and Berger, 2000). Disruption of *Gcn5* and *Pcaf* genes revealed that they play distinct but functionally overlapping roles during embryogenesis (Yamauchi et al., 2000). Other well-characterized coactivators possessing HAT activity are CBP and P300, which are ubiquitously expressed global transcriptional coactivators that have critical roles in a wide variety of cellular processes, including development (Giles et al., 1998; Giordano and Avantaggiati, 1999; Yao et al., 1998).

Recent studies have demonstrated that some environmental pollutants affect the hormonal system and produce adverse effects on animals and probably also humans (Colborn et al., 1996; Van der Kraak et al., 1992). These pollutants are referred to as endocrine-disrupting chemicals (EDCs). The major targets of EDCs are nuclear hormone receptors, which bind steroid hormones and regulate transcription of their target genes (Nishihara et al., 2000; Nishikawa et al., 1999). For ligand-dependent gene activation, nuclear hormone receptors require coactivators that link the basal transcriptional machinery with the hormone receptors (Chen, 2000). Recent studies have shown that the nuclear hormone receptor coactivators possess HAT activity and recruit two other types of HATs, CBP and PCAF (Chen et al., 1997; Spencer et al., 1997). Hormone-dependent gene activation mediated by nuclear receptors involves the mutual recruitment of at least three classes of HATs.

These observations raise the possibility that HATs may be the targets of EDCs, and we tested the effects of suspected EDCs on HAT activity. Interestingly tributyltin (TBT) and triphenyltin (TPT) enhanced HAT activity, but other EDCs did not. These organotin chemicals have been used in such applications as wood preservation and as antifouling agents in marine paints, and are ubiquitous in the environment. TBT and TPT have been found to induce imposex (the superimposition of male sex organs in female gastropods) in the rock shell *Thais clavigera* and are known EDCs in marine species (Horiguchi et al., 1997). These compounds are also reported to affect not only the hormone system but also embryogenesis in mammals (Harazono et al., 1998; Nakanishi et al., 2002). Organotins caused behavioral and neurological symptoms and pancreatic and hepatic toxicities in rodents (Brown et al., 1979; Merkord et al., 2001). In the immune system, at low doses TBT inhibits immature thymocyte proliferation, whereas at higher doses in particular TBT induces apoptotic cell death (Gennari et al., 2002). However, the biological mechanism of the effects of organotin compounds on marine species and mammals awaits further characterization. The present study showed that some organotin compounds enhanced HAT activity when both core and nucleosomal histones were used as substrates. These data suggest that the varied toxicities of the organotin compounds may be caused by aberrant gene expression following altered histone acetylation.

2. Materials and methods

2.1. Chemicals

All chemicals were dissolved in dimethyl sulfoxide (DMSO) (Wako Pure Chemicals, Osaka, Japan). Organotin and related chemicals tested are listed in Table 1.

2.2. Preparation of rat liver nuclear extracts and HAT fraction

All animal care and handling procedures were approved by the animal care and use committee of Osaka University. Rat liver nuclear extracts (RLNE) were prepared as described previously (Osada et al., 1995). For binding RLNE to Ni^{2+}-NTA agarose (Qiagen, Hilden, Germany), nuclei were suspended in a nuclear lysis buffer (10 mM HEPES, pH 7.6, 100 mM KCl, 10% glycerol, 3 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and

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.

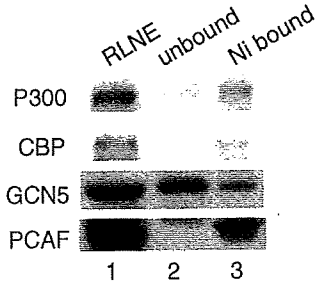

Fig. 1. Partial purification of HATs from RLNE. Western blots were performed with RLNE (lane 1), flow-through from a Ni^{2+}-NTA agarose column (unbound fraction, lane 2), and eluate from a Ni^{2+}-NTA agarose column (Ni-bound, lane 3) using antibodies against P300, CBP, GCN5, and PCAF.

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-

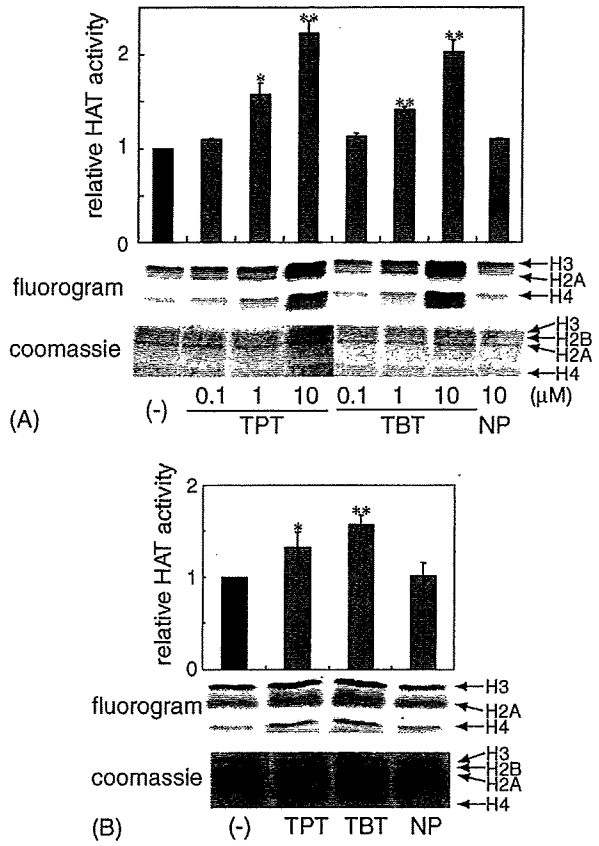

Fig. 2. Effect of TPT and TBT on HAT activity. (A) HAT assays with core histones as substrates and partly purified HAT fraction from RLNE as enzyme. The bar graph shows mean HAT activities (±S.D.) relative to a control treated without chemical (three independent experiments). 4-nonylphenol (NP) was used 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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Cloning and Characterization of a cDNA Encoding the Histone Acetyltransferase Monocytic Leukemia Zinc Finger Protein (MOZ) 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, monocytic leukemia zinc finger protein, transcription, c-Jun, CCAAT/enhancer binding protein, basic region leucine zipper 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. Monocytic leukemia zinc finger protein (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

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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

(Invitrogen, Carlsbad, CA, U.S.A.) 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, U.S.A.) 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, U.S.A.), 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 [contains full-length (FL) Jun], GST-c-Jun [contains the Jun transactivation domain (AD)], and GST-c-Jun [includes the DNA-binding domain (DBD) 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, U.S.A.). 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 hr 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 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,

(A)

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   1 MVKLANPLYTQWILEAIKKVKKQKQRPSEERICNAVSSSHGLDRKTVLEQLELSVKDGTILKVSNKGLNSYKDPDNPGRIALPKPRNHGKLDNKQSVDWNKLLKRAFEGLAESGGSTLKS
 121 IERFLKSQKDVSAACGGTAASGFHQQLRLAIKRAVGHGRLLKDGPLYRLNTKAANAEGKEGCESLSCLPPVSLLPHEKDKPVAEPIPICSFCLGTKEQNREKKPEDLISCADCGNSGHPS
 241 CLKFSPELTVRVRALRWQCIECKTCSSCRDQGKNADNMLFCDSCDRGFHMECCDPPLTRMPKGMWICQICRPRKKGRKLLQKKAAQIKRRYANPIGRPKNRLKKQSTVSKGPFSKVRTGP
 361 GRGRKRKITVSSQSASSSEEGYLERIDGLDFCRDSSAPLKFNKKTKGLIDGLTKFFTPSPDGRKARGEAVDYSELRIRKKGNRKSSTSHWPTDNQDGWESKQESEERLFGSQEIMTERDM
 481 ELFRDIQEQALQKVGVTGPPDPQVRCPSVIEFGKYEIHTWYSSPYPQEYSRLPKLYLCEFCLKYMKSRTILQQHMKKCGWFHPPANEIYRKNNISVFEVDGNVSTIYCQNLCLLAKLFLD
 601 HKTLYYDVEPFLFYVLTQNDVKGCHLVGYFSKEKHCQQKYNVSCIMILPQYQRKGYGRFLIDFSYLLSKREGQAGSPEKPLSDLGRLSYMAYWKSVILECLYHQNDKQISIKKLSKLTGV
 721 CPQDITSTLHHLRMLDFRSDQFVIIRREKLIQDHMAKLQLNLRPVDVDPECLRWTPVIVSNSVVSEEEDEEADDGEKEEPQGQERELETRERVGKSVSRENKDQDSSSLIESEKKPEVKE
 841 LASSSRLSKQALVRDSLPANSQPPRRGRCGRKNRKTQERFGDKDSKMLVGETLSTSQEQYGECEEKSAASRERYTEVGEQPAAPQAQADGNPDIPKGRFSESADLWRGQLKKSPETLKCR
 961 LPEGNDRLPCCYTDGDRAVFRGFSESSEEEEEPESPRSNSPPVLTKPTLKRKKPILHRRRRVRKRKHHNSSVVTETISETTEVLDEPFEDSDSERPMPRLEPTFEIEEEEEEEDENELFP
1081 RGYFHCLSSQDILRCQASSKRTASKDEEEEEEESDDADDTPVLKPVSLLRKCDVNSASLEPDTSTPMKKKKGWPKGKSRKPIHWKKRPGRKPGFKLNQDIIAVSTQECIVEPIVPIKPGR
1201 KPRTQESEELVEVKEGLVEERKEEMHTEADEEAEEEEDAASSDIRAMSPLDSSNSPDADPKEPEAEEEEEKPLDDPRQSEEEPQELEEQEQEEEDEVTAEANQNEDHDADDEDDGHLDSL
1321 KTKEPEGQPAREDGTEEPGTQESFLDASIQDSRENAKDKDETEADSEEEQPSHEASVGSETMPGSEEDHEEDSNTKEELIELKEEEEIPHSELDLETVQAVQSLTQEESSEHEGAYQDCE
1441 ETLAACQTLQSYTHTDEDPQMSMVEDCHASEHNSPISSIPSHPSQSVRSVSSPSMPALESGYTQISPEQGSLSAPSMQNMETSPMMDVPSVSDHSQQVVDSGFSDLGSIESTTENYENPS
1561 SYDSTMGSSICGNNSSQSSCSYGGLSSSSSLTQNSCVVTQQMASMGNSCSMLQQNSVQPATNCNIKSPQTCVVERPPSNQQPPPPPPPPPPQQPQPQPQQQAAPQPPPPQPQQQPPPPP
1681 QQQPQPPPPPQQQPPLSQCSMNNSFTAAPMIMEIPESGGTGNISIYERIPGDFGAGSYSQPSATFSLAKLQQLTNTIMDPHAMPYSHSPAVTSYATSVSLSNTGLAQLAPSHPLAGTPQA
1801 QATMTPPPNLAPTTMNLTSPLLQCNMSATNIGIPHTQRLQGQMPVKGHISIRSKSAPLPSATAHQQQLYGRSPPAVAMQAGPRALAVQRGMNMGVNLMPTPAYNVNSMNMNTLNAMNSYR
1921 MTQPMMNSSYHSNPAYMNQTAQYPMQMQMGMMGSQAYTQQPMQPNPHGNMMYTGPSHHSYMNAAGVPKQSLNGPYMRR*1998
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(B)

(C)

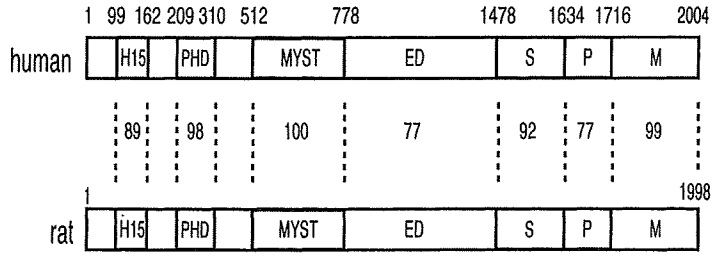

Fig. 1. Molecular Cloning of CDNA Encoding rnMOZ

(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.

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* interac-

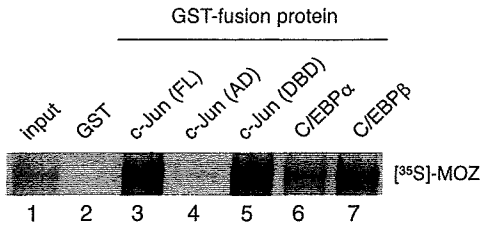

Fig. 2. Association of rnMOZ with bZIP-Type Transcription Factors

[^{35}S]-MOZ was incubated with GST (lane 2) or GST-fused c-Jun (FL), c-Jun (AD), c-Jun (DBD), C/EBPα. and CIEBPβ (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.

tion 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 AD and the 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.

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Trialkyltin Compounds Bind Retinoid X Receptor to Alter Human Placental Endocrine Functions

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Retinoid X receptor (RXR) is a nuclear receptor that plays important and multiple roles in mammalian development and homeostasis. We previously reported that, in human choriocarcinoma cells, tributyltin chloride and triphenyltin hydroxide, which are typical environmental contaminants and cause masculinization in female mollusks, are potent stimulators of human chorionic gonadotropin production and aromatase activity, which play key endocrine functions in maintaining pregnancy and fetal development. However, the molecular mechanism through which these compounds stimulate these endocrine functions remains unclear. Our current study shows that trialkyltin compounds, including tributyltin chloride and triphenyltin hydroxide, function as RXR agonists. Trialkyltins directly bind to the ligand-binding domain of RXR with high affinity and function as transcriptional activators. Unlike the natural RXR ligand, 9-*cis*-retinoic acid, the activity of trialkyltins is RXR specific and does not activate the retinoic acid receptor pathway. In addition, trialkyltins activate RXR to stimulate the expression of a luciferase reporter gene containing the human placental promoter I.1 sequence of aromatase, suggesting that trialkyltins stimulate human placental endocrine functions through RXR-dependent signaling pathways. Therefore, our results suggest that activation of RXR may be a novel mechanism by which trialkyltins alter human endocrine functions. (*Molecular Endocrinology* 19: 2502–2516, 2005)

THE RETINOID X receptors RXRα, RXRβ, and RXRγ, which are type II nuclear receptors, are thought to be key factors in several nuclear receptor signaling pathways. These molecules specifically bind 9-*cis*-retinoic acid (9cRA) and thus may be directly involved in the transduction of retinoid signals. In transfected cultured cells, as well as established cell lines, RXRs can act either as homodimers or heterodimeric partners of several other nuclear receptors, including retinoic acid receptors (RARα, -β, and -γ), thyroid hormone receptors (TRα and -β), vitamin D receptor (VDR), peroxisome proliferator-activated receptors (PPARα, -γ, and -δ), and a number of orphan receptors (1–3). Therefore, RXRs may be central to the modulation of several hormonal signals.

The placenta is a transient, but vital, organ for maintaining pregnancy in mammals. Its functions range from nutrient and gaseous exchange to hormone and growth factor production. Several observations suggest that RXRs play indispensable roles in placental development and physiology. In mice, RXRα transcripts are strongly expressed in the ectoplacental cone and, at later stages, in giant trophoblastic cells and the labyrinthine zone of the chorioallantoic placenta (4). RXR knock-out experiments in mice have revealed that RXRα and RXRβ are involved in the formation of the chorioallantoic placenta (5, 6). In particular, the inactivation of RXRα ligand-dependent transcriptional activation function 2, but not ligand-independent transcriptional activation function 1, prevents the formation of labyrinthine trophoblasts and leads to fetal death during the late fetal period or

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Abbreviations: atRA, All *trans*-retinoic acid; CDCA, chenodeoxycholic acid; CG, chorionic gonadotropin; 9cRA, 9 *cis*-retinoic acid; 15dPGJ$_2$, 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$; DBTCl$_2$, dibutyltin dichloride; DHA, docosahexaenoic acid; DMSO, dimethyl sulfoxide; DPTCl$_2$, diphenyltin dichloride; DR, direct repeat; FCS, fetal calf serum; FXR, farnesoid X-activated receptor; GST, glutathione *S*-transferase; LBD, ligand-binding domain; LUC, luciferase; MBTCl$_3$, butyltin trichloride; MPTCl$_3$, phenyltin trichloride; PPAR, peroxisome proliferator-activated receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; RXRE, RXR response element; TBT, tributyltin; TBTBr, tributyltin bromide; TBTCl, tributyltin chloride; TBTH, tributyltin hydride; TBVT, tributylvinyltin; TChTOH, tricyclohexyltin hydroxide; TeBT, tetrabutyltin; TETBr, triethyltin bromide; TMTCl, trimethyltin chloride; TOTH, trioctyltin hydride; TPBS, Tween 20-PBS; TPrTCl, tripropyltin chloride; TPT, triphenyltin; TPTCl, triphenyltin chloride; TPTOH, triphenyltin hydroxide; TR, thyroid hormone receptor; VDR, vitamin D receptor.

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shortly after birth (7). These placental abnormalities are similar to those found in the placentas of vitamin A-deficient rats (8). These observations suggest that the ligand-dependent transcriptional activation of RXRα is physiologically required for placentation in rodents.

RXRα mRNA and protein have been detected in human cytotrophoblasts and choriocarcinoma cells (9–11). Treatment of these cells with 9cRA and synthetic RXR-specific ligands increases the level of mRNA expression of steroidogenic enzymes, such as aromatase and human (h) chorionic gonadotropin (CG) (9, 10, 12–15). Estrogens and hCG are the principal hormones produced by the placenta during human pregnancy. These hormones are essential for several important events in the establishment and maintenance of pregnancy. Biosynthesis of estrogens requires the catalytic activity of an aromatase enzyme complex, which converts androgenic to estrogenic steroids (16). The human placenta exhibits a high level of aromatase activity and therefore regulates the balance of estrogens *in utero* (17). Altering aromatase function *in utero* can cause permanent effects in human embryos; the lack of placental aromatase causes female pseudohermaphroditism, as is seen in patients with aromatase deficiency (16, 18).

hCG is a luteotropic factor and the primary marker of pregnancy in humans. Stimulation by hCG governs not only progesterone production in the corpus luteum during the first trimester (19) but also testosterone production within the fetal testes (20). Given the pivotal functional roles of aromatase and hCG in sexual development and reproduction, the extant retinoid signals of RXR-mediated transcription in the placenta may greatly alter fetal development because of their disruption of these endocrine functions.

Organotin compounds have been used widely as biocides, agricultural fungicides, wood preservatives, disinfecting agents in circulating industrial cooling waters, and antifouling paints for marine vessels (21, 22). There are many reports of the biological effects of organotin compounds, which vary in their toxic effects to eukaryotes. One of the most notable toxicities in sexual development and reproduction is that of tributyltin (TBT)- and triphenyltin (TPT)-mediated endocrine disruption in some species of gastropods (23, 24). This phenomenon is known as "imposex," the superimposition of male genitalia on female. Therefore, these trialkyltin compounds are suspected to cause endocrine-disrupting effects in mammals, including humans. Human exposure to organotin compounds may result from the consumption of organotin-contaminated meat and fish products, occupational exposure during the manufacture and formulation of organotin compounds, or the application and removal of organotin-containing paints (25, 26). The possible exposure of humans to organotins has therefore aroused great concern about potential toxicities.

Previously, we reported that both tributyltin chloride (TBTCl) and triphenyltin hydroxide (TPTOH) enhance hCG secretion and aromatase activity in human choriocarcinoma cells. In addition, these compounds cause dose-related increases in the steady-state mRNA levels of both hCGβ and aromatase in human choriocarcinoma Jar cells after their exposure to nontoxic concentrations (27). These results suggest that these trialkyltin compounds are potent stimulators of human placental hCG production and aromatase activity *in vitro* and act as endocrine disruptors, the effects of which might alter local hCG and estrogen concentrations in pregnant women. However, the molecular mechanism underlying trialkyltin-induced alterations of human placental endocrine functions remains unclear. To extend our knowledge of the correlation between the structure of organotin compounds and their endocrine-disrupting effects, we assessed the effects of 17 tin compounds on hCG secretion, aromatase activity, and the mRNA levels of hCG and aromatase in Jar cells. We found that the effects of organotin compounds are related to both the number and length of their alkyl chains, suggesting that organotin compounds might interact with a target molecule in a fashion similar to that by which environmental estrogenic chemicals interact with estrogen receptors (28–34). Further, the promoter sequences of both human placental hCGβ and aromatase have several common half-site sequences (T/AGGTCA), of which nuclear receptor response elements typically are composed (13, 14). In addition, expression of both human placental hCG and aromatase is induced by specific RXR ligands (9, 10, 13, 14). In light of all of these results, we hypothesize that organotin compounds interact with RXRs to alter placental endocrine functions. Here we demonstrate that trialkyltin compounds bind to RXRs with high affinity and stimulate transcription through these receptors to alter endocrine functions in human choriocarcinoma cells.

RESULTS

Effects of Organotin Compounds on hCG Production and Aromatase Activity in Human Choriocarcinoma Cells

Previously, we reported that both TBTCl and TPTOH enhance hCG secretion and aromatase activity in human choriocarcinoma cells (27). To extend our knowledge of the correlation between the structures of organotin compounds and their endocrine-disrupting effects, we assessed the effects of 17 tin compounds (Fig. 1) on hCG secretion from, and aromatase activity in, Jar choriocarcinoma cells after their exposure to nontoxic concentrations of these compounds, which were determined from the results of [^{3}H]thymidine uptake assays (data not shown). The most active compounds were TBT or TPT derivatives (Fig. 2, Group III). Exposure to $\geq$ 10 nM of each of these trialkyltin compounds caused statistically significant increases in hCG production by Jar cells. Aromatase activity also

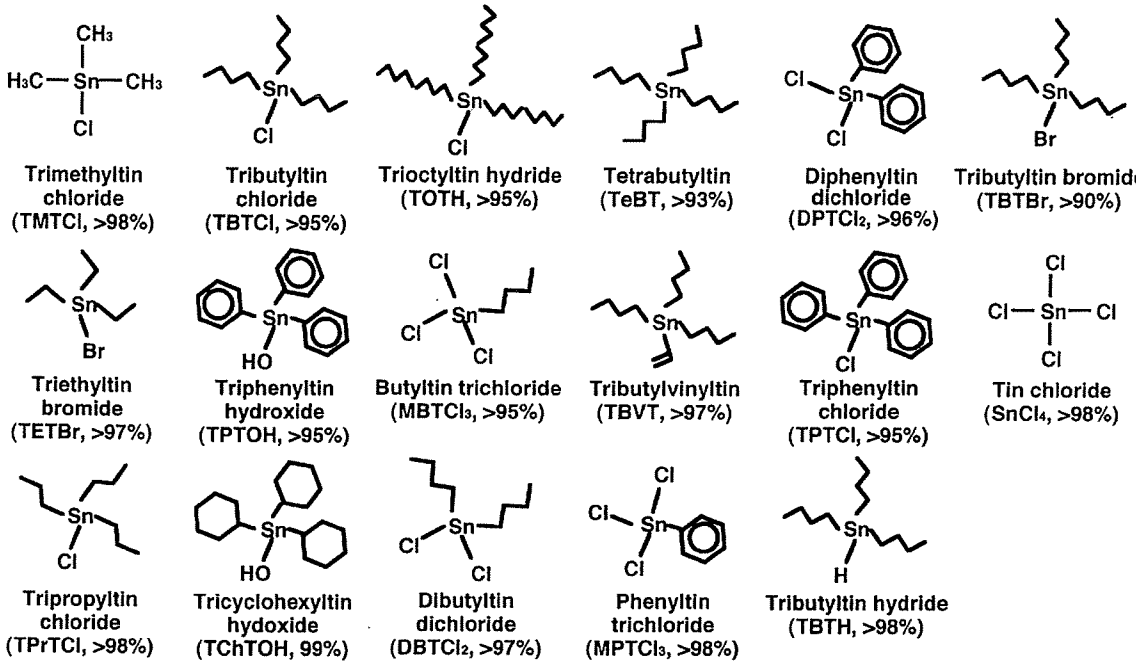

Fig. 1. Structures of the Tin Compounds Used in This Study
The abbreviation for, and purity of, each compound used are indicated in *parentheses*.

increased significantly as the concentrations of the TBT derivatives increased beyond 10 nM or those of the TPT derivatives increased in excess of 30 nM. Among the other trialkyltin compounds (Group I), tripropyltin chloride (TPrTCl) and tricyclohexyltin hydroxide (TChTOH) were active. Like the TBT and TPT compounds, TPrTCl stimulated both hCG production and aromatase activity, whereas TChTOH stimulated hCG production but not aromatase activity. Among the butyltin and phenyltin derivatives (Group II), neither of the mono-alkyltin compounds altered hCG production or aromatase activity. Dibutyltin dichloride ($DBTCl_2$) stimulated aromatase activity at 30 nM but failed to induce hCG production at any of the concentrations tested. In contrast, diphenyltin dichloride ($DPTCl_2$) stimulated hCG production at 30 nM but not aromatase activity at any tested concentration.

There were no significant differences in hCG production and aromatase activity among the TBT and TPT derivatives (Group III), suggesting that the ligand on the trialkyltin (as long as it is not an alkyl group) is relatively unimportant to the stimulation of these endocrine functions. However, the presence of a fourth alkyl group on the tin atom decreased the stimulus potency of the alkyltin compounds on hCG production and aromatase activity, because both tetrabutyltin (TeBT) and tributylvinyltin (TBVT) failed to stimulate these placental functions at doses less than 100 nM (Fig. 2, Group III). These results suggest that the potency of the effects induced by alkyltin compounds is related to both the number and length of the alkyl groups.

In addition, we investigated the tin compound-induced mRNA expression of hCGβ and aromatase at either the concentration that elicited the greatest response in each endocrine function or the maximal nontoxic concentration. The changes in hCGβ and aromatase mRNA expression were almost parallel to those in hCG secretion and aromatase activity (Fig. 3). These results indicate that the observed alkyltin-induced alterations in these placental functions are both caused by regulation at the mRNA level. Such overt correlation between the mRNA expression induced by alkyltin compounds and their structure led us to hypothesize that alkyltin compounds may interact with a nuclear receptor to alter placental endocrine functions; a similar mechanism has been demonstrated for environmental estrogenic chemicals that interact with estrogen receptors (28–34).

Interaction of Alkyltin Compounds with the Hormone-Binding Domain of RXRs

In human placental cells, both hCG production and aromatase activity are controlled by cAMP-dependent intracellular signal pathways. However, in our previous study, neither TBTCl nor TPTOH exerted any effect on cAMP production (27). After a literature search to identify a signaling pathway common to the mRNA expression of both hCG and aromatase, we arrived at the hypothesis that alkyltin compounds act as ligands of RXRs to activate the transcription of hCG and aromatase, because these placental factors are both induced by specific ligands of RXRs (9, 10, 13, 14). In

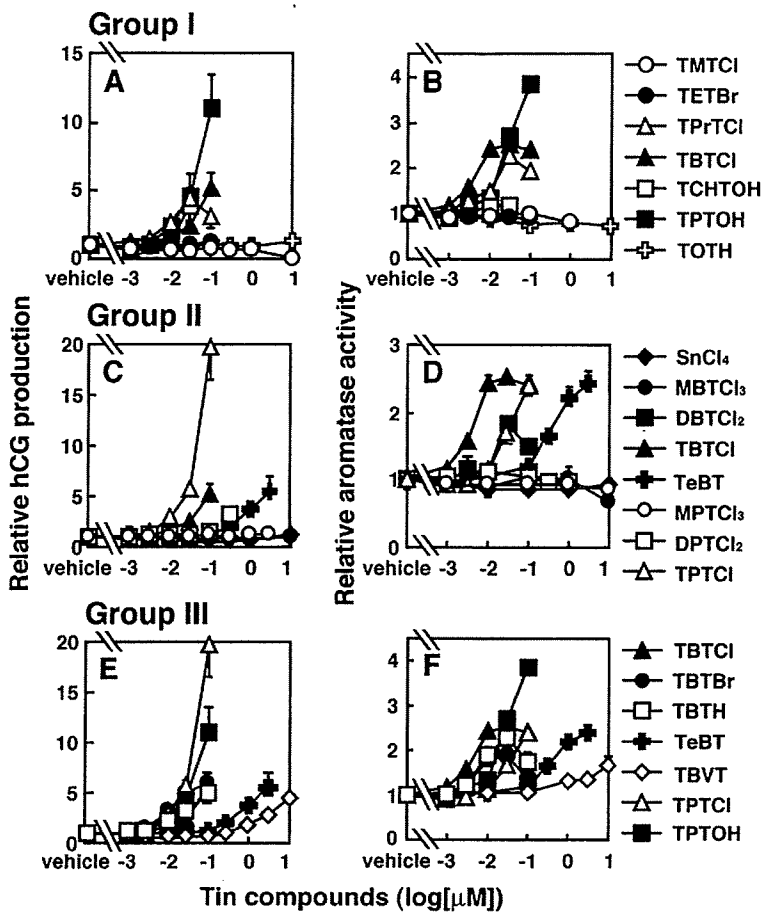

Fig. 2. Effects of Tin Compounds on hCG Secretion (A, C, and E) and Aromatase Activity (B, D, and F) in Jar Cells

Cells were treated with various nontoxic concentrations of tin compounds for 48 h. A nontoxic concentration of a tin compound was defined as a concentration at which the uptake of [^{3}H]thymidine was ≥ 80% that for the vehicle alone (data not shown). Results are expressed as mean ± 1 SD of triplicate cultures. Group I (A and B): comparison of different lengths of alkyl chains in trialkyltin compounds. Group II (C and D): comparison of different numbers of alkyl chains in butyltin and phenyltin compounds. Group III (E and F): comparison of different fourth function groups on the tin of TBT and TPT. The hCG production and aromatase activity in vehicle-only cells, calculated from all experiments, were 290.0 ± 85.3 mIU/well·24 h and 4.08 ± 0.91 pmol/well·4 h, respectively.

fact, we were able to confirm that a natural RXR ligand, 9cRA, and a synthetic RXR-specific ligand, LG100268, induced mRNA expression of both hCGβ and aromatase in Jar cells (Fig. 4).

Accordingly, we examined the dose-response effectiveness of TBTCl and TPTOH in stimulating RXR activity by using human choriocarcinoma JEG-3 cells cotransfected with a human RXRα expression plasmid and a luciferase (LUC) reporter plasmid containing an RXR response element (RXRE). RXR homodimers bind to direct repeat (DR)1 motifs (1–3). We constructed and used pTALDR1, which contains two DR1 sequences, as a reporter plasmid. Treatment with 1–100 nM 9cRA resulted in a 3- to 10-fold increase in LUC activity, whereas similar treatment with LG100268 led to a 3.3- to 4.5-fold increase (Fig. 5A). In addition, 100 nM 9cRA slightly stimulated the expression of LUC in JEG-3 cells transfected with a control plasmid, because JEG-3 cells express endogenous RXRα (10). Treatment of RXRα-transfected JEG-3 cells with 1–100 nM TBTCl stimulated LUC expression 1.5- to 9-fold, and exposing the cells to the same concentrations of TPTOH induced LUC expression 1.8- to 19-fold; these results suggest that low doses of these trialkyltin compounds activate RXR.

To identify the region of RXR involved in activation by alkyltin compounds, we used a chimeric receptor consisting of the DNA-binding domain of the yeast transcription factor GAL4 and the ligand-binding domain (LBD) of RXRα (GAL-RXR). Consistent with the results in Fig. 5A, the activity of the LUC reporter construct in JEG-3 cells cotransfected with the GAL-RXR chimeric receptor and the UAS-LUC reporter increased in a dose-dependent manner after incubation with not only 9cRA but also either TBTCl or TPTOH (Fig. 6). We then investigated the effect of the other tin compounds on activation of GAL-RXR chimeric receptors after exposure of the cells to nontoxic concentra-

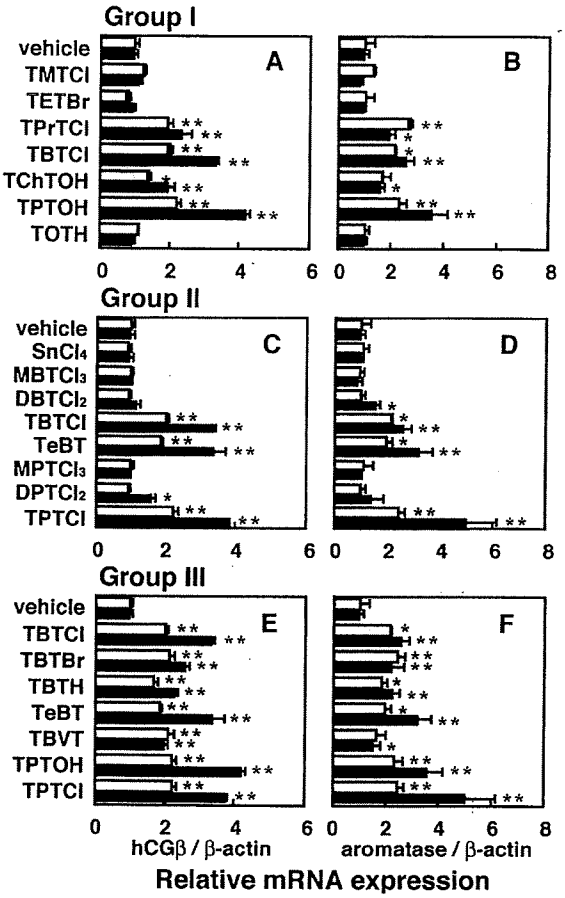

Fig. 3. Effects of Tin Compounds on the mRNA Expression of hCG β (A, C, and E) and Aromatase (B, D, and F) in Jar Cells

Total RNA isolated from Jar cells was treated with tin compounds for 24 h (*open bars*) and 48 h (*solid bars*). The concentrations of each compound were: 10 μM of TOTH, $SnCl_4$, $MBTCl_3$, and TBVT; 3 μM of $MPTCl_3$ and TeBT; 1 μM of 9cRA and TMTCl; 300 nM of $DPTCl_2$; 100 nM of TETBr, TBTCl, TPTOH, TPTCl, TBTBr, and TBTH; and 30 nM of TPrTCl, TChTOH, and $DBTCl_2$. The relative hCGβ and aromatase mRNA levels for each condition were determined by quantitative RT-PCR three times for each of the three independent cultures (see *Materials and Methods*). Results are expressed as mean ± 1 SD of three independent cultures. Groups I (A and B), II (C and D), and III (E and F) correspond to the groups described in the legend for Fig. 2. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.005$ *vs.* vehicle.

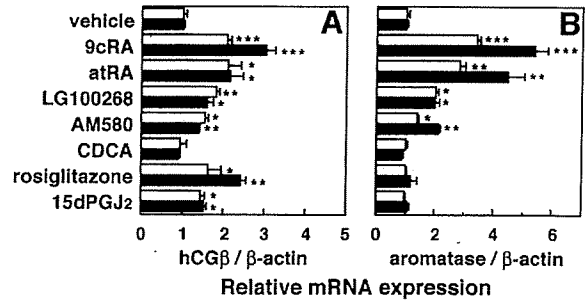

Fig. 4. Effects of Various Nuclear Receptor Agonists on the mRNA Expression of hCG β (A) and Aromatase (B) in Jar Cells

Total RNA isolated from Jar cells was treated with tin compounds for indicated time periods. The concentrations of each compound were: 10 μM of CDCA; 100 nM of 9cRA, atRA, rosiglitazone, or $15dPGJ_2$; and 100 nM of LG100268 or AM580. The relative hCGβ and aromatase mRNA levels for each condition were determined by quantitative RT-PCR three times for each of the three independent cultures (see *Materials and Methods*). Results are expressed as mean ± 1 SD of three independent cultures. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.005$ *vs.* vehicles.

tions. TPrTCl, TeBT, and TBVT, as well as the TBT and TPT derivatives, activated transcription through GAL-RXR. TChTOH also activated transcription though GAL-RXR markedly, but the level of activation was only slight compared with that induced by the TBT and TPT derivatives. The level of GAL-RXR activation induced by these tin compounds was almost parallel to the increase in mRNA expression of hCGβ or aromatase in JEG-3 cells treated with these compounds.

RXR serves as a common heterodimerization partner for several receptors (1–3). In addition to RXR homodimer, RXR-specific ligands can activate two types of complex. One type is the permissive heterodimers, such as PPAR-RXR (35–39) and farnesoid X-activated receptor (FXR)-RXR (40), which can be fully activated by a ligand of either RXR or its partner receptor and are activated synergistically in the presence of both ligands; the other is the conditionally permissive heterodimer RXR-RAR, which can be conditionally activated by RXR ligands only in the presence of an RAR agonist (38, 41). To identify the complexes involved in alkyltin-induced mRNA expression of both placental factors, we assessed the effects of the ligands of various RXR partners. The PPAR ligand 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 ($15dPGJ_2$) and the PPARγ-specific ligand rosiglitazone induced mRNA expression of hCGβ, but not aromatase, in Jar cells. Chenodeoxycholic acid (CDCA), an FXR agonist, failed to increase the mRNA expression of either placental factor. However, a natural RAR ligand, all-*trans*-retinoic acid (atRA), and a synthetic RARα-specific ligand, AM580, induced expression of the mRNA of both hCGβ and aromatase in human placental cells, as previously described (15). Furthermore, the natural RXR ligand 9cRA also can function as an RAR agonist to transactivate RXR-RAR heterodimer (1–3, 42). Accordingly, we examined whether typical trialkyltin compounds, which function as RXR agonists, can activate RXR-RAR transcription, as does 9cRA, by using LUC reporter plasmids containing an RAR response element (DR2 or DR5). Cells simultaneously transfected with human RXRα and RARβ expression vectors dramatically respond to atRA and 9cRA (Fig. 5B). Although TBTCl and TPTOH also stimulated the expression of LUC, the effectiveness of these organotin compounds was comparable to

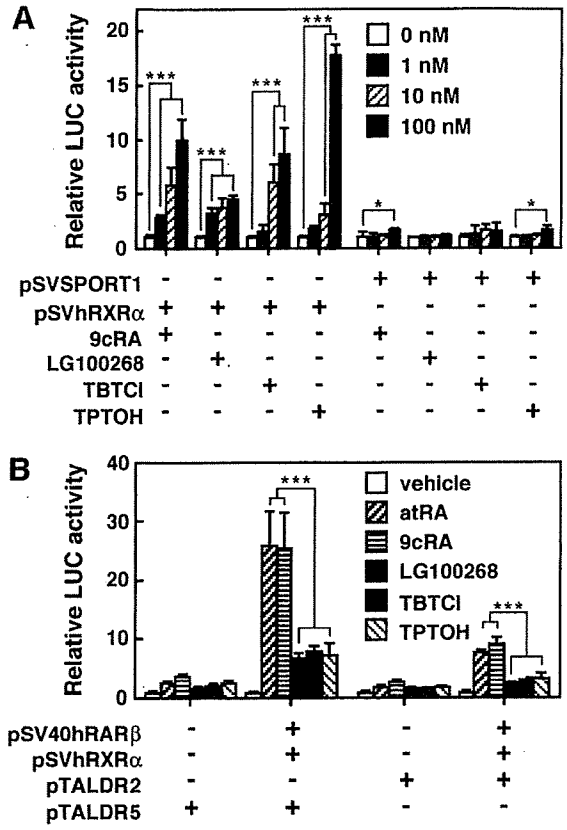

Fig. 5. Ability of TBTCl and TPTOH to Activate RXR and RAR

A, JEG-3 cells were cotransfected with 10 ng of either pSVhRXRα or pSVSPORT1 in addition to 0.1 μg pTALDR1 (see *Materials and Methods*) and then treated with various concentrations of 9cRA, LG100268, TBTCl, or TPTOH. B, JEG-3 cells were cotransfected with 10 ng each of pSVhRXRα and pSV40hRARβ in addition to 50 ng pTALDR2 or pTALDR5 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 are expressed as average fold activation ± 1 SD after normalization to *Renilla* LUC activity. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.005$.

that of LG100268, which does not transactivate RXR-RAR heterodimer (Fig. 5B). In addition, we examined the effect of TBTCl and TPTOH on activation of RARα, -β, and -γ by using chimeric receptors in which the GAL4 DNA binding domain was fused to the LBD of RARs (GAL-RARs) instead of RXRα. Neither alkyltin compound activated transcription through GAL-RARs (data not shown). These results suggest that the alkyltin compounds, which activate transcription of the reporter construct through GAL-RXR, interact with the LBD of RXR and transactivate RXR homodimer, but not RXR-RAR heterodimer, unlike 9cRA. Taken together, these results suggest that RXR homodimer may be involved in the alkyltin-induced mRNA expression of both placental factors, provided that the hCGβ induced also involves PPAR-RXR heterodimers.

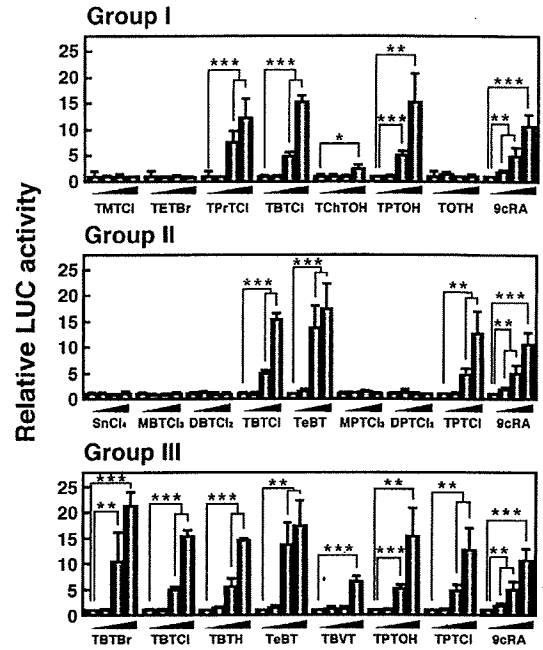

Fig. 6. Ability of Tin Compounds to Activate GAL-RXR

JEG-3 cells were cotransfected with 10 ng p4xUAS-tk-luc, 5 ng pBK-CMV-GAL4-hRXRα, and then treated with 9cRA or each of the tin compounds. The doses of each compound were: 0, 1, 10, or 100 nM of 9cRA, TETBr, TBTCl, TPTOH, TPTCl, TBTH, or TBTBr; 0, 0.1, 1, or 10 μM of TOTH, $SnCl_4$, $MBTCl_3$, or TBVT; 0, 1, 10, or 30 nM of TPrTCl, TChTOH, or $DBTCl_2$; 0, 0.1, 1, or 3 μM of $MPTCl_3$ or TeBT; 0, 10, 100, or 300 nM of $DPTCl_2$; and 0, 10, 100, or 1000 nM of TMTCl. pRL-TK (2 ng) was cotransfected as the control for normalization (see *Materials and Methods*). The results are expressed as average fold activation ± 1 SD after normalization to *Renilla* LUC activity. Groups I, II, and III correspond to the groups described in Fig. 2. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.005$.

Binding of Tin Compounds to RXR

To characterize the binding affinities of these tin compounds to RXR directly, we performed analyses of the saturation binding of [^{14}C]TPTOH and [^{3}H]9cRA to chimeric receptors, which consisted of glutathione *S*-transferase (GST) fused to the LBD of human RXRs (GST-RXRs). The binding of 9cRA to GST-RXRs was specific and saturative (Fig. 7). Scatchard analyses of the binding of [^{3}H]9cRA to GST-RXRα, -β, and -γ yielded dissociation constant (K_d) values of 11.7, 15.5, and 8.66 nM, respectively. These K_d values were similar to those previously reported (3, 42), suggesting that this system is useful for determining the binding affinity of alkyltin compounds to RXRs. Scatchard analyses of the binding of [^{14}C]TPTOH to RXRα, -β, and -γ yielded K_d values of 55.5, 241, and 95.3 nM, respectively (Fig. 7). Although the K_d values of TPTOH for RXRs were approximately 5- to 15-fold higher than those for 9cRA, our results indicate that TPTOH binds to the RXRs with high affinity in a saturable and specific manner.

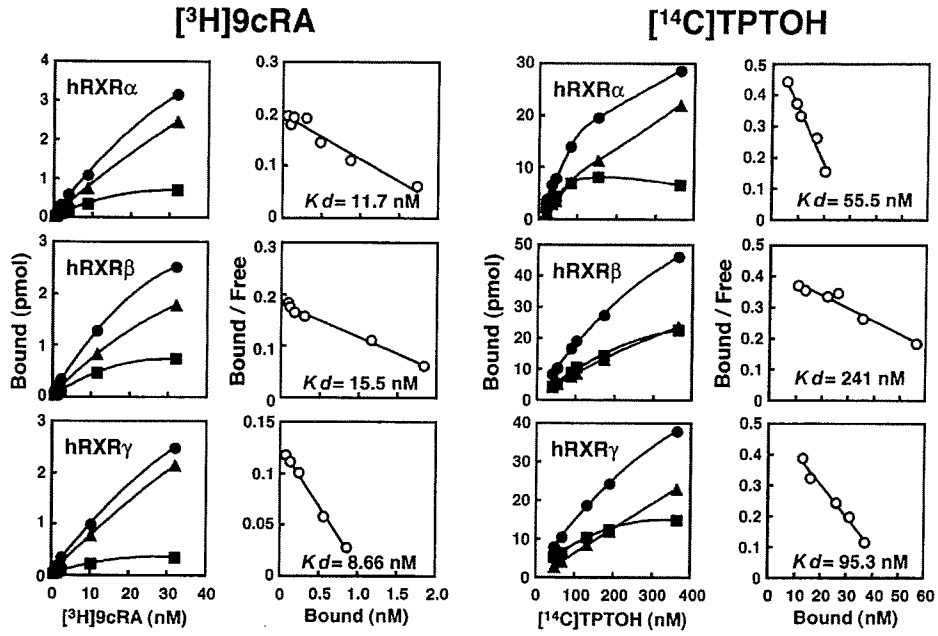

Fig. 7. Saturation Kinetics for the Binding of [^{3}H]9cRA and [^{14}C]TPTOH to hRXRα, -β, and -γ

Specific binding (*solid square*) is defined as total binding (*solid circle*) minus nonspecific binding (*solid triangle*). Scatchard analysis was performed on specific binding data (triplicates at each point) to yield the indicated dissociation constants (K_d value) for each receptor.

To further test which of the other tin compounds might bind to RXR as a ligand, we performed competitive ligand-binding assays. We measured the ability of [^{3}H]9cRA or [^{14}C]TPTOH to compete with the tin compounds for binding to GST-RXRα. The TBT and TPT derivatives all competed with [^{3}H]9cRA for binding to GST-RXRα in a concentration-dependent manner (Fig. 8). Consistent with the K_d value of TPTOH for RXRα, the IC_{50} value of TPTOH for binding [^{3}H]9cRA was approximately 5-fold higher than that of unlabeled 9cRA. The IC_{50} values of the other TBT and TPT derivatives for binding [^{3}H]9cRA were almost the same as that of TPTOH, demonstrating that these trialkyltin compounds bind directly to RXR. In contrast to the results of competitive assay for [^{3}H]9cRA, 9cRA failed to completely out compete [^{14}C]TPTOH for binding to GST-RXRα, whereas the TBT and TPT derivatives successfully competed for binding (Fig. 8B). The IC_{50} value of 9cRA for binding [^{14}C]TPTOH was more than 10-fold higher than those of TBTs and TPTs (Table 1). Further, TPrTCl bound to GST-RXRα as well as did the TBT and TPT derivatives, because its IC_{50} values for binding [^{3}H]9cRA and [^{14}C]TPTOH were almost the same as those of the TBT and TPT derivatives (Fig. 8 and Table 1). However, TChTOH, TeBT, and TBVT failed to compete with [^{3}H]9cRA for binding to GST-RXRα, despite their ability to activate RXRα. Although TChTOH out competed [^{14}C]TPTOH for binding to GST-RXRα, TeBT only slightly out competed [^{14}C]TPTOH, and TBVT failed to compete with [^{14}C]TPTOH for binding to GST-RXRα (Table 1). By contrast, triethyltin bromide (TETBr), which was unable to activate transcription through an RXR, bound weakly to GST-RXRα. The IC_{50} values of TETBr for binding [^{3}H]9cRA and [^{14}C]TPTOH were approximately 25- to 50-fold higher than those of the TBT and TPT derivatives (Table 1). The remaining tin compounds (trimethyltin chloride, TMTCl; trioctyltin hydride, TOTH; butyltin trichloride, $MBTCl_3$; phenyltin trichloride, $MPTCl_3$; $DBTCl_2$; $DPTCl_2$; and $SnCl_4$) did not compete successfully with either [^{3}H]9cRA or [^{14}C]TPTOH for binding to GST-RXRα, suggesting that they were unable to bind to RXRs.

Trialkyltin Compounds Stimulate the Expression of an LUC Construct Containing the Human Placental I.1 Sequence of Aromatase via Activation of RXRα

To determine whether trialkyltin compounds, which bind to and activate RXRs, induce aromatase expression in the human placenta via ligand-dependent activation of RXRs, we assessed the dose-response effectiveness of TBTCl and TPTOH by using JEG-3 cells cotransfected with a human RXRα expression plasmid and a LUC reporter plasmid containing the promoter sequence (−2295 to +107 bp) of exon I.1, which is the major promoter of human placental aromatase (Fig. 9A; and Refs. 16, 43, and 44). As expected, LG100268 stimulated the expression of LUC by 2.4- to 3.5-fold. These results suggest that the aromatase promoter is regulated by ligand-bound RXR (13). In addition, TBTCl and TPTOH stimulated the expression of LUC in

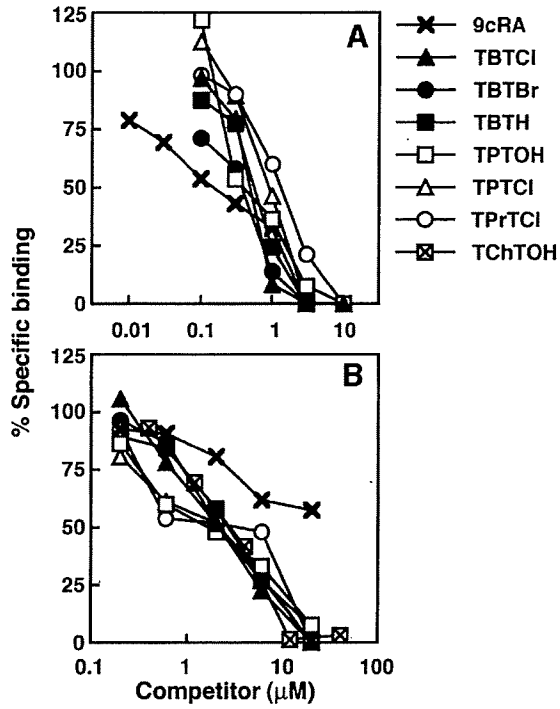

Fig. 8. Competition by 9cRA and Alkyltin Compounds with [^{3}H]9cRA (A) and [^{14}C]TPTOH (B) for Binding to the LBD of hRXRα

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.

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),

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.

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-

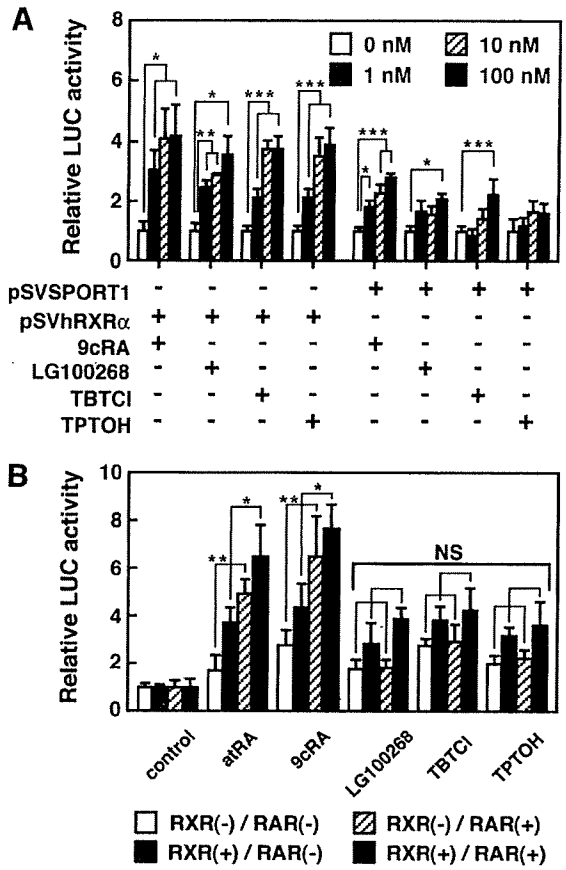

Fig. 9. Ability of TBTCl and TPTOH to Increase Transcription of an LUC Reporter Gene Containing the Human Placental Promoter I.1 Sequence of Aromatase via the Activation of RXR but not RAR

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