AN ABSTRACT OF THE THESIS OF

Kathy A. Mangold for the degree of Doctor of Philosophy in Toxicology presented on December 21, 1989

Title: Characterization of a *ras* Gene in Rainbow Trout.

Abstract approved: Redacted for Privacy

George S. Bailey, Major Professor

The rainbow trout (*Oncorhynchus mykiss*) model of chemical carcinogenesis is becoming increasingly important as a supplement to rodent studies. However, much of the molecular biology of the carcinogenic response is still unknown in the trout model. The *ras* gene family has been implicated in the tumorigenesis of both spontaneous and chemically-induced tumors in mammals. This study is the first to characterize a *ras* proto-oncogene in rainbow trout. To accomplish this, the *ras* gene sequence was amplified *in vitro* by using polymerase chain reaction (PCR).

Two synthetic and degenerative oligonucleotide sequences based on a consensus mammal/goldfish *ras* sequence were used as primers in the PCR procedure. An 800 base pair (bp) sequence was amplified from trout genomic DNA and hybridized with a human c-Ha-*ras*

sequence. The initial amplifications of trout liver cDNA using the PCR procedure with the synthetic *ras* primers resulted in a single product of approximately 216 bps. However, this amplified "trout" 216 bp product was subsequently shown to be an artifact of carryover from a human Ki-*ras* plasmid. Carryover is a common problem found in many laboratories involved with the PCR procedure, and extensive precautions were used to eliminate the problem in our laboratories.

The 800 bp PCR product was cloned and sequenced using Taq polymerase. RT-8, a clone containing the 800 bp insert, was shown to have 91% homology to the first two exons of mammalian c-Ha-*ras* gene and lesser homology to other *ras* genes. Amplification of trout liver cDNA using specific primers based on the RT-8 sequence resulted in the amplification of sequences identical to the sequence of the RT-8 insert without an intron, as well as unique sequences, which may represent additional trout *ras* genes. The PCR procedure was modified to identify sequence information immediately 3' of the known trout *ras* sequence. Partial sequences of at least two different trout *ras* genes are presented. With this new information, analysis of DNA sequence information from chemically initiated tumors may elucidate the role activation of *ras* genes plays in the trout model of carcinogenesis.

Characterization of a *ras* Gene in Rainbow Trout

by

Kathy A. Mangold

A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirements for

the degree of

Doctor of Philosophy

Completed December 21, 1989

Commencement June 1990

APPROVED:

Redacted for Privacy

Professor of Toxicology in charge of major

Redacted for Privacy

Chairman of Toxicology Program

Redacted for Privacy

Dean of Graduate School

Date thesis is presented December 21, 1989

Typed by researcher for Kathy A. Mangold

ACKNOWLEDGEMENT

I would like to thank the following people for their assistance in the completion of my thesis: Dr. George Bailey, for his guidance during the duration of my doctoral program; the members of my committee, for their assistance when I encountered difficulties in my research; Dr. Koenraad Mariën, for his instruction into the intricacies of DNA sequencing; Kate Mathews, for her assistance and friendship, both in and out of the laboratory; my many friends, for helping me to keep everything in perspective; and finally, my family, whose encouragement and support are greatly responsible for the accomplishment of this goal.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHARACTERIZATION OF A *ras* GENE IN RAINBOW TROUT

INTRODUCTION

Cancer is not a single disease, but a collection of diseases possibly with many different causes but with a common basis: uncontrolled cell growth often accompanied by loss of cell differentiation. Although epigenetic factors leading to cancerous growth are known (Reitz & Watanabe, 1983), genetic mechanisms continue to be the primary focus of cancer studies. Investigations ranging from whole animal studies to alterations in the DNA are in progress to probe into the cause, prevention and treatment of cancer.

The idea of a genetic mechanism for cancer is not a recent development. Evidence supporting a genetic basis for cancer include 1) familial clustering of many forms of cancer indicating genetic predisposition (Hansen & Cavenee, 1987); 2) specific chromosomal abnormalities associated with particular cancers (Sasaki, 1985; Klein, 1983; Baker et al., 1989); and 3) transfer of cell-free extracts from tumor cells into receptive animals resulting in the formation of tumors (Shih et al., 1979b). A proposal by Huebner and Todaro (1969) suggested the possibility of cellular genes in control of cellular differentiation and growth in normal cells going awry in neoplastic cells. An "oncogene" was later defined as being a eukaryotic gene

which 1) encodes for a protein, 2) is preserved in evolution and presumably fulfills an essential physiological role in the normal cell, and 3) has the potential to become a dominant oncogenic determinant (Vogt et al., 1985). The first oncogene to be isolated was the *src* gene found in the avian Rous sarcoma virus (Rous, 1911). The first oncogene to be implicated in human cancer was a member of the *ras* family (reviewed in Levinson, 1986; Barbacid, 1987; Lacal & Tronick, 1988; Santos et al., 1982).

The *ras* Oncogene

Members of the *ras* family were first identified as the transforming genes of seven highly oncogenic retroviruses. The first virus to be isolated was the Harvey-Murine Sarcoma Virus (MSV; Harvey, 1964) containing the v-Ha-*ras* oncogene. A second related *ras* oncogene, known as v-Ki-*ras*, was found in an isolate of the Kirsten-MSV (Kirsten & Mayer, 1987). Subsequently, five different viral isolates have been reported, each containing a form of the v-Ha-*ras* oncogene: BALB-MSV (Peters et al., 1974), Rasheed-MSV (Rasheed et al., 1978), AF-1 (Franz et al., 1985), and NS.C58 MSV-1 and -2 (Fredrickson et al., 1987). The structure of the viral *ras* genes and the analysis of deletion mutants of Ha-MSV (Cichutek & Duesberg, 1986) showed that the *ras* gene sequences were sufficient for transformation of the recipient cells to the oncogenic phenotype. It was

eventually shown that the retroviruses had captured distantly related cellular sequences from their rodent hosts (Ellis et al., 1981), and subsequently modified the sequences to conform with the viral genomes. There is no current evidence for any involvement of oncogenic viruses carrying *ras* genes in human cancer.

Two cellular homologs of both Ha-*ras* and Ki-*ras* were identified in mammalian genomes using the viral sequences as probes (Chang et al., 1982b). In each case, one locus contained a pseudogene derived from a processed mRNA (Miyoshi et al., 1984; Ruta et al., 1986a; McGrath et al., 1983). The pseudogenes (known as c-Ha-*ras*-2 and c-Ki-*ras*-1 respectively) lacked any intervening sequences and differed from the viral and active cellular sequences by base substitutions, insertions, and deletions resulting in several premature terminations in-frame. In humans, the locus for c-Ha-*ras*-2 is located on the X chromosome at Xpter-q28 (O'Brien et al., 1983), and c-Ki-*ras*-1 is located at 6p12-p23 (McBride et al., 1983; O'Brien et al., 1983). Active genes corresponding to Ha-*ras* and Ki-*ras* were located at the other loci identified with the respective viral probes. In the case of the Ha-*ras* proto-oncogene (known as c-Ha-*ras*-1), the gene is composed of 567 base pairs (bps) separated into four translated exons preceded by an untranslated exon, and is located on human chromosome 11 at p15.1-p15.5

(McBride et al., 1982; O'Brien et al., 1983). The entire gene spans less than six kilobases (Kbs) in length in all mammalian genomes (Capon et al., 1983).

No traditional promoter sequences, such as TATA or CAAT signals, have been found for c-Ha-*ras* but the 5' promoter region has a high GC content (Honkawa et al., 1987) and several GC boxes similar to the human EGF-receptor promoter (Ishii et al., 1985). The DNA binding protein Spl binds to the promoter region (Ishii et al., 1986), and the promoter region is also responsive to the phorbol ester TPA and to serum factors (Imler et al., 1988). Two separate enhancer regions containing positive and negative elements have been identified in the first intron of the human c-Ha-*ras* gene (Honkawa et al., 1987). An additional enhancer region located in the fourth intron was identified by a point mutation responsible for increased expression and transforming activity in the cloned T24 colon cancer oncogene (Cohen & Levinson, 1988). Also identified in the 3' end of the cloned T24 oncogene is a variable tandem repeat (VTR) sequence which has been shown to have transcriptional enhancer activity and to be highly polymorphic in the human population (Spandidos & Holmes, 1987). The polyadenylation signal of the human c-Ha-*ras* gene has been identified 407 bps downstream of the fourth exon, resulting in mRNA transcripts of 1.1-1.3 Kbs in length

(Gibbs et al., 1984; Goldfarb et al., 1982; Reddy, 1983; Ruta et al., 1986b).

The Ki-*ras* proto-oncogene (named c-Ki-*ras*-2) is also divided into four translated exons with an untranslated exon upstream of the first translated exon. However, there are two alternative fourth exons (identified as 4a and 4b) which encode for proteins of 188 and 189 amino acids respectively (McGrath et al., 1983). Two mRNA transcripts (5.2 and 2 Kbs) have been identified in humans as Ki-*ras* specific (Ruta et al., 1986b). The intervening sequences of the mammalian c-Ki-*ras*-2 gene are also much larger than c-Ha-*ras*-1 with the total gene spanning up to 50 Kbs. Possibly due to its large genomic size, the transcriptional regulatory elements of c-Ki-*ras*-2 have not been described in as much detail as the regulatory elements of the human c-Ha-*ras* gene. The human c-Ki-*ras*-2 gene has been mapped to 12p12.1-pter (McBride et al., 1983; O'Brien et al., 1983).

A third closely related member of the *ras* family was identified in a NIH 3T3 mouse cell line transfection assay (Shimizu et al., 1983). N-*ras* was originally isolated from a human neuroblastoma cell line, and to date no naturally occurring retrovirus has been isolated containing a viral homolog of N-*ras*. The structure of N-*ras* is comparable to the other *ras* proto-oncogenes, except that two additional

untranslated exons exist downstream of the fourth exon, and several RNA splicing options have been observed. Three mRNA transcripts of 5.0, 2.4, and 1.3 Kbs have been identified as N-*ras* specific (Ruta et al., 1986b). The promoter region of N-*ras* is similar to that of c-Ha-*ras*-1 with no obvious TATA or CAAT box but with a GGGCGG sequence upstream of the first exon. The human N-*ras* gene is located at 1p22-p32 (McBride et al., 1983; Davis et al., 1983; DeMartinville et al., 1983).

The cellular counterparts of the viral *ras* oncogenes are evolutionarily well-conserved and present in low copy numbers in the various genomes. In all vertebrates, the three classical *ras* genes are 100% homologous for the first eighty amino acid residues with greater than 80% homology at the DNA sequence level. Amino acid residues 80 through 165 have less homology, but are still very similar between the different *ras* gene products. The carboxy terminus has the greatest divergence among the *ras* genes, with the only constant being a cysteine residue followed by two hydrophobic residues at the extreme carboxy end. The exon sequences of several *ras* genes in various vertebrates are shown in Table 1.

Distantly related genes have been identified with 30-65% DNA sequence homology to the three primary *ras* genes, leading to the classification of *ras* as a

Table 1. Several vertebrate ras sequences.

EXON 1

```
                  10         20         30         40         50         60
Human H[1]  ATGACGGAATATAAGCTGGTGGTGGTGGGCGCCGGCGGTGTGGGCAAGAGTGCGCTGACC
Rat H[2]    -----A-----C-----T--------------T--A--C-----A--------C------
Chicken H[3]-----C--G--C-----------A-----A--T--A-----C--G-----C--TT----G
Human K[4]  -----T--------A--T-----A--T--A--T--T--C--A-----------CT----G
Rat K[5]    -----T--G-----A--T-----A--T--A--T--T--C--A-----------CT----G
Mouse K[6]  -----T--G-----A--T--------T--A--T--T--C--A--------C--CT----G
Goldfish[7] -----A----------T-----C--A--G--T--A--C--------------T--C--C
Human N[8]  -----T--G--C--A----------T--A--A--T-----T--G--A--C--A-----A
Mouse N[9]  -----T--G--C--A----------T--A--A--T-----T--G--A--C--C-----G

                  70         80         90        100        110
Human H     ATCCAGCTGATCCAGAACCATTTTGTGGACGAATACGACCCCACTATAGAG
Rat H       ------------------------------G--T--T-----------
Chicken H   --A-----C--T--------------T--T--G----------A------
Human K     --A-----A--T-----T-----------------T--T--A--A------
Rat K       --A-----A--T-----T--C--------T-----T--T--T--G------
Mouse K     --A-----A--T-----T--C--------T--G--------T--G------
Goldfish    -----A--C----------C--------------------A--C------
Human N     --------A----------C-----A--T-----T--T-----C------
Mouse N     --------A----------C--------T-----T--T-----C------
```

Table 1. Several vertebrate ras sequences (cont.).

EXON 2

```
                120       130       140       150       160       170       180
Human H   GATTCCTACCGGAAGCAGGTGGTCATTGATGGGGAGACGTGCCTGTTGGACATCCTGGATACCGCCGGC
Rat H     --C----------A-----A-------------------TT-AC--------T-A--C--A--A--T
Chicken   ---------A-A-----A--C-----C-----A-----C--TT--C----------------G--G--G
Human K   ---------A-------A--A--A--------A--A--C--T--C-----T--T--C--C--A--A--T
Rat K     --C------A----A--A--A--A--------A--A--C--T--C-----T--T--C--C--A--A--T
Mouse K   --C------A----A--A--A--A--------A--A--C--T--C-----T--T--C--C--A--A--T
Goldfish  --C------A------------G-----------------T-----------T----C--T--A--T
Human N   -----T---A-A--A--A-----T--A-----T--A--C--TT----------A--------A--T--A
Mouse N   -----T-----A-----A-----G--------T-----C------C-------A-----C--A--T--A

                190       200       210       220       230       240
Human H   CAGGAGGAGTACAGCGCCATGCGGGACCAGTACATGCGCACCGGGGAGGGCTTCCTGTGT
Rat H     --A--A-----T--T-------------------------A--------------C---
Chicken   --------------T--------A------------A-A--G-----A--A--------C
Human K   --A-----------T--A---A--------------A-G--T-----------T--T---
Rat K     --A-----------T--A---A--------------A-A--T-----------T--T---
Mouse K   --A-----------T--A---A--------------A-A--T-----------T--T---
Goldfish  --------------------A----T---------A-G--A--A-----------C---
Human N   --A--A--------T------A-A-----A------A-G--A--C--A--------C---
Mouse N   --A-----------T------A-A------------A-G--A--C--A--G-----C---

                250       260       270       280       290
Human H   GTGTTTGCCATCAACAACACCAAGTCTTTTGAGGACATCCACCAGTACAGG
Rat H     --A----------------------C-----A--------T---------
Chicken   --C--------T--------------C----------------------
Human K   --A--------A--T--T--T--A--A-----A--T--T-----T--T--A
Rat K     --A--------A--T--T--T--A--A-----A--T--T-----T--T--
Mouse K   --A--------A--T--T--T--A--A-----A--T--T-----T--T---
Goldfish  --C--C--------T----------C-----------T-----C-----A
Human N   --A-----------T--T-G------A----C---T--TA---TC------
Mouse N   --A-----------T--T-G---A--A----CA--T--TA---TC------
```

Table 1. Several vertebrate ras sequences (cont.).

EXON 3

```
                300       310       320       330       340       350
Human H    GAGCAGATCAAACGGGTGAAGGACTCGGATGACGTGCCCATGGTGCTGGTGGGGAACAAG
Rat H      ----------G--------A--T--A-----T-----A--------------C------
Chicken    ----------GA-------A-----A-----T--C--C--------------A--T--A
Human K    --A--A--T---A-A--T--------T--A--T--A--T-----C--A--A--A--T--A
Mouse K    --A--A--T---A-A--A--------T--A--T-----T-----C-----A-----T---
Goldfish   --------A-----A--A----------G-----C--------C----------T---
Human N    --------T--G--A--A--A----------T--A--T--------A-----A------
Mouse N    -----A--T--G--T-----A--T--T--T--T--C--------------A--C------

                360       370       380       390       400       410
Human H    TGTGACCTGGCTGCACGCACTGTGGAATCTCGGCAGGCTCAGGACCTCGCCCGAAGCTAC
Rat H      --------------T--------T--G----------C--------T--T--C-----T
Chicken    -----T---C-A-----G--A-----GA-C-----A--G--------G-----G--T---
Human K    -----TT--C--T-T--A--A--A--CA-AAAA----------T-A--AA----T--T
Mouse K    -----TT--C--T-TA-A--A--A--CA-GAAA----------GT-A--AA-G--T---
Goldfish   -----T--TC-GT-C----G------CA-CAAG----------TT-A--A--G------
Human N    -----TT--C-AA--A-G--A--T--TA-AAAA--A--C--C--A--G---AAG--T---
Mouse N    ------T--C-AA--A-G--A--T--CA-AAAG--A--C--C--A--G---AAG--T---

                420       430       440       450
Human H    GGCATCCCCTACATCGAGACCTCGGCCAAGACCCGGCAG
Rat H      --------------T--A--A--A---------------
Chicken    --G----------A--A--G--------A---A-A---
Human K    --A--T--T-TT--T--A--A--A--A-----AA-A---
Mouse K    --G--T--G-TC--T--------A--A-----AA-A---
Goldfish   ----------TT--A--------A--A-----GA-A---
Human N    --G--T--A-T---T--A-----A---------A-A---
Mouse N    --A--T--A-T---T--------A----------A---
```

Table 1. Several vertebrate ras sequences (cont.)

EXON 4

```
                460       470       480       490       500       510
Human H    GGAGTGGAGGATGCCTTCTACACGTTGGTGCGTGAGATCCGGCAGCACAAGCTGCGGAAG
Rat H      --T-------------------AC-A--A--------T--------T--A--------A
Chicken    --C--C--A-----------T--C--A-----G--------T-----T--A-----C---
Human Ka   A-------------T--T--T--A-------A-A--------A--AT---GAT--AAA--A
Mouse Ka   A-------------T--T--T--A------A-A--------A---T---GAT--AAA--A
Human Kb   G-T--T--T-----------T--A--A--T--A--A--T--AA-A--T--AGAAAAG-T-
Mouse Kb   --T--T--C-----------T--A--A--C--A--A--T--AA-A--T--AGAAAAG-T-
Goldfish   A-A-----A--------T--T--TC----A--G--A---A----AT--CG----A-A--A
Human N    --T--T--A-----T--T-----AC----AA-A--A--A--C---T--CGAA--AAA--A
Mouse N    --T--------------T-----AC----AA-G-----A--C---T--CGAT--AAA---

                520       530       540       550       560       570
Human H    CTGAACCCTCCTGATGAGAGTGGCCCCGGCTGCATGAGCTGCAAGTGTGTGCTCTCCTGA
Rat H      --------G----------------TT-------------------------G------
Chicken    --------A--A--------------T----------A---T--A--C---A-A--G---
Human Ka   A-C-G-AAAGAA--AA---C-CCTGGCT-TGTG-AA-TTAAA--A--CA-TA-AATG-A-
Mouse Ka   A-C-G-AAAGAA--AA---C-CCTGGCT-TGTG-AA-TTAAA--A--C--TA-AATG-A-
Human Kb   AGC--AGA-GG-A-AA---AGAAAAAGAAG-CA-A-...ACA--------AA-TATG-A-
Mouse Kb   AGC--AGA-GGGA-GA---AGAAGAAGAAG-CA-G-...ACA-G----ACAG-TATG-G-
Goldfish   --C-GTAAAGAA--A----CAACA-AAT--AT--A-CTT (End of insert)
Human N    --C---AGCAG------TG-GACT-AG--T--T---G-A-TGCCA------G-GATG-A-
Mouse N    --C---AGCAG---C--TG-CACT-AA--T--T---G-G-CGCCC--------GATG-AG
```

---

[1] Reddy, 1983; [2] Ruta et al., 1986b; [3] Westaway et al., 1986; [4] Chang et al., 1982 [5] Tahira et al., 1986 [6] George et al., 1985 [7] Nemoto et al., 1986 [8] Taparowsky et al., 1983 [9] Guerrero et al., 1985

superfamily of related genes. Members of the *ras* superfamily have been identified in mammals, birds, insects, fish, mollusks, plants, fungi, slime molds, and yeast. Probing a human genomic library at low stringency with v-Ha-*ras* identified R-*ras*, a gene that encodes for a 218 amino acid protein (Lowe et al., 1987). The homology between R-*ras* and the other mammalian *ras* genes is 55% at the amino acid level, and a mouse R-*ras* gene has also been identified. Screening of a simian B-cell cDNA library with a synthetic oligonucleotide probe based on a highly conserved region of the *ras* genes resulted in the isolation of the *ral* gene. The *ral* gene encodes for a 206 amino acid protein with 50% homology to the *ras* gene product (Chardin & Tavitian, 1987).

Among the lower eukaryotes with *ras*-related genes are *Drosophila* (D-*ras*-1, D-*ras*-2, and D-*ras*-3), *Dictyostelium* (Dd-*ras*), *Saccharomyces cerevisiae* (*RAS*-1, *RAS*-2, YPT), and *Schizosaccharomyces pombe* (*SPRAS*). *The homology of D-*ras*-1 to c-Ha-*ras*-1 is* greater than 90% for the first 120 amino acids, whereas D-*ras*-2 and D-*ras*-3 have less than 60% homology to c-Ha-*ras*-1 (Neuman-Silberberg et al., 1984; Schejter & Shilo, 1985). Dd-*ras* of *Dictyostelium* has an overall homology of 65% (Pawson et al., 1985; Reymond et al., 1984). The *ras* gene products from the yeast species all are larger than the vertebrate forms and range in

homology from 38% (YPT) to 65% (*RAS*-1, *RAS*-2, and *SPRAS*) for the first 165 amino acid residues (Gallwitz et al., 1983; DeFeo-Jones et al., 1983; Powers et al., 1984; Haubruck et al., 1987; Fukui & Kaziro, 1985).

Homology to the yeast YPT gene identified a family of genes in a rat brain cDNA library known as *rab*. The similarity between YPT and the rab isolates is 37-75%, but the homology between *rab* and mammalian *ras* falls to approximately 30%. The *rho* gene has 30-40% homology to the *ras* genes and was first isolated from the marine snail *Aplysia* (Madaule & Axel, 1985). The *rho* gene has since been found in human, rat, yeast and *Drosophila* genomes. Homology to *Drosophila* D-*ras*-3 gene has identified another related gene family in human cDNA libraries known as rap1A, rap1B, and rap2 with 50% homology to the classical *ras* genes (Pizon et al., 1988). The *ras* superfamily has also been extended to a prokaryotic gene. The *era* gene, isolated from *Escherichia coli*, has amino acid homology greater than 40% to the yeast *RAS*-1 and *RAS*-2 genes (Ahnn et al., 1986).

Expression of the *ras* Genes

Members of the *ras* gene family are expressed in nearly every cell at some level. Different mammalian organs have substantially different levels of expression of Ha-*ras*, Ki-*ras*, and N-*ras* (Leon et al., 1987). In mice, Ha-*ras* is strongly expressed in brain,

muscle, and skin with lower expression in liver and ovary. The highest expression of Ki-*ras* was found in the gut, lung, and thymus with lower expression found in liver, muscle, skin and ovary. Expression of N-*ras* is high in thymus and testis and lower in liver and kidney. Expression of the different *ras* genes also varied during development. Expression of Ha-*ras* was constant throughout fetal development, whereas expression of Ki-*ras* and N-*ras* peaked at 10-13 days of gestation and declined substantially by the end of pregnancy. Differential expression of the *ras* genes suggest varying roles for each gene in either proliferation or differentiation. Differential *ras* expression has also been observed in rat organs (Tanaka et al., 1986; 1987).

Activation of the *ras* Gene

The identification of *ras* as the first oncogene isolated in a human neoplasm led to the discovery of a single nucleotide substitution resulting in the activation of a proto-oncogene (Parada et al., 1982; Santos et al., 1982; Capon et al., 1983a; 1983b; Barnes, 1986; Tabin et al., 1982). In this case, activation is defined as a genetic alteration which causes misdirection of a normal cellular gene into producing an oncogenic phenotype. Activating mutations have been limited to codons 12 (Capon et al., 1983a; 1983b; Reddy et al., 1982; Tabin et al., 1982;

Taparowsky et al., 1982; Fasano et al., 1983), 13 (Bos et al., 1985; Hirai et al., 1987), 59 (Dhar et al., 1982; Tsuchida et al., 1982), and 61 (Taparowsky et al., 1983; Yamamoto & Perucho, 1984; Brown et al., 1984; Bos et al., 1984; Pelling et al., 1988) in naturally occurring tumors. Table 2 lists many human cancers in which the activation of a *ras* gene has been implicated in carcinogenesis, and Table 3 lists animal carcinogenesis studies showing *ras* activation. Additionally, *in vitro* mutagenesis of codons 63 (Fasano et al., 1984), 116 (Walter et al., 1986), and 119 (Sigal et al., 1986b) have been shown to activate the *ras* proto-oncogenes. The consequences of these mutations will be discussed later.

Although the majority of *ras* activations has been the result of a single base substitution without any further modification, overexpression or larger mutations such as deletions or gene amplification have also resulted in neoplastic transformation *in vitro* (McCoy et al., 1983). Insertion of retroviral regulatory elements near normal *ras* genes or the inclusion of multiple copies of normal *ras* genes caused malignant transformation in NIH 3T3 cells and resulted in 30-100 fold increase in *ras* expression (Chang et al., 1982a; Pulciani et al., 1985; Cohen et al., 1987). Elevated expression of *ras* genes has often been shown in a variety of tumors when compared to the expression

Table 2. Incidence of ras activation in human cancer.[1]

Malignancy	Cell Source[2]	Gene Type	Mutation Site	Reference
Bladder	C:EJ,T24	H	12	Capon et al., 1983a; Parada et al., 1982; Pulciani et al., 1982; Reddy et al., 1982; Santos et al., 1982;
	C:WEB	H		Marshall, 1985
	C:A1698	K	12	Barbacid, 1985
	C:A1163	K	12	Valenzuela & Groffen, 1986
	B (2/38)	H	61	Fujita et al., 1985
	B (1/38)	H	12	Fujita et al., 1985
	B (2/28)	H		Malone et al., 1985
	B (1/15)	H		Fujita et al., 1987
	B (4/24)	H		VisVanathan et al., 1988
	B	N	61	Kuzumaki et al., 1989
Breast	C:HS578T	H	12	Kraus et al., 1984
	C:MDA-MB231	K	13	Kozma et al., 1987; Rochlitz et al., 1989
	C:PE600I	K	12	Rochlitz et al., 1989
	B (0/16)			Kraus et al., 1984
	B (1/40)	K	13	Rochlitz et al., 1989
	B (2/24)	H	12	Spandidos, 1987
Cervix	B (0/30)			Bos, 1988
	B (7/76)			Riou et al., 1988

Table 2. Incidence of ras activation in human cancer (cont.)

Malignancy	Cell Source[2]	Gene Type	Mutation Site	Reference
Colon	C:SW480	K	12	Bos et al., 1986; Capon et al., 1983b; McCoy et al., 1984
	C:A2233	K		Pulciani et al., 1982
	C:Duke-2	K		McCoy et al., 1983
	C:SK-CO-1	K	12	Winter et al., 1985
	C:7060	N	61	Yuasa et al., 1986b
	C:CC20	H	12	Greenhalgh & Kinsella, 1985
	B	K	12	Yuasa et al., 1986a
	B	N		Murray et al., 1983
	B (9/27)	K	12	Bos et al., 1987a
	B (1/27)	K	61	Bos et al., 1987a
	B (1/27)	N	12	Bos et al., 1987a
	B (26/66)	K	12	Forrester et al., 1987
	B (26/40)	K	12	Burmer & Loeb, 1989
	B (6/9)	K	12	Burmer et al., 1989
	B (65/172)	K		Vogelstein et al., 1988
	B (5/75)	K		Farr et al., 1988a
	B (0/9)			Feinberg et al., 1983
Esophagus	B (0/25)			Hollstein et al., 1988
Fibrosarcoma	C:HT1080	N	61	Brown et al., 1984
Gall Bladder	C:A1604	K		Barbacid, 1985
	C:A1609	K		Pulciani et al., 1982
	B (0/5)			Almoguera et al., 1988

Table 2. Incidence of ras activation in human cancer (cont.)

Malignancy	Cell Source[2]	Gene Type	Mutation Site	Reference
Glioblastoma	C:Hu70	N		Gerosa et al., 1989
	C:Hu104	N		Gerosa et al., 1989
	C:Hu112	N		Gerosa et al., 1989
	C:Hu195	N		Gerosa et al., 1989
	C:Hu197	N		Gerosa et al., 1989
	B (0/30)			Bos, 1988
Kidney	B (1/16)	H	12	Fujita et al., 1988a
	B (1/16)	H	61	Fujita et al., 1988a
Liver	C:7402	N		Gu et al., 1986
	C:Hep G2	N		Barbacid, 1985
	B (3/10)	N		Gu et al., 1986
	B	N		Barbacid, 1985
	B	N		Yuan et al., 1988
	B	H		Zhang et al., 1987
Lung	C:HS242	H	61	Yuasa et al., 1983
	C:SK-LU-1	K	12	Winter et al., 1985
	C:Lx-1	K		Der et al., 1982
	C:A2182	K	12	Santos et al., 1984
	C:A427	K	12	Valenzuela & Groffen, 1986
	C:A549	K	12	Valenzuela & Groffen, 1986
	C:PR310	K	61	Yamamoto & Perucho, 1984; Yamamoto et al., 1985
	C:PR371	K	12	Nakano et al., 1984; Yamamoto et al., 1985

Table 2. Incidence of ras activation in human cancer (cont.)

Malignancy	Cell Source[2]	Gene Type	Mutation Site	Reference
Lung (cont.)	C:SW1271	N	61	Yuasa et al., 1984
	C:Lu-65	K	12	Taya et al., 1984
	C:Calu1	K	12	Capon et al., 1983b; Shimizu et al., 1983
	C:LC-12-JCK	K		Fukui et al., 1985
	C:QG56	K	61	Kagimoto et al., 1985
	B	K		Pulciani et al., 1982
	B	K	12	Santos et al., 1984
	B (15/45)	K	12	Rodenhuis et al., 1988
	B (0/10)			Feinberg et al., 1983
Melanoma	C:SK-MEL146	H		Albino et al., 1984
	C:SK-MEL93	N		Albino et al., 1984
	C:SK-MEL119	N		Albino et al., 1984
	C:SK-MEL147	N		Albino et al., 1984
	C:SK-MELSWIFT	N	61	Padua et al., 1985
	B	H	61	Sekiya et al., 1984
	B (3/8)	H		Ananthaswamy et al.,1988
	B (1/10)	H	61	Leon et al., 1988
	B (1/13)	N	61	Raybaud et al., 1988
	B (7/37)	N		van't Veer et al., 1989
Neuroblastoma	C:SK-N-SH	N	61	Taparowsky et al., 1983
	B (1/15)	N	13	Ireland, 1989
	B (1/15)	N	61	Ireland, 1989
	B (0/25)			Ballas et al., 1988

Table 2. Incidence of ras activation in human cancer (cont.)

Malignancy	Cell Source[2]	Gene Type	Mutation Site	Reference
Ovary	B (3/37)	K		van't Veer et al., 1988
Pancreas	C:All65	K	12	Valenzuela & Groffen, 1986
	C:PancI	K	12	Malone et al., 1985
	C:T3M-4	K	61	Hirai et al., 1985a
	B	K	12	Yamada et al., 1986
	B	K		Pulciani et al., 1982
	B (21/22)	K	12	Almoguera et al., 1988
	B (28/30)	K	12	Smit et al., 1988
	B (47/63)	K	12	Grunewald et al., 1989
Rhabdomyo-sarcoma	C:RD301	N	61	Chardin et al., 1985
	B	K		Pulciani et al., 1982
Skin (keratoacanthoma)	B	H	61	Leon et al., 1988
Stomach	B	K	12	Bos et al., 1984
	B	N	61	O'Hara. et al., 1986
	B (0/7)			Fujita et al., 1985
	B (0/26)			Sakato et al., 1986
Terato-carcinoma	C:PA1	N	12	Tainsky et al., 1984

Table 2. Incidence of ras activation in human cancer (cont.)

Malignancy	Cell Source[2]	Gene Type	Mutation Site	Reference
Thyroid	B (2/5)	H	61	Lemoine et al., 1988
Follicular	B (2/5)	K	12	Lemoine et al., 1988
	B (2/5)	N	61	Lemoine et al., 1988
Papillary	B (1/10)	K	13	Lemoine et al., 1988
	B (1/10)	N	61	Lemoine et al., 1988
	B (0/20)			Fusco et al., 1987
Acute	C:Rc-2a	N	12	Janssen et al., 1987
Myeloid	C:KG-1	N	12	Janssen et al., 1987
Leukemia	C:THP-1	N	12	Janssen et al., 1987
	C:HL60	N	61	Bos et al., 1987b
	C:PAC	N		Murray et al., 1983
	C:31-26-146	N		Eva et al., 1983
	B	N	12	Gambke et al., 1985
	B (3/6)	N		Needleman et al., 1986
	B (4/45)	N	61	Bos et al., 1987b
	B (6/45)	N	13	Bos et al., 1985
	B (2/45)	K	12	Bos et al., 1987b
	B (13/52)	N	12	Farr et al., 1988
	B (1/52)	N	13	Farr et al., 1988
	B (7/10)	N		Hirai et al., 1987
	B (1/9)	H	12	Janssen et al., 1987
	B (1/9)	N	12	Janssen et al., 1987
	B (3/9)	N	61	Janssen et al., 1987
	B (5/18)	N		Senn et al., 1988
	B (7/26)	N		Yunis et al., 1988
	B (15/57)	N		Bartram et al., 1989
	B (1/9)			Pedersen-Bjergaard et al., 1988

Table 2. Incidence of ras activation in human cancer (cont.)

Malignancy	Cell Source[2]	Gene Type	Mutation Site	Reference
Chronic Myeloid Leukemia	C:IM9	N		Eva et al., 1983
	B (0/25)			Bos, 1988
	B (0/26)			Janssen et al., 1987
	B (3/12)	H	12	Liu et al., 1988
	B (1/12)	N	12	Liu et al., 1988
	B (1/8)	N		Hirai et al., 1985b
Juvenile	B	K		Janssen et al., 1985
Myelo-dysplastic syndrome	B (1/19)	N	12	Janssen et al., 1987
	B (3/8)	N	13	Hirai et al., 1987
	B (20/50)	N/K		Padua et al., 1988
	B (11/27)	N		Yunis et al., 1988
	B (3/34)	K		Lyons et al., 1988
Myeloproli-ferative Syndrome	B (1/15)	N	12	Janssen et al., 1987
Acute Lymphoid Leukemia	C:RPMI	N		Souyri & Fleissner, 1983
	C:CCRF-CEM	K		Eva et al., 1983
	C:T-ALL1	N		Souyri & Fleissner, 1983
	C:p-12	N		Souyri & Fleissner, 1983
	C:MOLT4	N	12	Eva et al., 1983
	B (2/19)	N	12	Rodenhuis et al., 1986
	B (0/14)			Senn et al., 1988
	B (6/33)			Neri et al., 1988

Table 2. Incidence of ras activation in human cancer (cont.)

Malignancy	Cell Source[2]	Gene Type	Mutation Site	Reference
Burkitt	C:AW Ramos	N		Murray et al., 1983
Hodgkin	B (2/3)	N		Sklar & Kitchingman, 1985
Hairy cell	B (0/6)			Neri et al., 1988
Idiopathic Myelofibrosis	B (2/9)	N		Buschle et al., 1988

[1] Updated from data presented in Bos, 1988; 1989.
[2] C: cell line; B biopsy (incidence/total)

Table 3. Incidence of ras in animal models of carcinogenesis.

Species	Organ	Chemical	Gene	Mutation	Incidence	Reference
Mouse						
CD-1	Breast	dibenz(c,h)-acridine	H	61	1/1	Bizub et al., 1986
BALB/c	Fibro-sarcomas	3-methyl-cholanthrene	K N		4/14 2/11	Borrello et al., 1988
B6C3/Fl	Liver	spontaneous	H H		11/23 15/27	Reynolds et al., 1986 Reynolds et al., 1987
A/J	Liver	4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone			0/5	Belinsky et al., 1989
A/J	Liver	nitroso-dimethylamine	K	12	1/8	Belinsky et al., 1989
B6C3/Fl	Liver	nitroso-diethylamine	H	61	14/33	Stowers et al. 1988
B6C3/Fl	Liver	1-hydroxy-2',3'-dihydroesyragole	H K	61	10/11 1/11	Wiseman et al., 1986
B6C3/Fl	Liver	N-hydroxy-2-acetlaminoflurene	H	61	7/7	Wiseman et al., 1986

Table 3. Incidence of ras in animal models of carcinogenesis (cont.).

Species	Organ	Chemical	Gene	Mutation	Incidence	Reference
Mouse (cont.)						
B6C3/F1	Liver	vinyl carbamate	H	61	7/7	Wiseman et al., 1986
B6C3/F1	Liver	furan	H	61	5/29	Reynolds et al., 1987
			H	117	4/29	
			K		2/29	
B6C3/F1	Liver	furfural	H	13	2/16	Reynolds et al., 1987
			H	61	6/16	
			H	117	1/16	
			K		1/16	
A/J	Lung	spontaneous	K	12	6/10	Belinsky et al., 1989
			K	61	3/10	
	Lung	tetranitromethane	K	12		Guerrero & Pellicer, 1987
A/J	Lung	4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone	K	12	8/10	Belinsky et al., 1989
			K	61	2/10	
A/J	Lung	nitroso-dimethylamine	K	12	7/10	Belinsky et al., 1989
			K	61	3/10	

Table 3. Incidence of ras in animal models of carcinogenesis (cont.).

Species	Organ	Chemical	Gene	Mutation	Incidence	Reference
Mouse (cont.)						
SENCAR	Skin	spontaneous	H	61	7/9	Pelling et al., 1988
	Skin	B-propiolactone	H	61	1/6	Hochwalt et al., 1988
CD-1	Skin	7,12-dimethyl-benzanthracene	H	61	3/4	Bizub et al., 1986
			H			Balmain & Pragnell, 1983
CD-1	Skin	benzo(a)pyrene			0/3	Bizub et al., 1986
CD-1	Skin	dibenz(c,h)-acridine	H	61	4/5	Bizub et al., 1986
	Thymic lymphoma	nitrosomethyl-urea	N	61		Guerrero et al., 1985
Rat						
F344	Bladder	N-butyl-N-(4-hydroxybutyl)-nitrosamine	H	61	1/9	Fujita et al., 1988b

Table 3. Incidence of ras in animal models of carcinogenesis (cont.).

Species	Organ	Chemical	Gene	Mutation	Incidence	Reference
Rat (cont.)						
	Breast	spontaneous			0/26	Zarbl et al., 1985
	Breast	nitrosomethyl-urea	H	12	48/58	Zarbl et al., 1985
			H	12	61/71	Sukumar et al., 1983; Barbacid et al., 1986
	Breast	7,12-dimethyl-benzanthracene	H	61	3/14	Zarbl et al., 1985
			H	61	5/26	Barbacid et al., 1986
F344	Fibro-sarcoma	1,8-dinitro-pyrene	K	12	1/7	Tahira et al., 1986
F344/N	Liver	spontaneous			0/29	Reynolds et al., 1986
Fischer	Liver	aflatoxin B_1	K		2/11	McMahon et al., 1986
			K	12	3/4	Sinha et al., 1988
			N		1/4	
F344	Liver	nitrosodiethyl-nitrosamine			0/28	Stowers et al., 1988

in adjacent normal tissues (Spandidos & Kerr, 1984; Spandidos & Agnantis, 1984; Spandidos et al., 1985; Williams et al., 1985; Viola et al., 1985; Agnantis et al., 1986; Agnantis et al., 1988; Clair et al., 1987). Overexpression of the nonactivated c-Ha-*ras* gene, however, did not fully transform in Rat-1 cells, nor did the overexpression of the nonactivated form suppress the transformation by the activated c-Ha-*ras* gene (Ricketss & Levinson, 1988). Truncation of the 5' noncoding exon of a nonmutated Ha-*ras* gene has resulted in cellular transformation in some cases (Cichutek & Duesberg, 1986) but not in all cases (Tabin & Weinberg, 1985). The degree of DNA methylation, often involved in the regulation of specific gene expression, may play a role in controlling *ras* gene expression. *In vitro* methylation of an activated human Ha-*ras* gene decreased its transforming activity in the NIH 3T3 transfection assay approximately 80% (Borrello et al., 1987). The transforming activity was recovered after a 24 hour incubation in the presence of the DNA-demethylating agent 5-azacytidine. Hypomethylation of both c-Ha-*ras* and c-Ki-*ras* was achieved in rats by dietary methyl deficiency; however, all DNA samples showed hypomethylation regardless of the tissue source (normal, preneoplastic or neoplastic) or exposure to the carcinogen diethylnitrosamine (Bhave et al., 1988).

The activation of *ras* genes in tumor tissue but not in normal surrounding tissue (Kraus et al., 1984; Santos et al., 1984; Burmer et al., 1989) has strengthened the conclusion that *ras* is involved in human carcinogenesis. Precisely when *ras* involvement occurs may differ depending on the circumstances. In many cases, the activation of *ras* is seen as an early event in the carcinogenic process. Activated *ras* genes have been identified in preneoplastic lesions in the mouse skin carcinoma model (Balmain et al., 1984) and are predominant in rat mammary carcinoma initiated with a single dose of nitrosomethylurea (Sukumar et al., 1983; Zarbl et al., 1985). In human cancer, activated *ras* genes are found in benign and malignant regions of a human colon carcinoma (Bos et al., 1987a), and precede aneuploidy in human colon carcinoma (Burmer et al., 1989). An activated *ras* gene is sufficient to cause transformation of established preneoplastic cell lines such as the NIH 3T3 mouse cell line, but generally requires the presence of a second activated oncogene (Land et al., 1983) or proliferating agents (Imler et al., 1988; Marczynska et al., 1988; Pelling et al., 1988) for transformation of primary rodent cells. Without any secondary support, an activated *ras* gene cannot sustain total neoplastic transformation (Leon et al., 1988). A second activated oncogene is not required, however, for the transforma-

tion of normal embryonic cells (Spandidos & Wilkie, 1984). Activation of *ras* is not always associated with initiation or the early stages of carcinogenesis. One example of late activation in an animal model showed *ras* activation only in the later stages of mouse lymphoma (Vousden & Marshall, 1984). Studies of human myeloid leukemia (Janssen et al., 1987; Yunis et al., 1988) and melanoma (Albino et al., 1984; van't Veer et al., 1989) have also shown *ras* activation occurring in both early and late stages of neoplasia. Tumor promotion or tumor progression may be facilitated by the activation of *ras* in proliferating cells which have been initiated by other activated oncogenes (Albino et al., 1984).

The *ras* Gene Product

The *ras* genes in vertebrates code for a phosphoprotein of 21,000 Daltons known as p21 (Shih et al., 1986). A precurser protein of 23,000 Daltons (p23) has been identified in the biosynthesis of p21 (Shih, et al., 1982), and post-translational modification of the phosphoprotein involves the covalent binding of palmitic acid to the cysteine residue near the carboxy terminal (Buss & Sefton, 1986; Fujiyama & Tamanoi, 1986; Chen et al., 1985; Magee et al., 1987). p21 has been localized to the interior side of the cellular plasma membrane (Shih et al., 1979a; Shih et al., 1979c; Willingham et al., 1980) and the acylated

cysteine residue is required for membrane attachment (Willumsen et al., 1984a; 1984b) and transforming activity (Willumsen et al., 1984a), but acylation is not required for normal *RAS* function in *S. cerevisiae*. Specific monoclonal antibodies capable of recognizing the different *ras* p21 proteins have been produced (Wong et al., 1986; Yoshida et al., 1988). Microinjection of the *ras* p21 protein or its antibody has been shown to affect cellular morphology (Bar-Sagi & Feramisco, 1986; Stacey & Kung, 1984), division (Baltus et al., 1988; Birchmeier et al., Wigler, 1985; Sadler et al., 1986), or differentiation (Bar-Sagi & Feramisco, 1985; Haga et al., 1986; Satoh et al., 1987).

The conservation of the *ras* genes across such diverse species indicated a fundamental role in the cell life cycle. A common characteristic of all *ras* gene products was found to be the capacity to bind guanine nucleotides (Scolnick et al., 1979; Shih et al., 1979a; Trahey et al., 1987). This strong similarity to a class of proteins known as G proteins indicated a possible function for the *ras* proto-oncogene in the nontransformed cell (Hanley & Jackson, 1987; Halliday, 1984; Hurley et al., 1984; Lochrie et al., 1985). G proteins function as signal transducers across the cell membrane in a variety of cells (Pall, 1985; Gilman, 1987). Stimulus of a receptor on the

cell surface requires the signal to be transmitted across the membrane to the cytoplasm and possibly to the nucleus for cellular response (Barnes, 1986). The G protein is coupled to membrane receptors of extracellular signals and to effectors of intracellular second messenger systems (Sullivan et al., 1987; Chabre, 1987). The G protein is activated by replacement of a guanine diphosphate (GDP) molecule with a guanine triphosphate (GTP) molecule. Inactivation occurs when the GTP molecule is hydrolyzed to GDP by an intrinsic GTPase activity of the G protein. Some cellular processes regulated by G proteins include activation and inhibition of adenylyl cyclase (Robishaw et al., 1986; Masters et al., 1988; Mattera et al., 1989), stimulation of retinal cyclic GMP phosphodiesterase (Yatsunami & Khorana, 1985; Deterre et al., 1988; Brann, 1988), stimulation of phosphoinositide hydrolysis (Uhing et al., 1986; Cockcroft & Gowperts, 1985; Cockcroft, 1987), and regulation of ion channels (Scott & Dolphin, 1987; Caffrey et al., 1987; Cerbai et al., 1988; Van Dongen et al., 1988; Yatani et al., 1988; Mattera et al., 1989).

The *ras* gene product of the nonactivated proto-oncogene also has an intrinsic GTPase activity, and an associated protein, GTPase-activating protein (GAP), acts as an effector molecule (Adari et al., 1988;

Trahey & McCormick, 1987; Trahey et al., 1988; Hoshino et al., 1988; McCormick, 1989). GAP, a series of monomeric proteins of 100-125 kDaltons, has the ability to stimulate GTP hydrolysis by the normal p21 molecule 200-fold, yet has no effect on the oncogenic variants. The GTPase activity is generally absent or greatly reduced in the activated gene product (Gibbs et al., 1984; Finkel et al., 1984; Sweet et al., 1984; Manne et al., 1985). However, an activated p21 has been shown to retain a strong GTPase activity and yet still be able to transform NIH 3T3 cells (Papageorge et al., 1982; Lacal et al., 1986), leading to the conclusion that a function other than GTP hydrolysis is also involved in neoplastic transformation. The p21 molecule is also capable of autophosphorylation, especially evident in viral forms (Shih et al., 1979a). The site of autophosphorylation is the threonine residue at position 59, and in contrast with the GTPase activity, autophosphorylation activity is increased in the oncogenic variants (Gibbs et al., 1984).

The three-dimensional structure of p21 has recently been characterized (de Vos et al., 1988; Schlichting et al., 1989). The structure contains one β-sheet containing six strands, four α-helices, and nine connecting loops. The GDP or GTP molecule sits in a pocket formed by four of the nine loops (L1, L2, L7, and L9). The catalytic site for the hydrolysis of GTP

is most likely loop L1, with loop L2 the probable recognition site for the GAP molecule. Activating mutations of the *ras* genes affect guanine nucleotide binding capacity of the gene product (Feig et al., 1986; Der et al., 1986; Lacal & Aaronson, 1986; Lacal et al., 1986; Clanton et al., 1986; Sigal et al., 1986a; 1986b; Clanton et al., 1987). Mutants at residue 12 or 13 (located in loop L1) cause the loop to become enlarged and would explain the reduction in GTPase activity (Tong et al., 1989). Mutants at residue 61 alter the long loop L4 which spans the molecule, again enlarging the pocket for guanine nucleotide binding (Pincus et al., 1987). Another consequence of the reported base substitutions is a reduction in the guanine-binding strength, resulting in an enhancement of the exchange of GTP for GDP. The phosphorylation of threonine-59 stiffens the conformation of p21 around the guanine nucleotide molecule. The ultimate outcome in every case is an increase in the concentration of GTP-bound p21 molecules (Srivastava et al., 1985), prolonging the activated signal.

The precise function (or functions) of the *ras* genes in the cell is not yet known, although several possibilities are under investigation. In yeast, *RAS* proteins have been shown to control growth arrest by stimulating adenylyl cyclase activity (Toda et al.,

1985). Other cellular functions in yeast that require *RAS* activity include spore viability (Tatchell et al., 1984) and endocytosis (Mayorga et al., 1989). Nonmutated *RAS* genes require the CDC25 protein for optimum activity; mutant *RAS* genes have full activity without the CDC25 protein (Broek et al., 1985; 1987; Robinson et al., 1987). The CDC25 protein also controls other functions of the *RAS* genes in addition to the stimulation of adenylyl cyclase activity (Wigler et al., 1988).

Both mammalian and yeast *ras* gene products work in heterologous systems, indicating the conservation of *ras* gene functions over evolutionarily distant species (DeFeo-Jones, 1985; Papageorge et al., 1986; Temeles et al., 1985; Kataoka et al., 1985; Lowe et al., 1988). However, in contrast with its action in yeast, p21 does not appear to be part of the adenylyl cyclase pathway in mammalian or Xenopus cells (Beckner et al., 1985; Birchmeier et al., 1985) with the exception of two studies involving rat cell lines. Increased adenylyl cyclase activity was observed in rat thyroid epithelial cells transformed with viral *ras* genes (Spina et al., 1987), and rat kidney cells infected with a temperature sensitive viral Ki-*ras* had a stimulation of adenylyl cyclase as the cells entered early G_1 phase (Franks et al., 1985). However, in a vast majority of the studies, a separate second messenger signalling system

is the probable target of p21 function. Oncogenic forms of p21 stimulate inositol phospholipid breakdown and this pathway may be the target signalling system (Chiqrugi et al., 1985; Fleischman et al., 1986; Preiss et al., 1986; Lacal et al., 1987; Lacal et al., 1987; Wolfman & Macara, 1987). Phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis is stimulated by neurotransmitters, hormones, serum and growth factors (Berridge & Irvine, 1984; Joseph, 1984; Cockcroft, 1987; Hill et al., 1988; Coughlin et al., 1989). PIP_2 hydrolysis is catalyzed by polyphosphoinositide phosphodiesterase (a form of phospholipase C) to generate two intracellular signals: diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). As intracellular regulators of growth, DAG activates protein kinase C and IP_3 stimulates Ca^{2+} release from storage vesicles in the cytosol (Gill et al., 1986).

One G protein (G_p) specific for the PIP_2 pathway has been identified that is distinct from, yet has many similarities to, p21 (Cockcroft & Gowperts, 1985; Smith et al., 1986). DAG levels, but not IP_3 levels, were elevated in *ras*-transformed fibroblasts (Wolfman & Macara, 1987) and in a temperature-sensitive Ki-*ras* transformed cell line incubated at permissive temperatures (Preiss et al., 1986). These studies have strengthened the connection between phospholipase C and *ras* p21 (Yu et al., 1988;

Melin et al., 1986). Seuwen et al., however, have shown that phospholipase C is not the mediator of *ras*-transformed Chinese hamster lung fibroblasts (1988), and Benjamin et al. have shown decreased phospholipase C activity in *ras*-transformed NIH 3T3 cells (1987a; 1987b). Other second messenger systems are being investigated. One candidate for investigation is a direct interaction with protein kinase C (Ballester et al., 1987; Jeng et al., 1987).

The coupling of p21 to a transmembrane receptor is necessary for activity in the normal cell cycle. Several hormones and other molecules have been linked to receptor:*ras* p21 complexes, including platelet derived growth factor (PDGF) (Coughlin et al., 1989), epidermal growth factor (Kamata & Feramisco, 1987; Parries et al., 1987), bombesin (Wakelam et al., 1986), transferrin (Finkel & Cooper, 1984), and insulin (Deshpande & Kung, 1987; Korn et al., 1987; O'Brien et al., 1983; Kamata et al., 1987; Sacks et al., 1989).

In addition to controlling cell proliferation, *ras* has also been implicated in cell differentiation. Microinjection of normal and activated p21 into PC12 neural cells induced differentiation (Satoh et al., 1987). Transfection of the viral Ha-*ras* oncogene into human medullary thyroid carcinoma cells induced differentiation into functioning endocrine cells. In contrast, oncogenic p21 molecules, but not normal

forms, inhibit differentiation of mouse muscle cell line C2 into skeletal myoblast cells (Olson et al., 1987).

Trout Model of Carcinogenesis

Fish models are used in a wide variety of scientific disciplines, including genetics, developmental biology, neurobiology, and aquatic toxicology (reviewed in Powers, 1989). Many species can be utilized in both laboratory and field studies. The genetic background is well established for many fish species, including several species that have been manipulated to produce homozygous strains, either by inbreeding or constructed in the laboratory by physical means (Streisinger et al., 1981; Thorgaard et al., 1985).

Fish are used not only as representative species of an aquatic environment but also as important alternative vertebrate models for comparative biology. There are obvious differences which preclude complete comparisons between mammalian and fish physiology; for example, the absence of lung or mammary tissue in the fish species. However, basic embryology and biochemistry are a few of the many areas where knowledge can be increased by comparative studies across phyla. Depending on the requirements of the study, an ideal model can be found amongst the large number of diverse fish species. Most fish are very

prolific, producing hundreds of externally fertilized eggs, allowing ample opportunity to study embryonic development. Smaller aquarium fish have the advantages of short generation time and require small rearing areas, but are often too small to adequately obtain isolated tissue samples in quantities sufficient for enzymology or metabolism studies. Many fish species, such as rainbow trout (*Oncorhynchus mykiss*, formerly classified as *Salmo gairdneri*), grow much larger, and can be used more effectively in studies requiring large amounts of tissue, but have longer generation times and require larger areas for rearing. Other advantages and limitations of fish models in general and rainbow trout in particular, have been presented by Bailey et al. (1984).

Fish species have been used in carcinogenesis studies for over twenty-five years. A direct link between hepatic carcinoma in hatchery rainbow trout and aflatoxin B_1 contamination of the feed given to the trout established trout as a valuable and sensitive model for carcinogenicity testing (Wales & Sinnhuber, 1966; Ayres et al., 1971). Although several other species have been used in carcinogenesis studies (Toledo et al., 1987; Stanton, 1965; Khudoley, 1972; Sato et al., 1973; Kyona, 1978; Woodhead, Setlow, and Pond, 1982; Schultz and Schultz, 1982; Barkenow et al., 1982; Kimura et al., 1983; Smith et al., 1979;

Grizzle et al., 1981; Falkmer et al., 1977), the species used most extensively for this purpose has been rainbow trout. Rainbow trout are sensitive to a wide range of carcinogens (Meyers & Hendricks, 1982), can be exposed by several different routes (Hendricks et al., 1984; Bailey et al., 1987), and isolated tissue can easily be obtained for further biochemical and molecular studies (Williams & Buhler, 1983; Bailey et al., 1982; Loveland et al., 1984).

Although many aspects of carcinogenesis have been thoroughly investigated in the trout model, very little is known about the molecular biology of trout carcinogenesis. The only trout oncogene that has been previously characterized is the *myc* oncogene (Van Beneden et al., 1986). A form of the *src* gene identified in a hybrid of swordtails (*Xiphophorus*) is responsible for melanoma in that species. Other fish species in which *ras* has been reportedly identified are goldfish (Nemoto et al., 1986; 1987), tomcod (Wirgin et al., 1989; Wirgin et al., 1989) and winter flounder (McMahon, unpuplished data). This study will characterize a *ras* gene isolated from rainbow trout.

In Vitro Gene Amplification

A recent development in the field of molecular biology is a powerful tool known as polymerase chain reaction (PCR). This procedure allows the selective *in vitro* amplification of a particular sequence out of a

complex genome without cloning into a vector system. A schematic of the PCR procedure is illustrated in Figure 1. Basically, the technique repeatedly mimics the DNA synthesis procedure during a cell cycle: separating the double strands of the target DNA, annealing primer sequences for initiation of DNA synthesis, and extension by a DNA polymerase to copy the target DNA. By selecting primer sequences specific for a particular gene of interest and repeating the cycling 20-40 times, the gene (or a manageable portion of the gene) is amplified a million-fold. The original description of the PCR procedure used the heat-labile Klenow fragment of the *E. coli* DNA polymerase I enzyme (Saiki et al., 1985; 1986), and this required the replenishment of the enzyme after each denaturation step. Substitution of this heat-labile enzyme with the heat-stable Taq DNA polymerase (isolated from the bacterium *Thermus aquaticus*) greatly simplified the procedure and permitted the possible automation of the PCR process (Saiki et al., 1988). PCR has been used in a wide variety of studies, including the analysis of *ras* activation in cancer (McMahon et al., 1987; Farr et al., 1988b; Hollstein et al., 1988; Almoguera et al.,1988; Rodenhuis et al., 1988; Stevens et al., 1988; van't Veer et al., 1988; Burmer et al., 1989).

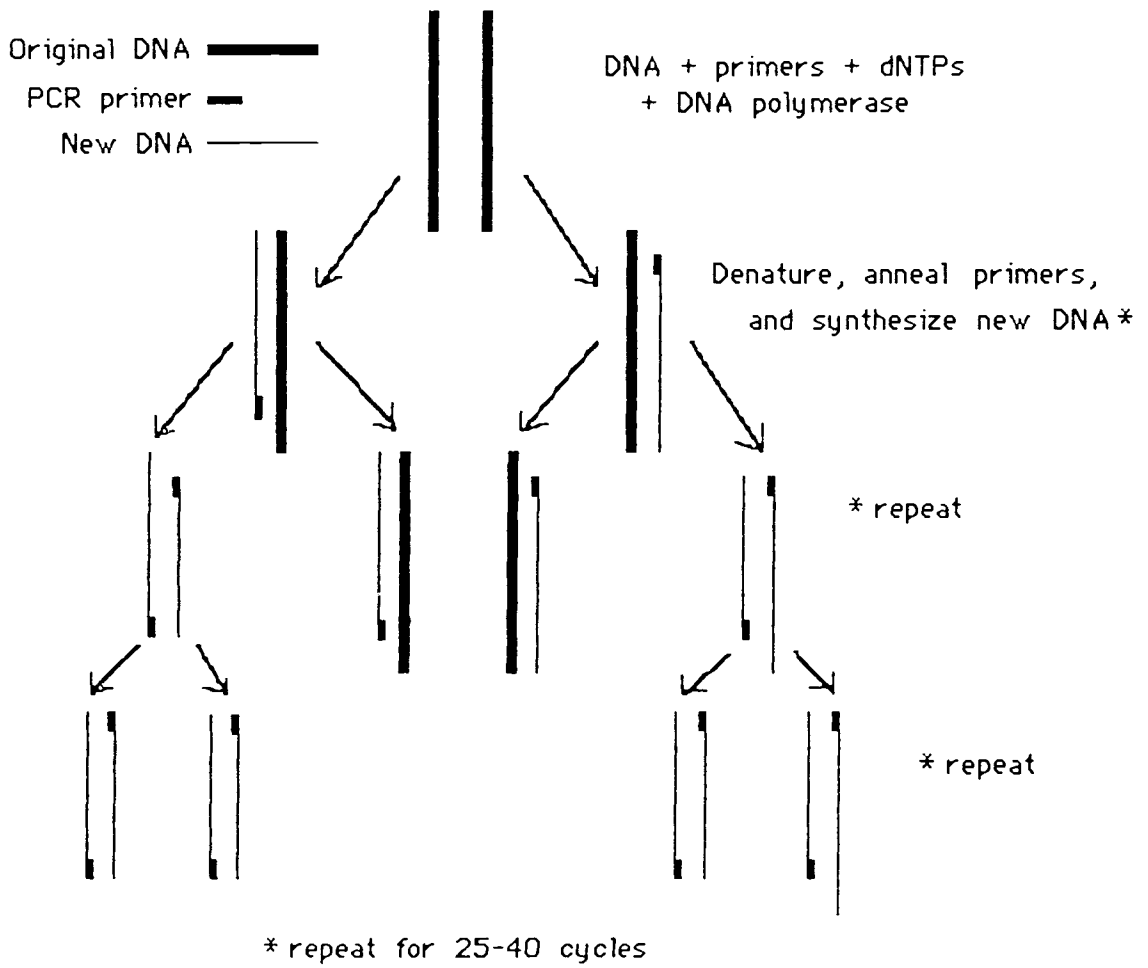


Figure 1. The polymerase chain reaction procedure.

Although no exact sequence information has been available for any trout *ras* gene, the highly conserved nature of *ras* genes between diverse species can be exploited in an attempt to locate the homologous gene(s) in trout. Specifically, the selection of primers derived from conserved regions of a consensus of the vertebrate *ras* genes would hopefully allow the *in vitro* amplification of one or more trout *ras* genes that can be further analyzed by several traditional methods. This structural information is essential for future studies on the role of *ras* genes in neoplasia in trout and other fish species.

MATERIALS AND METHODS

General methods used are described further in Davis et al., 1986. Unless otherwise specified, chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

DNA Isolation

Trout tissue samples were immediately frozen in liquid nitrogen and pulverized in a mortar with a pestle. The powder was suspended in 10 volumes RSB buffer (10 mM Tris, pH 7.4; 10 mM sodium chloride (NaCl); 25 mM EDTA). Sodium dodecyl sulfate was added to equal 1% final volume, and proteinase K was added at a concentration of 1 mg/ml. Each suspension was incubated at 37°C for 4-8 hours until the sample became uniformly viscous. Each sample was extracted with Tris-saturated phenol (Boehringer Mannheim Biochemicals, Indianapolis, IN):chloroform (International Biotechnologies, Inc., New Haven. CT):isoamyl alcohol (25:24:1), and the top aqueous layer transferred to a new tube without disturbing the interface. Each aqueous phase was extracted twice with chloroform: isoamyl alcohol (24:1) and once with diethyl ether. The DNA from each sample was precipitated with 2.5 volumes of cold 95% ethanol (Aldrich, Milwaukee, WI) and wound onto a glass rod. After transferring to a new tube, the DNA was air-dried. The DNA was

resuspended in TE buffer (10 mM Tris, pH 7.4, 1 mM EDTA) with 20 μg/ml DNase-free RNase A.

Messenger RNA (mRNA) Isolation

Tissue samples were immediately frozen in liquid nitrogen and pulverized in a mortar with a pestle. The powder was suspended in RNAzol (Cinna/Biotecx, Friendswood, TX) at a concentration of 100 mg pulverized tissue/2 ml RNAzol. Chloroform (1/10 volume) was added to the RNAzol suspension and mixed well. After incubation at 4°C for 15 minutes, the suspension was centrifuged at 10,000 x g for 15 minutes to separate the organic and aqueous phases. The top aqueous layer was transferred to a new tube, and total RNA was precipitated with 1 volume isopropanol at -20°C for 45 minutes and centrifuged at 10,000 x g for 15 minutes. The RNA pellet was resuspended in 1 ml sterile distilled water containing 40 units RNasin RNase inhibitor (Promega Biotec, Madison, WI). Messenger RNA (mRNA) was isolated by passage of total RNA through an oligo(dT)-cellulose column using a commercial mRNA isolation buffer set (5 Prime-3 Prime, West Chester, PA). The total RNA sample was heated to 65°C for 5 minutes. One volume of 2x Loading buffer provided in the isolation buffer set was added to the sample and reheated to 65°C. The sample was cooled to room temperature and applied to a prepared 4 ml oligo(dT)-cellulose column. After repeated washing to

remove unattached RNA, the poly(A)+ mRNA was eluted with the provided elution buffer. Nucleic acid in aliquots of the eluate was detected by mixing 2 μl of each aliquot with 10 μl dilute ethidium bromide solution (1 μg/ml), dotting the sample on cellophane wrap placed on a UV transilluminator, and observing the fluorescence. Fluorescence-positive aliquots were combined, and the mRNA was precipitated with 1/10 volume 2 M ammonium acetate and 3 volumes of 95% ethanol. The mRNA in ethanol was stored at -80°C until needed. At that time, the precipitated mRNA was centrifuged at 12,000 rpm, and the pellet vacuum dried. After resuspension in sterile distilled water containing RNasin RNase inhibitor, the mRNA was quantified by measuring an aliquot for absorbance at a wavelength of 260 nm (Concentration = 40 μg/ml x dilution factor x A_{260}).

cDNA Synthesis

cDNA was synthesized using a commercial cDNA synthesis system (Bethesda Research Laboratories, Gaithersburg, MD). First strand synthesis was executed at 37°C for 1 hour using 10 μg mRNA, Moloney MuLV reverse transcriptase and oligo$(dT)_{15}$ primer. Ten μl of the total 50 μl reaction was removed after first strand synthesis for use directly in the polymerase chain reaction (see below). Second strand synthesis was executed on the remainder of the first strand

reaction. Second strand synthesis buffer, E. coli polymerase I, and RNase H were added, and each reaction was incubated at 16°C for 2 hours. The completed cDNA was phenol/chloroform extracted, followed by ethanol precipitation. The precipitated cDNA was resuspended in 100 μl TE.

Polymerase Chain Reaction

In vitro gene amplification was accomplished using a modification of the polymerase chain reaction (PCR) protocol (Saiki et al., 1988). One μg of genomic DNA or 100 ng of cDNA (first strand or fully synthesized) was included in 50 μl reactions containing 50 mM KCl, 10 mM Tris, pH 8.3, 1.5 mM $MgCl_2$, 200 mM of each dNTP, 0.01% gelatin , 2-3 units Taq polymerase (Beckman, Inc. Fullerton, CA) and 60 pmoles of both a 5' and a 3' primer. In reactions designed to amplify the 3' end of cDNA, however, 90 pmoles of the poly$(dT)_{24}$ were used as the 3' primer to account for the large number of poly(dA) tails. The selection of primers was dependent on the region of *ras* to be amplified as illustrated in Figures 2-4. The actual primer sequences are listed in Table 4. Primers were synthesized at the Center for Gene Research at Oregon State University. Forty cycles of denaturing at 94°C for 0.5-1.0 minutes, primer annealing at 35-50°C for 0.5-1.0 minutes, and primer extension at 65-70°C for 2-6 minutes were executed for each PCR. To enrich for the correct PCR product

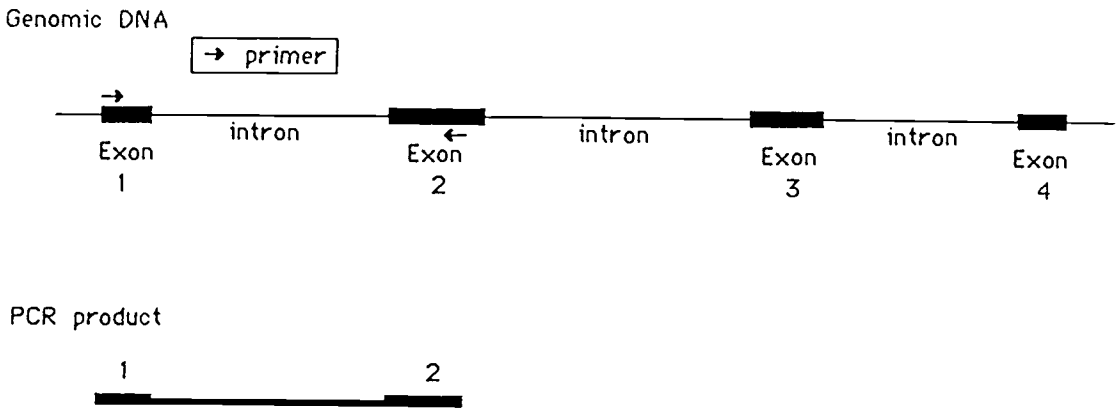


Figure 2. PCR amplification of a portion of a *ras* gene from genomic DNA. Two primers (one corresponding to the sense strand at the 5' end of the sequence to be amplified and one corresponding to the complementary strand at the 3' end of the sequence to be amplified) are indicated by arrows. The resulting PCR product after amplification is shown below the original DNA target.

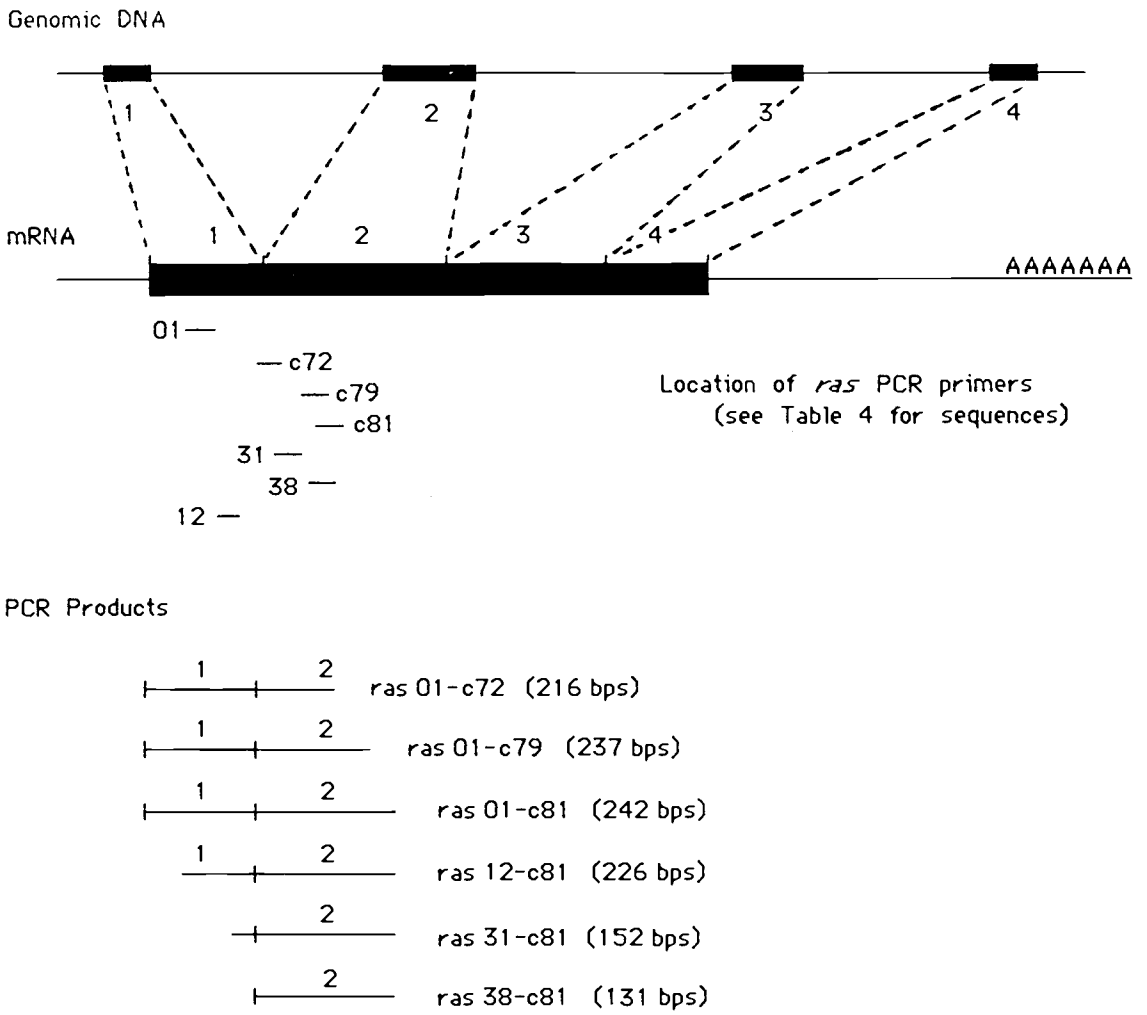


Figure 3. PCR amplification of a portion of a *ras* sequence from cDNA. The relative positions of several primers are shown, and the resultant PCR products are depicted in the bottom portion of the illustration. The actual sequences of the primers are listed in Table 4.

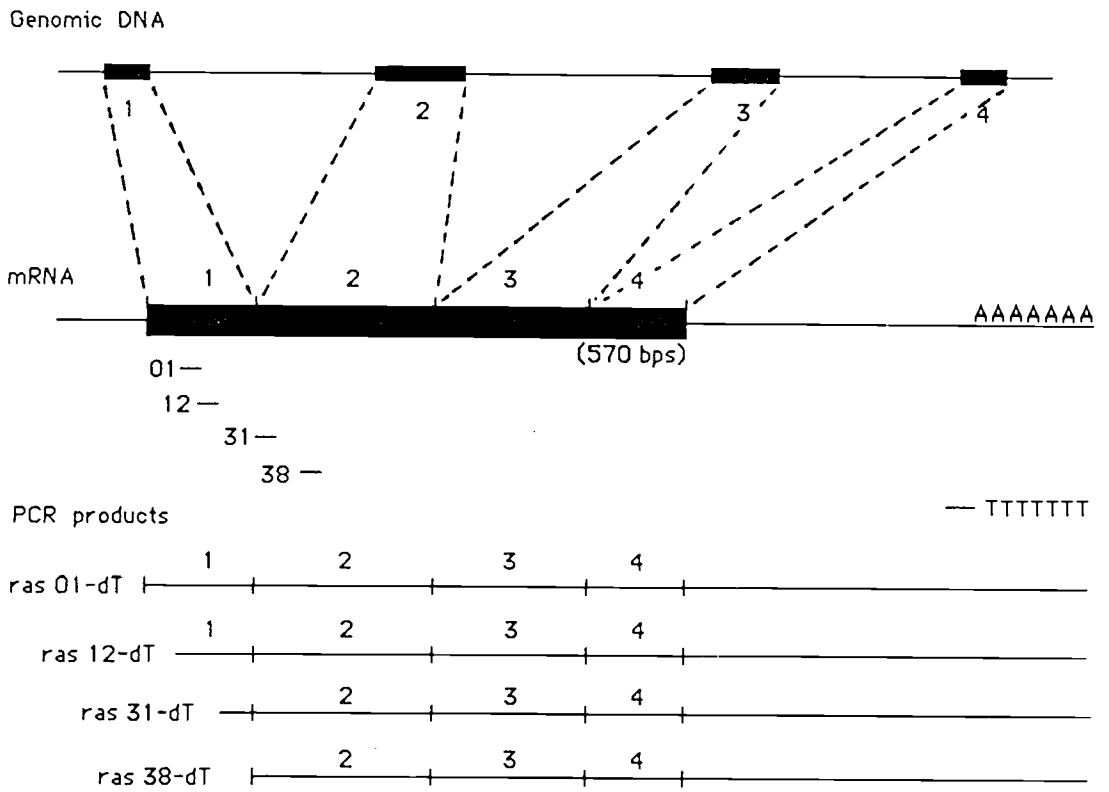


Figure 4. PCR amplification of the 3' portion of a *ras* sequence from cDNA. Amplification of the 3' end of cDNA is accomplished using a 5' primer and poly(dT)$_{24}$. The resultant PCR products (all >500 bps in length) are depicted in the bottom portion of the illustration.

Table 4. *ras* PCR primers. (5'-3')

*ras*01	ATG ACZ GAA TAT AAX CTV GTG
ratK*ras*01	ATG ACT GAG TAT AAA CTT GT
*ras*c72	CAT GTA CTG GTC CCV CAT VGC
*ras*c79	XAG XAA GCC CTC YCC ZCT WC
RT*ras*H01	ATG ACV GAA TAT AAG CTV GTG G
RT*ras*Hc81	ACA CAG AGG AAG CCC TCY CC
RT*ras*H12gly	GGG GGC AGG AGG TGT GGG CA
RT*ras*H31	GAA TAT GAC CCC ACC ATC GAG
RT*ras*H38	GAC TCG TAC AGG AAG CAG GTG G
poly $(dT)_{24}$	TTT TTT TTT TTT TTT TTT TTT TTT

A:adenine, C:cytosine, G:guanosine, T:thymidine, V:G or T, W:G or C, X:G or A, Y:C or T, Z:A, T, or G.

containing the 3' end of cDNA, two sequential PCRs were executed (Ohara et al., 1989). After an initial PCR amplification of either 10 or 40 cycles containing cDNA, a 5' primer, and the poly$(dT)_{24}$ primer, the PCR reaction mixture was washed and concentrated with a Centricon-100 (Amicon, Danvers, MA) and an aliquot used in a second PCR reaction containing an internal 5' primer and poly$(dT)_{24}$. The PCR products were examined by electrophoresis on a 4-6% acrylamide gel, transferred to a Zeta-Probe membrane (Bio-Rad Laboratories, Richmond, CA), hybridized and probed to identify *ras*-positive bands (see below).

Asymmetric PCR for direct sequencing was accomplished by limiting the amount of one of the two primers to 0.6 pmoles/50 μl reaction, 1/100 the concentration of the unlimited primer. The selection of the primer to be limited was in the same orientation as the sequencing primer.

Southern Transfer

A fraction (5-10 μl) of each PCR reaction was mixed with 1 μl of bromophenol blue/xylene cyanol/glycerol loading dye and loaded onto a vertical 4% acrylamide gel. Electrophoresis was accomplished at 15 v/cm for 40-60 minutes. The gel was stained with ethidium bromide and photographed with UV transillumination. The DNA was transferred in 1 M NaCl, 0.5 M NaOH to ZetaProbe membrane by capillary action for 16 hours. The membrane was rinsed in 2x SSC (0.3 M NaCl, 0.03 Na_3 citrate, pH 7.0) and baked for 2 hours at 80°C.

Genomic DNA was digested with various restriction enzymes (Bethesda Research Laboratories; Boehringer Mannheim Biochemicals; Pharmacia, Piscataway, NJ; New England BioLabs, Beverly, MA) using the appropriate 10x reaction buffer and 5 units of enzyme/μg DNA, and incubating the reactions for 16 hours at the appropriate temperature for the various enzymes. The concentration of DNA in the reaction was approximately 25 μg/500 μl total volume. The digested DNA was concentrated using Centricon-30 concentrators. One μl

of loading dye was added to 10 μg of digested DNA, and the sample was loaded into each well of a horizontal 0.8% agarose gel. Electrophoresis was carried out at 3 v/cm for 4-8 hours until the bromophenol blue dye reached 1 cm from the end. The gel was stained with ethidium bromide and photographed with UV transillumination. The gel was soaked with slight agitation for 15-20 minutes in 0.25 M HCl at room temperature to hydrolyze the DNA, and the DNA was transferred in 0.4 M NaOH to ZetaProbe membrane by capillary action for 16 hours. The membrane was rinsed in 2x SSC and baked for 2 hours at 80°C.

Radioactive Labeling of Hybridization Probe

Random primer labeling of DNA probes was achieved using α^{32}P-dATP (3000 Ci/mmol, 10 μCi/μl; Du Pont NEN, Boston, MA) and a commercial random labeling system (Bethesda Research Laboratories). Twenty-five ng of probe DNA was heat-denatured in a volume of 23 μl and placed on ice. Addition of 2 μl each of 0.5 mM dCTP, 0.5 mM dTTP, and 0.5 mM dGTP was followed by addition of 15 μl random primers buffer mixture, 5 μl α^{32}P-dATP, and 1 μl Klenow enzyme. The mixture was incubated at room temperature for 1-4 hours. The probe was heat-denatured before being added to the hybridization solution.

Hybridization

The membrane was prehybridized for 1-6 hours in hybridization buffer (50% formamide, 6x SSC, 100 μg/ml chicken DNA, 250 μg/ml yeast RNA, 0.5% Carnation non-fat dry milk, 1% SDS) at 42°C. Radioactive probe (25-100 ng) was added to the hybridization solution and incubated 14-30 hours at 42°C. The membrane was removed from the hybridization solution and washed with 2x SSC, 0.1% SDS at room temperature for 15 minutes, followed by washes of increasing stringency (2x SSC to 0.1x SSC and 42°C to 55°C) until a majority of the membrane had radioactivity levels near background. The membrane was blotted dry and wrapped in cellophane wrap. Autoradiography of the membrane was done with enhancer screens at room temperature, with exposure times of 2 hours to 3 days.

DNA Extraction from Acrylamide Gel Slices

DNA from PCR reactions was concentrated using Centricon-30 filtration units, mixed with 1/10 volume loading dye, and electrophoresed through a vertical 4% acrylamide gel at 15 v/cm for 1.0-1.5 hrs. The gel was lightly stained with ethidium bromide and photographed with UV transillumination. A section of the gel containing DNA of the desired size (determined by position relative to size markers) was sliced out using a new razor blade. The DNA was eluted from the gel slice into 0.5-1.0 ml of 0.5 M ammonium acetate by

overnight incubation at 37°C with shaking. The eluted DNA was concentrated using Centricon-30 filtration units and used for direct sequencing or for cloning.

Cloning

Digestion of pUC 10 vector was achieved with a 2 hour incubation at room temperature with the appropriate 10x reaction buffer and 5 units of Sma I restriction enzyme/μg DNA at a DNA concentration of 1 μg/20 μl total reaction volume. The reaction was heat-inactivated at 60°C for 15 minutes. Insert DNA was concentrated using Centricon-30 or Ultrafree MC filtration unit (Millipore, San Francisco, CA) and combined with vector DNA at a ratio of 100:1 to 250:1, and the DNA was precipitated with 1/10 volume 6.5 M ammonium acetate and 2.5 volumes 95% ethanol. After vacuum drying, the precipitated DNA was resuspended in 10 μl 1x ligase buffer containing 1 unit T4 ligase (Bethesda Research Laboratories) and incubated at 10-14°C for 16 hours. The ligation mixture was diluted 1:5 and 1 μl was used to transfect 25 μl of DH5α competent cells (Bethesda Research Laboratories). The transfected cells were plated onto LB agar plates containing ampicillin (100 μg/ml) with 10 μl 10% 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal; Boehringer Mannheim Biochemicals) in dimethylformamide and 10 μl isopropyl-β-D-thiogalactopyranoside (IPTG; Boehringer Mannheim Biochemicals). The plated cells

were incubated at 37°C overnight. White colonies were screened for DNA insert size by mini-preparation analysis and for probing by slot blot hybridization.

Plasmid Mini-preparation

After regrowth on an ampicillin grid plate, bacteria were resuspended in 0.5 ml glucose buffer (50 mM glucose, 25 mM Tris, pH 8.0, 10 mM EDTA) containing 1 mg/ml lysozyme and incubated at 25°C for 10 minutes. Ten μl of 10% SDS was added to each sample and mixed well. Fifty μl of 3 M potassium acetate (KAc) solution, pH 4.8 was added to each sample, mixed well, and incubated on ice for 30 minutes. The samples were centrifuged at 15,000 rpm in a Hermle tabletop microfuge for 30 minutes at 4°C. The supernatant was poured into a new tube, and the tube filled with 95% ethanol. The DNA was precipitated by incubation at -20°C for 15 minutes, followed by centrifugation at 12,000 rpm for 15 minutes. The DNA pellet was vacuum dried, followed by resuspension in 20 μl TE containing RNase A. Ten μl of each sample was added to 1 μl loading dye. The samples were electrophoresed through a 1% agarose gel at 10 v/cm for 30-45 minutes. The gel was stained with ethidium bromide and photographed with UV transillumination.

Slot blot hybridization

One μl of loading dye was mixed with 10 μl of plasmid mini-prep DNA, or alternatively with whole

cells resuspended in 10 μl TE buffer. Fifty μl of 0.5 M sodium hydroxide (NaOH) was added to each sample, and the mixture loaded onto a moistened ZetaProbe membrane mounted into a slot blot apparatus. After adsorption of the samples, the membrane was baked at 80°C for 1 hour before hybridization as mentioned above. Larger preparations of positive clones were made to obtain sequence-quality plasmid DNA.

Small Volume Plasmid Preparation

Bacteria containing probe-positive plasmids were inoculated into 5 ml LB broth containing 100 μg/ml ampicillin and incubated overnight at 37°C with shaking. The cells were pelleted by centrifugation at 3,000 x g. The cells were resuspended in 225 μl glucose buffer. Addition of 75 μl glucose buffer containing 8 mg/ml lysozyme was followed by incubation at 25°C for 5 minutes. Six hundred μl of 0.2 M NaOH, 1% SDS was added to each sample, mixed well, and incubated on ice for 5 minutes. Four hundred fifty μl of ice cold 3 M KAc, pH 4.8 was added to each sample. After mixing well, the samples were centrifuged for 10 minutes at 12,000 x g at 4°C. The supernatant was transferred to a new tube, avoiding all precipitate. Isopropanol (750 μl) was added to each sample, and the sample was mixed by vortexing. After incubation at -20°C for 15 minutes, the plasmid DNA was precipitated by centrifugation at 12,000 x g at 4°C. The DNA was

resuspended in 200 μl TE containing RNase A (25 μg/ml), and immediately phenol/chloroform extracted. The DNA was reprecipitated with 1/10 volume 6.5 M ammonium acetate and 3 volumes of 95% ethanol. The plasmid DNA was resuspended in 25 μl TE. One μl of the plasmid DNA sample was mixed with 1 μl loading dye and 5 μl TE, and the sample was electrophoresed through a 1% agarose gel at 10 v/cm for 30-45 minutes. The gel was stained with ethidium bromide and photographed with UV transillumination.

Large Volume Plasmid Preparation

Bacteria containing the desired plasmid were incubated overnight at 37°C with shaking in LB broth containing 100 μg/ml ampicillin. The cells were pelleted by centrifugation at 3,000 x g for 5 minutes, and the supernatant discarded. The cells were resuspended in 2 ml glucose buffer, followed by an addition of 0.5 ml glucose buffer containing 10 mg/ml lysozyme and incubation at room temperature for 5 minutes. Five ml 0.2 M NaOH, 1% SDS was added, mixed gently, and the suspension was incubated 10 minutes on ice. Four ml of ice-cold 3M KAc, pH 4.8 was added and mixed well, followed by an additional 10 minute incubation on ice. The samples were centrifuged for 10 minutes at 12,000 x g at 4°C. The supernatant was transferred to a new tube, avoiding all precipitate. Isopropanol (5.5 ml) was added to each sample, and the

sample was mixed by vortexing. After incubation at -20°C for 15 minutes, the plasmid DNA was precipitated by centrifugation at 12,000 x g at 4°C. The DNA was resuspended in 1 ml TE containing RNase A (25 μg/ml), and immediately phenol/chloroform extracted. The DNA was reprecipitated with 1/10 volume 6.5 M ammonium acetate and 3 volumes of 95% ethanol. The plasmid DNA was resuspended in 100 μl TE. One μl of the plasmid DNA sample was mixed with 1 μl loading dye and 5 μl TE, and the sample was electrophoresed through a 1% agarose gel at 10 v/cm for 30-45 minutes. The gel was stained with ethidium bromide and photographed with UV transillumination.

Restriction Mapping of Recombinant Plasmids

Each clone of interest was digested with 12-20 different restriction enzymes, with digestion using 1 or a combination of 2 enzymes/reaction. An aliquot of each reaction was electrophoresed through a 5% acrylamide gel at 20 v/cm for 45-60 minutes. The fragment sizes were estimated by comparison to marker band fragments.

Sequencing

Direct-sequencing of PCR products was achieved when a strong band was amplified during the asymmetric amplification. End-labeled primer was made using γ-^{32}P-ATP (3000 Ci/mmol, 10μCi/μl) and polynucleotide kinase at 37°C for 1 hour. Sequencing reactions were

executed according to a modification of Taq-Track sequencing system (Promega Biotec). Concentrated PCR products were suspended in 25 μl of 1x Taq sequencing buffer, heat-denatured at 95°C for 5 minutes and immediately placed on ice. Ten pmol of end-labeled primer and 4 units of Taq DNA polymerase were added to the denatured DNA. Six μl of the reaction mix was added to 1 μl of each deoxy-/dideoxy-nucleoside triphosphate (d/ddNTP) in an appropriately labeled tube and incubated at 70°C for 5 minutes. The sequencing reactions were stopped by the addition of 3 μl formaldehyde stop solution. The DNA was denatured at 95°C for 5 minutes before being loaded onto a 8 M urea, 5% acrylamide sequencing gel. The samples were electrophoresed for 2-4 hours at a constant power of 45 watts. After drying at 80°C on a slab dryer for 1 hour, autoradiography was carried out at room temperature with 2-10 hour exposure before film development.

Sequencing of probe-positive clones was also accomplished using a different modification of Taq-Track system (Promega Biotec). Plasmid DNA (100-2000 ng) was denatured in 0.4 M NaOH for 5 minutes at 25°C. M13/pUC primer (2 pmol) was added, followed by the addition of potassium acetate (3 M K, 5 M acetate, pH 4.8) to a final concentration of 0.45 M. Three volumes of 95% ethanol was added and the sample was centrifuged at 12,000 rpm for 30 minutes to precipitate the denatured

DNA. After air drying, the samples were resuspended in 5 μl 5x Taq buffer, 2 μl extension mix, and 11 μl sterile distilled water. Two μl of α^{32}P-dATP (3000 Ci/pmol, 10 mM) was added, followed by 1.5 μl Taq DNA polymerase (5 units/μl) and immediately incubated at 37°C for 3-10 minutes. One μl of each d/ddNTP solution was added to the appropriately labeled tube. Six μl of the reaction mix was added to each d/ddNTP and incubated at 70°C for 5 minutes. The sequencing reactions were stopped by the addition of 3 μl formaldehyde stop solution. The DNA was denatured at 95°C for 5 minutes before being loaded onto a 8 M urea, 5% acrylamide sequencing gel. The samples were electrophoresed for 2-4 hours at a constant power of 45 watts. After drying at 80°C on a slab dryer for 1 hour, autoradiography was carried out at room temperature with 2-10 hour exposure before film development.

RESULTS

PCR of Trout Genomic DNA

The use of degenerate primers in the PCR procedure to identify possible *ras*-like sequences in trout genomic DNA posed several problems. The annealing temperature during the PCR reaction was lowered to below 42°C to achieve visible products on ethidium bromide gels. The lower annealing temperatures and the degenerate primers resulted in nonspecific PCR reactions, with many diffuse bands visible in each reaction. Lowering the Mg^{2+} concentrations to a low level (1.5 mM) improved the specificity of the reactions to some extent (data not shown), but unique bands were not observed with any reactions utilizing degenerate primers to amplify *ras* sequences from genomic DNA. Although *ras*-positive PCR products were obtained from the positive control, mouse DNA, after extension times of 2-3 minutes per cycle, the larger *ras*-positive PCR products obtained from trout genomic DNA required extension times of 4-5 minutes per cycle. Figure 5 shows both an ethidium bromide-stained 1.2% agarose gel and an autoradiograph of the transfer membrane probed with a 1.0 Kb human c-Ha-*ras* probe. The primers used for the reactions in this illustration were *ras*01 and *ras*c72, and a similar *ras*-positive band was obtained using the primers *ras*01 and *ras*c79. The size of the *ras*-positive PCR product obtained from

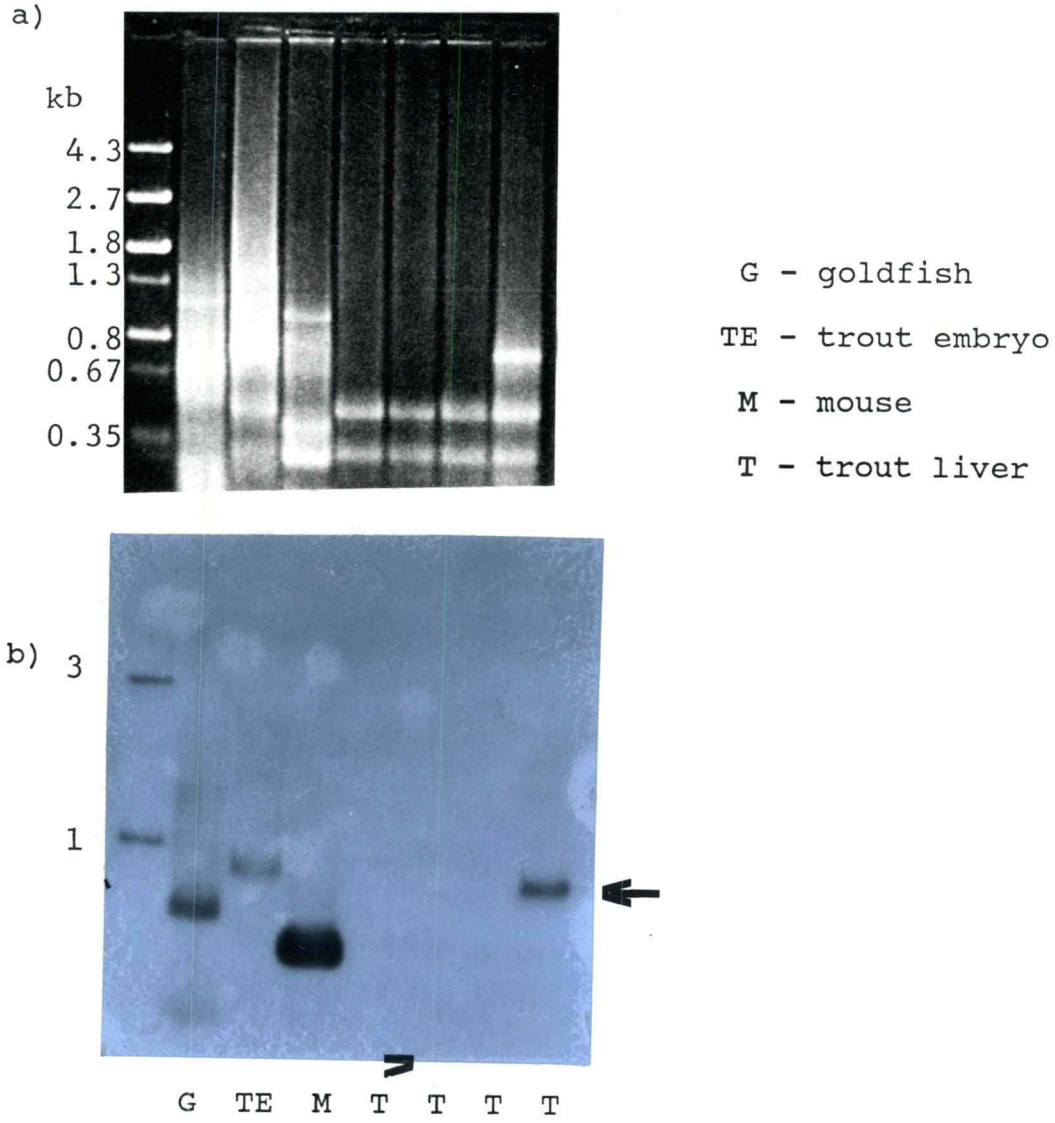


Figure 5. PCR products of genomic DNA (a) An ethidium bromide-stained 1.2% agarose gel displaying products of a PCR amplification using primers *ras*01 and *ras*c72, and an extension time of 5 minutes. (b) An autoradiograph of the transfer membrane probed with a 1.0 Kb human c-Ha-*ras* probe. The arrow indicates probe-positive bands of approximately 800 bps in several lanes of products from PCR amplifications of trout genomic DNA.

amplification of trout genomic DNA was calculated to be approximately 800 bases in length, suggesting an intron size of approximately 550 bases. This intron size is different from any vertebrate *ras* gene previously reported, indicating a sequence apparently unique to trout. Direct sequencing of the *ras*-positive trout genomic PCR product was not feasible due to the large number of nonspecific PCR products in the reaction. The DNA from reactions using the primers *ras*01 and *ras*c72 was sized on a 4% acrylamide as mentioned in the Materials and Methods section, and prepared for cloning into the Sma I site of pUC 10 vector.

PCR of Trout Liver cDNA--Possible Carryover Contamination

The PCR technique was used to amplify any *ras* sequences expressed in normal trout liver, some of which may have large introns to prohibit their amplification from genomic DNA. As a positive control, a linearized plasmid containing an activated human Ki-*ras* cDNA was used as a companion reaction. Figure 6 shows ethidium bromide-stained 3% Nu-Sieve (FMC BioProducts, Rockland, ME) agarose gel of both the genomic and cDNA PCR products, and the accompanying autoradiographs probed with either a 1.1 Kb human Ki-*ras* cDNA probe or a 1.0 Kb human c-Ha-*ras* probe. Both the trout genomic and liver cDNA PCR products contained a 216 bp band that probed positively with the Ki-*ras*

Figure 6. Comparison of PCR products from genomic DNA and cDNA. (a) An ethidium bromide-stained 3% Nu-Sieve agarose gel of both the genomic and cDNA PCR products. Lane 1 - isolated 800 bp trout genomic PCR product; Lane 2 - 216 bp "trout" cDNA PCR product; Lane 3 - 216 bp human Ki-*ras* cDNA PCR product; Lane 4 - 500 bp human Ha-*ras* genomic PCR product; Lane 5 - trout genomic PCR products ; Lane 6 - mouse genomic PCR products. (b) Autoradiograph of the transfer membrane probed with a 1.1 Kb human Ki-*ras* cDNA probe. Arrow indicates the probe-positive 216 bp product in several lanes. (c) Autoradiograph of the transfer membrane reprobed with a 1.0 Kb human c-Ha-*ras* probe. Arrow indicates the probe-positive 800 bp PCR product from amplifications of trout genomic DNA. (Note: exposure time for the autoradiograph of lanes 4-6 is 0.5 hr; the other portion of the autoradiograph is an exposure of 2 hr)

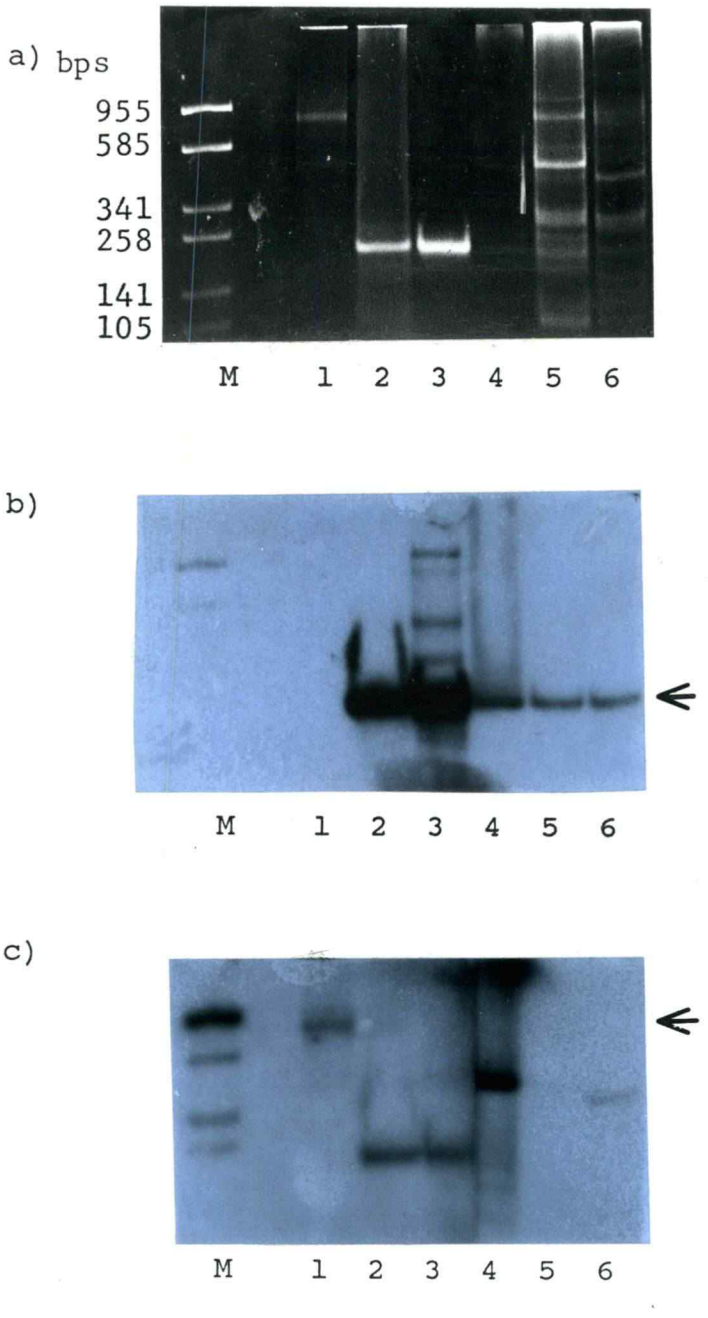


Figure 6.

probe but not with the Ha-*ras* product (Figure 7) revealed a sequence that was identical to the activated human Ki-*ras* cDNA used as a positive control in companion reactions. Since these species have been separated by 400 million years of evolution, it is unlikely that the *ras* gene sequences from the two species would be identical. Therefore, the "trout" sequence was believed to be in fact a human sequence resulting from the PCR amplification of the positive plasmid control contaminating the trout reactions. DNA from several different trout individuals as well as a control human placental DNA and rat liver DNA were amplified using the original primers and conditions. Sequencing of the human placental DNA PCR product revealed the normal Ki-*ras* proto-oncogene sequence, but the trout and rat PCR products again contained the activated human Ki-*ras* sequence (data not shown). Since the sequence of the rat Ki-*ras* is known to be different from that of the human Ki-*ras* gene, carryover contamination from the positive plasmid control was a probable cause for the incorrect sequence information.

Restriction Mapping of Trout Genomic 800 bp PCR Inserts of Recombinant Clones

Because of its unique intron size, the 800 bp PCR product would not be confused with any carryover product. To identify recombinant clones containing the trout genomic PCR product, several white colonies were

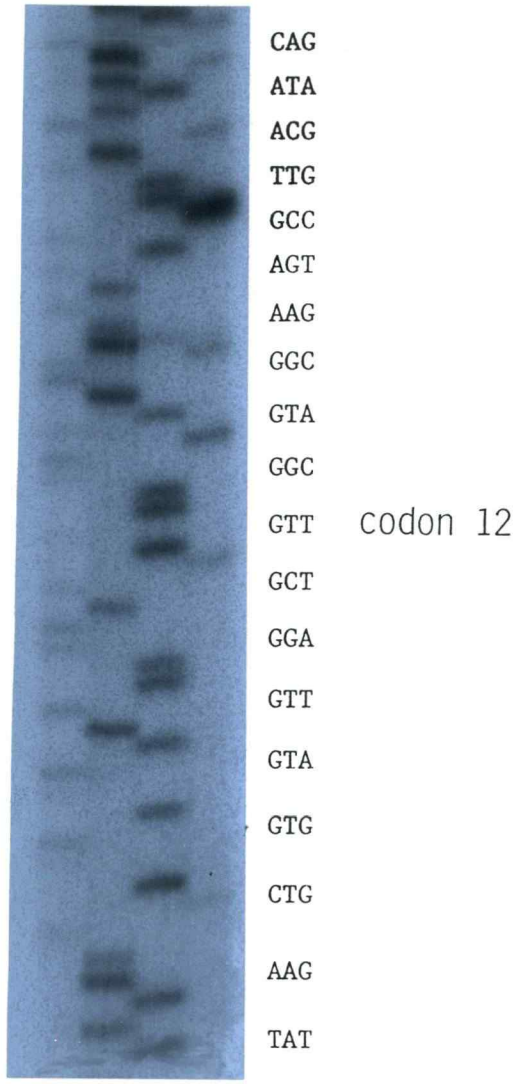


Figure 7. Sequencing gel autoradiograph showing carryover contamination. The sequence is of the 216 bp product from PCR amplification of trout liver cDNA. The sequence is identical to that of the human Ki-*ras* cDNA plasmid insert used as a positive control in the PCR reactions, and is believed to be carryover contamination in the PCR amplification of trout liver cDNA.

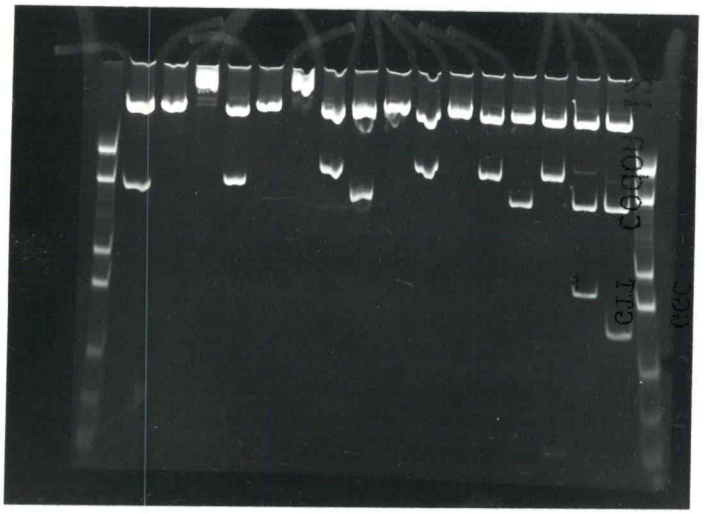
selected and sized by mini-preparation analysis for inserts larger than 600 bps. Five separate clones (Appendix Table I) were identified as possibly containing the trout PCR product. Two of the 5 clones were digested with 17 different enzymes, and Figure 8 shows an ethidium bromide-stained gel of many of the digested fragments of clone RT-8. Very few restriction sites were identified in the 800 bp inserts; however, an Xba I site was identified near the center of the insert. Sizing and positioning the digested fragments revealed a restriction map of RT-8 illustrated in Figure 9.

Sequencing of Trout Genomic PCR Product Inserts of Recombinant Clones

Partial sequencing of the 5 recombinant clones containing the trout genomic 800 bp PCR product revealed each contained a similar sequence with homology to mammalian *ras* genes. Clone RT-8 was identified as having a consensus sequence of the 5. Inserts in two clones (RT-6 and RT-8) were completely sequenced and the sequence of the RT-8 insert is shown in Figure 10. The insert contains the first exon and 131 bps of the second exon of a *ras* gene that is similar, but not identical, to vertebrate *ras* genes.

RT-8 Probing of Restriction Digested Trout Genomic DNA

Additional information on the structure of the gene containing the 800 bp sequence was obtained by



Lane 1 - Acc I	Lane 9 - Bam HI/Cla I
2 - Bam HI	10 - Bam HI/Eco RI
3 - Cla I	11 - Bam HI/Nco I
4 - Dra I	12 - Bam HI/Pst I
5 - Eco RI	13 - Bam HI/Xba I
6 - Nco I	14 - Hind III/Pst I
7 - Pst I	15 - Eco RI/Xba I
8 - Xba I	16 - Pst I/Xba I

Figure 8. Restriction digests of RT-8. Ethidium bromide-stained 6% acrylamide gel of clone RT-8 digested with several restriction enzymes. (Marker sizes: 955 bps; 585 bps; 341 bps; 258 bps; 141 bps; 105 bps; 78 bps; 75 bps)

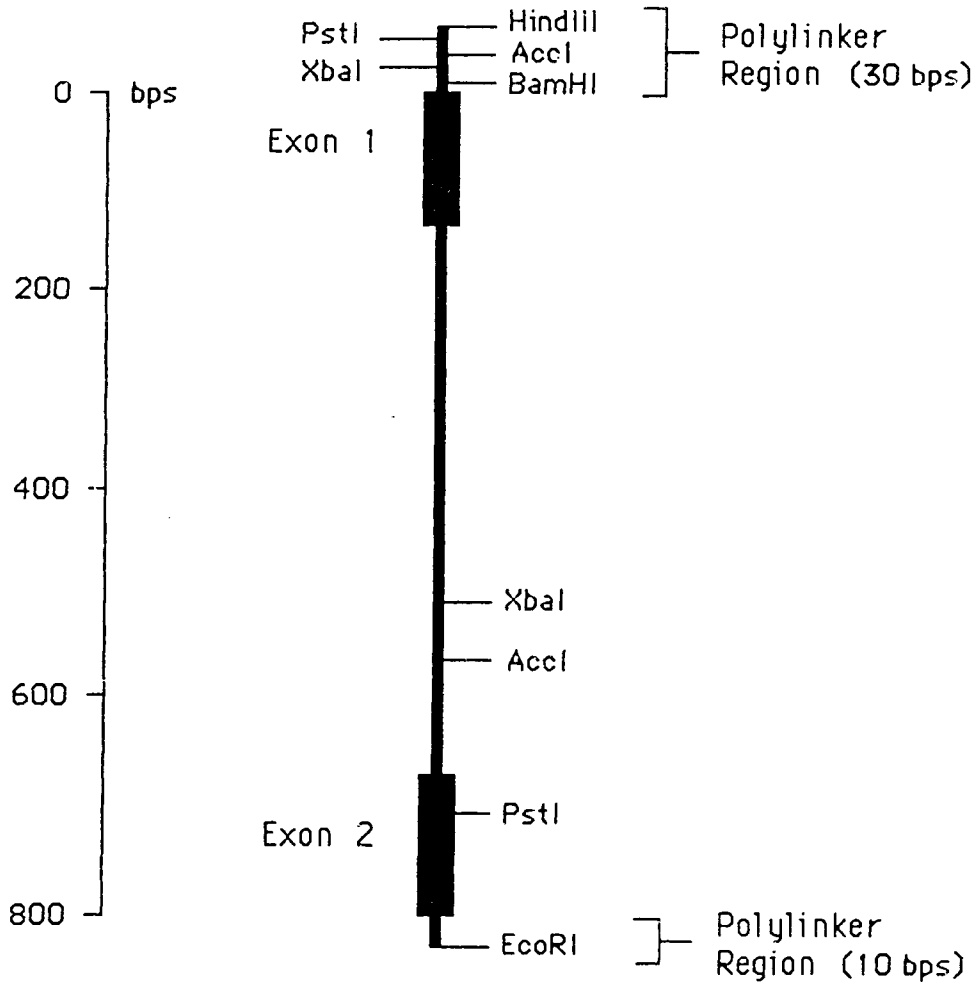


Figure 9. Restriction map of the RT-8 insert.

EXON 1 ATG ACG GAA TAT AAG CTT GTG GTG GTG GGG GCA GGA GGT GTG GGC AAG AGC GCG CTC ACC ATC CAG CTC ATT CAG AAC CAC TTT GTG GAT GAA TAT GAC CCC ACC ATC GAG

INTRON GTAATAAGTAGTTTCAGTACACCCTTGTCTCTTACTTCAGCAAGGCATGCCTGGGAATCGGGAGACAAAAATCTT TTAGAAATAAAGTTGTGTAAACAGGTTTCGATCATTTCTGATCATTTTCTGTTATTTGAATGCATGAATATTATA ATATCATAAACATGGTGTCTTTCAGTCAGTTTAATTTTGTTGGTTGATGAAAGATTACTATGTGATTAAATGCTT TAGTAGTTTCACATGTCAGATATTATTACTCAATTATACTTTCTGAAAGCTATCAATTTTATGACTGCTTGAAGA TTGAGTGCATTTTTTCCCTTGGTGCACATACAGTCGCTCTTAGTCATCCCATCCATTCTCTCTGATTCTAATATTA TTGAATTTCCTCAGCGACTTTATTAGATGAGCATTCTATGACCATATAGTTAGGTCTAGACATTCTGATTCCTAG TCAGGTAGACTGGACACCTATGAATATGTGATCATTCATAAGTGTCCAGTCTACCTGATTACCGCTAAGGTGCCC TCCTGACCCAACCCATCCCTCTCTTTCTCTCTCCCTCCTTCAG

EXON 2 GAC TCG TAC AGG AAG CAG GTG GTG ATT GAT GGG GAG ACA TGT CTG CTG GAC ATC CTG GAC ACT GCA GGT CAG GAG GAG TAC AGC GCC ATG AGG GAC CAA TAC ATG AGG ACA GGG GAG GGC TTC CTC (End of insert)

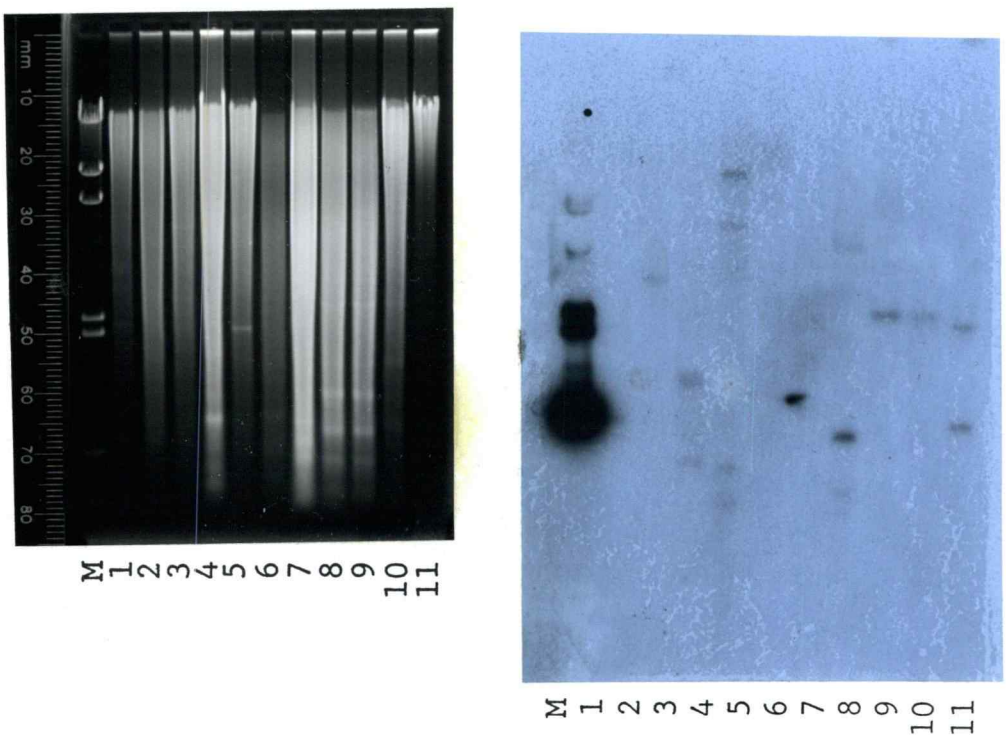
Figure 10. Sequence of the 800 bp PCR product insert of RT-8.

probing a southern transfer of restricted trout genomic DNA with the insert of RT-8. Previous attempts to identify trout *ras* genes on southern transfers using mammalian *ras* probes were unsuccessful. Trout genomic DNA was digested with 9 different enzymes, electrophoresed through a 0.8% agarose gel, transferred to Zeta-Probe membrane, and probed with ^{32}P-labeled RT-8 insert as described above with the RT-8 insert (Figure 11). Distinct bands were observed in many of the lanes. In general, the data obtained from the transfer corroborated the restriction map information. Discrepancies, such as the multiple bands in the lane containing the Hind III digested DNA, were attributed to incomplete digestion of the trout genomic DNA.

PCR of Trout Liver cDNA

Based on the unique trout sequence obtained from the clone RT-8, nondegenerate primers were synthesized for amplification of cDNA to determine whether this trout *ras* gene is expressed in liver cells. Amplification of uncontaminated trout liver cDNA using internal 5' primers derived from the RT-8 insert sequence resulted primarily in specific PCR products that probed positively with the RT-8 insert (Figure 12). A 215 bp trout liver cDNA PCR product (from a reaction using RT*ras*H12gly and RT*ras*Hc81 as primers) and a 130 bp trout liver cDNA PCR product (from a reaction using RT*ras*H38 and RT*ras*Hc81 as primers) were cloned as

(a) (b)



Lane 1 - Bam HI
2 - Bam HI/Bgl II
3 - Eco RI
4 - Hind III
5 - Kpn I
6 - Nci I
7 - Pst I
8 - Pvu II
9 - Pvu II
10 - Xba I
11 - Xho I

Figure 11. Southern transfer of trout genomic DNA probed with RT-8 insert. (a) Ethidium bromide-stained 0.8% agarose gel of trout genomic DNA digested with several restriction enzymes. (b) Autoradiograph of the southern transfer probed with the insert of RT-8.

(Marker - 23 kb; 9 kb; 6 kb; 2.3 kb; 2.0 kb; 0.5 kb)

previously described. The inserts of several recombinant clones were sequenced, and 3 of the 4 clones obtained from the reaction utilizing RT*ras*H12gly as the 5' primer had sequences identical to RT-8. The fourth clone, along with a clone obtained from the reaction containing RT*ras*H38, had 3 base differences from the RT-8 insert sequence.

After obtaining sequence information on the 5' end of a *ras* gene expressed in trout liver, a modification of the PCR technique which utilizes one specific primer and one nonspecific primer could be used to amplify the 3' end of that gene (Ohara et al., 1989). Using oligo$(dT)_{15}$ as the nonspecific primer with any of the specific 5' primers was not effective (data not shown), possibly due to insufficient hydrogen bonding between 15 dA:dT pairs. A single amplification of trout liver cDNA using poly$(dT)_{24}$ as the nonspecific primer with any of the trout specific 5' primers resulted in the production of a variety of product sizes; however, no probe-positive areas were visualized. In contrast, a second amplification of the first PCR products using an internal 5' primer plus poly$(dT)_{24}$ did result in two bands that probed positively with RT-8 (Figure 13). The corresponding area of a reaction amplified first using RT*ras*H31 and subsequently with RT*ras*H38 was isolated and cloned as above. Recombinant clones were screened by slot blot hybridization with the RT-8 insert

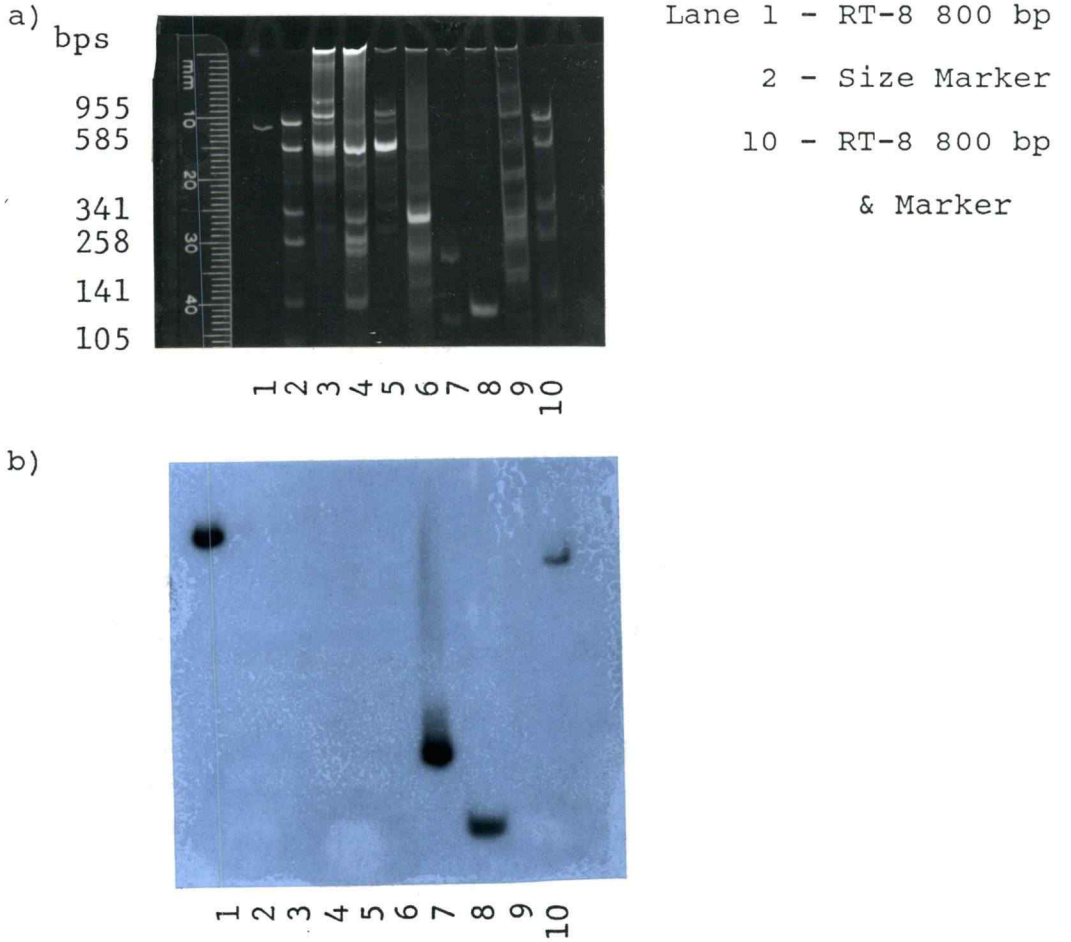


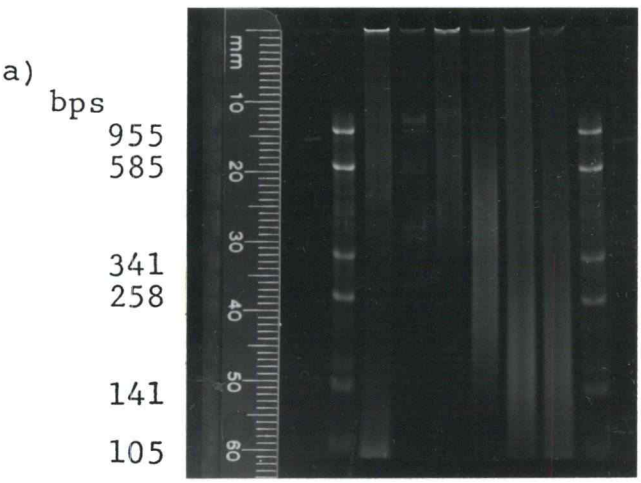
Figure 12. PCR products of trout liver cDNA. (a) An ethidium bromide-stained 4% acrylamide gel of products of an amplification of uncontaminated trout liver cDNA with trout-specific primers. (b) Autoradiograph of the southern transfer probed with the RT-8 insert. Lane 7 contains the 215 bp product from a PCR amplification of trout liver cDNA using RT*ras*H12gly and RT*ras*c81 primers. Lane 8 contains the 131 bp product from a PCR amplification of trout liver cDNA using RT*ras*38 and RT*ras*c81 primers.

(Figure 14). The sequence of one probe-positive clone (RT31/38-dT-8) matched the sequence of the two cDNA PCR product clones that differed from RT-8, and none of the differences caused a change in the amino acid sequence. In the region extending beyond codon 81, the sequence continued to resemble a *ras* gene through nucleotide 500 where it suddenly had a poly(dA) region, resulting in an unexpectedly short product. The coding region of the *ras* genes extends at least 567 bps, with additional 3' noncoding information included in the mRNA transcript before the poly(dA) tail. Analysis of this region in other vertebrate *ras* genes revealed an abundance of adenine bases in many of the sequences, indicating a possible annealing site for the poly$(dT)_{24}$ primer that could result in the truncated PCR product. Of the probe-positive clones analyzed, none contained an insert of a size equal to the larger (possibly nontruncated) probe-positive band identified on the southern transfer. Also, no clones were identified that matched exactly the sequence of RT-8, raising the possibility of several *ras* genes being expressed in trout liver.

In an attempt to isolate the 5' sequence of the trout *ras* gene that is linked to the sequence of RT31/38-dT-8, several different primer combinations were used in PCR amplifications of trout liver cDNA.

Figure 13. PCR products of the 3' end of trout *ras* cDNA. (a) An ethidium bromide-stained 4% acrylamide gel of products of an amplification of trout liver cDNA with trout-specific primers and poly(dT)$_{24}$. (b) Autoradiograph of the southern transfer probed with the RT-8 insert. Lane 2 contains 2 probe-positive PCR product bands of a reamplification using the poly(dT)$_{24}$ primer with RT*ras*38 after an original amplification using poly(dT)$_{24}$ primer with RT*ras*31.

a)


bps
955
585
341
258
141
105
mm
10
20
30
40
50
60
RT-8 M 1 2 3 4 5 6 M
800 bp

b)


RT-8 M 1 2 3 4 5 6 M
800 bp

Figure 13.

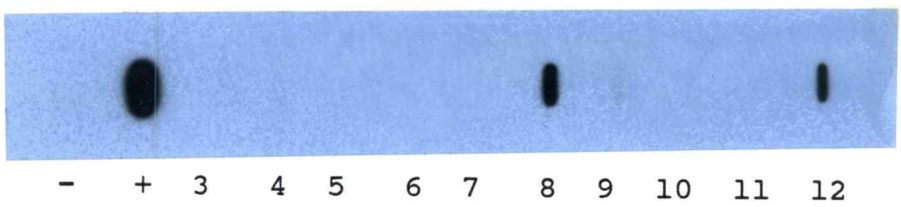
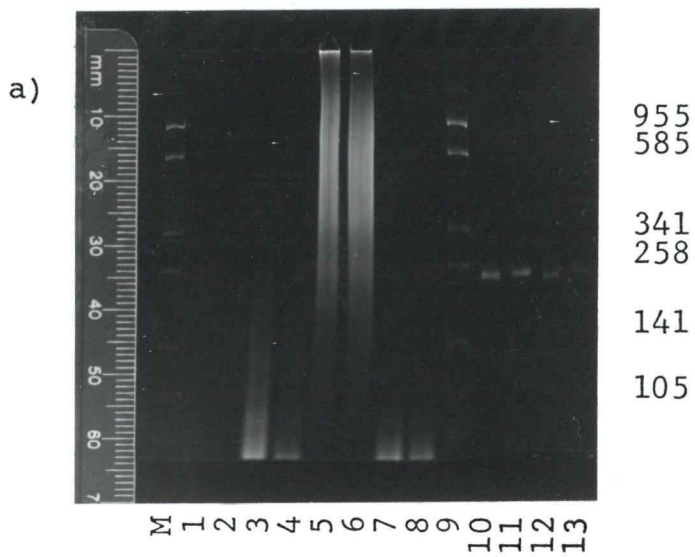


Figure 14. Autoradiograph of a slot blot of putative recombinant clones containing products from the PCR reamplification using the RT*ras*38 and poly(dT)$_{24}$ primers. Clones #8 and #12 probed positively with the RT-8 insert.

Primers derived from the first exon of rat Ki-*ras* gene sequence have recently been used in a PCR to obtain possible Ki-*ras* sequence information from winter flounder (McMahon et al., unpublished data). Amplification of trout liver cDNA using either the RT*ras*H01 or the rat*Kras*01 as the 5' primer and either *ras*c79 or RT*ras*Hc81 as the 3' primer resulted in the expected PCR products of 237 bps or 242 bps, respectively, which probed positively with the RT-8 insert (Figure 15). The PCR products were cloned as previously described, and several clones were sequenced. Two clones containing a PCR product from a reaction using the rat*Kras*01 and *ras*c79 have a unique *ras* sequence not previously described. A clone containing a PCR product from a reaction using RT*ras*H01 and *ras*c79 has a sequence that is similar to both RT-6 and RT-8, but has the same base substitutions found in RT31/38-dT-8. Table 5 displays the insert sequences of the different trout PCR recombinant clones.

Figure 15. PCR products of trout liver cDNA. a) Ethidium bromide-stained 4% acrylamide gel containing products from PCR amplifications of trout liver cDNA. Lanes 2 through 8 contain PCR products with specific 5' primers and poly(dT)$_{24}$. Lanes 10 through 13 contain PCR products with specific 5' and 3' primers. b) Autoradiograph of southern transfer probed with the RT-8 insert. The intense probe-positive bands are 237 bps (Lanes 10 & 12) or 242 bps (Lanes 11 & 13). Sequences of several clonal inserts are listed in Table 5.


a)
mm
10
20
30
40
50
60
955
585
341
258
141
105
M 1 2 3 4 5 6 7 8 9 10 11 12 13

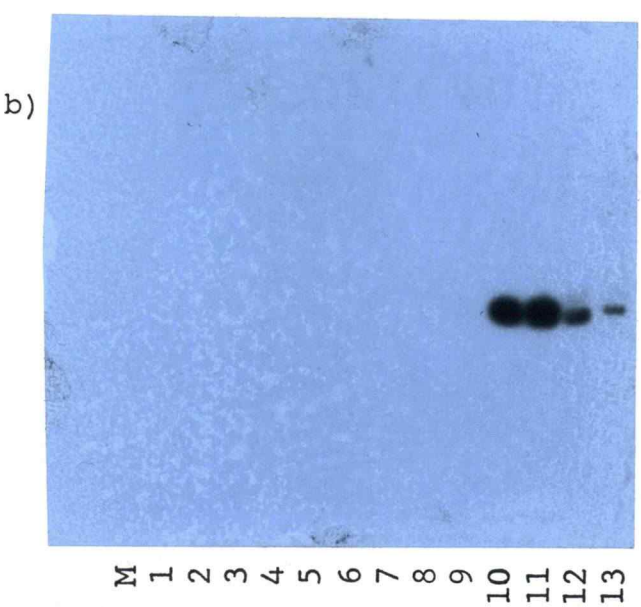

b)
M 1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 15.

Table 5. Trout ras PCR product sequences.

EXON 1

```
                10         20         30         40         50         60
Human H    ATGACGGAATATAAGCTGGTGGTGGTGGGCGCCGGCGGTGTGGGCAAGAGTGCGCTGACC
RT-6       -----T----------------------G--A--A--------------C-----C---
RT-8       ----------------T-----------G--A--A--------------C-----C---
H01c81-4   ----------------------------G--A--A--------------C-----C---
K01c79-1   -----T--G-----A--T--T--------T--T--A-----T--G---------CT-A--T
Human K    -----T--------A--T-----A--T--A--T--T--C--A-----------CT----G
Goldfish   -----A----------T-----C--A--G--T--A--C--------------T--C--C
Human N    -----T--G--C--A----------T--A--A--T-----T--G--A--C--A-----A

                70         80         90        100        110
Human H    ATCCAGCTGATCCAGAACCATTTTGTGGACGAATACGACCCCACTATAGAG
RT-6       --------C--------T--C---------T-----T--------C--C---
RT-8       --------C--T--------C--------T-----T--------C--C---
H01c81-4   --------C--------T--C--------T-----T--------C--C---
K01c79-1   --------C--------T--C--------T-----T--------C--C---
Human K    --A-----A--T-----T----------------T--T--A--A------
Goldfish   -----A--C----------C--------------------A--C------
Human N    --------A----------C-----A--T-----T--T-----C------
```

Table 5. Trout ras PCR product sequences (cont.).

EXON 2

```
                120       130       140       150       160       170       180
Human H   GATTCCTACCGGAAGCAGGTGGTCATTGATGGGGAGACGTGCCTGTTGGACATCCTGGATACCGCCGGC
RT-6      --C--G---A-------A-----G--------------A--T--CC-------------C--T--A--T
RT-8      --C--G---A-------------G--------------A--T---C-------------C--T--A--T
H01c81-4  --C--A---A-------------G-----C----------T---C-------------C--T--A--T
38dT-8    --C--G---A-------------G-----C----------T---C-------------C--T--A--T
38dT-12   --C--G---A-------------G-----C--A-------T---C-------------C--A--A--T
K01c79-1  --C--G---A-------------G-----C--A-------T---C-------------C--A--A--T
Human K   ---------A-------A--A--A--------A--A--C--T--C-----T--T--C--C--A--A--T
Goldfish  --C------A-------------G----------------T-----------T----C--T--A--T
Human N   -----T---A-A--A--A-----T--A-----T--A--C--TT----------A--------A--T--A

                190       200       210       220       230       240
Human H   CAGGAGGAGTACAGCGCCATGCGGGACCAGTACATGCGCACCGGGGAGGGCTTCCTGTGT
RT-6      --------------------A-------A------A-G--A--------------C (End of insert)
RT-8      --------------------A-------A------A-G--A--------------C (End of insert)
H01c81-4  --------------------A-------------A-G--A--------------C---
38dT-8    --------------------A-------------A-G--A--------------C---
38dT-12   --A-----------------A--A----------A-G--A-----A--------T---
K01c79-1  --A-----------------A--A----------A-G--A-----A--------  (End of insert)
Human K   --A----------T--A---A-------------A-G--T----------T--T---
Goldfish  --------------------A----T---------A-G--A--A----------C---
Human N   --A--A--------T------A-A-----A------A-G--A--C--A--------C---

                250       260       270       280       290
Human H   GTGTTTGCCATCAACAACACCAAGTCTTTTGAGGACATCCACCAGTACAGG
H01c81-4  -- (End of insert)
38dT-8    --C----------------------C--C-------------C--T--A
38dT-12   -----C-------------T------C---------G-T----T-------
Human K   --A--------A--T--T--T--A--A-----A--T--T-----T--T--A
Goldfish  --C--C--------T----------C----------T-----C-----A
Human N   --A----------T--T-G------A----C---T--TA---TC------
```

Table 5. Trout ras PCR product sequences (cont.).

EXON 3

```
                300        310        320        330        340
Human H    GAGCAGATCAAACGGGTGAAGGACTCGGATGACGTGCCCATGGTGCTGGTGGGG
38dT-8     -----------G--------A--------A----------------------A
38dT-12    --A--------CA----A-G-ACAG--TT-ACATG-G-AGCTCAAT---GATTT
Human K    --A--A--T---A-A--T--------T--A--T--A--T-----C--A--A--A
Goldfish   --------A-----A--A----------G-----C--------C---------
Human N    --------T--G--A--A--A----------T--A--T--------A-----A

             350        360        370        380        390
Human H    AACAAGTGTGACCTGGCTGCACGCACTGTGGAATCTCGGCAGGCT
38dT-8     ---------------C-GT-C--G--G-----CA-CAAA------
38dT-12    TTTTTTG-CA-AAGA----AT-TG-T--GTC--AAGACCAGTAG-
Human K    --T--A-----TT--C--T-T--A--A--A--CA-AAAA------
Goldfish   --T--------T--TC-GT-C----G------CA-CAA-------
Human N    ----------TT--C-AA--A-G--A--T--TA-AAAA--A--C

                400        410        420        430        440        450
Human H    CAGGACCTCGCCCGAAGCTACGGCATCCCCTACATCGAGACCTCGGCCAAGACCCGGCAG
38dT-8     ------T-G--G--C-CG--T----------T-----------------A---A-A---
38dT-12    G----AAATAT-A---TTC-G-CTGC-TGTGTG-A-ACGG---TATA-TGTTG--ATTTC
Human K    ------T-A--AA----T--T--A--T--T-TT--T--A--A--A--A-----AA-A---
Goldfish   -----TT-A--A--G---------------TT--A--------A--A-----GA-A---
Human N    --C--A--G---AAG--T-----G--T--A-T---T--A-----A---------A-A---
```

Table 5. Trout ras PCR product sequences (cont.).

EXON 4

```
                460        470        480        490        500        510
Human H    GGAGTGGAGGATGCCTTCTACACGTTGGTGCGTGAGATCCGGCAGCACAAGCTGCGGAAG
38dT-8     --T--C--T-----A--T-----A--A-----G--A-----AA-A------GAGAA--T-
38dT-12    TATAGGATCATGTCCATTATTAGGCTAGCTTTCAGTTCTAGCTTGAGGTCATTAAAGGAA
Human Ka   A-------------T--T--T--A------A-A--------A--AT---GAT--AAA--A
Human Kb   G-T--T--T-----------T--A--A--T--A--A--T--AA-A--T--AGAAAAG-T-
Goldfish   A-A-----A--------T--T--TC----A--G--A---A----AT--CG----A-A--A
Human N    --T--T--A-----T--T-----AC----AA-A--A--A--C---T--CGAA--AAA--A

                520        530        540        550        560        570
Human H    CTGAACCCTCCTGATGAGAGTGGCCCCGGCTGCATGAGCTGCAAGTGTGTGCTCTCCTGA
38dT-8     GCA-GGAGG-AAAAAAAAA
38dT-12    AGTGTGCACATGAAAAAAAAAA
Human Ka   A-C-G-AAAGAA--AA---C-CCTGGCT-TGTG-AA-TTAAA--A--CA-TA-AATG-A-
Human Kb   AGC--AGA-GG-A-AA---AGAAAAAGAAG-CA-A-...ACA--------AA-TATG-A-
Goldfish   --C-GTAAAGAA--A----CAACA-AAT--AT--A-CTT (End of insert)
Human N    --C---AGCAG------TG-GACT-AG--T--T---G-A-TGCCA------G-GATG-A-
Mouse N    --C---AGCAG---C--TG-CACT-AA--T--T---G-G-CGCCC--------GATG-AG
```

DISCUSSION

The Polymerase Chain Reaction

PCR is a very powerful and potentially dangerous tool. The system has the capability of amplifying two copies of a gene from a single cell into a million copies in only a few hours. The very real danger of amplifying a stray molecule from an unknown source has been documented (Kwok & Higuchi, 1989; Schochetman et al., 1988), and extreme precautions must be taken to avoid any carryover of DNA molecules from extraneous sources into the PCR environment. Previously amplified sequences and plasmid DNA are excellent substrates for the reaction, and preparation of the reactions should be executed in areas completely separate from the actual reaction and analysis areas. In this study, the first "trout" sequence obtained was identical to an activated human Ki-*ras* cDNA sequence contained in a plasmid used as a probe and as a positive control for the PCR system. Subsequent experiments showed the expected nonactivated sequence from human placental DNA, but the activated human Ki-*ras* sequence was obtained from amplifications of normal rat liver DNA and DNA isolated from 4 trout livers. The use of a separate laboratory and positive displacement pipets for the preparation of the reactions before amplification, isolating the PCR products from other reactions, and limiting the use of reamplification were

some of the precautions employed to eliminate carryover in the PCR.

Trout-Mammalian *ras* Homology

The first isolation of a PCR product of trout origin was the 800 bp PCR product amplified from genomic DNA. This PCR product was cloned and sequenced, revealing exon sequences that were highly homologous, but not identical, to vertebrate *ras* genes (Table 5). The sequencing also showed an intron of 550 bps, a size that differed from any reported vertebrate *ras* gene. These differences supported the conclusion that the sequence was of trout origin and not carryover of any type. Although a 90 bp *ras* sequence from winter flounder is reported to be identical to rat Ki-*ras* (McMahon, unpublished data), the DNA sequence homology of the 800 bp PCR product identified in this study (excluding primer regions) is 91% when compared with reported mammalian Ha-*ras* sequences, and has slightly less homology with other *ras* sequences (Table 6). The homology of RT-8 with human Ha-*ras* is 86%, and increases to 91% only after grouping all mammalian Ha-*ras* sequences together. Although base differences are present, the differences do not cause amino acid changes for the first 80 residues; therefore, this sequence could code for a portion of a functional trout *ras* gene. Three short stretches in the introns of both RT-8 and human Ha*ras* have greater than 70%

Table 6. Homology of trout *ras* PCR products to other vertebrate ras gene sequences.

	RT-8	H01c81:31/38dT
Human H	86%	85%
Mammalian H	91%	89%
Human K	80%	80%
Mammalian K	83%	81%
Human N	80%	82%
Mammalian N	85%	86%
Goldfish	90%	86%

homology, but very little is known about possible significance of these sequences. Although the sequence is closer to that of vertebrate Ha-*ras* genes than to Ki-*ras* or N-*ras*, classification of this trout sequence as one of the three classical *ras* genes would be inappropriate based on the limited sequence information available.

After identifying a *ras* gene sequence of trout origin, additional information about the entire gene and other related *ras* genes in trout can be obtained. Southern transfers of restriction enzyme-digested trout genomic DNA probed with human *ras* probes did not reveal any probe positive bands due to insufficient homology between the human and trout gene sequences (data not shown). Probing similar transfers with the

RT-8 insert, however, revealed distinct bands corresponding to restriction fragments containing the isolated trout *ras* sequence. For example, a single band (approximately 6 kb) is probe-positive in a BamHI digest of trout genomic DNA, while two bands (2 kb and 4 kb) are identified in an XbaI digest of the same DNA. The concordance of the southern transfer probing and the RT-8 restriction map shows the specificity of the RT-8 insert as a probe. Although some of the larger probe-positive restriction fragments may contain the entire trout *ras* gene, this possibility has not been confirmed.

Expression and Multiplicity of Trout *ras* Genes

PCR products from amplifying trout liver cDNA were cloned, sequenced, and found to have an identical sequence to RT-8 (except for being intronless). The *ras* gene represented by the RT-8 insert is actively expressed, and therefore a proto-oncogene and not a pseudogene. The PCR products were made using the trout specific 5' primers RT*ras*H12gly or RT*ras*H38 and the 3' primer RT*ras*Hc81. Although three separate clones identified contained the exact sequence of the RT-8 exons, several clones contained inserts with 2-3 base substitutions. The base substitutions were in the third position of specific codon triplets, and none resulted in amino acid changes after translation. The Taq DNA polymerase is known to have a higher degree of

error than other DNA polymerases (Saiki et al., 1988; Dunning et al., 1988); however, the consistency of the differences at positions which do not affect the amino acid sequence suggested the presence of several *ras* genes instead of the random positioning attributed to polymerase mistakes. The expression of more than one *ras* gene has been documented in several mammalian tissues, including liver (Leon et al., 1987). Trout, in particular, may have several very closely related *ras* genes, due to a relatively recent tetraploid event during evolution. During the tetraploid event, the chromosomal content per cell was doubled. Although several chromosomes have since fused, many duplicate genes remain, upon which selection and genetic drift can act. It is conceivable that several nearly identical *ras* genes exist in the trout genome and are expressed at some level in liver cells.

Strategies for Obtaining a Complete *ras* cDNA Sequence

The experiments described so far used a pair of specific primers to amplify a highly conserved region of one or more trout *ras* genes. The same approach would be less successful if applied to the 3' coding region due to greater divergence between the different *ras* genes and between species. However, using a primer derived from the known trout sequence at the 5' end and poly(dT)$_{24}$ to anneal to the poly(dA) tail of cDNA, the entire *ras* cDNA sequence from the 5' primer through the

poly(dA) tail can theoretically be amplified using a one-sided PCR technique (Ohara et al., 1989). This technique employs one specific primer (in this case, the 5' trout *ras* primer) and one nonspecific primer (the poly(dT)$_{24}$ primer). Since the poly(dT) primer will anneal to the poly(dA) tail of every cDNA transcript, the specificity of this reaction is less than reactions using two specific primers, or than reactions using degenerative primers for a specific sequence. To improve the specificity of the end product, a specific 5' primer slightly internal of the one employed in the first amplification was used along with the poly(dT)$_{24}$ primer in a second amplification of the first PCR products. The smaller of the two probe-positive bands visualized on a transfer of the PCR products is approximately 550 bps, the minimum size expected *a priori* for this reaction. The larger band is approximately 750 bps and is also a PCR product possibly containing the 3' region of a trout *ras* gene. Possible recombinant clones were screened by slot blot hybridization using the RT-8 insert as the probe, and two probe-positive clones were sequenced. Both contained *ras*-like sequences for the region corresponding to the second exon; however, neither contained the exact sequence of RT-6 or RT-8 in this region. Three to six base differences were identified in the 85 bp sequence in common between the trout

genomic PCR clones and the RT31/38dT clones. All of the differences occurred in the third position of codon triplets without changing the amino acid sequence. The clone with only three differences (RT31/38dT-8) did match the insert sequence of several recombinant clones that contained the PCR product from a PCR amplification using RT*ras*Hl2gly and RT*ras*Hc81 as specific primers. The clone RT31/38dT-12 diverged significantly from vertebrate *ras* sequences in the region corresponding to the third exon, and could be the result of a rearrangement after the PCR amplification. Clone RT31/38dT-8, on the other hand, agrees with the vertebrate *ras* sequences midway through the fourth exon, where a poly(dA) region occurred prematurely. Analysis of other vertebrate *ras* genes in the area of the premature poly(dA) reveals an adenine-rich region in several sequences; for example, mouse Ki-*ras* exon 4b has a stretch of 18 adenine bases out of a total of 25, beginning with 7 adenines in a row. At the low annealing temperatures used in the PCR procedure, this sequence could be an alternate priming site for $poly(dT)_{24}$, resulting in the smaller PCR product. The larger probe-positive band was not found in any of the isolated recombinant clones.

To clarify the sequence immediately 5' to the insert of clone RT31/38dT-8, additional PCR amplifications of trout liver cDNA were executed utilizing

several combinations of the available primers, including RT*ras*H01 and rat*Kras*01. Using two distinctly different primers corresponding to the first 20 bases of several *ras* genes in the PCR procedure resulted in at least two discrete products from trout liver cDNA. In comparison, primers derived from the RT-8 sequence resulted in the amplification of several different trout *ras* sequences, although a single primer pair was used in each reaction. The selection of the sequences used for the PCR amplifications had a dramatic effect on the nature of the product(s). RT*ras*H12gly, RT*ras*H31, and RT*ras*H38 were synthesized based on the sequence of the RT-8 insert, but their use in PCR amplifications resulted in several products in addition to the desired sequence. The selected sequences were, in many cases, in highly conserved regions of the different *ras* genes. The first 20 bases are distinctive between the different *ras* genes, and their use defined the resultant PCR product.

The sequence of PCR products from amplifications using rat*Kras*01 as the 5' primer was distinct from any previously reported trout *ras* PCR product with the exception of the 85 bp overlap with the clone RT31/38dT-12, which matched exactly. PCR products from amplifications using RT*ras*H01 as the 5' primer agreed exactly with the sequence of the 85 bp overlap with clone RT31/38dT-8. By linking the sequence of the

clone RT*ras*H01c81-4 with that of the clone RT31/38dT-8, 525 bps out of a theoretical 570 bps have been identified for one trout *ras* gene, and 300 bps out of the 570 bps of a second trout *ras* gene can be extrapolated from linking the clone ratK*ras*01c79-1 with the first 190 bps of the clone RT31/38dT-12 (Figure 16). RT-6 and RT-8 may be allelic forms of the first trout *ras* gene, or could represent entirely different genes. Further studies are required to distinguish between the two possibilities.

ras Genes and Carcinogenesis in Trout

The only fish species that has a published *ras* gene sequence is the goldfish (Nemoto et al., 1986; 1987). A 90 bp *ras* sequence from a PCR amplification of winter flounder DNA has been described (McMahon, unpublished data), with the flounder sequence being identical to the rat Ki-*ras* gene. Based on the results presented in this study and the feasibility of carryover contamination in the PCR procedure, identical sequences obtained from such diverse species as rat and winter flounder should be verified independently before publication.

The characterization of *ras* genes from rainbow trout establishes a foundation for many additional studies on the molecular biology of chemical carcinogenesis in this model system. It is now possible to

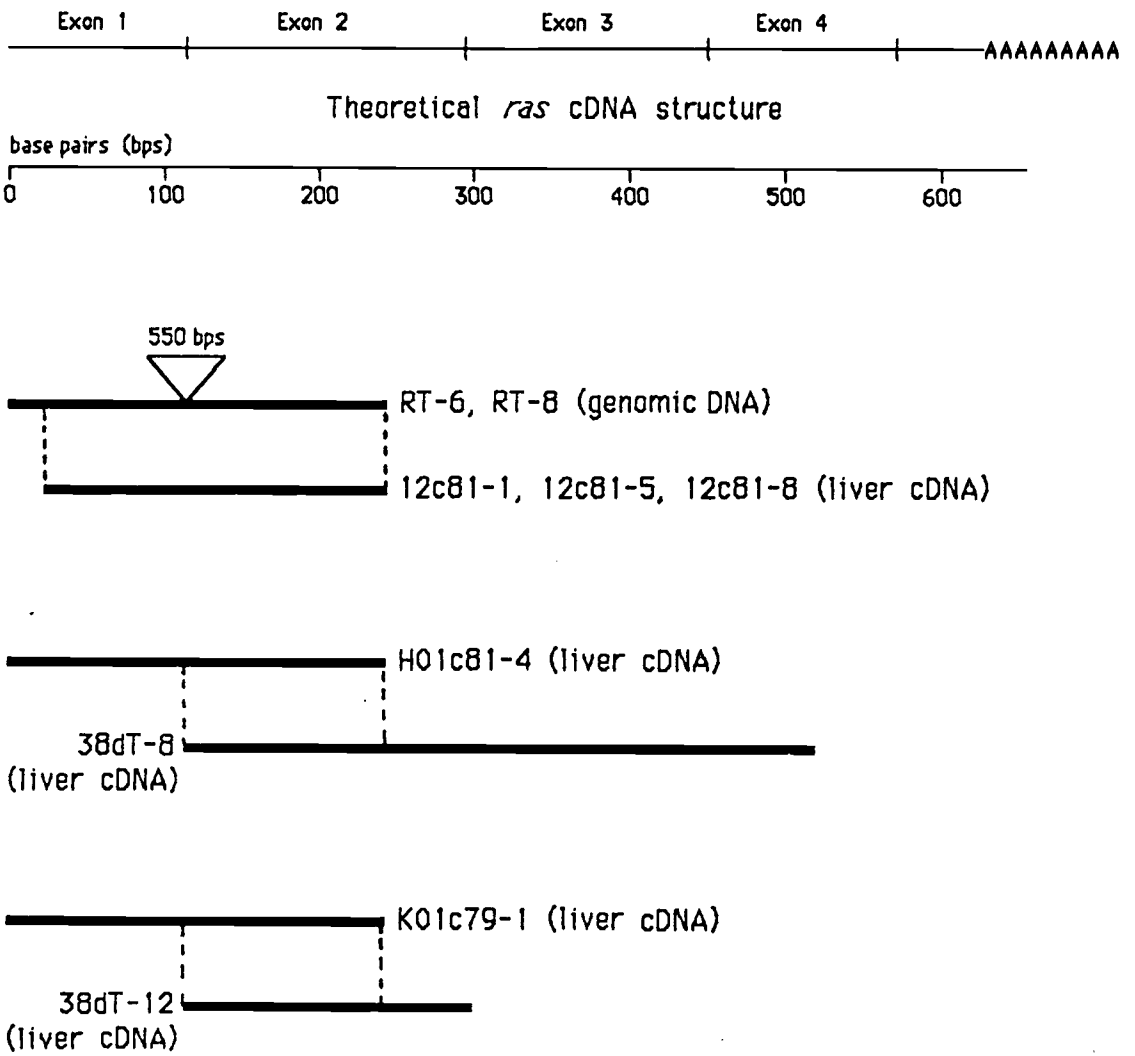


Figure 16. Alignment of the trout PCR products.

study the *ras* gene sequences in various carcinogen-initiated tumor tissues to determine if point mutations may be involved in trout neoplasia. The expression of the *ras* genes may be quantified in northern transfers of mRNA isolated from both normal and neoplastic tissues. *In vitro* mutagenesis studies could be employed to determine the distribution of carcinogen adducts along a trout *ras* sequence. Introduction of plasmids containing normal or mutated trout *ras* genes into cultured cells could increase knowledge of the basic cellular events involved in neoplastic transformation. Introduction of trout *ras* genes, along with other genes of interest, into transgenic animals also could increase our knowledge of the neoplastic process. The characterization of trout *ras* genes is only the beginning of valuable information that can be obtained on the molecular biology of trout neoplasia; many of which are dependent on knowing the sequence of a trout *ras* gene.

BIBLIOGRAPHY

Adari,H., Lowy,D.R., Willumsen,B.M., Der,C.J. and McCormick,F. (1988) Guanosine triphosphatase activating protein (GAP) interacts with the p21 *ras* effector binding domain. Science, 240, 518-521.

Agnantis,N.J., Petraki,C., Markoulatops,P. and Spandidos,D.A. (1986) Immunohistochemical study of the *ras* oncogene expression in human breast lesions. Anticancer Res., 6, 1157-1160.

Agnantis,N.J., Spandidos,D.A., Mahera,H., Parissi,P., Kakkanas,A., Pintzas,A. and Papaharalampous,N.X. (1988) Immunohistochemical study of *ras* oncogene expression in endometrial and cervical human lesion. Eur. J. Gynaecol. Oncol., 9, 360-365.

Ahnn,J., March,P.E., Takiff,H.E. and Inouye,M. (1986) A GTP-binding protein of Escherichia coli has homology to yeast *RAS* proteins. Proc. Natl. Acad. Sci. USA, 83, 8849-8853.

Albino,A.P., Le Strange,R., Oliff,A.I., Furth,M.E. and Old,L.J. (1984) Transforming *ras* genes from human melanoma: a manifestation of tumour heterogeneity?. Nature, 308, 69-72.

Almoguera,C., Shibata,D., Forrester,K., Martin,J., Arnheim,N. and Perucho,M. (1988) Most human carcinomas of the exocrine pancreas contain mutant c-K-*ras* genes. Cell, 53, 549-554.

Ananthaswamy,H.N., Price,J.E., Goldberg,L.H. and Bales,E.S. (1988) Detection and identification of activated oncogenes in human skin cancers occurring on sun-exposed body sites. Cancer Res., 48, 3341-3346.

Ayres,J.L., Lee,D.J., Wales,J.H. and Sinnhuber,R.O. (1971) Aflatoxin structure and hepatocarcinogenicity in rainbow trout (*Salmo gairdneri*). J. Natl. Cancer Inst., 46, 561-564.

Bailey,G., Selivonchick,D. and Hendricks,J. (1987) Initiation, promotion, and inhibition of carcinogenesis in rainbow trout. Environ. Health Perspect., 71, 147-153.

Bailey,G.S., Hendricks,J.D., Nixon,J.E. and Pawlowski,N.E. (1984) The Sensitivity of rainbow trout and other fish to carcinogens. Drug Metab. Rev., 15, 725-750.

Bailey,G.S., Taylor,M.J. and Selivonchick,D.P. (1982) Aflatoxin B_1 metabolism and DNA binding in isolated hepatocytes from rainbow trout (*Salmo gairdneri*). Carcinogenesis, 3, 511-518.

Baker,S.J., Fearon,E.R., Nigro,J.M., Hamilton,S.R., Preisinger,A.C., Jessup,J.M., Van Tuinen,P., Ledbetter,D.H., Barker,D.F., Nakamura,Y., White,R. and Vogelstein,B. (1989) Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science, 244, 217-221.

Ballas,K., Lyons,J., Janssen,J.W.G. and Bartram,C.R. (1988) Incidence of *ras* gene mutations in neuroblastoma. Eur. J. Pediatr., 147, 313-314.

Ballester,R., Furth,M.E. and Rosen,O.M. (1987) Phorbol ester- and protein kinase C-mediated phosphorylation of the cellular Kirsten *ras* gene product. J. Biol. Chem., 262, 2688-2695.

Balmain,A. and Pragnell,I.B. (1983) Mouse skin carcinomas induced in vivo by chemical carcinogens have a transforming Harvey-*ras* oncogene. Nature, 303, 72-74.

Balmain,A., Ramsden,M., Bowden,G.T. and Smith,J. (1984) Activation of the mouse cellular Harvey-*ras* gene in chemically-induced benign skin papillomas. Nature, 307, 658-660.

Baltus,E., Hanocq-Quertier,J., Hanocq,F. and Brachet,J. (1988) Injection of an antibody against a p21 c-Ha-*ras* protein inhibits cleavage in axolotyl eggs. Proc. Natl. Acad. Sci. USA, 85, 502-506.

Barbacid,M. (1985) Oncogenes in human cancers and in chemically induced animal tumors. Prog. med. Virol., 32, 86-100.

Barbacid,M. (1986) Oncogenes and human cancer: cause or consequence?. Carcinogenesis, 7, 1037-1042.

Barbacid,M. (1987) *ras* Genes. Ann. Rev. Biochem., 56, 779-827.

Barbacid,M., Sukumar,S. and Zarbl,H. (1986) Activation of *ras* oncogenes by chemical carcinogens. Gene Amplif. Anal., 4, 21-38.

Barkenow,A., Schartl,M., Anders,F. and Bauer,H. (1982) Identification of a fish protein associated with a kinase activity and related to the Rous sarcoma virus transforming protein. Cancer Res., 42, 2429-2433.

Barnes,D.M. (1986) How cells respond to signals. Science, 234, 286-288.

Bar-Sagi,D. and Feramisco,J.R. (1985) Microinjection of the *ras* oncogene protein into PC12 cells induces morphological differentiation. Cell, 42, 841-848.

Bar-Sagi,D. and Feramisco,J.R. (1986) Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by *ras* proteins. Science, 233, 1061-1068.

Bartram,C.R., Ludwig,W.-D., Hiddeman,W., Lyons,J., Buschle,M., Ritter,J., Harbott,J., Froehlich,A. and Janssen,J.W.G. (1989) Acute myeloid leukemia: analysis of *ras* gene mutations and clonality defined by polymorphic X-linked loci. Leukemia, 3, 247-356.

Beckner,S.K., Hatton,S. and Shih,T.Y. (1985) The *ras* oncogene product is not a regulatory component of adenylate cyclase. Nature, 317, 71-72.

Belinsky,S.A., Devereux,T.R., Maronpot,R.R., Stoner,G.D. and Anderson,M.W. (1989) Relationship between the formation of promutagenic adducts and the activation of the K-*ras* protooncogene in lung tumors from A/J mice treated with nitrosamines. Cancer Res., 49, 5305-5311.

Benjamin,C.W., Tarpley,W.G. and Gorman,R.R. (1987) The lack of PDGF-stimulated PGE2 release from *ras*-transformed NIH-3T3 cells results from reduced phospholipase C but not phospholipase A2 activity. Biochem. Biophys. Res. Comm., 145, 1254-1259.

Benjamin,C.W., Tarpley,W.G. and Gorman,R.R. (1987) Loss of platelet-derived growth factor-stimulated phospholipase activity in NIH-3T3 cells expressing the EJ-*ras* oncogene. Proc. Natl. Acad. Sci. USA, 84, 546-550.

Berridge,M.J. and Irvine,R.F. (1984) Inositol triphosphate, a novel second messenger in cellular signal transduction. Nature, 312, 315-321.

Bhave,M.R., Wilson,M.J. and Poirier,L.A. (1988) c-Ha-*ras* and c-Ki-*ras* gene hypomethylation in the livers and hepatomas of rats fed methyl-deficient, amino acid-defined diets. Carcinogenesis, 9, 343-348.

Birchmeier,C., Broek,D. and Wigler,M. (1985) *ras* proteins can induce meiosis in Xenopus oocytes. Cell, 43, 615-621.

Bishop,J.M. (1987) The Molecular genetics of cancer. Science, 235, 305-311.

Bizub,D., Wood,A.W. and Skalka,A.M. (1986) Mutagenesis of the Ha-*ras* oncogene in mouse skin tumors induced by polycyclic aromatic hydrocarbons. Proc. Natl. Acad. Sci. USA, 83, 6048-6052.

Borrello,M.G., Carbone,G., Pierotti,M.A., Molla,A. and Della Porta,G. (1988) Activated c-K-*ras* and c-N-*ras* oncogenes in 3-methylcholanthrene-induced BALB/ c fibrosarcomas. Carcinogenesis, 9, 1517-1519.

Borrello,M.G., Peirotti,M.A., Bongarzone,I., Donghi,R., Mondellini,P. and Della Porta,G. (1987) DNA methylation affecting the transforming activity of the human Ha-*ras* oncogene. Cancer Res., 47, 75-79.

Bos,J.L. (1988) The *ras* gene family and human carcinogenesis. Mutat. Res., 195, 255-271.

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

Bos,J.L., Fearon,E.R., Hamilton,S.R., Verlaan-de Vries,M., van Boom,J.H., van der Eb,A.J. and Vogelstein,B. (1987) Prevalence of *ras* gene mutations in human colorectal cancers. Nature, 327, 293-297.

Bos,J.L., Toksoz,D., Marshall,C.J., Verlaan-de Vries,M., Veeneman,G.H., van der Eb,A.J., van Boom,J.H., Janssen,J.W.G. and Steenvoorden,A.C.M. (1985) Amino-acid substitutions at codon 13 of the N-*ras* oncogene in human acute myeloid leukaemia. Nature, 315, 726-730.

Bos,J.L., Verlaan-de Vries,M., Jansen,A.M., Veeneman,G.H., van Boom,J.H. and van der Eb,A.J. (1984) Three different mutations in codon 61 of the human N-*ras* gene detected by synthetic oligonucleotide hybridization. Nucleic Acids Res., 12, 9155-9163.

Bos,J.L., Verlaan-de Vries,M., Marshall,C.J., Veeneman,G.H., van Boom,J.H. and van der Eb,A.J. (1986) A human gastric carcinoma contains a single mutated and an amplified normal allele of the Ki-*ras* oncogene. Nucleic Acids Res., 14, 1209-1217.

Bos,J.L., Verlaan-de Vries,M., van der Eb,A.J., Janssen,J.W.G., Delwel,R., Lowenberg,B. and Colly,L.P. (1987) Mutations in N-*ras* predominate in acute myeloid leukemia. Blood, 69, 1237-1241.

Brann,M.R. (1988) Expression of transducin in retinal rod photreceptor outer segments. Science, 241, 845-847.

Broek,D., Samiy,N., Fasano,O., Fujiyama,A., Tamanoi,F., Northup,J. and Wigler, M. (1985) Differential activation of yeast adenylate cyclase by wild-type and mutant *ras* proteins. Cell, 41, 763-769.

Broek,D., Toda,T., Michaeli,T., Levin,L., Birchmeier,C., Zoller,M., Powers,S. and Wigler,M. (1987) The S. cerevisiae CDC25 gene product regulates the *RAS/* adenylate cyclase pathway. Cell, 48, 789-799.

Brown,R., Marshall,C.J., Pennie,S.G. and Hall,A. (1984) Mechanism of activation of an N-*ras* gene in the human fibrosarcoma cell line HT1080. EMBO J., 3, 1321-1326.

Burmer,G.C. and Loeb,L.A. (1989) Mutations in the K*RAS*2 oncogene during progressive stages of human colon carcinoma. Proc. Natl. Acad. Sci. USA, 86, 2403-2407.

Burmer,G.C., Rabinovitch,P.S. and Loeb,L.A. (1989) Analysis of c-Ki-*ras* mutations in human colon carcinoma by cell sorting, polymerase chain reaction, and DNA sequencing. Cancer Res., 49, 2141-2146.

Buschle,M., Janssen,J.W.G., Drexler,H., Lyons,J., Anger,B. and Bartram,C.R. (1988) Evidence for pluripotent stem cell origin of idiopathic myelofibrosis: clonal analysis of a case characterized by a N-*ras* gene mutation. Leukemia, 2, 658-660.

Buss,J.E. and Sefton,B.M. (1986) Direct identification of palmitic acid as the lipid attached to p21*ras*. Mol. Cell. Biol., 6, 116-122.

Caffrey,J.M., Brown,A.M. and Schneider,M.D. (1987) Mitogens and oncogenes can block the induction of specific voltage-gated ion channels. Science, 236, 570-573.

Capon,D.J., Chen,E.Y., Levinson,A.D., Seeburg,P.H. and Goeddel,D.V. (1983) Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. Nature, 302, 33-37.

Capon,D.J., Seeburg,P.H., McGrath,J.P., Hayflick,J.S., Edman,U., Levinson,A.D. and Goeddel,D.V. (1983) Activation of Ki-*ras* 2 gene in human colon and lung carcinomas by two different point mutations. Nature, 304, 507-513.

Cerbai,E., Klockner,U. and Isenberg,G. (1988) The a subunit of the GTP binding protein activates muscarinic potassium channels of the atrium. Science, 240, 1782-1783.

Chabre,M. (1987) The G protein connection: Is it in the membrane or the cytoplasm?. TIBS, 12, 213-215.

Chang,E.H., Furth,M.E., Scolnick,E.M. and Lowy,D.R. (1982) Tumorigenic transformation of mammalian cells induced by a normal human gene homologous to the oncogene of Harvey sarcoma virus. Nature, 297, 479-483.

Chang,E.H., Gonda,M.A., Ellis,R.W., Scolnick,E.M. and Lowy,D.R. (1982) Human genome contains four genes homologous to transforming genes of Harvey and Kirsten murine sarcoma viruses. Proc. Natl. Acad. Sci. USA, 79, 4848-4852.

Chardin,P. and Tavitian,A. (1987) The ral gene: a new *ras*-related gene isolated by the use of a synthetic probe. EMBO J., 5, 2203-2208.

Chardin,P., Yeramian,P., Madaule,P. and Tavitian,A. (1985) N-*ras* gene activation in the RD human rhabdomyosarcoma cell line. Int. J. Cancer, 35, 647-652.

Chen,Z.-Q., Ulsh,L.S., DuBois,G. and Shih,T. (1985) Post-translational processing of p21 *ras* proteins involves palmitylation of the C-terminal tetrapeptide containing cysteine-186. J. Virol., 56, 607-612.

Chiarugi,V., Porciatti,F., Pasquali,F. and Bruni,P. (1985) Transformation of BALB/3T3 cells with EJ/T24/H-*ras* oncogene inhibits adenylate cyclase response to β-adrenergic agonist while increases muscarinic receptor dependent hydrolysis of inositol lipids. Biochem. Biophys. Res. Comm., 132, 900-907.

Cichutek,K. and Duesberg,P.H. (1986) Harvey *ras* genes transform without mutant codons apparently activated by truncation of a 5' exon (exon-1). Proc. Natl. Acad. Sci. USA, 83, 2340-2344.

Clair,T., Miller,W.R. and Cho-Chung,Y.S. (1987) Prognostic significance of the expression of a *ras* protein with a molecular weight of 21,000 by human breast cancer. Cancer Res., 47, 5290-5293.

Clanton,D.J., Hattori,S. and Shih,T.Y. (1986) Mutations of the *ras* gene product p21 that abolish guanine nucleotide binding. Proc. Natl. Acad. Sci. USA, 83, 5076-5080.

Clanton,D.J., Lu,Y., Blair,D.G. and Shih,T.Y. (1987) Structural significance of the GTP-binding domain of *ras* p21 studied by site-directed mutagenesis. Mol. Cell. Biol., 7, 3092-3097.

Cockcroft,S. (1987) Polyphosphoinositide phosphodiesterase: Regulation by a novel guanine nucleotide binding protein, Gp. TIBS, 12, 75-78.

Cockcroft,S. and Gowperts,B.D. (1985) Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase. Nature, 314, 534-536.

Cohen,J.B. and Levinson,A.D. (1988) A Point mutation in the last intron responsible for increased expression and transforming activity of the c-Ha-*ras* oncogene. Nature, 334, 119-124.

Cohen,J.B., Walter,M.V. and Levinson,A.D. (1987) A Repetitive sequence element 3' of the human c-Ha-*ras*1 gene has enhancer activity. J. Cell Physiol. (Suppl.), 5, 75-82.

Cooper,G.M. (1982) Cellular transforming genes. Science, 218, 801-806.

Coughlin,S.R., Escobedo,J.A. and Williams,L.T. (1989) Role of phosphatidylinositol kinase in PDGF receptor signal transduction. Science, 243, 1191-1194.

Davis,L.G., Dibner,M.D. and Battey,J.F. (1986) In: Basic Methods in Molecular Biology. Elsevier, New York, 388 pp.

Davis,M., Malcolm,S., Hall,A. and Marshall,C.J. (1983) Localisation of the human N-*ras* oncogene to chromosome ICEN-P21 by in situ hybridisation. EMBO J., 2, 2281-2283.

DeFeo-Jones,D., Scolnick,E.M., Koller,R. and Dhar,R. (1983) *ras*-Related gene sequences identified and isolated from Saccharomyces cerevisiae. Nature, 306, 707-709.

DeFeo-Jones,D., Tatchell,K., Robinson,L.C., Sigal,I.S., Vass,W.C., Lowy,D.R. and Scolnick,E.M. (1985) Mammalian and yeast *ras* gene products: biological function in their heterologous systems. Science, 228, 179-184.

De Martinville,B., Cunningham,J.M., Murray,M.J. and Francke,U. (1983) The N-*ras* oncogene assigned to the short arm of human chromosome 1. Nucleic Acids Res., 11, 5267-5275.

Der,C.J., Krontiris,T.G. and Cooper,G.M. (1982) Transforming genes of human bladder and lung carcinoma cell lines are homologous to the *ras* genes of Harvey and Kirsten sarcoma viruses. Proc. Natl. Acad. Sci. USA, 79, 3637-3640.

Der,C.J., Pan,B.-T. and Cooper,G.M. (1986) *ras*H mutants deficient in GTP binding. Mol. Cell. Biol., 6, 3291-3294.

Deshpande,A.K. and Kung,H. (1987) Insulin induction of Xenopus laevis oocyte maturation is inhibited by monoclonal antibody against p21 *ras* proteins. Mol. Cell. Biol., 7, 1285-1288.

Deterre,P., Bigay,J., Forquet,F., Robert,M. and Chabre,M. (1988) cGMP phosphodiesterase of retinal rods is regulated by two inhibitory subunits. Proc. Natl. Acad. Sci. USA, 85, 2424-2428.

de Vos,A.M., Tong,L., Milburn,M.V., Matias,P.M., Jancarik,J., Noguchi,S., Nishimura,S., Miura,K., Ohtsuka,E. and Kim,S.-H. (1988) Three-dimensional structure of an oncogene protein: Catalytic domain of human c-H-*ras* p21. Science, 239, 888-893.

Dhar,R., Ellis,R.W., Shih,T.Y., Oroszlan,S., Shapiro,B., Maizel,J., Lowy,D. and Scolnick,E. (1982) Nucleotide sequence of the p21 transforming protein of Harvey murine sarcoma virus. Science, 217, 935-936.

Dunning,A.M., Talmud,P. and Humphries,S.E. (1988) Errors in the polymerase chain reaction. Nucleic Acid Res., 16, 10393.

Ellis,R.W., DeFeo,D., Shih,T.Y., Gonda,M.A., Young,H.A., Tsuchida,N., Lowy,D.R. and Scolnick,E.M. (1981) The p21 src genes of Harvey and Kirsten sarcoma viruses originate from divergent members of a family of normal vertebrate genes. Nature, 292, 506-511.

Eva,A., Tronick,S.R., Gol,R.A., Pierce,J.H. and Aaronson,S.A. (1983) Transforming genes of human hematopoietic tumors: frequent detection of *ras*-related oncogenes whose activation appears to be independent of tumor phenotype. Proc. Natl. Acad. Sci. USA, 80, 4926-4930.

Falkmer,S., Marklund,S., Mattsson,P.E. and Roppe,C. (1977) Hepatomas and other neoplasms in the Atlantic hagfish (*Myxine glutinosa*): A histopatholic and chemical study. Ann. N. Y. Acad. Sci., 298, 342-355.

Farr,C.J., Marshall,C.J., Easty,D.J., Wright,N.A., Powell,S.C. and Paraskeva,C. (1988) A Study of *ras* gene mutations in colonic adenomas from familial polyposis coli patients. Oncogene, 3, 673-678.

Farr,C.J., Saiki,R.K., Erlich,H.A., McCormick,F. and Marshall,C.J. (1988) Analysis of *RAS* gene mutations in acute myeloid leukemia by polymerase chain reaction and oligonucleotide probes. Proc. Natl. Acad. Sci. USA, 85, 1629-1633.

Fasano,O., Aldrich,T., Tamanoi,F., Taparowsky,E., Furth,M. and Wigler,M. (1984) Analysis of the transforming potential of the human H-*ras* gene by random mutagenesis. Proc. Natl. Acad. Sci. USA, 81, 4008-4012.

Fasano,O., Taparowsky,E., Fiddes,J., Wigler,M. and Goldfarb,M. (1983) Sequence and structure of the coding region of the human H-*ras*-1 gene from T24 bladder carcinoma cells. J. Mol. Appl. Genet., 2, 173-180.

Feig,L.A., Pan,B.-T., Roberts,T.M. and Cooper,G.M. (1986) Isolation of *ras* GTP-binding mutants using an in situ colony-binding assay. Proc. Natl. Acad. Sci. USA, 83, 4607-4611.

Feinberg,A.P., Vogelstein,B., Droller,M.J., Baylin,S.B. and Nelkin,B.D. (1983) Mutation affecting the 12th amino acid of the c-Ha-*ras* oncogene product occurs infrequently in human cancer. Science, 220, 1175-1177.

Finkel,T. and Cooper,G.M. (1984) Detection of a molecular complex between *ras* proteins and transferrin receptor. Cell, 136, 1115-1121.

Finkel,T., Der,C.J. and Cooper,G.M. (1984) Activation of *ras* genes in human tumors does not affect localization, modification, or nucleotide binding properties of p21. Cell, 37, 151-158.

Fleischman,L.F., Chahwala,S.B. and Cantley,L. (1986) *ras*-transformed cells: altered levels of phosphatidylinositol-4,5-biphosphate and catabolites. Science, 231, 407-410.

Forrester,K., Almoguera,C., Han,K., Grizzle,W.E. and Perucho,M. (1987) Detection of high incidence of K-*ras* oncogenes during human colon tumorigenesis. Nature, 327, 298-303.

Franks,D.J., Whitfield,J.F. and Durkin,J.P. (1985) The mitogenic/oncogenic p21 Ki-*ras* protein stimulates adenylate cyclase activity early in the G1 phase of NRK rat kidney cells. Biochem. Biophys. Res. Comm., 132, 780-786.

Franz,T., Lohler,J., Fusco,A., Pragnell,I., Nobis,P., Padua,R. and Ostertag,W. (1985) Transformation of mononuclear phagocytes in vivo and malignant histiocytosis caused by a novel murine spleen focus-forming virus. Nature, 315, 149-151.

Fredrickson,T.N., O'Neill,R.R., Rutledge,R.A., Theodore,T.S., Martin,M.A., Ruscetti,S.K., Autsin,J.B. and Hartley,J.W. (1987) Biologic and molecular characterization of two newly isolated *ras*-containing murine leukemia viruses. J. Virol., 61, 2109-2119.

Fujita,J., Kraus,M.H., Onoue,H., Srivastava,S.K., Ebi,Y., Kitamura,Y. and Rhim, J.S. (1988) Activated H-*ras* oncogenes in human kidney tumors. Cancer Res., 48, 5251-5255.

Fujita,J., Ohuchi,N., Ito,N., Reynolds,S.H., Yoshida,O., Nakayama,H. and Kitamura,Y. (1988) Activation of H-*ras* oncogene in rat bladder tumors induced by N-butyl-N-(4-hydroxybutyl)nitrosamine. J. Natl. Cancer Inst., 80, 37-43.

Fujita,J., Srivastava,S.K., Kraus,M.H., Rhim,J.S., Tronick,S.R. and Aaronson,S. A. (1985) Frequency of molecular alterations affecting *ras* protooncogenes in human urinary tract tumors. Proc. Natl. Acad. Sci. USA, 82, 5954-5958.

Fujita,K., Ohuchi,N., Yao,T., Okumura,M., Fukushima,Y., Kanakura,V., Kitamura, Y. and Fujita,J. (1987) Frequent overexpression, but not activation by point mutation, of *ras* genes in primary human gastric cancers. Gastroenterology, 6, 1339-1345.

Fujiyama,A. and Tamanoi,A. (1986) Processing and fatty acid acylation of *ras* 1 and *ras* 2 proteins in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA, 83, 1266-1270.

Fukui,M., Yamamoto,T., Kawai,S., Maruo,K. and Toyoshima,K. (1985) Detection of a raf-related and two other transforming DNA tumors maintained in nude mice. Proc. Natl. Acad. Sci. USA, 82, 5954-5958.

Fukui,Y. and Kaziro,Y. (1985) Molecular cloning and sequence analysis of a *ras* gene from Schizosaccharomyces pombe. EMBO J., 4, 687-691.

Fusco,A., Grieco,M., Santoro,M., Berlingieri,M.J., Pilotti,S., Pierotti,M.A., Della Porta,G. and Vecchio,G. (1987) A New oncogene in human thyroid papillary carcinomas and their lymph-node metastases. Nature, 328, 170-172.

Gallwitz,D., Donath,C. and Sander,C. (1983) A Yeast gene encoding a protein homologous to the human c-has/bas proto-oncogene product. Nature, 306, 704-707.

Gambke,C., Hall,A. and Moroni,C. (1985) Activation of an N-*ras* gene in acute myeloblastic leukemia through somatic mutation in the first exon. Proc. Natl. Acad. Sci. USA, 82, 879-882.

Gerosa,M.A., Talarico,D., Fognani,C., Raimondi,E., Colombatti,M., Tridente,G., De Carli,L. and Della Valle,G. (1989) Overexpression of N-*ras* oncogene and epidermal growth factor receptor gene in human glioblastomas. J. Natl. Cancer Inst., 81, 63-67.

Gibbs,J.B., Ellis,R.W. and Scolnick,E.M. (1984) Autophosphorylation of v-Ha-*ras* p21 is modulated by amino acid residue 12. Proc. Natl. Acad. Sci. USA, 81, 2674-2678.

Gibbs,J.B., Sigal,I.S., Poe,M. and Scolnick,E.M. (1984) Intrinsic GTPase activity distinguishes normal and oncogenic *ras* p21 molecules. Proc. Natl. Acad. Sci. USA, 81, 5704-5708.

Gill,D.L., Veada,T., Chuch,S. and Noel,M.W. (1986) Ca2+ release from endoplasmic reticulum is mediated by a guanosine nucleotide regulatory mechanism. Nature, 320, 461-464.

Gilman,A.G. (1987) G proteins: Transducers of receptor-generated signals. Ann. Rev. Biochem., 56, 615-649.

Greenhalgh,D.A. and Kinsella,A.R. (1985) c-Ha-*ras* not c-Ki-*ras* activation in three colon tumour cell lines. Carcinogenesis, 6, 1533-1535.

Grizzle,J.M., Schwedler,T.E. and Scott,A.C. (1981) Papillomas of black bullheads (*Ictalurus melas*) (Rafinesque), living in a chlorinated sewage pond. J. Fish Dis., 4, 345-351.

Grunewald,K., Lyons,J., Froehlich,A., Feichtinger,H., Weger,R.A., Schwab,G., Janssen,J.W.G. and Bartram,C.R. (1989) High frequency of Ki-*ras* codon 12 mutations in pancreatic adenocarcinomas. Int. J. Cancer, 43, 1037-1041.

Gu,J.R., Hu,L.F., Cheng,Y.C. and Wan,D.F. (1986) Oncogenes in human primary hepatic cancer. J. Cell Physiol. (Suppl.), 4, 13-20.

Guerrero,I. and Pellicer,A. (1987) Mutational activation of oncogenes in animal model systems of carcinogenesis. Mutat. Res., 185, 293-308.

Guerrero,I., Villasante,A., Corces,V. and Pellicer,A. (1985) Loss of the normal N-*ras* allele in a mouse thymic lymphoma induced by a chemical carcinogen. Proc. Natl. Acad. Sci. USA, 82, 7810-7814.

Hagag,N., Haleguoa,S. and Viola,M.V. (1986) Microinjection of antibody to *ras* p21 inhibits nerve growth factor induced differentiation of PC12 cells. Nature, 319, 680-682.

Halliday,K.R. (1984) Regional homology in GTP-binding proto-oncogene products and elongation factors. J. Cyclic Nucleotide Prot. Phosphor. Res., 9, 435-448.

Hanley,M.R. and Jackson,T. (1987) The *ras* gene: Transformer and transducer. Nature, 328, 668-669.

Hansen,M.F. and Cavenee,W.K. (1987) Genetics of cancer predisposition. Cancer Res., 47, 5518-5527.

Harvey,J.J. (1964) An Unidentified virus which causes the rapid production of tumors in mice. Nature, 204, 1104-1105.

Haubruck,H., Disela,C., Wagner,P. and Gallwitz,D. (1987) The *ras*-related ypt protein is an ubiquitous eukaryotic protein: isolation and sequence analysis of mouse cDNA clones highly homologous to the yeast YPT-1 gene. EMBO J., 6, 4049-4053.

Hendricks,J.D., Meyers,T.R., Casteel,J.L., Nixon,J.E., Loveland,P.M. and Bailey,G.S. (1984) Rainbow trout embryos: Advantages and limitations for carcinogenesis research. Natl. Cancer Inst. Monogr., 65, 129-137.

Hill,T.D., Dean,N.M. and Boynton,A.L. (1988) Inositol 1,3,4,5-tetrakisphosphate induces Ca2+ sequestration in rat liver cells. Science, 242, 1176-1178.

Hirai,H., Kobayashi,Y., Mano,H., Hagiwara,K., Maru,Y., Omine,M., Mizoguchi,H., Nishida,J. and Takaku,F. (1987) A point mutation at codon 13 of the N-*ras* oncogene in myelodysplastic syndrome. Nature, 327, 430-432.

Hirai,H., Nishida,J. and Takaku,F. (1987) Highly frequent detection of transforming genes in acute leukemias by transfection using in vivo selection assays. Biochem. Biophys. Res. Comm., 147, 108-114.

Hirai,H., Okabe,T., Anraku,Y., Fujisawa,M., Urabe,A. and Takaku,F. (1985) Activation of the c-K-*ras* oncogene in a human pancreas carcinoma. Biochem. Biophys. Res. Comm., 127, 168-174.

Hirai,H., Tanaka,S., Azuma,M., Anraku,Y., Kobayashi,Y., Fujisawa,M., Okabe,T., Urabe,A. and Takaku,F. (1985) Transforming genes in human leukemia cells. Blood, 66, 1371-1378.

Hochwalt,A.E., Solomon,J.J. and Garte,S.J. (1988) Mechanism of H-*ras* oncogene activation in mouse squamous carcinoma induced by an alkylating agent. Cancer Res., 48, 556-558.

Hollstein,M.C., Smits,A.M., Galiana,C., Yamasaki,H., Bos,J.L., Mandard,A., Partensky,C. and Montesano,R. (1988) Amplification of epidermal growth factor receptor gene but no evidence of *ras* mutations in primary human esophageal cancers. Cancer Res., 48, 5119-5123.

Honkawa,H., Masahashi,W., Hashimoto,S. and Hashimoto-Gotoh,T. (1987) Identification of the principal promoter sequence of the c-H-*ras* transforming oncogene: Deletion analysis of the 5'-flanking region by focus formation assay. Mol. Cell. Biol., 7, 2933-2940.

Hoshino,M., Kawakita,M. and Hattori,S. (1988) Characterization of a factor that stimulates hydrolysis of GTP bound to *ras* gene product p21 (GTPase-activating protein) and correlation of its activity to cell density. Mol. Cell. Biol., 8, 4169-4173.

Huebner,R.J. and Todaro,G.J. (1969) Oncogenes of RNA tumor viruses as determinants of cancer. Proc. Natl. Acad. Sci. USA, 64, 1087-1094.

Hurley,J.B., Simon,M.I., Teplow,D.B., Robislaw,J.D. and Gilman,A.G. (1984) Homologies between signal transducing GI proteins and *ras* gene products. Science, 226, 860-862.

Imler,J.L., Schatz,C., Wasylyk,C., Chatton,B. and Wasylyk,B. (1988) A Harvey-*ras* responsive transcription element is also responsive to a tumour-promoter and to serum. Nature, 332, 275-278.

Ireland,C.M. (1989) Activated N-*ras* oncogenes in human neuroblastoma. Cancer Res., 49, 5530-5533.

Ishii,S., Kadonaga,J.T., Tjian,R., Brady,J.N., Merlino,G.T. and Pastan,I. (1986) Binding of the Spl transcription factor by the human Harvey *ras*1 proto-oncogene promoter. Science, 230, 1410-.

Ishii,S., Merlino,G.T. and Pastan,I. (1985) Promoter region of the human Harvey *ras* proto-oncogene: similarity to the EGF receptor proto-oncogene promoter. Science, 230, 1378-1381.

Janssen,J.W.G., Steenvoorden,A.C.M., Collard,J.G. and Nusse,R. (1985) Oncogene activation in human myeloid leukemia. Cancer Res., 45, 3262-3267.

Janssen,J.W.G., Steenvoorden,A.C.M., Lyons,J., Anger,B., Bohlke,J.U., Bos,J.L., Seliger,H. and Bartram,C.R. (1987) *RAS* gene mutations in acute and chronic myelocytic leukemias, chronic myeloproliferative disorders, and myelodysplatic syndromes. Proc. Natl. Acad. Sci. USA, 84, 9228-9232.

Jeng,A.Y., Srivastava,S.K., Lacal,J.C. and Blumberg,P.M. (1987) Phosphorylation of *ras* oncogene product by protein kinase C. Biochem. Biophys. Res. Comm., 145, 782-788.

Joseph,S.K. (1984) Inositol triphosphate: an intracellular messenger produced by Ca2+ mobilizing hormones. TIBS, 9, 420-421.

Kagimoto,M., Miyoshi,J., Tashiro,K., Naito,Y., Sakaki,Y., Sueishi,K., Tanaka,K. and Imamura,T. (1985) Isolation and characterization of an activated c-H-*ras*-1 gene from a squamous-cell lung carcinoma cell line. Int. J. Cancer, 35, 808-812.

Kamata,T. and Feramisco,J.R. (1987) Epidermal growth factor stimulates guanine nucleotide binding activity and phosphorylation of *ras* oncogene proteins. Nature, 310, 147-150.

Kamata,T., Kathuria,S. and Fujita-Yamaguchi,Y. (1987) Insulin stimulates the phosphorylation level of v-Ha-*ras* protein in membrane fraction. Biochem. Biophys. Res. Comm., 144, 19-25.

Kataoka,T., Powers,S., Cameron,S., Fasano,O., Goldfarb,M., Broach,J. and Wigler,M. (1985) Functional homology of mammalian and yeast *RAS* genes. Cell, 40, 19-26.

Khudoley,V.V. (1972) Induction of liver tumors by some azo compounds in aquarium fish (*Lebistes reticulatus*). J. Ichthyol., 12, 319-324.

Kimura,I., Morikawa,S., Kiriyama,T. and Kitaori,H. (1983) An epizootic occurrence of rhabdomyoma and a case of ganglioneuroma in hatchery-reared ayu *Plecoglossus altivelis* Temminch and Schlegel. J. Fish Dis., 6, 195-200.

Kirsten,W.H. and Mayer,L.A. (1967) Morphologic responses to a murine erythroblastosis virus. J. Natl. Cancer Inst., 39, 311-334.

Kitayama,H., Sugimoto,Y., Matsuzaki,T., Ikawa,Y. and Noda,M. (1989) A *ras*-related gene with transformation suppressor activity. Cell, 56, 77-84.

Klein,G. (1983) Specific chromosomal translocations and the genesis of B cell derived tumors in mice and men. Cell, 32, 311-315.

Korn,L.J., Siebel,C.W., McCormick,F. and Roth,R.A. (1987) *ras* p21 as a potential mediator of insulin action in Xenopus oocytes. Science, 236, 840-843.

Kozma,S.C., Bogaard,M.E., Buser,K., Saurer,S.M., Bos,J.L., Groner,B. and Hynes, N.E. (1987) The human c-Kirsten *ras* gene is activated by a novel mutation in codon 13 in the breast carcinoma cell line MDA-MB231. Nucleic Acids Res., 15, 5963-5971.

Kraus,M.H., Yuasa,Y. and Aaronson,S.A. (1984) A position 12-activated H-*ras* oncogene in all HS578T mammary carcinosarcoma cells but not normal mammary cells of the same patient. Proc. Natl. Acad. Sci. USA, 81, 5384-5388.

Kuzumaki,N., Ogiso,Y., Oda,A., Fujita,H., Suzuki,H., Sato,C. and Mullauer,L. (1989) Resistance to oncogenic transformation in revertant R1 of human *ras*-transformed NIH 3T3 cells. Mol. Cell. Biol., 9, 2258-2263.

Kwok,S. and Higuchi,R. (1989) Avoiding false positives with PCR. Nature, 339, 237-238.

Kyona,Y. (1978) Temperature effects during and after the diethylnitrosamine treatment on liver tumorigenesis in the fish, *Oryzias latipes*. Eur. J. Cancer, 14, 1089-1097.

Lacal,J.C. and Aaronson,S.A. (1986) *ras* p21 deletion mutants and monoclonal antibodies as tools for localization of structural domains relevant to p21 function. Proc. Natl. Acad. Sci. USA, 83, 5400-5404.

Lacal,J.C., Anderson,P.S. and Aaronson,S.A. (1986) Deletion mutants of Harvey *ras*-21 protein reveal the absolute requirement of at least two distant regions for GTP-binding and transforming activities. EMBO J., 5, 679-687.

Lacal,J.C., de la Pena,P., Moscat,J., Garcia-Barreno,P., Anderson,P.S. and Aaronson,S.A. (1987) Rapid stimulation of diacylglycerol production in Xenopus oocytes by microinjection of H-*ras* p21. Science, 238, 533-536.

Lacal,J.C., Moscat,J. and Aaronson,S.A. (1987) Novel source of 1,2-diacylglycerol elevated in cells transformed by H-*ras* oncogene. Nature, 330, 269-272.

Lacal,J.C., Srivastava,S.K., Anderson,P.S. and Aaronson,S.A. (1986) *ras* p21 proteins with high or low GTPase activity can efficiently transform NIH/3T3 cells. Cell, 44, 609-617.

Lacal,J.C. and Tronick,S.R. (1988) The *ras* oncogene. In Reddy,E.P., Skaika,A.M. and Curran,T. (eds.), The Oncogene Handbook. Elsevier, Amsterdam, pp. 257-304,535-551.

Land,H., Parada,L.F. and Weinberg,R.A. (1983) Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature, 304, 596-602.

Lemoine,N.R., Mayall,E.S., Wyllie,F.S., Farr,C.J., Hughes,D., Padua,R.A., Thurston,V., Williams,E.D. and Wynford-Thomas,D. (1988) Activated *ras* oncogenes in human thyroid cancers. Cancer Res., 48, 4459-4463.

Leon,J., Guerrero,I. and Pellicer,A. (1987) Differential expression of the *ras* gene family in mice. Mol. Cell. Biol., 7, 1535-1540.

Leon,J., Kamino,H., Steinberg,J.J. and Pellicer,A. (1988) H-*ras* activation in benign and self-regressing skin tumors (keratoacanthomas) in both humans and an animal model system. Mol. Cell. Biol., 8, 786-793.

Levinson,A.D. (1986) Normal and activated *ras* oncogenes and their encoded products. TIG, 2, 81-85.

Liu,E., Hjelle,B. and Bishop,J.M. (1988) Transforming genes in chronic myelogenous leukemia. Proc. Natl. Acad. Sci. USA, 85, 1952-1956.

Lochrie,M.A., Hurley,J.B. and Simon,M.I. (1985) Sequence of the alpha subunit of photoreceptor G protein: homologies between transducin, *ras*, and elongation factors. Science, 228, 96-99.

Loveland,P.M., Nixon,J.E. and Bailey,G.S. (1984) Glucoronides in bile of rainbow trout (Salmo gairdneri) injected with (3H)aflatoxin B_1 and the effects of dietary β-naphthoflavone. Comp. Biochem. Physiol., 78C, 13-19.

Lowe,D.G., Capon,D.J., Delwart,E., Sakaguchi,A.Y., Naylor,S.L. and Goeddel,D.V. (1987) Structure of the human and murine R-*ras* genes, novel genes closely related to *ras* proto-oncogenes. Cell, 48, 137-146.

Lowe,D.G., Ricketts,M., Levinson,A.D. and Goeddel,D.V. (1988) Chimeric proteins define variable and essential regions of Ha-*ras*-encoded protein. Proc. Natl. Acad. Sci. USA, 85, 1015-1019.

Lyons,J., Janssen,J.W.G., Bartram,C., Layton,M. and Mufti,G.J. (1988) Mutation of Ki-*ras* oncogenes in myelodysplastic syndromes. Blood, 71, 1707-1712.

Madaule,P. and Axel,R. (1985) A Novel *ras*-related gene family. Cell, 41, 31-40.

Magee,A.I., Gutierrez,L., McKay,I.A., Marshall,C.J. and Hall,A. (1987) Dynamic fatty acylation of p21N-*ras*. EMBO J., 6, 3353-3357.

Malone,P.R., Visvanathan,K.V., Ponder,B.A., Shearer,R.J. and Summerhayes,I.C. (1985) Oncogene and bladder cancer. Br. J. Urol., 57, 664-667.

Manne,V., Bekesi,E. and Kung,H. (1985) Ha-*ras* proteins exhibit GTPase activity: point mutations that activate Ha-*ras* gene products result in decreased GTPase activity. Proc. Natl. Acad. Sci. USA, 82, 376-380.

Marczynska,B., Hoijer,J., Spragia,L., Wilson,W., Nelson,R.L. and Khoobyarian,N. (1988) Chromosomal and c-Ki-*ras* oncogene alterations induced by a chemical carcinogen and phorbol ester in skin fibroblasts of individuals with familial polyposis coli. Carcinogenesis, 9, 1897-1900.

Marshall,C.J. (1985) Human oncogenes. In Weiss,R., Tetch,N., Varmus,H. and Coffin,J. (eds.), RNA Tumor Viruses second edition. Cold Spring Harbor Laboratory, Cold Spring Harbor,NY, pp. 487-558.

Masters,S.B., Sullivan,K.A., Miller,R.T., Beiderman,B., Lopez,N.G., Ramachandran,J. and Bourne,H.R. (1988) Carboxyl terminal domain of Gsa specifies coupling of receptors to stimulation of adenylyl cyclase. Science, 241, 448-451.

Mattera,R., Graziano,M.P., Yatani,A., Zhou,Z., Graf,R., Codina,J., Birnbaumer, L., Gilman,A.G. and Brown,A.M. (1989) Splice variants of the a subunit of the G protein Gs activate both adenylyl cyclase and calcium channels. Science, 243, 804-807.

Mayorga,L.S., Diaz,R. and Stahl,P.D. (1989) Regulatory role for GTP-binding proteins in endocytosis. Science, 244, 1475-1477.

McBride,O.W., Swan,D., Santos,M., Barbacid,M., Tronick,S.R. and Aaronson,S.A. (1982) Localization of the normal allele of T24 human bladder carcinoma oncogene to chromosome 11. Nature, 300, 773-774.

McBride,O.W., Swan,D.C., Tronick,S.R., Gol,R., Klimanis,D., Moore,D.E. and Aaronson,S.A. (1983) Regional chromosomal localization of N-*ras*,K-*ras*-1, K-*ras*-2 and myb oncogenes in human cells. Nucleic Acids Res., 11, 8221-8236.

McCormick,F. (1989) *ras* GTPase actvating protein: signal transmitter and signal terminator. Cell, 56, 5-8.

McCoy,M.S., Bargmann,C.I. and Weinberg,R.A. (1984) Human colon carcinoma Ki-*ras*2 oncogene and its corresponding proto-oncogene. Mol. Cell. Biol., 4, 1577-1582.

McCoy,M.S., Toole,J.J., Cunningham,J.M., Chang,E.H., Lowy,D.R. and Weinberg,R. A. (1983) Characterization of a human colon/lung carcinoma oncogene. Nature, 302, 79-81.

McGrath,J.P., Capon,D.J., Smith,D.H., Chen,E.Y., Seeburg,P.H., Goeddel,D.V. and Levinson,A.D. (1983) Structure and organization of the human Ki-*ras* proto-oncogene and a related processed pseudogene. Nature, 304, 501-506.

McMahon,G., Davis,E. and Wogan,G.N. (1987) Characterization of c-Ki-*ras* oncogene alleles by direct sequencing of enzymatically amplified DNA from carcinogen-induced tumors. Proc. Natl. Acad. Sci. USA, 84, 4974-4978.

McMahon,G., Hanson,L., Lee,J.-J. and Wogan,G.N. (1986) Identification of an activated c-Ki-*ras* oncogene in rat liver tumors induced bu aflatoxin B_1. Proc. Natl. Acad. Sci. USA, 83, 9418-9422.

Melin,P., Sundler,R. and Jergil,B. (1986) Phospholipase C in rat liver plasma membranes: phosphoinositide specificity and regulation by guanine nucleotides and calcium. FEBS Lett., 198, 85-88.

Meyers,T.R. and Hendricks,J.D. (1982) A Summary of tissue lesions in aquatic animals induced by controlled exposures to environmental contaminants, chemotherapeutic agents, and potential carcinogens. Marine Fish. Rev., 44, 1-17.

Miyoshi,J., Kagimoto,M., Soeda,E.-I. and Sakaki,Y. (1984) The Human c-Ha-*ras*-2 is a processed pseudogene inactivated by numerous base substitutions. Nucleic Acids Res., 12, 1821-1828.

Murray,M.J., Cunningham,J.M., Parada,L.F., Duatry,F., Lebowitz,P. and Weinberg, R.A. (1983) The HL-60 transforming sequence: a *ras* oncogene coexisting with altered *myc* genes in hematopoietic tumors. Cell, 33, 749-757.

Nakano,H., Yamamoto,F., Neville,C., Evans,D., Mizuno,T. and Perucho,M. (1984) Isolation of transforming sequences of two human lung carcinomas: structural and functional analysis of the activated c-K-*ras* oncogenes. Proc. Natl. Acad. Sci. USA, 81, 71-75.

Needleman,S.W., Kraus,M.H., Srivastava,S.K., Levine,P.H. and Aaronson,S.A. (1986) High frequency of N-*ras* activation in acute myelogenous leukemia. Blood, 67, 753-757.

Nemoto,N., Kodama,K., Tazawa,A., Matsumoto,J., Masahito,P. and Ishikawa,T. (1987) Nucleotide sequence comparison of the predicted first exonic region of goldfish *ras* gene between normal and neoplastic tissue. J. Cancer Res. Clin. Oncol., 113, 56-60.

Nemoto,N., Kodama,K.-I., Tazawa,A., Masahito,P. and Ishikawa,T. (1986) Extensive sequence homology of the goldfish *ras* gene to mammalian *ras* genes. Differentiation, 32, 17-23.

Neri,A., Knowles,D.M., Greco,A., McCormick,F. and Dalla-Favera,R. (1988) Analysis of *RAS* oncogene mutations in human lymphoid malignancies. Proc. Natl. Acad. Sci. USA, 85, 9268-9272.

Neuman-Silberberg,F.S., Schejter,E., Hoffman,F.M. and Shilo,B.Z. (1984) The Drosophila *ras* oncogenes: structure and nucleotide sequence. Cell, 37, 1027-1033.

O'Brian,R.M., Siddle,K., Honslay,M.D. and Hall,A. (1987) Interaction of the human insulin receptor with the *ras* oncogene product p21. FEBS Lett., 217, 253-259.

O'Brien,S.J., Nash,W.G., Goodwin,L., Lowy,D.R. and Chang,E.H. (1983) Dispersion of the *ras* family of transforming genes to four different chromosomes in man. Nature, 298, 839-842.

O'Hara,B.M., Oskarsson,M., Tainsky,M.A. and Blair,D.G. (1986) Mechanism of activation of human *ras* genes cloned from a gastric adenocarcinoma and a pancreatic carcinoma cell line. Cancer Res., 46, 4695-4700.

Ohara,O., Dorit,R.L. and Gilbert,W. (1989) One-sided polymerase chain reaction: The amplification of cDNA. Proc. Natl. Acad. Sci. USA, 86, 5673-5677.

Olson,E.N., Spizz,G. and Tainsky,M.A. (1987) The oncogenic forms of N-*ras* or H-*ras* prevent skeletal myoblast differentiation. Mol. Cell. Biol., 7, 2104-2111.

Padua,R.A., Barrass,N.C. and Currie,G.A. (1985) Activation of N-*ras* in a human melanoma cell line. Mol. Cell. Biol., 5, 582-585.

Padua,R.A., Carter,G., Hughes,D., Gow,J., Farr,C., Oscier,D., McCormick,F. and Jacobs,A. (1988) *ras* mutations in myelodysplasia detected by amplification, oligonucleotide hybridization, and transformation. Leukemia, 2, 503-510.

Pall,M.L. (1985) GTP: A central regulator of cellular anabolism. Curr. Topics Cell. Reg., 25, 1-20.

Papageorge,A., Lowy,D. and Scolnick,E.M. (1982) Comparative biochemical properties of p21 *ras* molecules coded for by viral and cellular *ras* genes. J. Virol., 44, 509-519.

Papageorge,A.G., Willumsen,B.M., Johnsen,M., Kung,H.-F., Stacey,D.W., Vass,W.C. and Lowy,D.R. (1986) A transforming *ras* gene can provide an essential function ordinarily supplied by an endogenous *ras* gene. Mol. Cell. Biol., 6, 1843-1846.

Parada,L.F., Tabin,C.J., Shih,C. and Weinberg,R.A. (1982) Human EJ bladder carcinoma oncogene is homologue of Harvey sarcoma virus *ras* gene. Nature, 297, 474-478.

Parries,G., Hoebel,R. and Racker,E. (1987) Opposing effects of a *ras* oncogene on growth factor-stimulated phosphoinositide hydrolysis: desensitization to platelet-derived growth factor and enhanced sensitivity to bradykinin. Proc. Natl. Acad. Sci. USA, 84, 2648-2652.

Pawson,T., Amiel,T., Hinze,E., Auersperg,N., Neave,N., Sobolewski,A. and Weeks, G. (1985) Regulation of a *ras*-related protein during development of Discyostelium discoideum. Mol. Cell. Biol., 5, 33-39.

Payne,P.A., Olson,E.N., Hsiau,P., Roberts,R., Perryman,M.B. and Schneider,M.D. (1987) An activated c-Ha-*ras* allele blocks the induction of muscle-specific genes whose expression is contingent on mitogen withdrawal. Proc. Natl. Acad. Sci. USA, 84, 8956-8960.

Pedersen-Bjergaard,J., Janssen,J.W.G., Lyons,J., Philip,P. and Bartram,C.R. (1988) Point mutation of the *ras* oncogenes and chromosome aberrations in acute nonlymphocytic leukemia and preleukemia related to therapy with alkylating agents. Cancer Res., 48, 1812-1817.

Pelling,J.C., Neades,R. and Strawhecker,J. (1988) Epidermal papillomas and carcinomas induced in uninitiated mouse skin by tumor promoters alone contain a point mutation in the 61st codon of the Ha-*ras* oncogene. Carcinogenesis, 9, 665-667.

Peters,R.L., Rabstein,L.S., Van Vleck,R., Kelloff,G.J. and Huebner,R.J. (1974) Naturally occurring sarcoma virus of the BALB/cCr mouse. J. Natl. Cancer Inst., 53, 1725-1729.

Pincus,M.R., Brandt-Rauf,P.W., Carty,R.P., Lubowsky,J., Avitable,M., Gibson,K.D. and Scheraga,H.A. (1987) Conformational effects of substituting amino acids for glutamine-61 on the central transforming region of the P21 proteins. Proc. Natl. Acad. Sci. USA, 84, 8375-8379.

Pizon,V., Lerosey,I., Chardin,P. and Tavitian,A. (1988) Nucleotide sequence of a human cDNA encoding a *ras*-related protein protein (rap1B)). Nucleic Acids Res., 16, 7719.

Powers,D.A. (1989) Fish as model systems. Science, 246, 352-358.

Powers,S., Kataoka,T., Fasano,O., Goldfarb,M., Strathern,J., Broach,J. and Wigler,M. (1984) Genes in S. cerevisiae encoding proteins with domains homologous to the mammalian *ras* proteins. Cell, 36, 607-612.

Preiss,J., Loomis,C.R., Bishop,W.R., Stein,R., Niedel,J.E. and Bell,R.M. (1986) Quantitative measurement of sn-1,2-diacylglycerols present in platelets, hepatocytes, and *ras*-and *sis*-transformed normal rat kidney cells. J. Biol. Chem., 261, 8597-8600.

Pulciani,S., Santos,E., Lauver,A.V., Long,L.K. and Barbacid,M. (1982) Transforming genes in human tumors. J. Cell. Biochem., 20, 51-61.

Pulciani,S., Santos,E., Long,L.K., Sorrentino,V. and Barbacid,M. (1985) *ras* gene amplification and malignant transformation. Mol. Cell. Biol., 5, 2836-2841.

Rasheed,S., Gardner,M.B. and Huebner,R.J. (1978) In vitro isolation of stable rat sarcoma viruses. Proc. Natl. Acad. Sci. USA, 75, 2972-2976.

Raybaud,F., Noguchi,T., Marics,I., Adelaide,J., Planche,J., Batoz,M., Aubert, C., de Lapeyriere,O. and Birnbaum,D. (1988) Detection of a low frequency of activated *ras* genes in human melanomas using a tumorigenicity assay. Cancer Res., 48, 950-953.

Reddy,E.P. (1983) Nucleotide sequence analysis of the T24 human bladder carcinoma oncogene. Science, 220, 1061-1063.

Reddy,E.P., Reynolds,R.K., Santos,E. and Barbacid,M. (1982) A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. Nature, 300, 149-152.

Reitz,R.H. and Watanabe,P.G. (1983) Importance of non-genetic mechanisms in carcinogenicity. In Hayes,A.W., Schnell,R.C. and Miya,T.S. (eds.), Developments in the Science and Practice of Toxicology. Elsevier, New York, pp. 163-172.

Reymond,C.D., Gomer,R.H., Mehdy,M.C. and Firtel,R.A. (1984) Developmental regulation of a Dictyostelium gene encoding a protein homologous to mammalian *ras* protein. Cell, 39, 141-148.

Reynolds,S.H., Stowers,S.J., Maronpot,R.R., Anderson,M.W. and Aaronson,S.A. (1986) Detection and identification of activated oncogenes in spontaneously occurring benign and malignant hepatocellular tumors of the B6C3F1 mouse. Proc. Natl. Acad. Sci. USA, 83, 33-37.

Reynolds,S.H., Stowers,S.J., Patterson,R.M., Maronpot,R.R., Aaronson,S.A. and Anderson,M.W. (1987) Activated oncogenes in B6C3F1 mouse liver tumors: Implications for risk assessment. Science, 237, 1309-1316.

Ricketts,M.H. and Levinson,A.D. (1988) High-level expression of c-H-*ras*1 fails to fully transform rat-1 cells. Mol. Cell. Biol., 8, 1460-1468.

Riou,G., Barrois,M., Sheng,Z.-M., Duvillard,P. and Lhomme,C. (1988) Somatic deletions and mutations of c-Ha-*ras* gene in human cervical cancers. Oncogene, 3, 329-333.

Robinson,L.C., Gibbs,J.B., Marshall,M.S., Sigal,I.S. and Tatchell,K. (1987) CDC25: A Component of the *RAS*-adenylate cyclase pathway in Saccharomyces cerevisiae. Science, 235, 1218-1221.

Robishaw,J.D., Russell,D.W., Harris,B.A., Smigel,M.D. and Gilman,A.G. (1986) Deduced primary structure of the a subunit of the GTP-binding stimulatory protein of adenylate cyclase. Proc. Natl. Acad. Sci. USA, 83, 1251-1255.

Rochlitz,C.F., Scott,G.K., Dodson,J.M., Liu,E., Dollbaum,C., Smith,H.S. and Benz,C.C. (1989) Incidence of activating *ras* oncogene mutations associated with primary and metastatic human breast cancer. Cancer Res., 49, 357-360.

Rodenhuis,S., Bos,J.L., Slater,R.M., Behrendt,H., van 't Veer,M. and Smets,L.A. (1986) Absence of oncogene amplifications and occasional activation of N-*ras* in lymphoblastic leukemia of childhood. Blood, 67, 1698-1704.

Rodenhuis,S., Slebos,R.J.C., Boot,A.J.M., Evers,S.G., Mooi,W.J., Wagenaar,S.S., van Bodegom,P.C. and Bos,J.L. (1988) Incidence and possible clinical significance of K-*ras* oncogene activation in adenocarcinoma of the human lung. Cancer Res., 48, 5738-5741.

Rous,P. (1911) A Sarcoma of the fowl transmissible by an agent separable from the tumor cell. J. Exp. Med., 13, 397-411.

Ruta,M., Wolford,R., DeFeo-Jones,D., Ellis,R.W. and Scolnick,E.M. (1986) Nucleotide sequence of the two rat cellular *ras*H genes. Mol. Cell. Biol., 6, 1706-1710.

Ruta,M., Wolford,R., Dhar,R., DeFeo-Jones,D., Ellis,R. and Scolnick,E.M. (1986) Nucleotide sequence of the two rat cellular *ras*H genes. Mol. Cell. Biol., 6, 1706-1710.

Sacks,D.B., Glenn,K.C. and McDonald,J.M. (1989) The Carboxyl terminal segment of the c-Ki-*ras* 2 gene product mediates insulin-stimulated phosphorylation of calmodulin and stimulates insulin-independent autophosphorylation of the insulin receptor. Biochem. Biophys. Res. Comm., 161, 399-405.

Sadler,S.E., Schechter,A.L., Tabin,C.J. and Maller,J.L. (1986) Antibodies to the *ras* gene product inhibit adenylate cyclase and accelerate progesterone-induced cell divivion in Xenopus laevis oocytes. Mol. Cell. Biol., 6, 719-722.

Saiki,R.K., Bugawan,T.L., Horn,G.T., Mullis,K.B. and Erlich,H.A. (1986) Analysis of enzymatically amplified β-globin and HLA-DQa DNA with allele-specific oligonucleotide probes. Nature, 324, 163-166.

Saiki,R.K., Gelfand,D.H., Stoffel,S., Scharf,S.J., Higuchi,R., Horn,G.T., Mullis,K.B. and Erlich,H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science, 239, 487-491.

Saiki,R.K., Scharf,S., Faloona,F., Mullis,K.B., Horn,G.T., Erlich,H.A. and Arnheim,N. (1985) Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science, 230, 1350-1354.

Sakato,H., Mori,M., Taira,M., Yoshida,T., Matsukawa,S., Shimizu,K., Sekiguchi, M., Terada,M. and Sugimura,T. (1986) Transforming gene from human stomach cancers and a noncancerous portion of stomach mucosa. Proc. Natl. Acad. Sci. USA, 83, 4679-4683.

Santos,E., Martin-Zanca,D., Reddy,E.P., Pierotti,M.A., Della Porta,G. and Barbacid,M. (1984) Malignant activation of a K-*ras* oncogene in lung carcinoma but not in normal tissue of the same patient. Science, 223, 661-664.

Santos,E., Tronick,S.R., Aaronson,S.A., Pulciani,S. and Barbacid,M. (1982) T24 human bladder carcinoma oncogene is an activated form of the normal human homologue of BALB- and Harvey-MSV transforming genes. Nature, 298, 343-347.

Sasaki,M.S. (1985) Chomosome abnormalities in cancer development. Environ. Sci. Res., 31, 35-59.

Sato,S., Matsushima,T., Tanaka,N., Sugimura,T. and Takashima,F. (1973) Hepatic tumors in the guppy (Lebistes reticulatus) induced by aflatoxin B_1, dimethylnitrosamine, and 2-acetylaminofluorene. J. Natl. Cancer Inst., 50, 767-778.

Satoh,T., Nakamura,S. and Kaziro,Y. (1987) Induction of neurite formation in PC12 cells by microinjection of proto-oncogenic Ha-*ras* protein preincubated with guanosine-5'-O(3-thiotriphosphate). Mol. Cell. Biol., 7, 4553-4556.

Schejter,E.D. and Shilo,B.-Z. (1985) Characterization of functional domains of p21 *ras* by use of chimeric genes. EMBO J., 4, 407-412.

Schlichting,I., Rapp,G., John,J., Wittinghofer,A., Pai,E.F. and Goody,R.S. (1989) Biochemical and crystallographic characterization of a complex of c-Ha-*ras* p21 and caged GTP with flash photolysis. Proc. Natl. Acad. Sci. USA, 86, 7687-7690.

Schochetman,G., Ou,C.Y. and Jones,W.K. (1988) Polymerase chain reaction. J. Infect. Dis., 158, 1154-1157.

Schultz,M.E. and Schultz,R.J. (1982) Induction of hepatic tumors with 7,12-dimethylbenz(a)anthracene in two species of viviparous fishes (genus Poeciliopsis). Environ. Res., 27, 337-351.

Scolnick,E.M., Papageorge,A.G. and Shih,T.Y. (1979) Guanine nucleotide-binding activity as an assay for src protein of rat-derived murine sarcoma viruses. Proc. Natl. Acad. Sci. USA, 76, 5355-5359.

Scott,R.H. and Dolphin,A.C. (1987) Activation of a G protein promotes agonist responses to calcium channel ligands. Nature, 330, 760-762.

Sekiya,T., Fushimi,M., Hori,H., Hirohashi,S., Nishimura,S. and Sugimura,T. (1984) Molecular cloning and the total nucleotide sequence of the human c-Ha-*ras*-1 gene activated in a melanoma from a Japanese patient. Proc. Natl. Acad. Sci. USA, 81, 4771-4775.

Senn,H.P., Tran-Thang,C., Wodnar-Filipowicz,A., Jirincy,J., Fopp,M., Gratwohl, A., Signer,E., Weber,W. and Moroni,C. (1988) Mutation analysis of the N-*ras* proto-oncogene in active and remission phase of human acute leukemias. Int. J. Cancer, 41, 59-64.

Seuwen,K., Lagarde,A. and Pouyssegur,J. (1988) Deregulation of hamster fibroblast proliferation by mutated *ras* oncogenes is not mediated by constitutive activation of phosphoinositide-specific phospholipase C. EMBO J., 7, 161-168.

Shih,T.Y., Hattori,S., Clanton,D.J., Ulsh,L.S., Chen,Z.-Q., Lautenberger,J.A. and Papas,T.S. (1986) Structure and function of p21 *ras* proteins. Gene Amplif. Anal., 4, 53-72.

Shih,T.Y., Papageorge,A.G., Stokes,P.E., Weeks,M.O. and Scolnick,E.M. (1979) Guanine nucleotide-binding and autophosphorylating activities associated with the p21 src protein of Harvey murine sarcoma virus. Nature, 287, 686-691.

Shih,T.Y., Shilo,B.Z., Goldfarb,M.P., Dannenberg,A. and Weinberg,R.A. (1979) Passage of phenotypes of chemically transformed cells via transfection of DNA and chromatin. Proc. Natl. Acad. Sci. USA, 76, 5714-5718.

Shih,T.Y., Weeks,M.O., Gruss,P., Dhar,R., Oroszlan,S. and Scolnick,E.M. (1982) Identification of a precursor in the biosynthesis of the p21 transforming protein of Harvey murine sarcoma virus. J. Virol., 42, 253-261.

Shih,T.Y., Weeks,M.O., Young,H.A. and Scolnick,E.M. (1979) Identification of a sarcoma virus-coded phosphoprotein in nonproducer cells transformed by Kirsten or Harvey murine sarcoma virus. Virology, 96, 64-79.

Shimizu,K., Birnbaum,D., Ruley,M.A., Fasano,O., Suard,Y., Edlund,L., Taprowsky, E., Goldfarb,M. and Wigler,M. (1983) Structure of the Ki-*ras* gene of the human lung carcinoma cell line Calu-1. Nature, 304, 497-500.

Shimizu,K., Goldfarb,M., Suard,Y., Perucho,M., Li,Y., Kamata,T., Feramisco,J., Stavnezer,E., Fogh,J. and Wigler,M.H. (1983) Three human transforming genes are related to the viral *ras* oncogenes. Proc. Natl. Acad. Sci. USA, 80, 2112-2116.

Sigal,I.S., Gibbs,J.B., D'Alonzo,J.S. and Scolnick,E.M. (1986) Identification of effector residues and a neutralizing epitope of H-*ras*-encoded p21. Proc. Natl. Acad. Sci. USA, 83, 4725-4729.

Sigal,I.S., Gibbs,J.B., D'Alonzo,J.S., Temeles,G.T., Wolanski,B.S., Socher,S.H. and Scolnick,E.M. (1986) Mutant *ras*-encoded proteins with altered nucleotide binding exert dominant biological effects. Proc. Natl. Acad. Sci. USA, 83, 952-956.

Sinha,S., Webber,C., Marshall,C.J., Knowles,M.A., Proctor,A., Barrass,N.C. and Neal,G.E. (1988) Activation of *ras* oncogene in aflatoxin-induced rat liver carcinogenesis. Proc. Natl. Acad. Sci. USA, 85, 3673-3677.

Sklar,M.D. and Kitchingman,G.R. (1985) Isolation of activated *ras* transforming genes from two patients with Hodgkin's disease. Int. J. Radiat. Oncol. Biol. Phys., 11, 49-55.

Smit,V.T.H.B.M., Boot,A.J.M., Smits,A.M.M., Fleuren,G.J., Cornelisse,C.J. and Bos,J.L. (1988) K-*ras* codon 12 mutations occur very frequently in pancreatic adenocarcinomas. Nucleic Acids Res., 16, 7773-7782.

Smith,C.D., Cox,C.C. and Snyderman,R. (1986) Receptor-coupled activation of phosphoinositide-specific phospholipase C by an N protein. Science, 232, 97-100.

Smith,C.E., Peck,T.H., Klanda,R.J. and McLaren,J.B. (1979) Hepatomas in Atlantic tomcod (*Microgaddus tomcod*), (Walbaum) collected in the Hudson River estuary in New York. J. Fish Dis., 2, 313-319.

Souyri,M. and Fleissner,E. (1983) Identification by transfection of transforming sequences in DNA of human T-cell leukemias. Proc. Natl. Acad. Sci. USA, 80, 6676-6679.

Spandidos,D.A. (1987) Oncogene activation in malignant transformation: a study of H-*ras* in human breast cancer. Anticancer Res., 7, 991-996.

Spandidos,D.A. and Agnantis,N.J. (1984) Human malignant tumors of the breast as compared to their respective normal tissue have elevated expression of the Harvey *ras* oncogene. Anticancer Res., 4, 269-272.

Spandidos,D.A. and Holmes,L. (1987) Transcriptional enhancer activity in the variable tandem repeat DNA sequence downstream of the human Ha-*ras*1 gene. FEBS Lett., 218, 41-46.

Spandidos,D.A. and Kerr,I.B. (1984) Elevated expression of the human *ras* oncogene family in premalignant and malignant tumors of the colorectum. Br. J. Cancer, 49, 681-688.

Spandidos,D.A., Lamothe,A. and Field,J.N. (1985) Multiple transcriptional activation of cellular oncogenes in the human head and neck solid tumors. Anticancer Res., 5, 221-224.

Spandidos,D.A. and Wilkie,N.M. (1984) Malignant transformation of early passage rodent cells by a single mutated human oncogene. Nature, 310, 469-475.

Spina,A., DiDonato,A., Collela,G., Illiano,G., Berlingieri,M.T., Fusco,A. and Grieco,M. (1987) Increased adenylate cyclase activity in rat thyroid epithelial cells expressing viral *ras* genes. Biochem. Biophys. Res. Comm., 142, 527-535.

Srivastava,S.K., Yuasa,Y., Reynolds,S.H. and Aaronson,S.A. (1985) Effects of two major activating lesions on the structure and conformation of human *ras* oncogene products. Proc. Natl. Acad. Sci. USA, 82, 38-42.

Stacey,D.W. and Kung,H.F. (1984) Transformation of NIH/3T3 cells by microinjection of Ha-*ras* p21 protein. Nature, 310, 508-511.

Stanton,M.F. (1965) Diethyl nitrosamine-induced hepatic degeneration and neoplasia in the aquarium fish, *Brachydanio rerio*. J. Natl. Cancer Inst., 34, 117-130.

Stevens,C.W., Manoharan,T.H. and Fahl,W.E. (1988) Characterization of mutagen-activated cellular oncogenes that confer anchorage independence to human fibroblasts and tumorigenicity to NIH 3T3 cells: Sequence analysis of an enzymatically amplified mutant H*RAS* allele. Proc. Natl. Acad. Sci. USA, 85, 3875-3879.

Stowers,S.J., Wiseman,R.W., Ward,J.M., Miller,E.C., Miller,J.A., Anderson,M.W. and Eva,A. (1988) Detection of activated proto-oncogenes in N-nitrosodiethylamine-induced liver tumors: A comparison between B6C3F1 mice and Fischer 344 rats. Carcinogenesis, 9, 271-276.

Streisinger,G., Walker,C., Dower,N., Knauber,D. and Singer,F. (1981) Production of clones of homozygous diploid zebrafish (*Brachydanio rerio*). Nature, 291, 293-296.

Sukumar,S., Notario,V., Martin-Zanca,D. and Barbacid,M. (1983) Induction of mammary carcinomas in rats by nitroso-methylurea involves malignant activation of H-*ras* locus by single point mutations. Nature, 306, 658-661.

Sullivan,K.A., Miller,R.T., Masters,S.B., Beiderman,B., Heideman,W. and Bourne, H.R. (1987) Identification of receptor contact site involved in receptor-G protein coupling. Nature, 330, 758-760.

Sweet,R.W., Yokoyama,S., Kamata,T., Feramisco,J.R., Rosenberg,M. and Gross,M. (1984) The product of *ras* is a GTPase and the T24 oncogenic mutant is deficient in this activity. Nature, 311, 273-275.

Tabin,C.J., Bradley,S.M., Bargmann,C.I., Weinberg,R.A., Papageorge,A.G., Scolnick,E.M., Dhar,R., Lowy,D.R. and Chang,E.H. (1982) Mechanism of activation of a human oncogene. Nature, 300, 143-149.

Tabin,C.J. and Weinberg,R.A. (1985) Analysis of viral and somatic activations of the c-Ha-*ras* gene. J. Virol., 53, 260-265.

Tahira,T., Hayashi,K., Ochiai,M., Tsuchida,N., Nagao,M. and Sugimura,T. (1986) Structure of the c-Ki-*ras* gene in a rat fibrosarcoma induced by 1,8-dinitropyrene. Mol. Cell. Biol., 6, 1349-1351.

Tainsky,M.A., Cooper,C.S., Giovanella,B.C. and Van de Woude,G.F. (1984) An activated *ras*N gene: detected in late but not early passage human PA1 teratocarcinoma cells. Science, 223, 643-645.

Tanaka,T., Ida,N., Shimoda,H., Waki,C., Slamon,D.J. and Cline,M.J. (1986) Organ specific expression of *ras* oncoproteins during growth and development of the rat. Mol. Cell. Biochem., 70, 97-104.

Tanaka,T., Ida,N., Waki,C., Shimoda,H., Slamon,D.J. and Cline,M.J. (1987) Cell type-specific expressions of c-*ras* gene products in the normal rat. Mol. Cell. Biochem., 75, 23-32.

Taparowsky,E., Shimizu,K., Goldfarb,M. and Wigler,M. (1983) Structure and activation of the human N-*ras* gene. Cell, 34, 581-586.

Taparowsky,E., Suard,Y., Fasano,O., Shimizu,K., Goldfarb,M. and Wigler,M. (1982) Activation of the T24 bladder carcinoma transforming gene is linked to a single amino acid change. Nature, 300, 762-765.

Tatchell,K., Chaleff,D.T., DeFeo-Jones,D. and Scolnick,E.M. (1984) Requirement of either of a pair of *ras*-related genes of Saccharomyces cerevisiae for spore viability. Nature, 309, 523-527.

Taya,Y., Hosogai,K., Hirohashi,S., Shimosato,Y., Tsuchiya,R., Tsuchida,N., Fushimi,M., Sekiya,T. and Nishimura,S. (1984) A novel combination of K-*ras* and *myc* amplification accompanied by point mutational activation of K-*ras* in human lung cancer. EMBO J., 3, 2943-2946.

Temeles,G.L., Gibbs,J.B., D'Alonzo,J.S., Sigal,I.S. and Scolnick,E.M. (1985) Yeast and mammalian *ras* proteins have conserved biochemical properties. Nature, 313, 700-703.

Thorgaard,G.H., Scheerer,P.D. and Parsons,J.E. (1985) Residual patenal inheritance in gynogenetic rainbow trout: Implications for gene transfer. Theor. Appl. Genet., 71, 119-121.

Toda,T., Uno,I., Ishikawa,T., Powers,S., Kataoka,T., Broek,D., Cameron,S., Broach,J., Matsumoto,K. and Wigler,M. (1985) In yeast, *RAS* proteins are controlling elements of adenylate cyclase. Cell, 40, 27-36.

Toledo,C., Hendricks,J., Loveland,P., Wilcox,J. and Bailey,G. (1987) Metabolism and DNA-binding in vivo of aflatoxin B_1 in medaka (Oryzias latipes). Comp. Biochem. Physiol., 87C, 275-281.

Tong,L., de Vos,A.M., Milburn,M.V., Jancarik,J., Noguchi,S., Nishimura,S., Miura,K., Ohtsuka,E. and Kim,S.-H. (1989) Structural differences between a *ras* oncogene protein and the normal protein. Nature, 337, 90-93.

Trahey,M. and McCormick,F. (1987) A Cytoplasmic protein stimulates normal N-*ras* p21 GTPase, but does not affect oncogenic mutants. Science, 238, 542-545.

Trahey,M., Milley,R.J., Cole,G.E., Innis,M., Paterson,H., Marshall,C.J., Hall, A. and McCormick,F. (1987) Biochemical and biological properties of the human N-*ras* p21 protein. Mol. Cell. Biol., 7, 541-544.

Trahey,M., Wong,G., Halenbeck,R., Rubinfeld,B., Martin,G.A., Ladner,M., Long,C. M., Crosier,W.J., Watt,K., Koths,K. and McCormick,F. (1988) Molecular cloning of two types of GAP complementary DNA from human placenta. Science, 242, 1697-1700.

Tsuchida,N., Ryder,T.R. and Ohtsubo,E. (1982) Nucleotide sequence of the oncogene encoding the p21 transforming protein of Kirsten murine sarcoma virus. Science, 217, 937-939.

Uhing,R.J., Prpic,V., Jiang,H. and Exton,J.H. (1986) Hormone-stimulated polyphosphoinositide breakdown in rat liver plasma membrane: roles of guanine nucleotides and calcium. J. Biol. Chem., 261, 2140-2146.

Valenzuela,D.M. and Groffen,J. (1986) Four human carcinoma cell lines with novel mutations in position 12 of c-K-*ras* oncogene. Nucleic Acids Res., 14, 843-852.

Van Beneden,R.J., Watson,D.K., Chen,T.T., Lautenberger,J.A. and Papas,T.S. (1986) Cellular *myc* (c-*myc*) in fish (rainbow trout): Its relationship to other vertebrate *myc* genes and to the transforming genes of the MC29 family of viruses. Proc. Natl. Acad. Sci. USA, 83, 3698-3702.

Van Dongen,A.M.K., Codina,J., Olate,J., Mattera,R., Joho,R., Birnbaumer,L. and Brown,A.M. (1988) Newly identified brain potassium channels gated by the guanine nucleotide binding protein Go. Science, 242, 1433-1437.

van 't Veer,L.J., Burgering,B.M., Versteeg,R., Boot,A.J.M., Ruiter,D.J., Osanto,S., Schrier,P.I. and Bos,J.L. (1989) N-*ras* mutations in human cutaneous melanoma correlated with sun exposure. Mol. Cell. Biol., 9, 3114-3116.

van 't Veer,L.J., Hermens,R., van den Berg-Bakker,L.A.M., Cheng,N.C., Fleuren, G.-J., Bos,J.L., Cleton,F.J. and Schrier,P.I. (1988) *ras* oncogene activation in human ovarian carcinoma. Oncogene, 2, 157-165.

Viola,M.V., Fromowitz,F., Oravez,S., Deb,S. and Schlom,J. (1985) *ras* oncogene p21 expression is increased in premalignant lesions and high grade bladder carcinoma. J. Exp. Med., 161, 1213-1218.

VisVanathan,K.V., Pocock,R.D. and Summerhayes,I.C. (1988) Preferential and novel activation of H-*ras* in human bladder carcinomas. Oncogene Res., 3, 77-86.

Vogelstein,B., Fearon,E.R., Hamilton,S.R., Kern,S.E., Preisinger,A.C., Leppert, M., Nakamura,Y., White,R., Smits,A.M.M. and Bos,J.L. (1988) Genetic alterations during colorectal-tumor development. New Engl. J. Med., 319, 525-532.

Vogt,P.K., Bister,K., Burny,A.L., Croce,C.M., Haseltine,W.A., Hayman,M.J., Hayward,W.S., Klein,G., Moelling,K., Neth,R.D., Pragnell,I.B. and Rowley,J.D. (1985) Genes and viruses able to transform hematopoietic cells. In Weissman, I.L. (ed.), Leukemia. Springer-Verlag, New York, pp. 275-292.

Vousden,K.H. and Marshall,C.J. (1984) Three different activated *ras* genes in mouse tumors; evidence for oncogene activation during progression of a mouse lymphoma. EMBO J., 3, 913-917.

Wakelam,M.J.O., Davies,S.A., Houslay,M.D., McKay,I., Marshall,C.J. and Hall,A. (1986) Normal p21N-*ras* couples bombesin and other growth factor receptors to inositol phosphate production. Nature, 323, 173-176.

Wales,J.H. and Sinnhuber,R.O. (1966) An Early hepatoma epizootic in rainbow trout, *Salmo gairdneri*. Calif. Fish Game, 52, 85-91.

Walter,M., Clark,S.G. and Levinson,A.D. (1986) The oncogenic activation of human p21*ras* by a novel mechanism. Science, 233, 649-652.

Westaway,D., Papkoff,J., Moskovici,C. and Varmus,H.E. (1986) Identification of a provirally activated c-Ha-*ras* oncogene in an avian nephroblastoma via a novel procedure: cDNA cloning of a chimaeric viral-host transcript. EMBO J., 5, 301-309.

Wigler,M., Field,J., Powers,S., Broek,D., Toda,T., Cameron,S., Nikawa,J., Michaeli,T., Colicelli,J. and Ferguson,K. (1988) Studies of *RAS* function in the yeast Saccharomyces cerevisiae. In (ed.), Cold Spring Harbor Symposia on Quantitative Biology: Molecular Biology of Signal Transduction (53). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 649-655.

Williams,A.R.W., Piris,J., Spandidos,D.A. and Wyllie,A.H. (1985) Immunohistochemical detection of the *ras* oncogene p21 product in an experimental tumor and in human colorectal neoplasms. Br. J. Cancer, 52, 687-693.

Williams,D.E. and Buhler,D.R. (1983) Purified form of cytochrome P-450 from rainbow trout with high activity toward conversion of aflatoxin B_1-2,3-epoxide. Cancer Res., 43, 4752-4756.

Willingham,M.C., Pastan,I., Shih,T.Y. and Scolnick,E.M. (1980) Localization of the src gene product of the Harvey strain of MSV to plasma membrane of transformed cells by electron microscopic immunocytochemistry. Cell, 19, 1005-1014.

Willumsen,B.M., Christensen,A., Hubbert,N.L., Papageorge,A.G. and Lowy,D.R. (1984) The p21 *ras* C-terminus is required for transformation and membrane association. Nature, 310, 583-586.

Willumsen,B.M., Norris,K., Papageorge,A.G., Hubbert,N.L. and Lowy,D.R. (1984) Harvey murine sarcoma virus p21 *ras* protein: biological and biochemical significance of the cysteine nearest the carboxy terminus. EMBO J., 3, 2581-2585.

Winter,E., Yamamoto,F., Almoguera,C. and Perucho,M. (1985) A method to detect and characterize point mutations in transcribed genes: amplification and overexpression of the mutant c-Ki-*ras* allele in human tumor cells. Proc. Natl. Acad. Sci. USA, 82, 7575-7579.

Wirgin,I.I., Currie,D., Grunwald,C. and Garte,S.J. (1989) Molecular mechanisms of carcinogenesis in a natural population of Hudson River fish. Proc. Annu. Meet. Amer. Assoc. Cancer Res., 30, A770.

Wirgin,I.I., Grunwald,C. and Garte,S.J. (1989) Oncogene activation in Hudson River tomcod. Proc. Amer. Fish. Soc., 119, 35.

Wiseman,R.W., Stowers,S.J., Miller,E.C., Anderson,M.W. and MIller,J.A. (1986) Activating mutations of the c-Ha-*ras* protooncogene in chemically induced hepatomas of the male B6C3 F1 mouse. Proc. Natl. Acad. Sci. USA, 83, 5825-5829.

Wolfman,A. and Macara,I.G. (1987) Elevated levels of diacylglycerol and decreased phorbol ester sensitivity in *ras*-transformed fibroblasts. Nature, 325, 359-361.

Wong,G., Arnheim,N., Clark,R., McCabe,P., Innis,M., Aldwin,L., Nitecki,D. and McCormick,F. (1986) Detection of activated Mr 21,000 protein, the product of *ras* oncogenes, using antibodies with specificity for amino acid 12. Cancer Res., 46, 6029-6033.

Woodhead,A.D., Setlow,R.B. and Pond,V. (1982) Effects of polycyclic aromatic hydrocarbons on the proliferation of ectopic thyroid tissue in *Poecilia formosa*, the Amazon molly. J. Fish. Biol., 20, 455-463.

Yamada,H., Sakamoto,H., Taira,M., Nishimura,S., Shimosato,Y., Terada,M. and Sugimura,T. (1986) Amplifications of both c-Ki-*ras* with a point mutation and c-*myc* in a primary pancreatic cancer and its metastatic tumors in lymph nodes. Jpn. J. Cancer Res., 77, 370-375.

Yamamoto,F., Nakano,H., Neville,C. and Perucho,M. (1985) Structure and mechanisms of activation of c-K-*ras* oncogenes in human lung cancer. Prog. med. Virol., 32, 101-114.

Yamamoto,F. and Perucho,M. (1984) Activation of a human c-K-*ras* oncogene. Nucleic Acids Res., 12, 8873-8885.

Yatani,A., Hamm,H., Codina,J., Mazzoni,M.R., Birnbaumer,L. and Brown,A.M. (1988) A Monoclonal antibody to the a subunit of Gk blocks muscarinic activation of atrial K+ channels. Science, 241, 828-831.

Yatsunami,K. and Khorana,H.G. (1985) GTPase of bovine rod outer segments: The amino acid sequence of the a subunit as derived from the cDNA sequence. Proc. Natl. Acad. Sci. USA, 82, 4316-4320.

Yoshida,K., Hamatani,K., Koide,H., Ikeda,H., Nakamura,N., Akiyama,M., Tsuchiyama,H., Nakayama,E. and Shiku,H. (1988) Preparation of anti-*ras* Mr 21,000 protein monoclonal antibodies and immunohistochemical analyses on expression of *ras* genes in human stomach and thyroid cancers. Cancer Res., 48, 5503-5509.

Yu,C.-L., Tsai,M.-H. and Stacey,D.W. (1988) Cellular *ras* activity and phospholipid metabolism. Cell, 52, 63-71.

Yuan,L., Tong-hua,L. and Shi-e,L. (1988) Expression of c-*myc* and n-*ras* oncogenes in human hepatocellular carcinoma and pancreatic adenocarcinoma. Chin. Med. J, 101, 523-528.

Yuasa,Y., Gol,R.A., Chang,A., Chiu,I.-M., Reddy,E.P., Tronick,S.R. and Aaronson,S.A. (1984) Mechanism of activation of an N-*ras* oncogene of SW-1271 human lung carcinoma cells. Proc. Natl. Acad. Sci. USA, 81, 3670-3674.

Yuasa,Y., Oto,M., Sato,C., Miyaki,M., Iwana,T., Tonomura,A. and Namba,M. (1986) Colon carcinoma K-*ras* 2 oncogene of a familial polyposis coli patient. Jpn. J. Cancer Res., 77, 901-907.

Yuasa,Y., Reddy,E.P., Rhim,J.S., Tronick,S.R. and Aaronson,S.A. (1986) Activated N-*ras* in a human rectal carcinoma cell line associated with clonal homozygosity in myb locus-restriction fragment polymorphism. Jpn. J. Cancer Res., 77, 639-647.

Yuasa,Y., Srivastava,S.K., Dunn,C.Y., Rhim,J.S., Reddy,E.P. and Aaronson,S.A. (1983) Acquisition of transforming properties by alternative point mutations within c-bas/has human proto-oncogene. Nature, 303, 775-779.

Yunis,J.J., Boot,A.J.M., Mayer,M.G. and Bos,J.L. (1988) Mechanism of *ras* mutation in myelodysplastic syndrome. Oncogene, 4, 609-614.

Zarbl,H., Sukumar,S., Arthur,A.V., Martin-Zanca,D. and Barbacid,M. (1985) Direct mutagenesis of Ha-*ras*-1 oncogenes by N-nitroso-N-methylurea during initiation of mammary carcinogenesis in rats. Nature, 315, 382-385.

Zhang,X.-K., Huang,D.-P., Chiu,D.-K. and Chiu,J.-F. (1987) The expression of oncogenes in human developing liver and hepatomas. Biochem. Biophys. Res. Comm., 142, 932-938.

APPENDIX

Appendix Table I. List of trout ras PCR product recombinant clones.

Name	Size	Comments
RT-1 through RT-5	<216 bp	Carryover Human Ki- ras cDNA
RT-6gen01-c79	804 bp	Incl. 1st intron, not identical to RT-8
RT-7gen01-c79 (15)	804 bp	Incl. 1st intron, matches RT-8, rev.
RT-8gen01-c79 (19)	804 bp	Incl. 1st intron, consensus sequence
RT-9gen01-c79 (14)	804 bp	Incl. 1st intron, matches RT-8
RT-10gen01-c79 (18)	804 bp	Incl. 1st intron, matches RT-8
RT-11genRat01-c30-a*,b,c,d	90 bp	Different sequence from RT-8
RT-12gen01-c79 (NB2)	804 bp	Different sequence from RT-8 or RT-11
RT-13gen01-c79 (NB6)	804 bp	Different sequence from RT-8 or RT-11
RT-20BamHI-XbaI	500 bp	Subclone of RT-6, Ist exon
RT-21XbaI-EcoRI	300 bp	Subclone of RT-6, 2nd exon
RT-30cDNA12-c81-1,5,8	219 bp	cDNA match of RT-8
RT-31cDNA12-c81-2	219 bp	does not match RT-8
RT-32cDNA38-c81-1	131 bp	does not match RT-8
RT-33cDNAH01-c79 (1)	237 bp	differs slightly from RT-6 & RT-8
RT-34cDNAH01-c81 (4)	242 bp	differs slightly from RT-6 & RT-8
RT-35cDNAK01-c79 (1,2)	237 bp	differs from RT-33, RT-34, matches RT-11
RT-40cDNA38-dT-8	~550 bp	3' of RT-33, RT-34, stops short of end
RT-41cDNA38-dT-12	~550 bp	3' of RT-35, ?rearranges in 3rd exon