Expression Profiling of Estrogenic Compounds Using a Sheepshead Minnow cDNA Macroarray

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A variety of anthropogenic compounds are capable of binding to the estrogen receptor (ER) of vertebrate species. Binding of these chemicals to the ER can interfere with homeostasis by altering normal gene expression patterns. The purpose of this study was to characterize the expression of 30 genes using a sheepshead minnow (*Cyprinodon variegatus*) cDNA macroarray. Many of the genes on the array were previously identified by differential display reverse transcriptase–polymerase chain reaction to be upregulated or downregulated in sheepshead minnows treated through aqueous exposure to known or suspected estrogenic chemicals. The results of this study show that 17β-estradiol (E_2), 17α-ethinyl estradiol (EE_2), diethylstilbestrol (DES), and methoxychlor (MXC) have similar genetic signatures for the 30 genes examined. The genetic signature of fish treated with *p*-nonylphenol was identical in pattern to that in fish treated with E_2, EE_2, DES, and MXC except for the additional upregulation of a cDNA clone that shares similarity to ubiquitin-conjugating enzyme 9. Endosulfan produced results that resembled the gene expression patterns of untreated control fish with exception of the upregulation of estrogen receptor α and the downregulation of a cDNA clone that shares similarity to 3-hydroxy-3-methylglutaryl–coenzyme A reductase. We show that our estrogen-responsive cDNA macroarray can detect dose-dependent changes in gene expression patterns in fish treated with EE_2. *Key words:* array, biomarkers, endocrine disruption, estrogen, fish, macroarray. *Environ Health Perspect* 111:839–846 (2003). doi:10.1289/txg.5752 available via *http://dx.doi.org/* [Online 6 November 2002]

Endocrine-disrupting compounds (EDCs) that mimic estrogens come from a variety of sources, including byproducts from manufacturing, effluent from wastewater treatment plants, and pesticides (Nimrod and Benson 1996a, 1996b; Solomon and Schettler 2000; Sumpter 1998). Exposure to these estrogenic EDCs may lead to a variety of physiologic problems in humans, including vaginal cancer, reproductive tract abnormalities, cryptorchidism, semen abnormalities, and hypospadias (Carlsen et al. 1993; Giusti et al. 1995; Giwercman et al. 1993; Sharpe and Skakkebaek 1993; Toppari 1996; Toppari et al. 1996).

A principal role of the native estrogen 17β-estradiol (E_2) in the liver of adult female fish is to activate the synthesis of specific gene transcripts that encode proteins required for reproduction by binding to the estrogen receptor (ER). Several genes known to be activated by this process include those that encode the ER itself, vitellogenins (Vtgs), and choriogenins (Arukwe et al. 2001; Bowman et al. 2000; Celius et al. 2000; Denslow et al. 2001a, 2001b; Flouriot et al. 1995, 1996, 1997; Folmar et al. 2000; Funkenstein et al. 2000; Hemmer et al. 2001; Lattier et al. 2001; Le Guellec et al. 1988; Lim et al. 1991; Murata et al. 1997). Vtgs, the egg yolk precursor proteins, and choriogenins, which are required for making the inner covering of the egg, normally increase in the circulation of females during oogenesis (Mommsen and Walsh 1988; Oppen-Berntsen et al. 1992; Specker and Sullivan 1994; Tyler and Sumpter 1996). However, in males, the normal endogenous levels of E_2 are sufficient to induce only very small amounts of plasma Vtgs and choriogenins (Arukwe et al. 2001; Copeland et al. 1986). When males are exposed to natural or anthropogenic estrogens, which can either enhance the steady-state concentrations of endogenous E_2 or bind directly to the ER, the result is an increase in the circulating levels of Vtg and choriogenin proteins. Vtg and choriogenin synthesis in male fish have therefore become accepted assays for measuring exposure to estrogenic chemicals (Arukwe et al. 1997; Bevans et al. 1996; Celius et al. 1999; Celius and Walther 1998; Denslow et al. 1996; Folmar et al. 1996, 2000; Hemmer et al. 2001; Heppell et al. 1996; Jobling et al. 1995; Orlando et al. 1999; Sumpter and Jobling 1995).

A number of natural and synthetic chemicals in the environment are estrogenic in *in vitro* and *in vivo* assays, including 17α-ethinyl estradiol (EE_2), diethylstilbestrol (DES), *p*-nonylphenol (PNP), methoxychlor (MXC), and endosulfan (ES) (Bowman et al. 2000; Bulger et al. 1978; Coldham et al. 1997; Denslow et al. 2001a, 2001b; Folmar et al. 2000; Hemmer et al. 2001; Larkin et al. in press; Nimrod and Benson 1997; Petit et al. 1997; Schlenk et al. 1998; Shelby et al. 1996; Soto et al. 1995; Vonier et al. 1996). EE_2 is currently used in oral contraceptives, and DES was once prescribed to prevent spontaneous abortion in early pregnancy (Herbst et al. 1971). PNP is the primary microbial degradation product of alkylphenol ethoxylates (Naylor et al. 1992), which are used as surfactants and emulsifiers in numerous industrial and commercial applications (Nimrod and Benson 1996a, 1996b). MXC and ES, which are both organochlorine pesticides, are used to control a variety of insects on food crops (Solomon and Schettler 2000).

There is emerging evidence that some estrogenic compounds may have additional modes of action independent of the ER. PNP, for example, enhances pregnane-X receptor-mediated transcription in COS-7 cells (Masuyama et al. 2000). Pregnane X is a nuclear receptor that regulates the expression of several genes, including cytochrome P450 3A (Bertilsson et al. 1998; Kliewer et al. 1998; Lehmann et al. 1998; Masuyama et al. 2000; Pascussi et al. 1999). Moreover, MXC induces gene expression in mice via a signaling pathway that does not involve ERα or ERβ (Ghosh et al. 1999; Waters et al. 2001). These studies suggest some genes may be differentially regulated by various estrogenic compounds and raises the possibility of specific genetic markers for some of the different

EDCs that mimic E_2. Using differential display reverse transcriptase–polymerase chain reaction (DD RT–PCR), we previously isolated 30 genes, several of which were upregulated or downregulated in sheepshead minnows (*Cyprinodon variegatus*) exposed to E_2. Other genes that appeared to be constitutive were added to the array for normalization purposes. In this study we have characterized the expression of these genes by macroarray analysis, using RNA from livers of male sheepshead minnows receiving an aqueous exposure to environmentally relevant concentrations of E_2, EE_2, DES, PNP, MXC, or ES.

Materials and Methods

Amplification of cDNA to Be Spotted on Macroarrays

Minipreps of 30 cDNA clones derived from DD RT–PCR analysis (Denslow et al. 2001a, 2001b) were PCR-amplified in a 300-μL reaction containing 1× PCR Buffer A (Promega, Madison, WI, USA), 2 mM $MgCl_2$ (Promega), 160 μM each deoxynucleotide triphosphate (Stratagene, La Jolla, CA, USA), 0.4 μM M13 primers (5′-GTT TTC CCA GTC ACG ACG TTG and 5′-GCG GAT AAC AAT TTC ACA CAG GA), and 1.25 units *Taq* polymerase (Promega). The PCR reaction conditions were as follows: 1 cycle at 80°C (1 min); 1 cycle at 94°C (2 min); 32 cycles at 94°C (1 min), 57°C (1 min), and 72°C (2 min); 1 cycle at 72°C (10 min), and then hold at 4°C. After completion of the PCR, the products were purified in a spin column (Qiagen, Chatsworth, CA, USA), then concentrated in a speed vacuum (Savant SVC100; Axon Instruments, Inc., Farmingdale, NY, USA). Aliquots of the PCR products were run on a 1.2% agarose gel containing 0.3 mM ethidium bromide. The gels were digitally imaged using a UVP Bio Doc-It camera (Ultra-Violet Laboratory Products, Upland, CA, USA), and the concentration of each PCR product was determined by comparing the intensity of each band to a standard curve derived from a low DNA mass ladder (Invitrogen Corp., Carlsbad, CA, USA). The PCR products were adjusted to a concentration of 160 ng/μL cDNA template.

Thirty unique clones were spotted on the array. Of these, 19 were identified based on their similarity to sequences in the National Center for Biotechnology Information (NCBI) database as determined by the Basic Local Alignment Search Tool (BLAST X) (Altschul et al. 1997). The highest expectation (E) value obtained is shown in parentheses. The 30 unique genes spotted include α_1-microglobulin/bikunin precursor protein (AMBP) (E = 1e-11), β-actin (GenBank accession no. AF253319), choriogenin 2 (E = 1e-45), choriogenin 3 (E = 4e-43), coagulation factor XI (E = 3e-12), ERα (E = 1e-71), glycosylate reductase (E = 3e-14), hepatic lipase precursor (E = 7e-6), 3-hydroxy-3-methylglutaryl–coenzyme A (CoA) reductase (E = 9e-51), low-molecular-mass protein 2 (E = 2e-12), transferrin (E = 8e-26), ubiquitin-conjugating enzyme 9 (the cDNA clone is 87% identical at the 5′ end to an EST clone (GenBank accession no. BJ028023) that has an E value of 1e-11 with ubiquitin-conjugating enzyme 9), unknown protein (E = 4E-23), Vtg 1 (GenBank accession no. AF239720), Vtg 2 (GenBank accession no. AF239721), ribosomal protein L8 (E = 7e-56), ribosomal protein S8 (E = 5e-29), and two unique genes similar to ribosomal protein S9 (E = 2e-45 and 4e-5). The remaining 11 clones, designated NDN1-A, ND1-E, ND9C-D2, ND10C-A, ND13C-E, ND15-B3, ND17-E3, ND96-C, ND98-E, ND102-A, and ND103-B, do not match any sequences in the database.

Array Controls

Various controls were also spotted onto the membranes, which provided information about cDNA labeling efficiency, blocking at the prehybridization step, and nonspecific binding. These controls included three *Arabidopsis thaliana* cDNA clones, Cot-1 repetitive sequences, poly A sequence (SpotReport 3; Stratagene), and an M13 sequence (vector but no cDNA insert). We also assessed the consistency of our technique by spotting on the array multiple cDNA products from the same genes amplified in separate PCR reactions. Genes spotted multiple times on different parts of the array include 3-hydroxy-3-methylglutaryl–CoA reductase, glycosylate reductase, choriogenin 2, a clone that shares homology to Unknown protein (GenBank accession no. AAH10857), and several unidentified clones (ND98-E, ND1-E, ND2C-A, ND62-B2, and ND102-A). In all, each membrane had 62 spots in duplicate that included the 30 unique genes described above, the multiple spotted genes, and the procedural controls.

Spotting of the Macroarrays

The PCR products were loaded into 96-well plates (Fisher Scientific, Pittsburgh, PA, USA), denatured with 3 M NaOH, heated to 65°C for 10 min, then immediately quenched on ice. Twenty times saline sodium citrate (SSC) (3 M NaCl, 0.3 M sodium citrate, pH 7.0) containing 0.01 mM bromophenol blue was added to the samples to yield a final concentration of 0.3 M NaOH, 6× SSC, and 100 ng/μL cDNA template. The PCR products were robotically spotted (Biomek 2000; Beckman Coulter, Fullerton, CA, USA) in duplicate onto 11.5 × 7.6 cm neutral nylon membranes (Fisher Scientific) using 100-nL pins. Membranes were ultraviolet cross-linked at 1×10^5 μJ (UV Stratalinker 1800; Stratagene) and stored under vacuum at room temperature until hybridization.

Sample Extraction

Total hepatic messenger ribonucleic acid (mRNA) was extracted using affinity columns (Qiagen) from adult male sheepshead minnows treated by aqueous exposure to either 65.14 ng/L E_2, 109 ng/L EE_2, 100 ng/L DES, 11.81 μg/L PNP, 590.3 ng/L ES, 5.59 μg/L MXC, or triethylene glycol (vehicle control) using a flow-through dosing apparatus as described previously (Folmar et al. 2000; Hemmer et al. 2001). All animals used in the research were treated humanely according to institutional guidelines (U.S. Environmental Protection Agency), with due consideration for the alleviation of distress and discomfort. Three fish were used per treatment group. Criteria for selection of samples from each compound tested were based on previously generated dose–response curves (Folmar et al. 2000; Hemmer et al. 2001) and chosen to give similar levels of expression of Vtg mRNA, a well-established estrogenic biomarker (Bowman et al. 2000; Sumpter and Jobling 1995). By selecting the concentration and length of exposure to yield similar Vtg mRNA expression levels, we accounted for differing potencies among the chemicals tested. On the basis of this criterion, length of exposure was 4 days for EE_2 and DES, 5 days for E_2 and PNP, and 13 days for MXC. ES treatment levels ranging from 68.8 ng/L to 788.33 ng/L failed to induce Vtg mRNA. We chose a treatment of 590.3 ng/L of ES for these analyses. This level of ES was slightly below the maximum acceptable toxicant concentration (MATC) derived for ES for sheepshead minnows (Hansen and Cripe 1991). ES is available commercially as a mixture of two ES isomers, α and β, which are typically supplied at the ratios of 65–70% α and 30–35% β.

Labeling of RNA and Hybridization

Radiolabeled probes were generated by random primer labeling of DNase-treated (DNA-free; Ambion, Inc., Austin, TX, USA) total RNA from male sheepshead minnow livers with $[\alpha\text{-}^{33}P]dATP$ (2'-deoxyadenosine 5'-triphosphate) (Strip-EZ RT, Ambion). The blots were prehybridized

with ULTRAArray hybridization buffer (Ambion) at 64°C for 3 hr. After prehybridization, each probe was diluted 20-fold with 10 mM disodium ethylenediaminetetraacetate, pH 8.0, to yield 1×10^6 cpm incorporated ^{33}P/mL hybridization solution. The diluted probes were heated to 95°C for 5 min, quenched on ice for 1 min, and added directly to the prehybridization buffer. The blots were then hybridized overnight at 64°C. After hybridization, the blots were washed $4 \times$ 15 min each with low ($2\times$ SSC, 0.5% sodium dodecyl sulfate [SDS]) and high ($0.5\times$ SSC and 0.5% SDS) stringency washes (Ambion) at 64°C.

Detection and Normalization

The membranes were exposed to a phosphor screen (Molecular Dynamics, Piscataway, NJ, USA) at room temperature for 48 hr. The blots were quantitatively evaluated using a Typhoon 8600 imaging system (Molecular Dynamics). For each cDNA clone the general background of each membrane was subtracted from the average value of the duplicate spots on the membrane. The values were normalized to the average value of 11 cDNA clones. These genes include ribosomal proteins L8 and S8, two unique genes similar to ribosomal protein S9, and several clones (designated NDN1-A, ND9C-D2, ND10C-A, ND13C-E, ND17-E3, ND102-A, and D103-B) that do not match any sequences in the NCBI database. These genes were chosen to normalize the data because they were equally expressed in control and treated fish by differential display analysis (data not shown) and/or they did not fluctuate more than 1.3-fold on macro arrays from E_2-treated and control fish (Larkin et al. 2002).

Gene array data were analyzed using linear regression and 1-way analysis of variance, with Tukey post hoc analysis (SPSS, Jandel, CA, USA).

Results

The advent of array technology has enabled researchers to analyze hundreds to thousands of genes on a single array. As a first step toward using array technology to assess exposure to environmental estrogens, we determined the variability between our macroarrays. To accomplish this, aliquots of identical RNA samples were hybridized onto two separate membranes (Figure 1A). Figure 1B illustrates a scatter plot correlating the intensity values from each spot from the two membranes. The data points in the graph cluster along a slope of one for all of the spots, including both the low and highly expressed cDNA clones. Similar values were observed in four replicate experiments (mean R^2 0.93, range 0.88–0.97).

cDNAs corresponding to 30 unique genes were spotted on the macroarrays. These genes were originally isolated from DD RT–PCR experiments by comparing gene expression profiles from control and E_2-treated fish. Hepatic mRNAs from exposed fish were converted to cDNAs and radiolabeled. The samples were individually hybridized to separate membranes to determine if fish treated with E_2, EE_2, DES, PNP, MXC, and ES shared similar expression profiles. Three separate fish were used for each treatment. Figure 2 contains representative membranes from the different treatments; Figure 3 shows a graphic representation of the data. Figure 3A illustrates the mean ± SEM intensity values for each of the cDNA clones arranged in order of their expression. Figure 3B illustrates the

mean intensity values for each of the cDNA clones for E_2, EE_2, DES, PNP, MXC, or ES divided by the mean intensity values of the respective cDNA clones from the untreated control fish.

Several of the genes spotted on the array were upregulated or downregulated in E_2-treated fish compared with controls. The genes were identified on the basis of the intensity values of the 11 constitutively expressed cDNA clones used to normalize the data (see "Materials and Methods"). To identify differentially regulated genes, the 11 reference genes were numerically ranked with respect to each other within a treatment. The upper confidence limit was set as the mean plus one standard deviation of the highest ranked value across the treatment groups (mean 1.38 ± 0.28). Likewise, the lower confidence limit was set as the mean plus one standard deviation of the lowest ranked value across the treatment groups (mean 0.63 ± 0.21). On the basis of these criteria, any cDNA clones in the macroarray experiments above a 1.66-fold induction were designated as upregulated with respect to control fish, and any cDNA clones that had a value below 0.42 were designated as downregulated. These expression levels of 1.66 and 0.42 are similar to the 2-fold change in expression used as cutoffs by other investigators (Coller et al. 2000; Wang et al. 2001). The upper (1.66) and lower (0.42) confidence limits used for these experiments are conservative, based on the observation that several genes whose expression levels fell within these values were identified as differentially regulated by DD RT–PCR (data not shown).

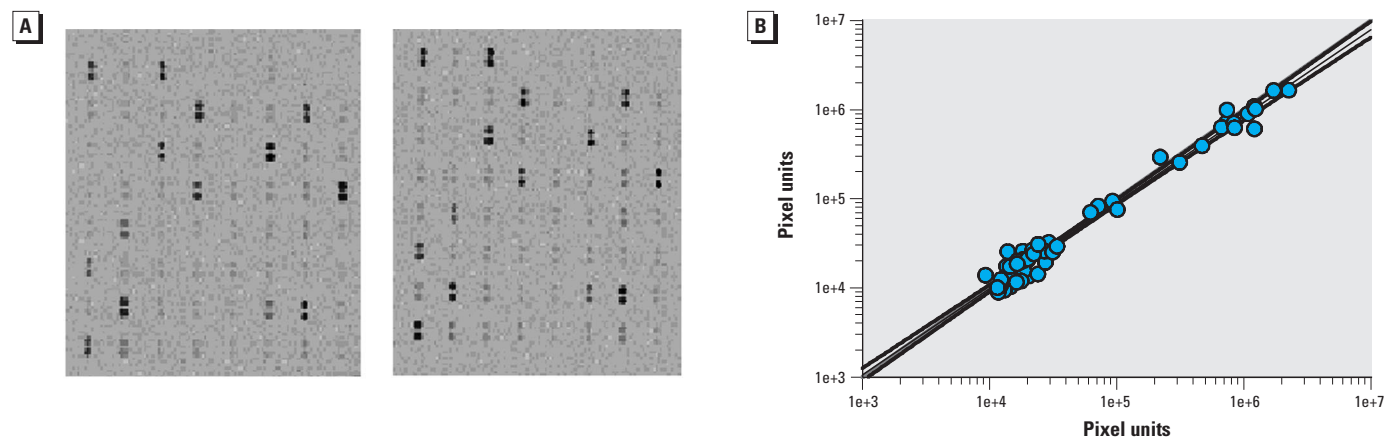Of the 30 genes used on our array, six genes were found to be upregulated by

Figure 1. Scatter plot of a self–self-hybridization. Aliquots of identical RNA samples were hybridized to two separate arrays shown in *A*. For each cDNA clone, the general background of each membrane was subtracted from the average value of the duplicate spots on the membrane. The values were then normalized to the average value of 11 cDNA clones (see "Materials and Methods"). The data points in the graph cluster along a slope of 1 from the low to the highly expressed cDNA clones (*B*), as verified by linear regression analysis (mean R^2 0.93, range 0.88–0.97) (SPSS, Jandel, CA). Ninety-five percent confidence intervals are shown on the graph. The data on both axes are plotted using a $\log_{10}$ scale.

E_2, including Vtgs 1 and 2, choriogenins 2 and 3, $ER\alpha$, and coagulation factor XI. Three genes found to be downregulated by E_2 were transferrin, β-actin, and AMBP. The remaining genes did not appear to be differentially regulated by E_2 when compared with controls. All the genes identified as upregulated or downregulated on the arrays showed identical expression patterns by DD RT–PCR (data not shown).

The 9 genes upregulated or downregulated by EE_2, DES, PNP, and MXC exposures showed a pattern of expression similar to that of the E_2 treatment. Interestingly, a cDNA clone that shares similarity to ubiquitin-conjugating enzyme 9 was significantly ($p < 0.05$) upregulated only in the PNP treatments. Eight of the 9 genes upregulated or downregulated for E_2, EE_2, DES, PNP, and MXC did not fluctuate for ES-treated fish but instead resembled the pattern observed in control fish. The only exception was $ER\alpha$, which appeared to be upregulated for all the compounds, including ES. An additional cDNA clone that shares similarity to 3-hydroxy-3-methylglutaryl–CoA reductase appeared to be slightly downregulated (decrease of 2.9-fold) in fish treated with ES compared with all the other treatments and the controls.

To determine if the gene expression profiles on the array could be verified by other techniques that monitor mRNA expression, we compared the expression profiles of several genes on the arrays (Vtg 2, choriogenin 2, and transferrin) to their profile by Northern blots and DD RT–PCR. Figure 4A, B shows that both Vtg 2 and choriogenin 2 mRNA levels increase in fish treated with E_2, as measured by Northern blots and DD RT–PCR. Figure 4C illustrates that transferrin decreases with E_2 treatment. We have not quantified the response by DD RT–PCR and Northern blot analysis compared with the arrays because limited amounts of samples required the use of different RNA samples in these experiments.

To assess whether the arrays could be used as a quantitative tool to measure the expression of multiple genes at varying concentrations of an estrogenic chemical, we examined male sheepshead minnows exposed for 4 days to nominal concentrations of 0, 20, 100, or 1000 ng/L EE_2 (Folmar et al. 2000; Hemmer et al. 2001). The measured concentrations were 24, 109, or 832 ng/L, respectively. Figure 5 contains representative arrays from the three EE_2 treatments. Figure 6 contains graphic illustrations of genes whose expression levels significantly changed by more than 2-fold in one or more of the three EE_2 concentrations examined ($p < 0.05$). Vtgs 1 and 2, choriogenins 2 and 3, $ER\alpha$, and coagulation factor XI increased in a concentration-dependent manner in the EE_2-exposed fish (Figure 6A). Three other genes, transferrin, AMBP, and β-actin, appeared to decrease in a dose-dependent manner (Figure 6B). These genes were the same genes that were upregulated or downregulated in the fish exposed to E_2, DES, PNP, and MXC (Figure 3).

Discussion

The goal of this study was to determine the expression profile of 30 estrogen-responsive genes in sheepshead minnows treated with both strong and weak estrogenic chemicals in a flow-through aquatic exposure system. The 30 genes arrayed were a subset of all genes inducible by E_2. These genes were isolated by DD RT–PCR in screening experiments where approximately 18% of RNA messages expressed in the livers of sheepshead minnows were tested (Denslow et al. 2001a, 2001b). In addition to genes that were upregulated and downregulated by E_2, the array also contained several constitutive genes. Although most of the genes on the array have been identified, we are continuing to screen our sheepshead minnow cDNA libraries to identify the remainder.

We evaluated the reproducibility of our printing process for the cDNAs by comparing the spot intensity for each of the duplicate spots for each gene on a membrane. The spot intensity varied on average by 6.5% between paired spots on a single membrane. When aliquots of RNA from identical samples were evaluated on membranes printed at the beginning, middle, and end of the printing process, we observed similar expression patterns on all membranes (data not shown). The interassay variability was minimal, as determined by the high R^2 value (mean 0.93) observed when aliquots of the same RNA samples were hybridized to independent membranes. Slightly more variability appeared to
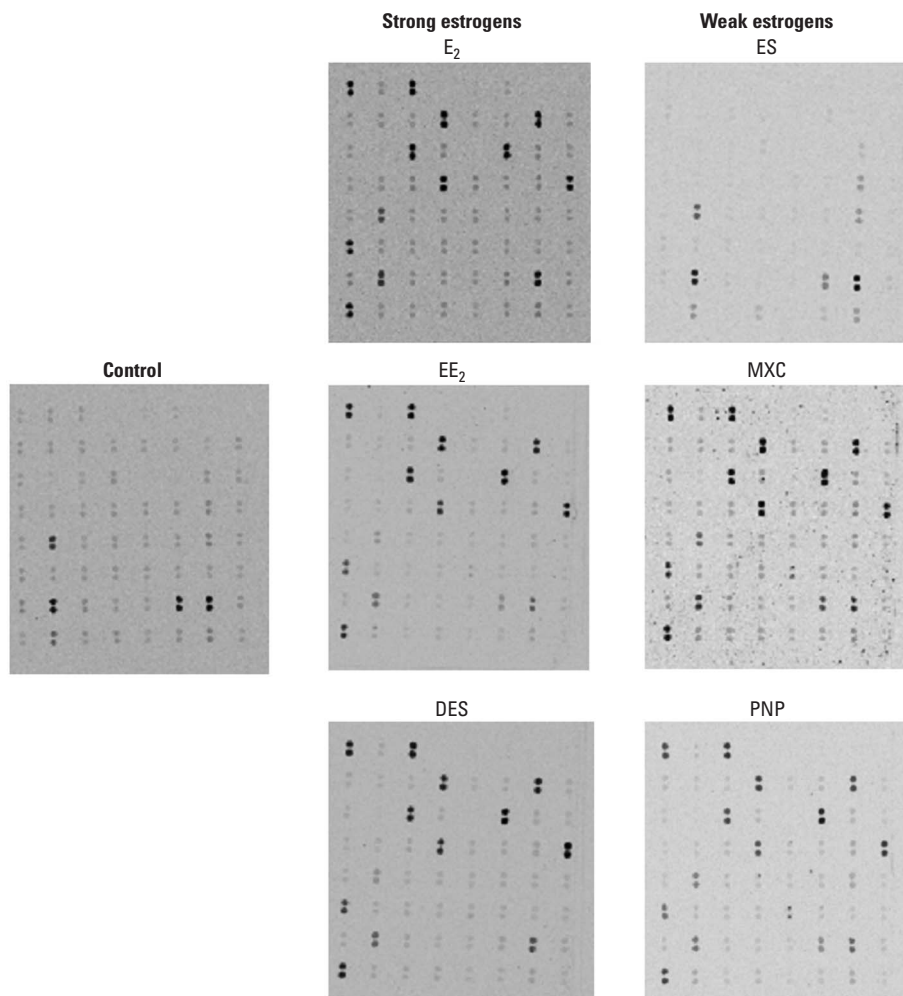

Figure 2. Macroarrays demonstrating gene expression profiles for untreated control fish and fish exposed to E_2, EE_2, DES, PNP, MXC, and ES. Three separate membranes were used for each treatment. A representative blot is shown.

be associated with the lower intensity values during the self–self hybridization test, a condition previously observed (Richmond et al. 1999). The cDNA labeling efficiency, blocking at the prehybridization step, and nonspecific binding also were consistent between the different treatments, based on similar expression of the various procedural controls present on each membrane.

Our results show similar expression patterns for the estrogen-responsive genes on our array (Figure 3A, B). Fish exposed to E_2, EE_2, DES, and MXC had identical genetic signatures for the 30 genes examined, whereas fish exposed to PNP differed by the increased expression of one

additional cDNA clone that shares similarity to ubiquitin-conjugating enzyme 9. Six genes (Vtgs 1 and 2, choriogenins 2 and 3, ERα, and coagulation factor XI) were upregulated in sheepshead minnows exposed to E_2, EE_2, DES, PNP, or MXC. The upregulation of the first 5 of the 6 identified genes was expected, considering their involvement in the estrogen-regulated process of oogenesis. The Vtgs, choriogenins, and ERα gene transcripts are induced by these chemicals in a variety of species (Arukwe et al. 2001; Bowman et al. 2000; Celius et al. 2000; Denslow et al. 2001a, 2001b; Folmar et al. 2000; Hemmer et al. 2001; Larkin et al. in press;
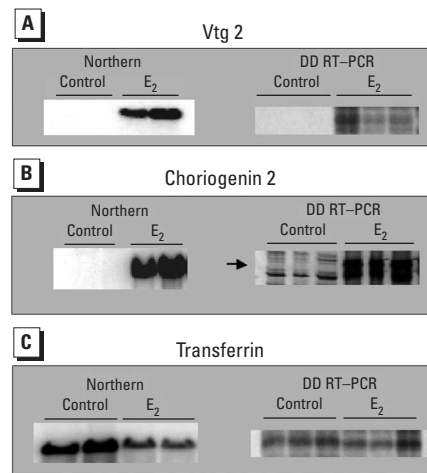
Figure 4. Comparison of Vtg 2, choriogenin 2, and transferrin expression levels by Northern blots and by DD RT–PCR data. Northern blots and DD RT–PCR were performed as described previously (Bowman et al. 2000; Denslow et al. 2001a).
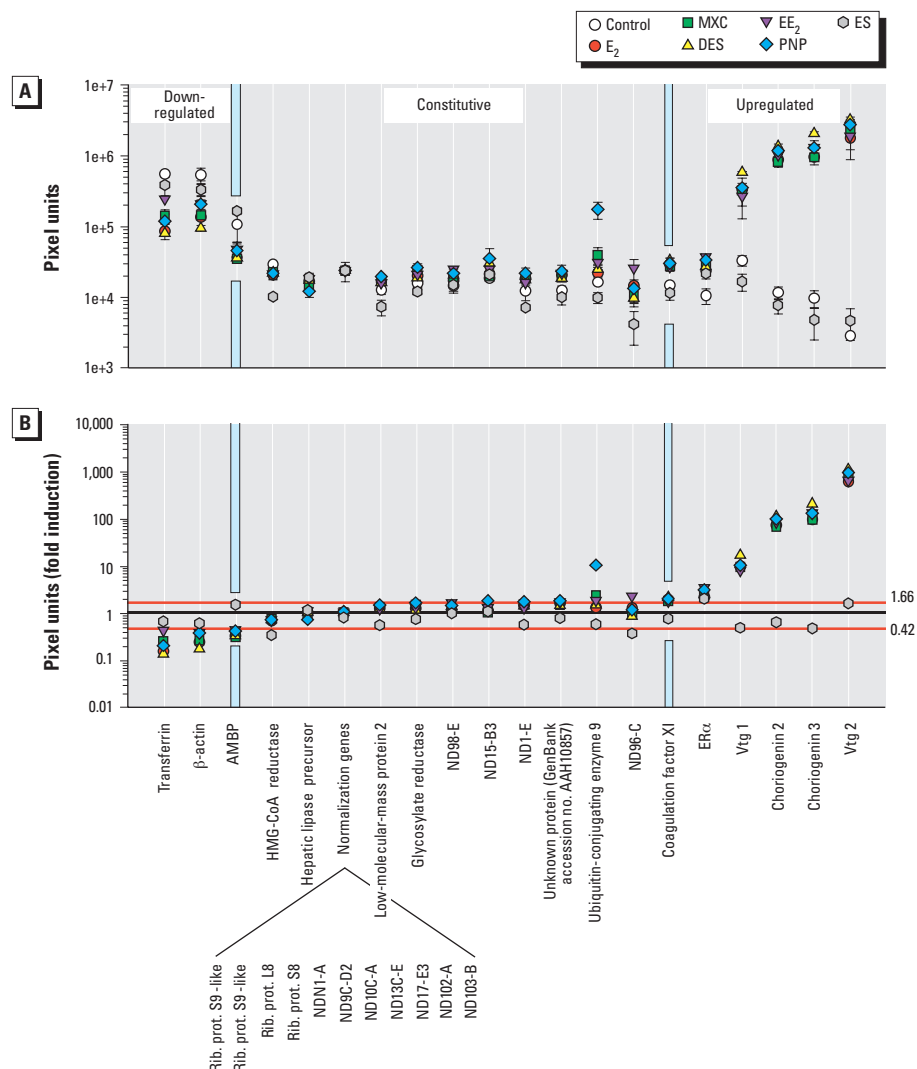

Figure 3. Quantification of the E_2, EE_2, DES, PNP, MXC, ES, and control arrays. Rib. prot., ribosomal protein. (*A*) Plot of the mean ± SEM intensity values for each of the cDNA clones arranged in order of their expression. (*B*) Plot of the mean intensity values for each of the cDNA clones for E_2, EE_2, DES, PNP, ES, or MXC divided by the mean intensity values of the respective cDNA clones for untreated control fish. The intensity values were normalized as described in the legend of Figure 1. Any clones above the red line labeled 1.66 were considered upregulated, any clones below the red line labeled 0.42 were considered downregulated, and any clones between the red lines were considered constitutive. Genes on the macroarray were designated as constitutive if their intensity values fell within the range of the mean plus 1 standard deviation of the highest and lowest values of the 11 clones used to normalize the data.
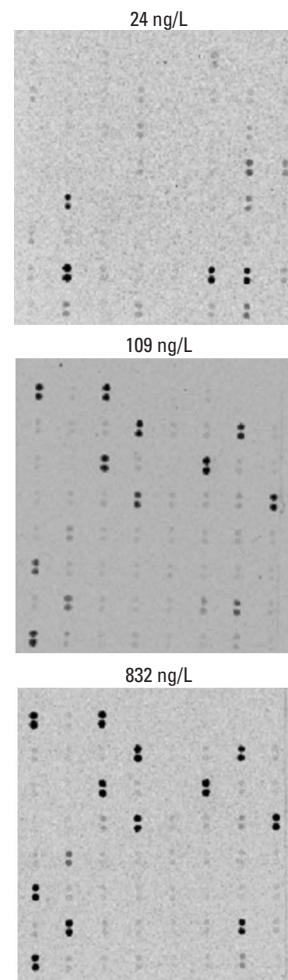

Figure 5. Gene expression profiles of fish exposed to various concentrations of EE_2. The nominal concentrations were 20, 100, or 1,000 ng/L EE_2, whereas the measured concentrations were 24, 109, or 832 ng/L, respectively. Three separate membranes were used for each treatment. Representative blots are shown.

Lattier et al. 2001). Because the ubiquitin-conjugating enzyme was not significantly upregulated to the same levels by the natural or pharmaceutical estrogens, its regulation may be related to another detoxification or metabolic pathway specific to alkylphenols. Ubiquitinated proteins are targets for proteolysis and other cellular functions, including protein trafficking and kinase activation. The different expression pattern for PNP may be valuable when trying to identify specific estrogenic agents in mixed effluents such as sewage treatment plant discharges.

Three genes (transferrin, β-actin, and AMBP) were downregulated in sheepshead minnows exposed to E_2, EE_2, DES, PNP, or MXC. Transferrin, a protein involved with iron transport, is downregulated by E_2 and other estrogenic compounds in the livers of largemouth bass (Larkin et al. in press); however, it is upregulated by E_2 in livers of chickens (Lee et al. 1978; McKnight et al. 1980). These observations suggest that transferrin may be regulated differently across vertebrate classes. β-Actin, a housekeeping gene commonly used to normalize gene expression assays, was also downregulated on our arrays for fish treated with E_2, EE_2, DES, PNP, and MXC. These results suggest that β-actin may not be a good housekeeping gene for estrogen-responsive arrays. The AMBP gives rise to two proteins, α_1-microglobulin and bikunin. The exact function of α_1-microglobulin is unknown. However, this protein is thought to be involved in immunoregulation (Akerstrom et al. 2000). Bikunin is the active subunit of protein/carbohydrate complexes that together comprise the inter-α-inhibitor protein family, which plays a major role in extracellular matrix stability and integrity (Bost et al. 1998). It is of interest to note that both β-actin and the precursor protein for bikunin, two proteins involved with the formation of the cytoskeleton, were both downregulated in fish treated with estrogenic chemicals. These data are consistent with the observations that several estrogenic compounds disrupt cytoskeleton components *in vitro* (Bocca et al. 2001; Chaudoreille et al. 1991; Sakakibara et al. 1991).

The gene expression profile of fish treated with ES did not resemble that of the fish treated with the other test chemicals but instead resembled more the expression pattern of the control fish. Several laboratories have shown that micromolar (or greater) concentrations of ES can induce MCF-7 cells to proliferate (Soto et al. 1995), interact with the trout ER (Petit et al. 1997), and induce Vtg mRNA in trout hepatocytes (Petit et al. 1997). However, in other studies, micromolar concentrations of ES were unable to interact with a mouse ER to transactivate a reporter construct in HeLa cells (Shelby et al. 1996) or compete well for binding to the mouse ER (Shelby et al. 1996). The doses used in the above *in vitro* assays would be considered pharmacological in a live animal (Hansen and Cripe 1991). Therefore, to determine whether ES was estrogenic in a live animal, we exposed sheepshead minnows to 590.3 ng/L ES, a
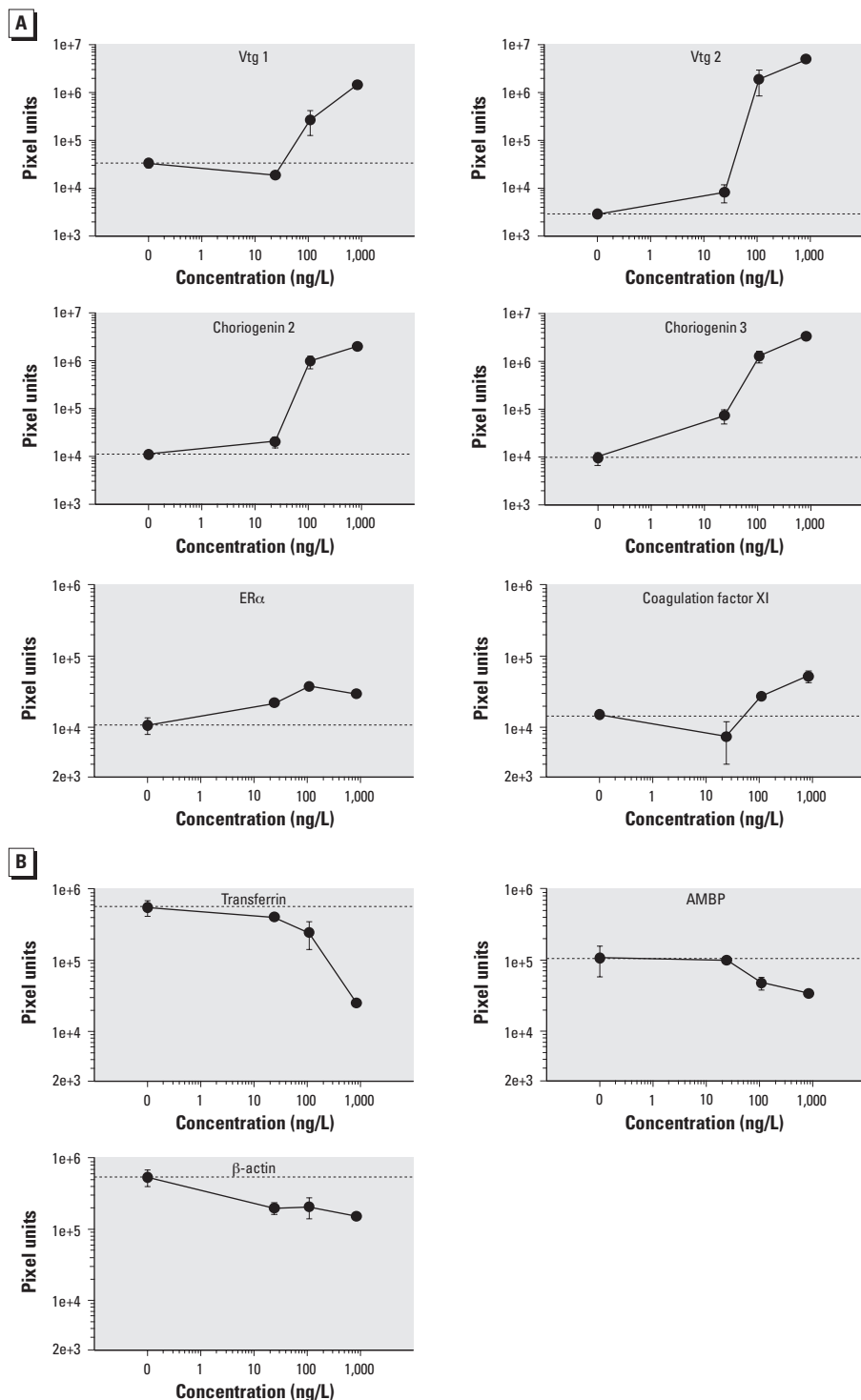

Figure 6. Quantification of the EE_2 dose–response arrays. Each graph contains a plot of a gene whose expression levels significantly increased (*A*) or decreased (*B*) more than 2-fold at one or more of the three EE_2 concentrations compared with those of controls, as revealed by one-way analysis of variance ($p < 0.05$). The data on both axes are plotted using a $\log_{10}$ scale. The measured concentrations (24, 109, or 832) were used to plot the data.

concentration within the MATC of 0.58–1.2 µg/L for aquatic animals. ES appears not to regulate (up or down) the same set of genes regulated by the other estrogenic compounds, with the exception of ERα, which was upregulated to a similar level in all treatment groups. This observation suggests the cascade of events downstream of the ER in the ES-exposed fish differs from that observed in the fish exposed to the natural and pharmaceutical estrogens and PNP. A second cDNA clone, 3-hydroxy-3-methylglutaryl–CoA reductase, appeared to be slightly downregulated in the ES-treated fish compared with all the other exposure groups and controls. This gene is involved in cholesterol and steroid biosynthesis. It will be important to confirm this downregulation by other methods. We are developing real-time PCR assays to do this.

Although in this study we saw no differences in gene expression between estradiol and MXC, we expect that we would see such differences in a larger array, as it has been noted by others that in mice MXC may stimulate some genes through pathways that do not involve ERα and ERβ (Ghosh et al. 1999; Waters et al. 2001).

Additionally, we investigated the sensitivity of array technology to detect and quantitate differences in gene expression by examining the expression profiles of the 30 arrayed genes in sheepshead minnows exposed to environmentally relevant (24 and 109 ng/L) and high (832 ng/L) doses of EE_2. The 24-ng/L dose represents the threshold concentration for Vtg protein induction in sheepshead minnows (Folmar et al. 2000). Our results show that estrogen-responsive genes vary in expression in a dose-dependent manner with increasing concentrations of EE_2 (Figures 5, 6). These genes include Vtgs 1 and 2, choriogenins 2 and 3, ERα, coagulation factor XI, transferrin, AMBP, and β-actin. These findings demonstrate the potential for use of this assay in screening programs (to establish lowest observable effect concentrations and no observed effect concentrations) and toxicologic mode of action studies for estrogenic chemicals.

In summary, our results indicate that gene arrays have potential as screening assays for new and existing chemicals to determine their potential estrogenic potency. Although the array used in this study was limited in the number of genes queried, our preliminary findings suggest EDCs that mimic estrogen will exhibit unique genetic fingerprints, indicating the usefulness of this technology to identify specific classes of chemicals capable of eliciting estrogenic responses in wild populations of fish.

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