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13. ABSTRACT *(Maximum 200 words)*

The objective of this research is to develop the medaka as a model system to better understand the molecular mechanisms of chemical carcinogenesis. Although the medaka has been widely used in carcinogenicity studies for the past two decades, almost no information is available at the molecular level. We have focussed our studies these past three years on the role of oncogenes and suppressor genes in tumorigenesis. We exposed medaka to three known carcinogens: diethylnitrosamine (DEN), methylazoxymethanol acetate (MAMAc) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Animals exposed to either DEN or MAMAc developed liver tumors. Medaka exposed to MNNG, however, developed a wide array of extra-hepatic lesions. Our studies have shown that DNAs from these chemically-induced tumors are able to transform NIH3T3 cells and induce tumors in nude mice. One animal with a DEN-induced cholangiocarcinoma appears to have a novel activated oncogene. Preliminary evidence indicates that DNA from MAMAc-induced hepatocellular cholangiocarcinomas may contain activated K-ras and/or inactivated p53 genes. More sensitive PCR-based techniques are being developed to analyze the DNA from the MNNG-induced tumors.

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Robert J Van Somme 6 July 94

PI - Signature Date

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INTRODUCTION

For over twenty years, tumors in fish and other lower vertebrates and invertebrates have been systematically documented by the Registry of Tumors in Lower Animals at the Smithsonian Institution in Washington DC. While an overwhelming amount of data has been accumulated which documents the high incidence of neoplasia in fish residing in areas high in anthropogenic pollution and numerous studies have reported the chemical induction of tumors in fish, very little is known about the molecular basis of carcinogenesis in these animals. It wasn't until 1986 that the first oncogenes from fish were cloned and sequenced from the goldfish (Nemoto *et al.* 1986) and the rainbow trout (Van Beneden *et al.* 1986). Since that time, however, interest in fish as model systems for molecular studies has risen exponentially.

The development of aquatic models for studies in molecular carcinogenesis and toxicological studies has focused primarily on the teleosts (bony fishes). This group is the largest and most diverse class of vertebrates, with over 20,000 described species. Their phylogenetic position relative to the other vertebrates makes them ideal for comparative carcinogenesis studies. One of the best developed laboratory models is the Japanese medaka (*Oryzias latipes*). The medaka has been used for carcinogenicity testing for well over a decade (Hoover, 1984). It is well suited for these experimental studies and offers numerous advantages over rodent models: they are highly sensitive to a variety of chemicals, tumors may be induced within a short period of time and their small size allows exposure of large, statistically relevant numbers of animals at considerably less expense than rodent studies. Medaka can easily be induced to breed year round, provide large numbers of eggs and are easy to culture. The genetics, developmental biology and embryology are well-documented (Yamamoto 1975). Induction of tumors has been reported in nearly every organ by agents known to be carcinogenic to humans. Neoplastic lesions have been induced in the liver (Ishikawa *et al.* 1975; Aoki and Matsudaira 1977, Hawkins *et al.* 1988a,b, Van Beneden *et al.* in press, a,b), gills (Brittelli *et al.* 1985), eye (Hawkins *et al.* 1986), striated muscle (Van Beneden *et al.* in press,a) and in the epidermis as melanomas (Hyodo-Taguchi and Matsudaira 1984). In addition to these advantages, the medaka model offers a unique system with which to specifically address the affects of *aquatic* toxicants which are being discovered at an alarming rate in the natural environment.

In spite of the vast amount of background material available on carcinogenicity testing in the medaka (Hoover, 1984), relatively few studies have addressed the molecular basis of tumor induction and progression. It has been well established that tumor development is a multistage process of initiation, promotion and progression (Pitot, 1990). Specific classes of genes - the oncogenes and the tumor suppressor genes - are believed to play key roles in the regulation of this process. We are addressing the role of these genes in the development of tumors in medaka exposed to known carcinogens: diethylnitrosamine (DEN), methylazoxymethanol acetate (MAMAc) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). The importance of this study lies in the integration of molecular approaches with classic studies of the effects of toxicants on environmental health. The use of fish models promises to provide important contributions to the field of cancer research.

The results of these studies conducted for the past three years on the

molecular basis of tumor induction in the medaka provide further clues in the ongoing investigation of the role of oncogenes in the development of chemically-induced tumors.

BODY

I. EXPERIMENTAL METHODS

A. Tumor Induction

Medaka were exposed to three known carcinogens in separate experiments as described below. Fifty animals were used for each exposure and control group:

(1) Fourteen-day-old medaka fry were exposed to MAMAc at 10mg/liter for 2 hours. Animals were then transferred to aquaria containing clean water. Fish were sacrificed at three and six months post-exposure. Livers were excised and a portion was preserved by fixation in Bouin's solution and subsequently stained with hematoxylin and eosin for histopathological analysis. The remaining tissue was immediately frozen in liquid nitrogen and stored at -70^{O} until DNA was extracted.

(2) Exposure of fourteen-day-old fry to DEN (200 mg/liter) and subsequent sacrifice of the fish was done using a similar procedure, described previously in detail (Van Beneden *et al.* 1990).

(3) In a third exposure test, fourteen-day old medaka fry were exposed to MNNG at 30 mg/liter for one hour. Unlike MAMAc and DEN, MNNG is a direct-acting carcinogen.

B. Transfection Analysis

A transfection assay using mouse fibroblast (NIH3T3) cells modified from Graham and van der Eb (1973), was used to identify oncogenes in fish tumors. DNA used in transfection studies was extracted by quick dounce homogenization (Van Beneden *et al.* 1988; Van Beneden *et al.* in press). Ten to twenty μgs of high molecular weight fish DNA was co-transfected with the pSV_2neo plasmid in the presence of calcium phosphate. Cells were grown in the presence of G418 (Geneticin; Gibco/BRL) for two weeks and drug resistant colonies harvested by trypsinization. The cells were pooled, and divided among three assays: (a) standard focus assay; (b) nude mouse assay; (c) colony selection assay. In some cases, the third assay was omitted.

In the standard focus assay, cells were replated and grown to confluency. Selected foci were picked, expanded and DNA isolated for further analysis. Cells from the same pool were injected into athymic mice at 1.5 x 10^6 cells/mouse with 1-2 mice injected per plate of cells (Blair *et al.* 1982). Mice were examined for tumor formation at the site of injection (usually in 6-8 weeks). In the colony-selection assay, cells were replated in a defined serum-free media (QBSF, Quality Biologicals) both in the presence and absence of a low amount (0.1%) of fetal calf serum. Transformed cells formed colonies in the absence of serum, usually within two weeks.

In order to confirm that cells picked as foci in the standard focus

assay or as colonies in QBSF selection were true transformants, we expanded these cells and grew them in soft agar (McPhearson and Montegnier, 1964). In this assay, cells are suspended in a soft agar media and examined for growth after two weeks. NIH3T3 cells which normally require a hard surface to attach are unable to grow in this media. Transformed cells will grow and form small colonies in the soft agar.

C. Cloning experiments

We have continued attempts to identify the novel transforming gene which was detected by transfection assay of DNA from a DEN-induced cholangiocarcinoma. A genomic DNA library was previously prepared in a lambda based vector (EMBL4) and screened using pSV_2neo as a probe (Van Beneden *et al.* in press). Several positive clones were identified including clone C-7 which contains a 17 kb insert. We have attempted to identify the medaka sequence within this clone using Sanger dideoxy sequencing methods (Sanger *et al.*, 1977).

D. Gene Expression Analysis

Animals were collected at different developmental stages for the isolation of RNA in order to establish the pattern of expression of cellular oncogenes during normal development. For the developmental studies, the entire animal (or egg) was used and animals were pooled in order to obtain sufficient tissue for analysis. Preliminary experiments were done using liver tissue from adult medaka.

Expression of oncogenes was determined by reverse-transcriptase PCR (RT-PCR). This allows optimization of the analysis of a small amount of tissue. Northern analysis of total RNA is often confusing due to interference from the large amount of rRNA present. The yield of mRNA from total RNA for fish tissue is so low (<0.2%), that amplification of specific messages from total RNA appears to be the best method for these studies with limited amounts of tissue. Accordingly, primers were prepared which were specific to conserved regions of the *myc*, *H-ras* and *K-ras* oncogenes and the *p53* suppressor gene. A cDNA copy of the specific mRNA of interest was made using reverse transcriptase and appropriate primers. The unique cDNA was then amplified using Amplitaq (Perkin Elmer).

In order to further examine oncogene and suppressor gene expression in the medaka, mRNA was isolated from over 400 normal medaka adult liver (Chirgwin *et al.*, 1975) and used to prepare a cDNA library. The cDNA was ligated to *EcoRI* adaptors and ligated into the *EcoRI* site of the cloning vector Lambda Zap (Stratagene). The library was screened for the medaka *p53* gene using the homologous trout clone.

II RESULTS

A. Tumor Induction

Histopathology of the medaka tissue was evaluated by Dr. Marilyn Wolfe at the Environmental Pathology Laboratory, Inc., in Herndon, VA. Medaka exposed to MAMAc or DEN developed liver tumors. In animals exposed to MNNG, the target tissues varied and included gill, eye, skin and scales. The

transfection assays are done as a blind study so that results were correlated with the histopathology only after the transfection analysis was completed.

B. Transfection Studies

(1) MAMAc-exposed animals - Transfection assays were used to analyze DNA samples from medaka which had been exposed to MAMAc and sacrificed at either 3 or 6 months post-exposure. At 3 months, the fish were so small we were unable to obtain much DNA for the assays. It was also difficult to get a representative sample for histology. We have focused our attention on the animals sacrificed at six months which had substantially larger livers and therefore provided more DNA. Later testing protocols were modified to eliminate the early sacrifice time.

3 month sacrifice

The histopathology of the three month samples is given in **Table I**. Results obtained in transfection TR23 are summarized in **Table II**. DNAs from medaka AA-91-351-5-19 (TR23-14) which contained a cholangiocarcinoma and medaka AA-91-351-4-4 (TR23-16) which had a mixed hepato-cholangiocarcinoma were positive in both the standard focus assay and the colony selection assay. DNA from medaka AA-91-351-4-18 (TR23-12/13) whose liver contained hepatocellular vaculation and moderately severe bile duct hyperplasia was also transformation positive, but to a lesser degree. DNA from medaka AA-91-351-4-17 (TR23-17) which was identified as possessing a cholangiocarcinoma was negative in our assay.

A Southern blot of DNA from NIH3T3 cells transfected with DNA isolated from MAMAc-exposed medaka (TR23) was done in order to identify activated oncogenes. Transfected cell DNA was digested with *PstI*, size fractionated on a 20x20cm 0.8% agarose gel and transferred to nitrocellulose. Duplicate sections were hybridized at low stringency (37^{o}C, 35% formamide) to either ^{32}-P labeled *p53* (human, Oncor; lanes 15-20), *c-myc* (the 1.5 kb *EcoRI -PstI* fragment from rainbow trout which contained exons II and III; lanes 9-14) or *K-ras* (from mouse, plasmid pHiHi3; lanes 1-7) probes. No apparent activation of *K-ras* or *myc* was observed. However, two amplified *PstI* bands were observed in digests from TR23-14 and TR23-16, the cholangiocarcinoma and the mixed hepatocellular carcinoma, respectively, which were hybridized to the *p53* probe. These results suggest that the *p53* gene may be amplified or mutated in these tumors. Further experiments to verify this hypothesis are in progress.

6 month samples

DNA was also isolated from MAMAC-exposed fish at 6-months post exposure and analyzed in transfection TR24. The histopathological identity of the livers used in these studies is given in **Table III**. Results of the standard transfection assay are given in **Table IV**. Tumorigenicity studies in nude mice are also reported in **Table V**. Due to the very low efficiency of incorporation of DNA in this test, it was repeated as TR25. Of the seven livers used for DNA isolation and transfection analysis, 5 were reported to have either a cholangiocarcinoma or a hepatocellular carcinoma. Only DNA from the hepatocellular carcinomas were able to induce significant numbers of foci in the Standard Focus Assay. Cells transfected by DNA from AA-92-85-4-3 (cholangiocarcinoma) and AA-92-85-5-4 (hepatocellular carcinoma were able to induce tumors in nude mice in 3-4 weeks (**Table V**). In summary, DNA isolated

from these tumors had relatively low activity in these assays. This could have been due to the very low efficiency of transfection or alternatively, the gene(s) altered by MAMAc may not be readily detectable by this type of assay.

Since the efficiency of this transfection was much lower that expected (data not shown), we repeated these studies as TR25. DNAs used in TR25 were transfected at high efficiencies into the NIH3T3 cells. Histopathological analysis of the tissues used in this transfection is given in **Table VI.** Results from the transfection analysis and tumorigenicity studies in nude mice are reported in **Tables VII and VIII**, respectively. We observed a significant improvement in the results of the Standard Focus Assay where 8/8 DNAs from tumor-bearing livers (cholangiocarcinomas, hepatocellular carcinomas and one mixed type) were able to induce significant numbers of foci. Significant numbers of foci were also seen when dexamethasone was added to the assay medium (5/8 tumor DNAs). This suggests that our first hypothesis was correct - that the low numbers of foci seen in TR23 was due to the very low efficiency of DNA entering the cell. Three of the cell lines transfected with tumor DNA were able to grow in the colony selection assay in low amounts of serum (**Table VII**). Most of the transformed cells were able to induce tumors in nude mice in less than 8 weeks post injection (**Table VIII**).

Secondary transfection assays (TR26) have been completed using DNA isolated from these primary transfectants. The efficiency of DNA transfer in this experiment was excellent. The results of the Standard Focus Assay and the Colony Selection Assay are reported in **Table IX.** Cells transfected with DNA from two primary foci (TR25-8-1-1 and TR25-13-A-1-1) were significantly transformed in both assays. The DNA originally used in the primary transfections came from livers containing both a cholangiocarcinoma and hepatocellular carcinoma. In contrast, cells transfected with DNA from TR25-14-B-2 showed only marginal transformation in both assays. The DNA used to transfect plate TR25-14 in the primary assay was originally isolated from a hepatocellular carcinoma. Although it induced tumors in the primary assay, it appears that either the transformation was not stable in the secondary assay or that this particular focus was not a true transformant. DNA from unexposed medaka controls was negative in the Standard Focus Assay and gave some background in the colony selection assay.

Results of the nude mouse assays are given in **Tables X and XI.** Cells transfected with DNA from one of the cholangiocarcinoma/hepatocellular carcinomas (TR25-8-1-1) was able to induce tumors in 3 of the 4 nude mice, with the earliest onset of only 2.5 weeks. This is indicative of a highly tumorigenic cell line. Cells transfected with DNA from a homologous tumor (TR26A, **Table XI**) also induced tumors in two animals within four weeks post-injection. Unfortunately, the technicians at the nude mouse facility incorrectly calculated the length of time that the animals had been housed (post-injection) and prematurely terminated the experiment at 5 weeks post-injection (We normally consider any tumor that begins by eight weeks post-injection to be a positive response and generally carry out the experiment for a total of twelve weeks post-injection). Cells transfected with DNA originally isolated from the hepatocellular carcinoma also induced tumors in 3 of 4 mice but with a longer latency period (4-5 weeks). Cells transfected with DNA originally from the hepatocellular carcinoma also caused tumors in the nude mice but with a relatively longer latent period. We also observed some background in 2 of the 5 control animals. It is unknown what is causing this high background in control animals. This problem has also appeared in control animals in the exper-

iments of other investigators using the Frederick Facility and is being investigated further. Speculations on the cause of the problem are that it may involve that source of the animals (ie they may be more sensitive to the development of tumors than animals previously obtained from other sources). A second possible cause is the large numbers ($1.5x10^6$) of cells injected into the animals. This is at the high end of the acceptable limit and may contribute to the high level of background tumors observed.

Southern blots were prepared using DNA digests of both primary (TR25) and secondary (TR26) DNA. These were probed both with oncogene sequences and with high molecular weight medaka liver DNA. Hybridization to oncogene sequences was done at low stringency (35% formamide and $37^{O}C$). Since genes of the *ras* family are those most often detected by transfection assays, we hybridized Southerns first to *K-ras* (from the plasmid pHiHi3 containing the mouse *c-ras*, provided by Dr. Tom Shih). As expected, fragments hybridizing to the mouse NIH3T3 *c-ras* are apparent in Pst I digests of all transfectants. Faint bands of approximately 1.9 and 0.9 kb which correspond to the two Pst I fragments in the medaka controls are also present in the transfectant digests. These may be either the medaka *K-ras* homolog or, since the signal was so weak, cross hybridization to another *ras*-related medaka gene.

Hybridization to medaka probes was done at high stringency (50% formamide, 42^{O}). We were unable to establish unambiguously the presence of medaka sequences in these transfectants. Too much background was present on these gels which obscured any bands which might have been present due to areas rich in medaka repetitive sequences. These data are not shown since these gels provided no useful information. In order to improve our assay methods, different restriction digests of medaka liver DNA were run on agarose gels and transferred to nitrocellulose. This Southern was hybridized to a ^{32}-P labeled medaka probe at high stringency. We were able to distinguish distinct bands in each digest which correspond to medaka repeat regions. This should enable us to isolate and identify these repeat regions which can then be used as more specific probes to confirm the presence of medaka sequences in the transformed NIH3T3 cells.

(2) MNNG-exposed animals - Unlike animals exposed to either DEN or MAMAc, who developed primarily liver tumors, the MNNG-treated fish exhibited a wide variety of external lesions. Tumors were detected on the scales, fins, epidermis, gills and in the eyes. Due to the extremely small size of these tumors, very small amounts of DNA were isolated. Histopathology data are given in **Table XII**. A transfection analysis was done using DNA from the larger samples and results are given in **Tables XIII** and **XIV**. Data from the nude mouse assay are reported in **Tables XV and XVI**. Although we looked at several tumor types, the results of both these assays were primarily negative. Low numbers of foci developed in plates transfected with DNA from a basal cell tumor (**Table XII**) and an adenoma (**Table XIV**). Tumors **developed in** nude mice but only after a very long latent period.

(3) DEN-exposed animals - Two studies using DNA isolated from DEN-exposed medaka were initiated to confirm the results of the preliminary experiments with DEN-treated animals. Histopathology data are given in **Tables XVIIA and XVIIB**. Results of primary transfections (TR21 and TR30) are summarized in **Tables XVIII and XIV**, respectively. The numbers of foci observed in the Standard Focus Assay were relatively low. DNA from two DEN-exposed individuals induced significant numbers of foci in NIH3T3 cells. Cells

transfected with DNA from the DEN-exposed fish were also positive in the colony selection assay. Tumorigenicity testing in nude mice using cells from TR21 and TR30 are reported in **Tables XIX and XVI**, respectively.

Results of the soft agar assay using cells expanded from foci isolated from transfection TR21 are summarized in **Table XX**. The numbers of colonies observed were relatively low. However, cells transfected with DNA from DEN-exposed fish # L88-308-4-2 was able to grow in this assay. Preliminary histopathological analysis indicated that tissue from this animal appeared normal.

In a second area, we have focussed our attention on identifying the novel transforming gene responsible for the DEN-induced cholangiocarcinoma (see also Cloning Studies). As part of this study, we investigated whether a positive clone isolated from a genomic library made from the DEN-induced cholangiocarcinoma transformed NIH3T3 cell line had retained biological activity. DNA isolated from the EMBL4 clone C-7 was used to transfect NIH3T3 cells together with carrier calf thymus DNA (**Tables IX; XXI-XXII**). This lambda clone contains a 17 kb insert, has homology to the marker pSV_2neo and presumably contains a novel medaka transforming gene (see Cloning experiments described below). Cells transfected with the construct developed numerous foci, were positive in the Colony Selection Assay (see **Table XXI**) and induced tumors in nude mice (**Table X**) with a short latent period. These results suggest that this clone has retained biological activity. DNA was isolated from these transformed cells and used in a secondary transfection cycle.

In order to determine which portion of this 17 kb clone contains this activity, 13 overlapping Bluescript subclones were transfected into NIH3T3 cells. We hope to be able to identify a portion of this large clone which still retains biological activity and may be more easily sequenced. The C-7 clone was digested with *Xba I*, which generates at least eleven fragments not recognized by EMBL4 or pSV_2neo. These fragments were subcloned into pBluescript and amplified. The plasmid DNA from these subclones and from the Sac 2 and Sac 3 subclones was used to transfect NIH3T3 cells. Analysis of the transfection data indicated that subclones C-7 Xba 6 (2 foci/plate), C-7 Xba 9 (5 foci/plate) and the Sac 2 subclone (2 foci/plate) possessed the ability to transform the NIH3T3 cells (See **Tables XXI-XXII)** Transfection analysis also indicated that the Sac 3 subclone had a high transforming ability (26 foci/plate). However, when the Sac 3 subclone was digested with *Eco R1* and re-subcloned, these fragments exhibited no ability to transform the cells.

C. Cloning experiments

A genomic DNA library was previously prepared in a lambda based vector (EMBL4) and a clone (C7) which contained a 17 kb insert and homology to pSV_2neo was isolated. Original attempts to characterize this clone centered on the sequence analysis and restriction mapping of the portions of the DNA which did not hybridize to the vector DNA (EMBL4) or to the co-transfectant (pSV_2neo). Three large fragments were obtained; the Sac 1 fragment, the Sac 2 fragment and the Sac 3 fragment. The Sac 2 and Sac 3 fragments were an appropriate size to subclone into pBluescript (SK-). These subclones have been mapped and a large portion of the DNA sequenced. Analysis of the nucleotide sequence revealed no significant homology to any gene family for either the Sac 2 or Sac 3 subclones. In order to determine whether this clone retained biological activity, it was transfected into NIH 3T3 cells. As shown

in **Table IX**, DNA from this clone was very active in both the Standard Focus Assay as well as the Colony Selection Assay. In order to localize the biological activity to a smaller unit of the clone, we transfected NIH3T3 cells with DNA from subclones which contained small portions of the C7 clone. **Table XXI** shows that DNA from a SacI clone (S3-4) still retained significant biological activity.

At this time the identity of the transforming gene from DEN-induced cholangiocarcinoma is still unknown. The C7Pst1 Band3 and Band 4 subclones were sequenced by the Sanger dideoxy chain-termination method. According to Dr. McClellan-Green, the postdoctoral fellow who did these experiments, both clones showed significant homology to the *Wnt-1* oncogene. However, we have been unable to confirm this analysis. After further sequencing was done on both fragments, the combined sequence data were analyzed using both Genbank and EMBL data bases. Our search revealed 100% sequence homology to Bluescript (the vector itself) and *E. coli* strain K12 (which was probably the result of bacterial contamination due to improper purification of the plasmid DNA) for the two subclones, respectively (**Figs. 1 and 2**). In spite of repeated attempts, we have not been able to duplicate the original analysis.

D. Gene Expression Studies

Developmental expression

Studies were initiated to examine expression of cellular oncogenes during normal development in the medaka. Total RNA has been isolated from 14 samples of 5 developmental stages of the medaka (see **Table XXIII**). Yield varied from 0.18 to 2.70 mg/gram of tissue. Quality was assessed by Northern analysis which showed the presence of the two expected ribosomal RNA (rRNA) 28 and 18s bands and good quality, high molecular weight mRNA.

Pilot studies on the RT-PCR method were done using the rainbow-trout derived *c-myc* primers. Amplified cDNAs were electrophoresed on agarose gels. Only the G6PDH positive control was visible. The gels were then transferred to nitrocellulose membranes and the resulting Southern blot hybridized to a ^{32}-P labeled *myc* probe. Both the random primer and the specific downstream primer worked to amplify *c-myc* in rainbow trout liver and RTG-2 (a cell line derived from rainbow trout gonad) total RNA. Bands were not visible for the medaka RNA samples from whole fry and adult liver. Random primers worked better than the specific primer. As expected, the RNA from rainbow trout gave a weak signal, since the *myc* gene is expressed at very low levels in the normal adult liver. These results indicate that the technique is working but that the conditions need to be optimized to detect the medaka sequences. We will take two approaches to do this. First, the primer annealing temperature will be decreased to allow for less specific binding. Second, we will try primers made to different regions of the gene.

Cloning of the *p53* gene

A cDNA library was prepared from RNA isolated from over 400 normal adult livers. This will serve as the standard for comparison to genes expressed during different developmental stages. It was screened for the medaka p53 suppressor gene using the trout *p53* clone obtained from Dr. Soussi (Soussi *et al.*, 1990). The entire cDNA clone has been sequenced and compared to other

p53 homologues. *p53* has 5 evolutionarily conserved domains, which are represented in **Fig. 3** by the one letter abbreviations for the amino acids they are predicted to code for. These regions are highly conserved in the medaka relative to both the rainbow trout and humans (as expected).

III. Discussion

Tumor development is a multistage process which may be subdivided into three stages: initiation, promotion and progression. Initiation is characterized by an irreversible change usually in DNA structure. Chemicals which act as initiators are also potent mutagens which react with cellular DNA either directly or after metabolic activation by the cell's biotransformation mechanisms. Although the permanent and heritable characteristics of initiated cells are well established, very little is actually known about the molecular basis of these changes. Specific mutations in proto-oncogenes have been proposed as a key step in this process. The second stage, promotion, is characterized by the reversible expansion of the initiated cell. Many chemicals which function as promoters act via receptor mechanisms, in a dose response manner. During progression, the cellular genome undergoes further irreversible alterations directly related to growth rate. (Pitot, 1990). Examination of these changes at the genetic level is just beginning.

Modern molecular oncology has focused on the interactive roles of two classes of genes involved in tumor development: the cellular oncogenes, dominant cellular genes with key roles in the control of cell growth and differentiation; and suppressor genes, recessive genes which act as negative regulators of cellular proliferation. The mechanisms of activation of cellular oncogenes include point mutations, inappropriate gene expression, chromosomal translocation and gene amplification (Bishop 1987). The functions of these genes have been extensively studied in human and other mammalian tumors, as well as *Drosophila*, *Xenopus* and yeast. Research at the molecular level in teleost fish, however, has lagged far behind. It wasn't until 1986 that the first oncogenes from fish, *ras* (Nemoto *et al.*, 1986) and *myc* (Van Beneden *et al.*, 1986a), were cloned and sequenced. Since that time the field of teleost oncogene research has exploded with efforts concentrated on the roles of these genes in the tumor formation (Van Beneden, in press). The use of fish models promises to provide important contributions to the field of cancer research.

The direct activation of oncogenes by chemical carcinogens was first described in detail in rodent models (Barbacid 1987; Sukumar, 1990). One of the best-defined systems is the activation of H-*ras*-1 in nitroso-methylurea (NMU)-induced mammary carcinoma in Buf/N rats (Sukumar *et al.* 1983) which is characterized by a specific G -> A transition of the second base of the 12th codon. NMU is known to specifically induce such mutations by methylation of the O^6 position of deoxyguanosine. The specificity of this response was supported by later studies (Zarbl *et al.* 1985) which showed that mammary carcinomas induced by dimethylbenz(a)-anthracene (DMBA) do not exhibit the same G -> A transition. DMBA, unlike NMU, forms large adducts with deoxyguanosine and deoxyadenosine that lead usually to excision repair and non-specific point mutations. In the DMBA-induced tumors in which the H-*ras*-1 locus was activated (23%), the mutations were localized to the two deoxyadenosine residues of codon 61. The repeated detection of activated oncogenes such as *ras* in animal tumors induced by

specific chemical carcinogens has important implications on the biological significance of oncogene activation in human cancers. The reproducible detection of specific transforming genes in animal model systems strongly suggests that these oncogenes have a significant role in development of certain tumors and that the mechanisms of carcinogenesis show remarkable conservation throughout phylogenetically distant models. This conservation at the molecular level validates the use of animal systems as models for carcinogenesis.

A. Transfection Analysis

MAMAc-Exposed Animals - MAMAc is the stable aqueous form of methylazoxymethanol (MAM), the active carcinogenic component of the naturally occurring glucoside carcinogen cycasin. MAMAc appears to be metabolically activated in tissues by esterases and NAD-dependent dehydrogenases (Grab *et al.*, 1977). The carcinogenicity of MAMAc in higher animals is well documented (Zedeck *et al.*, 1977; Sieber *et al.*, 1980). MAMAc has also been reported in previous studies to induce tumors in fish (Aoki and Matsudaira, 1981; Hawkins *et al.*, 1986; Fournie *et al.*, 1987; Van Beneden *et al.*, 1990).

The identification of the transforming gene detected in the MAMAc-induced tumors is still unknown. We have been able to successfully transform NIH3T3 cells with DNA from MAMAc-induced medaka tumors. DNA from the hepatocellular/cholangiocarcinomas was the most effective in causing transformation. Southern blots on the restriction digests of DNA from the transformed cells showed some indication that both the K-*ras* oncogene and the *p53* suppressor gene may be activated and/or amplified in these tumors. However, we have been unable to show that mutations have occurred at the codons known to be "hot spots" in these genes. In order to confirm that the transformation of NIH3T3 cells was due to fish sequences, restriction digests of DNA isolated from transfected cells were analyzed on Southern blots for the presence of fish-specific sequences. The results of these studies were inconclusive. High background obscured any bands suggestive of repetitive sequences which may have been present. We are continuing to develop new and more sensitive methods to analyze the transformed cells.

MNNG-exposed Animals - Unlike MAMAc and DEN, MNNG is a direct-acting carcinogen. The target organ of this carcinogen also varies in this fish. It appears to affect a number of organs, including gills, skin, eyes and scales. Transfection studies using DNA isolated from these tumors detected almost no transforming ability. There may be several explanations for these results. The most probable is that due to the very small size of the tumors, we were transfecting only one tenth of the amount of DNA that we normally need to detect changes in single copy genes. Molecular analysis of these MNNG-induced tumors is still in progress, using PCR analysis. We have been able to detect the *p53* gene but not mutations in the fifth conserved DNA-binding domain, which is a hot spot for mutational events in humans and rodents.

DEN-exposed Animals - DEN is one of the most potent and extensively studied mammalian liver carcinogens. Metabolic activation of DEN via α-hydroxylation results in an electrophilic metabolite which is able to ethylate a variety of sites in DNA. In a recent study (Stowers *et al.*, 1988), DNAs isolated from DEN-induced tumors in $B6C3F_1$ mice and Fisher 344 rats were examined for the presence of activated cellular oncogenes using a transfection technique similar to the one described here. Somewhat unexpectedly, the

incidence of activated *ras* oncogenes detected (14/33) in B6C3F1 mouse liver tumors was significantly lower than reported for other chemically-induced mouse liver tumors. The authors suggested that it is probable that multiple pathways exist for the formation of liver tumors in this strain of mouse. Activation of the H-*ras* oncogene may be one event in some but not all of these pathways. In contrast, DNA isolated from only one of the Fisher 344 rats was able to produce foci in NIH3T3 cells. These results were supported by data from previous studies which reported that *ras* activation was not consistently observed in tumors in Fisher rats induced by a variety of chemicals.

DEN has been used to induce a variety of tumors, also primarily of hepatic origin, in several species of fishes (Park and Kim, 1984; Schultz and Schultz, 1988; Grizzle and Thiyagarajah, 1988; Lee *et al.*, 1989; McCarthy *et al.*, 1991). Activated *ras* oncogenes have been detected by transfection analysis of DNA from several fish tumors. Other studies of molecular analysis of DEN-induced tumors in fish have not been reported. The gene detected in the DEN-induced cholangiocarcinoma does not appear to be homologous by Southern blot analysis to any of the known oncogenes that were used as probes. Sequence data to date support this conclusion. This strongly suggests that it may be a novel oncogene. This supports the conclusions of Stowers *et al.* (1988) of the existence of multiple pathways which do not involve the activation of *ras* genes. Work to determine the identity of this transforming gene is discussed below in the Cloning Studies section.

C. Cloning Studies

At this time the identity of the transforming gene from DEN-induced cholangiocarcinoma is still unknown. We were very disappointed that we were unable to validate the earlier findings reported by our laboratory that this gene appeared to have *wnt-1* homology. Analysis of the data collected at the Duke Laboratory indicated that two separate subclones of the C-7 clone possessed sequence homology to the *wnt-1* oncogene. However, when contiguous regions of the same DNA used at Duke was sequenced at the Dana Farber Cancer Center, no homology was detected. Furthermore, when we re-analyzed the sequence from the Duke study we detected no homology to the *wnt-1* gene. Both the original and the second homology searches were done using Genbank. What we did find was 100% homology to the *E. coli* sequence in one clone and 100% homology to the Bluescript subcloning vector in the second clone.

D. Gene Expression Studies

The genomes of higher organisms contain approximately 100,000 different genes, only a small fraction of which are expressed in each cell. The coordinated regulation of gene expression affects all processes of cell growth and differentiation. Pathological alterations in expression are the basis for many types of cancer. In the context of this study, gene expression may be affected by either a mutation in the regulatory region of a cellular oncogene, mutational inactivation of a suppressor gene or other mechanisms which deregulate gene transcription. Alteration of oncogene/suppressor gene expression has been observed in all stages of chemical carcinogenesis (Pitot 1990) and is believed to play a key role in the conversion of a normal cell to a neoplastic state. Determination of oncogene expression during development will provide the basis for the comparison of alteration in gene expression during tumorigenesis.

CONCLUSIONS

I. Significance of completed work

Results of the transfection analysis of tumor DNA from MAMAc, MNNG and DEN-exposed fish suggest that, like mammals, fish tumors have activated transforming genes which are able to transform NIH3T3 mouse fibroblasts *in vitro*.

Previous studies of a DEN-induced cholangiocarcinoma had indicated that a novel oncogene may have been activated in this tumor. Cloning and sequence analysis of this gene has not yet revealed significant homology to known genes. These preliminary findings are in support of our hypothesis that a novel transforming gene has been activated in the cholangiocarcinoma.

Analysis of the MAMAc-exposed fish is still in progress. The transfection data indicate that DNAs isolated from both a cholangiocarcinoma and a mixed cholangiohepatocellularcarcinoma are able to transform NIH3T3 cells. Southern analysis of DNA from transformed cells suggests that the suppressor gene, *p53*, may be amplified and that the *K-ras* gene may be activated. Further studies should indicate the molecular basis of these chemically-induced tumors.

The *p53* gene sequence has been determined for the normal gene. We are continuing to investigate whether mutations appear in any of the tumor samples.

II. Recommendations for future work

While our data provide a firm foundation for further studies of the molecular basis of tumorigenesis in the medaka, early termination of the contract prevented us from finishing all of our proposed tasks. It is recommended that the work continue along the directions detailed below. In addition, we suggest that the study be expanded to include suppressor genes and exposure to aquatic carcinogens. Suggested aquatic carcinogens include trichloroethylene, polycyclic aromatic hydrocarbons, polychlorinated biphenyls or dioxin-related compounds. Future studies should include exposure to more than one carcinogen, i.e. both an initiator and a promoter.

In order to complete this study, the following specific tasks will be addressed: (1) Continue analysis of MAMAc, MNNG- and DEN-exposed fish using both Southern analysis and PCR amplification and direct sequencing techniques; (2) begin again the sequence analysis of the C-7 clone; (3) continue to characterize the medaka *p53* gene; (4) continuation of oncogene expression studies during normal development of the medaka; (5) characterization of mutations in the medaka *p53* gene.

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TABLE I

Identification of tumors in livers of MAMAc-exposed fish used in transfection TR23[1]

Fish	Histopathology
Medaka controls	
AA-91-351-1-1	ND
AA-91-351-1-6	hepatocellular vaculation
AA-91-351-1-11	ND
AA-91-351-1-18	ND
MAMAc-exposed medaka	
AA-91-351-5-18	spindle cell proliferation cyst degeneration hepatocellular vaculation, mild one vaculated hepatocyte locus
AA-91-351-4-18	hepatocellular vaculation moderately severe bile duct hyperplasia
AA-91-351-5-19	cholangiocarcinoma
AA-91-351-4- 4	mixed hepato-cholangiocarcinoma
AA-91-351-4-17	cholangiocarcinoma
AA-91-351-4-21	hepatocellular vaculation, moderate bile duct hyperplasia
AA-91-351-5-1	ND

[1]Fish were sacrificed at 3 months post-exposure.
ND, not done

TABLE II

Transfection analysis of liver DNA from MAMAc-exposed medaka (TR23)[1]

DNA Source	Standard Focus Assay (# foci / μg DNA)	Colony Selection Assay[2] (QBSF + 0.1% serum)
Calf thymus	0	+
Medaka controls		
AA-91-351-1-1	0	ND
AA-91-351-1-6	0.07	ND
AA-91-351-1-11	0	++
AA-91-351-1-18	0	0
MAMAc-exposed medaka		
AA-91-351-4-18	0	0
AA-91-351-5-18	0.14	++
AA-91-351-5-19	26.9	++++
AA-91-351-4-4	2.0	+++
AA-91-351-4-17	0	0
AA-91-351-4-21	0	ND
AA-91-351-5-1	0	ND

[1] Fish were sacrificed at 3 months post exposure.
[2] Growth relative to *mos*-transformed NIH3T3 cells: ++++, numerous and large colonies; 0, no growth; ND, not done.

Table III

Histopathology of liver samples used in TR24 (MAMAc-exposed medaka)[1]

Transfection Plate #	DNA	Histopathology
TR24-2-3	CT[2]	-
TR24-6-9	AA-92-85-1-4	hepatocellular vaculation
TR24-10-12	AA-92-85-1-17	moderate cystic degeneration
TR24-13-16	AA-92-85-4-3	cholangiocarcinoma, hepatocellular carcinoma
TR24-17-20	AA-92-85-4-6	cholangiocarcinoma, hepatocellular carcinoma
TR24-21-24	AA-92-85-5-3	hepatocellular carcinoma
TR24-25-28	AA-92-85-5-4	hepatocellular carcinoma
TR24-29-30	AA-92-85-5-7	hepatocellular carcinoma
TR24-31-32[3]	L-88-308-4-4	hepatocellular carcinoma

[1]Fish were sacrificed at 6 months post exposure.
[2]Calf thymus DNA, negative control plate.
[3]This animal was exposed to diethylnitrosamine in a previous experiment. It was included in this study in order to compare the transformation efficiency.

Table IV

Results of Primary Transfection Analysis of DNA from MAMAc-exposed Medaka (TR24)[1]

Plate#	DNA[2]	SFA #Foci/plate	SFA/DEX #Foci/plate
TR24-2	calf thymus	0	0
TR24-3	" "	0	0
TR24-6	hepatocellular	8	2
TR24-7	vaculation	2	0
TR24-8	"	5	0
TR24-9	"	0	0
TR24-10	mod. cystic degen.	0	3
TR24-11	" "	19	0
TR24-12	" "	0	0
TR24-13/14	mixed tumor[3]	0	0
TR24-15/16	" "	0	0
TR24-17/18	cholangiocarcinoma	-	0
TR24-19/20	"	3	0
TR24-21	hepatocellular carcinoma	7	1
TR24-22	" "	1	10
TR24-23/24	" "	0	1
TR24-25/26	hepatocellular carcinoma	0	-
TR24-27/28	" "	1	25
TR24-29/30[4]	hepatocellular carcinoma	0	0
TR24-31/32	" "	10	1

[1] Animals were sacrificed 6 months post exposure.
[2] See previous Table for more complete description.
[3] Cholangiocarcinoma, hepatocellular carcinoma
[4] Tumor induced by exposure to diethylnitrosamine

Table V. Summary of tumorigenicity assay in nude mice (TR24)[1]

Plate #	DNA Source	# tumors/# mice	weeks to onset[2]
TR24-2	Calf Thymus	2/2	(3 wks,5 days)/(4 wks,5 days)
TR24-3	Calf Thymus	0/2	-
TR24-6	AA-92-85-1-4	1/1	4 wks
TR24-7	AA-92-85-1-4	1/1	9 wks, 5 days
TR24-8	AA-92-85-1-4	1/1	9 wks, 5 days
TR24-9	AA-92-85-1-4	0/1	-
TR24-10	AA-92-85-1-17	0/1	
TR24-11	AA-92-85-1-17	0/1	-
TR24-12	AA-92-85-1-17	0/2	-
TR24-13/14	AA-92-85-4-3	0/1	-
TR24-15/16	AA-92-85-4-3	2/2	(3 wks,5 days)+(4 wks,5 days)
TR24-17/18	AA-92-85-4-6	0/1	-
TR24-19/20	AA-92-85-4-6	0/2	-
TR24-21	AA-92-85-5-3	0/2	-
TR24-22	AA-92-85-5-3	0/1	-
TR24-23/24	AA-92-85-5-3	2/2	9 wks, 5 days
TR24-25/26	AA-92-85-5-4	1/1	4 wks, 5 days
TR24-27/28	AA-92-85-5-4	1/1	9 wks, 5 days
TR24-29/30	AA-92-85-5-7	0/1	-
TR24-31/32	L-88-308-4-4	0/1	-

[1] Animals were sacrificed 6 months post exposure. Those designated "AA" were exposed to MAMAc; the animal desigated "L" was exposed to DEN.

[2] Experiment terminated at 10 weeks, 5 days

Table VI. Histopathology of liver samples used in TR25 (MAMAc-exposed medaka)[1]

Transfection Plate #	DNA	Histopathology
TR25-2-3	CT	-
TR25-4	AA-92-85-1-4	hepatocellular vaculation
TR25-5	AA-92-85-1-17	moderate cystic degeneration slight/mild hepatocellular vaculation
TR25-6-9	AA-92-85-4-3	cholangiocarcinoma, hepatocellular carcinoma
TR25-10-13	AA-92-85-4-6	cholangiocarcinoma, hepatocellular carcinoma
TR25-14-15	AA-92-85-5-3	hepatocellular carcinoma
TR25-16-17	AA-92-85-5-4	hepatocellular carcinoma
TR25-18-19	AA-92-85-5-7	hepatocellular carcinoma
TR25-20-21	L-88-308-4-4	hepatocellular carcinoma
+TR25-22-25	TR23-14-1-SFA	cholangiocarcioma
+TR25-26-29	TR23-16-1-SFA	mixed hepatocholangiocarcinoma

[1]Animals were sacrificed 6 months post exposure.
+secondary transfection of AA-91-351-5-19 and AA-91-351-4-4, respectively

Table VII. Summary of Transfection TR25 (MAMAc-exposed medaka) [1]

Plate #	DNA	SFA (#foci/plate)	SFA/DEX[2] (#foci/plate)	QBSF + 0.1% serum[3] (relative growth)
TR25-(2-3)	calf thymus	0	0	0
TR25-(4-5)	unexposed control	0.25	0	
TR25-(6-9)	cholangiocarcinoma, hepatocellular carcinoma	0.29	2.4	
TR25-(10-13)	cholangiocarcinoma, hepatocellular carcinoma	6.5	1.4	0
TR25-(14-15)	hepatocellular carcinoma	2.0	0	+
TR25-(16-17)	hepatocellular carcinoma	1.5	0	0
TR25-(18-19)	cholangiocarcinoma	0.7	0	0
TR25-(20-21)	hepatocellular carcinoma	1.5	0.5	0
TR25-(22-25)	cholangiocarcinoma	7	1.1	+1/2
TR25-(26-29)	mixed hepatocholangio carcinoma	9.75	2.4	++1/2

[1] Animals were sacrificed 6 months post exposure.
[2] Dexamethasone was added to these plates.
[3] Only a few plates in each group were examined. In the colony selection assay, all QBSF plates (containing no serum) were discarded early in the experiment due to bacterial contamination.

Table VIII
Summary of tumorigenicity assay in nude mice (TR25)
(MAMAc-exposed medaka)

Plate #	DNA Source	# tumors/# mice	weeks to onset
TR25-2-3	Calf Thymus	0/2	-
TR25-4	AA-92-85-1-4	1/1	7.5 wks
TR25-5	AA-92-85-1-17	1/1	5.5 wks
TR25-6	AA-92-85-4-3	0/1	-
TR25-7	AA-92-85-4-3	1/1	4 wks
TR25-8	AA-92-85-4-3	1/1	5 wks
TR25-9	AA-92-85-4-3	0/1	-
TR25-10	AA-92-85-4-6	1/1	7 wks
TR25-11	AA-92-85-4-6	1/1	7 wks
TR25-12	AA-92-85-4-6	0/1	-
TR25-13	AA-92-85-4-6	1/1	6.5 wks
TR25-14	AA-92-85-5-3	1/1	7.5 wks
TR25-15	AA-92-85-5-3	2/2	4.6 wks + 8.4 wks
TR25-16	AA-92-85-5-4	0/1	-
TR25-17	AA-92-85-5-4	2/2	7.4 wks + 5 wks
TR25-18	AA-92-85-5-7	0/1	-
TR25-19	AA-92-85-5-7	1/1	7.4 wks
TR25-20	L88-308-4-4	0/1	-
TR25-21	L-88-308-4-4	0/1	
TR25-22	TR23-14-1-SFA	1/1	6 wks
TR25-23	TR23-14-1-SFA	1/1	
TR25-24	TR23-14-1-SFA	1/1	7.4 wks
TR25-25	TR23-14-1-SFA	1/1	4 wks
TR25-26	TR23-16-1-SFA	1/1	7.4 wks
TR25-27	TR23-16-1-SFA	0/1	-
TR25-28	TR23-16-1-SFA	0/1	-
TR25-29	TR23-16-1-SFA	1/1	5.4 wks

Experiment terminated at 8 weeks

Table IX
Transfection TR26: Secondary Transfection of DNA from MAMAc-exposed Medaka

Plate #	DNA Source[1]	Standard Focus Assay (#foci/plate) DMEM	DMEM/DEX	Colony Selection Assay QBSF + 0.1% serum
TR26-2/3	calf thymus	0	0	0
TR26-4	unexposed control (TR25-4-1)	0	0	7
TR26-(6-8)	cholangiocarcinoma/ hepatocellular carcinoma (TR25-8-1-1)	>24	17	6
TR26-(9-12)[2]	cholangiocarcinoma hepatocellular carcinoma (TR25-13-A-1)	tmtc	tmtc	12
TR26-(15-18)	hepatocellular carcinoma (TR25-14-B-2)	1	1	1
TR26-19[3]	C-7 (EMBL4 clone from DEN-induced cholangiocarcinoma)	24	14	10

[1]DNA was isolated from either expanded focus or colony cells from primary transfection TR25

[2] tmtc-too many to count. These cells run as transfection number TR26A.

[3] DNA isolated from clone C-7 which contains a putative transforming gene from a DEN-induced cholangiocarcinoma.

TABLE X

ANIMAL INOCULATIONS

Cell Line	9/14/92	9/21/92	9/28/92	10/5/92	10/7/92	10/12/92	10/19/92	10/26/92	11/2/92	11/9/92	11/16/92
IR26-2	-	-	-	+	+	+	+	H**			
IR26-3	-	-	-	Died							
IR26-4	-	-	-	-	-	-	+	+	+	+	H**
IR26-5-1	-	-	-	-	-	-	-	-	-	+	+
IR26-5 2	-	-	-	-	-	-		-	-	-	+
IR26-6	-	-	-	-	-	-	+	+	+(H,11/6)	H**	
IR26-7	-	-	-	-	-	-	-	-	-	+	+
IR26-8-1	-	-	+	+	+	+	H**				
IR26-8-2	-	-	-	+	+	+	H**				
IR26-9-10	-	-	-	-	-	-	-	-	-	-	-
IR26-13	-	-	-	-	-	-	-	+	+	+	H**
IR26-14-1	-	-	-	-	-	-	-	-	-	-	-
IR26-14-2	-	-	-	-	-	-	-	-	-	-	-
IR26-15	-	-	-	-	-	-	-	-	-	-	-
IR26-16	-	-	-	-	-	-	+	+	+(H,11/6)		
IR26-17	-	-	-	-	-	-	+	+	+	+	+
IR26-18	-	-	-	-	-	+	+	+	+(H,11/6)		
IR26-19-1	-	-	-	-	-	+	+	+	+(H,11/6)		
IR26-19-2*	-	-	-	-	-	+	+	H**			

\+ Tumor observed
\- No tumor

*** Injected 9/10; all others injected 9/8 with 1-3 x 10^6 cells/mouse.**

****Tumor harvested.**

TERMINATED 11/21/92.

TABLE XI

ANIMAL INOCULATIONS[1]

Cell Line	10/15/92	10/19/92	10/26/92	11/2/92	11/9/92	11/16/92	11/23/92
TR26A-2A	-	-	-	-	-	-	-
TR26A-2B	-	-	-	-		-	-
TR26A-3A	-	-	-	-	-	-	-
TR26A-3B	-	-	-	-	-	-	-
TR26A-4A	-	-	-	-	-	-	-
TR26A-4B	-	-	-	-	+	+	+
TR26A-5A	-	-	L*	L*		L*	
TR26A-5B	-	-	-	-	-	-	-
TR26A-6A	-	-	-	-	-	-	-
TR26A-6B	-	-	-	-	-	-	-

[1]Injected 10/15/92 @ 1-3 x 10^6 cells/mouse.

*Lump observed in groin area.
+ Tumor observed
- No tumor

Table XII
Histopathology of Tissues from MNNG-exposed Animals

Animal Number	Tissue	Pathology
BB-92-350-5-2	skin	sarcoma
BB-254-4-2	gills	sarcoma
BB-92-254-4-3	skeletal muscle	hemangiosarcoma
BB-92-006-4-1	gill	epithelial hyperplasia
BB-92-350-4-2	skin	squamous cell papilloma
BB-93-350-5-1	skin	cystic basal cell tumor
BB-93-54-5-6	abdominal mass	follicular cell adenocarcinoma (metastasized from the thyroid)
BB-92-116-5-2	red area on head	lepidocytoma
BB-93-54-5-19	gill	rhabdomyosarcoma

Table XIII

Summary: TR29, Primary transfection of DNA from MNNG-exposed animals

Plate #	DNA Source	#foci/plate	
		SFA	SFA/DEX
TR29-(2-3)	calf thymus	0	0
TR29-4	sarcoma	0	0
TR29-5	sarcoma	0	0
TR29-6	hemangiosarcoma	0	0
TR29-7	hyperplasia	0	0
TR29-8	papilloma	0	0
TR29-9	basal cell tumor	0	7
TR29-(10-11)	MD liver (unexposed controls)	2	0

Details of the histopathology are presented in Table XII.

Table XIV

TR30: Summary of Transfection Data: NIH3T3 Cells transfected with DNA from MNNG- and DEN-exposed Medaka

Plate #	DNA Source	#foci/plate SFA	#foci/plate SFA/DEX
TR30-2	Calf thymus	0	0
MNNG-exposed animals			
TR30-3	adenoma	18	12
TR30-4	"	1	23
TR30-5	squamous cell carcinoma	0	0
TR30-6	lepidocytoma	0	-
TR30-7	rhabdomyosarcoma	0	-
DEN-exposed animals			
TR30-8	vaculation	0	0
TR30-9	mod. vaculation	0	-
TR30-10	" "	0	-
TR30-11	mod/severe vaculation	0	-
TR30-12	cholangiocarcinoma	0	-
TR30-13	" "	0	-
TR30-14	adenoma	11	-
TR30-15	"	4	0
TR30-16	"	1	-
TR30-17	medaka liver control	0	0
TR30-18	" " "	0	0
TR30-19	medaka scales/skin control	0	0

Details of the histopathology are presented in Tables XII and XVII.

TABLE XV

Summary: TR29 Tumor Induction in Nude Mice by cells transfected with DNA from MNNG-exposed medaka and C-7 subclones

Plate #	DNA Source	# tumors/#mice	wks to onset
TR29-(2-3)	calf thymus	1/2	5.9 wks
MNNG-exposed Animals			
TR29-4	lepidocytoma	1/1	5.9 wks
TR29-5	sarcoma	1/1	4.9 wks
TR29-6	hemangiosarcoma	0/1	
TR29-7	hyperplasia	0/1	
TR29-8	papilloma	0/1	
TR29-9	cystic basal cell tumor	0/1	
TR29-(10-11)	MD liver (unexposed)	0/2	
C-7 Subclones			
TR29-(12-13)	Bluescript	0/2	
TR29-(14-15)	S2Pst 2	1/2	3.9 wks
TR29-(16-17)	S3Xba2	0/2	
TR29-(18-19)	S3Eco1	0/2	
TR29-20	S3Eco2	0/1	

Tumors appearing after 6 weeks were considered background.

Plates 4-9 contain DNA from MNNG-exposed animals.

Plates 12-20 were transfected with DNA from C-7 plasmid subclones, and the vector Bluescript.

Table XVI

TR30: Results from the Nude Mouse Assay: NIH3T3 Cells transfected with DNA from MNNG- and DEN-exposed Medaka

Plate #	DNA Source	#tumors/ #mice injected	weeks to onset
MNNG-exposed animals			
TR30-5	squamous cell carcinoma	1/1	11
TR30-6	lepidocytoma	1/1	13.1
TR30-7	rhabdomyosarcoma	1/1	8.3
DEN-exposed animals			
TR30-9	mod. vaculation	0/1	-
TR30-10	" "	0/1	-
TR30-11	mod./severe vaculation	1/1	12
TR30-12	cholangiocarcinoma	1/1	12
TR30-13	" "	1/1	8
TR30-14	hepatocellular adenoma	1/1	11
TR30-16	" "	0/1	-
TR30-17	medaka liver control	0/1	-
TR30-18	" " "	1/1	10
TR30-19	medaka scales/skin control	0/1	-

Liver tissue was used in all DEN-exposed animals.

Table XVIIA

Histopathology of DEN-exposed animals used in TR30

Animal #	Tissue Source	Pathology
CC21-29	liver	Slight hepatocellular vaculation
CC21-24	liver	Moderate hepatocellular vaculation
CC21-19	liver	Mod. Severe hepatocellular vaculation
CC21-13	liver	Cholangiocarcinoma
CC21-38	liver	Hepatocellular adenoma

Table XVIIB

Identification of tumors in livers of DEN-exposed fish used in transfection analysis (TR21)

Fish	Histopathology[1]
Medaka controls	
L88-308-2-3	normal
DEN-exposed medaka	
L88-308-4-2	normal
L88-308-4-4	hepatocellular carcinoma
L88-308-4-6	questionable
L88-308-4-8	cholangiocarcinoma
L88-308-4-10	questionable
K89-046-2-1	not examined
K89-046-3-6	not examined

[1]Pathology of samples was evaluated by Dr. Marilyn Wolfe, Experimental Pathology Laboratory, Inc., Herndon, VA. In two samples (4-6 and 4-10), the presence of neoplastic tissue was questionable. Samples K89-046-2-1 and K89-046-3-6 were not evaluated.

Table XVIII

Primary transfection of NIH3T3 cells with DNA from DEN[1]-exposed medaka (TR21): Growth of G418-selected cells in a standard focus assay (SFA) and colony selection assay

DNA Source	SFA (average # foci/plate)	Colony Selection (QBSF[2] + 0.1% serum)[3]
calf thymus	0.3	0
mos-transformed cells	ND	++++
medaka (untreated)	0.5	+1/2
DEN-exposed medaka		
L88-308-4-2	6	++
L88-308-4-6	11	++++
L88-308-4-8	1	ND
L88-308-4-10	1.5	0
K89-046-2-1	2	0
K89-046-3-6	1.5	+

[1]diethylnitrosamine
[2]Quality Biologicals Serum-free media
[3]Growth of colonies is measured relative to positive control cells (*mos*-transformed NIH3T3 cells); +++, growth similar to positive controls; 0, no growth observed.
ND - not done.

Table XIX

Results of Tumorigenicity Assay in Nude Mice: Primary transfection of DNA from DEN-exposed animals (TR21)

Pathology	#tumors/#mice injected	time[1]
normal medaka	1/4	7 wks
questionable[2]	1/3	9 wks
cholangiocarcinoma	0/1	--
questionable[2]	0/1	--
not examined	1/2	5.8 wks

[1]Time indicates the number of weeks from the day injected to the onset of tumor development.

[2]The presence of neoplastic tissue in these samples was questionable.

Table XX

Soft agar assay of NIH3T3 cells transfected with DNA from DEN-exposed medaka (TR21)

DNA Source	Relative Growth[1]
NIH3T3	0
mos-transformed cells	++++
Calf thymus	0
normal medaka	0
questionable neoplasm	1/2+
cholangiocarcinoma	0
questionable neoplasm	0
not examined	0
not examined	0

[1]Growth of colonies is measured relative to positive control cells (*mos*-transformed NIH3T3 cells); ++++, growth similar to positive controls; 0, no growth observed.

[2]The presence of neoplastic tissue in these samples was questionable.

TABLE XXI

Summary: TR28 transfection of subclones of Lambda clone C-7

Plate #	DNA Source	#foci/plate SFA	#foci/plate SFA/DEX
TR28-(2-3)	calf thymus	0	0
TR28-(4-5)	TR10	20	0
TR28- 6	TR14	0	0
TR28-(8-9)	Bluescript	0	0
TR28-(10-11)	C-7 Xba7	0	0
TR28-(12-13)	C-7 Xba9	5	1
TR28-(14-15)	C-7 Xba10	0	2
TR28-(16-17)	C-7 Xba6	2	0
TR28-(18-19)	C-7 Xba5	0	0
TR28-(20-21)	C-7 Xba4	0	0
TR28-(22-23)	C-7 Xba8	0	0
TR28-(24-25)	S3-4	26	4
TR28-(26-27)	S2-2	2	0

Plates 10-27 were transfected with DNA isolated from subclones (in plasmid Bluescript) from lambda clone C-7.

Table XXII
Summary: TR29, primary transfection of DNA from C-7 subclones

Plate #	DNA Source	#foci/plate	
		SFA	SFA/DEX
TR29-(2-3)	calf thymus	0	0
TR29-(12-13)	Bluescript	0	0
TR29-(14-15)	S2Pst2	5	0
TR29-(16-17)	S2Xba2	0	0
TR29-(18-19)	S3Eco1	0	0
TR29-20	S3Eco2	-	0

TABLE XXIII

Isolation of total RNA from Different Developmental Stages of Medaka

0 day eggs	
0.30 ug/ul	155 ug RNA/g tissue
0.76 ug/ul	633 ug/g
1.12 ug/ul	-
2 day eggs	
0.88 ug/ul	138 ug/g
0.97 ug/ul	-
0.23 ug/ul	-
5 day eggs	
not done	
7 day eggs	
1.90 ug/ul	413 ug/g
1 day fry	
0.74 ug/ul	185 ug/g
1.80 ug/ul	-
3.90 ug/ul	-
14 day fry	
1.90 ug/ul	2,700 ug/g
8.40 ug/ul	
1.99 ug/ul	
5.07 ug/ul	
Adult	
not done	

Concentration values are given for each pooled sample.
[-] indicates that these values are not available.

Figure legends

Fig. 1

Sequence identification of Clone 7 Pst I band 3 subclone

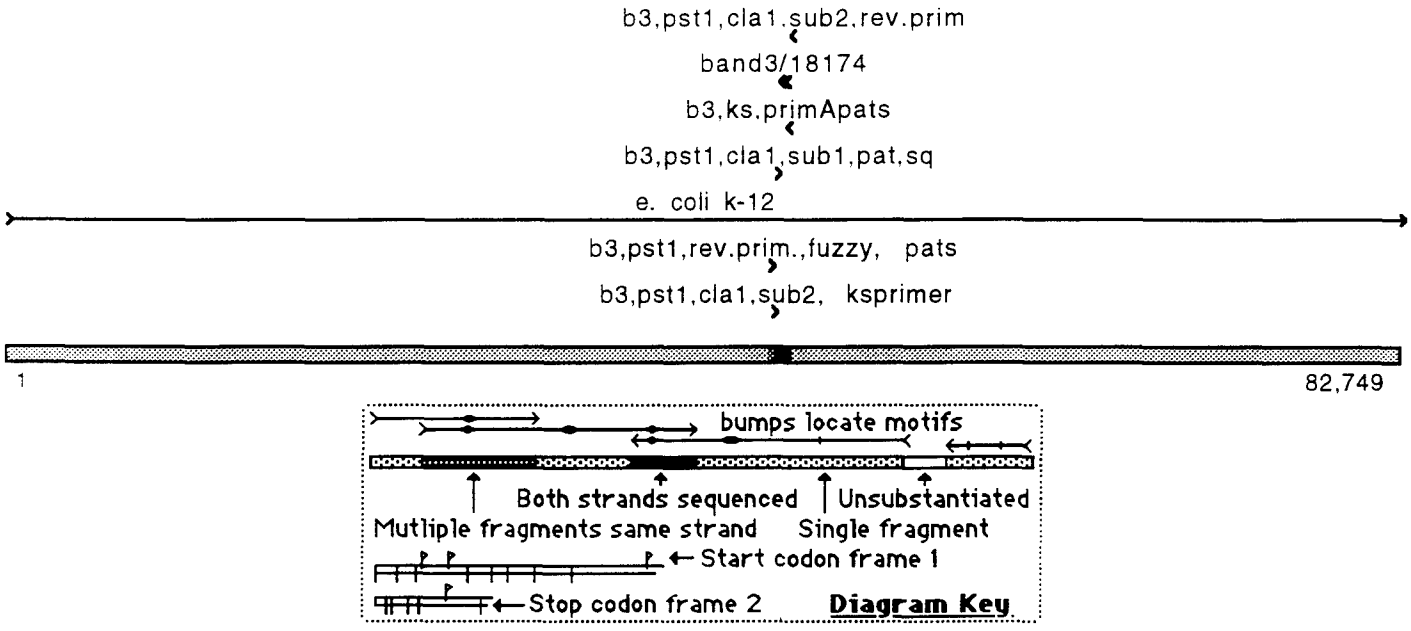
Fig. 2

Sequence identification of Clone 7 Pst I band 4 subclone

Fig. 3

[....] indicates other amino acids in the sequences between the conserved domains. Each dot indicates one amino acid; [----] indicates amino acid sequence identity (predicted from the cDNA nucleotides) to human and zebrafish;[*] indicates that either a deletion or insertion of a codon has occurred; italicized Roman numerals number the domains; the numbers at the ends of the lines correspond to the number of the last amino acid in that line for each of the 3 species. #1 is the start codon. Letters used are the standard, universal one-letter abbreviations for amino acids. A Table is attached as the final page of this document which defines these symbols.

Fig. 1


b3,pst1,cla1,sub2,rev.prim
band3/18174
b3,ks.primApats
b3,pst1,cla1,sub1,pat,sq
e. coli k-12
b3,pst1,rev.prim.,fuzzy, pats
b3,pst1,cla1,sub2, ksprimer
1
82,749

bumps locate motifs
Both strands sequenced
Unsubstantiated
Mutliple fragments same strand
Single fragment
Start codon frame 1
Stop codon frame 2
Diagram Key

Fig. 1 cont.


e. coli k-12 CTGACCGCCG TCCATATCAG CAGAAGCAGT ACCTGCAACC ATCGCCAGCA
b3,pst1,rev.pr… CTGA
b3,pst1,cla1,s… CTGACCGCCG TCCATATCAG CAGAAGCAGT ACCTGCAACC ATCGCCAGCA
#45401
CTGACCGCCG TCCATATCAG CAGAAGCAGT ACCTGCAACC ATCGCCAGCA
b3,pst1,cla1,s… TTTTT TAGACATAAA AATCCTTTAA
e. coli k-12 TAAGACCAGA CAGGGCAAAA CCTAATTTTT TAGACATAAA AATCCTTTAA
b3,pst1,cla1,s… TAAGACCAGA CAGGGCAAAA CCTAATTTTT TAGACAT
#45451
TAAGACCAGA CAGGGCAAAA CCTAATTTTT TAGACATAAA AATCCTTTAA
b3,pst1,cla1,s… TAAAATTCCA TTAGTCAGAC TACATGTTTG AAGAATGACT ATTCATGACA
e. coli k-12 TAAAATTCCA TTAGTCAGAC TACATGTTTG AAGAATGACT ATTCATGACA
#45501
TAAAATTCCA TTAGTCAGAC TACATGTTTG AAGAATGACT ATTCATGACA
b3,pst1,cla1,s… CAAATAGGAG AAACAAATGT TAGATATTAA TGAGCAATGA TATTTGTTAC
e. coli k-12 CAAATAGGAG AAACAAATGT TAGATATTAA TGAGCAATGA TATTTGTTAC
#45551
CAAATAGGAG AAACAAATGT TAGATATTAA TGAGCAATGA TATTTGTTAC
b3,pst1,cla1,s… CCAAATTTAC AACCATTGTT CATTAGGTCG CCTATTGTGC ACTTTAGAAG
e. coli k-12 CCAAATTTAC AACCATTGTT CATTAGGTCG CCTATTGTGC ACTTTAGAAG
#45601
CCAAATTTAC AACCATTGTT CATTAGGTCG CCTATTGTGC ACTTTAGAAG
b3,pst1,cla1,s… CTTTTGAACA AATTAAATTT ACTTAATTCA AAA
e. coli k-12 CTTTTGAACA AATTAAATTT ACTTAATTCA AAATTAAGTA AAAATAAGTT
#45651
CTTTTGAACA AATTAAATTT ACTTAATTCA AAATTAAGTA AAAATAAGTT
band3 18174 CA ATTGGTTAGG GTATGGAGAT GGGATTGATA TTTATTTATA
e. coli k-12 CACAAGTGCA ATTGGTTAGG GTATGGAGAT GGGATTGATA TTTATTTATA
#45701
CACAAGTGCA ATTGGTTAGG GTATGGAGAT GGGATTGATA TTTATTTATA

Fig. 2

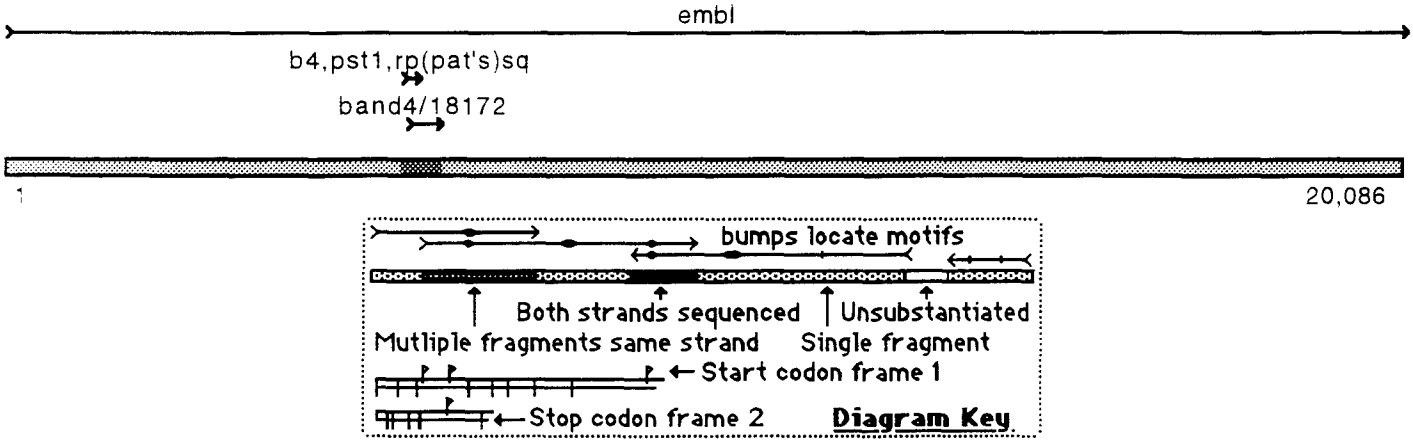

embl
b4,pst1,rp(pat's)sq
band4/18172
1
20,086
bumps locate motifs
Both strands sequenced
Unsubstantiated
Mutliple fragments same strand
Single fragment
Start codon frame 1
Stop codon frame 2
Diagram Key

Fig. 2 cont.


embl
ACGTGGTGCC AGCGACGGAG GGCGAGAACG CCAGCGCGGC
#5401
ACGTGGTGCC AGCGACGGAG GGCGAGAACG CCAGCGCGGC
embl
GCAGCCGGAC GTGAACGCGC AGATCACCGC AGCGGTTGCG
#5441
GCAGCCGGAC GTGAACGCGC AGATCACCGC AGCGGTTGCG
embl
GCAGAAAACA GCCGCATTAT GGCGATCCTC AACTGTGAGG
#5481
GCAGAAAACA GCCGCATTAT GGCGATCCTC AACTGTGAGG
embl
AGGCTCACGG ACGCGAAGAA CAGGCACGCG TGCTGGCAGA
#5521
AGGCTCACGG ACGCGAAGAA CAGGCACGCG TGCTGGCAGA
embl
AACCCCCGGT ATGACCGTGA AAACGGCCCG CCGCATTCTG
#5561
AACCCCCGGT ATGACCGTGA AAACGGCCCG CCGCATTCTG
embl
GCCGCAGCAC CACAGAGTGC ACAGGCGCGC AGTGACACTG
#5601
GCCGCAGCAC CACAGAGTGC ACAGGCGCGC AGTGACACTG
embl
CGCTGGATCG TCTGATGCAG GGGGCACCGG CACCGCTGGC
b4,pst1,rp(pat...
#5641
CGCTGGATCG TCTGATGCAG GGGGCACCGG CACCGCTGGC
embl
TGCAGGTAAC CCGGCATCTG AT:GCCGTTA ACGATTTGCT
b4,pst1,rp(pat...
TGCAGGTAAC NCGGCATCTG ATTNCCGTTA ACGATTTGCT
#5681
TGCAGGTAAC CCGGCATCTG ATTGCCGTTA ACGATTTGCT

Fig.3

COMPARISON OF PREDICTED AMINO ACID SEQUENCES of p53 CONSERVED DOMAINS

```
                  I
medaka      PESQGSF.....................................................  70
trout       -L--E--                                                       66
human       -L--ET-                                                       78

                                                        II
medaka      ..........................................SGTAKSVTSTYSETLNKLYCQLAKTSP  134
trout                                                 -*----------D----------C-  130
human                                                 *G------C---PA---M-------C-  142

                                          III
medaka      ..............................DVVRRCHHHQN.................................  205
trout                                     ----------S                                 206
human                                     E--------ER                                 217

                                      IV                                    V
medaka      ..................LSYMCNSSCMGGMNRRPILTILTLE..........FEVRICACPGRDRRTEE  274
trout                         YNF------------------I---          ----V--------K---  275
human                         YN------------------I---           ----V------------  286
```

Amino Acids

Amino acid	Single-letter code	Triple-letter code	Molecular weight (pH 7)	Side chain pK	α-NH_2 pK	α-COOH pK
Alanine	A	Ala	89		9.87	2.35
Arginine	R	Arg	174	13.2	9.09	2.18
Asparagine	N	Asn	132		8.8	2.02
Aspartic Acid	D	Asp	133	3.65	9.6	1.88
Cysteine	C	Cys	121	8.33	10.78	1.71
Glutamic Acid	E	Glu	147	4.25	9.67	2.19
Glutamine	Q	Gln	146		9.13	2.17
Glycine	G	Gly	75		9.6	2.34
Histidine	H	His	155	6.0	8.97	1.78
Isoleucine	I	Ile	131		9.76	2.32
Leucine	L	Leu	131		9.6	2.36
Lysine	K	Lys	146	10.28	8.9	2.2
Methionine	M	Met	149		9.21	2.28
Phenylalanine	F	Phe	165		9.24	2.58
Proline	P	Pro	115		10.6	1.99
Serine	S	Ser	105		9.15	2.21
Threonine	T	Thr	119		9.12	2.15
Tryptophan	W	Trp	204		9.39	2.38
Tyrosine	Y	Tyr	181	10.1	9.11	2.2
Valine	V	Val	117		9.72	2.29

Antibodies: A Laboratory Manual. Eds. E. Harlow and D. Lane, Cold Spring Harbor Laboratory Press, 1988.