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Hypomethylation of raf and Ha-ras in Mouse Liver Following Cell Proliferation and in Mouse Liver Tumors

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HYPOMETHYLATION OF raf AND Ha-ras IN MOUSE LIVER FOLLOWING CELL PROLIFERATION AND IN MOUSE LIVER TUMORS

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ABSTRACT

HYPOMETHYLATION OF raf AND Ha-ras IN MOUSE LIVER FOLLOWING CELL PROLIFERATION AND IN MOUSE LIVER TUMORS

By

Jean Sara Ray

The liver tumor-prone B6C3F1 male (C57BL/6 female x C3H/He male) mouse, the more liver tumor-prone paternal strain (C3H/He), and the relatively resistant maternal strain (C57BL/6), plus the liver tumor-prone CD-1 male mouse were employed for this study. The objective was to test the hypothesis that hypomethylation of DNA might lead to the aberrant expression of proto-oncogenes which, along with mutation, appears to play a role in carcinogenesis. This was accomplished by examination of the methylation status and mRNA levels of raf and Ha-ras in the nascent liver, following cell proliferation, and in liver tumors. Rat liver tumors induced by the injection of viral-Ha-ras infected rat liver epithelial cells were also examined. Methylation status of the proto-oncogenes raf and Ha-ras was determined by digestion of DNA with methylation sensitive restriction endonucleases and Southern blot analysis. Messenger RNA and p21 ras protein levels were assessed by Northern and Western blotting, respectively.

The raf gene was relatively hypomethylated in the tumor-prone C3H/He relative to the tumor-resistant C57BL/6. The B6C3F1 appears to inherit a raf allele from the C57BL/6 that is methylated at the external cytosine in a 5'-CCGG-3'

sequence, while the corresponding allele inherited from the C3H/He is not methylated at this site. With regard to the 5'-CCGG-3' site of interest in raf, the CD-1 exhibited a level of methylation similar to the B6C3F1 strain. Hypomethylation of raf was found in B6C3F1 and C57BL/6 following partial hepatectomy, and in the B6C3F1, but not the C57BL/6, following phenobarbital administration (75 mg/kg/day) for 14 days. Ha-ras and raf were found to be hypomethylated in mouse liver tumors. Elevated levels of raf mRNA were found in phenobarbital-induced tumors, but not spontaneous tumors, while increased Ha-ras mRNA levels were found in both.

The studies reported here support the notion that hypomethylation of DNA, an epigenetic change that is expected to result from threshold-exhibiting events, is involved in the multistep process underlying carcinogenesis.

This dissertation is dedicated to my husband, John Bergsma. My graduate studies would not have been possible without his help and support.

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LIST OF ABBREVIATIONS

5MeC	5-methylcytosine
bp	base pairs
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EGF-R	epidermal growth factor receptor
kb	kilobases
LTR	long terminal repeat sequence
mRNA	messenger ribonucleic acid
PB	phenobarbital
PH	partial hepatectomy
PKC	protein kinase C
PLC	phospholipase Cγ
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
TGF-α	transforming growth factor-α
TGF-β	transforming growth factor-β
TPA	12-*O*-tetradecanoylphorbol-13-acetate

INTRODUCTION

1. An Overview of Multistep Carcinogenesis

The development of cancer is a complex, multistep process. Carcinogenesis can be thought of as the progression of cells from a normal to an abnormal phenotype. At least 3 events must occur in the classical 3 stage carcinogenesis model: a genetic change for initiation, promotion by selective proliferation of initiated cells, and one or more additional genetic changes during progression (reviewed in Pitot and Sirca, 1980; Farber, 1984; Boyd and Barrett, 1990; Weinstein, 1988). Initiation, promotion and progression are not necessarily discreet steps; some chemical compounds can function as one or more steps in the carcinogenic pathway. Diethylnitrosamine, for example, when applied repeatedly, will result in the development of skin tumors in C57BL/6♀ mice without the application of additional chemicals (Reiners, *et al.*, 1984).

2. The Role of Proto-oncogenes in Carcinogenesis

Proto-oncogenes were first discovered as the cellular homologs of the oncogenes present in transforming retroviruses. Proto-oncogenes are cellular genes which are generally involved in functions such as proliferation and

differentiation (reviewed in Bishop, 1991). They can be categorized by the cellular compartment in which their protein product functions: transmembrane (e.g. erbB), cytoplasmic (e.g. the ras family and raf), or nuclear (e.g. fos and jun). Alternatively, proto-oncogenes can be categorized by biochemical function. There are proto-oncogenes whose protein products function as protein tyrosine kinases (e.g. src, erbB), protein serine threonine kinases (e.g. raf, mos), GTPases (e.g. the ras family), and transcription factors (e.g. fos and jun which, together, function as the transcription factor AP-1 and enhance transcription of genes containing AP-1 enhancer sites). Proto-oncogenes are termed activated oncogenes when they develop transforming ability by changes in gene expression or mutations in the protein products which remove the normal controls on their biochemical functions. While activation of a single oncogene is not generally capable of causing tumors by itself, two or more oncogenes can act together to transform cells. Generally, oncogenes which code for cytoplasmic components of signal transduction pathways (e.g. ras) complement oncogenes which code for nuclear proteins involved in cell proliferation (e.g. myc) (Land, *et al.*, 1983).

A. The role of Ha-ras in carcinogenesis

Activation of Ha-ras by point mutations, primarily in codon 61, has been implicated in liver tumors in the B6C3F1 mouse and the transforming capabilities and tumorigenicity of these DNA sequences (Reynolds, *et al.*, 1987; Stowers, *et al.*,

1987; Wiseman, *et al.*, 1986; Fox, *et al.*, 1990). In the human Ha-ras gene, the mutation responsible for conferring transforming ability is most often in codon 12 (Taparowsky, *et al.*, 1982; Tabin, *et al.*, 1982; Reddy, 1982). The T24 ras gene from a human bladder cell carcinoma contains a mutation at codon 12, and, when transfected into an immortal human epithelial cell line, caused transformation and the transfected cells were tumorigenic (Hurlin, *et al.*, 1989).

Increased expression of Ha-ras has also been implicated in the transforming abilities of transfected DNA (Chang, *et al.*, 1982; Pulciani, *et al.*, 1985; Rimoldi, *et al.*, 1991; Huber and Cordingley, 1988; Seyama, *et al.*, 1988). Chang and coworkers (1982) found that induction of high levels of c-Ha-ras mRNA by dexamethasone treatment of cells transfected with LTR-complexed c-Ha-ras DNA resulted in transformation. Increased expression of normal Ha-ras mRNA and p21 due to transfection with large amounts of DNA containing normal c-Ha-ras was shown by Pulciani and coworkers, (1985) to result in a transformed phenotype in NIH 3T3 cells; these cells were also capable of forming tumors when injected into NIH Swiss mice. 5-aza-2'deoxycytidine treatment of nontumorigenic interferon-induced revertants of NIH 3T3 cells transfected with LTR-activated cellular-(c-)Ha-ras resulted in increased expression of Ha-ras mRNA and exhibition of a transformed phenotype (Rimoldi, *et al.*, 1991). When injected into nude mice these cells gave rise to malignant and metastatic tumors.

Thus, it is apparent that activation of Ha-ras by point mutation and increased expression of the Ha-ras gene are each capable of causing a transformed phenotype, and these two mechanisms might act synergistically or additively to produce a qualitatively more transformed phenotype. Huber and Cordingley (1988) transfected rat epithelial cells with viral-(v-)Ha-ras (which is capable of transforming NIH 3T3 cells and contains mutations in codons 12 and 59) under the transcriptional control of the corticosteroid-inducible long terminal repeat (LTR) of the murine mammary tumor virus (MMTV). Low levels of v-Ha-ras mRNA were correlated with a transformed phenotype. However, the induction of expression by dexamethasone of this mutated ras gene caused the cells to exhibit more profound transformed characteristics such as increasingly bizarre cell morphology and larger colony formation than non-induced transfected cells.

Many rodent liver tumors do not have detectible mutations in the Ha-ras gene. In recent studies, mutated Ha-ras was detected in 63-64% of spontaneous B6C3F1♂ liver tumors (Fox, *et al.*, 1990; Dragani, *et al.*, 1991) and 29% of spontaneous C3H/He tumors (Rumsby, *et al.*, 1991). Benzidine-induced liver tumors contained mutated Ha-ras at a rate of 59% in B6C3F1♂ (Fox, *et al.*, 1990) while diethylnitrosamine induction resulted in mutated Ha-ras in 41% of C3H/He mice (Rumsby, *et al.*, 1991). It is reasonable to suspect that one or more other oncogenes might also be involved in carcinogenesis.

B. The role of raf in carcinogenesis

The raf proto-oncogene is a good candidate for involvement in carcinogenesis because the raf gene product (Raf-1) plays a pivotal role in signal transduction by reception of signals from membrane-associated growth factor receptors and transmission of these signals to nuclear targets (Rapp, 1991). Viral raf was originally characterized as the transforming oncogene present in the 3611-MSV (murine sarcoma virus) (Rapp, *et al.*, 1983a). The v-raf protein is a fusion protein consisting of the amino-terminal 384 amino acids of the viral *gag* gene and the carboxy-terminal 323 amino acids of mouse c-raf (Rapp, *et al.*, 1983a; Rapp, *et al.*, 1983b). When transfected into NIH-3T3 cells, 3611-MSV DNA caused transformation and these cells were tumorigenic when injected into newborn NFS/N mice. Transfection of immortalized human bladder epithelial cells with 3611-MSV DNA also resulted in malignant transformation (Skouv, *et al.*, 1989).

There are three known members of the raf proto-oncogene family. A-raf-1 and B-raf are expressed in urogenital and brain tissue, respectively, while c-raf-1 is expressed in all tissues (Storm, *et al.*, 1990). The raf oncogene is related by sequence homology to the tyrosine kinase class of oncogenes (v-src, v-fps, v-abl, and v-fes), but does not demonstrate any tyrosine kinase activity (Mark, *et al.*, 1984). Raf-1 is a serine/threonine kinase which is activated by phosphorylation (Moelling, *et al.*, 1984). The mechanism by which Raf-1 transmits its nuclear signal appears

to be mediated through the heterodimeric (composed of the protein products of the c-fos and c-jun proto-oncogenes) transcription factor AP-1 (Wasylyk, *et al.*, 1989). Thus, activation of Raf-1 results in increased transcription of genes with AP-1 enhancer elements.

The cellular raf proto-oncogenes have transforming potential. For example, loss or substitution of the regulatory amino terminal region of raf genes results in transformation of cells (Ikawa, *et al.*, 1988). Activated c-raf genes (i.e. capable of causing transformation in cells transfected with the tumor DNA) from human and rodent tumors are frequently found to be fused to other genes such that the amino-terminal portion of the c-raf gene is missing (Ishikawa, *et al.*, 1986; Stanton *et al.*, 1987; Heidecker, *et al.*, 1990). Increased expression of raf genes has been implicated in tumorigenesis. Cells cultured from tumors induced by injection of v-raf infected rat liver epithelial cells exhibited an increased level of v-raf mRNA relative to the injected cells (Worland, *et al.*, 1990). Elevated levels of raf mRNA have been found in PB-induced rat liver tumors (Beer, *et al.*, 1988).Increased expression of raf proto-oncogenes has been implicated in the transforming ability of raf genes by others, such as Beck, *et al.* (1987) who showed that the normal A-raf-1 gene was capable of transforming NIH 3T3 cells when over-expressed in a viral vector.

C. Signal transduction in hepatocytes

Normal cell replication depends on the transduction of an external signal to the nucleus. In many transformed cells, however, cell proliferation is uncoupled from the signal transduction pathway resulting in growth factor independence. The following discussion will focus on the events occurring after binding of the hepatocyte growth-stimulatory factors epidermal growth factor (EGF) (Richman, *et al.*, 1976) or transforming growth factor-α (TGF-α) (Fausto and Mead, 1989) to the epidermal growth factor receptor (EGF-R) (reviewed in Velu, 1990) and the potential roles of ras p21 and Raf-1 proteins in hepatocyte signal transduction (Figure 1).

EGF and TGF-α are endogenous ligands for the EGF-R; EGF-R is activated by ligand binding and functions as a tyrosine kinase. Autophosphorylation of the EGF-R on tyrosine or serine phosphorylation by protein kinase C (PKC) leads to internalization and degradation of EGF and EGF-R. The EGF-R tyrosine kinase also phosphorylates other cellular proteins, including phospholipase Cγ (PLC) which then becomes activated. The activation of PLC is thought to be mediated through a G-protein. The p21 protein product of the ras family of proto-oncogenes (Ha-ras, Ki-ras, and N-ras) acting at the cytoplasmic face of the cellular membrane, binds and hydrolyzes GTP. Thus, p21 appears to act in a manner analogous to the alpha subunit of the G-proteins in signal transduction (Sigal, *et al.*, 1988) and might be involved in

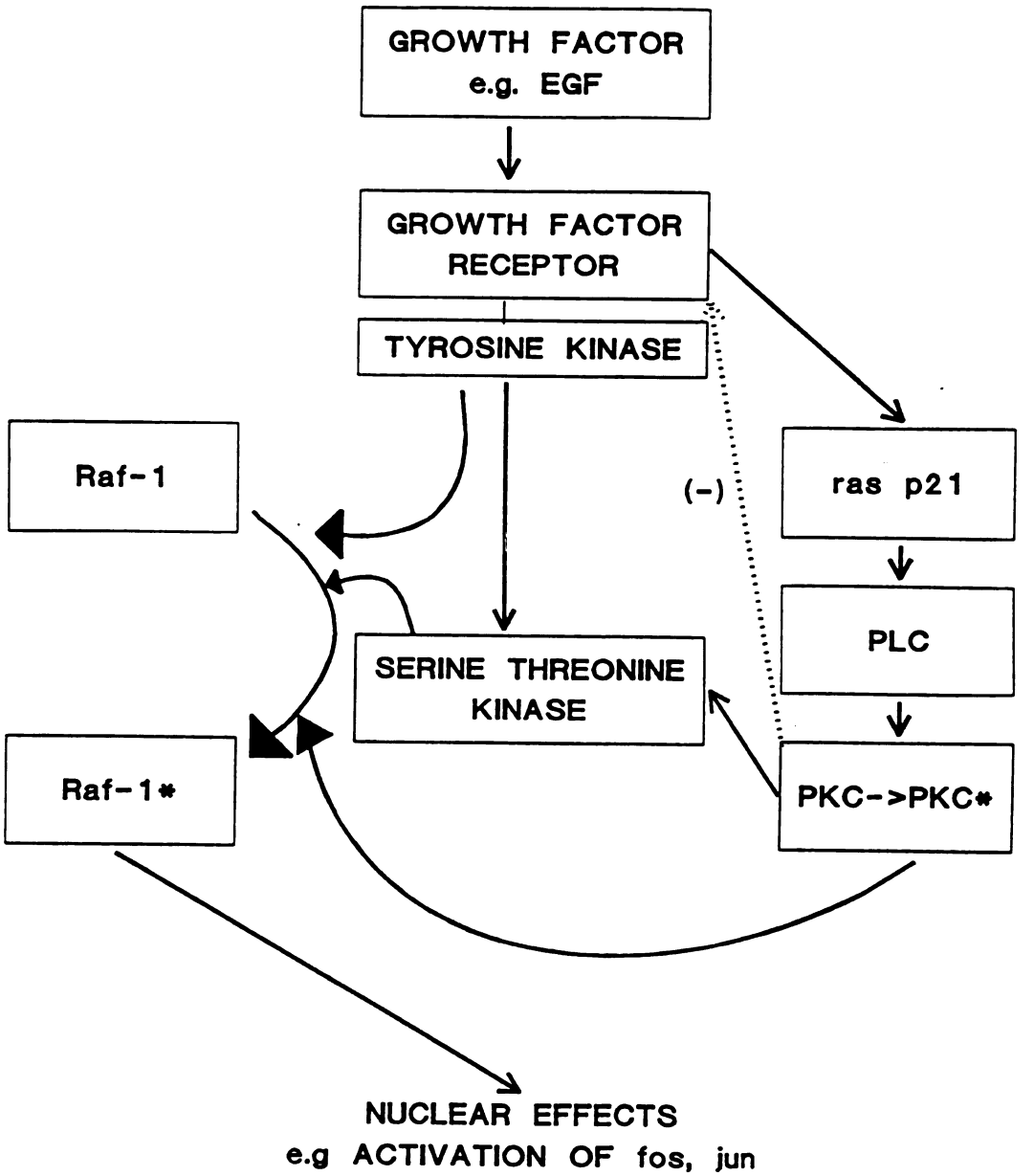


Figure 1. Hepatocyte signal transduction pathway. * denotes activated form of kinase.

the activation of PLC. PLC catalyzes the hydrolysis of phosphatidylinositol 4,5 bisphosphate into inositol 1,4,5 triphosphate, which functions to release intracellular stores of Ca^{++}, and diacylglycerol, which activates protein kinase C (PKC). Phorbol ester tumor promoters [e.g. 12-*O*-tetradecanoylphorbol-13-acetate (TPA)] can substitute for diacylglycerol and activate PKC (Nishizuka, *et al.*, 1986; Blumberg, 1991).

The serine/threonine kinase function of Raf-1 can be activated by phosphorylation on a tyrosine residue (Carroll, *et al.*, 1990; Morrison, *et al.*, 1989). However, phosphorylation in response to EGF has been demonstrated to occur on serine, not tyrosine residues (App, *et al.*, 1991). The most probable explanation for this apparent discrepancy is the presence of an intermediate serine/threonine kinase in the pathway between the EGF-R and Raf-1. In other words, stimulation of the EGF-R results in the activation of a serine/threonine kinase whose target is Raf-1. One candidate for the this intermediate kinase is PKC. T cell antigen mediated Raf-1 activation is dependent on phosphorylation by PKC (Siegel, *et al.*, 1990). Also, phorbol 12-myristate 13-acetate was shown to increase the activity of the raf protein through its action on PKC (Morrison, *et al.*, 1988). However, EGF stimulates serine phosphorylation of Raf-1 in cells which have been depleted of PKC (App, *et al.*, 1991), suggesting the presence of an as-yet-unidentified serine/threonine kinase. Another possibility for this intermediate serine/threonine

protein kinase is mitogen activated protein kinase (MAP-1 kinase), as suggested by the finding that MAP-1 kinase can phosphorylate Raf-1 *in vitro* and MAP-1 kinase can be activated by tyrosine and threonine phosphorylation (Anderson, *et al.*, 1991).

3. Carcinogenesis in Mouse Liver

A. The B6C3F1♂ mouse

The hybrid B6C3F1♂ mouse, along with the parental strains, C3H/He♂ and C57BL/6♀, is a good model to study the molecular mechanisms involved in the conversion of phenotypically normal cells to cancer cells. The B6C3F1♂ spontaneously develops liver tumors at a rate of approximately 30% (Becker, 1982, Buchmann, *et al.*, 1991). This is intermediate between the spontaneous liver tumor incidences of its parents, the C3H/He♂ at approximately 60% and the C57BL/6♀ with a negligible rate (Buchmann, *et al.*, 1991) which suggests a heritable factor influencing spontaneous tumor development. The susceptibility of the different strains (only regarding the sexes used in this study) to chemically-induced liver tumors is comparable to the incidence of spontaneous tumor development; B6C3F1♂ and C3H/He♂ are very sensitive to the chemical induction of liver tumors in response to both mutagens and many nonmutagenic compounds such as trichloroethylene (Clayson, 1987; Maronpot, *et al.*, 1987; Ashby and Tennant, 1988) while the C57BL/6♀ is relatively resistant (Buchmann, *et al.*, 1991). The B6C3F1♀ develops

fewer spontaneous liver tumors than the B6C3F1♂, but is equally, or even more sensitive to the chemical induction of liver tumors.

Clearly, there are one or more characteristics of the C3H/He which cause it to be more prone to liver tumor development than the C57BL/6. In histologically normal primary hepatocyte cultures from C3H/He mice, twice as many cells are synthesizing DNA (measured as ^{3}H-thymidine incorporation) as in hepatocytes derived from C57BL/6 mice (Hanigan, *et al.*, 1988). Primary C3H/HeNJcl hepatocyte cultures spontaneously develop multiple colonies, while C57BL/6NJcl hepatocyte cultures only rarely develop colonies (Lee, *et al.*, 1989). In a study of diethylnitrosamine-induced preneoplastic lesions in the livers of C3H/HeN<->C57BL/6N chimeric mice, it was found that relative to C57BL/6N cells, more (5x) C3H/HeN hepatocytes developed lesions and altered C3H/HeN cells exhibited a more malignant phenotype (Lee, *et al.*, 1991). There are intrinsic differences between the cells of the two strains which have the potential to influence susceptibility to liver tumors. One difference that has been found is that C3H/He, but not C57BL/6 mice possess the *Hcs* (hepatocarcinogen sensitivity) gene (Drinkwater and Ginsler, 1986). Another identified difference is a variation in methylation status. In comparison to the C3H/He♂ and B6C3F1♂, the C57BL/6♀ possesses at least one additional methylated site (5'-^{Me}CCGG-3') in the Ha-ras gene in the nascent liver (Vorce and Goodman, 1989a).

B. The CD-1♂ mouse

The CD-1♂ mouse is also a good model in which to study mouse liver carcinogenesis. The CD-1♂ develops spontaneous liver tumors at a rate similar to that of the B6C3F1♂ (Hoffmann LaRoche Laboratories, unpublished data) and is not related to either the C3H/He or C57BL/6. The CD-1 HaM/ICR mouse stock is a non-inbred subcolony of the minimally inbred (0.5% per generation) Icr:Ha(ICR) mouse stock (Eaton, *et al.*, 1980) which was developed from inbred Swiss mice (Lynch, 1969).

4. The Involvement of Cell Proliferation in Carcinogenesis

A. The role of cell proliferation in carcinogenesis

Cell proliferation plays a major role in carcinogenesis (Ames and Gold, 1990; Cohen and Ellwein, 1990). Epidemiologically, chronic mitogenic stimuli have been implicated in numerous human cancers (e.g. papilloma virus in cervical cancer, alcohol in liver cancer, cigarette smoke in lung cancer) (Preston-Martin, *et al.*, 1990). Carcinogen bioassays involve the administration of chemicals at the maximum tolerated dose. This high dose can cause cell death and thus act as a stimulus for mitogenesis, in a manner similar to chronic wounding and wound repair. Alternatively, some noncytotoxic compounds, such as PB, stimulate cell proliferation without causing cell death. Under conditions in which an increased number of cells are undergoing mitogenesis at the same time, there is a higher probability that errors

will occur. Increased demand for DNA repair enzymes, maintenance methylase, DNA binding proteins, and cofactors might alter DNA primary, secondary, or tertiary structure which might in turn alter gene function or expression and contribute to carcinogenesis.

B. Partial hepatectomy and cell proliferation

Partial hepatectomy (PH; surgical removal of 2/3 of the liver volume) stimulates regeneration of liver cells until the original liver mass is attained. In hepatocytes from B6C3F1♂ mice, 48 hours after PH, DNA synthesis is increased 14-fold over hepatocytes from an intact liver (Itze, *et al.*, 1973). The peak DNA synthesis occurs at approximately 30-36 hours post-PH in B6C3F1♂ mice (Itze, *et al.*, 1975), approximately 40 hours post-PH in B6AF1♂ and 46 hours post-PH in B6AF1♀ mice (Chernozemski and Warwick, 1970). Diurnal rhythm influences timing of DNA synthesis even after a major stimulatory event such as PH. Therefore, the time of day at which a PH is performed will influence the time of peak DNA synthesis.

Both raf and Ha-ras mRNA levels increase following PH. In the rat [peak DNA synthesis following PH in the rat is 24 hrs (Goyette, *et al.*, 1983)], raf mRNA levels peak at 3-5x basal levels 24 hours after PH and return to baseline by 72 hours (Silverman, *et al.*, 1989). Ha-ras mRNA levels are maximal (2-3x basal levels) between 18-36 hours after PH in the rat and have returned to normal levels by 72 hours (Goyette, *et al.*, 1983; Silverman, *et al.*, 1989).

C. Phenobarbital and cell proliferation

Phenobarbital (PB) is a liver tumor promoter in rats (Peraino, *et al.*, 1980; Pereira, *et al.*, 1986a; Pitot, *et al.*, 1987) and mice, including the B6C3F1 (Pereira, *et al.*, 1986b; Klaunig, *et al.*, 1987; Klaunig, *et al.*, 1988), and C3H but not the C57BL (Lee, *et al.*, 1989). Furthermore, PB (500 ppm in drinking water for 18 months) alone results in 100% liver tumor incidence in B6C3F1♂ and C3H/He♂, but not C57BL/6♀, mice (Becker, 1982). In aging (12 month old) C3H mice, administration of 500 ppm PB in the drinking water for 12, 24, or 36 weeks was found to increase the size and number of foci and neoplasms arising from alleged spontaneously initiated hepatocytes (Ward, *et al.*, 1988). In the presence of 2 mM PB, there was a ten-fold increase in the number of proliferative hepatocyte colonies which developed from initiated [by administration of methyl(acetoxymethyl)nitrosamine or benzo[a]pyrene-7,8-diol-9,10-epoxide(anti)] rat cells relative to control (not initiated, but treated with PB) cells (Kaufmann, *et al.*, 1986; Kaufmann, *et al.*, 1988).

PB administration results in a dose dependent increase in DNA synthesis as measured by ^{3}H-thymidine incorporation in DNA in primary hepatocyte cultures obtained from rats [Edwards and Lucas, 1985 (2 mM PB for 46 hours); Sawada, *et al.*, 1987 (maximum increase with 1 mM PB for 44 hours); Yusof and Edwards, 1990 (2 mM PB; maximum DNA synthesis at approximately 25 hours after addition of PB)], in hepatocytes obtained from PB-treated rats [Eckl, *et al.*, 1988

(0.1% PB in the drinking water for up to 30 days), *in vivo* in rats [Busser and Lutz, 1987 (1 dose of 0.1 mmole/kg PB 24 hours prior to ^{3}H-thymidine incorporation)], and in C57BL♂, C3H♂, B6C3F1♂, and C3B6F1♂ mice [Lin, *et al.*, 1989 (500 ppm PB in the drinking water for 4 days - all strains, or 28 days - C57BL/6♀ and C3B6F1♂)]. In B6C3F1♂ mice, 500 ppm, but not 20 ppm, PB in the drinking water stimulates DNA synthesis; this effect peaks at 2 weeks, then declines (Weghorst and Klaunig, 1989; Siglin, *et al.*, 1991; Klaunig, *et al.*; 1991; J.E. Klaunig, unpublished data). Chronic PB treatment (more than one month) *in vivo* has an inhibitory effect on hepatocyte proliferation (Eckl, *et al.*, 1988).

PB has additional effects on cell physiology. PB treatment results in increased unscheduled DNA synthesis (i.e. DNA repair) following initiation in cultured rat hepatocytes (Althaus, *et al.*, 1986). PB reduces gap junctional communication in a dose dependent manner (Ruch, *et al.*, 1987; Klaunig and Ruch, 1987; Klaunig, *et al.*, 1990) which might contribute to unregulated cell proliferation. Hepatocytes isolated from PB pre-treated rats (0.1% PB in the drinking water for 2 weeks) showed a 15-fold increase in clonogenicity relative to hepatocytes from non-PB treated rats when injected into the fat pad of recipient rats (Jirtle and Michalopoulos, 1986). The inhibitory effect of physiological concentrations of extracellular calcium is eliminated during the first month of PB treatment (Eckl, *et al.*, 1988). Hepatocyte EGF-R mRNA (Hseih, *et al.*, 1988 - 0.05% PB for 16 days) or number (Eckl,

et al., 1988 - 0.1% PB for 2 or 8 weeks) is decreased in PB treated rats.

5. The Role of 5-Methylcytosine in Mediating Gene Expression

A. The role of 5-methylcytosine in differentiation and aging

5-Methylcytosine (5MeC) is the only naturally occurring methylated base in mammalian DNA. 5MeC plays an important role in development and differentiation (reviewed in Michalowsky and Jones, 1989b; Cedar and Razin, 1990; Razin and Cedar, 1991). DNA in sperm is heavily methylated, while oocyte DNA is unmethylated. The early gamete has an intermediate methylation pattern, suggesting a mixture of methylated and unmethylated DNA. By the time the blastocyst stage is reached, the DNA is unmethylated. In the embryo, the genome is generally heavily methylated, with genes becoming hypomethylated only in the tissues in which they are expressed. Thus, as cells differentiate into specific tissues, the DNA undergoes active demethylation to activate genes and *de novo* methylation to inactivate genes resulting in the appropriate phenotypic expression.

Undifferentiated cells (e.g. transformed cells) can be stimulated to differentiate by treatment with a hypomethylating agent such as 5-azacytidine (Creusot, *et al.*, 1982; Jones and Taylor, 1980). Conversely, DNA which is unmethylated in normal cells might become methylated as the cell dedifferentiates during transformation. Jones and

coworkers (1990) found that the normally unmethylated CpG island upstream of the MyoD1 gene is methylated in immortalized cell lines.

There is a decrease in 5MeC content in the genome in general in aged animals (Wilson, *et al.*, 1987b; Singal, *et al.*, 1987). Aging and subsequent hypomethylation of DNA might predispose animals to carcinogenesis. There is also a decrease in overall 5MeC content with time *in vitro* as primary cell cultures senesce and the rate of loss correlates with the lifespan of a particular cell culture (Wilson and Jones, 1983a). Specific genes have been shown to become hypomethylated during aging. Mouse intracysternal A particle genes have lost both 5'-C^{Me}CGG-3' and 5'-^{Me}CCGG-3' sites, seen as increased digestion by both MspI and HpaII in DNA from 24-26 month old C57BL/6σ mice (Mays-Hoopes, *et al.*, 1983). The major mouse long interspersed sequence was found to have lost approximately 8% of its 5'-C^{Me}CGG-3' sites in C57BL/6J by the age of 27 months (Mays-Hoopes, *et al.*, 1986).

While the genome, in general, becomes hypomethylated with aging, some specific genes have been found to be hypermethylated in older animals. The 5' spacer region and external transcribed spacer of 18S and 28S rRNA genes in liver, brain, and spleen are more methylated in 18 month old than 6 month old CBA/Ca mice (Swisshelm, *et al.*, 1990). This hypermethylation is accompanied by a decrease in detectable activity of rRNA genes in some chromosomes which is restored by treatment with 5-azacytidine. While the methylation status

of the 5'-flanking region, first exon and first intron of the mouse c-fos gene remains constant from the age of one month, a site in exon 2 is partially methylated at one month and a progressively greater fraction of cells have this site in its methylated state in older mice (Uehara, *et al.*, 1989). Relative to 2 month old mice, the c-myc gene in 26 month old mice was found to be hypermethylated in liver DNA at sites 3' to the first intron (Ono, *et al.*, 1986; Ono, *et al.*, 1989). Following digestion of DNA with MspI, additional large fragments are detected, indicating that the hypermethylation of the myc gene is due to an increase in 5'-^{Me}CCGG-3' sites. This hypermethylation of myc was correlated with decreased levels of myc mRNA in the liver.

B. 5-Methylcytosine as a regulator of gene expression

Modification of DNA by methylation of cytosine is one mechanism postulated to be involved in the regulation of gene expression. The methylation status of a gene has been found to inversely correlate with its expression. In general, quiescent genes are hypermethylated while genes which are actively being expressed or have the potential to be expressed are relatively hypomethylated (Doerfler, 1983; Riggs and Jones, 1983; Jones, 1986; Cedar, 1988). Hypomethylation might facilitate the binding of transcription factors, thereby resulting in increased expression of the gene. The presence of cytosine rather than 5MeC results in a local destabilization of the double helix structure (Murchie and Lilley, 1989). The $\alpha_1$globin and MyoD1 genes in C3H 10T1/2

cells treated with 5-aza-2'deoxycytidine were hypomethylated and the chromatin was more accessible to nuclease digestion than in control cells (Michalowsky and Jones, 1989a), suggesting a more open chromatin structure.

5MeC can also negatively influence gene expression. The binding of some specific transcription factors has been found to be physically hindered by 5MeC (Watt and Molloy, 1988; Comb and Goodman, 1990; Antequera, *et al.*, 1989; Boyes and Bird; 1991). For example, Boyes and Bird, (1991) found that methylation of the murine myeloproliferative sarcoma virus promoter region had a direct inhibitory effect on transcription. Additionally, hemimethylated (i.e. 5MeC present in only one strand) sites were found to partially inhibit binding of the MLTF transcription factor in the adenovirus major late promoter region (Watt and Molloy, 1988). Another mechanism by which 5MeC interferes with transcription is by binding proteins (e.g. the methyl CpG binding protein) which inhibit transcription (Meehan, *et al.*, 1989; Levine, *et al.*, 1991; Boyes and Bird, 1991). A minimum number of 5MeC in a gene is required for binding the inhibitory proteins and the binding affinity of the inhibitory methyl CpG binding protein is increased in the presence of additional 5MeC (Boyes and Bird, 1991). Thus either the absolute number of methylated sites in a gene or the methylation status of a critical site involved in the binding of transcription factors can influence expression of a gene.

The methylation status of sites in the promoter and 5' flanking region of the gene is critical (Bird, 1986; Langner, *et al.*, 1984), but the methylation status of sites in the interior region of a gene has also been found to influence expression (Dizik, *et al.*, 1991; Langner, *et al.*, 1984). Langner and coworkers (1984) found that *in vitro* methylation of three 5'-CCGG-3' sites in the promoter and 5' flanking region of the human adenovirus type 2 *E2a* gene resulted in transcriptional inactivity. *In vitro* methylation of 11 5'-CCGG-3' sites in the coding region of the gene resulted in a decreased level of transcription (Langner, *et al.*, 1984). Therefore, a decrease in methylated sites in the interior of a gene might allow increased transcription. The 5'-CCGG-3' sites in the CG rich 5' flanking region of c-myc are unmethylated in normal rat liver DNA but the regions including the second and third exons are heavily methylated (Dizik, *et al.*, 1991). Following 1-4 weeks of a methyl-deficient diet, hypomethylation of the coding and 3' flanking regions was detected and c-myc mRNA levels were increased.

More than 90% of 5MeC occur in the sequence 5'-CG-3' (Riggs and Jones, 1983). However, 5MeC at other sites can also influence gene expression. Kong and coworkers (1991) found a decrease in methylation of the external, but not the internal, cytosine of 5'-CCGG-3' sites after treatment of cells with 5-azacytidine, a potent DNA maintenance methylase inhibitor, which resulted in the induction of expression of a previously quiescent gene.

C. The inheritance of methylation status

1) Somatic

The methylation status (i.e. number and position of 5MeC) of a gene is an epigenetic characteristic which is heritable through multiple rounds of cell division (Holliday, 1987; Holliday, 1990). There is a difference between maintenance methylation which results in the methylation of hemimethylated sites following DNA replication and the *de novo* methylation which takes place during differentiation. During DNA replication, an unmethylated daughter strand is synthesized using a parental strand as a template. CpG sites which were fully methylated (i.e. 5MeC on each strand) are now hemimethylated. DNA maintenance methylase recognizes this hemimethylated site and catalyzes the transfer of a methyl group from the cofactor S-adenosyl-methionine to the unmethylated cytosine (Wigler, *et al.*, 1981). Fully unmethylated sites are poor substrates for DNA maintenance methylase.

If there is some interference in the activity of the DNA maintenance methylase, such as inhibition of the enzyme, or decreased availability of the cofactor and the DNA replicates prior to methylation, a fully unmethylated site could result. Because some regions of chromatin are less accessible than others to DNA repair enzymes (Topal, 1988), it is reasonable to suspect that some hemimethylated sites might also be relatively inaccessible to maintenance methylase.

This scenario also has the potential to result in heritably unmethylated sites following DNA replication.

2) Through the germ line

Both the base sequence of a gene and secondary modifications of DNA such as methylation are heritable through the germ line. Alleles are variants in DNA base sequence at a single genetic locus. When the difference in base sequence results in a change in the cleavage pattern of restriction endonucleases, restriction fragment length polymorphisms (RFLP) are detectible with Southern blot analysis. The inheritance of alleles as detected by the presence of RFLP is diagrammed in Figure 2.

A variation in methylation at a genetic locus is not synonymous with different alleles because the base sequences at that locus might be identical. However, differences in methylation status which affect cleavage by methylation sensitive restriction endonucleases will also be seen as RFLP. An example of the inheritance of methylation status through the germ line is diagrammed in Figure 3. The methylation pattern at a specific genetic locus was found to be inherited in a Mendelian fashion (Silva and White, 1988; Ghazi, *et al.*, 1990; Chandler, *et al.*, 1987) Silva and White (1988) examined the methylation status of MspI sites in ten loci in related individuals. The methylation status of a particular allele was found to persist through at least 3 generations regardless of the sex of the parent through which the allele was transmitted. Differential methylation of

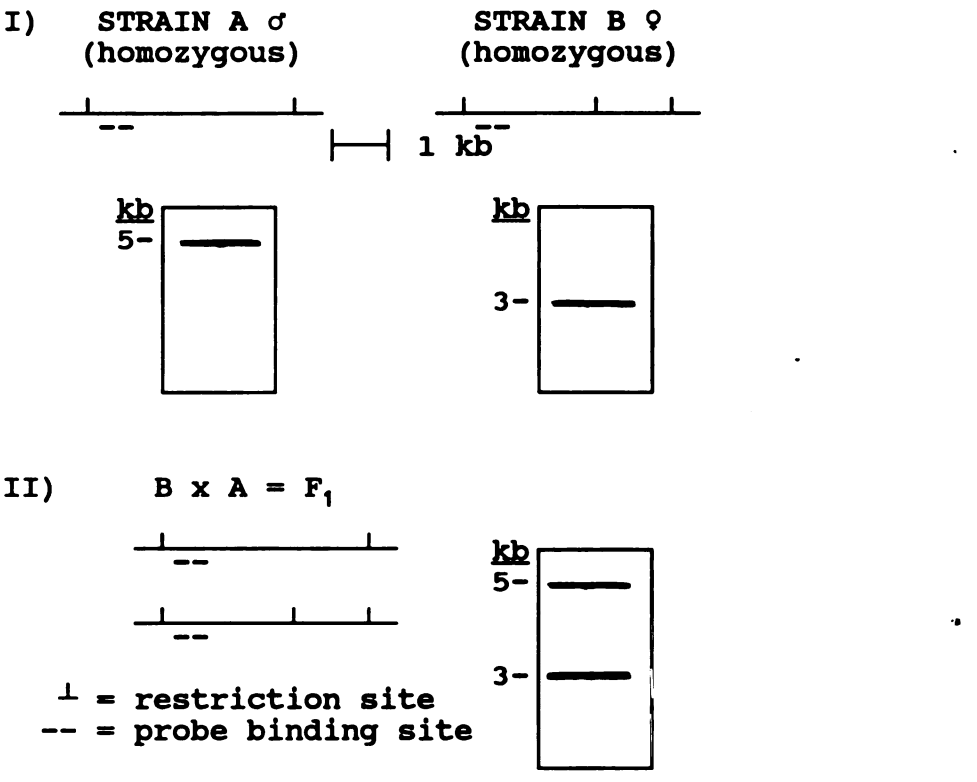
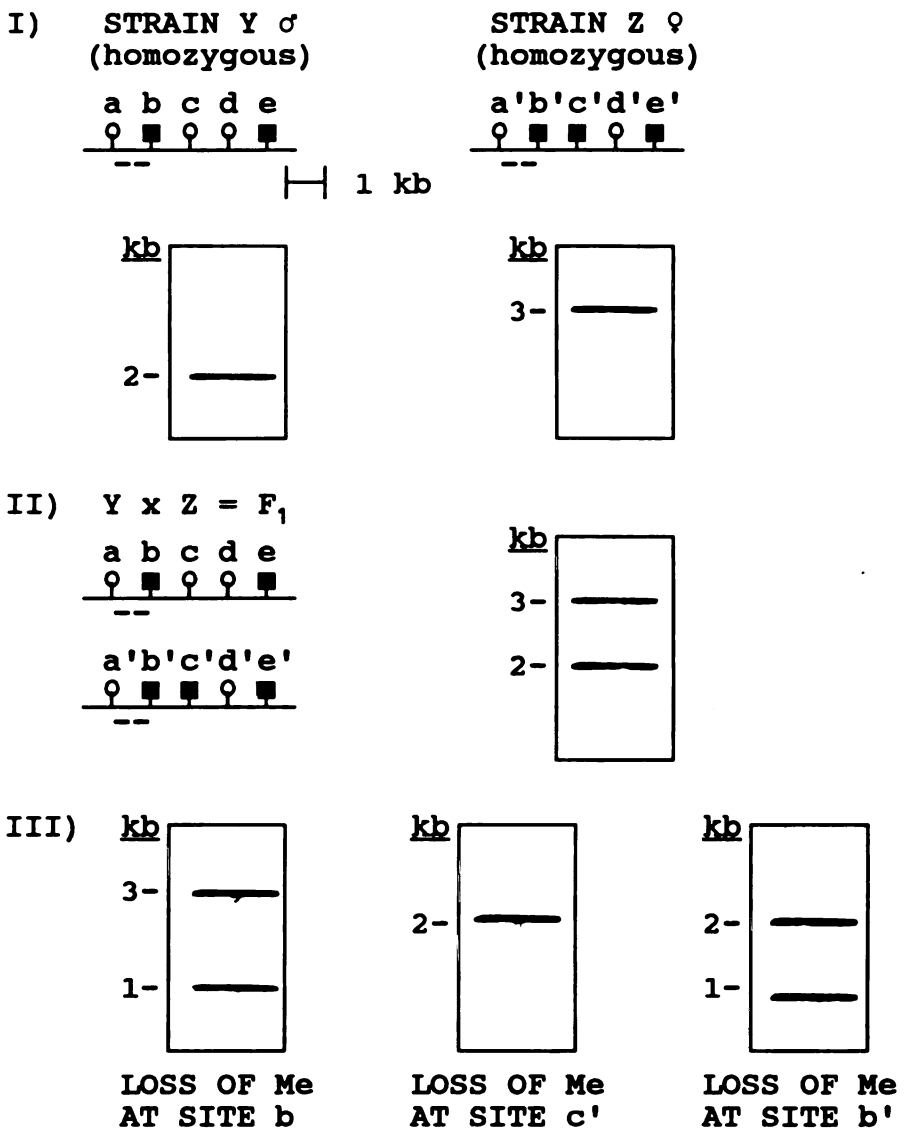


Figure 2. Illustration of the inheritance of alleles. I) The physical maps of a hypothetical genetic locus in two homozygous strains of mice are shown with recognition sites of a restriction endonuclease (⊥). The Southern blot showing the restriction pattern of the DNA probed for this gene is drawn below the maps. II) The physical map of the parental alleles in the F_1 generation is shown along with a Southern blot illustrating the restriction pattern.

Figure 3. Illustration of the inheritance of methylation status. I) The physical maps of a hypothetical genetic locus in two homozygous strains of mice are shown. The restriction endonuclease used to generate the map is methylation sensitive, i.e. it does not cleave when the recognition site contains a 5MeC (○ = unmethylated site, ■ = methylated site). A Southern blot showing the restriction pattern of the DNA probed for this gene is depicted below the map for each strain. II) The physical map of the genetic locus inherited from each parent in the F_1 generation is pictured along with the Southern blot showing the methylation sensitive restriction pattern. III) Southern blots illustrating the restriction patterns of Y x Z F_1 DNA in which one or more methylated sites have become unmethylated.


I)
STRAIN Y ♂
(homozygous)
a b c d e
STRAIN Z ♀
(homozygous)
a'b'c'd'e'
1 kb
kb
2-
kb
3-
II)
Y x Z = F1
a b c d e
a'b'c'd'e'
kb
3-
2-
III)
kb
3-
1-
LOSS OF Me
AT SITE b
kb
2-
LOSS OF Me
AT SITE c'
kb
2-
1-
LOSS OF Me
AT SITE b'

parental Ha-ras alleles was found in tissues (Ghazi, *et al.*1990) and in an immortal cell line (Chandler, *et al.*1987).

3) Partial methylation

A difference in the methylation status at a genetic locus between cells in a tissue (e.g. hepatocytes) is referred to as partial methylation at a site or mosaicism of methylation status (McGowan, *et al.*, 1989). Partial methylation is generally expressed as the percent of the specific loci which are methylated in a given cell population. Differential methylation of the two parental alleles (i.e. the maternal allele is methylated and the paternal allele is not) would result in a methylation value at that site of 50%. In a gene which is 25% methylated at a site, one allele out of every four would be methylated at that site (e.g. the maternal allele in every second cell is methylated and the paternal alleles are all unmethylated). While conclusive determination of methylation status of a particular site requires sequencing, partial methylation might be indicated by differing intensities of bands on a Southern blot of a DNA sample digested with a methylation sensitive restriction endonuclease. Decreased intensity of one band relative to the other bands in the same lane is not diagnostic of partial methylation, but partial methylation at a site, resulting in partial digestion by a methylation sensitive restriction endonuclease, is one possible explanation for decreased intensity of a band.

Partial methylation of a site is heritable both somatically and through the germ line. The methylation pattern of a CpG site 420 bp upstream of the mouse adenine phosphoribosyltransferase gene is stably maintained between different individuals (i.e. it is 44% methylated in liver DNA of CBA mice) and is not altered with aging (Turker, *et al.*, 1989). The degree of methylation at partially methylated sites has been found to influence the level of expression of that gene. McGowan, *et al.* (1989) studied the methylation status of the *lacZ* transgene in neural tube tissue of transgenic mice. The *lacZ* transgene was found to be in a highly methylated, unmethylated or intermediately methylated state in individual transgenic mice. The level of *lacZ* gene expression was inversely correlated with the methylation state of the gene. Mice exhibiting an intermediate methylation pattern of the transgene *lacZ* were found to express an intermediate amount of *lacZ*. This was interpreted as indicating a mosaic pattern of expressing and nonexpressing cells.

4) Imprinting

Imprinting refers to epigenetic modifications such as the relative hypermethylation of one parentally-derived allele that result in differential expression of genetic material, depending on the parent of origin. Imprinting by hypermethylation is thought to be responsible for the silencing of the inactive paternally-derived X chromosome. Genomic imprinting has been implicated in some

cancers such as familial retinoblastoma and osteosarcoma (Hall, 1990; Sapienza, 1991).

In accordance with the theory that methylation plays a role in imprinting, the methylation status of the inserted gene (transgene) in the offspring of transgenic mice has been found to be dependent on the sex of the parent through which the transgene was transmitted (Sapienza, *et al.*, 1989; Swain, *et al.*, 1987; Reik, *et al.*, 1987; Sapienza, *et al.*, 1987). In most of the studies, the transgene was not expressed regardless of its methylation status, but transgenes transmitted through the female parent were consistently hypermethylated relative to the same transgene at the same locus transmitted through the male parent. However, if the c-myc and the Rous sarcoma virus LTR transgene was inherited from the male, it was hypomethylated and expressed while it was not expressed if inherited (relatively hypermethylated) from the female (Swain, *et al.*, 1987).

D. DNA hypomethylation associated with carcinogen treatment

A major factor in multi-step carcinogenesis is the activation of previously quiescent genes, such as α-fetoprotein, which is expressed in some preneoplastic foci, but not in normal liver. Hypomethylation has the potential to alter the expression of genes. The Ha-ras proto-oncogene in B6C3F1♂ mouse liver tumors induced by benzidine, chloroform, or phenobarbital, or in spontaneous mouse liver tumors is hypomethylated relative to surrounding nontumorous tissue

(Vorce and Goodman, 1989a; Vorce and Goodman, 1989b) and Ha-ras mRNA levels are elevated in benzidine-induced tumors (Vorce and Goodman, 1989a).

The level of DNA methylation is decreased in many cancer cells and tumor tissues (Jones and Buckley, 1990). This may be due to the hypomethylation seen during aging, indeed, cancer is more prevalent in aged individuals. Alternatively, hypomethylation can be a result of chemical exposure. Treatment of normal dividing human bronchial epithelial cells with chemical carcinogens with diverse mechanisms of action was shown by Wilson, *et al.* (1987a) to significantly decrease the total 5MeC content of cellular DNA.

Chemicals which form DNA adducts such as acrolein (Cox, *et al.*, 1988) and antibenzo[a]pyrenediol epoxide (Ruchirawat, *et al.*, 1984), have been found to interfere with the activity of DNA maintenance methylase. The DNA adduct complexes might physically interfere with the enzyme's access to the hemimethylated sites or some chemicals, such as N-methyl-N-nitro-N-nitrosoguanidine or ethylnitrosourea form adducts on the enzyme itself (Wilson and Jones, 1983b). When incorporated into DNA, the cytidine analog 5-azacytidine binds and inhibits DNA maintenance methylase (Santi, *et al.*, 1984). Ethionine, a nongenotoxic carcinogen which is an antimetobolite of methionine, interferes with methylase function by competing with the cofactor S-adenosyl-methionine (Shivapurkar, *et al.*, 1984). There is also the possibility of active demethylation resulting in the occurrence of

demethylated sites on a gene (Gjerset and Martin, 1982; Razin, *et al.*, 1986). This action would be due to one or more demethylating enzymes, would not be a function of DNA maintenance methylase, and could occur in the absence of cell proliferation.

5MeC might also play a role in mutagenesis. Mutations are a major factor in carcinogenesis. Both initiation and progression frequently involve alterations in DNA base sequence. Methylated cytosines might protect DNA from mutations that occur during DNA replication by directing repair of a mismatched base to the newly synthesized unmethylated strand, thus preserving the parental sequence (Hare and Taylor, 1989). Conversely, the presence of 5MeC might increase mutations because spontaneous deamination of 5MeC results in a change from cytosine to thymine (Rideout, *et al.*, 1990). This possibility is supported by the fact that actual CpG dinucleotide content in most of the genome is lower than predicted statistically (Schorderet and Gartler, 1990). In contrast, GC-rich islands are generally unmethylated (Bird, 1986) and might thereby be protected against mutations.

6. Hypothesis and Objectives

The hypothesis underlying this study is that specific proto-oncogenes are hypomethylated in mouse liver tumors and that this hypomethylation results in elevated levels of the proto-oncogene mRNA. Hypomethylation is one mechanism by which transcription from particular genes might be

facillitated. Hypomethylation of proto-oncogenes has been found previously in tumors and increased expression of proto-oncogenes has been implicated in rodent liver tumors. The specific proto-oncogenes examined in this study were Ha-ras and raf because of their role in signal transduction and involvement in rodent liver tumors. The B6C3F1♂, its parental strains, the C3H/He♂ and the C57BL/6♀, and the CD-1♂ were chosen as the models in which to study alterations in raf and Ha-ras methylation and expression because of the differing susceptibilities to liver tumor development of each strain.

My first aim was to determine if there are intrinsic differences in the primary structure or methylation patterns of raf and Ha-ras between the C3H/He♂ and C57BL/6♀. The methylation status of the genes was analyzed by digestion with methylation sensitive restriction endonucleases, and the genes were screened for the presence of RFLPs by digestion with several other restriction endonucleases to detect differences in base sequence. The same parameters were examined in the B6C3F1♂ to determine if any differences found were hereditary. Additionally, raf and Ha-ras methylation and RFLP patterns were examined in the unrelated CD-1♂ strain.

Secondly, the effect of cell proliferation on the maintenance of methylation patterns of raf and Ha-ras was examined. PH or PB (500 ppm in the drinking water for 14 days) were used in separate groups of B6C3F1♂, C3H/He♂, and C57BL/6♀ mice to increase the liver cell growth fraction. The methylation status and mRNA levels of raf and Ha-ras from each

of these groups was measured to examine the relationship between changes in methylation status and expression of the genes.

The third aim of this study was to examine the relationship between tumorigenicity, hypomethylation, and mRNA levels. The methylation status of v-Ha-ras, Ha-ras mRNA and ras p21 levels were examined in rat liver tumors induced by injection of v-Ha-ras-infected rat liver epithelial cells ($WB^{Ha\text{-}ras}$ cells). Although the presence of the viral Ha-ras DNA might be the major factor governing the tumorigenicity of the transfected cells, it was important to determine the relationship between the methylation status of this DNA and its expression. The methylation status and mRNA levels of raf and Ha-ras in B6C3F1♂ and CD-1♂ liver tumors was analyzed to determine if there was a relationship between hypomethylation of proto-oncogenes, expression, and tumor formation *in vivo*.

Finally, to determine if PB-induced liver tumors were due to an acceleration in the development of spontaneous tumors or if PB administration resulted the clonal expansion of a different subset of cells, the methylation status and mRNA levels of raf and Ha-ras in PB-induced B6C3F1♂ liver tumors were compared with those of spontaneous B6C3F1♂ liver tumors.

MATERIALS AND METHODS

1. Animals: Maintenance and Treatment

A. Mice

Four strains of mice were used in these studies: the B6C3F1, C3H/He, C57BL/6, and CD-1. Young adult B6C3F1♂, C3H/He♂ and C57BL/6♀ mice (19-20 g) were obtained from Charles River Laboratories (Portage, MI). Male mice at 19-20 g were generally 4-5 weeks old, while female mice were approximately one to two weeks older. The mice were housed in a university laboratory animal care facility at constant temperature (70°F) and humidity (35-40%) with a reverse phase 12 hour light/dark cycle and allowed food and water *ad libitum*. Prior to any treatment or sacrifice, mice were acclimated to the environment for at least 6 days. All mice were euthanized by cervical dislocation. Following sacrifice, the entire liver was placed in liquid nitrogen, then stored at -80°C until use. The PB-induced B6C3F1♂ and CD-1♂ liver tumors were provided by from Hoffman LaRoche Laboratories (Nutley, NJ). To induce the tumors, mice were treated with 1000 ppm PB (150 mg/kg/day) in the diet for 24 months. Spontaneous B6C3F1♂ liver tumors, age-matched B6C3F1♂ and CD-1♂ liver tissue and young CD-1♂ liver tissue were also provided by Hoffman LaRoche Laboratories.

B. Cells

One study involved cultured cells. WB^{wt} cells are immortal rat liver epithelial cells. Cells were infected with the viral vector (pZip) containing the neomycin marker alone (WB^{neo}) or with pZip containing v-Ha-ras DNA ($WB^{Ha\text{-}ras}$). All WB cells were provided by Dr. James Trosko. Tumors were induced in Fischer 344 (F344) rats by injection of 1×10^6 $WB^{Ha\text{-}ras}$ cells into the portal vein. Rats were sacrificed after 3 weeks. Liver and lung tissue from an untreated rat, liver and lung tumors and nontumorous liver tissue from $WB^{Ha\text{-}ras}$-treated rats, and cells cultured from F344 liver tumors were provided by Dr. James Klaunig.

C. Phenobarbital administration

In the 14 day PB administration studies, the PB treated mice (B6C3F1♂, C3H/He♂, or C57BL/6♀) were given 0.002% (3 mg/kg/day) or 0.05% (75 mg/kg/day) (w/v) PB (free acid, Sigma Chemical Co., St. Louis, MO) in the drinking water. PB solutions were made fresh weekly and replaced twice weekly. Control animals were given distilled water.

D. Partial hepatectomy

PH were performed on B6C3F1♂, C3H/He♂, or C57BL/6♀ mice under ether anesthesia, modified from the procedure described by Higgins and Anderson (1931). A 1-1.5 cm ventral midline incision was made in the anesthetized mouse and the left and middle liver lobes were exteriorized. The lobes were ligated with 4-0 silk and resected. Removed liver was immediately placed in liquid nitrogen, broken into smaller

pieces and then stored at -80°C until use. The incision was closed with 4-0 silk in two layers. The animals were allowed to recover several hours at approximately 80°F by placing the cage about 24" under an incandescent light before returning the mice to the laboratory animal care facility. Viability following surgery ranged from <50% of the C57BL/6♀ to nearly 100% of the B6C3F1♂. Mice which were moribund or visibly jaundiced 7 days after PH were not used in these studies.

2. Isolation of DNA

DNA was isolated from frozen tissue or frozen tissue culture cells by a modification of the method of Strauss (1987). Approximately 100-300 mg of liver tissue was ground in liquid nitrogen, then homogenized in 5 ml of 100 mM sodium chloride (NaCl), 10 mM Tris (pH 8.0), 25 mM ethylenediaminetetraacetic acid (EDTA, pH 8.0), 0.5% sodium dodecyl sulfate (SDS) with 200 μg/ml Proteinase K (Boehringer Mannheim Biochemicals; BMB, Indianapolis, IN; Proteinase K is prepared as a 10 mg/ml solution in 50 mM Tris, 1 mM $CaCl_2$). Digestion proceeded overnight at 50°C. DNA was washed with an equal volume of a mixture of 50% equilibrated phenol [phenol is prepared by saturating solid redistilled phenol (BMB) with water, adding 0.1% 8-hydroxyquinoline as an antioxidant, and equilibrating with 100 mM Tris buffer(pH 8.0) so that the pH of the phenol was >7.6], and 50% chloroform (with 3% isoamyl alcohol). The layers were separated by centrifugation at 10,000 rpm (Sorvall centrifuge with SA-600 rotor), 10 minutes

at 25°C. The aqueous layer was removed and the DNA precipitated with 1/2 volume of 7.5 M ammonium acetate (pH 7.5), and 2 volumes of 95% ethanol. The DNA was rinsed in 70% ethanol and dissolved in 5 ml TE buffer [10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0)]. The samples were then digested with 400 μg/ml RNase A (Sigma Chemical Company; preboiled 10 minutes) in the presence of 0.01% SDS for 1 hour at 37°C, followed by 200 μg/ml Proteinase K for 1 hour at 50°C. The DNA was washed in phenol and chloroform and precipitated as above. DNA isolation from tissue culture cells was essentially the same, but smaller volumes were used and centrifugation was in a Brinkmann microfuge. After dissolution in TE buffer, the absorbances at 260 and 280 nm (Gilford Response spectrophotometer) were used to determine concentration (1 A_{260} = 50 μg DNA) and assess purity as the ratio of absorbance at 260nm to that at 280nm. Generally, acceptable A_{260}/A_{280} ratios were between 1.7 and 2.0, however DNA from some tumor samples had lower ratios.

3. Restriction Endonuclease Digestion, Agarose Gel Electrophoresis, and Southern Transfer

A. Restriction endonuclease digestion

Digestion of DNA with restriction endonucleases and electrophoresis was performed essentially as described by Vorce and Goodman (1987). Ten microgram aliquots of DNA were digested to completion with 5 units/μg MspI, HpaII, StuI, XhoI, or TaqI; or 3/units μg HhaI, HindIII, or EcoRI. See

Figure 4 for restriction sites. All enzymes were purchased from Bethesda Research Laboratories (BRL; Gaithersburg, MD) or BMB and were supplied with the appropriate 10X concentrated reaction buffer. The DNA, buffer, 1/2 the enzyme, and distilled water to 57.5 μl were mixed thoroughly and incubated at 37°C for 30 minutes, then the remaining enzyme was added, the reaction mixture was mixed thoroughly and incubated for an additional 90 minutes at 37°C. TaqI digestions were carried out at 65°C. For estimation of fragment size, 1 μl lambda phage HindIII fragments (BRL) diluted to 60 μl with water was prepared. A 5X marker dye (15% Ficoll 400, 0.125% bromphenol blue, 0.125% xylene cyanole ff, 5x TBE) was added to each sample and the samples were heat-treated at 65°C for 10 minutes.

B. Agarose gel electrophoresis

Electrophoresis was carried out in a model HO/H1 horizontal electrophoresis apparatus (BRL). Gels were prepared as 0.9% or 1.1% agarose (BRL) in 1X TBE by heating in a microwave until boiling, then stirring until approximately 50°C, pouring, and inserting the well former. After solidification, the well former was removed and the gel was submerged in 1X TBE (89 mM Tris, pH 8.3, 89 mM boric acid, 2.5 mM EDTA) in the electrophoresis apparatus. The samples were loaded using a Pipetman (Rainin Instrument Co.). Electrophoresis at 50 V proceeded for 16 hours or until the dye front was approximately 1 inch from the end of the gel.

ENZYME	RECOGNITION SITE	REFERENCES
EcoRI	G/AATTC*	Rubin and Modrich, 1980
HhaI	GC*G/C	Bird and Southern, 1977
Hind III	A/AGC*TT	Smith and Marley, 1980
HpaII	C/C*GG	Waalwijk and Flavell, 1978; Mann and Smith, 1977
MspI	C*/CGG	Sneider, 1980; van der Ploeg and Flavell, 1980
StuI	AGG/C*C*T	Shimotsu, *et al.*, 1980
TaqI	T/CGA	Streek, 1980
XhoI	C/TC*GAG	Gingeras, *et al.*, 1977

Figure 4. Restriction endonucleases and recognition sites. The restriction endonucleases used in these studies are listed, along with their recognition sites. If cleavage by the enzyme is inhibited by the presence of 5MeC, an * indicates those inhibitory sites.

The gel was removed from the apparatus, stained in 0.5 μg/ml ethidium bromide (Oncor, Gaithersburg, MD) for 15 minutes, then destained in glass distilled water for 15 minutes on a LabLine orbital shaker. The DNA was visualized on a UV light box (Fotodyne, New Berlin, WI) and photographed using a Polaroid MP-3 camera with a Kodak Wrattan No. 9 filter, an aperture of 6.8, exposure time of 1 second, and Polaroid 667 black and white film. A fluorescent ruler was photographed alongside the lane containing the lambda HindIII fragments. A graph of the fragment sizes on the ordinate of semilog paper as a function of the distance each fragment traveled was used to estimated the size of sample fragments.

C. Southern transfer

The gel was soaked in 0.25 N HCl for 10 minutes to fragment the DNA by depurination and facilitate the transfer of high molecular weight DNA. The DNA was denatured by soaking the gel in 0.6 M NaCl, 0.4 M NaOH for 30 minutes, then neutralized by soaking in 1.5 M NaCl, 0.5 M Tris (pH 7.5) for 30 minutes. Constant agitation was provided during all gel soaks by an Orbit Shaker (Lab Line). A piece of 3 MM paper (Whatman) served as a wick for the transfer buffer, 20X SSC (3 M NaCl, 1 M sodium citrate, pH 7.0). The gel was inverted onto the wick and a Hybond (Amersham, Arlington Heights, IL) nylon membrane, trimmed to size, was placed on the gel. Four pieces of trimmed 3 MM paper, wet in 20X SSC, were placed on the membrane and bubbles were removed by rolling a pipet gently over the stack. Trimmed blotting pads and paper towels

were added and a smooth plate and 500 g weight were placed on top. Wet pads were removed as needed and the entire apparatus was covered with plastic wrap overnight. Capillary transfer of DNA to the membrane was allowed to proceed for approximately 22 hours. After transfer, the membrane was placed face up on the damp 3 MM paper in a Stratlinker (Stratagene, LaJolla, CA) and irradiated with 120,000 μjoules of ultraviolet light, rinsed briefly in 5X SSC and baked for 2 hours in an 80°C vacuum oven. Membranes were stored in a hybridization bag at room temperature until use.

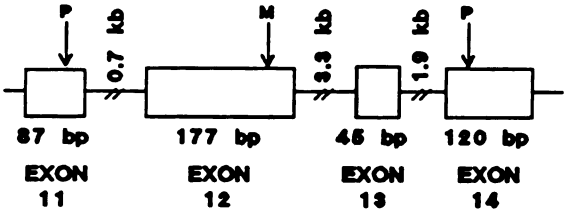
4. Labeling of Probe with ^{32}P by the Random Primers Method

A. Probes

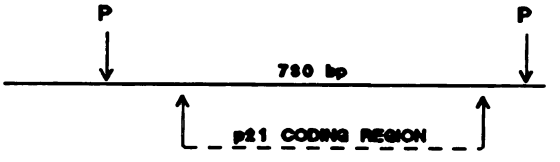
Oncogene probes were purchased from Oncor (Gaithersburg, MD). The raf probe is 290 base pairs long and consists of the StuI digestion fragment of v-raf that extends from the interior of exon 11 to the interior of exon 14. This is homologous to the region from base #1295 (a StuI site) to base #1564 (not a StuI site in human DNA) in the human raf gene (Bonner, *et al.*, 1986; Rapp, *et al.*, 1988; Devereux, *et al.*, 1984) (Figure 5A). The Ha-ras probe is the SstI/PstI digestion fragment of v-Ha-ras and is approximately 730 base pairs long. It includes the entire coding region and about 100 base pairs both 5' and 3' to the coding region (Dhar, *et al.*, 1982) (Figure 5B).

Figure 5. Schematic representation of probes. A) raf. The area of the human raf gene which is homologous to the viral probe is shown between the 2 arrows labeled with P. The sizes are for the human raf gene; the size of the introns in the mouse raf gene is not known. The arrow labeled with an M points to an MspI site known to be in exon 12 of human raf. B) Ha-ras. The SstI/PstI digestion fragment of the Harvey Murine Sarcoma Virus, used as the probe, is shown between the arrows labeled with P. The region which codes for the p21 protein is indicated by the dotted line.

a)


P
0.7 kb
M
3.3 kb
1.9 kb
P
87 bp
177 bp
45 bp
120 bp
EXON 11
EXON 12
EXON 13
EXON 14

b)


P
P
780 bp
p21 CODING REGION

B. Labeling

Probes were labeled using a random primer DNA labeling kit (BMB) which is a modification of the method originally described by Feinberg and Vogelstein (1983; 1984). Template DNA was diluted to 25 ng/10 µl in TE buffer and stored at -20°C in 25 ng aliquots. 25 ng of the template DNA was denatured by boiling 10 minutes, then immediately immersing in an ice water bath. The buffer containing assorted hexanucleotides (the random primers), dATP, dGTP, dTTP and α^{32}PdCTP (3000 Ci/mmol, New England Nuclear) were added to the template DNA. The large Klenow fragment of DNA polymerase was added to extend the primers and the reaction was carried out at 37°C for 30-60 minutes. The reaction was stopped by the addition of 1/5 volume 0.1 M EDTA (pH 8.0) and the probe was diluted to 50 µl with STE [10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0), 100 mM NaCl]. Unincorporated nucleotides were removed by passage through a BioSpin 30 column (BioRad) spun at 1,100 x g 4 minutes in a GLC-1 centrifuge. The specific activity of the labeled probes was determined by averaging the counts per minute (cpm) of two 2 µl aliquots counted in Safety-Solve in a Packard Tricarb 460C scintillation counter. The labeled probes were stored at -20°C until use and were discarded when one half-life of the ^{32}P (2 weeks) had elapsed.

5. Hybridization of DNA Affixed to Hybond Membranes

A modification of the method described by Seldon (1987) was used for hybridization of DNA affixed to nylon membranes.

Ten ml of pre-hybridization buffer [5X SSC, 25 mM KPO_4 (pH 7.4), 0.5% dextran sulfate, 1% SDS, 50% deionized formamide, 5X Denhardt's solution (0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin)] was added to the dry membranes and distributed throughout the bag, then the bag was sealed and placed in a 32°C water bath for 4 hours. Ha-ras (2×10^7 cpm) or raf (1×10^7 cpm) ^{32}P-labeled probe, diluted with 500 µl STE, and 1 g (final concentration - 100 mg/ml) sheared, sonicated salmon sperm DNA (Sigma Chemical Co.), was boiled 10 minutes, then immersed in ice water. The prehybridization buffer was completely removed from the bag by rolling a pipet over the open bag. Ten ml of hybridization buffer (the same as pre-hybridization buffer without Denhardt's solution) was quickly poured into the bag. The probe mixture was added to the hybridization buffer by pasteur pipette and rapidly dispersed over the membrane. The bag was sealed, then extensively massaged to distribute the probe. Hybridization was carried out in a 32°C water bath for 20 hours with the Ha-ras probe and 16 hours for the raf probe.

Following hybridization, the membranes were rinsed in 2X SSC, 0.1% SDS for 5 minutes, then washed, with agitation, in 2X SSC, 0.1% SDS for 15 minutes at room temperature, 0.5X SSC, 0.1% SDS 15 minutes at room temperature, 0.1X SSC, 0.1% SDS for 15 minutes at room temperature, and 0.1X SSC, 1.0% SDS for 30 minutes at 37°C. Membranes hybridized with the Ha-ras probe were washed an additional 10 minutes at room temperature in 0.1X SSC, 1.0% SDS. The membranes were then blotted on

3 MM paper and wrapped in Handi-Wrap (Dow Chemical Co.) for autoradiography.

The radiolabeled probe was removed from the membrane following autoradiography by pouring a 78-80°C solution of 10 mM Tris (ph 8.0), 1 mM EDTA (pH 8.0), 1% SDS on the membrane and agitating for 30 minutes, then repeating once.

6. Autoradiography

The plastic-wrapped membrane was placed between 2 Cronex Lightening Plus intensifying screens inside a Kodak cardboard exposure cassette. Kodak X-OMAT AR5 film was added between the membrane and the upper intensifying screen (in complete darkness), the cassette was wrapped in foil, and clamped between 2 clipboards. The film was exposed to the membrane for 1-7 days at -80°C.

The developing process was carried out in complete darkness. The film was removed from the cassettes, placed in a film holder, immersed in Kodak GBX developer for 4 minutes (at 72°F), rinsed briefly in running water, and immersed in Kodak GBX fixer for 4 minutes. The developed film was rinsed thoroughly in running water and dried for 45 minutes in a drying cabinet.

7. RNA Isolation

RNA isolation requires the use of RNase-free materials. Glassware was baked at 400°C for 4 hours, glass distilled water and buffers were treated with 0.1% diethylpyrocarbonate

(DEPC) and autoclaved, plasticware was assumed to be RNase-free if new. RNA was isolated by a modification of the method described by Chomczynski and Sacchi (1987). A frozen liver sample weighing from 50 to 300 mg was placed in 3 ml of 4 M guanidinium isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl with 21.6 μl β-mercaptoethanol and immediately homogenized in a Polytron tissue grinder (Brinkmann). The solution was poured into a new 15 ml Corning polypropylene tube. One-tenth volume of DEPC-treated, autoclaved 2 M sodium acetate (pH 4.0) was added and the solution was mixed gently. One volume of water-saturated (not equilibrated) phenol (with 0.1% 8-hydroxyquinoline) was added and the solution mixed gently, then one-third volume of chloroform (containing 3% isoamyl alcohol) was added. The solution was shaken vigorously for 10 seconds, transferred to a baked 15 ml Corex tube and placed on ice 15 minutes. The layers were separated by centrifugation at 8,100 x g (7,500 rpm in Sorvall centrifuge) 20 minutes at 0°C. The aqueous layer was removed by transfer pipet to another baked 15 ml Corex tube and the RNA was precipitated with 1 volume n-propanol at -20°C at least 1 hour. The RNA was pelleted by centrifugation at 8,100 x g (7,500 rpm) for 20 min at 0°C. The pellet was redissolved in 300 μl of the same guanidinium isothiocyanate-containing denaturing solution used initially, transferred to a new eppendorf tube, and the RNA was reprecipitated with 1 volume of n-propanol at -20°C for at least 1 hour. The RNA was again pelleted by centrifugation at 10,000 rpm for 10 minutes in an

Eppendorf centrifuge (Brinkmann) in a cold room. The pellet was rinsed in 75% ethanol and recentrifuged. After air drying, the pellet was dissolved in 100 µl of DEPC-treated 0.05% SDS at 65°C with frequent vortexing, then placed on ice for 10 minutes. Insoluble salts were removed by centrifugation in the Eppendorf centrifuge at 10,000 rpm 5 minutes. The supernatent containing the RNA was transferred to a new tube and concentration and purity were determined spectrophotometrically. Concentration was determined using the constant value of 1 A_{260} = 40 µg of RNA/ml. The A_{260}/A_{280} ratios in TE buffer routinely approximated 2.0. RNA was stored for future use at -80°C.

8. Simultaneous Isolation of DNA and RNA

When less than 200 mg of liver tissue was available (e.g. some tumor samples), DNA and RNA were isolated simultaneously by a modification of the method of Chirgwin, *et al.* (1979). Frozen liver tissue was pulverized in liquid nitrogen with a mortar and pestle, then stirred rapidly into 4 M guanidine isothiocyanate, 0.5% sarcosyl, 1 M sodium citrate (pH 7.0), 0.5% β-mercaptoethanol until homogeneous. This was layered over a 5.7 M/3 M CsCl [0.1 M EDTA (pH 8.0), DEPC-treated, and autoclaved) gradient and centrifuged in a Beckman ultracentrifuge in a SW41 rotor at 29,000 rpm for 22 hours.

A. DNA isolation

Following centrifugation, the guanidium layer was discarded. Using a transfer pipette, the viscous DNA

containing layer was removed from just below the 5.7 M/3 M CsCl interface and measured. One thousandth volume of RNase A (50 mg/ml in TE buffer, preboiled 10 minutes) was added to the DNA solution and the mixture was dialyzed against a solution of 10 mM Tris (pH 7.5), 1 mM EDTA (pH 7.5), 0.1% SDS for 1 hour at room temperature. One hundredth volume of Proteinase K (10 mg/ml in TE buffer) was added, the dialysis solution replaced, and the DNA was dialyzed another hour. The dialysis solution was then replaced with 10 mM Tris (pH 7.5), 1 mM EDTA. Following overnight dialysis at 4°C, the DNA was washed with phenol and chloroform, precipitated, and dissolved as in section 2.

B. RNA isolation

After removal of the DNA layer, the tube was rapidly emptied and inverted to drain. The portion of the tube containing the RNA pellet was cut and the upper portion of the tube discarded to avoid RNase contamination. The RNA was dissolved in DEPC-treated water and washed twice with an equal volume of 4:1 chloroform:butanol and brief centrifugation at 14,000 rpm in a Brinkmann microfuge. The top, RNA containing, layer was removed and precipitated with an equal volume of n-propanol at -20°C for at least 1 hour, centrifuged, washed, and dissolved in 0.5% SDS as in Section 7.

9. Assessment of RNA Integrity by Electrophoresis

Assessment of RNA was performed using either extra samples prepared at the time an electrophoresis for Northern

transfer was done (10 μg RNA/25 μl total volume) or 3.5 μl RNA (independent of concentration) denatured in 50% formamide, 0.5X MOPS (pH 7.0), 2.2 M formaldehyde. One μl of 10,000X ethidium bromide (Oncor) was added to each sample and the samples were heated at 60°C for 15 minutes then placed on ice. One-fifth volume of 5 X RNA loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromphenol blue, 0.4% xylene cyanol ff) was added to each sample and 7 μl of each sample was loaded on a 1% agarose, 0.66 M formaldehyde, 1X MOPS (pH 7.0) gel in a minigel apparatus (BRL). The running buffer was 1 X MOPS and electrophoresis was carried out at 80 V for approximately 1 hour. The gel was photographed as described in section 3B. The RNA was determined to be intact if 2 major bands were seen and if the band most proximal to the wells (the 28S ribosomal RNA band) was most intense.

10. Agarose Gel Electrophoresis of RNA and Northern Transfer

A. Agarose gel electrophoresis

RNA was diluted in DEPC-treated water to 10 μg in a total volume of 4.5 μl and denatured in 50% formamide, 0.5 X MOPS (pH 7.0), 2.2 M formaldehyde at 60°C for 15 minutes, then placed on ice. One-fifth volume of 5 X RNA loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromphenol blue, 0.4% xylene cyanol ff) was added. RNA ladder (BRL) was treated as one sample. The samples were loaded on a 1% agarose, 1X MOPS, 0.66M formaldehyde gel and electrophoresis was carried out in 1X MOPS at 80 V for 6-7 hours or until the dye front was

approximately 2/3 of the way between the wells and the end of the gel. The lane containing the RNA ladder was cut from the gel and stained with 1:1000 of 10,000X ethidium bromide for 20 minutes and destained in water 1-2 hours. During the staining and destaining procedure, sodium, not fluorescent, lights were used in the room to decrease uptake of ethidium bromide by the formaldehyde-containing gel and subsequent autofluorescence of the gel. The RNA ladder was photographed as in section 3B using 2A and 23A filters and a f-stop of 11.

B. Northern transfer

Prior to northern transfer, the gel was rinsed 5 times in DEPC-treated water. Gene Screen Plus (NEN) was cut to size, wet in water, then soaked in 10X SSPE [1.5 M NaCl, 0.1 M NaH_2PO_4, 0.01 M EDTA (pH 7.4)] for 15 minutes. The Northern transfer was set up exactly as in section 3C, except the transfer buffer was 10X SSPE. After transfer, the membrane was UV-crosslinked and baked as described in section 3C. If not hybridized immediately, the membrane was stored in a hybridization bag at -20°C.

11. Hybridization of RNA Affixed to Gene Screen Plus

Northern blots were prehybridized in 10 ml of 5X SSPE (pH 7.4), 50% deionized formamide, 2.5X Denhardt's solution, 10% dextran sulfate and 1% SDS at 42°C for 2-4 hours. Radiolabeled probe (1 x 10^7cpm/10 ml hybridization buffer) and 1 g sheared salmon sperm DNA were denatured by boiling add immersing in an ice water bath, then added to the

hybridization bag. After hybridization at 42°C for 16-18 hours, the membranes were washed with agitation twice in 2X SSPE for 15 minutes at room temperature, twice in 2X SSPE, 2% SDS at 65°C for 45 minutes, and twice in 0.1X SSPE at room temperature for 15 minutes. Autoradiography was as described in Section 6; exposure was for 3-14 days. The membranes were stripped of the radiolabeled probe by agitating the membrane in boiling 0.1% SDS 30 minutes.

12. 5' End-labeling of 28S Oligonucleotide

A 25-mer DNA oligonucleotide specific for 28S rRNA (5'-AAC GAT CAG AGT AGT GGT ATT TCA CC-3'; Barbu and Dautry, 1989) was synthesized by the Michigan State University Macromolecular Synthesis Facility. The oligonucleotide was diluted and the concentration determined spectrophotometrically. Based on the extinction coefficients of the dNTPs in this oligonucleotide, 1 A_{260}=29.4 μg/ml. The oligonucleotide was divided into aliquots of 20 μg/tube, dried in a Speed-vac (Savant) and stored dessicated. For use 20 μg was diluted with 100 μl sterile water. A 5' DNA terminus labeling system (BRL) was used to label the oligonucleotide. 1 μl (200 ng) of oligonucleotide was mixed with 12 μl of water, incubated at 65°C for 15 minutes, and removed to an ice-water bath. 5 μl forward reaction kinase buffer, 5 μl γ^{32}P ATP (3000 Ci/mmole, NEN) and 10 units T4 polynucleotide kinase were added. The solution was mixed gently, centrifuged briefly, and incubated at 37°C for 20 minutes. An additional

10 units of T4 polynucleotide kinase was added and the mixture was incubated for another 20 minutes. The volume was increased to 100 μl with STE buffer and unincorporated nucleotides were removed by centrifugation through a BioSpin 6 column (BioRad). The specific activity was determined by scintillation counting.

13. Hybridization of RNA Affixed to Gene Screen Plus with 28S Oligonucleotide

Northern blots were examined for levels of 28S rRNA as an internal control to correct for possible differences in the amount of RNA loaded in each lane. The membrane was prehybridized with 200X denatured unlabeled 28S oligonucleotide in 5X SSPE, 10X Denhardt's solution and 0.5% SDS at 48°C at least 15 minutes. Labeled probe (6.35×10^6 cpm/10 ml hybridization buffer) and 200X unlabeled 28S oligonucleotide were denatured by boiling and immersing in an ice water bath, then added to the hybridization bag. Following hybridization at 48°C for 16-18 hours, the membranes were rinsed in 2x SSPE, 0.1% Na pyrophosphate, 0.1% SDS for 5 minutes at room temperature, then washed in 2X SSPE, 0.1% Na pyrophosphate, 0.1% SDS in a 42°C incubator for 30 minutes. Autoradiography as described in Section 6 was accomplished in 1-24 hours.

14. Image Analysis of Autoradiographs

A. Southern blot analysis.

For analysis of alterations in methylation status of raf the ratio of the intensity of the band at 6.7 kb to the sum of the intensities of the bands at 5.1 and 3.0 kb in the MspI digests was determined using a BioQuant MEG IV VCMTE image analyzer (R&M Biometrics, Inc., Nashville, TN). The intensity (average density within a constant sized region multiplied by the number of pixels above background) was measured for each band of interest. An individual background correction was made for each band by averaging the density of the area above and below the band and subtracting that number from the band density. The ratios of the intensities of the bands rather than the absolute value for the 6.7 kb band was used to minimize misinterpretations that could occur if there were differences in the amount of DNA loaded per lane. Alterations in the methylation status of Ha-ras was examined similarly by measuring the intensity of the 2.6 kb band in HpaII digests and comparing that with the intensity of the major band at <2.0 kb in the same lane. Because ratios were determined within one lane, comparisons could be made between samples on different autoradiographs. One-way analysis of variance was employed for statistical analysis and significance was determined by the Newman-Keuls test ($p=0.05$).

B. Northern blot analysis

Analysis of Northerns blots was accomplished by comparison of the intensity of the hybridization of the raf or

Ha-ras probe to the intensity of the hybridization of the 28S oligonucleotide. Because ratios were determined from bands on different autoradiographs, comparisons could only be made between samples on the same autoradiograph.

15. Protein Isolation

Approximately 50 mg of frozen liver tissue was added to 1 ml Triton lysis buffer [150 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris (pH 7.4), 1% Triton X-100] containing 8.8 µl 2% phenylmethyl sulfonyl fluoride (PMSF; dissolved in ethanol) and 10 µl aprotinin, homogenized immediately, and centrifuged at 16,000 x g, 15 minutes at 4°C in an Eppendorf microfuge. The supernatent was transferred to a new tube. Protein concentration was determined spectrophotometrically with a Bradford protein assay using bovine serum albumin to generate a standard curve (Bradford, 1976).

16. Polyacrylamide Gel Electrophoresis and Western Blotting

A. Polyacrylamide gel electrophoresis

A 12% acrylamide, 0.5% bis-acrylamide gel was prepared in 375 mM Tris (pH 8.8) with 0.1% SDS and 0.1% ammonium persulfate. 12 µl N,N,N',N'-tetramethylethylenediamine (TEMED) (for a 30 ml gel) was added as a catalyst to polymerize the gel (modified from Laemmli, 1970). The gel was poured in a Hoeffer (San Francisco, CA) SE 650 vertical gel apparatus and allowed to polymerize about one hour. A 3.75% stacking gel (3.75% acrylamide, 0.1% bis-

acrylamide, 83 mM Tris (pH 6.8), 0.1% SDS, 0.1% ammonium persulfate with 6 μl TEMED (in a total volume of 6 ml) was layered over the 12% gel and allowed to polymerize overnight with the well-former in place.

Protein (150 μg) was diluted to 20 μl, mixed with one volume of 2X sample buffer [125 mM Tris (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.01% bromphenol blue], boiled for 3 minutes and loaded on the gel. Prestained protein markers (20 μl) were included as one sample to monitor the transfer and 20 μl unstained low molecular weight protein standards (BioRad; diluted 1:10) were included for estimation of protein size. The samples were stacked using 20 mA (constant current) for about 30 minutes, then resolved at 40 mA for 3-4 hours. The electrophoresis buffer was 25 mM Tris (pH 8.3), 192 mM glycine, 0.1% SDS.

B. Western blotting

Following electrophoresis, the stacking gel was removed and the resolving gel sandwiched against a sheet of nitrocellulose between 2 sheets of filter paper, 2 Scotch-Brite pads and 2 plastic supports. The proteins were transferred to the nitrocellulose electrophoretically overnight at 30 volts overnight in 25 mM Tris (pH 8.3), 192 mM glycine, 20% methanol (w/v) as described by Towbin, *et al.*, (1979). After the transfer was complete, the lanes containing the low molecular weight protein standards were removed and visualized by India Ink staining in a solution of 0.1% India

Ink, 0.3% Tween 20 in phosphate-buffered saline several hours to overnight.

17. Detection of p21 by immunoblotting

The nitrocellulose was incubated in 10 ml of incubation buffer [1X TTBS (100 mM Tris (pH 7.5), 0.9% NaCl, 0.1% Tween 20), 5% non-fat dry milk] per lane in a sealable sandwich bag for 1 hour on a Red Rocker mixer (Hoeffer). Fresh incubation buffer containing a 1:500 dilution of the monoclonal antibody Ab-1 (Oncogene Science, Manhasset, NY; from the clone Y13-259, Furth, *et al.*, 1987; Sigal, *et al.*, 1987) was added. Following overnight incubation, the membrane was washed in TTBS, placed in a new bag with fresh incubation buffer and the second antibody (biotinylated anti-mouse IgG; Vector Laboratories, Burlingame, CA), incubated overnight, then washed in TTBS. Then the membrane was incubated with the Vectastain reagent, consisting of a biotin-avidin-horseradish peroxidase complex (Vector), for 1 hour, and washed with TTBS. The diagnostic color change was catalyzed by incubation with a solution of diaminobenzidine, hydrogen peroxide, and NiCl (Vector). The color change is temporary (about 2 weeks if exposed to light), so the membranes were photographed to preserve the data.

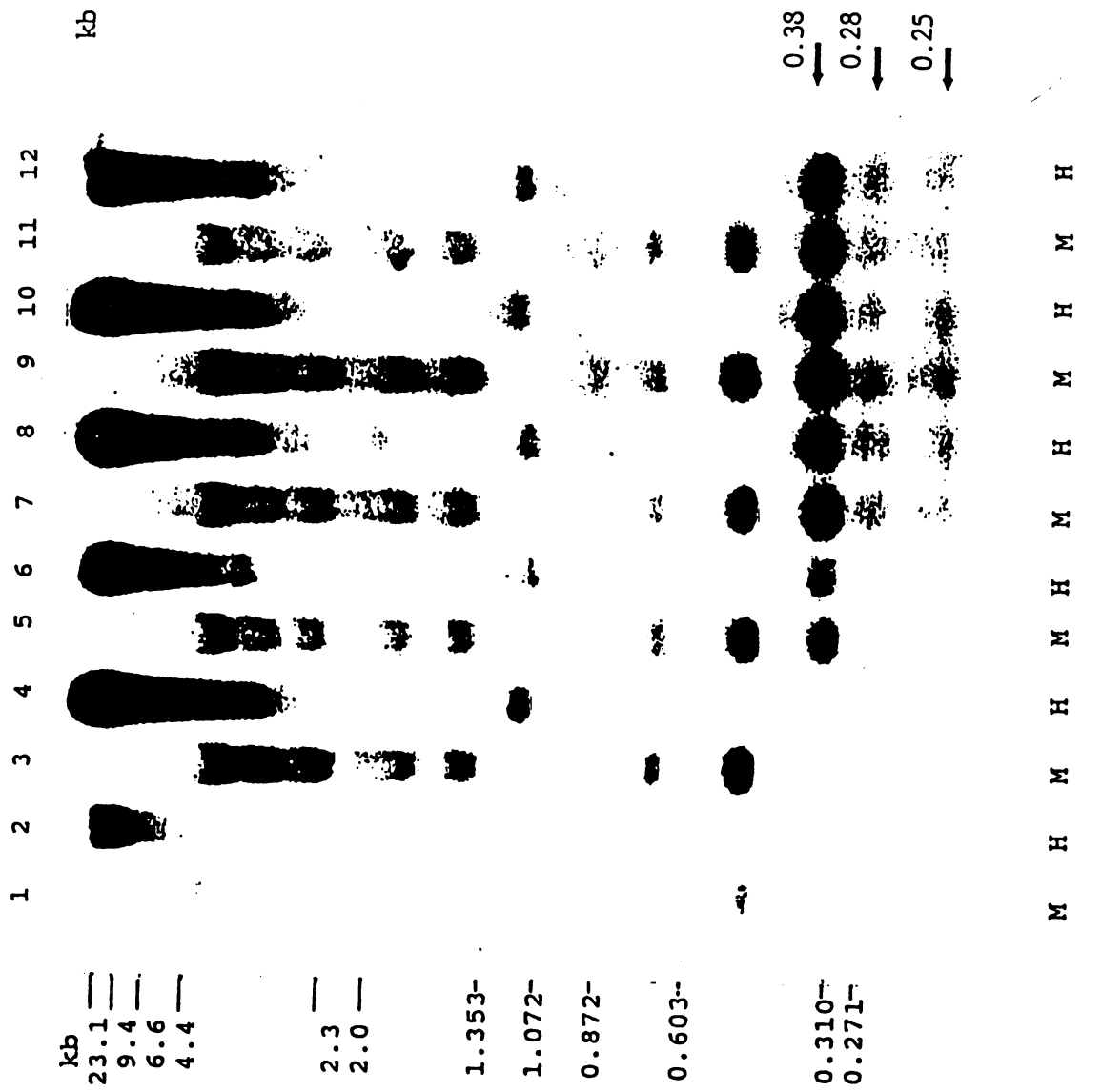
RESULTS

1. Methylation Status and mRNA Levels of Ha-ras in $WB^{Ha\text{-}ras}$ cell-induced Rat Liver Tumors

A. Assessment of methylation status of Ha-ras in WB cells

The methylation status of Ha-ras in DNA from WB^{wt}, WB^{neo}, and $WB^{Ha\text{-}ras}$ (cell lines described in the Methods section) was examined by MspI, HpaII, and HhaI digestion and hybridization with the ^{32}P-labeled v-Ha-ras probe. In both the WB^{wt} and the WB^{neo} cells, the DNA is largely undigested by HpaII (Figure 6, lanes 1-4), i.e. the majority of 5'-CCGG-3' sites are methylated on the internal cytosine (5'-C^{Me}CGG-3'). In DNA from $WB^{Ha\text{-}ras}$ cells, additional bands are detectible in the MspI digest at 280 and approximately 250 base pairs (Figure 6, lane 5) indicating the presence of foreign DNA (i.e. the virally-introduced v-Ha-ras). These bands are also present in the HpaII digest of the same DNA; additionally, a band at 380 bp which was present in MspI digests of WB^{wt} and WB^{neo} cells is now detectible in the HpaII digest of $WB^{Ha\text{-}ras}$ cells (Figure 6, lane 6). This indicates that the introduced Ha-ras DNA is hypomethylated since it is cleaved by both MspI and HpaII. A further indication of hypomethylation of the v-Ha-ras DNA is the detection of additional bands (1.9 and 1.1 kb) in Ha-ras

Figure 6. The methylation status of Ha-ras: WB cells; MspI and HpaII digests. DNA from WB^{wt}, WB^{neo}, $WB^{Ha\text{-}ras}$ cells and cells cultured from liver tumors from F344 rats which were injected with $WB^{Ha\text{-}ras}$ cells (WBK1, WBK2, and WBK3 cells) was digested with 5U/μg MspI (M) or HpaII (H), fractionated through a 1.1% agarose gel, and analyzed by Southern blotting, hybridization with the ^{32}P-labeled v-Ha-ras probe, and autoradiography. The arrows point to the additional bands seen in the HpaII digests of the Ha-ras infected cells and the cells cultured from tumors. Lanes 1 and 2: WB^{wt} cells; lanes 3 and 4: WB^{neo} cells; lanes 5 and 6: $WB^{Ha\text{-}ras}$ cells; lanes 7 and 8: WBK1 cells; lanes 9 and 10: WBK2 cells; lanes 11 and 12: WBK3 cells.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from $WB^{Ha\text{-}ras}$ cells digested with HhaI compared to WB^{wt} cells (Figure 7, lanes 1 and 2). Cells cultured from liver tumors in $WB^{Ha\text{-}ras}$-injected rats (WBK1, WBK2, and WBK3 cell lines) have the same HpaII (Figure 6, lanes 7-12) and HhaI (Figure 7, lanes 3-5) digestion patterns as the original injected cells, suggesting that the tumors did indeed evolve from the $WB^{Ha\text{-}ras}$ cells.

B. Assessment of methylation status of Ha-ras in F344 rats injected with $WB^{Ha\text{-}ras}$ cells

Figure 8 shows the results of MspI and HpaII digestion of DNA from the liver and lung of an untreated F344 rat and liver and lung tumor tissue and surrounding nontumorous liver tissue from one F344 rat given approximately 1×10^7 $WB^{Ha\text{-}ras}$ cells via portal vein injection 3 weeks previously. The Ha-ras gene is poorly digested by HpaII in the normal F344 rat liver (lanes 1 and 2) and lung DNA (lanes 7 and 8) and in DNA from the liver tissue surrounding the tumor in the treated rat (lanes 3 and 4) indicating that most 5'-CCGG-3' sites are methylated on the internal cytosine. The DNA isolated from liver (lanes 5 and 6) and lung tumors (lanes 9 and 10) has a relatively hypomethylated Ha-ras gene indicated by the presence of an additional band in the HpaII digests at 380 bp as seen in the $WB^{Ha\text{-}ras}$ cells. HhaI digestion of DNA from liver tumors from 2 rats injected with $WB^{Ha\text{-}ras}$ cells results in the detection of additional bands at approximately 1.9 and 1.1 kb, also indicating hypomethylation of Ha-ras in the tumor tissue relative to Ha-ras in untreated

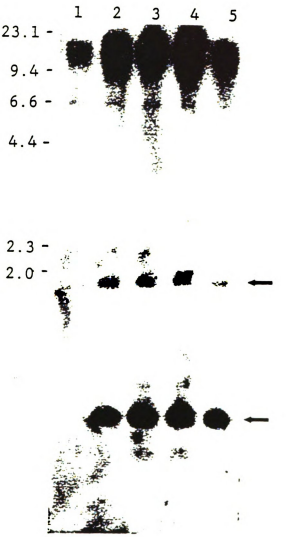
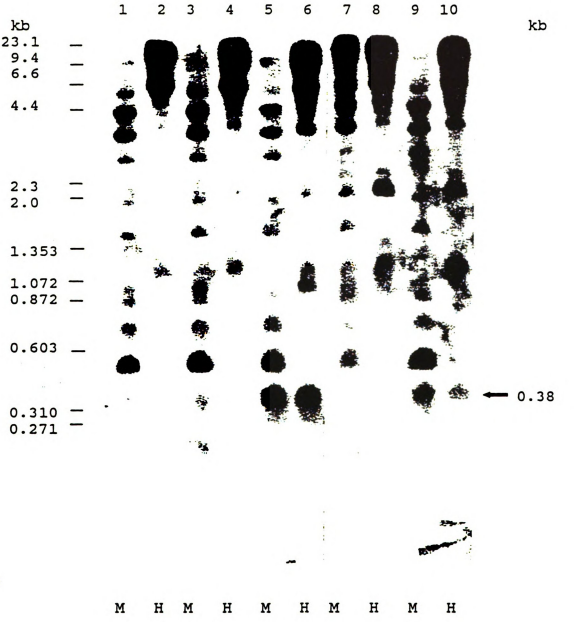


Figure 7. The methylation status of Ha-ras: WB cells; HhaI digests. DNA from WB^{wt}, $WB^{Ha\text{-}ras}$, WBK1, WBK2, WBK3 cells was digested with 3U/μg HhaI, fractionated through a 0.9% gel and analyzed as in Figure 6. The arrows point to the additional bands seen in the Ha-ras infected cells and the cells cultured from tumors. Lane 1: WB^{wt} cells; lane 2: $WB^{Ha\text{-}ras}$ cells; lane 3: WBK1 cells; lane 4: WBK2 cells; lane 5: WBK3 cells.

Figure 8. The methylation status of Ha-ras: F344 rat liver and lung tumors; MspI and HpaII digests. DNA from liver and lung tissue from an untreated F344 rat, liver and lung tumors from a F344 rat which had received 1 x 10^7 $WB^{Ha\text{-}ras}$ cells via the portal vein 3 weeks previously and nontumorous liver tissue surrounding the liver tumor was analyzed as in Figure 6. Lanes 1 and 2: normal liver; lanes 3 and 4: nontumorous liver surrounding a tumor; lanes 5 and 6: liver tumor; lanes 7 and 8: normal lung; lanes 9 and 10: lung tumor.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F344 rat liver and liver tissue surrounding tumor tissue (Figure 9).

C. Assessment of Ha-ras mRNA and ras p21 protein levels

Because hypomethylation of a gene has the potential to increase mRNA expression of that gene and increased expression of Ha-ras has been implicated in tumorigenesis, we examined Ha-ras mRNA levels in tumors from two F344 rats injected with $WB^{Ha\text{-}ras}$ cells (Figure 10a). The amount of Ha-ras mRNA in the adjacent nontumor tissue was similar to that seen in normal liver tissue from an untreated rat. The level of Ha-ras mRNA in both the tumors is greatly increased relative to nontumor tissue (Figure 10A). The level of p21 protein from a liver tumor was increased relative to control liver tissue (Figure 10B). Therefore there is a relationship between hypomethylated v-Ha-ras DNA, increased expression of Ha-ras (both mRNA and p21) and tumorigenicity.

2. Ha-ras and raf in the Nascent Liver of B6C3F1♂, C3H/He♂, and C57BL/6♀ Mice

A. RFLP screening

DNA from B6C3F1♂, C3H/He♂, and C57BL/6♀ mice was screened for RFLP. MspI digestion detected an RFLP (Figure 11); bands of 6.7, 5.1, 3.0, and 2.3 kb were seen in raf-probed C57BL/6♀ and B6C3F1♂ DNA, while the 6.7 kb band is not seen in C3H/He♂ DNA. To determine if the MspI RFLP was due to a change in base sequence or to a difference in methylation status, C57BL/6♀ DNA was digested with MspI and HpaII

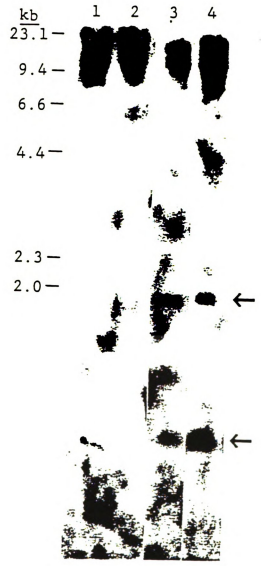
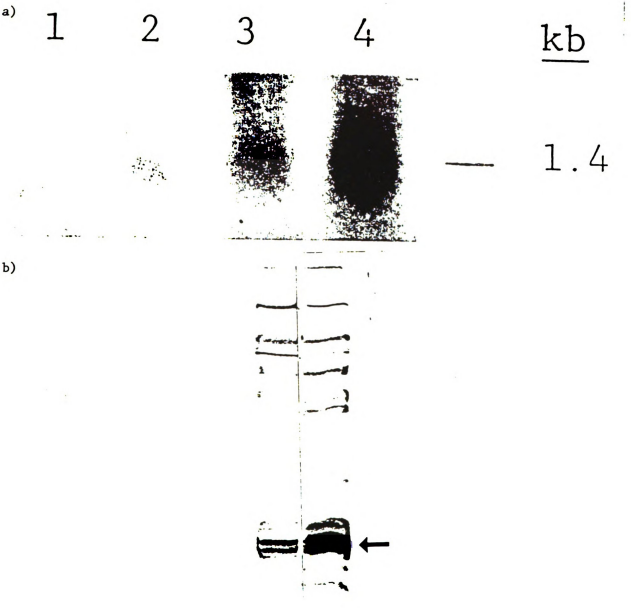


Figure 9. The methylation status of Ha-ras: F344 rat liver tumors; HhaI digests. DNA from liver tissue from an untreated F344 rat, liver tumors from 2 rats which had received 1 x 10^7 $WB^{Ha\text{-}ras}$ cells via the portal vein 3 weeks previously and nontumorous liver tissue adjacent to a liver tumor was analyzed as in Figure 7. Lane 1: normal liver; lane 2: nontumorous liver adjacent to a tumor; lanes 3 and 4: liver tumors from 2 rats.

Figure 10. Ha-ras mRNA and p21: F344 rat liver tumors. A) Ha-ras mRNA levels. RNA from liver tissue from an untreated F344 rat, liver tumors from 2 rats which had received 1 x 10^7 $WB^{Ha\text{-}ras}$ cells via the portal vein 3 weeks previously and nontumorous liver tissue adjacent to a liver tumor was analyzed by Northern blot analysis, hybridization with the ^{32}P-labeled v-Ha-ras probe, and autoradiography. Lane 1: normal liver; lane 2: nontumorous liver tissue adjacent to a tumor; lanes 3 and 4: liver tumors from 2 rats. B) p21 levels. Total protein from liver tissue from an untreated rat and from a liver tumor induced by injection of $WB^{Ha\text{-}ras}$ cells was fractionated by size through a polyacrylamide gel, transferred to nitrocellulose, and analyzed by immunoblotting with the Y13-259 ras p21 antibody. Lane 1: normal liver; lane 2: liver tumor.



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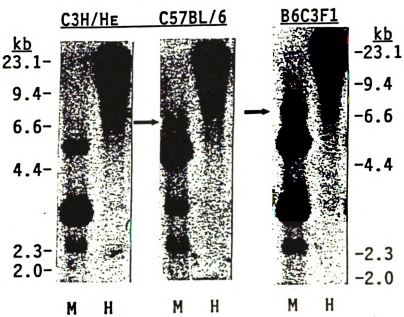


Figure 11. Restriction fragment analysis of raf: B6C3F1♂, C3H/He♂, and C57BL/6♀; MspI. DNA from C3H/He♂, C57BL/6♀, and B6C3F1♂ was digested with 5U/μg MspI (M) or HpaII (H) for 2 hours at 37°C, fractionated through a 0.9% agarose gel and analyzed by Southern blotting, hybridization with the ^{32}P-labeled raf probe, and autoradiography. The arrows point to the 6.7 kb band seen in C57BL/6♀ and B6C3F1♂, but not C3H/He♂ DNA. Lanes 1 and 2: B6C3F1♂; lanes 3 and 4: C3H/He♂; lanes 5 and 6: C57BL/6♀.

sequentially (Figure 12). The 6.7 kb band is not seen in the DNA digested with both MspI and HpaII (Figure 12, lane 3). Cleavage by MspI is inhibited by the presence of a methyl group on the external cytosine of its recognition sequence (Sneider, 1980; van der Ploeg and Flavell, 1980; Waalwijk and Flavell, 1978), while HpaII will cleave 5'-^{Me}CCGG-3', but not 5'-C^{Me}CGG-3' sites (Mann and Smith, 1977). This indicates the presence of a 5'-^{Me}CCGG-3' site within the fragment which is detected as a 6.7 kb band in MspI-digested, raf-probed C57BL/6♀ DNA. In the C3H/He♂, this site is not methylated since it is digested by MspI. Thus the raf gene in the tumor-prone C3H/He♂ is hypomethylated relative to the tumor-resistant C57BL/6♀.

An RFLP was detected in TaqI digested DNA (Figure 13). Hybridization with the raf probe results in 3.0 and 2.0 kb bands in C3H/He♂ DNA and 2.5 and 2.0 kb bands in C57BL/6♀ DNA. 3.0, 2.5, and 2.0 kb bands are detected in B6C3F1♂ DNA (Figure 13). Therefore, the raf gene in the 2 parental strains differs in base sequence and the B6C3F1♂ inherits one allele from each parent. RFLPs of the raf gene were not detected with EcoRI, HindIII, or StuI (Figure 13).

The published sequence of c-raf-1 was examined for recognition sites of the enzymes used in this study using the Map subprogram of the GCG sequence analysis program on the Vax computer system (Devereux, *et al.*, 1984). There is an MspI recognition site (5'-CCGG-3') in exon 12 approximately 0.8 kb

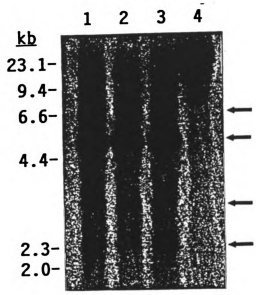
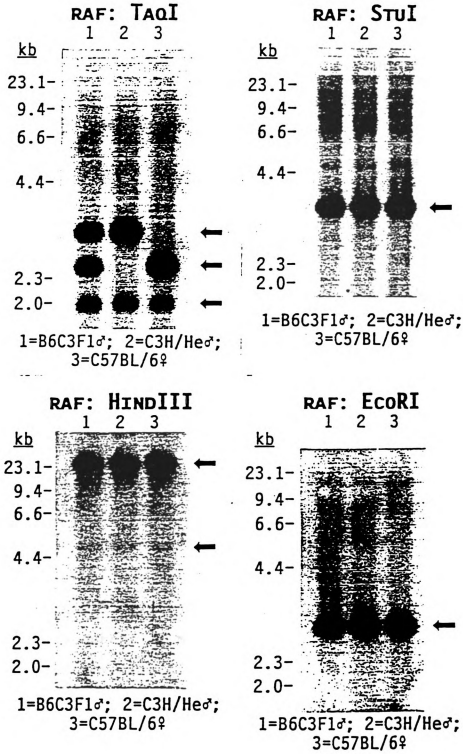


Figure 12. MspI and HpaII double digests of C57BL/6♀ DNA; raf. 10 μg of DNA was digested with 5U/μg MspI or HpaII in the appropriate buffer [MspI reaction buffer is 50 mM Tris (pH 8.0), 10 mM $MgCl_2$; HpaII reaction buffer is 20 mM Tris (pH 7.4), 10 mM $MgCl_2$] for 2 hours at 37°C. For the double digest, 10 μg of DNA was digested with HpaII in the buffer supplied by the manufacturer for 2 hours, then the buffer was adjusted to the proper concentrations [Tris (pH 8.0) added to a final concentration of 50 mM] for digestion with MspI which was carried out for 2 hours. Analysis was performed as in Figure 11. Arrows point to the 6.7, 5.1, 3.0, and 2.3 kb bands in the MspI digests. Lane 1: DNA digested with MspI in manufacturer's recommended buffer; lane 2: DNA digested with MspI in buffer recommended for HpaII, adjusted to 50 mM Tris; lane 3: DNA digested with HpaII, then MspI; lane 4: DNA digested with HpaII.

Figure 13. Restriction fragment analysis of raf: B6C3F1♂, C3H/He♂, and C57BL/6♀; TaqI, StuI, HindIII, EcoRI. DNA from B6C3F1♂ (lane 1), C3H/He♂ (lane 2), and C57BL/6♀ (lane 3) was digested with 5U/μg TaqI (2 hours at 65°C), 5U/μg StuI, 3U/μg HindIII, or 3U/μg EcoRI and analyzed as in Figure 11. The arrows point to the major bands.


RAF: TaqI
1 2 3
kb
23.1-
9.4-
6.6-
4.4-
2.3-
2.0-
1=B6C3F1♂; 2=C3H/He♂;
3=C57BL/6♀
RAF: StuI
1 2 3
kb
23.1-
9.4-
6.6-
4.4-
2.3-
2.0-
1=B6C3F1♂; 2=C3H/He♂;
3=C57BL/6♀
RAF: HindIII
1 2 3
kb
23.1-
9.4-
6.6-
4.4-
2.3-
2.0-
1=B6C3F1♂; 2=C3H/He♂;
3=C57BL/6♀
RAF: EcoRI
1 2 3
kb
23.1-
9.4-
6.6-
4.4-
2.3-
2.0-
1=B6C3F1♂; 2=C3H/He♂;
3=C57BL/6♀

from the StuI site at the 5' boundary of the area homologous to the probe (Figure 5A). StuI digestion of B6C3F1♂ DNA results in a 3.2 kb band when hybridized with the raf probe (Figure 14, lanes 1-6). The length of the human c-raf fragment homologous to the probe is approximately 6.2 kb (Rapp, *et al.*, 1988). Therefore there are differences in human and mouse c-raf intron size. The major bands detectable in a StuI and MspI double digest of B6C3F1♂ DNA are 2.4 and 0.8 kb (Figure 14, lanes 7-9), while double digestion with StuI and HpaII does not result in cleavage of the 3.2 kb fragment (Figure 14, lanes 10-12). This indicates that the 5'-CCGG-3' site in exon 12 is methylated on the internal cytosine (i.e., 5'-C^{Me}CGG-3') since it is digested by MspI but not HpaII.

Aside from the RFLP previously detected in the MspI digests of the 3 mice strains, seen as a 15 kb band in the C57BL/6♀, but not the B6C3F1♂ or C3H/He♂ (Vorce and Goodman, 1989a), no Ha-ras RFLPs were detected with EcoRI, HindIII, StuI, or TaqI (Figure 15). The methylation sensitive enzymes HhaI and XhoI did not detect any RFLPs in either raf or Ha-ras in any of the 3 mice strains (Figure 16).

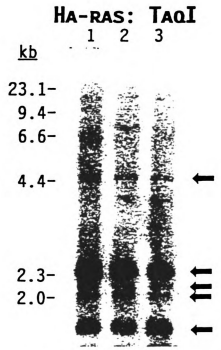
B. Assessment of mRNA in nascent liver

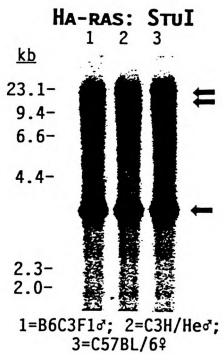
Northern blots of RNA from each strain were probed for raf and Ha-ras mRNA and 28S rRNA. The levels of each were approximately equal between the strains (Figure 17). Thus, despite the relative hypomethylation of raf and Ha-ras of

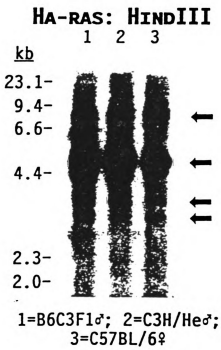
Figure 14. The methylation status of the CCGG site in exon 12 of raf: B6C3F1♂, C3H/He♂, andC57BL/6♀. DNA from B6C3F1♂ (lanes 1,4,7,10), C3H/He♂ (lanes 2,5,8,11) and C57BL/6♀ (lanes 3,6,9,12) was digested with StuI (lanes 1-6; lanes 1-3 were digested in the buffer supplied by the manufacturer, lanes 4-6 were digested in the buffer supplied with MspI adjusted to be optimum for StuI digestion), MspI then StuI (lanes 7-9), or HpaII then StuI (lanes 10-12). Double digests were performed with MspI or HpaII first in the recommended buffer, then the buffer was adjusted to 10 mM Tris (pH 8.0), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol for digestion with StuI. Southern blots were analyzed as in Figure 11. The arrows point to the 3.2 kb bands in StuI and StuI/HpaII double digests and to 2.4 and 0.8 kb bands in StuI/MspI double digests.

kb
23.1–
9.4–
6.6–
4.4–
2.3–
2.0–
1.35–
1.08–
0.87–
0.60–
StuI
1 2 3 4 5 6
MspI/StuI
7 8 9
HpaII/StuI
10 11 12

Figure 15. Restriction fragment analysis of Ha-ras: B6C3F1♂, C3H/He♂ and C57BL/6♀; TaqI, StuI, HindIII, EcoRI. The Southern blots pictured in Figure 13 were stripped, hybridized with the ^{32}P-labeled v-Ha-ras probe, and analyzed by autoradiography. The arrows point to the major bands.


Ha-ras: TaqI
1 2 3
kb
23.1-
9.4-
6.6-
4.4-
2.3-
2.0-
1=B6C3F1♂; 2=C3H/He♂;
3=C57BL/6♀


Ha-ras: StuI
1 2 3
kb
23.1-
9.4-
6.6-
4.4-
2.3-
2.0-
1=B6C3F1♂; 2=C3H/He♂;
3=C57BL/6♀


Ha-ras: HindIII
1 2 3
kb
23.1-
9.4-
6.6-
4.4-
2.3-
2.0-
1=B6C3F1♂; 2=C3H/He♂;
3=C57BL/6♀

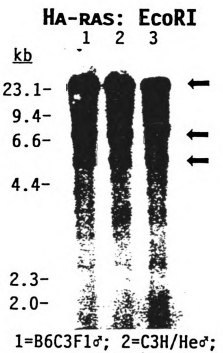
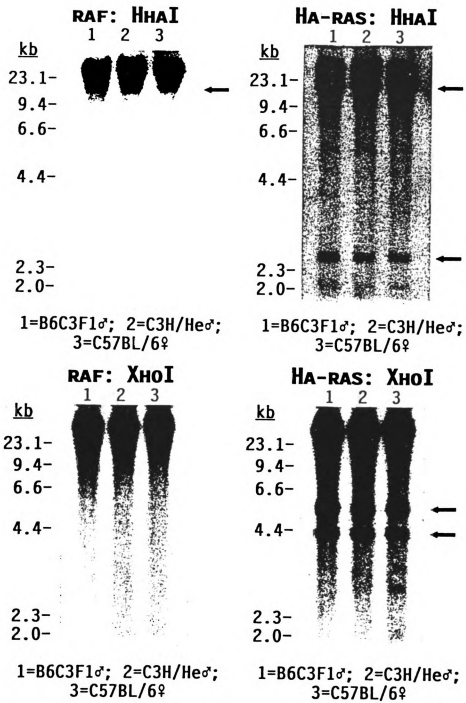

Ha-ras: EcoRI
1 2 3
kb
23.1-
9.4-
6.6-
4.4-
2.3-
2.0-
1=B6C3F1♂; 2=C3H/He♂;
3=C57BL/6♀

Figure 16. Restriction fragment analysis of raf and Ha-ras: B6C3F1♂, C3H/He♂, and C57BL/6♀; HhaI and XhoI. DNA from B6C3F1♂ (lane 1), C3H/He♂ (lane 2), and C57BL/6♀ (lane 3) was digested with 3U/μg HhaI, or 5U/μg XhoI and analyzed as in Figure 11 (raf) or Figure 15 (Ha-ras). The arrows point to the major bands.


RAF: HHAI
1 2 3
kb
23.1-
9.4-
6.6-
4.4-
2.3-
2.0-
1=B6C3F1♂; 2=C3H/He♂;
3=C57BL/6♀
HA-RAS: HHAI
1 2 3
kb
23.1-
9.4-
6.6-
4.4-
2.3-
2.0-
1=B6C3F1♂; 2=C3H/He♂;
3=C57BL/6♀
RAF: XHOI
1 2 3
kb
23.1-
9.4-
6.6-
4.4-
2.3-
2.0-
1=B6C3F1♂; 2=C3H/He♂;
3=C57BL/6♀
HA-RAS: XHOI
1 2 3
kb
23.1-
9.4-
6.6-
4.4-
2.3-
2.0-
1=B6C3F1♂; 2=C3H/He♂;
3=C57BL/6♀

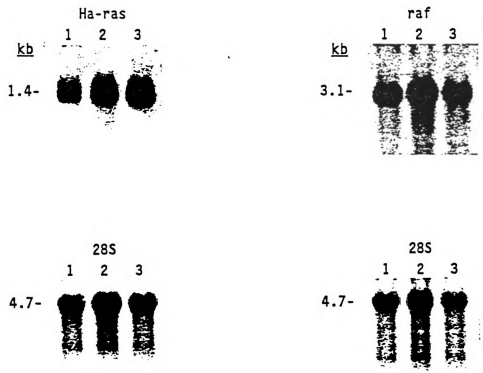


Figure 17. Ha-ras and raf mRNA levels: B6C3F1♂, C3H/He♂, and C57BL/6♀. RNA isolated from B6C3F1♂ (lane 1), C3H/He♂ (lane 2), or C57BL/6♀ (lane 3) was analyzed by Northern blotting, hybridization with the ^{32}P-labeled raf, Ha-ras, or 28S probes (membranes were stripped between subsequent probings), and autoradiography. The size of the RNA calculated as function of the distance traveled relative to an RNA ladder is given for each gene.

C3H/He♂ mice, there is no corresponding increase in mRNA levels.

3. Ha-ras and raf Following Phenobarbital Administration or Partial Hepatectomy in B6C3F1♂, C3H/He♂, and C57BL/6♀ Mice

A. Assessment of raf methylation status following partial hepatectomy

To determine the effect of hepatocyte proliferation on the methylation status of raf in the liver of B6C3F1♂, C3H/He♂, and C57BL/6♀ mice, a 2/3 surgical PH was performed, the mice were allowed to recover for 7 days and then sacrificed. Probing MspI-digested DNA with the ^{32}P-labeled raf probe results in the detection of 6.7, 5.1, 3.0, and 2.3 kb bands in B6C3F1♂ (Figure 18) and C57BL/6♀ (Figure 20), and 5.1, 3.0, and 2.3 kb bands in the C3H/He♂ (Figure 19). Figure 21 shows results of densitometric analysis of Figures 18, 19, 20. Because the relative intensities of the 5.1 and 3.0 kb bands varies between the 3 strains, the sum of the 2 bands was used for comparisons where possible. The 6.7 kb band is greatly diminished relative to the other major bands (5.1 and 3.0 kb) following PH of B6C3F1♂ mice (Figure 18, 21A). The C3H/He♂ lacks the 6.7 kb band which is altered in the B6C3F1♂, however, the 5.1 kb band is decreased relative to the 3.0 kb band following PH (Figure 19, Figure 21B). Like the B6C3F1♂, the C57BL/6♀ shows decreased intensity of the 6.7 kb band relative to the sum of the 5.1 and 3.0 kb bands following PH

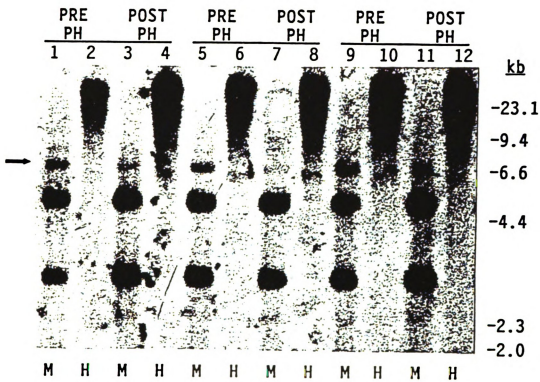


Figure 18. The methylation status of raf: B6C3F1♂ 7 days after PH; MspI and HpaII. Pre-PH refers to the liver sample removed at the time of PH, while post-PH refers to the liver sample removed 7 days after PH. The arrows indicate the bands seen following analysis as in Figure 11. Lanes 1 and 2, 5 and 6, and 9 and 10: DNA from 3 mice prior to PH; lanes 3 and 4, 7 and 8, and 11 and 12: DNA from the same 3 mice 7 days after PH.

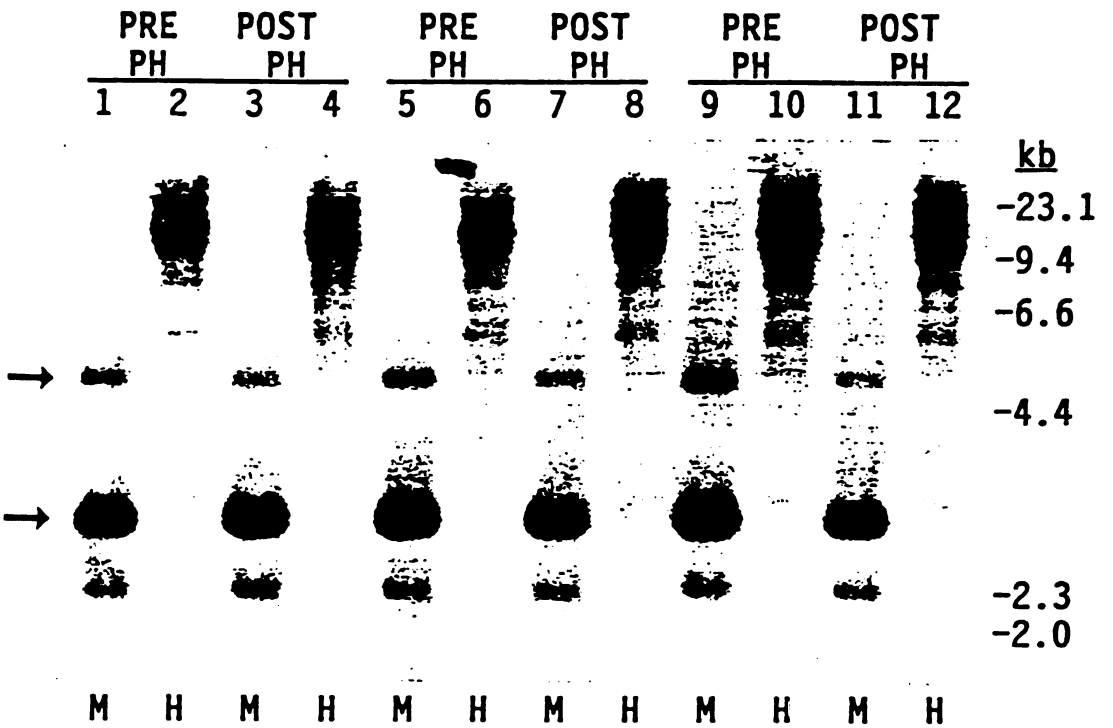


Figure 19. The methylation status of raf: C3H/Heσ 7 days after PH; MspI and HpaII. Pre-PH refers to the liver sample removed at the time of PH, while post-PH refers to the liver sample removed 7 days after PH. DNA was analyzed as in Figure 11. Arrows point to the 5.1 and 3.0 kb bands in the MspI digests. Lanes 1 and 2, 5 and 6, and 9 and 10: DNA removed from 3 mice at the time of PH; lanes 3 and 4, 7 and 8, and 11 and 12: DNA removed from the same 3 mice 7 days after PH.

Figure 20. The methylation status of raf: C57BL/6♀ 7 days after PH; MspI and HpaII. DNA was analyzed as in Figure 11. Arrows point to the 6.7, 5.1, and 3.0 kb bands in the MspI digests. Lanes 1 and 2, 5 and 6, 9 and 10, and 13 and 14: DNA removed from 4 mice at the time of PH; lanes 3 and 4, 7 and 8, 11 and 12, and 15 and 16: DNA removed from the same 4 mice 7 days after PH.

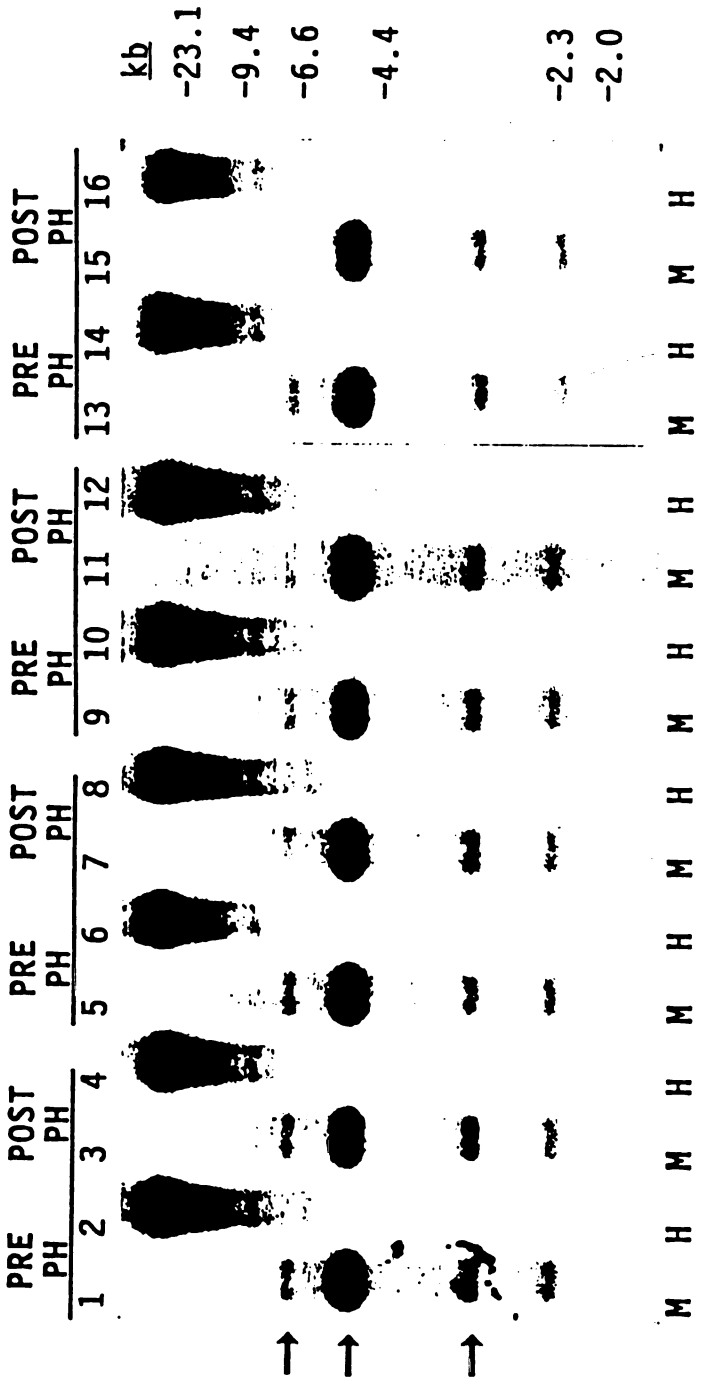
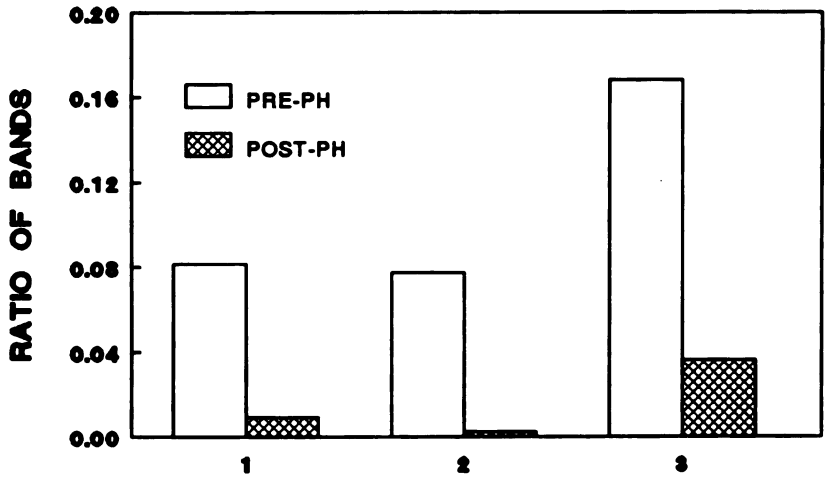
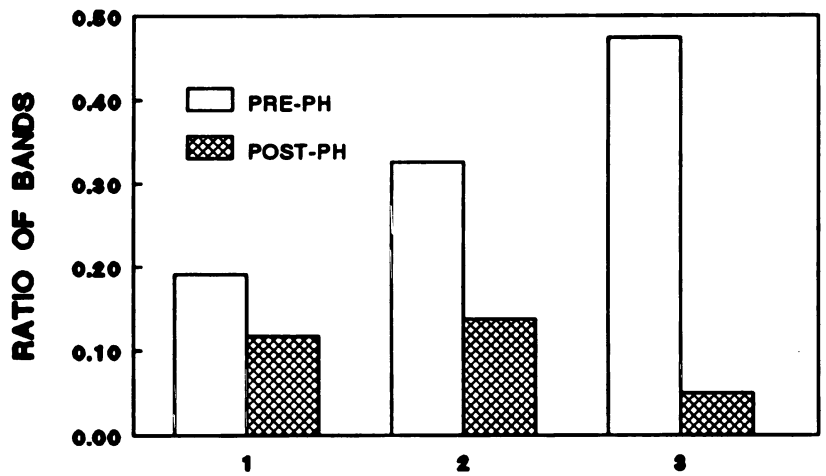

kb
-23.1
-9.4
-6.6
-4.4
-2.3
-2.0
PRE PH
POST PH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
M H M H M H M H M H M H M H M H M H

Figure 21. Relative changes in raf band intensities following PH. A) B6C3F1♂. The ratio of the intensity of the 6.7 kb band to the sum of the intensities of the 5.1 and 3.0 kb bands in the MspI digests on the autoradiograph pictured in Figure 18 was determined as described in the Methods section. The numbers along the x-axis refer to the individual mice as shown in Figure 18. The actual ratios determined by densitometric analysis are given in parentheses. 1 corresponds to lanes 1-4 (pre-PH: 0.0817, post-PH: 0.0091); 2 corresponds to lanes 5-8 (pre-PH: 0.0774, post-PH: 0.0023); and 3 corresponds to lanes 9-12 (pre-PH: 0.1678, post-PH: 0.0362). B) C3H/He♂. The ratio of the intensity of the 5.1 kb band to the intensity of the 3.0 kb band in the MspI digests on the autoradiograph pictured in Figure 19 was determined. 1 corresponds to lanes 1-4 (pre-PH: 0.191, post-PH: 0.117); 2 corresponds to lanes 5-8 (pre-PH: 0..326, post-PH: 0..137); and 3 corresponds to lanes 9-12 (pre-PH: 0..474, post-PH: 0..049). C) C57BL/6♀. The ratio of the intensity of the 6.7 kb band to the sum of the intensities of the 5.1 and 3.0 kb bands in the MspI digests on the autoradiograph pictured in Figure 20 was determined. The background in lane 11 interfered with image analysis, so lanes 9-12 are not included. 1 corresponds to lanes 1-4 (pre-PH: 0.015, post-PH: 0.014); 2 corresponds to lanes 5-8 (pre-PH: 0.020, post-PH: 0.005); and 3 corresponds to lanes 13-16 (pre-PH: 0.027, post-PH:0.008).

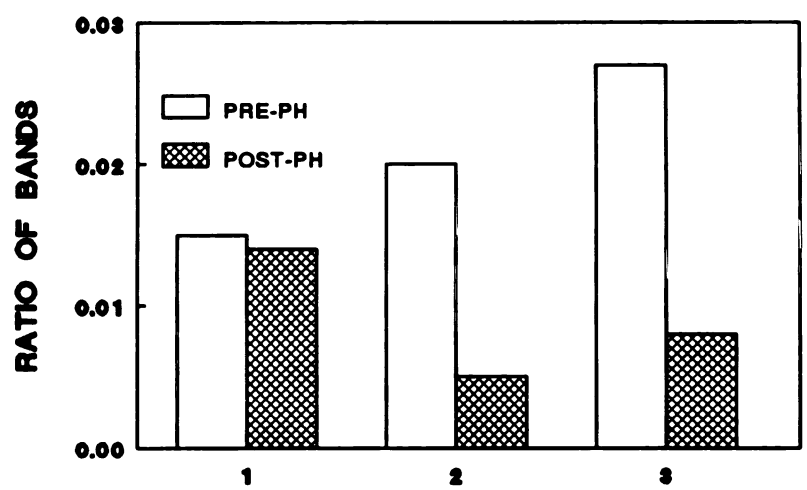
a)


RATIO OF BANDS
0.20
0.16
0.12
0.08
0.04
0.00
PRE-PH
POST-PH
1
2
3

b)


RATIO OF BANDS
0.50
0.40
0.30
0.20
0.10
0.00
PRE-PH
POST-PH
1
2
3

c)


RATIO OF BANDS
0.03
0.02
0.01
0.00
PRE-PH
POST-PH
1
2
3

(Figure 20, 21C). The loss (or decrease) of a band detected in MspI digested DNA indicates the loss of methylation at a 5'-^{Me}CCGG-3' site in some, but not all of the cells, i.e. there is mosaicism of methylation between liver cells. These results, summarized in Table 1, indicate that, in the 3 strains examined, raf becomes hypomethylated following cell proliferation stimulated by PH relative to the nascent liver.

B. Methylation status of Ha-ras 7 days after partial hepatectomy

To determine the methylation status of Ha-ras following PH, the blots pictured in Figure 18, 19, and 20 were stripped of the raf probe and hybridized with the ^{32}P-labeled v-Ha-ras probe. The MspI and HpaII digestion patterns are similar in all 3 strains (B6C3F1♂--Figure 22, C3H/He♂--Figure 23, C57BL/6♀--Figure 24). An approximately 15 kb band is detectible in the MspI digests of C57BL/6♀ DNA but not the other DNA as was found previously (Vorce and Goodman, 1989a); the intensity of this band is not altered by treatment. There is an increase in intensity of the 2.6 kb band in HpaII digests relative to the other, smaller (<2.0 kb) major band present in the HpaII digests in 2/3 of the B6C3F1♂ mice (Figure 22, 25A), 3/3 of the C3H/He♂ mice (Figure 23, 25B), and 2/4 of the C57BL/6♀ mice (Figure 24, 25C) following PH. A 5'-C^{Me}CGG-3' site which was present in the largely undigested DNA seen at the top of the lane has become unmethylated and is now cleaved by HpaII, resulting in increased intensity of the band at 2.6 kb. Clearly, this site is unmethylated in some

Table 1. Methylation Status and mRNA Levels of raf and Ha-ras Following Cell Proliferation.

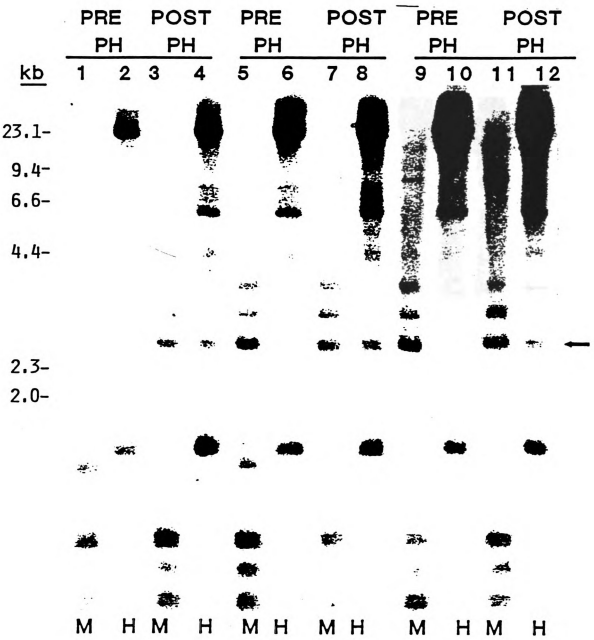
STRAIN	TREATMENT[1]	HYPOMETHYLATION OF raf[2]	HYPOMETHYLATION OF Ha-ras	INCREASE IN raf mRNA LEVEL[3]	INCREASE IN Ha-ras mRNA LEVEL
B6C3F1♂	PH	3/3	2/3	0/3	0/3
B6C3F1♂	PB	3/5	0/5	0/5	0/5
C3H/He♂	PH	4/4	3/3	0/3	0/3
C3H/He♂	PB	0/6	0/6	0/6	0/6
C57BL/6♀	PH	2/3	2/4	0/4	0/4
C57BL/6♀	PB	0/6	0/6	0/6	0/6

[1] PH refers to a 2/3 PH 7 days prior to sacrifice. The methylation status of the post-PH sample is compared to that of the sample removed from the same animal at the time of PH. PB refers to the administration of 75 mg PB/kg/day in the drinking water for 14 days prior to sacrifice. Control animals received distilled water.

[2] Relative hypomethylation was determined by densitometric analysis as described in the methods section.

[3] Levels of raf or Ha-ras mRNA were determined relative to the level of 28S rRNA in the same sample.

Figure 22. The methylation status of Ha-ras: B6C3F1♂ 7 days after PH; MspI and HpaII. The blot pictured in Figure 18 was stripped of the raf probe and analyzed as in Figure 15. The arrow points to the band at 2.6 kb in the HpaII digests. Lanes 1 and 2, 5 and 6, and 9 and 10: DNA from 3 mice at the time of PH; lanes 3 and 4, 7 and 8, and 11 and 12: DNA from the same 3 mice 7 days after PH.


PRE PH
POST PH
PRE PH
POST PH
PRE PH
POST PH
kb
1 2 3 4 5 6 7 8 9 10 11 12
23.1-
9.4-
6.6-
4.4-
2.3-
2.0-
M H M H M H M H M H M H

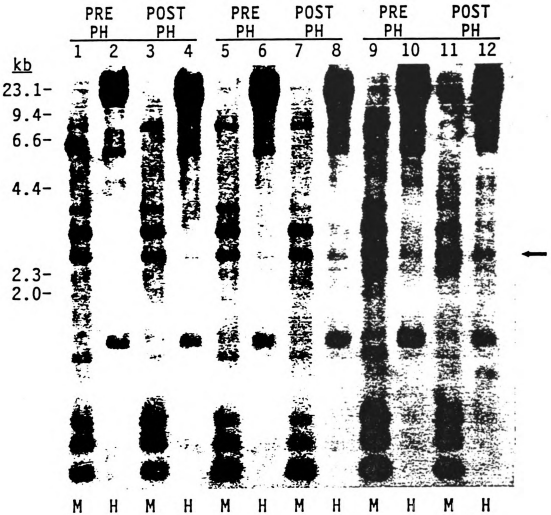


Figure 23. The methylation status of Ha-ras: C3H/Heơ 7 days after PH; MspI and HpaII. The blot pictured in Figure 19 was stripped of the raf probe and analyzed as in Figure 15 (Lanes 1-12 are pictured). The arrow points to the 2.6 kb band in the HpaII digests. Lanes 1 and 2, 5 and 6, 9 and 10: DNA from 3 mice at the time of PH; lanes 3 and 4, 7 and 8, and 11 and 12: DNA from the same 3 mice 7 days after PH.

Figure 24. The methylation status of Ha-ras: C57BL/6♀ 7 days after PH; MspI and HpaII. The blot pictured in Figure 20 was stripped of the raf probe and analyzed as in Figure 15. The arrow points to the 2.6 kb band in the HpaII digests. Lanes 1 and 2, 5 and 6, 9 and 10, and 13 and 14: DNA from 4 mice at the time of PH; lanes 3 and 4, 7 and 8, 11 and 12, and 15 and 16: DNA from the same 4 mice 7 days after PH.

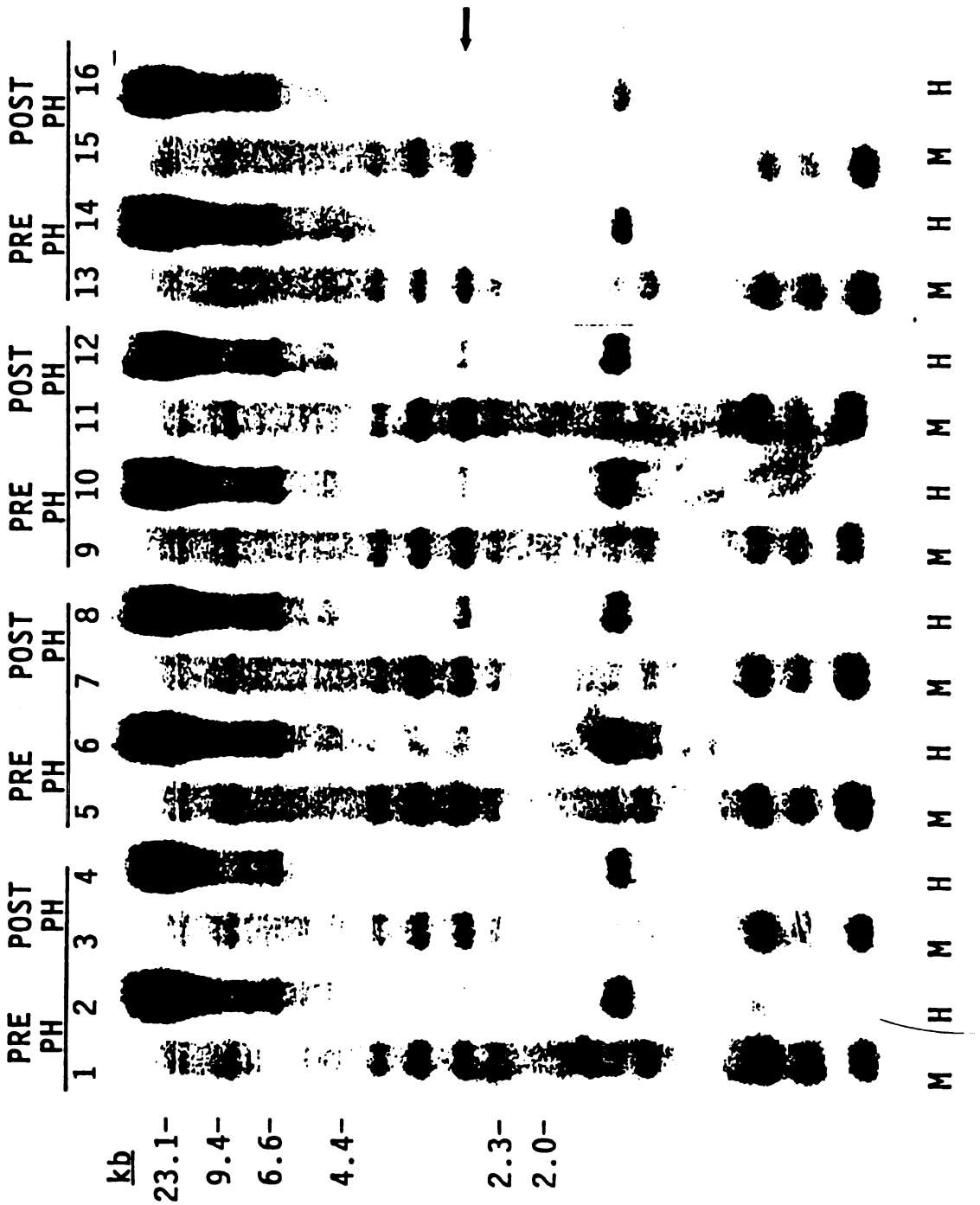
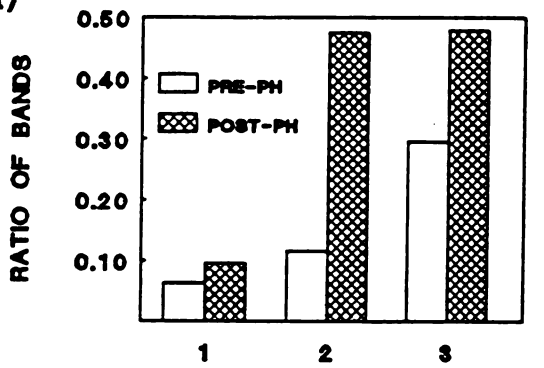
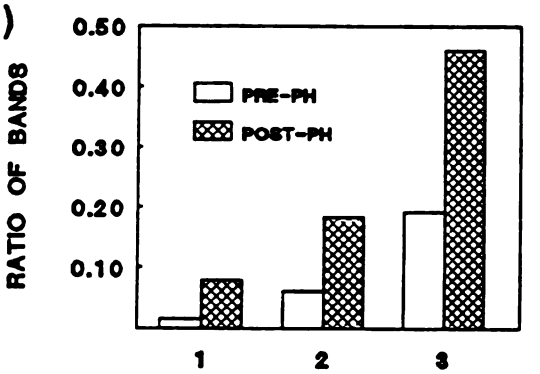

kb
23.1
9.4
6.6
4.4
2.3
2.0
PRE PH
POST PH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
M H M H M H M H M H M H M H M H

Figure 25. Relative changes in Ha-ras band intensities following PH. The ratio of the intensity of the 2.6 kb band to the intensity of the smaller major band in the HpaII digests on the autoradiographs pictured in Figures 22, 23, and 24 was determined as in the Methods section. The numbers along the x-axis refer to the individual mice as shown in the figures. The actual ratios determined by densitometric analysis are given in parentheses. A) 1 corresponds to Figure 22, lanes 1-4 (pre-PH: 0.063, post-PH: 0.096); 2 corresponds to lanes 5-8 (pre-PH: 0.115, post-PH: 0.495); 3 corresponds to lanes 9-12 (pre-PH: 0.295, post-PH: 0.479). B) C3H/He♂. 1 corresponds to Figure 23, lanes 1-4 (pre-PH: 0.016, post-PH: 0.079); 2 corresponds to lanes 5-8 (pre-PH: 0.061, post-PH: 0.183); and 3 corresponds to lanes 9-12 (pre-PH: 0.191, post-PH: 0.460). C) C57BL/6♀. 1 corresponds to Figure 24, lanes 1-4 (pre-PH: 0.040, post-PH: 0.042); 2 corresponds to lanes 5-8 (pre-PH: 0.058, post-PH: 0.295); 3 corresponds to lanes 9-12 (pre-PH: 0.046, post-PH: 0.087); and 4 corresponds to lanes 13-16 (pre-PH: 0.085, post-PH: 0.077).

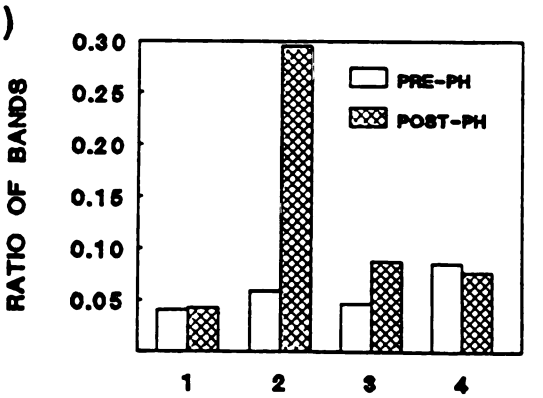
a)


RATIO OF BANDS
0.50
0.40
0.30
0.20
0.10
PRE-PH
POST-PH
1
2
3

b)


RATIO OF BANDS
0.50
0.40
0.30
0.20
0.10
PRE-PH
POST-PH
1
2
3

c)


RATIO OF BANDS
0.30
0.25
0.20
0.15
0.10
0.05
PRE-PH
POST-PH
1
2
3
4

cells in the nascent liver, since the 2.6 kb band is faintly visible in the control HpaII digests. However, some of the mice in each of the 3 strains had difficulty maintaining methylation at this particular site in the Ha-ras gene during cell proliferation stimulated by PH. These data are summarized in Table 1.

C. Methylation status of raf following 14 day phenobarbital treatment

To examine the capability of B6C3F1♂, C3H/He♂, and C57BL/6♀ mice to maintain the methylation status of the raf gene during the initial stimulation of DNA synthesis by 500 ppm PB in the drinking water, mice from each of the 3 strains were treated with 20 or 500 ppm PB in the drinking water for 14 days (results are summarized in Table 1). Figure 26A shows the results of MspI and HpaII digestion of DNA from B6C3F1♂ mice treated with 500 ppm PB for 14 days hybridized with the raf probe. Figure 26B shows results of MspI and HpaII digestion of DNA from B6C3F1♂ mice treated with 500 ppm PB for 14 days, 7 days prior to sacrifice. The control DNA shown in Figure 26B (lanes 1-4) is from the same animals as the DNA in lanes 11-14 of Figure 26A. The 6.7 kb band is decreased in intensity in 4/5 of the mice which were treated with 500 ppm PB for 14 days relative to the control mean (Figure 27A). Compared to the mean of the 2 controls shown in Figure 26B, the 6.7 kb band is decreased in intensity in MspI digests of DNA from 3/4 of the PB-treated B6C3F1♂ mice which were maintained 7 days following PB treatment (Figure 27B). These

Figure 26. The methylation status of raf: B6C3F1♂ following 500 ppm PB for 14 days; MspI and HpaII. A) Following 14 day PB treatment. DNA was analyzed as in Figure 11. Arrows point to the 6.7, 5.1, and 3.0 kb bands in the MspI digests. Lanes 1 and 2, 3 and 4, 11 and 12, and 13 and 14: DNA from control mice. Lanes 5 and 6, 7 and 8, 9 and 10, 15 and 16, and 17 and 18: DNA from 5 mice treated with 500 ppm PB for 14 days. B) Following administration of 500 ppm PB for 14 days ending 7 days prior to sacrifice. Lanes 1 and 2, and 3 and 4: DNA from 2 control mice; these are the same control samples shown in Figure 26A. Lanes 5 and 6, 7 and 8, 9 and 10, and 11 and 12: DNA from mice treated with 500 ppm PB in the drinking water for 14 days ending 7 days prior to sacrifice.

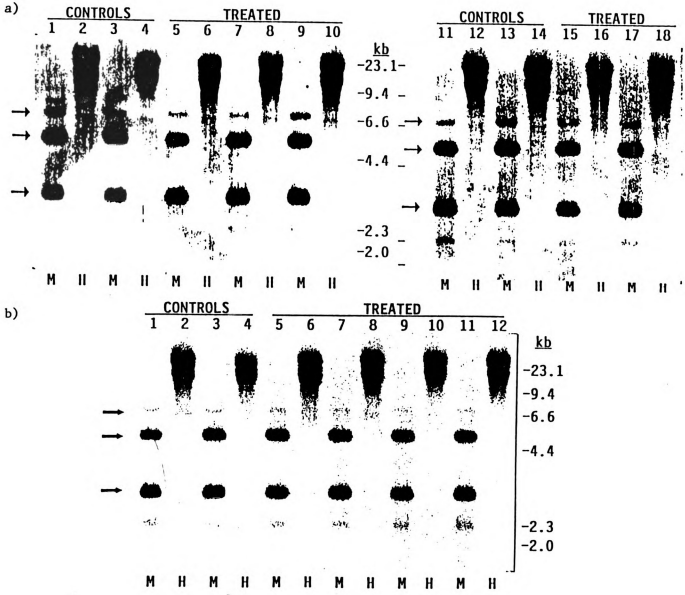
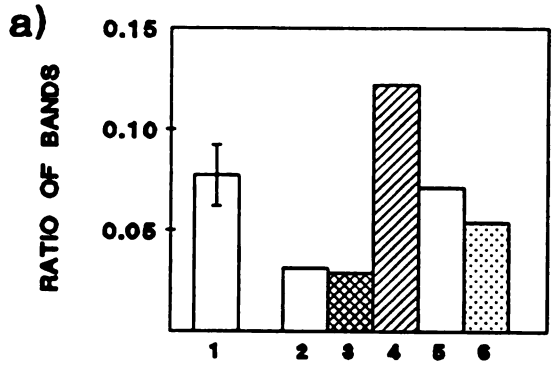
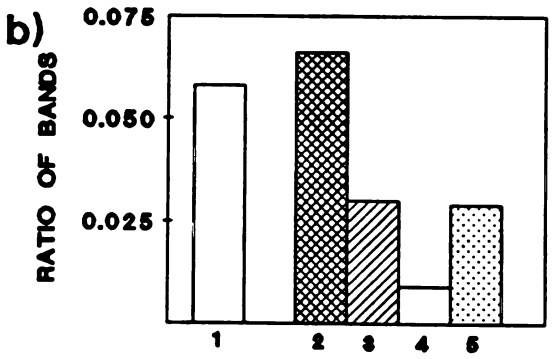

a)
CONTROLS
TREATED
1 2 3 4 5 6 7 8 9 10
kb
-23.1
-9.4
-6.6
-4.4
-2.3
-2.0
M H M H M H M H M H
CONTROLS
TREATED
11 12 13 14 15 16 17 18
M H M H M H M H
b)
CONTROLS
TREATED
1 2 3 4 5 6 7 8 9 10 11 12
kb
-23.1
-9.4
-6.6
-4.4
-2.3
-2.0
M H M H M H M H M H M H

Figure 27. Relative changes in raf band intensities following 500 ppm PB: B6C3F1♂. A) Following administration of 500 ppm PB in the drinking water for 14 days. The ratio of the intensity of the 6.7 kb band to the sum of the intensities of the 5.1 and 3.0 kb bands in the MspI digests on the autoradiograph pictured in Figure 26A was determined as in the Methods section. The actual ratios as determined by densitometric analysis are given in parentheses. 1 corresponds to the mean value of the control samples (with standard error), i.e. lanes 1,3,11,13 (0.077 ± 0.015). 2 corresponds to lane 5 (0.031), 3 to lane 7 (0.029), 4 to lane 9 (0.122), 5 to lane 15 (0.071), 6 to lane 17 (0.054). B) Following administration of 500 ppm PB in the drinking water for 14 days, ending 7 days prior to sacrifice. The ratio of the intensity of the 6.7 kb band to the sum of the intensities of the 5.1 and 3.0 kb bands in the MspI digests on the autoradiograph pictured in Figure 26B was determined. 1 corresponds to the control mean value, i.e. lanes 1 and 3 (0.058); 2 corresponds to lane 5 (0.066), 3 to lane 7 (0.030), 4 to lane 9 (0.009), and 5 to lane 11 (0.029).


a)
0.15
0.10
0.05
RATIO OF BANDS
1
2
3
4
5
6


b)
0.075
0.050
0.025
RATIO OF BANDS
1
2
3
4
5

data indicate that approximately 75% of these B6C3F1♂ mice were unable to maintain methylation at the site responsible for the detection of the 6.7 kb band and that this site does not become methylated again following the cessation of PB administration.

The effect of 500 ppm PB treatment on the methylation status of raf in C3H/He♂ and C57BL/6♀ mice is shown in Figures 28 and 29, respectively. There were 6 animals in each group (control, 20 ppm PB and 500 ppm PB) and the results shown on the pictured Southern blots are representative. No change in the ratio of the intensity of the 5.1 to 3.0 kb band was seen in the C3H/He♂ and no change in the intensity of the 6.7 kb band relative to the sum of the intensities of the 5.1 and 3.0 kb bands was detected in the C57BL/6♀ at either dose. The C3H/He♂ lacks the 6.7 kb band so it can not be directly compared to the B6C3F1♂. The C57BL/6♀ is apparently better able to maintain methylation at this site during PB treatment than the B6C3F1♂.

D. Methylation status of Ha-ras following phenobarbital administration.

To assess the ability of the B6C3F1♂, C3H/He♂, and C57BL/6♀ mice to maintain methylation of 5'-CCGG-3' sites in the Ha-ras gene, groups of 6 mice each were given 20 or 500 ppm PB in the drinking water. These were the same groups used for analysis of the methylation status of raf in C3H/He♂ and C57BL/6♀; an identical experiment was carried out with B6C3F1♂ mice. The results of MspI and HpaII digestion of liver DNA

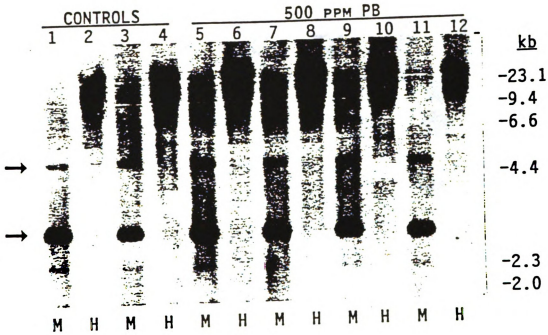


Figure 28. The methylation status of raf: C3H/Heơ after 14 days of PB treatment; MspI and HpaII. Mice were treated with 500 ppm PB in the drinking water for 14 days. DNA was analyzed as in Figure 11. The arrows point to the 5.1 and 3.0 kb bands seen in the MspI digests. Lanes 1-4: DNA from 2 control mice; lanes 5-12: DNA from 4 PB-treated mice.

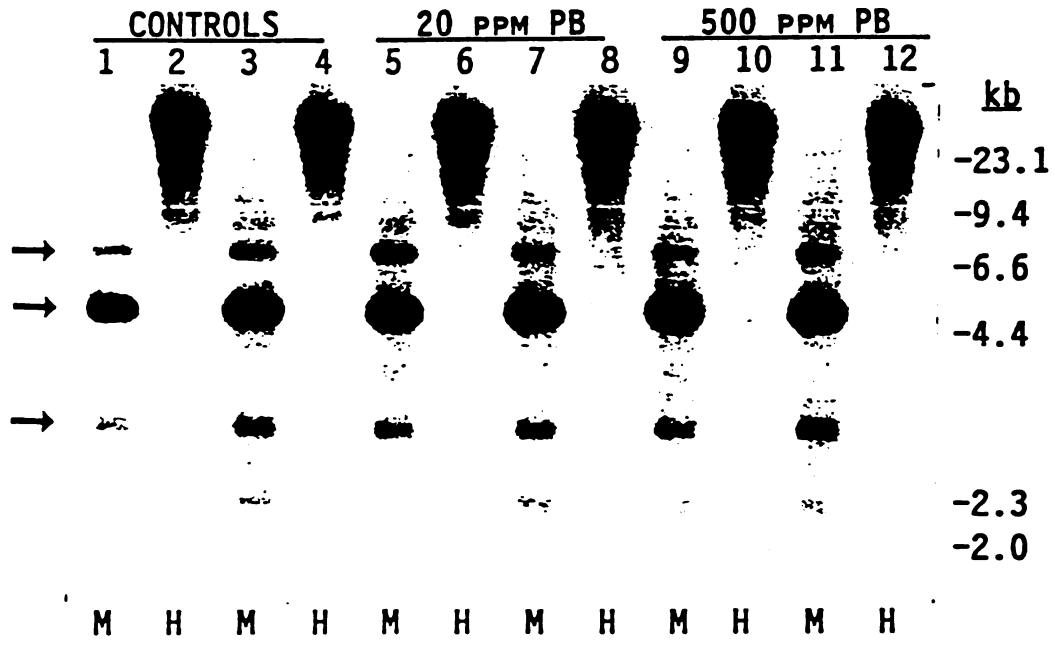


Figure 29. The methylation status of raf: C57BL/6♀ following 14 days of PB treatment; MspI and HpaII. Mice were treated with 20 or 500 ppm PB in the drinking water for 14 days. DNA was analyzed as in Figure 11. The arrows point to the 6.7, 5.1, and 3.0 kb bands in MspI digests. Lanes 1-4: DNA from 2 control mice; lanes 5-8: DNA from 2 mice treated with 20 ppm PB; lanes 9-12: DNA from 2 mice treated with 500 ppm PB.

from B6C3F1♂, C3H/He♂, and C57BL/6♀ mice hybridized with the Ha-ras probe are shown in Figures 30, 31, and 32, respectively. No change in the methylation status of Ha-ras was detected in any of the mice (Table 1).

E. Assessment of raf and Ha-ras mRNA levels

Northern analysis of RNA from each of the 3 strains was performed to quantitate raf and Ha-ras mRNA levels following treatment or between strains. No change in the levels of raf or Ha-ras mRNA was detected in any of the 3 strains following PB treatment or PH (data not shown; Table 1). Thus, the relative hypomethylation of raf and Ha-ras seen after PH in all 3 strains and of raf in B6C3F1♂ does not result in increased expression of the genes.

4. Ha-ras and raf in Phenobarbital-induced and Spontaneous B6C3F1♂ Liver Tumors

A. Methylation status of raf

B6C3F1♂ mice were fed phenobarbital at 1000 ppm in their diet for 24 months to induce liver tumor formation. Bands of 8.0, 5.1, 3.0, and 2.3 kb were detected in DNA from age-matched control liver tissue digested with MspI and probed with the raf probe (Figure 33). There were age-matched control livers from 10 mice and tumor tissue from 10 mice examined in this study. The results shown in Figure 33 are representative. Because these tissues were supplied by Hoffmann LaRoche Laboratories, the B6C3F1♂ mice were obtained from a different source than the young adult mice; this may

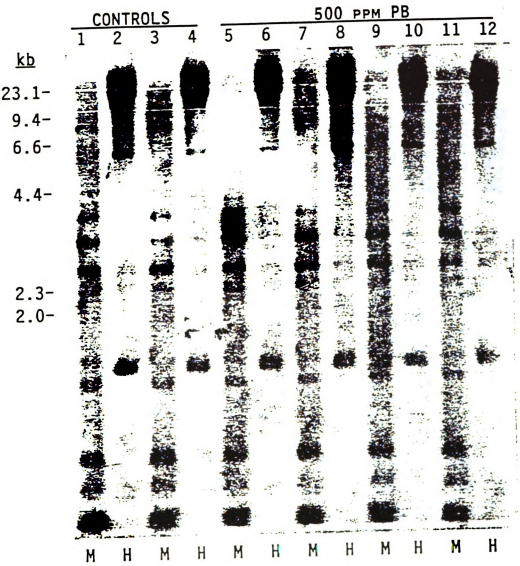


Figure 30. The methylation status of Ha-ras: B6C3F1♂ after 14 days of PB treatment; MspI and HpaII. 500 ppm PB was administered in the drinking water for 14 days. DNA was electrophoresed through a 0.9% agarose gel, then analyzed as in Figure 6. Lanes 1-4: DNA from 2 control mice; lanes 5-12: DNA from 4 PB-treated mice.

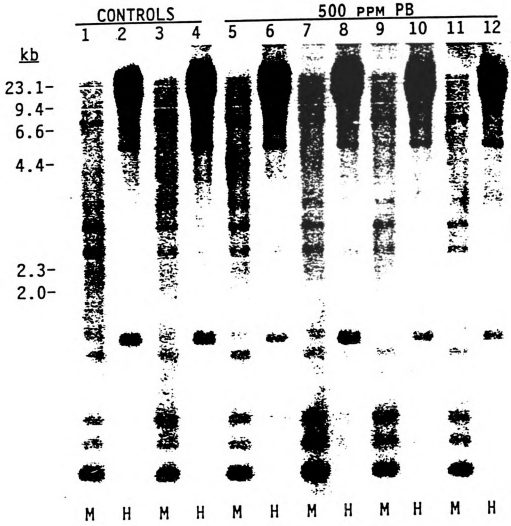


Figure 31. The methylation status of Ha-ras: C3H/Heơ following 14 days of PB treatment; MspI and HpaII. 500 ppm PB was administered in the drinking water for 14 days. DNA was analyzed as in Figure 30. Lanes 1-4: DNA from 2 control mice; lanes 5-12: DNA from 4 PB-treated mice.

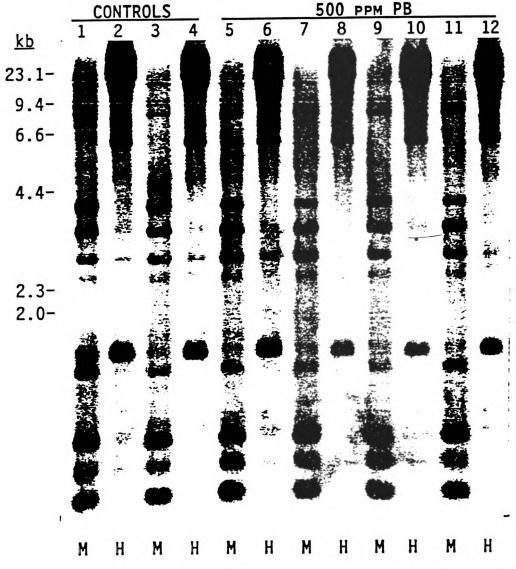
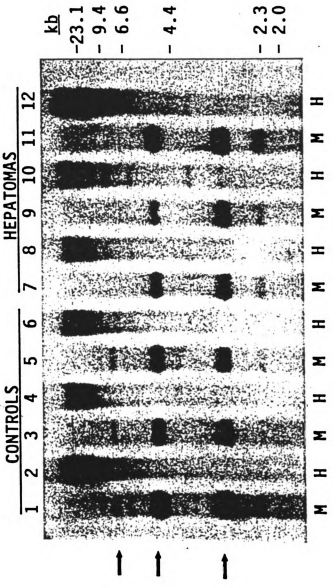


Figure 32. The methylation status of Ha-ras: C57BL/6♀ following 14 days of PB treatment; MspI and HpaII. 500 ppm PB was administered in the drinking water for 14 days. DNA was analyzed as in Figure 30. Lanes 1-4: DNA from 2 control mice; lanes 5-12: DNA from 4 PB-treated mice.

Figure 33. The methylation status of raf: B6C3F1♂ PB-induced tumors and age-matched controls; MspI and HpaII. DNA was analyzed as in Figure 11. The arrows point to the 8.0, 5.1, and 3.0 kb bands. Lanes 1-6: DNA from 3 age-matched controls; lanes 7-12: DNA from PB-induced tumors from 3 mice.



induced
DNA was
0, 5.1,
ntrols;

account for the difference in size of the largest MspI band. The largest band (8.0 kb) is undetectable in phenobarbital-induced liver tumors (Figure 33). The 8.0 kb band is absent or diminished in 4/5 spontaneous tumors (Figure 34). Thus, hypomethylation of raf occurs in both PB-induced and spontaneous tumors. These data are summarized in Table 2.

Digestion of DNA from age-matched controls (Figure 35, lanes 1-3), with StuI results in a 3.2 kb band and StuI/MspI double digestion results in the major digestion products of 2.4 and 0.8 kb as seen in the young B6C3F1♂ mice (Figure 14) when probed with the raf probe. The same pattern is seen in StuI digested DNA from PB-induced adenomas (Figure 35, lanes 4-6) or carcinomas (Figure 35, lanes 7-9), spontaneous adenomas (Figure 35, lanes 10-12) or spontaneous carcinomas (Figure 35, lanes 13-15). Therefore, the decrease in methylation detected by MspI digestion affects specific sites and is neither generalized nor random since this 5'-CCGG-3' site remains methylated in tumors.

B. Methylation status of Ha-ras

A decrease in the methylation status of the Ha-ras gene, detected as increased digestion by HpaII, was seen in 7/8 phenobarbital-induced tumors (Figure 36, Table 2) and 5/5 spontaneous tumors (Figure 37, Table 2), confirming earlier findings from our laboratory (Vorce and Goodman, 1989b). The same Southern blots used to analyze the methylation status of raf were used to analyze the methylation status of Ha-ras,

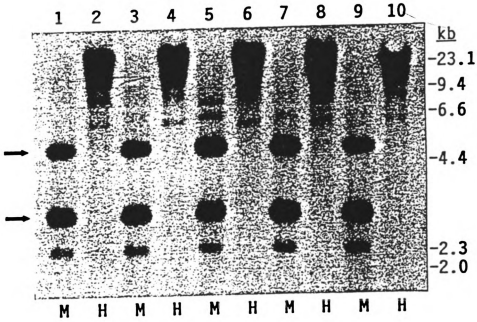


Figure 34. The methylation status of raf: B6C3F1♂ spontaneous tumors; MspI and HpaII. DNA was analyzed as in Figure 11. The arrows point to the 5.1, and 3.0 kb bands. Lanes 1 and 2, 3 and 4, 5 and 6, 7 and 8, and 9 and 10: DNA from spontaneous tumors from 5 mice.

Table 2. Methylation Status and mRNA Levels of raf and Ha-ras in B6C3F1♂ and CD-1♂ Liver Tumors.

STRAIN	TREATMENT[1]	HYPOMETHYLATION OF raf[2]	HYPOMETHYLATION OF Ha-ras	INCREASE IN raf mRNA LEVELS[3]	INCREASE IN Ha-ras mRNA LEVELS
B6C3F1♂	PB	10/10	7/8[4]	7/10	8/10
B6C3F1♂	NONE	4/5	5/5	1/5	4/5
CD-1♂	PB	4/5	5/5	4/5	5/5

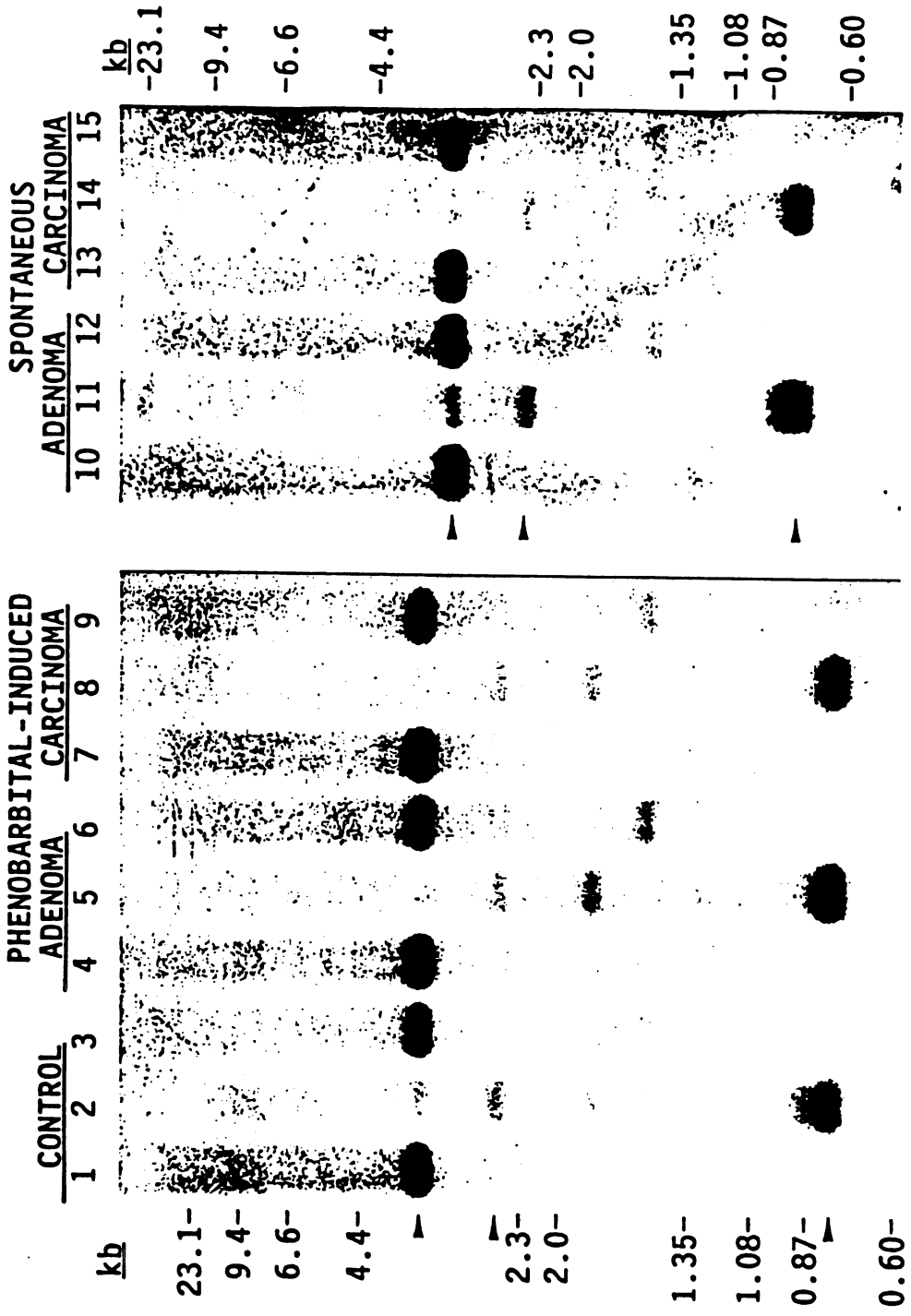
[1] Tumors were induced by the administration of 150 mg PB/kg/day in the diet for 26 months.

[2] Relative hypomethylation was determined by densitometric analysis as described in the methods section.

[3] Levels of raf or Ha-ras mRNA were determined relative to the level of 28S rRNA in the same sample.

[4] Southern blot analysis of Ha-ras for 2 samples was uninterpretable due to technical difficulties.

Figure 35. The methylation status of CCGG site in exon 12 of the raf gene in PB-induced and spontaneous B6C3F1♂ tumors. DNA from age-matched control liver (lanes 1-3), PB-induced adenoma (lanes 4-6), PB-induced carcinoma (lanes 7-9), spontaneous adenoma (lanes 10-12), or spontaneous carcinoma (lanes 13-15) was digested with StuI (lanes 1,4,7,10,13), MspI then StuI (lanes 2,5,8,11,14), or HpaII then StuI (lanes 3,6,9,12,15). Double digests were performed as in Figure 14. DNA was analyzed as in Figure 11. The arrows point to the 3.2 kb bands in StuI and StuI/HpaII double digests and to 2.4 and 0.8 kb bands in StuI/MspI double digests.


SPONTANEOUS
ADENOMA
CARCINOMA
10 11 12 13 14 15
kb
23.1
9.4
6.6
4.4
2.3
2.0
1.35
1.08
0.87
0.60
PHENOBARBITAL-INDUCED
CONTROL
ADENOMA
CARCINOMA
1 2 3 4 5 6 7 8 9
kb
23.1
9.4
6.6
4.4
2.3
2.0
1.35
1.08
0.87
0.60

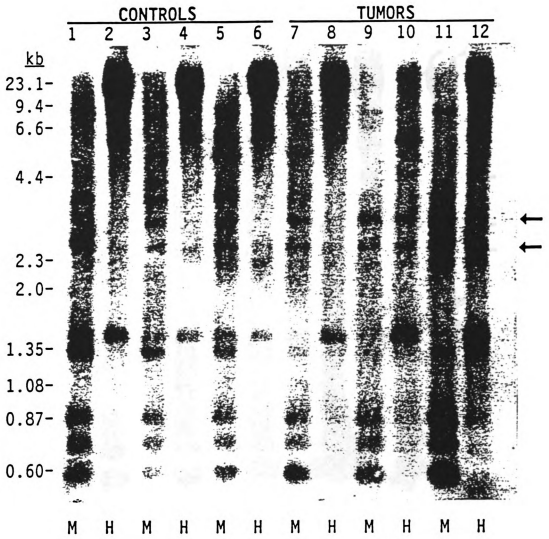


Figure 36. The methylation status of Ha-ras: B6C3F1♂ PB-induced tumors and age-matched controls; MspI and HpaII. The southern blot pictured in Figure 33 was stripped of the raf probe and analyzed as in Figure 15. The arrows point to the additional bands seen in the HpaII digests. Lanes 1-6: DNA from 3 age-matched controls; lanes 7-12: DNA from PB-induced tumors from 3 mice.

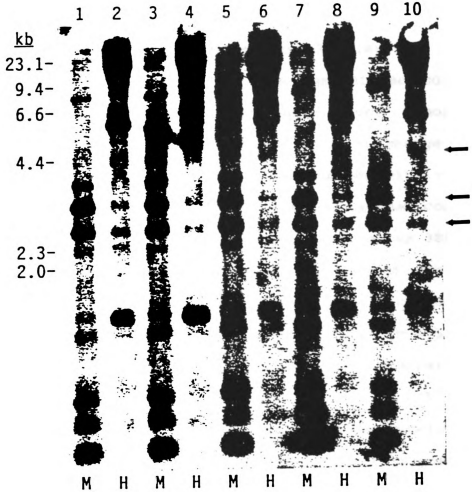


Figure 37. The methylation status of Ha-ras: B6C3F1♂ spontaneous tumors; MspI and HpaII. The southern blot pictured in Figure 33 was stripped of the raf probe and analyzed as in Figure 15. The arrows point to the additional bands seen in the HpaII digests. Lanes 1 and 2, 3 and 4, 5 and 6, 7 and 8, and 9 and 10: DNA from spontaneous tumors from 5 mice.

however, technical problems prevented the analysis of two samples. Thus, a decrease in methylation of both the raf and Ha-ras genes is a common feature of liver tumors in B6C3F1♂ mice.

C. Assessment of raf and Ha-ras mRNA levels

RNA was isolated from the age-matched controls, phenobarbital-induced, and spontaneous liver tumors and assessed by Northern analysis (Figure 38). No change in the size of the raf mRNA was detected in the phenobarbital-induced or spontaneous tumors. Relative to the age-matched controls, 3/5 of the phenobarbital-induced adenomas (Figures 38A, 39A, Table 2) and 4/5 of the phenobarbital-induced carcinomas (Figures 38B, 39B, Table 2) had increased levels of raf mRNA. In the same samples, Ha-ras mRNA was increased in 4/5 of the adenomas (Figures 38A, 39A, Table 2) and 4/5 of the carcinomas (Figures 38B, 39B, Table 2). However, only 1/5 of the spontaneous tumors (a carcinoma) had increased levels of raf mRNA, while 4/5 had elevated Ha-ras mRNA levels (Figures 38C, 39C, Table 2). This suggests that elevated expression of raf and Ha-ras genes may be involved in PB-induced mouse liver tumors. PB treatment, however appears to result in the selective expansion of a different population of cells than those involved in spontaneous tumors.

Figure 38. Ha-ras and raf mRNA: B6C3F1♂ PB-induced and spontaneous tumors and age-matched controls. Levels of mRNA were analyzed by Northern blotting, hybridization with the ^{32}P-labeled v-raf, v-Ha-ras or 28S rRNA probes, and autoradiography. Membranes were stripped between sequential hybridizations. Northern blots of RNA from age-matched control liver tissue (lanes 1-5), and phenobarbital-induced adenomas (lanes 6-10) hybridized with the raf, Ha-ras, and 28S probes are shown in panel A. In panel B is pictured the Northern blot of age-matched control liver tissue (lanes 1-5) and phenobarbital-induced carcinomas (lanes 6-10) hybridized with raf, Ha-ras, and 28S probes. The same control tissue RNA is on the Northern blot in panel C (lanes 1-10); values are comparable only with other lanes on the same blot, not between blots. RNA from spontaneous liver tumors (lanes 11-15) is pictured in panel C.

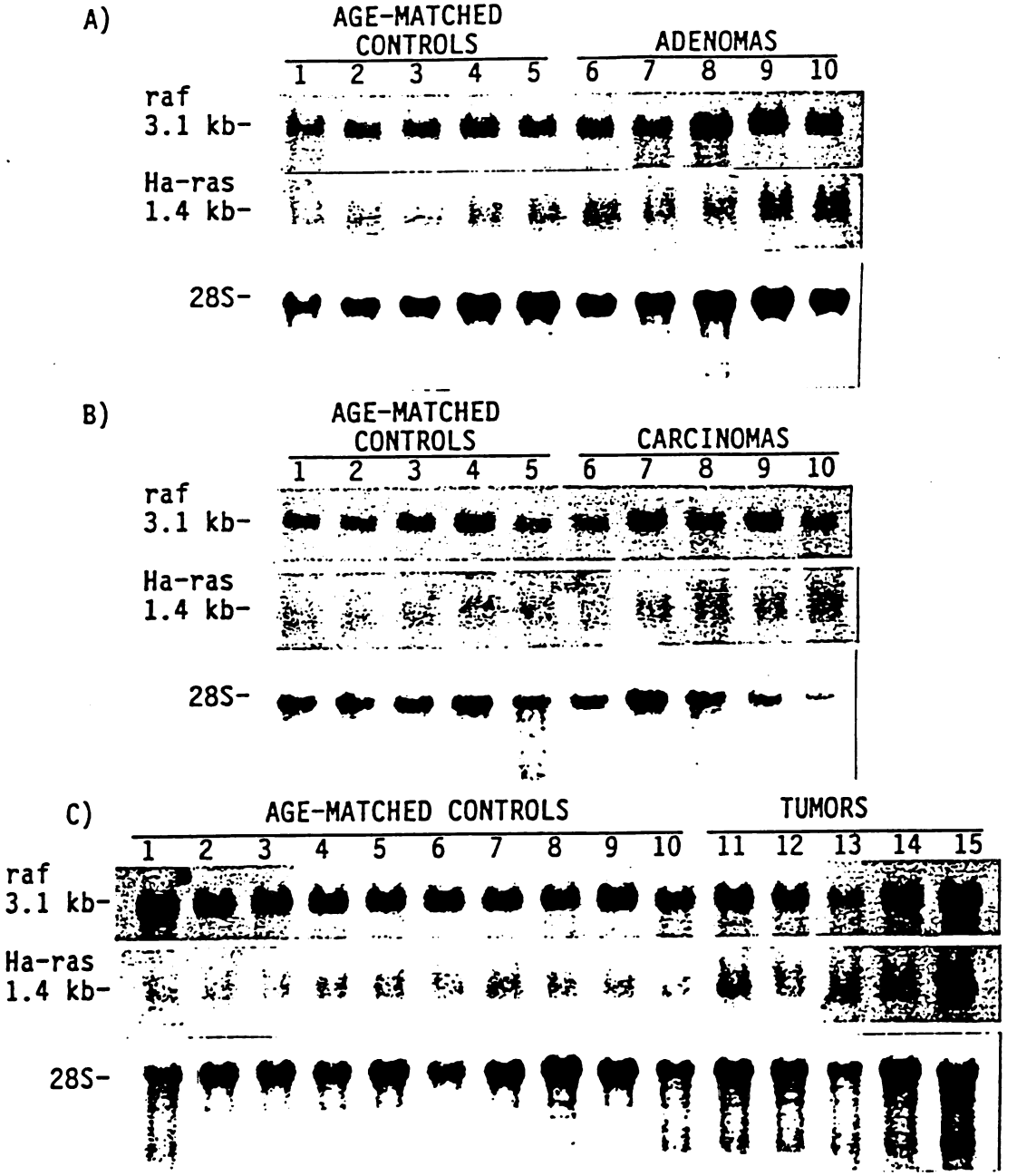
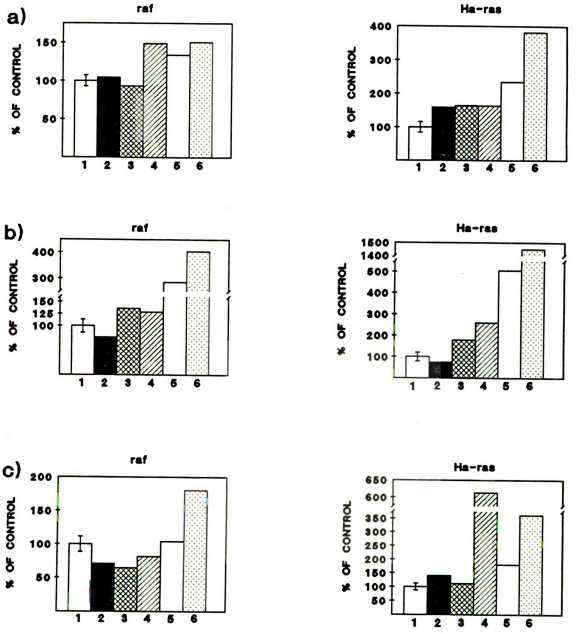

A)
AGE-MATCHED CONTROLS
ADENOMAS
1 2 3 4 5 6 7 8 9 10
raf 3.1 kb-
Ha-ras 1.4 kb-
28S-
B)
AGE-MATCHED CONTROLS
CARCINOMAS
1 2 3 4 5 6 7 8 9 10
raf 3.1 kb-
Ha-ras 1.4 kb-
28S-
C)
AGE-MATCHED CONTROLS
TUMORS
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
raf 3.1 kb-
Ha-ras 1.4 kb-
28S-

Figure 39. Relative amounts of raf and Ha-ras mRNA in B6C3F1♂ PB-induced and spontaneous tumors. Panels correspond to those in Figure 38. Control mean values are set at 100%. The actual ratios determined by densitometric analysis are given in parentheses. A) PB-induced adenomas. raf: 1 corresponds to controls pictured in Figure 38A, lanes 1-5 (0.561 ± 0.041); 2 to lane 6 (0.587); 3 to lane 7 (0.524); 4 to lane 8 (0.835); 5 to lane 9 (0.751); and 6 to lane 10 (0.844). Ha-ras: 1 corresponds to controls pictured in Figure 38A, lanes 1-5 (0.269 ± 0.043); 2 to lane 6 (0.695); 3 to lane 7 (0.441); 4 to lane 8 (0.438); 5 to lane 9 (0.631); and 6 to lane 10 (1.029). B) PB-induced carcinomas. raf: 1 corresponds to controls pictured in Figure 38B, lanes 1-5 (0.708 ± 0.098); 2 to lane 6 (0.548); 3 to lane 7 (0.965); 4 to lane 8 (0.913); 5 to lane 9 (2.019); and 6 to lane 10 (2.860). Ha-ras: 1 corresponds to controls pictured in Figure 38B, lanes 1-5 (0.206 ± 0.042); 2 to lane 6 (0.153); 3 to lane 7 (0.368); 4 to lane 8 (0..532); 5 to lane 9 (1.040); and 6 to lane 10 (2.986). C) Spontaneous tumors. raf: 1 corresponds to lanes 1-10 (0.928 ± 0.106); 2 to lane 11 (0.660); 3 to lane 12 (0.598); 4 to lane 13 (0.757); 5 to lane 14 (0.962); 6 to lane 15 (1.671). Ha-ras: 1 corresponds to lanes 1-10 (0.457 ± 0.058); 2 to lane 11 (0.645); 3 to lane 12 (0.507); 4 to lane 13 (2.803); 5 to lane 14 (0.825); 6 to lane 15 (1.651).


a)
raf
% OF CONTROL
150
100
50
1 2 3 4 5 6
Ha-ras
% OF CONTROL
400
300
200
100
1 2 3 4 5 6
b)
raf
% OF CONTROL
400
300
150
125
100
1 2 3 4 5 6
Ha-ras
% OF CONTROL
1500
1400
500
400
300
200
100
1 2 3 4 5 6
c)
raf
% OF CONTROL
200
150
100
50
1 2 3 4 5 6
Ha-ras
% OF CONTROL
650
600
350
300
250
200
150
100
50
1 2 3 4 5 6

5. Ha-ras and raf in the Nascent Liver of the CD-1♂ Mouse

A. Methylation status of raf

Bands of 6.7, 5.1, 3.0, and 2.3 kb are detected in raf-probed MspI digested DNA from 8 week old CD-1♂ mice (Figure 40), as in C57BL/6♀ and B6C3F1♂ mice (Figure 11). This indicates that the CD-1♂ also possesses the 5'-^{Me}CCGG-3' site which is present in the C57BL/6♀ and the B6C3F1♂, but not the C3H/He♂. There is some variation in the intensity of the 6.7 kb band between individuals which may be because the CD-1 is a non-inbred mouse stock. As determined by image analysis, the ratio of the intensity of the 6.7 kb band to the sum of the intensities of the other 2 bands (5.1 and 3.0 kb) for the CD-1♂ is most similar to that of the B6C3F1♂ (Figure 41). This suggests that the CD-1♂ may have differential methylation of raf alleles similar to the B6C3F1♂, mosaicism of methylation status of the raf gene between hepatocytes or the fragment responsible for the 6.7 kb band may bind the probe less avidly due to a lower sequence homology.

B. Methylation status of Ha-ras

The 5'-^{Me}CCGG-3' site in Ha-ras, responsible for the approximately 15 kb band in MspI digests probed with Ha-ras in C57BL/6♀ but not C3H/He♂ (Vorce and Goodman, 1989a), is detectible in 4/5 of the young adult CD-1♂ (Figure 42). Otherwise, the MspI and HpaII digests of CD-1♂ DNA, probed for Ha-ras, appear identical to the B6C3F1♂, C3H/He♂, and C57BL/6♀.

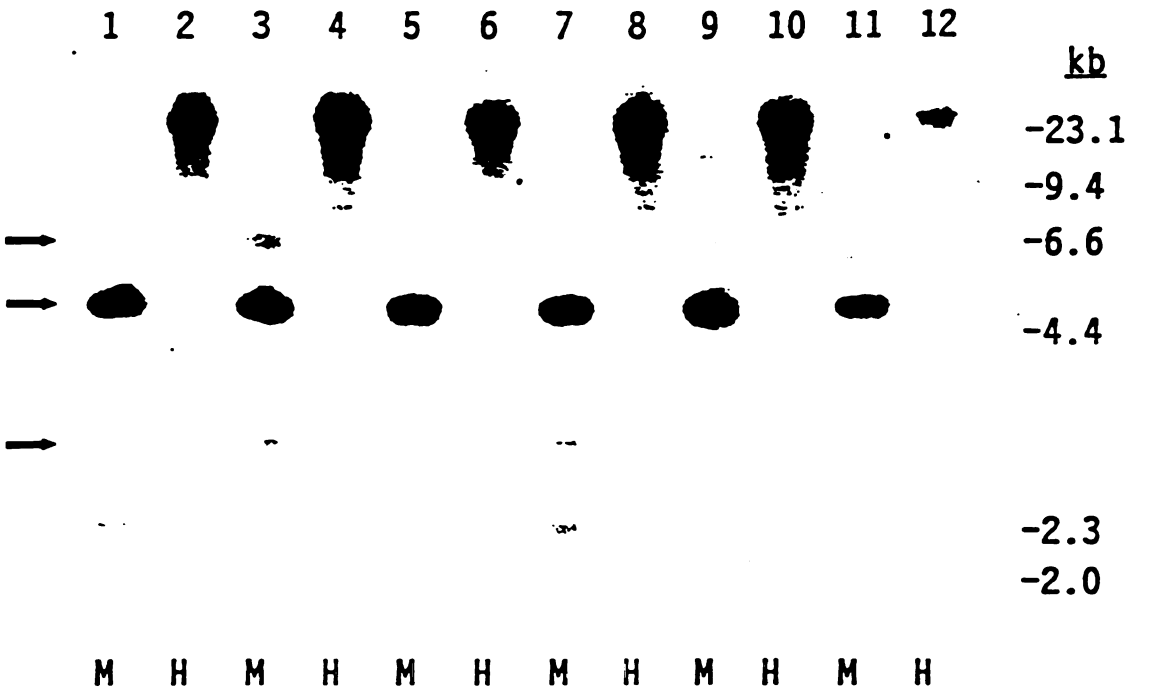


Figure 40. The methylation status of raf: CD-1σ; MspI and HpaII. DNA was analyzed as in Figure 11. Arrows point to the 6.7, 5.1, and 3.0 kb bands seen in the MspI digests. Lanes 1 and 2, 3 and 4, 5 and 6, 7 and 8, and 9 and 10: DNA from 5 CD-1σ mice.

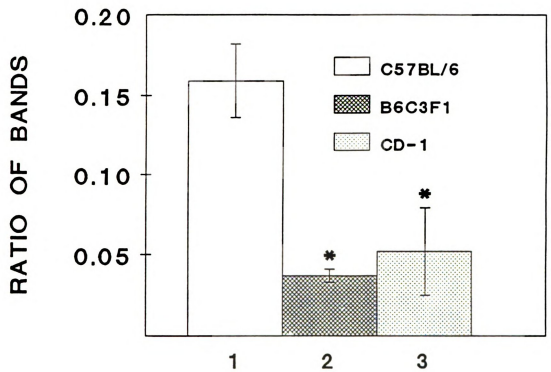


Figure 41. Relative intensity of the 6.7 kb band in the C57BL/6♀, B6C3F1♂, and CD-1♂ raf gene. The raf gene from 6 C57BL/6♀ (not shown), 6 B6C3F1♂ (not shown), and 5 CD-1♂ (Figure 40, lanes 1,3,5,7,9) was analyzed. The ratio of the intensity of the 6.7 kb band to the sum of the intensities of the 5.1 and 3.0 kb bands in the MspI digests was determined as described in the methods section. * indicates a significant difference in the ratios of the B6C3F1♂ and CD-1♂ bands relative to that of the C57BL/6♀. There is no difference between the ratios of the B6C3F1♂ and CD-1♂ bands.

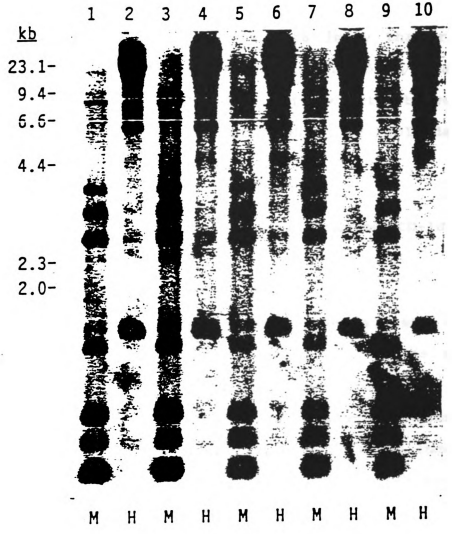


Figure 42. The methylation status of Ha-ras: CD-1♂; MspI and HpaII. DNA from the same mice as pictured in Figure 40, lanes 1-10 was analyzed as in Figure 30.

C. RFLP analysis of raf and Ha-ras in CD-1♂

CD-1♂ DNA contains the same TaqI restriction sites as C57BL/6♀ DNA in the region of the raf gene detected by the probe (Figure 43A). No RFLPs in the TaqI digested DNA were detected in Ha-ras in the CD-1♂ compared to B6C3F1♂, C3H/He♂, or C57BL/6♀ (Figure 43B). These data imply that raf and Ha-ras in the CD-1♂ are more similar to the C57BL/6♀ and the B6C3F1♂ than the C3H/He♂. No RFLPs were detected between the 4 strains with EcoRI, HindIII, StuI, HhaI, or XhoI (data not shown).

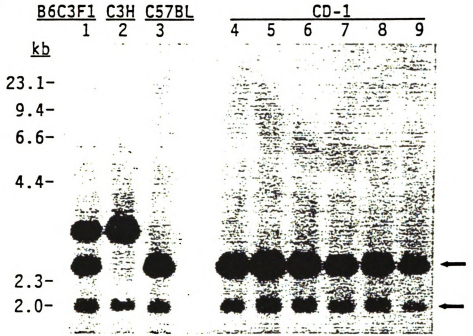
6. Ha-ras and raf in Phenobarbital-induced CD-1♂ Liver Tumors

A. Methylation status of raf

In 26 month old CD-1♂ mice, the raf gene is more methylated than it is in 2 month old mice; this is seen as additional bands in the MspI digests above the major 5.1 kb band in 5 out of 6 of the age-matched controls (Figure 44A, lanes 1,3,5; Figure 44B, lanes 3,5). To rule out incomplete digestion, this DNA was restricted again with 50% more MspI which was added in 3 aliquots with identical results (data not shown). MspI from the same lot was also shown to digest C57BL/6♀ liver DNA to completion under the same conditions (data not shown). The bands larger than 5.1 kb were either not detected or were greatly diminished in 4/5 PB-induced CD-1♂ liver tumors (Figure 44A, lanes 7,9,11; Figure 44B, lane 7, Table 2), indicating loss of 5'-^{Me}CCGG-3' sites in the tumor

Figure 43. Restriction fragment analysis of raf and Ha-ras: CD-1♂; TaqI. A) raf. DNA from B6C3F1♂ (lane 1), C3H/He♂ (lane 2), C57BL/6♀ (lane 3), or CD-1♂ (lanes 4-9) was digested with 5U/μg TaqI at 65°C and analyzed as in Figure 11. Arrows point to the major bands. B) Ha-ras. The same blot pictured in panel A was stripped of the raf probe and analyzed as in Figure 15. The arrows point to the major digestion products.

a)


B6C3F1
1
C3H
2
C57BL
3
CD-1
4 5 6 7 8 9
kb
23.1-
9.4-
6.6-
4.4-
2.3-
2.0-

b)

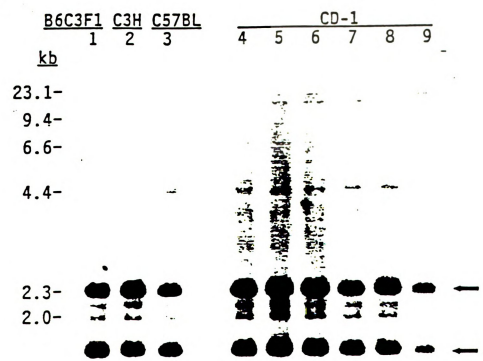
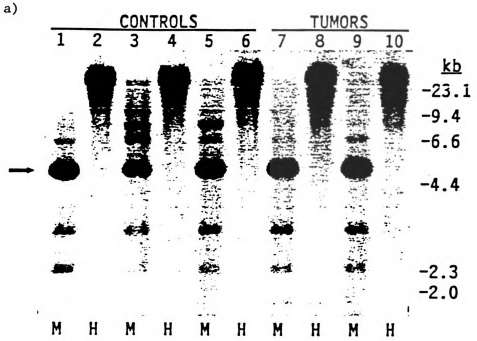

B6C3F1
1
C3H
2
C57BL
3
CD-1
4 5 6 7 8 9
kb
23.1-
9.4-
6.6-
4.4-
2.3-
2.0-

Figure 44. The methylation status of raf: CD-1♂ PB-induced tumors and age-matched controls; MspI and HpaII. DNA was analyzed as in Figure 11. The arrow points to the major 5.1 kb band in MspI-digested DNA. Note the multiple bands detectible above the 5.1 kb band in the age-matched control tissue. A) Lanes 1-6: DNA from 3 age-matched controls; lanes 7-10: DNA from PB-induced liver tumors from 2 mice. B) Lanes 1-6: DNA from 3 different age-matched controls; lanes 7-12: DNA from PB-induced liver tumors from 3 mice.


a)
CONTROLS
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

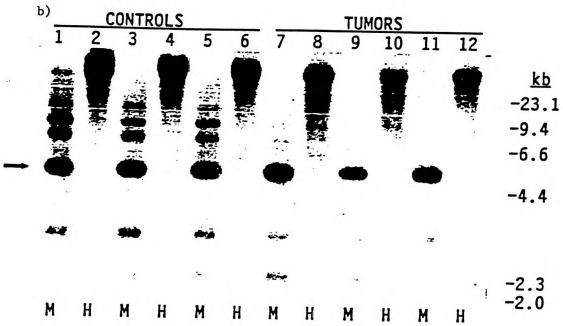
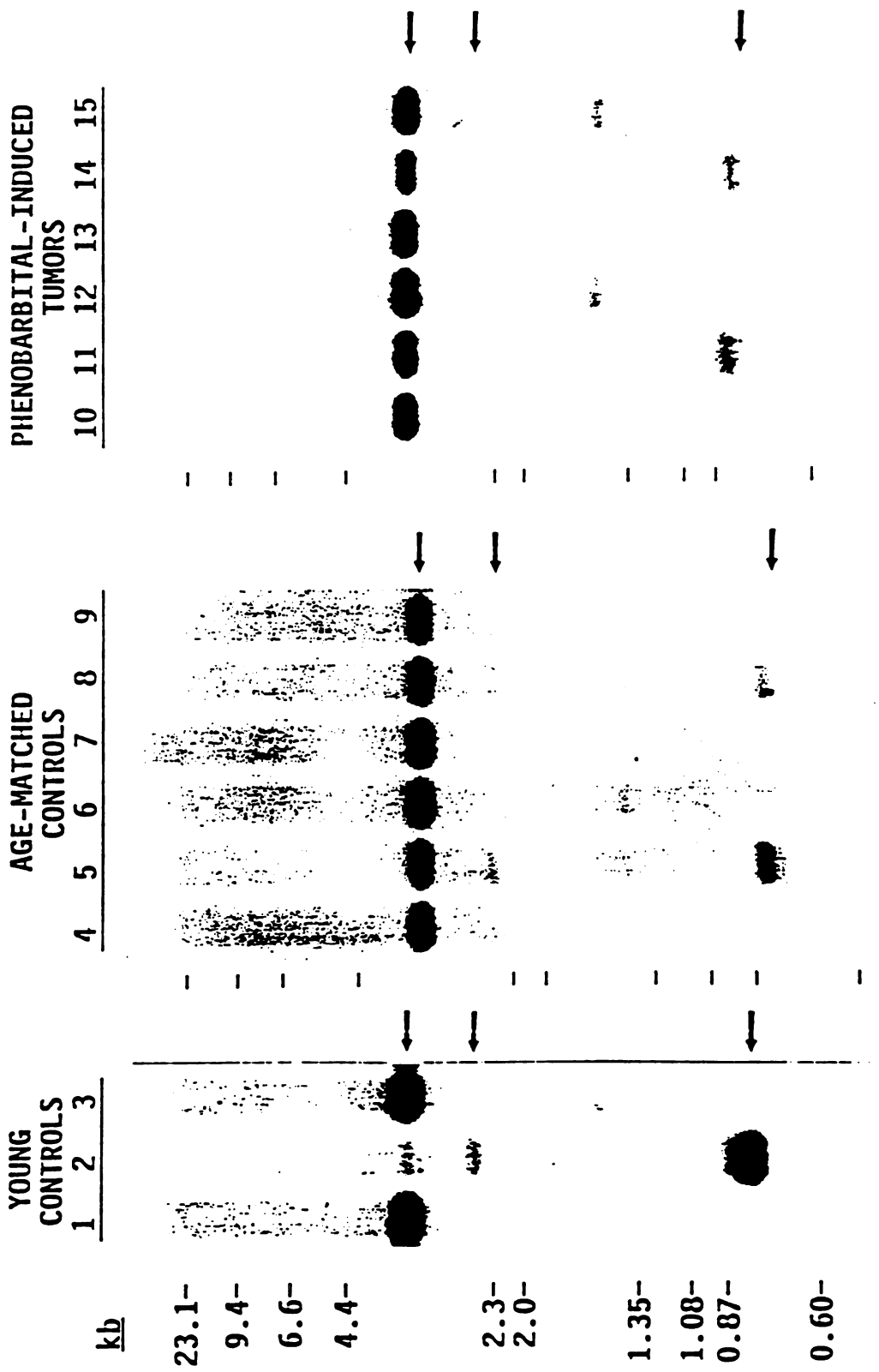

b)
CONTROLS
TUMORS
1 2 3 4 5 6 7 8 9 10 11 12
kb
-23.1
-9.4
-6.6
-4.4
-2.3
-2.0
M H M H M H M H M H M H

Figure 45. The methylation status of the CCGG site in exon 12 of the raf gene in CD-1♂ liver, PB-induced CD-1♂ tumors and age-matched controls. DNA from young adult CD-1♂ liver (lanes 1-3), age-matched control liver (lanes 4-9), and PB-induced liver tumors (lanes 10-15) was digested with StuI (lanes 1,4,7,10,13), MspI then StuI (lanes 2,5,8,11,14), or HpaII then StuI (lanes 3,6,9,12,15). Double digests were performed as in Figure 14. Southern blots were analyzed as in Figure 11. The major digestion products are noted by arrows.


YOUNG CONTROLS
1 2 3
AGE-MATCHED CONTROLS
4 5 6 7 8 9
PHENOBARBITAL-INDUCED TUMORS
10 11 12 13 14 15
kb
23.1-
9.4-
6.6-
4.4-
2.3-
2.0-
1.35-
1.08-
0.87-
0.60-

tissue. Thus, the raf gene in the CD-1♂, similarly to the B6C3F1♂, is hypomethylated in PB-induced liver tumors.

To determine the specificity of the hypomethylation, we examined the methylation of the MspI site in exon 12 of the raf gene. As in the B6C3F1♂ (Figure 14), this site is methylated on the internal cytosine (i.e. 5'-C^{Me}CGG-3') in young adult CD-1♂ mice (Figure 45, lanes 1-3). This site remains methylated at the internal cytosine and is also methylated at the external cytosine in a majority of the cells in the age-matched control liver tissue since it is largely uncleaved by MspI or HpaII (Figure 45, lanes 4-9). This is consistent with the increased methylation seen in the raf gene in the MspI digested DNA from the age-matched control mice (Figures 44A and 44B). The methylation status does not change in the tumor tissue (Figure 45, lanes 10-15). This indicates that the loss of methylated sites in the tumor tissue is specific and neither generalized nor random.

B. Methylation status of Ha-ras

Increased digestion of Ha-ras in tumor tissue by HpaII is seen as additional bands in the region between 2.3 and 4.4 kb (Figures 46, lanes 8 and 10, Figure 47, lanes 8, 10 and 12, Table 2) not seen in HpaII digests of age-matched control liver DNA. Therefore, there has been a loss of methylation at some specific 5'-C^{Me}CGG-3' sites in Ha-ras as was seen in PB-induced B6C3F1♂ liver tumors.

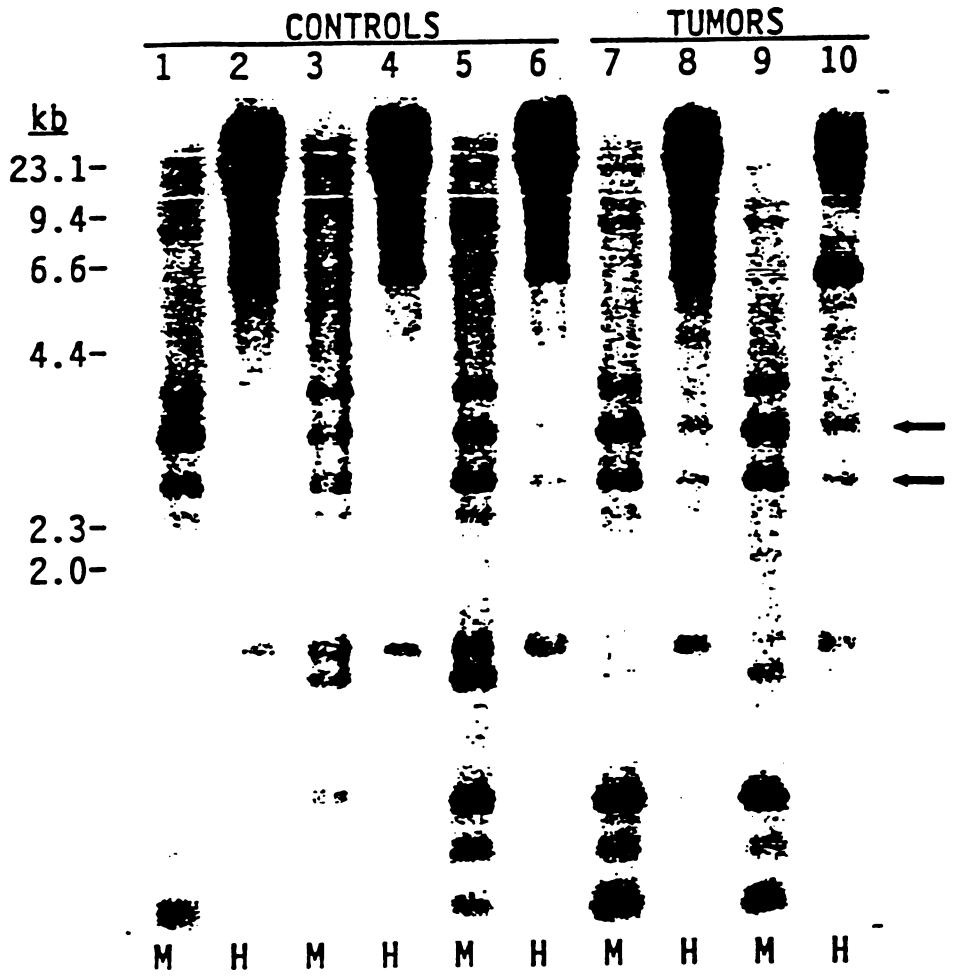


Figure 46. The methylation status of Ha-ras: CD-1σ PB-induced tumors and age-matched controls; MspI and HpaII. The blot in Figure 44A was stripped of the raf probe and analyzed as in Figure 15. The arrows point to the additional bands detected in the tumor tissue in the HpaII digests. Lanes 1-6: DNA from 3 age-matched controls; lanes 7-10: DNA from PB-induced liver tumors from 2 mice.

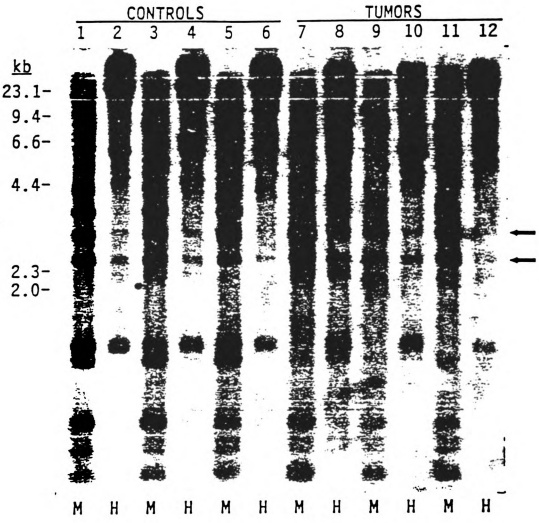


Figure 47. The methylation status of Ha-ras: CD-1σ PB-induced tumors and age-matched controls; MspI and HpaII. The blot in Figure 44B was stripped of the raf probe and analyzed as in Figure 15. The arrows point to the additional bands detected in the tumor tissue HpaII digests. Lanes 1-6: DNA from 3 different age-matched controls; lanes 7-12: DNA from PB-induced liver tumors from 3 mice.

C. Ha-ras and raf mRNA levels

Levels of raf mRNA are elevated in 4/5 CD-1♂ liver tumors vs. the age-matched CD-1♂ control liver tissue (Figures 48A and 49A, Table 2), while Ha-ras mRNA is elevated in 5/5 of the tumors (Figure 48B and 49B, Table 2). This indicates that the hypomethylation seen in raf and Ha-ras may play a role in CD-1♂ hepatocarcinogenesis through the stimulation of increased raf or Ha-ras expression.

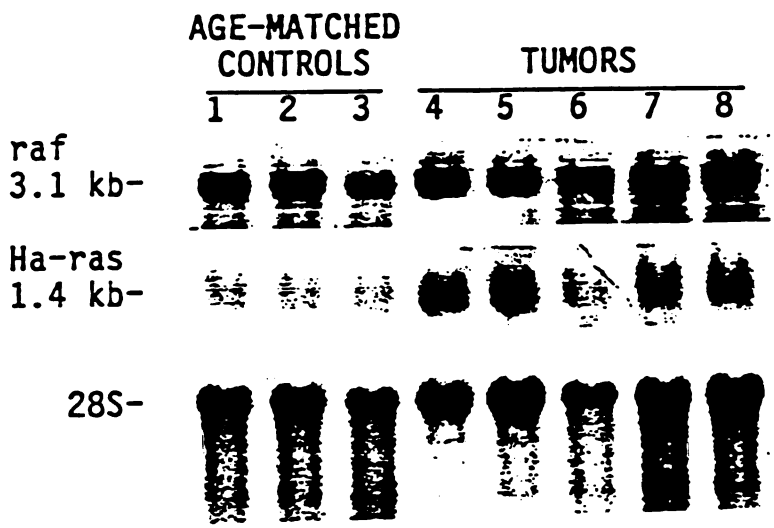
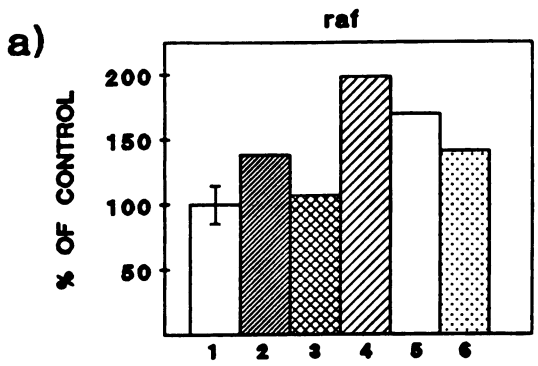
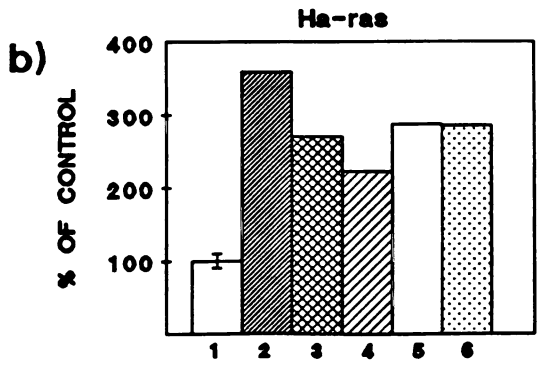


Figure 48. Ha-ras and raf mRNA: CD-1σ PB-induced tumors and age-matched controls. A Northern blot of RNA from age-matched control liver tissue (lanes 1-3) and PB-induced liver tumors (lanes 4-8) was analyzed as in Figure 38. Lane 1 corresponds to Figure 44B and 46B, lanes 1 and 2; lane 2 to Figure 44B and 46B, lanes 3 and 4; lane 3 to Figure 44B and 46B, lanes 5 and 6; lane 4 to Figures 44A and 46A, lanes 7 and 8; lane 5 to Figures 44A and 46A, lanes 9 and 10; lane 6 to Figures 44B and 46B, lanes 7 and 8; lane 7 to Figures 44B and 46B, lanes 9 and 10; lane 8 to Figures 44B and 46B, lanes 11 and 12.

Figure 49. Relative levels of raf and Ha-ras mRNA in CD-1♂ PB-induced and spontaneous tumors. Panels correspond to those in Figure 48. Control mean values are set at 100%. The actual ratios determined by densitometric analysis are given in parentheses. A) raf: 1 corresponds to the mean of lanes 1-3 with standard error (0.619 ± 0.087); 2 to lane 4 (0.856); 3 to lane 5 (0.665); 4 to lane 6 (1.230); 5 to lane 7 (1.049); and 6 to lane 8 (0.874) B) Ha-ras: 1 corresponds to the mean of lanes 1-3 with standard error (0.280 ± 0.028); 2 to lane 4 (1.005); 3 to lane 5 (0.757); 4 to lane 6 (0.622); 5 to lane 7 (0.804); 6 to lane 8 (0.800).


a)
raf
% OF CONTROL
200
150
100
50
1
2
3
4
5
6


b)
Ha-ras
% OF CONTROL
400
300
200
100
1
2
3
4
5
6

DISCUSSION

1. Factors Influencing the Regulation of raf Expression

Differences in both methylation status and DNA base sequence in the raf gene were found between the four strains of mice which were examined. In the most tumor-prone strain, the C3H/He♂, raf was found to be relatively hypomethylated as shown by the lack of a 6.7 kb band in the MspI digests of C3H/He♂ DNA probed for raf. This 6.7 kb band was present in the C57BL/6♀, B6C3F1♂, and CD-1♂ DNA. The B6C3F1♂ had an intermediate degree of methylation at the 5'-CCGG-3' site which was methylated on the external cytosine in the C57BL/6♀, but not the C3H/He♂.

The C3H/He♂ and C57BL/6♀ are each homozygous for different raf alleles as indicated by TaqI RFLP. The B6C3F1♂ apparently received one methylated allele from the C57BL/6♀ and a different, unmethylated allele from the C3H/He♂. The fact that the liver tumor incidence in the B6C3F1♂ is intermediate between its parents lends credence to the theory that intrinsic differences in the raf gene are involved in susceptibility to hepatocarcinogenesis. The methylation status of raf in the CD-1♂ was most similar to that of the B6C3F1♂. Because the spontaneous liver tumor incidences are similar between the B6C3F1♂ and the CD-1♂, these data suggest

a possible relationship between methylation status of raf and liver tumor susceptibility.

Based on current knowledge of the sequence of the raf gene in the area homologous to the probe, both the MspI site which was found to be differentially methylated between the strains and the TaqI site(s) responsible for the detection of different alleles are in introns. Intron sequences are important in the regulation of gene expression (Brinster, *et al.* 1988; Palmiter, *et al.* 1991). Expression of the p53 gene in tissue culture cells and transgenic mice is greatly enhanced by the presence of the p53 introns (Hinds, *et al.*, 1989; Lozano and Levine, 1991). Furthermore, two point mutations in intron 4 of the p53 gene prevented binding of an unidentified ubiquitous nuclear protein and resulted in decreased expression of the gene (Beenken, *et al.*, 1991). The methylation status of introns also has the potential to affect expression. Munzel and coworkers (1991) showed that *N*-nitrosomorpholine-induced hypomethylation of the c-myc gene at a site in the first intron (which contains a normally silent promoter region) resulted in increased levels of myc mRNA in liver nodules. These data suggest that a change as apparently minor as a variation in the position of a TaqI site or the loss of methylation at one site within an intron has the potential to greatly influence the expression of a gene.

The data obtained in this study suggest that there is a relationship between cell proliferation such as that induced by PH or 14 day PB treatment and subsequent hypomethylation of

raf, and to a lesser extent, Ha-ras. All three strains studied (B6C3F1♂, C3H/He♂, and C57BL/6♀) could not maintain methylation of raf following PH. Only some (3/3 of C3H/He♂, 2/3 of B6C3F1♂, and 2/4 of C57BL/6♀) lost methylated sites in Ha-ras in a sufficient number of cells for detection after PH. Following 75 mg PB/kg/day, a loss of methylated sites was detected only in the raf gene of the B6C3F1♂. While neither the intrinsic differences in the raf gene between the strains nor the hypomethylation induced in raf or Ha-ras by PH or 14 day PB treatment had any effect on the expression of raf mRNA, these alterations have the capability to increase the potential for elevated raf mRNA expression, which in turn might play a role in carcinogenesis.

Hypomethylation and gene expression are not always correlated. Becker, *et al.* (1987) studied the relationship between protein binding and methylation in the 5' flanking region of the tyrosine aminotransferase gene in expressing and nonexpressing cell lines. In expressing cells, the CpG sites are unmethylated, DNase hypersensitive sites are present, and cellular proteins bind in the area -1 kb upstream of the transcription start site. In cells which do not express the tyrosine aminotransferase gene, DNA footprinting indicated that protein binding does not occur, DNase hypersensitive sites are not detected, and all CpG sites in the region are methylated. Hypomethylation of CpG sites in the -1 kb region of the tyrosine aminotransferase gene in nonexpressing cells *in vivo* by 5-azacytidine treatment does not result in protein

binding, suggesting that hypomethylation alone is not permissive for binding of transcription factors (Weih, *et al.*, 1991). The 5' flanking region of the tyrosine aminotransferase gene in nonexpressing cells is organized into nucleosomes, and the nucleosomes persist in the hypomethylated 5-azacytidine treated cells. This suggests that hypomethylation of DNA which is already organized into an inactive chromosome structure does not increase accessibility of the gene to transcription factors. Other changes which result in an open, active chromosome conformation might be required for transcription to occur.

The methylation status of specific sites might be critical for expression of some genes while methylation or the lack of methylation at other sites might have no effect on expression. Weih, *et al.* (1991) used synthetic oligonucleotides homologous to the cAMP response element to demonstrate that methylation of CpG sites prevented binding of the CREB transcription factor while unmethylated oligonucleotides bound the transcription factor. These data suggest that the absence of methyl groups allows the binding of transcription factors which would facilitate transcription.

Binding of inhibitory proteins to methylated DNA requires a minimum number of 5MeC in a region of DNA (Meehan, *et al.*, 1989) and demethylation of a single site might not decrease the number of 5MeC below the this minimum number. Thus, the binding of inhibitory proteins would not be affected by loss of methylation at one site. These data fit well with the

multistep theory of carcinogenesis, i.e. that multiple alterations must occur for a phenotypically normal cell to become a cancer cell. In other words, other changes must occur besides the loss of methylation seen after cell proliferation before transcription of mRNA is increased and tumors form. These might include loss of additional methylated sites, point mutations, gene rearrangements, deletions, or amplifications.

The raf gene was found to be hypermethylated in 26 month old CD-1♂ mice relative to the young adult mice. There were an increased number of cells in which the 5'-CCGG-3' site in exon 12 was resistant to digestion by both MspI and HpaII, indicating that it is methylated on both the internal and external cytosines (5'-$^{Me}C^{Me}$CGG-3'). There was also evidence for the presence of additional 5'-^{Me}CCGG-3' sites in raf, seen as multiple bands above the 5.1 kb band in MspI digests. Hypermethylation of genes in aged animals has been correlated with decreased expression of these genes (Swisshelm, *et al.*, 1990; Ono, *et al.*, 1986; Ono, *et al.*, 1989). Indeed, the aged CD-1♂ control livers had lower levels of raf mRNA than the young CD-1♂ controls (data not shown). However, the mRNA levels from the tumor tissue were compared with mRNA levels from control mice of the same age.

The hypermethylation of the raf gene in aged CD-1♂ mice probably does not have any bearing on PB-induced carcinogenesis. While loss of the 5'-^{Me}CCGG-3' site responsible for the detection of the 6.7 (or 8.0) kb band in

the control B6C3F1♂ and CD-1♂ mice does appear to be involved in carcinogenesis, this 5'-^{Me}CCGG-3' site and the 5'-^{Me}CCGG-3' sites responsible for the other bands above 5.1 kb in the MspI digests are all lost in the PB-induced CD-1♂ tumors. The effect of the hypermethylation of the raf gene in aged CD-1♂ mice might be that additional hypomethylation is required (i.e., at least 2 more 5'-^{Me}CCGG-3' sites must be lost) for increased expression to occur. Because the methylation status of the MspI recognition site in exon 12 did not change under any of the conditions in this study (except the hypermethylation seen in the aged CD-1♂ mice), the methylation status of this site apparently has no role in carcinogenesis.

Based on the findings of this study, it appears that 5'-^{Me}CCGG-3' sites (rather than 5'-C^{Me}CGG-3' sites) have an important regulatory role in the expression of the raf gene. The hypomethylation of raf seen in the C3H/He♂ *vs.* the C57BL/6♀ was due to the lack of a 5'-^{Me}CCGG-3' site. This 5'-^{Me}CCGG-3' site was also lost in B6C3F1♂ and C57BL/6♀ following PH and in B6C3F1♂ following PB treatment. The difficulty in maintenance of methylation at this site might be because it is methylated through a different mechanism than 5'-C^{Me}CGG-3' sites or because of the physical position of this site in the DNA helical structure. There may be multiple maintenance methylase enzymes (i.e. there might be a maintenance methylase to methylate hemimethylated 5'-C^{Me}CGG-3' sites and a different one to methylate 5'-^{Me}CCGG-3' sites). Alternatively, the external cytosine is less accessible to the

maintenance methylase than the internal cytosine due to stearic hindrances or sequence-dependent variations in chromatin structure. Hemimethylated 5'-^{Me}CCGG-3' sites would then be more difficult to methylate, especially under conditions in which cells are repeatedly traversing the cell cycle. The C3H/He♂ and B6C3F1♂ might have a defect in the system responsible for methylating 5'-^{Me}CCGG-3' sites. To determine if such a defect exists, DNA maintenance methylase (or at least nuclear extract) isolated from C3H/He♂ and C57BL/6♀ mice should be compared for ability to methylate 5'-CCGG-3' sites both in naked DNA and in intact chromatin.

2. Correlation between Hypomethylation, Increased Expression and Tumorigenicity

The hypomethylation of raf seen in the B6C3F1♂ after a 14 day treatment with PB was found to be accompanied by increased amounts of raf mRNA in PB-induced tumors. Both raf and Ha-ras were hypomethylated in PB-induced B6C3F1♂ (raf--10/10, Ha-ras--7/8) and CD-1♂ (raf--4/5, Ha-ras--5/5) tumors. PB-induced tumors in B6C3F1♂ and CD-1♂ mice also had elevated levels of raf (B6C3F1♂--7/10, CD-1♂--4/5) and Ha-ras (B6C3F1♂--8/10, CD-1♂--5/5) mRNA. There might have been loss of additional methylated sites which were not detectable by MspI or HpaII digestion. The MspI site in exon 12 remained methylated on the internal cytosine in the B6C3F1♂ and methylated on both the internal and external cytosines in the CD-1♂, indicating that the loss of methylation was neither

generalized nor random. Alternatively, other changes such as relaxation of the chromatin structure or alterations in base sequence might have occurred to allow increased transcription. Loss of additional methylated sites occurred in the Ha-ras gene which might be directly responsible for the increased expression, or other changes might also have been involved.

The exogenous v-Ha-ras in $WB^{Ha\text{-}ras}$ cells and $WB^{Ha\text{-}ras}$ cell-induced F344 rat liver tumors was hypomethylated relative to c-Ha-ras. The hypomethylation of Ha-ras in tumors was correlated with increased expression which was seen as elevated levels of both Ha-ras mRNA and p21 ras protein. Because transformation of cells transfected with mutated Ha-ras is dependent on the use of hypomethylated Ha-ras DNA (Borrello, *et al.*, 1987), it follows that a major factor in the tumorigenicity of $WB^{Ha\text{-}ras}$ cells was the hypomethylated state of v-Ha-ras and subsequent increase in p21 ras protein levels.

Because hypomethylation has the potential to facilitate expression of genes, and there is a correlation between hypomethylation and expression in tumor tissue, it appears that the hypomethylation of these genes could play a role in carcinogenesis. Since hypomethylation seen after a 14 day administration of PB did not affect gene expression, hypomethylation alone is not sufficient alone to increase gene expression. Other, as-yet-unidentified alterations, occurred in the cells with hypomethylated proto-oncogenes during the progression to actual tumor formation.

3. Differences in the Mechanisms Involved in PB-induced and Spontaneous Tumorigenesis

PB is not simply accelerating the development of spontaneous tumors, but is selecting a different population of cells. In spontaneous B6C3F1♂ liver tumors, hypomethylation of raf was seen in 4/5 tumors, but raf mRNA levels were elevated in only one of the tumors. The methylation status and mRNA levels of Ha-ras in spontaneous tumors were similar to those in PB-induced tumors. Because there is a difference in the phenotype of spontaneous *vs.* PB-induced tumor cells, it is likely that the two tumor types arose via different mechanisms. In addition to the findings in this study involving differential levels of raf expression, others have found differences in the activation of the Ha-ras gene in PB-induced *vs.* spontaneous tumors (Fox, *et al.*, 1990; Rumsby, *et al.*, 1991).

Others have also detected intrinsic differences in the response of C3H/He♂ and C57BL/6♀ to PB promotion. C3H/He hepatocytes exhibit enhanced responsiveness to PB alone or to PB administration as a promoter following initiation by other chemicals. Primary C3H/HeNJcl hepatocyte cultures developed more (5x) colonies in the presence of 1.5 mM PB than C57BL/6NJcl hepatocytes which did not respond to PB administration with enhanced colony formation (Lee, *et al.*, 1989b). Lee and coworkers (1989a) found that the number of enzyme altered (glucose-6-phosphatase negative) foci was increased in both C3H/HeN and C57BL/6N mice which were

initiated with one injection of diethylnitrosamine 20 hours after a PH and then fed 500 ppm PB in the diet for 20 weeks *vs.* mice that did not receive PB. However, the foci were >100x larger in C3H/HeN mice than in C57BL/6N mice.

There has also been found to be interstrain variation in the response to TPA promotion of skin tumors. CD-1 and C3H mice are responsive, but C57BL/6 mice are resistant to TPA promotion of dimethylbenz(a)anthracene-induced skin lesions (DiGiovanni, *et al.*, 1988). Multiple applications of TPA (8 bi-weekly treatments) resulted in a 3 fold increase in epidermal thickness in SENCAR mice (which were developed by breeding CD-1 to STS mice for 8 generations and selecting those offspring which were most sensitive to phorbol ester promotion for breeding), but only a slight increase in epidermal thickness in C57BL/6 mice (DiGiovanni, *et al.*, 1991). In other words, TPA induces epidermal cell proliferation to a much greater extent in SENCAR mice than C57BL/6 mice. However, C57BL/6 mice respond to benzoyl peroxide promotion of dimethylbenz(a)anthracene-induced skin tumors (Reiners, *et al.*, 1984; DiGiovanni, *et al.*, 1991) suggesting that the signal transduction pathway mediated by PKC (and therefore activated by TPA) functions differently in C57BL/6 epidermal cells than in the other strains studied.

It might be possible to extrapolate the theory that the epidermal cell PKC pathway is altered in C57BL/6♀ relative to the other strains to hepatocytes. TPA's effects on hepatocytes *in vivo* cannot be determined because TPA is too

toxic to be administered internally. It is also not known if TPA and diacylglycerol, the endogenous activator, activate PKC through the same mechanism, although that is generally assumed to be the case. There are multiple isozymes of PKC and they might be present in different amounts in different cell types (e.g., epidermal *vs.* endothelial) or they might be activated by different mechanisms. Therefore, the theory that the PKC pathway in C57BL/6♀ hepatocytes is different than in the other strains relies on several assumptions and would require extensive additional testing for proof.

PB has an effect on the signal transduction pathway involving EGF, PKC, ras p21 and Raf-1 (Figure 1). Chronic PB administration such as that used in promotion protocols decreases the response of hepatocytes *in vivo* to the stimulatory effects of PH (Barbason, *et al.*, 1983) and the response of hepatocytes *in vitro* to EGF (Eckl, *et al.*, 1988). Hepatocytes from chronically treated animals have decreased numbers of EGF-R (Eckl, *et al.*, 1988; Meyer and Jirtle, 1989), decreased levels of EGF-R mRNA (Hsieh, *et al.*, 1988), do not respond to TPA treatment with activation and membrane translocation of PKC (Brockenbrough, *et al.*, 1991), and have elevated levels of transforming growth factor β1 (TGF-β1) (Meyer and Jirtle, 1991). TGF-β1 is produced by liver nonparenchymal cells, binds to a specific receptor on hepatocytes, and inhibits hepatocyte proliferation (Braun, *et al.*, 1988). Each of these effects of chronic PB treatment would result in decreased responsiveness to cell proliferative

stimuli in normal hepatocytes. Therefore, the population of cells which participate in PB-induced tumorigenesis must be able to overcome the inhibitory effects of chronic PB administration.

Cells with activated raf or Ha-ras have the potential to overcome PB's growth inhibition. Hampton and coworkers (1990) showed that tumor derived liver cells (cultured from tumors resulting from injection of v-raf transfected rat liver epithelial cells into athymic nude mice) could grow in medium without supplemental EGF, while the cells used to induce the tumors (transfected with v-raf) would not grow under the same conditions. Increased levels of normal or mutated Ha-ras mRNA have been found to stimulate increased expression of EGF-R mRNA which results in increased numbers of EGF-R on the cell surface (Theodorescu, *et al.*, 1990; Theodorescu, *et al.*, 1991). The EGF-R gene contains an AP-1 enhancer site about 200 bp downstream of the transcription start site (Theodorescu, *et al.*, 1991). Elevated levels of ras p21 protein or activation of Raf-1 have been implicated in the enhanced expression of genes with AP-1 enhancer sites.

The presence of either activated raf or Ha-ras also has the potential to interfere with the inhibitory effects of elevated TGF-β1 in hepatocytes due to chronic PB treatment. Following transfection of rat liver epithelial cells with v-raf, transformed cells are relatively resistant to the growth inhibitory effects of TGF-β1 (Huggett, *et al.*, 1990). Some of the transformed cells have a decreased number of

TGF-β1 receptors, while others have normal numbers of receptors, but post-receptor signalling pathways are perturbed and cell proliferation is not inhibited by TGF-β1. v-Ha-ras or T24 ras transfected rat liver epithelial cells are also resistant to the growth inhibitory effects of TGF-β (Houck, *et al.*, 1989). These studies examined the effects of oncogenes which were activated by mutation, but since increased expression of the corresponding proto-oncogenes can transform cells, it is likely that oncogenes activated by deregulation of expression might act similarly.

SUMMARY AND CONCLUSIONS

Four major findings resulted from this study. First, there are intrinsic differences in the raf gene between the C3H/He♂ and C57BL/6♀ mice which increase the potential for aberrant expression of the raf gene in the C3H/He♂. Therefore, the C3H/He♂ mouse might be one step further along the multistep pathway involved in carcinogenesis than the C57BL/6♀ due to hypomethylation of the raf proto-oncogene in the nascent liver. The B6C3F1♂ inherits a methylated raf allele from the C57BL/6♀ and an unmethylated allele form the C3H/He♂. This gives credence to the involvement of hypomethylated raf in carcinogenesis since the spontaneous liver tumor rate of the B6C3F1♂ is intermediate between that of the C3H/He♂ and the C57BL/6♀. B6C3F1♂ and CD-1♂ mice have similar methylation patterns of raf and similar incidences of spontaneous liver tumor development.

Second, methylation at the external cytosine of one or more 5'-CCGG-3' sites appears to be more important than sites which are methylated on the internal cytosine in the regulation of expression of the raf gene. The 5'-^{Me}CCGG-3' site which is present in the C57BL/6♀ but not the C3H/He♂ is apparently difficult to maintain. Methylation at this site is decreased following cell proliferation induced by PH in the

B6C3F1♂ and C57BL/6♀ and after 14 days of 500 ppm PB administration in the C57BL/6♀.

Third, there is a correlation between hypomethylation and increased expression of raf and Ha-ras in PB-induced tumors. Additionally, there is a correlation between hypomethylation of v-Ha-ras, levels of ras mRNA and p21, and tumorigenicity of cells transfected with v-Ha-ras ($WB^{Ha\text{-}ras}$ cells). While hypomethylation alone is not sufficient for increased expression, this suggests that increased expression of raf and/or Ha-ras genes, subsequent to hypomethylation and additional as-yet-unidentified cellular changes, is a mechanism involved in carcinogenesis.

The fourth major finding confirms the findings of others that PB administration does not simply accelerate the development of spontaneous liver tumors. While both raf and Ha-ras are hypomethylated in spontaneous B6C3F1♂ liver tumors, elevated levels of raf mRNA are not detected in the majority of the tumors, indicating a difference in the phenotype of the cells involved in spontaneous *vs.* PB-induced tumors. While it is possible that increased raf mRNA levels are a consequence of PB administration and not causal in tumorigenesis, it appears that PB administration results in selective expansion of cells with either increased potential for raf expression or actual elevated levels of raf mRNA.

Based on these results, it can be concluded that hypomethylation of proto-oncogenes plays a role in the potential of mice to develop liver tumors. There is an

intrinsic inability of the C3H/He♂ mouse to methylate 5'-^{Me}CCGG-3' sites in the raf gene relative to the C57BL/6♀ mouse. A defect in this pathway might be one of the mechanisms underlying the increased propensity of C3H/He♂ and B6C3F1♂ mice to develop tumors. Increased raf expression allows cells to overcome PB's growth inhibitory effects and thus appears to be a major factor in PB-induced mouse liver carcinogenesis.

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