POTENTIAL FOR ONCOGENE EXPRESSION IN THE LIVER AND IN SPONTANEOUS AND CHEMICALLY-INDUCED HEPATOMAS OF THE B6C3F1 MOUSE

By

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The male hybrid B6C3F1 mouse exhibits a 30% spontaneous hepatoma incidence, whereas the paternal C3H/He strain and the maternal C57BL/6 strain exhibit a 60% and a negligible incidence, respectively. The Ha-ras, Ki-ras, and myc oncogenes have been implicated in a variety of solid tumors. Specifically, Ha- and, less frequently, Ki-ras have been reported to be activated in B6C3F1 mouse liver tumors. The first objective of this study was to examine two possible points of transcriptional control of Ha-ras, Ki-ras, and myc in all three mouse strains, the hypothesis being that these oncogenes may be primed for expression in the nascent liver of those strains exhibiting a high spontaneous hepatoma incidence.

A positive correlation has been established between gene expression and both hypomethylation and the presence of DNase I hypersensitive sites. It was found that Ha-ras is hypomethylated in a site-specific manner in B6C3F1 and C3H/He mouse liver as compared to C57BL/6 mouse liver. DNase I hypersensitive sites were observed in the Ha-ras and myc oncogenes in the three mouse strains. However, Ha-ras appears to possess an additional site in B6C3F1 and C3H/He as compared to C57BL/6. Similarly, the Ki-ras oncogene exhibited a DNase I hypersensitive site only in B6C3F1 and C3H/He mouse liver. These results indicate that the hepatoma-prone strains (B6C3F1 and C3H/He) may have a greater potential for Ha- and Ki-ras expression than does the non-hepatoma-prone strain (C57BL/6). This may explain, in part, the high propensity of B6C3F1 and C3H/He mice toward hepatoma development.

It was also hypothesized that Ha-ras, Ki-ras and myc have an increased potential for expression in B6C3F1 mouse liver tumors. Therefore, the methylation states of these genes was examined in spontaneous liver tumors and in tumors induced by three diverse hepatocarcinogens: benzidine, phenobarbital, and chloroform. Ha-ras was found to be hypomethylated in all tumors examined, whereas Ki-ras was sometimes hypomethylated. The methylation state of myc usually was unaltered, although this gene appeared to be amplified in tumors. These results suggest that a component of the mechanism by which these oncogenes are activated in B6C3F1 mouse liver tumors involves an increased potential for expression, via hypomethylation of the ras oncogenes and amplification of myc. Therefore, it appears that common mechanisms may underlie the development of both spontaneous and chemically-induced B6C3F1 mouse liver tumors.

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LIST OF ABBREVIATIONS

DEN	diethylnitrosamine
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
Ha-ras	Harvey-ras
Ki-ras	Kirsten-ras
mRNA	messenger RNA
RNA	ribonucleic acid
RNase	ribonuclease
SAM	S-adenosylmethionine

INTRODUCTION

1. The B6C3F1 Mouse in Carcinogen Bioassays

The B6C3F1 mouse (male C3H/He x female C57BL/6) was developed by the National Cancer Institute in the 1960s for use in carcinogen bioassays. Several advantages exist for the choice of this strain in these long-term studies (Cameron et al., 1985). As a hybrid, the B6C3F1 mouse is genetically heterogeneous, a trait shared with the human population, and hybrids exhibit increased hardiness and longevity over inbred strains. In addition, the B6C3F1 mouse has a low incidence of mammary tumors and leukemia relative to many murine strains. However, the male B6C3F1 mouse has a spontaneous hepatoma incidence of approximately 30% (Becker, 1982; Maronpot et al., 1987); spontaneous hepatoma incidence in females is much lower at 7-8% (Maronpot et al., 1987). This characteristic is heritable and, in males, intermediate between that of the paternal C3H/He strain and the maternal C57BL/6 strain which exhibit 60% incidence and virtually zero incidence, respectively, at 18 months of age (Becker, 1982). Tumors exhibit a low level of malignancy, and neither metastasize nor kill the host.

The liver of the B6C3F1 mouse (both males and females) is extremely sensitive to tumor induction by a wide variety of chemicals, including classic tumor initiators such as diethylnitrosamine (DEN) (Vesselinovitch and Mihailovich, 1983; Stowers et al., 1988), N-hydroxy-2-acetylaminofluorene (Wiseman et al., 1986), and other genotoxicants (Ashby and Tennant, 1988). In addition, a number of Ames test-negative chemicals effectively induce liver tumors in this animal (Ashby and Tennant, 1988). An example of a chemical falling into this category is phenobarbital, a non-mutagenic rodent tumor promoter. This chemical is able to increase the hepatoma incidence in B6C3F1 mice to 100% when administered in drinking water at a concentration of 0.05% for one year (Becker, 1982). Furthermore, the B6C3F1 mouse liver appears to be sensitive to hepatoma induction by high doses of some chemicals, including trichloroethylene and other chlorinated ethane derivatives, which rarely produce tumors in other species or at other sites (Clayson, 1987). Another chemical falling into this category is butylated hydroxytoluene (BHT), a widely used food preservative which is not considered mutagenic. BHT has been reported to cause liver tumors in male, but not female, B6C3F1 mice at the maximum tolerated dose of 2% in the diet (Inai et al., 1988). In fact, a comprehensive survey evaluating 222 chemicals conducted by the U.S. National Cancer Institute/National Toxicology Program revealed that mouse liver accounts for 24% of all chemical/tissue reports of carcinogenicity among the 115 carcinogens plus the 24 equivocal carcinogens evaluated for carcinogenicity in B6C3F1 mice and Fisher 344 rats (Ashby and Tennant, 1988). This same survey found that only 30% of the mouse liver-specific carcinogens are mutagenic in the Ames test. Thus, it is obvious that some characteristic of B6C3F1 mouse liver confers upon it a predisposition to the development of tumors.

The high spontaneous hepatoma incidence and sensitivity to chemical induction of liver tumors in the B6C3F1 mouse have fueled the controversy over whether or not mouse liver tumors are a valid endpoint for carcinogen bioassays. The classification of mouse liver-positive chemicals as carcinogens, despite negative results in both a bioassay using another rodent species and the Ames test, is especially disconcerting. It is interesting to note that phenobarbital, were it bioassayed today, would be placed in the Environmental Protection Agency's Category I (probable human carcinogen), with zero tolerance and no threshold. In reality, epidemiological studies have produced no evidence that chronic admini stration of this drug is carcinogenic in humans (Clemmesen and Hjalgrim-Jensen, 1978a,b, 1980, 1981). In addition, Becker (1982) found that phenobarbital is unable to induce liver tumors in the non-hepatoma prone C57BL/6 mouse, although B6C3F1 and C3H/He mice, both of which are hepatoma-prone strains, showed a strong positive response. Thus, the simple extrapolation of the results of the carcinogen bioassay to a wide variety of species based on a positive response in mouse liver alone may be an overly conservative interpretation.

Based on the above discussion, data generated using the B6C3F1 mouse in carcinogen bioassays led to the suggestion that this animal exhibits an abnormal response during the promotion phase of tumor development. Supporting this view is the finding by Drinkwater and Ginsler (1986) and Hanigan and coworkers (1988) that the increased susceptibility of the paternal, hepatoma-prone C3H/He mouse over the maternal, non-hepatoma-prone C57BL/6 mouse to DEN induction of liver tumors is heritable and largely determined by a single genetic locus which appears to exert its effects during tumor promotion. Specifically, the proliferative rate of both normal and preneoplastic hepatocytes is increased in animals possessing this HCS (hepatocarcinogen sensitivity) locus. Similarly, Dragani et al. (1987) have hypothesized that the increased susceptibility to carcinogenesis induced by DEN/1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (a phenobarbital-like chemical) observed in B6C3F1 mice as compared to a non-hepatoma-prone murine strain is due to a higher proliferative rate of initiated B6C3F1 hepatocytes.

Because promotion is considered to consist of epigenetic alterations (i.e., changes in gene expression) which lead to a proliferative advantage of initiated cells, this study was designed to examine putative control points of transcriptional activity of specific oncogenes believed to be involved in hepatocarcinogenesis. The hepatoma-prone B6C3F1 mouse is an excellent model in which to study the molecular mechanisms by which a phenotypically normal cell is transformed into a malignant cell. In addition, it is hoped that the rational interpretation of carcinogen bioassay data will be facilitated by the results presented herein.

2. Oncogene Involvement in Tumorigenesis

A. Oncogenes as normal components of the genome

Oncogenes are a group of normal cellular genes which are highly conserved in evolution (Shilo, 1984; Shilo and Weinberg, 1981) and appear to play a vital role in normal growth and differentiation. For example, the myc oncogene is differentially expressed both temporally and spatially during development of the fetal and neonatal mouse (Slamon and Cline, 1984; Ruppert et al., 1986; Zimmerman et al., 1986) and in developing human placenta (Pfeifer-Ohlsson et al., 1984). The src oncogene has been implicated in the differentiation of neural tissue, and fos appears to be involved in the differentiation of cells of different hematopoietic lineage (Muller, 1986).

The expression of a number of oncogenes has been demonstrated to be cell cycle-dependent, i.e., expression of these genes is minimal or absent in quiescent cells, but greatly elevated in proliferating cells (for review see Kaczmarek, 1986). Oncogenes falling into this category include myc, fos, myb, Ha-ras, Ki-ras, and N-ras. The expression of two of these (myc and myb) have been found to vary in a manner dependent on the different stages of the cell cycle.

Although it is clear from the above discussion that oncogenes are somehow involved in cell proliferation and differentiation, the precise biochemical role of oncogene products remains unclear. In general, oncogenes can be classified into one of four categories (Weinberg, 1985; Pitot, 1986; Garrett, 1986). The protein products of a number of oncogenes, including src and abl, function as kinases, the unusual substrate being a tyrosine residue of various proteins. It is well established that phosphorylation of many enzymes serves to alter their kinetic properties, so it is likely that this oncogene functions in a similar manner. The products of a second group of oncogenes, such as Ha- and Ki-ras, are located on the plasma membrane and bind GTP. Thus, the function in these oncogene products is speculated to involve transmembrane signalling. The products of a third class of oncogenes binds DNA. The expression of two members of this class, myc and myb, is elevated just prior to DNA replication. These two traits make it likely that such oncogenes are necessary for DNA synthesis. The last class of oncogene products are homologs of growth factors (e.g., sis and platelet-derived growth factor) or growth factor receptors (e.g., erbB and the epidermal growth factor receptor). These oncogenes most likely play a role in the pathway of mitogen-induced cell proliferation.

B. Oncogenes in neoplastic growth

It has become increasingly obvious in recent years that oncogenes play a causal role in tumorigenesis (Land _et al._, 1983a; Cooper and Lane, 1984; Slamon _et al._, 1984; Spandidos, 1985). Two major lines of evidence lead to this conclusion. First, the study of acute transforming retroviruses has revealed that such viruses contain oncogenes (v-onc) which are responsible for the transformation of host cells into malignant cells. The discovery of homologous cellular (host) oncogenes (c-onc) (DeFeo _et al._, 1981) led to the subsequent finding that activation of such genes could occur during tumorigenesis (Der _et al._, 1982). It is interesting to note that retroviral oncogenes originated from the host and apparently were acquired as a consequence of the ability of retroviruses to reversibly integrate and recombine into the host's DNA. The second line of evidence has demonstrated that transfection of DNA from transformed cells into NIH3T3 cells results in their acquisition of the transformed phenotype. It has been shown that the activated oncogenes, primarily from the src and ras families, are the critical entity transferred to NIH3T3 cells in such cases.

A number of mechanisms have been discerned by which oncogenes are activated. The ras and src oncogenes are often activated by point mutation. For example, a mutation in the 12th (Reddy *et al.*, 1982; Tabin *et al.*, 1982; Taparowsky *et al.*, 1982) or 61st (Sekiya *et al.*, 1985) codon of the Ha-ras oncogene appears to be a requirement for conferring the transforming capacity on this gene. Mammary tumors induced by N-nitroso-N-methylurea have been reported to contain a specific point mutation, a G to A transition, in codon 12 of the Ha-ras oncogene (Zarbl *et al.*, 1985). The p21 protein product of such a mutated gene exhibits altered electrophoretic mobility as compared to the normal protein (Quintanilla *et al.*, 1986; Harper *et al.*, 1987), and it is believed that an accompanying alteration in biochemical function contributes to the malignant phenotype. Mutation has also been demonstrated to be the mechanism by which the Ki-ras oncogene is activated (Santos *et al.*, 1984; O'Hara *et al.*, 1986; Liu *et al.*, 1987). In contrast, overexpression of the myc oncogene is commonly seen in malignant cells. This occurrence may be due to gene amplification (Alitalo *et al.*, 1987), rearrangement to the region of the immunoglobulin heavy chain locus (Blick *et al.*, 1986; Murphy *et al.*, 1986), or unknown causes (Rothberg *et al.*, 1984; Erisman *et al.*, 1985; Yoshimoto *et al.*, 1986). Similarly, an increased transcription rate of Ha-ras is sufficient for transformation of NIH3T3 cells (Chang *et al.*, 1982; DeFeo, 1981).

The multistep nature of cancer points to multiple changes in multiple genes, and there is evidence that activation of more than one oncogene is required to effect transformation (Glaichenhaus *et al.*, 1985). The ras and myc oncogenes have qualitatively different effects which can act in a complementary fashion in the transformation process: the ras oncogene is potent in inducing refractile morphology, anchorage independence, and growth factor secretion and is weak in its ability to immortalize cells, whereas the myc oncogene is capable of immortalizing cells (Land et al., 1986). Due to these characteristics, an activated (mutated) Ha-ras oncogene is able to transform NIH3T3 cells when transfected into them, whereas transfection of myc, as expected, has no effect in this already immortal cell line. It has been shown that neither Ha-ras nor myc alone is able to transform rat embryo fibroblasts (REFs) in the in vitro transfection assay, whereas simultaneous introduction of these two oncogenes into REFs produces a high degree of transformation (Land et al., 1983b; Birrer et al., 1988; Storer et al., 1988). Similarly, an activated Ha-ras oncogene is able to transform hamster fibroblasts only if they have previously been immortalized by carcinogen treatment (Newbold and Overell, 1983). These results are corroborated by observations in vivo: an activated ras oncogene has been found together with an activated myc oncogene in a promelocytic leukemia and in an American Burkitt lymphoma (Murray et al., 1983), and two or more oncogenes were found to be transcriptionally active in a variety of human malignancies (Slamon et al., 1984).

C. Activated oncogenes in B6C3F1 mouse liver tumors

The presence of activated oncogenes was first identified in spontaneous B6C3F1 mouse liver tumors by Fox and Watanabe in 1985. To this end, tumor DNA was transfected into NIH3T3 cells; subsequent foci formation was taken as evidence that an activated oncogene was contained in the tumor DNA. Reynolds and coworkers (1986) identified Ha-ras as the activated oncogene species in the majority (11/13) of transfection assay-positive spontaneous tumors examined. The p21 protein product derived such tumors (i.e., those containing an activated Ha-ras oncogene) has been shown to exhibit altered electrophoretic mobility as compared to the normal protein (Reynolds et al., 1986; Wiseman et al., 1986; Reynolds et al., 1987; Stowers et al., 1988), suggesting that activation of Ha-ras is the result of a mutation in this gene.

As mentioned in the previous section, activation of the Ha-ras oncogene is often due to a mutation in codon 61. Hybridization of tumor DNA with oligonucleotide probes capable of detecting a single base change in codon 61 has revealed that this codon is mutated in 50/57 chemically-induced (Wiseman et al., 1986; Reynolds et al., 1987; Stowers et al., 1988) and 15/15 spontaneous (Reynolds et al., 1987) B6C3F1 mouse liver tumors containing an activated Ha-ras oncogene; mutations in codons 13 or 17 of Ha-ras have been seen in 7/57 chemically-induced tumors (Reynolds et al., 1987). In addition, an activated Ki-ras oncogene, as well as other oncogenes, occasionally has been observed (Reynolds et al., 1987). These results indicate that the presence of an activated oncogene in B6C3F1 mouse liver tumors is frequently associated with mutation of Ha-ras, usually within codon 61, regardless of whether the tumors were chemically-induced or spontaneous.

It must be noted that DNA from non-tumor B6C3F1 mouse liver has not been demonstrated to contain activated oncogenes in the aforementioned studies, suggesting that the frequently-observed mutation in codon 61 of Ha-ras is an acquired characteristic. Therefore, this mutation cannot be responsible for the predisposition of the B6C3F1 mouse to hepatoma development. In addition, a number of spontaneous and chemically-induced tumors have tested negative in the NIH3T3 transfection assay. This result suggests that a mechanism other than mutation of Ha-ras, either through disruption of transcriptional regulation of Ha-ras or activation of another oncogene, underlies hepatoma development in these cases.

The transformation of a phenotypically normal cell into a malignant cell is considered to consist of an initial mutagenic event, termed initiation, followed by epigenetic events leading to a selective growth advantage of initiated cells (promotion), putatively through changes in gene expression patterns. It has been demonstrated that mutational activation of Ha-ras is an early event in B6C3F1 mouse liver carcinogenesis (Wiseman et al., 1986), as well as mammary (Zarbl et al., 1985) and skin (Quintanilla et al., 1986; Bizub et al., 1986; and Pelling et al., 1986) carcinogenesis. Once a critical mutation occurs, deregulation of gene expression becomes pivotal in determining whether or not the cell proceeds to a phenotypically malignant state. The Ha-ras oncogene in the nascent liver of the B6C3F1 mouse may be primed for expression, leading to an increased possibility that an activating mutation will be expressed and will thereby result in the phenotypic alterations associated with malignancy. In addition, a compromised ability to control the transcriptional activity of Ha-ras may be the heritable factor responsible for the high spontaneous hepatoma incidence and the sensitivity to chemical induction of liver tumors in the B6C3F1 mouse.

Supporting this view is the fact that B6C3F1 mouse liver appears to respond abnormally during tumor promotion (see Section 1). In addition, it has been demonstrated that DEN-induced hepatomas in Fisher 344 rats do not possess activated Ha-ras oncogenes (0/28), whereas Ha-ras was activated in 14/14 DEN-induced hepatomas in B6C3F1 mice (Stowers et al., 1988). Since it is unlikely that DEN produces different mutation frequency and/or patterns in these two species, it seems reasonable to suspect that a critical mutation in Ha-ras of B6C3F1 mouse liver is more likely to exert phenotypic effects due to an increased probability of Ha-ras expression in this species. Therefore, the studies which comprise this thesis examined two parameters of gene expression with regard to the oncogenes putatively involved in B6C3F1 mouse hepatocarcinogenesis. First, the methylation state of these genes was examined due to the established correlation between hypomethylation of a gene and its potential for expression.

Second, these genes were assessed for the presence of deoxyribonuclease I (DNase I) hypersensitive sites, as transcriptionally active genes are known to contain sites which are exquisitely sensitive to the action of this enzyme.

3. Methylation and Transcriptional Activity of Genes

A. Methylation as a regulation point of gene transcription

In mammals, a number of regulatory mechanisms participate in the control of gene expression. At the DNA level, one of these mechanisms has been identified as the methylation state of a gene (Riggs and Jones, 1983; Jones, 1985). In general, a relatively low degree of gene methylation (hypomethylation) is associated with gene expression, whereas a relatively high degree of gene methylation (hypermethylation) acts to block transcription.

The only naturally-occurring methylated base found in mammalian DNA is 5-methylcytosine (5-MC). Approximately 3% of all cytosine residues exist in the form of 5-MC, and at least 90% of these methylated bases occur in the sequence 5'-CG-3' (termed "CpG islands"). Depending on the species and tissue, between 70 and 90% of such CG sequences are methylated. Furthermore, these sites are methylated in a symmetrical fashion, i.e., cytosine residues are methylated on both strands:

$$\begin{array}{c} 5'\text{-}\overset{\overset{\text{Me}}{\downarrow}}{\text{C}}\text{G -}3' \\ 3'\text{-G}\underset{\underset{\text{Me}}{|}}{\text{C}}\text{ -}5' \end{array}$$

It has been hypothesized that 5-MC exists at all sites that will ever be methylated early in embryonic development (Singer et al., 1979; Razin and Riggs, 1980). Tissue-specific patterns of gene expression then are established during the DNA replication accompanying cell division through selective inhibition of methylation of genes destined to be transcriptionally active or through active demethylation of specific genes (Razin et al., 1986). Once a methylation pattern is established within a cell's genome, it is propagated through successive rounds of cell division, as described below.

Replication of DNA containing a 5-MC residue results in the formation of hemimethylated DNA, i.e., DNA that is methylated on only the parental strand. It has been demonstrated that a maintenance methylase activity exists in mammalian cells which recognizes hemimethylated sites. Using S-adenosyl methionine (SAM) as the methyl group donor, this enzyme methylates the newly synthesized strand of DNA, thereby forming the fully methylated site. Thus, once a pattern of gene methylation is established, maintenance methylase activity allows somatic heritability of this trait.

Hypomethylation of a gene has been associated with its transcriptional activity in a variety of systems. The serum albumin gene is hypomethylated in normal rat hepatocytes, but hypermethylated in non-parenchymal cells (Vorce and Goodman, 1985), conditions consistent with expression in the former cell type and non-expression in the latter. Similarly, Ichinose and coworkers (1988) have demonstrated a correlation between hypomethylation of the pepsinogen gene and its expression in developing rat stomach. Conversely, it has been observed that CpG islands within housekeeping genes on the inactive mammalian X chromosome are methylated; methylation has therefore been proposed as the mechanism by which the inactivity of such genes is maintained (Bird, 1986). Supporting this hypothesis is the finding that demethylation of three CpG islands within the glucose-6-phosphate dehydrogenase gene on the inactive X chromosome is associated with activation of this gene (Toniolo et al., 1988).

Methylation of certain regions of a gene appears to be more critical than others in inhibiting gene expression. Specifically, methylation of the 5' end of a number of genes appears to regulate transcription. One such example is the hamster adenine phosphoribosyltransferase gene. When the body of this gene is artificially methylated, there is no inhibition of transcription, but when a region near the 5' end is methylated, transcription is significantly reduced (Keshet et al., 1985). A similar situation has been found in other systems: absence of methylation in the 5' region of the calcitonin gene has been correlated with its expression in a medullary thyroid carcinoma cell line (Baylin et al., 1986), and a region of the albumin gene extending from the 5' end to the middle is hypomethylated in a hepatoma cell line which synthesizes albumin, but methylated in a nonproducing variant (Orlofsky and Chasin, 1985; Ott et al., 1982). However, in other genes, different patterns of methylation appear to be necessary for gene expression. In S49 lymphoma cells, the metallothionine gene is heavily methylated and not expressed; when induced to synthesize metallothionine by UV irradiation, a region of one allele spanning this gene becomes demethylated (Lieberman et al., 1983). A variant of the mouse hepatoma cell line Hepa-1 has a high constitutive level of cytochrome P_1-450 expression which appears to be due to hypomethylation of a specific site in the middle of the P_1-450 gene (Peterson et al., 1986). Hypomethylation of regions near the 3' end of a gene has also been found to be necessary for expression. The myc oncogene is methylated at a site near the 3' end in normal cultured fibroblasts, but hypomethylated in three of five tumor cell lines (Cheah et al., 1984). Thus, hypomethylation of regions of genes near the 5' end, 3' end, and middle has been correlated with gene expression.

B. Gene activation through induction of a hypomethylated state

The cytidine analog 5-azacytidine (5-azaCR) is capable of inducing a hypomethylated state in proliferating cells by virtue of its ability to inhibit maintenance methylase irreversibly following its incorporation into DNA (Taylor et al., 1984; Jones, 1985). 5-AzaCR treatment of a variety of cell types has been demonstrated to activate previously quiescent, methylated genes (Jones, 1985). For example, 5-azaCR is able to induce expression of the hypoxanthine guanine phosphoribosyl transferase gene contained on the inactive X chromosome (Mohandas et al., 1981; Graves, 1982; Lester et al., 1982). Other genes whose expression has been induced by 5-azaCR treatment include prolactin, growth hormone, globin, and thymidine kinase (Jones, 1985).

5-AzaCR also has been shown to induce cellular differentiation (Jones, 1985). New phenotypes induced by 5-azaCR treatment of various cell lines include muscle cells, adipocytes, chrondrocytes, and others. Similarly, immunoresistance acquired by tumor cells via loss of tumor antigen expression can be eliminated through 5-azaCR treatment (Altevogt et al., 1986). These results indicate that inhibition of methylation by 5-azaCR results in derepression of transcription in a variety of genes.

5-AzaCR treatment has been shown to induce transformation of established cell lines (Yasutake et al., 1987; Hsiao et al., 1985) and primary rat tracheal epithelial cultures (Walker and Nettesheim, 1986). An increased ability to form experimental metastases was observed after 5-azaCR treatment of B16 melanoma cells (Trainer et al., 1985). Furthermore, there is evidence that 5-azaCR is carcinogenic in mice (Cavaliere et al., 1987). It thus appears that alteration of gene expression via 5-azaCR-induced gene hypomethylation plays a role in the transformation process. It follows that derangement of methylation patterns may play a role in in vivo carcinogenesis (Riggs and Jones, 1983; Jones, 1985; Boehm and Drahovsky, 1983).

Support for this hypothesis comes from dietary studies in which animals are fed a methyl-deficient diet. It has been observed that rats fed a diet deficient in methionine and choline develop liver tumors (Yokoyama et al., 1985; Ghoshal and Farber, 1984; Mikol et al., 1983). It has been demonstrated that a methyl-deficient diet results in a decrease in available methyl groups, i.e., the ratio of S-adenosyl methionine to S-adenosyl homocysteine is significantly lowered in animals fed the choline/methionine-deficient diet as compared to controls (Wilson et al., 1984). Furthermore, the 5-MC content of hepatic DNA, as assessed by high performance liquid chromatography, is significantly decreased in rats fed this methyl-deficient diet for 22 weeks (Wilson et al., 1984). A similar decrease in 5-MC content of hepatic DNA was seen in rats fed a choline-devoid diet for 14 months (Locker et al., 1986). In this case, restriction endonuclease analysis (using enzymes that can distinguish between methylated and unmethylated recognition site) showed hepatic DNA to be hypomethylated in both tumor and non-tumor tissue of animals fed the methyl-deficient diet as compared to hepatic DNA from animals fed the control diet. These results suggest that a methyl-deficient diet may be carcinogenic by virtue of its ability to transcriptionally activate critical genes through the induction of a generalized state of DNA hypomethylation. However, reversible alkali-labile lesions, indicating DNA damage and repair (Rushmore et al., 1986), and free-radical production (Rushmore et al., 1987) have been observed as early events resulting from commencent of a methyl-deficient diet. Thus, it is possible that mutations also result from this dietary regimen and may thereby contribute to carcinogenesis. It is interesting to note that a choline-devoid diet causes a high level of cell proliferation in the liver which persists after the reintroduction of choline, although the proliferative rate decreases with time (Chandar and Lombardi, 1988). This result suggests that DNA hypomethylation may activate genes involved in mitogenesis. Indeed, hypomethylation of Ha-ras and Ki-ras has been observed in preneoplastic and neoplastic livers of rats maintained on a methyl-deficient diet (Bhave et al., 1988), and ras gene transcription is enhanced during rat hepatocarcinogenesis induced by choline deficiency (Yaswen et al., 1985).

C. Hypomethylation and cancer

It has been proposed that modifications of DNA methylation are involved in the inheritance of epigenetic defects, including those seen in carcinogenesis (Holliday, 1987a,b). A number of studies have shown that the 5-MC content of DNA is lower in malignancies than in normal tissue (Riggs and Jones, 1983). A reduction in 5-MC was observed in both benign and malignant human colon tumors (Feinberg et al., 1988) and hamster kidney tumors (Lu et al., 1988) as quantitated by HPLC analysis. Similar results were obtained by restriction enzyme analysis: hypomethylation was observed in a variety of genes in DNA derived from benign and malignant human colon neoplasms relative to the methylation state of these genes in normal tissue (Goelz et al., 1985). Genomic hypomethylation was also observed in a number of metastatic variants selected from a poorly metastatic human melanoma cell lines (Liteplo and Kerbel, 1987). These results indicate that generalized DNA hypomethylation is a common phenomenon in transformed cells. This condition may facilitate aberrant gene expression, including those genes involved in the carcinogenic process.

Hypomethylation of specific oncogenes has been observed in numerous neoplasms. The Ha- and Ki-ras oncogenes were found to be hypomethylated in various human tumors (Feinberg and Vogelstein, 1983), and the 5' region of the Ha-ras oncogene is undermethylated in a leukemic cell line (Barbieri et al., 1987). Hypomethylation of the myc oncogene, but not two other genes, has been observed in human hepatocellular carcinomas (Kaneko et al., 1985). Furthermore, a specific site in the third exon of the myc oncogene is hypomethylated in human hepatocellular carcinoma (Nambu et al., 1987) and in a set of human tumor cell lines (Cheah et al., 1984). Thus, it appears that oncogenes thought to be involved in carcinogenesis often possess an increased potential for expression in tumors versus non-tumor tissue. Furthermore, site-specific hypomethylation may be sufficient to permit transcriptional activity of certain genes.

Treatment of cultured cells with a number of carcinogens results in a decrease in genomic 5-MC content (Wilson and Jones, 1984; Wilson et al., 1987a; Boehm et al., 1983). In vitro, the transfer of a methyl group from SAM to hemimethylated DNA is inhibited by a range of ultimate carcinogens (Wilson and Jones, 1983), a result suggesting that the activity of maintenance methylase may be inhibited by carcinogenic species. Paradoxically, methylase activity is higher in tumorgenic as compared to non-tumorigenic cell lines (Kautiainen and Jones, 1986); the activity of this enzyme is also higher in target rat tissues after in vivo treatment with N-methyl-N-nitrosourea (Pfohl-Leskowicz and Dirheimer, 1986). In agreement with this observation, de novo methylation is increased following treatment of cells with N-acetoxy-N-2-acetylaminofluorene, even though this chemical causes an initial dose-dependent decrease in maintenance methylase activity (Boehm et al., 1983). The physical and catalytic properties of methylase derived from normal rat liver and a transplantable hepatocellular carcinoma have been compared and found to be indistinguishable (Ruchirawat et al., 1985). Therefore, although many carcinogens possess the ability to inhibit methylation of DNA, the mechanism does not appear to involve long-term depression of methylase activity. However, transient inhibition of methylase by carcinogens during periods of hyperplasia could result in propogated DNA hypomethylation since the methylation state of genes is somatically heritable. It is also possible that the rebound increase in methylase activity results from increased transcription of the methylase gene due to its hypomethylation.

Although methylation of genes appears to be a control point of gene expression, hypomethylation alone is not considered sufficient to produce transcriptional activity. This is illustrated by the fact that some hepatoma cell line variants contain a hypomethylated serum albumin gene yet do not produce albumin. In addition, it has been shown that the stimulation of prolactin and growth hormone production by 5-azaCR is a function of time, i.e., maximal stimulation occurs 3 weeks after drug exposure (Laverriere et al., 1986). These observations indicate that further epigenetic alterations beyond hypomethylation are necessary for transcription to proceed.

D. Use of restriction endonucleases to assess gene methylation state

To assess the methylation state of a gene, the restriction endonucleases Msp I and Hpa II may be utilized. The Msp I/Hpa II isoschizomers cleave double-stranded DNA at the following recognition sequece: 5'-CCGG-3' (Figure 1). However, Msp I, but not Hpa II, is able to perform this function when the internal cytosine residue is methylated. Conversely, Hpa II, but not Msp I, will cleave this sequence when the external cytosine is methylated. Since more than 90% of 5-methylcytosine occurs in the sequence of 5'-CG-3' (Riggs and Jones, 1983), the methylation state of a gene may be assessed by comparing the restriction patterns produced by digestion with Msp I and Hpa II. If a gene is hypomethylated, very similar restriction patterns will be produced by digestion with either enzyme. However, if a gene is hypermethylated, digestion with Msp I will produce more bands of a smaller size than will digestion by Hpa II. In addition, the restriction enzyme Hha I can be used for methylation state assessment (Figure 1). This restriction enzyme cleaves the sequence 5'-GCGC-3' only when the internal cytosine residue is unmethylated. Since this recognition site also contains the 5'-CG-3' sequence at which most 5-MC occurs, Hha I will more readily cleave hypomethylated regions of DNA. Because an isoschizomer is not available which cleaves this site when methylated, Hha I analysis can only be used to compare the methylation state of a gene between samples.

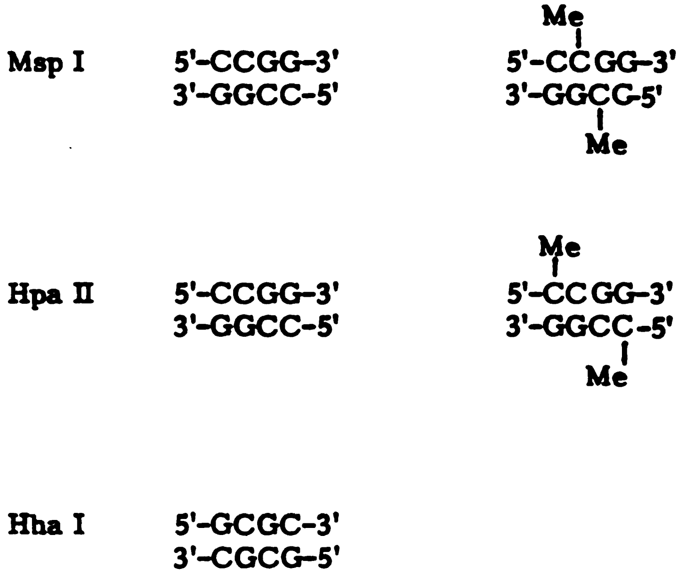

Figure 1. Recognition sites of the restriction endonucleases Msp I, Hpa II, and Hha I.

4. Deoxyribonuclease I Hypersensitivity and Transcriptional Activity of Genes

Deoxyribonuclease I (DNase I) is a non-specific endonuclease that splits phosphodiester linkages of DNA, producing 5' phosphate terminated polynucleo tides. Although purified DNA is randomly degraded by this enzyme, transcriptionally active regions of chromatin are approximately 10-fold more sensitive to DNase I digestion than are quiescent regions of chromatin (Gross and Garrard, 1988). This property is a consequence of the DNA/RNA/protein interactions which determine the conformation of DNA within chromatin. As can be seen in Figure 2A, chromatin in the region of quiescent genes is normally condensed. However, as a gene becomes prepared for transcription, the structure of chromatin becomes altered locally (stage 1). Full activation involves commencement of DNA transcription in this area of decondensed chromatin (stage 2).

Figure 2B illustrates the fact that both genes actively transcribing RNA and genes with the potential for transcriptional activity are preferentially sensitive to DNase I digestion, supposedly because such regions are more accessible to this enzyme.

It has been discovered that the 5' end of genes with the potential for transcription (whether transcribing RNA or not) contains sites which are exquisitely sensitive to DNase I digestion (Elgin, 1981, 1982; Gross and Garrard, 1988). Such sites are roughly two orders of magnitude more sensitive to DNase I than is bulk chromatin and are thought to represent areas which allow transcription factors (such as RNA polymerase and topoisomerases) to gain access to regulatory (e.g., promoter) regions of genes. These DNase I hypersensitive sites appear to be nucleosome-free and lack histone proteins, a condition which would be expected to promote DNA/transcription factor interactions, as well as DNA/DNase I interactions. However, not all genes possessing a DNase I hypersensitive site are actively engaged in RNA transcription.

Figure 2. Schematic diagram of chromatin alterations involved in gene activation and the relationship of these changes to sensitivity of genes to DNase I digestion. (A) Chromatin becomes decondensed as a gene becomes readied for transcription (Stage 1), and this change in conformation becomes more pronounced as the gene is actively transcribed (Stage 2). (B) Due to the decondensed conformation of chromatin in the region of genes with the potential for transcription and those genes actively transcribing DNA, DNase I can more readily degrade DNA in these areas than in highly condensed regions of chromatin containing quiescent genes. (Adapted from Molecular Biology of the Cell, Garland Publishing Company, New York, 1983.)

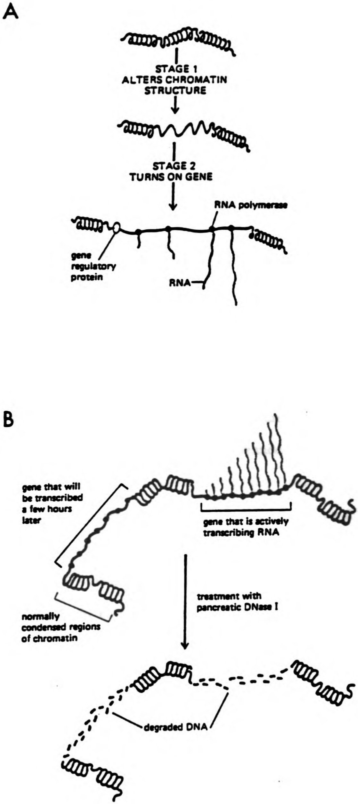
A
STAGE 1
ALTERS CHROMATIN
STRUCTURE
STAGE 2
TURNS ON GENE
RNA polymerase
gene
regulatory
protein
RNA
B
gene that will
be transcribed
a few hours
later
gene that is actively
transcribing RNA
normally
condensed regions
of chromatin
treatment with
pancreatic DNase I
degraded DNA

Figure 2

Depending on the gene, DNase I hypersensitive sites may be constitutive, inducible, developmental, or tissue-specific (Gross and Garrard, 1988). For example, DNase I hypersensitive sites appear in the metallothionein gene when cells are induced to express this enzyme by cadmium treatment (MacArthur and Lieberman, 1987). Similarly, a site at the 5' end of the β-major globin gene was observed after dimethylsulfoxide induction (Balcarek and McMorris, 1983). This site appears prior to observed increases in globin mRNA, suggesting that alterations in chromatin are necessary to allow transcription to commence. DNase I hypersensitive sites have been found to be regulated developmentally in the α-fetoprotein gene (Turcotte et al., 1986), and tissue-specific DNase I hypersensitive sites have been observed in the cardiac myosin light chain gene (Winter and Arnold, 1987). DNase I hypersensitive sites can be induced in the major chicken vitellogenin gene by hormone treatment, but only in specific tissues (Burch and Weintraub, 1983). Again, the appearance of hypersensitive sites in the gene precedes its expression.

Few studies have examined oncogenes with regard to DNase I hypersensitivity. DNase I hypersensitive sites have been observed 5' to the myc oncogene in HL-60 cells (Tuan and London, 1984) and a Burkitt lymphoma (Siebenlist et al., 1984) expressing myc. In some Burkitt lymphomas, translocation of myc to the immunoglobulin region is associated with the appearance of new DNase I hypersensitive sites, suggesting that sites normally involved in the regulation of the immunoglobulin gene may now exert influence over myc transcription (Dyson and Rabbits, 1985). A similar result was obtained in myc-transfected plasmacytoma cells (Feo et al., 1986) using S1 nuclease, an enzyme which closely resembles DNase I in its ability to recognize hypersensitive sites. A S1 nuclease-sensitive site was also detected in the myc oncogene in HL-60 cells upon chemical induction of differentiation, a regimen known to increase the expression of myc

(Grosso and Pitot, 1985). Conversely, induction of differentiation in HL-60 cells results in a decrease in transcriptional initiation of myc expression; this phenomenon was accompanied by a concurrent loss of two DNase I hypersensitive sites near the myc promoter (Siebenlist et al., 1988). Thus, there is precedent for the association of nuclease hypersensitive sites with oncogene expression.

5. Hypothesis and Experimental Objectives

The hypothesis underlying this study consists of two related parts. First, because the liver of the B6C3F1 mouse exhibits a high spontaneous tumor incidence and exceptional sensitivity to chemical induction of tumors, I have hypothesized that certain oncogenes possess a relatively high potential for transcriptional activity in the nascent liver of this animal. The oncogenes examined include Ha-ras and Ki-ras, both of which have been identified as activated oncogenes in B6C3F1 liver tumors, and myc, which has been shown to cooperate with ras oncogenes to effect transformation of normal cells. In addition, because of the heritable nature of hepatoma development, the potential for expression of these three oncogenes was assessed in the liver of the two parental strains, C3H/He and C57BL/6. In this manner, it is possible to compare the potential for expression of each oncogene among the three mouse strains with respect to their spontaneous hepatoma incidence. I predicted that those strains having a high spontaneous tumor incidence (B6C3F1 and C3H/He) possess an elevated potential for Ha-ras, Ki-ras, or myc expression versus the strain displaying a low spontaneous tumor incidence (C57BL/6).

Two different parameters of gene expression were examined for each oncogene: the methylation state of the gene and the presence or absence of DNase I hypersensitive sites. Restriction enzyme analysis was used to assess the methylation state of a gene, and an assay for DNase I hypersensitive sites was developed. Hypomethylation and the presence of DNase I hypersensitive sites appear to be necessary, but not sufficient, for gene expression. It follows that a state of relative hypomethylation of and/or DNase I hypersensitive sites in an oncogene in one mouse strain as compared to another strain would be indicative of a higher potential for expression of that gene in the first strain. This increased potential for transcriptional activity may facilitate aberrant gene expression, which may, in turn, contribute to hepatoma development. This approach may identify a heritable factor underlying the differential incidence of spontaneous hepatomas in the B6C3F1, C3H/He, and C57BL/6 mouse strains.

The second part of my hypothesis is that an increased potential for expression is a component of the mechanism by which the Ha-ras, Ki-ras, and myc oncogenes are activated in B6C3F1 mouse liver tumors. Experimental evidence suggests that a point mutation in the Ha-ras oncogene is often present in B6C3F1 mouse liver tumors. However, phenotypic effects of such a mutation cannot occur unless the gene is also expressed; the critical step then becomes deregulation of gene expression. Therefore, to determine whether or not the Ha-ras, Ki-ras, and myc oncogenes possess an increased potential for expression in B6C3F1 mouse liver tumors, the methylation state of each gene was assessed by restriction enzyme analysis.

It was also important to determine whether or not oncogene activation through an increased potential for expression is qualitatively similar in spontaneous and chemically-induced B6C3F1 mouse liver tumors. To this end, tumors were chemically-induced in three groups of mice, and spontaneously-arising tumors were harvested from a fourth mouse group. The three carcinogens used included: 1) benzidine, a mutagenic complete carcinogen; 2) chloroform, a non-mutagen which is capable of producing liver tumors in the B6C3F1 mouse; and 3) phenobarbital, a non-mutagenic rodent liver tumor promoter. This approach provided a group of tumors arising from diverse treatments, and thereby allows the determination of whether or not tumor development in the different groups shares common mechanisms of oncogene activation.

Lastly, a determination of transcriptional activity of each oncogene was made in control liver as well as in hepatoma tissue. To accomplish this, the amount of messenger RNA for Ha-ras, Ki-ras, and myc was assessed by Northern blot analysis. This portion of the study allows comparisons to be made between the potential for transcription displayed by each gene with the RNA product of transcription.

From these experiments, it is believed that the molecular mechanisms underlying hepatoma development in the B6C3F1 mouse, as well as carcinogenesis in general, has been further elucidated. In addition, insight has been gained regarding heritable factors underlying the propensity of the B6C3F1 mouse to develop spontaneous and chemically-induced hepatomas.

MATERIALS AND METHODS

1. Animals: Maintenance and Carcinogen Treatment

Primarily, male B6C3F1 male C3H/He, and female C57BL/6 mice were used in these studies. Where indicated, the opposite sex of these animals (female B6C3F1, female C3H/He, and male C57BL/6 mice) were employed. Young adult mice (18-19 g) were purchased from Charles River Laboratories (Portage, MI), housed in constant temperature and humidity conditions with a 12-hr light/dark cycle, and provided with food and water ad libitum.

Male B6C3F1 mice were used for carcinogen treatment. The benzidine-treated animals were given 120 ppm benzidine in drinking water for one year prior to sacrifice; this treatment resulted in an 85% tumor incidence. For the phenobarbital-treated group, animals received 0.05% (w/v) phenobarbital in drinking water for one year and were sacrificed six months later. Tumors were apparent in 68% of these animals. Tumors were induced with chloroform by administering 200 mg/kg of this chemical in a corn oil vehicle by gavage twice weekly for one year, a regimen resulting in a tumor incidence of 80%. A group of animals was allowed 24 months for the development of spontaneous tumors in the absence of any chemical treatment.

Partial hepatectomies were performed on one group of control B6C3F1 mice. Methoxyfluorene or ketamine was used for anesthesia, and surgery was performed as described by Higgins and Anderson (1931).

2. Isolation of DNA: Marmur Method

Hepatic nuclei were prepared by the method of Blobel and Potter (1966) as follows. Fresh liver was homogenized in 3 volumes of ice-cold 0.25 M sucrose, 50 mM Tris (pH 7.5), 25 mM KCl, 5 mM $MgCl_2$ (STKM) and filtered through cheese cloth. A 10-ml aliquot of homogenate was mixed with 2 ml of 20% Triton X-100, diluted with 8 ml STKM, and centrifuged at 750 x g for 10 min at 4°C. The resulting nuclear pellet was washed once with the same volumes of STKM and Triton X-100 and once with STKM alone.

High molecular weight DNA was isolated from nuclei by a modification of the method of Marmur (1961). Nuclei from 10 ml homogenate were suspended in 15 ml 10 mM Tris (pH 7.9), 0.1 M NaCl, 5 mM EDTA, 0.5 M $NaClO_4$, and 0.5% sodium dodecyl sulfate and incubated at 37°C for 40 minutes. An equal volume of chloroform:3% isoamyl alcohol was used to deproteinize the suspension, followed by centrifugation at 400 x g for 9 minutes; this wash was repeated once. DNA was precipitated from the upper (aqueous) fraction with 2 volumes of ice-cold 95% ethanol and pelleted by centrifugation at 12,100 x g for 10 minutes at 4°C. The DNA was redissolved in 10 mM Tris (pH 7.9), 5 mM EDTA, and ribonuclease A (Sigma; heat-treated at 80°C for 10 min to destroy DNase activity) was added to a final concentration of 200 μg/ml. After incubation at 37°C for 40 min, the NaCl concentration was adjusted to 0.1 M and 3 mg/ml protease (Sigma; heat-treated at 80°C for 10 minutes) was added. Incubation was continued at 37°C for 90 minutes. An equal volume of chloroform:3% isoamyl alcohol was used to deproteinize the solution, and this was followed by a wash with an equal volume of redistilled phenol. One-half volume of 7.5 M ammonium acetate and 2 volumes 95% ethanol were used to precipitate the DNA, which was stored overnight at -20°C. The DNA was dissolved in 5 ml 10 mM Tris (pH 7.8), 5 mM EDTA, reprecipitated with 0.5 volumes ammonium acetate and 2 volumes 95% ethanol, and again stored at

-20°C overnight. The DNA was then dissolved in 250 μl-1000 μl 5 mM Tris (pH 7.8), 0.5 mM EDTA. Absorbance at 260 nm was used to quantitate the concentration of DNA, as one A_{260} unit equals 50 μg DNA/ml. The A_{260}/A_{280} ratio was used to assess its purity, and the ratios obtained were approximately 1.7. (The ratio of a pure DNA solution is approximately 2.0, and this value decreases with increasing protein contamination.)

3. Restriction Enzyme Digestion, Agarose Gel Electrophoresis and Southern Transfer of DNA to Nitrocellulose Paper

A. Restriction enzyme digestion

Forty microgram aliquots of DNA were digested to completion with 5 units/μg Msp I or Hpa II (BRL, Gaithersburg, MD) at 37°C for 2 hours in a reaction volume of 120 μl. Reaction buffers were supplied as a 10X concentrate by BRL, and the final reaction mixture contained 50 mM Tris (pH 8.0), 10 mM $MgCl_2$ for Msp I, and 20 mM Tris (pH 7.4), 10 mM $MgCl_2$ for Hpa II. Following digestion, 10 μl of marker dye (composed of 50% glycerol, 0.25% bromophenol blue, and 0.25% xylene cyanole FF) was added. Samples, along with 2 μg lambda phage Hind III fragments (BRL, Gaithersburg, MD) diluted to 120 μl with running buffer, were heat-treated at 65°C for 10 minutes to dissociate "sticky ends" formed by restriction enzymes digestion, and cooled on ice.

B. Agarose gel electrophoresis

Electrophoresis was carried out in a model HO/H1 horizontal electrophoresis apparatus using TBE (89 mM Tris, pH 8.3, 89 mM boric acid, 2.5 mM EDTA) as the running buffer. Wick gels consisting of 1.4% agarose in TBE were poured and covered with TBE to a depth of approximately 2.5 cm. A 0.9% agarose gel was prepared in TBE by heating the solution to 100°C to dissolve the agarose, followed by cooling with stirring to 55-60°C. The edges of the gel support tray were sealed to the wick gels by pipetting liquid agarose into this space and allowing it to solidify. The remainder of the agarose was then poured, the well former was inserted, and the gel was allowed to solidify for at least one hour.

After careful removal of the well former, the samples were loaded into the wells using a Pasteur pipette; unused wells were filled with TBE. Electrophoresis was carried out at 40-50 V for 16 hours. The gel was then cut at the ends of the gel support tray and placed in a solution of 0.5 μg/ml ethidium bromide for 15 minutes in order to stain the DNA. The gel was destained in glass distilled water for 10 minutes. The DNA was visualized on a UV light box (Fotodyne, New Berlin, WI) and photographed through a Kodak No. 9 Wrattan gelatin filter at an aperture of 8 for 1 second using Type 667 black and white Polaroid film. A ruler was placed along the gel to assess the distance migrated by the lambda Hind III fragments. The molecular weights of these fragments, which range from 2.2 to 23.1 kilobases in size, were plotted on the ordinate of semilog paper as a function of distance.

C. Southern transfer

After being photographed, the gel was soaked in 0.25 N HCl for 10 minutes to fragment the DNA by random depurination; this facilitates the transfer of high molecular weight DNA (Wahl et al., 1979). Otherwise, the transfer procedure was performed according to the method of Southern (1975). The gel then was soaked in 1.5 M NaCl, 0.5 M NaOH for 2 hours with occasional agitation in order to denature the DNA. This solution was decanted and replaced with 1 M Tris (pH 8.0), 1.5 mM NaCl. Neutralization was carried out for 2 hours with occasional agitation. The gel was inverted and placed on a piece of 3 MM paper (Whatman) prewet in 10X SSC (1.5 M NaCl, 0.5 M sodium citrate, pH 7.0) with the ends of the 3 MM paper immersed in a tray of 10X SSC to serve as wicks. A piece of nitrocellulose paper (Schleicher and Schuell) was cut to the exact size of the gel and wet in glass distilled water. In the event that the nitrocellulose membrane failed to wet completely, it was covered with boiling water. The membrane then was immersed briefly in 10X SSC before being placed on the gel. Several blotting pads (BRL) and approximately 2 inches of paper towels (both cut to the same size as the gel) were stacked on top of the membrane. A 600 g weight was placed on top of the paper towels, and the entire assembly was covered with plastic wrap to prevent drying of the wick. Transfer of the DNA to the nitrocellulose by capillary action was carried out for 20-24 hours, during which time the wet paper towels were removed several times. At the end of the transfer period, the paper towels and blotting pads were discarded and the nitrocellulose was carefully peeled off the gel and air-dried on 3 MM paper. The DNA was affixed to the membrane by baking in a vacuum oven for 2 hours at 80°C.

4. Amplification and Isolation of Plasmids Containing pRSA 13 and myc Pst I Fragment

The pRSA 13 clone of serum albumin (Sargent et al., 1981) and the Pst I fragment of myc (Vennstrom et al., 1981) had been cloned into the pBR322 plasmid leaving an intact tetracycline resistance gene. The plasmids were amplified and isolated as described by Maniatis et al. (1982). Ten milliliters of M-9 glucose medium (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 0.5% glucose 0.5% Casamino acids, .002% thiamine-HCl, 1 mM $MgCl_2$) plus 0.015 mg/ml tetracycline was innoculated with E. coli strain HB101 containing the pBR322 plasmid. The innoculum was incubated overnight at 37°C with constant shaking; it was sometimes necessary to allow the incubation to proceed for an additional 24 hours. Following incubation, the entire 10 ml were added to 1 liter of M-9 medium, and the mixture was incubated and shaken at 37°C. When the absorbance at 600 nm reached 0.5-0.6, 150 mg of chloramphenicol (Sigma Chemical) was added per liter of culture, and the plasmid was allowed to amplify overnight. Cells were chilled on ice for 5 minutes, then centrifuged at 5,000 rpm, 10 minutes at 5°C. Cells were suspended in 40 ml of 10 mM Tris (pH=8.0) and 1 mM EDTA (washing buffer) and centrifuged at 5000 rpm, 10 minutes, 5°C. Cells were resuspended in 4 ml freshly prepared lysozyme (2 mg/ml), 50 mM glucose, 10 mm EDTA, and 25 mM Tris (pH=8.0) and incubated at 0°C for 30 minutes. Two volumes of alkaline SDS (0.2 N NaOH, 1% SDS) were added and the mixture was incubated at 0°C for 5 additional minutes. Following the addition of 1.5 volumes of 3 M sodium acetate (pH=4.8), the suspension was incubated at 0°C for 1 hour, followed by centrifugation at 15,000 rpm for 20 minutes. The supernatant was precipitated with 2 volumes of ethanol overnight at -20°C followed by centrifugation at 10,000 rpm for 15 minutes. The pellet was resuspended in 15 ml of sterile 10 mM Tris (pH=7.4) and 1 mM EDTA, and 15.8 g cesium chloride and 7.5 mg ethidium bromide was added. The suspension was centrifuged in a type 60 Ti fixed angle rotor at 22°C, 40,000 rpm for 48 hours. The supercoiled plasmid DNA (lower UV-visible band) was removed and the ethidium bromide was extracted with an equal volume of water-saturated butanol until the pink color of the top layer disappeared. The top layer (approximately 8 ml) was dialyzed overnight against 1 liter of 10 mM Tris, 0.1 mM EDTA, and the dialysis buffer was changed once. The dialysate was washed once with redistilled phenol and once with chloroform. The plasmid DNA was precipitated by the addition of 0.1 volume of 20% sodium acetate (pH 5.2) and 2 volumes of 95% ethanol and pelleted by centrifugation at 10,000 rpm for 20 minutes, 5°C. After decanting the supernatant, the pellet was solubilized in 500 μl of 10 mM Tris, 0.1 mM EDTA and stored at 20°C.

5. Nick Translation of the Ha-ras BS-9 Probe

The BS-9 clone of Ha-ras (Ellis _et al._, 1981) is a 450 base clone corresponding to the 5' region of the gene, commencing approximately 50 base pairs upstream from the N-terminus of the p21 protein (Dahr et al., 1982). This probe was ^{32}P-labelled using a nick translation kit purchased from BRL. On ice, 1 μg BS-9-containing plasmid DNA was mixed with 8 μl each α-^{32}P-labelled dATP, dCTP, dGTP, and dTTP (New England Nuclear; 10 mCi/ml; 800 Ci/mmol) and glass distilled water to a volume of 45 μl. After a brief mixing, 5 μl of a 10X reaction buffer containing DNA polymerase I and DNase I was added. Final composition of the reaction solution was 2.08 μM in each of the four radiolabelled nucleotides, 0.04 U/μl DNA polymerase I, 4 pg/μl DNase I, 5 mM Tris (pH 7.5), 0.5 mM magnesium acetate, 0.1 mM 2-mercaptoethanol, 0.01 mM PMSF (phenylmethylsulfonyl fluoride), 10 μg/ml bovine serum albumin and 5% glycerol. Nick translation was carried out at 15°C for one hour. The reaction vial then was placed on ice, and 38 μl STE and 12 μl of a bromophenol blue solution was added (final volume = 100 μl).

Separation of newly labelled probe from unincorporated nucleotides was accomplished by spun column chromatography (Maniatis et al., 1982). Columns were prepared by packing Sephadex G-50 Fine in a 1-ml syringe plugged with glass wool. To this end, Sephadex was equilibrated in STE (10 mM Tris, pH 8.0, 1 mM EDTA, 0.1 M NaCl), and transferred to the column with a Pasteur pipette. The column was placed in a 15-ml Corex tube and centrifuged (IEC Centra 7-R) at 2000 rpm for 8 minutes. Sephadex was added to the column and recentrifuged until the post-centrifugation volume reached 0.9 ml. Columns were washed at least twice with 100 μl STE before use.

An Eppendorf tube (cap removed) was placed in a 15-ml Corex tube, and the column was placed in the tube such that the tip was within the Eppendorf tube. The entire 100 μl nick translation mixture was applied to the top of the column, which was centrifuged at 2000 rpm for 8 minutes. The column, which retains unincorporated nucleotides along with bromophenol blue, was discarded. The labelled DNA contained in the Eppendorf tube was diluted with 100 µl STE, and 2 µl aliquots were counted in a Packard Tricarb Model 460C scintillation counter using Safety Solve (RPI, Mount Prospect, IL) scintillation cocktail.

6. Hybridization of DNA Affixed to Nitrocellulose Membranes

Membranes were wet in a minimum volume of hybridization buffer (50% formamide, 10 mM Hepes (pH 7.4), 3X SSC, 1 mg/ml yeast tRNA, 1X Denhardts buffer (0.02% each Ficoll 400, polyvinyl pyrollidone, and bovine serum albumin), and 100 µg/ml denatured salmon sperm DNA). After wrapping in plastic wrap, the membranes were prehybridized at 42°C for approximately 16 hours. The ^{32}P-labelled probe (prepared by nick translation) was denatured by boiling for 5 minutes in a water bath and 10^6 cpm were added to a small amount of hybridization buffer. The probe solution was spread on plastic wrap, and the nitrocellulose membrane was placed on it DNA side down. The membrane was rewrapped with plastic wrap and massaged to spread the probe evenly across the membrane. Hybridization was carried out at 42°C for 48 hours.

Membranes were washed 4 times with 2X SSC-0.1% SDS at room temperature for 5 minutes each, twice with 1X SSC-0.1% SDS at 55°C for 10-15 minutes each and, finally, 4 times with 0.1X SSC-0.1% SDS at 55°C for 10-15 minutes each. Membranes were allowed to air dry and wrapped in plastic wrap for autoradiography.

7. Simultaneous Isolation of DNA and RNA: CsCl Method

DNA and RNA were isolated simultaneously by an adaptation of the method described by Chirgwin et al. (1979). For this procedure, livers were excised and immediately frozen in liquid nitrogen. Tissue was either used immediately or stored at -85°C. Frozen tissue was ground to a fine powder in liquid nitrogen with a mortar and pestle. The frozen powder was then stirred into at least 24 volumes of 4.0 M guanidinium isothiocyanate, 0.5% sarcosyl, 1 M sodium citrate (pH 7.0), and 0.5% β-mercaptoethanol until a particle-free viscous liquid was produced. This was then layered onto a CsCl step gradient (3 ml 5.7 M CsCl, 2 ml 3.0 M CsCl; CsCl made in 0.1 M EDTA, treated overnight with 0.1% diethylpyrocarbonate, and autoclaved) and ultracentrifuged at 29,000 rpm for 22 hours at 20°C in a Beckman SW41 rotor. At the end of the centrifuge run, the upper guanidium-containing layer was removed and discarded.

A. DNA isolation

The DNA, which migrates to just below the 5.7 M/3.0 M CsCl interface, was removed with a wide-mouth pipette. RNase A (Sigma; preboiled 10 min) was added to the DNA-containing solution to a final concentration of 50 μg/ml and the DNA was dialyzed against 1 liter of 10 mM Tris/1 mM EDTA/0.1% SDS (pH 7.5) for one hour at room temperature. Proteinase K (BMB, Indianapolis, IN) was added to a final concentration of 50 μg/ml, the dialysis buffer was changed, and dialysis was continued for 1 hour. The DNA was then dialyzed against 2 liters of 10 mM Tris/1 mM EDTA (pH 7.5) at 4°C for 16-20 hours. An equal volume of ultra-pure phenol (equilibrated with 0.5 M Tris, pH 8.0 until the aqueous layer reached pH 7.6) was used to extract proteins, and this was followed by a wash with an equal volume of chloroform:3% isoamyl alcohol. The DNA was then precipitated with 0.5 volumes of 7.5 M NH_4OAc and 2 volumes of ethanol. After DNA was dissolved in TE (10 mM Tris/1 mM EDTA, pH 8.0), it was reprecipitated with NH_4OAc and ethanol and again dissolved in TE. DNA was quantitated by the A_{260} absorbance, and the A_{260}/A_{280} ratio routinely approached 1.8.

B. RNA isolation

After removal of the DNA fraction, the remaining CsCl solution was decanted by rapid inversion of the centrifuge tube, and the tube was allowed to drain for several minutes. A razor blade was used to cut the tube approximately 1.5 cm from the end, thus forming a "cup", the bottom of which contains the RNA pellet. The RNA was dissolved in diethyl pyrocarbonate-treated water (RNA from 1 g of liver in 500 μl water), and washed twice with an equal volume of chloroform:butanol (4:1). The phases were separated by centrifugation in a Brinkman microfuge at 14,000 rpm for 1 minute. The top, RNA-containing layer was removed to an RNase-free 15-ml Corex centrifuge tube. One-tenth volume 2.0 M potassium acetate (pH 5.2; DEPC-treated and autoclaved) and 2.5 volumes 100% ethanol were used to precipitate the RNA, which was then stored at -20°C.

8. Isolation of DNase I-Treated DNA

The methods of Burch and Weintraub (1983) were followed for preparation of nuclei, DNase I treatment, and DNA isolation. Preliminary studies identified 15°C as the optimum temperature for DNase I digestion as the control sample (no added DNase I) remains viscous after incubation at this temperature for 10 minutes.

Fresh mouse liver (approximately 1 g) was chopped finely in ice-cold SSCT (1X SSC, 10 mM Tris, pH 7.4). The liver pieces were washed several times with ice-cold SSCT to remove contaminating blood elements, and briefly (5 seconds) centrifuged at 1500 rpm in an IEC Centra-7R centrifuge at 4°C to pellet the tissue.

After decanting the supernatant, the liver was homogenized in 10 ml RSB (10 mM Tris, pH 7.4, 10 mM NaCl, and 3 mM $MgCl_2$) plus 0.5% Nonidet P-40 (NP-40) and 1 mM PMSF. Nuclei were pelleted by centrifugation at 1500 rpm for 10 minutes at 4°C (IEC Centra-7R centrifuge) and washed twice in RSB plus 0.5% NP-40. Nuclei from the livers of 5-6 mice were pooled in 7-ml RSB plus 0.1 mM $CaCl_2$. One milliliter aliquots of the nuclei suspension were added to tubes containing 0 (2 tubes), 28, 33, 38, 43, or 47 units DNase I. DNase I-treated samples were incubated at 15°C for 10 minutes; one control tube was incubated at 0°C, and one control tube was incubated at 15°C to assess intrinsic nuclease activity. The reaction was halted by the addition of an equal volume of stop buffer (1% SDS, 0.6 M NaCl, 20 mM Tris, pH 7.5, 10 mM EDTA, and 400 μg/ml proteinase K). Samples were incubated for 2 hours at 37°C and diluted to a volume of 4-5 ml for deproteination. A wash with an equal volume of phenol (neutral pH) was followed by a wash with an equal volume of chloroform:3% isoamyl alcohol; separation of the phases was accomplished by centrifugation at 400 x g for 15 minutes for the first wash and 10 minutes for the second wash. DNA was preciptiated by the addition of 0.5 volumes 7.5 M ammonium acetate and 2 volumes ethanol. The DNA was rinsed in 70% ethanol, allowed to dry briefly, and dissolved in 1 ml TE (10 mM Tris, pH 8.0, 1 mM EDTA). DNA concentration was assessed by absorbance at 260 nm.

To visualize the extent of DNase I digestion, 1 μg samples were diluted to a volume of 20 μl and 4 μl of 5X marker dye (15% Ficoll 400, 0.125% bromophenol blue, 0.125% xylene cyanode ff, and 5X TBE) was added. DNA was loaded onto a submerged 0.9% agarose baby gel in TBE (model H6, BRL) and electrophoresed for 2 hours at 30 V. The gel was stained with 1 μg/ml ethidium bromide for 15-30 minutes, destained overnight, and photographed as described in Section 3B.

9. Restriction Enzyme Digestion, Electrophoresis, and Southern Transfer of DNA to Gene Screen Plus

A. Restriction enzyme digestion

Restriction enzyme digestion of DNA was performed as described in Section 3A with the following modifications. Twenty microgram aliquots of DNA were digested with restriction enzyme in a reaction volume of 60 μl. A Ficoll-based marker dye was used (5X = 15% Ficoll 400, 0.125% bromophenol blue, 0.125% xylene cyanole ff, and 5X TBE).

Reaction buffers for Msp I and Hpa II were as described in Section 3A. For the other enzymes used, the reaction buffers (all supplied as a 10X concentrate by BRL) were as follows: Hha I, Hind III, and Pst I - 5 mM Tris (pH 8.0), 1 mM $MgCl_2$, and 5 mM NaCl; Eco R1 - 5 mM Tris (pH 8.0), 1 mM $MgCl_2$, and 10 mM NaCl. DNA was digested with 5 units Hha I per μg DNA, and 3 units Hind III, Pst I, or Eco R1 per μg DNA.

B. Agarose gel electrophoresis

Preparation of the agarose gel was performed as described in Section 3B with the following modifications. A 0.9% agarose gel (0.8% where indicated) in 300 ml TBE was prepared and poured in a gel support tray which had the ends taped to contain the agarose. After solidification and tape removal, the gel was placed in a H0/H1 electrophoresis unit and covered with TBE to a depth of approximately 5 mm. Samples were loaded into the wells using a Pipetman (Rainin). Electrophoresis, ethidium bromide staining, and photography were performed exactly was described in Section 3B.

C. Southern transfer

Transfer of DNA to Gene Screen Plus was performed as suggested by the manufacturer (NEN/DuPont). All gel soaks were performed with constant agitation on a Orbit Shaker (LabLine).

DNA was depurinated in 0.25 N HCl for 10 minutes and denatured in 0.4 N NaOH, 0.6 M NaCl for 30 minutes. The gel then was neutralized in 1.5 M NaCl, 0.5 M Tris (pH 7.5) for 30 minutes. The transfer was set up as described in Section 3C except that 5-6 pieces of 3 MM paper were used in place of blotting pads.

At the end of the transfer, the Gene Screen Plus membrane was peeled off the gel and briefly immersed in 0.4 N NaOH to denature the DNA; it was then neutralized by dipping in 0.2 M Tris, 2X SSC (pH 7.5). The membrane was allowed to air dry, and, in some cases, it was baked at 80°C for 2 hours in a vacuum oven.

10. Labelling of Probes with ^{32}P: Random Primers Method

Probes for Ha-ras, Ki-ras and myc were purchased from Oncor (Gaithersburg, MD). The Oncor Ha-ras probe differs from the BS-9 clone in that it is approximately 66% larger, extending about 300 base pairs further in the 3' direction than does the BS-9 clone. The 1.5 kb Pst I fragment of myc (Vennstrom _et_ _al_., 1981) and the pRSA 13 clone of serum albumin (Sargent _et_ _al_., 1981) were both utilized as inserts in pBR322. A random primers labelling kit was purchased from BRL. For each reaction, 25 ng of DNA was denatured by boiling for 5 minutes and immediately placed on ice. dATP, dGTP, and dTTP were added to a final concentration of 0.02 mM each, along with a 3.3X buffer concentrate (containing 18 A_{260} units/ml oligodeoxyribonucleotide hexamers, 0.67 M Hepes, 0.17 M Tris, 17 mM $MgCl_2$, 33 mM 2-mercaptoethanol, and 1.33 mg/ml BSA, pH 6.8), and GDW to 49 µl. Approximately 50 µCi of α-^{32}P dCTP (10 mCi/ml; 3000 Ci/mmol) was added and mixed briefly prior to the addition of 3 units of the Klenow fragment of DNA polymerase I. Labelling was performed at 25°C for one hour. The volume was increased to 100 µl by the addition of 38 µl STE and 12 µl of a bromophenol blue solution. Separation of newly labelled probe from unincorporated nucleotides was accomplished by spun column chromatography as described in Section 5.

11. Hybridization of DNA Affixed to Gene Screen Plus with ^{32}P-Labelled Probes

Prehybridization and hybridization were carried out in Scotch-pak (Kapak) heat-sealable bags placed in a 65°C water bath with constant agitation (as recommended by NEN/DuPont for Gene Screen Plus). Membranes were prehybridized for at least 15 min with approximately 50 $\mu l/cm^2$ of a solution containing 10% dextran sulfate, 1% SDS, and 1 M NaCl, preheated to 65°C. This solution was sometimes buffered with 50 mM Tris, pH 7.5. Denatured probe ($1 x 10^5$ cpm per ml hybridization solution; prepared by random primers method) and sonicated salmon sperm DNA (100 µg/ml) in 500 µl STE were then added and mixed thoroughly with the prehybridization solution by pulling the bag back and forth over an edge. Hybridization was carried out for approximately 16 hours. Membranes were washed twice with 2X SSC for 5 minutes at room temperature, twice with 2X SSC/1% SDS for 15 minutes each at 65°C, and twice with 0.1X SSC for 15 min each at room temperature (250 ml solution per wash), all with constant agitation. Membranes were then blotted with 3 mm paper (Whatman) and wrapped in Handi-Wrap (Dow Chemical Co.) in such a manner as to prevent drying.

Membranes that were to be rehybridized were stripped of probe after autoadiography (Section 15) by pouring boiling 10 mM Tris/1 mM EDTA/1% SDS, pH 7.5 over them and agitating until cool; this procedure was then repeated. Autoradiography was performed on the stripped membranes to ensure complete probe removal prior to rehybridization, which was performed as described above.

12. Electrophoresis and Northern Transfer of RNA

A. Preparation of RNA

Water used in the preparation of RNA was treated with 0.1% DEPC (diethyl pyrocarbonate), allowed to sit overnight and autoclaved. Concentrated running buffer was autoclaved before use. RNA preparation and electrophoresis was performed according to the method suggested by NEN/DuPont. RNA was pelleted by centrifugation at 17,600 x g for 20 minutes at -10°C. Pellets were washed with 1 ml absolute ethanol at room temperature and recentrifuged as above. RNA was dried under a stream of ethanol and dissolved in 100-250 µl water. The concentration of RNA was quantitated by absorbance at 260 nm (1 A_{260} unit = 40 µg RNA/ml). RNA was then diluted to a concentration of 4.4 µg/µl. A 3.7-µl aliquot was removed to an Eppendorf tube and stored at -85°C until assessment of RNA integrity was performed (Section 14).

A 4.5-µl aliquot of RNA (20 µg) was denatured in a solution of 1X MOPS (5X MOPS = 0.2 M morpholinopropanesulfonic acid, 50 mM sodium acetate and 5 mM EDTA, pH 7.0), 2.2 M formaldehyde and 50% deionized formamide in a volume of 20 µl. Samples were incubated at 60°C for 10 minutes, and 5 µl of sterile loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue and 0.4% xylene cyanode ff) was added.

B. Electrophoresis and Northern transfer

RNA samples were loaded onto a 1.0% agarose gel prepared in 2.2 M formaldehyde and 1X MOPS. Gels were submerged in 1X MOPS, and electrophoresis was carried out at 30 V for 18-20 hours.

Prior to transfer of RNA to Gene Screen Plus, the gel was rinsed 5 times in an equal volume of glass distilled water (1 minute per wash). The transfer was set up exactly as described in Section 3C, except that blotting pods were omitted; transfer was carried out for approximately 22 hours. After the membrane was removed from the gel, it was rinsed in 2X SSPE (0.3 M NaCl, 20 mM NaH_2PO_4, and 2 mM EDTA, pH 7.4), allowed to air dry, and baked at 80°C in a vacuum oven.

13. Hybridization of Northern Blots

Northern blots were hybridized in heat-sealable plastic bags (Scotch-pak, Kapak Corp.) according to the method described by NEN/DuPont. Membranes were prehybridized in a minimum volume (approximately 50 µl/cm^2) of prehybridization solution (5X SSPE, 50% deionized formamide, 5X Denhardt's solution, 10% dextran sulfate and 1% SDS) for 2-4 hours in a 42°C water bath with constant agitation. To hybridize, 4x10^5 cpm/ml denatured, ^{32}P-labelled probe (random primers method) was added and the bag was resealed. Mixing of the probe into the prehybridization solution was accomplished by pulling the bag back and forth over an edge several times. Hybridization was carried out for 16-18 hours at 42°C with constant agitation.

Membranes were washed twice with 250 ml 2X SSPE at room temperature, twice with 400 ml 2X SSPE/2% SDS at 65°C, and twice with 250 ml 0.1X SSPE at room temperature; all washes were 15 minutes in duration. After blotting on 3 MM paper, membranes were wrapped in Handi-Wrap in preparation for autoradiography (Section 15).

The resulting autoradiograph was scanned with an LKB UltroScan XL densitometer to assess the relative degree of hybridization of probe to RNA samples. The resulting peaks were cut out and weighed to quantitate this relationship.

14. Assessment of RNA Integrity

RNA samples were thawed and denatured in a mixture of 50% DMSO, 0.01 M NaH_2PO_4 (pH 7.0), and 6.8% deionized glyoxal at 50°C for 1 hour in a volume of 16 µl as described in Maniatis (1982). Four microliters of RNA marker dye were added, and a 13 µl (13 µg) aliquot of each sample was loaded onto a 1% agarose gel prepared and submerged in 0.01 M NaH_2PO_4 (pH 7.0). Electrophoresis was carried out at 30 V for 2 hours; running buffer was changed every 30 minutes. Gels were stained with 1 µg/ml ethidium bromide for 15 minutes and destained overnight in glass distilled water. Photography of the gel was performed as described in Section 3B.

15. Autoradiography

The Handi-Wrap-wrapped, hybridized membrane was taped to a Cronex Lightning Plus intensifying screen in a film holder. In complete darkness, a sheet of X-OMAT AR film was placed over the membrane, and another intensifying screen was placed over the film before the film holder was closed. The film holder was then wrapped in aluminum foil and clipped between two clipboards. Autoradiography was carried out at -85°C for 1-7 days.

Prior to film development, the autoradiographic apparatus was allowed to equilibrate to room temprature. In complete darkness, the film was removed from the film holder, and immersed in GBX developer (Kodak) for 5-6 minutes. The film was then rinsed in water for one minute, and immersed in GBX fixer (Kodak) for 5-6 minutes before being rinsed in water and allowed to dry.

RESULTS

1. Methylation State of the Ha-ras, Ki-ras, and myc Oncogenes in B6C3F1, C3H/He, and C57BL/6 Mouse Liver

The degree of methylation of Ha-ras in male B6C3F1, male C3H/He, and female C57BL/6 mouse liver was assessed by comparison of the restriction patterns obtained following digestion with either Msp I or Hpa II. If the patterns were similar the gene examined was considered relatively hypomethylated. If the patterns were dissimilar (resistant to digestion by Hpa II), the gene was considered relatively hypermethylated. In some cases, an intermediate degree of methylation was noted.

The methylation state of the Ha-ras oncogene was assessed using two different probes. The BS-9 clone, described by Ellis *et al.* (1981), covers approximately 450 base pairs toward the 5' end of the Ha-ras gene. The Oncor clone is approximately 66% larger, extending approximately 300 base pairs in the 3' direction.

The results of hybridizing Msp I- and Hpa II-digested DNA with the BS-9 probe for the Ha-ras oncogene are shown in Figure 3. When B6C3F1 mouse hepatic DNA is digested with Msp I (lane 1), a number of bands are produced, ranging in size from < 1.9 kilbases (kb) to 8.8 kb. Hpa II digestion of the same DNA (lane 2) produces some fragments of comparable size (i.e., 1.9 kb, 2.7 kb, 8.8 kb). In addition, hybridization is clearly evident at a molecular weight of 23.1 kb. These results indicate that the Ha-ras oncogene is methylated to an intermediate degree in the liver of the B6C3F1 mouse. Digestion of C3H/He hepatic DNA with Msp I (lane 3) and Hpa II (lane 4) produces very similar restriction maps, with

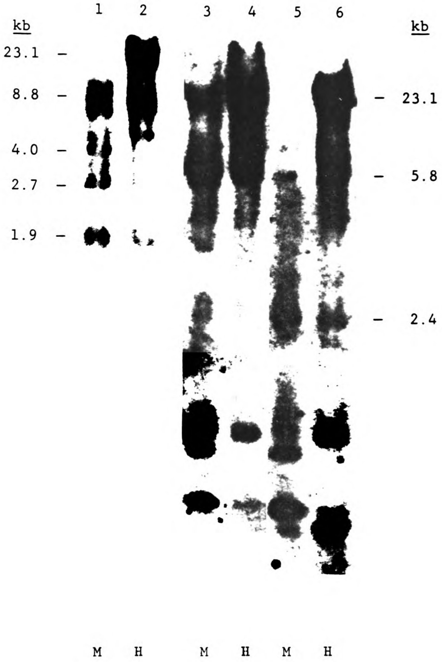

Figure 3. Methylation state of the Ha-ras oncogene in B6C3F1, C3H/He, and C57BL/6 mouse liver using the BS-9 clone. DNA was isolated by the method of Marmur from B6C3F1 (lanes 1 and 2), C3H/He (lanes 3 and 4), C57BL/6 (lanes 5 and 6) mouse liver and digested with Msp I (M) or Hpa II (H). The resulting fragments were electrophoresed and transferred to nitrocellulose paper. The DNA was then hybridized to the ^{32}P-labelled BS-9 probe for the Ha-ras oncogene, and regions of homology were visualized by autoradiography.

homologous bands occurring at 23.1 kb, 5.8 kb, and two sizes < 2.4 kb. It is thus evident that the Ha-ras oncogene is hypomethylated in the liver of the C3H/He mouse as compared to the B6C3F1 mouse. In contrast, hepatic DNA from the C57BL/6 mouse shows a different restriction pattern when digested by Msp I (lane 5) as compared to Hpa II (lane 6). Msp I digestion produces three major bands of a relatively low molecular weight, whereas Hpa II digestion produces several bands, two at relatively high molecular weights (23.1 kb and 5.8 kb), and only one corresponding to a band produced by Msp I digestion (2.4 kb). Because Msp I cleaves the Ha-ras oncogene in C57BL/6 liver DNA to much greater extent than does Hpa II, it is concluded that the Ha-ras oncogene is hypermethylated in the liver of this mouse.

The results of restriction enzyme analysis performed on a number of animals showed a consistent trend (Table 1). Ha-ras was either hypomethylated or methylated to an intermediate degree in 4/4 B6C3F1 mice and 5/7 C3H/He mice. In C57BL/6 mice Ha-ras was hypermethylated in 4 animals and methylated to an intermediate degree in 2 animals.

The results of experiments using the Oncor probe for methylation assessment are presented in Figure 4. In young adult animals, there is one difference in the methylation state of Ha-ras among the three strains. An additional 15 kb band is apparent in Msp I-digested C57BL/6 DNA (lanes 9 and 11; see arrow), but not in Msp I-digested DNA from B6C3F1 and C3H/He mice. This band is probably due to the relatively rare occurrence of a methyl group on the external cytosine residue of a 5'-CCGG-3' recognition site, thus preventing Msp I from completely cleaving this fragment. Because male mice of the B6C3F1 and C3H/He strains and female C57BL/6 mice were used for this experiment, it is possible that the site-specific methylation of the Ha-ras gene seen in C57BL/6 mouse liver results from sex differences. To address this possibility, hepatic DNA from female

TABLE 1

METHYLATION STATE OF THE Ha-ras ONCOGENE IN THREE MOUSE STRAINS

	Hypo-methylation	Intermediate Methylation	Hyper-methylation
B6C3F1	2*	2	0
C3H/He	2	3	2
C57BL/6	0	2	4

*Numbers refer to the number of individual animals displaying the indicated level of methylation.

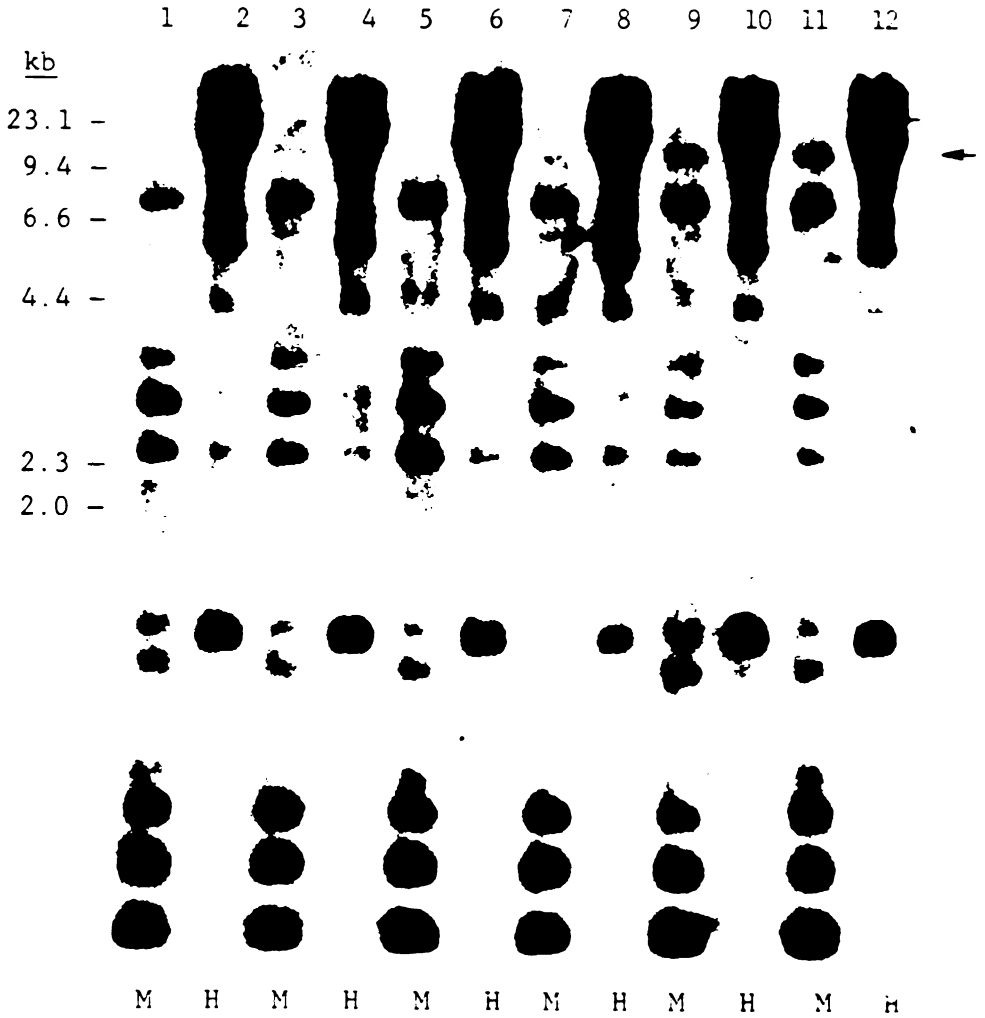

Figure 4. Methylation pattern of the Ha-ras oncogene in control mice. Hepatic DNA was isolated from male B6C3F1 (lanes 1-4), male C3H/He (lanes 5-8), and female C57BL/6 (lanes 9-12) mice on a CsCl gradient, digested with Msp I (M) and Hpa II (H), and electrophoresed in a 0.9% agarose gel. DNA was then transferred to Gene Screen Plus, hybridized to a ^{32}P-labelled probe for the Ha-ras oncogene (Oncor), and visualized by autoradiography. The arrow points to a 15 kb band present in Msp I-digested C57BL/6 DNA but absent in B6C3F1 and C3H/He DNA.

B6C3F1, female C3H/He, and male C57BL/6 (opposite sex) was subjected to restriction enzyme assessment of Ha-ras methylation state. As seen in Figure 5, a 15-kb band was observed in Msp I-digested C57BL/6 DNA, but not in Msp I-digested DNA from the other two strains, when probed with Ha-ras (see arrow). This result confirms the finding that the Ha-ras oncogene is hypomethylated in a site-specific manner in B6C3F1 and C3H/He mouse liver as compared to C57BL/6 mouse liver.

In other respects, the Ha-ras oncogene appears to be relatively hypermethylated in liver of mice of all three strains (Figures 4 and 5). This conclusion is based on the fact that Msp I digestion (M) is extensive, producing a number of bands ranging from a few hundred base pairs to ~9 kb, whereas the bulk of hybridization to Hpa II-digested DNA (H) occurs at very large ($\geq$ 23 kb) fragments, with 2 bands occurring at smaller molecular weights.

The importance of complete transfer of high molecular weight DNA in the determination of gene methylation state by Msp I/Hpa II analysis is illustrated in Figure 6. Although DNA samples used in Figures 4 and 6 were digested, electrophoresed, and transferred to nylon membranes concurrently, the depurination step of gel processing was omitted in the experiment shown in Figure 6. A high degree of hybridization at the region $\geq$ 23 kb is seen in Hpa II-digested DNA that underwent depurination (Figure 4, even-numbered lanes), whereas little hybridization is seen in this region when depurination was omitted (Figure 6, even-numbered lanes). It must be noted that the 15 kb band seen in Msp I-digested C57BL/6 DNA is not apparent in Figure 6. Thus, in order to assess accurately the methylation state of genes using restriction enzyme analysis, efficient transfer of large DNA fragments must be assured through inclusion of a depurination step.

The methylation state of the Ki-ras (Figure 7) and myc (Figure 8) oncogenes was also assessed in the three mouse strains. Comparison of the restriction

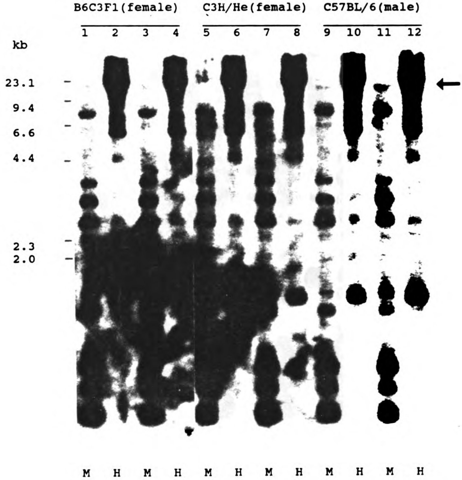

Figure 5. Methylation pattern of the Ha-ras oncogene in mice of the opposite sex. Hepatic DNA from female B6C3F1 (lanes 1-4), female C3H/He (lanes 5-8) and male C57BL/6 (lanes 9-12) was isolated and treated as described in Figure 4. The arrow points to a 15 kb band present in Msp I-digested C57BL/6 DNA but absent in B6C3F1 and C3H/He DNA.

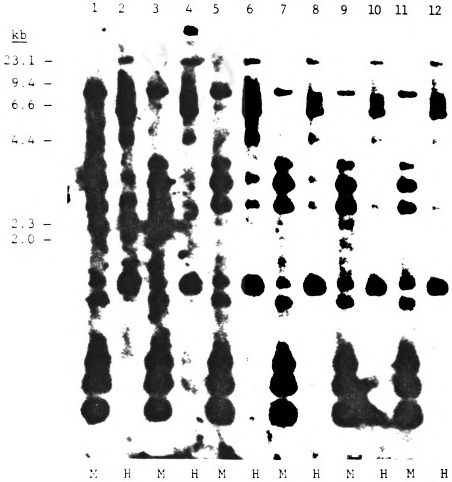

Figure 6. Effect of the omission of the depurination step on the transfer of large DNA fragments. DNA samples were Msp I (M)- or Hpa II (H)-digested, electrophoresed, and transferred to Gene Screen Plus concurrently with the samples shown in Figure 4, except that the HCl soak prior to Southern transfer was omitted. DNA was derived from male B6C3F1 (lanes 1-4), male C3H/He (lanes 5-8), and female C57BL/6 (lanes 9-12) mouse liver.

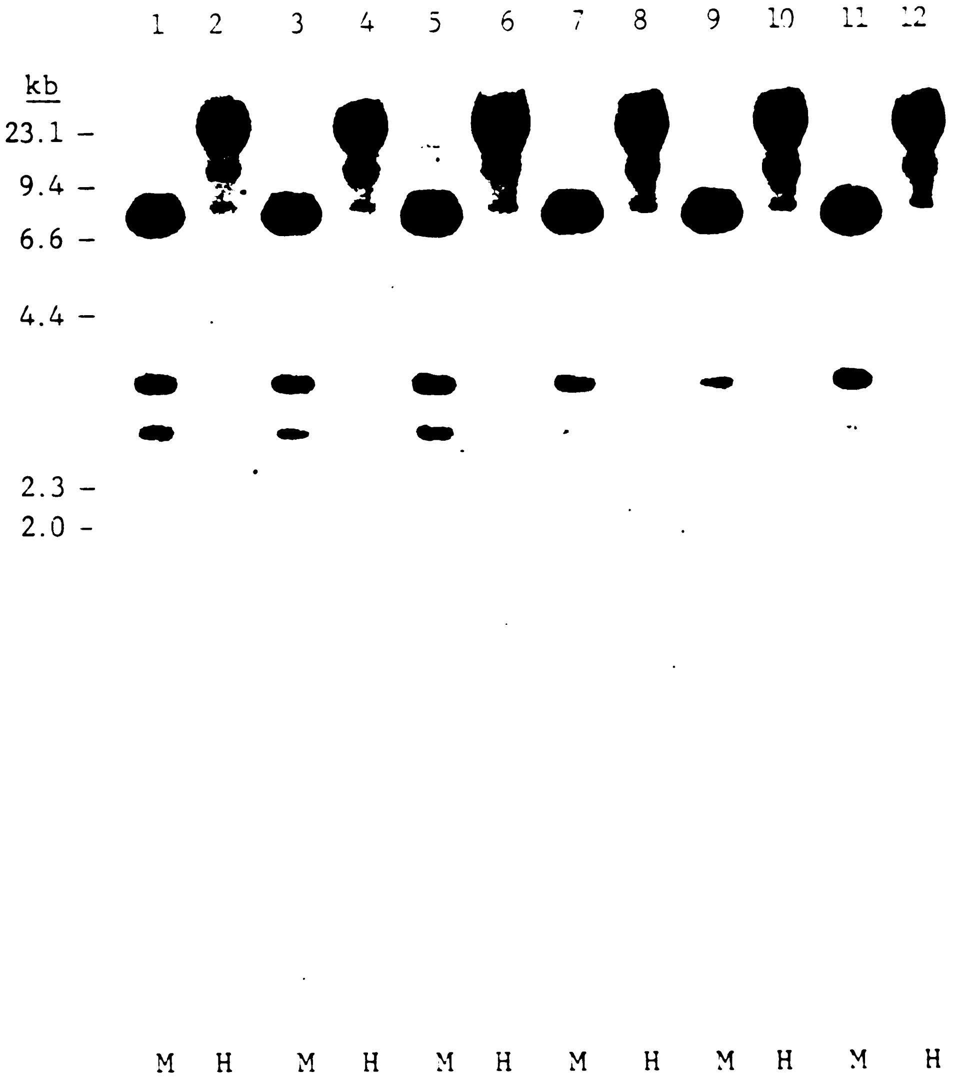

Figure 7. Methylation status of the Ki-ras oncogene in control mice. The membrane shown in Figure 2 was stripped of the Ha-ras probe and rehybridized to ^{32}P-labelled probe for Ki-ras. Autoradiography shows the methylation pattern of the Ki-ras oncogene to be similar in all three mouse strains.

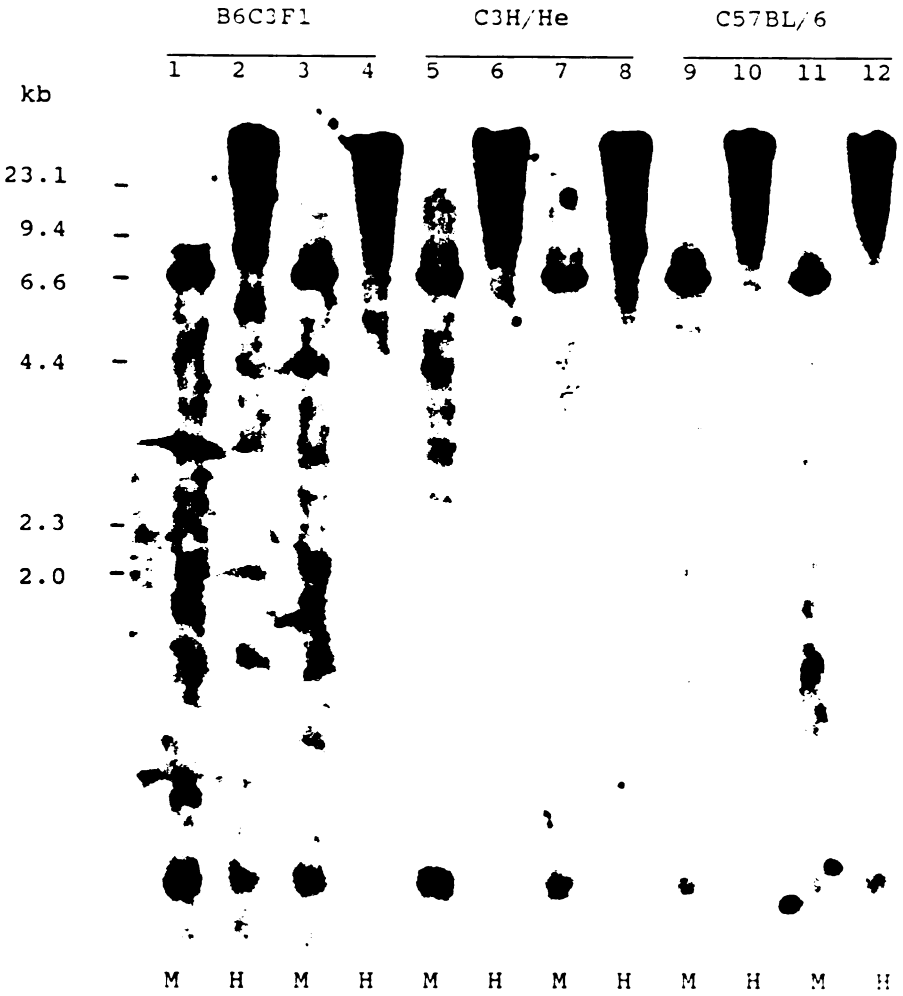

Figure 8. Methylation status of the myc oncogene in control mice. The membrane shown in Figure 7 was stripped of the Ki-ras probe and, rehybridized to a ^{32}P-labelled probe (Oncor) for myc. Autoradiography shows the methylation pattern of myc to be similar in all three mouse strains.

patterns produced by Msp I digestion of male B6C3F1, male C3H, and female C57BL/6 hepatic DNA shows no differences for Ki-ras (Figure 7, lanes 1, 3, 5, 7, 9, and 11) or myc (Figure 8, lanes 1, 3, 5, 7, 9, and 11); the same is true for Hpa II-digested DNA hybridized to Ki-ras (Figure 7, lanes 2, 4, 6, 8, 10, and 12) and myc (Figure 8, lanes 2, 4, 6, 8, 10, and 12). These results indicate that no differences exist between the three mouse strains with regard to the methylation state of the Ki-ras and myc oncogenes. Furthermore, the increased extent of digestion by Msp I versus Hpa II seen in both figures indicates that Ki-ras and myc are hypermethylated in male B6C3F1, male C3H/He, and female C57BL/6 mouse liver. Msp I/Hpa II analysis using hepatic DNA from mice of the opposite sex mice (female B6C3F1, female C3H/He, and male C57BL/6) produced an identical result for Ki-ras (Figure 9) and myc (Figure 10), indicating that these two oncogenes are hypermethylated to a similar extent in the liver of mice of the opposite sex. Within each strain, Msp I and Hpa II restriction patterns were observed to be very consistent when probed with Ha-ras; this was also true for Ki-ras and myc.

The serum albumin gene is known to be expressed in normal liver. Therefore, this gene was used as a positive control for restriction enzyme analysis of gene methylation state. Figure 11 shows that Msp I and Hpa II digestion of B6C3F1 (lanes 1-4), C3H/He (lanes 5-8) and C57BL/6 (lanes 9-12) DNA produces very similar restriction patterns when probed with the serum albumin clone pRSA 13 as evidenced by a major band at 6.0 kb in Msp I-digested samples (lanes 1, 3, 5, 7, 9, and 11) and 6.4 kb in Hpa II-digested samples (lanes 2, 4, 6, 8, 10, and 12). A similar result was seen in DNA from mice of the opposite sex (data not shown). These results not only indicate the expected result of hypomethylation of this gene, but also provide evidence that the conditions of restriction enzyme reactions were appropriate for complete digestion.

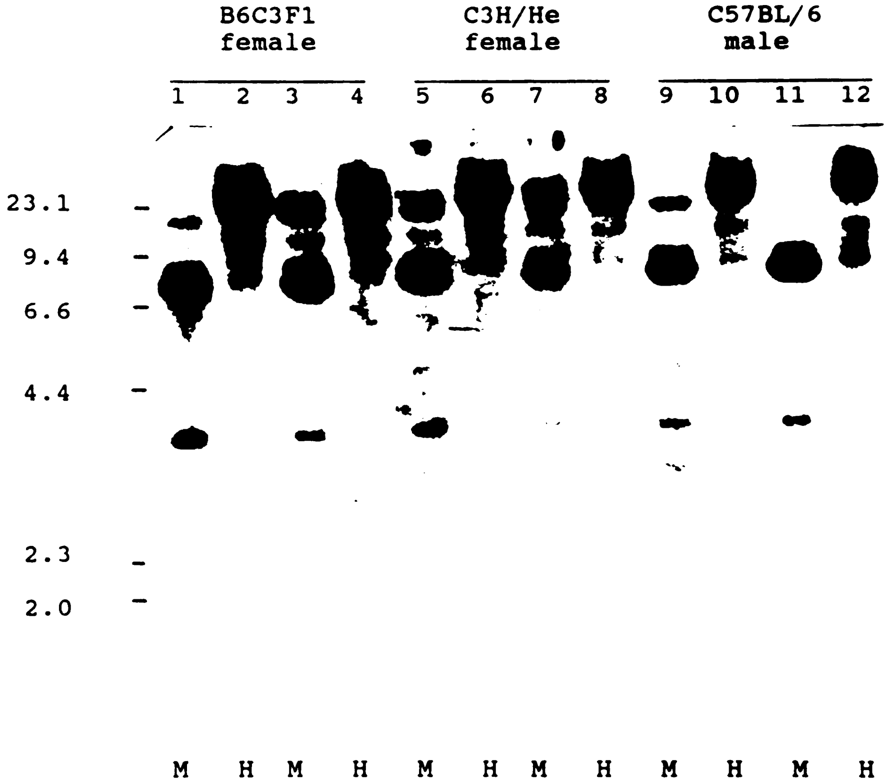

Figure 9. Methylation status of the Ki-ras oncogene in mice of the opposite sex. The membrane shown in Figure 5 was stripped of the Ha-ras probe and rehybridized to a ^{32}P-labelled probe for Ki-ras.

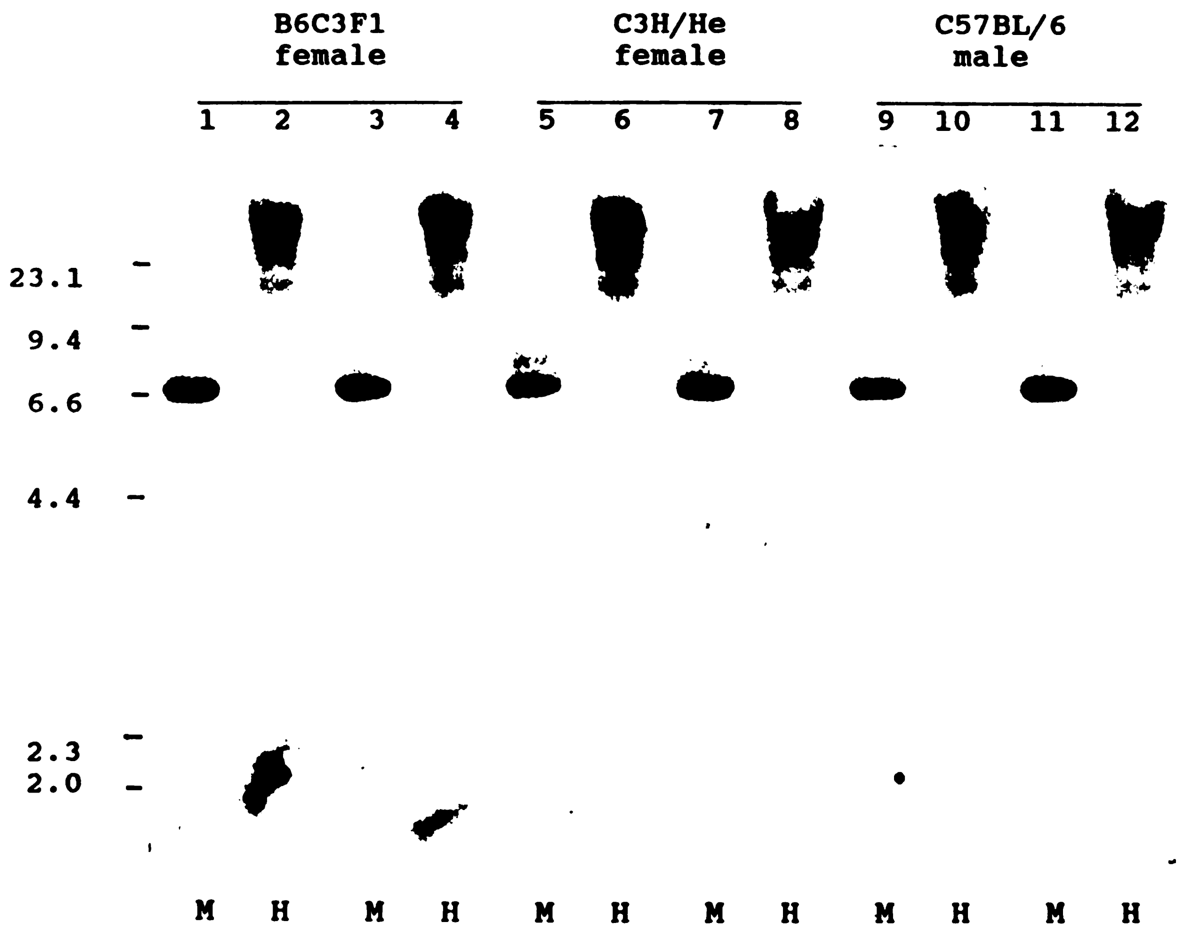

Figure 10. Methylation status of the myc oncogene in mice of the opposite sex. The membrane shown in Figure 9 was stripped of the Ki-ras probe and rehybridized to a ^{32}P-labelled probe for myc (Pst I fragment).

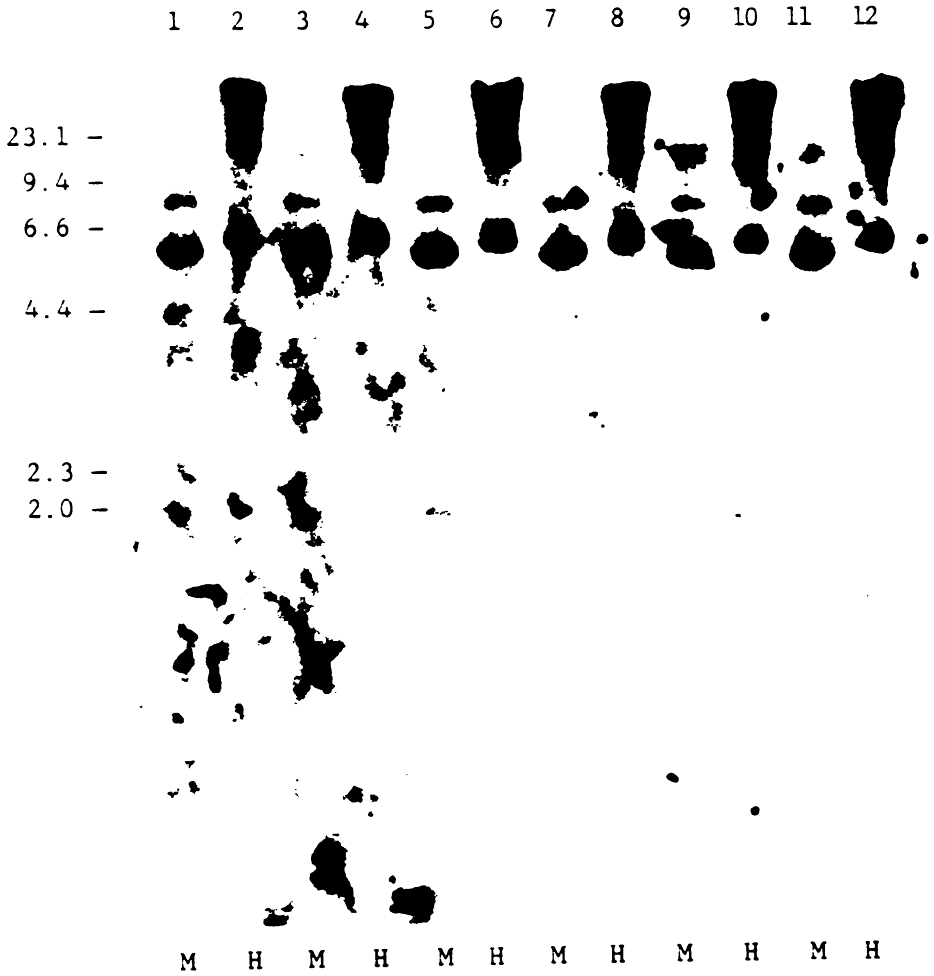

Figure 11. Methylation status of the serum albumin gene in control mice. The membrane shown in Figure 8 was stripped of the myc probe and rehybridized to a ^{32}P-labelled probe for serum albumin.

2. Oncogene Methylation State in B6C3F1 Mouse Liver Tumors

Figure 12 shows the results of hybridizing the probe for Ha-ras to benzidine-induced tumor (lanes 3, 4, 7, 8) and adjacent non-tumor (lanes 1, 2, 5, 6) DNA that has been digested with Msp I and Hpa II. A striking difference in the restriction pattern is obvious between Hpa II-digested tumor and non-tumor DNA. Hpa II digestion of tumor DNA produced intense bands at 4.4, 3.2, and 2.6 kb (lanes 4 and 8; see arrows) which are not apparent in non-tumor DNA (lanes 2 and 6). Furthermore, the smaller two of these three bands correspond to bands produced by Msp I digestion of tumor and non-tumor DNA, indicating that these bands result from a loss of methylation at 5'-C$\overset{\text{Me}}{\text{C}}$GG-3' sites. This result was observed in all 4 tumor/non-tumor pairs of samples examined from the benzidine-treated group of mice. Note that DNA derived from adjacent non-tumor liver tissues (lanes 1, 2, 5, and 6) produces restriction patterns nearly identical to those seen in control (young adult) animals (see Figure 4, lanes 1-4). Because Southern transfer of DNA from the benzidine treatment group was carried out without depurination, this experiment was repeated for Ha-ras with inclusion of the HCl treatment (Figure 13). Again, bands are seen in Hpa II-digested tumor DNA (lanes 4 and 8) which are not apparent in non-tumor DNA (lanes 2 and 6). Therefore, the results of experiments presented in Figures 12 and 13 are qualitatively similar, despite the lack of high molecular weight DNA transferred in the former.

A similar outcome for Ha-ras was produced by Msp I/Hpa II analysis of tumor DNA from the other three treatment groups. DNA derived from young adult male B6C3F1 mouse liver was used for controls in these experiments. The restriction patterns produced by Msp I and Hpa II digestion of control DNA were essentially identical to those seen in Figure 4 (lanes 1-4) in all cases. Hpa II digestion of phenobarbital-induced tumor DNA (Figure 14, lanes 4 and 6), chloroform-induced tumor DNA (Figure 14, lanes 8, 10, 12, and 14), and

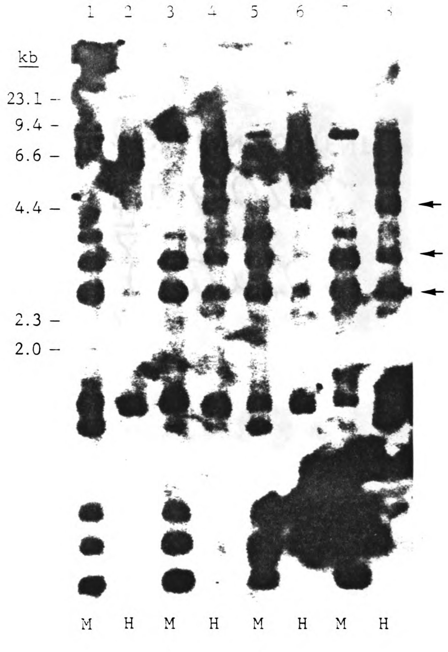

Figure 12. Methylation status of the Ha-ras oncogene in benzidine-induced hepatic tumors and adjacent non-tumor tissue. DNA was isolated from non-tumor hepatic tissue (lanes 1, 2, 5 and 6) or adjacent tumors (lanes 3, 4, 7 and 8) on a CsCl gradient and digested with Msp I (M) or Hpa II (H). Electrophoresis and Southern transfer to Gene Screen Plus were performed as described in Methods, except the depurination step was omitted. Lanes 1-4 represent DNA isolated from one animal, and lanes 5-8 represent DNA isolated from a second animal. Note the increased intensity of the bands marked by arrows in the Hpa I-digested tumor DNA (lanes 4 and 8) as compared to non-tumor DNA (lanes 2 and 6).

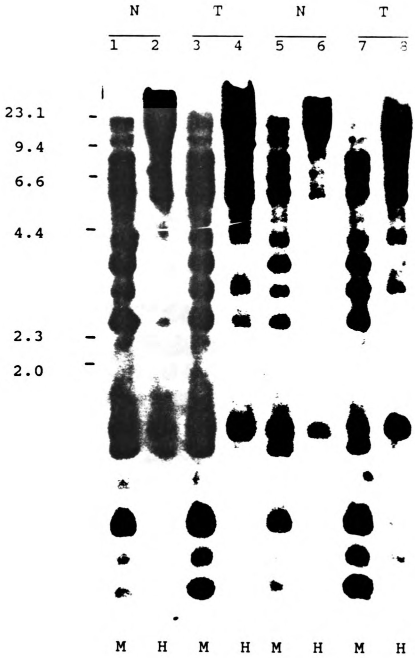

Figure 13. Lack of effect of depurination on the methylation assessment of Ha-ras in benzidine-induced tumor and non-tumor tissue. Tumor (T) and non-tumor (N)-derived DNA was digested with Msp I (M) or Hpa II (H), electrophoresed and transferred to Gene Screen Plus as described in Methods with inclusion of the depurinaton step. Note that extra bands are again observed in Hpa II-digested tumor DNA (lanes 4 and 8) as compared to non-tumor DNA (lanes 2 and 6).

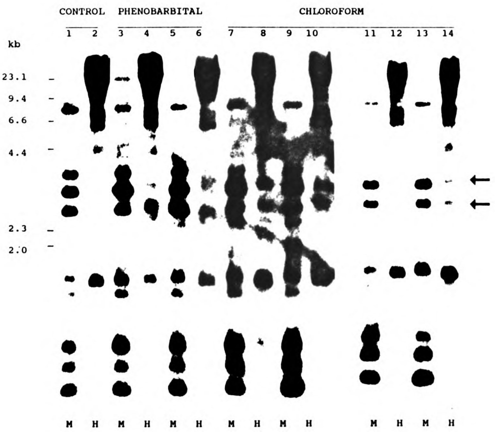

Figure 14. Methylation status of the Ha-ras oncogene in phenobarbital- and chloroform-induced hepatic tumors. DNA from phenobarbital-induced (lanes 3-6) and chloroform-induced (lanes 7-14) tumors was isolated and treated as described in Figure 4. Arrows point to bands observed in Hpa II-digested tumor DNA (lanes 4, 6, 8, 10, 12, and 14) which are not present in Hpa II-digested control DNA (lane 2).

spontaneous tumor DNA (Figure 15, lanes 4, 6, 8, and 10) produces additional bands at 4.4, 3.2, and 2.6 kb which are not seen in Hpa II-digested control DNA (Figures 14 and 15, lane 2). The appearance of these bands indicate that the Ha-ras oncogene is hypomethylated in all tumors examined, regardless of treatment group.

Methylation assessment of the Ki-ras oncogene in tissue from the benzidine group is presented in Figure 16. A 8.5 kb band is obvious in Hpa II-digested tumor DNA (lanes 4 and 8), whereas a band of this size is not readily apparent in non-tumor DNA (lanes 2 and 6). This band corresponds to a band produced by Msp I digestion of 5'-C$\overset{Me}{\mathrm{C}}$ GG-3' sites. Two out of four tumor/non-tumor pairs of samples analyzed from the benzidine-treated group showed this result. It thus appears that the Ki-ras oncogene is sometimes hypomethylated in benzidine-induced tumor DNA.

Experiments assessing the methylation state of Ki-ras in tumors from the other three treatment groups produced similar results. Hpa II-digested DNA from phenobarbital-induced tumors (Figure 17, lanes 4 and 6), chloroform-induced tumors (Figure 17, lanes 8, 10, 12, and 14) sometimes produces extra bands or bands of increased intensity as compared to control (lane 2) at 6.0 (lane 10), 5.0 (lanes 12 and 14) and 4.0 kb (lanes 4, 8, 10, 12, and 14). Spontaneous tumor DNA (Figure 18) digested with Hpa II sometimes produces a band of increased intensity at 8.5 kb (lane 8) and extra bands at 5.0 (lanes 4 and 8) and 3.0 kb (lanes 4, 6, 8, and 10). These results indicate that the Ki-ras oncogene is sometimes hypomethylated in tumors from all four treatment groups, but this phenomenon has a lesser degree of commonality than does hypomethylation of Ha-ras.

Because cell proliferation is a component of tumor development, it is important to determine whether or not the Ha- and Ki-ras oncogenes become hypomethylated during compensatory hyperplasia following partial hepatectomy.

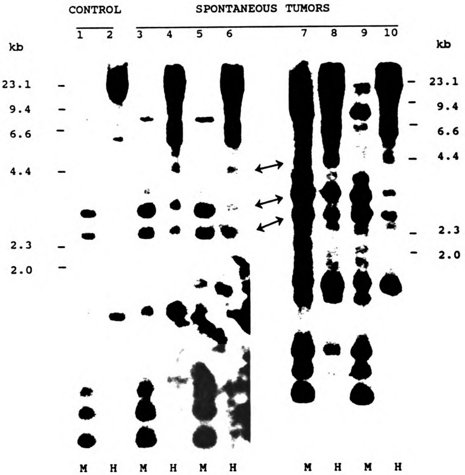

Figure 15. Methylation status of the Ha-ras oncogene in spontaneous hepatic tumors. DNA from spontaneous tumors was isolated and treated as described in Figure 4. Arrows point to bands observed in Hpa II-digested tumor DNA (lanes 2, 4, 6, 8, and 10) which are not present in Hpa II-digested control DNA (lane 2).

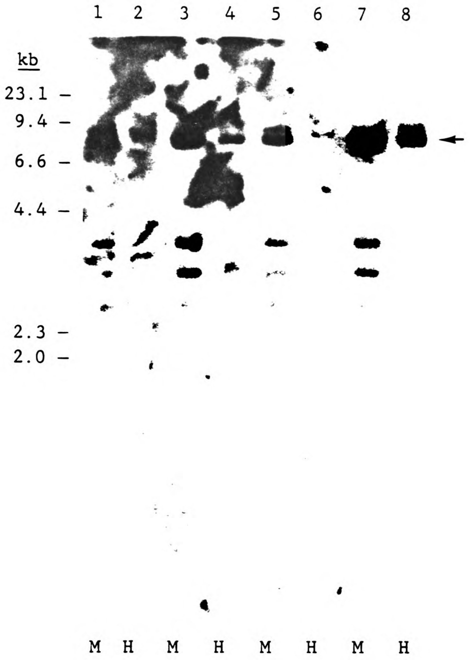

Figure 16. Methylation status of the Ki-ras oncogene in benzidine-induced hepatic tumors and adjacent non-tumor tissue. The membrane shown in Figure 12 was stripped of the Ha-ras probe and rehybridized with a ^{32}P-labelled probe for Ki-ras. The arrow shows a more intense band in Hpa II-digested tumor DNA (lanes 4 and 8) as compared to non-tumor DNA (lanes 2 and 6).

Figure 17. Methylation status of the Ki-ras oncogene in phenobarbital- and chloroform-induced hepatic tumors. DNA from phenobarbital-induced (lanes 3-6) and chloroform-induced (lanes 7-14) hepatic tumors was treated as described in Figure 4 except hybridization was to a ^{32}P-labelled probe for Ki-ras. The arrows point to bands whose intensity is greater in Hpa II-digested tumor DNA (lanes 4, 6, 8, 10, 12, and 14) than in control DNA (lane 2).

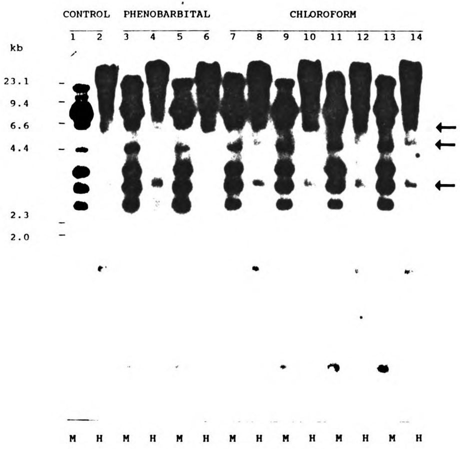
CONTROL
PHENOBARBITAL
CHLOROFORM
1 2 3 4 5 6 7 8 9 10 11 12 13 14
kb
23.1
9.4
6.6
4.4
2.3
2.0
M H M H M H M H M H M H M H

Figure 17

Figure 18. Methylation status of the Ki-ras oncogene in spontaneous hepatic tumors. DNA from spontaneous tumors was treated as described in Figure 4, except that a ^{32}P-labelled probe for Ki-ras was used for hybridization. The arrows point to bands whose intensity is greater in Hpa II-digested tumor DNA (lanes 4, 6, 8, and 10) than in Hpa II-digested control DNA (lane 2).

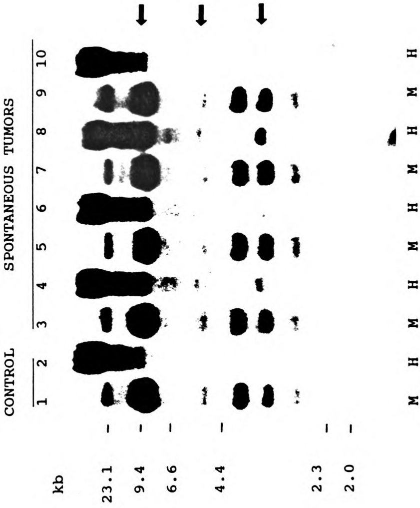
kb
23.1
9.4
6.6
4.4
2.3
2.0
CONTROL
SPONTANEOUS TUMORS
1
2
3
4
5
6
7
8
9
10
M
H
M
H
M
H
M
H
M
H

Figure 18

Figures 19 and 20 show the result of methylation analysis of Ha-ras and Ki-ras, respectively, 24 hours post-2/3 hepatectomy. Comparison of Hpa II-digested control DNA (lane 2, each figure) with Hpa II-digested DNA from proliferating tissue (lanes 4, 6, 8, and 10, each figure) reveals that the extra bands or bands of increased intensity seen in tumor DNA are not apparent in Hpa II-digested DNA from partially hepatectomized animals. These results indicate that the hypomethylation observed in tumor tissue is not due to cell proliferation per se. Thus, it can be concluded that the Ha-ras and Ki-ras oncogenes are often markedly less methylated in liver tumors than in non-tumor tissue or during compensatory hyperplasia following partial hepatectomy.

The methylation status of the myc oncogene in benzidine-induced tumors and non-tumor tissue was assessed and the results are presented in Figure 21. No differences were discerned in the banding pattern between non-tumor tissue (lanes 1, 2, 5, and 6) and tumor tissue (lanes 3, 4, 7, and 8). This result indicates that the myc oncogene is methylated to a similar degree in both normal and tumor tissue. However, hybridization of the myc probe to tumor-derived DNA was more intense than was hybridization to the non-tumor DNA, as evidenced by the darker bands in lanes 3 and 7 as opposed to the bands in lanes 1 and 5. This result may indicate that the myc oncogene is amplified in tumor DNA. As discussed in the Introduction, increased transcription of myc is often due to amplification.

The methylation state of myc was also assessed in DNA derived from phenobarbital-induced (Figure 22, lanes 3-6), chloroform-induced (Figure 22, lanes 7-14), and spontaneous (Figure 23, lanes 3-10) tumors. No difference was discerned in the restriction patterns of myc in tumors compared to control DNA (lanes 1 and 2, each figure), indicating that the methylation state is not altered in tumors. However, similar to the results seen in Figure 21, the degree of

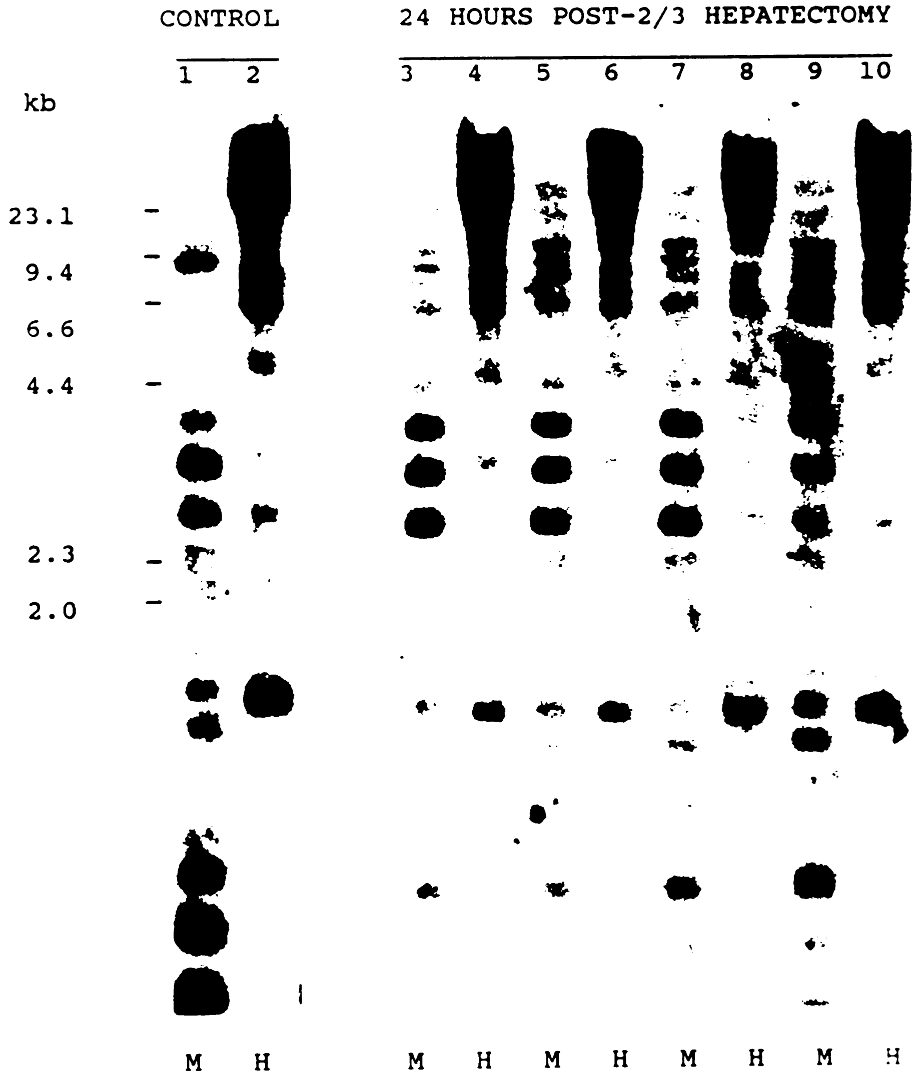

Figure 19. Methylation status of the Ha-ras oncogene following partial hepatectomy. Mice were sacrificed 24 hours following 2/3 hepatectomy. Hepatic DNA was isolated and treated as described in Figure 4. Note that Hpa II-digested DNA from partially heptectomized animals (lanes 4, 6, 8, and 10) in the region of 2.3 to 4.4 kb shows no difference as compared to Hpa II-digested control DNA (lane 2).

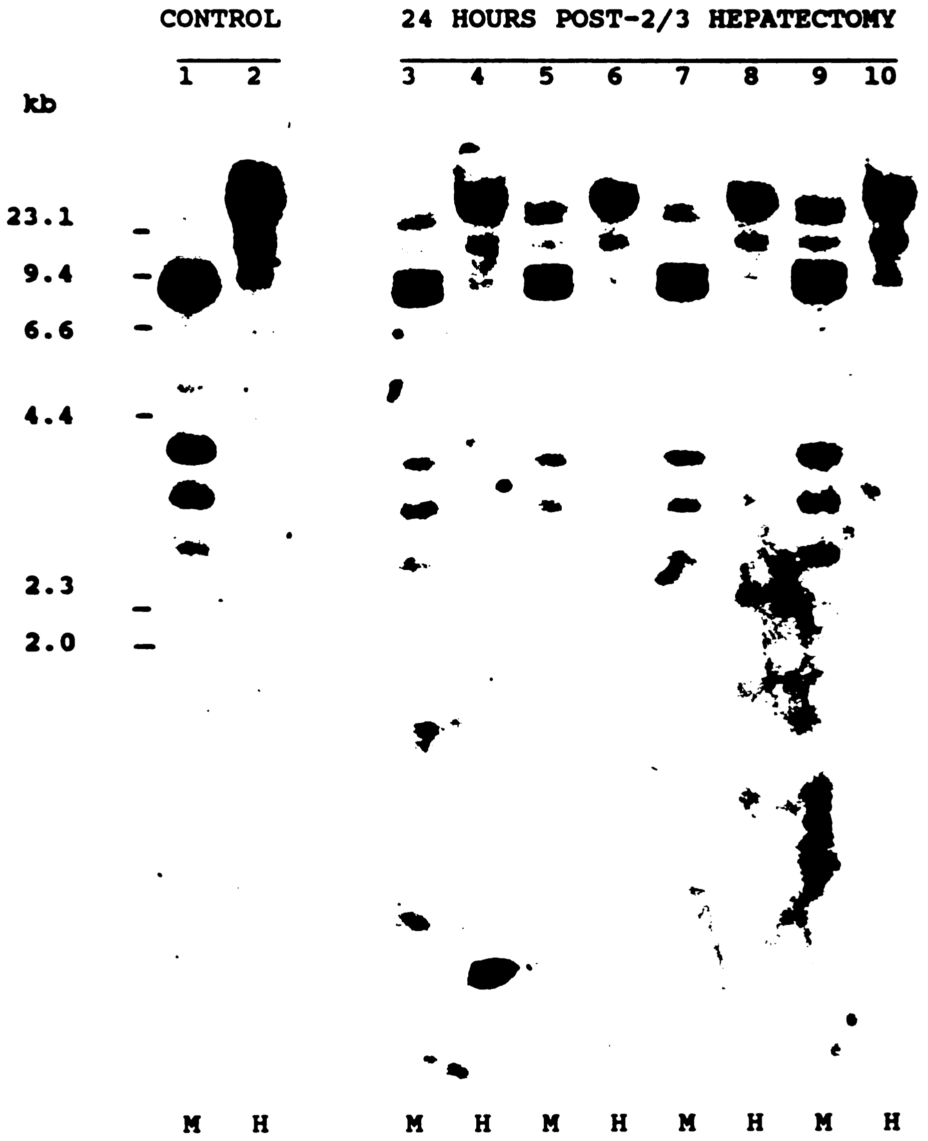

Figure 20. Methylation status of the Ki-ras oncogene following partial hepatectomy. The membrane shown in Figure 19 was stripped of Ha-ras probe and rehybridized with a ^{32}P-labelled probe for Ki-ras. Note that no differences exist between Hpa II-digested control DNA (lane 2) and DNA from partially hepatectomized animals (lane 4, 6, 8, and 10).

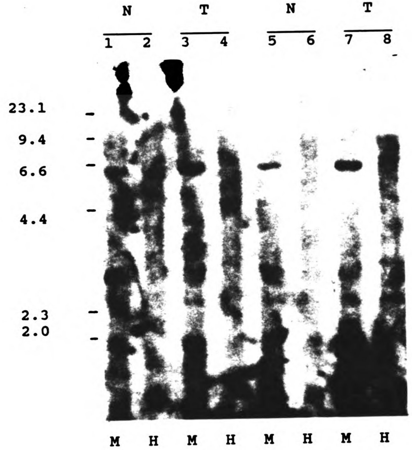

Figure 21. Methylation status of the myc oncogene in benzidine-induced hepatic tumors and adjacent non-tumor tissue. The membrane shown in Figure 16 was stripped of the Ki-ras probe and rehybridized with a ^{32}P-labelled probe for myc (Oncor). Note the increased intensity of bands in tumor DNA (lanes 3 and 7) as compared to non-tumor DNA (lanes 1 and 5).

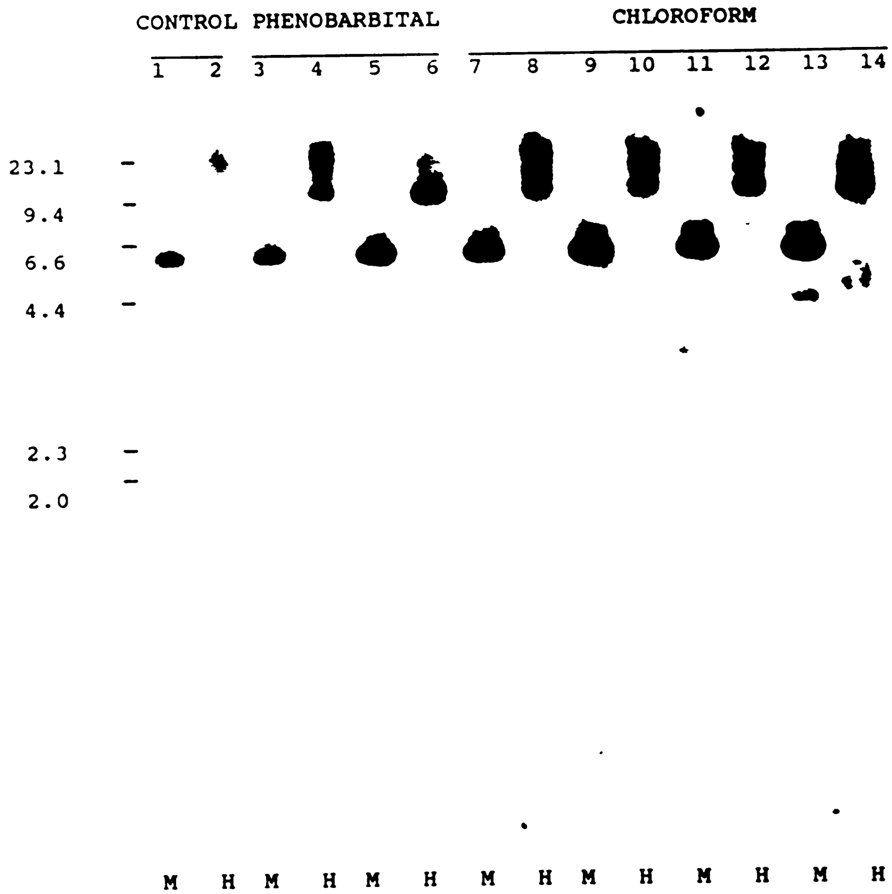

Figure 22. Methylation status of the myc oncogene in phenobarbital- and chloroform-induced hepatic tumors. The membrane shown in Figure 17 was stripped of the Ki-ras probe and rehybridized to a ^{32}P-labelled probe for myc (Pst I fragment). Note the increased intensity of bands in tumor-derived DNA (lanes 3-14) as compared to control DNA (lanes 1-2).

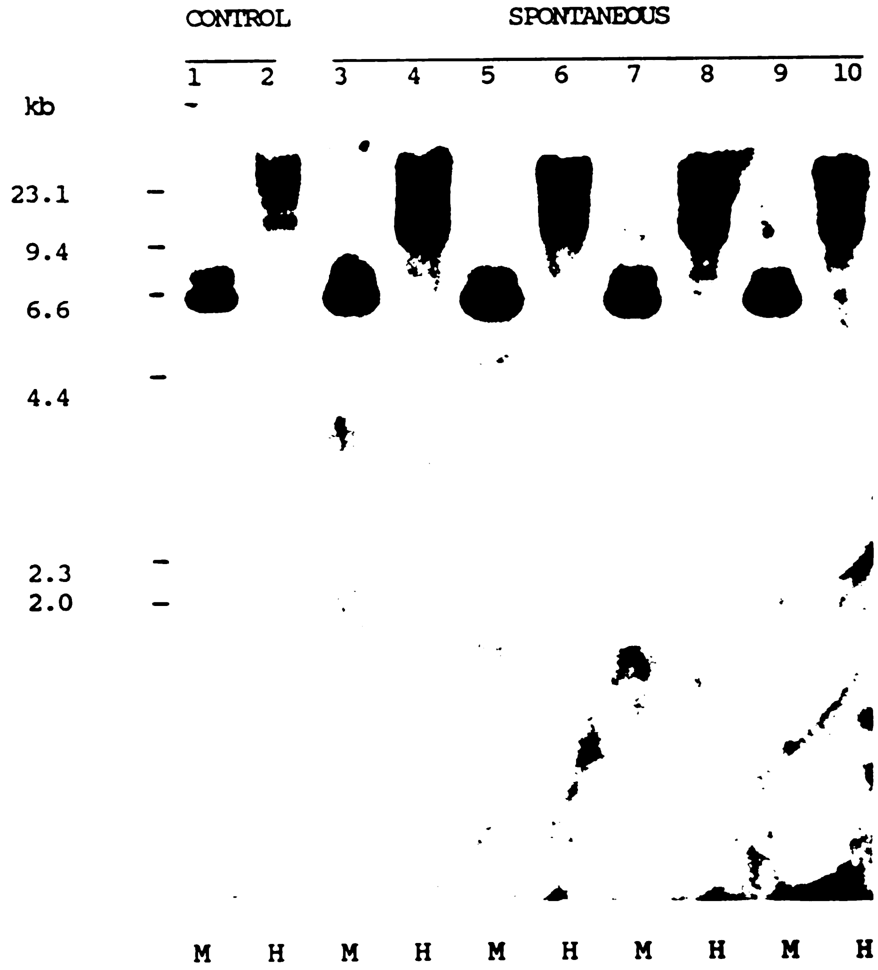

Figure 23. Methylation status of the myc oncogene in spontaneous hepatic tumors. DNA from spontaneous tumors was treated and described in Figure 4 except hybridization was to a ^{32}P-labelled probe for myc (Pst I fragment). Note the increased intensity of bands in tumor-derived DNA (lanes 3-10) as compared to control DNA (lanes 1-2).

hybridization of the myc probe to tumor DNA is greater than to control DNA. It thus appears that myc may be amplified in tumors from all four treatment groups.

As an internal control, the methylation state of the serum albumin gene was assessed in all samples. As can be seen in Figure 24, Msp I and Hpa II digestion of each sample from the benzidine group produces nearly identical restriction patterns. This result indicates that the serum albumin gene is hypomethylated in both tumor and non-tumor tissue. Similar results were seen when DNA from the phenobarbital (Figure 25, lanes 3-6), chloroform (Figure 25, lanes 7-14) and spontaneous (Figure 26, lanes 3-10) tumor groups were probed with pRSA 13. Thus, the nearly identical restriction patterns produced by Msp I and Hpa II digestion indicate that the serum albumin gene is uniformly hypomethylated in control DNA and tumor DNA from all four groups.

3. Assessment of Oncogene Methylation State with Hha I

The methylation state of each oncogene was also assessed in all samples relative to each other using the restriction endonuclease Hha I. Figure 27 shows the result of hybridizing the Ha-ras probe to Hha I-digested DNA derived from male B6C3F1 (lanes 1-4), male C3H/He (lanes 5-8), female C57BL/6 (lanes 9-12), female B6C3F1 (lanes 13-14), female C3H/He (lanes 15-16), and male C57BL/6 (lanes 17-18) mouse liver. It can be seen that a major band of very high molecular weight DNA ($\geq$ 23.1 kb) results in each case. Smaller bands at 6.2 and 2.6 kb of similar intensity in each samples were also produced, although this is more evident on the actual autoradiograph. These results indicate that the Ha-ras oncogene is methylated to a similar extent in the liver of both sexes of all three mouse strains.

When a similar experiment was performed using non-tumor and tumor DNA from the benzidine group, Hha I did not produce identical restriction patterns in

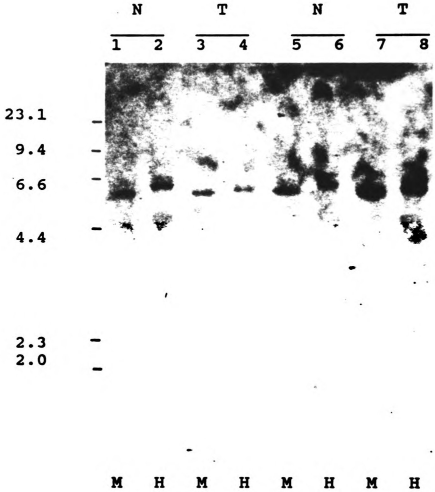

Figure 24. Methylation status of the serum albumin gene in benzidine-induced hepatic tumors and adjacent non-tumor tissue. The membrane shown in Figure 21 was stripped of the myc probe and rehybridized with a ^{32}P-labelled probe for serum albumin.

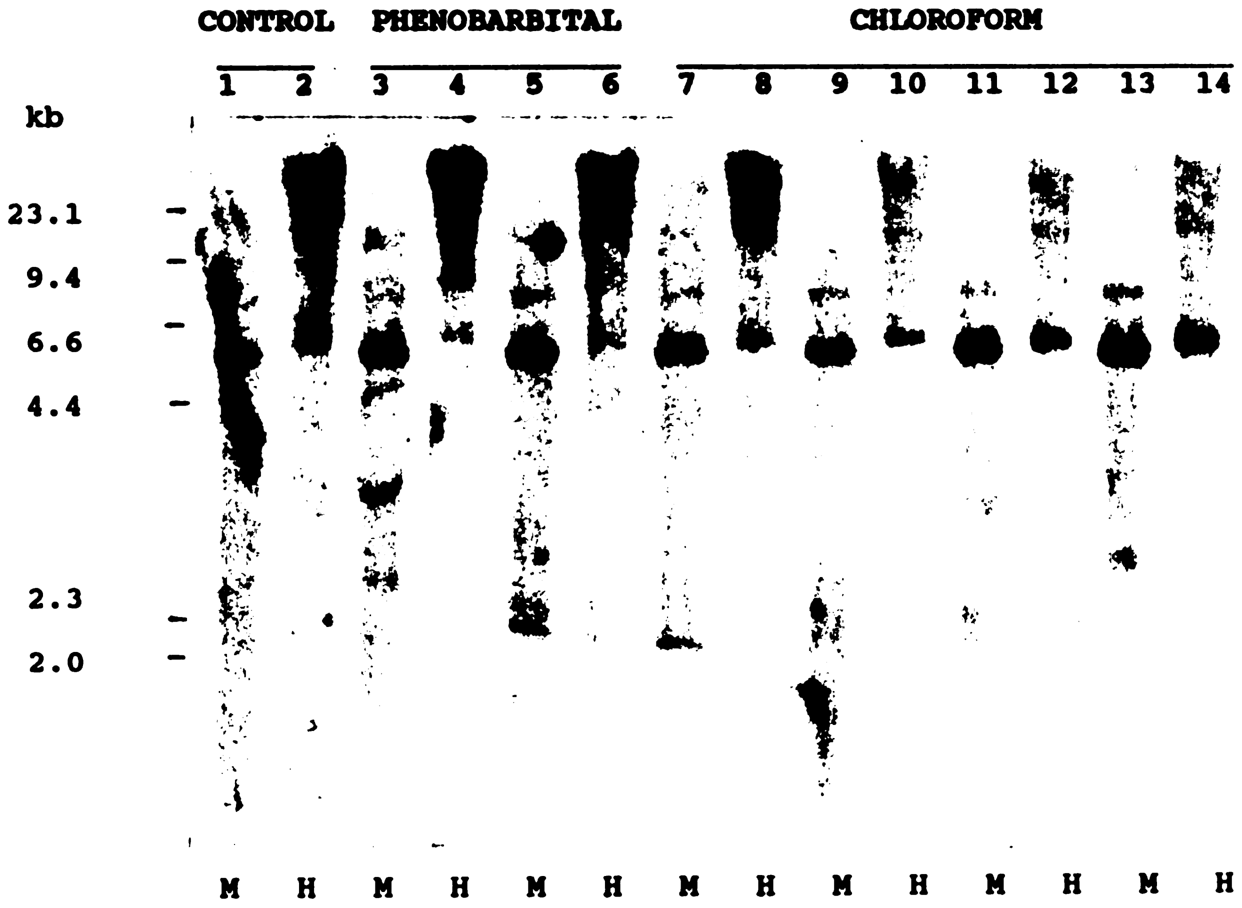

Figure 25. Methylation status of the serum albumin gene in phenobarbital- and chloroform-induced hepatic tumors. The membrane shown in Figure 22 was stripped of the myc probe and rehybridized to a probe for serum albumin.

Figure 26. Methylation status of the serum albumin gene in spontaneous hepatic tumors. The membrane shown in Figure 18 was stripped of the Ki-ras probe, rehybridized to and stripped of the myc probe, and finally rehybridized to a ^{32}P-labelled probe for serum albumin.

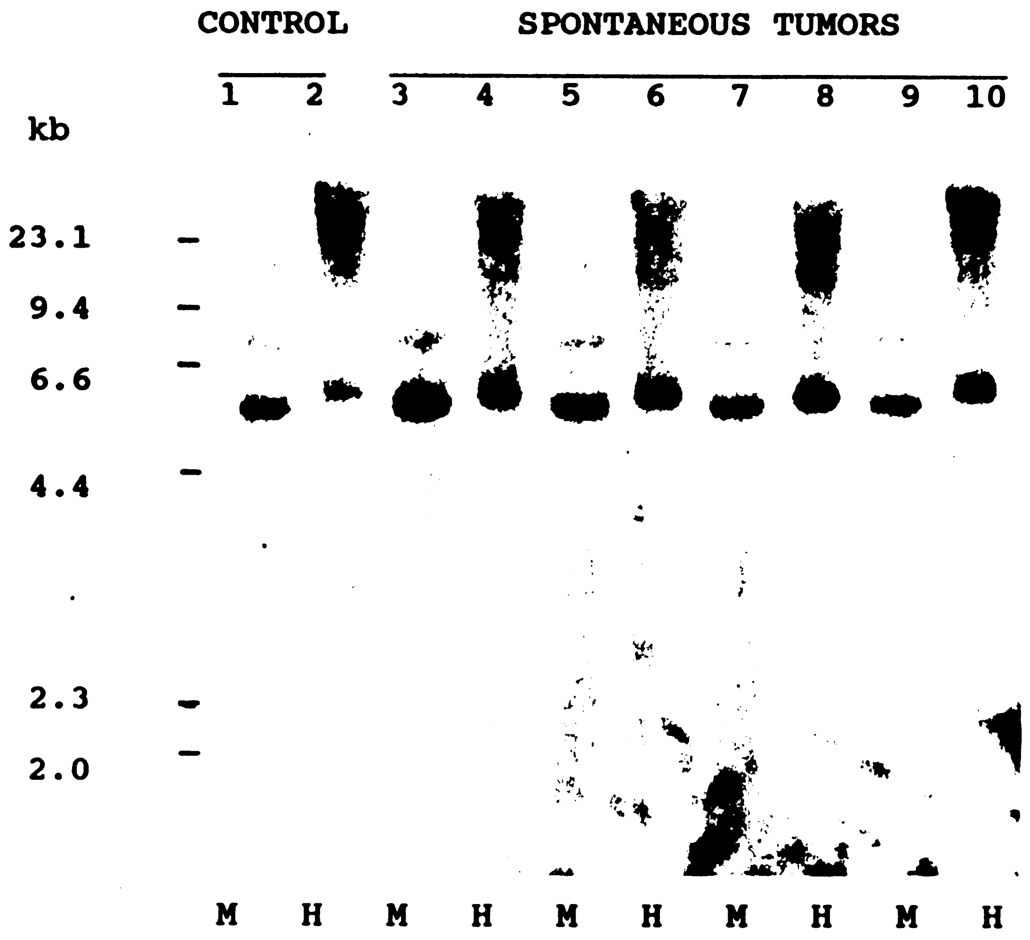
CONTROL
SPONTANEOUS TUMORS
1
2
3
4
5
6
7
8
9
10
kb
23.1
9.4
6.6
4.4
2.3
2.0
M
H
M
H
M
H
M
H
M
H

Figure 26

Figure 27. Hha I assessment of the methylation state of the Ha-ras oncogene in B6C3F1, C3H/He, and C57BL/6 mouse liver. DNA was isolated from male (lanes 1-4) and female (lanes 13-14) B6C3F1, male (lanes 5-8) and female (lanes 15-16) C3H/He, and female (lanes 9-12) and male (lanes 17-18) C57BL/6 mouse liver on a CsCl gradient. Samples were digested with Hha I, electrophoresed on a 0.9% agarose gel, and transferred to Gene Screen Plus. The DNA was hybridized to a ^{32}P-labelled probe for the Ha-ras oncogene (Oncor), and regions of homology were visualized by autoradiography.

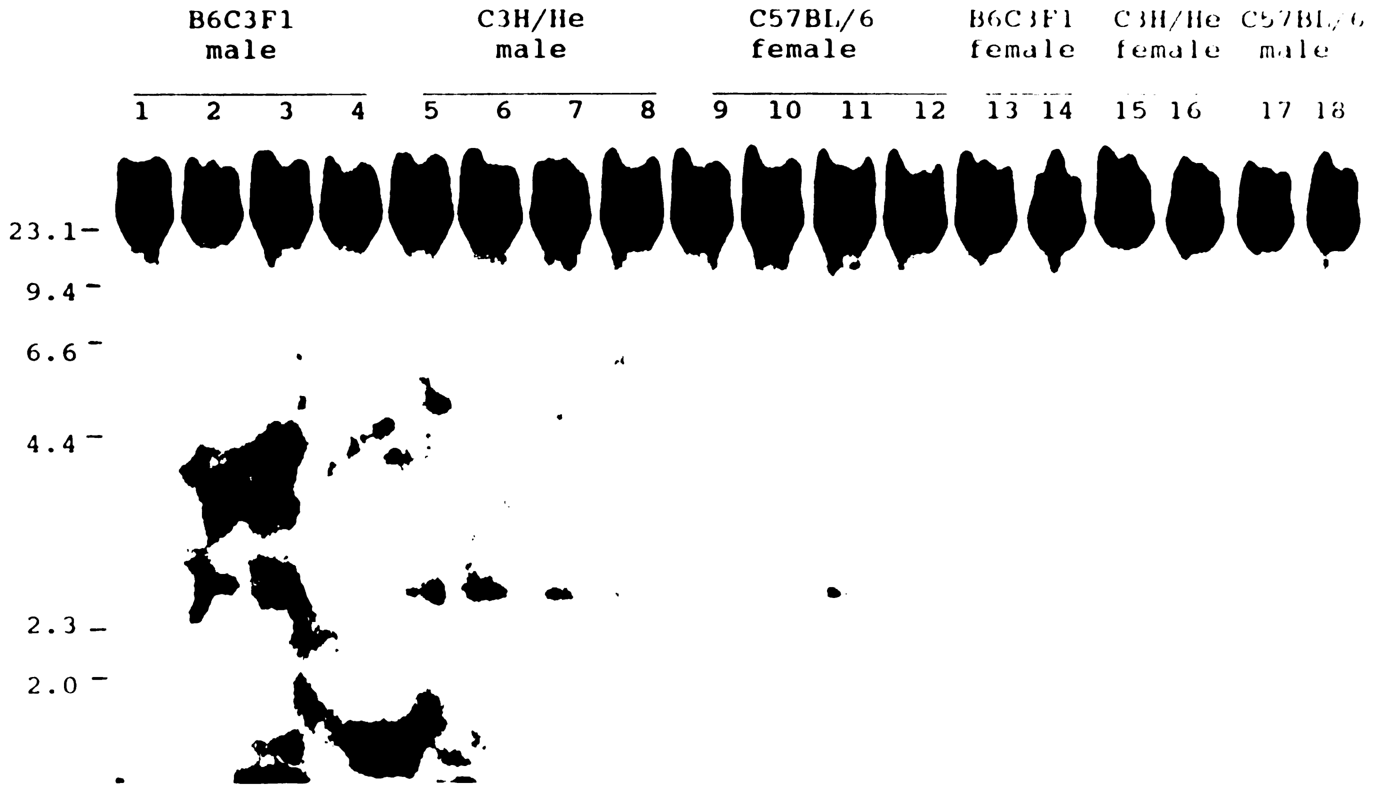
B6C3F1 male
C3H/He male
C57BL/6 female
B6C3F1 female
C3H/He female
C57BL/6 male
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
23.1
9.4
6.6
4.4
2.3
2.0

Figure 27

all cases (Figure 28). The high molecular weight band produced by Hha I digestion of tumor DNA (lanes 2, 4, 6, and 8) was observed to exhibit consistently greater electrophoretic mobility than the corresponding band produced in non-tumor tissue by this enzyme (lanes 1, 3, 5, and 7). This indicates that Hha I is able to cleave DNA in the area of Ha-ras to a greater extent in benzidine-induced tumors due to hypomethylation of sites in this tissue as compared to adjacent non-tumor tissue.

Similar results were obtained when DNA from phenobarbital-induced (Figure 29, lanes 2-3), chloroform-induced (Figure 29, lanes 4-7) and spontaneous (Figure 29, lanes 8-11) tumors were digested with Hha I. Compared to control DNA (lanes 1 and 12), the major band produced by Hha I-digested tumor DNA sometimes exhibited greater electrophoretic mobility (lanes 2 and 9) and a doublet was frequently produced (lanes 3, 6, 7, 8, and 10). An increased exposure time of the membrane shows a much greater intensity of band at 2.6 and 6.2 kb in all tumors as compared to controls (Figure 30). The greater ability of Hha I to cleave tumor DNA than control DNA indicates that the degree of methylation of Ha-ras is often decreased in tumor tissue as compared to control liver.

Hha I analysis was also used to examine the methylation state of Ki-ras (Figure 31) in male B6C3F1 (lanes 1-4), male C3H/He (lanes 5-8), female C57BL/6 (lanes 9-12), female B6C3F1 (lanes 13-14), female C3H/He (lanes 15-16) and male C57BL/6 (lanes 17-18) mouse liver. Two very high molecular weight bands ($\geq$23 kb) are produced by Hha I digestion of each sample indicating that no strain or sex differences exist in the methylation state of the Ki-ras oncogene.

When DNA samples from the benzidine group were analyzed with Hha I (Figure 32), greater mobility of the lower band was observed in two tumor samples (lanes 2 and 6) as compared to non-tumor samples (lanes 1, 3, and 5) when hybridized to Ki-ras. This result suggests that the Ki-ras oncogene is sometimes

Figure 28. Hha I assessment of the methylation state of the Ha-ras oncogene in benzidine-induced tumors and adjacent non-tumor tissue. DNA derived from tumors (lanes 2, 4, 6, and 8) and non-tumor tissue (lanes 1, 3, 5, and 7) was isolated and treated as described in Figure 27. Note the increased mobility of the band resulting from Hha I-digested tumor DNA versus non-tumor DNA.

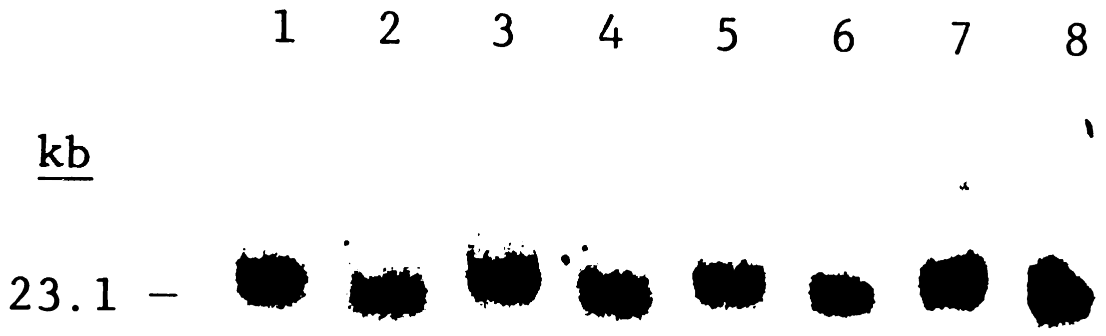

Figure 28

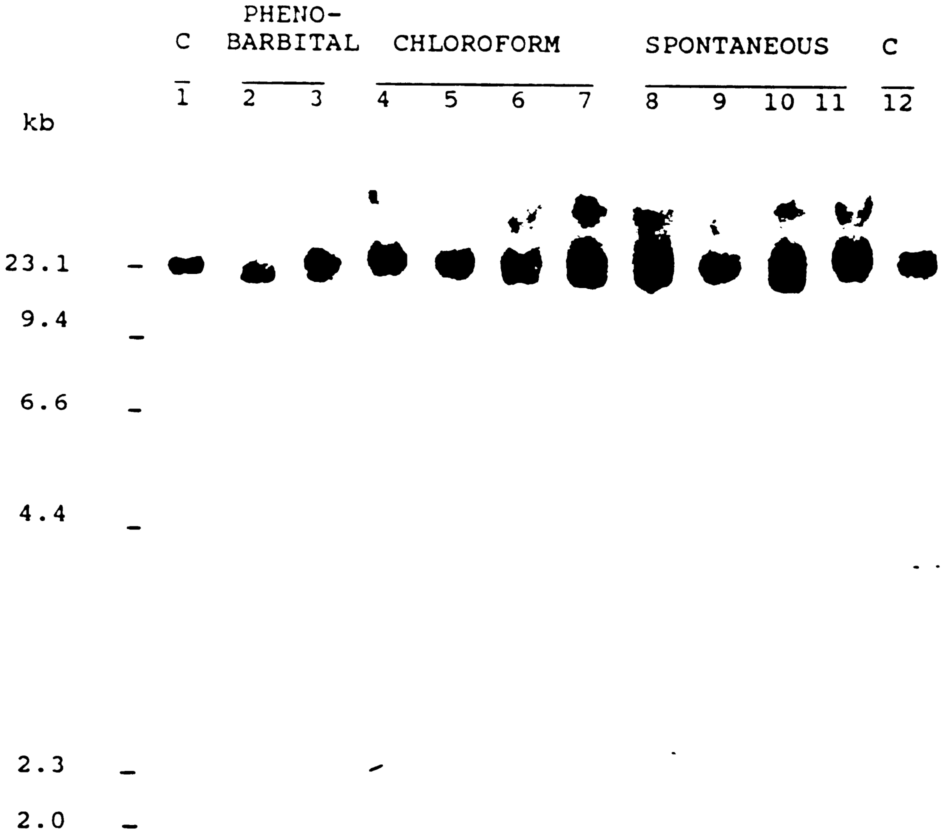

Figure 29. Hha I assessment of the methylation state of the Ha-ras oncogene in phenobarbital-induced, chloroform-induced, and spontaneous hepatic tumors (short exposure time). DNA derived from phenobarbital-induced (lanes 2-3), chloroform-induced (lanes 4-7), and spontaneous (lanes 8-11) tumors was isolated and treated as described in Figure 27, except that a 0.8% agarose gel was used and electrophoresis was carried out for an additional 4 hours. Note the altered mobility of some bands in tumor samples (lanes 2-11) as compared to control DNA (lanes 1 and 12).

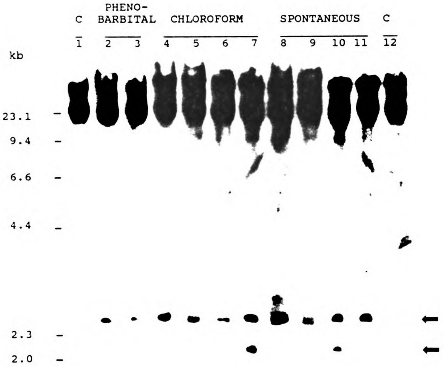

Figure 30. Hha I assessment of the methylation state of the Ha-ras oncogene in phenobarbital-induced, chloroform-induced, and spontaneous hepatic tumors (long exposure time). The membrane shown in Figure 29 was reexposed to x-ray film for double the amount of time. The arrows point to bands whose intensity is much greater in tumor DNA (lanes 2-11) than in control DNA (lanes 1 and 12).

Figure 31. Hha I assessment of the methylation state of the Ki-ras oncogene in B6C3F1, C3H/He, and C57BL/6 mouse liver. The membrane shown in Figure 27 was stripped of the Ha-ras probe and rehybridized to a ^{32}P-labelled probe for Ki-ras.

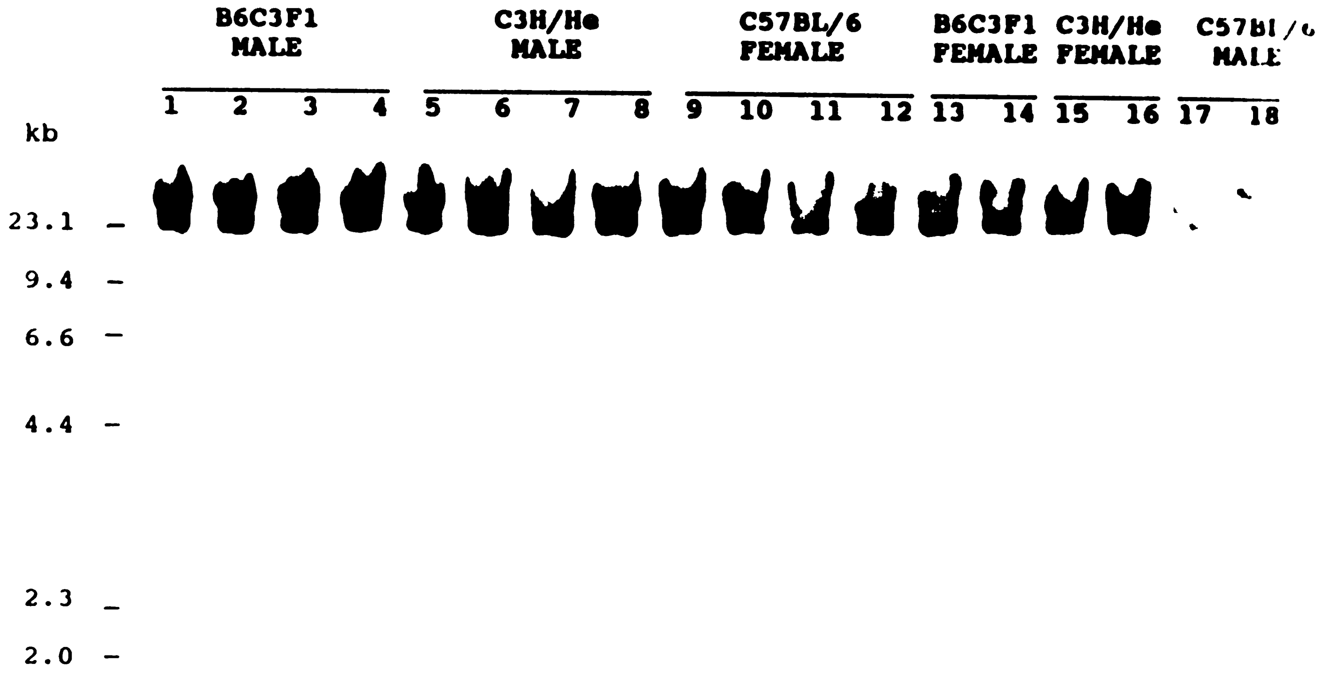
B6C3F1 MALE
C3H/He MALE
C57BL/6 FEMALE
B6C3F1 FEMALE
C3H/He FEMALE
C57Bl/6 MALE
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
kb
23.1
9.4
6.6
4.4
2.3
2.0

Figure 31

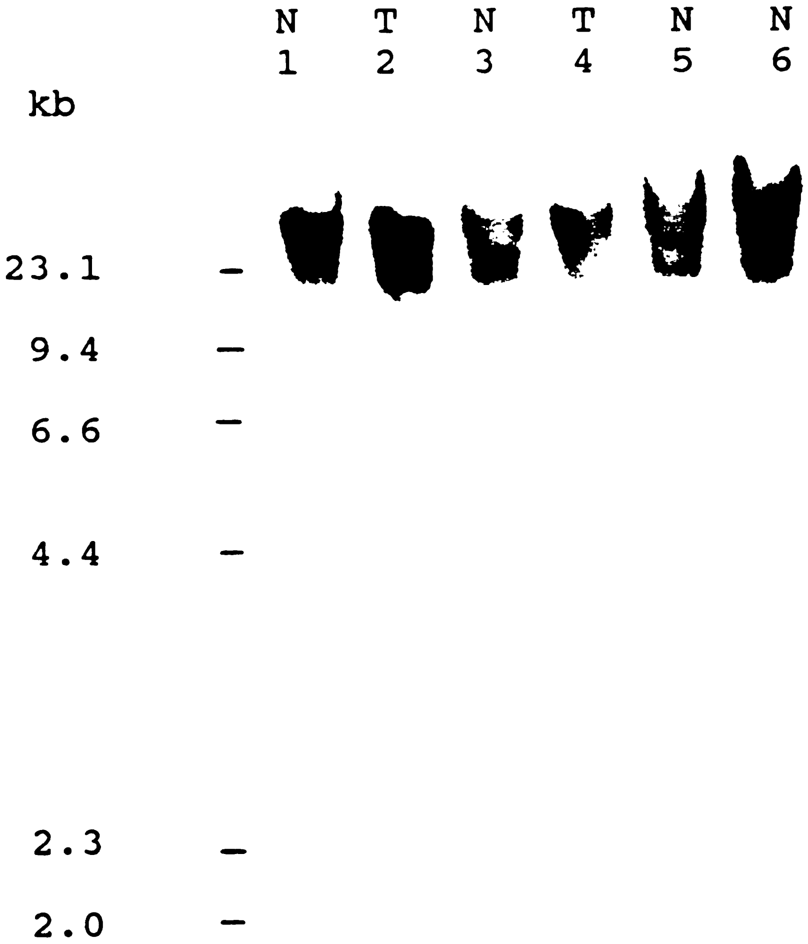

Figure 32. Hha I assessment of the methylation state of the Ki-ras oncogene in benzidine-induced tumors and adjacent non-tumor tissue. DNA derived from tumors (lanes 2, 4, and 6) and non-tumor tissue (lanes 1, 3, and 5) was isolated and treated as described in Figure 27. A ^{32}P-labelled probe for Ki-ras was used for hybridization.

hypomethylated at 5'-GCGC-5' sites in tumor versus non-tumor liver tissue. Similarly, when Hha I was used to digest tumor DNA from the phenobarbital, chloroform, and spontaneous groups (Figure 33), a band was sometimes observed at approximately 20 kb (lanes 2, 4, 6, 7, 8, and 10; see arrow) which was not observed in control DNA (lanes 1 and 12). These results indicate that the Ki-ras oncogene is sometimes hypomethylated in phenobarbital-induced, chloroform-induced, and spontaneous B6C3F1 mouse liver tumors.

Figure 34 shows the result of hybridizing Hha I-digested DNA from control and opposite sex mice to the myc probe. In each sample, a band is apparent at approximately 12 kb, although some non-specific hybridization occurs at higher molecular weights. It thus appears that myc is methylated to the same extent in B6C3F1, C3H/He, and C57BL/6 mice of both sexes.

Hha I was also used to assess the relative methylation state of myc in benzidine-induced tumors and non-tumor tissue (Figure 35). A band at 14.5 kb is apparent in non-tumor DNA (lanes 1, 3, and 5) whereas this band displays a size of 13.5 kb in tumor samples (lanes 2, 4, and 6). This decrease in band size indicates that some 5'-GCGC-3' sites have acquired a demethylated status in tumor tissue. An extra band was sometimes observed in Hha I-digested tumor DNA from the phenobarbital- and chloroform-induced and spontaneous tumors (Figure 36, lanes 3, 6, 7, and 10; see arrow) which was not present in control DNA (lanes 1) or in DNA from the other tumors. The presence of this $\geq$ 23 kb band may be indicative of myc hypomethylation in some tumors from the three treatment groups depicted in this figure.

Hybridization of the serum albumin probe to Hha I-digested control and opposite sex DNA is shown in Figure 37. In each sample, bands at 10 and 8 kb are produced. These results indicate that serum albumin is methylated to the same extent in male and female B6C3F1, C3H/He, and C57BL/6 mouse liver. A simlar

Figure 33. Hha I assessment of the methylation state of the Ki-ras oncogene in phenobarbital-induced, chloroform-induced, and spontaneous hepatic tumors. The membrane shown in Figure 29 was stripped of the Ha-ras probe and rehybridized to a ^{32}P-labelled probe for Ki-ras. Arrow points to a band of greater electrophoretic mobility that is sometimes seen in tumor DNA (lanes 2, 6, 7 and 10) but not in control DNA (lanes 1 and 12).

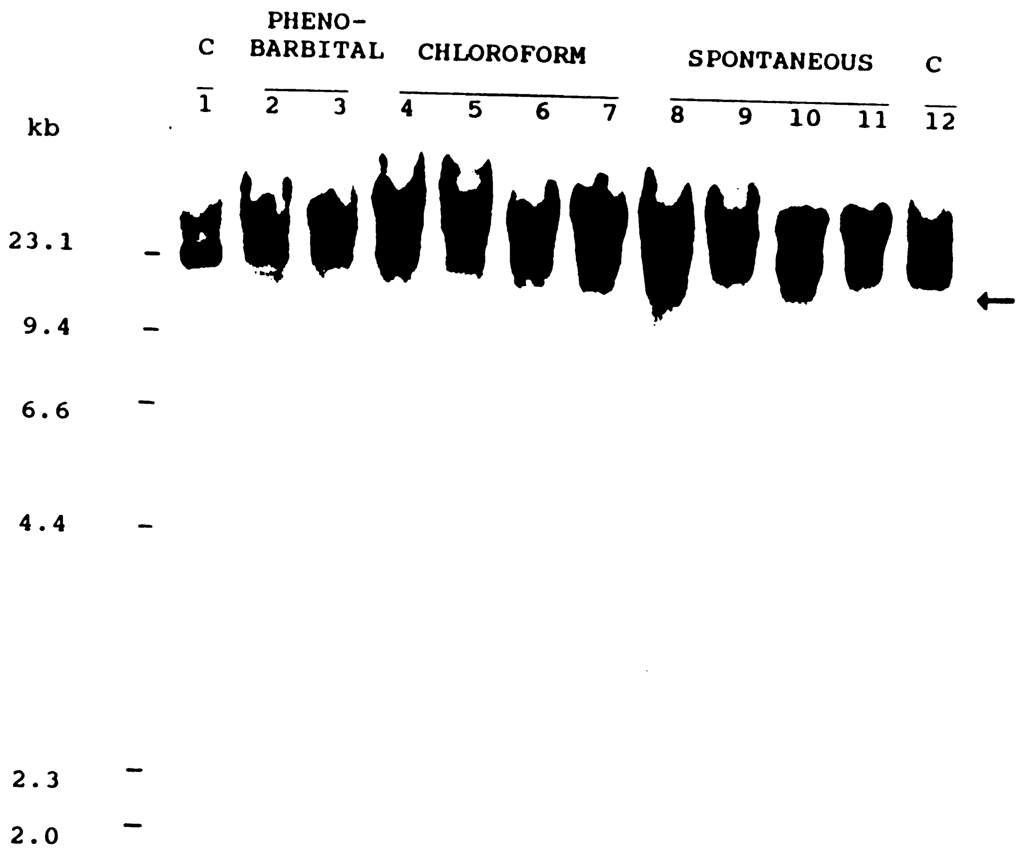
C
PHENO-
BARBITAL
CHLOROFORM
SPONTANEOUS
C
1
2
3
4
5
6
7
8
9
10
11
12
kb
23.1
9.4
6.6
4.4
2.3
2.0

Figure 33

Figure 34. Hha I assessment of the methylation state of the myc oncogene in B6C3F1, C3H/He, and C57BL/6 mouse liver. The membrane shown in Figure 31 was stripped of the Ki-ras probe and rehybridized to a ^{32}P-labelled probe for myc (Pst I fragment).

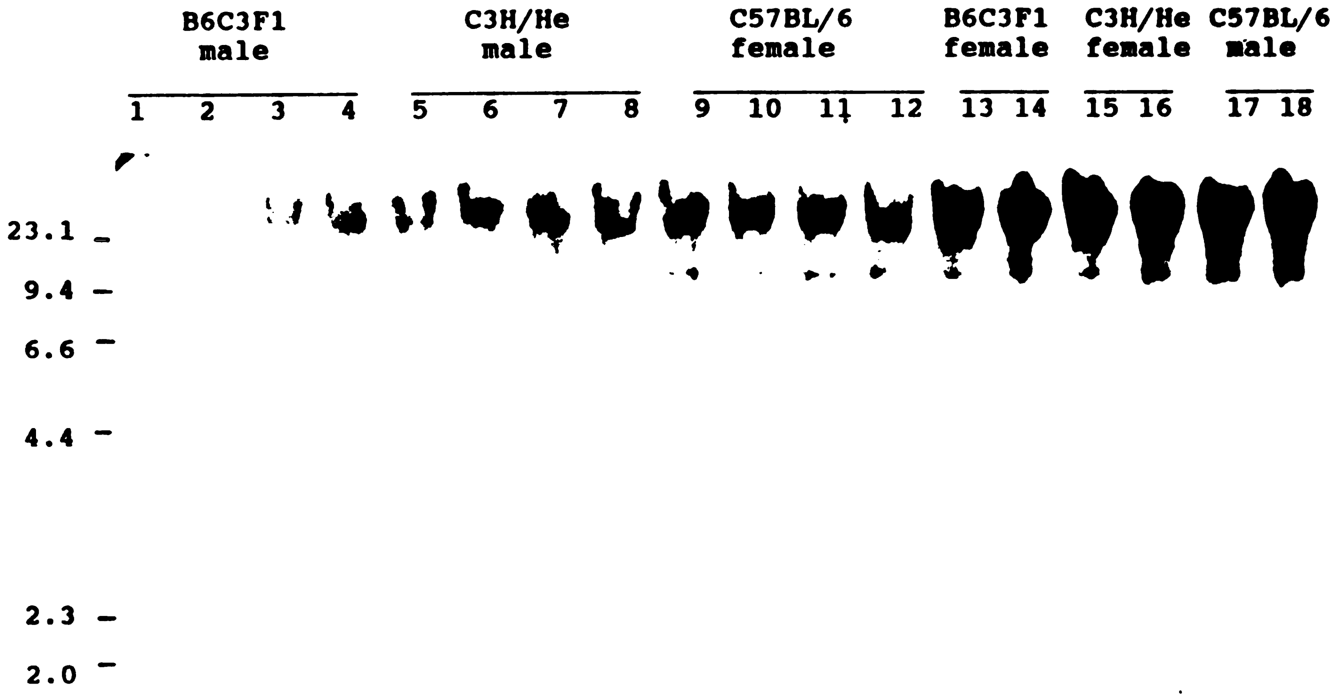
B6C3F1
male
C3H/He
male
C57BL/6
female
B6C3F1
female
C3H/He
female
C57BL/6
male
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
23.1
9.4
6.6
4.4
2.3
2.0

Figure 34

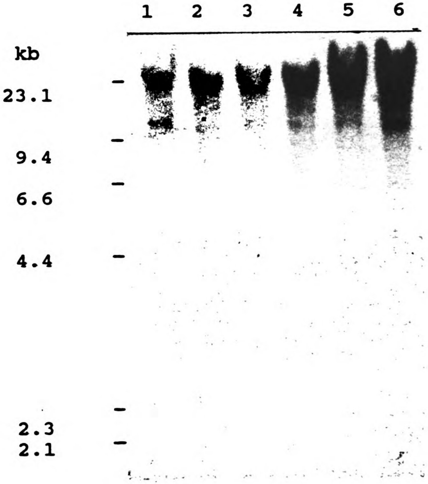

Figure 35. Hha I assessment of the methylation state of the myc oncogene in benzidine-induced tumors and adjacent non-tumor tissue. The membrane shown in Figure 32 was stripped of the Ki-ras probe and rehybridized to a ^{32}P-labelled probe for myc (Pst I fragment).

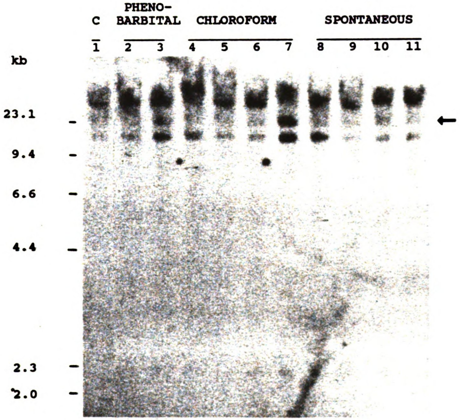

Figure 36. Hha I assessment of the methylation state of the myc oncogene in phenobarbital-induced, chloroform-induced, and spontaneous hepatic tumors. The membrane shown in Figure 33 was stripped of the Ki-ras probe and rehybridized to a ^{32}P-labelled probe for myc (Pst I fragment).

Figure 37. Hha I assessment of the methylation state of the serum albumin gene in B6C3F1, C3H/He, and C57BL/6 mouse liver. The membrane shown in Figure 34 was stripped of the myc probe and rehybridized to a ^{32}P-labelled probe for serum albumin.

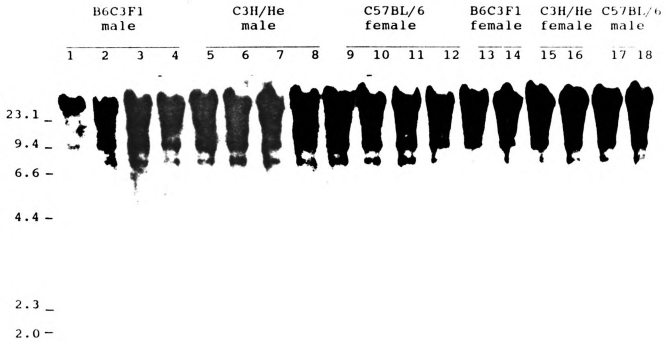
B6C3F1
male
C3H/He
male
C57BL/6
female
B6C3F1
female
C3H/He
female
C57BL/6
male
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
23.1
9.4
6.6
4.4
2.3
2.0

Figure 37

result was observed when the serum albumin probe was hybridized to Hha I-digested DNA from all four groups of tumors (data not shown). These results indicate that the methylation state of the serum albumin gene is not altered in tumor tissue as compared to normal liver.

4. DNase I Studies

A photograph of an ethidium-bromide-stained baby gel upon which was electrophoresed a set of DNase I-treated B6C3F1 DNA samples is presented in Figure 38. In this figure, as in the following DNase I figures (Figures 39-51), the 0°C control sample is in lane 1, the 15° control sample is in lane 2, and lanes 3-7 represent DNA treated with increasing concentrations of DNase I (28-47 units/ml). As can be seen in lane 1, electrophoresis of the 0°C control sample results in a band of very high molecular weight DNA. Similarly, the 15°C control DNA (lane 2) also produces a single band of high molecular weight, although DNA in this sample exhibits slightly greater electrophoretic mobility as compared to the 0°C control. The DNase I-treated samples show evidence of smaller DNA fragments in a concentration-dependent manner, as evidenced by a faint smear of DNA down the gel. These results indicate that the 0°C control DNA is undegraded, whereas the 15°C control DNA exhibits minimal degradation. Furthermore, it appears that a limited DNase I digestion has been achieved in the DNase I-treated samples.

Figure 39 shows the result of hybridizing EcoR1-digested DNase I-treated B6C3F1 DNA to the Ha-ras probe. It can be seen that two bands at 4.4 and 3.0 kb (see arrows) appear in the DNase I-treated samples (lanes 3-7) in a concentration-dependent manner; these bands are extremely faint (probably due to some endogenous nuclease activity) or not present in the controls (lanes 1-2). The appearance of these bands indicate the presence of DNase I hypersensitive sites in the Ha-ras oncogene B6C3F1 mouse liver.

Figure 38. Extent of DNA digestion in DNase I-treated nuclei. DNA was purified from nuclei incubated without DNase I at 0°C (lane 1) or 15°C (lane 2) or at 15°C with increasing concentrations of DNase I (lanes 3-7; 28-47 units/ml). One microgram aliquots were electrophoresed as described in Methods, and the gel was stained with ethidium bromide and photographed.

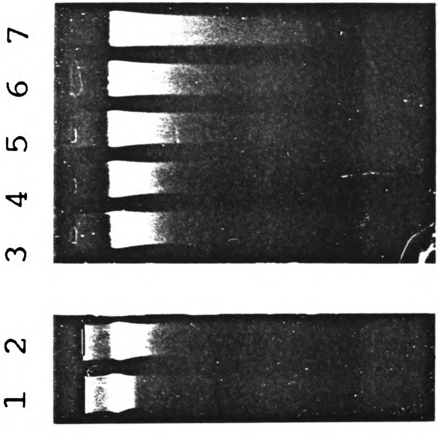

Figure 38

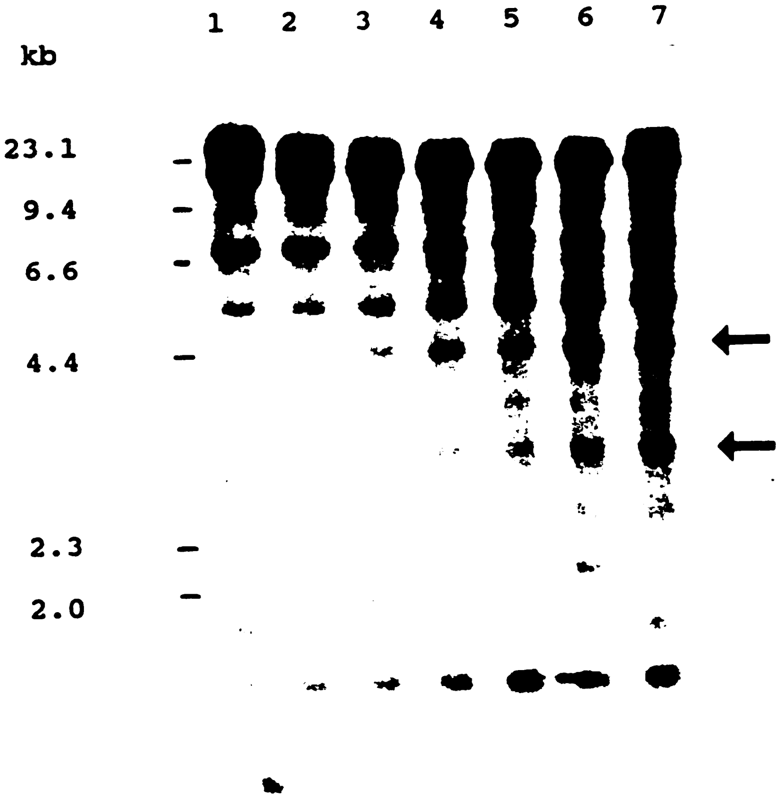

Figure 39. Assessment of the Ha-ras oncogene for the presence of DNase I hypersensitive sites in B6C3F1 mouse liver using EcoR1. DNA isolated from 0°C (lane 1) or 15°C (lane 2) control nuclei or nuclei treated with increasing concentrations of DNase I (lanes 3-7; 28-47 units/ml) was digested with EcoR1. Electrophoresis, Southern transfer, and hybridization to a ^{32}P-labelled probe for Ha-ras (Oncor) was performed as described in Methods. Bands resulting from digestion at DNase I hypersensitive sites are indicated by arrows.

Similar results were observed when DNase I-treated DNA from C3H/He (Figure 40) and C57BL/6 (Figure 41) mouse liver was digested with EcoR1 and hybridized to the Ha-ras probe. In each case, bands at approximately 4.4 and 3.0 kb were observed in the DNase I-treated samples (lanes 3-7) whereas these bands are absent in the 0°C control sample (lane 1). It thus appears that the Ha-ras oncogene possesses DNase I hypersensitive sites in all three mouse strains.

The enzyme Hind III was also used to examine the Ha-ras oncogene for the presence of DNase I hypersensitive sites. As seen in Figure 42, digestion of DNase I-treated B6C3F1 DNA (lanes 3-7) with this enzyme produces bands at 4.0 and 1.9 kb, whereas corresponding bands are not seen in 0°C control DNA (lane 1). The presence of the 1.9 kb and was corroborated in a repeat of this experiment (Figure 43). A similar result was seen in DNase I-treated C3H/He DNA (Figure 44). A 4.0 kb band is apparent only in the DNase I-treated samples (lanes 3-7); a 1.9 kb band is not visible. In contrast, no additional bands are seen when DNase I-treated C57BL/6 DNA was digested with Hind III (Figure 45) even after a longer exposure of the x-ray film (not shown). These results indicate that DNase I hypersensitive sites exist in the Ha-ras oncogene when Hind III is used for analysis in both B6C3F1 and C3H/He mouse liver, but appear not to be present in C57BL/6 mouse liver.

The Ki-ras oncogene was also examined for the presence of DNase I hypersensitive sites. When DNase I-treated B6C3F1 DNA was digested with Pst I, bands at 10 and 4.8 kb were observed in the DNase I-treated samples (Figure 46, lanes 3-7) but not in the control samples (lanes 1-2). Furthermore, the sizes of these two bands sum to 14.8 kb. It can be seen that a band of approximately 14.5 kb decreases with intensity as the concentration of DNase I is increased. It thus appears that the 10 and 4.8 kb bands may result from the action of DNase I at a hypersensitive site within the 14.5 kb fragment. These results indicate that the

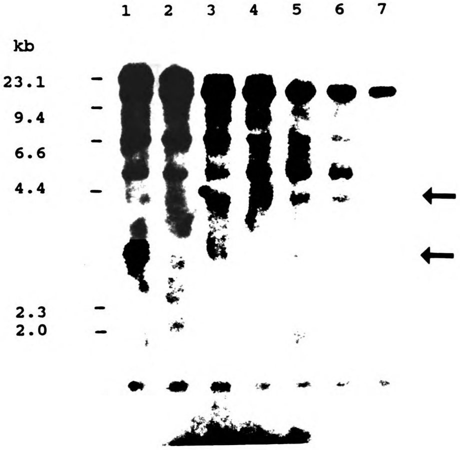

Figure 40. Assessment of the Ha-ras oncogene for the presence of DNase I hypersensitive sites in C3H/He mouse liver using EcoR1. DNA isolated from 0°C (lane 1) or 15°C (lane 2) control nuclei or nuclei digested with increasing concentrations of DNase I (lanes 3-7; 28-47 units/ml) was digested with EcoR1. Electrophoresis, Southern transfer, and hybridization to a ^{32}P-labelled probe for Ha-ras (Oncor) was performed as described in Methods. Bands resulting from digestion at DNase I hypersensitive sites are indicated by arrows.

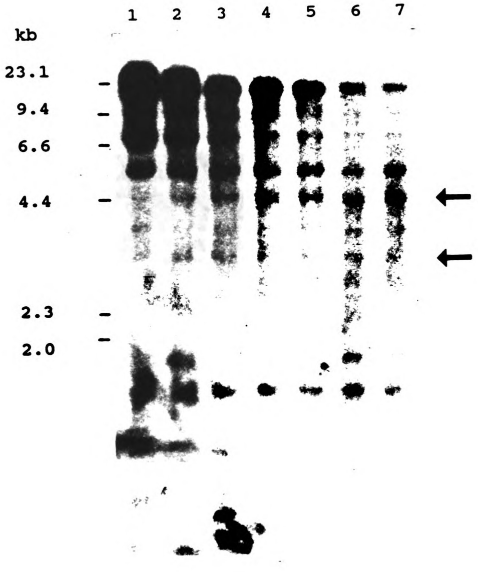

Figure 41. Assessment of the Ha-ras oncogene for the presence of DNase I hypersensitive sites in C57BL/6 mouse liver using EcoR1. DNA isolated from 0°C (lane 1) or 15°C (lane 2) control nuclei or nuclei treated with increasing concentrations of DNase I (lanes 3-7; 28-47 units/ml) was digested with EcoR1. Electrophoresis, Southern transfer, and hybridization to a ^{32}P-labelled probe for Ha-ras (Oncor) was performed as described in Methods. Bands resulting from digestion at DNase I hypersensitive sites are indicated by arrows.

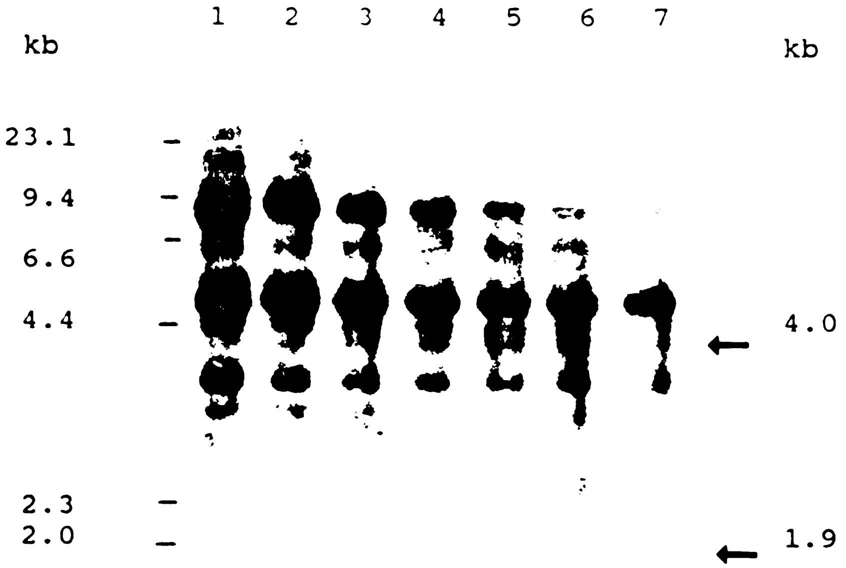

Figure 42. Assessment of the Ha-ras oncogene for DNase I hypersensitive sites in B6C3F1 mouse liver using Hind III. DNA was prepared and treated as described in Figure 39 except that Hind III was used for digestion. Lane 1, 0°C control; lane 2, 15°C control; lanes 3-7, DNA from nuclei treated with increasing concentrations of DNase I (28-47 units/ml). Bands resulting from digestion at DNase I hypersensitive sites are indicated by arrows.

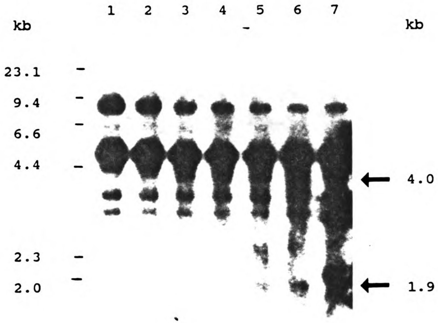

Figure 43. Confirmation of the presence of a Ha-ras band at 1.9 kb in DNase I-treated B6C3F1 mouse liver DNA digested with Hind III. DNA was prepared and treated exactly as described in Figure 42. Bands resulting from digestion at DNase I hypersensitive sites are indicated by arrows.

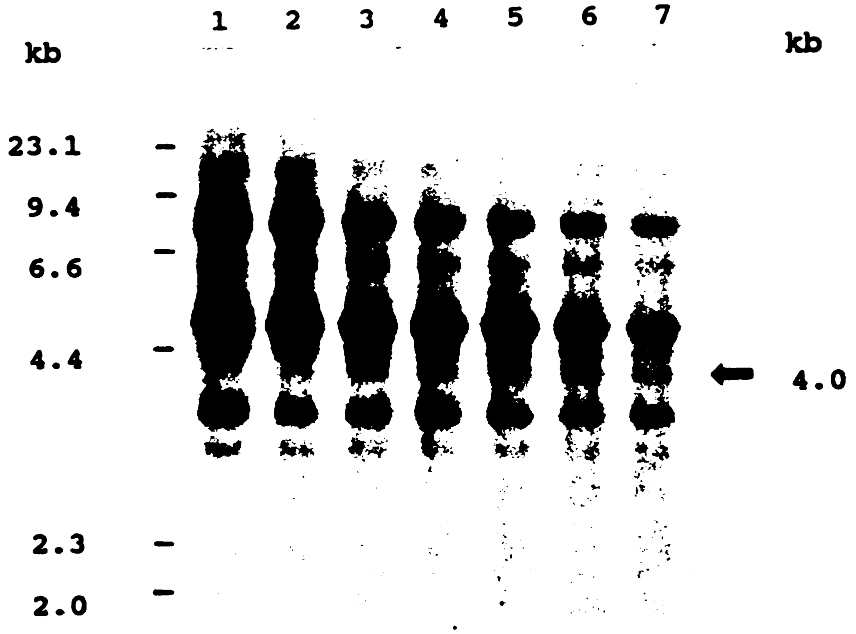

Figure 44. Assessment of the Ha-ras oncogene for the presence of DNase I hypersensitive sites in C3H/He mouse liver using Hind III. DNA was prepared and treated as described in Figure 40 except that Hind III was used for digestion. Lane 1, 0°C control; lane 2, 15°C control; lanes 3-7, DNA from nuclei treated with increasing concentrations of DNase I (28-47 units/ml). The arrow points to a band resulting from digestion at a DNase I hypersensitive site.

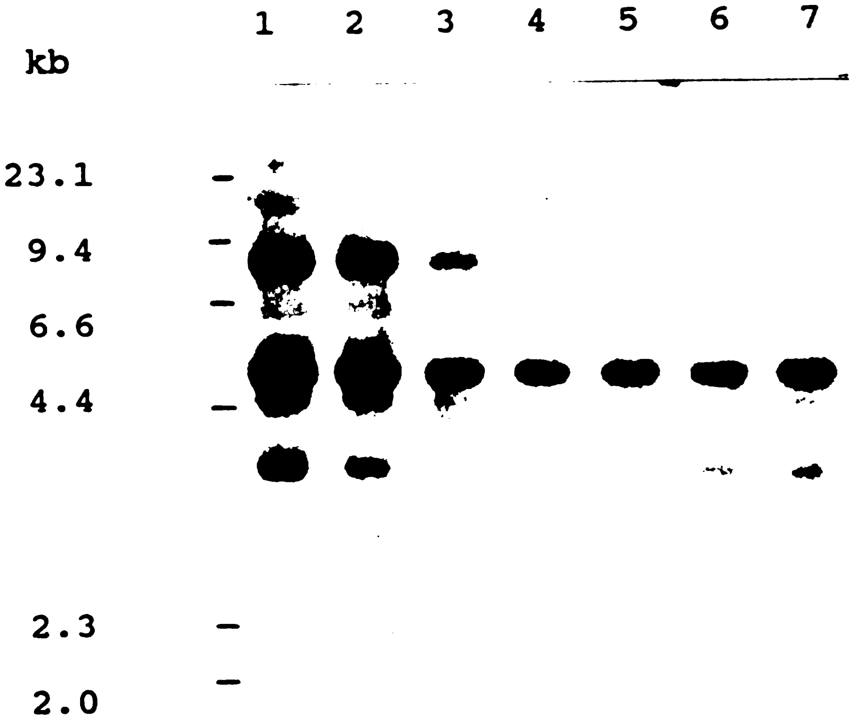

Figure 45. Assessment of the Ha-ras oncogene for the presence of DNase I hypersensitive sites in C57BL/6 mouse liver using Hind III. DNA was prepared and treated as described in Figure 41 except that Hind III was used for digestion. Lane 1, 0°C control; lane 2, 15° control; lanes 3-7, DNA from nuclei treated with increasing concentrations of DNase I (28-47 units/ml).

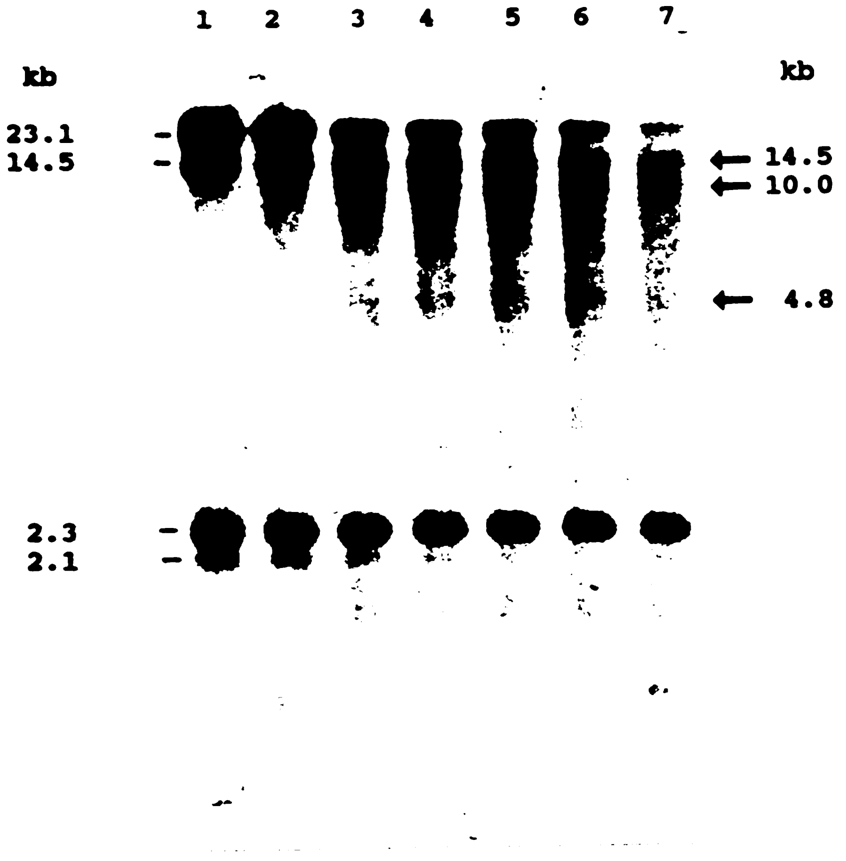

Figure 46. Assessment of the Ki-ras oncogene for the presence of DNase I hypersensitive sites in B6C3F1 mouse liver. DNA was prepared and treated as described in Figure 39 except that Pst I was used for digestion and the DNA was hybridized to a ^{32}P-lablled probe for Ki-ras. Lane 1, 0°C control; lane 2, 15°C control; lanes 3-7, DNA from nuclei treated with increasing concentrations of DNase I (28-47 units/ml). The 10.0 and 4.8 kb bands appear to result from cleavage of the 14.5 kb band at a DNase I hypersensitive site (see arrows).

Ki-ras oncogene possesses a DNase I hypersensitive site in B6C3F1 mouse liver. DNase I-treated C3H/He DNA digested with Pst I shows a similar result (Figure 47) with bands appearing at 10 and 4.8 kb only in the DNase I-treated samples. In contrast, bands corresponding to these sizes are not apparent in DNase I-treated C57BL/6 DNA digested with this enzyme (Figure 48). These results indicate that the Ki-ras oncogene possesses a DNase I hypersensitive site in B6C3F1 and C3H/He mouse liver, but this site appears to be absent in the liver of the C57BL/6 mouse.

The assessment of the myc oncogene for DNase I hypersensitive sites in B6C3F1 mouse liver is presented in Figure 49. When digested with Hind III, bands appear at 3.1 and 2.3 kb in DNase I-treated DNA (lanes 3-7), but these bands are not evident or faint (probably due to endogenous nuclease activity) in control DNA (lanes 1-2). It appears that these bands may result from the cleavage of the 5.1 kb band, which shows a decrease in intensity as the concentration of DNase I increases. Similar results were observed in DNase I-treated C3H/He (Figure 50) and C57BL/6 (Figure 51) DNA. In both cases, bands appear at approximately 3 adn 2 kb in the DNase I-treated DNA, whereas the 5 kb band shows a concurrent decrease in intensity. These results indicate that the myc oncogene exhibits a DNase I hypersensitive site in B6C3F1, C3H/He, and C57BL/6 mouse liver.

5. RNA Studies

The integrity of RNA samples was assessed by electrophoresis of glyoxal-denatured RNA followed by ethidium bromide staining (Figure 52). The 28S and 18S ribosomal RNA bands are clearly visible, with little evidence of RNA with greater electrophoretic mobility than these bands. It thus is evident that the RNA shows no indication of degradation and is therefore assumed to be intact.

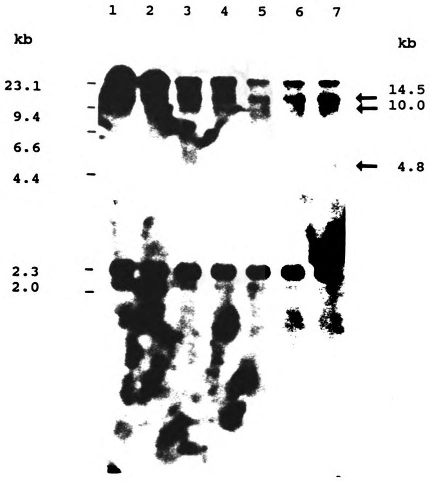

Figure 47. Assessment of the Ki-ras oncogene for the presence of DNase I hypersensitive sites in C3H/He mouse liver. DNA was prepared and treated s described in Figure 40 except that Pst I was used for digestion and the DNA was hybridized to a ^{32}P-labelled probe for Ki-ras. Lane 1, 0°C control; lane 2, 15°C control; lanes 3-7, DNA from nuclei treated with increasing concentrations of DNase I (28-47 units/ml). The 10.0 and 4.8 kb bands appear to result from cleavage of the 14.5 kb band at a DNase I hypersenstiive site.

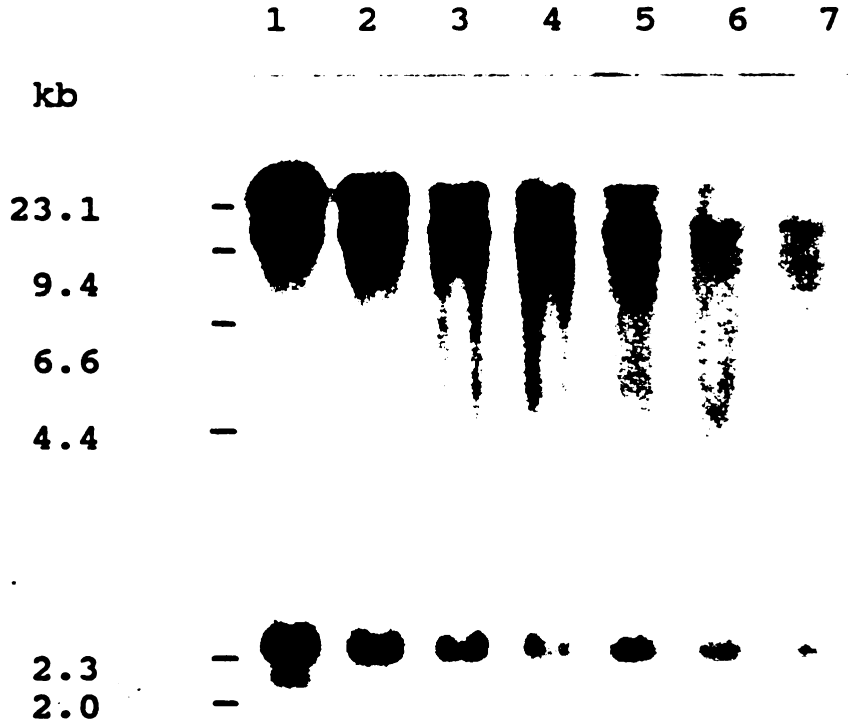

Figure 48. Assessment of the Ki-ras oncogene for the presence of DNase I hypersensitive sites in C57BL/6 mouse liver. DNA was prepared and treated as described in Figure 41 except that Pst I was used for digestion and the DNA was hybridized to a ^{32}P-labelled probe for Ki-ras. Lane 1, 0°C control; lane 2, 15°C control; lanes 3-7, DNA from nuclei treated with increasing concentrations of DNase I (28-47 units/ml).

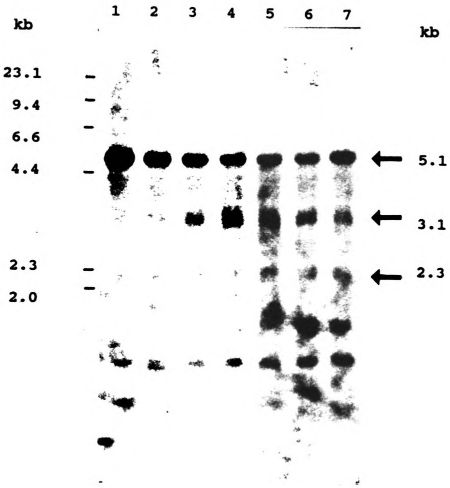

Figure 49. Assessment of the myc oncogene for the presence of DNase I hypersensitive sites in B6C3F1 mouse liver. DNA was prepared and treated as described in Figure 39 except that Hind III was used for digestion and the DNA was hybridized to a ^{32}P-labelled probe for myc (Pst I fragment). Lane 1, 0°C control; lane 2, 15°C control; lanes 3-7, DNA from nuclei treated with increasing concentrations of DNase I (28-47 units/ml). The bands of approximately 2 and 3 kb appear to result from cleavage of the 5 kb band at a DNase I hypersensitive site (see arrows).

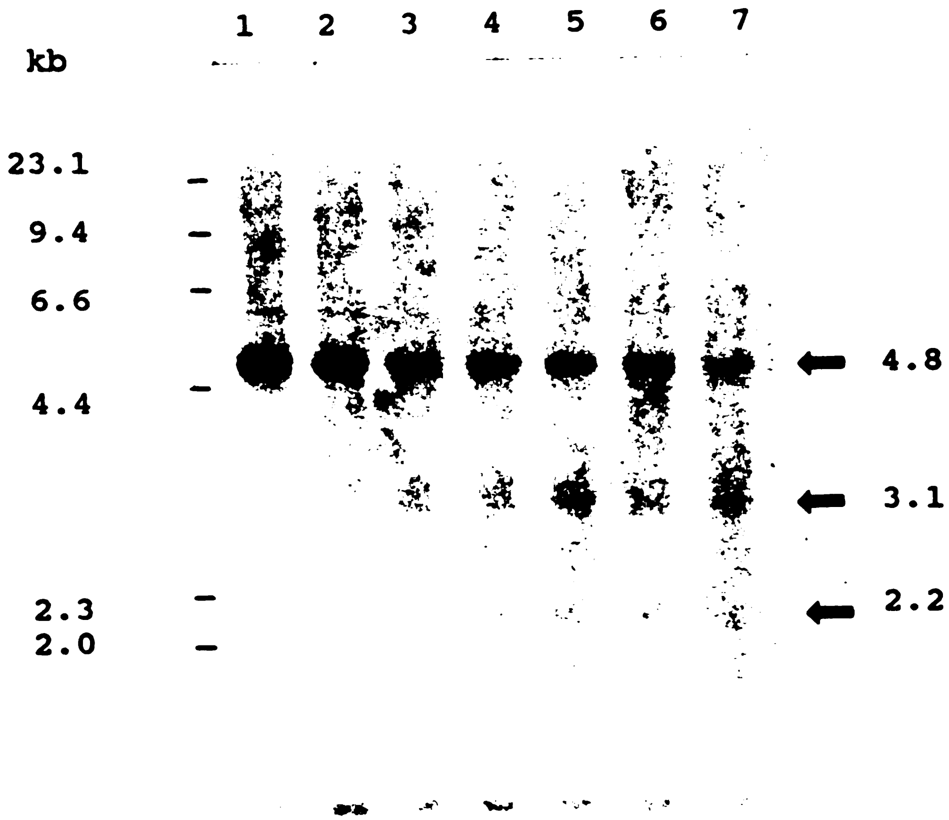

Figure 50. Assessment of the myc oncogene for the presence of DNase I hypersensitve sites in C3H/He mouse liver. DNA was prepared and treated as described in Figure 40 except that Hind III was used for digestion and the DNA was hybridized to a ^{32}P-labelled probe for myc (Pst I fragment). Lane 1, 0°C control; lane 2, 15°C control; lanes 3-7, DNA from nuclei treated with increasing concentrations of DNase I (28-47 units/ml). The bands of approximately 2 and 3 kb appear to result from cleavage of the band at approximately 5 kb at a DNase I hypersensitive site (see arrows).

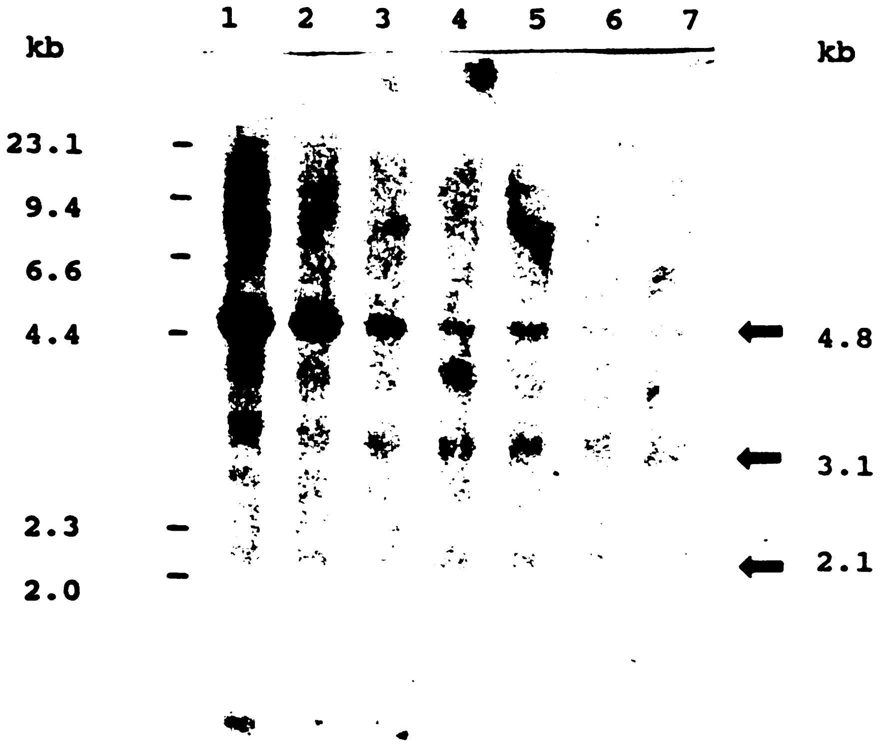

Figure 51. Assessment of the myc oncogene for the presence of DNase I hypersensitive sites in C57BL/6 mouse liver. DNA was prepared and treated as described in Figure 41 except that Hind III was used for digestion and the DNA was hybridized to a ^{32}P-labelled probe for myc. lane 1, 0°C control; lane 2, 15°C control; lanes 3-7, DNA from nuclei treated with increasing concentrations of DNase I (28-47 units/ml). The bands at approximately 2 and 3 kb appear to result from cleavage of the approximately 5 kb band at a DNase I hypersensitive site (see arrows).

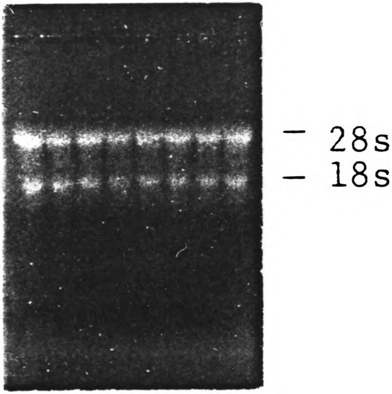

Figure 52. Assessment of RNA integrity. Total RNA was denatured with glyoxal, and 13 μg samples were electrophoresed on a 1.0% agarose gel. After ethidium bromide staining, the gel was illuminated with UV light and photographed. The figure shows a typical result, and RNA was assumed to be intact only if a similar result was obtained.

Figure 53 presents the result of hybridizing RNA derived from benzidine-induced tumors (lanes 2, 4, 6, and 8) and adjacent non-tumor tissue (lanes 1, 3, 5, and 7) to the ^{32}P-labelled Ha-ras probe. It can be seen that in 3 cases, RNA from tumors shows a slightly greater degree of hybridization (lanes 4, 6, and 8) than RNA from the adjacent non-tumor tissue (lanes 3, 5, and 7). Densitometry revealed that the intensity of the Ha-ras band is increased from 14% to 67% in tumor versus non-tumor in these three cases (Table 2). It thus appears that tumors sometimes contain an elevated level of Ha-ras mRNA as compared to non-tumor tissue.

When a duplicate Northern transfer was hybridized to the Ki-ras probe, very faint bands were produced, even after an extended exposure period (not shown). Hybridization of another duplicate Northern transfer to the myc probe produced no bands (not shown). These results indicate that there is a low level of Ki-ras mRNA in benzidine-induced tumors and adjacent non-tumor tissue, whereas the level of mRNA coresponding to myc, if present, is beyond the limits of detection by the method used.

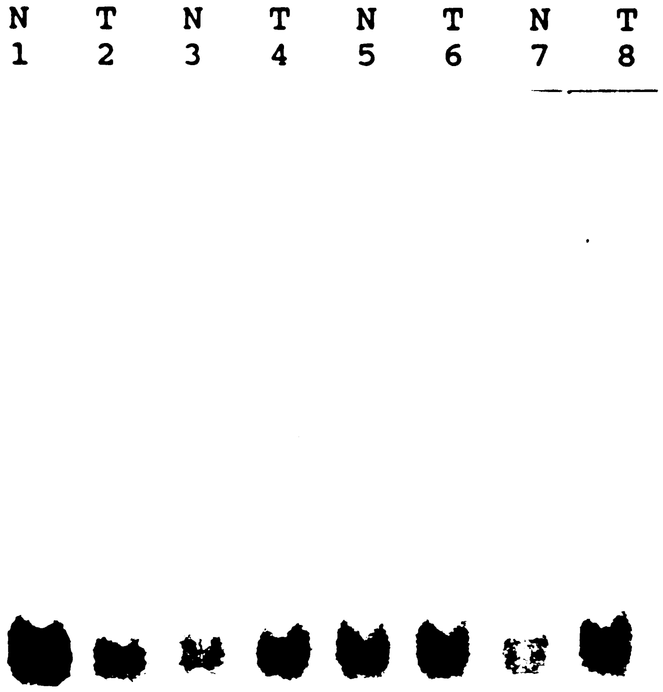

Figure 53. Levels of Ha-ras mRNA in benzidine-induced tumors and adjacent non-tumor tissue. RNA was isolated, denatured with formaldehyde, and electrophoresed as described in Methods. Lanes 1, 3, 5, and 7, non-tumor RNA; lanes 2, 4, 6, and 8, tumor RNA. Note the increased intensity of the hybridization in lanes 4, 6, and 8 as compared to lanes 3, 5, and 7, respectively.

TABLE 2

Ha-ras mRNA Levels in Benzidine-Induced B6C3F1 Mouse Liver Tumors and Adjacent Non-tumor Tissue

Lane Number	Sample[a]	Intensity[b]	% Change[c]
1	N-1	0.245	
2	T-1	0.137	-44
3	N-2	0.107	
4	T-2	0.152	+42
5	N-3	0.141	
6	T-3	0.160	+14
7	N-4	0.084	
8	T-4	0.140	+67

[a] N represents RNA isolated from non-tumor liver tissue; T represents RNA isolated from adjacent tumor tissue.

[b] Each lane on the autoradiograph presented in Figure 53 was scanned with an LKB UltroScan XL densitometer. The resulting peaks were cut out and weighed.

[c] The difference in intensity between non-tumor and corresponding tumor samples was divided by the intensity of the non-tumor sample.

DISCUSSION

1. Differential Potential for Expression of Oncogenes in B6C3F1, C3H/He, and C57BL/6 Mouse Liver

A. Hypomethylation of Ha-ras in B6C3F1 and C3H/He mouse liver

Use of the BS-9 probe for methylation state assessment revealed that the Ha-ras oncogene is differentially methylated in the liver of B6C3F1, C3H/He, and C57BL/6 mice. This gene is relatively hypermethylated in the C57BL/6 mouse, a condition consistent with quiescence. In contrast, the Ha-ras oncogene tends to be relatively hypomethylated in B6C3F1 and C3H/He mice, thus suggesting that this gene has a greater potential for activity in these two strains than in the C57BL/6 mouse.

Due to the frequency with which an activated Ha-ras oncogene has been observed in B6C3F1 mouse liver tumors (Reynolds et al., 1986; Wiseman et al., 1986; Reynolds et al., 1987; Stowers et al., 1988), it appears that Ha-ras activation may play a causal role in the genesis of hepatomas in this animal. It is possible that a state of relative hypomethylation of the Ha-ras oncogene facilitates its aberrant expression and thereby contributes to neoplastic growth. When considering the three strains of mice examined in this study, a positive correlation has been discerned between relative hypomethylation of Ha-ras and spontaneous hepatoma incidence. The Ha-ras oncogene is relatively hypomethylated in B6C3F1 and C3H/He mouse liver, and both of these strains have a high propensity for hepatoma development (both spontaneous and chemically-induced). In contrast, this gene is relatively hypermethylated in C57BL/6 mouse liver, and this strain exhibits a spontaneous hepatoma incidence of nearly zero and appears to be less sensitive to induction of liver tumors by chemicals such as phenobarbital (Becker, 1982). Thus, the methylation state of the Ha-ras oncogene in nascent mouse liver may be a determinant of the propensity of this gene to become activated.

The state of relative hypomethylation of Ha-ras in the liver of B6C3F1 and C3H/He mice as compared to the C57BL/6 mouse discerned using the BS-9 clone was corroborated by use of a Ha-ras probe from Oncor. These studies revealed that the Ha-ras oncogene is hypomethylated in a site-specific manner in B6C3F1 and C3H/He versus C57BL/6 mouse liver. This was observed in both sexes. This absence of a methylated site in the two hepatoma-prone strains may play a role in facilitating expression of this gene. Two mechanisms for this phenomenon are possible. First, the site under consideration may play a key role in the control of Ha-ras transcription. In other words, when this site is unmethylated, transcription of Ha-ras is permitted, whereas methylation acts to impede transcription. Second, to the extent that hypomethylation is one of the requirements for trancription of a gene, the absence of methylation at one critical site can be viewed as bringing the gene a step closer to the degree of hypomethylation required to permit enhanced transcription. Thus, the Ha-ras oncogene in B6C3F1 and C3H/He mouse liver may be primed for expression by virtue of its relatively hypomethylated state. However, the dissimilar restriction pattern produced by Msp I and Hpa II digestion indicate that the Ha-ras oncogene is generally hypermethylated and therefore has a low potential for expression.

This scenario is compatible with the view that a critical mutation is involved in the activation of the Ha-ras oncogene. It is axiomatic that an activating mutation cannot exert phenotypic effects unless the gene is also expressed. If the altered gene is quiescent, no effect will be seen, although the cell is now primed for transformation. At this point, the critical step becomes deregulation of gene expression. Only if the mutated gene achieves a state permissive for expression can phenotypic changes be seen. On the other hand, a mutation in an active gene or in a gene in a state permissive for expression may have a more immediate effect. This scenario is supported by the finding that the c-Ha-ras gene is hypomethylated in mouse epidermis, a cell type known to be susceptible to oncogenic conversion by a single-point mutation (Ramsden et al., 1985). It has been speculated that carcinogens can more easily access hypomethylated regions of DNA (Balmain et al., 1986); i.e., hypomethylated genes are mutational "hot spots". However, an alternate explanation is that hypomethylated genes are primed for expression. A mutation in such a gene would thus have a high probability of affecting the phenotype of the cell (Goodman et al., 1986).

Support for this hypothesis comes from the finding that the methylation state of the Ha-ras oncogene exerts a profound effect on its transforming ability. In an elegant study by Borrello and coworkers (1987), the pT24-C3 plasmid, which contains an Ha-ras oncogene possessing an activating mutation in codon 12, was evaluated for its ability to transform NIH 3T3 cells when methylated and unmethylated. It was found that methylation of the internal cytosines at all 5'-CCGG-3' and 5'-GCGC-3' sites results in an 80% decrease in the transforming efficiency of this plasmid, and a longer delay prior to foci formation was noted after transfection with the methylated versus the unmethylated oncogene. The promoter region was found to be demethylated in all transformants, whereas the body of the gene exhibited variable methylation, suggesting that hypomethylation of the promoter region, at minimum, is necessary for transcription to proceed. Furthermore, 5-azaCR treatment of NIH 3T3 cells transfected with the methylated plasmid increased the number of foci produced to the level of 5-azaCR treated NIH 3T3 cells transfected with an unmethylated oncogene. These results indicate that the ability of the Ha-ras oncogene to exert phenotypic effects is dependent on its expression. Inhibition of transcription via methylation abolishes the transforming capacity of pT24-C3, even though an activating mutation is present.

The relatively hypomethylated state of the Ha-ras oncogene in B6C3F1 and C3H/He mouse liver may result from an inability of this gene to be maintained in a methylated state. Two possible mechanisms, discussed below, may be pictured as underlying this phenomenon.

Because Ha-ras expression has been found to be elevated in developing (Zhang et al., 1987) and regenerating (Thompson et al., 1986) liver, the protein product of this gene appears to play a role in liver development. Therefore, it is likely that the Ha-ras gene exists in a hypomethylated state in fetal liver and acquires a hypermethylated state as the liver matures and Ha-ras expression diminishes; this is probably accompanied by Ha-ras methylation through de novo methylase activity. It is possible that the Ha-ras oncogene near the observed unmethylated site is a poor substrate for the methylase. One explanation for this possibility is alteration of the DNA sequence in this region of the Ha-ras gene in B6C3F1 and C3H/He as compared to C57BL/6 mice. As a consequence, the conformation of chromatin may be altered in such a way that the methylase cannot access this site.

The second possibility involves altered demethylase activity at the Ha-ras locus. Demethylation of DNA appears to be enzymatic (Gjerset and Martin, 1982; Razin et al., 1986) and has been proposed as a mechanism to activate tissue-specific genes during differentiation (Razin et al., 1986). The Ha-ras oncogene in B6C3F1 and C3H/He mouse liver may be a good substrate for the demethylase, resulting in a relatively hypomethylated state of this gene. Again, this could result from an alteration in base sequence near the observed unmethylated site in

Ha-ras, resulting in a chromatin configuration that facilitates demethyalse activity at this site.

Supporting the idea that a mutation can affect a gene's potential for transcriptional activity is the finding that the T24 form of the Ha-ras oncogene contains a mutation in an intervening sequence whose effect is to increase the expression level of this gene ten-fold (Cohen and Levinson, 1988). Because the mutation occurs in a non-coding region, it is unlikely that it results in an altered protein product; further experiments revealed no qualitative alterations in the p21 protein as a result of this mutation.

The possibility that an altered base sequence can affect the transcription rate of a gene without altering the protein product is intriguing for a number of reasons. First, a compromised ability of Ha-ras to be maintained in a methylated state as a result of an altered base sequence in the Ha-ras gene of B6C3F1 and C3H/He mice as compared to C57BL/6 mice is a heritable trait. Assuming that activation of Ha-ras is necessary for hepatoma development and hypomethylation of Ha-ras plays a role in this activation process, this possibility agrees with the observed heritable incidence of hepatoma development in B6C3F1 and C3H/He, and C57BL/6 mice. Second, it has been observed that transfection of multipotential murine stem cells with a twelfth codon-mutated Ha-ras oncogene, followed by induction of differentiation, produces fibroblasts displaying the transformed phenotye and expressing a high level of the p21 protein product (Bell et al., 1986). Other differentiated cells were non-transformed and expressed a low level of p21. However, transfection of the same gene into already differentiated fibroblasts produced no transformants. These results suggest that expression of Ha-ras in stem cells may be required for differentiated cells to be subject to Ha-ras-induced transformation and also indicate that cell type specificity exists for sensitivity to transformation. Deranged control of Ha-ras expression during liver development through altered methylation may predispose hepatocytes to Ha-ras-induced transformation. Furthermore, this study clearly demonstrated that an increased level of Ha-ras expression is required for transformation to occur.

B. Methylation state of the Ki-ras and myc oncogenes in B6C3F1, C3H/He, and C57BL/6 mouse liver

No differences in the methylation state of hepatic K-ras and myc oncogenes were discerned between B6C3F1, C3H/He, and C57BL/6 mice. These results indicate that there is no increased potential for expression of these oncogenes in the hepatoma-prone strains as compared to the non-hepatoma-prone strain. Furthermore, Ki-ras and myc were observed to be hypermethylated in all cases, suggsting that these genes possess a low potential for expression in normal mouse liver.

C. DNase I hypersensitive sites in oncogenes

Using the restriction endonuclease EcoR1, the Ha-ras oncogene was found to possess a DNase I hypersensitive site in B6C3F1, C3H/He, and C57BL/6 mouse liver, suggesting that this gene has some potential for expression in all three strains. However, use of Hind III for DNase I hypersensitive site detection revealed such a site only in B6C3F1 and C3H/He mouse liver. Although it is possible that detection of the same DNase I hypersensitive site was achieved by the two enzymes, this is unlikely due to the fact that a DNase I hypersensitive site in the Ha-ras gene of C57BL/6 mouse liver was observed following EcoR1 but not Hind III digestion. It thus appears that the Ha-ras oncogene possesses two DNase I hypersensitive sites in both hepatoma-prone strains, whereas this gene contains only one DNase I hypersensitive site in the non-hepatoma-prone strain C57BL/6. This result suggests that Ha-ras may have a greater potential for trancriptional activity in B6C3F1 and C3H/He mouse liver as compared to C57BL/6 mouse liver, a finding in agreement with the differences detected in the methylation status of this gene in the three strains (Section 1A). Indeed, the phenomena of relative hypomethylation of Ha-ras and an increased number of DNase I hypersensitive sites may be related. It has been found that an unmethylated gene, when transfected ito mouse L cells, integrates into the host DNA in a DNase I-sensitive conformation, whereas integration of a methylated gene occurs in a DNase I-insensitive conformation (Keshet et al., 1986). These results suggest that methylation of a gene affects chromatin structure by altering the interactions of its DNA, RNA, and protein components.

A number of studies have demonstrated a correlation between an increased number of DNase I hypersensitive sites in a gene and an increased potential for its expression. For example, both the major chicken vitellogenin gene (Burch and Weintraub, 1983) and the mouse metallothionein gene (MacArthur and Lieberman, 1987) exhibit one or more DNase I hypersensitive sites during periods of quiescence. When expression of these genes is induced, an additional set of DNase I hypersensitive sites becomes evident. These results indicate that an increase in the number of hypersensitive sites in a gene is associated with an increased potential for its expression. This relationship supports the assertion that an additional DNase I hypersensitive site in the Ha-ras oncogene of B6C3F1 and C3H/He mouse liver as compared with the C57BL/6 mouse liver may indicate an increased potential for its expression in the former two strains.

Similarly, the Ki-ras oncogene was found to possess a DNase I hypersensitive site in B6C3F1 and C3H/He mouse liver, but not in C57BL/6 mouse liver. Again, this result suggests a greater potential for Ki-ras expression in the two hepatoma-prone strains than in the non-hepatoma-prone strain. In contrast, the myc oncogene exhibits a DNase I hypersensitive site in the liver of all three mouse strains, suggesting an equal potential for transcriptional activity of myc in each. During compensatory hyperplasia following partial hyperplasia, expression of myc and ras oncogenes has been found to increase within 2 hours and 15 hours, respectively (Thompson et al., 1986). Therefore, these genes must possess some potential for transcriptional activity in normal adult liver in order for expression to commence so quickly. The presence of at least one DNase I hypersensitive site in Ha-ras and myc observed in this study may be indicative of this potential.

2. Increased Potential for Oncogene Expression in B6C3F1 Mouse Liver Tumors

A. Hypomethylation of the Ha-ras oncogene

Msp I/Hpa II restriction enzyme analysis revealed that the Ha-ras is hypomethylated in benzidine-, phenobarbital-, and chloroform-induced and spontaneous B6C3F1 mouse liver tumors as compared to non-tumor liver tissue. In DNA from each tumor, a shift in the Hpa II restriction pattern to more closely resemble that of Msp I indicates that Ha-ras has acquired a hypomethylated state, suggesting that this gene has a greater potential for expression in neoplastic tissue than in normal tissue. This conclusion is supported by the results of experiments using Hha I for methylation assessment. In benzidine-induced tumors, a consistent shift in the Ha-ras band to a smaller size was observed; in tumors from the other three treatment groups, a similar alteration was sometimes seen, along with the appearance of additional bands of a much smaller size. Thus, it appears that regulation of transcriptional activity of the Ha-ras oncogene is compromised in tumor tissue. This situation may serve to facilitate Ha-ras expression and thereby contribute to hepatoma development.

A number of investigators have found that overexpression of the normal (non-mutated) Ha-ras oncogene can cause transformation under certain conditions. The normal rat (DeFeo et al., 1981) and human (Chang et al., 1982) Ha-ras oncogenes, when transfected into NIH 3T3 cells, are unable to effect transformation. However, when these genes are ligated to an efficient viral promoter, the ability to transform these cells is acquired. In both cases, a high level of the p21 protein product was evident in foci. These results indicate that an increased level of expression of the normal cellular Ha-ras oncogene is sufficient for transformation in this paradigm.

Similarly, the normal Ha-ras oncogene can transform NIH 3T3 cells if a large amount (15-30 μg DNA/$2x10^5$ cells) of cloned oncogene is used for transfection (Pulciani et al., 1985). Foci were found to contain multiple copies of the Ha-ras gene, and expression was proportional to copy number. Further experiments revealed no evidence of an activating mutation. In contrast, overexpression of the normal Ha-ras oncogene in the rat embryo fibroblast cell line Rat-1 is capable of producing only partial transformation; a mutated Ha-ras oncogene was required for full transformation (Ricketts and Levinson, 1988). The differential ability of overexpression of Ha-ras to transform NIH 3T3 and Rat-1 cells may be due to the fact that NIH 3T3 cells display characteristics of a semi-transformed cell line and may be easier to transform. In addition, overexpression of the normal Ha-ras oncogene in early passage rat cells can rescue these cells from senescence (Spandidos and Wilkie, 1984). Taken together, these results indicate that increased expression of the normal Ha-ras oncogene contributes to transformation, although the degree to which this ability is manifested depends upon the characteristics of the cell system used.

The transforming efficiency of a mutation-activated Ha-ras oncogene also has been demonstrated to be influenced by its level of expression. The T24 Ha-ras oncogene, which contains a mutation in codon 12, can transform early passage rodent cells when linked to transcriptional enhancers (Spandidos and Wilkie, 1984; Spandidos, 1986). Other investigators have reported that T24 is unable to effect transformation in rat embryo fibroblasts unless a second, complementing oncogene is cotransfected into these normal cells (Land et al., 1983b; Ruley, 1983); this observation may be due to a low level of T24 Ha-ras expression. It thus appears that a mutant Ha-ras oncogene, when expressed at a high level, is sufficient for full transformation of normal cells, whereas a low level of expression requires further alterations supplied by a second oncogene. In addition, an increased level of T24 Ha-ras has been correlated with an increased metastatic potential in 10T1/2 and NIH 3T3 cells (Egan et al., 1987). The above results indicate that an elevated level of expression of a mutated Ha-ras oncogene may elicit a shift toward the phenotypic alterations characteristic of malignancy.

In vivo, the level of Ha-ras expression has been found to be elevated during carcinogenesis, most notably in skin (Balmain et al., 1984; Pelling et al., 1986, 1987) and liver (Makino et al., 1984a; Corcos et al., 1984; Cote et al., 1985; Yaswen et al., 1985). It appears that an increase in transcriptional activity is a relatively early event in the carcinogenic process, having been observed in benign skin papillomas (Balmain et al., 1984; Pelling et al., 1986, 1987), and in liver early after the commencement of a hepatocarcinogenic diet (Yaswen et al., 1985). Therefore, increased expression of Ha-ras may contribute to the transformation process. However, an activating point mutation has been detected in the Ha-ras oncogene concurrent with its overexpression (Pelling et al., 1987), suggesting that both alterations are required for full transformation.

B6C3F1 mouse liver tumors frequently possess an Ha-ras oncogene containing a critical mutation, often in codon 61 (Wiseman et al., 1986; Reynolds et al., 1986, 1987; Stowers et al., 1988). The p21 product of this mutated gene exhibits altered electrophoretic mobility and, presumably, altered biochemical activity compared to the normal p21 protein. Thus, it appears that Ha-ras activation in B6C3F1 mouse liver often involves mutation. However, these same studies show that some B6C3F1 liver tumors test negative in the NIH 3T3 cell transfection assay, indicating that a mutated Ha-ras oncogene does not exist in these tumors. This suggests that other biochemical mechanisms exist by which phenotypically normal mouse hepatocytes can be transformed into malignant cells. One such mechanism is the activation of Ha-ras through increased expression in the absence of a mutation that alters physical and biochemical characteristics of the p21 protein. The finding in this study that Ha-ras possesses a relatively high potential for expression in tumors supports this possibility. An increased potential for transcriptional activity also may contribute to the activation of Ha-ras in the presence of a critical mutation. Furthermore, the relatively high potential for Ha-ras expression may underlie the high frequency with which this gene is observed to be activated in B6C3F1 mouse as compared to rat liver tumors (Stowers et al., 1988).

B. Hypomethylation of the Ki-ras oncogene

Assessment of the methylation state of the Ki-ras oncogene in benzidine-, phenobarbital-, and chloroform-induced and spontaneous tumors revealed that this gene is frequently methylated to a lesser degree in tumors as compared to normal liver tissue. Msp I/Hpa II analysis showed that the Hpa II restriction pattern was often shifted to resemble more closely that of Msp I in tumor samples, whereas a shift to a smaller size was frequently observed in tumor DNA digested with Hha I. These results indicate that the Ki-ras oncogene is hypomethylated in tumors as compared to non-tumor tissue and therefore suggests that this oncogene possesses an increased potential for expression in tumor tissue. Therefore, the hypomethylated state of Ki-ras may contribute to the development of hepatomas by facilitating aberrant expression of this oncogene.

A variety of tumors have been demonstrated to contain an amplified Ki-ras oncogene (Winter et al., 1985; Winter and Perucho, 1986; Heighway and Hasleton, 1986; Bos et al., 1986). This observed increase in copy number is thought to result in an overall increase in transcription of the amplified gene. In addition, greatly increased expression of Ki-ras has been observed in a bone marrow-derived mouse cell line (George et al., 1986). Experimental evidence indicates that this is the result of integration of viral DNA containing an efficient promoter upstream from the first exon of Ki-ras. These results suggest that overexpression of Ki-ras may contribute to carcinogenesis.

In addition, a number of tumors have been observed to contain a mutated Ki-ras oncogene (Guerrero et al., 1984; Nakano et al., 1984; Shimizu et al., 1983; Tahira et al., 1986; Bos et al., 1986). Similar to the Ha-ras oncogene, activating point mutations have been most frequently observed in codons 12 and 61 of Ki-ras. This is not surprising since both ras oncogenes encode a protein of molecular weight 21 kD (p21) which binds GTP. Presumably, an activating mutation alters the biochemical activity of the Ki-ras protein product in a manner such that it acquires the transforming capacity. However, it is axiomatic that the Ki-ras oncogene must be expressed before the p21 protein product can exert its transforming effects on phenotype.

Supporting this idea is the finding that the degree of transformation of rat fibroblast cells containing a mutated Ki-ras oncogene is a function of the level of Ki-ras expression (Winter and Perucko, 1986). Specifically, transfection of a mutated Ki-ras into the Rat-4 cell line resulted in morphological transformation only when Ki-ras was amplified; expression was determined to be proportional to copy number. Furthermore, those cells with a low copy number were observed to exhibit a low ability to grow in soft agar, whereas a small increase (< 10-fold) in gene dosage resulted in a greatly increased degree of anchorage independence and the acquisition of tumorigenicity. Similarly, a mutant Ki-ras oncogene is overexpressed relative to the normal allele in lung carcinoma cell lines, and this is sometimes associated with moderate amplification of the mutant gene (Winter et al., 1985). It thus appears that quantitative changes in Ki-ras expression may be involved in the transformation process.

The above results indicate that a moderate increase in expression of Ki-ras can exert a profound effect on phenotype. Therefore, the finding that the Ki-ras oncogene is often hypomethylated in B6C3F1 mouse liver tumors is significant. The increased potential for expression of Ki-ras in tumors may facilitate transcriptional activity of this oncogene. Increased Ki-ras expression may, in turn, contribute to the phenotypic alterations associated with malignancy. Indeed, an increased level of Ki-ras expression has been observed during hepatocarcinogenesis (Corcos et al., 1984; Cote et al., 1985; Yaswen et al., 1985), providing further support for the hypothesis that an increased potential for Ki-ras expression may contribute to B6C3F1 mouse liver tumor development.

C. Amplification and hypomethylation of the myc oncogene

Hybridization of the myc probe to Msp I- and Hpa II-digested DNA showed an increased degree of hybridization to B6C3F1 mouse liver tumor DNA than to non-tumor DNA. This was observed in all tumor DNA samples, regardless of whether the tumors were chemically-induced or spontaneous. A probable explanation for this observation is amplification of the myc oncogene in tumors. It is possible that the increased intensity of myc bands in tumor-derived DNA is the result of a greater quantity of DNA affixed to the membrane. However, comparison of the general intensity of bands produced by hybridization of the Ha- and Ki-ras probes prior to and the pRSA 13 probe following myc hybridization make this scenario unlikely.

Because an increased copy number of a gene provides increased template available for transcription, it is thought that an amplified gene possesses the potential for enhanced expression. In addition, use of Hha I for methylation state assessment revealed that the myc oncogene is relatively hypomethylated in some tumors as compared to non-tumor liver tissue. These results suggest that

B6C3F1 mouse liver tumors have acquired an incrased potential for expression of the myc oncogene.

Many transformed cells exhibit an elevated myc expression level (Alitalo et al., 1987). This may result from amplification of the myc gene or alterations (including chromosomal rearrangement) such that myc is more efficiently transcribed. In addition, myc-containing retroviruses are thought to exert their transformating effects as a consequence of a high level of myc expression. Thus, it is clear that myc overexpression appears to participate in the carcinogenic process.

As mentioned in the Introduction, myc is thought to be involved in cell immortalization. The protein product encoded by the myc oncogene is known to bind DNA, and it has been demonstrated that the presence of myc protein is necessary for the commencement of DNA synthesis (Studzinski et al., 1986; Kaczmarek et al., 1985). A role for aberrant myc expression in transformed cells has been suggested by the finding that cell cycle control of myc expression is lost in a murine embryonic fibroblast cell line after chemically-induced transformation, although overexpression was not apparent (Campisi et al., 1984). Deregulated expression also has been found to inhibit differentiation of erythroleukemia cells (Prochownik and Kukowska, 1986; Dmitrovsky et al., 1986), suggesting that myc may play a role in the dedifferentiation often seen in malignancies.

Experimental evidence indicates that myc overepxression also may result in more overt signs of malignancy. The immortal cell line EK-3 requires transfection of both myc and ras to produce morphological transformation (Katz and Carter, 1986), suggesting that myc performs some additional function beyond immortalization in the carcinogenic process. Furthermore, overexpression of myc is sometimes sufficient to induce transformation. For example, a highly expressed myc oncogene in transgenic mice is thought to be reponsible for their frequent lymphoma development (Adams et al., 1985). Similarly, quail embryo fibroblasts exhibit a transformed morphology after infection with a highly expressed myc oncogene (Martin et al., 1986), and a high level of myc expression has been found to confer anchorage-independence and tumorigenicity upon rat fibroblasts (Pellegrini and Basilico, 1986).

An increased potential for myc expression has been noted in neoplastic tissue (Alitalo et al., 1987). This gene is hypomethylated in a number of human tumor cell lines (Cheah et al., 1984) and in human hepatocellular carcinoma (Nambu et al., 1987). Elevated myc expression has been noted in regenerating liver (Makino et al., 1984b) and during hepatocarcinogenesis (Makino et al., 1984a; Cote et al., 1985; Dragani et al., 1986). An elevated level of myc mRNA also has been noted in B6C3F1 mouse liver tumors (Dragani et al., 1986). These results suggest a causal role for the action of myc in hepatocarcinogenesis. Therefore, the finding in this study that myc has the potential for increased expression in B6C3F1 mouse liver tumors suggests that overexpression of myc may be involved in the development of these tumors.

Thus far, B6C3F1 mouse liver tumors have not been reported to contain an activated myc oncogene as assessed by the NIH 3T3 cell transfection assay, probably because this test is unable to detect overexpressed myc due to the fact that immortal cells are employed. Therefore, the novel finding that myc possesses an increased potential for expression in chemically-induced and spontaneous hepatomas is compatible with the results of studies utilizing NIH 3T3 cells for activated oncogene detection.

3. Ha-ras mRNA in Benzidine-Induced B6C3F1 Mouse Liver Tumors

Northern blot analysis of RNA in benzidine-induced liver tumors and adjacent non-tumor tissue from B6C3F1 mice revealed a moderately increased level of Ha-ras mRNA in the neoplastic tissue in three out of four cases. This result suggests that tumors have an increased rate of Ha-ras transcription. The observed elevated Ha-ras mRNA level supports the assertion that the Ha-ras oncogene has an enhanced potential for transcriptional activity in tumors versus non-tumor tissue. Therefore, the mechanism by which the Ha-ras oncogene is activated in B6C3F1 mouse liver tumors may include increased expression of this gene, ultimately resulting in an elevated level of the p21 protein product.

As discussed in Section 2A, an increase in the p21 protein of the Ha-ras oncogene can greatly increase its transforming capability. Conversely, it has been demonstrated that even a two-fold reduction in the level of an activated p21 protein from a closely related ras gene leads to reversion of the transformed phenotype in a spontaneous human fibrosarcoma cell line (Paterson et al., 1987). Therefore, the modest increase in Ha-ras mRNA levels observed in benzidine-induced tumors may have a profound impact on B6C3F1 mouse liver tumorigenesis.

It has been proposed that the Ha-ras oncogene is expressed at a basal level in a tightly controlled manner; expression then is subject to extensive modulation by a novel regulatory element (Cohen and Levinson, 1988). A number of studies have discerned a moderate increase (2-3 fold) in Ha-ras mRNA levels during rat hepatocarcinogenesis (Beer et al., 1986; Cote et al., 1985; Corcos et al., 1984; Makino et al., 1984a), suggesting that the escape of this gene from normal regulation is a common phenomenon during liver carcinogenesis. In agreement with this, a slightly elevated level of Ha-ras mRNA also has been observed in DEN-induced B6C3F1 mouse liver tumors (Dragani et al., 1986).

Due to the frequency with which a critical mutation has been detected in Ha-ras in B6C3F1 mouse liver tumors, it is probable that altered biochemical activity of the p21 protein product plays a role in tumorigenesis. However, an increased transcription rate of Ha-ras is likely to exacerbate the effects of such a mutation by increasing the amount of p21. Thus, the increased level of Ha-ras mRNA seen in three of four benzidine-induced B6C3F1 mouse liver tumors may be involved in the mechanism by which a normal cell is transformed into a malignant cell.

4. Implications of Increased Potential for Oncogene Expression

The fact that B6C3F1 and C3H/He mice possess a relatively hypomethylated Ha-ras oncogene suggests that transcriptional activation of this gene may require fewer steps than activation of the same gene in the C57BL/6 mouse; this characteristic may facilitate aberrant Ha-ras expression. Since Ha-ras appears to be primed for expression, the occurrence of an activating mutation within this gene would be expected to have a high probability of exerting phenotypic effects. In this manner, both a predisposition toward Ha-ras expression and a mutation at a critical site in the Ha-ras gene may contribute to the Ha-ras activation believed to be involved in the development of both spontaneous and chemically-induced liver tumors.

Tumorigenesis may result from pleiotropic effects of B6C3F1 mouse liver carcinogens. Many B6C3F1 mouse hepatocarcinogens are mutagens. Therefore, one obvious consequence of carcinogen treatment is the production of an activating mutation in the Ha-ras oncogene. However, over half of the chemicals which produce hepatomas in the B6C3F1 mouse are non-mutagens as assayed in the Ames test (Ashby and Tennant, 1988). Therefore, this group of B6C3F1 mouse hepatocarcinogens may exert their effects in an epigenetic manner, possibly by altering the methylation state of the Ha-ras gene.

It has been observed that treatment with a number of carcinogens results in decreased levels of DNA methylation (Wilson and Jones, 1983; Boehm et al., 1983;

Wilson et al., 1987a). Several mechanisms of carcinogen action may contribute to this phenomenon. First, carcinogen/DNA adducts may inhibit maintenance methylase from accessing hemimethylated sites. This effect on the Ha-ras oncogene may be exacerbated if, as previously proposed, a region of this gene is already a poor substrate for the methylase enzyme. Second, since some methylation of daughter strand DNA is delayed for up to several hours after DNA replication (Woodcock et al., 1986), it is possible that the hyperplasia caused by many carcinogens may result in a failure to complete this phase of methylation. Third, carcinogens may inhibit maintenance methylase activity, either by direct inhibition of the enzyme or by perturbing the substrate pool. Fourth, demethylase activity may be stimulated by carcinogen treatment, producing hypomethylation. If a region of Ha-ras is already a good substrate for the demethylase, an even greater degree of hypomethylation may result in this gene.

It is thus clear that a number of mechanisms exist by which carcinogens may interfere with DNA methylation. The possibilities involving an alteration of methylase or demethylase activity are especially intriguing, as these mechanisms involve an epigenetic event with a threshold effect level and are most likely to come into play during periods of cell proliferation. These three characteristics also are consistent with properties of chemicals known to be tumor promoters. As was noted in the Introduction, the B6C3F1 mouse appears to exhibit an altered response during the promotion phase of hepatocarcinogenesis compared to non-hepatoma-prone mice. Therefore, the B6C3F1 mouse liver may be especially sensitive to carcingen-induced DNA hypomethylation. In addition, due to the relatively hypomethylated state of the Ha-ras oncogene in B6C3F1 and C3H/He mouse liver, carcinogen-induced hypomethylation may more readily bring about the degree of hypomethylation required for enhanced transcriptional activity.

Spontaneous tumors in B6C3F1 and C3H/He mouse liver may arise from an Ha-ras oncogene which has been activated, in part, by hypomethylation in the absence of carcinogen treatment. The level of genomic 5-MC has been observed to decrease with age (Wilson et al., 1987b). It is possible that the Ha-ras oncogene, being relatively hypomethylated in young adult B6C3F1 and C3H/He animals, rapidly reaches the degree of hypomethylation permissive for enhanced transcription due to this normal aging phenomenon. Once again, a spontaneous mutation may be involved in full activation of the Ha-ras oncogene in this scenario.

Regardless of whether hypomethylation of Ha-ras occurs through the actions of carcinogens or through natural processes (e.g., aging), it appears that this phenomenon plays a role in B6C3F1 mouse liver carcinogenesis. This statement is based on the fact that Ha-ras was observed to be hypomethylated in all tumors examined, regardless of whether they were spontaneous or induced by benzidine, phenobarbital, or chloroform.

The presence of a greater number of DNase I hypersensitive sites in the Ha-ras and Ki-ras oncogenes in B6C3F1 and C3H/He as compared to C57BL/6 mouse liver suggests that these genes have a greater potential for expression in the former two strains. Due to the chromatin conformation associated with DNase I hypersensitive sites, transcription factors such as RNA polymerase and topoisomerases might more easily access these genes in B6C3F1 and C3H/He mouse liver. In this manner, the Ha- and Ki-ras oncogenes might be expressed more readily in these two hepatoma-prone strains. In addition, both the relative hypomethylation of Ha-ras and the presence of additional DNase I hypersensitive sites in this gene support the assertion that the Ha-ras oncogene possesses an increased potential for transcriptional activity in the liver of B6C3F1 and C3H/He mice as compared to the C57BL/6 mouse.

The finding that the myc oncogene is amplified and therefore has achieved a high potential for expression in B6C3F1 mouse liver tumors is significant in light of the observation that more than one oncogene is required for full transformation. As discussed in the Introduction, myc and ras oncogenes appear to behave in a complementary fashion, and the actions of both oncogene products are necessary to effect transformation of normal cells. Therefore, amplification of myc may contribute significantly to the tumorigenic process.

SUMMARY AND CONCLUSIONS

Six major findings have resulted from the studies presented in this thesis. First, the Ha-ras oncogene appears to possess an increased potential for expression in the liver of the B6C3F1 and C3H/He hepatoma-prone mouse strains than in the liver of the non-hepatoma-prone C57BL/6 mouse. This conclusion is based on the observation that the Ha-ras gene is hypomethylated in a site-specific manner and possesses an additional DNase I hypersensitive site in the liver of the B6C3F1 and C3H/H strains as compared to the C57BL/6 strain. Second, the fact that B6C3F1 and C3H/He mouse liver possesses a DNase I hypersensitive site in the Ki-ras oncogene whereas no evidence of this site was observed in C57BL/6 mouse liver suggests that Ki-ras also has an increased potential for expression in the two hepatoma-prone strains. Third, the Ha-ras oncogene was found to display a greatly increased potential for expression in B6C3F1 mouse liver tumors as compared to non-tumor liver tissue. Hypomethylation of Ha-ras was observed in all tumors from the benzidine-, phenobarbital- and chloroform-induced and spontaneous groups, suggesting that increased Ha-ras expression is a common phenomenon in Ha-ras activation in B6C3F1 mouse liver tumors. Fourth, Ki-ras was sometimes observed to have an increased potential for expression in B6C3F1 mouse liver tumors from all four treatment groups. This result suggests that activation of Ki-ras may contribute to hepatocarcinogenesis, but with a lesser degree of commonality than that seen with Ha-ras. Fifth, the myc oncogene was found to be amplified in all tumors, regardless of whether they were chemically-induced or spontaneous. Therefore, increased potential for myc expression appears to be a common event in tumorigenesis in B6C3F1 mouse liver. Sixth, an elevated level of Ha-ras mRNA was observed in three of four benzidine-induced liver tumors, providing evidence that the increased potential for expression observed in this gene indeed results in increased transcription.

Based on these results, it can be concluded that the Ha-ras oncogene might be frequently activated in B6C3F1 mouse liver tumors as a consequence of its basally high potential for transcriptional activity. According to this scenario, a critical mutation, whether spontaneous or chemically-induced, would have a high probability of producing transforming phenotypic effects. This characteristic may, in part, underlie the high propensity for hepatoma development exhibited by B6C3F1 and C3H/He mice. In addition, it appears that increased potential for expression of oncogenes might be an underlying mechanism by which their aberrant expression is brought about in the multistep process by which a normal cell is transformed into a cancer cell. Hypomethylation of Ha-ras and Ki-ras, as well as amplification of myc, appear to be common mechanisms by which these oncogenes are activated in B6C3F1 mouse liver tumors. This observation supports the assertion that tumors induced by different classes of carcinogens or arising spontaneously share common biochemical pathways of oncogene activation during tumorigenesis.

These conclusions illustrate the importance of examining epigenetic parameters of oncogene expression in studies aimed at elucidating the molecular mechanisms by which cancer develops. In addition, it is clear that information regarding basal oncogene potential for expression is vital in formulating a rational interpretation of carcinogen bioassay data.

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