Exposure to the Herbicide Acetochlor Alters Thyroid Hormone-Dependent Gene Expression and Metamorphosis in *Xenopus Laevis*

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A growing number of substances released into the environment disrupt normal endocrine mechanisms in a wide range of vertebrates. Little is known about the effects and identities of endocrine-disrupting chemicals (EDCs) that target thyroid hormone (TH) action, particularly at the cellular level. Frog tadpole metamorphosis depends completely on TH, which has led to the suggestion of a metamorphosis-based assay for screening potential EDCs. A major mechanism of TH action is the alteration of gene expression via hormone-bound nuclear receptors. To assess the gene expression profiles in the frog model, we designed a novel multispecies frog cDNA microarray. Recently, the preemergent herbicide acetochlor was shown to accelerate $3,5,3'$-triiodothyronine (T_3)-induced forelimb emergence and increase mRNA expression of thyroid hormone β receptors in ranid tadpoles. Here we show that T_3-induced metamorphosis of *Xenopus laevis*, a species commonly used in the laboratory, is accelerated upon acute exposure to an environmentally relevant level of acetochlor. The morphologic changes observed are preceded by alterations in gene expression profiles detected in the tadpole tail, and the nature of these profiles suggest a novel mechanism of action for acetochlor. *Key words:* acetochlor, cDNA array, endocrine disruptor, gene expression, herbicides, metamorphosis, persistent organic pollutants, tadpoles, thyroid hormone, *Xenopus laevis. Environ Health Perspect* 110:1199–1205 (2002). [Online 7 October 2002]
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Anuran metamorphosis is a rapid, complex postembryonic process in which the tadpole transforms into a juvenile frog. The dramatic structural and functional changes of larval tissues during metamorphosis depend completely on an elevation of endogenous thyroid hormones (TH), $3,5,3'$-triiodothyronine (T_3), and $3,5,3',5'$-tetraiodothyronine (T_4) (Gilbert et al. 1996; Shi 2000). The major mechanism of TH action involves hormone binding to nuclear TH receptors (TRα and TRβ), resulting in tissue-specific activation/repression of gene transcription (Helbing and Atkinson 1994; Helbing et al. 1992, 1996; Sachs et al. 2000; Shi 2000; Tata et al. 1993). Studies using subtractive hybridization have identified components of these genetic programs initiated during amphibian metamorphosis. In *Xenopus laevis*, TH differentially affects the expression of 45 genes in the tail, 34 genes in the brain, and more than 120 genes in the limb within the first 48 hr of precocious metamorphosis (Brown et al. 1996; Buckbinder and Brown 1992; Denver et al. 1997; Wang and Brown 1993). With the development of DNA microarray technology, high throughput analysis of the expression profiles of hundreds to thousands of genes altered during metamorphosis is possible. The absolute requirement for TH action has led to the consideration of amphibian metamorphosis as a standardized method for screening potential endocrine-disrupting chemicals (EDCs), many of which are environmental contaminants (Crump 2001; De Vito et al. 1999; Hutchinson et al. 2000). Indeed, DNA microarrays have been used to detect genotoxic effects after exposure to chemical contaminants (Bartosiewicz et al. 2001; Custodia et al. 2001; Lobenhofer et al. 2001; Nuwaysir et al. 1999).

The preemergent herbicide acetochlor [2-chloro-*N*-(ethoxy-methyl)-*N*-(2-ethyl-6-methylphenyl) acetamide] is a persistent organic pollutant (POP) that can be detected in shallow ground water 1 year after field application, and there is evidence that it can act as an EDC (Ashby et al. 1996; Barbash et al. 1999; Cheek et al. 1999a; Veldhoen and Helbing 2001; Wilson et al. 1996). Acetochlor was introduced in 1994, and its use in agriculture has increased approximately 32% measured as the number of pounds applied annually (from 1994 to 2000). The national area of application (crop acreage) has also risen by approximately 56% in the United States (Barbash et al. 1999; Service NAS 2001). Concurrently, acetochlor concentrations in streams within the midwestern United States have been increasing and have reached a median concentration of approximately 2.7 nM (730 ng/L), with concentrations up to 10 nM (2.7 μg/L) falling within the 80th percentile of sites sampled (Scribner et al. 2000).

Acetochlor has been shown to accelerate T_3-dependent metamorphosis in ranid species. In *Rana pipiens*, exposure to acetochlor during T_3-induced metamorphosis resulted in reduced time to forelimb emergence (Cheek et al. 1999a). In *R. catesbeiana*, exposure to acetochlor increased T_3-dependent expression of TRβ mRNA in tail fin tissue within 24 hr (Veldhoen and Helbing 2001).

On the basis of these observations, we hypothesized that acetochlor may disrupt T_3-dependent gene expression programs during metamorphosis of the pipid frog *X. laevis*. This frog species is used extensively as a laboratory model in developmental and toxicologic studies. Here we describe the development of a 420-gene cDNA array derived from known frog sequences and the use of this array to determine the effects of acetochlor on precocious metamorphosis of *X. laevis*. We show that acetochlor accelerates T_3-induced metamorphosis within 72 hr and demonstrate that alterations in gene expression can be detected before overt morphologic change.

Materials and Methods

Test organisms. The care and treatment of animals used in this study were in accordance with the guidelines of the Animal Care Committee, University of Victoria. Premetamorphic *X. laevis* tadpoles [Nieuwkoop Faber (NF) stage 46; Nieuwkoop and Faber 1956] were purchased from Xenopus I, Inc. (Dexter, MN, USA) and maintained under natural lighting conditions in a 360-L all-glass flow-through aquarium containing charcoal-filtered municipal water at 22 ± 1°C. Tadpoles were fed Nutrafin flakes (Rolf C. Hagen Inc, Montreal, Québec, Canada) daily.

Chemicals used in animal exposures. T_3 and DMSO were purchased from Sigma (St. Louis, MO, USA); methanol was purchased from J.T. Baker (Phillipsburg, NJ, USA); acetochlor was purchased from AccuStandard (New Haven, CT, USA); and tricaine

methanesulfonate (MS-222) was purchased from Syndel Laboratories Ltd. (Vancouver, British Columbia, Canada).

Chemical exposure conditions. Before exposure, test animals were acclimatized to laboratory conditions at 22 ± 1°C for 48 hr. During the acclimatization and exposure periods, animals were not fed. We exposed 4–10 tadpoles, NF stage 52–54, in 1–2 L of preaerated, dechlorinated water. Dissolved oxygen, pH, temperature, and ammonia levels were measured, and all values fell within the accepted guidelines (ASTM 2000). Chemical treatments included nominal applications of 100 nM (67 μg/L) T_3 dissolved in DMSO, 10 nM (2.7 μg/L) acetochlor dissolved in methanol, and a combination of 100 nM T_3 and 10 nM acetochlor. A vehicle treatment containing a combination of DMSO and methanol served as a control. We have previously determined that the response to each vehicle alone is equivalent. The ratio of vehicle to water was 1:10,000 (vol/vol). Chemical applications were not renewed during the test period. We euthanized tadpoles in 0.1% MS-222 and photographed them using a 35-mm camera. The images were digitized, and measurements were made using Adobe Photoshop 5.0 (Adobe Systems Inc., San Jose, CA, USA). For RNA preparation, we collected tadpole tail tissue by removing it at the fifth myomere and immediately stored the tissue in the RNA preservative RNAlater (Ambion Inc., Austin, TX, USA) at 4°C. Tail tissue from the 10 tadpoles per treatment were combined before RNA isolation.

Preparation of RNA. Total RNA was obtained from preserved tadpole tissue using TRIzol reagent as described by the manufacturer (Invitrogen Canada Inc., Burlington, Ontario, Canada). We subsequently resuspended isolated RNA in RNase-free water and stored it at –70°C. Poly(A)+ RNA was isolated from total RNA using the Oligotex mRNA isolation mini kit (Qiagen Inc., Mississauga, Ontario, Canada).

Array target sequence selection and primer design. The frog MAGEX (multispecies analysis of gene expression) cDNA array was designed using *Xenopus* and *Rana* complete cDNA information obtained from GenBank (GenBank 2001). The abundance of *Xenopus* cDNA sequences compared to *Rana* sequences on the array reflects their relative abundance in GenBank. The cDNA sequences chosen encode structurally and functionally important products involved in regulation of amphibian development (embryogenesis and metamorphosis) as well as basal cell metabolism (ViagenX Biotech, Inc. 2002). We selected *X. laevis* ribosomal L8 (GenBank accession no. U00920) cDNA for normalization, as its mRNA transcript levels remain relatively constant between different developmental stages (Shi and Liang 1994). We included 420 cDNA sequences on the array, of which 390 are *X. laevis* and 30 are *R. catesbeiana* in origin.

All primers were designed with Primer Premier V4.1 software (Premier Biosoft International, Palo Alto, CA, USA) and synthesized by AlphaDNA (Montreal, Québec, Canada). Primers of 18 to 20 base pairs with an optimal annealing temperature between 50° and 55°C were designed to amplify sequences of between 450 and 550 base pairs within each target cDNA. Primer pairs were combined and diluted to a final concentration of 10 μM.

Total cDNA preparation. We prepared individual cDNA target sequences which form the basis of the array clone bank from total RNA isolated from untreated *Xenopus* tadpoles (NF stages 47 and 58; Nieuwkoop and Faber 1956) and oocyte tissues and *Rana* tadpoles (stage XV; Taylor and Kollros 1946) using TRIzol reagent as described by the manufacturer (Invitrogen). Total RNA was annealed with 500 ng random hexamer oligonucleotide (Amersham Biosciences, Baie d'Urfe, Québec, Canada) and cDNA was generated using Superscript II RNase H-reverse transcriptase as described by the manufacturer (Invitrogen). The 20-μL reaction was incubated at 42°C for 2 hr and diluted 20-fold before DNA amplification.

Amplification, cloning, and verification of target cDNAs. Each 50-μl DNA amplification reaction contained polymerase chain reaction buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$), 200 μmol dNTP, 20 pmol of each gene specific primer, 1 μL of a 20-fold dilution of *X. laevis* or *R. catesbeiana* total cDNA, and 2.5 U Taq DNA polymerase (Invitrogen). Amplification was carried out in a DNA Thermal Cycler 480 (Perkin-Elmer, Wellesley, MA, USA). The thermocycle program included a denaturation step at 94°C (7 min); 45 cycles of 94°C (1 min), 52°C (1 min), and 72°C (1 min); and a final elongation step at 72°C (7 min). Amplified DNA products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining on a ChemiImager 4000 (Alpha Innotech Corp., San Leandro, CA, USA). We excised DNA bands within the correct size range (450–550 base pairs) from the gel and extracted them by freeze-thaw centrifugation (Smith 1980). We confirmed purified samples by electrophoresis on a 1.5% agarose gel.

Polymerase chain reaction products were cloned into pCR II-TOPO vector using the TOPO TA Cloning Kit (Invitrogen). We purified plasmid DNA using the QIAprep Spin Miniprep Kit (Qiagen) and confirmed the presence of insert sequence by restriction analysis using *Eco*RI (Amersham Biosciences). Cloned cDNA was sequenced and compared by BLAST to sequences available in GenBank. Clones that matched the correct target sequence were included in the plasmid clone bank.

Preparation of cDNA fragments for spotting on the array. We prepared specific cDNA fragments for the frog MAGEX cDNA array using the plasmid clone bank. Individual amplification reactions were assembled in 96-well amplification plates (VWR International, Mississauga, Ontario, Canada). Each 100-μl reaction was similar to that described above, except that the target DNA comprised 1 ng of each plasmid clone, and 1 U AmpliTaq polymerase (Applied Biosystems, Foster City, CA, USA) was used. Amplification was carried out in an MJ Research Gradient Cycler PTC-255 (MJ Research, San Francisco, CA, USA). The thermocycle program included a denaturation step at 95°C (2 min); 35 cycles of 95°C (30 sec), 52°C (30 sec), and 72°C (1 min); and a final elongation step at 72°C (10 min). We separated amplified DNA products by electrophoresis on a 1.5% agarose gel and purified them using the QIAquick PCR purification kit and the Qiagen Biorobot 3000. Purified cDNA fragments were resuspended in 3× SSC (20× SSC contains 3 M NaCl, 0.3 M Na citrate, pH 7.0) to a final concentration of 0.5 μg/μL. The prepared cDNA was then loaded into 384-well plates (VWR International), and 90 nL/spot was spotted in duplicate on Biodyne B positively charged nylon membranes (PALL Gelman Laboratory, Ann Arbor, MI, USA) using the Biorobotics Total Array System (Biorobotics Ltd., Woburn, MA, USA). The spotted membranes were UV-crosslinked and stored at –20°C. Before hybridization, membranes were overlayed for 5 min on Whatman filter paper soaked in a denaturation solution containing 0.5 M NaOH. The blots were then sequentially placed for 5 min on Whatman filters soaked in 1 M Tris-HCl, pH 7.5, and 0.5 M Tris-HCl, pH 7.5/1.25 M NaCl. We rinsed membranes briefly in 2× SSC after the neutralization step.

Probe preparation and labeling. Tadpole tail poly(A)+ RNA (300–400 ng) was annealed with 500 ng random hexamer oligonucleotide (Amersham Biosciences), and cDNA probes were prepared using M-MLV reverse transcriptase as described by the manufacturer (Invitrogen) with the following modifications: the dNTP mix consisted of 500 μmol dGTP, dTTP, and dCTP, and 4 μmol dATP, and 50μCi [α-^{32}P] dATP (Amersham Biosciences) was used for radioactive labeling. We removed RNA from the cDNA probe by adding the following in sequential order: 1 μL 10% SDS, 1 μL 0.5 M EDTA, and 3 μL 3 M NaOH, and incubating for 15 min at 68°C. We neutralized the probe by adding 10 μL 1 M Tris-HCl and 3 μL 2 N HCl and then purified it on a QIAquick PCR purification column

(Qiagen). Before hybridization, the cDNA probes were heat denatured for 5 min at 95°C and then quickly cooled on ice for 5 min.

Hybridization and scanning of the array. Hybridizations were carried out in 15 mL of hybridization solution containing 5× SSC, 5× Denhardt's solution (0.5 mg/mL each final concentration of Ficoll, polyvinylpyrrolidone, and bovine serum albumin; Sigma-Aldrich Corp., St. Louis, MO, USA), and 0.1% SDS. Sheared, heat-denatured calf thymus DNA and 50% (w/v) dextran sulfate were added to hybridization solution prewarmed to 65°C at final concentrations of 100 μg/mL and 200 μL/mL, respectively. We added this solution to a hybridization tube (35 mm inner diameter × 150 mm length; Amersham Biosciences) containing the array membrane and incubated at 65°C for 2 hr in a Mini 10 Thermo Hybaid oven (Thermo Hybaid, Franklin, MA, USA). To prevent direct exposure of the array to concentrated probe, we transferred the prehybridization solution to a 15-mL Falcon tube and added the probe to a final concentration of 1.5×10^6 cpm/mL. The hybridization solution was then reapplied to the hybridization tube and incubated overnight at 65°C.

After hybridization, membranes were rinsed with 50 mL 2× SSC at room temperature and then washed twice with 50 mL 2× SSC/0.1% SDS at 65°C for 15 min, once with 50 mL 0.1× SSC/0.1% SDS at 65°C for 25 min, and rinsed with 50 mL 0.1× SSC at room temperature. Arrays were placed on 3M Whatman filter paper soaked with ddH_2O and wrapped with plastic wrap. We exposed the membranes to phosphor screens (Molecular Dynamics Inc., Sunnyvale, CA, USA) for 1 week. We collected hybridization signals using a Storm 820 optical scanner phosphor-imaging system (Molecular Dynamics) at 50 μm resolution. The resulting image data were converted to a standard 8-bit TIFF file using Photoshop V5.0 (Adobe Systems). Both nonauto- and auto-level images were prepared for analysis to account for signal saturation.

Array data analysis. Array image analysis was performed using ScanAlyze V2.44 (Eisen et al. 1998). Gene expression determinations were done in duplicate. We determined spot intensities from the mean pixel values for each gene and blank position and corrected them for noise by subtracting the local median background. Signal intensities that were derived from areas of nonspecific hybridization on the array were not included in the final analysis. We determined ratios for all duplicate positions in both nonauto- and auto-level array images. Gene and blank positions with duplicate ratios (DR) > 1.5 or < 0.67 were removed from both nonauto- and auto-level data sets. Average intensity values were calculated for duplicate gene spots and for blank positions in both nonauto- and auto-level data sets. A

nonsignal background was determined from the average intensity value plus 1 standard deviation of blank positions across the auto-level data set. We adjusted gene positions exhibiting values below the nonsignal to this value. Both nonauto- and auto-level data were then normalized to the mean internal ribosomal protein L8 gene signal. Saturated gene positions identified in the auto-level data set were replaced by the corresponding values obtained in the nonauto-level analysis. We subsequently determined response to chemical exposure for each gene relative to the vehicle control and expressed this as a ratio. Treatment response ratios > 2 or < 0.5 were identified for further discussion. Scatterplots were generated using Excel 97 (Microsoft Corporation, Redmond, WA, USA).

Real-time quantitative polymerase chain reaction. Total cDNA was prepared from 50 ng of tadpole tail poly(A)$^+$ RNA and 500 ng random hexamer oligonucleotide (Amersham Biosciences) using Superscript II RNase H$^-$ reverse transcriptase as described by the manufacturer (Invitrogen). The 20-μL reaction was incubated at 42°C for 2 hr and diluted 30-fold before DNA amplification. For each treatment, the expression of individual gene targets was analyzed using an MX4000 real-time quantitative polymerase chain reaction system (Stratagene, La Jolla, CA, USA). Each 15-μL DNA amplification reaction contained 10 mM Tris HCl, 50 mM KCl, 3 mM $MgCl_2$, 0.01% Tween 20, 0.8% glycerol, 40,000-fold dilution of SYBR Green I (Molecular Probes Inc., Eugene, OR, USA), 200 μM dNTPs, 83.3 nM ROX reference dye (Stratagene), 10 pmol of each primer, 2 μL of diluted cDNA, and 1.0 U Platinum Taq DNA polymerase (Invitrogen). The thermocycle program included an enzyme activation step at 95°C (9 min) and 40 cycles of 95°C (15 sec), 55°C (30 sec), and 72°C (45 sec). We included controls lacking cDNA template or Taq DNA polymerase to determine the specificity of target cDNA amplification. Cycle threshold (Ct) values obtained were converted into copy number using standard plots of Ct versus log copy number. We generated standard plots for each target sequence using known amounts of plasmid containing the amplicon of interest. Triplicate data obtained for each target cDNA amplification were averaged and normalized to the invariant ribosomal L8 control. Standard errors were calculated using InStat V3.01 (GraphPad Software Inc., San Diego, CA, USA).

Results

To determine the effects of acetochlor on *X. laevis* tadpoles, premetamorphic animals were exposed to 10 nM acetochlor both in presence and absence of 100 nM T_3 for up to

72 hr. Premetamorphosis is the interval of development that precedes thyroid gland function and is mainly a period of growth. At this stage, the tadpole is competent to respond to TH but is functionally athyroid (Shi 2000). We took advantage of this fact to eliminate possible confounding effects of endogenous TH. Morphometric analyses were performed on tadpoles at both 48 and 72 hr after exposure to the various chemical treatments. No significant effect in body area was observed at 48 hr after treatment with T_3, acetochlor, or their combination (Figure 1A). Animals exposed to acetochlor alone displayed a modest decrease in body area at 72 hr, although this observation was statistically nonsignificant (Figure 1). However, tadpoles exposed to T_3 showed a significant reduction in body area ($p < 0.05$) and the adoption of an adult morphology within the head region when compared to control animals (Figure 1). The combination of acetochlor with T_3 resulted in a significant reduction in body area ($p < 0.01$), more extensive sculpting of the head, reduced tail fin quality, and an increase in pigmentation, suggesting an overall acceleration of the metamorphic process (Figure 1). Other morphometric measurements such as interocular distance, snout-vent length, and body-to-tail length ratio were collected, and no significant differences were observed between treatments at 48 or 72 hr (data not shown).

Several genes whose expression is TH dependent and are proposed to be involved in metamorphosis have been identified in *X. laevis* tadpole tail tissue (Brown et al. 1996). Although morphometric analyses identified no significant observable effect at 48 hr, a large proportion of these TH-dependent genes are known to be affected at the level of mRNA expression before any overt morphologic change (Shi and Ishizuya-Oka 2001). Therefore, we wanted to assess whether there was a measurable change in gene expression caused by acetochlor exposure within the tadpole tail at this time point. We have shown previously that acetochlor significantly enhances TH-induced TRβ mRNA levels in *R. catesbeiana* tail fin (Veldhoen and Helbing 2001) and thus first examined the expression levels of TRα and TRβ transcripts in *X. laevis* tail tissue. TRα transcript levels did not change significantly upon TH treatment (Figure 2A), whereas a significant 5-fold increase in TRβ transcripts was observed ($p < 0.01$; Figure 2B). The expression of both TRα and TRβ mRNA transcripts was not affected by acetochlor treatment alone. However, the combination of acetochlor and T_3 resulted in a significant increase in TRα transcript levels relative to the control ($p < 0.05$; Figure 2A) and in a further significant increase in TRβ transcript levels ($p < 0.001$

compared to control and $p < 0.05$ compared to TH; Figure 2B).

To screen other genes within tadpole tail tissue whose expression may be altered after exposure to acetochlor, we designed and used a cDNA array called the frog MAGEX array. In the premetamorphic tadpole tail, the MAGEX array detected 267 genes upon vehicle control treatment and 285 genes upon T_3 treatment for 48 hr. Figure 3A–D depicts the tadpole tail-specific gene expression pattern from a subsection of the array across the four chemical treatments (vehicle control, acetochlor, T_3, and a combination of acetochlor and T_3). An example of differential response to chemical treatment is highlighted by the TH-responsive genes TH/bZip and gene 18 (Figure 3A–D). The results from array analyses for these representative genes were independently confirmed by real-time quantitative polymerase chain reaction analyses and are shown in Figure 3E and F. The MAGEX array identified two distinct responses to acetochlor treatment: genes whose expression is altered in premetamorphic tadpoles and those that are affected in the context of precocious metamorphosis.

Eleven genes were identified in premetamorphic tadpole tail tissue whose steady-state mRNA levels were altered within 48 hr after exposure to acetochlor (Table 1). Ten of these genes were downregulated in response to acetochlor. A single gene (encoding GSK-3 binding protein) was upregulated in tadpole tail tissue after acetochlor exposure.

Morphometric analyses suggested acetochlor might act in concert with TH to accelerate metamorphosis. To address this potential interaction at the genetic level, we investigated the effects of acetochlor on T_3-dependent gene expression during precocious metamorphosis. Figure 4 shows a scatterplot of the relationship between the fold differences relative to control of T_3 treatment alone and T_3 plus acetochlor, where each data point represents a distinct gene. The herbicide modulated mRNA expression of 26 genes that were subdivided into 3 groups based on their initial level of response to T_3 (Table 2). Group 1 contains genes that were upregulated by 2-fold or more in response to T_3. A number of these genes (80%) have previously been identified as responsive to exogenous TH administration (Amano 1998; Brown et al. 1996), confirming the ability of the MAGEX array to detect modulation in gene expression associated with TH action. Combination treatment with acetochlor and T_3 resulted in a further increase in the response ratio values of genes within group 1 (Table 2). Notable exceptions are found for the B-50/GAP 43 and iodothyronine 5-deiodinase III genes, in which acetochlor reduced the T_3-mediated upregulation in mRNA expression. The

expression of deiodinase type II mRNA was not affected by T_3 and/or acetochlor treatment (data not shown). Genes within group 2 displayed little or no response to T_3 treatment alone (response ratios of < 2 or > 0.5; Table 2). In contrast, the presence of acetochlor and T_3 resulted in an elevation in mRNA expression within this group (Table 2). Group 3 comprises genes within tadpole tail tissue that are downregulated after exposure to exogenous T_3 (Table 2), and 50% of the genes identified within this group are known to be TH-responsive (Brown et al. 1996; Furlow et al. 1997; Miyatani et al. 1986). The presence of acetochlor resulted in an attenuation of this T_3-mediated gene repression. Finally, Table 3 lists genes that are T_3 responsive during precocious metamorphosis but not affected by acetochlor exposure.

Discussion

Before this study, evidence for endocrine-disrupting activity of the POP acetochlor in Amphibia was restricted to ranid species. Cheek et al. (1999a) observed that T_3-induced forelimb emergence in *R. pipiens* was accelerated by 7 days in the presence of an environmentally relevant concentration of acetochlor. Although no genetic observations were made, preconditioning the tadpoles with T_3 resulted in an accelerated response to

acetochlor, suggesting the involvement of T_3-dependent gene expression (Cheek et al. 1999a). A complementary observation was found for T_3-induced metamorphosis in *R. catesbeiana*, where exposure to acetochlor resulted in a significant increase in tail fin TRβ mRNA expression within 24 hr (Veldhoen and Helbing 2001).

We found that T_3-induced metamorphosis was accelerated by 72 hr in the pipid frog *X. laevis* after acute exposure to a concentration of acetochlor that falls within current levels (80th percentile) detected in streams in the midwestern United States (Scribner et al. 2000). Although the present study was not designed to assess the effect of acetochlor on *X. laevis* spontaneous metamorphosis, our 72-hr acute exposure did have a modest effect on premetamorphic tadpole body area, suggesting that this herbicide may affect natural metamorphosis in this species. However, Cheek et al. (1999a) found that acetochlor did not significantly affect spontaneous metamorphosis in *R. pipiens*. Whether spontaneous metamorphosis is altered in *X. laevis* and other frog species needs to be addressed further.

The initiation of amphibian metamorphosis depends on the presence of TH and is mediated through the hormone-dependent activation of the TR isoforms α and β (Eliceiri and Brown 1994; Kawahara et al.
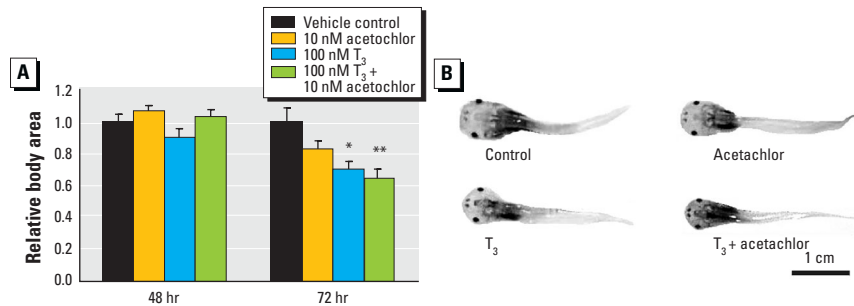
Figure 1. Morphometric analyses of premetamorphic *X. laevis* tadpoles exposed to acetochlor in the presence or absence of T_3. (*A*) Tadpoles were exposed to vehicle control, 10 nM acetochlor, 100 nM T_3, or 100 nM T_3 and 10 nM acetochlor for the times indicated. Body area was measured from digital images and expressed in graphic form relative to the vehicle control values. Each bar represents the mean ± SE of 8–10 animals, with the exception of 72 hr acetochlor (*n* = 4). (*B*) Representative tadpole morphology 72 hr after exposure to vehicle control, T_3, acetochlor, or T_3 and acetochlor.
*$p < 0.05$; **$p < 0.01$ relative to the control.
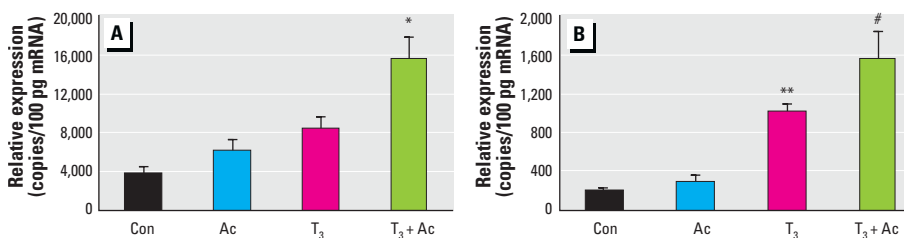
Figure 2. Relative expression levels of (*A*) TRα and (*B*) TRβ mRNA in *X. laevis* tadpole tail tissue 48 hr post-treatment as determined by polymerase chain reaction. The treatments indicated are vehicle control, 10 nM acetochlor, 100 nM T_3, and a combination of 100 nM T_3 and 10 nM acetochlor. Data are presented as the normalized number of copies per 100 pg of input mRNA. Three independent experiments were performed; error bars represent SEM.
*$p < 0.05$; **$p < 0.01$; #$p < 0.001$, relative to control.

1991; Yaoita et al. 1990). It has been proposed that TRα is required for competence to metamorphose, whereas TRβ is primarily responsible for modulating the expression of a number of genes whose products are involved in tissue remodeling during metamorphosis (Brown et al. 1996; Sachs et al. 2000). We chose gene expression in the tail for analysis because acetochlor has been shown to affect TRβ transcript levels in this tissue (Veldhoen and Helbing 2001), and the tail is readily isolated. In *X. laevis* tadpole tail tissue, exposure to 10 nM acetochlor disrupts T_3-dependent expression of both TR isoforms within 48 hr, resulting in an increase in steady-state levels of TR mRNA. These results correlate with an increased rate of metamorphosis and led us to investigate the effects of acetochlor exposure using a multigene approach.
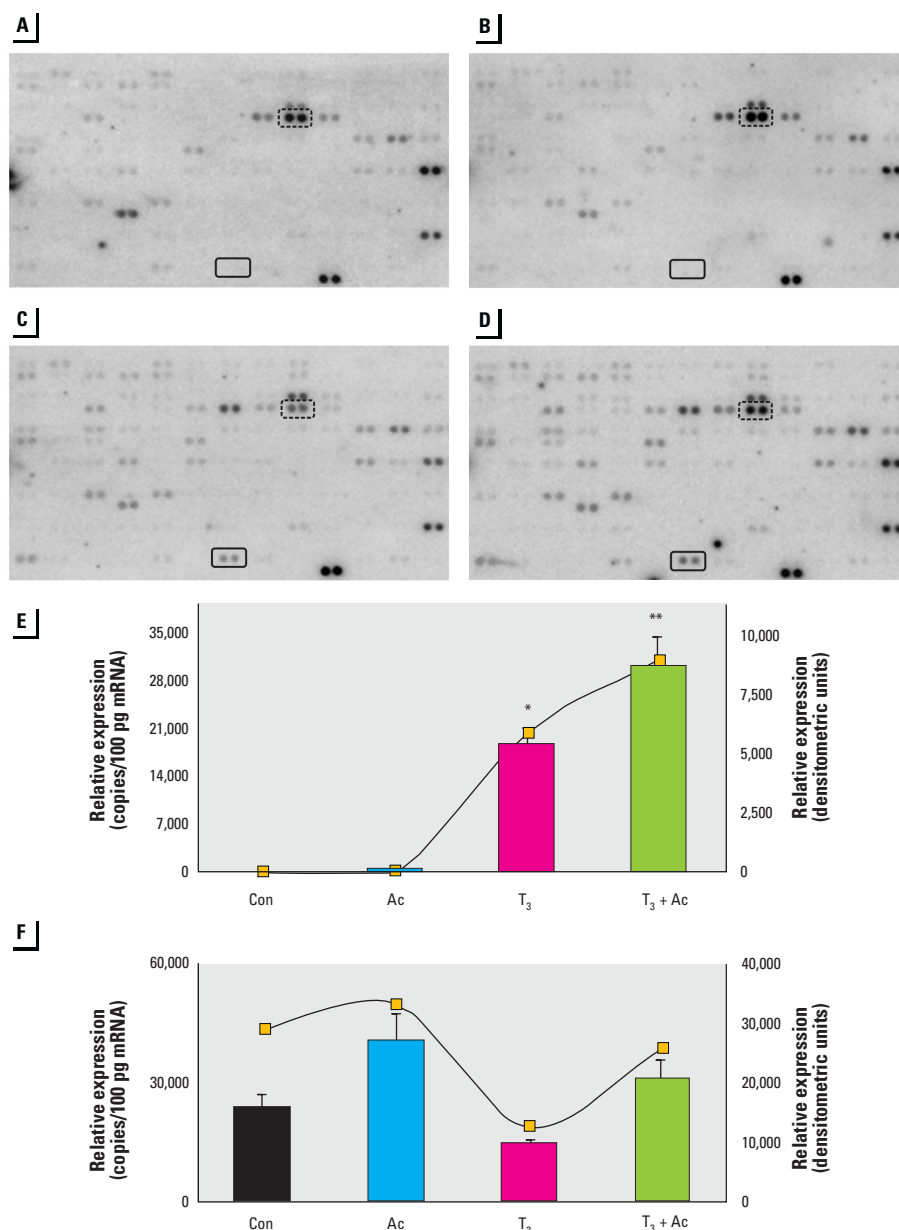
Using our novel MAGEX cDNA array, we identified several genes in the tail whose expression is altered upon acetochlor exposure. These include genes encoding transcription factors, signaling molecules, and apoptotic proteins (Nakashima et al. 1993; Thress et al. 1998). All genes except one (encoding GSK-3 binding protein) show a decrease in transcript levels compared to control. It is interesting to note that three of the genes (encoding PTEN, K-*ras* and GSK-3 binding protein) are part of the phosphatidyl 3-inositol kinase signaling pathway, and eight are important in the regulation of cell proliferation (Adachi and Laemmli 1992; Cantley and Neel 1999; Tonissen and Krieg 1994; Vriz et al. 1989; Wade et al. 1999; Yost et al. 1998). Only one of the genes, c-*myc*, is also repressed by T_3 treatment, although acetochlor does not further affect

Figure 3. Gene expression analysis using the frog MAGEX cDNA array. The expression profiles of mRNA isolated from tail tissue of *X. laevis* tadpoles exposed for 48 hr to (*A*) vehicle control, (*B*) 10 nM acetochlor, (*C*) 100 nM T_3, or (*D*) 100 nM T_3 and 10 nM acetochlor are shown on a representative subsection of the array. The positions of TH/bZIP (box) and gene 18 (dashed box) are identified on each array as examples of differential response to treatment. Relative expression levels are shown for (*E*) TH/bZIP and (*F*) gene 18 mRNA in *X. laevis* tadpole tail tissue 48 hr post-treatment as determined by real-time quantitative polymerase chain reaction. The treatments indicated are vehicle control, 10 nM acetochlor, 100 nM T_3, and a combination of 100 nM T_3 and 10 nM acetochlor. Bars indicate the normalized number of copies per 100 pg of input mRNA. Three independent experiments were performed; error bars represent SEM. Gene expression normalized to L8 ribosomal protein mRNA expressed as densitometric units determined from array analyses are shown as a line graph for comparison.
*$p < 0.01$; **$p < 0.001$, relative to the control.

Table 1. Effects of acetochlor on gene expression in tail tissue of premetamorphic *X. laevis* tadpoles.

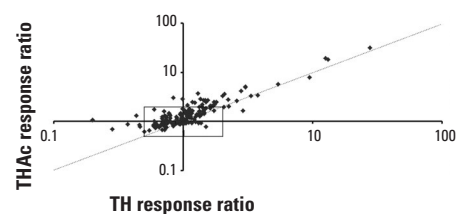| Gene name	GenBank accession no.	Response ratio[a]
DAD-1	D15059	0.31
Methyl-CpG binding protein	AF170347	0.32
Protein/lipid phosphatase PTEN	AF144732	0.32
*DLL*2	L09730	0.34
c-*myc* I	X14806	0.40
*Max*2 and *Max*4	L09738	0.43
*mdm*2	not submitted[b]	0.44
scythe	AF098511	0.52
K-*ras*	AF085280	0.53
Replication protein A	X67240	0.55
GSK-3 binding protein	AF062738	2.40

[a]Ratio of gene expression relative to the vehicle control. Ratios > 1.0 reflect fold induction, and those < 1.0 indicate the reciprocal of fold repression. [b]Previously identified thyroid hormone responsive gene.

Figure 4. The effects of acetochlor on TH-dependent gene expression in *X. laevis* tadpole tail tissue. Gene response ratios following treatment for 48 hr to TH treatment alone (TH response ratio) and after a combined treatment of TH with acetochlor (THAc response ratio) were determined relative to the vehicle control and compared by scatterplot analyses. All data were normalized using L8 ribosomal protein mRNA expression. Those data points (diamonds) that fall within the rectangle that represents a 2-fold response relative to vehicle control are defined as being nonresponsive to TH treatment. The diagonal dashed line represents no difference in gene expression response between TH and THAc treatments. Deviation from this diagonal line represents an effect by acetochlor on the expression of that gene.

its expression in the presence of T_3 (compare Table 1 to Table 3).

During precocious metamorphosis, exposure to acetochlor affects the expression of genes that exhibit similar cellular functions to those identified in premetamorphic tadpoles. The general effect of this herbicide was to enhance the accumulation of T_3-responsive gene transcripts. In addition, 10 genes that are normally refractory to TH treatment responded to exogenous T_3 administration in the presence of acetochlor. As was previously reported, iodothyronine 5-deiodinase type III (an enzyme important in the inactivation of T_3 and T_4) increased substantially after T_3 treatment, whereas the transcript levels of the type II deiodinase

that catalyzes the 5´-deiodination of T_4 to T_3 were unaffected (Becker et al. 1995, 1997; Huang et al. 1999, 2001; Marsh-Armstrong et al. 1999). In the presence of acetochlor, the hormone-dependent increase of deiodinase type III is slightly attenuated, with no effect on type II deiodinase. We do not know the relationship between deiodinase type III mRNA and the corresponding enzyme activity, but if they correlate, then a reduction in the rate of T_3 inactivation could manifest as an acceleration of metamorphosis. This idea is supported by the converse observation that overexpression of the type III deiodinase in transgenic X. laevis tadpoles inhibits metamorphosis (Huang et al. 1999).

The MAGEX array identified many genes that were previously reported to be under TH regulation and several that are novel targets for TH. Despite a clear association between TH responsiveness and acetochlor action for many genes, not all T_3-regulated genes are targets of acetochlor in X. laevis. In rats, studies have shown that acetochlor acts by increasing T_4 clearance from the liver, thereby reducing TH levels (Ashby et al. 1996; Wilson et al. 1996). This mechanism appears to be incompatible with our observations and those of others. In premetamorphic tadpoles, where no endogenous T_3 or T_4 is present (Leloup and Buscaglia 1977), acetochlor accelerated T_3-induced metamorphosis rather than inhibited it (Cheek et al. 1999a; Veldhoen and Helbing 2001). This herbicide may have an effect on tissue deiodinases; however, not all T_3-responsive genes were affected by acetochlor action. Thus these results suggest that the mode of action of acetochlor may be at the level of gene transcription. Acetochlor does not bind to human TRβ (Cheek et al. 1999b) and, although the same studies have not been done in frog, it is likely that similar results would be found due to the high degree of sequence conservation. The scope of the EDC effects of acetochlor may extend beyond TH-dependent regulation of gene expression. Acetochlor inhibited binding of [³H]estradiol-17β to rat uterine nuclear and cytoplasmic estrogen receptors (Rollerova et al. 2000), indicating that the herbicide can target multiple endocrine pathways using different modes of action and reinforcing the importance of considering crosstalk between TH and other hormone regulatory pathways (Vasudevan et al. 2001; Zhu et al. 1996).

The fact that acetochlor magnifies the T_3-induced response for many upregulated genes and attenuates the response for downregulated ones suggests a mechanism of disruption that involves transcriptional regulation. Because acetochlor does not bind to TRβ directly (Cheek et al. 1999b), we hypothesize that this herbicide may instead have epigenetic effects that change the nature of TR-containing complexes. Depending on the presence or absence of hormone and the cellular context, TRs associate with retinoid X receptors, histone deacetylases/acetylases, and other ancilliary factors (Shi and Ishizuya-Oka 2001). One or more of these regulatory components may be a target of acetochlor action, thus representing a novel mechanism for endocrine disruption.

Table 2. Effects of acetochlor on gene expression in tail tissue of X. laevis tadpoles during precocious metamorphosis.

Gene name	GenBank. accession no.	TH response ratio[a]	TH + acetochlor response ratio[a]
Group 1			
Gene 12	U41860[b]	27.73	31.21
TH/bZip	U37375[b]	13.12	18.09
Collagenase-3	U41824[b]	12.53	19.28
Iodothyronine 5-deiodinase III	L28111[b]	9.45	7.95[c]
Retinoic acid receptor α	X87365	3.04	5.09
fos-related antigen-2	U37374[b]	3.03	4.93
B-50/GAP 43	X87582	2.89	1.67[c]
BTEB	U35408[b]	2.80	4.14
Gelatinase A	Not submitted[b]	2.09	3.60
Fibronectin	M77820[b]	2.02	3.35
Group 2			
Mad2	L77885	1.60	2.71
Fragile mental retardation protein 1	U25164	1.49	2.69
Frizzled 7	AF159107	1.43	2.72
Caspase-9	AB038172	1.40	2.87
Nuclear factor I-C1	L43149	1.30	3.72
Transcription factor A	U35728	1.20	2.45
Transferrin		1.20	2.37
Transcription factor AP-2		1.10	2.15
Vitellogenin receptor	AB006906	1.00	2.90
Natriuretic peptide receptor type C	AF231035	0.80	3.04
Group 3			
51-kD cytokeratin type I	Y00968	0.46	1.60
Gene 18	U41839[b]	0.46	0.86
Glutamine synthetase	D50062	0.43	1.31
Gene 17	U41860[b]	0.37	0.87
Gene 19	U41861[b]	0.28	0.70
Gli2	AF109923	0.20	1.08

[a]Ratio of gene expression relative to the vehicle control. Ratios > 1.0 reflect fold induction, and those < 1.0 indicate the reciprocal of fold repression. [b]Previously identified thyroid hormone responsive genes. [c]TH response is diminished in the presence of acetochlor.

Table 3. Thyroid hormone-dependent genes not affected by acetochlor in tail tissue of X. laevis tadpoles during precocious metamorphosis.

Gene name	GenBank. accession no.	TH response ratio[a]	TH + acetochlor response ratio[a]
Fibroblast activation factor α	U41856[b]	5.42	5.70
CCAAT/enhancer (C/EBP)	S44193[b]	3.79	3.41
GSK-3 binding protein	AF062738	3.32	3.26
Peroxisome proliferator receptor α	M84161	2.58	2.65
Protein kinase C related kinase 2	AF027183	2.26	2.01
Negative regulator Id2	AB019520	2.23	2.77
MAP kinase MPK2	X80751	2.05	1.94
c-myc I	X14806	0.50	0.62

[a]Ratio of gene expression relative to the vehicle control. Ratios > 1.0 reflect fold induction, and those < 1.0 indicate the reciprocal of fold repression. [b]Previously identified thyroid hormone responsive genes.

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