MECHANISMS OF PROLACTIN AND ANDROGEN ACTION ON THE EXPRESSION OF THE PROLACTIN-INDUCIBLE PROTEIN (PIP)

by
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"All we really need to make us happy is something to be enthusiastic about"

- Charles Kingsley

LIST OF ABBREVIATIONS

ARE	androgen-responsive element
ATP	adenosine triphosphate
b	base pairs
BSA	bovine serum albumin
°C	degrees centrigrade
$CaCl_2$	calcium chloride
cDNA	complementary DNA
CM	complete medium
CsCl	cesium chloride
CTP	cytosine triphosphate
DEPC-H_2O	diethyl pyrocarbonate - H_2O
DHT	5α-dihydrotestosterone
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DRB	5,6-dichloro-1-ß-D-ribofuranosylbenzimidazole
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
FBS	fetal bovine serum
g	gram

GTP	guanosine triphosphate
HBC	human breast cancer
HCl	hydrochloric acid
HGH	human growth hormone
hnRNA	heteronuclear RNA
hr	hour
IPTG	isopropyl-ß-D-thiogalacopyranoside
KAc	potassium acetate
kb	kilobase
KCl	potassium chloride
L	litre
M	molar
mg	milligram
$MgCl_2$	magnesium chloride
min	minutes
ml	millilitre
mM	millimolar
MMTV	mouse mammary tumor virus
MOPS	3N-morpholino-propanesulfonic acid
mRNA	messenger RNA
NaCl	sodium chloride
NaOH	sodium hydroxide

NP-40	Nonidet P-40
OCS	organic counting scintillant
O/N	overnight
PBS	phosphate buffered saline
RE	restriction endonuclease
RNA	ribonucleic acid
RPM	revolutions per minute
SDS	sodium dodecyl sulfate
SSC	standard saline citrate
T	thymidine
TBE	Tris borate EDTA buffer
TCA	trichloroacetic acid
TE	tris-EDTA
tRNA	transfer RNA
ug	microgram
ul	microlitre
uM	micromolar
UTP	uridine triphosphate
UV	ultraviolet
vol	volume
v/v	volume per volume
w/v	weight per volume

x-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
%	percent

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INTRODUCTION

A. Hormones, Growth Factors and the Breast Epithelium

Hormonal Regulation of the Normal Mammary Gland

Several hormones play significant roles in the development and function of the mammary gland. These include the sex steroids, prolactin, placental lactogen, growth hormone, insulin, glucocorticoids, growth factors and others. Under the regulation of these hormones, the mammary gland undergoes a number of dramatic changes in the lifetime of an individual such as mammogenesis (growth and differentiation of the mammary gland) in the fetus and adolescent, and cyclical growth and regression in the nonpregnant adult. During pregnancy, the mammary glands undergo differentiation with extensive lobuloalveolar growth and the production of milk (lactation). Finally , following weaning, the glands regress to a state resembling that found in the nonpregnant adult (involution) (Neville, 1983).

Fetal mouse mammary gland development occurs independently of ovarian and pituitary hormones (Raynaud, 1950, Raynaud, 1971). In the male mouse fetus, androgens from the developing testes cause detachment of the gland from the nipple and preempt any further development (Raynaud, 1961). At birth, the mammary gland consists of a primary duct and a few primitive branched ducts (Vonderhaar, 1988). No further development occurs until puberty when the ducts begin to proliferate. Ductal growth requires estrogen and either growth hormone or prolactin (Topper and Freeman, 1980). Epidermal growth factor (EGF) as well as other as yet undefined hormones or growth

factors may also be required for maximal growth (Richards et al., 1982, Nandi, 1958). In the sexually mature female, the mammary duct structure extends throughout the fat pad with well-defined interductal spaces (Topper and Freeman, 1980). Peaks of ovarian steroids during the menstrual cycle result in limited, cyclical, lobuloalveolar growth but otherwise very little development occurs in the nonpregnant adult (Neville, 1983). Maximal differentiation of the mammary gland occurs during pregnancy. Lobuloalveolar structures fill in the interductal spaces, and the cells comprising the alveoli begin to generate the components of milk (Topper and Freeman, 1980). Lobuloalveolar growth in triply operated (ovariectomized, hypophysectomized, and adrenalectomized) mice requires treatment with estrogens, progesterone, prolactin and/or growth hormone, and deoxycorticosterone acetate (Nandi, 1958). Thyroid hormones have also been shown to stimulate alveolar growth in the mouse (Vonderhaar, 1979). Experiments by Ichinose and Nandi (1966) on whole organ cultures of virgin mouse mammary glands demonstrated that the organs needed to be primed by estrogen and progesterone for a number of days before development could occur. Development itself required insulin, prolactin, aldosterone, and hydrocortisone. Withdrawal of all hormones except insulin resulted in regression of the gland. Other researchers using a similar model demonstrated that EGF, transforming growth factor-α (TGF-α), and mammary-derived growth factor (MDGF), could also stimulate lobuloalveolar growth (Tonelli and Soroff, 1980, Schreiber et al., 1986, Vonderhaar, 1984). Placental lactogen can substitute effectively for prolactin in its effects on the mammary gland, and due to its high concentration, may play the greater role in mammary development in late pregnancy (Topper and Freeman, 1980).

Milk protein synthesis is stimulated by prolactin, glucocorticoids, and insulin in mouse mammary explant cultures (Guyette et al., 1979). Limited production of milk proteins begins in the second trimester of pregnancy, but secretion of milk is blocked until parturition by high levels of progesterone (Martin et al., 1980, Kuhn, 1977). Prolactin's exact role in stimulating milk production *in vivo* is not clear. Treatment of women with bromocriptine, which inhibits prolactin secretion, results in cessation of milk production. On the other hand, when prolactin levels have fallen to the non pregnant range, lactation is maintained for many months and years after parturition (Brun del Re et al., 1973, Gross and Eastman, 1979).

Involution following weaning appears to be controlled largely by factors other than hormones (Neville, 1983). Involution at menopause appears to be related to the decline in ovarian function (Vorherr, 1974).

Hormones, Growth Factors and Breast Cancer.

The role of hormones in the control of breast cancer was first demonstrated by Beatson in 1896. He observed regression of advanced tumours in premenopausal women following removal of the ovaries. Since that time a variety of endocrine based therapies, including adrenalectomy, hypophysectomy, antiestrogens, androgens, progestins, and glucocorticoids have been developed (Huggins and Bergenstal, 1952, Pearson and Ray, 1960, Henderson and Canellos, 1980). It is thought that hormones exert their effects on breast cancer by acting both directly, to affect gene expression in the cells themselves,

and indirectly, to affect the production of various mediators in the stroma and other endocrine tissues (Dickson and Lippman, 1988). Regardless of the mechanisms by which they act, a variety of hormones exert profound effects, both stimulatory and inhibitory, on breast cancer.

As stated, ovariectomy can result in the regression of advanced breast cancer in premenopausal women. In addition, breast cancer incidence is 100 times greater in women with intact ovaries than in men, or in women who have never had functional ovaries (Thomas, 1986). This suggests a major role for ovarian hormones in the etiology and/or progression of breast cancer. Approximately 60% of human breast tumours contain receptors for estrogen and two thirds of those respond to estrogen blocking therapies such as tamoxifen (McGuire, 1980). Estrogens have been shown to induce tumour cell growth both *in vitro*, using human breast cancer (HBC) cell lines, and in the nude mouse (Soule and McGrath, 1980, Lippman et al., 1976). In rats, administration of moderate doses of estrogen enhances the growth of 7,12-dimethylbenzanthracene (DMBA)-induced mammary tumours (Kiang and Kennedy, 1971).

There are a number of probable mechanisms by which estrogens induce growth of breast cancer cells. Estrogens have been shown to induce the synthesis of DNA polymerase, thymidine kinase, and uridine kinase in HBC cell lines (Edwards et al., 1980, Aitken and Lippman, 1983). As well, Dubik and Shiu (1988) have shown in the HBC cell line MCF-7, that estrogens cause a rapid increase in the transcription rate of the cell cycle competence gene c-myc. Thus there is some evidence that estrogens may act directly as a mitogen on HBC cells.

Considerable evidence exists that estrogens can also act indirectly on cell growth. In the DMBA rat model, estrogens are ineffective at stimulating growth or DNA synthesis in the tumour cells *in vitro* (Aspegren, 1975a, Aspegren, 1975b). Neither can estrogens stimulate the growth of the DMBA-induced tumour in hypophysectomized rats (Sterental et al., 1963). Estrogen's major action in the DMBA model may be to stimulate the production of prolactin, which in turn is growth stimulatory for the tumour (Kim, 1965). Estrogen is also capable of increasing prolactin levels in humans, although prolactin's effect in HBC is uncertain. Lipsett and Bergenstal (1960) suggested that a pituitary factor distinct from prolactin or growth hormone is required for estrogen action in HBC, while Shiu et al. (1986) have found insulin-like growth factor-II (IGF-II) to be the major component of an "estrogen potentiating factor" isolated from human pituitary.

In HBC cell lines, estrogens induce the expression of growth factors such as TGF-α, insulin-like growth factor I (IGF-I), IGF-II and EGF (Bates et al., 1988, Huff et al., 1988, Yee et al., 1988, Dickson and Lippman, 1987). These in turn are all mitogenic for HBC cells (Imai et.al., 1982, Myal et.al., 1984, Lippman and Dickson, 1989). Estrogens also stimulate the expression of progesterone receptors in HBC cells, and the likelihood of response to endocrine therapy is greatly increased when progesterone receptors are present (Horwitz and McGuire, 1978). Progesterone receptor expression in breast tumours then may indicate a functioning estrogen receptor pathway (McGuire, 1980).

Progestins are growth inhibitory in the T-47D HBC cell line but are growth stimulatory in the DMBA-induced rat mammary tumour (Horwitz and Freidenberg, 1985, Huggins et al., 1962). At least part of their action in HBC may be due to their down-

regulation of the estrogen receptor in the breast cancer cells (Read et al., 1989). Progestins such as megestrol acetate and medroxyprogesterone acetate (MPA) have been used clinically to treat advanced breast cancer and have achieved objective remission rates as high as 35% (Santen et al. 1990).

Objective remission rates of approximately 25% have been achieved in patients with advanced breast cancer using synthetic androgens (Cooperative Breast Cancer Group, 1961). Both a HBC cell line and the DMBA-induced tumour have been shown to be growth inhibited by physiological concentrations of androgens (Poulin et al., 1988, Ercoli and Briziarelli, 1961). Like progestins, androgens can decrease the levels of estrogen receptor in HBC cell lines and this may account for part of their inhibitory action (Poulin et al., 1989).

While androgens may appear to have an inhibitory effect on some breast cancers, they have a growth stimulatory effect on others. About 7% of breast cancers *in vitro* are androgen dependent (Hobbs et al., 1974). As well, one mouse mammary tumour cell line, the S115 cell line is also androgen dependent (Smith and King, 1972). Lea et al. (1989) have reported the presence of androgen receptors in as many as 85% of primary breast tumours, and both high and low serum androgen levels have been reported in women with breast cancer (Secreto et al., 1989, Wang et al., 1977). Thus the exact role of androgens in HBC remains unclear. Both Chalbos et al. (1987) and Murphy et al. (1987b) have identified androgen-regulated genes in HBC cell lines.

Glucocorticoids have also been used as second line endocrine therapy for advanced breast cancer. Approximately 50% of HBC biopsies contain receptors for

glucocorticoids but the mechanism of their inhibitory action, both *in vivo* and *in vitro*, is unclear (Allegra et al., 1979, Lippman et al., 1976). Only 18-21% of women with advanced breast cancer will respond to glucocorticoid treatment (Brennan and McMahan, 1976).

The role of prolactin in the DMBA-induced rat mammary tumour is well established. Suppression or removal of prolactin results in marked inhibition of development and/or growth of DMBA-induced tumours (Welsch, 1985). The role of prolactin in human breast cancer however is not as clear. Several studies have demonstrated a tendency toward elevated prolactin levels in breast cancer patients and their high-risk family members (Henderson and Pike, 1981). Other studies, however, do not support a connection between prolactin levels and breast cancer risk (Kwa et al., 1976). As well, breast cancer risk is not increased in those women with elevated prolactin levels due to pregnancy, lactation, or drug treatment (Vorherr, 1980). In fact, pregnancy and lactation appear to be major protective factors against breast cancer (McMahon et al., 1970).

While up to 60% of breast tumour biopsies and several HBC cell lines have been shown to possess prolactin receptors, only about 10% of breast tumours have been shown to be prolactin dependent to some degree (Pearson, 1978, Shiu, 1979, Hobbs et al., 1974). Prolactin has been shown in some studies to stimulate DNA synthesis, pentose monophosphate pathway activity, and cell proliferation in HBC cell lines (Salih, 1972, Simon et al., 1985). Other studies have found no effect of prolactin on the growth of HBC cells in culture (Shiu, 1981). Shiu et al. (1987) have shown that treatment of the

HBC cell line T-47D with physiological concentrations of prolactin results in shape change, increased lipid synthesis, decreased cell-substratum interaction, and the synthesis of a unique secretory protein called the prolactin-inducible protein (PIP). Despite all of these studies however, the exact role, if any, that prolactin plays in HBC *in vivo* remains unclear.

Insulin has been shown to be mitogenic for HBC cells and for DMBA-induced mammary tumours (Heusen et al., 1972, Barnes and Sato, 1979). Its action in HBC cells however may be due to cross reactivity with IGF-I and II receptors rather than through the insulin receptor itself (Myal et al., 1984).

Both vitamin D and retinoic acid have been found to suppress the growth of HBC cells although the clinical significance of this is not yet known (Lacroix and Lippman, 1980, Colston et al., 1989). Also unclear is the *in vivo* role of thyroid hormones in breast cancer. The thyroid hormones have been shown to be growth stimulatory for MCF-7 and T-47D cells (Shiu, 1981).

Locally produced growth factors may also exert profound effects on the growth of breast tumours. Estrogens stimulate the production of IGF-I, IGF-II, EGF, and TGF-α in HBC cells, which in turn are mitogenic for those cells. Platelet-derived growth factor (PDGF) and transforming growth factor ß (TGF-ß) are also expressed in many HBC cell lines (Rozengert, 1986, Wang and Hsu, 1986). PDGF stimulates the growth of stromal cells such as fibroblasts (Bronzert et al., 1987). Lippman and Dickson (1989) have hypothesized that the PDGF-stimulated fibroblasts may produce growth factors which act in a paracrine fashion to cause growth of the tumour cells. TGF-ß on the other

hand acts directly on HBC cells to inhibit their growth (Wang and Hsu, 1986). The fibroblast growth factors (FGF) may act in a manner similar to that hypothesized for PDGF - that is, by stimulating growth factor synthesis by fibroblasts, but they have also been shown to directly stimulate growth of HBC cell lines (Karey and Sirbasku, 1988). Recently two novel proteins, mammastatin and amphiregulin, have been identified. Both of these have been shown to be growth inhibitory for several breast tumour cell lines (Ervin et al., 1989, Plowman et al., 1990).

The Human Breast Cancer Cell Line T-47D

Human breast cancer cell lines present a useful model for the study of the hormonal regulation of gene expression. Due to selection pressures during the initiation and continuation of cell cultures, and the lack of a physiological hormonal milieu, the responses to hormones observed in culture may, or may not be representative of those responses *in vivo* (Lippman, 1981). Whether or not the molecular mechanisms by which the hormones elicit those responses remains unchanged is also unknown. Molecular pathways may be modified *in vivo* by the action of other hormones or by cell-substratum interactions. Nonetheless, the ability to grow cells in a defined medium presents one of the best opportunities to study the specific effects of a single hormone or growth factor on human tissue.

The T-47D cell line was derived from the pleural effusion of a 54 year old patient with infiltrating ductal carcinoma of the breast (Keydar et al., 1979). The cells exhibit

an epithelial morphology, have an aneuploid karyotype of 66 chromosomes and a doubling time of 32 hours. T-47D cells possess specific receptors for estrogens, androgens, progesterone, and glucocorticoids (Keydar et al., 1979). Cell surface receptors for prolactin are present in large quantities. In fact, the HBC cell line with the next highest level of prolactin receptor, MCF-7, contains only 1/3 of the amount present in T-47D (Shiu, 1979).

Zajchowski et.al. (1988) compared the levels of mRNAs of various growth factors and oncogenes in T-47D cells to those in cell strains derived from normal breast epithelium. They found the T-47D cells to contain lower mRNA levels for the EGF receptor, TGF-α, TGF-ß, c-myc and N-ras genes than the normal cells. Levels of erbB2 and Ha-ras mRNAs in T-47D cells were similar to those in the normal cells.

B. Regulation of Gene Expression

The study of the regulation of gene expression can be broadly divided into two areas. The first area deals with the study of tissue-specific genes; that is, the turning on and off of genes expressed only in restricted tissue types during differentiation and, in the case of neoplasia, dedifferentiation. Mechanisms of gene regulation in this area involve changes to the packaging of the chromatin and /or the presence of tissue-specific transcription factors. The second area deals with the regulation of genes that are known to be expressed in a particular cell type. Mechanisms utilized here include altering the

rate of transcription, changing the rate of hnRNA processing and transport, editing of the RNA, and modifying the stability of the RNA transcript, along with a variety of translational and post-translational controls. The control of gene expression by hormones and growth factors, and by signals received through cell-cell and cell-substratum interactions, is mediated through a combination of these mechanisms, and by these mechanisms the phenotype of any cell can be precisely defined.

Transcriptional Regulation - Chromatin Structure

While every different cell type, with a few exceptions, in a particular individual contains the same DNA sequences, it is obvious that all cell types do not express the same genes. It has become apparent that the way in which DNA is packaged into chromatin can help determine if that DNA is capable of being transcribed. Regions of chromatin containing DNA sequences that are capable of being expressed are referred to as active chromatin, while unexpressed sequences are contained in inactive chromatin. Active chromatin is distinct from inactive chromatin in a number of ways.

Transcriptionally active chromatin is known to be more sensitive to digestion by pancreatic DNase I (Reeves, 1984). Weintraub and Groudine (1976) studied the DNase I sensitivity of the globin gene in chick erythrocytes where it is expressed, and in oviduct cells where it is not expressed. They found the globin gene to be more sensitive to DNase I digestion in the erythrocytes than in the oviduct cells. Conversely, the ovalbumin gene was found to have greater DNase I sensitivity in oviduct cells than in

erythrocytes (Garel and Axel, 1976). The increased sensitivity of active chromatin to DNase I may be due to selective decondensation of the higher order chromatin structure (Reeves, 1984). This opening up of the chromatin is believed to facilitate the interaction of RNA polymerase, as well as other general and specific transcription factors with the DNA itself (Gross and Garrard, 1987).

A number of other features distinguish active from inactive chromatin. These features may contribute to the nuclease-sensitive characteristic of the active chromatin or they may have effects on transcription independent of that phenomena. Modifications to the DNA and their effect on transcription have been studied extensively. A large amount of that work has focused on the role of methylation in the control of gene expression (Weisbrod, 1982). In mammalian DNA, 2-7% of cytosine residues exist as 5-methylcytosine (5mC) (Razin and Riggs, 1980). However these methyl groups do not appear to be randomly distributed. Active genes have been found to be relatively hypomethylated, while inactive genes are usually well methylated (Weisbrod, 1982). Experimental demethylation of DNA using 5-azacytidine has resulted in the activation of several inactive genes (Compere and Palmiter, 1981, Groudine et al., 1981). Differences in methylation patterns have been hypothesized to affect gene expression by altering DNA-protein interactions and thus packaging of the DNA into chromatin (Chandler et al., 1987).

Differences in both histone and non-histone chromosomal proteins have also been observed between active and inactive chromatin. Weintraub (1984) has shown that histone H1 binds differently to active than to inactive chromatin. The H1 may bind the inactive

DNA sequences into tight "supranucleosomal" structures which restrict access by transcription factors and polymerases. Post-translational modifications to the histones also play a role in the conformation of the chromatin. The amino terminal region of the histone proteins are rich in the positively charged amino acids lysine, arginine and histidine (Vidali et al., 1978). Acetylation of the lysines in the amino terminus results in neutralization of the positive charge. This loss of charge changes the ability of the histones to interact with the DNA as well as the ability of the nucleosomes to interact with each other, probably resulting in decondensation of the chromatin in that region (Vidali et al., 1978, Doenecke and Gallwitz, 1982). Direct correlations exist between histone acetylation, DNase I sensitivity, and active chromatin (Ruiz-Carrillo et al., 1975, Hebbes et al., 1988). Other post-translational histone modifications such as ubiquitination, phosphorylation and poly(ADP)-ribosylation have also been suggested to affect higher order chromatin structure and thus possibly gene expression (Levinger and Varshavsky, 1982, Poirier et al., 1982).

Small nonhistone chromosomal proteins known as high mobility group (HMG) proteins may play some role in the formation or maintenance of active chromatin structure. While the HMG proteins have been found by some researchers to bind to inactive DNA, they tend to bind preferentially to transcriptionally active DNA sequences (Nicholas et al., 1983, Weisbrod and Weintraub, 1981, Gross and Garrard, 1987). When antibodies against some HMG proteins were microinjected into human fibroblasts, transcription was reduced in a concentration-dependent manner (Einck and Bustin, 1983).

Transcriptionally active chromatin has also been found to contain greater amounts

of topoisomerase I than does inactive chromatin (Gilmour et al., 1986). The action of the topoisomerase is required during transcription to relieve the torsional strain placed on the DNA as it is unwound (Wu et al., 1988). However, the topoisomerase may also act to make the DNA more accessible to polymerases by placing it under greater initial torsional strain than inactive DNA. This increased strain might result in the decondensation of specific sections of the DNA, in particular the promoter and start site regions (Gross and Garrard, 1987).

It is evident then that a number of different mechanisms must contribute to the formation and maintenance of the active chromatin state. These mechanisms appear to result in a less tightly packaged form of chromatin. This looser packaging may facilitate transcription by increasing the access of polymerases and transcription factors to the DNA sequences themselves.

Transcriptional Regulation - Controlling Sequences

It has become clear that the frequency of initiation of transcription is immediately regulated by factors which bind to specific DNA sequences in or around each gene. These sequences have been separated into two groups known as promoters and enhancers, however some sequences are difficult to place squarely into either category.

The promoter is responsible for accurate and efficient initiation of transcription. Promoters are typically about 100 bp in length and are usually located immediately upstream from the start site of transcription (Maniatis et al., 1987). Promoters consist

of a number of short elements ranging from 5 to 20 bp in length. The most commonly found element is an AT-rich sequence known as the TATA box, usually located 25-30 bp upstream from the start site (Breathnach and Chambon, 1981). The TATA box ensures that transcripts are initiated at the correct start site (Maniatis et al., 1987). In some genes which lack a TATA box, such as the terminal deoxynucleotidyltransferase gene, an element called the initiator (Inr) overlies the start site and ensures accurate initiation (Smale and Baltimore, 1989). Other TATA-less genes have promoters rich in GC boxes, and may have multiple initiation sites (Sehgal et al., 1988). Other elements found in the promoter are called upstream promoter elements (UPE). These act to increase the rate of initiation of transcription (Maniatis et al., 1987). UPEs commonly found in some, but not all promoters include GC boxes and CAAT boxes.

Several genes are now known which contain more than one promoter. These include the actin alcohol dehydrogenase and yolk protein genes in Drosophila, and the mouse α-fetoprotein gene (Fischer and Maniatis, 1986, Garabedian et al.,1986, Hammer et al., 1987). The different promoters are often utilized in different tissues resulting in tissue-specific mRNAs which differ only in their 5' exons.

Enhancers are DNA sequences which regulate the activity of *cis*-linked promoters. Enhancers can act from up to 30 kb away, on either side of the start site, and in an orientation-independent manner (Maniatis et al., 1987, Mitchell and Tjian, 1989). Like promoters, each enhancer is composed of a number of shorter elements spaced up to 50 bp apart. These are called enhancer modules. Each module in turn is composed of one or more short DNA sequences termed enhansons. It is believed that each enhanson

corresponds to an individual binding site for a transcriptional regulatory protein (Dynan, 1989). Factors binding to enhansons can both increase and decrease the activity of promoters and thus the rate of initiation of transcription (Maniatis et al., 1987).

The organization of the various promoter and enhancer elements appears to be unique to each gene. It is believed that the presence of, and the organization of these elements help confer on each gene its own unique pattern of transcriptional activity (Mitchell and Tjian, 1989).

Transcriptional Regulation - Transcription Factors

The extent to which the various promoter and enhancer elements affect the rate of transcription depends largely on the sequence-specific factors which bind to them. Changes in the concentration or activity of these transcription factors may alter their interactions with the DNA elements, or other regulatory proteins, and thus change the rate of transcription.

Regulation of the concentration of specific transcription factors is a common method used in the control of gene expression (Jones, 1990). The OCT 2 transcription factor is specifically found in lymphoid cells, and consequently, promoters and enhancers that depend upon OCT 2 for transcription are only active in such cells (Muller et al., 1988, Jones, 1990). The concentrations of the steroid hormone receptors - which act as transcription factors, also determine gene expression. Both progestins and androgens have been shown to antagonize the effects of estrogens in human breast cancer cells (Horwitz

and Freidenberg, 1985, Poulin et al., 1988). This effect is believed to be mediated in part, by the down-regulation of estrogen receptors by progestins and androgens (Read et al., 1989, Poulin et al., 1989). Thus by changing the level of estrogen receptors in these cells, the antagonistic steroids can affect the expression of estrogen-responsive genes.

It may be that the ratios of various transcription factors in the cell, plays as large a role in determining transcription rates as do their absolute concentration. In yeast, overexpression of the GAL 4 transcription factor results in decreased transcription of genes which are not regulated by GAL 4 (Gill and Ptashne, 1988). This effect, called squelching, may be the result of competition between GAL 4 and other transcriptional activators for specific proteins required for transcription (Ptashne, 1988). A similar mechanism has been proposed for the steroid hormone receptors (Meyer et al., 1989).

Different transcription factors can also compete with each other for binding to the same DNA enhancer elements (Schule et al., 1990). The vitamin D response element (VDRE) of the human osteocalcin gene has been shown to bind the receptors for both vitamins A and D (Kerner et al., 1989, Schule et al., 1990). Both vitamin receptors act through this element to increase the transcription of the osteocalcin gene. The transcription factors jun and fos, which inhibit transcription of this gene, also act by binding to the VDRE (Schule et al., 1990). Thus transcription factors with opposite effects on gene expression can compete for the same binding site on the DNA. Conversely, some transcription factors have been shown to bind cooperatively to enhancer elements and thus exert a synergistic effect on transcription (Tsai et al., 1989, Maekawa et al., 1989, Crenshaw et al., 1989).

The activity of many transcription factors is regulated in a manner that does not affect their concentration. Instead they are changed from a latent or inactive form - either by interactions with other proteins, or by post-translational modifications, into an active form capable of modulating transcription (Jones, 1990). This activation may enable them to bind to, or to dissociate from the enhancer elements, or it may affect transcription independent of DNA binding (Ptashne, 1988). Phosphorylation has been suggested as a control mechanism for several transcription factors. Exposure of yeast cells to heat activates transcription of a number of heat-shock genes through a specific factor called the heat-shock transcription factor (HSTF) (Pelham, 1985). Neither the amount of this factor nor its DNA binding activity are affected by the exposure to heat. Rather, the heat appears to result in phosphorylation of HSTF, thus presumably increasing its transcriptional activity (Sorger et al., 1987). Similar mechanisms have been suggested for the RNA polymerase III transcription factor TFIIIC, as well as for the cAMP response element binding protein (CREB) (Yamamoto et al., 1988, Hoeffler et al, 1988). Many transcription factors have been shown to be glycosylated. As such, glycosylation has been proposed as a mechanism for controlling the activity of transcription factors (Jackson and Tjian, 1988). However, this possible mechanism of regulation has yet to be explored in depth (Jones, 1990).

The interaction of transcription factors with other proteins or factors is an important mechanism for the regulation of their activity. The yeast transcription factor, GAL 4 is one example (Ptashne, 1988). The metabolism of galactose in yeast is controlled by a set of genes which are turned on by GAL 4 only in the presence of

galactose. When galactose is absent, another protein, GAL 80 binds to GAL 4 and represses its transcriptional activity. The addition of galactose causes the dissociation of GAL 80 from GAL 4, enabling the GAL 4 to activate the galactose metabolism genes. The steroid hormone receptors provide another example of this type of regulation. In the absence of their specific hormones the receptors form an inactive oligomeric complex which may contain several different proteins including a 90 kDa heat-shock protein, HSP 90 (Carson-Jurica et al., 1990). Binding of HSP 90 has been shown to maintain the receptor in an inactive state (Denis and Gustafsson, 1989). When the steroid binds to its receptor, HSP 90 is released and the receptor-hormone complex is activated (Carson-Jurica et al., 1990).

A further mechanism of regulation involves the synthesis of mutated or alternate forms of transcription factors. The erb-A oncogene is one such example. The viral erb-A oncogene encodes a cysteine-rich protein that is a derivative of the thyroid hormone receptor (Weinberger et al., 1986). Mutations in the ligand-binding domain of the protein have changed it into a constitutively active, thyroxin-independent transcription factor (Sap et al, 1986). As well, variant mRNAs for the estrogen receptor have been identified in human breast cancer tumour biopsies, and may encode alternate forms of the protein with alternate activities (Murphy, L.C. and Dotzlaw, H., 1989). It is obvious then that regulation of the activity, and of the concentration, of the various transcription factors is a potent mechanism for the control of transcription.

Transcriptional Regulation - Transcript Elongation / Attenuation

Evidence has been recently mounting that transcriptional regulation of gene expression involves more than merely regulating the rate of initiation of transcription. A number of genes have been identified in which transcription is properly initiated but is interrupted in the first half of the gene. The result is the synthesis of short mRNAs which do not code for a functional protein. Removal of the block to elongation results in the synthesis of full length transcripts. In bacteria, this process is known as transcriptional attenuation (Platt et al., 1986).

When the human promyelocytic leukaemia cell line, HL60, is induced to differentiate, the level of c-myc mRNA falls by more than 10 fold (Westin et al., 1982). Likewise, differentiation of the pre-B cell lymphoma line 70Z/3B is accompanied by a 10 to 100 fold decrease in c-myb mRNA (Bender et al., 1987). These declines in c-myc and c-myb mRNA levels in the differentiated cells are due to blocks to transcriptional elongation (Bentley and Groudine, 1986, Bender et al., 1987). The block to elongation in the c-myc gene has been localized to two T stretches near the 3' end of the first exon, and controlling sequences have been identified immediately upstream of these (Bentley and Groudine, 1988). The mechanism by which this block is turned on during differentiation is unknown.

It has been hypothesized that regulation of transcription elongation may provide a more sensitive mechanism for rapidly increasing or decreasing mRNA levels than regulation of transcription initiation (Bender et al., 1987). Other genes, such as c-fos, and ß-casein (see section C - Prolactin) have also been reported as being regulated in part

by a block to elongation of their transcripts (Fort et al., 1987, Rosen et al., 1989, Goodman and Rosen, 1990).

RNA Processing and Transport

The modifications made to a newly synthesized RNA molecule are collectively referred to as RNA processing. Processing results in the production of a mature mRNA which differs significantly from its precursor heteronuclear RNA (hnRNA). Processing includes the addition of a methylated G nucleotide at the 5' end of the transcript, cleavage and the addition of a poly-(A) tail at the 3' end, and removal of non-coding intron sequences (cis-splicing). Other modifications seen in a limited number of genes include editing of the nucleotide sequence, and the splicing together of exon sequences from different primary transcripts (trans-splicing).

One mechanism of RNA processing used to regulate gene expression is the use of alternate exons, or alternative splicing. This involves a change in the splicing pattern of the hnRNA so that normally excluded sequences are included and/or normally included sequences are excluded. This altered splicing pattern is usually seen in a restricted set of cells and is probably controlled by cell-specific, trans-acting factors (Smith et al., 1989). The calcitonin gene provides one example (Amara et al., 1982). In the C cells of the thyroid gland, the first five exons of the calcitonin hnRNA are spliced together to yield a mRNA coding for the calcitonin polypeptide. In the hypothalamus, the fifth exon of the hnRNA is replaced by a downstream exon used only in that tissue. The resulting

mRNA codes for a novel protein, the calcitonin-gene-related product (CGRP). Thus two unique mRNAs, coding for two unique polypeptides, are generated from the same hnRNA sequence.

Alternative splicing can affect gene expression in a number of ways. The use of an alternate 3' exon in the immunoglobulin μ (Igμ) heavy chain gene, allows for secretion of Igμ from mature B cells, rather than retention in the cell membrane as occurs in the pre-B cells (Early et al., 1980). Protein isoforms generated by alternative splicing may vary in their activity level, as is demonstrated by the different forms of the rat α-thyroid hormone receptor (Izumo and Mahdavi, 1988). In Drosophila, alternative splicing pathways result in the production of mRNAs encoding active or inactive proteins (Rio et al., 1986). The two protein isoforms may also have entirely different functions. For example, calcitonin regulates serum Ca^{++} levels while CGRP is thought to have both neuromodulatory and trophic activities (Fontaine et al., 1987). Finally, it has been proposed that the alternative use of untranslated exons may generate mRNAs with different stabilities and translational efficiencies, thus affecting the overall level of the protein product (Smith et al., 1989).

Another form of RNA processing that has been shown to affect the gene product is RNA editing. In the mitochondria of some protozoa, uridine nucleotides are deleted or inserted into several mRNAs producing a coding sequence different from that of the DNA (Shaw et al., 1988). Up to 50% of some mRNA sequences are created after transcription, by the process of RNA editing (Feagin et al., 1988). In mammals, RNA editing has been shown to occur in the hnRNA of the apolipoprotein B gene (apo-B).

Two different forms of apo-B have been identified; a long form expressed in liver (apo-B100), and a shorter form expressed in the intestine (apo-B48). The smaller apo-B48 is produced by a post-transcriptional C to U conversion in the open reading frame of the hnRNA. This conversion results in the creation of an in-frame stop codon and thus synthesis of the smaller protein (Powell et al., 1987).

Nematodes and some protozoa utilize trans-splicing as a form of gene regulation. Trans-splicing refers to the splicing together of exons from two independently transcribed hnRNAs. This process usually involves the addition of a short 5' leader sequence to hnRNAs, 30-70 bp upstream of the translation start site (Agabian, 1990). The list of genes known to be regulated, at least in part, by some form of RNA processing is growing steadily, and demonstrates the importance of processing in the regulation of gene expression.

The transport of processed mRNA molecules from the nucleus to the cytoplasm has been hypothesized as another important mechanism in the regulation of gene expression. Translocation of poly(A)$^+$ RNA through the nuclear pore complex is an energy-dependent process which may be regulated by hormones, lectins, and carcinogens (Schroder et al., 1987). Insulin, dexamethasone and cAMP have all been shown to increase RNA efflux from isolated nuclei *in vitro*. Their effects on transport *in vivo* however, are unknown (Schroder et al., 1987).

RNA Stability

The rate of degradation of mRNA is an important control point in the regulation of gene expression. Changes to the half-life of a specific mRNA can result in large changes in the cytoplasmic concentration of that message. For example, glucocorticoids are estimated to increase the expression of growth hormone 4-fold by stabilizing the growth hormone mRNA (Paek and Axel, 1987). As well, estrogen has been shown to have a similar effect on the vitellogenin mRNA (Brock and Shapiro, 1983).

The exact pathways of mRNA decay are largely unknown, as are the particular features which contribute to a highly stable or highly unstable transcript (Nielson and Shapiro, 1990). One area of great interest is the apparent regulation of mRNA decay by sequence-specific trans-acting factors. The most ubiquitous of these factors is probably the poly(A) binding protein (PABP). The PABP has been shown to bind to the poly(A) tail of mRNAs, forming an ordered, nucleosome-like complex with a periodicity of 25-27 nucleotides (Baer and Kornberg, 1980). Association of the PABP with the poly(A) tail has been shown to stabilize the mRNAs of a number of genes (Bernstein et al., 1989). As well, it has been demonstrated for several genes, such as growth hormone and insulin, that the stability of their mRNAs are related to the length of their poly(A) tail (Paek and Axel, 1987, Muschel et al., 1986). Presumably a shorter poly(A) tail cannot bind PABP as efficiently, and is thus susceptible to degradation (Nielson and Shapiro, 1990).

A second trans-acting factor that has been shown to be involved in RNA stability is the iron-responsive element binding protein (IRE-BP) (Mullner et al., 1989). IRE-BP

binds to a stem-loop structure in the 3' untranslated region (3'-UTR) of the transferrin receptor mRNA . The binding of IRE-BP is activated by low levels of iron in the cell, and results in stabilization of the mRNA.

In contrast to the stabilization of transcripts by PABP and IRE-BP, other factors have been shown to facilitate the degradation of transcripts. The histone mRNAs are autoregulated in this manner. Free histone proteins, leftover from DNA synthesis, will bind to a 3'-UTR stem-loop structure in the histone mRNA, making the message more susceptible to ribonucleases (Peltz and Ross, 1987).

At least one other 3'-UTR structure has been proposed to bind trans-acting factors and cause destabilization of the mRNA. An AU-rich sequence has been found in the 3'-UTR region of c-fos, c-myc, and several other short-lived mRNAs (Brewer and Ross, 1988, Wilson and Treisman, 1988, Shaw and Kamen, 1986). Deletion of this sequence from the c-fos mRNA results in stabilization of the transcript (Wilson and Treisman, 1988). The AU-sequence has been proposed to destabilize the mRNA either by signalling a shortening of the poly(A) tail, or by directing endonucleases to cut the mRNA directly (Wilson and Treisman, 1988, Brewer and Ross, 1988, Nielson and Shapiro, 1990). Whatever the mechanism, the AU-sequences must be only part of the regulation machinery as they have been found in the 3'-UTR of the stable ß-globin mRNA, and sequences contributing to decreased message stability have been identified in the 5' end of the c-fos mRNA (Brawerman, 1989, Kabnick and Houseman, 1988).

Control of ß-tubulin mRNA decay does not involve trans-acting factors recognizing specific RNA sequences. Instead, when tubulin levels are high in the cell,

free tubulin dimers will recognize the first four amino acids of a nascent tubulin polypeptide as it emerges from the polysome. This recognition signals the degradation of the tubulin mRNA by a ribosome-bound nuclease (Yen et al., 1988).

Translation

A growing number of eukaryotic genes have been shown to be regulated at the level of translation of the mRNA. Translational controls may involve changes to the translational machinery itself - and thus changes in the overall rate of protein synthesis in the cell, or changes in the translation rate of just one particular species of mRNA. Mammalian reticulocytes provide an example of the former type of regulation (Safer, 1983). In the absence of heme, the translation protein, eukaryotic initiation factor 2 (eIF2) is phosphorylated. Phosphorylation of eIF2 causes it to become complexed with a second protein and unable to perform its function in translation. As a result, translation as a whole slows down.

Control of the translation rate of individual mRNAs does not rely on changes to the translational machinery. Instead, it probably involves the interaction of trans-acting factors with specific sequences in the mRNA. These factors may act to block the binding, or the progress of the translational machinery along the mRNA, or they may facilitate initiation of translation via an effect on mRNA conformation (Jackson and Standart, 1990). Since the translation initiation complex first interacts with the 5' end of the message, it is reasonable that controlling sequences might be located in this region.

Indeed, the 5'-UTR of ferritin mRNA contains a stem-loop structure which, in the absence of iron, binds an iron-responsive element binding protein (IRE-BP). Binding of the IRE-BP to this stem-loop structure effectively blocks translation of the ferritin message (Walden et al., 1989).

Regulatory sequences however, are not limited to the 5' end of the mRNA. The poly(A) tail, poly(A) binding protein, and the 3'-UTR also seem to have a significant effect on mRNA translation. For example, the addition of long poly(A) tails to chymosin and lysozyme mRNAs increases the rate of their translation in Xenopus oocytes 10 and 20-fold respectively. Meanwhile, deadenylated mRNAs in the same system, are translated with only 50% of the efficiency of the poly(A) form (Drummond et al., 1985, Galili et al., 1988). It has been suggested that the poly(A) tail and poly(a) binding protein may promote interaction between the 5' and 3' ends of the mRNA. This then would increase the likelihood of ribosomes reinitiating translation on the same transcript (Jackson and Standart, 1990).

Another sequence shown to have an effect on translation is an AU-rich sequence in the 3'-UTR of c-fos and granulocyte-macrophage colony stimulating factor (GM-CSF). This is the same sequence that was previously shown to be at least partially responsible for the short half-lives of these mRNAs. The presence of the AU-rich sequence appears to decrease the translational efficiency of the c-fos and GM-CSF mRNAs in both Xenopus oocytes and reticulocyte lysates (Kruys et al., 1989).

The androgen-regulated gene, probasin is an example of another type of translational regulation (Spence et al., 1989). The probasin mRNA contains two in-frame

initiation codons separated by bases coding for a signal peptide. Initiation from the first AUG results in the presence of the signal peptide, and thus synthesis of a secreted protein. Conversely, use of the downstream AUG results in the exclusion of the signal peptide and the production of an intracellular protein. Two separate translation initiation sites are also present in the c-myc mRNA. Interestingly, the upstream initiation site in c-myc mRNA is encoded by a CUG rather than an AUG codon (Hann et al., 1988). The two forms of the c-myc protein are expressed coincidentally in murine erythroleukemia cells, however the ratio of their synthesis changes during drug-induced differentiation of these cells (Spotts and Hann, 1990). The mechanism and significance of this change is unknown. The odorant-binding protein, as well as the Ia antigen-associated invariant chain, are also regulated at the translational level by the use of multiple AUG initiation codons (Pevsner et al., 1988, Strubin et al., 1986).

Post-translational Modifications

Following translation, a large number of polypeptides undergo processing before they play an active role. One common type of processing is the removal of the signal peptide from proteins synthesized into the endoplasmic reticulum (Walters et al., 1984). Likewise, a N-terminal extension is removed from proteins translocated into mitochondria (Schatz and Butow, 1983). Many protein hormones such as insulin, are synthesized in an inactive prohormone form, and are later cleaved to yield a smaller bioactive protein (Fisher and Scheller, 1988). The proopiomelanocortin (POMC) polypeptide is cleaved

to yield a number of different biologically active proteins. Interestingly, different tissues have been found to process POMC in different ways to yield different, tissue-specific proteins (Lundblad and Roberts, 1988).

A large number of modifications such as phosphorylation, glycosylation, amidation etc. may also occur. These may change the activity level of the proteins or may specify localization within the cell (Fisher and Scheller, 1988). A final modification affecting gene expression is ubiquitination. The attachment of several ubiquitin proteins to a particular polypeptide has been shown to target that polypeptide for degradation, thus ending its expression in the cell (Ciechanover et al., 1984).

C. Regulation of Gene Expression by Androgens and Prolactin

Androgens

Androgens have been shown to regulate the expression of several specific genes. In the mouse kidney, these include the genes encoding kidney androgen-regulated protein (KAP), ornithine decarboxylase (ODC), ß-glucuronidase (GUS) and alcohol dehydrogenase (ADH) (Berger and Watson, 1989). Probasin - a rat prostatic protein, and several secretory proteins from the rat seminal vesicle have also been shown to be androgen regulated (Spence et al., 1989, Mansson et al., 1981, Williams et al., 1983, Harris et al., 1990). Two androgen-regulated proteins, including the prolactin-inducible protein have been identified in HBC cells (Murphy et al., 1987b, Chalbos et al., 1987).

Androgen action on gene expression is believed to be mediated through the androgen receptor (Berger and Watson, 1989). Like other members of the steroid hormone family, androgens are believed to bind to intracellular receptors. The steroid-receptor complex then interacts with enhancer-like, androgen-responsive elements (AREs) in the DNA, altering the transcription rate of a particular gene (Beato, 1989). Nuclear run-on assays have demonstrated that androgens increase the transcription rates of the ovalbumin and ovomucoid genes in the chick oviduct, the PIP gene in the T-47D HBC cell line, the rat prostatic binding protein (PBP), and the ODC, ADH and RP2 genes in the mouse kidney (Compere et al., 1981, Murphy et al., 1987b, Page and Parker, 1982, Rheaume et al., 1989, Felder et al., 1988). Androgens have also been shown to induce the synthesis of KAP and GUS mRNAs *in vivo* in the mouse (Watson and Paigen, 1988).

Attempts to identify androgen-responsive elements surrounding these genes have proven difficult. One ARE has been identified in the long terminal repeat of the mouse mammary tumour virus (MMTV-LTR) (Ham et al., 1988). It consists of four partial inverted repeats of the hexanucleotide TGTTCT and also serves as the response element for progestins and glucocorticoids. This hexanucleotide is present in the 5' flanking region of at least one of the eukaryotic androgen-regulated genes, but has not yet been shown to be functional as an ARE in this system (Myal et al., 1990). Analysis of the 5' flanking regions of androgen-responsive genes from the rat seminal vesicle and prostate, and of the rat renin genes failed to reveal any consensus sequence which could serve as an ARE (Williams et al., 1985). The ARE for these and other genes may either lie downstream of the start site, or else it may diverge significantly between genes.

Increases in the levels of RP2, ADH, ODC, PBP and GUS mRNAs, following androgen treatment, cannot be fully accounted for by increases in the transcription rates of these genes (Berger and Watson, 1989). This may reflect an additional effect of androgens, either direct or indirect, on the processing or stability of these transcripts. Alternatively, the discrepancy between the level of mRNA induction and the transcription rate may reflect a deficiency in the ability of nuclear run-ons to accurately measure real changes in transcription rates. Rheaume et al. (1988) have found that constitutive, and occasionally antisense, transcription in particular sections of genes, may mask true transcription rates as measured by nuclear run-on analysis.

While androgens have large effects on the mRNA levels of many genes, they can also alter gene expression by acting at the protein level. For example, androgens have been shown to increase ODC protein levels by stabilizing the protein in addition to stimulating transcription (Seely et al., 1982).

Prolactin

While over 85 distinct physiological effects of prolactin have been catalogued, the specific genes regulated by prolactin have been identified in only a few cases (Nicoll, 1974). These include genes encoding several proteins expressed in the mammary gland such as the caseins, the rodent-specific whey acidic protein (WAP), α-lactalbumin, PIP in HBC and the parathyroid hormone-like peptide (PTHLP) (Rosen et al., 1986, Shiu and Iwasiow, 1985, Thiede, 1989). Prolactin regulates the expression of calpactin in the

pigeon cropsac, and of aromatase mRNA in the rat ovarian granulosa and luteal cells (Horseman, 1989, Krasnow et al., 1990). In the rat Nb2 T lymphoma cell line, prolactin stimulates a number of growth-related genes including actin, c-myc, ornithine decarboxylase (ODC), interferon regulatory factor-1 (IRF-1), and a heat-shock protein 70 homologue (Yu-Lee, 1990, Fleming et al., 1985, deToledo et al., 1987). Finally, prolactin has been shown to regulate the concentration of androgen receptors in the rat lateral prostate as well as the concentration of its own receptor in rat liver (Prins, 1987, Barash et al., 1988).

Like other peptide hormones, prolactin initiates its action by binding to its specific receptor on the cell surface. The prolactin receptor is a member of a novel receptor family which includes the receptors for growth hormone, erythropoietin, granulocyte- and granulocyte-macrophage colony stimulating factors, and interleukins 2, 3, 4 and 6 (Fukunaga et al., 1990). Two distinct forms of the prolactin receptor have been cloned (Boutin et al., 1988, Edery et al., 1989). The two forms vary only in their cytoplasmic domains, and are believed to have arisen from alternative splicing of a single primary transcript (Shirota et al., 1990). Both forms of the receptor are expressed in rat ovary and liver with the short form predominating in the liver and the longer form predominating in the ovary. The two forms of the receptor appear to mediate different functions of prolactin in the same tissue, as only the long form is able to induce ß-casein expression (Lesueur et al, 1991).

Despite the cloning of the receptor, the signal transduction pathway used by prolactin remains unknown. No consensus sequence for a tyrosine kinase domain, nor

any potential phosphorylation site has been found in the receptor (Edery et al., 1989). Many compounds have been investigated as potential second messengers for prolactin. These include synlactin, cyclic nucleotides, prostaglandins, polyamines, protein kinase C and calcium ions (Nicoll et al., 1985, Matusik and Rosen, 1980, Rillema, 1975, Oka et al., 1978, Caulfield and Bolander, 1986). Several of these have been shown to elicit some effect in the target cells, yet none are sufficient to account for all of prolactin's effect.

The mechanisms induced by prolactin are only slightly better elucidated at the level of the gene. Prolactin's effect on the rat ß-casein gene has been studied extensively. Prolactin alone appears to increase the transcription rate of the ß-casein gene from two to six fold in both mammary explant cultures and mouse mammary cell lines (Eisenstein and Rosen, 1988, Ball et al., 1988, Rosen et al., 1986). This increase in transcription is sufficient to account for the prolactin-induced increase in ß-casein mRNA levels (Eisenstein and Rosen, 1988). Transfection assays utilizing rat ß-casein promoter-chloramphenicol acetyltransferase (CAT) fusion genes reveal that the sequences conferring transcriptional responsiveness to prolactin are within -2300 to +490 bp of the ß-casein start site (Lee et al., 1989, Doppler et al., 1989).

Prolactin's effect on ß-casein expression however is not limited to transcription alone. Incubation of mammary cell lines or explant cultures with glucocorticoids results in little or no increase in the level of ß-casein mRNA (Eisenstein and Rosen, 1988). Addition of prolactin however, results in an approximately 70 fold increase in ß-casein mRNA - far more than can be accounted for by prolactin's transcriptional effect alone.

Prolactin then, acts synergistically with glucocorticoids to increase ß-casein mRNA levels at some point other than transcription.

The synergism between prolactin and glucocorticoids is apparently mediated by intragenic sequences in the casein gene. In transgenic mice bearing rat ß-casein promoter-CAT fusion genes, CAT expression is induced only 1 to 4.5 fold by treatment with prolactin and glucocorticoids, while the endogenous mouse ß-casein mRNA levels rise 25 to 40 fold (Lee et al., 1989). Conversely, in mice bearing the entire rat ß-casein gene, prolactin and glucocorticoids induce the transgene to levels similar to that of the endogenous mouse gene.

While sequences within the rat ß-casein gene confer a large portion of the gene's responsiveness to prolactin, the nature of prolactin's effect on these sequences is unclear. Using pulse-chase analysis in explant cultures, Rosen et al. (1986) found the stability of ß-casein transcripts were increased by prolactin only when a short, 30 min. pulse was used. When a longer pulse was employed, no effect of prolactin on casein mRNA stability was seen. It was suggested that prolactin's action was to stabilize the nuclear casein transcripts, such that a greater proportion would reach the cytoplasm where they would be fully stable.

Nuclear run-on analysis employing genomic clones from different portions of the ß-casein gene indicated an effect of prolactin on the elongation of transcription (Rosen et al., 1989). The investigators hypothesized that in the absence of prolactin, a block to transcriptional elongation, or a "pausing" of transcription appears to occur near the 5' end of the ß-casein gene, with full length transcripts synthesized only in the presence of

prolactin. Goodman and Rosen (1990) confirmed the nuclear run-on results using ^{3}H-uridine pulse assays on intact cells, eliminating the possibility that the observation was an artifact of the *in vitro* assay. The investigators have hypothesized that the prematurely terminated ß-casein transcripts seen in the absence of prolactin, represent the unstable casein transcripts found in the earlier pulse-chase stability analysis.

The prolactin-inducible protein is induced slowly in HBC cell lines in response to prolactin (Shiu and Iwasiow, 1985). Approximately 24 hours of exposure to prolactin are required before PIP mRNA levels rise appreciably above control levels (Murphy et al., 1987b). Prolactin did not appear to stimulate transcription of the PIP gene as measured by nuclear run-on analysis (Murphy et al., 1987b).

In the rat Nb2 T-lymphoma cell line, prolactin stimulates the expression of a number of growth-related genes such as c-myc, ß-actin, ODC, HSP70 and IRF-1 (Yu-Lee et al., 1990). While prolactin may exert some posttranscriptional effect on the c-myc gene, nuclear run-on analysis suggests that prolactin acts primarily to increase the rate of transcription of all of these genes (Yu-Lee, 1990, Yu-Lee et al., 1990). Several lines of evidence however, suggest that the pathway employed by prolactin to stimulate these growth-related genes in Nb2 cells may be distinct from the pathways employed in the induction of the PIP and casein genes. First, the activation of these genes by prolactin occurs rapidly, with detectable increases in the transcription rates of ß-actin, c-myc and IRF-1 after only 15 minutes of hormone treatment (Yu-Lee et al., 1990, Yu-Lee, 1990). This is in sharp contrast with the ß-casein and PIP genes where increased expression is not seen until after several hours of exposure to prolactin (Eisenstein and Rosen, 1988,

Murphy et al., 1987b). It may be argued that the induction of the PIP and casein genes by prolactin requires *de novo* synthesis of specific regulatory factors, and that induction of these factors accounts for the lag time in PIP and casein expression (Yoshimura and Oka, 1990). It is unlikely however, that these early prolactin-induced factors, which may regulate PIP and casein expression, are the same proteins that are induced early in the Nb2 cells since the induction of ß-casein and PIP in mouse mammary explant cultures and the T-47D HBC cell line respectively does not involve changes to the level of ß-actin mRNA (Rosen et al., 1986, Murphy et al., 1987b). Finally, since prolactin's action as a powerful mitogen is unique to the Nb2 cell line, the activation of growth-related genes by prolactin may represent an unusual linkage of the prolactin receptor with mitogenic pathways in these cells (Gout et al., 1980, Shiu, 1980).

D. The Prolactin-Inducible Protein (PIP)

The prolactin-inducible protein (PIP) was independently isolated from the human breast cancer cell line T-47D (PIP), and from human breast gross cystic disease fluid (Gross Cystic Disease Fluid Protein-15 or GCDFP-15) (Shiu and Iwasiow, 1985, Haagensen et al., 1979). It is an 11 K secreted protein with both 14 K and 16 K glycosylated forms and an isoelectric point of 3.75 (Shiu and Iwasiow, 1985, Vandewalle et al., 1986). PIP has been shown to bind to fibrinogen with an estimated association constant of 7 X 10^7 litres/mole (Haagensen et al., 1990).

Using immunohistochemistry, Mazoujian et al. (1983) found PIP in the exocrine cells of the salivary, lacrimal, ceruminous and sweat glands. PIP-like immunoreactivity has also been found in submucosal glands of the bronchi, the modified sweat glands (of Moll) of the eyelid and the apocrine glands of the perineum. Silva et al. (1983) detected PIP protein by radioimmunoassay in extracts of "normal" human mammary glands. However, Mazoujian et al. (1983) failed to find PIP by immunoperoxidase staining in normal breast ductules and lobules, and Murphy et al. (1987a) were unable to detect PIP mRNA in the single normal breast sample investigated by them. PIP has been shown to be present in human milk and thus may be expressed by normal mammary glands during lactation (Mazoujian et al., 1983).

Low levels of PIP have been detected in the serum of healthy women while higher levels have been detected in the serum of patients with gross cystic breast disease and breast carcinoma (Haagensen et al., 1990, Murphy et al., 1987a). PIP is a major protein component of breast gross cystic disease fluid, and about 60% of human breast cancer biopsies contain detectable levels of PIP mRNA (Murphy et al., 1987a). PIP is also expressed by the estrogen receptor positive HBC cell lines T-47D, ZR-75-1, and BT-474 (Murphy et al., 1987b).

The PIP gene is conserved in a number of species including monkey, cow, dog, chicken, rabbit, rat and mouse (Myal et al., 1990). The mouse homolog of PIP shares a 67% homology with the human cDNA and was previously identified as a mouse submaxillary gland protein (mSMGP) (Windass et al., 1984, Murphy et al., 1987b). The human PIP gene is approximately 7 Kb in length and has been localized to the long arm

of chromosome seven (Myal et al. 1990, Myal et al., 1989a). The 5' end of the gene contains a "TATA" box-like and a "CAAT" box-like sequence, as well as four copies of the sequence TGTTCT which forms the core element of the androgen, glucocorticoid and progesterone responsive elements in the mouse mammary tumour virus (Myal et al., 1990, Ham, et al., 1988). Two restriction length polymorphisms (RFLPs) are also present in the 5' region of the gene (Myal et al., 1989b). Northern analysis indicates that both HBC cell lines and biopsy specimens contain a single mRNA species of about 900 bases (Murphy et al. 1987a&b).

PIP was originally identified by Shiu and Iwasiow (1985) as a protein synthesized in T-47D cells in response to incubation with human prolactin and hydrocortisone. Subsequent analysis has shown that dihydrotestosterone (DHT) is the most potent steroid at increasing PIP mRNA levels, and that human growth hormone (hGH), acting through the prolactin receptor is equipotent to human prolactin (Murphy et al., 1987b, Shiu, 1979). PIP mRNA levels in T-47D cells are increased approximately 5 fold after 5 days of prolactin or hGH treatment, while incubation with DHT for 3 days results in a 10 fold accumulation (Murphy et al., 1987b, Kelton and Monahan, 1986). Synthetic progestins and hydrocortisone can also cause PIP mRNA accumulation although they are 3-4 orders of magnitude less effective than DHT (Murphy et al., 1987b). In ZR-75-1 cells, estrogen inhibits both the basal and DHT-induced accumulation of PIP mRNA (Simard et al., 1989). Interestingly, androgens have been shown to inhibit both basal and estrogen-induced proliferation in these cells (Poulin et al., 1988). Nuclear run-on analysis by Murphy et al. (1987b) showed that while DHT increased the transcription rate of PIP 4-

fold, prolactin or hGH had no apparent effect on transcription.

Indirect, *in vivo* evidence of hormonal control of PIP gene expression also exists. Haagensen et al. (1981) induced elevated plasma levels of PIP in a portion of patients with advanced metastatic breast carcinoma by treatment with the synthetic androgen fluoxymesterone. The induction was particularly strong in patients with a high basal serum level of PIP. Silva et al. (1983), using a radioimmunoassay of cell lysates found that PIP protein levels in "normal" human breast tissue varied consistently during the menstrual cycle. Finally, PIP expression in HBC biopsies is well correlated to the estrogen receptor content of the tumour (Murphy et al., 1987a, Silva et al., 1982).

E. Research Objectives

The mechanisms by which prolactin and androgens regulate gene expression are largely unresolved. Expression of the prolactin-inducible protein in the HBC cell line T-47D is regulated by physiological concentrations of prolactin and androgen. PIP mRNA levels increase slowly over 5 days in response to the hormones - 5 fold in response to prolactin and 10 fold in response to androgens. The effect of both hormones on PIP expression is additive. Nuclear run-on analysis using cDNA probes indicated that androgens increase transcription 3.7 fold while prolactin has no apparent effect on transcription.

The PIP gene in T-47D cells presents a useful model to study the mechanisms by

which prolactin and androgens regulate gene expression. The following investigations will be performed to that end:

1. PIP mRNA Stability Analysis

Since prolactin does not appear to affect the transcription rate of the PIP gene, it may increase PIP gene expression by stabilizing the PIP mRNA. Androgens may also affect the stability of the PIP transcript in addition to their effect on transcription. The stability of the PIP mRNA under various hormonal conditions will be investigated.

2. Hormonal Regulation of PIP Heteronuclear RNA (hnRNA)

It has been proposed that prolactin may increase ß-casein expression by stabilizing the precursor hnRNA from degradation by ribonucleases, thereby allowing a greater proportion to be processed to mature transcripts (Rosen et.al., 1986). Changes to either the degradation or the processing rate of PIP hnRNA may be reflected by changes in the rate of its disappearance from the cell. The disappearance rate, or stability of the PIP hnRNA under various hormonal conditions will be examined.

3. Nuclear Run-On Analysis

Nuclear run-on analysis using PIP cDNA probes indicated that androgens increase transcription of the PIP gene. Prolactin however appeared to have little effect on transcription. In the time since these transcription assays were performed, reports have appeared which suggest that, constitutive transcription within a gene may mask changes in transcription rates as measured by cDNA probes in nuclear run-on analysis

(Rheaume et.al., 1988, Rheaume et.al., 1989). Thus prolactin may yet stimulate PIP gene transcription.

Nuclear run-on analysis using 5'- and 3'-specific genomic PIP probes will be conducted to determine if prolactin has effects on transcription that are not readily apparent using cDNA probes. Specifically, an effect on transcriptional elongation, such as has been shown for the casein gene, will be investigated.

Determination of the general mechanisms used by prolactin and androgens in the regulation of PIP expression, will allow further work to be carried out towards characterization of the sequences and factors which mediate these effects. This knowledge may lead to better insight into the role of prolactin and androgens in normal tissue, and in the pathogenesis of the human breast.

MATERIALS AND METHODS

A. Tissue Culture

The T-47D cell line was derived from the pleural effusion of a 54 year old patient with disseminating carcinoma of the breast and was obtained from the E.G. and G. Mason Research Institute (Rockville MD). The cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 10 μg/ml insulin, 50 μg/ml penicillin, 50 units/ml streptomycin, 3.5 g/litre glucose, and 2 mM glutamine. This medium is referred to as complete medium (CM). All of the above components were obtained from GIBCO. Just prior to the cells becoming confluent, trypsin-EDTA was used to detach the cells and remove them to fresh flasks at a lower density. Trypsin-EDTA was also purchased from GIBCO. Tissue culture plates and flasks were purchased from Corning or Canlab.

B. Hormone Treatment

For use in experiments, cells were collected using trypsin-EDTA and counted with a Coulter counter. Cells were then plated in CM, and allowed to attach to the plates. After 48 hours the medium was aspirated, the cells were washed in DMEM, and then given DMEM supplemented with penicillin, streptomycin, glucose, glutamine and 0.01% bovine serum albumin. This medium was referred to as serum-free medium (SFM). The cells then received either 1 μg/ml human growth hormone (hGH), 10^{-8} M

5α-dihydrotestosterone (DHT), both hormones together, or ethanol carrier only (None). Human growth hormone was the kind gift of Drs. I. Worsley and H.G. Friesen (Dept. of Physiology, University of Manitoba). Human growth hormone is equipotent to human prolactin in its effect on T-47D cells and is routinely used as the lactogen of choice as it is more readily available (Shiu, 1979). Incubation with hormones varied from 4 hours to 4 days depending on the experiment.

C. RNA Isolation

Total RNA was isolated using the guanidine thiocyanate/cesium chloride method (Chirgwin et al., 1979). Medium on the tissue culture plates was aspirated and the cells were scraped with a rubber policeman in a solution containing 4 M guanidine thiocyanate, 17 mM N-lauroyl sarcosine, 0.007% 2-mercaptoethanol, and 25 mM sodium citrate pH 7.0. This solution is referred to as guanidine thiocyanate solution (GuSCN) and all of its components were obtained from Sigma. The cells in GuSCN were aspirated several times through a 21-gauge needle and then layered over 5 mls of 5.7 M cesium chloride-0.1 M EDTA in Beckman Quickseal centrifuge tubes (16 x 76 mm). The RNA was pelleted on the basis of its bouyant density during centrifugation at 35000 RPM for 18 hours in a Ti75 rotor in a Beckman L-8 ultracentrifuge. Following centrifugation, the RNA pellet was resuspended in TE (10 mM Tris-Hcl, 1 mM EDTA) and then ethanol precipitated in 0.2 M NaAc and 2.2 vol. of ethanol at -70°C. The precipitated RNA was pelleted, dried, and then resuspended in double distilled H_2O treated with 0.1% diethyl

pyrocarbonate (DEPC-H_2O).

D. Northern Analysis

Northern analysis was performed largely as outlined in Maniatis et al. (1982). The essential features of this procedure are summarized below:

i. Gel Electrophoreses of RNA

Total RNA was denatured in 33% formamide and 2.2 M formaldehyde at 65°C for 15 minutes. The RNA was then electrophoresed in a 1% (w/v) agarose gel containing 2.2 M formaldehyde, 40 mM MOPS pH 7.0, 10 mM NaAc pH 5.2, 1 mM EDTA pH 8.0, and 0.06 μg/ml ethidium bromide. The RNA was transferred to a Nitroplus 2000 (Micron Separations Inc.) hybridization filter using 20 X SSC (1 X SSC = 0.15M NaCl, 0.015 M sodium citrate) and the filter was baked for 2 hours under vacuum at 80°C. Equal RNA loading was confirmed by visual inspection of ethidium bromide staining under ultraviolet (UV) light.

ii. Nick Translation and Hybridization

DNA probes were labelled by nick translation. Labelling was carried out using α^{32}P-dCTP (3000 Ci/mmol) and a nick translation kit obtained from Amersham. Filters containing RNA were placed into Seal-o-Meal bags (Sears Canada) and prehybridized for at least 2 hours at 42°C in hybridization solution containing 50% (v/v) formamide,

5 X Denhardt's solution (1 X Denhardt's = 0.02% w/v each of BSA, Ficoll,and polyvinylpyrrolidine), 5 X SSPE (1 X SSPE = 0.15 M NaCl, 0.01 M NaH_2PO_4, and 1 mM EDTA), 250 μg/ml denatured salmon sperm DNA, and 0.1% sodium dodecyl sulfate (SDS). Following prehybridization, the bags were opened and the nick translated probe was added. The bags were then resealed and placed in a 42°C shaking water bath for approximately 16 hours. Following hybridization, the filters were washed 2 times in 2 X SSC/0.1% SDS at room temperature for 20 minutes and then 2 times in 0.1 X SSC/0.1% SDS at 65°C for 30 minutes. The filters were then placed into Seal-O-Meal bags again and autoradiographed using Kodak XAR x-ray film and an intensifying screen at -70°C.

iii. RNA Probes and Hybridization

Antisense RNA probes were used to detect lower abundance RNAs. The probes were generated using α^{32}P-UTP (650-800 Ci/mmol, ICN) and a Transprobe T kit obtained from Pharmacia. The sequences to be used as probes were subcloned as double stranded DNA into the plasmid vector PVZ1 (Bluescribe vector modified by S. Heinkoff, Fred Hutchinson Cancer Research Centre). PVZ1 contains the T3 and T7 RNA polymerase promoters on either side of its multiple cloning site. Following subcloning, the orientation of the insert within the plasmid was determined using restriction endonucleases, and the plasmid was then linearized immediately 3' of the sense strand. The linearized plasmid was then incubated with a nucleotide buffer, α^{32}P-UTP, and 70 units of either T3 or T7 RNA polymerase, whichever was 5' of the sense strand of the

insert. The reaction proceeded for 30 min. at 37°C, during which time the RNA polymerase utilized the appropriate promoter to transcribe the sense strand of the DNA insert into antisense RNA containing the labelled UTP. The reaction was stopped by the addition of 15 units of DNase I (10 min., 37°C) which degraded the DNA template. The reaction mixture was then extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and the labelled RNA was ethanol precipitated. The RNA was pelleted, dried, and resuspended in DEPC-H_2O. The extent of the reaction was determined by electrophoresis of a small sample of the RNA on a 5% polyacrylamide gel and autoradiography.

Filters containing RNA were prehybridized for at least 2 hrs at 65°C in hybridization buffer containing 50% formamide, 5 X SSC, 50 mM Tris-HCl pH 7.5, 0.1% w/v sodium pyrophosphate, 1% SDS, 0.2% (w/v) each of polyvinylpyrrolidine and Ficoll, 5 mM EDTA, and 150 μg/ml denatured salmon sperm DNA. The RNA probes were denatured by boiling for 5 min. before hybridization. Probes were hybridized to the filters at 65°C for 16-24 hrs and then washed twice in 2 X SSC/0.1% SDS at 65°C for 20 min. and twice in 0.1 X SSC/0.1% SDS at 65°C for 30 min. before autoradiography.

E. RNA Stability Studies - Pulse Chase

T-47D cells were plated at 7.5 X 10^5 cells per 60 mm dish and allowed 48 hrs to attach. The cells were then incubated with hormones as described above. In addition to the hormones, 2.5 mCi of [5,6-^{3}H]-uridine (40 mCi/ml) (ICN) was added to each plate.

After 24 hours incubation, 5 mM unlabelled uridine was added to the medium. At various times following the addition of unlabelled uridine, the cells were collected, and RNA was isolated by the guanidine thiocyanate / cesium chloride method described above. The amount of isolated RNA was determined by spectrophotometry and scintillation counting.

For the detection of specific transcripts, denatured plasmid DNA was bound to nitrocellulose using a dot blot apparatus. The DNA was denatured by boiling for 10 min., after which 1.0 vol. of 1 M NaOH was added and the DNA was left at room temperature for 20 min.. The mixture was then neutralized with 0.5 vol. of neutralization solution (1 M NaCl, 0.3 M Na citrate, 0.5 M Tris-HCl pH 8.0, 1 M HCl) and placed on ice. The DNA was diluted with several vol. of 15 X SSC and applied to the nitrocellulose filters. The DNA was bound to the filters by baking at 80°C for 2 hrs under vacuum. The filters were prehybridized in Seal-o-Meal bags for at least 2 hours in hybridization buffer (see Sec. Dii - Nick Translation and Hybridization). Following prehybridization, the buffer was removed, replaced with fresh buffer, and equal amounts of RNA were added to each filter. Hybridization was carried out at 42°C for at least 16 hours. Following hybridization, the filters were washed 4 times in 2 X SSC at 65°C for 15 min., once for 30 min. in 2 X SSC / 10 μg/ml RNase A at 37°C, and once for 30 min. at 37°C in 2 X SSC alone. The filters were dried, the dots were cut out, and counted in OCS.

RNA Stability Studies - Act D/DRB

To determine the stabilities of specific transcripts, T-47D cells were plated at 2 X 10^6 cells per 150 mm plate, and treated with hGH, DHT, hGH and DHT, or carrier only as described in section B. After 2 to 4 days in SFM plus hormones, actinomycin D (Act D)(5 μg/ml) or 5,6-dichloro-1-ß-D-ribofuranosylbenzimidazole (DRB)(75μM) was added. Act D and DRB are transcription inhibitors - Act D inhibits transcription from all 3 polymerases while DRB is specific for RNA polymerase II. Cells were harvested as described in section 3, at various times following the addition of Act D or DRB. Any cells which were not collected until 24 hrs or more after the initial treatment with DRB were treated with a second equal dose of DRB at 24 hrs. Total RNA was isolated from the cells, and the amount of specific transcript remaining at various times after Act D or DRB treatment was detected by Northern hybridization using either nick translated DNA or antisense RNA probes. Autoradiograms were quantitated by densitometry. A slope was obtained for each hormone treatment, in each experiment, by linear regression analysis. Analysis of variance was performed on the slopes to determine significance.

F. Nuclear Run On Transcription Assay

i. Nuclei Isolation

Nuclei were isolated as outlined (Dubik and Shiu, 1988) with some modifications. T-47D cells were plated on 150 x 25 mm plates at 4 x 10^6 cells per plate, and treated

with DHT, hGH, DHT and hGH, or ethanol carrier only as described in Section B. The cells were treated with hormones for 24 to 72 hrs depending on the experiment. Following hormone treatment, the medium was aspirated and the cells were washed in 7 mls of ice-cold phosphate buffered saline (PBS). The PBS was removed, replaced with fresh PBS and the cells were collected by scraping with a rubber policeman. The cells were pelleted by centrifugation at 75 x g at 4°C in a Damon PR6000 centrifuge. The cell pellet was then resuspended in 3 mls of lysis buffer consisting of 10 mM KCl, 10 mM Tris-HCl pH 7.5, and 2 mM $MgCl_2$, and aspirated 5 times through a 21-gauge needle to disperse them fully. Following aspiration, Nonidet P-40 (NP-40) was added to a final concentration of 0.2% and the cells were aspirated again. The cells were then left on ice in the lysis mixture for 7 to 8 min. before being aspirated a third time in order to separate the nuclei from the cell membrane. The NP-40 was then diluted by the addition of 4 mls of lysis buffer and the mixture was centrifuged for 3 min. at 150 x g at 4°C. The nuclear pellet was resuspeded in 5 mls of wash buffer consisting of 10 mM Tris-HCl, 2 mM $MgCl_2$, 100 mM KCl,and 0.25 M sucrose, and recentrifuged at 150 x g for 3 min..

ii. Transcription Assay

The transcription assay was performed as described (Marzluff and Huang, 1985) with some modifications. Nuclei were incubated for 30 min. at 25°C in 20% (v/v) glycerol, 30 mM Tris-HCl pH 8.0, 2 mM $MgCl_2$, 100 mM KCl, 0.05 mM EDTA, 750 μCi/ml α-^{32}P UTP and 500 μM each of ATP, CTP and GTP. The transcription reaction

was terminated by the addition of 6 mls of GuSCN and aspiration through a 21-gauge needle. Forty micrograms of E. coli ribosomal RNA was added as a carrier, and the RNA was isolated by the guanidine thiocyanate/cesium chloride method as described in Section C. Following isolation, the yield of labelled RNA was determined by precipitation in trichloroacetic acid (TCA) and scintillation counting. TCA precipitation was carried out as described in Maniatis et al. (1982). Two microlitres of the RNA in DEPC-H_2O was spotted on each of 2 Whatman GF/C glass fibre filters and dried at room temperature. One filter was then washed 4 times in ice-cold 5% TCA/20 mM sodium pyrophosphate for 2 min., and twice briefly in 95% ethanol. The filter was then dried and both filters were placed in scintillation vials. The radioactivity on each filter was determined by scintillation counting in 5 mls of organic counting scintillant (OCS). The difference in radioactivity on each filter represents the amount of free ^{32}P-UTP isolated with the RNA.

iii. Hybridization

For the detection of specific transcripts, 5 ug each of PVZ1, PIP cDNA, gPIP 1.5, gPIP 0.7, and ß-actin DNA were bound to nitrocellulose using a Bio-Rad slot blot apparatus as described above (Section E). The nitrocellulose filters were prehybridized for at least 2 hrs in hybridization buffer (see Sec. Dii - Nick Translation and Hybridization). After prehybridization, the buffer was removed and replaced with fresh buffer. An equal number of counts of labelled RNA were added to each filter as probe. Prior to addition to the filters, the labelled RNA was boiled for 5 min. and then cooled

rapidly. Hybridization was carried out at 42°C for at least 16 hrs. Following hybridization the filters were washed 4 times in 2 X SSC/0.1%SDS for 5 min at room temperature and then twice in 1 X SSC/0.1%SDS for 30 min. at 65°C. Filters were then washed in 0.3 M NaCl with 10 μg/ml RNase A for 10 min. at 37°C, followed by 10 min. in 0.3 M NaCl at room temperature. The filters were exposed for various lengths of time to x-ray film at -70°C with an intensifying screen. Hybridization signals were quantitated by densitometry.

G. Subcloning of DNA Fragments

Plasmid DNA was digested at 37°C with restriction endonucleases (RE) obtained from either Pharmacia, Bethesda Research Laboratories (BRL), or Boehringer Mannheim. Either the buffer supplied with the enzyme, or the appropriate RE buffer as described in Maniatis et al.(1982) was used in the digestions. Digestions were usually no longer than 3 hrs in length. When appropriate, the RE were inactivated following digestion by heating at 65°C or 85°C for 20 min.. The method of Andrews et al.(1982) was used to separate the RE digested DNA fragments. The DNA was electrophoresed in horizontal gels of 1% (w/v) agarose dissolved in TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) with 0.2 μg/ml EtBr. The gels were run at room temperature in TBE and the DNA bands were visualized under UV light.

A small well, roughly corresponding to the size of the DNA band to be eluted, was cut out of the gel immediately in front of the band. The well was filled with 5 X TBE and

the DNA was electrophoresed further. When the DNA band had entered the well, the electrophoresis was stopped, and the DNA in 5 X TBE was eluted from the well. The DNA was extracted 3-4 times with 3-4 vol. of isoamyl alcohol and then ethanol precipitated. The DNA was pelleted and redissolved in TE.

DNA fragments isolated by electroelution were ligated into plasmid vectors in order to be amplified for use in experiments. Plasmids were digested with the appropriate RE and the enzyme was heat inactivated. The RE digested plasmid, the DNA fragment of interest, and T4 DNA ligase (BRL) were combined in an eppendorf tube in buffer supplied by the manufacturer. The ligation reaction was allowed to proceed for at least 2 hrs at room temperature. The majority of subcloning was done into the PVZ1 vector, a modified Bluescribe vector containing an ampicillin resistance gene.

Competent cells were generated for transformation by the following protocol. A single bacterial colony was used to inoculate 5 mls of LB broth (1 litre LB = 10 g bactotryptone, 5 g bacto yeast extract, 10 g NaCl) and was allowed to grow O/N at 37°C in a shaking incubator. One ml of the O/N culture was added to 100 mls of LB broth and grown for 1.5 hrs before being centrifuged for 10 min. at 2500 RPM at 4°C. The bacterial pellet was redissolved in 20 mls of sterile ice-cold 50 mM $CaCl_2$/10 mM Tris-HCl and left on ice for 30 min. The bacteria were pelleted again by centrifuging for 10 min. at 2500 RPM at 4°C and resuspended in 4 mls of the cold $CaCl_2$/Tris-HCl solution.

Two hundred ul of the competent cells were added to 10-50 ng of plasmid from the ligation mixture, mixed gently, and placed on ice for 30 min.. The bacteria were then placed at 42°C for 60 sec. before being returned to the ice for 2 min.. SOC broth (900

μl)(1 litre SOC = 20 g bactotryptone, 5 g bacto yeast extract, 10 mM NaCl, 2.5 mM KCl) was added to the bacteria and the tube was shaken at 37°C for 1 hr before being poured and spread onto LAX plates (LB broth with 15 g/L bacto agar, 200 μg/ml ampicillin, 50 μg/ml X-Gal, 100 mM IPTG). The plates were dried briefly in a laminar flow hood before being inverted and grown O/N at 37°C. Only those bacteria containing plasmid would grow in the antibiotic. Those colonies containing recombinant plasmids grew white, while those without inserts grew as blue colonies.

H. Small Scale Preparation of Plasmid DNA (Minipreps)

Minipreps were performed according to the method of Serghini et al.(1989). Single colonies of bacteria, transformed with plasmid were picked from plates and grown O/N in 5 mls of LB broth by shaking at 37°C at 250 RPM. The LB broth contained 45 μg/ml ampicillin to select for those bacteria containing plasmid. Following the O/N incubation, 1.5 mls of culture were removed to an eppendorf tube and the bacteria were pelleted by centrifugation. The bacterial pellet was resuspended in 50 μl of TNE (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA) and 50 μl of phenol/chloroform/isoamyl alcohol (25/24/1) was added. The mixture was vortexed and centrifuged and the aqueous layer was removed. The DNA in the aqueous layer was precipitated with 2 M (final concentration) ammonium acetate and 2 vol. of ethanol at -70°C. The precipitated DNA was then pelleted, dried and resuspended in 20 μl of TE. The DNA was analyzed by RE digestion and electrophoresis. DNase-free RNase (50 μg/ml) was added to the RE buffer

to degrade any RNA present in the samples.

I. Large Scale Preparation of Plasmid DNA

Plasmids were amplified for use in experiments by the method outlined in Maniatis et al.(1982). Bacteria, transformed with the plasmid of interest were grown O/N in 5 mls of LB broth containing ampicillin or 15 μg/ml tetracycline according to the resistance of the plasmid. The 5 ml cultures were added to 500 mls of fresh LB broth with antibiotic and grown until the optical density (OD_{600}) was greater than 0.6. Chloramphenicol was then added to a final concentration of 10 μg/ml, and the culture was grown for a further 16 hrs. The broth was then centrifuged at 6000 RPM for 10 min. in a Beckman JA-10 rotor. The bacterial pellet was either stored at -70°C or used immediately. The pellet was resuspended in 9.5 mls of lysis buffer (25 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM glucose), transferred to sterile 50 ml Oakridge tubes (Nalgene), and 0.5 mls of freshly prepared lysozyme (20 mg/ml) was added. The tubes were placed on their side on ice and shaken for 30 min.. Ten mls of a 0.2 M NaOH/0.2% SDS solution was then added and the tubes were left to shake for a further 30 min.. A final 10 mls of a 3 M KAc solution was added, the tubes shaken on ice for another 30 min., and the mixture was centrifuged in a Beckman JA-20 rotor for 30 min. at 17000 RPM at 4°C. Following centrifugation, the supernatant was removed and extracted once with 20 mls of phenol/chloroform/isoamyl alcohol (25/24/1). The aqueous and organic phases were separated by centrifugation at 3000 RPM in 50 ml centrifuge

tubes. The aqueous phase was then removed, and the DNA in it was precipitated by the addition of 0.6 vol. of isopropanol. The DNA was pelleted by centrifugation in a JA-20 rotor at 15000 RPM for 10 min. at 20°C, and then resuspended in 6.0 mls of TE. When all of the DNA had been resuspended, 6.6 g of 5.7 M cesium chloride (CsCl) was dissolved in the TE and the mixture was transferred to a Beckman Quickseal centrifuge tube (16 x 76 mm). The TE was overlayed with 0.2 ml of 10 mg/ml EtBr and the tube was filled to the top with mineral oil. The tubes were balanced, sealed, and centrifuged in a Beckman Ti75 rotor at 55000 RPM for 16 hrs at 20°C. After 16 hrs, the speed was reduced to 45000 RPM for 45 min.. Following centrifugation, the plasmid and bacterial genomic DNA bands were visualized with UV light. The plasmid band was removed using a needle and syringe, and extracted 4 times with 2-3 vol. of isoamyl alcohol to remove the EtBr. The DNA was then ethanol precipitated and the dried pellet redissolved in TE pH 7.5.

J. Recombinant DNA Probes

ß-Actin	Chicken actin cDNA (2 kb) in pBR322
PIP cDNA	PIP cDNA (577 bp) in PVZ1
gPIP 1.5	A 1.3 kb Eco R1 genomic fragment encompassing 458 bp of 5' flanking DNA, exon 1, and 387 bp of intron A of the PIP gene, in PVZ1
gPIP 0.7	A 700 bp Eco R1 - Xba 1 genomic fragment encompassing

	exons 3 and 4, and intron C of the PIP gene, in PUC 19
gPIP BE	a 670 bp Bam H1 - Eco R1 genomic PIP fragment from the 3' region of intron B, in PVZ1
28S	28S ribosomal RNA cDNA (5 kb) in pBR322

RESULTS

A. Effect of Hormones on PIP mRNA Stability

Experiments were conducted to determine if either lactogens or androgens increase PIP mRNA levels in T-47D cells by stabilizing the mature PIP transcript. Initial attempts to study PIP mRNA stability involved pulsing T-47D cells with ^{3}H-uridine, and then following the degradation of labelled PIP mRNA. Table 1 shows the results of one experiment where cells were incubated for 24 hours with 1 mCi/ml ^{3}H-uridine and then immediately collected. Only 696 DPMs hybridized to a 28S ribosomal RNA cDNA, while hybridization to the PIP cDNA was similar to background levels. Numerous repetitions, employing a variety of experimental conditions, were also unsuccessful. Thus, despite the use of relatively high concentrations of ^{3}H-uridine, we were repeatedly unable to label PIP mRNA to a specific activity detectable above background.

As the pulse-chase experiments proved unsuccessful, it was necessary to adopt a different approach to study PIP mRNA stability. Following 2-4 days of hormone treatment, 5 μg/ml of actinomycin D (Act D), a transcriptional inhibitor, was added to T-47D cells in order to inhibit synthesis of new transcripts. At various times following Act D treatment, the amount of remaining PIP mRNA was determined by Northern analysis. Figure I shows an autoradiogram from one experiment. The PIP mRNA appeared to be relatively stable in both hormone-treated and untreated cells.

Since Act D, a general RNA synthesis inhibitor, might have considerable cytotoxic effects with prolonged exposure, the experiments were repeated using 5,6-

dichloro-1-ß-D-ribofuranosylbenzimidazole (DRB) in place of Act D. DRB is an RNA polymerase II-specific inhibitor and was less toxic to the cells (Tamm et al., 1976). Figure II shows an autoradiogram from one experiment. The half-life of the PIP mRNA in cells treated with no hormones was found to be 17.0 ± 3.0 (SEM) hours. Treatment with hGH, DHT, and both hormones together yielded PIP mRNA half-lives of 18.3 ± 1.7, 14.3 ± 0.5 and 21.9 ± 2.1 hours respectively (Table 2). Analysis of variance indicated no significance difference ($p = 0.163$) in PIP mRNA half-life between the different hormone treatments.

B. Effect of Hormones on PIP hnRNA Stability

Previous work on the mouse ß-casein gene suggested that lactogens may increase gene expression by stabilizing the hnRNA in the nucleus (Rosen et al., 1986). In order to determine whether lactogens or androgens affect PIP hnRNA stability, the DRB experiments described above were repeated with the Northern blots probed for PIP hnRNA. A ^{32}P-labelled, antisense cRNA probe (gPIP BE), corresponding to a 670 bp region in the 3' end of intron B of the PIP gene (Figure III) was used to detect PIP hnRNA. Figure IV shows a single Northern blot probed with both the gPIP BE cRNA and a nick translated PIP cDNA probe. The uppermost band (approx. 7 Kb in size) indicated by the arrow is detected by both probes and thus most likely represents the full length PIP hnRNA. The cRNA probe was chosen for the stability studies because of its higher specific activity.

Figure V shows an autoradiogram from one PIP hnRNA stability experiment. As

can be seen in the figure, PIP hnRNA levels declined sharply following DRB treatment. In contrast, the level of mature PIP mRNA, detected in a subsequent hybridization with the PIP cDNA, did not decline appreciably over the course of the experiment. The half-life of the PIP hnRNA was determined for cells treated for 2-4 days with no hormones (49.1 $\pm$ 14.7 min.), hGH (31.2 $\pm$ 2.8 min.), DHT (36.9 $\pm$ 4.3 min), and hGH and DHT together (36.4 $\pm$ 7.8 min.) (Table 3). Analysis of variance indicated no significant difference ($p = 0.85$) in PIP hnRNA half-life between the various treatments.

Androgen stimulated the level of PIP hnRNA (Fig. IV) as it did for the mature PIP mRNA. However, the level of induction of hnRNA stimulated by hGH varied considerably from experiment to experiment. In Figure VI *Left,* for example, the level of hnRNA was stimulated 2.9 fold by hGH - the same level of induction seen in the mature mRNA. In Figure VI *Right* however, PIP hnRNA levels are reduced by 68% by hGH, while the mature mRNA is increased 4.23 fold. Table 4 shows the effect of hGH on both the hnRNA as well as the mature PIP mRNA in each experiment. Overall, the level of hnRNA in hGH treated cells was not significantly higher ($p = 0.26$) than in controls. The induction of PIP hnRNA by hGH, in the presence of androgens, was likewise inconsistent, with no overall significant difference in hnRNA levels between cells treated with hGH plus DHT and cells treated with DHT alone.

C. Effect of Hormones on PIP Transcription

Since neither androgens nor lactogens affected the stability of either the PIP hnRNA or mRNA, they must act to increase synthesis of the PIP transcript. Nuclear run-

on transcription assays were performed using both the PIP cDNA, as well as genomic fragments from different regions of the PIP gene as probes. Nuclei were isolated from T-47D cells treated with and without hGH, in either the presence or absence of androgens. The nuclei were incubated with ^{32}P-UTP, and the resulting labelled RNA was purified. TCA precipitation indicated that 100% of the CPMs purified were incorporated into RNA. An approximately 1.3 kb Eco R1 genomic PIP restriction fragment (gPIP 1.5), encompassing 458 bp of 5' flanking DNA, exon 1, and 387 bp of intron A was used as a 5'-specific probe, while a 700 bp Eco R1 - Xba 1 genomic restriction fragment (gPIP 0.7), encompassing exons 3 and 4, and intron C, was used as a 3'-specific probe (Figure VII Top).

The results of one experiment are shown in Figure VII (Bottom). In the figure, hybridization to the 5' gPIP 1.5 probe is increased in the presence of DHT, suggesting that androgens increased the rate of initiation of transcription. The results obtained with the PIP cDNA (not shown) are identical to those obtained with the 5' gPIP 1.5, and confirm earlier results showing that androgens increase PIP transcription by 3.7 fold (Murphy et al., 1987b). Figure VII also shows that hybridization to the 3' gPIP 0.7 fragment was higher in the presence of hGH, regardless of the presence of DHT. There was no effect of hGH, on hybridization to the 5' gPIP 1.5 fragment. This suggested that hGH was affecting transcription only in the 3' end of the PIP gene.

To confirm this result, the nuclear run-on experiment was repeated 5 times. In each experiment, the degree of hybridization to the PIP cDNA, the 3' gPIP 0.7, and the 5' gPIP 1.5 was determined and compared for cells treated with and without hGH. The

ratio of hybridization to the gPIP 0.7 versus hybridization to the gPIP 1.5 (3'/5' signal ratio) was also determined (Table 5). Paired T-test analysis showed no significant difference in hybridization to either the cDNA ($p = 0.74$) or the 5' gPIP 1.5 fragment ($p = 0.27$) in cells treated with and without hGH. Hybridization to the 3' gPIP 0.7 fragment however, was significantly increased ($p = 0.04$) in the presence of hGH, and the 3'/5' signal ratio in hGH-treated cells was significantly higher ($p = 0.014$) than that in the untreated cells. This confirmed the initial result showing that hGH increased transcription only in the 3' end of the PIP gene.

When cells treated with either DHT and hGH, or DHT alone were compared (Table 6), there was no significant difference between the two treatments in hybridization to either the cDNA ($p = 0.85$), the gPIP 1.5 ($p = 0.75$) or the gPIP 0.7 ($p = 0.22$). However, when the 3'/ 5' signal ratios were compared between the two treatments, cells treated with both hGH and DHT had a significantly higher ($p = 0.008$) ratio than cells treated with DHT alone. These results support the concept that lactogens act to increase transcription specifically in the 3' end of the PIP gene.

DISCUSSION

The PIP gene in T-47D HBC cells was used as a model to study the molecular mechanisms of action of both lactogens and androgens. The mature PIP mRNA was found to be relatively stable ($t_{1/2}$ = 17 hrs) and unaffected by treatment with either DHT or hGH. The half-life of the PIP hnRNA was found to be approximately 35 min. and also unaffected by treatment with either hormone. Nuclear run-on analysis indicated that androgens stimulate PIP mRNA accumulation by increasing the rate of initiation of transcription of the PIP gene, while lactogens appear to stimulate transcription elongation.

Two major approaches were employed in the study of PIP mRNA stability: pulse-chase studies, and experiments using transcriptional inhibitors. The major advantage of pulse-chase analysis is that RNA stability can be studied in the absence of drugs which block transcription. Thus, the cells are generally healthy, even during chases of several days duration. Pulse-chase studies have been successfully used in the analysis of ß-casein mRNA in mammary explant cultures, and globin mRNA in erythroleukemia cells, as well as several others (Rosen et al., 1986, Volloch and Housman, 1981, Welsh et al., 1985, Brock and Shapiro, 1983, Krowczynska et al., 1985). Repeated attempts to pulse-label PIP mRNA to a specific activity detectable above background were unsuccessful (Table 1). This was despite the use of a variety of experimental protocols, and the use of relatively high concentrations of ^{3}H-uridine. While the PIP mRNA is readily detectable

in Northern analysis, it is possible that its transcription rate is not sufficient to allow for a detectable level of specific labelling in this system. Many of the transcripts with which pulse-chase analysis has been used successfully, such as ß-casein and globin mRNAs, are highly abundant and make up a large percentage of the poly(A)$^+$ RNA in the cells studied (Rosen et al., 1986, Volloch and Housman, 1981). Although less abundant transcripts, such as actin, have been successfully analyzed, it required the use of concentrated cell suspensions (Krowczynska et al., 1985). Since basal PIP expression in T-47D cells appears to rise with increasing cell density (unpublished observations), highly concentrated cell cultures are inappropriate for the study of PIP gene expression.

As the pulse-chase experiments proved unsuccessful, it became necessary to use transcriptional inhibitors to study PIP mRNA stability. Actinomycin D is a commonly used drug in the study of RNA stability, as it rapidly and specifically inhibits RNA synthesis by all 3 RNA polymerases (Reich and Goldberg, 1964, Dubik and Shiu, 1988, Murphy et al., 1990, Jinno et al., 1988). Unfortunately, extended use of Act D in these experiments resulted in considerable cell death. This toxic effect, which has been reported by others, may be due to prolonged inhibition of transcription from all three RNA polymerases, or it may result from some other effect of Act D in these cells (Bacchetti and Whitmore, 1969, Pater and Mak, 1974, Reich and Goldberg, 1964, Jinno et al., 1988). Nevertheless, since the half-life of the PIP transcript was relatively long (Figure I), the toxicity of the Act D made it difficult to obtain sufficient quantities of RNA at late time points in the experiment. Thus it was necessary to use a different transcriptional inhibitor. DRB has been shown to specifically inhibit RNA polymerase

II transcription, and was found in these experiments, to be less toxic to the T-47D cells than was Act D (Tamm et al., 1976). As a result, it was possible to obtain consistent quantities of RNA throughout the experiment, and thereby determine the stability of the PIP transcript under different hormonal conditions (Table 2).

The use of transcriptional inhibitors in the study of RNA stability can confound results in two ways. First, cells in which transcription is inhibited for up to 2 days may likely exhibit characteristics, including RNA turnover rates, different from those in healthy growing cells. Thus the absolute value determined for the PIP mRNA half-life in these experiments may differ somewhat from its half-life in reality. Second, in theory, the cells may respond differently to DRB under different hormone conditions, such that the efficiency, or degree to which transcription is inhibited may not be same in each hormone treatment. Differences in the stability of the PIP transcript induced by the hormones then, may not be readily apparent. It is important to note though that there is no evidence to support the concept that DRB action is affected by hormone treatment. Therefore, as determined in these experiments, there is most likely no effect of either DHT or hGH on PIP mRNA stability.

Since neither lactogens nor androgens affect the degradation rate of the PIP mRNA, they must act to increase the synthesis of the mature mRNA. Rosen et al. (1986) have suggested that mRNA levels can be increased by stabilizing the precursor hnRNA in the nucleus from degradation by ribonucleases. This would allow a greater proportion of hnRNAs to be processed to mature mRNAs. In previous experiments, Northern blots, probed with nick-translated PIP cDNA probes and exposed for long periods of time,

would reveal hybridization to 2-3 bands of 5-8 kb in length, in addition to the 800 bp PIP transcript (Figure V). As the hybridizations were performed at high stringency, these bands were believed to represent intermediates in the processing of the PIP hnRNA, as well as the full length hnRNA itself. The identity of these bands was confirmed by hybridization to an intron-specific PIP probe (Figure V). The largest band was used in the hnRNA stability studies.

Since there was no measurable effect of hormones on the disappearance of the PIP hnRNA, one of two scenarios is possible: 1) there is no effect of hormones on either the processing or the degradation rate of the hnRNA, or 2) the hormones stabilize the hnRNA from degradation while increasing the processing rate, to yield no overall change in the disappearance rate of the hnRNA. Very little, if any information exists regarding control of the speed of RNA processing. As well, the chances are probably low that both the processing and the degradation rate would change in such a manner so as to yield the exact same stability of the hnRNA. Thus while neither of the above scenarios can be dismissed, the idea that neither of the hormones act on the PIP hnRNA is conceptually easier.

The level of PIP hnRNA induced by hGH varied dramatically from experiment to experiment (Table 4). In some experiments, the hnRNA induction under hGH was sufficient to account for the induced increases in PIP mRNA, while in other experiments, the hnRNA level was actually reduced under hGH treatment. Because of this variability, there was no overall statistically significant induction of PIP hnRNA by hGH. The lack of a consistent effect of hGH on PIP hnRNA most likely results from the complexity of

the experimental system. Little is known about the kinetics of hnRNA processing and degradation in the nucleus, or about the physiological conditions which affect them. For example, the variation in hnRNA may reflect dish to dish variations in cell density or basal PIP expression.

One further consideration in these experiments, is that the disappearance of hnRNA following DRB treatment may not have been a linear process, as was assumed in the calculation of hnRNA half-life. If this were the case, then effects of hGH or DHT on any initial, rapid declines in the hnRNA levels, may not have been detected in these experiments.

Since neither lactogens nor androgens appear to affect the half-life of either the mature PIP mRNA or the hnRNA, they must act to increase the rate of transcription. The nuclear run-on experiments confirmed earlier observations that androgens increase the transcription rate of the PIP gene (Murphy et al., 1987b). Hybridization to the cDNA and the 5' probe was increased in the presence of androgens (Figure VII), suggesting that androgens increase the rate of initiation of transcription. Androgens have been shown to regulate the transcription rate of several genes, and specifically, to increase initiation of transcription of the ODC gene in the mouse kidney, and of the mouse mammary tumour virus (Compere et al, 1981, Page and Parker, 1982, Felder et al., 1988, Rheaume et al., 1989, Ham et al., 1988). It is possible that androgens act directly through their receptor to stimulate transcription of the PIP gene. Four copies of the hexanucleotide TGTTCT, which serves as the androgen-responsive element in the MMTV, are present in the 5' flanking region and first exon of the PIP gene. Further studies are required to determine

whether these sequences are functional.

As in the previous nuclear run-on experiments by Murphy et al. (1987b), there was no apparent effect of hGH on transcription as detected with the PIP cDNA. Hybridization to the genomic probes however, revealed that transcription was specifically increased by hGH only in the 3' end of the PIP gene (Figure VII, Tables 5 and 6). This suggests that hGH may affect the elongation of transcription rather than its initiation. Recently, it has been shown that prolactin regulates expression of the rat ß-casein gene by elongating transcription (Rosen et al., 1989, Goodman and Rosen, 1990). In the absence of prolactin, a block to elongation results in the synthesis of truncated transcripts, while the addition of prolactin results in removal of the block and the synthesis of full length ß-casein RNAs. Since the action of hGH in T-47D cells is mediated through the prolactin receptor, transcription elongation may represent a common molecular mechanism of action of lactogenic hormones (Shiu, 1979).

Intragenic transcriptional blocks have also been described in such eukaryotic genes as the human histone H3:3 gene, the human c-myc and c-myb genes, and the hamster c-fos gene (Reines et al., 1987, Bentley and Groudine, 1986, Bender et al., 1987, Fort et al., 1987). Stretches of seven or more thymidine residues in the untranscribed DNA strand appear to occur at, or near the transcription termination site for several of these (Dedrick et al., 1987, Kerpolla and Kane, 1988, Reines et al., 1987). Analysis of the PIP gene reveals three runs of 13, 9 and 7 T residues respectively, within a 100 bp segment of the first intron (Myal et al. 1990). This region lies immediately upstream of the second exon and is between the 5' gPIP 1.5 and the 3' gPIP 0.7 probes. Further analysis

must be carried out to determine if a block to transcription elongation occurs in this region.

A second possible explanation for the observed increase in 3' transcription, is that prolactin stimulates antisense transcription in the 3' region. Antisense transcription, in limited regions of genes has been reported for the ODC, c-myb, c-myc and ß-casein genes (Rheaume et al., 1989, Bentley and Groudine, 1986, Bender et al., 1987, Goodman and Rosen, 1990). Several of these investigators have suggested that the antisense transcription may be an artifact of the *in vitro* assay. Goodman and Rosen (1990) however found antisense transcription present even during ^{3}H- uridine pulse transcription assays in intact cells, suggesting that the antisense transcription was physiological. Interestingly, in their experiments the antisense transcription was not affected by prolactin treatment.

Further analysis must also be carried out in order to resolve two discrepancies in the nuclear run-on results. In the presence of androgens, the 3'/5' signal ratio in the PIP gene was significantly increased in the presence of hGH, suggesting that hGH was still affecting transcription in the 3' end of the gene. However, when hybridization to the 3' probe alone is compared, there is no statistically significant difference ($p=0.22$) in the 3' signal between cells treated with and without hGH (Table 6). This discrepancy between the 3'/5' signal ratio and the 3' signal alone may be due to technical differences such as the number of cpms added to each filter, and/or differences in hybridization efficiencies between filters. Alternatively, the higher overall rate of transcription induced by androgens, may make it more difficult to detect the 3' specific increase in

transcription induced by hGH.

Changes in transcription should also be detected in nuclear run-on experiments, by an increase in hybridization to cDNA probes (Bender et al., 1987, Bentley and Groudine, 1986). However, both in these experiments, and in previous nuclear run-on experiments, hGH had no significant effect on hybridization to the PIP cDNA, regardless of the presence of androgens (Tables 5,6, Murphy et al., 1987). This phenomenon has been described previously for the ODC gene in the mouse kidney. Nuclear run-on analysis using cDNA probes indicated that androgens had no effect on the transcription rate of the ODC gene (Berger et al., 1986). Later experiments however, using a variety of single-stranded, sense and antisense genomic ODC probes, indicated that androgens did indeed increase transcription of the ODC gene, and that high, regional transcription in both the sense and antisense direction, masked detection of this effect by the cDNA probes (Rheaume et al., 1989).

SUMMARY AND FUTURE DIRECTIONS

The purpose of this research was to further elucidate the molecular mechanisms used by lactogens and androgens in their regulation of the PIP gene in T-47D HBC cells. Both hormones were found to affect transcription of the PIP gene. Androgens increased the rate of initiation of transcription, while lactogens appeared to stimulate transcription elongation. Neither hormone appeared to affect the stability of the PIP mRNA. Nor did

either appear to have any effect on the stability of the precursor hnRNA.

The PIP gene joins a large and growing list of eukaryotic genes regulated in part transcriptionally. Further nuclear run-ons can now be carried out in order to determine the exact nature of the prolactin-induced increase in 3' transcription. Single-stranded sense and antisense genomic probes from different regions of the PIP gene will be used to detect antisense transcription, if any, and to localize the sequences at which elongation may be regulated by lactogens. Also, the use of a variety of both sense and antisense probes may hopefully explain why the effect of lactogens on transcription elongation is not detected by the PIP cDNA in the nuclear run-ons. Since androgens increase the initiation of transcription of the PIP gene, experiments utilizing the PIP promoter linked to a reporter gene, should be pursued in order to localize the androgen-responsive sequences in the PIP gene. It can then be determined if the androgen receptor itself interacts with the these sequences, or whether androgens stimulate PIP transcription indirectly, through the regulation of a second gene.

The PIP gene continues to serve as a useful model to study the molecular mechanisms of lactogen and androgen action. It is hoped that the information gathered through the study of the PIP gene can be applied to the action of these hormones in other systems. It is also hoped that further elucidation of the mechanisms by which these hormones regulate gene expression may provide some clue as to their role in the pathophysiology of the human breast.

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TABLE 1: Pulse-Chase Analysis in T-47D Cells

	HORMONE TREATMENT	
Plasmid	None	hGH + DHT
28S	324.2	696.2
PIP cDNA	14.6	20.0
Background	19.9	7.9

T-47D cells were incubated with 1 mCi/ml ^{3}H-uridine for 24 hr in the presence or absence of 1 μg/ml hGH and 10^{-8} M DHT. The RNA was isolated and hybridized to 28S ribosomal RNA cDNA and PIP cDNA bound to nitrocellulose. The data represent DPMs obtained by scintillation counting. Background represents non-specific binding to blank nitrocellulose.

Table 2: Effects of Hormones on the Stability of PIP mRNA

HORMONE TREATMENT	mRNA $t_{\frac{1}{2}}$ (hr)
None	17.0 ± 3.0
hGH	18.3 ± 1.7
DHT	14.3 ± 0.5
hGH + DHT	21.9 ± 2.1

Linear regression analysis was performed on the experiments outlined in Figure 2. Data represent the mean half-life ± SEM of 7 experiments for each hormone treatment.

Table 3: Effects of Hormones on the Stability of PIP hnRNA

HORMONE TREATMENT	hnRNA $t_{\frac{1}{2}}$ (min)
None	49.1 ± 14.7
hGH	31.2 ± 2.8
DHT	36.9 ± 4.3
hGH + DHT	36.4 ± 7.8

Linear regression analysis was performed on the experiments outlined in Figure 5. Data represent the mean half-life ± SEM of 5 (None and hGH) and 6 (DHT and hGH + DHT) experiments.

Table 4: Effect of hGH on PIP mRNA and hnRNA

EXPERIMENT	CONTROL		hGH		FOLD EFFECT of hGH	
	hnRNA	mRNA	hnRNA	mRNA	hnRNA	mRNA
1	0.113	0.185	0.288	0.470	2.93	2.86
2	0.670	0.128	0.230	0.495	-3.13	4.23
3	0.120	0.043	0.270	0.358	1.48	11.36
4	0.400	0.250	0.270	0.330	-1,52	1.42
5	0.630	0.200	0.740	0.588	1.17	3.61

Levels of PIP hnRNA and mRNA in hGH-treated, and untreated cells immediately prior to the addition of 75 μM DRB as described in the legend to Figure VI. Data represent the optical densities obtained from the autoradiograms by densitometry.

Table 5: Effect of hGH on Hybridization to PIP Probes During Nuclear Run-on Analysis

	EXPERIMENT NUMBER 1		2		3		4		5		p value (G>N)
	None	hGH	None	hGH	None	hGH	None	hGH	None	hGH	
PIP cDNA	1.25	1.50	0.82	1.91	1.67	1.00	1.13	1.00	-	1.43	0.74
gPIP 1.5 (5')	3.38	5.75	1.64	2.09	3.67	4.00	1.50	1.88	4.75	4.25	0.27
gPIP 0.7 (3')	3.25	6.50	2.27	4.27	4.00	6.33	2.50	3.13	3.83	4.08	0.04
3'/5' ratio	0.96	1.13	1.38	2.04	1.09	1.58	1.67	1.67	0.81	0.96	0.01

Data represent the optical densities obtained by densitometry from nuclear run-on autoradiograms. The data in each experiment were normalized relative to hybridization to ß-actin. P values represent the results of paired Student T-tests, testing hGH > None.

Table 6: Effect of DHT and DHT + hGH on Hybridization to PIP Probes During Nuclear Run-on Analysis

	EXPERIMENT NUMBER 1		2		3		4		5		p value (D+G>D)
	DHT	D+G	DHT	D+G	DHT	D+G	DHT	D+G	DHT	D+G	
PIP cDNA	2.13	2.25	1.36	2.64	4.67	3.00	2.13	3.00	1.68	1.58	0.85
gPIP 1.5 (5')	5.88	4.50	3.55	4.55	4.67	8.33	4.88	4.75	6.08	4.58	0.75
gPIP 0.7 (3')	5.25	6.25	3.00	4.27	9.67	19.7	6.00	7.63	6.08	5.58	0.22
3'/5' ratio	0.89	1.39	0.85	0.94	2.07	2.36	1.23	1.61	1.00	1.22	<0.01

Data represent the optical densities obtained by densitometry from the nuclear run-on autoradiograms. The data in each experiment were normalized relative to hybridization to ß-actin. P values represent the results of paired Student T-tests, testing DHT + hGH > DHT.

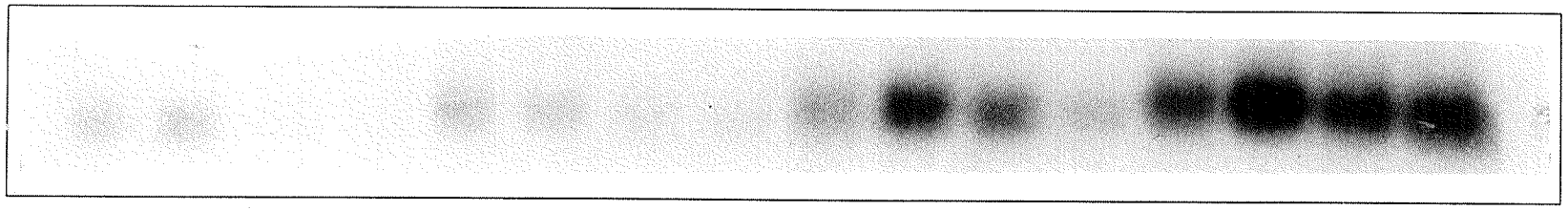
Figure I: Northern Analysis Showing the Effect of Hormones on the Stability of PIP mRNA Following Treatment With Actinomycin D

T-47D cells were treated with either no hormones (None), 1 μg/ml hGH, 10^{-8} M DHT, or both hormones together for 4 days prior to the addition of 5 μg/ml actinomycin D. At various times following treatment with Act D, cells were collected and the RNA was isolated. (*Top*) Ethidium bromide stain of 28S ribosomal RNA. Thirty μg of total RNA was loaded in each lane. Times represent hours following Act D treatment. (*Bottom*) The RNA was transferred to a nitrocellulose filter and probed with a nick-translated PIP cDNA to detect PIP mRNA.

28S

0 12 24 48 0 12 24 48 0 12 24 48 0 12 24 48

None hGH DHT hGH + DHT



0 12 24 48 0 12 24 48 0 12 24 48 0 12 24 48

None hGH DHT hGH + DHT

Figure II: Northern Analysis Showing the Effect of Hormones on the Stability of PIP mRNA Following Treatment with DRB

T-47D cells were treated as in Figure I except that 75 μM DRB was used in place of Act D. (*Left*) Ethidium bromide stain of 18S ribosomal RNA. Thirty μg of total RNA were loaded in each lane. (*Right*) The RNA was transferred to a nitrocellulose filter and probed with a nick-translated PIP cDNA probe to detect PIP mRNA. Autoradiograms for each hormone treatment were exposed for different lengths of time to optimize quantitation. Actual inductions of PIP mRNA by hGH, DHT, and both hormones together were approximately 5, 10 and 15 fold respectively.

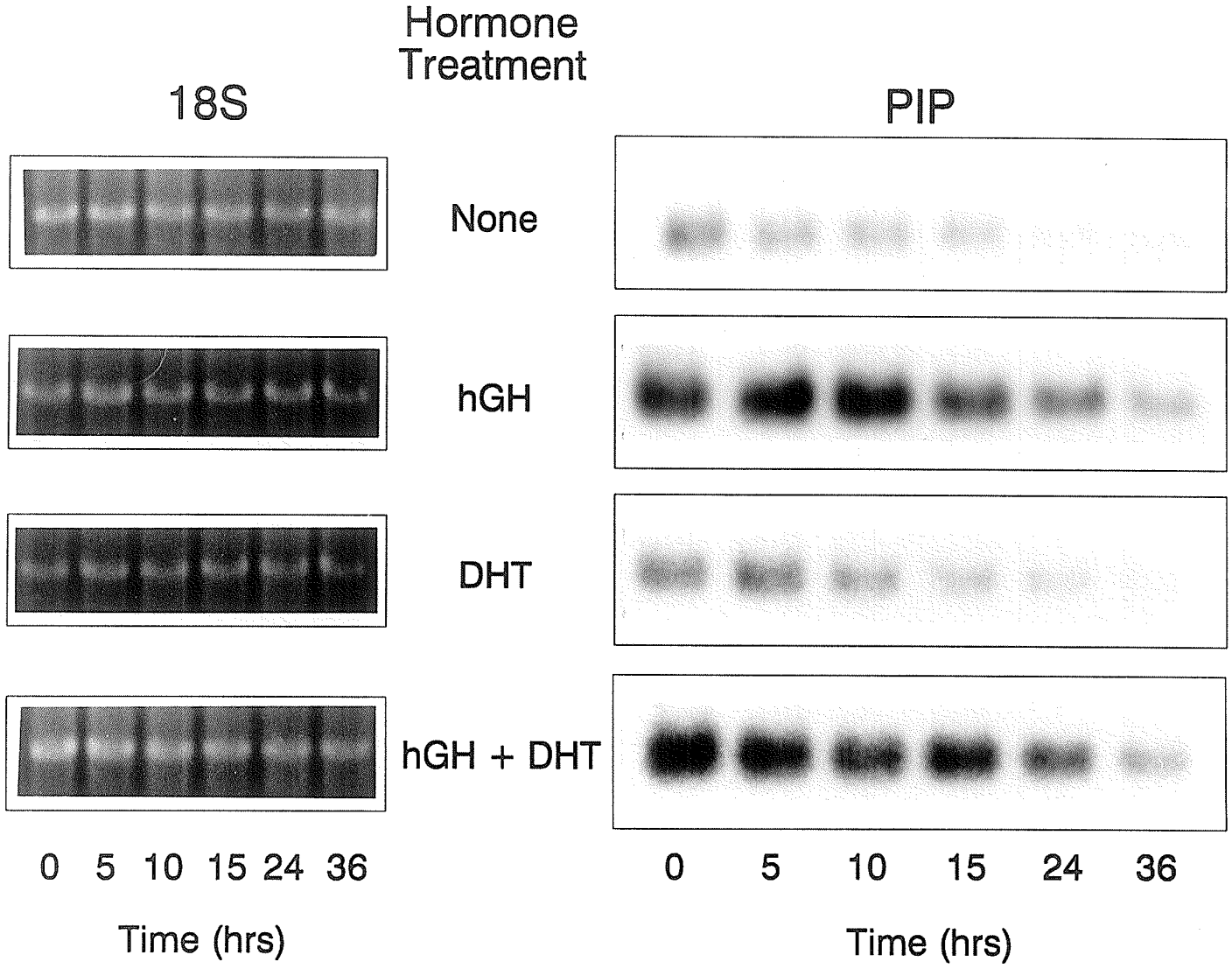

Hormone Treatment
18S
PIP
None
hGH
DHT
hGH + DHT
0 5 10 15 24 36
Time (hrs)
0 5 10 15 24 36
Time (hrs)

Figure III: Location of the gPIP BE fragment in the PIP Gene

Line drawing of the PIP gene showing the location of the gPIP BE fragment in relation to the four exons. The 670 bp Bam H1- Eco R1 fragment was subcloned into the PVZ1 plasmid and used to generate antisense cRNA probes for the detection of PIP hnRNA.

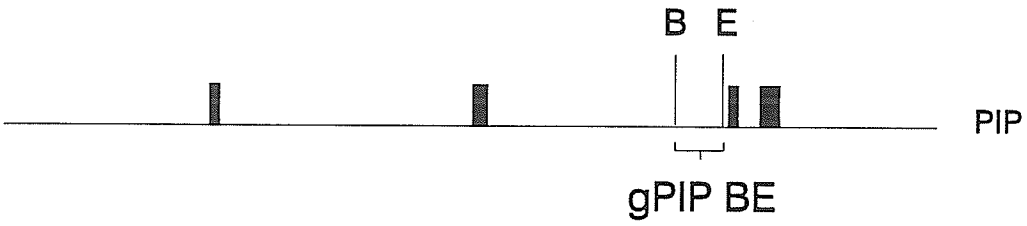

B
E
PIP
gPIP BE

Figure IV: Northern Analysis Showing Detection of PIP hnRNA by both the PIP cDNA and gPIP BE

Total RNA from hormone-treated T-47D cells was isolated and analyzed on Northern blot (30 μg RNA per lane). (*Left*) Northern blot probed with PIP cDNA. (*Right*) The same Northern blot was stripped and later reprobed with gPIP BE.

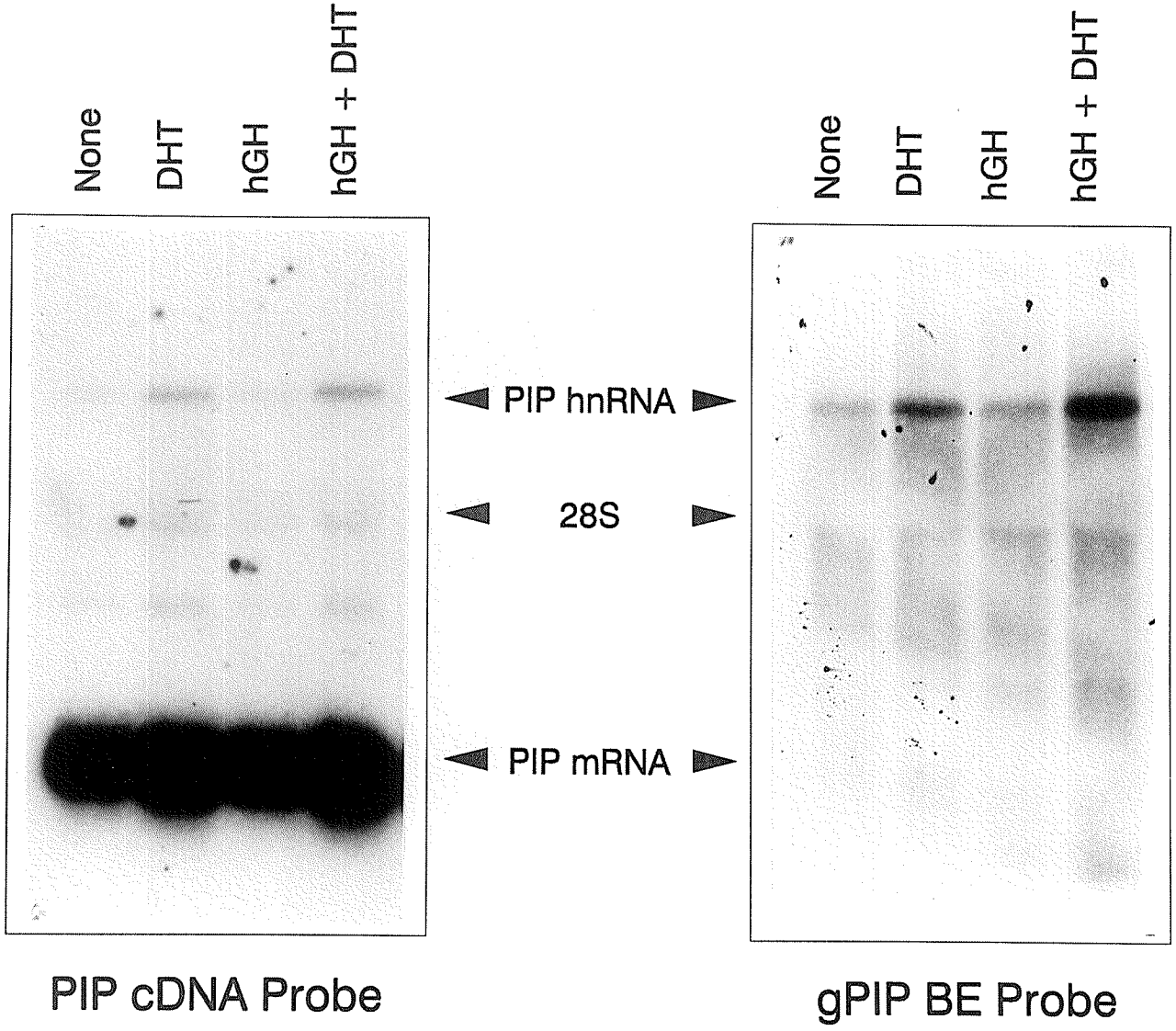

None
DHT
hGH
hGH + DHT
PIP hnRNA
28S
PIP mRNA
None
DHT
hGH
hGH + DHT
PIP cDNA Probe
gPIP BE Probe

Figure V: Northern Analysis Showing the Effect of Hormones on the Stability of PIP hnRNA Following Treatment With DRB

T-47D cells were treated with either no hormones (None), 1 μg/ml hGH, 10^{-8} M DHT, or both hormones together for 4 days prior to the addition of 75 μM DRB. At various times following DRB treatment, cells were collected and the RNA was analyzed by Northern blot (30 μg total RNA per lane). Times represent minutes following DRB treatment. (*Top*) Ethidium bromide stain of 18S ribosomal RNA. (*Middle*) The RNA was transferred to nitrocellulose filter and probed with an antisense, cRNA gPIP BE probe. (*Bottom*) The filter was stripped and reprobed with a nick-translated PIP cDNA.

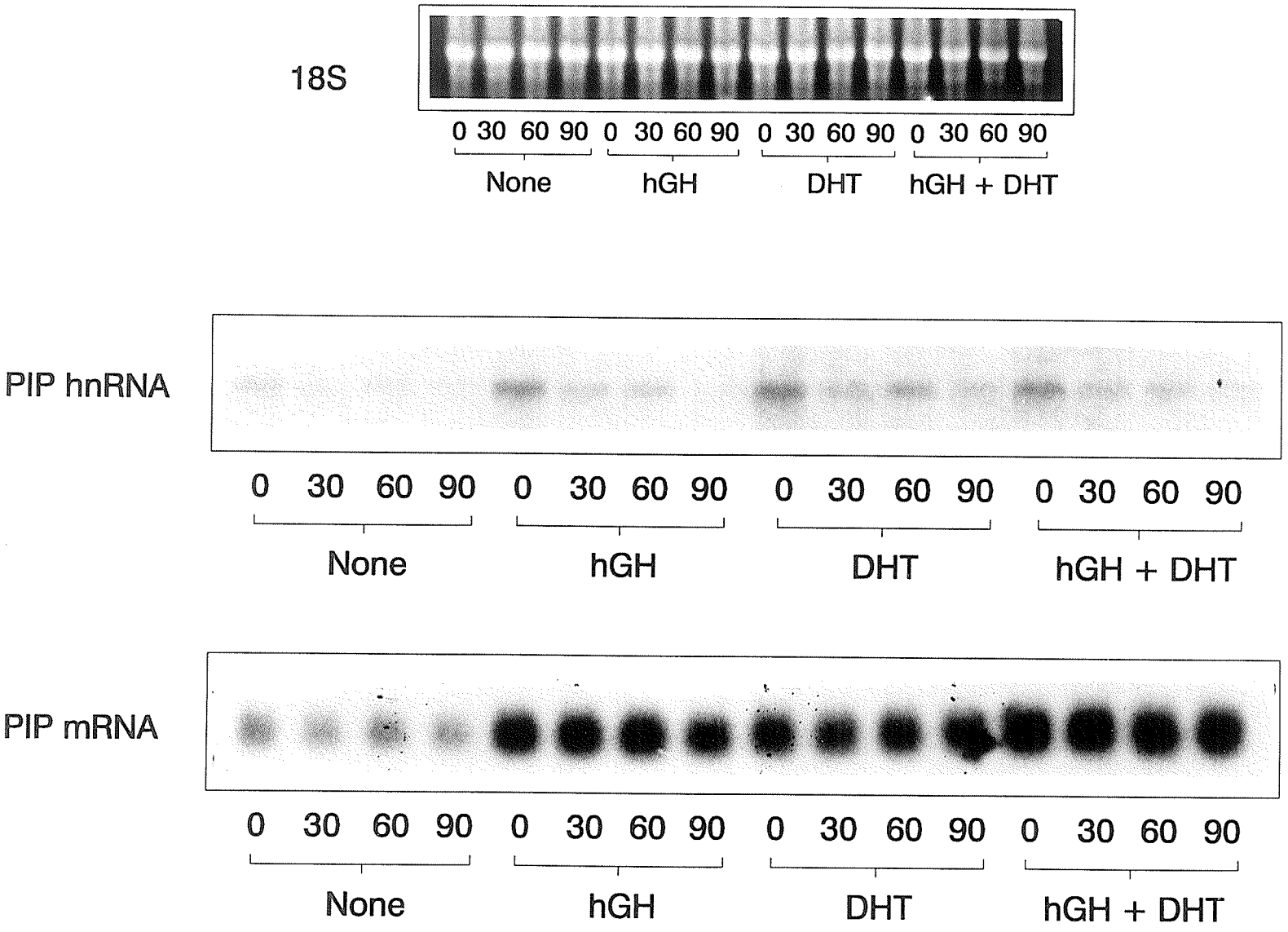
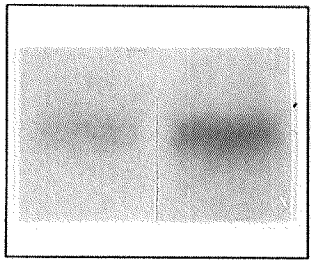

18S
0 30 60 90 0 30 60 90 0 30 60 90 0 30 60 90
None
hGH
DHT
hGH + DHT
PIP hnRNA
0 30 60 90 0 30 60 90 0 30 60 90 0 30 60 90
None
hGH
DHT
hGH + DHT
PIP mRNA
0 30 60 90 0 30 60 90 0 30 60 90 0 30 60 90
None
hGH
DHT
hGH + DHT

Figure VI: Northern Analysis Showing the Effect of hGH on the Level of PIP hnRNA in T-47D Cells.

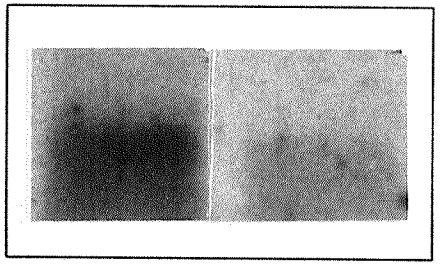
T-47D cells were treated as described in the legend to Figure V. The hnRNA was detected using an antisense cRNA gPIP BE probe, while a nick-translated PIP cDNA was used to detect the mature mRNA. The RNA in this figure was isolated immediately prior to the addition of DRB. (*Left*) Effect of hGH on the level of PIP hnRNA (*Top*) and mRNA (*Bottom*) in one experiment. (*Right*) Effect of hGH on PIP hnRNA (*Top*) and mRNA (*Bottom*) in a second identical experiment. The two experiments represented here correspond to experiments #1 and #2 in Table 4.

EXPT #1

EXPT #2

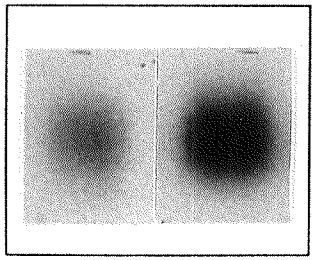


PIP hnRNA

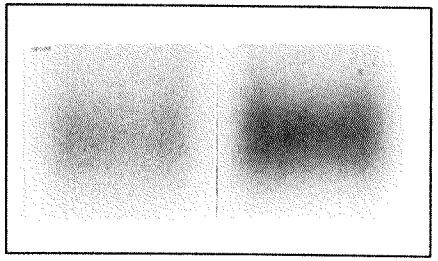


None hGH

None hGH



PIP mRNA

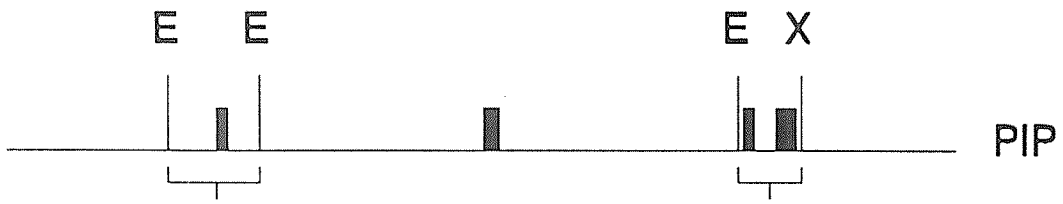


None hGH

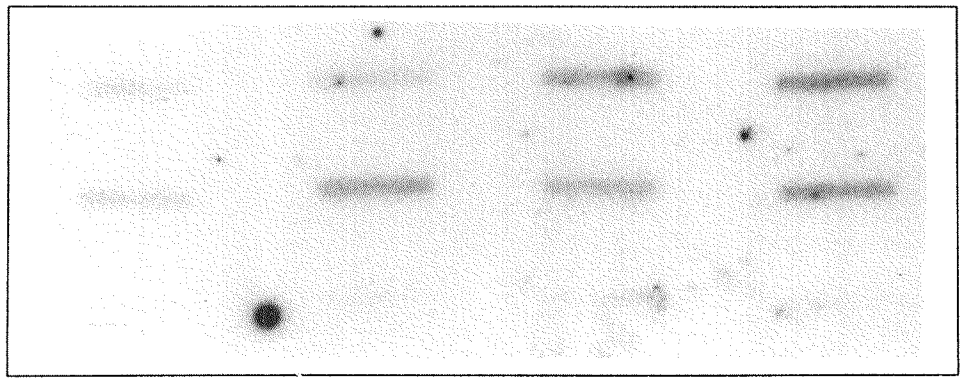
None hGH

Figure VII: Effect of Hormones on the Transcription Rate of the PIP Gene

(*Top*) Line drawing of the PIP gene showing the location of the gPIP 1.5 and gPIP 0.7 probes in relation to the four exons. (*Bottom*) Nuclei were isolated from T-47D cells treated for 24 hours with either no hormones (None), 1 μg/ml hGH, 10^{-8} M DHT, or both hormones together. The nuclei were incubated with ^{32}P-UTP and the resulting labelled RNA was hybridized to excess (5 ug) denatured PVZ1 plasmid, PIP cDNA, gPIP 1.5, gPIP 0.7, and ß-actin cDNA to determine specific transcription rates. There was no detectable hybridization to the PVZ1 plasmid (data not shown). Hybridization to the PIP cDNA (data not shown) was identical to that found with the gPIP 1.5.



gPIP 1.5 gPIP 0.7



None hGH DHT hGH + DHT