Bull. Environ. Contam. Toxicol. (1996) 56:287-294
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Bulletin of Environmental Contamination and Toxicology

Estrogenic Activity in Rainbow Trout Determined with a New cDNA Probe for Vitellogenesis, pSG5Vg1.1

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Received: 5 April 1995/Accepted: 25 June 1995

Estrogenic compounds have been shown to induce specific endocrine systems in various species. The protein vitellogenin (Vg) and its corresponding mRNA have been shown to be induced by estrogenic chemicals in aquatic species, but specific probes for the measurement of this induction are not available. In this investigation the probe, pSGSVg1.1, was produced for detection of Vg expression in rainbow trout. The probe was made by the construction of a Vg cDNA fragment which was generated by the reverse transcriptase polymerase chain reaction (RT-PCR) and placed into the plasmid, pSG5. Using northern blotting techniques, pSGSVg1.1 was able to detect responses in liver Vg mRNA in fish treated with estradiol, a known inducer of Vg expression in aquatic species. Nonylphenol and DDE, which are also thought to be estrogenic, were also shown to cause induction of trout liver Vg mRNA as detected by pSGSVg1.1. These results indicate that Vg mRNA in fish liver induced by estrogenic chemicals can be detected using the cDNA probe, and both DDE and nonylphenol have estrogenic effects which can be detected by pSG5Vg1.1.

Several halo-organic environmental chemicals have been shown to exhibit estrogenic or antiestrogenic activity, and their presence has been correlated with various human diseases including cancer, a decrease in fertility in women, disorders of the reproductive tract and lower sperm count in men (Safe 1994). Tissue levels of organochlorine chemicals DDE {bis(4-chlorobiphenyl)-1,1-dichloroethene} and PCBs (polychlorinated biphenyls) were 50-60% higher in women with breast cancer compared to those with nonmalignant disease (Safe 1994).

Estrogenic effects of DDE and nonylphenol in humans have not been addressed directly, although they are being studied in light of reports of disruption of breeding and fertility in wildlife species (Korach 1993). For example, DDE in an alligator population of about 0.1 mg/Kg body weight was insufficient to cause general toxic effects but did appear to affect sexual development. Even if DDE is 1,000 times less estrogenic that natural hormones, body burdens of DDE may be sufficient to explain the feminizing effects seen in the alligators (Hormonal Pollutants 1993). Another issue is that many estrogenic chemicals are neither mutagenic nor acutely toxic in some concentrations, but they may be present in

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the environment in concentrations sufficient to produce estrogen-like effects. An example of a chronic effect produced by estrogenic chemicals was seen with diethylstilbestrol (DES), a synthetic estrogen that was used to prevent spontaneous abortions in women from 1948 until 1971. Daughters whose mothers took DES (about 1 million or more women between 1960 and 1970) had an increased incident of reproductive organ dysfunction, abnormal pregnancies, a reduction in fertility, and reproductive tract cancer (Colborn et al. 1993).

Recently attention has been given to the development of chemical exposure biomarkers using antibodies and molecular probes (Goodwin et al. 1992). The use of biomarkers to detect chemical exposure and toxicological effects of such compounds has become a priority issue in assessing chemical hazards, both in humans and feral animals. Induction of hepatic cytochrome p450 (CYP1A) by polychlorinated biphenyls (PCB's) and polycyclic aromatic hydrocarbons in birds and fish is under evaluation as a biomarker through the use of microsomal enzyme assays, CYP1A antibodies and a cDNA probe (pfP¡450-3') to estimate CYP1A in livers from chemically exposed animals and in human leukocytes (Haasch et al. 1993 and Heilmann et al. 1988). Certain estrogen-like chemicals permit the estrogen receptor (ER) to bind the estrogen receptor element (ERE) in the upstream enhancer of Vg gene thereby enhancing gene expression (Burch et al. 1990). Antiestrogens, such as dioxin (TCDD, 2,3,7,8-tetrachlorodibenzo -p-dioxin) are thought to suppress Vg gene expression in several species (Pelissero et al. 1993). Because of the induction of Vg by certain xenoestrogens, the induced mRNA has the potential to serve as a biomarker for exposure to estrogen-like chemicals by monitoring fish and other aquatic species for the induction of Vg mRNA. We have cloned a 1,094 base pair segment of the Vg cDNA from rainbow trout into plasmid pSG5 and have successfully used this subclone (pSG5Vg1.1) for northern blot analysis of rainbow trout liver mRNA after treatment with DDE, nonylphenol, or estradiol. Although vitellogenin gene expression has been studied in estradiol stimulated male trout (Guellec et al. 1988), cDNA probes have not been investigated with respect to xenoestrogen stimulation of vitellogenin mRNA production.

MATERIALS AND METHODS

Rainbow trout (*Oncorrhynchus mykiss;* 100-200g) were purchased from Hideaway Springs Trout Farm (West Bend, Wisconsin). The trout were maintained in flow-through conditions in dechlorinated tap water at 10 to 15°C on a 12:12 hour light-dark photoperiod and fed daily with Silvercup trout pellets (Murray Elevators, Murray, Utah) at a rate approximately equal to 5% body weight per day for at least 3 wk before the experiment. The trout were maintained under conditions in compliance with the Aminal Care Committee of the University of Wisconsin Great Lakes Research Facility.

Two primers for the polymerase chain reaction (PCR) were synthesized by the Protein/Nucleic Acid Shared Facility at Medical College of Wisconsin. Reverse transcriptase PCR was performed using 1 µg mRNA and 0.15 mM primers (Vg1.1+: 5'-GAT CCT GGA AAC TGT GTG CTG ARG G-3' and Vg1.1-: 5'-GAT CCG GCT CAA CAG AGT AGC ATT TGG-3') for 40 cycles. The annealing temperature was 57°C (RT-PCR kit from Perkin Elmer, Norwalk, Connecticut). A 1.2% agarose gel slice of 1,100 base pair cDNA (Vg1.1) was cut out from low melting agarose gel (NuSieve GTG Agarose from FMC, Rockland, Maine) for further subcloning.

The 4.1 kb vector pSG5 (Stratagene, La Jolla, California) was prepared for subcloning by digestion with BamHI (New England BioLabs, Beverly, Massachusetts) overnight at 37°C and dephosphorylated with alkaline phosphatase (New England Biolabs, Beverly, Massachusetts) for 30 min. at 37°C. This was followed by phenol extraction and ethanol precipitation. To construct the pSG5Vg1.1 vector, 1 pmol of phosphorylated Vg1.1 cDNA in a slice of low melting gel and 5 pmol of dephosphorylated pSG5 were ligated with T4 DNA ligase (New England BioLabs, Bererly, Massachusetts) at room temperature overnight. The ligation products were transformed into competent AG1 *E.Coli* host cells (Stratagene, La Jolla, California) and applied onto LB-plates with ampicillin and grown overnight at 37°C. Thirty-two colonies were selected and grown for 16 hr in 15 mL tubes containing 5 ml LB medium at 37°C with gentle agitation. The plasmid preparation was accomplished using the QIAprep-8 Plasmid Kit (Qiagen, Chatswoth, California). Approximately 30 µg of DNA was recovered from each sample.

The pSG5Vg1.1 plasmid was labeled for use in northern blot analysis using Amersham Fluorescein Gene Images Labeling Kit (Amersham, Arlington Heights, Illinois). 100 ng of denatured DNA probe in 34 µL of water was added to 10 µL of nucleotide mix, 5 µL of primers and 1 µL of Klenow DNA polymerase. The mixture was then incubated at room temperature overnight. The reaction was terminated by adding EDTA to a final concentration of 20 mM and incubating for 15 min at 65°C. 50 ng of probe per gel was found to be sufficient for detection of the mRNA. The labeled probe can be stored at -20°C for several months.

Three rainbow trout in each group were injected ip with estradiol (17β-estradiol, Sigma Chemical Co., St. Louis, Missouri) in ethylene glycol carrier at 2 mg/ kg body weight. Control fish were injected with ethylene glycol only. DDE [2,2'-bis (4-chlorophenol)-1,1'-dichloroethylene], (Aldrich Chemical Co., Milwaukee, Wisconsin) treated fish were injected ip with 22 mg/kg or 44 mg/kg DDE in olive oil carrier. Control fish were injected with olive oil only. Nonylphenol (American Cyanamid Co., Wayne, New Jersey) treated fish were exposed under flow-through conditions at 100 µg nonylphenol / L of water. The nonylphenol was dissolved in dimethylformamide (DMFA), and control fish were exposed to DMFA alone, under identical flow-through conditions.

RNA, from treated and control fish, was purified using Trizol reagent (Gibco BRL, Gaithersburg, Maryland) according to the manufacturer's instructions. The reagent was used in the ratio of 1 mL/50 mg of tissue. 100 µg purified RNA from each sample was electrophoresed in a 1.2% agarose, formaldehyde gel. Following electrophoresis the gel was incubated in 50 mM NaOH for 10 min. and then transferred to a nylon membrane (Hybond-N^+ membrane from Amersham) with a vacuum blotter (Pharmacia, Piscataway, New Jersey). The RNA was immobilized using an UV-Cross Linker (Stragagene, La Jolla, California). The blot was then prehybridized in a sealed plastic bag with hybridization buffer (5X SSC, 0.1 % SDS, 5 % Dextran Sulfate, and 1:20 ratio of liquid block from Amersham) for 30 min at 60°C. 50 ng of labeled probe was added to the hybridization buffer and the membrane was incubated for 16 hr at 60°C. Two stringency washes were then performed. The first wash was done in 1X SSC, 0.1 % SDS for 15 min at 60°C. The second wash was done in 0.5X SSC, 0.1

% SDS for 15 min at 60°C. The membrane was then briefly rinsed in diluent buffer (100 mM Tris-HCl and 300 mM NaCl at pH 7.5). The membrane was then placed into 10 % (V/V) liquid blocking agent in diluent buffer and agitated for 1 hr. The membrane was rinsed with diluent buffer and then placed into a solution of a 5000- fold dilution (V/V) of anti-fluorescein-AP conjugate in diluent buffer (5 µL conjugate, 1.25 mL of 10 % (V/V) BSA in 25 mL diluent buffer) for 1 hr. The membrane was then washed 3 times with a solution of 0.3 % (V/V) Tween-20 in diluent buffer for 10 min each. The membrane was then sprayed with dioxetane detection reagent and placed into a film cassette for a 2 hr exposure.

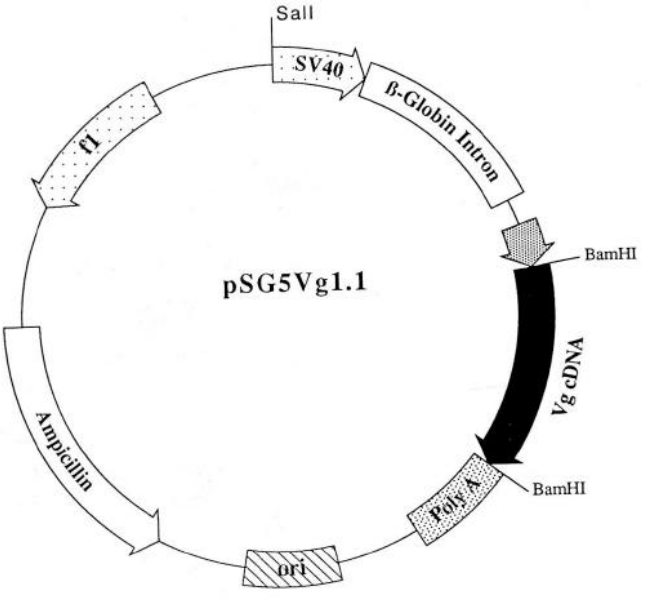


Figure 1. Construction of pSG5V1.1. 1,087 bp Vg cDNA was inserted into a pSG5 vector.

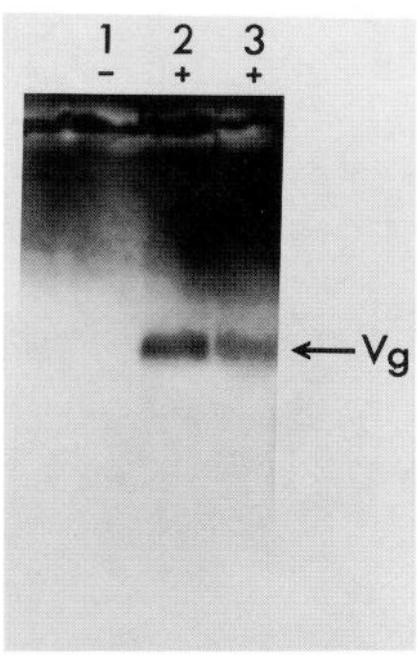
RESULTS AND DISCUSSION

One band of vitellogenin (Vg) cDNA, with 1,106 bp (Vg1.1) was generated by RT-PCR from estradiol treated rainbow trout liver (Figure 1). Vg1.1 was inserted in the vector pSG5 (4.1 kbp) to generate a probe for use in northern blot analysis. 400 bases from the 5' end of Vg1.1 were sequenced to verify the insertion (Automated DNA Sequencing Service, Department of Biological Sciences, University of Wisconsin-Milwaukee). The sequenced portion of the insertion matched the published rainbow trout Vg gene sequence at 92 % similarity (accession number: m27651).

Figures 2 and 3 show the results of northern blot analysis of RNA from the livers of fish treated with either estradiol or nonylphenol. Negative control used untreated trout liver mRNA and positive control used Vg cDNA. The Vg mRNA band located at same level of a double stranded 3,000 bp DNA in the same gel. Nonylphenol and estradiol showed clear estrogenic effects at the mRNA level in regards to vitellogenin. The control groups for both compounds showed no reaction with the probe, pSG5Vg1.1. The results show that the fish had a greater response to estradiol than to nonylphenol based on the northern blot analysis, but

rigorous quantitation of the responses remains to be done. There was no difference between male and female immature trout in response to estrogenic chemicals.

It was found that the response to the estrogenic compounds could be detected following RT-PCR of the RNA. Due to the relative ease of the PCR procedure, RT-PCR is routinely performed prior to the labor intensive northern analysis. The primers used for PCR detection (Vg 0.8+: 5'- GTT GCC ACA TCT GAA AAG AC-3' and Vg 0%: 5'- AGC ATC CAG GCA GAC AAC G-3') are different than the primers used for constructing the pSGSVg1.1 northern probe. The cDNA product is 827 bp long versus the 1,094 bp for the Vg1.1 cDNA. This second probe was designed to be used with multiple species of fish whereas



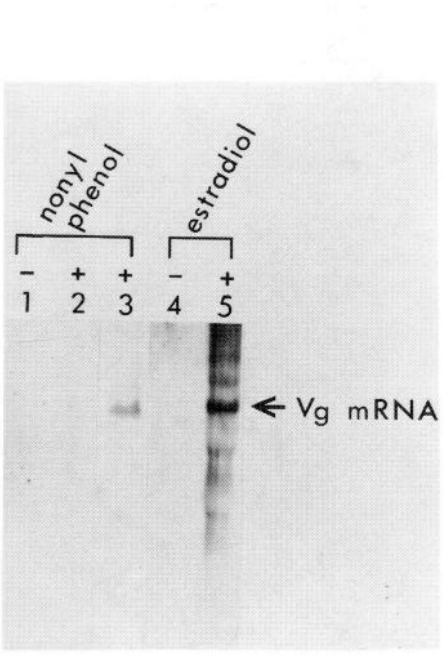


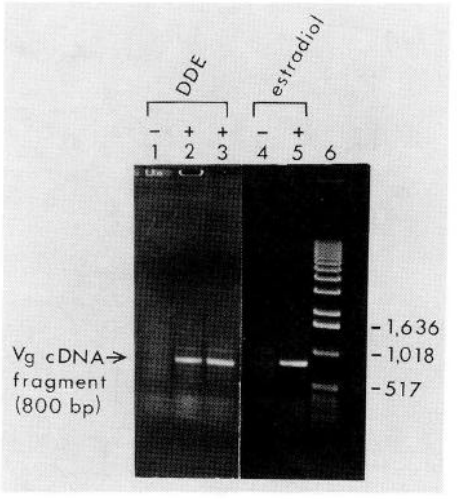
Figure 2 (left). Northern blot showing the Vg mRNA in trout treated with estradiol (lanes 2 and 3) and their control sample (lane 1). The probe used for detection was pSG5Vg1.1 labeled with fluorescein from Amersham. Exposed to film for 2 hr at room temperature.

Figure 3 (right). Northern blot showing the Vg mRNA in trout treated with nonylphenol (lanes 2 and 3) and their control sample (lane 1). Estradiol was also nm (lane 5) as well as an estradiol control sample (lane 4) as positive and negative controls. The probe used for detection was pSG5Vg1.1 labeled with fluorescein from Amersham. Exposed to film for 2 hr at room temperature.

the Vg1.1 set of primers was specific for the trout alone. Successful RT-PCR studies have been performed on catfish (data not shown) as well as trout with the Vg 0.8 primers.

Figures 4 and 5 show the RT-PCR results on a 1.2 % agarose gel. The 800 bp Vg cDNA products are only observed in the treated fish, either estradiol, DDE or nonylphenol (labeled with "+"), but not in the control fish ("-"). These results have been reproducible in immature male and female trout (under 150 g body weight), but not in mature female trout (with eggs, usually over 200 g). These results suggest that 1) vitellogenin is not normally present in sexually immature female or male trout and 2) the compounds DDE and nonylphenol have estrogenic activity.

Several recent reports have suggested that dietary and environmental estrogens may play a role in the increasing incidence of breast cancer in women as well as disorders of the male reproductive tract (Safe 1994). The need for a common biomarker to study estrogenic



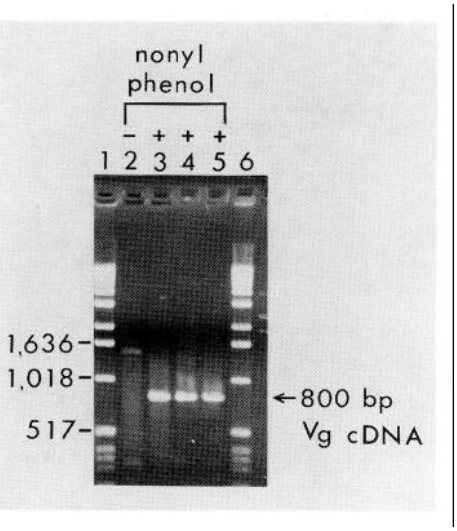


Figure 4 (left). 1.2% agarose gel shows RT-PCR results. Trout (immature) were treated DDE (lanes 2 and 3) and estradiol (lane 5). Lanes 1 and 4 are the respective controls. Lane 6 is a 1Kb DNA marker (BRL).

Figure 5 (right). 1% agarose gel shows RT-PCR results. Trout (immature) were treated with nonylphenol (lanes 3,4 and 5). Lane 2 shows the control fish. Lanes 1 and 6 are 1Kb marker (BRL).

chemicals is apparent. In oviparous species, estrogen induces Vitellogenin expression through the estrogen receptor, therefore vitellogenin has become a viable biomarker to be used to detect the presence of estrogen and xenoestrogens. A vitellogenin cDNA with 1,100 base pairs, from rainbow trout, was constructed into the plasmid pSG5. This probe was successfully used to detect vitellogenin mRNA upon northern blot analysis following exposure of rainbow trout to various estrogenic compounds. The goal is to use this probe for detecting

xenoestrogens in suspect aquatic environments by either northern blot analysis or PCR of fish liver mRNA.

DDE is a breakdown product of DDT and dicofol. Recently, DDE has been characterized as estrogenic and has been associated with breast cancer (Safe 1994). Our results show clearly that DDE induces vitellogenin and is therefore estrogenic. Nonylphenol is a surfactant which has also been described to have estrogenic activity. Nonylphenols are breakdown products of alkylphenol polyethoxylates (APE), a class of surfactant which has been demonstrated to bind to the estrogen receptor (White et al. 1994). The concentrations of DDE and nonylphenol that were tested were below acute toxic levels. The trout showed no physical effects after exposure to 44 mg DDE per kg of body weight via ip injection nor after a 3-d flow through exposure of 100 µg/L nonylphenol. These doses did cause significant estrogenic effects in the absence of overt toxicity. High concentrations of DDE and DDT are not unusual in environmental samples due to unusually large (50 to 100,000) bioaccumulation factors of these lipid soluble compounds.

Although the PCR technique is usually not used for the analysis of mRNA abundance, it was found that a good quality picture of the effects of the estrogenic compounds could be produced through the use of RT-PCR. In addition RT-PCR is a much less labor intensive procedure when compared to northern blot analysis, and would better lend itself for preliminary field screening of suspect aquatic sites.

The fish used in this study were either immature male or sexually immature female trout and sex-specific estrogenic effects were not observed through either PCR or Northern analysis. Fish vitellogenin is not absolutely sex-specific. The estrogen receptor and vitellogenin gene transcription may be active in normal male fish (Goodwin et al. 1992).

Acknowledgments. This work was supported by funds from the air force office of scientific research grand (F49620-94-1-0264). We appreciate the technical assistance of: Leslie Sutherland, Steven Lewis, Kris Kosteretz, Dr. Liane M. Mende-Mueller, and Brady Stoner in carrying out this study.

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