ABSTRACT OF THE THESIS OF

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George S. Bailey

A digoxigenin-labeled nonradioactive detection system was used to screen a zebrafish cDNA library for p53-like and ras-like genes. One clone was isolated and identified as an incomplete p53-like gene. The insert size of this clone is 1777 bp, which encodes part of evolutionarily conserved region II and all of regions III, IV, and V. A magnetically enriched whole zebrafish cDNA library was constructed to enhance possible recovery of ras-like genes in zebrafish. One clone, termed Zras-B1, carried an insert of 2592 bp with an open reading frame encoding a 188 amino acid residue ras p21 protein. Based on total protein sequence, this expressed zebrafish ras p21 is most closely related to human N-ras (91% homology), with lesser homology to Ha-ras (84%) and Ki-ras (85%). Preliminary partial sequence data obtained by genomic and reverase, transcriptase-polymerase chain reaction (RT-PCR) screening indicate the presence of at least one additional expressed ras gene in zebrafish.

The tumorigenicity and Ki-ras mutational properties of dietary 7,12-dimethylbenz[a]anthracene (DMBA) and dibenzo[a,l]pyrene

(DBP) were compared in rainbow trout. Both chemicals elicited predominantly 12(1)G→A and 12(2)G→T mutations in trout liver tumors. Two {12(1)G→T and 12(2)G→T} and one {12(1)G→A and 12(2)G→T} double mutation were also observed in DBP livers tumors, but not in DMBA liver tumors. Some stomach tumors from both chemicals exhibited so much DNA degradation that routine PCR amplification was not possible. Among sixteen DMBA stomach tumors with intact DNA, no Ki-ras mutations were found. Of sixteen DBP stomach tumors examined, one had 12(1)G→A and two had 13(1)G→C mutations. The observed G→T transversions are compatible with apurinic mutagenesis driven by unstable DNA adducts arising from one-electron oxidation, but this is not true for the major G→A transitions or G→C transversions and rare double mutations found in this study. The low sensitivity of direct sequencing may limit the frequency of ras mutant detection in this study.

Ras Oncogenes and p53 Suppressor Genes in Fish Carcinogenesis Models

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Ronshan Cheng, Author

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Ras Oncogenes and p53 Suppressor Genes in Fish Carcinogenesis Models

Chapter 1: Introduction

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INTRODUCTION

It has been appreciated for many years that chemical carcinogens after bioactivation interact with macromolecules to form specific adducts. DNA adduction can lead to the introduction of single base mutations, deletions, or rearrangements in the genetic material. Although many adducts resulting from treatment of DNA or cells with chemical carcinogens have been identified, the biological significance of specific adduct formation has not been clear. Some of the major adducts may be readily repaired, whereas minor adducts could be those responsible for the initiation of genetic alternations inside the cell. The chemical characteristics of each carcinogen determine the specific DNA adducts formed, and the nucleotides adducted. Bulky DNA adducts might affect the fidelity of DNA polymerase and DNA repair systems (Scicchitano et al., 1992). Indeed, carcinogen-mediated genetic alternations such as point mutations, translocations, or gene amplification have been implicated in the activation of proto-oncogenes to an oncogenic state or inactivation of tumor suppressor gene function (Hollstein et al., 1991; Lowy et al., 1993). The ras oncogene and p53 tumor suppressor gene are the most prevalent genetic alterations found in human and animal models. Chapter 2 is a literature review of the biological and biochemical functions of the ras proto-oncogene and p53 tumor suppressor gene. Ras oncogene mutation induced by chemical carcinogens, especially, 7, 12 diemthylbenz[a]anthracene (DMBA) and dibenzo[a,l]pyrene (DBP),

in animal tumors will be reviewed. Much of the research in this thesis used the trout as a surrogate vertebrate for cancer research. Among the several aquarium species also being developed (Ahmed, 1993), the zebrafish (*Brachydanio rerio*) is of great interest and potential as an environmental toxicology and carcinogenesis model (Stanton, 1965; Streisinger et al., 1981). Although the zebrafish has a well established development biology of embryogenesis, the role of proto-oncogene and tumor suppressor gene expression in differentiation and tumorigenesis is unknown. In chapters 3 and 4, we provide the first isolation of the p53 tumor suppressor gene and ras proto-oncogene from zebrafish cDNA libraries, for further studies of oncogene and tumor suppressor function in this species.

During the past decade, our knowledge of polycyclic aromatic hydrocarbons (PAH) in the aquatic and terrestrial environment has advanced substantially to encompass studies of bioavailability, metabolism, toxic effects, and their ecological and biological consequences (Varanasi, 1989). The impetus for these studies has come from reports of epizootics of diseases in animal population from PAH-contaminated areas, and a general awareness towards human health. Moreover, because many aquatic organisms reside in confined waters, where they may be exposed to xenobiotics over long periods of time, they may serve as good models for studies to establish cause and effect relationships between exposure to xenobiotics and subsequent biological effects (Bailey et al., 1989). Until recently, DMBA has been the most popular synthetic polycyclic aromatic hydrocarbon used in carcinogenesis

studies. However, recent attention has focused on dibenzo[a,l]pyrene, a natural polycyclic aromatic hydrocarbon and one of the most potent PAH carcinogens tested in rodent models (Cavalieri et al., 1989). In chapter 5, we investigate the tumorigenicity and Ki-ras mutational properties of these two compounds in dietary studies with rainbow trout. In the last chapter, I try to summarize the research results and propose some new directions for future research.

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CHAPTER 2: LITERATURE REVIEW

Malignant cells arise as an accumulation of genetic events that lead to oncogene activation, tumor suppressor gene inactivation, and genomic instability, which escape the cell cycle control checkpoints and finally result in indefinite life span. Recent results have indicated that the activation of cellular ras proto-oncogenes by specific base mutations or overexpression may participate in the transformation of normal cells to malignancy. Ras protein function in receptor-mediated proliferation or differentiation is determined by the cell (Feig, 1993). These two responses may share overlapping signaling mechanisms. In defining ras protein functions, it is necessary to identify the molecules in the cell that interact with p21ras, and to establish how this leads to the activation of signal transduction pathways coupled to cell proliferation and morphological transforamtion.

Ras signal transduction pathway

The ras genes belong to a GTPase superfamily encoding proteins of 188-189 amino acids that bind guanine nucleotides GDP and GTP with high affinity and possess intrinsic GTPase activity. Ras proteins cycle between an active GTP-bound form and an inactive GDP-bound state (Bourne et al., 1990). Mammals have three well-defined ras genes, Ha- , N-, and Ki-ras (Parada et al., 1982; Der et al., 1982; Taparowsky et al., 1983), which encode closely related 21 KDa guanine nucleotide-binding proteins thought to play a

central role in cellular signal transduction. Mammalian ras homologues have been found in yeast, mollusks, and fish (DeFeo et al., 1983; Swanson et al., 1986; Nemoto et al., 1986; McMahon et al., 1990; Mangold et al., 1991), and their functions are sufficiently conserved that they can be biologically active in heterologous systems (Barbacid, 1987). Based on their protein sequence, ras proteins can be divided into three regions: the first domain, including the first 86 amino acids of the N-terminal region is highly conserved; the next 80 amino acid regions contains the second domain and has diveraged slightly; the remaining amino acids are highly variable, except the CaaX (C= cysteine amino acid, a= aliphatic amino acid, X= any amino acid) motif in the C-terminal end (Schafer et al, 1989). Post-translational processing of ras proteins, which is required for cytoplasmic inner membrane localization, normal signal transduction or transformation ability, includes isoprenylation, proteolysis, and carboxyl methylation in the highly conserved CaaX box.

(A): Membrane receptor tyrosine kinase interaction and regulation of ras protein

Activating point mutations in the p21ras proteins have been found in a variety of animal tumors, thus implicating these proteins with other cellular proteins involved in cellular malignancies. Despite this evidence, the function of p21ras in the regulation of normal cell growth, mitogenic signal transduction, and cell differentiation is still unclear. Recent extensive genetic and biochemical evidence indicates that ras proteins can function

downstream of various growth or nongrowth factor receptor tyrosine kinases and upstream of cytosolic serine/threonine kinases (Abdellatif et al., 1994). Hydrolysis of GTP appears to be much faster in vivo than in vitro, suggesting that in vivo normal p21ras exists mainly in the inactive GDP form (Trahey et al., 1987). Indeed, in the cells ras activities are controlled by two types of proteins, guanine nucleotide releasing factors (GNRFs) and GTPase activating proteins (GAPs). The GNRFs and GAPs are tissue-, receptor-, and ras-specific in their regulation of ras activity (Pronk et al., 1994). The GNRPs serve as positive regulators by facilitating the release of GDP, thereby allowing GTP to bind to ras. The GAPs act as negative regulators by stimulating the low intrinsic GTPase activity of ras. Neurofibromatosis type 1 (NF-1), the tumor suppressor gene encoding a protein known as neurofibromin, has a region homologous to the catalytic domain of GAP and is more abundant in the nervous system. In the NF-1 derived malignant Schwannomas that lack neurofibromin but express normal levels of p120GAP, the p21ras-GTP is at normal level thus indicating null GAP activity (Basu et al 1992, DeClue et al 1992). So it seems that, in Schwann cells, p120GAP has little or no part in p21ras-GTPase activation, and that neurofibromin is the major negative regulator. Conversely, in mouse fibroblasts p120GAP activity is sufficient to maintain p21ras in its inactive state despite the presence of neurofibromin (Zhang et al, 1991). These observations imply that, although neurofibromin and p120GAP are both ubiquitous, one or the other predominates to determine the fraction of p21ras that is in the active GTP state.

The existence of different ras regulating proteins in specific cells might imply that ras mutations found in animal and human are tissue specific.

Studies have shown that p21ras-GTP can be regulated by signals generated from membrane receptors. There are two type of membrane receptors, which through ligand binding are able to stimulate the ras protein to its activate state. The first type are receptors with tyrosine kinase activity, like platelet-derived growth factor (PDGF), epidermal growth factor (EGF), nerve growth factor (NGF), and insulin receptors. The second type of receptors contain no intrinsic tyrosine kinase activity, but couple to non-receptor tyrosine kinase, such as T cell receptor for interleukins and erythropoietin receptor (Pronk et al., 1994). The stimulation of a variety of cell surface receptors promote the accumulation of the active, GTP-bound form of ras proteins in cells. To reach the active GTP-bound state, ras proteins must first release bound GDP and reduce the GTPase activity by inhibiting the GAP activity. This rate-limiting step in GTP binding is thought to be catalyzed by guanine nucleotide releasing factors (GNRFs). Several potential activators of ras have been discovered in a variety of animals, such as Sos gene for Drosophila, mSos-1 and mSos-2 for mouse, and $p140^{ras\text{-}GRF}$ for rat (Shou et al., 1992). In the case of growth factor receptors with tyrosine kinase activity, ligand activation will stimulate the autophosphorylation of receptors at tyrosine residues. These phosphorylated-tyrosine residues in turn trigger the binding of adaptor proteins, such as Grb2 protein, which will bring the guanine nucleotide exchange factor to the membrane to

activate the ras proteins (Pawson, 1994). The data in summary indicate that these adaptor proteins facilate the association of GNRFs with receptor tyrosine kinase.

Activation of tyrosine kinase receptors such as PDGF or EGF with the appropriate ligands, or transforming of cells with tyrosine kinase oncogene product (e. g., v-src), resulted in the phosphorylation of GAP at tyrosine residues and association with receptors. The association of receptors and GAP reduces the amount of free GAP in the cytosol and leaves ras protein in its activate state. Thus, the ras/GAP complex is regulated by the tyrosine kinase signal transduction cascade.

The association of GAP with two cellular proteins p62 and p190 suggests that GAP may actually serve a dual function, as an effector and an attenuator of ras (Downward, 1992). The interaction between GAP and the ras effector domain, amino acid residues 32-40, demonstrates clearly that GAP is an effector molecule of ras. These results support the concept that tyrosine phosphorylation of GAP results in the inhibition of its GTPase-stimulating activity, leading to increase GTP-bound p21ras, which might then affect mitogenic signaling through some as yet undetermined downstream component. Indeed, in some studies, no effect on nucleotide exchange activity in the cell was observed, with the decrease of GAP activity being directly responsible for the ras-GTP accumulation (Downward, 1992).

(B). Ras downstream signal transduction: cytoplasm to nucleus

Signals initiated at various receptors in the plasma membrane must traverse the cytoplasm and converge in the nucleus. The convergence of these signals alters the expression of specific genes and commits a cell to proliferation or differentiation. Ras proteins serve as pivotal molecules to transduce the signal from the membrane to activate the cytoplasmic serine/threonine protein kinase in response to many different growth factors (Kyriakis et al 1992). In 1986, Smith et al. discovered that inhibition of p21ras by neutralizing antibodies does not abolish v-raf-induced transformation, indicating either that v-raf acts as a downstream effector, or is independent of p21ras. Later on, Yamada et al. (1991), showed retransformation of revertants from oncogenic ras transformed NIH 3T3 cells by raf oncogene. This result implies that raf acts downstream of ras in signal transduction. Kyriakis et al. (1992) discovered a signalling mechanism based on a v-raf transformed cell line, in which mitogen-activated protein kinases p44MAPK and p42MAPK (MAPKs), also known as extracellular-signal-regulated kinases 1 and 2 (ERK1 and ERK2), and MAPK kinase are constitutively expressed and activated by c-raf at their serine/threonine residues, indicating that c-raf-1 is an immediately upstream activator of MAPKs in vivo. A direct association of raf-1 with the activate state of ras was later demonstrated that the N-terminal region of raf-1 protein was bound to the effector domain of ras protein.

The presence of MAPKs and members of MEK (MAPK or ERK Kinase) in the cytoplasm and nucleus of Hela cells after response to growth factors indicated that regulation of nuclear MAPKs and

MEK activities by growth factors is coordinated with immediate-early gene expression (Chen et al.,1992). Indeed, in vitro, MAPK and MEK phosphorylate histone H3 and the c-fos, c-jun transcription factors in a variety of cells. These in vitro studies raise the possibility that the MEK/MAPK signal transduction pathway represents a tyrosine, serine/threonine phosphorylation cascade for the rapid transmission of growth-regulating information from the membrane, through the cytoplasm, and to the nucleus.

AP-1 activity, which is composed of the jun and fos gene products, is stimulated by growth factors, tumor promoters, and transforming oncoproteins (Angel et al., 1988; Lamph et al., 1988; Schonthal et al., 1988; Wasylyk et al., 1989) and is required for proliferation of fibroblasts and their transformation by the ras oncogene (Lloyd et al., 1991). While part of the increase in AP-1 activity is due to increased jun and fos gene transcription, post-translational modifications by phosphorylation play an important role, at least in the initial phase of the response. In the case of c-jun, phosphorylation of two to three C-terminal sites next to its DNA binding domain negatively regulates DNA binding, whereas phosphorylation of two N-terminal sites residing within its activation domain stimulates its transactivation activity (Smeal et al, 1991). While the tumor promoter tetradecanoyl phorbol acetate (TPA) stimulates c-jun activity by inducing dephosphorylation of the inhibitory sites, transient or stable expression of the Ha-ras oncoprotein leads to both partial dephosphorylation of the inhibitory sites and hyperphosphorylation of the stimulatory sites

(Boyle et al, 1991). Other oncoproteins with distinct biochemical activities, Ha-ras, v-sis, v-src and an activated form raf-1 also display an identical effect on c-jun phosphorylation and activity (Smeal et al, 1991). Expression of all four proteins stimulates phosphorylation of serine 63 and 73 within the c-jun activation domain. Therefore, c-jun is a downstream target for a phosphorylation cascade involved in cell proliferation and transformation. Evidence may suggest that receptor tyrosine kinase-induced signalling through p21ras is a more or less simple linear pathway, however this does not appear to be the case (Pronk et al., 1994). A more divergent and complicated pathway will be cell and tissue dependent and also depend on certain physiological conditions. A generalized ras signaling pathway is presented in Figure 2.1.

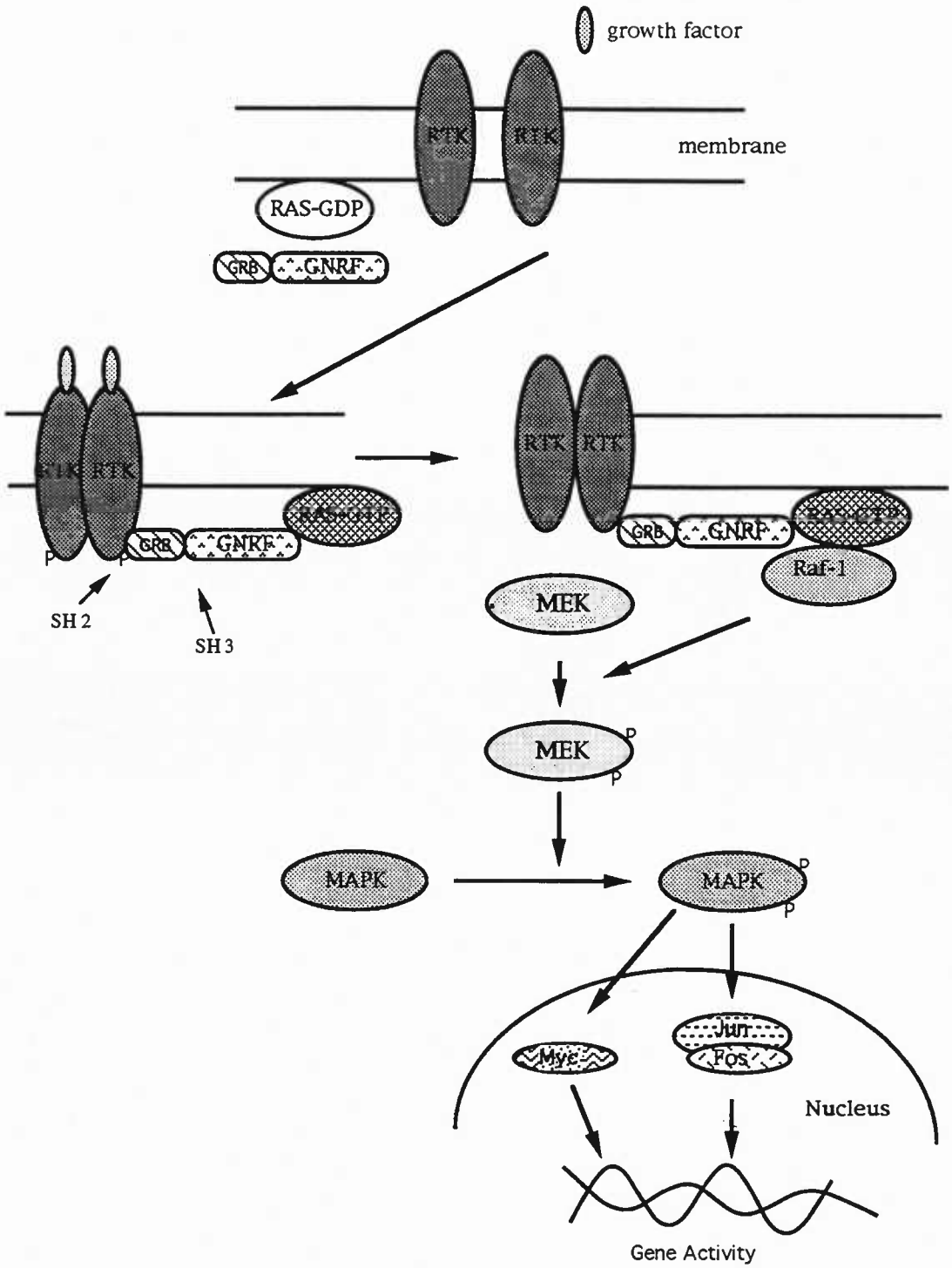

Figure 2.1: Ras signaling pathway

Biological consequences of oncogenic ras protein

Oncogenic ras is constitutively locked in the active GTP form, through mutations that either inhibit the hydrolysis of bound GTP or increase the rate of guanine nucleotide exchange, so favoring increased levels of p21ras-GTP. The ras oncogenes function by indirectly controlling expression of a set of undefined genes that are crucial for cell growth and differentiation. The downstream nuclear transcriptional factors, c-jun and c-fos have been associated with ras-induced gene expression. The discovery of gene promoters with AP-1 binding sites will be helpful to elucidate the biological functions induced by oncogenic ras genes. One such gene, the DNA methyltransferase has been identified (Rouleau et al., 1995), which contains several AP-1 sites in the 5' regulatory region. Introduction of an oncogenic Ha-ras into a adrenocortical tumor cell line, Y1, increased the level of DNA methylation and DNA methyltransferase activity (Macleod et al., 1995). The methylation activity has been associated with gene regulation, however, the biological consequence of increasing DNA methylation by oncogenic ras gene is still unclear. Several biological consequences have been related with the oncogenic activity of ras, such as up regulation of ornithine decarboxylase (Hurta et al., 1994), aberration of histone H1 protein and chromatin structure (Denko et al., 1994), and reduction of the cell surface and extracellular matrix proteins like fibronectin and type I collagen (Schonthal et al., 1988; Slack et al., 1992). Transformation with oncogenic ras and other oncogenic proteins

has been reported to repress transcription of the collagen gene and induce cells to synthesize high levels of matrix-degrading enzymes. A more surprising observation is an increase of genomic instability (Laitinen et al., 1995), which is associated with early genetic events in tumor progression and might imply a strong correlation with aberrant chromatin structure (Denko et al.,1994).

Stage of ras mutation relative to tumor formation

Initial evidence suggested that ras activation was a late event (Albino et al., 1984). These researchers reported that, among five melanoma cell lines originating from separate metastatic tumors, only one contained an activated H-ras gene. Similar conclusions were reached by Vousden et al. (1984), who found that a mouse T cell lymphoma contained a activated Ki-ras gene in a metastatic derivative of this lymphoma. This suggested that ras activation was not involved in tumor initiation or maintenance but occurred at the stage of metastasis. A similar finding was published recently by Sugio et al. (1994), who demonstrated that in lung cancer, K-ras mutation is a late event and may be associated with the malignant phenotype. This supported the previous suggestion that the ras mutation might be a late event for cancer formation. However, other data suggest that activation of ras genes can also be an early event in in vivo carcinogenesis (Zarbl et al., 1985; Quintanilla et al., 1986). In these studies, only a single dose of MNU or DMBA was applied, yet H-ras activation was consistently found, not only in carcinoma but also in premalignant papillomas. These findings are

consistent with the observation of Kumar et al (1990), in which mutated H-ras and K-ras alleles are found in mammary glands 2 weeks after the carcinogen treatment and 2 months prior to tumor appearance. Thus mutant ras oncogenes can remain dormant in the target tissue and require addition events before tumors develop. In humans, colorectal cancers were used to study the timing of activation of ras genes since this type of cancer has a well-defined premalignant stage in the form of adenomas or polyps. Histological analysis frequently shows that adjacent to carcinoma tissue tissue, adenoma tissue from the benign precursor lesion of the tumor may be present (Bos et al., 1987). In all carcinoma tissues examined, only one lacked an activated ras gene in the adenoma; in all others situations both the carcinoma and adenoma contained the same mutated ras gene. This indicated that ras activation may be an initiating event, but other genetic events are required to drive the cancerous cell to a futher degree of malignancy.

The biological and biochemical function of p53 tumor suppressor gene

The p53 tumor suppressor gene is highly conserved in the veterbrate kingdom (Soussi et al., 1990) and found in a variety of species including the human, mouse, rat, chicken, hamster, frog and trout (Oren et al., 1983; Matlashewski et al., 1984; Coulier et al., 1985; Louis et al., 1988; Smith et al., 1988; Legros et al., 1992; de Fromentel et al., 1992). Although there is sequence diversity of the p53 gene among species, the p53 coding sequence consists of five evolutionarily conserved domains that are believed to specify

the important functions of this protein (Soussi et al., 1990; Prives, 1994). Many lines of evidence suggest the importance of p53 in human carcinogenesis, chief among these being that mutations within the p53 gene are the most frequent genetic aberration thus far associated with human cancer (Nigro et al., 1989; Hollstein et al., 1991). The diversity of aberrant p53 proteins associated with a variety of tumors has provided important clues to the biological and biochemical functions of p53 proteins inside the cell. Based on the primary amino acid sequences, there are three functional domains of the p53 protein (Levine, 1993). The first 80 amino acids at the N-terminal end are acidic in nature and function as a transcriptional activator. The next 75 amino acids located in the central region of p53 protein are proline-rich and quite hydrophobic and reflect a crucial role in structural stability. The carboxyl domain, containing about 100 amino acid from the C-terminal end, has many basic amino acids and functions as a domain for p53 protein oligomerization, DNA-binding, and a motif for nucleus localization signal. The evolutionarily conserved domains are located between the N- and C-terminal functional domains, and contain about 200 amino acids including the proline-rich region. The p53 mutations identified in cancer cells are generally point mutations within the evolutionarily conserved domains (Hollstein et al., 1991; Harris, 1993). It is believed that wild type p53 acts as a transcriptional factor to modulate gene expression and functions as a cell cycle checkpoint at the G1/S phase to protect the integrity of genome in response to X-ray, oxidative stress, or drug-induced damage (Fields et al., 1990; Kern

et al., 1991; Lane, 1992; Funk et al., 1992; Seto et al., 1992; Zhan et al., 1993; Tishler et al., 1993). By contrast, cells with mutant p53 proteins do not pause in G1, but progress into S-phase with damaged DNA (Hicks et al. 1991). This results in genomic instability, increased mutation, and even selection of mutant clones having the properties of cancerous cells. p53 has also been shown to have negative effects on the transcription of various genes as well as to act as a DNA-binding transcriptional transactivator (Weintraub et al., 1991; Mercer et al., 1991; Shiio et al., 1992; Dutta et al., 1993; Dameron et al., 1994). Although p53 can block the progression of the cell cycle when artificially expressed at high levels, it appears to play little role in normal cell cycle control (Louis et al., 1988; Donehower et al., 1992), because p53-deficient mice develop normally but have an increased incidence of tumors. The molecular functions of the wild type p53 protein are still emerging. A outline of p53 protein biochemical properties and p53 mutation location found in various human tumor is given in Figure 2.2.

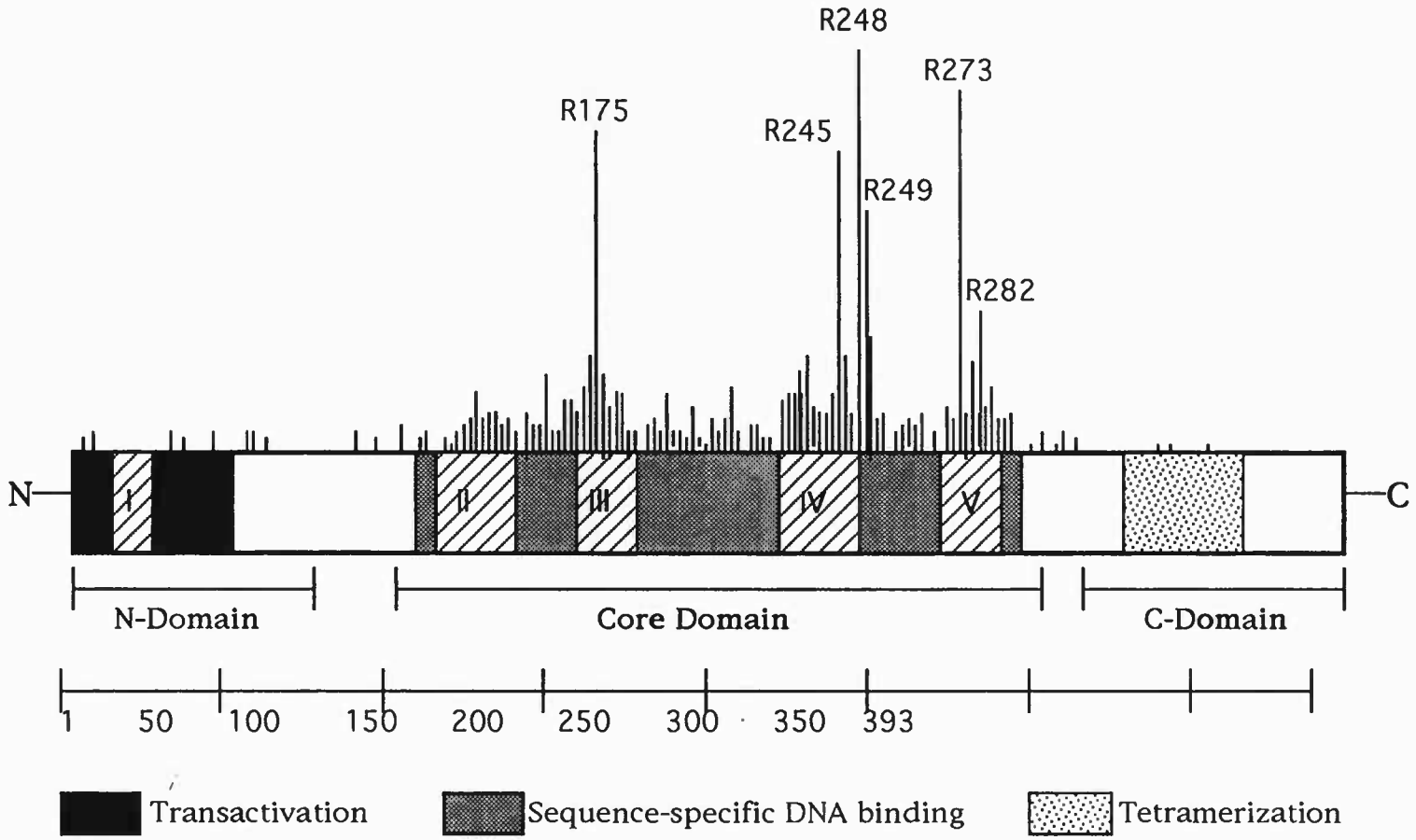

Figure 2.2: p53 functional domains and mutational locations.

Chemical carcinogenesis and ras mutation spectrum by polycyclic aromatic hydrocarbons

A large body of data has been published over the past years on ras activation in human and animal tumors and significant ras activation frequencies have been linked to several types of tumors (Guerrero et al., 1987; Bos, 1988). In human carcinogenesis, key parameters as to the carcinogenic agent and genetic background are frequently unknown. Therefore, a considerable number of animal model systems have been established to study carcinogenesis at the molecular level. Ras proto-oncogenes are activated by point mutations (Bos, 1988), insertion of repeat sequences, small deletions in the coding sequence (Higinbotham et al., 1994; Wiest et al., 1994), mutation at promoter regions (Chakraborty et al., 1991), or overexpression of ras protein by aberrant splicing (Cohen et al., 1988), all of which may result in a transformed cell in vivo or in vitro. Although mutations at other codons have been reported, the animal tumors mainly contain ras oncogenes activated by point mutation at codon 12, 13 or 61 (Barbacid, 1987). It is noteworthy in the case of chemically induced tumors that the type of carcinogenic compound used for tumor induction strongly influences the types of mutation found in ras gene. Whether ras oncogene mutations represent early initiating events in tumorigenesis or play a role at later stages of the process remains to be determined. However, ras activation alone appears insufficient to promote the continual growth of a tumor, and subsequent accumulation of additional genetic damage is required to make the tumor invasive and metastatic (Burmer et

al., 1989; Fearon et al., 1990). Regardless, evidence clearly indicates that the cellular ras protooncogene can serve as a target for environmental carcinogens, such as PAH, to initiate carcinogenesis under the influence of endogenous or exogenous promotion.

(A). Polycyclic aromatic hydrocarbons: bioactivation and DNA binding

The polycyclic aromatic hydrocarbons (PAH) are products formed by incomplete combustion of organic compounds. Sources of environmental PAH include industrial and domestic furnaces, gasoline and diesel engines and tobacco smoke. The basic hypothesis of chemical carcinogenesis by PAH is that formation of a covalent bond between the PAH and DNA represents the first essential step in the tumor initiation process. DNA adduction by promutagenic compounds may be non-complementary or lead to errors during DNA replication. Most PAH need metabolic activation to bind covalently to cellular macromolecules and the ultimate reactive species, which are electrophilic in character, react with nucleophilic groups of cellular macromolecules (Sims et al., 1981). Benzo[a]pyrene was the first PAH to be shown in cells to form a chemical reactive intermediate, diol-epoxide (Sims et al., 1974). Over the last decade, metabolism, mutagenicity, DNA-binding, and tumorigenicity studies have established that bay-region diol epoxides are the ultimate carcinogens of at least a dozen polycyclic aromatic hydrocarbons (Jerina et al., 1986). However, in some cases, activation to other intermediates have led other

investigators to postulate that radical cations arising from removal of one electron from PAH are important electrophilic species capable of reacting with cellular nucleophiles to form unstable DNA adducts (Cavalieri et al., 1985; Devanesan et al., 1990).

Early data lead to the belief that most carcinogens react preferentially with guanine residue in DNA (Jeffrey, 1985). However, experiments used DMBA and other polycyclic aromatic hydrocarbons by the transfection assay in NIH/3T3 cells demonstrated that an H-ras codon 61(2)A→T transversion was the exclusive mutation found in PAH-initiated mouse skin tumors (Bizub et al., 1986). More direct evidence for A→T transversion caused by DMBA was provided by Cheng et al. (1988), who reported that the *syn* 3,4-dihydrodiol 1,2-epoxide of DMBA reacts almost exclusively with deoxyadenosine in DNA. The diol epoxide and one electron oxidation pathways of PAH bioactivation, are both capable of providing initiated DNA adduct formation at guanine or adenine residues (Bigger et al., 1983; Cheng et al., 1988). The diol epoxides are believed to form stable DNA adducts, while one electron oxidation reportedly leads to unstable DNA adducts almost all of which are lost from DNA by spontaneous depurination (Devanesan et al., 1990). Until recently, DMBA, the synthetic PAH, was the most potent known carcinogenic PAH. However, the natural occurring dibenzo[a,l]pyrene (DBP) has been established recently as the strongest PAH carcinogen ever tested in rodent and human cell lines (Cavalieri et al., 1991). However, the in vivo genetic mechanisms including ras oncogene activation, induced by DBP have not been defined.

(B). Adduct formation is DNA context dependent

A significant number of chemical carcinogens are known to form adducts with DNA bases. Whereas some of these adducts are quickly repaired by the DNA repair system, others are highly mutagenic due to their miscoding properties, generation of apurinic sites, or the limited fidelity of repair polymerase (Downers et al., 1993; Hanawalt, 1994). In addition, there is increasing awareness of the heterogeneity of DNA structure, which may be a factor in such mutation targeting (Marien et al., 1989; Georgiadis et al., 1991; Hoffmann et al., 1993). Targeting of mutation, in principle, can be caused by bias of the damaging agent and damage repair activities for or against certain DNA sequences. The reproducible activation of ras oncogenes in carcinogen-induced tumors has made it possible to correlate their activating mutation with the known mutagenic effects of certain carcinogens. In the case of N-nitroso compounds, N-methyl-N-nitrosourea (MNU) methylates DNA at a number of sites, including the O^6 position of guanine. The resulting of O^6-methylguanine-DNA adduct is converted to a G→A point mutation in the second nucleotide of Ha-ras codon 12 during DNA synthesis (Zarbl et al., 1985). Tissue susceptibility to the carcinogenic effect of nitrosoureas shows a strong inverse relation with the amount of alkyltransferase repair activity (Dumenco et al., 1993). Unlike MNU or other alkylation agents, which produced adducts repaired by alkyltransferase, the PAH form large adducts with deoxyguanine and deoxyadenine residues that lead to excision

repair, and generate point mutations of undefined specificity. In the case of benzo[a]pyrene, the mutation spectrum is influenced by the rate of excision repair and the strand-specific preference of adduct position (Chen et al., 1992; Wei et al., 1995).

(C): The spectrum of ras mutation by DMBA, DBP

While it is clear that the nature of the initiating carcinogen can strongly affect the profile of ras mutations detected in tumors, it is also evident that other factors, such as species, tissue type, promoter, dosing regimen, could also exert important influence (Loktionov et al., 1990; Manam et al., 1992; Manam et al., 1995). For example, in the case of DMBA-induced mouse tumors, mammary and skin tumors have predominantly an Ha-ras codon 61(2)A→T transversion (Loktionov et al., 1990), liver tumors carry both the Ha-ras codon 61 mutation and a Ki-ras codon 13(1)G→C transversion, and lung tumors contain a variety of Ki-ras mutations but no Ha-ras mutation. This is consistent with other research showing a strong preference for Ki-ras gene activation in mouse rat and human lung tissue (Manam et al., 1992), and supports previous results that ras mRNA expression is different in different tissues of mouse (Leon et al., 1987). The ras mutation profile can also be affected by the genetic background of the species used. Spontaneous tumors in mice commonly carry ras genes activated by mutations in codons 12, 13 or 61 (Table 2.1). Therefore, the detection of ras mutations in chemically induced mouse tumors does not always demonstrate that the chemical in

Table 2.1: Ras mutation spectra of spontaneous tumor from mice.

Strain	Tumor	Oncogene (Frequency)	Mutation	Reference
B6C3F1	lung	K-ras (3/16)	$G^{35}\rightarrow A$	Candrian et al., 1991
B6C3F1	liver	H-ras (67/103)	$C^{181}\rightarrow A$; $A^{182}\rightarrow T,G$	Reynolds et al., 1987
B6C3F1	liver	H-ras (32/50)	$C^{181}\rightarrow A$; $A^{182}\rightarrow T,G$	Fox et al., 1990
B6C3F1	liver	H-ras (18/32)	$C^{181}\rightarrow A$; $A^{182}\rightarrow G$	ferreira et al., 1995
A/J	lung	K-ras (10/11)	$G^{34}\rightarrow C$; $G^{35}\rightarrow A,T$; $A^{182}\rightarrow G$; $A^{183}\rightarrow T$	You et al., 1989
A/J	lung	K-ras (17/19)	$G^{34}\rightarrow C$; $G^{35}\rightarrow A, T$; $A^{182}\rightarrow T, G$; $A^{183}\rightarrow T, C$	You et al., 1994
C57	liver	H-ras (2/12)	$A^{182}\rightarrow T$	Anderson et al., 1992
C3H/He	liver	H-ras (6/21)	$C^{181}\rightarrow A$	Rumsby et al., 1991
CD-1	lung	K-ras (17/20)	$G^{35}\rightarrow A,T$; $G^{38}\rightarrow A$; $A^{182}\rightarrow G,T$	Manam et al., 1992a
CD-1	liver	H-ras (3/36)	$C^{181}\rightarrow A$; $A^{182}\rightarrow G$	Manam et al., 1992b
CD-1	liver	H-ras (10/36)	$C^{181}\rightarrow A$; $A^{182}\rightarrow G$	Manam et al., 1995

question acts as a mutagen; instead, it might act as by a nongenotoxic mechanism through facilitating a clonal expansion of cells bearing a spontaneous mutation.

The administration of tumor promoters in carcinogenesis studies can also influence the frequency of ras mutations. For example, promotion with prolactin reduces the frequency of Ha-ras mutations among rat mammary tumors induced by MNU (Zhang et al., 1990). Also, when the promoters phenobarbital, dieldrin, ciprofibrate, or chloroform (Fox et al., 1990; Hegi et al., 1993), are given alone to mice, the liver tumors have a low frequency of Ha-ras codon 61 mutations compared with that found in spontaneous tumors. The molecular mechanisms behind the promotion effect are largely unclear, but appear to require cytotoxicity and tissue regeneration (Ledda-Columbano et al., 1993; Costa, 1995).

The carcinogen dosing schedule or dose level might also affect ras mutation frequency in chemically induced tumors. The frequency of Ha-ras codon 12 mutations among rat mammary carcinomas has been reported to decrease with increased MNU dose (Zhang et al., 1990). Likewise, the frequency of Ha-ras codon 61 mutation in C57B1/J6 mouse liver tumors reported varies inversely with dose of vinyl carbamate (Stanley et al., 1992). In B6C3F1 mouse DEN-induced liver tumors, the frequency and profile of Ha-ras codon 61 mutations was affected by both the dose and the time point at which the tumors were taken (Chen et al., 1993) and at low dose there was a high frequency of codon 61(2)A→G. Mouse thymic lymphomas induced by multiple doses of

MNU have about 4-fold higher frequency of ras mutations than lymphomas induced by a single dose (Corominas et al., 1991). In the case of DMBA, single or multiple doses were reported to induce the same frequency of ras mutation (Manam et al., 1995). An summary of reported DMBA- induced ras mutations is presented in Table 2.2.

Table 2.2: DMBA induced ras mutation spectra.

Animal	Strain	Tissue	Ras gene mutation	Reference
Mouse	NIH/Swiss	skin	H-ras, 61(2)A→T	Brown et al., 1990
	TG:AC	skin	H-ras, 61(2)A→T	Mills et al., 1993
	CD-1	skin	H-ras, 61(2)A→T	Bizub et al., 1986
	CD-1	lung	K-ras, 12(1)G→C; 12(2)G→T; 13(1)G→C; 61(2)A→G,T; 61(3)A→T,C	Manam et al., 1992a
	CD-1	liver	H-ras, 61(2)A→T. K-ras, 13(1)G→C	Manam et al., 1992b, 1995
	rasH2	lung spleen forestomach	H-ras, 61(2)A→T	Doi et al., 1994
Rat	S-D	mammary	H-ras, codon 61	Zarbl et al., 1985
Hamster	Syria	pouch	H-ras, 61(2)A→T	Gimenez et al., 1992
	Syria	skin	H-ras, 61(2)A→T	Robles et al., 1993
Rabbit		ear skin	H-ras, 61(2)A→T	Corominas et al., 1991
Trout	shasta	liver	K-ras, 12(1)G→A, 12(2)G→T 61(2)A→T	Fong et al., 1993

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CHAPTER 3

A p53-like clone from a zebrafish (*Brachydanio rerio*) cDNA library detected by a nonradioactive digoxigenin colorimetric system

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Abstract

A digoxigenin-labeled colorimetric detection system was used to screen a zebrafish cDNA library for p53-like genes. A polymerase chain reaction generated nonradioactive probe of the rainbow trout p53 conserved region IV was prepared with the incorporation of digoxigenin-dUTP into the chain reaction. Hybridization signals were then detected by a colorimetric system at room temperature for up to 2 days. A total of 2 x 10^5 p.f.u. were screened and one clone, termed Z-53, was isolated and identified as a p53-like gene. The insert size of this clone was 1777 bp, which encodes part of conserved region II and all of regions III, IV, and V. The deduced amino acid sequence of conserved regions III, IV, and V are 73%, 92%, and 100% homologous to rainbow trout and 100%, 96%, and 94% homologous to the human p53 gene. A cDNA clone carrying the complete 5' sequence of this gene has yet to be isolated.

Introduction

Mutation of the p53 gene appears to be a major genetic event in many forms of human cancer (reviewed in Hollstein et al., 1991). Several biochemical properties of the wild type p53 protein have been described. For example, the wild type p53 suppresses cell division and induces growth arrest at the G1 phase of the cell cycle, acts as a transcription factor to regulate certain genes in vivo, and acts as a cell cycle check point protein for the repair of DNA damage prior to DNA duplication (reviewed in Levine, 1993). These multiple functions of the p53 protein indicate that it acts as a guardian inside the cell to protect the integrity of the genome. p53 genes have been described from human, mouse, rat, chicken, hamster, frog and rainbow trout (Matlashewski et al., 1984; Oren et al., 1983; Coulier et al., 1985; Louis et al., 1988; Legros et al., 1992; de Fromentel et al., 1992). Though there is some sequence diversity of the p53 gene among species, the encoded protein consists of 5 highly conserved domains believed to involve the important function of this protein (reviewed in Soussi et al., 1990; Prives, 1994). Zebrafish, a small tropical freshwater fish, has received considerable attention as a model for vertebrate embryology and developmental biology (Weinberg, 1992), and was the first aquarium species reported to show a tumorigenic response following carcinogen treatment (Stanton, 1965). In spite of the important role of the p53 gene as a guardian of the genome, nothing is known about the function of this gene in zebrafish development or carcinogenesis. In this study we used a

digoxigenin-labeled probe to isolate a p53 clone from a zebrafish cDNA library. One such clone, which we termed Z-53, was quickly recovered from a total of 2 x 10^5 p.f.u. by a colorimetric detection system. The nucleotide sequence was established and contains the p53 highly conserved domain.

Materials and Methods

(A). Reagents

Sodium chloride, citric acid trisodium salt, sodium dodecyl sulfate, ethylenediaminetetraacetic acid, formamide, urea, boric acid, TRIZMA™ hydrochloride, and ammonium persulfate were purchased from Sigma (St. Louis, MO). RNase cocktail was manufactured by 5' to 3' (Boulder, CO). Acrylamide/bis-acrylamide solution, saturated phenol/chloroform, and chloroform were from AMRESCO (Solon, OH). X-OMAT AR film was from Kodak (Rochester, NY). ^{35}S-dATP was from NEN (Boston, MA). Proteinase K, and DIG-dNTP were from Boehringer Mannheim (Indianapolis, IN). Restriction enzymes EcoRI and Xba were from Stratagene (La Jolla, CA). Rainbow trout p53 cDNA clone was kindly supplied by Dr. Thierry Soussie (Laboratoire de Genetique Moleculaire, IRSC, France). The zebrafish cDNA library was kindly supplied by Dr. Philip McFadden, Department of Biophysics and Biochemistry, Oregon State University.

(B). RNA and DNA isolation

Whole adult zebrafish, obtained from the Oregon State University Food Toxicology and Nutrition Lab, were cut into small pieces and frozen immediately in liquid nitrogen. For DNA isolation, 0.1g of ground tissue was added per 1.2ml of lysis buffer (0.1M NaCl, 10mM Tris, 25mM EDTA, 0.5% SDS, and 0.1mg/ml of proteinase K) and incubated overnight at 37^0C. The DNA was extracted once with phenol/chloroform and chloroform, and the DNA was then precipitated with 2.5 volumes of 95% ethanol and final NaCl concentration of 0.2M. DNA pellets were dissolved in TE buffer and treated with RNase (Maniatis et al., 1989). For RNA isolation, the ground zebrafish tissue was homogenized in TRIzol™ reagent (GIBCO BRL, Gaithersburg, MD) and the company's protocol was followed for total RNA isolation. The concentrations of RNA and DNA were determined spectrophotometrically. Magnetic separation for poly(A) mRNA isolation was carried out using a Biomag™ mRNA purification kit following the manufacturer's protocol (Perseptive Diagnostics, Cambridge, MA), using **cZ53-BIII** primer for enrichment. The first strand cDNA was synthesized by a Superscript™ preamplification system (GIBCO BRL, Gaithersburg, MD) and directly used for PCR reaction without further purification.

(C). Primer

5' biotinylated **cZ53-BIII** primer (based on the zebrafish Z-53 clone nucleotide sequence): 5'- CTC CGG CCC AGC AAC TGA CCT TCC

TG- 3', which is anti-parallel to p53 mRNA, was purchased from Amitof Biotech (Boston, MA). Zebrafish **cZ53-137** primer: 5'- GGA CCA CTT CAG CCA CAT GC-3'; rainbow trout p53 **E7** primer (a forward primer located at the 5' end of exon 7): 5'- GGG ATC AGA GTG TAC CAC TG-3'; **cE7** Primer (a reverse primer located at the 3' end of exon 7): 5'-GTG TCT CCA GGG TGA TGA TG-3' and **20C** primer: 5'- CCC CCC CCC CCC CCC CCC CC-3' were synthesized by the Center for Gene Research and Biotechnology, Oregon State University. All primers were dissolved in water and used without further purification.

(D). Probe preparation by polymerase chain reaction (PCR) amplification

A nonradiolabeled probe based on conserved region IV of the rainbow trout p53 gene was PCR amplified using **E7** and **cE7** primers and the rainbow trout p53 cDNA clone as a template, with the incorporation of digoxigenin-dUTP and other dNTP into the chain reaction by Replitherm™ polymerase. PCR reaction conditions were as follows: one cycle of denaturing at 95^0C for 6 min; 2 cycles of denaturing at 95^0C for 2 min, annealing at 60^0C for 45 seconds, and extension at 74^0C for 25 seconds; 40 cycles of denaturing at 95^0C for 30 seconds, annealing at 60^0C for 45 seconds, and extension at 74^0C for 25 seconds. The PCR products were purified through a Microcon™-30 column (Amicon Division, Beverly, MA) to remove excess primer and dNTP.

(E). Southern blotting

Southern blot procedures (Maniatis et al., 1989) are briefly described as follows: rainbow trout and zebrafish genomic DNA (10μg each) were digested with 20 units of EcoRI or Xba at 37^0C for 3 hours and the digested DNA was then electrophoresed on 1% agarose. After electrophoresis, the gel was denatured at 0.2N HCl, then capillary transferred to the nylon membrane, and the DNA was fixed by UV-light (UV-Cross Link, Stratagene). The membrane was then soaked in 20 ml prehybridization solution (7% SDS, 5x SSC, 2% blocking agent, 0.1% N-lauryl sarcosine and 50mM sodium phosphate pH6.8) per 100 cm^2 membrane at 60^0C for 3 hours and hybridized with probes for 12 hours. The membranes then were stringently washed and signals were detected colorimetrically following the manufacturer's protocol.

(F). cDNA library screening and DNA sequencing

The plaque-lift hybridization methods for cDNA library screening are described elsewhere (Maniatis et al., 1989). The nonradiolabeled screening and detection system was from Boehringer Mannheim (Indianapolis, IN) and the method is described briefly as follows: duplicated membranes were prehybridized and then hybridized with digoxigenin-labeled probe in a concentration of 100ng/ml using the same method as for southern blotting described above. After hybridization, the membranes were stringently washed and incubated with anti-digoxigenin antibody, which was conjugated to alkaline

phosphatase. The membranes carrying the hybridized probe and bound antibody conjugate were reacted with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium salt (NBT) for up to 2 days at room temperature in the dark, and the color development was carefully monitored to reduce false signals. Suspected signals were confirmed by second and third screenings following the same procedures prior to isolation of plaques. The plasmid sequencing of isolated clones was carried out by Sequenase Version 2.0 (USB, Cleveland, OH) and final products were resolved on 5% acrylamide denaturing sequencing gel with ^{35}S-dATP as described elsewhere (Maniatis et al., 1989).

(G). 5' RACE (rapid amplification of cDNA end)

The enriched p53 mRNAs and their first strand cDNAs were synthesized by the Superscript™ preamplification system (GIBCO BRL, Gaithersburg, MD). After cDNA synthesis, the solutions were adjusted to 0.4N NaOH and incubated at 65^{0}C for 30 min. to destroy the RNA template. The reaction solutions were brought to 500µl and filtered through an Amicon-30 column to remove RNA, primers and salts. The retentate was reversibly spun and diluted to 11.8µl for terminal transferase reactions with the incorporation of poly(G) at the 3' end (Boehringer Mannheim, Indianapolis, IN). The terminal transferase reactions were precipitated and redissolved in water for PCR amplification to seek the 5' end unknown sequence of the p53 gene by **20C** and **cZ53-137** primers.

Results

(A). Southern blotting of the zebrafish p53-like gene

Zebrafish and rainbow trout genomic DNA were digested with restriction enzymes, electrophoresed on a 1% agarose gel, transferred to a nylon membrane, and hybridized with PCR-generated digoxigenin-labeled probes, based on the rainbow trout p53 gene conserved region IV. Hybridized signals were detected on zebrafish restriction enzyme digested genomic DNA after incubation at room temperature for 6 hours (Figure 3.1). The rainbow trout p53 cDNA positive control was quickly detected after 30 minutes incubation at room temperature, followed by detection of restriction enzyme digested rainbow trout genomic DNA. For restricted zebrafish DNA, hybridization signal bands were barely visible after 3 hours incubation at room temperature in the presence of a colorimetric reaction substrate, but the intensity of bands increased with incubation time. These results indicate the existence of a p53-like gene in the zebrafish genomic DNA.

(B). An incomplete p53 clone from the zebrafish cDNA library

The southern blot hybridization conditions and probe that were used to detect p53-like sequences were also used to screen the zebrafish cDNA library. A total of 2 x 10^5 p.f.u. were screened and

their duplicates were colorimetrically detected at room temperature for up to two days (see Materials and Methods).

Figure 3.1. The existence of a p53 gene in zebrafish genomic DNA by Southern blotting. Lane 1 is rainbow trout genomic DNA; lane 2 is rainbow trout p53 cDNA clone; lanes 3 and 5 are zebrafish genomic DNA digested with restriction enzyme EcoRI; lane 4 is zebrafish genomic DNA digested with restriction enzyme Xba.

Suspicious spots were located and isolated by contrasting the blue color intensity with the surrounding background color for second and third screening verification. After the third screening, the Z-53 plaque did show a strong hybridization signal. After further in vivo excision of the purified plaque into a phagemid (p^{bluescript},

Stratagene), the insert size was compared with the rainbow trout p53 cDNA (data not shown). Plasmid isolation and restriction enzyme analysis revealed the insert of the Z-53 clone was around 1.7 kb, which was smaller than the rainbow trout 2.2 Kb insert. DNA sequencing revealed the Z-53 clone contained a total nucleotide sequence of 1777 bp (Figure 3.2). The deduced amino acid sequence was then compared with the rainbow trout and human p53 gene conserved regions II, III, IV and V (Figure 3.3). The Z-53 in the conserved region III, IV, and V is 73%, 92%, and 100% homologous to rainbow trout and 100%, 96%, and 94% homologous to the human p53 gene. These results support previous indication that regions IV and V of the p53 gene are highly conserved during species evolution. The incomplete nucleotide sequence of this Z-53 clone might imply poor processivity of reverse transcriptase and incomplete synthesis of first strand cDNA, which is common and sometimes unavoidable during cDNA library construction.

Figure 3.2. The nucleotide sequence of the zebrafish Z-53 clone and deduced amino acid sequence. The incomplete Z-53 clone open reading frame encodes 276 amino acid residues, ranging from part of conserved region II to the end of the C-terminus.

```
                                                        5'CG
GCA CGA GAT AAA CTC TTC TGT CAG CTG GCA AAA ACT TGC CCC GTT
A   R   D   K   L   F   C   Q   L   A   K   T   C   P   V
CAA ATG GTG GTG GAC GTT GCC CCT CCA CAG GGC TCC GTG GTT CGA
Q   M   V   V   D   V   A   P   P   Q   G   S   V   V   R
GCC ACT GCC ATC TAT AAG AAG TCC GAG CAT GTG GCT GAA GTG GTC
A   T   A   I   Y   K   K   S   E   H   V   A   E   V   V
CGC AGA TGC CCC CAT CAT GAG CGA ACC CCG GAT GGA GAT AAC TTG
R   R   C   P   H   H   E   R   T   P   D   G   D   N   L
```

Figure 3.2 (Continued)

```
GCG CCT GCT GGT CAT TTG ATA AGA GTG GAG GGC AAT CAG CGA GCA
A   P   A   G   H   L   I   R   V   E   G   N   Q   R   A
AAT TAC AGG GAA GAT AAC ATC ACT TTA AGG CAT AGT GTT TTT GTC
N   Y   R   E   D   N   I   T   L   R   H   S   V   F   V
CCA TAT GAA GCA CCA CAG CTT GGT GCT GAA TGG ACA ACT GTG CTA
P   Y   E   A   P   Q   L   G   A   E   W   T   T   V   L
CTA AAC TAC ATG TGC AAT AGC AGC TGC ATG GGG GGG ATG AAC CGC
L   N   Y   M   C   N   S   S   C   M   G   G   M   N   R
AGG CCC ATC CTC ACA ATC ATC ACT CTG GAG ACT CAG GAA GGT CAG
R   P   I   L   T   I   I   T   L   E   T   Q   E   G   Q
TTG CTG GGC CGG AGG TCT TTT GAG GTG CGT GTG TGT GCA TGT CCA
L   L   G   R   R   S   F   E   V   R   V   C   A   C   P
GGC AGA GAC AGG AAA ACT GAG GAG AGC AAC TTC AAG AAA GAC CAA
G   R   D   R   K   T   E   E   S   N   F   K   K   D   Q
GAG ACC AAA ACC ATG GCC AAA ACC ACC ACT GGG ACC AAA CGT AGT
E   T   K   T   M   A   K   T   T   T   G   T   K   R   S
TTG GTG AAA GAA TCT TCT TCA GCT ACA TTA CGA CCT GAG GGG AGC
L   V   K   E   S   S   S   A   T   L   R   P   E   G   S
AAA AAG GCC AAG GGC TCC AGC AGC GAT GAG GAG ATC TTT ACC CTG
K   K   A   K   G   S   S   S   D   E   E   I   F   T   L
CAG GTG AGG GGC AGG GAG CGT TAT GAA ATT TTA AAG AAA TTG AAC
Q   V   R   G   R   E   R   Y   E   I   L   K   K   L   N
GAC AGT CTG GAG TTA AGT GAT GTG GTG CCT GCC TCA GAT GCT GAA
D   S   L   E   L   S   D   V   V   P   A   S   D   A   E
AAG TAT CGT CAG AAA TTC ATG ACA AAA AAC AAA AAA GAG AAT CGT
K   Y   R   Q   K   F   M   T   K   N   K   K   E   N   R
GAA TCA TCT GAG CCC AAA CAG GGA AAG AAG CTG ATG GTG AAG GAC
E   S   S   E   P   K   Q   G   K   K   L   M   V   K   D
GAA GGA AGA AGC GAC TCT GAT TAA
E   G   R   S   D   S   D
GGTGATGGGATGCTAAGAGAGAAAGAAACTGGGAGTTTTGCTCTTTTTGACTGCCTTTTTTGCTTTTAT
TACACCAAAAAAAAAAGCTTGTCACAGGGGTCATTTGGGGAACTGCGATACATGTGATCCATTAGTCCT
GTTTGTCATGTTTCTTCTTGTTCACTCCACTGGGACCTTTGTTGCTGGACACTGATTTTGCAGTGTTTT
ACCAGCACTGGCGAGGGCTAAGACTAGCCTGACGTTTTACAGACCCGTTAAATGAACGTTCAGGAACGA
AGTTTACCTGTGACTTTTGTAAATTGTAATAATGCCAGTATTCGGTTGTTTTGTAAGCAGCATTTTTTT
TTATTTTTATAAAGAAATGAGTATTAGACTAATTGGAACAAACCATCGGAATTGGTTGCAAGCCGATTA
TGTAATAAAAATACCAAAGCAAAACCGTTTTGTATCTTATATTTTTTTATGTTAATTACTGCTGTTCCT
ATTTTTGTATTTTAGCTAATAAGAGAAGCTGCCCTTTATAACACGCTTTTAGATTTAGTACAACCATTG
TTCTTTTTCAATTGAAGTAGTTGTTGATTTACTCTTGTGATTGTTGTTGAGTAATGTGGCACAGATAAG
CTCATAAGTGGCCAGTCTGTTTTGACATGTGCTTGTGCAAATGGTTATGGATTGGATGTCTAAATATGA
GCAAATGAAATGTCAAATACTCAGGGCATGTACAAGTCCCTCCTGGAAATCTTGATAAAACTCTTCTAC
TGTTTTACCTGCTATAGATTTTATGCTGGTTTTGTTTCTTATTACTGTTTACACGCATTTGCCTTTTAT
AAATATATAAGCGTGCATTTATTTGTAGCTAGCTGAATCATTTATAATTTTTCTTTTTAAGAGAAATGT
TTTAAAATACACTCTTTAAAAAAAACAAAAAAAAAAAAAAAAA
```

Figure 3.3. Comparsion of conserved regions among rainbow trout, human and the deduced amino acid of zebrafish p53 proteins. Part of region II and full regions of III, IV, and V were compared among these species. Amino acids identical to rainbow trout p53 protein are indicated by dashes.

```
                II
rainbow trout   DLNKLFCQLAKTCPVQIVVD  HPPPPGAVVRALAIYKKLSD
zebrafish       ARD-------------M---  VA--Q-S----T-----SEH
human           A---M-----------LW--  ST----TR---M----QSQH

                   III
rainbow trout   VADVVRRCPHHQSTSENNEG  PAPRGHLVRVEGNQRSEYME
zebrafish       --E--------ER-PDGDNL   --A---I-------AN-R-
human           MTE--------ERC-D/SDG  L--PQ--I-----L-V--LD

                                    IV
rainbow trout   DGNTLRHSVLVPYEPPQVGS  ECTTVLYNFMCNSSCMGGMN
zebrafish       -NI------F----A--L-A  -W----L-Y-----------
human           -R--F----V------E---  D---IH--Y-----------

                                  V
rainbow trout   RRPILTIITLETQEGQLLGR  RSFEVRVCACPGRDRKTEEI
zebrafish       --------------------  -------------------S
human           -----------DSS-N----  N--------------R---I
```

Discussion

In the past decade, traditional library screening has relied on radiolabeled probes produced either by nick translation or by end labeling of ^{32}p-dNTP. Such probes are known to detect genes with low copy numbers either in genomic DNA or in cDNA library screening, and they continue to be convenient tools for the research community. However, the short half-life of ^{32}p-labeled probes and concern about the hazard they present to the environment and to personnel, have encouraged researchers to develop safer techniques and to limit the use of radiolabeled probes. Nonradiolabeled screening and detection systems, using either biotin- or digoxigenin-labeled probes, have proven to be nonhazardous to personnel and to the environment. Furthermore, detection by chemiluminescence has shown sensitivity equivalent to ^{32}p-labeling (Wundrack et al, 1992). Although the sensitivity of the chemiluminescence method is better than the colorimetric detection method, the chemiluminescence detection method does have high background problems (authors' data not shown; Puchhammer-Stoeckl et al., 1992). This high background problem is attributed to the substrate, Lumi-Phos^{530} ™, which is highly sensitive to the presence of alkaline phosphatase. Even without the existence of a hybridized probe, nonspecific annealing may occur with longer probes. In the presence of Lumi-Phos^{530} ™, aseptic methods and technical skill are required to reduce the high background. Although detection sensitivity is less than the chemiluminescence system, the colorimetric detection system

required no longer than overnight to detect genes by southern blotting. Colorimetric detection can also offer an alternative method for screening cDNA libraries when room temperature incubation is extended up to 2 days in the dark. The existence of p53-like hybridized signals in the southern blot (Figure 3.1) suggests that the p53 gene is highly conserved among fish species. To examine its expression and open reading frame DNA contexts, a zebrafish cDNA library (a total of 2 x 10^5 p.f.u) was screened with conditions modified from southern blotting, but the color development was extended up to two days. During this prolonged color development, careful control of background was necessary in order to avoid ambiguous signals. A clone, termed Z-53, was readily isolated and revealed an insert of 1777 nucleotides (Figure 3.2) by in-house sequencing. The amino acid sequence deduced from open reading frame and maximum homology alignment revealed the Z-53 insert encoded an incomplete protein sequence starting from the middle of the p53 conserved region II domain and ending at the C-terminus by the stop codon TAA. The deduced amino acid sequence of conserved regions III, IV, and V are 73%, 92%, and 100% homologous to rainbow trout and 100%, 96%, and 94% homologous to the human p53 protein (Figure 3.3). The flanking regions between region IV and V are identical in rainbow trout and zebrafish. Incomplete clones such as isolated here are commonly found in cDNA library construction mainly due to the incomplete synthesis of first strand cDNA by reverse transcriptase (Adams et al., 1991). Attempts were made to complete the 5' end sequence by the 5' RACE (rapid amplification of cDNA end) method

of Frohman et al. (1988). After PCR reactions, the products were separated on a 5% acrylamide gel. Strongly smeared bands of 400bp to >1 Kb with certain specific bands around 800bp were clearly visible, and the 800bp bands were excised and eluted into water for cloning (data not shown). Using PCR, we were unable to verify the existence of a p53 clone.

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CHAPTER 4

Molecular cloning and sequencing of an expressed ras gene from an enriched Zebrafish (*Brachydanio rerio*) cDNA library

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Abstract

A magnetically enriched whole zebrafish (*Brachydanio rerio*) cDNA library was constructed for screening ras-related genes. Using this approach, a positive clone was readily identified from a single plate of 3 x 10^4 p.f.u.. The clone, termed Zras-B1, carried an insert of 2592 bp with an open reading frame encoding a 188 amino acid residue ras p21 protein. The deduced amino acid N-terminal region (residues 1-86), as well as the CAAX binding motif at the C-terminus of the protein are identical to mammalian N-, Ha-, and Ki-ras. Based on total protein sequence, this expressed zebrafish ras p21 is most closely related to human N-ras (91% homology), with lesser homology to Ha-ras (84%) and Ki-ras (85%). Preliminary partial sequence data obtained by genomic and RT-PCR screening indicate the presence of at least one additional expressed ras genes in zebrafish.

Introduction

There are three known ras proto-oncogenes in mammalian genomes, designated N-, Ha- and Ki-ras (Parada et al., 1982; Der et al., 1982; Taparowsky et al., 1983). These genes encode 21 KDa (p21) encode proteins of 188 or 189 amino acids, which undergo a number of post-translational modifications at the carboxy terminus including isoprenylation or palmitoylation essential to plasma membrane localization (Hancock et al., 1989). The discovery of activated ras genes in a variety of human and animal tumors stimulated researchers to examine their biochemical properties and biological function both in normal and transformed cells. Ras p21 functions as a signal transduction protein (Barbacid, 1987; Bourne et al., 1990; Downward, 1992) through binding of guanosine triphosphate (GTP) and its hydrolysis to guanosine diphosphate (GDP). Evidence indicates that the GTP-bound form of p21-ras is biologically active while the GDP-bound form is inactive. The GTP-bound conformation interacts with an effector molecule, and the life-span of this interaction is regulated either by the effector-mediated or the intrinsic GTPase activity of the protein. Ras-mediated signal transduction is believed to be involved in cell differentiation, development and oocyte maturation (Daar et al., 1991; Benito et al., 1991; Fortini et al., 1992). Many human and animal tumors carry ras p21 activated by point mutations at certain positions, such as amino acid residues 12, 13, and 61, to produce acutely transforming products defective in GTPase

activity and thus held in a chronically active configuration (Bos et al., 1988).

Mammalian ras homologues have been found in yeast, mollusks, and fish (DeFeo, et al., 1983; Swanson et al., 1986; Nemoto et al., 1986, 1987; McMahon et al., 1990; Mangold et al., 1991). Zebrafish (*Brachydanio rerio*), a small tropical freshwater fish, has received considerable attention as a model for vertebrate embryology and developmental biology (Streisinger, et al., 1981), and was the first aquarium species reported to show a tumorigenic response following carcinogen treatment (Stanton, 1965). However, no published reports have appeared describing ras gene sequence or their involvement in signal transduction, differentiation, or carcinogenesis in this species. In this initial study we report the construction of a magnetically enriched whole-fish cDNA library for isolation of ras-related sequence. One such gene, which we term Zras-B1, was quickly recovery from a single 3 x 10^4 p.f.u. plate by chemiluminescence detection, and the nucleotide sequence was established. The p21 protein encoded by this expressed gene shows strongest sequence homology (91%) to human N-ras.

Materials and Methods

(A). Reagents

Sodium chloride, citric acid trisodium salt, sodium dodecyl sulfate, ethylenediaminetetraacetic acid, formamide, urea, boric

acid, TRIZMA hydrochloride, and ammonium persulfate were purchased from Sigma (St. Louis, MO). RNase cocktail was from 5' to 3' (Boulder, CO). Acrylamide/bis-acrylamide solution, satured phenol/chloroform, and chloroform were from AMRESCO (Solon, OH). X-OMAT AR film was from Kodak (Rochester, NY). ^{35}S-dATP was from NEN (Boston, MA). Proteinase K was from Boehringer Mannheim (Indianapolis, IN).

(B). RNA and DNA isolation

Whole zebrafish, obtained from the Oregon State University Food Toxicology and Nutrition Lab, were cut into small pieces and frozen immediately in liquid nitrogen. For DNA isolation, 0.1g of ground tissue was added per 1.2ml of lysis buffer (0.1M NaCl, 10mM Tris, 25mM EDTA, 0.5% SDS, and 0.1mg/ml of proteinase K) and incubated overnight at 37^{0}C. The DNA was purified once with phenol/chloroform and chloroform, and precipitated with 2.5 volume of 95% ethanol at a final NaCl concentration of 0.2M. The DNA pellets were dissolved in TE buffer and treated with RNase (Maniatis et al., 1989). For RNA isolation, the ground zebrafish tissue was homogenized in TRIzol reagents (GIBCO BRL, Gaithersburg, MD) and the company's protocol was followed for total RNA isolation. The concentrations of RNA and DNA were determined by spectrophotometry. Magnetic separation for mRNA isolation was carried out using a Biomag mRNA purification kit following the manufacturer's protocol (Perseptive Diagnostics, Cambridge, MA). The first strand cDNA was synthesized by Superscript preamplification system (GIBCO BRL, Gaithersburg,

MD) and directly used for RT-PCR reaction without futher purification.

(C). Primers

Rainbow trout Ki-ras **H01** primer (a forward primer located at the 5' end of exon 1): 5'- ATG ACG GAA TAC AAG CTG-3'; **c37** Primer (a reverse primer located at the 3' end of exon 1): 5'- CTC GAT GGT GGG GTC ATA TT-3'; **sc67(1)G** primer (a reverse primer on exon 2 region): 5'- CAT GGC GCT GTA CTC CTC CTG-3'; and zebrafish ras-like sequence, the **HZras-08** primer (a zebrafish forward primer on exon 1 region) : 5'- TCG TGG GAG CTG GAG GCG TA-3' were synthesized by the Center for Gene Research and Biotechnology, Oregon State University. 5'-Biotinylated **cZRAS-BII** primer (based on the zebrafish Z-1 type ras gene on exon 1) : 5'- GTG AGA GCG CTT TTG CCT ACG CCT CC- 3', which is anti-parallel to ras mRNA, was purchased from Amitof Biotech (Boston, MA). All the primers were dissolved in water and used without futher purification. The primer concentrations used for PCR amplification were the same as described by Fong et al. (1993).

(D). Polymerase chain reaction (PCR) amplification, cloning and sequencing

The zebrafish genomic ras-like DNA sequence was amplified by nested PCR using Replitherm thermostable DNA polymerase (Epicentre Technologies, Madison, WI) and rainbow trout Ki-ras exon 1 **H01** and **c37** primers. PCR reaction conditions were as follows: one cycle of denaturing at 95^0C for 6 min; 2 cycles of

denaturing at 95^{0}C for 2 min, annealing at 55^{0}C for 45 seconds, and extension at 74^{0}C for 25 seconds; 40 cycles of denaturing at 95^{0}C for 30 seconds, annealing at 55^{0}C for 45 seconds, and extension at 74^{0}C for 25 seconds. RT-PCR reactions for zebrafish ras exon 1 and part of the exon 2 region were also carried out by nested PCR using **HZras-08** and **sc67(1)G** primers and first strand cDNA as templates. The PCR cycles were identical to the above conditions. The PCR products were purified through a Microcon-30 column (Amicon Division, Beverly, MA) to remove excess primer and dNTP. Genomic PCR and RT-PCR cloning reactions followed the protocol of Invitrogen (San Diego, CA) TA cloning kit and PCR products were finally transformed to *E. coli* competent cells. To screen positive clones, traces of single colony bacterial cells were picked from plates, suspended in Replitherm reaction buffer and directly amplified by nested PCR with the same primers used for amplification. The results were analyzed by gel electrophoresis and positive clones were isolated and recultured in LB broth with appropriate antibiotics for plasmid isolation. Plasmid sequencing reactions by Sequenase Version 2.0 (USB, Cleveland, OH) and final products were resolved on 5% acrylamide denaturing sequencing gels with ^{35}S-dATP as described elsewhere (Fong et al., 1993).

(E). Magnetically enriched ras cDNA library construction

About 900μg of total RNA were used for total mRNA isolation by magnetic separation. The final mRNAs were resuspended in

180µl of 0.5M NaCl and 50% formamide, then incubated with **cZRAS-BII** primer-bound streptavidin beads at room temperature (low stringency) to enrich ras mRNA by following the manufacturer's protocol (Biomag Streptavidin system, Perseptive Diagnostics, Cambridge, MA). The ras-enriched mRNAs were magnetically separated, and heat eluted in diethylpyrocarborate water. The mRNA quantitation was checked by DNA DipStick (San Diego, CA). mRNA (5µg) was precipitated by 0.1 volume of 3M sodium acetate and 2.5 volumes of 95% ethanol at -70^{0}C overnight and used for library construction with the ZAP Express cDNA synthesis kit (Stratagene, La Jolla, CA) by the manufacturer's protocol.

(F). Plaque hybridization and chemiluminescent detection

An RT-PCR clone with the insert of 180bp zebrafish exon 1 and part of exon 2 region, (PCR amplification by **HZras-08** and **sc67(1)G** primer) was used as a template and nonradioactive probes were prepared by incorporating digoxigenin-dUTP (DIG-dUTP, Boehringer Mannheim, Indianapolis, IN) and other dNTP into the PCR products under the same conditions used above. The plaque-lift hybridization methods were described by Maniatis et al., (1989). Lumi-Phos 530, a chemiluminescent formulation for alkaline phosphatase detection when using nonradioactive nucleic acid labeling, was used in cDNA library screening. The detection procedures followed the manufacturer's protocol (Boehringer Mannheim, Indianapolis, IN) as briefly described: duplicate

membranes on a 3 x 10^4 p.f.u. plate were hybridized with DIG probe in 7% SDS, 5x SSC, 2% blocking agent, 0.1% N-lauryl sarcosine 50mM and sodium phosphate pH 6.8 solution. After blocking, membranes were incubated with anti-DIG Fab fragments, which were conjugated to alkaline phosphatase. The membranes carrying the hybridized probe and bound antibody conjugate were reacted with Lumi-Phos 530 for 30 min. at 37^0C and finally exposed to X-OMAT AR film to record the chemiluminescent signal.

Results

(A). Initial screening for zebrafish ras-like sequences in genomic DNA and mRNA

Rainbow trout Ki-ras exon 1 primers (H01 and c37) were used initially to amplify zebrafish genomic DNA by PCR under low stringency conditions. PCR products were separated on 5% acrylamide gels, a DNA band migrating at the expected 110bp region was excised and DNA was eluted in water for PCR cloning (data not shown). Bacterial colonies were probed by a similar PCR, and 26 of 29 colonies (93%) were found to carry 110 bp inserts. Preliminary plasmid sequencing of 18 clones revealed unique ras-related sequences of 66bp located within these two primers; since the sequences differed at several sites from any trout or mammalian ras-containing plasmids in our laboratory we assume them to be of zebrafish origin. Figure 4.1 shows the two main categories (Z-1 and Z-5) of ras-related DNA sequence obtained from these 18 positive clones. Six out of 18 clones (33%) were Z-1

Figure 4.1. Nucleotide sequence comparison of rainbow trout ki-ras exon 1 region with two types of zebrafish ras-like sequences. Zebrafish genomic DNA was amplified by rainbow trout H01 and c37 primer and DNA sequences were compared between these two primers.

```
           8
RT Ki-ras  GTG GTG GGG GCA GGA GGT GTG GGC AAG AGC GCG CTC ACC
Z-1        --C --- --A --T --- --C --A --- --A --- --T --- ---
Z-5        --- --- --C GAC --T --C --A --- --- --- --A --- --T

           21
RT Ki-ras  ATC CAG CTC ATT CAG AAC CAC TTT GTG
Z-1        --- --A --- --C --- --- --- --- ---
Z-5        --- --- TTT TTC --- --G ATC --C ---
```

and 7 out of 18 clones (39%) were the Z-5 type. The remaining five clones had intra-primer DNA sequences unrelated to ras. The translated Z-1 sequence is identical in amino acid sequence to mammalian ras proteins within the central 22-codon region, whereas the Z-5 sequence contained four amino acid substitutions and may represent a related G-protein. To obtain initial sequence information on expressed ras-related sequences, total zebrafish poly(A)$^+$ mRNAs was isolated and first- strand cDNAs were synthesized by reverse transcriptase. First strand cDNAs served as templates for PCR reactions using HZras-08 and rainbow trout sc67(1)G primers under the same conditions used for genomic DNA amplification. The resulting PCR products were separated by gel electrophoresis and DNA bands migrating near the expected 200bp were excised and eluted in water for cloning (data not shown). Twenty-four out of 29 resulting colonies gave positive PCR reaction and, by in-house plasmid sequencing, all 24 clones were

the Z-1 sequence type within this 200 bp region. As seen in Figure 4.2, nucleotide sequencing revealed two Z-1 ras sequence (Z-RTPCR-1 and -2), differing by a single redundant nucleotide change at amino acid 34 codon, third base position. The deduced amino acid sequence of Z-RTPCR-1 and -2 were identical to mammalian ras proteins in this region.

Figure 4.2. Ras exon 1 and 2 region nucleotide sequence comparison of zebrafish RTPCR-1 and -2 clones, Zras-B1 cDNA clone and rainbow trout ki-ras. Zebrafish RT-PCR clones were generated by primers of HZras-08 and sc67(1)G under low stringency conditions. Deduced amino acids indicating a 100% homology shared by these ras proteins. Nucleotide sequences from amino acid residues 8 to 60 were compared to two RT-PCR clones and also with a complete cDNA clone isolated by enriched ras cDNA library and rainbow trout ki-ras. The RTPCR-1 and RTPCR-2 are only different in codon 34, third position. The Zras-B1 cDNA clone is different from RTPCR clones by nucleotide sequence.

```
            8
RT ki-ras   GTG  GTG  GGG  GCA  GGA  GGT  GTG  GGC  AAG  AGC  GCG  CTC  ACC
Z-RTPCR-1   --C  ---  --A  --T  ---  --C  --A  ---  --A  ---  --T  ---  ---
Z-RTPCR-2   --C  ---  --A  --T  ---  --C  --A  ---  --A  ---  --T  ---  ---
Zras-B1     --T  ---  --A  ---  ---  ---  --T  --G  ---  ---  ---  TTA  --A

            21
RT ki-ras   ATC  CAG  CTC  ATT  CAG  AAC  CAC  TTT  GTG  GAT  GAA  TAT  GAC
Z-RTPCR-1   ---  --A  ---  --C  ---  ---  ---  ---  ---  ---  ---  ---  ---
Z-RTPCR-2   ---  --A  ---  --C  ---  ---  ---  ---  ---  ---  ---  ---  ---
Zras-B1     ---  ---  ---  --C  ---  --T  ---  ---  ---  ---  ---  ---  ---

            34
RT ki-ras   CCC  ACC  ATC  GAG  GAC  TCG  TAC  AGG  AAG  CAG  GTG  GTG  ATT
Z-RTPCR-1   --A  --T  --T  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---
Z-RTPCR-2   --G  --T  --T  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---
Zras-B1     ---  --T  --T  ---  ---  --T  ---  ---  ---  ---  ---  ---  ---

            47
RT ki-ras   GAT  GGG  GAG  ACA  TGT  CTG  CTG  GAC  ATC  CTG  GAC  ACT  GCA
Z-RTPCT-1   --C  --A  ---  --G  ---  --A  ---  ---  ---  ---  ---  ---  ---
Z-RTPCR-2   --C  --A  ---  --G  ---  --A  ---  ---  ---  ---  ---  ---  ---
Zras-B1     --C  --C  ---  --G  ---  ---  ---  ---  ---  ---  ---  ---  ---
```

Figure 4.2 (Continued)

```
          60
RT Ki-ras GGT
Z-RTPCR-1 ---
Z-RTPCR-2 ---
Zras-B1   --C
```

(B). A full-length zebrafish ras clone isolated from enriched cDNA library

Z-RTPCR-1 provided a zebrafish-specific probe for the isolation of a full-length zebrafish ras cDNA. To synthesize a ras-enriched cDNA library, whole zebrafish poly(A)$^+$ mRNA was magnetically separated (see Methods) from total RNA, then incubated at room temperature in 50% formamide with a streptavidin-bound cZRAS-BII primer, derived from Z-RTPCR-1. This procedure was designed to enrich for ras specific mRNAs prior to cDNA library construction. A subsequent cDNA synthesis from this enriched mRNA fraction followed the manufacturer's protocol. After the complete synthesis, the presence of ras-related cDNA was verified and roughly quantified by external PCR. An appropriate amount of cDNAs was ligated into the ZAP Express system for later use in plaque screening. A non-radioactive DIG-labeled probe was made by PCR using Z-RTPCR-1 as a template and HZras-08 and sc67(1)G as primers. A single 3 x 10^4 p.f.u. plate was hybridized with the DIG-probe followed by chemiluminescence detection. Six potential clones were isolated for second and third screenings and 2 out 6 showed positive results. Inserts from these two clones (Zras-B1

and Zras-B5) were then excised into the pBK-CMV phagmid. Plasmid isolation and restriction enzyme analysis indicated that the insert sizes of Zras-B1 and Zras-B5 were 2.6Kb and 0.6 Kb, respectively (data not shown). Futher DNA sequencing showed only the Zras-B1 is the ras-related clone. The complete nucleotide sequence of the 2592 bp Zras-B1 insert is shown in Figure 4.3. The deduced amino acid sequence (Z-ras) of the Zras-B1 protein is compared with mammalian N-, Ha-, and Ki-ras protein in Figure 4.4. The Zras-B1 nucleotide sequence was not identical in the amino acid residue 8-60 overlap region to either of the RT-PCR fragments obtained by initial screening (Figure 2). Differences were observed in several positions, mainly in redundant third base codon positions. This preliminary evidence suggests that more than one ras-related protein may be expressed in the zebrafish.

Figure 4.3. Nucleotide sequence of Zras-B1 clone and deduced amino acid codon. The Zras-B1 clone open reading frame encodes 188 amino acid residues, which are indicated by the bold face letter under each nucleotide sequence.

```
GGCACGAGCGTGAATCGTGAAGTTCTGGGGAAGCTGCTTGCTGAACTCTTATTCAAAACTTTGGCCAGACTTGTTTAACGGCT
GTTTATATACCTGGATTTGTGGCCTCTCTACGAATCTGGTTAGCGCGGGTTACGCTGCCTCCAGACGGGTGTGAAGTGACTTT
TGGAGGGCTCCTCATGGCATTATTTTGGGCGAGGGAGCAGTAATAAGCAGAGCCCTGTTGATCAGATCAGCCAGAGGTCGCAG
AACTGCAGTGTAAGCGGTGGTCCCTCAGGACTGTAAG
  ATG  ACT  GAG  TAT  AAG  CTG  GTT  GTT  GTG  GGA  GCA  GGA  GGT  GTT  GGG
  M    T    E    Y    K    L    V    V    V    G    A    G    V    G
  AAG  AGC  GCG  TTA  ACA  ATC  CAG  CTC  ATC  CAG  AAT  CAC  TTT  GTG  GAT
  K    S    A    L    T    I    Q    L    I    Q    N    H    F    V    D
  GAA  TAT  GAC  CCC  ACT  ATT  GAG  GAC  TCT  TAC  AGG  AAG  CAG  GTG  GTG
  E    Y    D    P    T    I    E    D    S    Y    R    K    Q    V    V
  ATT  GAC  GGC  GAG  ACG  TGT  CTG  CTG  GAC  ATC  CTG  GAC  ACT  GCA  GGC
  I    D    G    E    T    C    L    L    D    I    L    D    T    A    G
  CAG  GAA  GAG  TAC  AGC  GCA  ATG  AGA  GAC  CAG  TAC  ATG  AGG  ACA  GGA
  Q    E    E    Y    S    A    M    R    D    Q    Y    M    R    T    G
```

Figure 4.3 (Continued)

```
GAG  GGT  TTC  CTC  TGC  GTC  TTC  GCT  ATC  AAC  AAC  AGC  AAA  TCC  TTC
E    G    F    L    C    V    F    A    I    N    N    S    K    S    F
GCC  GAC  GTG  CAT  TTG  TAC  AGA  GAG  CAG  ATC  AAG  CGT  GTG  AAG  GAC
A    D    V    H    L    Y    R    E    Q    I    K    R    V    K    D
TCG  GAT  GAT  GTT  CCC  ATG  GTC  CTA  GTG  GGG  AAC  ATT  TGT  GAT  TTG
S    D    D    V    P    M    V    L    V    G    N    I    C    D    L
GCA  AGG  ACT  GTG  GAC  ACC  AAG  CAA  GCT  CAG  GAA  CTT  GCC  AGA  AGC
A    R    T    V    D    T    K    Q    A    Q    E    L    A    R    S
TAC  GGT  ATT  GAG  TTT  GTA  GAA  ACC  TCT  GCC  AAA  ACC  AGA  CAG  GGA
Y    G    I    E    F    V    E    T    S    A    K    T    R    Q    G
GTC  GAG  GAT  GCT  TTC  TAC  ACC  CTT  GTT  CGT  GAG  ATC  CGG  CAT  TAT
V    E    D    A    F    Y    T    L    V    R    E    I    R    H    Y
CGC  ATG  AAA  AAG  CTC  AAC  AGC  AGA  GAA  GAC  AGG  AAG  CAG  GGC  TGT
R    M    K    K    L    N    S    R    E    D    R    K    Q    G    C
CTG  GGC  GTG  TCC  TGT  GAA  GTC  ATG  TGA
L    G    V    S    C    E    V    M
CCGCACTGTCTCCCTATTTTTGTGTTTGTTTTTTGGAAGCAGTTTGTTAAGCAGGCAGCTGGAAGCCTCATGACCACCCGTGT
GCATGCGGCAGATTTCGCCCTCTGCTGGCTGAAAGGGAATAATGCACAACTGGTCATCTTTCCGGGTTGGCCTGCTGTAATAT
ATATAAATGAGGCCGATGGACATTACATGTTTTAACCGAGGCTTAGTACTTAGGTTTTCCTTCTTCCTTTGGAAGAGCAACTT
AAAACGGGCATTGAGATTGGGCATTGGATGATTCTACATTCTCTTCTGACCCCTTATAAACACGGGATCATCAAGCAGATTGG
CATTGAGGAAAATAAATAAAAATTAATATTCATGGCACATTTTATCCCAAAATCTGAAGGATTTAAATTTTTTGTTTATATAC
AATTTATCACATTTACCCTCATTCTTATTATAATTTAGCTATTTTTAATATTCTCATCTTTTAATAGACATCAAATAAAGCCA
TTACCTATTACTGTGATGCATAAAAATGAATATATTATTTCCTCATTAACATAATGATAATTGAGAAGGAAATGTGCTTAACT
GCCAATCATCATTTGACATTAAAGCACCACAATGGCAAAAATTCTAGACGTATACCCACATATATATAATTTCTGATTAATTG
TTTTAATTTTGGGATTATATTTGGAGCTGTTCTTTGCAGGGTGTTATGACCCGTAGAATGTTTTACAATACATCTGTATGTCC
AGAAGAAGCTGTTTTCAAGTCATACACTTCAGGATTAGTATAATTTATGACTGTCATTTTCTCAGAATTTGTAAAGGCCTCAG
AAGAGAACGAGTGAAATGCCCTTTAATCTTAATCCGCAGGTTCAATGCTCAGGAATACTGTGAAACACAGAACTTCTTCTTCC
CCAAAAGTGTGGAATTTTTCTCTTCAGTTTTTTTTTAATTTGTTTGTTTTCAAGGTAGCCAAAGAGTTTTAGCATTTACCATT
TTTAGATACATTTGCTCTAGAGTTTCACCTTCTTTGCTCTCAGATGATAATTGTATATATTGATATATAATACTTAATGATTT
GGTTCAGCTGCAGACAATTGGATTGGATTTTAAGCATAGGGGTTTGTTGGATTTTACTTTTAAGGGCCTTTTGAATCATGGTG
CTGCTGGTGTTTATGCCAAATCTGCGTGCCGCCTTCTGTGTCCTTTATAGCATGACATCTTTAGCTCATATCAATGTCTTAAA
TCTTCCAACAAAAGAGTTTTTCTTGGAAGTGTAACGGATCAGAGTAAGGTTTGTTGCATTGTTTCCATGCCTGAGGTCTCCTC
TTAAGAGCCGCTGAAGGTTTCCCAAGAAGTTGAAATGATCTTTCTTGTTGACTGTCGTGTTATTTATATGGCATTTGTCTGAA
ACTGTGCCTGATTTCATTGAGAATGAAATCTGCAATGTGTTCTTGTTATTACTGCGGTTGCGTTTGGCCTTCTGTCCTCACAC
TAACAGAGGCAAGATGTTTGGTAGAAAATACAGACTAAGTTTGCCATTATGTTAACAAATCCTTTCTTTTTTTAAACTATATA
GAAACTTGTTTTTTGTATATTTGTTTTACCATTTTAGCACACTTTATCATTACCACCGACTATCTCTAATCAGAAAGTCATGT
GAAAACGAGCATTTCATGTCTTTTGGGGGAGGGTGGGGGACTATACATGTGTAACAGATGTGCCTACCATATTACAAGAAGTT
TAACCAGTAAATTAACTGGAATCTGAAAAAAAAAAAAAAAAA
```

Figure 4.4. Deduced amino acid of Z-ras protein compared to mammalian N-, Ha- and Ki-ras. Amino acid identical to N-ras protein is indicated by "-".

```
      1              13             25             37
N-ras  MTEYKLVVVGAG   GVGKSALTIQLI   QNHFVDEYDPTI   EDSYRKQVVIDG
Z-ras  ------------   ------------   ------------   ------------
Ha-ras ------------   ------------   ------------   ------------
Ki-ras ------------   ------------   ------------   ------------
```

Figure 4.4 (Continued)

```
      49              61              73              85
N-ras   ETCLLDILDTAG    QEEYSAMRDQYM    RTGEGFLCVFAI    NNSKSFADINLY
Z-ras   ------------    ------------    ------------    --------VH--
Ha-ras  ------------    ------------    ------------    --T---E--HQ-
Ki-ras  ------------    ------------    ------------    --T---E--HH-

      97              109             121             133
N-ras   REQIKRVKDSDD    VPMVLVGNKCDL    PTRTVDTKQAHE    LAKSYGIPFIET
Z-ras   ------------    --------I---    /A--------Q-    --R----E-V--

Ha-ras  ------------    ------------    AA---ESR--QD    --R-----Y---
Ki-ras  ----------E-    ------------    -S--------QD    --R---------

      145             157             169             181
N-ras   SAKTRQGVEDAF    YTLVREIRQYRM    KKLNSSDDGTQG    CMGLPCVVM
Z-ras   ------------    --------H---    -----RE-RK--    -L-VS-E--
Ha-ras  ------------    ---------HKL    R---PPE-SGP-    --SCK--LS
Ki-ras  ------------    -----------L    --ISKEEKTPGC    VKIKK-II-
```

Discussion

The techniques used to isolate ras-related genes have been reviewed by Chardin (1993). Methodologies based on traditional poly(A)$^+$ mRNA cDNA library synthesis without any specific enrichment of pertinent sequence often prove difficult for efficient isolation of mRNA of low copy number. Magnetic enrichment has shown promise in isolating low copy sequences from complex cDNA or genomic libraries (Morgan et al., 1992). Enrichment not only reduces the effort to screen a high background library, but also offers an alternative way to isolate sequence-related genes such as the ras superfamily from the same library. To obtain zebrafish-specific ras sequences, two rainbow trout Ki-ras primers were used at a low annealing temperature for PCR amplification. Cloning and sequencing analysis revealed two types of partial

genomic clones. Z-1, the intra-primer sequence is identical to mammalian ras in translated amino acid sequence, and Z-5, a fragment of similar length, which shares 77% homology has a conserved region around codons 12 and 13. Z-5 is presumed to represent a more distantly related ras-type GTP-binding protein in the zebrafish, though its total sequence and biological function remain to be established. DNA sequencing of RT-PCR clones Z-RTPCR-1 and -2 revealed heterogeneity at the third position of codon 34 in the Z-1 gene. It is presently unclear if this represents polyphorphism or duplication. Absence of Z-5 clones in the RT-PCR preparation suggests that this gene may not be expressed in the mature zebrafish used for mRNA preparation, though this was not investigated exhaustively. Efforts using trout ras primers and probes to obtain a full-length ras clone from a traditional zebrafish cDNA library were unsuccessful (data not shown). However, the alternative strategy of constructing a cDNA library enriched for expressed ras-related zebrafish genes through use of a 5'-biotinylated 26mer antiparallel to ras mRNA proved successful. By this approach several potential clones were readily isolated from a single 3 x 10^4 p.f.u. plate and finally identified a positive clone, Zras-B1. The first 86 amino acids of the Zras-B1 clone are identical in sequence to mammalian ras proteins, and the CaaX motif for C-terminal modification is maintained (Figure 4.4). The segment encompassing amino acids 87 to 164 is more divergent among mammalian ras proteins, and region 165-189 is hypervariable. Z-ras homologies with mammalian N-, Ha-, and Ki-ras proteins within region 87-164 are 88, 83, and 86%, while homologies in the

hypervariable C-terminal region are 64, 32, and 28% respectively (Figure 4.4). Overall, Z-ras is 91, 84, and 85% homologous to human N-, Ha-, and Ki-ras p21, respectively. Thus, by simple amino acid sequence comparison zebrafish Zras-B1 shows strongest homology to mammalian N-ras. However, the precise differences that confer biologically significant properties unique to N-, Ha-, Ki-ras p21 are not known, and we cannot be confident that Z-ras functions in zebrafish as an N-ras protein. Zras-B1 and the two RT-PCR fragments share extensive nucleic acid homology, with minor differences in the wobble base (Figure 4.2). Since the ras-related mRNAs were enriched under low stringency conditions, it was not surprising that similar nucleotide sequences would be present in the enriched cDNA library. This result is consistent with the known ras superfamily diversity in other species, and indicates the similar existence of multiple forms of ras-like proteins in the zebrafish. The functioning of these proteins in zebrafish cellular differentiation, embryogenesis, and tumorigenesis remain to be established. The PCR techniques and enriched cDNA library application used here offer a relatively quick and efficient way to search for additional ras-related genes in zebrafish.

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CHAPTER 5

Comparative tumorigenic potency and Ki-ras mutation by dietary 7,12-dimethylbenz[a]anthracene and dibenzo[a,l]pyrene in rainbow trout

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Abstract

The tumorigenicity of dietary 7,12-dimethylbenz[a]anthracene (DMBA) and dibenzo[a,l]pyrene (DBP) were compared in the rainbow trout model. Both chemicals are potent, dose-responsive, multiorgan carcinogens, eliciting adenopapillomas of the stomach and gas bladder, hepatocellular and cholangiocellular adenomas and carcinomas, and nephroblastomas at high doses. At 100 ppm, incidences were 3.6% and 65% in liver, 94% and 76% in stomach, and 0.9% and 29% in swim bladder for DMBA and DBP, respectively. The incidence and spectrum of Ki-ras oncogene mutations in liver and stomach tumors were also compared by direct sequencing of exon I and II PCR products. Both chemicals elicited predominantly 12(1)G→A and 12(2)G→T mutations in trout livers and no codon 61(2)A→T mutation, which is dominant in mouse skin, liver and lung tumors. In the sixteen DMBA-induced liver tumors, six were 12(1)G→A and one was 12(2)G→T. Of the twenty four DBP liver tumors, ten were 12(1)G→A and six were 12(2)G→T, while two double mutations of {12(1)G→T and 12(2)G→T} and one double mutation of {12(1)G→A and 12(2)G→T} also existed. Some stomach tumors from both chemicals were degraded and we were unable to amplify them by routine PCR reaction, so only those with recoverable DNA were used for mutational screening. Of the sixteen DMBA stomach tumors examined, no Ki-ras mutations were found. In the sixteen DBP stomach tumors, one had a 12(1)G→A and two had 13(1)G→C mutations. Three spontaneous liver tumors were also examined for

their ras activation by direct sequencing and no mutation was observed. The G→T transversions elicited by these carcinogens are partially compatible with apurinic mutagenesis driven by unstable DNA adducts arising from one-electron oxidation, but the major G→A transitions, G→C transversions and rare double mutations in this study are not compatible with a one-electron oxidation activating mechanism.

Introduction

The availability of an aquatic vertebrate model known to be responsive to carcinogens is especially important for understanding the environmental health significance of aquatic genotoxicants. Xenobiotics such as the polycyclic aromatic hydrocarbons (PAH) tend to persist in the aquatic enviroment and have raised concerns about the cause of neoplasms in some fish populations. The discovery of tumor epizootics in feral fish populations from PAH contaminated waters support the concerns (Murchelano et al., 1985; Malins et al., 1985; Malins et al., 1987). Among freshwater fishes used for tumor induction studies, the rainbow trout is the most thoroughly characterized model for carcinogenesis (Hendricks et al., 1984; Bailey et al., 1984), and it is able to metabolize and activate several PAH to induce tumor formation (Hendricks et al., 1985; Fong et al., 1993). DMBA is a synthetic PAH compound and has long been used to study PAH carcinogenesis mechanisms in animal models. Dibenzo[a,l]pyrene (DBP) is naturally found in the environment and in cigarette smoke condensate, and was shown to be a much more potent carcinogen than DMBA in mouse skin (Cavalieri et al., 1991). Over the past decade, metabolism, mutagenicity, DNA binding, and tumorigenicity studies have established that bay-region diol epoxides are the ultimate carcinogens of at least a dozen polycyclic aromatic hydrocarbons (Jerina et al., 1986). However, in some cases, activation to other intermediates has led other investigators to postulate that radical cations arising from removal of one

electron from PAH were the predominant electrophilic species and were capable of reacting with cellular nucleophiles to form unstable DNA adducts (Cavalieri et al., 1985; Devanesan et al., 1990; RamaKrishna et al., 1992). PAH bioactivation either by diol epoxide or one electron oxidation leads to major DNA adducts located on guanines or adenines (Bigger et al., 1983; RamaKrishna et al., 1992). The diol epoxides are believed to form stable DNA adducts, while one electron oxidation may lead to unstable DNA adducts, almost all which are lost from DNA by depurination (RamaKrishna et al., 1992). In the rodent models, DMBA-induced ras mutations spectra may be tissue dependent. For example, DMBA-induced mouse mammary and skin tumors carry predominantly Ha-ras codon 61(2)A→T transversions, whereas liver tumors have the Ha-ras codon 61(2)A→T mutation and Ki-ras codon 13(1)G→C transversions, and lung tumors contain a variety of Ki-ras mutations but no Ha-ras mutation (Loktionov et al., 1990). In hamster and rabbit of DMBA-induced skin tumors, the codon 61(2)A→T of H-ras was also the exclusive mutation found (Corominas et al., 1991; Robles et al., 1993), while in rainbow trout liver tumors, the Ki-ras12(1)G→A and 12(2)G→T are dominant (Fong et al., 1993). Until recently, DMBA was the most potent known carcinogenic PAH. However DBP has been established recently as the strongest PAH carcinogen ever tested in rodent and human cell lines (Ralston et al., 1994), but the role of ras activation in DBP carcinogenesis has received little attention. The objectives of the present study were to compare the relative tumorigenicity of DMBA and DBP in rainbow trout when

administered in the diet, and to examine the types and incidences of Ki-ras oncogenic mutations elicited by these two carcinogens.

Materials and Methods

(A). Reagents

DMBA was purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI) and DBP from Chemsyn Science Laboratories (Lenexa, KS). Purity of both compounds was >98%. Sodium chloride, citric acid trisodium salt, sodium dodecyl sulfate, ethylenediaminetetraacetic acid, formamide, urea, boric acid, TRIMA hydrochloride^{TM}, and ammonium persulfate were purchased from Sigma (St. Louis, MO). Acrylamide/bis-acrylamide solution, saturated phenol/chloroform, and chloroform were from AMRESCO (Solon, OH). X-OMAT AR film was from Kodak (Rochester, NY). ^{35}S-dATP and ^{35}S-dCTP were from NEN (Boston, MA). Proteinase K was from Boehringer Mannheim (Indianapolis, IN).

(B). Carcinogenesis study

Shasta strain rainbow trout (*Oncorhynchus mykiss*) were hatched and reared in 12^{0}C well water at our laboratory as previously described (Sinnhuber et al., 1976). Animals for each treatment group were reared in separate 100-gallon tanks. The fish were fed twice daily. Fish were given the control diet from swim-up to 14 weeks of age. Beginning at 14 weeks, fish were were fed diets containing 0, 10, 100, 500, or 1000 ppm DMBA or

DBP (dry diet basis). Each concentration was fed for 4 months to duplicate groups of 100 fish each (DMBA treatment) or 75 fish each (DBP treatment). The number of fish at necropsy, shown in Table 5.1, and 5.2 were in most cases lower due to occasional mortalities. High fish mortalities from toxicity were observed in the fish fed 1000ppm DMBA and DBP. All fish were sacrificed twelve months after start of exposure. After sacrifice, the livers, stomachs, and swim bladders of each fish were examined for grossly observable tumors. Portions of liver, stomach, and swim bladder were fixed in Bouin's solution for light microscopy. After 1 week in fixative, the tissues were processed by routine histological methods. Tumors were classified according to criteria established previously by Hendricks et al. (1984).

(C). PCR hygiene for tumor DNA isolation and amplification

Tumors larger than 3mm were collected and stored at -80 ^{0}C until analysis for Ki-ras mutation. Tumor DNA was isolated from each tumor tissue by incubation overnight with lysis buffer (50 mM Tris pH8.0, 1 mM EDTA, 0.5% NP40, 400µg/ml proteinase K) at 50^{0}C. The tumor DNA was then used directly for PCR amplification of Ki-ras exon I and exon II regions by nested primers. PCR hygiene for tumor DNA isolation and Ki-ras exons amplification was strictly followed to prevent any potential tumor DNA carry-over contamination. Filtered tips and gloves were used throughout the analysis. The working surface was routinely cleaned with 2%

Table 5.1. Twelve month tumor incidence of rainbow trout (Oncorhynchus mykiss) after 4 month dietary DMBA treatment.

Diet	Liver Tumors		Stomach Tumors		Swim Bladder Tumors		Other	
	No.	%	No.	%	No.	%	No.	%
Control	1/114	0.9	1/114	0.9	0/114	0	0	0
Control (DMSO)	0/120	0	0/120	0	0/120	0	0	0
10 ppm DMBA	0/126	0	6/126	4.8	0/126	0	0	0
100 ppm DMBA	4/112	3.6	105/112	94	1/112	0.9	0	0
1000 ppm[a] DMBA	30/41	73	41/41	100	21/41	51.2	3/41	7.3

[a]: High mortalitily (68%) was observed with the high dose DMBA.

Table 5.2. Twelve month tumor incidence of rainbow trout (*Oncorhynchus mykiss*) after 4 month dietary DBP treatment.

Diet	Liver Tumors		Stomach Tumors		Swim Bladder Tumors		Other	
	No.	%	No.	%	No.	%	No	%
Control (THF)	5/76	6.6	1/76	1.3	0/76	0	0	0
10 ppm DB[a,l]P	15/126	12	1/66	1.5	0/66	0	0	0
100 ppm DB[a,l]P	41/63	65	48/63	76	18/63	28.6	0	0
500 ppm DB[a,l]P	60/92	65	67/92	73	42/92	45.6	2/92	2.2
1000 ppm[a] DB[a,l]P	24/30	80	30/30	100	16/30	53	0	0

[a]: Dose was toxic. Most of the fish died. Stopped feeding DB[a,l]P diet after 17 days. Fed normal diet till termination.

sodium hypochloride and all pipet equipment was cleaned with 3% hydrogen peroxide at intervals. All reagent solutions used for tumor DNA isolation were newly purchased and aliquots stored until used and routinely checked for possible Ki-ras contamination by PCR. The PCR products were purified using differential precipitation as follows: one volume of PCR products and one volume of 5M ammonium acetate were mixed with 2 volumes of isopropanol incubated at room temperature for 5 minutes and immediately centrifuged at high speed (13,000 g). The supernatant was pipeted out and the DNA was air dried. The dry DNA was dissolved in the same volume of UV-treated sterile water and about 10 ng were used for direct sequencing.

(D). Primers

Rainbow trout Ki-ras **H01** primer (a forward primer located at the 5' end of exon 1): 5'- ATG ACG GAA TAC AAG CTG-3'; **c37** Primer (a reverse primer located at the 3' end of exon 1): 5'- CTC GAT GGT GGG GTC ATA TT-3'; **H38** primer (a forward primer located at the 5' end of exon II): 5'-GAC TCG TAC AGG AAG CAG GT-3'; **sc67(1)G** primer (a reverse primer on exon 2 region): 5'-CAT GGC GCT GTA CTC CTC CTG-3'. Before being used for Exon I and II amplification or for direct sequencing, all newly made primers were dissolved in water and then checked by 20% denaturing acrylamide gels for quality.

(E). PCR amplification and sequencing

For PCR amplification of exons, the reaction conditions were as follows: one cycle of denaturing at 95^0C for 6 min; 2 cycles of denaturing at 95^0C for 2 min, annealing at 60^0C for 45 seconds, and extension at 74^0C for 26 seconds; 40 cycles of denaturing at 95^0C for 40 seconds, annealing at 60^0C for 42 seconds, and extension at 74^0C for 26 seconds. For direct sequencing of exons, the reaction conditions were as follows: in the labeling steps one cycle of denaturing at 95^0C for 2 min; 50 cycles of denaturing at 95^0C for 28 seconds, annealing at 60^0C for 45 seconds, and extension at 72^0C for 12 seconds; in the extension steps one cycle of denaturing at 95^0C for 2 min; 30 cycles of denaturing at 95^0C for 30 seconds, annealing at 70^0C for 30 seconds, and extension at 72^0C for 20 seconds.

Results

(A). Tumor incidence

Rainbow trout fed various doses of DMBA and DBP produced tumors of the liver, stomach, and swim bladder (see Table 5.1 and 5.2). DMBA and DBP were both multiorgan carcinogens at all doses with the exception of DMBA at the low 10ppm dose where no liver tumors were observed. The primary target organ in rainbow trout for DMBA by diet is the stomach, followed by the liver and swim bladder. However, for DBP, the liver is the primary target organ at the low dose, with a slightly higher perference for stomach tumors at the higher doses. Both carcinogens, especially DBP showed

higher mortalities at the high dose of 1000 ppm, with most losses occurring within two weeks after the start of feeding. Dose related increases in liver, stomach, and swim bladder tumor incidence was generally observed for DMBA and DBP.

(B). Tumor multiplicity

Multiplicity of tumors (number of tumors per tumor-bearing animal (TBA)) increased with increasing dose of DMBA and DBP (Figure 5.1-5.4). The number of trout bearing one tumor/TBA decreased and those bearing ≥2 tumors/TBA in target organs increased as the carcinogen dose increased. This trend was particularly noticable in the stomach, with the number of trout bearing ≥5 tumors/TBA dramatically increasing at the higher doses of both DMBA and DBP. These results show that an increase in carcinogen dose increased both the tumor incidence and tumor multiplicity.

Figure 5.1. Dieatry DBP stomach tumor multiplicity

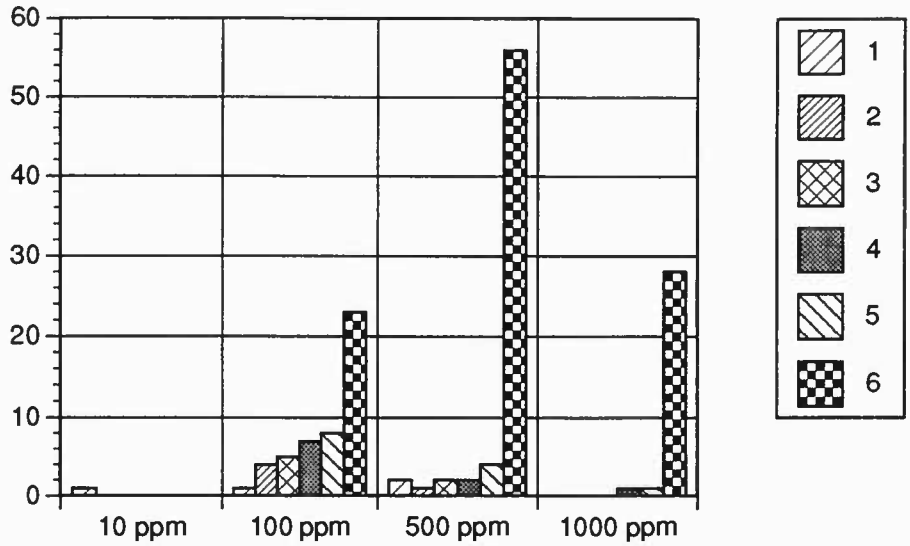


Figure 5.2. Dietary DBP liver tumor multiplicity

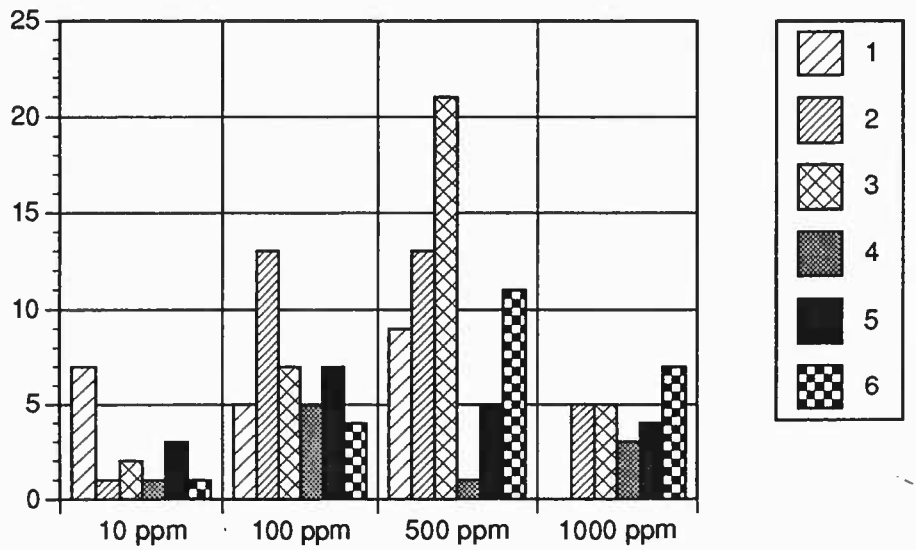


Figure 5.3. Dietary DMBA stomach tumor multiplicity

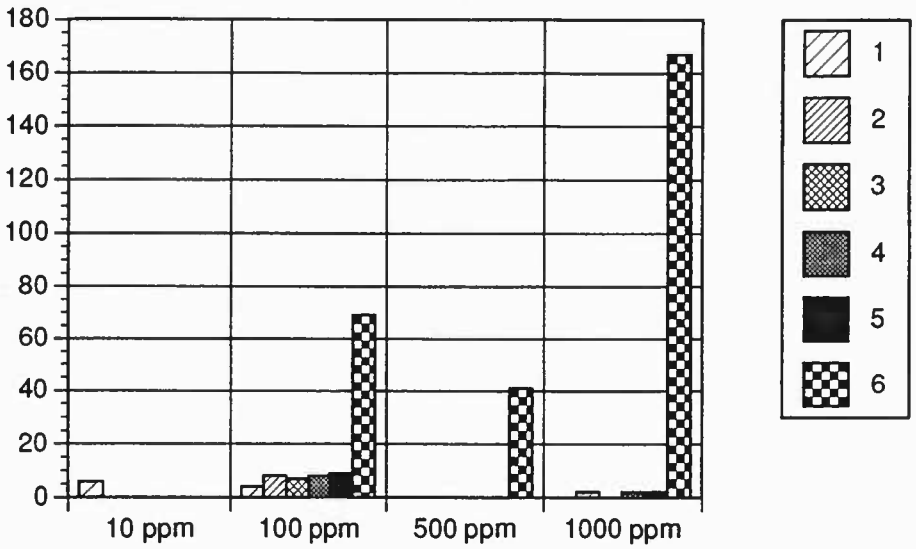
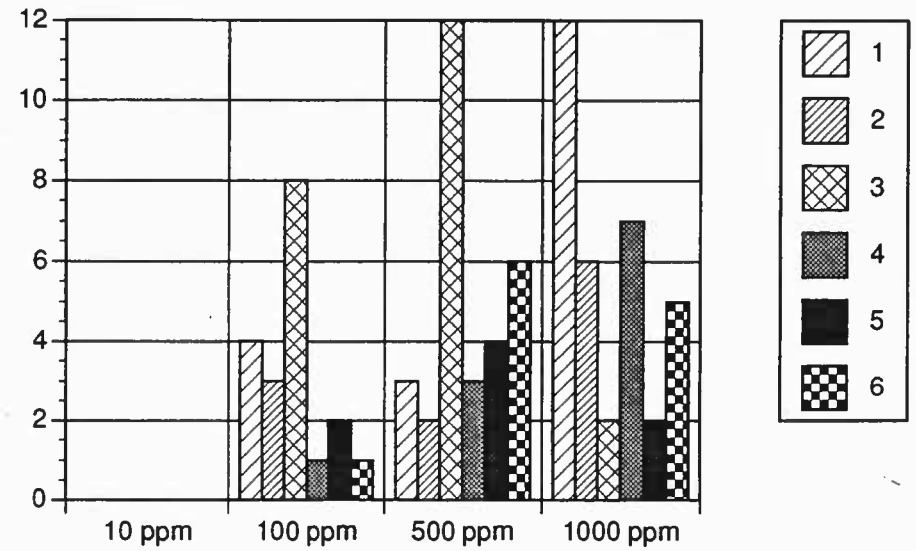


Figure 5.4. Dietary DMBA liver tumor multiplicity



(C). Ki-ras mutations

Hepatic and stomach tumors initiated by DMBA and DBP were examined for evidence of activating point mutations in codons 12, 13 and 61 of the Ki-ras protooncogene by direct sequencing (Tables 5.3, 5.4 and Figure 5.5). Of the tumors analyzed for Ki-ras mutations, 7/16 of DMBA initiated and 18/24 of DBP initiated hepatic tumors in trout were found to carry Ki-ras mutant allelles. DMBA initiated hepatic tumors were predominatly 12(1)G→A transitions in this study. DBP initiated hepatic tumors carried G→A transitions and G→T transversions in codon 12(1) and 12(2) at similar frequency. Even though the primary target organ of DMBA is the trout stomach, Ki-ras point mutations were not detected. However, DBP initiated stomach tumors showed evidence for 12(2)G→A transitions and 13(1)G→C transversions. Some of the DNA from DMBA or DBP initiated stomach tumors was difficult to amplify by PCR and showed severely smeared DNA patterns (data not shown), which might indicate degradation of the original genomic DNA. Only good recovery DNA was further analyzed for Ki-ras mutation by direct sequencing. The DMBA and DBP-initiated K-ras mutations seen here in trout differ from the study of DMBA and DBP-initiated mouse skin papillomas (Quintanilla et al., 1986; Chakravarti et al., 1994), which predominantly show H-ras codon 61(2)A→T transversion. This event also was not seen in a previous study of DMBA carcinogenesis in trout (Fong et al., 1993). The double mutation of 12(1)G→T and 12(2)G→T or 12(1)G→A and 12(2)G→T is found only in DBP-induced liver tumors.

Table 5.3. Mutation profile of Ki-ras gene induced by dietary DMBA in rainbow trout

Dose	Tumor tissue	Exon I 12(1)G→A	12(1)G→T	12(2)G→T	13(1)G→C	Exon II 61(2)A→T	Total
Control (DMSO)	no tumors	–	–	–	–	–	–
10 ppm	Liver	–	–	–	–	–	–
	Stomach	–	–	–	–	–	–
100 ppm	Liver	2/2	0/2	0/2	0/2	0/2	2/2
	Stomach	0/7	0/7	0/7	0/7	0/7	0/7
1000 ppm	Liver	4/14	0/14	1/14	0/14	0/14	5/14
	Stomach	0/9	0/9	0/9	0/9	0/9	0/9

Table 5.4. Mutation profile of Ki-ras gene induced by dietary DBP in rainbow trout

Dose	Tumor tissue	Exon I 12(1)G→A	12(1)G→T	12(2)G→T	13(1)G→C	Exon II 61(2)A→T	Total
Control(THF)	Liver	0/3	0/3	0/3	0/3	0/3	0/3
10 ppm	Liver	0/4	0/4	2/4	0/4	0/4	2/4
100 ppm	Liver	5/10	1/10[a]	3/10	0/10	0/10	8/10
	Stomach	1/10	0/10	0/10	0/10	0/10	1/10
500 ppm	Liver	5/10	1/10[a]	4/10	0/10	0/10	8/10
	Stomach	0/6	0/6	0/6	2/6	0/6	2/6

[a]: double mutation at 12(1)G→T and 12(2)G→T

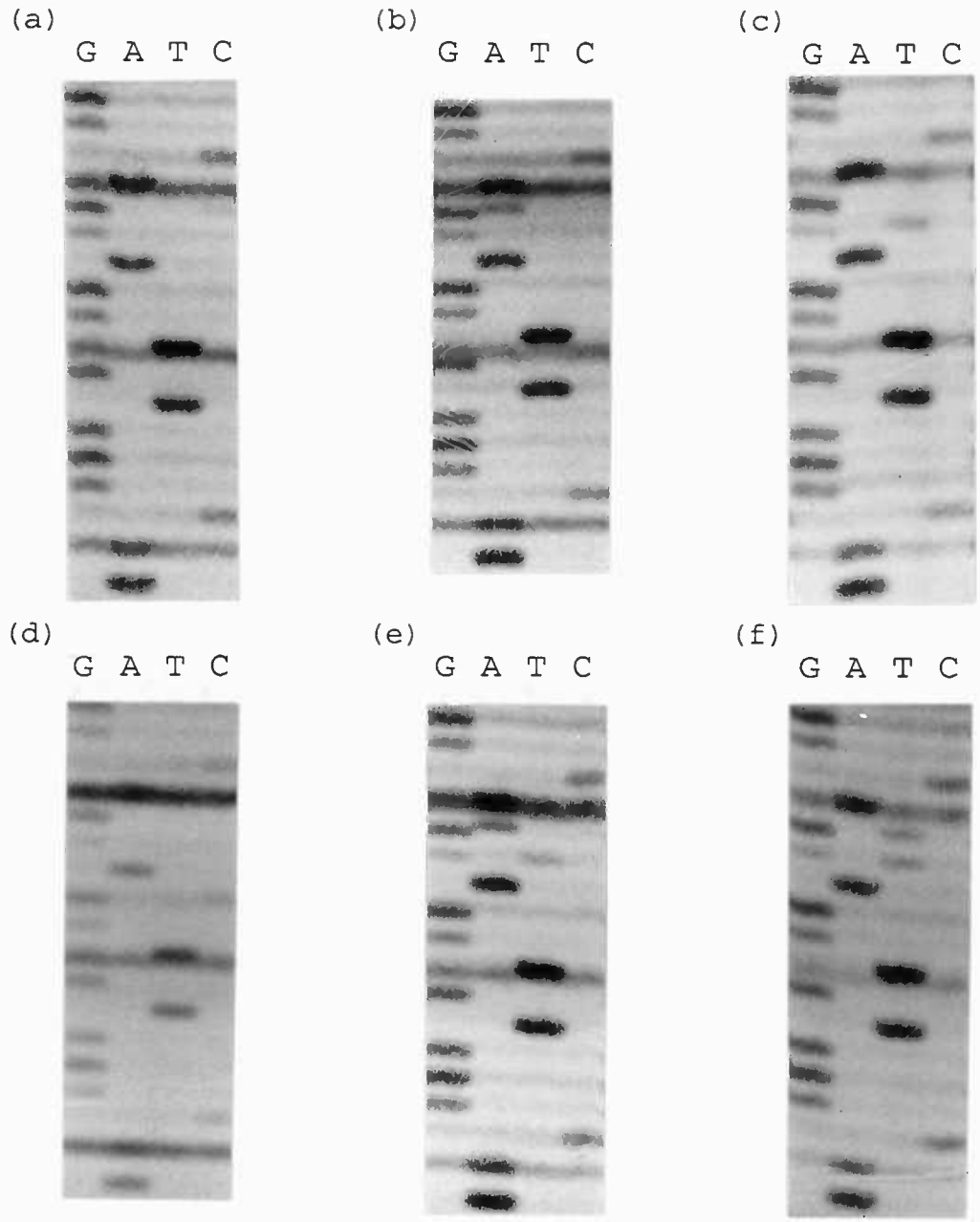


Figure 5.5. Direct sequencing of Ki-ras gene exon I region of rainbow trout tumor initiated by DMBA or DBP. (a) normal tissue (b) codon 12(1)G→A mutation (c) codon 12(2)G→T mutation (d) codon 13(1)G→C mutation (e) codon 12(1)G→A,12(2)G→T double mutation (f) codon 12(1)G→T,12(2)G→T double mutation.

Discussion

Development of malignancy is generally believed to involve multiple steps, in which cells acquire a series of genetic changes leading to progressive disruption of control mechanisms that govern cell growth. Cancers of different origins might share certain common mechanisms, while tumors in the same tissue may evolve from different pathways. In this study, tumors of different size or pathological stages might reflect diverse genetic pathways or consequences. For example, in this study, tumors of the liver did not always contain a Ki-ras mutation, indicating that non-ras initiated tumor progression can also occur. However, there was no evident relation between size and presence of a ras mutation. For the sixteen amplificable exon regions from DBP stomach tumor, Ki-ras mutations were rare and two 13(1)G→C mutations were observed. These were not found in liver tumors by direct sequencing. In this study of DMBA stomach tumors, sixteen DNA samples were screened for Ki-ras mutations and none had mutations detected by direct sequencing. This result might imply another ras gene beside Ki-ras is prevalent in this tissue, that ras mutation is a rare event of stomach cancer in the rainbow trout model, or that the percentage of cells in the tumor with mutant ras was below detection limits by direct sequencing. The 13(1)G→C mutation in stomach tumors found to be initiated by DBP in this study was also seen in a separate DMBA study (data not shown). Codon 13(1)G→C mutations in stomach tumors were less frequent than codon 12(1)G→A or 12(2)G→T mutations in the liver. The

reasons for this mutation in stomach, and swim bladder (data not shown) but not in liver are unclear, but might indicate specific ras mutations in different tissues.

If ras mutations are directly caused by interaction with the carcinogen as a part of the initiation process, then the type of mutation introduced should correlate with the known metabolism and DNA-binding characteristics of each initiator (Balmain et al., 1988). The first evidence for this came from the induction of rat mammary carcinomas by the methylating agent MNU (Zarbl et al., 1985). These carcinomas were found to contain an activated H-ras oncogene mutation at codon 12(2)G→A. This particular mutation would cause the generation of O^6-methylguanine, which is known to mispair with thymidine during DNA replication. However, that study did not consider that many carcinogens are also oxidants and may generate oxygen free radicals, which generate 8-hydroxyguanine and complement with thymine to produce a G→A mutation in DNA duplication (Shibutani et al., 1991). The polycyclic aromatic hydrocarbons, unlike MNU or other alkylation agents, form large adducts with deoxyguanine and deoxyadenine residues that lead to excision repair, thus generating point mutations of undefined specificity. In the case of benzo[a]pyrene, the mutation spectrum appears to be influenced by excision repair and the strand-specific preference of adduct position (Chen et al., 1992; Wei et al., 1995). It has been implied that mutation formation is due to the slow DNA repair of DNA adducts (Service, 1994), which is related to a restricted accessibility of DNA repair enzymes to certain gene structures. Marien et al. (1989) first showed that DNA

replication is strongly blocked by carcinogen-DNA adducts at the codon 12 region of the c-Ha-ras gene. In a later experiment, Hoffmann et al. (1993) found that a strong secondary structure around the codon 12 and 13 region of the H-ras gene impeded the DNA polymerase progression at these regions. The above reports support the hypothesis of transcription-couple excision repair proposed by Hanawalt (1994).

If PAH bioactivation occurs through one-electron oxidation (Cavalieri et al., 1985; RamaKrishna et al., 1992), then the majority of adducts in vitro mediated by induced rat liver microsomes are unstable adducts of guanine and adenine and could generate apurinic sites. The dAMP is most likely selected to incorporate opposite the apurinic sites to cause G→T mutations (Loeb, 1985). However, dAMP is not exclusively complementary to apurinic sites. It was suggested that dGMP is second most frequently incorporated by DNA polymerase at these positions. Thus, dGMP might explain the G→C transversion found in codon 13, but not the dominant mutation found in the codon 12(2) position. It is not yet possible to say whether these mutations are all directly induced by the carcinogenic agent or if they arise at a later stage of tumor development. The ras mutation spectra in spontaneous rodent tumors did indicate that ras mutations might arise from a subpopulation of cells harboring the mutated ras gene which were promoted in vivo (Reynolds et al., 1987; Belinsky et al., 1989; You et al., 1989). However, the historical frequency of spontaneous liver tumors in trout is about 0.1% much lower than in rodent systems. The few tumors examined so far contained no Ki-ras

mutation on codon 12, 13 and 61 (data not shown). This might imply that in the rainbow trout system, any spontaneously mutated ras subpopulation is very low or a significance number is still not achieved at this stage.

Acknowledgments

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CHAPTER 6: CONCLUSION

Summary

Two types of cDNA library constructions were used to screen for zebrafish ras protooncogene and p53 tumor suppressor gene sequences. These sequence data will be essential for future studies of chemical carcinogenesis by oncogene activation and tumor suppressor gene inactivation in this species. In the first attempt, a traditional cDNA library was used for p53 gene screening by nonradiolabeled-digoxigenin colorimetric detection (Chapter 3). An incomplete p53 clone was isolated by this method by screening 2 x 10^5 p.f.u. This p53 gene contained evolutionarily conserved regions III, IV, V and part of II, but lacked the expected 5' end sequence of 300-400 bp. The same library was screened (1 x 10^6 p.f.u.) for ras genes using rainbow trout Ki-ras exon I and II as probes by colorimetric or chemiluminescence detection, but with no success. In a second attempt, a ras-enriched cDNA library was constructed to as an approach to improve the probability for isolation of ras gene from zebrafish (Chapter 4). A clone was readily recovered by screening 3 x 10^4 p.f.u. of this enriched library, and a Z-ras cDNA was isolated with a translated amino acid sequence sharing 91% homology with the mammalian N-ras protein. The enriched cDNA library application used here offers a relatively quick and efficient way to search for ras-related genes in zebrafish. We also have attempted to complete the 5' end unknown sequence of zebrafish p53 by using the rapid

amplification of cDNA end (RACE) technique, but were not successful. We used only the Replitherm DNA polymerase to PCR the 5' end of the unknown region and the processivity of this enzyme might be not good for amplifying products of >500bp, compared with other thermostable enzymes.

The spectrum of rainbow trout Ki-ras oncogene mutations from DMBA and DBP induced liver and stomach tumors were compared by direct sequencing of the exon I and II regions to determine the specific ras mutations found in the tumors. Both chemicals elicited predominantly 12(1)G→A and 12(2)G→T mutations in liver tumors. The codon 61(2)A→T mutation, which is dominant in mouse skin, liver and lung tumors (Chapter 2, Table 2.2) by these carcinogens, was not detected. In addition to the above mutations, the DBP induced liver tumors also contained a few double mutations {12(1)G→T and 12(2)G→T} and {12(1)G→A and 12(2)G→T}, which did not occured in DMBA livers. The 13(1)G→C mutation was found only in stomach tumors induced by DBP in this study and by DMBA in another study (Table 6.1), at a frequency lower than the codon 12(1)G→A or 12(2)G→T. Failure to detect this mutation in liver might indicate the ras mutations are tissue-specific, or may simply reflect insufficient sampling in liver.

It is important to note that cells in an end-stage tumor containing a ras mutation are selected, and therefore, the type of mutation in them is also selected. This selection can be cell-type specific, and tissue-specific susceptibility for a particular mutagen as well as specificity of DNA damage and repair may influence the mutational spectrum observed in tumors. There are several

mechanisms by which carcinogen treatment could affect the ras mutation profile in tumor DNA (Harris et al., 1991). First, the carcinogen or its metabolites could cause mutations by direct chemical interaction with DNA. Second, the carcinogen could indirectly cause oxidative damage to DNA via oxygen free radical or lipid peroxidation produced radicals during carcinogen metabolism or cytotoxic injury. Third, the carcinogen could cause other genetic alterations which either render the tumor cells hypermutable or are selectively complemented by a particular type of ras mutation. Fourth, the carcinogen could selectively promote the growth or death of tumor cells that carry a specific ras type. The spontaneous tumor incidences and ras mutations found in some rodent model can be quite high (Table 2.1). Therefore, the detection of ras mutations in chemically induced mouse tumors does not necessarily demonstrate that the chemical in question acts as a mutagen in vivo; indeed, it might act as a nongenotoxic mechanism by facilitating a clonal expansion of cells bearing this spontaneous mutation. However, the historical frequency of spontaneous tumors in rainbow trout (<1%) is much lower than in these rodent systems. The spontaneous tumors (8 archive tissue, 3 frozen samples), mainly from liver, examined so far contained no ras mutations on codon 12, 13, or 61 (data not shown). The scarcity of spontaneous tumors in trout actually provides us a challenge to identify the direct relationship of carcinogen-induced ras mutation found in tumors.

Among the alkylating DNA adducts, O^6-methylguanine is known to mispair with thymidine and gives G→A mutation during DNA replication. This mispairing may explain the observation of ras G→A mutations by most of the alkylating agents that induced tumors, but does not explain the observation of 12(1)G→A mutations found with other carcinogens in this study and others. The most mutagenic compound that results from reactive oxygen species-induced damage to DNA in bacteria is 5-hydroxy-2'-deoxycytidine, which causes C→T transitions (Feig et al., 1994a; Feig et al., 1994b). If oxygen free radicals have reacted at the codon 12 region of rainbow trout Ki-ras, where the antisense strand reading is CCT, it would create a C→T transition and eventually generate a G→A mutation in the sense strand. This might indicate that indirect carcinogen-induced oxidative DNA damage during DMBA and DBP metabolism may contribute to the observed spectrum of ras mutations.

Metabolic activation of PAH could occur through one-electron oxidation, such that a majority of adducts are unstable adducts of guanine and adenine and then could generate apurinic sites. dAMP is most likely selected to incorporate opposite the apurinic sites to cause G→T mutations (Loeb, 1985). However, dAMP is not exclusively complementary to apurinic sites. It was suggested that dGMP is the second most frequently incorporated by DNA polymerase at these positions, an event that would cause G→C transversion. Thus, apurinic sites generated by DMBA or DBP might explain the G→T and G→C transversion found in this study and others (Table 6.1). However, 8-hydroxyguanine, one of the

oxygen free radicals, induces promutagenic lesions in DNA and mainly induces an in vitro G→T transversion followed by G→C mutation (Shibutani et al., 1991; Kamiya et al., 1995a), and therefore makes the interpretation of PAH specific ras mutation more ambiguous.

A parallel experiment of oxygen free radical treatment and carcinogen treatment might indicate whether the ras mutation is driven directly from the carcinogen itself or indirectly by free radicals generated by oxidative stress as a result of carcinogen metabolism.

Table 6.1. Chemical carcinogenesis induced Ki-ras mutation spectra in rainbow trout codon 12 (GGA), codon 13 (GGT), and codon 61 (CAG)

Carcinogen	Tumor	Mutation[Frequency]	Reference
DMBA[1]	liver[a]	12(1)G→A[2/9], 12(1)G→C[1/9], 13(1)G→C[1/9]	this study
	stomach[a,o]	12(1)G→C[1/9], 13(1)G→C[4/9], 61(2)A→T[4/9]	"
	swim bladder[a]	12(1)G→C[1/9], 13(1)G→C[3/9], 61(2)A→T[2/9]	"
DMBA[2]	liver[a]	12(1)G→A[5/27], 12(2)G→T[11/27]	this study
	stomach[a]	12(1)G→A[1/9]	"
DMBA[3]	liver[b]	12(1)G→A[4/11], 12(2)G→T[4/11], 61(2)A→T[1/11]	Fong, 1993
DMBA[2]	liver[c]	12(1)G→A[6/16], 12(2)G→T[1/16]	this study
	stomach[c]	no mutation[0/16]	"
DBP[2]	liver[d,p]	12(1)G→A[10/24], 12(2)G→T[9/24] 12(1)G→T and 12(2)G→T[2/24] 12(1)G→A and 12(2)G→T[1/24]	this study
	stomach[d]	12(1)G→A[1/16], 13(1)G→C[2/16]	"
AFB_1[4]	liver[e]	12(1)G→A[1/14], 12(2)G→T[7/14], 13(2)G→T[2/14]	Chang, 1991
AFB_1[1]	liver[f]	12(1)G→A[2/32], 12(2)G→T[22/32], 13(2)G→T[3/32]	Gayle, 1995
AFB_1[1]	liver[g]	12(1)G→A[1/29], 12(2)G→T[17/29], 13(2)G→T[7/29]	Gayle, 1995
DHEA[1]	liver[h]	12(1)G→A[8/25]	Gayle, 1995
DEN[1]	liver[i]	12(1)G→A[6/7]	Hendricks, 1994
MNNG[1]	liver[j]	12(1)G→A[3/30], 12(2)G→A[25/30]	unpublished
control[1]	spontaneous	no mutation[0/11]	unpublished

Table 6.1 (Continued)

1: detected by mismatch PCR and some confirmed by direct sequencing.
2: detected by direct sequencing only.
3: detected by mismatch PCR and confirmed by cloning and sequencing.
4: detected by allele hybridization and confirmed by cloning and sequencing.
a: DMBA fry bath exposure/aroclor 1254 promoter.
b: DMBA embryo bath exposure.
c: DMBA dietary.
d: DBP dietary.
e: AFB1 dietary
f: AFB1 fry bath exposure.
g: AFB1 fry bath exposure/DHEA promoter.
h: DHEA promoter only.
i: DEN bath exposure.
j: MNNG bath exposure
o: one double mutation 12(1)G→C and 13(1)G→C.
p: two double mutation at 12(1)G→T and 12(2)G→T.

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