Phenytoin-induced alterations of early embryonic gene expression

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Introduction

Over the past thirty years, both clinicians and epidemiologists have observed that women who suffer from seizure disorders are at an increased risk for giving birth to children with congenital defects (for review see: Dansky and Finnell, 1991). Many investigators have sought the possible cause of these malformations, resulting in a substantial body of evidence to suggest that the etiology of the problem lies not in the convulsant disorder, but rather in the teratogenicity of many anticonvulsant drugs. The vast majority of studies to date show that the incidence of congenital malformations amongst the offspring of drug treated women is approximately two to three times the rate observed in either non-treated epileptics or that of the general population (Dansky and Finnell, 1991; Kaneko et al, 1992). The nature of the adverse effects of anticonvulsant drugs on developing fetuses have been well documented in human clinical and epidemiological studies (Hanson et al.,1976; Dansky and Finnell, 1991; Kaneko et al, 1992; Lindhout, 1992), and have been replicated in experimental animal models (for review see Finnell and Dansky, 1991). The present study focuses on the possible underlying mechanisms involved in the adverse developmental effects of phenytoin, one of the most widely prescribed anti-epileptic drugs (Niedermeyer, 198-?).

Early embryonic exposure to phenytoin has been associated with a pattern of malformations often classified as the Fetal Antiepileptic Drug Syndrome, which some refer to as the Fetal Hydantoin Syndrome (Figure 1). Clinical characteristics of this teratogen-induced malformation complex include prenatal growth deficiency, nail and/or distal phalangeal hypoplasia, microcephaly, mental deficiency, and craniofacial abnormalities (Hanson et al., 1976, Hanson, 1986). The craniofacial dysmorphia include : midfacial hypoplasia, a short nose with anteverted nostrils, a broad depressed nasal bridge leading to redundant

inner canthal skinfolds, and a long philtrum ending in a bowed upper lip. These characteristic features occur in combination with other abnormalities as diverse as strabismus, ptosis, cleft palate, ventricular septal defects, hypospadius, and inquinal hernias (Bustamante et al., 1978, Schinzel, 1979, Smith, 1980, Finnell and DiLiberti, 1983). Since all children with this embryopathy had chronic exposure to phenytoin during organogenesis, the wide variety of congenital malformations has often made its diagnosis somewhat difficult. Some clinicians have gone so far as to question the diagnostic usefulness of the syndrome classification, based on the individual variability in the pattern of observed malformations (Apt and Gaffney, 1977; Janz, 1982). However, such variability in phenotypic expression is common among most drug-induced birth defects, and is believed to be a result of interactions between the teratogenic agent and the maternal and fetal genotypes (Fraser, 1977; Finnell and Chernoff, 1984).

It is unlikely that a single genetic etiology can explain the entire spectrum of the observed congenital malformations found amongst the offspring of epileptic women. Rather, the dysmorphia present in affected infants is probably multifactorial in origin, arising secondarily to the combined effects of genetic and environmental components (Fraser, 1977). Therefore, in order to offer effective means of prevention and/or treatment for these individuals it is first necessary to try to unravel the genetic basis of susceptibility to phenytoin-induced birth defects. Previous investigations of this nature on the molecular level have encountered insurmountable technological limitations. Even when working in an experimental animal model system, there has been an insufficient quantity of embryonic material from which to isolate mRNA necessary to create and screen $cDNA_1$ libraries. Recent advances in molecular biology now make it possible to circumvent this problem, so that now changes in gene expression during the

critical periods of embryogenesis can be monitored, and alterations in gene expression secondary to a teratogenic insult can be detected.

The vast majority of investigations into early murine morphogenesis have been restricted to demonstrating the ontological pattern of expression of individual genes, using *in situ* hybridization. However, it is unlikely that isolated individual genes independently control complex developmental events. It is far more reasonable to assume that the combined effects of a population or collection of genes, acting in concert with one another, control the patterned events of morphogenesis. As such, it may be more informative to study the coordinate regulation of multiple genes in order to better understand their function in the complex events involved in early embryonic morphogenesis. The present study was undertaken to examine the expression of a population of genes in SWV embryos that are known to be expressed and developmentally regulated during murine craniofacial and neural development. This population of candidate genes includes those that are believed to be centrally involved in directing or regulating a cascade of morphogenetic events critical to early embryogenesis. The genes studied include transcription (c-FOS, c-JUN, WNT-1, CREB, TRK) and growth (IGF-I, IGF-II, NGF, NT3, BDNF, CNTF, TGFα, TGFβ1) factors, various receptor subtypes (GABA-Aβ1, NIC, FOLATE) and voltage-sensitive ion membrane channels (calcium, sodium and potassium).

Since teratogen-induce congenital malformations are likely to result from changes in programmed gene expression, we have monitored the level of expression of a population of 20 candidate genes in order to identify any potentially altered patterns of gene expression occurring secondary to *in utero* phenytoin exposure that may contribute to the pathogenesis of the observed defects. This was accomplished using two newly developed procedures, in situ transcription and anti-sense RNA amplification (RT/aRNA) technology.

Quantification of the hybridization intensity of the aRNA probes generated through these procedures from gestational day 9:12 and day 10:0 embryos to cDNA clones of the selected candidate genes on slot blots allowed the relative abundance of the mRNAs of interest to be measured and statistically analyzed. Fluctuations in mRNA population levels during embryogenesis following maternal phenytoin exposure were monitored. This study provides the first composite examination of the coordinate changes in mRNA abundance for twenty candidate genes that may contribute to abnormal development resulting in phenytoin-induced congenital malformations.

Materials and Methods

Teratogenic treatments

The highly inbred SWV/Fnn mouse strain was selected for its demonstrated sensitivity to the induction of phenytoin-induced congenital malformations (Finnell 1981; Finnell et al, 1986, 1993). Table 1 shows the effect of various dosages of phenytoin on three different mouse strains. These mice were maintained on a 12 hour light cycle in the Laboratory Animal Research and Resource Center at Texas A&M University. Pathogen-free, virgin females, 60 to 90 days of age were used in this study. These females were mated overnight with experienced males and checked for the presence of a vaginal plug the following morning. The start of gestation was set at 10 p.m., the previous night, which is the midpoint of the dark cycle (Snell et al. 1948). Phenytoin was chronically administered to the animals through their drinking water, the pH of which was elevated to prevent the phenytoin from precipitating out of solution (Finnell, 1981). Phenytoin was administered at 60 mg/kg, a dosage which has resulted in a high response frequency of malformations in previous studies (Finnell, 1981; Finnell et al 1986, 1993) (Table 2). The control group received pH 10.3 drinking water, the carrier for the phenytoin solution. Drug treatment was started two weeks prior to mating, and continued throughout gestation until the dams were euthanized and the embryos recovered. For both the treated and the control group, no fewer than four dams provided embryos for these experiments.

Embryo collection

Embryos were collected at two critical gestational timepoints during early morphogenesis, day 9:12 (gestational day 9 plus 12 hours) and 10:0. At the assigned hour, pregnant dams were killed by cervical dislocation, the abdomen opened, and the uterine contents removed. The location of all viable embryos

and resorption sites were recorded. Using watchmaker's forceps, the embryos were dissected free of the decidual capsule and its chorion and amnion membranes while in Kreb-Ringers solution, under a Wild M8 dissecting microscope.

Embryo fixation and histological section preparation

Once freed of their extraembryonic membrane, gestational day 9:12 and 10:0 mouse embryos were immediately frozen in liquid nitrogen, and stored at -80°C until needed for the *in situ* transcription procedures. This processing did not adversely affect the *in situ* synthesis of cDNA. While frozen, the embryos were embedded in cold Tissue Tek* O.C.T. compound. Eight micron thick midsagital sections were cut on a cryostat (Slee, Pittsburgh, PA) at -30°C and mounted on diethylpyrocarbonate (DEPC)-treated, gelatin subbed slides. As a rule, four embryonic sections were mounted on each slide, which were stored at -20°C until used for *in situ* cDNA synthesis.

In situ transcription and aRNA amplification Procedures

Repeated applications of rubber cement were used to create sufficiently deep wells around each section to prevent mixing of the prehybridization buffer. A sufficient volume of buffer (25% formamide in 5X sodium citrate buffer (SSC)) was applied to completely cover each embryo section. In selected sections, an unlabeled oligo-dT-T7 amplification oligonucleotide (Eberwine et al, 1992) was added (100 ng) and allowed to hybridize at 37°C for 10-18 hours. At the completion of the hybridization period, the slides were washed at room temperature twice for 15 minutes in 2X SSC, followed by two 1 hour washes in 0.5X SSC. cDNA synthesis proceeded in the presence of reverse transcriptase (40-80 units; Seiguguiku, Bethesda, MD), labeled and unlabelled

deoxynucleotides (10mM), and transcription buffer at 37°C for 60 minutes (for details, see Eberwine et al. 1992a). This buffer was removed, and slides were carefully washed in SSC (2X and 0.5X) for 6 to 18 hours at room temperature. The slides were air dried, exposed to X-ray film, for approximately 12 hours at -80°C with an intensifying screen. Figure 2 represents a typical in situ transcription reaction on a gestational day 10:0 embryo. The embryo on the left (A) did not receive the oligo-dT-T7 primer, and demonstrates the endogenous background level of hybridization. Note the intensity of the hybridization in areas of rapid cellular proliferation in the embryo (B) that was allowed to hybridize in the presence of the oligo-dT primer. The in situ transcribed cDNAs were then recovered from the embryo sections for amplification by alkaline denaturation (0.5N NaOH and 0.1% SDS). This treatment disrupts the mRNA-cDNA hybrid formed by the in situ transcription procedure within the embryo sections (Eberwine et al., 1992a, Eberwine et al, 1992b). T4 DNA polymerase (5 units) and an equal amount of the Klenow fragment of *E. coli* DNA polymerase I were then added to drive second strand cDNA synthesis as has been described elsewhere (Eberwine et al., 1992a). To remove the hairpin loop formed as a result of self-priming, the cDNA was treated with S1 nuclease (2 units) for 15 minutes at 37°C. As the primer contains the promoter region for bacteriophage T7 RNA polymerase, the double stranded cDNAs also contain this promoter, which enabled the transcription of all downstream sequences when T7 RNA polymerase was added to the amplification reactions. These reactions were carried out for one hour at room temperature, followed by 3 hours at 37°C. The RNA that is synthesized is anti-sense in its orientation to the cDNA obtained from the original mRNA transcripts and although amplified several thousand fold, it was reamplified by conversion back into double stranded cDNA containing the T7 promoter site (Eberwine et al., 1992a). This repetition of the

amplification procedure made it possible to obtain yields in excess of one million fold amplification of the original embryonic mRNA. The reamplified aRNAs generally ranged in size from between 300 bp to 2 kb, somewhat smaller than the aRNA obtained from a single round of amplification. This material was used as a riboprobe to probe slot blots containing the equimolar concentrations of the cDNAs of interest. This procedure is summarized in Figure 3.

Slot blots and expression profiles

cDNA clones were immobilized on nylon membranes (Zetaprobe, BioRad) using a BioRad slotting apparatus. Each blot was prehybridized for 5 minutes in buffer (50% formamide; 0.12M Na2HPO4, pH 7.2; 0.25M NaCl; 7% (w/v) SDS) at 42°C. The heat denatured riboprobes were applied to the blots and hybridized for 24 hours. The slot blots were washed for 15 minutes at room temperature in each of the following solutions: 2XSSC/0.1% SDS, 0.5X SSC/0.1% SDS, 0.1X SSC/0.1% SDS. The dried slot blots were opposed to X-ray film overnight at -80°C with an intensifying screen. The autoradiograms of the slot blots were converted to relative transcription rates once they were scanned with a MicroImage radiographic optical densitometry system utilizing Image analysis software. The product of the area and mean density of each signal were measured. The individual signals were corrected for background obtained by the subtraction of the puc 13 plasmid vector and then all values were normalized to the tyrosine kinase gene expression. Tyrosine kinase expression was normalized to brain derived neruotrophic expression. The radiographic signal for each of the cDNAs is a direct reflection of the abundance of the mRNA that is present in the original embryo section. The hybridization pattern obtained from each embryo section derived riboprobe was referred to as its genetic expression profile. Figures 8 and 9 are examples of genetic expression profiles for two

embryos at gestational day 9:12, one was phenytoin-treated, the other was not. For each of the experimental groups, no fewer than 12 expression profiles were generated, with at least two different females providing two embryos each, from which up to three sections were subjected to *in situ* transcription to provide cDNA substrates.

Statistical Analysis

The mean was calculated for each product of area and mean density for each treatment at both timepoints. It was then assumed that the values for each gene were normally distributed. A two sample t-statistic assuming unequal standard deviations was performed to determine if there was a significant difference between the averages. The treated and untreated averages were compared at each timepoint, giving the effect of phenytion exposure on the relative level of gene expression. Significance was set at a comparisonwise error rate $\alpha = 0.002$. This level is set to ensure the entire experimentwise error rate is at the most $\alpha=0.05$, which is the probability of concluding that the means of each of the gene expressions are different. The error rates define the probability of a type I error which occurs when the means are found to be different when, in actuality they are not.

Results

Gene Expression Changes Under Control Conditions

In SWV embryos, several genes were found to be developmentally regulated as the embryos progressed through the 12 hours of morphogenesis between gestational day 9:12 and 10.0 (Figure 4). The only upregulation observed in gene expression among the 19 genes analyzed was seen as the obvious increase in TRK expression. The growth factors and receptors manifested a varies response where BDNF, CNTF, IGF-II, TGFα, and TGFβ1 were all downregulated while IGF-I, NGF, and NT3 were maintained at the same levels throughout these 12 hours of development. Although the expression of the oncogene c-FOS was not greatly changed, that of c-JUN was slightly decreased, but not significantly, as was the expression for the transcription factor WNT-1. The transcription factor CREB was also slightly downregulated. As a group, the expression of the ion channels were all suppressed as gestation proceeded from day 9:12 to 10:0. The calcium, potassium, and, to a lesser extent, the sodium ion channel gene were normally developmentally downregulated. Finally, GABA , FOLATE, and NIC receptors were slightly downregulated compared to embryos collected 12 hours earlier.

Gene Expression Changes Between Untreated and Phenytoin-Treated Embryos

Gestational Day 9:12

In embryos recovered on gestational day 9:12, the expression of several genes was visually and densitometrically found to be altered following *in utero*

phenytoin exposure (Table 3). However, closer analysis revealed that none of the alterations were statistically significant when compared to the pattern of gene expression observed in comparably staged control embryos. This was likely due to two factors, the variability observed in those genes that were limited in the intensity of their expression, and the small sample size of pH 10.3 observations.

Although there were no statistically significant alterations, the expression of several individual genes exhibited trends that may be of biological significance. The majority of gene expression at this gestational time point appeared to be lower than that in untreated embryos at the same age (Figure 5). The relative expression of the growth factors CNTF, NT3, and TGFβ_1 indicate such downregulations. In addition, the transcription factor WNT-1, the voltage sensitive ion channel regulator Ca^{2+}, and the nicotinic receptor show signs of lowered expression. Although not significant at α=0.002, these alterations are significant at a level of α=0.05. The levels of IGF-I, FOLATE, and GABA are not noticeably altered at gestational day 9:12 by *in utero* exposure to phenytoin. NT3, c-FOS, and c-JUN are slightly downregulated, while the expression of BDNF, IGF-II, TGFa, and KV1 is more noticeably decreased.

Gestational Day 10.0

Those embryos treated and recovered on gestational day 10.0 showed statistically significant differences in their pattern of gene expression as compared to controls (Table 4). Amongst the growth factors used, IGF-I, NGF, NT3, TGFα, TGFβ_1, and TRK all showed altered levels of gene abundance (Figure 6). All of these genes were upregulated, except TRK, which was significantly downregulated. The transcription factors, c-FOS, c-JUN, CREB, and

WNT-1 reflected the expression pattern of growth factors, and were significantly upregulated. The voltage sensitive ion channels, Ca^{2+}, Na^{+}, and KV_1 all had levels of expression higher than those in control embryos of similar gestational age. An interesting observation was that the levels of gene expression in phenytoin-treated embryos at gestational day 9:12 were similar to the levels found in untreated day 10:0 embryos. All of the candidate genes, except TRK, normally show a mean relative abundance at day 10:0 which the phenytoin-treated embryos showed 12 hours earlier.

Discussion

This study represents the initial investigation using the new techniques of in situ transcription and aRNA amplification to examine the changes of gene expression induced by *in utero* exposure to phenytion. Phenytoin has been shown to produce congenital defects in animals as well as human neonates (For review see Finnell, 1991). The data presented in this paper demonstrates that concommitant with the development of birth defects are changes in the relative levels of expression for several candidate genes. While alterations in the expression of individual genes is undoubtedly important, it is the functional consequences of alterations in mRNA abundance, taken as a composite, that are critical in regulating the normal physiological functioning of cells. It is therefore the action of all of these alterations, working in concert with one another, that leads to abnormal developmental processes which result in congenital defects.

Changes in the relative levels of gene expression for the candidate genes during 12 critical hours of morphogenesis in untreated and phenytoin treated embryos are detailed in Tables 3-4. It is important to realize when examining this data that no attempt was made to determine the absolute amounts of mRNA present. Rather, I have compared the levels of expression of individual genes in relation to the levels of other messages. This comparison of the relative abundance of mRNAs in untreated and phenytoin treated embryos yields new insights into the events which have occurred during this critical period of morphogenesis. Several changes in gene expression normally occur during development. The levels of expression for the various growth factors and receptors were varied, with a decrease in the levels of BDNF, CNTF, IGF-II, TGFα, and TGFβ, no alteration in IGF-I, NGF, of NT3, and a large increase in the levels TRK. The expression of transcription factors c-FOS, c-JUN, CREB, and WNT-1 decreased during the 12 hours from day 9:12 to 10:0, as did all of the

membrane ion channels. The FOLATE, GABA, and nicotinamide receptors were all slightly downregulated. All changes in gene expression have been normalized by dividing the product of hybridization intensity and area from the densitometry scans of all the cDNAs by the product of the tyrosine kinase gene. These data should not be interpreted to suggest that the changes observed in this study represent the limit of detection in the embryonic material. Rather, the use of sections through the entire embryo likely included cells that were not specifically involved in the morphogenic processes of neural tube closure, and their contribution to the mRNA populations may have obscured less robust changes in the expression profiles. The use of highly anatomically restricted cell populations may provide additional resolving power to detect these low abundance changes in gene expression.

The results of the molecular biology of this project show that treating embryos with phenytoin elicits genetic expression in day 9:12 embryos similar to those in untreated day 10:0 embryos (Figure 7). That is, the normal temporal pattern of gene expression has been altered, and the phenytoin-treated embryos on day 9:12 express the candidate genes at levels that are usually found on day 10:0 in untreated embryos. This accelerated development is marked by a reduction in the level of mRNA for most of the candidate genes at day 9:12, including transcription factors. For example, phenytoin treatment decreases the expression of TGFα. Transforming growth factors regulate cellular proliferation and differentiation during craniofacial development. Decreased mRNA levels , if paralleled by changes in the level of proteins synthesized, would induce lower levels of functional transcription factors which, in turn, may inappropriately activate or inactivate gene expression. Premature downregulation in this message could contribute to the overall abnormal temporal expression of genes which, acting together at a critical morphogenic stage, can cause the observed

growth deficiencies in fetal hydantoin syndrome. Phenytoin also maintains the expression of WNT-1; normally WNT-1 is downregulated after day 9:12, but in the presence of phenytoin , it is still expressed at day 10:0. WNT-1 is a protooncogene and a transcription factor which has a temporal and spatial pattern of expression in the murine neural tube that is consistent with a role in the early organization of this structure (McMahon and Bradley, 1990). This gene is not expressed prior to the formation of the neural plate (Wilkinson et al. 1987). However, by gestational day 9:12, WNT-1 mRNA transcripts extend from the presumptive mid brain caudally to the rhombencephalon and anterior spinal cord (Wilkinson et al. 1987, 1989; McMahon and Bradley, 1990). Whether this gene regulates some aspect of cell recognition in early neural development, or if it is involved in pattern regulation, WNT-1 likely plays a significant role in the establishment and development of a large portion of the mid brain and rostral hind brain (McMahon and Bradley, 1990). By artificially elevating WNT-1 mRNA levels at a time when expression would normally be decreasing, the teratogenic exposure may significantly disrupt the relationship of WNT-1 to selected growth factors. A difference in the ratio of functional growth factors which may be reflected by the RNA differences in the developing embryonic tissue would cause extended or delayed responses, potentially accounting for the observed morphological abnormalities.

The growth factors that were significantly altered, NGF, NT3, TGFa, and TGFb were all upregulated at gestational day 10:0, but TRK expression was decreased. These genes play an important role in regulating proliferation and differentiation during development (Sporn, et al, 1987; Sporn and Roberts, 1988). NGF also binds to the TRK family of growth factor receptors (Chao, 1992). NGF is a tropic factor involved in cell survival and the maintenance of viability. Its premature lowering in day 9:12 embryos may prevent the survival of cells

essential to normal development. Nerve growth factor has been localized by in situ hybridization studies to the neural tube during early neurogenesis, suggesting it may play a wider role than previously thought in the differentiation of cells that make up the future central nervous system (Hallbook et al., 1990), while TRK expression is thought to be restricted to neurons of the sensory spinal and cranial ganglia of neural crest cell origin (Martin-Zanca et al., 1990; Kaplan et al., 1991). Neurotropin-3 is another growth and tropic factor which binds to the TRK receptors, whose mRNA expression in gestational day 9:12 embryos is comparable to the mRNA levels expressed by control specimens on day 10:0. As with NGF, NT3 aids in the viability of developing nerve cells, thus any alteration in its expression could disrupt the sequence of events leading to normal brain development. TGFβ1 has been shown to regulate angiogenesis and the synthesis of extracellular matrix (ECM) proteins such as fibronectin and collagen, which are critical to normal craniofacial development (Roberts, et al, 1986; Ignotz, et al, 1987). The alteration of expression of growth factors and receptors can cause serious perturbations in the normal morphogenic pathway.

Phenytoin appears to also alter the expression of insulin growth factors I and II. Just as in the case of many of the other candidate genes, at day 9:12 the level of expression of IGF-I and IGF-II have shifted to those observed in untreated day 10:0 embryos. On gestational day 10:0 the treated embryos express significantly elevated levels of IGF-I.

The expression of the transcription factors is similarly marked by a temporal shift in the level of relative abundance. In embryos collected on day 9:12, expression of c-FOS, c-JUN, and WNT-1 was not detected. Similarly, untreated day 10:0 embryos showed no appreciable expression of these genes. CREB however was upregulated at day 10:0. Transcription factors, and protooncogenes, such as these, regulate both cellular proliferation and/or

differentiation. Altered levels of these messages may lead to abnormal amounts of functional transcription factors, which would result in premature activating or inactivating of other genes.

The three membrane ion channels which were included in my study all responded to phenytoin treatment by significantly upregulated expression at day 10:0. Correct functioning of these genes is important to the formation of synaptic pathways both in the central nervous system as well as in the periphery. The teratogenic treatment elicited day 10:0 levels of expression at day 9:12 for the calcium channel gene, and at day 10:0, the treated embryos had excessive expression of this gene. The abnormal activation of calcium channels may change the embryo's sensitivity and responsiveness to calcium, contributing to subsequent changes in gene expression. Indeed, there are a number of genes whose expression is regulated by calcium both directly via a calcium responsive element (CaRE) and indirectly via calcium induced changes in post-translational regulation of cytoplasmic protein functioning. At day 9:12, the sodium channel gene does not appear to be influenced by phenytoin treatment, but at day 10:0 it is significantly upregulated. Sodium ions are critical to the generation of action potentials; therefore an increased responsiveness to sodium may elicit more neuronal activity at an earlier stage of development which would likely result in aberrant synaptic connections being reinforced and strengthened. Alternatively, sodium responsiveness may make neuroepithelial cells more permeable to fluid intake from hypoosmolar extracellular compartments, and might result in hypervolemia and increased blood pressure within the developing embryo (Grabowski, 1963, 1970). This series of events could result in either excessive cell death. The excessive fluid volume could similarly result in a disruption in the vascular supply to the developing neuroepithelium which might restrict cellular proliferation (Stevenson et al., 1987; Vogel,1961). It is even possible that the

hypervolemia leads to the distention of hollow structures, such as the neural tube, resulting in a disruption of previously fused regions and the development of exencephaly in the fetuses. The potassium channel mRNA levels were extremely high amongst the day 10:0 treated embryos. The ligand gated ion channel GABA-Aβ1 receptor is significantly upregulated by phenytoin in day 10:0 embryos, although it remained rather constant during the 12 hours from gestational day 9:12 to 10:0. There are at least 11 other subunits of the GABA-A receptor, each of which may be different in their expression pattern from the b1 subunit. Regulation of the individual subunits is important in understanding the functioning of this receptor system, as multiple subunits interact to form homo- and hetero-oligomeric receptors, each of which may have distinct pharmacological properties (Schofield et al., 1990). Alterations in the level of individual subunit mRNA levels may be a way of altering the GABA responsiveness of the cells within the developing embryo (Mackler and Eberwine, unpublished results).

The nicotinic acetylcholine receptor is also a multi-subunit ligand gated channel in which the subunit composition is important for developmental regulation of acetylcholine responsiveness. The expression of the a1-subunit in the gestational day 9:12 embryos was barely detectable, as in control day 10:0 embryos. This low level of expression was maintained as well in treated day 10:0 embryos. These changes, as with the GABA-Aβ1 receptor, suggest that dramatic changes in the heteromeric receptor containing the α1 subunit is altered by the teratogenic stimuli. This has implications for muscle development, where the α1 subunit is an essential component of the mature receptor complex (Stroud and Finer-Moore, 1985).

These studies clarify several points. The expression of many embryonic genes is altered throughout normal morphogenesis. In utero phenytoin

exposure significantly changes these normal patterns of gene expression, shifting the normal level of gene expression at gestational day 9:12 to those usually occurring 12 hours later at day 10:0. The changes in mRNA levels that are seen suggest that the corresponding protein levels are altered in a similar manner. While this may not be true for all of the molecules examined in this study, for those in which it is true, a change in functional protein levels may result in significant alterations in normal embryonic development. These data support the hypothesis that no one single gene is responsible for teratogen-induced changes in normal morphogenesis. Rather, it is the coordinate change of several molecules, each of which by itself may not be sufficient to elicit detectable developmental changes, but when combined together produce the adverse phenotypic changes that prevent normal early embryonic development. Reactive phenytoin intermediates may be such molecules. They may covalently bind to genes that are important regulators of cellular proliferation, such as transcription factors (c-FOS, c-JUN, WNT-1) and growth factors (IGF-I and II, NT3). By downregulating their expression, phenytoin may delay the normal temporal pattern of embryonic development, which would result in the appearance of congenital malformations. It would be instructive to investigate the actual levels of proteins present through immunohistochemistry, radioimmunoassays, and functional protein assays to determine whether the changes we have documented translate into comparable differences in protein levels. Additionally, it would be beneficial to explore phenytoin induced alterations in gene expression in isolated regions of the embryo. Such expanded investigations are currently being pursued in Dr. Richard Finnell's laboratory.

Footnotes

1. Abbreviations used throughout this manuscript refer to the following:

   SWV - Swiss-Vancouver

   cDNA - complimentary deoxyribonucleic acid

   RNA - ribonucleic acid

   aRNA - antisense ribonucleic acid

   RT/aRNA - reverse transcriptase antisense ribonucleic acid

   GD - gestational day

   IGF I & II - insulin-like growth factor

   NT3 - neurotrophin three

   GABA-A_{b1} - gamma amino butyric acid

   CREB - cyclic AMP response element binding protein

   CNTF - ciliary neurotrophic factor

   BDNF - brain derived neurotrophic factor

   NIC - nicotinic acetylcholine receptor

   TRK - tyrosine kinase receptor

   mRNA - messenger ribonucleic acid

   DEPC - diethylpyrocarbonate

   SSC - sodium citrate buffer

   SDS - sodium lauryl sulfate

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Table 1. Effect of phenytoin treatment on implants, resorptions, fetal weights, abnormalities, and maternal plasma phenytoin concentration

Strain	Phenytoin treatment (mg/kg)	Implants (No.)	Resorptions (%)	Fetal weight (g) Mean ± SEM	Abnormalities (%)	Plasma phenytoin (μg/mL) Mean ± SEM
SWV*	0	121	11	0.81 ± .01	6	0
	20	119	15	0.73 ± .01	41	4.13 ± .51
	40	118	18	0.68 ± .02	53	7.77 ± .58
	60	115	14	0.64 ± .01	85	11.03 ± 1.48
LM/Bc*	0	102	4	0.99 ± .01	4	0
	20	102	13	0.86 ± .01	40	3.49 ± .42
	40	92	20	0.84 ± .01	40	7.13 ± .55
	60	94	15	0.82 ± .01	56	9.54 ± .42
C57BL/6J†	0	127	5	1.04 ± .02	3	0
	20	113	6	0.78 ± .02	38	3.34 ± .42
	40	116	11	0.75 ± .02	62	6.74 ± .50
	60	114	23	0.73 ± .01	75	11.83 ± .45

*Ten liters per treatment.
†Fifteen liters per treatment.

Table 2. Effects of phenytoin treatment in quaking dams on plasma phenytoin concentrations, seizure activity, and abnormalities

Phenytoin treatment (mg/kg)	Phenytoin plasma concentration (μg/mL) Mean ± SEM	Seizure activity (per mouse day) Mean ± SEM	Malformed fetuses (%)
0	0	2.06 ± .14	1
10	1.7 ± .02	2.04 ± .21	17
20	4.1 ± .05	1.96 ± .27	29
40	7.4 ± .06	0.79 ± .24	61
60	12.4 ± .19	0.34 ± .15	77

Table 3. Desitometric Analysis of Embryonic Gene Expression

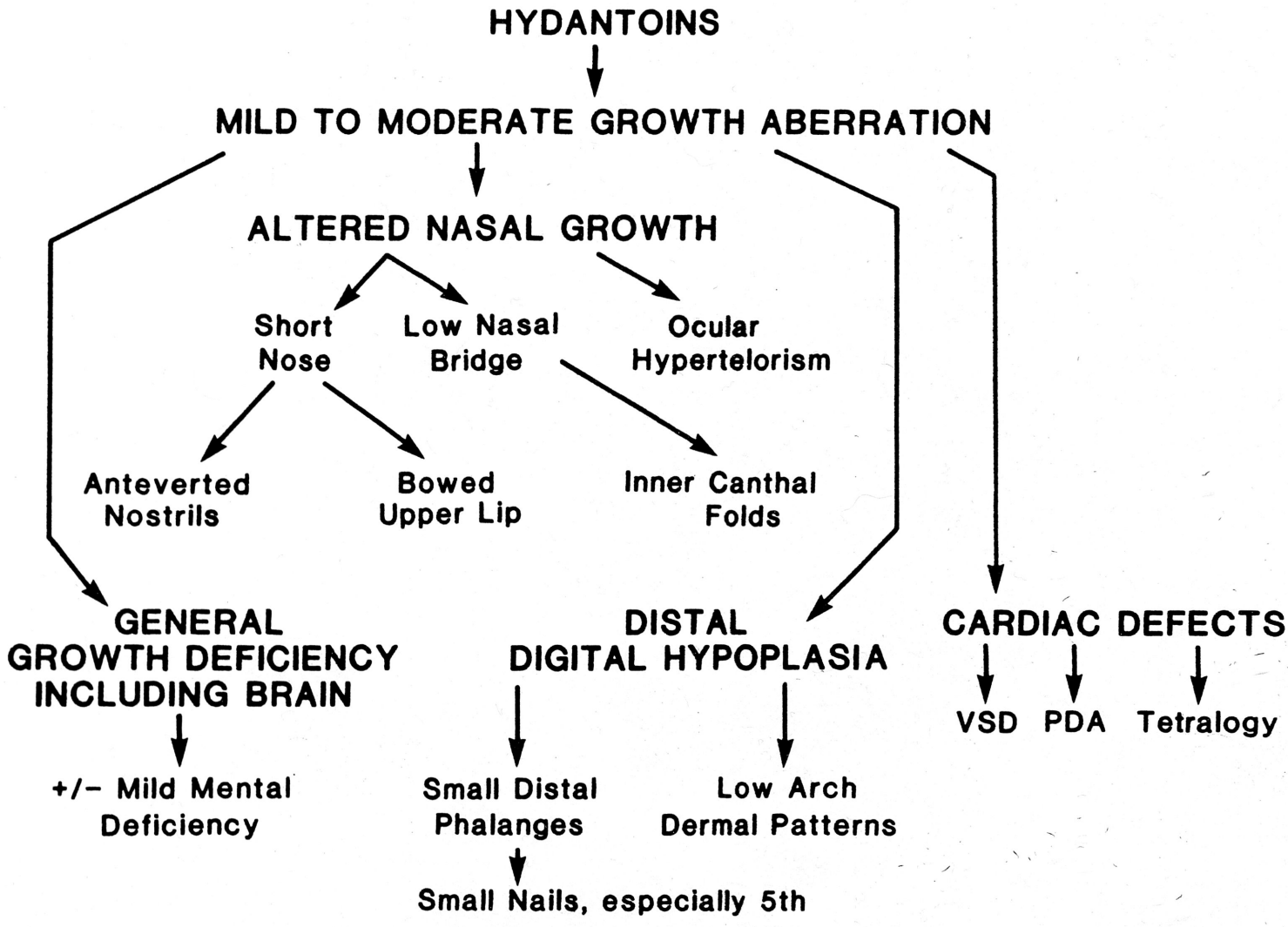
Candidate Genes	Gestational Day 9:12	
	Control mean ± SEM	Phenytoin Treated mean ± SEM
Transcription Factors		
C-FOS	0.178 ± 0.12	0.041 ± 0.02
C-JUN	0.279 ± 0.24	0.049 ± 0.03
CREB	0.995 ± 0.20	0.864 ± 0.09
WNT-1	0.957 ± 0.43	0.031 ± 0.02
FOLATE	0.358 ± 0.15	0.378 ± 0.13
GABA	0.296 ± 0.15	0.328 ± 0.11
NIC	0.335 ± 0.16	0.008 ± 0.00
Membrane Channels		
CA^{2+}	1.145 ± 0.52	0.043 ± 0.03
KV1	1.141 ± 0.45	0.396 ± 0.10
NA^{+}	0.927 ± 0.14	0.836 ± 0.11
Growth Factors & Receptors		
BDNF	1.308 ± 0.63	0.548 ± 0.10
CNTF	1.623 ± 0.65	0.313 ± 0.08
IGF-I	0.026 ± 0.03	0.067 ± 0.04
IGF-II	0.649 ± 0.39	0.025 ± 0.01
NGF	0.680 ± 0.15	0.955 ± 0.10
NT3	0.132 ± 0.05	0.012 ± 0.00
TGFα	0.962 ± 0.44	0.136 ± 0.04
TGFβ1	1.641 ± 0.69	0.267 ± 0.13
TRK	3.709 ± 2.52	2.996 ± 0.49

Table 4. Densitometric Analysis of Embryonic Gene Expression

Candidate Genes	Gestational Day 10:0	
	Control mean ± SEM	Phenytoin Treated mean ± SEM
Transcription Factors		
C-FOS	0.060 ± 0.02	0.702 ± 0.42
C-JUN	0.034 ± 0.01	0.603 ± 0.29
CREB	0.825 ± 0.11	1.097 ± 0.46
WNT-1	0.045 ± 0.02	1.343 ± 0.58
FOLATE	0.224 ± 0.06	0.233 ± 0.11
GABA	0.147 ± 0.04	0.416 ± 0.18
NIC	0.031 ± 0.02	0.097 ± 0.06
Membrane Channels		
CA^{2+}	0.097 ± 0.06	3.174 ± 1.96
KV1	0.211 ± 0.04	3.569 ± 2.04
NA^{+}	0.714 ± 0.07	0.874 ± 0.24
Growth Factors & Receptors		
BDNF	0.671 ± 0.10	0.974 ± 0.18
CNTF	0.348 ± 0.09	0.532 ± 0.18
IGF-I	0.003 ± 0.00	2.472 ± 2.16
IGF-II	0.037 ± 0.01	0.033 ± 0.01
NGF	0.658 ± 0.06	1.720 ± 0.63
NT3	0.100 ± 0.03	0.220 ± 0.12
TGFα	0.088 ± 0.02	1.094 ± 0.48
TGFβ1	0.082 ± 0.02	5.373 ± 2.96
TRK	6.997 ± 2.97	1.343 ± 0.49

Legend

Figure 1. Schematic Diagram of the Effects of Prenatal Hydantoin Exposure



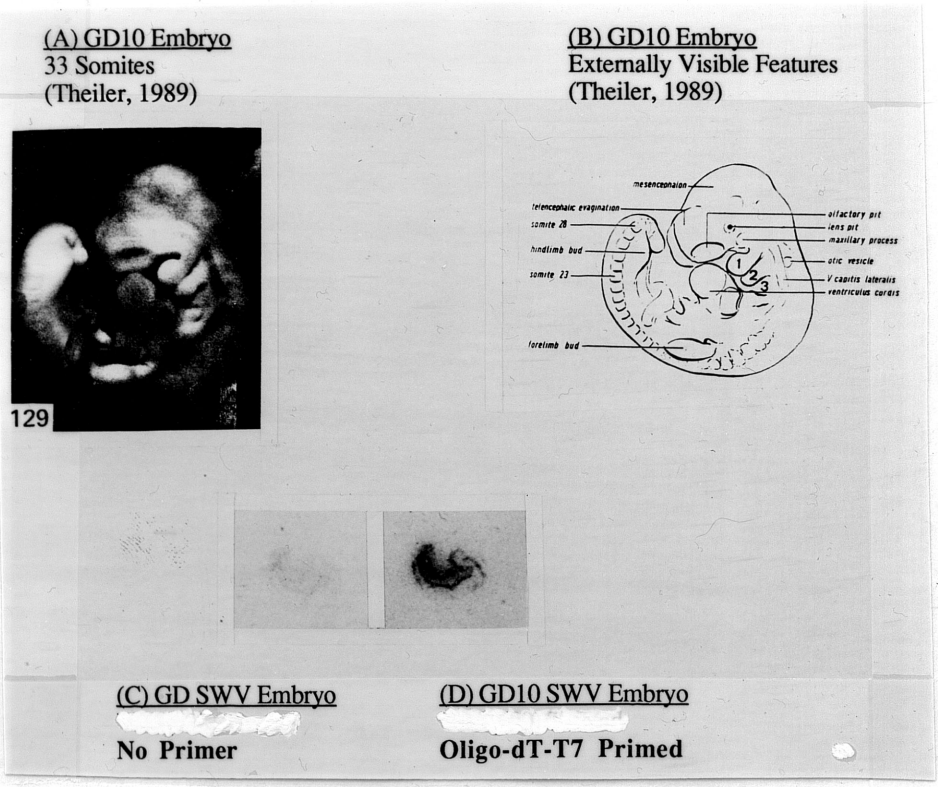


Figure 2. In situ transcription reactions on 8 μ thick mid-sagital section of gestational day 10:0 SWV embryos. (A) This section did not receive the oligo-dT-T7 amplification oligonucleotide, and only an endogenous level of binding is apparent. (B) This section was allowed to hybridize overnight at 37°C in the presence of the oligo-dT-T7 oligonucleotide. The intense binding of this embryo section in areas of rapid cellular proliferation represents areas of successful cDNA synthesis.

Figure 3a. Schematic of In Situ Transcription Procedure

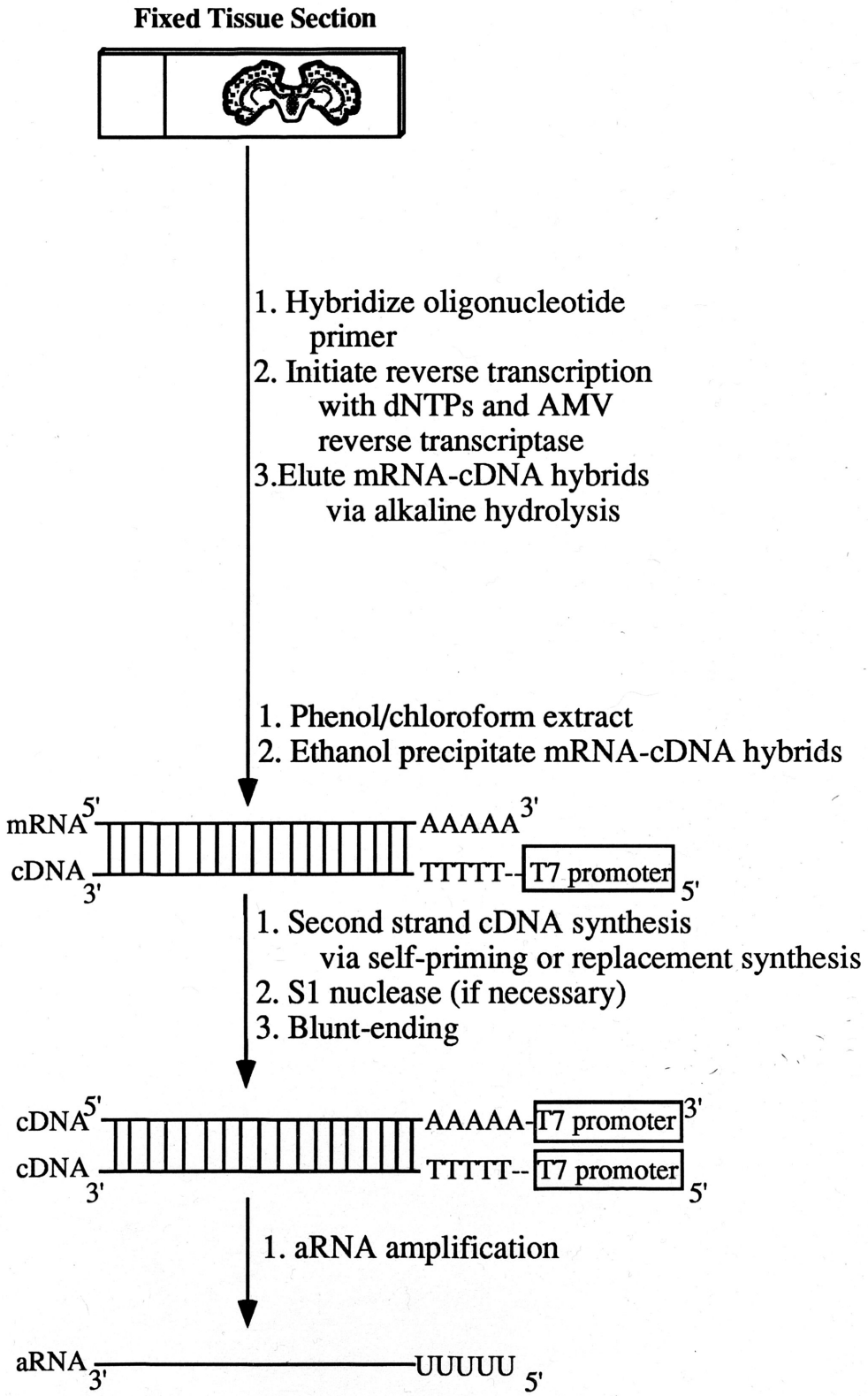
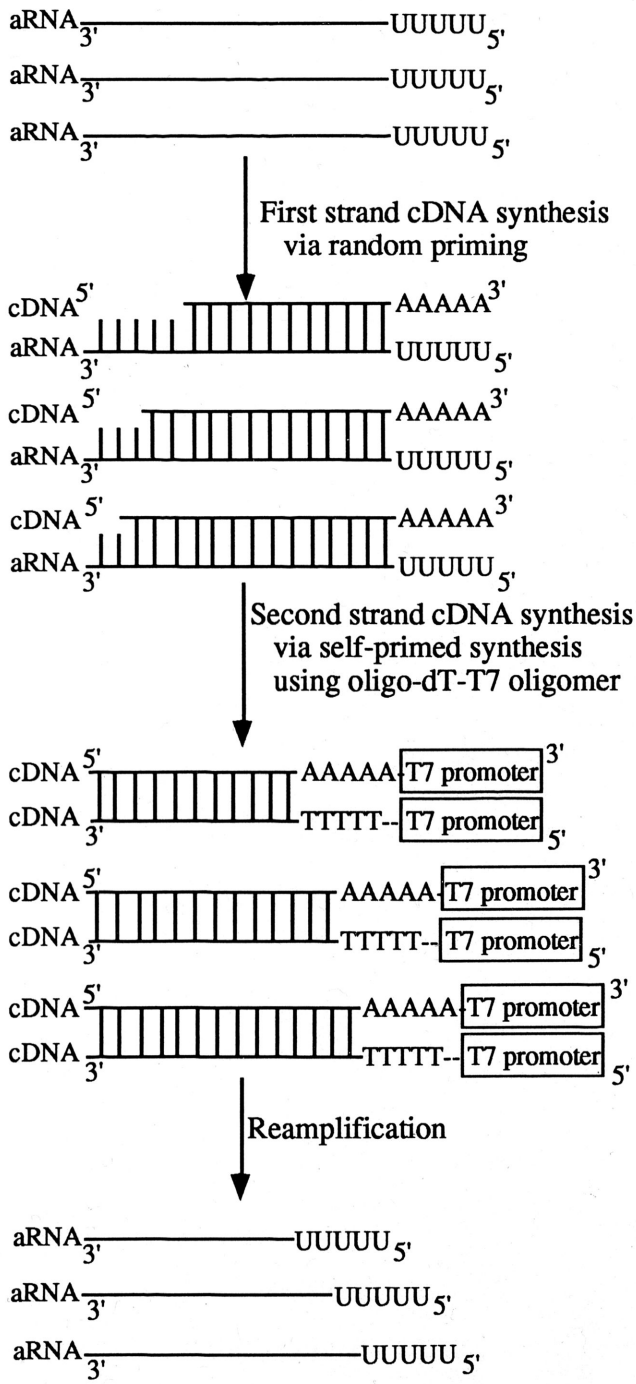


Figure 3b. Schematic of the aRNA Amplification Procedure



A schematic for reamplification of oligo-dT-T7 primed, IST-derived transcripts

Figure 4.

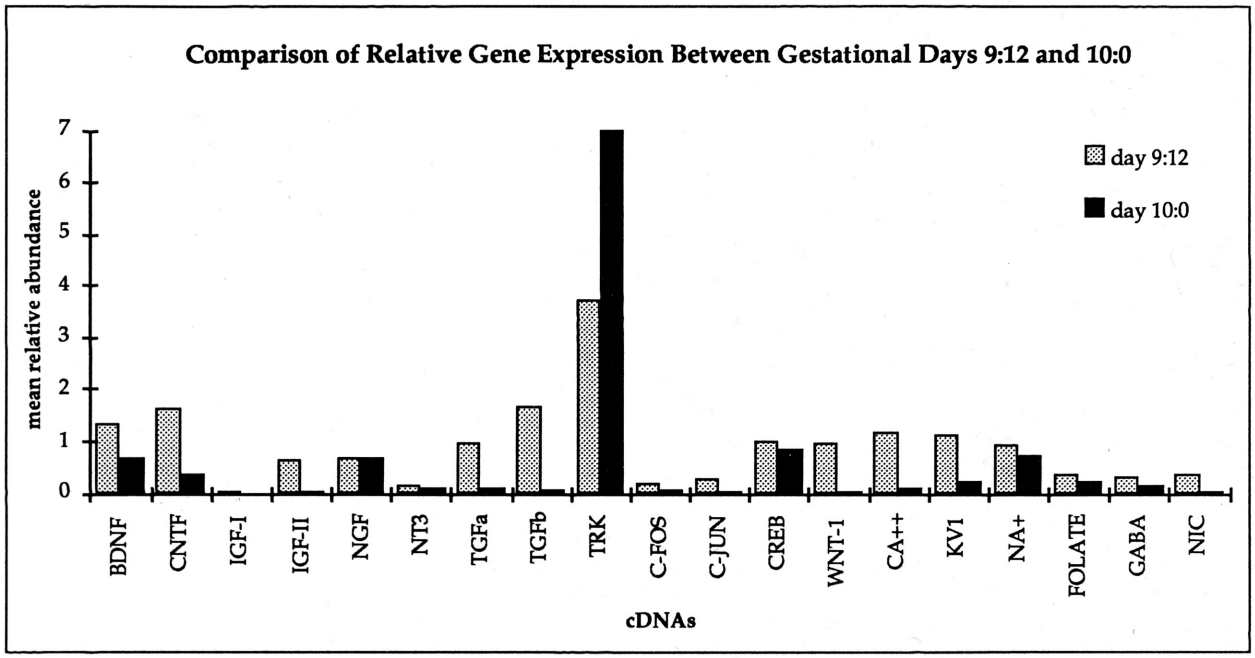
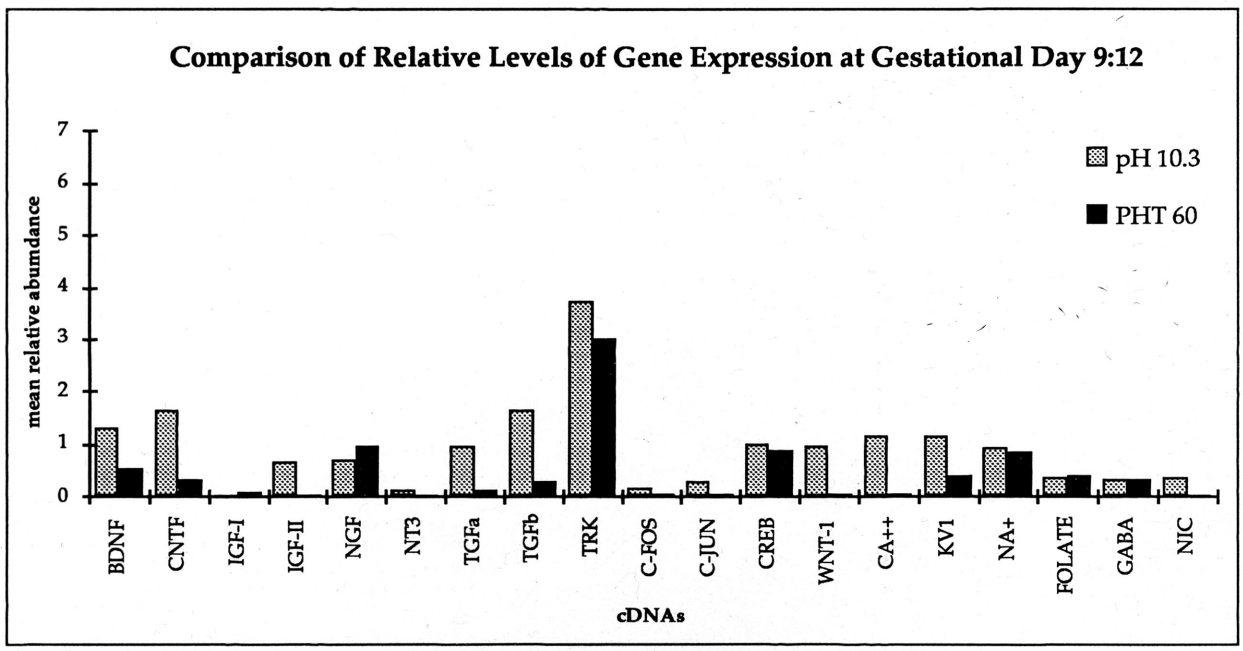


Figure 5.



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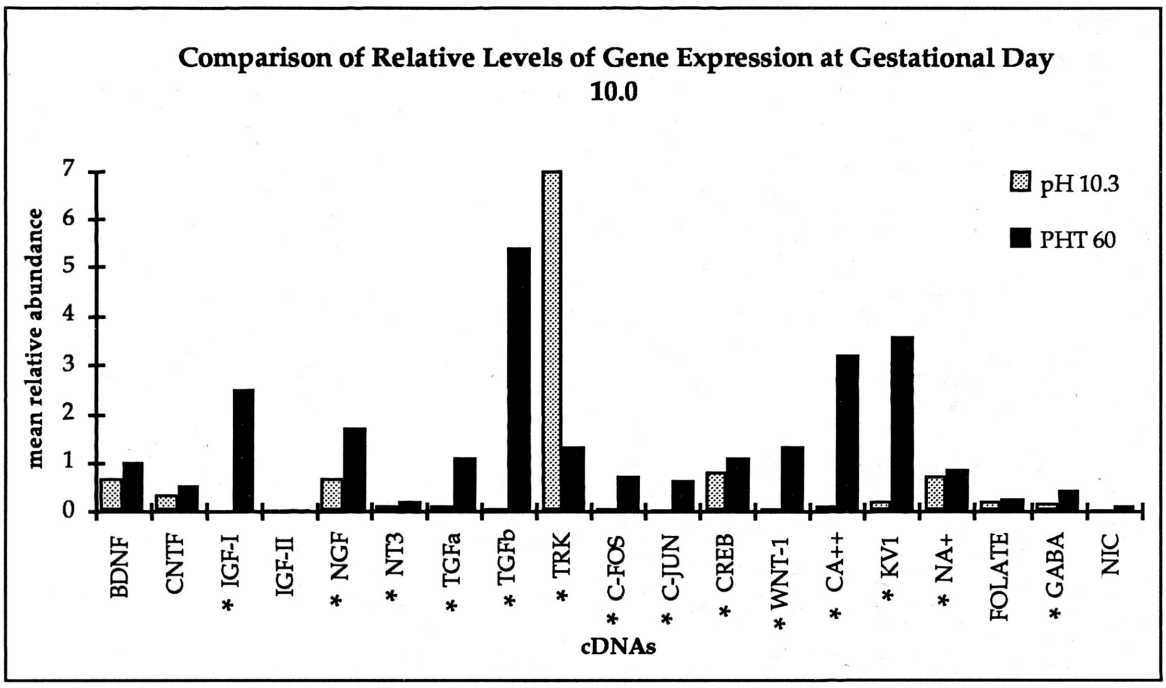

* denotes a statistically significant alteration

Figure 7.

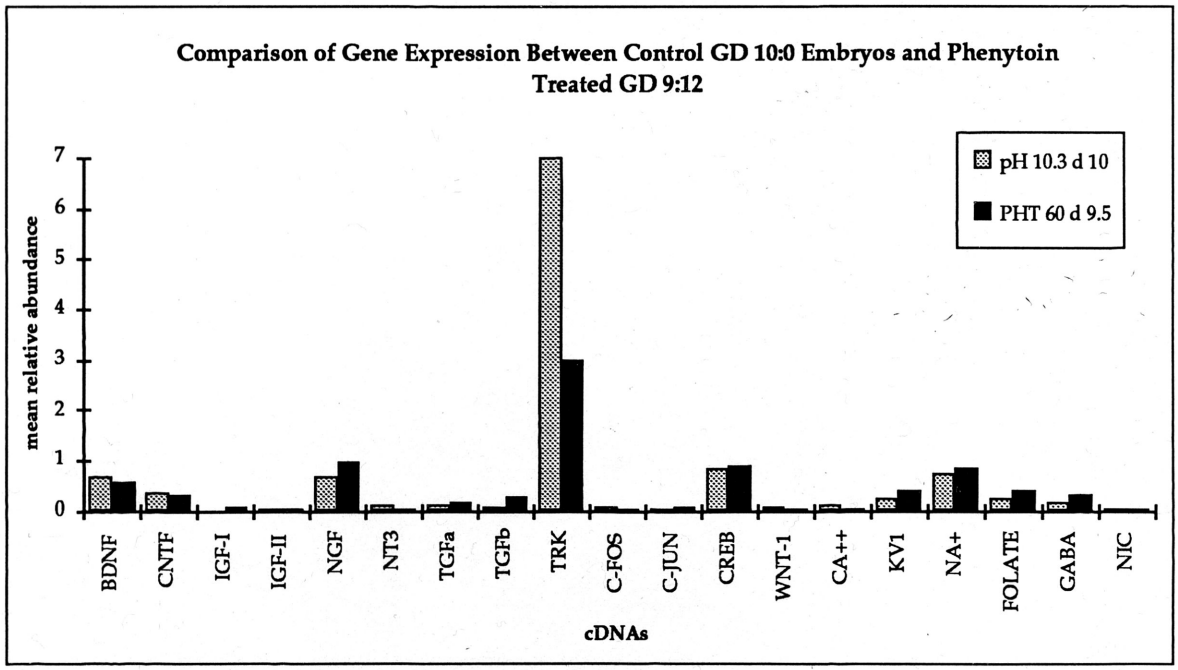

Figure 8. Expression profile for an untreated SWV embryo at gestational day 9.5

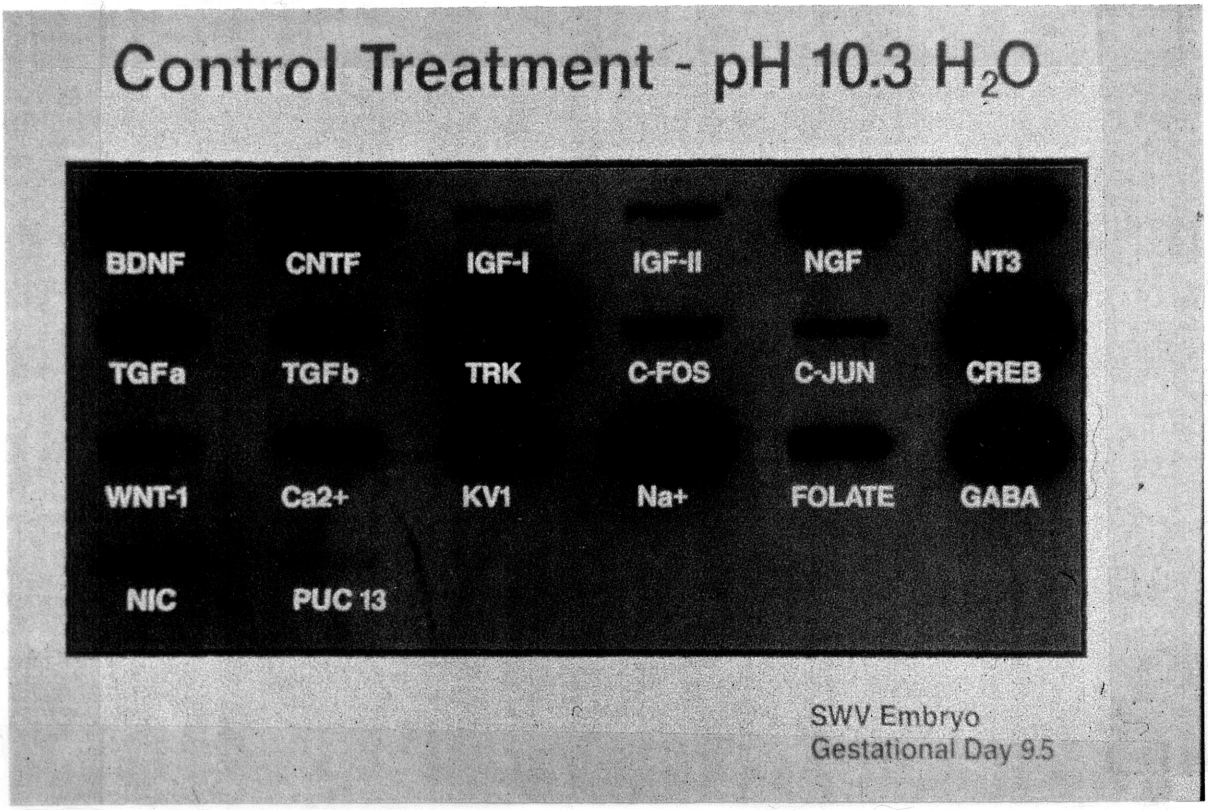

Figure 9. Expression profile for a phenytoin-treated SWV embryo at gestational day 9.5