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^{14}C methanol incorporation into DNA and proteins of organogenesis stage mouse embryos in vitro

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Abstract

Methanol (MeOH), a widely used industrial solvent and alternative motor fuel, has been shown to be mutagenic and teratogenic. We have demonstrated that methanol is teratogenic in mice in vivo and causes dysmorphogenesis in cultured organogenesis stage mouse embryos. Although MeOH is a product of endogenous metabolism in the gut and can be found in humans following consumption of various foods, elevated levels of methanol could lead to methylation of cellular macromolecules. DNA methylation has been demonstrated to suppress transcription of fetal genes and may also play an important role in genetic imprinting. Embryonal proteins are also potential targets for methanol-induced methylation. We investigated the potential of administered methanol to incorporate into and/or alter the methylation of embryonal DNA or to affect specific protein methylation. Gestational day 8 CD-1 mouse embryos were grown for 24 h in culture medium (CM) with 0, 4, or 8 mg MeOH + 20μCi ^{14}C-MeOH/mL. At the end of the culture period, yolk sacs and embryos were separated for each treatment group. The DNA was purified by cesium chloride gradient centrifugation in the presence of ethidium bromide and ^{14}C incorporation was determined. Methylation of a selected gene, Hoxc-8, was assessed by using methylation-specific restriction enzymes. The ^{14}C activity was found superimposed over the DNA-containing fraction, indicating incorporation. DNA from embryos treated with 4 mg MeOH/mL CM gave the highest incorporation of ^{14}C-MeOH (8 mg/mL was growth inhibiting). Methylation of Hoxc-8 appeared to be increased in embryos treated with 4 mg MeOH/mL CM, but not in embryos treated with 8 mg MeOH/mL. Lack of incorporation of methylation at the higher concentration may be due to the failure of embryos to grow at this concentration of MeOH. The incorporation of ^{14}C-MeOH into embryo proteins was investigated by polyacrylamide gel electrophoresis (PAGE) and autoradiography. Incorporation of ^{14}C-MeOH into specific proteins was observed but the labeling specificity was not methanol dose-related. These results indicate that methyl groups from ^{14}C-MeOH are incorporated into mouse embryo DNA and protein. Our results further suggest that methanol exposure may increase genomic methylation under certain conditions which could lead to altered gene expression.

1. Introduction

Methanol (MeOH) is widely used as an industrial and laboratory solvent and is used as a feed stock for the production of methyl tert-butyl ether, formaldehyde, acetic acid, and methacrylate. Methanol was the chemical with the highest release to the environment (air, water, and land) in the 1992 Toxic Release Inventory of 23,630 facilities [1], and is a component of widely used consumer products including antifreeze, windshield washer fluids, solvents for duplicating machines, and paint [2]. Methanol or MeOH/gasoline blends are also used as alternative automotive fuels in some settings. The extent of human developmental hazard from MeOH exposure has not been determined. Methanol is present in fresh fruits and vegetables, fruit juices, fermented beverages [3] and the artificial sweetener aspartame [4]. Serum values in humans have been shown to be as high as 2.6 mg/liter [5]. Methanol has been shown to be teratogenic in mice [6,7] and rats following inhalation [8] or oral exposure [6,9]. These studies showed that MeOH exposure resulted in fetal mortality, growth retardation, skeletal alterations, exencephaly and cleft palate. We previously reported that MeOH was directly dysmorphogenic to both mouse and rat embryos in vitro, and that mouse embryos were more sensitive to MeOH exposure than were rat embryos [10]. In both primates and rodents, MeOH is oxidized

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to formaldehyde, which is rapidly further oxidized to formic acid [11,12]. Formic acid is then oxidized to carbon dioxide through a folate-dependent one-carbon pathway. Tetrahydrofolic acid (THF) is central to the metabolism of methanol and species sensitivity to acute methanol toxicity has been inversely correlated with the tissue concentration of this crucial intermediate [13,14]. THF functions as a carrier and donor of one-carbon groups, including methyl groups for DNA methyl transferase, the enzyme responsible for the methylation of DNA. Many amino acids can be synthesized in part through THF one-carbon transfer as well. At least one of these, glycine, is subsequently incorporated into the purine ring. The importance of DNA methylation in embryogenesis has been well established. It is known to suppress transcription of some fetal genes, and elimination of the DNA methyl-transferase gene by homologous recombination results in embryo lethality at midgestation [15]. Also, genetic imprinting may, at least in part, result from differential DNA methylation of parental genes [16], and this process affects gene expression. The presence of MeOH or its metabolites in the embryo could affect DNA methylation either directly through enhancement or inhibition of the activity of DNA methyl-transferase or indirectly through effects on its substrate pool.

Previous research by us [17] and others [18] suggests that MeOH is the teratogenic agent in mice. We have shown that MeOH is not metabolized appreciably by rodent embryos in culture [10]. The acute high dose toxicity of methanol in humans is largely due to the metabolite formate, which accumulates when the oxidative pathway is saturated. However, at lower, non-accidental exposure levels, formate build-up is less likely, so methanol per se may be a greater concern in humans. The present study investigates the potential effects of MeOH exposure on DNA and protein in whole mouse embryo culture by exploring the degree of DNA methylation and incorporation of ^{14}C-MeOH into protein resulting from embryonic exposure to methanol. Embryonic MeOH exposure concentrations were chosen from our previous study [10] to include the highest concentration that was not significantly dysmorphogenic (4 mg MeOH/mL CM) and a dysmorphogenic concentration (8 mg MeoH/mL CM). Additionally, these concentrations are comparable to the peak serum MeOH concentrations found in pregnant mice following teratogenic inhalation exposures to 10,000 or 15,000 ppm MeOH for 7 h in vivo [17].

2. Materials and methods

2.1. Animal and whole embryo culture

Timed-pregnant CD-1 mice (Crl:CD-1 [ICR] BR) were purchased from Charles River Laboratories (Raleigh, NC). The presence of a copulation plug was considered day 0 of gestation. Embryos were explanted at the 3 to 5 somite stage on the morning of gestation day 8 and Reichert's membrane was removed. Embryos having an intact visceral yolk sac, ectoplacental cone and amnion were pooled in culture medium (CM; 75% filter-sterilized, heat-inactivated male rat serum/25% Tyrodes salt solution) and randomly selected for culture. They were cultured at 37°C for 24 h in sealed 30-ml serum bottles containing four embryos in 5 ml CM as previously described [10] with 0, 4, or 8 mg/mL MeOH. Both of these MeOH concentrations have previously been shown to cause significant embryonic growth retardation and the 8 mg/mL MeOH level also was shown to cause significant embryonic dysmorphology [10]. The bottles were gassed for 3 min with 5% O_2, 5% CO_2, and 90% N_2 at 0, 5, and 20 h. After 24 h, the embryos were removed from culture and evaluated for development and dysmorphogenesis. The embryos were then washed and the yolk sacs separated from the embryo under a dissecting microscope. Subsequently, the yolk sacs and embryos from each treatment group (N = 8/group) were pooled for DNA and protein analysis.

2.2. Southern blot analysis of ^{14}C-methanol labeled embryonic DNA

To study incorporation of MeOH into embryonic macromolecules, whole embryos were cultured in 20 μCi/mL ^{14}C-MeOH (specific activity 42 mCi/mmole) (Amersham) plus unlabeled MeOH at 4 mg/mL. A control group was exposed only to the ^{14}C-MeOH. Embryos were minced with forceps and lysed with 4 ml of 1% sodium sarcocele, 10 mM EDTA, and 1 mg/mL proteinase K and incubated at 37°C overnight. Cesium chloride was at a final density of 1.1 g/mL and 50 μg/mL ethidium bromide was added. The solution was centrifuged in a Beckman T65 rotor at 45k for 36 h at 20°C. After centrifugation, samples were fractionated from the bottom of the tube at 0.3 ml/fraction. Optical density and radioactivity were determined for each fraction. Purified DNA (10 mg) from each treatment group was digested with either *Hpa* II or its isoschizomer, *Msp* I. Both enzymes recognize the sequence, CCGG, but *Hpa* II is sensitive to methylation of the 5 position of cytosine in the CG dinucleotide while *Msp* I is insensitive. The digested DNA was fractionated by electrophoresis in a 1% agarose gel, blotted with 0.4 M NaOH onto Gene Screen Plus (DuPont-NEN), and hybridized with the 2.2 kb *Bam* I/*EcoR* I fragment of the mouse *Hoxc*-8 gene. The 2.2 kb fragment was isolated from plasmid pMo-EA (ATCC number 37554). This fragment was labeled with α-[^{32}P]ATP by random primer extension with T7 polymerase. Blots were washed with 0.1× SSC and 0.1% SDS at 37°C, dried and exposed to x-ray film. The appropriate sample lane was then cut from the nylon membrane and the amount of radioactivity determined in an imaging analysis system. The x-ray film was scanned using a flat bed scanner (UMAX Powerlook II) equipped with a transparency attachment. The re-

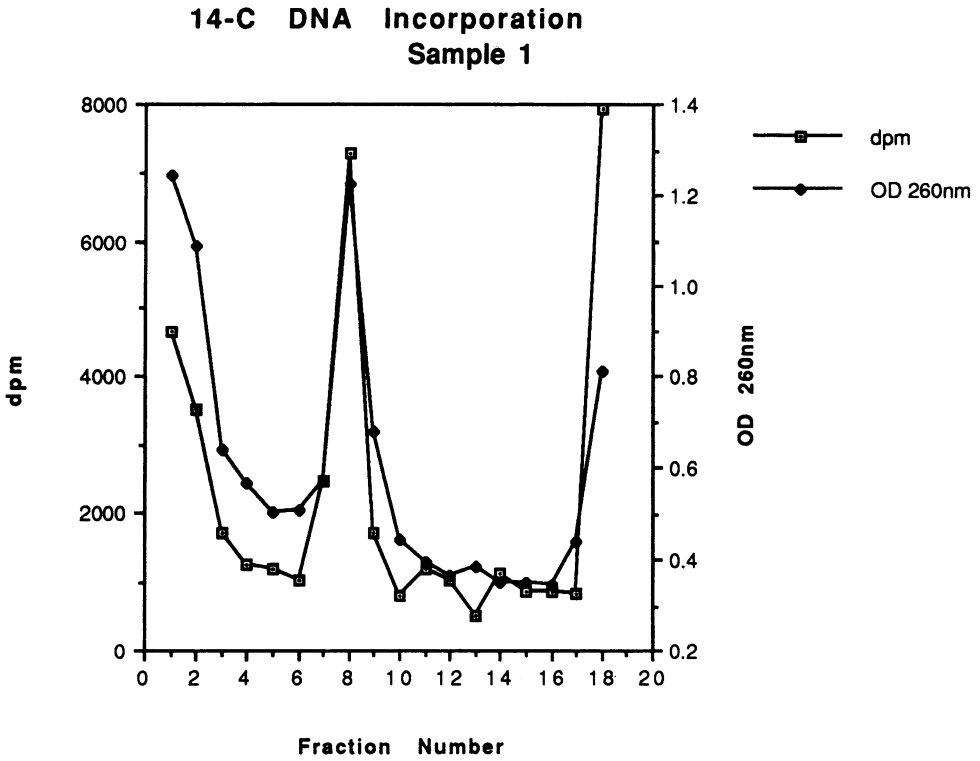

Fig. 1. Incorporation of ^{14}C-MeOH in mouse embryonic DNA. The profile of DNA is consistent with ^{14}C-activity.

sulting image was analyzed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/) using Andreas Becker's gel plotting macros (available on the same web site) to determine the area under the peaks.

2.3. Analysis of the protein profile by SDS-PAGE

The concepti were washed and yolk sacs and embryos carefully separated under a dissecting microscope. Pooled yolk sacs and embryos (N = 8/group) were lysed with 1% SDS, 1% β-mercaptoethanol, 0.025M Tris-HCl pH 6.8. Lysates were passed through a 30 gauge syringe needle 10 times and electrophoresed on 10% SDS-PAGE [19]. After electrophoresis, the gel was stained with Coomassie blue (0.25% Coomassie blue, 50% methanol, and 10% acetic acid) and destained (25% methanol,10% acetic acid) followed by autoradiography.

2.4. Incorporation of ^{14}C-methanol into embryonic protein

Embryos were cultured in CM containing ^{14}C-MeOH and processed as described above. Embryonic proteins were analyzed for incorporated radioactivity by SDS PAGE (PAGE) and autoradiography.

2.5. Analysis of ^{14}C-methanol and ^{35}S-methionine labeled, methanol exposed embryonic proteins

In order to investigate the specific effect of MeOH on protein synthesis in cultured embryos and to compare the protein profile for ^{14}C-methanol incorporation with the profile of ^{35}S incorporation into protein, embryos were cultured as described above for 24 h in the presence of ^{35}S-methionine at 20 μCi/mL plus methanol at 4 mg/mL. A control group was cultured in the absence of methanol. The embryos were collected as described above and the embryo protein profiles were analyzed for ^{35}S incorporation by SDS-PAGE and autoradiography.

3. Results

Mouse embryos cultured for 24 h in the presence of MeOH demonstrated a pattern of anomalies similar to that described earlier [10]. Briefly, embryos exposed to 4 mg MeOH/mL CM had crown-rump lengths, developmental scores, yolk sac diameters, and head lengths significantly lower than those found in controls. Embryos exposed to 8 mg MeOH/mL CM demonstrated more severe retardation of growth and development as well as significant increases in erratic neural seam, open neural tube and embryo lethality.

There was significant radiolabeling of DNA following embryonic exposure for 24 h to ^{14}C-MeOH as analyzed by

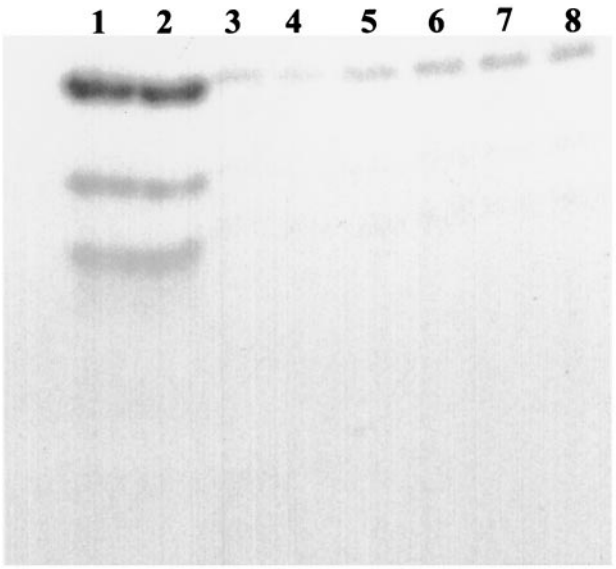

Fig. 2. Southern blot comparison of mouse embryonic DNAs treated with methylation-sensitive restriction endonuclease, *Hpa.* II and methylation insensitive *Msp.* I. Lane 1: plasmid pMo-EA containing mouse *Hoxc*8 gene cut with *Hpa.* II; lane 2: same plasmid as lane 1 cut with *Msp.* I; lane 3: control mouse embryonic DNA cut with *Hpa.* II; lane 4: mouse embryonic DNA treated with 4 mg/mL MeOH and cut with *Hpa.* II; lane 5: mouse embryonic DNA treated with 8 mg/mL MeOH and cut with *Hpa.* II; lane 6: same DNA as lane 3 but cut with *Msp.* I; lane 7: same DNA as lane 4 but cut with *Msp.* I; lane 8: same DNA as lane 5 but cut with *Msp.* I. DNA-methylation was significantly elevated in the 4 mg/mL MeOH exposed embryos.

cesium chloride density gradient centrifugation (Fig. 1). The embryonic DNA peak, as indicated by the optical density at 260 nm, was correlated with the ^{14}C activity, demonstrating that ^{14}C-MeOH was incorporated into DNA under these experimental conditions.

To investigate whether ^{14}C incorporation represented methylation of the DNA by MeOH, we analyzed genomic DNA methylation. Restriction enzyme digests of the DNA with the isoschizomers *Hpa*II (methyl sensitive) and *Msp*I (methyl insensitive) were transferred to nylon filters and probed with ^{32}P labeled probe for the *Hoxc8* gene (Fig. 2). At 4 mg MeOH/mL, embryonic DNA showed a decrease in *Hpa*II treated band intensity (lane 4) versus MspI treated DNA band intensity (lane 7). Methanol-induced changes in these band intensities of *Msp*1 and *Hpa*II in the Southern blot indicate increased methylation of DNA following embryonic exposure to 4 mg MeOH/mL culture medium. The embryonic DNAs had 30, 54, and 30% methylation in control, 4 mg MeOH/mL and 8 mg MeOH treatment groups respectively (Table 1). The failure of the highest concentration of MeOH to increase DNA methylation in these studies may be due to frank growth inhibition at this concentration.

Protein from embryos cultured with 4 mg MeOH and 20 μCi/mL ^{14}C- MeOH/mL culture medium were analyzed by SDS-PAGE. Protein fractions analyzed on SDS-PAGE and stained with Coomassie blue gave similar

Table 1
The effect of methanol on embryo DNA methylation as analyzed by methylation sensitive and insensitive endonucleases.

	Density Reading		Ratio*
Methanol (mg/mL)	*Msp*I	*Hpa*II	*Hpa*II/*Msp*I
0	6207	4406	70
4	5460	2518	46
8	5708	4092	70

* Ratio: % unmethylated fraction of DNA.

protein profiles in control and treated groups for both embryos and yolk sacs (Fig. 3a). No inhibition of specific protein synthesis was apparent at this concentration of MeOH. The protein autoradiograms gave evidence of specific incorporation of ^{14}C label into discrete proteins (Fig. 3b). The most obvious labeled proteins were those with apparent molecular weights of 72, 48, 44, 37, 26.5, 24, 18, 15, and 6 kDa. There was no evidence of a change in the level of synthesis of the labeled proteins. This profile was consistent whether the embryos were cultured with 4 mg MeOH/mL + 20 μCi ^{14}C-MeOH or with the 20 μCi ^{14}C-MeOH alone and was not influenced by MeOH concentration.

In an attempt to determine if the incorporation of ^{14}C into embryonic proteins was limited to nascent peptides, we investigated ^{14}C-MeOH incorporation in the presence of ^{35}S-methionine to determine the profile of incorporation of radio labels into the embryonic and yolk sac proteins (Fig. 4). Extensive radiolabeling was evident for all proteins

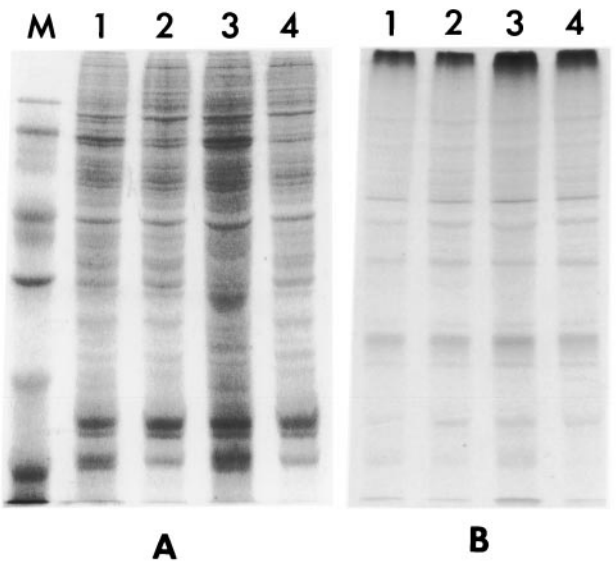

Fig. 3. Incorporation of ^{14}C-MeOH into mouse embryonic proteins. Panel A is an SDS-PAGE gel stained with Coomassie blue. M: molecular weight standards; Lane 1: yolk sac treated with 4 mg/mL of MeOH plus 20 μCi ^{14}C-MeOH; lane 2: yolk sac treated with 20 μCi ^{14}C-MeOH; lane 3: embryo treated with 4 mg/mL MeOH plus 20 μCi ^{14}C-MeOH; lane 4: embryo treated with 20 μCi ^{14}C MeOH. Panel B is the autoradiogram of the same SDS-PAGE gel. MeOH concentration did not influence the pattern of protein synthesis.

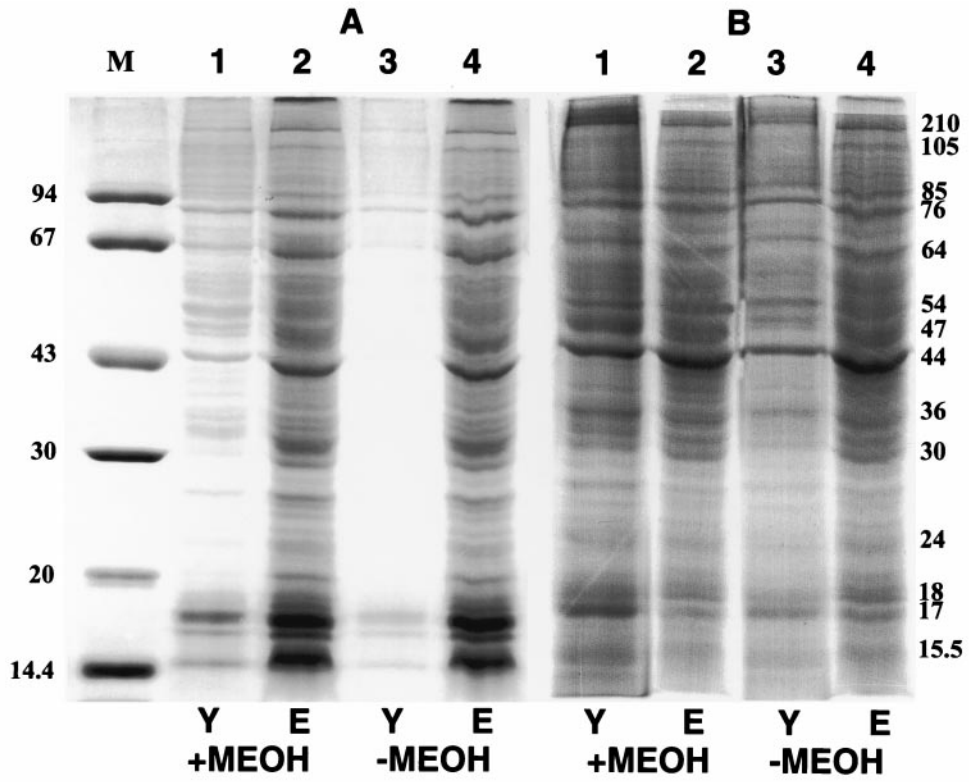

Fig. 4. Page gel depicting incorporation of ^{35}S-methionine into embryonic protein following 24 h of exposure of GD 8.5 mouse embryos. Y (Yolk Sac), E (Embryo). A. Coomassie blue stained gel which describes protein bands. B. Autoradiogram of A which describes ^{35}S-Methionine incorporation into protein as an indication of protein synthesis.

following ^{35}S-methionine exposure. There was no change in the protein profile compared to controls when embryos were cultured for 24 h in the presence of MeOH (Fig 4A2 vs 4A4). This profile was similar to the profile obtained following comparison of the autoradiogram with the respective Coomassie blue staining (Fig 5). Comparison of the radiolabeled protein profiles obtained from ^{35}S-methionine exposure and ^{14}C-MeOH exposure (Fig 3B vs 4B), indicated that all newly synthesized proteins were labeled by both radiolabels.

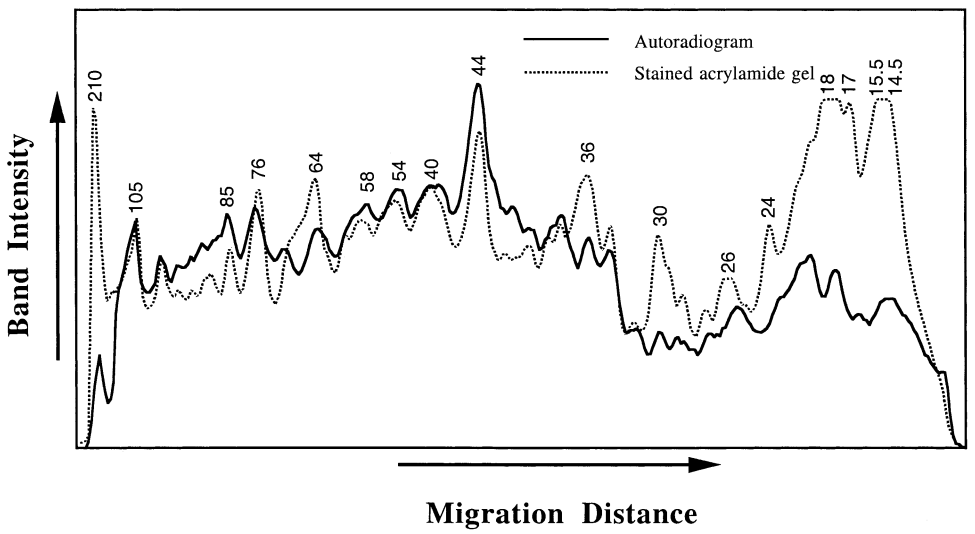

Fig. 5. When the gels in Fig. 4 were scanned, the resulting plots indicated that there were no significant differences between the profiles for the Coomassie blue stained proteins and the profile from the proteins on the autoradiogram and indicating that MeOH exposure did not result in an alteration in protein synthesis. For illustration, scans of 4A2 vs 4B2 are shown.

4. Discussion

These studies demonstrate that mouse embryos exposed in vitro for 24 h to dysmorphogenic concentrations of MeOH with tracer quantities of ^{14}C-MeOH exhibited radiolabeled DNA and protein. The observed malformations and growth retardation were similar to those we reported previously [10]. Methanol incorporation into DNA could be due to either direct methylation using MeOH as substrate or indirectly by the one carbon pathway through the conversion of MeOH to formate and subsequent participation in the tetrahydrofolate pathway.

By using the cytosine methylation discriminating isoschizomer endonuclease pair: *Msp*I (methylation insensitive) and *Hpa*II (methylation sensitive), we were able to estimate the degree to which embryonal DNA was methylated. The *Msp*I recognizes and cuts sequences both in methylated and unmethylated DNA and the *Hpa*II will cut only unmethylated DNA sequences. The results suggest that MeOH exposure did increase the frequency of DNA methylation in the region of the *Hoxc*-8 gene at the lower MeOH concentration. However, the 8 mg MeOH/mL exposure gave the same degree of methylation as the control. This lack of a dose-response for methylation could possibly be due to the severe embryo growth retardation (and presumed decreased DNA synthesis) at the 8 mg/mL concentration. Our results suggest that MeOH may be a substrate for DNA methylase, leading to the direct incorporation of the MeOH into cytosine in mouse embryonic DNA. The incorporation of radiolabel into the mouse embryonic DNA could also be through the formate-tetrahydrofolate pathway. In this pathway, THF functions as a coenzyme in the metabolism of embryonic cells by serving as a carrier of one carbon groups [20]. The one carbon derivatives of THF serve as donors of one-carbon units in synthesis of purines and certain pyrimidines [21].

Embryonic and yolk sac proteins showed similar patterns in control and methanol-treated embryos when analyzed by SDS-PAGE with Coomassie blue staining. Autoradiograms of proteins from embryos that were exposed to methanol plus 20 μCi ^{14}C-methanol/mL CM indicated there was specific incorporation of ^{14}C label into discrete proteins. The most obvious labeled proteins were those with apparent molecular weights of 72, 48, 44, 37, 26.5, 18, 15, and 6 kDa. When these autoradiograms were compared to the Coomassie blue-stained gels, the most intensely labeled proteins in the autoradiograms were, in most cases, not the most intensely Coomassie blue-stained proteins. This finding suggests that the MeOH exposure did not alter de novo protein synthesis but rather that MeOH was being incorporated into stage-specific embryonal proteins. The incorporation of radiolabel into the protein was independent of MeOH exposure.

In conclusion, when cultured embryos were exposed to radiolabeled MeOH, MeOH or its metabolites were incorporated into embryonic and yolk sac DNA and proteins. At the 4 mg MeOH/mL CM exposure, results suggested increased DNA methylation, indicating that the toxicologic effects of MeOH at this concentration may be due in part to a change in embryonic DNA methylation. However, at this exposure concentration, the incidence of embryo dysmorphology was not significantly elevated. At the 8 mg MeOH/mL CM concentration, there was greater embryotoxicity, along with significant dysmorphology, and growth inhibition may have masked any elevation in methylation. Incorporation of MeOH into embryonic proteins was evident following in vitro exposure to MeOH but no MeOH-induced de novo protein synthesis could be determined.

Acknowledgment

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