Biochemical Pharmacology, Vol. 53, pp. 1425–1434, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00084-1

Modulation of the Hepatic Expression of the Estrogen-Regulated mRNA Stabilizing Factor by Estrogenic and Antiestrogenic Nonsteroidal Xenobiotics

Ratneswaran Ratnasabapathy, Meldon Tom and Carole Post*

DIVISION OF PHARMACOLOGY, TOXICOLOGY AND MEDICINAL CHEMISTRY, ARNOLD AND MARIE SCHWARTZ COLLEGE OF PHARMACY, LONG ISLAND UNIVERSITY, BROOKLYN, NY 11201 U.S.A.

ABSTRACT. Estrogen-mediated accumulation of apolipoprotein II (apoII) mRNA in the avian liver is due, in part, to its stabilization. This stabilization appears to be due to the estrogen-regulated mRNA stabilizing factor (E-RmRNASF) that is expressed in response to estrogen. The E-RmRNASF protects the mRNA from targeted endonucleolytic degradation (Ratnasabapathy, *Cell Mol Biol Res* **41**: 583–594, 1995). To determine whether certain environmental xenobiotics altered the expression of the gene encoding E-RmRNASF by mimicking estrogen, roosters were given estrogen, tamoxifen, clomiphene, hexachlorophene, lindane, rotenone, chlordecone, dichlorodiphenyltrichloroethane (DDT), Araclor, methoxychlor, dieldrin, toxaphene, or bisphenol-A parenterally. Uniformly radiolabeled, capped, and polyadenylated apoII mRNA, incubated *in vitro* in the presence of liver cytosolic extracts from birds that received estrogen, tamoxifen, hexachlorophene, chlordecone, or Araclor, remained stable, indicating that these agents were estrogenic and stimulated the expression of E-RmRNASF. However, the same mRNA was degraded in similar extracts from control roosters and those treated with clomiphene, DDT, methoxychlor, dieldrin, rotenone, toxaphene, lindane, or bisphenol-A. To determine whether the latter agents were antiestrogenic, roosters were given a 1:5 molar combination of estrogen and each of the xenobiotics. ApoII mRNA showed degradation in liver extracts from roosters that received clomiphene, toxaphene, or bisphenol-A, indicating that these agents prevented estrogenic stimulation of expression of the E-RmRNASF and were antiestrogenic. However, the rest of the xenobiotics failed to antagonize estrogenic stimulation of *E-RmRNASF* gene expression. These results set a precedent in showing the estrogenic and antiestrogenic effects *in vivo* of environmental xenobiotics on the expression of a regulatory protein involved in estrogen-mediated mRNA stabilization. BIOCHEM PHARMACOL 53;10:1425–1434, 1997.

KEY WORDS. estrogen; xenobiotics; environmental toxicants; pollutants; apolipoprotein II; mRNA stability

Many environmental xenobiotics such as organochlorines have been shown to exert estrogenic or antiestrogenic effects at cellular and organismal levels [1–5]. These agents have been implicated in the burgeoning high incidence of hormonally related cancers, gender-bending effects, and infertility in human and animal populations [6–12]. For the past two to three decades, there has been widespread use worldwide of several organocyclic pesticides, including organochlorines. The organochlorine pesticides include chlorinated ethane derivatives such as DDT†, methoxychlor, cyclodienes such as chlordane, aldrin, dieldrin, heptachlor, and endrin, and other chlorinated cyclic hydrocarbons such as lindane (hexachlorocyclohexane), toxaphene, mirex, and chlordecone. Because they are biotransformed at extremely slow rates, many of these agents have accumulated in the vast majority of global ecosystems [10–13]. In addition to these pesticides, certain other industrial compounds such as bisphenol, and Aroclor, a PCB, also have accumulated in the environment due to the same reason. These lipophilic compounds have been biomagnified in the food web, often reaching dangerous concentrations in the animals higher up in the food chain [13–16]. DDT, for instance, has been a prime candidate for biomagnification, and is believed to be the cause of the near extinction of fish-eating birds such as the bald eagle [16, 17]. DDT and some of the other pesticides stimulate the microsomal cytochrome P450 enzymes [18], and are believed to increase steroid hormone metabolism, causing endocrine imbalance. Many of the environmental xenobiotics are known to

* Corresponding author: Ratneswaran Ratnasabapathy, Ph.D., Department of Pharmacology, AMS College of Pharmacy, Long Island University, Brooklyn, NY 11201. Tel. (718) 488-1499; FAX (718) 488-3437.

† *Abbreviations:* apoII, apolipoprotein II; E-RmRNASF, estrogen-regulated mRNA stabilizing factor; mRNP, messenger ribonucleoprotein (mRNA-binding protein); DDT, dichlorodiphenyltrichloroethane; PMSF, phenylmethylsulfonyl fluoride; and PCB, polychlorinated biphenyl.

Received 3 September 1996; accepted 21 November 1996.

produce a wide variety of toxic effects in humans and animals [1–19].

Whether or not these organochlorines exert estrogenic or antiestrogenic effects on the regulation of gene expression has not been demonstrated adequately. Studies on the effects of xenobiotics on transcriptional and posttranscriptional regulation of gene expression are essential for the understanding of the molecular basis of xenobiotic-induced pathogenesis. The only other report on altered mRNA stability due to a xenobiotic came from studies done by Aida and Negishi [20]. They showed that pyrazole, a non-environmental and non-therapeutic chemical, enhances the expression of coumarin-7-hydroxylase (P450coh) through stabilization of its mRNA, but the barbiturate-mediated induction of this enzyme did not follow the same mechanism. However, the mechanism of pyrazole-induced stabilization of P450coh mRNA has not been identified.

The regulation of mRNA turnover by stabilization or destabilization of mRNA is an important cytoplasmic control mechanism for modulating gene expression [21–23]. Various control factors have been shown to selectively regulate the rates of decay of specific mRNAs [21–26]. Estrogen regulates the stability of ovalbumin mRNA in the oviduct [21] and apoII and vitellogenin mRNAs in the chick liver [23, 25]. In addition to regulating the expression of apoII at the level of transcription, estrogen also alters the rate of turnover of the induced mRNA in the liver. The apoII mRNA has a $T^{1/2}$ of 13 hr in the presence of estrogen; withdrawal of estrogen after 14 days of treatment reduces the $T_{1/2}$ to 1.5 hr [25]. Unlike the mRNAs that undergo deadenylation [poly(A) shortening]-dependent degradation [26–28], apoII mRNA is targeted for degradation via endonucleolytic cleavage at 5′ AAU-3′/5′-UAA-3′ elements in single-stranded loop domains of its 3′ untranslated region (UTR) [29]. The cleavages occur in two larger domains of secondary structure of the 3′ UTR that bind specifically and independently to two apoII mRNPs of molecular weights 60 and 34 kDa [30]. Estrogen also induces the expression of a subset of apoII mRNA binding proteins of molecular sizes 32, 34, and 90 kDa that specifically bind apoII mRNA [31]. The estrogen-inducible mRNPs that bind the 3′ UTR of apoII mRNA also bind other estrogen-stabilized mRNAs, such as those encoding apo B and vitellogenin II, but not the non-estrogen-regulated mRNAs [32]. Dodson and Shapiro [24] have also demonstrated that estrogen induces proteins that bind specifically to a 27 *nt*, high-affinity binding site in the 3′ untranslated region of estrogen-stabilized *Xenopus* vitellogenin mRNA. They showed that two cytosolic proteins of molecular sizes 71 and 141 kDa bind to the 27 *nt* region. However, whether or not these estrogen-induced mRNPs have any function in the estrogen-mediated modulation of the respective mRNAs is not known. Estrogen has been shown to induce a cytosolic, biochemical activity in the rooster liver that protects specific mRNAs (including apoII mRNA) from endonucleolytic degradation [31]. This estrogen-mediated mRNA stabilizing activity was due to estrogen-dependent expression of a protein factor named E-RmRNASF in the liver [31]. In the absence of estrogen, there is no detectable hepatic expression of the E-RmRNASF. However, parenteral administration of estrogen stimulates the expression of E-RmRNASF in the rooster liver [31]. The molecular basis of estrogen-mediated stabilization of apoII mRNA is not fully understood and may involve the E-RmRNASF and possibly the estrogen-regulated mRNPs.

In the present study, the estrogenic and antiestrogenic effects of a few selected nonsteroidal compounds, including some environmental xenobiotics, on the regulation of *E-RmRNASF* gene expression were investigated. Estrogenic effects of these xenobiotic agents were determined by their ability to turn on the hepatic expression of the E-RmRNASF by mimicking estrogen, while the antiestrogenicity of these xenobiotics was determined by their ability to prevent stimulation of hepatic E-RmRNASF expression by estrogen. The hepatic expression of E-RmRNASF was biochemically detected by its ability to prevent the endonucleolytic degradation of apoII mRNA *in vitro* in cell-free liver cytosolic extracts. Similar cell-free systems have been employed for studies on the degradation of mRNAs [28, 31].

The following xenobiotic agents were examined for their estrogenic or antiestrogenic effects on the hepatic expression of E-RmRNASF: **tamoxifen** (Sigma Chemical Co., St. Louis, MO), {Nolvadex; ([Z] [*trans*] 4β-dimethylaminoethoxyphenyl) 1,2-diphenylbut-1-ene citrate; used in the treatment of breast cancer}; **clomiphene** (Sigma), {Clomid; a mixture of [Z] and [E] [41% *cis* and 57% *trans*] isomers of 2-chloro-1-(4β-diethylaminoethoxyphenyl)-1,2-diphenylethylene; used successfully for the induction of ovulation in subfertile women}; **hexachlorophene** (Sigma), {2,2′-methylenebis[3,4,6-trichlorophenol]; 2,2′-dihydroxy-3,3′,5,5′, 6,6′-hexachlorodiphenylmethane; $C_{13}H_6Cl_6O_2$; pHisohex, Dermadex, Exofene; used in germicidal soaps, topical antiinfectives, and anthelmintics [flukicide]}; **lindane** (Sigma), {Gammexane, Lindatox, Lorexene; {1α,2α,3β,4α,5α,6β-hexachlorocyclohexane; γ-HCH, γ-benzene hexachloride; $C_6H_6Cl_6$; used as an insecticide, pediculicide, scabicide, and ectoparasiticide in animals}; **rotenone** (Sigma), {Canex; [2*R*-(2α,6aα,12aα)]-1,2,12,12a-tetrahydro-8,9-dimethoxy-2-(1-methylethenyl)-[1]benzopyrano-[3,4-*b*]furo[2,3-*h*][1]benzopyran-6(6aH)-one; $C_{23}H_{22}O_6$; this powerful inhibitor of mitochondrial electron transport has been used as a pesticide, acaricide, and ectoparasiticide}; **chlordecone** (Chem Service, West Chester, PA), {Kepone; 1,1a,3,3a,4,5,5,5a,5b,6-decachlorooctahydro-1,3,4-metheno-2*H*-cyclobuta[*cd*]pentalen-2-one; $C_{10}Cl_{10}O$; used as an insecticide and fungicide, which is an environmental pollutant}; **Araclor 1254** (Chem Service), {PCB, a nonbiodegradable organochlorine for industrial use, which is an environmental pollutant}; **DDT** (Chem Service), {dichlorodiphenyltrichloroethane; 1,1′-(2,2,2-trichloroethylidene)bis[4-chlorobenzene]; $C_{14}H_9Cl_5$; chlorophenothane, used as a contact insecticide, ectoparasiticide, and pediculicide, this nonbiodegradable organochlorine is also an environmental pollutant}; **methoxychlor** (Chem

Service), {1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl)ethane; methoxy DDT, Marlate; $C_{16}H_{15}Cl_3O_3$; used as an insecticide and ectoparasiticide}; **toxaphene** (Chem Service), {chlorinated camphene; Alltox, Geniphene, Phenatox, $C_{10}H_{10}Cl_8$; used as an insecticide and is also an environmental pollutant}; **bisphenol-A** (Sigma), {4,4'-(methylethylidenediphenol; 2,2-bis(4-hydroxyphenyl)propane; $C_{15}H_{16}O_2$; this fungicide has also been used in the manufacture of epoxy resins and polycarbonates, and is an environmental pollutant}; **dieldrin** (Sigma), {(1aα,2β,2aα,3β,6β,6aα,7β,7aα) - 3,4,5,6,9, 9 - hexachloro - 1a,2,2a,3.6,6a,7,7a - octahydro - 2,7:3,6 - dimethanonaphth[2,3-*b*]oxirene; $C_{12}H_8Cl_6O$; used as an insecticide, and is also an environmental pollutant}.

MATERIALS AND METHODS

Synthesis of Uniformly Radiolabeled apoII mRNA

Uniformly radiolabeled, authentic capped, and polyadenylated apoII mRNA was synthesized by run-off transcription using an expression plasmid, pT7NAPOII [30, 31]. This expression plasmid contains the full-length apoII cDNA with a unique *Hin*dIII restriction site at the end of the poly(A) run of about 50 *nt*. Its construction and characterization have been described previously [30]. The plasmid was linearized by digestion with *Hin*dIII and transcribed with T7 RNA polymerase (0.8 U/μL) at 37° for 1 hr in a reaction containing 400 μM each of ATP, GTP, and CTP, 100 μM UTP, 2 mM ^{7Me}GpppG, [α-^{32}P]UTP (2 μCi/μL, 3000 Ci/mmol, Amersham Co., Arlington Heights, IL), 40 mM Tris–HCl, pH 8.0, 8 mM $MgCl_2$, 25 mM NaCl, 2 mM spermidine$(HCl)_3$, and 2 mM DDT. The RNA was purified by oligo(dT) affinity chromatography.

Preparation of Liver Cytosolic Extracts

Cytosolic extracts (S100) were prepared by a previously described procedure [30, 31] from livers of 3-week-old, white leghorn roosters (SPAFAS, Norwich, CT) that received an intramuscular injection of 50 μmol/kg 17β-estradiol (Sigma) (estrogen-treated extract), or 50 μmol/kg of the test xenobiotic compounds. To test for antiestrogenic effects, roosters received a combination of 50 μmol/kg of estrogen and 250 μmol/kg of test xenobiotics in peanut oil; control roosters received the same volume of the vehicle without estrogen (control extract). The animals were killed 3 days later. Estrogen and the lipophilic xenobiotics, when administered intramuscularly in peanut oil, would presumably partition into the tissues at a slower rate than by oral, intravenous, or intraperitoneal routes of administration. Maximal estrogenic induction of apolipoprotein B in rooster liver was achieved with 3 mg/kg (11 μmol/kg) of estrogen by Capony and Williams [33]. To safely achieve maximal estrogenic response, most of the previous studies have administered an intramuscular dose of 20 mg/kg (73.4 μmol/kg) of estrogen [25, 29–33]. Alternatively, some of these studies have employed subcutaneous implantation of constant-release tablets containing 100 mg of 17-β estradiol [29–33]. The levels of toxicants involved in chronic and acute exposures of humans and wildlife to highly contaminated food, water, or air in their normal habitats, or in factory workers during accidental or occupational exposures, may exceed the amount of xenobiotics used in the current study. However, the average human exposure to these environmental agents is considerably less than 50 μmol/kg. Chronic accumulation of some of these agents in the tissues from constant exposure could be higher than those achieved by a single acute treatment with 50 μmol/kg as employed in this study. All procedures were carried out at 0–4°. A 50% homogenate of liver was prepared with a hand-held, loose-fitting Dounce homogenizer in 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 5 μg/mL leupeptin, and 5 μg/mL aprotinin (Sigma). The homogenate was centrifuged for 10 min at 800 *g*, and the supernatant was removed and mixed with 0.11 vol. of 300 mM HEPES, pH 7.9, 1.4 M KCl, 30 mM $MgCl_2$, 0.5 mM PMSF, 0.5 mM DTT, 5 μg/mL leupeptin, and 5 μg/mL aprotinin. Following centrifugation at 100,000 *g* for 60 min in a Beckman SW 41 rotor, the supernatant was dialyzed for 5–8 hr against 20 vol. of 20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 30 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 5 μg/mL leupeptin, and 5 μg/mL aprotinin, flash-frozen in liquid N_2, and stored at −80°. Protein concentrations were estimated by standard procedures [34].

In Vitro Assay for Determination of apoII mRNA Stability

Radiolabeled apoII mRNA (2.5×10^5 cpm) was incubated in the presence of 10 mM HEPES, pH 7.9, 40 mM KCl, 6 mM $MgCl_2$, 1 mM DTT, 5 mg/mL heparin, 40 μg/mL yeast tRNA, and 20 μg protein of control or estrogen-treated liver cytosolic extracts at 26°, in time-course incubations. Results from experiments with extracts pooled from four similarly treated roosters were identical with that of each of the individual extracts. There appeared to be no individual variation among the different extracts. Reaction was stopped by the addition of 200 μL of stop solution containing 0.1% SDS and 4 mM EDTA. RNA was electrophoresed on 8 M urea-6% polyacrylamide gel and visualized by autoradiography. The results presented here are those of studies performed with individual roosters. Each of the experiments was repeated at least three times, and consistently gave the same results.

RESULTS

Stabilization of apoII mRNA in Liver Cytosolic Extracts from Estrogen-Treated Roosters

Figure 1 shows results of an experiment performed to demonstrate that treatment of roosters with estrogen results

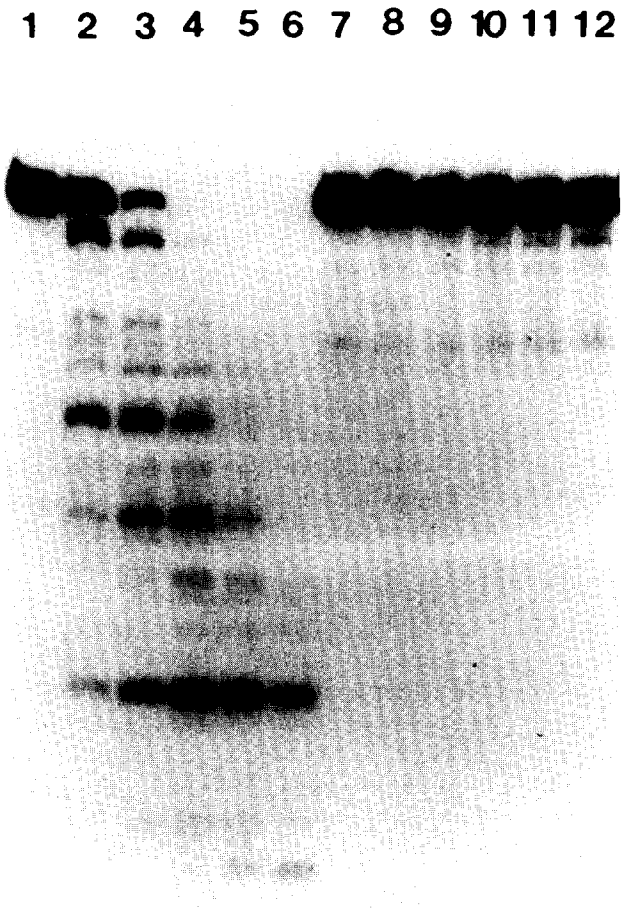

FIG. 1. Time-course incubations of apoII mRNA in the presence of S100 liver cytosolic fractions from roosters with or without estrogen treatment. An autoradiograph of an 8 M urea-6% polyacrylamide gel of uniformly radiolabeled, capped, and polyadenylated apoII mRNA incubated in the presence of 20 μg of S100 cytosolic fraction of liver (control) from rooster treated with only the vehicle (lanes 2–6), or from rooster treated with 50 μmol/kg estrogen (lanes 8–12) at 26° for 1 min (lanes 2 and 8), 2.5 min (lanes 3 and 9), 15 min (lanes 4 and 10), 30 min (lanes 5 and 11), and 60 min (lanes 6 and 12) or in reaction buffer in the absence of any extract for 60 min (lanes 1 and 7).

in the expression of the apoII mRNA stabilizing activity in the avian liver. Uniformly radiolabeled apoII mRNA was incubated in the presence of liver cytosolic extract from roosters that received only the vehicle (Fig. 1, lanes 2–6), or 50 μmol/kg estrogen (Fig. 1, lanes 8–12) at 26° for 1 min (Fig. 1, lanes 2 and 8), 2.5 min (Fig. 1, lanes 3 and 9), 15 min (Fig. 1, lanes 4 and 10), 30 min (Fig. 1, lanes 5 and 11), and 60 min (Fig. 1, lanes 6 and 12). In Fig. 1, lanes 1 and 7 show apoII mRNA incubated under the same conditions as above in reaction buffer in the absence of any extract for 60 min. The apoII mRNA underwent rapid endonucleolytic degradation in a time-dependent manner (Fig. 1, lanes 2–6), resulting in complete breakdown of all the full-length mRNA molecules into smaller fragments after 60 min of incubation (Fig. 1, lane 6). However, the same mRNA, when incubated for the same duration in the presence of the liver cytosolic extracts from roosters that received 50 μmol/kg β-estradiol, remained very stable at all the corresponding time points (Fig. 1, lanes 8–12). The mRNA stabilization was shown previously to be due to an estrogen-regulated mRNA stabilizing factor expressed in the liver upon estrogen stimulation [31]. The estrogenic stimulation of the mRNA stabilizing activity was inhibited by intramuscular administration of 7.5 mg/kg actinomycin-D 3 hr prior to estrogen treatment, indicating that estrogen stimulated transcription of the gene encoding the mRNA stabilizing factor (results not shown).

Estrogenic Effect of Xenobiotics on the Expression of the Estrogen-Regulated mRNA Stabilizing Activity

To determine whether certain commonly encountered xenobiotics purported to mimic estrogen have any effects on the expression of genes that are under estrogenic control, roosters were treated with 50 μmol/kg of estrogen or each xenobiotic compound. The control birds received only the vehicle. S100 cytosolic extracts prepared from the livers of these birds were then assayed for the activity of the estrogen-regulated mRNA stabilizing factor by incubating uniformly radiolabeled, capped, and polyadenylated apoII mRNA at 26° for 1 hr in the presence of each of these extracts. Figure 2 shows the results of uniformly radiolabeled, capped, and polyadenylated apoII mRNA incubated in 20 μg liver cytosolic extracts from roosters treated with 50 μmol/kg of estrogen or the xenobiotics. Lane 1 in Fig. 2 shows apoII mRNA incubated under the same conditions as

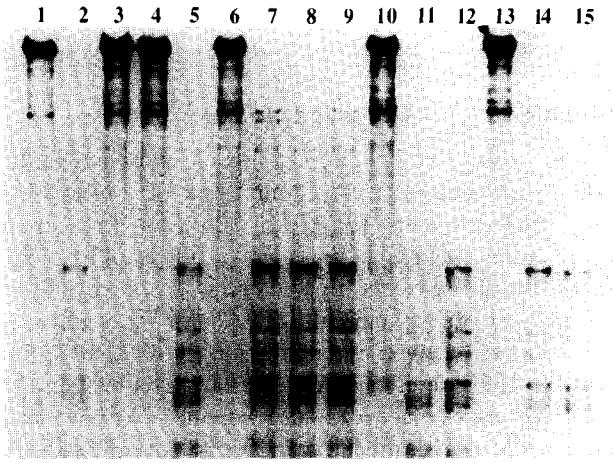

FIG. 2. Incubation of apoII mRNA with S100 liver cytosolic fractions from roosters treated with xenobiotic agents. An autoradiograph of an 8 M urea-6% polyacrylamide electrophoretic gel of uniformly radiolabeled, capped, and polyadenylated apoII mRNA incubated for 1 hr at 26°, in the presence of 20 μg of liver S100 cytosolic extract from roosters that received only the vehicle (lane 2) or 50 μmol/kg estrogen (lane 3), tamoxifen (lane 4), clomiphene (lane 5), Araclor (lane 6), methoxychlor (lane 7), DDT (lane 8), toxaphene (lane 9), Kepone (lane 10), dieldrin (lane 11), bisphenol-A (lane 12), hexachlorophene (lane 13), lindane (lane 14), or rotenone (lane 15). Lane 1 corresponds to apoII mRNA incubated under the same conditions as above, but in the absence of protein.

above, but in the absence of protein. The apoII mRNA was degraded in the control extract (Fig. 2, lane 2) as well as in extracts from roosters that received clomiphene (Fig. 2, lane 5), methoxychlor (Fig. 2, lane 7), DDT (Fig. 2, lane 8), toxaphene (Fig. 2, lane 9), dieldrin (Fig. 2, lane 11), bisphenol-A (Fig. 2, lane 12), lindane (Fig. 2, lane 14), and rotenone (Fig. 2, lane 15). However, the apoII mRNA remained stable in extracts from roosters treated with estrogen (Fig. 2, lane 3), tamoxifen (Fig. 2, lane 4), Araclor (Fig. 2, lane 6), Kepone (chlordecone) (Fig. 2, lane 10), and hexachlorophene (Fig. 2, lane 13). It is evident from these results that agents such as tamoxifen, Araclor, Kepone, and hexachlorophene are estrogenic agonists and exert an effect similar to that of estrogen on the expression of the estrogen-regulated mRNA stabilizing factor. Even at higher concentrations, toxaphene (60 mg/kg or 145 μmol/kg), DDT (100 mg/kg or 282 μmol/kg), methoxychlor (250 mg/kg or 723.2 μmol/kg), dieldrin (200 mg/kg or 525 μmol/kg), lindane (200 mg/kg or 687.6 μmol/kg), bisphenol-A (200 mg/kg or 876.1 μmol/kg), rotenone (200 mg/kg or 507 μmol/kg), or clomiphene (200 mg/kg or 492.6 μmol/kg) failed to stimulate E-RmRNASF expression (results not shown), indicating that these agents do not have any agonist activity even at very high concentrations. Durations of exposure to the same xenobiotics for other than 3 days also were tested. A lack of stimulation of E-RmRNASF was observed in roosters that were killed 1, 2, 4, 5, 6, or 7 days after intramuscular injections of 50 μmol/kg of the above xenobiotics, indicating that estrogenic response of the gene to the latter agents was not elicited for as long as 7 days after acute exposure.

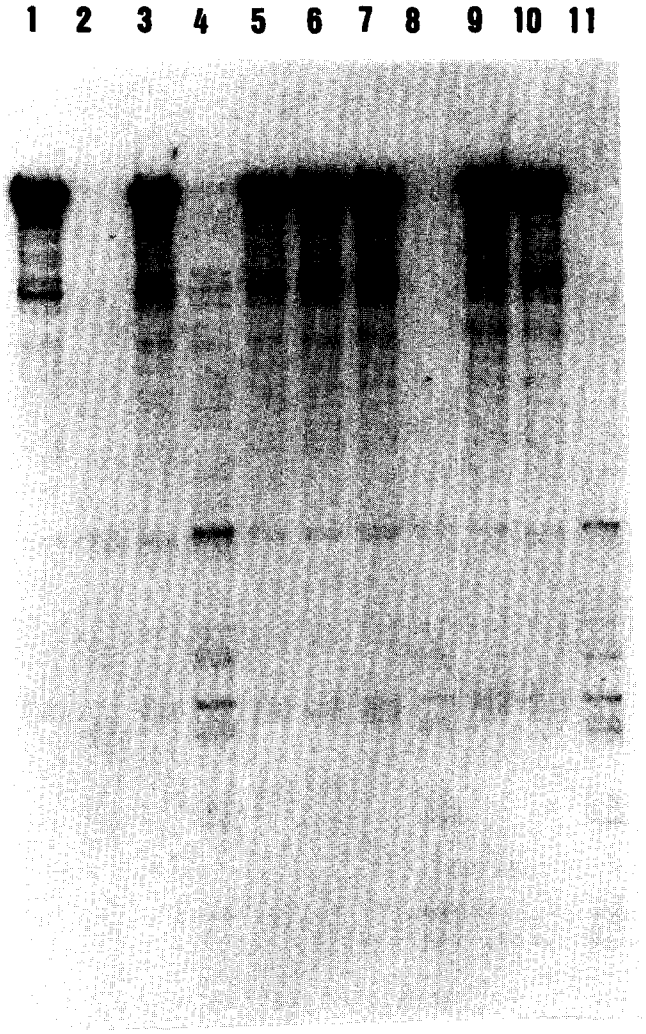

FIG. 3. Incubation of apoII mRNA with S100 liver cytosolic extracts from roosters treated with a combination of estrogen and each of the xenobiotic agents. An autoradiograph of an 8 M urea-6% polyacrylamide gel of uniformly radiolabeled, capped, and polyadenylated apoII mRNA incubated for 1 hr at 26°, in the presence of S100 liver cytosolic extracts from roosters that received only the vehicle (lane 2), or 50 μmol/kg estrogen (lane 3), 50 μmol/kg estrogen and 250 μmol/kg clomiphene (lane 4), 50 μmol/kg estrogen and 250 μmol/kg DDT (lane 5), 50 μmol/kg estrogen and 250 μmol/kg methoxychlor (lane 6), 50 μmol/kg estrogen and 250 μmol/kg lindane (lane 7), 50 μmol/kg estrogen and 250 μmol/kg toxaphene (lane 8), 50 μmol/kg estrogen and 250 μmol/kg dieldrin (lane 9), 50 μmol/kg estrogen and 250 μmol/kg rotenone (lane 10), or 50 μmol/kg estrogen and 250 μmol/kg bisphenol-A (lane 11). Lane 1 corresponds to apoII mRNA incubated under the same conditions as above, but in the absence of proteins.

Inhibition by Certain Xenobiotic Agents of Estrogenic Stimulation of Expression of the Estrogen-Regulated mRNA Stabilizing Activity

To determine which of the agents that failed to induce E-RmRNASF expression were truly antiestrogenic and which of them simply failed to interact with the estrogen receptor, roosters were treated with 50 μmol/kg of estrogen, a combination of 50 μmol/kg estrogen and 250 μmol/kg of the xenobiotic, or the vehicle alone parenterally 3 days prior to being killed. Liver cytosolic extracts prepared from these birds were then tested for the mRNA stabilizing activity. Figure 3 shows the results of uniformly radiolabeled, capped, and polyadenylated apoII mRNA incubated in the presence of the above extracts. The apoII mRNA remained stable in the presence of extracts from roosters treated with estrogen (lane 3) and combinations of estrogen and DDT (lane 5), estrogen and methoxychlor (lane 6), estrogen and lindane (lane 7), estrogen and dieldrin (lane 9), or estrogen and rotenone (lane 10). These results indicate that DDT, methoxychlor, lindane, and dieldrin were unable to antagonize the stimulation of expression of the mRNA stabilizing activity by estrogen. These agents failed to antagonize estrogen even at a 1:10 molar combination of estrogen:xenobiotic (results not shown). However, the apoII mRNA was degraded in extracts from roosters that received only the vehicle (lane 2) or combinations of estrogen and clomiphene (lane 4), estrogen and toxaphene (lane 8), or estrogen and bisphenol-A (lane 11). These results indicate that clomiphene, toxaphene, and bisphenol-A prevented estrogenic stimulation of the above gene. Clomiphene, toxaphene, and bisphenol-A exert an antiestrogenic effect on the expression of the mRNA stabilizing activity.

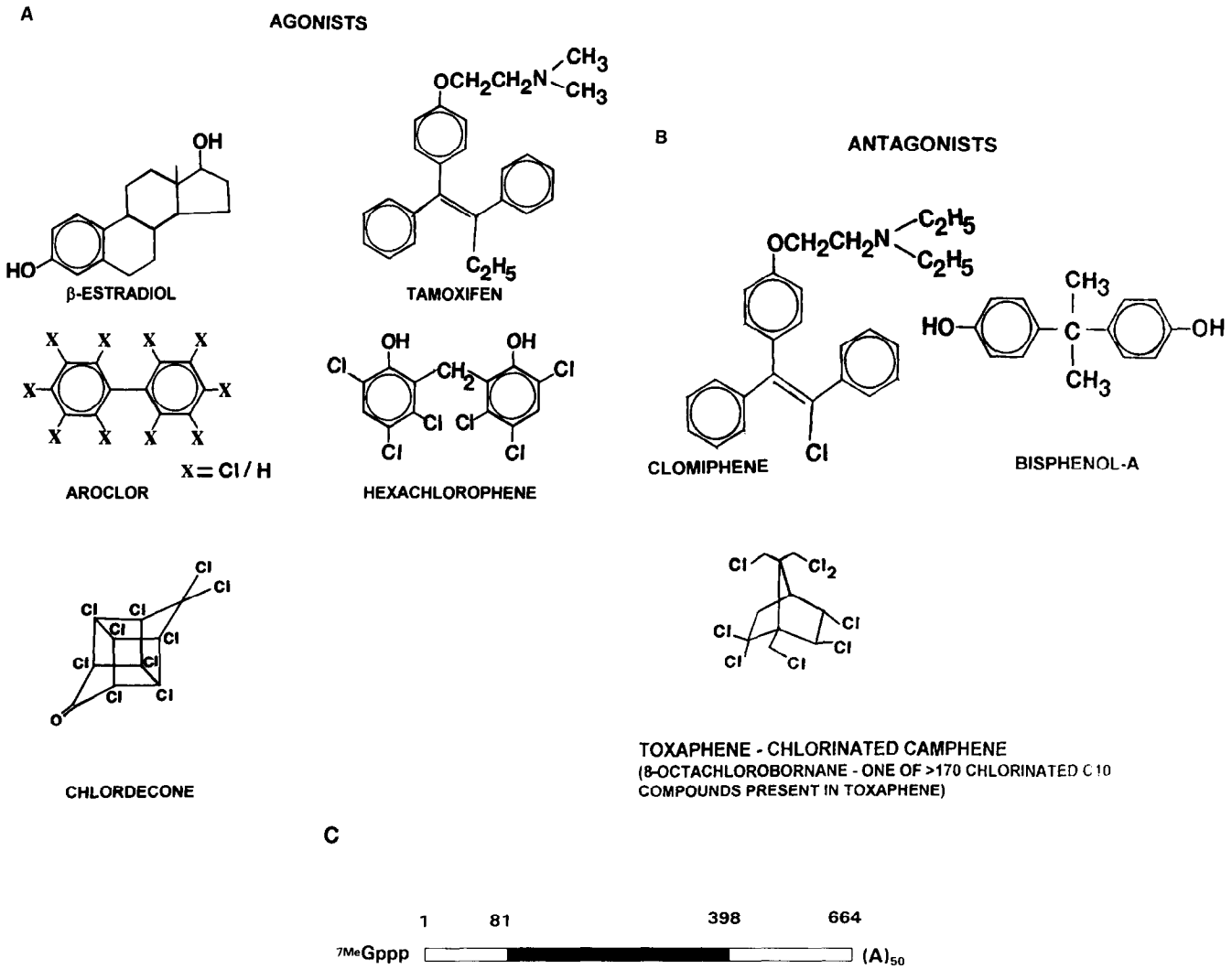

FIG. 4. Chemical structures of xenobiotic agents that stimulate or inhibit the expression of the hepatic estrogen-regulated mRNA stabilizing factor. The chemical structures of the estrogenic xenobiotics that stimulate (panel A) and antiestrogenic xenobiotics that inhibit (panel B) the hepatic expression of the estrogen-regulated mRNA stabilizing factor. Panel C shows the diagram of capped and polyadenylated synthetic apoII mRNA transcribed *in vitro* from pT7NAPOII expression vector, with nucleotide coordinates. The coding region is shaded.

Synthetic apoII mRNA

Figure 4C shows a diagram of apoII mRNA as transcribed from the pT7NAPOII vector. The characterization of this vector and the apoII mRNA transcribed *in vitro* from this vector was described previously [30]. ApoII mRNA transcribed from pT7NAPOII was full-length, capped, polyadenylated, and translationally active [30].

DISCUSSION

Determination of xenobiotic-induced changes in macromolecular structure or function such as gene expression, would enable early and rapid assessment of toxic effects of xenobiotics on biological systems. Very little is known to date about the effects of environmental toxins on transcriptional and posttranscriptional regulation of gene expression. Chronic toxicity resulting from some environmental xenobiotics has been attributed to xenobiotic-induced endocrinological dysfunction [6–8, 10–12, 16]. For example, the environmental xenobiotics that mimic steroidal hormones have been implicated in the burgeoning high incidence of breast cancer and other gender-specific disorders [6–12]. Exposure to these steroid-mimicking xenobiotics may also account for the infertility seen in wildlife as well as humans [6–9, 11]. A recent epidemiological study demonstrated a significant correlation between blood levels of 1,1-dichloro-2,2-bis-(*p*-chlorophenyl) ethylene (DDE) and the risk of breast cancer [12]. A positive correlation between breast cancer and exposure to PCBs has also been observed [10, 35].

Estrogen has been implicated in oncogenesis and maintenance of certain types of uterine and mammary carcinoma. The proto-oncogenes *fos*, *jun*, and *myc* are stimulated by estrogen [35]. Estrogen-dependent mRNA stability has

been shown to be an important component of this regulation [36]. *fos* and *jun* are transcription factors encoded by the immediate early genes, and are, in turn, involved in the regulation of other genes. It is therefore quite likely that xenobiotic agents that mimic estrogen predispose individuals to certain hormonally related cancers. Unlike endogenous hormones, plasma and tissue levels of xenobiotics cannot be regulated by homeostatic mechanisms. However, xenobiotic levels may, to some extent, be regulated by enzymes and/or cellular sequestration, which can indirectly maintain cellular homeostasis. Transdermal or inhalational absorption of xenobiotics or dietary intake of estrogenic xenobiotics through food and water would result in the accumulation and storage of these agents in fat depots in the body. Consequently, genes that are under hormonal regulation are subjected to abnormal stimulation or inhibition by these extraneous agents. High tissue levels of such estrogen-mimetic compounds could potentially promote the growth of breast tumors. MCF-7 human breast cancer cells, grown in medium devoid of estrogen, proliferate in response to exogenous estrogen [37]. This cellular response is used as an indicator for the screening of estrogenic xenobiotic compounds [5].

Several environmental toxicants, including organochlorine pesticides and polychlorinated biphenyls such as Aroclor, have been shown to produce estrogenic or antiestrogenic effects in animals [1–5, 16, 17]. Krishnan and Safe [3] have shown that PCBs, polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) decrease the expression of cathepsin-D in estrogen-sensitive MCF-7 cells. The present study sets a precedent in demonstrating the direct estrogenic or antiestrogenic effects *in vivo* of a variety of xenobiotic compounds on the expression of the E-RmRNASF in the avian liver and hence on mRNA stability.

Our results indicate that the liver cytosolic extracts from the control, mock-treated birds were devoid of E-RmRNASF activity due to the lack of estrogen induction. ApoII mRNA incubated in this extract was endonucleolytically cleaved at specific sites, resulting in discrete fragments. The sites of cleavage *in vitro* due to endonucleases in the cytosolic extracts correspond to those *in vivo* previously shown by Binder *et al.* [29], suggesting that the course of degradation of apoII mRNA *in vitro* [31] is similar to that *in vivo*. The addition of heparin and a nonspecific competitor such as tRNA (concentrations of 5 mg/mL and 40 μg/mL, respectively) to the incubation buffer reduces nonspecific nucleolytic activity [31]. Estrogen-induction of E-RmRNASF prevents degradation of apoII mRNA in liver cytosolic extracts of estrogen-treated roosters. Previously, we had shown that the specific endonuclease(s) and E-RmRNASF were both present in the estrogen-treated liver cytosolic extract, and in the presence of the latter the endonuclease(s) could not degrade the mRNA [31]. The estrogen induction of E-RmRNASF was inhibited by actinomycin-D and hence appeared to be encoded by an estrogen-responsive gene. One of the effects of the xenobiotics that mimic estrogen, therefore, would be to alter the expression of E-RmRNASF, and consequently to modify estrogenic regulation of mRNA stability. From the results shown in Fig. 2, it is evident that in addition to estrogen, agents such as tamoxifen, Aroclor, Kepone, and hexachlorophene also induce the expression of the E-RmRNASF in the rooster liver and prevent the endonucleolytic degradation of apoII mRNA. Again, the stimulation of E-RmRNASF expression in the rooster liver by the above xenobiotic agents was blocked by co-administration of actinomycin-D, indicating that these agents activated transcription of the gene (results not shown). Exactly the same results were obtained with regard to their effect on the apolipoprotein II gene expression as determined by northern blot analysis (unpublished results). It is highly probable that these agents, by mimicking estrogen, stimulate gene expression through their interaction with the estrogen receptor. Agents such as tamoxifen, Aroclor, Kepone, and hexachlorophene probably behave as agonists at the estrogen receptor; upon binding to the receptor they would cause the activation of the receptor. The xenobiotic–receptor complex will then bind ERE in the promoters of estrogen-regulated genes in a manner similar to that of the dimerized estrogen–receptor complex, and will stimulate transcription at these promoters [38]. Contrary to a number of reports on antiestrogenic activity of tamoxifen, our results, along with those of Blue and Williams [39], have shown tamoxifen to have a predominantly agonist activity at the chicken estrogen receptor. Similarly, there have been mixed reports regarding estrogenicity and antiestrogenicity of Aroclor [2, 9, 35]. Krishnan and Safe [3] demonstrated a lack of antiestrogenic effects in MCF-7 human mammary carcinoma cells. Several studies, including ours, have shown Aroclor to be estrogenic [35]. Estrogenicity and reproductive toxicity have been reported previously for Kepone (chlordecone) [1, 2, 15, 18]. Mirex, another extremely persistent chlorinated hydrocarbon insecticide, is believed to be converted into chlordecone following hepatic metabolism [15, 40], and would most likely function as an estrogenic agonist *in vivo*. Glucuronic acid conjugates of chlordecone alcohol are subjected to biliary excretion. From Fig. 2, it is also evident that agents such as clomiphene, methoxychlor, DDT, toxaphene, dieldrin, bisphenol-A, lindane, and rotenone failed to induce hepatic expression of E-RmRNASF. Of the agents that failed to stimulate E-RmRNASF expression, some may have functioned as antagonists, in which case they would have bound the receptor but failed to stimulate it, and some may have simply failed to interact with the receptor. From Fig. 3, it is evident that agents such as clomiphene, toxaphene and bisphenol-A blocked the stimulation of E-RmRNASF expression by estrogen. Thus, agents such as these must function as antagonists, competitively blocking the receptor from estrogen binding. These agents then could naturally be expected to inhibit estrogenic stimulation of estrogen-responsive genes. Toxaphene is a highly toxic agent [13] and was believed to have estrogenic activity

based on its proliferative effects on MCF-7 cells [5]. Our results show toxaphene to have a potent antiestrogenic activity in the rooster. Agents such as lindane, methoxychlor, DDT, dieldrin, and rotenone, which failed to inhibit estrogenic stimulation of E-RmRNASF expression, most likely failed to bind the estrogen receptor. These agents therefore were not capable of stimulating or inhibiting the expression of *E-RmRNASF* gene by themselves. Whether or not there is synergism between estrogen and these chemicals cannot be determined from the above studies. Contrary to previous reports, Laws *et al.* [41] also demonstrated the lack of estrogenic effects due to lindane in sexually immature or ovariectomized rats. Methoxychlor, DDT, and dieldrin have been reported to exert estrogenic effects in birds and mammals [1, 3–5, 7, 10, 15–17, 20, 24]. However, our results do not show any effect on the estrogen-dependent expression of E-RmRNASF in the rooster liver due to these agents.

Agents such as Kepone, Aroclor, toxaphene, and bisphenol-A are environmental toxins that have accumulated in high quantities in several ecosystems. From our studies, it is evident that these chemicals could alter the expression of estrogen-responsive genes in the birds. It is quite likely that they may have similar effects in the other vertebrates, including humans. Factory workers, particularly women, in industries that either produce or use these chemicals in large quantities may be at risk for breast cancer and reproductive disorders.

Hexachlorophene (pHisohex) has therapeutic use as a surgical scrub, a topical antiinfective, a bacteriostatic detergent cleanser against staphylococci and gram-positive bacteria, and an anthelmintic. Hexachlorophene is transdermally absorbed through skin; it is absorbed extremely rapidly through burned or denuded skin. Hexachlorophene is neurotoxic, is embryotoxic, produces teratogenic effects in rats, and causes impaired fertility in male rats following neonatal exposure. In the past, hexachlorophene was used widely, but presently in the U.S.A. it is dispensed only on prescription. This is the first report of its estrogenic properties. Because hexachlorophene could probably activate the human estrogen receptor, it may be expected to produce endocrinological disorders in humans. The drug is contraindicated in children and pregnant mothers.

Nonsteroidal antiestrogens such as clomiphene and tamoxifen are clinically useful drugs. The major clinical application of tamoxifen is in the treatment of advanced breast cancer in postmenopausal women. Estrogen and estrogen-like compounds promote growth of estrogen-receptor-positive breast tumors, and are antagonized by tamoxifen [42]. The [E] or the *cis* form of tamoxifen is an agonist, while its [Z] or *trans* form is an antagonist at the human estrogen receptor [43]. Contrary to results with the human estrogen receptor, the exclusively [Z] form of tamoxifen used in our studies appears to be an agonist at the chicken estrogen receptor and stimulates the expression of E-RmRNASF in the rooster liver. Blue and Williams [39] showed through radioimmunoassay that the basal serum apoII and vitellogenin levels in the rooster were raised 50- and 15-fold, respectively, by treatment with tamoxifen. They also showed that accumulation of these proteins was due to their increased hepatic synthesis in response to tamoxifen. The tamoxifen metabolites 4-hydroxytamoxifen and desmethyltamoxifen also increased serum apoII in roosters [39].

Clomiphene has been used successfully to treat infertility associated with anovulatory cycles [44]. Clomiphene acts as a competitive inhibitor of estrogen at estrogen receptors in the anterior pituitary and the hypothalamus, causing enhanced release of gonadotropin-releasing hormone and gonadotropin, and a consequent increase in gametogenesis and steroidogenesis in the ovaries. Unlike tamoxifen, a mixture of the *cis* and *trans* isomers of clomiphene used in our experiments failed to induce the hepatic expression of E-RmRNASF. Clomiphene was, in fact, a true antagonist and blocked the stimulation of E-RmRNASF expression in the liver by estrogen. Clomiphene and tamoxifen are both closely related triphenylethylene compounds (Fig. 4, A and B).

From the above studies we cannot distinguish whether the agents that altered the expression of E-RmRNASF were the parent compounds themselves and/or one or more of their metabolites. Therefore, we could conclude that xenobiotic agents such as tamoxifen, chlordecone, Aroclor, hexachlorophene, and/or their metabolites act as estrogenic agonists with regard to the hepatic expression of E-RmRNASF in the rooster. Agents such as clomiphene, bisphenol-A, toxaphene, and/or their metabolites act as estrogenic antagonists. The third category of agents, including DDT, methoxychlor, lindane, dieldrin, and rotenone, simply failed to show any estrogenic or antiestrogenic interactions with regard to gene expression in the rooster. By studying the effect of certain environmental chemicals on the expression of a reporter gene such as β-galactosidase in human estrogen receptor (hER)-containing yeast cells [yeast estrogen system (YES)], Arnold *et al.* [45], have shown that agents such as chlordecone, dieldrin, endosulfan, or toxaphene had very little effect on hER-mediated transactivation of β-galactosidase when used singly, but were a thousand times as potent when used in combination with one another. They also found a good correlation between the effect of these chemicals on the YES and their ability to bind hER [45]. Since these chemicals often occur as mixtures in the environment, they suggested that this synergistic interaction of chemical mixtures with the estrogen receptor may have profound environmental implications. Because most environmental chemicals are nonbiodegradable, they become concentrated in the food chain and tend to accumulate in the fat depots of the body. Cumulative exposure to multiple estrogenic and antiestrogenic agents could be hazardous to the health of humans and other life forms on this planet. Thus, this is potentially a very important issue that urgently needs to be addressed.

We thank Ravindra R. Raje for his support and helpful discussions, Sarin Sinawat and Shariq Ali for technical assistance, Joseph Morin for his support and use of certain laboratory equipment, and Mohini Ratnasabapathy and Ellen McCartney for library search and support.

References

1. Eroschenko VP, Estrogenic activity of the insecticide chlordecone in the reproductive tract of birds and mammals. *J Toxicol Environ Health* **8:** 731–742, 1981.
2. Johnson DC, Sen M and Dey SK, Differential effects of dichlorodiphenyl trichloroethane analogs, chlordecone, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on establishment of pregnancy in the hypophysectomized rat. *Proc Soc Exp Biol Med* **199:** 42–48, 1992.
3. Krishnan V and Safe S, Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) as antiestrogens in MCF-7 human breast cancer cells: Quantitative structure-activity relationships. *Toxicol Appl Pharmacol* **120:** 55–61, 1993.
4. Martinez EM and Swartz WJ, The effects of methoxychlor on the reproductive system of the adult female mouse. *Reproduct Toxicol* **5:** 139–147, 1991.
5. Soto AM, Chung KL and Sonnenschein C, The pesticides endosulfan, toxaphene and dieldrin have estrogenic effects on human estrogen-sensitive cells. *Environ Health Perspect* **102:** 380–383, 1994.
6. Auger J, Czygilk F, Kunsfmann JM and Jouannet P, Decline in semen quality among fertile men in Paris during the past 20 years. *N Engl J Med* **332:** 281–285, 1995.
7. Guillette LJ Jr, Reproductive impairment in the Florida panther: Nature or nurture. *Environ Health Perspect* **103:** 79–86, 1995.
8. Guillette LJ Jr, Gross TS, Gross DA, Rooney AA and Percival HF, Gonadal steroidogenesis *in vitro* from juvenile alligators obtained from contaminated or control lakes. *Environ Health Perspect* **103:** 31–36, 1995.
9. Kholkute SD, Rodriguez J and Dukelow WR, Reproductive toxicity of Aroclor-1254: Effects on oocyte, spermatozoa, *in vitro* fertilization, and embryo development in the mouse. *Reproduct Toxicol* **8:** 487–492, 1994.
10. Raloff J, Ecocancers: Do environmental factors underlie a breast cancer epidemic? *Science News* **144:** 10–14, 1993.
11. Safe S, Male sexual development in a sea of estrogens. *Lancet* **342:** 125, 1993.
12. Wolf MS, Blood levels of organochlorine residues and risk of breast cancer. *J Natl Cancer Inst* **85:** 648–652, 1993.
13. Saleh MA, Toxaphene: Chemistry, biochemistry, toxicity and environmental fate. *Rev Environ Contam Toxicol* **118:** 1–5, 1991.
14. Ankley GT, Niemi GJ and Lodge KB, Uptake of planar polychlorinated biphenyls and 2,3,7,8-substituted polychlorinated dibenzofurans and dibenzo-*p*-dioxins by birds nesting in the lower Fox river and Green Bay, Wisconsin. *Arch Environ Contam Toxicol* **24:** 332–344, 1993.
15. Waters EM, Huff JE and Gerstner HB, Mirex. An overview. *Environ Res* **14:** 212–222, 1977.
16. Lundholm CE, Thinning egg shells in birds by DDE: Mode of action on egg shell gland. *Comp Biochem Physiol* **88:** 1–22, 1987.
17. Lundholm CE, Influence of chlorinated hydrocarbons, Hg^{2+} and methyl-Hg^{2+} on steroid hormone receptors from eggshell gland mucosa of domestic fowls and ducks. *Arch Toxicol* **65:** 220–227, 1991.
18. Kocarek TA, Schuetz EG and Guzelian PS, Regulation of cytochrome P450 2B1/2 mRNAs by Kepone (chlordecone) and potent estrogens in primary cultures of adult rat hepatocytes on Matrigel. *Toxicol Lett* **71:** 183–196, 1994.
19. Cummings AM and Perreault SD, Methoxychlor accelerates embryo transport through the rat reproductive tract. *Toxicol Appl Pharmacol* **102:** 110–116, 1990.
20. Aida K and Negishi M, Posttranscriptional regulation of coumarin 7-hydroxylase induction by xenobiotics in mouse liver: mRNA stabilization by pyrazole. *Biochemistry* **30:** 8041–8045, 1991.
21. Brawerman G, Determinants of messenger RNA stability. *Cell* **48:** 5–6, 1987.
22. Klausner RD, Roualt TA and Harford JB, Regulating the fate of mRNA: the control of cellular metabolism. *Cell* **72:** 19–28, 1993.
23. Williams DL, Sensel M, McTigue M and Binder R, Hormonal and developmental regulation of mRNA turnover. In: *Control of mRNA Stability* (Eds. Brawerman G and Belasco J), pp. 161–197. Academic Press, San Diego, CA, 1993.
24. Dodson RE and Shapiro DJ, An estrogen-inducible protein binds specifically to a sequence in the 3′ untranslated region of estrogen-stabilized vitellogenin mRNA. *Mol Cell Biol* **14:** 3130–3138, 1994.
25. Gordon DA, Shellness GS, Nicosia M and Williams DL, Estrogen-induced destabilization of yolk precursor protein mRNAs in avian liver. *J Biol Chem* **263:** 2615–2631, 1988.
26. Wilson T and Treisman R, Removal of poly(A) and consequent degradation of c-fos mRNA facilitated by 3′ AU-rich sequences. *Nature* **336:** 396–399, 1988.
27. Beelman CA and Parker R, Degradation of mRNA in eukaryotes. *Cell* **81:** 179–183, 1995.
28. Brewer G and Ross J, Poly A shortening and degradation of the 3′ A+U rich sequence of human c-myc mRNA in a cell-free system. *Mol Cell Biol* **8:** 1697–1708, 1988.
29. Binder R, Hwang S-PL, Ratnasabapathy R and Williams DL, Degradation of apolipoprotein II mRNA occurs via endonucleolytic cleavage at 5′-AAU-3′/5′-UAA-3′ elements in single-stranded loop domains in the 3′-noncoding region. *J Biol Chem* **264:** 16910–16918, 1989.
30. Ratnasabapathy R, Hwang S-PL and Williams DL, The 3′ untranslated region of apolipoprotein II mRNA contains two independent domains that bind distinct cytosolic factors. *J Biol Chem* **265:** 14050–14055, 1990.
31. Ratnasabapathy R, *In vitro* characterization of an estrogen-regulated mRNA stabilizing activity in the avian liver. *Cell Mol Biol Res* **41:** 583–594, 1995.
32. Margo JB and Williams DL, Estrogen induces the assembly of a multiprotein messenger ribonucleoprotein complex on the 3′ untranslated region of chicken apolipoprotein II mRNA. *J Biol Chem* **271:** 4452–4460, 1996.
33. Capony F and Williams DL, Antiestrogen action in avian liver: The interaction of estrogens and antiestrogens in the regulation of apolipoprotein B synthesis. *Endocrinology* **108:** 1862–1868, 1981.
34. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurements using the Folin phenol reagent. *J Biol Chem* **193:** 265–275, 1951.
35. Li MH, Zhao YD and Hansen LG, Multiple dose toxicokinetic influence on the estrogenicity of 2,2′,4,4′,5,5′-hexachlorobiphenyl. *Bull Environ Contam Toxicol* **53:** 583–590, 1994.
36. Weisz A and Bresciani F, Estrogen-regulation of proto-oncogenes coding for nuclear proteins. *Crit Rev Oncog* **4:** 361–388, 1993.
37. Lippman M, Bolan G and Huff K, The effects of estrogens and antiestrogens on hormone-responsive human breast cancer in long-term tissue culture. *Cancer Res* **36:** 4595–4601, 1976.
38. Halachmi S, Marden E, Martin G, MacKay H, Abbondanza C and Brown M, Estrogen receptor-associated proteins: Possible

mediators of hormone-induced transcription. *Science* **264:** 1455–1458, 1994.
39. Blue M-L and Williams DL, Induction of avian serum apolipoprotein II and vitellogenin by tamoxifen. *Biochem Biophys Res Commun* **98:** 785–791, 1981.
40. Carlson DA, Konyhu KD, Wheeler WB, Marshal GP and Zaylskie RG, Mirex in the environment: Its degradation to kepone and related compounds. *Science* **94:** 939–941, 1976.
41. Laws SC, Carey SA, Hart DW and Cooper RL, Lindane does not alter estrogen receptor or the estrogen-dependent induction of progesterone receptors in sexually immature or ovariectomized adult rats. *Toxicology* **92:** 127–142, 1994.
42. Mouridsen H, Palshof T, Patterson J and Batersby L, Tamoxifen in advanced breast cancer. *Cancer Treat Rev* **5:** 131–141, 1978.
43. Lewis DFV, Parker MG and King RJB, Molecular modeling of the human estrogen receptor and ligand interactions based on site-directed mutagenesis and amino acid sequence homology. *J Steroid Biochem Mol Biol* **52:** 55–65, 1995.
44. Hupert LC, Induction of ovulation with clomiphene citrate. *Fertil Steril* **31:** 1–8, 1979.
45. Arnold SF, Klotz DM, Collins BM, Vonier PM, Guillette LJ Jr and MacLachlan JA, Synergistic activation of estrogen receptor with combinations of environmental chemicals. *Science* **272:** 1489–1492, 1996.