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LIVER GENE EXPRESSION DURING VITELLOGENESIS IN MALE RAINBOW TROUT

BY

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in

Biochemistry

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PREFACE

Patience - A minor form of suffering, of dispair, disguised as a virtue (unknown)

ABSTRACT

Estrogen has a marked effect on the expression of vitellogenin (Vg), the egg-yolk precursor protein, in the liver of egg-laying vertebrates. In this study we have examined the effects of estradiol on the *in vivo* stimulation of vitellogenesis in male rainbow trout (*Salmo gairdneri*). Vitellogenin appears to be encoded by a family of at least two genes in rainbow trout. In both the primary and secondary stimulations Vg mRNA is induced *de novo* by estradiol. The kinetics of induction are similar to that of *Xenopus* and chicken, in that the lag in the accumulation observed during primary stimulation is absent during secondary stimulation. Our results indicate that a decrease in serum estradiol levels is not required to induce maximum stimulation of vitellogenesis. *In vitro* translation of poly(A)$^+$ RNA extracted from the liver of control and estradiol-stimulated male trout indicates that while some messages are induced, the levels of several other liver mRNAs appear to be decreased by estradiol. Analysis of the levels of pSG 3.2 mRNA (a constitutively expressed trout liver mRNA) suggests that estradiol induces a general increase in the transcriptional activity of the liver. In contrast to pSG 3.2, high levels of estradiol act to maintain the levels of a second constitutively expressed liver mRNA (pSG 2.5) at a constant level relative to the amount of rRNA.

DEDICATION

This thesis is dedicated to my wife, Lydia, and to my parents in appreciation of their encouragement and support.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr M. Tenniswood, whose help and guidance has made this thesis possible. I would also like to acknowledge the help of Dr. K. Le Guellec for her help in the cloning and characterization of the vitellogenin clones and with the measurement of serum vitellogenin levels. I would also like to thank Dr. R. Le Guellec for his help with the two-dimensional gels and Dr A. Clark for arranging for the serum estradiol assays. Finally, I would like to thank the other graduate students in the lab, Jocelyne Léger, Mike Montpetit, and Paul Wong, for their friendship and help.

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INTRODUCTION

In oviparous vertebrates estradiol plays a central role in the production of the egg-yolk proteins by the liver. Vitellogenin, the precursor of these egg-yolk proteins, is synthesized in the liver of most egg-laying vertebrates (Pan, Bell, & Telfer, 1969). Vitellogenesis is a complex process in which the secretion of vitellogenin from the liver is closely coupled with oocyte maturation, the uptake of vitellogenin by the oocyte, and the specific proteolytic cleavage of the precursor protein into the respective yolk components. Vitellogenesis is a well conserved process and has been studied in a number of egg-laying species including chicken (Deeley, Gordon, Burns, Mullinix, Stein & Goldberger, 1977), Xenopus (Shapiro & Baker, 1979; Wahli & Dawid, 1980; Ng, Wolffe & Tata 1984) and several species of fish including catfish (Nath & Sundararaj, 1981) and rainbow trout (Salmo gairdneri) (Chen, 1983 ; Valotaire, Tenniswood, Le Guellec, & Tata, 1984). Vitellogenin-like proteins have also been studied in invertebrates such as Drosophila and Locusta (Bownes & Hames, 1977; Applebaum, James, Wreschner & Tata, 1981).

(A) Vitellogenesis in the female rainbow trout

Figure 1 outlines the general process of vitellogenesis in the female rainbow trout. Estradiol, which is produced

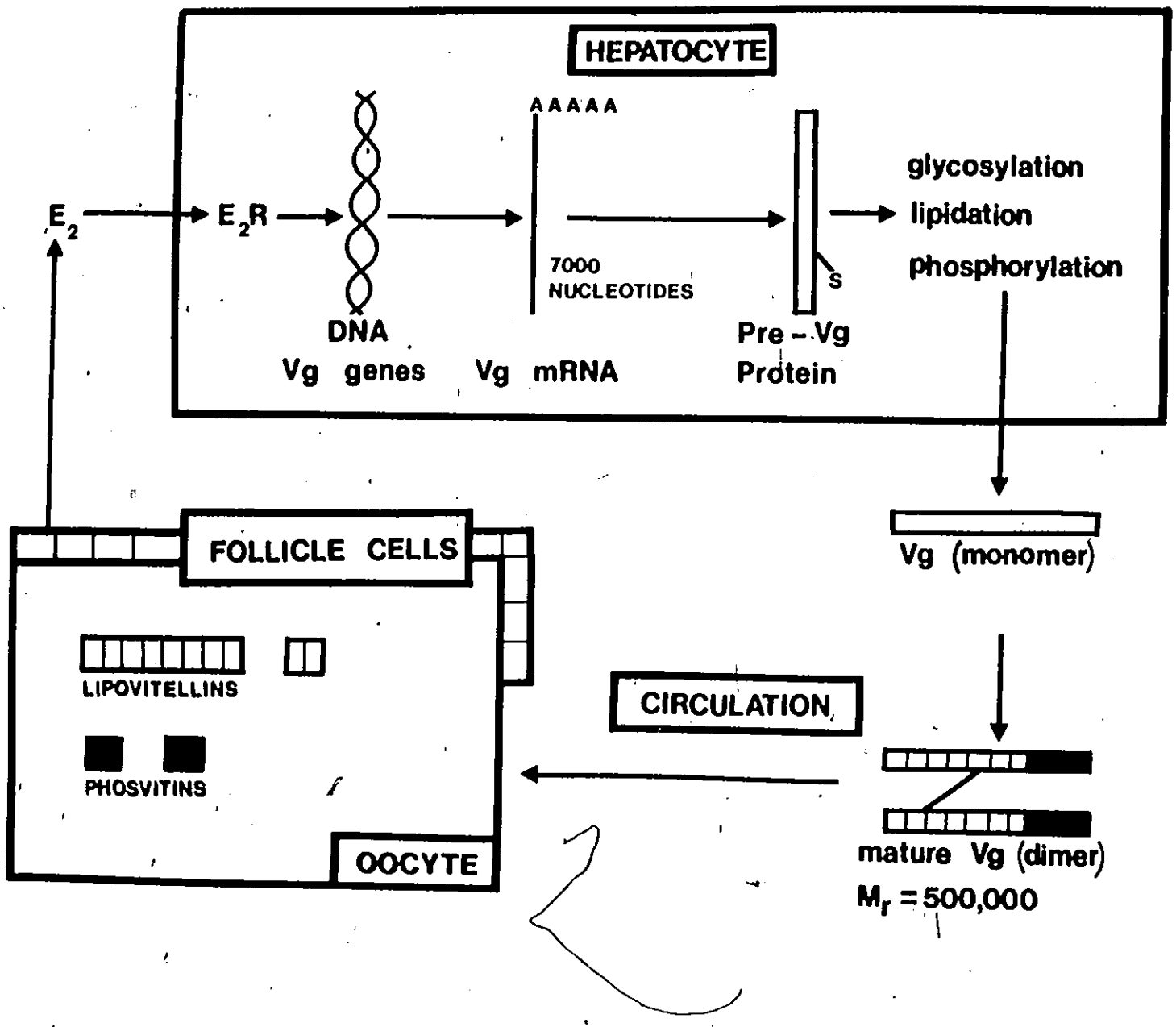
FIGURE 1: Summary of vitellogenesis in female rainbow trout, Salmo gairdneri.

E_2 : estradiol.

E_2R : estradiol-receptor complex.

Vg : vitellogenin.

VITELLOGENESIS IN RAINBOW TROUT



in the follicle cells of the ovary, is secreted into the circulation and transported to the liver where the steroid binds to a receptor protein. This initiates the transcription of the vitellogenin gene (or genes) and the production of vitellogenin messenger RNA (Vg mRNA) which is approximately 7000 nucleotides in length (Valotaire et al., 1984). The Vg mRNA is then translated to give a pre-vitellogenin protein which undergoes glycosylation, lipidation and phosphorylation before export into the circulation. Either during secretion from the liver, or immediately after entering the circulation, the vitellogenin monomer dimerizes to form mature vitellogenin which has a molecular weight of approximately 500,000 daltons (Maitre, Le Guellec, Derrien, Tenniswood & Valotaire, 1985). The mature dimer is transported to the oocyte where it is taken up by a specific transport system (Busson-Mabillot, 1984) and cleaved into the two major groups of egg-yolk proteins, loosely termed the phosvitins and the lipovitellins. These proteins serve as sources of phosphate and lipid respectively, for the developing embryo.

(B) Historical

The study of vitellogenesis began some fifty years ago, when Roepke and Hughes (1935) first demonstrated that a sex

specific phosphoprotein appears in the serum of domestic hens during yolk deposition in the oocyte. Flickinger and Rounds (1956) implicated estradiol in the production of these egg-yolk proteins. Several years later, Greengard, Gordon, Smith and Acs (1964) showed that the appearance of the phosvitin precursor in the serum represents de novo synthesis of the protein by the liver, and that RNA synthesis is a requirement for this process (Greengard, Sentenac and Acs, 1965). Although the yolk protein precursors are absent in the serum of the male, Vanestone, Dale, Oliver, and Common (1957) were the first to show that the administration of estrogen to roosters evoked the appearance of the phosvitin precursor in the serum. These results showed that vitellogenesis could be studied in the male of oviparous species without the potential problems associated with endogenous estrogens present in the female. It is now known that males of all egg-laying vertebrates including Xenopus (Follett & Redshaw, 1968; Wallace & Jared, 1968) and rainbow trout (Chen, 1980; Valotaire et al., 1984; Maitre et al., 1985) produce and secrete large amounts of vitellogenin into the circulation upon treatment with estradiol. Wallace (1970) first postulated that the egg-yolk proteins were synthesized in the liver as a large precursor. This was confirmed by cyanogen bromide cleavage of [^{3}H]-serine labeled in vitro translation products of Vg mRNA

(Gordon, Deeley, Burns, Patterson, Christmann and Goldberger, 1977). The fragments from the cyanogen bromide cleavage of the *in vitro* translation products were shown to be very similar to the fragments generated by cyanogen bromide cleavage of phosvitin and lipovitellin.

(C) The egg-yolk proteins

The lipovitellins are glyco-lipo-phospho-proteins with a very low content of acid-labile phosphorus, while the phosvitins are rich in phosphorus but lack lipid and carbohydrate. In rainbow trout there are two lipovitellins of 95,000 daltons and 24,000 daltons and two slightly different phosvitins of approximately 22,000 daltons each (Chen, 1980). In *Xenopus*, on the other hand, there are two lipovitellins of 121,000 and 34,000 daltons, one phosvitin of 32,000 daltons and two phosvettes of 19,000 and 14,000 daltons (Wiley, Opresko & Wallace, 1981). There is one lipovitellin (135,000 daltons) and two phosvitins of 34,000 and 28,000 daltons found in the chicken (Gordon *et al.*, 1977).

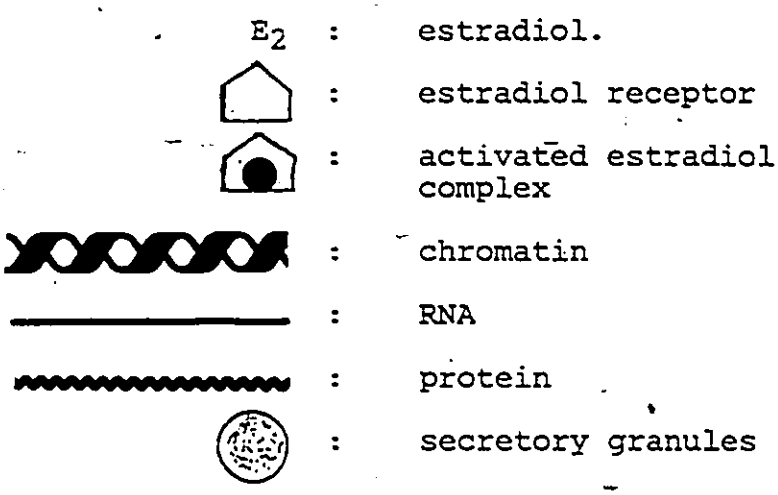
Trout lipovitellins have a lipid content of 25% and an alkali-labile phosphorus content of 0.007%, while trout phosvitins have an alkali-labile phosphorus content of 15.8%

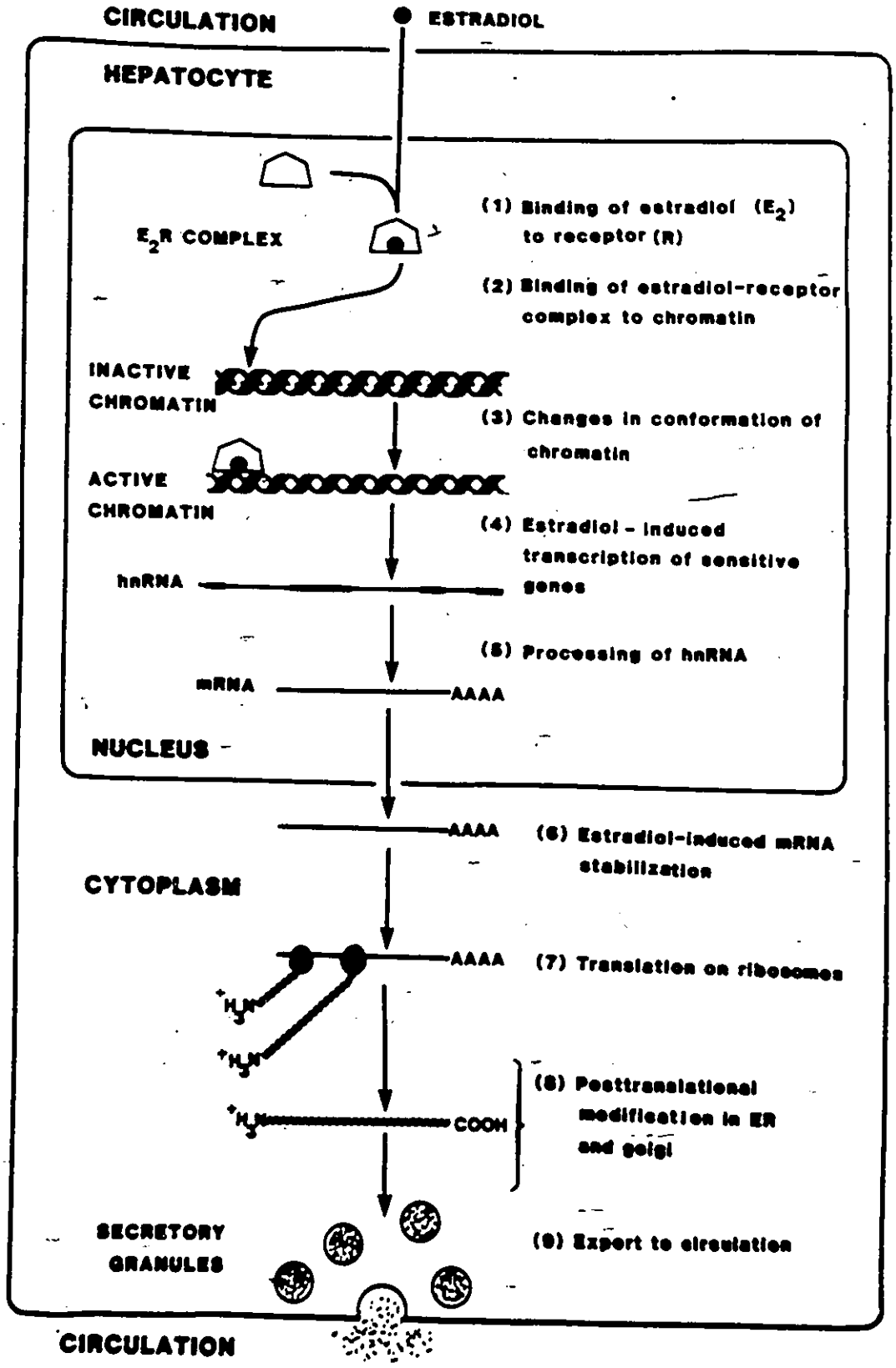
(Campbell & Idler, 1980). The amino acid composition of rainbow trout lipovitellin resembles that found in Xenopus except for some differences in serine and valine content (Campbell & Idler, 1980). The difficulties inherent in analyzing proteins containing large lipid and carbohydrate side chains has prevented the determination of the amino acid sequence of these proteins. The lipid content of trout lipovitellins is comparable to that in Xenopus (Redshaw & Follett, 1971). In salmonid phosvitins, serine is the predominant amino acid accounting for as much as 42% of the amino acid content, and exists almost exclusively in its phosphorylated form, phosphoserine (Mano & Yoshida, 1969; Campbell & Idler, 1980). These characteristics of teleost phosvitins are common to Xenopus and chicken phosvitins (Redshaw & Follett, 1971).

(D) Expression of the vitellogenin genes

Figure 2 outlines the mechanism of activation of estradiol responsive genes in hepatocytes. Estradiol circulates bound to proteins such as albumin and enters the cell, either by passive diffusion or facilitated transport (Gorski & Gannon, 1976). The hormone is translocated to the nucleus and binds to unoccupied receptors (King & Greene, 1984; Welshons, Lieberman & Gorski, 1984). The binding of

FIGURE 2: Summary of the mechanism of estradiol-induced gene expression.




CIRCULATION
ESTRADIOL
HEPATOCYTE
E_2R COMPLEX
(1) Binding of estradiol (E_2) to receptor (R)
(2) Binding of estradiol-receptor complex to chromatin
INACTIVE CHROMATIN
(3) Changes in conformation of chromatin
ACTIVE CHROMATIN
(4) Estradiol - induced transcription of sensitive genes
hnRNA
(5) Processing of hnRNA
mRNA
AAAA
NUCLEUS
AAAA
(6) Estradiol-induced mRNA stabilization
CYTOPLASM
AAAA
(7) Translation on ribosomes
COOH
(8) Posttranslational modification in ER and golgi
SECRETORY GRANULES
(9) Export to circulation
CIRCULATION

the activated hormone-receptor complex to chromatin initiates a change in the conformation of the chromatin from an inactive to an active state, resulting in the transcription of the estradiol sensitive genes (Williams & Tata, 1983). The primary RNA transcript is then capped, spliced, and polyadenylated to give the mature mRNA. In the cytoplasm the mature mRNA is protected from degradation by estradiol-induced stabilizing proteins (Brock & Shapiro, 1983a), and is translated to produce the pre-protein which undergoes post-translational modification in the endoplasmic reticulum and Golgi apparatus before packaging into secretory granules and export to the circulation (Tata & Smith, 1979).

(1) The Kinetics of induction:
Primary versus secondary stimulation

A general feature of the action of most growth and development hormones is a lag period preceding the onset of a given response (Tata, 1970). The latent period, which is important in explaining the early events underlying hormone action, can vary according to whether the animal has been previously primed with the hormone. In male Xenopus, vitellogenin starts to accumulate in the circulation approximately two days after the initial administration of

estradiol (Farmer, Henshaw, Berridge & Tata, 1978). If very large doses of estradiol are used for stimulation, vitellogenin is detectable in the serum at approximately 4-5 hours after treatment and the accumulation of Vg mRNA in the liver begins approximately 3-4 hours after treatment (Baker & Shapiro, 1977).

Upon withdrawal of the primary stimulus vitellogenin production and Vg mRNA levels gradually return to undetectable levels. If a second dose of estradiol is then administered, the lag in the accumulation of Vg mRNA is reduced to 1-2 hours (Baker & Shapiro, 1978) and the synthesis of vitellogenin is rapidly established at the maximum rate (Farmer et al., 1978). This is referred to as secondary stimulation. The rate of accumulation of Vg mRNA between four and six days after secondary stimulation is about four times greater than the primary induction during the equivalent time period. Levels of Vg mRNA during secondary stimulation are about twice those observed with the same dose during primary stimulation (Baker & Shapiro, 1978). This phenomenon has been termed the "memory effect" and has been observed in both Xenopus (Berridge, Farmer, Green, Henshaw & Tata, 1976; Farmer et al., 1978; Baker & Shapiro, 1977; Baker & Shapiro, 1978) and chicken (Beuving & Gruber, 1971; Maeenpaa, 1976). The length of the lag period

is dose dependent (Shapiro, 1982). The "memory effect" has also been demonstrated in organ culture (Green & Tata, 1976; Felber, Ryffel & Weber, 1978) and primary hepatocyte cultures (Searle & Tata, 1981; Tenniswood, Searle, Wolffe & Tata, 1983).

Subsequent studies have shown that estradiol regulates the absolute rate of vitellogenin gene transcription and the differences observed between primary and secondary stimulations are partly attributable to an increase in the rate of vitellogenin gene transcription (Brock & Shapiro, 1983b). There has been a considerable amount of research to determine the underlying cause of this "memory effect". It has been suggested that changes in the nuclear estradiol receptor populations, chromatin conformation, methylation of the vitellogenin genes or a combination of these processes may be involved in the regulation of vitellogenin gene expression and the establishment and maintenance of the "memory effect".

(2) Changes in the nuclear estradiol receptor populations

The original two step hypothesis of steroid receptor action proposes that the binding of the steroid to the unoccupied cytoplasmic receptor induces a conformational

change in the receptor protein which activates the steroid-receptor complex (Jensen, Suzuki, Kawashima, Stumpf, Jungblunt & Desombre, 1968). The steroid-receptor complex then translocates to the nucleus where it binds to acceptor sites on the chromatin, initiating transcription of the steroid-inducible genes. Recently, the estrogen receptor has been localized in the nucleus of estrogen responsive tissues by immunohistochemistry using monoclonal antibodies specific for the estrogen receptor (King & Greene, 1984; Welshons et al., 1984). Since there is little or no specific cytoplasmic staining for the receptor observed in any of the estrogen responsive tissues or tumour cells examined, there is now considerable debate as to whether cytoplasmic receptors are present in estrogen target tissues. These studies have led to the suggestion that the unoccupied receptors reside in the nucleus, not the cytoplasm. The occupied estrogen receptors which are localized in the nucleus are most probably bound to the nuclear matrix and chromatin, altering the expression of estradiol sensitive genes (Schrader, 1984).

In naive male Xenopus hepatocytes, there are several hundred estradiol receptors per cell, the majority of which are localized in the nucleus (Hayward, Mitchell & Shapiro, 1980). Upon stimulation with estradiol there is an

induction of the estradiol receptor. The total number of nuclear receptors increases to approximately 2000 receptors per cell, most of which are bound to estradiol. Although the nuclear receptor levels remain elevated up to 70 days after withdrawal of estradiol, only a few hundred remain bound to the nuclear matrix and chromatin. It has been suggested that the pool of free receptors may account for the rapid and enhanced secondary response to estradiol (Hayward et al., 1980), although other contributing factors have not been ruled out. The fact that estradiol-induced protein synthesis is required during the withdrawal period to induce a full secondary response suggests that new estradiol receptors continue to be synthesized even after hormone withdrawal (Perlman, Wolffe, Champion & Tata, 1984). It should be noted that de novo initiation of transcription is not dependent on new receptor synthesis, however receptor synthesis is required for continued transcription (Perlman et al., 1984).

(3) Estradiol-induced changes in chromatin conformation

Chromatin which is actively undergoing transcription, or has the potential to undergo active transcription, possesses a unique conformation (Weintraub & Groudine, 1976). This is reflected in enhanced susceptibility of the active chromatin to digestion by DNase I or micrococcal nuclease (Conklin &

Groudine, 1984). These changes in chromatin conformation are tissue specific. Limited digestion of nuclei isolated from Xenopus hepatocytes with DNase I, suggests that the vitellogenin genes of estradiol treated males are 2-fold more sensitive to digestion when compared to untreated males (Tata & Baker, 1979; Gerber-Huber, Felber, Weber & Ryffel, 1981). The sensitivity of the vitellogenin genes to DNase I occurs over the entire Xenopus vitellogenin gene locus and reflects the potential for transcription of the gene (Folger, Anderson, Hayward & Shapiro, 1983). Although no DNase I hypersensitive sites have been found in Xenopus, steroid-mediated transcription of the chicken vitellogenin II gene in the liver is accompanied by both a general increase in the sensitivity to DNase I and the appearance of two groups of nuclease hypersensitive sites in the region upstream of the vitellogenin gene (Burch & Weintraub, 1983) that maps to putative estradiol receptor binding sites (Burch & Weintraub, 1983; Burch, 1984).

The fact that the generalized sensitivity to DNase I in Xenopus persists as long as four months after the withdrawal of estradiol and the cessation of transcription has led to the suggestion that the change in chromatin conformation may play an important role in the maintenance of "memory" (Williams & Tata, 1983). Recently work by Burch and Evans (1986) demonstrates that the "memory effect " does not always correlate with DNase I sensitivity, suggesting that some other aspect of chromatin structure, which is not discernible by DNase I sensitivity may be, at least partially, responsible for the stability of "memory".

(4) Gene structure and expression

There are four vitellogenin genes expressed in Xenopus laevis (Wahli, Dawid, Wyler, Jaggi, Weber & Ryffel, 1979). The two main groups of mRNAs, A and B, differ in approximately 20% of their nucleotides. The A and B groups are each composed of two subgroups of sequences (A1 & A2; B1 & B2) which differ in about 5% of their nucleotides. The four mRNAs have a similar length (6000 nucleotides) and accumulate to high levels in stimulated animals (Wahli et al., 1979; Felber, Maurhofer, Jaggi, Wyler, Ryffel, Weber, 1980). Heteroduplex mapping has shown that the 6 kb coding region of the A1, A2 and B2 genes are interrupted 33 times

by intervening sequences, resulting in total gene lengths of 20.5 kb, 15.8 kb, and 17.5 kb respectively (Wahli & Dawid, 1980; Germond, Heggler, Schubiger, Walker, Westley & Wahli, 1980) The B1 gene is 16.6 kb in length and has 34 intervening sequences (Wahli & Dawid, 1980). Within each group of genes the lengths of the coding sequences do not change, only the length (and number) of introns. These similarities suggest that the four genes have arisen from a common ancestral gene by some form of duplication and are not independently derived (Wahli, Dawid, Ryffel & Weber, 1981).

During chronic stimulation all four genes are equally expressed and the messages are represented in equal amounts (Wahli et al., 1979). When male hepatocytes are stimulated by estradiol in vitro, only the B group of vitellogenin messages are detectable within the first 2-3 hours (Wolffe & Tata, 1983), the subsequent order of expression is B1, A1, A2 and B2. The B1:A1 ratio remains relatively constant throughout the first 12 hours. The A1 + B1 mRNAs are about 5-6 times more abundant than the A2 + B2 mRNAs during the first hour of stimulation (Ng et al., 1984). The fact that the B group of genes becomes DNase I sensitive at least 12 hours before the A group after stimulation (Williams & Tata,

1983), suggests that the B1 gene changes conformation and is transcribed before the A1 gene.

During secondary stimulation there is no lag in the expression of the A1 gene and all of the vitellogenin genes are equally expressed and equally sensitive to DNase I (Williams & Tata, 1983). The complex chromatin structural changes which account for this altered pattern of expression during the initial stages of primary induction take several hours to manifest themselves. These results indicate that the structural changes which occur during primary induction probably play an important role in the establishment and maintenance of "memory".

(5) Gene committment and DNA methylation

The tissue-specific methylation patterns that are created upon cell differentiation are part of the process termed "gene committment" (Compere & Palmiter, 1981). Early studies on methylation suggested that genes which are expressed in a particular cell type are undermethylated when compared to cells which do not express the gene (McGhee & Ginder, 1979; Mandel & Chambon, 1979; Bird & Taggart, 1980). Although this is true for several genes which are constitutively expressed (albumin in

hepatocytes) the demethylation of hormonally controlled genes is not universal. Nine of the twenty-one methylation sites within the transcribed region of the chicken vitellogenin II gene are demethylated in laying-hen liver (Philipsen, Gruber & Ab, 1985) while the Xenopus vitellogenin genes are expressed even when fully methylated (Folger et al., 1982; Gerber-Huber, May, Westley, Felber, Hosbach, Andres & Ryffel, 1983). This suggests that the demethylation of the vitellogenin genes may be species as well as tissue dependent.

The existence of female and liver specific demethylation at the 3'-end of the adult Xenopus B1 gene (Ng, Baker & Tata, 1984) and of expression-linked demethylation sites 5' to the chicken vitellogenin II gene (Burch & Weintraub, 1983; Philipsen et al., 1985) suggests that demethylation of cytosine residues outside of the coding region may also help to direct the expression of the vitellogenin genes in a sex and tissue-specific manner. However, the demethylation of the chicken vitellogenin occurs only after chronic stimulation of vitellogenesis and can not account for the enhanced expression of the vitellogenin genes upon secondary stimulation with estradiol.

(6) Other changes in liver gene expression

In addition to stimulating the production of vitellogenin, estrogen has a marked effect on the expression of a number of other genes in the liver. For example, stimulation of male Xenopus results in the induction of a small estrogen inducible message coding for a protein of about 22,000 daltons (Hayward, Barton and Shapiro, 1985). In rainbow trout the production of an estradiol responsive message of about 1800 nucleotides has been reported to increase several-fold upon induction with estradiol (Chen, 1983). In chicken, estradiol stimulates the production of the mRNA coding for apo very low density lipoprotein II (apo VLDL II mRNA), and secretion of apo VLDL II into the serum (Wiskocil, Bensky, Dower, Goldberger, Gordon and Deeley, 1980).

Estradiol also stimulates the production of several different vitamin binding proteins (Murthy & Adiga, 1978; Muniyappa & Adiga, 1980; Murty & Adiga, 1985). These vitamin binding proteins are synthesized de novo by avian liver and are minor, but important constituents of both the egg-white and egg-yolk. The kinetics of induction of these vitamin carriers during both primary and secondary

stimulation are qualitatively similar to that of vitellogenin (Murty & Adiga, 1985).

In contrast to the proteins mentioned above, chronic induction of vitellogenesis in male Xenopus results in the virtual disappearance of several proteins from the serum, including albumin (Follet & Redshaw, 1968; Follet & Redsaw, 1974). The level of albumin mRNA in male Xenopus liver declines rapidly following administration of estrogen (May, Ryffel, Weber & Westley, 1982). The decrease in the levels of albumin mRNA is due to a combination of a decreased rate of albumin gene transcription and an increased rate of albumin mRNA degradation (Wolffe, Glover, Martin, Tenniswood, Williams & Tata, 1985; Riegel, Martin & Schoenberg, 1986). The effect of estradiol on albumin gene transcription is transient and levels of albumin mRNA return to control after several days even in the presence of estradiol (Wolffe et al., 1986).

Estradiol also has an effect on the proteins required for transcription and translation. The chick oviduct is the site of egg-white protein synthesis and is one of many model systems used for the study of estradiol induced gene expression (Compere, McKnight & Palmiter, 1981; Palmiter, Mulvihill, Shepherd & McKnight, 1981). Stimulation of the

chick oviduct with estradiol results in an increase in overall RNA polymerase II levels prior to ovalbumin gene transcription (Taylor & Smith, 1986). This suggests that one of the first responses of the oviduct to estrogen is to increase RNA polymerase II levels in order to cope with the increased demands on the transcriptional machinery. It should be noted that while the increases in the level of RNA polymerase II activity is undoubtedly of physiological significance, it is orders of magnitude smaller than the effects of estradiol on the synthesis of vitellogenin mRNA (Shapiro, 1982).

In the chicken liver, administration of estradiol results in an increased rate of ribosomal RNA synthesis and a decrease in the amount of ribonuclease activity associated with the liver ribosomes (Bast, Garfield, Gehrke & Ilan, 1977; Penttila & Maenpaa, 1985). The levels of ribosomal RNA increase three to six-fold to meet the high demand placed on the system for translation of the estrogen responsive messages. The induction of vitellogenesis by estradiol is very complex, and involves not only the induction of the vitellogenin genes, but the coordinated control and expression of the transcriptional and translational machinery needed for the production of large amounts of vitellogenin (Tata & Smith, 1979).

(E) Rainbow trout as a model for vitellogenesis

Although the kinetics of induction of the vitellogenin genes has been thoroughly investigated in Xenopus and chicken, neither of these species are seasonal breeders. The reproductive cycle of the chicken is non-seasonal. When Xenopus are kept in captivity, the seasonal reproductive cycle is disrupted. Thus, the use of Xenopus and chicken as model systems for the study of vitellogenesis in seasonal species may not be appropriate. Since rainbow trout retain the seasonality of their reproductive cycle even in captivity, they are an appropriate model to study vitellogenesis in a seasonal species. Moreover, since female trout produce vast amounts of vitellogenin over a 4-5 month period, the control and expression of the vitellogenin genes may be different from that of Xenopus and chicken.

(1) The reproductive biology of the female rainbow trout

Rainbow trout spawn seasonally, producing one batch of eggs per year. First time spawners are two to three-years of age. In the young female trout, differentiation of the primordial germ cells into oogonia occurs at about three months of age. Throughout the life of the female, oogonia undergo mitosis to produce primary oocytes. Development of

the oocytes is arrested at the "prenucleolus" stage, by which time they have increased to over 1000 times their original volume. The secondary growth of the oocyte is synchronous and begins 18 months prior to spawning. This process can be divided into two phases: "endogenous vitellogenesis" and "exogenous vitellogenesis" (van Bohemen & Lambert, 1981). "Endogenous vitellogenesis" occurs primarily in the early stages of secondary growth and is characterized by the synthesis of a small amount of vitellogenin, apparently by the oocyte itself, and the appearance of yolk vesicles in the oocyte in February and March.

The levels of estradiol in the serum are very low during the previtellogenic period and begin to rise in May or June in response to longer periods of sunlight and warmer temperatures. The levels of estradiol peak at approximately 50 ng/ml in early October, one or two months before spawning, then return to previtellogenic levels just prior to ovulation (Scott, Bye & Baynes, 1980). As estradiol levels rise, "exogenous vitellogenesis" commences in the liver and continues until spawning in December. During this period vitellogenin, which is synthesized by the liver, is sequestered in the oocyte. By the end of this phase the oocytes have grown to a diameter of 5 mm and to a volume approximately 250 million-fold greater than that of the

oogonia. At ovulation, the ovaries constitute approximately 20% of the weight of the fish (Sumpter, 1984).

As these numbers might suggest estradiol has a pronounced effect on both liver metabolism and morphology. Estradiol induces hypertrophy of the liver in all teleost species studied so far, including rainbow trout (Campbell & Idler, 1980). During the early stages of the reproductive cycle the liver of the female trout is indistinguishable from the male liver; both show a moderate development of rough endoplasmic reticulum, the Golgi bodies are small and contain very little electron dense material. The livers of both sexes contain large cytoplasmic lipid and glycogen stores. As vitellogenesis progresses the cytoplasm is depleted of glycogen granules and lipid droplets, the rough endoplasmic reticulum increases dramatically and the Golgi bodies are enlarged with electron dense inclusions (Aida, Hirose & Yokote, 1973). As a result the liver weight increases 300% to meet the high demand for vitellogenin production (Sumpter, 1984). Similar changes occur in Xenopus after injection with estradiol (Lewis, Clemens & Tata, 1976).

(2) Role of pituitary hormones

Unlike the situation in Xenopus, pituitary hormones, particularly the gonadotropins, have a pronounced effect on vitellogenesis and oocyte maturation in teleost fishes. There are two types of gonadotropins in teleosts, the vitellogenic and maturation gonadotropins (Idler & Ng, 1979). The two are easily separated on a Concanavalin A Sepharose. The maturation hormone (ConA fraction II) is carbohydrate rich and is absorbed by the column. In contrast the vitellogenic hormone (ConA fraction I) is carbohydrate poor and is not absorbed. In the winter flounder, maturation hormone is present in follicle envelopes and interstitial tissue of the ovaries and in large immature oocytes (Ng, Idler & Burton, 1980). The maturation hormone is thought to be responsible for stimulating estrogen production by the ovary. The vitellogenic hormone has been shown to stimulate the uptake of vitellogenin into the ovary of hypophysectomized flounder which are actively undergoing vitellogenesis (Idler & Ng, 1979) and the in vitro incorporation of vitellogenin into immature chinook salmon oocytes (Campbell, 1978).

Several lines of evidence suggest that both pituitary factors are needed for normal vitellogenesis. First, unlike

whole pituitary extract, vitellogenic gonadotropin does not induce the ultrastructural changes in the ovary characteristic of exogenous vitellogenesis. Secondly, injection of an excess of anti-vitellogenic gonadotropin antibodies into vitellogenic flounder impairs, but does not prevent, vitellogenin accumulation in the oocyte (Wiegand & Idler, 1984), suggesting that both the ConA fraction I and ConA fraction II gonadotropins may be required for normal oocyte development and uptake of vitellogenin and lipids. The central nervous system-hypophysial axis is also the most likely trigger for the photoperiodic modulation of plasma estradiol in the female trout (Bromage, Whitehead & Breton, 1982).

(F) Specific aims

In the present study rainbow trout liver gene expression during vitellogenesis was examined with the following specific aims:

(1) to characterize the control of vitellogenesis in rainbow trout.

(2) to determine if any difference exists between the primary response of male trout liver to estradiol versus the secondary response.

(3) to determine the medium to long term effects of estradiol on the expression of non-vitellogenic messages in male trout liver.

METHODS

(1) ANIMALS

Two year old male rainbow trout (Salmo gairdneri), 175-600 g, were obtained from Thistle Springs Trout Farm, Ashton Ontario. The sex of each fish was determined on the basis of secondary sexual characteristics and later confirmed at autopsy. On arrival the fish were transferred to living streams filled with deionized, dechlorinated city water buffered with a small amount of sodium bicarbonate. The pH was maintained at pH 6.8-7.0 throughout the study. The light cycle was 12 hours on / 12 hours off. The fish were fed once a day with Purina Fish Chow, uneaten food was removed after 15 minutes of feeding. Fish were anesthetized by placing them in a bath of 100 mg/litre Tricane (ethyl 3-aminobenzoate, methanesulfonic acid) buffered to pH 7.0 with sodium bicarbonate. Blood samples were obtained from anesthetized fish via the caudal vein.

(2) ADMINISTRATION OF ESTRADIOL

The fish were divided into three groups. The first group (primary acute stimulation), was injected intraperitoneally with estradiol (3 mg/kg body weight) in warm cocoa butter (3 mg/ml) (Maitre et al., 1985). The second group of fish were implanted subcutaneously with

silastic tubes containing 20 mg estradiol (primary chronic stimulation). The remaining group was first injected with the cocoa butter-estradiol mix then 28 days later implanted with silastic tubing containing 20 mg estradiol (secondary chronic stimulation). At various times after injection or implantation, the fish were anesthetized, blood was sampled and the livers excised, frozen in liquid nitrogen and stored at -70° C. Body weights and liver weights were also measured. The primary acute stimulation was performed in late April, while the primary and secondary chronic stimulations were performed in June and August respectively.

(3) MEASUREMENT OF ESTRADIOL SERUM LEVELS

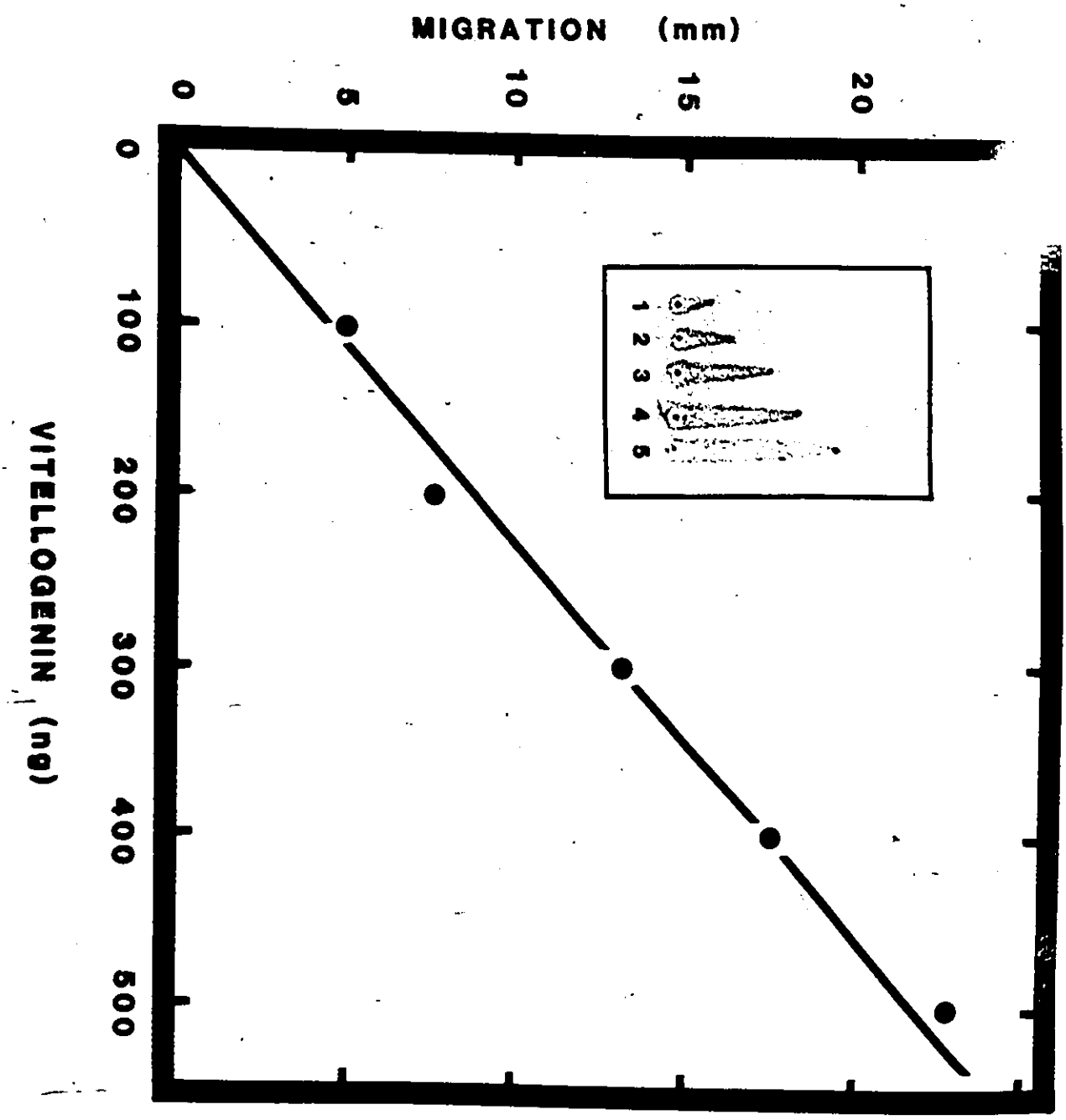
The serum levels of estradiol were measured by radio-immunoassay at the Kingston General Hospital (courtesy of Dr. Albert Clark). Sample determinations were performed in duplicate and compared to a prepared set of standards. The anti-serum used does not cross-react with estrone, estriol, progesterone or testosterone.

(4) MEASUREMENT OF VITELLOGENIN SERUM LEVELS

Serum vitellogenin was measured by rocket immunoelectrophoresis as described by Maitre et al. (1985),

FIGURE 3: Quantitative measurement of serum vitellogenin by rocket immunoelectrophoresis.

Rocket immunoelectrophoresis was performed on 5 X 5 cm gels containing 1% agarose, in 0.25 M Tris and 0.19 M glycine pH 9.2, containing monospecific anti-trout Vg antibody diluted 1:400. Inset: increasing amounts of purified vitellogenin (100-500 ng) were added to each well in 2 µl of gel buffer and electrophoresed for 18 hours at 80 volts. The graph shows the linear correlation between peak height and the amount of vitellogenin in each sample over the range of 0 to 500 ng. Each point represents a single determination.


MIGRATION (mm)
0
5
10
15
20
VITELLOGENIN (ng)
0
100
200
300
400
500
1
2
3
4
5

using anti-trout vitellogenin antibodies and purified trout vitellogenin as the standard. The purified vitellogenin standard was prepared from the serum of estradiol treated males by the method of Wiley, Opresko, and Wallace (1979), with modifications (Maitre et al., 1985). The precipitation band was visualized by staining with Coomassie blue (Maitre et al., 1985). A typical standard curve is shown in figure 3. The relationship between migration versus nanograms of vitellogenin is linear up to 500 ng. Unknown serum samples were diluted with saline to bring the concentration of vitellogenin within the linear range of the assay.

(5) EXTRACTION OF RNA

Total RNA was extracted from the livers of control and stimulated trout by the LiCl/urea procedure (Auffray & Rougeon, 1977) with minor modifications (Tenniswood & Simpson, 1982). The concentration of RNA was estimated by measuring optical density at 260 nm. The OD_{260}/OD_{280} ratios were all in the range of 1.5 - 1.7, indicating that the samples were essentially free of protein or phenol contamination. Typical yields were 3-10 mg of total RNA per gram of liver. Poly(A)$^+$ RNA was prepared by oligo(dT)-cellulose chromatography as described by Aviv and Leder (1972).

(6) AGAROSE GEL ELECTROPHORESIS OF RNA

Total and poly(A)$^+$ RNA were electrophoresed in 1.5% agarose slab gels under denaturing conditions (McMaster & Carmichael, 1977) using trout rRNA as size markers. Electrophoresis was carried out at room temperature at 100 volts for 4 hours, during the run the buffer was continually recirculated. The gels were stained with ethidium bromide (1 µg/ml) and photographed using 306 nm U.V. transillumination with an orange filter and Ilford FP4 film. Densiometric scans of the negatives were performed on a Beckman DU-8 spectrophotometer at a wavelength of 520 nm.

(7) PREPARATION OF cDNA LIBRARY FROM STIMULATED MALE TROUT LIVER

Trout liver cDNA clones were prepared using the procedure of Rutledge, Seligy, Côte, Dimock, Lewin and Tenniswood (1986). Poly(A)$^+$ RNA from the liver of an estradiol stimulated male trout, implanted 10 days previously with an estradiol containing silastic implant, was used for the cloning.

(i) Synthesis of [^{32}P]-single stranded-cDNA

[^{32}P]-ss-cDNA was synthesized as follows: 10 µg of poly(A)$^+$ RNA from the liver of an estradiol stimulated male was incubated in a final volume of 30 µl of a buffer containing 100 mM Tris-HCl pH 8.3, 130 mM KCl, 10 mM $MgCl_2$, 2.5 mM DTT, 4 µg oligo(dT)$_{10-18}$, 10 µg random hexanucleotide primer, 1mM dATP, 1mM TTP, 1mM dGTP, 1mM dCTP, and 50 µCi of [^{32}P]-dCTP. After heating to 60°C for 2 minutes, 24 units of AMV reverse transcriptase were added and the mix was incubated at 42°C for 30 minutes.

The [^{32}P]-ss-cDNA/RNA hybrids prepared above were incubated at 15°C for 1 hour with 400 units of T4 DNA ligase in a total volume of 100 µl of 50mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 20 mM DTT, 1 mM ATP, 50 µg/ml BSA. After incubation the sample was heated to 65° C for 15 minutes to inactivate the ligase.

(ii) Synthesis of [^{32}P]-double stranded-cDNA

RNase H (2 units) and DNA polymerase I (25 units) were added to the [^{32}P]-ss-cDNA/RNA hybrid and the reaction mixture was incubated at 15°C for 2 hours. At the end of the incubation period the sample was extracted with phenol/

chloroform/ isoamyl alcohol (25:24:1), and passed through a G-50 spin column (Maniatis, Fristsch, & Sambrook, 1982) to separate the free nucleotides from the incorporated radioactivity. The [^{32}P]-ds-cDNA in the void volume eluant from the G-50 columns (approximately 100 µl) was fractionated through a Sepharose 4B column packed in a 1 ml plastic pipette. One drop (30 µl) fractions were collected and counted in a Nuclear Chicago Isocap 300 scintillation counter.

One microlitre aliquots from the Sepharose 4B fractions were sized by electrophoresis on a 2% agarose slab gel in a Tris-acetate buffer. A Hind III digest of lambda DNA was used as a standard molecular weight marker. The gel was stained with ethidium bromide as outlined above and photographed. The gel was then dried down under vacuum on a Savant gel drier and autoradiographed using Cronex X-ray film in a Kodak X-Omatic X-ray cassette with intensifying screens.

(iii) Cloning of [^{32}P]-ds-cDNA into pUC 8

Fractions 8-12 from the Sepharose 4B column were pooled and precipitated at -20°C for 1 hour in alcohol with an equimolar amount of Sma I-digested calf intestinal

phosphatase-treated pUC 8. The sample was redissolved in a total volume of 10 µl in a buffer containing 50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 20 mM DTT, 1mM ATP, 50 µg/ml BSA and 400 units of T4 DNA ligase. The sample was incubated at 15°C for 16 hours. After dilution of the ligation mixtures to 100 µl with T.E. buffer, aliquots containing recombinant plasmids were used to transform E. coli DH1 cells, which were then grown on psi agar containing 100 µg/ml ampicillin. Plasmids containing cDNA inserts were identified by Grunstein-Hogness hybridization as described below. Plasmids of interest were prepared using a modification of the method described by Birnboim and Doly (1979).

(8) GRUNSTEIN-HOGNESS HYBRIDIZATION ANALYSIS

Ampicillin-resistant colonies were selected and characterized by colony hybridization to [^{32}P]-labeled cDNA (Grunstein & Hogness, 1975). The colonies were picked in duplicate onto nylon membranes, each of which was hybridized to [^{32}P]-cDNA complementary to estradiol stimulated or unstimulated male trout liver poly(A)$^+$ RNA. The intensities of the signals were compared, and the clones of interest were classified accordingly as constitutive or estradiol induced. Selected plasmids were further characterized by northern hybridization analysis.

(9) NORTHERN HYBRIDIZATION ANALYSIS

Duplicate lanes of poly(A)$^+$ RNA from control and stimulated male trout were electrophoresed as described previously, transferred to nylon membranes essentially as described by Thomas (1980) for nitrocellulose filters, and hybridized to nick-translated plasmids (average specific activity 8-10 x 10^6 cpm/200 ng plasmid). The filters were prehybridized for at least 6 hours at 42°C in a buffer containing 50% formamide, 5X SSC, 5X Denhardt's solution (0.2% bovine serum albumin, 0.2% ficoll, 0.25% polyvinylpyrrolidone) and 100 µg/ml heat denatured calf thymus DNA. The filters were then hybridized with [^{32}P]-labeled plasmids in the same buffer at 42°C for at least 18 hours. After hybridization the filters were washed twice in 2X SSC, 0.1% SDS at room temperature for 15 minutes, and twice in 1X SSC, 0.1% SDS at 42°C for 10 minutes. The filters were sealed in Saran Wrap and autoradiographed with Cronex X-ray film in a Kodak X-Omatic cassette with intensifying screens.

(10) PREPARATION OF RADIOACTIVE PROBES

[^{32}P]-cDNA complementary to poly(A)$^+$ RNA from unstimulated or stimulated trout used for Grunstein-Hogness

analysis was synthesized by the method of Williams and Penman (1975) with minor modifications (Smith, Searle, & Williams, 1979). Plasmids of interest were nick-translated following the standard procedure of Rigby, Dieckmann, Rhodes, and Berg (1977) or labeled using the oligo-nucleotide labeling technique described by Feinberg and Vogelstein (1983). [^{32}P]-5'-end labeling of 18S rRNA was performed using the method originally described by Richardson (1971) with modifications (Maniatis et al., 1982).

(11) RESTRICTION MAPPING OF CLONES

Restriction digests were performed on 5 or 10 µg of the plasmids of interest using conditions suggested by the enzyme supplier. Double digests were performed sequentially, using the enzyme requiring the lowest salt concentration first. The digestion products were separated on non-denaturing 2% Tris-acetate/agarose gels. A Hind III-Eco RI digest of lambda DNA or combination of Hind III digest of lambda DNA and Taq I digest of pBR 322 were used as standard molecular weight markers. After electrophoresis the gels were stained with ethidium bromide and photographed.

(12) SOUTHERN HYBRIDIZATION ANALYSIS

DNA was extracted from female trout liver using the method of Gross-Bellard, Oudet, and Chambon (1973). Restriction digests were performed on 40 µg of DNA, 10 µg of the digested DNA was electrophoresed on 0.8% Tris-acetate/agarose gels. After electrophoresis the gels were stained with ethidium bromide and photographed. The gels were then denatured for 30 minutes in 2.5 M NaCl, 0.5 M NaOH, and neutralized with 3M sodium acetate, pH 5.5 prior to transfer onto nylon membranes (Southern, 1975). After transfer the blots were baked at 80°C for 2 hours and then hybridized to [^{32}P]-labeled cDNA clones.

(13) QUANTITATION OF mRNA SPECIES

Total RNA extracted from the livers of control and stimulated trout was dot blotted in triplicate onto nylon membranes as described by Thomas (1983). A poly(A)$^+$ RNA preparation from control and stimulated male trout liver were used as standards. The amount of each of the messages in the poly(A)$^+$ RNA preparations was determined by scanning the negatives of an analytical gel of the standard poly(A)$^+$ RNA preparations (results not shown). Vg mRNA represented 13.5% of the stimulated poly(A)$^+$ RNA standard, while the mRNAs

corresponding to pSG 3.2 and pSG 2.5 represented 6.4% and 7.1% respectively of the poly(A)$^+$ RNA preparation from a control male trout. The nylon filters were prehybridized as previously described for Northern hybridization and hybridized with [^{32}P]-labeled plasmids at 42°C for at least 18 hours. After hybridization the filters were washed twice with 2X SSC, 0.1% SDS at room temperature for 15 minutes, then twice with 1X SSC, 0.1% SDS at 42°C for 10 minutes. The filters were sealed in Saran wrap and autoradiographed as previously described. The blots were then cut up and individual dots were placed in scintillation vials with 5 ml of Beckman Ready-Solv ET scintillation fluid and counted for 10 minutes using a Beckman LS1801 or Nuclear Chicago Isocap 300 scintillation counter. Ten micrograms of yeast total RNA was used to determine non-specific hybridization. Non-specific counts were subtracted from sample counts and then compared to the standards. The amount of each message is expressed as parts per million (ppm) of total RNA. A typical standard curve is shown in figure 4. Hybridization of [^{32}P]-labeled pSG Vg5.09 to Vg mRNA is linear up to 100 ng of Vg mRNA. The standard curves for pSG 3.2 and pSG 2.5 also showed a linear relationship between cpm hybridized and concentration of message, over the range assayed. A sample calculation is shown below.

Sample Calculation:

(sample cpm - non-specific) = cpm bound

extrapolated from standard curve (figure 4)

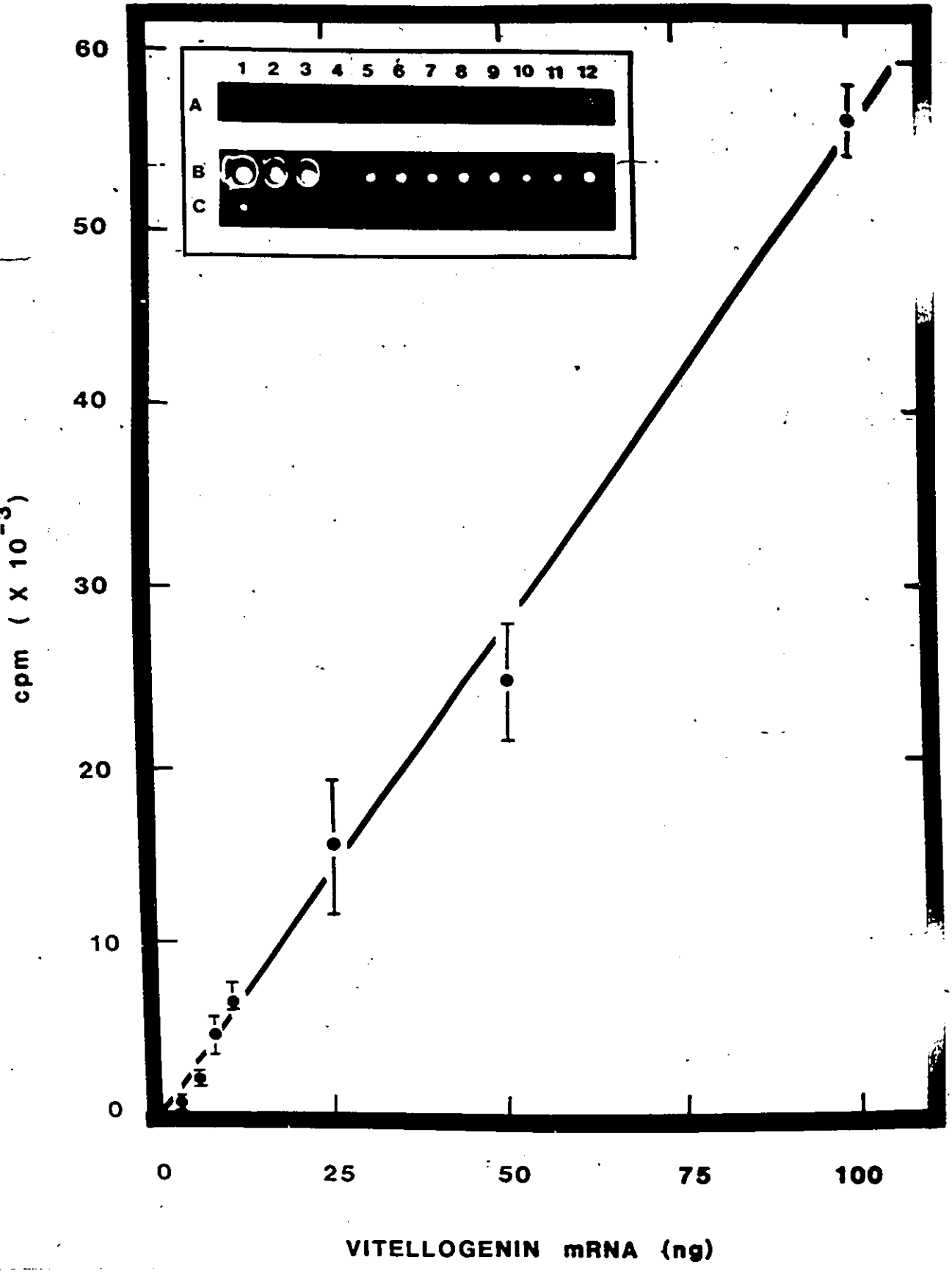
X cpm = Y ng of Vg mRNA

$$\frac{\text{Y ng Vg mRNA}}{\text{mg total RNA dot blotted}} = \text{ppm of Total RNA}$$

FIGURE 4: Quantitative measurement of Vg mRNA using dot blot assay.

Poly(A)$^+$ RNA standards from stimulated trout (+ 10 µg yeast total RNA) and yeast total RNA (control) were glyoxylated at 50°C for one hour prior to dot blotting onto nylon membrane. After baking the blots for two hours at 80°C, the filters were prehybridized for 6 hours at 42°C and then hybridized to [^{32}P]-pSG Vg 5.09 for 18 hours at 42°C. After hybridization the blots were washed twice in 2X SSC, 0.1% SDS at room temperature, then twice in 1X SSC, 0.1% SDS at 42°C and autoradiographed. The graph shows the linear relationship between cpm bound versus ng of Vg mRNA present. Points are the mean value of triplicates. The bars represent the range of the triplicates.

Inset:	A 1-12:	10	µg yeast total RNA
	B 1-3:	100	ng Vg mRNA
	B 4-6:	50	ng Vg mRNA
	B 7-9:	25	ng Vg mRNA
	B 10-12:	10	ng Vg mRNA
	C 1-3:	7.5	ng Vg mRNA
	C 4-6:	5.0	ng Vg mRNA
	C 7-9:	2.5	ng Vg mRNA
	C 10-12:	1.0	ng Vg mRNA.


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
60
50
40
30
20
10
0
cpm (X 10^{-3})
0
25
50
75
100
VITELLOGENIN mRNA (ng)

(14) BINDING AND RECOVERY OF 18S rRNA USING DEAE MEMBRANE

Duplicate lanes of non-glyoxalated poly(A)$^-$ RNA from male trout were electrophoresed in a 1.5% agarose gel using a 10 mM phosphate buffer at pH 6.8. One lane was stained with ethidium bromide and the position of the rRNA bands determined. The 18S and 28S rRNA bands in the duplicate lane were intercepted by placing a strip of Schleicher and Schuell DEAE membrane in front of the rRNA bands and electrophoresing the rRNA onto the membrane. The rRNA was eluted from the DEAE membrane by incubating the membrane at 65° C, in a formamide containing high salt buffer (55% formamide, 1.8 M sodium acetate, 2 mM EDTA, 0.2% SDS, pH 7.4) for 10 minutes. After incubation the buffer was diluted with water and the rRNA was precipitated with ethanol, microfuged and resuspended in a minimal amount of water.

(15) DOT BLOT MEASUREMENT OF THE CHANGES IN 18S + 28S rRNA CONTENT

Measurement of the qualitative changes in the levels of 18S and 28S rRNA was preformed using the dot blot assay previously described for the quantitation of mRNA species (section 13). The measurement of these changes was hampered

by a lack of available recombinant probes for trout rRNA. We considered using [^{32}P]-labeled cDNA prepared from isolated rRNA as a probe, but the synthesis was inhibited by the secondary structure of the rRNA (results not shown). However, the extensive secondary structure of the rRNA does allow for the hybridization of the rRNA to a [^{32}P]-5'-end labeled rRNA probe prepared from isolated 18S rRNA. The disadvantage of using [^{32}P]-labeled 18S rRNA as a probe is that it hybridizes to both 18S and 28S rRNA. This, coupled with the low specific activity of the probe (50 x 10^6 cpm/ 2.4 µg 18S rRNA) made it impossible to provide an absolute measure of the amount of rRNA dot blotted onto the membrane. However, the hybridizations were sufficient for determining if there are significant changes in the proportion of 18S and 28S rRNA in the samples. The levels of 18S and 28S rRNA are expressed as the amount of [^{32}P]-labeled probe hybridizing to 10 µg of total RNA.

(16) IN VITRO TRANSLATION AND TWO-DIMENSIONAL GELS

Poly(A)+ RNA from stimulated and control trout was translated in a micrococcal nuclease-treated reticulocyte lysate cell free translation system (Pelham & Jackson, 1976) using [^{35}S]-methionine as the radioactive label. After translation the [^{35}S]-labeled products were separated by two

dimensional electrophoresis (O'Farrell, 1975). The labeled translation products (400,000 cpm of TCA precipitable material), were first electrophoresed on isofocusing tube gels using LKB pH 3-10 ampholines, followed by SDS polyacrylamide slab gel electrophoresis (10% acrylamide) for the second dimension (Newbold, Boyle, Smith, & Brown, 1982). Isofocusing gels using unlabeled reticulocyte lysate were run concurrently, cut into 1 cm long discs, placed in 1 ml of distilled water and the pH measured. These served as a measure of pI for the sample gels. Bio-Rad low molecular weight SDS-PAGE protein markers were used as molecular weight standards. After electrophoresis the gels were stained with Coomassie blue, destained, and fixed with 7% acetic acid. After fixing, the gels were prepared for fluorography (Bonner & Laskey, 1974), dried down under vacuum and fluorographed using Cronex X-ray film in a Kodak X-Omatic cassette with intensifying screens.

RESULTS

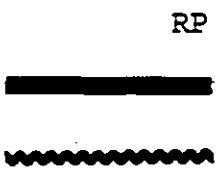
(A) CLONING AND CHARACTERIZATION OF cDNA CLONES

(1) Preparation of cDNA library from stimulated male trout liver

In order to study the effects of estradiol on liver gene expression, cDNA probes were needed to measure the levels of specific liver mRNA sequences. When this study was initiated very few trout liver cDNA clones were available. The cDNA plasmids specific for trout vitellogenin and other liver specific sequences which were available were very small (Tenniswood, Bailey, Valotaire & Tata, 1983b). This, coupled with the fact that these plasmids contained long tracts of cytodine and guanosine residues which hybridize nonspecifically, has made these recombinant probes very difficult to use. In order to measure the levels of liver specific messages it was therefore necessary to prepare another cDNA clone library made from trout liver poly(A)$^+$ RNA and to screen it for cDNA clones corresponding to Vg mRNA and other estradiol-inducible messages. Poly(A)$^+$ RNA, extracted from a male trout implanted 10 days previously with an estradiol-containing silastic tube, was used for cloning. This time point was selected because of the elevated levels of vitellogenin mRNA known to be present.

The cloning procedure is summarized in figure 5. The fractionation of the ds-cDNA on Sepharose 4B makes it possible to select specific size classes for cloning. The autoradiograph of the Tris-acetate gel of size fractionated ds-cDNA shows a number of distinct bands in the column profile, most of which represent full length ds-cDNA copies of mRNA species (figure 6) (Rutledge et al., 1986). In total 3.6 µg of ds-cDNA was prepared from 10 µg of poly(A)$^+$ RNA. Half of the ds-cDNA was size fractionated by Sepharose 4B chromatography and fractions 8-12 were pooled. Two hundred nanograms of ds-cDNA was recovered from these fractions and ranged in size from 1-5 kb. Forty nanograms of the size fractionated ds-cDNA was then used for ligation to pUC 8. Transformation of E. coli DH1 with the recombinant plasmids produced approximately 10,000 recombinant colonies. This size selected cDNA library should therefore represent clones for all abundant mRNA species greater than 1000 nucleotides. The background of colonies containing uninserted pUC 8 as determined by the ligation and transformation of phosphatase treated-Sma I digested pUC 8 into E. coli DH1 was less than 5%.

FIGURE 5: Cloning strategy (adapted from Rutledge et al., 1986).



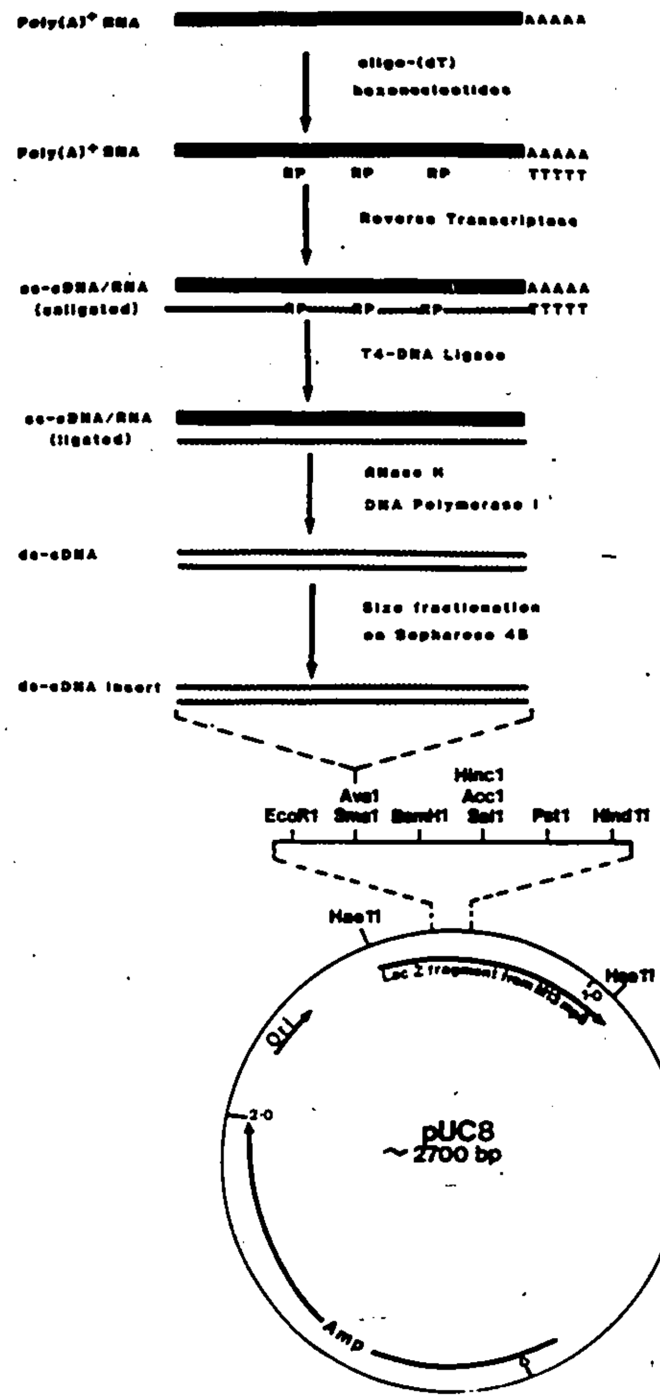
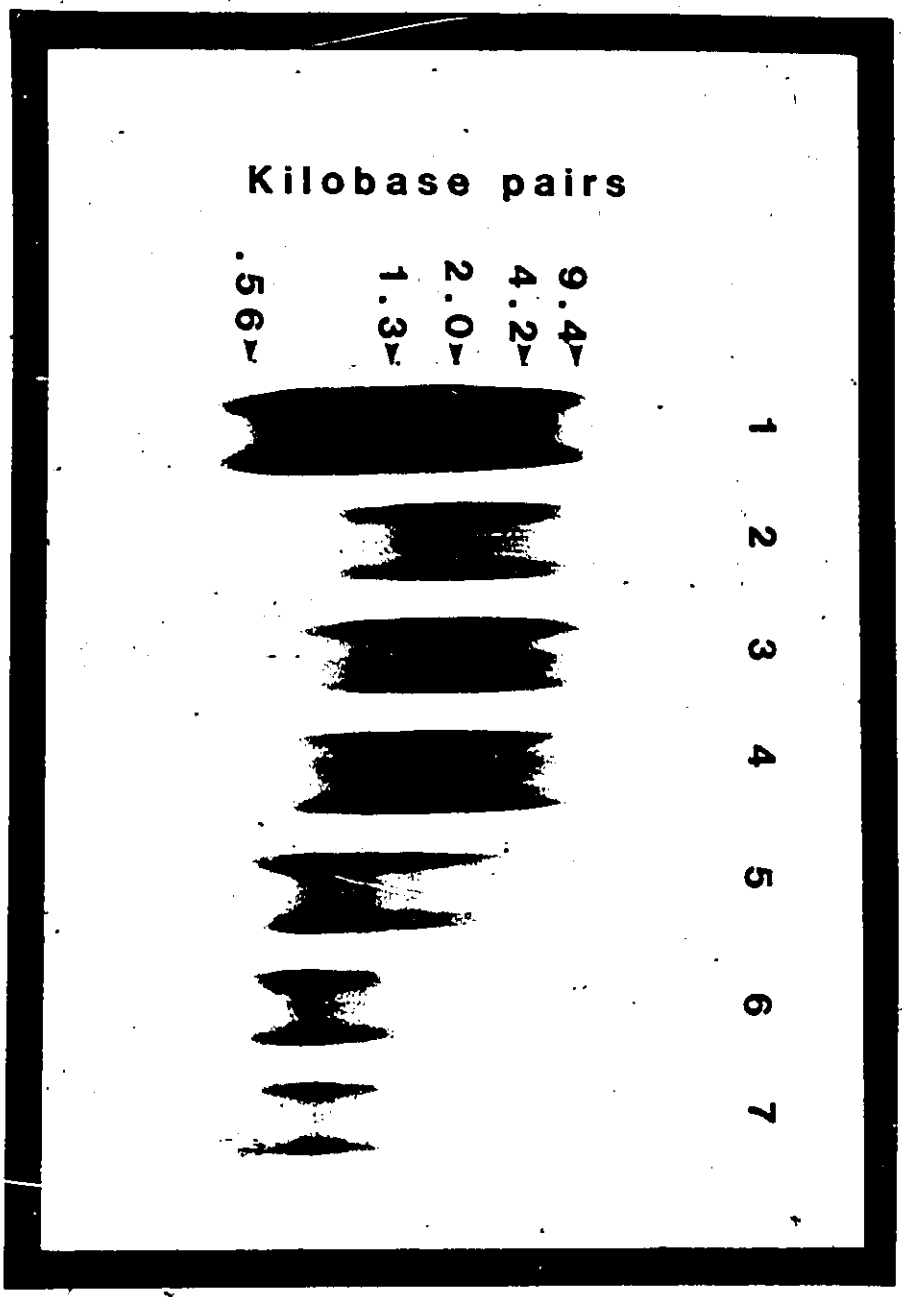

AAAAA
TTTTT
Hinc1
Acc1
Ava1
EcoR1
Sal1
Pst1
pUC8
~2700 bp
Amp
ori

FIGURE 6: Size fractionation of [^{32}P]-double stranded-cDNA by Sepharose 4B chromatography.

[^{32}P]-double stranded-cDNA, synthesized as described in methods section 7, was size fractionated on a 1 ml Sepharose 4B column (void volume-fraction 8). One micoliter aliquots from these fractions were electrophoresed on non-denaturing 2% Tris-acetate/agarose gels. A Hind III digest of lambda DNA was used as a molecular weight standard. After electrophoresis the gel was dried down and autoradiographed.

Lane 1: unfractionated cDNA
lane 2: fractions 9 & 10
lane 3: fractions 11 & 12
lane 4: fractions 13 & 14
lane 5: fractions 15 & 16
lane 6: fractions 17 & 18
lane 7: fractions 19 & 20.


Kilobase pairs
9.4
4.2
2.0
1.3
.56
1
2
3
4
5
6
7

(2) Grunstein-Hogness analysis

The cDNA clones specific for vitellogenin mRNA, were identified by differential hybridization. [^{32}P]-labeled ss-cDNA probes, prepared from control and stimulated trout, were hybridized to ampicillin-resistant colonies as described by Grunstein and Hogness (1975). Clones which hybridized to the [^{32}P]-labeled ss-cDNA from estradiol stimulated male trout but not the [^{32}P]-labeled ss-cDNA from control males were classified as estradiol-induced sequences. The clones which hybridized to both the control and stimulated cDNA probes were classified as being constitutively expressed. 1200 of the approximately 10,000 clones in the cDNA library were analyzed. Two hundred of these were classified as strongly estradiol-induced. Thirteen clones with the strongest hybridization were chosen for further analysis.

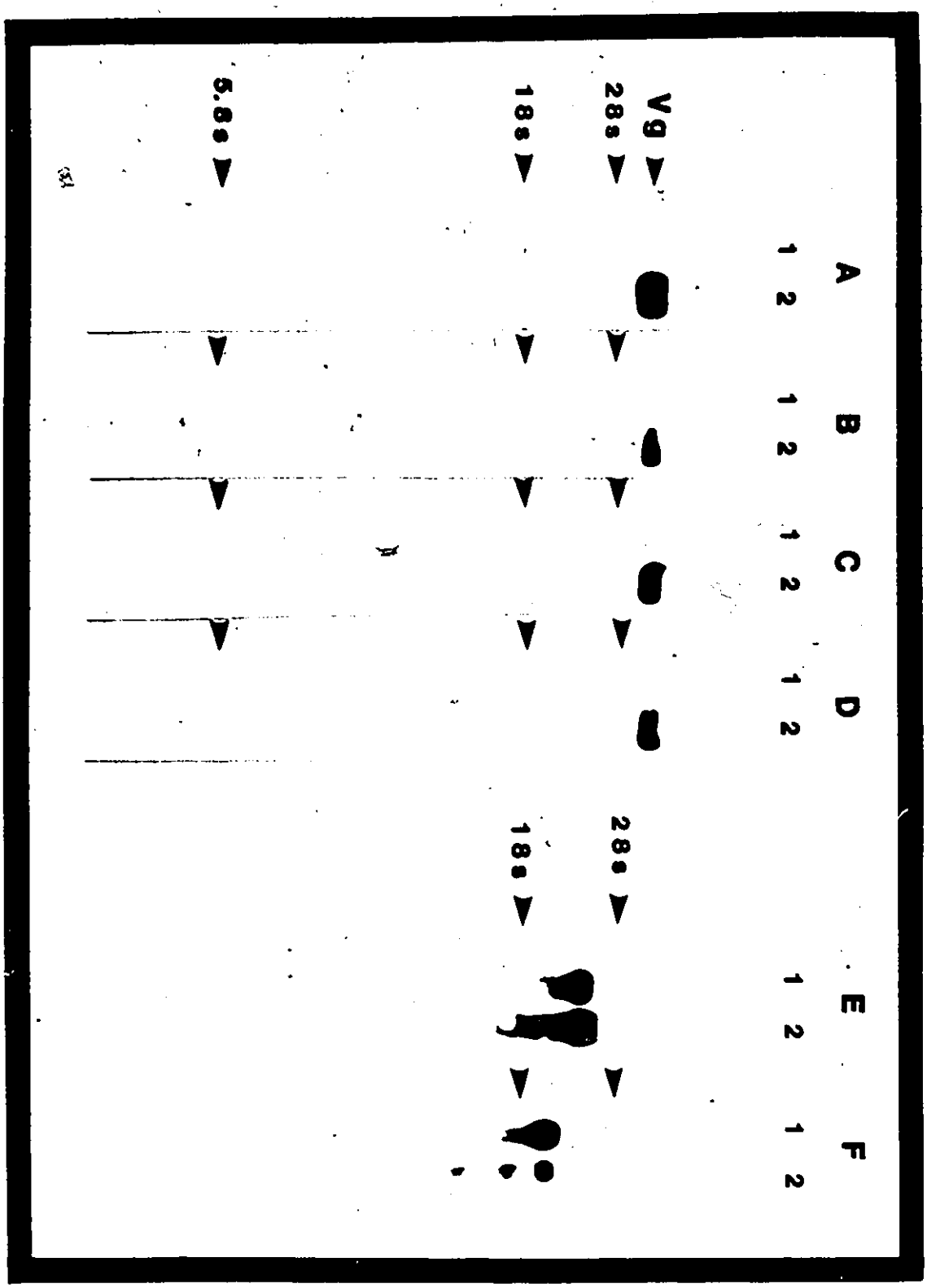
(3) Northern analysis of cDNA clones

The estradiol-induced cDNA clones were characterized by Northern analysis. Figure 7, panels A to D shows the hybridization of four [^{32}P]-labeled vitellogenin cDNA clones: pSG Vg 3.10, pSG Vg 4.14, pSG Vg 5.09 and pSG Vg 5.50. These plasmids hybridize to a 7000 nucleotide mRNA

FIGURE 7: Northern analysis of trout cDNA clones.

2 µg of poly(A)$^+$RNA from control (lane 1) and stimulated trout (lane 2) was glyoxylated and electrophoresed on analytical 1.5% agarose gels in 10 mM phosphate buffer pH 6.8 for 4 hours at 80 volts. Trout rRNA subunits were used as molecular weight markers. The RNA was blotted onto nylon membranes and hybridized to [^{32}P]-nick translated cDNA clones (average specific activity 8-10 X 10^6 cpm/200 ng). Poly(A)$^+$RNA from day 10 of the primary chronic stimulation was used for lane 2 of panels A-D. Poly(A)$^+$ RNA from day 4 of the primary acute stimulation was used for lane 2 of panels E and F.

panel A: pSG Vg 3.13
panel B: pSG Vg 4.14
panel C: pSG Vg 5.09
panel D: pSG Vg 5.50
panel E: pSG 3.2
panel F: pSG 2.5.


A
B
C
D
E
F
1 2
1 2
1 2
1 2
1 2
1 2
Vg
28s
18s
5.8s
28s
18s

from the liver of an estradiol treated male trout (lane 2), but do not hybridize to the RNA from the liver of unstimulated males (lane 1), demonstrating that the RNA is induced de novo by estradiol. The 7000 nucleotide band is the same as that obtained upon hybridization with pSG Vg 225, a trout vitellogenin specific cDNA plasmid (Tenniswood et al., 1983b), (not shown) and corresponds to the size of Vg mRNA reported by Valotaire et al. (1984). Northern analysis of three other estradiol-induced cDNA clones, designated pSG Vg 1.43, pSG Vg 3.13 and pSG Vg 14.12 showed similar results to those obtained above (results not shown).

Panels E and F (figure 7) represent the Northern analysis of two of the constitutively expressed cDNA clones designated pSG 3.2 and pSG 2.5 after the size of the message (in kilobases) to which they hybridize. Both messages are present in the livers of both unstimulated (lane 1) and stimulated (lane 2) male trout. At present the identity of the proteins encoded by pSG 3.2 and pSG 2.5 is not known.

(4) Restriction mapping of cDNA clones

The cDNA clones were characterized by restriction mapping. Figure 8-A represents a Bam HI - Eco RI digest of six of the vitellogenin clones and a pUC 8 control. The band

at approximately 2700 base pairs, present in all lanes corresponds to the Eco RI-Bam HI linearization of the pUC 8 vector (2722 bp) (lane 7). The vitellogenin clones shown here contain inserts that range from 1350 to 2500 bp in total length. The largest clone, pSG 4.14 (lane 3), is 2500 base pairs in length and represents over one third of the mature vitellogenin mRNA. The presence of extra bands on the gel is indicative of internal Eco RI or Bam HI sites. A summary of insert size and mRNA species size for the clones is shown in Table 1. The restriction maps of these vitellogenin clones are shown in figure 9. All the large clones, except for pSG Vg 5.50, contain a common Bam HI - Eco RI fragment of 1080 bp in length and a common Pst I site. pSG Vg 5.09 and pSG Vg 4.14 also contain a second common Pst I site. pSG Vg 3.10 and pSG Vg 4.14 are linearized upon digestion with Ava I suggesting either an internal Ava I site, or regeneration of one of the Sma I/Ava I sites upon ligation of the insert to the Sma I digested pUC 8.

The restriction map of pSG Vg 5.50 is distinctly different from the other vitellogenin clones. The insert contains three Pst I sites, a Hind III site, an Eco RI site and a Bam HI site adjacent to the Bam HI site in the multicloning cloning region of pUC 8. The relationship between pSG Vg 5.50 and the other vitellogenin clones has

FIGURE 8: 2% Tris acetate/agarose gel of Bam HI-Eco RI digested cDNA clones.

Ten micrograms of Bam HI-Eco RI digested plasmids were run on non-denaturing 2% Tris-acetate/agarose gel for 16 hours at 40 volts, using either a Hind III-Eco RI digest of lambda DNA or a Taq I digest of pBR 322 as molecular weight standards. After electrophoresis the gels were stained with ethidium bromide and photographed. The number designation for the vitellogenin specific clones is arbitrary.

Panel A:

lane 1: pSG Vg 3.10
lane 2: pSG Vg 3.13
lane 3: pSG Vg 4.14
lane 4: pSG Vg 5.09
lane 5: pSG Vg 5.50
lane 6: pSG Vg 14.12
lane 7: pUC 8

Panel B:

lane 1: pSG 3.2
lane 2: pSG 2.5.

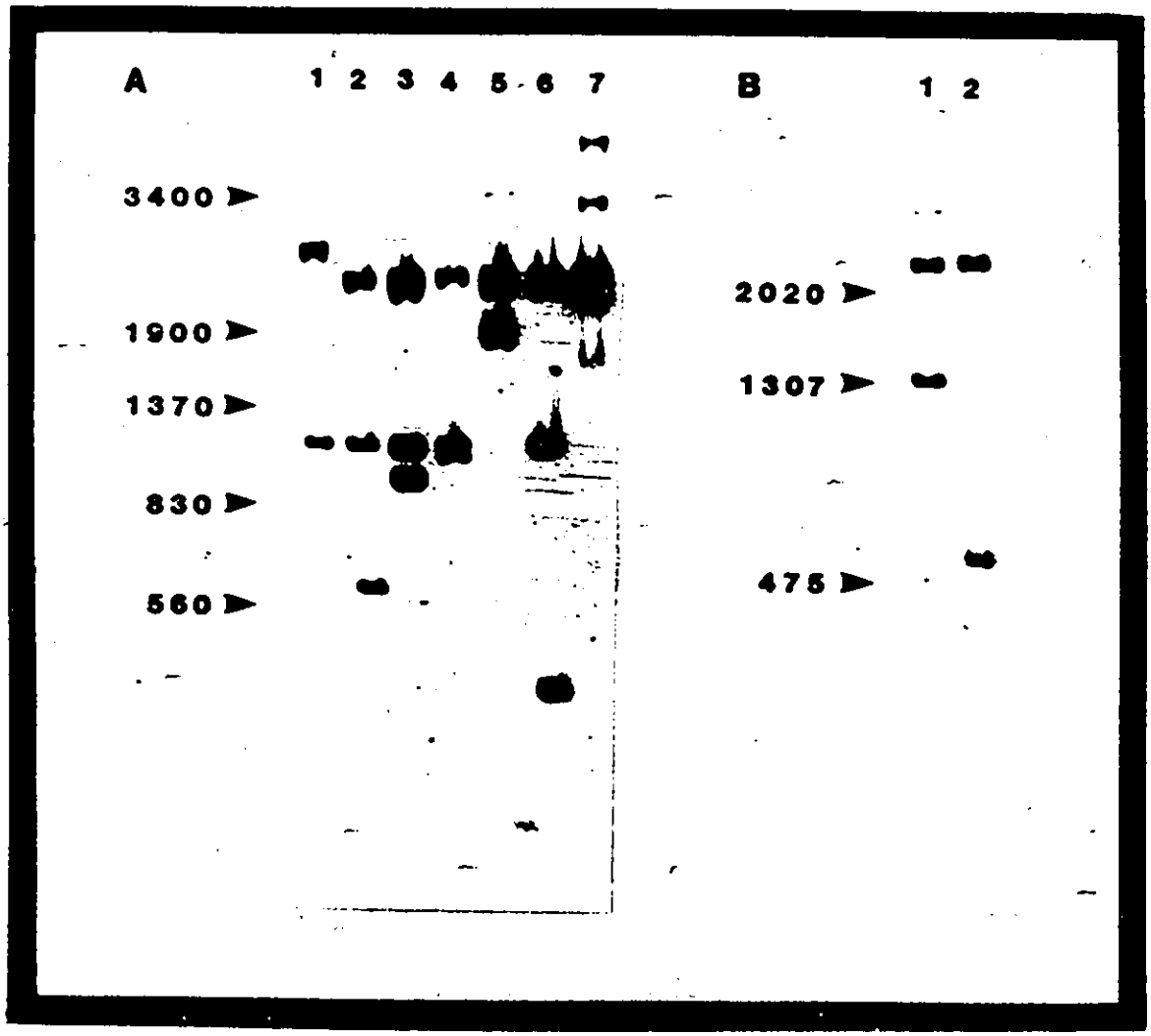

A
1 2 3 4 5 6 7
3400
1900
1370
830
560
B
1 2
2020
1307
475

FIGURE 9: Restriction Maps of vitellogenin cDNA clones.

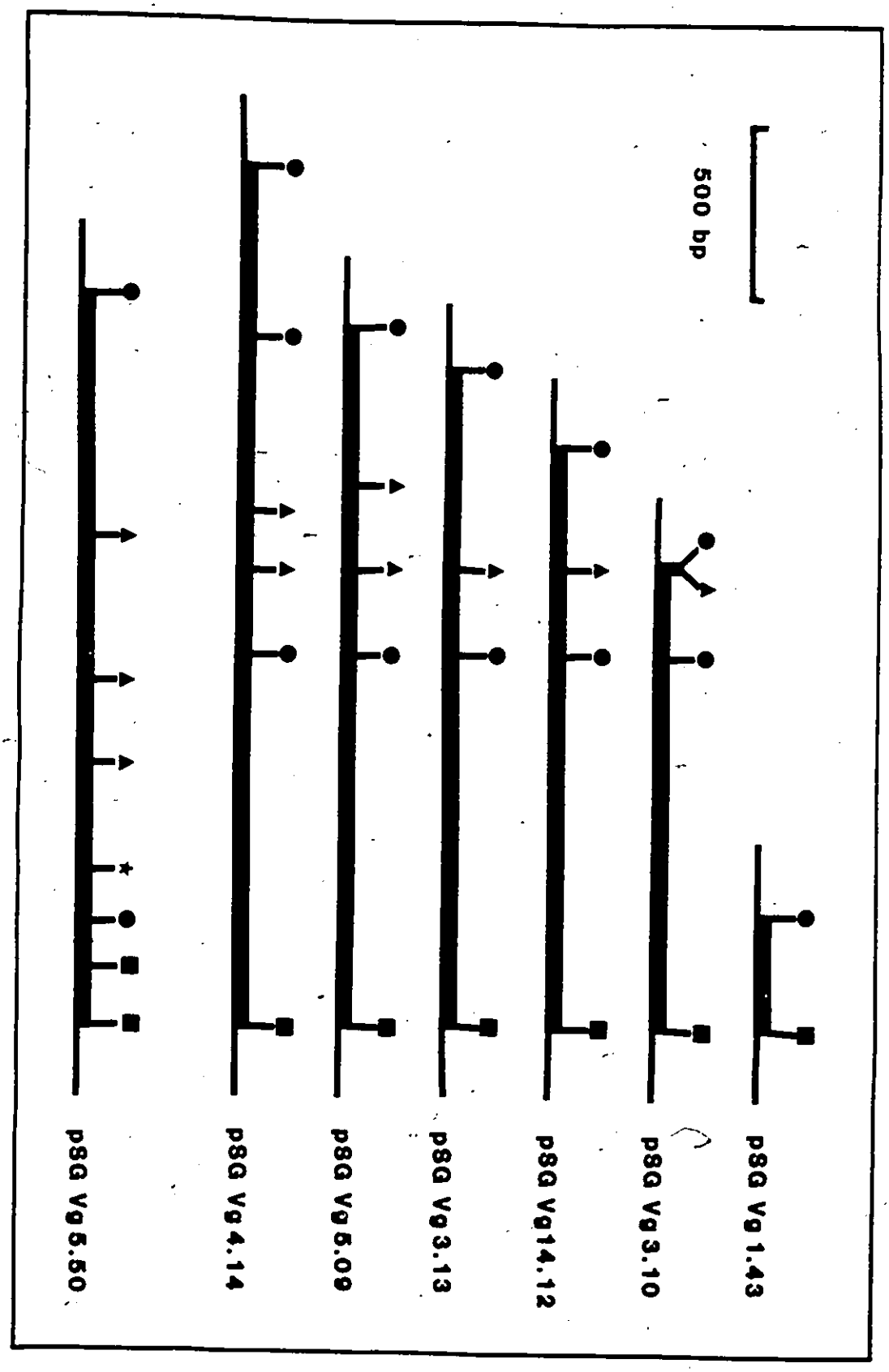
Restriction digests were performed as outlined in Methods (section 11). The vitellogenin cDNA clones are aligned with the Bam HI site found in the multicloning region of the pUC 8 vector. pSG Vg 3.10 and pSG Vg 4.14 were linearized with Ava I suggesting either an internal Ava I site, or regeneration of one of the Sma I /Ava I sites upon ligation of the insert to the Sma I digested pUC 8.

■ : Bam HI

● : Eco RI

★ : Hind III

▲ : Pst I


500 bp
pSG Vg 1.43
pSG Vg3.10
pSG Vg14.12
pSG Vg3.13
pSG Vg5.09
pSG Vg4.14
pSG Vg5.50

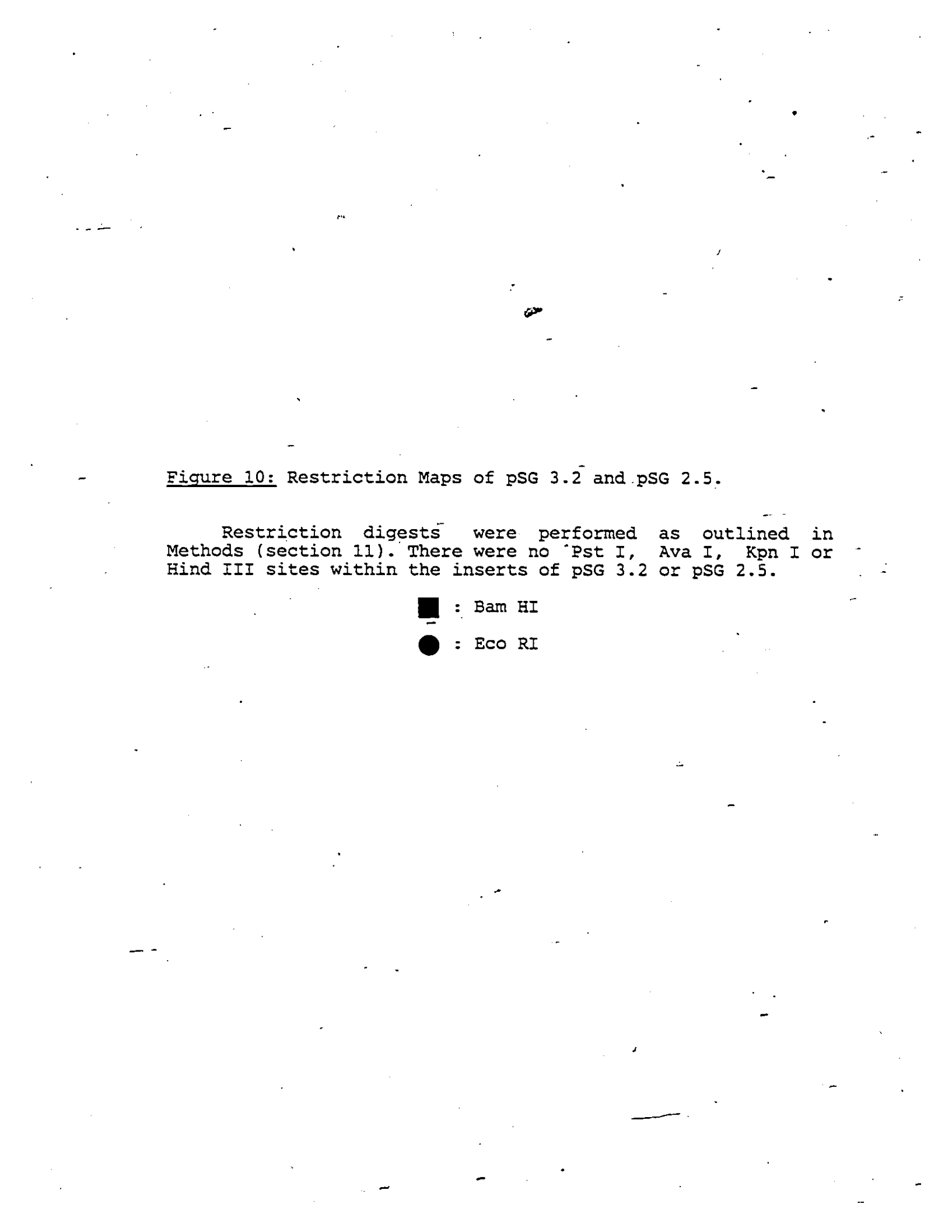


Figure 10: Restriction Maps of pSG 3.2 and pSG 2.5.

Restriction digests were performed as outlined in Methods (section 11). There were no Pst I, Ava I, Kpn I or Hind III sites within the inserts of pSG 3.2 or pSG 2.5.

■ : Bam HI

● : Eco RI

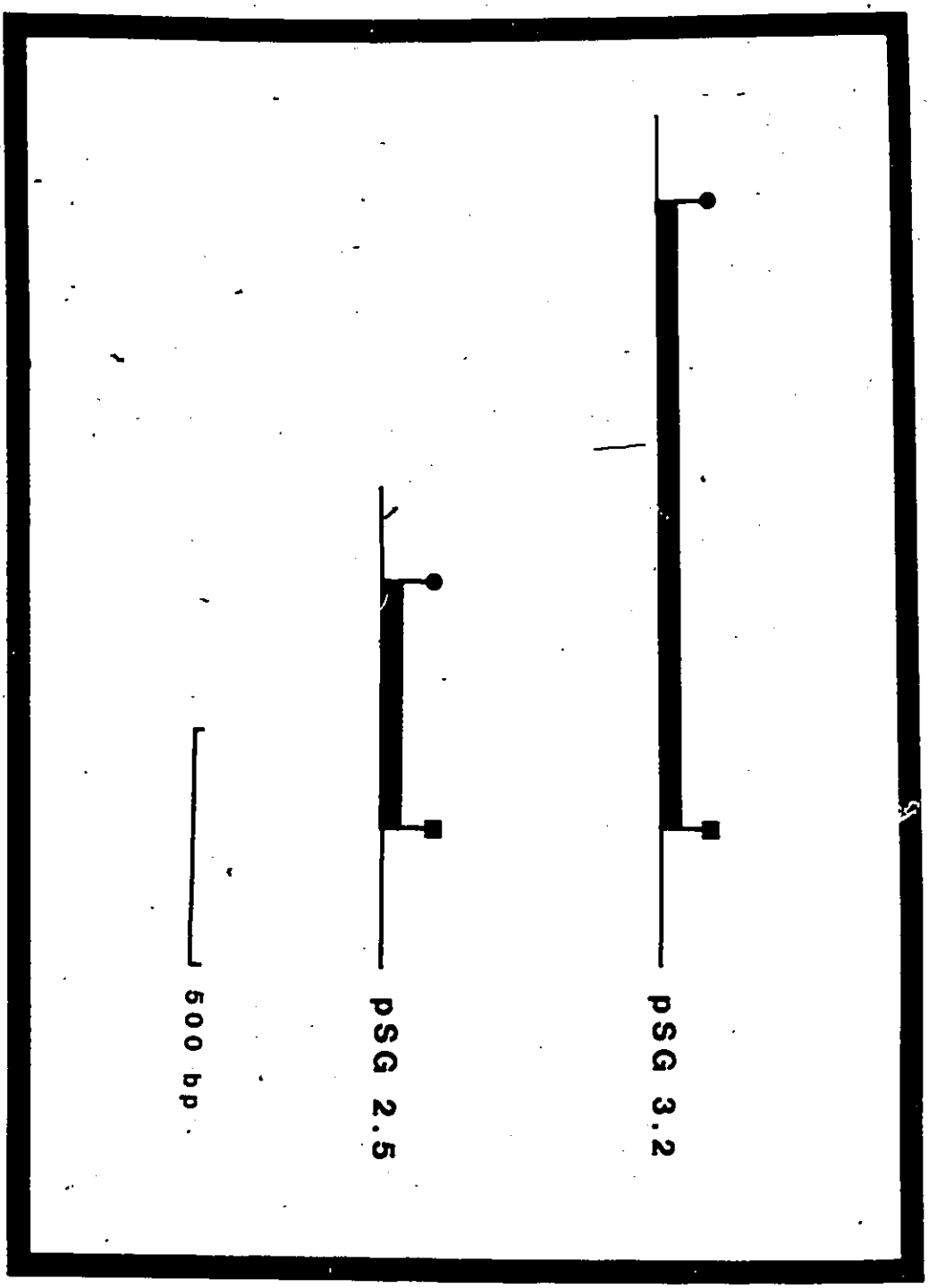

500 bp
PSG 2.5
PSG 3.2

TABLE 1: Summary of the total insert size of characterized plasmids.

SUMMARY OF CHARACTERIZED PLASMIDS

PLASMID	INSERT SIZE (bp)	mRNA SPECIES (nucleotides)	% MATURE mRNA
pSG Vg 1.43	340	7000	5 %
pSG Vg 3.10	1350	7000	19 %
pSG Vg 14.12	1690	7000	24 %
pSG Vg 3.13	1920	7000	27 %
pSG Vg 5.09	2030	7000	29 %
pSG Vg 5.50	2120	7000	30 %
pSG Vg 4.14	2500	7000	36 %
pSG 3.2	1330	3200	42 %
pSG 2.5	530	2500	21 %

not been firmly established, but it could represent either a clone specific for a different region of the same RNA, or a clone specific for a different Vg mRNA.

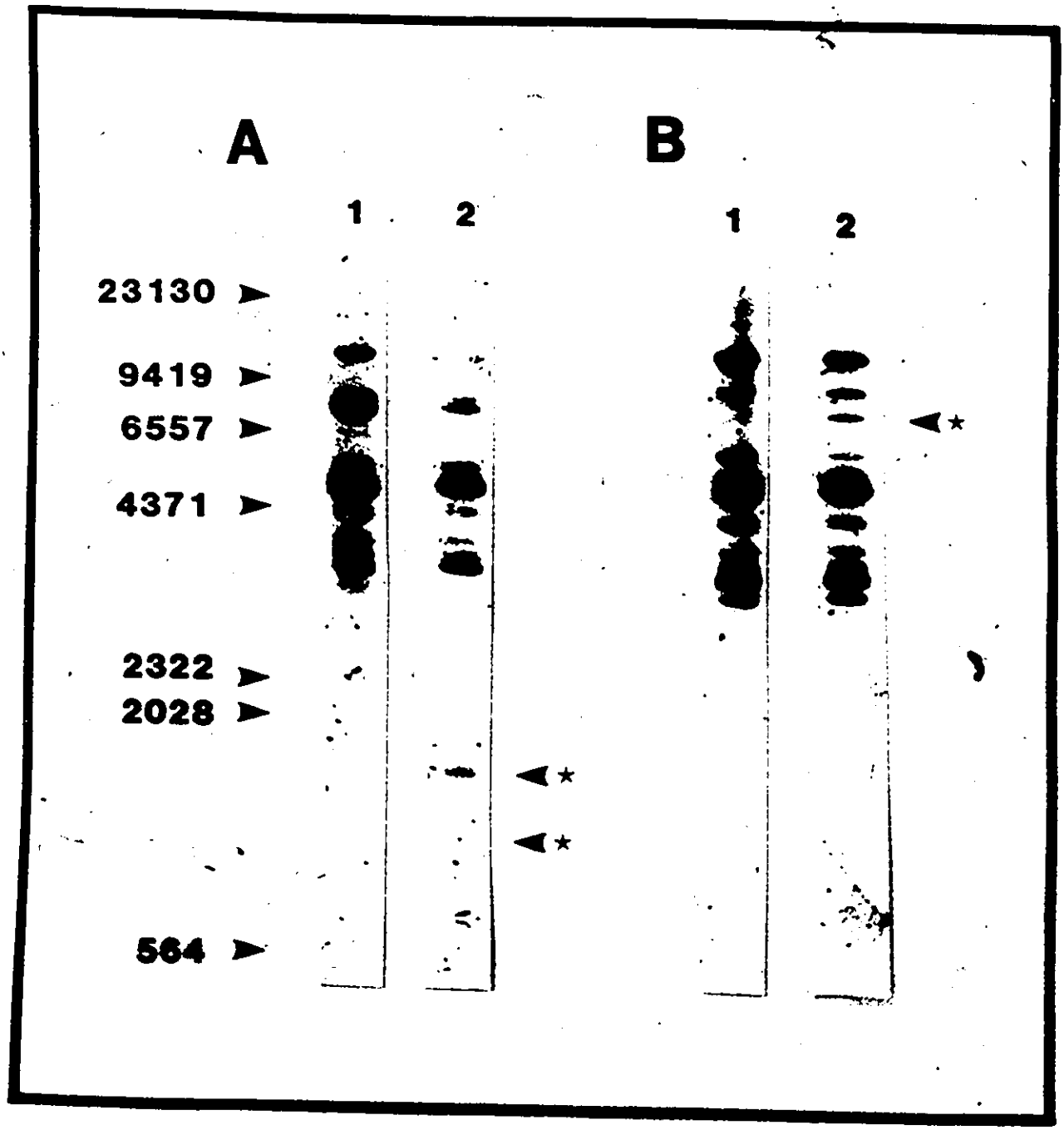
The Bam HI-Eco RI digestion of the two constitutively expressed cDNA clones, pSG 3.2 and pSG 2.5, is shown in figure 8-B. pSG 3.2 has an insert size of 1330 base pairs, or 41% of the mature sequence. The cDNA insert in pSG 2.5 is 530 base pairs in length corresponding to 21% of the mature message (Table 1). The restriction maps for these two cDNA clones are shown in figure 10. There are no internal Bam HI, Eco RI, Pst I, Ava I, Hind III or Kpn I restriction sites in either of the inserts of these two clones, however pSG 3.2 does contain several Hae III sites, the position of which are still to be determined.

(5) Southern analysis

In an attempt to determine the relationship between pSG Vg 5.50 and the other Vg plasmids, Southern analysis was performed on Hind III and Bam HI digests of female trout liver DNA (figure 11). Except for a number of minor differences, the hybridization pattern of [^{32}P]-labeled pSG Vg 5.50 (lane 1) and [^{32}P]-labeled pSG Vg 5.09 (lane 2) to Hind III digests of female trout DNA (panel A) is

FIGURE 11: Southern analysis of trout DNA using [^{32}P]-labeled pSG Vg 5.09 and pSG Vg 5.50 as probes.

Duplicate lanes of 10 µg of Hind III digested (panel A) and Bam HI digested (panel B) female trout liver DNA was electrophoresed on a 0.8% Tris-acetate/agarose gel at 40 volts for 16 hours. After denaturation the DNA was blotted onto nylon membranes and hybridized to [^{32}P]-labeled pSG Vg5.09 (lane 1) or pSG Vg 5.50 (lane 2), with an average specific activity of 30 x 10^6 cpm/100 ng in 10 ml of hybridization buffer. After hybridization the blots were washed and autoradiographed. The stars indicate differences in the hybridization banding patterns of pSG Vg5.09 and pSG Vg5.50.


A
B
1
2
1
2
23130
9419
6557
4371
2322
2028
564

substantially the same. When probed with pSG Vg 5.50, the large band at approximately 16 kilobase pairs is much fainter than in the lane probed with pSG Vg 5.09. This suggests that pSG Vg 5.09 may contain a larger proportion of the sequences contained in this band than pSG Vg 5.50. Two other bands of approximately 1.5 and 1.0 kilobase pairs are present only when probed with pSG 5.50. Southern analysis of the Bam HI digested DNA (panel B) shows only a single band difference (7.5 kilobase pairs) in the pattern of hybridization between the two probes. The fact that the sum of the major hybridization bands of the Hind III digested DNA is in excess of 43 kb suggests that both probes are hybridizing to two separate genes in the genome.

(B) VITELLOGENIN GENE EXPRESSION

(1) Serum estradiol levels

As described in the introduction, the synthesis of vitellogenin in the liver of egg-laying vertebrates is under the control of estradiol. We set out to characterize vitellogenin gene expression in trout because as a seasonally spawning species, rainbow trout must produce large amounts of vitellogenin over a 4-5 month period. Since the "memory effect" has been described in non-seasonal species, we wanted to determine if a seasonal species such as trout exhibits the same kinetics of induction. One of the aims of this study is to determine if a drop in serum estradiol is required to bring about maximum stimulation of vitellogenesis. To this end, we have used silastic implants containing estradiol to provide chronic stimulation of vitellogenesis. The treatment groups were characterized as follows:

(a) Primary "acute" stimulation: intraperitoneal injection of estradiol (3mg/kg body weight) in cocoa butter.

(b) Primary "chronic" stimulation: subcutaneous implantation of a silastic implant containing 20 mg of estradiol.

(c) Secondary "chronic" stimulation: intraperitoneal injection of estradiol (3mg/kg body weight) in cocoa butter 28 days prior to implantation of silastic implant containing 20 mg of estradiol.

It has been reported that the injection of estradiol in cocoa butter at a dose of 3 mg/kg body weight (primary acute stimulation), results in a large, but short lived rise in serum estradiol levels, that is sufficient to induce vitellogenesis in male rainbow trout (Maitre et al., 1985). Implantation of 3 cm silastic implants containing 20 mg estradiol either alone, or 28 days after a primary dose of estradiol, provides a continuous supply of estradiol to the serum. The levels of estradiol rise rapidly from control values and are maintained at approximately 30 ng/ml throughout the time course of the experiment (results not shown).

(2) Serum vitellogenin levels

In order to establish that these doses of estradiol are sufficient to induce vitellogenesis, we measured the serum vitellogenin levels by rocket immunoelectrophoresis. As shown in figure 12, acute primary stimulation using a single injection of estradiol results in a transient rise in serum

vitellogenin, increasing from undetectable levels on day 2 to a maximum of 2.4 µg/µl on day 9, declining to 1 µg/µl by day 12. In the primary chronic stimulation similar kinetics are observed up to day 7, but between day 7 and day 10 instead of declining, serum vitellogenin levels increase to 17 µg/µl. The levels of serum vitellogenin continue to increase after day 10 and reach approximately 19 µg/µl by day 21. In contrast to the primary chronic stimulation, there is no lag phase observed in the accumulation of vitellogenin in the serum during the secondary chronic stimulation. Serum vitellogenin begins to rise immediately after implantation, reaching a maximum of 19 µg/µl by day 7. The increases in the level of vitellogenin are similar to that seen between day 7 and 10 of the primary chronic stimulation.

(3) Liver Vg mRNA levels

To characterize the early events of vitellogenesis and to determine the extent of the induction of Vg mRNA, total RNA samples from the livers of stimulated trout were analyzed by Northern analysis. Figure 13 shows the hybridization of [^{32}P]-labeled pSG Vg 5.09 to total RNA from primary chronic estradiol stimulated trout. The hybridization signal of the [^{32}P]-labeled probe is visible by day 3 and

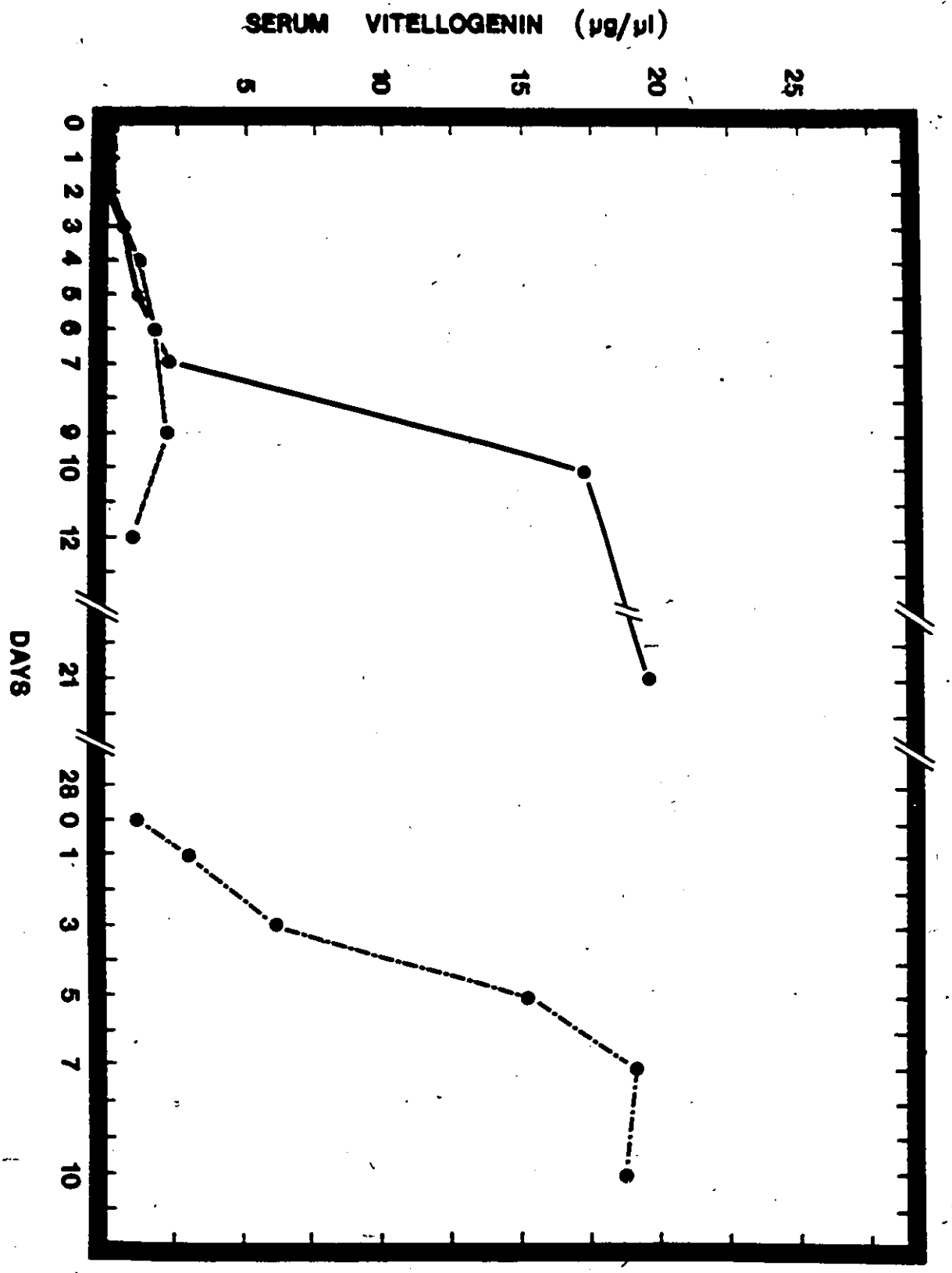
FIGURE 12: Serum vitellogenin levels in estradiol treated male rainbow trout.

Serum vitellogenin was measured by rocket immunoelectrophoresis using anti-trout Vg antibodies and purified vitellogenin as a standard (see Methods section 4). Each point represents the average value of two fish. Serum levels of individual fish varied by no more than 10% of mean values, except for day 21 of the primary chronic stimulation which varied by 14%.

●– – – – – – – – – ● : primary acute stimulation

●———————● : primary chronic stimulation

●– – –·– – –·– – – ● : secondary chronic stimulation.


SERUM VITELLOGENIN (µg/µl)
5
10
15
20
25
DAYS
0 1 2 3 4 5 6 7 9 10 12 21 28 0 1 3 5 7 10

increases to its maximum intensity by day 10. Although these results show a massive induction of Vg mRNA, they are difficult to analyze on the basis of scanning densitometry due to streaking and the necessity of obtaining signal in the linear exposure range of the autoradiogram. Therefore, the steady-state levels of Vg mRNA were measured using a dot blot assay (Methods, section 13). As shown in figure 14, primary acute stimulation with estradiol produces a transient rise in Vg mRNA, reaching a maximum of 260 ppm of total RNA on day 2, before dropping to undetectable levels by day 6. When silastic implants are used for primary stimulation, there is an initial lag of 2 days, after which the levels of Vg mRNA rise slowly to 520 ppm of total RNA on day 7. By day 10, these levels increase to 2750 ppm of the total RNA. This is five-fold higher than the levels seen at day 7 and a full 12-fold higher than maximum levels of Vg mRNA achieved by a single injection of estradiol. Between days 10 and 21 of the primary chronic stimulation levels of Vg mRNA drop by about half to 1450 ppm. Secondary stimulation with silastic implants containing estradiol results in a more rapid accumulation of the Vg mRNA. By day 1 the level of Vg mRNA are 500 ppm of total RNA, and rise steadily to 1200 ppm over the next 9 days. This is only half the maximum level observed with the primary stimulation using implants. Since both the primary and the secondary

FIGURE 13: Northern analysis of the induction of Vg mRNA during primary chronic stimulation.

Total RNA samples (10 µg/lane) extracted from the liver on different days after primary chronic stimulation were glyoxylated and run on a 1.5 % agarose gel in a 10 mM phosphate buffer pH 6.8 (see Methods section 9). After electrophoresis the RNA was blotted onto a nylon membrane and hybridized to [^{32}P]-labeled pSG Vg5.09 (20 x 10^6 cpm/200 ng). The membrane was then washed and autoradiographed. The positions of the 28S, 18S, and 5.8S rRNA subunits were determined from a set of duplicate lanes which were stained with ethidium bromide and photographed.

lane 1: 10 µg total RNA from day 0
lane 2: 10 µg total RNA from day 3
lane 3: 10 µg total RNA from day 5
lane 4: 10 µg total RNA from day 7
lane 5: 10 µg total RNA from day 10

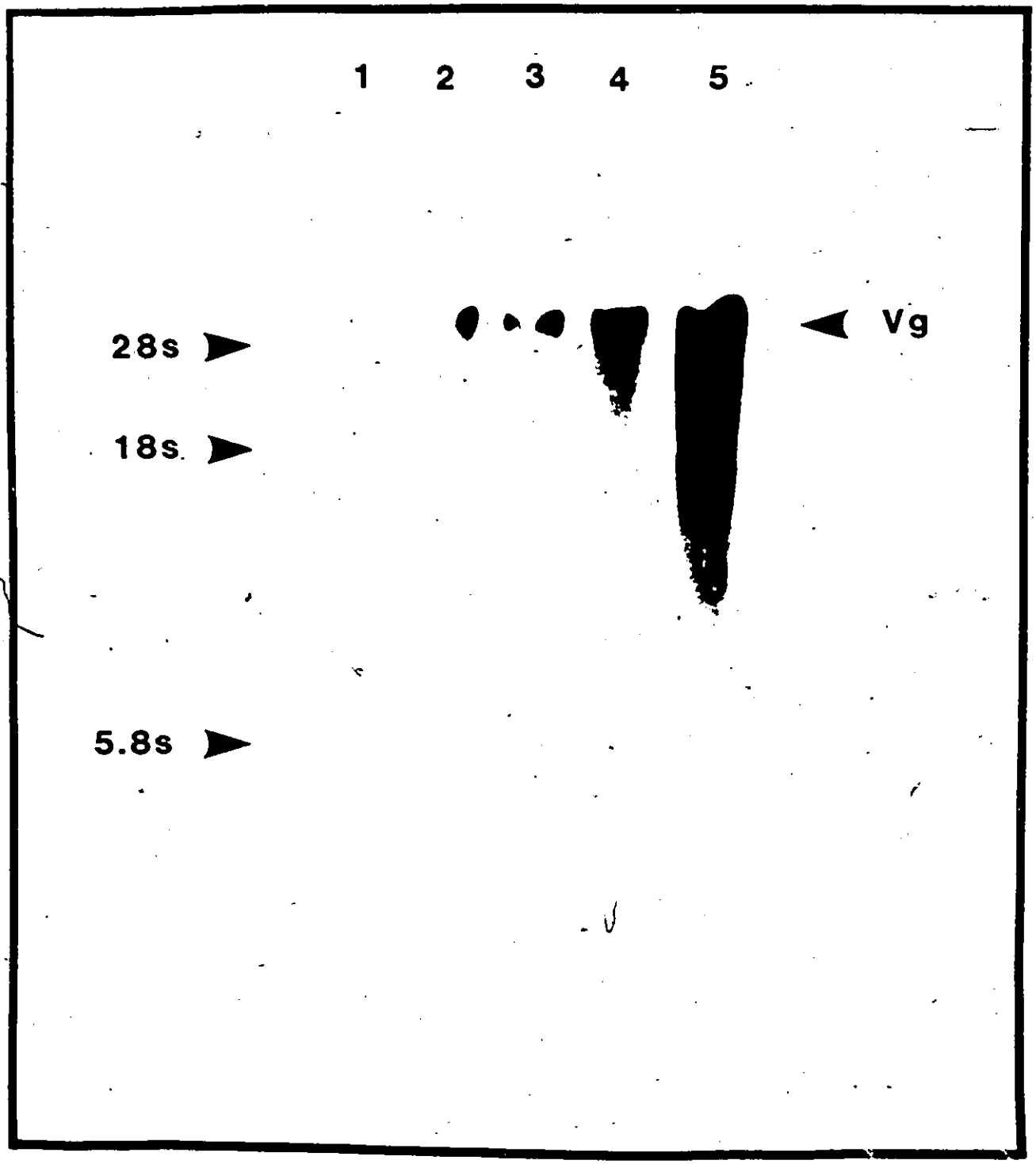
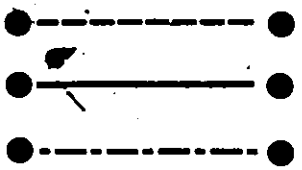

1
2
3
4
5
Vg
28s
18s
5.8s

FIGURE 14: Steady-state levels of Vg mRNA in the liver of estradiol treated male rainbow trout.

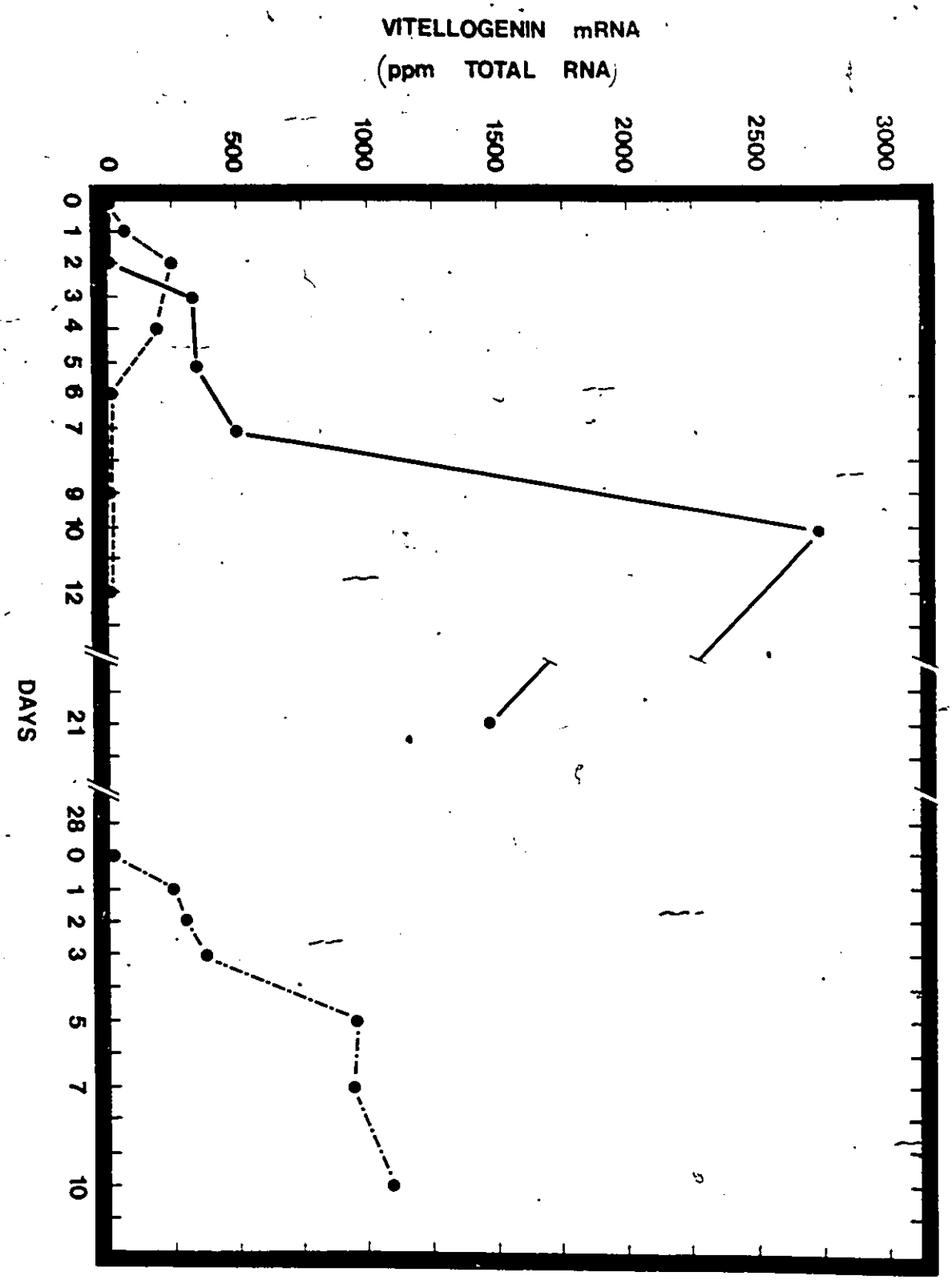
Total RNA samples extracted from the livers of stimulated trout were glyoxylated, dot blotted onto nylon memebranes, and hybridized to [^{32}P]-labeled pSG Vg 5.09 (50×10^6 cpm/200 ng plasmid). Poly(A)$^+$ RNA from a stimulated male was used as a standard and yeast total RNA to determine non-specific binding. Non-specific counts were subtracted from sample counts and then compared to the standards (see Methods section 13). Each point represents the mean value for two fish. The range of values for individual trout varied by no more than 10% of the mean.



: primary acute stimulation

: primary chronic stimulation

: secondary chronic stimulation.

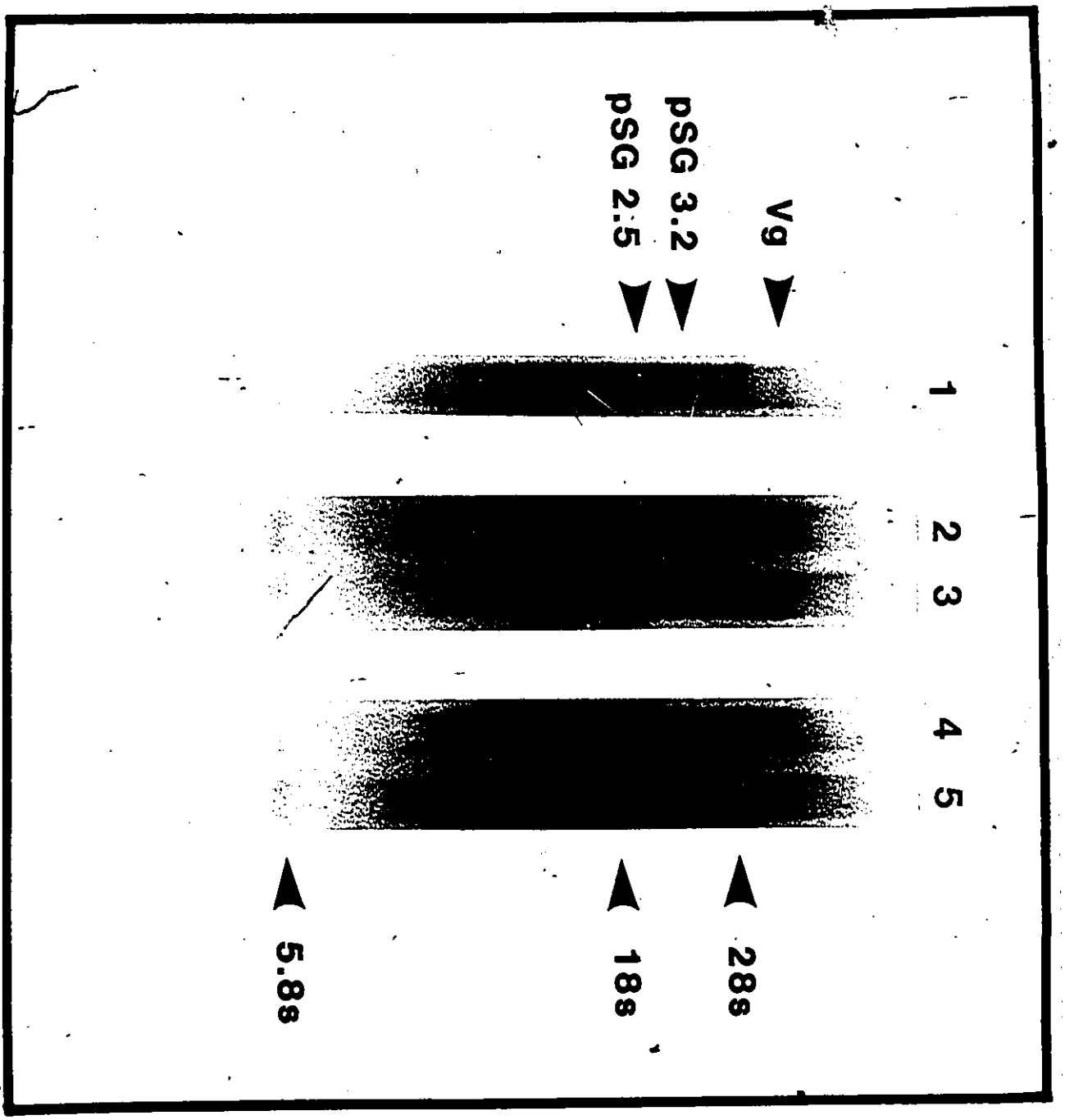

VITELLOGENIN mRNA
(ppm TOTAL RNA)
0
500
1000
1500
2000
2500
3000
DAYS
0 1 2 3 4 5 6 7 9 10 12 21 28 0 1 2 3 5 7 10

stimulations with silastic implants result in the same estradiol and vitellogenin serum concentrations this result is somewhat surprising.

To determine if there is a two-fold difference in the maximum levels of Vg mRNA, we isolated poly(A)$^+$ RNA from primary and secondary chronic stimulations. Figure 15 shows an analytical agarose gel of poly(A)$^+$ RNA from control, primary and secondary chronic stimulations. Densiometric scans of the negatives show that Vg mRNA makes up about 13% of all the messages ~~of~~ the poly (A)$^+$ RNA of day 10 (primary chronic stimulation) and about 10.5% of day 5 (secondary chronic stimulation). Vg mRNA therefore represents approximately the same level of the mRNA in both the primary and secondary response. There are two possible explanations for the apparent discrepancy in the differences between the two methods. Firstly, there may be a loss of Vg mRNA during oligo(dT)-cellulose chromatography of the day 10 primary chronic samples. Secondly, since estradiol is known to induce the synthesis of rRNA in other species (Lewis et al., 1976; Bast et al., 1977), there may be differences in the ribosomal RNA content of the total RNA sample between the primary and secondary chronic stimulations. Since the

FIGURE 15: Analytical gel electrophoresis of Poly(A)$^+$ RNA from control and estradiol stimulated male rainbow trout.

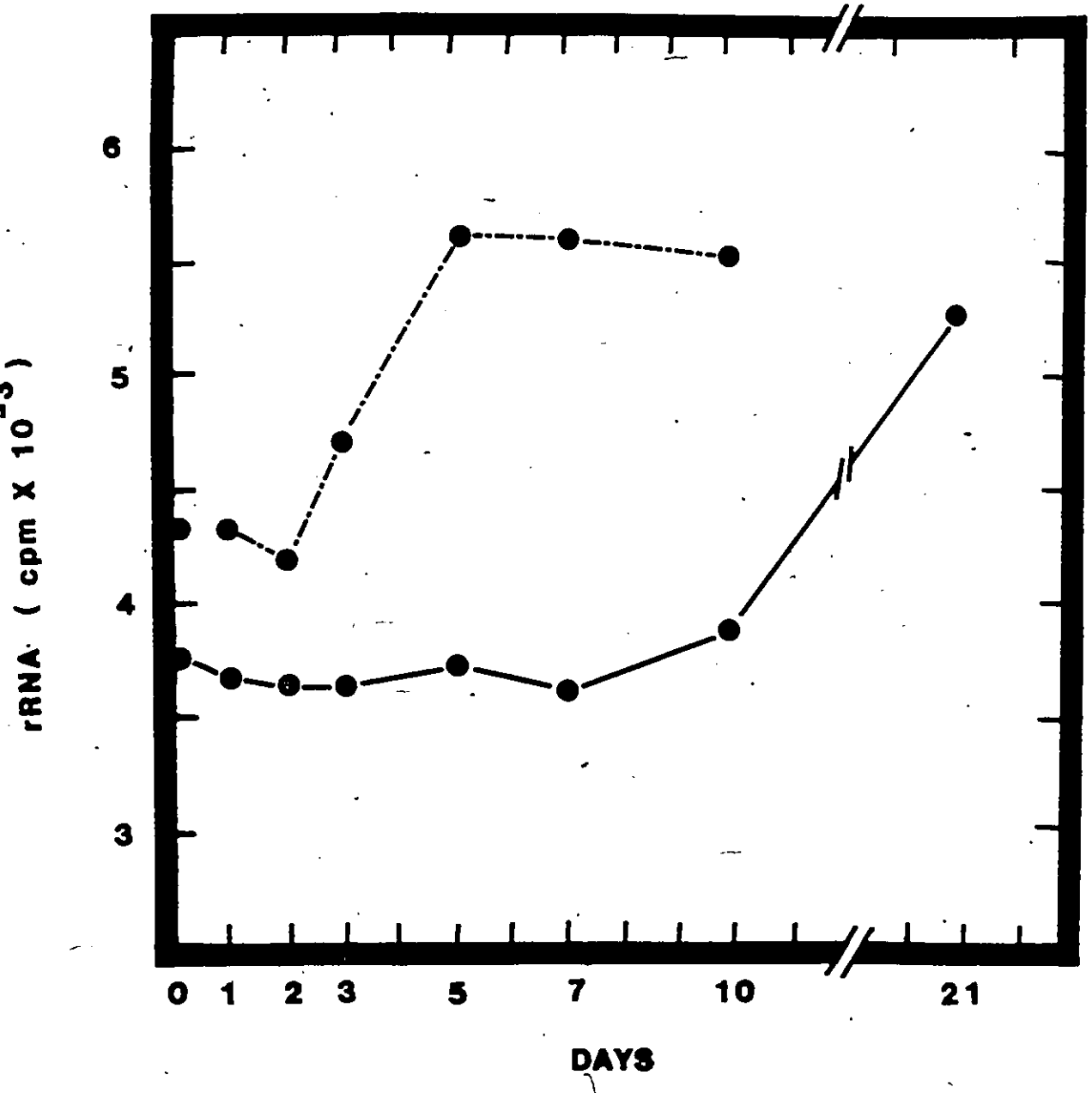
10 µg of poly(A)$^+$ RNA from control (lane 1), day 10 primary chronic stimulation (lanes 2 & 3), and day 5 secondary chronic stimulation (lanes 4 & 5) were glyoxylated and run on a 1.5% agarose gel in 10 mM. phosphate buffer pH 6.8 for 4 hours at 80 volts. After electrophoresis the gel was denatured, stained with ethidium bromide and photographed by 306 nm U.V. transillumination using an orange filter.


Vg
pSG 3.2
pSG 2.5
1
2
3
4
5
28s
18s
5.8s

selective loss of Vg mRNA during oligo(dT)-cellulose chromatography is unlikely we decided to measure the effects of estradiol on ribosomal RNA levels.

(4) Changes in ribosomal RNA content

Ribosomal RNA makes up a significant proportion of the total RNA. A several-fold increase in the level of rRNA in the cell effectively reduces the concentration of the other RNA species. Figure 16 summarizes the results of the rRNA dot blot experiments. Equal amounts of total RNA were dot blotted onto nylon membranes and hybridized to isolated 18S rRNA which had been alkali digested and 5'-end labeled using gamma-[^{32}P]-ATP (Methods, section 15). Despite several attempts it was not possible to produce a standard curve of cpm bound versus nanograms rRNA which was linear within the range necessary for the assay (4-10 µg/sample). We attributed this problem to the unusually high concentration of the [^{32}P]-labeled rRNA used in the hybridization assay, which effectively altered the kinetics of hybridization since the RNA bound to the filter was not the only "driver" in the hybridization reaction. The level of rRNA in the samples could not be quantified due to the inherent problems associated with using [^{32}P]-5'-end labeled 18S rRNA as a probe (see methods, section 15). However, the amount of


6
5
4
3
rRNA (cpm X 10^{-3})
0 1 2 3 5 7 10 21
DAYS

[^{32}P]-labeled probe hybridized does give an indication of the changes in the steady-state levels of 18S and 28S rRNA, since all the hybridizations were performed at the same time with the same probe.

During primary chronic stimulation the levels of [^{32}P]-labeled 18S rRNA bound to the total RNA remains constant at approximately 3,750 cpm until day 10, after which levels increase to a maximum of 5,250 cpm by day 21. In contrast, secondary stimulation results in a much more rapid accumulation of 18S and 28S rRNA. The levels of [^{32}P]-labeled probe hybridizing remains between 4,400 and 4,300 cpm until day 2, after which the levels increase to reach a maximum of 5,650 cpm by day 5. The relatively high levels of probe bound is maintained even to day 10. These results indicate that 18S and 28S rRNA occupies a greater proportion of the total RNA on day 5 (secondary chronic stimulation) than the day 10 (primary chronic stimulation). Since Vg mRNA represents approximately the same proportion of the poly(A)$^+$ RNA (figure 15), the apparent differences in the maximum levels of Vg mRNA (when expressed as ppm total RNA) between the primary chronic and the secondary chronic stimulation must be due to the differences in the rRNA content.

(C) OTHER ESTRADIOL-INDUCED CHANGES IN LIVER

(1) Changes in the liver weight/body weight ratios

The effect of estradiol on the liver is not restricted to the induction of vitellogenin and the changes in rRNA content shown above. As discussed in the introduction, estradiol in known to have a marked effect on the liver weight and morphology. Figure 17 shows the differences in the liver weight/body weight (LW/BW) ratios in response to the three treatment regimes. Acute primary estradiol stimulation shows no significant change in the liver to body weight ratio over the study period. In contrast both the primary and secondary stimulations using silastic implants show nearly a two fold increase in the LW/BW ratio over the same time period. These results indicate that the duration of the estradiol dose must be sufficiently long to induce the changes in the liver characterized by the gain in weight. The estradiol-induced changes in the LW/BW ratios plateau by day 10 of the primary chronic stimulation. By comparison, the changes in the LW/BW ratios during secondary chronic stimulation plateau by day 5 of the treatment. These results correlate with our earlier findings, in that the maximum accumulation of vitellogenin in the serum occurs when the changes in LW/BW ratios are complete (figure 12). The

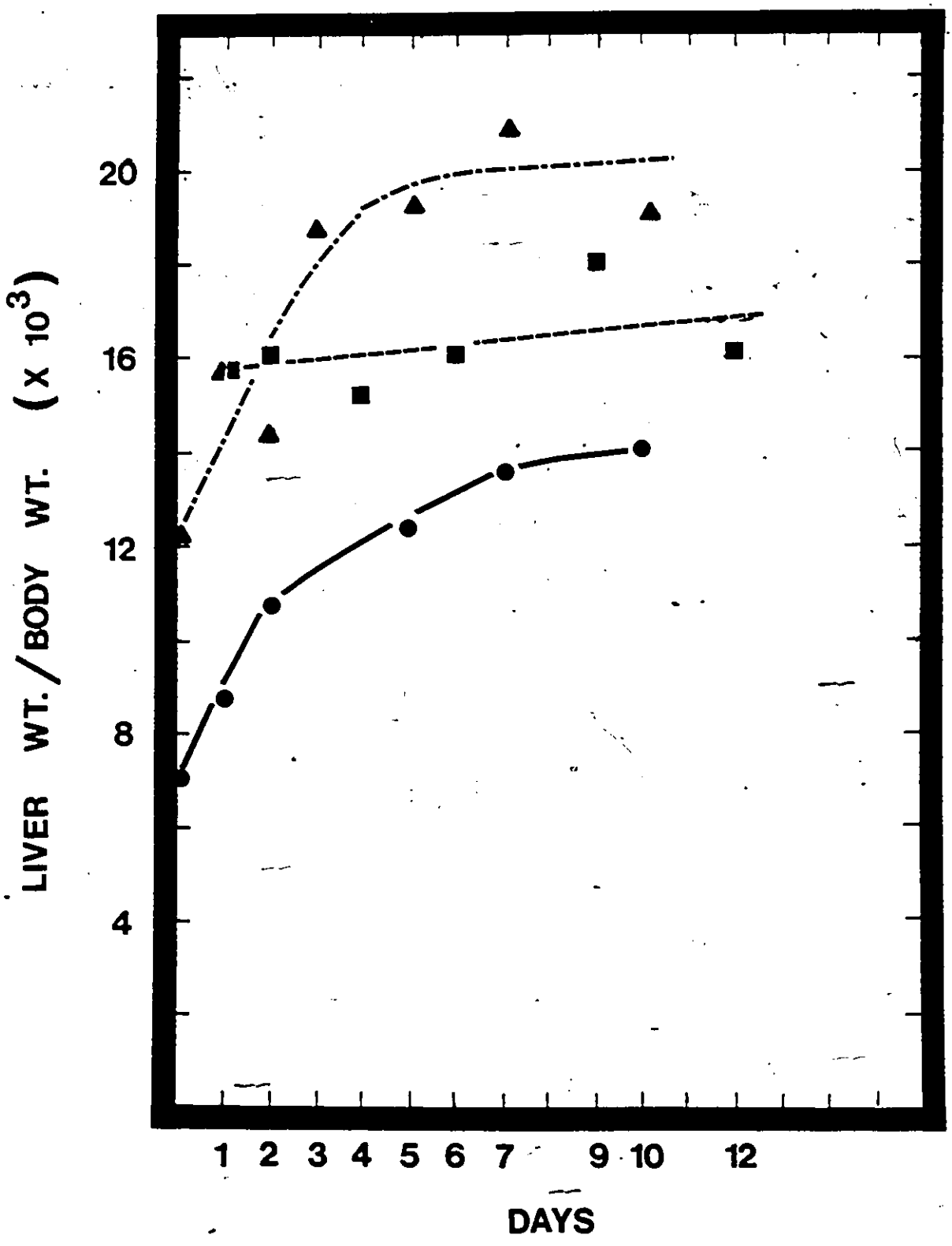
FIGURE 17: Liver/Body weight ratios of estradiol treated male rainbow trout.

Individual trout were weighted and the livers excised and weighted. Paired liver weight/body weight ratios were then determined.

■———————■ : primary acute stimulation

●———————● : primary chronic stimulation

▲-·-·-·-·-▲ : secondary chronic stimulation.


LIVER WT./BODY WT. (X 10³)
20
16
12
8
4
1 2 3 4 5 6 7 9 10 12
DAYS

differences in the initial LW/BW ratios of the primary and secondary chronic stimulations may be a reflection of the growth of the trout over the 28 days between the injection of estradiol and the implantation of the estradiol implants.

(2) In vitro translation products

The changes which occur in trout liver upon stimulation with estradiol are complex. In order to determine the scope of these changes, poly(A)$^+$ RNA from control and stimulated trout was translated in an in vitro rabbit reticulocyte translation system using [^{35}S]-methionine to label nascent proteins. Figures 18 and 19 represent the autoradiograms of two dimensional gels of the in vitro translation products of mRNA extracted from control and stimulated trout. The predominant vitellogenin band (M.W. 160,000 daltons, pI 8.2), is present in the stimulated trout, but completely absent in the unstimulated animal. A second protein (M.W. 65,000 daltons, pI 8.1) probably corresponds to the translation product of the small estrogen-inducible message described by Chen (1983). In addition to the estradiol-induced messages there are a number of translation products which are greatly reduced upon stimulation with estradiol. These include a group of translation products having a molecular weight of between 75,000 and 86,000 daltons and an isoelectric point

FIGURE 18: Two dimensional gel electrophoresis of [^{35}S]-labeled *in vitro* translation products of poly(A)$^+$ RNA from the liver of an unstimulated male trout.

Poly(A)$^+$ RNA from a control male was translated in a cell-free reticulocyte *in vitro* translation system using [^{35}S]-methionine to label the synthesized proteins. 400,000 TCA-precipitable cpm were loaded onto isofocusing tube gels. A 10% polyacrylamide gel was used for the second dimension (see Methods section 16). After electrophoresis the gel was flourographed at -70°C for 5 days. Bio-Rad Low Molecular Weight SDS-PAGE markers were used as molecular weight standards.

Molecular weight standards:

92.5 kDa :	phosphorylase B
66.2 kDa :	bovine serum albumin
45.0 kDa :	ovalbumin
31.0 kDa :	carbonic anhydrase
21.5 kDa :	soybean trypsin inhibitor

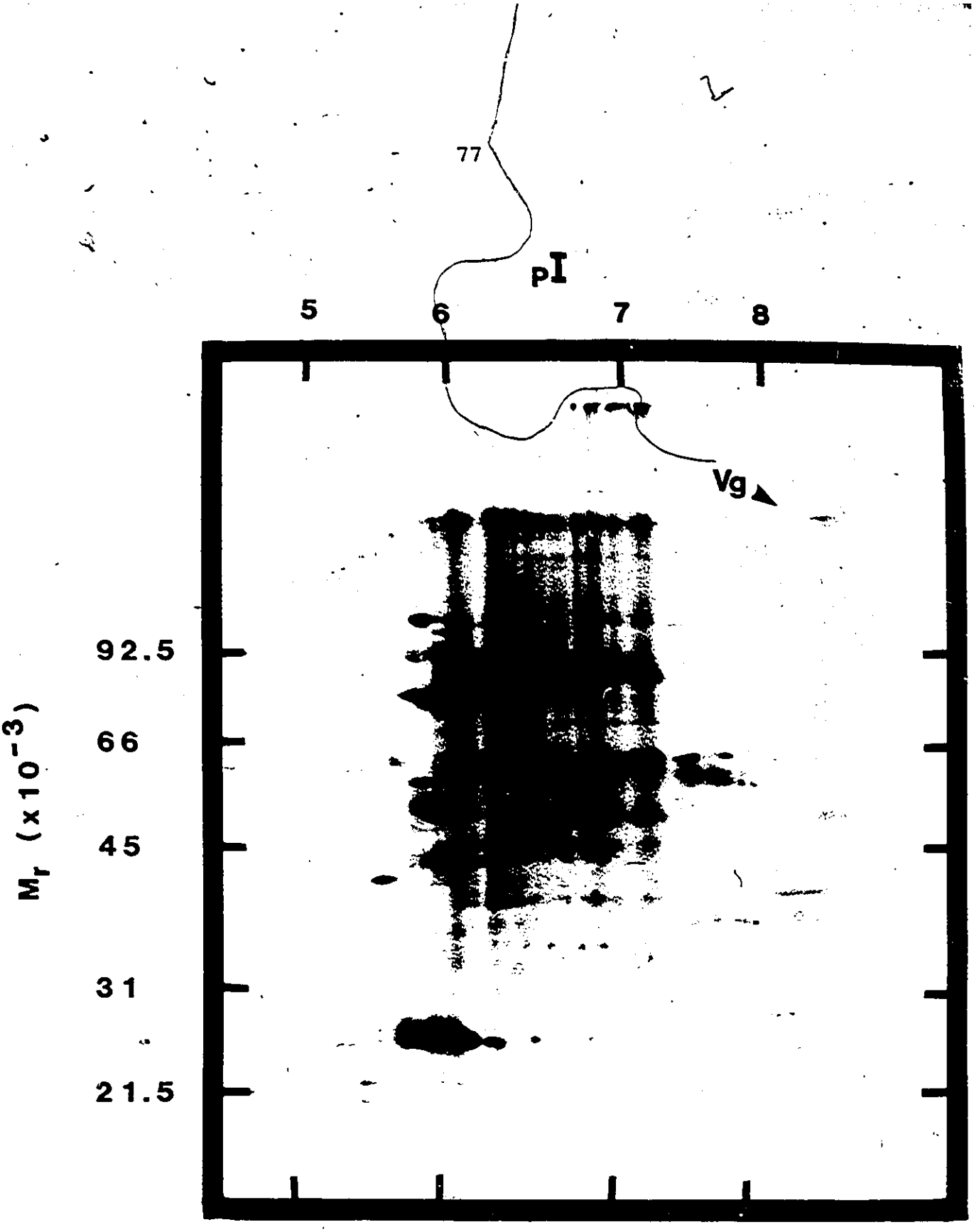
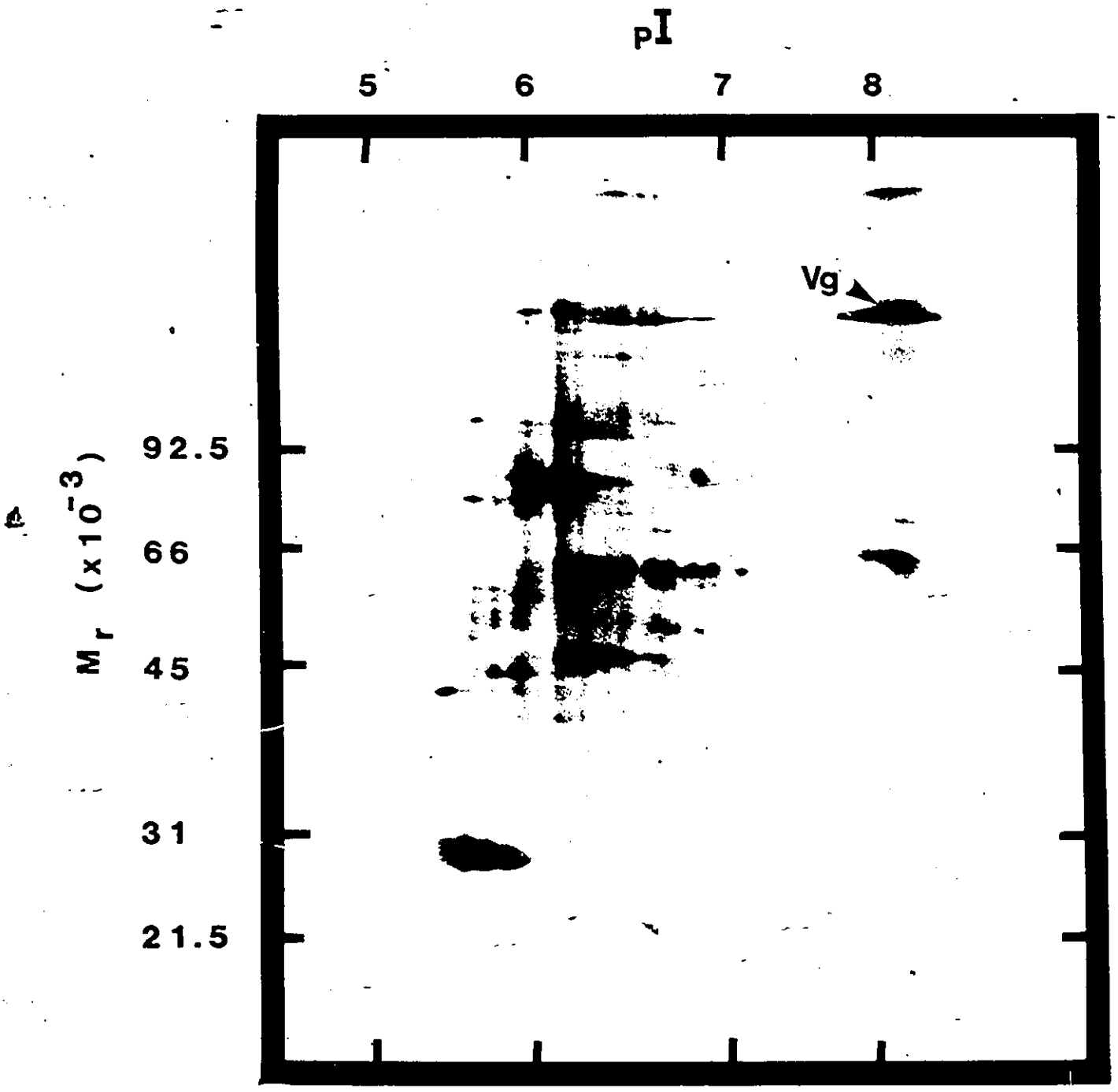

77
pI
5
6
7
8
Vg
92.5
66
45
31
21.5
M_r ($\times 10^{-3}$)

FIGURE 19: Two dimensional gel electrophoresis of [^{35}S]-labeled *in vitro* translation products of poly(A)$^+$ RNA from the liver of an estradiol stimulated male trout.

Poly(A)$^+$ RNA from a stimulated male trout was translated in a cell-free reticulocyte lysate *in vitro* translation system using [^{35}S]-methionine to label synthesized proteins. 400,000 TCA-precipitable cpm were loaded onto isofocusing tube gels. A 10% polyacrylamide gel was used for the second dimension (see Methods section 16). After electrophoresis the gel was flourographed at -70°C for 5 days. Bio-Rad Low Molecular Weight SDS-PAGE markers were used as molecular weight standards.

Molecular weight standards:

92.5 kDa :	phosphorylase B
66.2 kDa :	bovine serum albumin
45.0 kDa :	ovalbumin
31.0 kDa :	carbonic anhydrase
21.5 kDa :	soybean trypsin inhibitor


pI
5
6
7
8
Vg
M_r ($\times 10^{-3}$)
92.5
66
45
31
21.5

between 6 and 6.2. These results suggest that in addition to stimulating the synthesis of Vg mRNA, it also decreases the levels of other liver mRNAs.

(3) Changes in pSG 3.2 and pSG 2.5 mRNA levels

Because the changes in liver gene expression upon stimulation with estradiol are complex, we decided to examine the expression of two liver messages which are expressed in the male but which appear to be estrogen sensitive. We chose to study the messages for pSG 3.2 and pSG 2.5 because the levels of their messages appeared to be altered upon treatment with estradiol (figure 7). Hybrid selected translation has shown that pSG 3.2 codes for a protein of approximately 100,000 daltons and pSG 2.5 codes for a protein of approximately 83,000 daltons (Dr. K. Le Guellec, personal communication). Figure 20 shows the changes in pSG 3.2 and pSG 2.5 mRNA levels during primary acute stimulation. At day 0 both messages are present at appreciable levels, pSG 3.2 mRNA levels are 1000 ppm of total RNA and pSG 2.5 mRNA levels are approximately 600 ppm. After estradiol administration the level of pSG 3.2 and pSG 2.5 mRNAs rise to approximately three times their original values to 3100 ppm (day 2) and 2400 ppm (day 9) respectively. The increases in both messages are not sustained, and fall

FIGURE 20: Steady state levels of pSG 3.2, pSG 2.5 and Vg mRNA during primary acute stimulation.

Total RNA samples extracted from the livers of male trout from the primary acute treatment group were dot blotted onto nylon membranes and hybridized to [^{32}P]-labeled pSG 3.2 or pSG 2.5. Poly(A$^+$) RNA from a control male was used as a standard for both messages. Yeast total RNA was used to determine non-specific binding. After hybridization the filters were washed, and the amount of radioactivity bound to each of the dots was measured. Non-specific cpm were subtracted from sample cpm and compared to the standards (see Methods section 13). Since the same total RNA samples were also used to determine Vg mRNA levels (figure 15), the levels of Vg mRNA are reproduced for comparison purposes. Each point represents the mean value for two fish. The range of values of individual fish for Vg, pSG 3.2 and pSG 2.5 mRNAs were within 12% of the mean.

■--------■ : pSG 3.2 mRNA

▲--------▲ : pSG 2.5 mRNA

●--------● : Vg mRNA

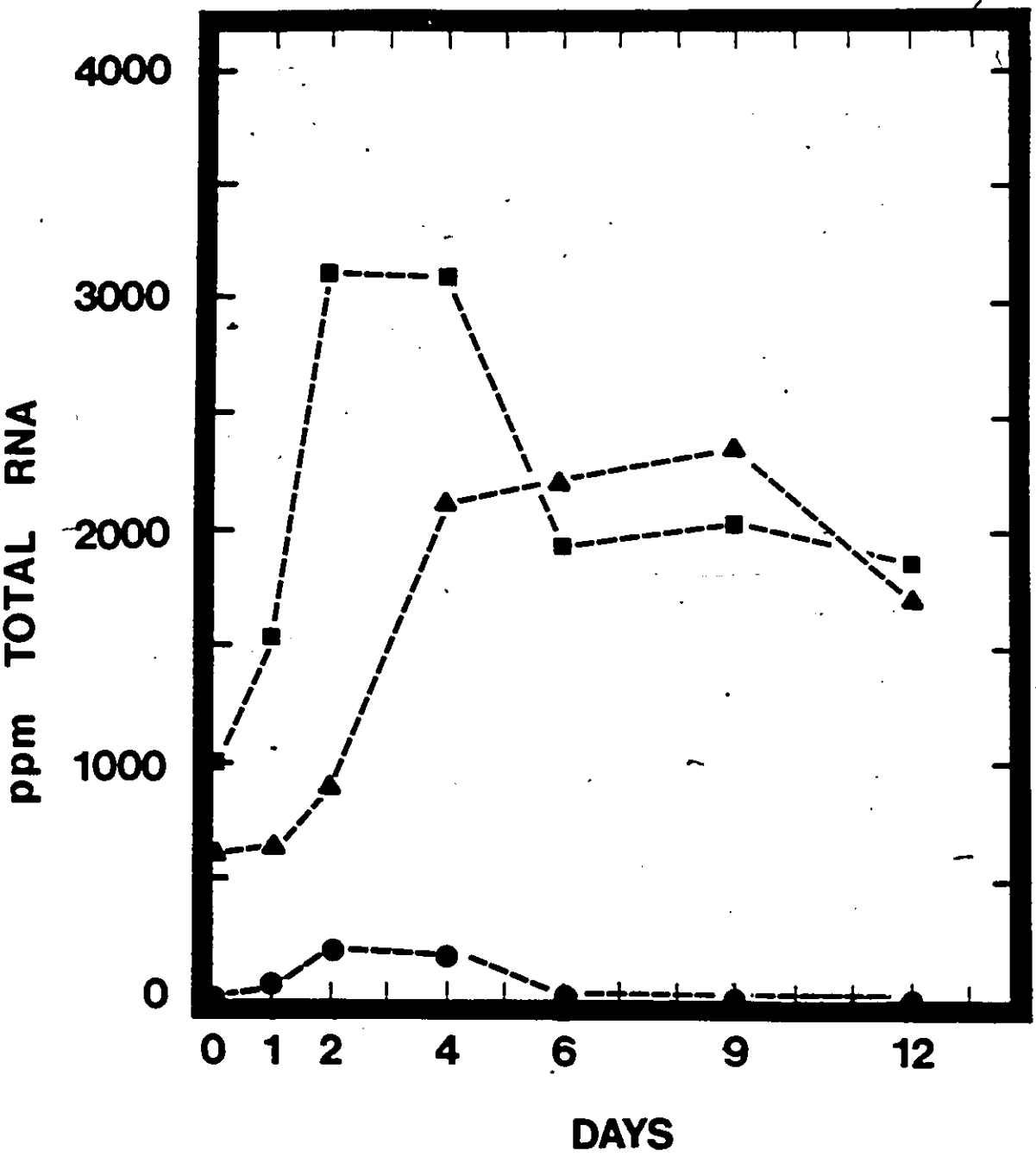

4000
3000
2000
1000
0
ppm TOTAL RNA
0 1 2 4 6 9 12
DAYS

FIGURE 21: Steady state levels of pSG 3.2 mRNA and pSG 2.5 mRNA during primary chronic stimulation.

Total RNA samples extracted from the livers of male trout from the primary chronic treatment group were dot blotted onto nylon membranes and hybridized to [^{32}P]-labeled pSG 3.2 or pSG 2.5 (see Methods section 13). Since the same total RNA samples were also used to determine Vg mRNA levels (figure 15), the levels of Vg mRNA are reproduced for comparison purposes. Each point represents the mean value for two fish. The range of values of individual fish for Vg, pSG 3.2 and pSG 2.5 mRNAs were within 12% of the mean.

■——■: pSG 3.2 mRNA

▲——▲: pSG 2.5 mRNA

●——●: Vg mRNA

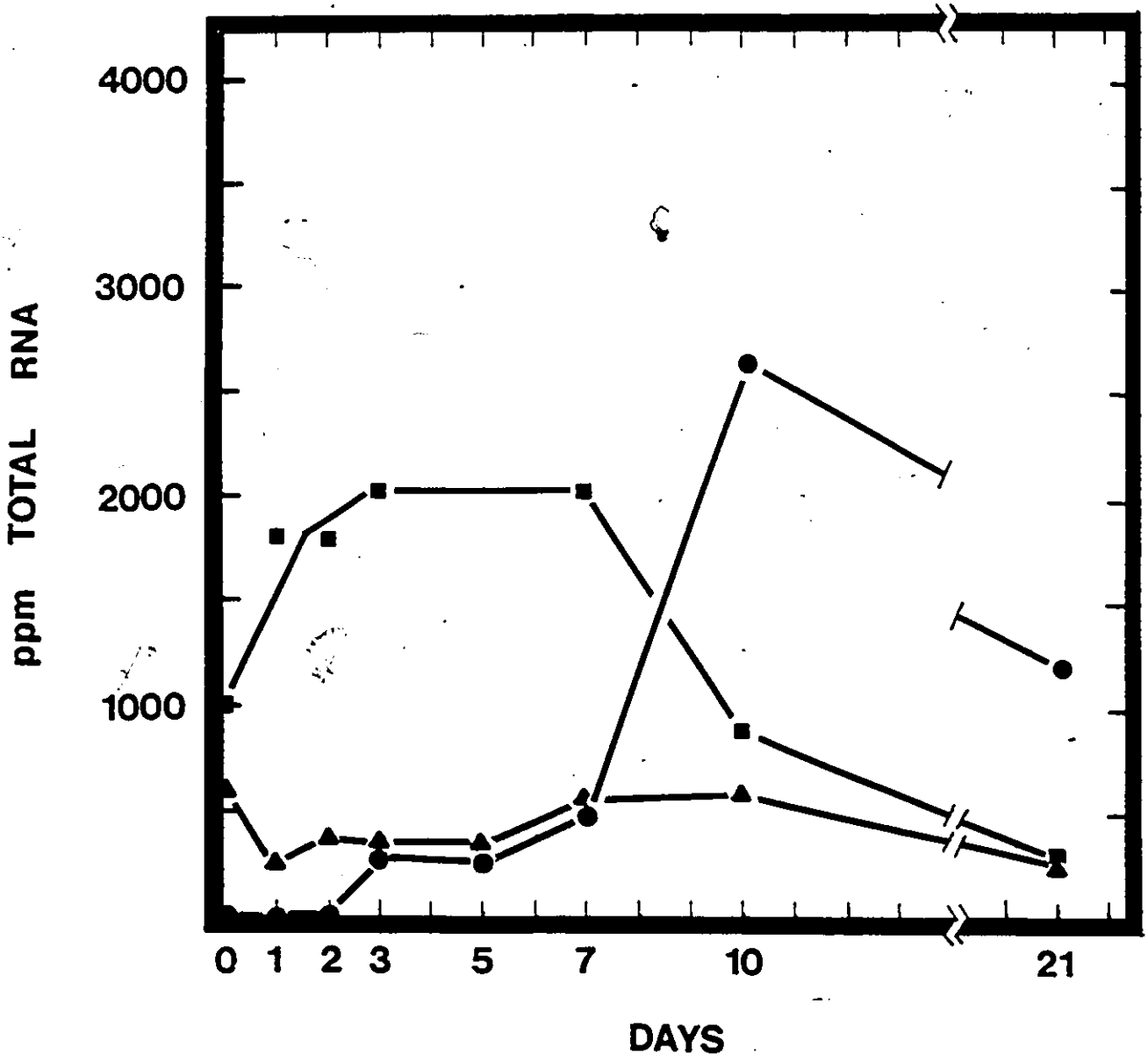

4000
3000
2000
1000
ppm TOTAL RNA
0 1 2 3 5 7 10 21
DAYS

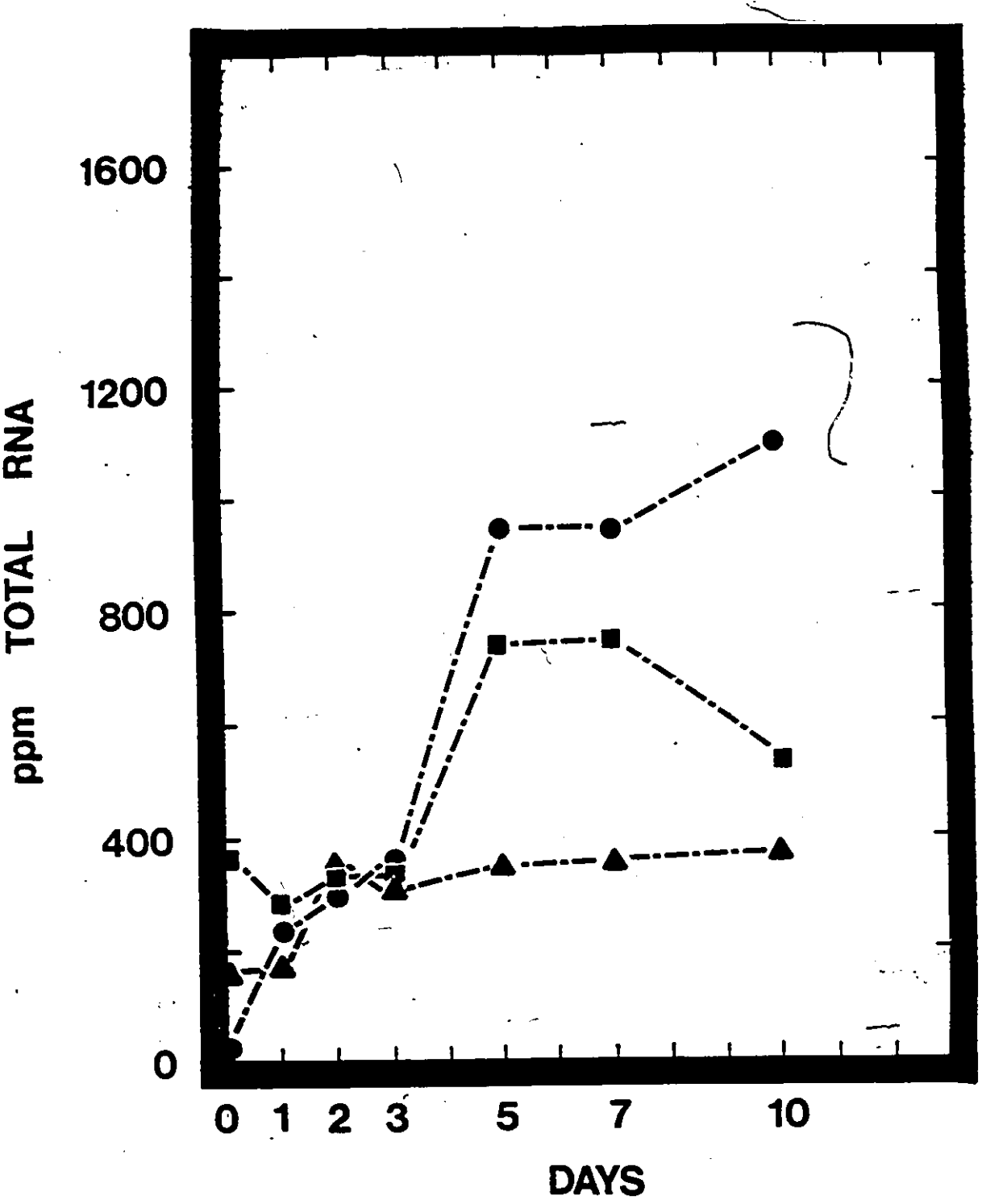
FIGURE 22: Steady state levels of pSG 3.2 mRNA and pSG 2.5 mRNA during secondary chronic stimulation.

Total RNA samples extracted from the livers of male trout from the secondary chronic treatment group were dot blotted onto nylon membranes and hybridized to [^{32}P]-labeled pSG-3.2 or pSG 2.5 (see Methods section 13). Since the same total RNA samples were also used to determine Vg mRNA levels (figure 15), the levels of Vg mRNA are reproduced for comparison purposes. Each point represents the mean value for two fish. The range of values for Vg, pSG 3.2 and pSG 2.5 mRNAs were within 12% of the mean.

■—·—·—■ : pSG 3.2 mRNA

▲—·—·—▲ : pSG 2.5 mRNA

●—·—·—● : Vg mRNA


1600
1200
800
400
0
ppm TOTAL RNA
0 1 2 3 5 7 10
DAYS

to 1850 ppm and 1700 ppm respectively by day 12. In comparison Vg mRNA levels rise transiently and return to undetectable levels as shown previously (figure 14).

During primary chronic stimulation using silastic implants (figure 21) pSG 3.2 mRNA levels rise quickly from an initial level of 1000 ppm to 2000 ppm on day 3. These levels are not maintained and fall to 900 ppm by day 10. The drop in the level of pSG 3.2 mRNA occurs at the same time as the level of Vg mRNA is increasing at a maximum rate. In contrast to pSG 3.2 mRNA, the levels of pSG 2.5 mRNA remain relatively constant at 600 ppm of the total RNA. A further decline in the level of both messages occurs between day 10 and 21, during which time the rRNA content of the hepatocytes is increasing (figure 16).

Secondary chronic stimulation (figure 22) shows a different time course of events, the levels of pSG 3.2 mRNA increase at a much slower rate than the primary chronic stimulation. The level of pSG 3.2 mRNA remains constant at approximately 350 ppm until day 3, after which levels increase to a maximum of 760 ppm by day 5. The increase in the level of pSG 3.2 is not sustained and the levels of the message decline to 580 ppm by day 10. In contrast to the lag in pSG 3.2 mRNA accumulation, the levels of pSG 2.5 mRNA

rise from an initial value of 250 ppm to 380 ppm on day 3, after which the levels of pSG 2.5 remain relatively constant. The relatively constant level of pSG 2.5 between days two and five suggest that the rate of pSG 2.5 mRNA accumulation is the same as that of rRNA. The concentrations of pSG 3.2 and pSG 2.5 mRNAs on day 0 of the secondary chronic stimulation are approximately 40% of the primary acute stimulations. The differences between the initial values of the two treatment groups probably reflect both a decrease in the size of the poly(A)$^+$ RNA pool and increases in rRNA content which occurs during the 28 days between the primary injection of estradiol and the implantation of the silastic implants containing estradiol.

DISCUSSION

(A) Cloning and characterization of cDNA clones

In order to characterize the control of vitellogenesis in rainbow trout, it was necessary to prepare a cDNA clone library from an estradiol stimulated trout and screen the library for vitellogenin and other liver mRNAs. We choose to use the cloning procedure developed by Rutledge et al. (1986), because it has been shown to have several advantages over conventional cloning procedures. These include the use of random hexanucleotides and oligo(dT) to prime the first strand synthesis, which in conjunction with the ligation reaction, has been shown to produce large cDNA clones (Rutledge et al., 1986). The use of RNase H and DNA polymerase I during the second strand synthesis eliminates the need to use S1 nuclease which results in the loss of some of the 5'-terminal sequences of the mRNA (Sippel, Land, Lindenmaier, Nguyen-Huu, Wurtz, Timmis, Giesecke and Schultz, 1978). Finally, the size selection of the ds-cDNA prior to cloning by Sepharose 4B chromatography decreases the amount of screening necessary to select for large clones.

The screening procedure which was used to select for the vitellogenin clones is based on the observation that

vitellogenin is induced de novo by estradiol in male rainbow trout (Maitre et al., 1985). The mRNA for Vg is therefore not expressed in male trout liver. This makes it possible to use differential hybridization to identify clones specific for estradiol-induced sequences. The colonies which hybridize specifically to [^{32}P]-labeled cDNA from estradiol-stimulated male trout and not with [^{32}P]-labeled cDNA from unstimulated male trout were classified as being estradiol-induced. The vitellogenin clones were selected from the estradiol-induced clones on the basis of Northern hybridization to the 7000 nucleotide Vg mRNA (figure 8). Hybrid selected translation of Vg mRNA using pSG Vg 5.09 produces a protein which is immunoprecipitable by anti-trout vitellogenin antibodies (Dr. K. Le Guellec, personal communication).

All of the large vitellogenin clones, with the possible exception of pSG Vg 5.50, appear to be related. Restriction mapping shows that these clones have common Eco RI and Pst I restriction sites (figure 9). Since we have used both random hexanucleotides and oligo(dT) to prime the first strand synthesis, these clones could represent sequences throughout the vitellogenin message. The number of vitellogenin clones containing the 1080 bp Bam HI-Eco RI restriction fragment suggests that these clones represent sequences from the

3'-end of the same Vg mRNA. This has been confirmed recently by sequencing of these clones (Dr. K. Le Guellec, personal communication).

In contrast to the clones above, the restriction map of pSG Vg 5.50 is distinctly different and contains internal Bam HI, Hind III, Eco RI and three Pst I restriction sites (figure 9). At present the relationship between pSG Vg 5.50 and the other vitellogenin clones remains unclear. Southern analysis of Hind III and Bam HI digested genomic DNA indicates that pSG Vg 5.09 and pSG Vg 5.50 share regions of homology (figure 11). The similarity in the banding pattern can be interpreted as suggesting that these two clones represent different regions of the same mRNA. However, the fact that the sum of the lengths of the major hybridization bands in the Hind III digest is in excess of 43 kb (figure 11), suggests that these two probes are hybridizing to at least two separate genes in the genome. In Xenopus the vitellogenin genes range in size from 15.8 to 20.5 kb (Wahli & Dawid, 1980; Germond et al., 1980). Comparison of the Xenopus A2 gene with the 22 kb chicken vitellogenin II gene indicates that the number and length of exons of the vitellogenin genes is conserved during evolution (Arnberg, Meijlink, Mulder, Van Bruggen, Gruber & Ab, 1981). Since trout Vg mRNA is only a few hundred base pairs larger than

Xenopus Vg mRNA (Valotaire et al., 1984), it is not unreasonable to expect the trout vitellogenin genes to be similar in size. Sequencing of the vitellogenin cDNA clones should establish whether pSG Vg 5.50 represents a related mRNA species or a different portion of the same mRNA.

At present the identities of the proteins encoded by pSG 3.2 and pSG 2.5 has not been determined. pSG 3.2 is a single copy gene in atlantic salmon, a species closely related to rainbow trout (Dr. W. Davidson, Memorial University of Newfoundland, personal communication). The protein encoded by pSG 3.2 is thought to be approximately the same size as teleost serum albumin and studies are underway to determine if this is the case. When the sequences of these plasmids have been determined, they will be compared to liver messages from other species to determine the nature of these sequences.

(B) Gene expression during estradiol-induced vitellogenesis

The results reported in this thesis (results section B) suggest that the control of vitellogenesis in rainbow trout is similar to that previously described in Xenopus and chicken (Baker & Shapiro, 1977; Maeenpaa, 1976), in that the lag in the induction of vitellogenin observed upon primary stimulation is absent during secondary stimulation. Although vitellogenesis has been thoroughly investigated in a number of species, this is the first time the "memory effect" has been demonstrated in teleosts. This is also the first time that there has been a distinction between the response of the liver to a primary acute versus a primary chronic stimulation with estradiol.

Previous studies have shown that injection of estradiol at a dose of 3 mg/kg body weight in cocoa butter results in a large but short-lived rise in estradiol serum levels sufficient to induce vitellogenesis in adult male rainbow trout (Maitre et al., 1985). As shown in figure 12, primary acute stimulation of juvenile male trout results in a transient rise in serum vitellogenin levels. The lag in the synthesis and secretion of vitellogenin is similar to that reported by Maitre et al. (1985) for adult male trout stimulated with a much lower dose of estradiol. Adult male

trout stimulated with the same dose of estradiol show serum vitellogenin levels approximately 20-fold higher than that of juvenile trout. The differences in the responses to estradiol is probably due to the high rate of estrogen metabolism in the juvenile trout (Hansson & Rafter, 1983).

During the primary acute stimulation, serum estradiol levels begin to decline on or by day 2 (Maitre et al., 1985). The fact that Vg mRNA levels also increase transiently (figure 14), is not surprising since estradiol is required for the continued transcription and stability of Vg mRNA in Xenopus (Brock & Shapiro, 1983a; Brock & Shapiro, 1983b). The maintenance of serum vitellogenin levels after the decline in Vg mRNA levels is due to the long half life of vitellogenin in the circulation of male vertebrates (Wallace & Jared, 1968).

In both the primary and secondary chronic stimulations serum estradiol levels are maintained at levels close to 30 ng/ml using silastic implants containing estradiol. The maximum vitellogenin serum concentrations (19 µg/µl) are approximately the same for both primary and secondary chronic stimulations. These levels are almost twice that of vitellogenic females with estradiol serum concentrations of 60 ng/ml (van Bohemen & Lambert, 1981). The higher

vitellogenin serum concentrations in the stimulated male can be attributed to the lack of clearance of vitellogenin from the serum of males (Wallace & Jared, 1968).

The kinetics of vitellogenin accumulation during primary chronic stimulation appears to be a composite of both the primary acute and the secondary chronic stimulations (figure 12). The early phase of the primary chronic stimulation is characterized by a lag in the secretion of vitellogenin into the circulation, similar to that observed during primary acute stimulation. The levels of vitellogenin rise slowly until day 7, after which the levels increase several-fold. The lag in the accumulation of vitellogenin observed in both the primary acute and primary chronic stimulations is similar to that observed after in vivo stimulation of male Xenopus by estradiol (Farmer et al., 1976). The rapid rise in serum vitellogenin after day 7 is similar to that seen during secondary chronic stimulation. These results suggest that a decrease in serum estradiol levels is not required to induce the high levels of serum vitellogenin observed upon secondary stimulation.

The increases in Vg mRNA levels during primary and secondary chronic stimulations parallel the increases in serum vitellogenin levels. The biphasic nature of the primary chronic response suggests that the changes which are responsible for the establishment of the "memory effect" occurs between days 0 and 7. The existence of a pool of unoccupied estrogen receptors after primary acute stimulation of male Xenopus has led to the suggestion that the establishment of "memory" may be dependent on the number of available estrogen receptors (Hayward et al., 1980). The rapid increase in Vg mRNA levels following day 7 of the primary chronic stimulation may be due to an increase in the number of available estrogen receptors. This model is consistent with the results obtained in Xenopus hepatocyte cultures which have established that the level of occupied estrogen receptors determines the maximum rate of vitellogenin gene transcription and that the receptor is induced de novo by estradiol (Perlman et al., 1984). A similar result has been observed in the chick oviduct, which shows a strong correlation between nuclear estrogen receptor concentrations, template engaged RNA polymerase II, and ovalbumin gene transcription (Taylor & Smith, 1986).

The enhanced response of the liver to estradiol during the primary chronic stimulation (between days 7 and 10) and

the secondary chronic stimulation may also reflect changes in the chromatin conformation. Although chromatin which is actively being transcribed, or has the potential to undergo active transcription is more susceptible to DNase I than inactive chromatin (Weintraub & Groudine, 1976; Conklin & Groudine, 1984), recent work by Burch and Evans (1986) suggests that the establishment of the "memory effect" does not always correlate with changes in DNase I sensitivity. These results imply that although the changes in chromatin structure discernible by DNase I sensitivity may be important for continued expression of the vitellogenin genes they are not necessarily responsible for the establishment and stability of hepatic "memory". Similarly, although estradiol may induce changes in the methylation pattern of the vitellogenin genes it is generally believed that demethylation is not directly involved in the establishment of "memory" (Burch & Evans, 1986).

Whereas the maximum serum vitellogenin concentrations are the same for both chronic stimulations, the level of Vg mRNA (expressed as ppm of total RNA) in the secondary stimulation is only half that of the primary chronic stimulation (figure 13). This result was somewhat surprising since both the primary and secondary chronic stimulations resulted in approximately the same Vg mRNA levels when

expressed as a proportion of the poly(A)$^+$ RNA (figure 14). The apparent two-fold difference in Vg mRNA levels is due to differences in the rRNA content of the total RNA between the primary and secondary chronic stimulations. The stimulation of rRNA synthesis by estradiol has been shown to occur in the livers of both Xenopus and chicken (Lewis et al., 1976; Bast et al., 1977). The effect of increasing the proportion of the rRNA in the samples is demonstrated by the decline in the levels of Vg mRNA, pSG 3.2 and pSG 2.5 after day 10 of the primary chronic stimulation (figure 21), a time when the rRNA content is increasing to maximum values (figure 16). Since the induction of 18S and 28S rRNA occurs much sooner in the secondary chronic stimulation than in the primary chronic stimulation (figure 16), the proportion of Vg mRNA in the total RNA from the secondary chronic stimulation must be lower than that of the primary chronic stimulation. These results imply that the changes in rRNA content account for the apparent differences in the levels of Vg mRNA. Since the synthesis of vitellogenin is a function of the level of its mRNA (Baker & Shapiro, 1977, 1978; Deeley et al., 1977; Ryffel et al., 1977; Smith & Tata, 1979) the number of Vg mRNA molecules per cell is most likely the same in both the primary and secondary chronic stimulations.

The increases in liver weight which are observed upon chronic stimulation of vitellogenesis (figure 17) has been well documented in both rainbow trout and Xenopus (Aida, Hirose & Yokote, 1973; Lewis et al., 1976). In Xenopus, the increases in liver weight have been associated with proliferation of the endoplasmic reticulum and Golgi apparatus and not cell proliferation (Lewis et al., 1976). Since there is very little cell division in primary cultures of male trout hepatocytes stimulated with estradiol (Maitre, Valotaire & Guguen-Guillouzo, 1986), it is unlikely that the increases in liver weight observed upon chronic stimulation are the result of cell proliferation. Thus, the increases in Vg mRNA observed upon stimulation with estradiol is the result of an increased rate of transcription and not due to an increase in the number of cells expressing the vitellogenin gene(s).

The induction of vitellogenesis is a complex process requiring the coordinated control and expression of a number of liver gene products (Tata & Smith, 1979). The translation profiles from stimulated and unstimulated male trout show that in addition to stimulating the accumulation of Vg mRNA, estradiol decreases the steady-state levels of several liver mRNAs (figures 18 & 19). In order to better understand these mechanisms we examined the changes in the levels of the mRNAs

for pSG 3.2 and pSG 2.5. Both these messages are present in the liver of the male and appear to have altered levels of expression upon induction of vitellogenesis with estradiol (figure 7).

Primary acute stimulation results in a transient rise in the steady-state levels of pSG 2.5 mRNA (figure 20). This contrasts the primary and secondary chronic stimulations in which the levels remain relatively constant (figures 21 & 22). The reason for the differential expression of pSG 2.5 in acute versus chronic stimulation is not known. However, the increases in the level of pSG 2.5 occurs only after day 2 of the primary acute stimulation, a time when the levels of serum estradiol are declining. These results suggest that estradiol may act to maintain the levels of pSG 2.5 at a constant level relative to the amount of rRNA, presumably by altering the rate of transcription and/or mRNA stability. These results contrast the induction of a small estrogen inducible mRNA (Hayward et al., 1985) and the deinduction of albumin mRNA in Xenopus liver upon induction of vitellogenesis (May et al., 1982). The rapid increase in pSG 2.5 levels after day 2 of the primary acute stimulation is consistent with the elevation of RNA polymerase II activity in the liver. This has shown to occur in chicken liver and the chick oviduct upon induction with estradiol

(Kastern, Christmann, Eldridge & Mullinix, 1981; Taylor & Smith, 1986).

The increases in the levels of pSG 3.2 mRNA upon stimulation with estradiol is probably a reflection of the increased transcriptional activity of the liver and not necessarily a specific effect of estradiol (figures 20, 21, 22). Two other trout liver mRNA clones which were characterized, but not presented displayed a similar pattern of expression. The rapid decline in the levels of pSG 3.2 between days 7 and 10 of the primary chronic stimulation is of particular interest because it correlates with the maximum induction of Vg mRNA (figure 21) and not with the increases in the rRNA content of the hepatocytes (figure 16). The rapid decline of pSG 3.2 upon maximum induction of the vitellogenin genes is similar to the deinduction of the albumin genes in male Xenopus. The decline in the steady-state levels of albumin mRNA in Xenopus have been shown in vivo and in vitro to be due to both a decrease in albumin mRNA stability and a decrease in albumin gene transcription (Riegel et al., 1986; Jackson & Shapiro, 1986; Wolffe et al., 1986). The decreased rate of albumin gene transcription is believed to be due to competition with the vitellogenin genes for the transcriptional apparatus (Wolffe et al., 1986) The decline in the levels of pSG 3.2 and pSG

2.5 after day 9 of the primary acute stimulation is probably due to a decline in the transcriptional activity of the liver which has been shown to occur upon withdrawal of estradiol (Kastern et al., 1981; Taylor & Smith, 1986).

CONCLUSION

The results reported in this thesis are a study of vitellogenesis in male rainbow trout. In both the primary and secondary stimulations Vg mRNA is induced de novo by estradiol. The kinetics of induction of the vitellogenin genes are similar to that of Xenopus and chicken, in that the lag in the accumulation of Vg mRNA present during the primary stimulation, is absent during the secondary stimulation. This is the first time that the "memory effect" has been demonstrated in teleosts. Although the induction of vitellogenesis occurs much sooner in the secondary chronic than the primary chronic stimulation, both of these forms of stimulation result in the same levels of vitellogenin in the serum.

The complexity of the induction of vitellogenesis is demonstrated by the changes in liver weight and the induction of rRNA which occurs upon administration of estradiol. The translation profiles indicate that while some messages are induced by estradiol, the levels of several messages appear to be reduced upon stimulation with estradiol. pSG 2.5 represents a constitutively expressed mRNA whose levels appear to be maintained at a constant level relative to that of rRNA by high levels of estradiol. The

exact mechanism has not been elucidated, but may involve altering the stability of the mRNA or the rate of pSG 2.5 transcription. The second message characterized, pSG 3.2, represents a constitutively expressed mRNA which appears to be induced upon stimulation with estradiol. We believe that this is a reflection of the general increase in RNA polymerase II levels and not a specific effect of estradiol.

Although the control and expression of vitellogenesis in rainbow trout is similar to that of non-seasonal species (Xenopus and chicken), this system remains an attractive model for the study of the expression of other liver mRNAs during vitellogenesis. Further characterization of the transcription and stability of the mRNAs for Vg, pSG 3.2 and pSG 2.5 should help elucidate the effects of estrogen on trout liver gene expression.

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APPENDIX 1:

LIST OF ABBREVIATIONS

BSA	bovine serum albumin
DNA	deoxyribonucleic acid
cDNA	complementary DNA
ds-cDNA	double-stranded cDNA
ss-cDNA	single-stranded cDNA
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
DEAE cellulose	diethylaminoethyl cellulose
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
estradiol-(-17β)	1,3,5(10)-estratriene-3, 17β-diol
LW/BW	liver weight/body weight ratio
PAGE	polyacrylamide gel electrophoresis
pH	$\log_{10}$ $[H^+]$
poly(A)$^+$ RNA	polyadenylated RNA
poly(A)$^-$ RNA	RNA not retained on oligo(dT)-cellulose column
ppm	parts per million
RNA	ribonucleic acid
mRNA	messenger RNA
rRNA	ribosomal RNA
tRNA	transfer RNA
SDS	sodium dodecyl sulphate
SSC (X1)	0.3 M NaCl/0.03 M sodium citrate
T.E. buffer	1 mM EDTA, 10 mM Tris-HCl, pH 8.0

Tris	2-amino-2-hydromethylpropane-1, 3-diol
TTP	thymidine 5'-triphosphate
Vg	vitellogenin (protein)
Vg mRNA	vitellogenin mRNA

APPENDIX: 2

MATERIALS

The following items were supplied by Amersham Corp. (Oakville, Ont.); α-[^{32}P]-dCTP (3000 Ci/mmol), gamma-[^{32}P]-dATP (3000 Ci/mmol), [^{35}S]-methionine (>800 Ci/mmol), Nick-translation kit, Multiprime labeling kit, and rabbit reticulocyte lysate. Estradiol-17β, dCTP , dATP, dGTP, TTP, oligo(dT)$_{12-18}$, acrylamide, bis-acrylamide, TEMED, and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, Mo.). The following products and enzymes were supplied by Boehringer Mannheim Canada Ltd (Dorval, Que.): random hexanucleotides, calf thymus DNA, DNA polymerase I, T4 ligase, polynucleotide kinase, calf intestinal phosphatase, Ava I, Bam HI, Eco RI, Hae III, Hind III, Kpn I, Pst I, Sal I, and Sma I. AMV reverse transcriptase was obtained from Life Sciences (St. Petersberg, Fl.). RNase H was supplied by BRL Canada Ltd (Burlington, Ont.). Electrophoresis grade agarose, Pall membrane, and the Hind III-lambda DNA molecular weight markers were obtained from International Biotechnologies Inc (Toronto, Ont.). AG 501-X8D mixed bed resin and the SDS-PAGE low molecular weight standards were supplied by Bio-Rad (Mississauga, Ont.). Agar, tryptone, and bacto-yeast extract were supplied by Oxoid Canada (Nepean, Ont.). Parmacia (Dorval, Que) supplied the following: oligo(dT)-cellulose, Sephadex G-50, and Sepharose 4B. Silastic tubing was supplied by Down's Surgical Supply (Mississauga, Ont.). Ficoll and polyvinylpyrrolidone were

supplied by BDH Canada (Toronto, Ont.). Ready-Solv ET Scintillation fluid was obtained from Beckman Canada (Mississauga, Ont.). Picker Int. (Ottawa, Ont) supplied the Cronex X-ray film. All other chemicals were reagent grade and were obtained from Fisher Scientifc Co. Ltd. (Nepean, Ont.).

APPENDIX: 3

CURRICULUM VITAE

Curriculum Vitae

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EDUCATION

05/84-present MSc. in Biochemistry,
University of Ottawa, Ottawa, Ontario.
Thesis Title: Liver gene expression during vitellogenesis in male rainbow trout.

09/79-09/83 BSc. Hon. in Biochemistry,
University of Ottawa, Ottawa, Ontario.

09/74-06/79 Ontario Secondary High School Diploma
South Carleton H.S., Richmond, Ontario

SPECIAL COURSES

02/84 Biological sampling and handling, University of Ottawa, Ottawa, Ontario.

AWARDS

09/79 South Carleton H.S. Staff Scholarship

RELEVANT EXPERIENCE

05/84-01/87 MSc. Student, (Dr. M Tenniswood supervisor)
Dept. of Biochemistry, University of Ottawa
Study involved the preparation and screening of a cDNA clone library for vitellogenin clones. The clones were used in the study of the induction of vitellogenesis in male rainbow trout liver by estradiol.

05/82-09/82 Laboratory Technician, Dept. of Biochemistry, University of Ottawa,
Ottawa Ontario.
Participated in a study on gene expression in the rat ventral prostate; effect of castration on prostate gene expression, discovery of androgen-repressed prostate messages (TRPMs)

05/82-09/82	COSAP Student, Animal Research Institute, Agriculture Canada, Ottawa, Ontario Participated in study on insecticide metabolism in chicken.
09/82-present	Undergraduate Lab Demonstrator, Dept. of Biochemistry, University of Ottawa, Ottawa, Ontario.

Other Positions

05/81-04/82	V.P. Academics, Science Students Association, University of Ottawa, Ottawa, Ontario.
09/82-09/83	4th year representitive, Dept. of Biochemistry council, University of Ottawa, Ottawa, Ontario.
05/85-02/87	Graduate student representitive, Dept. of Biochemistry, University of Ottawa, Ottawa, Ontario.

PUBLICATIONS

(1) Monpetit, M.L., Lawless, K.R. and Tenniswood, M. (1986) Androgen-repressed messages in the rat ventral prostate. The Prostate 8, 25-36

(2) Lawless, K.R., Le Guellec, K., and Tenniswood, M. (1987) Primary and secondary in vivo stimulation of yitellogenesis in male rainbow trout by estradiol. General and Comparative Endocrinology (in preparation)

(3) Le Guellec, K., Lawless, K.R., Valotaire, Y. and Tenniswood, M. (1987). Cloning and characterization of vitellogenin cDNA message from rainbow trout liver. General and Comparative Endocrinology (in preparation)

(4) Lawless, K.R., and Tenniswood, M. (1987) Expression of two abundant messages of rainbow trout liver upon the induction of vitellogenesis in male rainbow trout. (in preparation)

ABSTRACTS

(1) Lawless, K.R., Le Guellec, K. and Tenniswood, M. Primary and secondary *in vivo* stimulation of vitellogenesis in male rainbow trout. Presented at the 29th annual meeting of the Canadian Federation of Biological Societies, Guelph, June 1986.

(2) Lawless, K.R., Le Guellec, K. and Tenniswood, M. Vitellogenesis in male rainbow trout. Presented at the Ottawa Reproductive Biology Workshop, Ottawa, May 1986.

(3) Lawless, K.R., Le Guellec, K. and Tenniswood, M. Stimulation of vitellogenesis in male rainbow trout using estradiol-17β silastic implants. Presented at the 14th annual Southern Ontario Reproductive Biology Conference, London, May 1986

(4) Le Guellec, K., Lawless, K.R., Vaillant, C., Valotaire, Y. and Tenniswood, M. Vitellogenin gene expression in rainbow trout hepatocyte cell culture. Presented at the 13th annual Southern Ontario Reproductive Biology Conference, Kingston, May 1985