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Title : Analysis of *Ras* Gene Mutations in Rainbow Trout Tumors.

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George S. Bailey

For ras gene mutation analysis in the rainbow trout (Oncorhynchus mykiss) model system, a partial trout ras sequence was identified using the polymerase chain reaction (PCR). Two synthetic oligonucleotides based on rat K-ras gene sequence were used as primers for the PCR procedure. A 90 base pair (bp) sequence, referred to as the trout K-ras, was amplified from trout genomic DNA and cDNA. Cloned 90 bp PCR products from several normal liver tissues were sequenced resulting in the same sequence. Large-sized PCR products, 111 and 237 bp, were also cloned and sequenced indicating that these fragments included the 90 bp sequence information expressed in mRNA. This 5'-terminal partial trout K-ras nucleotide sequence was 88% homologous to that of the goldfish ras gene, and less homologous to those of mammalian ras genes.

Based on the partial sequence information of two trout ras genes, K-ras and H-ras, DNA from trout tumors induced by chemical

carcinogens, aflatoxin B1 (AFB1) and N-methyl-N'-nitro-N-nitrosoguanidene (MNNG), were analyzed for the presence of point mutations. Using the PCR and oligonucleotide hybridization methods, a high proportion (10/14) of the AFB1-initiated liver tumor DNA indicated evidence for ras point mutations. Of the 10 mutant ras genotypes, seven were probed as G to T transversions at the second position of codon 12, two were G to T transversions at the second position of codon 13, and one was a G to A transition at the first position of codon 12. Nucleotide sequence analysis of cloned PCR products from four of these tumor DNAs provided definitive mutation evidence in each case, which seemed to occur in only a fraction of the neoplastic cells. However, no mutations were detected in exon 1 of the trout K-ras gene, nor in DNA from trout normal livers. Results indicated that the hepatocarcinogen AFB1 induced similar ras gene mutations in trout as in rat liver tumors. By comparison, the mutation specificity of MNNG in trout liver tumors was for G to A transitions, but no ras mutations were detected in trout kidney tumors. This investigation was the initial study of experimentally induced ras gene point mutations in a lower vertebrate fish model.

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Typed by Yung-Jin Chang

To mother and Eyunmee.

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ABBREVIATION

AAAF : N-acetoxy-2-acetylaminofluorene

AFB1 : Aflatoxin B1

AML : Acute lymphoblastic leukemia

BaPDE : Benzo[a]pyrene diol epoxide

bp : base pair

DEN : Diethylnitrosoamine

DMBA : 7,12-Dimethylbenz[a]anthracene

EMS : Ethylmethanesulphonate

FAPY : Formamidopyrimidine

HCC : Hepatocellular carcinoma

MFO : Mixed function oxidases

MNNG : N-methyl-N'-nitro-N-nitrosoguanidene

MNU : N-methyl-N-nitrosourea

NMAB : N-nitroso-N-methyl-N-alpha-acetoxybenzylamine

PCR : Polymerization chain reaction

RFLP : Restriction fragment length polymorphism

SSC : Sodium-chloride and sodium-citrate

Taq : *Thermus aquaticus*

Td : Dissociation temperature

TMAC : Tetramethylammonium chloride (Me_4NCl)

ANALYSIS OF RAS GENE MUTATIONS IN RAINBOW TROUT TUMORS

INTRODUCTION

Ras oncogene and p21.

Oncogenes disrupt the control systems of cell growth and/or cell differentiation and in this way contribute to the development of cancer (Bishop, 1987). Identified oncogenes of viral and cellular origin were grouped on the basis of the nuclear or cytoplasmic localization of the oncogene encoded proteins (Weinberg, 1985). In the cytoplasm, these proteins may regulate levels of critical second messenger molecules; in the nucleus, these proteins may modulate the activity of the cell's transcriptional factors. Oncogene products can also be related to a signaling pathway for the cell's response to growth-stimulating factors (Barbacid, 1987). The ras gene family of 3 closely related genes, K-ras, H-ras and N-ras, has been extensively studied and appears to play an important role in a significant portion of human cancers (Barbacid, 1987). The gene family members are located in different chromosomes in human; the N-ras gene at chromosome 1, the H-ras gene at chromosome 11, the K-ras gene at chromosome 12 (see Lacal and Tronick, 1988). The ras oncogenes are almost invariably activated by single base changes (or point mutations) at specific positions, usually assayed by their ability to induce the malignant transformation of NIH3T3 cells. Ras gene mutational activation then results in amino acid substitutions in the p21 ras protein (Der et al., 1982; Reddy et al., 1982) without an affect on the

level of expression of these genes (Tabin et al., 1982; Taparowsky et al., 1982).

Identification.

It was only in 1982 that isolation of the human oncogene, ras, was reported (Goldfarb et al., 1982; Santos et al., 1982; Shih and Weinberg, 1982; Parada et al., 1982). Originally, ras genes were identified as the transforming principle of the Harvey and Kirsten strains of rat sarcoma viruses, two acute transforming retroviruses generated by transduction of the rat H-ras1 (Dhar et al., 1982) and K-ras2 (Tsuchida et al., 1982) cellular genes, respectively. The homologue of the viral Kirsten ras (v-K-ras) gene was then found in the human lung carcinoma cell line, Calu-1 (Shimizu et al., 1983c), and that of the viral Harvey ras (v-H-ras) was in the human bladder carcinoma cell lines, T24 and EJ (Reddy et al., 1982; Taparowsky et al., 1982; Tabin et al., 1982). The N-ras locus found in SK-N-SH neuroblastoma cells, however, has not been transduced by retroviruses (Taparowsky et al., 1983; Shimizu et al., 1983b).

Highly conserved sequence.

Three ras genes have been identified in several mammalian genomes; H-ras1 in human (Tabin et al., 1982; Goldfarb et al., 1982; Shih and Weinberg, 1982; Capon et al., 1983a; Pulciani et al., 1982b; Reddy, 1983; Yuasa et al., 1983) and in rat (Ruta et al., 1986; Sukumar et al., 1983; DeFeo et al., 1981), K-ras2 in human (McGrath et al., 1983; Shimizu et al., 1983a; Nakano et al., 1984; Capon et al., 1983b; McCoy et al., 1984) and in rat (Tahira et al., 1986) and in mouse (George et al., 1985), and N-ras in human (Shimizu et al., 1983b; Hall et al., 1983; Taparowsky et al., 1983; Brown et al., 1984; Padua et al.,

1985), in mouse (Guerrero et al., 1984; Guerrero et al., 1985) and in rat (McMahon et al., 1990b).

Ras gene family shares regions of nucleotide homology and nucleotide divergence within coding sequences and have a common intron/exon structure, indicating that they may have evolved from a similarly spliced ancestral gene. There is strong conservation of the entire amino acid sequence between homologous ras genes in distant species, despite a high frequency of third-base neutral changes (Barbacid, 1987). For instance, the nucleotide sequence of goldfish ras protein shows nearly 80% homology with the sequences of mammalian H-, K-, and N-ras genes (Nemoto et al., 1986).

p21 and its activation.

The ras gene products have been identified as proteins of 188 or 189 amino acid residues, generally known as p21. They are located at the inner side of the plasma membrane where they are anchored into the lipid bilayer by a covalently attached palmitic acid at the carboxy terminus. The proteins can bind GTP and GDP with high affinity and possess GTPase activity (McGrath et al., 1984). They share limited amino acid homology with G-like proteins, which are known to transduce signals from various cell-surface receptors to adenylate cyclase (Lacal and Tronick, 1988).

The high degree of evolutionary conservation of ras genes and their products implies that p21 may play a fundamental role in cellular proliferation among vertebrates (Barbacid, 1986). In particular, the N-terminal 85 amino acid residues of p21 is highly conserved, and thus is believed to be an essential region for the function of p21. The region about positions 59-63 adjoins a site for autophosphorylation by

GTP, whereas the sequence around amino acids 12 and 13 may neighbor the GTP binding site, since this region has amino acid homology to known nucleotide binding proteins (Wierenga and Hol, 1988; Shih et al., 1982). These regions might therefore be expected to be involved as target sites for carcinogenic activities of ras gene. Sequences of ras gene in exon 1 and 2 among several species are compared in Table 1.

It has been postulated that normal ras proteins exist in an equilibrium between 'excited' and 'relaxed' states depending on whether they bind GTP or GDP, respectively (Weinberg, 1985). Conversion of the 'excited' to the 'relaxed' state is thought to be mediated by an intrinsic GTPase activity of ras proteins. Point mutations to activate normal ras proto-oncogenes into oncogenes either block the intrinsic GTPase activity, thus preventing the normal deactivation of these proteins (Sweet et al., 1984) or reduce the affinity for GDP and GTP (Weinberg, 1985) to produce a constitutively activated ras gene product.

The availability of large quantities of purified p21 protein through the expression of ras genes in bacteria made it possible to more readily investigate the relationship of GTP binding and hydrolysis to biological function. Microinjection of purified mutant p21 proteins into NIH3T3 mouse fibroblasts led to transient morphologic transformation and cell proliferation (McGrath et al., 1984). The presence of a Gly residue at position 12 appears to be necessary for the normal function of ras proteins (Tabin et al., 1982). Substitution of Gly12 by any other amino acid residue (with the exception of Pro) results in a significant structural change in an essential domain of the

Table 1. Sequence comparison in exon 1 and part of exon 2 of ras gene among several species.

exon 1 :

```
     1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19
   MetThrGluTyrLysLeuValValValGlyAlaGlyGlyValGlyLysSerAlaLeu

HK:ATGACTGAATATAAACTTGTGGTAGTTGGAGCTGGTGGCGTAGGCAAGAGTGCCTTG
RK:--------G-----------------------------------------------
MK:--------G-----------G-----------------------C------

HH:-----G--------G--G-----G--G--C--C--C--T--G----------GC--
RH:-----A-----C--G--------G--G--C-----A-----G--A---------C--

HN:--------G--C-----G-----G--------A-----T--T--G--A--C--AC--
MN:--------G--C-----G-----G--------A-----T--T--G--A--C---C--

Gf:-----A--------G--------C--A--G-----A-----G----------TC-C
Ch:-----C--G--C--G--G--------G--------A--T--C--G-----C--T---


    20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37
   ThrIleGlnLeuIleGlnAsnHisPheValAspGluTyrAspProThrIleGlu

HK:ACGATACAGCTAATTCAGAATCATTTTGTGGACGAATATGATCCAACAATAGAG
RK:----------------------C--------T----------T--G------
MK:----------------------C--------T--G--C--C--T--G------

HH:--C--C-----G--C-----C----------------C--C--C--T------
RH:--C--------G--C-----C--------------G--------C--T------

HN:--A--C--------C-----C--C-----A--T----------C--C------
MN:-----C--------C-----C--C--------T----------C--C------

Gf:--C--C--A--C--C-----C--C--------------C--C-----C------
Ch:----------C--------C--------T--T--G--C--C--C----------
```

Table 1. Sequence comparison in exon 1 and part of exon 2 of ras gene among several species (Cont.).

part of exon 2 :

```
    38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56
   AspSerTyrArgLysGlnValValIleAspGlyGluThrCysLeuLeuAspIleLeu

HK:GATTCCTACAGGAAGCAAGTAGTAATTGATGGAGAAACCTGTCTCTTGGATATTCTC
RK:--C----------A-------------------------------------------
MK:--C----------A-------------------------------------------

HH:---------C-------G--G--C--------G--G--G--C--G-----C--C--G
RH:--C------C----A--G-----C--------G--G--G---T-AC----C--CT-A

HN:-----T-----A--A-----G--T--A-----T---------T-G-----C--A--G
MN:-----T---C-A--------G--G--------T--G-----C--GC----C--A--G

Gf:--C--------------G--G--G--------G--G--G-----G-----C--CT-G
Ch:----------A--------C--C--C--------G------T-GC----C--C--G


    57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75
   AspThrAlaGlyGlnGluGluTyrSerAlaMetArgAspGlnTyrMetArgThrGly

HK:GACACAGCAGGTCAAGAGGAGTACAGTGCAATGAGGGACCAGTACATGAGGACTGGG
RK:-------------------------------------------------A------
MK:-------------------------------------------------A------

HH:--T--C--C--C--G----------C--C---C-------------C-C--C---
RH:----------------A-----T-----C---C-------------C-C--A---

HN:--T-----T--A-----A----------C-----A-----A----------A--C
MN:--------T--A----------------C-----A----------------A--C

Gf:-----T--------G----------C--C--------T-------------A--A
Ch:--T--G--G--G--G-------------C---C-A-------------A--G---
```

Table 1. Sequence comparison in exon 1 and part of exon 2 of ras gene among several species (Cont.).

```
     76 77 78 79
    GluGlyPheLeu

HK:GAGGGCTTTCTT
RK:------------
MK:------------

HH:--------C--G
RH:--------C--C

HN:--A--G--C--C
MN:--A--G--C--C

Gf:--------C--C
Ch:--A--A--C--G
```

Ref.

HK : Human K-ras (Capon et al., 1983b; McCoy et al., 1984)
RK : Rat K-ras (Tahira et al., 1986)
MK : Mouse K-ras (George et al., 1985)

HH : Human H-ras (Tabin et al., 1982; Capon et al., 1983a)
RH : Rat H-ras (Sukumar et al., 1983; Ruta et al., 1986)

HN : Human N-ras (Brown et al., 1984; Gambke et al., 1985)
MN : Mouse N-ras (Guerrero et al., 1985)

Gf : Goldfish ras (Nemoto et al., 1986)
Ch : Chicken ras (Westaway et al., 1986)

p21 protein and causes the oncogenic activation of these molecules, suggesting a requirement for an alpha-helical polypeptide structure in this region (Seeburg et al., 1984). Similarly any replacements of the normal glutamine at codon 61 of H-ras, except by glutamic acid and proline, was found to cause activation (Der et al., 1986).

Mutational analysis of ras oncogene.

Most of these transforming genes have been identified as mutated alleles of cellular ras genes using DNA transfection of the NIH3T3 mouse fibroblast cell line. Generally, the activating mutations occur in the codons for amino acid residues 12, 13 or 61 of the protein (Seeburg et al., 1984; Bos et al., 1985; Brown et al., 1984), although potential sites for activation have also been identified at codons 59 (Fasano et al., 1984), 116 (Walter et al., 1986), and 119 (Sigal et al., 1986a and 1986b) by in vitro mutagenesis studies. Not all mutations at different sites may be equally effective at inducing tumorigenic transformation (Fasano et al., 1984). Table 2 describes the mutational activations of ras genes in human primary tumors. Tumor cell lines were not included because of the possibility that mutation occurred during later passages of cell lines (Pulciani et al., 1982a; Albino et al., 1984; Janssen et al., 1987a) or that cell lines obtained may represent only a subset of a particular tumor type (Tainsky et al., 1984).

Tissue-specific mutation incidence.

It can be seen from Table 2 that the various target tissues showed prevalent activation of one particular type of ras oncogene at certain incidence. Smit et al. (1988) mentioned that "tissue-specific factors" such as susceptibility of the various genes to particular chemical mutagens or activity of certain DNA repair mechanisms could

Table 2. Human ras oncogene mutations.

Tissue	Mutation Site(s)	Frequency (%)	Mutation Events	Detection Methods	Ref.
Lung	K 12	5/10 (50)	early	Oligo/PCR	[1]
Colon	K 12	6/6 (100)	early	Seq/PCR	[2]
	K 12	9/27	early	Oligo/PCR	[3]
	K 61	1/27			
	N 12	1/27			
		11/27 (41)			
	K 12		early	Seq/PCR	[4]
adenoma		9/12 (75)			
carcinoma		26/40 (65)			
	K 12	26/66 (40)	early(D)	RNAse	[5]
		68/172 (40)	-	Oligo/PCR	[6]
adenoma	K 12	17/80			
	K 13	6/80			
	K61, N12	2/80			
		25/80 (31)			
carcinoma	K 12	30/92			
	K 13	7/92			
	N 13	2/92			
	N 61	2/92			
	K61, N12	2/92			
		43/92 (47)			
Pancreas	K 12	47/63 (75)	both(D)	Oligo/PCR	[7]
	K 12	28/30 (93)	-	Oligo/PCR	[8]
	K 12	21/22 (95)	early	RNAse/PCR RFLP/Oligo	[9]
ALL	N 12	4/33	both(D)	Oligo/PCR	[10]
	N 13	3/33			
		6/33 (18)			
	N 12	2/19 (11)	-	Oligo	[11]

Table 2. Human ras oncogene mutations (cont.).

Tissue	Mutation Site(s)	Frequency (%)	Mutation Events	Detection Methods	Ref.
AML	N 61 N 12 H 12	3/9 1/9 1/9 5/9 (55)	-	Oligo/PCR	[12]
	N 13 N 61 K 12	6/45 4/45 2/45 12/45 (27)	-	Oligo	[13]
	N 12 N 13	13/52 1/52 14/52 (27)	both(D)	Oligo/PCR	[14]
	N 13	5/8 (62)	-	Oligo/PCR	[15]
	N	3/6		Transf	[16]
CML	H 12	0/25 (0)	none	RFLP	[17]
	ras	0/26 (0)		Oligo/PCR	[12]
MDS	N 13	3/8 (38)	early	Transf	[18]
	N 12	1/15 (7)	-	Oligo/PCR	[12]
	K 13	1/1	early	Transf Oligo/PCR	[19]
Breast	K 13	1/40 (2)	none	Oligo/PCR	[20]
	ras	0/16 (0)	-	Transf/RFLP	[21]
Stomach	ras	0/37 (0)	-	Transf	[22]
Esophagus	ras	0/25 (0)	-	Oligo/PCR	[23]
Urinary Tract	H 61	2/38 (5)	-	Transf	[24]
Kidney	H 12 H 61	1/16 1/16 2/16 (13)	-	RFLP	[25]

Table 2. Human ras oncogene mutations (cont.).

Tissue	Mutation Site(s)	Frequency (%)	Mutation Events	Detection Methods	Ref.
Liver	K12,N61	2/34 (6)	none	Oligo/PCR	[26]a
	N	3/10	-	Transf	[27]
	ras	0/12	none	Seq/PCR	[28]a
	K 12	5/9	-		[28]b
	K 61	1/9			
		6/9 (67)			
Skin	N12,13,61	7/37 (19)	early(D)	Oligo/PCR	[29]c
	H 61	1/10 (10)	early	Transf	[30]d
	N 61	1/13 (8)	later	Transf	[31]
Thyroid	K, H, N		early	Oligo/PCR	[32]
adenoma	N 61	3/24			
	H 61	2/24			
	H 12	1/24			
	K 12	1/24			
	N 12	1/24			
		8/24 (33)			
carcinoma	H 12	3/20			
	H 61	3/20			
	K 12	2/20			
	N 12	1/20			
	N61	1/20			
		10/20 (50)			

a : Hepatocellular carcinoma, b : Cholangiocarcinomas, c : Melanomas, d : Keratoacanthomas.

ALL : Acute Lymphoblastic Leukemia; CML : Chronic Myeloid Leukemia; MDS : Myelodysplastic syndrome (or preleukaemia).

D : simultaneous Double Mutations.

RFLP : Restriction Endonuclease Digestion; Oligo: Mutation-specific Oligonucleotide Hybridization; Transf : NIH3T3 Transfection Biological Assay; Seq : Direct Sequencing of PCR products; RNAse : Mismatches Cleavage.Assay

Ref.

[1] Rodenhuis et al., (1987); [2] Burmer et al., (1989); [3] Bos et al., (1987a); [4] Burmer and Loeb, (1989); [5] Forrester et al., (1987); [6] Vogelstein et al., (1988); [7] Grunewald et al., (1989); [8] Smit et al., (1988); [9] Almoguera et al., (1988); [10] Neri et al., (1988); [11] Rodenhuis et al., (1986); [12] Janssen et al., (1987b); [13] Bos et al., (1987b); [14] Farr et al., (1988); [15] Bos et al., (1985); [16] Needleman et al., (1986); [17] Browett et al., (1989); [18] Hirai et al., (1987); [19] Liu et al., (1987); [20] Rochlitz et al., (1989); [21] Kraus et al., (1984); [22] Sakamoto et al., (1986); [23] Hollstein et al., (1988); [24] Fujita et al., (1985); [25] Fujita et al., (1988); [26] Tsuda et al., (1989); [27] Gu et al., (1986); [28] Tada et al., (1990); [29] van't Veer et al., (1989); [30] Leon et al., (1988); [31] Raybaud et al., (1988); [32] Lemoine et al., (1989).

influence the incidence of ras activation among various target organs. Another explanation for tissue-specific activation was based on the linkage between the growth factor receptors and p21, which indicates that hormonal status or the differentiation state of the target tissue is important in determining the specificity (Balmain and Brown, 1988). Neri et. al. (1988) suggested that important differences in the frequency of ras gene mutation may exist between tumors derived from different tissues or between histopathological subtypes of tumors derived from the same tissues. However, Bos (1988) concluded in his review that there is no strict specificity for activation of particular ras genes in tumors from particular tissue. In many malignancies any of the three ras genes can be activated, particularly in all stages of human thyroid tumorigenesis (Lemoine et al., 1989) and human embryonic rhabdomyosarcomas showing features of skeletal muscle differentiation (Stratton et al., 1989).

A considerable number of activated ras genes have been detected in DNA from lung, colon, and pancreatic carcinomas (Pulciani et al., 1982a). In most cases the K-ras gene was found to be activated at a high incidence rate. In a recent animal study with individuals chronically exposed by inhalation to tetranitromethane, which mimics human exposure to a chemical in the workplace, all of the rat and mouse lung tumors tested had a K-ras oncogene mutation in the second base of codon 12 (Stowers et al., 1987). In other animal studies causing tumor formation in lung tissue, K-ras oncogene mutations were consistently involved (Belinsky et al., 1989; You et al., 1989). Mutational K-ras activation in these tissues was suggested to be an important early event in the pathogenesis of adenocarcinoma

(Rodenhuis et al., 1987; Burmer et al., 1989; Bos et al., 1987a; Burmer and Loeb, 1989; Forrester et al., 1987). Concurrent double mutations observed in the same tumor, however, suggested that some mutations do arise after the initial transforming mutation event (Grunewald et al., 1989).

The ras gene activation in hematopoietic malignancies, except chronic myeloid leukemia (CML), predominantly involves the N-ras gene at a substantial frequency (20-50%) (Neri et al., 1988; Janssen et al., 1987b; Bos et al., 1987b; Farr et al., 1988; Bos et al., 1985; Brown et al., 1984). The H-ras activation is rare in these malignancies (Browett et al., 1989). A high incidence of N-ras activation by the chemical carcinogen N-methyl-N-nitrosourea (MNU) was also reported to occur in mouse lymphomas (Guerrero et al., 1984), which suggests that N-ras, rather than the other ras genes, may be critically involved in the control of proliferation and differentiation of cells in the haematopoietic lineage. This observation may also indicate that environmental chemical agents may be responsible for the induction of the mutations (Bos, 1989).

Ras gene activation is not, however, believed to be involved in all human tumors. A very low percentage or none of urinary tract (Fujita et al., 1985), breast (Rochlitz et al., 1989; Kraus et al., 1984) and stomach carcinomas (Sakamoto et al., 1986) examined contained ras gene mutations, which suggested that tumorigenesis in different tissues have different pathogenic mechanisms with different frequencies of ras gene mutations (Bos et al., 1987a).

The very limited studies of human hepatocellular carcinoma (HCC) to date have indicated that point mutations of ras genes were very rare

or not found at all, suggesting that ras mutation may not be involved in all human HCC (Tsuda et al., 1989), and that a new transforming gene may be involved (Ochiya et al., 1986). However, cholangiocarcinomas arising from the epithelium of the intrahepatic bile duct showed a high frequency of K-ras mutation (Tada et al., 1990). The observed simultaneous mutation of N-ras and c-myc suggested their cooperation for the malignant phenotypic alteration in some human HCC (Gu et al., 1986).

Particularly in the cases of low mutation incidence in liver and stomach primary tumors, the activation of ras genes is argued not to be an obligatory event and not the only event in tumor formation (Bos, 1989). Other unknown activating event(s) may have the same ultimate effect (Ochiya et al., 1986; Sakamoto et al., 1986). But when a ras gene mutation occurs it can contribute to both early and advanced stages of human carcinogenesis (Bos, 1988). Cells with a mutated ras gene and thus the type of mutation are selected. This selection may be cell type specific and influence the tissue-specific mutation spectrum (Bos, 1989).

In an increasing number of malignancies it has been shown that one allele of a recessive "tumor suppressor" has been deleted and in this way chromosomal loss may contribute to the development of bladder (Fearon et al., 1985) and breast carcinomas (Theillet et al., 1986). A tumor-suppressor gene located on the same chromosome as H-ras, chromosome 11, can suppress the tumorigenic phenotype (Saxon et al., 1986). The loss of an H-ras allele as well as the tumor-suppressor gene correlates with the most aggressive primary carcinomas. The

incidence of mutationally activated H-ras alone is quite low in bladder and breast carcinoma.

As shown in Table 2, activated ras genes are found in most, if not all, types of solid tumors but the incidence rates vary considerably (Bos, 1988). No apparent correlation was found between the presence of mutant oncogenes and the degree of invasiveness of the tumors (Forrester et al., 1987).

Coexistence of normal and mutated alleles.

Many human primary tumors contain an activated ras gene allele as well as the corresponding normal allele (Stowers et al., 1987; Neri et al., 1988; Rochlitz et al., 1989). Since a single copy of the mutated ras oncogene is sufficient to impart oncogenicity even expressed under a weak transcriptional promoter, the mutated allele behaves in a dominant fashion (Tabin and Weinberg, 1985). It was reported that during progression of a tumor the ratio between the normal and the mutant allele in the tumor cell population shifts towards the mutated allele (Bos et al., 1987a; Almoguera et al., 1988). That the ras mutation has been seen to be acquired by a subpopulation of malignant cells (Neri et al., 1988) suggests a selective growth advantage (Rodenhuis et al., 1987).

Alternatively over-expression of normal p21 can induce the complete cellular transformation, but apparently no increase in mutant p21 expression is required (McKay et al., 1986). In a gastric carcinoma, for instance, a single mutated K-ras allele was found as well as a 30-50 fold amplified normal allele (Bos et al., 1986). The elevated expression of normal ras protein can play at least a permissive role in the induction of tumors (Spandidos and Wilkie, 1984; Cohen and

Levinson, 1988). Therefore, it seems likely that the ratio between normal and activated alleles or, eventually, a concentration of the mutated gene product is important for the phenotype of a tumor cell (Bos, 1988).

In normal tissue of a patient with activated K-ras oncogene in lung carcinoma, ras gene mutations were not found (Santos et al., 1984). Therefore, significant numbers of non-neoplastic cells, usually mixed in solid tumor specimens, would dilute any positive signal in the transfection assay (Verlaan-de Vries et al., 1986) and show the apparent heterozygosity. Or selective pressure may actually favor the loss of the ras oncogene during tumor development and the mutated allele is selectively lost (Neri et al., 1988). Sometimes as an experimental error the DNA from primary human tumors are slightly degraded during surgery and subsequent tissue handling (Bos et al., 1987a), perhaps obscuring the presence of activated ras.

The temporal relationship between the ras gene activation event and the disease process; i.e., whether activation occurs before, at the time of, or after the establishment of the malignant clone is an intriguing question. Barbacid (1986) strongly suggested in his review that ras gene activation plays a causative role in the development of neoplasia. The notion that mutant ras genes are involved in the initiation of tumorigenesis has been fostered by several studies; (1) Adenoma-Carcinoma Concept : K-ras mutations have been uncovered in premalignant tissue adjacent to human colon carcinomas (Bos et al., 1987a; Forrester et al., 1987; Burmer and Loeb, 1989), in very small lung tumors that had not metastasized (Rodenhuis et al., 1987) and in the preleukaemic state of a patient who progressed to acute leukaemia

(Liu et al., 1987). (2) Chemical Carcinogenesis : Based on the fact that the transforming allele was not present in the germ line but was generated somatically (Gambke et al., 1985; Kraus et al., 1984; Liu et al., 1987), animal studies using chemical carcinogenesis showed reproducible and high incidences of ras gene mutations (see Table 3). A single dose of chemical having a short half-life and the induced predictable base changes by its intrinsic chemical reactivity indicates that the direct reaction between DNA and chemical intermediates initiate the ras gene mutation (Zarbl et al., 1985). (3) Ubiquitous Presence : Particularly in exocrine pancreatic carcinomas, mutant K-ras alleles were discovered at a high incidence, in histologically different tumor regions, and in both primary tumors and their corresponding metastases (Almoguera et al., 1988).

Apparently, activation of a ras gene is an important step in tumor progression which predisposes a particular cell to become a tumor (Bos, 1988). However, this mutation already exists in most of the premalignant lesions (Balmain et al., 1984) and a subsequent secondary (or complementary) genetic event may be required for carcinogenesis to progress (Klein and Klein, 1984; Bos, 1988). Alternatively, certain epigenetic factors such as tumor promoters may overcome the normal control state (Barbacid, 1986). For instance, longitudinal study at several different stages of colorectal tumor development suggests mutant ras genes are actively involved and continuously contribute to the process of tumor progression including the metastatic process (Vogelstein et al., 1988). In addition the simultaneous presence of two differently mutated ras gene alleles in the same tumor suggests that the activating event can occur at several

points in the process of tumor formation (Rodenhuis et al., 1987; Grunewald et al., 1989).

By contrast, ras activation is involved only in the final stages of tumor progression in breast cancer where activating ras mutations are rarely associated with tumor initiation or maintenance but occur at the stage of metastatic progression (Rochlitz et al., 1989). Similar conclusion was reached by Albino et al. (1984).

Double mutations in one tumor sample.

Concurrent activation of two ras gene sequences or two different positions by point mutations is known to occur in human carcinoma cell lines (see Table 2). The simultaneous presence of activated N-ras and K-ras oncogenes in the same tumor DNA has been reported in human acute myelocytic leukemia (AML) cell line (Janssen et al., 1987a) and in human colon carcinoma (Forrester et al., 1987). Two mutations detected in both codons 12 and 13 (Neri et al., 1988) or in both codons 12 and 61 (Farr et al., 1988) of an N-ras gene were present in two distinct alleles of the same AML tumor.

Probably there are more than two complementation groups among the known oncogenes (Klein and Klein, 1984) and an activated ras gene alone cannot sustain the total neoplastic transformation (Leon et al., 1988; Land et al., 1983). In the multistep process of carcinogenesis, different oncogenes are sequentially, or concurrently, activated and the interactions of oncogene products determine the cell's phenotype (Husain et al., 1989; Levinson, 1987). Like the cooperation between ras and myc oncogenes (Land et al., 1983) one activated oncogene is complemented by other altered member of oncogenes (Weinberg, 1989). Alternatively, a concurrent point

mutation in codon 12 and 61 could significantly increase the transforming ability of respective proteins as compared to the ras protein altered solely in position 12 or 61 (Farr et al., 1988). Interestingly, triple mutation in a single allele of rat N-ras gene was reported at codon 8, 13 and 18, which are the unusual mutation sites of the ras gene family (McMahon et al., 1990b).

Target sequence of carcinogens.

It is generally accepted that many carcinogens are mutagens. The mixed function oxidases (MFO), located in the endoplasmic reticulum of most cells, generally metabolize the carcinogens to yield activated epoxides. This membrane bound system requires cytochrome P-450 and has greatest activity in liver (see Wade and Dharwadkar, 1986). A significant number of chemical carcinogens are known to react with DNA bases to form adducts, some of which are highly mutagenic due to their miscoding properties or the limited fidelity of repair polymerases (reviewed in Singer and Kusmierek, 1982). Topal (1988) suggested the "positional mutation bias" in his review that some DNA sequence may present particular base positions as easy targets to the mutagen and/or keep mutagenic lesions from repair activities so that the lesions persist.

As a target sequence of carcinogens, the ras gene mutations are often involved in chemically-induced tumors. The reproducible activation of ras oncogenes in carcinogen-induced tumors has made it possible to correlate their activating mutations with the known mutagenic effects of certain carcinogens (Table 3). Recently, Guerrero and Pellicer (1987) reviewed the activation of ras oncogenes in carcinogen-induced animal tumors.

Table 3. Point mutations in ras genes by chemical carcinogens.

Mutagen	Species & Tissue	*ras* Gene / Sites	Base Change	Incidence Rate (%)	Ref.
AFB1	rat liver	K12/1,2	G->A, G->T	2/11 (18)	[1]
AFB1	rat liver	K12/2 N	G->A -	1/4 (25) 3/4 4/4 (100)	[2]
AFB1	rat liver	K12/1 K12/2 N13	G->T G->A G->A, G->T	1/8 2/8 5/5 8/13 (62)	[3]
AFB1	E. coli	-	G->T G->A	169/187 (89) 12/187(6)	[4]
AFB1	E. coli	-	G->T G->A	66/126 (52) 54/126 (43)	[5]
Polycyclic Hydrocarbons					
DMBA	mouse skin	H61/2	A->T	33/37 (90)	[6]
DMBA	mouse skin	H61/2	A->T	3/4 (75)	[7]
DMBA	rabbit skin	H61/2	A->T	- (60)	[8]
MCA	mouse thymomas	K	-	- (83)	[9]
BaP	mouse lung (strain A)	K12/1 K12/2 K12/2	G->T G->T G->A	8/13 1/13 4/13 13/13 (100)	[10]
BaPDE	CHO cell line	-	G->T	10/14 (71)	[11]
BaPDE	human fibroblast cell line	H12/2	G->T	2/11 (18)	[12]
BaPDE	monkey kidney cell line	-	G->T G->C G->A	- (34) - (34) - (22)	[13]
BaPDE	E. coli	-	G->T	62/96 (65)	[14]

Table 3. Point mutations in ras genes by chemical carcinogens (cont.).

Mutagen	Species & Tissue	*ras* Gene / Sites	Base Change	Incidence Rate (%)	Ref.
HO-AAF	mouse liver (B6C3F1)	H61/1	C->A	7/7 (100)	[15]
AF	E. coli	-	G->T	14/17 (82)	[16]
AcO-TFA -AF	human kidney cell line	-	G->T G->A G->C	32/49 (65) 9/49 (18) 8/49 (16)	[17]
DNP	rat fibrosarcoma	K12/1	G->T	1/7 (14)	[18]
Spontaneous					
	mouse liver (B6C3F1)	H61/1 H61/2 H61/2	C->A A->G A->T	9/27 3/27 3/27 15/27 (56)	[19]
	mouse liver (B6C3F1)	H61/1 H61/2 H61/2	C->A A->G A->T	19/50 11/50 2/50 32/50 (64)	[20]
	mouse lung (strain A)	K12/2 K12/2 K12/1 K61/2 K61/3	G->A G->T G->C A->G A->T	3/10 2/10 1/10 2/10 1/10 9/10 (90)	[10]
Nitroso Compounds					
1) Nitrosamines					
DEN	mouse liver (B6C3F1)	H61/1 H61/2 H61/2	C->A A->G A->T	7/33 3/33 4/33 14/33 (42)	[21]

Table 3. Point mutations in ras genes by chemical carcinogens (cont.).

Mutagen	Species & Tissue	*ras* Gene / Sites	Base Change	Incidence Rate (%)	Ref.
DMN-OAc	rat liver				[22]
	adenoma	K12/2	G->A	2/137	
	carcinoma	K12/2	G->A	7/93	
	cholangioma	K12/2	G->A	2/10	
				11/240 (5)	
NMAB	E. coli	-	G->A	169/171 (99)	[23]
MBNA	rat esophagus	H12/2	G->A	18/18 (100)	[24]
2) Nitrosoureas					
MNU	rat mammary	H12/2	G->A	9/9 (100)	[25]
MNU	rat mammary	H12/2	G->A	36/36 (100)	[26]
MNU	mouse lung (strain A)	K12/2	G->A	15/15 (100)	[10]
ENU	E. coli	-	A->C	12/29 (46)	[27]
3) Nitrosoguanidine					
MNNG	E. coli	-	G->A	164/167 (98)	[28]
MNNG	E. coli	-	G->A	121/123 (98)	[29]
MNNG	E. coli	-	G->A	34/37 (92)	[30]
PNNG	E. coli	-	G->A	65/89 (73)	[29]
Carbamate Compounds					
Urethane	mouse skin	H61/2	A->T	13/14 (93)	[31]
vinyl carbamate	mouse liver (B6C3F1)	H61/2	A->T	6/7	[15]
		H61/2	A->G	1/7	
				7/7 (100)	

Table 3. Point mutations in ras genes by chemical carcinogens (cont.).

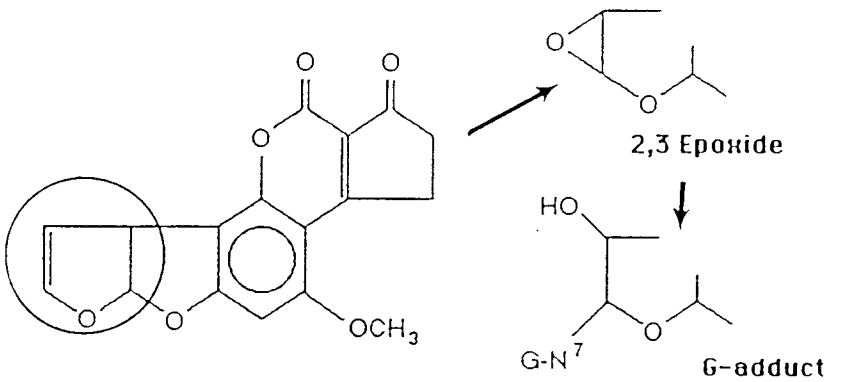
Mutagen	Species & Tissue	*ras* Gene / Sites	Base Change	Incidence Rate (%)	Ref.
ethyl carbamate	mouse lung (strain A)	K61/1 K61/2 K12/2	A->T A->G A->G	7/10 2/10 1/10 10/10 (100)	[10]

CHO cell line : Chinese Hamster Ovary cell line.
MCA : 3-methylcholanthrene; HO-AAF : N-hydroxy-2-acetylaminofluorene; AF : N-2-aminofluorene; AcO-TFA-AF : N-acetoxy-N-trifluoroacetyl-2-aminofluorene; DNP : 1,8-dinitropyrene; DEN : N-nitrosodiethylamine; DMN-OAc : methyl(acetoxymethyl)nitrosamine; NMAB : N-nitroso-N-methyl-N-acetoxybenzylamine; MBNA : methylbenzyl-nitrosamine; ENU : N-ethyl-N-nitrosourea; PNNG : N-propyl-N'-nitro-N-nitrosoguanidine.

Ref.

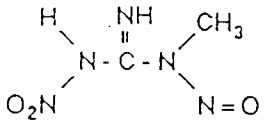
[1] McMahon et al., (1987); [2] Sinha et al., (1988); [3] McMahon et al., (1990b); [4] Foster et al., (1983); [5] Sambamurti et al., (1988); [6] Quintanilla et al., (1986); [7] Bizub et al., (1986); [8] Leon et al., (1988); [9] Eva and Trimmer, (1986); [10] You et al., (1989); [11] Carothers and Grumberger, (1990); [12] Stevens et al., (1988); [13] Roilides et al., (1988); [14] Eisenstadt et al., (1982); [15] Wiseman et al., (1986); [16] Bichara and Fuchs, (1985); [17] Mah et al., (1989); [18] Tahira et al., (1986); [19] Reynolds et al., (1986 and 1987); [20] Fox et al., (1990); [21] Stowers et al., (1988); [22] Watatani et al., (1989); [23] Horsfall and Glickman, (1988); [24] Wang et al., (1990); [25] Sukumar et al., (1983); [26] Zarbl et al., (1985); [27] Eckert et al., (1989); [28] Burns et al., (1987); [29] van der Vliet et al., (1989); [30] Richardson et al., (1987); [31] Bonham et al., (1989).

Direct evidence that exposure to chemical carcinogens can cause activation of ras genes was provided by the demonstration that in vitro modification of a plasmid containing the normal cellular H-ras proto-oncogene with ultimate carcinogens generates transforming oncogenes when the modified DNA is transfected into NIH3T3 cells (Eisenstadt et al., 1982; Marshall et al., 1984; Vousden et al., 1986; Ireland et al., 1988). However, spontaneous point mutations occur after the transfection of this vector into the NIH 3T3 cell line. This results from the DNA replication infidelity during repair of the transfected DNA damaged by cellular nucleases (Hauser et al., 1987). The findings that certain chemicals induced somatic mutations of ras oncogenes in several experimental animal systems provide evidence that direct interaction of these chemicals with DNA may be crucial to the initiation of carcinogenesis (Bos, 1988). The type of mutation observed has been shown to correlate strongly with the known chemical behavior of the carcinogen used (Bizub et al., 1986; Sukumar et al., 1983; Wiseman et al., 1986; Quintanilla et al., 1986). In a recent review, Basu and Essigmann (1988) pointed out the necessity of novel synthetic approaches that will use site-specifically modified oligonucleotides as probes for the structural and biological effects of chemical DNA adducts. The approach for establishing the relationship between adduct structure and biological effects is to situate individual adducts or their intermediate products at defined sites in genomes, allow enzymatic processing to occur in vivo, and then assess the resulting genetic changes. The structures of three carcinogens involved in this project are illustrated in Figure 1.



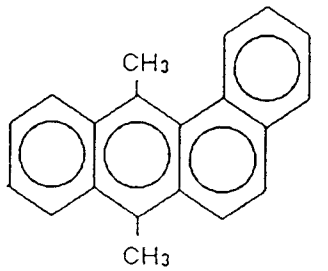
Aflatoxin B1

(AFB1)



N-methyl-N'-nitro-N-nitrosoguanidine

(MNNG)



7,12 - Dimethylbenzanthracene

(DMBA)

Figure 1. Structure of chemical carcinogens.

Aflatoxin B1 (AFB1)

AFB1 is a metabolic product of the fungal genus Aspergillus, and a potent hepatocarcinogen in the most susceptible animals as an alkylating mutagen. Modification of DNA by AFB1 requires metabolic activation by MFO producing AFB1-8,9-epoxide. This reactive electrophile attacks almost exclusively the N7 atom of guanine in the double-stranded DNA molecule forming an bulky N7-guanyl AFB1 adduct (Refolo et al., 1985; Misra et al., 1983; Groopman et al., 1981; Croy et al., 1978; Croy and Wogan, 1981).

Planar aromatic molecules such as anthracenes and benzopyrenes present in soot are able to intercalate between the stacked bases of the DNA double helix and bring about its partial unwinding. Intercalation of activated aflatoxins may be a significant transition state preceding AFB1-DNA covalent bond formation (Yu et al., 1990) because the overall rate of adduct formation with single-stranded DNA is greatly diminished (Marien et al., 1987) and the intercalating dye, ethidium bromide, inhibits the reaction of AFB1 with DNA (Misra et al., 1983).

A 10-fold variation in the frequency of AFB1-8,9-epoxide attack at particular guanyl sites was observed, and was suggested to be dependent on the flanking nucleotides of the site. A strong site for AFB1 modification is considered to be a guanine residue that has one or more adjacent G:C base pairs (Refolo et al., 1985). The observed in vitro sequence specificity is likely to occur in vivo (Muench et al., 1983; Marien et al., 1987).

Inhibition of DNA replication by AFB1 is directly attributable to N7-guanyl-AFB1 adducts or their secondary reaction products, such as

imidazole ring-opened formamidopyrimidine (FAPY)-aflatoxin (Croy and Wogan, 1981). The primary N7-guanine lesion has an in vitro half-life of only 8 hrs at 37 ^{o}C at pH 7.3 (Wang and Cerutti, 1980). It has been postulated, therefore, that this primary lesion may be lost through three major pathways (Wang and Cerutti, 1980): (1) simple removal of aflatoxin dihydrodiol, leaving an intact DNA guanine behind (detoxification); (2) guanine imidazole ring opening to yield the more stable FAPY lesions (Sambamurti et al., 1988); and (3) loss of guanine to yield an apurinic (AP) site (Groopman et al., 1981; Sinha et al., 1988). The FAPY-aflatoxin adduct has been detected in human hepatocarcinoma DNA (Lee et al., 1989), and has been shown to be nearly equally mutagenic as the primary adduct (Sambamurti et al., 1988).

Insight into the effect of damage to genetic information requires analysis of the interactions of cellular replication and repair processes with specific lesions in DNA. Induction of error-prone repair following replication block may be a major means by which bulky adducts lead to mutation (Schaaper et al., 1982). The basic concept for this mutational mechanism is that when the normal progress of DNA replication is stalled at bulky adducts (Reardon et al., 1990), a set of new proteins is induced which allows DNA synthesis to proceed past the blocking lesion, resulting in increased survival and markedly increased mutagenesis (Figure 2). The stringent fidelity requirements of the normal replicating complexes may not allow the stable incorporation of a nucleotide opposite the lesion. Under SOS conditions, however, these stringent requirements become relaxed. Blockage of DNA replication followed by SOS-induction in bacteria

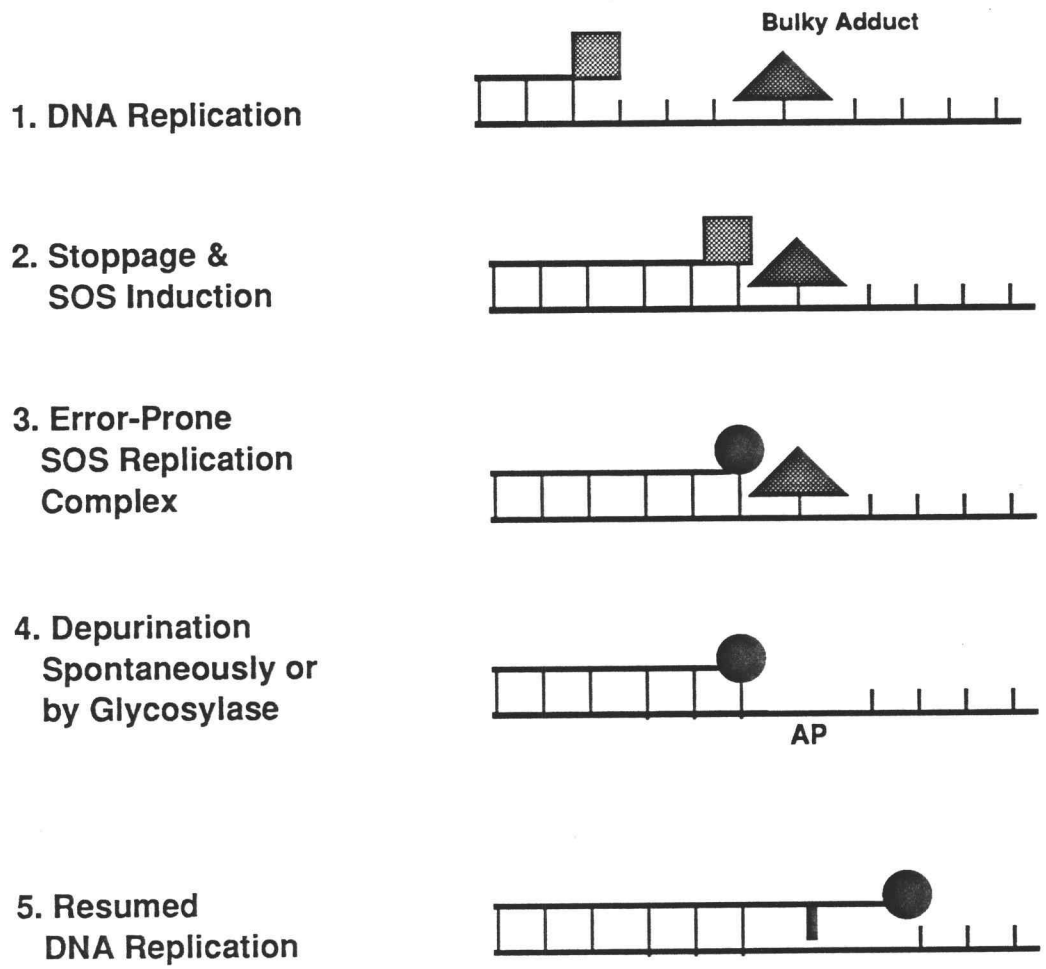


Figure 2. Mutagenesis by chemical carcinogens via depurination.

cells seems to take place in eukaryotic cells (Schaaper and Loeb, 1981).

Modification of bases in DNA also leads to enhanced rates of depurination (Singer and Kusmierek, 1982), as exemplified by the potent mutagen AFB1 (Schaaper and Loeb, 1981). The primary N7-guanyl AFB1 adduct is expected to weaken the N-glycoside bond of the nucleotide such that elevated pH and temperature would result in depurination, generating AP sites (Muench et al., 1983). Their depurination, either spontaneously or enzymatically by specific N-glycosylases removing damaged or unusual bases from the DNA, could provide a much better substrate for error-prone bypass than does the original blocking lesion (Schaaper et al., 1982).

AP sites are noninstructive, that is, devoid of information for Watson-Crick base pairing. In the absence of template information, the mutational specificity of AP sites may be depend on the properties of polymerase and its affinity for particular deoxyribonucleoside triphosphate (Strauss et al., 1982; Schaaper et al., 1983). Their enhanced mutagenicity is largely dependent on induction of the error-prone SOS response (Sambamurti et al., 1988). Site-specifically modified oligonucleotides resembling the product of bulky carcinogen adduct and a guanine breakdown product have been used for in vitro polymerization experiments, or in an E. coli system involving a damage-induced SOS response (O'Connor and Stohrer, 1985). Results showed that there is an overwhelming and unexpected specificity for the insertion of adenine nucleotides at such sites. This specificity correlated well with those which have AP sites as intermediates because there is a strong preference for insertion of dAMP at AP sites,

leading to distinctive G to T or A to T transversions (Kunkel, 1984; Sagher and Strauss, 1983). Sequence-level in vitro studies were carried out for the specificity of mutagenesis by activated aflatoxin (Foster et al., 1983; Sambamurti et al., 1988). Using in vitro modification of the lac gene followed by transfection into appropriate E. coli cells, resulting forward mutations in the lac gene segment were identified by DNA sequencing. The results showed that the major base-substitutions induced by AFB1 was a G to T transversion. G to A transitions were reported as well.

Other carcinogens causing bulky DNA adducts, benzo[a]pyrene-diol-epoxide(BaPDE) and N-acetoxy-2-acetylaminofluorene (AAAF), also specifically induce G to T transversions (Foster et al., 1983; Eisenstadt et al., 1982). Their similar mutagenic specificities may simply derive from their common ability to create AP sites in DNA and form the preferential insertion of adenine opposite such lesions, although the sites that are best induced are not the same (Drinkwater et al., 1980).

Activation in the c-K-ras gene, not in H-ras and N-ras, has been detected in liver tumors of rats chronically exposed to AFB1 (McMahon et al., 1986) and identified as both G to A and G to T base changes in codon 12 (McMahon et al., 1987). Sinha et al. (1988) reported that activated ras oncogenes were detected in all AFB1-induced rat liver tumors, and N-ras activation (3 out of 4 tumors) is most frequent as well as K-ras activation being found. Recently, McMahon et al. (1990b) presented an "unexpected" report that N-ras mutation is involved in all AFB1-induced rat liver tumors (5 of 5) as well as in all normal rat liver (3 of 3).

N-methyl-N'-nitro-N-nitrosoguanidene (MNNG)

MNNG is a direct-acting methylating agent that specifically (>90%) induces G to A transitions due to the methylation of the O6 position of guanine and the predicted mispairing property of O^6-methylguanine (O^6-MeG) adducts (Kohalmi and Kunz, 1988; Burns et al., 1987). Consistent with methylating agents whose principal mutagenic product is O6-methylguanine lesion, N-methyl-N-nitrosourea (MNU) (Zarbl et al., 1985) and N-nitroso-N-methyl-N-å-acetoxybenzylamine (NMAB) (Horsfall and Glickman, 1988) cause the G to A transition mutation preferentially.

N-nitroso compounds such as MNNG and MNU have a common alkylating intermediate, the methyl diazonium ion, following metabolic activation (Horsfall et al., 1990). They react primarily with oxygen (Singer and Kusmierek, 1982) and may have the same site specificity in mammalian cells as in E. coli (Burns et al., 1987).

The molecular electrostatic potential of the O6 position of guanine can be influenced by the adjacent base pairs and the accessibility of a particular O6 position to a reactive methylating species would be expected to be influenced by the local DNA helix geometry. In addition, the 5' nucleotide may influence the degree to which a thymine residue is incorporated opposite an O^6-MeG, or the extent to which the 3' to 5' editing function of the polymerase is able to remove the mismatched base. The relative reactivity of the O6 position of guanine is increased substantially when a purine occupies the 5' flanking position (Richardson et al., 1987). The 5' flanking sequence appears to influence the mutability of SN1-type direct-acting alkylating agents (Dolan et al., 1988). G to A transitions were found to be six

times more likely to occur at G:C base pairs at which the guanine residues were flanked (5') by a purine, particularly guanine, than at those preceded by a pyrimidine (Burns et al., 1987). MNU, for example, exclusively activates the H-ras proto-oncogene of rat by a G to A transition at the second guanine residue in codon 12 (GGC to GAC) (Zarbl et al., 1985), even though the same event at the first guanine in codon 12 also produces an activated phenotype (Seeburg et al., 1984).

Adaptation of E. coli involves the induction of the ada gene which codes for O^6-MeG-methyltransferase, which repairs alkylating agent-induced DNA lesions (Richardson et al., 1987). Evidence for an adaptive response in mammals which paralleled the E. coli response was identified in mammals liver tissues and was more ambiguous (Yarosh, 1985).

Most mutations induced by MNNG are the result of the altered templating property of the O^6-MeG lesion. This major premutagenic lesion, is able to mispair with thymine yielding a G to A transition at the subsequent round of replication (Richardson et al., 1987; Burns et al., 1987; Basu and Essigmann, 1988), but this mispairing is competitive with the normal incorporation of dCMP (Abbott and Saffhill, 1979).

An activated c-H-ras gene was identified in 10T1/2 cells transformed by exposure of a parental, wild-type population to MNNG (Smith and Grisham, 1987). The specificity of MNNG and MNU for activation of ras proto-oncogenes only by mutation of codon 12 may be explained by the fact that the major mutation induced in codon 61 by methylating agents results in a termination codon which would be

biologically inactive (Vousden et al., 1986). MNU seems to be directly responsible for the activation of H-ras1 because of its highly labile nature under physiological conditions (Zarbl et al., 1985).

The case of A:T to G:C transitions might be explained by the incorporation of O6-methyldeoxyguanosine (MeG) triphosphate from the alkylated nucleotide pools, opposite thymidine residues in the template strand (Singer and Kusmierek, 1982).

7,12-dimethylbenz[a]anthracene (DMBA)

While most carcinogen metabolites react preferentially with guanine residues, the in vivo metabolite of DMBA generated in mouse skin reacts almost exclusively with adenine residues (Cheng et al., 1988). From the cultured mouse embryo cells exposed to 7,12-DMBA, substantial amounts of adenosine adducts were formed (Dipple et al., 1983). Over 90% of mouse skin tumors initiated with DMBA have a specific A to T transversion at the second nucleotide of codon 61 of H-ras gene (Quintanilla et al., 1986; Balmain and Pragnell, 1983). The frequency of this mutation was dependent on the initiating agent used, but not on the promoter, suggesting that the mutation occurs at the time of initiation. The same type of mutation, A to T transversion at the second nucleotide of codon 61 of H-ras, was reported in human melanoma tissue (Sekiya et al., 1984) as well as in mice skin tumors induced by nitroso-compound (Anderson et al., 1989).

Polymerase chain reaction (PCR).

PCR is an in vitro DNA amplification procedure and circumvents to a great extent the need for subcloning and traditional biological plasmid amplification. A schematic diagram for the PCR principle is illustrated in Figure 3. The method is, like the natural DNA

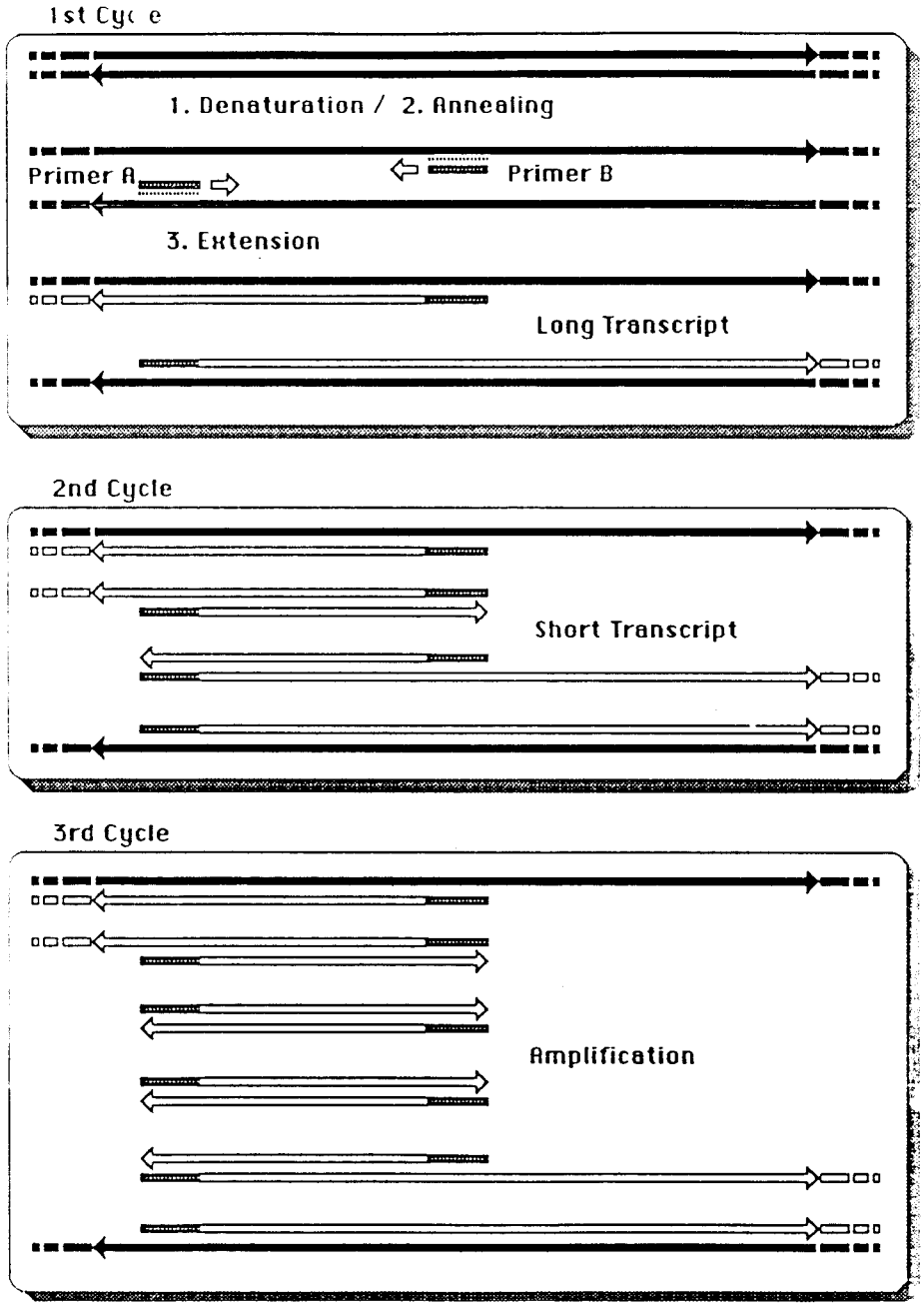


Figure 3. Schematic diagram for the PCR principle.

replication process, based on the repetition of a set of three steps : denaturation of templates, annealing of primers and primer extension (Oste, 1988). The primers flanking the region to be amplified are hybridized to different strands of a DNA template in a relative orientation such that their extension products overlap (Mullis et al., 1986). The amplified target sequence (or short transcript) becomes overwhelmingly dominant by its exponential accumulation. However, the amount of long transcript will increase only arithmetically because the quantity of original template remains constant. Thermostable DNA polymerase from the bacterium Thermus aquaticus (Taq) retains its activity after heat denaturation of the DNA, which simplifies the PCR reaction. In addition its higher optimum temperature (70 - 75 ^{o}C) significantly increases the specificity, yield, and length of targets to be amplified. Recently, PCR has been widely used, particularly in the analysis of ras gene activation in cancer.

Total precision of amplification parameters remained elusive. As a result, the concentration of each component, the temperature and time for given cycle segments are all subject to minute variations (Erlich, 1989). PCR primers are typically 20-25 bases in length having an average GC content (around 50%) and random base distribution avoiding substantial secondary structure. They have different sequences and are not complementary to each other, particularly at the 3' ends. Primer dimer concatenation is an amplification artifact often observed, especially when many cycles of amplification are performed on a sample containing very few initial copies of template. Sequences not complementary to the template can be added to the 5' end of the primers. These noncomplementary

5' extension sequences become incorporated into the double stranded PCR product and provide a means of introducing restriction sites (Scharf et al., 1986) or regulatory elements (e.g., promoters) (Stoflet et al., 1988) at the ends of the amplified target sequence without decrease of the amplification efficiency. In the later cycles of amplification these primers anneal primarily to the amplified products rather than to the original genomic sequences, and are therefore fully complementary. Degenerative oligonucleotides primers, a mixture of oligonucleotides equal in size but containing various sequences, allowed significant amplification relative to unique oligonucleotides for PCR (Mack and Sninsky, 1988) in the case where the exact target sequence is unknown. For highly degenerate primers, it is preferable that the most unambiguous sequence be situated at the 3'-end of the primer (Ehlen and Dubeau. 1989).

The concentration of $MgCl_2$ can have a profound effect on the specificity and yield of an amplification by Taq DNA polymerase and should be adjusted in parallel with high concentrations of sample DNA and dNTP's usually containing EDTA chelator. Generally, excess Mg^{+2} will result in the accumulation of non-specific amplification products and insufficient Mg^{+2} will reduce the yield.

Boiling and quenching on ice of the high molecular weight target DNA prior to PCR greatly enhances the amplification process, because full denaturation of the chromosomal DNA in the first few critical rounds of amplification determines the quantity of the final product. Insufficient heating during the denaturation step is one of the most common causes of failure in a manual PCR reaction. In addition, it

would inactivate proteases in DNA samples, if any, and prevent degradation of the Taq polymerase.

Because the primers are present in large excess over the DNA template, the formation of the primer-template complex will be favored over the reassociation of the two DNA strands when the temperature is lowered. Higher annealing temperatures generally result in much more specific product with higher yield, because poorly matched primer-template hybrids dissociate and there are fewer nonspecific extension products to compete for the polymerase.

The exponential accumulation of PCR amplification products is not an unlimited process. Eventually, a level of amplification is reached where more primer-template substrate has accumulated than the amount of enzyme present is capable of completely extending in the allotted time (Saiki et al., 1988). PCR can amplify DNA segments by million fold, and potentially as high as billion fold. The fact that the PCR technique allows the specific amplification of discrete fragments of DNA makes it much easier to detect nucleic acid fragments (Oste, 1988), reducing the nucleic acid complexity by nearly the same factor. Therefore, specific hybridization with much shorter oligonucleotide probes than is typically feasible and decreased stringencies of hybridization are possible (Mack and Sninsky, 1988).

PCR is also directly coupled with existing DNA sequencing methods because the amplified fragment typically becomes the most prominent species in the sample. This eliminates the need for cloning and purifying the DNA sample to be sequenced (Engelke et al., 1988; Wong et al., 1987). By using unequal molar amounts of the two amplification primers, it is possible in a single step to amplify a single-copy gene

and produce an excess of single-stranded DNA of a chosen strand for direct sequencing or for use as a hybridization probe (Gyllensten and Erlich, 1988).

The misincorporation rate of PCR DNA synthesis is extremely low (1 for each 9,000 nucleotides polymerized) (Tindall and Kunkel, 1988). Sequence analysis of directly cloned PCR products allowed the quantitative evaluation of its specificity and fidelity (Scharf et al., 1986). During PCR many types of DNA damage such as baseless sites, inter-and intramolecular cross-links would slow down or completely block the enzyme polymerization. Thus damaged DNA molecules in the amplification reaction can be expected either not to be replicated at all or to be at a replicative disadvantage, so that intact molecules will amplify preferentially (Paabo and Wilson, 1988).

The extremely high sensitivity of the PCR technique easily results in amplification of trace amounts of contaminants. "Carryover" of contaminating DNA is a most serious problem in PCR, and usually occurs by aerosolization of amplified samples when tightly capped tubes are once opened. Pipetting devices which do not physically separate tips from the material being pipetted is another source of "carryover" problem because even a single copy of DNA carrying known "control" sequences can be inadvertently amplified a million fold. It is strongly recommended to set up PCR reaction mixes in areas separate from PCR product handling and not to open the capped tubes being amplified until PCR is complete (Lo et al., 1988; Kwok and Higuchi, 1989). Each room should be completely equipped with its own set of appropriate pipetting and storage devices. Dedicated pipettes, vessels and solutions for DNA preparation, reaction mixing and sample

analysis will minimize across contamination. Nothing should be exchanged between rooms. It was recommend to use positive displacement pipetting devices which have disposable tips and plungers when pipetting the DNA. Contamination with target DNA is much less likely since target DNA, as opposed to amplified DNA, is generally of high molecular weight. As a strategy for detection of contamination, it was suggested that a negative control consisting of all PCR reagents minus the DNA template should be included in every amplification (Schochetman et al., 1988).

Detection methods for point mutations.

Gene transfer assays and Southern blot hybridization have been major experimental approaches to detect and characterize the genetic variation in human oncogenes (Varmus, 1984). Mutated ras genes were first identified by their ability to transform the established mouse cell line NIH3T3 after DNA transfection (Goldfarb et al., 1982, Santos et al., 1982, Shih and Weinberg, 1982). Mainly due to the laboriousness of the assay, however, the transfection assay was not suitable for the analysis of large number of tumors. Biological assay of gene transfection has relative low sensitivity of detection particularly for large size genomic DNA such as the K-ras gene (Bos et al., 1987a) and DNA transfected into mammalian cells was reported to be mutated at high frequency (Calos et al., 1983). NIH3T3 transfection assay cannot discriminate whether a mutation is present in one or in both alleles because of its inability to detect the normal alleles (Bos et al., 1984).

Recently, physical methods that detect base substitutions (or point mutation) causing genetic diseases or oncogene activation have

become important tools in several studies as well as ras gene mutation in cancer research. Five of the methods most frequently used to detect point mutations are briefly described as followings.

Restriction endonuclease digestion.

An end-labeled oligonucleotide probe with the wild-type sequence is hybridized to an in vitro amplified target genomic sequence. A mismatch within the restriction site prevents cleavage of the duplex, revealed by the appearance of different size of labeled fragments (Embury et al., 1987, Saiki et al., 1985). Using a similar method, mutations of the human c-H-ras oncogene at codon 12 were distinguished from the normal cellular counterpart by the loss of a restriction endonuclease site thus creating a diagnostic restriction fragment length polymorphism (RFLP). A mutation eliminating CCGG or GAGG sequences that is specifically recognized by the restriction endonuclease Hpa II/Msp I or Mnl I, respectively, destroys the cleavage site at codon 11 (GCC), codon 12 (GGC) in human H-ras (Feinberg et al., 1983, Muschel et al., 1983; Kraus et al., 1984) and codon 12 (GGA), codon 13 (GGC) in normal rat (Zarbl et al., 1985). Or as a non-radioactive assay, the amplified fragment by PCR was then directly digested to detect RFLP (Deng, 1988). In the case of the human K-ras proto-oncogene, however, the normal sequence of codon 12 was not part of any sequence specifically recognized. Instead, two specific mutations create new restriction sites (Santos et al., 1984). A similar case was also reported in codon 61 of H-ras during mouse skin chemical-induced carcinogenesis (Quintanilla et al., 1986).

Due to the easy handling of this technique, it has been often used for mutational screening assay. However, these specific sequences for

certain restriction endonuclease sites are limited to a few cases. Many point-mutations do not lead to an altered restriction site. Therefore, only a limited set of mutations, if any, can be detected (Valenzuela and Groffen, 1986).

Denaturing gradient gel electrophoresis.

DNA molecules that differ by single-base substitutions can be separated from one another by electrophoresis through an ascending gradient of denaturants in a polyacrylamide gel. The method can determine if DNA duplex contains any mismatch. Where DNA molecules migrate into a gradient of ascending concentration of denaturant, they undergo an abrupt decrease in mobility at a characteristic depth, resulting in specific positions and patterns on the gel running. Even a single-base substitution can cause two DNA molecules to melt at significantly different temperatures (Fischer and Lerman, 1983, Myers et al., 1987). The gradient of denaturants used in the system, urea and formamide, appears to be fully equivalent to a temperature gradient. The structural difference caused by a mismatch in heteroduplex substantially lowers the melting temperature or equivalently lowers the denaturant concentration at which melting takes place.

Mismatch cleavage.

RNAse A or S1 nuclease cleaves single stranded RNA or DNA, respectively. Total cellular RNA or genomic DNA is hybridized to end-labeled normal antisense RNA molecules. Mismatches within RNA-RNA or RNA-DNA heteroduplex caused by mutations in cellular RNA or in genomic DNA, respectively, leaves the single strand generating the labeled short fragment of RNA by RNAse A (Forrester et al., 1987,

Winter et al., 1985). Similarly labeled normal DNA molecules can be hybridized to genomic DNA and any mismatch at a mutation site is cleaved by S1 nuclease (Myers et al., 1985). Heteroduplex DNAs containing mismatched base pairs were also cleaved chemically following the treatment with a single strand specific modifying chemical agent (e.g., hydroxylamine for cytosine-specific modification) (Cotton et al., 1988; Montandon et al., 1989). These approaches are useful in the localization and diagnosis of mutations and in the estimation of expression level or mutation ratio between normal and mutant alleles in the same cell. However, mismatch cleavage assays do not provide the actual sequence at the mutation site and some mismatches in certain sequence are not detected (Engelke et al., 1988).

Direct sequencing of PCR products.

As a direct way to detect any base changes, sequencing of specific regions of genomic DNA became possible after the invention of PCR (Engelke et al., 1988). Addition of a internal third primer for the sequencing reaction (Wrischnik et al., 1987) and the use of unequal molar amounts of PCR primers to generate a predominant single strand (Gyllensten and Erlich, 1988) greatly improved the resolution of direct sequencing results and facilitated the automation for large-scale sequencing projects (Innis et al., 1988). Recently, ras oncogene alleles were characterized from carcinogen-induced animal tumors used direct sequencing of PCR product without purifying and cloning (McMahon et al., 1987). Furthermore, concurrent signals in one base were used as an estimation of mutation ratio. However, some direct sequencing resulted in sequencing gels with high background bands

that obscured the reading of the sequence (Wrischnik et al., 1987). Therefore, usually the results are confirmed by oligonucleotide hybridization analysis (Wong et al., 1987; Neri et al., 1988).

Mutation-specific oligonucleotide hybridization.

Usually the binding of the oligonucleotide probe depends on its length and GC content (Wood et al., 1985). Tetramethylammonium chloride (TMAC, Me_4NCl) binds preferentially to A:T base pairs (Shapiro et al., 1969) and raises the melting temperature (Chang et al., 1974). At 3 M Me_4NCl, this displacement is sufficient to shift the melting temperature of A:T base pairs to that of G:C base pairs (Melchior and von Hippel, 1973), reducing the problem to a simple dependence on length of the hybrid (Wood et al., 1985). For different oligonucleotides, therefore, empirical determination of a suitable hybridization and wash temperature is unnecessary (Conner et al., 1983, Verlaan-de Vries et al., 1986). Additionally, for the melting of long DNA, this shift results in remarkable sharpening of the melting profile, i.e., natural DNAs that melt over a range of 5 to 10 ^{o}C in the presence of sodium ion , melt within 1 ^{o}C in Me_4NCl (Chang et al., 1974). This is caused by the fact that unequal relative base pair stability with different G:C contents are responsible for most of the breadth of the melting transition (Melchior and von Hippel, 1973).

Since even a single-base-pair internal mismatch within a 17-mer probe decreases the Td of the hybrid 5 to 10 ^{o}C (Wallace et al., 1981, Wallace et al., 1979, Wood et al., 1985), correct mutants can be found relatively easily. After hybridization with a "mutant" probe of known sequence, the filters are washed with 6X sodium-chloride and sodium-citrate (SSC) buffer to remove the unbound labeled probe, then rinsed

with Me_4NCl at room temperature to substantially remove SSC because Na^+ will compete for Me_4N^+ binding (Orosz and Wetmur, 1977).

Originally, the procedure for mutation analysis was developed based on selective hybridization of mutation-specific oligodeoxynucleotide probes to genomic DNA without in vitro amplification (Bos et al., 1984, Conner et al., 1983). Unambiguous conclusions were difficult when screening highly complex genomic DNA of low abundance because detection level is limited (Bos et al., 1984, Wood et al., 1985; Valenzuela and Groffen, 1986) and the intensity of labeling at the hybridized gene fragment for each probe was significantly proportional to gene dosage (Conner et al., 1983). The procedure has been greatly improved both in sensitivity and speed by an in vitro amplification step (Verlaan-de Vries et al., 1986; Kozma et al., 1987). Allele-specific oligonucleotide hybridization procedures can then be used to detect the presence of single base changes in short segments of DNA if the exact nature of the mutation is known in advance (Vousden et al., 1986). The procedure can also be applied advantageously whenever an exact match to an oligonucleotide probe is desired, a pool of oligonucleotide probes are used (Wood et al., 1985), or PCR produces spurious bands on gel separation. The use of non-radioactive oligonucleotide probes provides an easier way to screen the mutations (Bugawan et al., 1988).

This procedure, widely used for identifying the mutation in conjunction with PCR, provides qualitative data on the type of mutations in positive cases as well as conclusive identification of the negative ones (Verlaan-de Vries et al., 1986; Kozma et al., 1987),

which are not readily achievable with biological assays involving NIH3T3 cell-transformation (Bos et al., 1987a).

Fish models.

Fish models are useful not only as environmental in situ monitors of aquatic pollution but as alternative vertebrate non-mammal species for comparative carcinogenesis studies (Powers, 1989). The rainbow trout (Oncorhynchus mykiss) has been most extensively used for toxicologic and carcinogenic research since the early 1960's. Even though trout have a long generation time (2 - 3 years) and have no homologue for several mammalian organs (e.g., lung, mammary glands and urinary bladder), they have unique characteristics highly desirable for most biomedical research and specific research problems (see Bailey et al., 1984, Table 4). In comparison with mammalian systems, however, little is known about the molecular basis of carcinogenesis, particularly any involvement of oncogenes in trout neoplasia. Recently, there have been several fish studies at the molecular level. The ras gene was cloned from goldfish liver DNA (Nemoto et al., 1986) and the c-myc gene was isolated from rainbow trout (van Beneden et al., 1986), which showed a remarkable similarity to their mammalian counterparts. Without the identification of the ras gene sequence, ras gene mutations were reported in Atlantic tomcod (Wirgin et al., 1989) and winter flounder (McMahon et al., 1990a). Because of the ubiquitous occurrence, and the evolutionary high conservation of ras proto-oncogene in eukaryotes, ras gene activations have often been detected in chemical carcinogenesis animal studies. Therefore the isolation and the mutational analysis of a trout ras gene could prove to be significant in this chemical carcinogenesis model. The primary

Table 4. Features of rainbow trout system.

1) Up to 6,000 offspring are available.
2) One hundred or more individuals can be housed in the same rearing unit.
3) The very low rearing cost is conducive to random sampling and statistical comparisons.
4) Diet requirements are known and can be carefully controlled from the time of hatching.
5) Growth and metabolism can be altered by changing water temperature.
6) Several routes of exposures to chemicals are available.
7) Trout can endure long periods of starvation without illness or mortality.
8) Trout remain sexually immature for approximately 18 months and do not reproduce until 2 or more years of age. This permits experiments to be run without the influence of sexual hormones.
9) Trout is very sensitive to the carcinogenicity of AFB1.
10) The spontaneous rate of tumor incidence is very low.

focus of our study was to identify the partial ras gene in normal trout liver DNA using in vitro gene amplification and then to investigate several carcinogen-initiated tumors in rainbow trout for evidence of ras gene point mutations. We used the oligonucleotide hybridization technique with position-specific degenerative and allele-specific 20-mer probes to screen whole PCR mixtures of each sample and confirmed findings using dideoxynucleotide sequencing of cloned PCR products.

MATERIALS AND METHODS

Tissue samples and DNA extraction.

Rainbow trout reared at the Toxicology and Nutrition Hatchery of Oregon State University were treated with 80 ppm AFB1 (Calbiochem) in the diet for two weeks. Nine months after AFB1 exposure, test fish were sacrificed and tissue samples were immediately frozen in liquid nitrogen. Fish treated with 50 ppm MNNG for 30 min and one that was treated with 5 ppm DMBA for 24 hours by bath exposure were sacrificed 18 months later. Chemical carcinogen waste was deactivated and disposed according to Armour et al. (1986). DNA was extracted from homogenized samples of normal tissues and primary liver tumors by lysis in buffer containing 10 mM EDTA, 100 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2% NaDodSO4, and proteinase K (100 µg/ml) overnight at 37 ^{o}C with mild shaking. DNA was then purified by phenol/chloroform (Boehringer Mannheim Biochemicals) extraction and 95% EtOH (Aldrich) precipitation. Resuspended samples in TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) were treated with RNAse (100 µg/ml, Sigma). Phenol/chloroform extraction and EtOH precipitation were repeated.

cDNA synthesis and random primer labeling.

Total RNA was extracted from normal trout liver tissue using commercial RNAzol (Cinna/Biotecx) and passed through an oligo(dT)-cellulose column (5Prime-3Prime) to isolate the mRNA. cDNA was then synthesized using a commercial cDNA synthesis kit from Bethesda Research Laboratories. The first strand of cDNA synthesized

was used as the template DNA for PCR. Random primers labeling of DNA probes was performed using [alpha-^{32}P] dATP (NEN; 3000 Ci/mmol) and a commercial random labeling kit (Bethesda Research Laboratories).

Oligonucleotide synthesis.

All the oligonucleotides used in this study were synthesized at the Gene Research Center of Oregon State University using an Applied Biosystems 380A DNA synthesizer.

PCR.

To amplify the fragment sequences of previously identified trout H- and K-ras gene, we used the panel of oligonucleotide primers shown in Figure 4 and Table 5. Sequences spanning 90 base pairs (bp) and 111 bp includes codons 12 and 13 of the ras genes of trout genomic DNA or cDNA. For the sequence analysis around codon 61, a 126 bp sequence between primer 38 and c79D was amplified. Using trout liver cDNA as a PCR template, a 237 bp PCR product covering exon 1 and part of exon 2 in trout ras gene was amplified between R01 and c79D primers. For each PCR reaction, 1 μg of DNA and 30 pmol of each of the two primers were added to a 50 μl reaction mixture containing 10 nmol of each dNTP (Pharmacia) and 1 unit of Taq DNA polymerase (Beckman) in PCR reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2). Forty-five cycles of denaturation at 94 ^{o}C for 30 sec, annealing at 45 ^{o}C for 30 sec, and extension at 70 ^{o}C for 30 sec were done on an automated heat-block (Ericomp). In the first 2 cycles, however, the denaturation step was done for 1.5 min per cycle and the DNA sample was boiled for 5 min prior to adding Taq polymerase for the complete denaturation of genomic DNA and for

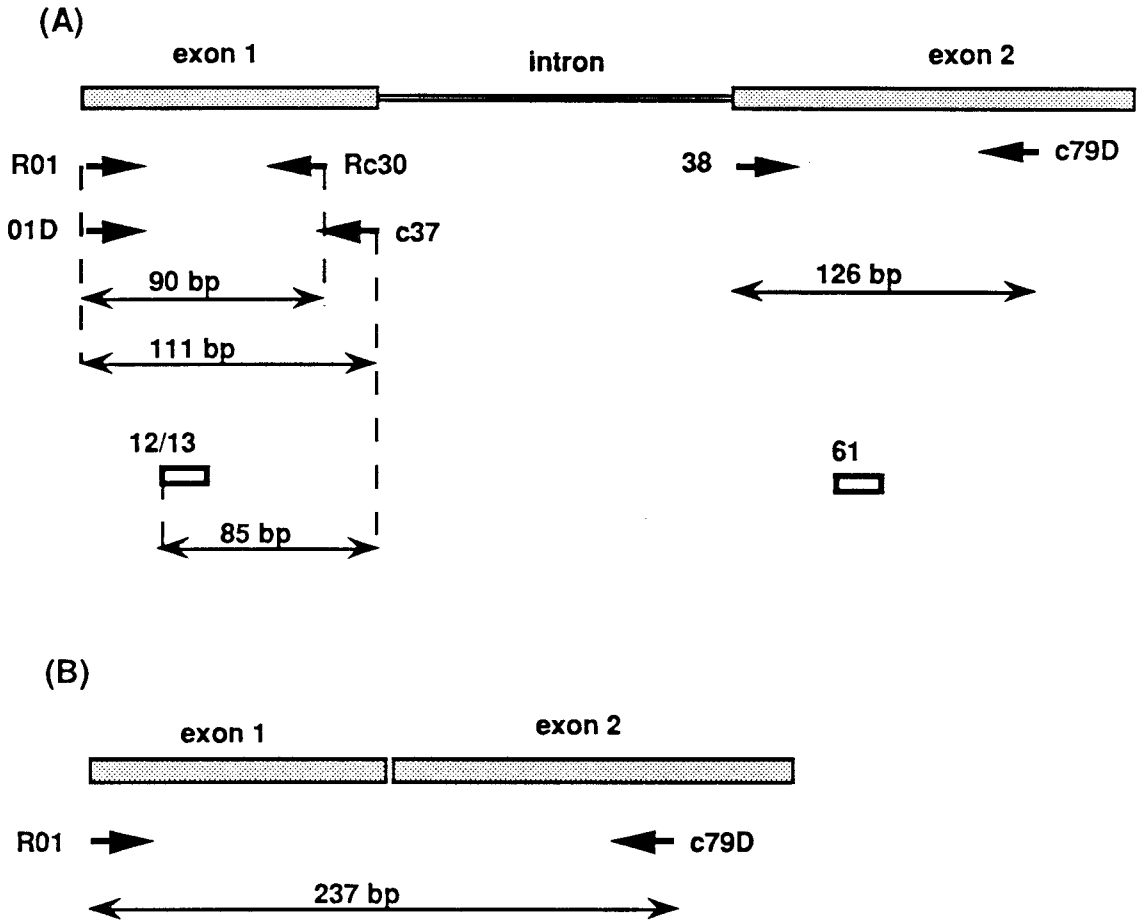


Figure 4. PCR amplification of a portion of a ras gene. Primers are indicated by arrows and probes are by squares; The expected size of PCR products from genomic DNA (A) and cDNA (B) are represented with base pairs (bp); and "R" indicates a rat sequence, "c" indicates a complementary sequence, and "D" indicates a degenerate sequence.

Table 5. PCR primers sequence. Degenerate sequences are presented as small capital letters.

R01	ATG ACT GAG TAT AAA CTT GT
Rc30	TTC AGA ATC ACT TTG TGG AT
01D	ATG ACTG GAA TAT AAG CTTG GTG G
c37	CTC GAT GGT GGG GTC ATA TTC
38	GAC TCG TAC AGG AAG CAG GTG G
c79D	GAAG GAAA GCC CTC TCCC AGTCT GCC

Table 6. Position- & allele-specific probes for oligonucleotide hybridization.

					D : A,C,T			
				12	13			
H normal	:	G	GGG	GCA	GGA	GGT	GTG	GGC A
H12 D1	:	-	---	---	D--	---	---	--- -
H12 D2	:	-	---	---	-D-	---	---	--- -
H13 D1	:	-	---	---	---	D--	---	--- -
H13 D2	:	-	---	---	---	-D-	---	--- -
H12 T1	:	-	---	---	T--	---	---	--- -
H12 A1	:	-	---	---	A--	---	---	--- -
H12 T2	:	-	---	---	-T-	---	---	--- -
H12 A2	:	-	---	---	-A-	---	---	--- -
H13 T2	:	-	---	---	---	-T-	---	--- -
H13 A2	:	-	---	---	---	-A-	---	--- -
K normal	:	G	GGT	GCT	GGA	GGT	GTT	GGG A
K12 D1	:	-	---	---	D--	---	---	--- -
K12 D2	:	-	---	---	-D-	---	---	--- -
K13 D1	:	-	---	---	---	D--	---	--- -
K13 D2	:	-	---	---	---	-D-	---	--- -

		61
H normal	:	GCA GGT CAG GAG GAG TAC AG

the inactivation of DNAse, if any. PCR products were then mixed with 0.1 volume of loading dye, and separated through 4% acrylamide slab gel electrophoresis. The gel was stained with ethidium bromide (1 μg/ml) and photographed with UV transillumination.

Oligonucleotide hybridization.

Three to five μl aliquots of the PCR mixtures were transferred to Zeta-Probe nylon filter (Bio-Rad) with a slot-blot manifold (Bethesda Research Laboratories) or by Southern blotting with 0.4 M NaOH (Reed and Mann, 1985). Replicate blots were simultaneously hybridized to a panel of 20-mer synthetic oligonucleotide probes shown in Table 6. Oligonucleotide probes (30 pmoles) were end-labeled with 30 pmoles of [gamma-^{32}P] ATP (NEN; 3000 Ci/mmol) by means of T4 polynucleotide kinase (Pharmacia or Promega) and purified through a Quick-Spin G-25 Sephadex column (Boehringer Mannheim Biochemicals). Washing of filters was performed in a 3 M TMAC (Aldrich) solution (50 mM Tris-HCl, pH 7.6, 2 mM EDTA, 0.1% NaDodSO4) at 61 ^{o}C with constant shaking (Verlaan-de Vries et al., 1986; Wood et al., 1985) slightly modified from MutaLyzer™2 manual (ClonTech). Following the final washing with 6X SSC, the blot was exposed to X-ray film (Kodak, X-Omat/AR) for 40 min or overnight at room temperature.

Cloning and plasmid preparation.

After the digestion of pUC10 vector with Sma I restriction enzyme, the linearized pUC10 with blunt ends was combined with PCR products concentrated using Centricon-30 filtration units (Amicon) at a molecular ratio of 1:100 and ligated using 1 unit of T4 DNA ligase (Bethesda Research Laboratories) in 4 ^{o}C for overnight (Ferretti and

Sgaramella, 1981). One µl of the 5 times-diluted ligation mixture was used to transfect 25 µl of DH 5 alpha competent cells (Bethesda Research Laboratories).

Selected white colonies on LB agar plate containing 100 µg/ ml of ampicillin, 10 µl of 10% X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside : Bethesda Research Laboratories), and 10 µl of 100 mM IPTG (isopropylthio-beta-galactoside : Bethesda Research Laboratories) were screened by the oligonucleotide hybridization method. Plasmid DNAs from probe-positive clones were prepared according to the procedures described in Davis et al. (1986).

Sequencing.

Sequencing reactions were done according to a modification of Taq-Track sequencing system (Promega). One µg of plasmid DNA was denatured in 0.4 M NaOH and neutralized with potassium acetate. After the precipitation with EtOH, 2 pmol of M13/pUC primer was added in the resuspended sample in Taq buffer. A 25 µl mixture was prepared containing 2 µl of extension mix, 2 µl of [alpha-^{32}P] dATP (NEN; 3,000 Ci/pmol), 5 units of Taq DNA polymerase. After incubation at 37 ^{o}C for 5 min, 6 µl of reaction mixture was added to 1 µl of each deoxy-/dideoxy-nucleoside triphosphate in the separate tubes. After incubation at 70 ^{o}C for 5 min, the sequencing reactions were stopped by the addition of 3 µl of 80% formamide dye mixture. Four µl of the heat-denatured reaction mixtures were loaded onto a 8 M urea/5% acrylamide gel. Following the electrophoresis in 50 ^{o}C, the gel was dried and exposed to X-ray film (Kodak, X-Omat/RP) at room temperature.

RESULTS

I. Identification of a *ras* gene in trout liver.

New set up for PCR.

Following the occurrence of a PCR "carryover" problem in the laboratory, the PCR experiment was resumed in a room which was physically separated from that in which the PCR products were handled. All preparations for PCR and all DNA extractions from the tissue samples were completed in a room which had not been used for DNA manipulation. Stock solutions were entirely replaced. PCR buffer was prepared without the general use of gelatin and was maintained as frozen aliquots. New DNA samples were prepared and a new set of primers was synthesized. When the DNA samples were taken, positive displacement pipet tips (Gilson Medical Electronics, France: reordered from Rainin Instrument Co. Inc.) were used to prevent carryover from sample to sample. For each PCR run, a negative control without a DNA template was performed along with samples to detect any trace amounts of carryover.

Since PCR is an elusive reaction, the "standard" PCR condition is summarized in Table 7. To obtain maximum yield and improved quality, this condition was slightly modified in accordance with specific cases. New primers, R01 and Rc30, based on the rat K-ras sequence (Table 5), were synthesized and used for 90 bp PCR amplification for the first exon of trout genomic DNA (McMahon et al., 1990a). The G:C base pair contents of the R01 and Rc30 primer were low (respectively, 30 and 35%), and the annealing temperature of PCR

Table 7. "Standard" PCR condition for 50 µl reaction.

Tris-HCl (pH 8.3)	10 mM
KCl	50 mM
$MgCl_2$	1.5 mM
Each Primer	0.6 µM
Each dNTP	0.2 mM
Taq. enzyme	4 U
Template DNA	0.5 µg
Mineral oil	35 µl

Template DNA at 100 °C for 5 min, then on ice.

Step-Cycle program (45 cycles)

45 °C	30 sec	
70 °C	30 sec	* 4.5 min (last cycle)
94 °C	30 sec	* 1.5 min (first 2 cycles)

at 45 °C produced a major 90 bp band on acrylamide gel electrophoresis. To obtain the information on the entire mRNA sequence, a PCR using R01 and $(dT)_{24}$ primers with cDNA template was performed, but failed to yield a discrete band. Reamplification of the above reaction with a third primer (Ohara et al., 1989) produced no clearer result.

To optimize the PCR, different amounts of template DNA and dNTP were tested (Figure 5). An increase in PCR products was observed as the template amounts were increased. Fractionation of the DNA template by physical shearing through the syringe or by vigorous vortexing was helpful for complete DNA denaturation. At dNTP concentrations less than 0.2 mM, no PCR products were detected on the gel. A newly prepared dNTP mixture which failed to produce any PCR product was found to have an absorbance peak at 270 nm. An equal ratio of each dNTP was mixed again and tested for the PCR, which yielded expected PCR products and had an absorbance peak at 260 nm. Although it was not known what happened in the former dNTP mixture, scanning of the dNTP mixture to assure an absorbance peak at 260 nm was essential to assure good PCR reactions (Figure 6). Higher annealing temperature (i.e., from 37 °C to 45 °C) improved the reaction yield to some extent (data not shown).

Oligonucleotide hybridization.

To establish the oligonucleotide hybridization technique in the laboratory, a preliminary experiment with a human K-ras sequence was performed until the trout ras sequence could be identified. As indicated by Verlaan-de Vries et al. (1986), 111 bp of the first exon was amplified by PCR from normal human and rat genomic DNA with a

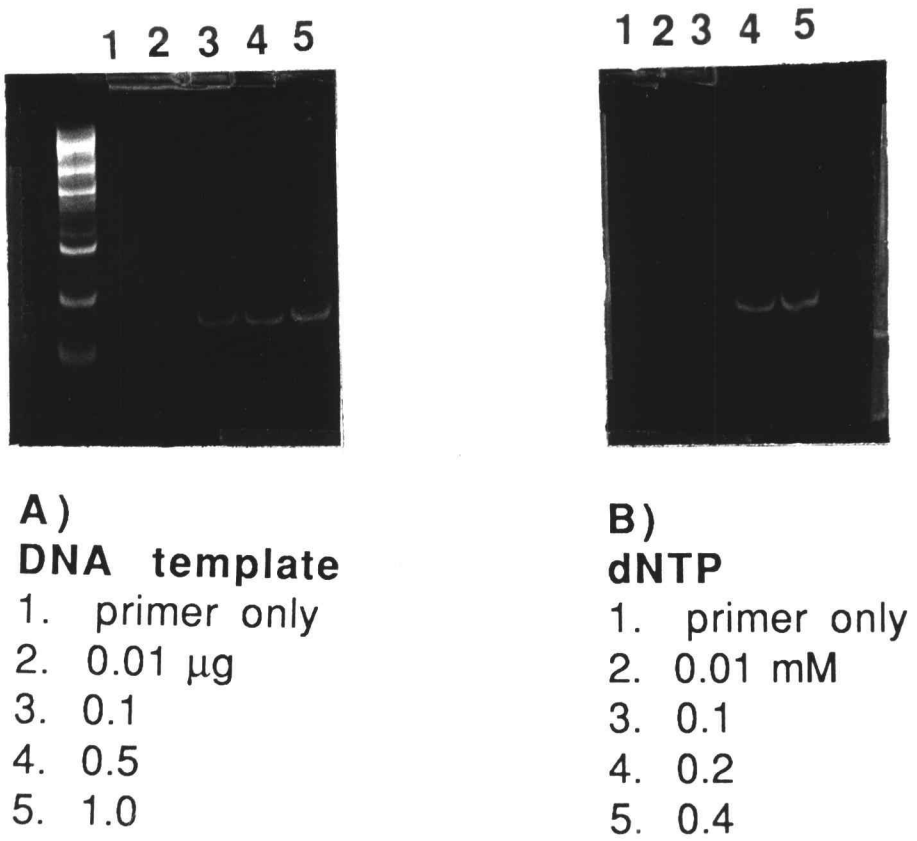


Figure 5. PCR at different amounts of template DNA and dNTP. An increase in PCR products was observed as the template amounts were increased (A). At dNTP concentrations less than 0.2 mM, no PCR products were detected on gel (B).

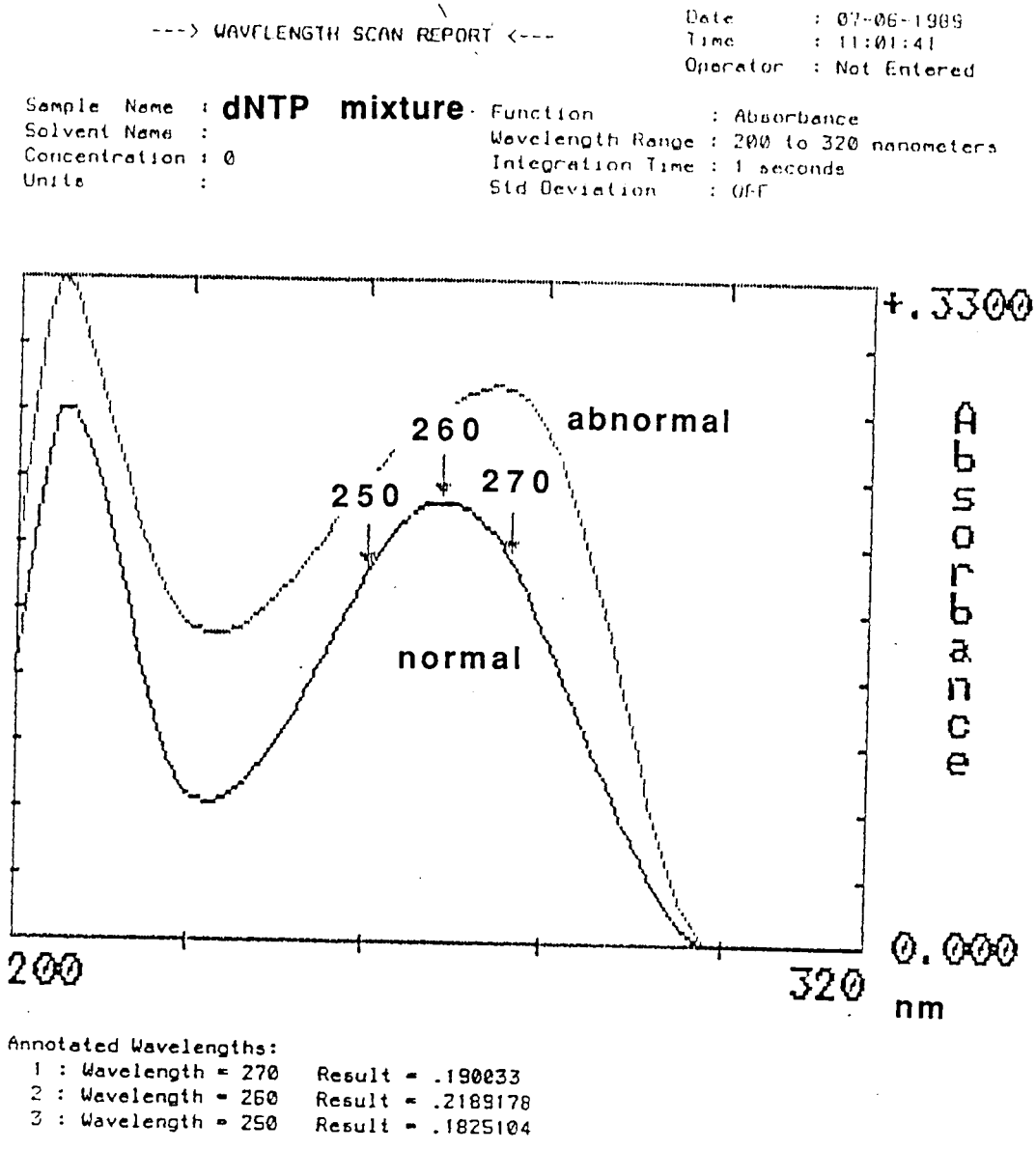


Figure 6. dNTP scanning between 200 and 320 nm wavelength. The dNTP mixture which failed to produce PCR product had an absorbance peak at 270 nm. The dNTP mixture which had an absorbance peak at 260 nm yielded expected PCR products.

GGT (Gly) sequence at codon 12 of the ras gene. For the mutated sequence controls, the plasmids containing the human mutated K-ras for GTT (Val) and the viral K-ras for AGT (Ser), were used as the PCR templates. From the viral K-ras template, a 216 bp PCR product was prepared, using 01D and c72D (5'-CAT GTA CTG GTC CCGT CAT GTGC-3') primers. Based on the human K-ras sequence, oligonucleotide probes (Gly : 5'-GGA GCT GGT GGC GTA GGC AA-3' and Val : 5'-GGA GCT GTT GGC GTA GGC AA-3') were purchased (ClonTech), each with a single base difference at the second position of codon 12 of the ras gene. The amounts of PCR products were estimated by ethidium bromide staining of the gel (Figure 7). The results indicated that each oligonucleotide probe was specifically bound to its unique sequence, but not to alleles differing by one nucleotide. A ser-specific probe (5'-GGA GCT AGT GGC GTA GGC AA-3') also showed specific binding (data not shown).

Washing of the blots was seemingly a critical step in sequence differentiation, and strict temperature controls and constant shaking were important factors. However, oligonucleotide probe binding was often not absolutely specific and some cross-binding among the negative controls was found to produce a background signal. Scanning with the densitometer was useful for quantification estimation and is subsequently described in Figure 14. The presence of large amounts of the normal allele sequence in PCR mixtures seems to have enhanced the extent of background binding of mutated oligonucleotide probes (for example, see Figure 8, lane 3).

90 bp trout sequence.

Since the rat sequence was used for the primer synthesis, as

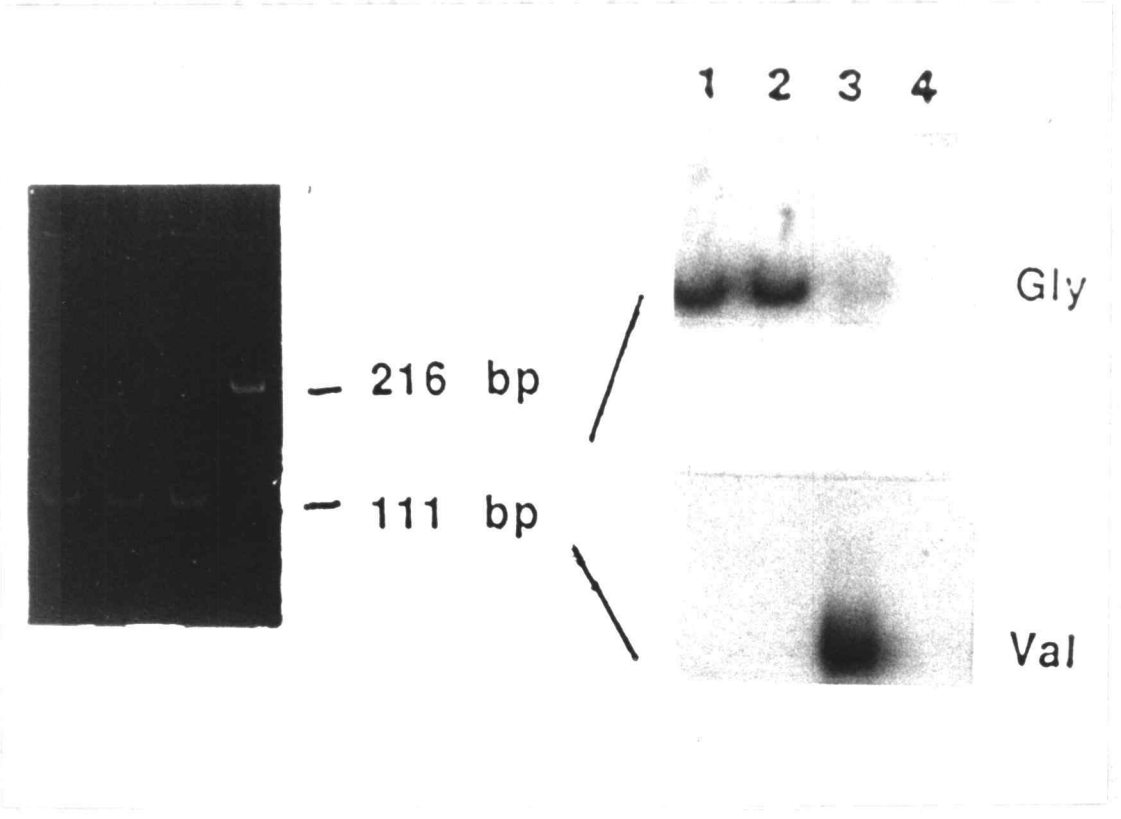


Figure 7. Specificity of oligonucleotide hybridization. PCR products of normal human genomic DNA (lane 1), normal rat genomic DNA (lane 2), human mutated K-ras plasmid (lane 3), and viral K-ras plasmid (lane 4) were hybridized with 20-mer probes specific for the codon 12 point mutation (Gly : GGT and Val : GTT) of the human K-ras sequence.

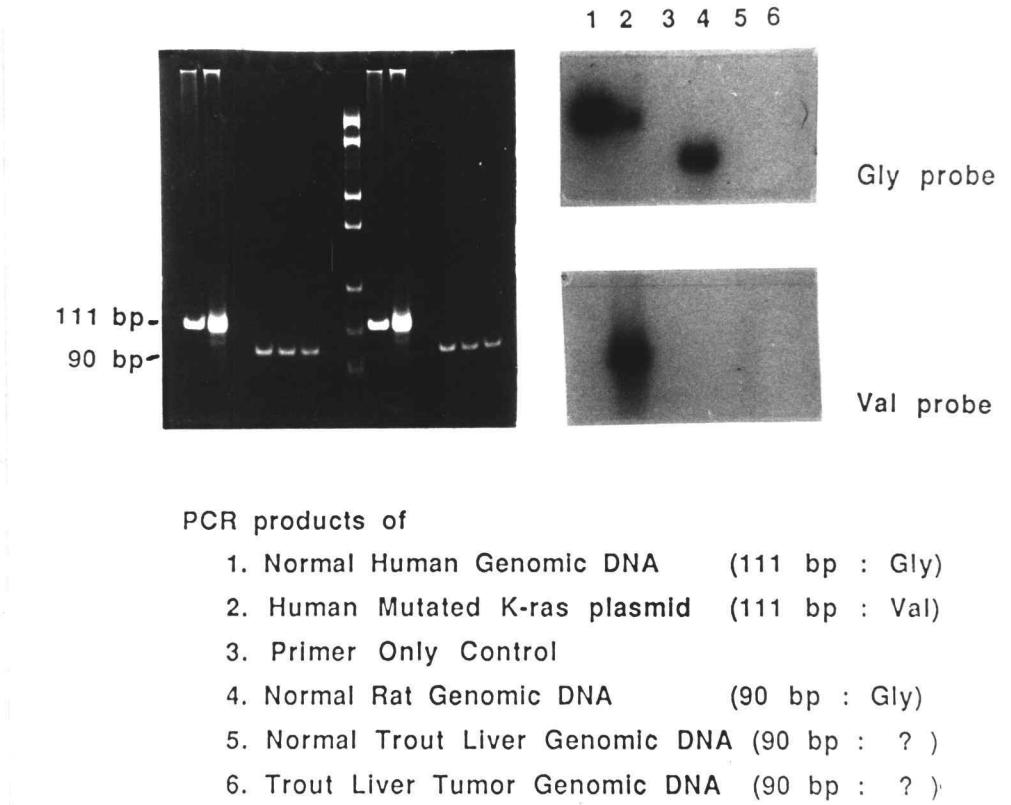


Figure 8. Oligonucleotide hybridization of trout PCR products. PCR products of normal human genomic DNA (lane 1), human mutated K-ras plasmid (lane 2), negative control of primer-only PCR product (lane 3), normal rat genomic DNA (lane 4), normal trout liver DNA (lane 5), and trout liver tumor DNA (lane 6) were hybridized with 20-mer probes specific for the codon 12 point mutation (Gly : GGT, Val : GTT) of the human K-ras sequence.

described by Wogan (McMahon et al., 1990a), this investigation ought to determine how the "trout DNA" PCR product compared to the rat sequence. Using normal rat liver DNA as a positive control, normal trout liver DNA and trout liver tumor DNA were used for the PCR template. Separation by acrylamide gel electrophoresis indicated that the trout as well as mammalian PCR had one major band with a clear background, and that the negative control generated no products (Figure 8, lane 3). PCR products from normal human genomic DNA and mutated human K-ras plasmid were then used as controls for oligonucleotide hybridization. Oligonucleotide hybridization with probes derived from normal (GGT) and mutated (GTT) human K-ras showed that the expected PCR products were obtained using the mammalian DNA template and that the trout sequence differed from mammalian sequences in at least one base in the region of the probe, such that no hybridization occurred.

Direct sequencing of PCR products was performed for the trout ras sequence, but did not yield readable sequence. To sequence the PCR product indirectly, it was ligated with a blunt end pUC10 plasmid for transformation. Clones selected with random labeled PCR product probe were then sequenced. The sequencing result for the 90 bp PCR product from normal trout liver genomic DNA is shown in Figure 9, compared with the rat K-ras and the goldfish ras gene. Since it was identified using rat K-ras primers, this sequence is hereafter referred to as the trout K-ras. However, its entire sequence or functional homology to mammalian ras genes is still unknown.

Several ras sequences from different species were compared around codons 12 and 13 (Table 8), suggesting that each species has a

```
R01-Rc30 : ATG ACT GAG TAT AAA  CTT GTT GTG GTG GGT
Rat K-ras: --- --- --- --- ---  --- --G --A --T --A
Goldfish : --- --A --A --- --G  --- --G --C --A --G
     (R01)-*** *** *** *** ***  *** **

R01-Rc30 : GCT GGA GGT GTT GGG  AAG AGT GCC TTA ACT
Rat K-ras: --- --T --C --A --C  --- --- --- --G --G
Goldfish : --- --- --C --G --C  --- --- --T C-C --C

R01-Rc30 : ATC CAG CTC ATT CAG  AAT CAC TTT GTG GAT
Rat K-ras: --A --- --A --- ---  --- --- --- --- ---
Goldfish : --- --A --- --C ---  --C --- --- --- --C
                      ** ***  *** *** *** *** ***

                                          -(Rc30)
```

Figure 9. Trout sequence of the 90 bp PCR product with primers R01 and Rc30. Star marks represent the positions of the primer sequence.

Table 8. Sequence comparison around codons 12 and 13 of ras genes.

```
                              12  13
Trout K-ras     : G GGT GCT GGA GGT GTT GGG A
Human K-ras     : T --A --- --T --C --A --C -
Rat K-ras       : T --A --- --T --C --A --C -
Mouse K-ras     : T --A --- --T --C --A --C -
Trout H-ras     : - --G --A --- --- --G --C -
Human H-ras     : - --C --C --C --- --G --C -
Rat H-ras       : - --C --- --- --C --G --A -
Human N-ras     : T --A --A --T --- --- --- -
Mouse N-ras     : T --A --A --T --- --- --- -
Goldfish ras    : A --G --- --- --C --G --C -
Chicken ras     : - --A --- --- --- --C --- -
```

distinct ras sequence which can be differentiated by oligonucleotide hybridization when at least two of the base pairs are different. The oligonucleotide probes, reflected in Figures 5 and 6, are one base shifted toward the 3'-end. This information can be used for the identification of a specific ras gene, as well as the point mutations of trout tumor DNA. An additional trout ras sequence, referred to as trout H-ras (Mangold et al., in preparation), was identified with consensus degenerative primers (01D). Trout H-ras differs from trout K-ras at more than two bases in the oligonucleotide probe region.

For confirmation of the 90 bp sequence, newly prepared DNA from a tumor and two normal liver tissues were used. These PCR products showed the same sequence as previously described in Figure 7. To investigate the expression of this sequence in mRNA, cDNA was used as a PCR template, producing the 90 bp products shown in Figure 10, lane 2. Determining the same sequence from cDNA confirmed the presence in genomic DNA as well as the expression in mRNA. With a genomic DNA template, a 111 bp PCR product was prepared using the two pairs of R01 - c37 and 01D - c37 primers (Figure 10, respectively, lanes 3 and 4). The whole mixture of PCR products was then blotted onto a nylon membrane in 0.4 M NaOH and probed with oligonucleotides specific for trout H-ras and trout K-ras sequences, indicating the base difference. The results indicate that R01 and 01D 5' primers were able to produce different sequences of PCR products from the same trout genomic DNA. A 237 bp PCR product was also made with the cDNA template and a pair of R01 and c79D primers. This PCR product was then ligated into pUC10 by a blunt-end ligation reaction and used for transformation. Though cloning occurred at a

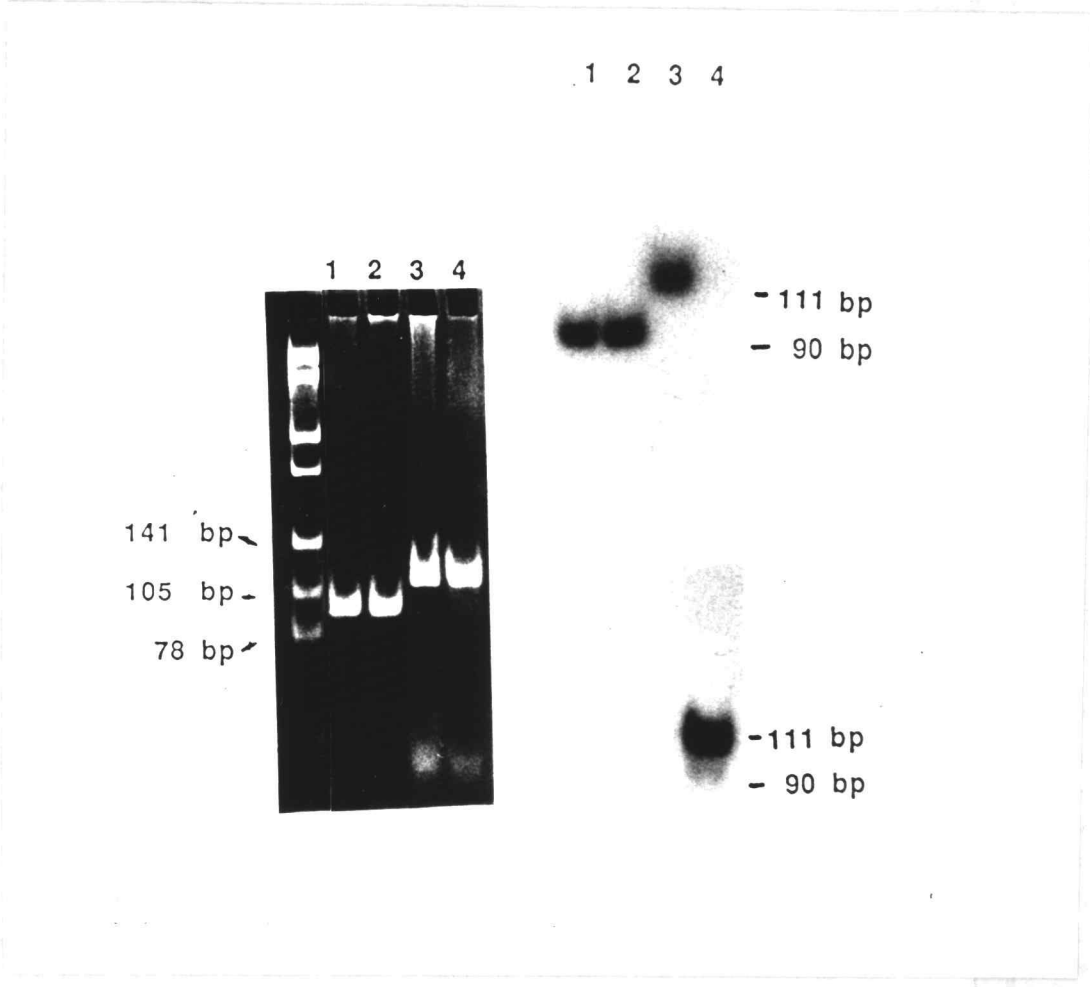


Figure 10. Oligonucleotide hybridization of trout PCR products using different primers and templates. PCR products (left panel) of genomic DNA with R01-Rc30 (lane 1), cDNA with R01-Rc30 (lane 2), genomic DNA with R01-c37 (lane 3), and genomic DNA with 01D-c37 (lane 4) were hybridized with trout K-ras (upper right panel) and H-ras (lower right panel) specific 20-mer oligonucleotide probes.

low degree of efficiency, the clones containing the PCR product insert were selected by PCR product probe labeled by the random priming method. Selected clones with the 237 bp PCR product inserted were sequenced, proving inclusion of a 90 bp sequence (Figure 11).

The nucleotide sequence was then compared, and the homologous percentages among several ras genes in their counterparts of actually amplified sequences (197 bp), excluding the primer sequences at both ends, are shown in Table 9. Most were homologous to 80% or greater; Trout K- and H-ras genes showed more than 90% homology, and were more closely related to the goldfish ras gene than to either the mammalian ras or the chicken ras genes. The predicted amino acid sequence of trout K-ras, compared to the other ras sequences, showed only one residue difference at codon 69 where asparagine replaced asparate. In the case of chicken ras (Westaway et al., 1986) and goldfish ras (Nemoto et al., 1986), the predicted amino acids sequence did not show any difference from the mammalian ras gene.

Table 9. Nucleotide sequence homology of trout ras PCR products to other vertebrate ras gene sequences within identified region.

	Homology Percentage		
	Trout K-*ras*	Trout H-*ras*	Goldfish *ras*
Human K-*ras*	83	82	82
Rat K-*ras*	84	83	82
Human H-*ras*	83	86	89
Human N-*ras*	81	82	78
Chicken *ras*	82	82	81
Goldfish *ras*	88	89	
Trout H-*ras*	91		

```
                                                                  10
R01-c79D     : ATG ACT GAG TAT AAA  CTT GTT GTG GTG GGT
Rat K-ras    : --- --- --- --- ---  --- --G --A --T --A
Human H-ras  : --- --G --A --- --G  --G --G --- --- --C
         (R01)-*** *** *** *** ***  *** **
                                                                  20
R01-c79D     : GCT GGA GGT GTT GGG  AAG AGT GCC TTA ACT
Rat K-ras    : --- --T --C --A --C  --- --- --- --G --G
Human H-ras  : --C --C --- --G --C  --- --- --G C-G --C
                                                                  30
R01-c79D     : ATC CAG CTC ATC CAG  AAT CAC TTT GTG GAT
Rat K-ras    : --A --- --A --T ---  --- --- --- --- ---
Human H-ras  : --- --- --G --- ---  --C --T --- --- --C
                                                                  40
R01-c79D     : GAA TAT GAC CCC ACC  ATC GAG GAC TCG TAC
Rat K-ras    : --- --- --T --T --G  --A --- --- --C ---
Human H-ras  : --- --C --- --- --T  --A --- --T --C ---
                                                                  50
R01-c79D     : AGG AAG CAG GTG GTG  ATT GAC GGA GAG ACG
Rat K-ras    : --- --A --A --A --A  --- --T --- --A --C
Human H-ras  : C-- --- --- --- --C  --- --T --G --- ---
                                                                  60
R01-c79D     : TGT CTG CTG GAC ATC  CTG GAC ACA GCA GGT
Rat K-ras    : --- --C --- --T --T  --C --- --- --- ---
Human H-ras  : --C --- T-- --- ---  --- --T --C --C --C
                                                                  70
R01-c79D     : CAA GAG GAG TAC AGC  GCC ATG AGG AAC CAG
Rat K-ras    : --- --- --- --- --T  --A --- --- G-- ---
Human H-ras  : --G --- --- --- ---  --- --- C-- G-- ---

R01-c79D     : TAC ATG AGG ACA GGG  G
Rat K-ras    : --- --- --A --T ---  -AG GGC TTT CTT
Human H-ras  : --- --- C-C --C ---  --- --- --C --G
                       ** *** ***  *** *** *** ***_
                                                   (c79D)
```

Figure 11. Trout sequence of the 237 bp PCR product with primers R01 and c79D.

II. Mutational analysis of trout *ras* genes.

PCR scheme.

DNA samples from trout tumors initiated by several chemical carcinogens, including AFB1, MNNG and DMBA, were subjected to the PCR to amplify the fragment of the trout H-ras gene, as illustrated in Figure 4 and Table 5. In previous studies, the two ras genes, trout H-ras and K-ras, from the genomic DNA and cDNA of normal liver tissues were identified (Mangold et al., in preparation). Based on the partial trout ras sequence information, the presence of ras gene mutations in codons 12 and 13 was analyzed by the combination of the PCR and the oligonucleotide hybridization assay. For in vitro amplification of the target sequence in exon 1, a set of oligonucleotides (01D - c37 or R01 - c37) was used as primer pairs, and trout tumor genomic DNA was used as template DNA. To amplify the exon 1 sequence of the trout H-ras gene from genomic DNA, the first intron sequence was once used for the synthesis of the 3'-primer oligonucleotide. However, the PCR yield using the 01D and the intron-sequenced 3'-primer was low with many diffuse bands, such that c37 primer was used.

Five sets of 20-mer oligonucleotides with four degenerative position-specific and a normal sequence were synthesized for the probes around codons 12 and 13. Allele-specific oligonucleotide probes were then synthesized for the identification of specific point mutations in accordance with the results of position-specific oligonucleotide hybridization. Systematic analysis of the trout ras genes mutations made it easy to screen the incidences and the specificities in a large number of tumor samples (Figure 12). To

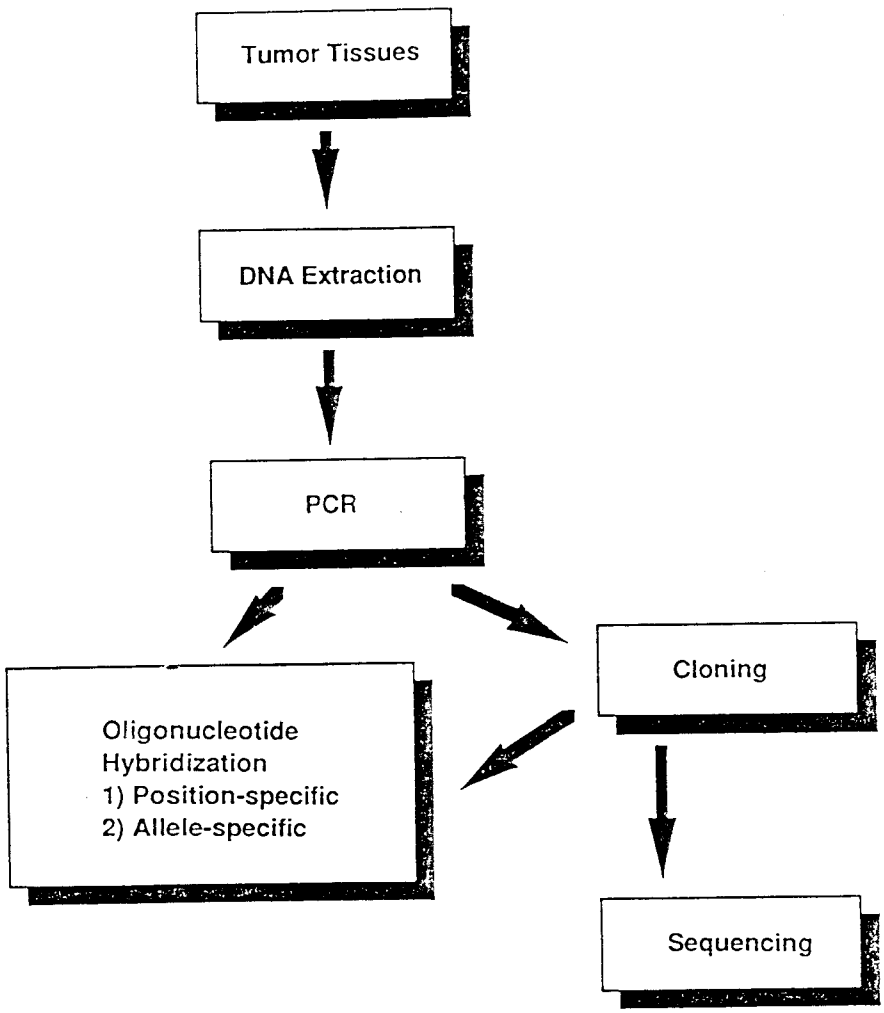


Figure 12. Schematic diagram for systematic mutational analysis. Extracted DNA from chemically-induced trout tumors were used as PCR templates. Whole mixtures of amplified PCR fragment were analyzed for evidence of point mutations using position- and allele-specific oligonucleotide hybridization. PCR products ligated into plasmid were cloned and selected by oligonucleotide hybridization. Mutant positive clones were then sequenced.

measure the ratio between normal and mutated allele, the PCR products were cloned and the colonies were hybridized with both normal and mutated specific oligonucleotide probes. To confirm the result obtained by oligonucleotide hybridization assay of the whole mixture of PCR products, selected colonies with a mutated probe were then sequenced.

Controls for oligonucleotide hybridization.

Because the oligonucleotide hybridization technique poses difficulties for the availability of positive mutated sequence controls, the controls for each probe were enzymatically polymerized by using the probe as a 5'-primer (Rochlitz et al., 1988) and the amplified 111 bp PCR product as a template DNA. This reaction yielded a major single band (85 bp PCR product), into which each probe was physically incorporated, with a clean PCR product background. Five reamplified PCR products, including four position-specific mutated sequences and a normal sequence, were separated on a 4% acrylamide slab gel electrophoresis and stained with ethidium bromide (Figure 13). Five replicate blots, each containing five control PCR products, were then simultaneously hybridized with each of the probes. There was no substantial cross-reaction among each of the PCR products. Reamplified 85 bp PCR products were then used for mutated sequence controls in the first and the second positions of codons 12/13 to screen the point mutations in the primary tumor PCR samples. As an alternative control sequence, allele-specific probes were used as primers for reamplification of the 111 bp PCR products, yielding the 85 bp PCR products.

Normal and mutated sequence controls (90 bp) were also

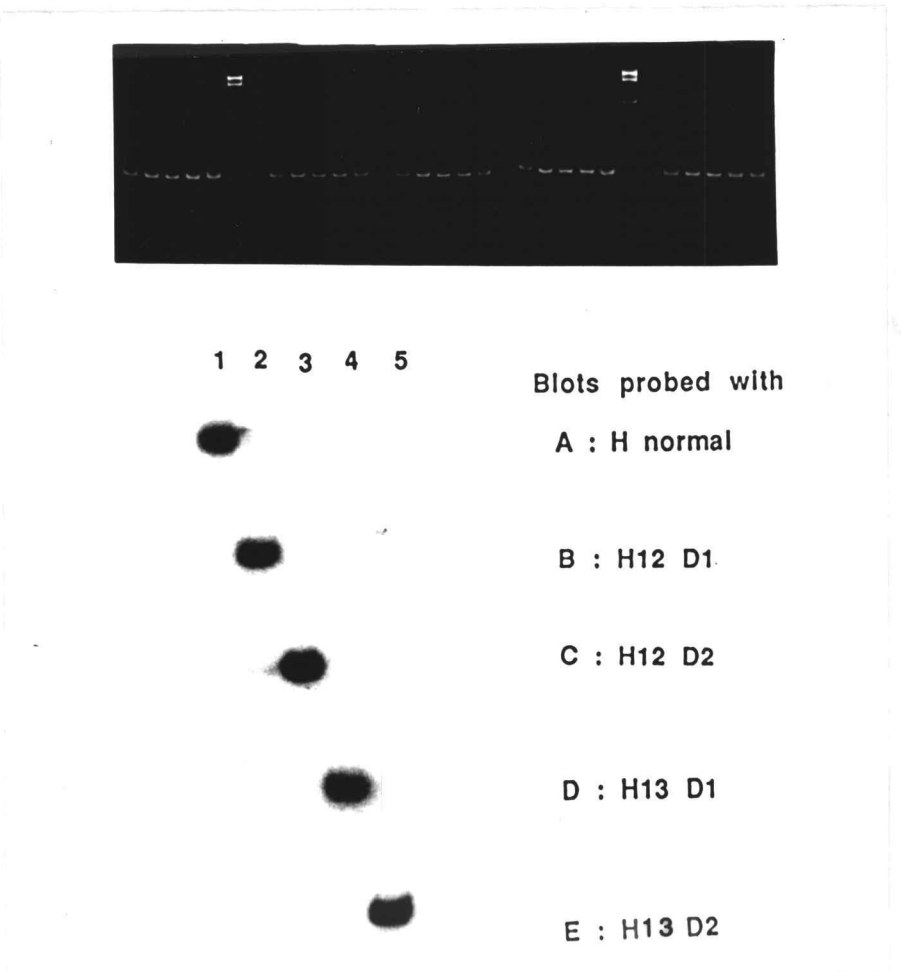


Figure 13. Positive controls for position-specific ras probes. Reamplification, using oligonucleotide probe around codon 12/13 as a 5'-end primer, produced 85 bp PCR products, which were stained with ethidium bromide on 4% acrylamide gel. These PCR products incorporated by H normal, H12 D1, H12 D2, H13 D1, and H13 D2 probes, respectively, are numbered as 1 through 5. The upper panel shows five replicate acrylamide gel of the five PCR products. The lower panel shows the specific probing of the five gel blots.

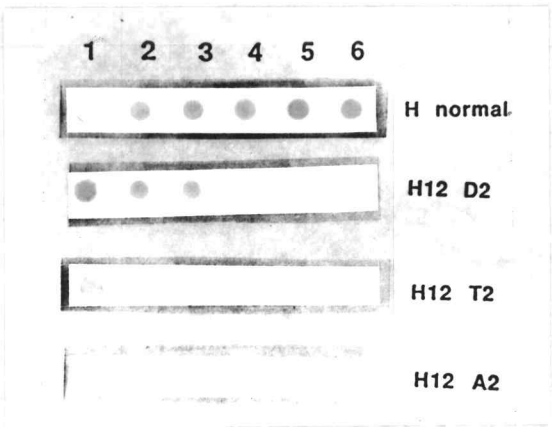
enzymatically polymerized from PCR product inserted plasmids with R01 and Rc30 primers. These PCR product controls were mixed and hybridized with oligonucleotide probes to investigate the linear relationship between signal strength and the control amounts (Figure 14). A dot blot autoradiogram was scanned and the heights were measured. Subtracting the background signals caused by the cross reactions, the heights of the signal peak were plotted against the each of control amounts. The slope in the graph presents the specific activity of the oligonucleotide probe, and the binding of two probes (H normal and H12 T2) was dependent upon the control amounts. This experiment demonstrated a possibility that the amount of each allele in each sample can be calculated quantitatively.

AFB1-induced trout liver tumors.

Southern blot.

Similar to the procedure for the control blots, five replicate Southern blots of whole PCR product mixtures from 14 primary trout tumors initiated by AFB1 were simultaneously hybridized with five different mutated codon 12/13 probes for trout H-ras mutational analysis. Only one band responded with a clear background (Figure 15). It was of interest to note that the normal ras gene sequence was dominant in the tumor DNA PCR products. However, it could not be determined whether the normal allele was excessively amplified in the tumor tissue, or if only a small portion of the cell population within the primary tumor carried a mutated ras allele.

Of the 14 trout liver tumors induced by a single AFB1 exposure, 10 samples (72%) showed evidence for a mutated allele in codons 12 or 13 of the trout H-ras gene. Specifically, probe H13 D2 was positive for



mixture	control PCR products (μl)		oligonucleotide probe hyb.	
	normal	H12 T2	normal	H12 T2
1	0	6	0.0	101.0
2	4	4	51.7	51.5
3	5	3	74.5	42.6
4	6	2	95.3	20.8
5	7	1	118.2	9.9
6	8	0	131.0	0.0

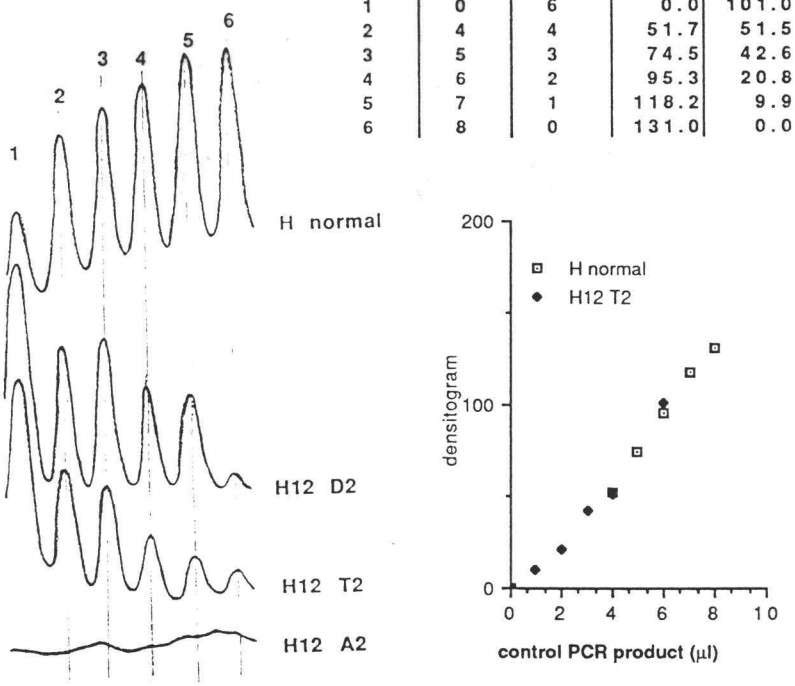


Figure 14. Densitogram of control PCR products mixtures. PCR product controls (H normal and H12 T2) were mixed at certain ratios, and hybridized with H normal, H12 D2, H12 T2, and H12 A2. Densitometry of dot blot autoradiogram, after the subtraction of background signal, showed that the bindings of oligonucleotides are nearly proportional to the amounts of PCR products.

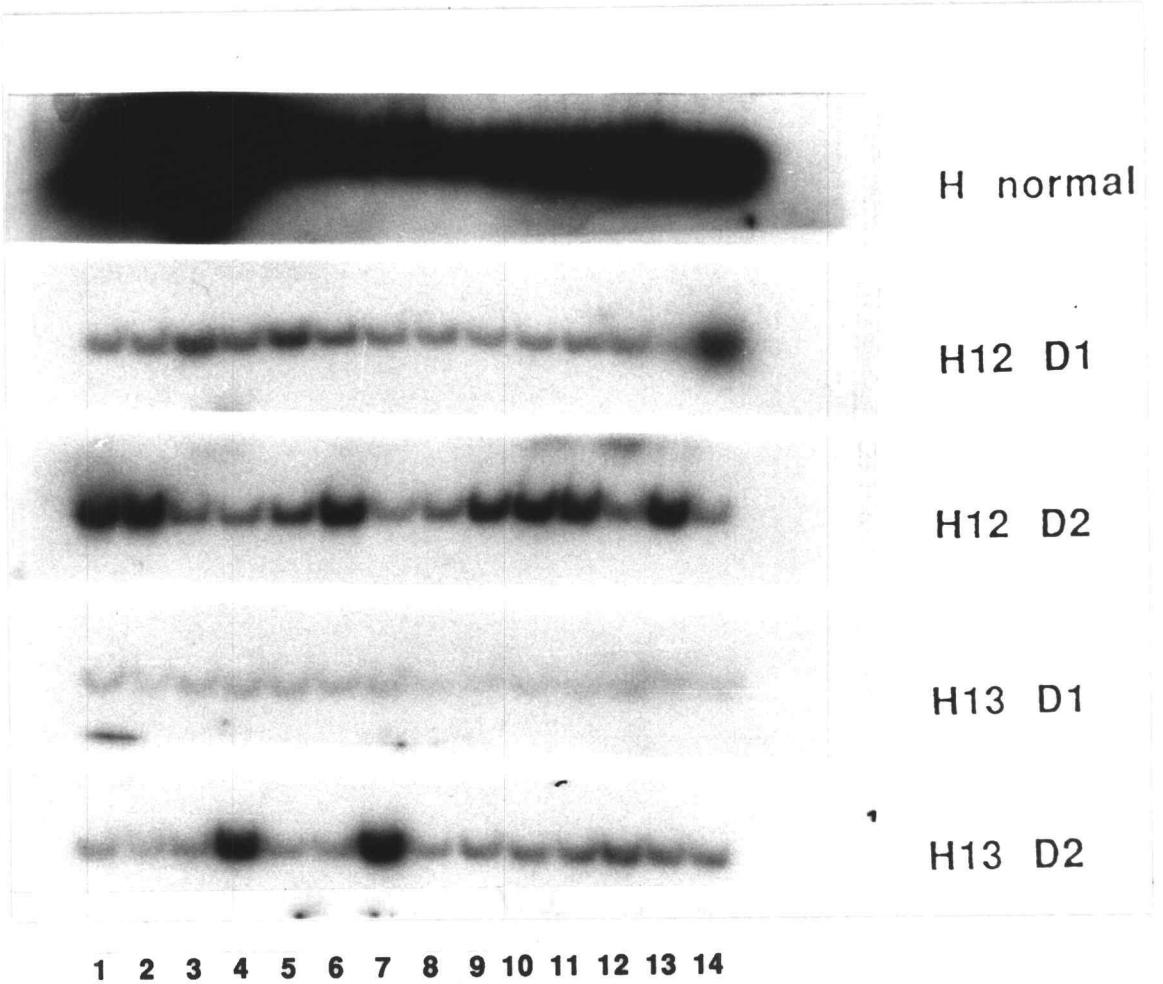


Figure 15. Position-specific probing of tumor DNA PCR products. PCR products (111 bp) of trout liver DNA induced by AFB1 were separated on 4% acrylamide gel. Five replicates of each Southern blot were hybridized with each position-specific probe. Samples were numbered arbitrarily from left (1) to right (14).

tumor DNAs 4 and 7, with all other DNAs showing some binding which was interpreted as background. Probe H13 D1 was negative for all DNAs, whereas probe H12 D2 indicated strong binding to DNAs 1, 2, 6, 9, 10, 11, and 13, and possibly 5. H12 D1 bound strongly to DNA 14 and possibly to 3 and 5. This initial binding study thus provided evidence for 7 of 10 mutations (70%) in the second position of codon 12, which is an often mutated site for ras-protein activation and tumor induction. No mutations were indicated in the first position of codon 13 for any of the tumors. Of these mutations, two were indicated in the second position of codon 13 and one mutation in the first position of codon 12.

Dot blot.

To repeat the previous oligonucleotide hybridization result, a dot blot assay with newly prepared PCR products was performed with results similar to that for the Southern blot assay. This time, allele-specific probes were included for the identification of specific mutations. However, only the A and T mutations for the allele-specific oligonucleotides were tested since it was known from prior report (McMahon et al., 1987) that the possible mutations caused by AFB1 are either A or T. Allele-specific hybridization for 14 primary tumor PCR products supported the results of the previous experiment with position-specific hybridization (Figure 16). It was of interest that all of the mutations in the second position of codons 12 and 13 indicated a G to T transversion mutation. However, a mutation in the first position of codon 12 indicated a G to A transition mutation, which is in agreement with previous reports on the rat liver model (McMahon et al., 1987; Sinha et al., 1988; McMahon et al., 1990b). To improve

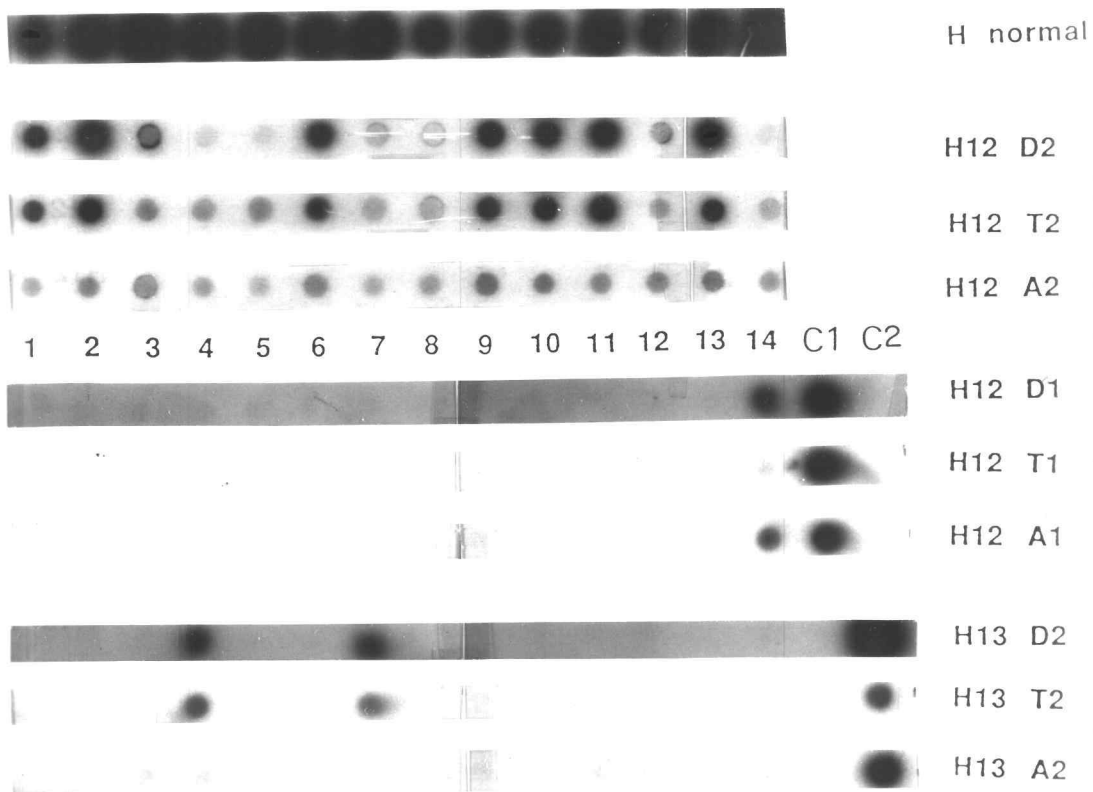


Figure 16. Position- and allele-specific dot blot analysis. Newly prepared PCR products of trout liver DNA induced by AFB1 were applied to five duplicate dot blots, which were then hybridized with position- and allele-specific oligonucleotide probes. Numbering for 14 samples follows the pattern established in Figure 15. Positive controls for H12 D1 (C1) and H13 D2 (C2) were applied.

representation, a densitogram was employed for the hybridized blot shown in Figure 16 (Figure 17). From the densitogram, a variation of H-ras normal probe binding was obviously observed among the tumor DNA PCR products. On the other hand, comparison of the H12 A2 probe binding with the H12 T2 probe binding indicated that the signals in H12 A2 could be considered as a background, which was not clear in the autoradiogram picture (Figure 16). In this respect, H12 D2 binding at PCR products of tumor DNA 3 was considered as a background. Because of the background problem in the H12 A2 probe shown in Figure 16, a dot blot assay with new PCR products from the same samples was repeated, including assay of the allele-specific control PCR products (Figure 18). This result confirmed the previously obtained results, and allele-specific controls for H12 T2 and H12 A2 failed to bind with normal probe, but bound with H12 D2 probes.

Additional 16 samples of AFB1-induced liver tumors were analyzed for trout H-ras mutations, using the same methodology detailed in Figure 16 (Figure 19). Of 16 tumor samples tested, 10 (63%) appeared to carry mutant alleles, and 8 samples indicated a mutation in the second position of codon 12, showing a G to T transition mutation. Two by each of the H12 D1 and H13 D2 probes showed mutations, but the H13 D1 and allele-specific mutations were not analyzed. Normal liver genomic DNA was also analyzed to determine the spontaneous mutations of the trout ras genes (Figure 20). In the normal counterpart tissue from the same individual containing the chemically induced tumors, no trout H-ras gene mutations around codons 12 and 13 were found.

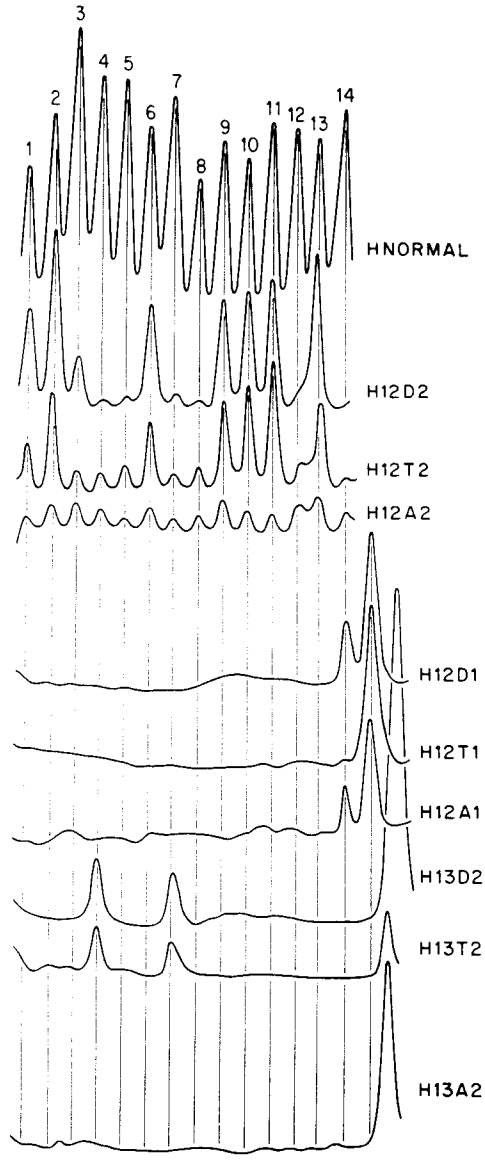


Figure 17. Densitogram of dot blot analysis. For improved resolution, the autoradiogram from figure 16 was scanned with the densitometer.

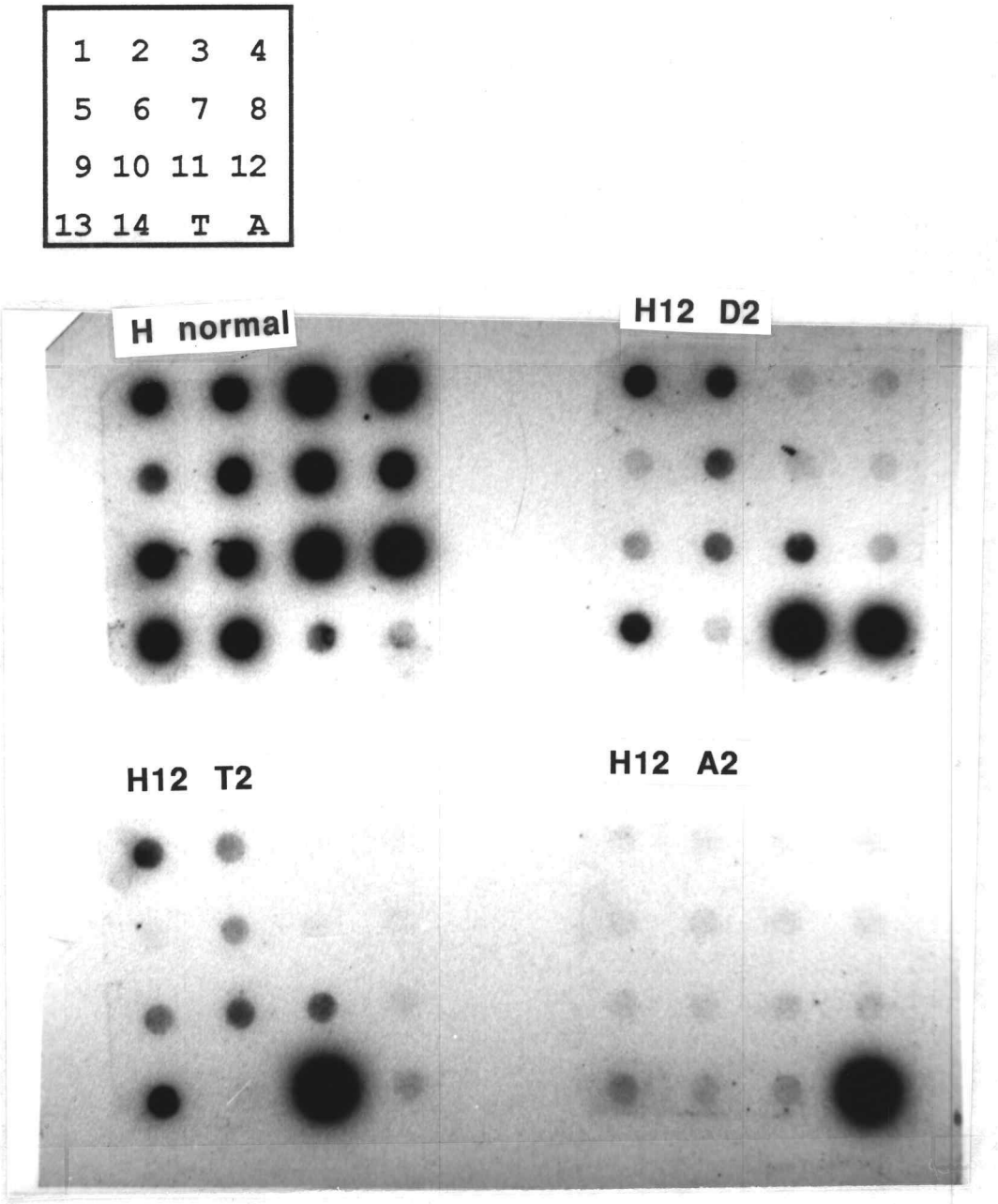


Figure 18. Dot blot analysis in the second position of codon 12. To repeat the results shown in Figures 15 and 16, PCR products were prepared and hybridized with the probes specific to the second position of codon 12. Controls were allele-specific H12 T2 (T) and H12 A2 (A).

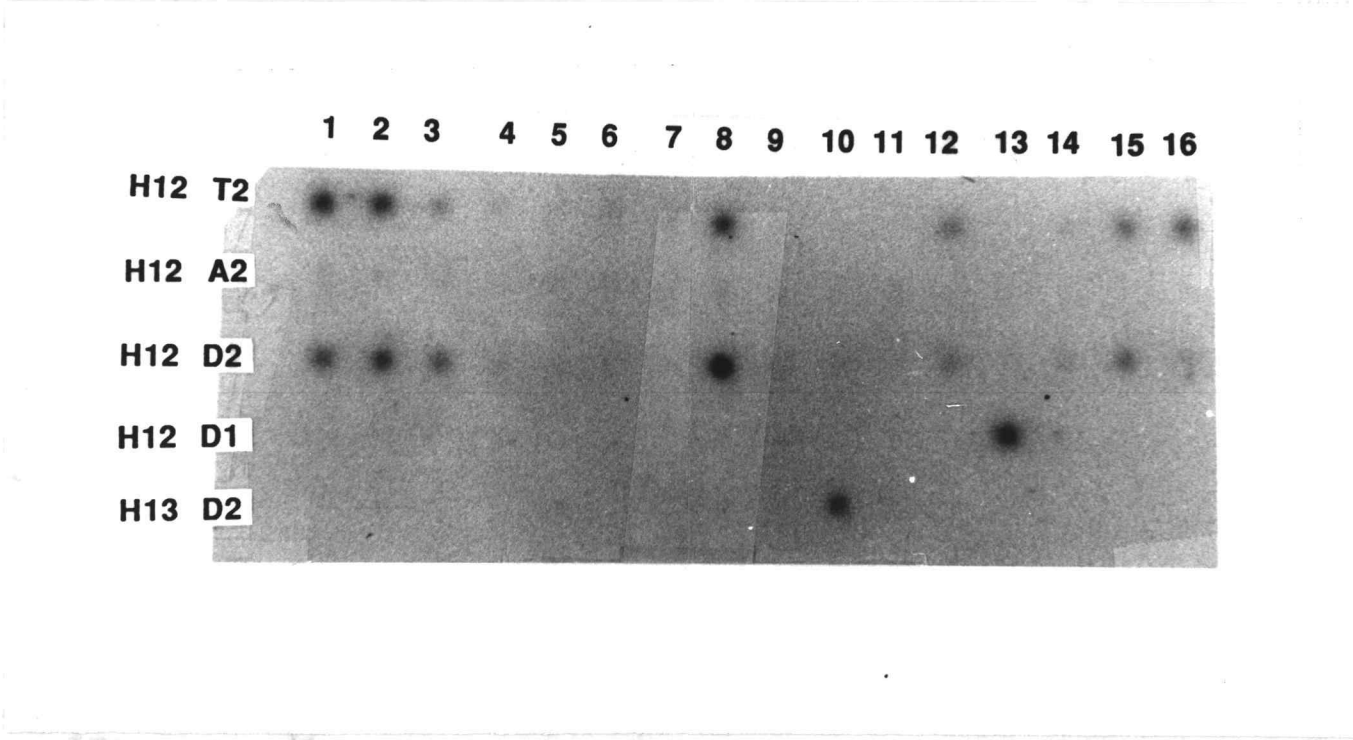


Figure 19. Dot blot analysis of 16 samples of AFB1-induced liver tumors. The procedure was identical to that established in Figure 16.

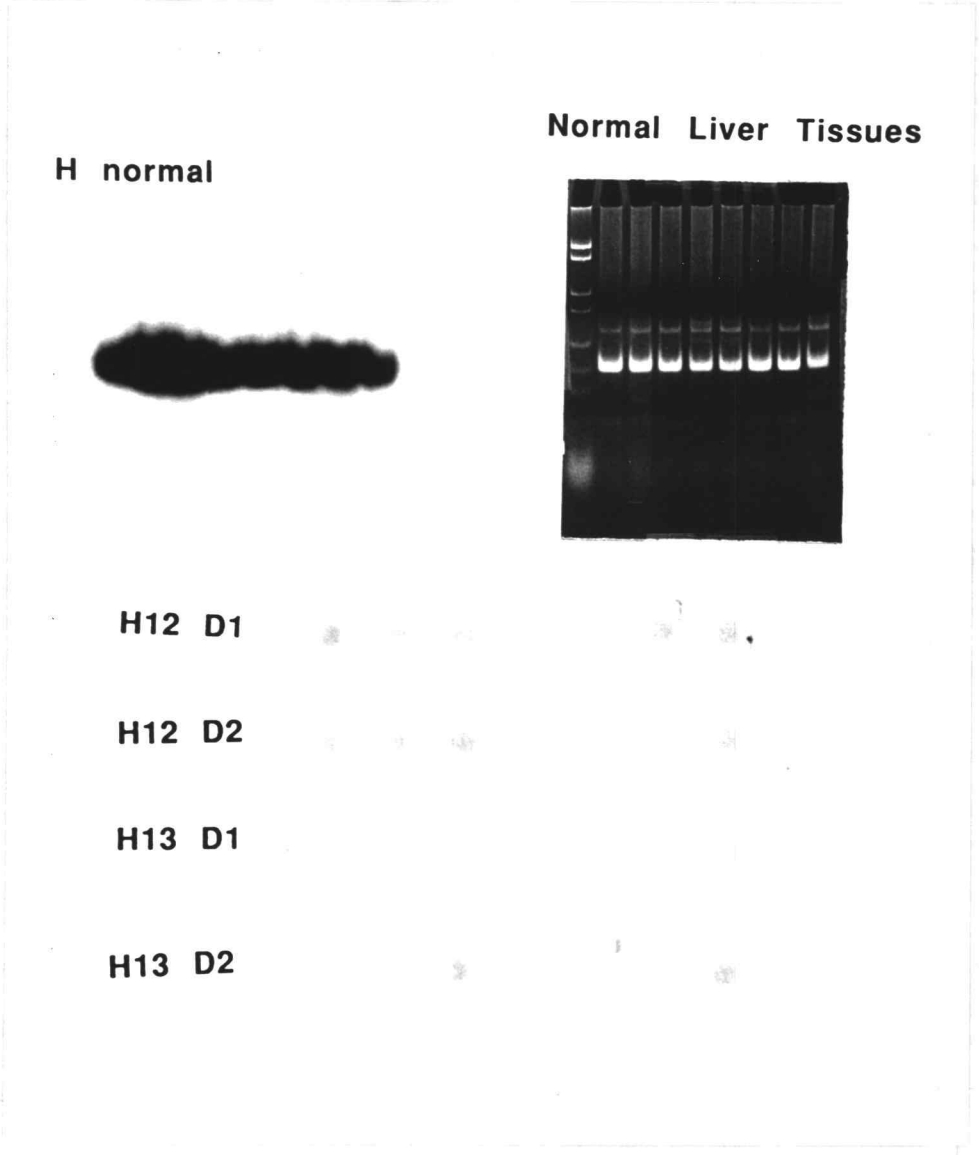


Figure 20. Mutational analysis of normal trout liver tissues. PCR products of eight normal liver tissues were separated on acrylamide gel (upper right panel), and after Southern transfer, probed with trout normal H-ras probe (upper left panel). The lower four dot blots were probed with the trout H-ras position-specific probes as indicated, showing no mutation in this region.

PCR clones screening and dideoxy sequencing.

To determine the ratio between normal and mutated allele in tumor tissues, the PCR clone screening method was applied. The whole mixture of PCR products from tumor DNA 6 was directly cloned into pUC19 vector by blunt-end ligation. Individual clones were then screened by position-specific probes, as well as a H normal probe, to select the base-changed PCR inserted clones and to determine the ratio between normal and mutated allele (Figure 21). In tumor DNA 6, only 1 of 20 H-ras positive colonies indicated a mutated sequence. These clones were used for dideoxy sequencing in order to confirm the results by allele-specific oligonucleotide hybridization of whole PCR mixture samples (Figure 22).

The same strategy was applied to DNAs 2, 7, and 14. The evidence for H-ras mutation in 14 trout liver tumors induced by AFB1 is summarized in Table 10. The PCR clone screening assay was used to confirm the presence and distribution of mutated alleles. This may have contributed substantially to the accuracy of the quantitative estimations of allelic frequency in the primary tumor DNA. Of the recombinant clones containing PCR products, most revealed a normal sequence, as was also reported for AFB1-induced rat liver tumor DNA by McMahon (1990b). PCR products containing a point mutation were only a small proportion of the total reaction population, which suggests that the majority of the cells within the tumor still contained normal ras sequence alleles or that non-neoplastic cells were mixed in the tumor tissue. Direct sequencing of the PCR products may not reveal the point mutations indicated by sequence-specific oligonucleotide hybridization in situations where a minor portion of

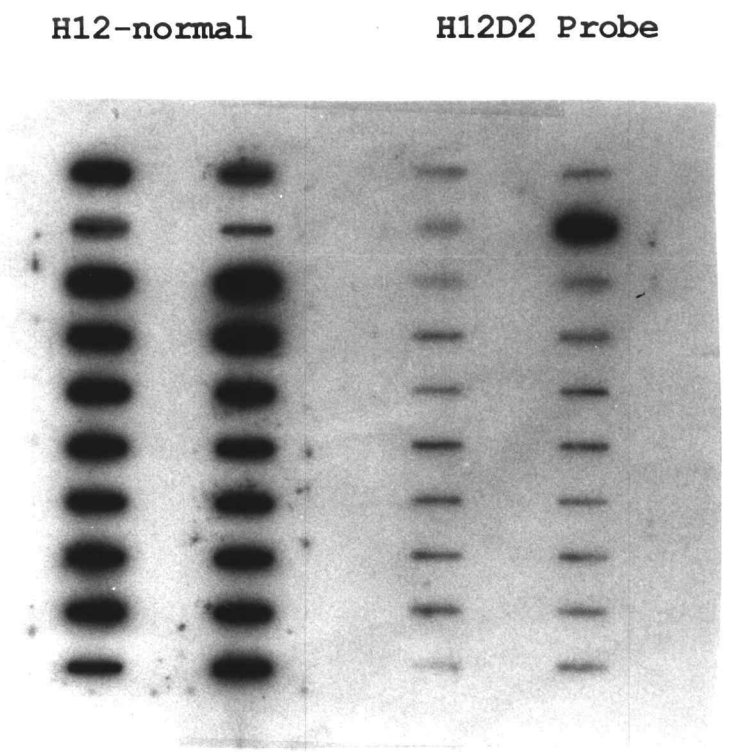


Figure 21. Position-specific probing of cloned PCR product from single tumor DNA. Individual clones were hybridized with normal and H12 D2 probes.

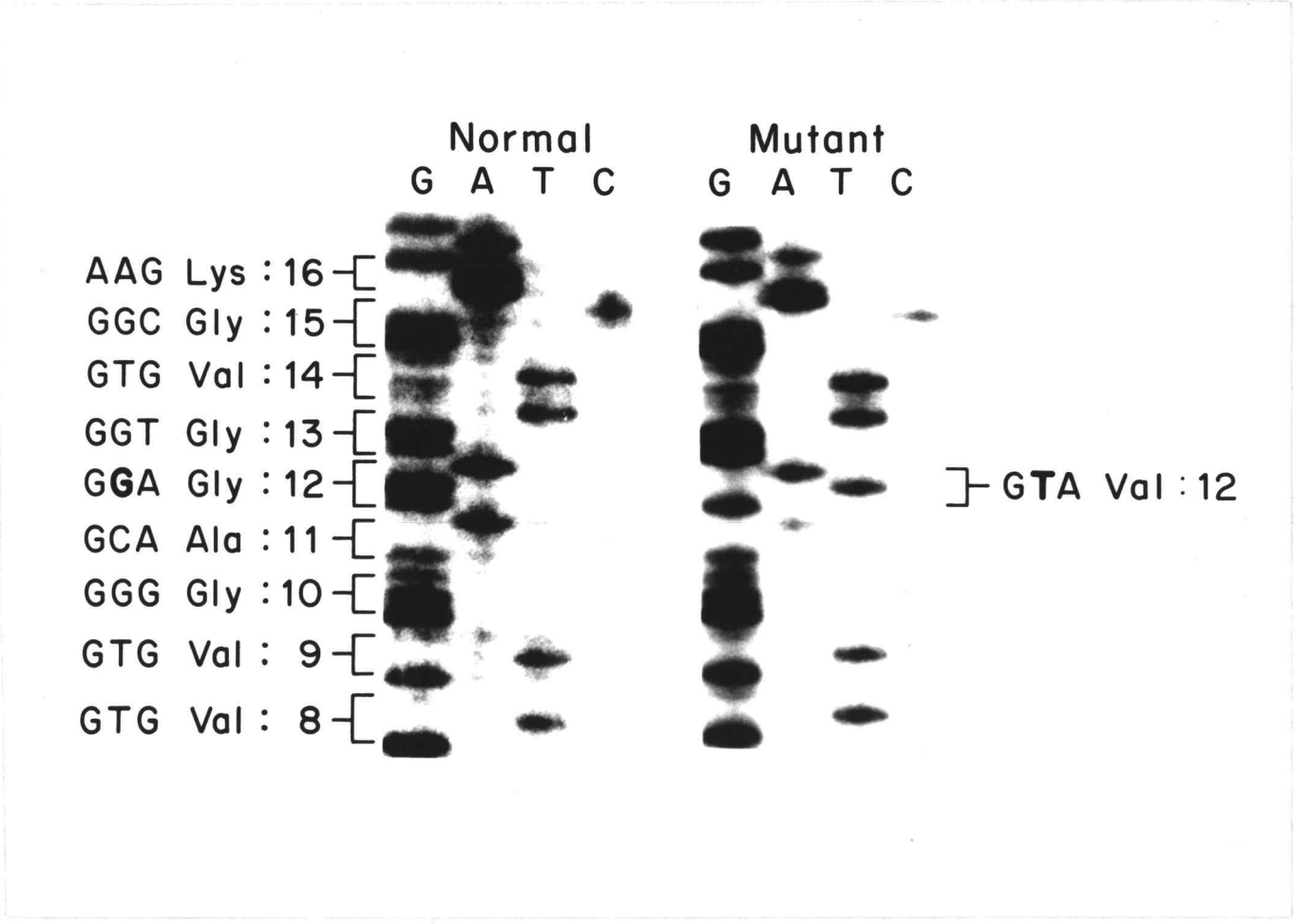


Figure 22. DNA sequence analysis of cloned PCR products. The positive clone with a H12 D2 probe (Figure 21) was sequenced (codon 12 GTA) with a normal sequence clone (codon 12 GGA), confirming the results for an allele-specific probe (H12 T2) hybridization (Figure 16).

Table 10. Summary of oligonucleotide probing and sequencing analysis. Trout H-ras gene mutations in AFB1-induced liver tumors were analyzed, based on position- and allele-specific oligonucleotide hybridization, PCR product colonies screening, and sequencing.

Tumor DNA	Total PCR DNA (Position-specific probes)	Total PCR DNA (Allele-specific probes)	Mutant : Normal Colonies (Position-specific probes)	Mutant Colonies (Sequencing)
14	Codon 12 : ?GA	AGA	1 : 8	Codon 12 : AGA
1	Codon 12 : G?A	GTA		
2	G?A	GTA	1 : 8	Codon 12 : GTA
6	G?A	GTA	1 : 19	Codon 12 : GTA
9	G?A	GTA		
10	G?A	GTA		
11	G?A	GTA		
13	G?A	GTA		
4	Codon 13 : G?T	GTT		
7	G?T	GTT	4 : 18	Codon 13 : GTT
3	Normal : GGA GGT			
5	GGA GGT			
8	GGA GGT			
12	GGA GGT			

the ras gene alleles are mutated.

Trout K-*ras* mutation.

The same methodology adopted for the trout H-ras mutation analysis was applied for the analysis of the trout K-ras mutations in the same 14 AFB1-induced trout liver tumors. PCR fragments (90 bp) with a pair of R01 and Rc30 primers were analyzed by five sets of position-specific oligonucleotide probes, including normal K-ras probe (Figure 23). In a Southern blot for the trout K-ras normal probe, three 85 bp negative controls, K12 D1 (N1), K12 D2 (N2), and K13 D2 (N3) and one 85 bp positive control (P) were included. All tumor DNA PCR products bound strongly with the K-ras normal probe suggesting that the PCR of liver tumor DNAs amplified the trout K-ras sequence differing from trout H-ras. For the mutated sequence probes, one negative (N) and one positive (P) control PCR products were used in dot blot assays. Since binding of 4 position-specific probes showed no variations among tumor DNA PCR products, this binding was interpreted as background. Although these tumor samples showed a high frequency of trout H-ras mutations, no mutations were observed in the trout K-ras gene. This result was not surprising since each tissue had the specific type of ras gene mutation as previously reflected in studies of human and animal carcinogenesis (Tables 2 and 3).

MNNG- and DMBA-induced trout tumors.

Mutational analysis of 15 trout liver tumors induced by MNNG was performed with the trout H-ras sequence, indicating that 13 of the samples (87%) were mutated. The specific point mutation was a G to A transition mutation in the first (3 of 13) and the second (10 of 13)

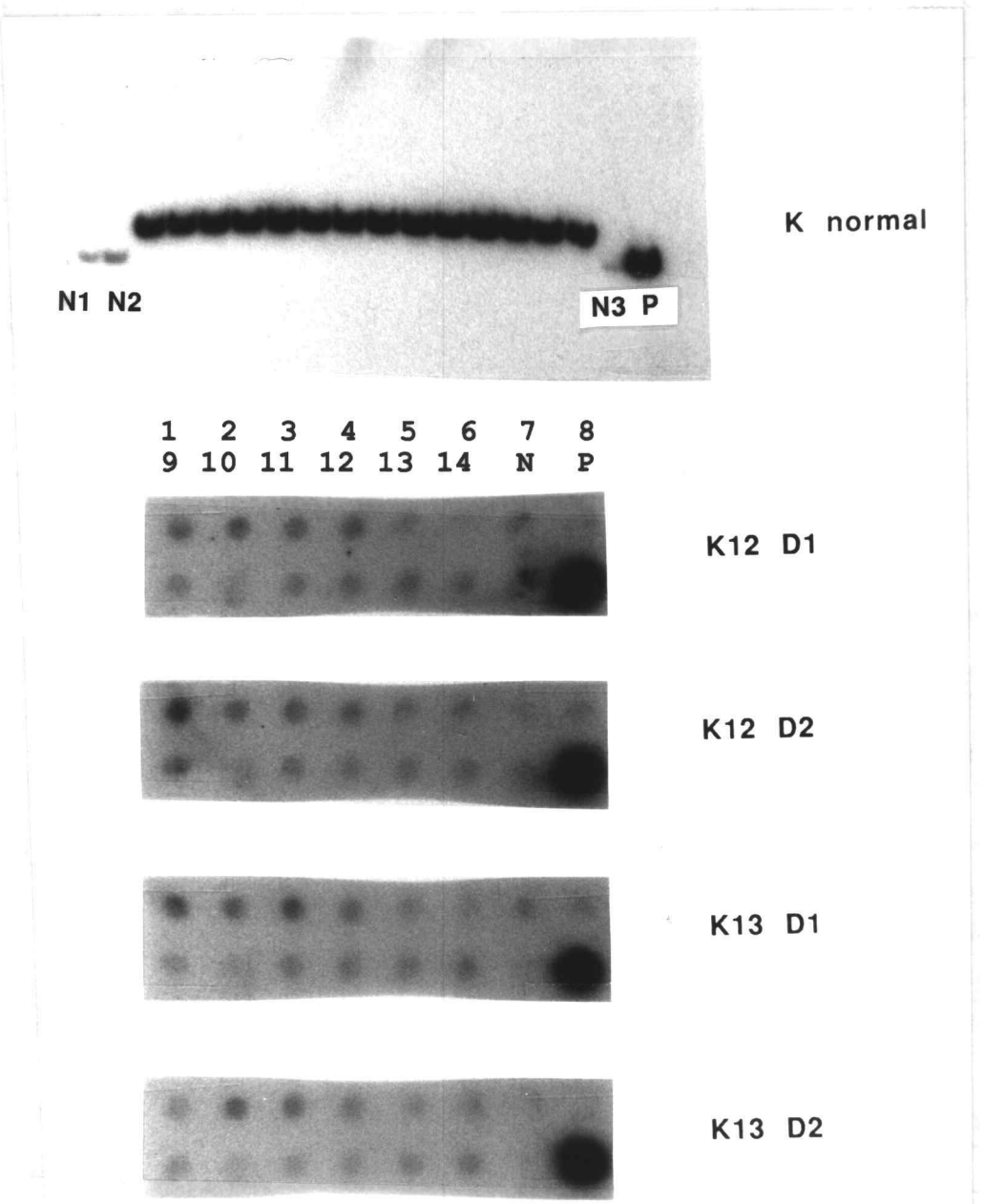


Figure 23. Dot blot analysis of trout K-ras mutation. Mutations in codons 12 and 13 of trout K-ras were analyzed with the samples identical to those in Figures 15 and 16. The upper panel shows an autoradiogram of the 14 tumor DNA PCR products after gel resolution, Southern transfer, and probing with K normal probe. The lower four dot blots were probed with the trout K-ras position-specific probes as indicated.

positions of codon 12 (Figure 24). However, 12 trout kidney tumors induced by MNNG failed to reveal mutations of the trout H-ras (Figure 25). It was of interest to note that a high incidence of the trout H-ras mutations in liver tumors by MNNG was not detected in kidney tumors.

Mutation analysis at codon 61 was not completed owing to the loss of most DMBA tumors, but PCR amplification spanning 126 bp between the 38 and c79D primers was successful with other minor bands. The PCR products of four DMBA-induced liver tumors and two normal liver tissues were hybridized with a normal allele oligonucleotide probe of codon 61 (Table 6), showing positive signal at 126 bp on autoradiogram (Figure 26).

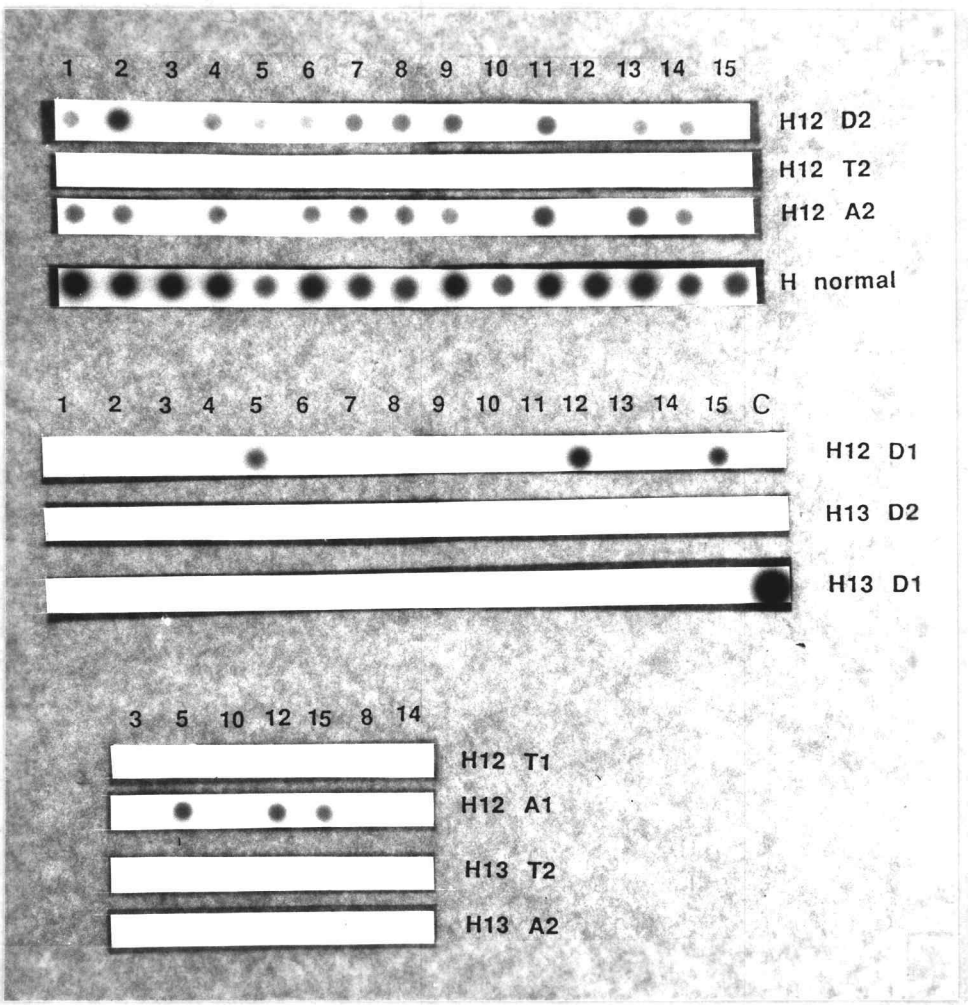


Figure 24. Dot blot analysis of MNNG-induced liver tumor DNA. Mutations in codons 12 and 13 of trout H-ras were analyzed with 15 trout liver tumors induced by MNNG.

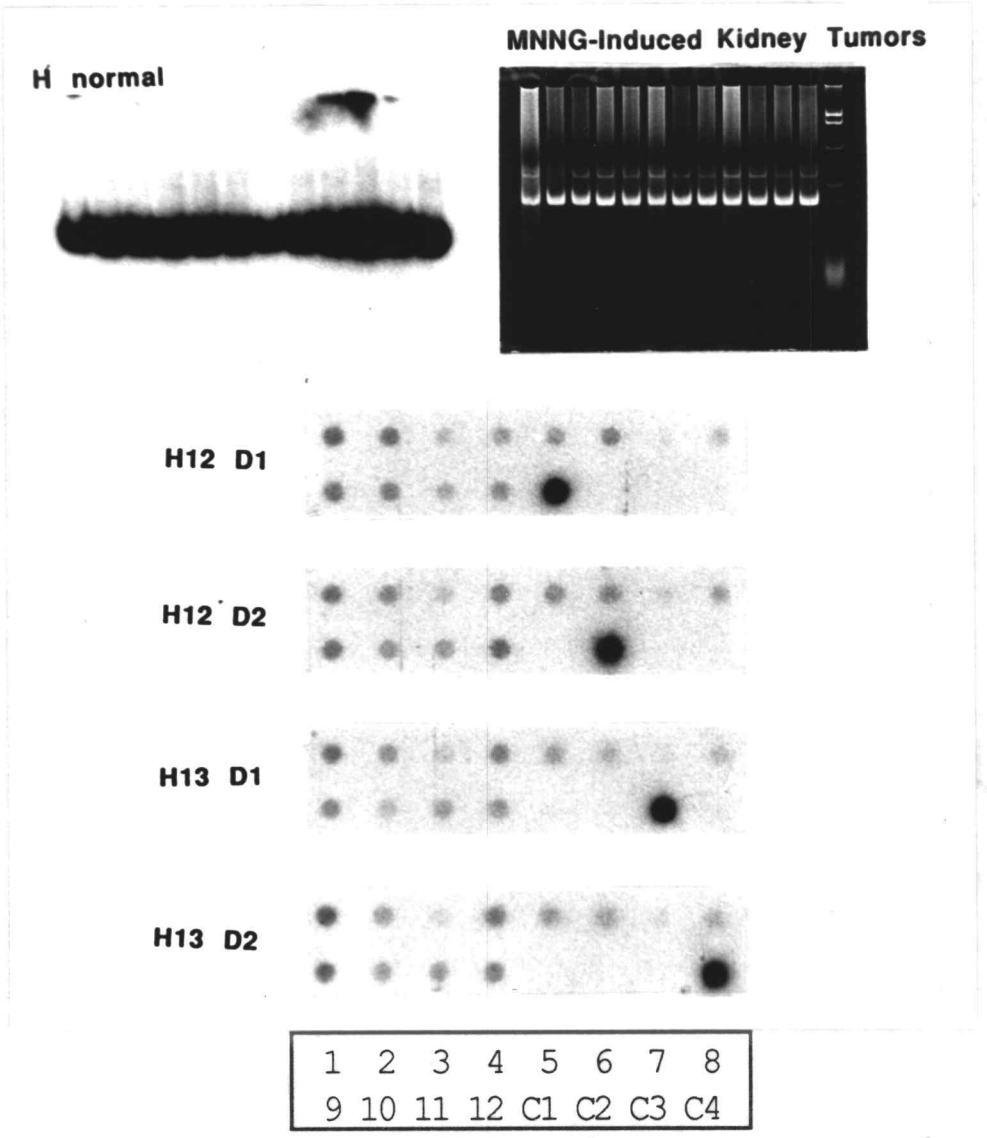


Figure 25. Dot blot analysis of MNNG-induced kidney tumor DNA. Mutations in codons 12 and 13 of trout H-ras were analyzed with PCR products of 12 trout kidney tumors induced by MNNG (upper right panel). The upper left panel shows Southern blot hybridization with trout H-ras normal probe. In the dot blot assay (lower panel), four mutated PCR product controls, H12 D1 (C1), H12 D2 (C2), H13 D1 (C3), and H13 D2 (C4), were used.

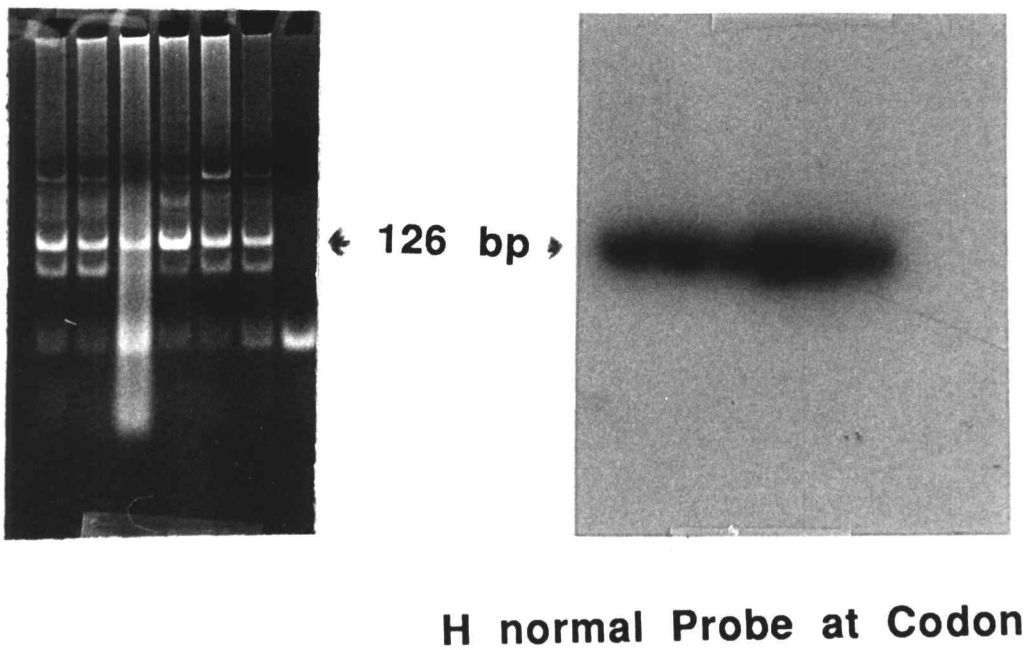


Figure 26. Oligonucleotide probing of trout H-ras sequence around codon 61. Following the amplification of exon 2 of trout H-ras from DMBA-induced trout liver tumors and normal livers, 126 bp of PCR products were separated on acrylamide gel (left panel). Southern blot of PCR products were probed with a normal sequence oligonucleotide probe around codon 61 (right panel).

DISCUSSION

Trout model for carcinogenesis studies.

Since the activated ras oncogene was first identified in human tumor cells in 1982 (Table 1), the mutation analysis of oncogenes has received great attention. The principal research questions have been directed at the causes of oncogene activation and the sequential molecular events associated with the different stages of carcinogenesis. Because the etiology of tumor initiation and development can be carefully controlled in animal model systems, mice and rats have been widely used for the studies of chemical carcinogenesis. Knowledge of the reproducible induction of specific types of mutations by particular chemical carcinogens has been accumulating, wherein the ras gene family has proved to be a highly prevalent target sequence in animal models as well as in human tumorigenesis (Tables 2 and 3). The trout model system possesses certain unique features (Table 5), including essentially zero background tumor incidence and persistent carcinogen-DNA adduct due to poor DNA repair activity (Walton et al., 1984). Furthermore, with the lower research cost, a sufficient number of animals can be used to provide a firm statistical basis for the comparison of data. Moreover, this approach reduces the need to use rats or other mammalian species, supporting the important basic research processes from the studies of non-mammalian species. At the molecular level, studies of trout, separated from mammals by 400 million years of evolutionary divergence, can be used to provide an

essential basis for comparative research with rodent models.

Unfortunately, the molecular genetics of carcinogenesis has been little studied in fish systems. Recently, several fish model studies at the molecular level have been reported. A ras gene was cloned and sequenced from goldfish (Nemoto et al., 1986). The sequence of the myc gene has been identified from the rainbow trout (Van Beneden et al., 1986). Mutations of K-ras genes in winter flounder (McMahon et al., 1990a) and Atlantic tomcod liver tumors (Wirgin et al., 1989) have been reported, but without firm knowledge of the ras sequences involved. Prior to the analysis of ras gene mutation in rainbow trout tumors, substantiated information on the ras gene sequence was required. In this study, a ras gene in trout liver was identified using in vitro PCR amplification. Based upon this information on the trout ras gene, mutations in experimentally induced trout tumors were analyzed with a combination of the PCR and oligonucleotide hybridization methods.

PCR carryover.

The ability to amplify discrete DNA fragments by factors of millions to billions by PCR provides a great advantage for the detection and manipulation of nucleic acid in trace amounts. However, extreme precautions must be taken with the PCR preparation and environment (Lo et al., 1988; Kwok and Higuchi, 1989). From this aspect, there have been a number of interesting reports using the PCR. In spite of their large evolutionary divergence, McMahon et al. (1990a) reported an identical ras gene sequence for winter flounder and the rat. In addition, it was reported that N-ras mutations were detected in all normal rat livers as well as in AFB1-induced rat liver tumors

(McMahon et al., 1990b). A clear possibility is that these puzzling findings reflect PCR plasmid carryover problems.

Identification of trout K-ras.

To confirm the sequencing result for the 90 bp PCR product using R01 and Rc30 primer pairs, the experiment was repeated using newly prepared trout genomic DNA from normal livers as well as liver tumors. The primers were also changed to produce large sizes of PCR products, 111 bp and 237 bp, using R01 and c37 from genomic DNA template or R01 and c79 from cDNA template, respectively. These PCR products included the identical sequence to the 90 bp PCR product, suggesting that this sequence was a real trout sequence expressed in mRNA. For complete sequence information on the trout K-ras gene, it will be useful to screen for clones within a trout liver cDNA library.

For further investigation of the identity of the ras gene, the Southern blot of trout genomic DNA could be probed with trout PCR product probes. Preliminary experimentation using the PCR product of human K-ras plasmid demonstrated a signal in the Southern hybridization of trout genomic DNA. Although a tomcod tumor DNA transfectant also was detectable using a mammalian K-ras probe, this assay may not be quite specific for the identification of the trout ras sequence, particularly in the N-terminal conserved sequences; thus, no definitive conclusions were drawn from this experiment. Another method of obtaining the transforming sequence from trout tumors is the NIH3T3 transformation assay with trout tumor genomic DNA. However, the application of the PCR with transformed NIH3T3 DNA template and R01/Rc30 primers produced mouse-specific PCR

products which failed to hybridize with trout K-ras specific oligonucleotide probes.

Oligonucleotide hybridization.

For the detection or screening of point mutations in human tumors or animal systems, it is important to select an appropriate method from among several available techniques. PCR is a favorite method for target sequence amplification. As shown in Tables 2 and 3, it is obvious that oligonucleotide hybridization method has contributed much to the screening of point mutations. In addition, the relative amounts of normal and mutated alleles could be determined by scanning the signals of the normal and mutated probes. The oligonucleotide sequence of an identified trout H-ras gene was compared with the reported sequences from several vertebrate animals (Table 8). Since all species differ by more than one base, each specific sequence can be determined by the oligonucleotide hybridization technique.

Positive controls for the hybridization of each oligonucleotide probe were prepared by the reamplification of diluted 111 bp PCR products as a template, rather than using genomic DNA (Rochlitz et al., 1988). The physical incorporation of each oligonucleotide probe into 85 bp PCR products provided a better yield and good quality products (Figure 13). Because of their smaller size and the lack of the 5'-primer sequence, these control PCR products eliminated experimental errors of false identification or the amplification of carryover DNA. This approach provides the advantages that large numbers of samples can be screened quickly and sensitively. Moreover, it allows the detection of all possible single-base mutations

at every possible position and an allele-carrying two or more base substitutions as long as the mutation-specific oligonucleotide probes are synthesized (McMahon et al., 1990b). This technique could also be applied to sequence-specific quantification in combination with densitography since each oligonucleotide probe binds proportionally to the target sequence (Table 14).

Mutation analysis of trout tumors.

DNA from 10 of 14 (72%) trout liver tumors initiated by AFB1 were mutated in codons 12 and 13 of the trout ras genes based on a combination of PCR and oligonucleotide hybridization methods. The major point mutation of the ras gene, for 7 of 10 point mutations, was a G to T transversion at the second position of codon 12. This confirmed that codon 12 is one of the critical positions for ras gene activation and the guanine base at the 5'-flanking position influences the carcinogenic activity of the second base of codon 12 (5'-GGA-3'). Two of the point mutations also had a G to T transversion mutation at the second position of codon 13. However, one tumor showed evidence of a G to A transition point mutation at the first position of codon 12, and there were no mutations in the first position of codon 13. An additional 16 trout liver tumors initiated by AFB1 were analyzed and demonstrated a similar pattern. A total of 10 samples (63%) showed evidence of mutations, 8 of which were major G to T mutations in the second position of codon 12. One each from the H12 D1 and H13 D2 probes also indicated a mutation. By comparison, the same experiment with normal liver tissues showed no trout H-ras mutations (Figure 20). Similar mutation results were reported in aflatoxin B1-initiated rat tumors (McMahon et al., 1987; Sinha et al.,

1988; McMahon et al., 1990b). This AFB1 mutational spectrum in both trout and rat liver tissue has important implications for patients with high chronic AFB1 exposure. Further analysis of the subtype populations of ras gene mutation within individual human liver tumors and mutation frequencies at various stages of tumor development following AFB1 initiation are suggested.

From the results of oligonucleotide hybridization (Figures 15 and 16), it was obvious that PCR products containing point mutations were only a small proportion of the total reaction population. This indicated that the majority of the cells in the tumor mass had a normal ras gene sequence. Of the recombinant clones containing PCR products previously identified by sequence specific hybridization as containing point mutations, most revealed a normal sequence (Figure 21 and Table 10). PCR-clones screening assay confirmed the presence and distribution of mutated alleles with respect to the total number of clones screened The ratio between normal and mutated allele in trout liver tumor induced by AFB1 ranged up to about 20%, which is comparable to the ratio for the rat (McMahon et al., 1990b). This finding may offer a substantial contribution to the quantitative estimation of the allelic frequency in the primary tumor DNA, but further systematic examination is suggested.

This apparent low frequency of mutated sequences in tumor tissues can be explained as follows; Activating point mutations occur in only one malignant cell type within the tumor (Watatani et al., 1989). Clonal expansion of malignant cells containing activated ras gene then give rise to the adenoma (You et al., 1989). Alternatively, connective tissue or other non-neoplastic cells within tumors may significantly

reduce the intensities of the mutation-specific signals (Grunewald et al., 1989). For instance, K-ras gene mutations were not found in hepatocellular carcinoma, but have been found in cholangiocarcinoma (Tada et al., 1990). In this respect, the trout liver tumors without ras gene mutations in this study may contain pure hepatocellular carcinoma. Methods are now available to test this hypothesis.

Double mutations of ras genes have been reported in different alleles (Neri et al., 1988, Farr et al., 1988). Recently, triple ras gene mutations within the same allele have been reported (McMahon et al., 1990b). However, the results of this study failed to reveal the existence of double mutations.

For tissue-specific mutations, it was found that trout liver tumors induced by AFB1 revealed H-ras mutations, but there were no K-ras mutations (Figures 15 and 23). Moreover, in the case of MNNG carcinogenesis, trout liver tumors revealed a high frequency of H-ras mutation, but at the same time there were no H-ras mutations in the trout kidney tumors (Figures 24 and 25). These results provide support for the concept that specific ras genes are involved in certain tissues, wherein tissue-specific factors are involved in the determination of carcinogen-specific mutation spectrum (Balmain and Brown, 1988). This could offer an explanation of the ubiquitous incidence of the ras gene mutations in eukaryotes, the importance of the ras gene products in cell physiology, and the conservation of ras gene family throughout the course of evolution. It is interesting that the treatment of mouse (B6C3F1) and rat (Fischer 344) liver tumors with an ethylating agent, diethylnitrosoamine (DEN), has shown a high incidence of ras gene mutation (42%) in the mouse, but no mutations

in the rat (Stowers et al., 1988). The repeated A to T transversion in the second position of codon 61 of the H-ras gene has occurred in mouse and rabbit skin tumors initiated by DMBA (Quintanilla et al., 1986; Bizub et al., 1986; Leon et al., 1988) suggesting that certain types of ras gene mutations are involved in tumor formation within specific tissues.

The results of this investigation indicate that molecular events in fish carcinogenesis resemble those of the higher vertebrates, including man. This is the first substantiated study of ras gene mutations in experimentally designed fish models. A high percentage of consistently activating point mutations in the absence of spontaneous mutations would indicate that fish could be an inexpensive and acceptable alternative chemical carcinogenesis model system (Powers et al., 1989). Which factors determine the type of ras gene mutation is still open to be answered, but the reproducible activation by chemical carcinogens suggests that DNA adduct formation is a principal factor in causing mutations. Further analysis of the expression of the mutated mRNA allele and its ratio to normal mRNA allele is suggested. The mutational spectrum of various carcinogens in a variety of trout tissues, in comparison with those of rodent animal systems, could increase our knowledge of the molecular events involved in neoplastic transformations.

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