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Richard J Van Benedek 10/28/92

PI - Signature Date

July 6, 1992 Annual Report (6/13/91 - 6/9/92)

Molecular analysis of medaka tumors: New models for carcinogenicity testing

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The broad, long-term objective of our laboratory is to use fish as a model system to better understand factors which promote tumor production and to develop a reliable and sensitive means of detecting carcinogens in aqueous environments. Studies of oncogene activation and tumor production in fish will contribute to our understanding of the molecular basis of carcinogenesis. Increased knowledge of molecular mechanisms of tumor progression in vivo may be used in the development of sensitive systems for the detection of potentially harmful environmental contaminants. In these studies we have examined the role of oncogene activation in tumors in Japanese medaka (Oryzias latipes) induced by specific carcinogens. The medaka has been widely used for carcinogenicity testing in the past decade. In studies described here, we used fish which were exposed to diethylnitrosamine (DEN) and methylazoxymethanol acetate (MAMAc). A significant proportion of exposed animals developed a variety of liver tumors. DNA extracted from these livers was analyzed in transfection assays for the presence of transforming genes. We have also initiated studies on oncogene expression in normal tissue and are continuing the analysis of the sequence of a novel oncogene detected in a DEN-induced cholangiocarcinoma.

chemical carcinogenesis, oncogenes, fish model, aquatic toxicology, lab animals, environmental health, RAIII

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INTRODUCTION

The study of oncogene activation in chemically-induced tumors in the Japanese medaka is part of an ongoing study to evaluate the use of non-mammalian species for carcinogenicity testing. The medaka, which has been used for carcinogenicity testing for well over a decade (Hoover, 1984), is well suited for these studies. Its small size allows treatment of large numbers of animals in a small space which provides statistically relevant numbers. Medaka can be induced to breed year round, provide large numbers of eggs and are easy to culture. Induction of tumors has been reported in nearly every organ by agents known to be carcinogenic to humans. Unlike some strains of rodents now used for testing, the incidence of spontaneous tumor formation in medaka is nearly zero (Hawkins, personal communication). A low incidence of spontaneous lymphomas has been reported in medaka (Battalora *et al.*, 1990).

Examination of changes at the genetic level, however, 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 functions of these genes have been extensively studied in human and other mammalian tumors, *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.*, 1986) - were cloned and sequenced. Since that time the field of teleost oncogene research has virtually exploded (Van Beneden, in press) with efforts concentrated on the roles of these genes in the tumor formation. The use of fish models promises to provide important contributions to the field of cancer research.

The results of the studies reported here 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. The data further indicate that the medaka would be an excellent candidate for the testing of the potentially carcinogenic effects of water-borne toxicants.

BODY

I. EXPERIMENTAL METHODS

A. Tumor induction

Fourteen-day-old medaka fry were exposed to MAMAc at 20mg/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.

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

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). Twenty ug 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) 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 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.

C. Sequence Analysis

A genomic DNA library was previously prepared in a lambda based vector (EMBL4) using DNA from NIH3T3 cells co-transfected with DEN-induced medaka tumor DNA and pSV_2neo DNA. Several positive clones were isolated using ^{32}P-labeled pSV_2neo as a probe. Further screening of this library and investigation into the DNA sequence responsible for the transformation of the NIH3T3 cells has proceeded in two directions.

First, the screening of the genomic library using pSV_2neo has continued. The first screens were performed under low stringency conditions (35% formamide at 37^oC) using PvuII-digested pSV_2neo DNA which was labeled with ^{32}P-dATP and ^{32}P-dCTP by the random primer method (Feinberg and Volgelstein, 1983).

Second, a clone designated as C-7 isolated in a previous screen was examined for the presence of DNA fragments which did not hybridize to either the co-transfected pSV_2neo DNA or to the EMBL4 vector DNA. It is assumed that these fragments would contain a portion of the DNA sequence responsible for the transformation of the NIH3T3 cells. Phage DNA isolated from the C-7 clone was subjected to restriction analysis using various enzymes which do not cut pSV_2neo and which only have 1 or 2 restriction sites in lambda DNA (see Table I). These digests were analyzed on a 1% agarose, 1X TBE gel (Figure 1) and transferred to a nitrocellulose filter. The filter was then hybridized with

the pSV_2neo probe and exposed to film. The probe was stripped from the filter and the filter rehybridized to ^{32}P labeled EMBL4 DNA. The largest fragment which was produced by digestion with the restriction endonuclease SacI was isolated and purified by agarose gel electrophoresis and adsorption to glass beads, using the Gene Clean Kit (Promega). It was then subcloned into dephosphorylated, SacI digested pBluescript KS. Recombinants were selected by a combination of two methods. First transformed XL1-Blue cells were plated on X-gal/IPTG, LB_{amp} plates. White or recombinant colonies were then selected and grown for isolation of DNA. The DNA was then run on an agarose gel and transferred to a nitrocellulose membrane. This was then hybridized using the C-7 fragment and positives selected.

C. Gene expression Analysis

Tissues were collected from different developmental stages of medaka for the isolation of RNA in order to establish the pattern of expression of cellular oncogenes during normal development. Preliminary experiments were done using liver tissue from adult medaka. RNA was isolated from normal medaka adult liver (Chirgwin *et al.*, 1975) and used to prepare a cDNA library. Due to difficulties in isolating sufficient quantities of mRNA, total RNA was used in these initial attempts to make a cDNA library. The cDNA was ligated to *EcoRI* adaptors and ligated into the *EcoRI* site of the cloning vector lambda GT10 (Stratagene).

In other experiments, RT-PCR (reverse-transcriptase polymerase chain reaction) was used to amplify a 157 bp segment of the p53 suppressor gene. Primers were prepared using regions conserved among rainbow trout and higher organisms as templates.

II RESULTS

A. transfection studies

(1). DEN-exposed animals

Studies using DNA isolated from DEN-exposed medaka were initiated to confirm the results of the preliminary experiments with DEN-treated animals. These assays are done as a blind study so that results were correlated with the histopathology only after the transfection analysis was completed (Table II).

Efficiency of the transfection assay was measured by the number of cells which survive drug treatment (i.e. exposure to G418). Cells exposed to G418 (a neomycin analog) will grow only if they have incorporated DNA from the plasmid pSV_2neo. This plasmid contains a neomycin resistance gene and was cotransfected with the fish tumor DNA. Efficiencies of a secondary transfection (TR20) and a primary transfection (TR21) are reported in Tables III and IV respectively. The transfection efficiency of the various fish tumor DNAs is compared to that of a calf thymus DNA standard. As is evident from these data, the transfection efficiencies have greatly improved. In transfection TR21, the numbers of drug-resistant cells containing fish tumor DNA was actually higher than the calf thymus control. This may be attributed both to better quality of DNA as well as modifications in the transfection procedure.

Results of a primary transfection (TR21) using medaka tumor DNA are summarized in Table V. 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 are reported in Table VI. This assay is still in progress for cells from transfection experiment TR21 and we are unable to draw any conclusions at this time. We have recovered tumors from three animals injected with cells from transfection TR16, after a long time of incubation. Two of these were histologically normal. The third (L88-308-4-4) was diagnosed as having a hepatocellular carcinoma.

Results of the soft agar assay using cells expanded from foci isolated from transfection TR21 are summarized in Table VII. 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.

(2) MAMAc-exposed animals

Transfection assays were also done using DNA samples from medaka which had been exposed to MAMAc and sacrificed 3 or 6 months post-exposure. The histopathology of the three month samples is given in **Table VIII**. Results obtained in transfection TR23 are summarized in **Table IX**. Tumorigenicity studies in nude mice are still in progress. 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) is shown in **Figure 3**. 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°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.

DNA was also isolated from MAMAC-exposed fish at 6-months post exposure and analyzed in transfection TR24. **Table X** shows that the efficiency of this transfection was much lower than expected. Results of the standard transfection assay are given in **Table XI**. The histopathological identity of these samples has not yet been determined. Tumorigenicity studies in nude

mice are also still in progress. Due to the very low efficiency of this test, it is currently being repeated.

B. Sequence analysis

The determination of the gene sequence responsible for the transformation of NIH3T3 cells transfected with DEN-induced medaka tumor DNA has been following two lines. The first of these is the continued screening of the genomic library. 67 recombinant clones which hybridize to the co-transfectant pSV_2neo under low stringency conditions were isolated. However, when these clones were rescreened under high stringency conditions (50% formamide at 42°C) no positive clones were detected. Analysis of pSV_2neo DNA used as probe revealed three bands by agarose gel electrophoresis, indicating a reannealing or possibility degradation of the DNA. Attempts to reculture the clone from glycerol stocks were unsuccessful. A new culture of pSV_2neo in pBR322 was obtained and plasmid DNA was isolated on a CsCl gradient. This DNA will be used to continue the screening of the genomic library.

The second aspect of the identification of the transforming gene involves the identification and characterization of the C-7 clone. The clone was digested with the restriction enzyme Sac I and three fragments not hybridizing to either EMBL 4 or pSV_2neo were isolated and subcloned (see **Figures 1 and 2**). The large Sac I fragment, Sacl, is approximately 9-12 kb and was subcloned into pBluescript KS. Because Sacl is near the limit for insert size of the plasmid it has been difficult to maintain the fidelity of the subclone. Therefore, Sacl has been divided into more manageable fragments using the restriction enzymes, Xba I and Bam Hl. Digestion with Xba I resulted in two fragments possessing Xba/Xba ends. These were subcloned and are identified as Xba3 and Xba7.

Xba3 is 533 nucleotides long and has been completely sequenced in one direction and is currently being verified in the reverse direction. The location of this fragment in Sacl is unknown at this time. In addition, it appears that Xba3 contains a characteristic C-A rich region. Data searches of Genebank and EMBL have shown that similar C-A regions have been identified in other fish genes as well as in many mammals and other organisms, most often in the regulatory portion of the genomes. However, no homology greater than 35% was identified to any other reported gene.

Xba 7 is approximately 6.0-6.5 kb and is currently being sequenced in both directions. The 5' end of the Xba7 fragment occurs at base 192-198 of Sacl and will be used to extend the sequence of this fragment. No comparable sequence has been identified in Genebank or EMBL. Digestion with Bam Hl resulted in the subcloning of one fragment with Bam Hl/Bam Hl ends. This subclone, Baml, is approximately 4-5 kb and is currently being sequenced in both directions. The position of this subclone in Sacl is unknown at this time.

The other two Sac I fragments, Sac2 and Sac3 (see **Figure 2**), have been subcloned into pBluescript SK. Sac2 is approximately 3.5-4.5 kb and Sac3 is slightly smaller at approximately 3-4 kb. Both subclones are currently being sequenced in both directions with approximately 1/3 of the sequences identified.

The size of the C-7 gene is approximately 17,000 nucleotides suggesting that only a fragment of the total gene has been isolated. Therefore the library will be screened using the new pSV_2neo DNA preparation. Sequence analysis will also continue utilizing the six subclones of the C-7 gene isolated and inserted into pBluescript SK. Additional experiments will include identification of fish DNA in the transformed 3T3 cells.

C. Gene Expression Studies

Trial packaging experiments indicated that very low numbers of recombinants were present in the cDNA libraries derived from adult liver tissue. This suggests a problem at the ligation step and will require that the experiment be repeated.

Initial attempts to identify expression of the medaka *p53* gene in adult liver using RT-PCR were also unsuccessful. Modifications of the protocols are now in progress. Primers are also being prepared to other known oncogenes, such as *ras*, *myc* and *ets*.

III. Discussion

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 Matsu daira, 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 also still unknown. In order to confirm that the transformation of NIH3T3 cells is due to fish sequences, restriction digests of DNA isolated from transfected cells will be analyzed on Southern blots for the presence of fish-specific sequences. Duplicate Southern blots will also be hybridized to radiolabelled probes of known oncogenes in order to identify activated oncogenes. These studies are in progress.

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 that 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 Schutlz, 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.

CONCLUSIONS

I. Significance of completed work

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

Analysis of DEN-exposed medaka revealed very few tumors in this transfection study. The examination of transfect DNA on Southern blots did not indicate activated oncogene homologs. Studies to date are inconclusive. A second exposure to DEN has just been initiated which will provide more tumor tissue for further transfection analysis. 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. Further studies should indicate the molecular basis of these chemically-induced tumors.

II. Recommendations for future work

It is recommended that the work continue along the directions detailed below. In addition, we suggest that the study include analysis of suppressor genes and expand the chemical exposure studies to include aquatic carcinogens. Suggested aquatic carcinogens include trichloroethylene, polycyclic aromatic hydrocarbons, polychlorinated biphenyls or dioxin-related compounds. Future studies may include exposure to more than one carcinogen, i.e. both an initiator and a promoter.

III. Work to be performed in next reporting period

Studies will continue along the following lines: (1) Continue Southern blot analysis of DNA from transformed cells and nude mouse tumors;

(2) continuation of transfection studies using DNA from MAMAc-exposed fish: (3) continuation of sequence analysis of the C-7 clone; (4) continuation of oncogene expression studies during development.

(1) Southern blot analysis - In order to confirm that the transformation of NIH3T3 cells is due to fish sequences, we will examine DNA restriction digests of transfected cells on Southern blots for the presence of fish-specific sequences. We will also hybridize them to known oncogene radiolabelled probes in order to identify known activated oncogenes. We will repeat the Southerns of TR23 DNA in order to confirm the presence of a mutated *p53* gene in two of the transfectants. These studies are in progress.

(3) Transfection experiments - Exposure of medaka to DEN and MNNG, in separate experiments, are currently in progress. Livers and other tumor-bearing tissue will be excised from the fish, a portion preserved for histopathological analysis and the remainder frozen for DNA extraction. DNA will be extracted from these fish and analyzed by transfection analysis as described previously. Primary transfections of MAMAc-exposed fish (from 6-month growout) will continue. We will follow these with secondary transfections.

(4) Cloning experiments - During the next year, efforts will be concentrated on obtaining the sequence for the C-7 clone. The *BamHI* and *XbaI* subclones will be sequenced in an effort to obtain information on the internal nucleotide composition of the clone. In addition, the genomic library will be rescreened with a new pSV_2neo DNA probe.

(5) Oncogene expression - We will continue efforts to prepare a cDNA library from normal adult liver tissue. This will serve as the standard for comparison to genes expressed during different developmental stages. We will also continue to develop methods using reverse-transcriptase PCR to identify transcripts of oncogenes and suppressor genes in RNA isolated from different developmental stages.

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Table I

Restriction sites in pSV_2neo and Lambda DNA

Enzyme	Cuts in neo	Cuts in Lambda
Apa I	unknown	1
Bam Hl	unknown	2
Eco Rl	unknown	2
Kpn 1	0	2
Sac I	0	2
Xba I	0	1
Xho I	0	1

In order to determine restriction sites appropriate for subcloning fragments of the C7 clone, several restriction enzymes were used to digest pSV_2neo DNA and lambda DNA. Enzymes which did not cut within the pSV_2neo sequence and did cut ≤ 2 times within the lambda DNA (i.e. SacI, XbaI, XhoI) were used to prepare fragments for subcloning into Bluescript. (See also Figs. 1-2).

Table II

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

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 III

Efficiency of a secondary transfection (TR20) of NIH3T3 cells.

DNA Source	Transfection Efficiency (# drug-selected cells/plate)[1]
Calf thymus DNA	9.2×10^5
TR16-5-1 (untreated medaka)	1.4×10^5

[1]Average of 4 plates; 20 ug genomic DNA transfected per plate. Original source of DNA for primary transfection is given in parentheses.

Table IV

Efficiency of primary transfection (TR21) of DNA from medaka tissues

DNA Source	Transfection efficiency (# drug-selected cells/plate)[1]
Calf thymus	5.2×10^5
Medaka controls	
L88-308-2-3	10.1×10^5
DEN[2]-exposed medaka	
L88-308-4-2	7.2×10^5
L88-308-4-6	9.5×10^5
L88-308-4-8	38.2×10^5
L88-308-4-10	10.7×10^5
K89-046-2-1	8.8×10^5
K89-046-3-6	7.4×10^5

[1]average of 4 plates; 20 ug genomic DNA transfected in each.
[2]diethylnitrosamine

Table V

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
medaka (untreated)	0.5	+1/2
DEN-exposed medaka		
L88-308-4-2	6	++
L88-308-4-6	11	+++
L88-308-4-8	1	-
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.

All exposed medaka show numbers of foci and growth in soft agar at levels significantly higher than background (cells transfected with calf thymus or unexposed medaka DNA)

Table VI

Results of Tumorigenicity Assay in Nude Mice: Primary transfection of DNA from DEN-exposed animals (TR16 and TR21)

DNA Source	#tumors/#mice injected	time
calf thymus	0/1	
TR16-10	1/1	10.5 wks
TR16-9	1/1	13.5 wks
TR16-7,8	0/1	14.5 wks
TR21-2	--	
TR21-3,4	2/2	5.5 wks
TR21-6-1-1	--	
TR21-8-1-1	--	
TR21-8-2-1	--	
TR21-9-2	--	
TR21-10-1	--	
TR21-10-2	--	
TR21-11	--	
TR21-14-3	--	
TR21-15-1	--	
TR21-16	1/1	4 wks

Time indicates the number of weeks from the day injected to the onset of tumor development. (--) indicates undetermined- these experiments are only in their fourth week.

Sample identification (i.e. the plate number in the transfection assay and the code number of the medaka from which DNA was extracted) is as follows: TR16-7,8, L88-308-4-4; TR16-9, L88-308-4-5; TR16-10, L88-308-2-1; TR21-2,3, and 4, calf thymus DNA; TR21-6, L88-308-2-3; TR21-8, L88-308-4-2; TR21-9,10, L88-308-4-2; TR21-11, L88-308-4-8; TR21-14, L88-308-4-8; TR21-15,16, K89-046-2-1.

Table VII

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

Cell Source	Relative Growth[1]
NIH3T3	0
mos-transformed cells	++++
TR21-2	0
TR21-6-1-1	
TR21-8-1-1	1/2+
TR21-8-2-1	0
TR21-9-2	1/2+
TR21-10-1	0
TR21-10-2	0
TR21-11	0
TR21-14-3	0
TR21-15-1	0
TR21-16	0
TR21-17-1	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.

Sample identification is as follows:
TR21-2, calf thymus DNA; TR21-6, L88-308-2-3; TR21-8, L88-308-4-2; TR21-9,10, L88-308-4-2; TR21-11, L88-308-4-8; TR21-14, L88-308-4-8; TR21-15,16, K89-046-2-1; TR21-17, K89-046-3-6.

TABLE VIII

Identification of tumors in livers of MAMAc-exposed fish used in transfection

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

TABLE IX

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

DNA Source	Standard Focus Assay (# foci / ug DNA)	Colony Selection Assay (QBSF + 0.1% serum)
Calf thymus	0	+
AA-91-351-1-1	0	-
AA-91-351-1-6	0.07	-
AA-91-351-5-18	0.14	++
AA-91-351-1-11	0	++
AA-91-351-4-18	0	0
AA-91-351-5-19	26.9	++++
AA-91-351-1-18	0	0
AA-91-351-4-4	2.0	+++
AA-91-351-4-17	0	0
AA-91-351-4-21	0	-
AA-91-351-5-1	0	-

[1] Growth relative to mos-transformed NIH3T3 cells

Table X

Transfection Efficiency of DNA from MAMAc exposed Fish (TR24)

Plate Number	DNA Source	Average # of Colonies/plate
1	Calf thymus[1]	0
2-3	Calf thymus	19
4-5	Calf thymus	26
6-9	AA-92-85-1-4	8
10-12	AA-92-85-1-17	11
13-16	AA-92-85-4-3	3
17-20	AA-92-85-4-6	3
21-24	AA-92-85-5-3	12
25-28	AA-92-85-5-4	4
29-30	AA-92-85-5-7	5
31-32	L-88-308-4-4[2]	10

[1] No pSV_2neo was added to this negative control plate.

[2] DEN-induced hepatocellular carcinoma.

Table XI

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

Plate#	SFA #Foci/plate	SFA/DEX #Foci/plate
TR24-2	0	0
TR24-3	0	0
TR24-6	8	2
TR24-7	2	0
TR24-8	5	0
TR24-9	0	0
TR24-10	0	3
TR24-11	19	0
TR24-12	0	0
TR24-13/14	0	0
TR24-15/16	0	0
TR24-17/18	-	0
TR24-19/20	3	0
TR24-21	7	1
TR24-22	1	10
TR24-23/24	0	1
TR24-25/26	0	-
TR24-27/28	1	25
TR24-29/30	0	0
TR24-31/32	10	1

Figure 1. Restriction digest of C-7 DNA. This figure shows the restriction analysis of the C-7 clone. C-7 phage DNA (10 ug) was digested with various restriction endonucleases and characterized on a 1% agarose, 1X Tris-Borate-EDTA gel. Lane 1 (Lambda-HindIII), lane 2 (Apa I), lane 3 (Bam H1), lane 4 (Eco R1), lane 5 (Kpn I), lane 6 (Sac I), lane 7 (Xba I), lane 8 (Xho I) and lane 9 (Lambda-HindIII).

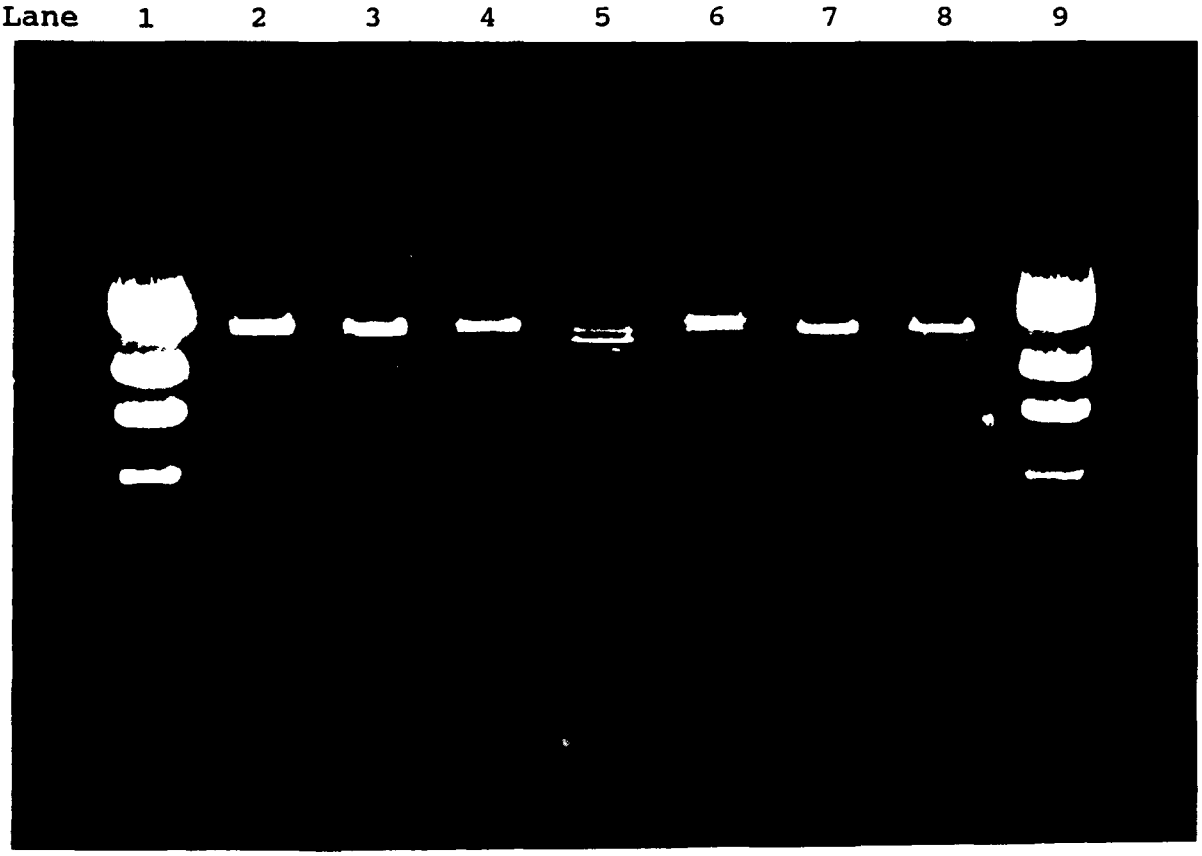
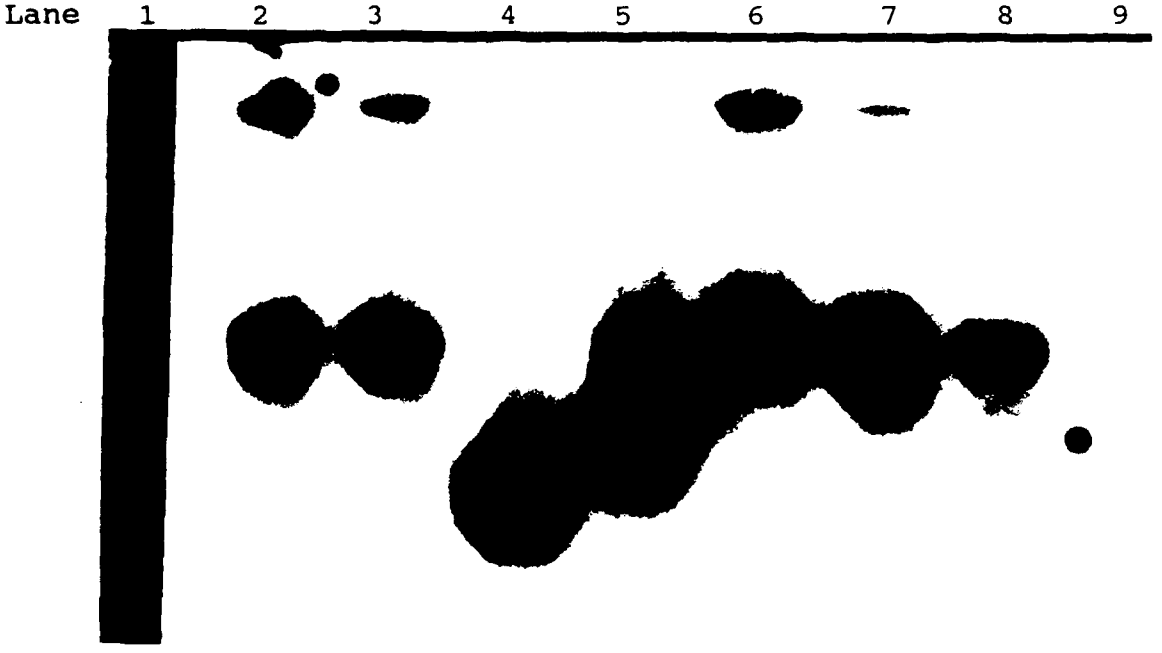


Figure 2. Hybridization of restriction digest products of C-7 DNA to pSV_2neo and EMBL 4. C-7 phage DNA (10ug) was digested with various restriction endonucleases and the products separated by agarose gel electrophoresis (see Figure 1). The DNA was transferred to nitrocellulose by Southern blotting and probed using ^{32}P-labelled pSV_2neo (Figure 2A) or EMBL 4 (Figure 2B). Lane 1 (Lambda-HindIII), lane 2 (Apa I), lane 3 (Bam H1), lane 4 (Eco R1), lane 5 (Kpn I), lane 6 (Sac I), lane 7 (Xba I), lane 8 (Xho I) and lane 9 (Lambda-HindIII).

2A



2B

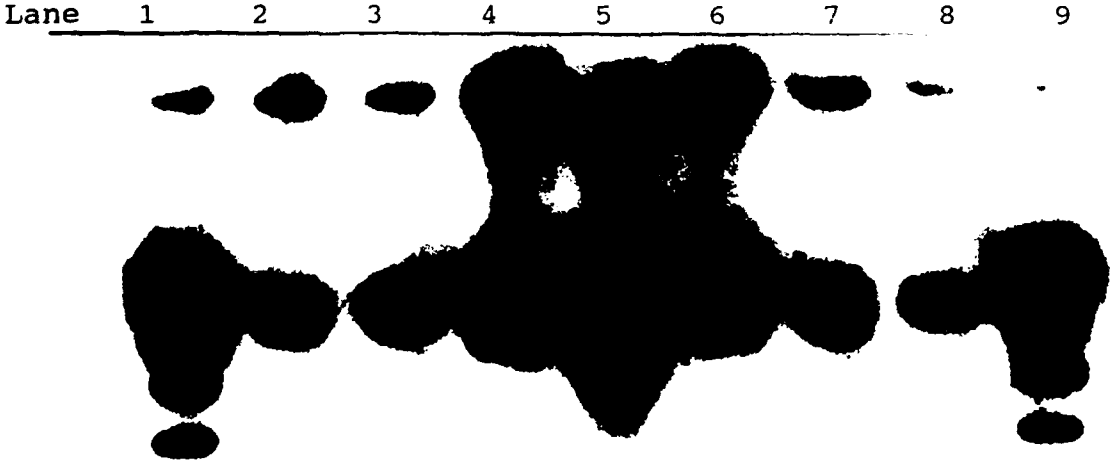


Figure 3. *Southern blot of DNA from NIH3T3 cells transfected with DNA isolated from MAMAc-exposed medaka.* Transfected cell DNA was digested with *PstI*, size fractionated on a 20x20cm 0.8% agarose gel and transferred to nitrocellulose. Duplicate sections were hybridized at low stringency (37°C, 35% formamide) to either ^{32}-P labeled *p53* (lanes 15-20), *c-myc* (lanes 9-14) or *K-ras* (lanes 2-7) probes. Lanes 2 and 20 show non-specific binding to the lambda marker DNA. Lanes 3,9,15 - NIH3T3 DNA; lanes 4,10,16 - TR23-9-1 DNA (spindle cell proliferation,hepatocellular vaculation); lanes 5,11,17 - TR23-14-1 DNA (cholangiocarcinoma); lanes 6,12,18 - TR23-16-1 DNA (mixed hepato-cholangiocarcinoma); lanes 7,13,19 - normal medaka liver DNA.