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"Activation of H-ras Oncogene by Drinking Water Disinfectant By-Products"

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16. Abstract (Limit: 200 words) The frequency and spectrum of H-ras oncogene activation by the water disinfection by products 2-chloroacetaldehyde (107200) (CAA), chloral-hydrate (302170) (CH), and dichloroacetic-acid (79436) (DCA) was investigated in the livers of male B6C3F1-mice. Mice had been administered high doses of these chemicals over a 2 year period. No mutations were noted in the H-ras oncogene in any normal liver tissue samples taken from mice with hepatocellular carcinomas. Codon 12/13 mutations were not present in any samples of either tumor tissues or normal liver. A relatively low frequency of H-ras activation was noted for liver tumors in mice dosed with CAA. For mice administered CH, a very low frequency of H-ras activation was found for liver tumors. A higher frequency of H-ras mutation was found for liver tumors in mice administered DCA. The authors conclude that the induction of the hepatocellular carcinomas by CAA does not involve the activation of the H-ras oncogene. The authors suggest that while DCA is most likely not directly causing these H-ras mutations, the proliferative action of DCA on the liver may be preferentially promoting cells containing ras mutations.

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List of Abbreviations

AAF	Acetylamino fluorene
ATP	Adenosine Triphosphate
B(a)P	Benzo(a)pyrene
bw	body weight
c12	Codon 12
c61	Codon 61
CAA	2-Chloroacetaldehyde
CH	Chloral Hydrate
CNS	Central Nervous System
DB(c,h)A	Dibenz(c,h)acridine
DBP	Disinfection By-Product
DCA	Dichloroacetic Acid
DMBA	7,12-Dimethylbenz(a)anthracene
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediamine Tetraacetic Acid
EPA	United States Environmental Protection Agency
NaCl	Sodium Chloride
NCI	National Cancer Institute
NMU	Nitrosomethylurea
NTP	National Toxicology Program
PIC	Phenol : Isoamyl Alcohol : Chloroform (25:1:24)
SDS	Sodium Dodecyl Sulfate
TCA	Trichloroacetic Acid
TCE	Trichloroethanol

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Significant Findings

1. As expected, no mutations in the H-*ras* oncogene (codons 12/13 or 61) were found in any samples of normal liver tissue taken from mice with hepatocellular carcinomas.
2. Codon 12/13 mutations were not found in any samples of either tumor tissues or normal liver. This is consistent with other reports in the literature.
3. A relatively low frequency of H-*ras* activation was found for liver tumors in mice administered 2-chloroacetaldehyde (2/6), and the two mutations that were seen (one C→A at the first position of codon 61 and one A→T at the second position) are consistent with the spectrum of mutations seen in spontaneous liver tumors of B6C3F1 mice. This suggests that the induction of the hepatocellular carcinomas by CAA does not involve the activation of the H-*ras* oncogene. This is contrary to the original hypothesis, which stated that a high frequency of activation of H-*ras* related to the genotoxicity of CAA (i.e., C→T transitions) would be expected in CAA-induced tumors.
4. A very low frequency of H-*ras* activation was found for liver tumors in mice administered chloral hydrate (1/7; an A→T transversion at the second position of codon 61). As with CAA-induced tumors, this suggests that the induction of the hepatocellular carcinomas by CH does not involve the activation of the H-*ras* oncogene. The findings for CH-induced tumors support the original hypothesis that a low frequency of activation of H-*ras* would be found, consistent

with other reports of mouse liver carcinogens that do not cause point mutations.

5. A higher frequency of H-*ras* mutation was found for liver tumors in mice administered dichloroacetic acid (3/5). This is contrary to the hypothesis that a low frequency of activation would be seen in these tumors, based on multiple reports in the literature showing a lower frequency of H-*ras* activation of tumors induced by nongenotoxic carcinogens, and the assumption that DCA induces liver cancer by a nongenotoxic mechanisms. The specific mutations seen in these tumors (2 CAA → AAA; 1 CAA → CTA) is consistent with the spectrum of mutations seen in spontaneous tumors. It appears, then, that while DCA is most likely not directly causing these H-*ras* mutations, the proliferative action of DCA on the liver may be preferentially promoting cells containing *ras* mutations.

Usefulness of Findings

The possible carcinogenicity of drinking water disinfection by-products (DBPs) is of significant concern because of the widespread exposure to humans. In addition to their formation as DBPs, occupational and medicinal uses of these chemicals or their precursors may lead to additional exposures. For several DBPs, the most significant neoplastic finding (and sometimes the only neoplastic finding) in studies using laboratory animals is an increased incidence of liver tumors in B6C3F1 mice. Because this strain of mouse, in particular males, is highly susceptible to both chemically-induced and spontaneous liver cancer, the relevance of these tumors to human cancer has been questioned. In order to answer these questions, a better understanding of the etiology of these tumors is critical.

Many factors have been implicated in the increased susceptibility of male B6C3F1 mouse liver to cancer, including a high rate of activation of the H-*ras* oncogene. Several investigators have shown a high frequency of H-*ras* activation in spontaneous tumors (roughly 2/3, with a consistent spectrum of mutations seen in codon 61), a lower frequency of activation in tumors induced by nongenotoxic carcinogens, and for some genotoxic carcinogens, a very high frequency of H-*ras* activation with a specific mutation in the oncogene that correlates with the known mutagenicity of the chemical carcinogen.

This research has shown that for each of the three DBPs, a low to moderate frequency of H-*ras* activation occurred. For CH and

CAA, the frequency was 14% and 33%, respectively, and the specific mutations observed were entirely consistent with those seen in spontaneous liver tumors. For DCA, a higher frequency of H-*ras* activation was observed (60%), but again, the spectrum of mutations was consistent with those seen in spontaneous tumors.

It appears, then, that the etiology of the liver tumors induced by these three DBPs does <u>not</u> generally involve carcinogen-mediated activation of the H-*ras* oncogene. The slightly higher frequency of H-*ras* activation seen in tumors induced by DCA (supported by findings in another laboratory) suggests that perhaps exposure to DCA is selectively promoting liver cells containing spontaneous mutations in the H-*ras* oncogene.

Abstract

Considerable evidence exists to suggest that the activation of cellular oncogenes may play a causal role in the carcinogenic process. The *ras* oncogenes (H, N, and K-) code for a G protein termed p21 which is an integral part of cross-membrane signal transduction pathways involved in growth regulation. Point mutations in "hot spots" of the gene (i.e., codons 12 and 61) lead to a single amino acid change which results in the acquisition of transforming properties. The *ras* oncogenes are activated in 10-20% of all human tumors, and are activated in many animal tumor models.

This research has focused on the liver of the male B6C3F1 mouse, a strain which is highly susceptible to the development of cancer, and which has a high frequency of H-*ras* activation in both chemically-induced and spontaneous tumors. In a 2-year bioassay, Dr. Daniel of the U.S. EPA administered mice high doses of drinking water disinfection by-products, specifically: 2-chloroacetaldehyde (CAA), chloral hydrate (CH), and dichloroacetic acid (DCA). The hypothesis of this research was that the frequency and spectrum H-*ras* mutations in the liver tumors will correlate with the type of DNA damage known to be caused by the chemicals under study. For CAA, a genotoxic carcinogen, it was hypothesized that a high frequency of activation would occur via mutations seen *in vitro* (i.e., C→T transitions). For CH, a spindle poison, no specific point mutation and a low frequency of H-*ras* activation was expected based on reports in the literature. For DCA, purportedly a non-genotoxic carcinogen, a low frequency of activation was expected.

High molecular weight DNA was prepared from normal liver and tumor tissue, and segments of about 130 base pairs surrounding codons 12 and 61 were amplified using the polymerase chain reaction (PCR). These amplified DNA fragments were then sequenced using the dideoxy method, and were analyzed for the presence of point mutations in the H-*ras* gene. For CAA, 2/6 tumors had mutations (1 C→A and 1 A→T); it does not appear that its genotoxic potential seen *in vitro* is contributing to the activation of the H-*ras* oncogene; indeed, the 2 mutations observed are the 2 most common mutations observed in spontaneous tumors. For CH, only 1/7 tumors had a mutation (A→T), suggesting that H-*ras* activation is not an important mechanism contributing to the etiology of these tumors. Finally, for DCA, 3/5 tumors had mutations (2 C→A and 1 A→T). Again, the frequency and spectrum of these mutations is consistent with those observed in spontaneous tumors.

Report

INTRODUCTION

Viral oncogenes were first identified as sequences in the genome of retroviruses that were necessary for the carcinogenicity of these viruses. It was subsequently found that homologous genes exit in human and animal genomes and that these cellular, or proto-oncogenes may play a role in the carcinogenic process (see Bishop, 1987 for review). While proto-oncogenes are believed to be involved in normal cellular functions, activation of these genes appears to be responsible, at least in part, for the loss of normal growth control mechanisms. Activation may occur by DNA alterations such as chromosomal translocations, gene amplification, and point mutations. It has been hypothesized that DNA damage induced by chemical carcinogens may result in the activation of these oncogenes, thereby contributing to the development of cancer.

The *ras* oncogene family, which includes N-, K-, and H-*ras*, is found to be activated in a wide variety of human cancers, with the overall frequency estimated to be 20 to 30% (Barbacid, 1987; Bos, 1989). As with all oncogenes, the *ras* genes are normal cellular genes that are believed to be involved in normal growth, development, and maintenance. The protein product of the ras genes is a 21 kdalton protein (p21) that functions as a G protein involved in regulatory signal transduction pathways. The transforming potential of the *ras* genes is elicited by point mutations in "hot spots" of the gene, most notably codons 12 and 61, leading to a single, critical amino acid change in the encoded

p21 protein. Some studies have demonstrated a direct relationship between the administration of a carcinogen and activation of the *ras* gene via specific point mutations (see Table 1).

Because activation of the H-*ras* oncogene has been found to be associated with mouse liver cancers, this research was designed to investigate the hypothesis that an association exists between the activation of the H-*ras* oncogene in these liver tumors (i.e., the frequency and spectrum of mutations) and the known mutagenic potential of the three carcinogens under study. The liver tumors under investigation in this research were hepatocellular carcinomas in male B6C3F1 mice that were generated in a 2 year bioassay conducted by the U.S. EPA (Daniel et al., 1992). Groups of male B6C3F1 mice were provided with drinking water containing the following disinfectant by-products: chloral hydrate (CH; 1 g/l), 2-chloroacetaldehyde (CAA; 0.1 g/l) and dichloroacetic acid (DCA; 0.5 g/l). A negative control group was maintained on distilled water.

The liver was the primary target organ for all three chemicals, with an increase being reported for absolute and relative liver weights, hepatocellular necrosis, and the incidence of liver tumors. The combined incidence of hepatocellular adenomas and carcinomas was 75% for DCA, 71% for CH, 38% for CAA and 15% for controls. The tissues from this study that were made available for this research included both normal liver tissue and tumor tissue (hepatocellular carcinomas) from selected mice. Upon sacrifice, sections of these tissues were taken for histopathology and the

TABLE 1

Activation of the H-*ras* Oncogene by Chemical Carcinogens

Reference	Target Organ	Chemical Agent[1]	Mutation Observed[2] Codon	#
Wiseman et al., 1986	Neonatal mouse liver	N-OH-2-AAF	c61/1 C→A	7/7
		Vinyl carbamate	c61/2 A→T	6/7
			c61/2 A→G	1/7
		1'-OH-2',3'-Dehydroestragole	c61/2 A→T	5/10
			c61/2 A→G	5/10
Zarbl et al., 1985	Rat mammary	NMU	c12/2 G→A	61/61
		DMBA	c61/2 A→N	5/5
			c61/3 A→N	
Sukumar et al. 1983	Rat mammary	NMU	c12/2 G→A	9/9
Quintanilla et al., '86	Mouse skin[3]	DMBA[4]	c61/2 A→T	33/37
Bizub et al., 1986	Mouse skin	DB(c,h)A	c61/2 A→T	5/6
		B(a)P	-	0/3
		DMBA	C61/2 A→T	3/4
Bailleul et al., 1989	Mouse skin	MNNG	c12/2 G→A	12/23
		DMBA[4]	c61/2 A→T	58/68
You et al., 1989	Strain A mouse lung	DMBA	c61/2 A→T	10/10
		MNU	c12/2 G→A	15/15
		Spontaneous	c12/2 G→T	4/18
			c12/2 G→A	4/18
			c12/1 G→C	1/18
			c61/2 A→G	7/18
			c61/2 A→T	2/18

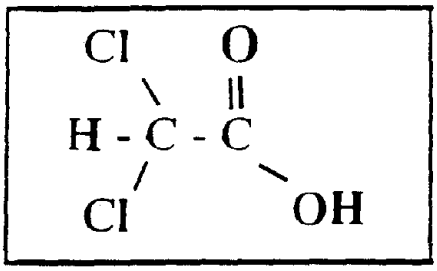
[1] See List of Abbreviations for full chemical names
[2] c61 = codon 61; c12 = codon 12
/1, /2 or /3 indicates the position (1st, 2nd, or 3rd base) within that codon
[3] 3 strains of mice tested: NIH, SENCAR, and NMRI
[4] These tumors were promoted with TPA or chrysarobin

rest was frozen in liquid nitrogen and stored at -70°C.

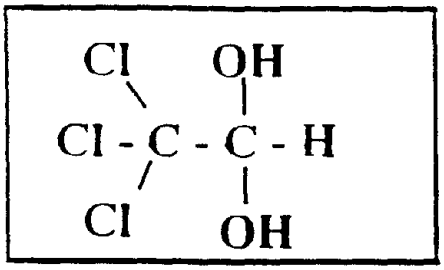
The three carcinogens (see Fig. 1) administered to mice in the study by Daniel et al. (1992) are of concern to humans because of their formation in drinking water as by-products of chlorine disinfection. Occupational and medicinal uses of these chemicals or their metabolic precursors provide additional sources of possible exposure.

The chlorinated acetates are among the most prevalent of the disinfection by-products (DBPs). Dichloroacetic acid (DCA) has been found in finished drinking water at concentrations of 8-79 μg/l (Singer and Chang, 1989). DCA is also a metabolite of trichloroethylene, a widely used solvent and itself a frequent water contaminant (Waters et al., 1977; Krasner et al., 1989) (see Figure 2). Finally, medicinal uses of DCA provide another source of potential exposure to humans; in specific, DCA is used in the treatment of lactic acidosis and as an antidiabetic agent which reduces blood glucose and lipids (Stacpoole et al., 1992; Stacpoole and Greene, 1992).

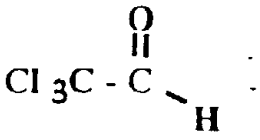
DCA has been demonstrated by several investigators to be a hepatocarcinogen in the B6C3F1 mouse (Herren-Freund et al., 1987; DeAngelo et al., 1991), with males being more sensitive than females (Bull et al., 1990). There is, however, little evidence to suggest that DCA is genotoxic and it has been postulated that the carcinogenic activity of DCA is closely tied to the severe hepatomegaly and cytomegaly it induces in the male mouse (Bull et al., 1990; Daniel et al., 1992).

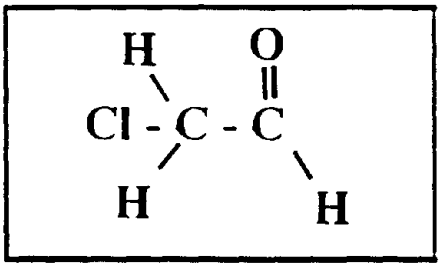


Dichloroacetic Acid



Chloral Hydrate
(hydrated form of Trichloroacetaldehyde)





2-Chloroacetaldehyde

Figure 1. Chemical Structures of DCA, CH, and CAA

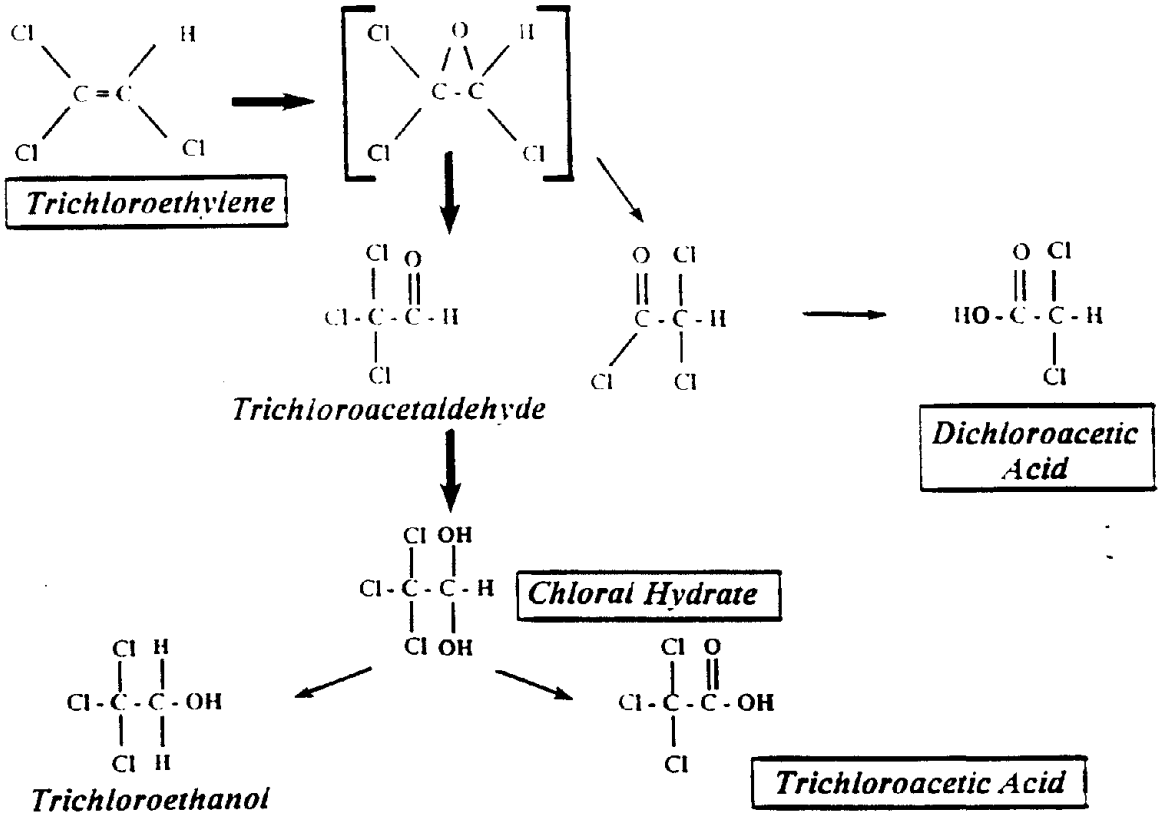


Figure 2. Metabolism of Trichloroethylene to Dichloroacetic Acid

Chloral hydrate (CH),also known as trichloroacetaldehyde, is used as an intermediate in the production of pesticides and plastics (Sax and Lewis, 1987) and is a metabolite of trichloroethylene (see Figure 2). CH has also been used historically as a sedative or anesthetic (Windholz et al., 1983) and is used in pediatric medicine and dentistry (McEvoy, 1985; Smith, 1990).

Health effects data for chloral hydrate show that it is mutagenic in *Salmonella* and fungi (Bignami et al., 1980), yeast (Crebelli et al., 1990), and Drosophila (Yoon et al., 1985). Several genotoxic effects have also been reported in mammalian systems (Gu et al., 1981; Vagnarelli et al., 1990; Degrassi and Tanzarella, 1988). DNA adduct formation has not been demonstrated for CH; rather, its genotoxicity appears to be related to its action as a spindle poison (Vagnarelli et al., 1990). Toxicologic studies have shown that the liver is the target organ for CH and that male mice are more sensitive than females (Rijhsinghani et al., 1986; Sanders et al., 1982; Daniel et al., 1992).

2-Chloroacetaldehyde (CAA) is of concern because of its presence in disinfected drinking water, but also because it is a metabolite of vinyl chloride, a major industrial chemical and known human liver carcinogen (Barbin and Bartsch, 1985). CAA is both a potent toxicant and an established genotoxicant (Lawrence et al., 1972). The mutagenicity of CAA is believed to result from its reaction with cytosine and adenosine residues of DNA to form ethėno adducts (Barbin et al., 1981). Jacobsen et al. (1989) demonstrated

in *E. coli* that CAA preferentially forms mutations at cytosines, less at adenines, and none at guanines or thymines. Of the mutations induced at cytosine residues, 80% were C→T transitions and 20% were C→A transversions.

The three chemicals of study provide an interesting variety because, while all three are hepatocarcinogens in the male B6C3F1 mouse, one is believed to involve epigenetic mechanisms (DCA), one is a spindle poison (CH), and one forms DNA adducts associated with its mutagenicity (CAA). This research was conducted to determine the frequency and spectrum of mutations in the H-*ras* oncogene in DNA isolated from hepatocellular carcinomas induced by these chemicals. Comparison of these findings with the frequency and spectrum of mutations reported in the literature for spontaneous B6C3F1 mouse liver tumors, and tumors induced by nongenotoxic and genotoxic carcinogens, can then provide valuable information about the etiology of these tumors.

Activation of the H-*ras* oncogene in mouse liver tumors has been investigated by several researchers. Buchmann et al. (1991) have reported a close correlation between the mutational activation of the H-*ras* gene in liver tumors of various strains of rodents and their susceptibility to development of liver cancer. The B6C3F1 mouse, used in the NTP and NCI carcinogenesis bioassays since 1971, is noted for its high incidence of spontaneous liver tumors (Tarone et al., 1981). Several investigators have reported that the mutation frequency for the H-*ras* gene (codon 61) ranged from 50-80% in spontaneous hepatocellular carcinomas in B6C3F1 mice (Reynolds

et al., 1986, 1987; Fox et al., 1990; Dragani et al., 1991).

The spectrum of mutations seen in spontaneous tumors is ~60% C→A transversions at the first position of codon 61, with the remaining mutations being either A→T transversions or A→G transitions at the second position of codon 61. In contrast, different mutational frequencies and spectra have been observed for liver cancers induced by various chemical carcinogens (see Table 1). It has been hypothesized by Fox et al. (1990) that, based on their finding of a much lower frequency of H-*ras* activation in liver tumors induced by nongenotoxic carcinogens (phenobarbital: 7%; chloroform: 21%; ciprofibrate: 21%) as compared to the frequency in either spontaneous tumors (64%) or those induced by a genotoxic compound (benzidine·2HCl: 59%), that the etiology of these tumors is likely to be different.

This research, involving liver carcinogens all thought to induce cancer by different mechanisms, was conducted to elucidate the relationship between the activation of the H-*ras* oncogene and the induction of liver cancer in the B6C3F1 mouse by the DCA, CAA, and CH.

MATERIALS AND METHODS

Mouse Liver Tissues

Normal and cancerous sections of liver from male B6C3F1 mice were generously provided by Dr. Daniel of the U.S. EPA. Details of the study from which these hepatocellular carcinomas were obtained are described above.

Isolation of High Molecular Weight DNA from Mouse Liver

Samples of mouse liver and liver tumor tissue were previously frozen at -70°C. For each mouse, both tumor and normal liver tissue were available. From these samples, pieces weighing approximately 150 mg were cut off and minced finely with scissors. Isolation of DNA was carried out, as follows, in a procedure adapted from Maniatis et al. (1982). The minced liver tissue was suspended in microcentrifuge tubes containing 700 μl of digestion buffer (10 mM Tris, pH 7.4; 10 mM NaCl; 25 mM EDTA, pH 8.0), 1 mg proteinase K, and 70 μl of 10% SDS. This mixture was placed in a shaking water bath at 37°C overnight. An equal volume of PIC (Phenol:Isoamyl Alcohol:Chloroform, 25:1:24) was then added to each tube and extractions were carried out by shaking the tubes gently for several minutes. The phenol and aqueous layers were subsequently separated by spinning the samples for 5 minutes in a microcentrifuge, the aqueous layer being removed to a clean tube following each separation. Three to four extractions were performed on each sample and the final aqueous layer (about 600 μl) was transferred to a 15 ml tube. NaCl was added to a final concentration of 0.5M, followed by 2 1/2 volumes of ice-cold ethanol. Upon the addition of the ethanol, the tubes were slowly inverted several times and the DNA became apparent as a white precipitate. A bent glass rod was used to spool out the DNA which was then transferred to a microcentrifuge tube containing 200 μl of TE buffer (10 mM Tris, pH 7.4; 1 mM EDTA, pH 8.0). DNA samples were then stored at -20°C.

Quantification of DNA

Prior to quantifying the isolated DNA, a qualitative determination of the integrity of the DNA was performed by agarose gel electrophoresis (see Maniatis et al., 1982). Using the methods of isolation previously described, DNA of ≥ 50 Kb could be obtained routinely. Quantification and further characterization were performed by running scans in a UV spectrophotometer and determining absorbance at 260 nm, where 20 O.D. units correspond to a 1 mg/ml concentration of double-stranded DNA. It was typically found that about 0.3% of the starting weight of the liver sample (~450 μg from a 150 mg piece of tissue) was recovered as high molecular weight DNA.

Polymerase Chain Reaction (PCR) Amplification of Mouse Liver DNA.

PCR was employed to amplify regions surrounding codons 12 and 61 of the H-ras gene. The technique used is an adaptation of that originally described by Saiki et al. (1985). *Thermus aquaticus (Taq)* DNA polymerase (2.5 units, Cetus) was added to a 50 ul reaction mixture containing approximately 1 ug of liver DNA, 0.2 mM dGTP, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM cCTP, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris/Hcl (pH 8.3) and 0.5 ug of 23- or 24-mer nucleotide primers. The primers, obtained from Midland Chemical Corp., were chosen to span a region of ~120 base pairs surrounding codons 12 (exon 1) and 61 (exon 2) of the H-ras gene. The primers for exon 1 were:

5'-CTTGGATAAGTGTGCTTCTCATT-3' (primer A) and

5'-CACCTCTGGCAGGTAGGCAGAGCT-3' (primer B). The primers used for amplifying the region of interest in exon 2 were:
5'-CTAAGCCTGTTGTTTTGCAGGACC-3' (primer A) and
5'-GGTAGCCATAGGTGGCTCACCTGT-3' (primer B). The reaction mixture was overlaid with 20 μl of mineral oil (Sigma) and subjected to an initial denaturation of 3 minutes at 94°C followed by 30 cycles consisting of an annealing step (2 minutes at 60°C), a polymerization step (3 minutes at 72°C), and a denaturation step (1 minute at 94°C). Following the 30 cycles, a final extension of 7 minutes at 72°C was carried out before the reaction was terminated by going to 4°C. In some cases, the "hot start" method was employed to prevent the dimerization of the primers. This entailed mixing all reaction components except the dNTPs; these were added after the initial denaturization step.

Purification of PCR Products

The DNA fragments generated by PCR amplification were purified and concentrated prior to being subjected to DNA sequencing. The entire PCR product (50 μl) was removed from under the mineral oil overlay, and to this 5 ul of 10x loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 40% sucrose, 5% glycerol) was added. The samples were electrophoresed through a 1.5% agarose gel (about one hour at 100 volts) containing ethidium bromide (0.5 ng per ml of gel). To visualize the location of the DNA fragments, the gel was placed on top of an ultraviolet light source and a piece of the gel containing the band of interest was sliced out using a

scalpel. The pieces of gel were placed in individual pieces of dialysis tubing (MW exclusion 6,000-8,000) containing 2 ml of TBE buffer and the DNA was electrophoresed out of the gel (35 volts overnight or 100 volts for 2 hours). The current was then reversed for 1 minute at 100 volts to help separate the DNA fragments from the dialysis tubing. The tubing was then cut open and the buffer containing the DNA was withdrawn and placed in a Centricon 30 tube (MW cut-off of 30,000). These were centrifuged at 5000 rpm for 50 minutes at 4°C in a Sorvall centrifuge, SS34 rotor. An additional 2 ml of ddH_2O was added to each centricon-30 tube and the centrifugation was repeated. The buffer in the bottom chamber was then discarded, the tubes were inverted and one drop of ddH_2O was added to elute the DNA from the centricon membrane. A third centrifugation was carried out for 5 minutes at 5000 rpm, 4°C, and the final solution was transferred to a microfuge tube, the contents of which were subsequently dried using a speed vac.

Direct DNA Sequence Analysis of PCR-Amplified DNA:

The dideoxy method of DNA sequencing (Sanger et al., 1977) was followed, using a ^{32}P-end-labelled primer. The primers used for sequencing Region 1 (surrounding codon 12) and Region 2 (surrounding codon 61) were the same as those used for PCR, but were further purified by gel electrophoresis. All reagents described below were used from the Sequenase DNA Sequencing kit available from U.S. Biochemical.

About 70 ng of primer DNA was used per sequencing reaction.

Dry samples of the primer DNA were resuspended in double-distilled water to a concentration of 17.6 ng/μl. Radiolabelling of the primer was carried out by adding the following in a microcentrifuge tube: 4 μl of the primer (about 70 ng total), 4 μl of gamma-^{32}P-ATP (about 50 μCi; specific activity $\approx$ 3000 Ci/mmol, from Amersham), 1 μl T4-polynucleotide kinase (PNK) and 1 μl T4-PNK 10X buffer. These reagents were given a quick spin in a microcentrifuge and then were placed in a thermocycler programmed for 30 minutes at 37°C, 2 minutes at 95°C and ending at 4°C. This end-labelled primer could then be used immediately in the subsequent sequencing reactions, or could be stored for up to a week at -20°C.

The second step of the sequencing procedure is to anneal the ^{32}P-end-labelled primer to the DNA template (purified PCR product) to be sequenced. For each sequencing reaction, about 50-100 ng of DNA template was used. The PCR and subsequent purification reactions described above yielded enough DNA to perform two separate reactions. To the dried PCR products, 21 μl of PCR-grade water was added, 10.5 μl being used for each sequencing reaction. Behind appropriate shielding, the following was added together in a microcentrifuge tube: 10.5 μl PCR product, 2.5 μl 5X reaction buffer (from Sequenase kit) and 2 μl of the ^{32}P- end-labelled primer. Following a quick spin in a microcentrifuge, this mixture was placed in the thermocycler programmed for 5 minutes at 95°C to denature the DNA and then was placed on ice for 5 minutes. One μl of dithiothreitol (DTT) and 1 μl of sequenase were then added to the tube, bringing the final volume to 17 μl, and it was again

given a quick spin in the microcentrifuge.

For each sequencing reaction, 4 microcentrifuge tubes were labelled "G", "A", "T", and "C" respectively, and to each, 3 μl of the appropriate dNTP/ddNTP nucleotide mixture from the Sequenase kit was added. To each of these tubes, 3 μl of the ^{32}P-labelled primer annealed to the DNA template was added, microcentrifuged, and put in the thermocycler at 37°C for 4 minutes. To terminate the sequencing reaction, 4 μl of stop solution was added to each tube and again was subjected to microcentrifugation.

An 8% acrylamide/bis-acrylamide sequencing gel was prepared according to the protocol provided by BioRad. The gel was pre-run for at least one hour at 2200 volts and the samples were prepared for loading onto the gel by denaturing at 95°C for 5 minutes and then cooling on ice for a few minutes. A pasteur pipette was used to clean the wells and sequencing pipette tips were then used to introduce 3 μl of the G, A, T and C samples into adjacent wells. The gel was then run at ~2300 volts for 3 hours and 40 minutes. The apparatus was then disassembled, cool water was run over the plates to cool them down, the plates were carefully separated, and 3M (Whatman) paper was used to remove the acrylamide gel from the glass plate it was adhered to. The gel was then dried under vacuum at 80°C for about 45 minutes, placed in an X-ray cassette with a piece of Kodak film, and then put at -70°C for 48-72 hours (or longer if the radioactivity was not as fresh). Following this exposure, the film was developed using an automatic developer.

Results

High molecular weight DNA was isolated from both normal and tumor tissue from all mouse livers, with a yield of about 0.3% of the weight of the starting material. Amplification of about 120 base pairs surrounding codons 12 (Region 1) and 61 (Region 2) was highly successful using the polymerase chain reaction. Starting with about 1 μg of genomic liver DNA, about 200 ng of target DNA was routinely obtained.

The DNA sequences for codons 12, 13, and 61 of each individual tumor are shown in Table 2 below. Table 3 presents these data in a summary format of how many of each mutation were seen in each chemical group.

TABLE 2

Spectra of Mutations in the H-*ras* Oncogene

Chemical	Sample	Codon 12 WT=GGA	Codon 13 WT = GGC	Codon 61 WT = CAA
DCA	1	G G A	G G C	C **A/T** A
n=5	2	G G A	G G C	C A A
	3 A	G G A	G G C	**C/A** A A
	3 B	G G A	G G C	**C/A** A A
	4	G G A	G G C	C A A
CAA	1	G G A	G G C	C **A/T** A
n=6	2	G G A	G G C	C A A
	3	G G A	G G C	C A A
	4	G G A	G G C	C A A
	5	G G A	G G C	C A A
	6	G G A	G G C	**C/A** A A
CH	1 A	G G A	G G C	C A A
n=7	1 B	G G A	G G C	C A A
	2 A	G G A	G G C	C A A
	2 B	G G A	G G C	C **A/T** A
	3	G G A	G G C	C A A
	4	G G A	G G C	C A A
	5	G G A	G G C	C A A
Water	1	G G A	G G C	C A A

CODON 61: GCA (59) - GGT (60) - CAA (61) - GAA (62) - GAG (63)

CODON 12: GGC (10) - GCT (11) - GGA (12) - GGC (13) - GTG (14)

TABLE 3

SUMMARY OF CODON 61 MUTATIONS

CHEMICAL	CODON 61 MUTATIONS (wt = CAA)		
	C A A	A A A	C T A
CAA n=6	4	1	1
CH n=7	6	0	1
DCA n=5	2	2	1

Following are representative sequences of codon 12/13 (Figure 3) and codon 61 (Figure 4). No mutations in codon 12/13 were seen in any sample (both normal liver tissue and tumor tissue). The wild type sequence of these two codons, as seen in Figure 3, is GGA (codon 12) and GGC (codon 13). The wild type sequence of codon 61 (as seen in Figure 4) is CAA. Figures 5, 6, and 7, show sequences exhibiting codon 61 mutations in tumors induced by DCA, CAA, and CH, respectively.

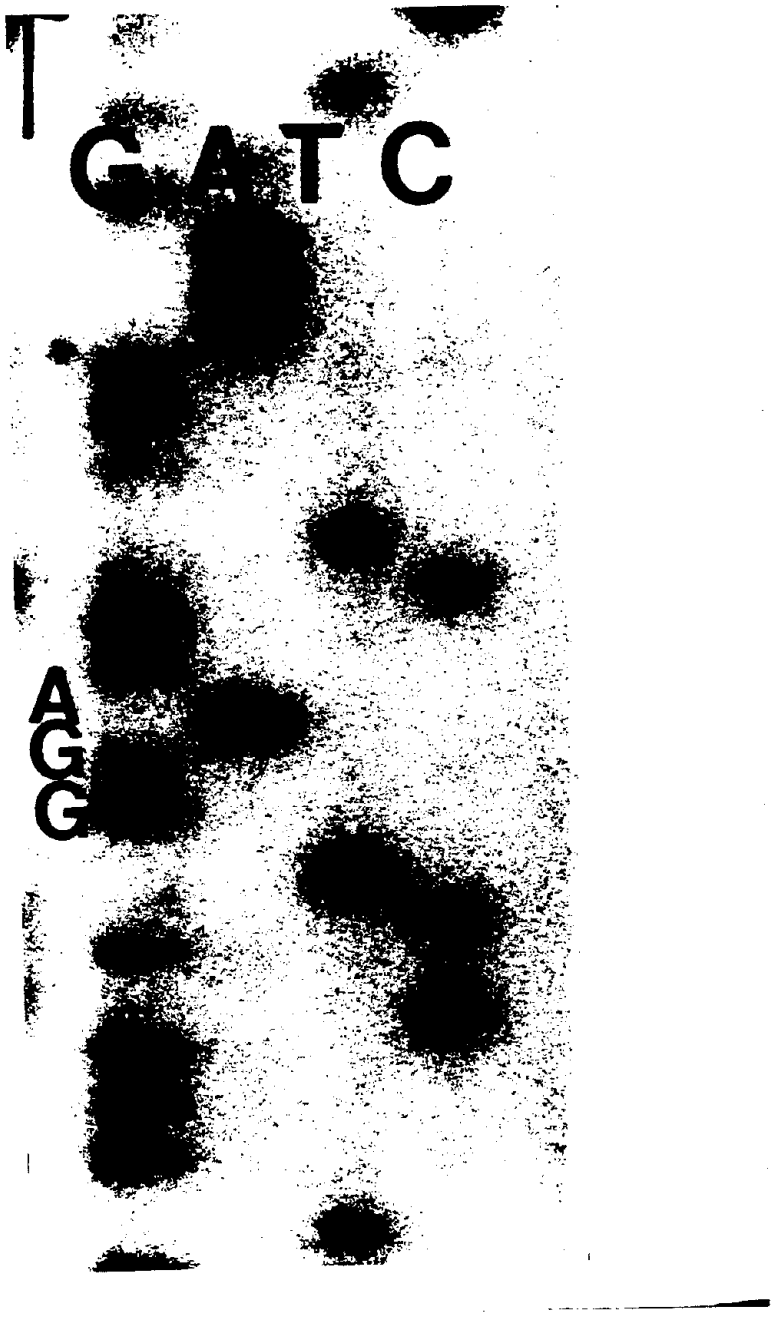


Figure 3. Representative sequence of Codons 12/13

codon 12 = GGA codon 13 = GGC

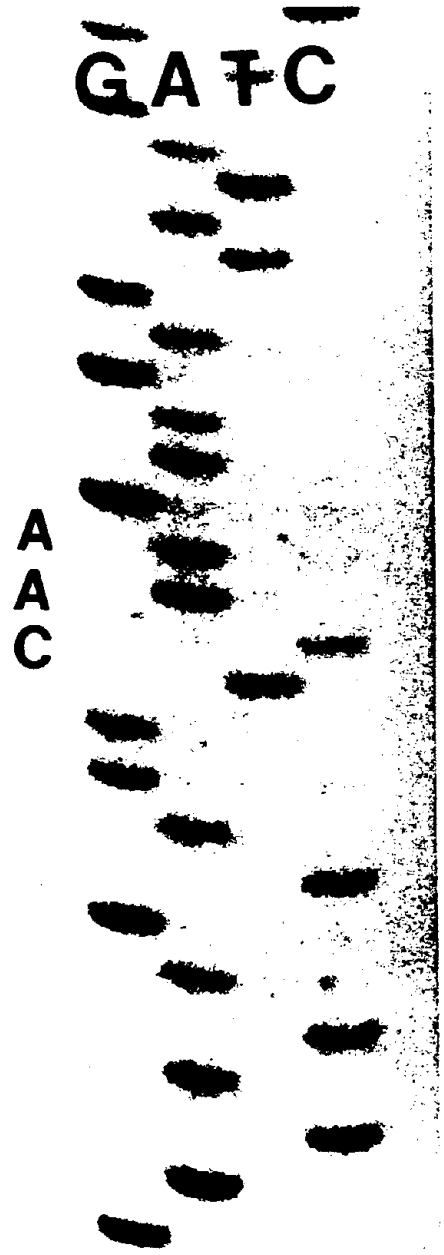


Figure 4. Representative Sequence of Codon 61

codon 61 = CAA (Wild Type)

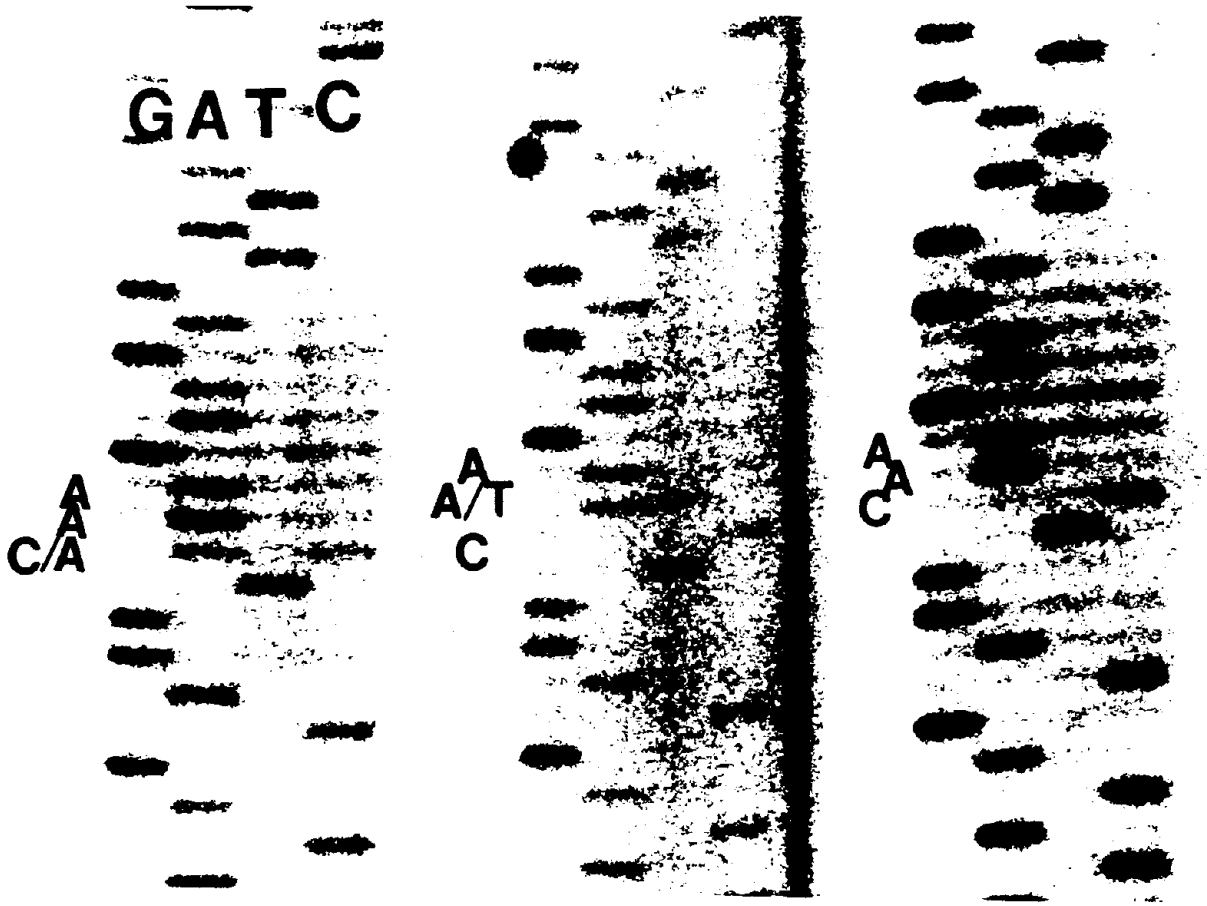


Figure 5. Sequence of Codon 61 Showing Mutations in DCA - Treated Mice.
Sequence #1 = C/A A A
Sequence #2 = C A/T A
Sequence #3 = C A A
(Two additional sequences not shown;
1 = C A A and 1 = C/A A A)

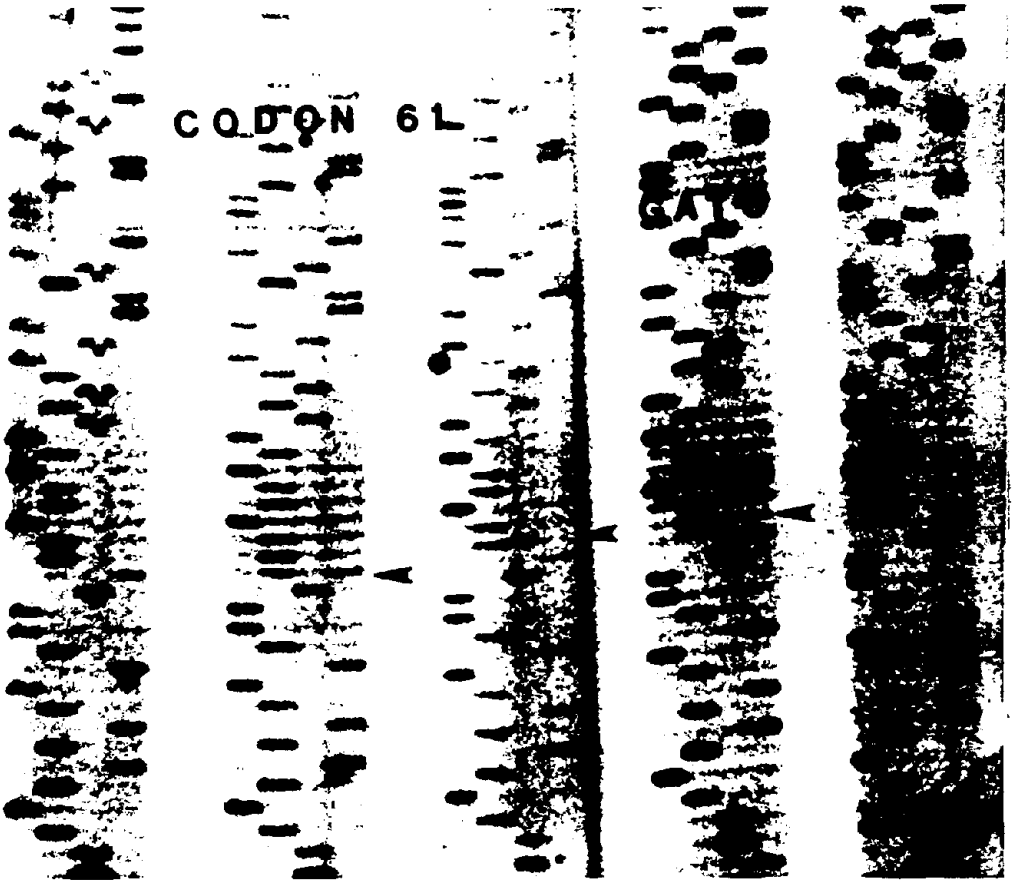


Figure 6. Sequence of Codon 61 Showing Mutations in CAA - Induced Tumors
Sequence #1 = C A A (no mutation)
Sequence #2 = C/A A A
Sequence #3 = C A/T A
Sequence #4 = C A A
Sequence #5 = C A A
(one additional sequence not shown, genotype = CAA)

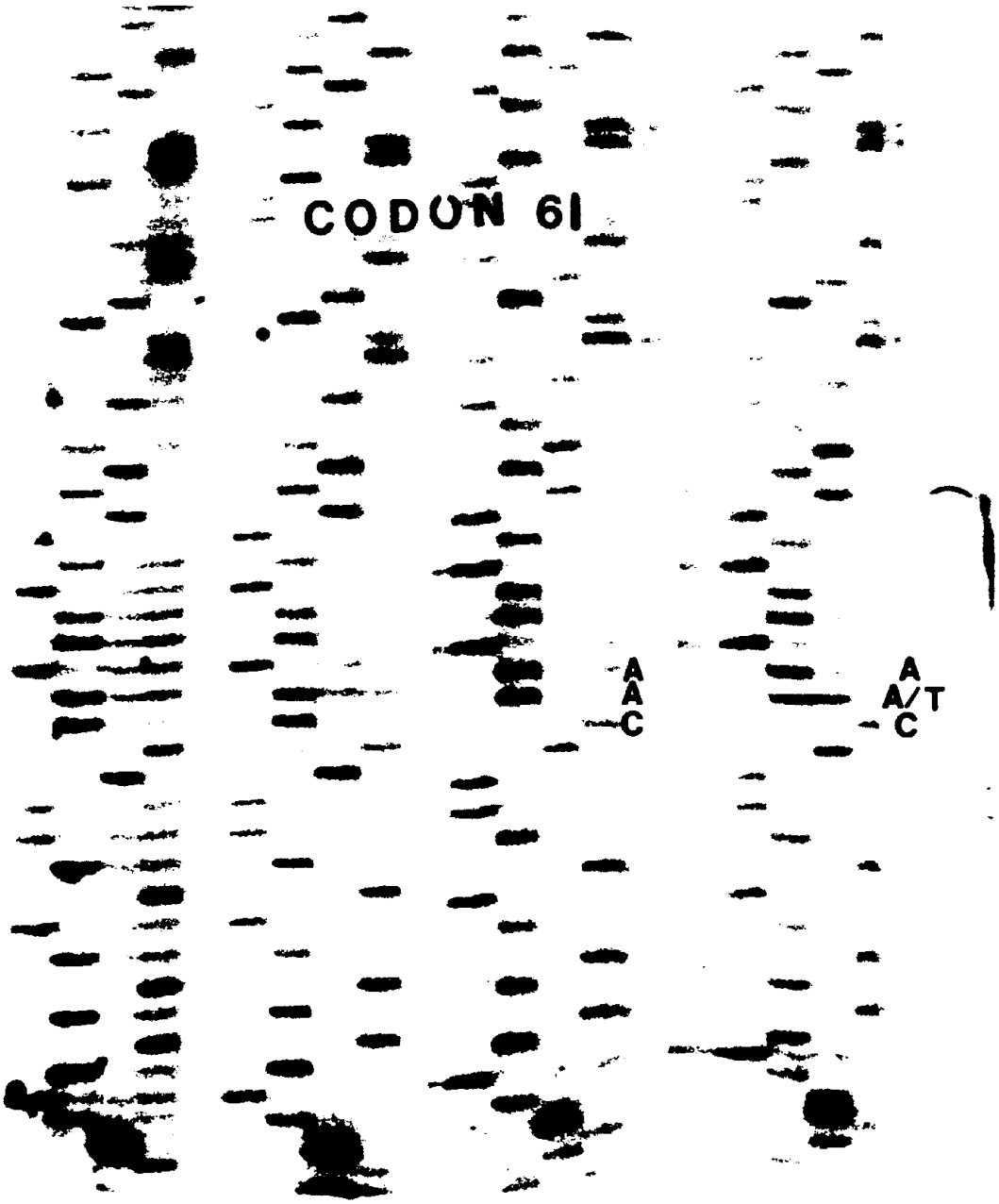


Figure 7. Sequence of Codon 61 Showing Mutations in CH - Induced Tumors
Sequences #1 - 3 = C A A (wild type)
Sequence # 4 = C A/T A
(Three additional sequences not shown, all three were wild type, C A A)

VIII. DISCUSSION

A. Comparison of Spectra of H-*ras* Mutations Observed

Several observations can be made regarding the spectra of H-*ras* mutations observed in the mouse liver tumors under study. First, it is noted that no mutations were observed in codons 12 or 13 for any group of mice. This is entirely consistent with the findings of several other researchers who likewise reported a high degree of codon 61 mutation in the absence of codon 12 mutations. The reason for this is not known. While both codon 12 and codon 61 mutations are observed in some tumor systems (e.g., mouse skin; rat mammary gland; Strain A mouse lung); other target organs exhibit a high degree of specificity for mutations in only one of the two hot spots. This may be because of differential repair capabilities, or alternatively, it may be that one codon is more prone to mutation than the other. Either of these possibilities may hold true for mouse liver. The high frequency of codon 61 mutations and lack of codon 12 mutations in spontaneous tumors as well as chemically-induced tumors may be the result of either increased susceptibility of codon 61 to being altered, or decreased repair capability at this site compared to codon 12. In the case of tumors induced by genotoxic agents, the ability of the carcinogen to interact with the DNA may be influenced by secondary structures in the regions of codons 12 and 61 such that codon 61 is more vulnerable to adduct formation.

Following is a discussion of the mutational spectra seen in the mouse liver tumors induced by DCA, CAA, and CH.

A.1 Dichloroacetic Acid

It was hypothesized that a low frequency of H-*ras* activation would be seen in tumors induced by DCA, by virtue of its classification as a nongenotoxic carcinogen. The overall rate of activation seen in this study was 60% (3 out of 5 tumors). Two of the mutations were C→A transversions in the first position of codon 61, and one was a A→T transversion in the second position. This spectrum is identical to that seen for spontaneous tumors. Unpublished results from another laboratory corroborate this finding: 31 out of 43 (72%) mouse liver tumors induced by DCA were found to have an activated H-*ras* gene (M. Pereira[1]). The spectrum of mutations in carcinomas observed was also quite similar to that seen in spontaneous tumors (i.e., c61/1 C→A predominating, with lesser frequencies of c61/2 A→G and A→T).

A.2 2-Chloroacetaldehyde

Of the six tumors induced by CAA, there were 2 mutations in codon 61 of the H-*ras* gene: one C→A transversion at the first position, and one A→T transversion at the second position. This is contrary to the original hypothesis that the activation of H-*ras* would be correlated to the known mutagenicity of each carcinogen. As discussed previously, CAA is genotoxic, forming etheno adducts primarily on cytosine residues, most of which result in C→T transitions. No mutations of this sort, however, were seen in any of the CAA-induced mouse liver tumors. Indeed, the mutations

[1] Information presented at the Department of Defense Risk Assessment Conference in Dayton, Ohio, April, 1993.

observed in CAA-induced tumors are consistent with the spectrum of mutations seen in spontaneous liver tumors of B6C3F1 mice, suggesting that the induction of these hepatocellular carcinomas by CAA does not involve the activation of the H-*ras* oncogene.

There are several possible explanations for why there appears to be no activation of H-*ras* associated with CAA treatment. It is quite possible that the mutations observed in *in vitro* studies (e.g., in *E. coli*) are not relevant to mammalian exposures *in vivo*. Other investigators have also shown discordance between the *in vitro* mutagenicity of a chemical carcinogen and the mutations seen in oncogenes in vivo. For example, diethylnitrosamine and benzidine·2HCl are both potent genotoxic carcinogens that induce liver tumors in B6C3F1 mice, which exhibit a frequency and spectrum of H-*ras* mutations that is identical to that seen for spontaneous tumors (Stowers et al., 1988; Rumsby et al., 1991; Fox et al., 1990). Another example is provided by aflatoxins, which have been shown to induce a specific mutation *in vitro* of GC→TA transversions in codon 249 of the p53 gene (Puisieux et al., 1991), nonspecific mutations were seen in an *in vivo* animal model (Hulla et al., 1993).

It may also be relevant that site-specificity has been demonstrated within mammalian chromatin for adduct formation by chloroacetaldehyde. Experiments by Kohwi-Shigematsu and Nelson (1988) found that during active transcription of a gene, CAA reacted with a unique DNA site. In cells that did not actively transcribe this gene, no adducts were detected. The authors

suggest that the interaction of the carcinogen with DNA requires a non-B DNA structure that contains unpaired DNA bases at specific sites within the chromatin. This was supported by work by Vogt et al. (1988), which demonstrated the potential for CAA to react with DNA in regions of transition from B-DNA to Z-DNA. Such conformational changes of chromatin, leading to an increased potential for interaction with chemical carcinogens, may also be a factor in the propensity for oncogene activation. In the case of CAA-induced mouse liver tumors, it appears that the carcinogen is not interacting with the hot spots of the H-*ras* oncogene, perhaps as a consequence of chromatin conformation.

A.3 Chloral Hydrate

Of the seven tumors induced by chloral hydrate, only one had an H-*ras* mutation (an A→T transversion at the second position of codon 61). These results clearly suggest that the etiology of CH-induced liver tumors does not involve the activation of the H-*ras* oncogene. The frequency of mutations is quite low (1/7, or 14%), which is consistent with the low frequency of H-*ras* activation reported for mouse liver tumors induced by other nongenotoxic carcinogens.

The mechanism by which CH induces liver cancer is not known. *In vitro*, CH is a clastogenic agent which acts as a spindle poison. One would not expect, then, to see specific point mutations in the H-*ras* gene as a result of CH administration.

The relevance of the effects of CH *in vitro* to effects seen *in*

vivo is not clear. Chloral hydrate is rapidly and completely absorbed when ingested by humans. It is also rapidly metabolized to trichloroethanol (TCE; formed by reduction of CH) and trichloroacetic acid (TCA; formed by oxidation of CH or, secondarily, TCE). The extent of this metabolism is such that no chloral hydrate could be detected in blood samples of humans drawn as early as 15 minutes after administration of an oral dose ranging from 15 to 60 mg CH/kg bw (Marshall and Owens, 1954). It was determined that the CNS depression resulting from administration of chloral hydrate is actually due to the action of one of its metabolites, TCE. It has been suggested that the carcinogenicity of CH may be actually due to its metabolite trichloroacetic aid, which is believed to induce hepatocarcinogenesis in mouse liver by a mechanism involving peroxisome proliferation.

Regardless of whether the carcinogenic outcome following CH administration is due to the parent compound or to the metabolite TCA, a low frequency of H-*ras* activation would be expected. It would be interesting to see whether a similarly low frequency of H-*ras* activation is found for TCA-induced mouse liver tumors. This research is currently in progress (DeAngelo, 1994; personal communication).

In summary, for each of the three DBPs studied, carcinogen-induced activation of the H-*ras* oncogene does not appear to be a contributing factor to the development of liver cancer in B6C3F1 mice. The frequency of H-*ras* activation was low for CH- and CAA-induced tumors (1/7 and 2/6, respectively). For DCA-induced

tumors, the frequency was higher (3/5), but this frequency and also the spectrum of mutations are identical to that seen for spontaneous tumors. It appears then, that there is some selective growth advantage for cells containing spontaneous H-*ras* mutations; and the lower frequency of mutations seen in CH- and CAA-induced tumors may be a reflection of induction of neoplastic mechanisms which are independent of the H-*ras* gene.

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References

Barbacid, M. 1987. *ras* genes. Annu. Rev. Biochem., 56: 779-827.

Barbin, A. and H. Bartsch. 1985. Mutagenic and promutagenic properties of DNA adducts formed by vinyl chloride metabolites. In: B. Singer and H. Bartsch (eds.), The Role of Cyclic Nucleic Acid Adducts in Carcinogenesis and Mutagenesis, IARC Scientific Publication No. 70, International Agency for Research on Cancer, Lyon.

Barbin, A., H. Bartsch, P. LeComte and M. Radman. 1981. Studies on the miscoding properties of 1,N^6-ethenoadenine and 3,N^4ethenocytosine, DNA reaction products of vinyl chloride metabolites, during *in vitro* DNA synthesis. Nucleic Acids Res., 9: 375-387.

Bignami, M., G. Conti, R. Conti, F. Crebelli, A. Puglia, R. Randazzo, G. Sciandrello and A. Carere. 1980. Mutagenicity of halogenated aliphatic hydrocarbons in Salmonella typhimurium, Streptomyces coelicolor and Aspergillus nidulans. Chem. Biol. Interact., 30: 9-23.

Bishop, J.M. 1987. The molecular genetics of cancer. Science, 235: 305-311.

Bos, J.L. 1989. *ras* oncogenes in human cancer: a review. Cancer Res., 49: 4682-4689.

Buchmann, A., R. Bauer-Hofmann, J. Mahr, N.R. Drinkwater, A. Luz and M. Schwarz. 1991. Mutational activation of the c-Ha-*ras* gene in liver tumors of different rodent strains: Correlation with susceptibility to hepatocarcinogenesis. Proc. Natl. Acad. Sci. USA, 88: 911-915.

Bull, R.J., I.M. Sanchez, M.A. Nelson, J.L. Larson and A.J. Lansing. 1990. Liver tumor induction in B6C3F1 mice by dichloroacetate and trichloroacetate. Toxicology, 63: 341-359.

Crebelli, R., G. Conti, L. Conti and A. Carere. 1990. Chloroacetaldehyde is a powerful inducer of mitotic aneuploidy in *Aspergillus nidulans*. Mutagenesis, 5: 165-168.

Daniel, F.B., A.B. DeAngelo, J.A. Stober, G.R. Olson and N.P. Page. 1992. Hepatocarcinogenicity of chloral hydrate, 2-chloroacetaldehyde, and dichloroacetic acid in the male B6C3F1 mouse. Fundam. Appl. Toxicol., 19: 159-168.

DeAngelo, A.B. 1994. Personal communication. March 11.

DeAngelo, A.B., F.B. Daniel, J.A. Stober and G.R. Olson. 1991. The carcinogenicity of dichloroacetic acid in the male B6C3F1

mouse. Fundam. Appl. Toxicol., 16: 337-347.

Degrassi, F. and C. Tanzarella. 1988. Immunofluorescent staining of kinetochores in micronuclei: A new assay for detection of aneuploidy. Mutat. Res., 203: 339-345.

Dragani, T.A., G. Manenti, B.M. Colombo, F.S. Falvella, M. Gariboldi, M.A. Pierotti and G. Della Porta. 1991. Incidence of mutations at codon 61 of the Ha-*ras* gene in liver tumors of mice susceptible and resistant to hepatocarcinogenesis. Oncogene, 6:333-338.

Fox, T.R., A.M. Schumann, P.G. Watanabe, B.L. Yano, V.M. Maher and J.J. McCormick. 1990. Mutational analysis of the H-ras oncogene in spontaneous C57BL/6 x C3H/He mouse liver tumors and tumors induced with genotoxic and nongenotoxic hepatocarcinogens. Cancer Res., 50: 4014-4019.

Gu, W.Z., B. Sele, P. Jalbert, M. Vincent, C. Chmara, J. Faure and C. Marka. 1981. Induction of sister chromatid exchange by trichloroethylene and its metabolites. Toxicol. Eur. Res., 3: 63-67.

Herren-Freund, S.L., M.A. Pereira, M.D. Khoury and G. Olson. 1987. The carcinogenicity of trichloroethylene and its metabolites, trichloroacetic acid and dichloroacetic acid in mouse liver. Toxicol. Appl. Pharmacol., 90: 183-189.

Hulla, J.E., Z.Y. Chen and D.L. Eaton. 1993. Aflatoxin B_1-induced rat hepatic hyperplastic nodules do not exhibit a site-specific mutation within the *p53* gene. Cancer Res., 53: 9-11.

Kohwi-Shigematsu, T. and J.A. Nelson. 1988. The chemical carcinogen, chloroacetaldehyde, modifies a specific site within the regulatory sequence of human cytomegalovirus major immediate early gene *in vivo*. Molec. Carcinog., 1:20-25.

Krasner, S.W., M.J. McGuire, J.G. Jacangelo, N.L. Patania, K.M. Reagen and E.M. Aieta. 1989. The occurrence of disinfection by-products in U.S. drinking water. J. Am. Water Works Assn., 81: 41-53.

Lawrence, W.H., E.O. Dillingham, J.E. Turner and J. Autian. 1972. Toxicity profile of chloroacetaldehyde. J. Pharmaceut., 61: 19-25.

Maniatis, T., E.F. Fritsch and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Marshall Jr., E.K. and A.H. Owens, Jr. 1954. Absorption, excretion and metabolic fate of chloral hydrate and trichloroethanol. Johns Hopkins Hospital Bull., 95: 1-18.

McEvoy, G.K. (ed.). 1985. Drug Information, American Hospital Formulary Service, American Society of Hospital Pharmacists. Bethesda, MD. p. 937.

Pereira, M. 1993. Poster presented at the Department of Defense Risk Assessment Conference. Dayton, OH. April.

Puisieux, A., S. Lim, J. Groopman and M. Ozturk. 1991. Selective targeting of *p53* gene mutational hot spots in human cancers by etiologically defined carcinogens. Cancer Res., 51: 6185-6189.

Reynolds, S.H., S.J. Stowers, R.R. Maronpot, S.A. Aaronson and M.W. Anderson. 1986. Detection and identification of activated oncogenes in spontaneously occurring benign and malignant hepatocellular tumors of B6C3F_1 mouse. Proc. Natl. Acad. Sci. USA, 83: 33-37.

Reynolds, S.H., S.J. Stowers, R. Patterson, R.R. Maronpot, S.A. Aaronson and M.W. Anderson. 1987. Activated oncogenes in B6C3F_1 mouse liver tumors: implications for risk assessment. Science: 288: 596-597.

Rijhsinghani, K.S., C. Abrahams, M.A. Swerdlow, K.V. Rao and T. Ghose. 1986. Induction of neoplastic lesions in the livers of C57Bl X C3HF1 mice by chloral hydrate. Cancer Detect. Prev., 9: 279-288.

Rumsby, P.C., N.C. Barrass, H.E. Phillimore and J.G. Evans. 1991. Analysis of the Ha-*ras* oncogene in C3H/He mouse liver tumours derived spontaneously or induced with diethylnitrosamine or phenobarbitone. Carcinogenesis, 12: 2331-2336.

Saiki, R.K., S. Scharf, F. Faloona et al. 1985. Enzymatic amplification of ß-globulin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science, 230: 1350-1354.

Sanders, V.M., B.M. Kauffmann, K.L. White, K.A. Douglas, D.W. Barnes, L.E. Sain, T.J. Bradshaw, J.F. Borzelleca and A.E. Munson. 1982. Toxicology of chloral hydrate in the mouse. Environ. Health Perspect., 44: 137-146.

Sanger, F., S. Nicklen, A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA, 74: 5463-5467.

Sax, N. and R. Lewis. 1987. Hawley's Condensed Chemical Dictionary. Eleventh Edition. Van Nostrand Reinhold Company, New York.

Singer, P.C. and S.D. Chang. 1989. Correlations between trihalomethanes and total organic halides formed during water

treatment. J. AWWA, 81: 61-65.

Smith, M.T. 1990. Chloral hydrate warning. Science, October 19, 1990: 359.

Stacpoole, P.W. and Y.J. Greene. 1992. Dichloroacetate. Diabetes Care, 15: 785-791.

Stacpoole, P.W., E.C. Wright, T.G. Baumgartner, R.M. Bersin, S. Buchalter, S.H. Curry, et al. 1992. A controlled clinical trial of dichloroacetate for treatment of lactic acidosis in adults. N. Engl. J. Med., 327: 1564-1569.

Stowers, S.J., R.W. Wiseman, J.M. Ward, et al., 1988. Detection of activated proto-oncogenes in N-nitrosodiethylamine-induced liver tumors: a comparison between B6C3F_1 mice and Fischer 344 rats. Carcinogenesis, 9: 271-276.

Tarone, R.E., K.C. Chu and J.M. Ward. 1981. Variability in the rates of some common naturally occurring tumors in Fischer 344 rats and (C57BL/6N x C3H/HeN)F_1 (B6C3F1) mice. J. Natl. Cancer Inst., 66:1175-1181.

Vagnarelli, P., A. DeSario and L. DeCarli. 1990. Aneuploidy induced by chloral hydrate detected in human lymphocytes with the Y92 probe. Mutagenesis, 5: 591-592.

Vogt, N., L. Marrot, N. Rousseau, B. Malfoy and M. Leng. 1988. Chloroacetaldehyde reacts with Z-DNA. J. Mol. Biol., 201: 773-776.

Waters, E.M., H.B. Gerstner and J.E. Huff. 1977. Trichloroethylene. I. An overview. J. Toxicol. Environ. Health, 2: 671-707.

Windholz, J., S. Budavari, R. Blumetti and E. Otterbein. 1983. The Merck Index. Tenth edition. Merck & Co., Inc., Rahyway, NJ. P. 288.

Wiseman, R.W., S.J. Stowers, E.C. Miller, M.W. Anderson and J.A. Miller. 1986. Activating mutations of the Ha-ras proto-oncogene in chemically induced hepatomas of the male B6C3F_1 mouse. Proc. Natl. Acad. Sci. USA, 83: 5825-5829.

Yoon, J., J. Mason, R. Valencia, R. Woodruff and S. Zimmering. 1985. Chemical mutagenesis testing in Drosophila. IV. Results of 45 coded compounds tested for the National Toxicology Program. Environ. Mutagen., 7: 349-367.