Screening for reproductive toxicity in *Fundulus heteroclitus* by genetic expression profiling

J.C. Craig, M. E. Westerman, G. D. Bennett, L. DiMichele and R. H. Finnell

Potentially teratogenic agents enter the environment at a rate that greatly exceeds current capabilities to effectively evaluate their reproductive toxicities. This is due, in part, to costly, labour-intensive methodologies involving mammalian embryonic screening assays that are currently in use worldwide. Therefore, we sought to develop a rapid, less expensive screening system with which to identify molecular biomarkers of teratogenicity using a non-mammalian system. Embryos of the topminnow, *Fundulus heteroclitus*, offer several advantages in terms of reproductive toxicity screening efficiency as compared with mammalian embryonic systems. These embryos are easily manipulated and develop normally at ambient temperature in air, water, or air-saturated mineral oils, making them readily adapted for field studies. In the present study, developing *F. heteroclitus* embryos were exposed to teratogenic concentrations of sodium valproate (VPA) or arsenic acid (arsenate), and the frequency and types of induced malformations were evaluated. Using *in situ* transcription and antisense RNA (aRNA) amplification procedures (IST/aRNA), we attempted to correlate the teratogenic outcomes to specific alterations in the expression of a panel of developmentally regulated genes. Preliminary studies identified treatment concentrations of arsenate and VPA that induced abnormal development in 95% of the surviving embryos. Among the *F. heteroclitus* embryos, the structural defects most commonly induced by these compounds were cardiac and neural tube malformations. The genetic expression profiles revealed a number of genes whose expression levels were significantly altered by exposure to the test compounds. Molecular analysis of *F. heteroclitus* embryonic development represents a novel, inexpensive approach to screen for potential teratogens, and identify genes whose expression patterns may be used as biomarkers, or indicators, of teratogenicity.

Keywords: *genetic expression profiling, sodium valproate, arsenate, teratology, in vitro assay.*

Introduction

The rate that potentially hazardous reproductive toxicants (teratogens), enter the environment is rapidly outpacing the ability to effectively evaluate their threat to human populations. In the US, the production of organic pesticides and synthetic organic chemicals more than tripled from 1960 to 1977 (Casarett and Doull 1980, 1996). This increased production results in a concurrent increased exposure of the general population to various chemical risks. Teratogenic assessment of these compounds poses a serious challenge to the biomedical research community, as federal guidelines typically require *in vivo* whole animal studies. These studies are not only time consuming, inefficient, and expensive, but often fail to adequately predict human risks. Furthermore, the costs involved in the toxicity testing of candidate pharmaceutical products may serve as an impediment to the development of new medications. Therefore, the development of an inexpensive and sensitive pre-screening assay for the initial detection of teratogens prior to testing in an *in vivo* rodent model is essential to remedy this potentially hazardous situation.

Screening assays currently in use have not been designed to determine a molecular basis for the induction of congenital defects. The application of newly developed techniques such as *in situ* transcription and antisense RNA amplification (IST/aRNA) for use as a screening system has great potential, as the teratogenic predictability would be based on subtle differences in embryonic gene expression. This methodology was originally developed to examine gene expression in limited, anatomically restricted tissue samples, and involves the hybridization of an amplification oligonucleotide to the polyadenylated messages in the target tissue and their subsequent amplification by T7 RNA polymerase (Eberwine *et al.* 1992 a,b, Miyashiro *et al.* 1993, Taylor *et al.* 1995, Mackler *et al.* 1996). Previous attempts to develop rapid teratogen screening assays have been unsuccessful in relating teratogenic activity to chemical structure (Schumacher 1975, Zimmerman 1975). The FETAX system was developed to screen for teratogenicity on the basis of chemical function, utilizing embryos of the South African clawed frog *Xenopus laevis* (DeYoung *et al.* 1991). This assay works in conjunction with an *in vitro* metabolic activation system to exploit relationships between compound function and teratogenic activity. However, the FETAX assay lacks an essential unifying premise, such as defining a reliable relationship between teratogenic activity and the induction of developmental delay, upon which to base its predictions.

Herein we describe a novel procedure for screening chemical teratogens using teleost embryos and modern molecular biological approaches. This method addresses the inefficiencies inherent in the mammalian embryo system by circumventing the high cost and extensive time commitments associated with traditional teratology studies, while providing fundamental information on the molecular mechanism(s) by which a test compound disrupts normal development. Embryos of the topminnow, *Fundulus heteroclitus*, were chosen as a potential non-mammalian screening system based on inherent qualities of the eggs such as large size, heartiness, manageability and transparency.

Initial experiments were designed to demonstrate the teratogenicity of the anticonvulsant drug, sodium valproate (VPA) (Depekane, Abbott Laboratories), and the heavy metal arsenate in *F. heteroclitus* embryos at concentrations previously shown to have adverse effects on normal murine

J. C. Craig, G. D. Bennett, R. H. Finnell *(author for correspondence) are in the Department of Veterinary Anatomy and Public Health, Texas A&M University, College Station, Texas 77843, USA;* **M. E. Westerman and L. DiMichele** *are in the Department of Wildlife and Fisheries, Texas A&M University, College Station, Texas 77843, USA.*

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development (Kao *et al.* 1981, Morrissey and Mottet 1983, Nau 1985, Sonada *et al.* 1993). Subsequent experiments were designed to identify those genes whose expression levels were consistently altered in response to these two compounds. Sodium valproate and arsenate were chosen on the basis of their suspected and documented teratogenic potential in humans and animals (Shepard 1992). Sodium valproate has a well established teratogenic potential in humans and a wide variety of animal species, most commonly inducing neural tube defects (for review see Finnell *et al.* 1995).

The teratogenic potential of arsenic has recently been established in mammalian animal models (Shalet *et al.* 1996). Organic and inorganic forms of arsenic are environmental contaminants frequently utilized as wood-treatment preservatives, herbicides or pesticides, and are found as byproducts of the smelting of metal ores (Casarett and Doull 1980, 1996). Sodium arsenate appears to be less toxic than inorganic arsenic; however, its teratogenic potential has been well established in laboratory animals. Both *in utero* and cell culture studies using a murine model have demonstrated increased frequency of resorptions, foetal death, growth retardation and neural tube defects (Mottet and Ferm 1983, Whillhite and Ferm 1984) in response to arsenate exposure.

In the present study, a dual-phase approach that examined both the morphological and genetic responses in *F. heteroclitus* was performed. Teratology studies were conducted to determine the dose at which congenital malformations could be induced, without significantly decreasing embryonic survival. These were followed by molecular studies conducted to examine how these teratogens altered gene expression in exposed embryos by using IST/aRNA methodologies.

METHODS AND EXPERIMENTAL PROCEDURE

Fundulus heteroclitus

Collection and maintenance

Adult *F. heteroclitus* (topminnow) were commercially obtained from the Whitney Marine Laboratory (WML, Marineland, Florida). Adults were maintained for a period of 4 years (1990–1994) in recirculating 1200 L tanks filled to capacity with 15 parts per thousand (ppt) seawater. They were fed a diet of commercial dried flake food which was supplemented with chopped, locally farm-raised shrimp and roe from locally farm-raised fish to avoid potential confounding of the experiments by the introduction of additional heavy metals. Water quality (ammonia, pH and salinity) was monitored routinely. Ammonia levels were maintained at > 0.08 mg L^{-1}, pH was maintained between 8.2 and 8.3, and salinity was maintained ± 2 %. Water temperatures were varied throughout the year. Water chillers were used to maintain a winter low temperature of 10 °C, and summer high temperatures of 23 °C. The fish were maintained on a 12 h light and dark cycle throughout our investigation. Under these conditions, female *F. heteroclitus* produced mature oocytes repeatedly over a 7–8 month period, usually between March and October, which roughly corresponds to their natural spawning season (DiMichele, personal communication).

Fertilization

Fertilization was performed by first removing the testes of two or three adult male *F. heteroclitus* and placing them in 30 ml of sterile seawater while the oocytes were being prepared. Mature oocytes were manually expressed from 10–20 adult female *F. heteroclitus* into a dry sterile 110 mm petri dish. Once the oocytes were collected, the testes were rapidly minced with a razor blade and the resultant sperm suspension was manually swirled into the oocytes to obtain an essentially synchronous fertilization of all mature oocytes. After approximately 2–3 min, embryos were washed with several changes of seawater to remove any excess sperm. Fertilizations that were less than 90 % efficient were discarded. Upon examination under a dissecting microscope, unfertilized oocytes and defective embryos were immediately removed. Viable embryos were randomly divided, placed in sterile 110 mm petri dishes, and assigned to a treatment group. Embryos were then covered with approximately 30 ml of sterile seawater (30 ppt) and placed into an incubator at 25 °C until the treatment was initiated, at 19 h post-fertilization for VPA treatment, or immediately injected with arsenate.

Teratology studies

Teratology procedures

For each test compound, three separate dose–response experiments were performed. For each study, 200–300 fertilized eggs were randomly divided into treatment and control populations. The VPA study was comprised of three treatment groups, each utilizing between 80 and 100 eggs per experiment. The arsenate study consisted of four treatment groups, each utilizing between 65 and 75 eggs per experiment. Sodium valproate and arsenate concentrations were chosen in accordance with murine embryonic doses previously shown to have adverse effects on normal development (Kao *et al.* 1981, Morrissey and Mottet 1983, Nau 1985, Sonada *et al.* 1993). The concentrations of VPA (1.8 mM and 3.6 mM) used in these studies were comparable to the murine embryonic concentrations (approximately 2.25–3.5 mM) reported to result from murine maternal exposure concentrations (4.5–7 mM) following intraperitoneal injection (Nau and Loscher 1986, Nau 1994). Sodium valproate was administered directly to the artificial seawater surrounding the eggs, beginning at 19 h post-fertilization.

The concentrations chosen for arsenate, 0.05 mM and 0.1 mM, correspond to the minimum and maximum teratogenic maternal plasma levels observed in mice (Morrissey and Mottet 1983, Hood *et al.* 1987, Copp *et al.* 1990). Owing to solubility issues, arsenate was directly microinjected (20 nl) into the yolk sac underlying the embryo immediately following fertilization using an IM-200 microinjector (Narishige USA, Inc.). This necessitated a second set of sham control samples for the injection procedure which were similarly injected with 20 nl of saline (pH = 7.5).

The embryos were developmentally staged and examined for the presence of any gross malformations or developmental delay, under a Wild Leitz (MPS 53) dissecting microscope. These examinations were conducted every 2–4 h for the first day (through the first 16–18 stages, representing cleavage through late gastrulation), and then every 12 h for the next 9–10 days, or until the fish hatched (stage 34, Armstrong and Child 1965). Percentages of abnormal embryos were calculated immediately prior to normal hatching time. Comparisons between treated and control embryos were conducted at the same time, rather than at the same developmental stage, in an effort to accurately assess the rate of development. All adverse developmental effects were recorded. Statistical computations were performed by SAS (Statistical Analysis Systems 1990). ANOVA was performed using the general linear models (GLM) procedure to: compute the mean percentages of malformations; determine the level of significance for these means among treatment classes, while adjusting for unequal sample sizes; make comparisons among treatment groups at the same developmental stage (Armstrong and Child 1965) using the Duncan's option. Statistical significance was set at the alpha 0.05 ($P < 0.05$) level.

Molecular studies

Sampling procedure

Once the teratology studies had been completed, three separate molecular experiments were performed. For each of these experiments, 200–300 fertilized

embryos were randomly divided into treatment and control groups and exposed to either VPA or arsenate. The number of eggs per treatment group, treatment concentrations, mode of teratogen administration and developmental staging were as described for the dose–response studies. The sampling period began at neurulation (stage 16), approximately 21 h post-fertilization (Armstrong and Child 1965). Embryos were randomly sampled at each developmental stage (n = 3/sample), every 12 h for 9 days, from stage 16 until the embryos hatched at stage 34 (Armstrong and Child 1965). Therefore, a total of 54 embryos per treatment group, representing all three experiments, were sampled over 18 developmental stages. A subgroup of these embryos (n = 5–9 per treatment group) from the three experiments at developmental stage 16 comprised the molecular studies. Sampling and subsequent profiling comparisons between treated and control embryos were performed at the same time, rather than at the same developmental stage to accurately assess any delay in development at the molecular level.

In situ transcription and aRNA procedures

These techniques were performed according to the procedures outlined by Eberwine *et al.* (1992a,b), Miyashiro *et al.* (1993), Taylor *et al.* (1995) and Mackler *et al.* (1996). Briefly, the procedure involves the use of an oligo-d(T) primer engineered to include a T7 RNA polymerase promoter at its 5' end. This oligonucleotide is allowed to hybridize to the embryonic poly-A + mRNA population in the presence of reverse transcriptase and dNTPs in order for cDNA synthesis to occur (Eberwine *et al.* 1992a,b, Miyashiro *et al.* 1993, Taylor *et al.* 1995). Following the synthesis of first strand of cDNA, which is heat-separated from the mRNA, the second cDNA strand is generated by hairpin priming of the first strand at its 3' end. The single-stranded hairpin is subsequently cleaved with S1 nuclease, and the template is blunt-ended using T4 DNA polymerase, Klenow fragment and additional dNTPs. The resulting double-stranded cDNA template can be used for [^{32}P-CTP] radiolabelled aRNA amplification using T7 RNA polymerase that will recognize the incorporated T7 RNA promoter. The amplified controls at each timepoint will ensure accurate assessment of the relative abundance and original distribution of the endogenous mRNA. The amplified aRNA will be antisense to the poly-A + mRNA, and can be used directly as a riboprobe for hybridization to the cDNAs of interest.

Genetic expression profiling

Equimolar concentrations of 47 candidate cDNA clones were immobilized on a nylon membrane (Zetaprobe, Biorad, Richmond, CA) by means of a Biorad slotting apparatus following manufacturer's protocol. The selected cDNAs span a wide variety of functional classes of genes that are known to play significant roles in developmental events, and are highly conserved among vertebrate taxa. Following a 15 min prehybridization at 37 °C, these 'reverse northern blots' were probed with radiolabelled, amplified antisense RNA (aRNA), obtained from the IST/aRNA amplification procedures outlined above. The prehybridization buffer contained 7 % SDS, 0.12 M Na_2HPO_4 (pH 7.2), 0.25 M NaCl, and 50 % formamide. The heat-denatured riboprobes were allowed to hybridize to the nylon membrane overnight at 37 °C, after which the blots were washed with a series of increasingly stringent solutions (2X SSC to 0.1X SSC). The washed, moist blots were then wrapped in saran wrap and scanned by an Ambis 101 two-dimensional direct beta detector (Scanalytics, Billerica, MA). The numeric values representing the cpm values or hybridization intensities for each gene were recorded from the individual blots. The value for the vector cDNA pUC13 (American Type Culture Collection) served as the background of non-specific binding, while the value for the cDNA clone encoding the neurofilament protein actin was used as an internal reference value with which to normalize all hybridization intensities. The normalization procedure enabled us to make direct comparisons from among blots. All statistical computations were performed by SAS (Statistical Analysis Systems 1990). ANOVA was performed using the general linear models (GLM) procedure to: compute the mean relative hybridization intensities (to actin) obtained for each of the 47 cDNA clones; determine the level of significance for these means among treatment classes, while adjusting for unequal sample sizes; make comparisons among treatment groups within a single developmental stage (Armstrong and Child 1965) using the Duncan's option. Statistical significance was set at the alpha 0.05 ($P < 0.05$) level.

Results

Teratology studies

Initial experiments were conducted to determine whether concentrations of VPA or arsenate, shown to induce congenital malformations in foetal mice, would similarly disrupt normal development in *F. heteroclitus* embryos. Administration of 1.8 mM and 3.6 mM VPA resulted in statistically significant increases in embryonic malformations ($P < 0.05$; Figure 1). The frequency of the various types of malformations were significantly elevated in both VPA treatment groups, as compared with control embryos ($P < 0.05$; Table 1). Under control (untreated) conditions in the VPA study, the mean percentage of embryos with gross malformations was 1 %. The frequency of gross malformations increased to 5 % in the 1.8 mM treatment group and 25 % in the 3.6 mM treatment group. The gross malformations induced by VPA exposure included cardiac, ocular, neural tube (Figure 2) and notochordal malformations. The cardiac anomalies involved extreme hypoplasia of the heart, reduction or atrophy of the ventricles, and/or a decreased vascularization of the anterior chamber. The only observed ocular defect was microphthalmia. The neural tube and notochord malformations occurred immediately caudal to the hindbrain, and usually appeared as an abrupt curvature at the midline fusion points of the neural folds. Embryos treated at either dose also manifested developmental delay, as compared with control embryos at the same time. Figure 1 also illustrates the relationship between the VPA dose and developmental delay. In the control population only 10 % of the embryos had failed to reach stage 34 (the hatching stage per Armstrong and Child 1965), while 50 % and 95 % of embryos in the 1.8 mM and 3.6 mM treatment groups, respectively, failed to reach the same stage. Developmental delay was characterized by a uniform retardation of growth and structural differentiation. In addition to smaller overall size, VPA-treated embryos possessed a rudimentary cardiovascular system, lacked normal body and eye pigmentation and coordinated movement (fin flexure, eye rotation), characteristics indicative of a slower progression through morphogenesis.

Administration of 0.05 mM and 0.1 mM arsenate resulted in statistically significant increases in embryonic malformations and overall developmental delay, as compared with embryos maintained under control conditions at the same time ($P < 0.05$; Figure 1). The frequencies of the various types of malformations were significantly elevated in both arsenate treatment groups, as compared with control embryos ($P < 0.05$; Table 1). A mean of 2 % of the non-injected control embryos, and a mean of 5 % of the sham-injected control embryos was

Teratogen	Treatment	Neural tube defects	Cardiovascular defects	Ocular defects	Other defects	Total malformations[a]
Arsenate	Control	0%	2%	0%	0%	2%
	Sham	0%	3%	1%	1%	5%
	0.05 mM	3%*	10%*	5%*	4%*	22%*
	0.1 mM	21%*	40%*	12%*	4.5%*	77.5%*
VPA	Control	0%	1%	0%	0%	1%
	1.8 mM	2%*	4%*	2%*	1%	9%*
	3.6 mM	25%*	17%*	5%	0.5%	47.5%*

Table 1. Teratological data for *Fundulus heteroclitus* embryos exposed to arsenic acid and sodium valproate.

Neural tube, cardiovascular, occular, and other defects were evaluated in response to arsenate (top panel) or VPA (bottom panel) and calculated as percentages of the total sample size.

[a] Malformations of more than one type occurred in the same embryos.

* Significant differences from sham-injected control and control embryos ($P < 0.05$).

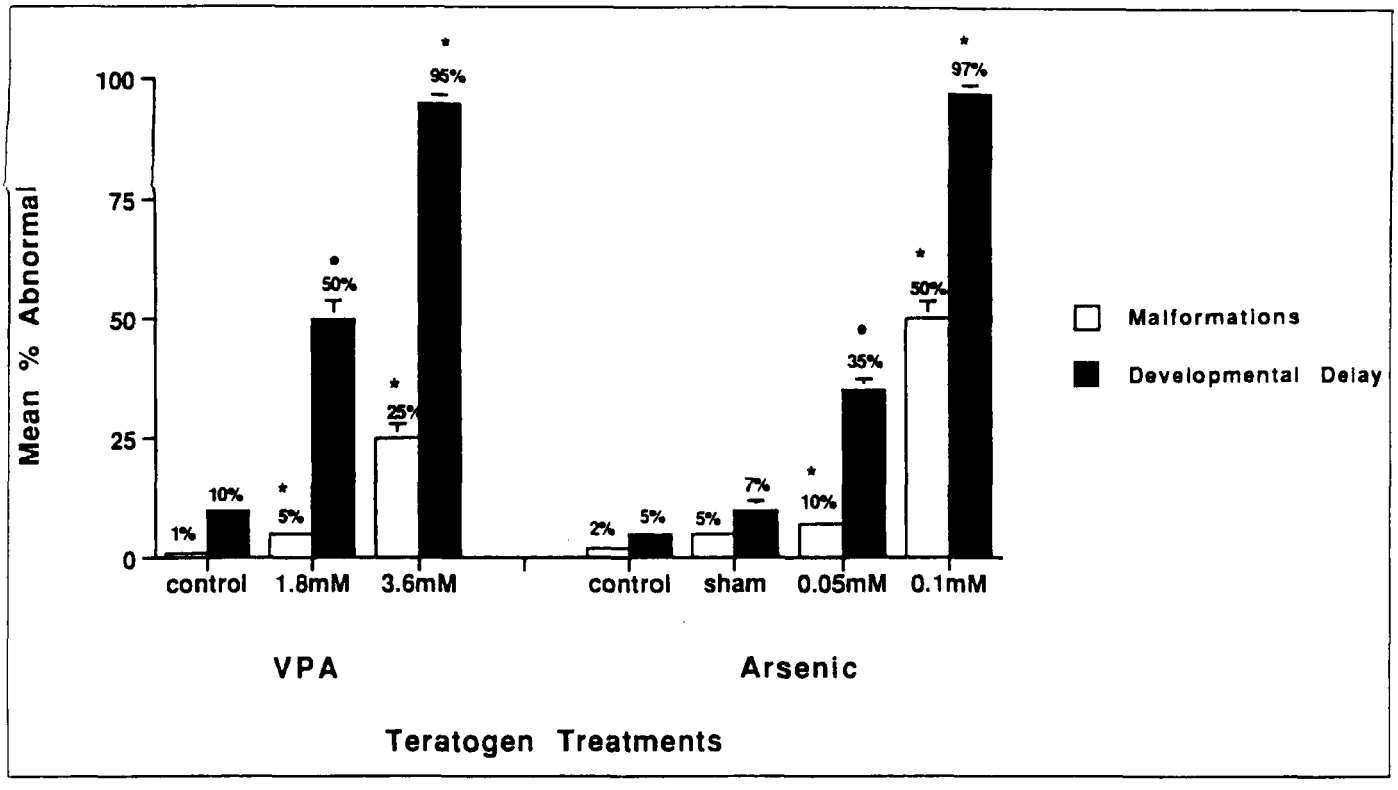

Figure 1. Dose–response effects of sodium valproate (VPA) (left) and arsenate (right). In the VPA studies, *Fundulus heteroclitus* embryos were maintained under control (untreated) conditions ($n = 273$), or treated with 1.8 mM ($n = 273$) or 3.6 mM VPA ($n = 273$). In the arsenate studies, *F. heteroclitus* embryos were maintained under control (untreated) conditions ($n = 216$), sham-injected with saline to control for the injection process ($n = 216$), or treated with 0.05 mM ($n = 216$) or 0.1 mM arsenate ($n = 216$). Each treatment group was a composite of three individual experiments. The mean percent of abnormal embryos were obtained from averaging the percentage of affected embryos from corresponding treatment groups in each experiment. Standard errors are given for all treatment groups, though the error bars are not visible when the standard error is negligible. * Significant differences from control embryos ($P < 0.05$).

grossly malformed. The values for the sham controls were not significantly different from the non-injected controls ($P > 0.05$). The frequency of gross malformations increased to 10 % in the 0.05 mM arsenate treatment group, and 50 % following exposure to 0.1 mM arsenate. Arsenate-induced defects were observed over the entire developmental period and included cardiac, neural tube, ocular and notochord malformations, truncated tail or midsection, and twinning anomalies (Table 1). The cardiac, neural tube and notochordal malformations were all similar to those described above for the VPA response. The ocular defects included cyclopia or microphthalmia, both of which occurred in response to either arsenate dosage. The

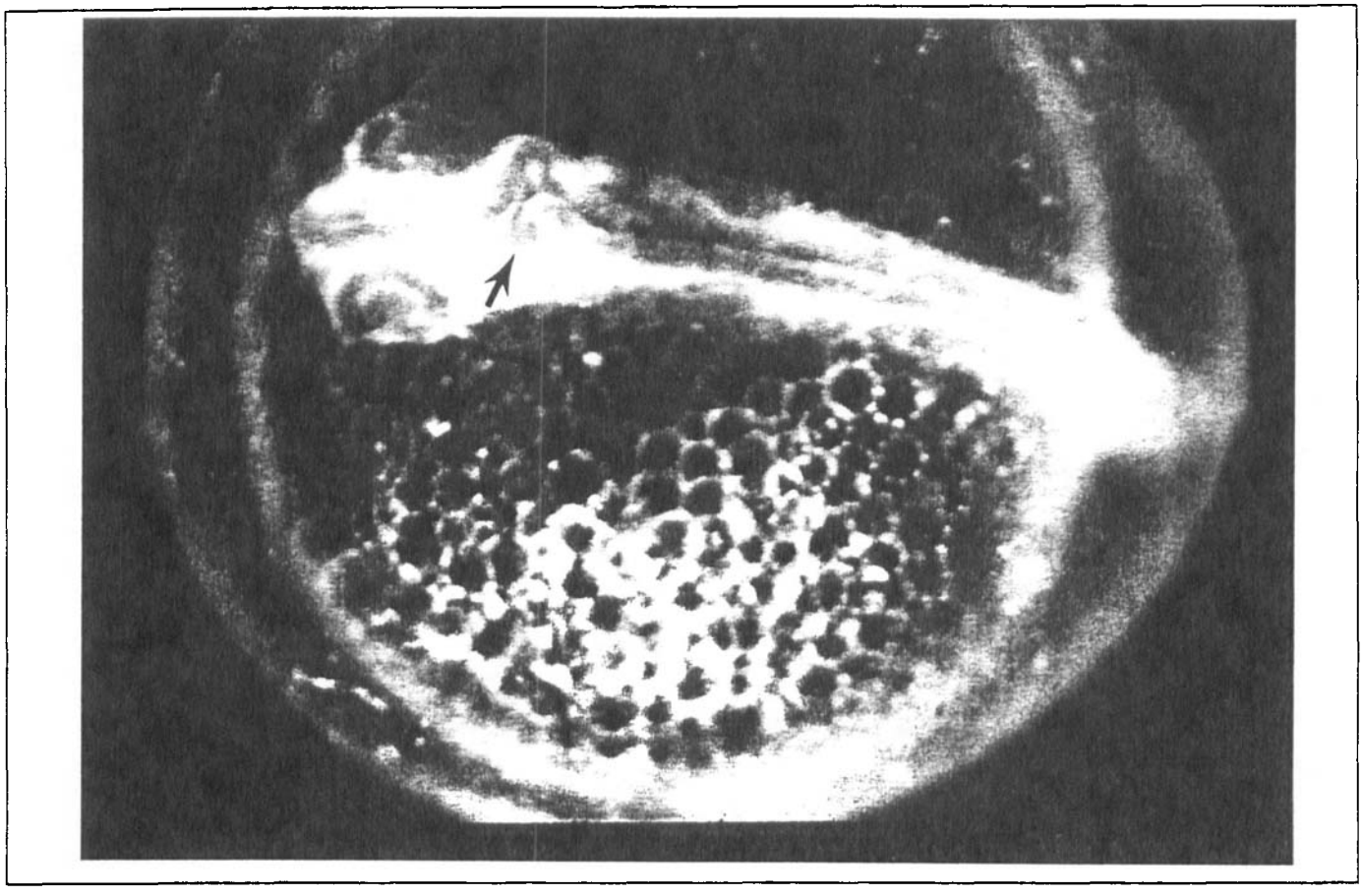

Figure 2. Characteristic neural tube lesions in response to sodium valproate (VPA) or arsenate. Three-day-old *Fundulus heteroclitus* embryo, developmental stage 24, incubated in 3.6 mM VPA.

truncated tail and midsection anomalies appeared to involve partial to complete absence of the fins, tail and/or body, while the head continued growth in a disproportionate manner to the final developmental stages. Neither normal ventral envelopment of the heart nor hatching occurred in these embryos. As with VPA, the primary effect of arsenate on the exposed embryos was developmental delay. Figure 1 illustrates that when 90 % of the control embryos had reached stage 34, only 65 % and 3 % of the embryos comprising the 0.05 mM and 0.1 mM arsenate treatment groups, respectively, had reached the same stage of development. The characteristics of the developmentally delayed arsenate-exposed embryos were similar to those seen among embryos exposed to VPA.

Molecular studies

To identify genes whose levels of expression were consistently altered in response to either VPA or arsenate exposure, genetic expression profiles using IST/aRNA technology were generated. Figure 3 is a representative reverse northern blot probed with aRNA prepared from an untreated whole embryo at stage 16, the initiation of neurulation in *F. heteroclitus* (Armstrong and Child 1965). Intense hybridization can be seen for *Pax-3*, *CNTF*, *NFL*, *IGF II*, *PDGF-R*, *Nic*, *Actin*, *pCHMR1*, *Sonic Hh*, *bcl-2*, *CK II*, and *tie-2*, while other genes were either quiescent, or only marginally expressed at this gestational stage.

As depicted in Figure 4(a and b), significant transcriptional alterations were observed in the *F. heteroclitus* embryos in response to the VPA treatments, among the following genes: paired box *Pax-3*, *c-fos*, cyclic AMP binding protein (*CREB*), tyrosine kinase receptor (*trk*), transforming growth factor alpha (*TGFα*), insulin-like growth factor 2 (*IGF-2*), basic fibroblast growth factor (*bFGF*), neurotrophin-3 (*NT3*), phospholipase 2 (*Pla-2*), *Wee-1*, glucocorticoid receptor (*GCR*), and the cellular retinal binding proteins 1 and 2 (*CRBP-1* and *CRBP-2*) ($P < 0.05$). Compared with control levels, the expression of: *Pax-3*, *CREB*, *trk*, *TGFα*, *bFGF*, *NT3*, *Pla-2*, *GCR*, and *CRBP-1* and *2* were significantly increased in response to the 1.8 mM dose of VPA ($P < 0.05$), but were not significantly altered from control levels in response to the 3.6 mM dose (Figure 4(a and b); $P > 0.05$). The expression of *IGF II* was significantly increased in response to either the 1.8 mM or 3.6 mM doses of VPA as compared with controls ($P < 0.05$). *Wee-1* expression significantly increased in a dose responsive manner as compared with controls ($P < 0.05$) and a dose-dependent decrease in *c-fos* expression was observed, although the reduced expression was only significant following 3.6 mM

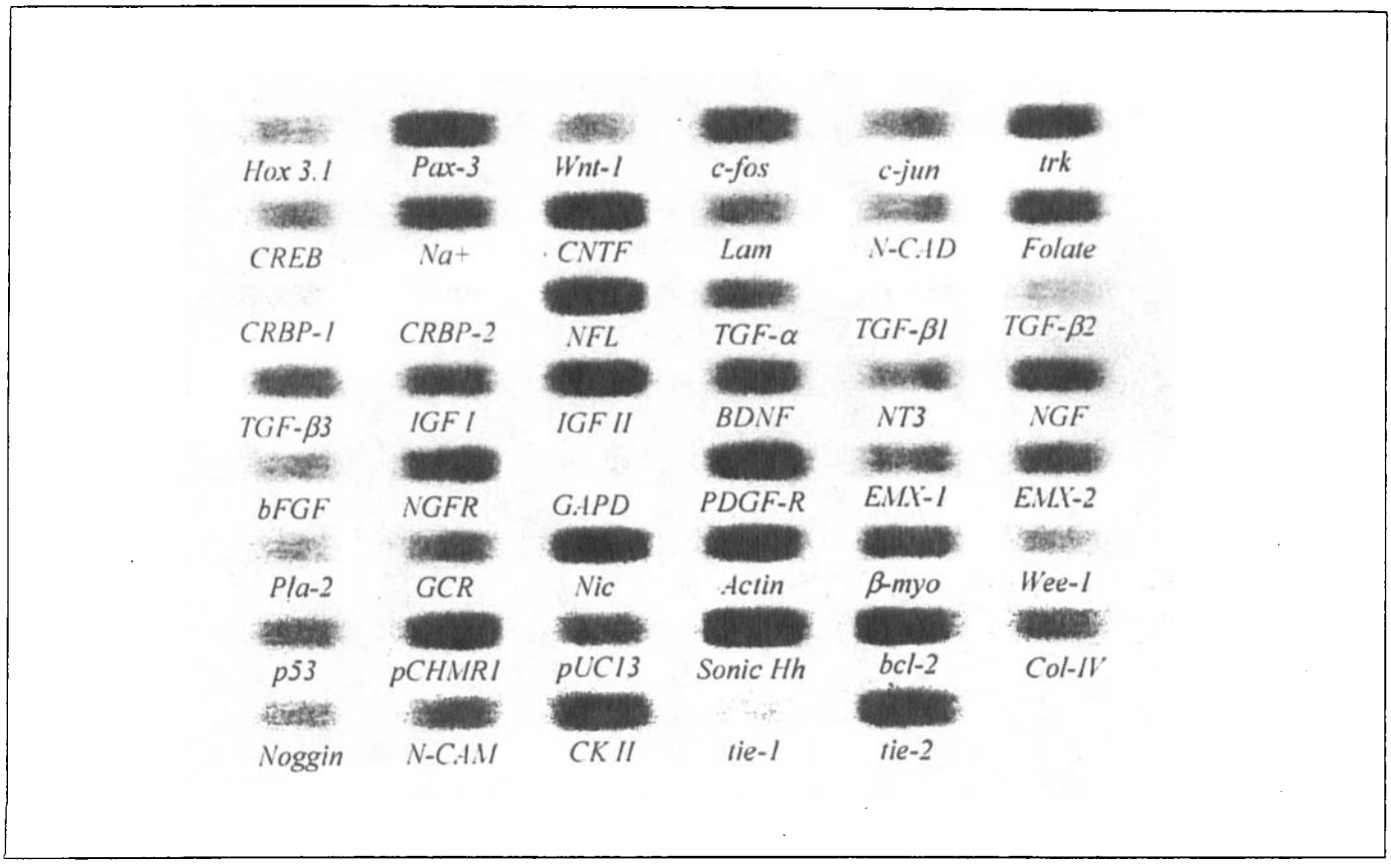

Figure 3. Reverse northern blot representing the genetic expression profile for 47 candidate cDNAs from an individual embryo under control conditions at developmental stage 16 (Armstrong and Child 1965).

VPA treatment ($P < 0.05$). Comparison of the expression profiles of our candidate genes between the two VPA treatments revealed a significant decrease in the expression level of *Pax-3*, *CREB*, *TGFα*, and *CRBP-1* in response to 3.6 mM VPA (Figure 4(a and b); $P < 0.05$).

As depicted in Figure 5(a and b), significant transcriptional alterations in response to arsenate treatments were observed among the following genes: the murine homologue of the *Drosophila* empty spiracle Hox gene (*EMX-1*), transforming growth factors β1 and 2 (*TGF-β1* and *TGF-β2*), neuronal cadherin (*N-CAD*), neural cell adhesion molecule (*N-CAM*), glyceraldehyde-3-phosphate dehydrogenase (*GAPD*), β-myosin (*β-myo*), the vertebrate homologue of the *Drosophila* segment polarity gene, sonic hedgehog (*SHh*), *CRBP-1*, *CRBP-2*, and *GCR* ($P < 0.05$). As with VPA, the lower arsenate treatment resulted in a more profound effect on gene expression than that observed with the higher arsenate concentration. Specifically, the expression levels of all genes in Figure 5(a and b) were significantly decreased in response to 0.05 mM arsenate relative to controls, with the exception of *GAPD* which significantly increased in a dose-responsive manner ($P < 0.05$). In response to 0.1 mM arsenate, the expression levels of *EMX-1* and *TGF-β2* were significantly decreased, while *N-CAM* was significantly increased relative to controls ($P < 0.05$). As compared with 0.5 mM arsenate treatment expression levels, significant increases in the expression levels of *TGF-β2*, *β-myo*, *SHh*, *GCR* and *CRBP-2* were noted in response to 0.1 mM arsenate ($P < 0.05$). Of the 47 genes examined, only three, the cellular retinal binding proteins (*CRBP-1* and *CRBP-2*), and the glucocorticoid receptor (*GCR*), were significantly altered by both of the test compounds (Figure 6). In response to 1.8 mM VPA, these three genes were significantly increased, whereas they were significantly decreased in response to 0.05 mM arsenate.

Discussion

The results obtained from these studies indicate that concentrations of either VPA or arsenate that have been shown to produce adverse effects on normal murine development can profoundly affect development of *F. heteroclitus* embryos. These teratogenic concentrations of either compound resulted not only in gross malformations in the developing fish embryos, but significantly altered the expression of a number of genes involved in regulating development. Sodium valproate (VPA) is one of several antiepileptic drugs used to control seizure disorders that has a well documented teratogenic potential in both humans and experimental animals (Robert 1982, Rosa *et al.* 1986, Lammer *et al.* 1987, Finnell *et al.* 1988, 1995, Finnell 1991). Administration of 400–600 mg kg^{-1} VPA to pregnant mice results in maternal plasma concentrations of 4.5–7 mM, 50 % (2.25–3.5 mM) of

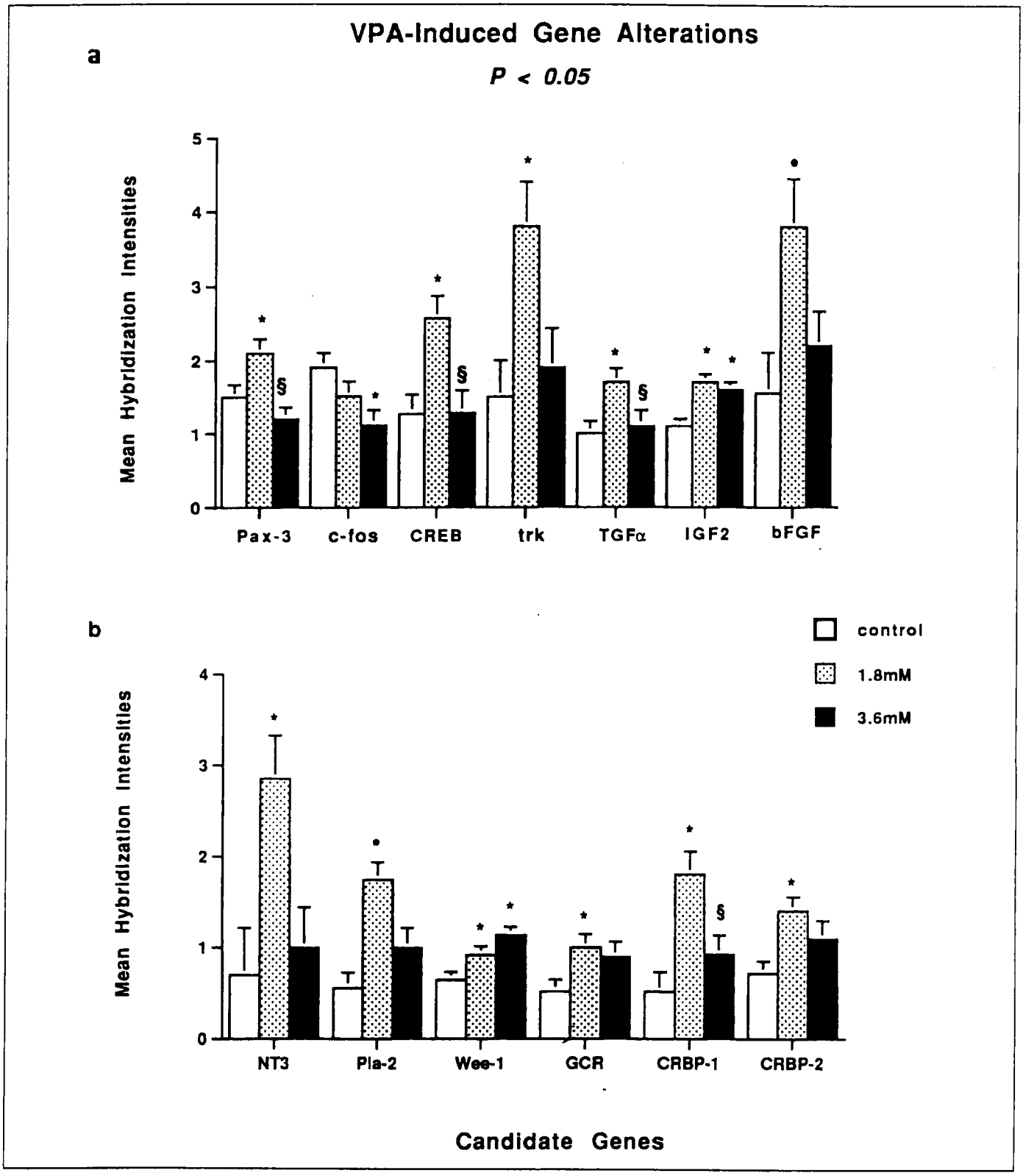

Figure 4. The mean expression level values, under control (untreated, $n = 8$) or VPA-treated (1.8 mM, $n = 7$; 3.6 mM, $n = 7$) conditions. Expression values are reported as the mean hybridization intensities relative to actin, our internal standard. (a) Expression values for transcription and growth factor genes. (b) Expression values for genes encoding neurotrophic factors, enzymes, cell cycle factors, receptors and binding proteins. * Significant differences from control values ($P < 0.05$); § Significant differences between the mean expression values for the two treatment concentrations ($P < 0.05$).

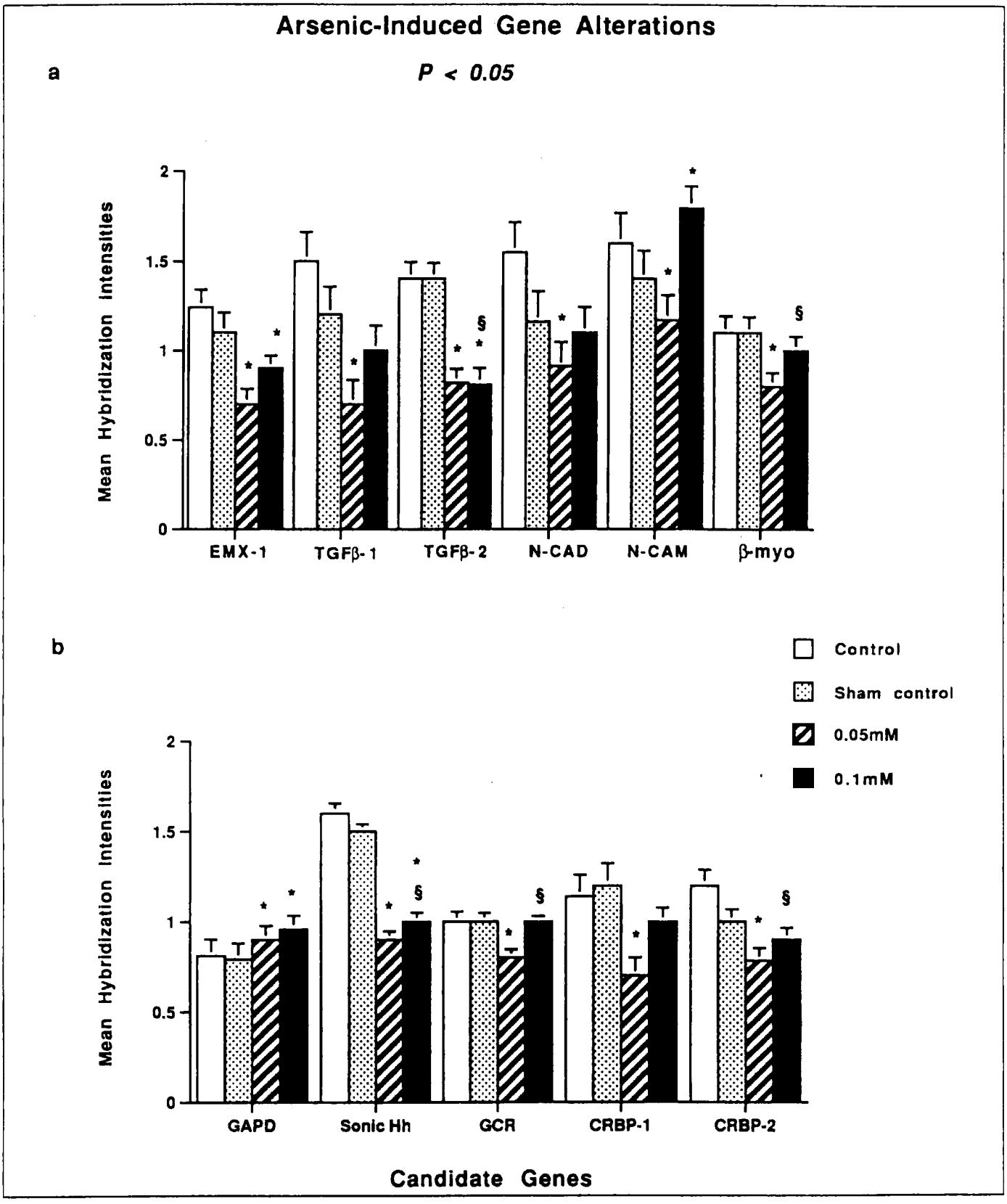

Figure 5. The mean expression level values, under control (untreated, $n = 6$), sham-injected control ($n = 7$), or arsenate-treated (0.05 mM, $n = 9$; 0.1 mM, $n = 9$) conditions. Expression values are reported as the mean hybridization intensities relative to actin, our internal standard. (a) Expression values for transcription, growth factors and cytoskeletal genes. (b) Expression values for enzymes, segment polarity genes, receptors and binding proteins. * Significant differences from control values ($P < 0.05$); § Significant differences between the mean expression values for the two treatment concentrations ($P < 0.05$).

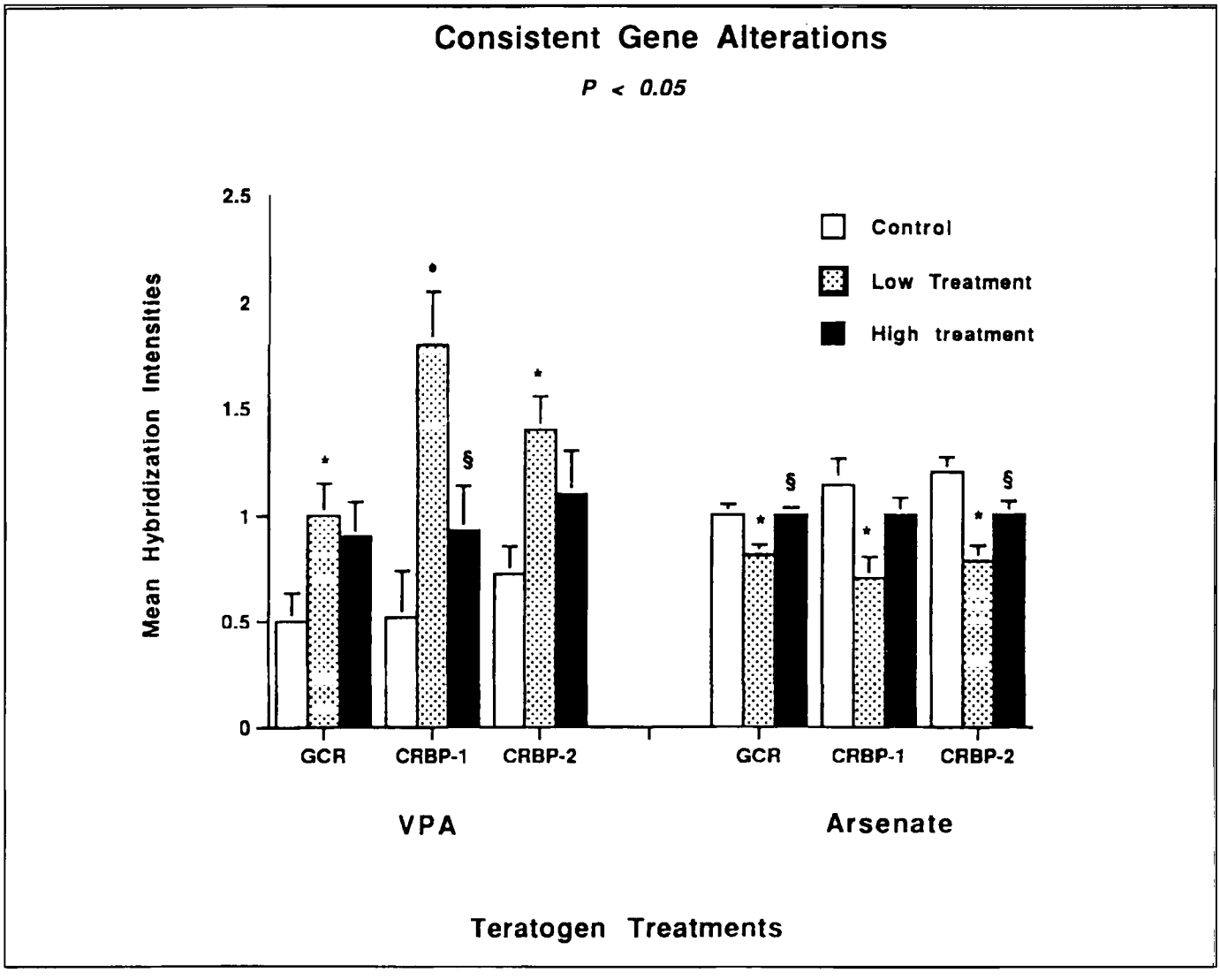

Figure 6. Consistent alterations in gene expression induced by sodium valproate (VPA) (left side) or arsenate (right side). Gene expression values, under control (untreated, $n = 8$ for VPA; total $n = 13$ for combined arsenate controls) VPA (1.8 mM, $n = 7$; 3.6 mM, $n = 7$) or arsenate (0.05 mM, $n = 9$; 0.1 mM, $n = 9$) treated conditions, are reported as mean hybridization intensities relative to actin, our internal standard. * Significant differences from control values ($P < 0.05$); § Significant differences between the mean expression values for the two treatment concentrations ($P < 0.05$).

which accumulates in the murine embryo, and induces neural tube and other congenital defects (Nau 1985, Loscher 1992). The experiments in the present study demonstrated that comparable dose levels of VPA (1.8 mM and 3.6 mM) induced congenital malformations in the *F. heteroclitus* embryos that were similar to those reported in murine embryos. Arsenate is also a well established teratogenic agent in laboratory animals, producing significant structural defects in murine embryos when administered at doses that result in maternal blood concentrations of 0.05–0.1 mM (Morrissey and Mottet 1983, Hood *et al.* 1987, Copp *et al.* 1990). The experiments presented here demonstrated that these same levels of arsenate (0.05 mM and 0.1 mM) also adversely affected *F. heteroclitus* development, and did so in a manner very similar to that seen in mice.

The most common response to VPA or arsenate was a delay in growth and development, which affected approximately 90 % of the surviving embryos. Since normal embryogenesis relies upon a precisely coordinated series of morphogenic events that are temporally and spatially regulated, developmental delay has been proposed as a teratogenic mechanism common to many different compounds. While the mechanism(s) by which developmental delay is induced remains unclear, one recently proposed hypothesis ('Embryonic Stress Hypothesis') contends that the induction of the heat shock, or stress response in embryos causes a delay in the transcription and translation of essential genes at critical developmental timepoints which can lead to congenital malformations (German 1984). However, this hypothesis does not adequately explain all teratogens, such as cortisone, which can induce a developmental delay without activating a heat shock response, and dinitrophenol, which can induce a

developmental delay without altering normal development (Gibson 1973). Therefore, it is important to delineate the specific, and more subtle, molecular alterations that lead to both developmental delay and congenital defects, in order to develop an effective screening system for identifying biomarkers of teratogenicity.

In the current study, the most common VPA-induced malformation was a lesion in the developing neural tube, usually located immediately caudal to the hindbrain (Figure 2). In addition, there was an abnormal curvature of the spinal cord in the posterior region of many of the affected embryos. Congenital defects secondary to *in utero* VPA exposure in humans include a multitude of craniofacial anomalies, as well as spina bifida (Nau and Loscher 1986, Loscher 1992, Padmanabhan and Hameed 1994). Experimental studies on rhesus monkeys (Mast *et al.* 1986) mice (Kao *et al.* 1981, Nau and Loscher 1986, Finnell *et al.* 1988, Sonada *et al.* 1993, Padmanabhan and Hameed 1994), rats (Ong *et al.* 1983) and rabbits (Whittle 1976) have shown that VPA is teratogenic, inducing craniofacial, skeletal, renal, neural tube and cardiovascular defects, as well as growth retardation. The most common defect reported following *in utero* exposure to VPA is exencephaly, which occurs in 14–44 % of exposed murine embryos (Finnell *et al.* 1988, Padmanabhan and Hameed 1994, Sato *et al.* 1995). This may be due to the proclivity of VPA to preferentially accumulate in the embryonic neuroepithelium (Dencker *et al.* 1990). Congenital abnormalities due to *in utero* arsenate treatment also include exencephaly, which occurs in 65 % of exposed murine embryos (Casarett and Doull 1980, 1996, Morrissey and Mottet 1983, Copp *et al.* 1990). Other arsenate-induced effects include microvascular injury (Tseng 1977), skeletal malformations, decreased growth and increased mortality (Morrissey and Mottet 1983). In rats and hamsters, renal agenesis has also been reported in response to teratogenic concentrations of arsenate (Hood *et al.* 1987).

Exposure of *F. heteroclitus* embryos to teratogenic concentrations of either VPA or arsenate produced a significant alteration in the expression of several genes within our initial genetic expression profiling study. Of the 47 genes analysed, VPA and arsenate significantly altered the expression of 13 and 11 genes, respectively. Interestingly, the lower doses of either VPA or arsenate (1.8 mM or 0.05 mM, respectively) elicited the greatest response among most of the altered genes (Figure 4(a and b), Figure 5(a and b)). The high treatment doses of either VPA or arsenate (3.6 mM or 0.1 mM, respectively) failed to significantly effect all but a small subset of these genes. One possible explanation for the differing dose effects could be related to the ability of these compounds to induce metallothionein (MT) (Keen *et al.* 1995). Once induced, MT is capable of scavenging cytoplasmic zinc (and other heavy metals), leading to embryonic zinc deficiencies and consequent disruption of zinc-dependent processes. Such disruptions may compromise essential housekeeping functions, including: nucleic acid metabolism, gene transcription, protein synthesis, tubulin polymerization, oxidative defence systems, and enzyme functions. Disruption of these vital processes may negatively impact normal embryonic development (Keen *et al.* 1995). The high concentrations of either compound might be sufficient to induce MT without stimulating observable effects on transcription by operating through another mechanism. For example, VPA has been shown to interfere with folate metabolism to produce neural tube defects (NTDs) among exposed foetuses (Nau 1994). The folate pathway is integral to *de novo* nucleic acid biosynthesis and occurs in any cell that is undergoing replication and division (Goodman and Gilman 1993). Disruption of folate metabolism by VPA is thought to result in elevated tetrahydrofolate (THF) levels, and may cause a transitory increase in available nucleotides via an increase in thymidylic acid. Enhanced *de novo* nucleic acid biosynthesis may ultimately cause an over-utilization of the cell's available nucleic acid stores. The high VPA dose may, therefore, cause the expected initial increase in nucleic acids via perturbation of folate metabolism, and induce MT simultaneously. If this were to occur, transcriptional effects would not be expected at stage 16 (owing to the short time interval between VPA dosing and embryo sampling) even though nucleotide levels may be high. Once MT induction has occurred, however, zinc levels will decrease, causing a debilitation of zinc-dependent transcriptional processes. Therefore, the transcriptional effects would be observed only after the nucleotide stores are depleted and cellular zinc has been eliminated. Consequently, the high VPA dose may be setting the stage for ensuing transcriptional alterations following a longer exposure period. Sampling at later timepoints may bear out transcriptional kinetics of these alterations. In contrast, the low levels of VPA used in these experiments may not be sufficient to induce MT. Therefore, zinc levels may not be disturbed and VPA can freely associate with the nucleus to directly perturb normal gene expression, resulting in the disruption of normal development.

In the case of arsenate, the high concentration and longer exposure time prior to sampling may be sufficient to induce MT. At the high dosage, MT may preferentially scavenge arsenate, thereby rendering this compound biologically inactive within the nucleus, and free to damage cellular membranes upon entry. Like the low VPA dose, the low arsenate dose may fail to induce MT, allowing the compound free nuclear access.

Consequently, at the early sampling stages and the lower doses of VPA or arsenate, abnormal embryonic development might be the result of perturbations of gene transcription and normal cytosolic events, while abnormalities seen in response to the high doses of either compound may be primarily the result of cellular damage. While we currently have insufficient data to support this hypothesis, it is interesting to contemplate the possible roles cellular metabolism and physiology play in the embryotoxicity of these compounds.

In an effort to streamline current screening efforts, one goal of this study was to identify a set of developmental genes with altered expression in response to a number of different teratogens. Of the 47 genes examined in this study, only three, cellular retinal binding proteins (*CRBP-1* and *CRBP-2*), and the glucocorticoid receptor (*GCR*), were significantly affected by both of the test compounds (Figure 6). These genes are known to play diverse roles in embryogenesis. The role of the CRBPs is to bind and sequester retinol in the plasma for its ultimate

conversion to retinoic acid (RA) and to transport it across the placental membrane (Kraft *et al.* 1989). In this manner, the amount of RA that reaches the embryo is strictly regulated. Because RA is an important morphogen during embryogenesis, any excess or deficiency in embryonic RA levels could compromise normal embryogenesis and lead to such NTDs as exencephaly, spina bifida, and microcephaly (Copp *et al.* 1990), as well as malformations of the heart, brain and thymus (Casarett and Doull 1996). Therefore, the observed perturbation in the *CRBP-1* and *CRBP-2* mRNA levels (either up- or down-regulated) could result in disproportionate CRBP protein levels, which may adversely alter the available free pools of cellular and nuclear RA. An alteration of RA levels in the *F. heteroclitus* embryo might subsequently contribute to the observed neural tube, notochordal and cardiac malformations, as has been demonstrated in murine models (Theodosis and Fraser 1978, Copp *et al.* 1990).

It is known that GCRs participate in the regulation of cellular proliferation events and induction of differentiation processes in neuroepithelia (Bohn 1980, Fuxe *et al.* 1994), retinal tissue (Vardimon *et al.* 1993, Flint *et al.* 1994) and cardiovascular tissues (Kalinyak *et al.* 1987) by activating the expression of steroid specific genes involved in cellular growth control (Doppler *et al.* 1989). Normal activation of GCRs during development contributes to the coordinate changes in cellular growth and differentiation of target tissues, resulting in the regulated changes during development and maturation (Bohn 1980). These processes are accomplished via interactions of GCR with components of AP-1 (c-Fos and c-Jun) that together modulate the expression of such important housekeeping genes as: transcription factors, growth factors (Weinberg 1985), and steroid hormone receptors (Funder 1993, Pearce and Yamamoto 1993). Such signalling modulations in gene activity are essential for normal development and for physiological homeostasis (Miesfeld *et al.* 1986). While the mechanism of modulation is presently unknown, it is currently thought that GCRs enhance transcription from the glucocorticoid responsive element (GRE) when AP-1 is composed of c-Jun homodimers. Alternatively, GCR represses transcription when AP-1 is composed of c-Jun/c-Fos heterodimers, and lacks activity in an intermediate ratio of c-Jun to c-Fos (Funder 1993). In the current study, the level of *GCR* expression was significantly increased in response to the low VPA treatment, while *c-fos* expression levels were significantly decreased. Alternatively, the level of *GCR* expression is significantly decreased in response to the low arsenate treatment, while *c-fos* and *c-jun* expression levels are unchanged. According to the above reports, either situation might lead to enhanced transcription of steroid specific downstream genes. Such ectopic gene expression during precisely timed developmental stages could have an adverse impact on target tissues resulting in the observed abnormalities. In addition, prolonged exposure of these embryos to either compound could affect GCR synthesis over time, as glucocorticoids are known to affect growth and protein synthesis (Svec 1985). Alterations in GCR levels could result in a loss of maximum biological activity in target tissues.

The inconsistent gene expression patterns in response to the low doses of VPA or arsenate that produce similar morphological outcomes may be further explained by the mechanistic actions of these two compounds. For example, the low VPA concentration may be effectively interrupting folate metabolism, causing the transitory enhancement of *de novo* nucleic acid biosynthesis and the resulting augmentation of available nucleic acid stores. The biological response may be an immediate consumption of these reserves in transcriptional processes leading to an overall increase in gene transcription. Therefore, elevated transcription may be a direct effect of VPA at the low concentration.

Based on the nature of arsenate toxicity, the low dose of this compound may be reducing transcription of selected genes in several ways. Biochemically, arsenate can uncouple oxidation and phosphorylation during glycolysis and the citric acid (or TCA) cycle, leading to decreased production of ATP and an overall energy imbalance. These imbalances could lead to decreased transcription and teratogenesis by adversely effecting such downstream events as normal cellular growth and proliferation via disruption of cell cycle regulatory kinases. In addition, perturbation of nucleic acid synthesizing kinases could lead to down-regulation of genes at the transcription level via a decrease in nucleic acid pools necessary for DNA repair. For example, normal coordinated regulation of GCR activity is determined by availability of ATP (Svec 1985). Depletion of ATP could disrupt the regulation of GCR binding, resulting in cellular and morphological disturbances. At the nuclear level, arsenate causes DNA damage via chromosomal breaks and DNA binding (Casarett and Doull 1980, 1996). Together with the metallothionein hypothesis, these scenarios could potentially explain the decreased gene expression and accompanying abnormal development in response to the lower arsenate levels documented in the present study. The net result of exposure to the low concentrations of VPA or arsenate would be to adversely affect normal regulation of gene transcription, which may have significant and detrimental biological effects on the developing neural tube, heart and eye.

There are multiple ways in which the teratogenic action(s) of arsenate or VPA may exert their adverse effects in order to produce similar developmental endpoints, in spite of the differences in gene expression patterns. While the expression patterns of *GCR*, *CRBP-1* and *CRBP-2* are dissimilar in response to the test compounds, their altered expression is important in the strict sense that their hormonal regulation (in the case of *GCR*), or their morphogen transport capability (in the case of *CRBP-1* and *CRBP-2*) was altered in response to both teratogenic compounds. With either compound, the lower doses may be associating with the nucleus, causing alterations in gene transcription and resultant morphological abnormalities via different mechanisms. Conversely, the high doses of either compound may be overloading the system in some manner that prevents access of the compounds to the nucleus, leaving it to render cellular damage and eventually contributing to the development of morphological abnormalities.

In summary, these preliminary findings suggest that *F. heteroclitus* embryos together with IST/aRNA analysis may be a useful method of pre-screening potential teratogens. Because

of its relative low cost, rapid results and high sensitivity, this method is ideally suited as a pre-screen for reproductive toxicity among candidate compounds in their early stages of development. Those compounds which alter embryonic gene expression could be identified and reassessed for further development, prior to testing with whole, *in vivo*, mammalian systems. Moreover, the method can also be utilized to help discern molecular mechanisms involved in disrupting normal morphogenesis. The ability to quickly and economically screen potential teratogens will not only reduce the risk these compounds may pose to the general public, but at the same time suggest alternative formulations to alleviate potential reproductive toxicity. Future work will be directed at the parallel assessment of morphological and transcriptional alterations occurring later in *F. heteroclitus* development. It is clear that treatment with either VPA or arsenate alters gene expression. However, whether the observed abnormalities are causally related to the gene changes requires further investigation. Furthermore, because overlap among gene responses to either VPA or arsenate was anticipated, the predictability of the *GCR*, *CRBP-1* and *CRBP-2* responses must be confirmed by different test compounds. In addition, comparative validation studies utilizing a variety of known teratogens and non-teratogens (possibly harmless metabolites of the former) in both the fish and murine systems need to be performed. An expanded panel of candidate genes to develop a more robust screening assay is also essential. Such studies are currently in progress in our laboratory.

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