

National Library of Canada Bibliothèque nationale du Canada

Canadian Theses Service

AND DECK AND DECK AND DECK

Ottawa, Canada K1A 0N4 Service des thèses canadiennes

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments. AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséguents.

NL-339 (r. 88/04) c

ESTRADIOL-INDUCED HEPATIC GENE EXPRESSION IN THE RAINBOW TROUT (Oncorhynchus mykiss)

ţ,

ţ,

h

2

by

Mairi Elizabeth MacKay

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry

at

Dalhousie University Halifax, Nova Scotia

March, 1990

© Copyright by Mairi Elizabeth MacKay, 1990

_

ļ

SHEEL.

. .

i

Canad'a

National Library of Canada Bibliothèque nationale du Canada

Canadian Theses Service

Ottawa, Canada KIA ON4 Service des thèses canadiennes

The author has granted an irrevocable nonexclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission. L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

I

ISBN 0-315-64394-3

DEDICATION

This thesis is dedicated to the memory of my father, James MacKay. For his constant support and belief in my abilities, for his pride in my achievements and for his love despite my failings, I will be grateful all my life.

And to my mother, for all of the same reasons, and for the strength to carry on.

"We must tackle and grasp the larger, encompassing themes of our universe, but we make our best approach through small curiosities that rivet our attention- all those pretty pebbles on the shoreline of knowledge. For the ocean of truth washes over the pebbles with every wave, and they rattle and click with a most wondrous din."

> Stephen Jay Gould "Wonderful Life"

> > ۲

P

TABLE OF CONTENTS

,

LIST OF FIGURES AND TABLES	viii.
ABSTRACT	xii.
LIST OF ABBREVIATIONS	xiii.
ACKNOWLEDGEMENTS	xv.
I. INTRODUCTION	
A. Vitellogenesis as a model system for steroid hormone action	
B. Estrogen receptors and the steroid hormone receptor family	
1. "Estrophilin" and the two-step model of steroid hormone action	
2. Structure/function relationships in the steroid receptor superfamily	
3. Hormonal control of estrogen receptor levels	
C. Estradiol-induced genes and their expression in liver	14.
1. Vitellogenin genes and transcripts	14.
2. Vitellogenin proteins	
3. Estradiol-induced apolipoproteins	
4. Estrogen-induced hepatic changes associated with vitellogenesis	20.
D. Reproductive endocrinology of the female rainbow trout	20.
E. <u>Oncorynchus mykiss</u> as a model for vitellogenesis in teleosts	23.
F. Aims of this thesis	
II. MATERIALS AND METHODS	
A. Experimental animals	25.
1. Rainbow trout	25.
2. Other species of fish	27.
3. Chickens and frogs	27.
4. Tissue culture	27.
B. Preparation of liver fractions for estrogen receptor assays	28.
C. DNA and RNA assays	29.
D. Estrogen receptor assays	29.

E. Estradiol binding by plasma	31.
F. Tamoxifen aziridine-labeling and interaction with H222	31.
G. Measurement of serum vitellogenin levels	33.
H. Isolation of RNA	39.
I. Preparation and labeling of cDNA clones	40.
J. Oligonucleotide probes	41.
K. Northern and slot blot analysis of RNA	42.
L. Quantitation of mRNA levels	43.
M. Preparation of liver genomic DNA and Southern blotting	44.
N. Sequencing of pSG Vg 5.50	45.

III. RESULTS

.

A. Characterization of estrogen-binding proteins from trout liver and set	rum47.
1. Demonstration of hepatic nuclear and cytosol estrogen receptors	47.
a. Nuclear ER	48.
b. Cytosol ER	
2. E2 binding by plasma	
3. Optimization of ER assay conditions	
a. Concentrations of [3H]-E2 and protein in assays	
b. Assay temperature and exchange time	
c Charcoal treatment	
i. Prior to assay	
ii. Post-assay stripping	
4. Comparison of chicken and trout hepatic estrogen receptors	
a. Labeling with tamoxifen aziridine	
b. Interaction with H222	
5. Distribution of ER, DNA and protein from frest, and frozen tissue	
B. Effects of estradiol-treatment on livers of trout maintained at	
different temperatures	
1. Hepatic estrogen receptor concentration and distribution	
a. Time course at 4°C	
b. Comparison of time courses at 9°C and 15°C	
2. Other E2-induced changes in trout liver	
3. Serum vitellogenin	

4. Vitellugenin mRNA	113.
a. Characterzation of the vitellogenin message	
b. Quantitation of vitellogenin mRNA	
c. Temperature effects on the induction of vitellogenin mRNA	120.
d. Ratios of Vg protein:mRNA and Vg mRNA:nuclear ER	
5. Regulation of ER mRNA	128.
a. Identification and quantitation of the ER message	
b. Hepatic ER mRNA levels in trout kept at different temperatures	138.
6. pSG 3.2 mRNA	147.
C. Effects of tamoxifen on vitellogenin and hepatic ER mRNA levels in the rainbow trout	153.
D. Hepatic estrogen-binding activity and Vg and ER mRNAs in other species of fish	
1. Concentration and distribution of hepatic E2-binding activity	157.
2. Vitellogenin and estrogen receptor mRNAs	
E. Characterization of pSG Vg 5.50	
1. Southern and Northern analysis using pSG Vg 5.09 and 5.50	161.
2. Sequence analysis of pSG Vg 5.50	163.
IV. DISCUSSION	
A. The hepatic estrogen receptor of rainbow trout	174.
B. Induction and intracellular distribution of hepatic ER activity after E2-treatment	
1. Comparison with Salmo salar	182.
2. Effects of temperature	
C. E2-induction of hepatic ER mRNA and vitellogenesis	
1. Detection and quantitation of vitellogenin and ER mRNA transcripts	
2. ER mRNA and hepatic ER activity	190.
3. Induction of vitellogenin mRNA and serum vitellogenin	195.
D. Summary and conclusions	201.
1. Effects of temperature on vitellogenesis	201.
2. A model for the control of vitellogenesis in rainbow trout and comparison to <i>Xenopus</i>	203.
REFERENCES	

ł

LIST OF FIGURES AND TABLES

FIGURES:

Figure 1. Schematic representation of estradiol-induction of vitellogenin production	3.
Figure 2. Schematic representations of the estrogen receptor and its response element	9.
Figure 3. Quantitative measurement of trout serum vitellogenin by rocket immunoelectrophysesis	35.
Figure 4. Molecular weight determination of trout vitellogenin standard and reactivity with anti-vitellogenin antibody	
Figure 5. [³ H]-E2 binding by a trout liver nuclear salt-extract	
Figure 6. Estrogen binding specificity of nuclear salt extracts of trout liver	52.
Figure 7. [³ H]-E2 binding by trout liver cytosol	
Figure 8. Estrogen binding specificity of trout liver cytosol	60.
Figure 9. [³ H]-E2 binding by trout plasma	
Figure 10. Steroid binding specificity of trout plasma	64.
Figure 11. Binding of [³ H]-E2 to various concentrations of trout liver cytosol and nuclear salt-extracts	67.
Figure 12. Association kinetics of [³ H]-E2 binding to trout liver cytosol	71.
Figure 13. Dissociation kinetics of [³ H]-E2 from pre-labeled trout liver cytosol	73.
Figure 14. SDS polyacrylamide gel of [³ H]-TAZ-labeled chicken and trout liver nuclear salt-extracts	81.
Figur 15. Sucrose density gradient profiles of [³ H]-TAZ-labeled chicken liver nuclear salt-extracts	84.
Figure 16. Sucrose density gradient profiles of [³ H]-TAZ-labeled trout liver cytosol and nuclear salt-extracts	86.
Figure 17. Comparison of nuclear and cytosol preparations from fresh and frozen livers of E2-treated trout	92.
Figure 18. Time course of hepatic ER concentrations in E2-treated trout maintained at 4°C	96.

1

-

1

5

1

t

a.

I

Figure 19. Comparison of hepatic ER concentrations in E2-treated trout maintained at 9°C or 15°C	103.
Figure 20. Comparison of hepatic ER concentrations in E2-treated trout maintained at 4°C, 9°C and 15°C	
Figure 21. Comparisons of %LSI, DNA and RNA concentrations and RNA:DNA ratios in E2-treated trout at 9°C and 15°C	107.
Figure 22. Time course of serum vitellogenin concentrations in E2-treated trout maintained at 9°C and 15°C	111.
Figure 23. Northern blot of total liver RNA from E2-treated and control trout prcbed with pSG Vg 5.09	116.
Figure 24. Northern blot of total liver RNA from E2-treated trout probed with a trout testis tubulin cDNA	
Figure 25. Relative vitellogenin mRNA levels in livers of E2-treated trout maintained at 9°C and 15°C	
Figure 26. Northern blot of RNA from chicken oviduct and trout liver probed for ER mRNA	130.
Figure 27. Northern blot of total RNA from trout and <u>Xenopus</u> liver and MCF7 cells probed for ER mRNA	132.
Figure 28. Nucleotide sequences complementary to oligonucleotide probes "C" and "E"	
Figure 29. Northern blot of total RNA from livers of E2-treated trout probed for ER mRNA	136.
Figure 30. Slot blots of total RNA hybridized to oligonucleotide "probe E" and oligo(dT) ₁₅	<u>140</u> .
Figure 31. Relative ER mRNA levels in livers of E2-treated trout maintained at 9°C and 15°C	142.
Figure 32. Northern blots of total liver RNA from control and E2-treated trout hybridized to pSG 3.2	149.
Figure 33. Relative levels of 3.2 mRNA in livers of E2-treated trout maintained at 9°C and 15°C	
Figure 34. Northern blot of total liver RNA from control, E2-treated and tamoxifen-treated trout, probed with pSG Vg 5.09	
Figure 35. Northern blots of total RNA from various species of fish, probed with pSG Vg 5.09 and ER oligonucleotide probes	

-

P

Figure 36. Southern blot of trout liver genomic DNA hybridized to pSG Vg 5.09 and pSG Vg 5.50	
Figure 37. Partial nucleotide sequences of the 5'- and 3'-ends of the pSG Vg 5.50 insert	167.
Figure 38 Restriction maps of pSG Vg 5.09 and pSG Vg 5.50 inserts and hybridization of Vg 5.50 fragment to trout liver RNA	
Figure 39. Hybridization of pSG Vg 5.09 and pSG Vg 5.50 fragment to restriction digests of plasmid DNA	172.
Figure 40. Comparison of derived amino acid sequences of the steroid-binding domains of estrogen receptors from rainbow trout, <u>Xenopus</u> , chicken and human	
Figure 41. Comparison of hepatic ER concentrations after E2-treatment of Salmo salar and Oncorhynchus mykiss	184.
TABLES:	
Table 1. Binding specificity of trout hepatic ER and plasma	
Table 2. Enrichment and yield of trout liver ER by precipitation with 30%-saturated ammonium sulfate	
Table 3. Effect of temperature on specific binding of [³ H]-E2 by trout liver cytosol	
Table 4. Effect of charcoal pre-treatment on binding of [³ H]-E2 to trout liver fractions	75.
Table 5. Effect of charcoal-stripping on specific binding of [³ H]-E2 to cytosol pre-incubated with E2	
Table 6. Effect of time of charcoal-treatment on removal of [³ H]-E2 from cytosol	
Table 7. Effect of assay conditions on specific [³ H]-TAZ binding by trout and chicken liver ER	82.
Table 8. Immunoprecipitation of [³ H]-TAZ-labeled chicken and trout ER with H222	
Table 9. Comparison of fresh and frozen livers from E2-treated trout maintained at 9°C	90.
Table 10. Comparison of %LSI, RNA/DNA ratios and serum vitellogenin concentrations in E2-treated trout maintained at 9°C and 15°C	105.

х

١.

Tat le 11. Relative vitellogenin mRNA levels after single and multiple injections of E2 in trout maintained at different temperatures	123.
Table 12. Ratios of serum Vg:Vg mRNA and Vg mRNA:Nuclear EP. in E2-treated trout at 9°C and 15°C	_127.
Table 13. Relative hepatic ER mRNA levels after single and multiple injections of E2 in trout maintained at different temperatures	144.
Table 14. Ratios of total hepatic E2-binding activity:ER mRNA in trout treated with E2 at different temperatures	145.
Table 15. Relative levels of vitellogenin and ER mRNAs in livers of E2-treated and tamoxifen-treated trout	156.
Table 16. Specific estradiol-binding activity in liver cytosol and nuclear extracts of various species of fish	156.
Table 17. Comparison of relative intensities of hybridization of pSG Vg 5.09 and pSG Vg 5.50 to RNA from E2-treated trout	162.
Table 18. Comparison of binding affinities and competitors for E2 of estrogen receptors and plasma steroid-binders in various species of fish	1 7 5.

į

Ĩ

· *****

xi

I

ABSTRACT

In order to gain a more complete understanding of the process of vitellogenesis in fish, we have studied the effects of estradiol (E2) on the levels of hepatic estrogen receptors (ER), and on the induction of ER mRNA and vitellogenin (Vg) mRNA and protein in the rainbow trout, <u>Oncorhynchus mykiss</u>. We have characterized high-affinity estrogenbinding sites (K_d 1-3 nM) in cytosol and nuclear extracts from livers of juvenile trout. These sites are specific for estrogens and bind antiestrogens with high relative affinity. These features are common to estrogen receptors in other species and distinguish the hepatic estradiol-binding activity from the lower-affinity, less specific steroid-binding components of plasma.

While examining the concentration and distribution of hepatic ER following E2treatment, we noted a marked effect of environmental temperature on ER dynamics. In fish maintained at 15°C, nuclear ER increased 10-fold within the first 24 hours after injection. Similar levels of nuclear ER accumulated in fish kept at 9°C or 4°C, but the response was slower. Levels of cytosol ER rose 4- to 5-fold after treatment, also with a delayed response at cooler temperatures. Up-regulation of ER mRNA was demonstrated as well, with increased amounts of message appearing rapidly after treatment at all temperatures. The enhanced expression of ER mRNA preceded the appearance of Vg mRNA and remained 3to 10-fold higher than in untreated controls over the time course examined. However, even in animals receiving multiple hormone injections, elevated levels of hepatic estrogenbinding activity were not maintained and had decreased almost to pretreatment levels by 10 days. This was notably different from the pattern of ER induction seen previously in Atlantic salmon.

i

The initial appearance and the amounts of Vg mRNA and protein induced by E2 were affected by acclimation temperature, as well as by hormone dosage, and generally paralleled the inc finitial error in the second protein induced by a rapid increase between 7 and 10 days. The second phase coincided with pronounced increases in hepato-somatic index and total liver RNA, but at this time hepatic ER activity was no greater than in untreated fish. This is clearly different from the situation in Xenopus liver, where high concentrations of nuclear ER appear to be necessary for the maintenance of vitellogenesis. We suggest that factors other than the estrogen receptor may be induced in trout liver by high levels of E2 and may play a role in maintaining and amplifying the output of Vg following the initial ER-mediated response.

LIST OF ABBREVIATIONS

*

.

3

apoB and apoII:	apoproteins of serum very low-density lipoproteins
17,20ßP:	17α,20B-dihydroxy-4-pregnen-3-one
bp:	base pair
Denhardt's:	0.02% polyvinylpyrrolidone, 0.02% Ficoll 400, 0.02% bovine serum albumin
DES:	diethylstilbestrol
DHT:	dihydrotestosterone
DTT:	dithiothreitol
E1:	estrone
E2:	estradiol-17B
E3:	estriol
EDTA:	ethylenediaminetetraacetic acid
ER:	estrogen receptor
GAR:	goat anti-rat IgG
HC:	hydrocortisone
kb:	kilobase
K _d :	equilibrium dissociation constant
11-KT:	11-ketotestosterone
LMP-agarose:	low melting point agarose
%LSI:	% liver-somatic index (% liver weight/body weight)
MOPS:	3-[N-morpholino]-propanesulfonic acid
MOPS buffer:	20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0
OHT:	4-hydroxytamoxifen
ORF:	open reading frame
PBS:	phosphate buffered saline

PEG:	polyethylene glycol
PMSF:	phenylmethylsulfonyl fluoride
Prog:	progesterone
RIA:	radioimmunoassay
SDS:	sodium dodecyl sulfate (lauryl sulfate)
SSC:	1.5 M NaCl, 1.5 M sodium citrate, pH 7.0
STE:	20 mM Tris (pH 7.4), 1 mM EDTA, 10 mM NaCl
TAE:	0.04 M Tris (pH 7.2), 20 mM sodium acetate, 1 mM EDTA
Tam:	tamoxifen (trans-1-(p-ß-dimethylamino-ethoxyphenyl)- 1,2-diphenylbut-1-ene)
TAZ:	tamoxifen aziridine ((Z)-1-[4-(2-[N-aziridinyl]-ethoxy)phenyl])- 1,2-diphenyl-1-butene)
TCA:	trichloroacetic acid
TE:	10 mM Tris (pH 7.0), 10 mM EDTA
TES:	N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid
TES-B2:	0.5 M KCl, 10 mM TES (pH 7.5), 1.5 mM EDTA, 10 mM monothioglycerol
TET:	10 mM Tris (pH 7.5), 1.5 mM EDTA, 10 mM monothioglycerol
Tris:	tris[hydroxymethyl]aminomethane
Vg:	vitellogenin
VLDL:	very low-density lipoprotein

......

-- •

And the second second

ACKNOWLEDGEMENTS

First of all, I owe many thanks to Dr. Tom Mommsen, presently at the University of Victoria, who has been invaluable as a resource person throughout the present study, providing materials as well as innumerable helpful suggestions regarding the care and physiology of <u>Oncorhynchus mykiss/Salmo gairdnerii</u>.

Thanks are also due to Dr. A. Farrell of Simon Fraser University, for provision of temperature-regulated facilities, and to Dr. P. Hochachka and Dr. H. Kasinsky for use of their lab space and equipment at UBC. I'd also like to mention my sisters and friends in Vancouver who lent a hand on the frequently cold and rainy days when "samples" needed to be taken. Dr. Martin Tenniswood (University of Ottawa) has also been extremely helpful, providing the rainbow trout Vg cDNA clones and the anti-Vg antibody, as well as advice and preprints of his papers.

The faculty, staff and students of the Department of Biochemistry at Dalhousie University have been unfailing in their support and assistance during the course of this project. I would especially like to thank Dr. John Raelson for refining the protocols used with the ER oligonucleotide probes, Ms. Elizabeth Campbell for preparing the dozens of RNA samples used in this study, Ms. Kim Lonergan for showing me how to do receptor assays, and Tom Digby and Pat Cho for help with sequencing. And to everyone else who lent me equipment and gave me technical advice, especially Drs. Mezei, Fenwick, Singer, Dolphin, Doolittle, Mr ger and Gray and their technicians and students, my gratitude.

Most important of all, I would like to express my deep appreciation to Dr. Catherine Lazier for her constant moral, intellectual, technical and financial support. She has confirmed my suspicions that curiosity, hard work and a sense of humour are the key positive regulatory elements of a good career in science and a fulfilling and happy life...and that a little estrogen doesn't hurt either!

This research was supported, in part, by the MRC Biotechnology Training Program.

I. INTRODUCTION

Station of the Table

a right beneficiated with

*

A. Vitellogenesis as a model system for steroid hormone action

Vitellogenensis is the estrogen-regulated production of egg-yolk precursor and nutrient-carrier proteins by the liver of oviparous vertebrates. These proteins include: vitellogenin, a phospholipoglycoprotein which associates with calcium in the serum and is cleaved into the major yolk proteins, lipovitellin and phosvitin, upon entry into the cocyte; apolipoproteins, components of the plasma very low-density and low-density lipoprotein particles responsible for the transport of triglycerides and cholesterol to the egg; and several vitamin- and mineral-binding proteins, such as riobflavin-, biotin- and retinol-binding proteins and transferrin, which carry a variety of micronutrients to the developing oocyte. Together, these proteins and their associated biochemicals provide the essential nutritional requirements for the embyro between the time of fertilization of the egg and independent feeding. The production and secretion of these egg-yolk constiuents is closely coupled to oocyte maturation and the process involves a integrated series of hormonal effects by pituitary factors and ovarian steroids, which in turn influence gene expression in the liver.

Vitellogenesis has provided an excellent model system for the study of tissuespecific steroid hormone action. The production of many of the hepatic yolk-precursor proteins is entirely dependent on estradiol, which acts via a high-affinity receptor protein to both induce and enhance the expression of very high levels of vitellogenin mRNA and other hepatic gene products. The process has been studied extensively in the South African frog, <u>Xenopus laevis</u>, both <u>in vivo</u> and in hepatocyte cultures, as well as in the chicken, but other oviparous vertebrates (fish, reptiles and other amphibians) are continually being added to the list. Vitellogenesis appears to be a highly conserved process, with differences in detail, among both seasonal and non-seasonal breeders (Ho, 1987).

The standard model of vitellogenesis, based largely on studies in <u>Xenopus</u> and chickens, is outlined in Fig. 1 (see Tata and Smith, 1979; Shapiro, 1982; Wahli, 1988 for reviews). Estradiol (E2) is produced by the follicle cells of the ovary (1), in response to circulating pituitary gonadotropins. In the serum (2), estrogen associates with and is carried by low-affinity sex-steroid binding proteins and/or by serum albumins (Callard and Callard, 1987). The hormone is thought to enter hepatocytes (and the cells of other target tissues) by diffusion (3), or perhaps by a plasma membrane carrier-mediated mechanism (Milgrom <u>et al.</u>, 1973).

Once inside hepatocytes, E2 binds to high-affinity receptor proteins (4), causing structural changes (activation) which allow the hormone/receptor complex to associate with sites in chromatin. These sites, known as estrogen response elements (5), are located upstream of, or within, hormone responsive genes. Activation of transcription of previously silent loci (e.g. vitellogenin and apoII) and enhanced transcription of other genes (e.g. apoB and retinol-binding protein as well as ribosomal RNA) ensues as a result of this association (6). The accumulation of high levels of induced mRNAs is accomplished by a combination of increased transcription and selective stabilization of messages (Brock and Shapiro, 1983a and 1983b).

Changes in hepatocyte morphology, such as proliferation of the rough endoplasmic reticulum and Golgi apparatus, also accompany estrogen stimulation. Translation of vitellogenin mRNA is associated with the rough endoplasmic reticulum (7), and extensive modification of the precursor protein (phosphorylation, glycosylation and lipidation) occurs within the endoplasmic reticulum and Golgi (8). The modified protein forms dimers (9), is rapidly secreted into serum and is transported to the ovary, where it is taken up by oocytes via a receptor-mediated mechanism (10). Vitellogenin is then processed by enzymatic cleavage into lipovitellin and phosvitin (11), the major proteinaceous components of the yolk.



ih,

Figure 1. Schematic representation of estradiol-induction of vitellogenin production.

Í,

an many further a state

س معيد در 12 م. 20 م. مدير در معيدي ومعيدي مردم الارس مالي مالي الدرايين. مدير معيد المالية

ì

÷

- ----

?

Advantages of vitellogenesis as a model system for the study of steroid hormone action include the fact that previously unexpressed genes are induced to very high levels by estrogen-stimulation and that this is a reversible phenomenon which ceases after the withdrawal of hormone. Vitellogenesis is also tissue-specific and does not appear to require cell division or substantial differentiation of the liver. The induction of vitellogenin can be accomplished by treatment of males, immature females and even embryos with estradiol, and can also be observed in hepatocyte and organ cuitures. This has greatly facilitated the study of the development of competence to express estrogen-responsive genes in the liver, as well as the involvement of other hormones and factors in the process. The abundance of the gene products has aided the development of antibodies and the cloning of their cDNAs. Genomic cloning has made possible the identification of common regulatory elements among the various estrogen-responsive genes (Walker et al., 1984) and the study of the interaction of estrogen receptors and other transcription factors with these regulatory elements (Martinez et al., 1987; Corthesy et al., 1988). Also, the cloning of estrogen receptor cDNAs and the development of expression systems for both ER and vitellogenin-promoter constructs have elucidated many of the structure/function relationships between the receptor and the regulation of gene expression at the molecular level.

B. Estrogen receptors and the steroid hormone receptor family

1. "Estrophilin" and the two-step model of steroid hormone action

The initial description of "estrophilin", a high-affinity estrogen-binding protein, was made by Jensen and Jacobson (1960), who noted the specific uptake and retention of $[^{3}H]$ -estradiol by female reproductive tissues in immature rats. The subsequent demonstration that antiestrogens, such as nafoxidine, clomiphene and tamoxifen, could inhibit the binding of estradiol to estrophilin and block its effects on uterine growth and differentiation implicated the hormone/receptor complex in the early steps of this process.

Inhibitors of RNA and protein synthesis (actinomycin D and puromycin) could also block the uterotropic response without inhibiting the binding of hormone to its receptor, suggesting that the receptor somehow mediated the action of hormone at the level of transcription and/o⁻⁻ translation (see Jensen <u>et al.</u>, 1982).

High-affinity receptors, specific for natural estrogens and related compounds, have since been identified and studied in many estrogen target tissues in a variety of organisms. Some of the more extensively studied systems include rat uterus, human breast cancer cells, especially the MCF-7 cell line, chicken oviduct and liver, and <u>Xenopus</u> liver. The estrogen receptor is a protein of approximately 67 kDa in molecular weight, and is thought to undergo a conformational change, known as activation or transformation, in response to binding of hormone. This alters the sedimentation coefficient of the protein in sucrose gradients and increases its affinity for chromatin and DNA (Gorski and Gannon, 1976; Jensen <u>et al.</u>, 1982). Some of the proposed conformational changes induced by hormone-binding include dimerization (Notides and Nielsen, 1975; Little <u>et al.</u>, 1975) and dissociation from a receptor-binding heat shock protein, hsp 90, which may block the nuclear translocation of the receptor in its unoccupied state (Joab <u>et al.</u>, 1984; Sanchez <u>et al.</u>, 1985).

A two-step translocation model was proposed by Jensen <u>et al.</u> (1968) to explain the location of unactivated estrogen receptors in the cytosol prior to their association with hormone, and their accumulation in the nucleus after hormone treatment. In this model the activation of receptors by hormone causes their translocation to the nucleus where they are able to interact with chromatin and influence gene expression. However recent literature, citing both immunochemical (King and Greene, 1984) and biochemical evidence (Welshons <u>et al.</u>, 1984), suggests that the appearance of ER in the cytosol may be an artefact of homogenization and that both the unoccupied and steroid-activated forms of the receptor reside in the nucleus at all times, as do the receptors for thyroid hormone and

÷

Ì

- Hacks - Car

Arry's Array Silling

vitamin D (Oppenheimer, 1985; Walters <u>et al.</u>, 1980). The modified model proposes that unoccupied estrogen receptors are loosely-associated with chromatin but, upon activation by binding to estradiol, they become more tightly-bound to specific hormone response elements and thus become extractable from nuclei only with high salt. Hence the term "cytosol receptor" is an operational term, referring to the unoccupied or unactivated form of the receptor which is detectable in the cytosol fraction after homogenization of tissue. However, the discovery that phenol red, a common pH indicator in tissue culture media, acts as a weak estrogen has kept the intracellular location of "unoccupied" estrogen receptors in cultured cells a subject of controversy (Berthois <u>et al.</u>, 1986). The glucocorticoid receptor, on the other hand, does appear to exist predominantly in the cytosol prior to hormone-binding (Picard and Yamamoto, 1987).

Regardless of their location before hormone binding, ligand-dependent activation of steroid receptors results in their tight association with nuclear components. Whether receptors bind to specific DNA sequences or require protein "acceptor" sites in chromatin is also a subject of some debate. The most compelling evidence for the existence of chromatin acceptor sites comes from studies of the chicken oviduct progesterone receptor (Spelsberg <u>et al.</u>, 1971 and 1983). Cell-free incubation experiments showed that progesterone-receptor complexes bound to chromatin of oviduct to a greater extent than to chromatin of non-target tissues, and that reconstituting oviduct chromatin with non-histone proteins from non-target chromosomes abolished this binding, and vice versa. Fractionation of the acidic chromatin proteins demonstrated that a group, designated AP₃, seemed to be the most effective in conferring the progesterone receptor-binding ability to non-target chromatin. Other fractions, AP₁ and AP₂, appeared to mask this high-affinity binding (Spelsberg <u>et al.</u>, 1976), an observation which has implications for the tissue specificity of steroid hormone action.

On the other hand, there is increasing evidence that steroid-activated receptors bind

with high-affinity to specific nucleotide sequences which mediate their actions on transcription. Examination of the 5'-sequences upstream of estrogen-reponsive genes has revealed several regions of nucleotide homology (Walker et al., 1984). From deletion studies using cloned promoter elements, several groups have established a consensus nucleotide sequence for the "estrogen response element", shown in Fig. 2A. The demonstration that both natural EREs and synthetic oligonucleotides corresponding to the consensus ERE can confer hormone/receptor dependent regulation of transcription to reporter plasmid constructs (Martinez et al., 1987; Klock et al., 1987) certainly points to the importance of specific DNA sequences in mediating the action of the estrogen receptor. Gel retardation assays using synthetic oligonucleotide response elements indicate that the glucocorticoid and progesterone receptors (Tsai et al., 1988) and the estrogen receptor (Kumar and Chambon, 1988) can bind, as dimers, to specific DNA sequences in the absence of other chromosomal proteins. Several mutations in the 13 bp palindromic ERE sequence can abolish the ability of the estrogen receptor to bind and induce transcription from a heterologous promoter. Also, the fact that two nucleotide changes can convert an ERE to a glucocorticoid respose element (Klock et al., 1987; Martinez et al., 1987) indicates that it is the precise nucleotide sequence which determines the specificity of the steroid receptor/DNA interaction.

Comparison of the 5'-upstream sequences of the apoII and vitellogenin genes has revealed other areas of homology (van het Schip <u>et al.</u>, 1986). One of these sequences may represent a signal for liver-specific expression, since it is not found in the 5'-flanking regions of estrogen-induced genes expressed in oviduct. As well, a liver-specific repressor of vitellogenin gene transcription has been noted in nuclear extracts made from male <u>Xenopus</u> (Corthesy <u>et al.</u>, 1989). This factor appears to bind to a negative regulatory element close to the transcription initiation site of the B1 promoter. Removal of this element, or depletion of the repressor protein, results in a low constitutive expression from

ł

Figure 2. Schematic representations of the estrogen receptor and its response element.

- A. ERE= consensus sequence for the estrogen response element.
 - GRE= consensus sequence for the glucocorticoid response element.
 - * nucleotides governing the specificity of the ERE and GRE.
- B. Representation of the domain structure of the estrogen receptor. Amino acid positions are indicated above and functional regions are indicated below the figure.
- C. Representation of the DNA-binding domain of the estrogen receptor showing proposed "finger" structure coordinated by zinc ions. Amino acid changes which can convert the first finger into a GRE-recognizing structure are indicated, as are the positions of the conserved cysteine residues in the primary amino acid sequence.

Α.

.

Β.



C.



ĸ

the vitellogenin promoter in the absence of hormone and an elevated response to estradiol. Extracts from non-liver tissues also bind to this site but the complexes are structurally and functionally different and cannot repress basal activity from the vitellogenin promoter. Hence it appears that the expression of genes in a hormone- and tissue-specific manner requires the interaction of several transcription factors, as well as the activated hormonereceptor complex, with a variety of regulatory sequences.

2. Structure/function relationships in the steroid receptor superfamily

In the last few years the cloning of the cDNAs for all of the major steroid receptors and a number of closely related proteins has been accomplished (see Green and Chambon, 1986; Evans, 1988; Beato, 1989 for reviews). This has resulted in a virtual explosion of information on the structure/function and evolutionary relationships between these proteins, based on sequence analysis, deletion-mapping studies and <u>in vitro</u> expression systems using cloned promoter elements of steroid responsive genes.

Nucleotide and derived protein sequence comparisons have revealed that estrogen and other steroid hormone receptors appear to belong to a family of genes encoding a variety of ligand-activated DNA-binding proteins, including the receptors for thyroid hormone (c-erb A oncogene), vitamin D₃ and retinoic acid, as well as several gene products for which no ligand has yet been identified. General structural features of this family of proteins are shown in Fig. 2B and include: a highly variable N-terminal domain; a central, highly conserved DNA-binding domain, rich in cysteine and basic amino acid residues; and a variable ligand-binding domain within the C-terminal region, where the extent of homology between the various receptors generally parallels the relatedness of the hormones or compounds to which they bind. In addition a nuclear localization signal, similar to that found in the SV40 T-antigen, appears to be present adjacent to the DNA-binding domain (Picard and Yamamoto, 1987).

The DNA-binding domain is the most highly conserved region among the steroid

receptor family. Of 65 amino acid residues, 20 are invariant, with 9 of the invariant residues being cysteines. The positioning of the Cys residues is similar to a motif observed in the Xenopus 5S gene transcription factor, TFIIIA (Miller et al., 1985), and the mammalian transcription factor Sp1 (Kadonaga et al., 1987), in which multiple Cys- and His-rich repeating units fold into "finger" structures, coordinated by zinc ions, which are proposed to interact with half turns of the DNA helix. The steroid receptors contain two such "zinc fingers" which have been shown by deletion analysis and site-directed mutagenesis to be necessary for their DNA-binding ability (Kumar et al., 1986; Giguere et al., 1986; Green and Chambon, 1987). Furthermore, in "finger-swapping" experiments between estrogen and glucocorticoid receptors, Green et al. (1988) have demonstrated that the first of the two zinc fingers is responsible for the specific recognition of hormone response elements, and that three amino acids at the root of this finger structure are responsible for the specificity (Mader et al., 1989) (Fig. 2C). Four of the conserved Cys residues in each finger are essential for DNA binding and are thought to coordinate with zinc ions in a tetrahedral arrangement which stabilizes the finger structure (Freedman et al., 1988).

Deletion-mapping and site-specific Lutagenesis studies of the estrogen receptor have revealed that the hormone-binding domain is essential for transcriptional activation (Kumar <u>et al.</u>, 1987; Webster <u>et al.</u>, 1988) and receptor dimerization (Kumar and Chambon, 1988). Interestingly, the N-terminal region appears to be important for transcriptional activation of some genes (pS2) but not others (reporter constructs fused to vitellogenin promoter elements) (Kumar <u>et al.</u>, 1987). A small acidic region within the N-terminal domain of the glucocorticoid receptor has also been shown to modulate the receptor's ability to bind to specific DNA sequences and to reduce non-specific DNA-binding (Danielson <u>et al.</u>, 1987). The major region responsible for transcriptional activation appears to be located within the hormone-binding domain of the estrogen States - Ministry

1447-44-54 S

the sub-section when

8. +13.er

receptor, although deletion of this domain of the glucocorticoid receptor results in constitutive activation of responsive genes in the absence of hormone (Danielsen et al., 1987; Godowski et al., 1987). These authors suggest that deletion of this region removes the signal for binding of hsp90, thus allowing the receptor to associate with DNA and activate transcription, an effect which is accomplished in the wild type receptor by binding of hormone. However, evidence that DNA-binding alone is not sufficient for transcriptional activation comes from experiments using fusion constructs of the hormonebinding domain of the ER to the yeast transcription factor GAL4 DNA-binding sequences (Webster et al., 1988). These constructs bound to a GAL4-responsive promoter in the presence of both estradiol and tamoxifen, but transcription was activated only with estradiol. Similar results were obtained with a constuct containing the GR steroid-binding domain using dexamethasone or RU486, a glucocoticoid antagonist. This suggests that steroid hormone antagonists act by blocking transcriptional activation by receptors rather than by preventing their interaction with steroid response elements. Estrogen receptors deleted in the hormone binding domain are also able to form dimers and to bind with response elements, as shown by gel retardation assays, but the complexes formed are weaker than those formed by hormone-activated wild type receptors (Kumar and Chambon, 1988), suggesting that, at least with the estrogen receptor, both the DNA- and hormone-binding domains are necessary for optimum function.

3. Hormonal control of estrogen receptor levels

Because the sensitivity of a given tissue to hormone may depend on the number of receptors present, the modulation of receptor levels has been an important area of investigation. The cloning of estrogen receptor cDNAs from a number of organisms and the development of anti-ER antibodies have allowed the examination of estrogen receptor regulation without the total reliance on hormone-binding *z*_says. The regulation of estrogen receptor levels has been examined in a variety of organisms and cell lines, and control

mechanisms appear to vary with the target organ in question. Effects of androgens, progesterone, estrogens and pituitary factors h^{-1} all been noted.

Androgens appear to down regulate ER mRNA and immunoreactive ER protein in the human breast cancer cell line ZR-75-1 (Poulin et al., 1989), while progesterone downregulates both ER mRNA and protein in MCF-7 and T47D cell lines (Berkenstam et al., 1989; Read et al., 1989) Estradiol itself can regulate the levels of ER mRNA and protein in cultured breast cancer cells, but the effect appears to depend on the growth status or the previous hormonal exposure of the cells. In confluent MCF-7 cells, estradiol treatment lowers the amount of immunoreactive protein and the steady-state level of ER mRNA (Read et al., 1989; Berkenstam et al., 1989), although actual transcription of ER mRNA may be increased (Saceda et al., 1988). These effects were dose-dependent and inhibited by tamoxifen, suggesting an ER-dependent mechanism. Futhermore, T47D cells, which have defective estrogen receptors, showed no change in ER protein or mRNA levels after E2 treatment, again suggesting that the effect is mediated by the estrogen receptor (Berkenstam et al., 1989). Interestingly, MCF-7 cells which had been maintained for several years in the absence of phenol red and with charcoal-stripped fetal calf serum showed no change in ER mRNA or protein levels after E2-treatment, although these cells were still able to respond to estradiol by proliferation (Read et al., 1989). Piva et al. (1988) reported that when estradiol was added to proliferating MCF-7 cells, ER mRNA levels increased by two-fold, suggesting that the response to estradiol in breast cancer cell lines is a function of their growth status as well as their previous hormonal history.

In the rat, regulation of estrogen receptor and ER mRNA levels appears to be tissuespecific. Ovariectomy results in an increase in ER mRNA in uterus but a decrease in both liver and pituitary, effects that were reversed by injection of estradiol (Shupnik <u>et al.</u>, 1989). Effects of both pituitary and adrenal hormones on the levels of E2-binding activity in the rat liver have also been demonstrated (Norstedt <u>et al.</u>, 1981). Ovariectomy increased 1

the levels of cytosol ER in rat liver, but hypophysectomy or adrenalectomy reduced these levels to 10% and 42%, respectively, of the control value. Prolactin or growth hormone were shown to restore levels to 37% of control when given to hypohysectomized animals, and when combined with dexamethasone, full hepatic ER activity was restored. Thus it appears that the maintenance of normal ER levels in the rat liver is controlled by both pituitary factors and glucocorticoids.

In general estrogen receptors appear to be up-regulated by estrogen in the livers of oviparous vertebrates such as the chicken and <u>Xenopus</u>, which appear to require very high levels of hormone activated receptor for the maintenance of the vitellogenic response (Lazier and Haggarty, 1979; Hayward <u>et al.</u>, 1980). In <u>Xenopus</u>, expression of the ER mRNA remains elevated for several months after a single transient dose of E2, as does the level of E2-binding activity in the cytosol (Barton and Shapiro, 1988). This may play a role in the more rapid re-induction of vitellogenin mRNA transcription with subsequent hormone treatment.

Hepatic ER activity in oviparous vertebrates may also be under multihormonal control. In the turtle, <u>Chrysemis picta</u>, hypophysectomy results in a virtual disappearance of E2-binding activity in the liver, which can be partially restored by treatment with growth hormone (Riley <u>et al.</u>, 1987). Effects of growth hormone, thyroid hormone and dexamethasone on the ability of estradiol to induce a full vitellogenic response have also been noted in <u>Xenopus</u> hepatocytes (Wangh, 1982) and in reptiles (Callard <u>et al.</u>, 1972). In light of the evidence discussed above, it is quite possible that these hormones act by regulating the amount of hepatic estrogen receptor.

C. Estradiol-induced genes and their expression in liver

1. Vitellogenin genes and transcripts

The vitellogenin genes of <u>Xenopus</u>, chicken, <u>Drosophila</u> and the nematode <u>Caenorhabditis</u> have been cloned and sequenced (see Wahli, 1988). They are all encoded 1

I

į.

. .

by small gene families which appear to have arisen by gene duplications. The vitellogenin genes of vertebrates share a common intron/exon organization, but their nucleotide and derived amino acid sequences vary extensively along the genes. The regions that are most conserved between <u>Xenopus</u> and chicken also appear to be the most conserved between <u>Xenopus</u> and <u>Caenorhabditis</u>, suggesting selective pressures on the regions of these proteins involved in post-translational modifications, uptake and cleavage by oocytes and storage in the yolk. Conservation of cysteine clusters in the amino- and carboxy-terminal regions also indicate a critical role for disulfide bonds in yolk protein structures. Vitellogenin genes of vertebrates are expressed only in the liver and only in response to estradiol.

Xenopus laevis has four vitellogenin genes, falling into two linkage groups, designated A1, A2 and B1, and B2. Within the A and B gene pairs the sequence divergence is about 5%, and is about 20% between the two groups. All four Xenopus vitellogenin transcripts are approximately 6-6.5 kb in length but the sizes of the genes themselves vary from 15.8 kb (A2) to 20.5 kb (A1), due to differences in the lengths of introns. The A1, A2 and B2 genes each have 33 introns located at homologous positions within the coding sequences (Germond et al., 1983; Gerber-Huber et al., 1987). Primary treatment with estradiol causes differential activation of transcription of the four genes in liver and in hepatocyte cultures, with the B1 mRNA appearing first, followed sequentially by the A1, A2 and B2 transcripts. Eventually, however, all four genes are expressed at equal rates and are induced with similar kinetics during secondary stimulation (Williams and Tata, 1983). Estradiol treatment evokes a massive increase in vitellogenin mRNA transcripts, which can account for up to 70% of the mRNA in maximally induced livers (Skipper and Hamilton, 1977). High steady-state levels appear to be a result of both increased transcription and stabilization of the vitellogenin mRNA in the presence of estradiol, and withdrawal of estrogen results in the rapid destabilization and disappearance of the message (Brock and z

È

÷

والمتلف والمركز والمراجع والم

4

A STATE OF A STATE OF

Shapiro, 1983a and 1983b).

The chicken has at least three vitellogenin genes, one of which (vtgII) has been cloned and sequenced (van het Schip <u>et al.</u>, 1986). This gene also contains 33 introns and the organization of exons appears to be homologous to the <u>Xenopus</u> vitellogenin genes. The mRNA encoded by the vtgII gene is 5.8 kb in length, coding for a protein of 1850 amino acids. cDNAs for the other two chicken vitellogenin mRNAs have been cloned and hybridize to two disitinct transcripts, one of which is slightly larger and the other slightly smaller than the vtgII message (Evans <u>et al.</u>, 1988). The three mRNAs, as well as the proteins they encode, are expressed in a ratio of approximately 3:10:1 for vtgI, vtgII and vtgIII in both the laying hen and estrogen-treated chicks, and an increased rate of induction or "memory effect" upon secondary treatment has been noted for all three transcripts.

A family of cDNA clones for rainbow trout vitellogenin have also been described (Le Guellec <u>et al.</u>, 1988). Most of these clones showed identical restriction digestion patterns, but one clone, pSG Vg 5.50, was distinctly different in its restriction map and hybridized to additional genomic DNA fragments on Southern blots (Lawless, 1987), suggesting that it might represent the product of a second vitellogenin gene. The partial nucleotide sequence of the pSG Vg 5.50 clone is presented as part of this thesis. All of the cDNAs hybridized to an mRNA of approximately 6.6 kb on Northern blots of total liver RNA from estrogen-treated, but not untreated, juvenile rainbow trout. Sequence analysis of one of the clones, pSG Vg 5.09, showed that the putative amino acid sequence bears considerable homology to the sequences of <u>Xenopus</u> and chicken vitellogenins (Le Guellec <u>et al.</u>, 1988). Expression of the trout vitellogenin mRNA in liver was induced by estradiol-treatment and the response was apparently more rapid upon secondary treatment, suggesting a memory effect similar to that seen witt the chicken and <u>Xenopus</u> vitellogenin mRNAs. pSG Vg 5.09 has been used as a probe to isolate a vitellogenin cDNA clone from another fish, <u>Oreochromis aureus</u> (tilapia) (Ding <u>et al.</u>, 1988), and has been shown in the

present thesis to hybridize to distinct hepatic mRNAs in other species of fish as well, suggesting significant homology among the vitellogenins of teleosts.

2. Vitellogenin proteins

The vitellogenins found in the serum of both <u>Xenopus</u> and chickens are large, highly modified proteins which exist as dimers complexed with large amounts of calcium ions and can make up to 40% of serum proteins in a fully-induced animal. Vitellogenin monomers have apparent molecular weights of approximately 200 kDa and are highly phosphorylated, glycosylated and lipidated in various regions of the polypeptide before secretion from the liver. The synthetic and secretory pathway of vitellogenin has been analysed in E2-treated frogs by protein A-gold immunochemistry and electron microscopy and was shown to progress through the rough endoplasmic reticulum, Golgi apparatus and secretory granules of liver parenchymal cells (Herbener <u>et al.</u>, 1984). Vitellogenin does not appear to stored in hepatocytes to any appreciable degree but is rapid secreted into the serum or culture medium. The protein is also rapidly cleared from the serum, at least in mature female animals, by receptor-mediated uptake by oocytes (Opresko <u>et al.</u>, 1981), where it is proteolytically cleaved into the egg-yolk components phosvitin and lipovitellin (Follett and Redshaw, 1974).

As would be expected from the similarity of their genes, the chicken and <u>Xenopus</u> vitellogenins show considerable amino acid sequence homology (van het Schip <u>et al.</u>, 1987; Gerber-Huber <u>et al.</u>, 1987). At the extreme N-terminal there is a hydrophobic signal peptide sequence of 15 amino acids, responsible for targeting the protein to the rough endoplasmic reticulum for translation and processing. This is followed by a stretch of about 1100 amino acids thought to encode lipovitellin I, a protein of 120 kDa in size. Immediately following this comes a highly serine-rich domain, approximately 217 amino acids in length, responsible for the highly phosphorylated yolk-protein, phosvitin. In <u>Xenopus</u>, the phosvitin molecule may undergo a second cleavage in the oocyte to yield two low

1

ş

molecular weight phosvettes. The final 500 amino acid domain is more than adequate to encode the 30 kDa lipovitellin II molecule, which has been placed at the extreme carboxy-terminal end of the precursor protein based on its amino acid composition. This leaves enough coding capacity for a putative polypeptide of 10-20 kDa, which is either lost during vitellogenin cleavage or has yet to be identified as an egg-yolk component (Gerber-Huber <u>et al.</u>, 1987).

The vitellogenins of several species of fish have been isolated and partially characterized (see Mommsen and Walsh, 1988; Weigand, 1982 for reviews). They appear to display a wider variety of molecular weights and degrees of phosphorylation and lipidation than seen between Xenopus and chicken vitellogenins. A several estimates for the molecular weight of serum vitellogenin in the rainbow trout have been reported, ranging from 350 kDa to 600 kDa, depending on the technique used. However the monomer produced by in vitro translation of purified trout vitellogenin mRNA is between 170-200 kDa (Chen, 1983; Valotaire et al., 1984). As in other species, the trout vitellogenin is cleaved in the oocyte to form phosvitin and lipovitellin. The lipid content of the lipovitellins is approximately 25%, while the alkali-labile phosphorous content of phosvitin is .58% (Campbell and Idler, 1980). In general, alanine, glutamic acid and leucine are the most abundant amino acids in fish vitellogenins and they are lower in serinecontent than their counterparts in chicken and Xenopus, which is reflected in a 50% lower protein-bound phosphate content. The phosphate content of fish vitellogenins is still high enough to give these proteins considerable ion-binding capacity and they have been shown to be efficient carriers of calcium, magnesium and iron (Hara, 1976). However the bulk of phosphorous in the unfertilized egg is in the form of inorganic phosphate and phospholipid rather than protein-bound.

The amount of lipid material carried on the vitellogenin molecules in fish is generally about twice as high as for other vertebrate groups, ranging around 20% by weight for various fish. Most is polar lipid and goes to make up the lipovitellin component of the yolk (Hori <u>et al.</u>, 1979). In rainbow trout, two-thirds of the vitellogenin-associated lipid is phospholipid, with the remainder consisting of triglycerides and cholesterol (Norberg and Haux, 1985). Generally the mature oocyte contains a much larger percentage of triglycerides, steryl-esters, sterols and wax esters, so there are probably sources of nonpolar lipids for the oocyte other than lipovitellin.

3. Estradiol-induced apolipoproteins

In addition to vitellogenin gene expression, the estrogen induction of the avian apolipoproteins, apo-VLDL-II and apo-VLDL-B, has also been extensively studied. These proteins, components of the very low-density lipoproteins in chicken serum, are secreted in large amounts by the liver in response to estrogen treatment. In addition, apoB is constitutively expressed in the liver, kidney and intestine (Blue <u>et al.</u>, 1980), but a 5- to 6-fold induction of the apoB mRNA after estrogen treatment occurs only in the liver (Kirchgessner <u>et al.</u>, 1987). ApoII mRNA demonstrates a lag in accumulation within the first few hours after primary estrogen treatment, although this is not as pronounced as with the vitellogenin mRNA. Like vitellogenin mRNA, apoII message is induced more rapidly with secondary stimulation (Wiskocil <u>et al.</u>, 1980; Noteborn <u>et al.</u>, 1986). ApoB, on the other hand, shows no appreciable lag and similar kinetics of accumulation with both primary and secondary treatment (Kirchgessner <u>et al.</u>, 1987).

While not much is known about the regulation of apolipoprotein synthesis during the reproductive cycle in fish, there is some evidence that sex hormones may be involved in the development of arteriosclerosis in salmonids. Development of degenerative lesions in the coronary arteries appears to be accelerated during spawning migrations and House <u>et al.</u> (1979) have noted that juvenile rainbow trout injected with human chorionic gonadotropin, estradiol or testosterone developed lesions resembling those seen in mature fish. However the high incidence of arteriosclerosis in males suggests that increases in plasma lipoproteins associated with vitellogenesis are not necessarily responsible.

4. Estrogen-induced hepatic changes associated with vitellogenesis

As would be expected, estradiol-treatment induces changes in liver morphology associated with the synthesis of large amounts of secreted proteins. These include proliferation of the endoplasmic reticulum and Golgi apparatus as well as increased synthesis of ribosomal RNAs and proteins (Lewis <u>et al.</u>, 1976; Maenpaa, 1976; Skipper and Hamilton, 1977; Bast <u>et al.</u>, 1977; van Bohemen <u>et al.</u>, 1981). Increased activity of RNA polymerases I, II and III have all been noted following E2 administration, resulting in a generalized up-regulation of transcription as well as induction of specific estradiolresponsive genes (Tata and Smith, 1979). Estradiol also has pronounced lipogenic effects on the liver (Luskey <u>et al.</u>, 1974; Emmersen <u>et al.</u>, 1979), in keeping with its effects on membrane synthesis, secretion of very low-density lipoproteins and lipidation of the vitellogenin molecule.

Estradiol also inhibits the expression of some liver genes. In <u>Xenopus</u>, the synthesis of serum albumin is almost completely abolished, due to a decreased transcription rate and destabilization of the albumin message (Reigel <u>et al.</u>, 1986b and 1987), although this effect on albumin mRNA is not observed in chickens (Wiskocil <u>et al.</u>, 1980).

D. Reproductive endocrinology of the female rainbow trout

The rainbow trout, <u>Oncorhynchus mykiss</u> (formerly <u>Salmo gairdnerii</u>; Smith and Stearley, 1989) is a seasonal breeder, spawning in the fall, winter or early spring, depending on the strain (MacKay, 1963). Photoperiod is believed to be the major environmental factor controlling reproductive function, with increasing daylength apparently triggering the release of gonadotropins from the pituitary (Whitehead <u>et al.</u>, 1978; Bromage <u>et al.</u>, 1982). Strain differences in responsiveness have been observed as well, suggesting that endogenous rhythms may modify the overall response of trout to light (Elliott <u>et al.</u>, 1984). Temperature may also modulate vitellogenesis and may have a major

į

21

;

!

1

.....

1

and the summary subset of

ì

Ì

role in regula...ig the vitellogenic responses of other species of fish (Yaron <u>et al.</u>, 1980; Manning and Kime, 1984; Korsgaard <u>et al.</u>, 1986).

Female rainbow trout become reproductively active between 2-3 years of age. The annual reproductive cycle in the female trout can be roughly divided into four physiological stages (van Bohemen <u>et al.</u>, 1981). During the first, pre-vitellogenic phase, the reproductive system appears to be quiescent, with very low levels of sex steroids detectable in the plasma. This usually occurs, in mature fish, during the 2-3 months following spawning. In the second stage, endogenous vitellogenesis, the oocytes themselves actively synthesize a proteinaceous material which is stored within yolk vesicles. This occurs at a time when serum estradiol levels are still very low, and there is some controversy about whether gonadotropin levels are increased during this period (Bromage <u>et al.</u>, 1982; Scott and Sumpter, 1983). Presently there is no evidence indicating that the endogenously produced protein bears any resemblance to hepatic vitellogenin, and the vesicles which fill the oocyte during these early stages are pushed to the periphery when exogenous vitellogenin is later sequestered from the blood.

The third stage, exogenous vitellogenesis, is marked by the rise in levels of serum estradiol, estrone and testosterone (Scott <u>et al.</u>, 1980), as well as increases in serum vitellogenin, the hepatosomatic index and the gonadosomatic index, indicating growth or hypertrophy of both the liver and the gonads (van Bohemen <u>et al.</u>, 1981). Liver morphology also changes during this period, with proliferation of the endoplasmic reticulum and Golgi and depletion of glycogen granules (van Bohemen <u>et al.</u>, 1981). Changes in liver morphology and production of vitellogenin are under the control of estradiol and estrone appears to potentiate the effects of E2 (van Bohemen <u>et al.</u>, 1982). This has been clearly demonstrated by treating immature or male animals with these hormones. The role of the high levels of testosterone is not clear, although it may simply reflect the general increase in steroid output by the ovaries. Alternatively testosterone may
serve some function, possibly as an estrogen precursor, in regulating the pituitary (Scott <u>et</u> <u>al.</u>, 1980).

Control of ovarian steroid production and uptake of vitellogenin from the serum is achieved by the pituitary gonadotropins. There are two forms of gonadotropins in fish, separable by chromatography on ConA-Sepharose, both of which are necessary for normal ovarian development. The carbohydrate-rich maturational form appears to be responsible for stimulating estradiol production by the follicle cells of the ovary, while the carbohydrate-poor vitellogenic fraction appears to be necessary for proper uptake of vitellogenin by the oocytes (Idler and Campbell, 1980). Injection of pituitary extracts has been shown to stimulate the synthesis of estrogen by the ovaries and to induce vitellogenesis in immature female fish of many species.

Exogenous vitellogenesis occurs over a period of approximately six months, during which the ovary increases dramatically in size, comprising up to 20% of the body weight at its peak. Individual oocytes increase in size by 250 million times before ovulation. During the latter part of this phase, E2 peaks at approximately 40-50 ng/ml, then drops dramatically a month or so before spawning. However levels of serum vitellogenin remain high for another 2-3 months after estradiol levels have declined.

The final stage of the reproductive cycle includes oocyte maturation and ovulation. There is a general agreement that secretion of gonadotropin by the pituitary is a procequisite for the resumption of meiosis and release of mature oocytes into the body cavity (Scott and Sumpter, 1983). Its action is probably mediated by the steroids 17α -hydroxy-20B-dihydroprogesterone (17,20BP) and 17α -hydroxyprogesterone, which rise in concentration immediately prior to ovulation and are potent inducers of trout oocyte maturation in vitro (Campbell et al., 1980).

Ovulation and spawning occur in winter or early spring when water temperatures are still low. Degradation of oocytes in the body cavity and poor survival of fertilized eggs 1

Ì

have been noted if water temperatures exceed 14-15°C (Scott and Sumpter, 1983). Under natural conditions, trout will spawn in loose gravel, in rapidly moving, well oxygenated streams. Upwards of 6000-7000 eggs may be deposited in a single breeding season and the fertilized eggs develop slowly over a period of several months. It is clear, therefore, that the tremendous amounts of vitellogenin synthesized by the liver are necessary to maintain the development of thousands of embryos for the prolonged period of time before they are able to feed independently, and that an understanding of the endocrine factors controlling vitellogenesis in fish may have serious impli, ations for aquaculture.

E. Oncorynchus mykiss as a model for vitellogenesis in teleosts

There are several advantages to using the rainbow trout as a model organism for the study of vitellogenesis in fish. Not least among them is the trout's hardiness in captivity, a characteristic that has led to its wide-spread use as a hatchery fish and as a laboratory animal in a variety of physiological and biochemical studies. Much is known about its physiology and endocrinology throughout the reproductive cycle and under a variety of environmental conditions. With respect to the study contact logenesis, many of the tools necessary for examination of the process at the molecular level have been developed. Antivitellogenin antibodies are available, as well as vitellogenin and estrogen receptor cDNA clones, and vitellogenin synthesis has been successfully induced in hepatocyte cultures. Therefore, like <u>Xenopus laevis</u>, the rainbow trout is an attractive model system for working out the details of hormone-induced gene expression.

F. Aims of this thesis

While the estrogen-induced synthesis of vitellogenin mRNA and protein have been demonstrated in the rainbow trout, no information was available on the relationship of hepatic estrogen receptors to this process. To obtain a better understanding of estradiolmediated control of vitellogenesis it was necessary to examine both the levels of hepatic estrogen receptors and their intracellular distribution following estradiol-treatment. To this end, we have further characterized the rainbow trow hepatic estrogen receptor, in both its nuclear and cytosolic forms, and have examined its indiction by estradiol at the mRNA level and by hormone binding assays. We have correlated these findings to the induction of vitellogenin mRNA and protein, as well as to total RNA levels in the liver following single and multiple injections of E2. We have also discovered that the temperature at which fish are maintained during treatment can modulate the effects of estradiol treatment and have therefore examined the induction of estrogen receptors and vitellogenin, at both the mRNA and protein levels, at different temperatures. Compared to the vitellogenic responses seen in <u>Xenopus</u> and closely related species of fish, <u>Salmo salar</u>, we have noted some intriguing differences which have implications for future studies of vitellogenesis in teleost fish.

Preliminary reports of this work have been presented in the following meetings:

Mann, M.E., Mommsen, T.P. and Lazier, C.B. (1987) <u>In vivo</u> stimulation of hepatic estrogen receptors in immature rainbow trout (<u>Salmo gairdnerii</u>). Canadian Federation of Biological Sciences Meetings, Winnipeg, Man.

Mann, M., Wiktorowicz, M., Mormsen, T.P. and Lazier, C.B. (1987) <u>In vivo</u> stimulation of hepatic estrogen receptors in various species of fish. Third International Symposium on the Reproductive Physiology of Fish, St. John's, Nfld.

Lazier, C.B., Mann, M. and Mommsen, T.P. (1987) Estrogen receptors and regulation of vitellogenesis. Third International Symposium on the Reproductive Physiology of Fish, St. John's, Nfld.

MacKay (Mann), M.E. and Lazier, C.B. (1988) Hepatic estrogen receptor dynamics in the rainbow trout, <u>Salmo gairdnerii</u>. First International Symposium on Fish Endocrinology, Edmonton, Alta.

MacKay, M.E. and Lazier, C.B. (1989) Environmental effects on estradiol-induced vitellogenin gene expression in the rainbow trout (<u>Salmo gairdnerii</u>). Canadian Federation of Biological Sciences Meetings, Calgary, Alta.

II. MATERIALS AND METHODS

A. Experimental animals

1. Rainbow trout

1

Male and female sexually immature rainbow trout, <u>Oncorhynchus mykiss</u> (formerly <u>Salmo gairdnerii</u>) weighing 200-400 grams were obtained from Sun Valley Trout Farm, Aldergrove, B.C. Animals were maintained in circulating fresh water tanks and fed once a day with Moore and Clarke's New Age Fish Food until injection, after which no food was given. Animals were sacrificed by decapitation and the livers were removed and immediately frozen in liquid nitrogen for storage at -70°C, or kept in ice cold saline and processed within 30 minutes. Each animal was weighed and the sex determined by examination of internal sex organs. Fish from groups (D) and (E) were anaesthetized with MS222 (ethyl m-aminobenzoate, Sigma) and blood (2-4 ml) was collected from the caudal vein into tubes containing 50 μ l of 10,000 U/ml heparin. Samples were spun 10 minutes in a microcentrifuge and the plasma was drawn off, frozen in liquid nitrogen and stored at -70°C.

Experimental groups were as follows:

(A). <u>Time course after a single injection (4°C)</u>

This experiment was performed in December. The water temperature was 4°C and the day length was 8 hours. Fish were kept in outdoor tanks and given a single intraperitoneal injection of 5 mg/kg estradiol-17 β (E2) in 50% ethanol/saline. Groups of 10 anima's were sacrificed immediately (controls) and at 4, 8, 24, 48, 72, 96 and 120 hours after injection. Livers were quick-frozen and stored at -70°C.

(B). Fresh vs. frozen (9°C)

This experiment was performed in September with a day length of 13.5 hours. Fish were kept in outdoor tanks with a water temperature of 9.5°C. Groups of 8 animals were

sacrificed immediately (controls) or 2, 5 and 7 days after injection with E2 (5 mg/kg). The livers from four animals from each group were processed immediately (fresh) for estrogen receptor assays, while the others were quick-frozen and stored at -70°C for processing a few weeks later.

(C). Tamoxifen vs. E2 (9°C)

Performed at the same time as group (B), animals (10 per group) in this experiment were treated with a single injection of E2 (5 mg/kg), tamoxifen citrate (25 mg/kg), or 50% ethanol/saline vehicle alone (controls) and sacrificed 6 days later.

(D). <u>Temperature comparison (multiple injections)</u>

Fish were kept indoors in circulating fresh water tanks maintained at either 9°C or 15° C. The light cycle was 13.5 hours light, 10.5 hours dark. Animals were allowed to acclimate to their respective temperatures for two weeks prior to treatment. To maintain high levels of serum E2, fish were given four injections of E2 (5 mg/kg) at 2 or 3 day intervals over the course of 10 days. Sample groups of 4 fish were taken immediately (controls), 8, 24, 48, 72, 96, 168 and 240 hours after the initial injection.

(E). <u>Single injection time course (15°C)</u>

Fish were kept in outdoor tanks during September with a water temperature of 15°C and a day length of 13.5 hours. Animals (4 per group) were given a single injection of E2 (5 mg/kg) and sacrificed immediately (controls) or 8, 24, 48 hours and 10 days later.

Results for estrogen receptor, %LSI and mRNA quantitations were expressed as means of values obtained from 3-5 animals per treatment group \pm SEM (standard error of the mean). Results were compared by an unpaired Student's T-test using Macintosh StatviewTM software. Levels of p ≤ 0.05 were taken as significant.

No significant differences were found between male and female fish in any of the parameters investigated. Therefore these results have been pooled. Also, since there was no significant difference between them, the results for the control, 8, 24 and 48 hour time points obtained from group (E) have been pooled with those of the 15°C experiment in group (D).

2. Other species of fish

Livers from <u>Hemitripterus americanus</u> (sea raven), <u>Myoxocephalus</u> octodecimspinosus (sculpin), <u>Macrozoarces americanus</u> (ocean pout), <u>Pseudopleuronectes</u> <u>americanus</u> (winter flounder) and <u>Gadus morhua</u> (Atlantic cod) were provided by Dr. T.P. Mommsen, from stocks maintained at the Huntsman Marine Laboratory, St. Andrews, N.B. Treated animals had been given a single injection of E2 (2 mg/kg) in ethanol/saline and were sacrificed four days later. Controls had received ethanol/saline alone. Animals were maintained at 9°C during treatment. Livers had been quick-frozen in liquid nitrogen and were stored at -70°C.

3. Chickens and frogs

White leghorn cockerels were injected once into the breast muscle with 25 mg/kg of E2 in propylene glycol. Livers and kidneys were taken 24 hours later for preparation of ER fractions and RNA. White leghorn hens were injected with 25 mg/kg E2 every second day for two weeks to induce development of the oviduct, which was also used for preparation of ER fractions and RNA. Livers, kidneys and oviducts were also obtained from uninjected animals.

Immature <u>Xenopus laevis</u> (approximately 25 g) were given four injections, at 48 hour intervals, of 1 mg E2 in propylene glycol into the dorsal lymph sac. Animals were sacrificed 2 weeks after the first injection and livers were taken for preparation of RNA. Livers from uninjected controls were also used for RNA preparation.

4. Tissue culture

MCF-7 (human breast carcinoma) cells were obtained from American Type Culture Collection and grown to confluence in Earles Minimal Essential Medium supplemented L

in second of and one of a second in

1

;

TAK' SHIEL

,**"**

4

•

Adda to the second the base of the

the set

with 10% fetal calf serum, 100 IU/ml penicillin, 100 μ g/ml streptomycin, 4 mM glutamine. Confluent cells were treated twice at 24 hour intervals with 20 nM E2 dissolved in ethanol (Eckert and Katzenellenbogen, 1982). Treated and untreated cells were harvested and lysed in 7.3 M guanidine hydrochloride (Sigma) for preparation of RNA (see section H below).

B. Preparation of liver fractions for estrogen receptor assays

1

Cytosol and salt-extractable nuclear fractions were prepared as described in Lazier et al. (1985). All procedures were carried out on ice or at 4°C. Livers were weighed, broken or chopped into small pieces and homogenized with 3-4 strokes of a motor-driven Teflon/glass homogenizer in 2.5 ml/g of homogenization buffer (20 mM Tris (pH 7.9), 3 mM MgCl₂, 0.33 M sucrose, 10 mM monothioglycerol). A crude nuclear pellet was removed by centrifugation at 800 X g for 20 minutes in a Sorvall SS-34 rotor, and the supernatant was centrifuged at 105,000 X g for 60 minutes in a Beckman Ti50 rotor. The resulting clear supernatant beneath the layer of lipid was removed with a Pasteur pipette and retained as the "cytosol" fraction.

The crude nuclear pellet was washed three times in washing buffer (5 mM Tris (pH 7.4), 3 mM MgCl₂, 0.25 M sucrose, 10 mM monothioglycerol), resuspending before each wash with one stroke of the motor driven homogenizer. Washed nuclei were suspended with one stroke of the motor driven homogenizer in 1 ml/g tissue TES-B2 buffer (0.5 M KCl, 10 mM TES (pH 7.5), 1.5 mM EDTA, 10 mM monothioglycerol), frozen at -70°C overnight, thawed on ice and centrifuged at 37,000 X g for 30 minutes. The resulting supernatant comprised the salt-extractable nuclear fraction. The remaining pellet was resuspended in 1 ml/g TET (10 mM Tris (pH 7.5), 1.5 mM EDTA, 10 mM EDTA, 10 mM monothioglycerol) and assayed as the salt-insoluble nuclear fraction.

In some cases aliquots of cytosol were partially purified by the dropwise addition of one half volume of cold saturated ammonium sulfate in homogenization buffer, bringing the final ammonium sulfate concentration to 30% saturation. Proteins were allowed to 1

precipitate for 15 minutes on ice and were collected by centrifugation at 31,000 X g for 15 minutes. The pellet was then dissolved in a volume of TES-B2 buffer equivalent to the original volume of the sample.

Aliquots of the original homogenate and the final nuclear suspensions, from which salt extracts were obtained, were stored at -20°C for subsequent measurement of DNA and RNA. Cytosol and nuclear fractions were stored at -70°C and thawed on ice for measurement of E2-binding activity.

C. DNA and RNA assays

The DNA contents of homogenates and nuclear suspensions were determined by the perchloric acid extraction and diphenylamine procedure of Burton (1956). Alternatively both the RNA and DNA contents of these fractions were measured by extraction of aliquo.s with 10% TCA, as described by Williams <u>et al.</u> (1986), and detection of RNA by the orcinol procedure (Kabat and Mayer, 1961) and DNA by the diphenylamine test (Burton, 1956).

D. Estrogen receptor assays

 $[^{3}H]$ -E2-binding activity in cytosol and nuclear fractions was measured by a charcoal adsorption assay (Lazier and Haggarty, 1979) after treatment of the fractions for 30 minutes with dextran-coated charcoal [1% (w/v) acid-washed charcoal (Norit-A, Fisher Scientific), 0.1% (w/v) Dextran T-70 (Pharmacia)] to remove endogenous steroids. All procedures were performed on ice or at 4°C.

Single-point binding assays were carried out by incubating the liver fractions, in duplicate, with 5-20 nM [2,4,6,7- 3 H(N)]-17 β -estradiol (104-115 Ci/mmole; New England Nuclear) in a volume of 300 µl. Cytosol fractions were diluted 6- to 12-fold with homogenization buffer while nuclear extracts were diluted 3-fold or used undiluted. Tubes containing 100-fold excess of radioinert diethylstilbestrol (DES) were incubated in parallel for determination of non-specific binding. Exchange was allowed to occur for 18-24 hours

at 4°C. Bound steroid was separated from free by treatment for 30 minutes with an equal volume of 0.5% charcoal/0.05% dextran in homogenization buffer (for cytosol fractions) or in TES-B2 (for nuclear fractions), with shaking every 10 minutes, followed by centrifugation for 3 minutes at 13,000 X g. Protein bound [³H]-E2 in the supernatant was quantitated by liquid scintillation counting in Beckman ReadySolv HP/b scintillation fluid, using a Beckman LS-3810 scintillation counter with automatic conversion of tritium counts per minute (cpm) to disintegrations per minute (dpm).

Assays of the salt-insoluble nuclear fraction were adapted from the procedure of Snow <u>et al.</u> (1978). 10 nM [³H]-E2 \pm 1 μ M DES was added to undiluted insoluble nuclear fractions and exchange was allowed to occur for 18-24 hours at 4°C. Nuclear material was collected by centrifugation at 13,000 X g for 3 minutes and the pellets were resuspended in, and washed 3 times with, 1 ml TET by alternately vortexing and spinning for 3 minutes. The final pellet was extracted with 1 ml absolute ethanol for 30 minutes at 30°C, with vortexing every 10 minutes. After a final 3 minute spin, the supernatant was collected and counted in ReadySolv HP/b.

Results of single-point assays were corrected for saturation with the following equation:

$$\frac{sB}{F} = \frac{1}{K_d} (n-sB)$$

where:

sB= specific radioactive hormone bound F= free hormone (total added minus bound hormone) n= total number of binding sites Kd= equilibrium dissociation constant (cytosol 3.4 nM; nuclear 1.4 nM)

Saturation analyses of nuclear and cytosol fractions were carried out as above, using a range of 1-30 nM [³H]-E2 \pm 100-fold excess of DES. Equilibrium dissociation constants (K_d) were determined by the method of Scatchard (1949). Competition studies were also performed on both types of fractions using 10 nM [³H]-E2 and increasing concentrations (1-10⁴ nM) of estradiol-17B (E2), estrone (E1), estriol (E3),

diethylstibestrol (DES), progesterone, 17α ,20ß-dihydroxy-4-pregnen-3-one (17,20ßP), dihydro-testosterone (DHT), tamoxifen, 4-hydroxy-tamoxifen (OHT) and hydrocortisone. Results were expressed as % specific binding, calculated as total binding in the presence of competitor minus that remaining in the presence of 100-fold excess of E2, divided by the specific binding in the absence of competitor. Association kinetics were determined by incubation of cytosol with 10 nM [³H]-E2 ± 1 μ M DES at 4°C and charcoal-treating 300 μ l samples at various times over a 30 hour period. Dissociation kinetics were determined by with 10 nM [³H]-E2 to cytosol fractions which had been preincubated for 24 hours with 10 nM [³H]-E2 ± 1 μ M DES, and then charcoal-treating samples at various times over a 20 hour period.

E. E2 binding by plasma

Trout serum was diluted 50-fold with homogenization buffer, treated with 1% charcoal/0.1% dextran and assayed for estradiol-binding activity in a volume of 300 μ l. Saturation analysis was performed by incubating serum with increasing concentrations (1- 500 nM) of [³H]-E2, in the presence or absence of 100-fold excess radioinert E2, for 18-24 hours at 4°C. Competition studies were performed using 10-fold diluted serum, 20 nM [³H]-E2 and varying concentrations (1-10⁵ nM) of radioinert E2, DES, DHT, testosterone, 11-ketotestosterone, progesterone, 17,20BP, OHT, tamoxifen and hydrocortisone.

F. Tamoxifen aziridine-labeling and interaction of ER with H222

Tamoxifen aziridine (TAZ), an electrophilic ligand, has been used to covalently label estrogen receptors from rat uterus and MCF-7 cells (Katzenellenbogen <u>et al.</u>, 1983) and was used to characterize chicken and trout ER. Chicken oviduct and liver nuclear saltextracts and trout cytosol and nuclear salt-extracts were incubated in a volume of 300 μ l with 20 nM [³H]-TAZ (22 Ci/mmole; Amersham) ± 6000 nM DES for 1 hour at 22°C or 30°C, or for 18 hours at 4°C, then charcoal-stripped with 0.25% charcoal/ 0.025% dextran for 15 minutes at 37°C.

The monoclonal antibody, H222 (a gift of Dr. C. Nolan, Abbott Laboratories, Chicago, Ill.), which is specific for an epitope in the steroid-binding domain of the human ER and known to cross-react with the chicken oviduct ER (Greene et al., 1984) was used in attempts to immunoprecipitate the chicken and trout liver ER. 250 μ l of charcoal-stripped $[^{3}H]$ -TAZ-labeled preparations were incubated with 1.25-25 µg of H222, or equivalent amounts of non-specific rat IgG (Sigma), for 6 hours at 4°C. Where the amount of H222 was less than 10 µg, non-specific rat IgG was added as a carrier to increase the concentration of antibody for efficient immunoprecipitiation. The amount of goat anti-rat IgG (GAR, Sigma) sufficient to immunoprecipitate 10 µg of H22_ complexed with labeled ER had to be determined experimentally as the rat IgG/anti-rat IgG complex appeared to have a very narrow precipitin curve. The optimum amount of GAR to precipitate 10 μ g of H222 was found to be 1.75 mg, with the amount of labeled ER recoverable in the final pellet dropping off rapidly in areas of antigen or antibody excess. For 25 µg of H222, 3.5 mg of GAR was found to be optimum. GAR was allowed to interact with the ER-antibody complex for 18 hours at 4°C and the precipitate was collected by centrifugation in an Eppendorf microcentrifuge for 2 minutes. The pellet was washed 3 times with phosphate buffered saline (PBS) and solubilized in 0.1 N NaOH for liquid scintillation counting.

 $[^{3}H]$ -TAZ and $[^{3}H]$ -moxestrol-labeled chicken oviduct and liver and trout liver extracts, and their H222 complexes, were analysed by sucrose density gradient centrifugation. Labeled preparations were charcoal-stripped and layered over 5-20% continuous sucrose gradients made up in TES-B2 buffer (containing 0.5 M KCl) and centrifuged at 50,000 rpm for 15 hours in an Beckman SW55Ti rotor. $[^{14}C]$ -Ovalbumin and $[^{14}C]$ - γ -globulin were used as internal markers. Fractions of 5 drops each were collected from the bottom of the tubes and counted in ReadySolv HP/b using a Beckman LS-3801 liquid scintillation counter with a dual $[^{3}H]/[^{14}C]$ program which corrected for $[^{14}C]$ crossover into the $[^{3}H]$ channel.

[³H]-TAZ-labeled chicken and trout liver nuclear extracts were also examined by electrophoresis in 10% SDS-polyacrylamide gels (Laemmli, 1970). Labeled samples were precipitated with 10% TCA or immunoprecipitated with H222 and solubilized by boiling in sample buffer (10% glycerol, 5% 2-mercaptoethanol, 3% SDS in 0.1 M Tris, pH 6.8) and electrophoresed for 1 hour at 200 V in a BioRad Protean 1 Model slab gel apparatus (gel size 14 X 17 X 0.2 cm) with 25 mM Tris (pH 7.6), 0.19 M glycine, 1% SDS as the running buffer. Gels were soaked for 1 hour in Amplify[™] (Amersham), dried under vacuum and exposed to pre-flashed Kodak XAR 5 film for 1-2 weeks at -70°C. Mobility of bands was calculated by comparison to markers of known molecular weight which were run in the same gel, stained with 0.25% Coomassie Blue in 50% methanol/10% acetic acid and dried under vacuum beside the [³H]-labeled portion of the gel to facilitate comparison.

G. Measurement of serum vitellogenin levels

Serum vitellogenin was measured by "rocket" immunoelectrophoresis as described by Maitre <u>et al.</u> (1985a) with modifications. Anti-rainbow trout vitellogenin antiserum was generously provided by Dr. M. Tenniswood, Ottawa University. Gels (110 mm X 205 mm X 1.5 mm) contained antiserum diluted 1:300 in 1% agarose (SeaKem LE, FMC Bioproducts, Mandel Scientific), 1% PEG (polyethylene glycol, MW 8000, Sigma), 0.1% thimerosal (Sigma) in electrophoresis buffer (0.25 M Tris (pH 9.2), 0.19 M glycine). 10 µl samples were applied in 4 mm diameter wells and the gels were electrophoresed in a BioRad Model 1415 Electrophoresis cell for 18 hours at 4 V/cm, at 15°C. Gels were then dried and stained with 0.2% Coomassie Blue in 40% methanol/ 10% acetic acid. Concentrations of serum vitellogenin were calculated by comparing peak heights to a standard curve of purified vitellogenin standards (50-500 ng), prepared as

- Figure 3. Quantitative measurement of trout serum vitellogenin by rocket immunoelectrophoresis.
 - A. Representative rockets of standards and serum samples from E2-treated trout. Anti-trout vitellogenin was diluted 1:300 in gels containing 1% agarose, 1% PEG, 0.1% thimerosal in 0.25 M Tris (pH 9.2), 0.19 M glycine. Electrophoresis was performed for 18 h at 4 V/cm at 15°C. Gels were dried and stained with Coomassie blue.

Lanes 1-6: trout vitellogenin standards (50-500 ng);

- Lanes 7-10: sera from individual E2-treated trout (96 h, 15°C; diluted 1:200);
- Lanes 11-14: sera from individual E2-treated trout (96 h, 9°C; diluted 1:100);
- Lanes 15-18: sera from individual E2-treated trout (72 h, 15°C; diluted 1:100).
- B. Representative standard curve of purified trout vitellogenin (50-500 ng) vs rocket peak height.

K.







Figure 3.

described below. A typical standard curve is shown in Fig. 3B. Serum samples, to which 100 μ g/ml PMSF had been added, were diluted with electrophoresis buffer to bring the vitellogenin concentration to within the linear range of the assay. A representative gel is shown in Fig. 3A.

For standards, vitellogenin from the serum of an E2-treated male trout was purified by the method of Wiley <u>et al</u>. (1979), as modified by Maitre <u>et al</u>. (1985a). Briefly, serum was thawed on ice and 4 volumes of 20 mM EDTA (pH 7.7) were added. After mixing, 0.5 M MgCl₂ was added to make the final concentration 0.03 M. The resulting precipitate was collected by centrifugation for 15 minutes in a clinical centrifuge and resuspended in one half the original plasma volume of 1 M NaCl, 50 mM Tris (pH 7.5). After a 30 minute centrifugation to remove insoluble material, one quarter volume of distilled water was added to the supernatant, bringing the salt concentration to 0.8 M. The resulting precipitate was collected by centifugation, resuspended again in 1 M NaCl, 50 mM Tris (pH 7.5), dialysed overnight at 4°C against the same buffer, and then for 24 hours against several changes of 50 mM Tris (pH 7.5). The final concentration of protein was determined with a BioRad Protein Assay kit (Bradford, 1976), using bovine serum albumin as a standard.

he purity of the vitellogenin standard was determined by non-denaturing gradient gel electrophoresis in precast 4-30% polyacrylamide gels (Pharmacia) using 90 mM Tris (pH 9.2), 80 mM borate, 3 mM EDTA as the running buffer. Although the yield of vitellogenin was low (3.7 mg/1.9 ml serum), it appeared to be free of low molecular weight contaminants and migrated as three closely spaced bands of molecular weights 538 kDa, 500 kDa and 465 kDa (Fig. 4A). Reactivity of these bands with the anti-vitellogenin antibody was demonstrated by alkaline phosphatase immunostaining (BioRad). Protein was transferred from gels to nitrocellulose membranes in a BioRad Transblot apparatus, overnight at 4°C, at 30 V with 25 mM Tris (pH 9.2), 192 mM glycine, 20% methanol, 0.02% SDS. The blot was treated overnight with 20 µl of anti-vitellogenin antibody in 3 ml Figure 4. Molecular weight determination of trout vitellogenin standard and reactivity with anti-vitellogenin antibody.

Vitellogenin was purified from the serum of an E2-treated trout as described and electrophoresed in non-denaturing 4-30% polyacrylamide gels.

A. Coomassie blue-stained gels.

Lanes 1 and 4: Molecular weight markers: thyroglobulin (669 kDa), apoferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and albumin (67 kDa);

Lane 2: purified trout vitellogenin $(2.5 \mu g)$;

Lane 3: 2.5 µg purified vitellogenin in control male serum (10 µg total protein);

Lane 5: purified trout vitellogenin (10 μ g);

Lane 6: control male serum (48 µg protein);

Lane 7: serum from an E2-treated trout (50 μ g protein).

B. Western blot of a native polyacrylamide gradient gel as shown in (A), immunostained with anti-trout vitellogenin antibody and alkaline phosphatase-conjugated anti-IgG.

Lane 1: serum from E2-treated trout (5 μ g protein);

Lane 2: control male serum (10 µg protein);

Lane 3: purified trout vitellogenin $(2.5 \mu g)$;

Lane 4: 2.5 µg purified vitellogenin in control male serum (10 µg total protein).





Ĭ

1% gelatin, then immunostained with anti-rabbit IgG-alkaline phosphatase conjugate. All three bands reacted with the anti-vitellogenin antibody (Fig. 4B) and migrated in the molecular weight range of the major induced protein present in the serum of E2-treated animals (535 kDa, Maitre <u>et al.</u>, 1985a), which also reacted with the anti-vitellogenin antibody.

H. Isolation of RNA

Total RNA was isolated from frozen livers and other tissues by the guanidine hydrocloride method of Protter et al. (1982). Briefly, livers were pulverized under liquid nitrogen in a cold mortar and pestle and homogenized in 20 ml per gram 7.3 M guanidine hydrochloride, 0.02 M potassium acetate, 5 mM EDTA, 1 mM dithiothreitol, using four 30 second bursts of a Polytron homogenizer, with cooling in liquid nitrogen between bursts. Cultured cells were homogenized in a volume of 20 ml per four 8 X 15 cm dishes. One half volume of cold 95% ethanol was added and nucleic acids were allowed to precipitate for 2 hours at -20°C. Precipitated matter was collected by centrifugation at 6500 rpm (Sorvall Hb-4 rotor) for 30 minutes at -10°C. The pellet was resuspended in 10 ml/g guanidine-HCl solution with a 30 second burst of the Polytron, precipitated once again with a half volume of 95% ethanol and stored overnight at -20°C. Precipitates were collected as before and resuspended in 1 ml/g 50 mM Tris (pH 7.5), 100 mM NaCl, 10 mM EDTA, 0.5% SDS. The solution was extracted 3 times with phenol:chloroform: isoamyl alcohol (25:24:1), with 3 back-extractions of the protein layer at the interface. The pH of the pooled aqueous phases was adjusted to 6.0-7.5 with glacial acetic acid, brought to 0.3 M with sodium acetate and the nucleic acids were precipitated by addition of 2 volumes of 95% ethanol and storage overnight at -20°C. Pellets were collected by centrifugation at 8000 rpm (Sorvall Hb-4 rotor), brought up in TE buffer (10 mM Tris (pH 7.0), 10 mM EDTA), and reprecipitated twice at -20°C, once with the addition of one-tenth volume of 3 M sodium acetate and 2 volumes of ethanol and once without the

addition of salt. The final RNA pellet was washed twice with 70% ethanol, dried under vacuum and dissolved in TE. Optical densities at 260 and 280 nm were determined, with the 260/280 ratio being in the range of 1.9-2.0 for RNA samples free of protein.

 $Poly(A)^+$ RNA was prepared by two rounds of chromatography through columns of oligo(dT)-cellulose (Pharmacia) (Aviv and Leder, 1972). The yield of $poly(A)^+$ RNA was typically in the range of 0.5-3% of the total RNA initially applied.

I. Preparation and labeling of cDNA clones

1

Plasmids pSG Vg 5.09, pSG Vg 5.50 and pSG 3.2, from an estradiol-induced rainbow trout liver cDNA library in pUC8, were a kind gift of Dr. Martin Tenniswood, University of Ottawa. The cDNA clone pSG Vg 5.09 has been sequenced and shows considerable homology to <u>Xenopus</u> and chicken vitellogenin cDNAs. Both pSG Vg 5.09 and pSG Vg 5.50 hybridize to the same 6660 nucleotide RNA from livers of E2-treated fish. However pSG Vg 5.50 has a different restriction enzyme digestion pattern from pSG Vg 5.09 (Le Guellec <u>et al.</u>, 1988). Its pattern of hybridization to Southern blots of trout genomic DNA and its sequence were analysed as part of this thesis. The sequence of pSG 3.2 has shown homology to β -lactalbumin (M. Tenniswood, personal communication.)

pOR8, a human estrogen receptor cDNA clone in pBR322 (Greene <u>et al.</u>, 1986; Green <u>et al.</u>, 1986), was a gift of Dr. Geoffrey Greene, University of Chicago; pRT-ER (Pakdel <u>et al.</u>, 1989), a rainbow trout estrogen receptor cDNA clone in Bluescript (Stratagene, San Diego, Ca.), was a gift of Dr. Yves Valotaire, Universite de Rennes, France; TUB5 ORI2, a rainbow trout testis α -tubulin cDNA clone in pTZ19R, was a gift of Dr. A. Garber, University of Calgary (Garber <u>et al.</u>, in preparation). All plasmids carried the gene for ampicillin resistance.

<u>E. coli</u> DH5- α cells containing plasmids were grown overnight in 500 ml cultures of 2 X YT broth (16 g tryptone, 10 g yeast extract, 5 g NaCl, 50 mM Tris (pH 7.5) per

. . .

liter) containing 100 μ g/ml ampicillin. Plasmids were isolated by alkaline lysis and CsCl gradient ultracentrifugation (Maniatis <u>et al.</u>, 1982) or by lysis with 2% Triton X-100 and purification through columns of QIAGENTM as outlined in the instruction manual (Qiagen Inc., Studio City, Ca.).

Inserts of pOR8 (2100 base pairs), pRT-ER (2602 base pairs) and TUB5 ORI2 (1532 base pairs) were prepared by *Eco* RI digestion of plasmids and purification in low melting point agarose gels. Bands of the appropriate size were cut out of the gels and purified by phenol extraction and ethanol precipitation, or treated with GENECLEANTM (Bio101, La Jolla, Ca.). Inserts were labeled with α -[³²P]-dCTP (3000 Ci/mmole, Dupont) by the random hexanucleotide primer method (Feinberg and Vogelstein, 1983), using kits from Pharmacia or Bethesda Research Labs (BRL, Bethesda, Md.). The plasmids pSG Vg 5.09, pSG Vg 5.5 and pSG 3.2 were linearized by digestion with *Eco* RI and *Bam* HI and were labeled without previous isolation of inserts. Labeled cDNA probes were purified through columns of Sephadex G-50 (Pharmacia), spun in a clinical centrifuge for 10 minutes, as described by Maniatis <u>et al.</u> (1982).

J. Oligonucleotide probes

ł

Oligonucleotides (30-mers), complementary to conserved regions of the putative DNA-binding domain (probe C) and steroid-binding domain (probe E) of the rainbow trout estrogen receptor cDNA (Pakdel <u>et al.</u>, 1989), were obtained from the Regional DNA Synthesis Laboratory, University of Calgary. Their sequences are shown in Fig. 28. Oligo(dT)15, used for quantitation of poly(A)⁺ RNA, was obtained from Boehringer Mannheim. The oligonucleotides (15 pmoles) were end-labeled for 3-4 hours at 37°C with 200 μ Ci γ -[³²P]-ATP (6000 Ci/mmole, Dupont), using 2 units of T4 polynucleotide kinase (Bethesda Research Labs) in 0.05 M Tris (pH 7.4), 10 mM MgCl₂, 2 mM dithiothreitol, 0.5 mM spermidine in a volume of 50 μ L Labeled probes were purified through Sephadex G-50 spin columns (Maniatis <u>et al.</u>, 1982). Centrifugation for 20 minutes was

necessary to recover the labeled $oligo(dT)_{15}$.

K. Northern and slot blot analysis of RNA

Total RNA (10-30 μ g) and poly(A)⁺ RNA (2-10 μ g) was denatured in 33% deionized formamide, 6% formaldehyde, 1 X MOPS buffer at 65°C for 10 minutes and separated by electrophoresis in formaldehyde/agarose gels at 25 V for 16-18 hours (Lehrach <u>et al.</u>, 1977). Gels contained 1.3% agarose (BioRad Ultrapure DNA Grade), 6% formaldehyde (Fisher Scientific, 37% solution), 1 X MOPS buffer (20 mM 3-[N-morpholino]-propanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0) and 5 μ g/ml ethidium bromide. 1 X MOPS buffer was used as the running buffer. Gels were photographed on a UV transilluminator, denatured for 45 minutes in 0.05 M NaOH/ 0.01 M NaCl, neutralized 45 minutes in 10 mM Tris (pH 7.5) and the RNA was transferred to GeneScreenTM membranes (Dupont) with 25 mM NaH₂PO4 (pH 6.5) for 18 hours by the capillary blot procedure (Southern, 1975). Membranes were rinsed in 25 mM NaH₂PO4, air dried and baked at 80°C for 2 hours.

For slot blots, total RNA was denatured at 65°C for 15 minutes in 7.5% formaldehyde in 10 X SSC (1.5 M NaCl, 1.5 M sodium citrate, pH 7.0), serially diluted with 10 X SSC (10-0.125 μ g/200 'l) and applied directly to GeneScreenTM membranes using a BioRad BioDot SFTM slot blot apparatus. Membranes were rinsed with 10 X SSC, air dried and baked for 2 hours at 80°C.

Hybridization of filters to labeled cDNA clones was carried out according to the procedure of Thomas (1983). Filters were prehybridized overnight at 42°C in ZiplocTM freezer bags (Dow Consumer Products) with 20 ml prehybridization buffer which contained 50% formamide (high stringency) or 35% formamide (low stringency), 50 mM NaH₂PO₄ (pH 6.5), 5 X SSC, 500 µg/ml yeast RNA, 1 X Denhardt's (0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.02% Ficoll 400) and 1% SDS. Radioactively labeled probes (0.5-1 X 10⁷ cpm/bag) were boiled for 10 minutes,

cooled on ice and added to the prehybridization mixture. Blots were allowed to hybridize for 2 days at 42°C. After hybridization, blots were washed twice for 10 minutes at room temperature in 2 X SSC, twice for 30 minutes in 2 X SSC/ 0.1% SDS a_1 50°C, and twice for 30 minutes at room temperature in 0.1 X SSC. Filters were autoradiographed using Kodak XAR 5 film and a single intensifying screen (Dupont CronexTM) at -70°C.

The procedure for hybridization of oligonucleotide probes "C" and "E" was based on that of Zeff and Gelieber (1987). Blots were prehybridized for 2 hours at 55°C in 3 X SSC, 20 mM sodium phosphate (pH 7.0), 10 X Denhardt's, 500 μ g/ml yeast RNA, 7% SDS. Filters were hybridized to end-labeled probes (10⁷ cpm/bag in 20 ml of the prehybridization buffer) for 18 hours at 55°C, and washed at 65°C for 60 minutes in 2 X SSC, 10 mM phosphate, 10 X Denhardt's, 5% SDS and then for 60 minutes at 65°C with 1 X SSC, 1% SDS.

Hybridization of slot blots to end-labeled oligo(dT)15 was carried out according to Harley (1987). Filters were presoaked for at least 10 minutes in 5 X SSC, then hybridized overnight at room temperature with 10^{5} - 10^{6} cpm of probe in 20 ml 5 X SSC, 5 X Denhardt's, 10 mM sodium phosphate (pH 7.0). Washing was performed at room temperature in four changes of 2 X SSC. Blots were autoradiographed with Kodak XAR 5 film at room temperature for 2-12 hours.

To reprobe blots with a second cDNA or oligonucleotide probe, filters were first stripped by two treatments with boiling 0.1 X SSC/1% SDS, agitating for 2 hrs at 80°C. Filters were rinsed for 30 minutes with 0.1 X SSC at room temperature and autoradiographed for several days to ensure that the previous signal had been removed.

L. Quantitation of mRNA levels

Levels of mRNAs were quantitated by scanning the autoradiographs of slot blots using a BioRad Model 620 Video Densitometer with 1-D Analyst[™] software. Peak areas within the linear range of the exposure were used to calculate the signal density per µg of

43

a mouth an

ŧ

÷

RNA, using linear regression analysis with Macintosh StatViewTM software. Correlation coefficients for peak area vs. μ g RNA were typically 0.9 or greater.

To correct for variations in the amount of RNA applied to the slot blots, values were normalized to the amount of $poly(A)^+$ RNA present in each sample. This was determined by reprobing the blots with end-labeled $oligo(dT)_{15}$ and scanning the autoradiographs as above. Concentrations of the various mRNAs were expressed relative to the signal density per µg RNA obtained with a proparation of total liver RNA from a single trout (maintained at 9°C and sacrificed 48 hours after E2-treatment), included as a standard on all slot blots.

E2 treatment has been shown to increase the concentration of rRNA in trout liver, resulting in dilution of mRNA sequences in a given amount of total RNA (Le Guellec <u>et al.</u>, 1988). In order to compensate for this dilution effect and to give a more accurate estimation of the amount of a particular mRNA per hepatocyte, relative concentrations of mRNAs were also corrected for the ratio of total RNA:DNA in each liver, relative to the RNA:DNA ratio in the standard. A sample calculation is shown below.

Sample (168 h, 15°C, #1): <u>1.8 (Vg mRNA)</u> X 9.0 (RNA:DNA) = 9.5 units/ μ g DNA 1.7 (poly(A)+ RNA)

Standard (48 h, 9°C, #1): 0.57 (Vg mRNA) X 5.6 (RNA:DNA) = 2.1 units/µg DNA 1.5 (poly(A)+ RNA)

Sample relative to standard: 9.5/2.1 = 4.5 relative units

M. Preparation of liver genomic DNA and Southern blotting

High molecular weight DNA was prepared from trout liver nuclei by the method of Gross-Bellard <u>et al.</u> (1973). Liver was homogenized in 20 mM Tris (pH 7.9), 3 mM MgCl₂, 0.33 M sucrose, 10 mM thioglycerol and a crude nuclear pellet was removed by centrifugation at 800 X g for 20 minutes in a Sorvall SS-34 rotor. The pellet was rinsed twice with STE (20 mM Tris (pH 7.4), 1 mM EDTA, 10 mM NaCl), with spinning at 800 X g between each wash, and the final pellet was brought up in STE to which one tenth volume of 10% SDS was added. Proteinase K was added to a final concentration of 0.2 mg/ml and the tube was slowly rotated overnight at 37°C to lyse nuclei and digest protein. The solution was extracted with gentle rotation for 20 minutes with an equal volume of phenol, followed by similar extractions with phenol:chloroform (1:1) and then chloroform alone, and the DNA was precipitated from the aqueous phase by slow addition of cold 95% ethanol with constant gentle swirling. After leaving the precipitate overnight at -20°C, DNA was removed with a sterile glass hook, air dried and allowed to dissolve for several weeks at 4°C in STE. Integrity of the high molecular weight DNA was checked by electrophoresis in 0.8% agarose gels with TAE (0.04 M Tris (pH 7.2), 20 mM sodium acetate, 1 mM EDTA) as the running buffer.

Digestion of 10 μ g DNA with various restriction enzymes was performed for 3-4 hours at 37°C in a volume of 80 μ l, using 2-3 units of enzyme per μ g of DNA. Digested DNA was separated in 1.5% agarose gels for 18 hours at 30 V, using TAE as the running buffer. Transfer of DNA to GeneScreenTM membranes was performed by the procedure of Southern (1975), after hydrolysing the DNA in 0.25 M HCl (2 X 15 minutes), denaturing in 0.5 N NaOH/1.5 M NaCl (2 X 20 minutes) and neutralizing in 1 M ammonium acetate/ 0.02 N NaOH (2 X 30 minutes). Southern blots were hybridized to radio-labeled probes as decribed above.

N. Sequencing of pSG Vg 5.50

17 17 171

ł

-

Double-stranded sequencing of both ends of the pSG Vg 5.50 insert was performed by the dideoxy method (Sanger et al., 1977), from the forward and reverse M13 primer sequences present in the pUC8 vector. Three μ g of double-stranded plasmid, purified as outlined above, was denatured by adding 4 μ l 1 M NaOH, 4 μ l 1 mM EDTA and distilled H₂O up to 10 μ l. After 5 minutes at room temperature the solution was neutralized by the addition of 2 μ l 2 M ammonium acetate (pH 4.6) quickly followed by 60 μ l cold 95% ethanol. After mixing, the precipitate was collected by centrifugation in a microcentrifuge for 20 minutes (at 4°C), washed with 80% ethanol and air dried (Zhang et al., 1988).

Dideoxy sequencing reactions were performed according to instructions provided with the SequenaseTM 2.0 sequencing kit (Tabor and Richardson, 1987). Primer annealing was achieved by heating a mixture of 2 µg denatured plasmid with 0.5 pmoles sequencing primer (either forward or reverse) in 2 X SequenaseTM reaction buffer (10 µl final volume) for 2 minutes at 65°C. Tubes were slowly cooled in a water bath to 37°C over a period of approximately 30 minutes. To the annealed primer/template mix was added 1 µl of 0.1 M DTT, 2 µl of 5 X diluted labeling mix, 0.5 µl of α -[³⁵S]-dATP (500 Ci/mmole, Dupont) and 2 µl of diluted SequenaseTM enzyme. After 5 minutes at room temperature, 3.5 µl of labeling mix was added to pre-warmed tubes (37°C) of termination reactions were performed for 10 minutes at 37°C and stopped with 4 µl of stop solution. Immediately prior to loading, reaction mixtures were heated to 75°C and cooled rapidly on ice.

Electrophoresis of reaction products was performed in 6% polyacrylamide gels (1:19 crosslinking) containing 7 M urea, for 2-3 hours (short-run) or 5-6 hours (long-run) at 1900 V. Gels were vaccuum-dried and exposed to Kodak XK-1 film for 18-60 hours.

Nucleotide and derived amino acid sequences were compared to those contained in the GenBank (Release 58.0) nucleic acid or NBRF Protein (Release 19.0) databases using the University of Wisconsin Genetics Computer Group software (Version 5.3, 1988) (Devereux <u>et al.</u>, 1984) through the Biomolecular Data Base System of the Canada Institute for Scientific and Technical Information (CISTI), National Research Council, Ottawa.

III. RESULTS

A. Characterization of estrogen-binding proteins from trout liver and serum

1. Demonstration of hepatic nuclear and cytosol estrogen receptors

By definition, estrogen receptors are proteins which bind estradiol and other estrogens with high affinity and specificity and with limited capacity in estrogen responsive target tissues. The liver of the rainbow trout has been shown to respond to E2 (Maitre <u>et al.</u>, 1985a and 1985b; Le Guellec <u>et al.</u>, 1988) and ER activity has been demonstrated and partially characterized in trout liver cytosol (Maitre <u>et al.</u>, 1985b). One of the aims of the present project was to quantitate the levels and examine the intracellular distribution of hepatic ER in response to E2 treatment. Therefore it was important to demonstrate the presence of receptors in both nuclear and cytosolic fractions of liver homogenates and to develop an assay system suitable for measuring ER activity in both fractions.

Conditions for the isolation and characterization of nuclear and cytosolic E2-binding activities were based on procedures optimized for the Atlantic salmon, <u>Salmo salar</u> (Lazier <u>et al.</u>, 1985). Liver tissue was homogenized in buffer containing 0.33 M sucrose and 3 mM MgCl₂ to maintain integrity of nuclei, which were collected by low speed centrifugation. The reducing agent monothioglycerol was included in all buffers to inhibit the potential activity of "type II receptors", a class of medium-affinity E2-binding proteins found in mammalian estrogen target tissues, distinct from the classical ER and known to be highly labile in the presence of reducing agents (Markaverich <u>et al.</u>, 1981). Crude nuclear preparations were resuspended in buffer containing 0.5 M KCl, then frozen and thawed, conditions which are known to release activated receptors from their association with chromatin in many other organisms (Mester and Baulieu, 1972; Lazier, 1975; Lazier <u>et al.</u>, 1985; Smith and Thomas, 1990). All binding assays were carried out at 4°C and all preparations were charcoal-stripped prior to assay to remove potential

interference by endogenous steroids (Mester and Baulieu, 1972).

a. Nuclear ER

.

ER-like binding activity was demonstrated in nuclear salt extracts of E2-treated fish by saturation analysis and competition studies. Fig. 5A and B show a typical binding curve and Scatchard plot of such a nuclear salt extract. Specific binding, defined as total binding of [³H]-E2 minus non-specific binding in the presence of 100-fold excess of DES, showed a single class of binding sites, saturable in the range of 10-12 nM, with an equilibrium dissociation constant (K_d) of 1.4 nM. The mean K_d, determined from four independent Scatchard plots, was 1.9 ± 0.33 nM.

Curves derived from competition studies using nuclear salt extracts and 10 nM [³H]-E2 plus increasing concentrations of competing steroids or antiestrogens are shown in Fig. 6A and B. DES and 4-hydroxytamoxifen showed very high affinity for the nuclear ER, 3- to 10-fold higher than E2 itself. Estrone and estriol also bound but with approximately 20- and 50-fold less affinity, respectively, than E2, based on their competition at 50% inhibition of specific binding. Surprisingly, both progesterone and 17α ,20ß-dihydroxy-4-pregnen-3-one (17,20BP) competed to a significant degree, albeit in the μ M range. Smith and Thomas (1990) also noted some affinity of 17,20BP for the nuclear ER of the spotted seatrout. The affinity of tamoxifen was also within this range, much lower than that of 4-hydroxytamoxifen, suggesting that in the trout, as in other organisms, the 4-hydroxy-metabolite of tamoxifen is the active agent binding the receptor. Dihydrotestosterone (DHT), testosterone and hydrocortisone did not compete to any significant degree (Table 1).

Attempts were made to quantitate salt-insoluble nuclear ER by the method of Snow et al. (1978). Non-specific binding was very high in proportion to the total binding and the variability between replicates within a given experiment ranged as high as 30-40%. It was therefore impossible to accurately quantitate the ER activity that may have been present in

Figure 5. [³H]-E2 binding by a trout liver nuclear salt-extract.

A. Binding of increasing concentrations of [³H]-E2 to a salt-extract of liver nuclei from a trout sacrificed 24 h after treatment with 5 mg/kg E2.

Total = total [³H]-E2 bound; Non-specific = binding in the presence of 100-fold excess of DES; Specific = total minus non-specific.

- B. Scatchard plot of data in (5A).
 - B =specific bound; F =unbound (free) [³H]-E2.



Figure 5.

-

1

Figure 6. Estrogen binding specificity of nuclear salt-extracts of trout liver.

Increasing concentrations of competitors and 10 nM [³H]-E2 were incubated with salt-extracts from nuclei of E2-treated trout. Results are expressed as % specific binding, calculated as the net binding suppressible by 1 μ M E2.

- A. Competitors: estradiol-17β (E2); diethylstilbestrol (DES); estrone (E1); estriol (E3); dihydrotestosterone (DHT).
- B. Competitors: estradiol-17β (E2); 4-hydroxy-tamoxifen (OHT); tamoxifen (tam); progesterone (prog); 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP).

ļ

te - - an area after after and an

4

F ł

н , т , у

ľ

-



•

.



;

7





•

P

D.



- --

Competitor	% Relative Affinity*				
	Nuclear	<u>Cytosol</u>	<u>Plasma</u>		
Estradiol	100	100	100		
DES	750	370	0.1		
Turone	5	0.25	n.d.		
Estriol	2	0.37	n.d.		
Testosterone	0	0	50		
DHT	0	2.2	2.0		
11-ketotestosterone	n.d.	n.d.	3.3		
Progesterone	4	0	0.1		
17,20BP	0.75	0	n.d.		
Tamoxifen	1.5	1.1	0		
4-Hydroxytamoxifen	300	180	0		

Table 1. Binding specificity of trout hepatic ER and plasma

* % relative affinity was calculated from the concentration of E2 required for 50% inhibition of $[^{3}H]$ -E2 binding divided by the concentration of competitor required for 50% inhibition.

n.d.= not determined.

1

1

It was therefore impossible to accurately quantitate the ER activity that may have been present in the salt-insoluble fraction, at least by this method. However, when detectable, amounts of E2 binding were low in this fraction (0-100 fmoles/ml), suggesting that most of the nuclear ER had been extracted by the salt treatment.

b. Cytosol ER

Results of saturation studies of the E2-binding component of crude liver cytosol were more difficult to interpret due to the high level of non-specific binding which frequently led to non-linear Scatchard plots. However saturation could be demonstrated in the range of 10-15 nM. Fig. 7A and B show a binding curve and Scatchard plot from a high-activity cytosol preparation, demonstrating a single class of binding sites with a Kd of 1.8 nM. The mean Kd, determined from four independent plots, was found to be 3.4 ± 1.0 nM. This was in fairly good agreement with the Kd of 1.5 nM determined by Maitre et al. (1985b), who also noted difficulties in obtaining linear Scatchard plots with crude cytosol preparations. These authors found that cytosol receptor could be purified about 10-fold with precipitation in 30%-saturated ammonium sulfate and that purification step significantly reduced non-specific binding. We also found that precipitation with ammonium sulfate could yield an approximately 10-fold purification of binding activity on a per mg protein basis (Table 2). However non-specific binding was still close to or greater than 50% of the total and yields of specific binding activity varied from 30 to 70%, a range which was felt to be unacceptable for comparing levels of ER from a number of different individuals. On the other hand, the fact that at least a portion of the binding activity is precipitable with 30%-saturated ammonium sulfate lent support to the conclusion that this protein was an estrogen receptor, since plasma steroid binding proteins usually require ammonium sulfate concentrations of 50% saturation or greater for precipitation (Puca et al., 1975; Lazier, 1979).

Figure 7. [³H]-E2 binding by trout liver cytosol.

A. Binding of increasing concentrations of [³H]-E2 to cytosol from a trout sacrificed 24 h after treatment with 5 mg/kg E2.

Total = total [³H]-E2 bound; Non-specific = binding in the presence of 100-foldexcess of DES; Specific = total minus non-specific.

k

ł

B. Scatchard plot of data in (7A). B = specific bound; F = unbound (free) $[^{3}H]$ -E2. ~ 1

** **

Press of



.

ŧ

ŝ

Figure 7.

56

₽,

<u>Sample</u>	Specific Binding	Specific Binding	Enrichment	<u>Yield</u>
	(before precipitation)	(after preciptation)		
4 h cyt	1465 fmole/ml	977 fmole/ml		67%
(4°C)	50 fmole/mg	499 fmole/mg	10 X	
24 h cyt (9°C)	940 fmole/ml	653 fmole/ml		69%
24 h cyt (9°C)	1721 fmole/ml	542 fmole/ml		31%
24 h cyt (15°C)	2451 fmole/ml	1279 fmole/ml		52%
24 h cyt (15°C)	3006 fmole/ml	1324 fmole/ml		44%
72 h SE	135 fmole/ml	28 fmole/ml		21%
(4°C)	241 fmole/mg	350 fmole/mg	1.5 X	
4 h SE	144 fmole/ml	229 fmole/ml		159%
(4°C)	164 fmole/mg	1355 fmole/mg	8 X	

Table 2.	Enrichment and	vield of tr	out liver	ER	by r	precipitation	with	<u>30%-s</u> ;	aturated
	ammonium	1 sulfate				-			

cyt= cytosol; SE= nuclear salt extract; h= time after first E2 injection; $^{\circ}C$ = temperature at which fish were maintained during treatment; fmole/ml= fmoles specific [³H]-E2 bound per ml of untreated extract; fmole/mg= fmoles specific [³H]-E2 bound per mg protein in each assay.

1

Competition studies (Fig. 8A and B and Table 1) showed that, like the nuclear ER form, DES and 4-hydroxytamoxifen were potent competitors for E2-binding sites of the cytosol, having relative affinities approximately 2- to 4-fold greater than E2. Tamoxifen and dihydrotestosterone were better competitors for the cytosol sites than for the nuclear form, while estriol and estrone were somewhat less efficient, and progesterone and 17,20BP did not compete to any significant degree in concentrations up to 10 μ M. Hydrocortisone and testosterone also did not compete.

2. E2 binding by plasma

4

Saturation and competition studies were performed on serum, diluted with homogenization buffer, to characterize the plasma E2 binder and compare it to hepatic cytosol and nuclear ER. As shown in Fig. 9A and B, trout plasma did contain significant amounts of E2-binding activity. However this binding was saturable at much higher concentrations of E2 than the cytosol or nuclear components (80-100 nM), with a K_d in the range of 50 nM. Fig. 10A and B and Table 1 show that there was significant competition by testosterone, DHT and 11-ketotestosterone for these plasma binding sites and DES had a much lower affinity than E2 or testosterone. Tamoxifen and 4-hydroxytamoxifen did not compete with E2 up to concentrations of 10 μ M. These results indicated that the serum E2binder was quite distinct in its properties from the cytosol binder, resembling the sex steroid-binding protein previously reported in trout plasma (Fostier and Breton, 1975).

It can be seen in Fig. 8A that approximately 10% of the cytosol binding sites labeled with [³H]-E2 were not competable by DES, which could indicate a small degree of contamination of this fraction with serum binding sites. Hence in the single point assays used to quantitate ER levels, DES was used as the competitor for demonstration of non-specific binding, to exclude the possible contribution of serum sites in the calculation of specific E2 binding.

ł

Figure 8. Estrogen binding specificity of trout liver cytosol.

Increasing concentrations of competitors and 10 nM [³H]-E2 were incubated with cytosol of E2-treated trout. Results are expressed as % specific binding, calculated as the net binding suppressible by 1 μ M E2.

- A. Competitors: estradiol-17β (E2); diethylstilbestrol (DES); estrone (E1); estriol (E3); dihydrotestosterone (DHT).
- B. Competitors: estradiol-17β (E2); 4-hydroxytamoxifen (OHT); tamoxifen (tam); progesterone (prog); hydrocortisone (HC).

;

;



ł

(log) competitor concentration (nM)



Figure 8.

Figure 9. [³H]-E2 binding by trout plasma.

| 1

.

.

A. Binding of increasing concentrations of $[^{3}H]$ -E2 to trout plasma diluted 50-fold with homogenization buffer.

Total = total [³H]-E2 bound; Non-specific = binding in the presence of 100-fold excess of DES; Specific = total minus non-specific.

B. Scatchard plot of data in (9A). B = specific bound; F = unbound $[^{3}H]$ -E2.





 \mathbf{r}_{i}^{t}

オールをする てんしょう しきろう える



Figure 9.

Figure 10. Steroid binding specificity of trout plasma.

ĺ

Increasing concentrations of competitors and 20 nM [³H]-E2 were incubated with charcoal-stripped plasma. Results are expressed as % specific binding, calculated as the net binding suppressible by 2.5 μ M E2.

- A. Competitors: estradiol-17ß (E2); testosterone (T); 11-ketotestosterone (11KT); dihydrotestosterone (DHT).
- B. Competitors: estradiol-17β (E2); diethylstilbestrol (DES); progesterone (prog).

11

ł



(log) competitor concentration (nM)





Figure 10.

3. Optimization of ER assay conditions

a. <u>Concentrations of [³H]-E2 and protein in assays</u>

Single point binding assays containing 5 nM or 10 nM [³H]-E2 were used to determine the levels of ER in cytosol extracts prepared from individual fish sacrificed at various times after hormone injection. Single point assays containing 10 nM or 20 nM [³H]-E2 were used for nuclear extracts. These concentrations of E2 were chosen to minimize the amount of non-specific binding while saturating 60-90% of the receptor sites under equilibrium conditions. All results were corrected for saturation using a K_d of 1.9 nM for the nuclear sites and a K_d of 3.4 nM for the cytosol sites. Differences in corrected values between assays performed with 5 nM and 10 nM E2 (approximately 20%) were within the range of intra-assay variability (10-20%), determined from replicates assayed at the same time, and inter-assay variability (5-20%), determined using different aliquots of the same sample and the same concentration of [³H]-E2. Furthermore, for a given sample, corrected values for the number of binding sites determined by single point assays were within 10% of the number of binding sites determined by Scatchard analysis.

To determine the optimal concentrations of extract to use in binding assays, various dilutions of cytosol (protein concentration 0.24-2.4 mg/ml) or nuclear salt extract (protein concentration 0.08-0.45 mg/ml) were added to the standard assay volume of 300 μ l. Fig. 11A and B show that specific binding of 10 nM [³H]-E2 was linear in the range of 10-50 μ l of crude cytosol or 50-290 μ l of nuclear salt extract. Routinely, dilutions of 25-50 μ l of cytosol in 300 μ l of homogenization buffer (approximately 0.5-1.0 mg/ml final protein concentration) or 100-290 μ l of salt-extract in TES-B2 buffer (approximately 0.1-0.3 mg/ml final protein) were used in all binding assays.

b. Assay temperature and exchange time

65

ſ

31

And the standards

- 1993 - 1993

the second of the second

Figure 11. Binding of [³H]-E2 to various concentrations of trout liver cytosol and nuclear salt-extracts.

10 nM [³H]-E2 \pm 1 μ M DES were incubated with increasing amounts of cytosol (A) or nuclear salt-extract (B) in a volume of 300 μ l for 18 hours at 4°C.

.

ĉ





.]

_

Figure 11.

0.3

67

;

ĩ

incubation at 25°C or higher. Similar tests with trout cytosol fractions showed that pretreatment of the incubation mixture for 2 hours at 30°C caused an approximately 40% reduction in the number of detectable specific binding sites (Table 3). It was also noted that a 2 hour incubation at 15°C or 25°C was insufficient to saturate these cytosolic sites, although saturation could be achieved with continued incubation at 4°C for 18 hours. Therefore incubations for 18-24 hours at 4°C were routinely peformed.

To determine whether equilibrium binding had been reached under these conditions, an association curve was plotted (Fig. 12A), showing the specific E2-binding activity determined at various times after addition of 10 nM [³H]-E2 to charcoal-stripped cytosol preparations. The curve shows that at least 90% of the detectable sites were saturated by 24 hours of incubation at 4°C. A value of 1.98 X 10⁵ M⁻¹ min⁻¹ was obtained for the second order association rate constant (k+1), calculated from the slope of the function in Fig. 12B.

Dissociation kinetics were determined after an 18 hour preincubation period at 4°C with 10 nM [³H] E2 \pm 100-fold excess of DES. Dissociation began with the addition of 1 μ M radioinert E2. Incubation was continued at 4°C and terminated at various times by charcoal-dextran treatment of samples. Fig. 13A shows the remaining specific binding, expressed as the log% of specific binding at time 0, at each sample time over a 20 hour period. Control incubations, to which no radioinert E2 was added, were performed in parallel and showed no loss of specific binding activity over the same period. The first order dissociation rate constant (k₋₁), determined from the plot of ln(% bound) vs. time, was calculated as 0.0085 min⁻¹ (Fig. 13B), with a half-life for the complex of 81 minutes. It would thus appear that the incubation conditions chosen (18-24 hours at 4°C) would be adequate for equilibrium to be reached and for substantial exchange of steroids to occur in the assay mixtures.

Ľ,

Preincubation temperature	Specific bin	<u>ding (dpm)</u>	% Specifi	% Specific binding	
	2hrs	18hrs	2hrs	18hrs	
30°C	5203	4043	74	57	
25°C	4764	7556	68	107	
15°C	6405	7298	91	104	
4°C		7025	59*	100	

Table 3. Effect of temperature on specific binding of [³H]-E2 by trout liver cytosol

* obtained in a separate experiment

3

.

er.

1 [] Figure 12. Association kinetics of [³H]-E2 binding to trout liver cytosol.

.

- A. Specific binding of 10 nM [³H]-E2 to charcoal-stripped cytosol as a function of time.
- B. Specific-binding from (12A) was transformed by the formula: $1/(T-S) \ln(S(T-X)/T(S-X))$ and plotted against time of incubation. The slope of the graph yields a second order association rate constant (k+1) of 1.98 X 10⁵ M⁻¹ min⁻¹.

÷.









Figure 12.

f

Figure 13. Dissociation kinetics of [³H]-E2 from pre-labeled trout liver cytosol.

)

5

- -

- A. Cytosol was preincubated for 18 h with 16 nM [³H]-E2 $\pm 1 \mu$ M DES. Specific binding was measured at various times after addition of 1 μ M E2 and expressed as % specific bound at time 0. Control = incubations to which no excess E2 was added.
- B. $\ln(\% \text{ bound})$ vs dissociation time. The slope of the graph yields a first order dissociation rate constant (k_{-1}) of 0.0085 min⁻¹.





Figure 13.

The Kd for the cytosol fraction calculated from the kinetic studies (k_{-1}/k_{+1}) was 42 nM, 10-fold higher than the Kd obtained from equilibrium measurements. The high concentration of sucro cytosol dilution buffer and the low temperature under which these experiments were performed may have contributed to a 10-fold lower association rate than that observed with salmon nuclear ER (1.98 X 10⁵ M⁻¹ min⁻¹ for trout cytosol vs. 1.1 X10⁶ M⁻¹ min⁻¹ for salmon nuclear extracts), where sucrose was not included in the incubation medium (Lazier <u>et al.</u>, 1985). Smith and Thomas (1990) also noted that the association rate of seatrout cytosol ER was slower in buffers containing glycerol than without, possibly due to the high viscosity of the medium at low temperature. Dissociation, on the other hand, may be accelerated by the presence of μ M concentrations of radioinert steroids used to displace the specifically-bound E2 (Borgna and Ladrech, 1982), leading to sizable differences in the Kd calculated kinetically and by equilibrium studies. Other authors have also noted discrepencies between the Kd's calculated kinetically and at equilibrium in other systems (Truong and Baulieu, 1971; Capony and Rochefort, 1978).

- c. Charcoal treatment
 - i. Prior to assay

Table 4 shows that the amount of specific $[^{3}H]$ -E2 binding detectable in ER preparations treated with dextran-coated charcoal prior to assay was often greater than that observed in unstripped preparations. This suggested that endogenous estrogens could be masking some or all of the receptor sites when exchange assays were performed on unstripped preparations. The effect was especially notable in extracts made from livers of E2-injected animals (96 hour cpm sol preparations), but even cytosol from control animals showed a 25-50% increase in detectable ER after charcoal treatment. Although presumably few "cytosol" receptor sites are occupied by ligand in the cell, homogenization may disrupt the natural division of hormone between cellular compartments and extracellular carrier proteins, with redistribution of steroids to the high affinity binders found in the cytosol

		Specific Bound				
<u>Sample</u>	Stript	oed		Unstripped		
	(dpm/assay)	(fmole/ml)	(dpm/assay)	(fmole/ml)	% of Stripped	
<u>Control</u>						
cyt 1 (15°C) cyt 4 (15°C) cyt 3 (9°C) cyt 4 (9°C)	9162 11002 4246 3588	718 862 333 281	6636 7006 1827 1714	520 554 143 134	72 64 43 48	
<u>96 h</u>						
cyt 1 (9°C) cyt 2 (9°C) cyt 3 (9°C) cyt 4 (9°C) cyt 3 (15°C)	21526 18958 17784 21834 15141	1688 1487 1395 1712 1187	484 11958 9593 0 1681	38 938 752 0 131	2 63 54 0 11	
<u>168 h</u>						
cyt 1 (15°C) cyt 2 (15°C)	4713 8475	409 737	5475 8527	476 741	100 100	
SE 1 (15°C) SE 2 (15°C)	3982 1367	173 59	1849 0	80 0	46 0	
<u>240 h</u>						
cyt 1 (15°C) cyt 3 (15°C) SE 1 (15°C)	9255 11286 3243	805 981 141	14279 12233 2637	1241 1064 115	100 100 82	
SE 3 (15°C)	1011	44	1762	77	100	

Table 4. Effect of charcoal pre-treatment on binding of [³H]-E2 to trout liver fractions

cyt = cytosol; SE = nuclear salt extract; h = time after first E2 injection; °C = temperature at which fish were maintained during treatment.

Ì

fraction. Significant amounts of free unlabeled estrogens would also tend to compete with the $[^{3}H]$ -E2 in exchange assays, lowering the number of ER sites detectable by this method. Hence all preparations were routinely stripped with 1% charcoal/ 0.1% dextran for 30 minutes prior to performing binding studies.

One other observation of these data should be made. Charcoal-suripping of cytosol extracts prepared from 7 and 10 day animals, which had received 3 and 4 injections of E2 respectively, did not appear to affect the subsequent number of detectable E2-binding sites. This would suggest that not much endogenous estrogen was present in the livers of these animals to interfere with the exchange assay. One possible explanation for this finding is that by 3 days after a given injection most of the E2 had been metabolized. Maitre et al. (1985a and 1985b) noted that an injection of E2 in warm cocoa butter (3 mg/kg) produced a rapid rise in serum E2 which fell gradually over the next 20 days. However they believe that the cocoa butter medium solidifies in the peritoneal cavity and provides a slow release of hormone to the system. Injection of E2 in saline/ethanol may provide a less sustained increase in serum E2 which is cleared more rapidly by the liver (Pankurst et al., 1986). This might also explain why the levels of nuclear and cytosol ER had returned to control values in the samples taken at day 7 and 10, both of which had received injections 3 days previously. Unfortunately radioinmunoassays of serum E2 levels were hampered by etherextractable components in control serum which interfered with the anti-E2 antibody, resulting in non-linear standard curves. However the suppression of specific binding in unstripped cytosol preparations might provide an indication of the relative amount of E2 present in the liver.

To test that charcoal-treatment was capable of removing the high concentrations of E2 potentially present in animals which had received multiple hormone injections, charcoalstripped receptor preparations were incubated for 18 hours with various concentrations (0-1 μ M) of radioinert E2 and then assayed with 10 nM [³H]-E2 ± 1 μ M DES. Results presented in Table 5 show that full specific binding activity was restored by charcoal treatment of preparations which had been preincubated with up to 1 μ M E2. This experiment also showed that binding sites appeared to be lost after 18 hours in the absence of steroid. However as long as E2 was present, cytosol and nuclear sites were stable at 4°C for at least 2 days. Therefore preparations were only charcoal-treated immediately prior to assay.

ii. Post-assay stripping

To ensure that the charcoal-stripping procedure removed all of the unbound hormone at the end of exchange assays, samples to which 10 nM [³H]-E2 and 1 μ M DES had been added were immediately treated with 0.5% charcoal/ 0.05% dextran for varying lengths of time. Table 6A shows that 10-30 minutes of treatment was sufficient to remove 94% of the added radioactivity. Due to the rapid dissociation kinetics of E2 from the receptor, it was also important to ensure that 30 minutes of charcoal treatment was not causing significant loss of bound hormone and therefore an underestimation of ER sites. Table 6B shows that the same number of specific binding sites were detectable with 20 or 30 minutes of charcoal treatment, with less non-specific binding evident after a 30 rninute treatment. However the non-specific binding remaining after 10 minutes of charcoal-treatment was high enough to cause a serious underestimation of specific sites.

4. Comparison of chicken and trout hepatic estrogen receptors

a. Labeling with tamoxifen aziridine

The electrophilic ligand tamoxifen aziridine (Katzenellenbogen <u>et al.</u>, 1983) has been successfully used as covalent label for ER from rat uterus and MCF-7 human breast cancer cells and has been useful for studying the structural properties of ER proteins by such techniques as sucrose density gradient centrifugation and SDS polyacrylamide gel electrophoresis. Optimal conditions for labeling of the chicken liver nuclear ER wer. * なしと 安全市ま

とうてい しょう いっていたまままま

Concentration of E2	% Specific Bound		
in pre-incubation* Stri	pped Unstripped		
0 nM	37		
0 nM	60		
10 nM 1	100 72		
50 nM 1	61		
100 nM	100 16		
1 μM 1	0 0		

II.

Table 5	. Effect of charcoal-stripping or	specific binding	of [³ H]-E2 to cytosol
	pre-incubated with E2		

* Charcoal-stripped cytosol was pre-incubated for 18 h at 4°C with radioinert E2.

			<u>a</u>
m 11 /		1 1	1 65 5771 730 6 1
TODIA A	Littant At times At	chorecal tractment on	romount of JJHI W I from outogol
TADIC U.	ETTECT OF THEFT OF	Unaituai=iicaimiciii un	
	<u></u>		

Length of charcoal treament (min)	DPM Remaining	%[³ H]-E2 Removed
0	590065	0
5	53680	91
10	43658	92
20	33572	94
30	32979	94

A. Before exchange:*

B. After 18 h of exchange:**

Length of charcoal treament (min)	Total bound (dpm)_	Non-specific (dpr1)	Specific (dpm)	Specific (fniole/ml)	
10	52054	48696	3358	438	
20	45273	36164	9108	1188	
30	39856	30981	8875	1158	

* 10 nM [³H] plus 1 μ M DES were added to cytosol which was immediately stipped with charcoal/dextran suspension;

** cytosol was incubated with 10 nM [³H]-E2 \pm 1 μ M DES for 18 h at 4°C.

۱.

determined to be 1 hour of incubation with 20 nM [³H]-TAZ at 30°C (Lazier and Lonergan, in preparation). Prominent bands with molecular weights of 56 kDa, 46 kDa, and 34 kDa and minor bands of 36 kDa and 29 kDa could be observed after SDS gel electrophoresis and autoradiography of labeled nuclear preparations (Fig. 14, lane 3).

Labeling of the trout nuclear and cytosol ER forms with $[^{3}H]$ -TAZ was also attempted. Some specific binding could be demonstrated with nuclear preparations labeled at 4°C for 18 hours, but not at 22°C or 30°C (Table 7). Cytosol preparations showed no specific binding of $[^{3}H]$ -TAZ under any of the conditions tried. No labeled protein could be demonstrated after SDS gel electrophoresis of nuclear or cytosolic forms (Fig. 14, lanes 1 and 2). All of the label migrated with the dye front of this gel, suggesting that the protein applied was not covalently labeled.

 $[^{3}H]$ -TAZ-labeled chicken and trout liver nuclear preparations were also compared by density gradient centrifugation in 5-20% sucrose gradients containing 0.5 M KCl. The gradient profile of chicken nuclear extract showed a peak of specific binding activity at 4.7S, with a small peak of non-specific binding in the 3.6S region (Fig. 15A). This was very similar to the 4.5S peak observed for $[^{3}H]$ -E2-labeled chicken nuclear ER (Lazier and Alford, 1977), and much like the high salt gradient profile of rat uterine cytosol, which showed a peak of $[^{3}H]$ -TAZ label at 5.6S and a small amount of non-specific binding in the 4S region (Katzenellenbogen <u>et al.</u>, 1983). The trout nuclear preparation, on the other hand, showed a broad band of partially DES competable binding activity sedimenting at about 6.6S, as well as a high level of non-specific binding (not competable with DES) in this region of the gradient (Fig. 1^cA and B). Trout cytosol showed only the peak of noncompetable binding in the 6.6S region (Fig. 16C and D).

Figure 14. SDS polyacrylamide gel of [³H]-TAZ-labeled chicken and trout liver nuclear salt-extracts.

Extracts were labeled with 20 nM [³H]-TAZ \pm 6 μ M DES for 1 h at 37°C (chicken) or 18 h at 4°C (trout) followed by charcoal-stripping for 30 min at 4°C. Electrophoresis was carried out for 1 h at 200 V. The gel was autoradiographed for 3 weeks.

Molecular weight markers indicated: phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa) and soybean trypsin inhibitor (21.5 kDa).

Lane 1: Trout nuclear salt-extract -DES.

Lane 2: Trout nuclear salt-extract +DES.

Lane 3: Chicken nuclear salt-extract -DES.

Lane 4: Chicken nuclear salt-extract +DES.



Figure 14.

l

,

r.

ł

Incubation time	Specific Bound				
and temperature	[³ H]-	-E2*	[³ H]-Т	[³ HI-TAZ*	
	(dpm/assay)	(fmole/ml)	(dpm/assay)	(fmole/ml)	
Trout Nuclear					
24 h, 4°C	19829 18930	440 420	3067 5899	39'7 764	
1 h, 22°C	3857	175	0	0	
1 h, 30°C	3439	159	0	0	
Trout cytosol					
24 h, 4°C	7588 2528	3907 650	0 0	0 0	
Chicken nuclear					
1 h, 30°C	12359	843	1537	483	

I

Table 7. Effect of assay conditions on specific [³HI-TAZ binding by trout and chicken liver ER

* Extracts were incubated with 10 nM [³H]-E2 \pm 1 μ M DES or 20 nM [³H]-TAZ \pm 6 μ M DES.

The second

:

 \mathbb{R}^{n}

Figure 15. Sucrose density gradient profiles of [³H]-TAZ-labeled chicken liver nuclear salt-extracts.

Ś

10

[³H]-TAZ-labeled nuclear preparations were applied to 5-20% sucrose gradients (containing 0.5 M KCl) and centrifuged for 15 h at 50,000 rpm.

- A. Total = preparation labeled with 20 nM [3 H]-TAZ alone; No₄₁-specific = labeling in the presence of 6 μ M DES.
- B. Labeled preparations were incubated for 18 h with 0.125 μ g H222 (-o-) or non-specific rat IgG (- \blacksquare -) before application to gradients.

Internal markers: $O = [^{14}C]$ -ovalbumin (3.6S); $\gamma = [^{14}C]$ - γ -globulin (6.6S).



Figure 15.

84

i

:

ł

,

ł

Figure 16. Sucross density gradient profiles of [³H]-TAZ-labeled trout liver cytosol and nuclear sa't-extracts.

Cytosol and nuclear preparations were labeled with 20 nM [³H]-TAZ \pm 6 μ M DES, applied to 5-20% sucrose gradients (containing 0.5 M KCl) and centrifuged for 15 h at 50,000 rpm.

- A. Nuclear preparation (total binding).
- B. Nuclear preparation + DES (non-specific binding).
- C. Cytosol (total binding).
- D. Cytosol + DES (non-specific binding).

Internal markers: $O = [{}^{14}C]$ -ovalbumin (3.6S); $\gamma = [{}^{14}C]$ - γ globulin (6.6S).



Figure 16.

86

the state of the s

ł

×,

-

b. Interaction with H222

The monoclonal antibody, H222, which is known to react with an epitope in the steroid-binding region of the human and chicken FR (Greene et al., 1984) was capable of immunoprecipitating the chicken liver ER (Table 8) and increased the sedimentation value of the [³H]-TAZ-labeled chicken nuclear receptor from 4.7S to 8S in sucrose gradients (Fig. 15B). On the other hand, trout nuclear preparations labeled with [³H]-TAZ and incubated with as much as 25 μ g of H222 were not immunoprecipitable, nor did the antibody alter the sucrose gradient sedimentation profile of the [³H]-TAZ-binding activity. This would suggest that either [³H]-TAZ was labeling something other than the trout ER or that H222 did not cross react with the trout ER. Attempts were also made to determine the sedimentation value and to immunoprecipitate the trout ER with H222 using [³H]-E2- or [³H]-moxestrol-labeled preparations (data not shown). However the rapid dissociation of ligand from the labeled complex under non-equilibrium conditions made detection of ER impossible under the long periods of centrifugation or extensive washing procedures involved in each of these protocols.

Clearly the t-out ER has different properties from the chicken or human receptors with respect to [³H]-TAZ-labeling and/or interaction with the antibody H222, either of which could be due to subtle differences in the steroid-binding domains of these proteins.

5. Distribution of ER, D^{**}A and protein from fresh and frozen tissue

In the time course experiments described below (see Figs. R14 and R15) it was noted that the concentration of cytosol ER (expressed on a fmole/gram tissue basis) was usually several-fold higher than the concentration of nuclear ER at any given time point after injection of E2. This was in marked contrast to the distribution of ER in the Atlantic salmon where, after a single dose of E2, nuclear ER gradually increased from 500 fmole/g to over 11,000 fmole/g by 120 hours, while cytosol ER levels remained at approximately

Sample		DPM/Pellet*		Specific DPM	
		<u>H222</u>	<u>Rat IgG</u>	(from exchange assay)	
Chicken	, , , , , , , , , , , , , , , , , , ,	<u></u>	an a		
Oviduct SE	Total NS Specific	7271 3030 4609	2662 3646 0	4090	
Liver SE	Total NS Specific	16608 2690 13968		11694	
Trout					
Liver SE	Total NS Specific	1534 1350 184	899 1538 0	3067	

Table 8. Immunoprecipitation of [³HI-TAZ-labeled chicken and trout ER with H222

* Samples were labeled with 20 nM [³H]-TAZ $\pm 6 \mu$ M DES for 1 h at 30°C (chicken) or for 24 h at 4°C (trout), charcoal-stripped and then incubated for 6 h at 4°C with 1.25 μ g H222 plus 10 μ g rat IgG (or rat IgG alone). NS = non-specific binding.

ł

Ļ

1000-2000 fmole/g (Lazier et al., 1985). One difference between the two studies was that the Atlantic salmon ER extracts had been prepared from fresh tissue while the trout extracts were prepared from frozen livers.

In order to test the possibility that the subcellular distribution of ER after homogenization of trout liver had been altered by freezing of the tissue, an experiment was performed in which two groups of fish were injected with E2 and sacrificed at various times after injection. Livers from one group of animals were frozen in liquid nitrogen before preparation of nuclear and cytosol fractions while fresh livers were used to prepare ER fractions from the other group. There was no significant difference in the average body or liver weights between the two groups of animals and it was noted that the amount of DNA/g liver decreased while the %LSI (% liver weight/total body weight) increased after E2 treatment in both groups (Table 9). This was consistent with hypertrophy of the liver after E2 treatment which has been noted by others (Lawless, 1987; van Boheman <u>et al.</u>, 1981).

Microscopic examination of the pellets used for salt extraction of nuclear ER showed that the preparations from fresh tissue contained intact nuclei as well as a substantial number of intact erythrocytes. On the other hand, a large proportion of the nuclei from frozen livers were broken, with strands of loose chromatin evident when stained with methylene blue. This was reflected in the amount of DNA/g tissue determined for nuclear preparations from the two groups (Table 9 and Fig. 17A). While with fresh tissue there was a loss (15-40%) of DNA between the original homogenate and the nuclear fraction used for salt extraction of ER, loss of DNA from nuclei prepared from frozen tissue was much greater (60-90%), especially when the livers were taken from E2-treated animals. Evidently E2-treatment appears to promote the loss of DNA from nuclei of frozen hepatocytes, perhaps by altering the composition of the nuclear membrane, making it more susceptible to lysis by freezing. Proliferation of the membranous organelles of

Controls 2 Days* 5 Days* 7 Days* Frozen Fresh Frozen Fresh Fresh Fresh Frozen Frozen 141±9.6 Body weight (g) 149±20 150±10 149±15 127±11 136±14 141±6 n.d. 1.64 ± 0.22 Liver weight (g) 1.4 ± 0.24 1.1 ± 0.34 1.8±0.09 2.1±0.23 1.8 ± 0.30 2.8 ± 0.12 1.6 ± 0.68 %LSI 0.91±0.04 0.8±0.23 1.2 ± 0.07 1.1 ± 0.07 $1.6 \pm .017$ 1.3 ± 0.11 2.0 ± 0.14 n.d. DNA (mg/g) Homogenate 3.4 ± 0.23 2.5±0.13 1.8±0.07 2.1±0.63 1.3±0.25 2.11±0.17 0.6±0.20 0.98±0.20 0.20±0.07 Nuclear pellet 1.36 ± 0.10 1.27 ± 0.21 0.34±0.23 Nuclear salt-extract ER (fmole/g) 145±12 35±19 1285±176 859±321 889±137 70±23 469±154 60±3 ER (fmole/mg DNA) 110±17 24±13 601±34 994±272 286±86 375±105 419±171 336±234 ER (fmole/mg protein) 32±7 31±18 343±46 435±23 251±32 258±123 118±44 103±10 5.3±1.3 1.1±0.2 3.8±0.3 1.1 ± 0.3 3.5±0.3 0.36±0.06 4.13±0.23 0.39±0.03 Protein (mg/g)Cytosol ER (fmole/g) 3814±989 3169±678 4294±1186 3461±808 2050±552 2181±396 2787±496 2940±1226 ER (fmole/mg DNA) 1122+291 936±203 1718±474 1373±275 1139±307 1204±231 1327±236 1360±186 ER (fmole/mg protein) 102±21 54±12 149±32 64±12 106±31 56±13 126±24 63±19 36.5±2.9 52.5±2.9 20.3±1.8 41.0±3.0 22.3±0.4 44.6±5.9 Protein (mg/g) 62.3±9.5 27.6±2.3

Table 9. Comparison of fresh and frozen livers from E2-treated trout maintained at 9°C

. .

*Animals (4 per group) were injected with 5 mg/kg E2 and sacrificed at the times indicated. (± SEM)

Figure 17. Comparison of nuclear and cytosol preparations from fresh and frozen livers of E2-treated trout.

Trout were treated with 5 mg/kg E2 and sacrificed at the times indicated. Livers were either processed immediately dresh) or quick-frozen and stored at -70°C before processing (frozen).

I

A. DNA content of homogenates and nuclear preparations (mg/g liver).

B. Nuclear ER expressed as fmole/g liver.

C. Nuclear ER expressed as fmole/mg DNA.

D. Cytosol ER expressed as fmole/g liver.



~

فاجت ال

_ -

.

Figure 17.

.

ı

E2-stimulated hepatocytes, such as the endoplasmic reticulum and Coigi apparatus (van Bohemen <u>et al.</u>, 1981), may also make recovery of nuclear components more difficult. We have noted that the nuclei of Atlantic cod hepatocytes, which contain large amongs of stored lipids, are very difficult to isolate by low speed centrifugation of liver homogenates (unpublished observations). Hayward and Shapiro (1981) have also noted difficulties in collecting nuclei from livers of E2-stimulated Xenopus by centrifugation through sucrose.

Paralleling the loss of DNA from frozen nuclei, the second activate the operation of the second activate the differences in nuclear ER concentrations between fresh and frozen tissue was fresh or frozen, concentrations of nuclear ER expressed on a per gram liver basis were probably underestimates of the amount of chromatin-bound ER actually present in the original homogenate. Therefore it would appear to be more appropriate to express results for nuclear ER as fmoles/ng DNA rather than as fmoles/g liver, or to correct values to take into account the loss of DNA and ER from the nuclear fraction after homogenization and subsequent washing of nuclei.

Concentrations of cytosol ER activity from fresh and frozen tissue were not significantly different from each other when expressed as either fmoles/g liver (Fig. 17D) or as fmoles/mg DNA (Table 9). However if the results were expressed as fmoles/mg protein there appeared to be significantly less ER in the cytosol fraction from frozen tissue compared to fresh (Table 9). This is explained by the fact that the concentration of protein in the cytosol of frozen tissue was approximately twice that seen in fractions prepared from fresh tissue, due perhaps in part to the lysis of erthrocytes by freezing and recovery of hemoglobin in this fraction. Conversely, the concentration of protein in the nuclear fractions from fresh tissue were several-fold higher than those from frozen tissue, perhaps

93

F.

E 1
reflecting the presence of intact erythrocytes in the fresh nuclear pellet and substantial amounts of hemoglobin in the subsequent salt-extract. It is evident that caution should be used when comparing concentrations of ER expressed as fmoles/mg protein, as freezing of the tissue prior to homogenization may significantly alter the distribution of protein recovered in the various subcellular fractions.

The major conclusion to be made from this experiment is that while freezing of tissue prior to homogenization does result in loss of DNA and ER from the nuclear compartment, this cannot completely account for the high levels of ER detectable in the cytosol fraction after treatment of rainbow trout with E2. When expressed as fmoles/g tissue or as fmoles/mg DNA, cytosol ER concentrations were consistently several-fold higher than nuclear, whether or not the livers had been frozen. In this respect the apparent intracellular distribution of ER in the rainbow trout liver after E2 treatment was clearly different from that seen in the Atlantic salmon (Lazier <u>et al.</u>, 1985), but similar to the situation seen in the spotted seatrout (Cynoscion nebulosus) where the concentrations of cytosol ER in both non-vitellogenic and vitellogenic females were much greater than those found in the corresponding nuclear fractions (Smith and Thomas, 1990).

B. Effects of E2 treatment on livers of trout maintained at different temperatures

1. Hepatic ER concentration and distribution

a. Time course at $4^{\circ}C$

Fig. 18A and B show the concentrations of hepatic cytosol and nuclear ER, from animals maintained at 4°C, .t various times after a single injection of E2. Nuclear ER concentrations were calculated as fmoles/mg DNA while cytosol ER levels were expressed as fmoles/g liver. Unfortunately, homogenate DNA concentrations were not determined for all preparations, therefore the cytosol and nuclear results were not directly comparable. However the trend within each group was clear. Nuclear ER increased slowly from

94

and the second

ţ

-

4

ĥ

A REAL PROPERTY.

Figure 18. Time course of hepatic ER concentrations in E2-treated trout maintained at 4°C.

Trout were given a single injection of E2 (5 mg/kg).

- A. Nuclear ER (fmole/mg DNA)
- B. Cytosol ER (fmole/g liver)

3

C. Comparison of nuclear, cytosol and total ER concentrations. (Nuclear concentrations were corrected for an average value of 2 mg DNA/g liver).

f

ł

.

~





96

ι

í,

the with the

7

and the second second

8

the second

ŧ

¥

ı

ここの 聖 の 天-中子者 人

AND ADDRESS AND ADDRESS ADDRESS

Nuclear ER increased slowly from 31 ± 3 fmole/mg DNA in control animals to a final concentration of 1618 ± 135 fmole/mg DNA at 120 hours after injection. Cytosol ER levels also rose, from 1364 ± 537 fmole/g in controls to 6951 ± 842 fmole/g at 120 hours. Like in the Atlantic salmon (Lazier et al., 1985), the total concentration of hepatic ER increased after E2 treatment, although the distribution of ER after homogenization was quite different in the two species. The maximum amount of nuclear ER per gram tissue in the trout (3.5 pmole/g), correcting for an average value of 2 mg DNA per gram of liver, was not as great as in <u>Salmo salar</u> (12 pmole/g). However the total amount of trout hepatic ER 5 days after a single injection (10.2 pmole/g) compared favorably with the amount induced in the salmon. As in the chicken (Lazier and Haggarty, 1979; Lazier, 1979) and Xenopus (Hayward et al., 1980), E2 appeared to stimulate a pronounced up regulation of own receptor in the livers of treated animals. However, even if one considers a 50% i loss of nuclear ER from freezing, homogenization and washing procedures, the concentration of cytosol ER was always several fold greater than the concentration of nuclear ER at each time point (Fig. 18C).

b. Comparison of Time Courses at 9°C and 15°C

Initial experiments attempting to duplicate the above results with fish kept at 9.5°C (see Fig. 17B and C) revealed that both cytosol and nuclear E2-binding activity appeared to peak earlier (48 hours) than at 4°C (120 hours). Korsgaard <u>et al.</u> (1986) showed that the induction of serum vitellogenin by E2 treatment was markedly affected by the temperature at which the animals were maintained. This led us to hypothesize that temperature may also have an effect on the dynamics of ER distribution and induction in the liver of the trout. Therefore a comparison was made between two groups of animals, injected with the same dose of E2 (5 mg/kg) and kept at two different temperatures (9°C and 15°C) under the same light cycle regime. Previously published work (Lawless, 1987; Maitre <u>et al.</u>, 1985a and

1985b) suggested that a single injection of hormone caused only a transient rise in serum E2 which was insufficient to sustain maximal vitellogenic activity. Since we also wanted to study the dynamics of vitellogenin transcription, the fish in this experiment were given four injections of E2 at 2 or 3 day intervals in an attempt to maintain high serum E2 levels.

Fig. 19A and B compare hepatic nuclear and cytosol ER levels in the two groups of fish. Nuclear ER concentrations were calculated as fmoles/mg DNA, then corrected for the concentration of DNA in each homogenate to give a value expressed as fmoles/g liver. In the 15°C group, nuclear ER rose very rapidly from 323 ± 47 fmole/g (219 ± 61 fmole/mg DNA) in untreated fish to 5275 ± 873 fmole/g (1681 ± 252 fmole/mg DNA) by 24 hours after the first injection. There was a second peak of receptor activity at 72 hours (24 hours after the second injection). ER levels then gradually fell over the course of the next few days, despite subsequent injections of hormone. Values obtained at 168 hours and 240 hours were only slightly higher than controls, although these samples were taken 3 days after an injection and peaks of E2-binding activity between these time points may have been missed.

At 9°C the accumulation of nuclear ER was more gradual, reaching a peak of 4799 ± 1587 fmole/g (1778 \pm 588 fmole/mg DNA) at 72 hours, after the second hormone injection. Levels fell after this time point, although once again any rise in E2-binding activity between the third and fourth injections may have been overlooked.

Comparison of cytosol ER concentrations per gram liver at the two temperatures (Fig. 19B) revealed a three- to four-fold increase in ER activity after the first hormone injection in both cases. However, like the nuclear ER, the peak of cytosol binding occurred earlier at 15°C (24 hours) than at 9°C (48 hours). Levels in both groups oscillated after the second hormone injection, falling at 72 hours, then rising slightly by 96 hours. At 168 hours cytosol ER concentrations were still elevated, but by the third day after the last hormone injection (240 hours) binding activity had fallen back to control values in both

Figure 19. Comparison of hepatic ER concentrations in E2-treated trout maintained at 9°C or 15°C.

Animals were given injections of E2 (5 mg/kg) at the times indicated.

- A. Nuclear ER (fmole/g liver). *Values were calculated as fmoles/mg DNA and then corrected for the amount of DNA per gram liver determined for each sample homogenate.
- B. Cytosol ER (fmole/g liver).
- C. Total ER per gram liver.

.

* *





100

ı.

I the same second spinor grant

ł

.

•

5, L .

groups of animals. The oscillations in nuclear and cytosol ER concentrations may reflect irregular concentrations of E2 reaching the liver, which might be expected with a series of intraperitoneal injections. It would be very interesting to compare the levels of ER seen here to those which might be induced in animals implanted with slow release silastic capsules, where serum E2 is maintained at a more constant elevated level (Le Guellec <u>et al.</u>, 1988).

Concentrations of total ER per gram at 9°C and 15°C are compared in Fig. 19C. A peak of approximately 11.3 pmoles/g, a 3- to 4-fold induction above control levels, was observed at both temperatures. Once again this occurred 24 hours earlier at 15°C than at 9°C. However multiple injections of E2 capable of inducing sustained steady-state levels of vitellogenin and ER mRNAs and production of vitellogenin protein (see below) did not seem to be able to maintain induced levels of ER protein, at least as detectable by the binding assays used in this study.

The overall conclusion to be made from this study is that the temperature at which trout are maintained does appear to affect the timing of ER redistribution and induction after hormone treatment. Comparing the concentrations of nuclear ER at three temperatures, after a single injection of E2, Fig. 20A shows that the rise in salt-extractable ER is more rapid at 15° C than at either 9°C or 4°C. In fish maintained at 4°C it took 120 hours for nuclear ER to reach concentrations equivalent to those seen at 24-48 hours in the higher temperature groups. The same held true for cytosol ER (Fig. 20B). Maximum levels of 8000 fmole/g, an approximate four-fold increase over control concentrations, were seen in all three groups but peak cytosol ER levels were seen by 24 hours at 15° C, 48 hours at 9°C and not until 120 hours at 4°C. At 9°C cytosol ER had returned to control levels by 120 hours after a single hormone injection and even in animals receiving multiple injections of hormone, cytosol ER levels had fallen by 240 hours, three days after the last injection, at both 9°C and 15°C.

Figure 20. Comparison of hepatic ER concentrations in E2-treated trout maintained at 4°C, 9°C and 15°C.

Animals had received a single injection of E2 (5mg/kg).

A. Nuclear ER (fmole/mg DNA).

B. Cytosol ER (fmole/g liver).

- * Animals were from a different experimental group from the others maintained at 9°C.
- ¥ Animals had received 4 injections of E2.

1

1

1

1





1



.....



t

Various mechanisms for the temperature effects, such as increased hormone/receptor affinity or more rapid transport of hormone to the liver at warmer temperatures, could be suggested and are discussed in more detail later. However, regardless of the mechanism, differences in timing of both the accumulation of nuclear ER and induction of total liver ER have been observed at different temperatures. This in turn might influence the dynamics of expression of hormone-responsive genes, such as vitellogenin, which, at least in animals such as <u>Xenopus</u> and chicken, require high concentrations of the nuclear hormone/receptor complex for activation and maintenance of transcription. The slower rise in nuclear ER at 9°C and 4°C may be related to the delay in activation of vitellogenin gene expression at these temperatures, as discussed below.

2. Other E2-induced changes in trout liver

E2 treatment has been shown to have a marked effect on liver weight, morphology and hepatic DNA and RNA concentrations in the rainbow trout (van Bohemen <u>et al.</u>, 1981 and 1982; Lawless, 1987) and in other teleosts (Emmersen <u>et al.</u>, 1979; Korsgaard <u>et al.</u>, 1986). Fig. 21A-D and Table 10 summarize the changes in % liver-somatic index (%LSI, calculated as % liver weight/body weight), RNA and DNA per gram tissue and the RNA:DNA ratio in livers of rainbow trout treated with multiple injections of E2 at 9°C and 15°C. As noted by others (Lawless, 1987; Bjornsson <u>et al.</u>, 1989; Olsson <u>et al.</u>, 1989), the %LSI gradually increased with time after E2 treatment in both temperature groups (Fig. 21A), rising from $0.89 \pm 0.05\%$ in controls to $1.73 \pm 0.08\%$ by 240 hours at 9°C, and from $0.95 \pm 0.15\%$ to $2.5 \pm 0.05\%$ at 15°C. In both groups %LSI was significantly higher than controls ($p \le 0.05$) at all time points 48 hours or more after injection. In addition, by 96 hours or later, %LSI was significantly greater at 15°C than at 9°C ($p \le 0.05$). Note also that a single injection of E2 at 15°C was able to stimulate a significant rise in %LSI by 240 hours after treatment, although this increase was not as great as when

ş

ş

a sub-

Time	% Liver-Somatic Index		RNA/DNA		Serum Vitellogenin (mg/n ⁻¹)	
	<u>9°C</u>	<u>15°C</u>	<u>9°C</u>	<u>15°C</u>	<u>9°C</u>	<u>15°C</u>
<u>Control</u>	0.89±0.05	0.95±0.15	4.0±0.50	5.3±0.90	0	0
<u>24 hour</u>	1.02±0.06	0.87±0.02	4.1±0.63	2.5±0.35	0	0.17±0.14
<u>48 hour</u>	1.06±0.03	1.28±0.08	4.2±0.71	4.7±0.73	0	0.80±0.16
<u>72 hour</u>	1.19±0.04	1.24±0.04	N.D.	2.4±0.20	0.35±0.12	2.70±0.34
<u>96 hour</u>	1.19±0.05	1.44±0.08*	4.7±0.47	3.6±0.43	0.79±0.08	6.60±0.76
<u>168 hour</u>	1.48±0.16	1.98±0.25*	6.1±0.33	6.9±1.1	3.18±0.58	27.1±3.7
<u>240 hour</u>	1.73±0.08	2.50±0.05*	7.3±0.70	12.1±2.7*	8.90±0.22	70.1±2.1
240 hour (single injection)		1.65±0.11		4.1±0.27		38.0±7.1

Table 10. Comparison of %LSI, RNA/DNA ratios and serum vitellogenin concentrations in E2-treated trout maintained at 9°C and 15°C

.

* Significantly higher than 9°C (p \leq 0.05). (± SEM)

.

- -

\$

1

I.

.

Figure 21. Comparisons of %LSI, DNA and RNA concentrations and RNA:DNA ratios in 72-treated trout at 9°C and 15°C.

÷

A. % Liver-Somatic Index (%LSI).

B. RNA (mg/g liver).

ł

à

C. DNA (mg/g liver).

D. RNA:DNA ratio.

* Significantly different from 9°C ($p \le 0.05$). ¥ Significantly different from untreated controls ($p \le 0.05$).

۰,

;



Figure 21.

,

ł

Т

.

multiple injections were given (Table 10).

Although there was some fluctuation over the time course, the amount of RNA/g liver was significantly greater than control values by 168-240 hours of treatment in both temperature groups (Fig. 21B). On the other hand, after an initial rise between 24-48 hours after treatment, there was an overall decline in the concentration of DNA/g by 168-240 hours (Fig. 21C), suggesting that the increase in liver weight was due to hypertrophy of the tissue rather than cell division. This has been observed in other teleosts (Emmersen et al., 1979) as well as in Xenopus (Follett and Redshaw, 1968). The RNA:DNA ratio at 168-240 hours after treatment was significantly higher than in controls at both temperatures (Fig. 21D). Since much of the RNA in the cell is presumably ribosomal RNA, this increase in the RNA:DNA ratio may represent induction of rRNA, observed by Le Guellec et al. (1988) to occur around 10 days after treatment. An increase in E2-dependent mRNA transcripts would also be expected. At 240 hours the RNA:DNA ratio was significantly higher at 15°C than at 9°C, suggesting perhaps a more pronouced induction of rRNA at the higher temperature. Note however that by 240 hours after a single injection of E2 at 15°C, the RNA:DNA ratio was not significantly different from controls (Table 10), suggesting that continuous exposure to E2 might be necessary for this increase to occur.

3. Serum vitellogenin

İ

The appearance of vitellogenin in the serum of rainbow trout after treatment with E2 has been well documented (van Bohemen <u>et al.</u>, 1982; Maitre <u>et al.</u>, 1985a; Le Guellec <u>et al.</u>, 1988) and estrogen-stimulated synthesis and secretion of vitellogenin by trout hepatocytes has been demonstrated <u>in vitro</u> (Maitre <u>et al.</u>, 1986; Vaillant <u>et al.</u>, 1988). In view of the observations of Korsgaard <u>et al.</u> (1986), who showed temperature dependence in the vitellogenic response of Atlantic salmon, we wanted to examine the

effect of temperature on the appearance of vitellogenin in the serum of E2-treated rainbow trout.

As shown in Table 10 and Fig. 22, vitellogenin was first detectable in serum by 24 hours after E2 treatment at 15°C, but not until 72 hours at 9°C. The induction of serum vitellogenin was very rapid at 15°C, with concentrations rising from undetectable in controls to over 70 mg/ml by 240 hours in animals which had received multiple injections of hormone. This was several-fold greater than the concentration seen in the serum of vitellogenic female trout (approximately 13 mg/ml, van Bohemen <u>et al.</u>, 1981), although the higher concentrations in immature fish may result from the lack of clearance of vitellogenin by the ovaries (Wallace and Jared, 1968). A single injection of E2 was also able to induce high levels of serum vitellogenin by 240 hours after treatment at 15°C, although the concentration (38 mg/ml) was approximately half of that seen in the animals which had received four injections. In contrast, the rise in serum vitellogenin was much slower and less pronounced at 9°C, with levels increasing from undectectable 0-48 hours after treatment to 8.9 mg/ml at 240 hours.

The pattern of vitellogenin appearance at 9°C was similar to that observed by Le Guellec <u>et al.</u> (1988) in chronically stimulated trout. This group showed that, in animals which had received E2 in the form of a slow release silastic implant, viteflogenin was first detectable in serum by 3 days after treatment, rose slowly to a concentration of 9 mg/ml by day 7 and then increased rapidly to 17 mg/ml between days 7 and 10. On the other hand, animals which had received only a single injection of E2 (3 mg/kg) showed similar levels of serum vitellogenin up to day 7, after which concentrations declined to 1 mg/ml by day 12. This is in contrast to the results presented here, where 38 mg/ml of vitellogenin was observed 10 days after a single injection of E2 (5 mg/kg) at 15° C. However, similar to our results, Maitre <u>et al.</u> (1985a and 1985b) reported that up to 45 mg/ml of vitellogenin was present in the serum of mature male trout 15-25 days after a

Figure 22. Time course of serum vitellogenin concentrations in E2-treated trout maintained at 9°C and 15°C.

Serum samples were obtained from animals which had received hormone injections at the indicated times and vitellogenin concentrations (mg/ml) were determined by rocket immunoelectrophoresis. .

4

t



Figure 22.

ب به جدیف بودنیود.

.

.

سومت حصدهان

أسبب مثاكله

single injection of hormone, despite a steady decline in the level of E2 in the blood after the first day. Le Guellec <u>et al.</u> (1988) postulated that the difference in results could be due to the more rapid metabolism of E2 in juvenile trout (Hansson and Rafter, 1983). However the results presented here, also obtained with juveniles, suggest that other mechanisms might be involved as well.

Seasonal differences in the response of rainbow trout to E2 have been reported. Elliott <u>et al.</u> (1980) noted that immature fish were most responsive to injections of E2 during the months of the breeding cycle when serum E2 and vitellogenin levels were normally rising or high in mature fish (April to October). In November to March, when mature fish would be spawning and serum E2 is low, E2 treatment could not stimulate the same high levels of vitellogenin synthesis. Baroiller <u>et al.</u> (1987) have noted that the metabolic clearance rate of E2 was low during the period of natural exogenous vitellogenesis and increased dramatically near the onset of oocyte maturation and ovulation in trout. They suggested that seasonal variation in serum sex steroid-binding globulin concentrations, which are under pituitary control, might affect the availability of E2 to hepatic metabolism. This might explain the difference in the observations of Le Guellec <u>et al.</u> (1988), who perfomed their single injection experiments in April, and the results of our experiments which were performed in September. Examination of basal concentrations of E2-binding activity throughout the breeding cycle, both in serum and in liver, might be informative.

Another comparison between our results and those of Le Guellec <u>et al.</u> (1988) is worthy of mention. When they administered an E2-containing silastic implant to fish which had received a primary injection of E2 (3 mg/kg) several weeks previously, a more rapid accumulation of serum vitellogenin was observed than with a primary implant. Vitellogenin was detectable in serum within the first day after implantation and rose to 20 mg/ml by 7-10 days. The authors concluded that this demonstrated a "memory effect" in the response to hormone, similar to that seen with the re-induction of Xenopus or chicken vitellogenin after estrogen withdrawal (Baker and Shapiro, 1978; Maeenpa, 1976). However this "secondary response" looks strikingly similar to the pattern of serum vitellogenin induction with chronic treatment at 15°C seen in the present study, obtained with animals which had not previously received E2. After an initial 24 hour lag period, our results paralleled the "secondary response" curve until 7 days, and by 10 days our serum vitellogenin levels had increased to 70 mg/ml compared to 20 mg/ml in the "secondary response" experiment. Le Guellec et al. (1988) state only that the primary and secondary response experiments were performed in June and August, respectively. As can be seen from the temperature experiments performed here, a difference in water temperature of only 6° C may have a significant effect on both the timing and the quantity of serum vitellogenin induced by a given dose of E2. Therefore the temperature at which the experiments are performed, as well as the time of year, appear to be important factors influencing the duration of the lag period in hormone responsiveness, as well as the magnitude of the response. Water temperature should be recorded if accurate comparisons are to be made between different experiments.

4. Vitellogenin mRNA

a. Characterization of the vitellogenin message

Fig. 23 shows a Northern blot of total trout liver RNA (20 μ g per lane) from E2-treated and control trout, hybridized to the rainbow trout vitellogenin cDNA clone pSG Vg 5.09. A prominant band of approximately 6 kilobases was apparent in samples from treated animals (lanes 3-6) while no hybridization signal was evident in the samples from untreated animals (lanes 1, 2, 7 and 8). The relative abundance of vitellogenin message was greater in samples prepared from E2-treated animals kept at 15°C (lanes 5 and 6) than in those prepared from animals kept at 9°C (lanes 3 and 4). Three

higher molecular weight bands (7.5 kb, 8.1 kb, and 10.4 kb) could also be seen in lanes 5 and 6. These bands appeared in several of the RNA samples analyzed on Northern blots, increasing in abundance as the major vitellogenin band increased. They may represent high molecular weight unspliced precursor RNAs. The diffuse areas of radioactivity seen in the regions between the 28S and 18s rRNAs, and below the position of the 18S rRNA (lanes 5 and 6), were only visible in over-exposed autoradiographs and probably represent degradation products of the vitellogenin mRNA.

The size calculated for the major vitellogenin message, based on the sizes of 28S (4.1 kb) and 18S (1.8 kb) rRNAs (Londei <u>et al.</u>, 1982) or using an RNA ladder of known sizes, ranged from 5.3 to 7.2 kb with an average size of 6.6 kb. This was in good agreement with the 6660 nucleotides reported for the vitellogenin message by Le Guellec <u>et al.</u> (1988), and the 6.3 kb and 7.2 kb sizes reported by Chen (1983) and Valotaire <u>et al.</u> (1984) respectively. The major bands of hybridizaton to pSG Vg 5.09 increased in intensity with time after E2 treatment, characteristic of a true vitellogenin mRNA, and a second putative vitellogenin cDNA clone, pSG Vg 5.50, also hybridized to RNA bands of the same size (see section E).

b. Quantitation of vitellogenin mRNA

Vitellogenin mRNA was quantitated by scanning densitometry of the autoradiographs of slot blots containing serial dilutions of total liver RNA and hybridized to pSG Vg 5.09. Results were normalized to the amount of $poly(A)^+$ RNA in each sample, as detected by hybridization of end-labeled $oligo(dT)_{15}$ to blots which had been stripped of vitellogenin probe. The results were expressed relative to the amount of hybridization to a standard sample of total liver RNA from a single E2-treated animal which was included on all the slot blots (see Materials and Methods, section L).

Figure 23. Northern blot of total liver RNA from E2-treated and control trout probed with pSG Vg 5.09.

RNA was isolated from trout maintained at 9°C or 15°C. Each lane contained 20 μ g of total RNA. Positions of the 28S (4.1 kb) and 18S (1.8 kb) rRNAs and the vitellogenin mRNA (V) are indicated.

Lanes 1 & 2: Controls (9°C);

Lanes 3 & 4: E2-treated (9°C) 48 h after injection;

Lanes 5 & 6: E2-treated (15°C) 48 h after injection;

Lanes 7 & 8: Controls (15°C).



Figure 23.

Several other probes were also tried, unsuccessfully, in an attempt to normalize the results to an RNA species which did not change in response to E2 treatment. These included a rainbow trout testis tubulin cDNA, which had been used by Olsson <u>et al</u>. (1989) to normalize results for metallothionein mRNA expression in E2-treated trout. This probe, when labeled by the random hexanucleotide procedure, hybridized to two RNA bands, 2.1 kb and 1.6 kb in size, on Northern blots containing 20 μ g of total liver RNA (Fig. 24). However the signal was visible only after 2-3 weeks of exposure, even though the probe was labeled to high specific activity (10⁹ cpm/ μ g). Hybridization of this probe to slot blots, which contained only 0.125-4.0 μ g of total RNA, showed only background levels of signal after 3 weeks of exposure. It was subsequently confirmed that, while abundant in brain and testis, this is a relatively rare message in trout liver (A. Garber, personal communication). Because of the low signal to background ratio, the tubulin cDNA clone was unsuitable for normalizing the results from the slot blot assays used in the present study.

Hybridizations with a chicken actin cDNA (Cleveland <u>et al.</u>, 1980) and a chicken 28S rRNA cDNA (S. Gupta, Dalhousie University) were also attempted but produced very high levels of non-specific binding in Northern blots and uneven hybridization to both Northerns and slot blots (data not shown). These clones were also judged unsuitable as probes for the purpose of normalizing the trout vitellogenin mRNA data. In the end, although the level of poly(A)⁺ RNA per liver cell was expected to rise with time after E2 treatment, oligo(dT)15 was chosen because of the rapid exposure times (4-12 hours) and very low background levels obtained in hybridizations with this probe. Normalizing to poly(A)⁺ RNA lowered the variability between samples at any given experimental time point, although relative values per μg of total RNA calculated for vitellogenin message may be underestimated at times \neg hen the total cellular content of poly(A)⁺ was greater than in the standard preparation.

Figure 24. Northern blot of total liver RNA from E2-treated trout probed with a trout testis tubulin cDNA.

RNA was isolated from trout maintained at 9°C and 15°C. Each lane contained 20 μ g of total RNA. Positions of the 28S (4.1 kb) and 18S (1.8 kb) rRNAs and the tubulin mRNAs (T) are indicated.

24 h after injection (Lane 1: 15°C; Lane 2: 9°C); 48 h after injection (Lane 3: 15°C; Lane 4: 9°C); 72 h after injection (Lane 5: 15°C; Lane 6: 9°C). ٩.

Statesto a astronome

New Courses And Annual Section

*

;

10°0 - 1 21 - 10°

•

ALC: NOT THE OWNER OF THE OWNER OF



• •

•

,640 IL 840

-

Figure 24.

c. Temperature effects on the induction of vitellogenin mRNA

• 1

As shown in Fig. 25A and Table 11, the induction of vitellogenin mRNA after treatment with E2 was very rapid at 15°C, with significant amounts of message present 8-24 hours after injection. The appearance of vitellogenin mRNA was delayed until 24 hours in fish treated at 9°C. In both groups of animals, given multiple injections, levels of vitellogenin message continued to increase in a somewhat stepwise manner, with the magnitude of the steps being greater at 15°C than at 9°C.

Table 11A compares the relative amounts of vitellogenin mRNA from the single injection experiment performed at 4°C with the amounts detected after the first E2 injection at 9°C and 15°C. The delay in appearance of vitellogenin message was more pronounced at 4°C than at 9°C. Significant levels were not detectable until 48 hours after injection. After a prolonged plateau, levels of message doubled between 96 and 120 hours at 4°C, but only reached levels seen between 8-24 hours at 15°C or at 48 hours at 9°C. The initial appearance and the early stages of induction of vitellogenin mRNA appear to correlate with the timing of increased nuclear E2-binding activity in the three temperature groups. While maximum nuclear ER was detected by 24 hours in the 15°C group, the increase in nuclear ER levels was slower at 9°C and slower still at 4°C (Fig. 20A), paralleling the delayed appearance and accumulation of vitellogenin message at the lower temperatures.

Table 11B shows that a single injection of E2 (5mg/kg in ethanol/saline) was able to stimulate significant levels of vitellogenin mRNA synthesis, detectable in total liver RNA 7 days (at 9°C) and 10 days (at 15°C) after treatment. In fact, apparent levels after the single injections were higher than those found in the livers of fish treated with multiple hormone injections. This may be due to the dilution of mRNA sequences by rRNA in the livers of animals which had received several hormone injections, as discussed below. Like the serum vitellogenin results, the detection of significant levels of vitellogenin mRNA 7-10 days after a single hormone injection conflict with the results of Le Guellec

÷

Figure 25. Relative vitellogenin mRNA levels in livers of E2-treated trout maintained at 9°C and 15°C.

Vitellog in mRNA was quantitated by analysis of slot blots containing 0.125-4 $\partial \mu g$ of total RNA probed with pSG Vg 5.09. Results were normalized to the relative amount of poly(A)⁺ RNA in each sample and expressed relative to a standard sample from a single individual included on each blot.

A. Relative amounts of vitellogenin mRNA at 9°C and 15°C.

B. Results from (25A) corrected for the RNA:DNA ratio for each sample and expressed as relative Vg mRNA/mg DNA.



٠,

.

× н.ч. ц.‡Ж. ч

. .

**

.

٠

c^

Figure 25.

یشد سه ۲

مود و ۱۳۵۵ مرد بر بر از ا^{یند} بر از ایک

.

Table 11. Relative vitellogenin mRNA levels after single and multiple injections of E2 in trout maintained at different temperatures

A. Single injection:

The area and a set of the set of the

ANY SUPPORT

Ĭ

ころうちょう 長、 人名

	Vitellogenin mRNA (relative units)			
Time	4°C	9°C	15°C	
Control	0	0	0	
8 hours	0	0	0.09±0.09	
24 hours	0	0.11±0.04	0.76±0.16	
48 hours	0.22±0.08	0.60±0.14	2.28±0.46	
72 hours	0.20±0.02	n.d.	n.d.	
96 hours	0.28±0.05	n.d.	n.d.	
120 hours	0.47±0.13	n.d.	n.d.	

B. Multiple injections vs single injections:

Time and Temperature	Multiple injections	Single injections
7 days (9°C)	1.28±0.12	6.4±0.90
(corrected)*	1.38±0.05	n.d.
10 days (15°C)	4.95±0.70	8.75±1.83
(corrected)*	10.46±1.23	4.9±0.61

* Results were corrected for the RNA:DNA ratio for each sample, as described in Materials and Methods (section L). (\pm SEM)

n.d.= not determined.

<u>et al.</u> (1988), who observed only a transient rise in vitellogenin message 2 days after a single injection and a steady decrease to undetectable levels by 6 days. Once again, this could be due to seasonal differences in hormone responsiveness.

As can be seen in Fig. 25A, there appeared to be a significant drop in vitellogenin mRNA levels at the 168 hour time point at 15°C, although concentrations increased once again by 240 hours. There are at least two possible explanations for the apparent decrease. First, serum E2 levels may have fallen sufficiently by three days after the previous injection to result in destabilization and loss of vitellogenin mRNA sequences. However the subsequent increase in vitellogenin mRNA at 240 hours, also three days after a hormone injection, and the observation that vitellogenin mRNA levels are high 10 days after a single injection of E2 (Table 11B), make this explanation unlikely. On the other hand, as was noted in Table 10, the ratio of RNA per mg DNA had approximately doubled by 168 hours at 15°C. An induction of rRNA was observed by Le Guellec et al. (1988) in both their primary and secondary response experiments. As noted by this group, an increase in rRNA content could dilute mRNA sequences per μg of total RNA, resulting in an underestimation of the actual concentration of vitellogenin transcripts per cell using slot blot assays. If we converted the relative concentration of vitellogenin message per μ g of total RNA to relative concentration per mg of DNA (see Materials and Methods, section L), we find that, in the 15°C group, the amount of vitellogenin mRNA remained relatively constant between 48 and 168 hours, then increased approximately 3- to 4-fold between 168 and 240 hours (Fig. 25B). This is very similar to the pattern of vitellogenin mRNA induction noted by Le Guellec et al. (1988) in animals given primary silastic implants of E2. Although their RNA values were expressed in different units (parts per million rather than units relative to a standard induced sample), they also observed an initial rise in vitellogenin mRNA starting 2 days after treatment, a plateau at about 500 ppm between 3 and 7 days of treatment and then a rapid five-fold increase in vitellogenin message levels between

4

1

a suit r und

-

7-10 days.

The RNA:DNA ratio did not increase as dramatically in the 9°C chronic treatment group as in the 15°C group (Table 10). Hence correction for the ratio of RNA to DNA did not significantly alter the apparent relative levels of vitellogenin mRNA in the 9°C group (Fig. 25B). As was noted above, vitellogenin mRNA sequences accumulated more slowly at 9°C than at 15°C and the steady-state levels remained more or less constant between 96-240 hours. The relative concentration of vitellogenin mRNA at 168 hours was approximately half of that seen in the 15°C group and a rapid increase in concentration between 168 and 240 hours was not observed.

Also previously noted, the hepatic RNA:DNA ratio had not changed significantly by 240 hours after a single injection of E2 at 15°C. Table 11B shows that the relative vitellogenin mRNA value in the animals receiving a single hormone injection, after correction for the RNA:DNA ratio, was approximately half of that seen in the group receiving multiple injections at 15°C. This agrees well with the serum vitellogenin data which showed that, 10 days after a single injection at 15°C, the concentration of vitellogenin protein was only half of that present in serum from animals which had received four injections (Table 10).

In conclusion, the temperature at which trout were maintained during estrogen treatment appeared to have marked effects on both the timing of appearance of vitellogenin mRNA in the liver and the amount of message which accumulated over the course of the treatment period. Temperature also appeared to affect the increase in the amount of total RNA in the liver relative to the amount of DNA. This increase resulted in the dilution of vitellogenin message at the later time points in the group of fish which had received multiple injections of E2 at 15°C, but not in the 9°C group. Single doses of hormone were able to induce substantial amounts of vitellogenin mRNA transcripts at all temperatures, although, at least at 15°C, the ratio of RNA:DNA in the liver was not greater at 10 days

after treatment than it was in controls. This suggests that sustained levels of E2 might be necessary for an increase in total liver RNA, presumably most of it rRNA, but not for the induction and accumulation of vitellogenin mRNA.

d. Ratios of Vg protein:mRNA and Vg mRNA:nuclear ER

Table 12 compares the ratios of serum vitellogenin and vitellogenin mRNA levels in the 9°C and 15°C treatment groups. At 15°C there appeared to be a significant increase in the vitellogenin protein:mRNA ratio between 168 and 240 hours. This would suggest that the dramatic increase in serum vitellogenin at this time was due not only to increased accumulation of vitellogenin message but also to an increase in the translational and/or posttranslational capacity of hepatocytes. One of the obvious candidates for this role would be increased numbers of ribosomes, reflected in the higher RNA:DNA ratios at these time points (Table 10). However animals which had received a single injection of E2 at 15°C also had a vitellogenin protein: RNA ratio equivalent to that seen in the group receiving multiple injections, even though the RNA:DNA ratio was not greater than in controls. Thus there may be other E2-induced factors which also contribute to the increased efficiency of translation of the vitellogenin message and/or the post-translational processing and secretion of the protein into the blood. We also note that at 240 hours at 9°C, when the amount of vitellogenin mRNA was equivalent to that seen at 168 hours at 15°C, the vitellogenin protein:RNA ratio was less than half of that seen at the higher temperature. This suggests that the hepatic changes responsible for the enhancement of vitellogenin protein synthesis and secretion might be temperature sensitive as well.

Table 12 also examines the ratios of vitellogenin mRNA to nuclear ER levels. It is interesting to note that vitellogenin mRNA sequences still appeared to accumulate even after the concentration of detectable nuclear ER had fallen at 15°C. Again this

Time	Serum Vitellogenin	Vitellogenin mRNA	Ratio	Nuclear ER	Ratio
9°C	(mg/ml)	(relative units/mg DNA)	(Serum Vg:Vg mRNA)	(pmole/mg DNA)	(Vg mRNA:Nuclear ER)
Control	0	0	0	0.56±0.11	0
24 hours	0	0.09±0.09	0	1.14±0.23	0.1
48 hours	0	0.47±0.18	0	1.11±0.31	0.4
96 hours	0.79±0.08	1.69±0.32	0.5	1.24±0.17	1.4
168 hours	3.18±0.58	1.38±0.05	2.3	1.07±0.22	1.3
240 hours	8.90±0.22	2.94±0.18	3.1	1.49±0.24	2.0
15°C					
Control	0	0	0	0.58±0.23	0
24 hours	0.17±0.14	0.27±0.06	0.6	1.82±0.32	0.2
48 hours	0.80±0.16	1.63±0.16	0.5	1.29±0.23	1.3
96 hours	6.60±0.76	2.50±0.42	2.6	1.09±0.20	2.3
168 hours	27.1±3.7	3.26±0.73	8.3	0.99±0.59	3.3
240 hours	70.1±2.1	10.5±1.23	6.7	0.93±0.24	11.2
240 hours (single injectio	38.0±7.1	4.9±0.61	7.7	0.37±0.02	13.3

Table 12. Ratios of serum Vg:Vg mRNA and Vg mRNA:Nuclear ER in E2-treated trout at 9°C and 15°C

.

-

was especially notable in the group which had received a single hormone injection 10 days previously, where detectable nuclear ER was no greater than in controls. This argues that increased amounts of nuclear ER may act as a 'rigger for vitellogenin mRNA transcription but that sustained occupancy of estrogen response elements by high levels of hormoneactivated receptor, which appear to be required for continued transcription of the vitellogenin genes in Xenopus (Shapiro, 1982), may not be necessary in the trout.

5. Regulation of ER mRNA

a. Identification and quantitation of the ER message

Initial attempts to identify the rainbow trout ER mRNA were made using the cDNA clone pOR8, which contains the entire coding sequence of the human estrogen receptor (Greene et al., 1986; Green et al., 1986). Northern blots, hybridized and washed under low stringency conditions (35% formamide and washing at 42°C in 2 X SSC) showed considerable non-specific hybridization to both the 28S and 18S rRNAs in human (MCF7 cells), Xenopus liver, chicken oviduct and trout liver total RNA preparations (Fig. 26A, Fig. 27A and Fig. 29A). Hybridization to 28S rRNA was especially notable in trout liver poly(A)⁻ RNA fractions (Fig. 26A). The probe also hybridized to a 6.2 kb band in RNA from MCF7 cells and to a 7.5 kb band in chicken oviduct total RNA, corresponding to the sizes of the ER mRNAs previously reported for these species (Walter et al., 1985). A diffuse smear of binding between the ribosomal RNAs was apparent in the trout poly(A)⁺ RNA (Fig. 26A), although a discreet band of approximately 1.7 kb could also be observed. Higher temperature washing (50°C) and a longer exposure of the same blot resulted in the disappearance of most of the non-specific rRNA binding in chicken total and trout poly(A)⁻ RNA and revealed a 3.9 kb band as well as the 1.7 kb band in the poly(A)⁺ RNA from livers of E2-treated trout (Fig. 26B). These were felt to be good candidates for the rainbow trout ER message, although they were

Figure 26. Northern blot of RNA from chicken oviduct and trout liver probed for ER mRNA.

- A. Hybridized to pOR8 insert in 35% formamide with washing at 40°C in 2 X SSC (low stringency);
- B. Washed at 50°C in 2 X SSC;
- C. Reprobed with oligonucleotide "probe C" at 42°C (low stringency);
- D. Probed with "C" and washed at 70°C.

Lane 1: total RNA from chicken oviduct $(30 \mu g)$;

Lane 2: liver poly(A) + RNA (7 μ g) from untreated trout;

Lanes 3 & 5: poly(A)⁺ RNA (20 µg) from E2-treated trout;

Lane 4: poly(A)⁻ RNA (10 µg) from E2-treated trout.

cER = chicken oviduct ER mRNA


in.

130

-

Figure 27. Northern blot of total RNA from trout and <u>Xenopus</u> liver and MCF7 cells probed for ER mRNA.

RNA (20 μ g/lane) was isolated from livers of E2-treated rainbow trout (lanes 1-4), MCF7 human breast carcinoma cells (lanes 5 & 6) and E2-treated <u>Xenopus</u> liver (lane 7).

- A. Hybridized to pOR8 insert in 35% formamide with washing at 42°C in 2 X SSC (low stringency);
- B. Reprobed with oligonucleotide "probe C" at 42°C (low stringency).

hER = human ER mRNA; rtER = trout ER mRNAs.



.

1

.,

.

132

considerably smaller than the ER messages found in human (6.2 kb), chicken (7.5 kb), mouse (6.5 kb), and rat (6 kb). <u>Xenopus</u>, on the other hand, has a family of ER mRNAs, two of them quite small (9 kb, 6.5 kb, 2.8 kb and 2.5 kb; Weiler <u>et al.</u>, 1987).

:3)

H

The cloning of the rainbow trout ER cDNA by Pakdel <u>et al.</u> (1989) made further low stringency hybridizations with heterologous probes unnecessary. Using the published nucleotide sequence of the trout ER cDNA, two oligonucleotide probes, complementary to regions encoding the presumptive DNA-binding domain (probe "C") and the steroidbinding domain (probe "E"), were synthesized. The deduced amino acid sequences of these regions are identical in the estrogen receptors of human, chicken, <u>Xenopus</u> and trout. The 33-nucleotide regions chosen also represented areas containing the fewast possible nucleotide differences between the various ER cDNAs and so could potentially be used as probes for ER messages in other species. The nucleotide sequences complementary to probes "C" and "E" are shown in Fig. 28, with differences between the rainbow trout sequence and those of human, chicken and <u>Xenopus</u> ER cDNAs indicated.

High st 3 ency hybridization and washing of Northern blots with either of the oligonucleotiae probes revealed a strong 3.9 kb signal in both poly(A)⁺ and total RNA from trout livers (Fig. 26D and Fig. 29B and C). The insert of the trout ER cDNA clone, pRT-ER, also hybridized to the same size band (Fig. 29D). Occasionally a faint 4.5 kb band could be observed in poly(A)⁺ RNA preparations from E2-treated animals (Fig. 26C). The 3.9 kb size determined for the major trout ER mRNA in these experiments was somewhat larger than the 3.5 kb message reported by Pakdel <u>et al.</u> (1989), although the minor 4.5 kb band was the same size in both studies. Differences in mRNA sizes between different strains of fish is a posssible explanation.

The major band of hybridization to probes "C" and "E" migrated just in front of the 28S rRNA in total RNA preparations. Given the non-specific hybridization of pOR8 to rRNA seen under lower stringency conditions, we were concerned that the presence of

134

i

<u>OLIGO C:</u> (complement)

۷.

L1

1 1 1

1

1

1

1 1 1

· Cyrran Arres

١

Trout	⁵ 'CAC	TAC	GG	A GT	t tgg `	тсс то	GC GA	G G	GC TO	3C3,
<u>Human</u>	Т	• •	T		С		т			
<u>Xenopus</u>	Т		Т	Т	С	Т	т	A	т	
<u>Chicken</u>			т	G	С	т	Т			

OLIGO E:(complement)

Trout	⁵ 'ATG	AGC	AAC	AAA	GGC	ATG	GAG	CAC	СТТ	тас ^{3'}
<u>Human</u>		т						т	G	
<u>Xenopus</u>			т	G	Ì		A		ΤG	
<u>Chicken</u>					A	L			G	

Figure 28. Nucleotide sequences complementary to oligonucleotide probes "C" and "E".

Differences between the rainbow trout ER sequence and the sequences from the corresponding regions of the human, <u>Xenopus</u> and chicken ER cDNAs are shown.

Figure 29. Northern blot of total RNA from livers of E2-treated trout probed for ER mRNA.

Each lane contains 20 μ g total RNA. Before reprobing, the blot was stripped with 0.1 X SSC/1% SDS at 80°C and autoradiographed to ensure that the previous signal had been removed.

- A. Hybridized to pOR8 insert at low stringency;
- B. Reprobed with oligonucleotide "probe C" (high stringency);
- C. Reprobed with oligonucleotide "probe E" (high stringency);
- D. Hybridized to pRT-ER insert at high stringency.



ĥ

ribosomal RNA might interfere with the quantitation of the ER message in total RNA. However several observations led us to believe that the hybridization of the oligonucleotide probes to total RNA represented only the ER message. Very little hybridization could be detected in trout liver poly(A)⁻ RNA, even under low stringency conditions (Fig. 26C and D). In cases where significant hybridization to trout "(A)-" RNA was observed, subsequent probing with end-labeled oligo(dT)15 revealed the presence of significant amounts of $poly(A)^+$ sequences which had not been retained on the oligo(dT)-cellulose column used to prepare this fraction (Fig. 30C and D). In addition, neither probe hybridized to rRNA from human, chicken or Xenopus, even under low stringency conditions (Fig. 26C and Fig. 27B). Furthermore, probes "C" and "E" did not cross hybridize with the ER mRNAs of chicken oviduct (Fig. 26C and D), MCF7 cells or Xenopus liver (Fig. 27B). This illustrated the high specificity of these oligonucleotide probes for trout ER sequences, which differ from the corresponding chicken and human ER sequences by only 2-5 nucleotides. This would also suggest that the 1.7 kb band visible in trout $poly(A)^+$ RNA, which hybridized to probe "C" under low stringency conditions (Fig. 26C), might be more closely related in sequence to the trout ER message than are the ER messages from the other species.

Both probes "C" and "E", as well as the pRT-ER insert, hybridized in the same relative proportions to the 3.9 kb bands in total RNA preparations from livers of different individuals. This band was quite sharp compared to the non-specific binding seen with lower stringency hybridizations with pOR8 (Fig. 29A-D). Both the 3.9 kb and 4.5 kb bands were enriched in $poly(A)^+$ RNA, but the ability of the end-labeled oligonucleotides to produce a strong signal in a relatively short time (2-7 days) permitted reliable quantitation of ER message levels in total RNA.

Probe "E" was used to quantitate the ER message using slot blots of total liver RNA from animals in the various treatment groups and the results were normalized to the amount

of $poly(A)^+$ RNA in each sample. As was observed by Pakdel <u>et al.</u> (1989), the levels of ER mRNA in control livers were lower than those seen in the livers of E2-treated trout (Fig. 30A). Reprobing of the same blots with oligo(dT)15 revealed that equivalent amounts of $poly(A)^+$ RNA were present in each preparation (Fig. 30C). Once again, no hybridization of probe "E" to total RNA from <u>Xenopus</u> liver or chicken liver, kidney or oviduct was noted (Fig. 30B).

b. Hepatic ER mRNA levels in trout kept at different temperatures

Fig. 31 compares the relative amounts of ER message over time in the 9°C and 15°C treatment groups, normalized to the amount of $poly(A)^+$ RNA in each sample (A) and corrected for the relative RNA:DNA ratio at each time point (B). Control values in both groups were very low, almost at the limit of detection of the slot blot assays. In the 15°C group, levels of ER message were induced approximately 10-fold by 8 hours after hormone treatment and by approximately 30-fold at 24 hours. In trout kept at 9°C, injection of E2 also induced a 10-fold increase in ER message by 8 hours, although at 24 hours levels were approximately half of those seen in the 15°C group. At 48 hours, ER mRNA levels were significantly higher ($p \le 0.05$) in the 9°C experiment than in the 15°C group and appeared to remain higher up to 168 hours of the treatment period. However caution should be used when interpreting these data because vitellogenin mRNA molecules were also accumulating during this time and made up a greater percentage of the $poly(A)^+$ -containing sequences at 15°C than at 9°C. Therefore, if we consider the possible dilution of ER mRNA, as a function of poly(A)⁺ RNA, by vitellogenin mRNA sequences, the differences between the 9°C and 15°C groups may not be as significant as they appear. Maximum induction of ER mRNA (approximately 200-fold above control values) was apparent at 168 hours at both temperatures. By 240 hours, the relative concentrations of ER message appeared to have fallen in both groups but were still about 20-30 times greater than control

Figure 30. Slot blots of total RNA hybridized to oligonucleotide "probe E" and oligo(dT)15.

Slots contained serial dilutions of 4.0-0.125 μ g RNA and were probed with oligonucleotide "probe E" (A & B), then reprobed with end-labeled oligo(dT)₁₅ (C & D).

- A & C. Total RNA from livers of E2-treated (1 & 6) and untreated (2-5) rainbow trout.
- B & D. RNA from:

E2-treated rainbow trout liver (1: poly(A)⁻ RNA; 2: total RNA), <u>Xenopus</u> liver (3) and chicken liver (4), kidney (5) and oviduct (6). í.

ł

ł



jero:

Figure 30.

* * 1

Figure 31. Relative ER mRNA levels in livers of E2-treated trout maintained at 9°C and 15°C.

ER mRNA was quantitated by analysis of slot blots containing $0.16-10.0 \mu g$ of total RNA probed with oligonucleotide "probe E". Results were normalized to the relative amount of poly(A)⁺ RNA in each sample and expressed relative to a standard sample from a single animal included on each blot.

- A. Relative amounts of ER mRNA at 9°C and 15°C;
- B. Results from (31A) corrected for the RNA:DNA ratio for each sample and expressed as relative ER mRNA/mg DNA.



i.



Figure 31.

•

values. Once again, considering the high relative concentrations of vitellogenin mRNA by this time, as well as the probable increase in rRNA, the actual concentrations of ER mRNA per cell may be much higher. The important conclusion to be made, however, is that ER mRNA was induced in response to E2 treatment and remained induced despite an overall decline in the total E2-binding activity of the liver (see Fig. 19C and Table 14). Trout which had received a single injection of E2 also showed higher relative levels of ER mRNA than controls (Table 13B), although detectable ER concentrations had completely subsided to pretreatment levels in the livers of these animals.

A comparison of relative ER mRNA levels after a single injection of E2 in fish maintained at 4°C, 9°C and 15°C is shown in Table 13A. In fish kept at 4°C, the ER message was induced at least 3 to 10-fold by 8 hours after treatment. Relative levels of ER mRNA, as a proportion of poly(A)⁺ RNA, were equivalent to or greater than the values in the 9°C and 15°C groups at 8 and 24 hours after injection, before significant accumulation of vitellogenin mRNA sequences. As with vitellogenin mRNA induction, this might reflect a seasonal difference in hormone responsiveness, as the 4°C experiments were performed in December while the higher temperature experiments were performed in September. The higher relative levels of ER mRNA in the animals kept at 4°C could also be a reflection of differing concentrations of poly(A)⁺ and/or total RNA in hepatocytes of the different groups. Without data on the concentration of hepatic RNA/mg DNA in the 4°C treatment group, the relative concentrations of ER mRNA in this experiment may not actually be equivalent to the results of the 9°C and 15°C experiments. However the trends within each group may still be compared.

As can be seen in Table 14, the ratio of total hepatic ER activity to relative ER mRNA concentration remained constant over the time course at 4°C. Increases in ER message paralleled the induction of E2-binding activity, with maximum levels of both ER activity and ER mRNA being seen 120 hours after injection. In the 9°C group, the

Table 13. Relative hepatic ER mRNA levels after single and multiple injections of E2 in trout maintained at different temperatures

A. Single injection:

<u></u>		ER mRNA (relative units)			
Time	4°C	9°C	15°C		
Control	0.18±0.18	0.07±0.05	0.01±0.005		
8 hours	0.59±0.18	0.17±0.07	0.18±0.18		
24 hours	0.47±0.21	0.15±0.08	0.58±0.07		
48 hours	0.53±0.10	0.82±0.13	0.46±0.06		
72 hours	0.91±0.23	n.d.	n.d.		
96 hours	0.95±0.24	n.d.	n.d.		
120 hours	1.38±0.28	n.d.	n.d.		

B. Multiple injections vs single injections:

Time and temperature	Multiple injections	Single injections
7 days (9°C)	1.05±0.03	0.45±0.12
(corrected)*	0.83±0.14	n.d.
10 days (15°C)	0.33±0.02	0.23±0.02
(corrected)*	0.72±0.13	0.17±0.02

* Results were corrected for the RNA:DNA ratio for each sample, as described in Materials and Methods (section L). (\pm SEM)

n.d.= not determined.

temperature	Total ER activity	ER mRNA	Ratio
<u>4°C</u>	(pmole/g)	(relative units)	(ER:ER mRNA)
Control	1.43 ± 0.54	0.18 ± 0.18	7.9
24 hours	3.40±0.32	0.47 ± 0.21	7.2
48 hours	3.86±0.92	0.53±0.10	7.3
72 hours	7.16±1.61	0.91±0.23	7.9
<u>96 hours</u>	7.49±1.57	0.95±0.24	7.9
<u>120 hours</u>	10.2±1.11	1.38±0.28	7.4
<u>9°C</u>	(pmole/mg DNA)	(relative units/mg DNA)	(ER:ER mRNA)
<u>Control</u>	1.40±0.10	0.06±0.04	23
24 hours	2.41±0.32	0.11±0.08	22
48 hours	4.17±0.21	0.66±0.17	6.3
<u>96 hours</u>	3.34±0.20	0.69±0.23	4.8
<u>168 hours</u>	3.05±0.02	1.15±0.09	2.6
240 hours	2.55±0.14	0.83±0.14	3.1
15°C	(pmole/mg DNA)	(relative units/mg DNA)	(ER:ER mRNA)
<u>Control</u>	2.10±0.46	0.01±0.005	200
24 hours	5.05±0.88	0.22 ± 0.02	23
48 hours	3.38±0.43	0.28±0.08	12
<u>96 hours</u>	2.84±0.31	0.24±0.07	12
<u>168 hours</u>	3.62±0.71	1.36±0.75	2.7
<u>240 hours</u>	2.01±0.24	0.72±0.13	2.8
<u>240 hours</u> (single injection)	0.95±0.17	0.17±0.02	5.6

Table 14. Ratios of total hepatic E2-binding activity:ER mRNA in trout treated with E2					
at different temperatures					

ť

relative levels of ER mRNA at 24 hours after treatment were lower than in the 15°C group, which appeared to correlate with the more gradual increase in detectable ER activity at 9°C (see Fig. 19). On the other hand, in animals maintained at both 9°C and 15°C, the ratios of total hepatic ER activity to ER mRNA gradually fell over the course of the treatment period. This might suggest that the efficiency of translation of the ER message was decreasing over the time course in these groups. This contrasts with the vitellogenin protein to mRNA ratios (Table 12) which increased over the course of the experiment. One explanation could be that as increasing numbers of ribosomes were directed towards the endoplasmic reticulum by secretory signals present in the abundant vitellogenin messages, less were available for translation of non-secreted proteins such as the estrogen receptor. Another possibility is increased degradation of the ER protein, possibly as a function of falling E2 concentrations between injections. Once again, comparison of the present results with experiments performed using slow release silastic capsules, which provide sustained levels of serum E2, would be interesting.

In both the 4°C and 9°C experiments, ER mRNA levels increased before the appearance of vitellogenin transcripts. Pakdel <u>et al.</u> (1989) also showed induction of trout ER mRNA within 3-6 hours after injection of fish, while detectable levels of vitellogenin transcript did not appear until between 6 and 12 hours. Therefore it would appear that enhanced accumulation of ER mRNA after E2 treatment is both more rapid and less temperature sensitive than is the induction of vitellogenin mRNA. It would also be interesting to see if the ER message remains induced after vitellogenin transcription has ceased. Barton and Shapiro (1988) have observed that, in Xenopus, a single transient dose of E2 was capable of eliciting the induction of hepatic ER mRNA for at least 4 months after the initial treatment, long after the disappearance of vitellogenin transcripts.

In summary, treatment of trout with E2 appeared to enhance the expression of the hepatic ER gene, resulting in both increased levels of ER mRNA and increased

concentrations of E2-binding activity in the livers of treated animals. This appeared to occur more rapidly than the induction of vitellogenin mRNA, at least in the groups of animals treated at 4°C and 9°C. As was noted with vitellogenin mRNA, relative levels of ER mRNA remained higher in treated fish than in controls, despite falling concentrations of nuclear ER activity and an eventual decline in the amount of detectable hepatic ER.

6. <u>3.2 mRNA</u>

The cDNA clone pSG 3.2, isolated from a cDNA library prepared from an E2-induced trout liver, has some sequence homology to B-lactalbumin and may code for an albumin-like protein (M. Tenniswood and W. Davidson, personal communications). pSG 3.2 hybridized to a 2.6 kb band in Northern blots of total liver RNA prepared from both control and treate' trout (Fig. 32A). This is somewhat smaller than the 3.2 kb size originally reported (Lawless, 1987) but may simply reflect differences in RNA isolation methods and gel systems used. Low stringency washes in 2 X SSC and long exposures of Northern blots revealed other higher molecular weight bands which also hybridized to pSG 3.2 in RNA preparations from some individuals, both treated and untreated (Fig. 32B). These bands, which varied in size between individuals, disappeared with higher stringency washing in 0.1 X S C. Davidson et al. (1988 and 1989) have described two fatty acid-binding proteins in trout plasma (approximate molecular weight 68 kDa), similar in amino acid composition to albumins of higher vertebrates. They have suggested that the two forms may exist in different proportions in different individuals. The observation that pSG 3.2, a putative albumin cDNA, cross-hybridizes at lower stringency to a second mRNA species which appears in only some individuals is certainly suggestive of two related forms of albumin mRNA, expressed in different amounts in different individuals. Xenopus laevis also has two forms of serum albumin, encoded by two related but different mRNAs which are expressed in different amounts in the liver (Westley et al.,

147

the street

Figure 32. Northern blots of total liver RNA from control and E2-treated trout hybridized to pSG 3.2.

Ne

ŗ

- A. Total RNA (20 μg/lane) from untreated (lane 1) and E2-treated trout maintained at 4°C (lanes 2 & 3), 9°C (lane 4) and 15°C (lane 5) hybridized to pSG Vg 5.09 and pSG 3.2 under high stringency conditions;
- B. Total RNA (20 µg/lane) from untreated (lanes 1 & 2), E2-treated (lanes 3-6) and tamoxifen-treated trout (lanes 7-10) probed with pSG 3.2 and washed in 2 X SSC (low stringency).

Positions of the 28S (4.1 kb) and 18S (1.8 kb) rRNAs, the vitellogenin mRNA (V) and 3.2 mRNA (3.2) are indicated.



.

-

Figure 32.

k ₽ 1981). The variation in size of the second mRNA species seen here might also suggest some polymorphism between individuals. This would not be surprising since albumins appear to be relatively rapidly evolving proteins (Wilson <u>et al.</u>, 1977).

Lawless (1987) noted that the steady-state levels of the mRNA hybridizing to pSG 3.2 varied after treatment of trout with E2. Animals receiving either a single injection or a silastic implant of E2 demonstrated a two- to three-fold increase in 3.2 mRNA steady-state levels within 48 hours following treatment, then a steady decline in levels after 4-7 days. The author suggested that the initial increase in 3.2 mRNA was probably a reflection of \neg_v erall increase in transcriptional activity of the liver and not a specific effect of E2. On the other hand, he suggested that the decline in 3.2 mRNA levels during the period of maximum induction of one vitellogenin message might reflect competition for the transcriptional apparatus and/or destabilization of 3.2 sequences, as in the case of albumin transcripts in Xenopus (Wolffe et al., 1985; Reigel et al., 1986b and 1987).

The results of slot blot quantitation of 3.2 mRNA levels in livers of trout, treated with E2 and maintained at 9°C and 15°C, are shown in Fig. 33. No significant difference in mRNA levels, normalized to the amount of $poly(A)^+$ RNA in each sample, could be seen over the time course at either temperature, except for a drop in relative concentration at 240 hours in the 15°C group. As was noted for vitellogenin mRNA, this might be explained by dilution of message due to increased amounts of rRNA in the liver at this time. In fact, when corrected for RNA:DNA ratios, there was no significant difference between controls and the 240 hour samples in the 15°C group. These results are somewhat different from those of Lawless (1987) in that we did not observe an initial increase in 3.2 mRNA levels relative to controls. However, normalizing to the amount of poly(A)⁺ RNA per sample may have masked any rise due to overall increases in hepatic transcriptional activity. Also, we did not observe a decline in the steady-state levels of 3.2 mRNA which would suggest destabilization of this message. If 3.2 is in fact an

Figure 33. Relative levels of 3.2 mRNA in livers of E2-treated trout maintained at 9°C and 15°C.

...

RNA levels were quantitated by analysis of slot blots containing 0.125-4.0 μg of total liver RNA and normalized to the amount of poly(A)⁺ RNA in each sample. Results are expressed relative to a standard RNA preparation included on all blots. * Results were corrected for the RNA:DNA ratio of the samples.

i



i

Figure 33.

ı

т 1

¥ ...

albumin mRNA, this would represent another difference between the pattern of RNA expression during vitellogenesis in the rainbow trout and <u>Xenopus</u>.

C. Effects of tamoxifen on vitellogenin and hepatic ER mRNA levels in the rainbow trout

To determine if the antiestrogen, tamoxifen, had any effects on vitellogenesis, a group of fish was treated with 25 mg/kg tamoxifen citrate. Fig. 34 shows a Northern blot of total liver RNA from control, E2-treated and tamoxifen citrate-treated trout, maintained at 9°C and sacrificed six days after injection, probed with pSG Vg 5.09. Vitellogenin mRNA appeared to be induced in the livers of tamoxifen-treated animals but not to the extent observed in the E2-treated group. This was confirmed by slot blot quantitation of vitellogenin mRNA. As shown in Table 15, the amount of vitellogenin mRNA present in control livers was negligible, while in E2-treated fish it was ap⁻ coximately 15-fold greater than in the tamoxifen-treated group. On the other hand, the amount of ER mRNA in the E2-treated group was 2-fold greater than in tamoxifen-treated fish (level of significance $p \le 0.1$) and 3-fold greater than in controls (level of significance $p \le 0.05$). ER mRNA levels in the tamoxifen-treated group were not significantly different from the controls.

It can be concluded that, like in <u>Xenopus</u> liver (Reigel <u>et al.</u>, 1986a) and in contrast to the chicken (Sutherland <u>et al.</u>, 1977; Lazier <u>et al.</u>, 1981), tamoxifen appears to act as a partial estrogen agonist in the rainbow trout liver, at least with respect to vitellogenin mRNA induction. On the basis of the high affinity of 4-hydroxytamoxifen for the trout hepatic ER, we would predict that, as in other systems, OHT is the active metabolite (Jordan <u>et al.</u>, 1977). Because of its weak agonist effects, we also predict that tamoxifen might act as an estrogen antagonist in the trout. Tamoxifen or OHT are known to bind to estrogen receptors of other species but inhibit the receptor's ability to activate transcription. If tamoxifen also acts as an antiestrogen in the trout, it would be a very helpful tool for identifying and examining the ER-mediated aspects of vitellogenesis in this animal. Figure 34. Northern blot of total liver RNA from control, E2-treated and tamoxifentreated trout, probed with pSG Vg 5.09.

Total liver RNA ($20\mu g$ /lane) was isolated from fish sacrificed 6 days after a single injection. Sizes of markers (BRL RNA ladder) and the position of the vitellogenin mRNA (V) are indicated.

Lanes 1-4: controls (ethanol/saline-injected);

Lanes 5-8: E2-treated (5 mg/kg);

Lanes 9-12: tamoxifen citrate-treated (25 mg/kg).

î

......

1

1

1

:





Figure 34.

.

,

1

.

Table 15. Relative levels of vitellogenin and ER mRNAs in livers of E2-treated and tamoxifen-treated rout

E2-treated	Tamoxifen-treated
C () 00	
6.4 ± 0.9	0.42 ± 0.17
0.45 ± 0.12	0.23 ± 0.06
	0.45 ± 0.12

* normalized to the amount of poly A+ RNA in each sample. (± SEM)

Table 16. Specific E2-binding activity in liver cytosol and nuclear extracts of various species of fish

Species	Nuclear		Cyte	osol
	Control	Treated	Control	Treated
<u>Hemitripterus</u> <u>americanus</u> (Sea Raven)	88 ± 8.0	912 ± 37	1265 ± 8.0	1042 ± 52
<u>Myoxocephalus</u> <u>octodecimspinosus</u> (Sculpin)	44 ± 31	207 ± 0.7	839 ± 143	437 ± 121
<u>Macrozoarces</u> <u>americanus</u> (Ocean Pout)	15 ±10	34 ± 18	1457 ± 88	2471 ± 477
<u>Oncorhynchus mykiss</u> (Rainbow Trout)	31 ± 20	410 ± 107	2453 ± 853	2012 ± 479
<u>Salmo salar</u> (Atlantic Salmon)*	600	9100	510 ± 50	640 ± 70

Specific [³H]-E2 bound (fmole/g) (±SEM)

Treated animals, maintained at 9°C, were sacrificed 4 days after a single injection of E2 (2 mg/kg). Fractions were prepared from frozen livers and assayed with 10 nM [³H]-E2 \pm 1 μ M DES.

* Data from Lazier et al. (1985).

D. Hepatic E2-binding activity and Vg and ER mRNAs in other species of fish

1. Concentration and distribution of hepatic E2-binding activity

Table 16 compares the levels of DES-competable E2-binding activity in liver cytosol and nuclear salt-extracts from various species of fish. Treated animals had been given a single intraperitoneal injection (2 mg/kg) of E2 in ethanol/saline four days before sacrifice. Hepatic cyosol and nuclear salt-extracts were prepared according the procedure of Lazier et al. (1985) and single point binding assays were performed using $10 \text{ nM} [^3\text{H}]$ -E2 ± 1 μ M DES.

As in the rainbow trout, DES-competable E2-binding activity was found in nuclear extracts and cytosol fractions of control and treated <u>Hemitripterus americanus</u> (sea raven), <u>Mvoxocephalus octodecimspinosus</u> (sculpin) and <u>Macrozoarces americanus</u> (ocean pout). Concentrations of ER were low in the nuclear extracts of untreated fish and appeared to be induced after E2 treatment in all but the ocean pout, although none showed the very high levels of activity seen in the Altantic salmon. However, as was noted previously, peaks of binding activity may have occured earlier than four days after injection of E2 in animals maintained at 9°C. Also as in the trout, and unlike the Atlantic salmon, the cytosol form $c \in$ E2-binding activity appeared to predominate in both control and untreated animals. This was also found to be the case in <u>Cynoscion nebulosus</u> (Smith and Thomas, 1990). It can be concluded therefore that the induction of E2-binding activity after E2 treatment, and the apparent intracellular distribution of this activity in response to the same homogenizationconditions, may differ among teleost species.

2. Vitellogenin and ER mRNAs

Fig. 35A-C show Northern blots of total liver RNA from E2-treated sculpin (<u>Myoxocephalus octodecimspinosus</u>), sea raven (<u>Hemitripterus americanus</u>), winter flounder (<u>Pseudopleuronectes americanus</u>) and Atlantic cod (<u>Gadus morhua</u>). Fig. 35A

shows that, under high stringency conditions, the rainbow trout vitellogenin cDNA clone, pSG Vg 5.09, hybridized strongly to a banc if appproximately 7 kb in flounder and sculpin RNA, and weakly to a band of the same size in cod and sea raven RNA. This would suggest that the vitellogenin mRNAs of flounder and sculpin are more closely related in sequence to the rainbow trout mRNA than are those of the cod and sea raven.

Northern blots probed with oligonucleotides "C" and "E", complementary to regions of the rainbow trout ER cDNA which encode the putative DNA-binding and steroid-binding domains, are shown in Fig. 35B and C. Both oligonucleotides hybridized strongly to bands of similar size in total RNA from livers of rainbow trout, sea raven and sculpin, and to a slightly smaller band in liver RNA of the winter flounder. A very faint signal was also observed in the RNA preparation from Atlantic cod liver, probed with oligonucleotide "E". As was noted previously, no signal could be observed in RNA preparations obtained from chicken oviduct or <u>Xenopus</u> liver, even though the sequences of the ER mRNAs in these species differ from that of the rainbow trout by only a few nucleotides (Fig. 28). It can be concluded, therefore, that the ER mRNAs of the sea raven, sculpin and flounder must be very closely related in sequence to the ER message of the rainbow trout, at least within the highly conserved regions coding for the DNA- and steroid-binding domains.

In conclusion, the livers of several species of non-salmonid fish contained measurable quantities of DES-competable E2-binding activity, as well as mRNAs which appear to be closely related to the rainbow trout ER message. Livers of animals treated with E2 also contained mRNAs which hybridized to the trout vitellogenin cDNA clone. The ability of rainbow trout sequences to cross-hybridize with messages in these species should facilitate the cloning of their vitellogenin and ER cDNAs. These would be valuable tools for studying the evolutionary relationships among teleost vitellogenins and estrogen receptors, and for examining the regulation of vitellogenesis at the molecular level in these fish.

Figure 35. Northern blots of total RNA from various species of fish, probed with pSG Vg 5.09 and ER oligonucleotide probes.

RNA samples were prepared from livers of rainbow trout (RT), sea raven (R), sculpin (S), cod (C), winter flounder (F) and <u>Xenopus laevis</u> (X). RNA was also obtained from chicken oviduct (O). Sizes of markers (BRL RNA ladder) are indicated.

Total liver RNA (20 μ g/lane) was isolated from E2-treated animals and hybridized under high stringency conditions with:

- A: pSG Vg 5.09 (trout vitellogen in cDNA);
- B: "probe "E" (trout ER, steroid-binding region);
- C: "probe C" (trout ER, DNA-binding region).



-1.35

-1.35

•

....

; 2



Figure 35.

ų

E. Characterization of pSG Vg 5.50

1. Southern and Northern analysis using pSG Vg 5.09 and 5.50

As described in the introduction, Le Guellec <u>et al.</u> (1988) have sequenced pSG Vg 5.09 and shown that it represents the 3'-end of a message with significant homology to chicken and <u>Xenopus</u> vitellogenin cDNAs. They also described a family of cDNA clones with the same restriction digest pattern as pSG Vg 5.09. On the other hand, pSG Vg 5.50, although hybridizing to the same 6660 nucleotide RNA as pSG Vg 5.09, had a different restriction enzyme digestion pattern from the pSG Vg 5.09 family. Furthermore, Southern analysis of trout genomic DNA, restricted with *Bam* HI or *Hind* III, showed that the two probes hybridized to several common bands but that pSG Vg 5.50 also hybridized to at least three unique genomic fragments (Lawless, 1987). The suggestions made to explain these results were: a) that pSG Vg 5.09 and pSG Vg 5.50 could represent cDNA clones derived from different regions of the same RNA; b) that pSG Vg 5.09 and pSG Vg 5.50 were cDNA clones derived from mRNAs transcribed from different vitellogenin genes; or c) that pSG Vg 5.50 contained sequences homologous to pSG Vg 5.09 but also sequences derived from the blunt-end ligation of two unrelated messages during the cloning process.

In the present study, it was also found that pSG Vg 5.50 hybridized to the same 6 kb and higher molecular weight RNA bands as pSG Vg 5.09 (see Fig. 23). Comparison of densitometric scans of a Northern blot probed with pSG Vg 5.09, stripped and then reprobed with pSG Vg 5.50, showed that the relative intensities of hybridization to the 6 kb band of RNA preparations from several E2-treated animals were virtually equal for both probes (Table 17). Long exposure times or hybridization of pSG Vg 5.50 to RNA from control animals did not reveal any additional bands, suggesting that both probes were hybridizing to the same message, which was present only in the livers of E2-treated animals.

RNA sample*	Relative intensity of	Relative intensity of hybridization		
	pSG Vg 5.09	pSG_Vg 5.50		
<u>48 h #1</u>	1.0	1.0	1.0	
<u>48 h #7</u>	0.74	0.76	0.97	
<u>72 h #1</u>	1.25	1.16	1.08	
<u>72 h #3</u>	0.73	0.66	1.1	
<u>72 h #6</u>	1.24	1.30	0.95	
<u>96 h #9</u>	2.02	1.61	1.25	
<u>96 h #10</u>	2.64	2.51	1.05	
<u>120 h #10</u>	3.56	3.55	1.0	
<u>120 h #6</u>	4.03	3.77	1.07	

Table 17. Comparison of relative intensities of hybridization of pSG Vg 5.09 andpSG Vg 5.50 to RNA from E2-treated trout

* RNA samples were prepared from livers E2-treated trout maintained at 4°C and sacrificed at the indicated times. Relative intensity of hybridization was determined by scanning densitometry of a Northern blot sequentially hybridized with the two probes.

No. of the local diversion of the local diver

94 2⁴ 5

ç

Southern blots of trout liver genomic DNA, digested with various restriction enzymes and probed with pSG Vg 5.09 and pSG Vg 5.50 are compared in Fig.36A and B. With slight differences in intensity, the two probes hybridized to the same three bands in *Bam* HI digested DNA and the same four bands in *Eco* RI digested DNA. However, while hybridizing to the same bands as pSG Vg 5.09 in *Hind* III, *Pst* I and *Xba* I digested DNA, pSG Vg 5.50 also hybridized to several additional bands in these lanes. This would suggest that pSG Vg 5.50 has at least some sequence homology to pSG Vg 5.09 but also contains additional sequences.

It is not unreasonable to propose that there are at least two genes coding for vitellogenin in the rainbow trout. Multiple copies of vitellogenin genes are present in <u>Xenopus</u> and chicken as well as in insects (Wahli, 1988). The sum of the lengths of the major hybridization bands in the *Bam* HI digest (12.2 kb, 11.5 kb and 10.2 kb) is approximately 33.9 kb. It is possible that the 2 kb pSG Vg 5.09 probe could be hybridizing to fragments of a single gene, as the last 2 kb of both the chicken vtgII and <u>Xenopus</u> vitellogenin A2 cDNAs cover more than 10 xb of geneomic sequence. However vitellogenin genes in <u>Xenopus</u> and chicken are in the order of 16-20 kb in length (Gerber-Huber et al., 1987; van het Schip et al., 1987). The single 8.6 kb *Hind* III and *Xba* I bands and the small *Pst* I bands (total length 2-4 kb) which hybridize to pSG Vg 5.09 are better reconciled with the idea that there are at least two genes coding for vitellogenin in the trout. Since salmonids are known to be stable tetraploids (Ohno et al., 1969), it would not be surprising to find at least two genes for both albumin and vitellogenin.

2. Sequence analysis of pSG Vg 5.50

-

To further characterize the pSG Vg 5.50 clone, both ends were sequenced in one direction from the forward and reverse universal primer sequences present in the pUC8 vector. As shown in Fig. 37A, the 350 nucleotides at the 5'-end of pSG Vg 5.50 were

Figure 36. Southern blot of trout liver genomic DNA hybridized to pSG Vg 5.09 and pSG Vg 5.50.

DNA (10 μ g/lane) was digested with *Bam* HI, *Eco* RI, *Hind* III, *Pst* I or *Xba* I and the blot was hybridized with pSG Vg 5.09 (A), stripped and reprobed with pSG Vg 5.50 (B). Sizes of markers (*Hind* III digested lambda phage DNA) are indicated.

164

1

ŧ

t antitic



.

•

2

Α.

Bam

۰.

#

Eco

Hind Pstl Xbal Bam Eco

B.

Barn Eco Hind Pstl Xbal

-





Figure 36.

165
Figure 37. Partial nucleotide sequences of the 5'- and 3'-ends of the pSG Vg 5.50 insert.

Dideoxy sequencing of the ends of the pSG Vg 5.50 insert was performed with SequenaseTM 2.0 from the forward and reverse M13 primer sequences in the pUC8 vector.

A. Partial sequence determined for the 5'-end of the cDNA, using the reverse M13 primer reaction. Restriction sites and differences in sequence between pSG Vg 5.50 and pSG Vg 5.09 are indicated. Numbers refer to the positions of nucleotides in the pSG Vg 5.09 cDNA sequence (Le Guellec et al., 1988).

***: nucleotides missing in pSG Vg 5.09.

>>>>>: direction of sequencing.

B. Partial sequence determined for the putative anti-sense strand at the 3'-end of pSG Vg 5.50, using the forward M13 primer reaction. The derived amino acid sequence of a potential open reading frame in the coding direction is indicated above the nucleotide sequence. Restriction sites and sequencing ambiguities (+++) are also shown.

<<<<<: direction of sequencing.

Α.	>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>
	5'TC CAA AAA <u>GTC tac</u> tit grt arg tac tca tgg agg att aaa gtt gtg gac tgg atg aag gga cag acc tgt 881
	GGA CTC TGT GGA ARG GCT GAT GGC GAA CAC AGA CAG GAG TAC CGT ACA CCC AGT GGC CGC CTG ACC AA <u>g agc tc</u> a A G Sac 1
	GTC AGC TTT GCG CAT TCC TGG GTG CTT CCT TCT GAT AGC TGC CGT GAT GGG TCT GAA TGT CTC ATG AAG <u>CTC GAG</u> *** C Ava 1
	TCT GTG AAG CTG GAG AAG CAG GTG ATT GTT GAT GAC AGG GAG TCC AAA TGC TAC TCT GTT GAG CTT GTG CTG CGT C
	TGT CTG CCT GGA TGC TCT CCA GTG AGG ACC ACC CCC ATC ACC ATC GGT TTC CAC TGC 1230
В.	GA ATT CTG CTA GAA GTA CCT GGC CAC GGC ACA TAC ACT GGT CTA CTA AGT CCT AGA AGT GT(C/G) GAC T(C/G)T GCA
	Gly His Leu Glx Asn Leu Ser Gly Lys Leu lle Pro Asp Gly Ser. (C/G)CA A(C/G)A CTA GAA TTC CTC (T/G)AC TTA CCC GTA <u>GAC CT(C/G)</u> TTA GAG TCA CCG TTT GAG TAT GGC CTA <u>CCT AGG</u> ++ + Pst 1(?) Bam H1
	Thr Val Asn lie Val lie lie Gly Asp Asn Asn Trp lie Leu Leu Asp Leu Asn Glu Ser Gln His Leu Phe Cys Phe TGC CAC TIG TAT CAA TAA TAG CCC CTG TTA TTA A <u>CC TAG G</u> AA GAA CTA GAG TTG CTT TCA GTT GTA AAC AAA ACG AAG3' Bam Hi
	Figure 57.

· .

. .

167

I

Ł

virtually identical to nucleotides 881-1230 of the pSG Vg 5.09 clone. The differences are four changed nucleotides, which might simply reflect ambiguities in the sequencing data, and an insertion of three nucleotides (1 extra codon), which would not change the reading frame of the message and might be attributed to microheterogeneity at a splicing junction or allelic polymorphism in the vitellogenin message. An *Ava* I site, not originally reported in the restriction map of pSG Vg 5.50 (Le Guellec <u>et al.</u>, 1988), was found by sequencing, in a position identical to a site found in pSG Vg 5.09. The putative alignment of the two cDNA clones is presented in Fig. 38A and shows that at least 1.5 kb of the pSG Vg 5.50 cDNA clone would have to lie 3'- to the position of the poly(A)⁺ tail of pSG Vg 5.09.

The sequence determined for 218 nucleotides at the 3'-end of pSG Vg 5.50 is given in Fig. 37B. A potential open reacing frame of at least 41 amino acids, in the same orientation as the ORF at the 5'-end of the clone, is indicated. Interestingly, a search of the GenBank database revealed some nucleotide sequence homology between the sequence of the 3'-end of pSG Vg 5.50 and the coding sequence of chicken vitellogenin II. However no clear homology was evident at the level of the derived protein sequence.

To see if the 3'-end of pSG Vg 5.50 actually hybridized to the vitellogenin mRNA, the plasmid was digested with *Hind* III and a 340 base pair fragment, which lies at the extreme 3'-end of the insert (indicated in Fig. 38A), was isolated in a low melting point agarose gel. This fragment was labeled with α -[³²P]-dCTP and hybridized to a Northern blot of total liver RNA from E2-treated and control fish. As shown in Fig. 38B, a very faint hybridization signal at the 5.8 kb position of the vitellogenin message could be detected after a 48 hour exposure. However, as shown in Fig. 39B, the same probe also gave a very weak signal from the 1150 base pair band derived from an *Eco* RI/*Bam* HI digest of pSG Vg 5.09. It also hybridized weakly to the 600 base pair *Sac* I/*Sac* I fragment of pSG Vg 5.50. Neither of these fragments should contain sequences homologous to the probe. This indicates that the labeled *Hind* III fragment might contain some contaminating

- Figure 38. Restriction maps of pSG Vg 5.09 and pSG Vg 5.50 inserts and hybridization of Vg 5.50 fragment to trout liver RNA.
 - A. Restriction maps of pSG Vg 5.09 and pSG Vg 5.5 inserts, derived from Le Guellec <u>et al.</u> (1988) and modified according to sequencing data in Fig. 37.

* position of poly(A)⁺ addition signal in pSG Vg 5.09. R1= Eco RI sites; B= Bam HI sites; H= Hind III sites.

B. Northern blot of total liver RNA (20 μg/lane) from control (C) and E2-treated trout (E), probed with the 340 bp *Hind* III fragment of pSG Vg 5.50 indicated in Fig. 38A (hatched box). 48 h exposure.

ŀ.

۱





Α.

£ 1%.

....



.

-- V



1**7**0

ţ

Ŧ

- Figure 39. Hybridization of pSG Vg 5.09 and pSG Vg 5.50 fragment to restriction digests of plasmid DNA.
 - A. Ethidium bromide stained agarose gel of restriction fragments of pSG Vg 5.09 and pSG Vg 5.50.
 - Lane 1: Eco RI/Bam HI digest of pSG Vg 5.50;
 - Lane 2: Eco RI/Bam HI digest of pSG Vg 5.09;
 - Lane 3: 600 bp fragment of *Sac I/Eco* RI digested pSG Vg 5.50 (LMP-agarose gel-purified);
 - Lane 4: 3.1 kb fragment of *Sac I/Eco* RI digested pSG Vg 5.50 (LMP-agarose gel-purified);

M: markers (Hind III digested lambda.phage).

41

- B. Southern blot of gel shown in (A), probed with the 340 bp *Kind* III fragment of pSG Vg 5.50.
- C. Restriction maps of pSG Vg 5.50 and pSG Vg 5.09 and fragment sizes are shown for reference.

* position of equivalent Ava I sites in the two plasmids; hatched box= position of Hind III fragment used as probe; R1=Eco RI sites; B=Bam HI sites; S=Sac I sites.



ļ



Figure 39.

sequences, not unexpected since the DNA was only gel-purified once. In contrast, the probe gave an intense signal from both the 2.1 kb and 210 base pair fragments derived from *Eco* RI/*Bam* HI digested pSG Vg 5.50 (lane 1), and from the 3.1 kb *Sac* I/*Eco* RI fragment of pSG Vg 5.50 (lane 3), all of which contain at least part of the *Hind* III fragment sequences. A faint signal was also obtained from the 2.7 kb vector fragment (lanes 1 and 2), which was expected since the probe contained a few nucleotides of the pUC8 multiple cloning site.

From the results of this experiment it may be concluded that the sequence at the 3'-end of pSG Vg 5.50 is not contained in the vitellogenin mRNA band, or if present, is in very low abundance. Without subcloning, it was not possible to establish whether or not pSG Vg 5.50 contained sequences homologous to the stop codon, poly(A)⁺-addition signal or poly(A)⁺ tract seen in pSG Vg 5.09. This information would further help to establish the character of the pSG Vg 5.50 clone. As it stands, it appears that if pSG Vg 5.50 represents the cDNA of a genuine vitellogenin message, this message is rare and would probably have an extra 1.5 kb in its 3'-untranslated region. This is unlikely in view of the fact that the 3'-untranslated ends of both chicken and Xenopus vitellogenins are short (221 and 182 nucleotides respectively; van het Schip et al., 1987; Gerber-Huber et al., 1987). Furthermore, because it hybridizes to the same size message as pSG Vg 5.09, an mRNA hybridizing to pSG Vg 5.50 would have to be missing 1.5 kb upstream of the putative 3'-untranslated region. This would make the message too small to code for a protein of 160-200 kDa, the size of the unmodified vitellogenin protein obtained by in vitro translation (Chen, 1983; Valotaire et al., 1984). This adds further support to the hypothesis that the clone was derived from artifactual blunt-end ligation of two unrelated sequences, one of which was a 3'-fragment of the vitellogenin mRNA. However, further sequencing of pSG Vg 5.50 and of the trout vitellogenin gene(s) are still necessary to resolve this problem.

IV. DISCUSSION

1

A. The hepatic estrogen receptor of rainbow trout

Estrogen receptor activity has been demonstrated in both nuclear and cytosol fractions prepared from livers of E2-treated and untreated trout. Specific binding of $[^{3}H]$ -E2 was shown to be high affinity (K_d 1-4 nM), saturable with nM concentrations of ligand and specific for estrogens and antiestrogens. In these respects the trout hepatic ER behaved like the classical estrogen receptors described in many other organisms. Estrogen receptors from cytosol and/or nuclear fractions have been identifed in several species of fish and all appear to have similar K_ds, ranging from about 1-5 nM (Table 18). Most show high specificities for natural estrogens, as well as for DES and antiestrogens. Several, including the rainbow trout ER, have been partially purified by precipitation with ammonium sulfate at 30% saturation. These features distinguish hepatic ER from plasma steroid-binding proteins.

As in several other species of fish, the E2-binding component of trout serum was saturable at much higher concentrations of E2 than the receptor and had very little affinity for DES or 4-hydroxytamoxifen. On the other hand, the plasma binder had a higher affinity for testosterone and its derivatives. resembling the sex-steroid binding protein previously described in trout plasma (Fostier and Breton, 1975) and the plasma steroid-binding activities in other fish (Table 18). Competition studies revealed a small amount of binding in trout liver cytosol that was not competable with DES. This could represent a minor degree of contamination of the cytosol by serum proteins. However, using high concentrations of DES to demonstrate non-specific binding should have negated the problem of serum contamination in single point binding assays.

The concentration of E2-binding activity in cytosol fractions from livers of untreated trout was very high (2 pmole/g or 15,000 sites per cell based on a diploid DNA content of

Species		K _d (nM)	Competitors*	Reference	
Oncorhynchus mykiss (rainbow trout)	nuclear cytosol	1.9 ± 0.33 3.4 ± 1.0	DES, OHT DES, OHT	This thesis	
	plasma	50	T, DHT, II-KT		
	cytosol¥	1.41-1.52	DES	Maitre <u>et al.</u> , 1985	
<u>Salmo salar</u> (Atlantic salmon)	nuclear cytosol¥	2.9 ± 1.1 5.9 ± 0.3	DES, OHT, tam DES, OHT	Lazier <u>et al.,</u> 1985	
	plasma	13	DHT, Prog		
<u>Salmo trutta</u> (brown trout)	cytosol¥ plasma	4.2 ± 0.16 48-67	E1 T,11-KT	Pottinger, 1986	
<u>Salvelinus fontinalis</u> (brook char)	nuclear cytosol	1.63 ± 0.15 7.5 ± 1.1	DES, E1 DES, E1	McPherson <u>et al.</u> , 1988	
	plasma		DHT, Prog		
Cynoscion nebulosus (spotted seatrout)	nuclear cytosol plasma	1.96 ± 0.42 1.26 ± 0.55 none	DES, tam DES, tam	Smith and Thomas, 1990	
Pseudopleuronectes americanus (winter flou	cytosol¥ inder)	0.6	DES, DHT, Prog	Sloop <u>et al.,</u> 1984	
Gobius niger	nuclear cytosol	0.63 ± 0.4 1.9 ± 0.3	DES, tam, DHT DHT	Le Menn <u>et al.,</u> 1980	
	plasma	3.5	DHT		
<u>Squalus acantias</u> (spiny dogfish)	nuclear cytosol	1.53 none	E1	Callard and Mak, 1985	
<u>Eptatretus stouti</u> (Pacific hagfish)	nuclear	0.38	DES, E1, E3	Turner <u>et al.,</u> 1981	

Table 18. Comparison of binding affinities and competitors for E2 of estrogen receptors and plasma steroid-binders in various species of fish

* Major competitors of [³H]-E2.

{

anan a' arandahar afi ungin t

.

¥ indicates that partial purification could be achieved with 30%-saturated ammonium sulfate.

13 pg/cell, Mirsky and Ris, 1949) when compared to the almost negligible amounts of cytosol ER found in the livers of untreated Xenopus or chickens (Hayward et al., 1980, Gschwendt, 1977; Lazier and Haggarty, 1979). High levels of non-specific binding encountered in crude cytosol preparations tended to complicate the positive identification of these sites as estrogen receptors by binding studies and Scatchard analysis. This problem was partially resolved by precipitation of cytosol with 30%-saturated ammonium sulfate, although the yields of E2-binding activity from livers of different individuals were too variable for routine use of ammonium sulfate precipitation as a purification step. We did observe a single class of high-affinity sites in crude cytosol preparations from E2-treated animals, where specific binding of [³H]-E2 was high relative to binding in the presence of excess DES. However we cannot absolutely rule out the presence of non-receptor E2-binding sites in cytosol from untreated trout. By Scatchard analysis of crude trout liver cytosol, Maitre et al. (1985b) noted a second, lower-affinity component which varied in concentration between animals, possibly as a function of different physiological states. Xenopus liver cytosol also contains large amounts of a medium-affinity E2 binder (K_d =40 nM) with high specificity for estrogens, including DES (Hayward and Shapiro, 1981). This protein differs from the ER in that it does not increase in concentration or translocate to the nucleus after E2-treament, and it perhaps has a role in concentrating E2 in hepatocytes and protecting the hormone from metabolism (Hayward et al., 1982). The Kd of 42 nM obtained by kinetic analysis of our crude cytosol preparations was suggestive of a middle-affinity site, although differences in Kds calculated kinetically and by equilibrium studies have been noted in other studies (Capony and Rochefort, 1978). Although we cannot eliminate the possibility that the E2-binding activity found in cytosols from untreated trout might represent an estrogen-specific middle-affinity binder, the demonstration of a single class of high-affinity sites in E2-treated animals suggests that a second estrogen-binding protein is either not present or in very low concentration. DNA-

176

cellulose columns have been used to resolve the two estrogen-binding activities in reptilian target tissues (Salhanick <u>et al.</u>, 1979) and might be useful for purification of trout cytosol receptors as well. However, we do note that similarly high concentrations of high-affinity E2-binding sites have been found in cytosols of other teleosts, both in males and untreated females (Lazier <u>et al.</u>, 1985; Smith and Thomas, 1990; McPherson <u>et al.</u>, 1988).

We have demonstrated that ER-activity can be detected in extracts made from frozen livers, although freezing did affect the distribution of DNA and protein between the nuclear and cytosol fractions. Only 20-50% of the DNA present in the original homogenates was recovered in nuclei prepared from frozen tissue, considerably less than from fresh tissue. An equivalent amount of chromatin-bound receptor was probably lost from this fraction as well. However, when expressed as per mg/DNA, the concentrations of nuclear ER were not significantly different when extracts prepared from fresh and frozen tissue were compared. If the concentration of DNA/g tissue was known, then quantities of ER in the nuclear and cytosol fractions could be directly compared. Freezing also resulted in considerable redistribution of protein in the various subcellular fractions. There was much more protein in nuclear fractions prepared from fresh tissue, attributable, at least in part, to substantial numbers of whole erythrocytes which co-sedimented with the nuclei. Conversely, the cytosols prepared from frozen tissue contained higher concentrations of protein, coincident with the recovery of large amounts hemoglobin in this fraction. ER concentrations, often expressed per mg protein, must therefore be interpreted with this in mind.

While freezing of the livers did result in substantial loss of DNA and ER from the nuclear compartment upon subsequent homogenization, this could not completely account for the large amounts of ER detectable in trout liver cytosol. No significant difference was found between fresh and frozen tissue when the concentrations of cytosol ER were expressed as either fmoles per gram liver or as fmoles per mg DNA. Although the

distribution of receptor differed from that observed in E2-treated Atlantic salmon (Lazier <u>et</u> <u>al.</u>, 1985), high concentrations of cytosol ER have been reported for other teleosts during times of vitellogenin synthesis. In the spotted seatrout (<u>Cynoscion nebulosus</u>), hepatic ER activity was more concentrated in the cytosol than in nuclear fractions of both vitellogenic and non-vitellogenic females (Smith and Thomas, 1990), and in the brown trout (<u>Salmo</u> <u>trutta</u>), as well as the seatrout, cytosol ER levels were twice as high in mature females as in males (Pottinger, 1986).

Both cytosol and nuclear forms of the trout liver ER could bind the antiestrogens tamoxifen and 4-hydroxytamoxifen. A high dose of tamoxifen was shown to have weak agonist activity in the trout liver, at least in terms of vitellogenin mRNA induction. The very high relative affinity of 4-hydroxytamoxifen for the trout ER might suggest that OHT, rather than tamoxifen itself, was the active form of the antiestrogen. This has been shown to be the case in mammals, <u>Xenopus</u> and chickens (Jordan <u>et al.</u>, 1977; Reigel <u>et al.</u>, 1986a; Borgna and Rochefort, 1981). We do not yet know if tamoxifen or OHT have any antiestrogenic effects in the trout, but on the basis of their high affinity for the ER and tamoxifen's weak agonist properties, we would predict so. This would be very useful for positively identifying and examining the ER-mediated aspects of vitellogenesis, both <u>in vivo</u> and in hepatocyte cultures.

Given the relatively high affinity of tamoxifen for the trout ER, the apparent lack of specific interaction of liver fractions with $[^{3}H]$ -tamoxifen aziridine was somewhat puzzling. It has been demonstrated that $[^{3}H]$ -TAZ covalently labels the human ER by electrophilic attack on cysteine 530, located in the steroid-binding site (Harlow <u>et al.</u>, 1989). Fig. 40 compares the amino acid sequences of the steroid-binding domains of chicken, human and trout ERs, derived from cDNA sequences. Cys 530 has been conserved in all three species, but in the trout two of the flanking amino acids, Ile 428 and Lys 533, are different from Met 428 and Val 533 found in the other proteins. Conceivably

Figure 40. Comparison of derived amino acid sequences of the steroid-binding domains of estrogen receptors from rainbow trout, <u>Xenopus</u>, chicken and human.

۲. ۳ ۱

Amino acid sequences of estrogen receptors were derived from the nucleotide sequences of cDNA clones and aligned to show maximum homology (Pakdel <u>et al.</u>, 1989).

Tamoxifen aziridine reacts with cystcine 530 in the human ER to form a covalent bond (Harlow et al., 1989).

The underlined sequence, from amino acids 463-528 in the human ER, comprises the epitope for the H222 antibody (Kumar et al., 1986).

The boxed sequence is the region encoded by the complement of our oligonucleotide "E".

ı.

G

₩.

Rainbow trout <u>Xenopus</u> Chicken Human	LNPO S S S	AFSF VYT VYT VYT	=CS L L L	SNSVES STL STLK ST <i>LK</i>	LHNS ED EEF EEF	SSAV TDLI RDYI <i>KDHI</i>	/ESMI H HRV <i>HRV</i>	DNI K K <i>K</i>	TDALI I T V T <i>T</i>	HH I SH S FMAK LMAK <i>LMAKA</i>	GASVQO LL LL <i>LTL</i>	QQPRR Q H HQ
				453				530				
	QAQI	LLLL	.SF	IIRHM	SNKG	MEH	ЩYSI	кC	KNKV	PLYDLLLE	MLDGH	RLQAP
	L	1					N	Λ	V		Α	I HT
	L	I					N	M	V		Α	Н
	L	1					Γ	Ν	V		Α	Н
							- 52	.8				

Figure 40.

スカンマールシーチョ

¥

the altered sequence, especially the value to lysine substitut on, might disrupt the local conformation necessary for efficient covalent attachment of the aziridine group.

Sucrose density gradient analysis of the chicken nuclear ER, labeled with ³H]-TAZ, showed a component sedimenting at 4.7S, as well as a small amount of non-specific binding in the 4S region. The mobility of 4.7S peak increased to 8S after incubation of the nuclear fraction with the ER-specific monoclonal antibody, H222. On the other hand, trout liver cytosol showed a relatively broad area of [³H]-TAZ distribution in high salt-containing sucrose gradients, with much of the label found near the top of the gradient. This observation, and the fact that no labeled protein was apparent in SDS gels, also suggested that the trout ER was not covalently labeled. A peak of [³H]-TAZ activity, only partially (nuclear extracts) or not competable by DES (cytosol), sedimented at about 6.5S in high-salt gradients. This was much larger than the 4.7S peak observed using chicken nuclear extracts, the 5.6S peak seen with MCF-7 cell extracts (Katzenellenbogen et al., 1983), or the 3.6S peak of [³H]-E2-labeled nuclear extracts from salmon (Lazier et al., 1985). The mobility of this peak did not alter in the presence of H222, further suggesting that it might not represent the trout ER. Could the 6.6S peak represent an antiestrogen-binding site? High-affinity binding sites, specific for triphenylethylene derivatives, have been demonstrated in microsomal fractions of MCF-7 cells, rat liver and in chicken liver cytosol (Watts et al., 1986). It has been postulated that they may play a role in the anti-tumor effects of these drugs. However the component from chicken liver cytosol migrates between 10-30S in sucrose gradients (Lazier and Murphy, 1986). Further investigation of the ability of E2 and other estrogens to compete with [3H]-TAZ or ^{[3}H]-tamoxifen might help to clarify the nature of the 6.6S binding-activity in trout liver.

We were not able to definitively determine whether the trout ER cross-reacted with the H222 antibody because of the rapid dissociation of [³H]-E2 from the receptor under the conditions required for sucrose gradient centrifugation or immunoprecipitation. Sucrose gradients containing a uniform distribution of $[{}^{3}H]$ -E2 or post-labeling of fractions, or use of a vertical-tube rotor (Beckman VTi 65) which would reduce centrifugation time to a couple of hours, might resolve this problem. However, as shown in Fig. 40, much of the region representing the H222 epitope (Kumar <u>et al.</u>, 1986) is not as well conserved in the trout ER as it is in other species.

The E2-binding activity of trout liver differed from the chicken liver ER in other respects as well. The trout ER was less heat-stable than that of the chicken, making steroid exchange assays at high temperature unpractical. This was similar to the heat-sensitivity observed with the salmon and seatrout receptors (Lazier <u>et al.</u>, 1985; Smith and Thomas, 1990). We noted, however, that the trout ER is slightly more heat-tolerant than its counterpart in salmon and that it remained stable at -70°C for years, both in whole liver and in subcellular fractions. The binding-activity was also stable for 2-3 days at 4°C in the presence of E2, although it rapidly declined in the absence of steroid. This instability might partially explain the decline in hepatic E2-binding activity observed in fish which had received E2 injections several days previously, despite the presence of induced levels of ER mRNA in these animals.

B. Induction and intracellular distribution of hepatic ER activity after E2-treatment

1. Comparison with Salmo salar

The results presented in this thesis have shown that E2-treatment stimulated a 3- to 4-fold increase the total amount of hepatic E2-binding activity in the rainbow trout (see Figs. 18 and 19). However the intracellular distribution and the dynamics of induction of hepatic ER in the rainbow trout contrasted with the situation observed in E2-treated Atlantic salmon maintained at 6-9°C (Fig. 41A). Over a 5 day period following a single injection of hormone, very high levels of ER accumulated in the nuclear fractions of salmon livers, while cytosol ER concentrations showed only a modest increase during this time (Lazier et al., 1985). In trout, on the other hand, maximum levels of ER were observed between

ť

- Figure 41. Comparison of hepatic ER concentrations after E2-treatment of <u>Salmo salar</u> and <u>Oncorhynchus mykiss</u>.
 - A. Time course of cytosol and nuclear ER levels in Atlantic salmon (<u>Salmo salar</u>) after a single injection of E2 (5 mg/kg). Animals were maintained at 5-9°C during treatment. Data from Lazier <u>et al.</u>, 1985.
 - B. Time course of cytosol and nuclear ER levels in rainbow trout (<u>Oncorhynchus mykiss</u>) after a single injection of E2 (5 mg/kg). Animals were maintained at 4°C during treatment.
 - C. Time course of cytosol and nuclear ER levels in rainbow trout (<u>Oncorhynchus mykiss</u>) maintained at 9°C. Animals received injections of E2 (5 mg/kg) at 0, 48, 96 and 168 hours.

1



Figure 41.

I

184

1

. .

24-48 hours after injection (in the groups treated at 9°C or 15°C), or by 5 days after

atment at 4°C, and only a portion of the E2-binding activity appeared to be located in the salt-extractable nuclear fraction. As was discussed above, the high levels of cytosol receptor were not simply due to loss of ER from nuclei of frozen tissue.

Differences in E2 uptake and metabolism between the species may be responsible for the slower accumulation of hepatic ER in salmon given the same dose of E2 and maintained at the same temperature as the trout (9°C) (Fig. 41A and C). Although no direct comparisons of E2 clearance rates at a given temperature have been made, there is some suggestive evidence that differences exist between the two species. Bjornsson et al. (1989) demonstrated that in Atlantic salmon, maintained at 10°C, serum E2 levels of about 4 ng/ml were observed 24 hours after injection of E2 (10 mg/kg in peanut oil), and were significantly higher than controls 10 days later. Flett and Leatherland (1939), citing unpublished observations, noted that when rainbow trout were given a dose of E2 (5 mg/kg in peanut oil), serum levels reached 100 ng/ml within 1 hour then fell to baseline levels by 24 hours. Atlantic salmon have also been observed to be less tolerant of high doses of E2 than rainbow trout, especially at high temperature. Korsgaard et al. (1986) noted the toxic effects of E2-treatment (five injections of 1 mg/kg) in salmon maintained at 15°C, while in our experiments with trout maintained at 15°C and given a higher dose of E2 (4 injections of 5 mg/kg), the animals appeared healthy throughout the trea.ment period. These observations might be explained by a more rapid metabolism of E2 by trout than by salmon, hence its lower toxicity and less prolonged presence in trout serum. Obviously direct comparisons of E2 metabolism in the two species should be made. However the effects of E2-treatment do appear to be more rapid and shorter-lived in the trout than in Salmo salar. Bjornsson et al. (1989) have observed that the induction of calcitonin in reponse to E2-treatment was faster in trout than in salmon, even though the trout were given a lower dose of E2. Peak calcitonin levels were observed in trout within five days of

i

185

injection, while in salmon the levels continued to rise over a period of 10 days following treatment. We have also observed this phenomenon at the level of hepatic ER induction, where maximum levels of ER were observed within 48 hours of treatment in trout maintained at 9°C, but not for several days in the Atlantic salmon (Fig. 41).

Sale-

ŝ

Slower metabolisen of E2 by Salmo salar might also explain the high levels of nuclear ER present in livers from treated animals. Steroid receptors which partition into the cytosol are thought to be in their unactivated form and not occupied by hormone. If, in Salmo salar, concentrations of E2 were sufficiently high to fill hepatic estrogen receptors, this might explain their predominantly nuclear location several days after injection. On the other hand, if E2 was being metabolized in the trout as rapidly as Flett and Leatherland (1989) have suggested (within the first 24 hours at 9°C), then we might expect the accumulation of ER in the cytosol as hormone levels became depleted. As can be seen in Fig. 41C, peak levels of cytosol ER occured 48 hours after the first injection of E2, at a time when serum E2 concentrations may have been falling. At 72 hours, 24 hours after the second injection, high concentrations of the hepatic ER were located in the nuclear fraction, coincident with a drop in cytosol ER levels. This would suggest "translocation" of hormone-activated receptor. ER had accumulated in the cytosol once more by 96 hours, when E2 levels may have fallen again. On the other hand, in trout maintained at 4°C, a single injection of E2 produced a slow rise in both nuclear and cytosol ER levels (Fig. 41C), perhaps due to the very slow transport of E2 to the liver at this temperature. Although levels of nuclear ER were relatively low, this was sufficient for the induction of both ER mRNA and E2-binding activity, which remained primarily in the cytosol.

Determination of serum E2 levels would be important to support this explanation. However our attempts to measure E2 concentrations by standard RIA were frustrated by the presence of a cross-reacting substance in trout serum. Values obtained for control male trout serum were greater than 50 ng/ml, the concentration of E2 observed by others in vitellogenic females (Scott <u>et al.</u>, 1980; van Bohemen and Lambert, 1981). Resting levels of E2 in untreated males or immature females should have been in the order of 1-2 ng/ml. Comparison of charcoal-stripped and unstripped cytosol preparations (Table 4) did indicate that there was enough endogenous estrogen in liver homoge: ates to block 50-100% of the cytosol sites in the 96 hour samples, at least under the exchange assay conditions employed. However, since there can be considerable redistribution of hormone to high affinity sites after homogenization of the tissue, this cannot really tell us if the cytosol sites were actually occupied by hormone <u>in situ</u>. Confirmation might be obtained by homogenizing the tissue in the presence of [³H]-E2, under conditions which would allow binding of labeled hormone to the unoccupied receptors but would prevent exchange with ligand already present in the occupied sites (Clark <u>et al.</u>, 1987). However the relatively long association time and the rapid dissociation kinetics of E2 from the trout receptor could complicate this procedure.

Charcoal pre-treatment of cytosols from 10 day samples did not reveal any additional binding sites, suggesting that by three days after an injection the amount of endogenous E2 was negligible. We also noted that at this time the levels of E2-binding activity in livers from fish maintained at 9°C and 15°C were no greater than in controls. This might suggest that hepatic estrogen receptors were unstable in the absence of hormone, an effect that was also noted in our binding assays in vitro.

The multiple injection routine used in our experiments may not have been the optimum method for administration of hormone, although this technique has been employed by many other investigators and vitellogenin synchesis was induced very effectively. A slower release method of hormone administration, such as silastic implants or warm cocoa butter which forms a soft pellet in the peritoneal cavity, might have been a better choice for observation of hepatic ER dynamics. These methods are known to provide sustained levels of serum E2 in a more physiological range, rather than the pulsatile, short-

í

R.

lived and potentially toxic pharmacological levels of hormone obtained when E2 is administered in saline or peanut oil (Pankhurst <u>et al.</u>, 1986; Maitre <u>et al.</u>, 1985a; Lawless, 1987). A single treatment also would reduce the amount of handling and stress on the fish which might alter their responsiveness to hormone treatment.

2. Effects of temperature

The temperature at which trout were maintained during E2-treatment had a marked effect on the induction of hepatic E2-binding activity. Maximum levels of hepatic ER were observed within 24 hours after treatment at 15°C and by 48 hours at 9°C, but not until 5 days after treatment at 4°C (Fig. 20). We also noted that in animals kept at 9°C and 15°C, the concentration of detectable hepatic ER appeared to decline over the treatment period, despite multiple injections of hormone, and that this decline was more pronounced at 15°C than at 9°C. On the other hand, total hepatic ER activity continued to increase over the 5 day treatment period at 4°C. The rate of transport of E2 to the liver, as well as differences in hormone metabolism, might be responsible for these effects. Cook and Peter (1980) have noted that the rate of uptake of intraperitoneally injected gonadotropin was considerably greater in golfish maintained at 20°C than at 12°C. Trout are known to have increased cardiac output at higher temperatures, to cope with the lowered oxygen content of the water (Houston, 1982). This could influence the rate of transport of hormone to the liver. More rapid metabolism of steroids by the liver with increasing temperature has also been observed in fish (Kime and Saksena, 1980). Both factors may have contributed to the more rapid induction of receptor and the faster decay of the response in trout maintained at 15°C than at 9°C. In animals maintained at 4°C, a combination of low cardiac output and precipitation of E2 in the body cavity, resembling a slow release form of hormone treatment, could be partly responsible for the slower accumulation of hepatic ER.

C. E2-induction of hepatic ER mRNA and vitellogenesis

1. Detection and quantitation of vuellogenin and ER mRNA transrcipts

Both vitellogenin and ER mRNAs were identified on Northern blots of total and $poly(A)^+$ RNA from trout livers. Using the cDNA clones pSG Vg 5.09 and pSG Vg 5.50 (Le Guellec <u>et al.</u>, 1988), a 6-7 kilobase vitellogenin mRNA was detected only in livers from E2-treated animals. Bands of approximately the same size were also observed in liver RNA preparations from E2-treated sculpin (<u>Myoxocephalus octodecimspinosus</u>), winter flounder (<u>Pseudopleuronectes americanus</u>), sea raven (<u>Hemitripterus americanus</u>) and Atlantic cod (<u>Gadus morhua</u>).

ER mRNA was detected using oligonucleotide probes complementary to regions coding for the putative DNA-binding and steroid-binding domains of the rainbow trout ER cDNA (Pakdel <u>et al.</u>, .989). Low levels of a 3.9 kilobase ER message were present in total liver RNA preparations from untreated trout as well as from treated animals. Both oligonucleotide probes appeared to be very specific for the trout ER message under high stringency hybridization conditions. They did not cross-h⁻¹bridize with rRNA or ER mRNAs from chicken oviduct, <u>Xenopus</u> liver or MCF-7 cells, even though the homologous regions of the receptor mRNAs differ in sequence by only a few nucleotides. Thus it was quite surprising to find that these probes could detect RNA transcripts in livers of various species of fish (sea raven, sculpin and winter flounder). This indicates that the regions complementary to our oligonucleotides are very highly conserved among the teleosts examined.

To study the dynamics of induction of vitellogenin and ER mRNAs under different temperature and dosage cond¹¹¹ ns, relative concentrations of each were quantitated by slot blot analysis of total liver RNA from untreated and E2-treated individuals. As discussed previously, direct comparisons of the relative mRNA levels between the groups of trout have to be interpreted with caution. We have noted that increased concentrations of rRNA

I

١

189

may dilute the amount of mRNA per unit of total RNA. This effect may be overcome by correcting for the ratio of RNA to DNA in the original tissue sample. However we have normalized our data to the amount of $poly(A)^+$ RNA in each preparation. Increasing amounts of vitellogenin transcripts, which can comprise up to 20% or more of the $poly(A)^+$ -containing sequences in E2-induced livers, could result in considerable dilution of the relatively rare ER message. Ideally data should be normalized to a transcript which does not change in abundance with E2-treatment. Actin and tubulin cDNAs 1 where used by others (Pakdel et al., 1989; Olsson et al., 1989), but our attempts to hybridize these clones to slot blots of total RNA from trout liver were largely unsuccessful. Perhaps a histone cDNA might be more useful, since proliferation of liver cells does not appear to occur after E2-treatment.

Slot blot analysis of total RNA only gives us an indication of the steady-state concentration of a given RNA. Accumulation of E2-induced transcripts usually reflects a balance between an increase in the absolute transcription rate of a responsive gene and selective stabilization of the mRNA (Brock and Shapiro, 1983a and 1983b; Wiskocil <u>et al.</u>, 1980). However, with these technical limitations in mind, we can still make some important observations regarding the timing and extent of induction of the two messages under different temperature and treatment regimes, and the relationship between steady-state message levels and the relative abundance of their translation products.

2. <u>ER mRNA and hepatic ER activity</u>

Т

Low 'evels of ER mRNA, as well as cytosol E2-binding activity, were detectable in livers of untreated juvenile rainbow trout. E2-treatment resulted in an approximately 10-fold increase in the relative levels of the ER message within the first 8 hours after injection in all temperature groups. Barton and Shapiro (1988) have also observed the induction of hepatic ER mRNA following E2 treatment of <u>Xenopus</u>, both <u>in vivo</u> and in cultured liver cubes. Induction was inhibited by tamoxifen and therefore appears to be an ER-mediated phenomenon and not dependent on the production of extra-hepatic factors.

_

Maximum levels of hepatic ER mRNA in our multiple injection experiments were at least 200-fold greater than in controls by 7-10 days of treatment, and were also greater than pretreatment levels in animals given a single injection of E2 10 days previously. However by this time both nuclear and cytosol E2-binding activity had decreased nearly to control levels. This observation could imply that post-transcriptional control mechanisms operate in modulating the levels of ER activity in the trout liver. As was discussed previously, the decreasing ratio of detectable hepatic ER to ER mRNA could be partly an effect of the overwhelming abundance of vitellogenin messages, hence the redirection of the translational apparatus towards synthesis and secretion of vitellogenin rather than non-secreted proteins. Comparison with the rate of synthesis of other non-secreted proteins in vitellogenic livers would be helpful. Rapid degradation of the receptor protein with declining hormone levels has also been suggested, a hypothesis which could be tested by using a slow release form of hormone administration to maintain elevated serum E2 levels for a longer period of time.

Other **F** ssibilities are also testable by established methods. In vitro translation of RNA could indicate if the ER message was anslatable. Several ER-specific monoclonal and polyclonal antibodies are currently available which might cross-react with more highly conserved epitopes of the trout ER. This could permit use of a variety of immunochemical techniques for quantitation of receptors which might be modified or masked in some way and could not be detected by standard hormone-binding assays (Greene, 1984). ER might also be associated with the microsomal fraction, which was largely separated from the cytosol with the high speed centrifugation step during preparation. We have observed ER activity associated with microsomes in LMH-cells, an estrogen-responsive chicken liver cell line, which had been transfected with a chicken ER expression plasmid (C.B. Lazier, unpublished observations). Binding assays could easily be performed on microsomal

191

fractions from livers of E2-treated trout.

÷

Induced levels of ER mRNA were apparent well before the appearance of vitellogenin mRNA, at least in the animals maintained at 4°C. Pakdel <u>et al.</u> (1989) also noted that ER mRNA levels increased in trout livers within three hours of injection and preceded the appearance of the vitellogenin message by several hours. We observed that the pattern of induction of ER mRNA paralleled the increases in nuclear ER activity in the 9°C and 15°C treatment groups, but induction preceded any substantial increase in nuclear ER in the 4°C group. This might suggest that relatively low concentrations of nuclear ER were required for an increase in the steady-state level of the constitutively expressed ER message.

A similar case of dissociation in the kinetics of E2-induction has been observed with the apolipoproteins, apo-VLDL-B and apo-VLDL-II, and vitellogenin mRNAs in the chicken liver. ApoB is constitutively expressed in the liver and increased transcription occurs rapidly and reaches maximal levels between 12 and 24 hours following administration of E2 (Kirchgessner et al., 1987). On the other hand apoll and vitellogenin mRNAs accumulate much more slowly in the first few hours following estrogen treatment (Wiskocil et al., 1980). The immediate increase in transcription rate for the constitutively expressed apoB mRNA, which appears to be similar to the rapid induction of the ER mRNA in the trout, could reflect a more competent chromatin configuration, ready for enhanced transcription in the presence of receptor. This might be reflected in a generalized sensitivity to nuclease digestion, a phenomenon which has been observed in many transcriptionally active genes (Weintraub and Groudine, 1976). The precise mechanism and significance of nuclease sensitivity is not completely understood. However it does appear to reflect the potential for a given gene to be transcribed and is not necessarily a result of active transcription itself. The vitellogenin genes of Xenopus liver become sensitive to limited DNAse I digestion only after hormone treatment (Gerger-Huber et al., 1981, Folger

et al., 1983), but this may persist for 2-3 months after estrogen withdrawal, long after active transcription has ceased (Dimitriadis and Tata, 1982). Such an "open" state for chromatin has been implicated in the more rapid response of estrogen-inducible genes to secondary stimulation with hormone, constituting the so-called "memory effect" (Williams and Tata, 1983).

The accumulation of ER mRNA in trout liver at a time which precedes the maximally induced levels of nuclear ER and the onset of vitellogenin gene transcription also appears to be compatible with a model such as that proposed by Palmiter et al. (1981) for the differential estrogen-mediated activation of the conalbumin and ovalbumin genes in chicken oviduct. Whereas the rate of transcription of the conalbumin message is directly proportional to the amount of chromatin-bound ER, the kinetics of ovalbumin mRNA induction suggest that higher levels of nuclear ER are necessary for full activation of transcription, as appears to be the case with vitellogenin mRNA. The model proposes that occupation of a single estrogen response element is sufficient for induction of conalbumin transcription. On the other hand, the cooperative effect of multiple receptors binding to multiple response elements upstream of the ovalbumin gene would be required for its full induction. The discovery of multiple estrogen response elements upstream of the <u>Xenopus</u> and chicken vitellogenin genes, and the apo-VLDL-II gene of the chicken, certainly lends support to this idea (Walker et al., 1984; Klein-Hitpass et al., 1988). The coopprative interaction of hormone response elements has been demonstrated for the vitellogenin B1 promoter, using constructs containing either one or two of the imperfect palindromic sequences which comprise the EREs upstream of this gene (Seiler-Tuyns et al., 1986). When fused to a thymidine kinase-chloramphenicol acetyltransferase (tk-CAT) reporter construct, a single imperfect ERE was not able to confer estrogen-responsiveness to the tk promoter in transient transfection assays using MCF-7 cells. However two or more of these imperfect palindromes acted in synergy to form a strong ERE, allowing E2-induction

193

Ì

of the CAT message (Martinez <u>et al.</u>, 1987). Once they have been cloned, sequence analysis of the upstream regions of the ER genes and of the trout vitellogenin gene(s), as well as deletion analysis of cloned promoter elements fused to reporter constructs, should enable us to tell whether this kind of control system is involved in the differential induction of the two messages.

The rapid induction by E2 of other factors necessary for full transcriptional activation of more slowly responding genes may also play a role. Of course, one of these rapidly induced factors may be the estrogen receptor itself. E2-dependent up-regulation of its own receptor ir liver appears to be common among oviparous vertebrates (Hayward et al., 1980; Lazier, 1975; Lazier and Haggarty, 1979). Shapiro and co-workers have postulated that the high levels of ER induced by primary hormone treatment are necessary for full activation of the vitellogenin genes and are responsible for the more rapid secondary response after estrogen withdrawal (Shapiro, 1982). Barton and Shapiro (1988) have recently reported that induced levels of the ER message were present in male Xenopus for up to 4 months after a single dose of E2. Similar levels of ER mRNA were seen in liver cultures maintained in the presence of very low levels of E2, equivalent to the amount of hormone normally circulating in the male (aproximately 0.2 nM) and sufficient to occupy only about 25% of the estrogen receptors present. Since tamoxifen was able to reverse this persistent induction, the authors suggested that very low levels of hormone-occupied receptor are sufficient to allow transcription of the ER message. Thus primary estrogen treatment establishes an auto-regulatory loop maintaining induced levels of ER protein after estrogen withdrawal. The higher levels of receptor available for binding when a second dose of hormone is administered allows rapid reactivation of the vitellogenin genes. This model is also compatible with the hypothesis that the ER gene may possess fewer or stronger estrogen response elements than the vitellogenin genes.

In view of the auto-regulatory loop model, and in light of our results showing a

decline in total hepatic E2-binding activity over the course of our experiments, it would be very interesting to see if the ER mRNA remains induced for a prolonged period in the trout liver. However we note that levels of ER mRNA and cytosol ER in untreated trout were much higher than in untreated Xenopus, suggesting that the control mechanisms governing basal levels of ER in the two species may be quite different. The continued expression of vitellogenin mRNA in the trout, despite declining hormone levels and low concentrations of nuclear ER, also indicates that the details of E2-regulation of hepatic gene expression in teleosts may be distinct from those of Xenopus.

3. Induction of vitellogenin mRNA and serum vitellogenin

The temperature at which fish were maintained during E2 treatment influenced the time of first appearance of the vitellogenin mRNA, as well as the relative amount of message which accumlated in a given period. In trout treated at 15°C the induction of vitellogenin mRNA was very rapid, with significant amounts of message appearing within 8 hours of injection. Vitellogenin transcripts first appeared by 24 hours in fish maintained at 9°C, while in the animals kept at 4°C significant levels were not detectable until 48 hours (Table 11). In general, the demonstration of a lag period before the accumulation of vitellogenin message in the trout agrees with the delayed induction fo¹lowing primary E2-treatment in the chicken and in <u>Xenopus</u> (Wiskocil <u>et al.</u>, 1980; Baker and Shapiro, 1978). The effect of temperature on the initial induction of vitellogenin mRNA also seems to correlate with the timing of increased amounts of nuclear ER in the three groups.

The pattern of vitellogenin mRNA accumulation in our multiple injection experiment at 15°C strongly resembled the pattern observed by Le Guellec <u>et al.</u> (1988) in chronically treated trout. We noted an extended plateau between 2 and 7 days of treatment, then a 3- to 5-fold increase in steady-state levels by 10 days. At cooler temperatures the plateau period was more prolonged and lower relative levels of vitellogenin transcripts were observed. This might reflect a general lowering of transcription rates with cooler temperatures, or a ļ

an actually

A

dose response effect due to slower transport of E2 to the liver as discussed above. We also noted, contrary to the results of Le Guellec <u>et al.</u> (1988), that vitellogenin mRNA levels were moderately high 10 days after a single injection of E2 and have suggested that seasonal or strain differences in estrogen responsiveness might be responsible for the conflicting observations (Elliott <u>et al.</u>, 1979).

The appearance of detectable amounts of vitellogenin protein in serum followed the induction of the mRNA by several hours, probably reflecting the time necessary for translation of the message and processing of the protein before secretion. Accumulation of serum vitellogenin was also influenced by the temperature at which the fish were maintained during treatment and showed a longer lag before appearance at 9°C than at 15°C. The ratio of vitellogenin protein to mRNA and was low during the early part of the treatment period at 15°C, but rapidly increased by 7-10 days in trout which had received multiple hormone injections. This appeared to correlate with major increases in the %LSI (liver hypertrophy) and the RNA to DNA ratio of the liver. These changes were not as profound in the 9°C group, or in the animals which received a single injection of E2 at 15°C. We suggest, therefore, that the induction of the hepatic translational and secretory apparatus is a temperature- and/or dose-sensitive phenomenon. This agrees with the observations made by Korsgaard and Mommsen (1986), who noted both temperature and dose effects on the amount of serum vitellogenin induced in E2-treated Atlantic salmon. Another interesting observation was that the rapid accumulation of high levels of serum vitellogenin in our 15°C chronically treated group corresponded to the pattern of induction after secondary stimulation in the experiments of Le Guellec et al. (1988), while their primary induction pattern resembled our results at 9°C. Thus it is important to state not only the season and dose of E2, but also the water temperature during treatment if accurate comparisons are to be made between different experiments.

There appears to be a correlation between the length of time when high levels of ER

were located in liver nuclei and the enhanced production of vitellogenin several days later. Since most of the changes related to this phenomenon take place at a time when nuclear ER concentrations are less than half of their maximum levels, we can speculate that the high levels of chromatin-bound receptor earlier in the treatment period are responsible for the induction of factors, other than the estrogen receptor itself, which amplify the vitellogenic response in the rainbow trout. We have also noted, with both the multiple and single injection experiments, that the steady state levels of vitellogenin mPNA continued to increase despite the drop in salt-extractable nuclear E2-binding activity. This was very different from the situation observed in Xenopus, where continuous high concentrations of E2 and nuclear ER appear to be necessary to maintain vitellogenin mRNA transcription and stability (Baker and Shapiro, 1978; Brock and Shapiro, 1983a). It could be that a portion of the chromatin-bound receptor in treated trout could not be extracted from nuc' with 0.5 M KCl, although we were not able to detect appreciable levels of ER activity in the saltinsoluble fraction and the presence of reducing agents in all buffers should have released any nuclear matrix-associated receptor. However several observations by other groups suggest that the maintenance of vitellogenesis in teleosts may not be as strictly E2dependent as in Xenopus.

Elliott <u>et al.</u> (1979) have noted that a single injection of E2 into immature rainbow trout resulted in peak serum vitellogenin concentrations 21 days later, in spite of the fact that E2 concentrations had dropped to basal levels after 5 days. Indeed, total serum phosphoprotein phosphorous and calcium, indicators of serum vitellogenin, did not return to basal levels until 5 months after the single E2 treatment. Similarly, in the male flounder, vitellogenin accumulated at a constant rate for over 28 days following a single injection of E2 (Korsgaard <u>et al.</u>, 1983). Vasius and Fletcher (1988), examining female winter flounders which had been hypophysectomized after the onset of vitellogenesis, detected active transcription of vitellogenin mRNA in liver nuclei thirty days or more after surgery, despite extremely low levels of serum E2 by this time. Furthermore, during the natural breeding cycle of the trout, peak E2 concentrations precede maximal serum vitellogenin levels by 1-3 months (Whitehead <u>et al.</u>, 1978). These observations a. jue that high concentrations of nuclear ER may act as a "trigger" for vitellogenesis and that sustained occupancy of estrogen response elements by high levels hormone-activated receptor may not be necessary for continued transcription of the vitellogenin gene in fish. This also agrees with the hypothesis that E2 induces other factors which are responsible for the long-term stimulation of vitellogenesis.

Nuclear "type II" E2-binding sites have been implicated in the long-term effects of estrogen in other systems. These intermediate-affinity sites (K_d 10-20 nM), first identified in rat uterus, accumulate in nuclei after E2 treatment and their concentration remains elevated after the decline of high-affinity ER levels. The induction of type II sites can be correlated with the stimulation of uterine growth and other long-term uterotropic effects in rats (Clark and Markaverich, 1981). A strong correlation also appears to exist between the concentration of type II binding sites in the nucleolus and increased synthesis of rRNA in uterine nuclei (Whelly, 1986). Type I receptors were not found in appreciable concentrations in isolated nucleoli, hence it is tempting to speculate that the type II sites were somehow involved in this process.

Deeley and co-workers have hypothesized that both type I and type II E2-binding sites are involved in the enhanced expression of the various apolipoproteins produced by HepG2 human hepatoma cells (Tam et al., 1986a and 1986b). Exposure of HepG2 cells to low concentrations of E2 for 48 hours induced a 14- to 15-fold increase in high-affinity type I estrogen receptors and, after a lag of several hours, a comparable increase in the levels of type II sites. In contrast to the rapid disappearance of type I receptors on estrogen withdrawal, high levels of the type II sites persisted for more than 10 cell divisions in the absence of hormone. However, primary treatment with E2 for only 6 hours induced type I

ER to about 80% of the maximum but failed to induce substantial quantities of type II sites. This suggests that high and constant levels of E2-activated type I receptors are necessary for the initial induction of type II sites but not for their later amplification. Levels of type II binding sites correlated well with the slow kinetics of primary induction of apoE and apoB mRNAs in HepG2 cells, and with their ability to show a memory-effect in response to secondary estrogen stimulation. A similar relationship between nuclear ER levels, longterm induction and propagation of type II binding sites, and a more rapid response to secondary hormone treatment was observed with the E2-induction of vitellogenin and apoII mRNAs in adua and embryonic chickens (Hache et al., 1987). A model proposed for the regulation of type II sites and the memory-effect involves ER-mediated induction of threshold levels of type II binding sites, which are then capable of self-stimulation in the absence of hormone, much like the auto-regulatory loop mechanism proposed for the estrogen receptor itself in Xenopus (Barton and Shapiro, 1988). In this model, type II sites, in combination with hormone-activated ER, are necessary for the induction of genes such as vitellogenin and the apolipoproteins, which characteristically display a lag period during primary estrogen treatment. These genes then show a more rapid response to secondary stimulation when high levels of type II sites are already present. Interestingly, high levels of type II binding sites alone appeared to be able to maintain transcription of the vitellogenin and apoII mRNAs in embryonic chick liver, in the absence of significant amounts of nuclear type I ER (Hache et al., 1987). The authors suggested that E2metabolites with high affinity for the type II sites might accumulate in the egg and serve to promote expression of these genes via a type II "receptor"-mediated mechanism. The rapid decline of apoII and vitellogenin mRNAs after hatching is consistent with this proposal.

A type II-like model might explain several of the observations made in our experiments with rainbow trout, as well as observations made by other groups. First of all, trout which received multiple hormone injections at 15°C, or silastic implants of E2

(Le Guellec <u>et al.</u>, 1988), demonstrated an extended submaximal accumulation of vitellogenin transcripts for the first seven days, then a sudden 3- to 5-fold increase in mRNA, at a time when rRNA appeared to be increasing as well. This occurred when nuclear ER concentrations had declined to approximately half of their fully induced levels. We also observed that a single injection of E2, or multiple injections at 9°C, failed to promote either the induction of total RNA in the liver or the later increase in vitellogenin transcripts, at least within the 10 day sampling period. With cultured trout hepatocytes, Vaillant <u>et al.</u> (1988) observed that the amount of vitellogenin mRNA induced by a secondary treatment with E2 was a function of the time of primary stimulation. They suggested that the amount of ER induced during the primary treatment might be responsible for the rate of secondary induction. However we suggest that another factor, perhaps a type II-like protein, may also be induced by initially high levels of nuclear ER and might be responsible for the later maintenance and amplification of the vitellogenic response in the absence of high receptor concentrations.

The existence of type II sites in trout liver, as well as the identity of ligands which might activate ER-independent transcription, have yet to be determined. Type II E2-binding sites can usually be detected in nuclei isolated in the absence of reducing agents, although sometimes a brief digestion with DNAse I is necessary to render them salt-extractable (Hache <u>et al.</u>, 1987). It would be very interesting to see if these sites are present in trout livers and if their induction follows an E2-dependent time course or parallels the induction of vitellogenin mRNA or rRNA. An examination of tamoxifen's antagonist properties and its effects on various aspects of the vitellogenic response in fish might also prove to be a fruitful area of investigation. In addition, other steroids (estrone, cortisol and progesterone), pituitary factors (growth hormone and prolactin) and thyroid hormone have been shown to influence the extent of the vitellogenic response <u>in vivo</u> and in hepatocyte cultures of several species (Ho, 1987). The availability of methods for maintaining primary

cultures of trout hepatocytes and the demonstration of vitellogenin induction in vitro (Maitre et al., 1986; Vaillant <u>et al.</u>, 1988) should greatly facilitate the exploration of possible multihormonal control mechanisms involved in this process. Also the development of methods for analyzing the effects of nuclear extracts on <u>in vitro</u> transcription of cloned vitellogenin promoter elements (Corthesy <u>et al.</u>, 1988) might prove to be a useful tool in the search for both positive and negative regulatory transcription factors.

D. Summary and conclusions

1. Effects of temperature on vitellogenesis

Our experiments have shown that the temperature at which trout are maintained influences the overall dynamics of the E2-induced vitellogenic response in liver. Temperature effects were noted at several levels of the pathway, from the induction of hepatic estrogen receptors to the transcription of the vitellogenin message and its subsequent translation and secretion into the serum.

We have noted that temperature may act at any of several places in the E2-regulated pathway. One of the major effects. most consistent with all of our observations, is that temperature influences the amount of E2 which is transported to the liver after intraperitoneal injection. Increased cardiac output at higher temperatures could be responsible for a very rapid rise in the local concentration of hormone in the liver, reflected in the earlier appearance of ER in the nuclear fraction of hepatocytes. There might also be influences on the transport of hormone into cells, either by simple diffusion or by plasma membrane-associated transport mechanisms. Cooler temperatures might also affect the interaction of hormone with ER and therefore their subsequent binding to chromatin. Hochachka and Somero (1973) discuss numerous instances of "negative thermal modulation" of enzymes in the rainbow trout and suggest that disruption of hydrophobic interactions in the cold may be responsible. Since the interaction of E2 with its receptor is largely hydrophobic in nature, this could be a serious consideration. However we do note I,
that hormone/receptor interactions take place at 4°C in vitro, although the association rate was somewhat slower than at 15°C.

Accelerated transcription rates with increasing temperature may also play a role in the more rapid induction of vitelogenin mRNA and its higher steady-state levels in animals treated at 15°C. However we note that increases in ER mRNA levels do not show an appreciable lag at 4°C, suggesting that the induction of its transcription is not particularly temperature sensitive and takes place even without high concentrations of nuclear ER complex. The generally lower steady-state levels of vitellogenin mRNA in the 9°C and 4°C treatment groups may reflect a dosage effect rather than an effect of temperature on transcription, since single injection experiments performed at 15°C showed approximately the same levels of vitellogenin mRNA as in chronically treated animals maintained at 9°C.

Perhaps the most profound temperature effect we observed was on the very high levels of serum vitellogenin which accumulated during chronic treatment at 15°C. This did not simply reflect the lower amounts of message present in animals treated at 9°C, since the ratio of vitellogenin protein to mRNA was markedly higher in the 15°C group. Cold-acclimated fish generally appear to be able to adjust their rate of protein synthesis to compensate for decreased reaction rates at low temperature (Haschemeyer, 1973). In the toadfish (Opsanus tau), this appears to be a result of the induction of aminoacyl-tRNA transferase, hence the overall rate of protein synthesis in 10°C-acclimated fish appears higher than that of 20°C-acclimated fish when measured in vitro. However, while animals may partially compensate their normal protein synthesis.

We suggest that increases in the hepatic translational and secretional apparatus are necessary for the production of high levels of serum vitellogenin and that this may represent a major temperature sensitive step. We have observed a significant increase in the ratio of RNA to DNA in livers between 7 and 10 days of chronic treatment at 15°C, coinciding with a general increase in liver weight reflecting hypertrophy of the organ. The output of vitellogenin protein into the serum also increased dramatically at this time. We suggest that the induction of rRNA, as well as amplification of the encoplasmic reticulum and Golgi apparatus, could be responsible for the enhanced production of vitellogenin at 15°C. Bast <u>et al.</u> (1977) have observed that the kinetics of hepatic ribosome synthesis and their recruitment into polysome are closely coordinated with the rate of vitellogenin synthesis in E2-treated chicks. Interestingly, Nishio <u>et al.</u> (1978) have observed a preferential decline in the synthesis of the 40S rRNA precursor in Xenopus neurula cells at 10°C. However, we hypothesize that the high concentration of nuclear ER early in the chronic treatment period at 15°C may be responsible for inducing factors which must accumulate before significant changes in liver physiology can occur. Chronic treatment at 9°C or a single injection of hormone do seem to be sufficient to induce these later changes, perhaps due to lower effective concentrations of hormone in the liver.

Finally, we would like to repeat that, since they appear to have very marked effects on the ability of the liver to respond to E2, any investigation of the regulation of vitellogenesis in fish should consider the environmental temperature as well as the season in the interpretation and comparison of experimental results.

2. A model for the control of vitellogenesis in rainbow trout and comparison to Xenopus

Our experimental results have suggested that the control of vitellogenesis in the trout may be quite different in detail from that of <u>Xenopus</u>. The most important observation we have made is that vitellogenin mRNA and protein apparently continue to accumulate in the absence of high levels of E2 and nuclear ER. This suggest: at the control mechanisms involved are less dependent on the presence of continuous high levels of hormone than they are in <u>Xenopus</u>.

The induction and maintenance of vitellogenin transcripts in X. laevis appears to be totally dependent on E2. The model proposes that high levels of ER are induced by E2

treatment via up-regulation of the ER message. High levels of hormone-occupied receptor accumulate and bind to multiple response elements upstream of the vitellogenin genes, thereby activating their transcription. As long as hormone levels remain high, vitellogenin gene transcription can continue, but once E2 is removed, levels of message rapidly decline through a combination of decreased transcription and destabilization of the message. Low levels of E2 are able to maintain the persistent induction of some genes, such as that of the retinol-binding protein (McKearin and Shapiro, 1988), possibly because these genes have fewer estrogen response elements and can be activated by lower concentrations of nuclear ER. Transcription of the ER gene also remains activated in the presence of very low hormone concentrations, maintaining induced levels of receptor protein, which resides in the cytosol fraction in its unoccupied state. Restimulation of the liver with E2 causes the rapid translocation of high levels of receptor to the nucleus and the immediate reactivation of the vitellogenin genes. Hence it appears that the induction and maintenance of the highaffinity ER alone is sufficient for the memory effect observed upon secondary stimulation.

In the rainbow trout we have found high concentrations of ER in the cytosol fractions of livers from untreated juvenile animals. Our estimate of 12,000 sites per cell is much higher than the concentrations observed in hormonally naive <u>Xenopus</u> (approximately 500 sites per cell), but corresponds well with the levels reported for other teleosts (Lazier <u>et al.</u>, 1985; Smith and Thomas, 1990). Like in <u>Xenopus</u>, the induction of the ER message was very rapid following E2-treatment. Our experiments at different temperatures demonstrated that the ER message was induced much more rapidly than that of vitellogenin and with much lower concentrations of nuclear ER. Levels of both nuclear and cytosol ER activity also rapidly increased, to maximum combined levels of about 10 pmole/g or 60,000 sites per cell. Once again this was several-fold higher than the levels induced in <u>Xenopus</u> but equivalent to concentrations seen n E2-treated Atlantic salmon (Lazier <u>et al.</u>, 1985). The appearance of vitellogenin transc 'pts followed the increase in

nuclear ER, showing a lag time that appeared to be temperature or dose dependent. Over the course of our 10 day treatment period, we observed that the levels of hepatic ER activity declined, even in animals which had received multiple injections of hormone. However steady-state levels of vitellogenin mRNA continued to increase, even in trout which had received a single injection. This suggests that the trout vitellogenin message is stable and that, once induced, its transcription can be maintained in the absence of appreciable hormone-activated nuclear ER, quite different from the situation seen in Xenopus. A memory effect has been observed in vivo (Le Guellec et al., 1988) and in cultured trout hepatocytes (Vaillant et al., 1988), and the magnitude of the secondary response has been correlated to the length of primary stimulation in culture. We postulate that higher levels of induced ER may not necessarily correlate with a greater secondary response. We have hypothesized that E2-treatment induces factors in trout liver, other than the ER itself, which are responsible for the maintenance of vitellogenin gene transcription and the amplification of the translational apparatus (rRNA and secretory membranes), even though hormone levels have declined and nuclear ER concentrations have diminismed. The identity of these other factors needs to be established. However type II estrogen-binding sites have been implicated in similar effects in HepG2 cells and in chicken liver so their presence and possible involvement in the regulation of vitellogenesis in fish should be explored.

REFERENCES

Aviv, H. and Leder, P. (1972) Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA <u>69</u>: 1408-1412.

Baker, H.J. and Shapiro, D.J. (1978) Rapid accumulation of vitellogenin messenger RNA during secondary stimulation of <u>Xenopus laevis</u>. J. Biol. Chem. <u>253</u>: 4521-4524.

Baroiller, J.F., Fostier, A., Zohar, Y. and Marcuzzi, O. (1987) The metabolic clearance rate of estradiol-17ß in rainbow trout, <u>Salmo gairdnerii</u> R., estimated by both single injection and constant infusion methods: increase during oocyte maturation. Gen. Comp. Endecrinol. <u>66</u>: 85-94.

Barton, M.C. and Shapiro, D.J. (1988) Transient administration of estradiol-17ß establishes an autoregulatory loop permanently inducing estrogen receptor mRNA. Proc. Natl. Acad. Sci. USA <u>85</u>: 7119-7123.

Bast, R.E., Garfield, S.A., Gehrke, L. and Ilan, J. (1977) Coordination of ribosome content and polysome formation during estradiol stimulation of vitellogenin synthesis in immature male chick livers. Proc. Natl. Acad. Sci. USA <u>74(8)</u>: 3133-3137.

Beato, M. (1989) Gene regulation by steroid hormones. Cell 56: 335-344.

Berkenstam, A., Glaumann, H., Martin, M., Gustafsson, J.A. and Norstedt, G. (1989) Hormonal regulation of estrogen receptor messenger ribonucleic acid in T47D_{co} and MCF-7 breast cancer cells. Mol. Endocrinol. $\underline{3}(1)$: 22-28.

Berthois, Y., Katzenellenbogen, J.A. and Katzenellenbogen, B.S. (1986) Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc. Natl. Acad. Sci. USA <u>83</u>: 2496-2500.

Bjornsson, B.T., Haux, C., Bern, H.A. and Deftos, L.J. (1989) 17ß-Estradiol increases plasma calcitonin levels in salmonid fish. Endocrinol. <u>125</u>(4): 1754-1760.

Blue, M.L., Protter, A.A. and Williams, D.L. (1980) Biosynthesis of apolipoprotein B in rooster kidney, intestine and liver. J. Biol. Chem. <u>255</u>: 10048-10051.

Boehm, K.D., Hood, R.L. and Ilan, J. (1988) Induction of vitellogenin in primary monolayer cultures of cockerel hepatocytes. Proc. Natl. Acad. Sci. USA <u>85</u>: 3450-3454.

Borgna, J.L. and Ladrech, S. (1982) The dissociation of estrogen receptor-ligand complexes by high concentrations of steroids and antiestrogens. Molec. Cell. Endocrinol. <u>27</u>: 1-15.

Borgna, J.L. and Rochefort, H. (1981) Hydroxylated metabolites of tamoxifen are formed in vivo and bound to estrogen receptor in target tissues. J. Biol. Chem. <u>256</u>: 859-868. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. <u>72</u>: 248-254.

Brock, M.L. and Shapiro, D.J. (1983a) Estrogen stabilizes vitellogenin mRNA against cytoplasmic degradation. Cell <u>34</u>: 207-214.

Brock, M.L. and Shapiro, D.J. (1983b) Estrogen regulates the absolute rate of transcription of the <u>Xenopus laevis</u> vitellogenin genes. J. Biol. Chem. <u>258(9)</u>: 5449-5455.

Bromage, N.R., Whitehead, C. and Breton, B. (1982) Relationships between serum levels of gonadotropin, oestradiol-17 β , and vitellogenin in the control of ovarian development in the rainbow trout. II. The effects of environmental photoperiod. Gen. Comp. Endocrinol. <u>47</u>: 366-376.

Burch, J.B.E. and Weintraub, H. (1983) Temporal order of chromatin structural changes associated with the activation of the major chicken vitellogenin gene. Cell <u>33</u>: 65-76.

Burton, K. (1956) A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. <u>62</u>; 315-323.

Callard, G.V. and Mak, P. (1985) Exclusive nuclear localization of estrogen receptors in <u>Squalus</u> testis. Proc. Natl. Acad. Sci. USA. <u>82</u>: 1336-1340.

Callard, I.P., Banks, S.H. and Banks, W.L. (1972) Hepatic protein and nucleic acid content in <u>Diposaurus dorsalis</u> following hypophysectomy and treatment with estradiol-17ß and growth hormone. Comp. Biochem. Physiol. <u>41B</u>: 503-510.

Callard, I.P. and Callard, G.V. (1987) Sex steroid receptors and non-receptor binding proteins. In: "Hormones and Reproduction in Fishes, Amphibians and Reptiles" (D.O. Norris and R.E. Jones, eds.), pp. 355-384. Plenum Press, New York.

Capony, F. and Rochefert, H. (1978) High-affinity binding of the anti-estrogen [³H]-tamoxifen to the 8S estradiol receptor. Molec. Cell. Endocrinol. 11: 181-198.

Campbell, C.M. and Idler, D.R. (1980) Characterization of an estradiol-induced protein from rainbow trout serum as vitellogenin by composition and radioimmunological cross-reactivity to ovarian yolk fractions. Biol. Reprod. <u>22</u>: 605-617.

Chen, T.T. (1983) Identification and characterization of estrogen responsive gene products in the liver of the rainbow trout. Can. J. Biochem. Cell Biol. <u>61</u>: 802-810.

Clark, J.H. and Markaverich, B.M. (1981) Relationships between type I and type II estradiol binding sites and estrogen induced responses. J. Steroid Biochem. 15: 49-54.

Clark, J.H., Peck, E.J. and Markaverich, B.M. (1987) Steroid hormone receptors: basic principles and measurement. In: "Laboratory Methods Manual for Hormone Action and Molecular Endocrinology" (W.T. Schrader and B.W. O'Malley, eds.), Department of Cell Biology, Baylor College of Medicine, Houston, Texas.

į

Cleveland, D.W., Lopata, M.A., MacDonald, R.J., Cowan, N.J., Rutter, W.J. and Kirschner, M.W. (1980) Number and evolutionary conservation of α - and β -tubulin and cytoplasmic β - and γ -actin genes using specific cloned cDNA probes. Cell <u>20</u>: 95-105.

Cook, A.F. and Peter, R.E. (1980) The effect of temperature on the clearance of intraperitoneally-injected goradotropin in the goldfish, <u>Carassius auratus</u>. Aquaculture <u>19</u>: 275-285.

Corthesy, B., Hipskind, R., Theulaz, I. and Wahli, W. (1988) Estrogen-dependent in vitro transcription from the vitellogenin promoter in liver nuclear extracts. Science 239: 1137-1139.

Corthesy, B., Cardinaux, J.R., Claret, F.X. and Wahli, W. (1989) A nuclear factor I-like activity and a liver-specific repressor govern estrogen-regulated in vitro transcription from the Xenopus laevis vitellogenin B1 promoter. Molec. Cell. Biol. 9(12): 5548-5562.

Danielsen, M., Northrop, J.P., Jonklaas, J. and Ringold, G.M. (1987) Domains of the glucocorticoid receptor involved in specific and non-specific deoxyribonucleic acid binding, hormone activation, and transcriptional enhancement. Molec. Endocrinol. 1(11): 816-822.

Davidson, W.S., Birt, V.L., Birt, T.P. and Green, J.M. (1988) Palmitate-binding, serum albumin-like proteins in salmonids. FEBS Lett. 233(2): 299-302.

Davidson, W.S., Bartlett, S.E., Birt, T.P., Birt, V.L. and Green, J.M. (1989) Identification and purification of serum albumin from rainbow trout (<u>Salmo gairdnerii</u>) Comp. Biochem. Physiol. <u>93B</u> (1): 5-9.

Devereux, J., Haeberli, P. and Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. Nucl. Acids Res. <u>12(1)</u>: 387-395.

Ding, J.L., Valotaire, Y., Le Guellec, K., Ho, B. and Lam, T.J. (1988) Cloning the vitellogenin cDNA of <u>Oreochromis aureus</u>. Abstract P1. First International Symposium on Fish Endocrinology, Edmonton, Alberta.

Dimitriadis, G.J. and Tata, J.R. (1982) Differential sensitization to deoxyribonuclease I of <u>Xenopus</u> vitellogenin and albumin genes during primary and secondary induction of vitellogenesis by oestradiol. Biochem. J. <u>202</u>: 491-497.

Eckert, R.L. and Katzenellenbogen, B.S. (1982) Physical properties of estrogen receptor complexes in MCF-7 human breast cancer cells: differences with anti-estrogen and estrogen. J. Biol. Chem. <u>257</u>(15): 8840-8846.

Elliott, J.A.K., Bromage, N.R. and Whitehead, C. (1979) Effects of oestradiol-17ß on serum calcium and vitellogenin levels in rainbow trout. J. Endocrinol. <u>83</u>: 54-55.

Elliott, J.A.K., Bromage, N.R. and Whitehead, C. (1980) Oestradiol-17ß and vitellogenesis in the rainbow trout. J. Endocrinol. <u>87</u>: 65-66.

Elliott, J.A.K., Bromage, N.R. and Springate, J.R.C. (1984) Changes in reproductive function of three strains of rainbow trout exposed to constant and seasonally-changing light cycles. Aquaculture <u>43</u>: 23-34.

Emmersen, J., Korsgaard, B and Petersen, I. (1979) Dose response kinetics of serum vitellogenin, liver DNA, RNA, protein and lipid after induction by estradiol-17ß in male flounders (<u>Platichthys flesus</u> L.). Comp. Biochem. Physiol. <u>63B</u>: 1-6.

Evans, M.I., Silva, R. and Burch, J.B.E. (1988) Isolation of chicken vitellogenin I and III cDNAs and the developmental regulation of five estrogen-responsive genes in the embryonic liver. Genes and Devel. <u>2</u>: 116-124.

Evans, R. (1988) The steroid and thyroid hormone receptor superfamily. Science 240: 889-895.

Feinberg, A.P. and Vogelstein, B. (1983) A technique for radio-labeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. <u>132</u>: 6-13.

Flett, P.A. and Leatherland, J.F. (1989) <u>In vitro</u> hepatic monodeiodination of L-thyroxine and the temporal effect of 17B-estradiol on deiodination in rainbow trout (<u>Salmo gairdnerii</u> Richardson) Fish Physiol. Biochem. <u>6</u>(3): 129-138.

Folger, K., Anderson, J.N., Hayward, M.A. and Shapiro, D.J. (1983) Nuclease sensitivity and DNA methylation in estrogen regulation of <u>Xenopus laevis</u> vitellogenin gene expression. J. Biol. Chem. <u>258</u>(14): 8908-8914.

Follett, B.K. and Redshaw, M.R. (1968) The effects of oestrogen and gonadotrophins on lipid and protein metabolism in <u>Xenopus laevis</u>. J. Endocrinol. <u>40</u>: 439-449.

Follett, B.K. and Redshaw, M.R. (1974) The physiology of vitellogenesis. In: "Physiology of Amphibia" (B. Lofts, ed.). Academic Press, New York.

Fostier, A. and Breton, B. (1975) Binding of steroids by plasma of a teleost: the rainbow trout, <u>Salmo gairdnerii</u>. J. Ster. Biochem. <u>6</u>: 345-351.

Freedman, L.P., Luisi, B.F., Korszun, Z.R., Basavappa, R., Sigler, P.B. and Yamamoto, K.R. (1988) The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. Nature <u>334</u>: 543-546.

Gerber-Huber, S., Felber, B.K., Weber, R. and Ryffel, G.U. (1981) Estrogen induces tissue specific changes in the chromatin conformation of the vitellogenin genes in <u>Xenopus</u>. Nucl. Acids Res. 9(11): 2475-2494.

Gerber-Huber, S., Nardelli, D., Haefliger, J.A., Cooper, D.N., Givel, F., Germond, J.E., Engel, J., Green, N.M. and Wahli, W. (1987) Precursor-product relationship between vitellogenin and the yolk proteins as derived from the complete sequence of a <u>Xenopus</u> vitellogenin gene. Nucl. Acids Res. 15(12): 4737-4753.

Germond, J.E., ten Heggeler, B., Schubiger, J.L., Walker, P., Westley, B. and Wahli, W. (1983) Vitellogenin B2 gene in <u>Xenopus laevis</u>: isolation, <u>in vitro</u> transcription and relation to other vitellogenin genes. Nucl. Acids Res. <u>11</u>(10): 2979-2997.

Giguere, V., Hollenberg, S.M., Rosenfeld, M.G. and Evans, R.M. (1986) Functional domains of the human glucocorticoid receptor. Cell <u>46</u>: 645-652.

Godowski, P.J., Rusconi, S., Meisfeld, R. and Yamamoto, K.R. (1987) Glucocorticoid receptor mutants that are constitutive activators of transcriptional enhancement. Nature 325: 365-368.

Gorski, J. and Gannon, F. (1976) Current models of steroid hormone action: a critique. Ann. Rev. Physiol. <u>38</u>: 425-450.

Green, S. and Chambon, P. (1986) A superfamily of potentially oncogenic hormone receptors. Nature <u>324</u>: 615-617.

Green, S. and Chambon, P. (1987) Oestradiol induction of a glucocorticoid-responsive gene by a chimeric receptor. Nature <u>325</u>: 75-78.

Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J.M., Argos, P. and Chambon, P. (1986) Human oestrogen receptor cDNA: sequence, expression and homology to v-*erb*-A. Nature <u>320</u>; 134-139.

Green, S., Kumar, V., Theulaz, I., Wahli, W. and Chambon, P. (1988) The N-terminal DNA-binding 'zinc finger' of the oestrogen and glucocorticoid receptors determines target gene s1 ecificity. EMBO J. 7(10); 3037-3044.

Greene, G.L. (1984) Application of immunochemical techniques to the analysis of estrogen receptor structure and function. In: "Biochemical Actions of Hormones" (G. Litwack, ed.), Vol. XI, pp. 207-239. Academic Press, New York.

Greene, G.L., Sobel, N.B., King, W.J. and Jensen, E.V. (1984) Immunochemical studies of estrogen receptors. J. Steroid Biochem. 20(1): 51-56.

Greene, G.L., Gilna, P., Waterfield, M., Baker, A., Hort, Y. and Shine, J. (1986) Sequence and expression of human estrogen receptor complementary DNA. Science 231: 1150-1154.

Gross-Bellard. M., Oudet, P. and Chambon, P. (1973) Isolation of high-molecularweight DNA from mammalian cells. Eur. J. Biochem. <u>36</u>: 32-38.

Gshwendt, M. (1975) The effect of anti-estrogens on egg yolk protein synthesis and estrogen-binding to chromatin in the rooster liver. Biochim. Biophys. Acta <u>399</u>: 395-402.

Hache, R.J.G., Tam, S.P., Cochrane, A., Nesheim, M. and Deeley, R.G. (1987) Long-term effects of estrogen an avian liver: estrogen-inducible switch in expression of nuclear hormone-binding proteins. Molec. Cell. Biol. <u>7</u>(10): 3538-3547.

Hansson, T. and Rafter, J. (1983) <u>In vitro</u> metabolism of estradiol-17ß by liver microsomes from juvenile rainbow trout, <u>Salmo gairdnerii</u>. Gen. Comp. Endocrinol. <u>49</u>: 490-495.

Hara, A. (1976) Iron-binding activity of female-specific serum proteins of rainbow trout (<u>Salmo gairdnerii</u>) and chum salmon (<u>Oncorhynchus keta</u>). Biochim. Biophys. Acta <u>437</u>: 549-557.

Harley, C.B. (1987) Hybridization of oligo(dT) to RNA on nitrocellulose. Gene Anal. Techn. <u>4</u>: 17-22.

Harlow, K.W., Smith, D.N., Katzenellenbogen, J.A., Greene, G.L. and Katzenellenbogen, B.S. (1989) Identification of cysteine 530 as the covalent attachment site of an affinity-labeling estrogen (ketononestrol aziridine) and antiestrogen (tamoxifen aziridine) in the human estrogen receptor. J. Biol. Chem. <u>264</u>(29): 17476-17485.

Haschemeyer, A. (1973) Control of protein synthesis in the acclimation of fish to environmental temperature changes. In: "Responses of Fish to Environmental Changes" (W. Chavin, ed.), pp. 3-30. Charles C. Thomas, Springfield, Ill.

Hayward, M.A., Mitchell, T.A. and Shapiro, D.J. (1980) Induction of estrogen receptor and reversal of the nuclear/cytoplasmic receptor ratio during vitellogenin synthesis and withdrawal in <u>Xenopus laevis</u>. J. Biol. Chem. <u>255</u>(23): 11308-11312.

Hayward, M.A. and Shapiro, D.J. (1981) A middle-affinity estrogen-specific binding protein in livers of vitellogenic and non-vitellogenic <u>Xenopus laevis</u>. Devel. Biol. 88: 333-340.

Herbener, G.H., Bendayan, M. and Feldhoff, R.C. (1984) The intracellular pathway of vitellogenin secretion in the frog hepatocyte as revealed by protein A-gold immunochemistry. J. Histochem. Cytochem. <u>32</u>(7): 697-704.

Ho, S. (1987) Endocrinology of vitellogenesis. In: "Hormones and Reproduction in Fishes, Amphibians and Reptiles" (D.O. Norris and R.E. Jones, eds.), pp. 145-169. Plenum Press, New York.

Hori, S.H., Kodama, T. and Tanahashi, K. (1979) Induction of vitellogenin synthesis in golfish by massive doses of androgen. Gen. Comp. Endocrinol. <u>37</u>: 306-320.

Hochachka, P.W. and Somero, G.N. (1973) "Strategies of Biochemical Adaptation" W.B. Saunders Co., Philadelphia.

House, E.W., Dornauer, R.J. and van Lenten, B.J. (1979) Production of coronary arteriosclerosis with sex hormones and human chorionic gonadotropin (HCG) in juvenile steelhead and rainbow trout, <u>Salmo gairdnerii</u>. Atherosclerosis <u>34</u>: 197-206.

Houston, A.M. (1982) "Thermal Effects Upon Fishes" National Reseach Council of Canada, Ottawa, Ont.

Idler, D.R. and Campbell, C.M. (1980) Gonadotropin stimulation of estrogen and yolk precursor synthesis in juvenile rainbow trout. Gen. Comp. Endocrinol. <u>41</u>: 384-391.

Jensen, E.V. and Jacobson, H.I. (1960) Fate of steroid estrogens in target tissues. In: "Biological Activities of Steroids in Relation to Cancer" (G. Pincus and E.P Vollmer, eds.), pp. 161-178. Academic Press, New York.

Jensen, E.V., Suzuki, T., Kawashima, T., Stumpf, W.E., Jungblut, P.W. and DeSombre, E.R. (1968) A two-step mechanism for the interaction of estradiol with rat uterus. Proc. Natl. Acad. Sci. USA <u>59</u>: 632-638.

Jensen, E.V., Greene, G.L., Closs, L.E., DeSombre, E.R. and Nadji, M. (1982) Receptors reconsidered: a 20-year perspective. Rec. Prog. Hormone Res. <u>38</u>: 1-40. Joab, I., Radanyi, C., Renoir, M., Buchou, T., Catelli, M.G., Binart, N., Mester, J. and Baulieu, E.E. (1984) Common non-hormone binding component in non-transformed chick oviduct receptors of four steroid hormones. Nature <u>308</u>: 850-853.

Jordan, V.C., Collins, M.M., Rowsby, L. and Prestwich, G. (1977) A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J. Endocrinol. <u>75</u>: 305-316.

Kabat, E.A. and Mayer, M.M. (1961) "Experimental Immunochemistry" Thomas, Springfield, Ill.

Kadonaga, J.T., Carner, K.R., Masiarz, F.R. and Tjian, R. (1987) Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell 51: 1079-1090.

Katzenellenbogen, J.A., Carlson, K.E., Heiman, D.F., Robertson, D.W., Wei, L.L. and Katzenellenbogen, B.S. (1983) Efficient and highly selective covalent labeling of the estrogen receptor with [³H]-tamoxifen aziridine. J. Biol. Chem. <u>258</u>(6): 3487-3495.

Kime, D.E. and Saksena, D.N. (1980) The effect of temperature on the hepatic catabolism of testosterone in the rainbow trout (<u>Salmo gairdnerii</u>) and the golfish (<u>Carassius auratus</u>). Gen. Comp. Endocrinol. <u>42</u>: 228-234.

King, W.J., and Greene, G.L. (1984) Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. Nature <u>307</u>: 745-747.

Kirchgessner, T.G., Heinzmann, C., Svenson, K.L., Gordon, D.A., Nicosia, M., Lebherz, H.G., Lusis, A.J. and Williams, D.L. (1987) Regulation of chicken apolipoprotein B: cloning, tissue distribution, and estrogen induction of mRNA. Gene 59: 241-251.

Klein-Hitpass, L., Ryffel, G.U., Heitlinger, E. and Cato, A.C.B. (1988) A 13 bp palindrome is a functional estrogen responsive element and interacts specifically with estrogen receptor. Nucl. Acids Res. <u>16</u>: 647-663.

Klock, G., Strahle, U. and Schutz, G. (1987) Oestrogen and glucocorticoid responsive elements are closely related but distinct. Nature <u>329</u>: 734-736.

Koike, S., Sakai, M. and Muramatsu, M. (1987) Molecular cloning and characterization of the rat estrogen receptor cDNA. Nucl. Acids Res. 15(6): 2499-2513.

Kok, K., Snippe, L., AB, G. and Gruber, M. (1985) Nuclease-hypersensitive sites in chromatin of the estrogen-inducible apo VLDL II gene of chicken. Nucl. Acids Res. 13: 5189-5202.

Korsgaard, B., Emmersen, J. and Petersen, I. (1983) Estradiol-induced hepatic protein synthesis and transaminase activity in the male flounder, <u>Platichthys flesus</u> L. Gen. Comp. Endocrinol. <u>50</u>: 11-17.

Korsgaard, B., Mommsen, T.P. and Saunders, R.L. (1986) The effect of temperature on the vitellogenic response in Atlantic salmon post-smolts (<u>Salmo salar</u>). Gen. Comp. Endocrinol. <u>62</u>: 193-201.

Krust, A., Green, S., Argos, P., Kumar, V., Bornert, J.M. and Chambon, P. (1986) The chicken oestrogen receptor sequence: homology with v-*erb*-A and the human oestrogen and glucocorticoid receptors. EMBO J. 5(5): 891-897.

Kumar, S. and Chambon, P. (1988) The estrogen receptor binds tightly to its responsive element as a ligand-induced dimer. Cell <u>55</u>: 145-156.

Kumar, V., Green, S., Staub, A. and Chambon, P. (1986) Localization of the oestradiolbinding and putative DNA-binding domains of the human oestrogen receptor. EMBO J. 5: 2231-2236.

Kumar, V., Green, S., Stack, G., Berry, M., Jin, J.R. and Chambon, P. (1987) Functional domains of the human estrogen receptor. Cell <u>55</u>: 941-951.

Laemmli, U. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature <u>227</u>: 680-685.

Lawless, K. (1987) Liver gene expression during vitellogenesis in the male rainbow trout. M.Sc. Thesis, University of Ottawa, Ottawa, Ont.

Lazier, C.B. (1975) [³H]-Estradiol binding by chick liver nuclear extracts: mechanism of increase in binding following estradiol injection. Steroids <u>26(3)</u>: 281-298.

Lazier, C.B. (1979) Estrogen-binding proteins in avian liver: characteristics, regulation and ontogenesis. In: "Ontogeny of Receptors and Reproductive Hormone Action" (T.H. Hamilton, J.H. Clark and W.A. Sadler, eds.), pp. 353-370. Raven Press, New York.

Lazier, C.B. and Alford, W.S. (1977) Interaction of the antioestrogen, nafoxidinehydrochloride, with the soluble nuclear oestradiol-binding protein in chick liver. Biochem. J. <u>164</u>: 659-667.

Lazier, C.B. and Haggarty, A.J. (1979) A high-affinity oestrogen-binding protein in cockerel liver cytosol. Biochem. J. <u>180</u>: 347-353.

Lazier, C.B., Capony, F. and Williams, D.L. (1981) Antioestrogen action in chick liver: effects on oestrogen receptors and oestrogen-induced proteins. In: "Non-steroidal Antioestrogens: Molecular Pharmacology and Antitumor Activity" (R.L. Sutherland and V.C. Jordan, eds.), pp. 215-229. Academic Press, Sydney, NSW.

Lazier, C.B., Lonergan, K. and Mommsen, T.P. (1985) Hepatic estrogen receptors and plasma estrogen-binding activity in the Atlantic salmon. Gen. Comp. Endocrinol. 57: 234-245.

Lazier, C.B. and Murphy, P.R. (1986) Antiestrogen action in the chicken. In: "Estrogen/Antiestrogen Action and Breast Cancer Therapy" (V.C. Jordan, ed.), pp. 93-114. University of Wisconsin Press, Madison, WI.

Le Guellec, K., Lawless, K., Valotaire, Y., Kress, M. and Tenniswood, M. (1988) Vitellogenin gene expression in male rainbow trout (<u>Salmo gairdnerii</u>). Gen. Comp. Endocrinol. <u>71</u>: 359-371.

Le Menn, F., Rochefort, H. and Garcia, M. (1980) Effects of androgen mediated by the estrogen receptor of fish liver: vitellogenin accumulation. Steroids <u>35</u>: 2592-328.

Lehrach, H., Diamond, D., Wozney, J.M. and Boedtker, H. (1977) RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochem. <u>16(21)</u>: 4743-4751.

Lewis, J.A., Clemens, M.J. and Tata, J.R. (1976) Morphological and biochemical changes in the hepatic endoplasmic reticulum and Golgi apparatus of male <u>Xenopus laevis</u> after induction of egg-yolk protein synthesis by oestradiol-17ß, Molec. Cell. Endocrinol. <u>4</u>: 311-329.

Little, M., Szendro, P., Teran, C., Hughes, A. and Jungblut, P.W. (1975) Biosynthesis and transformation of microsomal and cytosol estradiol estrogen receptors. J. Steroid Biochem. <u>6</u>: 493-500.

Londei, P., Cammarano, P., Mazzei, F. and Romeo, A. (1982) Size heterogeneity of ribosomal RNA in eukaryote evolution- 1. rRNA molecular weights in species containing intact large ribosomal subunit RNA. Comp Biochem. Physiol. <u>73B</u>: 423-434.

Luskey, S.D., Brown, M.S. and Goldstein, J.L. (1974) Stimulation of synthesis of very low density lipoproteins in rooster liver by estradiol. J. Biol. Chem. <u>249</u>: 5939-5947.

MacKay, H.H. (1963) "Fishes of Ontario" Ontario Dept. Lands and Forests, Bryant Press, Toronto, Ont.

Mader, S., Kumar, V., de Verneuil, H. and Chambon, P. (1989) Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid-responsive element. Nature <u>338</u>: 271-274.

Maenpaa, P.H. (1976) Vitellogenin synthesis in rooster liver: changes in liver polysomes in relation to the activation of nucleolar RNA polymerase and vitellogenin synthesis. Biochem. Biophys. Res. Comm. <u>72</u>: 347-354.

Maitre, J.L., LeGuellec, C., Derrien, S., Tenniswood, M. and Valotaire, Y. (1985a) Measurement of vitellogenin from rainbow trout by rocket immunoelectrophoresis: application to the kinetic analysis of estrogen stimulation in the male. Can. J. Biochem. Cell Biol. <u>63</u>: 982-987

Maitre, J.L., Mercier, L., Dolo, L. and Valotaire, Y. (1985b) Caracterisation de recepteurs specifiques a l'oestradiol, inducton de la vitellogenine et de son mRNA dans le foie de triute arc-en-ciel (Salmo gairdnerii). Biochimie <u>67</u>: 215-225.

Maitre, J.L., Valotaire, Y. and Guguen-Guillouzo, C. (1986) Estradiol-17ß stimulation of vitellogenin synthesis in primary culture of male rainbow trout hepatocytes. In Vitro Cell. Devel. Biol. <u>22</u>(6): 337-343.

Maniatis, T., Fritsch, E. and Sambrook, J. (1982) "Molecular Cloning- A Laboratory Manual" Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Manning, N.J. and Kime, D.E. (1984) Temperature regulation of ovarian steroid production in the common carp, <u>Cyprinus carpio</u> L., in vivo and in vitro. Gen. Comp. Endocrinol. <u>56</u>:376-388.

Markaverich, B.M., Williams, M., Upchurch, S. and Clark, J.H. (1981) Heterogeneity of nuclear estrogen-binding sites in rat uterus: a simple method for the quantitation of type I and type II sites by [³H]-estradiol exchange. Endocrinol. <u>109</u>: 62-69.

Martinez, E., Givel, F. and Wahli, W. (1987) The estrogen-responsive element as an inducible enhancer: DNA sequence requirements and conversion to a glucocorticoid-responsive element. EMBO J. $\underline{6}(12)$: 3719-3727.

Maxwell, B.L., McDonnell, D.P., Conneely, O.M., Schulz, T.Z., Greene, G.L. and O'Malley, B.W. (1987) Structural organization and regulation of the chicken estrogen receptor. Mol. Endocrinol. $\underline{1}(1)$: 25-35.

McKearin, D.M. and Shapiro, D.J. (1988) Persistent induction of hepatic <u>Xenopus laevis</u> serum retinol binding protein mRNA. J. Biol. Chem. <u>263</u>(7): 3261-3265.

McPherson, R., Hannum, J. and Greco, T. (1988) An investigation of an estrogen-binding component in the liver and plasma of brook char, <u>Salvelinus fontinalis</u>. Comp. Biochem. Physiol. <u>89A</u> (4): 615-619.

Mester, J. and Baulieu, E.E. (1972) Nuclear estrogen receptor of chick liver. Biochim. Biophys. Acta <u>261</u>: 236-244.

Milgrom, E., Atger, M. and Baulieu, E.E. (1973) Studies on estrogen entry into uterine cells and on the estradiol-receptor complex attachment to the nucleus: Is the entry of estrogen into uterine cells a protein-mediated process? Biochim. Biophys. Acta 320: 267-283.

Miller, J., McLachlan, A.D. and Klug, A. (1985) Repetitive zinc-binding domains in the protein transcription factor IIIA from <u>Xenopus</u> oocytes. EMBO J. <u>4</u>: 1609-1614.

Mirsky, A.E. and Ris, H. (1949) Variable and constant components of chromosomes. Nature <u>163</u>: 666-667.

Mommsen, T.P. and Walsh, P.J. (1988) Vitellogenesis and oocyte assembly. In: "Fish Physiology" (W.S. Hoar and D.J. Randall, eds.), Vol. 11A, pp. 347-406. Academic Press, San Diego.

Nishio, Y., Shiokawa, K. and Yamana, K. (1978) Effects of temperature on the pattern of RNA synthesis in <u>Xenopus laevis</u> neurula cells. Devel. Growth and Differ. <u>20(1)</u>: 35-40.

Norberg, B. and Haux, C. (1985) Induction, isolation and a characterization of the lipid content of plasma vitellogenin from two <u>Salmo</u> species: rainbow trout (<u>Salmo gairdnerii</u>) and sea trout (<u>Salmo trutta</u>). Comp. Biochem. Physiol. <u>81B</u>: 869-876.

Notides, A.C. and Neilsen, S. (1975) A molecular and kinetic analysis of estrogen receptor transformation. J. Ster. Biochem. <u>6</u>: 483-486.

Noteborn, M.H.M., Bakker, O., de Jonge, M.A.W., Gruber, M. and AB, G. (1986) Differential estrogen responsiveness of vitellogenin and apo very low density lipoprotein II genes in the rooster liver. J. Steroid Biochem. 24(1): 281-285.

Norstedt, G., Wrange, O. and Gustafsson, J.A. (1981) Multihormonal regulation of the estrogen receptor in rat liver. Endocrinol. <u>108</u>(4): 1190-1196.

Ohno, S., Muramoto, J., Klein, J. and Atkin, N.B. (1969) Diploid-tetraploid relationships in clupeoid and salmonoid fish. In: "Chromosomes Today" (C.D. Darlington and K.R. Lewis, eds.), Vol. 2, pp. 139-147. Oliver and Boyd, Edinburgh.

Olsson, P.E., Zafarullah, M. and Gedamu, L. (1989) A role of metallothionein in zinc regulation after oestradiol induction of vitellogenin synthesis in rainbow trout, <u>Salmo</u> gairdnerii. Biochem. J. <u>257</u>: 555-559.

Oppenheimer, J.H. (1985) Thyroid hormone action at the nuclear level. Annals Int. Med. 102: 374-384.

Opresko, L., Wiley, H.S. and Wallace, R.A. (1981) Receptor-mediated binding and internalization of vitellogenin by <u>Xenopus</u> oocytes. J. Cell Biol. <u>91</u>: 218a.

Pakdel, F., Le Guellec, K., Vaillant, C., Le Roux, M.G. and Valotaire, Y. (1989) Identification and estrogen induction of two estrogen receptor (ER) messenger ribonucleic acids in the rainbow trout liver: sequence homology with other ERs. Mol. Endocrinol. 3(1): 44-51.

Palmiter, R.D., Mulvihill, E.R., Shepherd, J.H. and McKnight, G.S. (1981) Steroid hormone regulation of ovalbumin and conalbumin gene transcription: a model based upon multiple regulatory sites and intermediary proteins. J. Biol. Chem. <u>256</u>(15): 7910-7916.

Pankurst, N.W., Stacey, N.E. and Peter, R.E. (1986) An evaluation of techniques for the administration of 17B-estradiol to teleosts. Aquaculture <u>52</u>: 145-155.

Picard, D. and Yar amoto, K. (1987) Two signals mediate hormone-dependent nuclear localization of the givecocorticoid receptor. EMBO J. <u>6</u>(11): 3333-3340.

Piva, R., Bianchini, E., Kumar, V.L., Chambon, P. and del Senno, L. (1988) Estrogen induced increase of estrogen receptor RNA in human breast cancer cells. Biochem. Biophys. Res. Comm. <u>155</u>(2): 943-949.

Pottinger, T.G. (1986) Estrogen-binding sites in the liver of sexually mature male and female brown trout, <u>Salmo trutta</u> L. Gen. Comp. Endocrinol. <u>61</u>: 120-126.

Poulin, R., Simard, J., Labrie, C., Petitclerc, L., Dumont, M., Lagrace, L. and Labrie, F. (1989) Down-regulation of estrogen receptors by androgens in the ZR-75-1 human breast cancer cell line. Endocrinol. <u>125</u>: 392-399.

Protter, A.A., Wang, S.Y., Shelness, G.S., Ostapchuk, P. and Williams, D.L. (1982) Isolation and characterization of a cDNA clone specific for avian vitellogenin II. Nucl. Acids Res. <u>10</u>(16): 4935-4950.

Puca, G.A., Nola, E., Sica, V. and Brescani, F. (1975) Purification of estrogen receptors. In: "Methods in Enzymology" (B.W. O'Malley and J.G. Hardman, eds.), Vol. 36 pp. 331-349. Academic Press, New York.

Read, L.D., Greene, G.L. and Katzenellenbogen, B.S. (1989) Regulation of estrogen receptor messenger ribonucleic acid and protein levels in human breast cancer cell lines by sex steroid hormones, their antagonists, and growth factors. Mol. Endocrinol. 3(2): 295-304.

Reigel, A.T., Jordan, V.C., Bain, R.R. and Schoenberg, D.R. (1986a) Effects of antiestrogens on the induction of vitellogenin and its mRNA in <u>Xenopus laevis</u>. J. Steroid Biochem. <u>24</u>: 1141-1149.

Reigel, A.T., Martin, M.B. and Schoenberg, D.R. (1986b) Transcriptional and posttranscriptional inhibition of albumin gene expression by estrogen in <u>Xenopus</u> liver. Molec. Cell. Endocrinol. <u>44</u>: 201-209.

Reigel, A.T., Aitken, S.C., Martin, M.B. and Schoenberg, D.R. (1987) Posttranscriptional regulation of albumin gene expression in <u>Xenopus</u> liver: evidence for an estrogen receptor-dependent mechanism. Mol. Endocrinol. <u>1</u>(2): 160-167.

Riley, D., Heisermann, G.J., MacPherson, R. and Callard, I.F. (1987) Hepatic estrogen receptor in the turtle, <u>Chrysemys picta</u>: partial characterization, seasonal changes and pituitary dependence. J. Steroid Biochem. <u>26</u>(1): 41-47.

Saceda, M., Lippmann, M.E., Chambon, P., Lindsey, R.L., Ponglikitmongkol, M., Puente, M. and Martin, M.B. (1988) Regulation of the estrogen receptor in MCF-7 cells by estradiol. Mol. Endocrinol. <u>2</u>(12): 1157-1162.

Salhanick, A.R., Vito, C.C., Fox, T.O. and Callard, I.P. (1979) Estrogen-binding proteins in the oviduct of the turtle, <u>Chrysemys picta</u>: evidence for a receptor species. Endocrinol. <u>105</u>: 1388-1395.

Sanchez, E.R., Toft, D.O., Schlesinger, M.J. and Pratt, W.B. (1985) Evidence that the 90-kDa phosphoprotein associated with the untransformed L-cell glucocorticoid receptor is a murine heat shock protein. J. Biol. Chem. <u>260</u>(23): 12398-12401.

Sanger, F., Miklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA <u>74</u>: 5463-5467.

Scatchard, G. (1949) The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. <u>51</u>; 660-672.

Scott, A.P., Bye, V.J. and Baynes, S.M. (1980) Seasonal variations in sex steroids of the female rainbow trout (Salmo gairdnerii Richardson) J. Fish Biol. <u>17</u>: 587-592.

Scott, A.P. and Sumpter, J.P. (1983) The control of trout reproduction: basic and applied research on hormones. In: "Control Processes in Fish Physiology" (J.C. Rankin, T.J. Pitcher and R.T. Duggan, eds.), pp. 200-220. John Wiley and Sons, New York.

Seiler-Tuyns, A., Walker, P., Martinez, E., Merillat, A.M., Givel, F. and Wahli, W. (1986) Identification of estrogen-responsive sequences by transient transfection

Statistics.

experiments in a human breast cancer cell line. Nucl. Acids Res. 14(22): 8755-8770.

Shapiro, D. (1982) Steroid hormone regulation of vitellogenin gene expression. CRC Crit. Rev. Biochem. <u>12</u>: 187-203.

Shupnik, M.A., Gaordon, M.S. and Chin, W.W. (1989) Tissue-specific regulation of rat estrogen receptor mRNAs. Mol. Endocrinol. <u>3</u>(4): 660-665.

Skipper, J.K. and Hamilton, T.H. (1977) Regulation by estrogen of the vitellogenin gene. Proc. Natl. Acad. Sci. USA <u>74(6)</u>: 2384-2388.

Sloop, T.C., Sherman, B. and Lucier, G.W. (1984) Characterization of a cytosolic steroid-binding protein in the liver of the winter flounder, <u>Pseudopleuronectes americanus</u>. Gen. Comp. Endocrinol. <u>55</u>: 157-169

Smith, G.R. and Stearley, R.F. (1913) The classification and scientific names of rainbow and cutthroat trouts. Fisheries 14(1): 4-10.

Smith, J.S. and Thomas, P. (1990) Binding characteristics of the hepatic estrogen receptor of the spotted seatrout, <u>Cynoscion nebulosus</u>. Gen. Comp. Endocrinol. <u>77</u>: 29-42.

Snow, L.D., Eriksson, H., Hardin, J.W., Chan, L., Jackson, R.L., Clark, J.H. and Means, A.R. (1978) Nuclear estrogen receptor in the avian liver: correlation with biologic response. J. Ster. Biochem. <u>9</u>:1017-1026.

Spelsberg, T.C., Steggles, A.W., and O'Malley, B.W. (1971) Progesterone binding components of chick oviduct. III. Chromatin acceptor sites. J. Biol. Chem. 246: 4188-4197.

Spelsberg, T.C., Webster, R.A. and Pikler, G.M. (1976) Chromosomal proteins regulate steroid binding to chromatin. Nature <u>262</u>: 65-67.

Spelsberg, T.C., Littlefield, B.A., Seelke, R., Dani, G.M., Toyoda, H., Boyd-Leinen, P., Thrall, C. and Kon, O.L. (1983) Role of specific chromosomal proteins and DNA sequences in the nuclear binding sites for steroid receptors. Rec. Prog. Hormone Res. <u>39</u>: 463-513.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. <u>98</u>: 503-517.

Sutherland, R., Mester, J. and Baulieu, E.E. (1977) Tamoxifen is a potent "pure" antioestrogen in chick oviduct. Nature <u>267</u>: 434-435.

Tabor, S. and Richardson, C. (1987) DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc. Natl. Acad. Sci. USA <u>84</u>(14): 4767-4771.

Tam, S.P., Archer, T.K. and Deeley, R.G. (1986a) Biphasic effects of estrogen on apolipoprotein synthesis in human hepatoma cells: mechanism of antagonism by testosterone. Proc. Natl. Acad. Sci. USA <u>83</u>: 3111-3115.

Tam, S.P., Hache, R.J.G. and Deeley, R.G. (1986b) Estrogen memory effect in human hepatocytes during repeated cell division without hormone. Science <u>234</u>:1234-1237.

Tata, J.R. and Smith, D.F. (1979) Vitellogenesis: a versatile model for hormonal regulation of gene expression. Rec. Prog. Hormone Res. <u>35</u>: 47-95.

4 (M) = 14

Thomas, P. (1983) Quantitation of DNA and RNA by dot blot hybridization. In: "Methods in Enzymology" (R. Wu, L. Grossman and U. Moldave, eds.), Vol. 100 pp. 255-267. Academic Press, New York.

Truong, H. and Balieu, E.E. (1971) Interaction of uterus cytosol receptor with estradiolequilibrium and kinetic studies. Biochim. Biophys. Acta <u>237</u>: 167-172.

Tsai, S. Y., Carlstedt-Duke, J., Weigel, N.L., Dahlman, K., Gustafsson, J.A., Tsai, M.J. and O'Malley, B.W. (1988) Molecular interactions of steroid hormone receptor with its enhancer element: evidence for receptor dimer formation. Cell <u>55</u>: 361-369.

Turner, R.T., Dickhoff, W. and Gorbman, A. (1981) Estrogen binding to hepatic nuclei of Pacific hagfish, <u>Eptatretus stouti</u>. Gen. Comp. Endocrinol. <u>45</u>: 26-29.

Vaillant, C., Le Guellec, C., Pakdel, F. and Valotaire, Y. (1988) Vitellogenin gene expression in primary culture of male rainbow trout hepatocytes. Gen. Comp. Endocrinol. <u>70</u>: 284-290.

Valotaire, Y., Tenniswood, M., Le Guellec, C. and Tata, J.R. (1984) The preparation and characterization of vitellogenin messenger RNA from rainbow trout (<u>Salmo gairdnerii</u>). Biochem. J. <u>217</u>: 73-77.

van Bohemen, C.G. and Lambert, J.G.D. (1981) Oestrogen synthesis in relation to oestrone, oestradiol and vitellogenin plasma levels during the reproductive cycle of the female rainbow trout, <u>Salmo gairdnerii</u>. Gen. Comp. Endocrinol. <u>45</u>: 105-114.

van Bohemen, C.G., Lambert, J.G.D. and Pleute, J. (1981) Annual changes in plasma and liver in relation to vitellogenesis in the female rainbow trout, <u>Salmo gairdnerii</u>. Gen. Comp. Endocrinol. <u>44</u>: 94-107.

van Bohemen, C.G., Lambert, J.G.D., Goos, H.J.T. and van Oordt, P.G.W.J. (1982) Estrone and estradiol participation during exogenous vitellogenesis in the female rainbow trout, <u>Salmo gairdnerii</u>. Gen. Comp. Endocrinol. <u>46</u>: 81-92.

van het Schip, F.D., Samalo, J., Broos, J., Ophuis, J., Mojet, M., Gruber, M. and AB, G. (1987) Nucleotide sequence of a chicken vitellogenin gene and derived amino acid sequence of the encoded yolk precursor protein. J. Mol. Biol. <u>196</u>: 245-260.

Vasius, A. and Fletcher, G. (1988) Transcription of a vitellogenin gene in winter flounder continues after hypophysectomy. Abstract P3. First International Symposium on Fish Endocrinology, Edmonton, Alberta.

Wahli, W. (1988) Evolution and expression of vitellogenin genes. Trends in Genet. 4(8): 227-232.

Wangh, L. (1982) Glucocorticoids act together with estrogens and thyroid hormones in regulating the synthesis and secretion of <u>Xenopus</u> vitellogenin, serum albumin, and fibrinogen. Devel. Biol. <u>89</u>: 294-298.

i

Walker, P., Germond, J.E., Brown-Leudi, M., Givel, F. and Wahli, W. (1984) Sequence homologies in the region preceding the transcription initiation site of the liver estrogen-responsive vitellogenin and apo-VLDLII genes. Nucl. Acids Res. <u>12</u>(22): 8611-8626.

Wallace, R.A. and Jared, D.W. (1968) Studies on amphibian yolk VII. Serum phosphoprotein synthesis by vitellogenic females and estradiol-treated males of <u>Xenopus</u> laevis, Can. J. Biochem. <u>46</u>: 953-959.

Walter, P., Green, S., Greene, G., Krust, A., Bornert, J.M., Jeltsch, J.M., Stuab, A., Jensen, E., Scrace, G., Waterfield, M. and Chambon, P. (1985) Cloning of the human estrogen receptor cDNA. Proc. Natl. Acad. Sci. USA <u>82</u>: 7889-7893.

Walters, M.R., Hunzike, W. and Norman, A. (1980) Unoccupied 1,25-dihydroxy-vitamin D3 receptors: nuclear/cytosol ratio depends on ionic strength. J. Biol. Chem. 255: 6799-6805.

Watts, C.K.W., Murphy, L.C. and Sutherland, R.L. (1986) Properties of high affinity intracellular binding sites for antiestogens. In: "Estrogen/Antiestrogen Action and Breast Cancer Therapy" (V.C. Jordan, ed.), pp. 93-114. University of Wisconsin Press, Madison, WI.

Webster, N.J.G., Green, S., Jin, J.R. and Chambon, P. (1988) The hormone-binding domains of the estrogen and glucocorticoid receptors contain an inducible transcription activation function. Cell <u>54</u>: 199-207.

Weigand, M.D. (1982) Vitellogenesis in fishes. In: "Proceedings of the International Symposium on Reproductive Physiology of Fish" (C.J.J. Richter and H.J.T. Goos, eds.), pp. 136-146. Centre for Agricultural Publishing and Documentation, Wageningen.

Weiler, I.J., Lew, D. and Shapiro, D.J. (1987) The <u>Xenopus laevis</u> estrogen receptor: sequence homology with human and avian receptors and identification of multiple estrogen receptor messenger ribonucleic acids. Mol. Endocrinol. <u>1</u>(5): 355-362.

Weintraub, H. and Groudine, M. (1975) Chromosomal subunits in active genes have an altered conformation. Science <u>193</u>: 848-856.

Welshons, W.V., Lieberman, M.E. and Gorski, J. (1984) Nuclear localization of unoccupied oestrogen receptors. Nature <u>307</u>: 747-749.

Westley, B., Wyler, T., Ryffel, G. and Weber, R. (1981) <u>Xenopus laevis</u> serum albumins are encoded in two closely related genes. Nucl. Acids Res. 9(15): 3557-3574.

Whelly, S. (1986) Estradiol regulation of uterine nucleolar estradiol binding sites. Biochim. Biophys. Acta <u>880</u>: 179-188.

White, R., Lees, J.A., Needham, M., Ham, J. and Parker, M. (1987) Structural organization and expression of the mouse estrogen receptor. Mol. Endocrinol. 1(10): 735-744.

Whitehead, C., Bromage, N.R. and Forster, J.R.M. (1978) Seasonal changes in the reproductive function of the rainbow trout (Salmo gairdnerii). J. Fish Biol. <u>12</u>: 601-608.

1

Whitehead, C., Bromage, N.R., Forster, J.R.M. and Matty, A.J. (1978) The effects of alterations in photoperiod on ovarian development and spawning time in the rainbow trout (Salmo gairdnerii). Ann. Biol. Anim. Bioch. Biophys. <u>18</u>(4): 1035-1043.

Wiley, H.S., Opresko, L. and Wallace, R.A. (1979) New methods for the purification of vertebrate vitellogenin. Anal. Biochem. <u>97</u>: 145-152.

Williams, D.L., Newman, T.C., Shelness, G.S. and Gordon, D.A. (1986) Measurement of apolipoprotein mRNA by DNA-excess solution hybridization with single-stranded probes. In: "Methods in Enzymology" (J.P. Segrest and J.J. Albers, eds.), Vol. 128 pp. 671-689. Academic Press, New York.

Williams, J.L. and Tata, J.R. (1983) Simultaneous analysis of conformation and transcription of A and B groups of vitellogenin genes in male and female <u>Xenopus</u> during primary and secondary activation by estrogen. Nucl. Acids Res. <u>11</u>(4): 1151-1166.

Wilson, A.C., Carlson, S.S. and White, T.J. (1977) Biochemical evolution. Ann. Rev. Biochem. <u>40</u>: 573-649.

Wiskocil, R., Bensky, P., Dower, W., Goldberger, R.F., Gordon, J.I. and Deeley, R.G. (1980) Coordinate regulation of two estrogen-dependent genes in avian liver. Proc. Natl. Acad. Sci. USA <u>77(8)</u>: 4474-4478.

Wolffe, A.P., Glover, J.F., Martin, S.C., Tenniswood, M.P.R., Williams, J.L. and Tata, J.R. (1985) Deinduction of transcription of <u>Xenopus</u> 74-kDa albumin genes and destabilization of mRNA by estrogen in vivo and in hepatocyte cultures. Eur. J. Biochem. <u>146</u>: 489-496.

Yaron, Z., Cocos, M. and Salzer, H. (1980) Effects of temperature and photoperiod on ovarian recrudescence in the cyprinid fish <u>Mirogrex terrae-sanctae</u>. J. Fish Biol. <u>16</u>: 371-382.

Yusko, S., Roth, T.F. and Smith, T. (1981) Receptor-mediated vitellogenin binding to chicken oocyte. Biochem. J. 200: 43-50.

Zeff, R.A. and Geleiber, J. (1987) Oligonucleotide probes for genomic DNA blots. Focus 9(2): 1-2.

Zhang, H., Scholl, R., Browse, J. and Somerville, C. (1988) Double stranded DNA sequencing as a choice for DNA sequencing. Nucl. Acids Res. <u>16(3)</u>: 1220.