TERATOLOGY 54:284–297 (1996)

Valproic Acid-Induced Changes in Gene Expression During Neurulation in a Mouse Model

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ABSTRACT The teratogenic potential of valproic acid has been well established both in experimental models and in human clinical studies. As with all human teratogens, there are genetically determined differences in individual susceptibility to the induction of congenital defects. Using a mouse model of valproate-induced neural tube defects, a study was undertaken to examine differential changes in gene expression for selected transcription factor (*Pax-3, Emx-1, Emx-2, c-fos, c-jun, creb*) and cell cycle checkpoint genes (*bcl-2, p53, wee-1*) during neural tube closure. In general, exposure to teratogenic concentrations of valproic acid elicited GD 9:12 control levels of transcription factor mRNA expression in GD 9:0 embryos of both strains. This accelerated developmental profile is marked by significant elevation of *Emx-1, Emx-2, c-fos, c-jun,* and *creb* expression. There was also a significant overexpression of the cell cycle genes *p53* and *bcl-2* in the LM/Bc embryos in response to the teratogenic insult. Examination of the ratio of expression of these genes clearly favored *bcl-2*, which supports the hypothesis that altered neuroepithelial cell proliferation rates, rather than increased apoptosis, is the underlying mechanism by which valproic acid alters normal neural tube morphogenesis. An investigation into interactive effects of these genes on the molecular profile of GD 9:0 embryos further validated this observation. That is, the overall proliferative state among the control embryos was prematurely modified into a more differentiated state following teratogenic insult. These results suggest that alterations in the expression of multiple genes are most likely responsible for valproic acid-induced neural tube defects. *Teratology 54:284–297, 1996.* © 1997 Wiley-Liss, Inc.

Teratogens are believed to act in a number of different pathways exploiting multiple targets and mechanisms to alter normal embryogenesis (Wilson, '77). Relatively little is known about the consequences of a teratogenic insult on the molecular homeostasis of the developing embryo. Such information is only now being rapidly acquired. What is apparent from a number of different experimental systems is that a teratogenic insult can disrupt important developmental processes controlled by multiple genes under an elaborate regulatory structure. One approach that has been successfully applied to investigate complex developmental events and their coordinate genetic regulation has been to disrupt normal morphogenesis using specific teratogenic agents and then to analyze carefully the physiological and molecular consequences. We used this approach to examine the expression patterns for selected transcription factors and cell cycle checkpoint genes and attempted to correlate any changes in gene expression with observations at the morphological levels following teratogenic treatment with the anticonvulsant medication, valproic acid (Depakene, Abbott Laboratories, Abbott Park, IL). The period during which neural tube closure occurs was selected as the target developmental timeframe, as the well-documented morphogenetic events occurring during this period of development demands complex tissue organization within rigid temporal and spatial requirements, and underscores the complexity of the processes involved (Geelen and Langman, '77; Kaufman, '79; Macdonald et al., '89; Finnell, '91; Golden and Chernoff, '93).

Structurally unrelated to any of the other anticonvulsant medications, valproic acid (VPA) has a demonstrated teratogenic effect in a wide variety of animal species, including the mouse, rat, hamster, rabbit, and rhesus monkey (for review, see Nau and Hendrickx '87). The principal malformation associated with VPA exposure in utero in experimental animals has been neural tube defects (NTDs), including exencephaly and spina bifida. Nau ('85) observed exencephaly in mouse embryos from dams exposed to sufficiently high dosages of

Contract grant sponsor: National Institute of Dental Research, contract grant number DE 11303; contract grant sponsor: National Institute of Environmental Health Sciences, contract grant number ES07165; contract grant sponsor: NIH.

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Received 12 September 1996; Accepted 17 December 1996

VPA to produce maternal plasma concentrations in excess of 230 μg/ml, irrespective of the route of administration. This concentration represents a two- to fivefold increase over the recommended human therapeutic level (Niedermeyer, '83). Ehlers et al. ('92a,b) demonstrated that VPA administered (200 mg/kg, IP) 6 hr apart, beginning on gestational day 9, produced a 10% response frequency of spina bifida occulta, which increased to 95% affected fetuses concomitant with a VPA dosage increase to 500 mg/kg body weight. A significant degree of malformation of the ribs and vertebrae was apparent when the above-treated fetuses were examined following Alcian blue–alizarin red skeletal staining (Ehlers et al., '92a,b). A low frequency (4–6%) of spina bifida aperta was also induced by the same VPA treatment regimen in the Han:NMRI mouse strain, resulting in a highly disorganized and necrotic spinal cord within the vertebral canal in the lumbosacral region of the developing fetus. The absence of neuronal tissue in these affected fetuses indicates an almost complete localized ablation of the neural tube in the VPA-exposed fetuses (Ehlers et al., '92b).

Clinically, infants exposed in utero to VPA are at elevated risk of an NTD (Robert, '82; Robert and Guibaud, '82; Lindhout et al., '92). An estimated 2% of all infants exposed to VPA during early pregnancy will have spina bifida, a 20-fold increased prevalence over that observed in the general population (Lammer et al., '87). In addition to NTDs, a characteristic pattern of craniofacial abnormalities, including a flat nasal bridge with upturned nasal tip, thin vermilion borders, a shallow philtrum, and downturning mouth, has been described among valproate-exposed infants (DiLiberti et al., '84; Ardinger et al., '88). As the mechanism by which VPA induces NTDs is poorly understood, it remains difficult to predict which infants exposed in utero to the drug will develop spina bifida. Clearly, fetuses that have some genetically determined predisposition are at increased risk.

Efforts to learn more about the genetic basis of susceptibility to VPA-induced NTDs have relied on data obtained from murine model systems. Finnell et al. ('88) treated dams from several inbred mouse strains with a single IP injection of VPA (600 mg/kg/day) on gestational day 8:12, and observed a strain-dependent hierarchy of susceptibility to NTDs that paralleled the pattern of susceptibility observed for mice exposed to elevated intrauterine temperatures (Finnell et al., '86). That is, the SWV mice were very sensitive, with 35% of fetuses exencephalic, while the LM/Bc embryos were much more resistant (20% NTDs), and the C57BL/6J and the DBA/2J mice were completely resistant (Finnell et al., '88). The underlying basis of this strain difference was examined with respect to folate metabolism. Wegner and Nau ('91, '92) determined total folate and several folate metabolite concentrations in mice following teratogenic exposure to VPA. These investigators found a significant alteration in the concentrations of several formylated tetrahydrofolates, specifically the 5- and 10-formyl-THF, as well as the 5-CH3-THF metabolites. When these metabolites were measured in VPA-exposed embryos from the NTD-sensitive (SWV) or -resistant (DBA/2J) strains, a 86–92% inhibition of the 5-CHO-THF and 5-CH3-THF metabolites was found in the SWV embryos, while the DBA/2J embryos had no alterations in their 5-CH3-THF, and only a 50% inhibition in the 5-CHO-THF metabolite. The LM/Bc strain embryos were intermediate between the SWV and DBA/2J embryos both in susceptibility to VPA-induced NTDs and in their profile of metabolic inhibition (R.H. Finnell and H. Nau, unpublished data). This drug-induced alteration in folate metabolism could potentially result in a decreased rate of methylation of essential developmentally regulated genes during critical periods of embryogenesis. This would significantly curtail the expression of certain genes, which in turn might enhance the sensitivity of the embryos to specific malformations. Such a difference in the methylation patterns between embryos of different strains might explain their differential sensitivity to VPA-induced NTDs.

The present study was undertaken to monitor the expression level of a population of six transcription factor and three cell cycle checkpoint genes in SWV and LM/Bc embryos in order to (1) document changes in gene expression that occur over time in normal murine embryos during a period of rapid morphogenesis, (2) identify altered patterns of gene expression occurring subsequent to a teratogenic exposure to VPA that may contribute to the abnormal pathogenesis observed in embryos, and (3) determine a possible molecular basis for the strain difference in susceptibility to VPA-induced NTDs. The six transcription factor genes (*Pax-3, Emx-1, Emx-2,* c-*fos,* c-*jun,* and *creb*) selected for investigation are all believed to be centrally involved in directing or regulating a cascade of morphogenetic events critical to early embryogenesis. The cell cycle checkpoint genes examined in these embryos included *bcl-2, p53,* and *wee-1.* These genes are known to be important modifiers of cell proliferation and are developmentally regulated in the mouse embryo.

MATERIALS AND METHODS

Experimental animals

The highly inbred SWV and LM/Bc mouse strains were selected for these studies on the basis of their known differences in sensitivity to VPA-induced NTDs (Finnell et al., '88). The mice were maintained on a 12-h light cycle in the Laboratory Animal Resources and Research Facility at the College of Veterinary Medicine, Texas A&M University. The animals were pathogen-free with no apparent health problems. Up to five healthy females were housed per polycarbonate cage and were allowed free access to Wayne TekLad rodent chow and tap water. Virgin females, 40–60 days of age, were bred overnight to experienced males of their respective strain. Females were examined the next morning for the presence of vaginal plugs. The begin-

ning of gestation (day 0) was set at 10 PM of the previous evening, the midpoint of the dark cycle (Snell et al., '48).

Teratogen treatments

For the teratology studies, no less than five dams were randomly assigned to each treatment group and exposed to a single intraperitoneal injection of VPA (600 mg/kg) or the distilled water vehicle at GD 8.5. The drug was dissolved in distilled water immediately before use and administered in volumes of 0.1 ml/10 g body weight. Following treatment, the dams are returned to their home cages until the desired gestational day for the embryo collection. At the assigned hour (GD 8:18, 9.0, and 9:12), pregnant dams were killed by cervical dislocation, the abdomen opened, and the uterine contents removed. The location of all viable embryos or fetuses and resorption sites were recorded. Using watchmaker's forceps, the embryos were dissected free of the decidual capsule and its chorion and amnion while in cold phosphate-buffered saline (PBS), under a Wild M8 dissecting microscope (Heerbrugg, Switzerland).

Embryo collection and morphological staging

Embryos were collected and grossly examined morphologically to facilitate their classification on the basis of their stage of neural tube closure, using previously described standardized staging criteria (Cole and Trasler, '80; Macdonald et al., '89; Golden and Chernoff, '93). Embryos with obviously delayed neural tube closure, or completely open neural folds that clearly were developing NTDs, were selected from the teratogen-exposed litters for use in the in situ transcription studies. All control embryos for these experiments were developing normally toward the completion of anterior neural tube closure.

Removal of neural tube from neural tube closure stage embryos

For the gene expression studies, no less than three embryos per litter from a minimum of five litters per treatment group were used as the basis of the dataset. The embryos were collected as described above. With the aid of watchmaker's forceps, the neural tube proper was dissected away from supporting paraxial mesodermal tissue under the dissecting microscope (Taylor et al., '95; Wlodarczyk et al., '96). Once dissected free, it was examined to ensure that only the intact neural tube tissue had been collected, free from extraneous tissues. In preparation for the in situ studies, this tissue was placed in a hybridization buffer containing 5 mM DTT, 100 units RNasin (Promega, Madison, WI), and 0.1% digitonin. Following a brief pulse with a sonic dismembranator, an additional 50 units of RNasin was added to the buffer, and the tissue was frozen at −80°C until further processed.

In situ transcription and aRNA amplification

In situ transcription and antisense RNA amplification (RT/aRNA) procedures were performed according to methods that have been described in detail elsewhere (Eberwine et al., '92a,b; Taylor et al., '95; Wlodarczyk et al., '96). The initial steps involved making an RNA/DNA hybrid molecule from the population of mRNAs present in each of the isolated neural tube samples with the addition of avian myeloblast reverse transcriptase (Seikagaku America, Bethesda, MD), and an oligo-dT-T7 oligonucleotide primer that hybridizes to the poly-A tail of the mRNAs. The purified single stranded cDNA was then made double stranded by the Gubler–Hoffman method, the resulting hairpin loop was digested with S1 nuclease, and then blunt-ended using standard procedures. The cDNA template was further purified by a spin column procedure, and then used to produce radiolabeled, amplified, antisense RNA (aRNA) by the addition of T7 RNA polymerase (Epicentre Technologies, Madison, WI) in the presence of [^{32}P]-CTP. The aRNA, representing the entire population of mRNAs from the neural tube tissue, was then hybridized to "reverse" Northern blots in order to determine quantitatively the pattern of gene expression for the candidate genes selected for investigation (see below). This approach was selected over more conventional reverse transcriptase–polymerase chain reaction (RT–PCR) procedures, given the linear nature of the RT/aRNA amplifications.

Genetic expression profiling

Equimolar concentrations of the cDNA clones of the nine genes of interest (*Pax-3, Emx-1, Emx-2,* c-*fos,* c-*jun, creb, bcl-2, p53,* and *wee-1*) were immobilized on a nylon membrane (Zetaprobe, BioRad, Richmond, CA), along with an internal control cDNA (cyclophilin), using a BioRad slotting apparatus following the manufacturer's protocol. The resulting slot blots were hybridized with an aRNA probe using conditions detailed previously by Taylor et al. ('95). Following hybridization, the slot blots were washed at medium stringency down to 0.1× SSC containing 0.1% sodium dodecyl sulfate (SDS) at 42°C, dried, wrapped in plastic wrap, and placed in an Ambis 101 two-dimensional radioanalytics imaging detector (Scanalytics, Billerica, MA) that directly measured the radioactivity (CPMs) of each slot on the reverse Northern blots. The individual signals were normalized to cyclophilin gene expression. The selection of cyclophilin as the normalizing cDNA simply enables us to make comparisons between different blots, as the individual hybridization intensities of individual cDNA on each blot can be expressed as a ratio of its expression to cyclophilin. There is no particular significance to the selection of this gene, other than the fact that it is constituitively expressed and found in high abundance in the mouse embryo (Danielson et al., '88). This makes it an excellent internal standard for use in multiprobe assays when examining low abundance messages.

Basis for selection of candidate genes

The nine cDNA clones selected for this particular analysis encode three cell cycle genes (*wee-1, p53,* and

bcl-2) and six transcription factor genes (*Pax-3, Emx-1, Emx-2,* c-*fos,* c-*jun, creb*). The basis for selection of the cell cycle cDNA clones, as well as c-*fos,* c-*jun,* and *creb* was their documented interactive effects on epithelial cell differentiation and proliferation through the cell cycle. The remaining transcription factors, *Pax-3, Emx-1,* and *Emx-2* are known to play important roles in patterning during early embryogenesis. The selected cell cycle genes encode checkpoint proteins that operate at the G_2 (*wee-1*) or the G_1 (*p53* and *bcl-2*) cell cycle phases, and are involved in various aspects of assessment and regulation of DNA replication and repair (Ellege and Lee, '95). *wee-1* encodes a protein kinase that negatively regulates entry into mitosis by inactivation of the maturation promoting factor (MPF) (Davey et al., '95). Overexpression of *wee-1* inhibits the cellular transition from G_2 to mitosis in the cell cycle, ultimately leading to apoptotic cell death (Igarashi et al., '91; Davey et al., '95). At the G_1 cell cycle phase, it has been reported that the protein product of *bcl-2* mediates the growth-inhibiting and apoptotic effects of *p53* protein product (Miyashita et al., '94). All three of these cell cycle genes are expressed in a number of different tissues, especially in "epithelium-like" cells. *p53* is expressed in the developing neural tube and has important implications in orchestrating neural tube closure, via cell cycle regulation (Sah et al., '95), while *bcl-2* is normally highly expressed in fetal central nervous system (CNS) tissues, as well as in rapidly proliferating epithelial cells (Hockenberry, '95; Veis-Novak and Korsmeyer, '94).

The transcription factors *Pax3, creb, Emx-1,* and *Emx-2* serve as the initial triggers in a cascade of molecular events that result in changing the relative abundance of many responsive downstream genes. With the exception of *creb,* all these genes are involved in embryonic pattern formation. *Emx-1* and *Emx-2* are both homeobox-containing genes expressed in restriction regions of the developing forebrain, including the presumptive cerebral cortex (Simeone et al., '92). The products of these homeobox containing genes, namely homeodomain proteins, serve to regulate gene expression by directly binding to *cis*-acting DNA sequences (Breier et al., '88). The *Pax3* gene encodes a 56-kd protein that contains a paired-type homeodomain in addition to the paired box domain (Goulding et al., '91). Throughout neural development, *Pax3* expression was coincident with regions of the neural tube from which neural crest cells are known to arise and migrate.

c-*jun* has the general properties of most oncogenes in that it is involved in regulating either cellular proliferation or differentiation (Weinberg, '85). Specifically, c-*jun* encodes for a nuclear phosphoprotein that is a transcription factor, comprising a major component of the AP-1 transcription complex, which also includes the Fos protein (Angel et al., '88). These immediate–early-response genes are believed to play a vital role in cellular proliferation and the regulation of the cell cycle, as it was demonstrated that these genes are induced within minutes of a mitogenic stimulation (Kovary and Bravo, '91). These transcription factors bind to specific DNA consensus sequences known as AP-1 recognition sites, or TPA-response elements (Bohmann et al., '87). This binding enables the preferential binding of RNA polymerase, and thus the rapid transcription of specific genes that have bound these factors (Ptashne, '88). Taken together, the interassociations of these nine genes may aid in regulating cellular proliferation and establishing future embryonic axis patterning and polarity in the developing neural tube. Such interactions may have important implications for neuroepithelial cell function during neural plate folding and neural fold fusion, as well as organization of future CNS differentiation events.

Statistical analysis

The data for the statistical analysis were generated from LM/Bc/Fnn and SWV/Fnn embryos collected at GDs 8:12, 9:0, and 9:12, under control and VPA treatment conditions. All statistical analysis of these data was performed using SAS (Statistical Analysis System) and completed in three stages. The first of these stages involved morphological analysis of somite pairs and determination of neural tube closure stage frequency distribution. The second and third stages involved univariate and multivariate analyses, respectively, of the candidate genes.

Morphological analysis

The mean number of somite pairs per embryo, as well as the frequency distribution of the stages of neural tube closure were subjected to a two-way analysis of variance (ANOVA) and a chi-square goodness of fit test. This was done in order to determine any VPA treatment effects at a given timepoint on embryonic growth and development (Sokal and Rohlf, '81). Follow-up analyses of significant effects observed in the ANOVA testing were performed using a Bonferroni multiple comparison test.

Univariate analysis

These procedures involved determination of statistical significance ($P < 0.05$) in the gene expression profiling studies, both within and between strains. Because of the factorial nature of the treatment combinations, the testing of these experiments required investigation of the interactive effects of treatment, strain and gestational stage. Statistical tests to determine treatment differences within both strains at each of the timepoints, followed by tests to determine treatment differences across strains and timepoints and simple ratios of gene expression, were conducted. All strain, treatment, and temporal interaction comparisons were evaluated by analysis of variance and the least-square means (LSMEANS) option in the general linear models (GLM) procedure. The LSMEANS option computes model-based estimates of arithmetic means when there are unequal sizes of the experimental groups. Therefore,

GLM was used to determine the level of significance for means among treatment classes, while adjusting for unbalanced sample sizes. For comparisons within each strain, LSMEANS was used to calculate the adjusted means for each control and VPA-treated experimental group, and contrasted these treatment means separately for each timepoint. To examine strain differences in terms of transcriptional activity, the LSMEANS procedure calculated the adjusted means of the differences between the control and the teratogen-treated means separately for each timepoint and strain. This procedure then contrasted these mean differences (representing specific treatments, timepoints and strains) to test the hypothesis that the effect of VPA treatment on transcription factor or cell cycle checkpoint gene expression is consistent across strains. Statistical significance for all of the univariate analyses was set at the ($P < 0.05$) level (Sokal and Rohlf, '81). For a description of the least square estimation formula, see Neter et al. ('89), and for a summary of the LSMEANS option see SAS (SAS Institute, '90). In order to avoid confusion, the adjusted means computed by LSMEANS in these analyses will be referred to throughout the remainder of the text as "means."

Multivariate analysis

Principal components analysis (PCA), an exploratory multivariate procedure, was performed in this study to examine the coordinate interaction of the transcription factor and cell cycle checkpoint genes, for the purpose of gaining new insight into their potential combined influence during neural tube development. PCA is a statistical technique that looks at a group of data, with the goal of creating new variables: (1) a recombination of the original data, and (2) capable of providing the same information as the original data, but which is also more insightful when trying to assess interdependence among the original measurements. This technique is concerned with finding linear, or additive combinations of the original measurements, called principal components (PCs), which are based on the eigenvectors of the covariance matrix based on the original dataset. The goal is to find such a transformation, which is both biologically and statistically meaningful. For a discussion of PCA see Rao ('73), Rohlf and Bookstein ('90), or Johnson and Wichern ('92).

As a technical footnote, PCA is usually performed using observations which are independent. This is not our situation, but due to the extensive controlled breeding program for these highly inbred strains, it is reasonable, in this exploratory setting, to set aside the embryo interdependence when looking for target gene relationships. A separate PCA was performed on the control embryos for each of the inbred strains. Since we started with expression data on nine genes, the PCA results consisted of nine new variables (PCs), which are linear combinations of the original measurements and, for convenience, sorted based on their variability. The first new variable (PC1), is the most variably expressed combination of the original measurements, whereas PC9 is the combination which is the least variably expressed. Therefore, the latter PCs are significantly more consistent in their respective values, among all embryos within the selected embryo group. As such, they make good candidates for describing interdependencies among the original measurements which are consistently expressed for all embryos within a given group. For each control group, PC1 through PC9 were computed, and the combination of genes going into each PC was assessed for biological significance. The SAS procedure PRINCOMP was used for these calculations.

Follow-up analysis

Once a specific PC was deemed to be informative, the interdependence was simplified by eliminating any genes that had minimal impact on the PC (eigenvector values <0.2). This new, reduced combination of the original measurements was then constructed as a multigene, or complex ratio, for all embryos, regardless of treatment. To graphically assess the change in the PC from control to VPA treated in each strain, a plot of the log of the numerator versus the log of the denominator was produced. If the PC distinguished among the treatment groups, the grouping for one treatment looked quite different from the other, with respect to either the location of the points or the pattern of the points within a treatment group. Simple descriptive statistics were calculated for this ratio, and a test of equal variances was performed using Hartley's F-Max test (Mason et al., '89) for each treatment group. To ensure that the exploratory process did not misstate the treatment relationship due to the lack of adjustment for correlation due to our sampling, the new PC was also assessed using the same univariate procedures described for the second stage.

RESULTS

Morphological studies

Mouse embryos from 99 litters collected between GD 8:18 and 9:12 were grossly examined for the number of somites present, and the stage or extent of neural tube closure achieved at each of the collection timepoints under control and teratogen treatment conditions (Table 1). For each of these treatment groups, no fewer than 33 embryos were examined. Any embryo whose neural folds had not yet fused at any site was considered "open." Embryonic growth, as determined by the mean number of somite pairs present in a given specimen, was found to differ significantly according to the gestational age ($P < 0.05$) and the teratogenic treatment ($P < 0.05$). VPA-treated embryos on GDs 8:18 and 9:0 had significantly fewer somite pairs relative to the controls (Table 1; $P < 0.05$). Of the SWV embryos examined at GD 8:18, the valproate-treated embryos were developmentally delayed as compared to the controls, with 33% of the embryos having completely open neural tubes, while 28% of the control embryos had progressed to the point where closure at site III had

TABLE 1. Progression of embryos through stages of neural tube closure[1]

Strain	Gestational age	Treatment	Litters (No.)	Embryos (No.)	Somites/embryo (mean ± SEM)	Closure sites (percentage of embryos) Open	I	II	III	IV	Closed	NTD
SWV	8:18	C	6	65	8.15 ± 0.23	11	61	0	28	0	0	0
		VPA	5	40	7.06 ± 0.20	33	62	0	5	0	0	0
	9:0	C	10	114	11.60 ± 0.28	4	13	0	33	21	29	0
		VPA	9	82	9.71 ± 0.24*	0	100	0	0	0	0	0
	9:12	C	11	132	18.39 ± 0.27	0	3	0	0	5	92	0
		VPA	12	130	17.45 ± 0.30	0	18	2	15	8	30	27
LM/Bc	8:18	C	6	33	9.10 ± 0.28	0	70	18	9	3	0	0
		VPA	12	68	5.72 ± 0.90*	18	54	25	3	0	0	0
	9:0	C	5	48	13.55 ± 0.28	0	5	28	65	2	0	0
		VPA	7	37	9.10 ± 0.37*	5	60	16	19	0	0	0
	9:12	C	5	49	24.87 ± 0.29	0	0	0	0	0	100	0
		VPA	11	50	20.74 ± 0.95	0	14	16	22	4	34	10

*$P < 0.05$ from controls.
[1]NTD, neural tube defect; VPA, valproic acid.

commenced. The growth and development of LM/Bc embryos at this stage were also significantly impacted, with the average number of somite pairs 5.7 compared with 9.1 in control embryos (Table 1; $P < 0.05$). In this strain under control conditions at GD 8:18, some degree of neural tube closure at sites I, II, or III was apparent in all of the embryos, while 72% of the VPA-treated embryos were either completely open or had, at most, progressed to closure site I (Table 1; $P < 0.05$).

By GD 9:0 the control SWV embryos had progressed to advanced stages of neural tube closure, with 83% having achieved at least closure III, while 21% of the embryos had begun closure IV (Table 1). By GD 9:0, 100% of the valproate-treated SWV embryos were still in the initial stages of neural tube closure and had significantly fewer somite pairs than those of the controls ($P < 0.05$). Two-thirds of the control LM/Bc embryos were at closure stage III by GD 9:0, whereas approximately as many VPA-treated embryos were still in the initial stages of neural tube closure. As with the SWV embryos, there was a significant reduction in somite formation in the drug-treated GD 9:0 LM/Bc embryos (Table 1; $P < 0.05$). By GD 9:12, 92% of the control SWV embryos had completed neural tube closure, while only 30% of the VPA-treated SWV embryos had completed the process, and it was clear from the splayed neural folds that at least 27% of the embryos would have NTDs (Table 1). The progression through neural tube closure was significantly delayed in the remaining 30% of the SWV embryos, although it was possible that some of these embryos might yet complete neural tube closure (Table 1; $P < 0.05$). The LM/Bc embryos exposed to VPA and examined at GD 9:12 were also significantly delayed in terms of neural tube closure (Table 1).

Gene expression studies

Univariate analysis. Analyses of variance were performed to compare levels of transcription factor and cell cycle checkpoint gene expression at each collection timepoint, both within and between the two inbred mouse strains. This was accomplished by calculating and comparing the means between control and VPA treatment groups for each timepoint and strain, as well as the mean differences between the two treatment groups, and then contrasting these mean differences for significance ($P < 0.05$) across strains. Each treatment group was represented by no less than 16 embryos for the gene expression studies. Table 2 summarizes the results of these observations. At GD 8:18, there were no significant differences observed in the SWV embryos for any of the six transcription factors in response to the VPA treatment (Table 2; $P > 0.05$). At 6 hr later, the expression of *Emx-1, Emx-2,* c-*fos,* c-*jun* and *creb* was upregulated in the VPA-exposed SWV embryos when compared to their control levels. However, the expression of all these genes in the GD 9:0 control group were significantly lower than those observed at GD 8:18 (Table 2; $P < 0.05$). This altered expression pattern was unique to the SWV embryos at this timepoint and underscored a significant difference between the two mouse strains with respect to the regulation of specific transcription factors. At GD 9:12, when neural tube closure was largely completed for the SWV embryos (Table 1), there were no significant differences observed in the expression of the transcription factor genes following a teratogenic exposure to VPA ($P > 0.05$). There was a significant difference in the response of the SWV embryos at this gestational stage for *Emx-2* expression, compared to that observed in the LM/Bc embryos ($P < 0.05$). While the embryos from both strains were nonsignificantly upregulated for this gene at GD 9:12 ($P > 0.05$), the relative level of *Emx-2* expression was greater in the SWV embryos compared to that in the LM/Bc embryos (Table 2; $P < 0.05$).

In terms of transcription factor gene expression in the LM/Bc embryos, the only statistically significant alteration was a downregulation in the expression level of the *Pax-3* gene at both GDs 8:18 and 9:0 (Table 2; $P < 0.05$). The level of expression for the other transcrip-

TABLE 2. Least-square mean response of transcription factor gene expression

		Gestational day					
		8:18 treatment		9:0 treatment		9:12 treatment	
Strain	Genes	Control	VPA	Control	VPA	Control	VPA
SWV	*Pax-3*	1.14	1.05	0.96	1.08[1]	1.09	1.2
	Emx-1	1.06	1.07	0.88	1.11*	1.15	1.16
	Emx-2	1.14	1.10	0.75	0.99*,[1]	0.97	1.13[1]
	c-*fos*	1.22	1.3	0.83	1.05*,[1]	1.11	1.17
	c-*jun*	1.21	1.11	0.80	1.06*,[1]	1.15	1.18
	creb	1.14	1.15	0.77	1.03*,[1]	1.15	1.19
LM/Bc	*Pax-3*	1.31	1.05*	1.4	1.0*	1.09	1.1
	Emx-1	1.13	1.13	1.16	1.07	1.07	1.05
	Emx-2	0.80	0.65	0.86	0.72	0.69	0.81
	c-*fos*	0.89	0.91	0.98	0.86	0.86	0.96
	c-*jun*	0.87	0.81	0.88	0.84	0.85	0.92
	creb	0.98	1.17	1.03	1.02	1.06	1.08

*$P < 0.05$ from controls.
[1]SWV transcriptional activity is significantly different from LM/Bc embryos.

TABLE 3. Least-square mean response of cell cycle gene expression

		Gestational day					
		8:18 treatment		9:0 treatment		9:12 treatment	
Strain	Genes	Control	VPA	Control	VPA	Control	VPA
SWV	*bcl-2*	2.65	1.70	1.55	1.75	3.50	3.10
	p53	1.92	1.31	1.12	1.40	2.60	2.40
	wee-1	2.77	2.40	1.50	1.93	5.20	3.50*
LM/Bc	*bcl-2*	4.80	10.70*,[1]	2.80	5.80*,[1]	1.60	4.30*,[1]
	p53	3.40	7.00*,[1]	1.30	4.05*,[1]	1.20	3.10*,[1]
	wee-1	1.89	1.98	2.08	2.36	3.5	2.60*

*$P < 0.05$ from controls.
[1]SWV transcriptional activity significantly different from that of LM/Bc embryos.

tion factors remained statistically unaltered, independent of treatment (Table 2; $P > 0.05$). At GD 9:0, there was a significant difference in the response to the VPA treatment between the two strains for *Pax-3* expression ($P < 0.05$). While the SWV strain embryos manifested a slight elevation in the expression of this gene, the LM/Bc embryos had a reduction of nearly 30% in *Pax-3* expression (Table 2).

The cell cycle checkpoint genes were similarly evaluated for changes in teratogen-induced expression (Table 3). Overall, the expression of these genes tended to be lower in the SWV embryos in response to VPA exposure at GD 8:18 and 9:12, although these reductions were not statistically significant (Table 3; $P > 0.05$). The sole exception was the significant reduction in the expression of *wee-1* at GD 9:12 ($P < 0.05$). Although the SWV embryos collected at GD 9:0 showed a trend towards increased gene expression of all three cell cycle checkpoint genes, none were significantly altered ($P > 0.05$).

With respect to the LM/Bc embryos, the VPA treatment significantly upregulated the expression of both *bcl-2* and *p53* at all three embryo collection timepoints (Table 3; $P < 0.05$). This was particularly pronounced at the earlier timepoints, with the expression increasing two- to threefold over that observed in the control embryos. Furthermore, the expression levels of *bcl-2* and *p53* in the drug-exposed LM/Bc embryos differed significantly from those observed in the SWV embryos at all three timepoints ($P < 0.05$). In the LM/Bc embryos, *wee-1* expression remained relatively constant following teratogenic treatment at the early timepoints, showing slight but nonsignificant increases (Table 3; $P > 0.05$). However, at GD 9:12, as was observed in the SWV strain, there was a significant decrease in the expression of this gene in the valproate-exposed embryos (Table 3; $P < 0.05$).

From the initial univariate analysis, it was clear that the functional consequences of alterations in mRNA abundance must be viewed as a composite, rather than focusing on isolated changes in individual gene expression in response to the VPA exposure. Such an overview was essential in order to dissect out the genetic interactions regulating the morphogenetic processes involved in neural tube closure. To attain such a profile, we compared the ratios of mRNA abundance of the three cell cycle genes, which were significantly altered at all three embryo collection timepoints in the LM/Bc strain. The transcription factor genes were not subjected to this gene ratio analysis, given that there were significant alterations in the gene expression at only one

TABLE 4. Ratios of least-square mean responses of cell cycle genes expression

		Gestational day					
		8:18		9:0		9:12	
Stain	Genes	Control	VPA	Control	VPA	Control	VPA
SWV	*bcl-2/p53*	1.37	1.27	1.39	1.25*,[1]	1.38	1.28
	wee-1/p53	1.50	2.04*,[1]	1.40	1.45[1]	2.3	1.90[1]
	bcl-2/wee-1	0.97	0.74[1]	1.06	0.90[1]	0.68	0.93
LM/Bc	*bcl-2/p53*	1.47	1.48	2.07	1.43*	1.37	1.36
	wee-1/p53	0.71	0.37	1.58	0.64*	3.05	0.93*
	bcl-2/wee-1	2.60	4.97*	1.35	2.52*	0.51	1.60*

*$P < 0.05$ from controls.
[1]SWV significantly different from LM/Bc embryos.

embryonic timepoint and only in the SWV embryos. In the GD 8:18 SWV embryos, there was a significant increase in the gene expression ratio of *wee-1* to *p53* (1.5 to 2.04; $P < 0.05$). Furthermore, the magnitude of the change was significantly different between the two strains, with the ratio of this gene pair significantly increasing in the SWV embryos ($P < 0.05$), while decreasing in the LM/Bc embryos. Just the opposite was observed at GD 8:18 for *bcl-2* to *wee-1* expression, which nonsignificantly decreased in the SWV embryos (Table 4; $P > 0.05$), and significantly increased in the LM/Bc embryos ($P < 0.05$) following the VPA treatment. The magnitude of the change differed significantly between the two strains ($P < 0.05$).

At GD 9:0, there was a significant decrease in the ratio of *bcl-2* to *p53* gene expression in both the SWV and the LM/Bc embryos (Table 4; $P < 0.05$). The remaining two ratios of cell cycle genes were not significantly altered in the valproic acid-exposed SWV embryos at this time point ($P > 0.05$). However, in the LM/Bc embryos at GD 9:0, the ratio of *wee-1* to *p53* expression was significantly decreased, while that of *bcl-2* to *wee-1* significantly increased ($P < 0.05$). All of the gene expression ratios examined in the LM/Bc embryos at this timepoint were significantly different from those observed in SWV embryos (Table 4; $P < 0.05$).

As neural tube closure progressed toward completion, there were no significant shifts in the ratio of cell cycle gene expression in the SWV embryos in response to the teratogenic insult ($P > 0.05$). However, in the LM/Bc embryos at GD 9:12, the gene expression ratio of *wee-1* to *p53*, and *bcl-2* to *wee-1* were both significantly altered ($P < 0.05$). That is, there was significantly more *p53* and *bcl-2* expression relative to *wee-1* in these embryos, and the magnitude of the expression change for these two genes differed significantly between the SWV and LM/Bc mouse strains (Table 4; $P < 0.05$).

Multivariate analysis. Given that it is not feasible to envision all possible relationships among the genes of interest by looking at each gene individually, PCA was used to provide insight into more complicated aspects of analyzing gene expression. From the nine original measurements, nine PCs were computed for the gene expression data generated from the LM/Bc and SWV/Fnn embryos collected at GD 9:0 under control conditions. The eigenvectors for the selected PCs, along with their respective percent of original variability, are given in Table 5. In the PCA using the LM/Bc control group, PC7 was deemed the most biologically interesting. Similarly, PC9 was selected for the PCA using the SWV control group. These PCs contained limited original variability, and thus are expressed at very consistent levels within all embryos comprising these groups. Inspecting the eigenvector weights associated with each gene, we decided that a simplification involving only *wee-1, Emx-2, Pax-3,* c-*fos,* and c-*jun* adequately represented PC7 for the LM/Bc group, while *bcl-2, p53,* and *Emx-2* represented PC9 for the SWV group. These gene subsets are used in all subsequent discussions of PCs 7 and 9. Thus, for the LM/Bc plot of ln(numerator) (natural log numerator) versus ln(denominator), *wee-1* and *Emx-2* are in the numerator and *Pax-3,* c-*fos,* and c-*jun* are in the denominator (Fig. 1a). Similarly, *bcl-2* is in the numerator, and *p53* and *Emx-2* are in the denominator of the SWV plot (Fig. 1b). The small variability associated with these combinations can be interpreted by observing that as expression of the numerator genes increases, so too does the expression in the denominator, thereby keeping the ratio constant.

TABLE 5. Eigenvector values for the influential PCs: PC7 and PC9 for the PCAs on LM/Bc and SWV control groups, respectively[1]

Strains	Eigenvectors	
Principal components (% variance)	LM/Bc PC7 (1.1%)	SWV PC9 (0.23%)
Genes		
wee-1	0.403[1]	0.148
bcl-2	0.061	0.637[1]
p53	0.161	−0.663[1]
Pax-3	−0.436[1]	0.107
c-*fos*	−0.339[1]	0.141
c-*jun*	−0.530[1]	−0.021
creb	0.158	0.105
Emx-1	0.197	−0.174
Emx-2	0.398[1]	−0.246[1]

[1]Heavily weighted genes. The PCAs are based on the expression values of the three cell cycle checkpoint and six transcription factor genes.

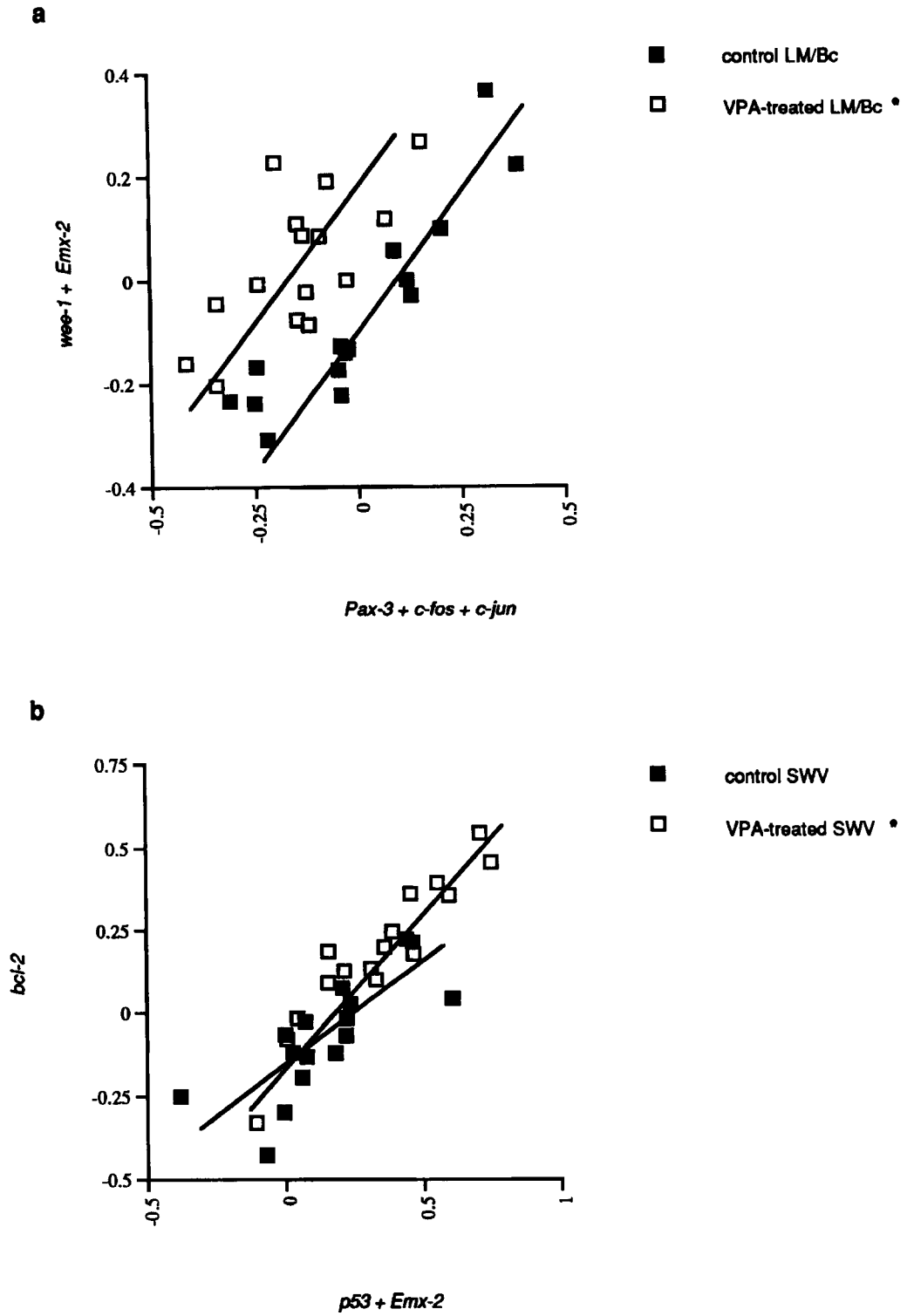

Fig. 1. a: PCA plot of valproic acid-treated and control LM/Bc embryos from GD 9:0 in the new coordinate space defined by PC7, using expression data for six transcription factor and three cell cycle genes. Lines drawn through the treatment groups identify the embryo distribution boundaries. *VPA-treated PCA ratio means significantly different ($P < 0.05$) from control PCA ratio means. **b:** PCA plot of valproic acid-treated and control SWV embryos from GD 9:0 in the new coordinate space defined by PC9, using expression data for six transcription factor and three cell cycle genes. The lines drawn through the treatment groups identify the embryo distribution boundaries. *VPA-treated PCA ratio means significantly different ($P < 0.05$) from control PCA ratio means. GD, gestational day; PC, principal components.

In Figure 1a,b, note that the control embryos for both strains are more tightly clustered around their respective line, indicating the consistency in the expression of this gene combination. Also note that the clusters for these control embryos are noticeably different than are the clusters for their respective treatment groups, indicating an alteration in the gene relationships. The variability of treatment and control groups for each strain are not significantly different ($P > 0.05$), as determined by Hartley's F-Max test for constant variance. However, a follow-up test for equality of means rejects at $P < 0.05$, with the mean for the GD 9:0 control embryos significantly distinct from the GD 9:0 VPA treatment means for both strains (Fig. 1a,b). Taken together, these findings suggest that the selected gene combinations for the control groups in both strains remain predictable following VPA exposure, as demonstrated by their unaltered distributions following treatment. However, their tolerance threshold limits appear to have been modified by VPA treatment, as suggested by the shift in the mean of the selected gene combinations.

DISCUSSION

Several hypotheses have been proposed to explain how VPA may disrupt normal morphogenetic mechanisms leading to the development of a NTD. In addition to the hypotheses concerning potential changes in embryonic folate metabolism (Wegner and Nau, '91, '92), it has also been suggested that VPA may exert its teratogenic effects by inducing cellular senescence and inhibiting normal proliferative events within the neural folds. This is based on work by Nau and Scott ('86, '87), who have proposed that weak acids, like VPA, can accumulate in the developing embryo because of its naturally alkaline medium, which is approximately 0.4 pH units above the maternal blood pH during the period of neural tube closure. The elevated fetal concentrations of VPA may decrease the pH of the embryonic environment below a threshold which allows for cell proliferation, although the precise mechanism by which this drug alters normal development remains highly speculative.

In the present study, we used molecular techniques for nucleic acid amplification in order to explore the possibility that the underlying basis for the difference in susceptibility to VPA-induced NTDs could be due to the differential expression of selected transcription factor or cell cycle checkpoint genes, which are molecular targets of VPA. Alterations in transcription factor gene expression were considered important because they serve as the initial triggers in a cascade of molecular events that result in changing the relative abundance of responsive genes. By altering the amount of any given transcription factor, the VPA treatment will likely alter the transcription rate of multiple downstream genes. This occurs because transcription factors function by binding to the promoter or enhancer region of a gene so as to alter the efficiency of RNA polymerase activity. Cell cycle checkpoint genes were considered important to include in this initial panel of candidate genes, given the previously published work from our laboratory, demonstrating a marked delay in embryonic development following exposure to teratogenic concentrations of VPA (Finnell, '91).

In the analysis of transcription factor mRNA changes, it is important to note that the corresponding proteins encoded by these genes can form multimeric functional proteins, either hetero- or homodimers (Kovary and Bravo, '91). The gene product of c-*fos* can form heterodimers with that of c-*jun,* while the c-*jun* protein product is also capable of forming heterodimers with the gene product of creb. Both c-*jun* and *creb* gene products can also form homodimers. If it is assumed that relative mRNA levels for these molecules parallel the protein levels, and that hetero- and homodimers of these molecules form with equal affinity, it is possible to predict the stochastic ratio of hetero- and homodimers in the population of transcription factors (Ryseck and Bravo, '91). Intuitively, if a single transcription factor that forms homodimers is altered, the transcription level of those genes regulated by this factor will be similarly altered. Since heterodimers are composed of two different transcription factors that have differing selectivity or affinity for specific *cis* DNA-binding sites, the alteration of any single transcription factor may significantly alter the relative amounts of hetero- and homodimers, which in turn can have a much broader molecular influence affecting a myriad of genes other than those simply regulated by the homodimers (Ryseck and Bravo, '91).

Based on gross morphological examination of neural tube closure, it appears as if VPA significantly delayed normal developmental events as well as impacting the overall growth of the embryo (Table 1). As a general rule, the molecular data presented in this study suggest that exposure to VPA consistently elicited GD 9:12 control levels of transcription factor mRNA expression in the embryos on GD 9:0. That is, the normal temporal pattern of gene expression is altered, and the teratogen-treated embryos express mRNA levels for candidate genes comparable to what would normally be observed 12 hr later under control conditions (Fig. 2). This was true for both inbred mouse strains, although it was more pronounced in the SWV embryos. This accelerated development was marked by a significant elevation in the level of mRNA for most of the transcription factors (*Emx-1, Emx-2,* c-*fos,* c-*jun,* and *creb*) in the SWV embryos, and a decrease in the expression of *Pax-3* in the LM/Bc embryos at GD 9:0 (Table 2). The increase in these transcription factors suggested a generalized activation of gene expression occurring at this gestational timepoint. Since the relative levels of gene expression are normalized to cyclophilin, which is present in all cells, the alteration in mRNA levels is in direct response to the teratogenic treatment and is not merely due to an increase in the number of cells within the developing embryo.

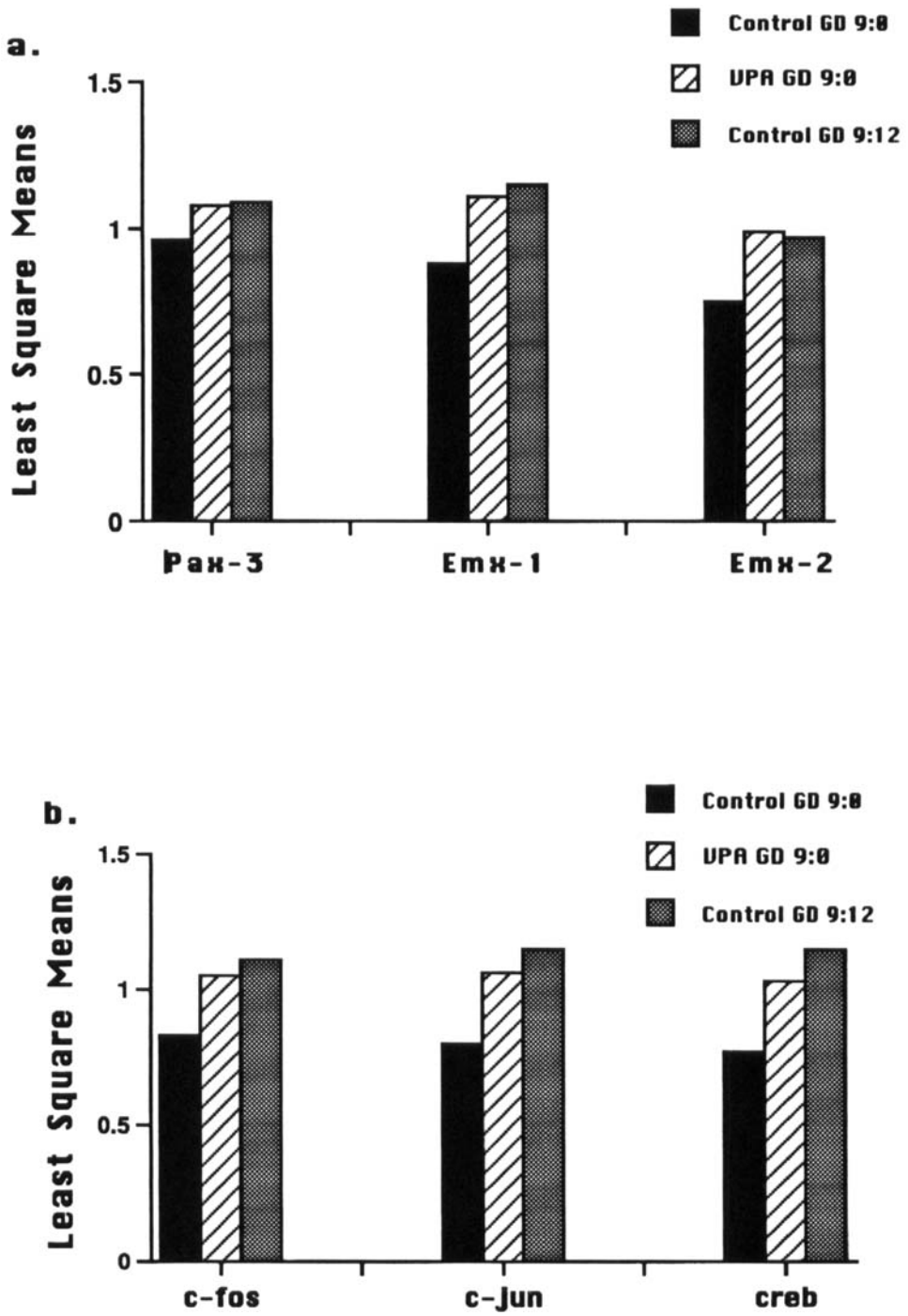

Fig. 2. Relative levels of transcription factor gene expression, depicted as least-square means, as observed in neuroepithelial tissue obtained from SWV embryos. **a,b:** Expression patterns of control GD 9:0 (*hatched*), valproic acid-treated GD 9:0 (*stippled*) and valproic acid-treated GD 9:12 embryos (*solid black*). GD, gestational day; PC, principal components.

In response to the teratogenic VPA exposure, we also observed a significant over-expression of the cell cycle checkpoint genes, *p53* and *bcl-2* in the neural tubes of LM/Bc embryos collected at all three gestational time-points. In the NTD-sensitive SWV embryos, the expression of these two genes was depressed at GD 8:18 and GD 9:12 and was only slightly elevated at GD 9:0 (Table 3). The peak expression of these genes occurs when the neural tube has initiated fusion at the prosencephalon–mesencephalon border. Coincidentally, this is also the

time when another teratogen, arsenate, produced a significant delay in neurulation in the SWV and LM/Bc embryos (Wlodarczyk et al., '96). Two cellular functions ascribed to the *p53* and *bcl-2* gene products are the inhibition of cellular proliferation, and the induction of apoptosis (Miyashita et al., '94). Although, no direct measurements of either apoptosis or cell proliferation were made in this study, the expression pattern of these two genes is consistent with a drug-induced inhibition in cell proliferation, rather than inducing apoptosis. The apoptotic functions of *p53* and *bcl-2* gene products are thought to be activated when the equilibrium between the expression of these genes favors *p53* (Miyashita et al., '94; Wang et al., '93). In the current study, the ratio of the expression of *bcl-2/p53* clearly favored *bcl-2* and argues against the induction of apoptosis (Table 4). The concomitant peak expression of *p53* and *bcl-2* observed in this study further discounts the involvement of an apoptotic pathway, since *p53*-induced apoptosis can be blocked by an elevation in the level of the *bcl-2* gene product (Wang et al., '93). Taken together, these data strongly suggest that teratogenic concentrations of VPA inhibit the cellular proliferation of the developing neuroepithelium during the early stages of neural tube closure. This produces a highly significant delay in development from which the embryo never fully recovers. The data from the present study support the hypothesis that a teratogenic treatment with VPA has highly specific effects on a number of different gene targets involved with the regulation of cell proliferation, in order to adversely affect morphogenetic processes critical to normal neural tube closure.

The PCA analysis provided an additional level of insight into the gene expression patterning, by revealing interactions among the transcription factors and cell cycle genes that distinguished among embryo treatment groups at GD 9:0. In addition, unlike the results obtained from the univariate analyses, we found that the mitotic inhibitor gene *wee-1* was a highly significant factor in characterizing the LM/Bc control GD 9:0 embryos. While the transcription factor genes were significantly upregulated in the VPA-exposed SWV embryos, as determined by the univariate analyses, their coordinate expression did not distinguish between the two treatment groups of this strain. The most noteworthy observation was the distinct shift in the mean location of the VPA-treated embryos, relative to the control group in both strains, with respect to their combined expression ratios (Fig. 1a,b). This shift may signify that a certain proportion of the embryos had fallen outside the control tolerance limits for this particular gene combination, and thus may be indicative of those embryos at an increased risk of developing an NTD. Therefore, it can be inferred that the gene associations revealed by these analyses must remain constant during neural tube closure to ensure normal morphogenesis.

These genes are potentially involved in regulating the transition between neuroepithelial differentiation and proliferation, via cell cycle regulation or dorsoventral (DV) and anteroposterior (AP) patterning to facilitate neural tube closure. The PCA yielded gene combination for each inbred strain that provided a biologically plausible interpretation of neuroepithelial cell activity. Had we relied solely on the univariate mean and ratio analyses (Table 2), it is likely that these combinations would have been overlooked. Furthermore, PCA revealed gene interassociations that broadened our view of the process of neural tube gene regulation and generated novel hypotheses concerning genetic control over the cell cycle in these tissues. PCA also enabled us to observe more complicated ratios that potentially have significantly greater resolving power, in terms of describing the behavior of genes during early embryogenesis. As such, the ratio relationship of these genes can be used to explain apparent contrasts in their regulatory behavior within an embryo group. Specifically, such an interpretation involves contrasting the behavior of the genes in the numerator with that of genes in the denominator. For example, all the GD 9:0 embryos in the LM/Bc control group responded similarly to the ratio of *wee-1, Emx-2,* to *Pax-3,* c-*fos,* and c-*jun,* such that if the numerator genes either increased or decreased their combined expression levels, the expression levels of the denominator genes would be altered in parallel, for each embryo. The significant mean differences between the control and VPA-treated groups in both strains for their respective ratios, indicated that these control embryos expressed a different tolerance threshold level of the gene combination. The similar variances between the two treatment groups in each strain illustrates that the strain-specific numerator and denominator relationships among these genes was expressed in parallel in all the embryos. Thus, the results of the analysis revealed a dramatic distinction in terms of tolerance limits for the combined expression of the respective ratio relationships between the GD 9:0 control and VPA treatment groups in both inbred strains.

At GD 9:0, proliferation is the main cellular state among the neuroepithelial cells of the neural tube. During this time, critical cell cycling and patterning events are occurring in preparation for postmitotic differentiation processes. The gene combinations selected to characterize active neural tube closure in each strain are consistent with cellular growth and the establishment of axis patterning in the developing neural tube. That is, the mathematical relationship of the genes selected to describe the LM/Bc control group, such that *wee-1,* and *Emx-2* are in the numerator and *Pax-3,* c-*fos,* and c-*jun* are in the denominator, can be aligned with their biological relationship to provide meaningful insight into cell cycle regulation and patterning in the neuroepithelium. Specifically, it has been shown that upregulation of *wee-1* results in the induction of apoptosis in certain cells lines (Davey et al., '95). Previous work indicates that *wee-1* expression is low at GD 9:0 in the LM/Bc strain (Wlodarczyk et al., '96).

Taken together, we can speculate that the low level of *wee-1* reflects the minimal impact of its protein product at G_2. This enables the neuroepithelial cells to rapidly progress through the cell cycle, thus complementing the growth inducing actions of the c-*fos* and c-*jun* gene products at G_1. Thus, the contrasting assemblage of these three genes could be reflective of a mitotic stimulation that counteractively decreases the activity of *wee-1* and stimulates the expression of c-*fos* and c-*jun,* thereby favoring cellular proliferation. The remaining two genes in the LM/Bc combination are *Pax-3* and *Emx-2,* both of which are involved in various aspects of embryonic patterning and are initially expressed early in mitotically active neuroepithelial cells. *Pax-3* expression is restricted to the dorsal neural tube along the entire length of the AP region delineating the hindbrain–midbrain boundary. This gene has also been implicated in influencing the DV polarity of the neural tube. *Emx-2* is expressed in the dorsal and ventral neural tube and plays a role in the patterning in the positional specification of cells along the DV axis. Therefore, the simultaneous contrasting expression of these two genes may be reflective of their opposing patterning roles during axis formation, with the gene product of *Pax-3* specifying the AP gradient, and the product of *Emx-2* regulating the DV axis. Taken together, the simultaneous expression of these five genes creates a picture of the cellular efforts necessary to establish and maintain neuroepithelial proliferation, while initiating patterning events vital to pre-neural tube closure embryos.

In the SWV embryos, the selected combination included *bcl-2* in the numerator and *p53* and *Emx-2* in the denominator. As with the LM/Bc embryos, this gene combination has implications in neuroepithelial cell proliferation and patterning. The contrasting regulatory effects of the *p53* and *bcl-2* gene products in the cell cycle are well documented and have been described above. The current multivariate analysis may be reflective of a regulatory counter balance of these two genes, such that the growth arrest effects of *p53* are held in check by *bcl-2,* enabling the cells to cycle uninhibited. Meanwhile, *Emx-2* may be functioning reciprocally to initiate cellular establishment of AV patterning, once the proliferative phase is complete.

From this study it is clear that the exposure to teratogenic concentrations of VPA results in a significant alteration in the relative expression of both transcription factors and cell cycle checkpoint genes. Overall, the data support the hypothesis that multiple gene targets of VPA are responsible for teratogen-induced changes during normal morphogenesis. It is the coordinate change of several molecules, which together are capable of producing the adverse phenotypic changes, that appear to be involved in disrupting embryogenesis during the period of neural tube closure. The mRNA levels observed in this study may be reflective of altered transcription rates of the corresponding genes, or of changes in mRNA stability. Irrespective of the mechanism, the fact that coordinate changes do occur secondary to an environmental influence on the developing murine embryo, suggests a number of areas of future investigation. It may be possible to limit or reduce the developmental influence of teratogens by artifically altering the embryonic expression profile in an attempt to retain or restore the normal embryonic pattern of gene expression. Experiments in which cDNA synthesis is restricted to individual cells of the neural tube, particularly in the region of closure site II, may permit greater sensitivity in detecting the most relevant changes in gene expression. Additionally, protein levels of expression may be examined using immunohistochemistry, radioimmunoassays, and functional protein assays to ascertain whether the changes we have documented in mRNA levels are translated into comparable differences in protein levels. Such comparisons deserve immediate experimental attention and are currently in progress in our laboratory.

ACKNOWLEDGMENTS

The contents of this work are solely the responsibility of the authors and do not necessarily represent the official views of the NIEHS. The authors express their appreciation to Ms. Aine Miller for her expert clerical and editorial assistance. We thank the following for providing cDNA clones: J.H. Eberwine, T. Curran, S.A. Mackler, M. Chao, E. Boncinelli, D. Trasler, M. Pisano, and S.J. Korsemeyer.

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