AN ABSTRACT OF THE DISSERTATION OF

Bryan L. Ford for the degree of Doctor of Philosophy in Toxicology presented on November 6, 2000. Title: Investigating Tumor Suppression in Triploid Trout

Signature redacted for privacy.

Abstract approved: _____

George S. Bailey

Previous work (Thorgaard, G. H. *et al.*, *Aquatic Toxicology* 46:121-126, 1999) showed triploid rainbow trout (*O. mykiss*) given embryonic carcinogen bath exposures had significant reduction of induced tumors relative to diploids. In the present study, trout were made triploid by thermal shock after fertilization. At age of 5 months they were given dietary carcinogen: aflatoxin B1 (AFB_1) or 7,12-dimethylbenz[*a*]anthracene (DMBA) for 30 or 120 days. The dietary exposures were at known tumorigenic levels (100, 200 and 300 ppb AFB_1; 250, 500 and 850 ppm DMBA). At about 16 months after fertilization the fish were sacrificed and tumor incidence and multiplicity were assessed. At all levels of carcinogen and in all tumorous organs tumor incidence was lower in the triploid fish. For DMBA-fed fish it was seen that the diploid:triploid incidence ratios ranged from 2.0 to 9.0 and for AFB_1 from 3.1 to 6.0. Weight class analyses dissociated the tumor incidence effects of growth from the effects of triploidy.Weight classes plotted against logit tumor incidence at all doses and durations showed parallel logistic lines. In every case the triploid curve was substantially lower than the diploid curve, showing the independent suppressive effect of triploidy.

Fifteen triploid DMBA liver tumors were examined by direct cycle-sequencing of p53 PCR products across the exons 5, 7 and 8 known to contain nearly all human tumor p53 mutations. There were no p53 mutations seen at, or above, the present threshold of detection, (for radiolabeled manual sequencing, under 5% of mutant in normal). Fluorescent sequencing of 15 stomach tumors, also showed no p53 mutations in the hotspot-containing exons. Mutation detection by sequencing the trout *Ki-ras1*

gene, ortholog human *KRAS2*, showed codons 12, and 61 mutations in DMBA-fed trout liver and stomach tumors. The DMBA liver tumor *Ki-ras1* mutation incidence showed no change by ploidy.There was a significant reduction in *Ki-ras1* exon 1 mutations in triploid stomach tumors (5% in triploids v. 33% in diploids, Fisher's Exact test $p<0.05$). AFB_1 liver tumors showed *Ki-ras1* mutation incidence of 75% (9/12) in diploids and 90% (9/10) triploids, nearly all in exon 1, this mutation difference with respect to ploidy did not reach significance.

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Investigating Tumor Suppression in Triploid Trout

by

Bryan L. Ford

A DISSERTATION

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Contribution of Authors

George S. Bailey, contributed guidance in formulating many aspects of the research reported in this Dissertation, further he provided critical assistance in its implementation. As Major Advisor to Bryan L. Ford, he provided editorial review of this Dissertation.

Ulrich Harttig, contributed in Chapter 2, the weight-class analyses and critical review of other portions of this Dissertation.

Jerry D. Hendricks, contributed in Chapter 2, fish necropsy and gross tumor pathology.

Catherine Z. Mathews, contributed in Chapter 4, sequence data on the post-translational portions of the *Ki-ras1* gene; and the design of several useful PCR primers.

Thierry Soussi, contributed in Chapter 5, the trout p53 cDNA sequence and a plasmid-containing a trout p53 cDNA clone.

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Glossary

Abbreviations, Acronyms and Special Terms Used in this Dissertation

A: Adenine base, deoxyadenosine nucleoside or nucleotide

ABI: Applied Biosystems Incorporated, Foster City; CA

AFB_1: Aflatoxin B_1

Arg: Amino acid arginine

C: Cytosine nucleic acid base, deoxycytidine nucleoside or nucleotide

cDNA: Complementary DNA, the reverse transcription product templated by mRNA

DMBA: 7,12-dimethlbenz[*a*]anthracene

dNTPs: All four deoxyribonucleoside triphosphates

dT: Thymidine

FAM: Fluorochrome, carboxyfluorescein

G: Guanine base, doxyguanosine nucleoside or nucleotide

Gly: Amino acid glycine

HEX: Fluorochrome, hexachlorofluorescein, Perkin-Elmer originated

His: Amino acid histidine

JOE: Fluorochrome, carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein

kbp: Kilo basepairs, measure of DNA length

Lys: Amino acid lysine

mRNA: Messenger RNA

Orthologs: Homologous genes in distantly related organisms, e.g. rat and trout p53

Paralogs: Homologous genes within a single organism, e.g. human HRAS and KRAS

PCR: Polymerase Chain Reaction

ROX: Carboxy-X-rhodamine

RT PCR: Reverse Transcription (and) PCR

Ser: Amino acid serine

T: Thymine base, thymidine nucleoside or nucleotide

Glossary (Continued)

TA and TA cloning: Refers to PCR cloning strategies based on propensity for Taq and some other non-proofreading thermostable DNA polymerases to leave an extra untemplated "A" overhang at the 3' terminus of a PCR product. The cloning operation makes use of the overhang to favor complementary annealing and ligation into a cloning vector engineered to have "T" overhangs

TAMRA: Fluorochrome, carboxytetramethylrhodamine, Molecular Probes product used byABI

Thr: Amino acid threonine

TOD: Threshold of detection (as for mutations)

Tris HCl: Buffer tris(hydroxymethyl)aminomethane hydrochloride

Trp: Amino acid tryptophan

Tyr: Amino acid tyrosine

Dedication

I dedicate this Dissertation to my Mother, Mrs. Mary Elizabeth Sweeney Ford. She has steadfastly and patiently drawn me to an understanding of biology for as long as I can remember, and doubtless longer.

INVESTIGATING TUMOR SUPPRESSION IN TRIPLOID TROUT

Chapter 1

Introduction and Literature Review

Cancer Biology Issues

Ploidy and carcinogenesis

It has been frequently observed that neoplastic tissues contain many aneuploid cells, so much so that this is a defining feature of advanced neoplasia. In addition, tumors and other neoplasia are often characterized by cells with fractional excess ploidy designated by the cytometric term "hyperdiploidy" (1-4). However, this association between abnormal ploidy and tumors does not generally extend to polyploidy, that is full genome multiplication. True polyploidy is not a feature of neoplastic tissues; instead it is a structural norm for some somatic cells, such as cardiomyocytes and megakaryocytes. In some mammalian cell types, such as hepatocytes, cells become polyploid as the animal develops and ages (5). The function of polyploidy in cells is a matter of speculation. In hepatocytes polyploidy could provide protective effects from genomic redundancy for these cells so likely to be directly exposed to metabolically activated genotoxins. The loss of such a protective mechanism may be reflected in reports that hepatocytes from advanced tumors are often found to have regressed to diploid or near diploid status (6, 7). The mechanistic underpinnings of the phenomenon of tumor ploidy reduction are not well understood. For liver, it may be hypothesized that the clonal expansion of subsets of transformed cells is intimately related to their reduced ploidy. That is, the reduction of ploidy, through yet undisclosed mechanisms, may release cells from restraints of extra copies of suppressor genes that normally provide redundant mitotic checkpoint controls in polyploid hepatocytes. In liver, injury, mutation or other oncogenic stimulation may result in the clonal expansion of pre-existing incidental diploid cells, or somehow induce production of diploid lineages of the hepatocyte or hepatocyte precursor cells. Hyperdiploidy is a cytometric category that is associated with many types of tumors. It results from non-mitotic selective chromosome or partial chromosome replications. It may be associated with the frequently observed tumor phenomenon of "gene multiplication" or "gene amplification." From whatever causes, there do appear to be

strong associations of fractional ploidy variation with tumor pathology, and conversely there are no reports of true whole genome ploidy duplication (or polyploidy) detected in tumor or other neoplasias in the oncology literature.

Our work, reported in this Dissertation, was undertaken to investigate tumorigenesis in triploid trout and may shed some light on these somewhat contrasting phenomena of aneuploidy, hyperdiploidy and polyploidy, by directly examining the effect of experimentally induced triploidy on tumor incidence and multiplicity. Further, we will examine the effects of experimental tumorigenesis on mutational spectra for two trout genes known to be orthologous to two of the most important human cancer genes, KRAS and TP53. These trout orthologs are respectively *Ki-Ras1* (8) and p53 (9), mutations of which have been shown to be associated with human tumors and with aneuploidy of human tumor cells (10, 11).

Triploidization and triploid animals

Successful experimental triploidization is currently limited to a diverse array of aquatic animal species including salmonids such as the rainbow trout (*Oncorhynchus mykiss*). There are also reports of triploidization of a number of other metazoans besides teleosts, including chondrichthys (12), molluscs (13, 14), and a species of crab (15). The apparent limitation to aquatic species may only reflect the relative ease of manipulating eggs from animals that externally fertilize. Numerous species of vertebrates and invertebrates are seen to be naturally triploid under certain conditions. Triploid chickens have been found, and some free-living lizards (16) are naturally triploid. It appears that there is little prospect of readily producing viable triploid eutherian mammals, since human, rat and mouse triploid embryos have a high rate of spontaneous abortion. Those few that progress to term suffer inevitable mortality shortly after birth, manifesting a variety of severe morphological and physiological defects (17), (18).

Methods used to induce triploidy often involve shocking a newly fertilized egg. Such shocks include timely administration of hyperthermia (19), cytochalasin B (20), ionic calcium (21), hydrostatic pressure (22), nitrous oxide (23), caffeine (24),

hypothermia (25), heat and electroshock (26), or 6-dimethylaminopurine (27). Most of these treatments disrupt normal spindle formation and cytokinesis during meiosis II, which then prevents the normal extrusion of a sequestered haploid genome-- itself a result of meiosis I-- as the second polar body (21, 22). Therefore, the haploid genome (1n) of the retained polar body adds to the normal zygotic diploid (2n) set to give a triploid genome (3n). All of the shock-induced triploidization methods result in triploid animals having a pair of maternal genomes and one paternal genome. By some measures (such as potential gene dosage), triploids may be considered to have undergone a profound genetic modification. While the triploids of a wide array of genera are often viable, with few if any exceptions they have difficulty producing fit gametes, since gametogenesis requires symmetrical segregation during meiotic reduction (28). Nevertheless, in many species, including salmonid fish, triploidization yields adult organisms with development and morphology very similar to their normal diploid siblings (19). The ready availability of such models invites the question of the effect of an extra copy of the genome on various aspects of the carcinogenesis process.

Reported effects of ploidy on DNA damage susceptibility

Evidence bearing on the effects of polyploidy with respect to gross DNA damage has long been available for one eukaryotic class of organisms: yeasts. Diploid yeast is far more resistant to radiation-induced genetic damage than are haploids (29). However, in yeast polyploids (3n or greater), radiation sensitivity increases with ploidy number from diploid through at least hexaploid. Interestingly, no degree of polyploidy up to at least 6n can make the yeast cells as susceptible as the haploids (30). This haploid susceptibility is attributed to the absence of a second genomic copy from which cellular DNA repair mechanisms can accurately redact damaged portions of the genome. The increased susceptibility of polyploids may result from the presence of extra genomes that proportionately increase the chance of incurring dominant lethal mutations (30). In metazoans, the analogous effect would be the increased likelihood that one or another dominant oncogenic mutation would occur. On this basis taken

alone, one might expect triploid and other polyploid metazoans, or polyploid cell types to be more susceptible to tumor initiation.

To this simple model based on genomic multiplicity in yeast has recently been added a report in *Science* (31), reviewed by Hieter and Griffiths (32) based on quantification of mRNA expression of all genes in *S. cerevisiae* using so-called "gene array" technology. This work shows that most yeast gene expression levels are unaffected by ploidy. However, a limited subset of genes in yeast is subject to substantial variation in expression with respect to ploidy. Just seven genes are proportionately repressed by a factor of 10-fold or more as ploidy increases from haploid up through tetraploid. These authors also found another subset of 10 genes to be induced by increasing degrees of ploidy. Such genome-wide expression profiling shows that complete understanding of the effects of ploidy on susceptibility to mutagens, at least in yeast, may well include factors beyond simple multiplicity of genomes. Presently, this genre of work cannot be conducted on ploidy-modulated metazoans. However, by extension and analogy, we may imagine that the effects of ploidy on carcinogenesis in metazoans might be ultimately explained not only by the presence of extra copies of tumor suppressor genes, but also by the modulation of the expression of certain genes analogous to those responsive to ploidy as are those now reported to be found in yeast.

Prior evidence of tumor suppression in triploid trout

The first report showing the effect of triploidy on carcinogenesis was published by Thorgaard *et al.* this past year (33). In those experiments, the triploid trout were found to exhibit markedly reduced tumor incidence following carcinogen initiation via a bath exposure of the trout at four months of age. Such tumor suppression together with results reported from the present work show that the yeast observations are not predictive of those seen in trout. Weight class analysis showed that the tumor suppressive effects of triploidy seen were apparently independent of the growth rates of the fish. The triploid tumor suppression occurred with each of the three carcinogens tested: aflatoxin B_1 (AFB_1), 7,12-dimethylbenz[*a*]anthracene (DMBA) and N-methyl-

N'-nitro-nitrosoguanidine (MNNG). The last result demonstrated that the suppression effect was independent of metabolic activation, since MNNG is highly mutagenic without such activation (34),(35).

Such a robust and consistent effect of ploidy in a representative vertebrate suggests that entirely different mechanisms may be involved than might have been inferred from the yeast. Such mechanisms, perhaps unique to metazoans, would likely be superimposed on, and possibly antagonistic to, the likely 50% increased exposure of proto-oncogenes to mutagenic processes that could result in higher levels of potentially dominant oncogenic mutations in triploids. In this regard, one salient difference between unicellular eukaryotes such as *Saccharomyces* and metazoans such as *Homo* and *Oncorhynchus* is the widespread presence of tumor suppressor genes (or anti-oncogenes) in the latter, and their absence or near absence in the former. At least 20 such suppressors have been characterized from humans, and there is good evidence for a substantially greater number (36). Unlike typical proto-oncogenes, which are normally recessive, tumor suppressors are normally dominant. Rather than gaining dominant function through selected sites of mutation, as most oncogenes do, the tumor suppressor genes, in most cases, lose function through mutation or deletion at any one of a larger number of sites within their coding sequence.

Theoretical effects of ploidy, a simplistic model

A simplistic, but useful, model of the potential effects of ploidy on the tumor suppressor genes and proto-oncogenes would be the following:

a) Increased genome copy number to 3n from the normal 2n increases the probability that a specific proto-oncogene may be activated by a factor of 1.5.

b) By contrast, inactivation of tumor suppressor genes with a normally dominant phenotype in a target cell would require deletion of function of each of the gene copies present.

Under certain assumptions*, a comparison of the probabilities of activation of an effective oncogene site by a single mutational event, versus that of complete

inactivation of a tumor suppressor site by such an event may be represented as follows:

	haploid	diploid	triploid
oncogene	$p_{ho} = x$	$p_{do} = 2x$	$p_{to} = 3x$
suppressor	$p_{hs} = y$	$p_{ds} = y^2$	$p_{ts} = y^3$

* These assumptions include:
1: activation/deactivation events are by single mutations
2: probability of such events is small
3: mutation probability and its repair are homogeneous with respect to base and site
4: mutations in different alleles of a gene are independent
5: mutation probabilities in all genes are independent

Thus, to a first order of approximation, one may expect that the effect of triploidy would be to diminish the probability of mutational abrogation of a given tumor suppressor gene's function by a factor of y^3/y^2 or simply y, the probability of mutating a single allele. On the other hand, the probability of mutation of a single allele, such as might activate a given proto-oncogene is increased by 50%, *i. e.* proportional to the presumed 50% increase in targets per cell. The assumption that y is a small number, far less than 1, accords well with previous estimates that have been empirically derived showing it to be substantially less than 10^{-6} even in an experimental carcinogenesis regime (37). y is of the same order of magnitude as x, but for any pair wise comparison of a given proto-oncogene and a tumor suppressor gene one would see that there are usually far more mutation paths to abrogating the function of a suppressor gene, than there are ways of activating a proto-oncogene. Furthermore, the ratio of effective potentially mutated sites per gene would surely vary in a comparison of any pair of cancer genes, regardless of their category. This model does not take into account the relative numbers of proto-oncogenes and tumor suppressor genes, nor the relative potency or efficacy of particular genes and their mutations in tumorigenesis. Since oncogenes have historically been much more readily identified than have suppressor genes, we cannot *ab initio* infer even an estimate of the relative numbers of the two types. Furthermore, we have little or no way to estimate their relative efficacy, taken in aggregate, toward enhancing tumor development. However,

this simple model shows how the observed robust tumor suppression in the triploid model can be predominantly due to the ability of factor *y* to offset the 50% greater exposure of proto-oncogenes by several fold. Such a model may assist us in generating a comparative assessment of the relative roles of these two categories of cancer genes. Such comparisons, allowing assessment of cumulated and interactive effects of large numbers of cancer-related genes under conditions of experimental tumorigenesis, would otherwise be technically and computationally unachievable.

Cancer Genes Examined in this Work

p53; a unique tumor suppressor

p53 is the single most frequently mutated gene observed in human tumors. In humans, the p53 protein has a diversity of signaling and control functions related to tumor surveillance and development. These include the monitoring of DNA damage, the detection of cellular redox status, and the integration of a variety of other signals related to tumor initiation, progression and metastasis. It further directly or indirectly participates in a variety of tumor suppressive activities. These include upregulation of DNA repair activities via transcriptional activation of GADD45 and SDI1 (38), and upregulation of apoptosis, for example via upregulation of the expression of Bax an antagonist member of the Bcl 2 family of genes (39). p53 has a diversity of other functions that are at least tangential to an antitumor role, but with a developmental theme, such as its ability to inhibit unscheduled homologous recombination (40), regulate mitotic spindle formation (41), regulate guanine nucleotide biosynthesis (42), and interact directly with the machinery of cell-cycle control (43). p53 acts as a transcription factor and upregulates a large number of genes containing duplicates 0 to 13 bases apart of the upstream p53-specific DNA binding domain: 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' (44). p53 acts to repress transcription of a large number of genes, including some TATA-box containing genes, that are repressed through p53's interaction with the TATA-binding protein (TBP) (45) and TATA-associated factors or TAFs (46). p53 also specifically down regulates the transcription of genes, including those transcribed by RNA polymerase II and RNA polymerase III

by repression of the promoters for those polymerases (47, 48). Recently (49), it has been shown that p53 may directly inhibit transcription by RNA polymerase I as well. The latter would have a particularly profound effect on cell proliferation by inhibiting general ribosomal RNA synthesis and thus down-regulating all protein synthesis.

Tumor suppressor p53, is so frequently found mutated in human tumors (50), that such mutations have become the subject of large accumulated databases. One such online database is maintained by Béroud and Soussi (51). The entries in this database are derived from peer-reviewed published reports of human p53 mutations confirmed by sequencing evidence, and currently number over 10,000. In this database (see Figure 1.1), it can be seen that nearly all cancer-associated mutations are seen at some 170 codons within the central region of the expressed portions of the gene coding for the central DNA-binding region of p53. The database shows that about 35% of p53 missense mutations are confined to just six codons, or mutational "hotspots" within this central region, codons 175, 245, 248, 249, 273 and 282. In liver tumors of all types, only codons 249 and 273 coincide with those six above.

Evolutionary conservation of the p53 gene

Another remarkable feature of p53 is the apparent universality of the gene in animals and the high degree of sequence conservation seen across classes and orders of metazoans. Figure 1.2 taken from an earlier publication of this laboratory (52) shows a multiple species alignment of the deduced amino acid sequences of p53 for 15 animals, including nine mammals, three fish and one invertebrate. The shaded boxes overlaying this alignment highlight the "conserved domains" originally identified by Caron de Fromentel *et al.* (9) as evolutionarily conserved in all known vertebrate p53 genes. These conserved motifs, known as the central DNA-binding region, code for portions of the p53 protein known to bind the aforementioned upstream p53-specific DNA binding domain crucial to the transcriptional transactivation functions of p53. These same regions also contain all of the mutational "hotspots" of p53 identified in human tumors. Orthologous p53 genes have now been identified in all three major branches of bilaterian metazoa. In addition to the p53 or a close homologue

Figure 1.1. Multi-species alignment of p53 peptide sequences. Adapted from Caron de Fromentel et al. (9) and from Cheng et al.(52), table assembled by Patricia. E. O'Neal of our laboratory, alignment using Clustal W and manual adjustments. Conserved "domains" I through V are emphasized by the shaded boxes.

Figure 1.1.

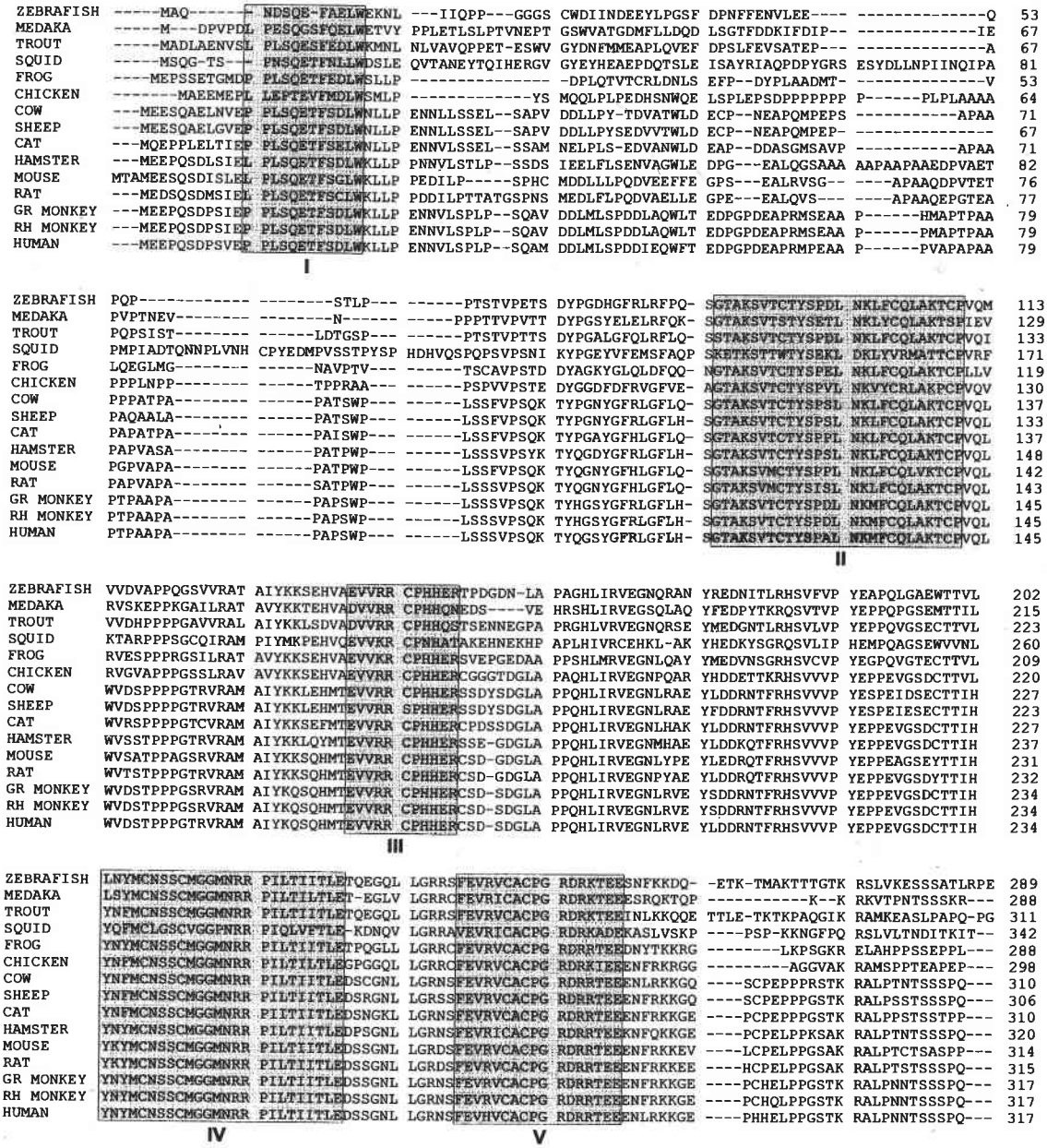
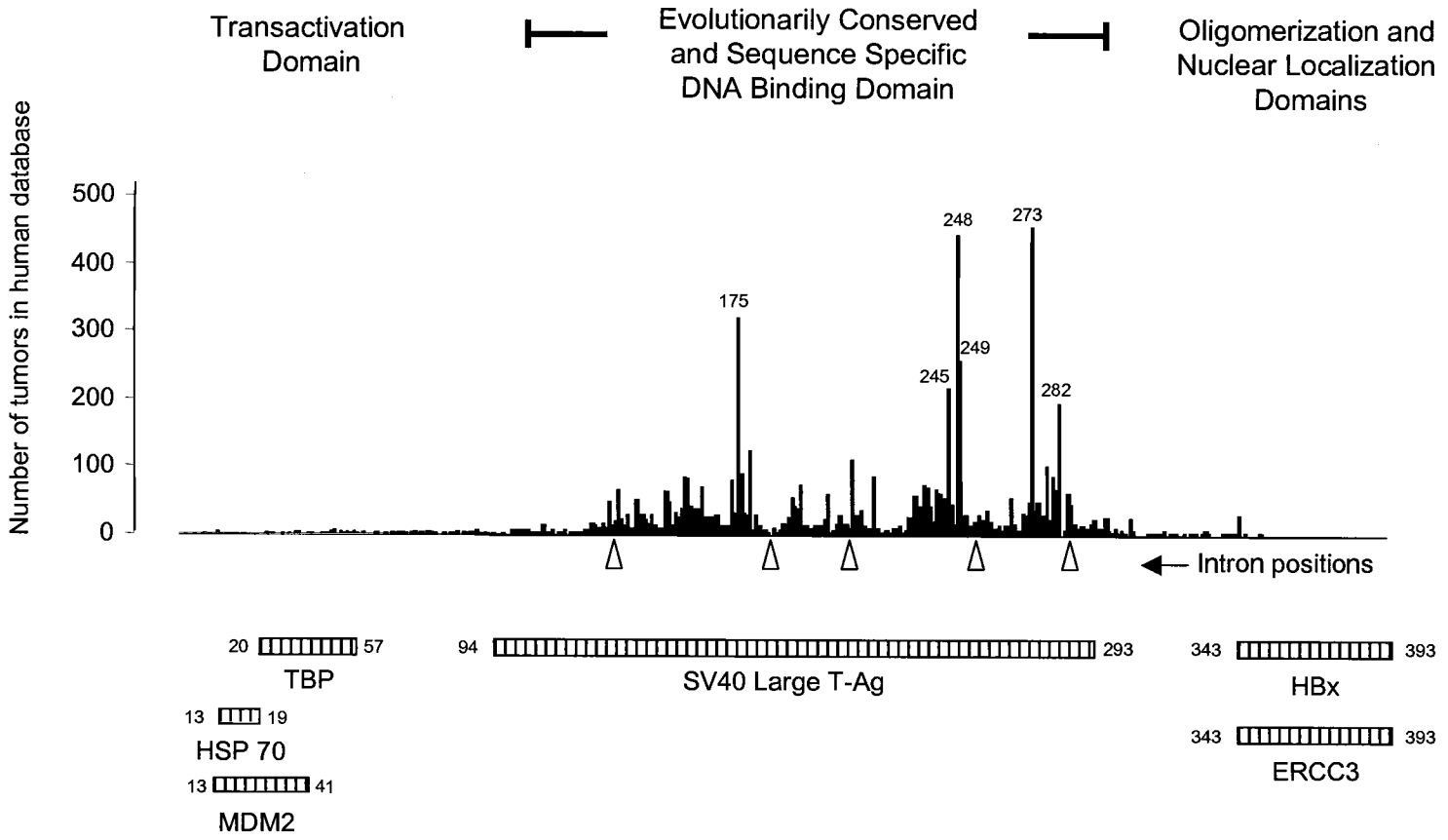

ZEBRAFISH
MEDAKA
TROUT
SQUID
FROG
CHICKEN
COW
SHEEP
CAT
HAMSTER
MOUSE
RAT
GR MONKEY
RH MONKEY
HUMAN
I
II
III
IV
V

Figure 1.2. p53 structure, function and human mutation spectrum. Adapted from (66) with human all-organ mutation spectrum from p53 database as of October 1998 (51). Hatched bands below the spectrum indicate approximate regions of the specified (TBP etc.) protein to p53 protein interaction.



characterized in molluscs (53, 54), an expressed gene with functional homology to p53 was identified several years ago in arthropods (55).

Recently a homologue of p53 has been characterized in *Drosophila melanogaster* (56) and has been shown to directly regulate the transcription of the proapoptotic gene *reaper* (57), thus showing that at least one important p53 function likely reaches back at least 600 million years to the proterostome/deuterostome divergence near the root of bilaterian metazoan evolution. However, p53 is absent in single-celled eukaryotes such as budding and fission yeasts (58) and no homologues are known in prokaryotes. p53 is only occasionally reported to be mutated in tumors of non-human mammalian species. The fact that mutational hotspots in human p53 are confined to the evolutionarily conserved regions (59) suggests that at least in naturally occurring tumors of non-human vertebrates one might expect substantial incidence of p53 mutations in tumors. In spite of this expectation, there are few reports of p53 mutations in tumors from wild animal populations. For fish, there has been only one such report in the peer-reviewed scientific literature: a liver tumor of a wild caught European flounder *Platichthys flesus* (60).

Possible importance of p53 gain-of-function mutations in triploids

p53 is distinct from all other known tumor suppressors in that it is claimed to have multiple sites thought to give rise to dominant oncogenic phenotypes through either dominant negative mechanisms (61) or true gain-of-function mutations (62). p53 dominant negative mechanism in some mutants is attributable to the formation of dysfunctional heterotetramers consisting of wildtype and mutant proteins. Such mutations in human cells include those altering the native arginine as follows: codon 175 $^{arg \rightarrow his}$, 248 $^{arg \rightarrow trp}$, 273 $^{arg \rightarrow his}$ or 285 $^{arg \rightarrow lys}$, as shown with human leukemia cells in SCID mice (63) and in SAOS-2 cells (64) in culture. These mutations are at p53 "hotspots," and correspond to some of the sites most frequently identified as mutated in human tumors (65,66). As mentioned above, it far less likely that mutation or allelic loss can abrogate all three alleles of a normally dominant gene such as a typical tumor suppressor. Further, a loss-of-function mutation of a single allele among three can be

can be expected to be somewhat less likely to generate a defective multimer of any normally dominant protein than it would as a single allele in a pair, thus tending to further protect the normal functions of such multimeric proteins as p53 in the triploid organisms. Thus, simple dominant negative mutations in p53 would not be expected to give altered frequency of tumors in triploid versus diploid animals. However, p53 is the only tumor suppressor reported to undergo true gain-of-function mutations. Such mutants could be sufficiently dominant that their effects may not be substantially diminished in the presence of two normal alleles of p53. Of particular interest for tumor suppression in triploids is the possibility that such p53 gain-of-function mutations may overwhelm even two normal doses of p53 suppressor function sufficiently to enable tumor development in triploids. Thus, the relatively few tumors seen in the triploid fish might be expected to exhibit increased likelihood of containing such dominant p53 mutations.

The human p53 tumor database (51) shows that the most frequently mutated sites in p53 are codons 175, 248 and 273 when all tumors, regardless of organ or system of origin, are counted. Among these general hotspots, there are particular amino acid substitutions that have proven or strongly suspected to have gain-of-function activity: codon 280 $^{arg\rightarrow thr}$ is "likely to be dominant in the heterozygous state," , and 273 $^{arg\rightarrow his}$ has "strong transactivating activity"(67). However, the mutations at 141 $^{thr\rightarrow tyr}$, 175 $^{arg\rightarrow his}$, and 248 $^{arg\rightarrow trp}$ when co-transfected with wild-type p53, each inhibited normal p53 transactivation and thus can be considered, in that assay at least, to be dominant negative mutations (68). Mutations at codons 248 $^{arg\rightarrow trp}$ and 273 $^{arg\rightarrow his}$ are so-called "contact mutants" that is those directly affecting the peptide or peptides actually contacting the p53 consensus binding domain of genes transcriptionally activated by p53. The majority of the other missense mutations are considered "structural mutants" that is those that alter amino acids that determine protein conformation but for which the coded peptides are not directly contacting DNA (69). The 175 $^{arg\rightarrow his}$ gain-of-function may be a consequence of its influence on p53 phosphorylation status (64).

Codon 249: a nearly exclusive liver p53 hotspot

In human liver, codon 249 $^{arg \rightarrow ser}$ alone accounts for 33% of the liver tumor p53 mutations, whereas the next most frequent liver site, codon 273, is the locus of just 5% of liver p53 mutations. There has been a great deal of effort to find animal models that reflect the same codon 249 prevalence seen in human tumors (70), (71), (72), but no animal model successfully reflects the apparent necessary and sufficient conditions of specific viral hepatitis and associated dietary aflatoxin exposure for development of human codon 249 mutations. If one discounts the approximately 80% of codon 249 mutation in the human p53 database that are known to be strongly associated with hepatitis and aflatoxin exposure, then the remaining codon 249 involvement still makes it a "hotspot" for liver but not a hotspot when scored against all p53 mutations in all tumor types and tissues. The epidemiology appears to suggest that 249 is not a strong hotspot in any population where there is only aflatoxin exposure or where there is only high incidence of hepatitis types B or C infection as measured by serum antibodies to these viruses. Codon 249 third base G→ T which gives a serine for the wildtype arginine, has been proposed as a biomarker of high exposure to dietary aflatoxins and to one or both of these hepatitis viruses (73, 74), but the evidence has been criticized as incomplete, and the status of codon 249 as a biomarker judged "premature". Whether or not it may subsequently be shown to be a useful human biomarker, presently we can only expect to report what is seen at this codon in aflatoxin B_1-fed trout (Chapters 3 and 4 of this Dissertation).

p53 and human/trout comparative carcinogenesis

The evolutionary conservation of p53 suggests its potential utility for comparative carcinogenesis research. Further this conservation indicates that the mutation spectral information from human tumors may provide some guidance for detection of mutations in the p53 from tumors of other species. Our initial impetus for examining p53 in trout came from the prevalence of p53 mutations in human tumors and from the apparent orthology of human and trout p53. The operating hypotheses

justifying the examination of p53 in triploid trout reported in this Dissertation also stems directly from characteristic mutational spectrum of human tumor p53, such as the aforementioned codon 249 mutations, the presence of distinct mutation "hotspots" and the presence of specific gain-of function mutations. We have proceeded with the caveat in mind that human and trout may not exhibit homologous p53 function, and that they are unlikely to exhibit identical mutagenesis and carcinogenesis processes. But, we have confidence that this approach would at least have heuristic value for comparative investigation of trout tumorigenesis. The human p53 database and other published quantitative information about human p53 incidence and organ-specificity, as well as the spectrum of mutations seen, are utilized in Chapter 4 of this Dissertation to guide our investigation of trout p53.

Structure and relevant novel functional aspects of p53

p53 is normally considered a tumor suppressor gene. Knudson's Rule (82) would predict that an oncogenic phenotype would ensue only if both alleles were inactive—that is oncogenic mutations would be recessive and loss-of-function. However, it is quite evident that p53 oncogenic mutations, at least, are very unlike ras in that they consist of quite specific amino acid substitutions. It is likely that p53 has this property because it is poised to integrate a variety of cellular signals, many of which transmit states of abnormal cell function. p53 has a negative effect on transcription of many genes, some of which is mediated through its association with the aforementioned TBP. However, p53 also enhances the transcription of a substantial number of genes carrying an upstream p53 consensus binding sequence. Many, if not all, of these positively p53-regulated genes encode proteins whose functions are important to blocking cell growth in one way or another. The most oncogenic mutations of p53 can be deduced from the published (51) mutational spectra of p53 that have been accumulated by tabulation of sequencings of this gene in thousands of human tumors. The incidence of the various mutations varies widely, affirming that there are indeed "hotspots" and that these differences very likely reflect on underlying mechanism(s) of oncogenicity conferred by the mutations.

p53 oligomerization; functional implications with respect to ploidy

In Chapter 4 of this Dissertation we conduct an informal analysis of the theoretical effects of triploidy on the relative number of defective p53 tetramers that would be expected if a single allele were mutated. On simple probabilities triploidy would make assembly of a tetramer containing a mutant monomer less likely by several fold, thus presumably increasing the likelihood that p53 would retain its normal anti-oncogenic phenotype in hypothetical but likely triploid mutants with the resulting 2:1 normal to mutant allelism, and hence make such mutants *less* likely to appear in tumors. However, true gain-of-function mutants, if they exist, may be expected to show oncogenic phenotype in the presence of even two normal p53 alleles, and in fact some oncogenic functions of mutant p53 are attributed to monomeric or dimeric forms. This quality should by definition be a hallmark of true gain-of-function mutations in a gene.

For one known p53 gain-of-function site, mutant codon $281^{asp \rightarrow gly}$, it was shown in cells transfected to express this p53 mutant and selected specific p53 deletions into nude mice, that some motif within the region of amino acids 393-327 was essential for at least the 281^{gly} gain-of-function (85). This suggests that for at least this hotspot there is oligomerization involvement since this region near the c-terminus defines the "oligomerization domain" (36). However, there is substantial evidence (86) that at least eight p53 mutations found in human tumors confer a true gain-of-function phenotype in a yeast assay. Interestingly, only one of these mutations corresponds to the strong human mutational hotspot, codon $273^{arg \rightarrow cys}$, an identical codon in human and trout. One should be cautious in over interpreting this result since the absence of other human hotspots seen in this assay may represent methodological or systematic bias. For example, there may have been inherent biases against potentially dominant mutations either in the experimental mutagenesis system used, or from an incompatibility of such mutants with yeast itself, which is sensitive to overexpression of exogenous normal p53 (87). Another potential explanation for gain-of-function behavior would rest on certain normal cellular functions regulated by p53 that may be capable of cooperating in oncogenesis (88). Such functions might then be

found to be coded by genes that do not possess the sequence-specific p53-binding domain, but would gain function by losing the normal negative regulation of p53 in some manner. An example may be seen in work by Ludes-Meyers *et al.* (89) in which constructs containing 4 of the human "hotspot" mutations: 175 $^{arg\rightarrow his}$, 248 $^{arg\rightarrow try}$, 273 $^{arg\rightarrow his}$, 281 $^{asp\rightarrow gly}$ and one "warmspot" mutation 143 $^{val\rightarrow arg}$, were each seen to efficiently transactivate a minimal EGFR promoter that does not contain the wildtype p53 consensus binding domain and which does not respond to wildtype p53.

Perhaps some of the answer to questions of p53 gain-of-function may actually be found in the widespread loss-of-function aspects of certain p53 mutations. Since wildtype p53 represses transcription of TATA-box regulated genes, through its binding of the TBP it may be that loss-of-normal function simply translates into gain in transcriptional efficiency for this large category of genes. Such genes include the nuclear oncogenes *Fos, Jun* and *Myb*b, DNA polymerase α, and PCNA and many others (36). Even a modest enhancement of transcriptional efficiency across the large number of such genes not associated with an upstream p53 consensus-binding domain, may have a tumor enhancing effect. If this were the case then it would suggest that the dominant negative mutations may well be rarer than the true gain-of-function mutations—that in fact the broad spectrum of p53 oncogenic mutations represents, by and large, an array of changes that are variably effective at dysregulating or unregulating a large number of growth-responsive or growth-enhancing genes. Only a few of these may actually disrupt the normal oligomerization of p53, these would be the dominant negatives. Perhaps those p53 mutations that exhibit both features are among the "hotspots".

Genetic aspects of p53

Mutations at putative dominant sites should require only one allele to give an oncogenic phenotype, whereas those at the putative loss-of-function sites would be recessive and hence would have to occur in both alleles. However, if one assumes that all of the sites exclusive of the six hotspots represent loss-of-function mutations, and that the hotspots themselves are dominant oncogenic sites, then the ratio of the number

of tumors from hotspots to those in the other categories ("coldspots" and "warmspots") is seen from Hollstein *et al.* (90) and from other data (51) to be between 3 to 1 and 20 to 1. Such ratios suggest that one or more mechanisms exist whereby single allele p53 mutations outside the hotspots can cause the loss of the second allele or conversion of the second allele to an identical mutation. One such mechanism that might explain the relatively high levels of mutations at these presumed loss-of-function sites would be from occasional "gene conversions" (91), that is mitotic recombination events that make both parental genomes homoallelic across particular loci. Such events are known to occur at a low but readily detectable level in all metazoans that have been examined.

A known normal function of p53 that is dissociated from its sequence-specific transactivation functions, is in inhibiting homologous recombination (40, 92, 93), thus it might be expected that loss or alteration of one allele itself could substantially increase the likelihood of loss of the second. Or put another way, the mutant proteins translated from transcripts containing even the less common p53 loss-of- function mutations could marginally increase the likelihood of mitotic crossover events and thus generate p53 homoallelic gene conversions or increase the likelihood of unrepaired functional loss of the second allele ultimately yielding what appears to be a dominant negative phenotype. There is evidence that at least 40 codons within human p53 have varying but measurable dominant negative activity (94), as measured in a transgenic p53 in yeast model expressing a spectrum of heterozygous mutant/normal human p53 DNA constructs. While Brachmann *et al.* (94) do not further functionally characterize these mutations, there are several well-characterized functions of p53, any of which alone or in concert might be expected to lead to greater likelihood of further mutation, including its functions in integrating and/or transducing signals of DNA damage, upregulating DNA repair, or regulating apoptosis of damaged cells.

It is important also to review positive evidence that at least one or another likely mutation of the "hotspots" of human p53 are clearly not "gain-of-function". One example of this is found in Chen *et al.* (95), where constructs containing wildtype and a selected p53 mutant ($279^{arg \rightarrow his}$) were transfected into p53-null Saos-2 cells that

were then assessed for neoplastic potential. These results showed clearly that the wildtype p53 was dominant with respect to this mutation. Nevertheless, this mutant has been identified as one with dominant function *in vivo*, with the ultimate phenotype being genomic instability (96).

Harris and Hollstein (97) make it clear that the character of p53 mutation spectra are quite novel amongst tumor suppressor genes. They report that about 80% of p53 mutations are missense. This is far higher than a typical tumor suppressor, for example, the comparable figure for the APC gene is just 9%. This further underscores the preference for p53 mutations that alter function rather than abolish it—another evidence for gain-of function or dominant negative mutants. Ford (98) shows clearly that at least for certain important mutations of p53, there is a definite gene dosage effect. That is, for cells in culture, the mutant homozygote is less efficient at DNA repair than the wildtype/mutant heterozygote that is in turn less efficient at repair than is the wildtype homozygote. p53 itself has a central role in maintaining genomic integrity, via regulation of apoptosis, and via recognition of DNA damage and upregulation of DNA repair and inhibition of untoward homologous recombination (99). So again it may not surprise us that certain first hits in p53 can have a high probability of leading to second hits in the same cellular genome, although the probability of such a subsequent mutation directly affecting precisely the other allele of the same genetic locus is admittedly much smaller.

Loeb (100) and (101) convincingly argues on epidemiological, mathematical and other grounds that there must be such positive feedback loops involved in human cancer, although in this case the subsequent mutations will typically occur in other genes. Loeb calls these predisposing mutations "mutator phenotypes". Without such predisposing events, Loeb argues, there would be a statistically inadequate chance to accumulate necessary multiple hits within any single cell lineage required for the transformation to malignancy to accord with the known data on human cancer incidence in light of the rates of accumulated unrepaired mutations.

If such positive feedback anomalies govern human cancer development, it would be likely to similarly affect trout tumorigenesis. Whether the gene dosage

effects operative in triploid trout could play a role in triploid tumor suppression underlies questions posed by the research reported in this dissertation.

Ras functional description

Ras proteins act as transient switches early in cellular signal transduction cascades (75, 76). Ras proteins are GTPases in which the "on" state is that in which the ras protein has bound a GTP molecule. The inherent GTPase activity of ras normally catalyzes rapid hydrolysis of GTP to GDP + P_i . The resulting GDP-bound ras is effectively "off" with respect to signal transduction. Ras is proximally downstream of membrane-associated cell surface receptors. Such cell surface receptors include the insulin, PDGF, EGF, FGF and bombesin receptors. All of these ras-associated receptors have growth-related functions, and each operates by activating intrinsic tyrosine kinase moieties in the cytoplasmic domain of the receptor. The receptors typically are dimeric in the absence of ligand and become tetrameric in the presence of ligand. The tetrameric conformation of growth factor receptors brings their cytoplasmic domains into a state that enables the specific tyrosyl residues to be phosphorylated. The autophosphorylated receptor/ligand complex can then directly activate the next stage of transducers, again via phosphorylation. Such transducers include GRB2 then sos1 or 2 in the case of PDGF. GRB2 has Src homology domains, SH2 and SH3 acting respectively as two poles of an adaptor molecule (77). These domains effectively transmit the growth factor receptor tyrosine phosphorylation status via phosphorylation to guanine nucleotide releasing proteins (GNRPs) such as sos 1 and 2. The GNRPs in turn cause ras to release bound GDP and to take up GTP thus placing the ras protein in the active or "on" state for initiating the rest of the signal transduction cascade. The active ras in turn activates a cascade of serine/threonine kinases, collectively referred to as MAP-kinases for "mitogenesis activated protein kinase". Often the first of these is itself a ras homologue-- "raf" or MAPKKK (MAP-kinase kinase kinase) (78). raf kinase then phosphorylates and thereby activates a MAPKK such as mek, which in turn phosphorylates and activates a final MAP-kinase such as erk. This final MAP kinase in turn acts to phosphorylate

certain nuclear proteins such as elk1 or sap1 and thereby activate them to in turn phosphorylate and activate transcription factors such as c-fos or c-jun in the case of oncogenic ras activation. Essentially the same signaling pathway has been characterized in diverse mammals, the nematode *Caenorhabditis elegans*, the fruitfly *Drosophila melanogaster* and in the fission yeast *Schizosaccharomyces pombe*. Ras functions consistently to transduce signals regulating growth and development in these diverse phyla. The strong conservation of ras signaling pathways is accompanied by a wide diversity of secondary ras effectors.

The above description reflects but one of several ras signaling cascades. Ras is also known to directly activate other signaling pathways, for example phosphatidylinositol-3-OH kinase (PI-3K) (79). Ras activation also potentiates apoptotic responses (80). This fact alone may explain some observations of the loss of ras in advanced tumors. Advanced tumors typically have a high level of genomic instability, which can signal GADD45-mediated apoptosis via p53.

Novel aspects of ras in human tumor mutation spectra

As with p53, ras possess genetic features that may place it outside conventional categorizations. Ras is ostensibly an oncogene and thus would be expected by Knudson's rule (81, 82) to be dominant, *i.e.* Ras proteins would become oncogenic through gain-of-function mutations. However, the codon 12, 13 and 61 mutations that are the nearly exclusively oncogenic ones, happen to be exceedingly permissive as to which amino acids may be substituted. For example at the normal codon12 ^{gly} virtually any amino acid except proline will result in activated ras. The situation is similar in codon 61^{gln} where only glutamate and proline are without activating effect. This permissiveness points rather to a very particular loss-of-function. These sites are intimately involved in the GTP hydrolysis function of ras, by virtue of their interaction with GAP (*G*TPase *A*ctivating *P*rotein) and almost any amino acid substitution will effectively inhibit GTP hydrolysis, thus rendering the ras in a constitutively "on" state. At the cell signaling level, this loss of native genetic

coding function results in a phenotypic gain-of-function of continuous growth stimulation (83, 84).

Outline of Relevant Methods and Applicable Technologies

Threshold of mutation detection

Advanced neoplasias are quite likely to be genetically unstable. They are often aneuploid and manifest defects in DNA repair. Additionally, neoplasias often consist of apposed tissue types and diverse cellular lineages. The genetic heterogeneity of advanced neoplastic tissues may effectively dilute those tumor suppressor or oncogene mutations involved in the initiation of the neoplasm. Detection of mutations in cancer-related genes can present the researcher with important questions: 1) what is the minimum content of mutations that may be causally related to the origin and/or development of the neoplasm? In addition: 2) what is the minimum content of a tumor-associated mutation that can be detected in a background of normal DNA sequence or sequence with non tumor-related mutations? In our work there has been considerable attention given to the second question, especially in view of the unexpected and marked absence of mutations seen in the p53 gene. The question is addressed in detail in Chapter 4 of this Dissertation.

There are a considerable number of methods available to assess the presence of mutations in neoplastic tissues. The detection of mutations in tumors is distinct from that of simply finding a polymorphism in a known gene. The latter is generally only a matter of detection at the 50% level since a diploid organism will have two normal alleles at this locus. For triploids the problem is only slightly more demanding, since the detection threshold for a minority allele must be greater than 33%. The demands on the detection technologies can be much more challenging when looking for mutational changes within genetic material from tumors. Advanced tumors are generally genetically heterogeneous. They consist of a variety of cells, only a variable fraction of which stem from the initial oncogenic lineage. They may contain

lineages that may be included within the apparent bounds of the tumor through the invasiveness of transformed cell lineages. Invasiveness may be reciprocal, for example, tissue macrophages can be attracted to tumors, especially if extensive apoptosis or necrosis is present.

Methods available for mutation detection

Methods that have been successfully used to detect mutations/polymorphisms in oncogenes and tumor suppressor genes include;

(Methods we have at least attempted to use):

1. SSCP (single-stranded conformation polymorphism)
2. direct sequencing
3. cloned sequencing
4. allele-specific PCR (primer mismatch assays)
5. ddF (dideoxy fingerprinting)
6. heteroduplex mobility shifts
7. chemical mismatch cleavage
8. SnuPE (single nucleotide primer extension)

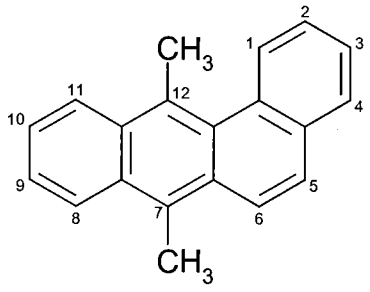
(And methods we have not attempted):

9. DGGE (denaturing gradient gel electrophoresis)
10. TGGE (temperature gradient gel electrophoresis)
11. functional assays in modified yeast system (86)
12. enzymatic mismatch cleavage
13. ligase-mediated PCR
14. single-stranded primer extension
15. "molecular beacons" realtime quantitative PCR
16. "Taqman" (102) realtime quantitative PCR

We presently feel that sequencing is a conservative and effective approach that can work well if the mutant cells are numerous enough in the tumor to reach a minimal threshold of detection. Site-specific assays (such as allele-specific PCR) can be rapid and simple but they require that one knows precisely where in the sequence to look. Unfortunately, using human p53 as an example, there are perhaps 6 or so "hotspots" and dozens of "warmspots" so one may not ever be in good position to do some of the site-specific assays effectively since each potentially mutated site requires as many as four site-specific primers. Combining the two approaches is a good strategy is many cases: by first sequencing one may then identify a (limited) spectrum of "hotspots", this could then allow the use of a simpler and quicker method, such as allele-specific PCR to identify the mutants in a large number of tumor samples, as an example.

Brief Reviews of Mutagens Used in this Work

7,12-Dimethylbenz[a]*anthracene (DMBA)*



DMBA was selected because earlier embryonic exposures in triploids utilized this carcinogen and because it reliably gives rise to multi-organ tumorigenesis in trout. Work by Harttig *et al.* (103) has shown that waterborne DMBA exposure of trout fry leads to greater stomach tumor incidence than liver (94% incidence in stomach at 100 ppm exposure). This result is similar to that seen in some comparable placental rodent DMBA exposures. DMBA was formerly thought to be the most potent carcinogen amongst the polycyclic aromatic hydrocarbons (PAH) (104), until the relatively recent

recognition that dibenzo[*a,l*]pyrene (DBP) holds this distinction. Unlike DBP, DMBA is a wholly synthetic carcinogen, not appearing in any known natural context. Perhaps because it is synthetic, there is less literature attempting to explain the exact mechanisms underlying its carcinogenic potential, than exists for some other polycyclic aromatics such as benzo[*a*]pyrene. This is unfortunate since mechanistic understanding of carcinogenesis is important regardless of the improbability of human exposure. To the present there are certain issues about DMBA metabolic activation that remain without satisfactory elucidation. Here we briefly outline features of coexisting theories of DMBA metabolism with respect to carcinogenesis.

Alternate pathways to carcinogenic activation, that is to an electrophile capable of covalent binding to DNA, have been characterized as one-electron and two-electron processes respectively. The former may be called "radical mediated" and the latter might be called "mono-oxygenase" mediated. However, it should be made clear that both pathways are well known as activators and that both can act sequentially on the same substrate. Under one reported metabolic scenario in rat liver (105), the phase I metabolism of DMBA proceeds initially to oxidize either one of the two methyls to a primary hydroxyl, which can then be directly phase II conjugated, or further oxidized to the carboxylic acid. The other methyl may then be oxidized and conjugated or further oxidized in a similar sequence to ultimately produce the dicarboxylate, which is also readily susceptible to phase II conjugation, for example by sulfate or glucuronide, and subsequent excretion via kidney or bile (105). In trout, DMBA may be metabolized in a similar manner (106). In models of DMBA bioactivation such as mouse skin and rat mammary gland, there are at least two potential routes to ultimate carcinogens. Cavalieri and co-workers (107) believe that cleavage of a sulfate from a methoxy can lead directly to formation of an electrophilic carbocation capable of DNA adduction. However, there are apparently other paths of intermediary oxidation present to give rise to other presumptive ultimate carcinogens such as 7,12-dimethylbenz[*a*]anthracene 3,4-diolepoxide.

In the human mammary cell culture line, MCF-7, it has been found that the primary DMBA adducts are to adenines. In trout, there appears to be a preference for

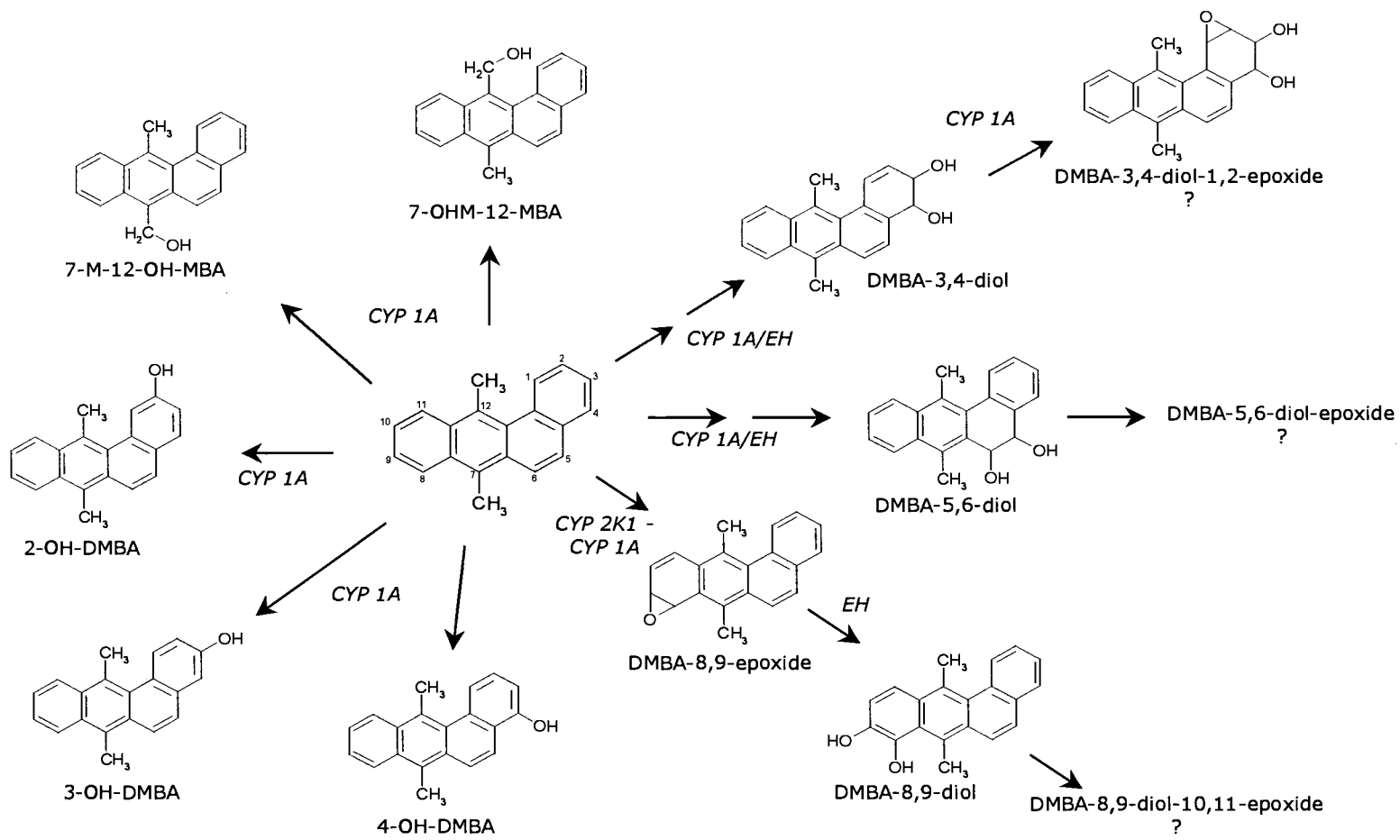
guanine adduction (108), in some contrast with the rat that is reported to have several times higher adduction to adenine versus guanine (109). This difference may be due to fact that trout make little or no use of the 3,4-dihydrodiol path to DMBA activation as seen in humans and rodents (110) (Figure 1.3).

In mammals, M. N. Gould's model would outline the activation of DMBA as follows: DMBA → DMBA epoxide → DMBA diol → DMBA diol epoxide (the ultimate carcinogen). Predominant adduct formation, identifiable through ^{32}P postlabeling, is the N7 of guanine and adenine bases. Activation through this path is supported by DNA binding studies utilizing labeled 3,4 epoxide, but activation via hydroxylation at a methyl group has not been ruled out.

While cytochrome P450 IA1, now known as CYP1A1, appears to produce the 3,4 dihydrodiol, the ultimate carcinogen in mice, it is not the only cytochrome P450 capable of doing so. Buters et al. (111) showed that embryonic fibroblasts were dependent on CYP1B1 to generate ultimate carcinogens from DMBA. It is noteworthy that P450s can also catalyze one-electron oxidations.

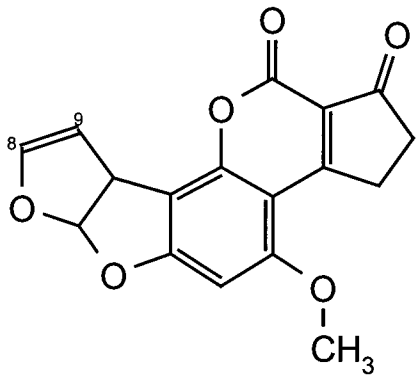
DMBA itself, without activation, may have promotional and/or epi-genotoxic effects. It has a high affinity for the Ah receptor and thereby induces pleiotropic gene expression regulated by that receptor in its agonist-liganded state. It also has a high potency of inhibition of the binding of epidermal growth factor (EGF) to 10T1/2 cells. Another feature that may be novel to DMBA amongst PAHs has been investigated by Matsuoka et al. (112, 113). These researchers have reported that both DMBA and benzo[*a*]pyrene (BP) can induce aneuploidy and polyploidy directly in a cell line in the absence of S9 microsomal activation. They showed that DMBA was by far the more potent aneugen of the two. Their work suggests that these PAHs can directly interact with the mitotic spindle apparatus or with key regulatory elements controlling spindle formation. Such a result might at first appear puzzling since DMBA, like many other PAHs, is quite insoluble in water. However, the physical chemistry of DMBA shows a property that may explain some of these puzzling interactions with

Figure1.3. Primary routes of DMBA biotransformation in trout liver.
From: Miranda, C.L. et al. (110)



chromatin: it can be solubilized in water by association with purines and their nucleosides, such as adenosine and guanosine (114, 115). This affinity for purines may increase association of this PAH with DNA as well as increase its effective concentration in a chromatin context. Furthermore, such affinity may lead to DNA strand separation and thence to greater vulnerability to strand invasion and crossing-over, as well as increasing vulnerability to adductive mutagenesis (113, 116).

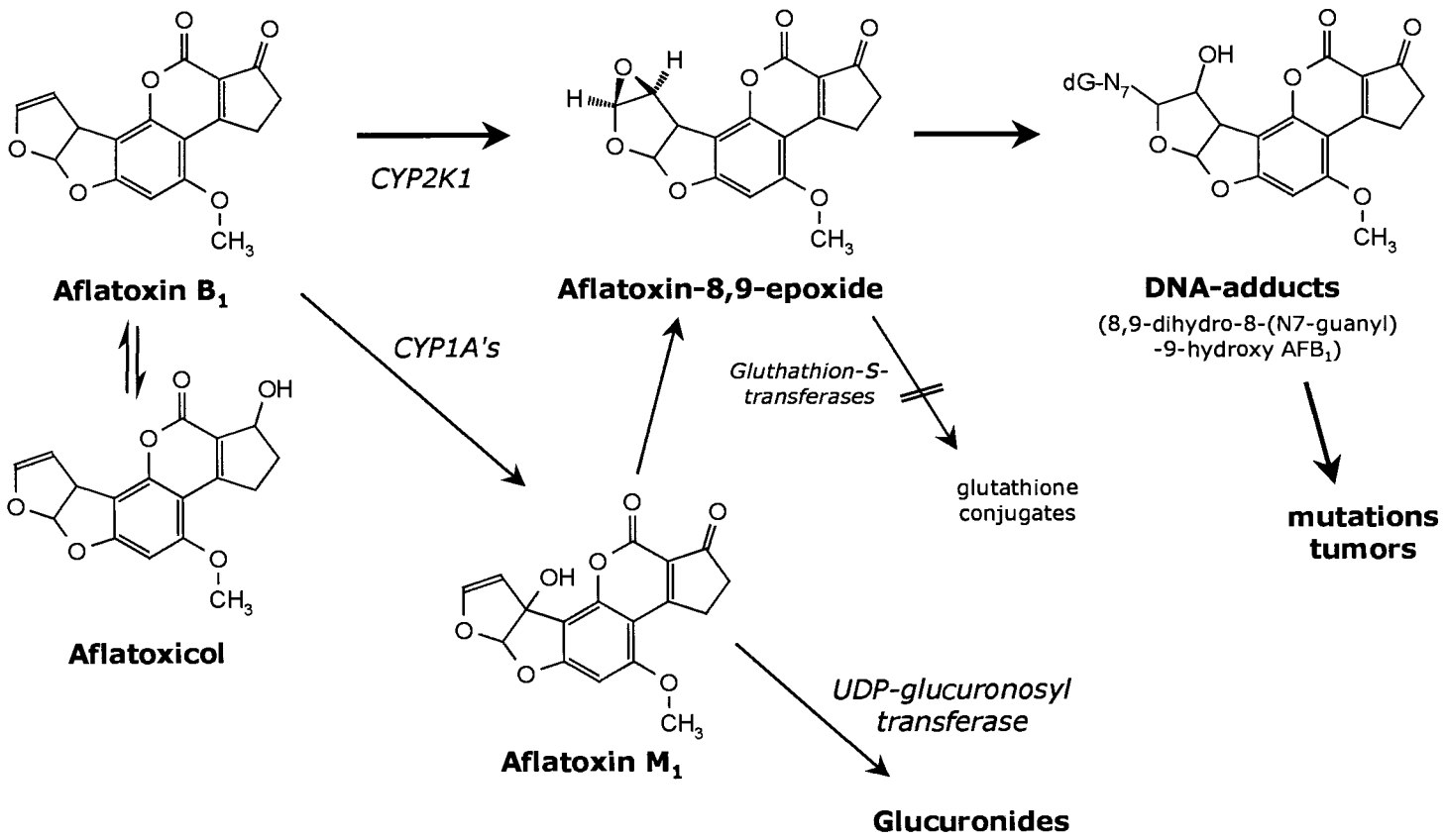
Aflatoxin B_1 (AFB_1):



AFB_1 is a fungal mycotoxin produced by *Aspergillus* and *Penicillium* species, especially *A. flavus, A. parasiticus, A. nominus* and *P. puberulum* (117) via the polyketide synthetic pathway beginning with norsolorinic acid through numerous intermediates and ultimately through sterigmatocystin (118). Aflatoxins are chemically classed as furanocoumarins, a class that includes other toxins such as the psoralens, however a second furan ring further distinguishes aflatoxin as a bis-furanocoumarin. Aflatoxin B_1 is the most potent promutagen of the dozen or so aflatoxins that have been well studied. It is a known hepatocarcinogen for many species, and has been epidemiologically implicated, in conjunction with hepatitis viral infections, in elevated incidence of human liver tumors. AFB_1 has been judged by the International Agency for Research on Cancer (IARC) as showing sufficient evidence to be categorized as a class 1 human carcinogen (119). The predominant and rather novel mutation in such tumors is a codon 249 G to T transversion in the p53 tumor suppressor gene (120). Trout are very sensitive to aflatoxin in that dietary exposures in the lower parts per billion range result in high and dose-dependent liver tumor

incidences (121, 122). Aflatoxins are typically detoxified by phase II conjugation with glutathione, glucuronate, sulfate, amino acids, or bile salts. However, even the phase I hydroxylation at the 8,9 double bond in the terminal furan via cytochrome p450 (for example, via CYP 2B1 or CYP 2C11 in humans and via CYP 1A1 in trout) is effectively detoxifying by preventing further epoxidation across the 8,9 bond. Another phase I detoxication is aflatoxin M1, the product of AFB_1-hydroxylation by CYP 1As at the C10 shared by the terminal furans. AFM_1 has only about 10% of the tumorigenic potential of AFB_1 in trout (123). The CYP 2K1 is a route to the generation of the resulting aflatoxin B_1 *exo*-8,9-epoxide appears to be the ultimate adducting species that typically attacks the N7 position of guanine (Figure 1.4). In both humans and trout, such adducts typically result in an apurinic site that is most often repaired to give the G to T transversion (124). The 8,9 *exo*-epoxide can also adduct amino acids with ϵ-amino moieties such as that of lysine, present in tissues and serum albumin, thus providing for convenient bioassays of time-integrated animal or human exposures to this mycotoxin (125).

Figure 1.4. Aflatoxin B_1 metabolism in rainbow trout



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Chapter 2

Tumor Suppression in Triploid Rainbow Trout

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Abstract

Previous work (Thorgaard, G. H. *et al.*, *Aquatic Toxicology* 46:121-126, 1999) showed that triploid rainbow trout given embryonic carcinogen bath exposures had substantial reduction of induced tumors relative to diploids. In the present study, rainbow trout (*Oncorhynchus mykiss*) were made triploid by brief thermal shock shortly after fertilization, as in the earlier work. Beginning at 5 months after fertilization they were given dietary carcinogen: either aflatoxin B1 (AFB_1) for 30 or 120 days or 7,12-dimethylbenz[*a*]anthracene (DMBA) for 30 or 120 days. The dietary exposures were at levels known to be tumorigenic (100, 200 and 300 ppb AFB_1; 250, 500 and 850 ppm DMBA) but below levels known to cause elevated mortality. At approximately 16 months after fertilization, the fish were sacrificed and tumor incidence and multiplicity were assessed. At all levels of carcinogen and in all organs (liver for AFB_1; liver, stomach, swim bladder and kidney for DMBA) tumor incidence was lower in the triploid fish. For DMBA fed fish it was seen that the diploid:triploid incidence ratios ranged from 2.0 to 9.0 and for AFB_1 from 3.1 to 6.0. For both carcinogens in liver and stomach at all doses and durations, each had a diploid:triploid log odds ratio (LOR) substantially greater than one. The mean LOR forAFB_1 livers was 2.5; mean LOR for DMBA livers 1.80; stomachs 3.9; and swim bladders 1.9. The mean weight of the triploid fish in every dose and duration was less than that of the diploids. Weight class analyses were conducted to dissociate the likely tumor suppressive effects of retarded growth from the effects of triploidy *per se*. Quintile weight classes plotted against logit tumor incidence in most dose and duration regimes showed the positive slopes s of both diploid and triploid logistic curves. In every case, the triploid curve was lower than the diploid curve, indicating a robust tumor suppressive effect of triploidy independent of that attributable to triploid growth retardation. Mean LORs after nullifying weight related tumor incidence showed a reduction of 47 ±2.6 logit % in AFB_1-fed triploid livers; similarly a 19.5 ±5 logit % reduction in DMBA-fed triploid livers; and a 65 ±4.2% logit % reduction in DMBA-fed stomach tumor incidence.

Introduction

Thorgaard *et al.*(1) recently reported consistent reduction in tumor incidence in carcinogen-treated triploid trout relative to diploids. This tumor suppression was seen across the three carcinogens tested, and across all organ systems in which experimental trout tumors are usually observed. These observations constitute a substantial body of evidence of an important effect in a viable model of experimental cancer and cancer chemoprevention and modulation (2). We sought to determine if these observations could be generalized to dietary exposures. We also sought to gain some understanding of the mechanistic underpinnings of triploid suppression. At the least it is curious that such a simple modification as adding a single genome copy to the cells of a vertebrate leads to robust reduction in tumor incidence. A further understanding of this triploid tumor suppressive effect may have profound implications for our understanding of the mechanisms common to all vertebrates by which neoplasias are normally prevented, remitted or otherwise kept in check.

In Thorgaard's work, experimental fish bathed in N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) for 1 hour and then examined 9 months later showed consistent and significant tumor reduction in triploids relative to diploids, as did those triploids receiving AFB_1 and DMBA treatments. Unlike AFB_1 and DMBA, MNNG requires no specific metabolic activation to form DNA-adductive species. This result, together with the consistency of tumor suppression across carcinogen, dose and modality when the present study is included, suggests that metabolic differences between the two ploidies are unlikely to be a substantial factor in any explanation of the triploid tumor suppression. The most evident difference, the presence of the extra genome itself, is an obvious place to begin seeking an explanation.

Experimental animal triploidization is currently confined to a diverse array of teleosts including zebrafish (*Danio rerio*) and the rainbow trout (*Oncorhynchus mykiss*). In addition, there are reports of triploidization of a number of other metazoans, including sharks (3), oysters and mussels (4,5). Many species of vertebrates and invertebrates are naturally triploid under certain conditions: triploid

chickens have been reported and some parthenogenic free-living lizards (6) are naturally triploid. Eutherian mammals including humans are likely to be indefinitely beyond the reach of experimentation with respect to triploidy. Triploid mammalian fetuses have low viability. Those few that are not spontaneously aborted have very high perinatal mortality, and those that survive delivery die soon after of numerous developmental abnormalities. Thus the trout model, already well-characterized with respect to carcinogenesis, provides a viable and relatively low cost means to investigate issues of tumor biochemistry and molecular biology, and their relationship to triploidy.

A number of experimental methods are available for producing triploidy. Such methods typically apply some form of shock to a newly fertilized egg. For our work we selected the method used by Thorgaard (7), consisting of a brief hyperthermic shock shortly after fertilization, both for reasons of continuity with that earlier work and because this method is the simplest, one of the safest and least expensive. Hyperthermia and all of the other triploidization methods induce the disruption of normal spindle formation and cytokinesis during meiosis II, which then prevents the normal extrusion of the second polar body, a sequestered haploid genome itself a result of meiosis I (8, 9). A consequence of this is the addition of the haploid genome (1n) of the retained polar body to normal zygotic diploid (2n) set to give a triploid genome (3n).

In theThorgaard *et al.*, (1) demonstration that triploidy reduces tumor incidence, it was also seen that the triploids were subject to reduced mean growth rate when compared with diploids. It is well known that growth rate and/or caloric intake within a species can strongly influence tumor response. Caloric restriction dietary regimes are among the most well studied phenomena in cancer prevention. Inverse correlations between caloric intake and both natural and experimental tumor incidence, are reported in a variety of tissue types and under a variety of tumor initiators and protocols in rats and mice. For one extensive review of this work that extends back over 90 years, see Pariza (10) and for 10 relevant articles see a special issue of Mutation Research published in 1993 (11). Human epidemiology strongly

suggests that the same robust relationship is present in humans. The effect has been described in fish but has not been extensively investigated (12). The fact that triploid trout tend to grow more slowly than diploids presented a potential confound to interpretation of the experimental results of triploid tumor suppression reported in Thorgaard *et al.* (1). This confound is largely nullified by a straightforward weight class analysis. They found triploid tumor suppression persists even if the triploids are only compared with diploids within the same weight class.

In the present work, we set out to confirm and potentially extend these recent results. Further, we wished to determine if the phenomenon of triploid suppression is seen in older animals under dietary exposure protocols of various doses and durations. We increased the numbers of experimental animals to give adequate data for a more comprehensive weight class analysis incorporating regression curve parallelism, for several organ systems in the case of DMBA and for liver tumors alone in the case of AFB_1. The larger numbers also provide sufficient tumor and normal tissue samples for molecular analysis of p53 and Ki-ras mutation spectra, and sufficient redundancy in sampling to address hypotheses relating to the potential involvement of tumor suppressor genes.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma (St. Louis, MO) unless specified otherwise. The carcinogens, DMBA and AFB_1, were purchased from Aldrich (Milwaukee, WI).

Animals

For each of the two studies, several monogamous spawnings containing at least 3000 eggs of the Shasta strain of *O. mykiss* were divided into two subsets consisting of approximately 40% and 60% of the fertilized eggs. The larger subsets were subjected to hyperthermic triploidization (13, 14) as follows: at 10 minutes after fertilization the

eggs were placed in a water bath at 29 °C for 10 more minutes, then they were placed in the incubators at the usual temperature of about 13 °C. The smaller of the subsets (normal diploids) were not shocked, but were placed directly in the 13 °C incubators. After the fish reached the feeding stage, sibling lots of diploid and triploid fish were selected to continue through the experiments.

Feeding and carcinogen exposure

The diploid and triploid rainbow trout were raised in the customary manner at the Food Toxicology and Nutrition Laboratory (FTNL), Oregon State University (Corvallis, OR) as previously described (15). All fish were raised and treated under guidelines and protocols approved by the Oregon State University Institutional Animal Care and Use Committee. The trout were raised to the age of about five months on Oregon Test Diet (OTD) (15) then, before carcinogen exposure, the triploidized fish and their normal siblings destined for DMBA diets were pooled together during exposure. They were assigned to their respective ploidy after sacrifice and necropsy using flow cytometry. Fish exposed to AFB_1 were segregated at triploidization and kept separate throughout the entire experiment and necropsy. Commencing at about 5 months after spawning, several days before beginning dietary carcinogen exposures, all fish were divided into subsets housed in separate tanks by their respective carcinogen dosage and duration. As much as practically possible the dose/duration subsets were represented by duplicate tanks, although limitations in resources prevented this in some cases. At the outset of the carcinogen exposures, the 770 fish of each ploidy in the DMBA study, and the 880 of each ploidy in the AFB_1 study received their respective carcinogen as incorporated into the lipid component of the OTD formulations. Dietary carcinogen concentration and duration of dietary exposures are indicated in the Results section tables.

Tumor sampling, pathology and storage

At 12 to 13 months after the cessation of dietary carcinogen exposure, successive small lots of the experimental fish were euthanized with an overdose of

tricaine methanesulfonate anesthetic. At necropsy the fish were individually weighed, dissected, and sexed, and the livers removed and weighed. Tumors in all tissues (liver in all fish, stomach, swim bladder, kidney in those fed DMBA diets) were individually assessed, counted and sized by an experienced fish pathologist (16). Customary fixation with Bouin's fluid for subsequent histopathology was limited to very few samples in this study, since such fixed tissues would have been refractory to subsequent molecular analysis. To maximize the availability of tumor tissues for subsequent protein and nucleic acid analysis, tumor and normal organ tissue samples from both the DMBA and AFB_1 experiments were rapidly frozen in liquid nitrogen and stored at -80 °C. In DMBA-exposed fish the stomach, swim bladder, and kidney were also carefully examined, tumors present were noted, and at least one tumor from each of those tissues bearing tumors was frozen and stored at -80 °C for later molecular analysis.

Statistical analyses

The weight class analysis was performed as first described in Harttig *et al.* (17). Some typographical errors in that description are removed from an abbreviated version quoted here: " Logistic regression analysis of the effect of ploidy on tumor incidence was performed using the GENMOD procedure of SAS for Windows, Version 6.10 (SAS Institute, Inc. Cary NC). The response data were modeled assuming a binomial distribution. The logit function was defined as the link function. Data showed little or no evidence of tank-dependent effects so data from duplicate tanks were pooled. The logit model used is described by: logit $(P) = \alpha + \beta * t$, where P = tumor response, t = ploidy variable (0 for diploid and 1 for triploid). α and β are the estimates for the response of different treatment groups. Similarly, the model used to estimate the influence of body weight on final tumor incidence is described by: *logit* $(P) = \alpha + \beta * t + \gamma * w$, where γ is the estimated response per gram of body weight, and w is the body weight at time of necropsy. Tests of interaction between ploidy and weight demonstrated that the fit to a parallel linear logistic model was satisfactory. Individual treatment groups were compared within a given model by using the

Contrast statement under GENMOD. Treatment effects on the multiplicity of tumors in individual fish were assessed by the non-parametric Wilcoxon test. Treatment effects on body weight and liver index (ratio of liver weight to body weight) were tested using ANOVA procedures."

Flow cytometry

Flow cytometry, based on the method of Thorgaard *et al.* (18), was used to assess the ploidy of all fish in the DMBA-exposed lots, and was used to sample and verify the effectiveness of triploidization of the AFB_1-exposed fish. Blood samples of 20 to 30 µl were taken shortly after death and added to 500 µl Alsever's anticoagulant buffer (2% glucose, 0.8% trisodium citrate, and 0.4% sodium chloride) at 4 °C. Blood samples were then frozen in liquid nitrogen for storage at -80 °C. For cytometry, the samples were thawed on crushed ice and then 100 µl of each were filtered through 40 µm monofilament nylon mesh (Small Parts, Miami Lake FL) into 400 µl of ice-cold stock solution consisting of 0.05% Triton X-100 and 0.25 M Na_2EDTA in phosphate buffered saline. At least 20 minutes prior to cytometry, 50 µl of a 500 µg/µl propidium iodide solution was added with gentle mixing to each diluted sample. Cytometry was conducted using an EPICS V cytometer (Coulter Electronics, Hialeah FL) with 488 nm argon laser fluorescence excitation. Data were analyzed for integrated fluorescence on a CICERO system (Cytomation, Ft. Collins CO). Integrated fluorescence histograms of a minimum of 1000 cells were found to unequivocally demonstrate ploidy for each sample. In all the approximately 1300 fish examined, except one, the integrated fluorescence was interpretable as showing that the sample represented either diploid or triploid fish. In that single exception, the integrated fluorescence was readily interpreted as indicating tetraploidy.

Results

Fish mortality

Early mortalities required that at least 20% more eggs be triploidized from a given monogamous sibling lot in order to have approximately equal numbers of triploid and diploid fish at the age carcinogen exposure commenced. The majority of these early mortalities occurred in the first few days after fertilization. Mortalities during and after AFB_1 exposure were low, representing in no case more than 4% of the population of any category of AFB_1 exposure, regardless of ploidy. In the DMBA exposures, the mortality was as high as 35% for the 500 ppm, 120-day exposures. The mean mortality across all DMBA experiments was 12%.

Tumor incidences

In the fish receiving DMBA dietary exposures, tumor incidences in the livers of the diploids ranged from 35% to 73% depending on dose and duration (Table 2.1). In stomachs, tumor incidences of 90% and higher were seen in the fish given DMBA regardless of dose and duration of exposure. In the swim bladders of diploids, the tumor incidence ranged from 12% to 52%. On the other hand, triploids fed DMBA showed incidences ranging from 8% to 30% in livers, 15% to 27% in stomachs, and 2% to 10% in swim bladders. There were consistently fewer tumors in every category of carcinogen exposure in the triploids compared to the diploids, with triploid showing one half to one ninth the incidence seen in diploid fish. In the AFB_1 exposures, the tumor incidences in liver ranged from 47% to 95% in diploids, and from 11% to 42% in the triploids (Table 2.2). Again, the diploid:triploid tumor incidence ratios were consistently less in livers of either carcinogen treatment. Figures 2.1, B - D display the summary data of tumor incidences versus dosage and duration of DMBA exposure in liver, stomach and swim bladder, and Figure 2.1 A shows that for AFB_1 in the only affected organ, liver.

Figure 2.1. Tumor incidence after dietary carcinogen treatments.
A) AFB1-induced liver tumors, B) DMBA-induced liver tumors, C) DMBA-induced swim bladder tumors, D) DMBA-induced stomach tumors.

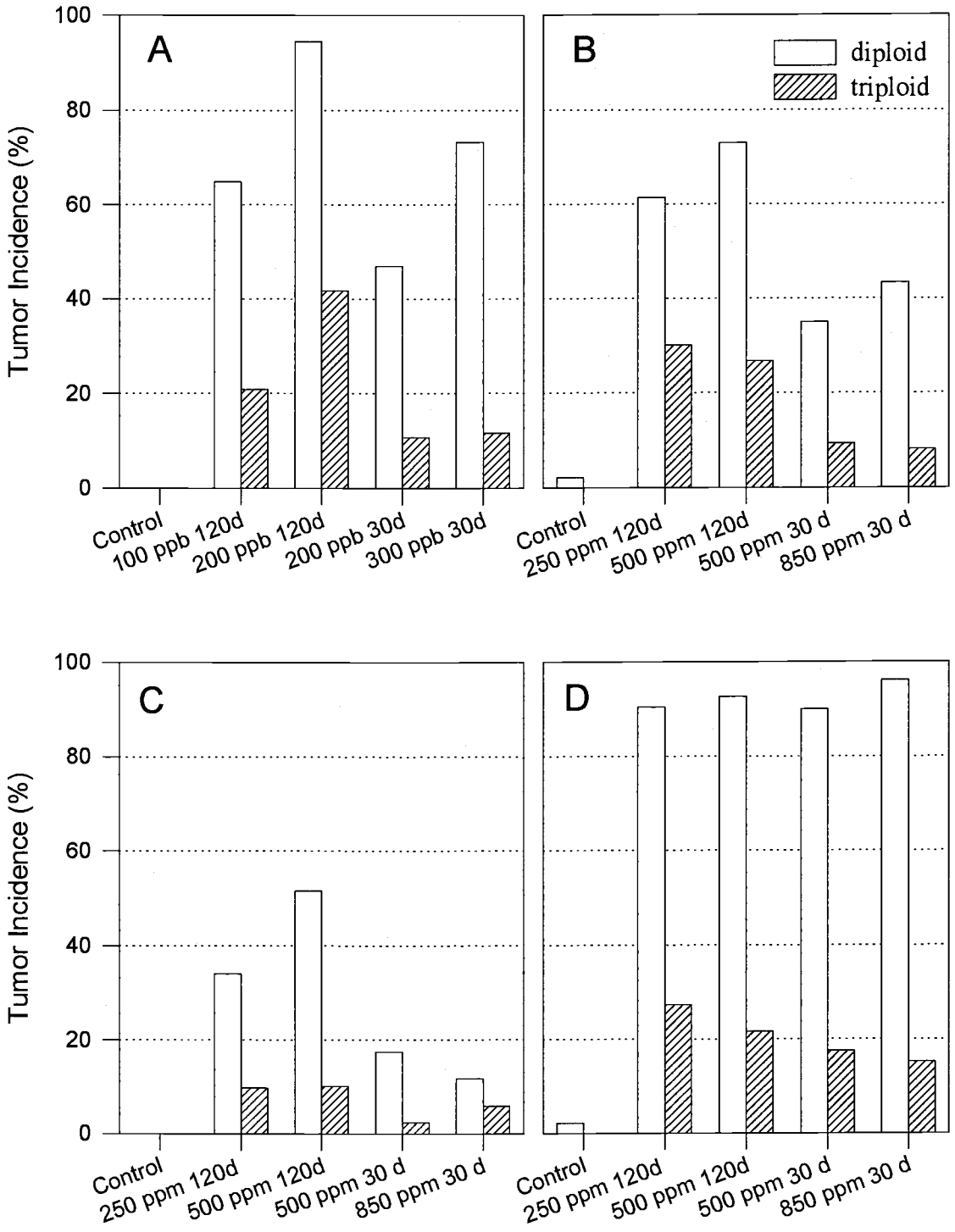


Table 2.1. Aflatoxin B_1 dietary dose, duration of dietary exposure, ploidy, final weight at necropsy and tumor incidence for livers.

AFB_1 (ppb)	Days AFB_1	ploidy	weight (g)	Liver tumor incidence %	multiplicity
100	120	diploid	138 ± 72	65 (124/191)	3.3 ± 3.0
100	120	triploid	140 ± 59	21 (37/177)[a]	2.8 ± 2.0
200	30	diploid	144 ± 66	47 (86/183)	9.5 ± 3.8
200	30	triploid	136 ± 67	11 (20/186)[a]	7.6 ± 3.2[b]
200	120	diploid	140 ± 56	95 (174/184)	8.3 ± 4.2
200	120	triploid	138 ± 58	42 (73/175)[a]	9.0 ± 3.8
300	30	diploid	138 ± 74	73 (143/195)	9.6 ± 3.7
300[§]	30	triploid	140 ± 65	12 (11/94)[a]	1.2 ± 0.4[a]

In this table "incidence" is the % tumor-bearing animals (number of tumor-bearing fish/sampled fish)
multiplicity: average number of tumors per tumor bearing fish , "±" is standard deviation

[a]: different from diploid groups with $P < 0.001$, [b]: $P < 0.05$

[§] One complete duplicate tank (100 triploid fish) in 300 ppb regime lost due to a water pump failure

Table 2.2. 7,12-dimethylbenz[*a*]anthracene dietary dose, duration of dietary exposure, cytometric ploidy, final weight at necropsy and tumor incidence for the three most frequently affected organs.

DMBA ppm	Days DMBA	Ploidy	Mean fish wt. ± SD (g)	Liver		Stomach		Swim Bladder	
				Incidence %	multiplicity	incidence %	mult.	incidence %	mult.
250	120	diploid	219 ±103	62 (96/156)	4.5	90 (141/156)	9.0	34 (53/156)	1.1
250	120	triploid	165 ±71	30 (43/143)	1.9	27 (39/143)	3.4	10 (14/143)	1.2
500	120	diploid	240 ±108	73 (89/122)	5.2	93 (113/122)	10.7	52 (63/122)	1.1
500	120	triploid	152 ±70	27 (37/138)	1.6	22 (30/138)	3.6	10 (14/138)	1.5
500	30	diploid	195 ±78	35 (28/80)	2.6	90 (72/80)	6.1	18 (14/80)	1.0
500	30	triploid	149 ±51	9 (8/85)	2.1	18 (15/85)	2.7	2 (2/85)	1.0
850	30	diploid	210 ±91	43 (33/76)	2.3	96 (73/76)	6.5	12 (9/76)	1.0
850	30	triploid	155 ±58	8 (7/85)	1.3	15 (13/85)	2.8	6 (5/85)	1.0
0	-	diploid	213 ±87	2 (1/45)	1.0	2 (1/45)	1.0	0 (0/45)	-
0	-	triploid	141 ±58	0 (0/41)	0	0 (0/41)	0	0 (0/41)	-

incidence: % tumor-bearing animals (number of tumor bearing fish/sampled fish)
multiplicity and "mult.": average number of tumors found per tumor-bearing organ

From the preceding tables it is clear that there was a substantial reduction in tumor incidence in the triploid trout relative to the diploids. At all levels of carcinogen and in all organs (liver for AFB_1; liver, stomach, swim bladder and kidney for DMBA) tumor incidence was lower in the triploid fish.Table 2.3 a, b shows two quantitative expressions of the triploid trout tumor suppression. First as simple diploid:triploid tumor incidence ratios and second as the log odds ratios (LORs)..

Table 2.3 a, b. Incidence Ratios and Log Odds Ratios (*LORs*) of diploid:triploid tumors

a. AFB_1 Liver

days	ppb	ratio	*LOR*
120	100	3.1	*1.94*
120	200	4.3	*3.27*
30	200	2.3	*1.87*
30	300	6.0	*2.98*

b. DMBA

		Liver		**Stomach**		**Swimbladder**	
days	ppm	ratio	*LOR*	ratio	*LOR*	ratio	*LOR*
120	250	2.1	*1.34*	3.3	*3.19*	3.4	*1.56*
120	500	2.7	*1.99*	4.2	*3.84*	5.2	*2.98*
30	500	3.9	*1.70*	5.0	*3.71*	9.0	*2.38*
30	850	5.6	*2.16*	6.4	*4.91*	2.0	*0.72*

In these tables "ratio" is the diploid:triploid tumor incidence ratio calculated as d/t; where d and t are the diploid and triploid fractional tumor incidences respectively.

"LOR" is the log odds ratio, calculated as $\{ ln(d/1-d\} - \{ln(t/1-t) \}$;

Weight class analyses

While triploidy was shown to be consistently associated with large reductions in tumor incidence, the differential growth weights of the two ploidies potentially confound facile interpretation of the results. However, logistic regression analysis demonstrates that the triploid suppression effect is independent of the growth effects on tumor incidence. These results are most robust for dosages and organs for which there were the largest numbers of tumors. Regardless of carcinogen, dose or length of dietary exposure, the regression curves fitted through the quintile weight classes, plotted as body weight versus logit of tumor incidence, are always shifted downward with respect to the incidence axis in the triploids, and in nearly all cases the regression curves are successfully modeled as parallel (Figures 2.2 through 2.5). Additionally, in all but three cases these curves show a substantial positive slope indicating that the known effects of growth on tumor incidence are consistently seen here as well.

Figure 2.2. AFB_1 -induced liver tumorigenesis in diploid (●) and triploid (□) trout. AFB_1 dose and duration of dietary exposure were: A) 100 ppb, 120 days, B) 200 ppb, 30 days, C) 200 ppb, 120 days, D) 300 ppb, 30 days.

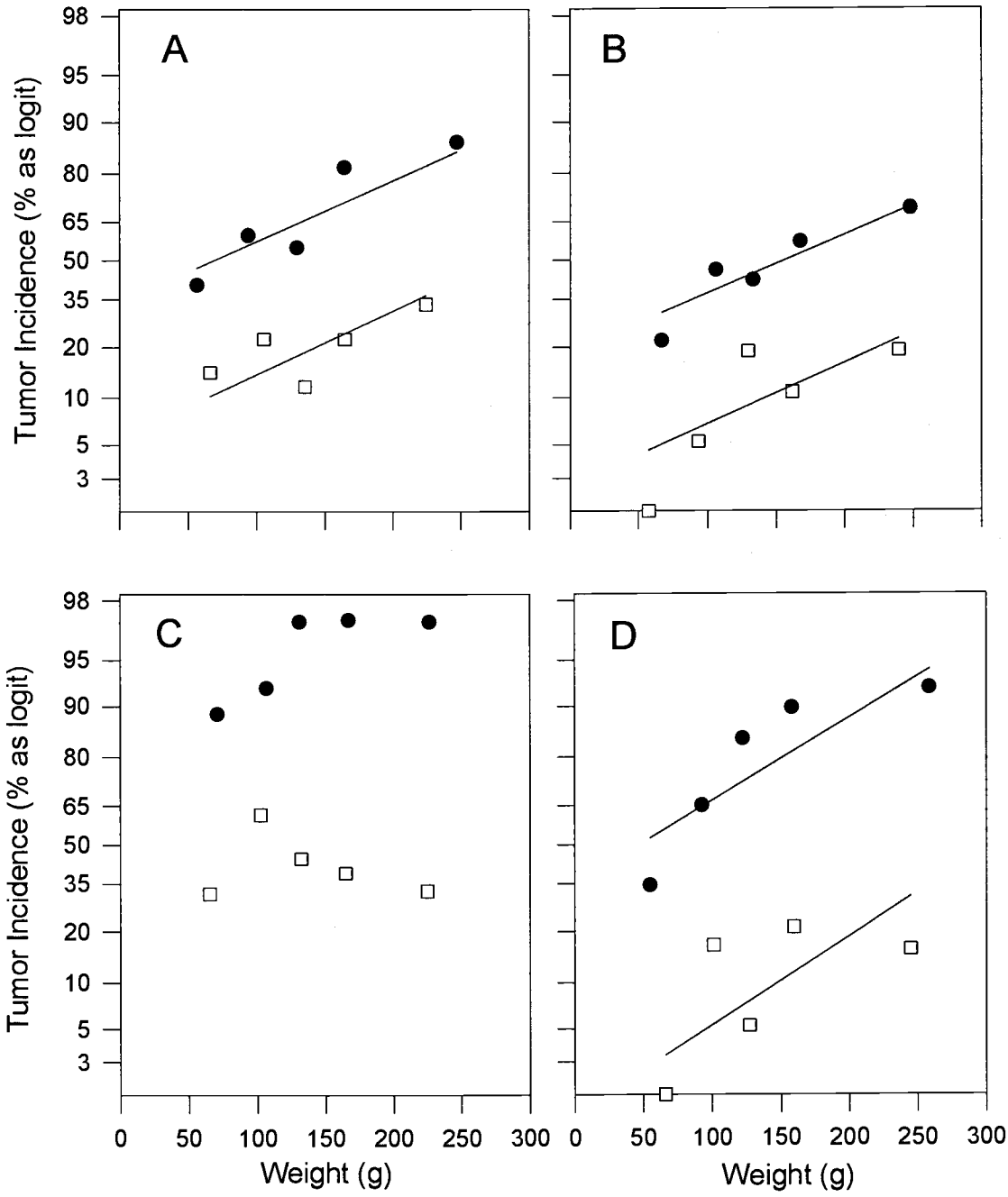


Figure 2.3. DMBA-induced liver tumorigenesis in diploid (●) and triploid (□) trout. DMBA dose and duration of dietary exposure were: A) 250 ppm, 120 days, B) 500 ppm, 120 days, C) 500 ppm, 30 days, D) 850 ppm, 30 days.

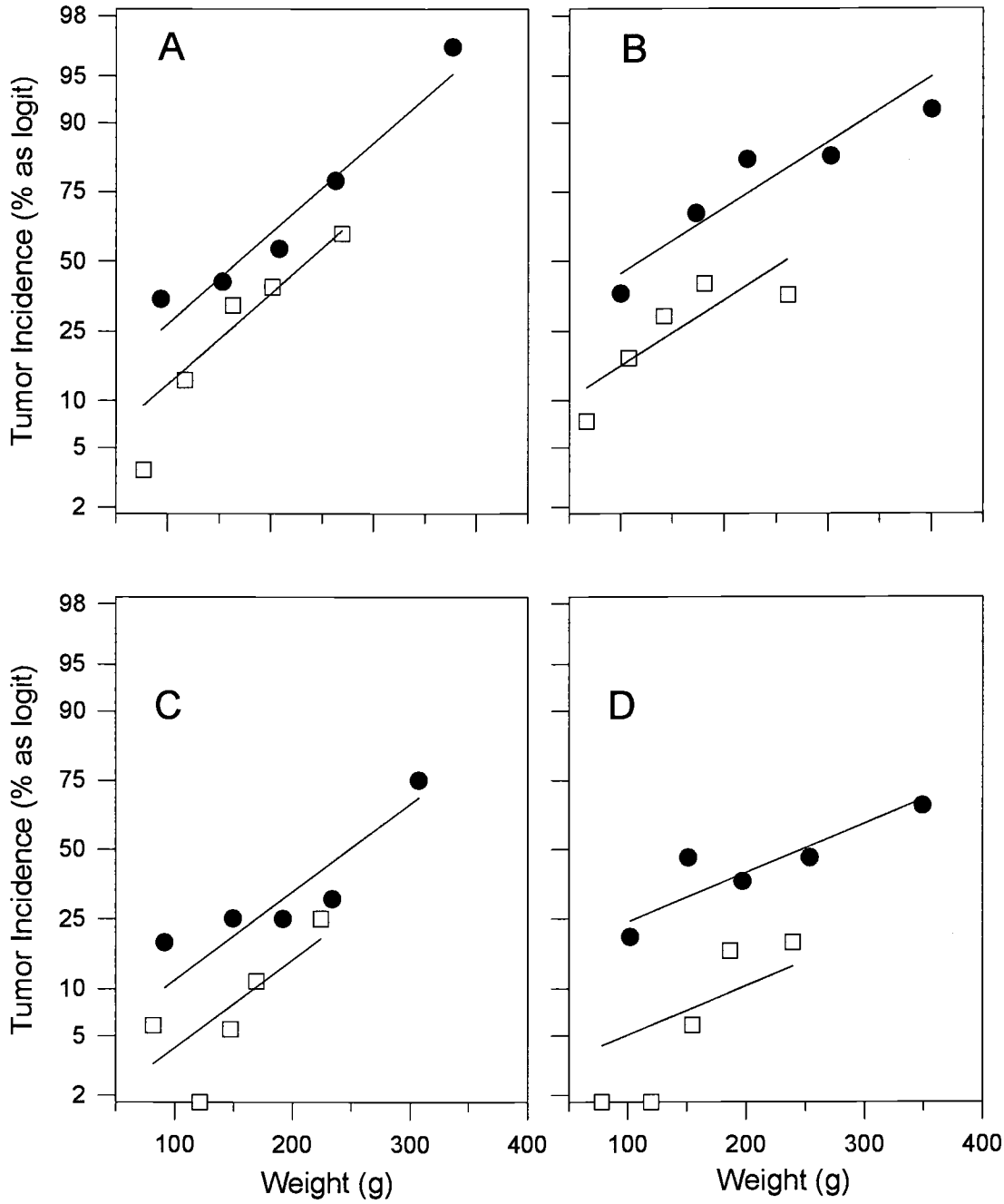


Figure 2.4. DMBA-induced stomach tumorigenesis in diploid (●) and triploid (□) trout. DMBA dose and duration of dietary exposure were: A) 250 ppm, 120 days, B) 500 ppm, 120 days, C) 500 ppm, 30 days, D) 850 ppm, 30 days.

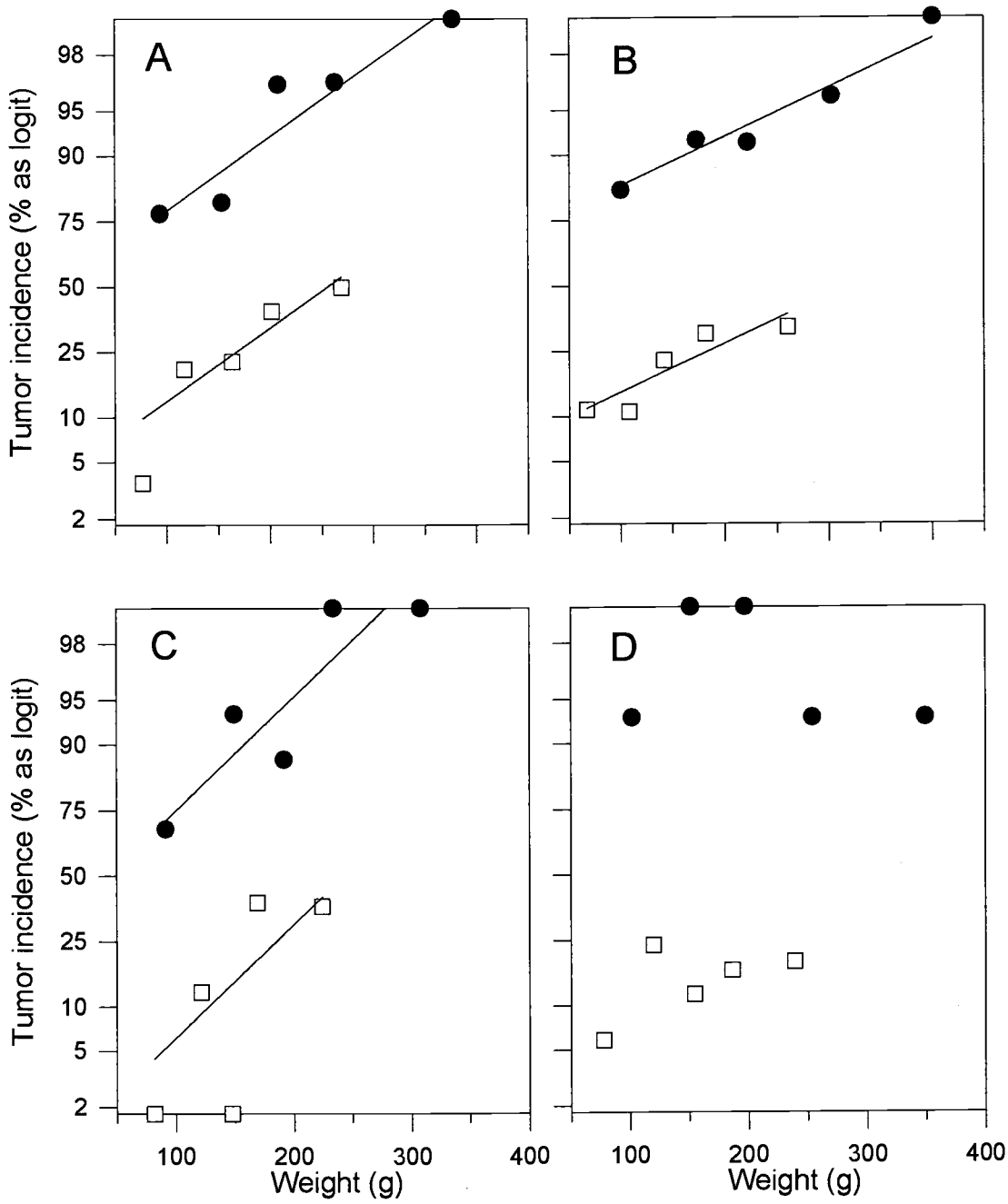
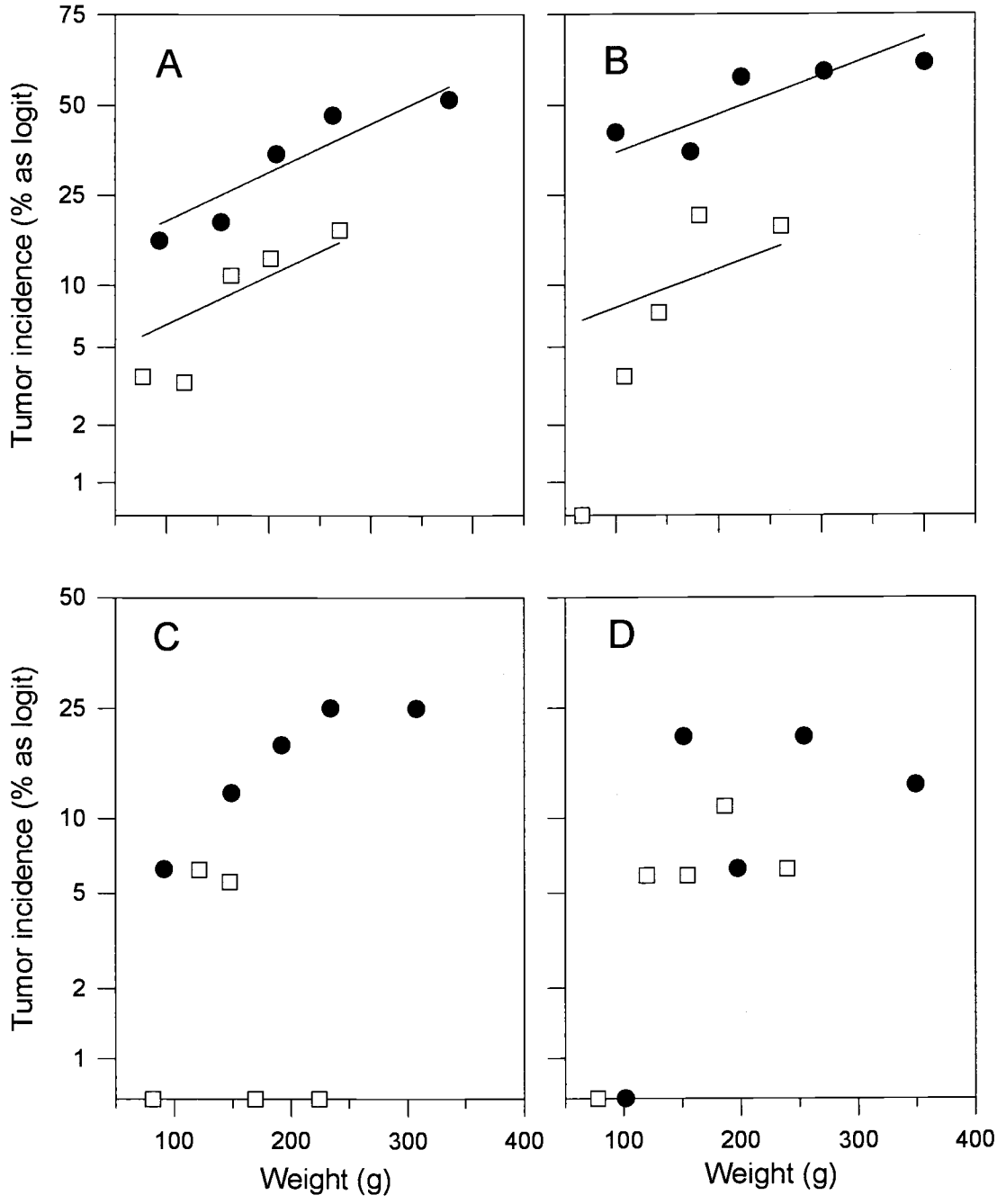


Figure 2.5. DMBA-induced swim bladder tumorigenesis in diploid (●) and triploid (□) trout. DMBA dose and duration of dietary exposure were: A) 250 ppm, 120 days, B) 500 ppm, 120 days, C) 500 ppm, 30 days, D) 850 ppm, 30 days.



Weight-compensated "logit y-shifts"

The weight class analysis seen in Figures 2.2 to 2.5 nullify the effect of the potential confounding variable of fish weight and hence the known positive effects of caloric intake and growth rate on tumor incidence. Table 2.4 a, b displays the quantitative consequence of the weight class analysis, *i.e.* the triploid suppression without that attributable to the lower weight gain in the triploid trout

Table 2.4 a, b. Diploid:triploid differential logit % or "logit y-shifts". Differential logit percents were calculated by subtracting the logit % (rounded to the nearest %) for triploid tumor incidence from that of diploids for each of the quintile weight classes, then averaging these differences to give mean logit % differentials for each of the dosage and duration categories reported here. Weight classes not represented in both ploidies were omitted from these calculations, and where applicable the differential logit % was deduced from the fitted logistic regression lines (Figures 2.2 through 2.5).

a. AFB_1

days	ppb	Liver
120	100	*49*
120	200	*52*
30	200	*38*
30	300	*48*
Mean ±s.e.m.:		47 ±5.2

b. DMBA

days	ppm	Liver	Stomach	Swimbladder
120	250	*16*	*54*	*23*
120	500	*18*	*67*	*36*
30	500	*16*	*61*	*7*
30	850	*28*	*77*	*9*
Mean ±s.e.m.:		19.5 ±5	65 ±8.4	19 ±11.8

From Table 2.4 it is clear that the suppression from triploidy alone is least in DMBA livers, intermediate in AFB_1 and greatest for DMBA stomach tumors.

Tumor multiplicity

Figure 2.6 shows, for comparison purposes, Thorgaard's (1) multiplicity distributions as percent for the stomachs in both DMBA and MNNG bath exposures. In our work, (Figures 2.7, 2.8, 2.9) some of the multiplicities follow similar trends. In general, for DMBA it is seen that triploids frequently have less tumors per tumor-bearing organ, so that the arithmetic mode is 1 or 2 tumors per tumor-bearing organ (Figures 2.7 and 2.8). By contrast, the mode in diploid stomachs appears to be between 6 and 10 or more tumors (Figure 2.7). This difference between diploids and triploids we will refer to as "modal disparity". There is little or no modal disparity by ploidy for most DMBA doses in liver (Figure 2.8). All of the stomach DMBA multiplicities exhibit modal disparities parallel to those seen in Thorgaard's DMBA and MNNG work. In the AFB_1-exposed fish livers in our experiments (Figure 2.9) the multiplicities show no strong modal disparity by ploidy. We note, however, that there was a nearly level distribution in diploids at 200 ppb for 120 days, and that 100% of the tumors in the triploids of the 300 ppb for 30 days were of a multiplicity of one to two per tumor bearing liver. These generally show the same downward trend by multiplicity as we see in the DMBA-fed tumor multiplicities of livers.

Figure 2.6. Stomach tumor multiplicity percentage distribution, from Thorgaard *et al.*, 1999 (1)

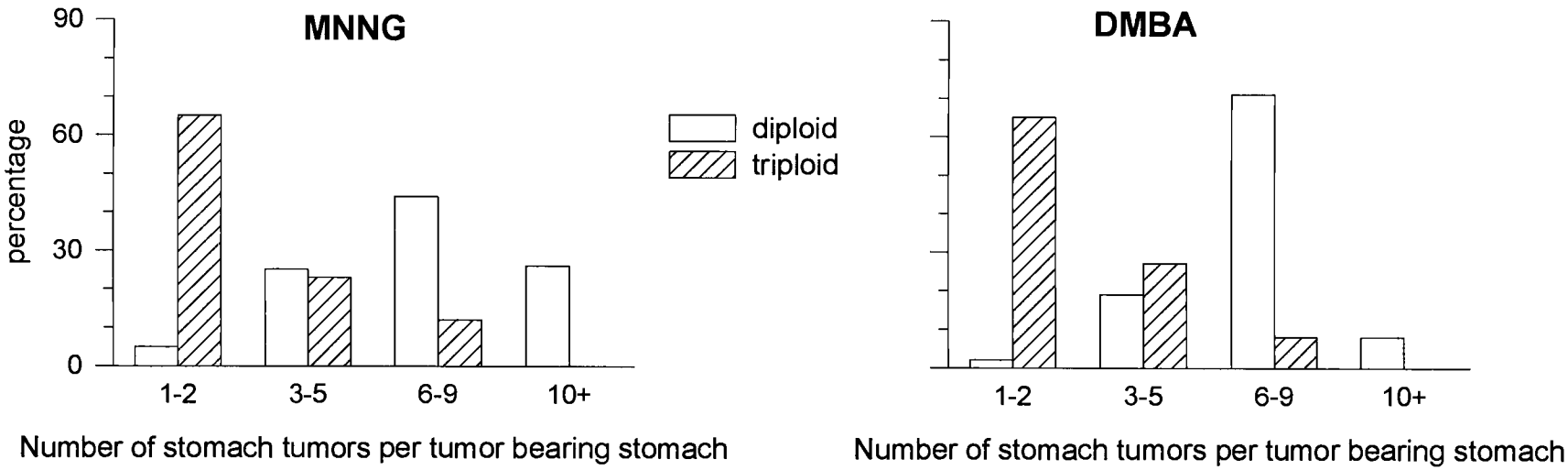


Figure 2.7. DMBA-induced liver tumor multiplicity percentage distributions. DMBA dose and duration of dietary exposure were: A) 250 ppm, 120 days, B) 500 ppm, 120 days, C) 500 ppm, 30 days, D) 850 ppm, 30 days.

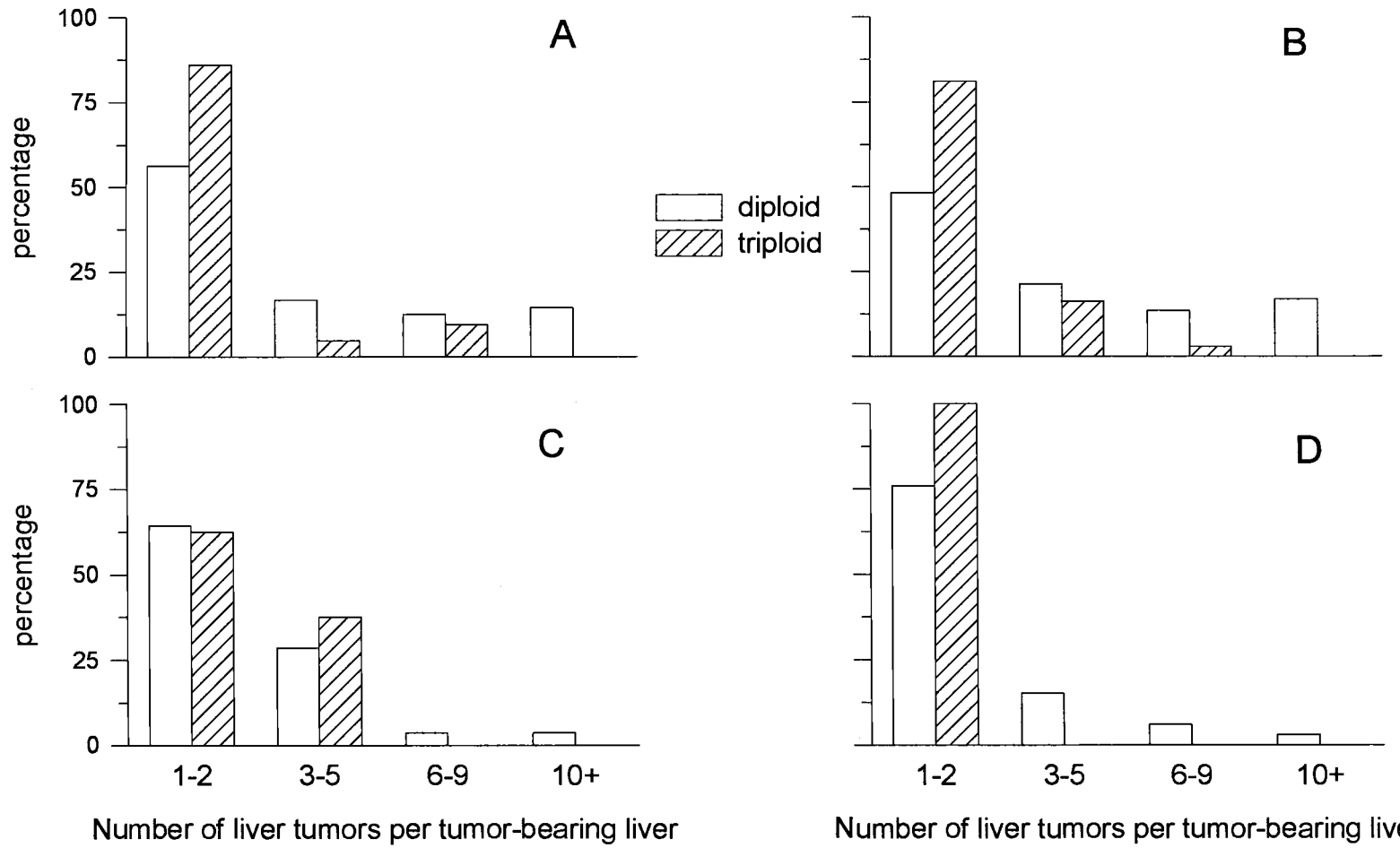


Figure 2.8. DMBA-induced stomach tumor multiplicity percentage distributions. DMBA dose and duration of dietary exposure were: A) 250 ppm, 120 days, B) 500 ppm, 120 days, C) 500 ppm, 30 days, D) 850 ppm, 30 days.

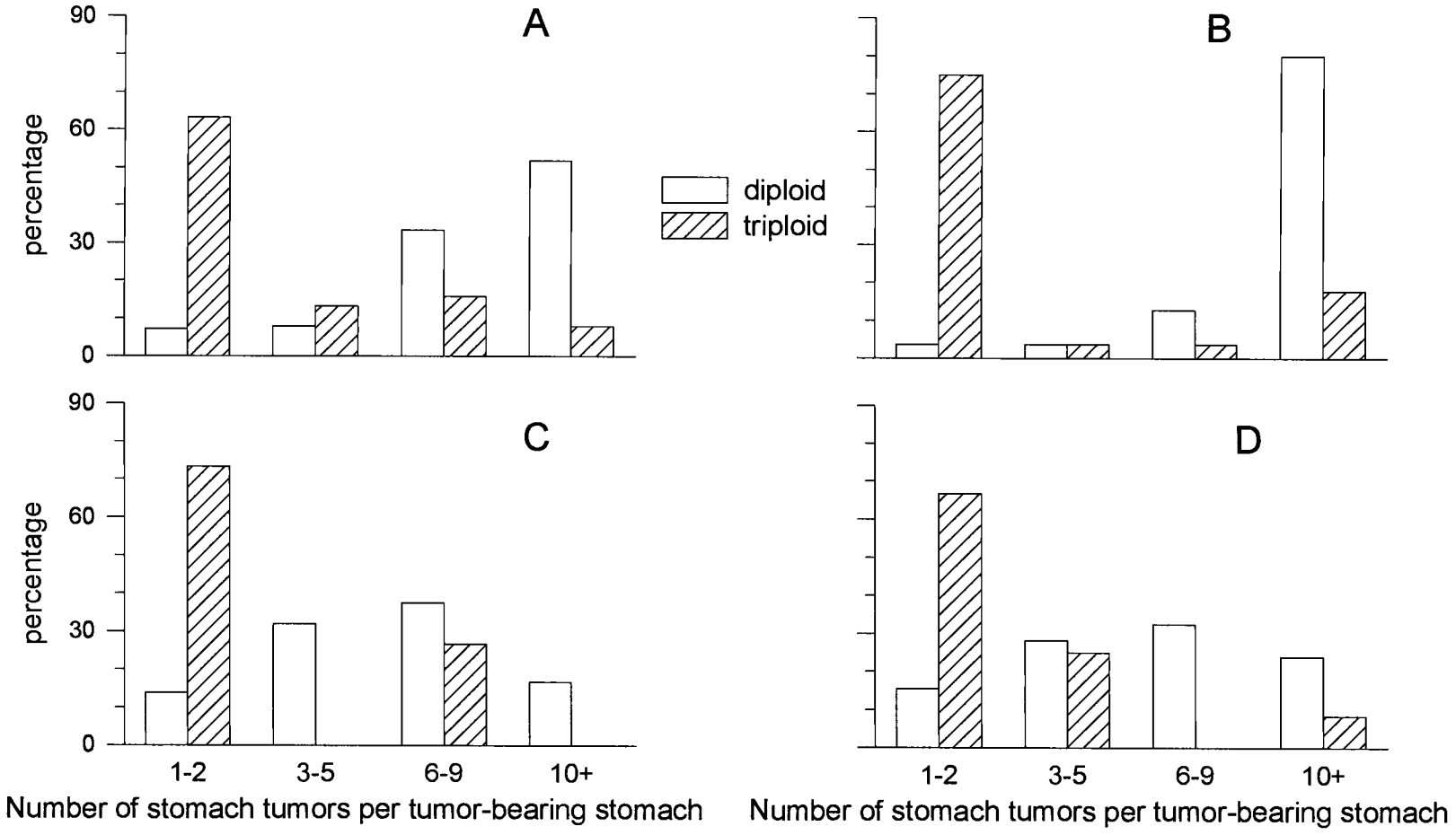
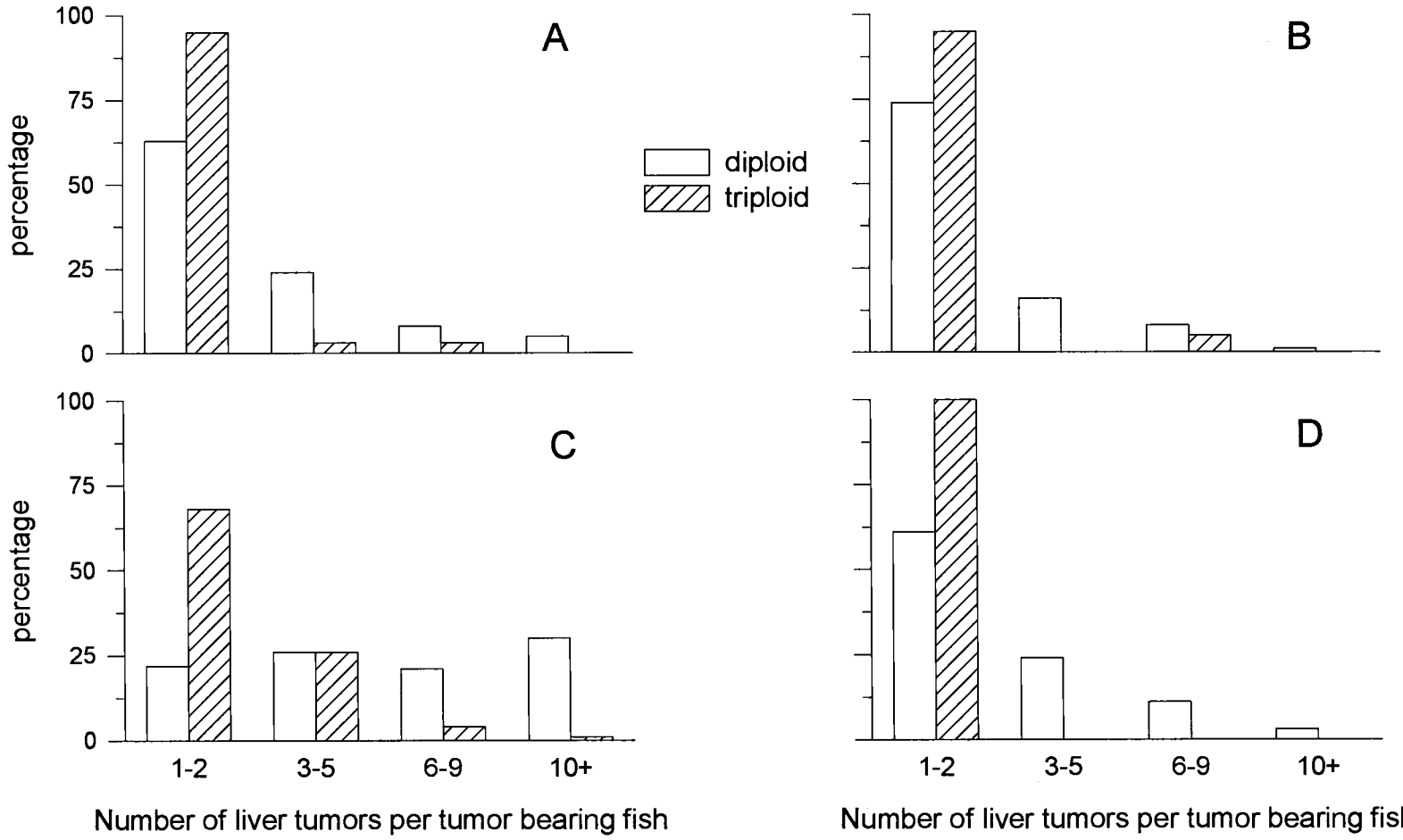


Figure 2.9. AFB_1 -induced liver tumor multiplicity percentage distributions. AFB_1 dose and duration of dietary exposure were: A) 100 ppb, 120 days, B) 200ppb, 30 days, C) 200ppb, 120 days, C) 300 ppb, 30 days.



Discussion

Effects of carcinogen and exposure duration

The data without weight compensation (Tables 2.1, 2.2 and Figure 2.1) show that, except for the 30 day swimbladder tumors, the suppression ratios (diploid incidence divided by triploid incidence) rise consistently with dose, where such comparisons are possible. This relationship also applies to the weight-compensated "logit y-shifts" of Table 2.4 a, b, but here swim bladders also show dose dependence. Duration of dietary carcinogen exposure also has a consistent effect on the non-weight compensated suppression ratios in that longer durations consistently reduce the suppression ratio in every case but that for 30 day swimbladders, which showed a pronounced contrary effect in the weight compensated data. These observations are similar to those for the log-odds analysis shown in Table 2.3 a, b. The degree of concordance between these three distinct analyses is consistent with a robust underlying phenomena of triploid suppression augmented by the uniform tumor enhancing effects of growth rate.

A possible explanation of the lower suppression seen in DMBA livers versus DMBA stomachs may be found in inherent developmental polyploidy of liver hepatocytes reported in many vertebrates (19). However this explanation would have to be reconciled with the apparent reduced incidences also seen in DMBA swim-bladders. The incidence data for swim bladders are distinct in that they appear to cluster at high and low suppression depending on the duration of carcinogen exposure. However, it should be noted that the relatively low numerical tumor incidence compared to those in livers and stomachs, gives much less certainty to any such inferences involving swimbladders.

Tumor multiplicity distributions

The DMBA-fed fish show a robust modal disparity with respect to ploidy for stomach tumor multiplicity distributions. A somewhat less robust example of this

disparity was also seen in stomach tumor counts from Thorgaard's DMBA and MNNG embryo bath-exposed diploid and triploids (see Figure 2.6). By contrast, the AFB_1 and DMBA liver tumor multiplicity distributions show no evidence of this modal disparity, nearly all doses and durations exhibiting declining percentage with increasing multiplicity, regardless of ploidy. The presence of distinctly different multiplicity percent distributions with respect to ploidy may reflect on the underlying mechanistic differences between the development of diploid and triploid tumors, and hence may reveal mechanistic underpinnings of triploid tumor suppression. For example, the number and location of mutations activating or inactivating genes required to initiate, promote and progress a triploid somatic cell lineage through to a tumorous clonal expansion may be different in a diploid than a triploid.

Conclusion

The results reported here provide expanded evidence for a profound effect of triploidy on tumor incidence. Such a robust and consistent effect on tumor incidence in a representative vertebrate suggests that entirely different mechanisms may be superimposed on, and act antagonistically to, the expected 50% increased exposure of target oncogenes to produce potentially dominant oncogenic mutations. In this regard, one salient difference between unicellular eukaryotes such as *Saccharomyces* and metazoans such as *Homo* and *Oncorhynchus*, is the widespread presence of tumor suppressor genes in the latter. At least 21 such genes have been well-characterized in humans (20) and there is good evidence for many more (21). Most tumor suppressor genes are presumed to be dominant and thus require loss of both alleles to confer an oncogenic phenotype (22). Rather than gaining oncogenic function through a mutation at a few specific sites, as proto-oncogenes often do, the tumor suppressor genes generally lose normal anti-oncogenic function through mutation or deletion at any one of a larger number of sites. This reflects the specificity required to gain new function versus the numerous ways available for a protein to lose function. A simplistic description of the potential effects of ploidy on the tumor suppressor genes and proto-oncogenes would be the following:

1. Increasing the genome copy number to 3n from the normal 2n increases the probability that a specific proto-oncogene may be activated by a factor of 1.5.
2. By contrast, inactivation of tumor suppressor genes typically requires disruptive mutation of both copies in diploids and presumably all three copies in triploids.

Chapters 4 and 5 of this Dissertation examine some of the possible influences of triploidy on mutation incidence and spectra within the p53 and *Ki-ras1* genes. Regardless of the mutation effects on those two specific cancer genes, there are doubtless other gene expression and/or protein functional correlates of triploidy, if the evidence of ploidy-related gene expression in yeast, recently reported by Galitski *et al.* (23) and reviewed by Hieter and Griffiths (24) is any indication. For example, Galitski's group reported that ploidy increases the mRNA expression by 10-fold or more in just 10 genes, and reduced expression to one tenth or less in 7 other genes. They found that increased ploidy decreases yeast agar invasiveness and led to a more fusiform morphology. They found that increased ploidy reduced the level of G1 cyclins and thus resulted in delayed G1 to S transition, which resulted in larger cell size. However, this work also shows that most yeast gene expression levels are unaffected by ploidy.

It is possible that some of the relative carcinogen resistance of the triploids is due to several causes acting in concert. In addition to the greater redundancy of the dominant tumor suppressor genes, some other possibilities may obtain. One is that the mass of DNA per unit of cell volume may be greater in the triploids, due either to the 50% increase in genome content per cell, or to a possible smaller cell volume due to diminished mitotic rates, or to some combination of both. This could be examined to some statistical certainty by comparing genome copy numbers contained in equal quantities of otherwise equivalent diploid and triploid tissue samples. Such an examination could be undertaken with flow cytometry on liver cells for example, wherein exact cell counts can be made and thus allow calculation of quite precise cell densities. Further, the existence of natural hepatocyte polyploidy has not been examined in trout livers. If trout hepatocytes are naturally polyploid, as they are

reported to be in several mammalian species, then this would be expected to reduce the protective effects of triploidy under the hypothesis that triploid suppression is due to the extra copy of tumor suppressor genes.

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Chapter 3

Absence of Mutations in Codon 249 of the p53 Tumor Suppressor Gene in Trout Hepatic Tumors Initiated by Short-term Aflatoxin B1 Exposure

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Abstract

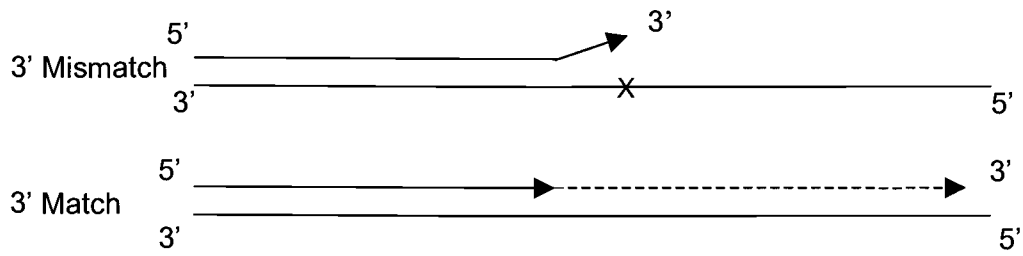
A variety of human tumors have been reported to possess p53 tumor suppressor gene alleles carrying point mutations within highly conserved regions of the structural gene. Among the 80 or more mutated sites so far identified, one has been associated with human hepatocellular carcinomas of possible aflatoxin origin. Approximately 50% of the tumors examined from individuals chronically exposed to high dietary aflatoxin appear to carry G to T transversions in p53 codon 249, located within a region highly conserved across several vertebrate orders. This observation is consistent with the known mutagenic specificity of aflatoxin B1, but does not demonstrate its involvement in p53 mutation. We have used the rainbow trout hepatocarcinogenesis model to investigate whether this mutation can be induced experimentally in hepatic tumors in aflatoxin-exposed animals. The orthologous codon 249 of the rainbow trout p53 gene is AGA and thus has one potential site for aflatoxin adduction. DNA extracted from 30 juvenile trout livers nine months after a 14 day exposure to aflatoxin B1, and screened for the presence of any mutation at position 2 (G) of codon 249 using a 3' primer-mismatch polymerase chain reaction (PCR)-based detection method. Under suitably stringent temperature and deoxynucleoside triphosphate concentrations, it was found that all 30 tumor DNAs only amplified to a gel-visible product with the normal complementing, 3' C-containing, PCR primer. Each of the 3' A-, G-, and T-containing primers (those complementing mutants) gave visible products under similar conditions with corresponding synthetic oligonucleotide control templates, but not with any tumor DNAs as template. A similar screening strategy failed to detect mutations within the codon 248 CGC sequence. Thus, brief exposure of trout to AFB_1 does not result in detectable p53 alleles carrying mutations in codon 248 or 249 guanines in whole liver tumor DNA nine months after initiation. This result may reflect the fact that AFB_1 exposure in these studies was early in development, whereas AFB_1-related p53 mutations detected in human tumors may reflect late events in tumor progression observable only with chronic AFB_1 exposure.

Introduction

Accumulating evidence indicates that most, if not all, cancers in humans and experimental animals involve activation of cellular proto-oncogenes and inactivation of tumor suppressor genes. The specific genes involved, their temporal relation to stages of tumor development, and the individual mechanisms of activation vary among tumor types, tissue types and organisms. In experimental carcinogenesis, these characteristics may also be a function of the protocols followed. The most common gene mutations so far reported in human tumors involves allelic deletion and point mutation events in the p53 tumor suppressor gene (1). Unlike other known tumor suppressor genes, some mutants of p53 function as a "dominant negative" in that loss of normal function in only one allele can be associated with oncogenic transformation (2) The expressed p53 protein is known to translocate to the nucleus and is suspected to be closely associated with cell cycle regulatory systems. Normal p53 appears to block the G1 to S transition of the eukaryotic cell cycle. Amino acid sequences inferred from p53 cDNAs of diverse vertebrates contain five highly conserved domains. Several important functional motifs have been identified within the expressed p53 gene, these include a nuclear localization sequence, probable helix-loop-helix motifs, a central DNA-binding domain, a powerful transcription activator (3) and possible signaled phosphorylation sites (4).

Recent analyses of human liver tumors taken from patients in Qidong province, China (5) and in a separate study in southern Africa (6), have shown that a high percentage of liver tumors (hepatocellular carcinomas) have a specific mutation of guanine to thymine at codon 249 of the p53 gene. Aflatoxin preferentially leads to G:C to T:A transversions, and aflatoxin is a prevalent dietary toxin in these regions of China and Africa. This suggests that the specific mechanism of aflatoxin carcinogenesis may well directly involve p53 at some level. Amongst the few available animal models for readily investigating this question is the rainbow trout, *Oncorhynchus mykiss* (formerly *Salmo gairdneri*). The suitability of rainbow trout for studies of carcinogenesis is well-established and has been reviewed several times (7, 8).

Figure 3.1: Allele specific mutation detection by primer mismatch



To investigate the incidence of mutation in codon 249 of the p53 gene we used DNA isolated from liver tumors of *O. mykiss*, as previously described (9). These fish had been treated as fingerlings with aflatoxin B1 (AFB_1) at 80 ppb for 2 weeks, and reared nine months for tumor development, as detailed elsewhere (9, 10). DNA extracted from each tumor was individually amplified in a 50 microliter reaction containing 10mM Tris-HCl, pH 8.3; 1.5 mM magnesium chloride, 50 mM KCl; 0.005% each of Tween 20 and NP-40 (Calbiochem); 0.001% gelatin; an upstream primer, and one of the four downstream "3'-mismatch" primers each at 400 nM (see Table 3.1); deoxyribonucleotide triphosphates (dNTPs) at 100 pmole each, 0.1 to 1.5 units of Replitherm polymerase (Epicentre Technologies, Madison, WI, but no longer listed in 2001 catalog) were used per individual reaction. We found that 100 pg of target DNA in the reaction was functional and possibly optimal. Amounts of DNA substantially greater than 3 ng were unsatisfactory. Thermocycling was conducted in an Ericomp ™ heat block, (San Diego, CA). The cycling regime consisted of two cycles containing an initial long denaturation at 94 °C for 5 minutes, annealing at 64 °C for 1 minute and extension at 74 °C for 1 minute. Following that, 35 cycles consisting of: 94 °C for 30 seconds, 64 °C for 30 seconds and 74 °C for 1 minute. Finally, a long extension at 74 °C for 5 minutes to finish synthesis of any prematurely truncated strands. In preparation for gel electrophoresis, 10 microliters of the amplified products were placed in the wells of a 80 by 100 by 0.77 mm vertical, 19:1 acrylamide-bisacrylamide polymer gel. The products were electrophoresed at about 100 volts (15-20 mA) for 40 minutes. The gels were stained for 1 minute with

ethidium bromide at a relatively high concentration of 5 to 10 μg/ml, to minimize elution loss of product DNAs. The gel was then destained for 2 to 5 minutes in running buffer (Tris-acetate-EDTA) to minimize the background gel and residual ethidium fluorescences. Fluorescence of ethidium-DNA was excited by UV transillumination at a peak of 302 nm and photographed through Kodak Wrattan ™ #9 and #23A filters in series. Exposures were typically 2 to 4 seconds at an aperture of *f*5.6 on Polaroid 3000 ASA type 667 film.

Table 3.1: PCR Primers

#	Name	5'genomic situs	Primer sequence 5'→ 3'	~Anneal °C
1	E7	U642	GGGATCAGAGTGTACCACTG	64
2	CE7A	L779	GTGTCTCCAGGGTGATGATG	68
3	RTP53-A	L764	TGATGATGGTGAGGATGGGTA	64
4	RTP53-C	L764	TGATGATGGTGAGGATGGGTC	65
5	RTP53-G	L764	TGATGATGGTGAGGATGGGTG	65
6	RTP53-T	L764	TGATGATGGTGAGGATGGGTT	64
7	2481-A	L768	ATGGTGAGGATGGGTCTGAA	62
8	2481-C	L768	ATGGTGAGGATGGGTCTGAC	62
9	2481-N	L768	ATGGTGAGGATGGGTCTGAG	62
10	2481-T	L768	ATGGTGAGGATGGGTCTGAT	62
11	2482A	L764	TGATGGTGAGGATGGGTCTGA	64
12	2482G	L764	TGATGGTGAGGATGGGTCTGG	64
13	2482N	L764	TGATGGTGAGGATGGGTCTGC	64
14	2482T	L764	TGATGGTGAGGATGGGTCTGT	64

Figure 3.2: Sequence of p53 exon 7, codons 248 and 249 in bold

gtg gga tca gag tgt acc act gtg ctc tac aac ttc atg tgc
aac agc tcc tgt atg gga ggg atg aac **cgc aga** ccc atc ctc
acc atc atc acc ctg gag aca caa gag

Position-specific detection of mutation was accomplished by appropriate adjustment of conditions so that a polymerase chain reaction (PCR) product consistently appears, or does not, based solely on the presence or absence, respectively, of a complementary match between the 3' base of one primer and the

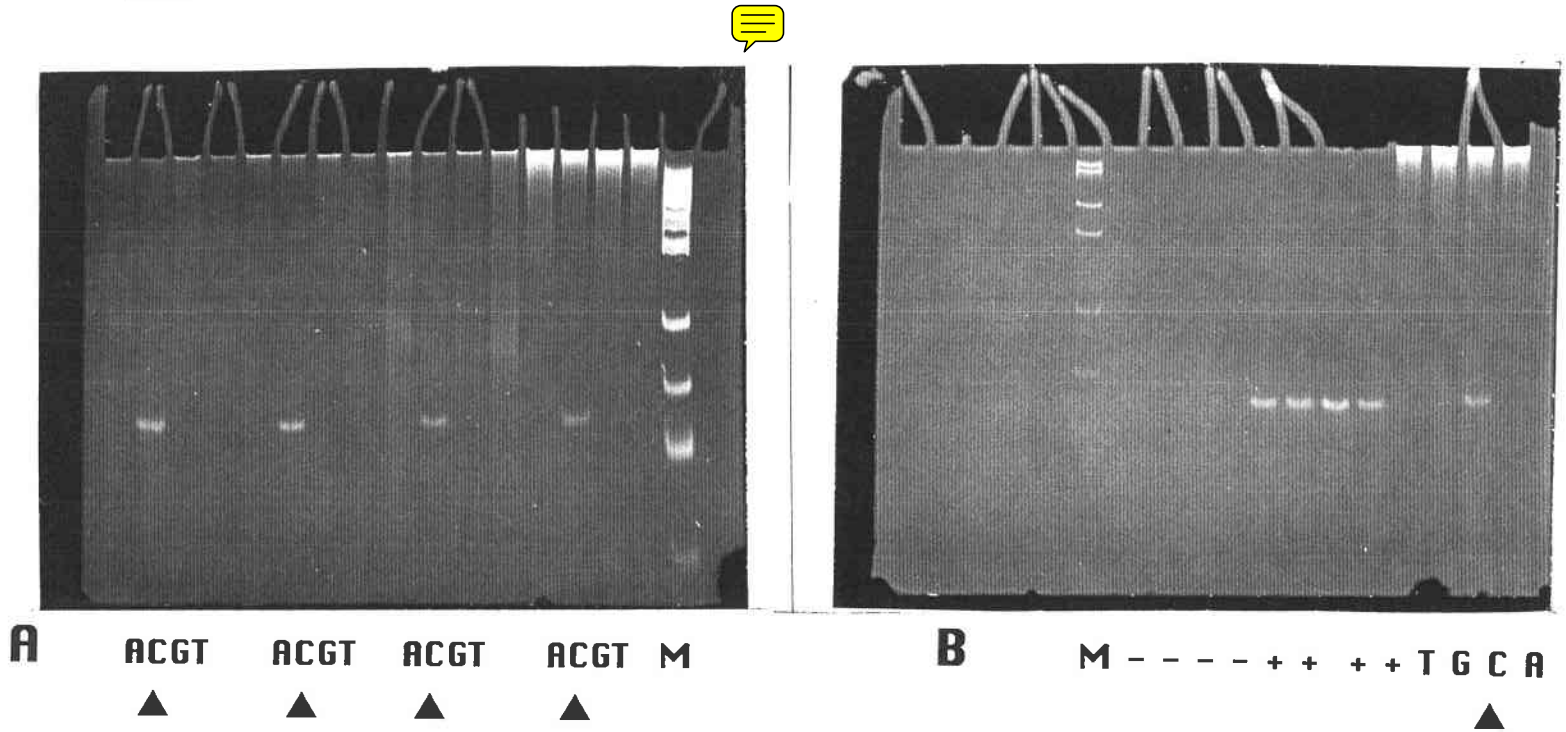
corresponding base of interest in the target DNA, as described by Ehlen and Dubeau in 1989 (11). Their method utilizes the known propensity of Taq polymerase to be less likely to synthesize DNA when a primer has a 3' mismatch. The method becomes able to detect single base mismatches if conditions are made stringent enough, *i.e.* very low dNTP concentrations (100-fold lower than normal) and by using the highest possible annealing temperatures. Our technique differs from that of Ehlen and Dubeau, in that we were able to directly detect the products (or their absence) with conventional UV-fluorescence of ethidium bromide/DNA on an acrylamide gel. We utilized optimized staining conditions, and a destaining rinse to lower background and enhance the relative strength of the ethidium stained UV fluorescence signal. By these means we were able to quickly screen tumor DNAs from aflatoxin-treated trout for the presence of any of the three possible base changes at a specific locus in the p53 gene (Figure 3.3).

To provide such positive controls for this PCR-based detection method, mutated DNAs were synthesized using PCR site-directed mutagenesis. This consisted of extending each of the three 3' mismatch primes under normal and less stringent conditions (estimated 55 °C annealing temperature, 200 μM dNTPs). The mutant PCR amplification products were excised directly from their respective electrophoresis lanes on polyacrylamide gel and eluted in 30 μL water. When probing these synthetic mutants using the 3'-mismatch method of Ehlen and Dubeau, it was found that an annealing temperature 3 to 5 °C higher was required to eliminate false positive signals. We had earlier observed a similar phenomenon with plasmids containing known (sequenced) mutations. This may be due to three factors: *a*) the greater mobility of the short strands than that of genomic DNA, *b*) the much higher number of targets per ng of DNA, and/or *c*) the terminal position of the potential mismatch on the template DNA.

Figure 3.3: Example of five allele-specific mismatch assays

Gels A and B. All but the control lanes show reaction products templated by a known normal trout p53. These alone provide the positive signals seen in the lanes labeled "C", those lanes showing the assay for ""A", "G" and "T" show no signal. Size marker "M" is a *Sau*3a digest of λ phage. Positive "+" and negative "_" control lanes are so indicated. Control reactions were templated by a p53 normal and four primer-based mutant constructs synthesized as described in the text.

Figure 3.3.



In each of the trout tumor DNAs, the second position of codon 249 appeared to be without mutation. As a confirmation of these results, potential mutations at this position were screened using more conventional ^{32}P radiolabeled oligonucleotide probe hybridizations, on a subset of 18 of these same tumor DNAs. The DNAs were PCR amplified and examined by Southern analysis oligonucleotide hybridization as described in Chang *et al.* (9). This technique utilizes tetramethylammonium chloride (TMAC) to improve the discrimination of probe-to-target hybridization so that single base mismatches can generally be detected. As for the 3'-mismatch method, only normal p53 codon 249 AGA sequences were detected in any of the 18 tumors examined. Because the adjacent codon 248 (CGC) is a frequently mutated in human tumors, we conducted similar 3'-mismatch analysis on positions 1 and 2 for this site. Of the trout tumors examined, all gave detectable PCR products only using the normal sequence primers (data not shown). We therefore conclude that brief AFB_1 exposure during the initiation phase of hepatocarcinogenesis does not produce tumors bearing AFB_1-related point mutations in trout p53 codon 248 or 249.

Several testable hypotheses may explain the apparent absence of AFB_1-related p53 mutations in this study. For example, Murokami *et al.*(12) provided evidence that p53 mutagenic activation occurs in a late stage of human hepatocellular carcinoma. If trout follow this pattern then we may expect significant incidences of AFB_1-driven p53 mutations only in tumors developed by chronic AFB_1 treatment, rather than those initiated by brief early aflatoxin exposure. This is reminiscent of a report by Tamura *et al.* (13) that found mutations in p53 were related to alterations in ploidy at comparatively late stages of carcinogenesis. It is possible that allelic deletions and point mutations in the p53 gene play no role in initiation, promotion, or progression of hepatic tumors in trout.

These initial studies with the trout model focused on the codon 248-249 arginyl-arginine sequence. It is interesting to note that four out of the five most frequently mutated "hotspots" in human p53 occur within codons specifying an arginine pair, and that all five hotspots normally code for an arginine. Arginines confer strong positive charge to their peptide locus. Double arginines have also been identified both as genuine phosphorylation signals and as pseudosubstrates with a

presumptive inhibitory interaction with kinase active sites (4, 14). Double arginines are also associated with helix-loop- helix motifs and DNA-binding functions (15). Thus, it seems likely that these arginines may not be mere coincidences. Such arginines may have particularly important effects on the specific function of the translated normal p53 product. Similarly, hotspot mutations at these arginines may have profound effects on the p53 product, possibly beyond a reduction or removal of the normal tumor suppressor activity. These effects might extend to the conversion of p53 from a tumor suppressor (anti-oncogene) to a positively functioning oncogene, as suggested by Michalovitz and colleagues (16). The fact that these arginine sites are all conserved in trout p53 offers potential to experimentally examine their functionality in p53 mutagenic transformation.

Our modification of the useful method of Ehlen and Dubeau allows visualization of the presence or absence of a site-specific mutation without the use of radiolabels, complex labeling protocols and/or extended x-ray film exposures or other development delays. Since PCR or another amplification schema is already required in these types of detection protocols it would appear to be advantageous to make this requisite amplification serve directly as the means of detection as well. The only limitations are that some of the normal gene sequence flanking the specified position of a possible mutation of interest be known.

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Chapter 4

Screening Tumors from Triploid Trout for p53 Mutations

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Abstract

As described in Chapter 2 of this Dissertation, it has been shown that the tumor incidence in triploid rainbow trout induced by dietary exposure to 7,12-dimethylbenz-[*a*]anthracene (DMBA) or aflatoxin B_1 (AFB_1) was substantially reduced compared to that in the diploid sibling trout. It was seen that the diploid:triploid tumor incidence ratios ranged from 2.0 to 9.0 and that the triploid suppression remained when growth effects were accounted for by weight class analysis. Such a substantial reduction of tumors in triploid extends earlier work and suggests that the 50% increased redundancy of any or all tumor suppressor genes may be a factor. To begin to explore this possibility, fifteen triploid liver tumors from the DMBA-fed animals were examined by direct cycle-sequencing of PCR products from all of exons 5, 7 and 8 of the p53 tumor suppressor gene, those containing the bulk of hotspots found in mammalian tumors. These showed no p53 mutations at, or above, the present threshold of detection, (which, for radiolabeled manual sequencing, we showed may be less than 5% of mutated in normal template). We also showed the threshold of mutation detection for fluorescent automated sequencing was consistently as low as 20% of mutated template in normal, and sometimes as low as 10%. Use of fluorescent automated sequencing technology for the examination of 15 stomach tumors, also showed no p53 mutations in the hotspot-containing p53 exons 5, 7 and 8 at or above the threshold of detection (10 to 20%). In an attempt to rapidly assess the mutation spectra of the AFB_1-fed fish, we examined 200 liver tumors simultaneously, by PCR and cycle-sequencing directly from a collective 200 tumor cDNA library. This tumor library showed no p53 mutational hotspots pronounced enough to register at or above the fluorescent threshold of mutation detection. The library also showed no evidence of alternatively spliced messages, as have occasionally been reported for p53 and the related gene p73. In addition, this library showed no p53 mutational hotspots pronounced enough to register at the 10 to 20% threshold of mutation detection in automated fluorescent sequencing.

Introduction

A recent paper by Thorgaard *et al.* (1) shows suppression of experimental tumor incidence in triploid trout, independent of carcinogen or growth modulation. Their study utilized brief bath exposures of sac-fry to three different carcinogens, aflatoxin B_1 (AFB_1), 7,12-dimethylbenz[*a*]anthracene (DMBA) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). In the present work, we utilized two of the same carcinogens, AFB_1 and DMBA, but in our study, carcinogen exposure was by diet over 30-day and 120-day periods. We used larger numbers of experimental animals in an effort to improve statistical power of the resulting influences on tumor incidence. In the present work, we sought mutations in a tumor suppressor gene and/or an oncogene might reveal evidence reflecting on the mechanism of triploid tumor suppression.

As reported in Chapter 2 we saw a strong suppression of tumor incidence in triploid trout when compared with their co-exposed diploid siblings. We hypothesized that the relatively few tumors occurring in the triploids would be relatively enriched for any gain-of-function and possibly any dominant negative oncogenic mutations. The argument was: loss-of-suppressor-function mutations in tumors from triploids would necessarily require the mutation or deletion of each of the other two alleles present, whereas the dominant, true gain-of-function sites would lead to an oncogenic phenotype with only a single mutated allele, whether in diploid or triploid context. Thus, a corollary to our hypothesis would be an expectation also of a *reduction* in triploids of the occurrence of loss-of-function mutations able to contribute to oncogenesis.

p53 is frequently reported as mutated in many human tumors (2). Over 70% of the tumor-associated mutations in human p53 are confined to an evolutionarily conserved 88 codons coding for the central consensus DNA binding portion of the p53 protein. Five mutational "hotspot" codons, accounting for 35% of p53 missense mutations, have been identified within this conserved central portion of the gene. These five mutational hotspots are in turn coincident with the five most highly

species may also include tumor suppression as in humans. Some of these same p53 mutation hotspots have been shown in experimental models to give rise to oncogenic phenotypes through either dominant negative mechanisms (4) or by apparent gain-of-function (5). It is this feature of p53 that may provide an approach to understanding triploid tumor suppression.

Importance of p53 gain-of-function mutations

p53 is the only known tumor suppressor gene that has multiple well-characterized sites thought to give rise to dominant oncogenic phenotypes through either dominant negative mechanisms (4) or true gain-of-function mutations (5). The p53 dominant negative mechanism in some mutants is attributable to the formation of dysfunctional heterotetramers consisting of wildtype and mutant proteins. Such mutations in human cells have been claimed to include those altering specific native arginine sites as follows: codon 175 $^{arg\rightarrow his}$, 248 $^{arg\rightarrow trp}$, 273 $^{arg\rightarrow his}$ or 285 $^{arg\rightarrow lys}$, as shown with human leukemia cells in SCID mice (6) and in SAOS-2 cells (7) in culture. These mutations correspond to p53 sites epidemiologically identified as mutated in human tumors (8), (9). As mentioned above, it would seem far less likely in a triploid cell that mutation or allelic loss can abrogate all three alleles of a normally dominant gene such as a typical tumor suppressor. Further, a loss-of-function mutation of a single allele among three can be expected to be somewhat less likely to generate a defective multimer of any normally dominant protein than it would as a single allele in a pair, thus tending to further protect the normal functions of such multimeric proteins as p53 in the triploid organisms. Thus, simple dominant negative mutations acting under a mechanism of defective multimers of p53 might be expected to give fewer tumors in triploid versus diploid animals. However, p53 true gain-of-function mutations could yield phenotypes sufficiently dominant that their effects may not be substantially diminished in the presence of even two normal alleles.

thought to express antimitotic, anti-oncogenic or antiproliferative functional proteins. The majority of the other missense mutations are considered "structural mutants," that is those that alter amino acids that determine structure but for which the coded peptides are not directly contacting DNA (15).

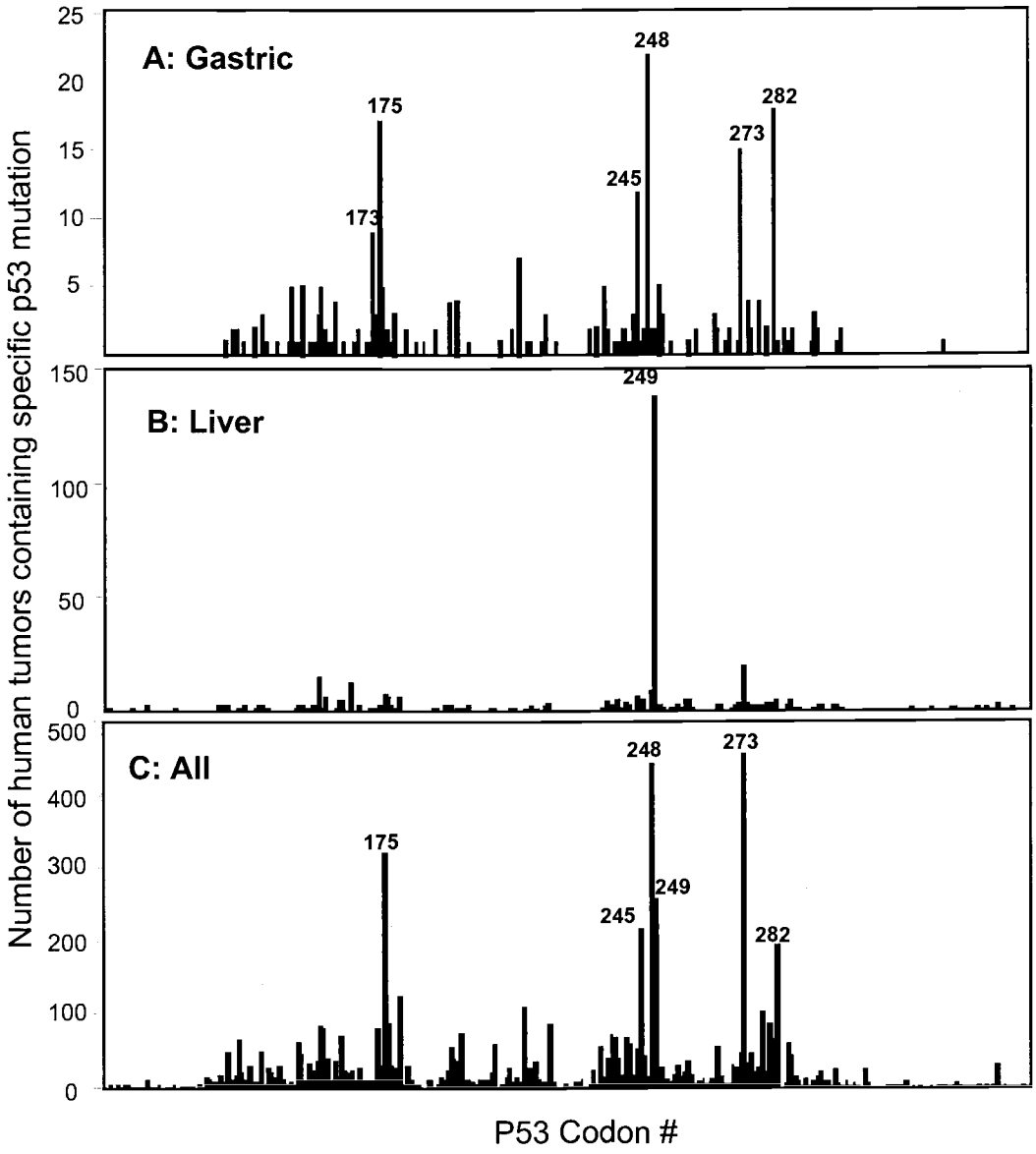
Codon 249: a nearly exclusive liver p53 hotspot

Among the six leading hotspots, codons 175, 245, 248, 249, 273 and 282 in tumors of all organs and types, only codons 249 and 273 coincide with those six in human liver cancer(16). In liver, $249^{arg \rightarrow ser}$ alone accounts for 33% of the liver tumor p53 mutations, whereas the next most frequent liver site, codon 273, is the locus of just 5% of liver p53 mutations. Approximately 80% of codon 249 mutations in the human p53 database are from liver tumors (Figure 4.1), and are believed to be due to hepatitis and aflatoxin co-exposure (17). For comparative purposes, we should note that there are no known trout homologues to either hepatitis B or C viral etiologic agents, and certainly none known to be enzootic at our experimental facilities. The absence of either hepatitis or aflatoxin markedly reduces not only liver cancer incidence but also codon 249 involvement in typical human tumorigenesis (18, 19). Discounting codon 249 liver tumor involvement in the human p53 database would remove this codon from the list of hotspots. In this specific instance we can expect that the human liver p53 incidence is likely to fail to predict that in trout liver p53.

Other p53 hotspots

Codon 175 is identical at the nucleotide level in trout and humans. Likewise, codon 273 is completely homologous between these two representatives of vertebrate classes. Both of these codons lie within areas of very strong evolutionary conservation. The percentage of hepatic tumors in the human p53 tumor mutation database (10) carrying these mutations is 0.25 % and 4.9 % respectively. By contrast, the database shows that amongst human gastric tumors, 6.4 % are codon 175 and 5.6 % are codon 273.

Figure 4.1. Three human p53 mutation spectra. Three spectra deduced from the p53 tumor mutation database (8) (as of October 1, 1998). Total tumors with p53 mutations in these categories were A: Gastric = 210, B: Liver = 466 and C: All = 6447.



The human mutational spectrum for all organs shows that another 25% of the mutations are found among some 16 additional codons at levels suggesting they might be called "warmspots". The remaining 40% of mutations are found broadly distributed across the same central region of the gene. Few reported gain-of-function or dominant negative characters have been attributed to sites outside of the hotspots (20).

Evolutionary conservation of the p53 gene

Figure 4.2 shows the coding and peptide sequences of the three selected "conserved domains" of human and trout p53 identified by Caron de Fromentel *et al.* (21) as evolutionarily conserved in all then known vertebrate p53 genes, and extended in Cheng *et al.* in 1997 (22). Conserved domains III, IV and V are also determinants of the central DNA binding region of p53. The hotspots in bold in Figure 4.2 also correspond to peptide structures proximal to specific nucleotides of the p53 consensus binding domain found upstream of all genes known to be transcriptionally activated by interaction with wildtype p53 tetramers. From Figure 4.2 and Table 4.2 one sees that the top five hotspots in human p53 code for arginine, and that the orthologous trout positions to these hotspots also code for arginine. From Table 4.2 it is evident that the conserved regions of p53 in humans and trout are highly similar, and that the amino residues of all 5 of the human mutational hotspots and their trout orthologs are identical. The regions containing the mutation hotspots in humans were referred to by Caron de Fromentel as "conserved domains" show overall identity of amino acids of 94% between trout and humans. The percentage identity based on DNA coding sequence is 84%. From published literature (22), it is also evident that these three conserved domains are at least 87.5% conserved in representatives from representative phyletic classes of vertebrates as diverse as zebrafish and chimpanzee.

Figure 4.2. Trout/Human p53 conserved "domain" alignments

Key: Conserved domains III, IV and V, showing Trout/*Human* orthologous coding sequences, AMINO ACIDS, and the top five human ***hotspots***. Trout nucleobases and peptide residues differing from human are underlined.

III, Exon 5, (hotspot codon **175**):

	D	V	V	R	R	C	P	H	H	Q	S
Trout:	gac	gtg	gtg	aga	cgc	tgc	cct	cac	cac	cag	agc
Human:	*gag*	*gtt*	*gtg*	*agg*	***cgc***	*tgc*	*ccc*	*cac*	*cat*	*gag*	*cgc*
	E	*V*	*V*	*R*	***R***	*C*	*P*	*H*	*H*	*E*	*R*

IV, Exon 7, (hotspot codons **248, 249**):

Y	N	F	M	C	N	S	S	C	M	G	G	M	N	R	R	P
tac	aac	ttc	atg	tgc	aac	agc	tcc	tgt	atg	gga	ggg	atg	aac	cgc	aga	ccc
tac	*aac*	*tac*	*atg*	*tgt*	*aac*	*agt*	*tcc*	*tgc*	*atg*	*ggc*	*ggc*	*atg*	*aac*	***cgg***	***agg***	*ccc*
Y	*N*	*Y*	*M*	*C*	*N*	*S*	*S*	*C*	*M*	*G*	*G*	*M*	*N*	***R***	***R***	*P*

I	L	T	I	I	T	L	E
atc	ctc	acc	atc	atc	acc	ctg	gag
atc	*ctc*	*acc*	*atc*	*atc*	*aca*	*ctg*	*gaa*
I	*L*	*T*	*I*	*I*	*T*	*L*	*E*

V, Exon 8, (hotspot codons **273, 282**):

F	E	V	R	V	C	A	C	P	G	R	D	R	K	T	E	E
ttt	gag	gtg	cgt	gtg	tgt	gcc	tgt	cct	ggt	cga	gac	agg	aag	aca	gag	gag
ttt	*gag*	*gtg*	***cgt***	*gtt*	*tgt*	*gcc*	*tgt*	*cct*	*ggg*	*aga*	*gac*	***cgg***	*cgc*	*aca*	*gag*	*gaa*
F	*E*	*V*	***R***	*V*	*C*	*A*	*C*	*P*	*G*	*R*	*D*	***R***	*R*	*T*	*E*	*E*

Table 4.2. Trout/Human p53 peptide similarities. Trout/Human percentage identity across the orthologous peptide and coding sequences of the three conserved domains, and associated mutation hotspots, for the central DNA binding region of the p53 protein and gene.

	AA conservation	DNA conservation
Domain III	73%	79%
Codon 175	identical	identical
Domain IV	96%	87%
Codon 249	identical	3rd base change
Codon 248	identical	3rd base change
Domain V	94%	84%
Codon 273	identical	identical
Codon 282	identical	1st base change
All three domains	**94%**	**84%**
All five hotspots	**Identical**	**80%**

What parts of trout tumor p53 should be examined, and how extensively?

Our impetus for examining p53 in trout derives from the prevalence of p53 mutations in human tumors. The operating hypotheses justifying the present effort in triploid trout also stems directly from features of the human p53 mutation spectrum, such as the aforementioned codon 249 mutations, the presence of distinct mutational "hotspots" and the putative presence of specific gain-of function mutations. The human p53 database and other published quantitative information about p53 mutation incidence, spectrum and organ-specificity were used below to provide a framework for focusing and directing our examination of trout tumor p53.

In Table 4.3 we tabulate the results of estimates of the number of human tumors it would be necessary to analyze to provide a specified certainty of finding particular p53 mutations. These calculations (for further details see Appendix B of this Dissertation) were based on mutation position spectral data taken from the human p53 database (10) and on overall estimates of organ-specific p53 involvement. If one assumes homologous function and presumes identical mutagenesis and carcinogenesis

processes between human and trout, then we can consider this one way to constrain the search for trout p53 mutations.

The sample size n necessary to provide 95% certainty of detecting a specific mutation or set of mutations can be derived from solving for n in the expression $(1-\mu)^n \leq 0.05$, where μ is the specific rate of a particular mutation (such as a specific site within human p53) in tumors of a particular organ in a known large population.

Table 4.3: Summary table: depth of tumor sampling required to reach a 95% confidence expectation (c. e.) of finding a specified human p53 mutation.

p53 position or region:	*Liver*		*Stomach*	
	$N_L/410 = f_L$ §	95% c.e. sample size n	$N_S/266 = f_S$ §	95% c.e. sample size n
Codons 175 & 273	0.0659	181	0.1203	61
Codon 248	0.0195	613	0.0827	90
Codon 249	0.3366	35	0.0075	995
Codons 248 & 249	0.3561	33	0.0902	82
All 5 top hotspots*	0.4976	23	0.3158	23
Exon 5	0.2512	47	0.4023	18
Exon 7	0.4853	24	0.2707	27
Exon 8	0.1732	68	0.2556	28
All of exons 5, 7, 8	0.9097	12	0.9286	6
Whole coding region	1.0000	11	1.0000	6
Exons 5, 7, 8.- 249adj#	0.6414	18	0.9211	7
Whole coding-249adj#	0.7317	15	0.9925	6

§ Here N_L and N_S are the number of liver and stomach tumor p53 mutations reported for this position or region of the human p53 gene as reported in the Human p53 database (10) as of September 1998.The total number of tumors for liver at that time was 410, for stomach 266.

*For liver tumors: codons 157, 166, 248. 249, 273; for stomach tumors: 175, 245, 248, 273, 282

#Here codon 249 liver incidence in humans is adjusted to reflect estimated likelihood that at least 0.80 of human liver 249 involvement is due to a specific carcinogenic interaction in human liver of aflatoxin in diet and hepatitis B or C infection, for which no viral parallels are known in trout.

The human mutation database does not provide the rate μ.. To derive this rate μ we take the product of f, the organ specific frequency of p53 mutations in the published human database, and a coefficient p that estimates the organ-specific frequency of all p53 mutations in human tumors of a particular organ. The $f \cdot p$ product is essential to computing the rate μ, since the p53 database itself tabulates only tumors with p53 mutations (f) and says nothing about the number of tumors that may have been examined to find the subset containing the mutations reported. Table 4.3 summarizes the inferred sample sizes necessary under the above assumptions, to have a 95% confidence expectation of finding at least one member of the sample with a mutation among the organ-specified class of p53 mutations based on human incidences. The overall organ-specific incidences (p_x) are inferred from the extant publications, many, if not all of which are represented in the same Béroud and Soussi p53 database. For reported studies of p53 mutations in gastric tumors of all types, we find p53 mutational incidences of 43% (n=12) and 50% (n=18) (23); 50% (n=90) (24); 28% (n=116) (25); 42% (n=59) and 37% (n=59) (26). These incidences are consistent with estimates of overall human tumor p53 mutation ranging from 30 to 50% and provide an estimate of expectation of 40% for overall gastric tumor p53 mutation in our own analysis. For the liver tumors, we find reported studies of hepatic tumor p53 mutation incidences to be even less informative than that for stomachs. Ueda *et al.* (27) suggest that liver tumor p53 involvement is quite rare in the absence of hepatitis. In the absence of conventional published data on human liver p53 involvement, we have used data given by Soussi, the original and currently co-publisher of the human p53 mutation database. In Soussi's p53 database-associated *World Wide Web* pages (16) he states that the incidence of p53 alteration observed in stomach tumors is 45% and in liver tumors is 25%. For the purposes of our analysis here, based on the limited data available, we have assumed that stomach p53 mutation incidence is at the mean of the values given in the sources cited above, that is 40%. For liver we will directly use the figure asserted by Soussi, which is that 25% of human liver tumors show p53 mutations. Thus, for the purposes at hand, we assumed p for stomach, or $p_S = 0.4$ and that p for liver, or $p_L = 0.25$.

From Table 4.3, one can see that based on human p53 mutation positional incidence, examining exons 5, 7 and 8 would likely detect 11/12 (> 91%) of the p53 mutations present. Further, one can see that elimination of 80% (the estimated contribution of hepatitis and aflatoxin) of the codon 249 involvement has the effect of increasing the liver tumor sampling requirement to reach the 95% confidence level from 12 to18 tumors, or by 50%. Eliminating 80% of 249 involvement in stomach would only increase the number of stomach tumors required from 6 to 7 or by ~17 %. This table suggests that our sample numbers may have been generous for stomach where we examined 15 tumors for exons 5, 7 and 8. The table also shows that our sampling of 15 liver tumors would have been adequate without the adjustment for likely reduced involvement of codon 249. In Table 4.3, we also show estimates of the likelihood of finding p53 mutations at specific codons or across larger regions of the human p53 tumor mutation database. We cannot rectify the likely sampling biases engendered by tendencies for researchers to look at regions of p53 that have proven to be productive in earlier work. These biases are unavoidable, but their effects may be minimized by the fact that p53 is a relatively small gene and protein so that a substantial number of the published p53 mutation spectra resulted from global examinations of the whole cDNA. The intensity of ongoing p53 research makes it unlikely that important human mutations have been completely overlooked.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma (St. Louis, MO) unless specified otherwise. The carcinogens, DMBA and AFB_1 were purchased from Aldrich (Milwaukee, WI).

Animals

Several monogamous spawnings containing at least 3000 eggs of the Shasta strain of *O. mykiss* were each asymmetrically divided into two lots consisting of approximately 35% and 65% of the fertilized eggs each. The larger sub lots were subjected to hyperthermic triploidization as follows: At 10 minutes after fertilization the eggs were placed in a water bath at 29 °C for 10 more minutes. Following this, they were placed in the incubators at the usual temperature of about 13 °C. The smaller of the sub lots (normal diploids) were not shocked, but were placed directly in the 13 °C incubators. After the fish reached the feeding stage, sibling lots of diploid and triploid fish were selected to continue through the experiments. For the DMBA experiments 880 fish of each ploidy, and for the AFB1 experiments 770 fish of each ploidy were utilized.

Care, feeding and carcinogen exposure

The diploid and triploid fish were raised in the customary manner at the Food Toxicology and Nutrition Laboratory (FTNL), Oregon State University (Corvallis, OR) as previously described (28). All animals were treated in accord with Federal guidelines (29) and protocols approved by the Oregon State University Institutional Animal Care and Use Committee. The trout were raised to the age of about 5 months before initiating the dietary carcinogen exposures. The diet for the fish was either standard Oregon Test Diet, (OTD) (28) or OTD in which carcinogen had been incorporated into the lipid component of the OTD formulation. The fish were then divided into equal numbered lots housed in separate tanks. Dietary carcinogen concentrations and lengths of exposure are indicated in Tables 2.1 and 2.2 of Chapter 2 of this Dissertation.

Tumor sampling and storage

At about16 months after the beginning of dietary carcinogen exposure, the fish were sacrificed by an overdose of tricaine methanesulfonate anesthetic. The fish were individually weighed, dissected, sexed, the liver removed and weighed. Tumors were

individually assessed, counted and sized by the on-site pathologist. For both the DMBA- and AFB_1-exposed animals, at least one tumor from each tumor-bearing fish liver was frozen in liquid nitrogen for storage at -80 °C. In DMBA-exposed fish the stomach, swim bladder, and kidney were also carefully examined, tumors present were noted, and at least one tumor from each of those tissues bearing tumors was frozen and stored at –80 °C.

Nucleic acid isolation

Genomic DNA was isolated using the QIAamp tissue kit (Qiagen, Inc., Santa Clarita, CA) following their published protocol. The yield of DNA from each isolation, was assessed by agarose gel electrophoresis and suitable dilutions were made to give working solutions of approximately equal concentration for use in subsequent PCR amplifications. For mRNA we utilized only liver tumors, since these are susceptible to gentle disruption thus permitting us to isolate cytoplasmic RNA. Cytoplasmic RNA has the advantage of exclusion of nuclear DNA and immature mRNA, while giving a good yield of mature mRNA. We followed most aspects of the Qiagen RNAeasy "Cytoplasmic RNA" protocol usually applied to cultured cells. This method utilizes ~ 4 °C lysis buffer containing the mild detergent "nonidet P40" (Calbiochem-Novabiochem, San Diego CA), that disrupts cell membranes while leaving nuclei intact. We added to that protocol gentle but thorough mechanical tissue disruption with conical plastic pestles (Kontes, Vineland NJ) manually pre-selected for close fit into Sorenson Bioscience, Inc (Salt Lake City UT)1700 μL "pre-siliconized Multi-safeseal, Item # 11700" conical microcentrifuge tubes. (Note: Of many brands examined, this manufacturer's tube was found to give the closest fit to the Kontes pestles.) This mechanical disruption was conducted in the cold lysis buffer in the presence of the "Prime" RNAase inhibitor (Eppendorf, Westbury NY), at 30 units per mL. The lysed and disrupted homogenate was then spun briefly at 300Xg in a precooled 4 °C centrifuge to pellet the nuclear and other insoluble components. This pellet was saved in TE buffer and rapidly frozen and stored at -80 °C, for possible later analysis. The supernatant, containing mature mRNA, was then processed according to

the Qiagen RNAeasy protocol. RNA isolations were not directly visualized by gel electrophoresis, but instead were used directly as templates in subsequent reverse transcription reactions. The purified RNA samples were stored in RNAase-free water at -80 °C.

Flow cytometry

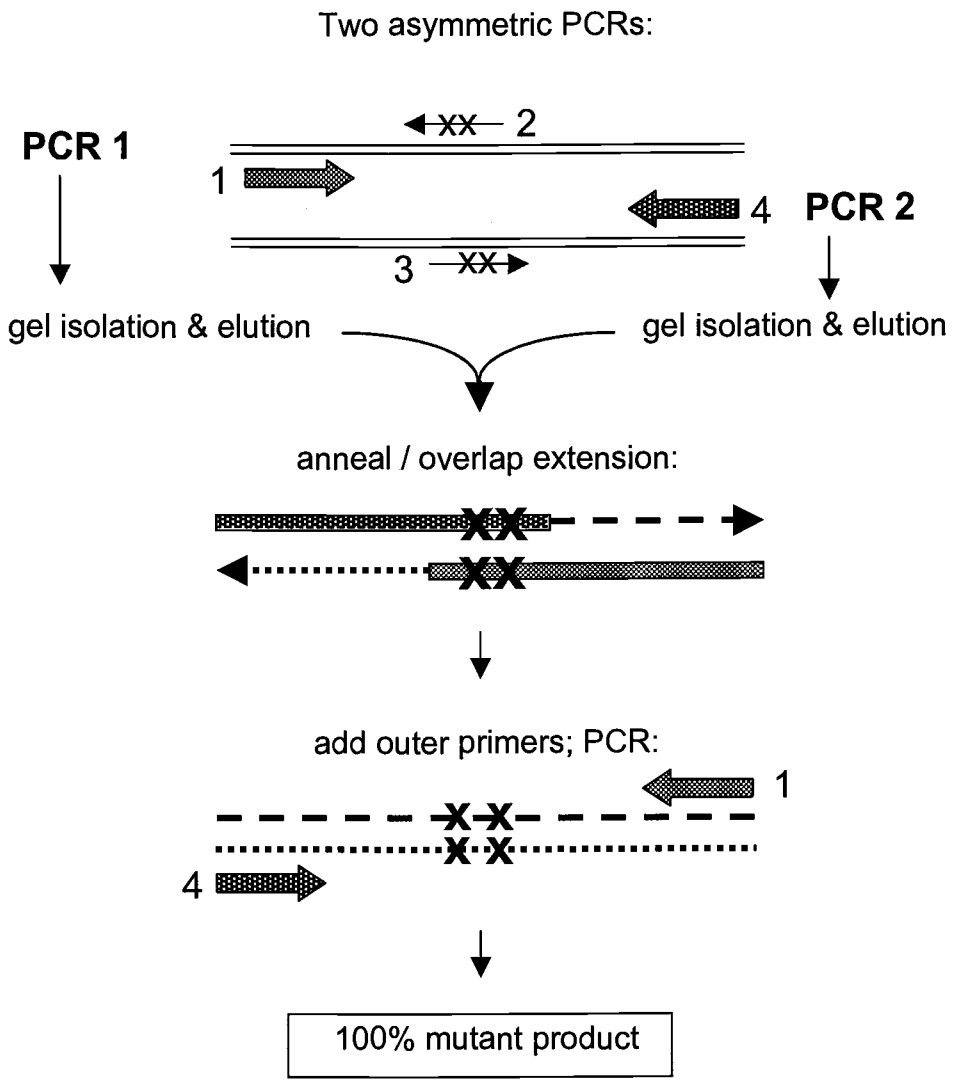
Flow cytometry, based on the method described by Thorgaard *et al.* (30), was used to assess the ploidy of all fish in the DMBA-exposed lots. Blood samples were taken shortly after death by expressing 20-30 µl from an incised gill into 500 µl Alsever's anticoagulant buffer (2% glucose, 0.8% trisodium citrate, and 0.4% sodium chloride) at 4 °C. Blood samples were then frozen in liquid nitrogen for storage at -80 °C. For cytometry, the samples were thawed on crushed ice and then 100 µl of each filtered through a 40 µm nylon mesh (Small Parts, Miami Lake FL) into 400 µl of an ice-cold stock solution consisting of 0.05% Triton X-100 and 0.25 M aqueous Na_2EDTA in PBS. At least 20 minutes prior to cytometry, 50 µl of a 500 µg/µl propidium iodide solution was added with gentle mixing to each diluted sample. Flow cytometry was conducted using an EPICS V cytometer (Coulter Electronics, Hialeah FL) with 488 nm argon laser fluorescence excitation. Data were analyzed for integrated fluorescence on a CICERO system (Cytomation, Ft. Collins CO). Integrated fluorescence histograms of a minimum of 1000 cells were found to unequivocally demonstrate ploidy. In all the approximately 1300 fish examined, save one, the integrated fluorescence was interpretable as showing that the sample represented either diploid or triploid fish. In a single exception, the integrated fluorescence was readily interpreted as indicating tetraploidy.

Site-directed mutagenesis

We sought validation of our primary mutation detection method of direct sequencing of PCR products for the limiting case of the p53 gene. There are no known trout p53 DNA templates containing mutations. Our approach required the synthetic generation of such mutations. However, to circumvent the disastrous consequences of

contaminating our labs with a synthetic mutated template, we sought to make a p53 construct containing a specific mutation that was useful as a "typical" mutation but that would have negligible possibility of occurring in nature. This was accomplished by creating a mutated sequence containing the archetypal liver mutation at codon 249, but conjoining it with a second mutation six basepairs downstream. We achieved this using a low cost PCR-based method that was conducted in such a way as to assure that no contaminating normal p53 template would be present in the final construct. Overlap extension has at least been theoretically a viable means to accomplish site-directed mutagenesis since 1989 (31). We utilized asymmetric PCR to generate excesses of the overlapping components (Figure 4.3). An approach very similar to this was subsequently published by Warrens *et al.*(32). Our method differs in substance from theirs only in that we utilize a simple and efficient acrylamide gel isolation of the asymmetric PCR product. However, this difference assures that the desired single-stranded DNAs are the only ones participating in the subsequent overlap extension reactions. Our own experience, and that of another laboratory we are in contact with, has shown that it is often difficult to successfully overlap-extend such PCR products unless they are gel purified. The reason for this may be due, at least in part, to carryover of primary amplification primers, which then compete to make shorter and undesired products.

Figure 4.3. PCR-based site-directed 100% mutagenesis. Outboard primers 1 and 2 are used in at least 20-fold excess to drive formation of a large excess of single stranded products complementary to the sense of the mutagenic primers 3 and 4. Mutation sites are designated with "X". Gel isolation refers to acrylamide only, since agarose will not resolve the ssDNA products of the initial asymmetric PCRs.



Scanning fluorescent densitometry-relative quantification procedure

A hexachlorofluorescein- ("HEX", Perkin-Elmer Corp., Foster City CA) labeled primer (*i.e.*:5'- HEX-TTAGGTTTTGGTTGAAGA) was synthesized and polyacrylamide gel-purified by Life Technologies (Gibco BRL, Gaithersburg MD). This fluorochrome-labeled primer was used for PCR in separate reactions from templates containing the desired mutated and normal sequences respectively. Sets of 8 reactions each, of the resulting mutated and normal fluorescent PCR products were then pooled to give mutated and normal pools of PCR product. Samples of each of the respective pools of product were loaded into alternate lanes in sixteen lanes of an acrylamide mini-gel. These were then electrophoresed, typically at 10 volts/cm for one hour. After electrophoresis the gel was briefly destained in distilled/deionized water for twenty minutes, using care to avoid any contaminants with strong fluorescent components. The respective sets of bands were quantified using a laser-excited scanning fluorescent densitometer, the Hitachi FMBIOII (San Francisco CA) Fluorescent Image Scanning Unit running FMBIO2 ReadImage version 1.1 and FMBIO Analysis version 6.0.11 software. To improve uniformity, fluorescence signals on gels were typically read twice, once from each side. The mean integrated fluorescence of the two readings for each band representing a given sample pool was then averaged across typically 8 similar lanes to give the mean working fluorescence value. This mean value was then compared to a standard curve generated by quantifying the integrated fluorescence of known concentration increments of an essentially identical HEX-labeled PCR product. The FMBIOII emission filter selected for all of this work had a transmission maximum at 585 nm, and full-width-at-half-maximum (FWHM) bandwidth of 40 nm. For the radioactive sequencing TOD templates, the gel fluorescence quantifications were conducted at a minimum in triplicate, and the mean values were then used to adjust the concentration by dilution so that the two purified products of the PCRs (normal and mutated) had the same measurable concentration of HEX-labeled DNA. For the fluorescent automated sequencing TOD effort, the template quantification was conducted in a similar

manner. Experiments indicated that the presence of the HEX label on sequencing templates was without quantifiable effect on the results of the ABI 373 sequencing output.

Threshold of mutation detection

For the radioactive manual sequencing, mixtures of mutated and normal PCR products were prepared to give final template mutated/normal ratios of 1:10, 1:20, 1:60 and 1:120, then directly sequenced as usual with Thermosequenase (as described below). The resulting dried gel blot was exposed to a storage phosphor image plate and then read on a Molecular Dynamics Phosphorimager following their protocols.

For the fluorescent automated sequencing with fluorochrome-labeled dideoxy-terminators run on an ABI 373 instrument, a similar series of mixtures were made utilizing a positive displacement microdispenser capable of precisely delivering submicroliter volumes (Drummond, Broomall PA). These were done in two series, one in which the normal template was the dominant component and another in which the mutated template was the dominant component. This additional complication was introduced to reveal any allele-specific PCR bias that might be present, and as a precaution to provide redundant measure of the thresholds and as a further check on the relative quantification of the input mutated and normal templates.

RT-PCR (cDNA syntheses)

Qiagen reagents for cDNA synthesis were used as described in their protocol for reverse transcription. Again, these reactions were conducted in the presence of the Prime RNAase inhibitor. A random 9-mer (Stratagene, La Jolla CA) oligonucleotide mixture was used for random priming general cDNA synthesis from the mRNA template. Subsequent specific PCR was conducted using the Expand High Fidelity PCR system from Roche Molecular Biochemicals (Indianapolis IN) as described in the following section. Concentrations, purity and size identity of the PCR products were estimated using 6% native 19:1 acrylamide/bisacrylamide mini-gel electrophoresis

with either taurine/EDTA or TAE buffer. Archive imaging was by conventional photography of UV fluorescence of the ethidium bromide-stained DNA products.

Polymerase chain reactions (PCRs)

With occasional exceptions noted elsewhere, all PCRs shared the following: Boehringer-Mannheim/Roche Molecular Biochemicals (Indianapolis IN)"Expand High Fidelity" polymerase and dNTPs, gradient or linear block 96-well thermocyclers (Stratagene, La Jolla CA), and primer sets as described below. Reaction volumes were typically 10 to 16 μL. One "drop" (15-20 μL) of molecular biology grade mineral oil is used on each reaction, "hot-top" oil-free PCRs were avoided to conserve on reagent costs. Unless otherwise noted, a typical 10 μL reaction had 0.2 to 0.3 units of the polymerase, 20 μM of each dNTP, 30 nM of each primer, 10-50 ng of genomic DNA template, one μL of the 10X buffer with $MgCl_2$ to a final Mg^{2+} concentration of 1.5 mM. Cycling parameters were typically as follows: an initial preheat at 95 °C for 3-5 minutes, followed by 36 cycles, each consisting of; denaturation at 95 °C for 45 s, and appropriate anneal temperature (50 °C to 70 °C, see Tables 4.4 and 4.5) for 60 s, extension at 72 °C for 45 s plus 60 s for each kbp of expected product length. At the end of the cycling, a single final extension at 72 °C for twice the duration of the penultimate extension period.

PCR cloning

PCR products were cloned using Invitrogen (San Diego, CA) TA cloning kit, following the manufacturer's protocols with the exception that half-size transformations were frequently used. Transformants were generally selected on kanamycin/LB plates, and positives were further selected on ampicillin/LB in 3 mL overnight agitated cultures. Colonies and overnight cultures were screened for appropriately sized inserts by PCR and gel electrophoresis, using primers PCRIIF and PCRIIR (see Table 4.5). These primers anneal to the p*CRII* TA cloning vector in the regions immediately flanking both sides of the insert site. Plasmid isolation was with Qiagen's QiaPrep kits, following the manufacturer's instructions.

PCR primers

All oligonucleotide primers were synthesized at the Central Services Laboratory of the Center for Gene Research and Biotechnology at Oregon State University (Corvallis OR), on Perkin Elmer, Applied Biosystems Division 380 or 394 DNA synthesizers using cyano-ethyl phosphoramidite chemistry and controlled pore glass bead columns, with cleavage in 30% NH_4OH and deblocking at 55 °C overnight. Without further purification, the primers were dissolved in 0.5X TE for high concentration (150 to 300 μM) frozen storage, and diluted to 10 μM in distilled and deionized water, stored at 4 °C for working stocks. Table 4.4 shows data relating to the PCR primers used in our p53 work. Figure 4.4 provides a primer map to show the approximate positions of these p53 primers.

Sequencing

Sequencing of DNA samples from liver tumors and some stomach tumors was conducted using United States Biochemical (Now USB, Cleveland OH) kits. In most of our p53 work, sequencing was done with their DeltaTaq kit; later their Thermosequenase ^{33}P-radiolabeled dideoxy-terminator kit was used. In early work sequencing templates were prepared from PCR primary amplifications using a modification of Qiagen's Qiaquick protocol (essentially a doubling of all binding and rinse steps, and very careful removal of residual ethanol prior to the elution step). In all work since mid-1997, PCR products to be sequenced have been digested with exonuclease I (to digest all single-stranded DNA and thus remove PCR primer carryover) and simultaneously with shrimp alkaline phosphatase (to dephosphorylate residual dNTPs). Both enzymes are available from Amersham as their "PCR Product Pre-sequencing Kit". After electrophoresis, sequencing gels were rinsed in 20 % methanol plus 7-10 % acetic acid fixative solution to remove urea before vacuum drying on filter paper. The dried gels were exposed to Molecular Dynamics (Sunnyvale, CA) storage phosphor screens for 10 to 36 hours, then read on a

Table 4.4. Primers used in PCRs of trout p53[§].

#:	Name	Exon	5'cDNA situs	Primer sequence 5'→ 3'	~Anneal °C
	Exonic primers:				
1	5'UTRA	Pretr.	U-45	CTTTTCTTCCCTGCGTGCTGCTTTGAGGTGCTA	70
2	2E5	5	U418	AAGTTGTTCTGCCAGTTGGC	60
3	cE5C	5	L426	CCCCAGGAGGAGGAGGGTGGTC	69
4	E5	5	U427	GCAGTGGTACGAGCCCTGG	62
5	2E5	5	L519	GTTCTCGCTGGTGCTCTGGTG	58
6	E6new	6	U547	CTGGTCAGAGTTGAGGGGAAC	64
7	cE6	6	L576	GATCGCTGGTTCCCCTCAAC	62
8	EX6-7U	6	U606	CACAGTGTGCTCGTCCCCTATG	68
9	E7	7	U642	GGGATCAGAGTGTACCACTG	64
10	cE7	7	L745	TGTGTCTCCAGGGTGATGATG	62
11	E8	8	U757	CTCCTGGGTCGGCGCTCCTTT	64
12	cE8	8	L863	GGTCTCCAGGGTTGTCTCCTG	67
13	3'UTRA	Posttr.	L1222	AATGACATGGAACCGAATCTCGCTCTCCTTCTC	70
	Intronic primers (situs relative to nearest intron/exon boundary):				
A	EX5PreU3		exon 5 – 82	ACACTGGGATTAGAACAA	49
B	EX5SEQD		exon 5 + 83	CTATACTGCATCAAAGCACATTCTAG	70
C	EX5PreR2		exon 5 + 147	TCATTCAATAGGCAACAA	49
D	EX7PreU2		exon 7 – 191	TTAGGTTTTGGTTGAAGA	48
D'	HEX-EX7PreU2		exon 7 – 191	*TTAGGTTTTGGTTGAAGA	48
E	p53I7REV		exon 7 + 45	ACAATCAATGGAGCAACAGTCTA	70
F	EX7SEQD		exon 7 + 48	AAGACAATCAATGGAGCAACAGTC	60
G	EX7PreD4		exon 7 + 117	GACTGAATGTAAATGGAA	48
H	EX8PreU2		exon 8 – 158	CCATTTACATTCAGTCAT	50
I	EX8SEQU2		exon 8 – 64	GACCCCCAACTAATGTCTTGTC	66
J	EX8PreD2		exon 8 + 146	CTCAGAGTCATAGGTCAA	50

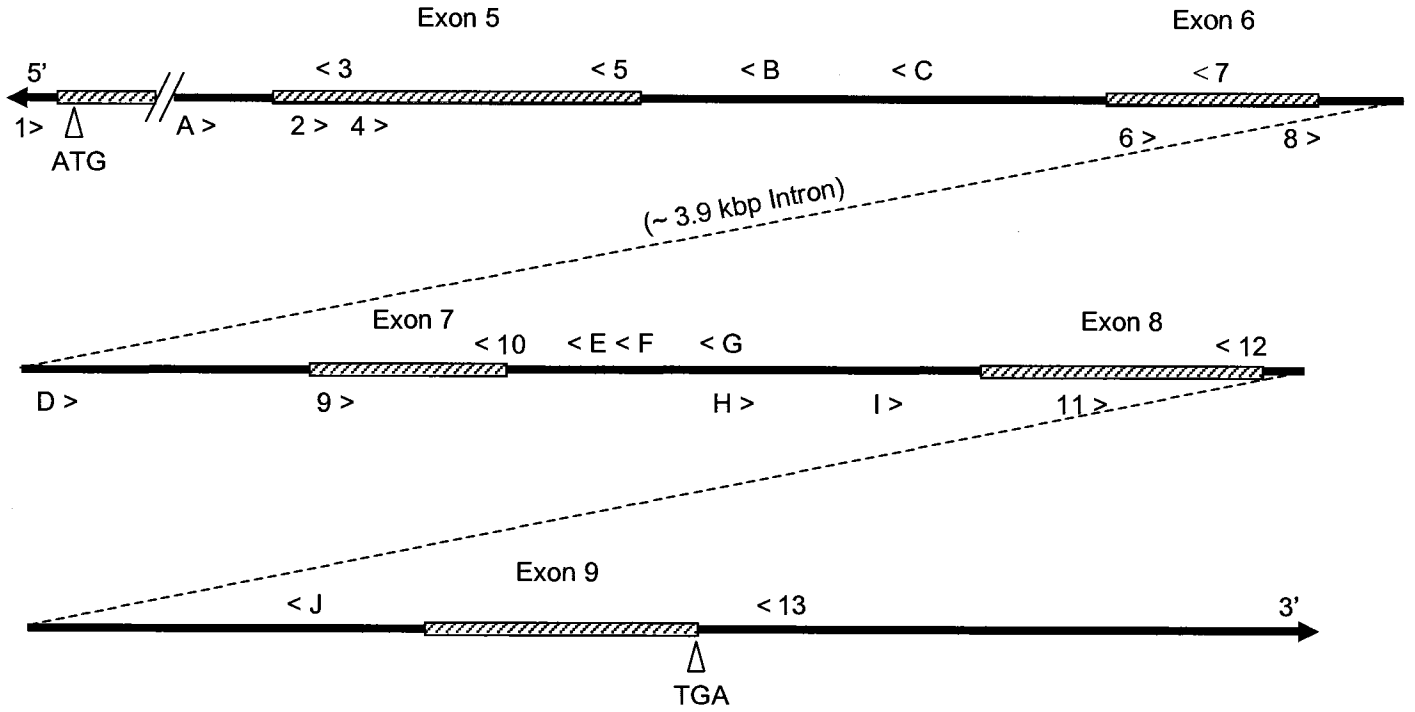
§ Sequences based on three sources: *a)* (21), *b*) (33) and *c*) flanking portion of the trout p53 intron lying 5' of exon 5, see Figure 3.10A; and the intron lying between exons 6 and 7, see Figures 3.9 and 3.10B "#:" heads the column containing alphanumeric designators which show each approximate 5' position on the primer map: Figure 3.2 "*" indicates 5' labeling with the fluorochrome, hexachlorofluorescein, "HEX".

Table 4.5. Primers* useful in diagnostic PCR of TA clones.

Name	Primer sequence 5'→ 3'
PCRIIF	TACCGAGCTCGGATCCACT
PCRIIR	CCAGTGTGATGGATATCTG

*:These anneal flanking the insert site of the p*CRII* TA cloning vector (Invitrogen, San Diego CA) (34).

Figure 4.4. Trout p53 PCR primer map. The horizontal portions of the map are drawn to scale, the dashed diagonal lines are not. The map omits most of the structure between exons 1 and the intron preceding exon 5. The numbers (annealing to exons) and letters (annealing to introns) approximate the physical positions of the respective primers, and refer to the designations in Table 4.4. The ">" and "<" symbols indicate primers annealing to the lower and upper strands respectively.



Molecular Dynamics PhosphorimagerSI workstation running ImageQuant version 5 software. In cases where quantitation of bands was necessary, the Molecular Dynamics ImageQuant Tools version 2.0 program was implemented. Fluorescent automated sequencing reactions and electrophoreses of all other stomach tumor DNA samples was done by the Oregon State University core facility: the Central Services Laboratory of the Center for Gene Research and Biotechnology. All templates for fluorescent sequencing were prepared by digesting candidate PCR products with exonuclease I and shrimp alkaline phosphatase as described above. These sequencings were conducted using standard ABI Prism™ (Foster City CA) 373A DNA Sequencer Stretch with 370A automated electrophoresis/detection unit and ABI 373A data collection software, version 1.2.1 and Sequencing Analysis software version 3.0. Quantification of individual peaks was accomplished by porting the 373A data over to ABI Genescan® software, v. 2.1.1 with appropriate matrix installation. For comparative purposes or when mutation detection was not an issue, the 377 DNA Sequencer was sometimes utilized with ABI Prism™ 377XL Collection software (Foster City CA). All fluorescent sequencing made use of "BigDye™ Terminator Cycle Sequencing chemistry and AmpliTaq DNA polymerase, type "FS".

Transintronic PCR for intron sequence characterization

In several instances, we required information about the sequences in flanking introns that were unknown or unavailable from any known source. To acquire these data we used known effective opposing primers from the respective flanking exons to PCR across the intervening intron. The resulting PCR products were in turn "probed" by PCR reamplification using various other available primers, some of which would have been expected to give a product of a specific length and other primers would not have been expected to anneal within the original PCR product. Such probing is very convenient and very reliable when numerous exonic primers are available, as in this case. Transintronic products that proved to be good candidates were then either

directly cycle-sequenced, or in some cases the product was TA cloned before sequencing.

Partial p53 intronic sequence data was published by Kusser *et al.* in 1994 (33), but was lacking sequence data for one intron flanking exons 4 and 5 and one intron flanking the 5' end of exon 7. We believed that it was important to have intronic preamplification primers for both flanking introns of the three hotspot-containing exons (5, 7 and 8). The direct approach to this problem was available since we had already gained some of this valuable intronic sequence data through transintronic PCRs and direct sequencing before the Kusser publication. With the development of facile "long PCR" also in 1994 (35), it became a simple matter for us to conduct PCR across the previously elusive, 3.9 kbp intron between exons 6 and 7 (Figure 4.4) that had apparently also eluded others (33). The relevant sequence data from this work is reported under "Results of transintronic PCRs" below (Figure 4.8).

Results

Threshold of mutation detection

For radiolabeled sequencing, Figure 4.5 clearly shows, at least in our model, a threshold of detection at or below 5% of mutant in normal. The fluorescent automated sequencing results shown in Figure 4.6 suggest that under that now common place technology the threshold is considerably higher. Certainly 20% is visible and readily interpretable (Figure 4.6). However 10% appears at the limit of the present reach of this technology, at least in this demonstration. Efforts to improve the fluorescent 10% and 5% reads by re-analyzing the electrophoretic data using Perkin-Elmer ABI Genescan software, while showing modest improvement (data not shown) was insufficient to confidently change the threshold estimate of 10 to 20 %.

Figure 4.5. Radiolabeled threshold of mutation detection. Sequencings conducted as described in the text. Terminated reactions loaded as six adjacent lanes per base (left to right: 6A, 6C, 6G, 6T) containing indicated percentage of mutant template, each balance consisting of normal. The double mutant construct shows 100% C →A at the far left illustration (A lanes), and 100% A →T in the first of the T lanes. The mutated template is visible down to 5%. For this work image processing was conducted without removal of background signal. Mutation containing band sets are indicated by arrows.

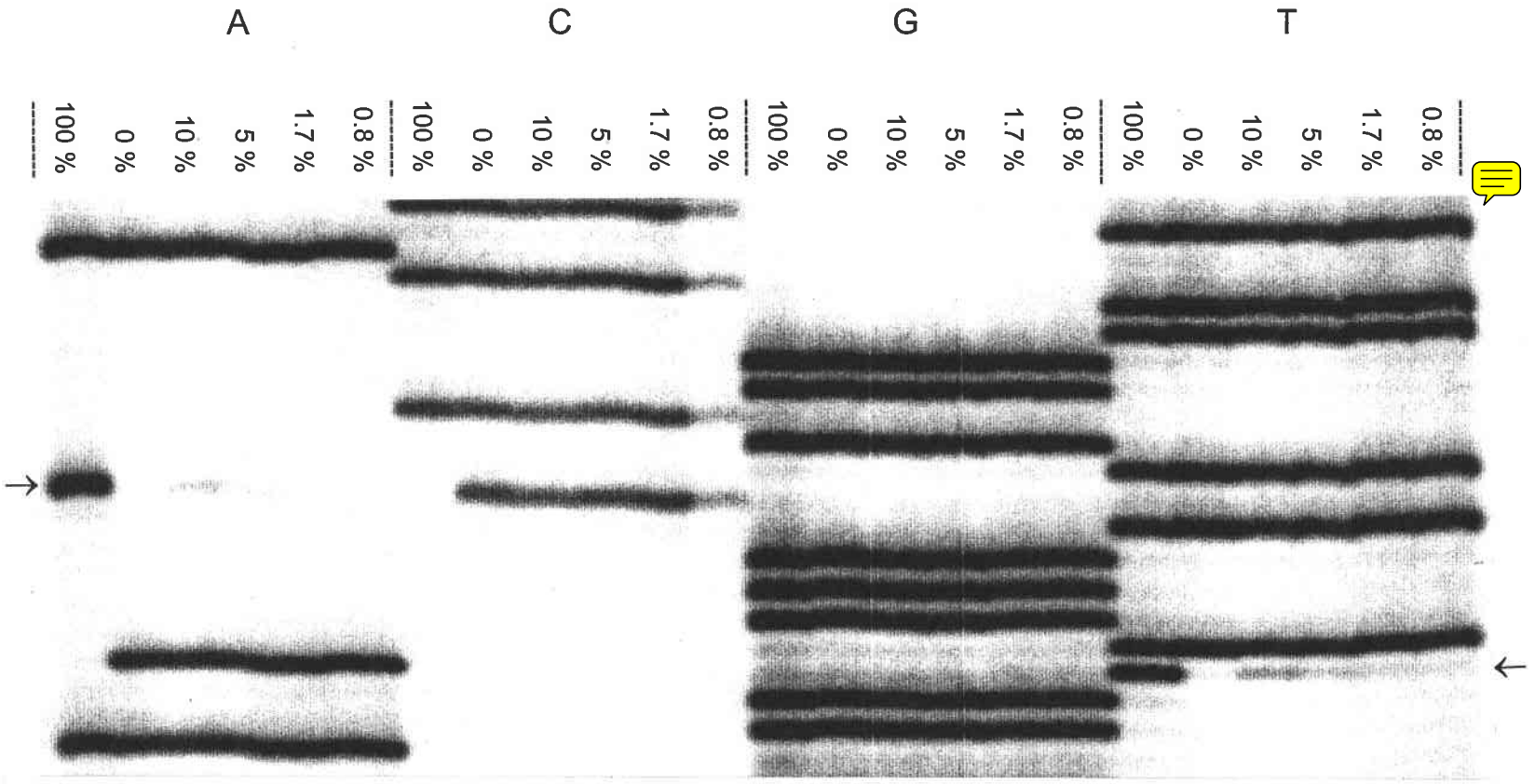
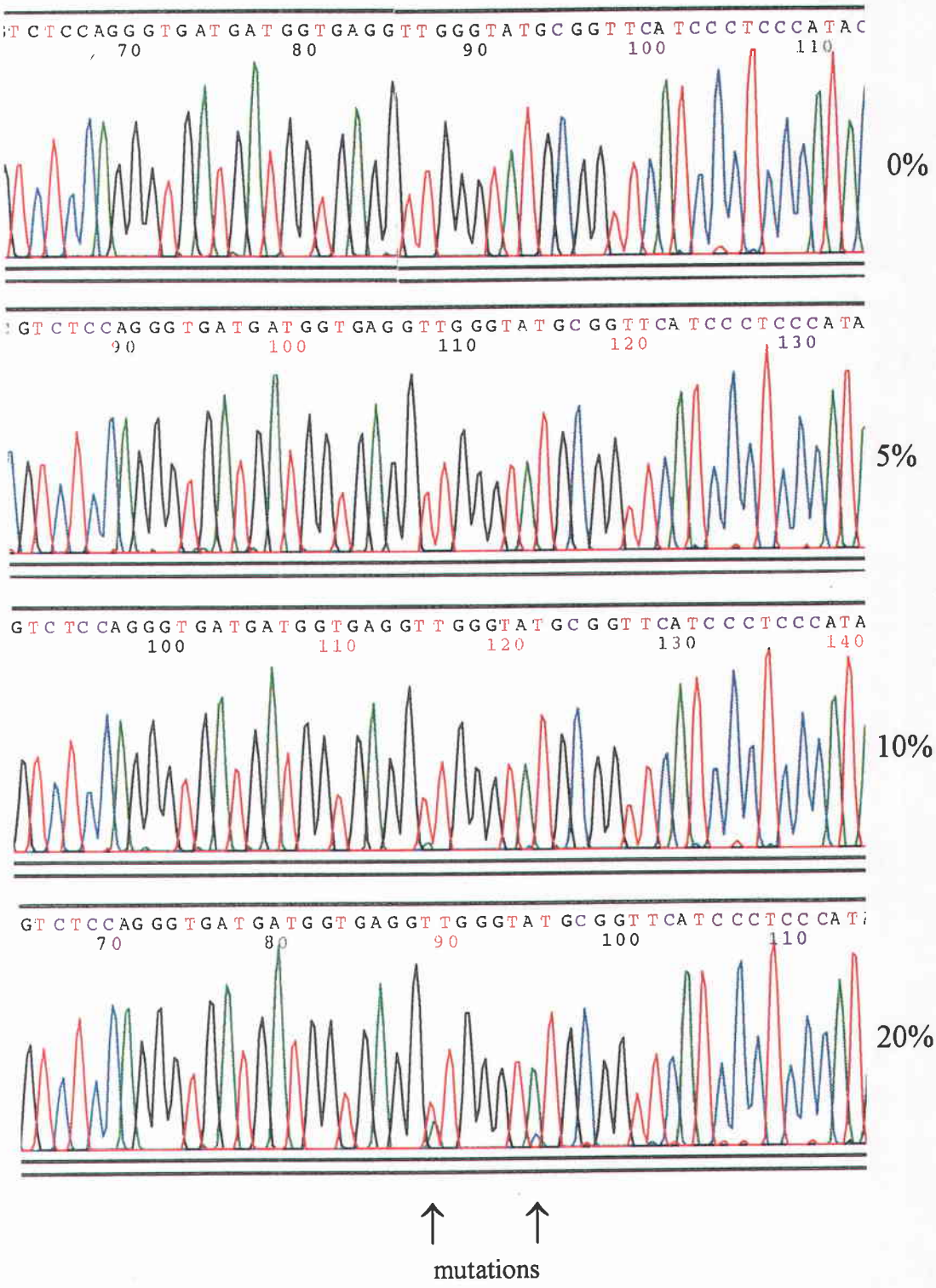


Figure 4.6. Fluorescent threshold of detection

Sequencings conducted as described in the text. The set of reactions visualized here consist of normal p53 template up to 20% in against a mutant background. Arrows point to the two synthetic polymorphic positions.

Figure 4.6. Fluorescent threshold of detection.



Results of transintronic PCRs

The transintronic PCR product (not shown) between exons 4 and 5 was directly sequenced to yield the useful partial sequence shown in Figure 4.8 A, heretofore unpublished (33). The "long" transintronic PCR (see Materials and Methods of Chapter 4) product between exons 6 and 7 of trout p53 was found to be ~3.9 kbp (Figure 4.7). This PCR product was TA cloned and partially sequenced to establish the useful flanking intronic sequence adjacent to the 3' end of exon 6 and the 5' end of exon 7, the latter shown in Figure 4.8 B.

Figure 4.7. Gel image of p53 exon 6 to exon 7 transintronic PCR product. Shows the right most and leftmost marker lanes (*Bst* EII digests of λ phage, band sizes indicated to right of image) and the two adjacent "index" lanes in apposed gel slabs so that the 3.9 kbp bands appear as one. Only these portions of the gel were imaged to avoid UV exposure of the excised 3.9 kbp product. Direct sequencing of the product enabled design of a flanking intronic and sequencing primers for low noise sequencing of p53 exon 7, which contains hotspot codons 248 and 249.

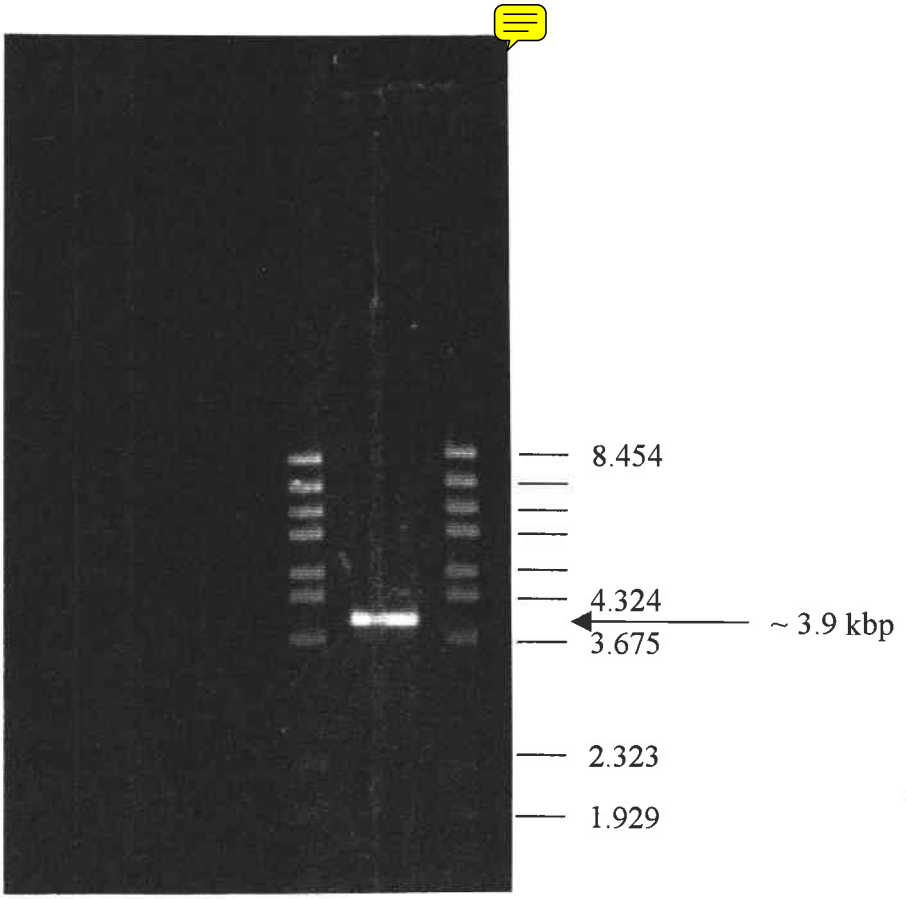


Figure 4.8. Trout p53 5'-flanking intronic sequences for exons 5 and 7

A: Exon 5, 5' flanking sequence

gttgtccata atatctatca cactgggatt agaacaatgc tgacaggtta
aactaagacc taagtattgt ttttgattta acaaactctt tctctctcc**a**
gtactcgcca gacctgaaca

B: Exon 7, 5' flanking sequence

cttttcaca caagggtatt gggtgctgca taaatttgtt tatgtaagtg
ttaatcattc gggttccctt tatctaatat taggttttgg ttgaagatct
gcttaacatt ccgcttcnca aatatncaga agtagagagg atcagagagg
gggcacatac tttttcacag cactgtatat gtttacactg tatgtgtttt
gtgttgtatg ggggagtgtg tgtttggggg tgtccatcag tcacagctgt
ctctctatct ggtttccc**ag** gtgggatcag agtgtaccac tgtgctctac

Note that the 3' intron excision terminal motif "**ag**" immediately precedes 5' portion of each previously characterized exon.

Absence of detectable mutations in p53

PCR products from liver tumor DNAs of DMBA-exposed triploids, amplified across all of exons 5, 7 and 8 (those containing the bulk of the mutational hotspots in mammalian tumors) showed no mutations, at or above the present limit of detection by radiolabeled sequencing (5% mutant template detected against 95% background of normal). PCR and direct automated fluorescent sequencing of the 200 liver tumor collective cDNA library (Figure 4.9) taken from the aflatoxin B1-exposed diploids also showed no hotspots approaching the apparent 10% to 20% threshold of fluorescent automated detection, nor did it reveal any indication of alternative splicing of the p53 gene as has been reported in some species. Examination of 16 stomach tumors across the same 3 exons showed no p53 mutations. Analysis of p53 mutations in the liver tumors of AFB_1-initiated fish was only done by sequencing a collective cDNA library representing ~200 liver tumors. There was no evidence for p53 mutations observed in the ~200 tumor collective library (Figure 4.9)—suggesting that

no hotspot for p53 was present at or above our 10 to 20% threshold of detection for mutated templates by fluorescent sequencing. As in the liver, no mutated p53 was detected in the DMBA-initiated stomach tumors of either ploidy. Our analysis based on the human mutation spectrum of p53 suggested that this might be the most likely organ in which to find tumors with such p53 mutations. However, in an examination of 16 stomach tumors, from 8 triploid and 8 diploid fish, there were no p53 mutational hotspots in exons 5, 7 or 8 showing detectable mutations on fluorescent sequencing rising to our 10-20% threshold of detection. Further, no p53 mutations were detected in triploid liver tumors for these same exons down to our lower manual radioactive sequencing TOD of 5%.

Discussion

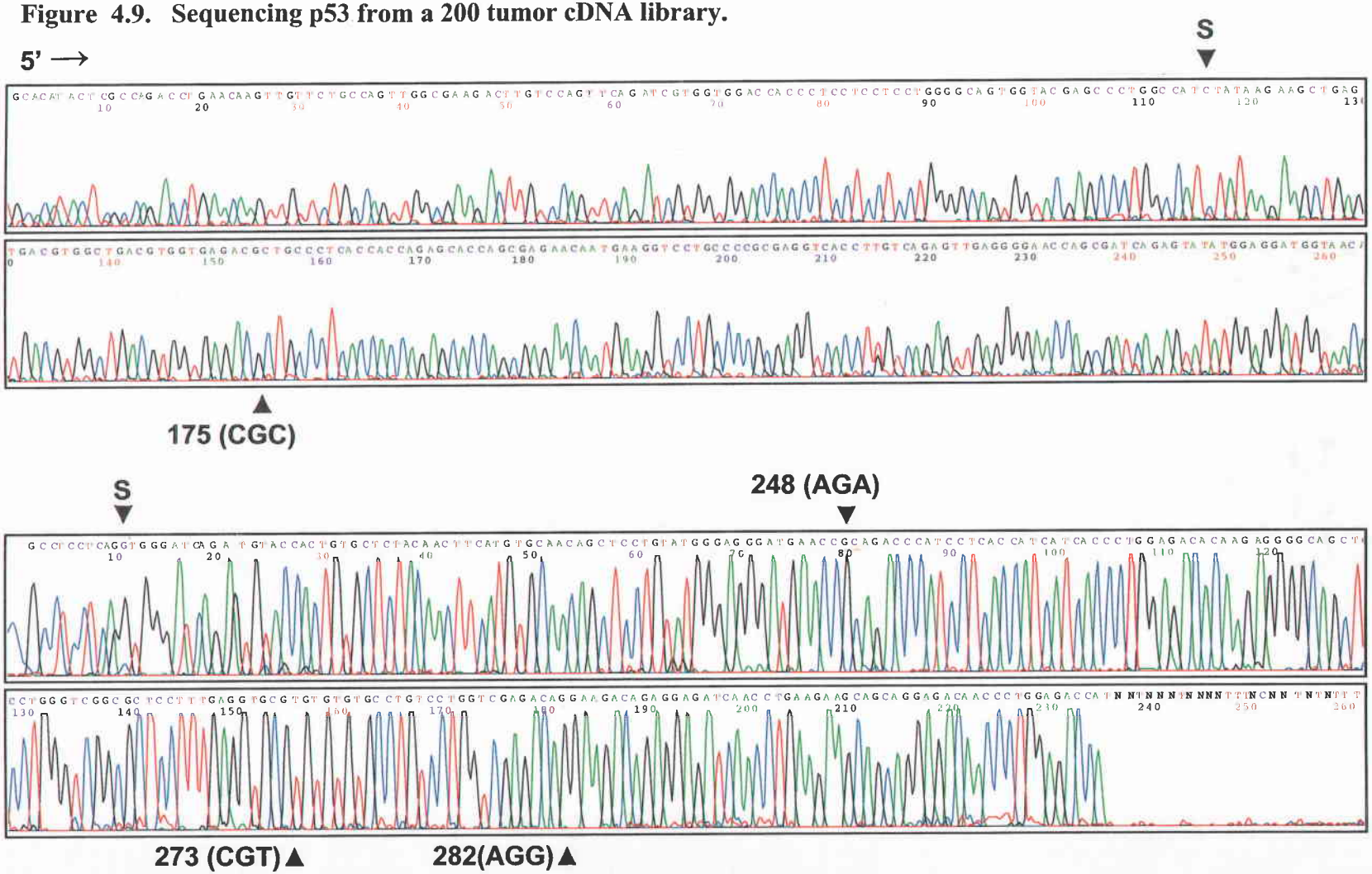
The aim of this study was to determine the effect of trout triploid tumor suppression mutation incidence and position within the trout p53 gene. In spite of some evidence that this trout p53 is a true functional ortholog of the human p53 (21), we found no p53 mutations in DNA from any liver or stomach tumor, nor in any liver cDNA, regardless of ploidy. p53 and the closely related gene p73 have been reported to be subject to developmentally related alternative mRNA splicing. Such splice alterations might be expected to be found in tumors. But, the direct PCR sequencing of the 200 tumor library (Figure 4.9) showed no evidence of alternative splicing at a level that would reach the 10 to 20% threshold of detection. We conclude that this trout 53 does not exhibit substantial alternative splicing in tumors. This does not necessarily preclude the possibility that alternative p53 splicing may still play a role in development.

Threshold of mutation detection

Our results (Figure 4.5) for radiolabeled sequencing threshold of detection at 5% or lower are clearly better than conventional estimates of 30% or more. We believe that a substantial portion of the improvement stems from the use of

Figure 4.9. Sequencing trout p53 from a 200 tumor cDNA library. All four panels are sequencings from the 5' direction. The upper pair of panels sequence from exon 4 and the lower pair from exon 6. All p53 human hotspots are covered, of those 175, 248, 273, 282 are so marked. "S" marks indicate probable silent polymorphisms that most likely represent interindividual genetic variation.

Figure 4.9. Sequencing p53 from a 200 tumor cDNA library.



the labeled dideoxy terminator method and from the use of phosphorimaging rather than film. Considerable effort was made to eliminate spurious templates from interfering at both the primary amplification and sequencing extension stages of the direct cycle-sequencing protocols. Transintronic PCRs were used to identify flanking intronic regions of p53 for design of both primary amplification PCR primers and for the nested sequencing primers, even though there was little evidence of competing pseudogenes or paralogs seen in numerous transintronic PCR products from that gene.

Our result with the fluorescent automated sequencing threshold on detection at 10 to 20% are less impressive, but are consistent with at least one reported effort using "dye-on-primer" technology (36), in which a 10% threshold was apparent. As in radioactive manual sequencing,, the fluorescent system also uses labeled dideoxy terminators, and hence might have been expected to deliver similarly enhanced performance. However, fluorescent automated sequencing is very often the purview of an institutional core facility, where uniformity and productivity are paramount considerations. Furthermore, the competition amongst sequencing apparatus manufacturers to meet those demands may combine to assure that sequencing systems are not optimized for low level mutation detection against a background of normal. The vast majority of sequencing projects for which such equipment and techniques are designed is simply calling bases for which one occasionally sees a 50:50 polymorphism. For such base calling the systems are admirably efficient and cost effective. The ABI 373 and 377 systems detect all four fluorochrome labels within the same lane, unlike conventional radiolabeled sequencing in which each base ladder occurs in separate lanes. Each of the four fluorochromes (TAMRA, ROX, JOE and FAM) used in the ABI system produces some fluorescence in substantial overlap with each of the other fluorochromes. Deconvolving the fluorescence emissions of the four fluorochromes necessarily involves compromises at the optical and at the signal processing level since cost of the system and velocity of base calling are primary constraints on design. We believe the net result is a considerable compromise in detection efficiency with respect to noise and background signals seen in our 10 to 20% threshold of detection. This conclusion is hypothetical and certainly could be put

to a relatively simple experimental test by simply using single lanes for each of the four fluorochromes. In performing automated sequencing this way it is likely that the necessary number of pipetting steps, extension reactions, and gel loading labor would increase by as much as a factor of four. One can surmise that such experiments would be somewhat costly but the labor involved would be no more than conventional radiolabeled manual sequencing.

The Perkin-Elmer ABI Genescan software analytical tools allow the each of the four individual signal graphs to be visually represented separately. They also enable one to magnify either or both axes. Applying this feature to the vertical axis can readily increase the apparent amplitude of small peaks, while applying it to the horizontal allows focus on a particular peak or portion thereof. These tools also allow one to highlight a peak and compute integrated area. In spite of such capabilities, there was a lack of substantial improvement from re-analyzing the electrophoretic data using Genescan software, due largely to the fact that the background or noise signals are also expanded by such manipulations. The results thus may indicate either practical or fundamental limits to discriminating singular, low level events from a background of noise and noisy signals. Nevertheless, such tools might be useful in specific situations, particularly if one able to predict exactly where on an electropherigram to look. Such situations may include, for example, *ras* mutation analysis.

Can collective tumor material provide a valid mutation spectrum?

The absence of p53 mutational hotspots that rose to the 10-20% threshold of detection in the collective sequencing of the 200 tumor cDNA library should not be construed to suggest that there were no p53 mutations there. However had these been human tumor cDNAs from liver tumors, it is likely that at least codon 249 would have exceeded the threshold. The prospect of collective screening to establish mutation spectra from large numbers of tumors, while promising, must be tempered with some reservations. The first is the difficulty in assessing the relative contribution of a particular mutant oncogene or tumor suppressor to such a collective tissue pool where

gene amplification may affect a particular gene or its mutationally activated form. Gene amplification will typically selectively elevate mRNA and hence the cDNA from such a pool. Similarly gene deletion or gene inactivation are known to occur, especially in tumors—such events can bias any results and conclusions that may be drawn from such collective library analyses. Further, there is the difficulty in assuring that all the tumors are of the same mass and that all are equally contributing to the nucleic acid isolation, whether such isolation may be conducted collectively or done on individual tumors. Finally, we must note the remarkable and quite likely non-representative result (Chapter 5, Figure 5.4) seen in our own examination of a collective tumor library, which at least should give caution to any future efforts in this regard. Although it should be noted that the cDNA library examined was a clonally amplified product of the original unamplified library, for which there is the manufacturer's cautionary note that "amplified libraries may substantially alter the proportion of representative cDNA clones present"; referencing Sambrook et al (37).

What may explain the lack of detected p53 mutations?

It was reported that codon 249 mutations were more prevalent in specific areas of the world where hepatitis B virus is also endemic (17). This report and others suggested that there might be a synergistic relationship between aflatoxin mutagenesis and some aspect of these viral infections that cooperates to lead to, or select for, this particular mutation. In a thorough metanalysis, Lasker and Magder found that the commonly assumed causal nexus between human dietary aflatoxin exposure and codon 249 $^{arg \rightarrow ser}$ mutation was yet to be rigorously demonstrated (38). They report a weakness in nearly all the epidemiology in that most of the published studies failed to use a definitive available means to assess adductive aflatoxin exposure, for example by lysine adduction (39). Thus from a comparative standpoint, even assuming all aspects of the carcinogenesis process were homologous, simply giving aflatoxin itself might not be expected to give rise to a comparable level codon 249 mutations without the equivalent cocarcinogenic addition of the equivalent of chronic viral hepatitis.

The apparent absence of any mutations in trout p53 raises a number of other possibilities. Plausible explanations include the possibility that *this* p53 (21) is not involved in trout tumor suppression. It may be that another p53 or p53-like gene in trout expresses the anti-oncogenic functions we associate with human p53. It is possible that we will yet see that there are other hotspots in the trout p53 outside of the human prevalence in exons 5, 7 and 8. There is a distinct possibility that alternative splicing might abrogate normal p53 function since such modifications are reported to modify p53 function in other species. However, our analysis of the 200 tumor cDNA library showed absolutely no evidence of any trout p53 splicing other than the canonical version published by Caron de Fromentel *et al.* (21).

Another area of uncertainty is whether the extra genome of triploids is even functional at the level of expression. The best answer that can be given at this point is that there is no evidence whatsoever that it is *not* active. There are conceivable mechanisms that might inactivate a third genome, such as extensive methylation, but there is no obvious opportunity for such inactivation to occur under the conditions of triploidization employed. The fact that the third genome arises from a polar body, rather than a normal gamete, might be of some concern. However, the genomes contained in polar bodies, such as those retained by our triploidization protocol, have been transferred to enucleated eggs in mammalian species and have proven to viably develop into normal adults (40).

p53 oligomerization; functional implications with respect to ploidy

p53 may be unique amongst tumor suppressor genes in that some of its mutations appear to generate true gain-of-function mutant products (6), including those giving a histidine at codon 175, a tryptophan at 248, and histidine at 273. These three "hotspots" are identical to those mutations of p53 most frequently identified in human tumors (9, 41). Human p53, based on results of another assay (42), is claimed to have over 40 dominant negative mutants, again including codons 175, 248 and 273. The most prevalent explanation for dominant negatives attributes dominance to disruption of the function of the normal tetramer by inclusion of one or more of the mutant monomer products. Since p53 acts as tetramer in many of its cellular roles, it

has been deduced that the dominance of some mutations may be a consequence of reduced functionality of the oligomers containing one or more abnormal monomers (43). Certainly, most, if not all of these and numerous other p53-related mechanisms are dependent on tetrameric p53 (44), which forms via dimerization of dimers (the dimers themselves have few known functions as such (45). One route to diminished function may include failure to oligomerize properly or to oligomerize at all. And certainly most if not all functions of p53 as a transcription factor require the tetrameric structure that brings the DNA contact domains of the protein into functional apposition to the aforementioned p53 consensus binding domain of genes positively regulated by p53. At least for some mutants of the p53 protein, a single mutant monomer complexed with three normal p53 monomers may yield a functionally disrupted tetramer, by inactivating the complex (46). A cursory analysis of the theoretical implications of such a heteromeric mechanism with respect to triploidy, indicates that it may be possible that a reduced gene dosage of mutant p53 in triploids could yield a substantial reduction in the probability of loss-of-normal-function. This in turn could raise the threshold for a cell with a potentially oncogenic mutant p53 to lose normal function or gain abnormal function. In our cursory analysis, which should be viewed only as illustrative, the probabilities are considered for the limiting case of assembling an all normal p53 tetramer containing only normal monomers from a pool representing a triploid cell with a single mutant p53 allele, containing 2/3 normal and 1/3 are mutant monomers. This probability is nominally $(2/3)^4$ or 0.1975. Similarly, the analogous probability in a diploid cell is $(1/2)^4$ or 0.0625. Thus, we may expect that the presence of two normal genomic alleles and one defective allele will inevitably substantially increase the number, and hence the cellular dosage, of the "all normal" tetrameric p53 proteins relative to that expected in a hemizygously mutated diploid. There are many factors not considered in this cursory analysis, such as the nuclear half-life of mutant p53, or the influence of defective monomers on p53 oligomeric assembly rates, or the transport kinetics, subcellular trafficking and compartmentalization, phosphorylation, acetylation or influences on the ubiquitination rates and so on. Nevertheless, the analysis suggests that regardless of the specific

factors that may have been overlooked, one might well expect several times the number of fully normal functional p53 tetramers in a p53 mutant-containing triploid cell than in the analogous diploid cell. One implication of this is that it may be more difficult to observe any kind of dominant negative or gain-of-function activity attributable to p53 in triploid trout tumors.

Using human p53 mutation data to predict trout tumor sample size

In the absence of any prior salmonid data to provide guidelines in sampling p53, the sampling was guided by comparison with human p53 tumor incidence using spectrum and incidence data of human p53 mutations in neoplasia (10). Using these data and other data indicating the organ-specific prevalence of p53 mutations in human tumors enabled us to generate predictions of sampling depth necessary to reach a 95% confidence of detecting at least one p53 mutation (Table 4.3). While the projected required sample sizes in the table were used to guide our experimental focus, the approach has clear shortcomings. Any inferences based on similarity or dissimilarity of the respective human and trout organ-specific incidences and spectra of mutations must be tempered by several caveats: *a)* The human p53 database consists of tumors of largely spontaneous origin, probably resulting mainly from incidental endogenous mutations or mutations resulting from one or more relatively low-level environmental or dietary mutagen exposure. *b)* The trout experimental tumors are products of orders of magnitude greater doses of carcinogen than any likely human exposure. *c)* The human tumors were likely to have been promoted by any of several endogenous or exogenous factors, any or all of which may not be present in trout. *d)* Natural and endogenous promotion in humans is not paralleled by the promotional effects of high carcinogen doses used in these trout experiments. *e)* Fundamental toxicology and tumor biology are likely to be at least somewhat divergent in two species with such different natural histories, occupying such differing habitats and separated by an estimated 380 million years of evolution (47, 48).

Nevertheless, a table such as this may well allow one to answer a number of important comparative carcinogenesis questions. For example, for any interspecies

comparison, is the observed disparity of two intraspecific organ systems greater or less than the disparity seen between two species for any or all of the features in the table? Similarly, one might ask if the disparities (or concordances) in such comparisons are skewed towards certain portions of p53 (or any other cancer gene for that matter). Or, if an experimental model possesses relatively frequent tumor p53 mutations, then such a table can simplify establishment of more universal understanding of p53 structure and function by pointing out the regions whose function is most conserved. In one sense, this has already been done by Soussi and May (49). In their approach, they argue qualitatively from the empirical human database but their comparisons are generally to the expressed structure and function motifs themselves.

Are we examining the right p53?

Since 1997 there have been several p53-like genes identified in the human genome (50-52). At least some of these paralogous proteins are known to have several functions, some of which overlap that of the original p53. It is possible that the p53 gene known in trout might be functioning in a paralogous manner and that there is a yet undetected "real" p53 in these fish. One hypothetical explanation for the lack of apparent p53 involvement would assert that the known trout p53 may actually a processed pseudogene, giving rise to an mRNA but not a translated one. However, evidence to the contrary is available: Caron de Fromentel *et al.* (21) reported that this trout p53 gene indeed codes for the "p53-like" protein with homologous function to the human p53. These investigators, who identified the trout p53, showed that the protein generated by bacterial expression of the trout cDNA, strongly bound the SV40 large T antigen just as mammalian p53 protein does (21). Second, our own p53 sequence data from numerous trout show identical sequences in every case, that is, a complete lack of polymorphisms in exons 5, 7 and 8. Such data are consistent with this presumptive trout p53 gene at least coding for an active functional protein, and probably a vital one under strongly constraining selection pressure.

A final caveat

In spite of the great evolutionary conservation of p53, and its clear involvement in human carcinogenesis, it is only variably reported to be mutated in the tumors of some non-human mammalian species, but not in others (53, 54). The impression we have, from reviewing the available literature, is that p53 mutations are rare in tumors of wild animal populations, but that they may be more common in domestic animal and especially in domestic pets. If this were epizootiologically accurate, it would be consistent with the notion that p53 mutations are most often relatively late events in the carcinogenesis process and hence may only be frequent in animals living to the majority of a mean expected "domestic" lifespan.

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Chapter 5

Ki-ras1 Mutation Analysis of Tumors from Triploid Trout

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Abstract

Tumor incidence induced by dietary exposure to aflatoxin B_1 (AFB_1) or to 7,12-dimethylbenz[*a*]anthracene (DMBA) is consistently and substantially reduced in triploid rainbow trout (*Oncorhynchus mykiss)* compared to their diploid siblings. As part of an effort to understand the contribution of oncogenes to the phenomenon of triploid tumor suppression, we examined the trout *Ki-ras1* gene, an ortholog of the frequently mutated human *KRAS2* gene. We used gene-specific non-exonic primers for *Ki-ras1* to generate primary PCR amplifications from tumor DNA. These were then sequenced by nested non-exonic primers using both radiolabeled manual and fluorescent automated sequencing. Mutation detection by direct sequencing showed a spectrum of codon 12 and codon 61 mutations in the DMBA-fed trout liver and stomach tumors. The total incidence of *Ki-ras1* mutations from exons 1 and 2 showed no significant ploidy-related change in DMBA liver tumor incidence (47% in diploids, 40% in triploids). However, there was a significant ($P< 0.05$) reduction of *Ki-ras1* exon 1 mutations in triploid DMBA stomach tumors from 33% in diploids (6/18) to 5% in triploids (1/20). In an attempt to rapidly assess the mutation spectra of the AFB_1-fed fish, we examined 200 liver tumors (taken from diploid trout in the same experiment) simultaneously, by PCR and cycle-sequencing directly from a collective 200 tumor cDNA library. Direct sequencing for *Ki-ras1* gene-specific PCRs on this collective library revealed a very high level of double mutation in codon 12 positions 1 and 2 of GG → TT dominating the normal signals, suggesting an artifactual result. Further examination of 22 liver tumor cDNAs from these aflatoxin B_1-fed fish, showed a conventional spectrum of codon 12 and codon 61 mutations, but also included a 14% incidence of the 12(1,2) GG → TT double mutant. In the liver tumors from these AFB_1-fed fish a non-significant ploidy difference in the high *Ki-ras1* mutation incidence was seen (75% in diploids, 90% in triploids).

Introduction

A recent study by Thorgaard *et al.* (1) shows suppression of experimental tumor incidence in triploid trout (*Oncorhynchus mykiss*), independent of carcinogen or growth modulation. Their study utilized brief bath exposures of sac-fry to three different carcinogens, aflatoxin B_1 (AFB_1), 7,12-dimethylbenz[*a*]anthracene (DMBA) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). In the present work, we utilized two of the same carcinogens, AFB_1 and DMBA, but in our study, carcinogen exposure was by diet over 30-day and 120-day periods. We used larger numbers of experimental animals in an effort to improve statistical power of the resulting influences on tumor incidence, and to provide sufficient numbers of tumors for molecular analysis.

In Chapters 3 and 4 of this Dissertation, we examined DNA and cDNA of trout stomach and liver tumors for p53 mutations and found none. Here we examine mutation spectra in trout stomach and liver tumors for exons 1 and 2 of for the oncogene *Ki-ras1 (*formerly referred to as Ras A (2-4)). This *ras* gene is an apparent ortholog to human *KRAS2,* one three p21 ras oncogenes found to be frequently mutationally activated in human tumors (human *KRAS1* is a pseudogene).

Ras involvement in mammalian and salmonid carcinogenesis

Ras involvement in the generation of rainbow trout (*O. mykiss*) tumors is inferred by the persistent presence of activating mutations of codons 12 or 13 in exon 1 or codon 61 in exon 2 of the *Ki-ras1* gene (3, 4) in such tumors. These are seen in tumors from trout exposed to a variety of carcinogens and under a great variety of tumor modulating influences (5). The *Ki-ras1* mutations seen in trout tumors of liver and stomach are almost exclusively confined to these three ras "hotspots" regardless of carcinogen or mode of initiation (4, 6). These same hotspots characterize the *KRAS2* mutations reported from human tumors of the pancreas and colon. In humans,

and other mammals for which data are available, liver and stomach tumors are predominantly characterized by mutations of the related p21 ras gene, *HRAS1* rather than *KRAS2*. Again, the mutation spectrum in humans consists almost exclusively of the same codon 12, 13 and 61 mutational hotspots.

General issues relating to successful gene-specific mutation detection

Among the factors that can confound mutation detection, is the presence of pseudogenes within the experimental subject organism. The most common form of these anomalies is the "processed pseudogene" which is thought to typically enter the genome over evolutionary timescales via recombinant insertion of a virally generated reverse transcript (cDNA) into a germ-line cell of an ancestral host and thereby becoming a permanent feature within the descendent population. There are reported examples of processed ras pseudogenes containing activating mutations (7). The presence of such confounding templates cannot be dismissed unless a thorough investigation demonstrates their absence. Another potential source of confounding signals in some forms of mutation detection would be from closely related genes within the individual experimental organism, such intra-organism homologues are referred to as paralogs. In trout, it is apparent that there are at least two closely related *Ki-ras* paralogs (4). If humans and rodents are representative, then other vertebrates may also be expected to have numerous ras-like genes. Mammals are thought to have at least 50 ras-related guanine nucleotide binding proteins (8)

Approaches to "low noise" sequencing for detection of mutations

Any attempt to detect mutations or to otherwise precisely examine selected targets within the context of gigabases of DNA template likely to contain at least 30,000 distinct genes, should be approached with caution. The risk of simultaneously detecting two or more paralogs during mutation detection might be expected to pose some problems for the selective hybridization of oligonucleotide probes and for generation of unique PCR products representing single *ras* or *ras*-related entities. Such entities may readily cause background bands or peaks in sequencing

electropherograms. In any PCR-based analysis, there is the risk of forming chimeras of closely related genes that happen to share strong identity at the primer annealing sites. This tendency to form chimeras can be ameliorated by precautions in conducting the PCR. Such precautions may include using one or another form of "hot start" technology, that is adding some crucial ingredient of the PCR reaction *after* the reactions has reached a denaturing temperature. Such crucial ingredients could be magnesium salt, deoxyribonucleotide triphosphates (dNTPs), the thermostable polymerase or the template. In practice, we have found it is often convenient to add the Mg^{2+}. However, such precautions as "hot start" alone, while helpful, cannot guarantee absence of chimeras. Any partially extended primer whose 3' end happens to be identical to, or nearly identical to, enough unintended complementary sequence in another gene to anneal and extend can give rise to a chimeric product. To avoid such inappropriate extensions, it is often prudent to allow generous extension times in programming PCR thermocycling parameters. Even with "hot start" and generous extension times, it may also be imperative to determine if the candidate primers are positioned to be selective for the desired paralog. One test for the presence of such confounding pseudogenes or close paralogs, is to examine the products of transintronic polymerase chain reactions (PCRs) using primers already known to work well within adjacent exons or in separated exons. If electrophoresis of such PCR products show several band sizes, and subsequent sequencing shows paralogous gene sequences, then the test is positive. In our judgment, and that of others (9), the prescription in these circumstances requires identification of enough sequence from all the paralogs to enable one to design primers (or probes) that can strongly discriminate for the desired gene and against the paralogous entities, whether they are genes or pseudogenes.

Overview of Ki-ras1 upstream analysis

Based on prior work in our laboratory with trout ras (2, 3, 10), we undertook to examine some of the possible paralogous ras genes that might be present in the trout. Transintronic PCRs using known effective *ras* primers (see Results) showed that

there were at least three candidate products possessing sufficient common exonic sequence to readily act as templates for PCR from some primers formerly thought to give unique *ras* products. The transintronic products had sufficiently different included intron lengths to allow electrophoretic resolution and discrimination based on size (data not shown). This finding formed the basis for cloning and sequencing and identification of at least two distinct trout ras genes (4), one of which had been sequenced previously (11), and subsequently designated as "ras B". Another, now designated *Ki-ras1,* has been found to have most, if not all, of the ras mutations heretofore found in trout experimental tumors (4). We utilized the exonic sequence for the *Ki-ras1* gene (2, 3, 10) to conduct two inverse PCR efforts reported below. This inverse PCR revealed for the first time upstream pretranslational genomic sequence for *Ki-ras1*. For another similar effort, we utilized nested sets of *Ki-ras1* specific primers to conduct PCR from template of circularly ligated restriction digests of a trout liver cDNA library. Sequencing the resulting "long inverse PCR" revealed a large pretranslational intron just upstream of the *Ki-ras1* translation start site.

Materials and methods

Chemicals

All chemicals were purchased from Sigma (St. Louis, MO) unless specified otherwise. The carcinogens, DMBA and AFB_1 were purchased from Aldrich (Milwaukee, WI).

Animals

For each of the two studies, several monogamous spawnings containing at least 3000 eggs of the Shasta strain of rainbow trout (*O. mykiss)* were divided into two lots consisting of approximately 35% and 65% of the fertilized eggs. The larger of the lots was subjected to hyperthermic triploidization as follows: At 10 minutes after fertilization the eggs were placed in a water bath at 29 °C for 10 more minutes.

Following this, the fertilized eggs were placed in the incubators at the usual temperature of about 13 °C. The fertilized eggs in the smaller of the lots (normal diploids) were not shocked, but were placed directly in the 13 °C incubators. After the fish reached the feeding stage, sibling lots of diploid and triploid fish were selected to continue through the experiments. For the DMBA experiments 880 fish of each ploidy, and for the AFB1 experiments 770 fish of each ploidy were utilized.

Care, feeding and carcinogen exposure

The diploid and triploid fish were raised in the customary manner at the Food Toxicology and Nutrition Laboratory (FTNL), Oregon State University (Corvallis, OR) as previously described (12). All animals were treated in accord with Federal guidelines (13) and protocols approved by the Oregon State University Institutional Animal Care and Use Committee. The trout were raised to the age of about 5 months before initiating the dietary carcinogen exposures. The diet for the fish was either standard Oregon Test Diet, (OTD) (12) or OTD in which carcinogen had been incorporated into the lipid component of the OTD formulation. The fish were then subdivided into equal number lots housed in separate tanks. Dietary carcinogen concentrations and lengths of exposure are indicated in Tables 2.1 and 2.2 of Chapter 2 of this Dissertation.

Tumor Sampling and Storage

At about 16 months after the start of dietary carcinogen exposure, the fish were euthanized by an overdose of tricaine methanesulfonate anesthetic. The fish were individually weighed, dissected, sexed, the liver removed and weighed. Tumors were individually assessed, counted and sized by the on-site pathologist. For both the DMBA- and AFB_1-exposed animals, at least one tumor from each tumor-bearing fish liver was frozen in liquid nitrogen for storage at -80 °C. In DMBA-exposed fish the stomach, swim bladder, and kidney were also carefully examined, tumors present were noted, and tumor samples were frozen in liquid nitrogen and stored at –80 °C.

Nucleic acid isolation

Genomic DNA was isolated using the QIAamp tissue kit (Qiagen, Inc., Santa Clarita, CA) following their published protocols. The yield of DNA from each isolation was assessed by agarose gel electrophoresis and suitable dilutions were made to give working solutions of approximately equal concentration for use as template in subsequent PCR amplifications. Total RNA was isolated either by pulverizing the tissue/tumor samples at -196 °C in liquid nitrogen in the presence of the chaotrope guanidinium isothiocyanate. Alternatively, in tissues that would permit gentle disruption, such as most of these experimental liver tumors, our preference was to isolate cytoplasmic RNA. Cytoplasmic RNA preparations customarily are used for cells in culture and reportedly avoid pre-mRNA and most nuclear DNA contamination by sparing the cell nuclei from disruption, while yielding mainly mature polyadenylated mRNA from the cytoplasm. We followed some aspects of the "Cytoplasmic RNA" protocol in the Qiagen RNAeasy kit, in which a cold lysis buffer "RLN" containing 50 mM Tris at pH 8.0, 140 mM NaCl, 1.5 mM $MgCl_2$, and the detergent nonidet P-40 (Calbiochem-Novabiochem, San Diego CA), disrupts cell membranes while leaving nuclei intact. We modified that protocol by adding gentle but thorough mechanical tissue disruption with conical plastic pestles (Kontes, Vineland NJ) manually pre-selected for close fit into Sorenson Bioscience, Inc (Salt Lake City UT) 1700 μL "pre-siliconized Multi-safeseal, Item # 11700" conical microcentrifuge tubes. (Note: Of many brands examined, this manufacturer's tube was found to give the closest fit to the Kontes pestles.) This mechanical disruption was conducted in the lysis buffer at ~4 °C, in the presence of the "Prime" RNAase Inhibitor (Eppendorf, Westbury NY) at 30 units per mL. The lysed and disrupted homogenate was then spun briefly at 300Xg in a precooled 4 °C centrifuge to pellet the nuclear and other insoluble components. This pellet was saved in TE buffer and rapidly frozen and stored at -80 °C, for possible later analysis. The supernatant, containing mature mRNA, was then processed according to the Qiagen RNAeasy protocol. When polyadenylated mRNA was required, it was isolated from the total RNA using Oligotex, an oligo dT-linked matrix, also from Qiagen. RNA isolations

were not directly visualized by gel electrophoresis, but instead were used directly as templates in subsequent reverse transcription reactions. The purified RNA samples were stored in RNAase-free water at -80 °C.

Polymerase chain reactions (PCRs)

With occasional exceptions noted elsewhere, all PCRs shared the following: Boehringer-Mannheim/Roche Molecular Biochemicals (Indianapolis IN)"Expand High Fidelity" polymerase and dNTPs, gradient or linear block 96-well thermocyclers (Stratagene, La Jolla CA), and primer sets as described in the relevant text and in Table and Figure 5.1). Reaction volumes were typically 10 to 16 μL. One "drop" (15-20 μL) of molecular biology grade mineral oil is used on each reaction, "hot-top" oil-free PCRs were avoided to conserve on reagent costs. Unless otherwise noted, a typical 10 μL reaction had 0.2 to 0.3 units of the polymerase, 20 μM of each dNTP, 30 nM of each primer, 10-50 ng of genomic DNA template, 1 μL of the 10X buffer with $MgCl_2$ to a final Mg^{2+} concentration of 1.5 mM. Cycling parameters were typically as follows: an initial preheat at 95 °C for 3-5 minutes, followed by 36 cycles, each consisting of; denaturation at 95 °C for 45 s, and appropriate anneal temperature (50 °C to 70 °C , see Table 5.1) for 1 minute, extension at 72 °C for 45 s plus 1min for each kbp of expected product length. At the end of the cycling, a single final extension at 72 °C for twice the duration of the last cycled extension period.

"Long" PCR

Long PCRs, here defined as those PCRs giving expected product lengths in excess of 3.5 kbp, were initially conducted substantially as described in Barnes (14) with the exception that *Pfu* exo-minus and *Pfu* polymerases (Stratagene, La Jolla CA) were used to provide the conventional and error-correcting thermostable polymerase functions respectively. Empirically, it was found that using 0.5X concentration of the *Pfu* buffer gave the strongest products on PCRs as long as 9.6 kbp. Extension times in excess of 1 minute per kbp of expected product were found to improve yields. On all of our long PCRs, the technique of rapid denaturations was used largely as described

in Barnes' protocols, with modifications to accommodate 0.2 mL tubes and smaller reaction volumes. This form of denaturation requires use of a robotic thermocycler so that the reaction tubes remain in the 99 °C block for just enough time to reach the desired 95 °C denaturation temperature but then are immediately withdrawn and shifted to the annealing block. This assures that the total accumulated time above the extension temperature of 68 °C and time at or near the denaturing temperature is minimized, thus preserving enzyme function and perhaps more importantly, minimizing depurinations of template and product DNA.

Determination of the actual time necessary to reach 95 °C required somewhat unconventional applications of direct temperature measurement. The mass and thermal conductivity of the miniature bimetallic thermocouple probe easily overwhelmed the mass of the working volumes PCR reagents and mineral oil subject to temperature measurement. To overcome this difficulty the thermocouple probe was preheated to 95 °C in a separate heat block, then placed rapidly into the experimental sample tube at numerous time points following the robotic insertion of the sample tube into the 99 °C block. This process was repeated successively until a residence time was found that gave little or no measured variation from the 95 °C targeted denaturation temperature. By repeatedly measuring the time v. temperature in this way it was empirically determined that, starting from the 68 °C extension temperature recommended by Barnes for very long PCRs, mixtures reached 95 °C in about 12 seconds under the conditions described. Denaturation block residence times of 13 seconds were generally used in such PCRs to accommodate unforeseen variations in performance of the PCR system. The possibility of repeated failure of denaturation was considered by us to present more risk to a successful long PCR than would the extra one second per cycle residence in the denaturing block.

Transintronic PCRs

In several instances, we required information about the sequences in flanking introns that were unknown or unavailable from any known source. Transintronic PCRs were conducted to characterize such whole or partial intronic sequences. To

accomplish these, we used primers from the respective flanking exons that had previously been found to be effective in exonic PCRs to conduct PCR across the intervening intron between exons. After gel electrophoresis, candidate transintronic PCR products were isolated and further "probed" by PCR reamplification using various other available primers, some of which would have been expected to give a product of a specific length and other primers would not have been expected to anneal within the original PCR product. Such probing conveniently and reliably allowed us to discriminate genuine transintronic products from PCR artifacts and non-viable candidates. Such an approach is very straightforward when a variety of exonic primers are available, as in this case. These PCR products were then gel-isolated and either directly sequenced (see "sequencing" below) or cloned and sequenced.

Inverse PCR

To assure that a single *ras* gene was being examined when amplifying and sequencing to detect mutations within the first *Ki-ras1* exon, it was considered prudent to identify a substantial portion of the 5' untranslated region of the Ki-ras1 previously reported from our laboratory. This precaution was taken to enhance discrimination against known and unknown *ras* or *ras*-like genes or pseudogenes having very similar exon I nucleotide sequences (4, 11). Transintronic PCRs (15) and subsequent PCR on cDNA libraries showed that there were at least three ras genes or pseudogenes in *O. mykiss*. One of these, subsequently designated *Ki-ras1*, proved to carry most, if not all, of the codon 12, 13 and 61 mutations found in tumors from a variety of initiation protocols in yet unpublished work from our lab. REX2SEQU (Table 5.1 and Figure 5.1), a primer annealing in the intron between the first two exons of *Ki-ras1* that had been empirically found to strongly discriminate against other known and unknown targets while consistently amplifying *Ki-ras1*, was used as the downstream outwardly directed primer in the inverse PCR. Another primer, cINT-1A, also empirically found specific for Ki- ras1, was used as the upstream outwardly-directed primer for this inverse PCR. *Rebase*, http://rebase.neb.com (16) was used to identify suitable restriction enzymes that would not digest within the already known portions of trout

Ki-ras1 (4) upstream of primer cINT1A corresponding to a portion of the first posttranslational intron plus all of the first exon. The *Rebase* search identified several candidate restriction enzymes including *Pst* I, which were used to digest trout genomic DNA. The digests were then diluted 1:100 treated with T4 DNA ligase (New England Biolabs, Beverly MA). The ligated product was then used in a PCR with the above-described outwardly oriented primers, yielding a PCR product that enabled reading of upstream, previously unknown, sequence described in the Results section below. A similar approach was also successfully used on the 200 tumor collective cDNA library to gain 400 basepairs of 5' untranslated cDNA sequence. Comparison of this cDNA sequence with the upstream genomic sequence allowed us to detect the large pretranslational intron described in the Results section.

PCR Cloning

PCR products were cloned using Invitrogen (San Diego, CA) TA cloning kit, following the manufacturer's protocols. Transformants were typically selected on kanamycin/LB plates, and positives were further selected on ampicillin/LB in 3 mL overnight agitated cultures. Colonies and overnight cultures were screened for appropriately sized inserts by PCR and gel electrophoresis, using primers PCRIIF and PCRIIR (see Chapter 4). These primers anneal to the pCRII TA cloning vector in the regions immediately flanking both sides of the insert site. Plasmid isolation was with Qiagen's QiaPrep kits, following the manufacturer's instructions.

PCR Primers

All oligonucleotide primers were synthesized at the Central Services Laboratory of the Center for Gene Research and Biotechnology at Oregon State University (Corvallis OR), on Perkin Elmer, Applied Biosystems Division 380 or 394 DNA synthesizers using cyano-ethyl phosphoramidite chemistry and controlled pore glass bead columns, with cleavage in 30% NH_4OH and deblocking at 55 °C overnight. Without further purification, the primers were dissolved in 0.5X TE for high

concentration (150 to 300 μM) frozen storage, and diluted to 10 μM in distilled /deionized water for working stocks.

Table 5.1: Primers used in PCRs of trout *Ki-ras1*. (cf. Figure 5.1)

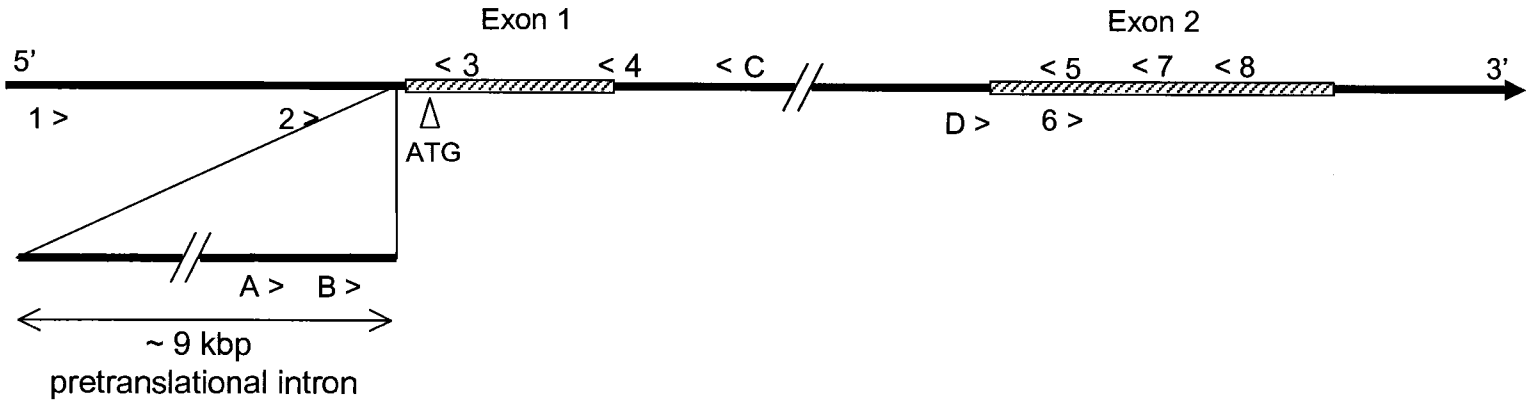
#:	Name	5 'genomic situs		Primer sequence 5'→ 3'	~Anneal °C
	Pretranslational primers				
1	UTRPREU1	5'UTR	U-206‡	GAGAAACGCTGTATCAAATGG	56
2	5'UTRSEQ	5'UTR	U-86‡	GATCGTGTGAAAAAGGAGGATTATCTG	65
	Exonic primers				
3	RTRS 122T	1	L57	GCGCGCTCTTGCCCACACCTA	64
4	RTRASc37	1	L111	CTCGATGGTGGGGTCATATTC	59
5	cRT44SEQ	2	L724	ACACGTCTCCCCGTCAATCACCAC	65
6	RT44	2	U744	GTGGTGATTGACGGGGAGACG	66
7	RTRASc76	2	L820	CCCCTGTCCTCATGTAC	52
8	RTc95sb	2	L872	ATGTCCTCGAAGGACTTT	52
	Non-exonic primers (including in pretranslational intron "-1"):				
A	RAS1PRU3	intron"-1"	U-91*	TGTTGAGTCGATAGTTGG	52
B	RAS1PREU	intron"-1"	U-44*	ATTCACTGACTGTATTCCTG	54
C	cINT1A	intron"1"	L155	CATGCCTTGTAGTAAAAGACACGT	65
C	cINT1As	intron"1"	L155	CCTTGTAGTAAAAGACACGT	56
D	REX2SEQU	intron"1"	U679	CATAGGTGTCCAGTCCTGAACCAT	64

#: indicates ≈position on the primer map Figure 5.1; U and L are upper and lower respective annealing strands, followed by 5' position relative to first nucleotide in the *atg* start codon; situs numbers containing "-" refer to pretranslational positions .

‡ these two primers anneal within the *Ki-ras1* 5'-UTR sequence.

* these two primers anneal within the pretranslational intron sequence.

Figure 5.1. Upstream trout Ki-ras 1 map showing primer positions from Table 5.1. Represents the pretranslational genomic and messenger mRNA structure deduced from the evidence cited in the text. The horizontal exonic portions of the map are drawn to scale. The map omits portions of introns and most of the structure beyond exon 2. The numbers (annealing to exons) and letters (annealing to introns) approximate the physical positions of the respective primers, and refer to the designations in Table 5.1. The ">" and "<" symbols indicate primers annealing to the lower and upper strands respectively.



A 200 tumor cDNA library

Two hundred liver tumors from the diploid AFB_1-fed trout (total weight 0.5 g) were used to generate a commercial cDNA library (Stratagene, La Jolla CA). The protocols employed were similar to those described in their "Lambda Zap" literature (17), except that due to an error at Stratagene, reportedly only the random primed portion of the reverse transcription reaction was utilized to make phage inserts. This limitation appears not to have reduced the utility of the library for experiments undertaken here, since both the Soussi p53 and *Ki-ras1* are abundantly represented in the library. PCR amplifications and sequencings of this library were conducted as described in the relevant headings in this section. We sought to examine this 200 tumor cDNA library as a means to collectively determine the *Ki-ras1*mutation spectrum. To accomplish this we utilized *Ki-ras1* specific primers, UTRPREU1 annealing upstream of the ~9 kbp intron anneal and cRTC95sb, whose 3' end discriminates against some known *Ki-ras1* paralogs.

RT-PCR (cDNA syntheses)

Qiagen reagents for cDNA synthesis were used as described in their protocol for reverse transcription. Again, these reactions were conducted in the presence of the Prime RNAase inhibitor. A random 9-mer (Stratagene, La Jolla CA) oligonucleotide was used for random priming general cDNA synthesis from the mRNA template. This random primed cDNA is then used as template for subsequent specific PCR was conducted using the Expand High Fidelity PCR system from Roche Molecular Biochemicals (Indianapolis IN) as described in the following section. Concentrations, purity and size identity of the PCR products were estimated using 6% native 19:1 acrylamide/bisacrylamide mini-gel electrophoresis with either taurine/EDTA or TAE buffer. Archived imaging was by conventional photography of UV fluorescence of the ethidium bromide-stained DNA products. In an effort to validate and understand the results of the above collective 200 tumor cDNA sequencing, we isolated mRNAs from a set of tumors from the same AFB_1-fed fish, reverse transcribed them to cDNAs with a similar 9-mer random primed reverse transcription used in production of the

commercial library. As with the above-mentioned PCR using the 200 tumor collective library, we amplified the random primed cDNA with the same *Ki-ras1*- and cDNA-selective primers, that is UTRPREU1 and RTc95sb. Further specific details continue in the Results section below.

Sequencing

Fluorescent automated sequencing reactions and electrophoreses of all other stomach tumor DNA samples was done by the Oregon State University core facility: the Central Services Laboratory of the Center for Gene Research and Biotechnology. All templates for fluorescent sequencing were prepared by digesting candidate PCR products with exonuclease I and shrimp alkaline phosphatase as described above. These sequencings were conducted using standard ABI Prism™ (Foster City CA) 373A DNA Sequencer Stretch with 370A automated electophoresis/detection unit and ABI 373A data collection software, version 1.2.1 and Sequencing Analysis software version 3.0. Quantification of individual peaks was accomplished by porting the 373A data over to ABI Genescan® software, v. 2.1.1 with appropriate matrix installation. For comparative purposes or when mutation detection was not an issue, the 377 DNA Sequencer was sometimes utilized with ABI Prism™ 377XL Collection software (Foster City CA). All fluorescent sequencing made use of "BigDye™ Terminator Cycle Sequencing chemistry and AmpliTaq DNA polymerase, FS.

Ki-ras1-specific sequencing primers were used whenever possible, for example, in sequencing genomic samples we utilized the specificity of 3' portions of the upstream ~9 kbp intron for selective annealing of the ad hoc sequencing primer RAS1PREU. For sequencing the 200 tumor cDNA library, and in sequencing the individually transcribed cDNAs, we utilized 5'UTRSEQ for upstream and cRT44SEQ for downstream sequencing primers respectively.

Results

Inverse PCR: structure of Ki-ras1 5' untranslated region

The inverse PCR conducted using primers known to be very specific for *Ki-ras1*, generated a single electrophoresis band of approximately 780 bp. Sequencing of this product revealed 180 bp of the hitherto unidentified 5' region immediately preceding the putative translation start. This sequence enabled design of an upstream non-exonic primary amplification primer for reliable and gene-specific generation of genomic templates of *Ki-ras1* for exon I (codons 12 and 13) mutation detection. Similarly, an inverse PCR templated by our 200 liver tumor cDNA library, revealed about 1.2 kbp of upstream *Ki-ras1* sequence now designated as the 5' UTR (untranslated region). Sequencing the 3' end of the 5'UTR and comparing it with the upstream genomic 5' sequence revealed evidence of a pretranslational intron. PCR on genomic DNA, using a primer annealing in this 5'UTR against a primer annealing in the exonic portion of *Ki-ras1,* gave a ~9 kbp product (Figure 5.2), suggesting an intron present at near that length. The 9 kbp genomic product was gel isolated and directly cycle sequenced from both ends (see Figure 5.3), and compared with the shorter corresponding cDNA sequence, revealing a region of identity between the two abutting hitherto unidentified sequence beginning as a *5'-**ag***.... Similarly the sequence from the 3' end again revealed sequence identical to the genomic abutting unknown sequence that ended adjacent to a ...***gt**-3'*. The presence of these intron excision consensus terminal sites (18, 19), the abutting known *Ki-ras1* cDNA sequence at either end of the 9 kbp genomic product (Figure 5.3) and successful identification of *Ki-ras1* mutations from tumor cDNAs using primers annealing only within this upstream cDNA sequence, were together considered sufficient evidence to confirm the presence of the intervening ~ 9 kbp as a pretranslational intron. We have designated this as *Ki-ras1* intron "-1". The two 175 bp variants (single base polymorphs) of the upstream genomic sequence proximal to the translation start of *Ki-ras1* were submitted to GenBank as accession numbers AF173858 and AF173859 (20). The pretranslational portion of the cDNA sequence was also submitted to GenBank as

accession number AF 301535 (21). (Appendix C shows facsimiles of these three GenBank submissions).

Figure 5.2. Long PCR of an apparent 9 kbp pretranslational intron in *Ki-ras1*. Agarose gel of gradient "long" PCR products, annealing temperatures at the two extremes indicated, gradient is 1 °C per lane. The marker lanes are M_1: a 1kbp ladder (maximum 12 kbp) and M_2: a *Bst* EII digest of λ phage respectively.

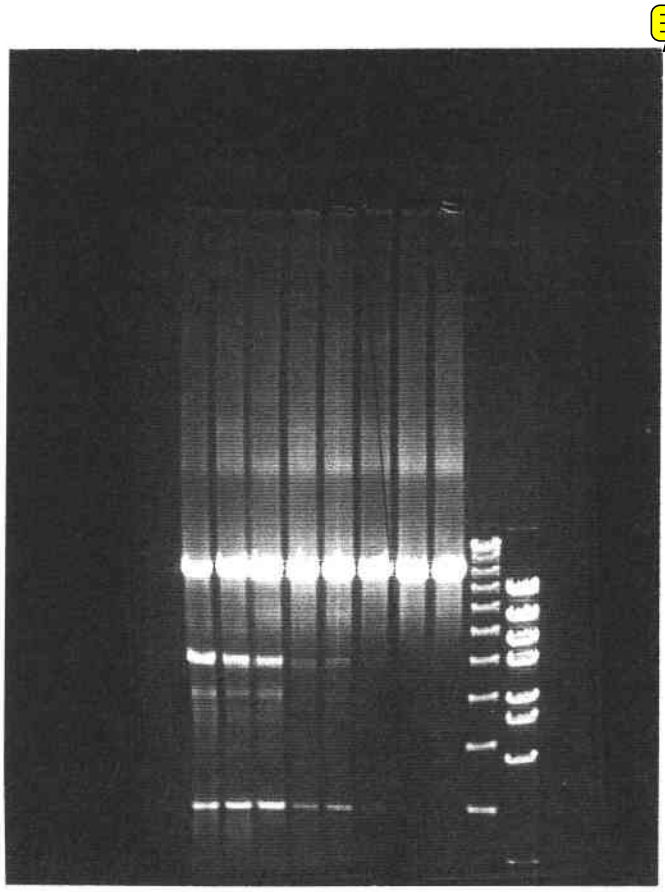


Figure 5.3: The upstream genomic structure of trout *Ki-ras1* (cf. Figures 5.1 and 5.2). Deduced from direct cycle-sequencing from both ends of a single 9 kbp *Ki-ras1*-specific PCR product using *Ki-ras1*-specific sequencing primers. The sequences respectively show in bold the universal intron excision motifs for the 5' (**gt**) and 3' (**ag**) junctions of a deduced pretranslational intron of ~9 kbp length. The first 50 bases shown are identical to bases 343 to 393 of GenBank AF301535, submitted August 30, 2000, an inverse PCR-generated sequence derived using *Ki-ras1*-specific primers on a *Ki-ras1*-enriched cDNA library (see Appendix D1 for 400 bp 5' UTR sequence electropherogramof this inverse cDNA PCR). Following the intron 3' terminal **"ag"** is sequence identical to bases 169 through 175 of GenBank AF173859 and AF173858 submissions of July 28, 1999 and revised March 21, 2000, showing identity with this portion of the *Ki-ras1* genomic sequence. The remainder beginning at **"*atg*"** is identical to the posttranslational portion of *Ki-ras1*, as reported in (4), all of exon 1 and part of the succeeding intron (second **"gt"** 17 bases from the end) were also read and are included here as verification of gene context, (20, 21)

5'tgacttct tatttggtcg ctacttcgga cgcggaactc cacacgctat
ag**gt**aaaaca attggaaaga tcactacact tgtatcaatt tacacaatgg
atacggggcg acattgtgtc ctctcgccta tatcttaatt tgagggttgt
tgcctgtaat gatttcataa tttcctgtgc aattgcagct aaaagtatcg
ttaatacaaa ctacgcccaa ttgttaacct aatgtctggc cctttaatgc
gcagacctat gcggtattta tggcggcttc aaactcatcc gctctcccat
gaatgctgca ggttctaccg ggtttacgca tagcattcaa tccattgtgc
taaagaaatt tgcacaagac aatcctagtt cactaacaat tatgggatct
gcatatgcta ggcttctcta taattgtgga cctactccag ttaattgtag

. . . . . (continues for a deduced ~8.5 kbp not sequenced and not shown) . . . .

aaatcccagg tggagaattc cctgttggtg gatttacctc ccagtttcct
gattctagga atcctccaac tggaaaacat gggcattttt ggaaggatac
tggaattctg caaccctata tatgtgactt tcttgttgag tcgatagttg
gtgtgkagct catgaaatgt ttatttttat attcactgac tgtattcctg
tgtcttctgt cccac**ag**gtg aacg***atg***acc gaatacaagc tggtggtggt
gggggcagga ggtgtgggca agagcgcgct caccatcacc atccagctca
tccagaatca ctttgtggat gaatatgacc ccaccatcga g**gt**aaaaagt
tgtttcagta

DMBA-induced tumor Ki-ras1 mutations

In tumors of DMBA-fed animals, a significant (Fisher's exact $p<0.05$) ploidy-related difference was seen in stomach tumor exon 1 *Ki-ras1* mutations. No other ploidy-related difference was seen in the DMBA-fed animals. associated ploidy neither carcinogen elicited differences in spectrum or incidence of *Ki-ras1* mutations (Tables 5.2 and 5.3). Sequencing of *Ki-ras1* (or p53) from RTPCR-generated cDNAs of stomach tumors was not undertaken since the yield of mRNAs from the stomach samples was low and inconsistent.

Table 5.2: Summary of *Ki-ras1* tumor analyses for DMBA-fed trout

	Diploids		Triploids		Totals	
	mut/n	%	mut/n	%	mut/n	%
Stomach tumors	19/20	95	12/20	60	31/40	78
Exon 1	6/18	33	1/20	5	7/38	18
Exon 2	13/20	65	11/20	55	24/40	60
Liver tumors	7/15	47	6/15	40	13/30	43
Exon 1	6/15	40	6/15	40	12/30	40
Exon 2	1/13	8	0/15	0	1/28	3

Numerators are number of detected mutations, denominators are the number of tumors analyzed.

"%" is the percent of tumors sequenced giving a detected mutation.

Those tumors with adjacent double mutations are scored as a single mutation.

For detailed data on these mutations see Appendix C, Tables C.1 and C.2.

AFB_1-induced liver tumor Ki-ras1 cDNA mutations

Analysis of *Ki-ras1* mutations in other liver tumors from AFB_1-initiated fish in this study was conducted using individual tumor mRNA isolation, then individual (RT PCR) with *Ki-ras1*-specific primers, then cDNA sequencing. The latter individual *Ki-ras1* cDNAs showed single base *Ki-ras 1* mutations were often present (18/22 or 82% mutated) predominantly in exon 1. Not reflected in the table, but of interest: about 9% (2/22) of these AFB_1-initiated liver tumors showed codon 12(2) G → T mutations and about 14% (3/22) had apparent codon 12 (1,2) GG → TT double mutations. It should be noted that these three apparent double mutations were clearly at or less than 50% mutant-in-normal molarity-- consistent with historic *Ki-ras1* mutations observed in our laboratory (data not shown).

Table 5.3. Summary of *Ki-ras1* liver tumor mutations in AFB_1-fed trout

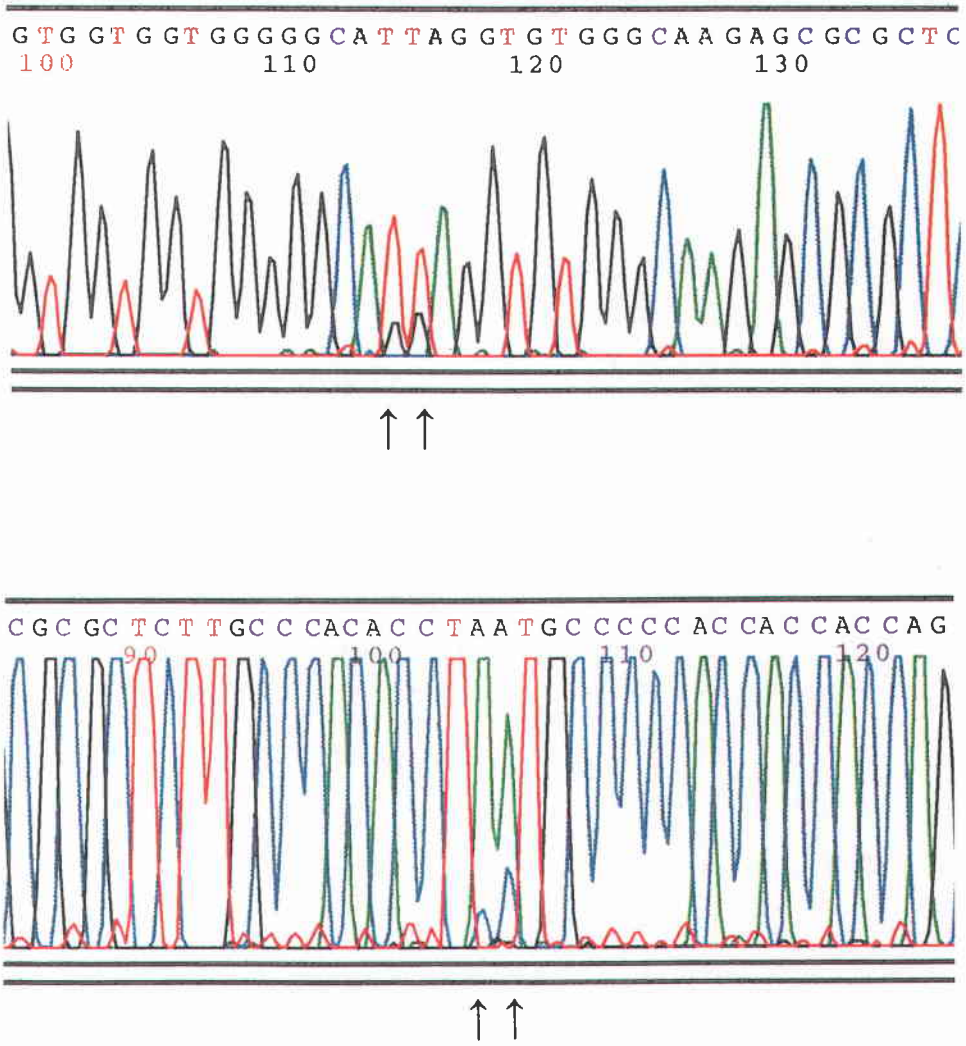
	Diploid		Triploid		Totals	
	mut/n	%	mut/n	%	mut/n	%
Liver tumors	9/12	75	9/10	90	18/22	82
Exon 1	7/12	58	9/10	90	16/22	73
Exon 2	2/12	17	0/10	0	2/22	9

Here "mut" is the number of *Ki-ras1* mutated tumors; "n" total number of tumors examined. These mutations were all detected by direct fluorescent sequencing of cDNAs. For detailed notes on these mutations see Appendix C, Table C.3.

Double mutations in the 200 liver tumor cDNA library

Collective analysis of mutations in the AFB_1-fed fish by *Ki-ras1*-specific PCR and *Ki-ras*-specific direct sequencing of the 200 tumor cDNA library showed pronounced representation of codon 12 position 1 and 2 mutations. That is, in both of these positions the electropherogram shows the mutant deoxythymidine predominated over the native deoxyguanosine (Figure 5.4). Sequencing of this same collective library using a downstream sequencing primer gave essentially the same remarkable result.

Figure 5.4. ***Ki-ras1*** **codon 12(1,2) GG → TT from the 200 tumor library.** The upper electropherogram panel shows the mutation of "TT" in *Ki-ras1* codon 12 positions1 and 2 (upper double arrows) at apparent concentrations greater than the normal "GG" at these positions. Similarly the lower electropherogram shows the complementary sequence across the same two positions, and again the mutations "AA" appears at greater concentration than the normal "CC".



Discussion

Transintronic PCRs: paralog identification

We regularly used PCR reamplification or "primer probing" to identify or confirm true positive products amongst several potential transintronic PCR candidates. Such an approach can save time and effort, since the presence or absence of a reamplification product provides a logical nexus to confirm or deny identity. Further, the product lengths can give a strong indication of identity. These methods can often exclude many false candidates that either fail to re-amplify or have length differing from the desired product. We believe that using such methods in the effort to eliminate paralogs and pseudogenes may have contributed an improvement in the performance of the *Ki-ras1* sequencing and mutation detection, although this has not been specifically addressed in our research.

In our opinion, the transintronic approach is essential to any effort where threshold of detection for mutated alleles in a background of normal is potentially an issue, such as in oncogene assay in tumors using PCR methods. In such assays the fraction of cells carrying activating oncogenic mutations is generally never more than 50% and may well be far less than this. Only by using primers that discriminate against unintended templates of primary PCR amplification or sequencing can one provide maximum suppression of interfering false templates

Threshold of detection

Based on prior work done in this laboratory it appears that at least 98% of tumor-associated trout ras mutations are likely to occur in just 3 codons, that is 12, 13 and 61 (4, 5). This is consistent with published mutation data examining ras mutations in mammalian species. Such site-specificity is helpful when hunting for mutations either using site-specific methods such as 3' primer mismatch assays (see Chapter 3) or if one were attempting to assess mutations using ABI 373 electrophoretic data,

whether by visual examination or by Genescan analysis. Fortunately there appeared to be little need for this capability when analyzing Ki-ras1 in the tumor DNAs or cDNAs.

Our threshold of detection (TOD) was between 10 and 20% for fluorescent automated sequencing, whereas with radiolabeled manual sequencing, under ideal conditions and with optimized methodology including use of radiolabeled dideoxy-terminators and phosphorimaging, showed a TOD of 5% (Chapter 4 Results). There were numerous mutations detected in *Ki-ras1* using the fluorescent method, apparently matching the historic incidence observed using radioactive sequencing in detection in AFB1-initiated tumors , suggesting that at least for work such as this, the fluorescent automated method may be at least adequate in spite of the likely decreased sensitivity.

Mutation "spectroscopy" by sequencing of collective tumor DNA or cDNA libraries

Our effort to generate a mutation spectrum in a single step by sequencing the 200 tumor collective cDNA library produced the remarkable result seen in Figure 5.4. The further effort to investigate the sources of this novel result, through independent isolation of tumor mRNA, reverse transcription, PCR amplification and direct cycle sequencing of individual tumors from the same cohort of AFB_1-fed fish resulted in an array of mutations consisting of a more conventional spectrum of AFB_1 mutations. But, the separately isolated and reverse transcribed RT PCR products indeed showed 14% (3/22) of the 12(1,2) GG → TT mutations so prevalent in the collective library. However, in those tumors the mutations were present with at least as much of the normal unmutated allele, thus the effective aggregate molar 12(1,2) GG → TT content of the 22 tumors is less than 7 %. Even though the 22 tumor sample was relatively small and subject to substantial sample variation, it is difficult to imagine how even several multiples of a 7 % molar concentration of these double mutations could be accurately represented by the robust finding of 60 to 85% molar concentration of double mutation seen in the cDNA library. There is little possibility that the difference could somehow be made up of high prevalence of 12(1) G → T, together with 12(2) G → T mutations, since the former would represent a stop codon (UGA) and thus render that mRNA not translatable to produce Ki-ras1 ras protein, active or otherwise.

Further, the 12(2) G → T itself had an unimpressive incidence of two in the 22 individual tumors. Again neither of these two tumors showed any more than the expected molar concentration of less than 1:1 mutated:normal, giving an overall molar prevalence of under 5%. Taken together the evidence makes it improbable that the observed high 12 (1,2) GG → TT represents anything other than the aggregate of true doubly mutated cDNAs in the 200 tumor library. Further investigation of this unusual phenomenon may be worthwhile.

Organ-specific variations in Ki-ras1 mutation incidence

The individually analyzed tumors from the AFB_1-fed trout showed 83% *Ki-ras1* mutations. There was a predominance of exon 1 mutations, only two out of 25 liver tumor cDNAs showed codon 61 mutations. As in the livers of DMBA-fed trout, AFB_1 did not elicit any significant ploidy-related differences in spectrum or incidence of *Ki-ras1* liver tumor mutations. These sequencing results indicate that the overall incidence of *Ki-ras1* mutations in liver tumors of the AFB_1-initiated fish was nearly twice (82% v. 43%, see Tables 5.2 and 5.3) that of the DMBA-initiated animals. This result suggests that our detection sensitivity using fluorescent automated sequencing is at least adequate, since the 82% Ki-ras1 liver tumor mutation incidence in AFB_1-fed trout exceeds the71% reported in the only other comparable study (3). The DMBA-fed *Ki-ras1* mutation incidence of 43%, exactly corresponds with that seen from earlier work at 43% (5). Showing that neither the likely greater age at necropsy (16 months) nor the higher level of DMBA in the diet, appeared to alter the tumor incidence. There can be no AFB_1 / DMBA comparison for stomachs, since AFB_1 only initiates liver tumors. Sequencing of *Ki-ras1* or p53 from cDNAs of DMBA-initiated stomach tumors was not undertaken since the yield of mRNAs from the stomach samples was low and inconsistent. These data were instead derived from sequencing of genomic DNA templates.

Ki-ras1 ploidy-related mutation incidence

There was an apparent but non-significant difference in *Ki-ras1*mutation incidence in tumors from livers of triploid versus diploid trout. The overall incidence of *Ki-ras1* mutants in livers, regardless of ploidy, is also consistent with that reported previously (3, 4). However, in stomach tumors the significant reduction, from 33% (6/18) containing exon 1 mutations in the diploid stomach tumors down to 5% (1/20) in the triploid stomach tumors, represents the first report of oncogene mutation spectrum variation with respect to ploidy. The lack of any similar ploidy-related mutational incidence in liver tumors may eventually provide a means to elucidate the mechanisms underlying the reduction in *Ki-ras1* exon 1 mutations in triploid stomach tumors. This lack of exon I involvement in triploid stomachs may provide a justification to narrow future research efforts for tumor suppressor mechanisms involving the stomach and not the liver. The fact the triploid incidence here is much lower than the diploid, conjoined with the fact that it is confined to exon 1, suggests the possibility of involvement of a Ki-ras-specific tumor suppressor gene. At least one such mechanism has been well documented, that is the evident ability of Rap 1A/Krev1/smg p21A to reverse the oncogenic effects of activated KRAS (22). Whether a trout ortholog of Rap 1A could explain a portion of the trout triploid tumor suppression seen in our work will remain for further investigations.

Acknowledgements

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Chapter 6: Summary and Conclusions

Summary of Results

At all levels of carcinogen and in all organs (liver for AFB_1; liver, stomach, swim bladder and kidney for DMBA), there was substantial reduction of tumor incidence in the triploids. These results demonstrate triploid tumor suppression effect in dietary carcinogen exposures of 30 and 120 days. The results confirm and extend those of Thorgaard *et al.* (1) and provide further justification for using polyploid models to investigate cancer gene function in carcinogenesis. We found no mutations of the trout p53 tumor suppressor gene in tumors of liver or stomach regardless of trout ploidy. We did identify numerous activating *Ki-ras1* mutations in tumors of liver and stomach. Further, we found a substantial and significant difference between the frequency of diploid and triploid exon 1 *Ki-ras1* mutations confined to stomach tumors.

Some Alternative Explanations for the Triploid Tumor Suppression

Possible explanations of the triploid tumor suppression phenomenon, in addition to the third genomic copy of all tumor suppressor genes include:

1. There are reports that polyploid cells are often larger than their diploid counterparts are. If such changes represented substantial changes in cell volume, it might contribute to, for example, differences in transport toxicokinetics, which in turn might lower ultimate carcinogen concentration in a nuclear context.

2. On the other hand, if cell size is not different in triploids, then it is conceivable that 50% greater DNA mass relative to nuclear or cellular volume might contribute to some degree of triploid tumor suppression by offering a larger effective target for a what could be a relatively constant number of ultimate carcinogen molecules per cell.

3. A reduced likelihood of assembling defective multimeric suppressor protein when the cellular monomer pool is one third mutant relative to that when it is one half mutant. This proposed mechanism is discussed in chapter 4 of this Dissertation.

4. Whether or not the extra genome of triploids is fully functional at the level of expression. If the third genome were simply idle chromatin, it might serve to dilute the effective concentration of ultimate carcinogen.

Implications of the Low Level or Absence of p53 Mutations

The apparent absence of any mutations in p53 raises several questions, which can only be laid to rest by further research. Among the possible explanations are that tumor-associated p53 mutations may relatively late events and that they may be most likely to be seen in advanced metastatic malignancies. There remains a possibility that this particular p53 (Caron de Fromentel, 1992) is not involved in trout tumor suppression and that another p53 or p53-like gene in trout expresses the anti-oncogenic functions we associate with human p53

We cannot interpret the lack of mutations in trout p53 as overwhelming evidence that none exist, but our analysis is sufficient to discount the notion of strong involvement of mutations of *this* p53 gene in trout tumors at any level paralleling that seen in human tumors. Our analysis based on the human tumor p53 mutation database suggests that the absence of p53 mutations in liver stomach tumors might indicate that functional differences between the human and trout p53 exist. We can conclude that the complete absence of any polymorphism in this p53 gene provides good evidence that at least it codes for a functional protein, and likely a protein whose structure is under very strong constraining selective pressure.

Implications of the Mutation Analysis of *Ki-ras1*

We reported that there was a modest reduction of *Ki-ras1* mutation incidence in tumors from livers of triploid trout. There was however, a profound reduction (to 5% from about 33%) in the incidence of triploid stomach tumors containing exon 1 *Ki-ras1* mutants. This also means that the tumors without ras activation are more prevalent in triploids. The mere dosage effect in triploids bearing an activated *ras* allele and two recessive normal alleles would be unlikely to explain the substantial

reduction in *Ki-ras1*-involved tumor incidence seen in the stomachs of diploids. The reduced incidence of *Ki-ras1* mutated triploid stomachs suggests instead that the redundancy of at least one tumor suppressor gene may be involved and further that the influence on *Ki-ras1* involvement suggests some possible close functional coupling to *Ki-ras1* itself.

Recommendations for Future Research

Further investigation is necessary to reveal whether the differential between stomach and liver tumor triploid suppression may be due to inherent polyploidy of liver hepatocytes. This should be relatively easy to accomplish by flow cytometric analysis of cellular versus nuclear DNA content in diploid and triploid hepatocytes.

To further compare and validate the relative efficacy fluorescent sequencing for mutation detection, it would be relatively easy to sequence thirty or more tumor DNAs for which Ki-ras1 mutations were detected using radiolabeled manual sequencing. Such a comparison could include tumors seen to give relatively weak signals with the radiolabeled sequencing and thus provide an independent validation of the relative thresholds of the two approaches in a completely realistic context.

There were some large and invasive gastrointestinal tumors from the DMBA-fed portion of this work that were examined by fish tumor histopathologist Dr. Jan Spitsbergen of Oregon State University and identified as novel in trout carcinogenesis. These unfortunately had only been preserved with Bouin's fixative. Bouin's fixed tissues have heretofore generally been refractory to recovery of PCR-competent template DNA. These may now be susceptible to genetic analysis using new techniques developed by us. Since p53 mutations are thought to often be late events in many human cancers, it may be productive to examine these novel advanced tumors for such mutations.

Techniques of differential display or subtractive hybridization could be used to identify genes whose message expression is differentially modulated when comparing diploid to triploid tumors. Work of this sort would need reference non-tumor material from the adjacent tissues. Fortunately, such reference material is available. The fact

that tumor samples have been retained at -80 °C makes it quite likely that useful mRNA can be recovered from them. Work reported in this thesis shows that for frozen liver tumors this is a facile process.

The triploid model potentially provides a means to *in vivo* assessment of the relative contributions of oncogenes and tumor suppressor genes to the carcinogenesis process. Such comparisons may otherwise be technically and computationally unachievable. Triploidy may also provides a means to identify new tumor suppressor genes, a task that has always been exceedingly slow and difficult using cancer-prone family pedigree analysis, karyotype-driven positional cloning, loss-of-heterozygosity analysis probably combined with a substantial contribution of good fortune. The presence of tumors from sibling triploids and diploids and their adjacent normal tissues provides a remarkable and novel opportunity for subtractive hybridization and/or differential display approaches to identifying new tumor suppressor genes.

The presence of a significant reduction in triploids of *Ki-ras1* exon 1 involvement in stomach tumors is intriguing. There is the possibility that some tumor suppressor function, perhaps associated with surveillance of the presumed mitogenic stimulation of activated Ki-ras1, is better preserved by the redundancy of the triploid genome. Such a result shows but one of the ways the triploid trout model may elucidate previously unidentified tumor suppressor mechanisms.

The remarkable result showing a predominant *Ki-ras1* codon 12 (1,2) GG → TT tandem mutation deserves some research attention for two reasons. First, the unexpected results seen in this may provide a window revealing biases that may affect examination of cDNA libraries. Second, collective sequencing of many tumors or cDNAs at once could be a route to reduced labor, time and costs for generating mutation spectra.

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Appendices

Appendix A: Computational details for depth of tumor sampling necessary to reach 95% confidence expectation (c. e.) of finding a specified human p53 mutation. (Expansion of Table 4.3)

p53 position or region:	Liver tumors					Stomach tumors				
	N_L†	f_L (N_L/410)	μ_L ($f_L p_L$)	1-μ_L §	95% c.e. sample size n	N_S†	f_S (N_S/266)	μ_S ($f_S p_S$)	1-μ_S §	95% c.e. sample size n
Codon 175	7	0.017073	0.0042682	0.995732	701	17	0.063910	0.0255639	0.974436	116
Codon 273	20	0.048780	0.0121951	0.987805	245	15	0.056391	0.0225564	0.977444	132
Codons 175 & 273	27	0.065853	0.0164634	0.983537	181	32	0.120301	0.0481203	0.951879	61
Codon 157	15	0.036585	0.0091463	0.990854	327	1	0.003759	0.0015038	0.998496	1991
Codon 166	13	0.031707	0.0079268	0.992073	377	0	0.000000	0.0000000	1.000000	(n.a.)
Codon 245	5	0.012195	0.0030488	0.996951	982	12	0.045113	0.0180451	0.981954	165
Codon 248	8	0.019512	0.0048780	0.995122	613	22	0.082707	0.0330827	0.966917	90
Codon 249	138	0.336585	0.0841463	0.915854	35	2	0.007519	0.0030075	0.996993	995
Codons 248 & 249	146	0.356098	0.0890244	0.910976	33	24	0.090226	0.0360902	0.963910	82
Codon 282	5	0.012195	0.0030488	0.996951	982	18	0.067669	0.0270677	0.972932	110
All 5 top hotspots*	204	0.497561	0.1243902	0.875610	23	84	0.315790	0.1263158	0.873684	23
Conserved domain III	28	0.068293	0.0170732	0.982927	174	42	0.157895	0.0631579	0.936842	46
Conserved domain IV	198	0.482927	0.1207317	0.879268	24	60	0.225564	0.0902256	0.909774	32
Conserved domain V	61	0.148781	0.0371951	0.962805	80	52	0.195489	0.0781955	0.921805	37
All of domains III -V	287	0.700000	0.1750000	0.825000	16	154	0.578947	0.2315789	0.768421	12
Exon 5	103	0.251220	0.0628049	0.937195	47	107	0.402256	0.1609023	0.839098	18
Exon 7	199	0.485366	0.1213415	0.878658	24	72	0.270677	0.1082707	0.891729	27
Exon 8	71	0.173171	0.0432927	0.956707	68	68	0.255639	0.1022556	0.897744	28
All of exons 5, 7, 8	373	0.909756	0.2274390	0.772561	12	247	0.928571	0.3714286	0.628571	6
Whole coding region	410	1.000000	0.2500000	0.750000	11	266	1.000000	0.4000000	0.600000	6
Codon 249adj#	28	0.068293	0.0170732	0.982927	174	2	0.007519	0.0030075	0.99699	995
Codons 248 & 249adj#	36	0.087805	0.0219512	0.964878	84	24	0.090226	0.0360902	0.96391	82
Exons 5, 7, 8.- 249adj#	263	0.641463	0.1603659	0.839634	18	245	0.921053	0.3684211	0.631579	7
Whole coding-249adj#	300	0.731707	0.1829268	0.817073	15	264	0.992481	0.3969925	0.603008	6

Appendix A (Continued)

Number of liver or stomach tumors with this mutation from the Human p53 tumor mutation database (October, 1998). Total number of tumors for liver at that time was 410, for stomach 266.

§ Here N_L and N_S are the number of liver and stomach tumor p53 mutations reported for this position or region of the human p53 gene as reported in the Human p53 database (1) as of October 1998.

The colums f_L and f_S refer to the fraction of p53 mutations at the specified p53 position or the mutations within the specified region, the fraction denominators being 410 for liver and 266 for stomach respectively taken from the Human p53 database at the time of this analysis. The 95% confidence level sample size n, was calculated solving by $(1-\mu)^n \leq 0.05$ for n. Where μ is the inferred site-specific or region-specific rate of p53 mutations in the human population based on the site- or region-specific database incidences of p53 mutations multiplied by the respective estimates of the frequencies of all p53 mutations in the human population for the organ in question. Thus, for any particular organ "o", $\mu_o = f_o p_o$. The respective factors p_L and p_S were based on estimates taken from published literature sources (see text). For this analysis, p_L was set at 0.25 and p_S was set at 0.40.

*For liver tumors: codons 157, 166, 248. 249, 273; for stomach tumors: 175, 245, 248, 273, 282

#Here codon 249 liver incidence in humans is adjusted to reflect estimated likelihood that at least 0.80 of human liver 249 involvement is due to a specific carcinogenic interaction in human liver of aflatoxin and hepatitis B or C, for which no viral parallels are known in trout.

1. Béroud, C., Dehouche, K., and Soussi, T. The p53 Database. Website: http://perso.curie.fr/Theirry.Soussi/p53_databaseWh.htm, 1999.

Appendix B: Tables of *Ki-ras1* Tumor Mutations Detected

Table B1. Ki-ras1 mutations in DMBA-induced liver tumors

Book	Pages	Lot	Number	Ploidy	Codon	Mutation
19	36(#1), 41b	L1	5b	D	12 (2)	G → T
16	66, 70B	L1	5	D	exon 1	ND
19	34(#2), 35	L1	15	D	exon 1	ND
19	34(#6), 38b	L1	47	D	12 (2)	G → T
19	33(#5), 35	L1	60	D	exon 1	ND
16	66, 67B	L2	5	T	12 (1)	G → T (noisy)
19	7, 8B	L2	21	T	exon 1	ND
19	31, 32B	L2	21b	T	12 (1)	G → A
19	34(#5), 38b	L2	27	D	12 (1)	G → A
19	34(#3), 35	L2	29a	D	12 (2)	G → T
19	36(#2), 38b	L2	42	T	exon 1	ND
19	36(#2), 41b	L2	51	D	exon 1	ND
19	36(#1), 38b	L2	80	T	12 (1)	G → A
16	66, 70B	L2	86	D	exon 1	ND
19	31, 32B	L2	93	T	exon 1	ND
19	36(#7), 41b	M1	3b	D	exon 1	ND
16	60, B	M1	11	T	13 (1)	G → C
19	31, 32B	M1	15	D	12 (2)	G → T
19	31, 32B	M1	16	D	exon 1	ND
16	66, 67B	M2	5	D	exon 1	ND
19	31, 32B	M2	21	T	exon 1	ND
16	66, 67B	M2	25	D	exon 1	ND
19	36(#6), 41b	M2	57	T	12 (2)	G → T ?
19	36(#4), 41b	M3	128	T	12 (1)	G → A, G → C
19	36(#5), 41b	M3	158	T	exon 1	ND
19	33(#3), 35	N1	7	T	exon 1	ND
19	34(#7), 38b	N1	28	T	exon 1	ND
19	33(#2), 35	N1	43	T	exon 1	ND
19	33(#4), 35	N1	50	T	exon 1	ND
19	36(#8), 41b	N1	65	D	12 (2)	G → T
16	60,B	L1	5*	D	exon 1	ND
19	7,8B	L1	15*	D	exon 1	ND
19	7,8B	L1	60*	D	exon 1	ND
19	31, 32B	L1	60**	D	exon 1	ND
19	31, 32B	L2	29a*	D	12 (2)	G → T
16	66, 67B	L2	42*	T	exon 1	ND
16	66, 70B	L2	51*	D	exon 1	ND
19	7,8B	L2	93*	T	exon 1	ND
19	7,8B	M2	21*	T	exon 1	ND
19	7,8B	N1	7*	T	exon 1	ND
19	7,8B	N1	43*	T	exon 1	ND
19	7,8B	N1	50*	T	exon 1	ND
16	66, 67B	N1	50*	T	exon 1	ND
16	66, 70B	N1	65*	D	12 (1)	ND

(continued next page)

Book	Pages	Lot	Number	Ploidy	Codon	Mutation
14	79, B	L1	5	D	exon 2	ND
14	79, B	L1	7	D	exon 2	ND
17	22, B	L1	15	D	exon 2	ND
14	81, B	L1	47	D	exon 2	ND
14	79, B	L1	60	D	exon 2	ND
17	22, B	L2	5	T	exon 2	ND
17	20, 26	L2	20	T	exon 2	ND
17	20, 26	L2	21	T	exon 2	ND
14	81, B	L2	23	D	61 (3)	G → T
17	22, B	L2	27	D	exon 2	ND
17	22, B	L2	29	D	exon 2	ND
17	20, 26	L2	37	T	exon 2	ND
17	22, B	L2	42	T	exon 2	ND
17	23, 25B	L2	93	T	exon 2	ND
14	81, B	M1	3	D	exon 2	ND
17	20, 21B	M1	12	T	exon 2	ND
17	20, 21B	M2	21	T	exon 2	ND
17	22, B	M2	57	T	exon 2	ND
17	23, 25B	M3	43	T	exon 2	ND
17	23, 25B	M3	128	T	exon 2	ND
17	23, 25B	M3	158a	T	exon 2	ND
17	20, 26	N1	7	T	exon 2	ND
17	23, 25B	N1	16	D	exon 2	ND
17	23, 25B	N1	25	D	exon 2	ND
17	20, 21B	N1	28	T	exon 2	ND
17	20, 21B	N1	43	T	exon 2	ND
17	22, B	N1	50	T(normal?)	exon 2	ND
17	23, 25B	N1	62	D	exon 2	ND
14	81, B	N1	65	D	exon 2	ND
14	79, B	L1	15*	D	exon 2	ND
17	22, B	N1	16*	D	exon 2	ND

(Note: in these tables a "*" indicates a duplicated sequencing

Table B2: Ki-ras1 mutations in DMBA-induced stomach tumors

Book	Pages	Lot	Number	Ploidy	Codon	Mutation
19	34(#4), 38B	M1	15	D	12 (2)	G → T
19	33(#7), 38B	M1	26	D	12 (2)	G → T
16	60, B	M3	114	T	exon 1	ND
16	60, B	M3	162	T	exon 1	ND
17	48, 63B	L1	15	D	exon 2	ND
17	50, 63, 43	L1	24	D	exon 2	ND
17	48, 63B	L1	35	D	exon 2	ND
17	48, 63B	L1	41	D	61 (2)	A → T
17	65, 74	L2	17a(1 :100)	T	exon 2	ND
17	65, 74B, 75B	L2	17b (1:1)	T	61 (2)	A → T
17	65, 74	L2	9	D	61 (2)	A → T
17	50, 63, 65, 74	L2	13	D	exon 2	ND
17	65, 74	L2	15a	D	61 (2)	A → T
17	29, B	L2	17	T	exon 2	ND
17	65, 74	L2	20	D	61 (2)	A → T
17	65, 74B, 75B	L2	24	T	61 (2)	A → T
17	65, 74B, 75B	L2	37	T	61 (2)	A → T
17	65, 74B, 75B	L2	44	T	exon 2	ND
17	7, 9, B	M1	11	T	61 (2)	A → T
17	50, 63, 43	M1	14	D	61 (2)	A → T
17	29, B	M1	15	D	exon 2	ND
17	50, 63, 43	M1	16	D	61(2),(1)	A → T, C → T
17	7, 9, B	M1	18	D	61 (2)	A → T
17	50, 63, 43	M1	24	D	exon 2	ND
17	29, B	M1	26*	D	61 (2)	A → T
17	7, 9, B	M1	27	T	61 (2)	A → T
17	7, 9, B	M1	33	D	exon 2	ND
17	7, 9B, 29B	M1	34	T	exon 2	ND
17	31, 32B	M1	92	T	61 (2)	A → T
17	31, 32B	M1	95	T	exon 2	ND
17	31, 32B	M1	113	T	exon 2	ND
17	65, 74B, 75B	M1	122	T	61 (2)	A → T
17	31, 32B	M1	126	T	61 (2)	A → T
17	31, 32B	M1	130	T	exon 2	ND
17	31, 32B	M1	134	T	61 (2)	A → T
17	31, 32B	M1	139	T	exon 2	ND
17	31, 32B	M1	147	T	61(2),(3)	A → T, G → T
17	48, 63B	M2	25	D	61 (2)	A → T
17	29, B	M3	114	T	61 (2)	A → T
17	74B, 75B	M3	138	T	exon 2	ND
17	48, 63B	M3	155	D	61 (2)	A → T
17	48, 63B	M3	156	D	61 (2)	A → T
17	29B. 65, 74	M3	158	D	61 (2)	A → T
17	65, 74	M3	162	T	exon 2	ND
17	50, 63, 43	M3	168	D	61 (2)	A → T
17	29, B	L2	9*	D	61 (2)	A → T

Book	Pages	Lot	Number	Ploidy	Codon	Mutation
17	50, 63, 43	L2	15*	D	61 (2)	A → T (faint)
17	50, 63	L2	20*	D	61 (2)	A → T
17	7, 9, B	M1	14*	D	61 (2)	A → T (faint)
14	81, B	M1	33*	D	exon 2	ND (cf book 17,p.9)
17	65, 74B, 75B	M3	114*	T	61 (2)	A → T (faint)
17	48, 63B	M3	158	D ?	exon 2	A → T (faint)

Book	Pages	Lot	Number	Ploidy	Codon	Mutation
(New) :	10-9-0-1	M3	155	D	exon 1	ND
↓	10-9-0-2	M3	168	D	exon 1	ND
	10-9-0-3	M3	156	D	exon 1	ND
	10-9-0-4	M3	158	D	15(2)	A → T ?
	10-9-0-5	L1	35	D	13(1)	G → C
	10-9-0-6	M1	18	D	exon 1	ND
	10-9-0-7	M1	24	D	12(1)	G→ A
	10-9-0-8	L1	9	D	13(1)	G → C
	10-9-0-9	M3	133	T	exon 1	ND
	10-9-0-10	M1	126	T	exon 1	ND
	10-9-0-12	M1	11 b	T	exon 1	ND
	10-9-0-13	M1	139	T	exon 1	ND
	10-9-0-14	M3	162 *	T	exon 1	ND
	10-9-0-15	M1	134	T	exon 1	ND
	10-9-0-16	M1	130	T	exon 1	ND
	10-19-0-1	L1	41	D	exon 1	ND
	10-19-0-2	M1	15 *	D	12(2)	G → T
	10-19-0-3	M1	16	D	exon 1	ND
	10-19-0-4	M1	26 *	D	exon 1	ND
	10-19-0-5	M1	34 b	T	13(1)	G → C
	10-19-0-6	M1	92	T	exon 1	ND
	10-19-0-7	M1	27	T	exon 1	ND
	10-19-0-8	M1	95	T	exon 1	ND
	10-19-0-9	L2	37	T	exon 1	ND
	10-19-0-10	M3	138	T	exon 1	ND
	10-19-0-11	L2	24	T	exon 1	ND
	10-19-0-12	L2	17 a	T	exon 1	ND
	10-19-0-13	L2	44	T	exon 1	ND
	10-19-0-14	M1	122	T	exon 1	ND
	10-19-0-15	M3	114 a * ?	T	exon 1	ND
	10-31-0-1	L1	24	D	exon 1	1bp del at codon18
	10-31-0-2	M2	25	D	exon 1	ND
	10-31-0-3	M1	14	D	exon 1	ND
	10-31-0-4	L2	13	D	13(1)	G → C
	10-31-0-5	L1	24	D	exon 1	ND
	10-31-0-6	L2	15	D	exon 1	ND
	10-31-0-7	M1	33	D	exon 1	ND
	10-31-0-8	M1	147	T	exon 1	ND

(Note: in these tables a "*" indicates a duplicated sequencing)

Table B3. Ki-ras1 mutations in cDNAs from AFB_1-induced liver tumors

₪CSL# :	Page	Lot	Fish #	Ploidy	Codon	Mutation
6-26-0-1	28	24B	6	T	exons1,2	ND
6-26-0-2	28	21B	5	T	12 (1,2)	GG→TT (represents 5 tumors)
6-26-0-3	28	4B	59	T	12 (1)	G→C (?)
6-26-0-4	28	4B	43	T	12 (1)	G→C
6-26-0-5	28	4B	20	T	12 (1)	G→A (?)
6-26-0-6	28	4B	68	T	12 (1)	G→A
7-3-0-1	29	5A	41	D	12 (2)	G→T
7-3-0-2	29	5A	33	D	12 (1)	G→A
7-3-0-3	29	5A	45	D	12 (2)	G→T
7-3-0-4	29	5A	46	D	12 (1)	G→C and G→A
7-3-0-5	29	5A	40	D	12 (1,2)	GG→TT
7-3-0-6	29	5A	19	D	exons1,2	ND
7-11-0-1	42	5B	3	D	12 (1)	G→C
7-11-0-2	42	5B	8	D	12 (1,2)	GG→TT
7-11-0-3	42	6B	20	T		failed
7-11-0-4	42	6B	4	T	11 (3)	A→T ?, 12 (1,2) prob. normal
7-11-0-5	42	6B	21	T	12 (1)	G→C
7-11-0-6	42	6B?	46	T	12 (2)	G→C, and 12 (1) ?
7-25-0-1	46	5B	9	D	exons1,2	ND
7-25-0-2	46	5B	6	D	61 (2,3)	A→G and G→T [△]
7-25-0-3	46	5B	1	D	61 (2,3)	A→G and G→T [△]
7-25-0-4	46	5B	12	D	exons1,2	ND
7-25-0-5	46	6A	78	T	12 (1)	G→C
7-25-0-6	46	6A	78*	T	12 (1)	G→C
7-25-0-8	42,46	6B	20*	(T)	12 (1,2)	GG→TT (redo of "failed" above)

Notes: These sequencings are of cDNAs prepared from mRNA isolations from AFB1 initiated trout liver tumors, they assess both exons in a single reading. All primary records are Oregon State University, Center for Gene Research and Biotechnology, Central Services Laboratory printouts. Preparative work is described in Bryan L. Ford's Notebook #21)

In these tables a "*" indicates a duplicated sequencing

₪ All CSL (Central Services Lab) numbers constructed as BLF-... followed by date and serial digit.

Excluding row 2, which represents 5 tumors sequenced collectively and one duplicated sequencing, there are Ki-ras1 mutations in 18 out of 22 of these liver cDNAs, or 82% incidence. There are three instances of a GG→TT double mutation, excluding that in row 2, for 14% overall incidence..

[△]This result in one sample is remarkable enough, but the fact that it is duplicated in an adjacent sample suggests that at some stage of the process the two samples may have been co-mingled, if so then they most likely would have been an A →T at codon 61(2) and a G →T at 61(2). However, there is at least one other example of such a combination seen in our genomic data: the stomach tumor analysis in Table C2, p. 147, Lot # M1 fish # 147.

Appendix C: Upstream *Ki-ras1* sequence data: GenBank submissions

Facsimile C1. GenBank accession AF173858 (cf. pp, 150 - 152 of this Dissertation).

☐ 1: AF173858 **Oncorhynchus mykiss Ki-Ras1 gene, 5' UTR partial sequence**

```
LOCUS       AF173858      175 bp    DNA             VRT       21-MAR-2000
DEFINITION  Oncorhynchus mykiss Ki-Ras1 gene, 5' UTR partial sequence.
ACCESSION   AF173858
VERSION     AF173858.2  GI:7272184
KEYWORDS    .
SOURCE      rainbow trout.
  ORGANISM  Oncorhynchus mykiss
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Euteleostei;
            Protacanthopterygii; Salmoniformes; Salmonidae; Oncorhynchus.
REFERENCE   1  (bases 1 to 175)
  AUTHORS   Ford,B.L.
  TITLE     Analysis of tumor suppression, ras activation and p53 mutation in
            triploid rainbow trout
  JOURNAL   Thesis (1999) Oregon State University
REFERENCE   2  (bases 1 to 175)
  AUTHORS   Ford,B.L., Cohen,A.L., Hendricks,J.D. and Bailey,G.S.
  TITLE     Analysis of tumor suppression, ras activation and p53 mutation in
            triploid rainbow trout
  JOURNAL   Unpublished
REFERENCE   3  (bases 1 to 175)
  AUTHORS   Ford,B.L. and Bailey,G.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (28-JUL-1999) Marine/Freshwater Biomedical Sciences
            Center, Oregon State University, Wiegand Hall, Room 100, Corvallis,
            OR 97331-6603, USA
REFERENCE   4  (bases 1 to 175)
  AUTHORS   Ford,B.L. and Bailey,G.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (21-MAR-2000) Marine/Freshwater Biomedical Sciences
            Center, Oregon State University, Wiegand Hall, Room 100, Corvallis,
            OR 97331-6603, USA
  REMARK    Sequence update by submitter
COMMENT     On Mar 21, 2000 this sequence version replaced gi:5825513.
FEATURES             Location/Qualifiers
     source          1..175
                     /organism="Oncorhynchus mykiss"
                     /strain="Shasta"
                     /db_xref="taxon:8022"
     gene            1..>175
                     /gene="Ki-Ras1"
     misc_feature    1..175
                     /gene="Ki-Ras1"
                     /note="includes 5'UTR and region upstream of translation
                     start site"
     variation       107
                     /gene="Ki-Ras1"
                     /note="compared to Ki-Ras1 sequence presented in GenBank
                     Accession Number AF173859"
                     /replace="t"
BASE COUNT       42 a     33 c     39 g     61 t


ORIGIN
        1 cctgattcta ggaacctcca acggaaaaca tgggcatttt tggaaggata ctggaattct
       61 gcaaccctat atatgtgact ttcttgttga gtcgatagtt ggtgtggagc tcatgaaatg
      121 tttatttta tattcactga ctgtattcct gtgtcttctg tcccacaggt gaacg
```

Appendix C (Continued): ***Facsimile C3. GenBank accession AF301535***
(cf. pp 150 -152 of this Dissertation)

☐ 1: AF301535 **Oncorhynchus mykiss Ki-ras1 gene, 5' UTR**

```
LOCUS       AF301535      399 bp    mRNA            VRT       01-NOV-2000
DEFINITION  Oncorhynchus mykiss Ki-ras1 gene, 5' UTR.
ACCESSION   AF301535
VERSION     AF301535.1  GI:11066946
KEYWORDS    .
SOURCE      rainbow trout.
  ORGANISM  Oncorhynchus mykiss
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Euteleostei;
            Protacanthopterygii; Salmoniformes; Salmonidae; Oncorhynchus.
REFERENCE   1  (bases 1 to 399)
  AUTHORS   Ford,B.L. and Bailey,G.S.
  TITLE     Ki-ras1 5'UTR from 200 tumor collective cDNA
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 399)
  AUTHORS   Ford,B.L. and Bailey,G.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-AUG-2000) Environmental and Molecular Toxicology,
            Oregon State University, Wiegand Hall 100, Corvallis, OR
            97331-6602, USA
FEATURES             Location/Qualifiers
     source          1..399
                     /organism="Oncorhynchus mykiss"
                     /strain="Shasta"
                     /db_xref="taxon:8022"
                     /tissue_type="approximately 200 pooled liver tumors"
                     /note="tumor initiated by dietary aflatoxin B1"
     gene            <1..>399
                     /gene="Ki-ras1"
     5'UTR           1..399
                     /gene="Ki-ras1"
BASE COUNT      104 a     87 c     99 g    108 t      1 others
ORIGIN
        1 tccccctaga gcagttgggg tgaggatccc acttgaaggt cctgctctnt tcagcaccca
       61 gaccacagtg tgtactggca gccttcacat ggcctggctg tgaactggtt cgactcagct
      121 agagtcacct tcaccatgat acacttggag aggtagttga acactgtggc cttgagtgtg
      181 acgagagtag caggccaaac gctgtatcaa atggaaagtt aaatcgatta atgtccttac
      241 ttttgtctgc ttttttaaaa gaatatacat ttacacaaat gcggccgttt cagtgtaaag
      301 taggaaatgc agagatcgtg tgaaaaagga ggattatctg cttgacttct tatttggtcg
      361 ctacttcgga cgcggaactc cacacgctat aggtgaacg
//
```

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Figure D1. ***Ki-ras1*** **pretranslational distal genomic sequence, from upstream of intron "-1" (cf. Figures 5.2 and 5.3).** Templated from a long inverse PCR on genomic DNA using *Ki-ras1*-selective primers. Direct cycle-sequencing of this inverse PCR was conducted using a *Ki-ras1*-selective primer (see Table and Figure 5.1). Comparison of this sequence with that generated in Figure D3, shows evidence of the ~9 kbp intron "-1" which begins at position 53 of this electropherogram. The apparent "**gt**" there corresponds to the consensus 5' intron terminal motif.

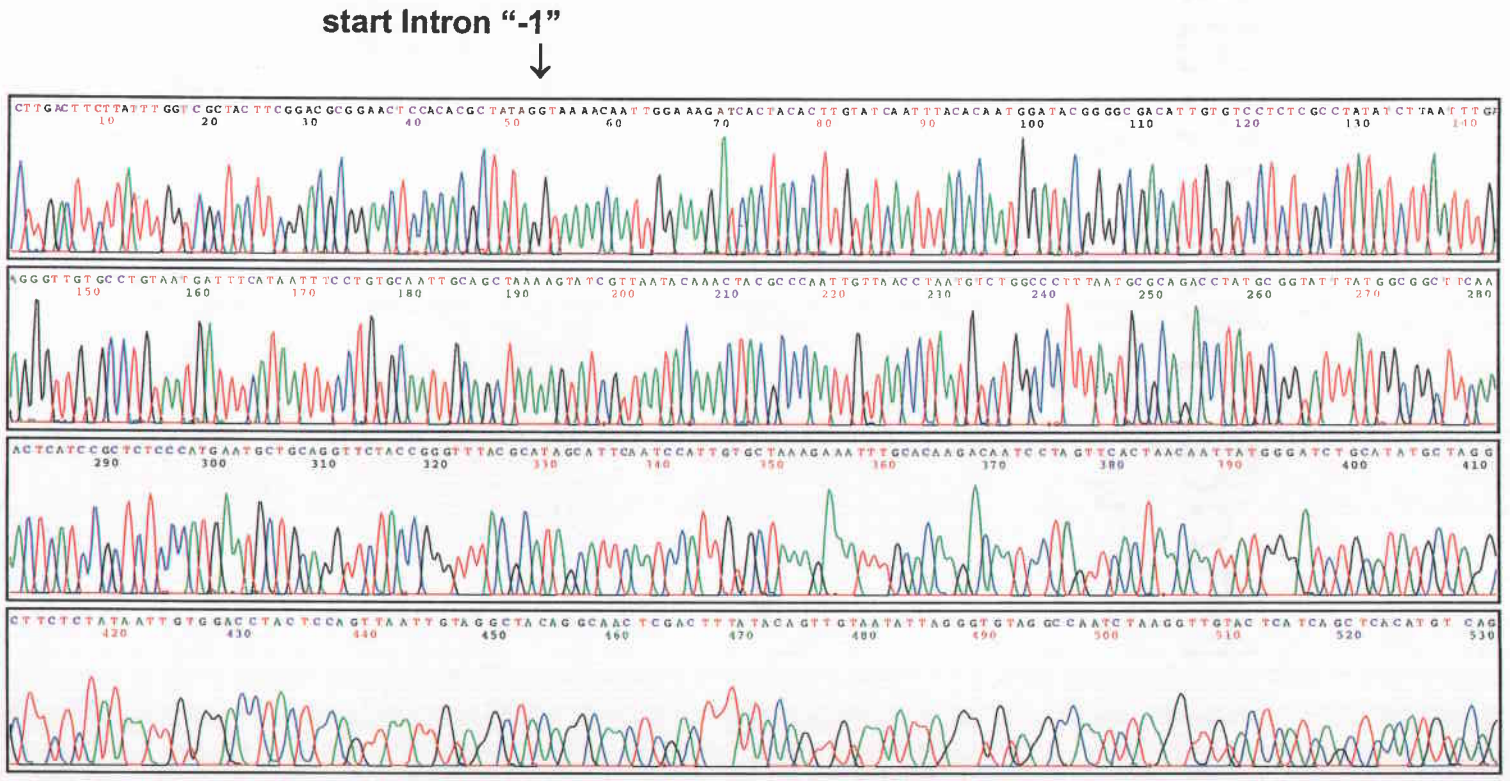

Figure D2. ***Ki-ras1* pretranslational proximal genomic sequence, from downstream of intron "-1" (cf. Figures 5.2 and 5.3).** From long inverse PCR on genomic DNA using *Ki-ras1*-selective primers. Sequencing also conducted using a *Ki-ras1*-selective primer (see Table and Figure 5.1). Comparison of this sequence with the exon 1 sequence known for *Ki-ras1*shows the inverse complement of the "**atg**" translation start at position 136 ("**cat**"). Comparison of this sequence with that generated in Figure D3, shows that the deduced ~9 kbp pretranslational intron "-1" ends at position 144 of this electropherogram. The inverse complement of the 3'consensus terminal motif "**ag**" is seen there as "**ct**".

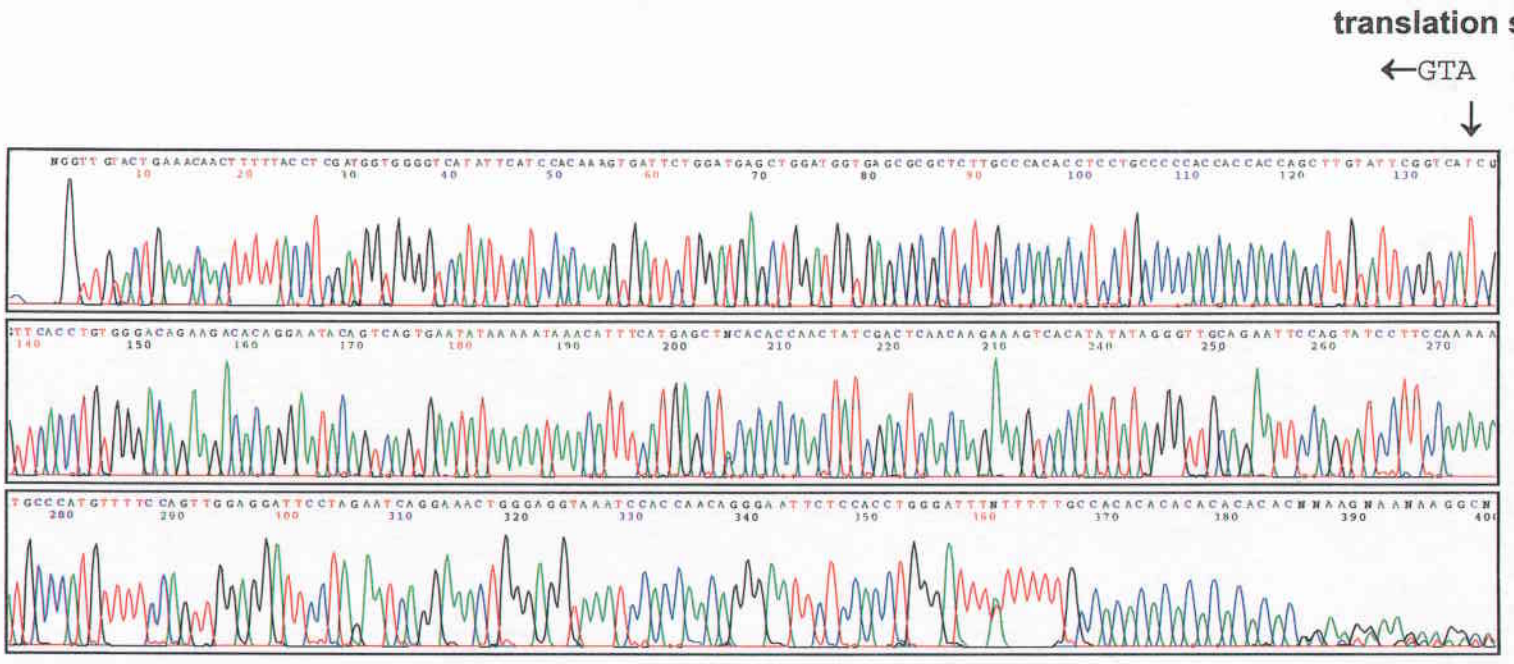

Figure D3. ***Ki-ras1* proximal 5' UTR sequence from inverse PCR of cDNA, (cf. Appendix facsimile C3).** Direct cycle sequencing conducted using a *Ki-ras1*-specific primer on a template of PCR product, itself generated from cDNA template using *Ki-ras1*-specific primers (see Table and Figure 5.1). The inverse complement of the "atg" at the translation start is apparent at position 31 in this electropherogram. The junction made by cellular ligation of the mature message, after processing excision of the 9 kbp intron "-1" of *Ki-ras1* pre-mRNA, is evident by sequence comparisons at positions 38 and 39 (*i.e.* at "/ " in **...catcgttcac/ctatagcgtg...**) of this electropherogram.