Gene expression domains as markers in developmental toxicity studies using mammalian embryo culture

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ABSTRACT We are examining the hypothesis that expression domains of developmental control genes may be informative markers in mammalian embryo culture studies of developmental toxicity. Expression domains might be altered directly by chemical exposure, or might reflect developmental abnormality prior to any overt morphological defect. Whole-mount *in situ* hybridization using digoxygenin-labeled RNA probes was used to monitor the regions of expression of *Hoxb-4*, *Pax-3* and *Emx-2*. These genes were selected because of their different restrictions within the developing CNS; *Hoxb-4* for its anterior margin in the hindbrain, *Pax-3* for its dorso-ventral pattern in the spinal cord, and *Emx-2* for being restricted to a portion of the forebrain. Valproic acid was used as a prototype developmental toxicant because of its known actions on neural tube closure and on segmentation. For patterns of expression, we made three comparisons, between: rat *in vivo* developed embryos and published descriptions for mouse; rat cultured and *in vivo*; control and valproate exposed. For these genes, there were no differences between domains of expression in rat and mouse, nor between rat cultured and *in vivo* embryos. In valproate-exposed embryos, some domains were spatially abnormal, for example *Pax-3* in the neural crest, but this was coincident with structural defects induced by the treatment. There was no indication, for these three genes, and this teratogen, that treatment caused any shifts in boundaries of expression, nor induced any ectopic domains, even though exposures induced overt malformation.

KEY WORDS: *expression domains, Hoxb-4, Pax-3, Emx-2, valproic acid, whole-mount in situ hybridization, embryo culture*

Introduction

Since a simple and reliable method of culturing mammalian embryos was devised by New and his colleagues (New *et al.*, 1973) a very large number of studies have used this approach to examine the effects of chemicals on development. Culture studies are of obvious benefit to mechanistic, metabolism and structure-activity investigations, but whole mammalian embryo culture is also a method of choice in screening for potential developmental toxicity (see Brown *et al.*, 1995). One restriction of screening by this method is the limited culture period, which means that only the early stages of organogenesis can be observed. Clearly, chemicals could induce developmental abnormality that would cause structural defects in late gestation, but have no morphological manifestation by the end of a culture experiment. One way to reduce this limitation may be to use sub-morphological markers of development.

Recent years have seen stunning progress in the identification of the molecular components that control development. In the early development of organs, regional specification is the result of tightly regulated spatial and temporal gene expression which precedes cellular and morphological change. Thus, gene expression domain boundaries, which can be monitored by *in situ* hybridization techniques, predict future pathways of normal or abnormal development. In a collaborative project, we are using whole-mount *in situ* hybridization to examine the use of expression domains as markers of chemically-induced developmental abnormality. Here, we present one section of that project, in which we have examined the effect of valproic acid on the expression domains of *Hoxb-4, Pax-3 and Emx-2*.

Valproic acid (VPA, 2-propyl pentanoic acid) was first identified as a teratogen by orthodox animal testing (Brown *et al.*, 1980) and subsequently was found to induce spina bifida (and other defects) in humans (Robert and Guibaud, 1982). VPA acts directly in whole embryo culture, at clinically-relevant concentrations (Kao *et al.*, 1981), and by manipulation of the period and level of exposure in

Abbreviations used in this paper: BCIP, 5-bromo-4-chloro-3-indolylphosphate; NBT, nitro blue tetrazolium; PBS, phosphate buffered saline; SSC, saline sodium citrate; VPA, valproic acid; r, rhombomere.

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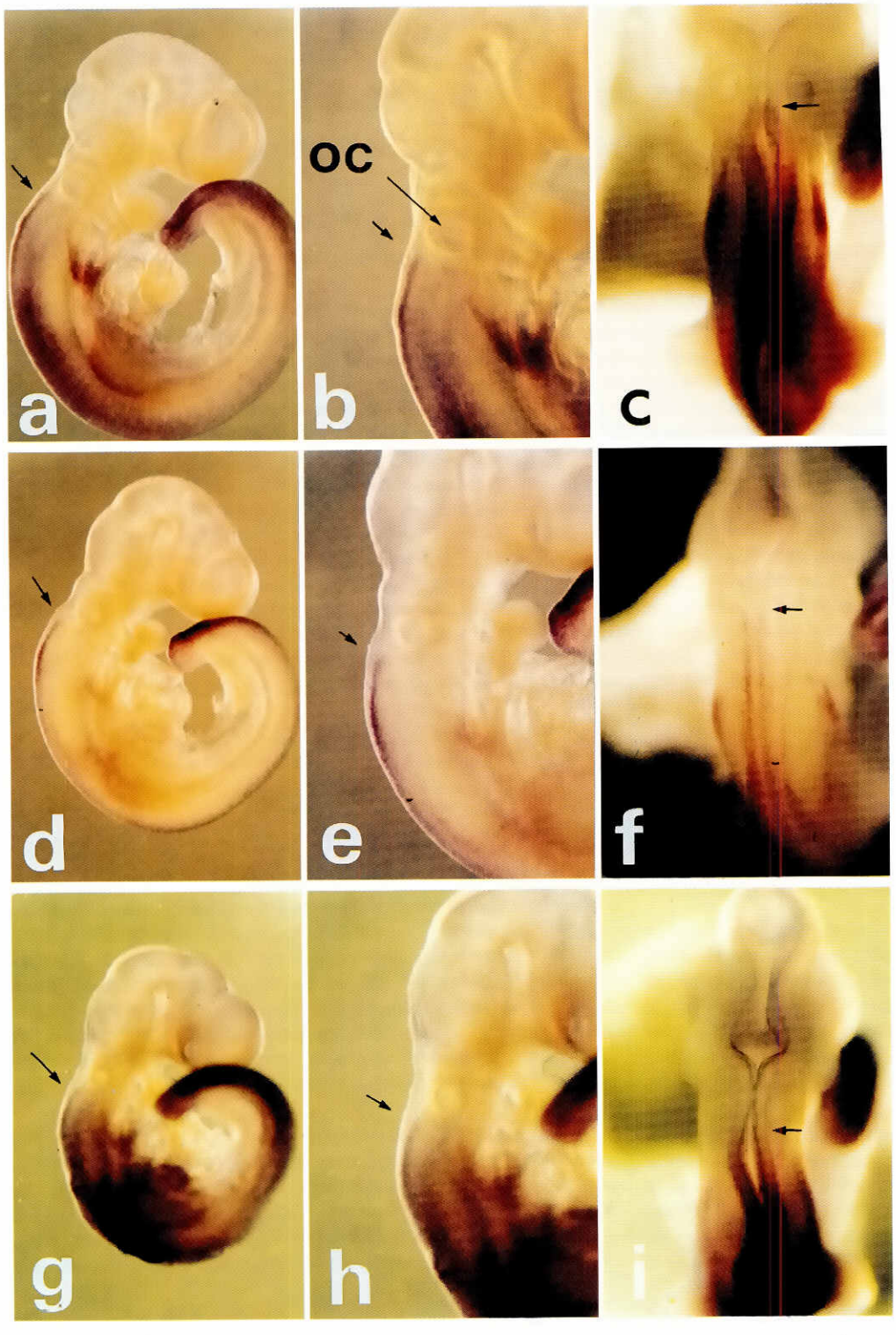

Fig. 1. Whole-mount *in situ* hybridization of *Hoxb-4* in 11.5 day rat embryos *in vivo* (a,b and c), cultured control embryos (d,e and f), and embryos exposed to 1.5 mM VPA for 16 h from 9.5 days (g,h and i). *Right lateral views at low (x13, a,d,g) and higher (x21, b,e,h) magnification. Dorsal views (c,f,i) at higher magnification only. The anterior boundary of expression in the neural tube is marked with a short arrow in each panel. The position of the otocyst (OC), overlaying rhombomeres 5 and 6, is marked with a long arrow in* **(b)**. *Note that expression extends from r6/7 posteriorly into the tail-bud in all embryos. Note also that the anterior margin is more posterior in mesoderm than neural tube.*

culture, the structural defects induced *in vivo* can be reproduced in culture (Brown *et al.*, 1991). The hallmarks of VPA-induced dysmorphogenesis in culture are open neural tube, irregular neural suture line, abnormal somite segmentation, and retarded otic vesicle closure (Kao *et al.*, 1981; Brown *et al.*, 1991). The molecular mechanism of VPA teratogenicity is unknown. Treatment of rodent embryos induces changes in membrane lipid metabolism (Brown *et al.*, 1991) and in folate metabolism, and folate supplementation can partially protect against VPA teratogenicity (Wegner and Nau, 1992), but the initial teratogenic insult remains obscure.

The selected genes each encode transcription factors that are expressed in restricted domains in the developing CNS. The *Hox* genes are well-known for their role in antero-posterior specification. *Hoxb-4* has an anterior margin of expression at the boundary

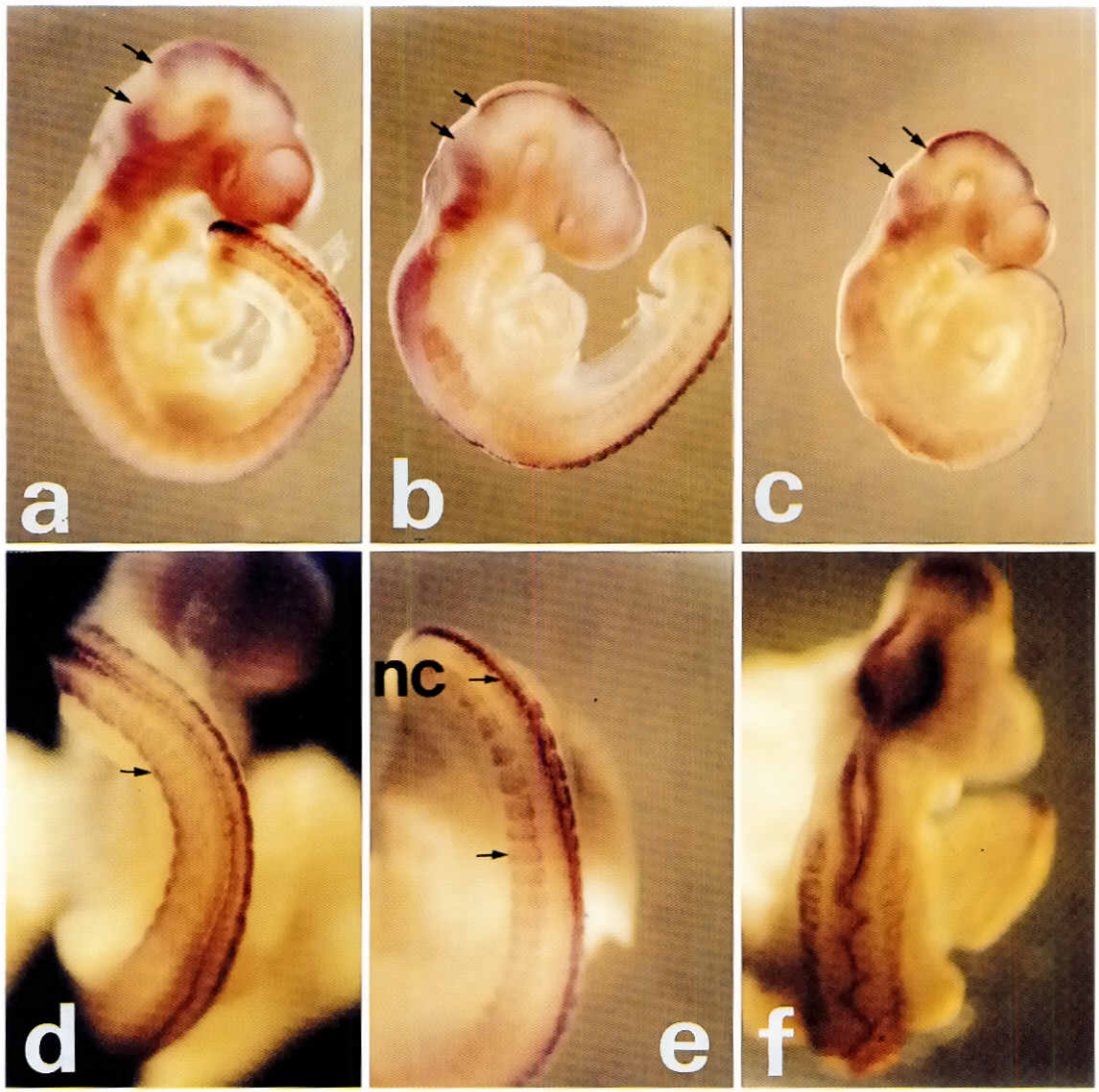

Fig. 2. Whole-mount *in situ* hybridization of *Pax-3* in 11.5 day rat embryos *in vivo* (a,d), cultured control embryos (b,e), and embryos exposed to 1.5 mM VPA for 16 h from 9.5 days (c,f). *Right lateral views* **(a,b,c)** *at low (x13) magnification. Dorsal views* **(d,e,f)** *at higher (x21) magnification of trunk region in* **(d,e)** *and cranial region in* **(f)**. *Double arrows in* **(a,b and c)** *mark a region of apparent non-expression in the hind-brain. Expression becomes greater in the more lateral edges of the somites, compare arrows in* **(d and e)**. *Neural crest (nc) expression is marked by an arrow in* **(e)**. *Note the pronounced zig-zag neural suture line in the VPA-treated embryo in* **(f)**.

between rhombomeres (r) 6 and 7 in the hindbrain. Mice with a null mutation in *Hoxb-4* have homeotic transformations in the cervical region (Ramirez-Solis *et al.*, 1993). The paired box gene *Pax-3* is expressed along the length of the developing spinal cord, in the dorsal-most neural tube that gives rise to neural crest cells (Goulding *et al.*, 1991). Mutations in the mouse *Pax-3* gene result in the *Splotch* phenotype, with abnormalities of neural tube closure, and in trunk neural crest derivatives (Tremblay *et al.*, 1995). One form of human Waardenburg syndrome is due to a *Pax-3* mutation (Tassabehji *et al.*, 1995). The homeobox gene *Emx-2* is related to the *Drosophila* gene *empty spiracles* and is expressed in a discrete domain of the anterior-most neural tube that gives rise to part of the fore-brain (Simeone *et al.*, 1992a).

Results

Morphology

As has been demonstrated in many previous studies, the morphology of 9.5 day rat embryos cultured untreated for 48 h was very like that of embryos developed *in vivo* (not shown). Control embryos had 24-28 somite pairs at the end of culture. All embryos exposed to 1.5 mM VPA for 16 h from 9.5 days, and examined at 48 h, showed characteristic morphological abnormalities (Kao *et al.*, 1981; Brown *et al.*, 1991). Wavy neural suture line and irregular somites (size and/or shape) were present in 100% of exposed embryos, whilst unclosed otic vesicles were observed in approximately 50%. VPA-exposed embryos were also smaller than untreated controls, again as previously described, although in this study we did not collect objective size measurements. At least 20 embryos were processed for *in situ* hybridization for each treatment group and for each gene. Representative embryos are shown in Figures 1,2 and 3.

Hoxb-4

In rat embryos *in vivo*, *Hoxb-4* was expressed from the tail bud posteriorly, throughout the neural tube, with an anterior margin just more caudal than the rim of the otocyst (Fig. 1a,b,c). In rat, as in mouse, the otocyst at this stage is adjacent to rhombomeres 5 and 6, suggesting that the anterior margin of *Hoxb-4* expression is at r6/7 in rat, as in mouse (Hunt *et al.*, 1991a). Expression was distinctly stronger in the most caudal tail bud, and was more caudal in mesoderm than in the neural tube. There were no observable

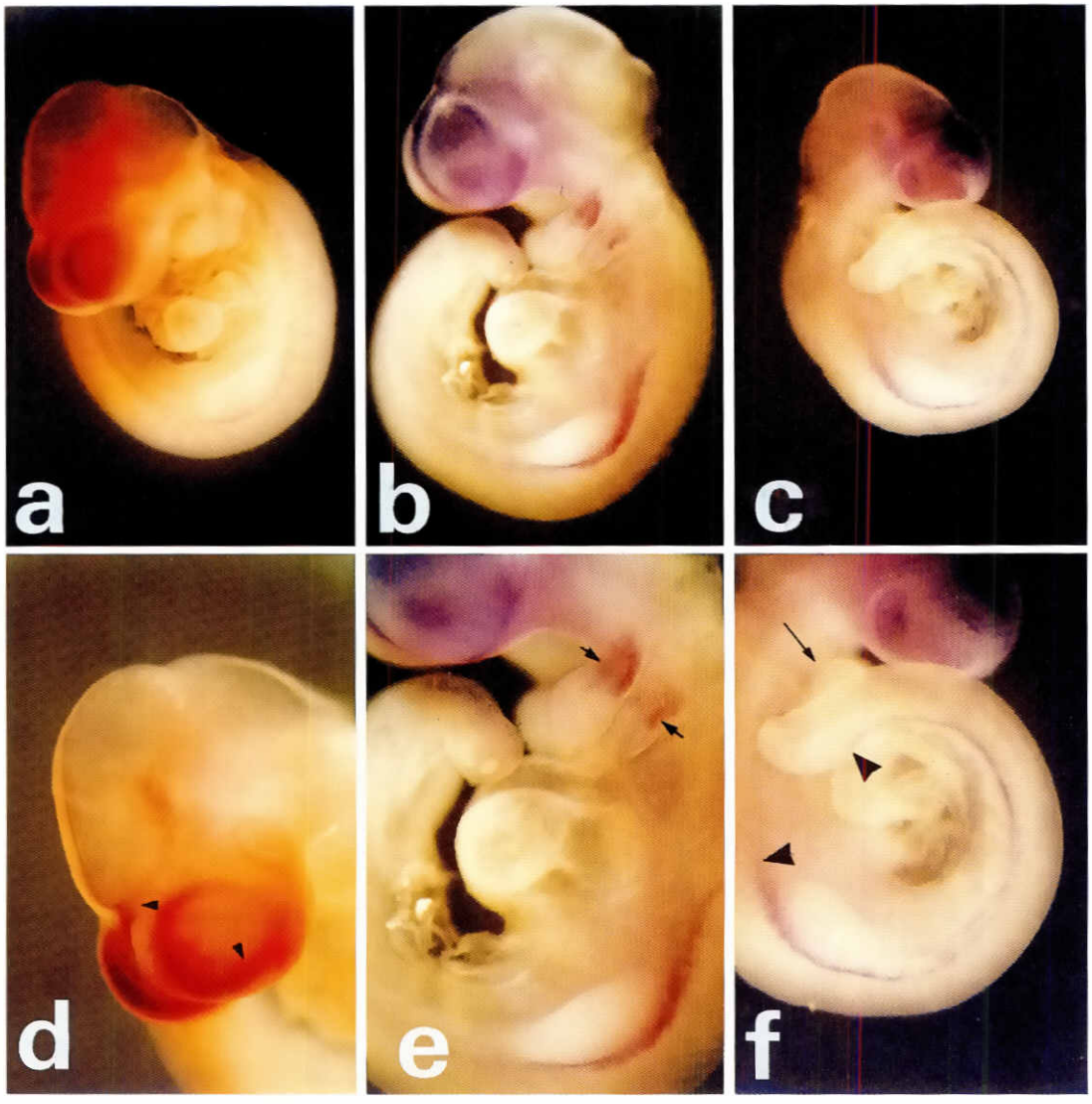

Fig. 3. Whole-mount *in situ* hybridization of Emx-2 in 11.5 day rat embryos *in vivo* (a,d), cultured control embryos (b,e), and embryos exposed to 1.5 mM VPA for 16 h from 9.5 days (c,f). *Left* **(a,b,d,e)** *and right* **(c,f)** *lateral views are at x13* **(a,b and c)** *and x21* **(d,e and f)** *magnification. Expression in the first (mandibular) and second (hyoid) pharyngeal arches is marked by arrows in* **(e)**. *Arrowheads in* **(f)** *mark the anterior (just rostral to the forelimb bud) and posterior boundaries of lateral expression, apparently in the Wolffian ridge. Also marked in* **(f)** *is the first pharyngeal arch, which is not yet expressing Emx-2 in this relatively retarded VPA-treated embryo.*

differences in this expression pattern in cultured compared to *in vivo* embryos (Fig. 1d,e,f). Similarly, there was no apparent differences in VPA exposed embryos, neither in the anterior margin; relative expression anterior to posterior; nor in neural tube compared to mesoderm. The anterior margin was preserved even when the suture line in the hind-brain region was markedly wavy (Fig. 1i). Characteristic features of VPA-induced abnormal development shown in Figure 1g,h, and i include irregular somites, a severely affected neural suture line, and an immature otic vesicle (the endolymphatic duct is not visible).

Pax-3

In rat embryos, *Pax-3* was most strongly expressed in the dorsal-most neural tube, in the trunk region, just parlateral of the neural suture line (*in vivo*, Fig. 2a; after culture, nc arrow in Fig. 2e). Expression was also clear in the somites, and within the cranial neural tube. In more advanced somites, expression is stronger in the ventro-lateral part (Fig. 2d arrow). Within the neural tube, there is an apparent region of none or low expression, with an anterior border at the mesencephalon/rhombencephalon boundary, and a posterior boundary within the rhombencephalon (arrows in Fig. 2), a region that may represent the cerebellum. The expression domains in neural crest, somites and cranial neural tube did not differ in *in vivo*, cultured control, and VPA-exposed embryos. The trunk neural crest expression highlighted the wavy neural suture in some VPA-treated embryos (Fig. 2f), but even in these embryos, expression domains did not appear to be ectopic or otherwise abnormal. The VPA-treated embryo shown in Figure 2c and 2f shows a severely irregular neural suture line, irregular somites, and an immature otic vesicle.

Emx-2

In rat embryos at 11.5 days, *Emx-2* was expressed in the forebrain, pharyngeal arches and in the lateral body (Fig. 3). In the forebrain, there was a boundary at the mesencephalon/ prosencephalon (upper arrowhead in Fig. 3d) border, with strong expression in both the posterior (diencephalic) and anterior (telencephalic) parts of the dorsal prosencephalon, and lesser expression in the ventral prosencephalon (lower arrow in Fig. 3d). In more advanced embryos, there was superficial expression in pharyngeal arches 1

(mandibular) and 2 (hyoid), in their most lateral parts (small arrows in Fig. 3e). Another superficial domain of expression ran in a lateral line from just anterior to the fore-limb bud to within the region of presomitic mesoderm (arrowheads in Fig. 3f). This appears to be within the ectodermal ridge sometimes called the Wolffian ridge. Once again, expression domains in all areas did not differ in *in vivo*, cultured control and VPA-exposed embryos. The VPA-exposed embryo in Figure 3 (c and f) does not have the pharyngeal arch expression domain, but this is stage-related, not treatment-related. The wavy neural suture line characteristic of VPA treatment can be observed in Figure 3 (c and f).

Discussion

The expression domains of *Hoxb-4*, *Pax-3*, and *Emx-2* in the rat embryo at 11.5 days, as we have observed by whole-mount *in situ* hybridization, are wholly compatible with those previously reported for equivalent stages of the mouse. In addition, the probes used were generated from mouse cDNA clones, demonstrating effective cross-hybridization. Although not previously reported for these genes, these similarities are to be expected, given the known evolutionary conservation of sequences for developmental control transcription factors, and the closeness of these two species. We have also shown that the expression domains of these genes are indistinguishable in embryos cultured for 48 h from neural plate stages, compared to *in vivo* embryos. Again, this has not been demonstrated previously, but is also unsurprising, given that morphological development in culture over this phase parallels that *in utero* very closely. Although some pathways of intermediary metabolism differ in culture and *in vivo* (e.g. Brown *et al.*, 1991), the explanation is likely to be the differing nutritional environments, and there are few indications of any fundamental differences in developmental processes *in vivo* and in culture.

Only one chemical, retinoic acid, has been extensively studied for its ability to alter the domains of gene expression in mammalian embryos, both *in vivo* and in culture. Exogenous retinoic acid has been shown to alter expression patterns, either shifting normal margins of expression, inducing ectopic expression, or abolishing usual domains (e.g. Hunt *et al.*, 1991b; Morriss-Kay *et al.*, 1991; Conlon and Rossant, 1992; and many others). However, retinoic acid should not be used as an example of a typical developmental toxicant, because of its role as an endogenous signaling molecule in many developmental processes. There is little indication that any other developmental toxicant can directly modulate developmental gene expression, although few have been explicitly tested for such effects. Our studies are the first to examine, systematically, chemical effects on expression domains in whole mammalian embryos in culture. Our expectation was not that chemicals might affect expression directly, but that domains would act as markers of submorphological populations of cells. In addition, and of great current interest, is the possibility that developmental toxicants might mediate their effects by affecting endogenous retinoid status, and thereby influencing gene expression (Williams *et al.*, 1996).

The main embryonic tissues affected by VPA in culture have been well described previously (Kao *et al.*, 1981; Brown *et al.*, 1991) and confirmed here: the neural tube, particularly the neural suture line; somites; and the otic primordium. The genes studied here were selected with these effects in mind. *Hoxb-4* marks anterior-posterior positional specification within the neural tube (and paraxial mesoderm), with an anterior boundary just posterior to the otocyst (Graham *et al.*, 1988). *Pax-3* marks the neural crest and somites, with initial expression in dorsal neuroepithelium, prior to expression in the roof and alar plates, dorsal neural tube and spinal neural crest (Goulding *et al.*, 1991). In paraxial mesoderm, *Pax-3* is evenly distributed over newly formed somites, then becomes restricted to the most ventrolateral and caudal portions of each somite (Bober *et al.*, 1994). *Emx-2* marks rostral neural tube, pharyngeal arches and the lateral ectodermal (Wolffian) ridge (Simeone *et al.*, 1992a). It is possible that this latter region partially corresponds with the coelomic epithelium covering the mesonephric column, which is *Emx-2* positive in the mouse (Simeone *et al.*, 1992a).

We observed two types of expression alterations in VPA-treated embryos, associated with either retardation or structural abnormality. The spatial distribution of transcripts is very dynamic, so patterns change with only small increments of developmental stage. Since VPA causes general retardation, we observed several examples of delayed expression. For example, the redistribution of *Pax-3* expression towards the ventro-lateral portion of somites was often not apparent in VPA-treated embryos. This is reminiscent of aspects of *Pax-3* expression in *Splotch* mutant embryos (Bober *et al.*, 1994). Similarly, VPA exposed embryos often lacked *Emx-2* expression in the pharyngeal arch region. This area of *Emx-2* expression was first apparent in control embryos at about the 25 somite stage, which was often not reached by VPA-treated embryos. We observed no obvious heterochrony in VPA-treated embryos, that is abnormal synchrony between morphological and molecular landmarks, but we did not examine this objectively.

VPA-treated embryos with structural defects showed spatially abnormal expression domains in the affected tissues. This was most dramatic in the dorsal neural tube, in which expression of *Pax-3* in the neural crest graphically illustrated the irregularity of the neural suture line induced by VPA. Similarly, there were overt changes in the shape of the head in VPA-treated embryos, which were reflected in differing areas of *Emx-2* expression in the forebrain. In all cases, however, the domains faithfully reflected the normal populations of expression, even though these were within an abnormal structure. In addition, in those VPA embryos of usual structure, domains were normal.

The absence of any other observable effects in this study does not mean that the overall approach of using gene expression as a marker in developmental toxicology is necessarily uninformative. We have examined only one prototype toxicant and three genes. It is certain that developmental toxicants exert their effects by many differing mechanisms, so other chemicals may affect the expression domains of other genes. Perhaps the most likely explanation of the lack of effect of VPA in this study is that the chemical acts 'downstream' of the genes studied. These genes are involved in initial cell specification events, so perhaps VPA alters later morphogenetic processes, such as proliferation, adhesion, migration, and so on. A comprehensive systematic study, monitoring the expression domains of genes involved in these processes, would reveal whether this were the case, although alternative strategies such as differential screening would be more likely to identify responsive genes.

Although the whole-mount approach to *in situ* hybridization yields easily interpretable 3-dimensional patterns, objective comparison of embryos for subtle differences in margins of expression is not straightforward. More information can be obtained by sec-

tioning whole-mount embryos, but the preservation of histology is not good. It is possible that a more productive overall strategy is to perform orthodox *in situ* hybridization on serial sections. This allows for multiple probes to be used on a single embryo, and for adjacent sections to be used for histological reference. Our colleagues in the collaborative project are evaluating this strategy (Picard *et al.*, 1996).

The results in this study illustrate a strategy for monitoring the effects of teratogens during early development using whole-mount *in situ* hybridizations. This technique can be used to screen for early, subtle effects on mammalian development using expression domains as sub-morphological markers of specific cell populations. It may be possible to pinpoint the spatial and temporal targets of developmental toxicants in embryos with this approach.

Materials and Methods

Embryo culture techniques were carried out as described previously (Brown *et al.*, 1991). In brief, Wistar rat conceptuses were explanted in the afternoon of the 10th day of gestation (plug= 1), at the early neural plate stage, with 0 to 3 somites. These were cultured for 48 h (4 conceptuses per 4 ml medium) in 50 ml culture bottles, on rollers at 38°C. The medium culture was 75% immediately centrifuged, heat-inactivated male rat serum, 25% Eagle's minimal essential medium with Earle's salts. Embryos are gassed initially with 5% O_2, changed to 20% at 16 h and 40% at 26 h, with 5% CO_2 and the balance N_2.

Our strategy was to begin with an exposure that caused overt structural defects, for which we chose 16 h at 1.5 mM. If alterations in domains were observed, we would move to lower exposures without morphological effect. VPA (as the sodium salt) was dissolved in MEM, then diluted to a medium concentration of 1.5 mM. Embryos were cultured in this medium for 16 h, washed 3 times, then cultured in fresh control medium for the remaining 32 h.

Embryos from *in vivo* and *in vitro* sources were washed in PBS and fixed in 4% (w/v) paraformaldehyde overnight. Whole-mount *in situ* hybridization was performed essentially as described by Wilkinson and Green (1990). Digoxygenin-labeled antisense RNA probes were synthesized from the following cDNA fragments: *Pax-3* (519bp Pst1/HindIII fragment, Goulding *et al.*, 1991), *Hoxb-4* (1200bp BamHI/HindIII fragment, Ramirez-Solis *et al.*, 1993), *Emx-2* (600bp HindIII/EcoRI fragment, Simeone *et al.*, 1992b). RNA probes were added to hybridization mix before overnight incubation with embryos at 70°C. After a series of washes in formamide/SSC the embryos were incubated again overnight with anti-digoxygenin antibody and stained up in the presence of NBT/BCIP. There were noticeable differences in the intensity of staining between embryos, however, there was similar variation in all groups, so we do not consider these differences to be related to culture or valproic acid exposure.

Acknowledgments

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