EXPRESSION OF ESTROGEN-RESPONSIVE GENES IN RAINBOW TROUT

A STUDY ON THE EXPRESSION OF ESTROGEN-RESPONSIVE GENES IN RAINBOW TROUT, SALMO GAIRDNERI

By

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ABSTRACT

The expression of the estradiol-responsive genes in the liver of rainbow trout, Salmo gairdnerii, has been studied as a system which may permit quantitative measure of adverse sublethal effects of various xenobiotics.

Upon administration of 17 β-estradiol to male or immature female rainbow trout, the expression of two genes are markedly enhanced. One of these genes codes for the egg yolk precursor protein, vitellogenin, whereas the other codes for another, as yet unidentified, protein. Complementary DNA (cDNA) genes of these two proteins have been employed as probes to characterize the dose-response relationship as well as the time course induction of these two classes of transcripts in liver both in vivo and in vitro. The maximal net transcription of pRTC 2 occurs at 20 ug β-estradiol per 100 gram fish body weight whereas those encoded for by pRTC 5 display maximal transcription at 5 μg β-estradiol per 100 gram fish body weight. With doses exceeding the dose required for maximal induction of transcripts, both classes of transcripts reach their maximal levels between 4 and 8 hours after primary and secondary induction. Transcripts homologous to pRTC 5 return to control levels by 16 hours and 2 days after primary and secondary induction respectively. Those homologous to pRTC 2 however, were not observed to recover to normal levels in the primary induction even 16 days after stimulation with estradiol. However, upon secondary induction pRTC 2 transcripts returned to control levels 8 days post-inoculation with estradiol.

The effect of various xenobiotics on the induction of these two estradiol-responsive genes was investigated. Pretreatment with β-naphthoflavone resulted in neither the induction or repression of both classes of transcripts. Administration of Kepone prior to a subsequent stimulation with estradiol, resulted in a decrease in the accumulation of pRTC 2 transcripts only. In contrast, an isomer of DDT, p,p'-DDT, was found to enhance the expression of pRTC 5 transcripts in fish subsequently administered β-estradiol.

Oncogenes have been found to be activated in transformed cells or cells treated with various xenobiotics. In this study, I have shown that the cellular oncogene, pRTC-myc 1-81, is expressed in the liver at an elevated level in response to estradiol treatment. The two classes of polyadenylated transcripts were detected (2.5 kb and 5.6 kb).

The three estradiol-responsive genes mentioned above (pRTC 2, pRTC 5, and pRTC-myc 1-81) were also shown to be transcribed in an established rainbow trout hepatoma cell line. Transcriptional activities of all three genes, as well as the translational activity of vitellogenin, were diminished upon growth in serum stripped of endogenous hormones.

Further characterization of this rainbow trout hepatoma cell line is warranted so that it may be employed in a sensitive and quantitative bioassay for assessing the pathobiochemical effects of environmental xenobiotics on fish reproduction. Such an index may be beneficial for establishing adequate guidelines for water contaminants that potentially could affect aquatic and human reproductive success.

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INTRODUCTION

The widespread use of polyhalogenated and polynuclear hydrocarbons as pesticides or for industrial purpose in recent years has resulted in the serious accumulation of these compounds or their metabolites in the aquatic ecosystems (Pichirallo, 1971; Moccia et al., 1978; Norstrom et al., 1978; Reinert, 1979; Sonstegard et al., 1983). Fish affected with such compounds display a wide range of pathobiological effects. These include disturbance in Ca^{++} metabolism (Leatherland and Sonstegard, 1981), induction of liver mixed function oxidases (Grant et al., 1974; Wassermann et al., 1979; Stanton and Khan, 1973; Kulkarni et al., 1975; Ahokas et al., 1976; Forlin and Strik, 1978; Elcombe and Lech, 1979; Addison et al., 1979), degeneration of heptocytes (Wassermann et al., 1979; Sivarajah et al., 1978a), and reduction of egg deposition and egg hatch (Wassermann et al., 1979; Johnsson et al., 1970). Unfortunately, detailed biochemical analysis on the toxic effects of halogenated and/or polynuclear aromatic hydrocarbons on fish reproduction are unresolved. Although there are methods currently available for detecting the presence of these compounds in the environment, these methods cannot be used for quantitative assessment of the earliest pathobiological effects in fish caused by pollutants at sublethal levels. Hence, there is an urgent need for establishing a rapid, reliable, and sensitive bioassay for detecting the adverse effects of chemical pollutants in fish at sublethal levels. Advances made over the past ten years in recombinant DNA technology permit differential gene expression

to be employed in the above mentioned bioassay. Establishment of such a bioassay is essential for generating information for the assessment and management of polluted lakes and rivers since the nature of the risks taken by people (especially children and pregnant women) consuming fish has not yet been determined.

I. ESTROGEN CONTROL OF VITELLOGENIN SYNTHESIS IN OVIPAROUS VERTEBRATES

A. Vitellogenins and Egg Yolk Proteins

Vitellogenin in all species studied is a glycoprotein that is synthesized in the liver, secreted into the circulation, and is then taken up by the developing oocytes (Bergink et al., 1974; Deeley et al., 1975, 1977a, 1977b; Christmann et al., 1977; Ohlendorf et al., 1977) where it is cleaved into lipovitellin and phosvitin. Some chemical and physical properties of lipovitellin, phosvitin, and the precursor protein vitellogenin from amphibian, avian, and fish are summarized in Table 1.

Phosvitin contains a large amount of serine (50-55% of the total amino acid content) all of which are phosphorylated in their hydroxyl side chains. Phosvitin also has a moderate number of carbohydrate moieties (10 percent by weight) consisting of three mannose, three galactose, five N-acetylglucosamine and two sialic acid residues arranged in a branched structure attached to the polypeptide via asparagine residues (Shainkin and Perlmann, 1971a, 1971b). In contrast, lipovitellin has a low serine and carbohydrate content and a moderately high lipid content (about 20% by weight), 75% of which are phospholipids (Ohlendorf et al., 1977). Multiple phosvitin and lipovitellin components which differ with respect to their molecular weights have been detected.

TABLE 1

Some Chemical and Physical Properties of Egg Yolk Proteins and Their Precursors in Some Oviporous Vertebrates

Proteins	Animal	Molecular Weight x 10^{-3}		Amino Acid Residues (mol%)		P*	Lipid	$(CH_2O)n$**	
		Native	Subunits	Serine	Methionine	(%)	(%)	(%)	
Phosvitin	Rainbow Trout		22	-	-	9.55	-	-	(1,2)
	Xenopus		32	56	0.4	10.0	0.5	10.0	(3)
	Xenopus		17	56	-	10.0	-	-	(4)
	Chicken		32	56	0.4	10.1	0.3	11.3	(3)
	Chicken		34,28	57,50	-	9.5,9.2	-	-	(5)
Lipovitellin	Rainbow Trout		95,24	-	-	-	-	-	(2)
	Xenopus		115	6	2.2	0.5	20.0	0.3	(3)
	Xenopus		105,35.5,32	-	-	-	-	-	(4)
	Chicken		130	5	2.1	0.4	21.1	0.3	(3)
	Chicken	170	140,30	-	-	-	17.5	-	(5)
Vitellogenin	Rainbow Trout		160,170	-	-	-	-	-	(2)
	Xenopus	490-600	215	12	2	1.6	12	2	(3)
	Chicken	500	240	14	2	2.4	12	2	(3)

(1) Wallace et al., (1966)
(2) Chen, T.T., (1983)
(3) Tata and Smith, (1979)
(4) Ohlendorf et al., (1977)
(5) Christmann et al., (1977)

* Phosphorous
** Carbohydrate
\- Not determined

However, this variation has been attributed to varying degrees of phosphorylation, glycosylation, and lipidation of the same polypeptide (Tata and Smith, 1979). Vitellogenin serves not only as a storage protein for phosphate, glucose, and fats but also for calcium ions (Ca^{++}) in the developing embryo (Munday et al., 1968).

The main properties of amphibian, avian, and fish vitellogenins are also summarized in Table 1. In frogs, the native vitellogenin (M.W. 4.9-6.0 X 10^5) is composed of two identical subunits of 2.1-2.2 X 10^5 daltons as judged by polyacrylamide gel electrophoresis under denaturing conditions (Bergink and Wallace, 1974; Clemens, 1974; Berridge et al., 1976; Tata, 1976; Penning et al., 1977). Similarly, chicken vitellogenin has been reported to be a protein dimer with a subunit size of 2.4-2.5 X 10^5 daltons (Deeley et al., 1975; Wetekam et al., 1975; Gruber et al., 1976; Jost and Pehling, 1976b; Christmann et al., 1977; Gordon et al., 1977). In rainbow trout however, vitellogenin is composed of smaller subunits being 1.6-1.7 X 10^5 daltons (Chen, 1983). In Xenopus, vitellogenin is composed of three distinct lipovitellin subunits (M.W. 105,000, 35,000, and 32,000) and one phosvitin moiety (M.W. 17,000) (Bergink and Wallace, 1974; Ohlendorf et al., 1977, 1978). Rainbow trout vitellogenin appears to be composed of two lipovitellins (M.W. 95,000 and 24,000) and two phosvitin aggregates (M.W. 22,000) (Wallace et al., 1966; Chen, 1983). In contrast to both of the above organisms, chicken vitellogenin appears to have one lipovitellin component (M.W. 170,000) and two distinct phosvitin polypeptide residues (M.W. 34,000 and 28,000) (Christmann et al., 1977).

B. Primary and Secondary Induction of Vitellogenin

The induction of vitellogenin synthesis by estrogen in the liver of male or female Xenopus, chicken, and fish have been well characterized (Follet and Redshaw, 1968; Wallace and Dumont, 1968; Wallace and Jared, 1968a, 1968b; Clemens, 1974; Bergink et al., 1974; Wallace and Bergink, 1974; Gruber et al., 1976; Plack et al., 1971; Chen, 1983; Chen et al., 1984a). Since males normally do not produce this protein, the estrogen-induced vitellogenin synthesis in males offers an ideal system for the analysis of the primary response of the target tissue following hormone treatment. With an injection of oestradiol benzoate, vitellogenin was first detected in the serum of cod after 2 days and the concentration increased rapidly during the next 3 days and more slowly the following 5 days (Plack et al., 1971). With a similar injection, vitellogenin was first detected in the serum of male Xenopus in 12 hours, becoming maximal after 8 to 10 days, and being undetectable after 30 days (Wallace and Dumont, 1968; Wallace and Jared, 1968a). In roosters, a similar but more rapid response was observed. Vitellogenin was first detected between 4 to 6 hours after hormone stimulation, was maximal by 2 to 4 days and depletes to normal levels by 12 to 14 days (Clemens, 1974; Bergink et al., 1974; Gruber et al., 1976).

The production of vitellogenin in the liver following estrogen treatment may be a result of the synthesis of the corresponding mRNA or the translation of stored vitellogenin mRNA. Initially, the presence of vitellogenin mRNA in Xenopus (Shapiro et al., 1976; Skipper and Hamilton, 1977; Farmer et al., 1978) and in chicken liver (Mullinex et al., 1976) was quantified by its translational activity in vitro. The

appearance of vitellogenin mRNA in these systems was identified using the ability of vitellogenin mRNA-containing polysomes or the polysomal mRNA to direct the synthesis of immunoprecipitable vitellogenin polypeptides in a cell-free protein synthesizing system (Berridge et al., 1976; Jost and Pehling, 1976a; Mullinix et al., 1976; Roskam et al., 1976a, 1976b; Bast et al., 1977; Skipper and Hamilton, 1977). These studies showed that translationally active mRNA accumulated in the cytoplasm only after hormonal stimulation. However, these studies did not test for the presence of vitellogenin mRNA which could be inactive as a template for in vitro translation prior to estrogen treatment.

By preparing complementary DNA (cDNA) from vitellogenin mRNA specific sequences it was possible to determine the number of vitellogenin mRNA sequences present (Baker and Shapiro, 1977; Deeley et al., 1977a, 1977b; Felber et al., 1978; Jost et al., 1978; Ryffel et al., 1977; Baker and Shapiro, 1978). In most of these studies the rapid accumulation of mRNA has been directly correlated with the rate of synthesis of vitellogenin. This suggests that the synthesis of vitellogenin is a function of the appearance of newly transcribed mRNA and not due to selective translation of vitellogenin mRNA transcripts.

Another feature of the action of most developmental hormones is a lag phase preceding the onset of the synthesis of specific proteins in the target tissue (Tata, 1970). In birds and amphibians, a lag phase exists bewteen the time of administration of the estrogen to the onset of vitellogenin synthesis. The duration of this lag phase depends on whether the induction is in vivo or in vitro, whether the protein is

measured in the blood or inside the cell, and upon the sensitivity of the assay methods used to determine the levels of mRNA or protein (Plack, 1971; Clemens et al., 1975; Berridge et al., 1976; Green and Tata, 1976; Gruber et al., 1976; Baker and Shapiro, 1978; Farmer et al., 1978). After primary stimulation, administration of a second dose of estrogen produces several significant differences. First the lag period is dramatically shortened and protein synthetic capacity at peak production is several fold greater than that in primary stimulation. Secondly, protein synthesis reaches its maximum more rapidly. This suggests that the existence of rate-limiting steps or factors essential for the specific expression of the vitellogenin genes occur at the transcriptional and/or translational levels.

The Xenopus system has lent itself to be exploited for the analysis of these controls. In Xenopus, vitellogenin is encoded by a multigene family of four actively expressed genes, made up of two genes each in the A and B groups (Wahli et al., 1979, 1981; Felber et al., 1980). Hormonal manipulation was employed to determine whether or not the individual members of the multigene family were coordinately or separately expressed (Williams and Tata, 1983). In male Xenopus, the primary administration of estradiol results in the non-coordinated activation in the liver of the A and B groups of vitellogenin genes, judged by transcription and DNAase I sensitivity in isolated nuclei, with B group genes being preferentially activated in the first 20 hours. In contrast however, secondary induction in males results in coordinated and equal transcription of these two gene groups. The elevated transcriptional activity following primary stimulation returned to low

levels rapidly but the high DNAase I sensitivity of these genes persisted for 2-3 months. However, a non-coordinated activation of the A and B groups of vitellogenin genes is re-established 8 months after a primary stimulation with estradiol (Williams and Tata, 1983). Furthermore, vitellogenin genes are not transcribed in the absence of estrogen (Brock and Shaprio, 1983a). Studies on the kinetics of vitellogenin mRNA accumulation during primary and secondary induction have revealed that the rate of mRNA accumulation (Baker and Shapiro, 1977, 1978; Deeley et al., 1977a, 1977b) and absolute rate of total nuclear RNA synthesis (Brock and Shapiro, 1983a) in the secondary stimulation is higher than that of the primary stimulation. The increased rate of vitellogenin mRNA accumulation has partially been attributed to the presence of estrogen which appears to selectively stabilize the mRNA from cytoplasmic degradation (Brock and Shapiro, 1983b). The induction of vitellogenin synthesis is accompanied by a massive proliferation of the endoplasmic reticulum (Lewis et al., 1976). These varied responses of the hepatocytes to estrogen stimulation indicate that many levels of control are being exerted upon vitellogenesis. Thus, the vitellogenin system offers a means of dissecting modes of gene regulation.

Estrogen has been shown to also induce vitellogenin synthesis in liver organ culture and hepatocyte culture (Wangh and Knowland, 1975; Green and Tata, 1976; Felber et al., 1978). These cultures offer several advantages over the in vivo studies. Perhaps the most important contribution that these have made to hormone research since their employment is that estrogen is the only controlling factor necessary of vitellogenin synthesis.

II. STEROID METABOLISM IN FISH

Estradiol-17B has been identified as a major plasma estrogen in female fish (Ozon, 1972a; Wingfield and Grimm, 1976; Yaron et al., 1977; Fostier et al., 1978). Many phenomena may be influencedby steroids in salmonid fish (e.g., reproductive function, migration, and osmoregulation) (Chester et al., 1972; Ozon, 1972a, 1972b; Bentley, 1976a, 1976b). Most species of teleosts have an annual reproductive cycle with gamete development and spawning following periods of gonadal regression and inactivity (Dodd, 1975). In female fish, levels of estradiol-17B increase during the maturation period but decrease before the onset of spawning (Billard et al., 1978; Lambert et al., 1978; Whitehead et al., 1978). It is during this maturation period that vitellogenin is produced in the liver under influence of 17 β-estradiol (Emmerson and Petersen, 1976; Whitehead et al., 1978; Elliot et al., 1979; Campbell and Idler, 1980; Chen, 1983). It is in the liver also where steroid metabolism occurs. Generally, hepatic metabolism makes the parent steroid more hydrophillic and less biologically active. There are two means by which this may be accomplished - by reduction or by oxidation. Together these pathways generate a spectrum of metabolites, the majority of which are further conjugated to glucuronides or sulphates before being excreted in the urine or bile (Gower, 1975). The hepatic metabolism of estrogens has received little attention in spite of an increasing interest in the role of 17 β-estradiol in the reproductive function.

The microsomal mixed function oxidase system is one of several key enzyme systems involved in the metabolism of steroids as well as

many other endogenous compounds such as sterols, fatty acids, and bile acids (reviewed by Cooper et al., 1979; Lu, 1979). The hemoprotein cytochrome P-450 catalyzes the hydroxylation reactions of steroids which require NADPH and molecular oxygen for activity (Conney and Kuntzman, 1971; Estabrook et al., 1971). Cytochrome P-450 has also been shown to have an important role in the biosynthesis of steroid hormones in the testes, ovaries, adrenals, and placenta where it is found in mitochondrial and microsomal fractions (reviewed by Cooper et al., 1979; Lu, 1979). Highly purified cytochrome P-450 from rat and rabbit liver microsomes have been shown to exhibit remarkable regioselectivity and stereospecificity for the substrates (Levin et al., 1974; Haugen et al., 1975; Ryan et al., 1979; Gustafsson and Ingelman-Sundberg, 1976) suggesting that different cytochrome P-450 subspecies may be involved in the hydroxylation of different sites of the steroid molecules.

Variations in the cytochrome P-450 mediated metabolism in mammals have also been associated with differences in biological (e.g. species, age, sex) and environmental factors (e.g. diet, season) (Kato, 1974; Blumberg, 1978; Walker, 1978; Hansson and Gustafsson, 1981). In addition to endogenous compounds, cytochrome P-450 also catalyzes the metabolism of a variety of xenobiotic compounds such as drugs, insecticides, and carcinogens (reviewed by Cooper et al., 1979; Lu, 1979). Such a broad substrate specificity of cytochrome P-450 can only be attributed to the presence of multiple forms of hemoproteins each with different substrate specificites as well as differential sensitivities to inhibitors and inducers (Haugan et al., 1975; Ryan et al., 1979; Lu and West, 1978; Lu, 1979).

Similarly, multiple forms of hepatic cytochrome P-450 species are present in fish (Elcombe and Lech, 1979; Forlin, 1980; James and Bend, 1980) which are capable of performing many of the same biotransformations as mammalian species however, usually with lower activity (Chambers and Yarbrough, 1976; Lech and Bend, 1980; Franklin et al., 1980; Forlin and Lidman, 1981). Exposure to the wide array of xenobiotics in the environment drastically alters the levels of the mixed-function oxidases (Elcombe and Lech, 1978, 1979), an alteration being an induction or repression of cytochrome P-450 activity. The most potent inducers of cytochrome P-450 activity in fish appear to be various polyaromatic hydrocarbons (PAH) (Bend et al., 1977; Gruger et al., 1977; Gerhart and Carlson, 1978; Statham et al., 1978; Balk et al., 1980; Forlin, 1980; James and Bend, 1980), polychlorinated biphenyls (PCB) and polybrominated biphenyls (PBB) (Hill et al., 1976; Lidman et al., 1976; Gruger et al., 1977; Elcombe and Lech, 1978), 5,6-naphthoflavone (Elcombe and Lech, 1979; James and Bend, 1980), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Bend et al., 1977), and 3-methylchloranthrene (3-MC) (Hansson et al., 1980). In contrast, phenobarbital (PB), DDT, and phenylbutazone failed to induce the cytochrome P-450 activity studied in fish (Buhler and Rasmusson, 1968; Addison et al., 1977; Bend et al., 1977; Elcombe and Lech, 1979; Balk et al., 1980; Forlin, 1980; Fabacher, 1982).

In the past decade, PAH and PCB compounds have been extensively used as pesticides and/or industrial purposes. Consequently, because of their widespread use and long half-life, these compounds and their metabolites have accumulated in aquatic ecosystems (Pichirallo, 1971;

Johnsson, 1970). There are numerous reports documenting various detrimental effects of organic environmental pollutants and their metabolites on humans (Jones, 1941), and on wildlife such as birds (Holmes et al., 1967; Johnsson et al., 1970) and fish (Johnsson et al., 1970; Donaldson and Scherer, 1981). Three independent reports have shown that birds eating fish, subsequently accumulating high levels of PAH and PBB compounds, produced eggs with extra thin shells suggesting the dysfunction of calcium homeostasis. Furthermore, these eggs produced a low hatch (Hickey and Anderson, 1968; Peakall, 1970). In recent studies on serum calcium levels of Great Lake coho salmon and chinook salmon, Leatherland and Sonstegard (1983) have observed hypocalcaemia in coho salmon which may be environmentally induced by PAH and PBB contaminated compounds. In addition to the observed calcium imbalance, morphological changes to the gonads (Donaldson and Scherer, 1981) and hepatocytes (Sivarajah et al., 1978a, 1978b) have observed in contaminated fish. In addition, it has been suggested that PAH and PBB contaminated compounds interfere with animal reproduction by disturbing the hepatic metabolism of steroid hormones (Nowicki and Norman, 1972; Orberg and Lundberg, 1974) by increasing the levels of cytochrome P-450. Correlations between the induction of liver cytochrome P-450 and the decrease in plasma steroid levels in fish have been reported (Sivarajah et al., 1978b). In support of these findings, the feeding of juvenile trout with polychlorinated biphenyls and/or Mirex contamined diets resulted in the reduction of vitellogenin synthesis by exogenous estradiol as detected by rocket immunoelectrophoresis (Chen, in preparation).

III. THE MYC ONCOGENE

The genomes of the rapidly transforming retroviruses contain transforming genes (v-onc genes) that are derived from a set of chromosomal sequences (c-onc or proto-onc genes). To date, molecular analyses of these viruses has led to the positive identification and characterization of more than 15 different v-onc genes which can induce various types of tumors *in vivo* and transform cells *in vitro* (reviewed by Cooper, 1982, Bishop, 1982, 1983). The cellular homologs of these viral sequences are highly conserved among vertebrate species (Eva *et al.*, 1983; Westin *et al.*, 1982; Shilo and Weinberg, 1981a, 1981b; Andersson *et al.*, 1979; Spector *et al.*, 1978, 1981a, 1981b; Stehelin *et al.*, 1976). The proto-onc genes are thought to function in normal cellular and developmental processes (Doolittle et al., 1983; Muller *et al.*, 1982; Gonda *et al.*, 1982; Langbeheim *et al.*, 1980). They may be activated by treatment with adverse chemicals such as carbon tetrachloride (Goyette *et al.*, 1983) and 3-MC (Eva and Aaronson, 1983). The coding regions of proto-onc genes are like those of other cellular genes, being separated by intervening sequences (Collett *et al.*, 1979).

One such nucleic acid sequence, referred to as *myc*, was the first identified as part of the transforming gene of avian myelocytomatosis virus MC29 (Duesberg *et al.*, 1977; Mellon *et al.*, 1978). Further analyses revealed that this 1.6 kb. nucleic acid sequence was associated with three other avian tumor viruses, namely MH2 (Duesberg and Vogt, 1979), CMII (Bister *et al.*, 1979), and OK10 (Bister *et al.*, 1980). These *myc* containing avian tumor viruses display a broad oncogenic spectra- they

cause acute leukemia, carcinomas, and sarcomas and transform firbroblasts as well as hematopoietic cells in tissue culture (Weiss et al., 1982). Like other onc-related sequences, those similar to myc have been found in DNA of normal avian and mammalian sequences including man (Sheiness et al., 1980; Taub et al., 1982; Watson et al., 1983a, 1983b). Furthermore, cellular myc related sequences are expressed in normal cells (Vennstrom et al., 1982; Gonda et al., 1982; Muller et al., 1982; Eva et al., 1982). Since cellular myc sequences are also expressed or in some cases overexpressed or rearranged in certain tumor cells (Erickson et al., 1983; Maguire et al., 1983; ar-Rushdi et al., 1983; Payne et al., 1982; Marcu et al., 1982; Westin et al., 1982; Hayward et al., 1981) and viral myc sequence has been reported to play a role in converting primary rat cells to cell lines in vitro (Land et al., 1983), it has been speculated that cellular myc related genes may have an oncogenic potential like their viral counterparts. At this time, however, their is no direct evidence that c-myc sequences have an oncogenic function comparable to viral myc sequences.

It has been proposed that upstream regulation of proto-myc by retroviral promoter insertion (Hayward et al., 1981) leads to oncogenic transformation. Translocation of c-myc may cause B cell lymphomas (Erikson et al., 1983; Marcu et al., 1983; Taub et al., 1982) and amplification of proto-myc may cause other tumors including myelocytic leukemias (Dalla-Favera et al., 1982; Collins et al., 1982). It should be noted however, that although there is equivalent evidence consistent with this view inasmuch as an elevated expression of proto-myc is not consistently found in the respective tumors and that such tumors display

a different phenotype from those caused by virus (e.g. lymphomas *versus* the carinomas, acute leukemias, and sarcomas caused by the viruses of the MC29 subgroup).

Recently, Papas *et al.*, (1983) have suggested an alternate explanation for the functional dissimilarities between proto-myc and viral *myc* genes. Although both classes of genes share a significant functional domain encoded at the 3' terminal of the conserved 1.6 kb. sequence, qualitative structural changes are present at the 5' terminal. The major structural differences between the viral and cellular *myc* related sequences are the virus-specific *gag* regions of the *onc* genes of MC29, CMII, and OK10. It is these structural changes that are thought to alter the stereochemistry of the *onc* gene products compared to the cellular myc products (Papas *et al.*, 1983).

IV. RATIONALE OF RESEARCH

From the above review, it is clear that the synthesis of vitellogenin induced by β-estradiol in rainbow trout may be used as a biochemical parameter for assessing and screening environmental xenobiotics which may be toxic to heptocytes or capable of affecting reproduction. To understand the molecular mechanisms of the toxic effects of environmental xenobiotics, it is essential to elucidate at which level the expression of the vitellogenin gene is affected. Therefore, the main purpose of this thesis research was to develop a bioassay to study the effects of some model environmental xenobiotics on the expression of estradiol-responsive genes (e.g. vitellogenin gene) in the liver of rainbow trout *in vivo*.

Northern blot analysis of RNAs of 17 β-estradiol treated juvenile rainbow trout revealed the presence of at least two high frequency mRNA species (Chen, 1983). The sizes of the two most abundant mRNAs are 6000 and 2000 bases. The cDNA genes of these two mRNA sequences have been cloned and are named pRTC 2 and pRTC 5 (Chen, 1983; Chen et al., 1984a). The sequences of pRTC 2 are complementary to vitellogenin mRNA which is 6000 bases, whereas the sequences of pRTC 5 are complementary to the second mRNA. The polypeptide encoded by pRTC 5 mRNA awaits to be determined. Nevertheless, these two estrogen-responsive cDNAs can be used as molecular probes for the above mentioned analyses.

The literature review also suggests that chemical carcinogens may induce tumors by activating previously inactive onc genes. In other words, increased expression of c-onc genes in animals may be viewed as an early sign of carcinogenesis. It also suggests that chemicals which can increase or activate the expression of c-onc genes should be considered carcinogens. Hence a new screening method for chemical carcinogens. Hence a new screening method for chemical carcinogens may be developed by evaluating the potential of activating known c-onc genes. Recently, a c-myc oncogene has been isolated from the rainbow trout gene bank in our laboratory (Chen et al., 1984b).

The objectives of this thesis research are two-fold:

1. In vivo and in vitro characterization of the estrogen-responsive genes induced by 17 β-estradiol using pRTC 2, pRTC 5, and pRTC-myc 1-81 as probes.

2. Elucidating the effects of selected model environmental xenobiotics on the expression of the pRTC 2 and pRTC 5 genes.

MATERIALS AND METHODS

Fish

Stocks of rainbow trout (Salmo gairdnerii) each weighing between 30-100 grams, obtained from Spring Valley Trout Farm (Petersburg, Ontario) or Goosens Trout Farm (Otterville, Ontario), were reared in flowing, charcoal filtered, dechlorinated water maintained at 14°C. A twelve hour day-night cycle was also used. A high protein diet (Martin Feed Mills Ltd., Tavistock, Ontario) was provided twice daily.

Rainbow Trout Hepatoma Cell Line

This cell line, which originated from an aflotoxin-induced hepatoma, was obtained from Dr. John Fryer (Oregon State University, Department of Microbiology). This cell line was passaged as monolayer cultures in sealed polypropylene flasks (Corning, 75 cm^2). Monolayer cultures were maintained in Alpha Minimal Essential Medium (α-MEM) (Gibco Canada, Burlington, Ontario) supplemented with 10% fetal calf serum (Bocknek Organic Materials, Rexdale, Ontario), containing penicillin-streptomycin (Gibco; 100 units/ml), kanomycin (Gibco; 100 µg/ml), and 25 mM Hepes buffer (Gibco) at 20°C. Cells were routinely subcultured when confluence was reached by trypsinization and re-seeded into sterile flasks.

Chemical Treatment

A. In vivo. 17 ß-estradiol (Sigma Chemical Company, St. Louis, Missouri) was dissolved in propylene glycol (Kodak Chemicals, Rochester,

New York). All other chemicals were dissolved in cod liver oil. Kepone (decachlorooctahydro-1,3,4-methen-2H-cyclobuta(cd)pentalen-2-one), p,p'-DDT (1,1-bis(p-chlorophenyl)-2,2,2-trichloroethane), and β-naphthoflavone (5,6-benzoflavone) were obtained from Sigma Chemicals. Fish were administered all chemicals by a single intraperitoneal injection by a Hamilton microsyringe. In chemically pretreated fish, the pretreatment occurred over a 4 day interval at which time 17 β-estradiol was administered. Fish were sacrificed 2 days post-estradiol administration by a blow to the head and the liver was excised and immediately immersed in liquid nitrogen or cold RNA extraction buffer.

B. In vitro. Confluent flasks (Corning, 75 cm^2) were used for all in vitro experiments. Alpha MEM containing either normal fetal calf serum, stripped serum, or stripped serum containing the desired concentration of exogenous estradiol. Serum was stripped of endogenous steroid hormones by treatment with dextran-coated charcoal as described by Horwitz and McGuire (1978). Estradiol-17B was dissolved initially in propylene glycol (1 mg/ml) and then diluted to the appropriate concentration with 0.5% ethanol. Chemicals were then added to α-MEM so that each flask contained 10 ml as a final volume. Cells were incubated at 20°C for the required duration, at which time the cells were trypsinized and pelleted prior to RNA extraction.

RNA Extraction

Total RNA was extracted from livers of fish by the method of phenol chloroform-SDS extraction described by Chen (1980). All chemicals were provided by BDH chemicals (Toronto, Ontario) unless

otherwise indicated. To 1 gm of liver, 10 ml of 0.1 M Sodium Acetate buffer (pH 5.0) containing 25 mM NaCl, 35 mM $MgCl_2$, 25 mM EGTA, 25 µg/ml polyvinyl sulfate (Sigma), 35 µg/ml spermine (Sigma), 0.5% sodium dodecyl sulphate (SDS), and 10 ml of buffer saturated double distilled phenol. After homogenization, the mixture was shaken for 10 minutes at room temperature, and then 20 ml of chloroform was added and shaken for another 10 minutes. The aqueous phase was subsequently extracted with equal volumes of chloroform for 5 times. The RNA was then precipitated by the addition of 2.5 volumes of ethanol at -20°C. The crude RNA pellet was washed 5 times in 3 M Sodium acetate (pH 6.0), twice in 70% ethanol containing 0.3 M Sodium acetate and then dissolved in distilled water.

In other instances, total RNA was prepared following the method of Lithium Chloride-Urea extraction described by Searle and Tata (1981). Each gram of liver was homogenized in 10 ml of LU1 extraction buffer (3 M lithium chloride, 6 M Urea, 200 µg/ml heparin, 0.05% SDS, 50 mM Sodium acetate; pH 5.6) and the RNA was allowed to precipitate overnight at 4°C. The RNA was pelleted at 17,000xg for 30 minutes, resuspended in LU2 buffer (4 M LiCl, 8 M Urea, 50 mM Sodium acetate at pH 5.6) and pelleted as above. The RNA was resuspended in TKE buffer (200 mM Tris-HCl, 50 mM KCl, 10 mM EDTA, 0.1% SDS; pH 8.5), extracted three times with the equal volume of TKE saturated phenol and chloroform and then followed by two extractions with chloroform. The RNA in the aqueous phase was then precipitated at -20°C in 2.5 volumes of ethanol containing 0.1 M Sodium acetate for 24 hours. Following centrifugation at 17000xg for 10 minutes, the RNA pellet was washed three times with 3 M Sodium acetate, once in 70% ethanol containing 0.1 M Sodium acetate, and once

in 100% ethanol. Upon drying, the RNA was finally resuspended in water or the appropriate buffer for further processing.

A third method for the extraction of RNA was employed for *in vitro* work only (Borun *et al.*, 1967). Cells were pelleted after trypsinization and then resuspended in 45 µl of 10 mM Tris (pH 7.0) and 1.0 mM EDTA (4°C). Cells were then lysed with 5 µl of 5.0% NP-40 (Shell Chemicals, Toronto, Ontario) and mixed on ice for five minutes. Nuclei were pelleted by centrifugation in an eppendorf centrifuge for 2.5 minutes and the supernatant transferred to a 1.5 ml eppendorf tube containing 30 µl of 20XSSC (1XSSC = 0.15 M NaCl, 0.015 M Trisodium citrate) plus 20 µl of 37% (w/v) formaldehyde. The mixture was incubated at 60°C for 15 minutes and either applied to nitrocellulose or frozen at -70°C for later analyses.

Enrichment of Poly A-Containing RNA

Poly A-containing RNA was enriched either by oligo(dT) cellulose (Type 3) or poly(U) Sephadex G-10 affinity chromatography (Collaborative Research Inc., Waltham, Massachusetts). The former employed the methods described by Shapiro and Baker (1977). RNA was dissolved in 25 mM Hepes, 10 mM EDTA, and 1% SDS (pH 7.5) at 20 A_{260} units of RNA per ml. This solution was heated at 68°C for 10 minutes and then cooled quickly on ice to room temperature. Lithium chloride was added to a concentration of 0.5 M. RNA was mixed with oligo(dT) cellulose at a ratio of 20 mg oligo(dT) cellulose per mg RNA and stirred for 20 minutes. The slurry was then transferred to a glass column and was washed with 20 ml of binding buffer (25 mM Hepes, 10 mM EDTA, 500 mM LiCl, 1% SDS; pH 7.5).

The effluent was reapplied three times to assume maximum binding of poly A-containing RNA to the oligo(dT) cellulose. The column was then washed with 20 ml of wash buffer (25 mM Hepes, 2 mM EDTA, 100 mM LiCl, 1% SDS; pH 7.5). The bound poly A-containing RNA was eluted in Hepes buffer (10 mM, pH 7.5) containing, 1 mM EDTA and 0.1% SDS at 37°C. After the addition of purified yeast transfer RNA to a final concentration of 50 µg/ml, the RNA was precipitated with ethanol. The RNA was repurified by oligo(dT) cellulose chromatography.

The conditions for poly(U) Sephadex G-10 affinity chromatography are as described by Lizardi (1976). RNA was dissolved in TES-25 buffer (10 mM Tris buffer, 0.25 M NaCl, 0.5 mM EDTA, 0.4% SDS; pH 7.4) and was heat denatured at 65°C for 10 minutes. For each mililiter bed volume of poly(U) Sephadex, 50 A_{260} units of total cellular RNA was applied. Hybridization was performed at room temperature and the unbound RNA removed by washing with eight bed volumes of TES-2 buffer (10 mM Tris, 0.02 M NaCl, 0.5 mM EDTA, 0.4% SDS; pH 7.4). Bound RNA was eluted by raising the column temperature to 53°C and washing with an elution buffer (5 mM Tris, 45% formamide (w/v), 0.5 mM EDTA, 0.4% SDS; pH 7.4).

Binding of RNA to Nitrocellulose

A. Agarose Gel Method

RNA samples were denatured and electrophoresed in 1.2% agarose horizontal gels (16.5 x 17.0 x 0.6 cm). RNA electrophoresis buffer (E-buffer) contained 50 mM Boric acid, 5 mM Sodium borate, 10 mM Sodium sulphate, 1 mM EDTA (pH 8.19). Agarose (1.2 w/v) was melted in E-buffer and cooled to 75°C. Methyl mercury hydroxide (10 mM) and Ethidium

bromide (5 µg/ml) were added, mixed, and poured into the gel apparatus. The RNA samples were diluted in water containing 5 mM methyl mercury hydroxide to the desired concentrations and then adjusted to 10% glycerol and 0.005% bromophenol blue. This mixture was heated for ten minutes at 65°C and electrophoresed at 2 volts/cm for 10 hours.

The transfer of RNA from the agarose gel to the nitrocellulose filter was a modification of the methods described by Southern (1975). Following electrophoresis, the gel was incubated with intermittent shaking in 50 mM NaOH twice for 20 minutes at room temperature. The gel was then washed in 0.5 M Tris-HCl (pH 7.4) containing 3 M NaCl for 30 minutes each time with intermittent shaking. The gel was placed on two sheets of Whatmann 3MM chromatography paper saturated with 20XSSC and the borders of the gel and paper were covered with sheets of plastic Saran Wrap. The nitrocellulose paper (Sartorius, 0.45 µm pore size) soaked in 20XSSC was placed on the gel, then overlayed with two sheets of Whatmann 3MM paper dampened with 20XSSC and overlayed with a stack of paper towels. A light weight was placed on top. After 10 hours, the nitrocellulose filter was removed and baked at 80°C for 2 hours.

B. The Hybridot Method

Total RNA was diluted in 5XSSC containing 10 mM methyl mercury hydroxide to various concentrations. A nitrocellulose filter, after being soaked for 1 hour, was placed on top of two sheets of presoaked Whatmann 3 MM filter paper laid on the Hybridot Manifold (BRL, Bethesda, Maryland). Samples were then applied in 30 µl volumes, each containing between 0.05 and 12.5 µg RNA, under suction. The sample wells were

then washed with 100 µl of 20XSSC to ensure that all RNA became bound to the nitrocellulose. The nitrocellulose filter was then removed from the apparatus and dried at 80°C under vacuum for 2 hours.

Extraction of Plasmid DNA

Plasmid DNA was prepared essentially following the method of Birnboim and Doly (1979) with a few modifications. Clones containing recombinant plasmids (namely pRTC 2, pRTC 5, and pRTC-myc 1-81) were kindly provided by Dr. T.T. Chen (McMaster University). Overnight cultures were inoculated in Luria broth containing 20 µg/ml tetracycline. Cells were pelleted and lysed thereby releasing the cellular constituents. Protein, high molecular weight RNA, and chromosomal DNA were selectively precipitated and pelleted in 3 M Sodium acetate (pH 4.8). The plasmid DNA, present in the supernatant, was removed, transferred to a second eppendorf tube, and then precipitated twice in ice cold ethanol. Following washing in 80% ethanol, the pellet was dissolved in TNE buffer (10 mM Tris, 10 mM NaCl, 1 mM EDTA; pH 7.6) and then reprecipitated in ethanol. After centrifugation, the plasmid DNA was dissolved in TNE buffer.

Nick Translation of Plasmid DNAs

The various plasmid DNAs were labelled with (alpha-^{32}P)dCTP by nick translation following the method described by Southern (1975). Approximately 1.0 µg of DNA was incubated for 10 minutes at 37°C with 0.25 ng of DNAase I (Boehringer Mannheim) in 100 µl of 25 mM Tris-HCl (pH 7.9), 25 mM $MgCl_2$, 5 mM β-mercaptoethanol, 5 µg bovine serum albumin, and 2 mM each of dTTP, dATP, and dGTP. The mixture was then

combined with 100 μCi of (alpha-^{32}P)dCTP in 10 mM. Tricine buffer (New England Nuclear, specific activity 800 Ci/mM) and 6 units of E. coli DNA polymerase I (Boehringer Mannheim) and incubated for 80 minutes at 12°C. Where all four bases were radiolabelled, no cold triphosphates were added. The reaction was stopped by addition of EDTA to a final concentration of 25 mM. The labelled DNA was separated from unincorporated (alpha-^{32}P)dCTP by spin dialysis through a 1.5 ml Sephadex G-50 column. The radiolabelled DNA was denatured for 5 minutes at 100°C and then hybridized to the Northern blots.

Hybridization of Radiolabelled DNA to Northern Blots

Hybridization of radiolabelled DNA to RNA bound to nitrocellulose filter was carried out in two steps. The filter was placed in a polyethylene "freezer" bag in the presence of approximately 10 ml of prehybridization buffer containing 30-50% formamide, 5XSSC, 5X Denhardt's Solution (0.02% w/v bovine serum albumin, 0.02% w/v polyvinyl pyrollidone, 0.02% ficoll), 50 mM Sodium phosphate (pH 6.5), and 250 μg/ml of sheared and denatured salmon sperm DNA. The prehybridization was carried out at 42°C for 2 hours. The preincubation buffer was removed and replaced by 10 ml of hybridization buffer (30-50% formamide, 5XSSC, 1X Denhardt's reagent, 20 mM Sodium phosphate (pH 6.5), 100 μg/ml sonicated and enatured salmon sperm DNA, and 10% Sodium dextran sulphate) and 5X10^{7} cpm heat denatured (alpha-^{32}P)-labelled DNA. The bag was sealed and incubated at 42°C for 24 to 48 hours. After hybridization, the filter was removed from the bag and washed 4 times each with 250 ml of 2XSSC, 0.1% SDS, for 5 minutes at

room temperature. The filter was then washed 3 times each with 250 ml of 0.1XSSC, 0.1% SDS at 50°C for 15 minutes. The filters were then dried in a vacuum oven at 80°C for 2 hours and exposed to Kodak X-Omat film. The intensity of the signal on the film was measured by densitometry and the area under each peak was measured with the Zeiss MOP III Digital Integrator System (Zeiss Ltd., Germany).

Labelling Estradiol-Responsive Protein Products in the Rainbow Trout Heptoma Cell Line

Cells were grown in a 20 ml α-MEM medium under standard conditions until confluence was reached. This medium was aspirated off and replaced by 20 ml α-MEM medium containing either normal fetal calf serum, stripped fetal calf serum, or stripped fetal calf serum containing the desired concentration of exogenous β-estradiol. Cells were incubated 24 hours with the medium being replaced with fresh medium every 4 hours. At 24 hours, the medium was removed and replaced with 4 ml α-MEM (Joklik's modified; Gibco) medium containing 50 uCi (^{35}S)-labelled methionine. The cells were gently shaken for 4 hours at 20°C. The medium was aspirated off, the cells were lysed with 1.0 ml of Tris-HCl buffer, and the two solutions combined together for centrifugation at 17000xg for 5 minutes. After centrifugation, 20 µl of each sample was spotted on 3MM filter discs and left to dry at room temperature. The discs were washed in 100 ml 20% TCA at 0°C for 10 minutes followed by washing in 100 ml 5% TCA. The latter solution was heated to 90°C for 20 minutes and then left to cool on ice. The discs were washed briefly in 5% TCA followed by a wash in 1 part ethanol to 1 part ether. The

filters were allowed to dry and then were counted in 5 ml aqueous scintillation counter fluid (Caledon Laboratories, Georgetown, Ontario).

The (^{35}S)-labelled vitellogenin polypeptides in each sample were precipitated with antivitellin serum and carrier vitellin essentially as described by Chen et al. (1979). To the medium extracted from the hepatoma cell line was added 300 µl antiserum and 120 ul lipovitellin (1 part globular protein to 50 parts buffer). After overnight incubation at 4°C, the mixture was washed twice in 0.4 ml sodium phosphate buffer (0.1 M, pH 7.5) containing 0.9% NaCl, 1% Triton X-100, and 1.0% deoxycholate, vortexed and centrifuged again at 17000xg for 5 minutes. The immunoprecipitate was resuspended in a buffer containing 0.2 M Tris-HCl (pH 8.0), 0.5% SDS, 10% glycerol, and 5% β-mercaptoethanol. Eighty microliters of each sample was then counted in 5 ml of scintillator fluid, and the remaining samples were analyzed by SDS-PAGE.

SDS (0.1%)-Polyacrylamide (5%) gels were prepared according to Chen et al. (1978). Bromophenol blue was added to the above protein samples to a final concentration of 0.002%. The samples were denatured at 100°C for 3 minutes and 50 µl of each denatured sample was applied to each well. Electrophoresis was carried out in a Tris-glycine buffer (25 mM Tris, 0.19 M glycine containing 0.1% SDS) at a constant voltage of 30 Volts.

After electrophoresis, polyacrylamide gels were prepared for fluorography by the method of Laskey and Mills (1975). The gels were fixed in 7% acetic acid, equilibrated with dimethylsulfoxide (DMSO) and impregnated in a solution of 22.2% (w/v) Omnifluor in DMSO. Gels were then washed in water for at least 2 hours, dried under vacuum at 90-100°C

and exposed to Kodak RPX-Omat film at -70°C. Films were developed in a Kodak automatic film processor.

Statistical Analyses

All results are expressed as a mean ± standard deviation for each group of fish. Statistical analyses were performed using a computer statistics package provided kindly by Dr. Douglas Davidson (McMaster University). Statistical analyses were performed using the Student's t-test and the level of significance was set at $P<0.05$.

RESULTS

Development of Quantitative Dot Hybridization Method

Quantitation of estrogen-responsive mRNA sequences by hybridization offers several advantages over quantitation by *in vitro* translation. These are: (a) the method does not require intact mRNA molecules, hence any nicked mRNA molecules generated during isolation can still be detected; (b) it offers high sensitivity so even rare species of mRNA can be detected; (c) quantitation of mRNA molecules by hybridization is independent of the translational efficiency of individual mRNA preparations in the *in vitro* translation system, hence all samples can be determined under equal efficiency. Since at least 30% of the total export proteins synthesized by the liver of reproductively active rainbow trout are estrogen-responsive (T.T. Chen, personal communication), the successful extraction and recovery of these specific mRNAs is essential for quantitative purposes. The large size of vitellogenin mRNA and the high levels of ribonuclease activity in the liver make extraction of undegraded vitellogenin mRNA difficult. For this reason, two different methods for the extraction of RNA were tried. Both procedures, Phenol chloroform-Sodium dodecyl sulphate and Lithium chloride-Urea, resulted in similar yields of intact RNA being recovered (Table 2 and Fig. 1).

The most reliable and sensitive method employed to quantify mRNA is by molecular hybridization carried out in liquid using cDNA, derived by reverse transcribing mRNA, as a probe. Conventionally, this

TABLE 2

Recovery of RNA Extracted From Rainbow Trout Liver By
The Lithium Chloride-Urea Method and the Phenol Chloroform-SDS Method

Extraction Method	Sample Number	RNA Recovery (mg RNA/gm liver ± S.D.)[a]
Lithium Chloride-Urea	5	9.81 ± 1.10
Phenol Chloroform-SDS	5	8.17 ± 1.48

[a] Standard derviation

Figure 1. Agarose gel electrophoresis of total liver RNA extracted by the lithium chloride-urea method and the Phenol chloroform-SDS method. RNA was extracted from the liver of juvenile rainbow trout by the lithium chloride-urea method (a) or the phenol chloroform-SDS method (b) as described in Materials and Methods, and then subjected to electrophoresis on á 1.2% agarose gel.


a
b
28S
18S

type of hybridization is done in liquid (Baker and Shapiro, 1977). The major drawback of this method is the lengthy operation. To simplify this method, RNA was bound irreversibly to nitrocellulose filter and then hybridized to (^{32}P)-labelled pRTC 2 and pRTC 5 following the conditions described by Chen (1983). Upon washing off any unhybridized probe, the radioactivity on each spot was determined either by direct scintillation counting or by autoradiography followed by subsequent densitometric scanning of the autoradiogram. Figure 2 is a representative autoradiogram of such an experiment. The total amount of mRNA hybridizing to the radiolabelled probe was proportional to the input of induced total RNA in each sample spot (Figs. 3A and 3B). Furthermore, addition of uninduced total RNA to the induced RNA had no effect on this proportionality (results not shown). In both cases, scintillation counting and densitometry, the hybridizations were found to be linear from 0-3 µg of the total induced RNA input. From many repetitive trials, the success of this method was found to be in the removal of unhybridized radioactive probe thoroughly following hybridization so that it would not contribute to a high background thereby interfering with samples that have a low degree of hybridization. In the experiments following, only points present within the extremes of the standard curve were used.

In vivo Induction of Estrogen-Responsive Genes

Results from previous studies in our laboratory indicated that doses as low as 20 µg β-estradiol per 100 gm fish body weight was able to induce vitellogenin to a detectable level in the serum by rocket immunoelectrophoresis (T.T. Chen, personal communication). Since the

Figure 2. A representative autoradiogram of quantitative dot-hybridization (Hybridot) of nick translated probe to increasing liver RNA. Various amounts of induced liver RNA in 30 µl was applied to nitrocellulose filter and hybridized to (^{32}P)-pRTC 2 (A) and (^{32}P)-pRTC 5 (B) under the conditions described in Materials and Methods.

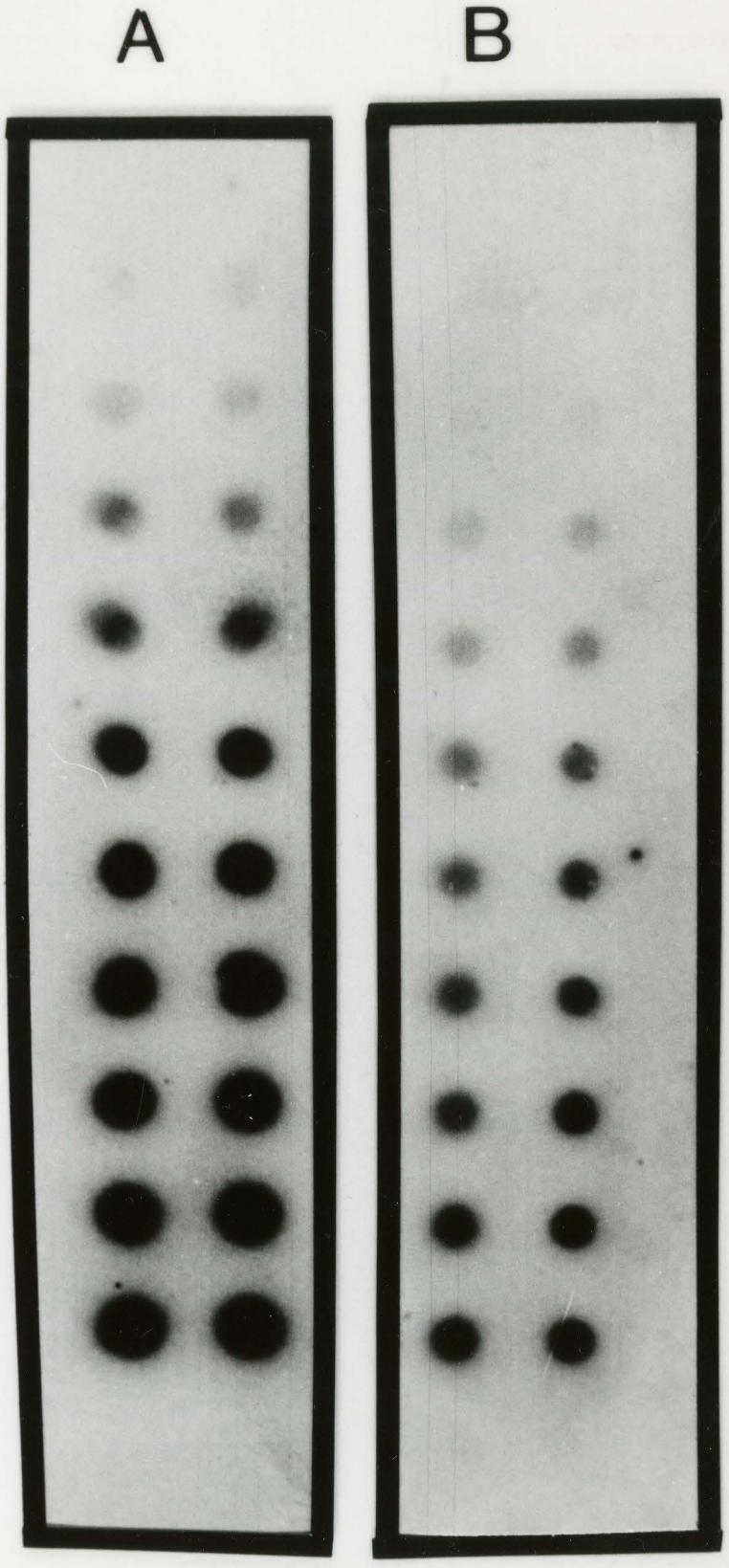
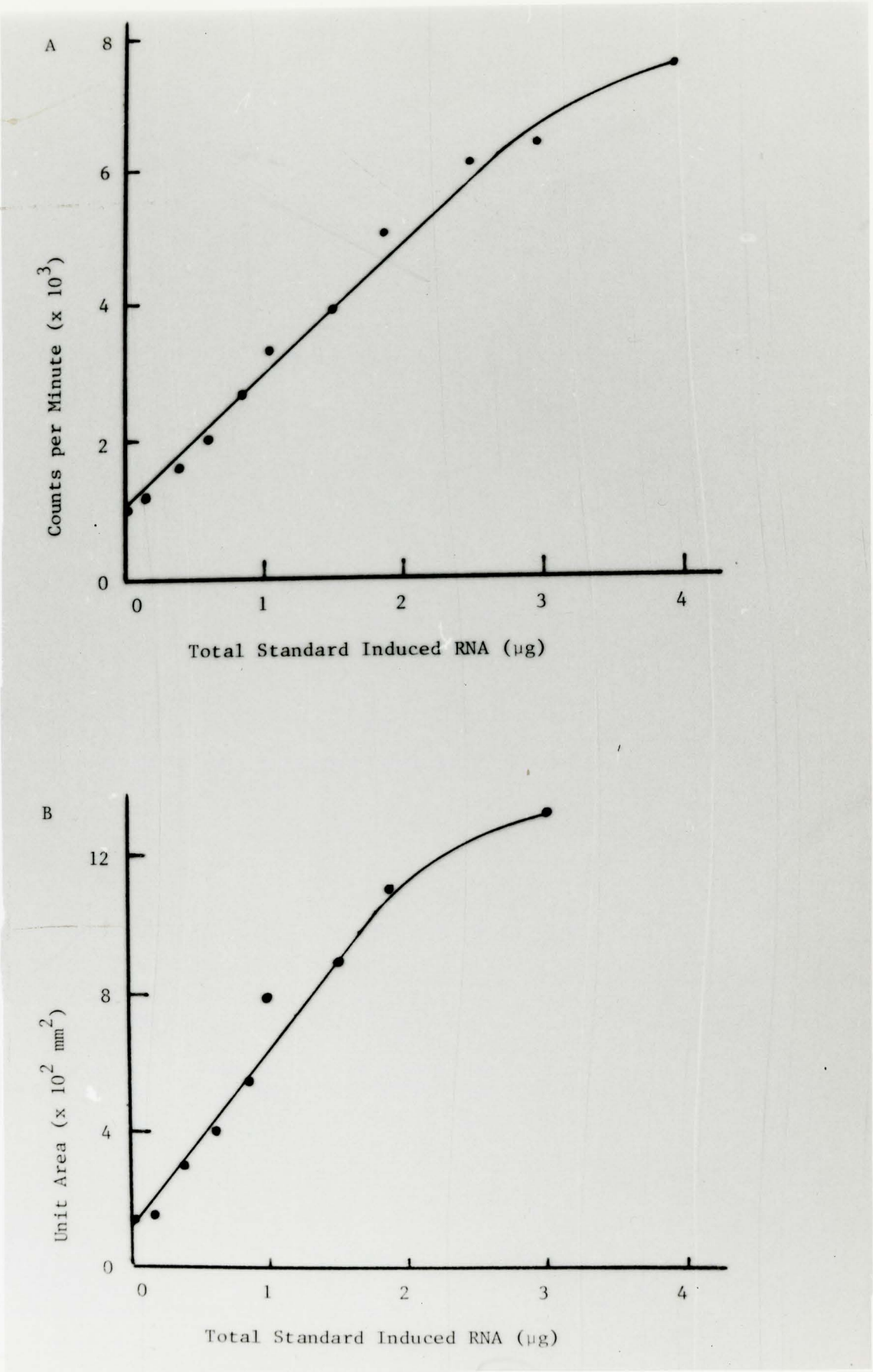

A
B

Figure 3. Standard curves derived from a representative Hybridot shown in Figure 2. (A) Radioactivity as a function of the amount of total induced RNA in each spot determine by scintillation counting. (B) Area under each peak as a function of the amount of total induced RNA in each spot determined by densitometry. The above are the average of 2 separate determinations.


A
8
6
4
2
0
Counts per Minute (x 10^3)
0
1
2
3
4
Total Standard Induced RNA (µg)
B
12
8
4
0
Unit Area (x 10^2 mm^2)
0
1
2
3
4
Total Standard Induced RNA (µg)

quantitative dot hybridization method if far more sensitive than rocket immunoelectrophoresis, one may be able to detect the effect of β-estradiol on gene expression in the liver at lower doses of hormone. Maximal induction of pRTC 2 mRNAs occurred at levels of 20 μg estradiol per 100 gm fish whereas the dose required for the maximal expression of pRTC 5 mRNAs was much less at 5 μg per 100 gm fish (Figs. 4 and 5).

The quantitative dot hybridization method was also employed to study the rate of accumulation of RNA transcripts homologous to pRTC 2 and pRTC 5. Maximal accumulation of transcripts homologous to pRTC 2 occurs within 8 hours after stimulation with 80 μg/100 gm body weight of estradiol (Fig. 6). The transcript level decreased rapidly between 8 and 24 hours whereby it slowly decreased thereafter. However, even 16 days after the intraperitoneal injection, the level of vitellogenin RNA transcripts never resumes the control level. Upon a second administration of estradiol, maximal accumulation of vitellogenin mRNA was reached 12 hours after hormone treatment, and the level of induction maintains at maximum for about 6 days (Fig. 7). The transcript level decreases slowly at this point such that even 20 days after secondary induction, the levels of vitellogenin mRNA are still above the control levels.

In many ways the accumulation of mRNA transcripts homologous to pRTC 5 mimics those encoding for vitellogenin. Upon primary induction, the transcripts were found to be present within 8 hours thereby decreasing rapidly for the next 8 hours (Fig. 6). By 16 hours post-inoculation, the transcript levels returned to those of control. Upon secondary induction, mRNAs homologous to pRTC 5 appear to reach maximal levels at 8 hours

Figure 4. Dose response relationship of the accumulation of pRTC 2 transcripts *in vivo* induced by estradiol. Juvenile rainbow trout were treated by intraperitoneal injection with varying concentrations of β-estradiol solubilized in propylene glycol. RNA was extracted from the livers 24 hours later and quantitated by the Hybridot procedure. A minimum of 5 fish per dose of estradiol were used.

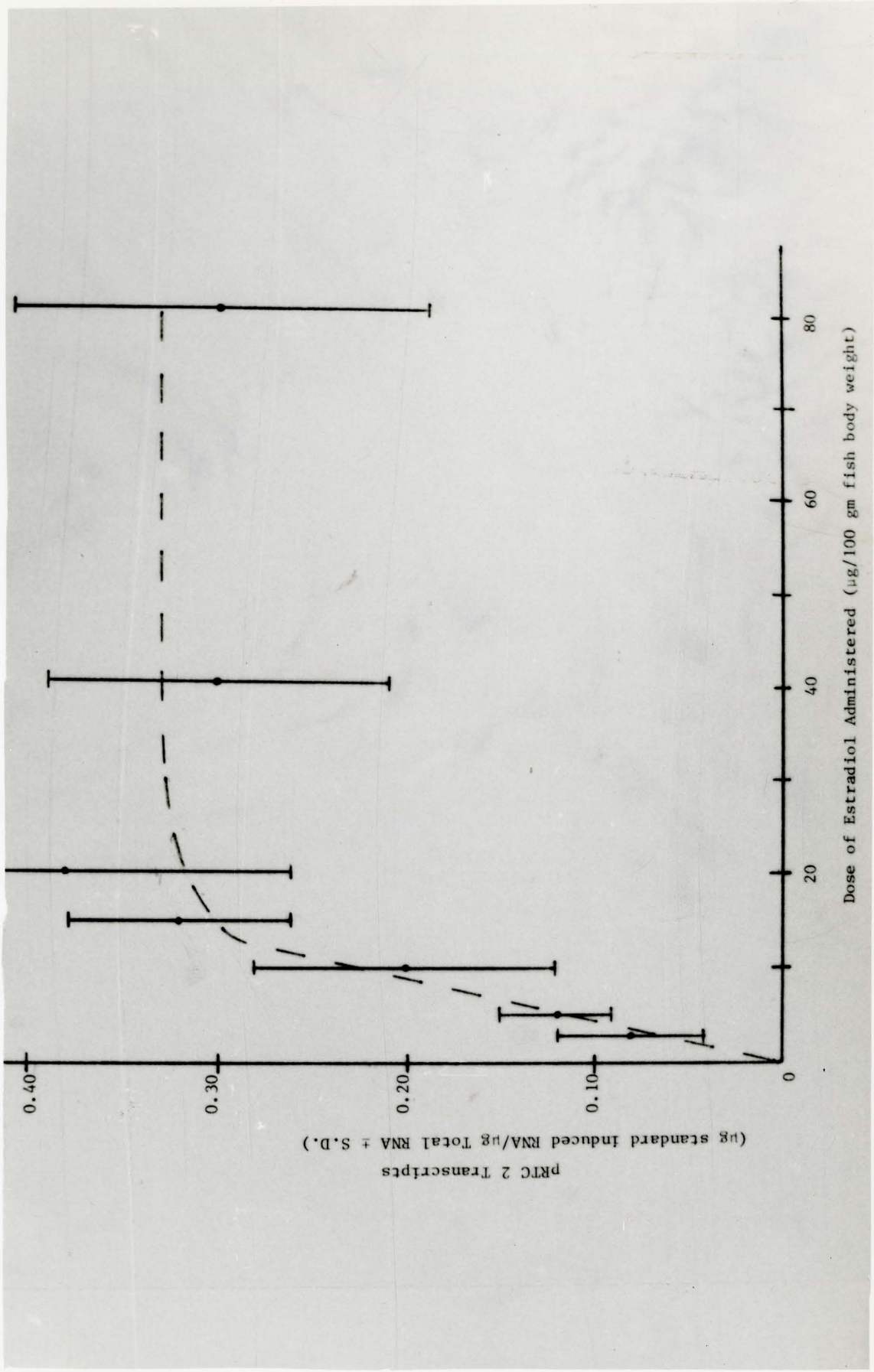

pRTC 2 Transcripts
(µg standard induced RNA/µg Total RNA ± S.D.)
0.40
0.30
0.20
0.10
0
20
40
60
80
Dose of Estradiol Administered (µg/100 gm fish body weight)

Figure 5. Dose response relationship of the accumulation of pRTC 5 transcripts *in vivo* induced by estradiol. Juvenile rainbow trout were administered by intraperitoneal injection varying concentrations of β-estradiol solubilized in prolylene glycol. RNA was extracted from the livers 24 hours post-inoculation and quantitated by Hybridot. A minimum of 5 fish per dose of estradiol were used.

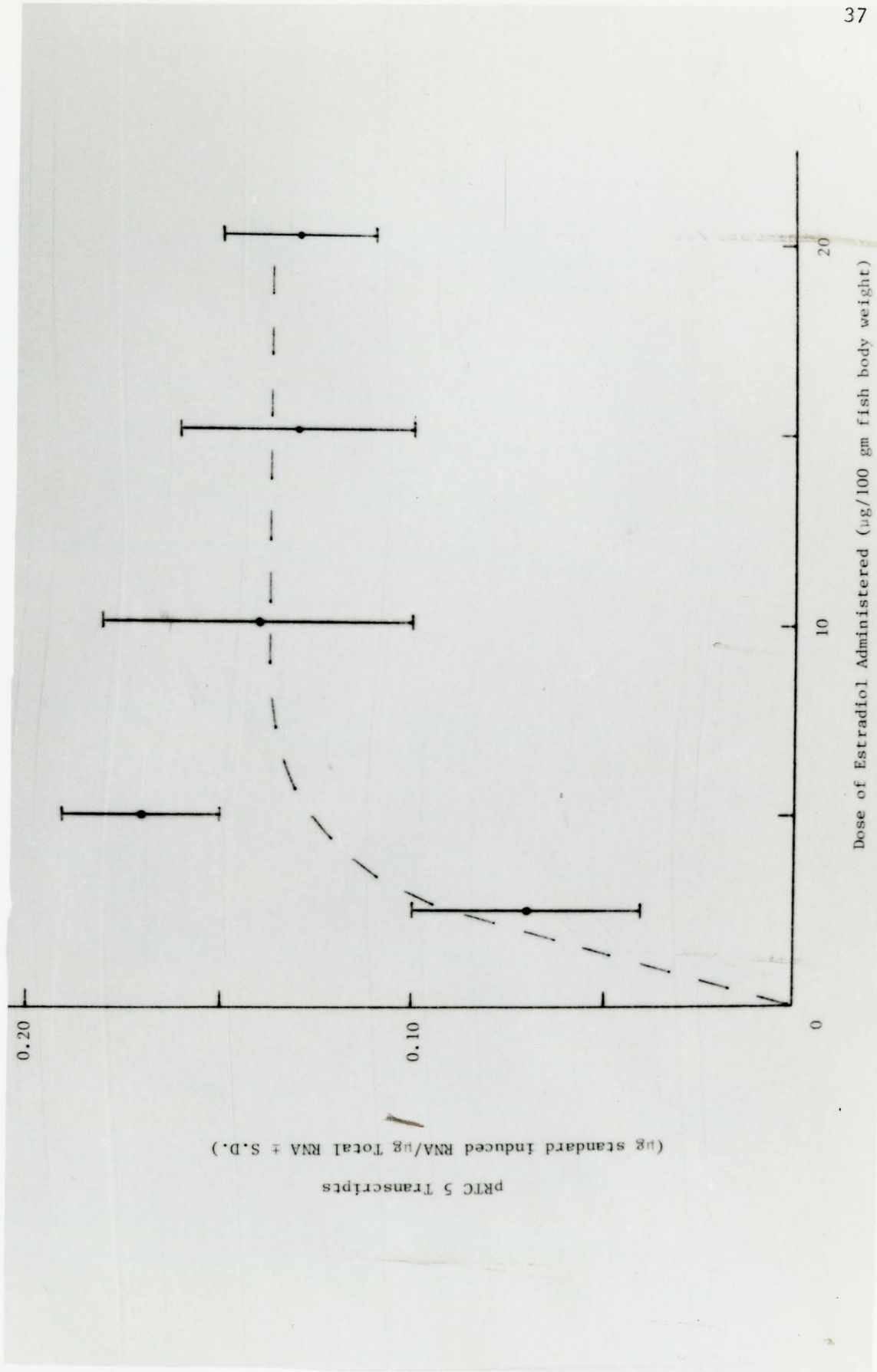

0.20
0.10
0
10
20
Dose of Estradiol Administered (µg/100 gm fish body weight)
pRTC 5 Transcripts
(µg standard induced RNA/µg Total RNA ± S.D.)

Figure 6. The time course of the accumulation of estrogen-responsive mRNAs incuded by β-estradiol in the primary stimulation in vivo. Juvenile rainbow trout were administered with 80 μg β-estradiol/ 100 gm fish body weight and were sacrificed at the appropriate time point. RNA was extracted from the livers and analyzed by the Hybridot procedure. All time points are the average of 5 fish. ●——●, pRTC 2 transcripts; ■——■, pRTC 5 transcripts.

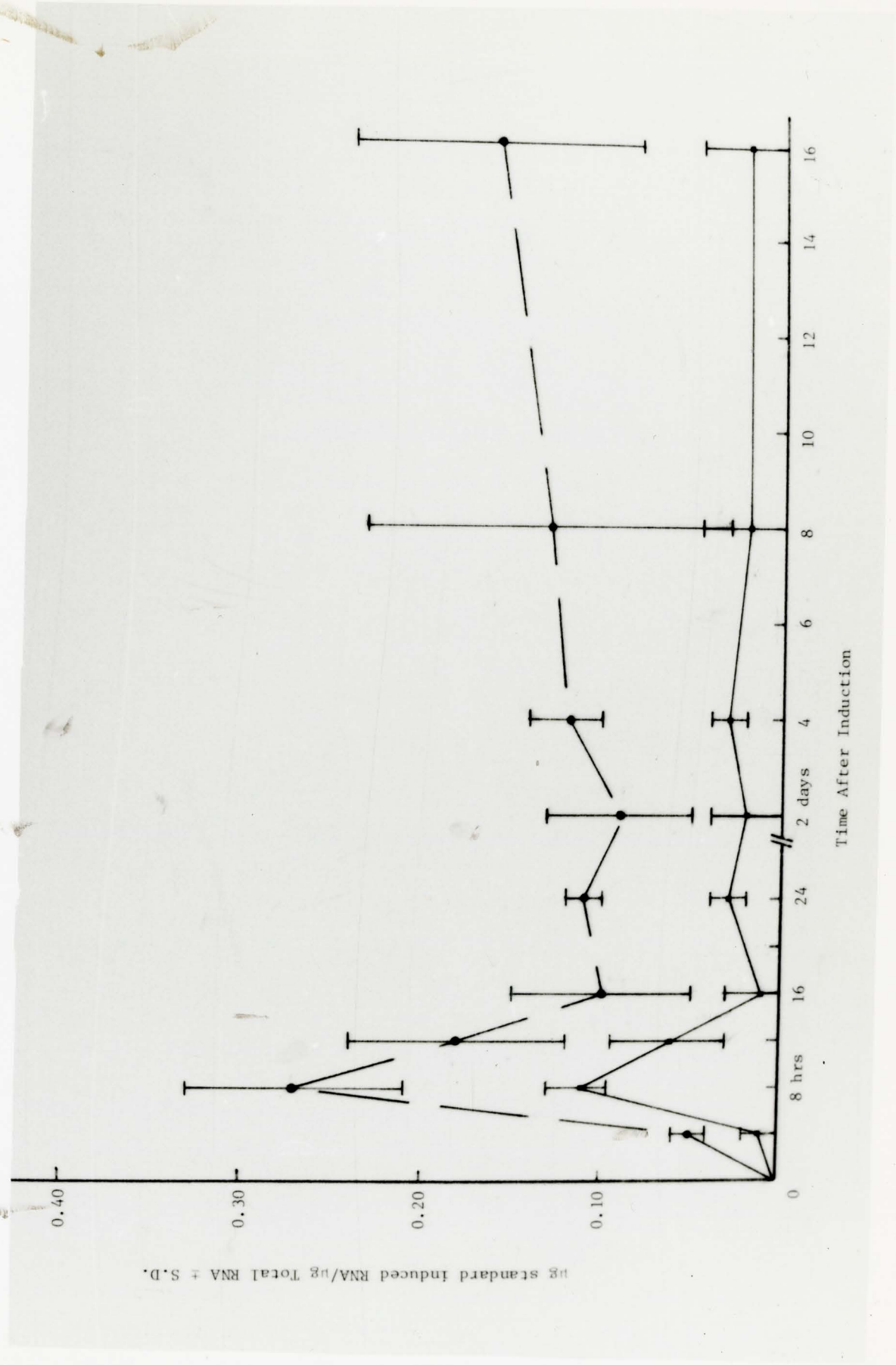
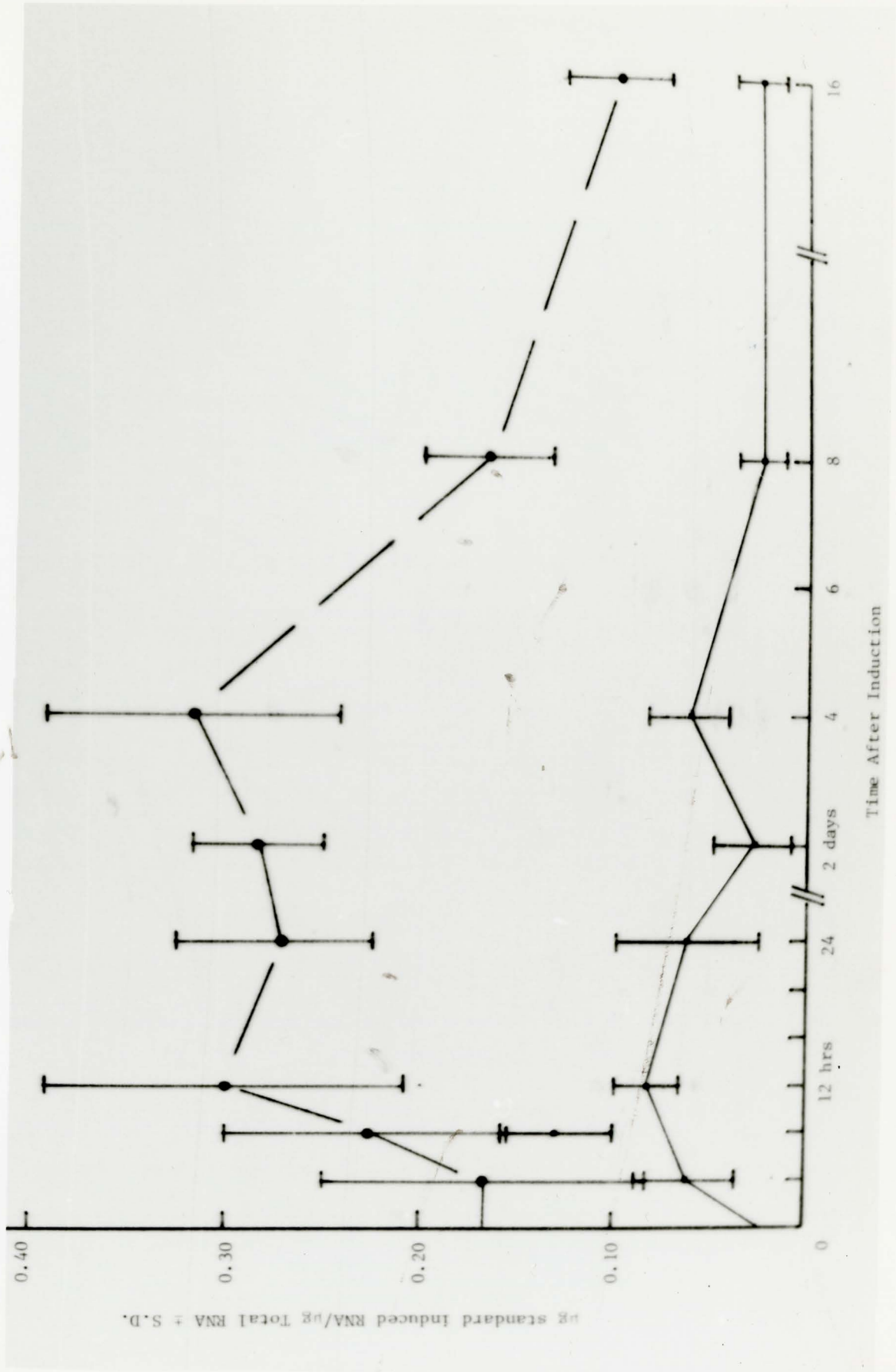

μg standard induced RNA/μg Total RNA ± S.D.
0.40
0.30
0.20
0.10
0
8 hrs
16
24
2 days
4
6
8
10
12
14
16
Time After Induction

Figure 7. The time course of the accumulation of estrogen-responsive mRNAs incuded by β-estradiol in the secondary stimulation *in vivo*. Juvenile rainbow trout were administered with 80 μg β-estradiol/ 100 gm fish body weight and subsequently injected a second time with the same dose twenty days after the primary injection. RNA was extracted from the livers and analyzed for homlogous transcripts to nick translated probes of pRTC 2 or pRTC 5 by the Hybridot procedure. All time points are the average of 5 fish. ●——●, pRTC 2 transcripts; ■——■, pRTC 5 transcripts.


μg standard induced RNA/μg Total RNA ± S.D.
0.40
0.30
0.20
0.10
0
12 hrs
24
2 days
4
6
8
16
Time After Induction

(Fig. 7). Levels of pRTC5 transcripts decreased slowly from 8 hours to 8 days after hormone administration at which time the levels were approximately that of the control.

Induction of Estrogen-Responsive Genes in Hepatoma Cells

An attempt was made to stimulate the expression of pRTC 2 and pRTC 5 transcripts in a hepatoma cell line by the addition of exogenous estradiol. In α-MEM containing 10% fetal calf serum at 20°C, the hepatoma cells have a doubling time of 74 hours (Fig. 8). Transcripts of pRTC 2 and pRTC 5 are detected in cells cultured in α-MEM containing 10% fetal calf serum (Fig. 9). Because fetal calf serum contains endogenous steroids, an attempt was made to strip them from the serum using dextran-coated charcoal. Transcripts of pRTC 2 and pRTC 5 are undetectable when cells are cultured in the stripped fetal calf serum (results not shown). However, cells grown in the stripped serum containing exogenous estradiol, in doses as low as 10^{-8} M, display the presence of both classes of these transcripts (Fig. (9). SDS-PAGE reveals that at least one of these mRNAs, presumably that encoding for vitellogenin, is translated into protein (Fig. 10).

Detection of c-myc mRNA

A plasmid clone containing v-myc, derived from avian myelocytomatosis virus MC29, was obtained and employed as a probe to screen the rainbow trout gene bank. From this bank, a sequence (RT c-myc) homologous to v-myc was isolated and subcloned into plasmid pBR325 (pRTC-myc 1-81) (Chen et al., 1984b). Northern blot analysis revealed

Figure 8. Growth curve of the rainbow trout hepatoma (RTH) cells. Cells were grown on α-MEM supplemented with 10% fetal calf serum (FCS), 25 mM Hepes buffer, Penicillin-Streptomycin (100 units/ml) and Kanomycin (100 ug/ml). At the appropriate time points, cells in pairs of flasks were counted and the average of both were recorded.

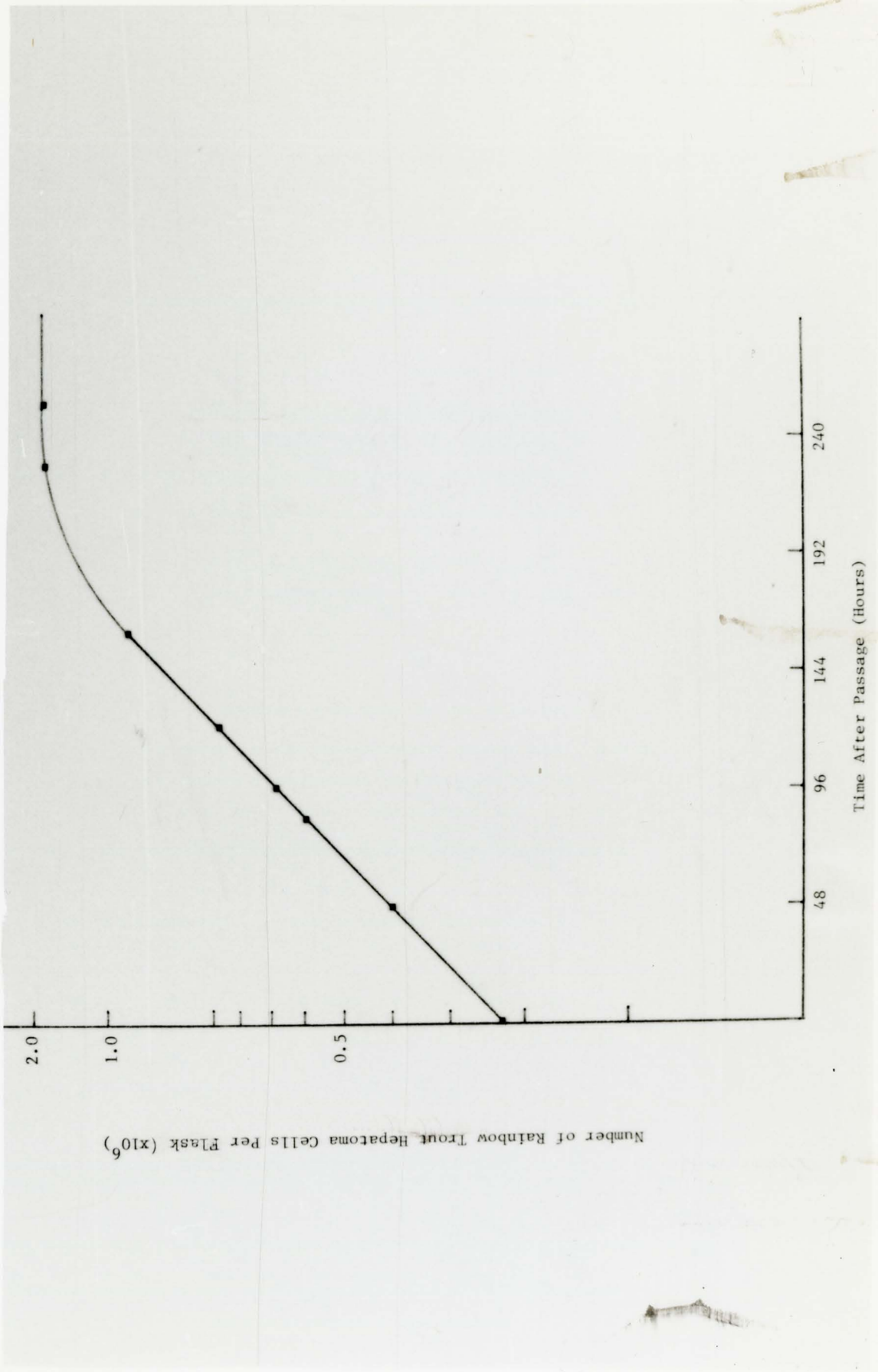

Number of Rainbow Trout Hepatoma Cells Per Flask ($x10^6$)
2.0
1.0
0.5
48
96
144
192
240
Time After Passage (Hours)

Figure 9. Quantitation of pRTC 2 and pRTC 5 transcripts in RTH cells by Hybridot. Cells were grown on α-MEM supplemented with 10% FCS, 25 mM Hepes, 100 units/ml penicillin-streptomycin, and 100 μg/ml kanomycin. As cells reached confluence, triplicate flasks were maintained in either α-MEM supplemented with normal fetal calf serum or α-MEM supplemented with charcoal-dextran stripped fetal calf serum containing varying concentrations of 17 β-estradiol for 24 hours. RNA was extracted from each sample, dissolved in 100 μl of water and 30 μl of each was dot blotted and hybridized with pRTC 5 (lane A) or pRTC 2 (lane B) under standard conditions. a, cells maintained on 10% FCS; b, stripped fetal calf serum (SFCS) with 10^{-10} M β-estradiol; c, SFCS supplemented with 10^{-9} M β-estradiol; d, SFCS supplmented with 10^{-8} M β-estradiol. It should be noted that SFCS alone did not induce transcripts homologous to pRTC 2 or pRTC 5 (results not shown).

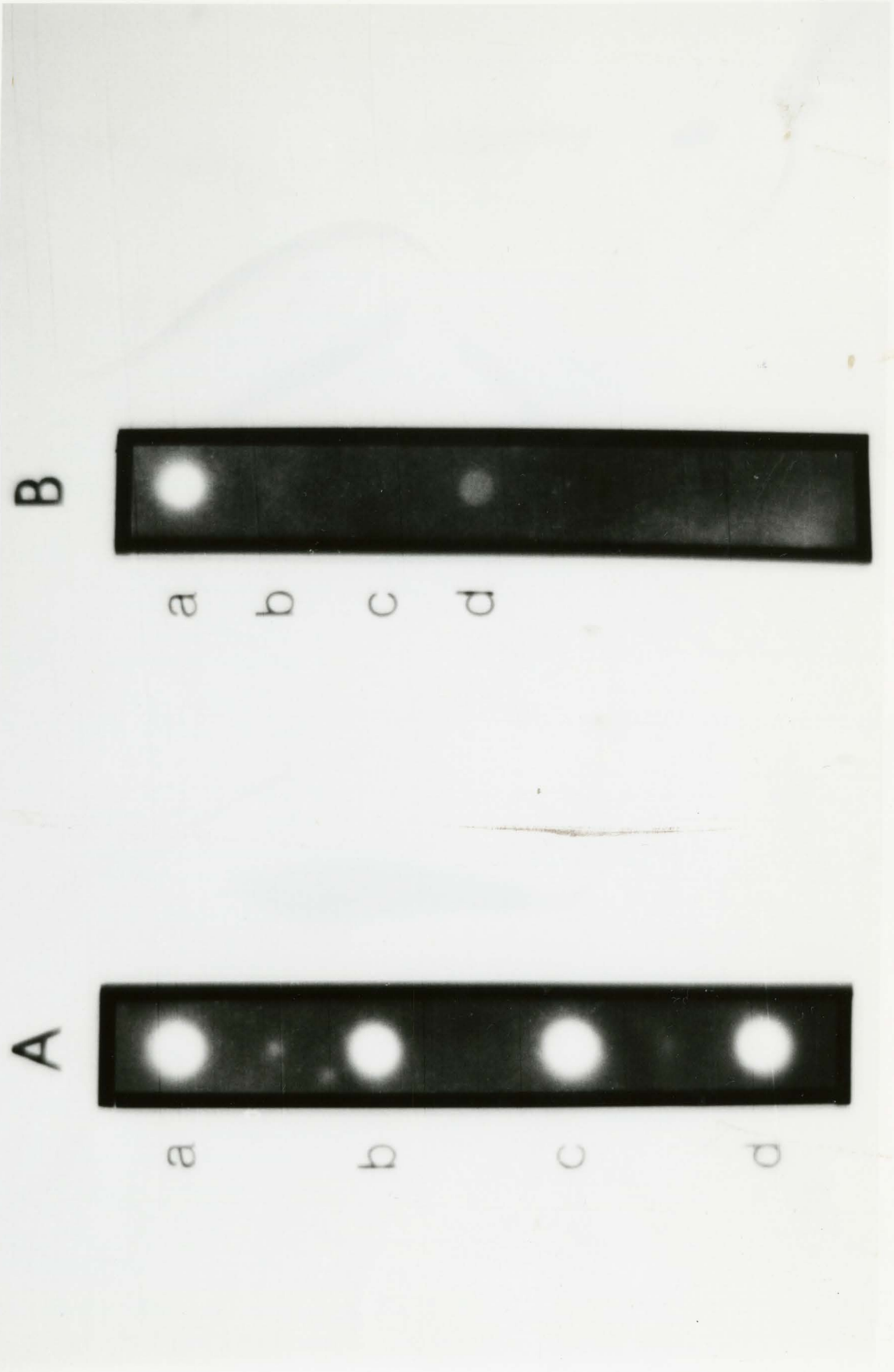
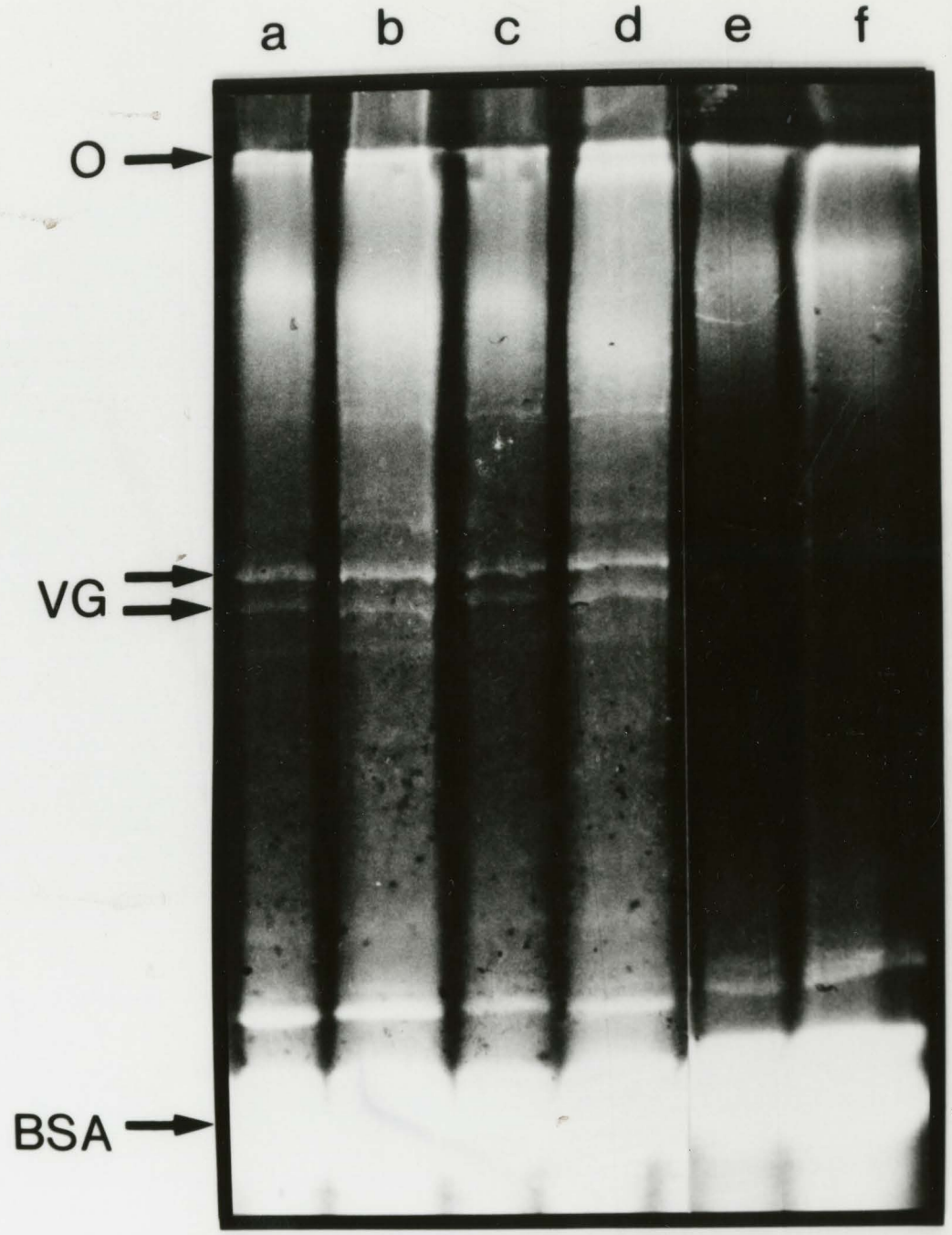

B
a b c d
A
a b c d

Figure 10. SDS-PAGE of immunoprecipitable products isolated from the RTH cell line. Cells were grown until confluence under standard conditions and then stripped of their medium. Fresh medium either containing normal fetal calf serum or stripped fetal calf serum or stripped fetal calf serum supplemented with 10^{-8} M β-estradiol was added every 4 hours for 24 hours. Cells were then labelled with (^{35}S)-methionine (100 µCi/flask) in 3 mls Joklik's modified medium for 4 hours. Cells were then lysed and the proteins were immuno-precipitated with anti-lipovitellin serum as described by Chen (1983) and the products analyzed on 5% SDS-polyacrylamide gels. a, (20 µl); b, (40µl), cells maintained in 10% fetal calf serum; c, (20 µl); d (40 µl), cells grown in stripped fetal calf serum supplemented with 10^{-8} M β-estradiol; e, (20 µl); f, (40 µl); cells grown in stripped fetal calf serum. O, origin of separation gel; VG, the presumptive vitellogenin peptides; BSA, bovine serum albumin marker.


a
b
c
d
e
f
O
VG
BSA

the presence of RT c-myc transcripts of approximately 2.5 and 5.6 kb (Fig. 11) in the poly A^+ RNA (Fig. 12) isolated from the livers of rainbow trout. The administration of estradiol increased the number of RT c-myc transcripts in the liver (Table 3 and Fig.13). Furthermore, similar results were observed in the hepatoma cell line (Table 3 and Fig. 13).

Effects of Xenobiotics on the Expression of Estrogen-Responsive Genes In Vivo

The heptosomatic indices (HSI) of the various schedules of pretreatment of representative xenobiotics are shown in Table 4. Rainbow trout treated with p,p'-DDT showed no significant differences to the controls in this respect. The HSI values in fish treated with large doses of either β-naphthoflavone or Kepone were found to be significantly larger ($p<0.005$ and $p<0.025$ respectively) than the controls.

The quantitative dot hybridization was also employed in the analysis of the effects of the above three mentioned chemicals on the induction of the estrogen-responsive mRNAs induced by β-estradiol. β-naphthoflavone at 7.5% or 75% LD_{50} neither induces or represses the expression of both estrogen-responsive genes (Table 5). Kepone at 7.5% significantly decreased the number of transcripts homologous to pRTC 2 at the lower dose (20 μg) of β-estradiol. In contrast, Kepone at 7.5% LD_{50} appears to have no effect on the expression of pRTC 5 transcipts (Table 6). The isomer of DDT, p,p'-DDT, at 7.5% LD_{50} appears to enhance the expression of pRTC 5 transcripts only upon subsequent

Figure 11. Northern blot analysis of RNA homologous to rainbow trout c-myc sequences. RNA was extracted from the liver of control fish (lane b) or fish treated with 200 μg β-estradiol/100 gm fish body weight, for both the primary and secondary inductions (lane a). Total RNA of 30 μg was run on a 1.2% agarose gel and transferred to nitrocellulose prior to hybridization with nick translated pRTC-myc 1-81. The hybridization was carried out under standard conditions. o, origin.

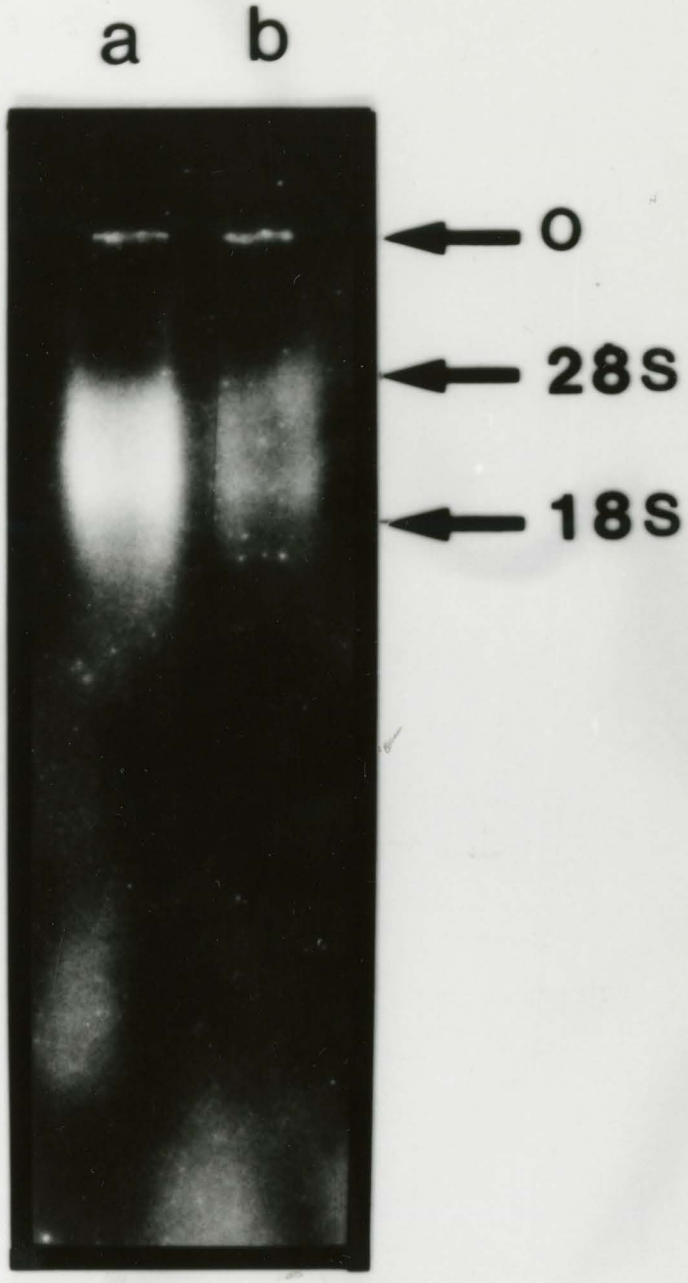
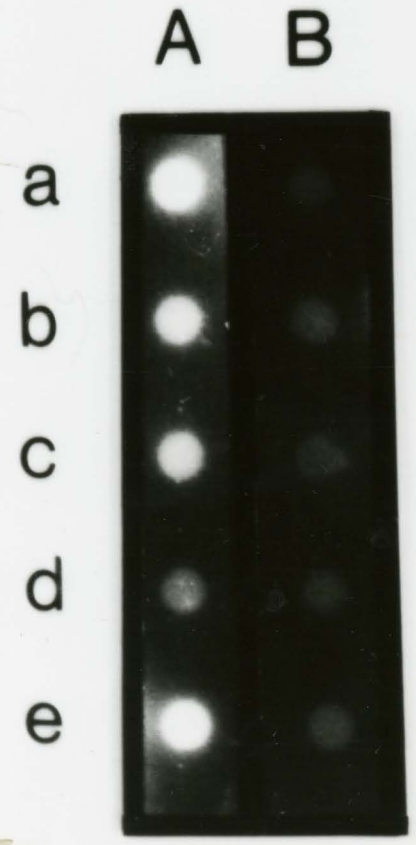

a
b
O
28S
18S

Figure 12. Detection of RNA sequence homologous to RT c-myc by Hybridot. Juvenile rainbow trout were administered with 200 µg β-estradiol/ 100 gm fish body weight for primary and secondary inductions and left 4 days until sacrifice. Poly A^+ RNA was prepared by oligo(dT) column chromatography. Pituitary RNA was obtained from Lou Agellon. After hybridization, filters were washed 4 times in 2XSSC containing 0.1% SDS at room temperature (filter set I) and then followed by 4 washes in 0.1XSSC containing 0.1% SDS at 37°C and 50°C (filter set II). All washes were 10 minutes in duration. Each dot had 5 µg RNA applied to it. A, probed with (^{32}P)-labelled pRTC-myc 1-81; B, probed with (^{32}P)-labelled pBR325. a, total induced liver RNA; b, control liver RNA; c, induced liver poly A^+ RNA; d, induced liver poly A^- RNA; e. pituitary poly A^- RNA.

I.



II.

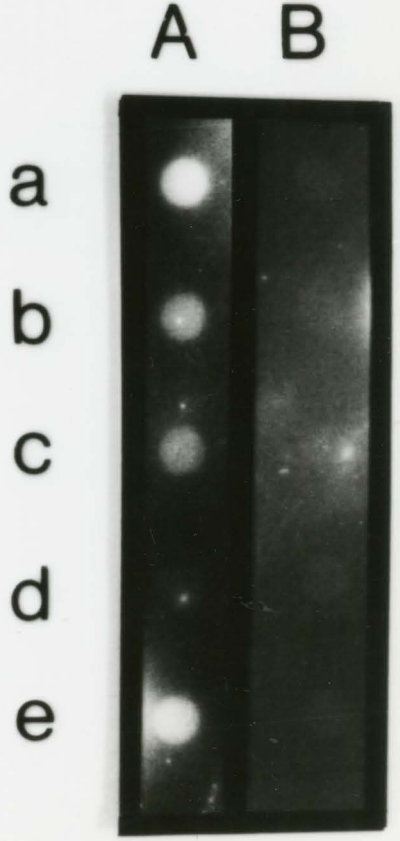


Figure 13. Quantitative determination of RT c-myc transcripts by Hybridot. Juvenile rainbow trout were administered with 200 µg β-estradiol/100 gm fish body weight for both the primary and secondary stimulations and left 4 days prior to being sacrificed. Rainbow trout hepatoma cells were grown until confluence and then grown for a further 24 hours in α-MEM supplemented with normal fetal calf serum containing various concentrations of β-estradiol. RNA was extracted and employed in the Hybridot procedure. Pituitary RNA (poly A^-), the kind gift of Lou Agellon, was used as a control. Lane A is the cellular probe pRTC-myc 1-81 and lane B is the probe for its viral counterpart MC29 v-myc. a, pituitary poly A^- RNA (5 µg); b, control liver RNA (5 µg); c, induced total liver RNA (5 µg); d, rainbow trout hepatoma (RTH), grown in 10% fetal calf serum (FCS), total RNA; e, RTH, grown in 10% stripped fetal calf serum (SFCS), total RNA; f, RTH, grown in SFCS supplemented with 10^{-10} M β-estradiol, total RNA; g, RTH, grown in 10% SFCS supplemented with 10^{-8} M β-estradiol, total RNA.

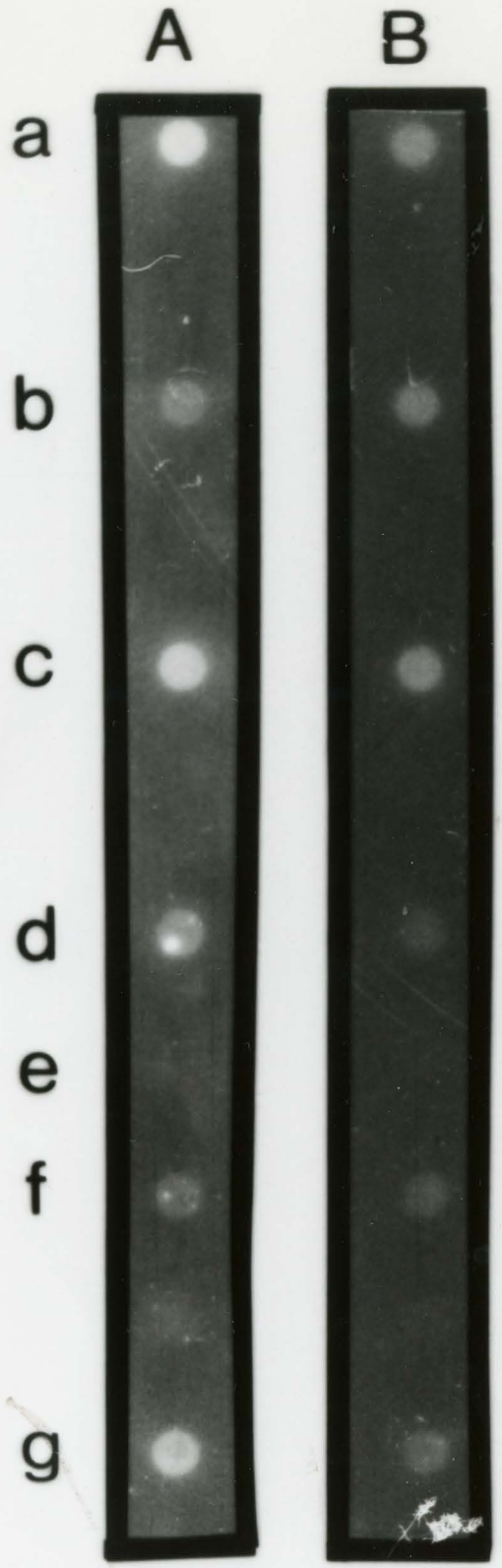

A
B
a
b
c
d
e
f
g

TABLE 3

Messenger RNA of c-myc in Rainbow Trout Liver and RTH Cell Line

Method of Hybridization	Source of RNA Samples	Densitometry Units (mm^2)	Ratios of induced/control
Northern blot with pRTC-myc 1-81	Induced liver	5927	2.29
	Control liver	2590	
Northern blot with MC-29	Induced liver	6717	1.36
	Control liver	4927	
Hybridot with pRTC-myc 1-81	Induced liver	1402	2.21
	Control liver	634	
	RTH in NFCS	936	3.9
	RTH in SFCS (control)	240	
	RTH in SFCS + 10^{-10}m Bestradiol	340	
	RTH in SFCS + 10^{-8}m Bestradiol	869	
Hybridot with MC-29	Induced liver	591	1.38
	Control liver	427	
	RTH in NFCS	231	4.40
	RTH in SFCS (control)	52	
	RTH in SFCS + 10^{-10}m Bestradiol	194	
	RTH in SFCS + 10^{-8}m Bestradiol	288	

RNA samples of rainbow trout liver or RTH cell line were applied on nitrocellulose filter and were hybridized to [^{32}P]-labelled v-myc (MC-29) or pRTC-myc 1-81 under standard conditions. The amount of hybridization was determined by autoradiography and densitometry.

NFCS - Normal Fetal Calf Serum
SFCS - Stripped Fetal Calf Serum

TABLE 4

Effects of Selected Xenobiotics on Hepatosomatic Indices in Rainbow Trout

Chemical	Hepatosomatic Indices ± S.D.[a] (Liver Wt/Body Wt x 100)		
	20 µg β-estradiol	40 µg β-estradiol	80 µg β-estradiol
	(per 100 gm fish body wt)		
β-napthoflavone[b]			
Control	1.08 ± 0.13 (14)		1.14 ± 0.16 (15)
7.5% LD_{50} dose	1.21 ± 0.19 (15)[e]		1.31 ± 0.32 (13)[f]
75% LD_{50} dose	1.38 ± 0.18 (15)[i]		1.35 ± 0.17 (15)[i]
Kepone[c]			
Control	1.31 ± 0.31 (15)	1.11 ± 0.09 (12)	
7.5% LD_{50} dose	1.28 ± 0.23 (14)	1.16 ± 0.10 (14)	
75% LD_{50} dose	1.77 ± 0.40 (4)[g]	1.61 ± 0.27 (6)[i]	
p, p'-DDT[d]			
Control	1.53 ± 0.21 (10)	1.40 ± 0.23 (10)	
7.5% LD_{50} dose	1.51 ± 0.18 (10)	1.38 ± 0.35 (10)	

Fish were administered intraperitoneally by a single injection the selected dose of chemical. Four days later, fish were administered by a second injection, the selected dose of β-estradiol. Two days later the fish were sacrificed, the livers excised, and weighed.
Number of fish used in each experiment is indicated in the parentheses.
a, S.D., Standard Deviation
b, LD_{50} of β-naphthoflavone 133.33mg/kg body weight
c, LD_{50} of kepone 55.8 g/kg body weight
d, LD_{50} of p,p'-DDT 1.339 g/kg body weight
Student's t-test analysis was employed to determine statistical significance.
e, $p<0.10$ when compared to corresponding control group
f, $p<0.05$; g, $p<0.025$; h, $p<0.010$; i, $p<0.005$

TABLE 5

Effects of β-naphthoflavone on the Expression of the Estradiol-Responsive Genes Induced by β-Estradiol

Treatment	Estrogen Responsive mRNAs			
	pRTC 2		pRTC 5	
	(μg standard induced RNA/μg total RNA) ± S.D.			
	20 μg β-estradiol (per 100 gm fish body wt)	80 μg β-estradiol	20 μg β-estradiol (per 100 gm fish body wt)	80 μg β-estradiol
Control	0.21 ± 0.15 (8)	0.24 ± 0.21 (8)	0.08 ± 0.04 (8)	0.08 ± 0.03 (8)
7.5% LD_{50}	0.13 ± 0.08 (8)	0.25 ± 0.26 (8)	0.09 ± 0.04 (8)	0.13 ± 0.08 (7)[a]
75% LD_{50}	0.17 ± 0.13 (8)	0.20 ± 0.14 (8)	0.07 ± 0.04 (8)	0.10 ± 0.05 (8)

Fish were administered intraperitoneally by a single injection, the selected dose of β-naphthoflavone. Four days later, fish were administered by a second injection the selected dose β-estradiol and then left for 2 days at which time they were sacrificed and the livers excised. Liver RNA samples were applied on nitrocellulose filter and were hybridized to [^{32}P]-labelled pRTC 2 or pRTC 5 under standard conditions. The amount of hybridization was determined by autoradiography and densitometry.
Number of fish used in each experiment is indicated in the parentheses.
Values are means ± standard deviation.
Student's t-test analyses were employed to determine statistical significance
a, $p<0.10$.
LD_{50} of β-naphthoflavone is 133.33 mg/kg body weight.

TABLE 6

Effect of Kepone on the Expression of the Estradiol-Responsive Genes Induced by β-estradiol

Treatment	Estrogen Responsive mRNAs					
	pRTC 2			pRTC 5		
	(μg standard induced RNA/μg total RNA) ± S.D.					
	0 μg β-estradiol	20 μg β-estradiol	40 μg β-estradiol	0 μg β-estradiol	20 μg β-estradiol	40 μg β-estradiol
	(per 100 gm fish body wt)			(per 100 gm fish body wt)		
Control	[0.30 ± 0.25 (6)]	0.30 ± 0.14 (7)	0.18 ± 0.08 (6)	[0.09 ± 0.04 (9)]	0.06 ± 0.05 (5)	0.06 ± 0.06 (4)
7.5% LD_{50}	-	0.10 ± 0.08 (8)[d]	0.22 ± 0.16 (7)	-	0.12 ± 0.05 (6)[a]	0.07 ± 0.05 (9)

Fish were administered intraperitoneally by a single injection, the selected dose of kepone. Four days later, fish were administered by a second injection the selected dose of β-estradiol and then left for 2 days at which time they were sacrificed and their livers excised. Liver RNA samples were applied on nitrocellulose filter and were hybridized to [^{32}P]-labelled pRTC 2 or pRTC 5 under standard conditions. The amount of hybridization was determined by autoradiography and densitometry.
Number of fish used in each group is indicated in the parentheses.
Values are means ± standard deviation.
The square parentheses indicate the background values which were subtracted from the test values.
Student's t-test analysis was employed for statistical significance determination
a, $p<0.10$ when compared to corresponding control group
d, $p<0.01$
LD_{50} of kepone is 55.8 g/kg fish body weight

administration of estradiol (Table 7). However, p,p'-DDT has no detectable estrogenic effect by itself (Table 8).

TABLE 7

Effect of p,p'-DDT on the Expression of the Estradiol-Responsive Genes Induced by β-estradiol

Treatment	Estrogen Responsive mRNAs					
	pRTC 2			pRTC 5		
	(μg standard induced RNA/μg total RNA) ± S.D.					
	0 μg β-estradiol	20 μg β-estradiol	40 μg β-estradiol	0 μg β-estradiol	20 μg β-estradiol	40 μg β-estradiol
	(per 100 gm fish body wt)			(per 100 gm fish body wt)		
Control	[0.30 ± 0.25 (6)]	0.30 ± 0.14 (7)	0.18 ± 0.08 (6)	[0.09 ± 0.04 (9)]	0.06 ± 0.05 (5)	0.06 ± 0.06 (4)
7 5% LD_{50}	-	0.18 ± 0.14 (10)[a]	0.28 ± 0.16 (9)	-	0.12 ± 0.05 (10)[b]	0.11 ± 0.05 (9)[a]

Fish were administered intraperitoneally by a single injection, the selected dose of p,p'-DDT. Four days later fish were administered a second injection containing the selected dose of β-estradiol. Two days later, fish were sacrificed and their livers excised for RNA extraction. Liver RNA samples were applied on nitrocellulose filter and were hybridized to [^{32}P]-labelled pRTC 2 or pRTC 5 under standard conditions. The amount of hybridization was determined by autoradiography and densitometry.
Number of fish used in each group is indicated in the parentheses.
Values are means ± standard deviation.
The square parentheses indicate the background values which were subtracted from the test values.
Student's t-test was employed for statistical significance determinations.
a, $p<0.10$ when compared to the corresponding control group
b, $p<0.05$
LD_{50} of p,p'-DDT is 1.339 g/kg fish body weight

TABLE 8

Effect of p,p'-DDT Alone on the Expression of the Estradiol-Responsive Genes Induced by β-estradiol

Treatment	Estrogen-Responsive mRNA	
	pRTC 2	pRTC 5
	(μg standard induced RNA/μg total RNA) ± S.D.	
Mock Injected	0.02 ± 0.11 (9)	0.04 ± 0.11 (13)
7.5% LD_{50} p,p'-DDT	N.D. (14)	N.D. (14)

Fish were administered by intraperitoneal injection, the selected dose of p,p'-DDT. Six days later, fish were sacrificed and their livers excised for RNA extraction. Liver RNA samples were applied on nitrocellulose filter and were hybridized to [^{32}P]-labelled pRTC 2 or pRTC 5 under standard conditions. The amount of hybridization was determined by autoradiography and densitometry.
Number of fish used in each group is indicated in the parentheses.
Values are means ± standard deviation.
LD_{50} of p,p'-DDT is 1.339 g/kg fish body weight
N.D. = not detectable

DISCUSSION

The estrogen-induced synthesis of vitellogenin in the liver of egg-laying vertebrates provides an excellent system for analyzing the response of a tissue to a hormone for several reasons. First, vitellogenin is synthesized in large amounts by a well characterized and homogeneous population of cells in response to a clearly-defined hormonal stimulus (Wangh and Knowland, 1975; Green and Tata, 1976; Gruber et al., 1976; Jost and Pehling, 1976a, 1976b; Tata, 1976). Secondly, males normally do not produce vitellogenin and may therefore be important to elucidating the control mechanisms of differential gene expression during development and differentiation (Shapiro, 1982). And finally, vitellogenin synthesis is independent of cellular proliferation (Mehta and Banerjee, 1975; Green and Tata, 1976), protein synthesis (Hayward et al., 1982), and DNA synthesis (Green and Tata, 1976). Because sportfishing is a population pasttime for many, and generates approximately 20 million dollars in related trades and businesses in Canada each year, the rainbow trout liver system is an ideal system well suited for the study of regulated gene expression.

Definition of conditions for the isolation and analysis of mRNA populations homologous to the estradiol-responsive cDNAs are essential for elucidation of controls that are exerted by estrogen at the transcriptional level (Shapiro and Baker, 1979). Isolation of intact RNA is a prerequisite for the quantitative analysis of the early molecular events involved in the induction of vitellogenin

synthesis in the liver of rainbow trout by β-estradiol. It is necessary to determine the accumulation of specific vitellogenin mRNA sequences during hormone stimulation in order to distinguish between possible transcriptional and/or translational control mechanisms. Similar information has been obtained for other hormone-induced proteins through the use of cell-free systems for translation and by DNA-RNA hybridization studies with the corresponding cDNA probe (reviewed by Palmiter, 1975; Ryffel, 1978; O'Malley et al., 1979; Tata and Smith, 1979; Westley, 1979).

The sensitivity of the immunological assay for vitellogenin synthesis has been shown to be somewhat unreliable unless the immunoprecipitable translation products are critically evaluated (Mullinix et al., 1976; Burns et al., 1978). More recently the use of translational analysis as a quantitative tool in the estimation of mRNA sequences has been superceded by cDNA hybridization analysis (reviewed by O'Malley et al., 1979; Tata and Smith, 1979). It is this latter method that I selected for employment in my investigations.

Recently, Chen (1983) demonstrated that the administration of estradiol to juvenile rainbow trout results in the induction of two major proteins in the liver. These two proteins are vitellogenin (the major egg yolk precursor protein) and an unidentified liver protein. This finding was further confirmed by hybridization studies involving the cDNA prepared from estradiol-treated and control liver RNAs which revealed the presence of at least two mRNA species in abundant sequences (Chen, 1983). A cDNA library of the estradiol-responsive genes has been constructed (T.T. Chen, personal communication). From this library, cDNA sequences of the above mentioned mRNA speices have been isolated

and designated as pRTC 2 and pRTC 5.

In this study, using pRTC 2 and pRTC 5 as probes, a quantitative hybridization method for estrogen-responsive mRNAs was developed. It was necessary to extract maximal amounts of intact RNA in order to make this analysis valuable. The phenol chloroform-SDS and lithium chloride-urea RNA extraction methods gave high yields of intact RNA. Hence, both of these RNA extraction protocols were employed throughout this thesis project. A reference RNA sample was extracted from the livers of rainbow trout four days following secondary induction with 200 µg estradiol/100 gm and was used as the standard.

The quantitative hybridization technique (Hybridot) used in these studies, employing the reference RNA standard, facilitated a rapid and accurate quantitation to 3 µg total RNA input or a little as 1 ng of specific mRNA. This permitted greater sensitivity for detecting specific mRNA sequences than the *in vitro* translation technique offers. Furthermore, Hybridot permits the rapid screening of large numbers of RNA samples, a feat that would be next to impossible using molecular hybridization in liquid. The limitation of the Hybridot technique appears to be the level of non-specific hybridization. This background effect was minimized by repeated washings of the nitrocellulose filter at elevated temperatures. Similar results were obtained by Searle and Tata (1981) in their investigations to determine the kinetics of vitellogenin synthesis in hepatocytes induced by β-estradiol *in vitro*.

In our investigations, duplicate samples were used to construct a standard curve for test samples. Furthermore, only those test samples present on the linear protion of the standard curve were used in

the analyses. These values were then converted to the units of μg standard induced RNA/μg total RNA and therefore reflect only the relative abundance of both classes of transcripts. However, the results reported herein could also be expressed in terms of molecules mRNA/cell if the size of the inserted cDNA into the respective recombinant plasmid and the number of hepatocytes per gram of liver are known.

Using Hybridot, the expression of the estradiol-responsive genes in juvenile rainbow trout was investigated. Transcripts complementary to pRTC 2 and pRTC 5 were maximal when rainbow trout were administered a dose of 20 μg/100 gm fish body weight and 5 μg/100 gm fish body weight respectively. Serum levels of 17 β-estradiol over the course of ovulation in female trout may reach 20 ng/ml (Scott et al., 1983) with the onset of vitellogenesis occurring at serum levels of 0.5 ng estradiol/ml (Breton et al., 1983).

To date, only large doses of estradiol have been administered in the studies of vitellogenesis. The published minimum value known to induced vitellogenin in Xenopus is 1 mg β-estradiol/100 gm body weight (Clemens, 1974). The discrepancy between these values of estradiol required for the onset of vitellogenin synthesis and the values described above for the maximum induction of pRTC 2 and pRTC 5 transcripts are most likely due to differences in the experimental techniques employed (i.e. route of administration of estradiol, method used for detecting vitellogenesis). In addition, because of the proximal location of the liver, chemicals administered through intraperitoneal injections or implants often undergo rapid transformations to less active forms (Gower, 1975; Cooper et al., 1979; Lu, 1979). For example, estradiol

implants containing 25 mg β-estradiol gave serum levels of only 2.7 ng β-estradiol/ml in fish 40 to 100 gm body weight (Hansson and Gustafsson, 1981). If this also holds true for intraperitoneal injections of estradiol, then the data in this study suggests that maximal induction of the transcripts homologous to pRTC 2 and pRTC 5 actually occurs at levels of 0.5 pg and 2.0 pg respectively which are several fold less than the minimum published values for vitellogenesis. Hence, Hybridot permits a more accurate determination of the dose-response relationship that exists during vitellogenesis.

For the following assays of the induction of the estrogen-responsive genes, doses exceeding 20 μg β-estradiol/100 gm fish body weight were routinely used to ensure maximum induction. In time course studies post primary induction, both sets of transcripts were found to reach their maximal levels within 8 hours. This is in contrast to the systems of chicken and Xenopus which reach their maximal levels within 80 hours (Deeley et al., 1977a, 1977b) and 12 days (Baker and Shapiro, 1977) respectively. During the course of the next 9 hours, the levels of both mRNAs drops dramatically with those homologous to pRTC 5 returning to control levels. However, even 16 days after primary induction the levels of transcripts homologous to pRTC 2 still do not return to those of the control. Upon secondary induction, maximal levels of both classes of transcripts occurs by 8 hours. This is in contrast to both the avian and amphibian systems which require 80 hours (Deeley et al., 1977a) and 6 days (Baker and Shapiro, 1977) respectively for maximal expression. Transcripts homologous to pRTC 2 decline in

their appearance at a much slower rate after secondary versus primary induction. This decline is also present, though less obvious, in the pRTC 5 transcripts. Within 8 days, pRTC 5 transcripts return to control levels whereas those transcripts homologous to pRTC 2 remain well above the control values. Similar results have been documented in chickens (Deeley *et al.*, 1977a, 1977b). At necroscopy, estradiol was observed to be precipitating out of the propylene glycol in the body cavity. This is likely the cause of the large fluctuations observed in the pRTC 2 and pRTC 5 transcripts in the latter part of the induction curves.

One interesting feature of the response of the pRTC 2 and pRTC 5 transcripts was the lack of a lag phase preceding the onset of mRNA and protein synthesis in the target tissue. It is possible however, that shorter time increments after estradiol administration would have detected such a phase. If under these conditions the lag phase was still undetectable, it would suggest that translational controls are predominant during vitellogenesis in rainbow trout since egg proteins remain undetectable several days after estradiol administration.

There are several inherent problems associated with the *in vivo* rainbow trout bioassays described in our investigations. These include: (a) trauma of fish resulting from the injection of hormone; (b) failure of hepatocytes to receive defined levels of hormone; (c) variation in levels of hormone retained in each fish due to leakage from the site of injection; (d) variations in time required for the hormone to reach the target tissue; and (e) relatively large amounts of hormone are required to elicit the required response. To overcome these problems, an *in vitro*

bioassay using a rainbow trout hepatoma (RTH) cell line was developed. The RTH cell line, when grown in α-MEM supplemented with 10% fetal calf serum (FCS) at 20°C, has a cell doubling time of 74 hours to reach confluence at $1.9X10^6$ cells/flask. Furthermore, RTH cells grown under standard conditions with 10% normal FCS display the presence of pRTC 2 and pRTC 5 transcripts. To determine if this is due to the endogenous steroids present in the FCS, an attempt was made to strip such steroids from the FCS by dextran-coated charcoal as described by Horwitz and McGuire (1978). Cells incubated in α-MEM supplemented with the stripped FCS did not display the presence of either class of transcripts. The minimal concentration of exogenous β-estradiol required in the stripped FCS for the expression of the estradiol-responsive transcripts is 10^{-8} M. Furthermore, this same dose of exogenous estradiol stimulated the production of the presumptive vitellogenin protein. This value is comparable to values observed in Xenopus liver organ cultures (Green and Tata, 1976). By further characterizing the vitellogenic response of these RTH cells, it will be possible to employ them in a rapid, sensitive, and quantitative bioassay for assessing and correlating the sublethal pathobiological effects of xenobiotics on fish (e.g. reproductive capabilities by employing pRTC 2 and pRTC 5).

The literature suggests that several different factors may be involved in cancerous growth. The early work with tumor viruses have been fundamental in the understanding of the cancer problem. However, the majority of human cancers are not caused by viruses (Cooper, 1982). Instead many types of cancers are the result of the expoure to various

types of chemical carcinogens (Goyette et al., 1983; Balmain and Pragnell, 1983; Eva and Aaronson, 1983). These carcinogens are thought to cause mutations in normal cellular DNA thereby converting the normal cells to cancerous growth. The discovery of oncogenes, genes believed to be important in malignant transformation, in retroviruses has been instrumental for the elucidation of mechanisms involved in oncogenesis (Bishop, 1982, 1983). Cellular DNA sequences homologous to the oncogenes sequences of the acute tumor viruses (v-onc) have been shown to be highly conserved within vertebrates and are referred to as proto-oncogenes (c-onc) (Shilo and Weinberg, 1981a, 1981b; Eva et al., 1982). Using v-myc of the avian myelocytomatosis virus MC29 as a probe, the c-myc (pRTC-myc 1-81) has recently been isolated from a rainbow trout gene bank (Chen et al., 1984b). The close conservation of the cellular genes suggests that they probably play essential roles in cellular differentiation or in the regulation of cell division (Collet et al., 1979; Sefton et al., 1980).

Northern blot analyses of liver tissues revealed two classes of RT c-myc transcripts (approximately 2.5 and 5.6 kb). In addition, hybridization of pRTC-myc 1-81 to the poly(A^+) or poly(A^-) fractions of RNA revealed that these transcripts are polyadenylated and specific for RT c-myc versus pBR325. Similar results have also been observed in normal cells where myc-related mRNAs ranged from 2.5 to 6. 0 kb (Sheiness et al., 1980; Gonda et al., 1982). However, because of the high GC content present in the probe, and therefore its ability to hybridize to several messenger RNA sequences, the upper band corresponding to 5.6 kb is thought to be an artifact (Watson et al., 1983a, 1983b).

A two-fold enhancement in the number of RT c-myc transcripts was observed in livers treated with 17 β-estradiol with respect to the control livers. It should be noted that the RT c-myc transcriptional activity appears to parallel that of estradiol-responsive genes (pRTC 2 and pRTC 5). The RTH cell line grown in stripped medium for 24 hours do not express transcripts unless the medium was originally supplemented with exogenous estradiol. In this case, 10^{-10} M β-estradiol is enough to cause the expression of c-myc transcripts thus mimicking pRTC 5 transcripts more closely than pRTC 2 transcripts. These results are supported by published studies that have shown that treatment of normal cells with various xenobiotics have enhanced the expression of specific oncogenes (Goyette et al., 1983; Balmain and Pragnell, 1983; Eva and Aaronson, 1983; Makino et al., 1984) and that the expression of the cellular oncogenes is enhanced upon transformation (Eva et al., 1982, 1983; Augenlicht and Kobrin, 1982; Schwab et al., 1983). Furthermore, estradiol has been shown to cause cancer in animals and in man when administered chronically at high levels or when present through disturbance of normal endocrine balances in unphysiologic amounts for long periods of time (Herbst, in Hiatt et al., 1977; Weisburger and Williams, 1979). However, because the enhanced expression is several fold greater in transformed tissues than what was observed in this study, the results reported herein could be a general effect exerted by estradiol on genes involved in cell maintenance. Future studies should be aimed at determining normal levels of RT c-myc transcript levels in the RTH cell line, as well as in vivo, and their induction time course by estradiol in order to provide possible insight into the mechanisms of

tumor promotion. Expanded investigations utilizing a battery of all the known cellular oncogenes would make it possible to seek correlations between the administration of a specific xenobiotic and the activation of a cellular oncogene.

In the final part of this thesis, three model compounds were assayed for their effects on the level of transcripts homologous to pRTC 2 and pRTC 5 upon induction by 17 β-estradiol. These three compounds have been shown to exert varying effects on the hemoprotein cytochrome P-450 and the mixed function oxidases. β-naphthoflavone (5,6-benzoflavone), belongs to a class of chemicals known as flavones. Flavones occur naturally in fruits and vegetables and have been implicated in being an inhibitor of chemically induced carcinogenesis. Furthermore, the inhibitory effects of chemically-induced neoplasia paralleled the employed flavone's ability to induce monooxygenase activites (e.g. arylhydrocarbon hydroxylase; Wattenberg and Leong, 1968, 1970). It is thought that the high levels of monooxygenase activities after induction leads to a rapid detoxication and/or degradation of the xenobiotic thereby extinguishing its carcinogenic effect. β-naphthoflavone, although a synthetic flavone, has been shown to be a potent enhancer of the monooxygenase activities in rainbow trout (Elcombe and Lech, 1979). The other two test chemicals, Kepone and p,p'-DDT are organochlorine pesticides and have been shown to be capable of inducing the mixed function oxidases in rats (Kaminski et al., 1978; Mehendale et al., 1978; Fabacher and Hodgson, 1976) and in rats and quail (Bunyan et al., 1972) respectively. Although no studies concerning the effects of kepone on the MFO system in fish have been

published three independent reports have shown that its sister organochlorine p,p'-DDT when administered in relatively massive doses to rainbow trout is incapable of inducing the mixed function oxidases (Addison et al., 1977; Fabacher, 1982; Buhler and Rasmussen, 1968). It is difficult, however, to compare the induction capabilities of all three of these chemicals to one another because of differences in the experimental approaches (i.e. species, environmental, chemical concentrations, vehicle used to solubilize chemicals). For this reason, the doses employed in this study were 7.5% and 75% of the lethal dose of 50% (LD_{50}).

Because the estradiol-responsive mRNAs contribute to approximately 30% of the total RNA content synthesized by the liver of induced rainbow trout, one would expect to observe a similar accumulation in the proteins for which they encode. Any appreciable increase in liver weight could inevitably be attributed to such a response. Hepatosomatic indices (HSI=liver weight/body weight X 100) are often used for a crude measure of the relative protein synthesis activity of the liver and hence were employed in addition to the hybridot detection of transcripts. Data suggests that fish treated with large doses of β-naphthoflavone or kepone significantly increase their HSI values with respect to the controls. In contrast, fish pretreated with p,p'-DDT prior to estradiol administration exhibit no increases in the HSI versus the controls. In looking at the levels of the estradiol-responsive mRNAs of control and treated fish, one soon realizes that there appears to be no direct relationship between the HSI values and

the levels of pRTC 2 and/pr pRTC 5 transcripts. For in the case of β-naphthoflavone pretreatment, the HSI values significantly increase whereas the relative levels of the estradiol-responsive mRNAs does not change. This suggests that β-naphthoflavone may be inducing the transcription and subsequent translation of some other genes or that although the pRTC 2 and pRTC 5 levels are unaffected, their translation rates may be increased. This could be resolved by SDS-PAGE of the total proteins in the liver following β-naphthoflavone treatment. Kepone treatment prior to the administration of estradiol appears to have no effect on the induction of pRTC 5 transcripts with respect to the controls. In contrast, pRTC 2 transcripts were significantly repressed ($p < 0.01$) in their expression upon kepone treatment prior to estradiol (20 μg/100 gm fish weight) administration. In contrast, its sister organochlorine pesticide, p,p'-DDT, pretreatment appears to increase the expression of the transcripts complementary to pRTC 5, although only significantly at the lower dose of estradiol ($p < 0.05$). However, no effect was observed on the expression of pRTC 2 transcripts with DDT pretreatment. Thus chemical pretreatment may lead to a synergistic effect on the estradiol-responsive genes, in this case pRTC 5. This is not surprising inasmuch as p,p'-DDT and kepone have been shown to have an estrogen-like action in the uterus of the mouse (Eroschenko and Mousa, 1979; Lundberg, 1973), the quail oviduct (McFarland and Lacy, 1969; Eroschenko and Wilson, 1975) and the chicken oviduct (Palmiter and Mulvihill, 1978; Singhal et al., 1970). Furthermore, the above results suggest that different controls are exerted on the expression

of each of the estradiol-responsive gene(s) inasmuch as each chemical is capable of eliciting a completely different response in their transcripts (i.e. no effect on the pRTC 2 transcripts and increasing the number of pRTC 5 transcripts).

There are several ways in which estrogenic activity may be elicited by these organochlorine pesticides. One is that such compounds mimic estrogenic steroids by binding to and activating the receptor molecules that normally mediate estrogenic activity. Kepone and o,p'-DDT (an isomer of p,'p'-DDT) have been found to competitively inhibit the binding of β-estradiol to the chicken oviduct nuclear receptor (Palmiter and Mulvihill, 1978) and the rat uterine cytosolic receptor (Nelson, 1974) respectively in vitro. In addition, o,p'-DDT was able to translocate the uterine cytosolic receptor in a manner similar to estradiol (Kupfer and Bulger, 1976). Similarily, p,p'-DDT has been shown to competitively bind (e.g. 30%) to the cytosolic receptor in the rainbow trout liver in vitro (personal communication, L. Carr).

Thus, it appears that p,p'-DDT and Kepone may impinge directly on the rainbow trout liver by the mechanism mentioned above. To confirm the same, experiments were performed whereby fish were treated only with p,p'-DDT. The results indicate that the levels of both classes of transcripts are significantly less ($p < 0.025$) in treated versus the control fish. In the case of pRTC 2 transcripts, any vitellogenin messenger present would be less than 1.2% of that induced by estradiol. These results are in contrast to those observed in the chicken oviduct where kepone alone induced ovalbumin and conalbumin

syntheses (Palmiter and Mulvihill, 1978). Kepone has been shown to increase the concentration of other active steroids by altering the normal synthetic and degradative pathways in steroid metabolism (Palmiter and Mulvihill, 1978); chemical carcinogens have been shown to inhibit DNA methylation *in vitro* (Wilson and Jones, 1983); estrogen has been shown to stabilize vitellogenin mRNA against cytoplasmic degradation (Brock and Shapiro, 1983); Mirex (an analog of kepone) and kepone have been shown to induce hepatic mixed function oxidase profiles that differ from one another and the classical inducers, PB and 3-MC (Kaminski *et al.*, 1978) - all of the above could also account for the observed estrogenic effects exerted by p,p'-DDT *in vivo*. Further experiments, perhaps even *in vitro*, are required to resolve this ambiguity.

CONCLUDING REMARKS

In this thesis, I have tried to integrate two diverse fields of biological science, molecular biology and toxicology, in order to develop a potential bioassay for detecting the adverse effects of environmental contaminants on fish reproduction. Future *in vivo* studies with rainbow trout should also include serum tests for determining the levels of estradiol present, thereby eliminating any confusion between induced and uninduced fish. The rainbow trout hepatoma cell line employed in the studies encompassed in this thesis appears to mimic the *in vivo* system with respect to the induction of all three of the estradiol-responsive mRNAs. Furthermore, the RTH cell line permits a better controlled quantitative, rapid, and sensitive system for measuring and assessing the earliest toxic effects caused by environmental xenobiotics. With such an index, reliable guidelines for the management of one of this country's greatest resources, the waterways, can be established.

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