Differential Display Identified Changes in mRNA Levels in Regenerating Livers From Chloroform-Treated Mice

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The nongenotoxic-cytotoxic carcinogen chloroform induces liver necrosis, regenerative cell proliferation, and, eventually, liver tumors in female B6C3F1 mice when administered by gavage at doses of 238 or 477 mg/kg/d. Administration of 1800 ppm of chloroform in the drinking water results in similar daily doses but does not produce liver toxicity or cancer. The differential-display technique was used to compare the expression of a subset of mRNAs in normal (control) and regenerating liver after chloroform-induced toxicity to define the proportion of genes whose expression changes under hepatotoxic conditions and to identify the genes that might play a role in regeneration and perhaps cancer. RNA was purified from the livers of female B6C3F1 mice after 4 d or 3 wk of gavage treatment with 3, 238, or 477 mg/kg/d of chloroform or treatment with 1800 ppm chloroform in drinking water. There was a remarkably high degree of consistency of gene expression among the animals and across dose and treatment groups as visualized by the differential-display technique. Of the 387 bands observed, only four (about 1%) changed expression in regenerating liver. The genes were assigned locus names by GenBank after sequence submission. The genes with increased mRNA levels as confirmed by northern blot analysis were MUSTIS21, a mouse primary response gene induced by growth factors and tumor promoters; MUSMRNAH, a gene highly homologous to a human gene isolated from a prostate carcinoma cell line; and MUSFRA, a novel gene. The novel gene MUSFRB exhibited decreased mRNA levels. No change in expression was seen among control mice given the nontoxic regimens of 3 mg/kg/d chloroform or 1800 ppm chloroform in drinking water, indicating that changes in expression were associated with toxicity and regeneration rather than chloroform per se. These genes and others that may be identified by expanding this approach may play a role in regeneration and perhaps in the process of chloroform-induced carcinogenesis in rodent liver. Mol. Carcinog. 20:288–297, 1997. © 1997 Wiley-Liss, Inc.

Key words: gene expression; differential display; polymerase chain reaction; cytotoxic chemicals; carcinogenesis; B6C3F1, mouse

INTRODUCTION

Chloroform (CHCl₃) is a trace contaminant generated as a by-product of drinking water and swimming pool chlorination [1,2] and of some paper-bleaching processes [3,4]. Chloroform induces cancer in rodents by a nongenotoxic-cytotoxic mode of action [5]. Neither chloroform nor its metabolites react directly with DNA [6]. Rather, tumor formation is secondary to events associated with cytotoxicity and regenerative cell proliferation [7]. For example, when given by bolus gavage in corn oil at doses of 238 or 477 mg/kg/d to female B6C3F1 mice, chloroform induces liver necrosis, regenerative cell proliferation, and, eventually, hepatocellular carcinoma [8,9]. However, when similar daily doses of chloroform are given in the drinking water, no increase in liver toxicity or liver tumors is observed [9,10]. Thus, although chloroform is a rodent carcinogen, it is probably not a human carcinogen because of its high-dose–only, nongenotoxic-cytotoxic mode of action and because it is found only in trace amounts in the environment [5].

Numerous events associated with toxicity and regeneration may be involved in carcinogenesis. Nucleases can be released that may damage DNA. Increased cell turnover can increase the rate of conversion of DNA adducts from endogenous and exogenous sources to mutations before DNA repair can occur. In addition, many genes responsible for growth control are also oncogenes and may exhibit increased expression in regenerating tissue [11–14]. Alteration in the sequence or expression of such genes can be involved in carcinogenesis [15,16]. Furthermore, during transcription the DNA is unwound, thus making these genes more susceptible to spontaneous mutations [17]. In stimulating regeneration, increased expression of growth-control genes may

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also provide a selective growth advantage to spontaneously initiated precancerous cells [18,19]. It has been estimated that some mammalian cells express about 15,000 genes [20]. Thus, it is a daunting task to identify those genes that change expression because of toxicity and regenerative cell proliferation and that may play a role in carcinogenesis. Differential display is a rapid, sensitive, and reproducible technique that can identify genes that are expressed at different levels in one population of cells compared with another [20]. Pairs of primers are chosen to amplify partial cDNA sequences from subsets of the total population of mRNAs by reverse transcription and polymerase chain reaction (PCR). The 3′ primers include a poly(T) sequence to select and anchor the primer at the 3′ end of the poly(A) tail present on most eukaryotic mRNAs. Two additional bases on the 3′ end of the poly(T) sequence restrict the primer to a subset of mRNAs. An internal 5′ primer is chosen to further restrict amplification to only cDNA sequences from a small subpopulation of mRNAs.

Our laboratory has described extensive necrosis and regenerative proliferation in the livers of female B6C3F1 mice given chloroform by gavage at the carcinogenic doses of 238 and 477 mg/kg/d (but not at 3 mg/kg/d) for periods of 4 d or 3 wk [9]. We also described the lack of liver toxicity when B6C3F1 mice were given 1800 ppm of chloroform in drinking water even though that regimen yields a dose of 329 mg/kg/d chloroform when averaged over a 3-wk treatment period [9]. The differential-display technique was used to analyze frozen liver samples from these studies to provide an initial estimate of the proportion of genes that change expression under hepatotoxic-carcinogenic conditions. Any genes so identified may be associated with molecular events underlying the initial stages of chloroform-induced hepatocarcinogenesis in female mice.

MATERIALS AND METHODS

Chemicals and DNA Probes
Chloroform, >99.5% pure and stabilized with 0.006% amylene, was obtained from Fluka Chemical Corp. (Ronkonkoma, NY). The rat albumin probe (a 1.0-kb PstI fragment) was a gift from Dr. J. Staudinger (Glaxo-Wellcome, Research Triangle Park, NC). All reagents for polyacrylamide gel electrophoresis were from Amresco (Solon, OH). Radiochemicals were obtained from Amersham Corp. (Arlington Heights, IL). HybriSorb hybridization solution and 20× standard saline citrate (SSC) stock were obtained from Oncor (Gaithersburg, MD). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), except as otherwise noted. All chemicals were of the highest purity available, and products characterized as “ultra pure” or “molecular biology grade” were used when possible.

Animals
This study was conducted under federal guidelines for the humane care and use of laboratory animals [21] and was approved by the Chemical Industry Institute of Toxicology Institutional Animal Care and Use Committee. The animals were housed in humidity- and temperature-controlled HEPA-filtered, mass air-displacement rooms in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care. The animal care procedures used have been detailed previously [9]. Briefly, female B6C3F1 mice (B6C3F1/CrlBR) were obtained from Charles River Breeding Laboratories, Inc. (Raleigh, NC). The rodents were provided with NIH-07 rodent chow (Ziegler Bros., Gardener, PA) and deionized, filtered tap water ad libitum. Lighting was provided on a 12-h light-dark cycle. Rodents were acclimated to the animal facility for 2 wk before use and were 9 wk old at the beginning of these studies. This age was chosen to duplicate the original conditions of the gavage bioassay [8]. Background cell proliferation rates were low and consistent from animal to animal at this age (data not shown). The animals were randomized by weight into treatment groups. Sentinel animals were housed in the animal facility as part of an ongoing program to monitor them for parasitic, bacterial, and viral infections and were pathogen free throughout the study.

Dosing and Necropsy of Animals
All liver tissue used was from a previously described study [9] and had been stored frozen at −70°C. No deterioration of the mRNA in the liver tissues was noted under these conditions (data not shown). The mice were given chloroform either daily by gavage in corn oil or in drinking water ad libitum. Chloroform in corn oil was given at doses of 0, 3, 238, or 477 mg/kg/d for either 4 consecutive days or for 5 d/wk for 3 wk. Chloroform in drinking water was provided ad libitum at concentrations of 0 or 1800 ppm for either 4 consecutive days or every day for 3 wk. At necropsy, the livers to be used for RNA purification were frozen in liquid nitrogen and stored frozen as described above.

The cell replication data from this experiment was published previously [9]. Groups of concurrently treated animals separate from those used to analyze gene expression were treated with 5-bromo-2′-deoxyuridine (BrdU) by osmotic pump for 3.5 d before necropsy to label cells in S phase. Regenerative cell proliferation was then quantitated immunohistochemically from liver sections.

Purification of RNA and Preparation of Northern Blots
Total cellular RNA was purified from approximately 0.5 g of liver tissue; each RNA preparation included
tissue from several different lobes of the liver. The RNAzol reagent was used for RNA purification according to the instructions of the manufacturer (Tel-Test, Friendswood, TX). RNA from at least two different mice was isolated for each individual data point. Furthermore, the degree of expression was compared across dose and treatment groups to confirm a consistent pattern of change. The consistent lack of response in the 3 mg/kg/d (low dose) and 1800 ppm drinking water groups served as additional internal controls. One hundred micrometers of total RNA was stored frozen at −70°C at a concentration of 1 µg/mL for use in the differential-display technique. Poly(A)+ mRNA was isolated and used to prepare northern blots as described previously [11]. After electrophoresis to separate the mRNA according to size, the RNA was transferred to a nylon membrane and then cross-linked to the membrane with a Stratalinker UV cross-linker (Stratagene, La Jolla, CA). The membranes were stored in sealed bags at 4°C until used.

Differential Display

Total cellular RNA isolated as described above was used to synthesize portions of cDNA, and those cDNAs were subsequently amplified by PCR by using the conditions of Liang and Pardee [20] with minor modifications. The sequences of the terminal primers used in the 12 different display reactions were T1-CN, T2-GN, or T3-AN (where N is A, T, G, or C) and are collectively referred to as the poly(T) primers. In all cases, the internal primer used was LTK3 (5′-CTTGATTGCC-3′). Reaction mixes of 10× buffer (100 mM Tris, pH 8.8; 500 mM KCl; and 15 mM MgCl₂; Perkin Elmer, Branchburg, NJ), dNTPs (2.5 mM final), 5 U of AmpliTaq, and 50 pmol each of the vector primers were used to initially amplify the fragment. The amplified fragments were cloned directly into the pCRII vector by using the TA Cloning kit (Invitrogen, San Diego, CA) according to the directions of the manufacturer. Clones were checked for correct insert size by colony PCR amplification from individual bacterial colonies in 10× buffer (Perkin Elmer), MgCl₂ (2 mM final; Perkin Elmer), dNTPs (320 µM final), 5 U of AmpliTaq, and 50 pmol each of the vector primers SP6 (5′-CCAGCTATTTAGTGAACATATG-3′) and T7 (5′-ATTGTAAATACGTACACATTAGG-3′). The PCR-amplified DNAs were purified for sequencing and probe preparation according to the manufacturer’s recommendations by using the Sephaglas BandPrep kit (Pharmacia Inc.). Each clone was isolated from at least two different colonies of bacteria and analyzed separately to verify the sequence of the clone. The clones were then used as probes to hybridize northern blots containing RNA from the livers of chloroform-treated animals as described above [11,22].

Northern Blot Hybridization and Analysis

Filters containing separated mRNAs were prehybridized with 6 mL of Hybrisol for 4 h at 42°C in a Robbins hybridization oven. The cDNA clones were labeled with [35S]dCTP by using the Prime-it II random priming kit (Stratagene and 50 ng of template. The probes were labeled to a minimum specific activity of 10⁸ cpm/µg. A total of 1 × 10⁶ incorporated counts was then added to each prehybridization solution. After hybridization for 16–38 h at 42°C, the filters were washed as previously described [11,22]. After autoradiography, each cDNA probe was removed from the membrane by two 15-min incubations in stripping solution (1× SSC, 0.25% sodium dodecyl sulfate, and 50% formamide) at 65°C followed by two brief washes in 2× SSC at room temperature. The same blot was then probed with an albumin DNA fragment to control for potential variations in mRNA loading. The autoradiographs were scanned with a laser densitometer, and quantitation was performed as previously described [11] with an
LKB Ultrascan XL Enhanced Laser Densitometer (LKB Biotechnology, Uppsala, Sweden). The samples on each autoradiograph were normalized to the values on the corresponding albumin autoradiograph. Gene expression was then calculated relative to levels of that gene in control animals.

Sequencing

DNA sequencing was performed using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) according to manufacturer's directions. Fifty picomoles of the SP6 and T7 primers was added to a premix of buffer, dNTPs, DyeDeoxy terminators, and AmpliTaq DNA polymerase. One microgram of template DNA was added to the mixture and amplified as follows: 96°C for 30 s, 42°C for 30 s, and 60°C for 4 min for 25 cycles. The reactions were then purified with a Centri-Sep column (Princeton Separations, Adelphia, NJ) and ethanol precipitated. They were resuspended in a 5:1 mixture of formamide and EDTA, loaded onto a 6% denaturing sequencing gel, and analyzed by using the Applied Biosystems Sequencing System (Applied Biosystems).

RESULTS

Hepatotoxicity and Cellular Changes

The patterns of chloroform-induced necrosis and regenerative cell proliferation that occurred after chloroform treatment in this study, have been described previously [9]. Briefly, there were no significant changes from controls in either histology or BrdU labeling index (percentage of hepatocytes in S-phase) of the livers of animals treated with either 1800 ppm chloroform in drinking water or 3 mg/kg/d chloroform administered by corn-oil gavage. Histological changes at higher doses of chloroform administered by corn-oil gavage ranged from scattered centrilobular and subcapsular hepatocyte necrosis at 4 d of 238 mg/kg/d to severe centrilobular hepatic necrosis at 3 wk of 5 d/wk 238 mg/kg/d to severe centrilobular coagulative necrosis with some inflammatory cells at 4 d of 477 mg/kg/d. Finally, at 3 wk of 5 d/wk 477 mg/kg/d, the central zone was populated by vacuolated and regenerating hepatocytes. There was a dose-related increase in labeling index over control levels at all 238 and 477 mg/kg/d time points, with the greatest increase following administration of chloroform for 4 d at 477 mg/kg/d.

Differential Display

Each of the 12 primer pairs (LTK3, paired with each poly(T)primer) resulted in amplification of about 30 cDNA fragments of about 100–500 bp. One of the most remarkable observations from these studies was the high degree of consistency of gene expression from animal to animal and across dose and treatment groups, as visualized by the differential-display technique. Of the 387 bands observed, only nine showed consistent increases or decreases in band intensity between at least one chloroform treatment and controls (Table 1). This consistency confirms the reliability and reproducibility of this technique. These clones were named to indicate the internal primer used, the poly(T)primer used, and the approximate observed size of the band from the polyacrylamide gel. For example, a clone of approximately 238 bp originally amplified in a PCR reaction using the LTK3 and the T11CG primers was named LTK3-T11CG-238. These fragments ranged in size from approximately 120 to 228 bp. Listed in Table 1 are the genes that were subsequently confirmed by northern blot analysis as changing expression. No differences in mRNA levels were observed between control animals and animals treated with 3 mg/kg/d chloroform in corn oil or 1800 ppm chloroform in drinking water.

Identification of Chloroform-Responsive Clones

All the fragments that initially demonstrated chloroform-induced changes in expression were isolated and cloned, and the inserts were used as sequencing templates. The sequences were checked for homologies to known sequences by using DNA databases at the National Library of Medicine and the BLAST server [23].

Four genes appeared to change expression when band intensities were compared on the differential-

<table>
<thead>
<tr>
<th>Isolated band</th>
<th>Locus name*</th>
<th>Accession number*</th>
<th>Length (bp)$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTK3-T11CG-238</td>
<td>MUSTIS21</td>
<td>M64292</td>
<td>224</td>
</tr>
<tr>
<td>LTK3-T11GG-230</td>
<td>MUSMRNAH†</td>
<td>L37047</td>
<td>228</td>
</tr>
<tr>
<td>LTK3-T11CG-240</td>
<td>MUSMRNAH†</td>
<td>L37047</td>
<td>228</td>
</tr>
<tr>
<td>LTK3-T11CG-134</td>
<td>MUSFRA†</td>
<td>L37148</td>
<td>126</td>
</tr>
<tr>
<td>LTK3-T11CG-205</td>
<td>MUSFRB‡</td>
<td>L37149</td>
<td>197</td>
</tr>
</tbody>
</table>

$^*$Accession number and locus number were assigned by GenBank after sequence submission. Only genes with confirmed changes in expression by subsequent northern blot analysis are shown in this table. The clones noted in the text (MUSFN, MUSFRC, MUSHEP, and MMU0443) failed to exhibit the same treatment-related changes in mRNA levels when hybridized to northern blots as initially observed on the differential-display gels. We consider these clones to be false positives.

†These cDNAs are novel clones isolated in this study. LTK3-T11GG-230 and LTK3-T11CG-240 were found to be identical except for a single base-pair variation.

‡Length of the cDNA sequence submitted to GenBank. The total reported length of MUSTIS21 is 4886 bp.
display gel, but no changes in mRNA levels were seen with subsequent northern blot analysis. The band designation, locus name (identification), GenBank accession number, and length of submitted fragment for these false positives were LTK*-T11 CG-214, MUSFN (mouse fibronectin), M18194, 217 bp (905 bp reported total length); LTK*-T11 CG-126, MMU04443 (mouse non-muscle myosin light chain), U04443, 120 bp (654 bp reported total length); LTK*-T11 CG-235, MUSFRC (novel), L37150, 191 bp; and LTK*-T11 CG-157, MUSHFP (novel), L37349, 148 bp, respectively.

Clone LTK*-T11 CG-238 was 97.4% homologous to MUSTIS21, a mouse primary response gene induced by growth factors and tumor promoters. Clones LTK*-T11 GG-230 and LTK*-T11 CG-240 appeared to be the same gene fragment except for a single base mismatch and had 97% homology to a portion of the human gene HSCDN12 and 96% homology to the human gene M78700 (figure 1). This sequence was submitted to the GenBank database and was given the locus name MUSMRNAH. HSCDN12 is a human gene originally isolated from LNCaP, a prostate carcinoma cell line [24]. M78700 is a human hippocampus cDNA that has not been well characterized [25]. Clone LTK*-T11 CG-214 was homologous to mouse fibronectin, and clone LTK*-T11 CG-126 was homologous to mouse non-muscle myosin light chain.

MUSFRA, MUSFRB, MUSFRC, and MUSHFP had no significant homology to known sequences in GenBank and were submitted and given locus names and accession numbers as noted above and in Table 1. (Note that “FR” in the gene names stands for fragment.) The sequences of these clones are shown in figure 2. Clone MUSFRB did not hybridize to RNA taken from rat liver 30 min after partial hepatectomy (data not shown) and may be mouse specific.

Northern Blot Analysis

The isolated clones were used as probes to confirm altered expression by northern blot analysis, examples of which are shown in figure 3. Of the eight bands originally identified from the differential-display gels, only four exhibited significantly changed expression by northern blot analysis (Table 2). Thus, of the 387 bands originally observed, just four bands (about 1%) could be confirmed as exhibiting changes in mRNA levels in regenerating tissue.

Three clones showed an increase in mRNA levels: MUSTIS21 (figure 4a), MUSMRNAH (figure 4b), and MUSFRA (figure 4c). One of the isolated clones, MUSFRB, showed a decrease in mRNA levels (figure 4d).

All four of the confirmed positive clones were also used as probes to hybridize northern blots containing mRNA from the livers of female mice treated for up to 3 wk with 1800 ppm chloroform in drinking water [9]. No treatment-related changes in mRNA levels of any of the four messages were observed at any time point (data not shown).

The clone originally identified as LTK*-T11 CG-238 had a high degree of homology to a 228-bp segment of the MUSTIS21 gene in its 3’ untranslated region.

Figure 1. Sequence homologies of MUSMRNAH to the human genes HSCDN12 and M78700. The entire MUSMRNAH sequence is compared with homologous portions of the human genes. Sequences that differ are surrounded by boxes. Asterisks (*) indicate nucleotides that are deleted. Possible stop codon sites (TAA) are indicated in bold at positions 145 and 188 of MUSMRNAH. The first 10 nt from the 5’ end are the sequence of the LTK3 primer.
However, the message sizes were different (3.6 kb for MUSTIS21 and 2.5 and 1.05 kb for LTK3-T11CG-238). To determine if LTK3-T11CG-238 was in fact MUSTIS21, a 600-bp fragment (TlS21) of a MUSTIS21 clone was used as a probe to hybridize the same northern blot on which the differential expression of LTK3-T11CG-238 was confirmed. The TlS21 probe hybridized to messages of the same size as LTK3-T11CG-238, indicating that LTK3-T11CG-238 was homologous to MUSTIS21 (data not shown).

**DISCUSSION**

Evidence to date indicates the chloroform produces cancer in rodents by a nongenotoxic-cytotoxic mode of action [5,7]. A great deal more of supportive mechanistic research is needed to identify the specific mechanisms that play a role in this mode of action. One area of interest is the toxicity-mediated induction and subsequent mutation or over-expression of genes participating in regenerative growth. These genes are often oncogenes and may be more susceptible to spontaneous or chemically induced mutations during expression or may provide a selective growth advantage to precancerous cells. Identification of such genes may provide insight into some of the mechanisms involved in chloroform-mediated carcinogenesis.

It has been estimated that some mammalian cells express 15 000 genes [20,26]. Identifying genes whose expression changes because of toxicity and regenerative cell proliferation and that may play a role in the carcinogenic process therefore presents a serious challenge. One goal of our studies was to determine the proportion of genes

**Figure 2.** Nucleotide sequences of MUSFRA (a), MUSFRB (b), MUSFRC (c), MUSHEP (d). The 5’ and 3’ primers are underlined.
expressed at different levels in the livers of mice treated under carcinogenic conditions with chloroform. Using the 12 primer sets described here, we amplified portions of 387 mRNA species, or about 2.6% of those that might be expressed in a cell. Of these 387 bands, only four, or about 1%, changed mRNA levels under regenerative or carcinogenic conditions. An important observation from these studies was the high degree of consistency of gene expression from animal to animal and across dose and treatment groups, as visualized by the differential-display technique. This consistency confirms the reliability and reproducibility of the technique. The degree of expression was compared across dose and treatment groups to confirm the consistent pattern of change. The consistent lack of response in the 3 mg/kg/d (low dose) and 1800 ppm drinking water groups served as additional internal controls. Although the differential display technique appears to be a valu-

**Table 2. Differentially Expressed Clones Confirmed by Northern Blot Analysis**

<table>
<thead>
<tr>
<th>Locus name</th>
<th>Observed differences*</th>
<th>Intensity difference*</th>
<th>mRNA transcript (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUSTIS21</td>
<td>3 wk, 238 mg/kg/d</td>
<td>3x</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>4 d, 477 mg/kg/d</td>
<td>3.5x</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 wk, 477 mg/kg/d</td>
<td>6x</td>
<td></td>
</tr>
<tr>
<td>MUSMRNAH</td>
<td>4 d, 238 mg/kg/d</td>
<td>4x</td>
<td>2.8 and 1.4</td>
</tr>
<tr>
<td></td>
<td>3 wk, 477 mg/kg/d</td>
<td>9x</td>
<td></td>
</tr>
<tr>
<td>MUSFRA</td>
<td>4 d, 477 mg/kg/d</td>
<td>3x</td>
<td>3.1†</td>
</tr>
<tr>
<td></td>
<td>3 wk, 477 mg/kg/d</td>
<td>7x</td>
<td></td>
</tr>
<tr>
<td>MUSFRB</td>
<td>3 wk, 238 mg/kg/d</td>
<td>0.06x</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>4 d, 477 mg/kg/d</td>
<td>0.06x</td>
<td></td>
</tr>
</tbody>
</table>

*Observed differences are reported as time point and dose. Intensities were analyzed from the northern blots as compared with controls and were calculated as described in Materials and Methods.

†There was an additional 1.2-kb band at each of these doses and time points that was not in any of the other samples.

§There was an additional band at 1.8 kb in all the samples, but there was no difference in expression levels.
Figure 4. Expression of isolated genes in the livers of female mice after exposure to chloroform by gavage for 4 d (shaded bars) or 3 wk (solid bars). Gene expression was assessed by northern blot hybridization, quantitated by densitometry, and measured relative to control levels as described in Materials and Methods. Standard error is indicated by the error bars. (a) MUSTIS21; (b) MUSMRNAH (2.8 kb message); (c) MUSFRA; and (d) MUSFRB.

able tool for examining changes in expression of the remainder of the genes, that task remains very large. Interestingly, changes in expression in the four genes identified occurred only under regenerative or carcinogenic conditions. Treatment with 3 mg/kg/d of chloroform or 1800 ppm chloroform in the drinking water (329 mg/kg/d), which does not produce tumors, also did not produce changes in mRNA expression, patterns indicating that changes in expression are associated with toxicity and regeneration rather than chloroform per se.

From the 12 amplification reactions, we initially isolated nine bands that appeared to be differentially expressed between control mice and mice treated with 238 or 477 mg/kg/d of chloroform by gavage. Confirmation of differential expression by northern blot analysis revealed that while all of the positive clones hybridized to at least one distinct mRNA, four of the initial clones did not show a change in expression between control and treated animals. Such false positives are common in the use of the differential-display technique [27,28]. We feel that because duplicate samples across all dose and treatment groups showed no change in expression, it is unlikely that there were many false-negative responses. Of the five remaining clones, sequencing revealed that two had the same cDNA. Almost all of our positive clones were isolated from reactions involving the primer T11CG, a result also observed by Mou et al. [29]. It is not known why this occurred. Clearly, there were hundreds of reproducible bands amplified from the other primers, so this does not appear to be a weakness of either the amplification or isolation procedures.

MUSTIS21 is a primary response gene induced rapidly and transiently in 3T3 cells by the tumor promoter and mitogen tetradecanoyl phorbol acetate [30]. This gene’s product may mediate extracellular signals by modulation of RNA splicing [31] and may also be involved in programmed cell death [32]. We observed increases in the levels of MUSTIS21 mRNA after 3 wk of gavage treatment with 238 mg/kg/d of
chloroform and 4 d and 3 wk of treatment with 477 mg/kg/d of chloroform. Each of these time points was characterized by the presence of areas of liver necrosis [9]. With so many changes occurring in the liver, it is difficult at this point to assign a probable function (such as the initiation of regenerative cell proliferation or a response to cell killing), to this or any of the other genes. The difference observed between the size of the MUSTIS21 message originally reported by Fletcher et al. [30] of 3.6 kb and the size of the transcript homologous to MUSTIS21 in our study may be due to different splicing or processing of this message in mouse liver versus 3T3 cells, from which the MUSTIS21 clone was originally isolated.

The MUSMRNAH clone hybridizes to two mRNA transcripts expressed in mouse liver, one 2.8 kb in size and the other 1.4 kb. These mRNAs may be the products of two different genes with a high degree of homology, or both transcripts may be alternatively spliced products of the same gene. We observed a large increase in the levels of both mRNAs after 3 wk of gavage treatment with 477 mg/kg/d of chloroform and smaller increases after 4 d of gavage treatment with 238 mg/kg/d of chloroform. At these time points and doses, cell replication levels are moderately elevated, but the widespread necrosis seen at either 4 d of 477 mg/kg or 3 wk of 238 mg/kg/d is not present [9]. Interestingly, the human gene to which MUSMRNAH has the greatest homology, HSCDN12, was also originally isolated by using the differential-display technique with the same primers (LT1 and T1,GG) [24]. The transcript sizes are similar and suggest that we may have isolated the mouse homologue to this human gene. HSCDN12 was originally isolated from human LNCaP cells and was found to exhibit minor but reproducible changes in expression between prostate cancer cell lines [24]. The other close human homologue, M78700, is an uncharacterized gene isolated from the human hippocampus [25].

We observed elevated levels of the MUSFRA transcript after 4 d and 3 wk of treatment with 477 mg/kg/d of chloroform. This pattern of expression does not correlate clearly with either the histopathological changes or the changes in cell proliferation seen over the course of chloroform treatment. However, that the increase in mRNA levels was observed exclusively in the animals that received the high dose indicates that the change in expression may be a response to the extreme toxicity of this treatment.

The decrease in expression of clone MUSFRB at 3 wk, 238 mg/kg/d, and 4 d, 477 mg/kg/d occurred in the tissues with the highest degree of necrosis [9]. However, there was no clear correlation between the decrease in MUSFRB expression and the amount of cell proliferation in these tissues. Differences observed at one time point or one dose but not another emphasize the complex nature of the changes taking place. It was surprising that no genes related to the cell cycle or cell proliferation were identified in these studies.

Acute injury is followed by an orderly process in which expression of key genes increases as long as regeneration is necessary and returns to control levels when the tissue lost to toxicity has been replaced. The constant proliferative stimulus provided by chronic injury provides an environment in which these genes may acquire mutations or in which the mechanisms that keep their expression in check become damaged [7,16]. Studies of the type described here need to be extended to longer periods of exposure. Comparisons of dose-response relationships in the target organs of animals ranging from no observed effects to precancerous lesions may reveal the underlying molecular events associated with tumor formation. Further examination of the clones isolated in this study and of the many as yet unidentified genes that may be changing expression may clarify the relationships between chloroform-induced toxicity and the subsequent events that result in tumor development.

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