

1992

Androgen Regulation of Gene Expression Associated with Cell Growth and Neural Plasticity: Studies in Songbird Brain and the S115 Cell Line

Kent L. Nastiuk

Follow this and additional works at: [http://digitalcommons.rockefeller.edu/
student_theses_and_dissertations](http://digitalcommons.rockefeller.edu/student_theses_and_dissertations)

 Part of the [Life Sciences Commons](#)

Recommended Citation

Nastiuk, Kent L., "Androgen Regulation of Gene Expression Associated with Cell Growth and Neural Plasticity: Studies in Songbird Brain and the S115 Cell Line" (1992). *Student Theses and Dissertations*. 372.
http://digitalcommons.rockefeller.edu/student_theses_and_dissertations/372

This Thesis is brought to you for free and open access by Digital Commons @ RU. It has been accepted for inclusion in Student Theses and Dissertations by an authorized administrator of Digital Commons @ RU. For more information, please contact mcsweej@mail.rockefeller.edu.

LD4711.6
N269
c.1
RES



THE LIBRARY

LD 4711.6 N269 1992 c.1 RES
Nastiuk, Kent L.
Androgen regulation of gene
expression associated with

Rockefeller University Library
1230 York Avenue
New York, NY 10021-6399



Androgen Regulation of Gene Expression Associated with Cell Growth
and Neural Plasticity: Studies in Songbird Brain and the S115 Cell Line.

Kent L. Nastiuk

A thesis submitted to the faculty of the Rockefeller University in partial
fulfillment of the requirement for the degree of Doctor of Philosophy

New York, N.Y. 1992

Acknowledgements

I am deeply grateful to my research advisor, David Clayton, who has been many things to those of us in the lab in addition to a great mentor. It is a special relationship being the first student of a scientist starting his career, with less experience to guide us through this tortuous path, but in its place, David has always been ready with extra enthusiasm, encouragement, and friendship.

I also owe a great deal to those members of the group who have made the long hours in the lab endurable, and even enjoyable. And especially for putting up with me in general.

Fernando Nottebohm has been extraordinarily generous with both his time, wisdom, and canaries, without which this work could not have taken place. And last, but by no means least, a hearty thank you to the birdkeepers at Millbrook who kept my birds alive so long, and nursed so many back to health.

TABLE OF CONTENTS

	page no.
ABSTRACTP	1
CHAPTER 1: INTRODUCTION	2
Molecular Mechanisms of Steroid Action	3
Non-Genomic Mechanisms of Steroid Action	3
RNA Stabilization by Steroids	4
Steroid Receptor Regulation of Gene Expression	5
Regulation of Gonadal Steroid Receptors	5
Regulation of Steroid Receptors in the Brain	7
Regulation of Other Genes by Steroid Receptors	7
Steroids molecular actions on the Brain	9
Steroid Action on Brain Development and Function	10
Development of Sexual Dimorphism	10
Steroid regulation of structure and function	11
Brain, Hormones and Behavior: Songbirds	12
Song specialization of the forebrain	12
Development of song control nuclei	16
Seasonal regulation of singing	
and the song control nuclei	16
Steroid Receptors are Found in Song Control Nuclei	17
Progesterone and Estrogen Receptors	17
Androgen Receptor	18
Hormonal control of song control nuclei	19
Prospects for Research	21

CHAPTER 2: THE CANARY ANDROGEN RECEPTOR.....	22
A: CLONING OF THE CANARY ANDROGEN RECEPTOR.....	22
Results	22
PCR Cloning the CDE Region of the AR	23
Cloning the Remainder of the AR	30
Genomic Cloning the First Exon of the AR	39
Sequencing the AR	44
B: LOCALIZATION OF THE CANARY ANDROGEN RECEPTOR.....	50
Results	50
Localization of the cAR in Testis	50
Localization of the cAR in Brain	56
C: REGULATION OF THE CANARY ANDROGEN RECEPTOR.....	67
Results	67
Spring versus Fall	71
Androgens Regulate the AR mRNA in Canaries	76
Androgens Regulate the AR in Zebra Finch	80
D: DISCUSSION	83
The Canary Androgen Receptor	83
Sequence Analysis and Evolution of the Androgen Receptor	86
in situ Hybridization of the cAR	87
Localization of the cAR in Testis	87
Localization of the cAR in Brain	88
Regulation of the cAR in Songbirds	89
Regulation of the cAR in the Brain	90
E: SUMMARY	91

CHAPTER 3: ANDROGEN REGULATED GENE EXPRESSION IN CANARIES	92
A: CLONING OF CANARY C-JUN	93
RESULTS	94
Homology of canary c-jun	94
B: ANDROGEN INDUCTION OF GENES IN THE CANARY BRAIN.....	104
RESULTS	107
HORMONES	107
Androgen Induction of Genes:	110
1: Preliminary Assay of Androgen Effects	110
2: Quantitative Assay of Androgen Effects	113
C: DISCUSSION	127
Canary c-jun	127
Induction of Genes by Androgen in the Canary Brain	127
Hormone Treatment	127
Anatomical Location of Tissue Samples	131
Androgen Regulation of Gene Expression in the Canary Song System	131
D: SUMMARY	133
CHAPTER 4: ANDROGEN REGULATED GENE EXPRESSION IN S115 CELLS	134
RESULTS	135
A: Androgen Regulation of Proliferation	135
B: Direct Effects of Androgen on Gene Expression	139
C: Androgen Modulation of Induced Gene Expression	146
Forskolin	146
TPA	146
A23187	147
DISCUSSION...	155
Androgen Regulation of Gene Expression in S115 Cells	157
SUMMARY	159

CHAPTER 5: CONCLUSIONS	160
CHAPTER 6: METHODS	163
cDNA Cloning- Direct Screening	163
PCR	164
Labeled Oligonucleotide Preparation	165
Southern Blot Analysis	166
Genomic Cloning and Screening	167
Sequence Analysis	168
Riboprobe Preparation	169
In Situ Hybridization	169
Northern Blot Analysis	172
Quantitative PCR	172
Animals- surgery, treatments, tissue isolation	177
Radioimmunoassays for steroid hormones	178
S115 cells	179
RNA preparation	179
cDNA-dots hybridizations	180
cDNA Probe Preparation	180
Plasmids	181
CHAPTER 7: REFERENCES	183

ABSTRACT

In order to characterize androgens' effects on the brain and behavior I identified the canary cDNA coding for the nuclear receptor for androgen. The sequence is very conserved over functional domains, but shows some divergence from the only other sequences known, those for rodents and man. The AR mRNA can be localized by in situ hybridization in the canary to the testis and the song nuclei HVc, RA, and MAN. The AR mRNA is regulated by androgens in the periphery in a tissue specific manner. Using a sensitive semi-quantitative pcr assay, regulation of the AR mRNA by androgen treatment and by natural fluctuations of circulating androgens is seen in the song control nucleus HVc.

I used the information about receptor location and the timing of androgen effects to examine androgen induced changes in genes likely be involved in neuronal plasticity. I cloned the canary homolog of the c-jun proto-oncogene, and confirmed its identity based on complete conservation of the important functional domains. I prepared RNA from steroid responsive parts of the brain from ovariectomized canaries treated with testosterone using a simple dot hybridization assay. I could detect a small but rapid induction by androgen of c-jun and two other proto-oncogenes (c-myc and n-myc) in RNA from tissue containing HVc and surrounding telencephalon.

Because of this lack of a more dramatic effect in the complex tissue of the brain, I also quantified changes in gene expression induced by androgens in a simpler system. Acting in relative isolation, testosterone can dramatically increase the rate of proliferation of the cells, while estradiol is without effect. Testosterone can increase the mRNA levels of several structural genes, including homologs of genes regulated in the canary brain, and represses several transcription factors in addition to its own receptor. Testosterone also interacts synergistically with the Ca^{++} ionophore A23187 to induce c-myc and histone H1 expression, where neither alone will do so. In addition, androgen pretreatment represses the TPA-induced increase of these same genes.

INTRODUCTION

The steroid hormones have long been interesting and productive objects of study by both biochemists and biologists. Biochemically, the steroids, with their receptors, comprise one of the earliest identified classes of transcription regulatory factors (Beato, 1989). Biologically, they have widespread actions ranging from the function of glucocorticoids as anti-inflammatory agents (Miesfeld, 1990) to the profound effects of gonadal steroids on the central nervous system (Goy and McEwen, 1980). They establish the sexual dimorphism in the structure of the reproductive tract and brain of many species (Harris, 1964). They also cause the expression of many behaviors, ranging from female receptivity in the rat (Parsons et al., 1980) to male aggression in rodents (Goy and McEwen, 1980) and birds (Wingfield et al., 1990). The steroids bring about these actions via intracellular receptors which alter the expression of responsive genes, although for very rapid effects, they may act at the cell membrane.

Androgens induce many of the changes in brain and behavior seen in male canaries during the reproductive season in spring. Specific areas of the brain responsible for song production and perception undergo changes in size (Nottebohm and Arnold, 1976) and connectivity (spine density and dendritic length (Devoogd and Nottebohm, 1981; Devoogd, 1986)) which are correlated with plasma steroid levels as well as singing behavior. These changes can be induced at other times of the year (and in females) by treatment with exogenous testosterone, and the magnitude of anatomical change witnessed is much greater than that observed in other gonadal steroid dependent neural systems (Tobet and Fox, 1989; Swaab and Fliers, 1985). The large changes in brain structures associated with song, and the dependence of these changes on androgen levels, make the canary a particularly good system to examine mechanisms of steroid hormone interaction with the nervous system. In the research presented later in this thesis, I have

INTRODUCTION

used a variety of approaches to characterize the effects of androgens on gene expression associated with cell growth and plasticity, focusing on songbirds as a model system.

Molecular Mechanisms of Steroid Action

Steroid hormone effects are mediated by nuclear receptors which bind DNA to regulate transcription of responsive genes (Slater et al., 1990). Steroid hormones and their receptors are well established models for transcriptional regulation (Yamamoto, 1985; Ham and Parker, 1989). The steroid receptor superfamily includes receptors active in gonadal, homeostatic, and morphogenic functions (Evans, 1988). These molecules share many structural features with well defined roles among them a cysteine rich DNA binding domain and a steroid binding domain (Berg, 1989; O'Malley, 1990). Steroid hormones bind to their receptors on a C-terminal domain (Green et al., 1988; Kumar et al., 1987), causing a change in the conformation of this region which promotes dimerization of receptors between specific hydrophobic regions (Fawell et al., 1990). The receptor dimer has a high affinity for specific DNA sequences, called hormone response elements (HREs) (Green et al., 1988; Beato, 1989; Giguere et al., 1986). These HREs are enhancer elements in the DNA that target the transcriptional effects of the receptors to genes nearby on the chromosome (reviewed in Yamamoto, 1985). When the receptors are bound to these enhancers, several other regions of the steroid hormone receptor, the so called "transcriptional activation functions" (TAF), can regulate transcription within minutes (Tora et al., 1989; Tasset et al., 1990).

Non-Genomic Mechanisms of Steroid Action

Transcription modulation by the steroid hormone receptors may be supplemented by faster, non-genomic actions of the hormones. Rapid membrane effects have been seen for each of the gonadal steroids (reviewed in Schumacher, 1990). These rapid effects may be due to direct actions on the plasma membrane, to membrane bound receptors

INTRODUCTION

which alter cyclic nucleotide concentration, or to direct effects on receptors for classical neurotransmitters. Steroids may alter the electrical characteristics of the plasma membrane by intercalating in the phospholipid bilayer (Gewirtz et al., 1989).

Testosterone induces increases the duration of action potentials in the electric fish *Sternopygus*, possibly by altering the electric organs' passive membrane properties (Mills and Zakon, 1991). Specific, saturable membrane bound receptors for each of the gonadal steroids (Towle and Sze, 1983) and corticosteroids (Orchinik et al., 1991) have been characterized, with binding to the glucocorticoid receptor (GR) directly associated with a suppression of sexual behavior in amphibians. Finally, there are a number of reports of rapid modulation by steroid hormones of ion channels (Nabekura et al., 1986), peptide hormone/transmitters (Schumacher et al., 1990), opiod receptors (Su et al., 1988) and classical transmitters such as GABA (Majewska et al., 1986). While such non-transcriptional mechanisms may be important for many of the rapid effects of the steroids, such as progestins induction of lordosis, most long term effects of the steroid hormones appear to be mediated via their nuclear receptors (Evans and Arriza, 1989).

RNA stabilization by Steroids

The regulation of mRNA levels has been largely simplified to control of transcription, but there is a balance maintained in the cell between synthesis and degradation which can be affected by the steroids. The steroids regulate the mRNA levels of a variety of genes which are not under direct transcriptional control by influencing their half-life in the cytoplasm. This regulation of mRNA stability is important for many cell cycle related genes (Brawerman, 1989). The steroids act on mRNA stability through primarily two mechanisms, increasing stability, or inducing degradative enzymes. Glucocorticoids enhance growth hormone mRNA stability by lengthening the polyA tail (Paek and Axel, 1987). Glucocorticoids also induce stabilization factors which extend the half-life of PEPCK mRNA (reviewed in Nielsen and Shapiro, 1990a). Estrogen, acting on estrogen receptor (ER) negative cells, stabilizes

INTRODUCTION

vitellogenin mRNA when the ER gene is co-transfected, but estrogen treatment without the ER does not destabilize the transcript (Nielsen and Shapiro, 1990b). A form of LDL mRNA is stabilized by estrogens by increasing the length of the polyA tail, but estrogen withdrawal induces a degradative system which rapidly clears the transcript (reviewed in Nielsen and Shapiro, 1990a). A final caveat must be included when describing steroid stabilization of mRNAs, as the effects have been observed in most systems using high, pharmacological levels of the hormones (1 μ M), whereas a recent examination using estrogen at 3 nM, a physiological concentration, failed to show stabilization of vitellogenin mRNA (McKenzie and Knowland, 1990).

Steroid Receptor Regulation of Gene Expression

Regulation of Gonadal Steroid Receptors

ER mRNA levels are regulated by changes in the levels of plasma estrogens in a tissue specific manner. Ovariectomy (ovx), which lowers plasma estrogens, induces the ER mRNA four fold in the uterus, and depresses it by two thirds in the liver (Shupnik et al., 1989). This effect is reversed one day after a single injection of estrogen (Shupnik et al., 1989). The estrogen receptor in the pituitary is similarly down-regulated by ovx in the rat (Shupnik et al., 1989). In addition, in the human breast cancer cell line EFM-19, ER mRNA is reduced five fold by estrogen, as long as the cells have not progressed to steroid insensitivity (Westley and May, 1988), and the ER mRNA is similarly regulated by progestins (Alexander et al., 1990). In an osteosarcoma cell line, the ER mRNA level is similarly down-regulated by estrogens, and also by testosterone (Komm et al., 1988). The progesterone receptor (PR) is regulated in an analogous manner, where its ligands, the progestins, down-regulate PR mRNA levels in both the pituitary (Shupnik et al., 1989) and the oviduct (Conneely et al., 1986). The PR is also regulated by estrogens, which induce the PR mRNA in the oviduct (Conneely et al., 1986), pituitary,

INTRODUCTION

hypothalamus (Lauber et al., 1991; Bayliss and Millhorn, 1991) and in breast cancer cells (Wei et al., 1988; Read et al., 1988; Poulin et al., 1989; Lee et al., 1989). Estrogens can also down regulate the glucocorticoid receptor in pituitary (Peiffer and Barden, 1987). Androgen receptor (AR) mRNA is regulated much like the ER in the reproductive accessory tissues of the rat. In the epididymis and coagulating gland, the AR mRNA is increased several fold by castration, and this induction is inhibited by exogenous testosterone (Quarmby et al., 1990). In the ventral prostate, a castration induced increase of the rAR (2 to 3 fold) is apparent within one day (Quarmby et al., 1990; Shan et al., 1990), and testosterone reduces AR mRNA below control levels within eight hours after an intramuscular injection (Quarmby et al., 1990). In the LNCaP human prostate cancer cell line, more dramatic repression (ten-fold) of the AR is seen in the transition from steroid-free medium to one containing 30nM testosterone (Quarmby et al., 1990). The AR mRNA is similarly regulated in the liver cell line HepG2 (Shan et al., 1990).

Whether the AR mRNA in the brain fluctuates in any way related to the natural cycling of androgens has not been examined in any species up to now. It is likely that in canary, like the rat, the receptors in the hypothalamus and other diencephalic regions control reproduction and are regulated by circulating levels of gonadal steroids. The AR mRNA levels in rat whole brain RNA preps are increased several fold and then return to near normal following treatment with exogenous testosterone (Quarmby et al., 1990), but there is no information on the anatomical localization of these effects. While there has been less analysis of the natural fluctuations of plasma androgen than estrogens or progestins, androgens are under some reproductive control, as can be seen in animals with defined breeding seasons such as canaries (Nottebohm et al., 1987). These fluctuations in circulating steroids can regulate the AR mRNA, at least in rats (Shan et al., 1990). Androgen levels can also be manipulated experimentally by surgical or chemically induced castration, which greatly reduce the level of plasma androgen (Jackson et al., 1986; Morris et al., 1986; Jackson and Jackson, 1984). Alternatively, the

INTRODUCTION

effective level of androgens can be manipulated with antagonists (Poyet and Labrie, 1985).

Regulation of Steroid Receptors in the Brain

The ER is regulated in brain centers associated with reproduction much like it is in the periphery (reviewed in McEwen et al., 1979). ER mRNA levels in the brain are negatively regulated by estrogens. In both the VMH and Arcuate nucleus, exogenous estrogens appear to reduce the amount of message per cell (Simerly and Young, 1991). The ER is not induced by ovx, perhaps because the normal circulating level of estrogens is so low, but the ER is repressed by exogenous estradiol administration (Simerly and Young, 1991). It is unclear whether ER levels cycle with the normal reproductive fluctuation of estrogen levels in the rat but ER mediated effects on synaptic morphology do fluctuate (Woolley et al., 1990). Estrogens induce the PR mRNA in the mPOA, Arcuate Nucleus, and VMH, but have no effect on mRNA levels in the cortex (Bogic et al., 1988).

Most animals have steroid hormone receptors in the evolutionarily ancient structures of the brain which regulate reproductive function (reviewed in McEwen et al., 1979). As shown later in this thesis, the androgen receptor is also present in parts of the canary telencephalon. The location of androgen receptor in the forebrain of songbirds has a parallel in the rat (Simerly et al., 1990), which has a moderate level of AR mRNA in the evolutionarily new cortical layers 2 and 3, and the hippocampus, and a high level in the older lateral septal nuclei. The regulation of the steroid receptors in the forebrain has not been examined, but may be novel since they are not part of the homeostatic control apparatus for the reproductive system.

Regulation of Other Genes by Steroid Receptors

Steroids, their receptors, and the genes they regulate have long been a model system to study transcription (Yamamoto, 1985). The *Drosophila* steroid hormone

INTRODUCTION

ecdysone provides a particularly elegant model of transcriptional regulation (Ashburner, 1990). Ecdysone induces puffing in a series of genes on the polytene chromosomes which puff maximally at different times (Ashburner, 1990). In addition, it has recently been demonstrated that some of these developmentally important genes are not only induced by the hormone, but are subsequently repressed by it (Thummel et al., 1990). One of these regulated genes, E74, even appears to encode a transcription regulatory protein (Burtis et al., 1990). This leads to the hypothesis that there are genes regulated initially by the steroids which go on to regulate other genes in a large network over time.

The glucocorticoid receptor is perhaps the most studied steroid receptor.

Glucocorticoids regulate a large variety of genes and in different ways. A number of genes have been characterized which respond immediately and directly to the steroid (even in the presence of the protein synthesis inhibitor cyclohexamide) including the mouse mammary tumor virus, the tyrosine amino transferase gene, and the growth hormone gene (reviewed in Yamamoto, 1985; Miesfeld, 1990). There are other genes which are directly responsive to glucocorticoid, but which respond much more slowly like collagenase (Jonat et al., 1990), Osteocalcin (Schule et al., 1990b), and proliferin (Diamond et al., 1990). In addition, there are a number of genes which appear to be "secondary response" genes, which do not have GREs in their enhancers, but which are nonetheless regulated by glucocorticoids, such as α -2 microglobulin, tryptophan dioxygenase, and α -1 acid glycoprotein (reviewed in Miesfeld, 1990).

The induction of common structural protein and enzyme genes in peripheral tissues by androgen has been well characterized (Watson and Paigen, 1990 and references therein). Very little information on the regulation of potential proliferation related genes by androgens exists. Androgens may direct transient increases in c-myc, c-fos, and hsp70 mRNAs seen following castration in regressing rodent prostate (Buttayan et al., 1988) and implanted tumor cells (Rennie et al., 1988), but these effects may be secondary to the regression of the tissues. Recently, Persson, et al. (1990) showed the NGF

INTRODUCTION

receptor in Sertoli cells to be down-regulated by androgen in vivo over several days.

Estrogen effects on proliferation related genes have been described in several systems. The levels of several proto-oncogenes are regulated by estrogen in vivo, including c-myc in the oviduct (Rempel and Johnston, 1988), and c-jun (Weisz et al., 1990), and c-fos in the uterus with estrogen in vivo (Gibbs et al., 1990b; Loose-Mitchell et al., 1988). In addition, the EGF receptor is induced rapidly by estrogen in the uterus (Lingham et al., 1988). Two days of estrogen treatment of osteosarcoma cells induces both TGF- β and procollagen two to three fold (Komm et al., 1988).

Steroids' Actions on Genes in the Brain

Gonadal steroids appear to regulate some proliferation related genes in the nervous system. Estrogen induces a 70 Kd protein which may be hsp70 (Mobbs et al., 1990), but fails to increase the levels of c-fos mRNA in the rat hypothalamus (Gibbs et al., 1990b), even though c-fos is seen to be induced developmentally and with seizure inducing drugs (Gibbs et al., 1990a). Estrogens also quickly induce a variety of structural and nerve function regulatory genes. For example, estrogen increases the transcription of ribosomal RNA in the VMH within 30 minutes, reaching a maximum 100% stimulation at two hours (Jones et al., 1990). Estrogen strongly induces mRNA encoding a neuropeptide, galanin, in the pituitary of both male and female rats (Kaplan et al., 1988), and the microtubule stabilizing factor τ (Ferreira and Caceres, 1991). Finally, estrogen increases the level of the receptor for the neuropeptide oxytocin within six hours in the hypothalamus (Coirini et al., 1991).

Reports of androgen effects on proliferation related genes in the brain are sparse. Stanley and Fink (1985; 1986) examined androgen induced molecular differences in the rat brain during development and found changes in several proteins, including actin and tubulin, by two dimensional electrophoresis of extracts from the sexually dimorphic hypothalamus and pre-optic areas. There is a sexual dimorphism of NGF receptor

INTRODUCTION

expression in the hippocampus of developing rats (Kornack et al., 1991).

The role of androgenic versus estrogenic actions in brain development is further confused by the potential metabolism of testosterone into estrogen. The estrogenic effects are mediated by the ER, when it is stimulated by estrogen, which can be produced in the periphery or formed in the brain by aromatization of testosterone. The AR mediates the androgenic effects when it is activated by testosterone, but it can also be activated by dihydrotestosterone, a non-aromatizable metabolite produced by 5α reduction. In the rat CNS, enzymes for both avenues of metabolism are found in the steroid receptor containing areas (reviewed in (Goy and McEwen, 1980). In male songbirds, the brain is the only site of aromatase activity, while in females, both the brain and the ovary can aromatize testosterone (Schlinger and Arnold, 1991).

Steroid Action on Brain Development and Function

Development of Sexual Dimorphism

In mammals and birds, differentiation of gonads is under genetic control. The subsequent differentiation of the reproductive system and brain is controlled by gonadal steroid secretion (reviewed in (Dohler, 1987). Early hormone exposure in mammals establishes persistent differences in both structure and function of the brain (Pfeiffer, 1936). There is a sensitive developmental period when estrogens or androgens specify the dimorphism in the hypothalamus and pre-optic area of mammals (Harris, 1970; Harris, 1964). Female rats given androgens before day 10 will develop masculinized reproductive systems (Barraclough, 1961) and brains (Gorski et al., 1978). The masculinization depends on both androgenic actions, and estrogenic stimulation from aromatization of the testosterone (Dohler et al., 1986). Feminization is also a hormone dependent event. Estrogen is necessary for the capacity to have cyclic release of GnRH and sexual behavior (reviewed in Goy and McEwen, 1980), but quantitative regulation of estrogen is necessary, as high levels will masculinize the brain (reviewed in Dohler,

INTRODUCTION

1987). These early events in neural development cause profound changes in the structure of the brain and the behavior exhibited later.

Steroid regulation of structure and function.

Testosterone has long been considered the primary inducer of sexual differentiation of the brain (Pfeiffer, 1936; Barraclough, 1961; Harris, 1964). Testosterone's profound effects on brain structure in many species likely derive from genomic events, since they can often be blocked by an inhibitor of RNA polymerase II, α -amanitin (Salaman and Birkett, 1974). Testosterone can cause neurite outgrowth in explants (Toran-Allerand, 1976; Toran-Allerand, 1980). They can also increase the re-establishment of connections in damaged nerves (Kujawa et al., 1991; Yu, 1989). It appears that it is the aromatization of androgens which provides the estrogens necessary for many of these neuronal effects.

There are sex differences in neuron number and the size of several brain nuclei in rodents (Gorski et al., 1978), (Gorski et al., 1980). Developmentally, estrogens are responsible for the growth of the pre-optic area, and rats given the estrogen antagonist tamoxifen will have female-like (small) POAs (Dohler, 1987). Estrogen may do this by regulating neurite outgrowth, as it does in explants (Ferreira and Caceres, 1991; Toran-Allerand, 1980; Toran-Allerand, 1976). Later, as adults, female rats show changes in synaptic anatomy with the cyclic change in the levels of estrogens during the female estrous cycle (Wooley, et al. 1990).

A particularly robust example of hormone mediated preservation of nerve cells is seen in the rat. The spinal nucleus of the bulbocavernosus (SNB) is a group of several hundred motor neurons in the rat which innervate the anus and penis of male rats (Nordeen et al., 1985). This group of neurons concentrates labeled androgens. Female rats develop these cells, but they die during development (Goldstein et al., 1990). If androgens are administered to females, these neurons are selectively preserved (Nordeen

INTRODUCTION

et al., 1985). The endogenous estrogens of females would not be effective at preserving the neurons in females, since it is sequestered by the estrogen binding alpha-fetoprotein circulating in the cerebrospinal fluid (Goy and McEwen, 1980). The muscles which are innervated by these cells also regress in females, but can be selectively preserved by prenatal androgen treatment (Breedlove, 1985). The hormonal specificity of this effect dramatic: When the non-aromatizable androgen dihydrotestosterone is administered, the muscles are preserved but the neurons die, implying that the muscle preservation is an androgenic effect, and the neuron preservation an estrogenic one (Breedlove, 1985).

Brain, Hormones and Behavior: Songbirds

As a part of their reproductive strategy, songbirds, *oscines* of the order Passerines, vocalize in order to establish territory and for courtship (reviewed in Konishi et al., 1989). These songs are learned from other adults in proximity, usually the father (Thorpe, 1958). In the so called "closed learners" such as finches and sparrows, whose song is crystallized at an early age, only the males are capable of singing a vigorous, stereotyped song (Immelmann, 1969). If these closed learners are deafened before their song is crystallized, they will "sing" a very garbled song, but if they are deafened after, their song will maintain its structure for years (Konishi, 1965). In birds capable of altering their song in adulthood, such as the canary (Nottebohm and Nottebohm, 1978) and the red wing blackbird (Marler et al., 1972), singing by the male predominates, although female canaries sing to a very limited extent (F. Nottebohm, unpublished observations). These "open ended learners" (at least the canaries) gradually "forget" their song after deafening (Nottebohm et al., 1976). For zebra finches, specific areas of the brain have been associated with both the plasticity in learning, and the stereotyped behavior which develops (Scharff and Nottebohm, 1991), and similar studies in the canary have suggested a similar localization of function (F. Nottebohm, unpublished).

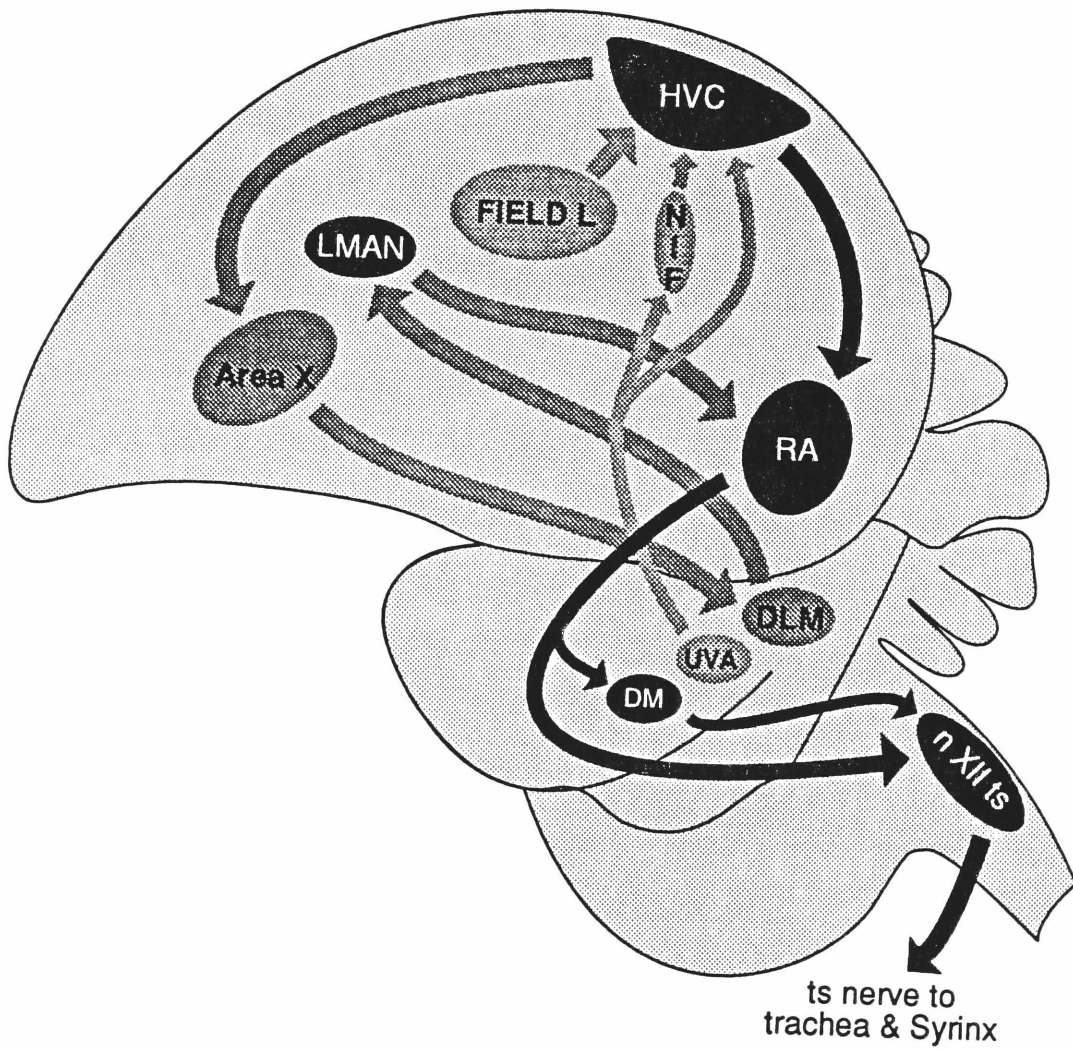
Song specialization of the forebrain

INTRODUCTION

Early studies by Nottebohm and colleagues (reviewed in Nottebohm, 1980a), defined the motor pathway by which the canary produces its song (see Figure 1). Using a combination of anatomical and electrophysiological techniques, a number of interconnected brain nuclei were shown to respond to auditory information and form a motor pathway from the forebrain to the vocal organ, the syrinx (Kelley and Nottebohm, 1979). A large nucleus in the dorsal telencephalon, the Hyperstriatum ventral pars caudale (HVc), and later called the higher vocal center (HVC), appears to integrate auditory information from field L and lateral MAN (lMAN), the latter also innervating the robust nucleus of the archistriatum (RA). RA is principally innervated by HVc, as part of the principal motor pathway controlling vocalization. The motor pathway from RA then bifurcates. The principle projection continues into the caudal half of the hypoglossal nucleus (nXIIIts), which directly innervates the vocal organ of birds, the syrinx. A secondary projection from RA innervates the dorsal medial (DM) nucleus of the intercollicular region(ICO). The first large difference discovered between the brains of male and female in any species was in the song control system of the canary (Nottebohm and Arnold, 1976). This discovery prompted re-examination of the rat and subsequently Gorski et al. found the sexually dimorphic nucleus of the pre-optic area in rodents (Gorski et al., 1978).

INTRODUCTION

Figure 1. Anatomy of the Songbird Brain. A schematic diagram of the song control circuit in the brain showing ^3H -testosterone concentrating nuclei and their axonal projections (dark). The other nuclei of the circuit, and the directions of their axonal connections are indicated (lighter shades).



= 3H-T concentrating areas

INTRODUCTION

Development of Song Control Nuclei

Although the song control nuclei of songbirds show a greater dimorphism than the SDN-POA, their development appears to be similar. Steroid responsive areas of the females songbird brain lose neurons to cell death during development, but in males the neurons survive and there is an increase in size and connectivity of these areas (Konishi and Akutagawa, 1985; Konishi et al., 1989). There is a critical period for steroid exposure, much like the SDN-POA in rodents, and it is also during this critical period that song is learned (Gurney and Konishi, 1980; Gurney, 1982). In canaries, the sexual dimorphism in the song nuclei HVC and RA develops more slowly as the bird matures (Nottebohm et al., 1986). In the first months after hatching, the canary sings an ill-formed song, akin to babbling, and their song control nuclei are one third to one tenth the size of adults. As the bird, and its song, matures, the nuclei grow to their adult dimensions. Finally, in adulthood the disparity between male and female song control nuclei ranges from 33% for nXIIIts to almost four-fold for area X (Nottebohm and Arnold, 1976). There has been a great deal of speculation that the development of the nuclei correlates with the learning of stable song (Nottebohm et al., 1981). However, seasonal changes in song center anatomy have been detected in some species without measurable changes in song behavior (Brenowitz et al., 1991).

Seasonal Regulation of Singing and the Song Control Nuclei

Singing by the canaries is a reproductive behavior which is most highly expressed during breeding in the spring. The song of juvenile canaries becomes both stereotyped and complex at the end of winter. At the end of the first breeding season, and subsequent breeding seasons, the canaries' song goes through a period of instability, which peaks in August and September. It is during this time that new songs are added to the canaries repertoire (Nottebohm et al., 1986). The size of the song control nuclei HVC and RA as defined by cresyl violet staining parallels the complexity of the song (Nottebohm et al., 1986). The brain of male canaries is larger in the first spring when they are singing

INTRODUCTION

prodigiously (aged 12 months) than the following fall when they are relatively silent (aged 17 months), by approximately 20% (Nottebohm, 1981). Normalizing to this change in brain size, the changes seen between spring and fall canary HVC is 1.9 fold and for RA it is 1.6 fold (Nottebohm, 1981). The size of the brain as a whole, and the song control nuclei particularly, rebounds to previous spring levels in following years (Nottebohm et al., 1981). This seasonal change in the size of the song control nuclei is accompanied by ten-fold higher spring levels of in plasma testosterone (Nottebohm, 1981), as might be expected since breeding is confined to the spring.

Steroid Receptors are Found in Song Control Nuclei

Progesterone and Estrogen Receptors

Gonadal steroids clearly play an important role in regulating song in the Passerine birds. Since the steroids act through a receptor, a number of studies have investigated the location of receptors for the gonadal steroids in the forebrain. Receptors for tritiated progestins are found primarily in the hypothalamus, and they do not appear to bind receptors in any of the forebrain song control nuclei Hvc, RA, MAN, or area X (Lubischer and Arnold, 1990). Receptors for labeled estrogens are also very rare in the songbird forebrain. Tritiated estradiol labels only a few percent of cells in MAN and Hvc (Nordeen et al., 1987). A large number of cells ventral to HVC were labeled, as were many hypothalamic neurons (Nordeen et al., 1987). A monoclonal antibody directed against the steroid binding domain of the human estrogen receptor has recently been used to successfully localize the receptor protein itself in the canary brain. The antibody reveals substantially the same pattern of estrogen receptor localization, except no receptors are found in MAN (Gahr et al., 1987). The antibody reveals an interesting developmental pattern of receptor expression in the Zebra finch, where receptor antibody positive cells decline in number 90% inside HVC proper and 75% in the surround

INTRODUCTION

between day thirty, before the critical period, and adulthood (Gahr and Konishi, 1988). Interestingly, the estrogen receptor positive cells in the area surrounding HVc appear to be those incorporated when HVc is at its maximum during the breeding season (Gahr, 1990b). This lack of seasonal change in the size of the estrogen receptor containing cell population stands in contrast to the cresyl violet staining defined seasonal changes in HVc volume (see above), but the significance of this difference remains to be explored. The ER containing neurons appear to send axonal projections to the song control nucleus area X, as they are double labeled when retrograde tracer injections into area X are combined with monoclonal ER antibody staining (Gahr, 1990b). Combining the ER antibody and tritiated androgen, Gahr (1990) examined whether the ER containing cells in HVc also contain the AR. He found that although in both ICO and the hypothalamus a substantial fraction of the cells were double labeled (30% and 15% respectively), no cells in HVc were so labeled (Gahr, 1990a).

Androgen Receptor

Since the large dimorphism was noticed in 1976, a large number of investigators have examined the distribution of androgen concentrating cells. In addition to non-song control areas (such as the hypothalamus) the song control nuclei nXIIIts, ICO, MAN, and HVc bind tritiated androgen (Evans and Arriza, 1989; Arnold, 1981; Arnold and Saltiel, 1979; Bottjer, 1987; Korsia and Bottjer, 1989; Sohrabji et al., 1989; Nordeen et al., 1986). When the brains were more closely examined, it became apparent that RA was also labeled (Arnold, 1981). Comparing male and female zebra finches, a three-fold difference in labeled androgen binding was found in HVc and MAN, but not the evolutionarily older ICO, nXIIIts, or the non-song control PVM of the hypothalamus (Arnold and Saltiel, 1979). During development of the zebra finch brain, the number of cells in both HVc and MAN decrease by several fold but the androgen concentrating cells are selectively preserved, such that there is no net loss of such cells during development (Bottjer, 1987) [but see (Korsia and Bottjer, 1989) which has absolutely contradictory

INTRODUCTION

data for IMAN T-cell survival and percent of labeled cells]. Just as many of the ER containing cells project to area X, a large number of projection cells in HVc appear to concentrate androgens (Sohrabji et al., 1989). Approximately 60% of RA projecting cells and 40% of area X projecting cells concentrate tritiated androgens. Recently, Balthazart, et al (Balthazart et al., 1992) have successfully used a human antibody to the n-terminal region of the receptor to localize it in zebra finch, canary, and quail. Their results, though preliminary, appear to agree with the labeled androgen binding data.

Hormonal control of song control nuclei

Just as the sexual dimorphism in brain and behavior can be reversed in rodents with early hormone manipulation, the male pattern of song control nuclei size and singing can be induced in female songbirds by treatment with gonadal steroids (Gurney, 1981; Gurney and Konishi, 1980; Gurney, 1982). Like the rodent, where estrogen has been shown to be the active gonadal steroid in preserving SDN-POA cells, systemic estrogen in female hatchling zebra finches increases the number of androgen concentrating cells in both HVc and MAN close to the levels seen in untreated males while having no effect on a non-dimorphic nucleus (SL) (Nordeen et al., 1986). Estrogen also increases the volume of HVc, RA, and area X, but only to a size intermediate between males and females (Simpson and Vicario, 1991b). These females also sing male typical songs, but their singing is much less frequent and of poorer quality than males (Simpson and Vicario, 1991a). Subsequent treatment with testosterone further masculinizes the song of the zebra finch (Simpson and Vicario, 1991a), but has little effect on the brain structures (Simpson and Vicario, 1991b). For the closed period learners, such as the Zebra finch, the sensitive period for estrogen actions extends from hatching to one month, when the cell-death induced dimorphism begins to become apparent, and by 45 days, gonadal steroid treatment loses all effect (Konishi and Akutagawa, 1988). Canaries, which are capable of continuous learning, show no age of treatment dependency. Adult female

INTRODUCTION

canaries, treated systemically with testosterone, sing vigorously in the spring (Nottebohm, 1980b; Devoogd et al., 1985), and the sizes of HVC and RA, in addition to synaptic connectivity, increase significantly (Nottebohm, 1980b; Devoogd and Nottebohm, 1981). Although there is no critical period for this effect, seasonal effects remain a consideration, as implanted females fail to sing in the fall (Devoogd et al., 1985), but changes in RA volume and some synaptic properties still occur (Devoogd et al., 1985).

In these androgen treated females, there is a large increase in protein synthesis in a number of song control nuclei (Konishi and Akutagawa, 1981). The increase in protein synthesis in these testosterone treated females was greatest after six days continuous exposure to androgens. It was also a purely androgenic effect, as estrogen had no effect on this system. Interestingly, Konishi and Akutagawa (1981) found increased protein synthesis in area X, a song control nucleus which does not accumulate labeled androgen, in addition to the androgen binding nuclei MAN, RA, and HVC, implying that the effect of the hormone treatment was transmitted by some intermediary, at least in this part of the songbird brain.

INTRODUCTION

Prospects for Research

Androgens' profound effects on the brain and behavior of canaries give us a very good system to examine the mechanisms of steroid hormone regulation of the neuronal plasticity. Based on evidence from many other experimental systems and from similar steroid hormones, the most probable locus for androgenic regulation is at the level of gene expression, via actions of the androgen receptor. An important step in characterizing androgen regulation in songbird brain, therefore, will be to confirm the identity of the nuclear receptor for androgen and determine its location in the brain of the canary. The changes in the structure of the canary brain between fall and spring or in testosterone treated females are likely to require the synthesis of new components of nerve cells, such as synaptic proteins, and may also involve changes in expression of the AR itself. However, the time course of potential molecular changes is unclear, and could be rapid, as is the case for immediate-early gene induction by estrogen in the oviduct, or it could occur on a similar time course as the behavioral effects, which in canaries require one week of continuous androgen treatment. An adequate analysis of androgen effects on gene expression will therefore include a range of response times to hormone stimulation. Androgens both induce and repress genes in several rodent organs. It is unclear how they may regulate gene expression in the brain, whether they induce genes responsible for song learning and production, suppress genes responsible for variability, or some combination of the two. One approach to analyzing this would be to obtain probes for a variety of candidate genes for androgen regulation (such as structural components of neurons, immediate early genes, and the AR itself), and look for evidence of either induction or repression following androgen treatment. Finally, in the brain, there are a large variety of biochemical signals acting on the cells concurrent with androgen, additionally complicating the examination of androgen effects, since the changes could be due to synergistic effects of all the stimuli. Analysis of interactions between androgen regulation and other signaling pathways active in neural tissue may benefit from exploration of simpler in vitro systems or cell lines as well.

CHAPTER 2: THE CANARY ANDROGEN RECEPTOR

A: CLONING OF THE CANARY ANDROGEN RECEPTOR

An essential step in understanding the mechanism of androgen action in the canary brain is to characterize its receptor. It is evident from a variety of studies that the gonadal steroids both organize the neuronal substrate for singing and activate the behavior in canaries (see introduction). In addition, ^3H -testosterone binds to many of the nuclei which control song production in situ, though it remains unclear whether the ^3H -steroid is binding to an authentic androgen receptor. The availability of molecular probes for the AR would allow tests of the hypothesis that the changes in behavior seen seasonally and with pharmacological manipulation of the gonadal steroids may be correlated with changes in the androgen receptor in the brain. To pursue this characterization, I have cloned the canary androgen receptor (cAR) and examined its localization and regulation.

Results

Initially, noting the high conservation between the rat and human androgen receptors, I directly screened several libraries already in the lab using reduced stringency hybridization. I obtained a cDNA clone of the rat Androgen Receptor (rAR) and isolated the region containing the CDE domains, radiolabeled this fragment and directly screened one million independent cDNA clones in a library derived from canary HVc (George and Clayton, 1992). None of these were positive in the initial screen. I next screened three hundred thousand independent cDNA clones in a library derived from the canary liver, and again failed to detect any positives. Since 1.) HVc contains androgen binding activity, 2.) liver contains the AR in other species, and 3.) I had screened the libraries extensively (see discussion), I concluded that the abundance of clones of the receptor in our libraries was vanishingly low.

PCR Cloning the CDE Region of the AR

To circumvent the low abundance of the receptor in our libraries, I next used the information about the conservation of several domains of the steroid receptors (see introduction) to make primers for polymerase chain reaction (pcr) amplifications. Specifically, I used the fasta paradigm (Pearson and Lipman, 1988) to identify regions identical in nucleic acid sequence between the rat and human receptors in the C (DNA binding) and E (hormone binding) regions and with this information designed oligonucleotide probes whose sequences were likely to be conserved in the canary gene (see Figure 2). Hybridization of these oligonucleotides to Southern blots of rat and canary genomic DNA gave a pattern of bands, some of which are identical to bands seen with the rat CDE probe (data not shown (dns)). I next reverse transcribed total RNA from canary testis using random primer and pcr amplified using the oligonucleotides ("RT-PCR", see methods and Figure 2). When these reactions were separated on an agarose gel, the reverse transcript of the canary testis RNA was the same size as the rat cDNA clone (see Figure 3), as would be predicted given the expected conservation of this functional domain. When Southern blotted and probe with the rAR cDNA, this one kb band from the canary RNA was identical in size and thermal stability to both the rat cDNA pcr and reverse transcribed rat testis RNA (dns), also consistent with the expected conservation of this part of the coding sequence. When DNA from canary and rat is probed with the pcr amplified product, a large number of the autoradiographic bands are identical between the two species (see Figure 4). This is additional evidence supports the hypothesis that the amplified product is the canary homolog of the rat androgen receptor.

THE CANARY ANDROGEN RECEPTOR

Figure 2. PCR Cloning of the AR. Diagrammatic representation of the cloning strategy for the canary androgen receptor. The locations of the "DNA Binding" and "Steroid Binding" primers used to clone the canary androgen receptor are as indicated (see methods for primer sequences).

Cloning the Canary Androgen Receptor Using PCR

Testis RNA was reverse transcribed using pdN(6) and oligo's conserved between rat and human were used to amplify the prescribed canary cDNA.

Steroid Receptor :

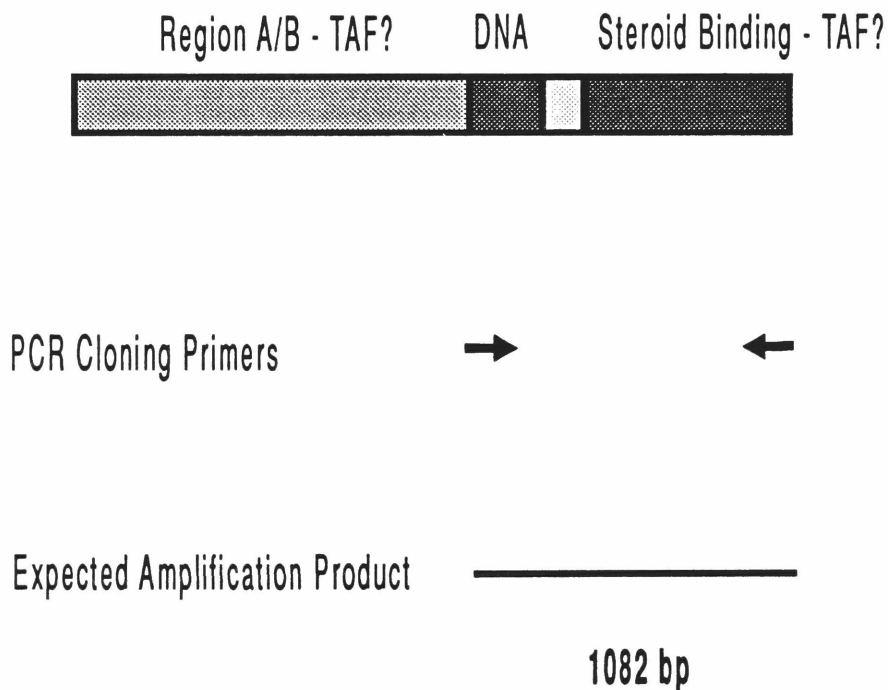


Figure 3. RT-PCR for cAR. Negative image from a scan of a photograph of 2% agarose gel stained with ethidium bromide. Canary testis total RNA (2 μ g) was reverse transcribed using either 0.6 μ g oligo dT or poly dN₆ (high is 1 μ g, low is 0.1 μ g). These samples, one identical to the high pdN₆ without reverse transcriptase, and 0.1 pg of the rat androgen receptor cDNA were separately PCR amplified using the "C" and "E" primers.

Reverse Transcription-PCR for Canary Androgen Receptor

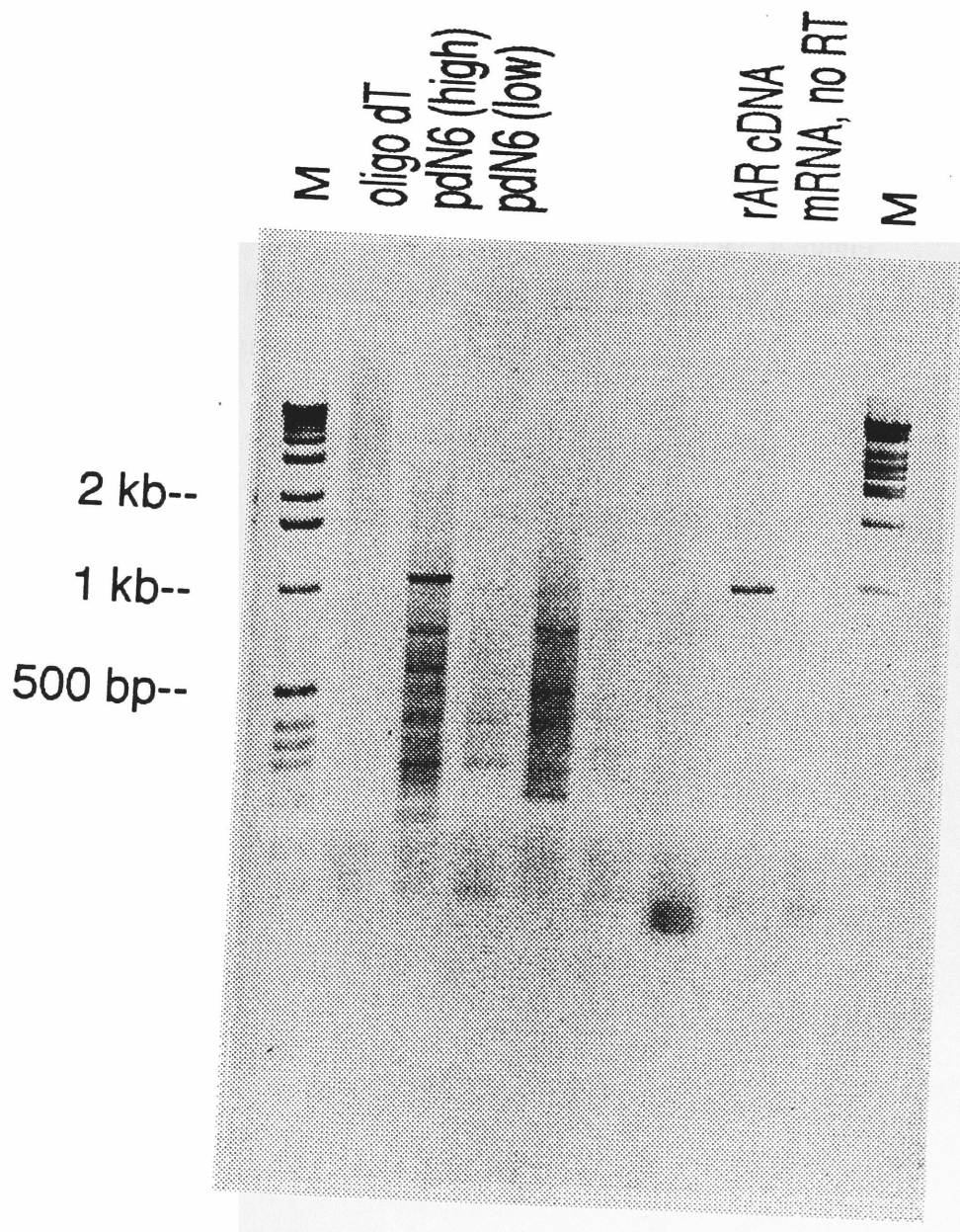
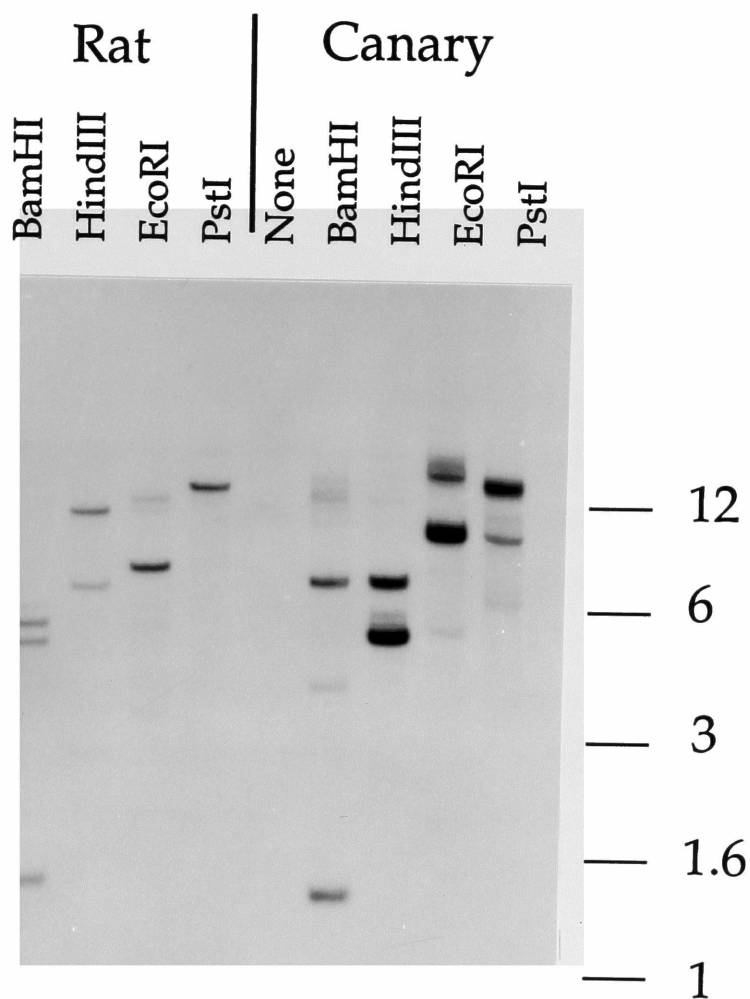


Figure 4. Genomic Southern Blot for the Androgen Receptor. The amplified product of the RT-PCR for the cAR (excised from a gel like in Figure 3) was used to probe digested genomic DNA from rat and canary.

Genomic DNA:

Endonuclease:



Cloning the Remainder of the AR

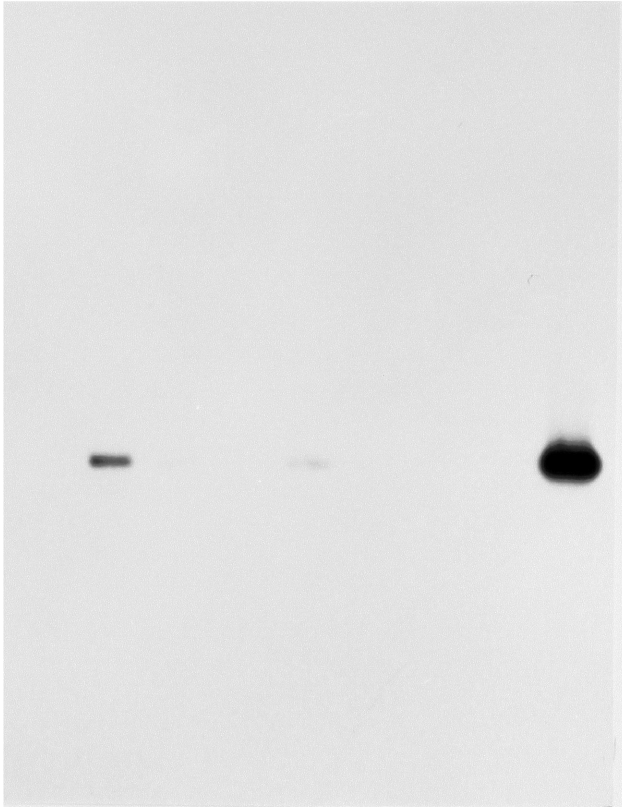
I next re-examined the DNA from the libraries screened earlier to determine whether they contain the same molecule I cloned by pcr, and perhaps, the other parts of the molecule not yet cloned. Although I failed to detect the CDE region in the original direct screen, I was able to amplify this fragment of the receptor gene from the HVc and non-forebrain libraries, but not the liver library. As it was for the direct screen, the signal was very low for the amplification from the libraries relative to the 10 pg rAR cDNA (see Figure 5). I tried to amplify the parts of the gene 5' and 3' to the pcr defined region of the cAR using one of the specific oligonucleotides and a flanking vector primer, but was unable to amplify any region other than that defined by the two original oligonucleotides (see Figure 6).

I have attempted to isolate the part of the cDNA coding for A/B region of the receptor by RT-PCR using the primer in the C region coupled with five different degenerate oligos upstream (see Figure 7). Unfortunately, this amplification was unsuccessful using canary RNA as template (see Figure 8). In addition, I have tried an anchored pcr technique (RACE) (Frohman et al., 1988) which despite working for controls, did not work for the canary androgen receptor. In subsequent experiments, it has become clear that there are strong reverse transcriptase "stops" on the 5' end of the C region, which I have been unable to overcome using either methylmercury or higher temperatures (dns).

Figure 5. Amplification of cAR from Canary Libraries. 20 µg of cesium chloride purified phage DNA was pcr amplified, separated on a 2% agarose gel, and transferred onto a nylon membrane. The membrane was probed with the rAR cDNA. F>R or C>E: 35 cycles. F>R, C>E: 35 cycles using F and R, subsequently re-amplified 35 cycles using C and E. Primers: F, λGT10 forward; R, λGT10 reverse; C, AR "C" region; E, AR "E" region. HVc, Non-Forebrain (NF), and Liver phage libraries as described in methods.

Template: --HVC-- | --NF-- | Liver

Primers: F>R C>E F>R, C>E 3>7 C>E 3>7, C>E C>E F>R C>E rAR



4.4
3.5
2.5
1.9
1.1

Figure 6. PCR for cAR Ends. PCR amplification of the λ GT10 HVc cDNA library, as in Figure 5.

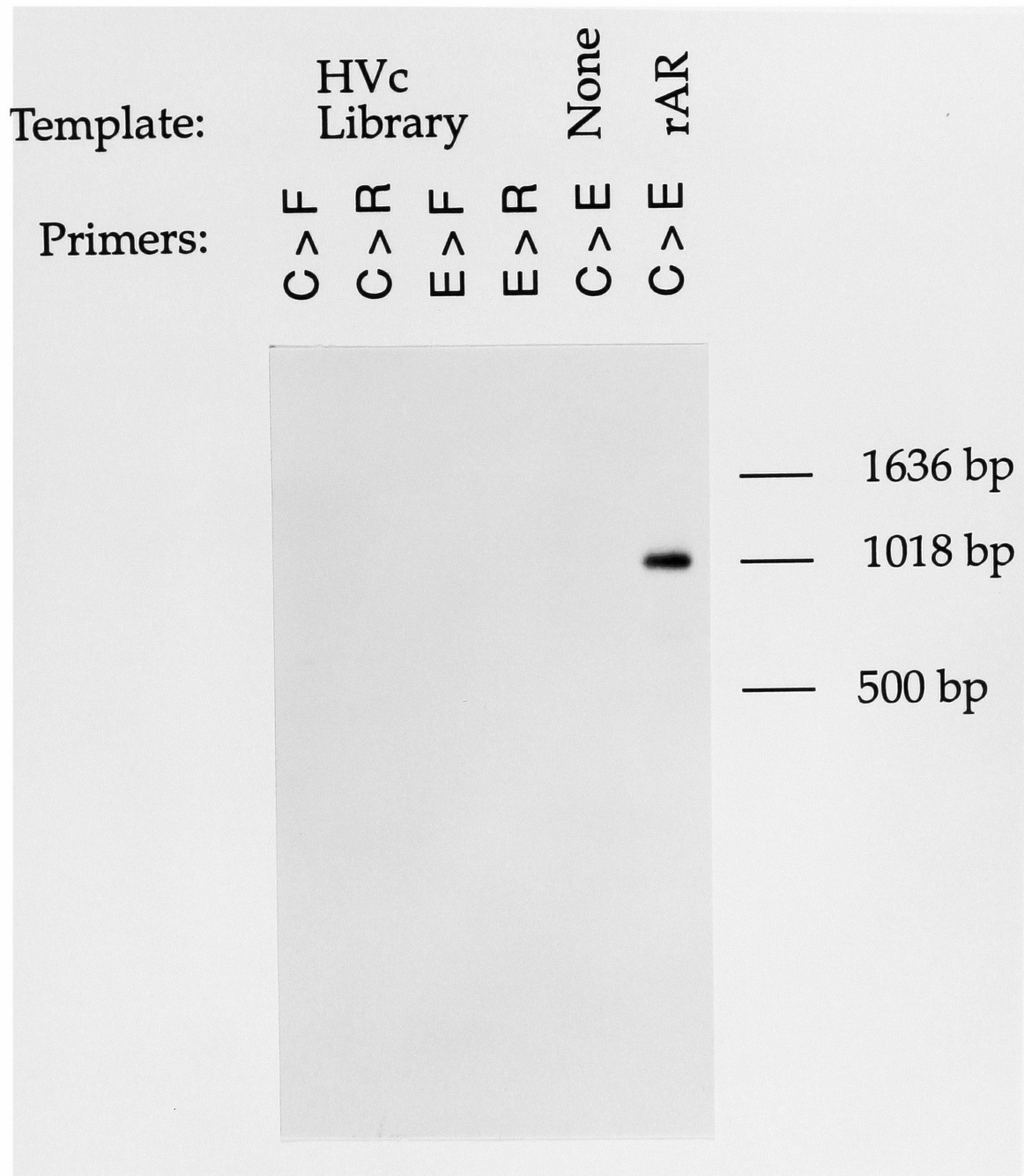
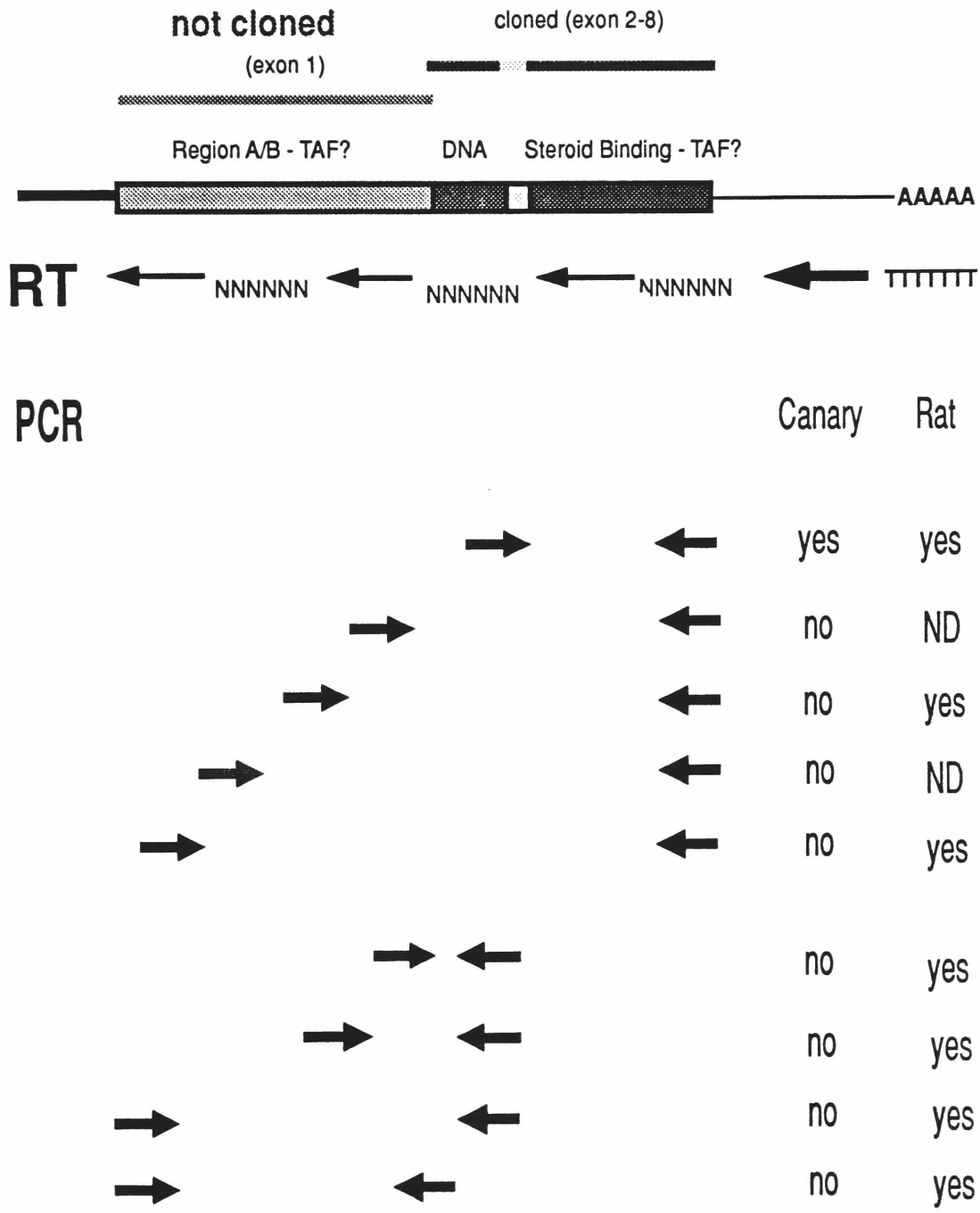


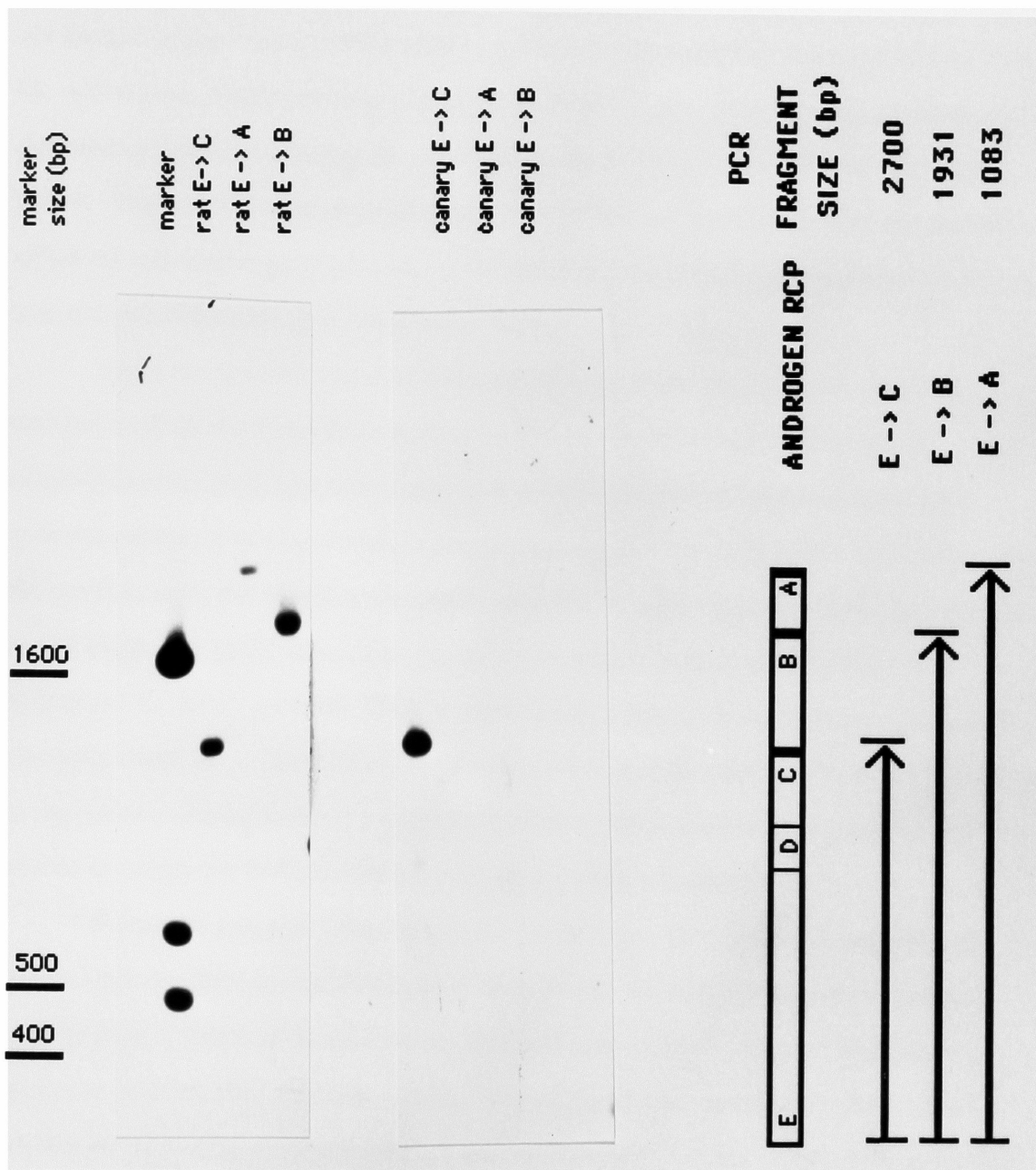
Figure 7. RT-PCR for the 5' End of the cAR. Top: Diagram of the structure of androgen receptors. Bottom: Diagram of RT using a combination of oligo dT and pdN₆, followed by PCR using a variety of combinations of primers. Results of the amplification (presence/absence of a band of the appropriate size by Southern blot) listed on right. Primers as described in methods.

Cloning the 5' end of the canary Androgen Receptor using RT-PCR



THE CANARY ANDROGEN RECEPTOR

Figure 8. Example of RT-PCR Diagramed in Figure 7. Three primer pairs were used to amplify the 5' end of the AR from either reverse transcribed testis RNA or a rat AR cDNA. The reactions were separated on a 2% agarose gel, Southern blotted, and probed with the rat AR cDNA.



Genomic Cloning the First Exon of the AR

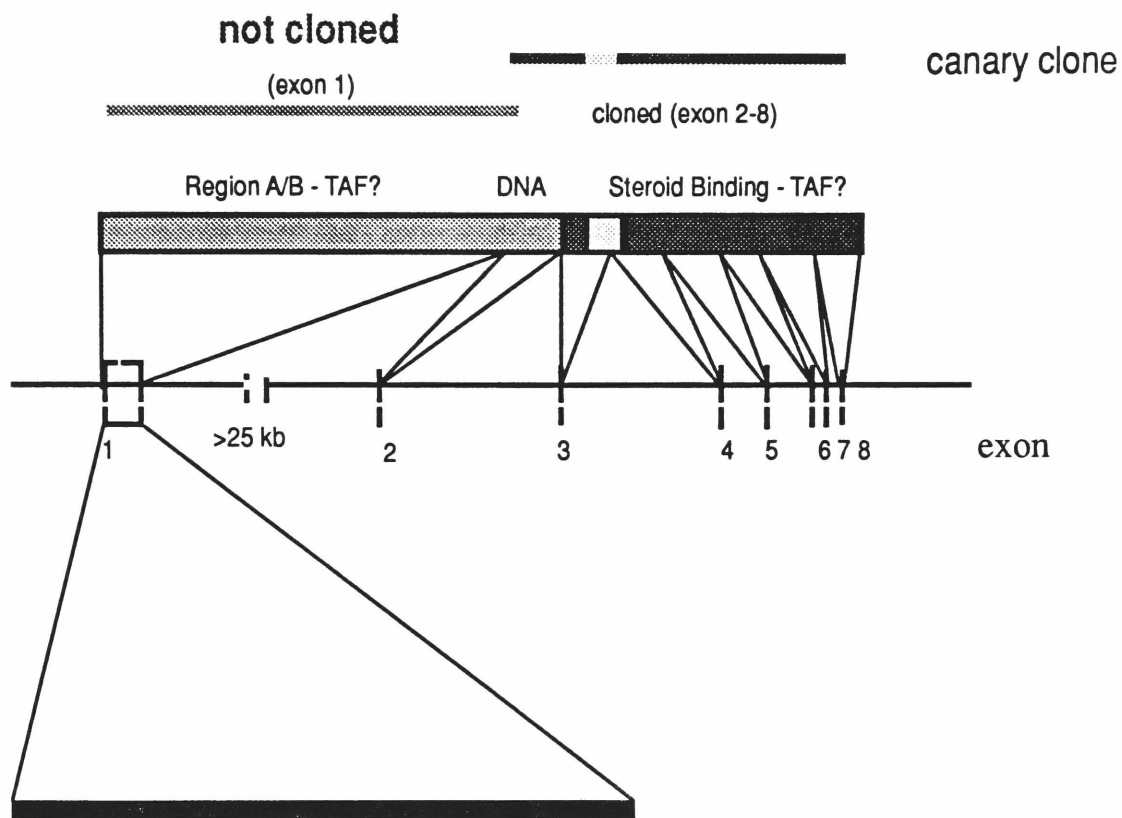
I continued my attempt to isolate pieces of the 5' end of the cDNA, which in humans is encoded on a single large exon (see Figure 9), by using the degenerate upstream oligos (see Figure 8, and methods) in pcr of genomic DNA. This approach is not limited by the "stops" which prevent the reverse transcription from proceeding into the A/B region. Unfortunately, this approach failed to give amplification products, as detected by Southern blotting the pcr reaction and probing with the 5' end of the rat cDNA. The pairs of degenerate oligonucleotides derived from this part of the sequence of the rat and human gene must be a poor match for the canary sequence even under the low stringency genomic pcr conditions.

Since the genomic pcr conditions failed to give any amplification product, I decided to clone the 5' end from a genomic library. Even though the first exon oligonucleotides didn't generate a pcr product which could be used as a probe for a genomic screen, single upstream oligos might still hybridize to genomic DNA even when the pcr fails since pcr requires reasonable matches from two primers. Indeed, Figure 10 shows oligo 134, the 5' most oligo for the rat androgen receptor coding sequence, hybridized to canary genomic DNA at moderate stringency. A second oligo (213, dns) also hybridized to the same bands. Since the two oligonucleotides (134 and 213) appear to recognize a unique band on a Southern blot of a genomic digest, they may be useful probes to isolate the first exon of the cAR from canary genomic DNA.

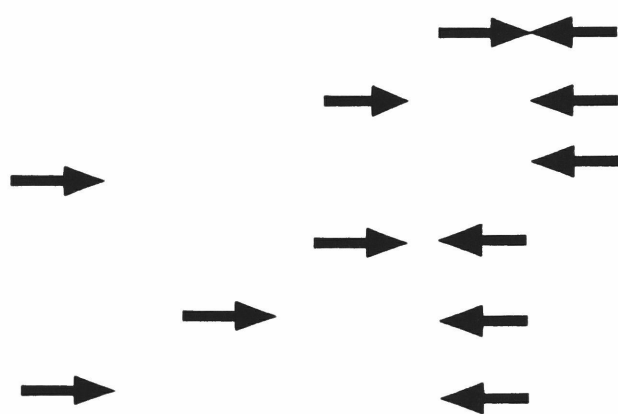
No canary genomic libraries existed at this time. I therefore constructed one, which has more than one million independent clones. I initially found seven positive plaques from a screen of approximately 200,000 unamplified clones. Five of these remained positive after a tertiary screen. Two of the clones contain only the 5' most portion of the coding sequence (dns). I am continuing my investigation of the remainder of the clones.

Figure 9. Genomic PCR for the cAR First Exon. Top: Diagram of the genomic structure of the human AR (adapted from Kuiper, et al., 1989). Bottom: Diagram of PCR amplifications from canary and rat genomic DNA using a variety of primer pairs. Results of the amplification (presence/absence of a band of the appropriate size by Southern blot) listed on right. Primers as described in methods.

Genomic structure of the human Androgen Receptor



GENOMIC PCR



Canary Rat

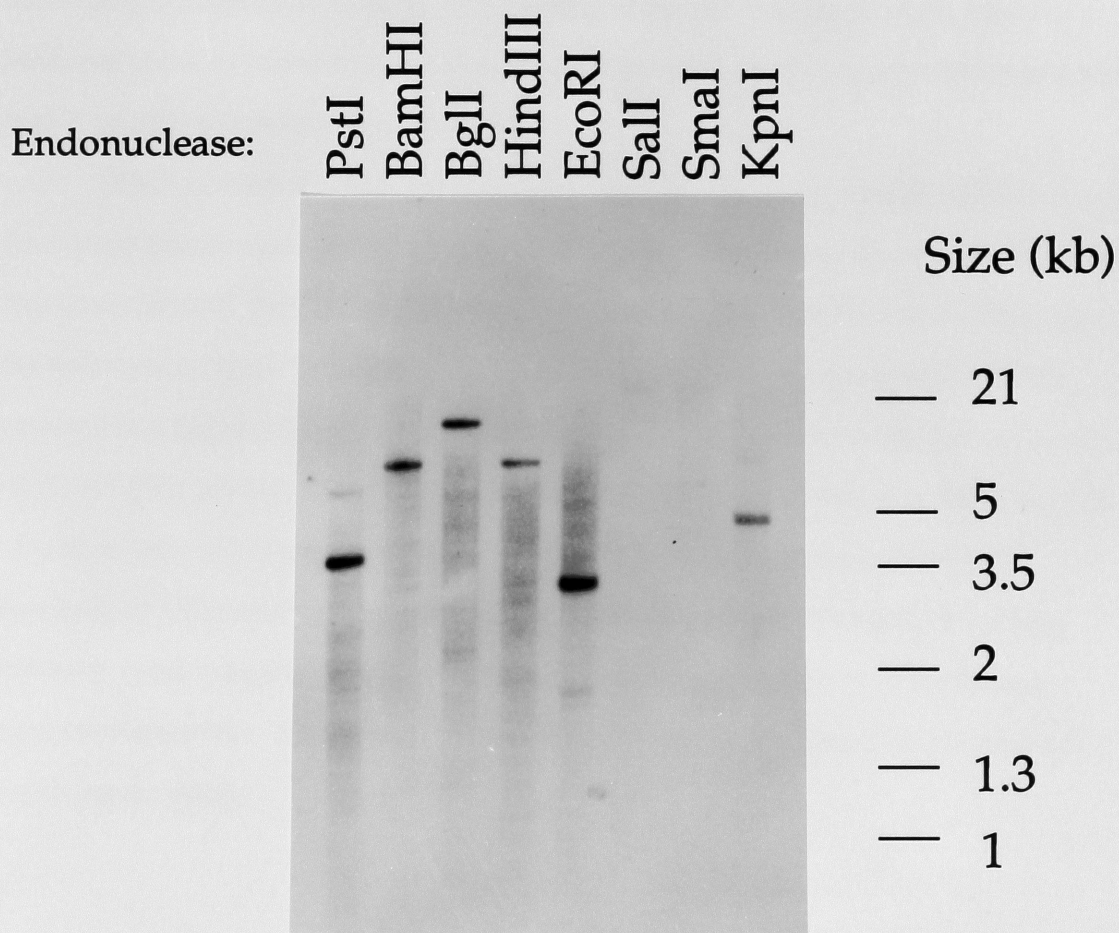
no	ND
no	yes
no	yes
no	ND
no	yes
no	yes

THE CANARY ANDROGEN RECEPTOR

Figure 10. Canary Genomic Southern Blot. Scan of photograph of autoradiogram showing canary genomic DNA which was digested with the indicated restriction endonuclease and separated on a 0.5% agarose gel, then transferred onto a nylon membrane. The membrane was probed with the 5' most degenerate oligonucleotide for exon 1 of androgen receptors which had been tailed with ^{32}P dCTP. The membrane was washed up to 49°C, 1X SSC.

Canary Genomic Southern Blot

AR 5' End Oligo Probe



Sequencing the AR

In order to confirm the band seen in the Southern blot as the canary androgen receptor, I compared it to the other steroid receptors by sequencing it. I isolated the pcr product and sequenced it directly using an end-labeled primer. The sequence from the pcr product, which represents the sequence of a very large number of molecules, and probably a large number of reverse transcribed mRNAs, indicated a strong similarity to the rat and human androgen receptors. I then isolated three independent clones of the positively hybridizing band and sequenced them by standard dideoxy techniques. By combining the sequence from the four clones and the pcr reaction product, I believe I have overcome any error introduced in the amplification by the taq polymerase and have highly reliable sequence (see Figure 11).

When I compare this sequence with known molecules, it is highly related to both the canary and rat androgen receptors and other steroid receptors. The molecule cloned represents most of the CDE region of the receptors and is clearly the canary homolog of the androgen receptor (see Figure 12). A more detailed comparison reveals that the conservation has areas of local modulation which define the structurally defined portions of the receptor protein. The DNA binding region, "C", and the steroid binding domain, "E", have been highly conserved between canaries and rats (see table 1) over the hundreds of millions of years since birds and other vertebrates diverged. It is clear, however, from examining the DNA sequence comparison that many of the codons contain mismatches. The conservation of the sequence in the "hinge" in particular, is much poorer (dns).

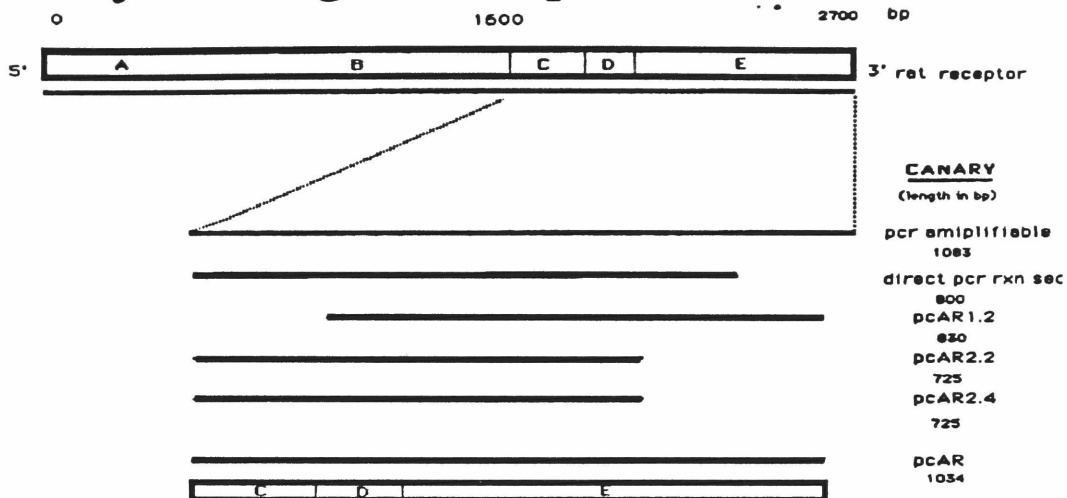
Table 1. Comparison of Canary and Rat Androgen Receptors

Region	AA Identity	AA Conservation	DNA Identity
"C" (DNA)	100%	100%	81%
"D" (hinge)	57%	95%	66%
"E" (steroid)	93%	100%	80%

THE CANARY ANDROGEN RECEPTOR

Figure 11. The cAR cDNA. Top: Diagram of the rat AR and the cAR cDNA isolates sequenced, and an indication of the sequence defined by direct sequencing (see methods). Bottom: Sequence of the cAR CDE composite clone, with the encoded amino acids and surmised exon/intro boundaries.

Canary Androgen Receptor cDNA



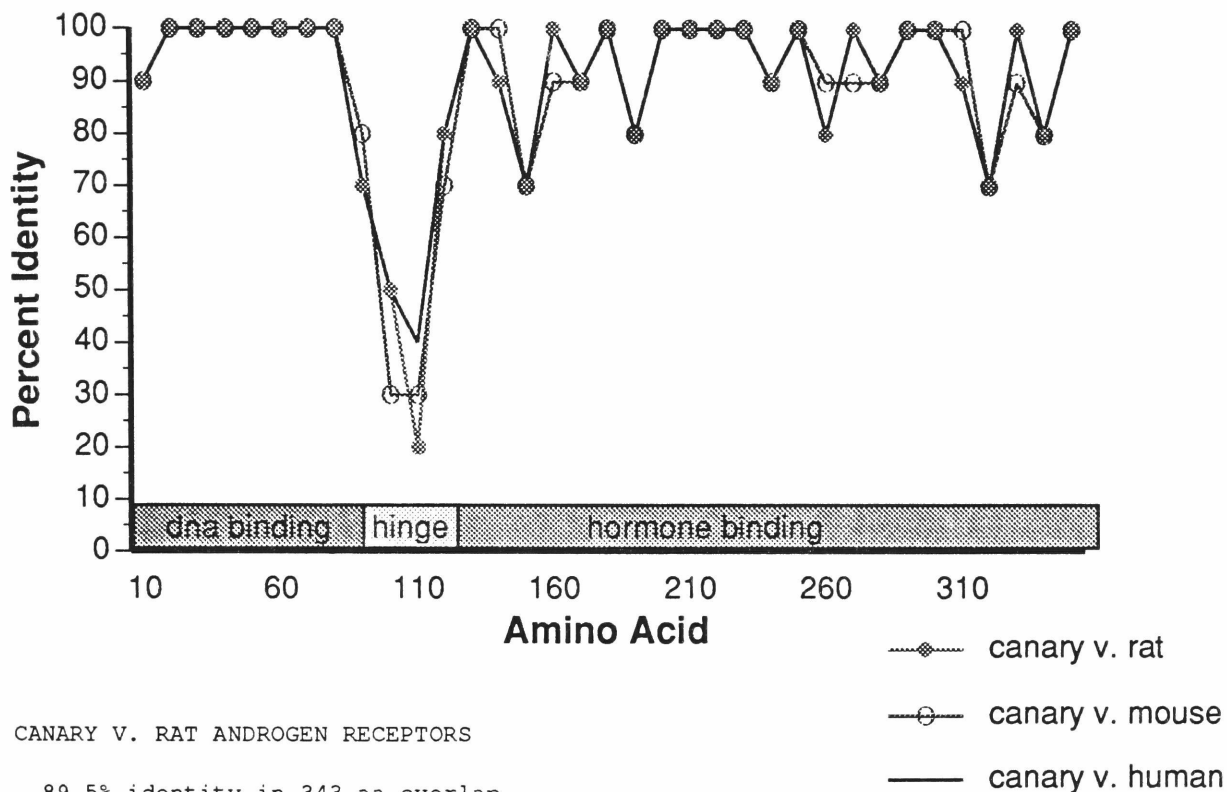
Canary Androgen Receptor (CDE)

19	AAG	ACC	TGC	CTG	ATC	TGT	GGA	GAT	Glu	Ala	Ser	Gly	Cys	His	Tyr	Gly	
9	Ala	Leu	Thr	Cys	Gly	Ser	Cys	Lys	Val	Phe	Phe	Lys	Arg	Ala	Ala	Glu	66
67	GCC	CTG	ACG	TGT	GGG	AGC	TGC	AAA	GTG	TTC	TTC	AAA	CGG	GCA	GCT	GAA	24
25	Gly	Lys	Gln	Lys	Tyr	Leu	Cys	Ala	Ser	Arg	Asn	Asp	Cys	Thr	Ile	Asp	114
115	GCT	AAA	CAG	AAG	TAC	CTC	TGT	GCC	AGC	CGC	AAC	GAC	TGC	ACC	ATC	GAC	40
41	Lys	Phe	Arg	Arg	Lys	Asn	Cys	Pro	Ser	Cys	Arg	Leu	Arg	Lys	Cys	Tyr	162
163	AAG	TTC	CGG	CGG	AAA	AAC	TGC	CCC	TCC	TGC	CGC	CTG	CGC	AAG	TGC	TAC	56
57	Glu	Ala	Gly	Met	Thr	Leu	Gly	Ala	Arg	Lys	Leu	Lys	Lys	Leu	Gly	Asn	72
211	GAG	GCC	GGG	ATG	ACG	CTT	GGA	GCC	CGC	AAG	CTG	AAG	AAA	CTG	GGT	AAC	258
73	Leu	Lys	Ala	Gln	Asp	Asp	Ile	Glu	Gly	Ala	Ser	Ser	Ser	Ser	Pro	Thr	88
259	CTG	AAG	GCA	CAG	GAC	GAC	ATA	GAG	GGA	GCC	AGC	TCG	TCC	AGC	CCA	ACG	306
89	Glu	Glu	Gln	Ala	Pro	Lys	Leu	Val	Met	Thr	Arg	Ile	Asp	Gly	Tyr	Glu	104
307	GAG	GAG	CAA	GCT	CCC	AAG	CTG	GTG	ATG	ACA	CGC	ATT	GAT	GGC	TAC	GAG	354
105	Cys	Gln	Pro	Ile	Phe	Leu	Asn	Val	Leu	Glu	Ala	Ile	Glu	Pro	Gly	Val	120
355	TGC	CAG	CCC	ATC	TTC	CTC	AAC	GTC	CTG	GAG	GCC	ATC	GAG	CCT	GGG	GTG	402
121	Val	Cys	Ala	Gly	His	Asp	Asn	Ser	Gln	Pro	Asp	Ser	Phe	Ser	Asn	Leu	136
403	GTG	TGT	GCT	GGC	CAT	GAC	AAC	AGC	CAG	CCT	GAC	TCC	TTC	TCC	AAC	CTG	450
137	Leu	Thr	Ser	Leu	Asn	Glu	Leu	Gly	Glu	Arg	Gln	Leu	Val	Tyr	Val	Val	152
451	CTG	ACC	AGC	CTG	AAT	GAG	CTT	GGG	GAG	AGG	CAG	CTG	GTC	TAC	GTG	GTC	498
153	Lys	Trp	Ala	Lys	Ala	Leu	Pro	Gly	Phe	Arg	Asn	Leu	His	Val	Asp	Asp	168
499	AAA	TGG	GCA	AAG	GCT	CTG	CCA	GGA	TTT	CGC	AAC	CTG	CAT	GTG	GAT	GAC	546
169	Gln	Met	Ser	Ile	Ile	Gln	Tyr	Ser	Trp	Met	Gly	Leu	Met	Val	Phe	Ala	184
547	CAG	ATG	TCA	ATA	ATC	CAG	TAC	TCT	TGG	ATG	GGC	CTG	ATG	GTG	TTT	GCT	594
185	Met	Gly	Trp	Arg	Ser	Phe	Thr	Asn	Val	Asn	Ser	Arg	Met	Leu	Tyr	Phe	200
595	ATG	GGT	TGG	AGA	TCC	TTC	ACC	AAC	GTC	AAT	TCC	AGG	ATG	CTT	TAC	TTT	642
201	Ala	Pro	Asp	Leu	Val	Phe	Asn	Glu	Tyr	Arg	Met	His	Lys	Ser	Arg	Met	216
643	GCT	CCA	GAC	CTG	GTC	TTC	AAT	GAG	TAC	CGC	ATG	CAC	AAA	TCC	AGG	ATG	690
217	Tyr	Ser	Gln	Cys	Ile	Arg	Met	Arg	His	Leu	Ser	Gln	Glu	Phe	Gly	Trp	232
691	TAC	AGC	GAC	TGC	ATC	AGG	ATG	CGG	CAC	CTC	TCC	CAG	GAA	TTC	GGG	TGG	738
233	Leu	Gln	Ile	Thr	Pro	Gln	Gly	Phe	Leu	Cys	Met	Lys	Ala	Leu	Leu	Phe	248
739	CTT	CAG	ATC	ACA	CCC	CAG	GGG	TTC	CTC	TGT	ATG	AAG	GCT	CTC	CTC	TTC	786
249	Phe	Ser	Ile	Ile	Pro	Val	Asp	Gly	Leu	Lys	Asn	Gln	Lys	Leu	Phe	Asp	264
787	TTC	AGT	ATT	ATT	CCA	GTG	GAT	GGC	CTG	AAG	AAC	CAG	AAG	CTC	TTC	GAT	834
265	Glu	Leu	Arg	Met	Asn	Tyr	Ile	Lys	Glu	Leu	Asp	Arg	Ile	Ile	Ala	Cys	280
835	GAG	CTC	CGC	ATG	AAT	TAC	ATC	AAG	GAA	CTT	GAC	CGT	ATC	ATT	GCC	TGC	882
281	Lys	Arg	Lys	Asn	Pro	Thr	Ser	Cys	Ser	Arg	Arg	Phe	Tyr	Gln	Leu	Thr	296
883	AAG	AGG	AAG	AAC	CCC	ACC	TCA	TGC	TCC	CGG	AGG	TTT	TAC	CAG	CTC	ACC	930
297	Lys	Val	Leu	Asp	Ser	Val	His	Pro	Ile	Ala	Lys	Asp	Leu	His	Gln	Phe	312
931	AAG	GTC	CTG	GAC	TCC	GTG	CAT	CC	ATT	GCC	AAG	GAC	CTG	CAT	CAG	TTT	978
313	Thr	Phe	Asp	Leu	Leu	Ile	Lys	Ala	His	Met	Val	Ser	Val	Asp	Tyr	Pro	328
979	ACA	TTT	GAT	CTC	CTA	ATC	AAG	GCG	CAC	HIS	ATG	GTG	AGC	GTG	TAC	CCA	1026
329	Glu	Met	Met														
1027	GAA	ATG	ATG	GC													

THE CANARY ANDROGEN RECEPTOR

Figure 12. Homology of the cAR with other ARs. Top: Graph of percent identity of amino acids (10 AA window) between canary and other receptors reported. Bottom: Amino acid comparison of canary and rat androgen receptors.

HOMOLOGY OF CANARY ANDROGEN RECEPTOR WITH MAMMALIAN RECEPTORS



CANARY V. RAT ANDROGEN RECEPTORS

89.5% identity in 343 aa overlap

```

CANARY 10      20      30      40      50      60      70      80      90
EFPLQKTCLICGDEASGCHYGALTCGSKVFFKRAAEGKQKYLCA SRNDCTIDKFR RNCP SCRLRKCYEAGMTLGARKLKKLG N LKAQDD
X: .....
YFPPQKTCLICGDEASGCHYGALTCGSKVFFKRAAEGKQKYLCA SRNDCTIDKFR RNCP SCRLRKCYEAGMTLGARKLKKLG N LKLQEE
RAT      550      560      570      580      590      600      610      620

100      110      120      130      140      150      160      170      180
IEGASSSSPTTEE QAPKLV MTRIDGYECQPIFLNVLEAIEPGVVCAGHDNSQPD SF SNLLTSLNELGERQLVYVVKWAKALPGFRNLHVDD
.....
GENSSAGSPTEDPSQKMTVSHIEGYECQPIFLNVLEAIEPGVVCAGHDNNQPD SFAALLSSINELGERQLVHVVKWAKALPGFRNLHVDD
630      640      650      660      670      680      690      700      710

190      200      210      220      230      240      250      260      270
QMSIIQYSWMGLMVFAMGWR SFTNVNSRMLYFAPDLVFNEYRMHKSRMYSQCIRMRHLSQEFGLWQITPQGFLCMKALLFFSIIPVDGLK
.....
QMAVIQYSWMGLMVFAMGWR SFTNVNSRMLYFAPDLVFNEYRMHKSRMYSQCVRMRHLSQEFGLWQITPQEFGLCMKALLFFSIIPVDGLK
720      730      740      750      760      770      780      790      800

280      290      300      310      320      330      340
NQKLFDELRMNYIKELDRIIACKRK NPTSCSRRFYQLTKVLDSVHPIAKDLHQFTFDLLIKAHMSVDYPEMM
.....:X
NQKFFDELRMNYIKELDRIIACKRK NPTSCSRRFYQLTKLLDSVQPIARELHQFTFDLLIKSHMSVDFPEMMAEIIISVQVPKILSGKVKPIQ
810      820      830      840      850      860      870      880      890
    
```

B: LOCALIZATION OF THE CAR IN THE CANARY BRAIN BY IN SITU HYBRIDIZATION

Since testosterone exerts its effects on gene expression through its receptor, the location of the receptor in the canary brain should provide information about the location of androgen action. The location of the receptor has been inferred from the binding of ^3H -testosterone to slices of canary brain, but this technique is necessarily indirect, since the labeled androgen might bind to a protein that is not a steroid receptor or be metabolized and bind the estrogen or other receptor proteins. Antibodies to the rat androgen receptor were thought not to react with canary tissue (M. Gahr, personal communication). Last year, one group was successful in using a human anti-AR antibody to localize the receptor in bird brain (Balthazart et al., 1992). In order to localize the site of receptor synthesis in the brain, and perhaps as a means of examining its regulation on a cellular level, the canary cDNA was labeled and hybridized to brain sections. This technique of in situ hybridization provides a measure of the location and quantity of androgen receptor message in specific cells.

Results

Localization of the cAR in Testis

As a first step, the cAR was hybridized to sections of the testis, which have a large number of cells which contain cAR mRNA (Isomma et al., 1985). Using a ^{35}S riboprobe on 10 μm sections of canary testis, a tubular structure is revealed with dark field illumination after hybridization and exposure under NTB-3 emulsion for one month (see part a. of Figure 13). Parts c. and d. of Figure 13 show high magnification bright field photomicrographs of the testis which reveal that it is the Sertoli cells lining the seminiferous tubules that have a large number of silver grains throughout their rather diffuse cytoplasm. The Leydig cells do not appear to have cAR mRNA, nor does the vascular epithelia, fibroblasts or the macrophages of the intertubular region and the

tubular wall. The Spermatogonia and spermatids are interdigitated in the cytoplasm of the Sertoli cells, and so it is difficult to exclude the possibility that they have cAR mRNA, but it appears that they do not. I have also successfully used digoxigenin labeled cRNA as a probe for the cAR, and these results are identical (see Figure 14). Although the sense strand appears to give some signal (Figure 14a.), this is only residual coloring reagent (see methods). This labeling technique has at least two advantages over ^{35}S : the results can be visualized after one day, and the subcellular localization is much better than the ^{35}S , which has a resolution no better than five microns.

Figure 13. ^{35}S -AR Testis. In situ hybridization of 10 μm fall testis from zebra finch probed with ^{35}S labeled antisense cAR. a, low magnification showing whole testis in dark field, bar = 500 μm ; b, medium magnification light field view of several tubules, with cellular labeling on the periphery of each tubule, bar = 200 μm ; c and d, high magnification light field view of labeled cells, primarily sertoli cells, bar = 20 μm .

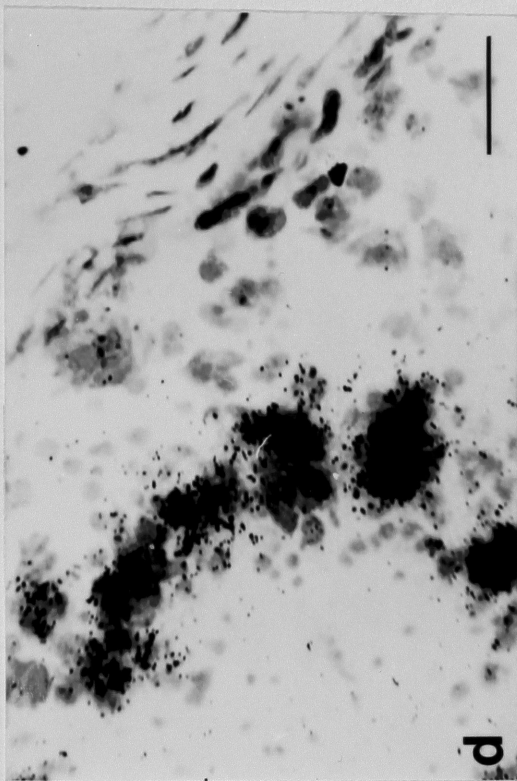
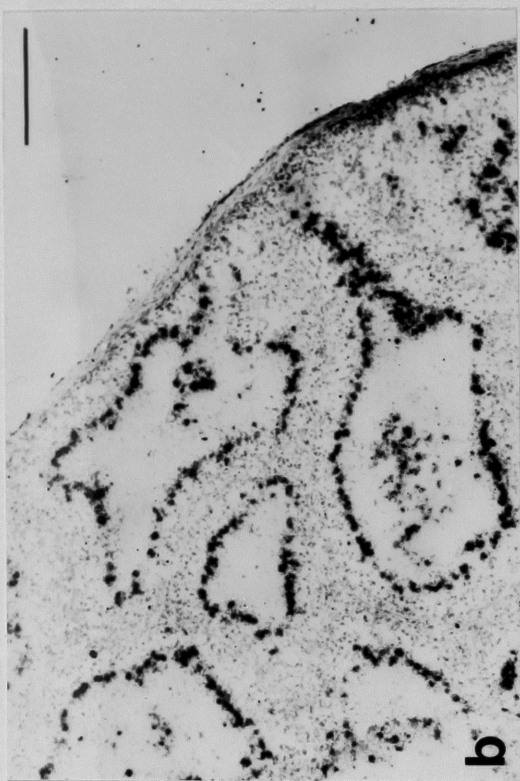
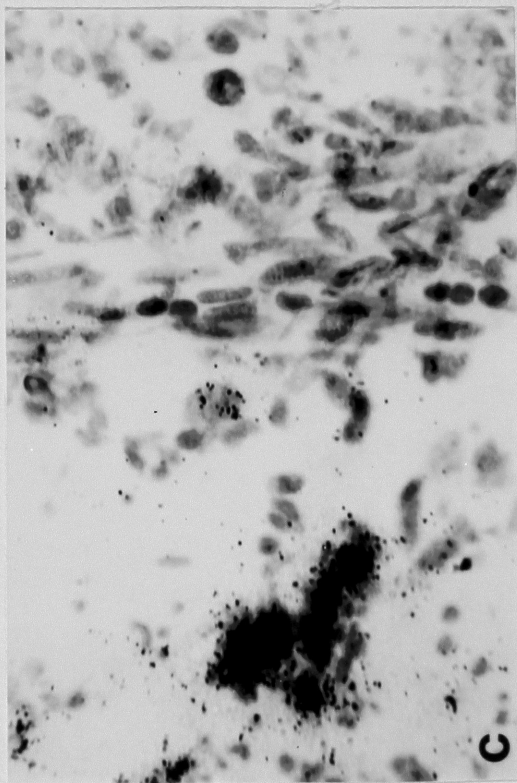
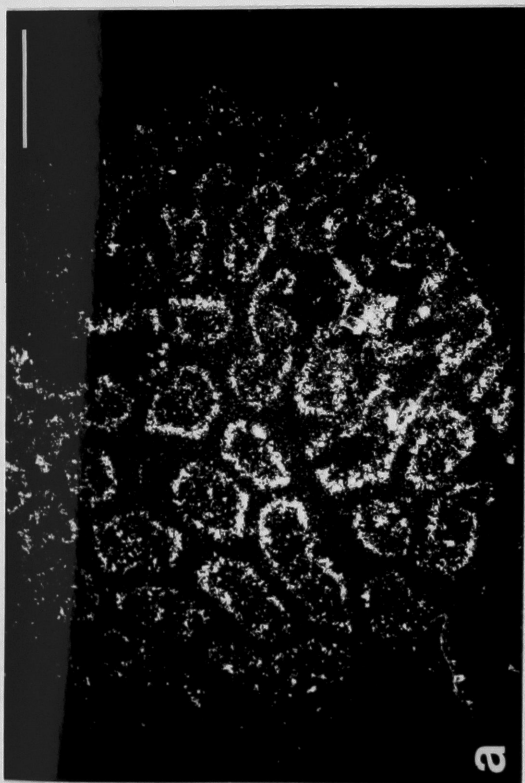
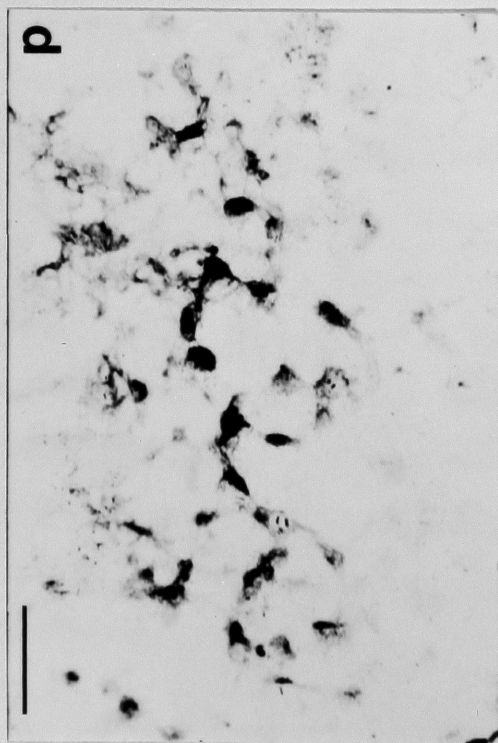
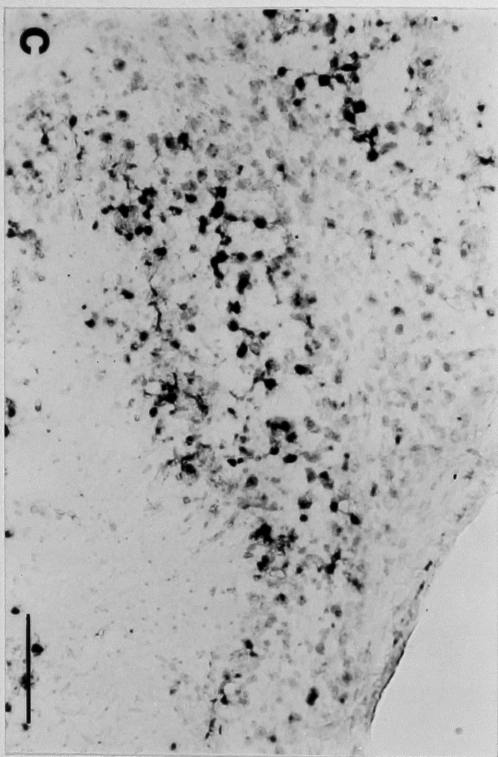
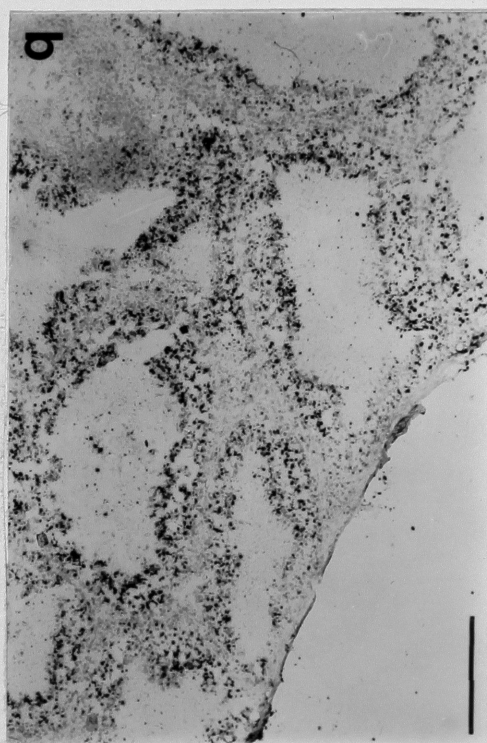
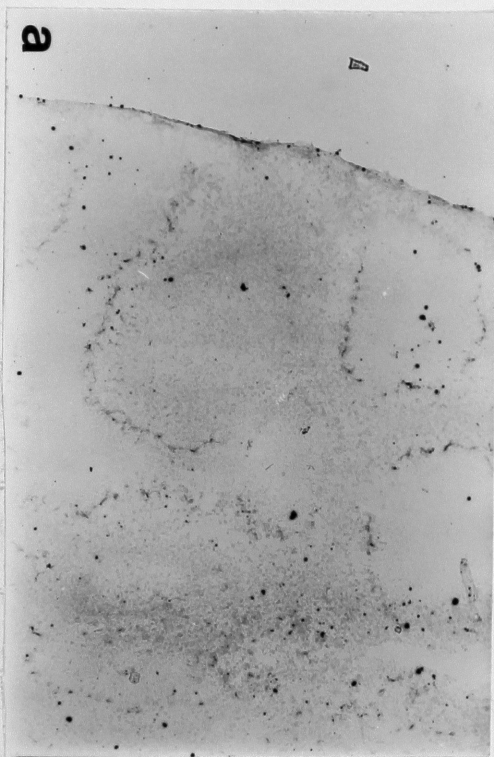


Figure 14. DIG-AR Testis. In situ hybridization of 10 μ m Fall testis from zebra finch probed with Digoxigenin labeled cAR. a, medium magnification showing whole testis hybridized with sense strand cAR; b, adjacent section hybridized with antisense probe, showing several tubules with cellular labeling on the periphery of each tubule, a and b, bar = 200 μ m; c and d, higher magnification light field view of labeled cells, primarily sertoli cells, c bar = 50 μ m; d, bar = 20 μ m.



Localization of the cAR in Brain

By comparison with testis, the brain contains much less AR. Figure 15 shows typical results of testis and brain sections hybridized with ^{35}S -labeled cAR. The number of cells labeled and grains per cell are very low in the brain. In order to optimize the signal to noise ratio for the brain sections, RNase was used to reduce the non-specific binding of the probe, but this unfortunately eliminates the cytoplasmic staining of cresyl violet, which relies on the cellular RNA. After one month under emulsion, sections of the canary brain hybridized with the ^{35}S -labeled probe show some cells in HVc with silver grains over their cytoplasm, but cells in the other song control nuclei are not labeled (see HVc in Figure 16, dns for others). The labeled cells in HVc have three to six grains over their cytoplasm, with the surrounding region being relatively devoid of signal. The digoxigenin labeled probe, which is less sensitive, does not react detectably with cells in the canary song control system. This indicates that the cAR mRNA is very rare in the canary forebrain, but that by optimizing the signal, useful results might be obtained.

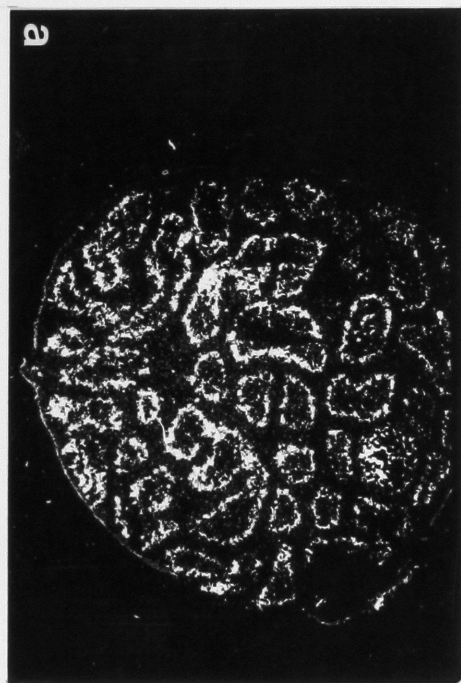
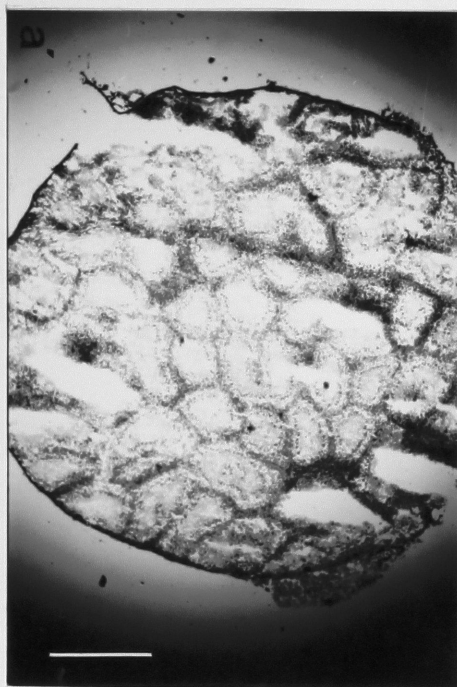
Three months exposure of the canary brain sections shows an accumulation of silver grains over all the song control nuclei of the canary known to bind ^3H -testosterone. Under dark field illumination at low magnification, HVc is clearly labeled, and there appears to be an increased level of grains over RA and MAN (see Figure 17). The clearest example of cellular labeling is in nucleus HVc, in concordance with the results obtained from one month exposure of the autoradiograms (Figure 18). RA and MAN are both labeled, but not nearly so well (see Figure 19). The background in the forebrain is generally quite low after the RNase treatment (see a and c from the previous figures). In addition, several areas of the hypothalamus, midbrain, and brainstem were clearly labeled by both the ^{35}S -labeled and digoxigenin labeled probe (dns), but these areas were not further characterized.

Figure 15. Testis versus HVC. 10 μ m sections of Fall zebra finch testis and canary HVc hybridized with ³⁵S labeled cAR in situ, processed in parallel and exposed one month. a, dark field view of tubular labeling by cAR; a', adjacent section stained with cresyl violet, a and a', bar = 500 μ m; b, dark field view of canary HVc showing no apparent labeling; b', same section stained with cresyl violet, b and b', bar = 200 μ m.

CRESYL VIOLET

AR mRNA

TESTIS



HVC

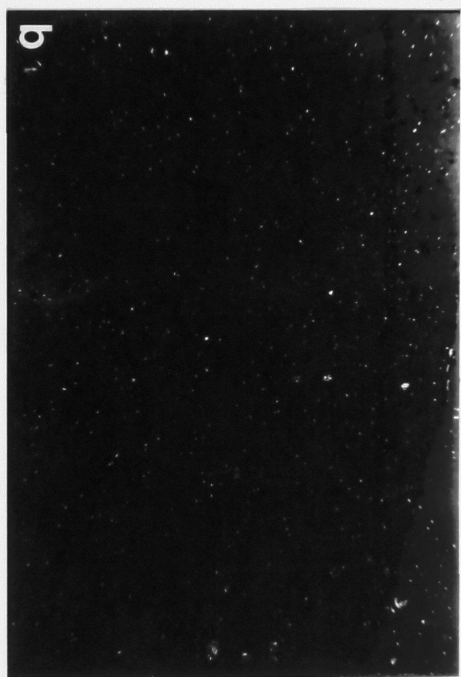
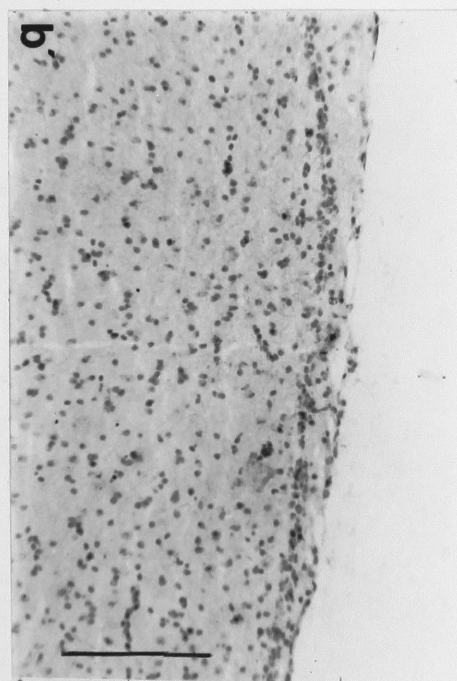


Figure 16. HVC 1 Month Exposure. High magnification view of canary HVc hybridized with ^{35}S labeled cAr as in Figure 13. A few lightly labeled cells are apparent (arrows) in a, b, d; c, adjacent field across lateral ventricle from HVc, showing low background; bar = 20 μm for all photos.

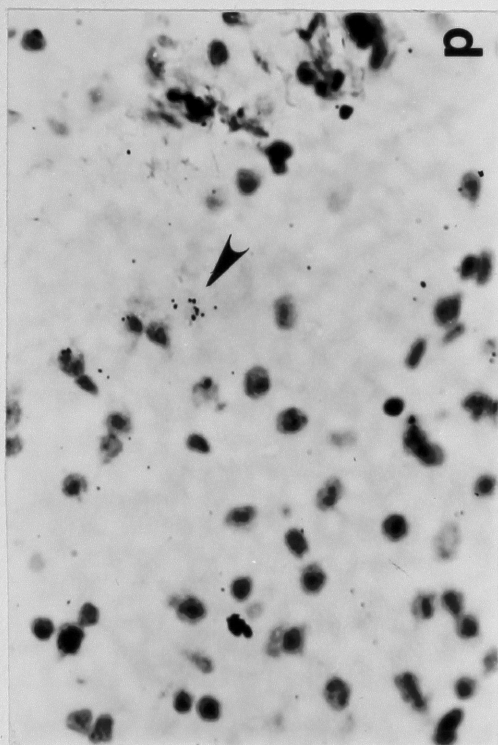
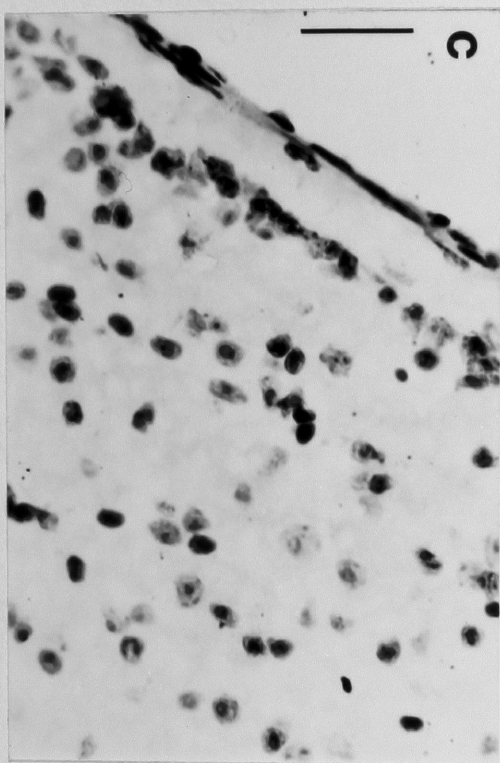
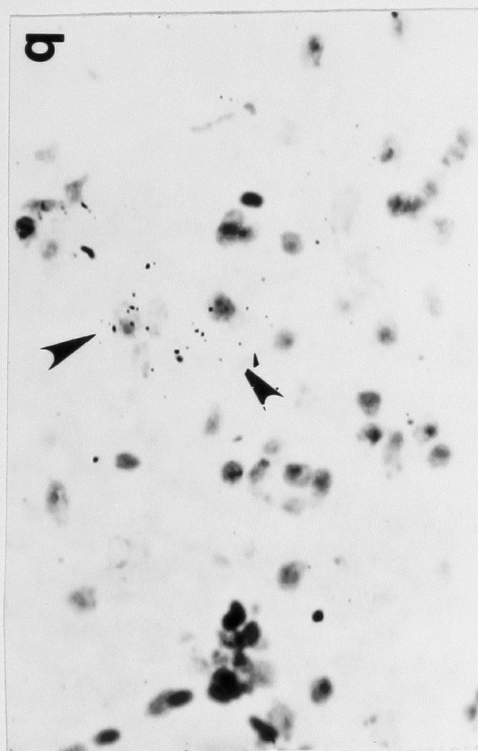
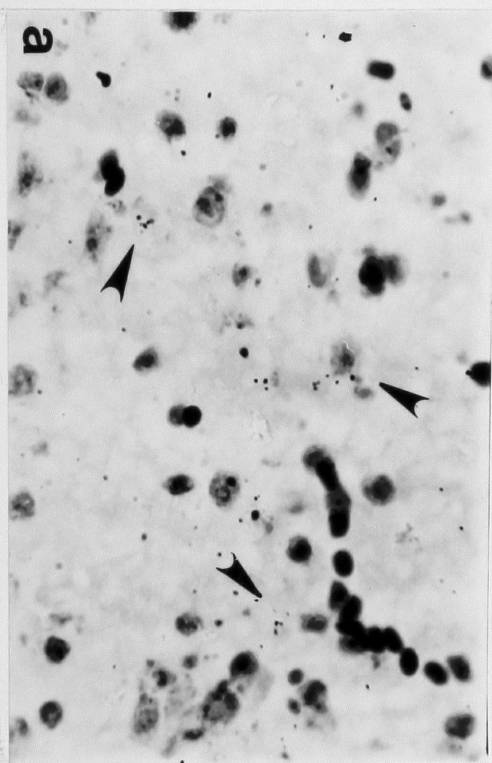


Figure 17. HVC, RA, MAN, 3 Months Exposure. Light field and dark field views of in situ hybridization with ^{35}S labeled cAR of 10 μm canary brain section, and exposed three months. a, a', nucleus RA, bar = 400 μm ; b, b', nucleus HVC; c, c', nucleus MAN; b and c bar = 200 μm .

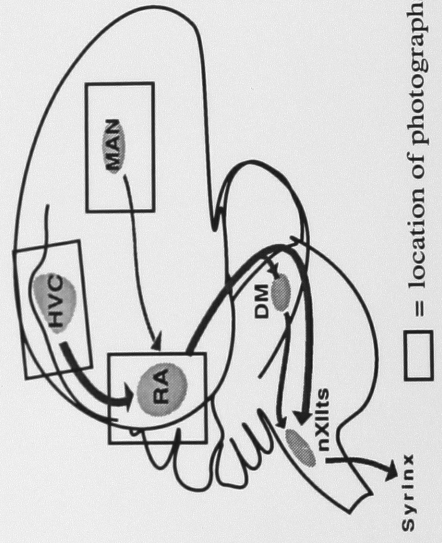
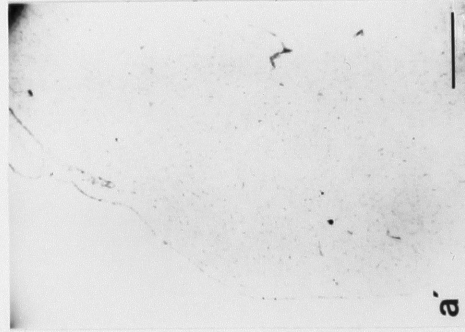
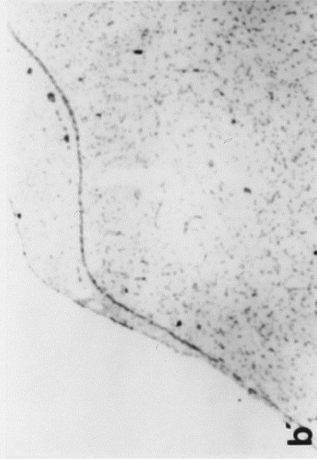
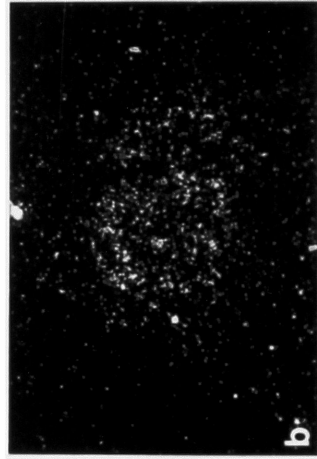
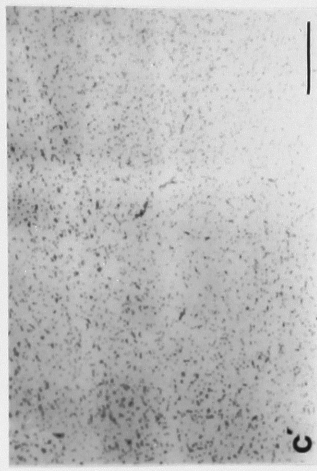
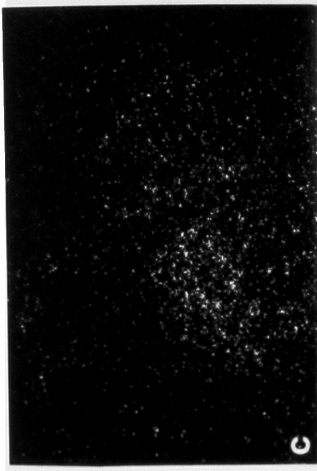


Figure 18. HVC, 3 Months Exposure. High magnification views of canary brain hybridized with ^{35}S labeled cAR, exposed three months. a, dorsal to lateral ventricle; b, ventral to lateral ventricle in nucleus HVc from the same section, note the high number of grains surrounding a subset of the nuclei visible; c, as in a from another bird in a separate hybridization, d, as in b; bar = 20 μm .

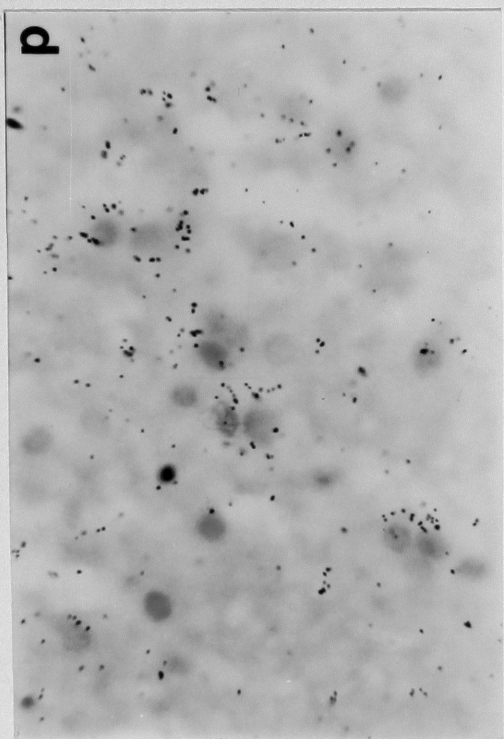
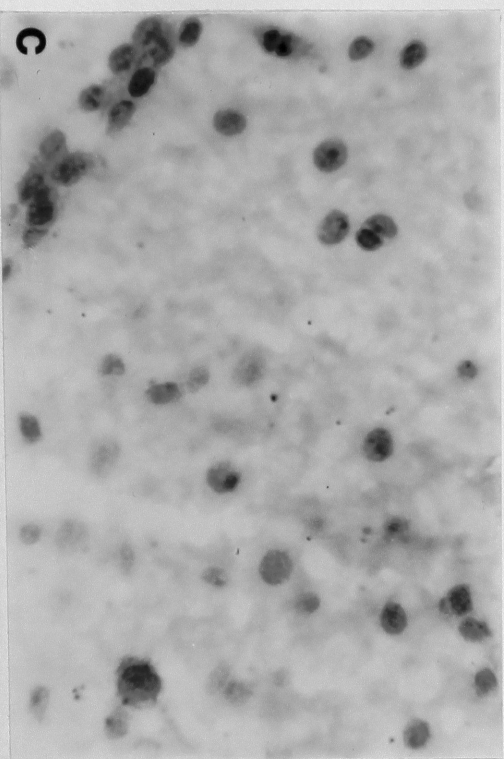
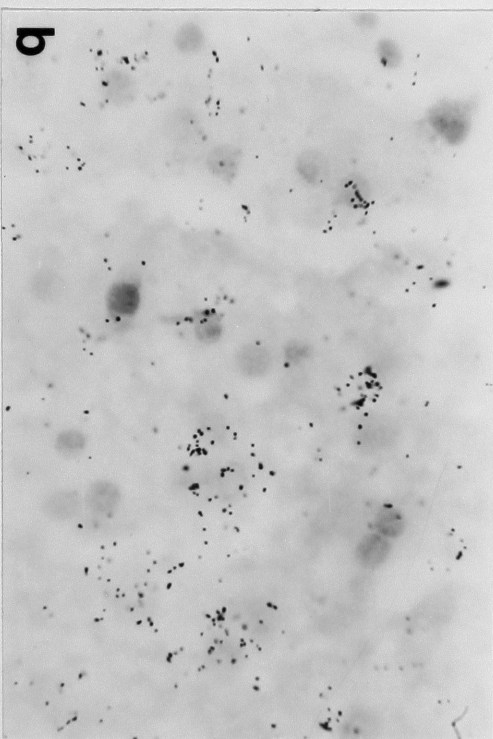
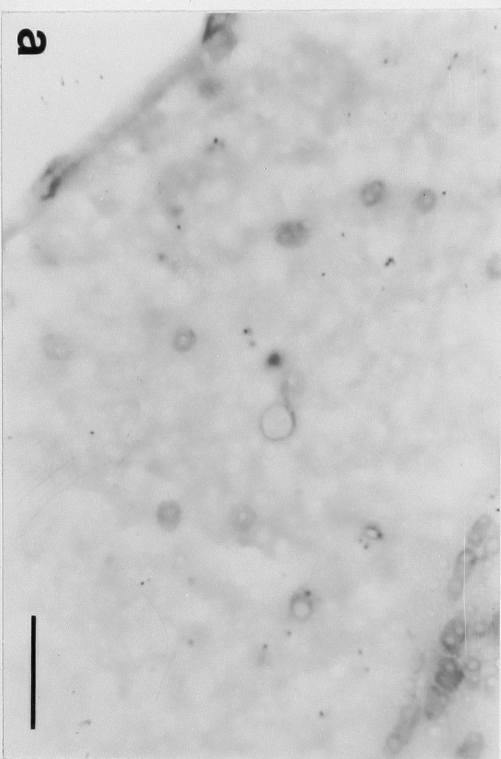
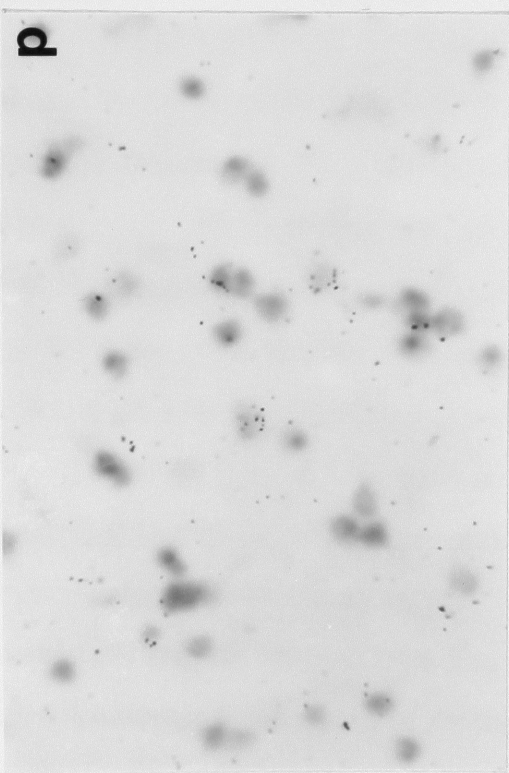
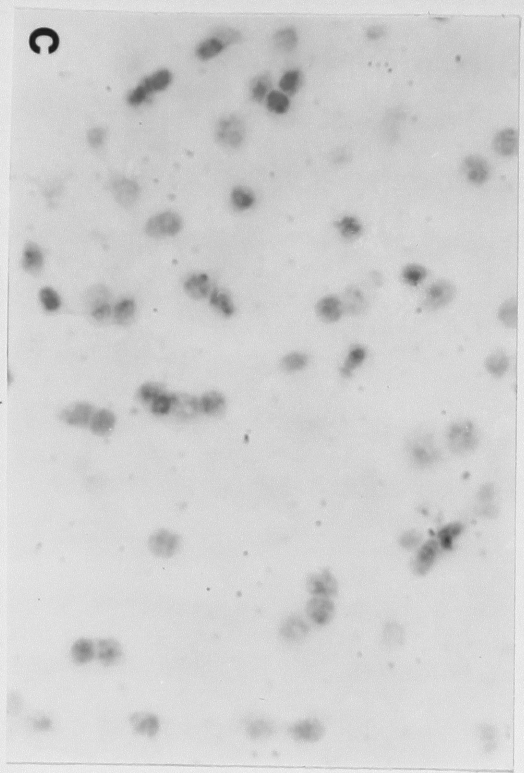
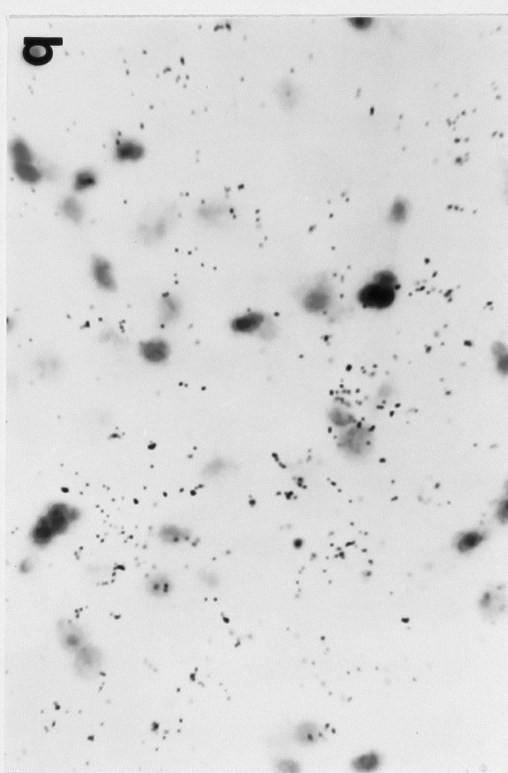
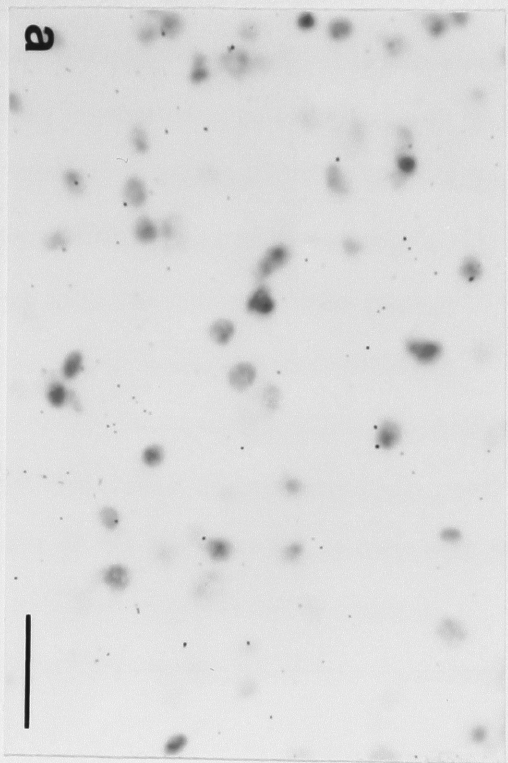


Figure 19. MAN, RA, 3 Months. High magnification views of canary brain hybridized with ^{35}S labeled cAR, exposed three months. a, anterior to nucleus MAN; b, in nucleus MAN from the same section; c, anterior to nucleus RA, d, in nucleus RA; bar = 20 μm .



C: REGULATION OF THE CANARY ANDROGEN RECEPTOR

The low levels of androgen seen in the fall (non-breeding season) in canaries may induce the levels of the receptor as a compensatory mechanism (see introduction). For example, in the rat hypothalamus the androgen receptor is up-regulated by the low levels of androgen seen following castration (Shan et al., 1990). However, it is completely unexamined how the receptor mRNA in the avian song control nuclei may change seasonally, if it does at all. Since the two receptor populations have such different functions, it is possible that the forebrain receptors may be regulated differently from the hypothalamic receptors. In addition, it is possible that different peripheral organs will show different patterns of tissue specific regulation of the AR message, as seen with the ER in rat (Shupnik et al., 1989). A comparison of the androgen receptor levels in canaries between fall and spring and following hormonal manipulation may therefore yield interesting results.

Results

The cAR mRNA is very large and has a very low abundance. When RNA from several regions of canary brain were northern blotted, the RNA appears to be between eight and ten kilobases in length (see Figure 20). The AR mRNA could also be seen to be partially degraded, since there is a trail below the prominent large band. The results of many such northern blots were quite inconsistent, due to either poor quality RNA (probably due to the very small amount of starting material from dissected brain regions), or due to the very low abundance of the transcript.

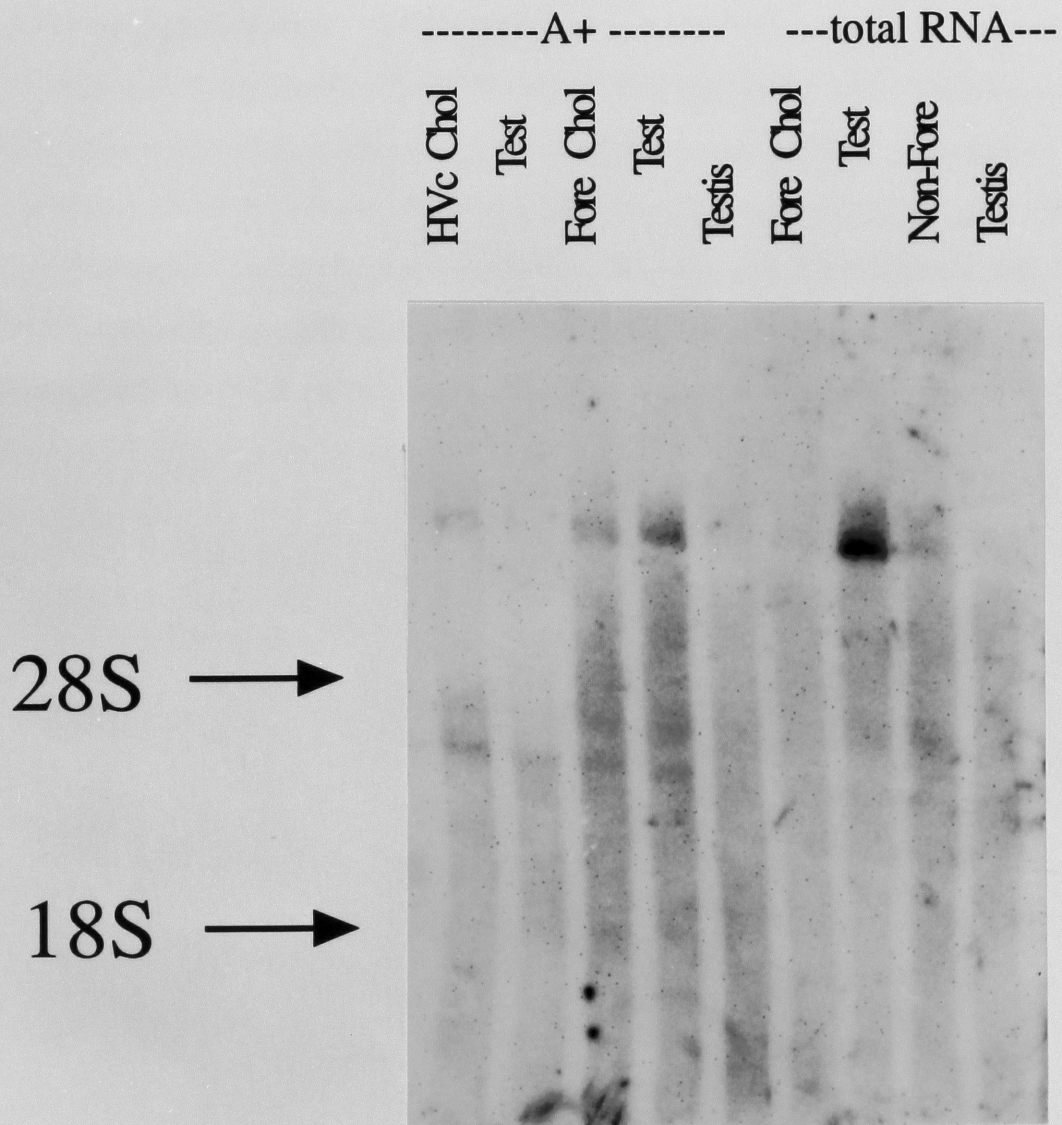
Since the level of AR mRNA is very low in every tissue examined and to partially overcome the problem of degradation, I have developed an assay using PCR of reverse transcribed RNA from canary tissues. This assay is sensitive to fg of AR mRNA as currently in use (see methods for a description of the assay). The primers for signal amplification are in the middle of the transcript, and so the assay should be relatively unaffected by small amounts of 3' or 5' exonuclease degradation (see discussion). This

THE CANARY ANDROGEN RECEPTOR

semi-quantitative pcr (qpcr) assay has proven to be an ideal tool to analyze the levels of cAR message in a variety of samples as it is quick, relatively quantitative, and reproducible.

Figure 20. Northern Blot for cAR. Scan of photograph of autoradiogram showing the detection of the cAR mRNA. Five μg poly A⁺ RNA or 20 μg total RNA was separated on a 1.2% formaldehyde/agarose gel and transferred to a nylon membrane. The membrane was probed with a riboprobe encoding cAR 2.2. The membrane was washed to 68°C, "11 mM sodium" (see methods). Samples: HVc or Fore(brain) from ovariectomized canaries treated for six hours with Chol(esterol) or Test(osterone); Testis from normal spring male canaries; Non-Fore(brain) from cholesterol treated ovariectomized canary.

Canary Androgen Receptor Northern Blot

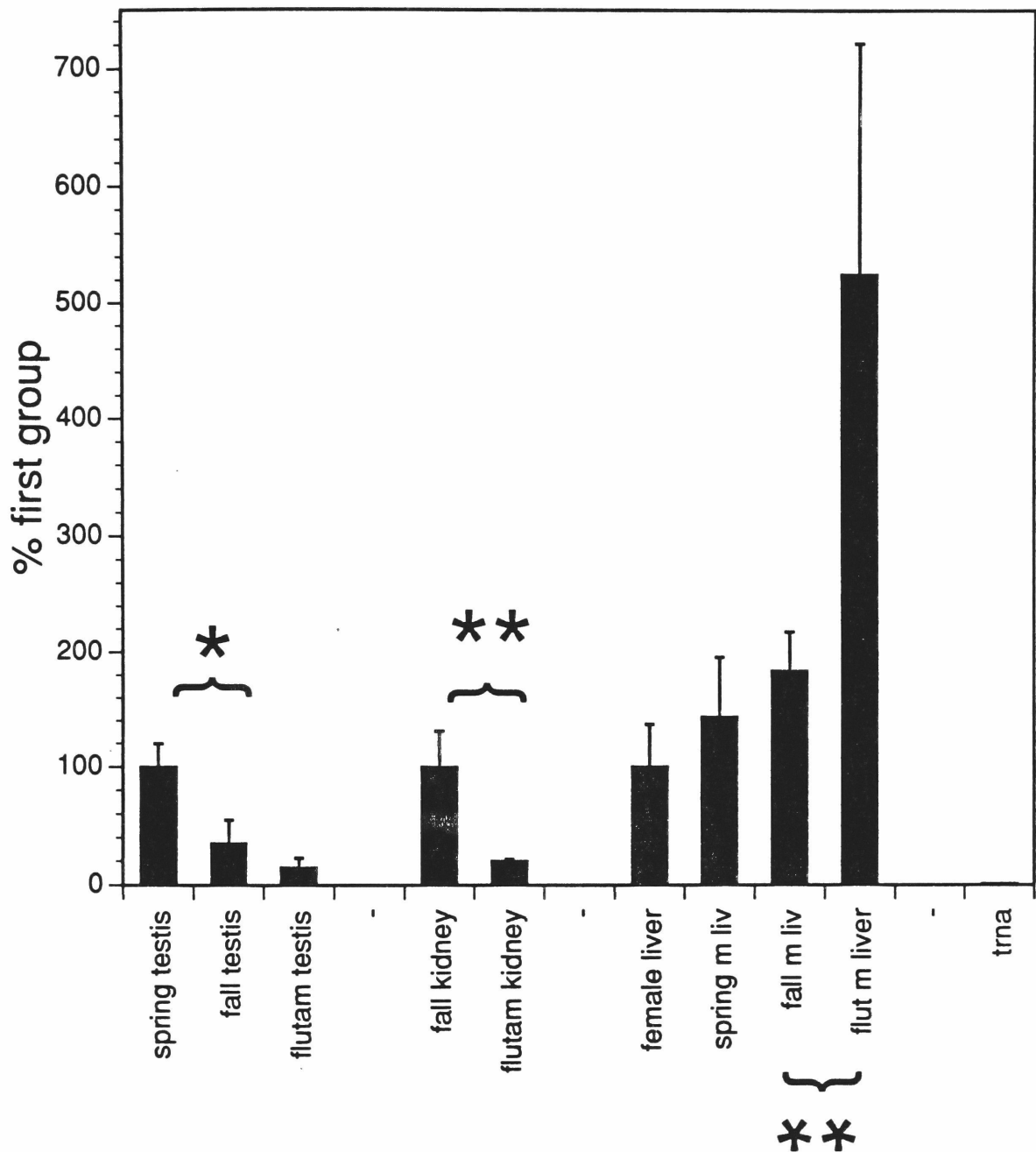


Spring versus Fall

There is a significant difference in cAR mRNA content between spring and fall in the testis samples have examined using qpcr. RNA from the testis of more than twenty spring canaries was isolated, as were the testis of four normal fall canaries. Spring testis from canaries are 100 fold larger than fall (Nottebohm, 1981). I measured the levels of circulating androgens from the fall birds using a testosterone RIA (see methods). Androgen levels are 160 +/- 30 pg/ml in the fall (October), one tenth that seen in the spring (spring levels are 3.2 +/- 0.5 ng/ml (Luine, et al, 1980)). Correspondingly, when the amount of endogenous cAR mRNA measured by qpcr in the testis, normalized to the spiked deletion control, is analyzed by ANOVA of repeated measures, there is a significant difference between the groups ($F(2,20)=7.756$, $p=0.0069$). The cAR mRNA is three-fold more abundant by proportional mass in spring than fall (Fisher's Paired Least Significant Difference (FPLSD), $p<0.05$, see Figure 21). There was not a significant seasonal effect on cAR mRNA levels in the liver of these canaries ($p>0.7$ by FPLSD).

Figure 21. Regulation of cAR in Peripheral Tissues. Graph of relative values for androgen receptor content determined by qpcr assays for the cAR in testis, kidney, and liver from male canaries (and female liver). The data (seven determinations per point) are presented as a percent of the mean of the seven determinations of the "control" group for each tissue (i.e. spring testis, fall kidney, or female liver) in the graph for visual comparison of the treatments by tissue. Mean +/- SE.

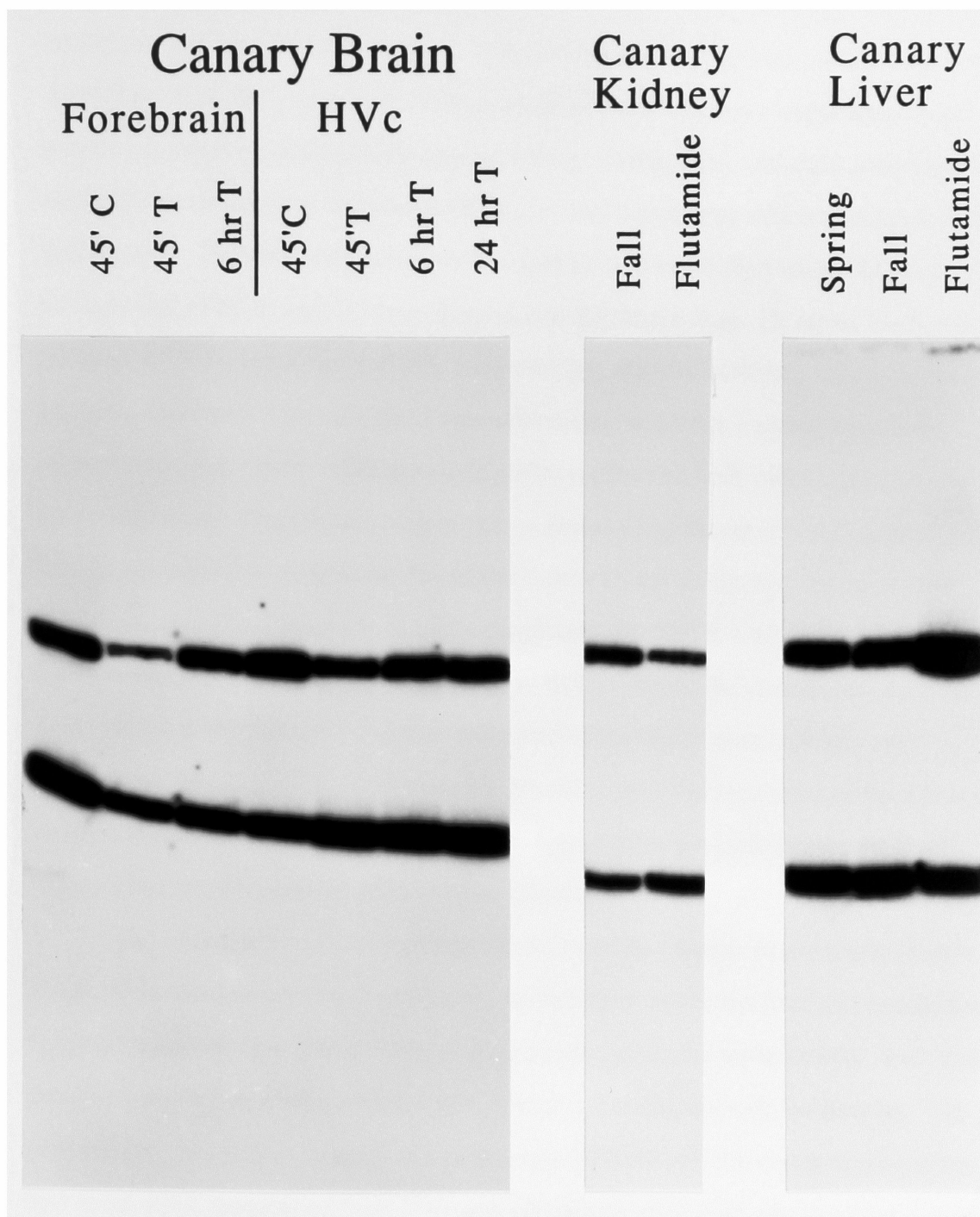
Canary Androgen Receptor mRNA levels



* $p < 0.05$ vs control

** $p < 0.01$ vs control

Figure 22. Example of QPCR. One sample set, an example of the eight RT-PCR assays compiled in Figures 21 and 23. Top bands are the endogenous cAR mRNA signals. Bottom bands are from the cAR deletion cRNA spiked into the reverse transcriptase reactions, to which the data were normalized (see methods for a complete description of the assay and its analysis).



Androgens Regulate the AR mRNA in Canaries

In order to define how androgens regulate the cAR, I administered several drugs which are known to affect androgens in rats and assayed the cAR mRNA with the qpcr technique. Sixteen fall male canaries were divided into four groups: four birds were left untreated (these birds were used in the analysis above), four were implanted with a silastic capsules containing flutamide, an androgen antagonist, and eight were injected with ethylene dimethane sulphonate (EDS), an anti-tumor drug which selectively kills Leydig cells. Unfortunately, seven of the eight EDS canaries died within 24 hours after the injection of EDS, apparently of dehydration due to the drug. (It seems likely that the remaining EDS treated bird was injected in the gut, thereby receiving only a partial dose of EDS.) The birds were sacrificed two weeks later, and RNA isolated from testis, kidney, liver, and spleen. EDS had no significant effect on cAR mRNA levels in the surviving canary. Qpcr assays of these tissue reveal a significant (FPLSD, $p < 0.01$) five-fold down-regulation of cAR mRNA in the kidney by the antagonist flutamide (see Figures 21 and 22). Flutamide caused a significant increase in cAR mRNA levels in the liver (FPLSD, $p < 0.01$). Flutamide appears to further reduce the already low levels of cAR mRNA in the fall testis, but due to the variability of the sample, it was not significant (FPLSD $p > 0.3$). It appears that the tissue specific regulation of the ER in the uterus and liver (Shupnik et al., 1989) is also apparent for the cAR in the testis and kidney (decreased by low T) and liver (increased).

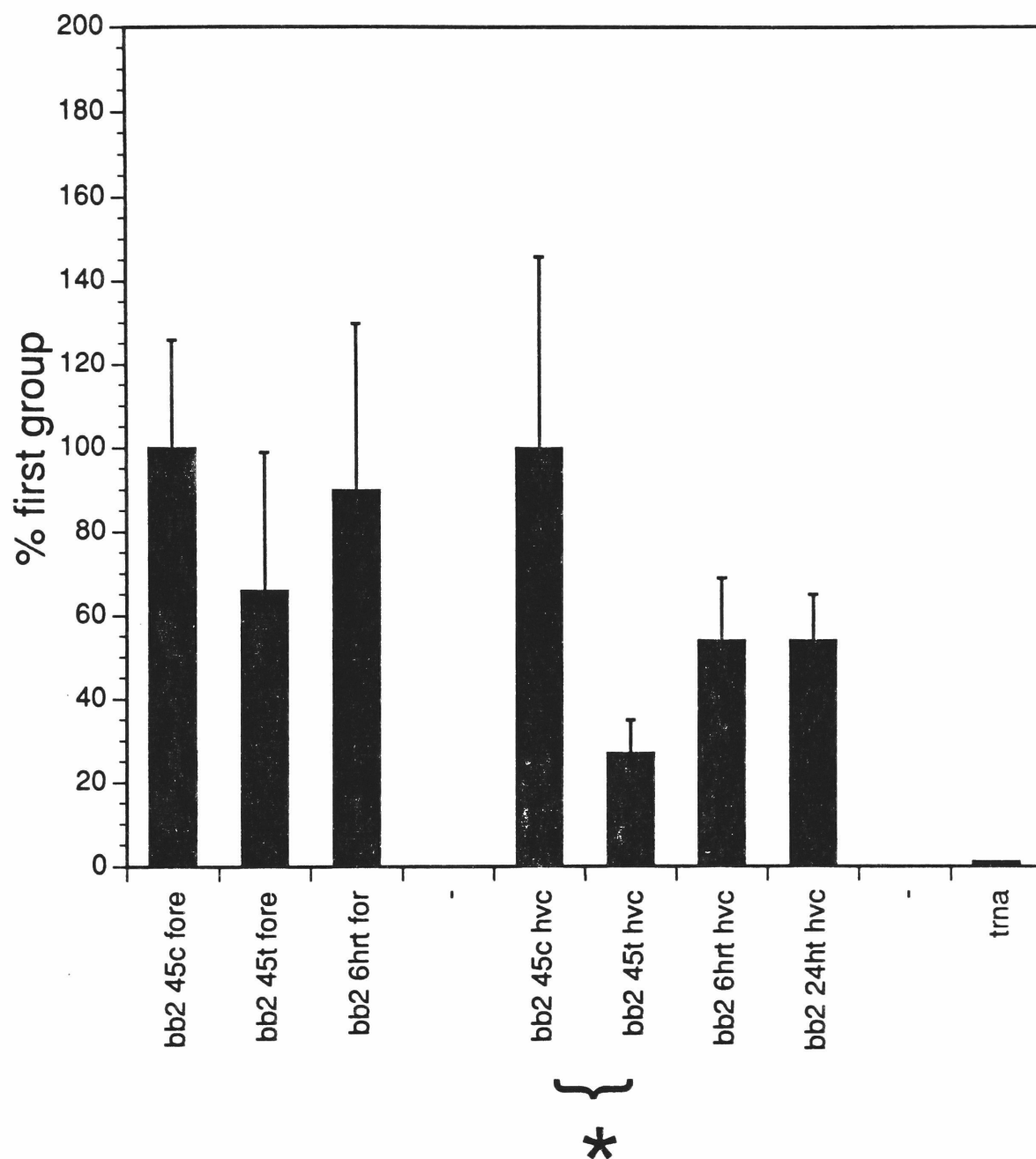
The cAR mRNA is also regulated in the brain by changes in plasma androgen levels. One hundred seventy five female canaries were ovariectomized and treated for varying lengths of time with androgen (ten birds per group for those shown), their brains dissected, pooled, and RNA isolated (see chapter three and methods for details). The cAR mRNA, as assayed by qpcr, is significantly (FPLSD $p < 0.05$) down-regulated two fold in the RNA preparation from HVc and surrounding tissue 45 minutes after a single injection of 2 μg testosterone (Figure 23 and 22). This regulation may be transient, as by six hours after the injection, cAR mRNA is reduced less than 50% (not significant)

THE CANARY ANDROGEN RECEPTOR

relative to control. This regulation is not apparent when the remainder of the forebrain is similarly assayed ($F(2,20)=0.427$, $p=0.66$), though this may be due in part to the greater variability in this sample (Figure 23).

Figure 23. Regulation of cAR in Brain. Graph of results of seven qpcr assays for the cAR HVc or forebrain from c(cholesterol) or t(estosterone) treated ovariectomized canaries. The data are presented as the percent of the mean of the "control" for each tissue (45 minute cholesterol treated forebrain or HVc). Mean \pm SE.

Canary Androgen Receptor mRNA levels

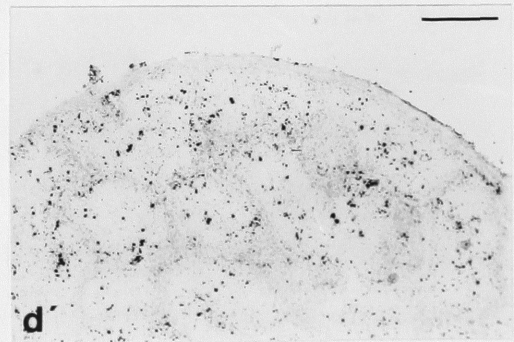
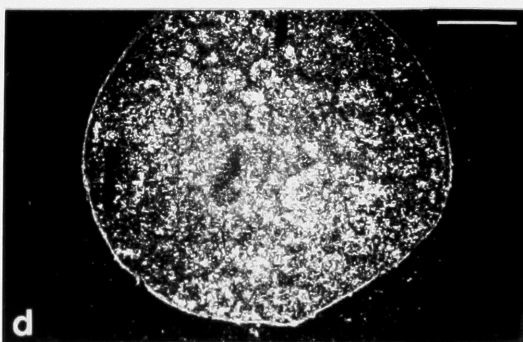
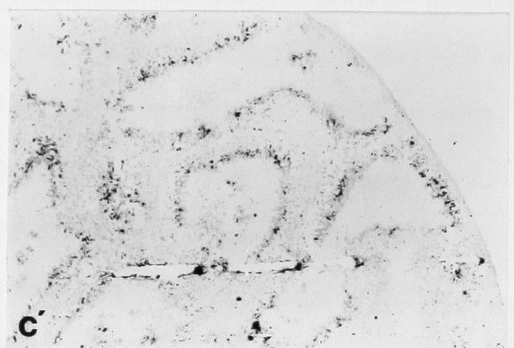
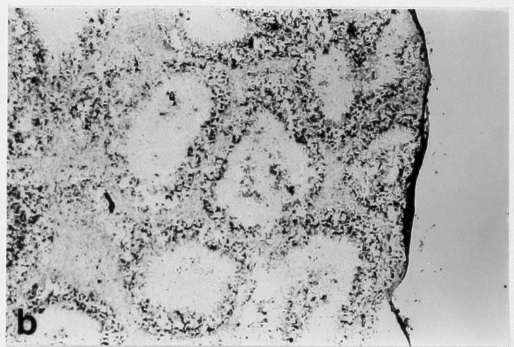
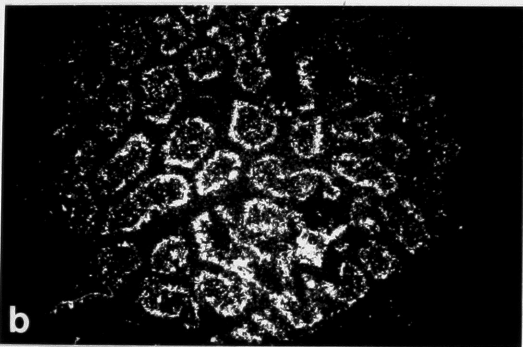
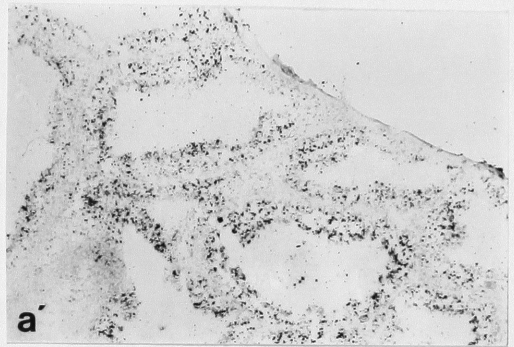
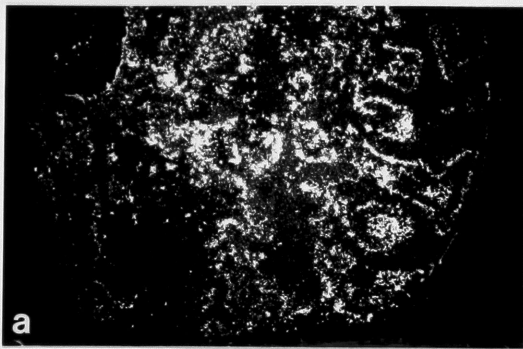


* $p < 0.05$

Androgens Regulate the AR in Zebra Finch

In addition, I analyzed cAR mRNA from two separate sets of zebra finches. One set was equally divided into four groups: castrated, castrated and treated with flutamide, castrated treated with a drug which selectively destroys Leydig cells, EDS, and castrated with testosterone replacement. The second set was normal males, equally divided between groups left intact, treated with flutamide, treated with EDS, and EDS treated and given testosterone replacement. Unlike the canaries, all of the zebra finches survived the EDS treatment, perhaps due to their adaption to the scarce water conditions in their native desert environment. Sections of testis from these birds were hybridized with ^{35}S -labeled cAR (see Figure 24). As with the canary, the level of cAR mRNA appears to not be affected by flutamide, relative to the fall (see Figure 24 a versus b). EDS appears to decrease the signal in the testis (see Figure 24c). Testosterone replacement in EDS birds dramatically increases the signal, but the internal structure of the testis is disorganized (compare Figure 24 a,b,c versus d). Although the canary primers for the qPCR appear to give a single band on acrylamide gels, the results of these assays have been quite variable.

Figure 24. Regulation of the cAR in Zebra Finch Testis. Sections from birds untreated (a, a'), treated with flutamide (b, b'), treated with EDS (c, c'), or treated with EDS, then given testosterone (d, d'). a,b,c,d dark field view showing modulation of signal from in situ hybridization for the cAR, bar = 500 μ mm. a',b',c',d' bright field view at higher magnification of adjacent sections hybridized with digoxigenin labeled cAR showing the accompanying changes in tubular morphology and digoxigenin distribution, bar = 200 μ mm.



DISCUSSION

The Canary Androgen Receptor

The AR was originally cloned in rats and humans in 1988 (Chang et al., 1988a; Tilley et al., 1989; Chang et al., 1988b; Lubahn et al., 1988). This characterization was done by cloning a panel of likely candidates based on the homology between the steroid receptor genes, and then testing their ability to bind androgens. I attempted to clone likely candidates for the canary androgen receptor by their homology to the rat gene.

I screened a canary male HVc library extensively but was unable to isolate a positive clone for the androgen receptor. The canary brain contains at least 30 thousand, and as many as 100 thousand mRNAs (Clayton and Huecas, 1990) and the androgen receptor is both very low abundance, and in only a small percentage of the cells in the "HVc" tissue that the library was made from. Therefore in order to assure a 95% probability of finding the cAR if it is represented only once in 1,000,000 independent mRNAs represented in the HVc library, I would need to screen almost five million clones (Sambrook et al., 1989). Since I only screened one million clones, it is perhaps not surprising that I did not find a clone in my direct screen of the library. The pcr amplification of the library shows that it is in the library. In my screening of the male liver library, assuming it is less complex and contains only 20 thousand independent mRNAs of the rare class (a high/conservative estimate), in order to assure 99% probability of finding a single positive, I would need to screen 275 thousand clones, and I screened 300 thousand. This indicates that not only was I unlucky, but the clone is of very low abundance in general, which we expected, and even lower in the cDNA libraries (in fact, absent from the liver library, see Figure 5), probably due to several structural features which reduced its representation, such as the long 3' UTR and the reverse transcriptase stops.

Since the abundance of the receptor and its representation in cDNA libraries was so low, I isolated the receptor using methods which are not dependent on the initial concentration of the receptor transcript. Genomic cloning, where the gene would be

THE CANARY ANDROGEN RECEPTOR

represented in equal proportion to all others, was not deemed practical for much of the cloning due to the large size (>100kb) (Kuiper et al., 1989) and many exons in the human transcription unit (Faber et al., 1991) (see Figure 9). The conservation of this genomic structure for other steroid receptors and across species (see (Kuiper et al., 1989) for references) suggests that a similar challenging genomic structure would be encountered in the canary androgen receptor. I therefore used the polymerase chain reaction and information about short regions of relatively high homology among the two androgen receptors and between the steroid receptors to eventually clone a large part of the cAR.

Rt-pcr eliminated many of the problems encountered in screening the canary cDNA libraries. By reverse transcribing RNA using random primer (poly dN₆), both the problems with reverse transcription stops 3' of the coding sequence and the length of the 3' UTR are eliminated. The amplification of the target cDNA by pcr eliminates the problem of the low abundance of the transcript in the source RNA. The amplification primers I originally designed were based on the conservation of the human and rat sequences. My objectives in designing the primers were two-fold. First, I wanted to define the largest possible amplification product. Second, since rodents and humans are both mammals, I had to assume that the homology between them would be higher than for canaries. I therefore chose to make one primer from a structural region which is conserved between steroid receptors, the DNA binding region ("C"). The amino acid sequence encoded by the primer for the DNA binding region is the most conserved between steroid receptors, especially the class I receptors (Miesfeld, 1990). The final six encoded amino acids are identical to the progesterone receptor, but the first two are unique to androgen receptors (Beato, 1989). The other primer is derived from the steroid binding domain ("E"), in a region identical between rat and human, but which has little homology to other receptors. Using testis as a source of RNA, believing that the cAR transcript would be relatively abundant there, I was able to isolate a clone for the cAR.

The canary clone includes 994 bp of androgen receptor coding sequence between the pcr primers. This includes the DNA binding region, the "hinge" and the steroid

binding domain. The clone is incomplete on both the 3' and 5' ends. If the canary receptor matches the rat and human coding sequence in length and structure, the clone is missing 70 bp of coding sequence from the 3' end of the eighth exon. It will be necessary to clone this end from the canary genomic library, as the 3' UTR is 6.8 kb in humans (Faber et al., 1991) and similar in rat (Quarmby et al., 1990; Lubahn et al., 1988). Either the length of the long 3' end or secondary structure in it prevents reverse transcriptase primed from the polyA tail from transcribing into the coding region (dns). The clone is also missing 77 bp from the 5' end of the second exon. This region is less well conserved between rat and human (Chang et al., 1988a) and even more poorly conserved in canaries (see results). The upstream pcr cloning primer contains the 5' end of the first zinc finger, which accounts for 20 bp of the uncloned region, and this region appears to be well conserved, as a non-degenerate primer amplifies efficiently. The rest of the uncloned 5' end of the second exon appears to be very poorly conserved, as two separate degenerate primers fail to amplify by RT-PCR (dns).

The entire first exon homolog in the canary remains uncloned. This region of the mRNA is quite large, 1566 bp in the rat and 1583 in humans (Chang et al., 1988a) of coding sequence, plus 1.1 kb 5'UTR (Faber et al., 1991). This region also shows the least conservation between androgen receptors in mammals (Chang et al., 1988a; He et al., 1990). Using six separate degenerate primers from this region, I have not been able to amplify any cAR sequences from genomic (dns) or reverse transcribed (see Figure 8) template. The absence of amplification product from the genomic pcr indicates that none of the degenerate primers as a pair are sufficiently homologous to the canary sequence. RT-PCR using random primed RNA and a combination of degenerate primers or one degenerate and one known primer for the amplification, also indicates that the primers for the first exon are poor matches. In addition, the lack of product using an anchored primer approach, where a primer from the defined sequence is used to prime one direction and a synthetic tail is added to the cDNA to prime the other, is a clear indication that secondary structure interferes with the progress of the reverse transcriptase. All of this information

THE CANARY ANDROGEN RECEPTOR

about the first exon indicates that the homology between the canary and the other species whose sequences have been reported is poor.

Since two oligonucleotides appear to recognize a unique band on a Southern blot of a genomic digest, they are currently being used to isolate the first exon of the cAR from a library of canary genomic DNA. Since no songbird genomic libraries existed, I constructed one which has more than one million independent clones. This library is representative of the canary genome, since the average insert size is almost 20 kb, which gives a complexity of 20×10^9 bp, and the canary genome is likely to be less than one sixth of that. From a screen of approximately 200,000 unamplified clones a priori, one would expect a positive clone for every genome equivalent screened by each oligo. Hence, if one assumes the canary genome is 2×10^9 bp, four positives would be expected for the two oligos I screened with, and I got five. Two of the clones contain only the 5' most end of the exon. The remainder of the clones have not yet been analyzed.

Sequence Analysis and Evolution of the Androgen Receptor

Although it has been reported that a small fragment of the xenopus AR has apparently been cloned, less than one hundred bp of sequence has been reported (He et al., 1990). Therefore although the canary receptor clone is not the only non-mammalian androgen receptor cloned and sequenced, it is by far the most complete, and the only receptor where we have information on regions other than the highly conserved DNA binding zinc fingers.

Various measures were taken to assure no taq polymerase induced mutations are included in the sequence for the canary androgen receptor. First, I isolated the pcr product and sequenced it directly using an end-labeled primer. The sequence from the pcr product, which represents the sequence of a very large number of molecules, showed similarity to the rat and human androgen receptors. I then isolated four independent clones of the band and sequenced them by standard dideoxy techniques. By combining the sequence from the four clones and the pcr reaction product, I believe I have overcome any error introduced in the amplification by the taq polymerase and have highly reliable

sequence (see Figure 11).

When I compare this sequence with known molecules, it is highly related to both the canary and rat androgen receptors and other steroid receptors. (see Figure 12). This homology is particularly strong in the DNA binding domain, which is completely identical in amino acid sequence to all of the mammalian homologs (Gaspar et al., 1990). This is quite remarkable, considering the hundreds of millions of years since birds and other vertebrates diverged, though a similar identity is seen between the chicken and human ER (Krust et al., 1986). It is clear, however, from comparing the DNA sequences of the ARs between the species that the codons have been randomly mixed over the years since these species diverged. The identity of the sequence in the "hinge" is much poorer, 57%, but accounting for conservative substitutions, the conservation is 95%. The high identity levels return in the steroid binding domain, where the species are 93% identical.

in situ Hybridization of the cAR

Since testosterone exerts its effects on gene expression through its receptor, the convincing localization of the receptor in the parts of the brain which control song suggests that androgen is at least capable of regulating transcription there. The location of binding of ^3H -testosterone to sections of canary brain (Evans and Arriza, 1989; Arnold, 1981; Sohrabji et al., 1989) matches the location of cAR mRNA I find in the brain. Antibodies to the rat androgen receptor also reveal a similar distribution of AR protein in bird brain (Balthazart et al., 1992). The in situ hybridizations for the cAR therefore provide another means of localizing the receptor, but one which has the potential to also measure quantity of androgen receptor message.

Localization of the cAR in Testis

The Sertoli cells of the testis, the cells lining the seminiferous tubules, have a large number of silver grains throughout their rather diffuse cytoplasm when hybridized to ^{35}S -labeled antisense cAR cRNA. The Leydig cells do not appear to have cAR

mRNA, nor does the vascular epithelia, fibroblasts or the macrophages of the intertubular region and the tubular wall. The Spermatogonia and spermatids are interdigitated in the cytoplasm of the Sertoli cells, and so it is difficult to exclude the possibility that they have cAR mRNA, but it appears that they do not. This localization is identical whether ^{35}S or digoxigenin labeled cRNA is used as a probe for the cAR, and there is no specific staining (and very low background) with the sense strand probe. The localization of AR to the Sertoli cells is identical in the rat using antibodies (Sar et al., 1990) or nuclear exchange assay (Sanborn et al., 1975), but there is a striking localization of the protein to the nuclei of the cells, rather than the diffuse cytoplasmic staining seen for the mRNA. However, Sar et al. (1990) report intense staining of the peritubular myoid cells and in some cells in the intertubular region, which I see no evidence of in the canary or zebra finch.

Localization of the cAR in Brain

The androgen receptor is much more rare in the brain. When canary brain sections are hybridized to cAR under the same conditions as the testis, using either ^{35}S or digoxigenin labeled probes, no specific labeling can be detected (see Figure 15). When the hybridization conditions are altered to include RNase treatment, the apparent non-specific binding (NSB) of the probe is reduced. Using standard RNase treatment conditions (Clayton et al., 1988), there is little reduction of the NSB. I therefore increased the concentration of RNases four fold, the incubation temperature to 37°C, and the duration of incubation to one hour, in addition to a higher stringency pre-wash (Clayton et al., (1988) uses 2X SSC, 50°C, but I used 2X SSC, 65°C, 50% formamide) and a high very high stringency post-wash (up to 11 mM sodium, 68°C-see methods).

Using these conditions, and exposing the sections for three months, I was able to detect relatively strong signals in all of the song control nuclei shown by labeled ligand binding to concentrate androgen (see Figure 17). At the cellular level, up to 20% of the neurons in HVc appear to be labeled (see Figure 18). The number of labeled cells in MAN is probably higher than HVc, but the NSB in this part of the forebrain is also higher

and the cells more closely packed than elsewhere, leaving some ambiguity as to the percentage of labeled cells. The signal in RA is much lower than either HVc or MAN, but cells labeled above background can be seen (Figure 19, c and d). It is interesting to note that despite a great deal of effort by colleagues in my lab to discover genes differentially expressed in an anatomically relevant fashion in the canary brain (Clayton et al., 1988; George and Clayton, 1992), the cAR is the only message discovered by us which is highly enriched in the song control nuclei relative to the rest of the brain, although one anonymous gene is slightly enriched (D. Clayton, unpublished observations).

Regulation of the cAR in Songbirds

The cAR is regulated both seasonally and pharmacologically in a tissue specific manner. This is the first report of a natural fluctuation in the mRNA levels of the androgen receptor. I expected that the receptor mRNA would be up-regulated in response to the low serum testosterone seen in the fall (Nottebohm, 1989; Nottebohm et al., 1987). The testis has a lower level of cAR mRNA in the fall than the spring (see Figure 21), mirroring the low level of circulating testosterone I measured in these birds (see results). This pattern of regulation extends to the androgen antagonist flutamide. In fact, the liver is the only organ tested where the AR is regulated in the expected direction, being higher after treatment with the androgen antagonist flutamide, but this change was not seen between spring and fall in the liver. The cAR mRNA levels are also reduced in the kidney when the feedback through the receptor is blocked by the competitive inhibitor.

This pattern of down-regulation seen in the testis and kidney was unexpected. The AR mRNA had been reported to increase 1.5 fold in the rat kidney after castration (Quarmby et al., 1990), but these results are from Northern blots, where the signal appears both very low and messy after twelve day exposure (see Figure 1a in Quarmby et al., 1990). Quarmby, et al. also report a significant positive regulation of the AR in the

THE CANARY ANDROGEN RECEPTOR

reproductive accessory tissues after castration, and although the testis is not strictly an accessory tissue, the results are contradictory to my results. The quantitative RT-PCR assay which I developed is an improvement over their assay, as the signal is robust, and the primers for the cAR, having a low melting point, do not suffer from the cross reaction seen in the northern blots. In addition, because the primers are several kilobases from the ends of the AR mRNA, the assay is relatively insensitive to partial exonuclease digestion. I therefore believe my results to be more reliable than those reported by Quarmby et al. (1990). It is also possible that the mRNA is regulated differently in canary than rat, a possibility which I have not directly examined.

The increase in cAR mRNA I find in the liver with the antagonist flutamide agrees with published reports of regulation by circulating steroid levels. AR mRNA increases in vitro in the liver derived HepG2 cells grown without serum and this induction is reversed when 100 nM testosterone is added back (Shan et al., 1990). In at least this one tissue the regulation seen between canary in vivo, and the human AR in vitro, agree in direction and magnitude of the response (about four fold in each). Since flutamide increases cAR mRNA, I expected a similar up-regulation in the fall, when the circulating levels of testosterone are low. It remains to be determined why such a change wasn't detected.

This tissue specific regulation of the AR is not surprising. Although such regulation has not been seen for the AR, similar regulation is seen for the ER in the rat (Shupnik et al., 1989). Ovariectomy reduced the levels of ER mRNA to about one third control levels in both the liver and pituitary, while uterine ER mRNA is increased four fold by ovx. While the specific tissues vary for the AR, a reduction is seen for the AR in the nearly regressed fall testis and in the kidney following flutamide administration, and the AR mRNA is increased in the male reproductive tissues in the rat and the canary liver.

Regulation of the cAR in the Brain

The only report of regulation of the AR in brain again comes from Quarmby, et al.

THE CANARY ANDROGEN RECEPTOR

(1990). Their northern blot shows a three fold induction of AR mRNA in castrated versus normal total brain RNA, which is abolished by a large injection of testosterone. This blot was much cleaner and agrees with my results. In HVC from ovariectomized canaries, I saw a reduction of the AR mRNA soon after androgen injection, which is abolished by one day. In the forebrain, I do not see a reduction in the message. The forebrain results may be suspect, however, since the variability is high, but when I normalize the results to an apparently unregulated canary gene, HAT 2 (see chapter three), a large reduction in the message is apparent (dns). In contrast, the normalization of the HVC data did not alter the results. The regulation I see in the canary brain is therefore very similar to that seen in the rat.

SUMMARY

In order to better understand how androgens act on the songbird brain, I have analyzed the androgen receptor in the canary. I have cloned eight of the nine exons of the gene, including the portions which, by homology, bind to DNA, bind androgen, and affect transcription. The sequence is very conserved over these structural domains, but shows some divergence from the only other sequences known, those for rodents and man. The AR mRNA can be localized by in situ hybridization in the canary to the song nuclei HVC, RA, and MAN. Like the ER, the AR mRNA is regulated by androgens in the periphery in a tissue specific manner. Down regulation of the AR mRNA by androgens, can also be clearly detected in the song control nucleus HVC using a sensitive semi-quantitative pcr assay. In addition, this is the first report of AR mRNA regulation by natural fluctuations of circulating androgens.

CHAPTER 3: ANDROGEN REGULATED GENE EXPRESSION IN CANARIES

The experiments of the previous chapter confirm that the canary androgen receptor mRNA is expressed in many of the nuclei of the canary brain controlling song. Since the androgen receptor is known to modulate the transcription of genes (Govindan, 1990; Rundlett et al., 1990; Marcelli et al., 1990), it is likely that androgen changes the structure and function of the canary brain by regulating transcription of responsive genes. Female canaries given androgen implants begin to show behavioral changes after one week, but the time course for testosterone's actions at the molecular level is poorly understood. Many androgen regulated genes in the periphery are regulated over the course of several days (C3: (Zhang and Parker, 1985); TRPM: (Leger et al., 1987; Leger et al., 1988); GUS: (Watson and Catterall, 1986; Palmer et al., 1983); ODC, ADH: (Watson and Paigen, 1990)), some as fast as 12 hours (MAK: (Snider et al., 1985)). In the songbird brain, the only report is of effects on general protein synthesis after 6 days androgen treatment (Konishi and Akutagawa, 1981). Androgen effects on rat brain protein synthesis have been reported in as few as six hours (Stanley and Fink, 1985; Stanley and Fink, 1986). I have measured the androgen regulation of a variety of genes in the canary brain at a variety of times in order to define what genes androgens regulate in the brain and when this regulation occurs.

Androgen regulated genes important in the acquisition and production of song are likely to be rare as a percentage of total brain RNAs, as are most regulatory molecules. In order to overcome this difficulty, I hypothesized that although the behavior displayed by the canary is a very complex example of learning and memory, the genes regulated by androgens in the song system are likely to include a number of the genes regulated in simpler processes. In simple systems, such as conditioning in the *aplysia* (Glanzman et al., 1990; Buonomano and Byrne, 1990) and even more complex ones, like *ltp* in the rat

brain (Chang and Greenough, 1984) learning and memory have been associated with changes in neuronal connectivity, and these changes are dependent on protein synthesis. The changes in connectivity include both a structural remodeling, and an alteration of the signal transduction abilities of the neuronal system. Various structural genes (such as actin and gap43) have been shown to be regulated in these simpler neural systems and I therefore considered them to be good candidates for androgen regulation in the song system. Another class of genes of special interest are the "immediate early" genes (such as *ngf* Ia and Ib, *myc*, and *fos*) which are induced by various stimuli such as hormones and electrophysiological activation of neurons (reviewed in (Sheng and Greenberg, 1990)), and these genes are also likely candidates for investigation. The immediate early genes *fos* and *jun* (which as a heterodimer make up the immediate early transcription factor AP-1), are induced in neural and peripheral organs by a wide variety of stimuli, are particularly good candidates for regulation in the remodeling of the canary brain. I have attempted to quantify changes in the mRNA levels of a variety of these genes following manipulation of androgen levels in the canary.

A: CLONING OF CANARY C-JUN

Unfortunately, many of the genes associated with changes in neuronal plasticity in mammals, especially rodents, have not been characterized in birds. Songbirds have diverged enough from mammals and even chickens so that DNA probes from other species do not necessarily cross-react satisfactorily with songbird RNA or DNA. Fortunately, others in the lab have cloned canary homologs of a variety of the genes described above, including *egr*, *c-* and *n-myc*, vimentin, and Gap 43. Also available for analysis of androgen regulation are a number of genes which show patterns of differential expression within the song circuit (George and Clayton, 1992) and elsewhere in the forebrain (Clayton et al., 1988). In addition, a few genes from other species cross-hybridize to canary RNA. Finally, to augment this stable of genes which are potential targets of androgen regulation, I decided to clone the canary homolog of *c-jun*, a

component of AP-1.

In order to isolate the canary homology of c-jun, I used the relatively close evolutionary relationship between chickens and canaries to identify cross hybridizing clones. I obtained a chicken cDNA clone (a kind gift of Dr. D. Foster) containing the entire coding sequence of the c-jun gene and used it as a probe to identify homologous bands on a canary Southern genomic blot (see methods). Using this probe, I screened 400 thousand independent clones from a library of male canary HVc cDNA. One independent clone was isolated which contained an insert of approximately 2.0 kb. This insert was subcloned into pBluescript and sequenced to confirm its identity as c-jun (see methods).

RESULTS

Homology of canary c-jun

The canary cDNA clone is homologous to a variety of jun genes. The clone contains 1958 bp, with 306 bp of five prime untranslated sequence, 942 bp of presumptive coding sequence, and a 683 bp three prime untranslated region (3' UTR) (see Fig 24). The 3' UTR contains at least three polyA addition sites, the last one of which is used in this clone. In addition, the end of the cDNA clone contains the sequence ATTTA, a sequence found in many mRNAs which are rapidly degraded (Shaw and Kamen, 1986). Translation of the 942 bp open reading frame yields a protein of 314 amino acids (aa), with a predicted molecular mass of approximately 34,500 daltons, exactly matching the chicken and close to the 37 kd mass of the 337 amino acid murine c-jun (Lamph et al., 1988). The predicted aa sequence of the canary c-jun clone is 96.2 percent identical to the chicken c-jun sequence (Nishimura and Vogt, 1988; Maki et al., 1987), and 100% identical in the DNA binding-leucine zipper region (see Figure 25)]. The 100% identity is maintained when the canary is compared to the murine and human c-jun homologs, but the N-terminal 213 aa of the molecule is only 69.5% identical (see Figure 25).

ANDROGEN REGULATED GENE EXPRESSION IN CANARIES

I examined homology of the c-jun cDNA to other genes in the canary, in order to assure that the signal when I measured c-jun regulation by androgen was not due to cross reaction with other genes. I hybridized the cDNA insert to a Southern blot of restricted canary genomic DNA. Figure 27 shows that with some digests I did get two bands. Fortunately, when washed at higher stringency (60°C, 0.5X SSC, their number is reduced to one in each case (dns). When canary forebrain RNA is examined, only one band is present after washes at 65°C, .1X SSC (see Figure 28).

Figure 25. c-jun cDNA. Sequence of the canary c-jun clone, with the encoded amino acids and surmised glycosylation sites, the potential rapid degradation site, and the poly A addition signal sites indicated.

Canary c-jun cDNA Sequence

Canary c-jun cDNA

TNG AGC GAA CGA GCG CGA CTG AGT GCG GCC GCC GGG ACG GTG GAG CGG GAA TAG CGC GGA GCC GGG CAG GGA AAA GTA CTT GTG GCG GCA GAG CGG	96
CGT CCC GGC ACC ACC GGC ACG CGG AGG AGG AGG GCG GCG AGG CGT CCC GCC AGG CCG GGC GGG CTC TCA GCG CGG CGG CAG CAG CGA TCC AGT	192
AAG GCT CCG CGT TTC TCC TTC CTC GGC TCC GCG TTC CCC TTC CCC GGG AGG GTC GGG GTG CCC GCG GGG AGC TGA GAG GCG GCC GGG CAG CTT CCC	288
Met Ser Ala Lys Met Glu Pro Thr Phe Tyr Glu Asp Ala Leu Ser Ala Gly Phe Ala Pro Pro Glu Ser Gly Gly Tyr	26
CTT CCC GGA CTG TGT TCT ATG AGT GCA AAG ATG GAG CCT ACT TTC TAC GAG GAT GCG CTG AGC GCC GGC TTC GCG CCG CCG GAG AGC GGC GGG TAC	384
Gly Tyr Asn Asn Ala Lys Val Leu Lys Gln Asn Met Thr Leu Asn Leu Ser Asp Pro Ser Ser Asn Leu Lys Pro His Leu Arg Asn Lys Asn Ala	58
GGA TAC AAT AAC GCC AAG GTG CTG AAG CAG AAC ATG ACG CTG AAC CTG TCC GAC CCC TCC AGC AAC CTG AAG CCG CAC CTG AGG AAC AAG AAT GCC	480
Asp Ile Leu Thr Ser Pro Asp Val Gly Leu Leu Lys Leu Ala Ser Pro Glu Leu Glu Arg Leu Ile Ile Gln Ser Ser Asn Gly Leu Ile Thr Thr	90
GAC ATC CTC ACC TCG CCC GAC GTG GGG CTC CTC AAA CTG GCC TCG CCC GAG CTG GAG CGG CTC ATC ATC CAG TCC AGC AAC GGG CTG ATC ACC ACC	576
Thr Pro Thr Pro Thr Gln Phe Leu Cys Pro Lys Asn Val Thr Asp Glu Gln Glu Gly Phe Ala Glu Gly Phe Val Arg Ala Leu Ala Glu Leu His	122
ACG CCG ACC CCG ACG CAG TTC CTG TCC CCC AAG AAT GTC ACC GAC GAG CAG GAG GGG TTC GCC GAG GGC TTC GTG AGA GCT TTG GCT GAG CTG CAC	672
Asn Gln Asn Thr Leu Pro Ser Val Thr Ser Ala Ala Gln Pro Val Thr Ser Gly Met Ala Pro Val Ser Ser Met Ala Gly Ser Thr Ser Phe Asn	154
AAC CAG AAC ACG CTG CCC AGC GTC ACC TCG GCC GCC CAA CCT GTC ACC AGC GGG ATG GCA CCT GTG TCC TCC ATG GCC GGC AGC ACC AGC TTC AAC	768
Thr Ser Leu His Ser Glu Pro Pro Val Tyr Ala Asn Leu Ser Asn Phe Asn Pro Asn Ala Leu Ser Ser Ala Pro Asn Tyr Asn Ala Asn Ser Met	186
ACT AGT TTG CAC AGC GAG CCC CCG GTG TAC GCC AAC CTC AGC AAC TTC AAC CCC AAC GCG CTC AGC TCC GCT CCC AAC TAC AAC GCC AAC AGC ATG	864
Gly Tyr Ala Pro Gln His His Ile Asn Pro Gln Met Pro Val Gln His Pro Arg Leu Gln Ala Leu Lys Glu Glu Pro Gln Thr Val Pro Glu Met	218
GGA TAC GCG CCC CAG CAT CAC ATA AAC CCC CAG ATG CCC GTG CAG CAT CCC CCG CTT CAG GCT CTG AAA GAG GAG CCG CAG ACT GTA CCT GAA ATG	960
Pro Gly Glu Thr Pro Pro Leu Ser Pro Ile Asp Met Glu Ser Gln Glu Arg Ile Lys Ala Glu Arg Lys Arg Met Arg Asn Arg Ile Ala Ala Ser	250
CCG GGG GAA ACT CCT CCC CTG TCC CCC ATT GAC ATG GAG TCA CAG GAG AGA ATC AAA GCC GAG AGA AAG CGC ATG AGG AAC AGA ATC GCG GCG TCC	1056
Lys Cys Arg Lys Arg Lys Leu Glu Arg Ile Ala Arg Leu Glu Glu Lys Val Lys Thr Leu Lys Ala Gln Asn Ser Glu Leu Ala Ser Thr Ala Asn	282
AAA TGC CGG AAA AGG AAG TTG GAA AGG ATT GCC CGG TTG GAA GAA AAA GTG AAA ACT TTG AAA GCC CAG AAC TCA GAG CTG GCA TCC ACT GCC AAC	1152
Met Leu Arg Glu Gln Val Ala Gln Leu Lys Gln Lys Val Met Asn His Val Asn Ser Gly Cys Gln Leu Met Leu Thr Gln Gln Leu Gln Thr Phe	314
ATG CTC AGA GAA CAG GTT GCA CAG CTT AAG CAG AAG GTC ATG AAC CAC GTC AAC AGC GGG TGC CAG CTC ATG CTC ACA CAG CAG TTG CAG ACG TTT	1248
TGA AGA GAC TCT TCA GCG AGA ACT GTG TGT TGT GGT ACA ACT AAA ACG GGA AAA ATC CAA AGT GGC AGA GGC ATA AAG CTA AAG GCA AAA GCT GAG	1344
AGG CTG AGT CCT GCC TGT GCT CCG CAA AGC GCA TGT GTG GAA AGA CTG GCA AAG CCT TCA GCT GGA GCC GGG AGT GCA GCG GCC AGC GCT GCT CCG	1440
GGA AGT GCT GTT CCT GCT CCG ATG AGA TGT CAG ATC TTC GTT TAA CAT TGA CCA AGA CCT GCA TGG ACC TAA CAT TCG ATG ATC ATT CAG TAT TAA	1536
AGG TTA AAC TGC AAT AGA AAC TGT AGA TTG CTT TAT GTA GTA TTC CTT AAG AAA AAA AAA AGT GGG AGG GAG GTT TGT GGG AGG CTG ATA AAC AAA	1632
AAA AAA AAA GAA CTG TTC TGC CTG CCT TCA AGT AAA TTG TGT ATG TAC ATA TCT TTT TTT ATT TTA TTT TAT GAA AGT TGA TTA ATG TCA ATA AAC	1728
TAC TTC ATG ACT TTG TAA GTT ATT TTT ATG TTG TTT ATT TGG GCA CTG CCC AGT ATT GTT TGT AAA TAA GAG GCT TTT TTT AGC ACT CTG AGT TTA	1824
CCA TTT GTA ATA AAG TAT AAT TTT TTA ATG TTT CTG TTT CTG GAA AAA ATT CTA GAA GGT TCT ATT ATA TTT AGG AAA AAT AAA ATA ATT AAA ATG	1920
TAT TTC CCC TCA AAA AAA AAA AAA AAA AAA AAA AA	



Glycosylation



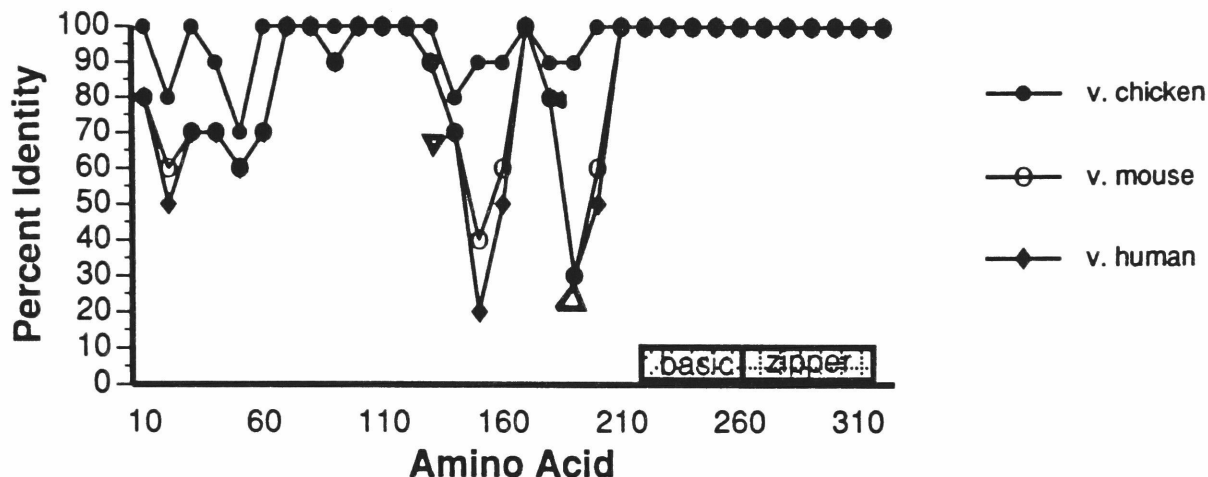
poly A



degradation

Figure 26. Homology of the canary c-jun with species' c-jun genes. Top: Graph of percent identity of amino acids (10 AA window) between canary and other receptors reported. Note the two regions of high homology. Bottom: Amino acid comparison of can(ary) and mur(ine) c-jun.

HOMOLOGY OF CANARY c-jun



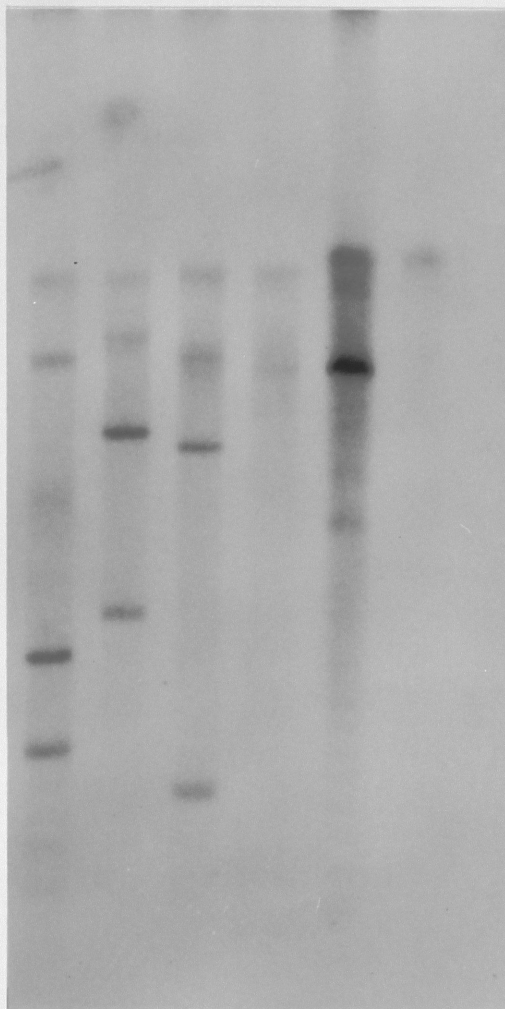
78.7% identity in 334 aa overlap

	10	20	30	40	50	60
CANJUN	MSAKMEPTFYEDALSAGFAPPESGGYGYNNAKVLKQNMTLNLSDPSSNLKPHLRNKNADI					
	..X:.....					
MURJUN	MTAKMETTFYDDALNASFLQSESGAYGYSNPKILKQSMTLNLADPVGSLKPHLRKNSDL					
	10	20	30	40	50	60
	70	80	90	100	110	120
CANJUN	LTSPDVGLLKLASPELERLIIQSSNGLITTTPTPTQFLCPKNVTDEQEGFAEGFVRALAE					
					
MURJUN	LTSPDVGLLKLASPELERLIIQSSNGHITTTPTPTQFLCPKNVTDEQEGFAEGFVRALAE					
	70	80	90	100	110	120
	130	140	150	160	170	
CANJUN	LHNQNTLPSVTSAAQP-----VTSGMAPVSSMAGSTSFNTSLHSEPPVYANLSNFPNPA					
v^.....			
MURJUN	LHSQNTLPSVTSAAQPVSGAGMVAPAVASVAGAGGGGGYSASLHSEPPVYANLSNFPNGA					
	130	140	150	160	170	180
	180	190	200	210	220	
CANJUN	LSS---APNYNANSMGY---A-----PQHHINPQMPVQHPRQLQALKEEPQTVPEMPG					
	:::		
MURJUN	LSSGGGAPSYGAAGLAFPSQPQQQQPPQPPHLPQQIPVQHPRQLQALKEEPQTVPEMPG					
	190	200	210	220	230	240
	230	240	250	260	270	280
CANJUN	ETPPLSPIDMESQERIKAEKRMNRNRIAASKCRKRKLERIALEEKVKTLKAQNSELAST					
					
MURJUN	ETPPLSPIDMESQERIKAEKRMNRNRIAASKCRKRKLERIALEEKVKTLKAQNSELAST					
	250	260	270	280	290	300
	290	300	310			
CANJUN	ANMLREQVAQLKQKVMNHVNSGCQLMLTQQLQTF					
					
MURJUN	ANMLREQVAQLKQKVMNHVNSGCQLMLTQQLQTF					

Figure 27. Canary Genomic Southern Blot. Scan of photograph of autoradiogram showing canary genomic DNA which was digested with the indicated restriction endonuclease and separated on a 0.8% agarose gel, then transferred onto a nylon membrane. The membrane was probed random primed canary c-jun. The membrane was washed up to 60°C, 1X SSC. Size of molecular weight markers indicated on right (in kb).

Canary Genomic DNA Hybridized with Canary c-jun

Endonuclease: PstI EcoRI HindIII BamHI KpnI Uncut



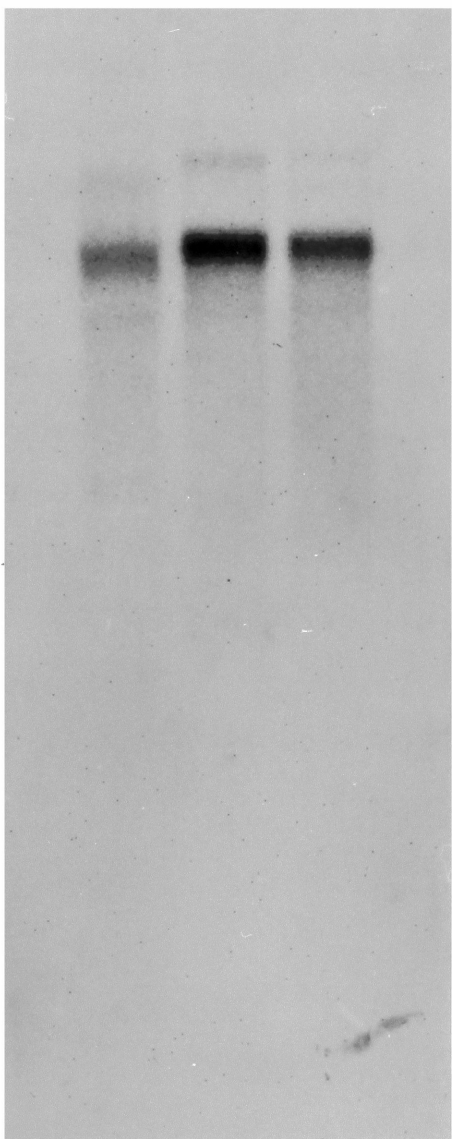
— 12
— 7
— 5
— 3
— 2
— 1.6

Figure 28. Northern Blot for c-jun. Scan of photograph of autoradiogram showing the detection of the c-jun mRNA. Three μg cytoplasmic poly A⁺ RNA or 30 μg total RNA was separated on a 1.2% formaldehyde/agarose gel and transferred to a nylon membrane. The membrane was probed with a riboprobe made from pcAR 2.2 (see Figure 11). The membrane was washed to 68°C, "11 mM sodium" (see methods).

Canary c-jun Northern Blot

Zebra Finch Fore. A+
Canary Forebrain A+
Canary Fore. total

28S →
18S →

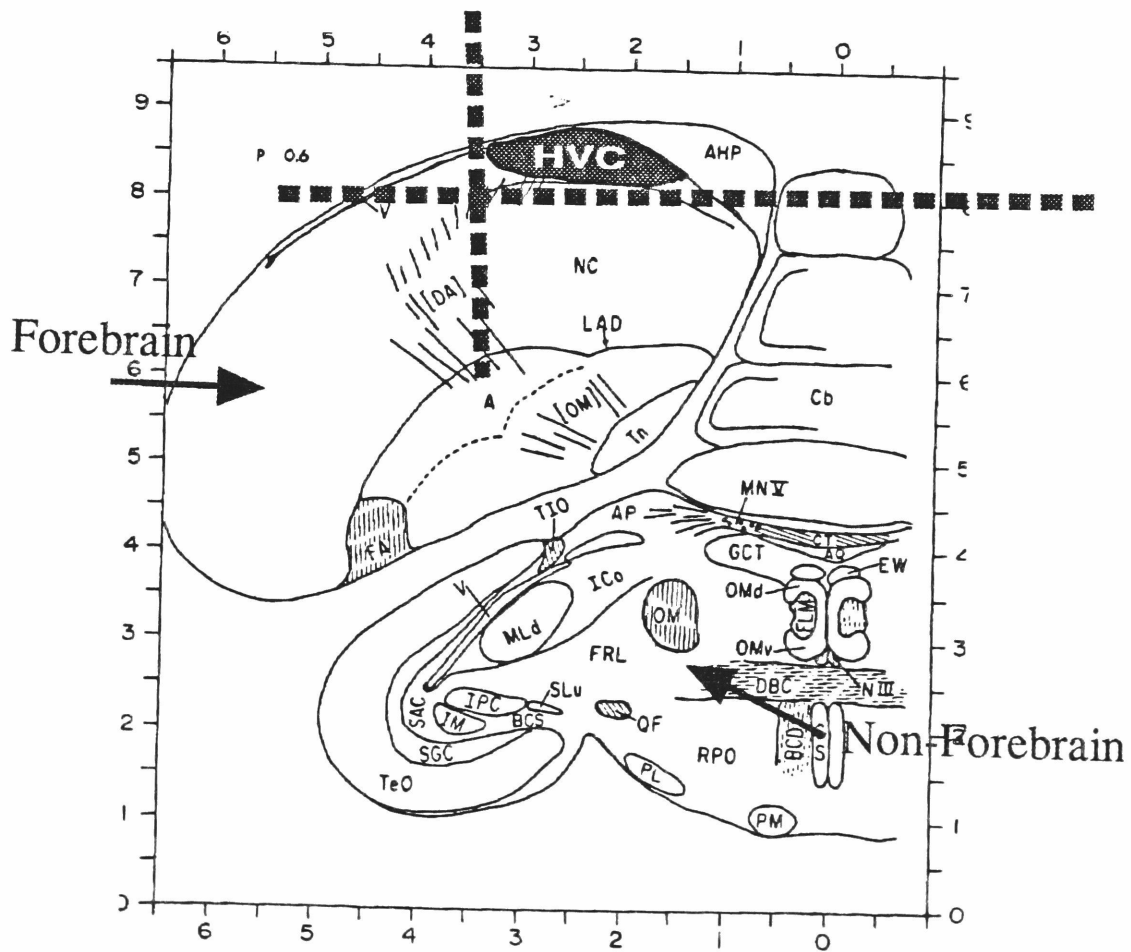
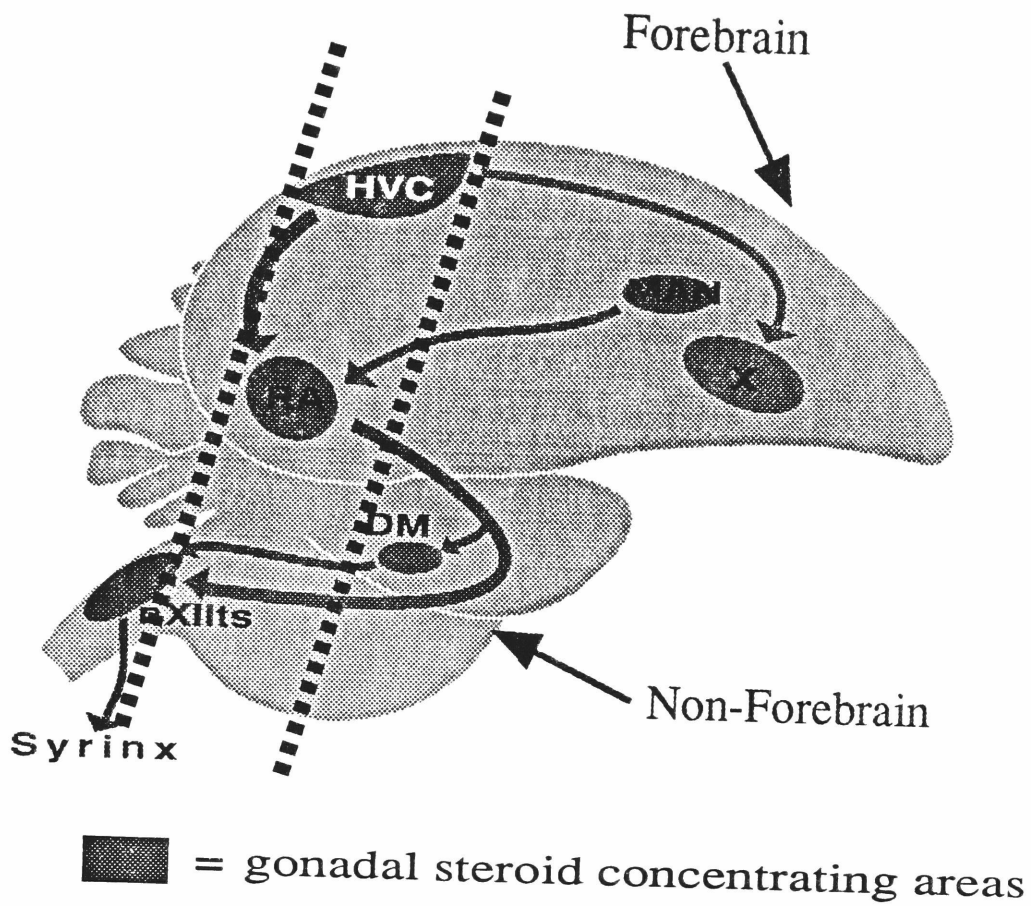


B: ANDROGEN INDUCTION OF GENES IN THE CANARY BRAIN

Since some of the song nuclei, including HVc, undergo changes in size and connectivity due to androgens, I have measured the levels of a variety of genes in androgen treated birds. In order to gain a preliminary understanding of these changes, I did a pilot set of experiments to optimize both the time-course of androgen administration and the assay conditions. Once the variables of androgen administration and tissue isolation were defined, I repeated the preparation of RNAs from canary HVc. Using this material, I carried out a comprehensive series of measurements of mRNA levels for the candidate genes (see above).

One hundred juvenile female canaries were ovariectomized and their circulating gonadal steroids were measured by radioimmunoassay (Wingfield and Farner, 1975) several months later to insure a steroid neutral background for the later treatment (see methods for surgical and hormone assays). After allowing one year for the birds to reach adulthood, they were then given either cholesterol (as a control) or testosterone. They were sacrificed from 30 minutes to seven days after the injection or implantation. Serum and liver were collected, in addition to various parts of the brain: "HVc" containing the song production nucleus HVc and surrounding tissue, "forebrain" (all of the telencephalon except "HVc", plus the anterior diencephalon), and "non-forebrain" (the remaining brain and a small amount of spinal cord) (see Figure 29). Conservatism is in order in interpreting the "HVc" data, as only 10 to 20 percent of the tissue is actually HVc proper (see discussion). RNA was prepared from these tissues and subsequently used as a probe of various message levels for a variety of genes.

Figure 29. Dissection of the Canary Brain. Diagram showing the dissection of "HVC", "rest of forebrain", and "non-forebrain", as described in the text and methods. Top: 3-D view of canary brain. Bottom: View of the indicated slice, and the dissection used for "HVC" (adapted from Stokes, et al., 1974).



RESULTS

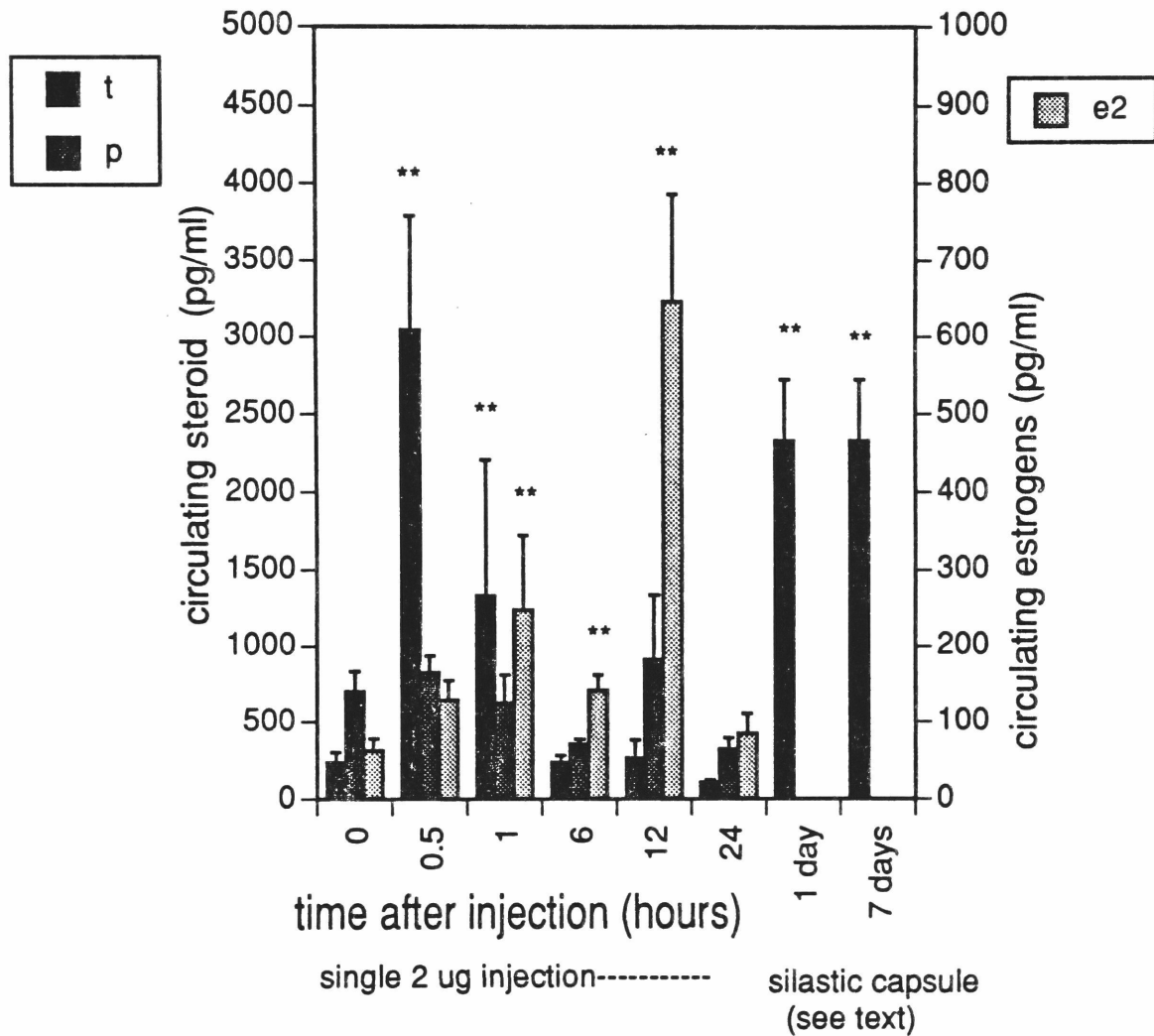
HORMONES

A RIA for estrogens was performed on the pretreatment serum (collected several months after the ovx) to exclude birds from the control groups in the experiment. High levels of serum estradiol were found in roughly 10% of the 200 ovariectomized females. These birds had a mean E2 level of 200 pg/ml, compared to less than 100 pg/ml for the whole group, which is near the limit of detection for this assay. If the assays indicated incomplete ovariectomy (or over-active adrenal) the birds were assigned to a long-surviving group where their low levels of circulating steroids would be masked by the high levels of exogenously added steroids.

The results of the post-sacrifice RIAs for estradiol, androgen and progesterone were analyzed by a one-way ANOVA to measure the effectiveness of the hormone treatments. The intramuscular injection of androgen profoundly affected serum testosterone levels ($F(7,91)=20.656$, $p<0.001$). Serum testosterone was elevated at thirty minutes ($3.0 \pm .75$ ng/ml, mean \pm se) and one hour ($1.3 \pm .8$ ng/ml, Fisher's Paired Leased Significant Difference (FPLSD) for either versus six hours cholesterol (237 ± 66 pg/ml), $p<0.001$), but the effect decayed by six hours to control levels ($6T=236 \pm 47$ pg/ml, FPLSD $6T$ v. $1T$ $p<0.001$, $6T$ v. $6C$ $F(1,73)=0.002$, $p=.987$; see Figure 30). Serum estradiol, which can be synthesized from free androgen through aromatization, follows a similar though delayed time course. Estradiol was significantly affected by the testosterone treatment, being elevated at one hour (250 ± 95 pg/ml), six hours (140 ± 20 pg/ml), and twelve hours (650 ± 140 pg/ml, all effects FPLSD $p<0.01$) and reduced to control levels ($6C=63 \pm 15$ pg/ml, FPLSD $24T$ vs $12T$ $p<0.001$, $24T$ vs $6C$ $p>0.5$) by twenty four hours following a single testosterone injection (all effects $p < 0.01$). Progesterone, which would indicate the steroid treatment's effects on the steroid synthesis ability of the adrenal or remnant gonad, showed no significant ($p < 0.1$) changes over the time course studied (see Figure 30).

Figure 30. Gonadal Steroid Levels after Testosterone Treatment. Graph of plasma levels of t(estosterone), p(rogestosterone), and e2 (estradiol) at the indicated times after injection of 2 μ g testosterone. Six hours cholesterol is represented as 0, the control. Mean \pm SE.

Plasma steroid levels after testosterone administration in gonadectomized female canaries



** $p < 0.01$ v. cholesterol (0)

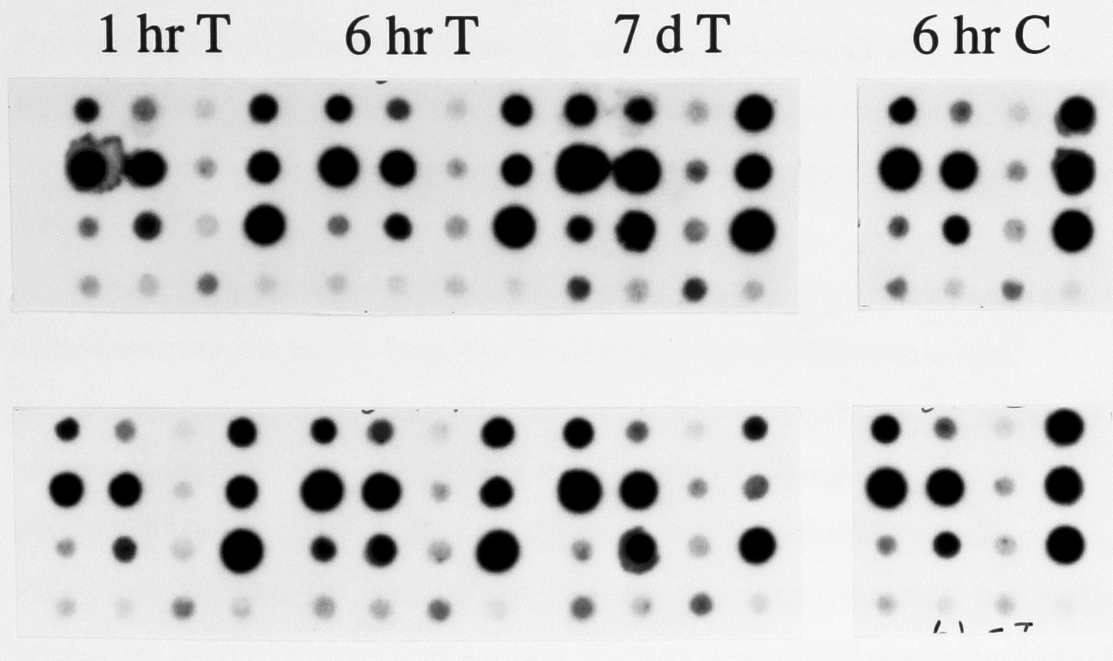
Preliminary Assay of Androgen Effects

Androgen effects on a variety of genes were first assessed in a preliminary experiment by semi-quantitative dot hybridization in order to assess which genes might be modulated by androgen and the appropriateness of the treatment time course. The canary homologues of several early-response transcription factors (c-jun, c-myc, n-myc, *egr*) were dotted onto duplicate membranes accompanied by several structural genes (canary gap-43, chicken actin, canary vimentin) and anonymous canary cDNAs (*cfos1*, *cfos4*, E1, E6, pCF17) in addition to vector controls. These filters were hybridized with ³²P labeled cDNA reverse transcribed to a high specific activity from RNAs isolated from song regions of one hour, six hour, and seven day post androgen injected birds (the seven day birds' testosterone level declined to control level by 24 hours, see discussion) as well as six hour post injection control birds.

The results of these preliminary experiments show no induction of any gene greater than three-fold (by visual inspection, see Figure 31). This led me to hypothesize several explanations for the apparent lack of positive results. First, subtle changes may be occurring in these genes which would require more rigorous quantification. The structural genes appear to show a slight induction at one hour and a subsequent reduction. The transcription factors may also exhibit some slight induction, but it is difficult to assess based on the autoradiographic signals from this early experiment. Second, it is also possible that none of the genes I assayed is in fact regulated by the androgens. Finally, it is possible that the treatment regimen was insufficient. It was clear from the post sacrifice RIA that the seven day survival following testosterone injection gave only a transient elevation of androgen levels, which may have been insufficient to elevate responsive gene levels if the induction required the continuous presence of steroid for several days. Using the information from this preliminary experiment, I designed a more rigorous assay of gene regulation in the canary brain.

Figure 31. Preliminary Assay for Androgen Regulation of Genes. DNAs arranged in a 4 X 4 matrix as indicated (bottom). Filters hybridized with labeled cDNA reverse transcribed from HVcs of ovariectomized canaries treated for the indicated times with T(estosterone) or C(holesterol).

Preliminary Assay of Gene Induction by Testosterone



A1	E1	pGEm3	c-jun
A4	E4	pBS	Gap-43
pCF-17	egr	lambda	Actin
n-myc	c-myc	pCF-37	pGem3

Quantitative Assay of Androgen Effects

Using the RIA data from above, I repeated the androgen treatment of ovariectomized female canaries using silastic implants containing androgen for survivals longer than six hours. Silastic capsules, when soaked prior to implantation, deliver their steroid contents at a constant level from within one hour post implantation for at least thirty days. Testosterone levels following implantation of the silastic capsules have been well characterized to be 2.33 ± 0.39 ng/ml within hours and persisting for up to one month (Nottebohm, 1980b) (see Figure 30). Seventy birds were divided into groups based on their pre-manipulation serum estradiol levels (as above) and given a 2 μ g injection of testosterone for forty five minute and six hour survival, or a silastic implant for one or seven days.

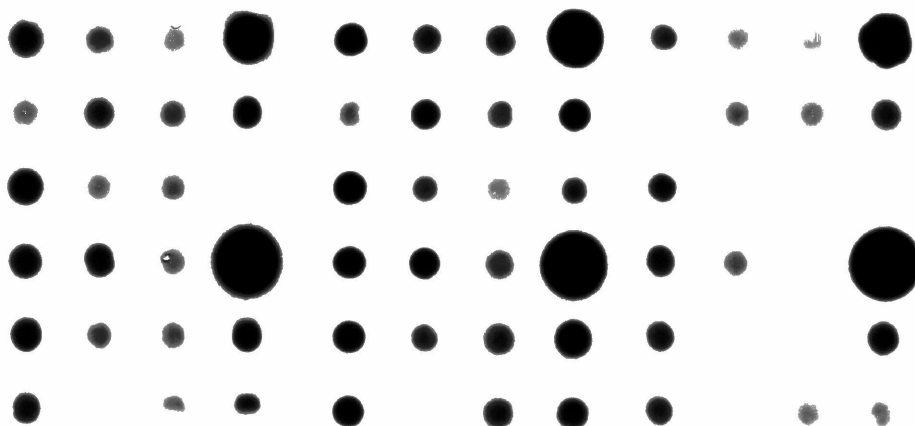
Using a combination of RNA from both the first (for shorter times only) and second hormone treatments, I repeated the cDNA dot hybridizations in a more quantitative manner. RNA from "HVC" of birds treated with androgen or cholesterol covering from seven days of cholesterol to seven days of androgen was reverse transcribed to a high specific activity and hybridized to dots of cDNA in triplicate for a variety canary genes. This group of genes was much more comprehensive than in the preliminary experiment, including a variety of canary genes which may be important for signal transduction in the brain, a larger variety of structural genes, and gonadal steroid receptors. The dots were washed stringently, exposed to film to detect obvious procedural artifact, and then individually excised from the filter and counted in a liquid scintillation counter (see methods). The signal intensity varied for the different classes of genes, but as we saw in the previous experiment there was no regulation apparent by visual comparison of the treatments (see Figure 32).

Figure 32. Example of Results of Quantitative cDNA:dot Assay for Androgen Regulation of Genes. DNAs arranged in a 6 X 4 matrix as indicated (bottom center). Filters hybridized with labeled cDNA reverse transcribed from HVcs of ovariectomized canaries treated for the indicated times (d = days, h = hours, () = minutes) with T(estosterone) or C(holesterol) (top row).

7d C

6h C

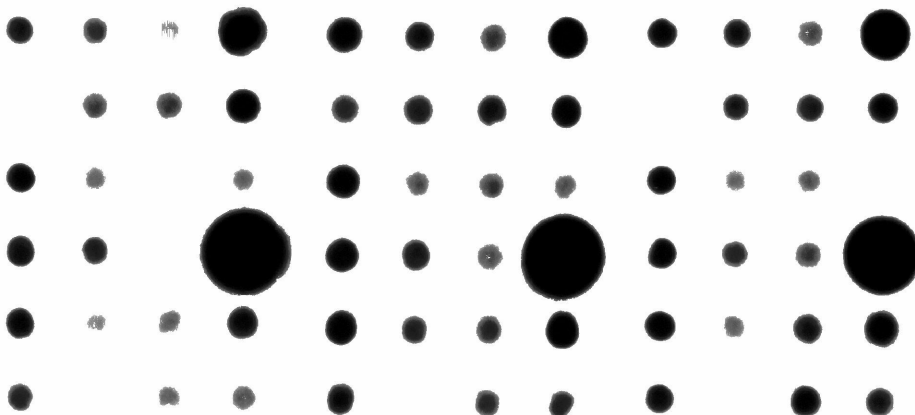
45 C



24h T

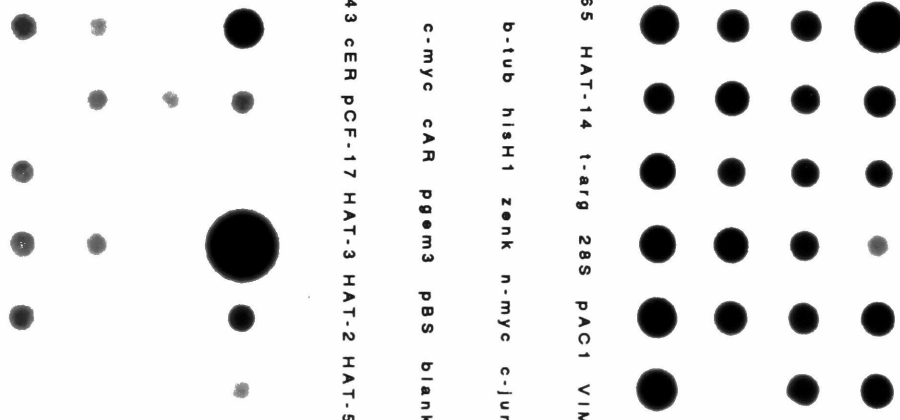
6h T

45 T



7d T

60 T



PCF-65 HAT-14 t-arg 28S PAC1 VIM
 a-tub b-tub h1sh1 zenk n-myc c-jun
 c-fos c-myc cAR pgem3 PBS blank
 GAP-43 CER PCF-17 HAT-3 HAT-2 HAT-5

ANDROGEN REGULATED GENE EXPRESSION IN CANARIES

These data represent the dpm from three separate hybridizations. Each probe for the time points used in these hybridizations was from a single pooled RNA sample of 10 to 40 "HVcs" from canaries treated with testosterone as described above. This pooling was necessary since a single bird yields a tissue sample of approximately 20 mg, and hence less than 20 μ g of total RNA. Such small amounts of RNA are difficult to isolate and more sensitive to degradation than larger samples, and so the samples were pooled. Such a pooling of the samples, however, reduces number of samples to one for each time point. Any biological variation in how different birds respond to the testosterone treatment is then averaged out of the results.

The data from each hybridization were first normalized to the adjusted mean cpm of genes thought not to be regulated by androgens, and then analyzed for significant changes after testosterone exposure. The normalization of the dpm for each gene served as a control for procedural differences in preparation of the tissues, efficiency of reverse transcription, and hybridization kinetics. Plasmids encoding α and β tubulin, which have been reported not to vary in other systems due to hormone manipulation (for estrogen (Loose-Mitchell et al., 1988; Ferreira and Caceres, 1991), were analyzed separately for significant changes in absolute cpm bound. There was no significant ($p < 0.1$) variation in any of the samples. Actin was not used as a control since there are reports of a small induction of transcription of this gene by androgens (Watson and Paigen, 1990). A one-way ANOVA was used to compare these data using an n of 3, which represents the assay variability. As stated above, any true biological variability is not adequately assessed in these experiments due to the pooling of RNA samples. In order for testosterone to be considered to have had a significant effect, the samples must differ from both the seven day cholesterol (which represents basal levels for the ovx canaries and any effects of the cholesterol alone) and the 45 minute cholesterol (which may also include effects of the stress" of the treatment paradigm) samples in the ANOVA at the $p < 0.05$ level. The six hour cholesterol time point was excluded from the comparison and all other analysis since the dpm values for the three hybridizations had very high variance. After the

ANDROGEN REGULATED GENE EXPRESSION IN CANARIES

statistical analysis, the data were expressed as a percent of the values for seven days cholesterol, a relatively easy to interpret graphical format (see Figures 33-35).

Examination of the RNA samples derived from the "HVc" dissection shows a potentially interesting array of changes in mRNA levels (see Figures 34 and 35). Several of the immediate early transcription factors appear to be induced by androgens (see Figure 35). The mRNA levels for c-myc and n-myc are significantly (FPLSD $p < 0.01$) increased after one hour androgen, relative to both seven days and forty five minutes cholesterol. At forty five minutes and one hour, c-jun mRNA levels are significantly above the controls (FPLSD $p < 0.05$ for 45 min T and $p < 0.01$ for one hour T). No significant changes are seen for either zenk ($F(9,18)=1.16$ $p=0.37$) or c-fos ($F(9,21)=0.73$ $p=0.68$) in HVc. Plotting these data as percent induction versus time shows the quick induction of these genes and their subsequent decline (see Figure 36).

None of the structural genes show any regulation in this tissue sample (see Figure 34). Histone H1 appears to be down-regulated in all groups relative to seven days cholesterol, but none of the differences achieve a statistical significance by Fisher's LSD method of $p < 0.05$ (see Figure 34). In addition, vimentin was up 70% at sixty minutes testosterone, but there was significant variation in the values for this gene due to the extremely low hybridization signal (see Figure 32).

None of the canary genes cloned for their differential anatomical expression, some of which were subsequently shown likely to be parts of different signal transduction pathways (HAT 2,3,5) (George and Clayton, 1992), nor gap-43 showed any regulation by androgens in HVc (see Figure 35). It is interesting to note, however, that the mRNA levels of both HAT2 and HAT5 were increased at both early T points (FPLSD $p < 0.05$) compared to the 7 day C control, but not the 45 min C control, perhaps indicating a "stress" response. Neither the androgen receptor (cAR2.4) nor the estrogen receptor appeared to be affected by the testosterone treatment.

Figure 33. Regulation of Various Genes in "Rest of Forebrain". Graph of results of quantitative cDNA:dot assays for the 45 minutes testosterone treated "rest of forebrain" as a percentage of 45 minute cholesterol treated "rest of forebrain" from ovariectomized canaries. Mean +/- SE.

Forebrain: mRNA levels after 45 minutes testosterone as a percent of 45 minutes cholesterol

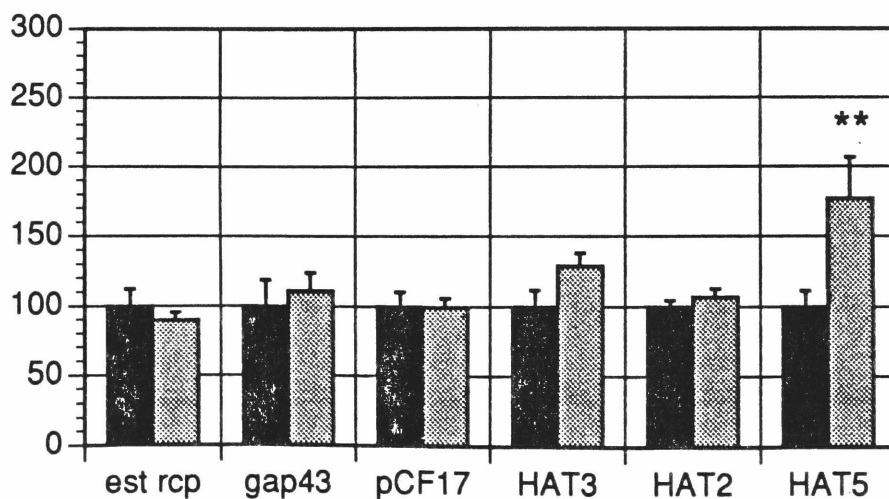
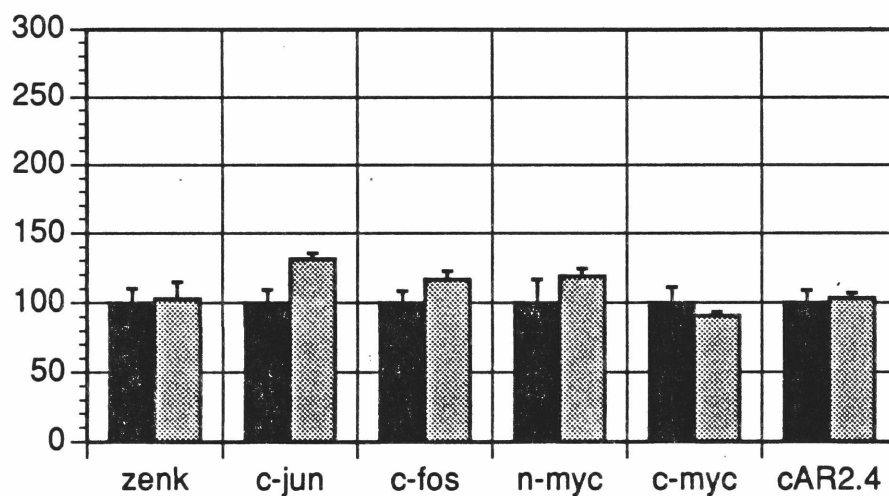
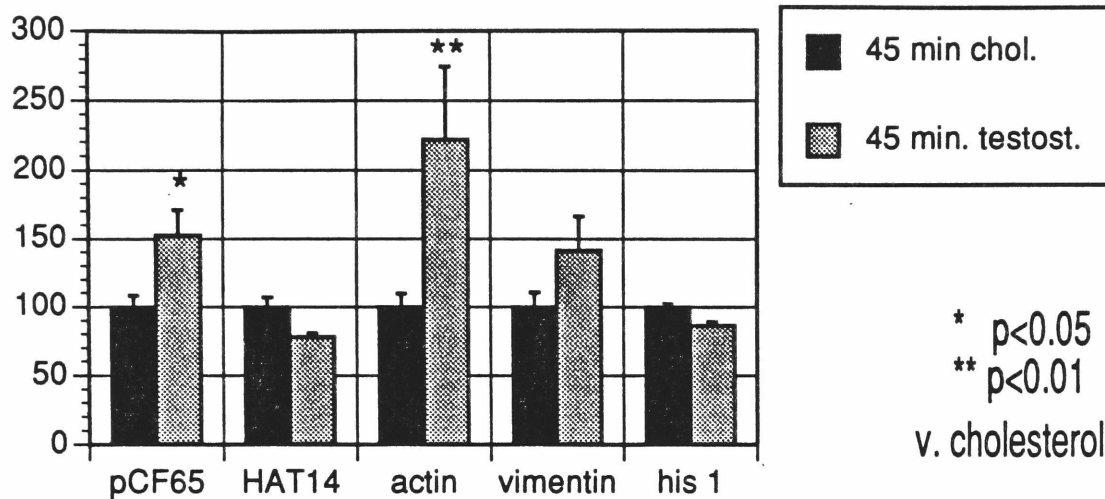
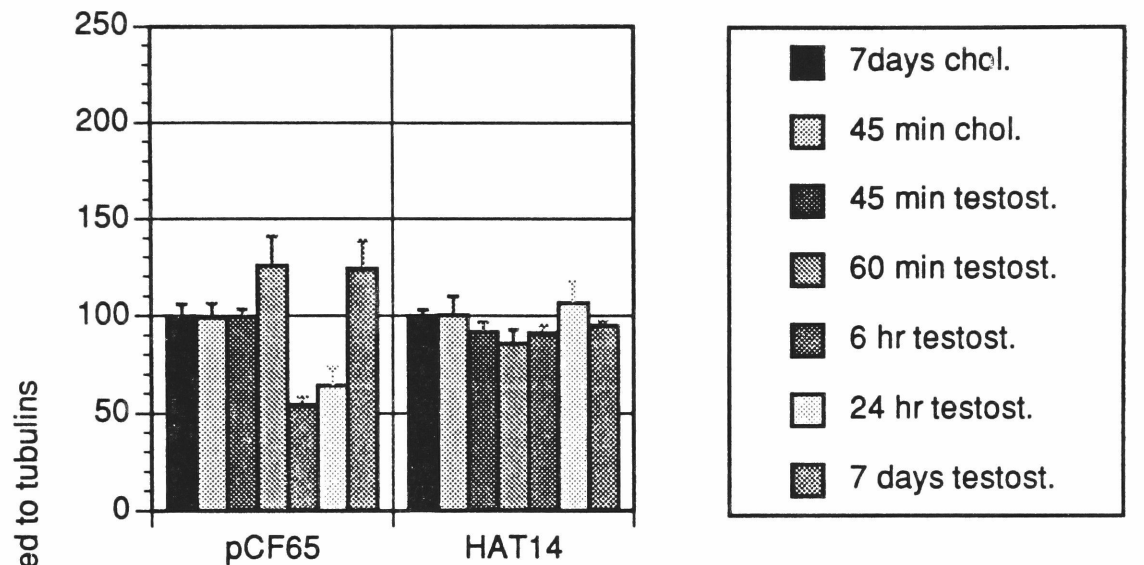


Figure 34. Regulation of Various Structural Genes in "HVc". Graph of results of quantitative cDNA:dot assays for various lengths of testosterone treatment as a percentage of 7 day cholesterol treated "HVc" from ovariectomized canaries. Mean +/- SE.

Structural Genes mRNA Levels in Canary HVc



* $p < 0.05$
 ** $p < 0.01$

v. cholesterol

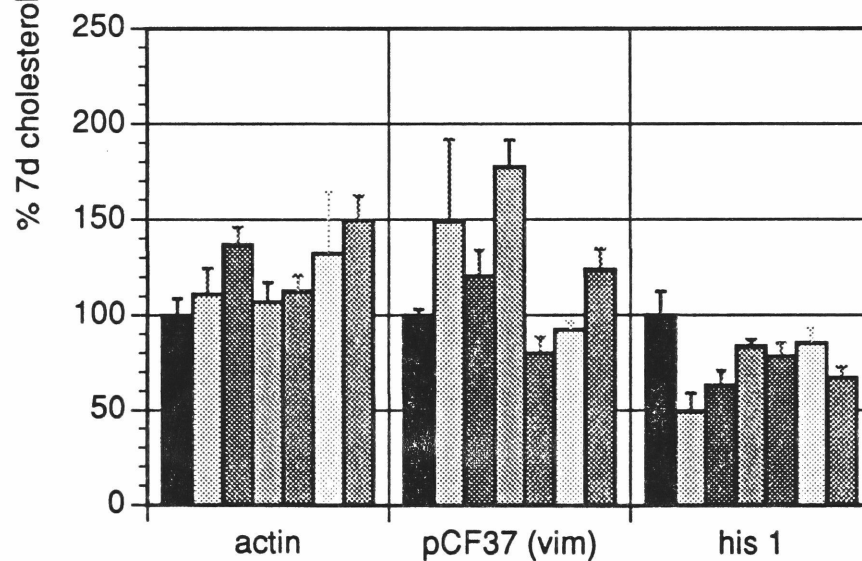


Figure 35. Regulation of Various Signal Transduction and Anonymous Genes in "HVC". Graph of results of quantitative cDNA:dot assays for various lengths of testosterone treatment as a percentage of 7 day cholesterol treated "HVC" from ovariectomized canaries. Mean \pm SE.

Signal Transduction Gene mRNA Levels in Canary HVc

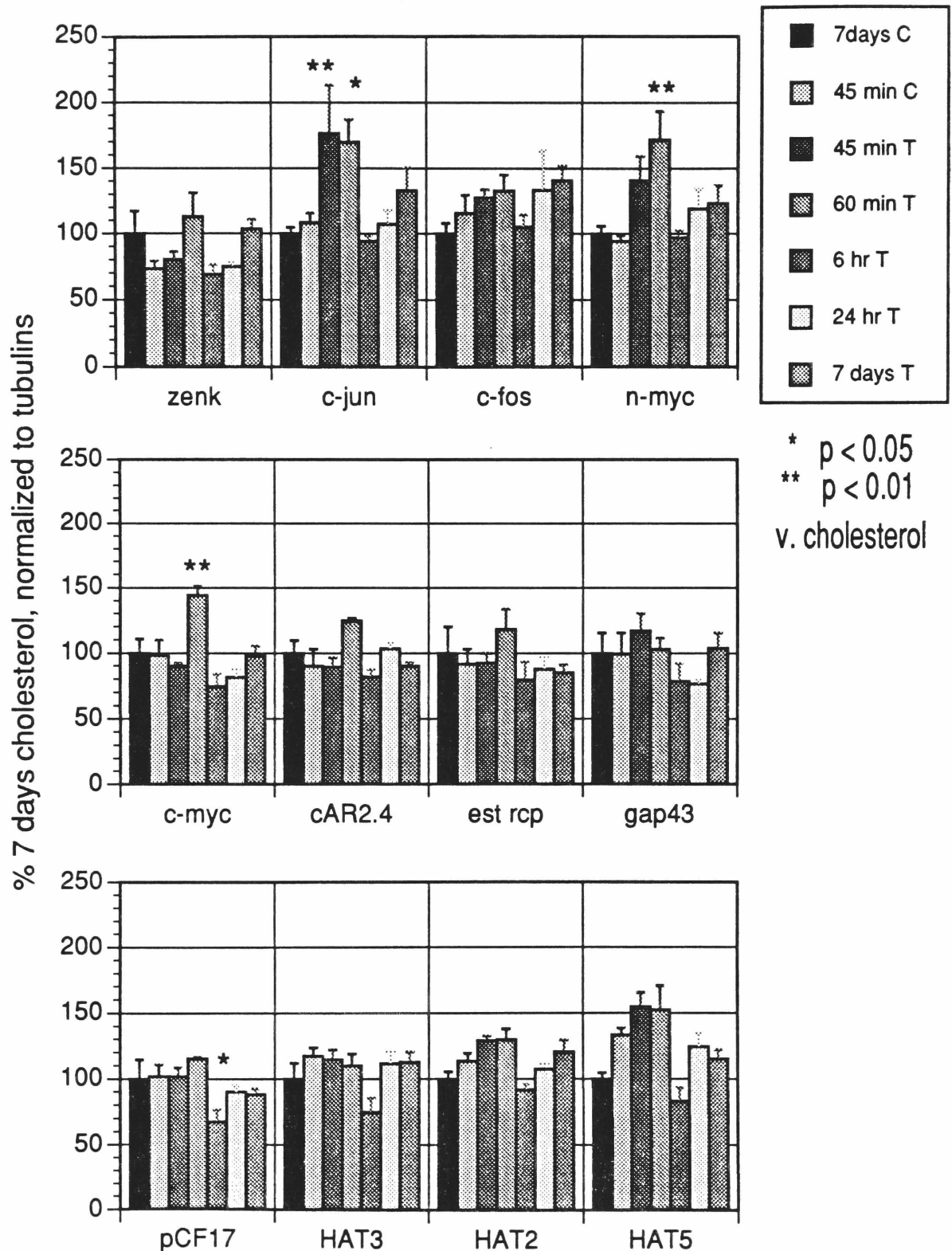
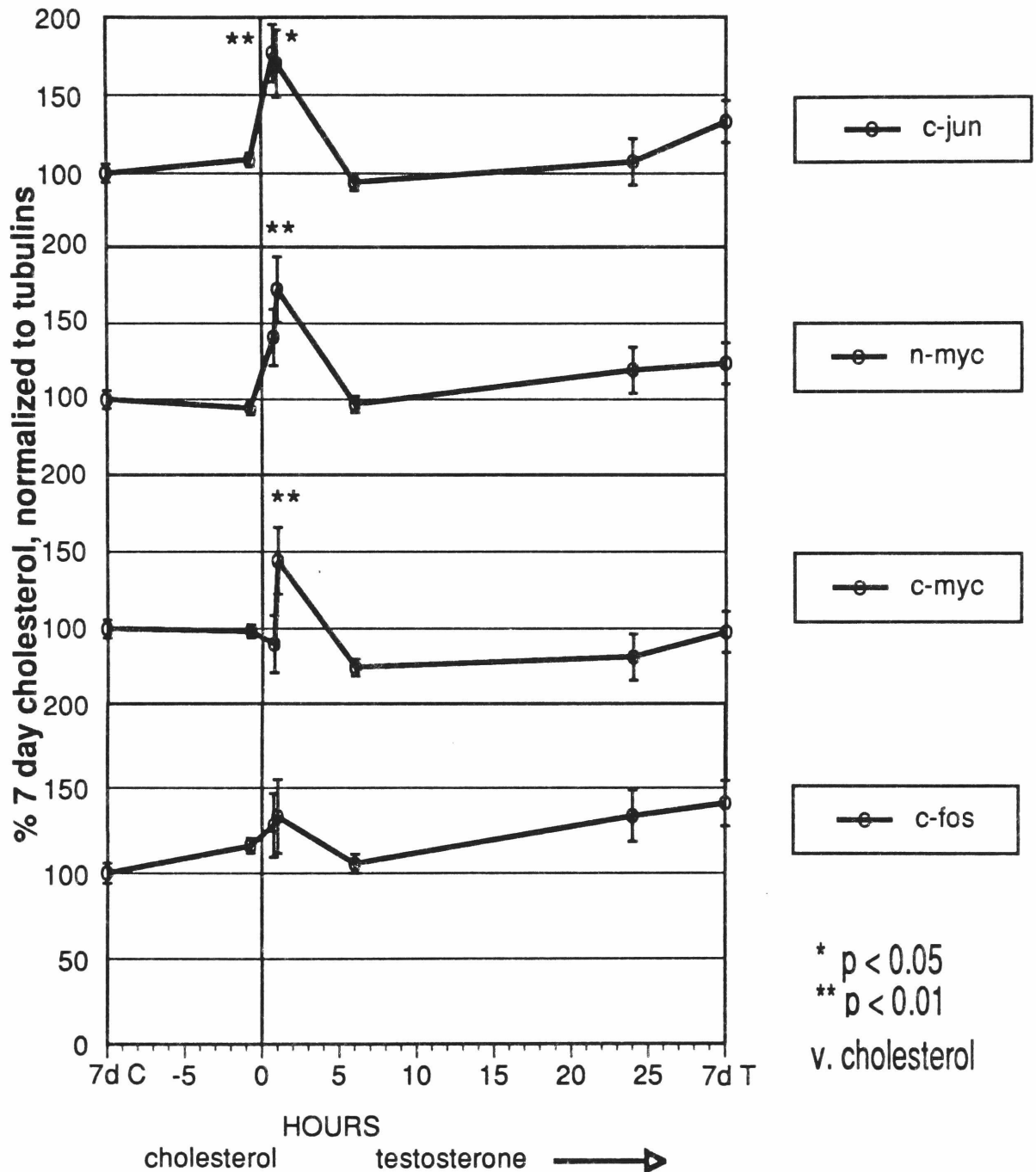


Figure 36. Regulation of "bZIP" Genes . Graph of results of quantitative cDNA:dot assays for various lengths of testosterone treatment as a percentage of 7 day cholesterol treated "HVc" from ovariectomized canaries. Presented as mean \pm SE with time on a linear scale (except 7 days). Notice the rapid induction and subsequent decline in the mRNA levels.

bZip mRNAs in Canary HVC after Testosterone



ANDROGEN REGULATED GENE EXPRESSION IN CANARIES

The HVc sample of canary forebrain should contain a high percentage of androgen receptor containing cells relative to the "rest of forebrain sample" based on my analysis of cAR mRNA expression in the brain (chapter 2). A comparison of the "rest of forebrain" versus the "HVc" sample should therefore serve as a further control for non specific effects or effects not mediated by the androgen receptor. A repeated measures ANOVA of all the data for androgen effects shows that there are no significant effects of testosterone on the mRNA levels of the three induced immediate early genes, c-myc, n-myc, or c-jun, or any of the other transcription factors examined in the forty five minute cholesterol versus testosterone (see Figure 33). The regulation of immediate early genes in the "HVc" sample, suggests that they may be direct targets of androgen regulation via the androgen receptor.

Actin and pCF-65 do, however, appear to be induced significantly in the "rest of forebrain" comparison, as does the anonymous canary clone HAT-5 (see Figure 33). The induction of actin and pCF-65 in the "rest of forebrain" sample is difficult to explain by nuclear receptor activation, but could be either 1.) evidence of a generalized non-receptor-mediated responsiveness or 2.) evidence for a very large response in one of the specific areas in the "rest of forebrain" sample which do have androgen receptors, such as IMAN or RA (see chap 1,A, and discussion). In order to define these effects more fully, in situ hybridization of each of these genes in brains treated for 45 minutes with cholesterol or testosterone is in progress.

C: DISCUSSION**Canary c-jun**

In order to assay this likely candidate gene for androgen regulation in the canary brain, I have cloned the canary c-jun. The canary homolog of c-jun is highly conserved between mammals, chickens and canaries. The protein domains encoded by the canary c-jun clone which bind to DNA (the basic region) and facilitate dimerization (the leucine zipper) are 100% identical to their homologs in these other species. Two regions of the amino terminal portion of the molecule are less well conserved. These regions are separated by another well conserved region, termed "homology region 1" by Ryder, et al., (1989).

Canary c-jun is expressed in the forebrain by both northern blot and in situ hybridization (dns). The entire coding sequence is on a single exon (Nishimura and Vogt, 1988), and PstI, EcoRI, and HindIII have no recognition sequence in the canary coding sequence, yet there are two bands in the genomic Southern blot for each of these enzymes after moderate stringency washes. For these reasons, the gene appears to either be represented more than once in the genome, or the transcript detects one of the related genes, jun-b or jun-d. When the filter was washed more stringently (60°C, 0.5X SSC) the second bands disappear, suggesting that they are due to cross hybridization, a likely possibility since the homology between c-jun, jun-b and jun-d over the two conserved regions (above) is about 75% (Ryder et al., 1989). It is clear from the northern blot (see Figure 28), however, that either the other gene isn't expressed, or isn't detected under those conditions.

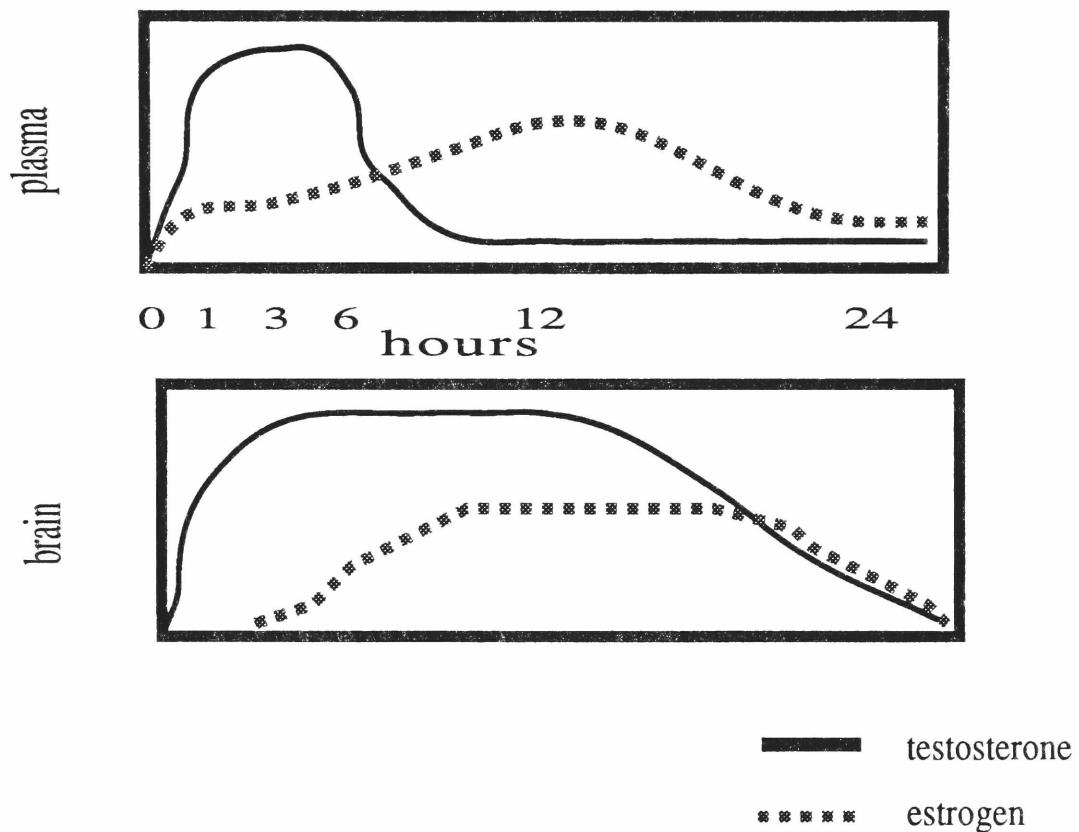
Regulation of Genes by Androgen in the Canary Brain*Hormone Treatment*

To better understand how androgen affects the neural circuit which controls song in the canary, I isolated tissues from castrated female canaries which had been treated with testosterone and measured the levels of a variety of genes which play structural and

functional roles in other systems. In a preliminary experiment, I castrated female canaries to create a steroid neutral background in the animal so the later androgen treatment would be relatively unaffected by endogenous signals. The ovariectomies were quite successful. I had a very high survival rate from the difficult surgery, and about 90% of the birds had very low levels of steroid hormones one year later. In this experiment, I injected the birds with two micrograms of testosterone in oil and sacrificed the birds some time later. I measured the effect of the injection on plasma steroid levels and found that the treatment was quite effective at one hour, but that the serum testosterone declined after that time. Estrogen levels, however, remained elevated up to twelve hours after the injection, and since the principle source of estrogens in ovariectomized songbirds is aromatization, which only takes place in the brain (Schlinger and Arnold, 1991), the intracerebral levels of testosterone must have been significantly elevated until this time, due to accumulation in the lipid-rich brain (see Figure 37). The six hour time point from these birds therefore probably have high intracranial levels of androgens. When I repeated the androgen stimulation of ovariectomized canaries the following spring, I substituted silastic implants for the injections for the one and seven day time points, thereby assuring a steady and high level of testosterone in circulation for the duration of the treatment.

Figure 37. Steroid Metabolism in the Canary. Top: Idealized results of Radioimmunoassays (Figure 30) for gonadal steroids in the plasma after testosterone injection. Bottom: Hypothesis of the gonadal steroid levels in the brain after the testosterone injections, based on the brain being the sole site of aromatization of testosterone into estrogen in the ovariectomized canary (see text).

CLEARANCE OF STEROIDS FROM PLASMA AND BRAIN



In Gonadectomized Female Songbirds,
All Aromatase Activity is in Brain

ANDROGEN REGULATED GENE EXPRESSION IN CANARIES

Anatomical Location of Tissue Samples

HVc is a small area of the canary forebrain whether defined by cresyl violet staining or biochemical markers. In female canaries, it is much smaller than in males (see introduction), and hence, my dissection of the "HVc" tissue sample necessarily included surrounding telencephalon (see Figure 29). HVc is approximately 1 mm³, and the "HVc" pairs I collected weighed an average of 20-30 mg. Even given the evidence (Gahr, 1990b) that HVc defined by estrogen receptor containing cells is larger, the true percentage of HVc in the "HVc" samples was between 10% and 20%, and between 30% to 40% of cells in HVc proper are androgen responsive. So in the "HVc" sample, it is possible that five percent of the cells are androgen responsive. The "rest of forebrain" sample contains much less androgen receptor mRNA, and therefore probably fewer androgen responsive cells (see chapter 2). This sample is probably much more consistent relative to the "HVc" since small differences in HVc dissection would have a minor effect on the content of androgen responsive cells in the larger sample. It is impossible to accurately estimate the percentage of such cells in this sample, but it is likely less than 0.1%. The "HVc" sample therefore represents a probable 10 to 50 fold enrichment for androgen responsiveness. It is for this reason that the focus of my analysis of androgen regulation of gene expression was on this sample.

Androgen Regulation of Gene Expression in the Canary Song System

Testosterone induces several genes in the tissue sample containing the song nucleus HVc, including the immediate early transcription factor component c-jun. It also induces c-myc and n-myc, genes which have been called "basic zipper" proteins, since their basic domains are sequence specific DNA binding proteins and when they dimerize at their "zipper", they are thought to regulate transcription (Blackwell et al., 1990). The myc family of genes are developmentally regulated in a variety of tissues (Zimmerman et al., 1986) and are expressed during both the normal development of the nervous system (Ruppert et al., 1986) and in neural tumors (Breakefield and Stern, 1986). The regulation of c-jun, c-myc and n-myc are specific to "HVc", as the surrounding forebrain does not

ANDROGEN REGULATED GENE EXPRESSION IN CANARIES

show this regulation of the genes. The mRNA levels of these genes is increased significantly within 60 minutes of testosterone treatment, and decline by six hours to control levels. The other half of the AP-1 heterodimer, c-fos, an immediate early transcription factor, does not show this regulation by androgen in the canary brain (but because I was unable to clone canary c-fos, I was forced to assay the chicken homolog, which may have distorted the results).

A variety of other genes, including a number of structural genes which are likely necessary for neuronal rearrangement, are not detectably increased in the "HVc" sample, even after seven days of androgen treatment, when significant changes in behavior are evident. This implies that the changes in these genes are likely to be small ones, which may nonetheless be significant over extended periods. It is also possible that these genes do not change and that the choice of genes which I thought likely regulated during the neuronal remodeling was incorrect.

Perhaps the most intriguing result is the induction of Actin, pCF-65, and HAT-5 in the "rest of forebrain" sample. Since this tissue has relatively few androgen receptor containing cells, it is possible that the androgen effects are indirect. Area X, the largest song nucleus in the brain, grows several fold in response to androgens, but contains no cAR. It is possible that the dramatic regulation of these genes in the "rest of forebrain" is related to the response of Area X. Perhaps some secondary diffusible factors or increased neural activity are responsible for the both induction of these genes and the regulation of Area X. The in situ hybridization of testosterone stimulated canary brains may begin to provide an answer to this question.

Finally, there are two caveats pertaining to these results. Since testosterone can be aromatized, I cannot rule out the possibility that these effects are due to estrogens, though the behavioral effects are strictly androgenic (see introduction). Second, a close examination of the autoradiograms from the hybridization shows that the signal for the "vector controls" is consistently higher than the signals for several of the very low abundance cDNAs assayed, including the two steroid receptors, t-argenine, histone H1,

and alpha tubulin (see Figure 32). In addition, the signal for n-myc in the un-induced condition is approximately the same as its vector. The vector samples must either be contaminated or the multi-linker reacts with some cDNA, as there is no other way to explain how the identical plasmids with a cDNA insert would be consistently lower than one without. For this reason, I did not subtract the vector dpm values from the experimentals, but instead subtracted only the filter background. Since each dot assayed for the different treatment contains exactly the same amount of DNA (they were made from the same dilution of stock at the same time) and I am reporting only relative changes in mRNA levels, the vector should not bias the results, though it is unsightly. Finally, it must be emphasized that the majority of genes, including the induced genes with the exception of n-myc, are unaffected, as their signals well exceeded that of their vectors.

D: SUMMARY

In order to further understand the mechanism by which androgens affect the song control system in canaries and other songbirds, I examined genes considered likely to play some role in the neuronal reorganization necessary for learning and memory. I cloned the canary homolog of the c-jun proto-oncogene, and confirmed its identity based on complete conservation of the important functional domains. I prepared RNA from steroid responsive parts of the canary brain which are known to regulate song, and used this to investigate the regulation of c-jun and a large number of other candidate genes. Using a simple dot hybridization assay, I could detect a small but rapid induction by androgen of c-jun and two other proto-oncogenes (c-myc and n-myc) in RNA from tissue containing HVC and surrounding telencephalon.

CHAPTER 4: ANDROGEN REGULATED GENE EXPRESSION IN S115 CELLS

The effects of androgens on the songbird brain and its behavior are profound, but the direct effects seen on gene expression appear to be small (see chapter 3). It is possible that the magnitude is small because in the previous experiments the fraction of the "HVC" sample which actually contains androgen responsive cells was also small (see discussion of chapter 3). Androgens may elicit a much more dramatic change in a purified population of androgen responsive cells. Another possibility is that androgens act to influence the transcriptional regulation of many genes *indirectly*. For example, androgens could enhance or repress the induction of genes by other regulatory pathways important in neural function, such as the dominant protein kinase signal transduction pathways. Evidence for this possibility in the song circuit was recently suggested by the finding that seizure activity induces an immediate early gene virtually everywhere in the songbird forebrain *except* the androgen concentrating song centers (Mello et al., 1991; Mello et al., 1992). In order to test these hypotheses, and given that there are no neuronal cell lines which are responsive to gonadal steroids, several sets of experiments were carried out to investigate the molecular response to androgens in the S115 mammary adenocarcinoma cell line whose proliferation and phenotype are regulated by androgen (Darbre and King, 1987b; Yates et al., 1980; Darbre et al., 1983; Smith and King, 1972; Darbre and King, 1987a). First, the specificity of the S115 cells growth responsiveness to androgens was confirmed. The S115 cells were then grown in a steroid-free environment and subsequently given androgen for varying lengths of time and the mRNA levels of a variety of structural genes and transcription factor genes were measured. Finally, I looked at androgens effects in combination with other stimuli which act through protein kinase signal transduction pathways.

RESULTS

A: Androgen Regulation of Proliferation

To ensure the integrity of the S115 cells, and the steroid-free nature of the culture conditions, I replicated the original characterization of the androgen responsiveness of the line (Darbre and King, 1987a) and further, examined the effects on estrogen on their growth. When cultured on non-adhesive dishes in dextran-charcoal stripped serum, the S115 cells fail to grow in suspension, and the few cells that survive grow in clumps on the few sticky surfaces of the petri dishes. When androgen is added, the cells grow in an anchorage independent manner, forming large balls of cells in the medium. The cells grown in androgen increase in number by two orders of magnitude over ten days, five times that seen in untreated or estradiol stimulated cells. In addition, the proliferation of cells grown without androgen appears to be limited by saturation of the available plate surface by day seven, when they begin to die off. This increase in cell number and saturation density is clearly evident in Figure 38a, although even the testosterone treated cells appear to die by two weeks, presumably due to the high cell density causing rapid exhaustion of the medium and its subsequent acidification. 17 β -estradiol is indistinguishable from the saline control with regards to its effects on S115 cell growth in suspension.

Cultured on an adhesive substrate, the S115 cells undergo a more dramatic transformation when testosterone is included in the medium. The cells form a monolayer with epithelial morphology in the absence of hormone but when testosterone is added, they assume a fibroblast like morphology. The proliferation rate is increased dramatically by testosterone. Figure 38b. illustrates this phenomenon, with the difference in cell number between the two groups clearly evident by day seven. The rate of proliferation of testosterone treated cells is more than twice that of the control, and they saturate their dish by day ten. As was the case for the cells grown in suspension, estrogens have no proliferative activity relative to the control.

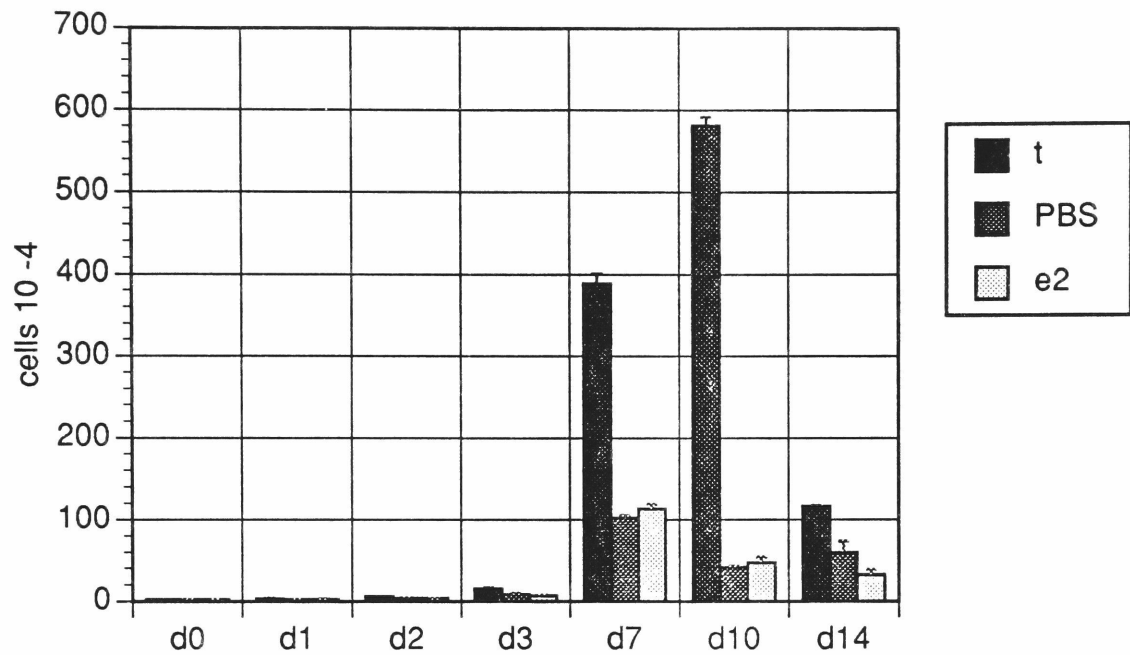
These experiments show that the cell line is indistinguishable in its hormone

ANDROGEN REGULATED GENES IN S115 CELLS

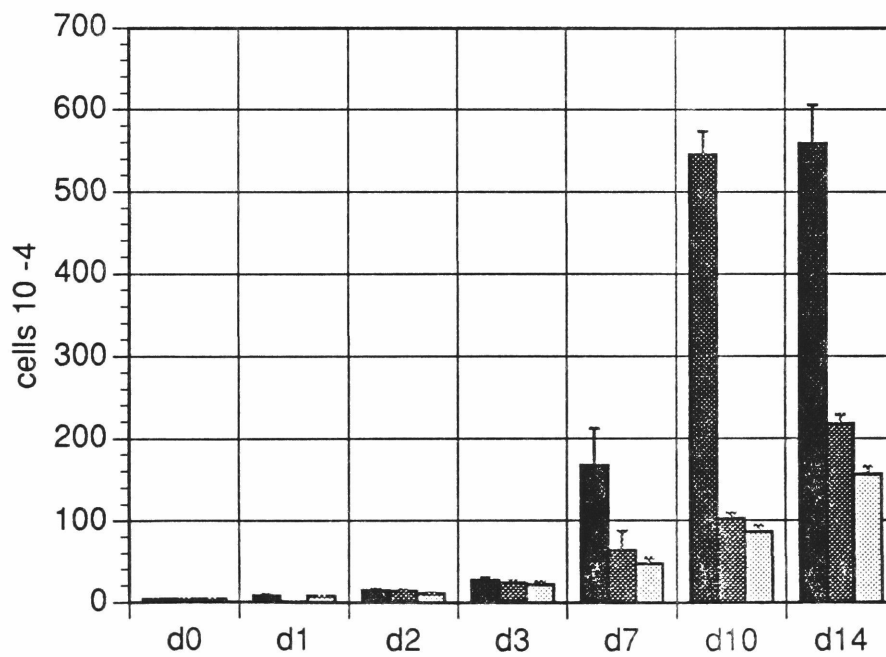
responsiveness from the earlier passage characterized (Darbre and King, 1987a). The hormone induced growth is mediated selectively by testosterone, with its receptor presumably mediating the specificity. 17 β -estradiol has no measurable effect on the morphology, proliferative rate, saturation density, nor the ability to grow in an anchorage independent manner. The line is therefore a reasonable model system to examine the effects of androgens on gene expression in a context of growth regulation, perhaps analogous to that seen in the canary brain.

Figure 38. Androgen Regulation of the Proliferation S115 Cells. Androgen, Estrogen, or PBS treatment of S115 cells grown in steroid-free medium. a. grown on petri dishes (in suspension) b. grown on adhesive (tissue culture) plates (as a monolayer). Each bar represents the mean \pm S.D. for three dishes for each treatment at each time point measured using a Coulter counter. Similar results were obtained in an identical experiment when the cells were counted in an hemocytometer.

a. S115 cells growin in suspension



b. S115 cells grown as a monolayer



B: Direct Effects of Androgen on Gene Expression

The S115 cells were examined for induction of structural and immediate early genes after steroid stimulation. I twice grew S115 cells in charcoal stripped medium to about 75 percent confluence and testosterone added for varying lengths of time. The cells were harvested at times between one and forty eight hours post androgen addition. Cytoplasmic RNA was prepared and used to synthesize labeled cDNA for hybridization in triplicate to DNA dots of structural and transcription factor genes. These dots were exposed to film, and the dpm subsequently quantified (see Figure 39). The mRNA level of each gene was analyzed by normalizing the signal to α and β tubulins to eliminate any experimental artifacts (see methods), and then the data was tested for significant effects of testosterone. Since the two independent treatments of the cells were slightly different (for instance, in cell density) I first performed a two-way ANOVA to determine whether there was a significant difference between the replications of the assay for each gene. If there was no significant difference, the data was pooled and analyzed for testosterone regulation using a one-way ANOVA. For those cases where there was significant inter-assay variation, the data were analyzed independently with a one-way anova, and where trends in the data existed (by Fisher's paired least significant difference (FPLSD) test) in the same direction, their probabilities were combined according to Sokal and Rohlf (1981) and analyzed for deviation from the chi-squared distribution. If the trends towards significance were in opposite directions (i.e. one set showed an increase and the other a decrease), the data were pooled and analyzed by a one-way ANOVA, since the inter-assay variance was not adding to create a significant more significant effect.

Figure 40 shows the results of two such assays, with the dpm at each time point expressed as a percent of the mRNA levels without testosterone treatment for each gene analyzed. The only large increase in the mRNA levels of any structural gene examined was vimentin at twenty four hours, (see table 2, FPLSD $p < 0.1$), indicating that the increase may have been artifactual, but the increase in vimentin mRNA was also seen in additional replications (chi squared $p < 0.01$, see section C.) Most of the structural gene

ANDROGEN REGULATED GENES IN S115 CELLS

mRNAs do not show any significant change (either increase or decrease) after up to two days treatment with testosterone. Shown in Figure 40 are the results for actin ($p=0.57$) and histone H1 ($p=0.29$). Not shown are the tubulin controls, which even without any adjustment for hybridization variability between filters show no significant regulation ($p=0.69$ for both). In addition, also not shown is the t-arginine encoding cDNA ($p=0.68$) and the anonymous housekeeping gene CHO_b ($p=0.42$).

The mRNA levels of many of the transcription factors also show little regulation (see Figure 40). Neither of two zinc finger-containing genes which are rapidly induced by serum and growth factors in a variety of cells, NGF Ia ($p=0.94$) nor NGF Ib ($p>0.3$, 48hr) were regulated by testosterone. The mRNA levels of the other immediate early transcription factors genes show little regulation, including pTIS21 ($p=0.14$), and c-jun ($p>0.1$, 48 hr). The c-myc mRNA levels were slightly depressed at one and 4 hours after testosterone treatment ($p<0.05$). The androgen receptor mRNA was down-regulated ($p<0.05$) immediately after testosterone addition, and then returns to control levels.

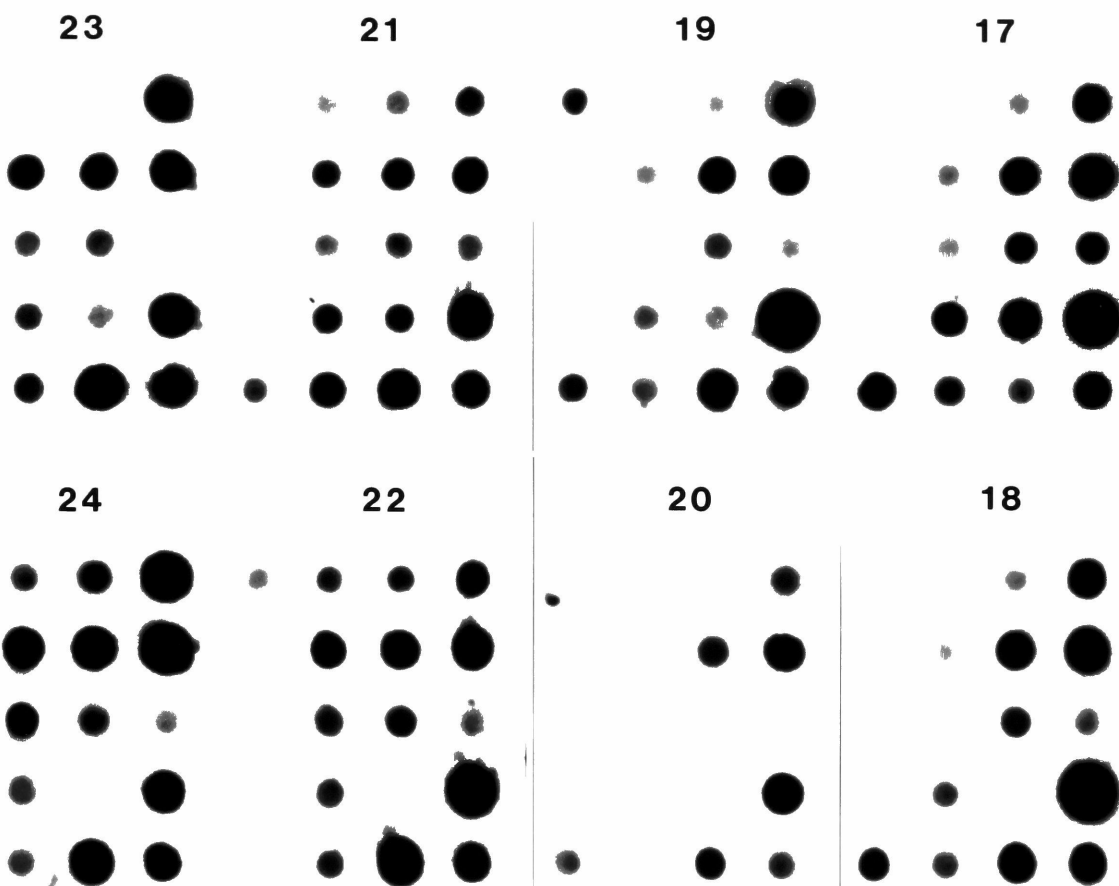
Androgen clearly regulates the growth rate and phenotype of the cells, and the androgen receptor mRNA was down-regulated by testosterone administration to the cells, indicating that the short treatment was effective. None of the structural genes assayed, with the exception of vimentin mRNA, are induced, and the only change in the "early" transcription factors was a slight repression of c-myc mRNA relative to identical cultures not stimulated by testosterone. However, these results do not appear to agree with the stimulation of transcription factors seen in canary HVc, especially for c-myc. In addition, the small effects seen do not appear to adequately account for androgens profound effects on the S115 cell line, or provide a good model for the equally profound effects in the canary. I therefore decided to examine androgens effects in a more physiological situation; in combination with other stimuli which might arise in the normal functioning of the central nervous system.

ANDROGEN REGULATED GENES IN S115 CELLS

Table 2. Two-way ANOVA for testosterone and assay interactions on S115 Cells

Gene	Assay			test	n	F value	p value
	F (x,y)	F value	p value				
CHOb	1,19	31.03	0.0001	F	4,24	1.36	0.422
t-arg	1,20	0.437	0.51	F	4,25	0.579	0.68
alpha tub	1,21	1.13	.30	F	4,26	0.56	0.69
beta tub	1,21	1.12	.30	F	4,26	0.56	0.69
Actin	1,20	20.58	0.0002	F	4,24	0.75	0.57
Vimentin	1,16	2.21	0.157	F	4,22	1.02	0.42
Histone H1	1,20	2.90	0.104	F	4,25	1.31	0.29
NGF Ia	1,21	5.94	0.024	F	4,26	0.20	0.94
NGF Ib	1,17	68.48	0.001	chi	2(4)	4.60	>0.3 (48hr)
c-jun	1,21	4.14	0.055	chi	2(4)	7.46	>0.1 (48hr)
pTIS21	1,20	1.87	0.187	F	4,25	1.89	0.14
c-myc	1,20	11.61	0.0028	chi	2(4)	14.61	<0.05 (1hr)
						15.76	<0.05 (4hr)
AR	1,20	10.18	.046	chi	2(4)	13.34	<0.05 (1hr)

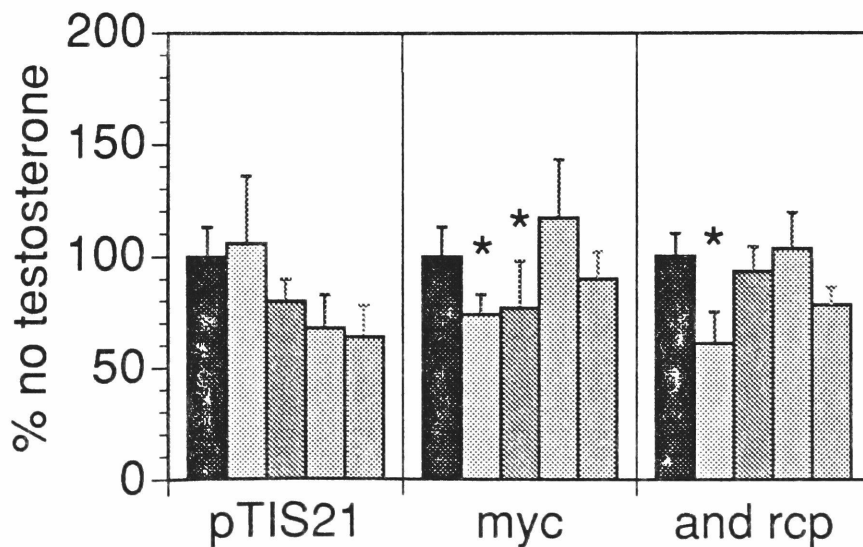
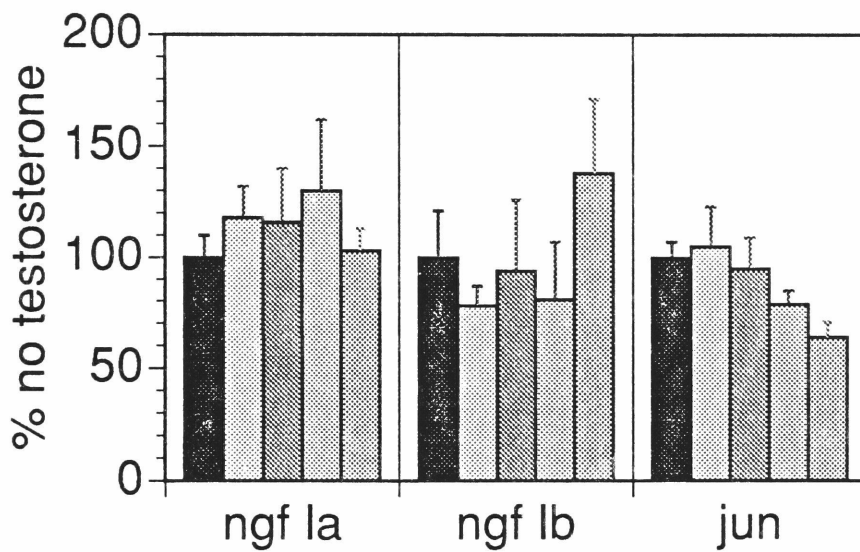
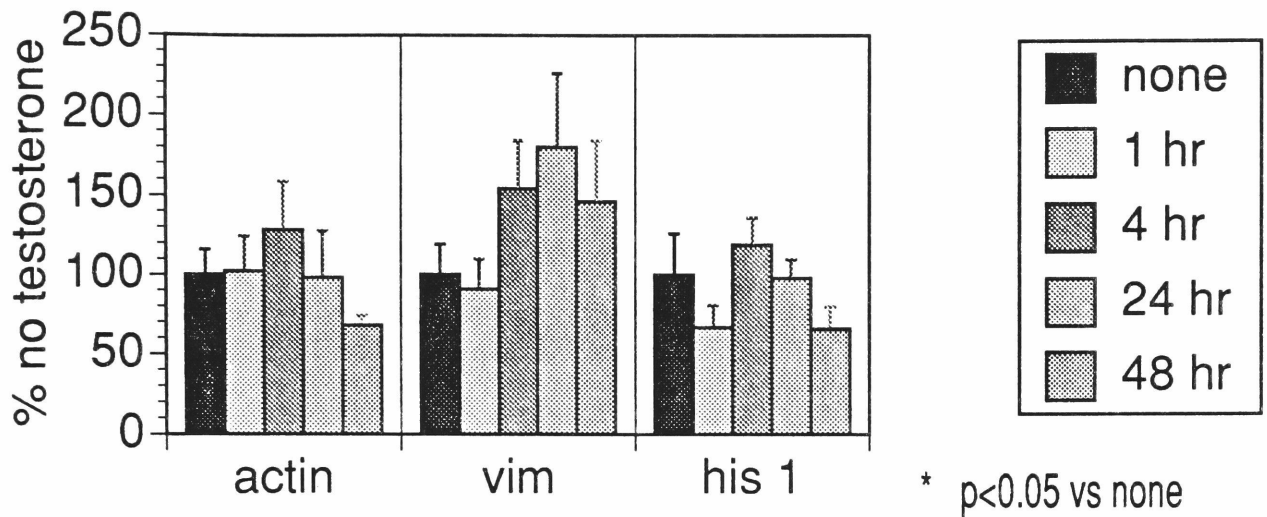
Figure 39. Assay for Androgen Regulation of Gene Expression. Results of cDNA:dot hybridization of RNA from S115 cells treated with various stimulators of signal transduction pathways. 17, no treatment; 18, one day testosterone; 19, forskolin; 20, testosterone pretreatment plus forskolin; 21, TPA; 22, testosterone plus TPA; 23, A23187; 24, testosterone plus A23187. Adjacent template of genes represented in the 4 X 5 matrix of cDNA dots. In filters 17-20, the c-jun cDNA is located in the 2nd "blank" position, and NGF Ib in the "c-jun" position on the filters.



CHO A	CHO B	t-arg	2ss	PAC1
Vim	a-tub	b-tub	h1h1	egr
NGF1-b	c-jun	PTIS21	c-myc	RAR
pgem3	pBR322	lambda	blank	blank

Figure 40. Regulation of Various Rodent Genes by Androgen in S115 Cells. Graph of quantitation of cDNA:dot hybridization assessing regulation of gene expression after various lengths of testosterone treatment. Mean \pm SE (as a percentage of PBS treatment) for two RNA samples for each time point, each sample hybridized to three filters.

Testosterone Regulation of Endogenous Gene mRNA Levels



C: Androgen Modulation of Induced Gene Expression

Cells in the central nervous system integrate information from a variety of sources, coming through a variety of signal transduction pathways. In order to determine whether androgens act synergistically with some of these other pathways to modulate gene expression, steroid deprived S115 cells were pretreated with testosterone for one day, then stimulated with activators of three separate signal transduction pathways which are active in the brain: Forskolin, which activates protein kinase A by raising the intracellular levels of cAMP; TPA, which activates protein kinase C by mimicking PI turnover; and A23187 which increases intracellular Ca^{++} and thereby activates Ca^{++} /Cam kinase. The cells were exposed to these drugs for one hour, then harvested. Cytoplasmic RNA was prepared from cells treated in this manner on two separate occasions and three hybridizations from each were carried out. The results quantified and analyzed for significant interactions as in the time study above (see table 3).

Forskolin

Forskolin does not have any effect on the message level of many of the genes studied. For the structural genes, the levels of message for histone H1 is raised (FPLSD $p < 0.01$), but there is no significant change in the levels of actin or vimentin. The messages of the transcription factors and the TPA responsive gene are not regulated by forskolin when compared to the control levels for these genes (chi-squared $p > 0.1$, FPLSD $p > 0.4$ see Figure 41). Forskolin addition after one day of testosterone treatment still increases histone mRNA levels, and no synergistic effect with testosterone is discernible, neither induction nor repression. Testosterone generally has no effect on the messages studied at twenty four hours when administered to deprived cells (except for vimentin after 24 hours testosterone alone, chi-squared $2(4)=18.42$, $p < 0.01$, see Figure 41, bar "t").

TPA

TPA does not affect the message levels of the structural genes (see Figure 42), except histone, which is significantly increased (FPLSD $p < 0.05$). This induction of histone is blocked by pre-testosterone treatment (FPLSD $p < 0.05$). Many of the

immediate early messages are induced by TPA treatment (chi-squared $2(4)=33.0$ $p<0.001$ for NGF Ia, chi squared $2(4)=12.23$, $p<0.05$ for c-jun, FPLSD $p<0.05$ for c-myc, see Figure 42). The message encoding NGF Ib showed a trend towards increasing (chi-squared $2(4)=8.82$, $0.1>p>0.05$). The mRNA levels of pTIS21 (which was isolated as a 3T3 cell TPA induced message by Lim et al., (1987) are also induced strongly (chi-squared $2(4)=26.2$ $p<0.001$). Androgen pretreatment blocks the increase in message levels for pTIS21 (chi $p<0.01$) and c-myc (FPLSD $p<0.05$ v. forskolin alone). In addition, there is some suggestion that it reduces the induction for the other TPA responsive genes. There was no effect on the message levels for the androgen receptor (chi $p>0.3$).

A23187

Intracellular Ca^{++} , increased by the ionophore A23187, induces many of the same genes as does TPA in the S115 cells. All of the immediate early genes except c-myc are greatly induced by A23187 (chi $p<0.001$ for NGF Ia, c-jun, and pTIS21, $p<0.05$ for NGF Ib, see Figure 43). Testosterone, in combination with the ionophore, synergistically induces many of the genes which it represses in combination with TPA. This is clearly seen for histone H1 and c-myc (FPLSD $p<0.01$ for H1 and $p<0.05$ for c-myc, compare Figures 42 and 43). pTIS21, which is well induced by Ca^{++} in addition to TPA, does not show regulation by testosterone with A2318, as it does for TPA.

ANDROGEN REGULATED GENES IN S115 CELLS

Table 3. Two-way ANOVA for testosterone/drug and assay interactions on S115 Cells

Gene	Assay		p value	test	n	F value	p value
	F (x,y)	F value					
Actin	1,33	45.00	0.0001	F	7,41	1.38	0.24
Vimentin	1,32	24.94	0.0001	chi	(see text)		
Histone H1	1,29	0.674	0.418	F	(see text)		
NGF Ia	1,33	56.98	0.0001	chi	(see text)		
NGF Ib	1,31	7.31	0.011	chi	(see text)		
c-jun	1,32	103.03	0.0001	chi	(see text)		
pTIS21	1,32	82.07	0.0001	chi	(see text)		
c-myc	1,32	1.39	0.248	F	(see text)		
AR	1,32	7.51	0.010	chi	2(4)	6.12	>0.1 (f)

Figure 41. Regulation of Various Rodent Genes by Forskolin with or without Androgen in S115 Cells. Graph of quantitation of cDNA:dot hybridization assessing regulation of gene expression after one hour forskolin treatment with or without one day pretreatment with testosterone. Mean \pm SE (as a percentage of PBS treatment) for two RNA samples for each condition, each sample hybridized to three filters.

Forskolin (cAMP) - testosterone effects on Endogenous mRNA Levels in S115 Cells

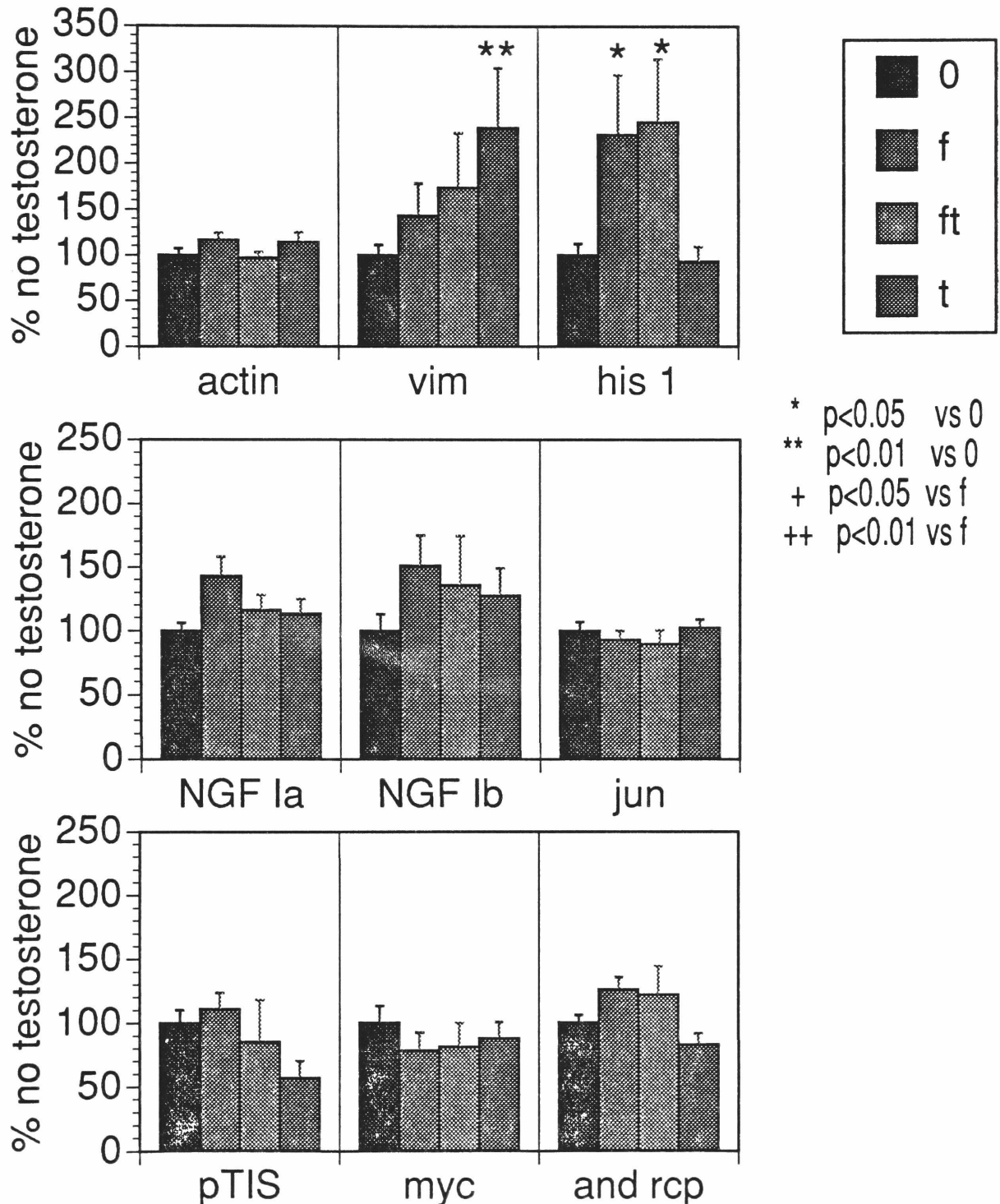


Figure 42. Regulation of Various Rodent Genes by TPA with or without Androgen in S115 Cells. Graph of quantitation of cDNA:dot hybridization assessing regulation of gene expression after one hour TPA treatment with or without one day pretreatment with testosterone. Mean \pm SE (as a percentage of PBS treatment) for two RNA samples for each condition, each sample hybridized to three filters.

TPA (PK C) - testosterone effects

on Endogenous mRNA Levels in S115 Cells

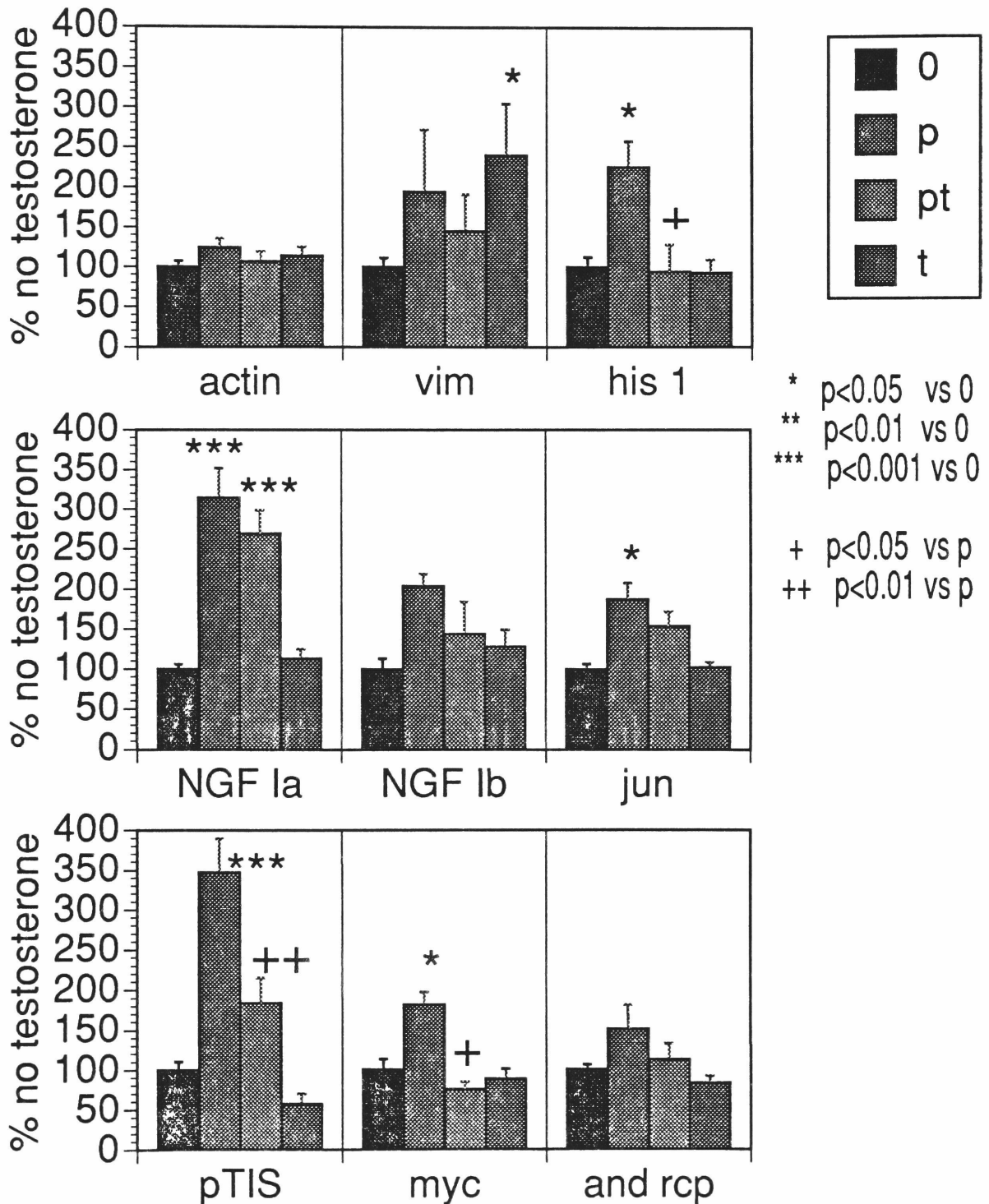
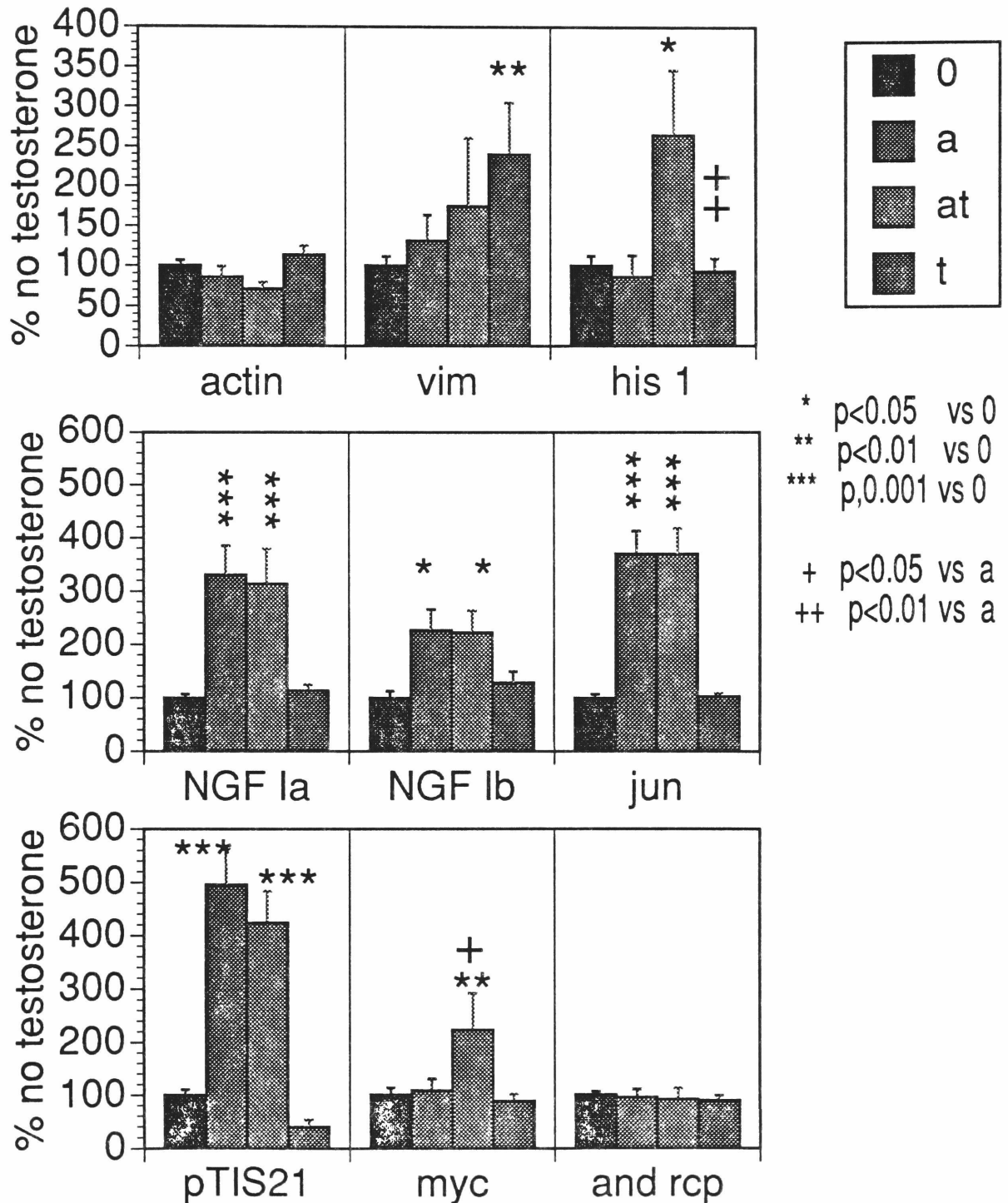


Figure 43. Regulation of Various Rodent Genes by Calcium with or without Androgen in S115 Cells. Graph of quantitation of cDNA:dot hybridization assessing regulation of gene expression after one hour A23187 treatment with or without one day pretreatment with testosterone. Mean \pm SE (as a percentage of PBS treatment) for two RNA samples for each condition, each sample hybridized to three filters.

A23187 (Ca++) - testosterone effects

on Endogenous mRNA Levels in S115 Cells



DISCUSSION

Androgen regulates both the growth and phenotype of the S115 mouse mammary tumor cell line in vitro (Darbre and King, 1987c; Smith and King, 1972). This cell line has been used as a model for the steroid dependence and subsequent independence of human tumors (Darbre and King, 1987a; Darbre and King, 1984; Darbre and King, 1987b). How androgen regulates the proliferation of the S115 cell line is unclear, but in general, the steroid hormones regulate the message levels of a wide variety of genes (Yamamoto, 1985; Beato, 1989). In addition to this direct regulation of mRNA levels, the steroid hormone receptors also autoregulate (Alexander et al., 1990; Shupnik et al., 1989; Quarmby et al., 1990) and regulate the transcriptional activation properties of at least some immediate early genes (c-jun and c-fos), and of other steroid receptors (Yang-Yen et al., 1990; Schule et al., 1990a; Diamond et al., 1990; Jonat et al., 1990; Schule et al., 1990b).

A variety of normal tissues and endocrine tumors are dependent on gonadal steroids for their growth. The mechanism of this regulation is unclear, though it is widely assumed that the steroids regulate the transcription of growth promotion or suppression genes. In the mouse mammary adenocarcinoma derived S115 cells, a likely candidate for a role in the increased proliferation in response to androgens are tumor viruses. Pieces of the most widely studied hormone responsive virus, the MMTV, are expressed in S115 cells. However, although transcription of the LTR of MMTV in S115 cells is regulated by androgens over a period of weeks (Darbre et al., 1983), this slow regulation fails to account for the rapid regulation of cell proliferation by androgens.

Cellular homologs of virally encoded tumor promoting genes are thought to play a role in the normal regulation of cell proliferation, and they often respond rapidly to proliferative stimuli (Bishop, 1985). These proto-oncogenes often encode pieces of signal transduction pathways, such as growth factor receptors, kinases, or transcription factors. The addition of serum or growth factors to cells or the stimulation of tissues in vivo increase the mRNA levels of these genes within minutes to hours (Mello et al.,

1991; Christy et al., 1988; Milbrandt, 1987; Hazel et al., 1988). The levels of mRNA for c-jun (Weisz et al., 1990), and the c-myc and c-fos proto-oncogenes increase rapidly in the uterus with estrogen in vivo (Rempel and Johnston, 1988). Direct effects of androgens on the expression of such genes are heretofore unknown, though their possible indirect effects in rodents were investigated by castration and examining c-myc and c-fos in regressing prostate (Buttayan et al., 1988) and implanted tumor cells (Rennie et al., 1988).

Interactions of steroid hormone receptors with immediate early genes have been described in several other systems. Diamond and colleagues (1990) show the TPA induction of the proliferin gene is regulated by glucocorticoids acting through their receptor. This regulation is dependent on the cellular environment. In CV-1 cells, TPA induced transcription from the proliferin promoter is blocked by dexamethasone, but in HeLa cells, the opposite effect is evident: TPA alone has little effect on the transcription, but when the hormone is added there is a three-fold induction of transcription from the promoter. Diamond, et al., (1990) say the glucocorticoid receptor (GR) regulates transcription at the proliferin enhancer, which contains binding sites for both the GR and AP-1. Induction or repression is determined by which components of the immediate early transcription factor AP-1, the jun-jun homodimer or fos-jun heterodimer, is interacting with the GR. The receptors for the steroid-like vitamin D and retinoic acid also interact with AP-1. The induction of the osteocalcin gene by both receptors is blocked by TPA treatment (Schule et al., 1990b). This interaction is also at the enhancer, but is not selective association of one subtype of AP-1, but competition for the GR binding site, which also contains a cryptic AP-1 binding site (Schule et al., 1990b). The collagenase gene is induced by glucocorticoids, like the osteocalcin gene, and the induction also blocked by TPA, but several groups have demonstrated that the repression is due to protein-protein interactions, between the dimerization domains of the AP-1 components and the hormone receptors (Yang-Yen et al., 1990; Schule et al., 1990a; Schule et al., 1990b). The transcriptional activation of the vitellogenin

promoter/enhancer by the estrogen receptor is also regulated by the constituent proteins of AP-1 in a protein-protein interaction mediated manner (Doucas et al., 1991). Finally, the rat prolactin gene is positively regulated by a tissue-specific regulatory factor, Pit-1, and negatively regulated by the GR. This negative regulation is due to protein-protein interactions between the receptor and Pit-1 (Adler et al., 1988). The mechanisms whereby immediate early genes regulate, and are regulated by, the steroid hormone receptors is clearly complex and may involve protein-protein interactions or be dependent on DNA elements to facilitate their interactions (or both). My finding that the androgen receptor modulates the mRNA levels of *endogenous genes* in S115 cells provides another example of the of both potential interactions between different classes of transcription factors and paradoxical cell environment-specific regulation of genes.

Androgen Regulation of Gene Expression in S115 Cells

This set of experiments clearly implies that the androgens' effects on this line stem less from its direct control of gene expression than from its action as a modifier of the response to other stimuli. Androgens alone do not appear to cause many significant ($p < 0.05$) changes gene expression, except for the androgen receptor. There were also small changes for vimentin and c-myc, but these changes were small. Other stimuli cause interesting changes, such as the induction of many of the immediate early genes, and some of these changes are quantitatively modified by the androgens (see Figures 42 and 43). This may be especially significant for c-myc and histone H1, two genes which are closely associated with proliferation in many systems (Laybourn and Kadonaga, 1991; Breakefield and Stern, 1986), but in no case was the absolute magnitude of these changes larger than about three-fold.

In addition to these effects, androgen could be affecting the mRNA levels of many genes a little. Such changes might have significant cumulative effects, but would be undetectable in this assay, since changes of less than 25% or so would be subsumed in the noise inherent in the assay. It is also possible that despite my efforts to select a panel

of genes likely to be androgen regulated, I have missed some classes of genes which are directly regulated to a greater extent than was seen here (3-fold). Another possibility is that despite having examined direct steroid action and three major pathways of signal transduction, a very different signal transduction mechanism is responsible for the change in growth exhibited by the S115 line. The regulation of mRNA levels by different signal transduction systems in the S115 line are not unlike the interactive models of steroid hormone action, such as estrogen-ovalbumin or the more complex PKC-corticosterone-collagenase model, and the relatively small magnitude of the changes observed may be biologically significant.

Finally, it must be emphasized when comparing my results with published reports of steroid hormone regulation of gene expression in other systems that I have measured the mRNA levels of *endogenous* genes, not constructs transfected into the cells, and measured regulation by the *endogenous* receptor, also not a construct transfected so as to over-express. The inter-assay variation I found between the replications was perhaps not surprising since the responsiveness of the cells to external stimuli is likely to be modulated by their internal state, which is difficult to duplicate (such as exact cell densities at the start of the experiments) on separate occasions. The magnitude of androgen induction of gene expression others report varies with the type of gene and tissue examined. The nerve growth factor receptor (NGFR) mRNA is induced almost fifty fold in the testis by EDS treatment or hypophysectomy (Persson et al., 1990). Testosterone induces a large number of kidney mRNAs five to ten fold, including ADH, ODC, GUS, KAP, RP2, and MK1440 (Watson and Paigen, 1990). Castration induces c-fos and c-myc approximately five fold in the prostate and androgen responsive tumors (Buttayan et al., 1988; Rennie et al., 1988). In the brain, the only reported effect is on the NGFR mRNA, which is twice as high in developing female rats than males. Finally, it is interesting to note that unlike the assays of messenger RNA levels in the canary brain, in this pure genotypically uniform population the levels of androgen receptor mRNA was sufficiently high to be measured with the cDNA:dot hybridization assay.

Since androgens alone are capable of causing the cells to undergo a dramatic transition in growth and proliferation, the effects here suggest a "complex" mode of androgen action. The subtleties of androgen regulation of gene expression in this cell line are consistent with the lack of large effects on gene expression measured in songbird brain, and suggest that this lack should not automatically be attributed to the cellular complexity of neural tissue. These cell line experiments support the hypothesis instead that androgens can act quietly, or indirectly, to influence the responsiveness of a variety of genes to various stimuli.

SUMMARY

I have shown how androgen regulates the expression of a panel of genes which I thought might be important for androgens effects on growth regulation and differentiation in the S115 mammary adenocarcinoma cell line. Acting in relative isolation, testosterone can dramatically increase the rate of proliferation of the cells, while estradiol is without effect. Testosterone can regulate the mRNA levels of structural genes, transcription factors, and its own receptor in these cells. Agents which stimulate protein kinase signaling pathways modulate the expression of many of these genes in S115 cells. Interestingly, androgens appear to interact synergistically with the Ca^{++} ionophore A23187 to induce c-myc and histone H1 expression, where neither alone will do so. In addition, androgen pretreatment represses the TPA-induced increase of these same genes. These results are potentially significant when extrapolated to the brain, where Ca^{++} is a significant signal and effector in many nervous system processes. In all cases, however, the measured effects of androgen on RNA levels were relatively small (less than three fold), even in this clonal cell line which shows significant growth regulation by androgen.

CHAPTER 5: CONCLUSIONS

Songbirds provide a unique opportunity to understand how sex differences in behavior are established and how the gonadal steroids bring about changes in brain and behavior in the adult. Many elegant studies have defined how the sexually dimorphic behaviors of singing in canaries and other songbirds develop and are controlled by estrogen and the androgens (reviewed in (Konishi, 1985; Nottebohm, 1989; Konishi et al., 1989; Konishi, 1989). I have carried out a series of investigations to begin to understand at a molecular level how androgens act on the canary brain.

I first cloned the canary androgen receptor, the only avian androgen receptor cloned. The first exon of the receptor remains uncloned despite my vigorous efforts. It is important to isolate this part of the receptor in order to better understand which domains of this large part of the protein have been conserved during evolution, since this will indicate the functionally important domains. It is difficult to determine such domains from a comparison of the rodent and human sequences (Muller et al., 1988; He et al., 1990) since they are highly conserved (~80%) and the regions of divergence are largely polyaminoacid strings whose functional significance is unclear. Other steroid receptors contain transcriptional activating domains in this region of the molecule (Tora et al., 1989), which may also be present in the androgen receptor. The genomic clones I have isolated appear by hybridization to contain at least portions of the exon. If the genomic clones I have isolated prove to be the first exon, the sequence may add further valuable functional information to our knowledge of the mechanism of transcriptional regulation by the androgen receptor.

The canary receptor cDNA has been useful as a probe in these studies to examine how changes in plasma androgens, either pharmacological, behavioral or seasonal, cause changes in receptor mRNA levels. I have been able to localize the synthesis of the receptor in anatomically defined areas of the brain, and thereby inferentially determine the locus of androgen action in the songbird brain. The location of cAR mRNA has in all

CONCLUSIONS

instances matched that of labeled ligand binding studies and the localization of the protein by antibodies. In addition, the cAR cDNA has proved to be a useful indicator of the time course of androgen effects on the canary brain.

I used the information about receptor location and the timing of androgen effects to examine androgen induced changes in gene expression in the canary brain.

Testosterone implants begin to induce singing behavior in female canaries after a week (F. Nottebohm, unpublished observations), so the changes in the brain induced by the androgens must be well underway or completed by this point. I therefore examined changes in the levels of a variety of genes which were likely to be involved in neural development and signal transduction from very short treatments up to one week.

Androgen did change gene expression at early times, but the magnitude of these changes was small, perhaps due to the low percentage of steroid responsive cells in the canary brain, the tissue sample dissection I used, or perhaps such small changes reflect the nature of androgens' effects. There was no discernible difference in gene expression after longer androgen stimulation.

Because of this lack of a more dramatic effect in the complex tissue of the brain, I also quantified changes in gene expression induced in a simpler system. I found that some of the same genes regulated in the canary brain are regulated in the androgen responsive S115 cell line. In addition, I found that androgens' role in the regulation of these genes is primarily modulatory, synergistically modifying the effects of other stimuli. Such a modulatory role had not been described before the experiments were carried out. All of the regulation by steroid hormones reported had been of induction (or repression) which could be associated, directly or through secondary factors, with androgen administration. My finding that the principle role of androgens was to modulate the regulation by other signal transduction pathways of endogenous genes in the S115 cell line was confirmed in essence by others who reported similar phenomenon for estrogens (Doucas et al., 1991), glucocorticoids (Yang-Yen et al., 1990; Schule et al., 1990a; Diamond et al., 1990; Jonat et al., 1990) and vitamin D/retinoic acid (Schule et al.,

CONCLUSIONS

1990b).

The role of androgen in bird song is certainly complex and involves the integration of many separate signals. Nevertheless, the effects on gene expression I measured in songbird brain were comparable in magnitude to what I measured in the S115 cell line. I believe the canary, remains a particularly a good model system for the examination of gonadal steroid effects on brain function. The differences between male and female have been directly associated with circulating androgen levels. The magnitude of the anatomical change seen between high and low testosterone is particularly large for neural systems, and well defined anatomically. In addition, there is a well defined behavioral correlate to the changes. Using the androgen receptor as a marker of both the location and effectiveness of androgen action in the canary brain, and the many interesting canary genes cloned by me and other members of the group, I have begun to explore the changes in gene expression which might induce, and certainly accompany, the establishment of the neural substrate for singing in the canary.

METHODS

cDNA Cloning- Direct Screening

cDNA libraries were constructed in lambda vectors, essentially as described by Watson and Jackson (1985). The liver library was synthesized from 2 µg spring male canary polyA+ RNA. The RNA was reverse transcribed using AMV reverse transcriptase and a XbaI-oligo dT primer-adaptor, and the complementary strand synthesized by E. coli DNA polymerase concurrent with RNase H treatment. The product was then methylated using EcoRI methylase, the ends filled using the Klenow fragment of DNA polymerase, and EcoRI linkers were added. The cDNA was then digested with EcoRI and XbaI, and size selected through a Sepharose CL-2B column, from which cDNA over 600 bp was collected and ligated into lambda Gem4 (Promega). The library was packaged (Promega), titered, and amplified (Sambrook et al., 1989). The Liver library contains approximately 5×10^6 independent clones.

The HVC library was constructed in lambda gt10, and contains approximately 3×10^6 independent clones (described in George and Clayton, (1992). The non-forebrain library was constructed in lambda Zap II, and contains approximately (1×10^6) independent clones (Oh and Clayton, unpublished).

Each Library was plated at approximately 50,000 pfu per 150 mm plate using the appropriate bacterial host. The plaques were allowed to develop to about 0.5 mm diameter, and lifted in duplicate onto nylon filters (Colony/Plaque Screen, NEN). The filters were hybridized essentially according to Westneat (1988). Briefly, they were rinsed in 2X SSC and pre-hybridized in a solution containing 7% SDS, 1 mM disodium EDTA, 263 mM sodium phosphate, and 1% BSA (763 mM final sodium concentration). Random primed (Feinberg and Vogelstein, 1983; Feinberg and Vogelstein, 1984) DNA probes ($>10^9$ dpm/µg) were denatured and added directly to the pre-hybridization solution, and incubated overnight in a shaking water bath. The filters were then rinsed in 2X SSC,

METHODS

0.1% SDS at room temperature and finally washed in 1X SSC, 0.1% SDS at the hybridization temperature. The filters were then exposed to XAR-5 film at -70°C with intensifying screens.

PCR

Polymerase chain reaction amplification of cDNA and RNA was carried out according to (Sambrook et al., 1989). A typical reaction for amplifying cDNA contained template DNA, 10 to 100 pmol each primer, 200 μ M each dNTP, and 1 U taq DNA polymerase in 100 μ l containing 50 mM potassium chloride, 10 mM Tris (pH 8.3), 1.5 mM magnesium chloride, and 0.01% gelatin. The reaction was overlaid with mineral oil and subjected to 35 cycles in an MJ Research thermal cycler. Templates were typically denatured at 94°C for 45 sec, followed by one min at the appropriate annealing temperature and three minutes at 72°C. Genomic DNA directed pcr was performed using 1 μ g Template and 100 pmol each primer, and cycled 45 times. Reverse transcriptase pcr for the cAR was performed using 200 U MMLV reverse transcriptase, 2 μ g total RNA from various tissues, 1 μ g pdN₆, 10 U RNasin, and 1 mM dNTPs in a 20 μ l volume and incubated at 37°C for one hour. Methyl mercury treatment of the RNA prior to reverse transcription and AMV reverse transcriptase use were according to standard protocols (Maniatis et al., 1982). Five μ l of this reaction was added to each pcr tube, which included 0.05% Nonidet P-40 (Weyant et al., 1990) and which had been irradiated with short-wave UV for 5 minutes to reduce possible contamination (Sarkar and Sommer, 1990).

Testis RNA was reverse transcribed and amplified using pcr, then cloned. Several reactions were pooled, chloroform extracted, treated with T4 DNA polymerase to blunt the ends (Huang and High, 1990), methylated with EcoRI methylase, EcoRI linkers added, and digested with EcoRI. The product was then gel isolated and cloned into lambda gt10 and screened using the rat AR clone (rAR 2830, a generous gift of C. Chang (Chang et al., 1988a)). This high efficiency cloning scheme was necessary due to the

METHODS

poor clonability of pcr products in general, and the cAR in particular. Subsequent to the isolation of the lambda clones (see cDNA cloning above), the inserts were subcloned into pBluescript by amplifying using lambda gt10 forward and reverse primers (Herrmann et al., 1990).

All primers were synthesized on a Applied Biosystems model 391 oligonucleotide synthesizer in the lab according to the manufacturer. T3, T7, SP6, and lambda primers were as indicated in the Promega "protocols and applications" manual. The AR DNA binding primer is 5'-AAGACCTGCCTGATCTGTGGAGA-3' and the steroid binding primer is 5'-GTGGAAATAGATGGGCTTGACTTT-3'. For quantitative pcr (see below), the forward primer is 5'-TACGAGGCCGGGATGACG-3' and the reverse primer is 5'-TCTCCACCCCATAGCAAA-3'. The primers for the first exon are: (in 5' to 3' order) 5'-GCCGAATTCTAGAGCTCATGGARGTXCARTTXGGXCTXGG-3', 5'-GGXCGXTTYCARAAYYTXTT-3', 5'-GCCGAATTCTAGAGCTCARCTXCGXGGXGAYTGXATGTA-3', 5'-GAYTAYTAYAAAYTTYCC-3', 5'-GARATGGGXCCXTGGATG-3', 5'-CATCCAXGGXCCATYTC-3', 5'-CGCATYTCXCCYTAXGGXCC-3'. All primers over 25 nt in length were affinity purified on an OPC column (Applied Biosystems), all other primers were used in a crude form. Typical stepwise synthesis efficiency was 99%.

Labeled Oligonucleotide Preparation

Oligonucleotide probes were labeled using either polynucleotide kinase or terminal deoxynucleotide transferase. For kinase labeling, 5 pmols oligonucleotide were labeled using 50 μ Ci [γ - 32 P]ATP (5000 Ci/mmol, Amersham) and 10 units T4 polynucleotide kinase (Biolabs) in a solution containing 50 mM Tris (pH 7.6), 10 mM magnesium chloride, and 5 mM DTT in a reaction volume of 10 μ l. For tailing, 5 pmols oligonucleotide were incubated in 50 μ Ci [α - 32 P]dCTP (3000 Ci/mmol, NEN) using 15 units terminal deoxynucleotide transferase (Pharmacia) in a solution containing 100 mM potassium cacodylate (pH 7.2), 2 mM cobalt chloride, and 0.2 mM DTT in a reaction

METHODS

volume of 10 μ l. Both reactions were incubated at 37°C for 45 min, followed by 5 min at 65°C to inactivate the enzyme. The probes were purified by column chromatography (Nensorb-20, NEN). The tailing protocol was optimized empirically to add three to five dCTPs to each oligonucleotide.

Southern Blot Analysis

Genomic DNA was analyzed by standard techniques (Maniatis et al., 1982). Canary genomic DNA was isolated from male livers by Dounce homogenization, followed by proteinase K digestion, RNase A treatment, phenol and chloroform extractions, and ethanol precipitation. Genomic DNA from other species was prepared in the same manner and all samples contained predominantly very high molecular weight DNA. Samples of DNA were digested by 10 units restriction endonucleases (Biolabs) in 10 μ l 1X appropriate restriction endonuclease buffer (Biolabs) per μ g DNA. The digestion reactions were incubated at the appropriate temperature (usually 37°C) for three hours and EDTA added to 15 mM to stop the reaction. The protein in the samples was then precipitated by adding ammonium acetate to 2.5 M and centrifugation at 15,000 X g for 15 min at room temperature. DNA in the supernatant was precipitated by adding 2.5 volumes ethanol and centrifugation at 15,000 X g for 15 min at 4°C. The pellet was washed with 70% ethanol and dried under vacuum.

The samples were resuspended at 0.1 μ g/ μ l, heated to 65°C for 10 min, cooled on ice, and loaded onto a 0.8% LE agarose gel and electrophoresed in TAE buffer (Maniatis et al., 1982). The DNA was transferred to a nylon membrane (Zeta-Probe, Biorad) according to Westneat (Westneat et al., 1988). Hybridizations using random primed probes were carried out exactly as for cDNA library screens (above). Hybridization with oligonucleotide probes were carried out using an empirically determined protocol. The filters were rinsed in 2X SSC and pre-hybridized overnight in a solution containing 5X SSPE, 7% SDS, 10% dextran sulfate, 10X Denhardt's reagent, and 100 μ g/ml sheared salmon testis DNA (stDNA). Oligonucleotide probes (see above) were added to a

METHODS

hybridization solution consisting of the prehybridization solution lacking stDNA and Denhardt's reagent, and incubated overnight at 45°C in a shaking water bath. The filters were then rinsed in 2X SSC, 0.1% SDS at room temperature and washed in 1X SSC, 0.1% SDS at the hybridization temperature. The filters were then exposed to XAR-5 film at -70°C with intensifying screens.

The hybridization of the 5' oligonucleotides to canary genomic DNA appears to be specific, and does not appear to be determined by the addition of 4 or 5 ³²P dCTPs. For example, an oligo to the 3' end of region C but labeled the same way gives a different pattern of bands than seen on a similar Southern genomic blot (such as Figure 10).

All non-genomic Southern blots were performed according to Reed and Mann (Reed and Mann, 1985). DNA samples were electrophoresed in LE agarose of varying concentrations in TAE buffer. The DNA was transferred in 0.4 N sodium hydroxide to nylon filters (Zeta-Probe, Biorad) after acid depurination. The filters were then rinsed in 2X SSC and hybridized according to Westneat (Westneat et al., 1988).

Genomic Cloning and Screening

The canary genomic library was constructed by standard procedures (Sambrook et al., 1989). High molecular weight genomic DNA was partially digested with BglII to a modal size of 20 kb, and the ends partially filled in with dGTP and dATP using the Klenow fragment of DNA polymerase I. This DNA was ligated into the partially filled (as above) XhoI site in lambda Fix II (Stratagene) at a 2:1 molar ratio. This reaction was packaged using Gigapack Gold (Stratagene) and titered using LE 392 bacteria previously infected with bacteriophage P2 to exclude non-recombinants. The library contains approximately 3 X 10⁶ independent clones.

The canary genomic library was screened in LE 392 bacteria grown on NZYM (Maniatis et al., 1982) plates. 40,000 pfu were plated for each 150 mm plate, and 5,000 pfu per 90 mm plate, and were allowed to grow to approximately 0.5 mm. These plates were lifted in duplicate onto nylon filters (see cDNA screening). The filters were

METHODS

hybridized with oligonucleotide probes as detailed above and exposed to XAR-5 for one week at -70°C using intensifying screens.

Sequence Analysis

Each cDNA clone was sequenced on both strands using Sequenase (USB) and synthetic oligonucleotide primers. Large preparations of each clone were grown, and purified by cesium density gradient centrifugation (Sambrook et al., 1989). The plasmids were sequenced from both the T7 and T3 primers, and subsequently from internal synthetic oligonucleotide primers (17 or 18 nt) determined by the previous sequencing results. Plasmid DNA (4 µg) was denatured using alkali according to the Promega "protocols and applications" manual. The primers were annealed to the denatured DNA and the labeling using [α -³⁵S]dATP and termination were according to the Sequenase V. 2.0 manual (USB). The products were denatured and electrophoresed on a 6% sequencing gel (Maniatis et al., 1982). The gels were fixed, dried, and exposed to XAR-5 film overnight at room temperature. The sequences were assembled on DNASIS (Hitachi) and all comparisons were carried out using the FASTA suite of programs for the PC (Pearson and Lipman, 1988).

The cAR pcr product was sequenced directly using end-labeled primers. Up to 10 100 µl rt-pcr reactions were pooled and the product isolated either by Centricon-30 (Amicon) filters or gel isolation and electroelution (Sambrook et al., 1989). An empirically determined amount of DNA was annealed to [γ -³²P]ATP labeled primer by boiling for 5 min, followed by cooling on ice for 10 min. The DNA was then sequenced by the dideoxy method using either the taq DNA polymerase sequencing kit (Promega) or Sequenase (USB). The taq kit was used according to the manufacturer. The Sequenase kit was modified according to (Meltzer et al., 1990). The termination buffers was altered included 80 µM each dNTP and 8 µM of the appropriate ddNTP. The reactions were then electrophoresed and analyzed like the standard thio labeled dideoxy sequencing (above).

Riboprobe Preparation

The ^{35}S labeled riboprobes used for in situ hybridizations for the cAR were synthesized out following the protocol of Clayton, et al. (1988). Briefly, riboprobes were synthesized from cesium chloride gradient purified preparations of pBluescript containing cAR 2.2 (see chapter. 2, and Figure 11) linearized with either BamHI for sense strand or HindIII for the antisense using either T7 or T3 RNA polymerase, respectively. A typical reaction contained 0.5 μg template, 0.5 mM each ATP, CTP, and GTP, 12 μM unlabeled UTP, 7.7 μM 5' [α - ^{35}S]UTP (50 μCi at 650 Ci/mmol in water, Amersham), 10 mM DTT, 40 mM Tris (pH 7.5), 6 mM magnesium chloride, 2mM spermidine, 10 mM sodium chloride, 10 units RNasin (Promega), and 20-50 units T3 (Pharmacia) or T7 (USB) RNA polymerase in a final volume of 10 μl . The reaction was incubated for 1 hr at 40°, then diluted to 50 μl with a buffer containing 10 mM Tris (pH7.5), 5 mM EDTA, 50 $\mu\text{g/ml}$ yeast tRNA, 0.1% SDS, and 150 mM sodium chloride. One μl was reserved to measure total radioactivity in the reaction, and the remainder was purified through a Sephadex G-50 spun column (Maniatis et al., 1982). Concentration of radiolabeled RNA product was determined from the percentage of incorporation of UTP. This reaction results in a specific activity of 5.9×10^8 dpm per μg synthesized RNA.

Digoxigenin labeled riboprobes were synthesized in a similar reaction, except that the nucleotide concentrations were increased and ^{35}S -labeled UTP eliminated from the reaction. ATP, CTP, and GTP were at 1 mM, unlabeled UTP was at 0.65 mM, and 0.35 mM dUTP labeled with the steroid hapten digoxigenin. The quality of each probe used for in situ hybridization was assessed by whether it met two standards: It had to be detected when spotted at a 10^{-10} dilution onto a nylon filter, and it had to detect less than one pg of the template cDNA in a filter hybridization of serial dilutions of the template DNA.

In Situ Hybridization

The in situ hybridizations were carried out largely as described in Clayton, et al.,

METHODS

(Clayton et al., 1988), with modifications to decrease the non-specific binding (nsb) of the probe to the tissue. For hybridization to ^{35}S -labeled probes, sections were first acetylated to reduce nsb by immersion for 10 min in fresh 100 mM triethanolamine and 25mm acetic anhydride (Hayashi et al., 1978). After rinsing the sections briefly in PBS, they were each covered with 16 μl of hybridization solution, consisting of 2X SSPE, 2 $\mu\text{g}/\mu\text{l}$ of tRNA, 0.4 $\mu\text{g}/\mu\text{l}$ BSA, 50% formamide, 1 $\mu\text{g}/\mu\text{l}$ of polyadenylic acid, 110 mM DTT, and 250,000 dpm radiolabeled RNA. A glass coverslip was placed on top of each section, and the slides were immersed in paraffin oil at 65°C. After 3 hr, slides were rinsed in chloroform to remove residual oil, and the coverslips were removed in 2X SSPE containing 0.1% β -mercaptoethanol (β -me). The slides were transferred to fresh 2X SSPE, 0.1% β -me and incubated one hour at room temperature. They were then incubated for one hour in 2X SSPE, 0.1% β -me, and 50% formamide at 65°C. This was followed by four 10 min washes in 2X SSC, 0.1% β -me at room temperature. The slides were then incubated in RNase A (40 $\mu\text{g}/\text{ml}$) and RNase T1 (200 U/ml) in 2X SSC for one hour at 37°C. The slides were then rinsed for 10 min at room temperature successively in 2X SSC, 1X SSC, and 0.5X SSC. Finally, they were washed at high stringency in the "11 mM sodium buffer", a solution containing 2 mM tetrasodium pyrophosphate, 1 mM sodium phosphate (pH 7.2), and 1 mM disodium EDTA (11 mM total sodium concentration) twice for 30 min at 65°C. The slides were then dehydrated in ethanol. Less stringent washing conditions resulted in high levels of nsb, which appears to be cell density specific and "nuclear", suggesting that the probes are hybridizing to ribosomal RNAs (see discussion in Shan et al., 1990).

The location of cRNA:mRNA hybrids was determined by autoradiography. The sections were first exposed to Kodak XAR-5 film for ten days. This film was developed according to the manufacturer. The slides were then delipidized by rinsing in xylene, and again dehydrated. The slides were then dipped in Kodak NTB-2 emulsion, exposed for one to three months at 4°C in a dehumidified atmosphere, developed, counterstained with cresyl violet following standard procedures (Rogers, 1979) and then coverslipped.

METHODS

Sections showing any signal on the film were subsequently shown by emulsion autoradiography to have high levels of nsb, and were generally discarded before dipping.

For the DIG-labeled probes, different hybridization conditions were used, but the washing protocol was largely similar. After acetylation, each section was covered with 200 μ l of pre-hybridization solution, consisting of 5X SSC, 50% formamide, 100 μ g/ml stDNA, 50 μ g/ml heparin, and 0.1% Tween-20. The slides were then incubated in a humidified atmosphere at 55°C for one hour. The slides were then rinsed in 2X SSC and each section was covered with 30 μ l hybridization solution, consisting of the pre-hybridization solution and approximately 300 pg DIG-labeled riboprobe. Each section was then covered with a piece of Parafilm and incubated overnight (~12 hr) at 55°C in a humidified atmosphere. The sections were washed exactly as for the ³⁵S-labeled in situ hybridization (omitting the chloroform rinse) and dehydrated.

DIG labeled hybrids were detected essentially as described in Boehringer Mannheim technical bulletin no. 9001631/5M. The slides were rinsed for 2 min in buffer 1 (consisting of 100 mM Tris (pH 7.5) and 150 mM sodium chloride), (all incubations at room temperature). They were then incubated for 30 min in buffer 1 containing 2% normal goat serum (ngs) and 0.3% Triton X-100. The buffer was then removed, and 100 μ l Anti-DIG antibody diluted 1:500 in buffer 1 containing 1% ngs and 0.3% Triton X-100 was applied to each slide. Parafilm coverslips were used to prevent evaporation, and the slides were incubated in a humidified atmosphere for 4 hr. The slides were then rinsed for 10 min in buffer 1, followed by 2 min in buffer 3 (100 mM Tris (pH 9.5), 50 mM magnesium chloride, and 100 mM sodium chloride). 200 μ l color solution, consisting of 334 μ g/ml NBT (nitroblue tetrazolium salt), 175 μ g/ml X-phosphate (5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt), 240 μ g/ml levamisole (L-)-2,3,5,6-Tetrahydro-6-phenyl-imidazo(2,1-b)-thiazole hydrochloride) in buffer 3. The slides with color solution were then covered with a Parafilm coverslip and incubated 18 hours in a humidified atmosphere. The slides were then rinsed in two changes buffer 3, followed by TE, counterstained with ethidium bromide, dehydrated and coverslipped.

METHODS

Northern Blot Analysis

Total or polyA+ RNA was electrophoresed in 1.2% LE agarose gels containing 2% formaldehyde and 67 ng/ml ethidium bromide. The RNA was partially hydrolyzed by soaking the gel in 50 mM sodium hydroxide, and subsequently transferred in 10X SSC to a nylon membrane (Zeta-Probe, Biorad) essentially as in (Maniatis et al., 1982). ³²P-labeled riboprobes were synthesized for the cAR as described above, using [α -³²P]UTP (800 Ci/mmol, NEN) instead of ³⁵S-labeled nucleotide, to a specific activity of approximately 6×10^8 dpm/ μ g. After purification through Sephadex, the probe was added directly to a hybridization solution, based on the solution used by Church and Gilbert (1984), as modified by Amasino (1986), containing 250 mM sodium chloride, 250 mM sodium phosphate (pH7.2), 7% SDS, 10% polyethylene glycol (MW 8000), 1 mM EDTA, 50% formamide, 20 μ g/ml polyadenylic acid, and approximately 10^6 dpm/ml probe. After hybridization overnight (>16 hr) at 65°C, the filters were washed initially in 0.5X SSC, 0.1% SDS at 65°C and exposed to XAR-5 for three days at -70°C with intensifying screens. If the nsb to ribosomal RNA was apparent, the blots were rewashed at 68°C in "11 mM sodium buffer" (see in situ hybridization washes) and exposed for two weeks. In order to assess the quality of the RNA samples, the integrity of the 28S rRNA band was noted, and the blots were subsequently re-hybridized with a random primed chicken actin clone (see cDNA screening for conditions).

Quantitative PCR

A semi-quantitative rt-pcr assay was developed in order to accurately determine the cAR mRNA levels in different samples. Total RNA or standards were reverse transcribed, amplified using the forward primer and [γ -³²P]ATP end labeled reverse primer, electrophoresed on a non-denaturing 6% acrylamide gel, dried and exposed to film. The bands were then excised and the dpm quantified by liquid scintillation counting. An exponential increase in signal is seen with increasing cycle number

METHODS

between 16 and 23 (dns) as has been observed for other mRNAs, including amyloid precursor protein (Golde et al., 1990), dopamine receptor (Dearry et al., 1990) and IL-1 α (Wang et al., 1989). Twenty three cycles gives good sensitivity (see Figure 44) and the assay is linear over at least five orders of magnitude.

The assays included controls for many of the confounding factors affecting the assessment of RNA levels. The efficiency of the reverse transcription was normalized by spiking each reverse transcription with a control RNA. An internal deletion of 158 bp was created in cAR 2.2 between the forward and reverse primers using the existing *BalI* and *BstEII* restriction endonuclease sites. This deletion was cloned into pBluescript, linearized, and used to transcribe cRNA. 333 fg of this RNA was included in each reverse transcription, and the endogenous cAR signal was normalized to it (see Figure 22). The presence of the internal deletion does not alter the levels of endogenous signal when identical reactions with or without the deletion transcript are assayed (dns). The only interference is at non-physiological levels of cAR, where the levels of the deletion signal is reduced, presumably due to limiting primer (dns). Others have observed both interference and unusual concatamerization at high levels of template, or with a large number of cycles (Golde et al., 1990). Such high levels of cAR cRNA were assayed only for a standard curve of transcribed sense strand cAR cRNA, to which the signals from all unknown RNA samples were compared, but although both interference and concatamerization were observed, when the signals were normalized to the internal deletion, they remained linear ($r^2 = .986$) with respect to the standard curve of lower concentrations cAR (see Figure 44). In addition, the brain samples were normalized to an anonymous canary brain cDNA, HAT-2, which was assayed using a similar protocol (dns).

Each unknown RNA sample was reverse transcribed and amplified at least three times. 500 ng total RNA was reverse transcribed in 10 μ l (conditions as described above), and one fifth of each reaction amplified in a 50 μ l reaction (above). 10 μ l of this pcr reaction was assayed by electrophoresis. In addition, tRNA, *E. coli* total RNA, and

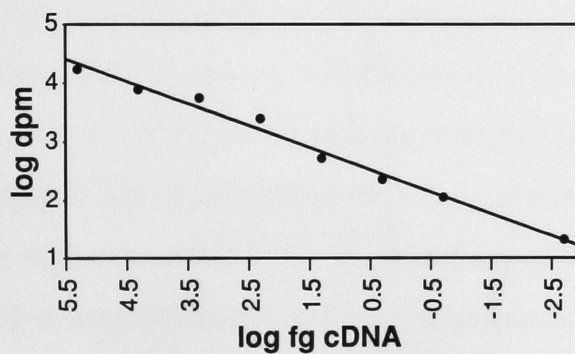
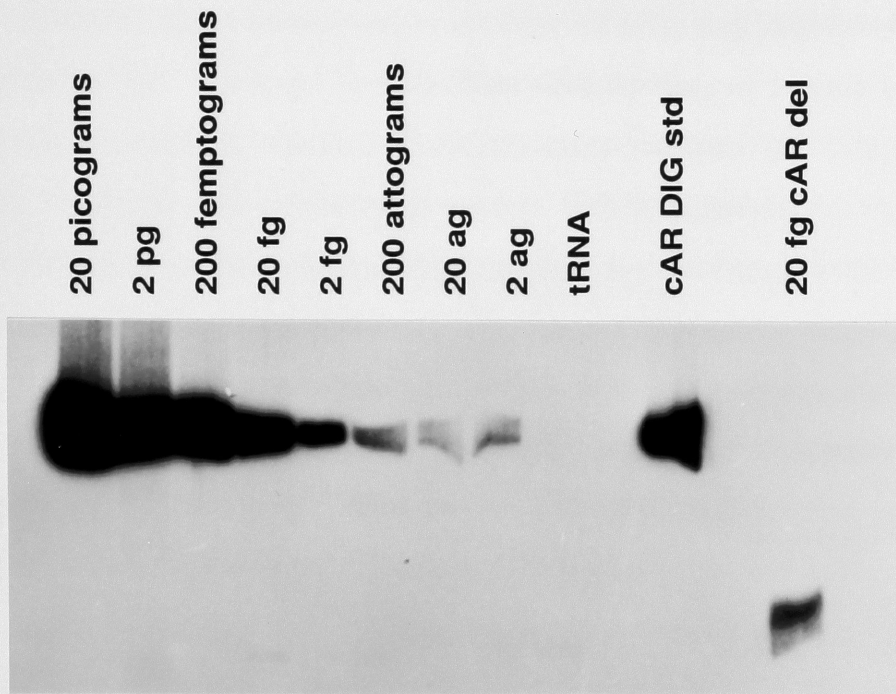
METHODS

canary and/or zebra finch spleen (which is AR (-) (Quarmby, et al., 1990)) RNAs were included in many of the amplifications. None of these sample gave signals of more than 4% of that seen for canary forebrain. All data were then normalized to the standard curve of cAR cRNA which was concurrently measured. The values were then normalized to the internal deletion. Four such reverse transcriptions were analyzed by two independent qpcr assays, and the resulting normalized values were analyzed for statistically significant differences by ANOVA of the repeated measures using the number of qpcr assays (8) and Fisher's paired least significant difference test to determine individual effects.

Figure 44. QPCR Standard Curve. Results of a typical reverse transcription-pcr of cAR cRNA used for the standard curve in each experiment. Note: The cAR deletion cRNA is usually included in all samples. Bottom, graph of dpm versus mass input.

Accuracy of RT-PCR quantitation of the canary androgen receptor

cAR 2.2 cRNA reverse transcribed



$$f(x) = 3.784239E-1 \cdot x + 2.333664E+0$$

$$R^2 = 9.862633E-1$$

METHODS

Animals- surgery, treatments, tissue isolation

Canaries were kindly supplied by F. Nottebohm. They are from a closed colony at Millbrook, NY which was established in 1967. Zebra finches were purchased from Canary Bird Farms, New Brunswick, NJ. All birds were kept on a New York light/dark schedule. The birds were housed in small groups for the duration of all experiments, except the ovariectomized canaries, which were housed with the rest of the colony for one year while they matured.

Juvenile female canaries were ovariectomized when they were between twenty and thirty days old. The surgery must be done when the birds are juvenile since the ovary quickly becomes heavily vascularized and the survival rate drops precipitously at later times (F. Nottebohm, unpublished observations). Within several days of fledging, the birds were anesthetized with Xylazine and Ketamine and their single ovary removed by electrocautery. The birds were fed soft food and kept under heating lamps for until they had recovered from the surgery (usually one day). If the birds developed post-operative infections, they were given oral antibiotics for one week or until their health improved. After three months, blood was collected from each bird for analysis of serum estradiol levels. The overall survival from almost two hundred surgeries was more than 90%.

When the ovariectomized canaries had matured (1 year), they were treated with testosterone or cholesterol. The treatments the first year were single injections into the breast muscle of 2 μ g testosterone (4-Androsten-17 β -ol-3-one, Steraloids, Wilton, NH) or cholesterol (5-Cholesten-3 β -ol, Sigma) in 50 μ l sesame oil. 42 birds were treated for six hours with cholesterol and 42 with testosterone, of which three of each were frozen for in situ hybridization. Four additional birds were treated for either 30 min, 60 min, 12 hr, 24 hr, or 7 days with testosterone. The remaining six hour birds, and all of the varied time point birds were sacrificed and their brains dissected for RNA preparations (see below). The brains were dissected by hand by cutting the brains coronally to remove the three mm wide slice which contained HVc. This portion was then removed by making one cut dorsal-ventral approximately 3.5 mm from the midline, and another medial-lateral 2-3

METHODS

mm ventral from the dorsal surface of the brain. This piece of tissue was called "HVC". The brain was then separated into forebrain and non-forebrain by separating along the third ventricle (see Figure 29). All of these tissues were stored in liquid nitrogen until RNA was made. Trunk blood was also collected for determination of plasma steroid levels.

Seventy canaries were treated with steroids the following spring using slightly different parameters. Thirteen birds were treated under each of the following conditions: 45 min testosterone, 45 min cholesterol, 6 hours testosterone, 24 hour testosterone, or 7 days testosterone. Three birds from each group had their brains frozen for in situ hybridization. The remainder were dissected as outlined above.

Male canaries and zebra finches were treated with androgen and drugs which interact with it. Ethane dimethane sulphonate (EDS), was a kind gift of H. Jackson of the University of Manchester. Flutamide was a gift from R. Neri of Schering Corp. The treatment of both types of birds followed the same protocol: Sixteen fall birds were divided into groups of four, one of which was left untreated. One group received a silastic implant (0.03/0.65 mm internal/external diameter, 5 mm long) of flutamide. The remaining two groups were injected i.p. with 50 µl PBS containing 1.875 mg EDS. All groups were returned to their cages for one week, when one of the EDS groups was implanted with testosterone silastics (same as flutamide). Unfortunately, seven of the eight canaries died, and so the testosterone implantation could not take place. After an additional week, the birds were sacrificed, their brains frozen for in situ hybridization. Kidney, liver, testis and spleen were collected from the birds for RNA preparation. In addition, trunk blood was collected to measure serum testosterone levels.

Radioimmunoassays for steroid hormones

Radioimmunoassays (RIA) for multiple hormones were performed exactly as detailed in (Wingfield and Farner, 1975). RIAs for estrogen alone or testosterone alone were performed exactly as described (Wingfield and Farner, 1975) except instead of

METHODS

eluting the steroid individually of Celite minicolumns, the plasma was only extracted once using dichloromethane and then assayed. This procedure did not measurably affect any aspect of the RIA.

S115 cells

S115 cells were a gift from P. Darbre. They were cultured and treated with steroids exactly as described (Darbre and King, 1987c). To measure the proliferation rate of the cells, they were plated in either petri or tissue culture 50 mm dishes (falcon) at approximately 5×10^5 cells per plate. The medium in tissue culture dishes was changed every three days, while the petri dishes had 1 ml fresh medium added every three days. Three dishes were lysed using zaponin and counted in a Coulter machine for each time point. The steroids used were those used to treat the birds, plus 17β -estradiol (1,3,5(10)-estratrien-3,17 β -diol, Steraloids).

Cells used for RNA preparation were added in 20 mls steroid-free medium at 10^5 cells/ml to 150 mm tissue culture dishes. After the cells had reached approximately 70% confluence, testosterone was added for 1, 4, 24, or 48 hours and then harvested (Sambrook et al., 1989). The control was allowed to grow 24 hours and then harvested. The cells treated with drugs were cultured the same way. At 70% confluence, testosterone was added to one half of the plates and they were allowed to grow for 24 hours. One half of the treated and untreated group were then treated with A23187, forskolin, or TPA (all Sigma). After one hour, all of the plates were harvested.

RNA preparation

Total cellular RNA was prepared from all canary tissues using either by the guanidinium-CsCl method (Kaplan et al., 1979; Glisin et al., 1974; Chirgwin et al., 1979) or by acid guanidinium thiocyanate phenol chloroform extraction (Chomczynski and Sacchi, 1987). RNA (measured by absorbance at 260 nm) was recovered at approximately 1mg per gram tissue. Poly A+ RNA was isolated by oligo dT

METHODS

chromatography (Maniatis et al., 1982). Total cytoplasmic RNA was prepared from the S115 cells as described (Sambrook, et al., 1989).

cDNA-dots hybridizations

Recombinant plasmid DNAs were applied to nylon filters (Zeta-Probe) using a Biorad dot blot apparatus. For each experiment, all of the filters used were made from the same dilutions of the plasmids, so each dot contains exactly the same amount of DNA, which was targeted to be 2 µg each. The filters were pre-hybridized in the same solution as the Northern blots, lacking the formamide, at 65°C for at least 8 hours. Identical dpm were added to each bag for each experiment (typically 5X⁶ dpm). Probes were allowed to hybridize at 65°C with shaking for 36 to 40 hours and then the filters were pre-washed in 2X SSC, 0.1% SDS. They were then washed at 65°C twice for one half hour in 0.5X SSC, 0.1% SDS. The filters were exposed to film to identify any areas of obvious nsb, and then cut out and quantified in a liquid scintillation counter.

Each value was then normalized to the adjusted mean dpm for α and β tubulins after the filter background had been subtracted. The tubulins do not vary in any condition analyzed, neither in terms of absolute dpm, nor when normalized to each other. This normalization is necessary, however, to reduce the variation from different hybridization conditions (different places in the waterbath, slight differences in input dpm, etc.). Differences in filter background due to inherent variations between filters "stickiness" or differences in washing conditions (stuck to the side versus free floating in the shaking waterbath) are eliminated by subtracting the absolute dpm value for the "water blank" dot. These adjusted, normalized data were then analyzed by factorial multi-comparison ANOVA using Statview.

cDNA Probe Preparation

Total RNA was used to synthesize labeled cDNA for hybridization. A typical reaction contained 2µg total RNA, 200 µCi [α-³²P]dCTP (3000 Ci/mmol, NEN), 10 U

METHODS

RNasin, and 9 U AMV reverse transcriptase (Life Sciences) in 5.3 μ l containing 100 mM Tris (pH 8.3), 12 mM magnesium chloride, 140 mM potassium chloride, 200 ng/ μ l oligo dT₁₂₋₁₈, 200 ng/ μ l Actinomycin D, 20 mM DTT, 2 mM d(AGT)TP, and 8 μ M dCTP. The reaction was incubated at 42°C for 60 min and was then stopped by diluting to 50 μ l with EDTA/water (f.c. 10 mM). Incorporation was measured using DE81 paper as described in Clayton et al., (Clayton et al., 1985a). The RNA was degraded in 100 mN sodium hydroxide at 65°C for 30 min, the solution neutralized and the RNA separated from unincorporated dCTP by Sephadex G-50 spun chromatography (see riboprobes). Incorporation rates for both canary and S115 RNA were approximately 5%.

Plasmids

Rodent DNAs used in the dot hybridizations were grown in 1 liter preps as described (Sambrook et al., 1989). DNAs were quantified by absorbance at 260 nm and checked for contaminating RNA and quantity by agarose gel electrophoresis. The pCF clones (including pCR-37, vimentin) and the chicken actin gene used are been described in Clayton et al., (1982) and the HAT clones in George and Clayton (1992). Canary Gap 43 was cloned by low stringency screening and subcloned into pBluescript. The canary mycs were also cloned into pBluescript (Breakefield and Stern, 1986). Canary egr (ZENK) was cloned by low stringency hybridization and subcloned into pBluescript. Other chicken genes use were the c-fos cDNA (a gift from D. Alcorta) which is in pBluescript, and the estrogen receptor (a gift from P. Chambon) which is in pEMBL. CHO A and B, t-argenine, α and β tubulin, and 28S are described in Clayton et al., (Clayton et al., 1985b). The rat AR is a generous gift of C. Chang and is in pGEM4 (Muller et al., 1988). The mouse Histone H1 gene is a gift from N. Heintz and is in pUC 18. Mouse NGFI-A and B (egr and nur77) were from J. Milbrandt and are described (Wilson et al., 1991; Milbrandt, 1987). pTIS21 was from D. Foster and was subcloned in pGEM4, as described in Lim, et al., (1987). Mouse c-myc contains the second and third exons with the intervening intron cloned in pBR322, a gift from R. Collum. Finally,

METHODS

mouse c-jun, a gift from D. Foster, is cloned in pIBI and is described in Lamph, et al., (Lamph et al., 1988). Vectors were from Promega (pGem3, lambda gt10, lambda gem2 and 4), Stratagene (pBluescript, lambda zap), and the Darnell lab (pBR322).

CHAPTER 7: REFERENCES

- Adler, S., Waterman, M.L., He, X., and Rosenfeld, M.G. (1988). Steroid Receptor-Mediated Inhibition of Rat Prolactin Gene Expression Does Not Require the Receptor DNA-Binding Domain. *Cell* **52**, 685-695.
- Alexander, I.E., Shine, J., and Sutherland, R.L. (1990). Progesterone Regulation of Estrogen Receptor Messenger RNA in Human Breast Cancer Cells. *Mol. Endocrinol.* **4**, 821-828.
- Amasino, R.M. (1986). Acceleration of nucleic acid hybridization rate by polyethylene glycol. *Analytical Biochemistry* **152**, 304-307.
- Arnold, A.P. (1981). Quantitative Analysis of Sex Differences in Hormone Accumulation in the Zebra Finch Brain: Methodological and Theoretical Issues. *J. Comp. Neurol.* **189**, 421-436.
- Arnold, A.P. and Saltiel, A. (1979). Sexual Differences in Pattern of Hormone Accumulation in the Brain of a Songbird. *Sci.* **205**, 702-705.
- Ashburner, M. (1990). Puffs, Genes, and Hormones Revisited. *Cell* **61**, 1-3.
- Balthazart, J., Foidart, A., Wilson, E.M., and Ball, G.F. (1992). Immunocytochemical Localization of Androgen Receptors in the male Songbird and Quail Brain. *J. Comp. Neurol.* (in press)
- Barracrough, C.A. (1961). Production of anovulatory, Sterile rats by single injections of testosterone propionate. *Endocrin.* **68**, 62-67.
- Bayliss, D.A. and Millhorn, D.E. (1991). Chronic estrogen exposure maintains elevated levels of progesterone receptor mRNA in Guinea pig hypothalamus. *Mol. Br. Res.* **10**, 167-172.
- Beato, M. (1989). Gene regulation by steroid hormones. *Cell* **56**, 335-344.

REFERENCES

- Berg, J.M. (1989). DNA Binding Specificity of Steroid Receptors. *Cell* **57**, 1065-1068.
- Bishop, J.M. (1985). Viral Oncogenes. *Cell* **42**, 23-38.
- Blackwell, T.K., Kretzner, L., Blackwood, E.M., Eisenman, R.N., and Weintraub, H. (1990). Sequence-Specific DNA Binding by the c-Myc Protein. *Sci.* **250**, 1149-1151.
- Bogic, J., Gerlach, J.L., and McEwen, B.S. (1988). The Ontogeny of Sex Differences in Estrogen-Induced Progesterone Receptors in Rat Brain. *Endocrin.* **122**, 2735-2741.
- Bottjer, S.W. (1987). Ontogenetic Changes in the Pattern of Androgen Accumulation in Song-control Nuclei of male Zebra Finches. *J. Neurobiol.* **18**, 125-139.
- Brawerman, G. (1989). mRNA Decay: Finding the Right Targets. *Cell* **57**, 9-10.
- Breakefield, X.O. and Stern, D.F. (1986). Oncogenes in neural tumors. *TINS* **9**, 150-155.
- Breedlove, S.M. (1985). Hormonal Control of the Anatomical Specificity of Motoneuron-to-Muscle Innervation in Rats. *Sci.* **227**, 1537-1539.
- Brenowitz, E.A., Nalls, B., Wingfield, J.C., and Kroodsma, D.E. (1991). Seasonal Changes in Avian Song Nuclei without Seasonal Changes in Song Repertoire. *J. Neurosci.* **11**, 1367-1374.
- Buonomano, D.V. and Byrne, J.H. (1990). Long-Term Synaptic Changes Produced by a Cellular Analog of Classical Conditioning in Aplysia. *Sci.* **249**, 420-423.
- Burtis, K.C., Thummel, C.S., Jones, C.W., Karim, F.D., and Hogness, D.S. (1990). The *Drosophila* 74EF Early Puff Contains E74, a Complex Ecdysone-Inducible Gene That Encodes Two ets-Related Proteins. *Cell* **61**, 85-99.
- Buttayan, R., Zakeri, Z., Lockshin, R., and Wolgemuth, D. (1988). Cascade Induction of c-fos, c-myc, and Heat Shock 70K Transcripts during Regression of the Rat Ventral Prostate Gland. *Mol. Endocrinol.* **2**, 650-657.

REFERENCES

- Chang, C., Kokontis, J., and Liao, S. (1988a). Structural analysis of complementary DNA and amino acid sequences of human and rat androgen receptors. *Proc. Natl. Acad. Sci. USA* **85**, 7211-7215.
- Chang, C., Kokontis, J., and Liao, S. (1988b). Molecular Cloning of Human and Rat Complementary DNA Encoding Androgen Receptors. *Sci.* **240**, 324-326.
- Chang, F.F. and Greenough, W.T. (1984). Transient and Enduring Morphological Correlates of Synaptic Activity and Efficacy Change in the Rat Hippocampal Slice. *Brain Res.* **309**, 35-46.
- Chirgwin, J.M., Przybyla, A., MacDonald, R., and Rutter, W.J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochem.* **18**, 5294-5299.
- Chomczynski, P. and Sacchi, N. (1987). Single-Step Method of RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction. *Analytical Biochemistry* **162**, 156-159.
- Christy, B.A., Lau, L.F., and Nathans, D. (1988). A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "zinc finger" sequences. *Proc. Natl. Acad. Sci. USA* **85**, 7857-7861.
- Church, G.M. and Gilbert, G. (1984). Genomic Sequencing. *Proc. Natl. Acad. Sci. USA* **81**, 1991-1995.
- Clayton, D.F., Harrelson, A., and Darnell, J.E.Jr. (1985a). Dependence of liver-specific transcription on tissue organization. *Mol. Cell. Biol.* **5**, 2623-2632.
- Clayton, D.F., Weiss, M., and Darnell, J.E.Jr. (1985b). Liver-Specific RNA Metabolism in Hepatoma Cells: Variations in Transcription Rates and mRNA Levels. *Mol. Cell. Biol.* **5**, 2633-2641.
- Clayton, D.F., Huecas, M.E., Sinclair-Thompson, E.Y., Nastiuk, K.L., and Nottebohm, F. (1988). Probes for rare mRNAs reveal distributed cell subsets in canary brain. *Neuron* **1**, 249-261.

REFERENCES

- Clayton, D.F. and Huecas, M.E. (1990). Forebrain-enriched RNAs of the canary: a population analysis using hybridization kinetics. *Mol. Br. Res.* **7**, 23-30.
- Coirini, H., Schumacher, M., Flanagan, L.M., and McEwen, B.S. (1991). Transport of Estrogen-induced Oxytocin Receptors in the Ventromedial Hypothalamus. *J. Neurosci.* **11**, 3317-3324.
- Conneely, O.M., Sullivan, W.P., Toft, D.O., Birnbaumer, M., Cook, R.G., Maxwell, B.L., Zarucki-Schulz, T., Greene, G.L., Schrader, W.T., and O'Malley, B.W. (1986). Molecular Cloning of the Chicken Progesterone Receptor. *Sci.* **233**, 767-770.
- Darbre, P.D., Dickson, C., Peters, G., Page, M., Curtis, S., and King, R.J.B. (1983). Androgen regulation of cell proliferation and expression of viral sequences in mouse mammary tumour cells. *Nature* **303**, 431-433.
- Darbre, P.D. and King, R.J.B. (1984). Progression to Steroid Autonomy in S115 Mouse Mammary Tumor Cells: role of DNA Methylation. *J. Cell Biol.* **9**, 1410-1415.
- Darbre, P.D. and King, R.J.B. (1987a). Differential Effects of Steroid Hormones on Parameters of Cell Growth. *Cancer Res.* **47**, 2937-2944.
- Darbre, P.D. and King, R.J.B. (1987b). Interaction of Different Steroid Hormones During Progression of Tumour Cells to Steroid Autonomy. *International Journal of Cancer* **40**, 802-806.
- Darbre, P.D. and King, R.J.B. (1987c). Progression to Steroid Insensitivity Can Occur irrespective of the Presence of Functional Steroid Receptors. *Cell* **51**, 521-528.
- Dearry, A., Gingrich, J.A., Falardeau, P., Freneau, R.T.Jr., Bates, M.D., and Caron, M.G. (1990). Molecular cloning and expression of the gene for a human D1 dopamine receptor. *Nature* **347**, 72-76.
- Devoogd, T.J., Nixdorf, B., and Nottebohm, F. (1985). Synaptogenesis and changes in synaptic morphology related to acquisition of a new behavior. *Brain Res.* **329**, 304-308.

REFERENCES

- Devoogd, T.J. (1986). Steroid Interactions with Structure and Function of Avian Song Control Regions. *J. Neurobiol.* **17**, 177-201.
- Devoogd, T.J. and Nottebohm, F. (1981). Gonadal Hormones Induce Dendritic Growth in the Adult Avian Brain. *Sci.* **214**, 202-204.
- Diamond, M.I., Miner, J.N., Yoshinaga, S.K., and Yamamoto, K.R. (1990). Transcription Factor Interactions: Selectors of Positive or Negative Regulation from a Single DNA Element. *Sci.* **249**, 1266-1272.
- Dohler, K.D., Coquelin, A., Davis, F., Hines, M., Shryne, J.E., Sickmoller, P.M., Jarzab, B., and Gorski, R.A. (1986). Pre- and Postnatal Influence of an Estrogen Antagonist and an Androgen Antagonist on Differentiation of the Sexually Dimorphic Nucleus of the Preoptic Area in Male and Female Rats. *Neuroendocrinology* **42**, 443-448.
- Dohler, K.D. (1987). The Special case of hormonal imprinting, the neonatal influence of sex. In *Development of Hormone Receptors*. G. Csaba, ed. (Basel: Birkhauser Verlag), pp. 103-124.
- Doucas, V., Spyrou, G., and Yaniv, M. (1991). Unregulated expression of c-Jun or c-fos proteins but not Jun D inhibits oestrogen receptor activity in human breast cancer derived cells. *EMBO* **10**, 2237-2245.
- Evans, R.M. (1988). the Steroid and Thyroid Hormone Receptor Superfamily. *Sci.* **240**, 889-895.
- Evans, R.M. and Arriza, J.L. (1989). A molecular framework for the actions of glucocorticoid hormones in the nervous system. *Neuron* **2**, 1105-1112.
- Faber, P.W., Van Rooij, H.C.J., Van der Korput, J.A.G.M., Baarends, W.M., Brinkmann, A.O., Grootegoed, J.A., and Trapman, J. (1991). Characterization of the Human Androgen Receptor Transcription Unit. *J. Biol. Chem.* **266**, 10743-10749.

REFERENCES

- Fawell, S.E., Lees, J.A., White, R., and Parker, M.G. (1990). Characterization and Colocalization of Steroid Binding and Dimerization Activities in the Mouse Estrogen Receptor. *Cell* **60**, 953-962.
- Feinberg, A.P. and Vogelstein, B. (1983). A Technique for Radiolabelling DNA Restriction Endonuclease Fragments to High Specific Activity. *Analytical Biochemistry* **132**, 6-13.
- Feinberg, A.P. and Vogelstein, B. (1984). A Technique for Radiolabelling DNA Restriction Endonuclease Fragments to High specific Activity: an Addendum. *Analytical Biochemistry* **137**, 266-267.
- Ferreira, A. and Caceres, A. (1991). Estrogen-Enhanced Neurite Growth: Evidence for a Selective Induction of Tau and Stable Microtubules. *J. Neurosci.* **11**, 392-400.
- Frohman, M.A., Dush, M.K., and Martin, G.R. (1988). Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* **85**, 8998-9002.
- Gahr, M., Fluge, G., and Guttinger, H.R. (1987). Immunocytochemical localization of estrogen binding neurons in the songbird brain. *Brain Res.* **402**, 173-177.
- Gahr, M. (1990a). Localization of androgen receptors and estrogen receptors in the same cells of the songbird brain. *Proc. Natl. Acad. Sci. USA* **87**, 9445-9448.
- Gahr, M. (1990b). Delineation of a Brain Nucleus: Comparisons of Cytochemical, Hodological, and Cytoarchitectural View of the Song Control Nucleus HVC of the Adult Canary. *J. Comp. Neurol.* **294**, 30-36.
- Gahr, M. and Konishi, M. (1988). Developmental changes in estrogen-sensitive neurons in the forebrain of the zebra finch. *Proc. Natl. Acad. Sci. USA* **85**, 7380-7383.
- Gaspar, M.L., Meo, T., and Tosi, M. (1990). Structure and Size Distribution of the Androgen Receptor mRNA in Wild-Type and Tfm/y Mutant Mice. *Mol. Endocrinol.* **4**, 1600-1610.

REFERENCES

- George, J.M. and Clayton, D.F. (1992). Differential regulation in the avian song control circuit of a mRNA predicting a highly conserved protein related to protein kinase C and the bcr oncogene. *Mol. Br. Res.* **12**, 323-329.
- Gewirtz, A.M., Anfossi, G., Venturelli, D., Valpreda, S., Sims, R., and Calabretta, B. (1989). G_i /S Transition in Normal Human t-Lymphocytes requires the Nuclear Protein Encoded by c-myc. *Sci.* **245**, 180-183.
- Gibbs, R.B., Lombardino, A., and Pfaff, D.W. (1990a). Sex Steroids and Fos Expression in the CNS of Prepubertal and Newborn Rats. *Mol. Cell. Neuro.* **1**, 250-261.
- Gibbs, R.B., Mobbs, C.V., and Pfaff, D.W. (1990b). Sex Steroids and Fos Expression in Rat Brain and Uterus. *Mol. Cell. Neuro.* **1**, 29-40.
- Giguere, V., Hollenberg, S.M., Rosenfeld, M.G., and Evans, R.M. (1986). Functional Domains of the Human Glucocorticoid Receptor. *Cell* **46**, 645-652.
- Glanzman, D.L., Kandel, E.R., and Schacher, S. (1990). Target-Dependent Structural Changes Accompanying Long-Term Synaptic Facilitation in Aplysia Neurons. *Sci.* **249**, 799-802.
- Glisin, V., Crkvenjakov, R., and Byus, C. (1974). Ribonucleic acid isolated by cesium chloride centrifugation. *Biochem.* **13**, 2633-2637.
- Golde, T.E., Estus, S., Usiak, M., Younkin, L.H., and Younkin, S.G. (1990). Expression of a Amyloid Protein Precursor mRNAs: Recognition of a Novel Alternatively Spliced Form and Quantitation in Alzheimers' Disease Using PCR. *Neuron* **4**, 253-267.
- Goldstein, L.A., Kurz, E.M., and Sengelaub, D.R. (1990). Androgen Regulation of Dendritic Growth and Retraction in the Development of a Sexually Dimorphic Spinal Nucleus. *J. Neurosci.* **10**, 935-946.
- Gorski, R.A., Gordon, J.H., Shryne, J.E., and Southam, A.M. (1978). Evidence for a morphological sex difference within the medial preoptic area of the rat brain. *Brain Res.* **148**, 333-346.

REFERENCES

- Gorski, R.A., Harlan, R.E., Jacobson, C.D., Shryne, J.E., and Southam, A.M. (1980). Evidence for the Existence of a Sexually Dimorphic Nucleus in the Preoptic Area of the Rat. *J. Comp. Neurol.* **193**, 529-539.
- Gould, E., Westlind-Danielsson, A., Frankfurt, M., and McEwen, B.S. (1990a). Sex Differences and Thyroid Hormone Sensitivity of Hippocampal Pyramidal Cells. *J. Neurosci.* **10**, 996-1003.
- Gould, E., Woolley, C.S., Frankfurt, M., and McEwen, B.S. (1990b). Gonadal Steroids Regulate Dendritic Spine Density in Hippocampal Pyramidal Cells in Adulthood. *J. Neurosci.* **10**, 1286-1291.
- Govindan, M.V. (1990). Specific Region in Hormone Binding Domain Is Essential for Hormone Binding and Trans-Activation by Human Androgen Receptor. *Mol. Endocrinol.* **4**, 417-427.
- Goy, R.W. and McEwen, B.S. (1980). *Sexual differentiation of the brain* (Cambridge, Mass.: MIT Press).
- 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 specificity. *EMBO Journal* **7**, 3037-3044.
- Gurney, M.E. (1981). Hormonal Control of Cell form and Number in the Zebra Finch Song System. *J. Neurosci.* **1**, 658-673.
- Gurney, M.E. (1982). Behavioral correlates of sexual differentiation in the zebra finch song system. *Brain Res.* **231**, 153-172.
- Gurney, M.E. and Konishi, M. (1980). Hormone-Induced Sexual Differentiation of Brain and Behavior in Zebra Finches. *Sci.* **208**, 1380-1383.
- Ham, J. and Parker, M.G. (1989). Regulation of gene expression by nuclear hormone receptors. *Curr. Opinion Cell Biol.* **1**, 503-511.

REFERENCES

- Harris, G.W. (1964). Sex Hormones, Brain Development and Brain Function. *Endocrin.* **75**, 627-648.
- Harris, G.W. (1970). Hormonal differentiation of the developing central nervous system with respect to patterns of endocrine function. *Phil. Trans. Roy. Soc. Lond. B.* **259**, 165-177.
- Hayashi, S., Gillam, I., Delaney, A., and Tener, G. (1978). Acetylation of chromosome squashes of *Drosophila melanogaster* decreases the background in autoradiographs from hybridization with ¹²⁵I-labeled RNA. *Journal of Histochemistry and Cytochemistry* **26**, 677-679.
- Hazel, T.G., Nathans, D., and Lau, L.F. (1988). A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. *Proc. Natl. Acad. Sci. USA* **85**, 8444-8448.
- He, W.W., Fischer, L.M., Sun, S., Bilhartz, D.L., Zhu, X., Young, C.Y-F., Kelley, D.B., and Tindall, D.J. (1990). Molecular Cloning of Androgen Receptors from Divergent species with a Polymerase Chain Reaction Technique: Complete cDNA Sequence of the Mouse Androgen Receptor and Isolation of Androgen Receptor cDNA Probes from Dog, Guinea Pig and Clawed Frog. *Biochem. Biophys. Res. Comm.* **171**, 697-704.
- Herrmann, J., lee, P., Saya, H., and Nakajima, M. (1990). Application of Polymerase Chain Reaction for Rapid Subcloning of cDNA Inserts from lambda gt11 Clones. *BioTechniques* **8**, 376-381.
- Huang, M.N. and High, K.A. (1990). Efficient Subcloning of DNA Fragment Amplified by Crude Oligonucleotides. *BioTechniques* **9**, 710-711.
- Immelmann, K. (1969). Song Development in the Zebra Finch and Other Estrildid Finches. In *Bird Vocalization*. R.A. Hinde, ed. (Cambridge: Cambridge University Press), pp. 61-74.

REFERENCES

- Isomma, V., Parvinen, M., Janne, O.A., and Bardin, C.W. (1985). Nuclear Androgen Receptors in Different Stages of the Seminiferous Epithelial Cycle and the Interstitial Tissue of Rat Testis. *Endocrin.* **116**, 132-137.
- Jackson, A.E., O'Leary, P.C., Ayers, M.M., and de Kretser, D.M. (1986). The Effects of Ethylene dimethane Sulphonate (EDS) on Rat Leydig Cells: Evidence to Support a Connective Tissue Origin of Leydig Cells. *Biol. Reprod.* **35**, 425-437.
- Jackson, C.M. and Jackson, H. (1984). Comparative protective actions of gonadotrophins and testosterone against the antispermatogenic action of ethane dimethanesulphonate. *J. Reprod. Fert.* **71**, 393-401.
- Jonat, C., Rahmsdorf, H.J., Park, K.-K., Cato, A.C.B., Gebel, S., Ponta, H., and Herrlich, P. (1990). Antitumor Promotion and Antiinflammation: Down-Modulation of AP-1 (Fos/Jun) Activity by Glucocorticoid Hormone. *Cell* **62**, 1189-1204.
- Jones, K.J., Harrington, C.A., Chikaraishi, D.M., and Pfaff, D.W. (1990). Steroid Hormone Regulation of Ribosomal RNA in Rat Hypothalamus: Early Detection Using in situ Hybridization and Precursor-Product Ribosomal DNA Probes. *J. Neurosci.* **10**, 1513-1521.
- Kaplan, B.B., Bernstein, S., and Gioio, A. (1979). An improved method for the rapid isolation of brain ribonucleic acid. *Biochem. J.* **183**, 181-184.
- Kaplan, L.M., Gabriel, S.M., Koenig, J.I., Sunday, M.E., Spindel, E.R., Martin, J.B., and Chin, W.W. (1988). Galanin is an estrogen-inducible, secretory product of the rat anterior pituitary. *Proc. Natl. Acad. Sci. USA* **85**, 7408-7412.
- Kelley, D.B. and Nottebohm, F. (1979). Projections of a Telencephalic Auditory Nucleus- Field L- in the Canary. *J. Comp. Neurol.* **183**, 455-470.
- Komm, B.S., Terpening, C.M., Benz, D.J., Graeme, K.A., Gallegos, A., Korc, M., Greene, G.L., O'Malley, B.W., and Haussler, M.R. (1988). Estrogen Binding, Receptor mRNA, and Biologic Response in Osteoblast-Like Osteosarcoma Cells. *Sci.* **241**, 81-84.

REFERENCES

- Konishi, M. (1965). The Role of Auditory Feedback in the Control of Vocalization in the White-Crowned Sparrow. *Z. Tierpsychol.* **22**, 770-783.
- Konishi, M. (1985). Birdsong: from behavior to neuron. *Annu. Rev. Neurosci.* **8**, 125-170.
- Konishi, M. (1989). Birdsong for Neurobiologists. *Neuron* **3**, 541-549.
- Konishi, M., Emlen, S.T., Ricklefs, R.E., and Wingfield, J.C. (1989). Contributions of Bird Studies to Biology. *Sci.* **246**, 465-472.
- Konishi, M. and Akutagawa, E. (1981). Androgen increases protein synthesis within the avian brain vocal control system. *Brain Res.* **222**, 442-446.
- Konishi, M. and Akutagawa, E. (1985). Neuronal growth, atrophy and death in a sexually dimorphic song nucleus in the zebra finch. *Nature* **315**, 145-147.
- Konishi, M. and Akutagawa, E. (1988). A critical period for estrogen action on neurons of the song control system in the zebra finch. *Proc. Natl. Acad. Sci. USA* **85**, 7006-7007.
- Kornack, D.R., Lu, B., and Black, I.B. (1991). Sexually dimorphic expression of the NGF receptor gene in the developing rat brain. *Brain Res.* **542**, 171-174.
- Korsia, S. and Bottjer, S.W. (1989). Developmental Changes in the Cellular Composition of a Brain Nucleus Involved with Song Learning in Zebra Finches. *Neuron* **3**, 451-460.
- Krust, A., Green, S., Argos, P., Kumar, V., Walter, P., Bornert, J.-M., and Chambon, P. (1986). The chicken oestrogen receptor sequence: homology with v-erbA and the human oestrogen and glucocorticoid receptors. *EMBO* **5**, 891-897.
- Kuiper, G.G.J.M., Faber, P.W., Van Rooij, H.C.J., Van der Korput, J.A.G.M., Ris-Stalpers, C., Klaassen, P., Trapman, J., and Brinkmann, A.O. (1989). Structural organization of the human androgen receptor gene. *J. Mol. Endocrinol.* **2**, R1-R4.

REFERENCES

- Kujawa, K.A., Emeric, E., and Jones, K.J. (1991). Testosterone Differentially Regulates the Regenerative Properties of Injured Hamster Facial Motoneurons. *J. Neurosci.* **11**, 3898-3906.
- Kumar, V., Green, S., Stack, G., Berry, M., Jin, J.R., and Chambon, P. (1987). Functional Domains of the Human Estrogen Receptor. *Cell* **51**, 941-951.
- Lamph, W.W., Wamsley, P., Sassone-Corsi, P., and Verma, I.M. (1988). Induction of proto-oncogene JUN/AP1 by serum and TPA. *Nature* **334**, 629-631.
- Lauber, A.H., Romano, G.J., and Pfaff, D.W. (1991). Sex Difference in Estradiol Regulation of Progestin Receptor mRNA in Rat Mediobasal Hypothalamus as Demonstrated by in situ Hybridization. *Neuroendocrinology* **53**, 608-613.
- Laybourn, P.J. and Kadonaga, J.T. (1991). Role of Nucleosomal Cores and Histone H1 in Regulation of Transcription by RNA Polymerase II. *Sci.* **254**, 238-245.
- Lee, C.S.L., Koga, M., and Sutherland, R.L. (1989). Modulation of estrogen receptor and epidermal growth factor receptor mRNAs by phorbol ester in mcf 7 breast cancer cells. *Biochem. Biophys. Res. Comm.* **162**, 415-421.
- Leger, J.G., Montpetit, M.L., and Tenniswood, M.P. (1987). Characterization and cloning of androgen-repressed mRNAs from rat ventral prostate. *Biochem. Biophys. Res. Comm.* **147**, 196-203.
- Leger, J.G., Le Guellec, R., and Tenniswood, M.P. (1988). Treatment With Antiandrogens Induces an Androgen-Repressed Gene in the Rat Ventral Prostate. *Prostate* **13**, 131-142.
- Lim, R.W., Varnum, B.C., and Herschman, H.R. (1987). Cloning of tetradecanoyl phorbol ester-induced 'primary response' sequences and their expression in density-arrested Swiss 3T3 cells and a TPA non-proliferative variant. *Oncogene* **1**, 263-270.

REFERENCES

- Lingham, R.B., Stancel, G.M., and Loose-Mitchell, D.S. (1988). Estrogen Regulation of Epidermal Growth Factor Messenger Ribonucleic Acid. *Mol. Endocrinol.* **2**, 230-235.
- Loose-Mitchell, D.S., Chiappetta, C., and Stancel, G.M. (1988). Estrogen Regulation of c-fos messenger Ribonucleic Acid. *Mol. Endocrinol.* **2**, 946-951.
- Lubahn, D.B., Joseph, D.R., Sullivan, P.M., Willard, H.F., French, F.S., and Wilson, E.M. (1988). Cloning of Human Androgen Receptor Complementary DNA and Localization to the X Chromosome. *Sci.* **240**, 327-330.
- Lubischer, J.I. and Arnold, A.P. (1990). Autoradiographic Localization of Progestin-Concentrating Cells in the Brain of the Zebra Finch. *J. Comp. Neurol.* **291**, 450-456.
- Luine, V., Nottebohm, F., Harding, C., and McEwen, B.S. (1980). Androgen Affects cholinergic Enzymes in Syringeal Motor Neurons and Muscle. *Brain Res.* **192**, 89-107.
- Majewska, M.D., Harrison, N.L., Schwartz, R.D., Barker, J.L., and Paul, S.M. (1986). Steroid Hormone Metabolites Are Barbiturate-Like Modulators of the GABA Receptor. *Sci.* **232**, 1004-1007.
- Maki, Y., Bos, T.J., Davis, C., Starbuck, M., and Vogt, P.K. (1987). Avian sarcoma virus 17 carries the jun oncogene. *Proc. Natl. Acad. Sci. USA* **84**, 2848-2852.
- Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).
- Marcelli, M., Tilley, W.D., Wilson, C.M., Griffin, J.E., Wilson, J.D., and McPhaul, M.J. (1990). Definition of the Human Androgen Receptor Gene Structure Permits the Identification of Mutations that Cause Androgen Resistance: Premature Termination of the Receptor Protein at Amino Acid Residue 588 Causes Complete Androgen Resistance. *Mol. Endocrinol.* **4**, 1105-1116.

REFERENCES

- Marler, P., Mundinger, P., Waser, M.S., and Lutjen, A. (1972). Effects of acoustical stimulation and deprivation on song development in red-winged blackbirds (*Agelaius phoeniceus*). *Anim. Behav.* **20**, 586-606.
- McEwen, B.S., Davis, P.G., Parsons, B., and Pfaff, D.W. (1979). The brain as a target for steroid hormone action. *Annu. Rev. Neurosci.* **2**, 65-112.
- McKenzie, E.A. and Knowland, J. (1990). High Concentrations of Estrogen Stabilize Vitellogenin mRNA against Cytoplasmic Degradation but Physiological Concentrations do not. *Mol. Endocrinol.* **4**, 807-811.
- Mello, C., Vicario, D.S., and Clayton, D.F. (1991). Song induces "immediate early" gene expression in songbird forebrain. *Soc. Neurosci. Abs.* **17**, 471.3.
- Mello, C., Vicario, D.S., and Clayton, D.F. (1992). Song induces "immediate early" gene expression in songbird forebrain. *Proc. Natl. Acad. Sci. USA*
- Meltzer, S.J., Mane, S.M., Wood, P.K., Johnson, L., and Needleman, W. (1990). Sequencing Products of the Polymerase Chain Reaction Directly, Without Purification. *BioTechniques* **8**, 142-148.
- Miesfeld, R.L. (1990). Molecular Genetics of Corticosteroid Action. *Am. Rev. Respir. Dis.* **141**, s11-s17.
- Milbrandt, J. (1987). A Nerve Growth Factor-Induced Gene Encodes a Possible Transcription Regulatory Factor. *Sci.* **238**, 797-799.
- Mills, A. and Zakon, H.H. (1991). Chronic Androgen Treatment Increases Action Potential Duration in the Electric organ of *Sternopygus*. *J. Neurosci.* **11**, 2349-2361.
- Mobbs, C.V., Fink, G., and Pfaff, D.W. (1990). HIP-70: A Protein Induced by Estrogen in the Brain and LH-RH in the Pituitary. *Sci.* **247**, 1477-1479.
- Morris, I.D., Phillips, D.M., and Bardin, C.W. (1986). Ethylene Dimethanesulfonate Destroys Leydig Cells in the Rat Testis. *Endocrin.* **118**, 709-719.

REFERENCES

- Muller, M.M., Gerster, T., and Schaffner, W. (1988). Enhancer sequences and the regulation of gene transcription. *Eur. J. Biochem.* **176**, 485-495.
- Nabekura, J., Oomura, Y., Minami, T., Mizuno, Y., and Fukuda, A. (1986). Mechanism of the Rapid Effect of 17 α -Estradiol on Medial Amygdala Neurons. *Sci.* **233**, 226-228.
- Nielsen, D.A. and Shapiro, D.J. (1990a). Insights into Hormonal Control of Messenger RNA Stability. *Mol. Endocrinol.* **4**, 953-957.
- Nielsen, D.A. and Shapiro, D.J. (1990b). Estradiol and Estrogen Receptor-Dependent Stabilization of a Mini-vitellogenin mRNA Lacking 5,100 Nucleotides of Coding Sequence. *Mol. Cell. Biol.* **10**, 371-376.
- Nishimura, T. and Vogt, P.K. (1988). The avian cellular homolog of the oncogene jun. *Oncogene* **3**, 659-663.
- Nordeen, E.J., Nordeen, K.W., Sengelaub, D.R., and Arnold, A.P. (1985). Androgens Prevent Normally Occurring Cell Death in a Sexually Dimorphic Spinal Nucleus. *Sci.* **229**, 671-673.
- Nordeen, K.W., Nordeen, E.J., and Arnold, A.P. (1986). Estrogen Establishes Sex Differences in Androgen Accumulation in Zebra Finch Brain. *J. Neurosci.* **6**, 734-738.
- Nordeen, K.W., Nordeen, E.J., and Arnold, A.P. (1987). Estrogen Accumulation in Zebra Finch Song Control Nuclei: Implications for Sexual Differentiation and Adult Activation of Song Behavior. *J. Neurobiol.* **18**, 569-582.
- Nottebohm, F., Stokes, T.M., and Leonard, C.M. (1976). Central Control of Song in the Canary, *Serinus canarius*. *J. Comp. Neurol.* **165**, 457-486.
- Nottebohm, F. (1980a). Brain pathways for vocal learning in birds: a review of the first 10 years. *Prog. Psychobiol. Physiol. Psychol.* **9**, 85-124.

REFERENCES

- Nottebohm, F. (1980b). Testosterone triggers growth of brain vocal control nuclei in adult female canaries. *Brain Res.* **189**, 429-436.
- Nottebohm, F. (1981). A Brain for All Seasons: Cyclical Anatomical Changes in Song Control Nuclei of the Canary Brain. *Sci.* **214**, 1368-1370.
- Nottebohm, F., Kasparian, S., and Pandazis, C. (1981). Brain Space for a Learned Task. *Brain Res.* **213**, 99-109.
- Nottebohm, F., Nottebohm, M.E., and Crane, L. (1986). Development and Seasonal Changes in Canary Song and Their Relation to Changes in the Anatomy of Song-Control Nuclei. *Beh. Neural Biol.* **46**, 445-471.
- Nottebohm, F., Nottebohm, M.E., Crane, L.A., and Wingfield, J.C. (1987). Seasonal Changes in Gonadal Hormone Levels of Adult Male Canaries and Their Relation to Song. *Beh. Neural Biol.* **47**, 197-211.
- Nottebohm, F. (1989). From bird song to neurogenesis. *Sci. Am.* **260**, 74-79.
- Nottebohm, F. and Arnold, A.P. (1976). Sexual Dimorphism in Vocal Control Areas of the Songbird Brain. *Sci.* **194**, 211-213.
- Nottebohm, F. and Nottebohm, M.E. (1978). Relationship between Song Repertoire and Age in the Canary, *Serinus canarius*. *Z. Tierpsychol.* **46**, 298-305.
- O'Malley, B.W. (1990). The Steroid Receptor Superfamily: More Excitement Predicted for the Future. *Mol. Endocrinol.* **4**, 363-369.
- Orchinik, M., Murray, T.F., and Moore, F.L. (1991). A Corticosteroid Receptor in Neuronal Membranes. *Sci.* **252**, 1848-1851.
- Paek, I. and Axel, R. (1987). Glucocorticoids Enhance Stability of Human Growth Hormone mRNA. *Mol. Cell. Biol.* **7**, 1496-1507.

REFERENCES

- Palmer, R., Gallagher, P.M., Boyko, W.L., and Ganschow, R.E. (1983). Genetic control of levels of murine kidney glucuronidase mRNA in response to androgen. *Proc. Natl. Acad. Sci. USA* **80**, 7596-7600.
- Parsons, B., MacLusky, J.F., Krey, L., Pfaff, D.W., and McEwen, B.S. (1980). The Temporal Relationship between Estrogen-Inducible Progesterone Receptors in the Female Rat Brain and the Time Course of Estrogen Activation of Mating Behavior. *Endocrin.* **107**, 774-779.
- Pearson, W.R. and Lipman, D.J. (1988). Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA* **85**, 2444-2448.
- Peiffer, A. and Barden, N. (1987). Estrogen-Induced Decrease of Glucocorticoid Receptor Messenger Ribonucleic Acid Concentration in Rat Anterior Pituitary Gland. *Mol. Endocrinol.* **1**, 435-440.
- Persson, H., Ayer-Le Lievre, C., Soder, O., Villar, M.J., Metsis, M., Olson, L., Ritzen, M., and Hokfelt, T. (1990). Expression of a-Nerve Growth Factor Receptor mRNA in Sertoli Cells Downregulated by Testosterone. *Sci.* **247**, 704-707.
- Pfeiffer, C.A. (1936). Sexual differences of the hypophyses and their determination by the gonads. *Amer. J. Anat.* **58**, 195-225.
- Poulin, R., Simard, J., Labrie, C., Petitclerc, L., Dumont, M., Lagace, L., and Labrie, F. (1989). Down-Regulation of Estrogen Receptors by Androgens in the ZR-75-1 Human Breast Cancer Cell Line. *Endocrin.* **125**, 392-399.
- Poyet, P. and Labrie, F. (1985). Comparison of the antiandrogenic/androgenic activities of flutamide, cyproterone acetate and megestrol acetate. *Mol. Cell. Endo.* **42**, 283-288.
- Quarmby, V.E., Yarbrough, W.G., Lubahn, D.B., French, F.S., and Wilson, E.M. (1990). Autologous Down-Regulation of Androgen Receptor Messenger Ribonucleic Acid. *Mol. Endocrinol.* **4**, 22-28.

REFERENCES

- Read, L.D., Snider, C.E., Miller, J.S., Greene, G.L., and Katzenellenbogen, B.S. (1988). Ligand-Modulated Regulation of Progesterone Receptor Messenger Ribonucleic Acid and Protein in Human Breast Cancer Cell Lines. *Mol. Endocrinol.* **2**, 263-271.
- Reed, K.C. and Mann, D.A. (1985). Rapid transfer of DNA from agarose gels to nylon membranes. *Nucleic Acids Res.* **13**, 7207-7221.
- Rempel, S.A. and Johnston, R.N. (1988). Steroid-induced cell proliferation in vivo is associated with increased c-myc proto-oncogene transcript abundance. *Development* **104**, 87-95.
- Rennie, P.S., Bruchovsky, N., Buttyan, R., Benson, M.C., and Cheng, H. (1988). Gene Expression during the Early Phases of Regression of the Androgen-dependent Shionogi Mouse Mammary Carcinoma. *Cancer Res.* **48**, 6309-6312.
- Rogers, A.W. (1979). *Techniques in Autoradiography* (New York: Elsevier Science Publishing, Inc.).
- Rundlett, S.E., Wu, X.P., and Miesfeld, R.L. (1990). Functional Characterizations of the Androgen Receptor Confirm that the Molecular Basis of Androgen Action is Transcriptional Regulation. *Mol. Endocrinol.* **4**, 708-714.
- Ruppert, C., Goldowitz, D., and Wille, W. (1986). Proto-oncogene c-myc is expressed in cerebellar neurons at different developmental stages. *EMBO* **5**, 1897-1901.
- Ryder, K., Lanahan, A., Perez-Albuern, E., and Nathans, D. (1989). Jun-D: A third member of the Jun gene family. *Proc. Natl. Acad. Sci. USA* **86**, 1500-1503.
- Salaman, D.F. and Birkett, S. (1974). Androgen-induced Sexual Differentiation of the Brain is Blocked by Inhibitors of DNA and RNA Sythesis. *Nature* **247**, 109-112.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular cloning: a laboratory manual* (Cold Spring Harbor: Cold Spring Harbor Laboratory Press).

REFERENCES

- Sanborn, B.M., Steinberger, A., Meistrich, M.L., and Steinberger, E. (1975). Androgen binding sites in testis cell fractions as measured by a nuclear exchange assay. *J. Steroid Biochem.* **6**, 1459-1465.
- Sar, M., Lubahn, D.B., French, F.S., and Wilson, E.M. (1990). Immunohistochemical Localization of the Androgen Receptor in Rat and Human Tissues. *Endocrin.* **127**, 3180-3186.
- Sarkar, G. and Sommer, S.S. (1990). Shedding light on PCR contamination. *Nature* **343**, 27.
- Scharff, C. and Nottebohm, F. (1991). A Comparative Study of the Behavioral Deficits following Lesions of Various Parts of the Zebra Finch Song System: Implications for Vocal Learning. *J. Neurosci.* **11**, 2896-2913.
- Schlinger, B.A. and Arnold, A.P. (1991). Brain is the major site of estrogen synthesis in a male songbird. *Proc. Natl. Acad. Sci. USA* **88**, 4191-4194.
- Schule, R., Rangarajan, P., Kliwer, S., Ransone, L.J., Bolado, J., Yang, N., Verma, I.M., and Evans, R.M. (1990a). Functional Antagonism between Oncoprotein c-Jun and the Glucocorticoid Receptor. *Cell* **62**, 1217-1226.
- Schule, R., Umesono, K., Mangelsdorf, D.J., Bolado, J., Pike, J.W., and Evans, R.M. (1990b). Jun-Fos and Receptors for Vitamins A and D Recognize a Common Response Element in the Human Osteocalcin Gene. *Cell* **61**, 497-504.
- Schumacher, M. (1990). Rapid membrane effects of steroid hormones: an emerging concept in neuroendocrinology. *TINS* **13**, 359-362.
- Schumacher, M., Coirini, H., Pfaff, D.W., and McEwen, B.S. (1990). Behavioral Effects of Progesterone Associated with Rapid Modulation of Oxytocin Receptors. *Sci.* **250**, 691-694.
- Shan, L.-X., Rodriguez, M.C., and Janne, O.A. (1990). Regulation of Androgen Receptor Protein and mRNA Concentrations by Androgens in Rat Ventral Prostate and Seminal Vesicles and in Human Hepatoma Cells. *Mol. Endocrinol.* **4**, 1636-1646.

REFERENCES

- Shaw, G. and Kamen, R. (1986). A Conserved AU Sequence from the 3' Untranslated Region of GM-CSF mRNA Mediates Selective mRNA Degradation. *Cell* **46**, 659-667.
- Sheng, M. and Greenberg, M.E. (1990). The Regulation and Function of c-fos and Other Immediate Early Genes in the Nervous System. *Neuron* **4**, 477-485.
- Shupnik, M.A., Gordon, M.S., and Chin, W.W. (1989). Tissue-Specific Regulation of Rat Estrogen Receptor mRNAs. *Mol. Endocrinol.* **3**, 660-665.
- Simerly, R.B., Chang, C., Muramatsu, M., and Swanson, L.W. (1990). Distribution of Androgen and Estrogen Receptor mRNA-Containing Cells in the Rat Brain: An In Situ Hybridization Study. *J. Comp. Neurol.* **294**, 76-95.
- Simerly, R.B. and Young, B.J. (1991). Regulation of Estrogen Receptor Messenger Ribonucleic Acid in Rat Hypothalamus by Sex Steroid Hormones. *Mol. Endocrinol.* **5**, 424-432.
- Simpson, H.B. and Vicario, D.S. (1991a). Early Estrogen Treatment Alone Causes Female Zebra Finches to Produce Learned, Male-Like Vocalizations. *J. Neurobiol.* **7**, 755-776.
- Simpson, H.B. and Vicario, D.S. (1991b). Early Estrogen Treatment of Female Zebra Finches Masculinizes the Brain Pathway for learned Vocalizations. *J. Neurobiol.* **22**, 777-793.
- Slater, E.P., Redeuihl, G., Theis, K., Suske, G., and Beato, M. (1990). The Uteroglobulin Promoter Contains a Noncanonical Estrogen Responsive Element. *Mol. Endocrinol.* **4**, 604-610.
- Smith, J.A. and King, R.J.B. (1972). Effects of steroids on growth of an androgen-dependent mouse mammary carcinoma in cell culture. *Exp. Cell Res.* **73**, 351-359.
- Snider, L.D., King, D., and Lingrel, J.B. (1985). Androgen Regulation of MAK mRNAs in Mouse Kidney. *J. Biol. Chem.* **260**, 9884-9893.

REFERENCES

- Sohrabji, F., Nordeen, K.W., and Nordeen, E.J. (1989). Projections of androgen-accumulating neurons in a nucleus controlling avian song. *Brain Res.* **488**, 253-259.
- Sokal, R.R. and Rohlf, F.J. (1981). *Biometry*. New York, NY.: Freeman.
- Stanley, H.F. and Fink, G. (1985). The Short-Term Effects of Testosterone on Brain Protein Synthesis in 4-Day-Old Rats: An Electrophoretic Study of Proteins following Intraventricular Injection of [³⁵S]Methionine. *Brain Res.* **358**, 241-248.
- Stanley, H.F. and Fink, G. (1986). Synthesis of Specific Brain Proteins is Influenced by Testosterone at mRNA Level in the Neonatal Rat. *Brain Res.* **370**, 223-231.
- Su, T.-P., London, E.D., and Jaffe, J.H. (1988). Steroid Binding at α Receptors Suggests a Link Between Endocrine, Nervous, and Immune Systems. *Sci.* **240**, 219-221.
- Swaab, D.F. and Fliers, E. (1985). A Sexually Dimorphic Nucleus in the Human Brain. *Sci.* **228**, 1112-1114.
- Tasset, D., Tora, L., Fromental, C., Scheer, E., and Chambon, P. (1990). Distinct Classes of Transcriptional Activating Domains Function by Different Mechanisms. *Cell* **62**, 1177-1187.
- Thorpe, W.H. (1958). The learning of song patterns by birds, with especial reference to the song of the chaffinch *Fringilla coelebs*. *IBIS* **100**, 535-570.
- Thummel, C.S., Burtis, K.C., and Hogness, D.S. (1990). Spatial and Temporal Patterns of E74 Transcription during *Drosophila* Development. *Cell* **61**, 101-111.
- Tilley, W.D., Marcelli, M., Wilson, J.D., and McPhaul, M.J. (1989). Characterization and expression of a cDNA encoding the human androgen receptor. *Proc. Natl. Acad. Sci. USA* **86**, 327-331.
- Tobet, S.A. and Fox, T.O. (1989). Sex- and hormone-dependent antigen immunoreactivity in developing rat hypothalamus. *Proc. Natl. Acad. Sci. USA* **86**, 382-386.

REFERENCES

- Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E., and Chambon, P. (1989). The Human Estrogen Receptor Has Two Independent Nonacidic Transcriptional Activation Functions. *Cell* **59**, 477-487.
- Toran-Allerand, C.D. (1976). Sex steroids and the development of the newborn mouse hypothalamus and preoptic area in vitro: implications for sexual differentiation. *Brain Res.* **106**, 407-412.
- Toran-Allerand, C.D. (1980). Sex steroids and the development of the newborn mouse hypothalamus and preoptic area in vitro. II. Morphological correlates and hormonal specificity. *Brain Res.* **189**, 413-427.
- Towle, A.C. and Sze, P.Y. (1983). Steroid binding to synaptic plasma membrane: Differential binding of glucocorticoids and gonadal steroids. *J. Steroid Biochem.* **18**, 135-143.
- Wang, A.M., Doyle, M.V., and Mark, D.F. (1989). Quantitation of mRNA by the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* **86**, 9717-9721.
- Watson, C.S. and Catterall, J.F. (1986). Genetic Regulation of Androgen-Induced Accumulation of Mouse Renal α -Glucuronidase Messenger Ribonucleic Acid. *Endocrin.* **118**, 1081-1086.
- Watson, C.J. and Jackson, J.F. (1985). An Alternative Procedure for the Synthesis of Double Stranded cDNA for Cloning in Phage and Plasmid Vectors. In *DNA cloning, Volume 1: a practical approach*. D.M. Glover, ed. (Oxford: IRL Press), pp. 79-88.
- Watson, G. and Paigen, K. (1990). Progressive induction of mRNA synthesis for androgen-responsive genes in mouse kidney. *Mol. Cell. Endo.* **68**, 67-74.
- Wei, L.L., Krett, N.L., Francis, M.D., Gordon, D.F., Wood, W.M., O'Malley, B.W., and Horwitz, K.B. (1988). Multiple Human Progesterone Receptor Messenger Ribonucleic Acids and Their Autoregulation by Progestin Agonists and Antagonists in Breast Cancer Cells. *Mol. Endocrinol.* **2**, 62-72.

REFERENCES

- Weisz, A., Cicatiello, L., Persico, E., Scalona, M., and Bresciani, F. (1990). Estrogen Stimulates Transcription of c-jun Protooncogene. *Mol. Endocrinol.* **4**, 1041-1050.
- Westneat, D.F., Noon, W.A., Reeve, H.K., and Aquadro, C.F. (1988). Improved hybridization conditions for DNA 'fingerprints' probed with M13. *Nucleic Acids Res.* **16**, 4161.
- Westley, B.R. and May, F.E.B. (1988). Oestrogen regulates oestrogen receptor mRNA levels in an oestrogen-responsive human breast cancer cell line. *Biochem. Biophys. Res. Commun.* **155**, 1113-1118.
- Weyant, R.S., Edmonds, P., and Swaminathan, B. (1990). Effect of Ionic and Nonionic Detergents on the Taq Polymerase. *BioTechniques* **9**, 308-309.
- Wilson, T.E., Fahrner, T.J., Johnston, M., and Milbrandt, J. (1991). Identification of the DNA Binding Site for NGFI-B by Genetic Selection in Yeast. *Sci.* **252**, 1296-1300.
- Wingfield, J.C., Hegner, R.E., Dufty, A.M.Jr., and Ball, G.F. (1990). The "challenge hypothesis": theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. *Amer. Nat.* **136**, 829-846.
- Wingfield, J.C. and Farner, D.S. (1975). The determination of five steroids in avian plasma by radioimmunoassay and competitive protein-binding. *Steroids* **26**, 311-327.
- Woolley, C.S., Gould, E., Frankfurt, M., and McEwen, B.S. (1990). Naturally Occurring Fluctuation in Dendritic Spine Density on Adult Hippocampal Pyramidal Neurons. *J. Neurosci.* **10**, 4035-4039.
- Yamamoto, K.R. (1985). Steroid Receptor Regulated Transcription of Specific Genes and Gene Networks. *Annu. Rev. Genet.* **19**, 209-252.

REFERENCES

- Yang-Yen, H.-F., Chambard, J.-C., Smeal, T., Schmidt, T.J., Drouin, J., and Karin, M. (1990). Transcriptional Interference between c-Jun and the Glucocorticoid Receptor: Mutual Inhibition of DNA Binding Due to Direct Protein-Protein Interaction. *Cell* **62**, 1205-1215.
- Yates, J., Coucouchman, J.R., and King, R.J.B. (1980). Androgen Effects on Growth, Morphology, and Sensitivity of S115 Mouse Mammary Tumor Cells in Culture. In *Hormones and Cancer*. S. Iacobelli and al. et, eds. (New York: Raven Press), pp. 31-39.
- Yu, W.A. (1989). Administration of Testosterone Attenuates Neuronal Loss Following Axotomy in the Brain-Stem Motor Nuclei of Female Rats. *J. Neurosci.* **9**, 3908-3914.
- Zhang, Y.-L. and Parker, M.G. (1985). Regulation of prostatic steroid binding protein mRNAs by testosterone. *Mol. Cell. Endo.* **43**, 151-154.
- Zimmerman, K.A., Yancopoulos, G.D., Collum, R.G., Smith, R.K., Kohl, N.E., Denis, K.A., Nau, M.M., Witte, O.N., Toran-Allerand, C.D., Gee, C.E., Minna, J.D., and Alt, F.W. (1986). Differential expression of myc family genes during murine development. *Nature* **319**, 780-783.



THE LIBRARY



19010000036914

End