TOXICOLOGICAL SCIENCES **54,** 399–407 (2000)
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Effect of Trichloroethylene and Its Metabolites, Dichloroacetic Acid and Trichloroacetic Acid, on the Methylation and Expression of c-*Jun* and c-*Myc* Protooncogenes in Mouse Liver: Prevention by Methionine

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Received September 8, 1999; accepted December 1, 1999

Trichloroethylene (TCE), dichloroacetic acid (DCA), and trichloroacetic acid (TCA) are environmental contaminants that are carcinogenic in mouse liver. 5-Methylcytosine (5-MeC) in DNA is a mechanism that controls the transcription of mRNA, including the protooncogenes, c-*jun* and c-*myc*. We have previously reported that TCE decreased methylation of the c-*jun* and c-*myc* genes and increased the level of their mRNAs. Decreased methylation of the protooncogenes could be a result of a deficiency in S-adenosylmethionine (SAM), so that methionine, by increasing the level of SAM, would prevent hypomethylation of the genes. For 5 days, female B6C3F1 mice were administered, daily by oral gavage, either 1000 mg/kg body weight of TCE or 500 mg/kg DCA or TCA. At 30 min after each dose of carcinogen, the mice received, by ip injection, 0-, 30-, 100-, 300-, or 450-mg/kg methionine. Mice were euthanized at 100 min after the last dose of DCA, TCA, or TCE. Decreased methylation in the promoter regions of the c-*jun* and c-*myc* genes and increased levels of their mRNA and proteins were found in livers of mice exposed to TCE, DCA, and TCA. Methionine prevented both the decreased methylation and the increased levels of the mRNA and proteins of the two protooncogenes. The prevention by methionine of DCA- TCA-, and TCE-induced DNA hypomethylation supports the hypothesis that these carcinogens act by depleting the availability of SAM. Hence, methionine would prevent DNA hypomethylation by maintaining the level of SAM. Furthermore, the results suggest that the dose of DCA, TCA, or TCE must be sufficient to decrease the level of SAM in order for these carcinogens to be active.

Key Words: **c-*myc*; c-*jun*; dichloroacetic acid; DNA methylation; trichloroacetic acid; trichloroethylene; mRNA expression; methionine; protooncogenes.**

Trichloroethylene (TCE) is a common industrial solvent that has been used for vapor degreasing of fabricated metal parts and as a lubricant, a low-temperature heat transfer medium, a fumigant, an extractant, and a disinfectant (Kaneko *et al.,* 1997; IARC, 1995; Maull *et al.,* 1997). Widespread use of TCE has resulted in the contamination of surface water, ground water, and hazardous waste disposal sites (Coleman *et al,* 1984; Conglio *et al,* 1980; Westerick *et al.,* 1984). Dichloroacetic acid (DCA) and trichloroacetic acid (TCA) are major metabolites of TCE (Bruckner *et al.,* 1989; IARC, 1995). The two chloroacetic acids are also found in chlorinated drinking water as by-products of chlorine disinfection (IARC, 1995; Coleman *et al,* 1984; Uden and Miller, 1983). Hence, there exists the potential for human exposure to TCE found in the environment and to DCA and TCA in chlorinated drinking water or from metabolism of TCE.

DCA, TCA, and TCE are peroxisome proliferators (Bruckner *et al.,* 1989; Elcombe, 1985; Goldsworthy and Popp, 1987; Odum *et al*., 1988) that induce foci of altered hepatocytes and hepatocellular adenomas and carcinomas in B6C3F1 mice (Bull *et al*., 1990; Herren-Freund *et al.,* 1987; Latendresse and Pereira, 1997; Pereira, 1996; Pereira *et al*., 1997; Pereira and Phelps, 1996). Mouse liver tumors induced by DCA, TCA, and TCE, relative to spontaneous tumors, have been shown not to contain a unique mutation in either the H- or K-*ras* oncogenes (Anna *et al*., 1994; Ferreira-Gonzalez *et al*., 1995; Fox *et al.,* 1996; Leavitt *et al.,* 1997; Schroeder *et al*., 1997). Due to their very weak genotoxicity in *in vitro* and *in vivo* assays (Chang *et al*., 1992; Fahrig *et al*., 1995; Fox *et al.,* 1996; Meier and Blazak, 1990), an epigenetic mechanism has been proposed for their carcinogenic activity that is associated with increased cell proliferation (Butterworth *et al.,* 1992; Goodman *et al.,* 1991; Roberts *et al.* 1997).

DCA, TCA, and TCE have been shown to induce cell proliferation in mouse liver (Bull *et al*., 1990; Channel *et al*., 1998; Pereira, 1996). The immediate-early protooncogenes, c-*jun* and c-*myc*, participate in the control of cell proliferation and apoptosis (Cole and McMahan, 1999; Fausto and Shank, 1983; Fausto and Webber, 1993; Sæter and Seglen, 1990). Increased expression of these two protooncogenes has been observed in the liver after partial hepatectomy and during treatment with epigenetic carcinogens (Fausto and Shank, 1983; Fausto and Webber, 1993; Sæter and Seglen, 1990). Increased levels of the mRNA and protein for c-*jun* and c-*myc* genes have been reported in liver and liver tumors from mice

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treated with DCA, TCA, or TCE (Latendresse and Pereira, 1997; Nelson *et al*, 1990; Tao *et al*., 1999). The c-*jun* and c-*fos* proteins form the transcription factor, AP-1, that primes cells for proliferation during liver regeneration following partial hepatectomy (Brenner, 1998; Fausto and Webber, 1993). c-*Myc* protein is required for efficient progression through the cell cycle (Cole and McMahon, 1999; Facchini and Penn, 1998). It forms a heteromeric complex with the murine Myn protein (Prendergast *et al.*, 1991) and the human homolog, Max protein (Blackwood and Eisenman, 1991) to activate transcription (Dang, 1999; Steiner *et al*., 1996). With respect to increased cell proliferation, target genes for up-regulation have been proposed to include *cdc25A* and cyclins A, D1, and E (Dang, 1999). c-*Myc* also regulates cyclin-dependent kinases (cdk) including inhibition of cdk inhibitors (Steiner *et al.*, 1996).

Methylation of DNA as 5-methylcytosine (5-MeC) in the promoter region of genes regulates mRNA expression, including that of the protooncogenes, c-*jun* and c-*myc* (Bird, 1986; Garcea *et al*, 1989; Herman *et al*., 1994; Jones and Buckley, 1990; Razin and Kafri, 1994; Stoger *et al*., 1993; Wainfan and Poirier, 1992). Decreased levels of 5MeC in DNA and in specific genes including c-*jun* and c-*myc* are frequently identified as early events in both human and mouse neoplasias (Gama-Sosa *et al*., 1983; Jones and Buckley, 1990; Lapeyre *et al*., 1981; Vogelstein *et al*., 1988). The extent of DNA methylation has been reported to decrease further with the progression from benign to metastatic neoplasm (Gama-Sosa *et al.*, 1983; Lapeyre *et al.*, 1981; Tao, *et al.*, 1998). In mouse liver, non-genotoxic carcinogens including phenobarbital, a choline-devoid and methionine-deficient diet, DCA, TCA, and TCE decreased the methylation of DNA (Christman *et al*., 1993; Counts and Goodman, 1995; Counts *et al*., 1996, 1997; Tao *et al*., 1998, 1999). Hence, DNA hypomethylation has been proposed as a mechanism for non-genotoxic carcinogens to increase cell proliferation and to induce tumors (Counts and Goodman, 1995).

Methionine is required for the synthesis of S-adenosylmethionine (SAM), the cofactor for DNA methylation. Choline-devoid and methionine-deficient diet has been shown to cause hepatocellular carcinomas in both rats and mice (Copeland and Salmon, 1946; Ghoshal and Farber, 1984; Mikol *et al*., 1983; Newberne *et al.*, 1982) and to induce hypomethylation of H-*ras*, c-*myc*, and c-*fos* genes (Henning and Swendseid, 1996; Wainfan and Poirier, 1992). Methionine has been reported to prevent aflatoxin B1-induced liver tumorigenesis (Newberne *et al.*, 1990) and diethylnitrosamine-initiated and phenobarbital-promoted liver carcinogenesis (Fullerton *et al.*, 1990). Thus, methionine deficiency appears to enhance liver carcinogenesis while supplemental methionine appears to prevent liver cancer. Furthermore, it appears that the cancer-enhancing activity of methionine deficiency is associated with DNA hypomethylation, including the hypomethylation of protooncogenes. Therefore, we evaluated the ability of methionine to prevent the decrease in the methylation of the c-*jun* and c-*myc* genes and the increase in the expression of their mRNA and proteins that are induced by DCA, TCA, and TCE.

MATERIALS AND METHODS

Chemicals and DNA probes. DCA and TCA were obtained from Aldrich Chemical Company, Inc (Milwaukee, WI). TCE, L-methionine, ribonuclease A type III-A and proteinase K were from Sigma Chemical Company, Inc (St. Louis, MO). TRIZOL Reagent was purchased from GIBCO BRL/Life Technologies, Inc. (Gaithersburg, MD). Oligonucleotide probes for c-*jun* and c-*myc* and monoclonal antibodies for c-*jun* (Ab-3) and c-*myc* (Ab-2) were obtained from Oncogene Research Products (Cambridge, MA). *Hpa II* was from New England BioLabs (Beverly, MA). Hybond^{TM}-N^{+} nylon membranes, (α-^{32}P) -dCTP (6000 Ci/mmol), (γ-^{32}P) -ATP (5000 Ci/mmol), enhanced chemiluminescence reagents and T_4 polynucleotide kinase were obtained from Amersham Corp. (Arlington Heights, IL). Prime-a-Gene-Labeling System was from Promega Corp. (Madison, WI). Anti-mouse IgG-HRP and protein molecular weight standards were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other chemicals used were molecular biology or HPLC grade, as commercially available.

Animals and treatments. VAF (viral antibody-free) B6C3F1 female mice were purchased from Charles River Breeding Laboratories (Portage, MI). Female mice were used, since the carcinogenic and tumor-promoting activities of DCA and TCA were first observed in this sex (Herren-Freund *et al*., 1987; Pereira and Phelps, 1996). Six-week-old mice arrived at the AAALAC-accredited facility of the Medical College of Ohio. They were housed in accordance with the U.S. Public Health Service "Guide for the Care and Use of Laboratory Animals." Deionized and filtered tap water and Laboratory Rodent Diet 5001 (J & B Feed, Toledo, OH) were provided *ad libitum* to the mice.

At 8 weeks-of-age, the mice were dosed by gavage 500 mg/kg DCA or TCA in water neutralized with sodium hydroxide to pH 6.5–7.5, or 1000 mg/kg TCE in corn oil. The doses of DCA, TCA, and TCE were chosen because they have been reported to increase liver growth, cell proliferation, and lipid peroxidation in mice (Channel *et al*., 1998; Dees and Travis, 1994; Larson and Bull, 1992; Styles *et al*., 1991). DCA, TCA, and TCE were administered once a day for 5 days. Vehicle-control mice received the same volume of water or corn oil. At 30 min after each dose of carcinogen or vehicle, the mice received 0, 30, 100, 300, or 450 mg/kg methionine by ip injection. The high dose level of 450 mg/kg was selected because it has been reported to prevent bromobenzene-induced decrease in hepatic SAM and alterations in DNA methylation (Lertratanangkoon *et al*., 1996, 1997). The mice were euthanized by carbon dioxide asphyxiation at 100 min after the fifth dose of carcinogen or vehicle. Sacrifice of the animals at 100 min after the last dose was chosen because it resulted in the peak increase in the level of the mRNA of the two protooncogenes after administering TCE (Tao *et al*., 1999). As a positive control for enhancement of mRNA expression of the protooncogenes, mice were administered by gavage a single 2 ml/kg dose of carbon tetrachloride (1:1 by weight in corn oil) and killed 100 min later. At necropsy, the liver was rapidly excised, weighed, frozen in liquid nitrogen and stored at –70°C.

Analysis of DNA methylation status in the promoter region for the c-*jun *and c-*myc *protooncogenes. Methylation status in the promoter region for c-*jun* and c-*myc* protooncogenes was evaluated using *Hpa* II restriction-enzyme digestion followed by Southern blot analysis, as described previously (Tao *et al*, 1999). Briefly, DNA was isolated from the liver tissues by digestion with 400 μg/ml RNase A and 200 μg/ml proteinase K followed by organic extraction with phenol, chloroform, and isoamyl alcohol. The isolated DNA was digested at 37°C with the methyl-sensitive restriction enzyme, *Hpa* II (10 U/μg DNA). *Hpa* II does not cut CCGG sites when the internal cytosine is methylated. The digested DNA was electrophoresed on 1% agarose gel and then transferred to Hybond^{TM}-N^{+} nylon membranes. *Hind* III produced DNA fragments of Lambda-phage were included with each gel as molecular size markers.

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DNA was cross-linked by ultraviolet irradiation with an UV Stratalinker 2400 (Strategene, La Jolla, CA) and hybridized with ^{32}P-labeled probes by random priming procedure. Probes for the promoter regions of the protooncogenes were produced by PCR amplification of normal mouse-liver genomic DNA using the following primers for c-*jun*, sense 5′-GTTGTAGTGGACCT-CATTCGCTCTA-3′ and antisense 5′-AGGGGTGTTCGCTTTGCCTC-CGAGG-3′ and for c-*myc*, sense 5′-TCTAGAACCAATGCACAGAG-CAAAAG-3′ and antisense 5′-GCCTCAGCCCGCAGTCCAGTACTCC-3′. The probes were designed from the GenBank database (Accession no. U60582 for c-*jun* and M12345 for c-*myc* genes) and contained, for c-*jun* and c-*myc*, the 1914–2422 and 1–1315 bp, respectively, in the promoter region of the genes.

Analysis for mRNA expression of protooncogenes. Expression of mRNA for c-*jun* and c-*myc* protooncogenes was evaluated by Northern blot analysis, as described previously (Tao *et al*, 1999). Briefly, total RNA was isolated from the liver tissue by using TRIZOL Reagent (Chomczynski, 1993). The yield, purity, and integrity of the RNA were assessed by absorbance at 260 nm, the A_{260}/A_{280} ratio (1.7–1.9), and agarose/formaldehyde gel electrophoresis, respectively. The RNA was electrophoresed on the denaturing formaldehyde gels and then transferred to Hybond^{TM}-N^{+} nylon membranes by downward alkaline capillary action (Ming *et al.*, 1994). Oligonucleotide probes for mouse c-*jun* and c-*myc* were labeled with (γ-^{32}P)-ATP by the 5′-end labeling procedure to a minimum specific activity of 10^8 cpm/μg and separated from unincorporated ATP using a Sephadex G-50 column. Using a Biometra Mini Hybridization Oven, OV3, (Biometra, Inc., Germany), the Northern blots were prehybridized at 65°C for 1 h in hybridization solution (1.0 M NaCl, 50 mM Tris-HCl, pH 7.5, 10% dextran sulfate, 1% SDS, and 100 μg/ml denatured nonhomologous DNA). The 5′ end-labeled probe (65 ng) was then added and hybridization proceeded overnight at 65°C. After hybridization, the membrane was washed 4× with 2 × SSC containing 0.1% SDS at room temperature, once for 30 min at hybridization temperature, and once again at room temperature for 5 min, followed by a brief wash with 2 × SSC. The membrane was dried and sealed in a plastic bag. Autoradiography was processed at –70°C using Kodak Biomax MR X-ray film with an intensifying screen.

Western blot analyses for c-*jun *and c-*myc *proteins. Liver tissue was homogenized in a buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM EGTA (pH 7.5), 1 mM EDTA (pH 8.0), 10 mM β-mercaptoethanol, 1 mM phenylmethyl-sulfonyl fluoride, 0.02% leupeptin, 0.04% trypsin inhibitor, 0.25M sucrose and 0.1% triton X-100, sonicated and centrifuged at 12,000g for 30 min at 40°C. Protein concentration in the supernatant was determined using the Bio-Rad Protein Assay (BioRad Corp. Richmond, CA). The supernatant (30 μg protein) was electrophoresed on 12% SDS-PAGE minigels under reducing conditions and blotted electrophoretically to Immobilon-P membranes. Detection of c-*jun* and c-*myc* was performed using 1:500 dilution of the mouse monoclonal antibodies, Ab-3 and Ab-2, respectively, and anti-mouse IgG-HRP. The blot was developed with enhanced chemiluminescence reagents.

Statistical evaluation. SigmaStat software, version 2.03, (Jandel Corp., San Rafeal, CA) was used to perform the statistical analysis. The results were analyzed for statistical significance by a 1-way analysis of variance followed by a Tukey test with a p-value < 0.05.

RESULTS

Mice treated for 5 days with TCE (1000 mg/kg), DCA (500 mg/kg), or TCA (500 mg/kg) had significantly increased liver/body weight ratios (×100) compared to vehicle-treated controls (Fig. 1). Methionine (300 or 450 mg/kg) did not significantly affect the increased liver/body weight ratios induced by DCA, TCA and TCE. Lower dose levels of 30 and 100 mg/kg methionine also did not affect the increased liver/body weight ratios induced by them (data not presented). Thus, methionine administered 30 min after each dose of DCA, TCA, and TCE did not prevent the increase in liver weight.

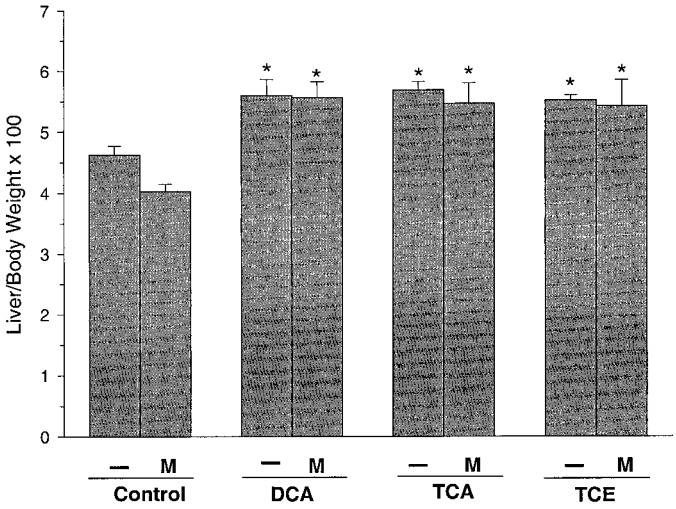

FIG. 1. Liver-to-body weight ratio in DCA, TCA and TCE-treated mice with (**M**) or without (-) methionine. Female B6C3F1 mice were treated for 5 days with 500 mg/kg DCA or TCA or 1000 mg/kg TCE by gavage followed 30 min later by an ip injection of methionine (300 mg/kg for TCE group and 450 mg/kg for DCA and TCA). Results are means ± SE for 4–6 animals/group. The asterisks denote significant difference from the vehicle control mice with (**M**) or without (-) methionine, p-value < 0.05.

*Effect of Methionine on DCA- and TCA-induced Hypomethylation of c-*Jun *and c-*Myc

The methylation-sensitive restriction endonuclease *Hpa* II and Southern blot analysis were used to assess the methylation status in the promoter regions of the c-*jun* and c-*myc* genes. *Hpa* II does not cut CCGG sites when the internal cytosine is methylated. The promoter regions for the c-*jun* and c-*myc* genes were hypomethylated after 5 days of exposure to DCA and TCA (Fig. 2). When treated with *Hpa* II, Southern blots probed for the promoter region of the c-*jun* gene contained bands of 3.2 and 1.5 kb that were present only in DNA from DCA- and TCA-treated mice. When probed for the promoter region of the c-*myc* gene, three bands of 0.5, 1.0 and 2.2 kb were present in *Hpa* II-digested DNA from DCA and TCA-treated mice. These bands were not present in undigested DNA from vehicle-control mice (Lane 1) or from mice treated with DCA and TCA (data not presented), or in *Hpa* II-digested DNA from vehicle-control mice (Lane 2). Thus DNA from control mice appeared to contain 5-MeC at the internal cytosine of CCGG sites that prevented digestion by *Hpa* II. In contrast, the external cytosine of the CCGG sites appears not to be methylated. When DNA from control mice was digested with *Msp* I, radioactivity was found between 100 and 600 bp when probed for c-*myc* (data not presented). This smear of radioactivity likely resulted from cuts at the 12 CCGG sites in the area probed. Thus, *Hpa* II digestion of DNA from

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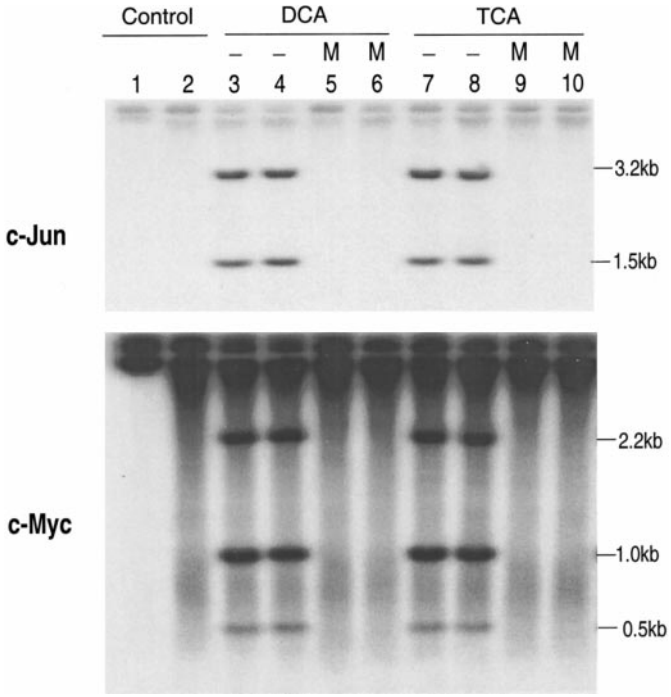

FIG. 2. Effect of methionine (M) on the methylation status of c-*jun* and c-*myc* gene promoters in the liver of mice administered DCA and TCA (500 mg/kg) for 5 days. Methionine (450 mg/kg) was administered ip at 30 min after each dose of carcinogen. DNA (30 μg), except for lane 1, was digested with *Hpa* II. The DNA was then electrophoresed in a 1% agarose gel, transferred to a Hybond-N$^+$ membrane, hybridized to ^{32}P-labeled probe for c-*jun* or c-*myc* promoter and visualized by autoradiography. Each DNA sample is from a different animal. Lanes 1 and 2 are from control. Lanes 3 to 6 and 7 to 10 are from mice treated by gavage with DCA and TCA for 5 days, respectively. The mice of lanes 5, 6, 9, and 10 were administered methionine (450 mg/kg) by ip injection at 30 min after each dose of the chloroacetic acids. The arrows in the right margin indicate the 3.2 and 1.5 kb bands and the 2.2, 1.0, and 0.5 kb bands for c-*jun* and c-*myc*, respectively.

mice treated with DCA, TCA, and TCE resulted in 3 distinct bands of greater size, than with *Msp* I digestion. This would indicate that many of the internal cytosine at CCGG sites in the region probed for c-*myc* remained methylated in DNA from mice treated with these chemicals. When *Msp* I-digested DNA was probed for c-*jun*, 2 bands were found that corresponded to the two *Hpa* II bands of DNA from DCA- and TCA-treated mice, indicating that the external cytosine was not methylated.

The ability of methionine (450 mg/kg), when administered 30 min after each dose of DCA and TCA to prevent the demethylation of DNA, was investigated. After *Hpa* II digestion, the 2 and 3 bands found when probed for c-*jun* and c-*myc*, respectively, were no longer present in DNA from DCA- and TCA-treated mice that had also received methionine (Fig. 2). Thus, methionine prevented DCA and TCA demethylation of the *Hpa* II-sensitive CCGG sites in the promoter regions of the 2 genes.

*Dose-Response for the Prevention by Methionine of TCE-, DCA- and TCA-Induced Hypomethylation of c-*Jun *and c-*Myc

The dose-response relationship for the prevention by methionine of DCA, TCA, and TCE-induced hypomethylation in the promoter regions of c-*jun* and c-*myc* genes is presented in Figure 3. Methionine at doses of 0, 30, 100, and 300 mg/kg was administered 30 min after each treatment of DCA, TCA, and TCE. Similar to the experiment presented in Figure 2, *Hpa* II digested DNA from DCA-, TCA- and TCE-treated mice contained 2 bands of 3.2 and 1.5 kb and 3 bands at 2.2, 1.0, and 0.5 kb after probing for c-*jun* and c-*myc*, respectively. Furthermore, the results confirm that TCE induces 2 and 3 bands in *Hpa* II-digested DNA probed for the promoter regions of the c-*jun* and c-*myc* genes, respectively (Tao *et al.*, 1999). These bands were absent in DNA from vehicle-treated control mice with/without treatment with methionine. When the mice received 300-mg/kg methionine after each dose of DCA, TCA, or TCE, the 2 and 3 bands after probing for c-*jun* and c-*myc*, respectively, were absent. When each dose of the DCA, TCA, and TCE was followed with 100 mg/kg methionine but not with 30 mg/kg methionine, the density of the bands after *Hpa* II digestion and probing for c-*jun* and c-*myc* was reduced. Hence, methionine exhibited a dose-dependent prevention of DCA-, TCA-, and TCE-induced demethylation of the promoter

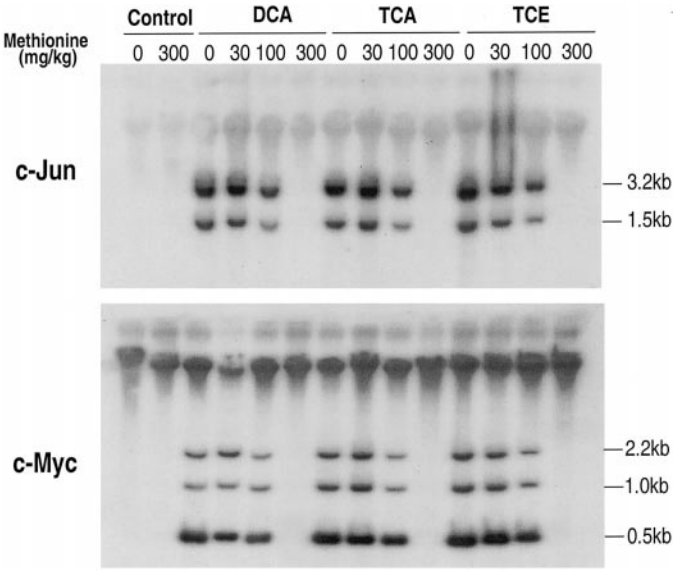

FIG. 3. Dose-response for the effect of methionine on the methylation of the c-*jun* and c-*myc* gene promoters in the liver of mice treated with DCA (500 mg/kg), TCA (500 mg/kg) and TCE (1000 mg/kg) for 5 days. The mice were administered methionine by ip injection (dose levels given in mg/kg) at 30 min after each dose of carcinogen. DNA (30 μg) was digested with *Hpa* II and electrophoresed in a 1% agarose gel. The DNA was then transferred to a Hybond-N$^+$ membrane, hybridized to ^{32}P-labeled probe for c-*jun* or c-*myc* promoter regions, and visualized by autoradiography. The arrows in the right margin indicate the size of bands.

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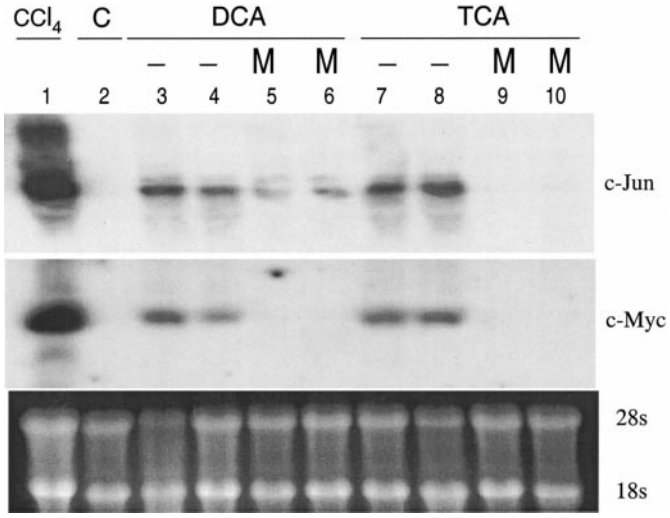

FIG. 4. Effect of methionine (M) on the expression of the mRNA for protooncogenes. The mice were administered DCA (500 mg/kg), TCA (500 mg/kg), and TCE (1000 mg/kg) for 5 days. Methionine (450 mg/kg) was administered ip at 30 min after each dose of carcinogen. Northern blot analysis of the RNA (30 μg) was performed using oligonucleotide probes for c-*jun* and c-*myc*. Lane 1 is the carbon tetrachloride (2 ml/kg) positive control for the enhancement of the expression of the protooncogenes. Lane 2 (C) is from a vehicle-control mouse.

regions of the c-*jun* and c-*myc* genes, with 30 mg/kg being an ineffective dose.

*Effect of Methionine on DCA- and TCA-Increased Expression of the mRNA of c-*Jun *and c-*Myc

Northern blot analysis of the mRNA for the c-*jun* and c-*myc* genes in the livers of mice that were treated with DCA and TCA, followed with/without methionine, is presented in Figure 4. The mRNA levels for the two protooncogenes were virtually undetectable in liver from vehicle-treated control mice with/without subsequent treatment with methionine. The expression of the mRNA for the c-*jun* and c-*myc* genes was increased at 100 min after administering the last doses of DCA and TCA (Lanes 3, 4, 7, and 8) and after a single 2 ml/kg dose of carbon tetrachloride (Lane 1, positive control). However, when each dose of either chloroacetic acid was followed in 30 min by 450 mg/kg methionine, the expression of mRNA for c-*jun* and c-*myc* was greatly attenuated or undetectable (Lanes 5, 6, 9, and 10). Methionine appeared to completely prevent the increase in the expression of the mRNA of both protooncogenes induced by TCA, and the increase in c-*myc* induced by DCA. Although not completely prevented, the DCA-induced increase in the mRNA expression of c-*jun* was reduced by methionine.

*Effect of Methionine on DCA- and TCA-Induced Increase in the Level of the c-*Jun *and c-*Myc *Proteins*

The c-*jun* and c-*myc* proteins were present in low levels in the livers of vehicle-treated control mice and their levels were not affected by methionine (Fig. 5). The c-*myc* protein migrated as 2 bands of 65 and 49 kDa. The upper band (65 kDa) is likely the phosphorylated form of c-*myc* (Persson *et al.*, 1984). DCA and TCA increased the yield of the c-*jun* and c-*myc* proteins including both forms of c-*myc*. c-*Jun* was increased to a greater extent than either form of c-*myc*. Methionine administered 30 min after each dose of DCA and TCA prevented the increase in the levels of the proteins for both protooncogenes. Thus, the level of the proteins for the 2 protooncogenes remained close to their levels in vehicle-treated control mice. Hence, the increased levels of the c-Jun and c-*myc* proteins induced by DCA and TCA were prevented by methionine.

DISCUSSION

Trichloroethylene, dichloroacetic acid, and trichloroacetic acid are environmental contaminants that are liver carcinogens in mice (Bull *et al.*, 1990; Herren-Freund *et al.*, 1987; IARC, 1995; National Toxicology Program, 1990; Pereira, 1996; Pereira and Phelps, 1996). Since they have demonstrated very weak if any genotoxic activity assays (Chang *et al.*, 1992; Fahrig *et al.*, 1995; Fox *et al.*, 1996; Meier and Blazak, 1990), it is likely that their carcinogenic mechanism is epigenetic and involves enhancement of cell proliferation and/or prevention of apoptosis. Nongenotoxic liver carcinogens have been proposed to act by altering both cell proliferation and apoptosis (Goodman *et al.*, 1991; Schulte-Herman *et al.*, 1983). During cell proliferation induced in the liver by chemicals or partial hepatectomy, the mRNA and protein levels of the immediate-early protooncogenes, including c-*jun* and c-*myc* are increased (Butterworth *et al.*, 1994; Coni *et al.*, 1993; Fausto and Shank,

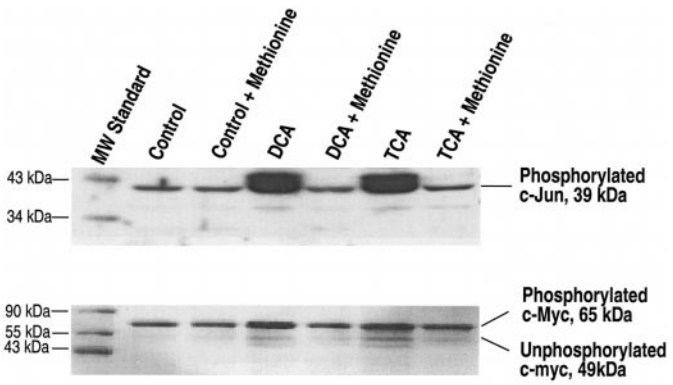

FIG. 5. Effect of methionine (M) on the expression of the protein for the protooncogenes. The mice were administered DCA (500 mg/kg), TCA (500 mg/kg), and TCE (1000 mg/kg) for 5 days. Methionine (450 mg/kg) was administered ip at 30 min after each dose of carcinogen. Western blot analysis of the protein (30 μg) was performed using the mouse monoclonal antibodies for c-*jun* and c-*myc* (1:500 dilution) and anti-mouse IgG-HRP. Blots were developed with enhanced chemiluminescence reagents. The arrows in the right margin indicate the size of the proteins and in the left margin the arrows indicate the molecular weight of the protein standards.

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1983; Fausto and Webber, 1993; Nelson *et al.*, 1990; Pitot, 1990; Sæter and Seglen, 1990; Schmiedeberg *et al.*, 1993; Wainfan and Poirier, 1992). Chemicals that increased the expression of the mRNA and proteins for the 2 protooncogenes include carbon tetrachloride, cyproterone acetate, ethylene dibromide, furan, lead nitrate, nafenopin, and phenobarbital (Butterworth *et al.*, 1994; Coni *et al.*, 1993; Pitot, 1990; Schmiedeberg *et al.*, 1993). We have previous reported that TCE increased the expression of the mRNA for the c-*jun* and c-*myc* genes (Tao *et al.*, 1999), and the current study has demonstrated that DCA and TCA also increase the level of their mRNA and proteins.

Elevated levels of c-*jun* and c-*myc* mRNA and protein have been demonstrated in human tumors in the absence of gene mutation and amplification (Erisman *et al.*, 1985; Vogt and Bos, 1990), which suggests that there exist some epigenetic mechanisms regulating gene expression. Methylation of CpG sites in the promoter region of genes, which are near, or directly within, transcription factor-binding motifs, can modulate the transcription (Baylin *et al.*, 1998). Hypomethylation of the immediate-early protooncogenes c-*jun* and c-*myc* has been associated with increased expression (Garcea *et al.*, 1989; Tao *et al.*, 1999; Wainfan and Poirier, 1992). This includes our previous demonstration that TCE decreased the methylation of the promoter regions of the c-*jun* and c-*myc* genes (Tao *et al.*, 1999). In the study presented here, the promoter regions of both protooncogenes were demonstrated to be hypomethylated in the livers of mice in response to DCA, TCA, or TCE exposure for 5 days, which was associated with increased populations of the mRNA and proteins of both genes. Increased mRNA and proteins of the 2 protooncogenes has been reported in DCA- and TCA-induced foci of altered hepatocytes and liver tumors (Latendresse and Pereira, 1997; Nelson *et al.*, 1990). Furthermore, these tumors also contained decreased methylation in the promoter regions of the c-*jun* and c-*myc* genes (unpublished results).

Other non-genotoxic carcinogens have been reported to cause hypomethylation and increased mRNA levels of protooncogenes in mouse liver. Phenobarbital decreased the methylation of the *raf* oncogene in B6C3F1 mouse liver (Ray *et al.*, 1994). In phenobarbital-induced and spontaneous liver tumors, *raf* and H-*ras* were hypomethylated (Counts *et al.*, 1997; Ray *et al.*, 1994; Vorce and Goodman, 1991). The H-*ras* gene was also hypomethylated in chloroform-induced liver tumors (Vorce and Goodman, 1991). Increased levels of H-*ras* and c-*myc* mRNA in response to chloroform have been reported (Sprankle *et al.*, 1996). Choline-devoid and methionine-deficient diet induced hypomethylation and increased expression of H-*ras*, c-*myc* and c-*fos* genes in mouse liver and tumors (Henning and Swendseid, 1996; Wainfan and Poirier, 1992). Hence, demethylation of protooncogenes and increased expression of their mRNA and proteins appears to be associated with treatment of non-genotoxic carcinogens that results in liver tumors. This has lead to the hypothesis that decreased methylation and increased expression of the protooncogenes plays a role in the mechanism of non-genotoxic carcinogens (Counts and Goodman, 1995). Our results indicate that DCA, TCA, and TCE can be added to the list of non-genotoxic carcinogens operating by this proposed mechanism.

DNA hypomethylation or demethylation can occur by three different mechanisms, which include (1) DNA demethylase activity, (2) removal of 5-MeC from DNA by 5-MeC-DNA glycosylase, and (3) prevention of the formation of 5-MeC in nascent DNA form during replication (Schmutte and Jones 1998; Wolffe *et al.*, 1999). Recently a DNA demethylase has been identified that hydrolyzes 5–MeC to cytosine and methanol (Bhattacharya *et al.*, 1999; Ramchandani *et al.*, 1999). The regulation of this enzymatic activity is unknown. However up-regulation by DCA, TCA, and TCE could be involved in the DNA hypomethylation induced by them. Two 5-MeC-DNA glycosylases have been described (Schmutte and Jones 1998). One is replication dependent and preferentially removes 5-MeC from hemi-methylated sites formed during DNA replication. Demethylation by this glycosylase is in competition with DNA methyltransferase (DNA MTase) for the hemi-methylated sites (Schmutte and Jones 1998). The up-regulation of DNA MTase that occurs concurrently with DNA hypomethylation should prevent demethylation of DNA by this glycosylase (Belinsky *et al.*, 1998; Lopatina *et al.*, 1998; Sun *et al.*, 1997). The other 5-MeC-DNA glycosylase is DNA replication-independent and appears to cause gene-specific demethylation. Since, DCA and TCA caused large decreases (35–50%) in global DNA methylation (Tao *et al.*, 1998) it is not likely that induction of gene specific 5-MeC-DNA glycosylases would be responsible for such extensive decrease in DNA methylation.

Prevention of the methylation of nascent DNA during replication could result from a down-regulation/inhibition of DNA MTase or from a decrease in the availability of SAM to act as substrate. However, elevated expression and activity of DNA MTase has been found along with DNA hypomethylation in many human and mouse tumors including liver tumors (Belinsky *et al.*, 1998; Lopatina *et al.*, 1998; Sun *et al.*, 1997). Thus, decreased DNA MTase activity is not the likely cause of DNA hypomethylation. Another way to decrease the formation of 5-MeC in DNA is to decrease the concentration of SAM and/or increase the concentration of SAH, an inhibitor of DNA MTase. Administration of a choline-devoid and methionine-deficient diet leads to the development of hepatocellular carcinomas in both rats and mice, which has been associated with hypomethylation and overexpression of H-*ras* and c-*myc* genes (Henning and Swendseid, 199; Wainfan and Poirier, 1992). Supplementation with both choline and methionine has prevented diethylnitrosamine-initiated and phenobarbital-promoted liver carcinoma formation in C3H/He mice (Fullerton *et al.*, 1990). This suggests that supplementation with methionine might prevent DNA hypomethylation. Hence, methionine prevented in a dose-dependent manner DCA-, TCA-, and TCE-

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induced hypomethylation of c-*jun* and c-*myc* protooncogenes. Methionine also prevented DCA-, TCA-, and TCE-induced increase in the expression of the mRNA and protein for the 2 protooncogenes, further indicating an association between hypomethylation of the genes and increased levels of their mRNA and protein. Since, hypomethylation and increased expression of the protooncogenes is associated with hepatocarcinogenesis, our results suggest that methionine would prevent liver cancer induced by DCA, TCA, and TCE.

In conclusion, DCA, TCA, and TCE decreased the methylation in the promoter regions for the c-*jun* and c-*myc* genes and increased the expression of their mRNA and proteins. Methionine, in a dose-dependent manner, prevented the decreased methylation and increased expression of the protooncogenes**.** Hence, the results are supportive of DNA hypomethylation as a mechanism for the carcinogenic activity of DCA, TCA, and TCE. Furthermore, the results suggest that the dose of DCA, TCA, and TCE must be sufficient to decrease the level of SAM in order for these carcinogens to be active.

ACKNOWLEDGMENT

This research was supported in part by the U.S. Environmental Protection Agency Grant No. R 825384–01–0.

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