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REGULATION OF THE Y_p SUBUNIT OF GLUTATHIONE *S*-TRANSFERASE P IN RAT EMBRYOS AND YOLK SACS DURING ORGANOGENESIS

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Abstract—Manipulation of the glutathione status of an embryo during organogenesis leads to abnormal development, as well as increasing the susceptibility of the embryo to insult by either xenobiotic or endogenous electrophiles. The glutathione *S*-transferases are a family of drug-metabolizing enzymes that catalyze the conjugation of reactive chemicals with glutathione, playing an important role in protecting cells against attack. The purpose of this study was to investigate the presence and regulation of one glutathione *S*-transferase, glutathione *S*-transferase P, a homodimer of the Y_p subunit, in the conceptus during organogenesis. Northern blot analysis of the RNA isolated from rat embryos and their yolk sacs on days 10, 11 and 12 of gestation revealed a single Y_p transcript. Steady-state concentrations of the Y_p mRNA in embryos did not change with either gestational age or culture for 24 hr (day 11 *in vitro*) or 45 hr (day 12 *in vitro*). In contrast, concentrations of this transcript in yolk sac increased 3-fold from day 10 to 12 of gestation and a further 3-fold with culture (day 12 *in vivo* compared with *in vitro*). Transcription of the rat Y_p subunit gene in cell lines is induced by treatment with phorbol esters. However, the addition of 12-*O*-tetradecanoylphorbol-13-acetate (TPA, 50 or 100 nM) to embryos in culture had no effect on the steady-state concentrations of the Y_p transcript. Thus, the glutathione *S*-transferase Y_p message is subject to tissue- and development-specific regulation in the conceptus during organogenesis. Moreover, culture of the embryos resulted in a further up-regulation of the steady-state concentrations of the Y_p transcript in yolk sac. Western blot analysis demonstrated that a single immunoreactive Y_p subunit band of 26 kDa was found in both embryos and yolk sacs. Neither age nor culture appeared to affect the concentrations of immunoreactive Y_p subunit in the yolk sac. Thus, glutathione *S*-transferase Y_p mRNA is translated in the conceptus during organogenesis. The apparent differences between the relative amounts of the message and immunoreactive protein in yolk sac suggest that this subunit may be subject to post-transcriptional as well as transcriptional regulation in this tissue. Immunohistochemical analysis of embryos cultured for 45 hr (day 12 *in vitro*) revealed that the Y_p reaction product was localized over the hepatic primordia, mesonephric ducts, otocyst, yolk sac and ectoplacental cone. Finally, glutathione *S*-transferase activity towards 1-chloro-2,4-dinitrobenzene, a substrate for many of the transferases, was present in rat embryos and their yolk sacs on day 12 of gestation. Culture of the embryos *in vitro* on day 10 of gestation for 45 hr (day 12 *in vitro*) did not alter the glutathione *S*-transferase activity toward this substrate in the embryo, but a 40% increase in this activity was observed in the yolk sac. Therefore glutathione *S*-transferase P is present in the embryo undergoing organogenesis and may have a role in protecting the conceptus against insult from toxic electrophiles. The mechanism(s) regulating the expression of the Y_p subunit of glutathione *S*-transferase P in the yolk sac deserves further investigation.

Key words: glutathione *S*-transferase; Y_p; embryo; yolk sac; organogenesis; rat

The tripeptide glutathione is a ubiquitous cellular nucleophile important in intermediary metabolism and enzyme regulation, as well as in the detoxification of reactive electrophiles. Several studies have shown that manipulation of glutathione in an embryo leads to abnormal development. Depletion of glutathione in rat embryos, either *in utero* or in culture, significantly increases the incidence of malformations [1, 2]. The exposure of rat conceptuses during organogenesis to diamide, a thiol oxidant, elicits embryo lethality and malformations [3].

Altering the glutathione status of the embryo can modify the teratogenicity of a variety of compounds, including quinones, aromatic nitro compounds, and alkylating agents [4–9]. Both the nature of the electrophilic intermediate and the glutathione status of the embryo are important in determining the consequent embryonic dysmorphogenesis [6]. One of the mechanisms by which glutathione may protect the conceptus against reactive electrophiles is by the formation of a glutathione conjugate, either spontaneously or enzymatically. The enzymatic reaction is catalyzed by the glutathione *S*-transferases [10–15]. Examples of reactive chemicals that can be detoxified by conjugation with glutathione include the K-region and bay-region diol epoxides from polycyclic aromatic hydrocarbons and lipid hydroperoxides [10–15].

The glutathione *S*-transferases are a family of drug-metabolizing enzymes found in many organisms

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[16]. The cytosolic glutathione *S*-transferases have been divided into five classes, based on biochemical, immunological and partial sequence characterizations; the three major classes are alpha (α), mu (μ) and pi (π) [16]. There is also a microsomal glutathione *S*-transferase; this enzyme contributes less to the total transferase activity and is unrelated to the cytosolic transferases [17].

The glutathione *S*-transferases are subject to both tissue- and developmentally-specific regulation [18, 19], but little is known with respect to the isozymes present in the rat conceptus during organogenesis. In one study [19], glutathione *S*-transferase activity toward 1-chloro-2,4-dinitrobenzene was first observed in liver on day 14 of gestation; this activity increased to a maximum in the fetus on day 21. One isozyme of particular interest with respect to development is the class π glutathione *S*-transferase P. Glutathione *S*-transferase P is a homodimer of the subunit, which has been known as Y_p (placental), Y_f (fetal) or rat subunit 7; this isozyme catalyzes the conjugation of substrates such as 1-chloro-2,4-dinitrobenzene, ethacrynic acid and benzo [α] pyrene 7,8-diol-9,10-epoxide with glutathione [10, 11, 20, 21]. The Y_p subunit transcript and protein are found in rat placenta, lung, kidney, epididymis, spleen, thymus, adrenal and smooth muscle [22]. Interestingly, Y_p is present in fetal liver and preneoplastic nodules from adult liver, but not in normal adult liver [20]. Steady-state mRNA concentrations and the rate of transcription of the Y_p transcript peak on days 16 and 17 of gestation in fetal rat liver [19].

Regulation of glutathione *S*-transferase P has been studied extensively [23–27]. The promoter region of the Y_p gene has both enhancer and silencer sequences, and includes a "TATA" box, Sp1 recognition site and AP-1 recognition sequence, conferring responsiveness to phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA*) [23–27].

Although the Y_p transcript is hardly detectable in normal adult liver or in freshly isolated hepatocytes, it rapidly accumulates in hepatocytes during culture (within 24 hr, and continuing for 72 hr) [28]. Early neural fold stage rat embryos are frequently cultured for 24–48 hr in the absence or presence of "suspect" teratogens to characterize their effects on development. During this culture period, development *in vitro* closely parallels that *in vivo* [29]. However, previous studies have found changes in the mRNA or protein concentrations of enzymes (ornithine decarboxylase [30]), cell adhesion molecules (cadherins [31]) and glutathione in cultured rat embryos [1]. Slott and Hales [1] showed that the glutathione content of day 10 embryos was decreased by half after 2 hr in culture. Any effect of culture on a glutathione *S*-transferase isozyme present in the embryo during organogenesis may alter the susceptibility of the embryo to insult.

The purpose of this study was to investigate the presence and possible regulation of glutathione *S*-transferase P in the rat conceptus during organogenesis. Rat embryos and yolk sacs were assessed for their glutathione *S*-transferase activity on day 12 of gestation and for their Y_p transcript and protein content on days 10, 11 and 12 of gestation. Immunohistochemical studies were done to localize the Y_p protein in embryos and their yolk sacs. Embryos *in vivo* were compared with embryos *in vitro* in the whole embryo culture system with respect to the same endpoints. Finally, cultured embryos were exposed to a phorbol ester, TPA to test the ability of the Y_p subunit gene in the embryo to respond to this inducing agent.

MATERIALS AND METHODS

Animals. Timed-gestation pregnant Sprague–Dawley rats (200–225 g) were purchased from Charles River Canada, Inc. (St. Constant, Quebec). The day on which spermatozoa were found in the vaginal smear was considered day zero of pregnancy. Rats were housed in the McIntyre Animal Centre (McGill University, Montréal, Québec) and given Purina rat chow and water *ad lib*. On days 10, 11 and 12 of gestation female rats were killed by ether overdose, the uteri were removed, and the embryos and yolk sacs were collected separately (E10, E11, E12 *in vivo*); liver and kidneys were also removed. Newborn rat pups were killed on the day after delivery, and liver and kidneys were removed. All tissues were frozen in liquid nitrogen and stored at −80°.

Embryo cultures. The embryo culture procedure used in this study was based on the system described by New [29]. On day 10 of gestation, embryos were removed from the uterus, placed in Hanks' balanced salt solution, and dissected free of decidua and Reichert's membrane, leaving the ectoplacental cone and yolk sac intact. Three embryos were placed in each sterile 60-mL culture bottle containing 4.8 mL of medium consisting of 90% heat-inactivated filtered rat serum and 10% Tyrode's saline (Gibco Laboratories). The contents of the bottles were gassed with a mixture of 20% O_2, 5% CO_2, 75% N_2. The bottles were placed in a rotator (New Brunswick Scientific Co., Edison, NJ) at 30 rpm, and the embryos were cultured for up to 45 hr at 37°. After the first 24 hr, the embryos were regassed with 95% O_2, 5% CO_2. Embryos and yolk sacs were collected separately after culture for 24 hr (E11 *in vitro*) and 45 hr (E12 *in vitro*).

In the induction experiments, phorbol ester (TPA), dissolved in DMSO, was added to the medium 1 hr after the initiation of the embryo cultures on day 10 of gestation. The final concentration of TPA in the culture medium was either 50 or 100 nM, added in 1 μL of DMSO/5 mL of medium. Control embryos received DMSO alone. Embryos and yolk sacs were collected 1, 6 and 24 hr later.

Northern blot analysis. RNA was extracted from the collected tissues using the phenol–chloroform extraction method [32]. Total RNA (10 or 20 μg per sample) was fractionated by electrophoresis in a 1% agarose gel containing 6% formaldehyde and blotted onto a Nylon membrane (Genescreen-plus, New England Nuclear, Missassauga, Ontario) with a

* Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; and AP-1, activator protein-1.

vacuum blotting system (Pharmacia LKB, Montréal, Québec) according to the instructions of the manufacturer. The membrane was baked in a vacuum oven at 80° for 2 hr. The specific cDNA probe for GST-Y_p (clone pGP5), provided by Dr. M. Muramatsu, contained the nucleotide sequence representing the total amino acid content of rat glutathione S-transferase P [21]. The probe was labeled with ^{32}P by random priming using an oligolabeling kit (Pharmacia, Montréal, Québec). The membrane was hybridized to the probe in 50% formamide, 1% SDS, 1 M NaCl, 10% dextran sulfate and 0.2 mg/mL denatured salmon sperm DNA at 42°. After hybridization, the membrane was washed with 2× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) and 1% SDS and exposed to a Kodak X-ray film at −80° using intensifying screens. To normalize the amount of RNA present in each lane, membranes were also hybridized to a synthetic oligonucleotide (24-mer) probe recognizing the 18S rRNA sequence [33]. Between hybridizations, the first probe was stripped by boiling the membrane in 0.01× SSC/0.1% SDS. Three to six Northern blots were done with RNA samples from independent batches of tissues. A 0.24–9.5 kb RNA ladder (Gibco Laboratories, Burlington, Ontario) was used as a molecular size marker.

Autoradiographs were scanned with an LKB laser densitometer (Pharmacia). Values obtained for the Y_p signals were normalized based on the intensity (areas of absorbance) obtained for the 18S rRNA signals. Values represent means ± SEM (N = 3–6). All absorbance readings obtained were within the linear range for this densitometer.

Western blot analysis. Embryos, yolk sacs and adult livers and kidneys were homogenized (1:10, w/v) in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) and centrifuged at 10,000 *g* for 60 min. The supernatants were frozen and stored at −80° until analysis. Samples (10 μg protein/lane) dissolved in sample loading buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.001% bromophenol blue) were boiled for 1–2 min; fractionation was by electrophoresis in 12% acrylamide discontinuous SDS/polyacrylamide gels [34]. Each gel was transferred to a nitrocellulose sheet [35]. The blot was blocked with 5% milk in TBS-T (137 mM NaCl, 20 mM Tris, pH 7.4, 0.1% Tween 20) at room temperature for 2 hr before incubation with Y_p antibody. The Y_p antibody is a polyclonal rabbit antibody, provided by Dr. J. Hayes [36]; the antibody was incubated with the blot at a dilution of 1:400 in 5% milk/TBS at 4° for 18 hr. The blot was then washed three times in TBS-T and incubated with biotinylated secondary antibody at room temperature for 2 hr. The antibody binding was visualized with streptavidin alkaline phosphatase using 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium as substrates (Amersham Canada, Oakville, Ontario).

Immunohistochemistry. Embryos and yolk sacs were fixed in Bouin's solution for 3 hr and were subsequently dehydrated and embedded in paraffin. Sections of 5 μm were cut and mounted on slides. The following staining procedure was done at room temperature. After rehydration, sections were incubated sequentially with 3% H_2O_2 in Tris-buffered saline (TBS, 20 mM Tris, 137 mM NaCl, 2.7 mM KCl, pH 7.4) for 30 min, washed in TBS for 20 min, and blocked with 1.5% goat serum for 20 min. Sections were then incubated for 3 hr with rabbit anti-rat-Y_p or control rabbit serum, both diluted 1:200 in TBS. After a 10-min wash, sections were incubated with biotinylated goat anti-rabbit immunoglobulin (Vector Laboratories, Burlingame, CA) for 30 min, washed for 10 min, and then incubated with avidin–biotin–peroxidase complex for 45 min. Sections were incubated subsequently with 0.01% H_2O_2 (in H_2O) and 0.05% diaminobenzidine tetrachloride (Sigma Chemical Co., St. Louis, MO) in 0.1 M Tris for 8 min, counterstained with haematoxylon, dehydrated, and mounted in Permount.

Enzyme assay. Embryos and yolk sacs from day 12 *in utero* and day 10 embryos after 45 hr in culture (day 12 *in vitro*) were homogenized (1:3, v/v) in 0.01 M potassium phosphate buffer, pH 6.5; samples were centrifuged at 100,000 *g* for 60 min at 4°. Assay conditions for the measurement of glutathione S-transferase catalytic activity toward 1-chloro-2,4-dinitrobenzene were identical to those of Habig *et al.* [37]. 1-Chloro-2,4-dinitrobenzene is a good substrate for glutathione S-transferase P, as well as most other glutathione S-transferases [10–14]. Assays were done at room temperature with both 1-chloro-2,4-dinitrobenzene and glutathione present at concentrations of 1 mM, in 0.1 M potassium phosphate buffer, pH 6.5; the change in absorbance at 340 nm was followed with a Beckman DU-7 spectrophotometer. Protein concentrations were measured by the method of Bradford [38], using the BioRad assay reagent and bovine serum albumin as a standard.

Statistical analyses. Comparisons among groups were done by one-way analysis of variance (ANOVA) using the Complete Statistical System (CSS) software program (Statsoft Inc., Tulsa, OK). The level of significance was $P \leq 0.05$.

RESULTS

Northern blot analysis of glutathione S-transferase Y_p mRNA concentrations. A Northern blot of glutathione S-transferase Y_p mRNA and 18S rRNA in embryos and yolk sacs is shown in Fig. 1. A single glutathione S-transferase Y_p mRNA transcript with a molecular size of 0.9 kb was found in both tissues. This glutathione S-transferase Y_p transcript was found in day 10 embryos and yolk sacs in similar concentrations. There was no significant change in the steady-state concentration of the Y_p mRNA in the embryos with either gestational age (day 11 or 12) or culture (C). In contrast, concentrations of the mRNA for Y_p in yolk sac increased with both age and culture.

The results of densitometric analysis of six individual Northern blots are depicted in Fig. 2. To normalize the results and allow the comparison of blots with different intensities of exposure, absorbance in the day 10 embryo was set at 1.0. As observed in Fig. 1, the steady-state concentrations

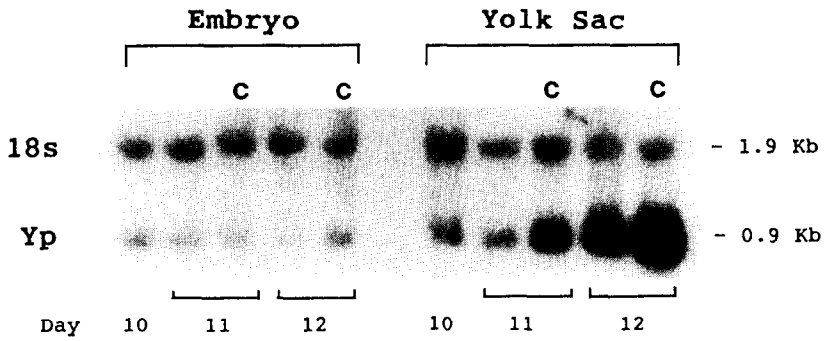

Fig. 1. Northern blot analysis using the rat glutathione *S*-transferase Y_p cDNA probe and an oligonucleotide probe for 18S rRNA. The sizes of the Y_p and 18S RNAs are shown on the right. Total RNA (20 μg) was loaded in each lane. The RNA was extracted from rat day 10 embryos (lane 1); day 11 embryos *in vivo* (lane 2) and *in vitro* (day 10 embryos after 24 hr of culture, C; lane 3); day 12 embryos *in vivo* (lane 4) and *in vitro* (day 10 embryos cultured for 45 hr, C; lane 5); day 10 yolk sacs (lane 6); day 11 yolk sacs *in vivo* (lane 7) and *in vitro* (day 10 yolk sacs after 24 hr of culture, C; lane 8); day 12 yolk sacs *in vivo* (lane 9) and *in vitro* (day 10 yolk sacs cultured for 45 hr, C; lane 10).

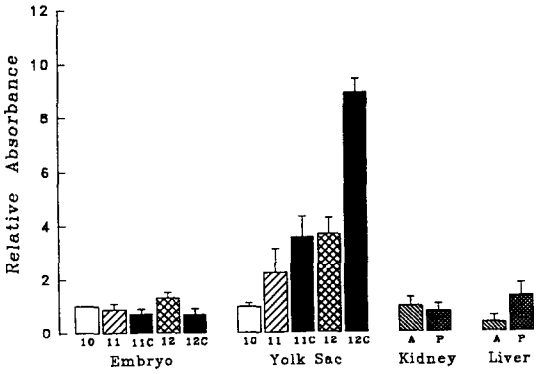

Fig. 2. Densitometric analysis of the glutathione *S*-transferase Y_p hybridization signals after standardization against the 18S rRNA signals. Values are means ± SEM (N = 6). The numbers beneath the columns indicate the day of gestation; 11C = day 10 embryos after 24 hr of culture, and 12C = day 10 embryos after 45 hr of culture. A = adult, and P = perinatal or newborn.

of the mRNA transcript for glutathione *S*-transferase Y_p in the embryo did not change with age. Moreover, concentrations of this message in embryos were similar to those in adult kidney from pregnant rats or in newborn kidney or liver. As expected, there was little transcript for this mRNA in the liver of adult animals. Unlike the results with embryos, the concentration of the mRNA transcript for the glutathione *S*-transferase Y_p subunit in yolk sac doubled by day 11 of gestation and tripled by day 12 of gestation *in vivo*. Although culture of the day 10 embryo for 24 hr (day 11 *in vitro*) did not alter significantly the steady-state concentrations of the Y_p transcript, culture for 45 hr (day 12 *in vitro*) dramatically potentiated the developmental increase in Y_p mRNA in the yolk sac. Y_p mRNA concentrations in yolk sacs from day 12 cultured embryos were 3-fold higher than those found in day 12 embryos *in vivo*. Thus, concentrations of the mRNA for glutathione *S*-transferase Y_p are regulated differentially in the embryo and yolk sac. The yolk sac appears to respond to a developmental trigger, as well as to some factor(s) in the culture system, which results in a dramatic increase in Y_p transcript concentrations.

The ability of the Y_p message in cultured embryos to respond to induction by a phorbol ester, TPA, was investigated (Fig. 3). Exposure to 50 or 100 nM TPA did not alter the steady-state concentrations of the mRNA for glutathione *S*-transferase Y_p in either the embryos or yolk sacs at any time that was studied (1 hr, 6 hr, 24 hr). Thus, Y_p in the embryos failed to respond to TPA induction. In Fig. 3 there appears to be a decrease in the steady-state concentration of the message for Y_p in the embryo after culture for 24 hr; when the Y_p transcript from three different blots was quantitated relative to the 18S rRNA, this decrease (approximately 20%) was not significant. As observed in Fig. 1 and 2, culture for 24 hr (day 11 *in vitro*) resulted in a greater than 3-fold increase in the concentration of Y_p mRNA in the yolk sac.

Western blot analysis of glutathione S*-transferase Y_p subunit protein concentrations.* Figure 4 is a Western blot of glutathione *S*-transferase Y_p immunoreactivity in embryos and yolk sacs. One predominant immunoreactive band with a molecular size of approximately 26 kDa was found in both tissues, as well as in adult kidney. A higher molecular weight band (approximately 51 kDa) was also found in adult liver. Although there were large variations among individual blots, similar concentrations of the 26 kDa immunoreactive Y_p subunit were found in embryos and yolk sacs on days 10, 11 and 12 *in vivo* and after culture *in vitro*. Thus, the Y_p mRNA is translated into an immunoreactive Y_p subunit in the conceptus during organogenesis. It is interesting that there does not appear to be a dramatic effect of either age or culture on the concentrations of the immunoreactive Y_p protein subunit.

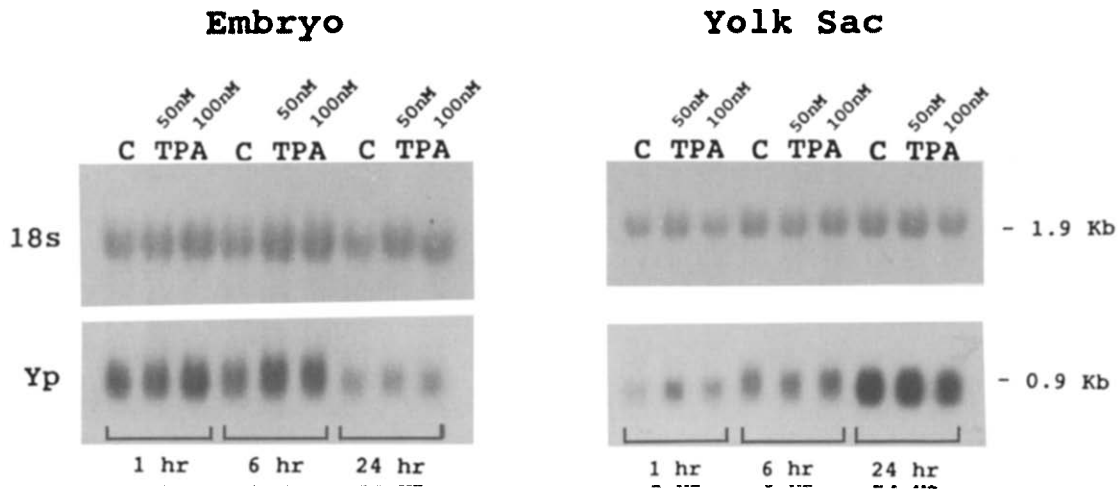

Fig. 3. Northern blot analysis of the steady-state concentrations of the mRNA for glutathione *S*-transferase Y_p in rat embryos and their yolk sacs cultured in the presence of 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Day 10 rat embryos were cultured for 1 hr, 6 hr or 24 hr with the addition of vehicle (DMSO control), or TPA (50 or 100 nM). Total RNA was extracted from embryos and yolk sacs separately, and 10 μg was loaded in each lane. The blots were probed with the cDNA probe for glutathione *S*-transferase Y_p and an oligonucleotide probe for 18S rRNA. The sizes of the Y_p and 18S RNAs are shown on the right. The blot on the left represents RNA from cultured rat embryos, and the blot on the right RNA from their yolk sacs. Exposure to TPA at concentrations of either 50 or 100 nM did not alter the steady-state concentrations of Y_p mRNA in either embryo or yolk sac.

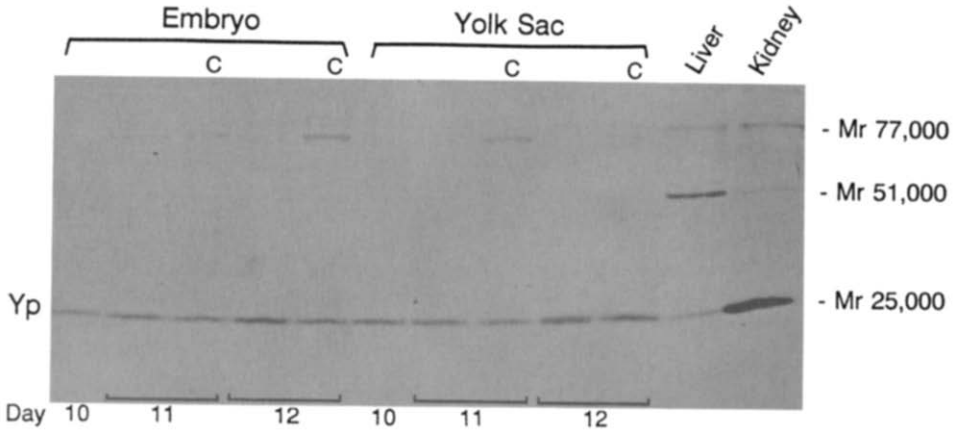

Fig. 4. Western blot analysis of the rat glutathione *S*-transferase Y_p protein subunit. Immunoreactive rat glutathione *S*-transferase Y_p protein was detected using a polyclonal rabbit antibody provided by Dr. J. Hayes. Protein (20 μg) was loaded in each lane as follows: lane 1, rat day 10 embryos; lane 2, day 11 embryos *in vivo*; lane 3, day 10 embryos after 24 hr of culture (C) (day 11 *in vitro*); lane 4, day 12 embryos *in vivo*; lane 5, day 10 embryos cultured for 45 hr (C) (day 12 *in vitro*); lane 6, day 10 yolk sacs; lane 7, day 11 yolk sacs *in vivo*; lane 8, day 10 yolk sacs after 24 hr of culture (C) (day 11 *in vitro*); lane 9, day 12 yolk sacs *in vivo*; lane 10, day 10 yolk sacs cultured for 45 hr (C) (day 12 *in vitro*); lane 11, adult liver; lane 12, adult kidney.

Immunohistochemistry. Embryos cultured for 45 hr showed a localized Y_p immunostaining reaction, primarily over the yolk sac, hepatic primordia, otocyst, and mesonephric tubules (Fig. 5, A and D). A strong immunostaining reaction can be seen clearly at higher magnification over the hepatic primordia (Fig. 5, B and E), otocyst (Fig. 5C), mesonephric tubules (Fig. 5F), ectoplacental cone (Fig. 5G), and yolk sac (Fig. 5H). The pattern of immunostaining was the same in embryos removed from the mother

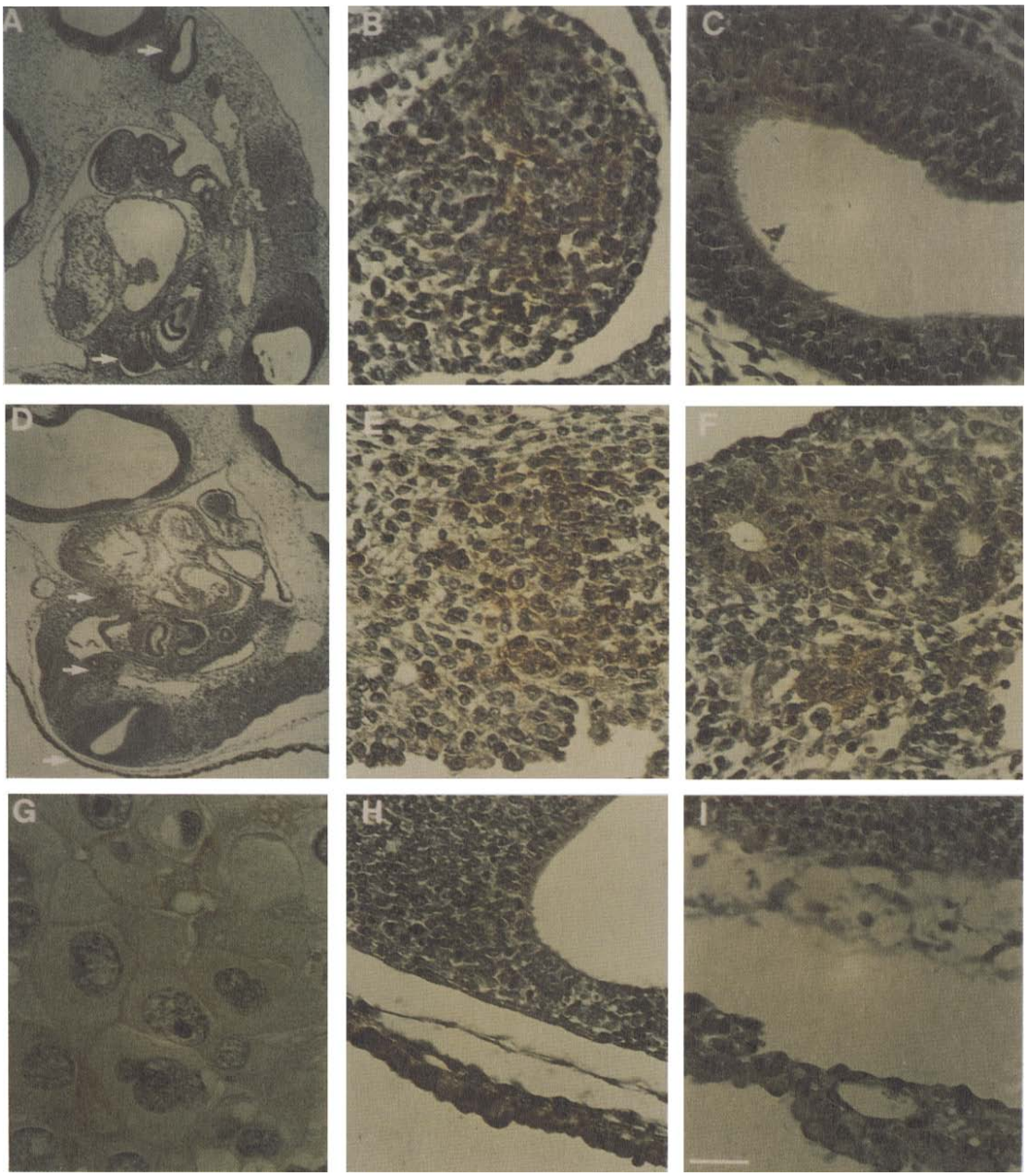

Fig. 5. Glutathione *S*-transferase Y_p immunohistochemical staining in day 10 rat embryos cultured for 45 hr. (A) Low magnification view of an embryo with arrows indicating the sites of the higher magnification views shown in (B) hepatic primordia, and (C) the otocyst. (D) Low magnification view of an embryo with arrows showing the locations of the higher magnification views shown in (E) hepatic primordia, (F) mesonephric ducts, and (H) yolk sac. (G) represents the Y_p immunochemical reaction products found in the ectoplacental cone. (I) is a section of the yolk sac incubated with normal rabbit serum; no immunostaining was found. The bar in (I) represents 375 μm in (A) and (D), and 37.5 μm in the other panels.

on day 12 of gestation (day 12 *in vivo*) as in those removed on day 10 and cultured for 45 hr (day 12 *in vitro*; data not shown). Control rabbit serum did not produce any immunoreaction product, as illustrated for the yolk sac in Fig. 5I.

Glutathione S-transferase catalytic activity. The low levels of enzyme activity and limited amount of tissues available made it difficult to assess glutathione *S*-transferase activity on days 10 and 11 of gestation. However, glutathione *S*-transferase activity toward 1-chloro-2,4-dinitrobenzene was detected in embryos and yolk sacs on day 12 of gestation *in vivo* and

Table 1. Glutathione S-transferase activity towards 1-chloro-2,4-dinitrobenzene in embryos and yolk sacs on day 12 of gestation *in vivo* and day 10 embryos after culture for 45 hr (day 12 *in vitro*)

Tissue	Glutathione S-transferase activity (nmol/min/mg protein)
Day 12 embryo	
In vivo	7.91 ± 0.94
In vitro	9.59 ± 0.33
Day 12 yolk sac	
In vivo	12.43 ± 0.90*
In vitro	17.60 ± 1.01†

Values are means ± SEM, N = 4.
* Significantly different ($P \leq 0.01$) from day 12 embryo.
† Significantly different ($P \leq 0.01$) from day 12 yolk sac *in vivo*.

after culture (day 12 *in vitro*) (Table 1). Glutathione S-transferase activity in yolk sacs on day 12 of gestation *in vivo* was approximately 50% higher than that in the embryo. Culture did not affect significantly the glutathione S-transferase activity in the embryo, but did result in a 40% increase in glutathione S-transferase activity in the yolk sac.

DISCUSSION

Glutathione S-transferase activity, as well as the Y_p protein and transcript, are present in both embryos and yolk sacs during organogenesis. Transferase activity toward 1-chloro-2,4-dinitrobenzene, a strong electrophile and good substrate for a number of the glutathione S-transferase isozymes, was approximately 5% of that previously reported for newborn rat liver cytosol [39]. Despite this low enzyme activity, steady-state concentrations of the immunoreactive glutathione S-transferase Y_p subunit in the embryo and yolk sac were approximately 50% of those found in the adult kidney. Interestingly, steady-state concentrations of the Y_p transcript in the embryo were in the same range as those in newborn rat liver or kidney; however, Y_p mRNA concentrations in the yolk sac were increased 3- to 9-fold, with age and culture, above those concentrations in the newborn and adult tissues studied. Thus, there is a discordance between the relative enzyme activity, Y_p subunit immunoreactive protein, and mRNA concentrations in the conceptus during organogenesis. The immunohistochemical localization of the Y_p subunit protein primarily to the yolk sac, ectoplacental cone, hepatic primordia and mesonephric ducts is compatible with other studies in which it has been reported to be in placenta and fetal liver, as well as in tissues derived from the mesonephric ducts such as the epididymis [22]. To the best of our knowledge, the presence of glutathione S-transferase P in tissues derived from the otocyst, such as the ear, has not been investigated.

Y_p subunit gene transcripts were selectively up-regulated in yolk sac with age and after culture *in vitro*. The increase in the steady-state concentration of the Y_p transcript in yolk sac after culture may be due to either increased synthesis or decreased degradation. Regulation of the Y_p transcript has been studied extensively. It is clear that GPEI, the enhancer of Y_p expression located approximately 2.5 kb upstream of the transcription start site, is activated by AP-1 [27]. However, footprinting analysis has revealed that nucleoproteins other than AP-1 bind to GPEI, and high levels of transcription of Y_p occur in cells lacking AP-1 [27]. Elegant transgenic rat experiments (with the incorporation of a Y_p gene promoter region-chloramphenicol acetyltransferase construct into the germ line) have revealed that a trans mechanism is involved in the activation of this gene during chemical carcinogenesis [40]. The trans-activator(s) has not been identified to date. Although the factor(s) responsible for the up-regulation of Y_p message in yolk sac in culture is not known either, the total lack of responsiveness to TPA would suggest that a protein kinase C mediated activation of AP-1 is not involved.

Other glutathione S-transferases, such as the Y_a subunit gene, as well as other drug-metabolizing enzymes, such as quinone reductase, also have two adjacent AP-1-like binding sites in their promoter sequences [14, 15, 41, 42]. These AP-1-like binding sites are responsive to induction by antioxidants (ARE, antioxidant response element, [41]) and electrophiles (EpRE, electrophile response element, [42]). There is some evidence that reactive oxygen may mediate the induction of these enzymes by a variety of compounds [43]; reactive oxygen serves as an inducing signal for AP-1 activity [44]. Depletion of endogenous nucleophiles such as glutathione in cultured embryos may trigger oxidative stress, and thus modify the status of thiols in protein transcription factors, turning on the oxidative stress response. Alternatively, growth factors or serum proteins present in the culture medium may be responsible for activating the transcription of the Y_p gene.

The loss of factor(s) responsible for the repression of Y_p in cultured yolk sac/liver may also be responsible for the increase in steady-state concentrations of this message. The Y_p gene has a negative regulatory region or silencer element approximately 300 bp upstream of the transcription start site [26]. Further studies are needed to elucidate the mechanism involved in the regulation of Y_p mRNA concentrations in yolk sac in culture.

It is noteworthy that increased Y_p mRNA expression in the yolk sac was apparently not reflected in a significant increase in immunoreactive Y_p protein concentration. The variability obtained using the Western blot technique may be too high to reveal a relatively small increase in protein content. This apparent lack of correspondence between the concentrations of Y_p transcript and protein would suggest that Y_p expression may be influenced by post-transcriptional events, as well as by transcription. In studies of glutathione S-transferase P as a marker enzyme for preneoplastic lesions arising during chemical carcinogenesis, concentrations of the Y_p translation product changed with mRNA levels in cultured hepatocytes [28]. A previous study of the developmental regulation of

glutathione *S*-transferase P expression also concluded that Y_p expression in fetal and neonatal rat liver was regulated predominantly by transcription [19].

There was also an increase in the glutathione *S*-transferase activity in the yolk sac after culture for 45 hr. This increase in enzyme activity was limited to approximately 40%, whereas the increase in Y_p transcript in cultured yolk sac was 150%. When this observation is considered together with the Western blot data on the concentrations of the immunoreactive Y_p subunit, it would seem possible that the increase in glutathione *S*-transferase activity toward 1-chloro-2,4-dinitrobenzene is not specific to glutathione *S*-transferase P.

In summary, glutathione *S*-transferase activity is present in rat embryos and their yolk sacs during organogenesis. The expression of the glutathione *S*-transferase Y_p subunit is differentially regulated with respect to tissue, gestational age and culture. The glutathione *S*-transferases may play an important role in protecting the embryos against insult.

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