Oxidative Stress Regulates the Expression and Activity of Transcription Factor Activator Protein-1 in Rat Conceptus1

TERENCE R. S. OZOLINŠ and BARBARA F. HALES
Department of Pharmacology and Therapeutics, McGill University, Montréal, Québec, Canada
Accepted for publication October 21, 1996

ABSTRACT
The transcription factor activator protein-1 (AP-1), composed of the Fos and Jun families of proto-oncogenes, is induced in response to extracellular signals as part of an immediate-early gene response. We hypothesize that teratogens such as oxidative stress induce AP-1 activity in the rat conceptus and that this AP-1 response may either trigger abnormal development or protect the embryo against insult. To test this hypothesis, the AP-1 response was assessed in whole embryos in culture. There was a significant elevation in the oxidized to reduced glutathione ratio in the embryo and yolk sac within 0.25 hr of the initiation of culture, peaking at 0.5 hr; this is indicative of heightened oxidative stress. At 0.5 hr protein oxidation was also enhanced, as demonstrated by increased protein reactivity with 2,4-dinitrophenylhydrazine. In the conceptus, the steady-state concentrations of c-fos, c-jun, junB and junD mRNAs were induced, peaking at 0.5 hr and returning to base line by 1 to 2 hr in the embryo and by 1 to 6 hr in the yolk sac. Electrophoretic mobility shift assays showed enhanced AP-1 DNA-binding activity in both the embryo (elevated by 0.5 hr and persisting for 1 hr) and the yolk sac (persisting for 3 hr). Thus, there are tissue-specific differences in the duration of the AP-1 response in the conceptus. Addition of the antioxidants catalase and superoxide dismutase, but not vitamin E, prevented the rise in the oxidized to reduced glutathione ratio and also inhibited the induction of AP-1 mRNAs and DNA-binding activity. The AP-1 response to oxidative stress may determine how the conceptus responds to insult.

Normal embryo development is contingent upon the tight regulation of a myriad of transcription factors to ensure the appropriate temporal and spatial activation of a number of developmentally important gene products. Thus, inappropriate gene expression may lead to abnormal embryo development. Many teratogens alter gene expression, but little is known about the transcription factors that initiate these changes. The fos and jun families of cellular proto-oncogenes encode nuclear proteins (Fos and Jun, respectively), which form heterodimers (Fos-Jun) or homodimers (Jun-Jun) referred to as AP-1. These dimers recognize the DNA consensus sequence TGACTCA, identified as the recognition sequence for AP-1 (Angel et al., 1988; Curran and Franza, 1988).

AP-1 is a model transcription factor for studies examining how embryos respond to teratogens, for a number of reasons. First, the use of antibody microinjection techniques (Nishikura and Murray, 1987; Kovary and Bravo, 1991) and antisense RNA (Holt et al., 1986; Riabowol et al., 1988; Smith and Prochownik, 1992) has implicated AP-1 as a key element in such basic developmental functions as cell cycle progression. Transfection (de Groot et al., 1990) and antisense RNA (Schlingensiepen et al., 1993) studies have demonstrated the importance of AP-1 during differentiation. Treatment of cells with heat shock (Andrews et al., 1987), heavy metals (Gubits and Fairhurst, 1988) and alkylating chemicals (Futschek and Erickson, 1990; Galtar et al., 1994), all of which are teratogens, induced AP-1 mRNA expression and DNA-binding activity. Furthermore, cell survival after UV irradiation was contingent upon the ability to elicit an AP-1 response (Devary et al., 1992). Together, these data suggest that redox-induced aberrations in AP-1 activity may either trigger abnormal development or evoke long-term transcriptional changes that protect embryos from oxidative stress.

Glutathione, the most abundant nonprotein thiol (Meister, 1976), exists in oxidized and reduced forms. The predominant cellular form is GSH. The GSSG:GSH ratio is tightly regulated, thus maintaining cellular redox balance. Depletion of GSH or the generation of GSSG increases the GSSG:GSH ratio, reflecting an increase in oxidative stress. Agents that induce oxidative stress (Harris et al., 1987; Wong and Wells, 1989) or deplete GSH (Slott and Hales, 1987; Wong et al., 1989) are embryotoxic. During cellular oxidative stress,

ABBREVIATIONS: AP-1, activator protein-1; Cat, catalase; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; GSH, reduced glutathione; GSSG, oxidized glutathione; kb, kilobases; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; SSC, standard saline citrate; TBS-T, Tris-buffered saline/Tween 20; VitE, vitamin E.
the preferential oxidation of GSH to GSSG protects cellular macromolecules and cell function. However, in embryos, glutathione and a number of antioxidant enzyme activities, such as Cat, SOD, and glutathione peroxidase (Di Ilio et al., 1986; El Hage and Singh, 1990; Serafini et al., 1991; Ozolins et al., 1996), are lower than in adults; embryonic proteins may not be protected adequately during transient rises in oxidative stress.

AP-1 is induced after in vivo treatment with oxidizing agents (Amstad et al., 1992; Maki et al., 1993; Meyer et al., 1993; Rao et al., 1993; Galter et al., 1994) or agents that deplete GSH (Futschek and Erickson, 1990; Bergelson et al., 1994). Aberrations in embryonic redox state may lead to transcriptional changes incompatible with normal development.

Therefore, the purpose of this study was to examine AP-1 regulation in the conceptus when glutathione homeostasis was altered. Conceptuses were studied from early organogenesis to mid-organogenesis, the period of development most sensitive to teratogenic insult.

Methods

Embryo culture. Timed-gestation pregnant Sprague-Dawley rats (200–225 g; Charles River Canada Ltd., St. Constant, QC, Canada) were housed in a temperature-controlled environment with an automatic 12-hr light/dark cycle (7:00 a.m. to 7:00 p.m.). Rat Chow (Purina, St. Louis, MO) and tap water were available ad libitum.

Analysis of proteinoxidation using Western blots. To assess the amount of carbonyl groups formed as a consequence of protein oxidation (Keller et al., 1993), after protein determination (Bio-Rad Laboratories Ltd.), 10 µg of protein was added to the sample loading buffer (62.5 mM Tris-HCl, pH 6.8, 12% glycerol, 2% SDS, 5% β-mercaptoethanol), boiled for 10 min and fractionated by using 10% polyacrylamide gel electrophoresis (Laemmli, 1970). Bio-tinylated low-molecular weight markers (1 µg; Amersham Canada Ltd.) were used as molecular weight standards. The proteins were transferred (Towbin et al., 1979) to Hybond PVDF membranes (Amersham Canada Ltd.). Blocking was performed overnight on an orbital shaker at 4°C with 5% skim milk powder and 1% bovine serum albumin fraction V (Sigma Chemical Co.) in TBS-T (137 mM NaCl, 20 mM Tris, pH 8, 0.1% Tween-20). The membrane was washed three times for 10 min in TBS-T and incubated for 1 hr at room temperature with anti-diphenylhydrazine antiserum (1:2500); Sigma Chemical Co.) in 1% skim milk powder in TBS-T. After two 10-min washes, the membrane was incubated for 1 hr at room temperature with horseradish peroxidase-linked anti-rabbit antibody (1:5000) and horseradish peroxidase-linked streptavidin in TBS-T; the signal was detected using enhanced chemiluminescence (Amersham Canada Ltd.).

Northern blot analysis. Total RNA (10 µg) from up to 12 embryos and yolk sacs was obtained using single-step guanidinium isothiocyanate extraction (Chomczynski and Sacchi, 1987). RNA (1 µg) from a neuroepithelial cell line chronically exposed to platelet-derived growth factor served as a positive control. RNA samples were fractionated on a 1% agarose gel containing 6% formaldehyde and were transferred onto a nylon membrane (GeneScreen-Plus; New England Nuclear, Mississauga, ON, Canada) with a vacuum blotting system (Pharmacia Biotech. Inc. Canada, Baie D’Urfe, QC, Canada), using 50 mM NaOH/10 mM NaCl for 20 min; 0.1 M Tris-HCl, pH 7.4, for 20 min and 20 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) for 90 min. The membrane was baked in a vacuum oven for 2 hr at 80°C. Northern blot analyses were done with cDNA probes for c-fos (Miller et al., 1984), c-jun (Ryder and Nathans, 1988), junB (Ryder et al., 1988) and junD (Ryder et al., 1989) obtained from the American Type Culture Collection. Probes were labeled by random priming with [32P]dCTP (Amersham Canada Ltd., Oakville, ON, Canada) with an oligolabeling kit (Pharmacia Biotech Inc. Canada). Hybridization was carried out overnight at 42°C in 50% formamide, 1% SDS, 1 M NaCl, 10% dextran sulfate, 0.2 mg/ml denatured salmon sperm DNA. The membrane was washed twice for 5 min at room temperature in 2 × SSC, followed by two washes for 20 min at 65°C in 2 × SSC/0.1% SDS. Autoradiography was done at ~80°C for 7 to 10 days, using intensifying screens. The membranes were exposed to 0.1 × SSC/1% SDS to permit reprobing. To normalize to the amount of RNA loaded in each lane, membranes were probed with a 32P-end-labeled (T4 kinase; Pharmacia Biotech. Inc. Canada) synthetic oligonucleotide (24-mer) recognizing the 18S rRNA sequence (Bzyf et al., 1990).

Quantification of autoradiograms. Three to five autoradiograms from separate experiments were probed for fos, jun and 18S rRNA and scanned with a laser densitometer (LKB Ultrascan laser densitometer; Pharmacia Biotech. Inc. Canada). The 18S rRNA signal was used to normalize to the amount of RNA loaded. The signals were then expressed relative to 0 hr.

EMSA. Embryo and yolk sac extracts were prepared as described by Scholler et al., (1989), using a modified lysis buffer that contained 25% glycerol, 450 mM NaCl, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.5 mM DTT, 0.2 mM EDTA, 40 µg/ml bestatin, 1.0 µg/ml aprotinin, 0.7 µg/ml pepstatin, 0.5 µg/ml leupeptin, 0.5 µg/ml calpain inhibitor peptide, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM NaF and 5 mM Na3VO4, pH 7.5. After several seconds of sonication with an ultrasonic processor (small tip, 25% output; Sonics and Materials, Inc., Danbury, CT), the extracts were cleared of particulate matter using an Eppendorf microfuge (15,000 × g for 30 sec). Protein content was determined in triplicate (Bradford, 1976) (Bio-Rad Laboratories, Mississauga, ON, Canada);
and extracts were adjusted to the same protein concentration with whole-cell lysis buffer. Samples were flash frozen in liquid N₂ and stored in aliquots at −80°C.

The DNA fragment (21-mer) containing the human collagenase AP-1 binding site (CGCTTGATGTCAGCCGGAA) (Angel et al., 1987) was 32P-labeled with Klenow fragment (Pharmacia Biotech, Inc. Canada) using a GCGAAC primer. Labeled oligonucleotides were purified from unincorporated nucleotides by chromatography on a Sephadex G-50 column.

Whole-cell extracts (15 μg protein) and nuclear extracts (5 μg protein) from HeLa cells (Promega Corp., Toronto, ON, Canada), which served as a positive control, were preincubated for 20 min at room temperature in 12% glycerol, 0.1% Nonidet P-40, 20 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid, 4 mM DTT, 3 mM MgCl₂, 1 mM EDTA, 2.5 mM poly(deoxyinosinic/cytidylic acid, 2.5 μg bovine serum albumin (RNase/DNase-free) (Chodosh, 1988). For the experiments performed with antioxidants, the binding buffer contained 0.4 mM DTT. Samples were reacted with approximately 15,000 cpm of oligonucleotide for 20 min at room temperature and electrophoresed on 6 or 8% polyacrylamide gels (250 mM Tris, 1.9 M glycine, 5 mM EDTA). Gels were dried and autoradiographed was done overnight at −80°C. Autoradiograms were quantified laser-densitometrically, and values were expressed relative to those obtained at 0 hr. Polyacrylamide gels from the experiments on the effects of antioxidants were transferred onto stacked PVDF membranes and Gene Screen membranes (Demczuk et al., 1993). The proteins, bound to PVDF membranes, were stained with Ponceau S and scanned laser-densitometrically to normalize to the amount of protein loaded in each lane. The radiolabeled oligonucleotide bound to Gene Screen membranes was detected by autoradiography. To confirm specificity of binding, samples were preincubated with a 400-fold molar excess of the corresponding unlabeled oligonucleotide, a mutated AP-1 oligonucleotide (CGCTTGATGTCAGCCGGAA) (Angel et al., 1987; Lee et al., 1987) or an AP-1 oligonucleotide (GATCGATCGGGGCGGGCGATC) (Anderson and Freytag, 1991).

**Statistical analysis.** Statistical analysis was performed by one-way analysis of variance using the Complete Statistics System computer program (Statssoft, Tulsa, OK), followed by post hoc Tukey’s test. The a priori level of significance was P < .05.

**Results**

**Oxidative stress in the conceptus as indicated by an increase in GSSG:GSH ratio.** The existence and time course of oxidative stress in the embryo were assessed by determining the redox state of glutathione (GSSG:GSH ratio). In the embryo (fig. 1, A and C), GSH was relatively constant, at approximately 20 nmol/mg protein during the culture period; a small but significant increase was observed at 24 hr (fig. 1A). To account for any possible compensatory increases in glutathione biosynthesis that may occur in response to redox changes, oxidative stress was expressed as the GSSG:GSH ratio. Before culture, in naive embryos (unexposed to medium gassed with 20% O₂), this ratio was 0.045 (fig. 1C). A significant increase in the GSSG:GSH ratio occurred within 0.25 hr of the initiation of culture and was sustained until 1 hr, after which the GSSG:GSH ratio returned to the 0.05 to 0.06 range. In the yolk sac during the 2-day culture period (fig. 1, B and D), GSH was constant and in the same range as for the embryo (fig. 1B). The magnitude and duration of the increased GSSG:GSH ratio in the yolk sac were similar to those noted in the embryo (fig. 1D).

**Increased oxidative stress resulting in the oxidation of cellular proteins.** The hypothesis that embryonic proteins may not be protected adequately during the transsient rise in the GSSG:GSH ratio was tested by examining the ability of embryo and yolk sac proteins to react with 2,4-dinitrophenylhydrazine; 2,4-dinitrophenylhydrazine forms a covalent bond with carbonyl moieties generated as a result of protein oxidation (Keller et al., 1993). The proteins derivatized with 2,4-dinitrophenylhydrazine were detected using anti-dinitrophenyl antiserum (fig. 2). In the embryo at 0 hr (fig. 2A), one protein band with an apparent molecular mass of 35 kDa reacted with the anti-dinitrophenyl antiserum, suggesting a basal level of protein oxidation. At 0.5 hr, three additional bands, at 40, 46 and 65 kDa, were detected and the signal at 35 kDa was intensified. Multiple protein bands reacted with 2,4-dinitrophenylhydrazine in the

![Fig. 1.](image1.png) **GSH and the GSSG:GSH ratio in embryos and yolk sacs at various times after the initiation of culture.** Day 10.5 whole-rat embryos were cultured in vitro for up to 48 hr and were analyzed at the specified times for total cellular GSH and GSSG. GSH in embryos (A) and yolk sacs (B) is expressed as nanomoles per milligram of protein. Oxidative stress, as reflected by increased GSSG production with no compensatory increase in GSH synthesis, is represented by the GSSG:GSH ratio (C and D). Each bar (mean ± S.E.) represents at least four separate embryo or yolk sac samples, assayed in triplicate. *, significant difference (P < .05) from 0 hr, as determined by analysis of variance.

![Fig. 2.](image2.png) **Protein oxidation in the embryo and the yolk sac.** Embryo (A) and yolk sac (B) samples were collected at time 0 and 0.5 hr after the initiation of culture, homogenized and incubated for 1 hr at room temperature in the presence of 0.5 mM 2,4-dinitrophenylhydrazine. Proteins samples (10 μg) were separated by polyacrylamide gel electrophoresis, followed by immunoblotting with anti-diphenylhydrazine antiserum and chemiluminescent detection. The positions of the molecular weight markers are indicated in the center.
yolk sac at 0 hr (fig. 2A). Four such bands displayed molecular masses similar to those in the embryo; there was an additional band at 27 kDa. The signals of all of the bands except the 27-kDa band were enhanced at 0.5 hr. Therefore, within 0.5 hr, coincident with the maximum rise in the GSSG:GSH ratio after the initiation of culture, significant protein oxidation did occur in both the embryo and the yolk sac. These data indicate that glutathione was unable to protect specific proteins from the oxidative effects of culture.

**Induction of AP-1 mRNAs in the rat conceptus.** To determine the time course of the AP-1 response to oxidative stress in rat conceptuses, embryos (fig. 3A) and their yolk sacs (fig. 3B) were removed from culture at different times between 0 and 48 hr. A platelet-derived growth factor-treated neuroepithelial cell line served as a positive control. Northern blot analysis showed single-molecular size transcripts for c-fos (2.2 kb), junB (2.1 kb) and junD (1.7 kb), whereas two bands (3.1 and 2.6 kb) were observed for c-jun. Transcripts for c-fos, c-jun and junB were present at low steady-state concentrations in the embryo at 0 hr (on day 10 of gestation); the mRNA for junD was present in the embryo constitutively at this stage of development. Similar to the embryo, the jun family members (c-jun, junB and junD) were all present in the day 10 (time 0) yolk sac.

Marked increases in the steady-state contents of c-fos, c-jun, junB and junD mRNAs were observed in both embryos and yolk sacs shortly after the initiation of the culture period. The results of quantification of the autoradiograms from three to five separate experiments are presented in figure 4. To permit examination of the relative induction, the mRNA content of each AP-1 member in the day 10 (0 hr) embryo or yolk sac was set at 1. Significant induction of the steady-state content of all AP-1 mRNAs was observed in the embryo shortly after the initiation of culture. This induction was maximal at 0.5 hr for all four transcripts (fig. 4). In the embryo (fig. 4A), the steady-state concentrations of the c-fos (8-fold) and c-jun (7-fold) mRNAs were most dramatically induced. The messages for junB (4-fold induction) and junD (5-fold) were less responsive. The steady-state mRNA concentrations of c-fos, c-jun and junB returned to base line by 1 hr, whereas junD returned only after 2 hr.

Similar to the embryo, the yolk sac AP-1 mRNAs were maximally induced at 0.5 hr after the initiation of embryo cultures (fig. 4B), but some of the responses were more pronounced and prolonged. Within 0.25 hr, c-fos mRNA demonstrated a significant induction that was maximal (12-fold) at 0.5 hr (fig. 4B). In contrast to the situation in the embryo, c-fos mRNA had not returned to basal levels by 3 hr. The increases in the steady-state concentrations of the mRNAs for c-jun, junB and junD at the 0.5-hr time point were 6-, 5- and 4-fold, respectively. Whereas c-jun message had returned to base line by 1 hr, junB and junD did so by 2 hr. These data suggest that there are tissue-specific differences in the AP-1 response of the embryo and the yolk sac during culture.

**Induction of AP-1 DNA-binding activity in the embryo and the yolk sac.** To investigate whether culture-induced oxidative stress stimulated AP-1 DNA-binding activity in the conceptus, as well as induction of fos and jun mRNAs, an in vitro assay for active Fos and Jun proteins was performed. EMSA was used to measure AP-1 DNA-binding activity with a double-stranded radiolabeled oligonucleotide containing the AP-1 consensus sequence (TGAGTCA). In the presence of either whole-cell (conceptus samples) or nuclear (HeLa cells) extracts, a single AP-1 DNA-binding complex was observed in the embryo (fig. 5A) and the yolk sac (fig.
In the absence of added protein (control), the migration of the radiolabeled oligonucleotide was unimpeached. Further specificity of the protein/DNA interaction was demonstrated in several ways; 1) the DNA binding was dependent upon the amount of nuclear protein, 2) a 200-fold molar excess of nonradiolabeled AP-1 oligonucleotide completely abolished the autoradiographic signal, whereas a 400-fold molar excess of an oligonucleotide containing the unrelated SP-1 consensus sequence had no effect on binding, and 3) an excess of a mutated AP-1 oligonucleotide only partially inhibited the binding (data not shown). When HeLa cells were used, this mutated sequence did not interfere with binding, perhaps reflecting species differences in AP-1 binding specificity. Thus, the retained complex formed with the radiolabeled AP-1 oligonucleotide and the proteins present within the embryo and yolk sac extracts is AP-1.

Quantiﬁcation of the EMSA data is depicted in ﬁgure 6. In the embryo, at 0.5 hr, there was a 6-fold increase in DNA-binding activity; binding activity remained elevated at 1.0 hr, returning to base line by 1.5 hr (ﬁg. 6A). DNA-binding activity in the yolk sac (ﬁg. 6B) also peaked at 0.5 hr (4-fold), but the increased binding activity persisted in this tissue until 3 hr after the initiation of culture.

**Effects of antioxidants on glutathione homeostasis and the AP-1 response in the conceptus.** To further explore the changes in glutathione homeostasis and in the AP-1 response in the conceptus at the onset of culture, embryos were cultured for either 30 or 90 min in the presence of one of three antioxidants, i.e., Cat, SOD or VitE. The ﬁrst time point (30 min) corresponds to the peak increase in the GSSG:GSH ratio and maximal induction of AP-1 mRNA concentrations and DNA-binding activity. The 90-min time point allows determination of whether antioxidants delay the onset of culture-induced changes in glutathione and the AP-1 response or prevent them outright.

Incubation with Cat, SOD or VitE did not significantly alter the content of GSH in the embryo (ﬁg. 7A). Compared with control embryos that were not cultured (0 min), there was a significant increase in the GSSG:GSH ratio after 30 or 90 min of culture (ﬁg. 7C). The presence of Cat or SOD, but not VitE, prevented the increase in the GSSG:GSH ratio. In the yolk sac, GSH content was also unaffected by antioxidant treatment (ﬁg. 7B). The increased GSSG:GSH ratio at 30 min in the yolk sac was prevented only by SOD (ﬁg. 7D). However, as in the embryo, the rise in the GSSG:GSH ratio at 90 min was prevented by both Cat and SOD, but not VitE.

In both the embryo and the yolk sac, the inductions of the mRNAs for c-fos, c-jun, junB and junD were maximal 30 min after the onset of culture (ﬁg. 4). At that time, the addition of Cat or SOD, but not VitE, signiﬁcantly inhibited the induction of fos and jun messages in the embryo (ﬁg. 8A and 9A). By 90 min, AP-1 mRNA contents were similar irrespective of the treatment, suggesting that Cat and SOD did not merely delay induction of fos and jun mRNAs. Similarly, in the yolk sac the induction of AP-1 mRNA at 30 min was prevented by Cat and SOD (ﬁgs. 8B and 9B). At 90 min, c-fos was still induced in the untreated control yolk sac (ﬁgs 4B and 9B). This longer-lasting induction was also inhibited by Cat and SOD but was unaffected by VitE. The inclusion of Cat and SOD had no signiﬁcant effect on the steady-state concentrations of c-jun, junB and junD at 90 min, again suggesting that there was no delay in the onset of mRNA induction. The concomitant protection from oxidative stress, as measured by the attenuated rise in the GSSG:GSH ratio and AP-1 mRNA concentrations, by antioxidants indicates that oxidative stress regulates AP-1 mRNA expression in the conceptus.

In the embryo, the peak DNA-binding activity of Fos and Jun proteins at 30 min (ﬁg. 6A) was inhibited by Cat and SOD (ﬁgs. 10A and 11A). By 90 min after the onset of culture, DNA-binding activity had returned to base line in all groups. Similarly, in the yolk sac, the peak in AP-1 DNA-binding activity at 30 and 90 min was abolished by the presence of the enzymic antioxidants Cat and SOD (ﬁgs. 10B and 11B).
Thus, the antioxidants that prevented oxidative stress in the conceptus also prevented the induction of an AP-1 response.

**Discussion**

We report here the presence of c-fos, c-jun, junB and junD mRNAs in the mid-organogenesis rat embryo (gestational days 10–12). Furthermore, we have demonstrated, using EMSA, that these proteins form functional AP-1 dimers that are active during mid-organogenesis. Previous work in rats, using Northern blot analysis, failed to demonstrate basal expression of c-fos on gestational day 12, 4 days after a single dose of whole-body irradiation of the dam (Higo et al., 1989).

In mice, c-fos transcripts were detected in unfertilized eggs, in preimplantation embryos (Pal et al., 1993) and from organogenesis through parturition (Müller et al., 1982). The inducibility of c-fos in early postimplantation embryos by growth factors (transforming growth factor-α, epithelial growth factor, platelet-derived growth factor and fibroblast growth factor) suggested a developmental role (Nielson et al., 1991). During terminal differentiation in the fetal period, c-fos transcripts were localized to the growth regions of bone (Dony and Gruss, 1987; Closs et al., 1990), the central nervous system (Caubet, 1989) and regions destined to undergo programmed cell death (Smeyne et al., 1993). In addition, immunohistochemistry studies have localized c-Fos to bone during osteogenesis (De Togni et al., 1988). Transgenic mice lacking the c-fos gene (Johnson et al., 1992; Wang et al., 1992) developed fairly normally in utero but presented postnatally with, among other features, skeletal malformations. During the fetal period in mice, c-junB was detected only on day 17.5. Localization of these transcripts revealed c-jun in developing cartilage, gut and central nervous system, but junB was...
restricted to differentiated epidermal and endodermal gut epithelium (Wilkinson et al., 1989). The c-jun knockout mice died in utero during the fetal period, between gestational days 14 and 16 (Hilberg et al., 1993).

At the beginning of the 2-day culture period (day 10.5 of gestation), the rat embryo is poorly differentiated, possessing 8 to 10 somites. At the end of culture, the embryo has limb buds, rudimentary organ systems and 30 to 35 somites. Because there are profound increases in the mRNAs of c-fos (Dony and Gruss, 1987) and c-jun and junB (Wilkinson et al., 1989) during differentiation, it was surprising that, other than the transient response shortly after the initiation of culture, there were no significant alterations in the expression patterns of the different AP-1 transcripts in the embryo or the yolk sac. This may reflect the fact that the tissues have not achieved a terminally differentiated state (Wilkinson et al., 1989). The stabilities of the fos and jun expression profiles during this period of organogenesis were also reflected at the protein level; EMSA did not reveal discernible changes in the banding pattern of the retarded oligonucleotide. This may indicate that there were no major changes in the AP-1 dimeric composition.

During this period of organogenesis, the embryo undergoes its most rapid growth and differentiation and consequently is highly susceptible to insult with many teratogens. Indeed, the whole-rat embryo culture system has been used extensively to characterize the effects of known and suspected teratogens (Hales, 1991). The present data provide the first evidence that, during this important developmental window, AP-1 acts as an immediate-early gene and can respond to stress or insult such as an oxidative stress. The immediate-early response of fos and jun mRNAs and AP-1 DNA-binding activity after oxidative stress was more prolonged in the yolk sac than in the embryo. Several factors may be involved. One may be the greater (3-fold) increase in the GSSG:GSH ratio in the yolk sac; the GSSG:GSH ratio only doubled in the embryo. Alternatively, signals that initiate immediate-early genes may be either down-regulated or absent in the embryo.

The time course of the AP-1 induction that we noted in the conceptus is very similar to that induced in cell cultures by a serum response. In cell culture, after 48 hr of serum deprivation and subsequent re-exposure to serum, fos (Müller et al., 1984) and jun (Ryder and Nathans, 1988) mRNAs are induced within approximately 0.5 hr. This increased expression of AP-1 has been termed the serum response. It is believed to be due to growth factors and hormones within the serum that signal transcriptional and posttranslational changes via various protein kinase C-driven pathways (for review, see Angel and Karin, 1991). In cell cultures, the removal of active oxygen species with antioxidants blocks c-fos mRNA induction by cytokines and growth factors (Lo and Cruz, 1995); conversely, c-fos induction in response to H2O2 may be blocked by pretreatment with protein kinase C antagonists (Maki et al., 1992; Rao et al., 1993). Therefore, there is cross-talk between the pathways underlying the serum response and the oxidative stress response. Indeed, studies in epithelial cells with stable transfections of segments of the c-fos promoter linked to a reporter construct indicated that the serum response element was required for the induction of AP-1 by serum and by active oxygen species (Amstad et al., 1992). Interestingly, only induction by oxidative stress required poly-ADP-ribosylation of chromosomal protein, suggesting that, although the same DNA promotor is targeted, separate transduction pathways are involved.

Posttranslational regulation may play an important role in the AP-1 response in the conceptus. The time course of mRNA induction did not completely parallel that of the enhancement of DNA-binding activity. DNA-binding activity in...
the embryo remained elevated until 1 hr, despite the return to base line of the transcripts for fos and for all of the jun messages except for junD. Even greater disparities were observed in the yolk sac; in this tissue, AP-1 DNA-binding activity persisted until 3 hr, by which time the transcripts for all jun family members had returned to base line. This was surprising, because c-Jun, rather than c-Fos, has been implicated as the major player in the cell stress response. Disparities between the mRNA expression patterns and the protein activity may also reflect the redox regulation of AP-1 DNA-binding activity (Abate et al., 1990).

In the present study, antioxidant enzymes (Cat and SOD) protected the conceptus against culture-induced increases in the GSSG:GSH ratio, in AP-1 mRNA expression and in DNA-binding activity in the conceptus. In previous studies, it was demonstrated that the addition of exogenous Cat and SOD to embryos in culture models protected against reactive oxygen species-induced DNA adduct formation (Winn and Wells, 1995). The ability of Cat and SOD to inhibit AP-1 induction supports the role of oxidative stress in inducing an AP-1 response in the embryo.

The inability of VitE to protect the conceptus against oxidative stress is interesting. In a previous report, the antioxidants ascorbate and GSH were found to protect embryos against reactive metabolites of 2-acetylaminofluorene, whereas α-tocopherol did not (Faustman-Watts et al., 1986). In this study, the time of exposure of the conceptus to VitE (90 min) may be insufficient to allow VitE to be absorbed and bioavailable; at 90 min in both the embryo and yolk sac, the GSSG:GSH ratio of the VitE-treated group was intermediate (P < .08) between that of the control group and that of Cat- or SOD-treated groups. An alternative explanation is that α-tocopherol and glutathione may be required to act synergistically to protect against free radical damage. Glutathione levels and the activities of many enzymes that defend against free radical damage are significantly lower in the embryo than in neonates or adult tissues (Di Ilio et al., 1986; El-Hage and Singh, 1990; Serafini et al., 1991; Ozolinš et al., 1996); these low levels of glutathione and related enzymes may prevent α-tocopherol from exerting its full antioxidant potential. In accordance with its inability to prevent oxidative stress in the conceptus, VitE also did not influence AP-1 mRNA expression or DNA-binding activity. This is not without precedent either; UV-A induction of AP-1 in human keratinocytes was prevented by α-tocopherol from exerting its full antioxidant potential (Djavaheri-Mergny et al., 1986).

Although AP-1 is not the only transcription factor regulated by redox changes (Toledano and Leonard, 1991), the AP-1 members play important roles in normal development and differentiation. Regulation of the activity of AP-1 may be one of the reasons why glutathione homeostasis is critical during rodent organogenesis (Harris et al., 1987; Slott and Hales, 1987; Wong et al., 1989), especially because it is during this period of development that there is a transition from anaerobic to aerobic metabolism (Tanimura and Shepard, 1970). The oxidation of GSH should spare the important cellular macromolecules, thereby protecting the cell. This was not the case in the conceptus, as demonstrated by the significant increase in protein oxidation after 0.5 hr of culture. DNA may also be a target (Winn and Wells, 1995). Therefore, despite the transient nature of the rise in the GSSG:GSH ratio, cellular damage and stress to the embryo and yolk sac clearly can result. The oxidative stress-induced AP-1 response in the embryo may serve to alter the profile of subsequent gene expression. Altered gene expression may protect the embryo against insult, but it may also lead to altered development, apoptosis or malformations. Identification of the gene targets responsive to AP-1 in the conceptus may provide clues to how embryos respond to different teratogens. Furthermore, if AP-1 does activate the transcription of cytoprotective gene products, then it may serve as a valuable target for pharmacological intervention aimed at protecting against developmental anomalies.

Acknowledgments
We thank Dr. G. Almanza for providing neuroepithelial cell extracts, Carmen Durham for excellent technical assistance and Dr. B. Robaire for valuable comments on this manuscript.

References
Djavaheri-Mergny, M., Mergny, J.-L., Bertrand, F., Santus, R., Maziere, C.,
MEISTER, A.: Glutathione and the...  
DONY, C. and GRUSS, P.: Proto-oncogene c-fos expression in growth regions of fetal...  
EL-HAGE, S. and SINGH, S. M.: Temporal expression of genes encoding free...  
JOHNSON, R. S., SPIEGELMAN, B. M. AND PAPAIONNIOU, V.: Pleiotropic effects of a...  
HARRIS, C., NAMKUNG, M. J. AND JUCHAU, M. R.: Regulation of intracellular...  
MILLER, A. D., CURRAN, T. AND VERMA, I. M.: c-Fos protein can induce cellular...  
LEE, W., MITCHEL, P. AND TJIAN, R.: Purified transcription factor AP-1 interacts...  
NISHIKURA, K. AND MURRAY, J. M.: Antisense RNA of proto-oncogene c-fos blocks...  