IDENTIFICATION OF THE RETINOBLASTOMA GENE AND RETINOBLASTOMA PROTEIN IN THE MEDAKA

(*Oryzias latipes*)

By

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BCIP	5-bromo-4-chloro-3-indolyl-phosphate
cDNA	complementary DNA
cdk	cyclin-dependent kinase
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DAB	3,3′-diaminobenzidine tetrahydrochloride
DEP	diethyl pyrocarbonate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleotide acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
dTTP	deoxythymidine triphosphate
EDTA	ethylenediamine tetracetic acid
EGF	epidermal growth factor
GST-RB	glutathionine S-transferase-retinoblastoma protein
ICE	interleukin 1β-converting enzyme
IFN-α	interferon-α
λ	lambda
LB	Luria-Bertani

mRNA	messenger ribonucleic acid
NBT	nitro blue tetrazolium
NGFI-A	nerve growth factor inducible A
PCR	polymerase chain reaction
PBS	phosphate buffered salinr
pfu	plaque forming bacteriophage units
Rb	retinoblastoma
RNA	ribonucleotide acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription and PCR
SA-PMPs	streptavidin-paramagnetic particles
SDS	sodium dodecyl sulfate
SSC	sodium chloride-sodium citrate
SSPE	sodium chloride-sodium phosphate-EDTA
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TE buffer	Tris-EDTA buffer
TGF-β	transforming growth factor-β
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
*X*Rb	*Xenopus* Rb

CHAPTER I

INTRODUCTION

Tumor Suppressor Genes

Cancer is the result of an accumulation of mutations that disrupt the homeostasis within a cell. Cancer cells posses the characteristics of continuous growth and an inability to undergo terminal differentiation (Lee, 1993). Normal cells require the interaction of positive and negative signals to maintain the balance. Positive signals are those responsible for promoting cell growth. Negative signals restrain cell growth.

The group of genes playing an essential role in negative signaling are described as recessive oncogenes, anti-oncogenes, or tumor suppressor genes (Lee, 1993; Klein,1993). Tumor suppressor genes act in normal cells to suppress cellular proliferation. The loss of tumor suppressor genes from a cell will remove normal constraints on cell growth. Tumor suppression was initially identified by somatic cell hybridization studies (Harris et al., 1969; Klein et al., 1971; Weiner et al., 1974). Fusion of normal cells to highly malignant cells resulted in the formation of stable hybrids. The hybrids contained normal chromosome complements and expressed the phenotype of the normal cell (Stanbridge et al., 1982). Other investigators (Stanbridge et al., 1981; Klinger et al., 1982) found a correlation of particular chromosomes with suppression of the tumorigenic phenotype. They used the HeLa x fibroblast hybrids to identify the chromosome (or chromosomes) responsible for

the nontumorigenic phenotype. Stanbridge (1981) demonstrated that after repeated inoculation of serially cultivated nontumorigenic hybrids into nude mice, tumorigenic segregants did appear. Comparison of chromosomal karyotypes between the nontumorigenic hybrids and their tumorigenic segregants revealed that loss of one copy each of both chromosomes 11 and 14, with a high degree of statistical significance, correlated with reexpression of tumorigenicity. Klinger (1982) reported similar findings, and chromosome 11 showed this same correlation with tumorigenicity. The current isolated and characterized human suppressor genes include the Rb (retinoblastoma), p53, WT (Wilms' Tumor gene), NF1 (neurofibromatosis type 1 gene), APC (adenomatous polyposis coli), DCC (deleted-in-colon-carcinoma gene) and DPC (delited in pancreatic cancer) (reviewed by Lee, 1993).

Retinoblastoma

A prototype biological model for the study of tumor suppressor genes is the retinoblastoma gene. Retinoblasts are the precursors of cells in the retina, the light sensitive screen at the back of the eye. Retinoblasts are destined to become the photoreceptor cells called cones which seem to be involved in retinoblastoma. In humans, a retinoblast differentiates to form a specialized retinal cell, it stops dividing and can no longer serve as a target for tumorigenesis (Weinberg, 1988).

Retinoblastoma is a malignancy of the eye occurring in infants and young children (1 in 20,000 children). It exists in both a familial (hereditary) and a sporadic form (Knudson, 1971). In familial retinoblastoma, deletion of one copy of the retinoblastoma

(Rb) gene in the germ line predisposes an individual to the cancer, and a second mutation in the normal gene causes eye tumors. Sporadic retinoblastoma is due to somatic mutations in both copies of the Rb gene. The fact that loss of both functional copies of the Rb gene underlies tumor formation suggests that this gene normally acts in the retinal cells to constrain cell proliferation (Weinberg, 1990).

Yunis et al. (1978) verified that the band responsible for a predisposition to retinoblastoma was in a specific small region on the q arm of chromosome 13 using interstitial deletions with two retinoblastoma patients. Other investigators (Sparks et al., 1980; Benedict et al., 1983; Godbout et al., 1983) also observed that the targets of two mutations in retinoblastoma were assigned to the q14 region on chromosome 13. Through the studies of families with hereditary retinoblastoma and analysis of patients with congenital chromosome abnormalities, the germ-line mutation responsible for the hereditary form of retinoblastoma has been assigned to the q14 region on chromosome 13 and closely linked to an enzyme, esterase D. The fact that this region was closely linked to esterase D was based on a successful approach to the isolation of the retinoblastoma gene.

Molecular Biology of Retinoblastoma

The retinoblastoma gene was cloned and characterized nearly simultaneously using a positional chromosome walking technique by three groups (Friend et al., 1986; Lee et al., 1987a; Fung et al., 1987). They cloned the correct genetic locus by comparing the structural changes in diseased and normal cells. Friend et al. (1986) cloned the Rb gene. They constructed a cDNA library from RNA of a human retinal line using the bacteriophage cloning vector lambda gt11 and found a 4.7 kilobases (kb) insert by

screening. The Rb gene was expressed in many tumors, including neuroblastoma, renal carcinoma and melanoma. However, there was no RNA transcript in retinoblastoma and osteosarcoma. Subsequently, the absent (or abnormal) Rb mRNA and/or protein was reported in other tumor types such as small cell lung cancer (SCLC), breast cancer (Harbour et al., 1988; Lee et al., 1988; Ryaard et al., 1990; Yokota et al., 1988), prostate cancer (Bookstein et al.,1990), bladder cancer (Logothetis et al.,1992), osteogenic and soft tissue sarcomas (Cance et al., 1990), and chronic lymphocyte leukemia (Kornblau et al., 1994). From these observations, it is clear that Rb inactivation may play a key role in the pathogenesis of several tumors, not just the exceedingly rare eye tumors. Lee et al. (1987a) reported a gene encoding a messenger RNA of 4.6 kb on the basis of chromosomal location, homozygous deletion, and tumor-specific alteration in expression. Transcription of this gene was abnormal in six of six retinoblastomas examined (Rb mRNA was not detectable or expressed variably in amount with a 4.0 kb size). The gene contained at least 12 exons distributed in a region of over 100 kb and could encode a hypothetical protein of 816 amino acids. In 1987, Fung et al. reported a 4.7 kb transcript. Sixteen of forty retinoblastomas examined had identifiable structural changes of the Rb gene including homozygous internal deletions with corresponding truncated transcripts. They identified possible hot spots for deletion within the Rb genomic locus. In all of the internal deletion cases examined, deletions involved either the 7.5 or 9.8 kb *Hind*III fragments. Mutations in the Rb gene were also reported by Dunn et al. (1989) and Horowitz et al. (1989). The mutations involved a small deletion, duplication or point mutation in Rb tumors. Lee et al. (1987b) reported a revised Rb cDNA sequence. It still maintained the same open reading frame as in the original clones, with an additional

methionine codon found at nucleotide 139. When this methionine was used as an initiation codon, the predicted Rb protein had 928 predict amino acids and Mr 110 Kda-identical to the apparent Mr by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Hong et al. (1989) and T'Ang et al. (1989) reported the genomic structure of the human retinoblastoma gene. The Rb transcript is encoded in 27 exons distributed over 200kb of genomic DNA. The length of individual exons ranges from 31 to 1889 nucleotides. The largest intron is more than 60 kb and the smallest one has 80 bp. Exon 1 contains 5′ untranslated sequences and encodes the first methionine. A 70 base pair promotor region which has a G+C rich region is located immediately upstream from the first exon. A putative "leucine-zipper" motif is exclusively encoded by exon 20. A typical upstream TATA box is not present. Toguchida et al. (1993) reported the complete genomic sequence of the human Rb gene. They used a directed prime-and-run approach to sequence about 180 kb encompassing the human Rb gene. This sequence extends from the G in the *Eco*R I site 2059 bp upstream of the first base of the initiation codon to the last T in a *Hin*dIII site 3008 bp down stream of the stop codon. Partial analysis of the sequence revealed a high (A+T)/(G+C) ratio and a high density of Line-1 repeat sequences. This favors a map location within a Giemsa-staining chromosome band or G-band. The sequence data indicative of a G-band location suggest an assignment to either 13q14.12 or 13q14.2. Toguchida et al. (1993) also found interspersed, repetitive DNA sequences (46 *Alu* and 17 Line -1 repeat sequence) within the180 kb contig.

The Rb cDNAs from mouse, frog, rat, and chicken have also been cloned (Bernards et al., 1989; Destree et al., 1992; Roy et al., 1993; Feinstein et al., 1994). In

1989, Bernards et al. isolated a cDNA clone of the murine homolog of the human retinoblastoma susceptibility gene. The length of the DNA was 4592 bp and it had an 84% similarity to the human Rb cDNA. The degree of conservation in the coading region (88.7%) was not much higher than the degree of conservation in the 3′ untranslated region of the cDNA (77.3%). The 3′ untranslated region of both the mouse and the human Rb cDNA contained a total of 12 ATTA motifs. The Rb cDNA contained a single large open reading frame from nucleotide 99 to a stop codon at nucleotide 2865. The predicted amino acid sequence of the mouse Rb protein is a 921-amino acid protein with a calculated molecular weight of 105Kda. Comparison of the predicted amino acid sequence of the mouse Rb protein with that of human Rb protein showed that the human Rb protein has a total of 7 additional amino acids. In total, 82 of 921 amino acids were different between mouse and human Rb proteins. This made the two proteins 91% identical. Bernards et al. (1989) also showed the conservation of the Rb gene in closely and distantly related organisms (lamprey, shark, swordtail, chicken, mouse, and human) by Southern blot analysis of *Hin*dIII-digested total genomic DNA using the 3.8 kb *Eco*R I fragment of the human Rb gene as a probe. All organisms examined contained sequences that crosshybridize with the human Rb gene probe. RNA analyses of the mouse showed a 4.7 kb Rb transcript in all tissues examined. Interestingly, an additional mRNA transcript (2.8 kb) was found in the testes of mice as the spermatids mature.

A *Xenopus* homolog (*X*Rb 1) of the human retinoblastoma susceptibility gene was cloned by Destree et al. (1992). The nucleotide sequence of *X*Rb has 65% similarity to that of mouse Rb. The *X*Rb sequence contains a single open reading frame of 2697 nucleotides which would encode an 899 amino acid protein. The predicted amino acid

sequence of the *Xenopus* retinoblastoma protein (p*X*Rb1) shows an overall 56% identity compared with murine and human Rb proteins. A "leucine-zipper" motif, consensus sequences for cdc2 kinase and potential sites for other kinases are conserved. The region of the protein homologous to the SV40 T antigen binding site and the basic region c-terminal to the E1A binding site are all conserved. There is a 75% amino acid identity between the human and the *Xenopus* proteins in the E1A binding domains (A and B). There is only a 37% identity of amino acids in the spacer region between the two domains. In mouse and human the Rb transcripts are 4.7 kb in size. However, 10 and 4.2 kb transcripts are present for all tissues and developmental stages in *Xenopus*. The level of expression of the *X*Rb gene varies greatly for the different tissues. The *Xenopus* 99-Kda protein (pXRb1) was detected using antibodies (XZ37, XZ78, and XZ160) raised against human p105Rb.

Roy et al. (1993) cloned the rat retinoblastoma cDNA. They sequenced a 2.1 kb cDNA fragment (R961). The rat R961 Rb cDNA sequence is homologous to the 3′ region of mouse and human Rb genes. Homology between rat and mouse is more than 92%, and between rat and human is approximately 81%. The amino acid sequence deduced from this cDNA is about 97% homologous with that of the human Rb protein sequence at the carboxyl terminus.

Feinstein et al. (1994) characterized a chicken cDNA encoding the retinoblastoma gene product. A full-length chicken Rb cDNA is approximately 4.5 kb and can be divided onto three *Eco*R I fragments of approximately 0.9 kb, 1.6 kb and 2.0 kb. The chicken Rb messenger RNA is predicted to contain a short GC-rich 5′ nontranslated region followed

by an AUG beginning at nucleotide 77 of the cDNA. The predicted chicken Rb protein is a 919 amino acid protein with a calculated molecular mass of 104 Kda. It is similar to that of the mouse, human and *Xenopus* Rb proteins in regions of known functions. In adenovirus E1A binding sites (amino acids 394-572 and 646-772 of human Rb) all four proteins have a total of 66% amino acid identity. However, chicken Rb has distinct species-specific differences near the N-terminus (from approximately amino acid 5 through 55 of chicken Rb). The mouse and human Rb proteins are similar in this region, the chicken and *Xenopus* Rb protein each have distinct sequences.

The Rb protein was identified by antibodies raised against a tryE-Rb fusion protein (Lee et al., 1987a). This protein, termed $p110^{Rb}$, migrates as multiple closely-spaced bands between Mr 110 to 116 Kda when sized on denaturing polyacrylamide gels. The protein Rb is a phosphoprotein and is located within the nucleus. The protein can be retained by and eluted from DNA-cellulose columns, suggesting that the protein has DNA binding activity. It is present in all cell lines expressing normal mRNA, but is absent from five retinoblastoma cell lines examined (Lee et al., 1987b).

Distinct domains of the Rb protein are important in understanding its biological functions. Riley et al. (1994) showed the schematic representation of the structure of the human Rb protein in the review paper as illustrated in figure 1. The Rb protein is organized into at least three distinct structural domains. The N-terminal domain (N) is important for oligomerization *in vitro* (Hensey et al., 1994). Two other domains (A and B) are frequently altered in human tumors (Hu et al., 1990; Huang et al., 1990) and are required for the binding of E2F. Several DNA tumor virus oncoproteins such as adenovirus E1A, SV40 large T antigen and human papillomavirus E7 protein can also bind

to these domains (Chellappan et al., 1992). These domains are also referred to as the "pocket " because they serve as receptors for proteins of a specific class (Kaelin et al., 1991). Nonspecific DNA binding is intrinsic to the carboxyl-terminal portion of the protein (Wang et al., 1990).

The Rb protein has been reported to be a phosphoprotein by biochemical studies (Lee et al., 1987b; Chen et al., 1989). Distinct electrophoretic bands migrating from 110 to 116 Kda have been shown to correspond to different phosphorylated forms of $p110^{Rb}$ (Xu et al., 1989; Ludlow et al., 1989). In normal cells, the Rb protein is expressed throughout the cell cycle but its functionally active states are determined by the degree of phosphorylation (DeCaprio et al., 1989; Chen et al., 1989; Ludlow et al., 1990). The under (un)phosphorylated Rb forms are present in G_0 and G_1. These forms seem to be involved in preventing the cell from undergoing transition from G_1 to S. Laiho et al. (1990) reported that TGF-β1 acts to retain Rb in the underphosphorylated growth-suppressive state. Whereas the more highly phosphorylated forms are found during S and G_2/M phases. The progressive phosphorylation of the Rb protein seemingly relieves the block and allows continuation of the cell cycle. Based on these observations, the underphosphorylated form of the Rb protein appears to have a key role in the growth-suppressive activity of Rb protein. This is supported by another study of role of the retinoblastoma Rb protein. This study was done with the products of DNA tumor viruses. Whyte et al. (1988) reported that adenovirus E1A and Rb protein form a specific complex. Similarly, DeCaprio et al. (1988) and Ludlow et al. (1989) observed that SV40 T forms a complex with this protein, $p110\text{-}114^{Rb}$. Like E1A and SV40 large T, Human papilloma virus E7 proteins form a specific complex with the Rb protein (Dyson et al., 1989; Munger

et al., 1989). These oncoproteins inactivate Rb at G_1 and allow quiescent cells to enter the cell cycle. Shortly, when Rb is complexed with an oncogene product, constraints to proliferation by activated Rb are removed and the unscheduled cellular proliferation characteristic of neoplasia can occur. This ensures that Rb is an anti-oncogene.

The cell cycle-dependent phosphorylation of Rb protein suggests that it may be a substrate of a cell cycle-regulated protein kinase. Recently, the kinases responsible for the phosphorylation of Rb protein have begun to be discovered. The cdc2 kinase (Lin et al., 1991) and cdc2-like kinase (Kitagawa et al., 1992) can phosphorylate the Rb protein *in vitro* on many of the same sites normally phosphorylated *in vivo*. Human cdc2 has also been shown to interact physically with Rb protein (Lin et al., 1991). Dephosphorylation of Rb protein can involve protein phosphatase 1. Durfee et al. (1993) reported that the Rb protein binds with the protein phophatase type 1 catalytic subunit. Moreover, dephosphorylation of Rb in M and G_1 phases by protein phosphatase 1 has been demonstrated by blocking protein phophatase 1 activity using phophatase inhibitors (Alberts et al., 1993; Kim et al., 1993; Ludlow et al., 1993).

Many studies have demonstrated that the Rb protein associates with cellular proteins. The best characterized cellular Rb-associated protein is the transcription factor E2F-1 (Chellappan et al., 1991; Chittenden et al., 1991). Chellappan et al. (1991) showed that the Rb protein is found in a complex with the E2F transcription factor and that only the underphosphorylated form of Rb is in the E2F complex. Moreover, E2F and the DNA tumor virus oncoproteins bind to the same regions of Rb and the E2F-Rb complex can be dissociated by these oncoproteins. Chittenden et al. (1991) reported that a DNA-binding site and enrichment procedure revealed a sequence-specific DNA-binding activity

selectively associated with glutathionine S-transferase-retinoblastoma protein chimeras (GST-RB) that had been incubated with a human cell extract. Appropriate mutant forms of GST-RB, incubated in equivalent extracts, did not associate this specific DNA-binding activity, and a peptide replica of the HPV E7 Rb binding segment selectively inhibited the association of GST-RB with sequence-specific DNA-binding protein. They suggest that Rb can associate specifically with the transcription factor E2F by sequence analysis of oligonucleotides with high affinity for GST-RB complexes and the results of competition binding studies. Results from both researchers suggest that the interaction of Rb with E2F is an important event in the control of cellular proliferation. When transcription factors are complexed with Rb and inactivated, transcription is inhibited and cell cycle progression is blocked. When they are free by oncoproteins, they can activate transcription of genes required for the S phase and beyond.

A family of Rb protein has been discovered based on sequence homologies of oncoprotein binding domains of the retinoblastoma. Two other proteins p107 and p130 have been isolated by their interaction with adenovirus E1A (Harlow et al., 1986; Dyson et al., 1989a; Mayol et al., 1993). p107 is a cellular protein which was identified by virtue of its ability to bind to the E1A, SV40 and JC virus large T-antigen oncoproteins. Sequence analysis of a human p107 cDNA clone identified a region of 564 amino acids with 57% identity to the so called "pocket domain" of the human Rb product (Ewen et al., 1991). Homology between the p107 and pRb pocket domain resides largely in two regions known as the A and B motifs, which are 178 and 124 amino acids in length, respectively. The A and B motifs are separated by non-homologous spacers of 211 amino acids in p107 and 73 amino acids in pRb. A third adenovirus E1A binding protein, designated p130 is

homologous with p107. Although there is less similarity between p130 and pRb (32%), the pocket domain sequence is highly conserved among all three proteins.

A Vertebrate Model for Retinoblastom

Although Rb is the best-known of all tumor suppressor genes, researchers are just beginning to understand the regulation and function of the Rb protein. It is necessary to determine how the Rb protein limits or shuts down cell proliferation in normal cells. A clear understanding of the ethiology of the Rb suppressor gene will provide an answer to this question and an opportunity to understand the tumorigenesis process in a variety of cancers.

Studies of retinoblastomas are limited to human autopsy and surgical material because of the lack of a vertebrate model capable of expressing retinoblastoma. Windle et al. (1990) and O'Brien et al. (1990) created a transgenic animal model of retinoblastoma. In this model, ocular retinoblastoma and central nervous system tumors with histological, ultrastructural and immunohistochemical characteristics identical to those of the human were observed in a mouse formed by the transgenic expression of the SV40 T-antigene. While this will no doubt be a valuable source of retinoblastoma tissue, owing to the way the mouse was engineered, it will not provide a means for understanding the genetics controlling expression of the disease under natural conditions. Therefore, we need a model to understand the mechanism of retinoblastoma involvement in tumorigenesis and other tumors under natural conditions.

Retinoblastoma protein has been reported in a number of fish species including medaka (Oryzias latipes) (Ostrander et al., 1992), coleacanth (Latimeria chalumnae),

rainbow trout (Oncorhynchus mykiss), and English sole (Parophyrs vetulus) (Van Beneden and Ostrander, 1994). Retinoblastomas of several fishes have been reported as single spontaneous cases (Fournie et al., 1985). Furthermore, Hawkins et al. (1986) reported the inducibility of intraocular neoplasms in Japanese medaka (Oryzias latipes) with methylazoxymethanol acetate (MAM-AC). The medaka was the only species to develop intraocular neoplasm among seven fish species examined. Ultimately, some of these lesions were determined to be retinoblastomas (Ostrander et al., 1992). Thus, a vertebrate model for retinoblastoma has been established using medaka. The medaka is a small aquarium fish (3-5 cm) that is easily maintained in the laboratory. The entire animal can be mounted on a paraffin block or frozen for later sectioning and histochemical analysis. The entire fish can also be exposed to antibodies or probes and all tissues simultaneously examined. Finally, retinoblastomas in the medaka can be induced as necessary (10% + incidence). Therefore, we have adequate samples to study retinoblastoma. For these reasons, a unique opportunity exists to study the ethiology of this malignancy in another vertebrate species. The similar pathology to mammalian models and unique mode of induction insure that information relevant to cancer in general will be gleaned from these studies.

The studies detailed below with the medaka provide a framework to investigate the normal function of the Rb protein. These studies form the basis for completion of experiments with medaka treated with carcinogens.

Specifically, I studied:

1. Detection of Rb mRNA and Rb protein during various developmental stages and in various tissues of normal medaka.

2. Construction and screening of a cDNA library with mRNA from medaka liver.

3. The effect of growth factors and hormones on the medaka Rb protein.

CHAPTER II

DETECTION OF Rb mRNA AND Rb PROTEIN DURING VARIOUS DEVELOPMENTAL STAGES AND IN VARIOUS TISSUES OF NORMAL MEDAKA

Introduction

Molecular analyses have shown that the retinoblastoma gene is evolutionarily conserved in vertebrates. Lee et al. (1987a) examined genomic DNA from four non-human vertebrate species by hybridizing with probe RB-5 (3.5 kb Rb cDNA). Under standard hybridization stringencies, homologous sequences were detected in all samples (human placenta, calf retina, cat fibroblasts, mouse NIH 3T3 fibroblasts and chicken embryo fibroblasts), with weaker hybridization intensity as evolutionary distance increased. Bernard et al. (1989) investigated the extent to which homologues of the human Rb are detectable in closely and distantly related organisms. They performed Southern blot analysis of *Hin*dIII-digested genomic DNA from the following organisms: amphioxus, brook lamprey, cat-shark, swordtail, chicken, mouse, and human. They used the 3.8-kilobase (kb) *Eco*R I fragment of the human Rb cDNA clone as a probe. All DNA samples, except for amphioxus, cross-hybridized with the human Rb gene probe. The gene is ubiquitously expressed in various organs of the mouse and frog (Bernards et al., 1989; Destree et al., 1992).

I hypothesized that the Rb gene exists in the medaka. To determine if medaka have the Rb gene and express a message, Southern blot analyses and Northern blot analyses were performed using a 505-base pair (bp) fragment from the human Rb cDNA. Northern blot analyses were performed to detect Rb mRNA in various organs of the medaka. Finally, Western blot analyses were used to detect the expression of Rb protein in developmental stages of eggs, fry, and various organs of adult medaka.

Materials and Methods

Animals and Cell Line--Medaka (*Oryzias latipes*) used in these studies were obtained from our breeding colony at Oklahoma State University which was initiated from fish obtained from Dr. William Hawkins at the Gulf Coast Research Laboratory. Fertilized 1 day-old eggs were collected every morning and stored at -85^{0}C. For later stages of egg development, the fertilized eggs were placed in embryo rearing solution until the appropriate time, then collected and stored at -85^{0}C. For fry, the eggs were raised in embryo rearing solution until hatched, then collected and stored at -85^{0}C. Chronic myelogenous leukemia cell line (K562) was purchased from American Type Culture Collection and used as a Rb-positive control.

Chemicals--Reagents (10X PCR buffer, $MgCl_2$, and dNTP) for PCR and AmpliTaq polymerase were from Perkin-Elmer Cetus (Branchburg, NJ). USBioclean MP Kit and Random Primed DNA Labeling Kit were from United States Biochemical Company (Cleveland, OH). TA Cloning™ Kit was from Invitrogen Company (San Diego, CA). Wizard Miniprep DNA Purification System, PolyATtract® mRNA Isolation System,

human genomic DNA, and *Hin*dIII restriction enzyme were from Promega (Madison, WI). Guanidium thiocyanate, diethyl pyrocarbonate (DEP), 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), and nitro blue tetrazolium (NBT) were from Sigma Chemical Company (St. Louis, MO). Monoclonal antibody (Rb Ab-1) against the Rb gene product was from Oncogene Science (Cambridge, MA). Alkaline phosphatase-conjugated affinipure rabbit anti-mouse IgG (H+L) was from Jackson Immuno Research (Westgrove, PA). Alpha-(^{32}P) dCTP (3000Ci/mmol) was from New England Nuclear (Boston, MA). All other reagents were of analytical grade or better.

Hybridization Probe--The 3.8 kb fragment of the human Rb cDNA was donated by Dr. Stephen H. Friend and Dr. Robert A. Weinberg. To make a 505 bp fragment corresponding to the conserved region of the Rb gene, polymerase chain reaction (PCR) was performed using the 3.8 kb fragment as a template. The oligonucleotides for the primers were synthesized by the Recombinant DNA/Protein Resource Facility at Oklahoma State University. The primer pair consisted of $^{5'}$ACTGCACAGTGAATCCAAAAG$^{3'}$ and $^{5'}$TAATAAGATCAAATAAAGGTG$^{3'}$.

The PCR reaction was prepared in a volume of 40 µl containing 10 mM Tris-HCl (pH 8.3)-50 mM KCl-0.001% (wt/vol) gelatin (1X PCR buffer; Perkin-Elmer Cetus, Norwalk, CT), 1.5 mM $MgCl_2$, 200 µM dNTP (dATP, dCTP, dGTP, and dTTP), 2 pmole/µl of each primer, 2 µl of cDNA, and 0.5 U of AmpliTaq polymerase (Perkin-Elmer Cetus). Amplification was performed using a Perkin-Elmer Cetus GeneAmp PCR System 9600. PCR was started at 94°C for 5 min. Then 35 cycles were performed of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. The samples were subjected to a 10 min

final extension at 72°C at the end of 35 cycles. Water was used as a negative control for PCR.

After the amplification was completed, the fragment was purified to clone into a vector using USBioclean MP kit (United States Biochemical Company). The sample was electrophoresed on a 1% low melting agarose gel in TAE buffer. The desired band was cut from the low melting agarose gel using a razor blade. The DNA:Agarose block was placed into a micro-centrifuge tube, three volumes of 6 M NaI solution were added to the agarose gel block and the tube was incubated at 55°C for 3-5 min to dissolve the agarose. Five µl of glass powder were added to the DNA:NaI solution for DNA binding. The glass powder with the bound DNA was microcentrifuged for 5-10 sec and rinsed 3 times with 50% ethanol rinse buffer. After the final centrifugation, the glass powder was suspended in 10 µl of sterile water. The DNA was eluted by incubating at 55°C for 2-5 min. The tube was microcentrifuged to recover the DNA. One or two µl of the elution was analyzed on a 1% agarose gel with a known concentration of DNA to approximate the concentration of the purified DNA.

After the purification, The PCR fragment was cloned to a pCR™ II vector using the TA Cloning™ kit (Invitrogen Company). The TA Cloning™ kit has the advantage that Amplitaq polymerase adds single deoxyadenosines to the 3′-end of all duplex amplified fragments. These A-overhangs are used to insert the PCR product into a specifically designed vector having T-overhangs. The composition of the ligation reaction was 5 µl of water, 1 µl of 10X ligation buffer, 2 µl of resuspended pCR™ II vector (25 ng/µl), 1 µl of purified PCR product, and 1 µl of T_4 DNA ligase. The amount (X ng) of PCR product of

"Y" base pairs (505 bp) to be ligated to the vector with a 1:3 molar ratio was calculated according to the equation below:

3X ng PCR product/Y bp PCR product = 50 ng pCR™ II vector/size in bp of the pCR™ vector (3932 bp).

The ligation reaction was incubated at 12°C overnight. Three µl of TA Cloning ligation reaction (containing ligated recombinant plasmid DNA) were combined with 50 µl ONE SHOT™ competent cells. After adding 450 µl of prewarmed SOC medium to the vial, the cells were incubated at 37°C for exactly 1 hr with shaking at 225 rpm in an orbit shaker incubator. Twenty five µl and 100 µl from the transformation vial were then plated out on the LB agar plates containing an antibiotic ampicillin (50 µg/ml) and X-gal (25 µl of 40 mg/ml X-gal stock was spread on the plates 1 hr before spreading the cells). After incubating at 37°C overnight, the white colonies were lifted and grown in 10 mls of Luria-Bertani(LB)-ampicillin medium (ampicillin concentration; 50 µg/ml) at 37°C overnight to purify the plasmid.

The Wizard Miniprep DNA Purification System from Promega was used to purify the plasmid. Five-six mls of cells from 10 ml cultures (1 ml was saved in 1 volume of LB medium containing 30% glycerol for frozen cell stocks) were treated with cell resuspension solution, cell lysis solution, then the neutralization solution and centrifuged to precipitate the cell debris. The plasmid in the supernatant was then purified using the Wizard Miniprep DNA purification resin and the Wizard minicolumn. The concentration of the plasmid was measured by a SHIMADZU spectrophotometer (model: UV 1201). The sequences of 505 bp human Rb cDNA was verified by the sequencing using the ABI

373A Automatic DNA sequencing system.

The DNA fragment to be used as the probe (505 bp fragment of the human Rb cDNA) was removed from the plasmid by restriction enzyme digestion and separated by low melting agarose gel electrophoresis. Final purification of the insert was done using the USBioclean MP kit as described above.

Southern Blot Analysis-- Intact genomic DNA from medaka must be prepared. Several methods were tried to extract the genomic DNA. Wirgin et al. (1990) reported that conventional DNA extraction procedures utilizing protease digestion used on fish tissues often yielded a predominance of sheared genomic DNA. The medaka genomic DNA was extracted by the Wirgin et al. (1990) method for Atlantic tomcod with a slight modification. Six whole medaka were used to prepare genomic DNA. After removing viscera, fins, and scales, the fish were ground to a fine powder in a mortar and pestle under liquid nitrogen. Approximately 100 mg of each sample was placed into 500 µl of 1 N NH_4OH/ 0.2% Triton X-100 in a microcentrifuge tube and mixed well with a spatula. This homogenate was incubated for 30-60 min at 37°C with periodic agitation to mix solution and sample well. One volume of phenol saturated with 0.1 M Tris, pH 8.0 buffer was added to each sample and mixed very gently. The samples were microcentrifuged for 6 min and the upper phase was transferred to a new tube using a pre-cut pipette tip. One more phenol extraction was performed. This was followed by two rounds of phenol-chloroform (1:1) (chloroform with isoamyl alcohol, 24:1, v:v), chloroform extractions (with isoamyl alcohol, 24:1, v:v). After the final chloroform extraction, the DNA in the supernatant was precipitated with 1/10 volume of 3 M sodium acetate (pH 7.0) and 2

volumes of 100% ethanol overnight at -20°C. DNA was pelleted by microcentrifugation at 16, 000 x g for 30 min, then washed with 70% ethanol and air dried. The purified genomic DNA was dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) at 4°C overnight and treated with RNase A to remove RNA. This was followed by one round of phenol-chloroform (1:1), chloroform extraction, and ethanol precipitation. DNA was resuspended in TE buffer. DNA concentration and purity were determined using a spectrophotometer. Aliquots of the genomic DNA were electrophoresed on a 0.5% agarose gel in order to check the DNA's integrity.

Human genomic DNA (from Promega) was used as a control. Medaka genomic DNA (10-15 μg) and human genomic DNA (10 μg) were digested with *Hin*dIII (Promega). In the digestion reaction, 1/10 of the total volume of enzyme buffer (30 μl) and 30-45 units of the enzyme were added. The digestion was performed at 37°C for 5-6 hr, then the samples were extracted with phenol:chloroform and precipitated with ethanol. The samples were dissolved in TE buffer, loaded on a 0.8% agarose gel, and electrophoresed in a GibcoBRL Horizon 11-14 unit at 40 volts with the lambda DNA-*Hin*dIII size maker. After electrophoresis, the DNA was transferred to a nylon membrane (Zeta Probe; Biorad) by capillary action in 0.4 M NaOH according to the manufacturer's instructions.

The DNA fragment to be used as the probe was prepared as described above under the section "Hybridization probe". The probe was labeled with a radioisotope (^{32}P) using a Random Primed DNA Labeling Kit (United States Biochemical). The cDNA (25 ng in a total volume of 9 μl) was heated at 95°C for 5 min in an eppendorf tube and then chilled on ice. The denatured DNA was combined with the following reagents on ice: 3 μl dATP,

dGTP, dTTP mixture, 2 µl reaction mixture (random hexanucleotide in 10X reaction buffer; 2 M Hepes, pH 6.6, 2 mM Tris-HCl, pH 7, 0.1 mM EDTA, 4 mg/ml bovine serum albumin), 5 µl α-^{32}P dCTP (50 µCi, 3000Ci/mmole, NEN), and 1 µl Klenow enzyme (2 units). The mixture was incubated at 37°C for 1 hr. The reaction was terminated by adding 2 µl of 0.2 M EDTA, pH 8. The unincorporated nucleotides were removed by ethanol precipitation or by chromatography on Sephadex G-50. The specific activity of the probe (2×10^9) was measured by DE 81 assay.

The filter was hybridized with 2×10^6 cpm/ml of α-^{32}P-labeled probe. The hybridization was performed in 40% deionized formamide, 5X SSC (1X SSC: 0.15 M NaCl, 0.015 M Na_3citrate-H_2O, pH 7), 7% sodium dodecyl sulfate (SDS), 1% polyethylene glycol (PEG) 20,000, 0.5% nonfat dried milk, and 0.3 mg/ml salmon-sperm DNA at 42°C overnight. The filter was then washed twice at room temperature for 20 min with vigorous agitation in 2X SSC/0.1% SDS followed by two washes at 60°C for 30 min in 0.2X SSC/0.1% SDS. After every wash, radioactivity on the filter was assessed with a mini Geiger counter. The filter was autoradiographed to Kodak XAR-5 film at -85°C with an intensifying screen.

Northern Blot Analysis-- For RNA isolation the modified Chomzinsky and Sacchi method (1987) was used. Various organs (liver, gastrointestinal tract, eye, gill, brain, muscle, skin, and spleen) were dissected out of medaka, collected in eppendorf tubes on dry ice, and stored at -85°C. Some organs (liver, gastrointestinal tract, and eye) were used fresh for RNA isolation. The organs were placed in 500 µl of the denaturing solution (solution D: 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl,

0.1 M 2-mercaptoethanol) and immediately homogenized. For RNA isolation from eggs, about 200 medaka eggs were ground to a fine powder in a mortar in the presence of liquid nitrogen. The ground sample (about 100 mg) was placed in 500 µl of denaturing solution (solution D) and homogenized. For fry, approximately 50 fry were homogenized in 500 µl denaturing solution. Sequentially, 50 µl of 2 M sodium acetate, pH 4, 500 µl of phenol (water saturated), and 100 µl of chloroform-isoamyl alcohol mixture (49:1) were added to the homogenate, with thorough mixing by inversion after the addition of each reagent. The final suspension was vortexed for 10 sec and cooled on ice for 15 min. Samples were microcentrifuged at 16,000 x g for 30 min at 4°C. After centrifugation, the aqueous phase was transferred to a fresh tube, mixed with 1 volume of isopropanol, and then placed at -20°C for 2 hr. Sedimentation at 16,000 x g for 30 min was again performed. The resulting RNA pellet was dissolved in 150 µl of solution D, transferred into a new tube, and precipitated with 1 volume of isopropanol at -20°C for 1 hr. After microcentrifugation for 10 min at 4°C, the RNA pellet was resuspended in 70% ethanol. The pellet was broken up by repeatedly pipetting it up and down in 70% ethanol. This wash was done at least twice. The pellet was dissolved in 75 µl of 1 mM EDTA, pH 8, treated with diethyl pyrocarbonate (DEP). The solution was precipitated with 1/10 volume of 2 M sodium acetate, pH 4, and 2 volumes of ethanol at -20°C overnight. RNA was pelleted by microcentrifugation at 16,000 x g for 30 min at 4°C, then washed in 70% ethanol and air dried. The RNA pellet was dissolved in DEP- treated H_2O. The quality of RNA was checked by spectrophotometer and the A_{260}/A_{280} ratio was 1.7-1.8. The K562 cell line and frog liver RNA were used for positive controls. Kornblau et al. (1994) used the K562 cell as a Rb-positive control in their experiment and this cell line expressed Rb protein well.

The frog (*Xenopus*) was obtained from Dr. John Bantle's laboratory at Oklahoma State University.

Poly (A) RNA isolation was done according to the procedure provided by Promega PolyATtract® mRNA isolation system. 500 µl of RNA solution (1 mg) were placed in a 65°C heating block for 10 min. Three µl of the Biotinylated-Oligo(dT) Probe and 13 µl of 20X SSC were added to RNA. The solution was mixed gently and incubated at room temperature until completely cooled. The Streptavidin-Paramagnetic Particles (SA-PMPs) were washed three times with 0.5X SSC using a magnetic stand and resuspended in 0.1 ml of 0.5X SSC during the incubation time. The entire contents of the annealing reaction were added to the tube containing the washed SA-PMPs and the tube was incubated at room temperature for 10 min. The supernatant was removed without disturbing the SA-PMPs pellet captured by the magnetic stand. The particles were washed four times with 0.1X SSC by gently flicking the bottom of the tube until all of the particles were resuspended. To elute the mRNA, the final SA-PMP pellet was resuspended in 0.1 ml of the RNase-Free water. The eluted mRNA aqueous phase was transferred to a sterile, RNase-free tube. The elution step was repeated with 0.15 ml of the RNase-Free water and pooled to the first elution. The A_{260}/A_{280} ratio was > 1.9.

Total RNA (20 µg) or poly(A)-enriched RNA (3-4 µg; Human K562 cell line <1µg) were electrophoresed on a 1.0% agarose gel containing 0.67 M formaldehyde (Davis et al., 1986). RNA was transferred in 50 mM NaOH to a nylon membrane (Zeta Probe; Biorad) by capillary action.

The radioisotope labeled 505 bp probe was prepared using the same method described above for the Southern blot analysis. The filter was hybridized with 3-5 x 10^6

cpm/ml of the probe. The hybridization was performed in 50% deionized formamide, 7% SDS, 1% PEG 20,000, 2X SSPE (1X SSPE: 0.15 M NaCl, 0.01 M $NaH_2PO_4 \cdot H_2O$, and 1 mM EDTA), 0.5% nonfat dried milk, and 0.5 mg/ml salmon sperm DNA. Washing was the same as for Southern blot analysis as described above. The filter was autoradiographed to Kodak XAR-5 film at -85°C with two intensifying screens.

Western Blot Analysis -- Organs were dissected out of 10 medaka and stored at -85°C. One hundred eggs of 1 day or over 6 days after fertilization were collected as described in the Materials section above and ground to a fine powder in a mortar under liquid nitrogen. Fifty fry were collected and stored at -85°C. The samples were homogenized in 150 µl-250 µl of modified EBC buffer (10 mM HEPES, pH 7.4, 4 M NaCl, 10 µg/ml aprotinin, 10 µg/ml phenylmethylsulfonyl fluoride, 2 mM EDTA, and 2 mM EGTA). The homogenates were spun for 20 min in a microcentrifuge at 16,000 x g at 4°C. The supernatants were transferred to new tubes. The protein concentration of the extracts was measured using the Bio-Rad Protein Assay based on the method of Bradford (1976). Bovine serum albumin was used as the standard. Proteins were resolved by electrophoresis on SDS-PAGE. One volume of each homogenate was combined with at least an equal volume of sample buffer (0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, and 10% 2-ß-mercaptoethanol) containing bromophenol blue. Twenty five-thirty µg of each sample were electrophoresed on 7.5% SDS polyacrylamide gels at 25 volts for 1.5 hr, 50 volts for 0.5 hr, 75 volts for 0.5 hr, and 100 volts for 0.5 hr using a mini gel apparatus (Bio-Rad Mini protean II). One gel was stained with coomassie blue and a duplicate gel was electroblotted to Immobilon® polyvinylidene difluoride microporous membrane

(Millipore, Bedford, MA). The membrane was blocked with blocking buffer (25 mM Tris, pH 6.8, 125 mM NaCl, 0.1% Tween 20, and 4% bovine serum albumin) for 1 hr at room temperature. Antibody against the Rb gene product was obtained from Oncogene Sciences (Rb Ab-1, # OP28) and was diluted in blocking buffer from the manufactured stock to a final concentration of 2.5 μg/ml. The antibody was clone C36 which is a mouse monoclonal antibody generated by immunizing BALB/c mice with cellular Rb and fusing its spleen cells with NS-1 mouse myeloma cells. This antibody recognizes an epitope between amino acid 300-380 of the human Rb protein. The membrane was incubated with primary antibody overnight at 4°C and rinsed 3 times for 30 min with PBS (10 mM Na_2HPO_4, 0.9% NaCl, pH 7.5). The membrane was incubated with secondary antibody (alkaline phosphatase-conjugated affinipure rabbit anti-mouse IgG (H+L)) obtained from Jackson Immuno Research (Westgrove, PA) for 1 hr at room temperature with gentle agitation and washed 3 times for 30 min with PBS. The protein bands were visualized by addition of 10 mM Tris, pH 9.5, 10 mM NaCl, 10 mM $Mg(C_2H_3O_2)_2 \cdot 4H_2O$, 100 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), 1 μg/ml nitro blue tetrazolium (NBT) and development was stopped upon transfer to deionized water. For a negative control, Western blot analyses were done without a primary antibody incubation step (Figs. 6B, 7C, and 8C).

Results

Conservation of Rb Gene in Medaka

To detect the Rb gene of medaka, a Southern blot analysis of *Hin*dIII digested genomic DNA from medaka whole tissues was performed by using a 505 bp probe

derived from the human Rb cDNA. The amino acids of 505 bp human cDNA fragment corresponded to one of two adenovirus E1A binding sites (amino acids 405-573). Human genomic DNA was used as a positive control (Fig. 2A). Digestion of genomic DNA with *Hin*dIII resulted in an approximately 4.0 kb segment for medaka compared to a 7.5 kb band for human (Fig. 2B). The results of this experiment indicated that medaka genomic DNA contained sequences that were sufficiently homologous to be cross-hybridized with the human Rb gene probe.

Detection of the Rb mRNA in Medaka

Since human Rb cDNA probe could cross-hybridize to medaka Rb gene, detection of the Rb transcript was tested by Northern blot analysis in preliminary experiments. As shown in Fig. 3A and 3B, the Rb mRNA was detected in medaka liver and viscera. The size of the Rb transcript appeared to be about 4.7 kb which is the same size as expected in human. The same result was obtained with poly (A)-enriched RNA (Fig. 4). The size identity of Northern blot band in medaka to that in human and presence of only one band suggested that Northern blot band detected by human Rb probe was genuine medaka Rb transcript. Furthermore, the hybridization conditions employed in these experiments sufficiently high stringency (50°C in the presence of 50% formamide) which could rule out nonspecific binding of human Rb probe. *Xenopus* RNA which has been known to contain Rb mRNA (Destree et al., 1992) was used as an additional positive control (Fig. 3B).

Next, I determined which organs in adult medaka expressed Rb gene (Fig. 5 A and B). Northern blot analysis was undertaken for the RNA extracted from various medaka tissues. The Rb transcript was detected in all the tissues examined (Fig. 5B). The level of

expression of the Rb gene varied for the different tissues. Even though the level of Rb gene expression varied among tissues, liver, gastrointestinal tract, muscle, skin, and spleen were found to express relatively high level of Rb gene.

Detection of the Rb Protein in Medaka

Western blot analysis was undertaken to detect medaka Rb protein by using commercially available monoclonal antibody against human Rb. Rainbow trout liver of which Rb could be detected by the same antibody was used as a positive control. The antibody recognized a protein with an apparent molecular weight of 97 kDa from medaka liver tissue. The 97 kDa protein was thought to be Rb protein based on comigration with the positive protein from rainbow trout and lack of 97 kDa band when the primary antibody was omitted (Fig. 6A and B). There appeared an additional immunoreactive proteins at 66 and 45 kDa from the lysates of both medaka and rainbow trout. It remains unclear whether these proteins are related to Rb protein. In medaka, a number of other bands were detected between 64 and 70 kDa which I considered non-specific proteins because of inconsistent detection especially by horse radish peroxidase visualization method (Data not shown). The medaka liver, in addition, had higher molecular weight bands of approximately 116 and 160 kDa. These proteins might be highly phosphorylated form of Rb. The phosphorylation status of Rb might affect migration mode on SDS-PAGE.

In order to determine the relative expression of Rb protein in various medaka tissues, the same amount of protein was loaded onto the SDS-PAGE gel (Fig. 7 A and B). Liver had relatively high levels of Rb protein. The 97, 66, and 45 kDa bands were found in liver. Gastrointestinal tract didn't have detectable Rb protein. Eye and gill had a 45 kDa

band. Brain had the 97, 66, and 45 kDa bands. The 66 kDa was predominant in this tissue. Skin and muscle showed approximately 64, 60, and 45 kDa bands, while a 180 kDa band was present in muscle. The intensities of 97 kDa band varied among tissues. Detection of 97 kDa protein was confined to specific tissues. The 45 kDa band was present in all the tissues examined except for gastrointestinal tract. The identity of 45 kDa protein remained unclear.

The expression of the Rb protein during early developmental stages was analyzed by Western blot analysis (Fig. 8 A and B). In the early (1 day after fertilization) and late egg stages (over 6 days after fertilization), a polypeptide of molecular weight of approximately 105 kDa was found. The 105 kDa protein might be highly phosphorylated form which preferentially expressed in highly proliferative cells. However, the 105 kDa band was not detected in the fry stage when cells become less proliferative. Again, the detection of Rb was specifically confined to the certain developmental stages. There were additional bands in late egg and fry stages at 80, 64, or 60 kDa. The 45 kDa band was present in both the late egg and fry stages.

Discussion

The first objective of my experiments was to determine whether the human Rb cDNA could be used as a probe for medaka Rb. For this end, I carried out a Southern blot analysis to detect Rb gene with medaka genomic DNA. I chose a probe from the human Rb cDNA from a region which has been reported to be highly conserved in among species (human, mouse, frog, and chicken) (Destree et al., 1992; Feinstein et al., 1994). This region covers exon 13-17 of human Rb cDNA which encodes one of two E1 A binding

regions. This human Rb probe with size of 505 bp showed 73% amino acid identity in *Xenopus* (Destree et al., 1992) and 80% in chicken (Feinstein et al, 1994). I expected that there might be a similar degree of identity with medaka. With this probe I was successfully able to detect Rb gene from medaka genomic DNA. The probe was verified by hybridization to a 7.5 kb *Hin*dIII fragment containing human Rb exons 13-17 as expected (Hong et al., 1989). This evidence suggests that the Rb gene is present in the medaka.

The next objective was to detect the Rb transcript in medaka with this probe by Northern blot analysis. Human K562 cell and *Xenopus* liver total RNA were used as positive controls. A message with nearly the same size as the human Rb mRNA (approximately 4.7 kb) was detected in the medaka. There was only one size of transcript in the medaka which ruled out alternative splicing. For human, mouse, and chicken, Rb transcript has been reported to be present as a size of 4.7/4.5 kb in all tissues except mouse testis, where a 2.8 kb RNA was also found (Bernards et al., 1989). However, in *Xenopus* two major Rb RNA species (4.2 kb and 10 kb) were found in all tissues and developmental stages. The relative ratio between the expression levels of the 4.2 and 10 kb transcripts varied significantly for the different tissues (Destree et al., 1992). *Xenopus* liver RNA was used as a positive control. The 10 kb transcript was not detected in my experiment. This was not unexpected, because the 10 kb transcript was barely detectable in *Xenopus* liver in original paper (Destree et al., 1992). The nature of these mRNAs with unusual size (2.8 kb and 10 kb) is unknown at present time. In *Xenopus*, it was suggested that the 10 kb transcript may be either a precursor of the 4.2 kb transcript or alternative splicing product (Destree et al., 1992).

Since the probe selected from the conserved region of human Rb cDNA could cross-hybridize with medaka Rb gene, I became interested in Rb expression patterns in the various organs of medaka. The RNA extracted from adult medaka were examined for expression of the Rb transcript by Northern blot analysis. The 4.7 kb transcript was detected in all the organs examined, even though the level of Rb mRNA varied for the different tissues. Relatively high levels of Rb mRNA were detected in liver, gastrointestinal tract, muscle, skin and spleen, whereas low levels in brain. For comparison purpose, an exactly same amount of total RNA (20 μg) was loaded on the agarose gel. In some organs two major RNA bands, 28S and 18S rRNA, were not intact on the formaldehyde gel. Therefore, it is possible that the different quality of total RNA in each organ may have affected my results. Considering RNA from skin and spleen with blurry 28S and 18S rRNA bands showed a relatively higher level of Rb expression compared with that from eye and gill containing clear rRNA bands, the quality of RNA may not decisively attribute to differential Rb expression in various organs in this experiment. If degradation of total RNA was a major contribution to differential levels of Northern blot bands, a streaking of bands resulting from degradation of RNA should have been apparent. The use of a housekeeping gene cDNA probe such as actin cDNA, as a internal control, could have confirmed my notion. Alternatively, *in situ* hybridization can be used to determine the exact spatial Rb expression patterns in tissues.

Northern blot analyses showed that the presence of Rb transcript was ubiquitous in all the tissues tested of the adult medaka. In order to examine the Rb RNA expression levels correlate with the Rb protein levels in medaka, I carried out Western blot analyses with a monoclonal antibody against human Rb. The molecular mass of the band detected

in adult medaka tissues by western blot analyses was approximately 97 kDa in size. The molecular weight of 110-114 kDa (Lee et al., 1987b), 115 kDa (Yokota et al., 1988) or 105 kDa (Whyte et al., 1988) Rb protein has been identified mainly by immunoprecipitation approaches using tumor cells and antibodies against a typeE-Rb fusion protein or synthetic peptides deduced from the Rb cDNA sequence. The synthetic peptide corresponded to the carboxyl-terminal 15 residues or internal regions of the deduced Rb protein sequence. Also, Lee et al. (1987b) reported that a spectrum of sizes of Rb between 108 and 128 kDa has been detected from quail, mouse, rat, and monkey. In *Xenopus*, the Rb with 99 kDa was the major species (Destree et al., 1992). The size of the Rb protein appeared variable among the organisms. The source of size variation for Rb protein determined by SDS-PAGE might be due to phosphorylation. Rb has been known to be phosphorylated at sites of serine and threonine (Riley et al., 1994). The degree of phosphorylation of Rb plays regulatory role for Rb function with respect to cell proliferation. In addition to the main band, I have also observed faster migrating bands on SDS-PAGE that react with the antibody. These bands have not been characterized. The faint bands between 60 and 70 kDa seemed to be non-specific binding bands. When the protein bands were visualized by peroxidase and DAB detection method, the bands at 68 and 70 kDa turned out to be nonspecific bands (Data not shown). However, the 66 and 45 kDa bands were detected by both visualization methods (alkaline phosphatase and peroxidase). It is possible that 66 and 45 kDa bands might be derived from proteolysis of the 97 kDa band. Therefore, three protein bands, 97, 66, and 45 kDa seemed to be either Rb or Rb-related proteins. This hypothesis is supported by a recent report (An and Dou, 1996) that hypophosphorylated Rb protein was cleaved to two fragments, 68 and 48 kDa

which are remarkably close to what I have observed. Proteolytic cleavage of Rb protein was implicated to involve in apoptosis (programmed cell death) (An and Dou, 1996). Therefore, identification of Rb proteins by Western blot analysis in various tissues would provide important information on the role of Rb in medaka.

The presence of the Rb protein was tested in various tissues that were used for Northern blot analysis. The relative detection levels between Rb transcript and protein were not correspondingly relevant in tissues tested. Liver had relatively high level of both Rb transcript and protein, whereas brain had high Rb protein but low Rb transcript. Rb protein (97 kDa) was weakly detected in muscle, skin, and spleen, even though the expression of the Rb transcript was high. In the gastrointestinal tract, Rb protein was not detected. Gastrointestinal tract showed very faint protein bands in coomassie blue stain compared with those of other organs even the same concentration of protein was used. Due to high contents of proteases in gastrointestinal tract, proteins in the organ might be degraded to small fragments. Therefore, the poor detection of Rb protein in the gastrointestinal tract might be due to loss by proteolytic degradation. Although Rb transcript was ubiquitously expressed in medaka, the Rb protein was not present in all medaka tissues examined. These observations suggest that regulation of the Rb function may not be at the level of transcription but at translation or post-translational modification, such as phosphorylation in medaka. The rate of translation might vary for the different tissues. Another possibility is that turn-over rate of Rb protein might be different in each tissues. Riley et al. (1994) insisted that since Rb protein is constitutively expressed in normal cells, has a half-life of at least 12 hr, and is present in all mammalian cells tested to date, regulation of Rb must be accomplished post-translationally. Rb is activated and

deactivated at appropriate times during cell cycle mainly through dephosphorylation and phosphorylation process. At the same time, apoptosis enhances cleavage of Rb protein by interleukin 1β-converting enzyme (ICE)-like protease (An and Dou, 1996). Therefore, medaka could provide an excellent system by which physiological role of Rb can be investigated in embryonic development, cell proliferation, and cell death.

The levels of Rb protein in early medaka development are shown in Fig. 8. I chose three developmental stages used by Kirchen and West (1976) to determine the expression of the Rb protein; early egg stage (1 day after fertilization), late egg stage (over 6 days after fertilization, most organs are formed) and fry stage (after hatching). The result showed that the 105 kDa band was detected in the egg stages, but not in the fry stage. The similar protein banding pattern was observed using peroxidase and DAB detecting method (Data not shown), suggesting that protein bands detected were all Rb-related. Interestingly in early egg stage, only one protein band (105 kDa) was detected. Later, several new bands appeared as embryonic development progressed to late egg and fry stage. Based on the result, I could draw a correlation that at early egg stage 105 kDa Rb was dominant and as tissue further developed up to fry stage, 105 kDa became cleaved to smaller fragments. This correlation was intriguing because 105 kDa Rb only appeared on the egg stage where cell proliferation is highly active.

One day after fertilization medaka embryos are in late gastrula or early neurula stage (Kirchen and West, 1976). The zygote nucleus undergoes a series of mitotic divisions after fertilization. The resulting daughter nuclei usually become partitioned off in separate cells fashioned from the cytoplasm of the zygote. This cleavage stage is followed by a morphogenesis stage. During the morphogenesis stage, the many cells produced by

cleavage continue to divide but segregate themselves into distinct layers and masses. Although the cells of the embryo during this phase of development are organized into distinct groups, they are all similar in structure. At this early stages, tissues (or organs) are not yet formed and cells have not reached to the point where the cell cycles are arrested to differentiate. At this high proliferative stage, Rb protein maintained as a 105 kDa in size, probably hyperphosphorylated Rb form. The hyperphosphorylated form of Rb, but not the hypophosphorylated form, has been suggested to involve in cell proliferation by others (DeCaprio et al., 1989; Chen et al, 1989).

In late egg stage (over 6 days after fertilization), the rudiments of most organs are developed by cell differentiation. This is the developmental stage where in addition to 105 kDa Rb new smaller forms of Rb, probably resulting from proteolytic cleavage, began to appear in medaka. Therefore, the timing of cleavage of Rb protein was coincident with the developmental stage where cell differentiation or apoptosis might occur.

All primitive organ systems are represented at fry stage. New smaller immunoreactive proteins also appeared at fry stage. The significance of these smaller proteins in late egg and fry stages remains to be determined at present. It is possible that faster migrating bands are due to proteolysis by interleukin 1β-converting (ICE)-like protease as proposed by An and Dou (1996). Therefore, it is possible that these bands are developmental stage-specific proteins. In *Xenopus*, additional immunoreactive bands at 60, 80, and 180 kDa besides the size of Rb have been detected in early developmental stages. Especially, the level of 180 kDa protein appears to be stage-specific in *Xenopus*. The low level of this protein in the unfertilized egg and in stage 3 embryos increased in embryos between stage 8 and stage 11.5 (Destree et al., 1992). In fry stage, 105 kDa band

completely disappeared and at the same time low molecular weight proteins appeared. The coincident timing of disappearance of the 105 kDa protein and appearance of the small size proteins suggested that the 105 kDa protein might be broken down to small fragments in this stage by protease. Based on the result, I can establish a hypothesis. The 105 kDa (hyperphosphorylated form) protein begin to hypophosphorylate (97 kDa) by phosphatase at late egg stage (apoptotic commitment stage). Then the Rb cleavage enzyme such as an ICE-like protease cleaves the newly formed hypophosphorylated Rb to small fragments at fry stage (apoptotic execution stage). Therefore, 105 kDa band disappears and small fragments appear at fry stage.

To date the description of functional biochemistry of Rb protein as a tumor suppressor has been delineated considerably. However, it has not been clear whether Rb plays an important role in normal embryonic development. Several groups (Lee et al., 1992; Jacks et al., 1992; Clarke et al., 1992) have reported that Rb is required for normal development. To understand the role of Rb in embryonic development they constructed a mouse strain in which one allele of Rb is disrupted. Homozygous mutants die between day 14 and 16 of gestation with multiple defects in neural and haematopoietic development while the heterozygous mice appeared normal without developing retinoblastoma. Thus Rb is essential for normal development.

An examination of the expression of the retinoblastoma gene and the regulation of its protein product in medaka should help us to understand the role of this tumor suppressor gene during embryonic development because medaka is the only species in which retinoblastoma can be induced by carcinogens. Thus this study could lead us to establish an excellent model with medaka for understanding cancer such as retinoblastoma.

Figure 1. Schematic representation of the structure of the human Rb protein.

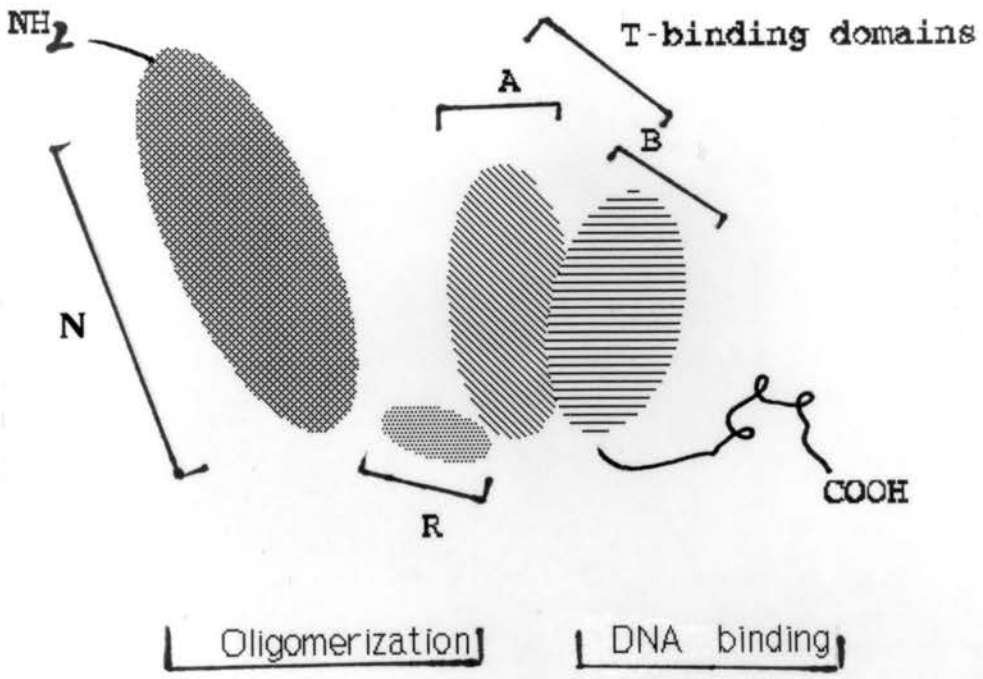
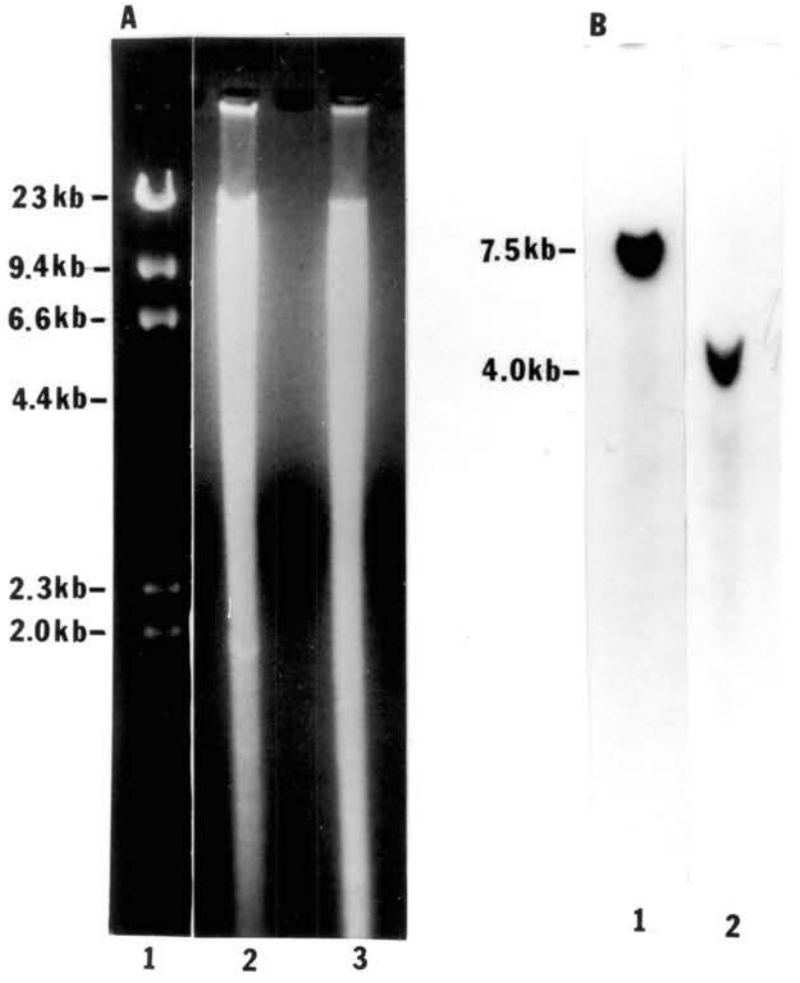

NH2
T-binding domains
A
B
N
R
COOH
Oligomerization
DNA binding

Figure 2. Southern blot analysis of *Hin*dIII-digested genomic DNA. (A) Human genomic DNA (10 μg) and medaka genomic DNA (10-15 μg) were digested with *Hin*dIII restriction enzyme. DNA was electrophoresed on a 0.8% agarose gel. Lanes:1, HindIII-digested phage λ DNA molecular size marker; 2, Human genomic DNA; 3, Medaka genomic DNA. (B) Blot was probed with a 505 bp fragment of human Rb cDNA. Hybridization was performed as described in Materials and Methods. Lanes:1, Human; 2, Medaka.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7.5kb
4.0kb
1
2

Figure 3. Detection of the Rb gene in various organisms. (A) RNA was isolate from each organism. Total RNA (20 μg) was fractionated on a 1% formaldehyde agarose gel. Position of the 28S and 18S ribosomal RNA are indicated (4.2 and 1.7 kb). (B) Blot was probed with the 505 bp fragment of the human Rb cDNA. Hybridization was performed as described in Materials and Methods. Lanes of (A) and (B):1, Human; 2, Medaka liver; 3, Medaka viscera 4, Rainbow trout; 5, Frog.

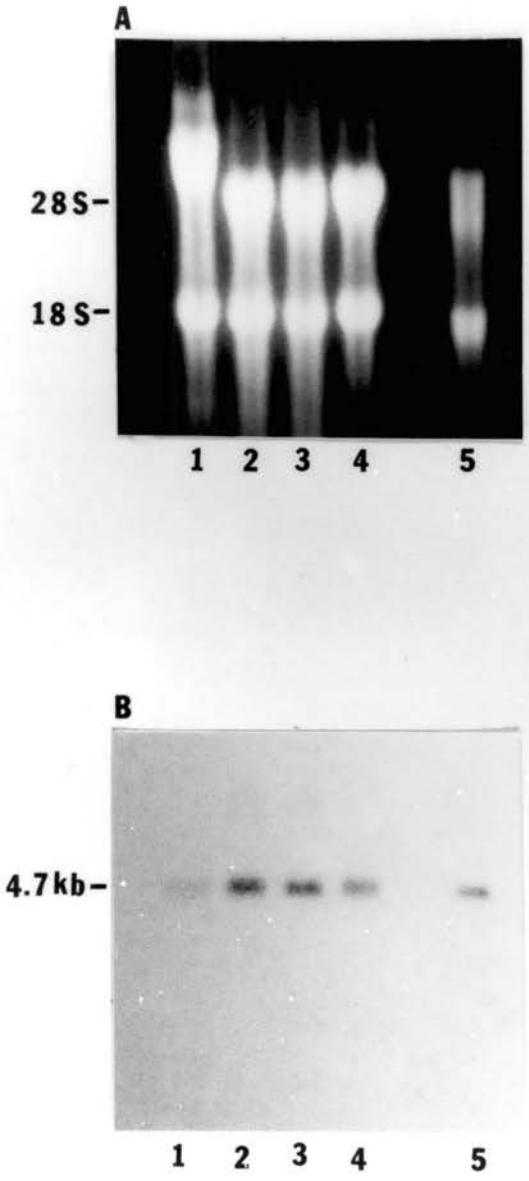

A
28S-
18S-
1 2 3 4 5
B
4.7kb-
1 2 3 4 5

Figure 4. Northern blot analysis of poly (A)-enriched RNA. mRNA was isolated from each organism and hybridization was performed as described in Materials and Methods. Lanes: 1, Human; 2, Medaka viscera; 3, Medaka liver; 4, Rainbow trout.

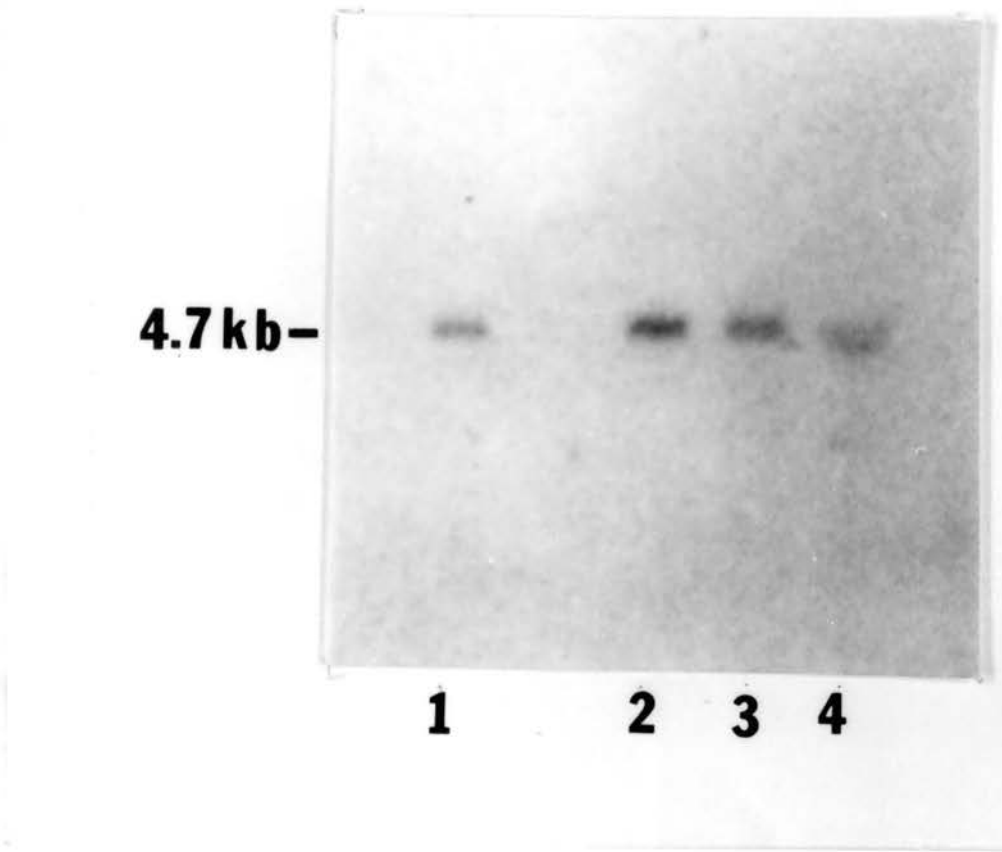

4.7kb–
1
2
3
4

Figure 5. Detection of the Rb mRNA in adult medaka. (A) RNA was isolated from organs of adult medaka. Total RNA (20 μg) was fractionated on a formaldehyde 1% agarose gel. Position of the 28S and 18S ribosomal RNA are indicated (4.2 and 1.7 kb). (B) Blot was probed with the 505 bp fragment of the human Rb cDNA. Lanes: 1, Human K562 cells; 2, Liver; 3, Gastrointestinal tract; 4, Eye; 5, Gill; 6, Brain; 7, Muscle; 8, Skin; 9, Spleen.

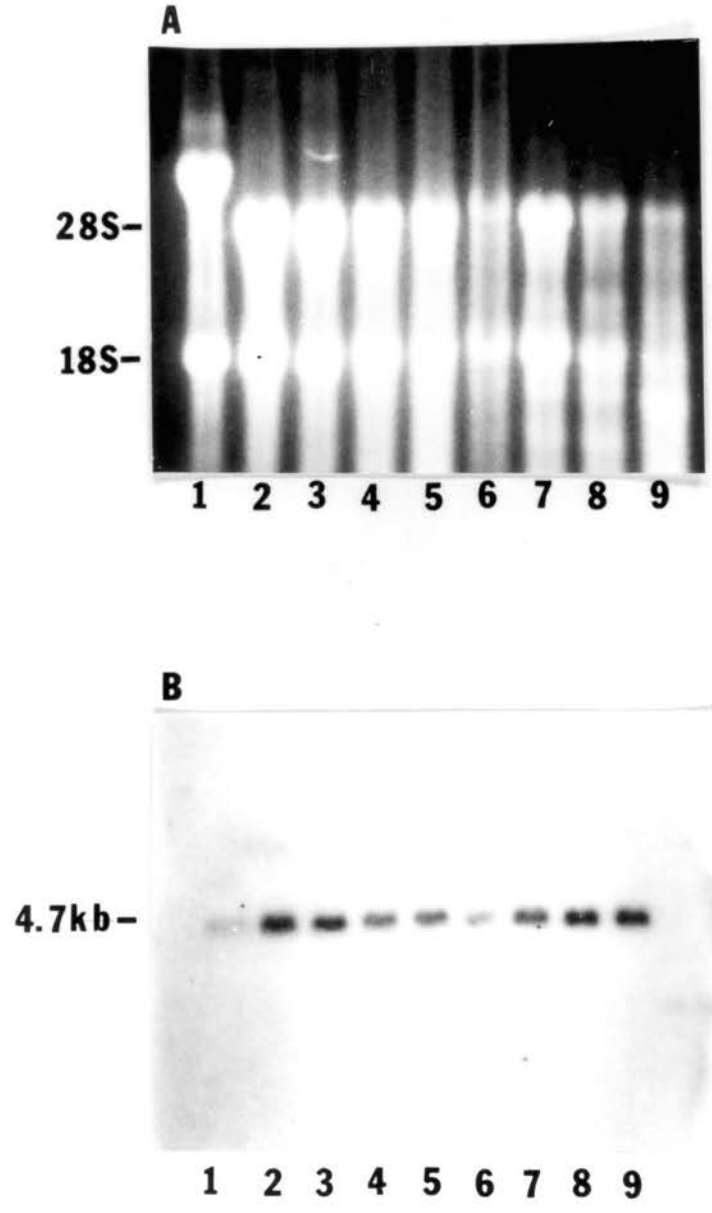

A
28S–
18S–
1 2 3 4 5 6 7 8 9
B
4.7kb–
1 2 3 4 5 6 7 8 9

Figure 6. Detection of the Rb protein in medaka. (A) 30 μg of the protein from each sample was electrophoresed on 7.5% SDS-PAGE followed by visualization with anti-Rb antibody. Lanes: 1, Molecular weight standards; 2, Rainbow trout; 3, Medaka liver; 4, Medaka eye. (B) Negative control in which samples were not incubated with primary antibody. Lanes: 1, Rainbow trout; 2, Medaka liver; 3, Medaka eye.

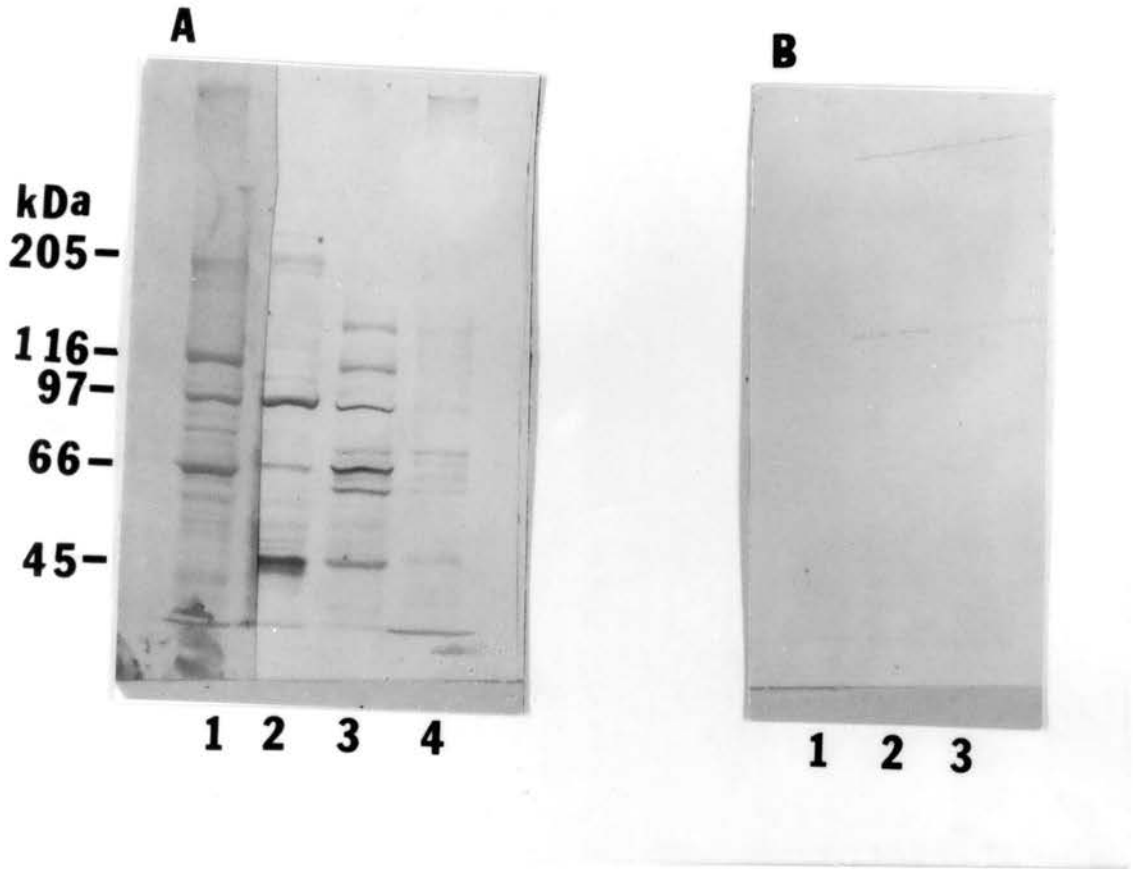

A
B
kDa
205-
116-
97-
66-
45-
1 2 3 4
1 2 3

Figure 7. Detection of the Rb protein in various organs of medaka. (A) 30 μg of protein from each organ was electrophoresed on SDS-PAGE and proteins were stained with coomassie blue. (B) Membrane was stained with anti-Rb antibody and visualized with alkaline phosphatase. (C) Negative control in which the membrane was not incubated with anti-Rb antibody. Lanes of (A), (B), and (C): 1, Liver; 2, Gastrointestinal tract; 3, Eye; 4, Gill; 5, Brain; 6, Spleen; 7, Skin; 8, Muscle.

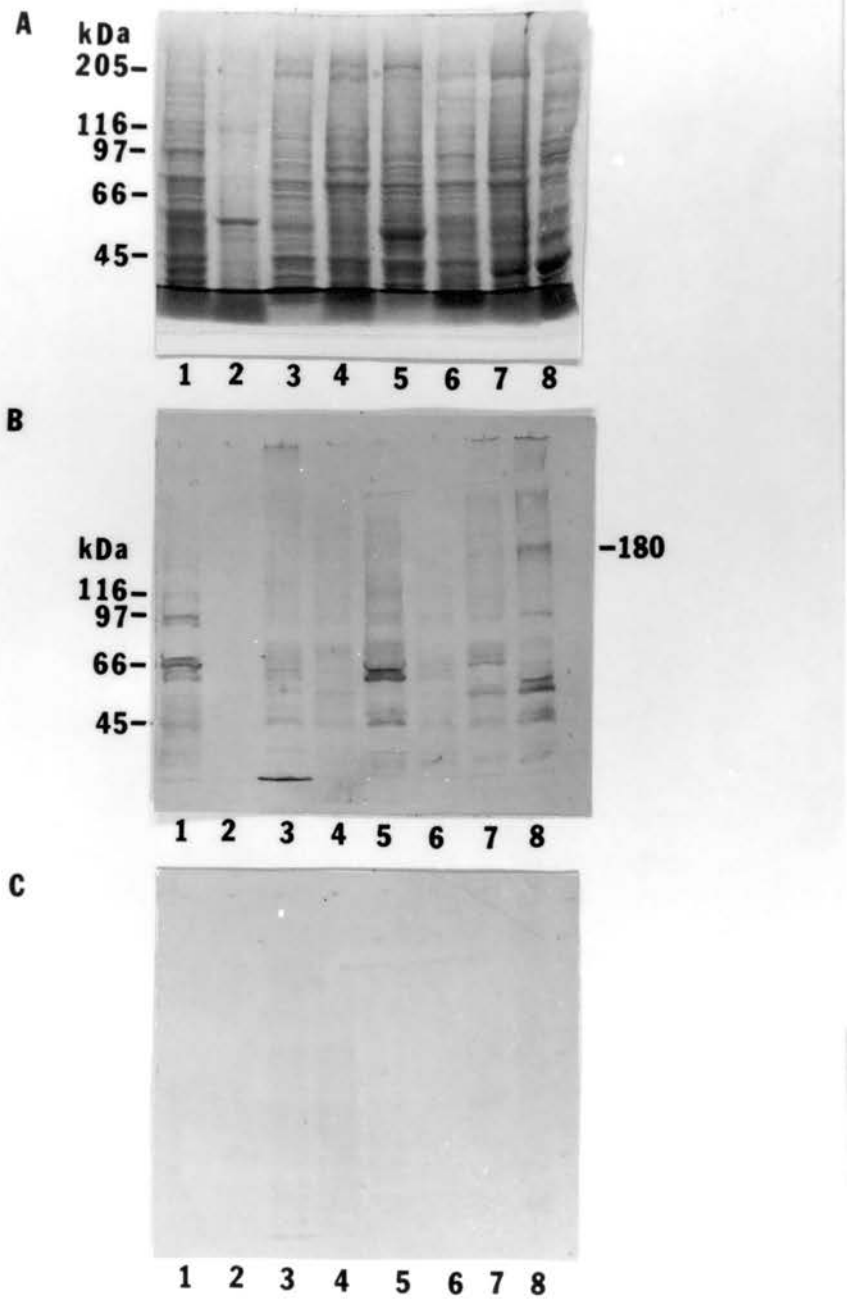
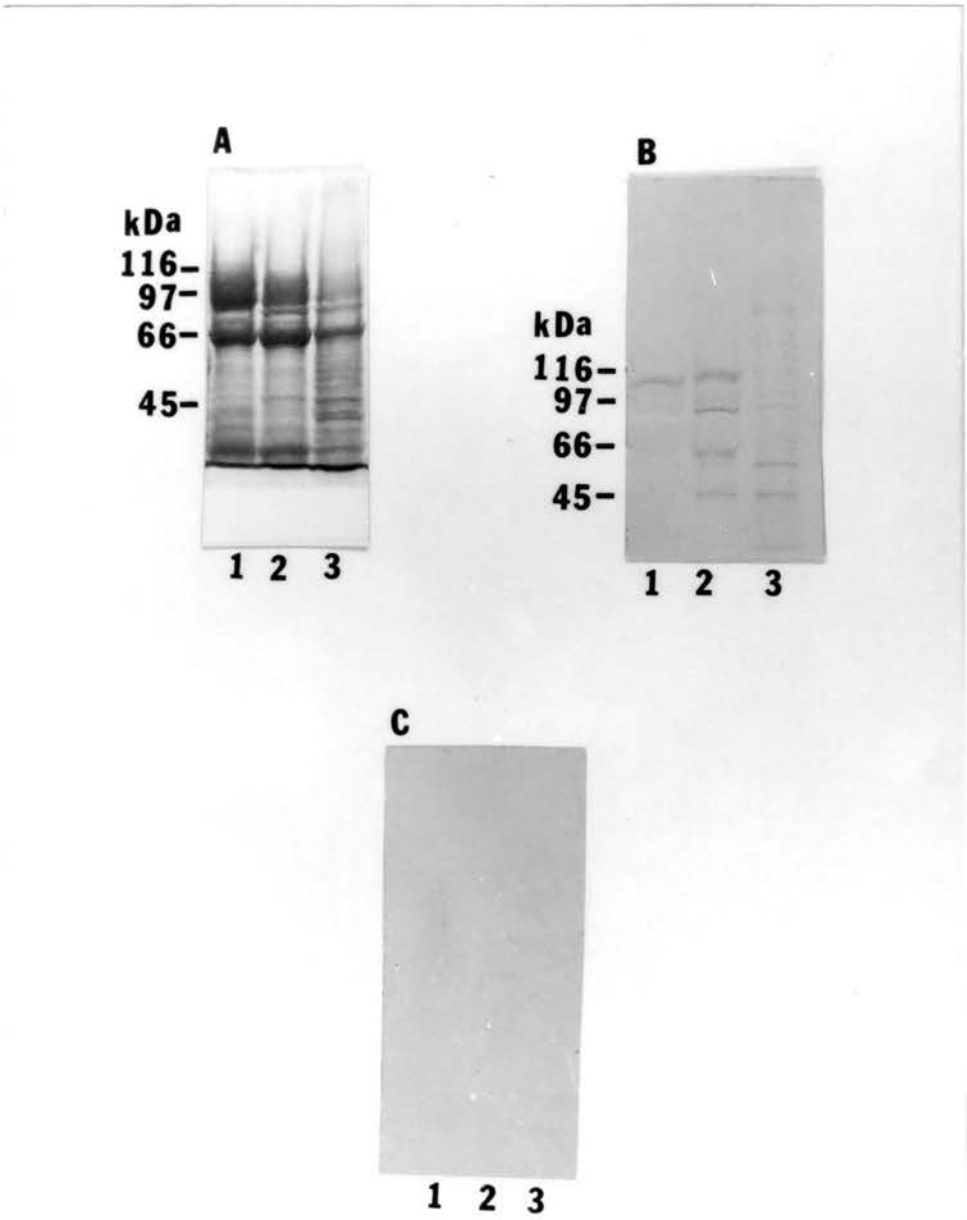

A
kDa
205-
116-
97-
66-
45-
1 2 3 4 5 6 7 8
B
kDa
116-
97-
66-
45-
-180
1 2 3 4 5 6 7 8
C
1 2 3 4 5 6 7 8

Figure 8. Detection of the Rb protein in the early developmental stages of medaka. (A) 30 μg of protein from each sample was electrophoresed on a 7.5% SDS-PAGE and proteins were stained with coomassie blue. (B) Membrane was stained with anti-Rb antibody and visualized with alkaline phosphatase. (C) Negative control in which the membrane was not incubated with anti-Rb antibody. Lanes of (A), (B),and (C): 1, early egg stage (1 day after fertilization); 2, late egg stage (over 6 days after fertilization); 3, fry stage.


A
kDa
116-
97-
66-
45-
1 2 3
B
kDa
116-
97-
66-
45-
1 2 3
C
1 2 3

CHAPTER III

CONSTRUCTION AND SCREENING OF A cDNA LIBRARY WITH mRNA FROM MEDAKA LIVER

Introduction

A candidate for the retinoblastoma suppressor gene (Rb) has been cloned and characterized (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987a). The isolation of the retinoblastoma gene has allowed a detailed analysis of the nature of the gene loss at a molecular level. After the identification of the first tumor suppressor gene, Rb, researchers have attempted to find new tumor suppressor genes and study their involvement in human cancer.

To date, full-length Rb cDNAs have been sequenced from human, mouse, frog, and chicken. Although Rb has been associated with cell cycle control, transcriptional control and growth inhibition, its exact functions have not been determined (Feinstein et al., 1994). To determine the evolutionary conservation of the Rb gene and to understand the molecular basis of Rb inactivation and its potential role in other tumors, I wanted to isolate and clone the Rb gene from medaka. The retinoblastoma was induced in medaka by a chemical carcinogen. A vertebrate model for retinoblastoma could be established using medaka.

Two cDNA libraries were used to screen the medaka's Rb gene; one was made in

Lambda ZAPII vector (Stratagene) by Dr. Becky Van Beneden's laboratory and the other was made in Lambda gt11 by our laboratory. As detailed below I screened both cDNA libraries with several different probes. During these experiments, I attempted to develop the medaka Rb cDNA probe using RT-PCR.

Materials and Methods

Animals-- Medaka (*Oryzias latipes*) used in this study were obtained from our breeding colony at Oklahoma State University.

Chemicals--A cDNA library was constructed by Dr. Becky Van Beneden using total RNA from medaka liver. The library was made in Lambda ZAPII (Stratagene). The titer of the amplified library was 3.17×10^6 pfu/µl. An aliquot of this library was donated by Dr. B. V. Beneden. PolyATtract® mRNA Isolation Systems was from Promega (Madison, WI). cDNA Synthesis kit, Lambda gt11 /*Eco*R I / CIAP-Treated Vector kit, and Gigapack ® II packaging extracts were obtained from Stratagene (Melville, NY). Super-Script Preamplification system for RT-PCR was obtained from GibcoBRL (Gaithersburg, MD). Guanidium thiocyanate and diethyl pyrocarbonate (DEP) were from Sigma Chemical Company (St. Louis, MO). Alpha-(^{32}P) dCTP (3000Ci/mmol) was from New England Nuclear (Boston, MA). All other reagents were of analytical grade or better.

Construction of a cDNA Library in Lambda gt11-- a cDNA library was made by cDNA Synthesis kit according to the manufacturer's protocol. The brief procedures were:

Poly (A) (7 μg) was used to synthesize cDNA using linker primer, random 9 mer-primer, and 5′-methyl-dCTP to make the first strand. After second-strand synthesis and ligation to *Eco*R I adapters, cDNA was size-fractionated through a Sephacryl S-400 column. The fraction eluted at > 0.5 kb was ligated into λ gt11 vector and packaged with Stratagene's Gigapack II Gold. The resulting library was amplified and used for the screening. All buffers for the construction of cDNA library were from the kit.

(1) RNA and mRNA Isolation-- Total RNA was isolated from 50 adult medaka livers by the method of Chomzynsky and Sacchi (1987) as described in Materials and Methods of Chapter II. mRNA was prepared using the PolyATtract® mRNA Isolation Systems. The detailed procedures were described in Chapter II. The A_{260}/A_{280} ratio of mRNA was ≈2.1. From 1 mg of total RNA, 8.5 μg of poly(A)-enriched RNA was obtained.

(2) Bacteria Strain and Media-- *E. coli* strain Y1088 (Stratagene: e14⁻(*mcrA*), *(lac)U 169, supE, supF, hsdR, metB, trpR, tonA21, proC::*Tn5 (kan^r) [pMC9$amp^r tet^r$], Note: pMC9 is pBR322 with $lacI^q$ inserted.) was used for all manipulation of the λ gt11 library. The following media were used for propagating *E. coli* and the bacteriophage λ: LB (per liter: 10 g tryptone, 5 g yeast extract, 5 g NaCl, pH 7.0), bottom agar (LB plus 15 g/liter agar with 0.2% maltose-10 mM $MgSO_4$), top agarose (LB plus 7 g/ liter agarose), and SM buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 10 mM $MgSO_4$, and 0.1% gelatin).

(3) First-Strand cDNA Synthesis-- Poly (A) (7 μg in 34 μl DEP H_2O) was added to 16 μl of the first strand synthesis reaction mixture (5μl 10X first-strand buffer, 3 μl first-strand methyl nucleotide mixture, 2 μl linker-primer (1.4 μg/μl), 1 μl random 9 mer-primer

(80 ng/μl), 1 μl RNase block I (40 U/μl), 0.5 μl α-^{32}P-labeled dATP (800 Ci/mmol), and 3.5 μl MMLV-RT (20 U/μl). The reaction mixture was incubated at 37°C for 1 hr and then kept on ice until the second-strand synthesis.

(4) Second-Strand Synthesis-- To 45 μl of first-strand reaction mixture, the following reagents were added in order: 20 μl 10X second-strand buffer, 6 μl second-strand nucleotide mixture, 106.2 μl sterile distilled water, 2 μl α-^{32}P-labeled dATP (800 Ci/mmol), 3.5 μl RNase H (0.9 U/μl), and 10 μl DNA polymerase I (10 U/μl). The mixture was incubated at 16°C for 2.5 hr and immediately placed on ice.

(5) Blunting the cDNA Termini-- 23 μl of blunting dNTP mix and 2 μl of cloned *Pfu* polymerase were added to the reaction tube. The reaction was incubated at 72°C for 30 min. The reaction was terminated by extraction with 200 μl phenol:chloroform (1:1 v/v). After extraction with another equal volume of chloroform, the cDNA was precipitated with 1/10 volume of 3 M sodium acetate and 2 volumes of ethanol overnight, then washed with 70% ethanol and dried. The pellet was resuspended in 9 μl of *Eco*R I adapters.

(6) Ligating the *Eco*R I Adapters-- The following components were added to the tube containing the blunted cDNA and the *Eco*R I adapters: 1μl 10X ligase buffer, 1 μl 10 mM γATP, and 1 μl T4 DNA ligase (4U/μl). The reaction mixture was incubated at 8°C overnight and the reaction was terminated by heating at 70°C for 30 min.

(7) Kinasing the *Eco*R I Ends-- The kinasing of the adapter ends was done by adding 1 μl 10X ligase buffer (500 mM Tris-HCl, pH 7.5, 70 mM $MgCl_2$, and 10 mM dithiothreitol), 2 μl 10 mM γATP, 6 μl sterile water, and 0.7 μl T4 polynucleotide kinase

(10 U/μl). The reaction mixture was incubated at 37°C for 30 min. The kinase was inactivated by heating at 70°C for 30 min.

(8) *Xho* I digestion-- cDNA Synthesis kit from Stratagene was designed for UniZAp™ vector. For this vector, *Xho* I restriction is needed for the orientation of insert DNA. However, λ gt11 vector arms with dephosphorylated *Eco*R I sites at each end were used and *Xho* I digestion was omitted. Tweenty eight μl of *Xho* I buffer were added to adjust salt concentration for subsequent steps and 3 μl of sterile water were substituted for *Xho* I enzyme to adjust the volum of reaction. Five μl of 10X STE buffer were added to the reaction mixture for Sephacryl S-400 column separation.

(9) Size Fractionation-- The cDNA was loaded onto a Sephacryl S-400 column and spun at 400 x g for 2 min. Fractions were collected four times. The size of the cDNA was checked by running a 1% agarose gel and visualizing using autoradiography. The cDNA was quantitated using the ethidium bromide plate assay according to the manufacturer's protocol. The fraction contained > 0.5 kb cDNA was used for ligating into the vector arms.

(10) Ligating cDNA into Lambda gt11/*Eco*R I/CIAP-treated Vector Arms-- The total ligation reaction volume included 1 μl Lambda gt11/*Eco*R I prepared arms(1 μg), 2.5 μl cDNA (50 ng/μl), 0.5 μl 10X ligation buffer, 0.5 μl 10 mM γATP, and 0.5 μl T4 ligase (4 U/μl). The ligation mixture was incubated at 12°C overnight.

(11) Packing-- The cDNA (1 μl ligation reaction) was immediately added to the Freeze-Thaw extract, then the Sonic extract was added to the Freeze-Thaw extract containing the DNA. The packaging mixture was incubated for 2 hr at room temperature.

The packaging was ended by adding 500 µl SM buffer and 20 µl chloroform, and then stored at 4°C.

(12) Plating and Titering the Primary cDNA Library-- The packaged ligation product (1 µl of 1:1 and 1:10) was mixed with 200 µl of Y1088 cells (A_{600} = 0.5) and incubated at 37°C for 30 min with gentle shaking. The 3 mls of top agarose (with 15 µl of 0.5 M IPTG and 50 µl X-gal of 250 mg/ml) were added and the bacteria were plated onto the LB plates. The plaques were counted after incubating at 37°C for 7-9 hr.

(13) Amplification of the λ gt11 Library-- The packaged mixture (20 aliquots) which contained 50,000 plaque-forming bacteriophage units (pfu) was mixed with 600 µl of A_{600} = 0.5 host cells (Y1088) and incubated at 37°C for 30 min. The 8 mls of top agarose were mixed with each aliquot of infected bacteria, which were then spread onto a freshly poured 150 mm plate of bottom agar. The harvesting of the bacteriophage was done after 6-8 hr incubation at 37°C by overlaying the plate with 10 ml of SM buffer and storing it at 4°C overnight. The recovery of the bacteriophage with 5% chloroform was followed by centrifugation. The amplified library was then stored at 4°C with 0.3% chloroform. Some aliquots were stored in 7% dimethylsulfoxide (DMSO) at -85°C. The remaining of aliquots were stored at 4°C. The titer of the amplified library was checked in the same way as for the primary library.

cDNA Library Screening

(1) Bacterial Strains-- *E. coli* strain XL 1-Blue (Stratagene: *recA1, endA1, gyrA96, hsdR17, supE44, relA1, lac,* [F′ *proAB, lac*^{q}*ZΔM15*, Tn *10* (tet^r)]) was used for the λ ZAPII library screening. *E coli* strain SOLR™ (Stratagene: e14$^-$ (*mcrA*$^-$), Δ(*mcrCB-*

hsdSMR⁻mrr) 171, *sbcC, recB, umuC::Tn 5* (kan^r), *uvrC, lac, gyrA96, relA1, endA1*, λ^R, [F′ *proAB lacI^{q}ZΔM15* Tn *10*] Su⁻ (nonsupressing)) was used for plating excised phagemids of the λ ZAPII library. *E. coli* stain Y1088 was used for the λ gt11 cDNA library screening.

(2) λ ZAPII cDNA Library Screening-- The amplified cDNA was plated on 150 mm LB plates to 50,000 pfu/plate with 600 μl XL 1 Blue cells at A_{600} = 0.5/plate and 8 mls of top agarose/plate (Twenty 150 mm plate used to screen 1 x 10^6 pfu). The plates were incubated at 37°C for 6-8 hr. Each individual plate with plaques was covered with a piece of labeled nitrocellulose filter for 2 min to transfer DNA. The filters were lifted off, denatured by submersion in a 1.5 M NaCl/0.5 M NaOH solution for 2 min, neutralized in a 1.5 M NaCl/0.5 M Trs-HCl (pH 8.0) solution for 5 min, and then rinsed in a 0.2 M Tris-HCl (pH 7.5) solution for 30 sec. The DNA was crosslinked to the membranes using the autocrosslink setting on the UV crosslinker (120,000 μJ of UV energy, UV Stratalinker™ 2400 from Stratagene). Hybridization and filter washes were performed using the same method described for Southern blot analysis in Chapter II. The positive plaques were identified by exposure to Kodak XAR-5 film. After film development, the putative clones were picked from master plates and stored in 1 ml of SM buffer including 20 μl of chloroform. The second and third screenings were done the same way as the first screening using small plates (100 mm).

(3) *In vivo* Excision of the pBluescript Phagemid from the λ ZAPII Vector-- An *in vivo* excision protocol using the ExAssist helper phage with SOLR strain (Stratagene) was used to isolate pBluescript phagemid containing the cloned insert from the vector. In a

50 ml polycarbonate tube, 200 µl of XL 1 Blue cells (A_{600} = 1) prepared with 10 mM $MgSO_4$, 250 µl of phage stock from the third screening (containing > 1 x 10^5 phage particles), and 1 µl of the ExAssist helper phage (> 1x 10^6 pfu/µl) were combined and incubated at 37°C for 15 min. Three mls of LB broth were added and the mixture was incubated for 2-2.5 hr at 37°C with shaking. The *in vivo* excision was ended by heating the mixture at 70°C for 15 min and centrifugation (4000 x g, 15 min). The phagemid stock was stored at 4°C. This stock (10 µl or 100 µl) was mixed with 200 µl of SOLR cells (A_{600} = 1) prepared with 10 mM $MgSO_4$, incubated at 37°C for 15 min, and plated onto 100 mm LB-ampicillin plates (50 µg/ml). The plates were incubated at 37°C overnight. Colonies appearing on the plate contain the pBluescript double-stranded phagemid with the cloned DNA insert. The colony was streaked on a new LB-ampicillin agar plate to maintain the pBluescript phagemid. A bacterial glycerol stock for each putative clone was prepared and stored at -85°C. Each phagemid contained the positive insert was prepared for Southern blot analysis and sequencing using Wizard™ Miniprep DNA Purification System (Promega).

(4) Lambda gt11 cDNA Library Screening-- The method for the screening of this library was the same as that of the screening of λ ZAPII cDNA. The Y1088 host strain was used instead of XL 1 Blue cell.

(5) The Preparation of Bacteriophage DNA from λ gt11 cDNA Library-- Lambda DNA was purified with Wizard™ Lambda Preps DNA Purification System (Promega). Phage lysate was prepared by adding 500 µl of the overnight host cell (Y1088) to a microcentrifuge tube containing 20 µl of the amplified cDNA library. The tube was

incubated at 37°C for 20 min. The infected culture was transferred to 50 mls of prewarmed LB broth supplemented with 0.5 ml of 1 M $MgSO_4$ and shaken with 200 rpm in an orbit environ shaker (Lab Line Instruments Inc.) at 37°C until cell lysis occurred. After lysis, 500 μl of chloroform were added to the lysate. The lysate was centrifuged at 8,000 x g for 10 min and the supernatant was transferred to a sterile tube for the lambda DNA purification. Forty μl of resuspended nuclease mixture (0.25 mg/ml RNase A, 0.25 mg/ml DNase I, 150 mM NaCl, and 50% glycerol) were added to 10 mls of lysate. The lysate was incubated at 37°C for 15 min. Four mls of phage precipitant (33% polyethylene glycol 8,000 and 3.3 M NaCl) were added to the tube. The mixture was placed on ice for 30 min and centrifuged at 8,000 x g for 10 min. The supernatant was removed and the pellet was resuspended in 500 μl of phage buffer (150 mM NaCl, 40 mM Tris-HCl, pH 7.4, and 10 mM $MgSO_4$). The resuspended phage was transferred to a microcentrifuge tube, treated with proteinase K, and centrifuged at 12,000 x g for 10 sec. The supernatant was transferred to a new tube and 1 ml of purification resin was added. Then lambda DNA containing the inserts was purified with a Wizard minicolumn. The purified lambda DNA was extracted using phenol:chloroform and precipitated with ethanol. The lambda DNA (30 μg) was digested with *Kpn* I and *Sac* I. The digested sample was separated using 1% agarose gel electrophoresis and then blotted to a nylon membrane. The Southern blot analysis was performed in 50% formamide, 4X SSPE, 7% SDS, 1% PEG 20,000, 0.5% blotto, and 0.5 mg/ml salmon sperm DNA. The λ gt11 vector itself was used as a negative control.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

(1) RNA Isolation-- The modified Chomzynsky and Sacchi method (1987) was used as described in Chapter II to isolate total RNA from medaka liver. Typical yield was 0.6-1 μg RNA/mg tissue. The quality of RNA was checked using a spectrophotometer. The A_{260}/A_{280} ratio was over 1.9 and the RNA was undegraded as judged by clear definition of the 28S and 18S rRNA bands.

(2) cDNA Synthesis-- The cDNA template for RT-PCR was synthesized from RNA by reverse transcription. cDNA synthesis was performed using Super-Script Preamplification System (GibcoBRL) according to the manufacturer's protocol using 43°C for reverse transcription and MMLV reverse transcriptase. Random primers (hexamers having all possible nucleotides at each position) that annealed randomly to the RNA molecules were used to prime the cDNA synthesis. A reaction mixture, which contained everything except for reverse transcriptase, was used as a negative control. As another negative control, a sample of the RNA to be used for RT-PCR was digested with RNase. Any bands generated would indicate that genomic DNA was present.

(3) PCR Amplification-- The RT-PCR primers were based on the sequence of the human Rb cDNA. Human Rb primers were donated by Dr. S.-H. Park at Whitehead Institute. The sequence of the primer pairs was as follows:

$^{5'}$CATCTAATGGACTTCCAGAG$^{3'}$ (R 54) and $^{3'}$TCAAGATTCTGAGTTGTACT$^{5'}$ (R 32) (corresponding to the human Rb cDNA exon 10 to 16)

$^{5'}$CAGCAGTTCGATATCTACTG$^{3'}$ (SH 51) and $^{3'}$TATAGAATCAGTCTGAAGAG$^{5'}$ (R34) (corresponding to the human Rb cDNA exon 6 to 10)

$^{5'}$CATCTAATGGACTTCCAGAG$^{3'}$ (R 54) and $^{3'}$TATAGAATCAGTCTGAAGAG$^{5'}$ (R 34) (corresponding to the human Rb cDNA exon 10).

Amplification reactions were prepared in volumes of 15 μl containing 1X PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 0.001% gelatin), 1 mM $MgCl_2$, 1.7 μM of each primer, 3 μl cDNA and 0.75 U of AmpliTaq polymerase (Perkin-Elmer Cetus). Amplification was performed using a Perkin-Elmer Cetus GeneAmp PCR System 9600; 2 cycles were performed of 2 min at 94°C, 30 sec at 55°C, and 2 min at 72°C. Forty cycles were then performed of 1 min at 94°C, 30 sec at 55°C, and 2 min at 72°C. A 30 sec ramp time was inserted between 55°C and 72°C, and 72°C and 94°C. A 1 min ramp was inserted between 94°C and 55°C. The samples were subjected to a 10 min final extension at 72°C at the end of 40 cycles. After the amplification was complete, 5-10 μl of the reaction mixture were analyzed on a 1% agarose gel. 123-bp and 1 kb DNA ladders (Bethsda Research Laboratories) were used as size standards.

(4) Hybridizations to p3.8 Rb in pUC 13 and/or p0.9 Rb in Sp 65--The RT- PCR fragments were labeled with PCR using 50 μCi α-^{32}P-dCTP and used as probes. One μg of p3.8 Rb in pUC 13 plasmid digested with *Eco*R I was loaded on a 1% agarose gel, electrophoresed, and transferred to a nylon membrane (Zeta Probe; Bio-Rad). Hybridization was performed at 42°C overnight in 6X SSC, 10 mM EDTA, 5X Denhardt's solution, 0.5% SDS, 100 μg/ml denatured salmon sperm DNA, 10% dextran sulphate, and 50% formamide. The filter was washed twice with 0.2X SSC/0.1% SDS at 42°C for 15 min, then twice with 0.1X SSC/0.1% SDS at 65°C for 15 min and exposed to Kodak XAR -5 film for 1-2 days at -85°C with an intensifying screen.

(5) Southern Blot Analysis with Medaka Genomic DNA -- Genomic DNA was extracted from whole medaka as described in Chapter II and digested with *Hin*dIII or *Eco*R I restriction enzymes. The RT-PCR fragments were used as probes. Hybridization was performed in 40% formamide, 6X SSC, 5X Denhardt's solution, 0.1 mg/ml salmon sperm DNA, 0.5% SDS, and 0.1 M phosphate buffer, pH 7.0 at 42°C overnight.

Results

Construction of medaka cDNA Library

RNA and mRNA were isolated from medaka liver to construct a cDNA library. Before making the cDNA library, the quality of the RNA and mRNA was analyzed by formaldehyde agarose gel electrophoresis and the existence of Rb transcript was checked by Northern blot analysis (Fig. 9 A and B). Total RNA and mRNA had a 4.7 kb of Rb transcript. The cDNA library of the medaka was constructed with 7 μg of poly (A) using cDNA Synthesis and Lambda gt11/*Eco*R I/CIAP-treated Vector Kit from Stratagene. The primary phage library (from 1/5 of ligation) packaged was about 0.8×10^6 pfu/μg of the vector. It is usually desirable to amplify libraries in lambda vectors to make a large, stable quantity of a high-titer stock of the library. To amplify 1×10^6 plaques, two primary phage library packaged were combined and used. The total titer for the amplified phage library was 1.7×10^9 pfu/ ml.

The Screening of Two cDNA Libraries

Two cDNA libraries were screened; one was made in λ ZAPII using RNA from medaka liver by Dr. B. Van. Beneden and the other was made in λ gt11 by me. The

libraries were screened with human a 505 bp Rb cDNA as a probe.

(1) The Screening of λ ZAPII cDNA Library

In the primary screening of the λ ZAPII cDNA library, 27 putative clones were detected. Following the second screening, a background problem arose. Some of the plaques were expected to show positive signals in the second screening. However, almost all plaques appeared to hybridize with the probe (the intensity of the signal varied). In the primary screening, the amplified cDNA was plated on 150 mm LB plate to 50,000 pfu/plate. The sizes of plaques in the primary screening are tiny and plaques are so confluent compared with that of the second screening. Therefore the background problem might not be detected in the primary screening. The plaques which had a stronger signal, compared to the other plaques, were chosen as putative clones. In the second screening, these positive plaques were too spatially close to the background plaques. The positive plaques were contaminated with non-positive plaques (background plaques) when they were picked. Thus, a tertiary screening was performed to obtain isolates. The same problem occurred in the tertiary screening. All plaques showed positive signals. Finally, 16 putative clones which had the strongest signal were chosen.

In vivo excision was performed on the isolates to obtain the insert-containing pBluescript phagemid for Southern blot analysis and sequencing. The Southern blot analysis was performed to check if the putative clones contained the Rb gene. Each phagemid was digested with *Eco*R I restriction enzyme and electrophoresed on agarose gel (Fig. 10). The hybridization was performed with human 505 bp Rb cDNA. The result of this experiment (Fig. 11) showed that pBluescript phagemids were crosshybridized with the probe. Therefore, the problem in the screening was due to the hybridization of the

phagemid to the probe. The other signals seemed to be from the undigested plasmids, not from inserts. Taken together, the final 16 clones selected from the tertiary screening did not contain Rb cDNA.

(2) The Screening of λ gt11 cDNA Library

The lambda gt11 library was screened using the same method as for the λ ZAPII cDNA library. The result was the same as that of the λ ZAPII cDNA library. The same background problem occurred in the λ gt11 cDNA library. While the library was being screened, a Southern blot analysis was performed to check if the cDNA library included the Rb cDNA insert. Lambda gt11 DNA containing inserts were isolated using the Wizard ™ Lambda Preps DNA Purification System and digested with *Kpn* I and *Sac* I (Fig. 12A). The Southern blot analysis was done using human 505 bp Rb cDNA as a probe. The λ gt11 which did not contain inserts was used as a negative control. The result is shown in Fig. 12 B. The probe hybridized to one of the bands from the DNA containing inserts (4.3 kb in size). Unfortunately, the same hybridization signal was present in the negative control. Therefore, the vector crosshybridized with the probe, not with any inserts. Similar to the λ ZAPII cDNA library, the background problem in this screening was due to the vector.

Development of Medaka Rb cDNA from RNA Using RT-PCR

Three fragments were obtained from RT-PCR and their identity was verified by hybridization experiments.

(1) Hybridization to the human Rb cDNA

To determine if the RT-PCR fragments were medaka Rb cDNA, the fragments

were labeled with ^{32}P and used as probes. The fragment produced by the primers R54 and R32 (corresponding to the human Rb cDNA exons 10-16) and the fragment made by the primers R54 and R34 (corresponding to exon 10) cross-hybridized to the human 3.8 kb Rb cDNA (containing exons 9-27) (Fig. 13 A and B). The fragment made by the primers SH51 and R34 (corresponding to the human Rb cDNA exons 6-10) crosshybridized to both human 0.9 (exons 1-9) and 3.8 (exons 9-27) kb Rb cDNA (Figs. 13C and 14A).

(2) Southern Blot Analysis with Medaka Genomic DNA

Further evidence attesting to the fidelity of RT-PCR fragments was obtained with a Southern blot analysis using the fragments as probes. With the medaka genomic DNA digested with *Eco*R I or *Hin*dIII, one hybridization signal was shown in each enzyme digested DNA with the 469 bp fragment probe made by R54and R32 primers. The size of each signal was 7 and 9.4 kb, respectively (Fig. 15). With the 493 bp fragment made by SH51and R32 primers, 2 signals in *Eco*R I digested DNA and 3 signals in *Hin*dIII digested DNA were obtained. The sizes of the signals were 13 and 3.8 kb in *Eco*R I and 5.2, 3.7, and 1.8 kb in *Hin*dIII, respectively (Fig. 16). The 349 fragment probe made by R54 and R34 primers didn't hybridize to medaka genomic DNA.

Discussion

Initially, λ ZAPII cDNA library was screened using several methods and probes. A time-saving method for screening cDNA published by Bloem and Yu (1990) was used. A replica of the plate made by lifting a nitrocellulose filter was placed plaque site up in a sterile petri-dish and rinsed with lambda dilution buffer. An aliquot of 20 μl was removed

from each filter rinse, and then used as the template for PCR. Human Rb primers were used for PCR. The advantage of this method was to reduction of the library's complexity by several orders of magnitude with only one and two rounds of PCR before any filter hybridization. A DNA band of the appropriate size was found in the primary screening, but this turned out to be a non-specific band in the second screening. This method was tried 4-5 times with various human Rb primers (Rb #1, Rb #2, Rb #5, SH51, R34, R54, and R32) . Unfortunately, the expected bands produced by the human primers were not found using this method. It seemed that the human Rb primers used did not have enough sequence homology with medaka DNA.

Another method for screening cDNA library was immunological screening using human Rb antibody. Commercial monoclonal antibodies developed against synthetic peptides representing a distinct region of the human Rb were used for Western blot analysis with medaka in our laboratory. The experiments were successful with these antibodies (Mh-Rb-02P1 from Pharmingen and Rb Ab-1 from Oncogene Sciences). Screening with the antibodies was unsuccessful. In the Western blot, protein is the primary structure. Therefore, it might be easy to detect Rb product. Protein produced in bacteriophage is folded. This structure can make it difficult to find the Rb product with monoclonal antibody in the screening. The commercial antibodies were expensive. Thus, antibodies for screening were limited. Our own Rb polyclonal antibodies developed from medaka could be used for screening cDNA library.

The third method for screening the cDNA library was using radioisotope labeled nucleotide probe. The human 3.8 kb Rb cDNA and *Xenopus* Rb cDNA were used as probes for screening. Both fragments did not detect positive plaques. Specifically,

Xenopus Rb cDNA showed background problem. From the second screening, all plaques showed hybridization signals (only some of the plaques were expected to hybridize with probe). In the λ ZAPII cDNA library, the cDNA was inserted into multiple cloning sites of pBluescript phagemid. *Xenopus* Rb cDNA was subcloned into pBluscript SK. It is possible that the probe was contaminated with the vector, even though the insert used as probe was isolated from vector after digestion with the restriction enzyme. To check the purity of the *X*Rb probe, Southern blot analysis was performed. Human 3.8 kb Rb in pUC 13, 1.2 kb Rb in pCRII, and 0.9 kb Rb in Sp 65 were digested with *Eco*R I. These fragments were tested to cross-hybridize with the *X*Rb probe. The results showed that all vectors and 3.8 kb Rb and 1.2 kb Rb strongly hybridized with the probe (data not shown). *X*Rb insert was purified three times to obtain a pure probe, but the result was poor again. The backbone of the pBluscript and the pCRII vectors is pUC plasmid. From these results, it was hypothesized that *Xenopus* Rb cDNA has sequence homology with the vector.

In preliminary experiments to find the Rb gene and Rb transcript, the human 3.8 kb Rb cDNA and *Xenopus* Rb cDNA did not detect them in medaka. Therefore, I tried to develop medaka Rb cDNA using RT-PCR in collaboration with Dr. Andrea Fekete. The RT-PCR is highly sensitive method that can be used for amplification of very low levels of specific mRNA. Only a small amount of RNA is required and it is not necessary to further isolate poly (A) RNA. However, the use of high quality RNA is critical for the successful RT-PCR analysis. The quality of RNA used in the experiments was checked by spectrophotometer and electrophoresis. The amplification reactions were done using

primers based on the human Rb cDNA. We guessed that there would be sequence homology to be able to amplify medaka cDNA fragments. Several fragments were obtained with RT-PCR and tested for back cross-hybridization to human Rb cDNA. Human 3.8 kb Rb in pUC 13 was digested with *Eco*R I to separate the insert from the vector and used for the experiments. As shown in figure 13, the fragments made by RT-PCR were specifically hybridized to human 3.8 kb Rb cDNA. The fidelity of these hybridizations was obtained from the experiment using the fragment made by primers Rb#1 and Rb#2. 0.9 kb Rb in Sp 65 was used as a control. The fragment made by primers SH51 and R34 (corresponding to the human Rb cDNA exons 6-10) was crosshybridized to both human 0.9 kb (exon 1-9) and 3.8 kb (exons 9-27) Rb cDNA as expected (Fig. 14A). However, the fragment made by primers Rb#1 and Rb#2 (corresponding to the human Rb cDNA exons 1-3) was only cross-hybridized to the 0.9 kb Rb cDNA (Fig. 14B).

Southern blot analyses were performed with these fragments as probes to verify that they were the Rb cDNA. The results of these experiments indicated that medaka genomic DNA contains sequences that crosshybridize with 469 bp (made by R54 and R32 primers) and 493 bp fragment (made by SH51 and R34 primers) probes, but not with the 349 bp fragment (made by R54 and R34) probe. As a control, human genomic DNA was used. Only one hybridization signal was shown with the 469 fragment probe. The signal was very weak (data not shown). In Southern blot analysis of *Hin*dIII-digested human genomic DNA, 6 of the *Hin*dIII restriction fragments (9.8, 7.5, 6.2, 5.3, 4.5 and 2.1 kb) were detectable with the 3.8 kb Rb cDNA probe. It was expected that the signal from human genomic DNA with the 469 bp fragment probe has the same size with one of 6

hybridization bands. Unfortunately, it did not match with any of the 6 signals. Also, RT-PCR fragment probes did not detect Rb transcript in Northern blot analysis. Therefore, It could not be determined if the fragments made by RT-PCR from medaka are real Rb genes. To be certain, the medaka Rb gene should be cloned, sequenced, and then compared with the fragments by RT-PCR.

The screening of the cDNA library was performed with the probe (human 505 bp Rb cDNA) made from the conserved A region. As shown in Fig. 11, pBluscript vector hybridized to this probe. The same experiment was done with an oligonucleotide probe made from the conserved B region. The probe was again hybridized with vector (data not shown). The sequence homology of probe to pBluscript was searched using Mac Vector 4.1.4. The probes did not show significant sequence homology to the vector.

A new cDNA library was constructed in the lambda gt11 vector. The library was screened with human 505 bp Rb cDNA. At the same time, lambda phage DNA which included the cDNA inserts was isolated and Southern blot analysis was performed to check for the presence of the Rb gene in the library. Both showed negative results. The same background problem of plaques in λ ZAPII occurred in the newly constructed library. In Southern blot analysis, lambda phage including inserts and the vector itself were digested with restriction enzymes and probed with human 505 bp Rb cDNA. The probe hybridized to the same fragments in both samples. This means the probe hybridized with the vector. Different hybridization conditions were used for the screening. The same result was obtained. This background problem has existed in all screening experiments and is unsolved at this time. Recently, a genomic DNA library has been screened and one

positive clone was isolated in our laboratory. The clone is being sequenced. If this clone turns out to be a Rb fragment, the cDNA library can be screened using this fragment.

Figure 9. Northern blot analysis with mRNA and total RNA of medaka. RNA and mRNA were isolated from medaka liver and viscera to construct a cDNA library. Before making cDNA library, the quality of the RNA and mRNA was analyzed by formaldehyde agarose electrophoresis (A) and the existence of Rb transcript was checked by Northern blot analysis (B). Lanes of (A) and (B): 1, mRNA (4 μg); 2, Human K562 cell (20 μg of total RNA); 3 Medaka liver (20 μg of total RNA); 4, Medaka viscera (20 μg of total RNA).

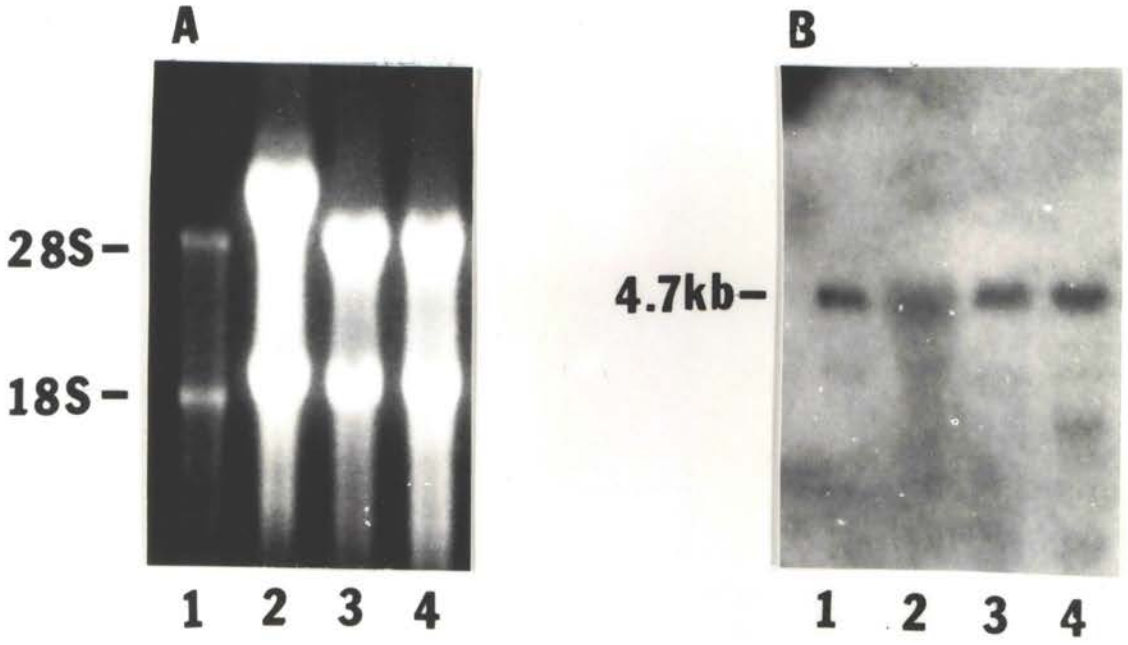

A
28S-
18S-
1 2 3 4
B
4.7kb-
1 2 3 4

Figure 10. Electrophoresis of phagemids from the putative clones. *In vivo* excision was performed on the isolates to obtain the insert-containing pBluescript phagemid from the putative clones. Each phagemid was digested with *Eco*R I restriction enzyme and electrophoresed on agarose gel. Lanes: 1-16, Positive clone 1-16. p indicates pBluescript vector.

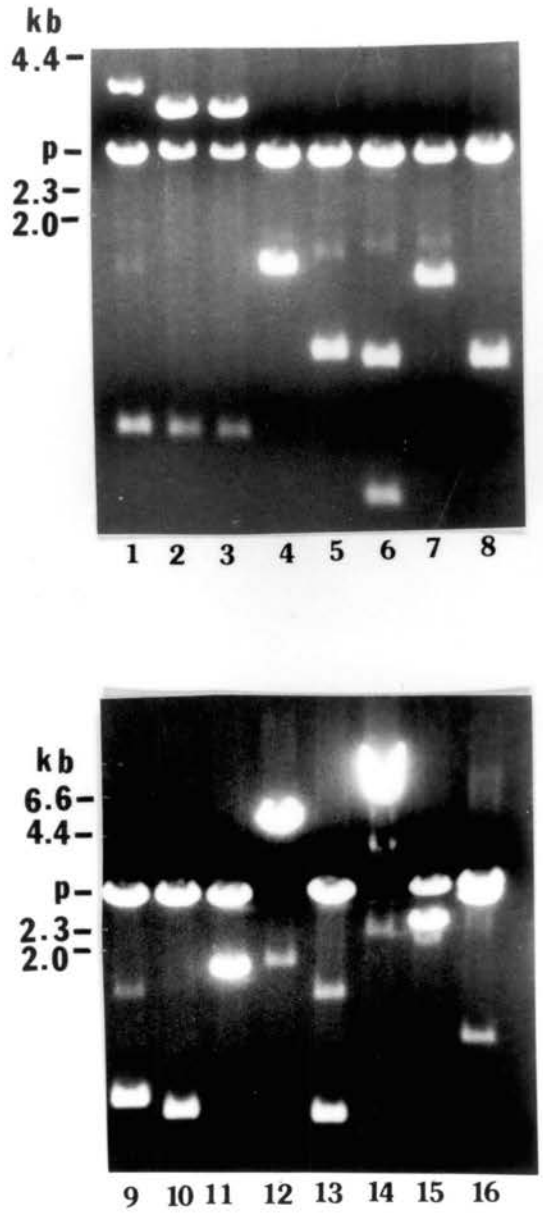
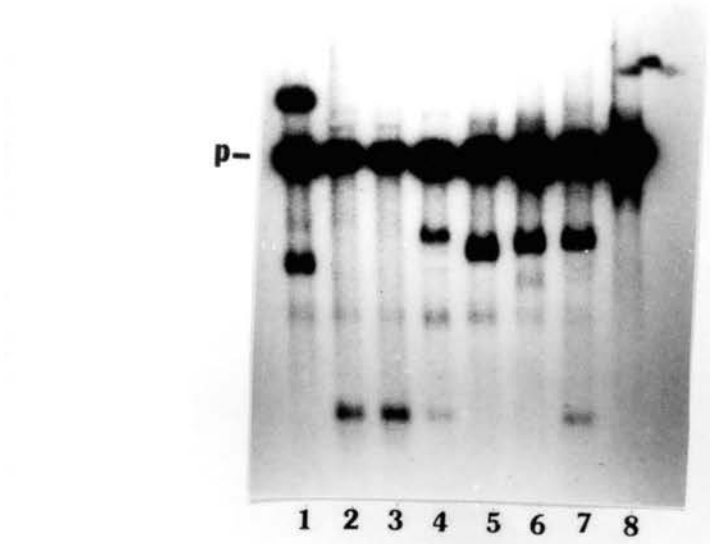

kb
4.4
p
2.3
2.0
1 2 3 4 5 6 7 8
kb
6.6
4.4
p
2.3
2.0
9 10 11 12 13 14 15 16

Figure 11. Southern blot analysis with phagemids from the positive clones. The hybridization was performed with human 505 bp Rb cDNA as described in Materials and Methods. Lanes:1-16, Positive clones 1-16. p indicates pBlue-script vector.


p–
1 2 3 4 5 6 7 8

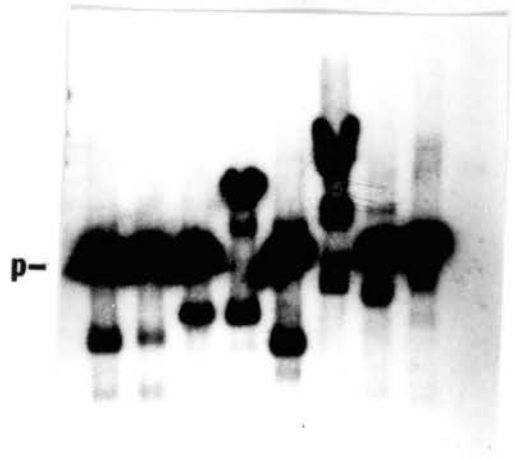

p–
9 10 11 12 13 14 15 16

Figure 12. Southern blot analysis with λ gt11 DNA containing inserts. Lambda gt11 DNA containing inserts were isolated using the Wizard Lambda Preps DNA Purification System and digested with *Kpn* I and *Sac* I (A). Lanes: 1, 1 kb DNA ladder; 2, λ gt11 vector; 3, λ gt11 DNA containing inserts. The Southern blot analysis was done using human 505 bp Rb cDNA as a probe (B). Lanes: 1, λ gt11 vector; 2, λ gt11 DNA containing inserts.

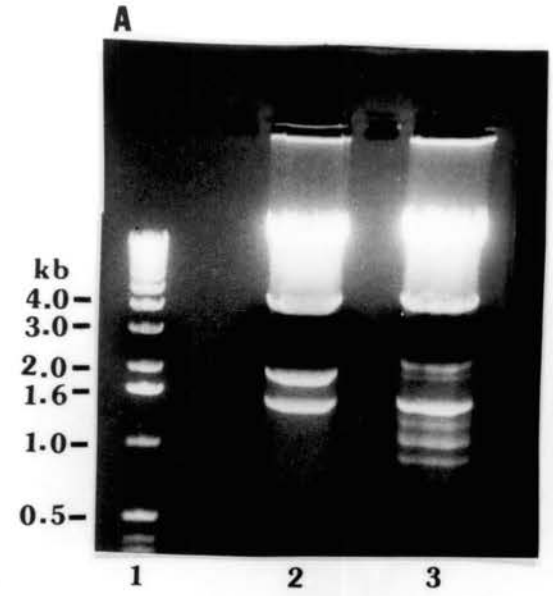

A
kb
4.0
3.0
2.0
1.6
1.0
0.5
1
2
3
B
kb
4.3
1
2

Figure 13. Crosshybridization of RT-PCR fragments to human 3.8 kb Rb cDNA. The fragment made by primers R54 and R32 (A), the fragment made by primers R54 and R34 (B), and the fragment made by primers SH51 and R34 (C) were crosshybridized to the human 3.8 kb Rb cDNA.

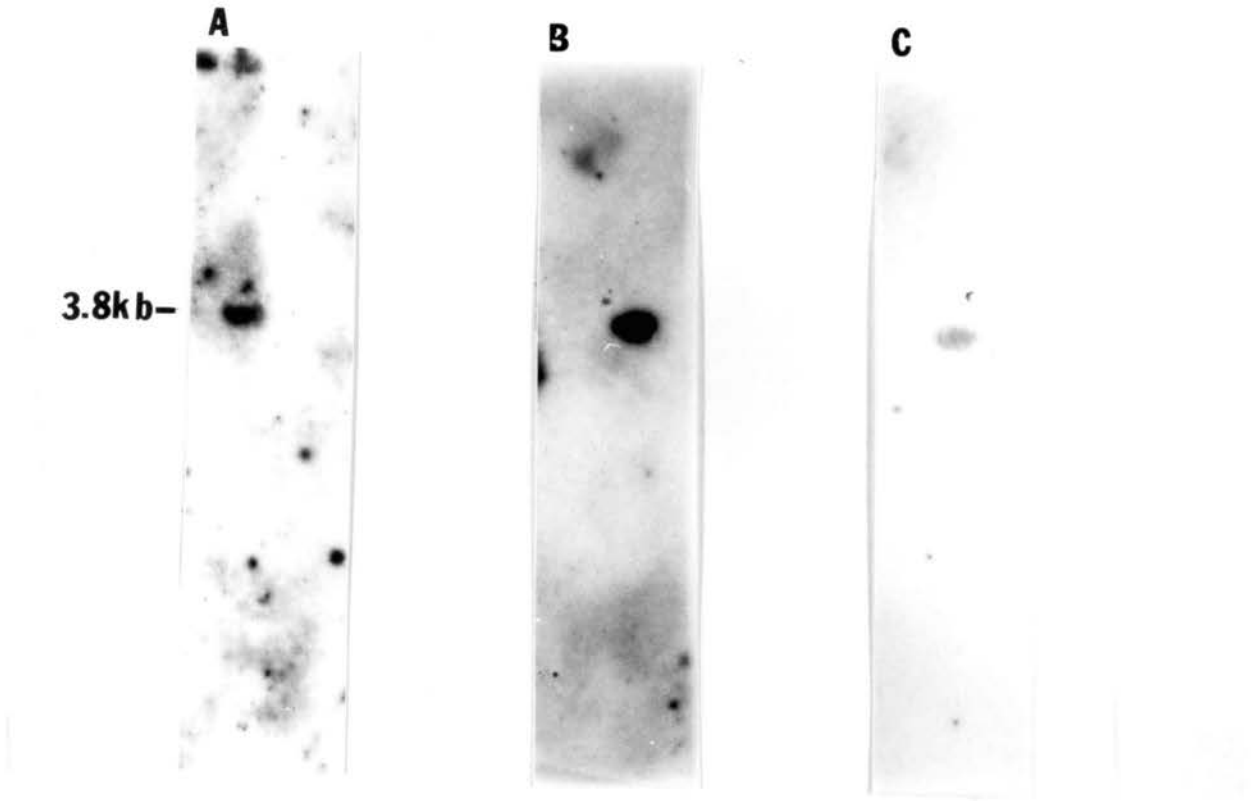

A
B
C
3.8kb–

Figure 14. Crosshybridization of RT-PCR fragments to human 3.8 kb and/or 0.9 kb Rb cDNA. The fragment made by primers SH51 and R34 (corresponding to the human Rb cDNA exons 6-10) (A) crosshybridized to both human 0.9 kb and 3.8 kb Rb cDNA. However, the fragment made by primers Rb#1 and Rb #2 (corresponding to the human Rb cDNA exons 1-3) (B) was only cross-hybridized to the 0.9 kb Rb cDNA.

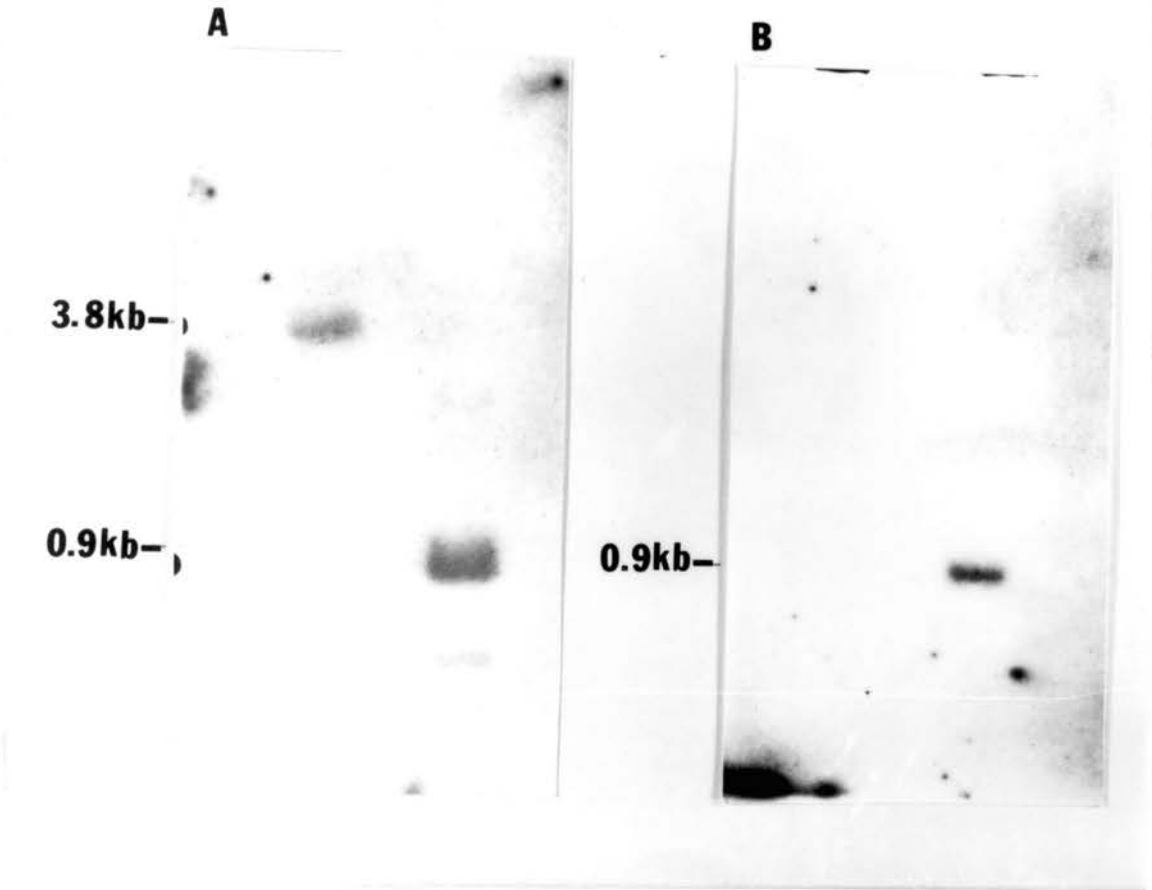

A
B
3.8kb
0.9kb
0.9kb

Figure 15. Southern blot analysis using the 469 bp fragment (made by R54 and R32 primers) as a probe. In the medaka genomic DNA digested with *Eco*R I (Lane 1) or *Hin*dIII (Lane 2), one hybridization signal was shown in each enzyme. The size of each signal was 7 and 9.4 kb, respectively.

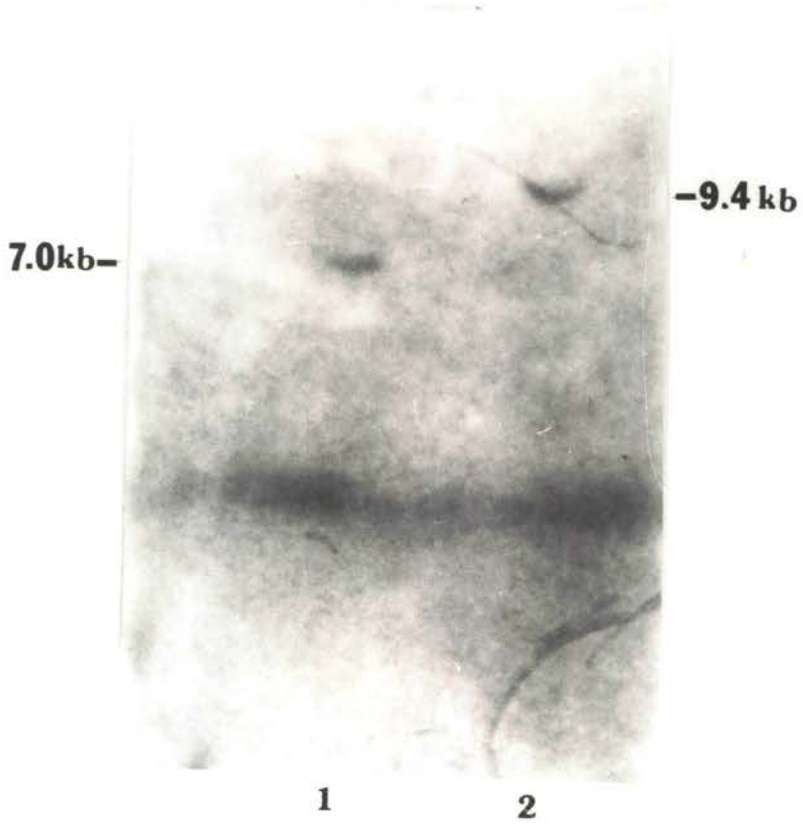
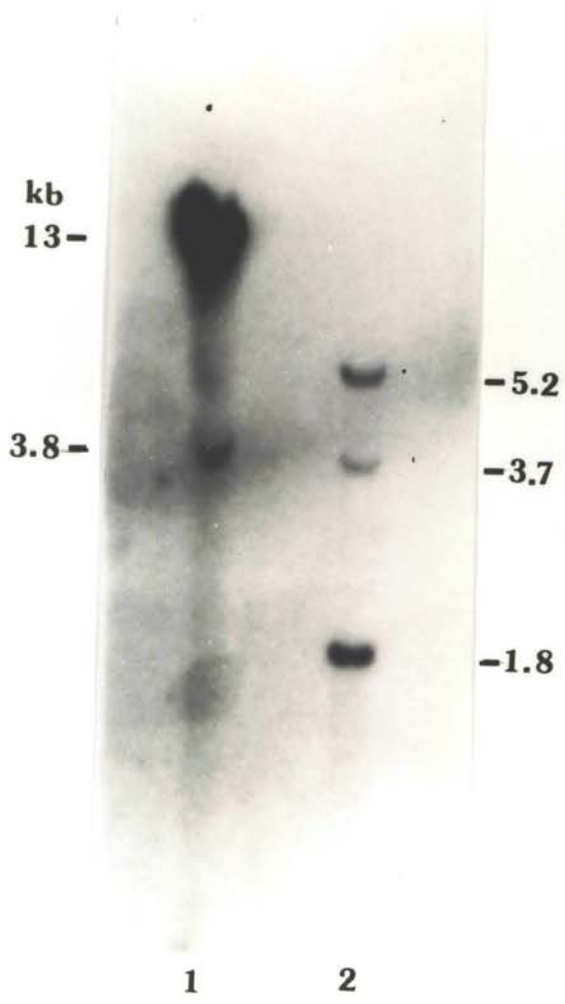

7.0kb
9.4 kb
1
2

Figure 16. Southern blot analysis using the 493 bp fragment (made by SH51 and R34 primers) as a probe. In the medaka genomic DNA digested with restriction enzymes, 2 signals in *Eco*R I digested DNA (Lane 1) and 3 signals in *Hin*dIII digested DNA (Lane 2) were obtained. The sizes of the signals were 13 and 3.8 kb in *Eco*R I and 5.2, 3.7, and 1.8 kb in *Hin*dIII, respectively.


kb
13
3.8
5.2
3.7
1.8
1
2

CHAPTER IV

THE EFFECT OF GROWTH FACTORS AND HORMONES ON MEDAKA Rb PROTEIN

Introduction

In normal cells, the Rb protein is expressed throughout the cell cycle but its functionally active states are determined by the degree of phosphorylation (DeCaprio et al., 1989; Chen et al., 1989; Ludlow et al., 1990). The unphospholylated Rb forms are present in G_0 and G_1. These forms seem to be involved in preventing the cell from under going transition from G_1 to S. Whereas the more highly phosphorylated forms are found during S and G_2/M phases. The progressive phosphorylation of the Rb protein seemingly relieves the block and allows continuation of the cell cycle. It has been reported that TGF-β (Transforming growth factor β) and IFNα (Interferon-α) can affect the phosphorylation of the Rb protein (Laiho et al., 1990; Thomas et al., 1991).

Laiho et al. (1990) showed that addition of the TGF-β1 to mink lung epithelial cells (Mv1Lu) in mid to late G_1 prevents phosphorylation of Rb scheduled for this cell cycle stage and arrests cells in late G_1. When TGF-β1 was added to cells in late G_1 or S phase, it did not prevent pRb phosphorylation at this stage of the cell cycle. In Mv1Lu cells, TGF-β1 inhibits cellular proliferation through its ability to downregulate the activity

of the cyclin-dependent kinases cdk2 and cdk4 (Ewen et al., 1993; Koff et al., 1993). Ewen et al. (1993) showed that TGF-β inactivates cdk4 by decreasing the level of cdk4 expression and constitutive expression of cdk4 renders Mv1Lu cells unresponsive to the effects of TGF-β. Koff et al. (1993) reported that TGF-β does not affect the levels of cdk2 expression but downregulates cdk2 activity by recruiting p27, a protein that is able to bind and inactivate cyclin E-cdk2 complexes. In 1994, Kim et al. reported that TGF-β and /or epidermal growth factor (EGF) stimulate by three to six-fold the level of Rb mRNA which is also reflected by the increased levels of p110Rb in C3H/10T1/2 mouse fibroblasts. Hyperphosphorylation of p110Rb by TGF-β can be observed when cells are in S phase. TGF-β stimulates by three to four-fold the activity of cdk2 kinase consistent with the observed phosphorylation of p110Rb. TGF-β as a growth stimulator induces the phosphorylation of p110Rb during cell cycle progression in mouse fibroblasts (Kim et al., 1994). Based on the results from Lahio et al. and Kim et al. it seems that the effect of TGF-β on cell growth is cell-type specific.

Other researchers reported that some growth factors affect the level of expression of Rb transcript and/or Rb protein (Day et al., 1993; Kim et al., 1994). Day et al. (1993) reported that the nerve growth factor inducible A gene (NGFI-A) in human prostate cancer cell line binds an element in the retinoblastoma gene promotor and that a 1.9-fold increase in Rb mRNA following castration that parallels a 2.7-fold induction of NGFI-A mRNA in gene expression studies of the rat ventral prostate. Based on these results, they suggested that the Rb gene may be transcriptionally regulated by NGFI-A in prostate cells.

Interferon-α (IFN-α) is a peptide hormone that regulate the physiology of a variety

of cell type (reviewed by Wang et al., 1994). Thomas et al. (1991) demonstrated that INF-α shows a growth-suppressing activity and leads to a cell cycle block in G1 in human Burkitt lymphoma cells Daudi. Cycling Daudi cells contain mainly hyperphosphorylated Rb. When the cells are exposed to IFN-α for 10 hr, unphosphorylated Rb becomes detectable. After exposure for 48 hr, all the Rb is the unphosphorylated form. Thus, IFN-α induces the complete dephosphorylation in this cell.

Rb transcript in medaka is the same size (4.7 kb) as in the human Rb. This is detected in all tissues examined in medaka as like mouse. Rb protein is also detected in various medaka tissues. It is possible that medaka Rb protein has a similar characteristic with that of mammalian Rb protein. Functionally active states of Rb protein are decided by the degree of phosphorylation. Growth factors and hormones can affect the phosphorylation of the Rb protein as mentioned above. The medaka is an excellent model to study retinoblastoma based on the reasons described in Chapter I. In order to study the potential effect of growth factors and hormones on the medaka Rb protein, primary tissue cultures from medaka liver were treated with EGF, TGF-β1, insulin, or estradiol.

Materials and Methods

Animals--Medaka (Oryzias latipes) used in this study were obtained from our breeding colony at Oklahoma State University.

Chemicals--Antibody(Rb Ab-1, Cat # OP28) was obtained from Oncogene Science (Cambridge, MA). Biotinylated horse antimouse antibody and peroxidase-conjugated avidin-biotin complex (ABC Elite Kit) were from Vector Laboratories (Burlingame, CA).

3,3′-Diaminobenzidine tetrahydrochloride, TGF-β1, insulin, and estradiol were from Sigma Chemical Co. (St. Louis, MO). EGF was obtained from GibcoBRL (Gaithersburg, MD). All other reagents were of analytical grade or better.

Western Blot Analysis--Primary cell cultures of medaka liver were prepared by Dr. YuAn Cao. Forty livers were dissected out of medaka and 8 livers were used for each treatment and a control. The primary cells were maintained in serum free media for 1 week to obtain culture growth. Cells were treated with growth factors or hormones for 1 day at the following concentrations: 100 ng/ml of EGF in 50 mM sodium phosphate, pH 7.2, 250 pM/l of TGF-β1 in 4 mM HCl containing 0.1% bovine serum albumin (BSA), 10^{-7} M/l of insulin in 10 mM HCl, or 10^{-7} M/l estradiol in ethanol. The samples were homogenized in 150 μl of modified EBC buffer (10 mM hepes, pH 7.4, 4 M NaCl, 10 μg/ ml aprotinin, 10 μg/ml phenylmethylsulfonyl fluoride, 2 mM EDTA, and 2 mM EGTA). The homogenates were spun for 20 min in a microcentrifuge at 4°C. The supernatants were transferred to new tubes. The protein concentration of the extracts was measured using the Bio-Rad Protein Assay based on the method of Bradford (1976). Bovine serum albumin was used as the standard. Proteins were resolved by electrophoresis on SDS-PAGE. One volume of each homogenate was combined with at least an equal volume of sample buffer (0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, and 10% 2-β-mercaptoethanol) containing bromophenol blue. Thirty μg of each sample were electrophoresed on 7.5% SDS polyacrylamide gels at 25 volts for 1.5 hr, 50 volts for 0.5 hr, 75 volts for 0.5 hr, and 100 volts for 0.5 hr using a mini gel apparatus (Bio-Rad Mini Protean II). One gel was stained with coomassie blue and a duplicated gel was

electroblotted to Immobilon polyvinylidene difluoride microporous membrane (Millipore, Bedford, MA). The membrane was blocked with Blocking buffer (25 mM Tris, pH 6.8, 125 mM NaCl, 0.1% Tween 20, and 4% bovine serum albumin) for 1 hr at room temperature. Antibody against the Rb gene product (Rb Ab-1) was diluted in blocking buffer to a final concentration of 2.5 μg/ml. The membrane was incubated with primary antibody overnight at 4°C and rinsed 3 times for 15 min with TTBS (25 mM Tris, pH 8.0, 125 mM NaCl, and 0.025% Tween 20). The membrane was incubated with biotinylated secondary antibody for 30 min at room temperature, washed 3 times for 15 min with TTBS, then incubated with avidine and biotinylated peroxidase complex (ABC Elite Kit) for 30 min at room temperature. The protein bands were visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB) for 45 sec.

Results

To investigate the effect of growth factors and hormones in the expression of Rb protein in medaka, primary tissue cultures from medaka liver were treated with EGF, TGF-β1, insulin, or estradiol (Fig. 17). The protein band pattern of each sample was shown in Fig. 17 A. The 97 kDa band was faint in all samples. The approximately 66 kDa protein was highly expressed in all samples compared with the 97 kDa band. The result indicated that growth factors and hormones did not affect the level of expression of Rb protein in medaka (Fig. 17B).

Discussion

Some growth factors and hormones affect the phosphorylation and the expression

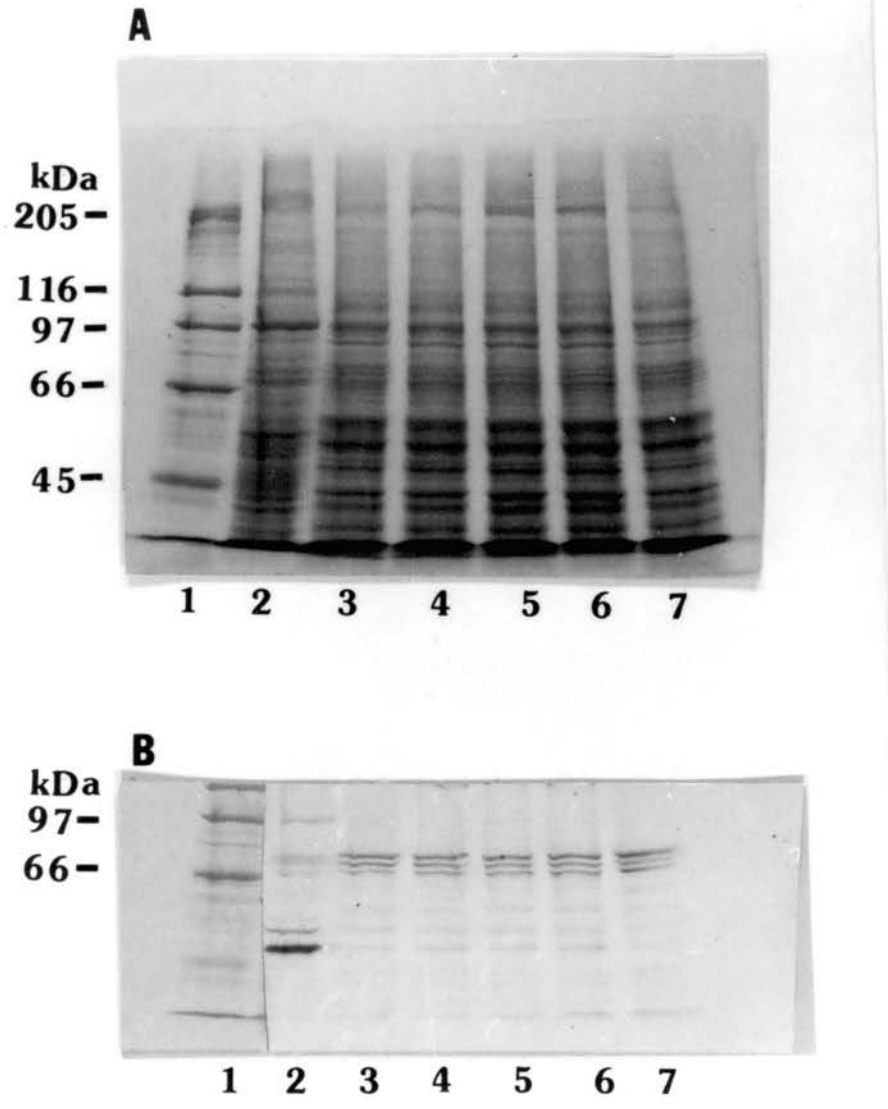
of Rb protein in mammalian cell cultures as mentioned in the introduction of this chapter. I expected the involvement of growth factors and hormones in medaka Rb protein to be as the mammalian systems. However, the result showed no change in medaka Rb protein after treatment with growth factors or hormones. In Western blot, the Rb protein of medaka and rainbow trout did not show multiple banding pattern on the SDS-PAGE. It is possible that the Western blot analysis in my experiment is not sensitive enough to detect the different phosphorylated forms or that the Rb protein of medaka exists as homogeneous forms.

Various growth factors have been found in fishes. For example, insulin like growth factor (IGF) was cloned in chum salmon and this has high sequence homology with a mammalian gene in the coding region (Moriama et al., 1993). A second IGF was identified in rainbow trout (Shamblott et al., 1992). Growth factors (fibroblast growth factor and insulin-like growth factor) have been shown to stimulate cell proliferation in fishes (Negish et al., 1993; Mack et al., 1993). Even the growth factors found in mammalian species have their origins in fishes evolutionarily, the growth factors I used , which are human recombinant, might not function in medaka. They might be species-specific. The receptors can not be existed in tne medaka. The experiment can be tried with growth factors prepared from fishes.

Fish are cold blooded animals and their metabolism might be slower than that of mammalian. Thus, a longer incubation time with growth factors and hormones might be needed for fish. My experiment with growth factors and hormones was preliminary work. The treatment concentrations were based on papers by Lahio et al. (1990) and Segner et al. (1994). A single concentration was used for each treatment. Cells were incubated with

treatments for 1 day. Experiments with various concentrations and incubation times might be needed to find out for optimal condition for medaka.

Figure 17. The effect of growth factors or hormones on the medaka Rb protein. The primary tissue cultures of medaka liver were grown for 1 wk in serum free media. Cells were incubated with 100 ng/ml of EGF, 250 pM/l of TGF-β1, 10^{-7} M/l of insulin, or 10^{-7} M/l of estradiol for 24 hr. Cells were lysed and lysates were analyzed by Western blot analysis with anti-Rb antibody. Lanes of (A) and (B): 1, standard molecular weight marker; 2, Rainbow trout liver; 3, Control (no treatment); 4, EGF; 5,TGF-β1; 6, Insulin; 7, Estradiol.


A
kDa
205
116
97
66
45
1 2 3 4 5 6 7
B
kDa
97
66
1 2 3 4 5 6 7

CHAPTER V

SUMMARY

To determine if medaka have the Rb gene, a Southern blot analysis was performed on DNA from whole medaka tissues using a 505 bp probe derived from the Human Rb cDNA. Medaka genomic DNA contained sequences that crosshybridized with the human Rb gene probe. Digestion with *Hind*III resulted in approximately 4.0 kb segment compared with 7.5 kb for the human.

Expression of the Rb message in medaka was verified by Northern blot analysis in preliminary experiments. The Rb transcript was detected in medaka liver and viscera. The size of the Rb transcript appears to be 4.7 kb in medaka tissues which is the same as that of human. The same result was obtained with poly (A)-enriched RNA. I determined which organs in adult medaka expressed Rb gene. Northern blot analysis was undertaken for the RNA extracted from various medaka tissues. The Rb transcript was detected in all the tissues examined. The level of expression of the Rb gene varied for the different tissues. Liver, gastrointestinal tract, muscle, skin, and spleen were found to have a relatively high level of Rb expression.

Western blot analysis was undertaken to detect medaka Rb protein by using commercially available monoclonal antibody against human Rb. Liver had relatively high levels of Rb protein. The 97, 66, and 45 kDa bands were detected in liver.

Gastrointestinal tract did not show any Rb protein band. Eye and gill showed a 45 kDa band. Brain showed the 97, 66, and 45 kDa bands. The 66 kDa band was highly detected in this tissue. Skin and muscle showed approximately 64, 60, and 45 kDa bands. Especially, about 180 kDa band was detected in muscle. In the early (1 day after fertilization) and late egg stage (over 6 days after fertilization), an approximately molecular weight of 105 kDa was detected. This band was not detected in the fry stage. There were additional bands in late egg and fry stage at 80, 64, or 60 kDa. The 45 kDa band was shown in both late egg and fry stage.

A cDNA library from medaka liver has been constructed. The primary phage library packaged was about 0.8×10^6 pfu/μg of vector. This was amplified and the total titer for the amplified phage library was 1.7×10^9 pfu/ml. Two cDNA libraries were screened by human 505 bp Rb cDNA. One was made in λ ZAPII by Dr. B. Van. Beneden and the other was made in λ gt11 by me. In the primary screening of the λ ZAPII, 27 putative clones were detected. Following the second screening, a background problem developed. All plaques showed positive signals. These signals in the screening were due to the hybridization of the pBluescript phagemid to the probe. Lambda gt11 cDNA library was screened using the same method as for the λ ZAPII cDNA library. The same background problem occurred in the λ gt11 cDNA library.

Medaka Rb cDNA probe was developed using RT-PCR. Three fragments were obtained and their identity was verified by hybridization experiments. To determine if the RT-PCR fragments were medaka Rb cDNA, the fragments were labeled with ^{32}P and used as probes. All three fragments crosshybridized to the human 3.8 kb Rb cDNA. Further

evidence attesting to the fidelity of RT-PCR was obtained with a Southern blot analysis using the fragments as probes. The 469 bp (made by R54 and R32 primers) and 493 bp (SH51 and R34 primers) fragments crosshybridized with the medaka genomic DNA.

To investigate the effect of growth factors and hormones on the expression of Rb protein in medaka, primary tissue cultures from medaka liver were treated with EGF, TGF-β1, insulin, or estradiol. The result indicated that growth factors and hormones did not affect the level of expression of Rb protein in medaka.

Future study should initially focus on clone of medaka Rb cDNA. A medaka genomic DNA library has been screened and one positive clone was isolate. The clone is being sequenced. If this clone turns out to be a Rb fragment, the cDNA library can be screened using the fragment. To answer the questions of where Rb mRNA is being synthesized and where it may be functioning, the sites where Rb mRNA accumulates should be determined by *in situ* hybridization using cloned medaka Rb cDNA as a probe. The ontogeny of neoplasia in the medaka following chemical carcinogens exposure should be explored. The expression of the Rb protein in all tissues during the neoplastic process could be traced using Western blot analysis and immunohistochemistry. Tissue culture cell line of medaka should be established for the detailed study of the basic properties of retinoblastoma.

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