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GENE EXPRESSION DURING ACTIVE CELL DEATH IN INVOLUTING PROSTATE AND MAMMARY GLAND: CLONING AND CHARACTERIZATION OF REGRESSION SELECTED GENES (RSG)

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ABSTRACT

Apoptosis or active cell death (ACD) is a physiological form of cellular suicide which occurs in a wide variety of situations during the normal development, growth and function of the various tissues of multicellular organisms. Both RNA and protein synthesis are required in the dying cell and until recently, very little was known about the molecules that participate in the process. Following hormonal ablation in the rat ventral prostate (via castration) or rat mammary gland (after weaning) the epithelial cells of both glands are eliminated by active cell death during the regression of the glands. Several genes including TRPM-2, (testosterone repressed prostate message), RVP.1, fos, and myc, have been shown to be induced in the prostate during this process. We have investigated the expression of several other genes that may be associated with apoptosis, including tissue transglutaminase (tTGase), poly (ADP-ribose) polymerase (PARP), and heat shock protein 27 (Hsp27). Northern hybridization has been used to determine the steady-state mRNA levels of these genes in both the regressing prostate and mammary glands. The time course of induction has been compared to changes in the steady state levels of several control genes including prostate steroid binding protein (PSBP) (prostate), β-casein (mammary), α-tubulin, and TRPM-2 (both prostate and mammary).

The results show that the mRNAs for PARP, tTGase, and Hsp27, in addition to TRPM-2, are induced by androgen ablation in the rat ventral prostate and reach maximum levels between days 3 and 4 after castration. The mRNAs for PARP, tTGase and TRPM-2 are transiently induced during ACD in mammary tissue, with their levels peaking between days 2 - 4 after weaning. In contrast to prostate regression the mRNA for Hsp27 was not significantly induced in the mammary gland during regression. Using *in-situ* hybridization we have established that these genes are expressed in the epithelial cells of the glands that are deleted during ACD; this result suggests that their gene products may be required in the dying cells to ensure that the biochemical and morphological processes of apoptosis are completed appropriately.

A novel cross-screening approach first described by Wong et al., 1989 was used to clone and characterize genes other than those described above which were induced during the regression of both rat prostate and mammary gland. Two regression selected genes (RSG) refered to as RSG-2, and RSG-8, were isolated, and their role in the active cell death process has been investigated. RSG-2 is homologous to cathepsin B, a thiol protease that has been previously identified as one of the extracellular proteases that is activated in metastatic cells. The steady-state levels for the RSG-2 mRNA are increased several fold in both regressing prostate and mammary gland, and this increase was shown by *in-situ* hybridization to localize to the epithelial cells that are deleted during the regression process. Analysis of the distribution of the cathepsin B protein by immunofluoresence microscopy demonstrates that both glands show diffuse punctate expression of the protein in all of the luminal cells. During regression the protein expression is redistributed to the basal aspect of the cells, with intense expression localized to the apoptotic bodies formed from dying cells. These results suggest that RSG-2, or

cathepsin B, is required for the local degradation of the basement membrane, which is one of the earliest morphologically recognizable events of active cell death.

RSG-8, is homologous to rat insulin like growth factor binding protein 5 (IGFBP-5), a member of a group of six related proteins that are known to mediate the effects of the growth factors IGF-I, and IGF-II. The steady state level of IGFBP-5 mRNA in the normal prostate is low but detectable. In the regressing prostate, the mRNA for IGFBP-5 mRNA is induced and its steady state peaks in the luminal epithelial cells of the prostate that undergo active cell death (ACD) between 3 and 4 days after castration. The gene is induced in a similar fashion in the regressing mammary gland following weaning. The expression of another IGFBP, namely IGFBP-2 contrasts that of IGFBP-5, with a high steady state level in both prostate and mammary gland, which increase only slightly during the regression process. The increase in mRNA steady-state levels for both IGFBP-2 and IGFBP-5 were shown by *in-situ* hybridization to localize to the epithelial cells that are deleted during the regression process. The differential expression of IGFBP-2 and IGFBP-5 suggest that modulating the epithelial cells response to the IGF growth factors may influence the active cell death process in hormonally dependent tissues such as the prostate and mammary gland.

DEDICATION

" And the end of all our exploring will be to arrive where we started and know the place for the first time. " T. S. Eliot

This thesis is dedicated to my grandmother the late Mrs. Myrtle Maclachlan whose love and support, as well as passion for knowledge instilled in me the courage necessary to complete this long journey.

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And finally I especially wish to thank my wife Karen and my family, especially Janie whose unflagging support and encouragment has never waned, these would have been tough times indeed without you.

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CHAPTER ONE

INTRODUCTION

1.100 - HISTORICAL OVERVIEW

It is now widely accepted that an integral part of a cell's potential response to its environment involves a form of cellular suicide referred to as apoptosis. The process of cellular destruction observed and described by researchers in a variety of fields, was termed necrosis and typically involved the destruction of cells in tissues which had been exposed to an environmental insult such as excessive heat, mechanical damage, or exposure to chemical toxins. The form of cell death which is now refereed to as apoptosis was considered to be a specialized case of necrosis, typically occurring during tissue remodeling events associated with the removal of unwanted cells during development.

Some of the earliest reports of what is now termed apoptosis described the deletion or removal of unwanted cells during development. These early studies began to characterize the morphological and biochemical features of apoptotic cell death that distinguish it from necrosis. The hormone induced regression of the tadpole tail during development was shown to require both protein and RNA synthesis for completion of the process (Beckingham-Smith and Tata, 1976; Tata 1965). The regression of mammary tumors in response to various chemicals was also associated with an increase in RNA synthesis and an increase in the activity of several proteins including proteases and lysosomal enzymes (Lanzerotti and Gullino 1972; Gullino et al., 1974). Several studies in other experimental systems such as chemically induced necrosis of the liver, intestine, and pre-natal tissues demonstrated that many "necrotic" cell deaths required protein synthesis (Schweichel and Merker 1973; Ying et al., 1980; Verbin et al., 1973). The accumulated data suggested that at least two types of cell death existed and that they can be distinguished based on their morphological and biochemical characteristics (Wyllie et al., 1972).

1.200 - MORPHOLOGY OF APOPTOSIS

Morphologically and biochemically, the process of necrosis and apoptosis are easily distinguishable. Necrosis does not require new protein or RNA synthesis, since the process occurs in the presence of inhibitors of macromolecular synthesis such as

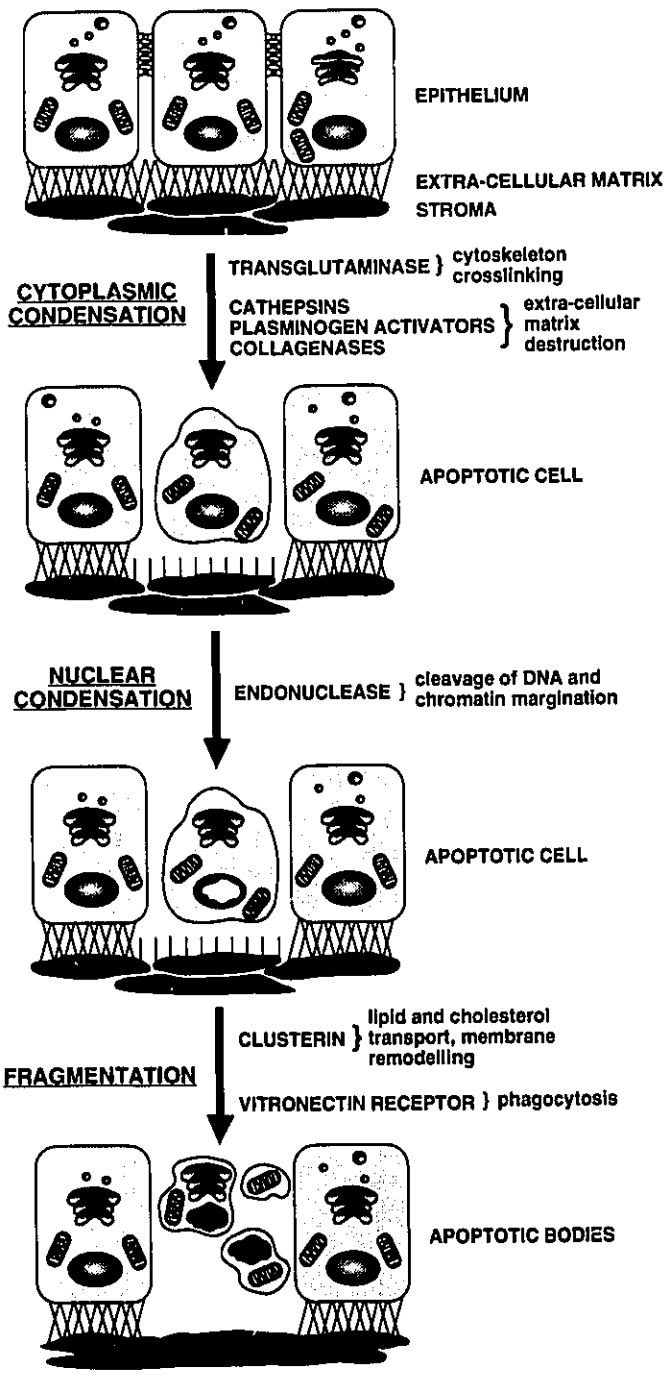
cycloheximide and actinomycin D. During necrosis the membrane integrity of the dying cell is compromised, resulting in swelling of the cell, disruption of the organelles and release of intracellular contents into the extracellular fluids. Typically, necrotic cell death results in the activation of an inflammatory response, with macrophage invasion and further damage to the surrounding cells.

Apoptosis in contrast, is an active process that usually requires new protein and RNA synthesis. We have referred to the apoptotic process as active cell death or ACD to distinguish the process from other forms of cell death (such as Fas-mediated cell killing) that do not seem to require macromolecular synthesis for completion of the process. We have used the term *thanatogens* from the Greek thanatos (meaning death) to describe genes that are involved in apoptosis. Thantogens fall into two broad categories: those that initiate the process of ACD (primary thanatogens) and those that ensure appropriate fragmentation and phagocytosis of the dying cells (secondary thanatogens) (Tenniswood et al., 1992,1994).

ACD can be broken down into a number of histologically distinct steps that appear to be common to the epithelial cells of the prostate, mammary gland and liver and probably most other epithelial cells (Bursch et al., 1990a; Bursch et al., 1990b) (Fig. 1-1). After the initial stimulus, there is a period, referred to as the "pre-condensation" phase, during which there is no histological evidence of ACD in individual cells. This period may range from only a few minutes to several hours or days. The initiation of ACD in the prostate and mammary gland, as a result of hormone withdrawal, is asynchronous, and only a small proportion of the susceptible cells in the tissue initiate ACD at a time. This asynchrony may be essential to ensure that the surviving cells of the tissue and the macrophages are capable of handling the apoptotic bodies produced by the dying cells. The first visible stage of ACD in the prostate and mammary glands is the loss of cell-cell interactions as the desmosomal contacts between the epithelial cells are lost. This "cytoplasmic condensation" is also characterized by the release of the cells from the basement membrane, and the disruption of the cytoskeleton of the affected cell, resulting

Figure 1-1: Morphological and Biochemical Stages of Apoptosis

The first visible stage of ACD in the prostate and mammary glands is the loss of cell-cell interactions as the desmosomal contacts between the epithelial cells are lost. This "cytoplasmic condensation" is also characterized by the release of the cells from the basement membrane, and the disruption of the cytoskeleton of the affected cell, resulting in the condensation of the intermediate filaments around the nucleus. In this way the cell undergoing ACD is isolated from its neighbours. "Chromatin condensation" occurs at about the same time and requires the activation of an endogenous Ca^{2+}/Mg^{2+} dependent endonuclease which degrades the nuclear DNA to produce the hyperchromatic, pyknotic nucleus typical of apoptotic bodies. The apoptotic cell is then fragmented into a number of smaller apoptotic bodies that contain portions of the fragmented nucleus and an array of intact organelles such as mitochondria and lysosomes.


EPITHELIUM
EXTRA-CELLULAR MATRIX
STROMA
CYTOPLASMIC CONDENSATION
TRANSGLUTAMINASE } cytoskeleton crosslinking
CATHEPSINS
PLASMINOGEN ACTIVATORS } extra-cellular matrix destruction
COLLAGENASES
APOPTOTIC CELL
NUCLEAR CONDENSATION
ENDONUCLEASE } cleavage of DNA and chromatin margination
APOPTOTIC CELL
FRAGMENTATION
CLUSTERIN } lipid and cholesterol transport, membrane remodelling
VITRONECTIN RECEPTOR } phagocytosis
APOPTOTIC BODIES

in the condensation of the intermediate filaments around the nucleus as originally described by Wyllie (Wyllie 1987). In this way the cell undergoing ACD is isolated from its neighbours. "Chromatin condensation" occurs at about the same time and requires the activation of an endogenous Ca^{2+}/Mg^{2+} dependent endonuclease that degrades the nuclear DNA to produce the hyperchromatic, pyknotic nucleus typical of apoptotic bodies (Wyllie et al., 1981; Wyllie et al., 1986; Kyprianou et al., 1988). The apoptotic cell is then fragmented into a number of smaller apoptotic bodies that contain portions of the fragmented nucleus and an array of intact organelles such as mitochondria and lysosomes. One of the critical features of the fragmentation process is that the cytoplasm and organelles of the apoptotic cell are essentially repackaged without the leakage of any of the cellular components into the extracellular space or bloodstream. This feature of apoptosis sets it apart from necrosis and complement induced cytolysis, since the intracellular enzymes are released into the circulation during the latter processes, resulting in an inflammatory response.

Accumulating evidence suggests that ACD is not a single phenomenon, but a series of morphologically and biochemically related processes (Gullino et al., 1972; Schweichel and Merker 1973; Ying et al., 1980; Clarke 1990; Zakeri et al., 1995). Cell death of lymphocytes and other cell of reticulo-endothelial origin is dominated by changes in nuclear morphology, while ACD of secretory epithelial cells involves profound cytoplasmic changes and alterations in the cell-cell and cell-substratum interactions typical of highly organized tissues (Clarke 1990; Cohen 1993; Zakeri et al., 1995). These changes are associated with alterations in nuclear morphology associated with endonuclease activation and the appearance of the characteristic DNA nucleosomal ladder and with lysosomal activation resulting in autophagic lysis (Bruchovsky et al., 1975; Kyprianou et al., 1988; Kyprianou and Issacs 1988; English et al., 1989; Dunn 1994). This is in marked contrast to the destruction of apoptotic lymphocytes, which are degraded following lysosomal activation in the engulfing macrophages (Walker et al., 1988; Cohen et al., 1992).

Recently, there has been some debate, based on the morphological examination of the final stages of ACD in several developmental systems and in pathological conditions, as to whether all forms of ACD proceed via identical pathways, and several attempts have been made to further classify the different forms of ACD (Clarke 1990). The final stage of ACD is the degradation of the apoptotic bodies by the lysosomes of the recipient cells. This process is referred to as heterophagy, or "type I" cell death, since it is apparent that lysosomal activation, if it occurs, occurs in the neighboring cell, rather than in the lysosomes of the dying cell. In some developing systems the lysosomes within the apoptotic body appear to be actively involved in the digestion of the apoptotic bodies, a process that has been designated as "autophagic" or "type II" cell death (Clarke 1990). In the event that the tissue undergoes substantial apoptosis (such as is likely to occur during the regression of hormone dependent tissues in the absence of the appropriate trophic hormone) the surviving cells may be unable to cope with all the apoptotic bodies, and those that remain are engulfed by macrophages which invade the dying tissue.

While it is clear that a large proportion of the epithelial cells in both the normal prostate and lactating mammary gland undergo ACD in response to hormone ablation, the two model systems are not strictly comparable. In the first place, the role of ACD in these two tissues is clearly very different. The regression of the lactating mammary gland that occurs after weaning is a physiologically controlled phenomenon that can be expected to occur several times during the lifespan of the animal. The specialized secretory cells in the mammary ducts that are required for milk production can be considered to be "programmed" for ACD since they are only required during lactation. The same cannot be said of the secretory epithelial cells of the prostate. Under physiological conditions these cells synthesize essential components of the seminal fluid throughout the lifespan of the male. The regression of the gland that occurs after castration cannot therefore be viewed as a physiologically normal phenomenon. However, since the secretory epithelial cells of both the prostate and lactating mammary gland undergo ACD even in the presence of the appropriate trophic hormones (at a much lower rate), it is clear that the induction of massive glandular regression after hormone ablation utilizes the same processes as are

induced by other lethal stresses in individual cells (Walker et al.,1988; Ferguson et al., 1988; Walker et al., 1989).

Secondly, several aspects of the morphology of ACD in these two systems are different. During ACD in the lactating mammary gland there are dramatic changes in the morphology of the epithelial cells in the luminal epithelium, including disruption of the rough endoplasmic reticulum and the disorganization of the Golgi bodies. Involution of the mammary gland is associated with intracellular trapping of secretory vesicles containing protein globules and lipid (Walker et al., 1989). The formation of these vesicles, originally described as cytosegresomes and cytosomes, coincides with, or precedes, the onset of the other morphological features of apoptosis including chromatin condensation and DNA fragmentation (Lascelles and Lee 1978; Helminen and Ericsson 1968). Although it is not yet clear whether the accumulation of these vesicles is unique to ACD in mammary epithelial cells, the morphology is clearly different from that seen in the prostate. The luminal epithelial cells of the prostate undergo ACD without any apparent accumulation of secretory vesicles, even though these cells also secrete substantial amounts of protein into the prostatic lumen (Kerr and Searle 1973; Mainwaring 1977; Clark et al., 1983; Sandford et al., 1984).

1.300 - BIOCHEMISTRY OF ACTIVE CELL DEATH (ACD)

1.310 - Ca^{2+} / Mg^{2+} Endonuclease

It was first shown by Wyllie in 1980 that the formation of the pyknotic nucleus in apoptotic thymocytes was co-incident with the fragmentation of DNA into oligonucleosomes. The nuclease involved in this DNA degradation requires Ca^{2+} / Mg^{2+} for its activity and was shown to be constituitively expressed in number of cell types (Wyllie, 1980). The typical oligonucleosome ladder of fragmented DNA has been used as a hallmark for apoptotic cell death in many experimental systems. However recent reports have shown that the degradation of the DNA into nucleosomal fragments is not an obligatory event for cells to undergo apoptosis (Cohen et al., 1992; Oberhammer et al., 1993; Walker et al., 1994). The primary cleavage event in the nucleus involves the

formation of DNA fragments 300 kbp in size, which occurs in all cells undergoing ACD and is cation independent (Walker et al., 1994). In some, but not all, cells the 300 kbp fragments are further degraded into fragments approximately 50 kbp in length by a Mg^{2+} dependent enzyme. A final stage which occurs in some cells involves the degradation of the 50 kbp fragments into the oligonucleosomal ladder and involves the Ca^{2+} / Mg^{2+} dependent endonuclease activity. The enzyme responsible for the initial degradation of the 300 kbp fragments into 50 kbp fragments was found to be distinct from the nuclease that generates the oligonucleosomes based on its cation requirements (Mg^{2+} alone) (Walker et al., 1994). The activity of this nuclease could be blocked by addition of a serine protease inhibitor, suggesting that proteolytic events are somehow involved in its activation (Walker et al., 1994). The formation of the various fragment sizes correlates with the morphology of the nucleus. Nuclei having little pyknosis contain 300 kbp fragments; Nuclei containing 50 kbp fragments show a higher degree of pyknosis; and nuclei containing oligonucleosomal DNA fragments showing highly condensed nuclei with the highest degree of pyknosis and margination.

1.320 - <u>Role of Calcium</u>

In thymocytes an early sustained increase in the levels of cytosolic Ca^{2+} occurs prior to endonuclease activation and DNA fragmentation in response to a variety of ACD inducers (McConkey et al., 1989b; Orrenius et al., 1989). Increases in the levels of Ca^{++} may serve as a second messenger for communicating the commitment of a cell to apoptosis since increases in nuclear calcium may be involved in activation of the Ca^{2+}/Mg^{2+} endonuclease. During necrosis the levels of intracellular calcium are also elevated as a result of the disruption of intermediary metabolism, and suggests that the magnitude of the surge in calcium may influence the fate of the dying cell (Wyllie et al., 1980). The release of Ca^{2+} from mitochondrion by pro-oxidants leads to an increase in Ca^{2+} cycling, an increased energy demand and a drop in the mitochondrial membrane potential. This leads to an decreased ability to retain Ca^{2+}, uncoupling of mitochondria and impairment of mitochondrial ATP synthesis. The lower levels of ATP impairs the function of the Ca^{2+} - sequestering ATPases, and as a consequence leads to a dramatic rise in cytosolic Ca^{2+}

levels (Richter, 1993). The transcription levels and activities of several genes including protein kinase C, insulin receptor tyrosine kinase, c-myc and c-fos are known to be regulated by calcium (Kass et al., 1989; Chan et al., 1986; Morgan and Curran, 1986) suggesting that some genes regulated by calcium may regulate or participate in the cell death process.

In the case of regressing prostate, treatment with Ca^{2+} antagonists alters the rate of cell death, delaying tissue regression and suppressing gene activity associated with cell death such as the induction of c-fos and TRPM-2 (Connor et al., 1988). The inolvement of a rise in intracellular calcium in all cell deaths may not be a neccessity. Treatment of three lymphoid derived, apoptosis sensitive cell lines (BM 13674, NS-1, and MOLT-4) with calcium chelators demonstrated that cell death can occur in these cell lines in presence of, or possibly even as a consequence of, decreased intracellular calcium (Kluck et al., 1994).

1.400 - GENE EXPRESSION DURING ACD

One of the most significant discoveries to emerge from cell death research is the observation that many of the genes previously described as oncogenes, with clearly defined roles in carcinogenesis, are somehow involved in suppressing apoptosis. Transfection of Rat-1 fibroblasts with c-myc leads to increased levels of apoptosis, particularly in the presence of low serum (Evan et al., 1992). The induction of apoptosis by high levels of c-myc in the presence of low serum can be prevented by over-expression of Bcl-2 without affecting the mitogenic function of c-myc, suggesting that there is a co-operative effect between these oncogenes (Fanidi et al., 1992; Bissonnette et al., 1992). The ability of various growth factors specifically IGF-I, IGF-II, and PDGF to prevent c-myc induced cell death, suggests that these cytokines act downstream of c-myc in the signalling pathway leading to the activation of cell death, and that these growth factors can block or abrogate the apoptotic signal. Other cytokines including EGF, bFGF, and bombesin are not capable of preventing cell death, suggesting that if they impinge on the apoptotic pathway at all, they probably act upstream of c-myc (Harrington et al., 1994).

Aberrant expression of the Bcl-2 gene is associated with many forms of leukemia. Expression of high levels of the Bcl-2 gene has been shown to correlate with the lymphocyte population resistant to cell death during normal development and in lymphoma derived cell lines (Vaux et al., 1988; Hockenbery et al., 1990; Alnemri et al., 1992a; Merino et al., 1994). The ability of Bcl-2 to protect cells from apoptosis extends to other cell types including T cells, Sf9 insect cells, primary kidney cell lines, neurons, and fibroblasts (Strasser et al., 1991; Alnemri et al., 1992b; Rao et al., 1992; Fanidi et al., 1992; Garcia et al., 1992; Kane et al., 1993). The Bcl-2 protein is localized in the mitochondrial membrane, and apparently can inhibit cell death induced by a variety of agents that generate free radicals and oxidants (Hockenbery et al., 1990; Hockenbery et al., 1993; Kane et al., 1993). Two structural domains required for Bcl-2 function have been identified but localization to the membrane does not appear to be required for its proper function (Borner et al., 1994). Bcl-2 belongs to a family of proteins including Bax, Bcl-X_L, Bcl-X_S, and Mcl-1 which share structural homology (Oltvai et al., 1993; Boise et al., 1993; Kozopas et al., 1993).

The family of Bcl like proteins share similarity in their sequences but apparently differ in their roles in cell death. Bax heterodimerizes *in vivo* with Bcl-2 and accelerates cell death in an IL-3 dependent cell line (Oltvai et al., 1993). Bcl-X_L inhibits cell death, while a splice variant of Bcl-X_L, Bcl-X_S accelerates the death of cells in the same model system (Boise et al., 1993). The large number of Bcl-2 homologs and their potential for hetero-dimerization provides a system that can regulate the cellular response to inducers of cell death by altering the pattern of Bcl-2 type protein expression. Several viral proteins also show homology or function similar to Bcl-2, including LMP-1, BHRF-1, E1B, p35 and iap, and suppress apoptosis of virally infected cells (Henderson et al., 1991; Rao et al., 1992; Henderson et al., 1993; Clem and Miller, 1994). The Bcl-2 gene is able to complement mutations in the p35 and iap genes suggesting that these viral proteins function in a similar fashion to other members of the Bcl-2 family.

It has been suggested that the tumor suppressor gene, p53, has a regulatory role in the induction of cell death. The presence of a functional p53 gene is essential for efficient initiation of the cell death program in lymphocytes in response to a variety of cytotoxic agents (Lowe et al., 1993), and for induction of apoptosis in a myeloid leukaemic cell line by withdrawal of IL-6 (Yonish-Rouach et al., 1991). The adenoviral E1A protein induces proliferation, and sensitizes cells to p53 induced cell death (Lowe et al., 1993). The apoptotic inducing properties of E1A can be inhibited by the adenoviral E1B 19kDa and less efficiently by E1B 55 kDa, proteins and by Bcl-2 (Rao et al., 1992). E1A induces apoptosis via a p53 dependent mechanism that can be blocked by either E1B or Bcl-2 (Chiou et al., 1994). Co-operation between c-myc and p53 in induction of cell death has been demonstrated in M1 leukemic cells and the prevention of cell death induced can be prevented by Bcl-2 over-expression (Lotem and Sachs, 1993).

The induction of apoptosis in a given cell or tissue requires some form of signal transduction which is initiated at the cell surface and must be transduced to initiate and complete the cell death pathway. In some lymphocyte cell lines the synthesis of new RNA and protein is not necessary as the cells already possess the machinery for cellular destruction. In these cells, the presence or absence of external signals may be the only requirement to initiate and complete the cell death pathway.

A monoclonal antibody directed against the B-cell surface antigen, APO-1, is capable of inducing apoptosis in both human lymphocytes and malignant human lymphocyte cell lines (Trauth et al., 1989). The APO-1 protein is identical to the Fas protein which also mediates apoptosis in cell lines that express the Fas cDNA and were treated with anti-Fas antibody (Itoh et al., 1991). The underlying genetic defect in *lpr* mice (a strain characterized by lympadenopathy and auto-immune disease) results in loss of expression of Fas protein, confirming the importance of Fas in regulating apoptosis in lymphocytes (Watanabe-Fukunaga et al., 1992). Other studies have shown that a functional Fas molecule on the target cell is required for induction of apoptosis by cytotoxic T cells (Ju et al., 1994). Fas is a member of a super-family of receptors that

includes TNF-R, NGFR, CD40 and OX40 that share many structural similarities. Tumor necrosis factor alpha (TNFα) has apoptotic effects on tumors and tumor cell lines, and mediates its effect through the TNF-R1 receptor (Kyprianou et al., 1991). Deletion mutagenesis of the TNF-R1 receptor has identified an 80 amino acid domain in the intracellular domain that is necessary for the cytotoxic signal (Tartaglia et al, 1993). Several amino acids in this region are also conserved in the intracellular domain of Fas, and suggest that a common "cell-death" signaling domain may exist in members of the TNF-R receptor super-family (Tartaglia et al., 1993). Other cell surface molecules such as the low affinity NGF receptor (another TNF-R super-family member), and the T cell receptor / α3 domain of class I MHC can deliver a cytotoxic signal to cells, but do not contain the same domain suggesting that there are several different signal cascades emanating from the surface that can initiate cell death (Sambhara and Miller, 1991; Rabizadeh et al., 1993).

Recent evidence suggests that TNF-α, Fas and ionizing radiation employ the sphingomyelin pathway to trigger apoptosis. The sphingomyelin pathway is initiated by hydrolysis of plasma membrane sphingomyelin to generate ceramide by the action of a sphingomyelinase. Ceramide serves as a second messenger stimulating a cascade of kinases and transcription factors that activate a final common pathway of programmed cell death. The extent to which this signalling system is used in ACD induced by other means is not known but accumulating evidence suggests that it may be a commonly employed pathway (Kolesnick et al., 1994 in press).

There is some evidence that some components of the G protein signalling system may be involved in transducing cell death signals. Growth factor receptor protein 2 (Grb2) links tyrosine kinase receptor proteins to the guanine nucleotide releasing factor, son of sevenless (Sos) by binding the receptor with its Src homology (SH2) domain and by attaching to Sos with two SH3 domains (Chardin et al., 1993; Egan et al., 1993; Rozakis-Adcock et al., 1993; Gale et al., 1993). A Grb-2 isoform, Grb3-3 which lacks an SH2 domain (and therefore cannot bind the EGF receptor) but still has SH3 domains

capable of binding Sos has recently been cloned. Grb3-3 expression inhibits EGF induced transactivation of a Ras-responsive element, and induces apoptosis in transfected fibroblasts. The Grb3-3 isoform is highly expressed in the thymus of rats at the age while negative selection of thymocytes is occurring (Fath et al., 1994). These results suggest that G proteins in conjunction with other signaling molecules may either stimulate growth or induce apoptosis, a process which can be modulated by other accessory factors such as Grb3-3. Most of the research directed at elucidating the signaling pathways involved in ACD have used lymphoid systems and it is not known to what extent these pathways control the signaling of ACD in more complex tissues such as the prostate and mammary glands.

The association of clusterin expression with apoptosis, or active cell death (ACD), was first made when TRPM-2 was identified as one of the major mRNA species induced during regression of the rat ventral prostate after castration (Montpetit et al., 1986). Subsequent cloning by differential hybridization and sequence analysis has demonstrated that TRPM-2 was identical to SGP-2 and clusterin (Leger et al., 1987; Collard and Griswold 1987; Tsuruta et al., 1990). The clusterin cDNA was subsequently identified and cloned from the regressing prostate using a variety of other screening strategies (Bettuzzi et al., 1989; Briehl and Miesfeld 1991).

The steady state level of clusterin mRNA is very low in the normal rat ventral prostate but is induced approximately 200 fold after castration (Leger et al., 1987; Furuya and Issacs 1993; Wong et al., 1993a). However, due to the complex ductal architecture of the rat ventral prostate, the analysis of changes in the steady state mRNA level in the whole tissue has limited usefulness. As originally described by Sugimura et al., and refined by Sensibar et al., the epithelial cells of the prostate display different morphologies and secretory activities depending on their anatomical location along the duct (Sugimura et al., 1986a; Sugimura et al., 1986b; Sensibar et al., 1991). In the adult male rat, the distal and intermediate zones of the prostatic ducts are lined with tall columnar secretory epithelial cells, while the lumen in the proximal region of the ducts is lined with cuboidal,

non-secretory epithelial cells (Lee et al., 1990). Prior to hormone ablation, a very small percentage (probably less than 0.3%) of these luminal epithelial cells undergo ACD each day. Electron microscopic analysis suggests that apoptotic bodies are scattered throughout the proximal, intermediate and distal zones of the ducts (Wong and Mooibroek, unpublished observations). On the other hand, immunoreactive clusterin is found almost exclusively associated with the luminal membranes of the epithelial cells in the proximal regions of the ducts, suggesting that either there is a higher incidence of ACD in the proximal region, or that clusterin accumulates in this region after secretion into the lumen from other regions of the ducts (Sensibar et al., 1991).

After hormone ablation there is a distinct increase in the expression of clusterin in the luminal epithelial cells in the distal and intermediate zones of the ducts, measured either by *in situ* hybridization, or by immunohistochemistry (Rouleau et al., 1990; Sensibar et al., 1991). This distinct population of secretory epithelial cells appears to be exquisitely dependent on the presence of androgens, while the cuboidal epithelial cells in the proximal region do not appear to require androgens for survival (Rouleau et al., 1990). The expression of clusterin correlates both temporally and anatomically with the region of the gland where ACD occurs. The sensitivity to ACD however does not correlate with the presence or absence of the androgen receptor, since the receptor appears to be present in all the luminal epithelial cells along the duct (Mooibroek, unpublished observations). This suggests that androgens have an indirect effect on clusterin expression, possibly mediated by other growth factors, or components of the extracellular matrix Tenniswood et al., 1990; Tenniswood et al., 1992).

The expression of clusterin also correlates with the induction of ACD in other hormone responsive tissues, particularly the seminal vesicles (Izawa 1990; Grima et al., 1990; Izawa 1991). Clusterin mRNA is induced *de novo* in the androgen-dependent Shionogi mouse mammary carcinoma, and becomes constitutively expressed in tumors that progress to androgen independence (Rennie et al., 1988; Rennie et al., 1990). Largely as result of the relationship between clusterin expression and tissue regression following

hormone ablation, clusterin expression has been widely used as a marker for ACD. While clusterin expression is increased in a wide variety of different systems undergoing hormone independent cell death, including prostate, kidney and liver, the expression of the gene does not correlate with ACD in a large number of tissues and cell lines, including testis and epididymis, bovine adrenal chromaffin granules, Madin-Darby canine kidney cells, and many neural tissues.(Kyprianou and Issacs 1989; Kyprianou et al., 1991; Schumer et al., 1992; Rosenberg and Paller 1991; Connor et al., 1991; Correa-Rotter et al., 1992; Helvering et al., 1993; Collard and Griswold 1987; Fritz et al., 1983; Cheng et al., 1988; Grima et al., 1992; Trasler et al., 1992; Sensibar et al., 1993; Zakeri et al., 1992; Mattmueller and Hinton 1991; Palmer and christie 1990; Fischer-Colbrie et al., 1982; Pilarsky and Koch-Brandt 1992; Danik et al., 1993; Michel et al., 1992; Garden et al., 1991) Furthermore, although clusterin mRNA has been reported to be transiently up regulated in thymocytes of rats treated with dexamethasone *in vivo* and in PHA stimulated lymphocytes, clusterin does not appear to be upregulated in the majority of inducible leucocyte models of ACD (Bettuzzi et al., 1991; Grassilli et al., 1991; Pearse et al., 1992). Further, clusterin expression in the human thymus is restricted to the epithelial cells of the thymic medulla, suggesting that its expression does not appear to be required for thymocyte cell death during negative selection (French et al., 1992).

Recent recognition that ACD can be subdivided into several categories based on morphological, biochemical and functional criteria, suggests that clusterin expression may be restricted to ACD of epithelial cells, during which the surface area of the cell decreases by approximately 50% (Zakeri et al., 1995). In this respect the secretory epithelial cells of the prostate and mammary glands (which have very large cytoplasmic/nuclear ratios) differ sharply from lymphocytes and thymocytes (which have very small cytoplasmic/nuclear ratios). Thus, if clusterin is directly involved in ACD, it is neither specific for the process, nor essential for all forms of ACD and may be required only in certain subsets of systems that undergo ACD.

1.500 - ACD DURING REGRESSION OF PROSTATE AND MAMMARY GLAND

Much of the research describing the molecular components of the cell death machinery has been performed in lymphocytes and in the lymphoid system. Although some of the thanatogens in lymphocytes are involved in other cell death systems, fundamental differences between lymphocyte models and more complex tissues exist. One obvious choice for studying apoptosis in tissues are systems in which regression can be induced by removal of the trophic hormones necessary for homeostasis. The rat prostate and mammary gland are two tissues which can be used to study hormonally based regression. The secretory epithelial cells of the prostate gland require androgens for homeostasis. Following orchiectomy the conversion of testosterone to its active metabolite 5α-DHT ceases and the epithelial cells undergo ACD (Bruchovsky et al.,1972; Sandford et al., 1984) . In contrast to the prostate the mammary gland enlarges during pregnancy due to a marked increase in the number of epithelial cells, which secrete milk proteins, primarily β-casein. Following weaning, the levels of prolactin hormone drop and the gland completes a restructuring involving extensive epithelial cell death, to return to its previous size (Battersby and Anderson, 1988; Walker et al., 1989). One of the major differences between cell death in these two tissues and lymphocytes is the presence of cell-cell contacts and the underlying extracellular matrix and stromal cells which interact with the secretory epithelium and which can effect the responsiveness and viability of the epithelial cells.

1.510 - Prostate Regression

In the rat ventral prostate, the nuclear androgen receptor decreases to undetectable levels within the first twelve hours after castration, followed by a marked reduction in prostatic size, and a dramatic decrease in androgen dependent protein synthesis (Heyns et al., 1977; Parker and Scrace,1979; Clark et al., 1983). Total RNA and DNA contents decrease 80-85% within 7 days of castration as a result of epithelial cell death (DeKlerk and Coffey, 1978; English et al., 1985; English et al., 1987). Paradoxically, the activity of a number of enzymes increases during this time. For example, the activity of cathepsin D is increased and reaches a peak between 5 and 7 days after castration (Tanabe et al., 1982;

Sensibar et al., 1990). The activity of acid ribonuclease (RNase) increases gradually, peaking between 5 and 7 days after castration. Treatment with actinomycin D retards this increase, and also decreases the rate of prostatic regression (Lee, 1981). The activity of both urokinase and tissue type plasminogen activator increases after castration and the peak activity correlates with the maximum rate of cell death in the gland (Freeman et al., 1990).

In addition to the increase in these enzymatic activities, several "castration-induced" mRNA species have been identified in the prostate (Montpetit et al., 1986; Lee and Sensibar, 1987), including the mRNA coding for the Yb1 subunit of glutathione S-transferase (Chang et al., 1987), TRPM-2 (clusterin/SGP-2) (Léger et al., 1987; Bettuzzi et al., 1989; Rouleau et al., 1990), and RVP.1 (Briehl and Miesfeld, 1991). Other genes, such as fos, myc and Hsp70 are also induced after castration (Buttyan et al., 1988) however to date only the expression of TRPM-2 (clusterin/SGP-2) mRNA and cathepsin D have been localized by *in situ* hybridization or immuno-histochemistry to the luminal secretory epithelial cells which undergo ACD (Rouleau et al., 1990; Sensibar et al., 1990; Sensibar et al., 1991).

1.520 - Mammary Regression

The morphological characteristics of ACD have been described in the resting breast, where cell death balances cell proliferation (Ferguson, 1988), and in the lactating mammary gland following weaning, where the reduction in mammary gland weight, the number and size of the ducts and DNA content is due to ACD (Battersby and Anderson, 1988; Walker et al., 1989). After weaning, the mammary gland ceases lactation and involutes. The wet weight of the gland decreases by 70% within 4 days of weaning. This involves significant tissue re-modelling as the ducts regress and return to the resting state. The presence of apoptotic bodies in the luminal epithelial compartment 2 to 3 days after weaning provides clear evidence that a substantial proportion of the regression is attributable to the induction of ACD of the epithelial cells. The time-course and magnitude of gene induction and the morphology of ACD during mammary gland regression and

re-modelling suggest that although the process is similar in general terms to that seen in the prostate, the individual stages of ACD may vary considerably.

1.530 - Regression in Both Systems

The activation and morphology of apoptosis in these systems reflects the fundamental differences described in sections 1.510 and 1.520 above. Both regressing prostate and mammary tissues activate the Ca^{2+}/Mg^{2+} endonuclease during regression and also show many of the classical morphological features. The apoptotic bodies formed during tissue regression are engulfed and degraded by normal neighboring cells which involves activation of the lysosomal compartment (Kyprianou and Issacs 1988; Walker et al., 1989). A variety of genes appear to be induced during regression of both tissues including TRPM-2, tissue transglutaminase, c-myc, c-fos, transin/stromelysin and TGF-β (Buttyan et al., 1988; Strange et al., 1992; Lefebvre et al., 1992; Guenette et al., 1994a and 1994b). Several proteases including cathepsin D, tissue and urokinase type plasminogen activators and collegenase increase in activity during the regression of epithelial cells in these tissues and are probably involved in degradation of the extracellular matrix (Sensibar et al., 1990; Freeman et al., 1990; Muntzing, 1981).

1.600 - SPECIFIC AIMS

One of the major goals of this thesis was to further characterize the changes in gene expression that occur during regression of both the rat ventral prostate and mammary gland hormonal ACD models, and identify novel genes involved in the cell death program in both tissues. One of the first genes described as a potential thanatogen was that of clusterin or TRPM-2 (see section 1.400). The expression of TRPM-2 during regression of both the prostate and mammary glands is similar, and suggests that common genetic events underlie ACD in both tissues. The first part of the thesis work characterized the expression of several genes namely poly (ADP-ribose) polymerase (PARP), heat shock protein 27 (Hsp27), and tissue transglutaminase (tTGase) which were postulated to have a potential role in the process of ACD. A number of genes were chosen to determine if the expression of any of these genes was different among the two systems. The second part of

the thesis involved identifying novel thanatogens expressed during ACD. Part of the rationale for looking for new thanatogens was the fact that one current marker of cell death, TRPM-2, is expressed in only some forms of apoptosis, and in many tissues not undergoing cell death. This suggests that the role of TRPM-2 as a marker for ACD is limited. The cloning and characterization of two new potential thanatogens, referred to as RSG-2, and RSG-8, are described and constitute the main work of the thesis.

1.700 - IDENTIFICATION OF THANATOGENS

The requirement for new mRNA synthesis and expression during ACD has resulted in a concerted effort to clone and characterize the genes involved in the molecular pathway of cell death. Identification of the primary thanatogens (genes that initiate ACD) has so far proven difficult.

Several of the genes that have been characterized were isolated using lymphocyte regression models and do not appear to be required in other systems. Furthermore the differences between lymphocytes and cells in a tissue may be reflected in the necessity to express a different set of molecules to complete ACD. The cloning and characterization of cDNAs which are induced during cell death of lymphocytes have been described by several groups. Two cDNAs, RP-2 and RP-8, have been cloned from regressing immature thymocytes by differential hybridization to subtractive cDNA libraries (Owens et al., 1991). Their cognate mRNAs show induction after exposure of immature thymocytes to gamma irradiation. RP-2 codes for a novel integral membrane protein, while RP-8 codes for a novel protein with a putative zinc finger domain and therefore might be involved in transcriptional regulation (Owens et al., 1991). The role of RP-2 and RP-8 in ACD in lymphocytes is unknown, since they are not induced in other models of ACD such as regression of hormonally dependent tissues like the prostate and mammary gland, and therefore it seems likely that their role will be tissue specific (Hacker and Vaux 1994).

Another cDNA referred to as PD-1 was cloned and characterized in another lymphocyte regression model using techniques similar to that used by Owens and

co-workers (Ishida et al., 1991). PD-1 is an integral membrane protein and a novel member of the immunoglobulin super-family, which is induced in several lymphocyte cell lines during ACD. PD-1 has a putative T-cell signalling motif in its cytoplasmic domain, but this region has not yet been demonstrated to be required for its function (Ishida et al., 1991).

The search for genes involved in cell death has not been limited to lymphocyte model systems. A temperature sensitive mutant tsBN7 of the BHK21 cell line undergoes apoptosis at the non-permissive temperature 39.5°C, but maintains normal growth properties at the permissive temperature of 33.5°C. The induction of cell death can be prevented by cycloheximide, suggesting that continued production of a protective protein is necessary to prevent cell death in the tsBN7 cell line. A cDNA which complements the tsBN7 mutation, DAD1, was cloned using DNA-mediated gene transfer techniques. DAD1 encodes a novel, 12 kDa protein which is highly hydrophobic but does not appear to be membrane bound (Nakashima et al., 1993). The tsBN7 mutation is a point mutation which leads to a single change in the amino acid sequence of the protein. In tsBN7 cells at the non-permissive temperature the DAD1 protein is rapidly degraded, leading to cell death. Apoptosis induced in tsBN7 cells is not prevented by over-expression of Bcl-2, suggesting a difference between the mechanism of Bcl-2 action and that of DAD1. The DAD1 protein has been well conserved during evolution with 91% similarity between the human and Xenopus amino acid sequence, and may imply an important role in regulating cell death .

Other groups have focused on isolating cDNAs involved in cell death from other model systems such as the rat ventral prostate during castration induced regression. Using a subtraction cDNA library made from regressing prostate and screening with a subtracted cDNA probe, Hoshikawa and co-workers identified several sequences that were castration induced (Hoshikawa et al., 1991). Three of these clones represented the Yb-1, Yb-2 and Yb-3 subunits of rat glutathione S-transferase, of which Yb-2 had already been shown to increase in mRNA steady state levels during prostatic regression (Chang et al., 1987). The

remaining sequences were unidentified after comparison to the DNA sequence data base Genbank, and their role in apoptosis has yet to be defined. Briehl and Miesfeld used a similar approach to that of Hoshikawa to isolate cDNAs induced during regression of the rat prostate (Briehl and Miesfeld, 1991). They identified a cDNA referred to as RVP.1 which is induced following castration in the prostate. The predicted protein from the RVP.1 cDNA shows no homology to known proteins or motifs currently in the Swiss Prot database. The role of RVP.1 in the cell death process remains unclear and requires further studies. One issue is pertinent for both the cDNAs described by Hoshikawa and RVP.1 regards their generality of expression during cell death (i.e. are they expressed in other systems undergoing cell death). Furthermore, given the multicellular nature of the tissues used to construct the cDNA libraries for screening it is important to establish that the increased expression of these genes is in the secretory epithelial cells of the prostate and mammary gland which die after removal of the appropriate trophic hormone.

1.800 - STRATEGIES FOR CLONING THANATOGENS

1.810 - Traditional Cloning Approaches

Novel genes involved in ACD have been identified using a variety of techniques. In model systems where cells in culture can be induced to undergo ACD relatively synchronously, differential hybridization and subtractive hybridization techniques have been used successfully. In some cases, based on the unique properties of existing mutant cell lines (tsBN7) or known chromosomal abnormalities (Bcl-2, E2A-PBX1), a more traditional and directed approach has resulted in the identification of the underlying genes responsible for the defects in the ACD pathway (Cleary et al., 1986; Dedera et al., 1993). The identification of novel thanatogens in model systems that are not based on cell culture methods proves to be more problematic. In particular, the two model systems chosen for the studies described in this thesis pose significant challenges to traditional techniques.

In hormonal models of ACD such as the prostate gland after castration and the mammary gland after weaning, the induction of cell death is asynchronous. At any time point during regression only a small percentage of the cells are under going ACD. This

means that only a very small proportion of mRNA isolated from these regressing tissues will be from cells currently activating the genes necessary for ACD. An additional problem with these model systems is their multi-cellular nature. Thus mRNA isolated from later stages of regression will not only reflect the loss of secretory epithelium, but also the significant increase in stromal components relative to epithelium as regression proceeds. Another problem arises when only one model system is used during the cloning process, since a gene induced in one model system may not be common to other model systems. To isolate thantogens that are expressed at lower levels and are associated with cell death in more than one model system, we decided to use an alternative approach to traditional methods.

1.820 - Lateral Cloning Strategy

Lateral cloning is a cross screening strategy that was developed to identify human mRNAs expressed on the X-chromosome, that may be candidates for several X-linked eye disorders (Wong et al., 1989). A library to library cross-screening strategy was used to isolate sequences that are common to both a human retinal cDNA library and a genomic library constructed from a hamster cell line that harbors a single human X chromosome. The cDNA inserts from the human retinal library were amplified using the polymerase chain reaction (PCR) and vector specific primers, which increases the amounts of rare messages. The amplified cDNA inserts were radiolabelled and used to screen the genomic library. The strategy resulted in the identification of a cDNA clone, R8, a human cDNA expressed in human retinal tissue that localizes specifically to the X-chromosome and may underlie X-linked retinitis pigmentosa (Wong et al., 1993b). The power of the lateral cloning approach is its ability to identify even rare messages that are expressed in common to two systems, using only standard library construction, PCR and standard hybridization techniques. This simplifies the initial stage of the project and eliminates the use of time consuming techniques such as subtractive hybridization which require numerous manipulations and often result in the loss of rarer messages.

We have used a modification of the lateral cloning technique described above to isolate novel thanatogens. We chose two hormonal models of cell death: the prostate gland after castration and the mammary gland after weaning. Theoretically, the morphological similarities of these two models of regression suggest that some genes induced during ACD (and therefore potential thanatogens) should be identical between the two systems (for a review on ACD in hormonally dependent tissues see Tenniswood et al., 1992). Preparing cDNA libraries from both regressing tissues and using lateral cross-screening to screen the libraries should result in the isolation of sequences common to both systems. Since the majority of messages present in both tissues would be tissue specific, the vast majority of sequences would not be identified in the screening process. Sequences common to both (which would be identified) represent either genes induced during ACD (a process occurring in both tissues) or constitutive or "housekeeping" genes. The latter can be removed by cross-screening with the cDNA inserts from a normal mammary or prostate library. Furthermore the use of PCR to amplify the inserts for screening enhances the probability of isolating even rare messages.

CHAPTER TWO

MATERIALS AND METHODS

2.100 - DNA

2.110 - Plasmid DNA Extraction

E. coli containing plasmids were grown in selective medium as defined by the specific suppliers. Plasmid DNA was extracted from 5 or 250 ml cultures using standard protocols (Birnboim and Doly, 1979; Holmes and Quigley, 1981) or the Qiagen mini or maxi-prep plasmid extraction kits and the protocol supplied by the manufacturer.

2.120 - Phage Clones and Libraries

Recombinant phage DNA was isolated from 50,000 independent clones from the unamplified MGC-10 and MGR2-10 cDNA library using a plate lysate technique (See Table 4-1 for library nomenclature). Phage DNA from isolated phage clones was prepared from lysates of small or large scale liquid cultures (Maniatis et al., 1982). The extraction of phage DNA from the lysate was performed using a modification of the protocol of Y. Myal (University of Manitoba, Department of Physiology, Winnipeg, Manitoba, unpublished). Host bacteria were infected with phage to obtain high density plating on L-agar plates, incubated at 37°C overnight, and the phage were recovered by elution into SM buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $MgSO_4$, 0.1% gelatin) (20 ml per plate). The phage lysate was incubated at 37°C with DNAse (100 μg/ml) and RNAse A (100 μg/ml) for 1 h. Cellular debris was pelleted by centrifugation at 3000 rpm for 30 min at 4°C. The supernatant was isolated, NaCl was added to 1 M, PEG 8000 was added to 1%, allowed to dissolve completely, and incubated on ice for 1 h. The sample was subsequently mixed on a rotary mixer at 4°C overnight.

Phage were pelleted by centrifugation at 11,000g for 10 min at 4°C and the pellet was redissolved in SM buffer. SDS was added to 0.5% and proteinase K was added to 300 μg/ml and incubated at 65°C for 1 h. The sample was gently extracted once with an equal volume of phenol saturated with TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA), once with phenol/chloroform (saturated with TE) and once with chloroform/isoamyl alcohol (24: 1). The DNA was precipitated with an equal volume of isopropanol for 30 min at -20°C, recovered by centrifugation, washed with 70% ethanol, and redissolved in TE.

2.130 - Measurement of DNA Concentration

The concentration of DNA was estimated by measuring the optical density (OD) at 260 nm wavelength. The ratio of OD_{260}/OD_{280} was used to assess purity. Ratios of 1.5 - 2.0 were considered to indicate negligible protein or phenol contamination.

2.200 - RNA

2.210- Isolation of Total RNA

Total RNA was isolated from fresh homogenized rat tissues using the LiCl/urea procedure (Auffray and Rougeon, 1979) with minor modifications (Tenniswood and Simpson, 1982). The tissue was weighed and homogenized on ice in a fixed initial volume of extraction buffer (20 ml/g of tissue: 3 M LiCl, 6 M urea, 50 mM NaOAc pH 5.5) and sonicated (3 x 15 sec bursts, with 1 minute cooling intervals between each burst). SDS was added to 0.1% and the sample was incubated at 4°C overnight. The sample was centrifuged at 16,000 rpm for 30 min and the pellet was recovered, drained, and resuspended in wash buffer (4 M LiCl, 8 M urea, 50 mM NaOAc pH 5.5, 0.1% SDS). The sample was centrifuged as described above and the pellet was recovered and dissolved in half the initial volume of 50 mM NaOAc, pH 5.5 and extracted with an equal volume of phenol (saturated with 50 mM NaOAc pH 5.5) and an equal volume of chloroform. The organic and aqueous phases were separated by centrifugation (4,200 rpm, 5 min, room temperature) and the aqueous phase was retained. The organic phase was re-extracted with 10 ml of 50 mM NaOAc and the aqueous phases were pooled. The combined aqueous phases were re-extracted in an equal volume of phenol/chloroform (saturated with 50 mM NaOAc pH 5.5) and once with chloroform. The RNA was precipitated from the aqueous phase by adding 1/10th volume 3 M NaOAc, pH 5.5 and 2.5 volumes of cold ethanol. The RNA was precipitated at -20°C overnight and pelleted at 10,000 rpm for 1 h at 0°C. The pellet was recovered, dried, redissolved in sterile double distilled water and stored at -70°C.

2.220 - Isolation of Poly(A)+ RNA

Poly(A)+ RNA was prepared using oligo(dT)cellulose chromatography (Aviv and Leder, 1972). Total RNA was diluted to a concentration of 2 mg/ml, boiled for 2 min and chilled on ice for 10 min. An equal volume of 2x binding buffer (40 mM Tris-HCl pH 7.6, 1 M NaCl, 2 mM EDTA) was added to the RNA, mixed with oligo(dT) cellulose and incubated at 37°C on a rotary mixer for 4 h. The slurry was packed into a column prepared in a 3 ml syringe. The poly(A)- fraction was eluted by passing a minimum of 10 bed volumes of binding buffer through the column. Poly(A)- RNA, was precipitated overnight at -20°C after addition of 1/10 volume of 3M NaOAc, pH 5.5, and 2.5 volumes cold ethanol. The poly(A)- RNA was centrifuged 13,000 rpm, 60 min, at 0°C and was redissolved in sterile double distilled water and stored at -70°C.

Poly(A)+ RNA was eluted from the column by passing 3 bed volumes of sterile water through the column and collecting the eluate. Poly(A)+ RNA was precipitated overnight at -20°C after addition of 1/10th volume 3 M NaOAc pH 5.5 and 2.5 volumes ethanol. The poly(A)+ RNA was pelleted at 10,000 rpm for 90 min at 0°C. The pellet was air dried, redissolved in sterile double distilled water and stored at -70°C.

2.230 - Quantification of RNA

The concentration of RNA was estimated by measuring optical density (OD) at 260 nm wavelength. The ratio of OD_{260}/OD_{280} was used to assess purity. Ratios of 1.5-2.0 were considered to indicate negligible protein or phenol contamination. Typical yields were approximately 250 µg of total RNA per gram wet weight of prostate or mammary tissue. Poly(A)+ RNA yields were approximately 2 - 5% by weight of total RNA.

2.240 - Characterization of RNA

The quality of total RNA samples was assessed by running 10 µg of each sample on 1.0% formaldehyde gels and staining with ethidium bromide (EtBr) as described in section 2.731. Intact ribosomal RNA (28S and 18S) and the absence of high molecular

weight DNA indicated high quality RNA samples. The integrity of poly(A)+ RNA was determined by northern analysis using a probe specific for rat α-tubulin.

2.300 - cDNA CLONING

2.310 - Construction of cDNA Libraries

2.311 - cDNA Synthesis:

Poly(A)+ RNA was used as the template for cDNA synthesis. cDNA wassynthesized using a modification (Rutledge et al., 1988) of the method described by Gubler and Hoffman (1983). For first strand synthesis, 6 μg of poly(A)+ RNA was incubated in a final volume of 30 μl with 100 mM Tris-HCl pH 8.3, 130 mM KCl, 10 mM $MgCl_2$, 2.5 mM DTT, 4 μg oligo(dT)10-18, 1 mM dATP, 1 mM dTTP, 1 mM dGTP, 1 mM dCTP, and 50 μCi of [α^{32}P]-dATP. After heating to 60°C for 2 min, AMV reverse transcriptase (24 U) was added and the mix was incubated at 42°C for 30 min. For second strand synthesis the reaction volume was brought up to 100 μl with 50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$ 20 mM DTT, 50 μg/ml BSA. RNase H (2 U) and DNA polymerase I (25 U) were added and the reaction was incubated at 15°C for 2 h.

The sample was then extracted with phenol/chloroform/isoamyl alcohol (25:24:1; saturated with TE) and passed through a 1 ml G-50 spun column to remove unincorporated nucleotides (Maniatis et al., 1982). A 1 μl aliquot of the eluate, in 5 ml of scintillation fluid was counted in a Beckman L1800 scintillation counter. The total incorporation of radioactivity was used to estimate the final yield of ds-cDNA using the equation:

$$\text{ds-cDNA yield (ng)} = \frac{\text{nmole cold dNTP added} \times 326 \times \text{total counts} \times 4}{\mu\text{Ci dNTP used} \times 2.2 \times 10^6}$$

2.312 - Size fractionation of cDNA:

Prior to cloning, the cDNA was fractionated on a 1 ml Sepharose 4B column to size select for the larger cDNA fragments. One drop (30 µl) fractions were collected and radioactivity measured by the Cerenkov method in a Nuclear Chicago Isocap 300 scintillation counter. One µl of the size fractionated cDNA pool was sized by electrophoresis on a 2% slab gel in a Tris-acetate buffer system. The gel was stained with ethidium bromide (l µg/ml), photographed, and dried down under vacuum on a Savant gel drier and autoradiographed using Cronex X-ray film in a Kodak X-Omatic X-ray cassette with intensifying screens. From the profile of incorporated counts vs fraction, the fractions containing ds cDNAs larger than 600 bp were pooled. The radioactivity associated with this pool was determined by counting a 1 µl aliquot in a Beckman L1800 scintillation counter and the total yield of ds-cDNA was estimated as above. The cDNA pool was ethanol precipitated at -20°C overnight in the presence of 0.3 M NaCl and redissolved in sterile double distilled water.

2.313 - Cloning into λgt10 and λgt11:

The size selected cDNA was inserted into the EcoRI site of the phage vector λgt10 or λgt11 (Hyunh et al., 1985) using a commercially available cloning kit (Amersham, Oakville, Ontario). The ds-cDNA (200-500 ng) was ligated to Eco RI adapters (250 pmole) in 1x ligase buffer (66 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP), 5 U T4 DNA ligase, and incubated at 15°C for 20 h. The reaction mix was extracted using Gene Clean™ (Bio101, LaJolla, CA) to remove un-incorporated adapters which bind irreversibly to the glass beads. Fifty ng of adapted ds-cDNA was ligated to 1.5 µg of lambda phage arms (either λgt10 or λgt11) with 2.5 U of T4 DNA ligase in 1x ligase buffer in a final reaction volume of 10 µl and incubated at 15°C for 16-20 h. The packaging extracts provided by Amersham were added directly to the ligation mix and incubated at 20°C for 2 h. Packaged libraries were stored in 0.5 ml of SM buffer containing 10 µl chloroform. The λgt10 or λgt11 libraries generated were plated as described below. A summary of the libraries constructed is presented as Table 4-1.

The bacterial host used for the λgt10 cDNA libraries was *E. coli* strain NM514. The *E. coli* strain L87 was used as a control for assaying cloning efficiency in λgt10. The bacterial host used for the λgt11 cDNA libraries was the *E. coli* strain Y1090. In all cases frozen liquid cell stocks of each cell line were established and stored at -70°C. Cells used for phage infection were grown in 50 ml of L-broth supplemented with 0.4% maltose at 37°C on a shaker until the OD_{600} reached 0.5, at which time the bacteria were cooled on ice, pelleted at 4°C and resuspended in 15 ml of cold 10 mM $MgSO_4$ or SM buffer. The cells were stored at 4°C and used within 3 days.

To plate recombinant phage a fixed number of phage particles was added to 200 - 1000 µl of plating cells and incubated at 37°C for 30 min. The phage and cells were added to melted top agar (3 ml for small plates and up to 50 ml for large plates), mixed and poured onto L-agar plates. The layer of top agar was allowed to solidify and the plate was incubated overnight at the appropriate temperature (37°C for λgt10, 43°C for λgt11).

2.400 - SCREENING OF cDNA LIBRARIES

Recombinant λgt10 DNA was isolated from $5x10^5$ independent clones from unamplified MGC-10 and MGR2-10 libraries (see Table 4-1 for nomenclature) using the plate lysate technique. The cDNA inserts from both libraries were amplified by PCR, using 1 ng of recombinant DNA from each library, and radiolabeled with [α^{32}P]-dCTP by random priming (NEN, Dupont, Lachine, Quebec). These two radiolabeled probes were used to screen the VPR2-11 library . Recombinants from the VPR2-11 library were plated (10,000 pfu/plate) using standard procedures and duplicate lifts were made on Hybond-N membranes. One lift was hybridized to the radiolabeled PCR-amplified cDNA inserts from the MGR2-10 library (100 ng, $5.0x10^7$ cpm). The other lift was hybridized to the PCR-amplified cDNA inserts from the MGC-10 library (100 ng, $5.0x10^7$ cpm). Plaques that hybridized to the MGR2-10 probe but not to the MGC-10 probe were plaque purified and stored as stocks in 100 µl SM buffer and 5 µl chloroform.

2.500 - SUBCLONING

2.510 - Subcloning of Individual Phage Clones

Selected λgt10 and λgt11 cDNA clones were subcloned into the plasmid vector pGEM7Zf-. Phage clones were digested with Eco RI to release the inserts. Fragments generated from 500 ng of intact recombinant phage DNA were ligated to Eco RI digested dephosphorylated vector (500 ng) with 1 U of T4 ligase in 1x ligase buffer and incubated at 16°C for 15h. The recombinant constructs were used to transform competent *E. coli* strain DH5αf' (see 2.520). In some cases, when the recombinant DNA proved difficult to digest, 1 ng of phage was amplified using lambda primers specific for the multiple cloning site in a standard PCR reaction (see section 2.610) and 1 μg of the product was digested with Eco RI and cloned as described above.

2.520 - Calcium Chloride Mediated Transformation

Competent cells, derived from a growing culture of *E. coli* strain DH5αf' were grown to an OD_{600} of 0.5 in 100 ml of 2YT broth (16 g bactotryptone/L, 10 g yeast extract/L, 5 g NaCl/L, pH 7.0), chilled on ice and pelleted by centrifugation. The cell pellet was resuspended in 40 ml of TfbI (30 mM KOAc pH 5.8, 100 mM $RbCl_2$, 10 mM $MnCl_2$, 15% glycerol) and incubated on ice for 5 min. The cells were pelleted, resuspended in 4 ml of TfbII (10 mM MOPS pH 5.8, 75 mM $CaCl_2$, 10 mM $RbCl_2$, 15% glycerol) and incubated on ice for 15 min. Competent cells were used directly for transformation or stored at -70°C and thawed prior to use.

Transformations were performed by adding 200 μl of competent cells to each ligation reaction and incubating on ice for 30 min followed by a 2 min heat shock at 42°C. 400 μl of 2YT broth was added to each tube and the cells were incubated at 37°C for 1 h, placed on ice for 5 min, plated out on LB-Amp plates containing isopropylthio-β-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and incubated at 37°C overnight.

2.530 - Electroporation Mediated Transformation

Electrocompetent cells were prepared from cultures of *E. coli* strain DH5αf' grown to OD_{600} of 0.5 in 500 ml of 2YT broth. The cells were chilled on ice and pelleted by centrifugation. Cells were then washed 2x in ice cold sterile double distilled water/10% glycerol solution, re-pelleted by centrifugation, suspended in 2 ml of ddH_2O/10% glycerol solution, dispensed in 100 µl aliquots and quick frozen in a dry-ice ethanol bath. Cells were used directly for transformation or stored at -70°C and thawed prior to use. 5 µl of the ligation mix, de-salted on a CL4B sepharose column, was mixed with 40 µl of electrocompetent cells, and samples were pulsed in Bio-Rad chambers (5 mm) gap at 2.5 kV, 25 µF, 200 ohms. 1 ml of 2YT medium was added to each tube and the cells were further incubated at 37°C for 1 h and then plated as described above.

2.600 - POLYMERASE CHAIN REACTION

2.610 - General Amplification of Inserts

Polymerase chain reactions were performed using the GeneAmp DNA amplification Reagent Kit (Perkin-Elmer Cetus, Ottawa, Ontario) essentially as described by Saiki et al., (1985) . The Mg^{2+} concentration was varied between 2 - 7 mM depending on the template and specificity of products obtained in the amplification. An excess of each primer (50 pmoles total) was added to each reaction and each nucleotide was present at a final concentration of 200 µM. Between 1 - 10 ng of template was added to each reaction, with the final volume of the reaction set at either 50 or 100 µl in 1x Taq polymerase buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl). Reaction mixes were denatured at 95°C for 5 min and 5U of Taq polymerase (BRL, Burlington, Ontario) was added to the reaction. The reactions were subjected to 30 cycles as follows: denaturation at 95°C for 2 min; annealling at 54°C for 2 min; extension at 72°C for 2 min. After the last cycle, the products were extended a final time at 72°C for 15 min and then stored at room temperature.

2.620 - RT-PCR Cloning of IGFBP-2 and IGFBP-5

200 ng of rat kidney poly(A)+ RNA was diluted to a final volume of 12 µl, incubated at 70°C for 5 min and then quick chilled on ice. 6 µl 5x RT Buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 2 µl of 100 mM DTT, 1 µl (10 µg) random hexamers, dNTPs (2 mM of each dNTP), and 1 µl (5 U) of Superscript™ (BRL, Burlington, Ontario) were added to each RNA sample. The reaction was incubated at 42°C for 1 h, and then at 50°C for 30 min. The products of the reverse transcription reaction were purified by passing the reaction mix over a CL4B sepharose column. 35 cycles of PCR were performed using the conditions detailed in section 2.610 using 5 µl of the purified reverse transcription reaction products with the exception that during the initial 3 annealing cycles the temperature was set to 42°C to facilitate primer/template interactions.. Primers specific for each IGFBP were designed with a 15 base match to the target sequences, and an overhang with EcoRI and NotI restriction sites. The reaction products were extracted with phenol/chloroform 1:1 (saturated with TE), ethanol precipitated, digested with EcoRI and subcloned into the pGEM7Zf- vector as described in section 2.510. The resulting clones were confirmed by sequence analysis (section 2.780).

2.700 - ANALYSIS OF NUCLEIC ACIDS

2.710 - Synthesis of DNA Probes

2.711 - Isolation on glass beads:

Clones were digested with specific restriction enzymes to release the inserts from the vector under the conditions suggested by the enzyme supplier. The digested samples were electrophoresed on a 0.7% agarose gel using a TAE buffer system to size fractionate the DNA fragments (Maniatis et al.,1982). The gel was stained with ethidium bromide (1 µg/ml) and the electrophoretic pattern was visualized on a 306 nm UV transilluminator. The fragments corresponding to the cloned inserts were cut out of the gel and retrieved by binding the DNA to glass beads (GeneClean™, Biol0l, La Jolla, CA) in the presence of sodium iodide, followed by elution into TE buffer pH 8.0 (Vogelstein and Gillespie, 1979).

2.712 - Polymerase Chain Reaction Isolation:

Template DNA (1 ng) was amplified using the appropriate primer pair in a standard PCR reaction using the conditions described in section 2.610. Reaction products were extracted once with phenol:chloroform 1:1 (saturated with TE), once with chloroform:isoamyl alcohol 24:1, precipitated with 2.5 volumes of ethanol, 1/10 volume 3 M NaOAc pH 5.5. The samples were dissolved in distilled water and small non-specific products (<500 bp), primer-dimers and un-incorporated primers were removed by spun-column purification over CL-4B sepharose.

2.713 - End labeling of oligonucleotides:

Oligonucleotides were synthesized by the Queen's University Biotechnology Research Institute (Kingston, Ontario) or on the Beckman Oligo 1000™ synthesizer. Oligonucleotides were end labeled according to Sambrook et al., (1989). 100 ng of oligonucleotide was mixed with 1x polynucleotide kinase buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine-HCl, 0.1 mM EDTA), 5 µl [γ^{32}-P]ATP, and 20 U of T4 polynucleotide kinase in a final volume of 50 µl and incubated for 30 min at 37°C. The reaction was stopped by the addition of 1 µl of 500 mM EDTA and unincorporated nucleotides were removed by passing the reaction over a Sephadex G50 spun column. The specific activity of the final probe ranged from 10^7-10^8 cpm/µg of DNA.

2.714 - Multiprime:

Isolated DNA was random primer labeled as described by Feinberg and Vogelstein (1983) using a commercially available kit (Amersham, Oakville, Ont.). In later experiments random primer labeling was performed using the NEN Random Primer extension labeling system (NEN, Dupont, Lachine, Quebec) or the T7 labeling system (Pharmacia, Baie D'Urfe, Quebec). The specific activity of the final probe ranged from 10^8-10^9 cpm/µg DNA.

2.720 - Clone Analysis

2.721 - Plaque lift analysis:

Plaque lifts of the respective phage libraries were prepared from plates which had been chilled at 4°C for 1 h before the lifts were made. Lifts were made using Hybond N filters (Amersham,Oakville, Ont) and were processed according to Allday and Jones (1987) or by denaturing for 3 minutes in 1.5 M NaCl, 0.5 M NaOH, neutralizing in 1.5 M NaCl, 0.5 M Tris-HCl pH 7.5 for 3 minutes, and fixing the DNA to the membrane using a Strata-linker (Stratagene, La Jolla, CA) (254 nm, 30 sec, 1,200 μJoules). Pre-hybridization, hybridization, wash conditions and autoradiography were performed as described in section 2.752, with the exception that sheared denatured *E. coli* DNA was included in the pre-hybridization and hybridization solutions at a final concentration of 60 μg/ml.

2.722 - Grunstein-Hogness Analysis:

Transformed bacterial colonies were picked onto Hybond-N membranes placed on top of L-agar plates containing 100 μg/ml ampicillin. The colonies were grown at 37°C overnight. Membranes were placed into 2x SSC, 5% SDS (colony side up) and incubated for 2 min and microwaved until dry to fix and denature the DNA onto the filter. Pre-hybridization, hybridization, wash conditions and autoradiography were performed as described in section 2.752 with the exception that sheared denatured *E. coli* DNA was included in the pre-hybridization and hybridization solutions at a final concentration of 60 μg/ml.

2.730 - Electrophoresis

2.731 - RNA:

RNA was electrophoresed in formaldehyde gels (Sambrook et al., 1989). Samples were prepared in a total volume of 40 μl lx MOPS buffer (20 mM morpholinopropanesulfonic acid pH 7.0, 5 mM NaOAc, 1 mM EDTA), 17.5% formaldehyde, 50% deionized formamide, incubated at 55°C for 15 min and quick chilled on ice. If the samples were to be visualized, ethidium bromide was added to a final

concentration of 1 μg/30 μl sample prior to incubation. 10 μl of loading buffer (50% glycerol, 0.25% bromophenol blue(w/v), 0.25% xylene cyanol(w/v), 1 mM EDTA pH 8.0) was added and the samples were electrophoresed in a 1.0% agarose gel (prepared in 18% formaldehyde and 1x MOPS buffer) at 30 V for 16 h. Ethidium bromide stained samples were visualized by UV transillumination at 306 nm and photographed using an orange filter and Ilford FP4 film.

2.732 - DNA:

DNA samples were diluted in 1/10th volume 10x TAE loading buffer (400 mM Tris-HCl pH 7.7, 200 mM EDTA, 2.3% glacial acetic acid, 50 % glycerol, 0.5 % xylene cyanol, 0.25% bromophenol blue) and incubated at 65°C for 5 min prior to loading. DNA samples were electrophoresed on 0.5-2.0% Tris-acetate/agarose gels depending on the specific experiment (Maniatis et al., 1982). After electrophoresis the gels were stained with ethidium bromide (1 μg/ml) and photographed as described above.

2.740 - <u>Northern analysis</u>

RNA samples fractionated on formaldehyde gels were processed and transferred directly to nylon membranes (ZetaBind, Cuno, Meriden, CT; or PALL Biodyne ICN, Irving, CA) by capillary blotting overnight using 20x SSC (0.3 M Na_3citrate pH 7.0, 3 M NaCl) as transfer buffer according to the protocols provided by the supplier. Membranes were baked at 80°C under vaccuum for 2 h, or cross-linked with UV using a Strata-linker (254 nm, 30 sec, 1,200 μJoules) prior to prehybridization. The membranes were prehybridized in 6x SSPE (20x SSPE is 50 mM NaH_2PO_4 pH 7.4, 3 M NaCl, 50 mM EDTA), 5x Denhardt's solution (100x Denhardts is 0.2% polyvinyl pyrrolidine, 0.2% BSA, 0.2% Ficoll), 50% Formamide and 120 μg/ml of heat denatured sonicated calf thymus DNA at 42°C for 4 h. Hybridization was in the same buffer with 100 ng of denatured radiolabeled probe (10^8 - 10^9 cpm/μg) at 42°C for 24 h. Membranes were subsequently washed at room temperature with four washes in 2x SSC, 0.1% SDS for 5 min, and once in 0.1x SSC for 30 min at 50°C if required. Membranes were wrapped in plastic wrap and autoradiographed using Cronex X-ray film in Kodak X-Omatic X-ray

cassettes with intensifying screens. The autoradiographs of the Northern blots were scanned using an LKB densitometer, and the values obtained for each point in the time-course were expressed as percentage of maximum expression. Errors for the densitometric scans of the northern were calculated using the following formula assuming the maximu error range of the instrument to be no greater than 5% of the maximum expression value obtained for each time course: % Error = (% max. expression x 5).

2.750 - Southern analysis

DNA samples, fractionated on agarose gels, were processed and transferred directly to nylon membranes (Genescreen Plus, NEN, Dupont, Lachine, Quebec) by capillary blotting using the protocol provided by the supplier. Prior to transfer the gels were soaked in 0.25 M HCl for 15 min, denatured in 1.5 M NaCl, 0.5 M NaOH for 30 min, and neutralized in 1.5 M NaCl, 0.5 M Tris-HCl pH 7.5, for 30 min. The transfer was carried out overnight using 20x SSC and the membrane was baked at 80°C under vaccuum for 2 h, or cross-linked with UV using a Strata-linker (254 nm, 30 sec, 1,200 μJoules) prior to prehybridization. In analysis of recombinant DNA the membranes were prehybridized in 6x SSPE, 5x Denhardt's solution, 0.5% SDS and 120 μg/ml of heat denatured sonicated calf thymus DNA at 65°C for 4 h. Hybridization was in the same buffer with 100 ng of denatured radiolabeled probe (10^8 - 10^9 cpm/μg) at 65°C for 24 h. In cases when the amount of target DNA was limiting, 10% dextran sulphate was added to both the prehybridization and hybridization solutions to enhance the degree of hybridization.

2.760 - Slot blot analysis

The Schleicher and Schuell slot blot apparatus (Keene, NH) was used for application of DNA and RNA samples to nylon membranes according to the manufacturer's instructions. The membranes were baked at 80°C for 2 h. Prehybridization, hybridization, wash conditions and autoradiography were performed as described in section 2.740 and section 2.750 for RNA and DNA respectively.

2.770 - Nucleic acid sequencing

Sequencing was performed by the chain termination method (Sanger et al., 1977) in the presence of [α^{35}S]-dATP and T7, SP6, or sequence specfic primers, using Sequenase™ (Tabor and Richardson, 1987). Samples were run on 5% Long-Ranger™ acrylamide (J.T. Baker, Phillipston, NJ), 8M urea, 1.2x TBE (10x TBE is 440 mM Tris-HCl pH 8.3, 440 mM boric acid, 200 mM EDTA) gels in 0.6x TBE buffer at 50W, 2000 V, and 30 mA for 2.5-5 h. The gels were lifted onto Whatman 3MM and dried at 80°C for 30 min on a BioRad Gel drier. Autoradiography was performed at room temperature with Cronex X-ray film in Kodak X-Omatic X-ray cassettes with intensifying screens. Computerized analysis of the sequences obtained and comparison with the EMBL, GenBank and Swiss-Prot databases was performed using PC-Gene™ computer software (Intelligenetics, Mountainview, CA).

2.780 - *In Situ* Hybridization

In situ hybridizations were performed as described by Lawrence and Singer (1986), with minor modifications (Rouleau et al., 1990). Freshly excised prostatic tissue was frozen in isopentane at -70°C, sectioned to a thickness of 8 µm and placed on silane treated slides (Rentrop et al., 1986). Frozen sections were fixed in 4% paraformaldehyde/PBS (0.14 M NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, 1.8 mM KH_2PO_4 pH 7.4) and then rehydrated for 10 min in PBS, 5 mM $MgCl_2$. Sections to be treated with RNase were rinsed briefly in 2x SSC, while experimental slides were placed in fresh PBS, 5 mM $MgCl_2$ until ready for the acetylation step. Negative controls were treated for 1 h at 37°C with 100 µl of RNase A (100 µg/ml). All slides (experimental and negative controls) were rinsed for 1 min in 70% ethanol, three times for 2 min in PBS, twice for 5 min in 2x SSC, and acetylated for 10 min at room temperature in 16 mM triethanolamine, 0.25% acetic anhydride pH 8. The sections were rinsed five times for 2 min in PBS, 10 mM DTT, twice for 5 min in 2x SSC, 10 mM DTT, and prehybridized for 1 h at room temperature in 40 µl 50% formamide, 2x SSC, 0.4% BSA, 30 mM DTT, 20 mM vanadyl ribonucleoside complex, 10 µg/ml heat denatured yeast tRNA and 200 µg/ml heat denatured salmon testes DNA. Hybridizations were performed at 42°C overnight with 40 µl of the above

mix supplemented with 10% dextran sulfate, and labeled probe ($1x10^6$ cpm/slide). The sections were washed twice for 30 min in 50% formamide, 2x SSC, 10 mM DTT at 50°C, three times for 20 min in 50% formamide, 1x SSC, 10 mM DTT at 50°C, three times for 20 min in 0.5x SSC, 10 mM DTT at room temperature. The slides were then dehydrated twice for 10 min in 70% ethanol and once for 5 min in 100% ethanol. The slides were dipped in nuclear track photographic emulsion, exposed for 4-6 days at 4°C, and were processed at 15°C as follows: 5 min in Kodak D-19 developer, 30 sec in 2% acetic acid, 5 min in Kodafix, and 30 min in running water. The slides were lightly counterstained with hematoxylin and eosin and examined on a Zeiss Axioskop microscope. Photographs were taken using Kodak Tmax 100 film for phase contrast images and Ilford PANF 50 film for the darkfield images. For each probe sections shown are from the same experiment, and were exposed for the same length of time, allowing direct comparison of the levels of expression at different times after castration.

To determine which cell type expressed the gene, each section has been analyzed to identify individual stromal and epithelial compartments. Between 3-5 sections for each probe were analyzed to determine the ratio of grains present over the epithelial cells versus the stromal cells.

2.800 - IMMUNOFLUORESCENCE

Frozen sections (8 μm) were air-dried before fixation in acetone at -20°C for 10 min. After rinsing in PBS, the tissue sections were incubated with undiluted donor horse serum (DHS) for 10 min at room temperature and subsequently with rabbit anti-rat cathepsin B (1:50 dilution in DHS) for 1 h at 37°C in a humidity chamber. The sections were rinsed with PBS and incubated in DHS for 10 min before incubation with fluorescein (FITC)-conjugated goat anti-rabbit IgG (1:50 dilution in DHS) for 40 min at room temperature. After washing 3 times for 5 min in PBS the tissue was mounted in 50% glycerol in PBS containing 1% p-phenylenediamine to reduce fading of the fluorescence.The sections were photographed on Kodak Ektachrome 400 film using a Zeiss Axioskop microscope equipped with epifluorescence.

2.900 - ANIMAL CARE

All procedures utilizing animals were approved by the University of Ottawa animal care committee and conformed to the standards required by the Canadian Council on Animal Care.

2.910 - Prostate gland

Male Sprague-Dawley rats, (250-300 g) were maintained in a controlled environment (14 h light, 10 h dark), and received Purina Rat Chow and water *ad libitum* The animals were castrated via the scrotal route under light halothane anaesthesia. Animals were sacrificed by cervical dislocation at different days after castration, and the prostate glands were excised and processed immediately. The groups varied in size from eight animals (normal controls) to thirty-eight animals (Day 4 after castration).

2.920 - Mammary gland

Timed pregnant female Sprague-Dawley rats were maintained in a controlled environment (14 h light: 10 h darkness), with Purina Rat Chow and water available *ad libitum*. The pups (7-16 pups per mother) were weaned between 14 and 24 days of age, when synthesis of β-casein is known to be maximal. The dams were killed in groups by cervical dislocation on different days after weaning, and the mammary glands were excised and processed immediately. The groups varied in size from three animals (lactating dams) to eight animals (Day 8 after weaning).

CHAPTER THREE

GENE EXPRESSION IN REGRESSING PROSTATE AND MAMMARY GLANDS

3.000 - INTRODUCTION

A preliminary set of studies was aimed at elucidating the expression levels of several genes that had already been characterized in other processes, but had the potential to be involved in ACD. Genes that are involved in ACD are termed thanatogens (from the Greek thanatos meaning death). Thanatogens fall into two broad categories; those that initiate the process (primary thanatogens), and those that ensure that the dying cell is fragmented and phagocytosed appropriately (secondary thanatogens) (Tenniswood et al., 1994). It is important to keep in mind that during the regression of hormone-dependent tissues such as the prostate and mammary gland, the steady-state levels of a number of mRNAs that are not involved in the apoptotic process may also increase in the cells that eventually undergo apoptosis. These include genes that may be induced in the dying epithelial cells, as part of a futile stress response mechanism after hormone ablation, and genes that are induced in a subset of the epithelial cells as part of a survival mechanism to ensure that the atrophied tissue can respond to restimulation by the trophic hormone.

Using Northern analysis and *in situ* hybridization, we have examined the expression of three genes during prostatic and mammary regression: tissue transglutaminase (tTGase), poly(ADP-ribose) polymerase (PARP) and heat shock protein 27 (Hsp27). Each of these genes may play a role in ACD, and they most likely represent primary or secondary thantogens remains to be determined. tTGase enzymatically cross-links proteins by the formation of ε-(γ-glutamyl)lysine bonds. Immunohistochemical methods have been used to localize the activity of tTGase to the apoptotic bodies produced from the fragmentation of dying hepatocytes after lead nitrate induced hyperplasia and subsequent liver regression (Fesus et al., 1987). PARP is a nuclear enzyme that is tightly bound to chromatin and catalyzes the poly(ADP)ribosylation of structural chromosomal proteins including histones, non-histones (Burzio et al., 1979; Poirier et al., 1982) and nuclear enzymes (Ohashi et al., 1983). PARP is highly stimulated by DNA strand breakage (Ferro and Olivera, 1982; Zahradka and Ebisuzaki, 1982) an event which occurs in apoptotic cells during the formation of a nucleosomal ladder after activation of an endogenous Ca^{2+}/Mg^{2+} endonuclease (Wyllie et al., 1986; Kyprianou et al.,1988b).

Hsp27 belongs to a family of proteins induced in response to environmental stress. It is induced in mammalian cells in the presence of arsenite and amino acid analogs as well as during hyperthermia (Crete and Landry, 1990; Welch, 1985). Following serum stimulation of growth, exposure to tumour promoters, or calcium ionophores there is an increase in the phosphorylation state of Hsp27 without increased synthesis of the protein (Welch, 1985). In chinese hamster O23 cells the induction of thermotolerance is correlated with an increased constitutive expression of the Hsp27 mRNA (Chretien and Landry, 1988), suggesting that Hsp 27 may serve a survival function in these cells.

3.100 - PROSTATE GLAND GENE EXPRESSION

3.110 - Expression of PSBP, TRPM-2 and α-tubulin After Castration

After castration, the steady state level of Prostate Steroid Binding Protein (PSBP) mRNA decreases steadily over the first four days (Fig. 3-1, panel A) and becomes essentially undetectable by day 6 (results not shown). The steady state level of TRPM-2 mRNA increases significantly over the same period, reaching a maximum on day 4 (Fig. 3-1, panel B) before declining to undetectable levels by day 8 after castration (results not shown), essentially in agreement with previous experiments (Heyns et al., 1977; Léger et al., 1987). The α-tubulin mRNA is expressed at a relatively low but easily detectable level in the rat ventral prostate, however the steady state levels do not change significantly after castration (Fig. 3-2, panels A and B). The α-tubulin mRNA is expressed in the epithelial cells in the rat ventral prostate (Fig. 3-3). Since the levels of α-tubulin do not appear to alter significantly during regression, and since the gene is expressed in the epithelial cells we have compared the expression of tTGase, PARP, and Hsp27 mRNAs against the expression of α-tubulin mRNA as observed by hybridization to duplicate nylon filters to correct for variations in loading.

Figure 3-1: Expression of PSBP and TRPM-2 in the Ventral Prostate After Castration.

Mature male adult rats were castrated and sacrificed on the days indicated. Poly(A)+ RNA was isolated from the rat ventral prostate as described in Material and Methods. 2 μg poly(A)+ RNA was loaded per lane, electrophoresed on formaldehyde gels, transferred to nylon membranes, and hybridized to radiolabeled cDNA probes ($1x10^8$ cpm/μg) specific for PSBP (panel A) exposed for 4 h; and TRPM-2 (panel B) exposed for 18 h. The positions of the 500 nucleotide PSBP mRNA and 1,900 nucleotide TRPM-2 mRNA were established by comparison to the migration of 18S and 28S ribosomal RNA.

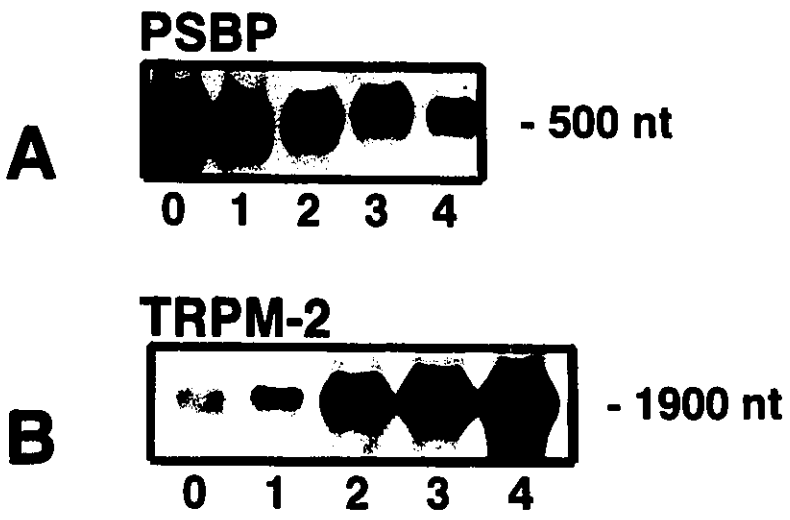

PSBP
A
- 500 nt
0 1 2 3 4
TRPM-2
B
- 1900 nt
0 1 2 3 4

Figure 3-2: Expression of α-tubulin mRNA in the Ventral Prostate After Castration

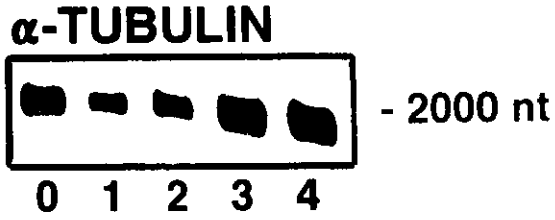
Panel A: Northern Analysis of α-tubulin Steady State mRNA Levels

Poly(A)+ RNA was extracted from the rat ventral prostate at different times after castration and 2 μg per lane were electrophoresed on formaldehyde gels, transferred to Nylon membranes, and hybridized to ^{32}P-labeled cDNA specific for α-tubulin ($1x10^8$ cpm/μg). The membranes were exposed for 18 h. The position of the 2,000 nucleotide α-tubulin mRNA was established by comparison to the migration of 18S and 28S ribosomal RNA.

Panel B: Bar graph Showing Changes in Steady State Levels of α-tubulin mRNA in the Prostate After Castration.

The autoradiographs of the Northern blots were scanned using an LKB densitometer, and the values obtained for each point in the time-course were expressed as percentage of maximum expression.

A


α-TUBULIN
- 2000 nt
0 1 2 3 4

B

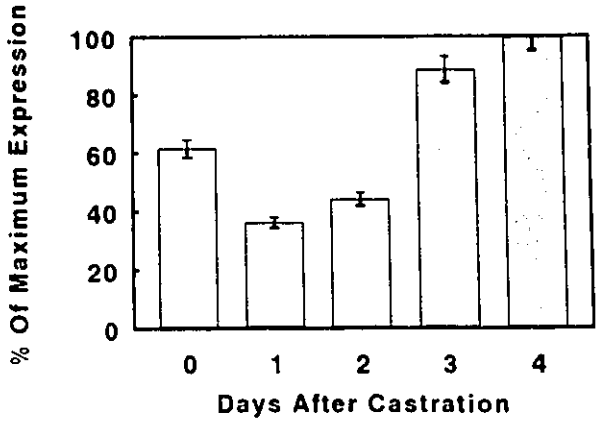

% Of Maximum Expression
100
80
60
40
20
0
0 1 2 3 4
Days After Castration

Figure 3-3: *In Situ* Hybridization of α-tubulin mRNA in the Ventral Prostate After Castration

Random sections from the rat ventral prostate excised at different times after castration were hybridized to ^{35}S-labeled cDNA insert specific for α-tubulin ($1x10^6$ cpm). The sections were exposed for 4 days. Magnification X20.

Panels A, C, E:	phase contrast showing morphology of sections
Panels B, D, F:	dark field illumination showing *in situ* hybridization
Panels A and B:	prostate from control animals
Panels C and D:	prostate 2 days after castration
Panels E and F:	prostate 4 days after castration

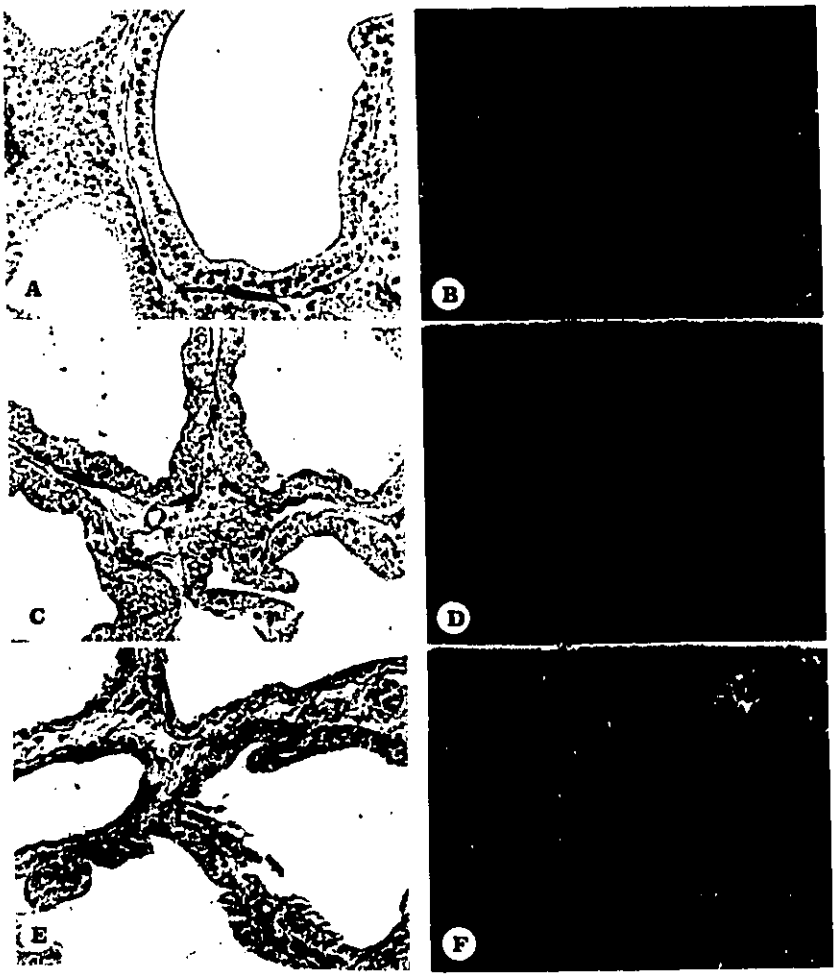

A
B
C
D
E
F

Figure 3-4: Expression of tTGase mRNA in the Ventral Prostate After Castration

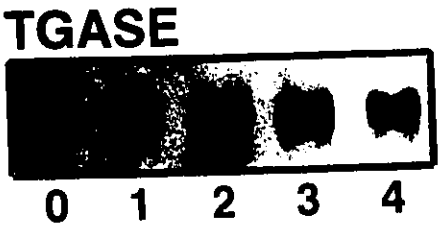
Panel A: Northern Analysis of tTGase Steady State mRNA Levels

Poly(A)+ RNA was extracted from the rat ventral prostate at different times after castration and 2 μg per lane were electrophoresed on formaldehyde gels, transferred to Nylon membranes, and hybridized to ^{32}P-labeled cDNA specific for tTGase ($1x10^8$ cpm/μg). The membranes were exposed for 7 days. The position of the 4,000 nucleotide tTGase mRNA was established by comparison to the migration of 18S and 28S ribosomal RNA.

Panel B: Bar graph Showing Changes in Steady State Levels of tTGase mRNA in the Prostate After Castration.

The autoradiographs of the Northern blots were scanned using an LKB densitometer, and the values obtained for each point in the time-course were expressed as percentage of maximum expression.

A


TGASE
- 4000 nt
0 1 2 3 4

B

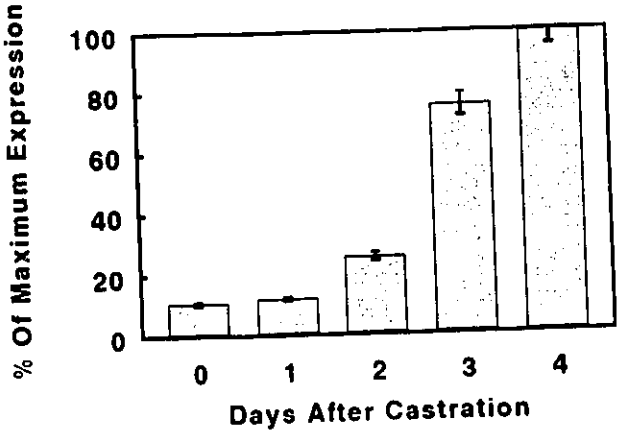

% Of Maximum Expression
100
80
60
40
20
0
0 1 2 3 4
Days After Castration

Figure 3-5: *In Situ* Hybridization of tTGase mRNA in the Ventral Prostate After Castration

Random sections from the rat ventral prostate excised at different times after castration were hybridized to ^{35}S-labeled cDNA insert specific for tTGase ($1x10^6$ cpm). The sections were exposed for 4 days. Magnification X20.

Panels A, C:	phase contrast showing morphology of sections
Panels B, D:	dark field illumination showing *in situ* hybridization
Panels A and B:	prostate from control animals
Panels C and D:	prostate 4 days after castration

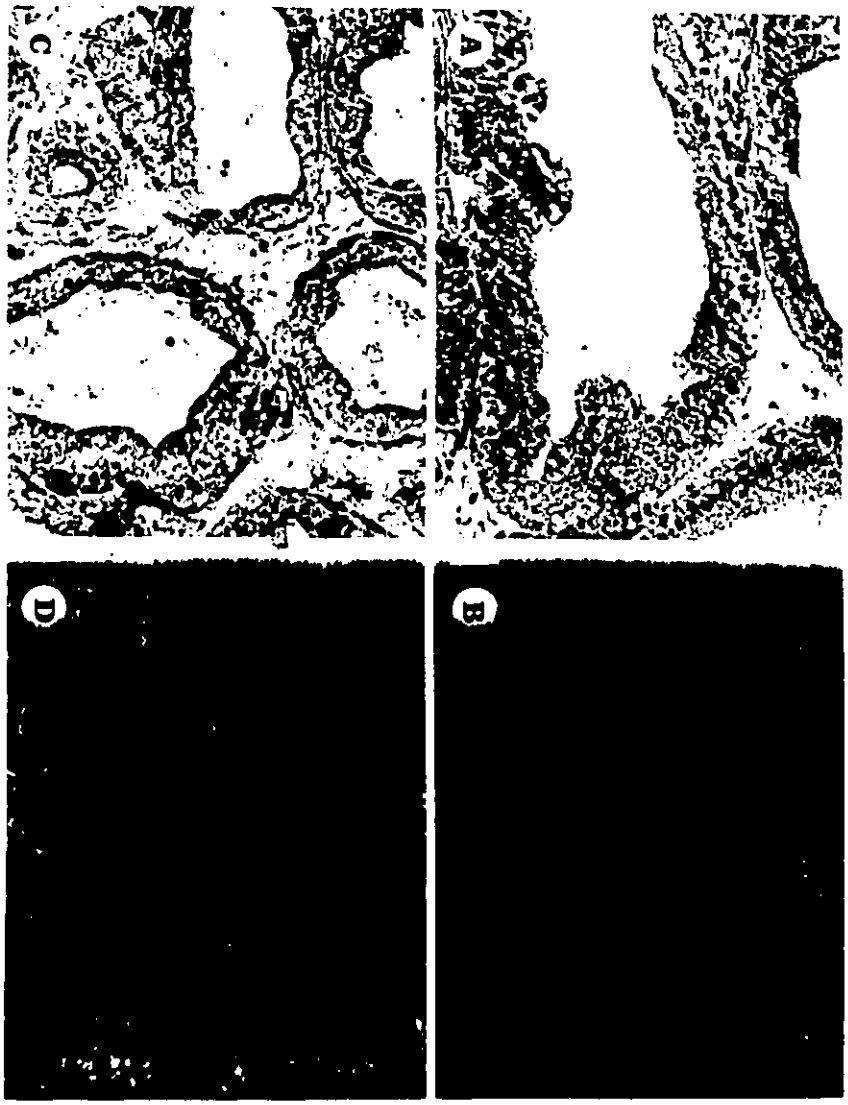

C
A
D
B

Figure 3-6: Expression of PARP mRNA in the Ventral Prostate After Castration

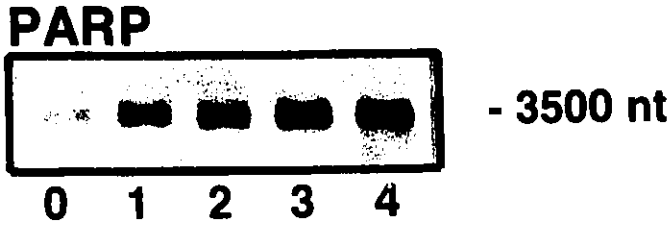
Panel A: Northern Analysis of PARP Steady State mRNA Levels

Poly(A)+ RNA was extracted from the rat ventral prostate at different times after castration and 2 μg per lane were electrophoresed on formaldehyde gels, transferred to Nylon membranes, and hybridized to ^{32}P-labeled cDNA specific for PARP ($1x10^8$ cpm/μg). The membranes were exposed for 4 h. The position of the 3,500 nucleotide PARP mRNA was established by comparison to the migration of 18S and 28S ribosomal RNA.

Panel B: Bar graph Showing Changes in Steady State Levels of PARP mRNA in the Prostate After Castration.

The autoradiographs of the Northern blots were scanned using an LKB densitometer, and the values obtained for each point in the time-course were expressed as percentage of maximum expression.

A


PARP
- 3500 nt
0 1 2 3 4

B

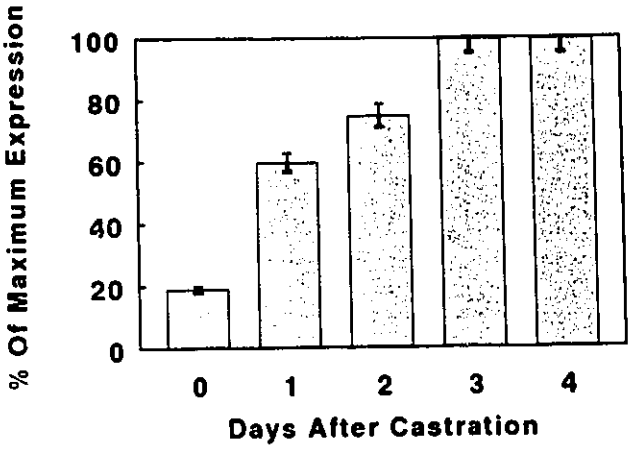

% Of Maximum Expression
100
80
60
40
20
0
0 1 2 3 4
Days After Castration

Figure 3-7: *In Situ* Hybridization of PARP mRNA in the Ventral Prostate After Castration

Random sections from the rat ventral prostate excised at different times after castration were hybridized to ^{35}S-labeled cDNA insert specific for PARP ($1x10^6$ cpm). The sections wereexposed for 4 days. Magnification X20.

Panels A, C, E:	phase contrast showing morphology of sections
Panels B, D, F:	dark field illumination showing *in situ* hybridization
Panels A and B:	prostate from control animals
Panels C and D:	prostate 2 days after castration
Panels E and F:	prostate 4 days after castration

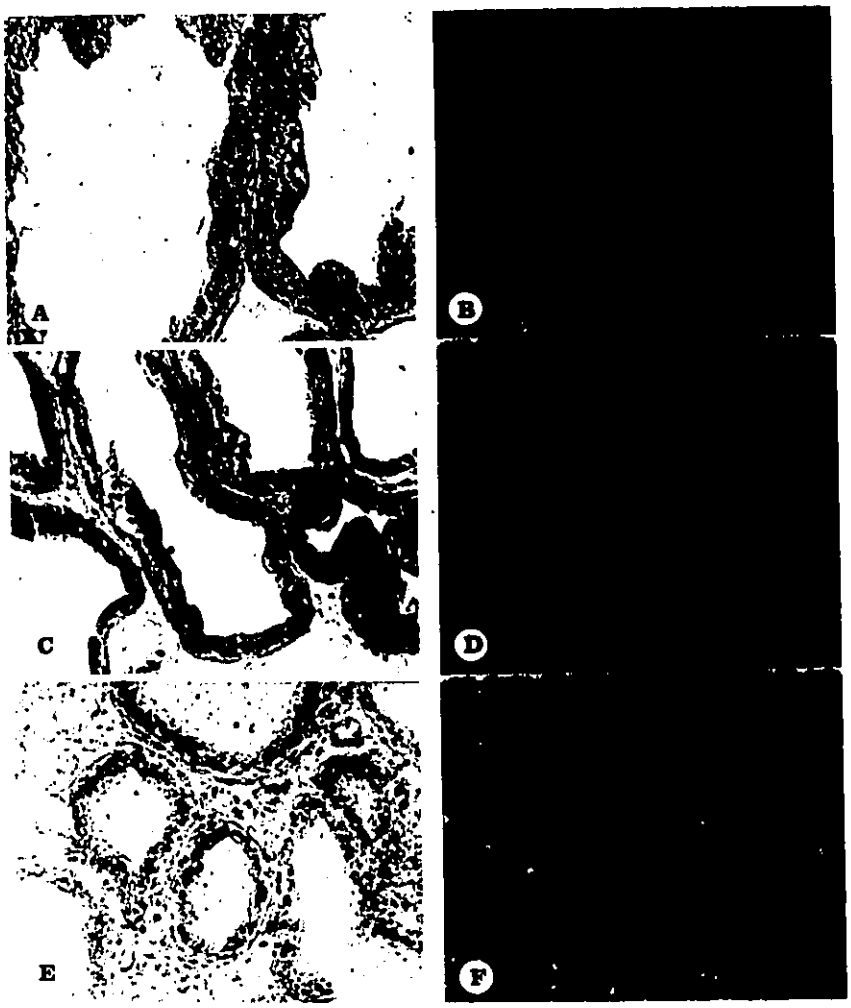

A
B
C
D
E
F

Figure 3-8: Expression of Hsp 27 mRNA in the Ventral Prostate After Castration

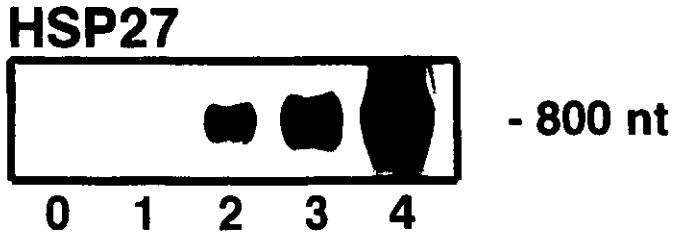
Panel A: Northern Analysis of Hsp 27 Steady State mRNA Levels

Poly(A)+ RNA was extracted from the rat ventral prostate at different times after castration and 2 μg per lane were electrophoresed on formaldehyde gels, transferred to nylon membranes, and hybridized to ^{32}P-labeled cDNA specific for Hsp 27 ($1x10^8$cpm/μg). The membranes were exposed for 18 h. The position of the 800 nucleotide Hsp27 mRNA was established by comparison to the migration of 18S and 28S ribosomal RNA.

Panel B: Bar graph Showing Changes in Steady State Levels of Hsp 27 mRNA in the Prostate After Castration.

The autoradiographs of the Northern blots were scanned using an LKB densitometer, and the values obtained for each point in the time-course were expressed as percentage of maximum expression.

A


HSP27
- 800 nt
0 1 2 3 4

B

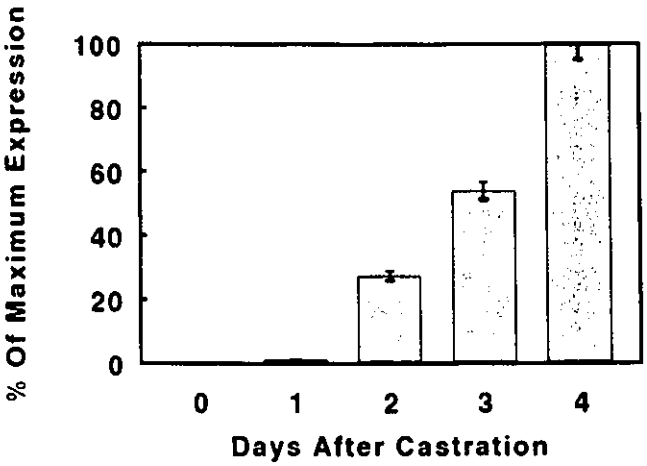
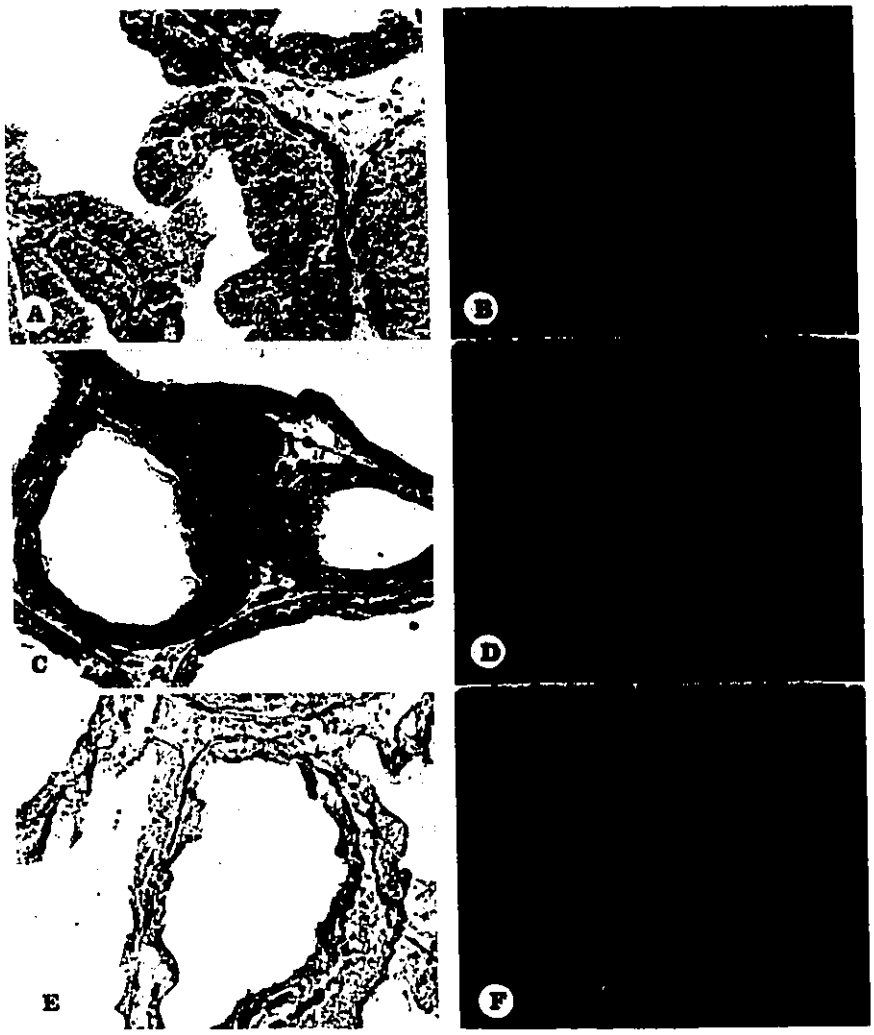

% Of Maximum Expression
100
80
60
40
20
0
0 1 2 3 4
Days After Castration

Figure 3-9: *In Situ* hybridization of Hsp 27 mRNA in the Ventral Prostate After Castration

Random sections from the rat ventral prostate excised at different times after castration were hybridized to ^{35}S-labeled cDNA insert specific for Hsp 27 ($1x10^6$ cpm). The sections were exposed for 4 days. Magnification X20.

Panels A, C, E:	phase contrast showing morphology of sections
Panels B, D, F:	dark field illumination showing *in situ* hybridization
Panels A and B:	prostate from control animals
Panels C and D:	prostate 2 days after castration
Panels E and F:	prostate 4 days after castration


A
B
C
D
E
F

3.120 - Expression of tTGase, PARP and Hsp27 After Castration

Tissue transglutaminase mRNA is detectable in the prostate prior to castration (Fig. 3-4, panels A and B), and increases significantly between days 2 and 4 after castration. *In situ* hybridization confirms that the mRNA is expressed at low levels in the epithelial cells of the prostate prior to castration and that the increase in the expression of the mRNA is localized to the epithelial cells of the gland (Fig. 3-5).

The expression of PARP mRNA in the prostate prior to castration is very limited, however the steady state levels increases substantially by day one after castration and the steady state level of the mRNA remains elevated for the next three days after castration (Fig. 3-6 panels A and B). As shown in Fig. 3-7 the gene is expressed in the epithelial cell compartment of the prostate after castration.

Hsp27 mRNA is not detectable in the prostate prior to castration or on day 1 after castration. The expression of the mRNA increases dramatically between days 2 and 4 after castration (Fig. 3-8 panels A and B). *In situ* hybridization confirms that the gene is induced in the prostate 4 days after castration, in the epithelial cells of the gland (Fig. 3-9).

With all of the probes used greater than 90% of the grains were attributed to hybridization in the epithelial cells. However it must be kept in mind that in all the sections there are ducts that have been sectioned at oblique angles, making it difficult to unequivocally assign the hybridization to one tissue compartment (see Appendix - In-Situ Quantification).

3.200 - MAMMARY GLAND GENE EXPRESSION

3.210 - Expression of β-casein and TRPM-2 After Weaning

To compare the expression of genes associated with lactation with ACD-related functions, Northern blots of poly(A)+ RNA extracted from the mammary gland at the indicated times after weaning were analysed to determine the relative steady-state levels of β-casein, TRPM-2, tTGase, PARP, and Hsp 27 mRNAs.

Figure 3-10: Expression of β-casein mRNA in the Mammary Gland After Weaning

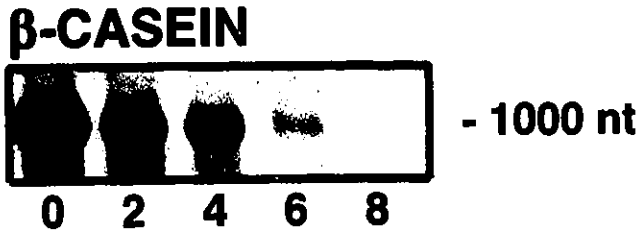
Panel A: Northern Analysis of β-casein Steady State mRNA Levels

Poly(A)+ RNA was extracted from the rat mammary gland at different times (0, 2, 4, 6, and 8 days) after weaning and 2 μg per lane were electrophoresed on formaldehyde gels, transferred to nylon membranes, and hybridized to a ^{32}P-labeled cDNA specific for β-casein ($1x10^6$ cpm/μg). The membranes were exposed for 18 h. The position of the 1,000 nucleotide β-casein mRNA was established by comparison to the migration of 18S and 28S ribosomal RNA.

Panel B: Bar Graph Showing Changes in Steady-State Levels of β-casein mRNA in the Mammary Gland After Weaning

The autoradiographs of the Northern blots were scanned using an LKB densitometer, and the values obtained for each point in the time-course were expressed as percentage of maximum expression.

A


β-CASEIN
- 1000 nt
0 2 4 6 8

B

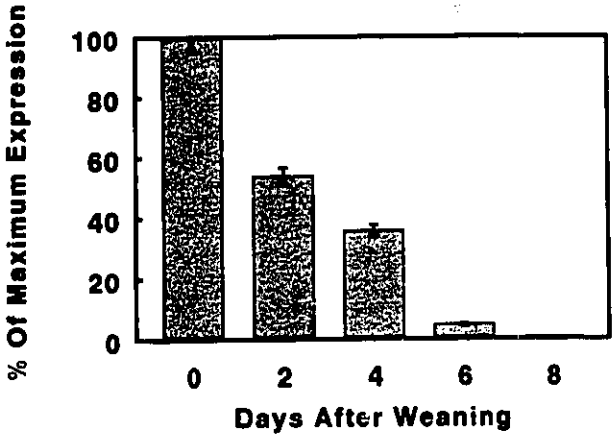

% Of Maximum Expression
100
80
60
40
20
0
0 2 4 6 8
Days After Weaning

Figure 3-11: *In Situ* Hybridization of β-casein mRNA

Random sections from the rat mammary gland excised at different times after weaning were hybridized to a ^{35}S-labeled cDNA insert specific for β-casein ($1x10^6$ cpm). The sections were exposed for 4 days. Magnification X20.

Panels A, C, E:	phase contrast showing morphology of sections
Panels B, D, F:	dark field illumination showing *in situ* hybridization
Panels A and B:	mammary glands from lactating dams
Panels C and D:	mammary gland 2 days after weaning
Panels E and F:	mammary gland 4 days after weaning

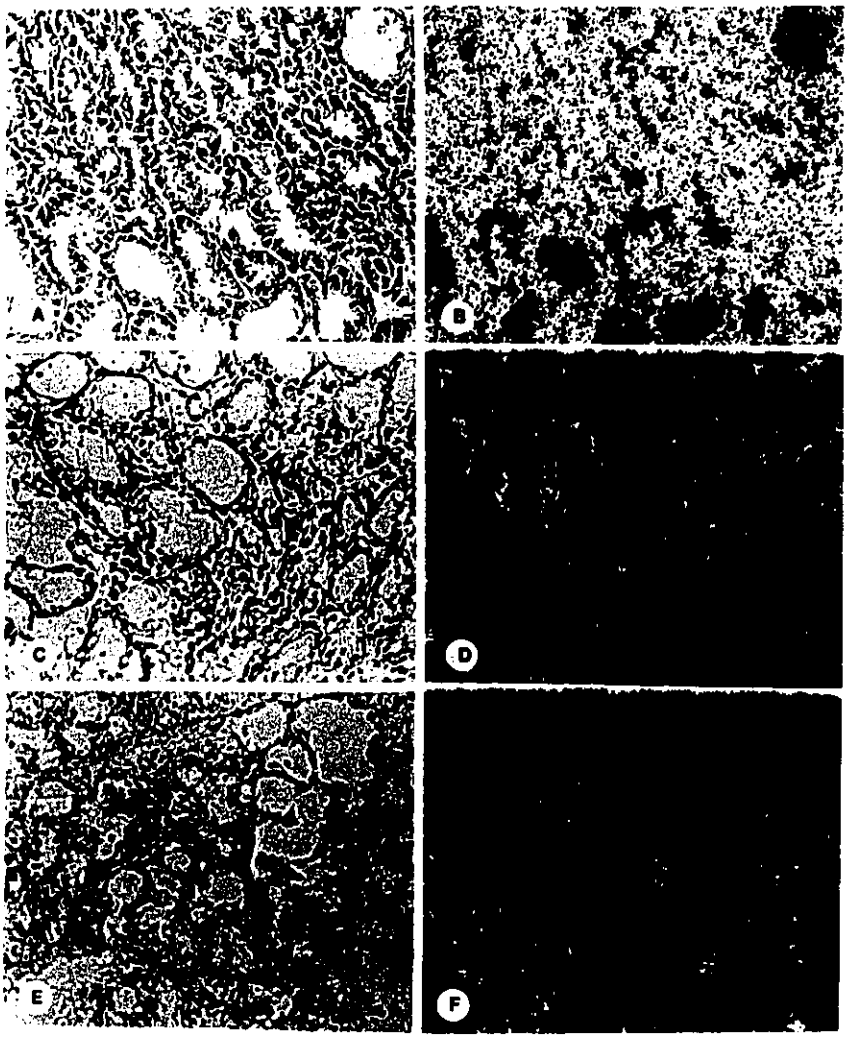

A
B
C
D
E
F

Figure 3-12: Expression of TRPM-2 mRNA in the Mammary Gland After Weaning

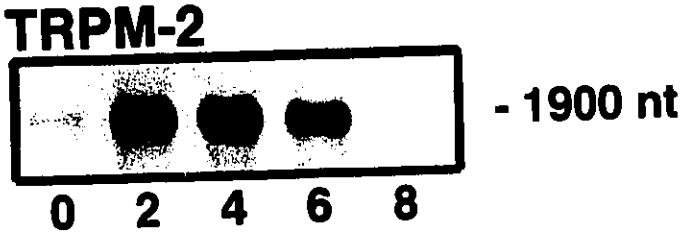
Panel A: Northern Analysis of TRPM-2 Steady State mRNA Levels

Poly(A)+ RNA was extracted from the rat mammary gland at different times (0, 2, 4 , 6 and 8 days) after weaning and 2 μg per lane were electrophoresed on formaldehyde gels, transferred to nylon membranes, and hybridized to a ^{32}P-labeled cDNA specific for TRPM-2 ($1x10^8$ cpm/μg). The membranes were exposed for 18 h. The position of the 1,900 nucleotide TRPM-2 mRNA was established by comparison to the migration of 18S and 28S ribosomal RNA.

Panel B: Bar Graph Showing Changes in Steady-State Levels of TRPM-2 mRNA in the Mammary Gland After Weaning

The autoradiographs of the Northern blots were scanned using an LKB densitometer, and the values obtained for each point in the time-course were expressed as percentage of maximum expression.

A


TRPM-2
- 1900 nt
0 2 4 6 8

B

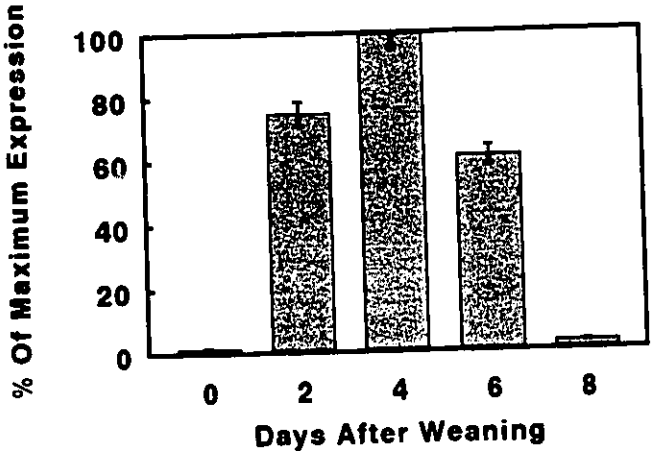
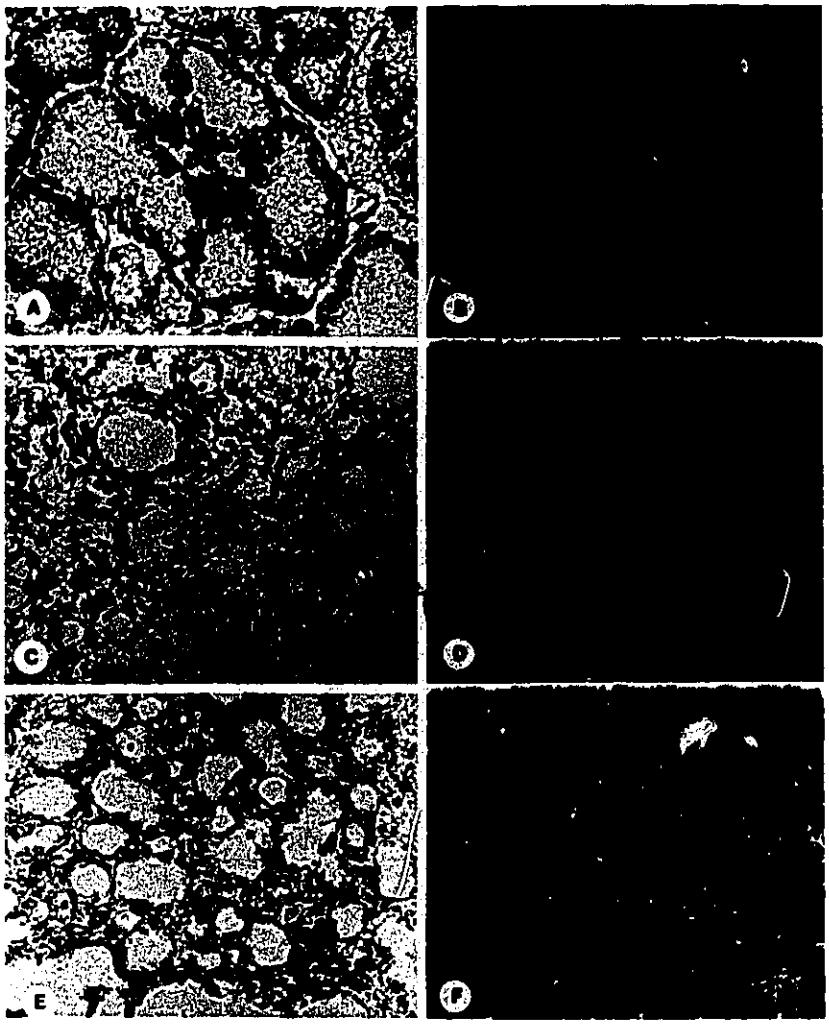

% Of Maximum Expression
100
80
60
40
20
0
0 2 4 6 8
Days After Weaning

Figure 3-13: *In Situ* Hybridization of TRPM-2 mRNA in the Mammary Gland After Weaning

Random sections from the rat mammary gland excised at different times after weaning were hybridized to a ^{35}S-labeled cDNA insert specific for TRPM-2 ($1x10^6$ cpm). The sections were exposed for 4 days. Magnification X20.

Panels A, C, E:	phase contrast showing morphology of sections
Panels B, D, F:	dark field illumination showing *in situ* hybridization
Panels A and B:	mammary glands from lactating dams
Panels C and D:	mammary gland 2 days after weaning
Panels E and F:	mammary gland 4 days after weaning


A
B
C
D
E
F

The steady-state level of β-casein mRNA declined steadily from the start of weaning, to less than 10% of the values seen during lactation by day 6 after the pups were removed, and the mRNA was not detected 8 days after weaning (Fig. 3-10, panels A and B). β-casein mRNA was expressed at very high levels in the luminal epithelial cells of the lactating mammary gland (Fig. 3-11, panel B). After weaning the expression of β-casein diminished in the luminal epithelium, and by days 2 and 4 the level of hybridization had decreased very significantly in the remaining luminal epithelial cells (Fig. 3-11, panels D and F). In keeping with the results of Northern analysis, by day 8 after weaning the level of β-casein mRNA present in the luminal epithelium was not distinguishable from the level of background hybridization (not shown).

TRPM-2 mRNA, on the other hand, was barely detectable in the lactating mammary gland prior to weaning but was expressed at significant levels between days 2 and 4 of involution (Fig. 3-12, panels A and B). The level of TRPM-2 mRNA diminished by day 6 of weaning and was barely detectable by day 8. *In situ* hybridization using the TRPM-2 cDNA probe showed that the gene was not expressed above background levels in any of the cell types present in the lactating duct (Fig. 3-13, panel B). On days 2 and 4 after weaning TRPM-2 mRNA was readily detectable in the luminal epithelial cells, while the level of hybridization in the stromal and endothelial cells was considerably lower (Fig. 3-13, panels D and F) . Even after 8 days of regression there were a few surviving luminal epithelial cells that expressed low levels of TRPM-2 mRNA (not shown). The hybridization of the TRPM-2 specific probe over the stromal cells was not significantly above background during the time-frame of the experiment. It was not possible to determine whether there was specific hybridization over the myoepithelial components of the mammary ducts, due to their relative scarcity and close proximity to the epithelial cells, which expressed high levels of TRPM-2 mRNA.

Figure 3-14: Expression of tTgase mRNA in the Mammary Gland After Weaning

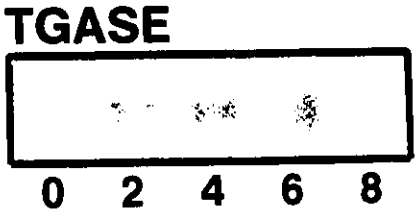
Panel A: Northern Analysis of tTgase Steady State mRNA Levels

Poly(A)+ RNA was extracted from the rat mammary gland at different times (0, 2, 4, 6 and 8 days) after weaning and 2 μg per lane were electrophoresed on formaldehyde gels, transferred to nylon membranes, and hybridized to a ^{32}P-labeled cDNA specific for TGase ($1x10^8$ cpm/μg). The membranes were exposed for 7 days. The position of the 4,000 nucleotide tTGase mRNA was established by comparison to the migration of 18S and 28S ribosomal RNA

Panel B: Bar Graph Showing Changes in Steady-State Levels of tTgase mRNA in the Mammary Gland After Weaning

The autoradiographs of the Northern blots were scanned using an LKB densitometer, and the values obtained for each point in the time-course were expressed as percentage of maximum expression.

A


TGASE
- 4000 nt
0 2 4 6 8

B

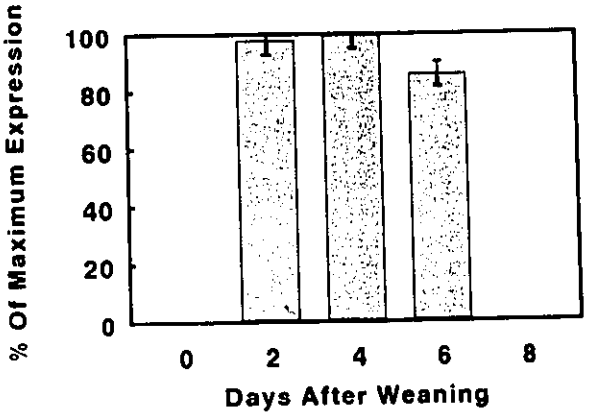

% Of Maximum Expression
100
80
60
40
20
0
0 2 4 6 8
Days After Weaning

Figure 3-15: *In Situ* Hybridization of tTgase mRNA in the Mammary Gland After Weaning

Random sections from the rat mammary gland excised at different times after weaning were hybridized to a ^{35}S-labeled cDNA insert specific for tTGase ($1x10^6$ cpm). The sections were exposed for 4 days. Magnification X20.

Panels A, C:	phase contrast showing morphology of sections
Panels B, D:	dark field illumination showing *in situ* hybridization
Panels A and B:	mammary glands from lactating dams
Panels C and D:	mammary gland 2 days after weaning

B
D
A
C

3.220 - Expression of tTGase, PARP and Hsp27 After Weaning

The steady-state level of tTGase mRNA was below the level of detection by Northern analysis in the lactating mammary gland, but increased significantly by day 2 after weaning. tTGase mRNA was present at low but readily detectable levels, in the regressing gland between days 2 and 6, but was not detectable in the mammary gland 8 days after weaning (Fig. 3-14, panels A and B). *In situ* hybridization showed that very low levels of tTGase mRNA could be detected in the secretory epithelium of the lactating mammary gland (Fig. 3-15, panel B). The increased expression of the gene on day 2 after weaning appeared to be restricted to the luminal epithelial cells of the mammary ducts (Fig. 3-15, panel D)

The steady state level of PARP mRNA in the mammary gland increased significantly after weaning, from barely detectable levels in the lactating gland to a maximum at 4 days after weaning, before decreasing rapidly so that by day 8 after weaning the PARP transcript was no longer detectable (Fig. 3-16, panels A and B). PARP mRNA was localized in the regressing epithelium of the mammary gland, and did not appear to be expressed in the stromal cells surrounding the epithelium (Fig. 3-17, panels D and F).

The expression of Hsp 27 mRNA was not detectable in the regressing mammary gland. *In situ* hybridization also failed to detect any evidence of Hsp 27 expression in the lactating or regressing mammary gland (results not shown).

With all of the probes used greater than 90% of the grains were attributed to hybridization in the epithelial cells. However it must be kept in mind that in all the sections there are ducts that have been sectioned at oblique angles, making it difficult to unequivocally assign the hybridization to one tissue compartment (see Appendix - In-Situ Quantification).

Figure 3-16: Expression of PARP mRNA in the Mammary Gland After Weaning

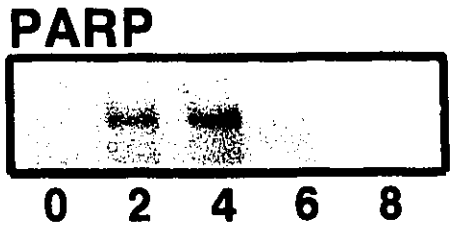
Panel A: Northern Analysis of PARP Steady State mRNA Levels

Poly(A)+ RNA was extracted from the rat mammary gland at different times (0, 2, 4, 6, and 8 days) after weaning and 2 μg per lane were electrophoresed on formaldehyde gels, transferred to nylon membranes, and hybridized to a ^{32}P-labeled cDNA specific for PARP ($1x10^8$ cpm/μg). The membranes were exposed for 2 days. The position of the 3,500 nucleotide PARP mRNA was established by comparison to the migration of 18S and 28S ribosomal RNA.

Panel B: Bar Graph Showing Changes in Steady-state Levels of PARP mRNA in the Mammary Gland After Weaning

The autoradiographs of the Northern blots were scanned using an LKB densitometer, and the values obtained for each point in the time-course were expressed as percentage of maximum expression.

A


PARP
- 3500 nt
0 2 4 6 8

B

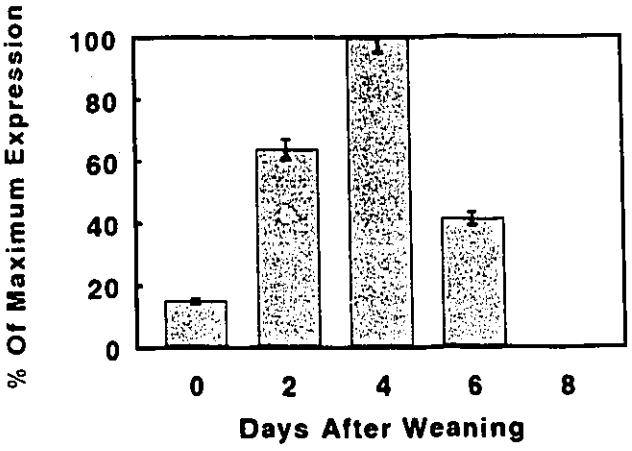

% Of Maximum Expression
100
80
60
40
20
0
0 2 4 6 8
Days After Weaning

Figure 3-17: *In Situ* Hybridization of PARP mRNA in the Mammary Gland After Weaning

Random sections from the rat mammary gland excised at different times (0, 2, 4, 6 and 8 days) after weaning were hybridized to a ^{35}S-labeled cDNA insert specific for PARP ($1x10^6$ cpm). The sections were exposed for 4 days. Magnification X20.

Panels A, C, E:	phase contrast showing morphology of sections
Panels B, D, F:	dark field illumination showing *in situ* hybridization
Panels A and B:	mammary glands from lactating dams
Panels C and D:	mammary gland 2 days after weaning
Panels E and F:	mammary gland 4 days after weaning

A
B
C
D
E
F

3.300 - DISCUSSION

The regression of the rat ventral prostate after castration is known to occur as a result of apoptotic cell death which occurs in approximately 90% of the epithelial cells of the gland within a 6 to 8 day period following castration (Sandford et al., 1984; English et al., 1985). It is now well established that the activity of a number of proteins increases during the regression of the gland, including cathepsin D, urokinase and tissue type plasminogen activators and RNase (Lee, 1981; Sensibar et al., 1990; Freeman et al., 1990). In addition a number of castration induced mRNA have been identified in the regressing prostate (Montpetit et al., 1986; Lee and Sensibar, 1987) and a number of these have now been cloned, including TRPM-2 (clusterin, SGP-2) (Léger et al., 1987; Wong et al., 1993a), RVP-1 (Briehl and Miesfeld, 1991) and the Yb sub unit of glutathione-S-transferase (Chang et al., 1987). The increase in the steady state levels of these genes and a number of others including p53, Rb, fos, and Hsp 70 has led to the suggestion that they are involved in the control of ACD (Buttyan et al., 1988; Colombel et al., 1992). However, as has been recently pointed out, increases in the steady state levels of mRNA in the regressing tissue, even if the expression is localized to the dying epithelium, need to be interpreted with caution, since transcription of these genes may be induced (or their steady state mRNA levels increased) for a variety of reasons that are not associated with the apoptotic process (Tenniswood et al., 1994). For example, genes may be induced in the dying cells as part of a futile stress response, or as part of a survival mechanism in cells that do not undergo ACD.

Indeed before genes can be categorized as primary or secondary thanatogens, corroborative evidence needs be obtained in other tissues, and a functional role in the apoptotic process needs to be firmly established. In our current experiments we have investigated the induction of three potential thanatogens in the rat ventral prostate after castration and mammary gland after weaning. Tissue transglutaminase has been implicated in ACD in the liver (Fesus and Thomazy, 1988) and thymocytes (Fesus et al., 1991a), and the product of the tTGase activity, the ε-(γ-glutamyl)lysine bonds, have been specifically identified in apoptotic bodies suggesting that tTGase activity is required to ensure the

increased rigidity of the apoptotic bodies (Fesus et al., 1989; Fesus et al., 1991b). In the prostate, tTGase mRNA is induced in the epithelial cells with kinetics that are very similar to those seen for TRPM-2 mRNA induction. Thus tTGase appears to be required for the normal completion of ACD, but is unlikely to be involved in the induction of the process, and as such represents a secondary thanatogen. To date tTGase appears to be the only gene (or gene product) that is required for all forms of ACD.

The induction of poly(ADP-ribose) polymerase mRNA in the epithelial cells of both the prostate and mammary gland after hormone ablation suggests that PARP is also a secondary thanatogen. However in glucocorticoid or calcium ionophore treated thymocytes (McConkey et al., 1989a; Hoshino et al., 1992; Ucker et al., 1992), PARP induction appears to be a futile attempt to repair the double strand DNA breaks introduced by the Ca^{2+}/Mg^{2+} dependent endonuclease (Cleaver and Morgan, 1991). The role of the poly(ADP-ribose) polymerase is not firmly resolved since the induction of poly(ADP-ribose) polymerase in the liver appears to be required for endonuclease activation (Jones et al., 1989), while in activated T-lymphocytes ADP-ribosylation of the endnuclease appears to suppress endonuclease and topoisomerase activity (Rice et al., 1992; Ferro et al., 1983). Because the PARP enzyme is activated by DNA strand breaks and is known to modify several of the enzymes involved in DNA repair, it is likely that the induction of PARP is an attempt by the dying cell to repair the damage caused by nuclease activation (Ohashi et al., 1983; Ueda and Hayaishi, 1985).

The induction of Hsp 27 mRNA in the epithelial cells of the prostate following hormone ablation is particularly interesting since this mRNA is induced with kinetics that are distinctly different from those seen for TRPM-2, tTGase and PARP mRNAs. Hsp27 mRNA is not detectable in the prostate before castration nor for the first 24 hours after castration. The gene is highly induced in the epithelial cells of the prostate between 24 and 48 hours and is expressed at elevated levels by day four after castration. This distinct time course of expression appears to represent a different response to castration, and may be either a futile response or be part of a survival strategy of a subset of the epithelial cells in

the gland. Since random sections were used for *in situ* hybridization it is not possible to determine whether the expression of the Hsp 27 gene is restricted to a particular subset of epithelial cells in the gland. However it is of interest that the Hsp 27 mRNA is not expressed in the lactating mammary gland after weaning suggesting that the expression of the gene may not occur in all tissues undergoing ACD. The secretory epithelium of the lactating mammary gland is presumably programmed to undergo ACD in response to the decline in the levels of trophic hormones that accompany weaning. Since the fluctuations in circulating androgen levels in the male are not profound, it is probable that the prostatic epithelium is not programmed to respond to massive changes in the trophic hormone in the same way, in which case the changes in Hsp27 mRNA levels may represent a futile stress response. It will be necessary to examine the expression of Hsp27 in other models of ACD to establish the relevance of this response in ACD.

The results presented here demonstrate that in addition to TRPM-2, several other genes are expressed in the prostatic and mammary epithelial cells that undergo ACD after castration and weaning respectively. The role of the gene products in ACD remains to be fully elucidated. While it is likely that tissue transglutaminase is a secondary thanatogen that is required for the completion of the apoptotic process, it is quite probable that Hsp27 and poly(ADP-ribose) polymerase represent either stress responses or futile responses to hormonal ablation.

CHAPTER FOUR

CROSS-SCREENING STRATEGY FOR THE ISOLATION OF REGRESSION SELECTED GENES (RSG)

4.000 - INTRODUCTION

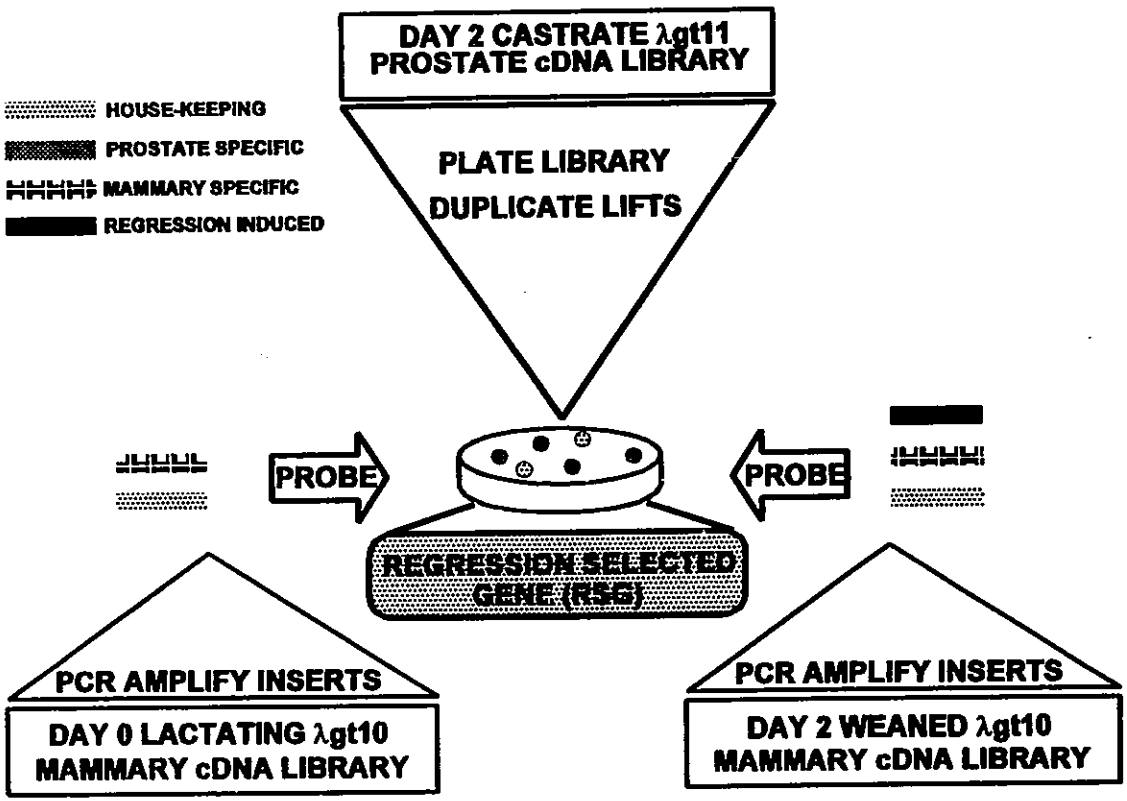
Within any particular cell, apoptosis or active cell death (ACD) occurs over a 3-4 hour time period. Tissue regression, on the other hand may last for several days. The induction of ACD in the individual cells of the regressing tissues is not synchronous, either due to the variability of the precondensation phase or due to the differential sensitivity of the cells to the induction stimulus. This greatly hampers the analysis of gene expression and the isolation of novel sequences that are involved in ACD.

To isolate sequences other than TRPM-2 that are expressed *de novo* during regression (and therefore are probably involved in apoptosis) we utilized a new strategy to cross screen lambda based cDNA libraries (Fig. 4-1) (Wong et al., 1989). The strategy is based on the fact that the cloning sites in the λ based vectors (in this case λgt10 and λgt11) are different. It is therefore possible to amplify the cDNA inserts in an entire λgt10 library, using the forward and reverse λgt10 sequencing primers and the polymerase chain reaction (PCR), and to screen an entire λgt11 library with the PCR amplified inserts. Since the λgt10 primers do not cross hybridize with the λgt11 arms, plaques that cross hybridize harbor sequences that are common to both libraries.

Thus by screening a λgt11 library prepared from regressing prostate with the PCR amplified cDNA inserts from a λgt10 library prepared from regressing mammary gland, sequences that are common to both regressing tissues will be identified. One of the major advantages of this method is that even rare mRNA species are sufficiently amplified to allow hybridization to plaques harboring homologous sequences. Plaques that show positive hybridization represent sequences that are expressed in both libraries. These may represent either constitutive sequences that are present in both tissues before and after hormonal manipulation, or sequences that are expressed in both regressing tissues after hormonal ablation. A second screen with amplified cDNA inserts from a λgt10 library constructed from normal mammary gland can be used to remove sequences expressed before hormonal manipulation. Clones which are up-regulated or expressed *de-novo* in

Figure 4-1: Cross-screening strategy For the Isolation of Regression Selected Genes (RSG)

Approximately 10,000 plaques from the VPR2-11 cDNA library were plated out and duplicate plaque lifts were made from these plates. Inserts from the MGR2-10 and MGC-10 libraries were PCR amplified, radio-labeled and used to screen the plaque lifts. Clones which were MGR2-10^{+} / MGC-10^{-} were cored and isolated. Inserts of the positive clones were PCR amplified, radio-labeled and used for northern analysis of regressing prostate and mammary gland poly(A)+ RNA. Clones showing induction in both systems were sub-cloned, sequenced and further characterized by *in situ* hybridization. Clones which were induced in both systems and localized to the epithelial cells of the gland were potential Regression Selected Genes.


HOUSE-KEEPING
PROSTATE SPECIFIC
MAMMARY SPECIFIC
REGRESSION INDUCED
DAY 2 CASTRATE λgt11
PROSTATE cDNA LIBRARY
PLATE LIBRARY
DUPLICATE LIFTS
PROBE
PROBE
REGRESSION SELECTED
GENE (RSG)
PCR AMPLIFY INSERTS
DAY 0 LACTATING λgt10
MAMMARY cDNA LIBRARY
PCR AMPLIFY INSERTS
DAY 2 WEANED λgt10
MAMMARY cDNA LIBRARY

both regressing tissues are more likely to be involved in ACD and can be classified as regression selected genes (RSGs). Further characterization of these clones as primary or secondary thanatogens can be made based on *in situ* hybridization and northern analysis to establish that these genes are expressed in the dying epithelial cells with the appropriate kinetics.

4.100 - THEORETICAL CONSIDERATIONS

A typical cell expresses approximately 37,000 different mRNA species (Williams, 1981). The level of expression of these mRNAs can be divided into three categories: abundant more than 12,000 copies per cell; middle abundant between 15 and 12,000 copies per cell (with a modal abundance of 300 copies per cell); and rare, less than 15 copies per cell. The precise number and type of messages expressed will vary from cell type to cell type. In the case of the epithelial cells of the prostate and lactating mammary gland, a large precentage of the messages expressed are devoted to the secretory functions of the gland. Since the process of ACD is asynchronous, only a small percentage of the cells (approximately 1% - 3%) are actively expressing the mRNA required for ACD at any one time. Therefore any library constructed from RNA isolated at any time during regression will potentially harbor regression selected genes in approximately 0.1% to 0.5% of the clones. The above estimates suggest that in every 1000 clones screened about 1-5 would potentially code for mRNAs involved in regression. The ability to amplify the probe using PCR significantly increases the chance that middle abundant and rare messages will be amplified sufficiently to provide enough of the probe to produce a detectable signal in the screening. While it is difficult to predict how many novel sequences would be selected by this screening procedure, on the basis of our past experience with libraries prepared from regressing tissues and R_0t analysis (Montpetit et al., 1986) we expect to identify somewhere between 10 and 15 unique, previously unidentified clones that hybridize specifically to the poly(A)+ RNA from the regressing tissues.

Table 4-1: cDNA Libraries Constructed for Cross-screening Isolation of Regression Selected Genes (RSG)

The construction of the cDNA libraries used in the cross-screening isolation of RSGs was as detailed in the materials and methods. The total number of plaques, % of recombinants, vector and cDNA source for each library is presented. The purpose of each library in the screening procedure is indicated in the text. The VPR3RP-10 library was made using random primers during cDNA synthesis, all other libraries were constructed from cDNA primed with oligo-dT_{17} primers.

NAME	VECTOR	TOTAL PFU	% REC.	cDNA SOURCE
VPR2-11	λgt11	185,000	90	Day 2 Regressing Prostate
VPR3-11	λgt11	152,500	92	Day 3 Regressing Prostate
VPR3-10	λgt10	145,000	N.D.	Day 3 Regressing Prostate
VPR3RP-10	λgt10	250,000	N.D.	Day 3 Regressing Prostate
MGC-10	λgt10	208,400	N.D.	Day 0 Lactating Mammary
MGR2-10	λgt10	138,500	N.D.	Day 2 Regressing Mammary

4.200 - CROSS SCREENING

4.210 - Library and Probe Construction

Using the techniques described in Chapter 2, three cDNA libraries were constructed and used for the screening procedure (Table 4-1). The first library referred to as VPR2-11 was made from poly(A)+ RNA isolated from prostate tissue at day 2 after castration and was cloned into λgtll. This library contains cDNAs specific for mRNAs induced during regression of the prostate and was used for screening. The second library referred to as MGR2-10, was made from poly(A)+ RNA isolated from the mammary gland 2 days after weaning and cloned into λgt10. This library contains cDNAs specific for mRNAs induced during regression of the mammary gland. The third library referred to as MGC-10, was made from poly(A)+ RNA isolated from the mammary gland of lactating dams prior to weaning and cloned into λgt10. This library contains cDNAs specific for hormone induced mRNAs and constitutively expressed mRNAs. Approximately 50,000 plaques from both the MGC-10 and MGR2-10 libraries were plated, and phage DNA from the resulting high titre lysate was isolated. The probe for cross-screening was prepared from 1 ng of each of the MGC-10 and MGR2-10 library phage DNA using a standard PCR reaction with λgt10 forward and reverse primers (Fig. 4-2).

4.220 - Isolation of Regression Selected Genes (RSG)

In a screen of duplicate lifts with 10^5 plaques from the VPR2-11 library with the PCR amplified MGR2-10 and MGC-10 cDNA inserts, 109 plaques hybridized to the MGR2-10 probe more strongly than they did with the MGC-10 probe. These MGR2-10^+/MGC-10^- VPR2-11 clones represent sequences that are potentially induced during regression in both tissues (Fig. 4-3 panels A and B). To eliminate any uncertainty in the primary screen, a second screen was performed. Triplicate slot blots containing equal amounts of λgt11 phage DNA for each of the positive clones were prepared and hybridized with the PCR amplified MGC-10 and MGR2-10 probes. The third slot-blot was hybridized with cDNA probes for genes known to be induced in ACD (including TRPM-2, poly(ADP-ribose) polymerase, transglutaminase and Hsp27). After this second

Figure 4-2: Preparation of MGR2-10 and MGC-10 cDNA Inserts

Fifty thousand plaques from each library were plated and phage DNA was prepared from the resulting lysates. PCR reactions using 1 ng of isolated phage DNA were used in a standard PCR amplification with the λgt10 forward and reverse primers. A total of 1 μg of amplified cDNA inserts from each reaction was analysed on a 1.0% agarose gel and stained with EtBr and photographed. Migration of the Hind III digested λ marker is indicated. The inserts range in size from 500-2,000 bp and were representative of the cDNA inserts contained in each library. The amplified cDNA inserts were used as probes in the cross-screening experiments.

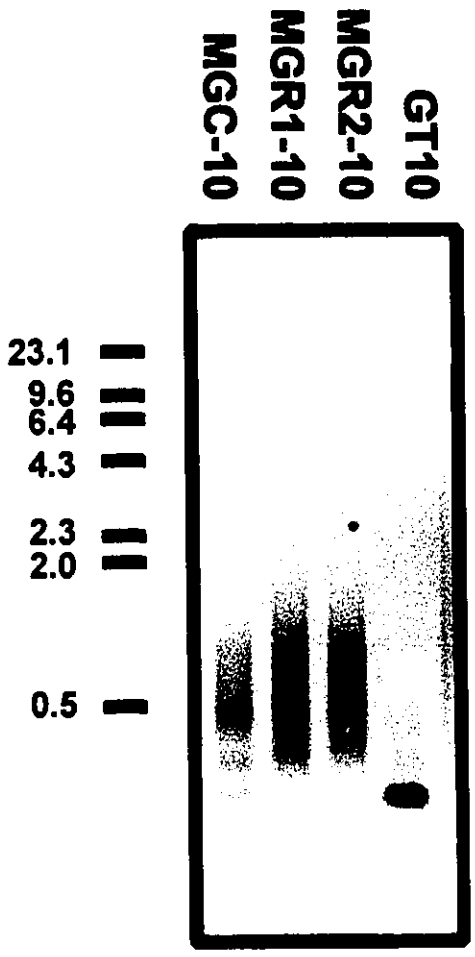
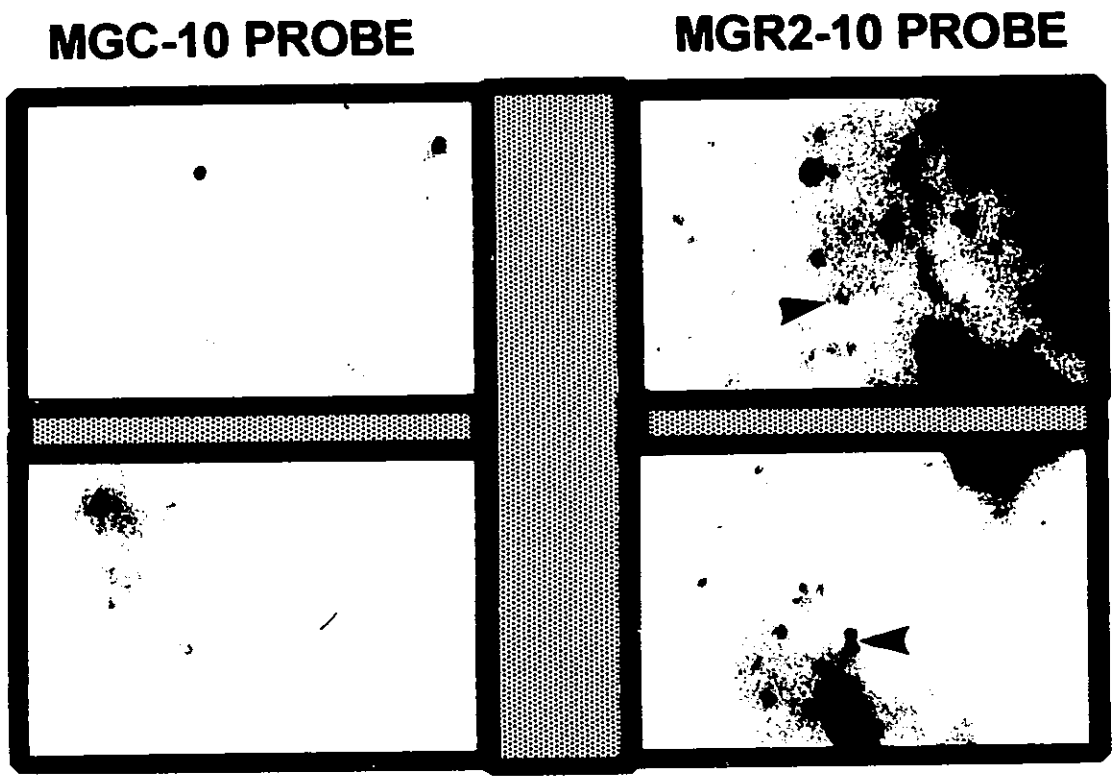

MGC-10
MGR1-10
MGR2-10
GT10
23.1
9.6
6.4
4.3
2.3
2.0
0.5

Figure 4-3: Screening of the VPR2-11 Library For Regression Selected Genes (RSG)

Plaque lifts of approximately 2,000 clones (10,000 total) from the VPR2-11 library were screened with the MGC-10 probe (specific activity; 10^8 cpm/μg). Duplicate plaque lifts were screened with the MGR2-10 probe (specific activity; 10^8 cpm/μg). The arrows point to 2 (of 109) VPR2-11 plaques which are MGR2-10^+ / MGC-10^-. These plaques are potential RSG clones and were isolated and further characterized. Labeling hybridization and wash conditions were as defined in the material and methods.


MGC-10 PROBE
MGR2-10 PROBE

round of screening, 8 clones remained which showed significantly increased hybridization to the MGR2-10 probe when compared to hybridization with the MGC-10 probe and which did not hybridize to the probes for mRNAs previously identified as ACD induced). These MGR2-10^{+}/MGC-10^{-} clones isolated from the VPR2-11 library represented potential regression selected genes (RSG) and were further characterized by Northern analysis. The clones were grouped according to their cognate mRNA size and each clone number was then replaced with and identified as indicated in Table 4-2.

4.300 - INITIAL ANALYSIS

4.310 - Northern Analysis

After plaque purification, the cDNA insert for each potential RSG was PCR amplified (Fig. 4-4) and hybridized to a screening panel consisting of poly(A)+ RNA extracted from the rat ventral prostate of normal males and males castrated 2 and 4 days previously and from the mammary gland of lactating dams and dams weaned 2 and 4 days previously. Four of the clones R1A, R1B, R1C, and R1D hybridize to 0.6 - 1.0 kb messages with up-regulated expression in the mammary gland, but down-regulation in the prostate gland during ACD (Fig. 4-5). The difference in expression patterns between the prostate and mammary gland for these messages made them unlikely candidates for a general role in ACD. The R2A, R2B, and R2C clones hybridize to messages approximately 2.0 kb in size which showed a significant up-regulation in both the prostate and mammary gland during regression (Fig. 4-6). The expression pattern for these clones indicated they were potential RSGs.The final clone R8A hybridizes to a large message approximately 6.0 kb in size which is expressed *de-novo* in both prostate and mammary gland during ACD (Fig. 4-6). The *de-novo* induction of this message in both regressing tissues made it an excellent candidate RSG.

Figure 4-4: Preparation of cDNA Inserts for RSG clones

Phage DNA was prepared from mini lysates of the plaque purified 8 RSG clones, R1A (41), R1B (47), R1C (53), R1D (76), R2A (43), R2B (97), R2C (102), R8A (62). PCR reactions using 1 ng of isolated phage DNA were used in a standard PCR amplification with the λgt11 forward and reverse primers. A total of 1/10 of the PCR product from each reaction was analysed on a 1.0% agarose gel and stained with EtBr and photographed. Migration of the Hind III digested λ marker is indicated. The inserts ranged in size from 500 -2,000 bp. The amplified cDNA inserts were used as probes in the northern hybridization experiments.

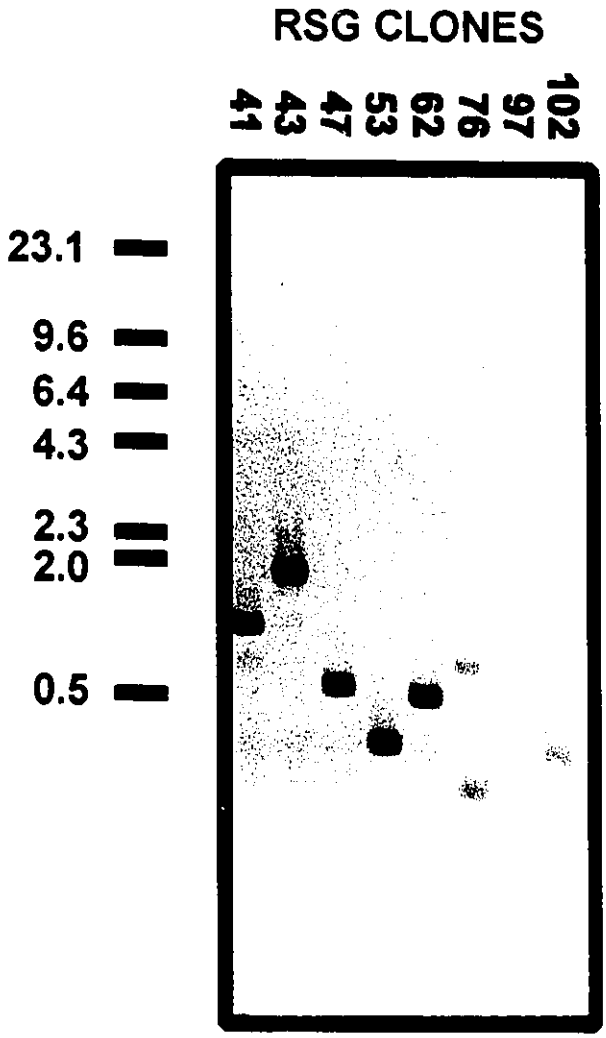
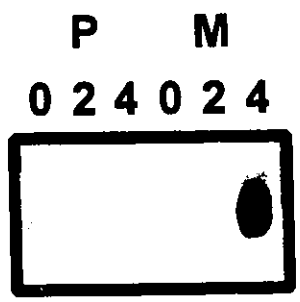
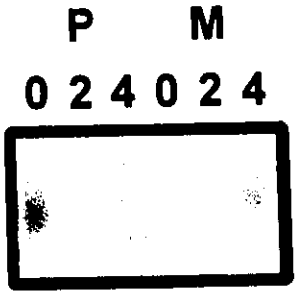

RSG CLONES
41
43
47
53
62
76
97
102
23.1
9.6
6.4
4.3
2.3
2.0
0.5

Figure 4-5: Northern Analysis of clones R1A, R1B, R1C, and R1D

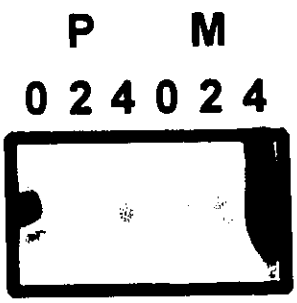
Poly(A)+ RNA was extracted from the rat prostate and mammary gland at different times (0, 2, and 4 days) after castration or weaning respectively and 2 μg per lane were electrophoresed on formaldehyde gels, transferred to nylon membranes, and hybridized to a ^{32}P-labeled PCR amplified cDNA specific for each RSG clone (specific activity; $1x10^8$ cpm/μg). The membranes were exposed for 18 h. The position of each mRNA was established by comparison to the migration of 18S and 28S ribosomal RNA.



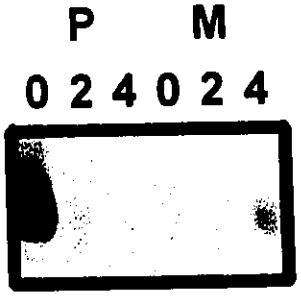
RSG-1A (900 nt)



RSG-1B (650 nt)



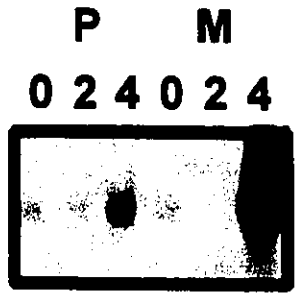
RSG-1C (900 nt)



RSG-1D (900 nt)

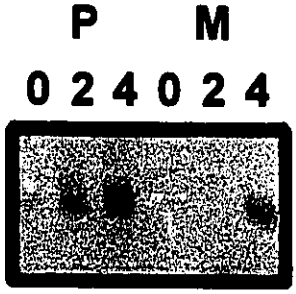
Figure 4-6: Northern Analysis of clones R2A, R2B, R2C, and R8A

Poly(A)+ RNA was extracted from the rat prostate and mammary gland at different times (0, 2, and 4 days) after castration or weaning respectively and 2 μg per lane were electrophoresed on formaldehyde gels, transferred to nylon membranes, and hybridized to a ^{32}P-labeled PCR amplified cDNA specific for each RSG clone (specific activity; $1x10^8$ cpm/μg). The membranes were exposed for 18 h. The position of each mRNA was established by comparison to the migration of 18S and 28S ribosomal RNA.

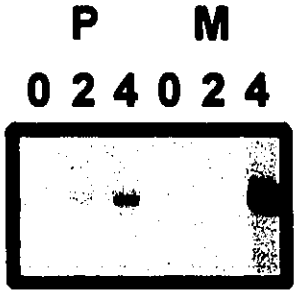


RSG-2A (1900 nt)

RSG-2B (1900 nt)



RSG-2C (1900 nt)



RSG-8 (6000 nt)

4.320 - Sequence Analysis

After the results of the Northern analysis the cDNA from each clone was subcloned into pGEM7Zf- and sequenced using the T7 and SP6 primers as described in methods. Approximately 100-200 bases of sequence was obtained from the long and short run of each primer reaction. The sequence data from each clone was then compared against the GenBank and EMBL database in both orientations to determine the identity of the clones. Two of the clones, R2B and R2C, code for β-actin and most likely represent constitutive messages which were inadvertently carried through during the screening process. We decided that the identification of this cDNA probably represents an artifact in the screening strategy either due to difficulties in standardizing the hybridization conditions during the screening or the change in the epithelial to stromal ratio during regression. While the observed induction of β-actin may reflect an actual change in the steady state levels of its message and an involvement in ACD, no further studies were conducted on the R2B and R2C clones. Sequence analysis for the R1A, R1C, and R1D clones indicated that they coded for the ferritin heavy chain. The involvement of ferritin in iron regulation in the cell and its differential expression in the prostate and mammary gland is intriguing but since it was unlikely to be a good canditate for a general role in ACD it was not studied any further. One clone, R1B codes for the rat homolog of the murine tumor transplantation antigen p198. This protein has homology to the L13P family of ribosomal proteins and may be part of the 60S ribosomal subunit. The expression and potential role for this clone in ACD was not obvious and therfore was not characterized any further. Neither the R2A or R8A clone showed any significant homology to sequences in the databases. Since both these clones showed the appropriate expression on the northern analysis, they were considered to be the most likely candidate RSGs and were studied further.

4.400 - DISCUSSION

Table 4-3 summarizes the results from the inital characterization of the 8 potential RSG clones. Using the PCR based cross-screening method described above, and after further characterization we had isolated 2 clones, R2A (RSG-2), and R8A (RSG-8) from

the VPR2-11 library which were induced during regression in both the prostate and mammary gland and were potential thanatogens. Northern analysis using the RSG cDNAs as probes indicates that these transcripts are induced in both the rat ventral prostate after castration and the rat mammary gland after weaning, at a time when there is significant ACD occurring in both organs. Comparison of preliminary sequence data for RSG-2 and RSG-8 to the Genbank Database revealed no significant homologies to existing sequences and indicated that they may code for novel proteins. Further research was aimed at the complete sequence analysis and characterization of the RSG-2 and RSG-8 clones. This is discussed in Chapters 5 and 6 respectively.

Table 4-2: Results From Characterization of RSG Clones

A summary of the information obtained during the characterization of the various RSG clones. Expression patterns in regressing prostate and mammary gland is indicated for each clone as well as the mRNA size and gene identity where appropriate.

Clone	#	Insert Size (nt)	mRNA Size (nt)	Prostate Expression	Mammary Expression	Gene
RSG-1A	41	900	900	-	+	Ferritin H.C
RSG-1B	47	700	650	-	+	Tumor Transplantation Antigen p198
RSG-1C	53	400	900	-	+	Ferritin H.C
RSG-1D	76	800	900	-	+	Ferritin H.C
RSG-2A	43	1,900	1,900	+	+	?
RSG-2B	97	400	1,900	+	+	β-actin
RSG-2C	102	400	1,900	+	+	β-actin
RSG-8A	62	600	8,000	+	+	?

CHAPTER FIVE

CHARACTERIZATION OF RSG-2

5.000 - INTRODUCTION

The cross-screen of the VPR2-11 library identified 8 clones which were potential thanatogens. Of these 8 clones, only RSG-2 (R2A) and RSG-8 (R8A) were novel, and showed the proper expression pattern in both regressing prostate and mammary glands. Both clones were chosen for further characterization. PCR amplification had demonstrated that the RSG-2 clone had a 1.9 kb insert, which appeared to be a full-length cDNA, based on a preliminary Northern analysis. To fully characterize RSG-2, we determined the full sequence of this clone, and characterized the expression of the gene by Northern analysis and *in situ* hybridization.

5.100 - Sequence Analysis of RSG-2

The 1.9 kb cDNA insert of the RSG-2 clone was subcloned into the EcoRI site of the pGEM7Zf- vector, and nested overlapping exonuclease III deletion clones were generated using the Erase-A-Base™ kit (Promega, Madison, WI). The overlapping clones were sequenced and 1904 bases of overlapping sequence for the RSG-2 clone was determined using sequence specific primers and the ABI-automated sequencer. Figure 5-1 shows the sequencing strategy for obtaining the cDNA sequence of RSG-2. Figure 5-2 shows the complete sequence of the RSG-2 cDNA including the 5' and 3' untranslated regions. The sequence was compared to the updated EMBL and Genbank databases and showed 100 % homology at the nucleotide level to the rat cathepsin B gene. Hereafter the RSG-2 clone was considered to be the cDNA for cathepsin B. The predicted protein sequence is shown below the DNA sequence and codes for a polypeptide of 339 amino acids. The conserved residues of the active site for cysteine proteases are underlined and are bold type. Several potential sites of glycosylation are also in bold. The protein predicted from the RSG-2 cDNA sequence has 92.3% and 79.9% homology to murine and human cathepsin B respectively (Fig. 5-3). All sequences obtained have been submitted to the appropriate databanks.

Figure 5-1: Sequencing Strategy for RSG-2 cDNA

The 2.0 kb cDNA insert of the RSG-2 clone was subcloned into the EcoRI site of the pGEM7Zf- vector. A set of overlapping Exonuclease III deletion clones were created from the RSG-2 clone using the Erase A Base™ kit (Promega, Madison, WI). The overlapping clones were sequenced and 1904 bases of overlapping sequence for the RSG-2 clone was determined using sequence specific primers and the ABI-automated sequencer.

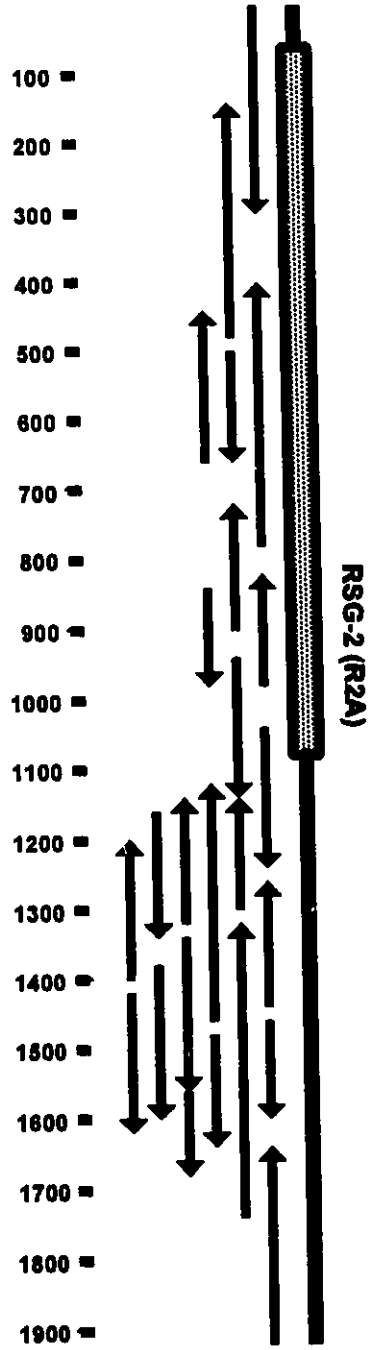

100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
1900
RSG-2 (R2A)

Figure 5-2: Complete Sequence of RSG-2 cDNA

The complete sequence (1904 nucleotides) of the RSG-2 cDNA including the 5' and 3' untranslated regions. The potential poly adenylation signal is underlined. Comparison to the EMBL and Genbank databases showed 100 % homology at the nucleotide level to the rat cathepsin B gene. Hereafter the RSG-2 clone was considered to be the cDNA for cathepsin B. The cDNA sequence predicts a protein of 339 amino acids. The conserved residues which form the active site in thiol proteases are underlined and in bold. Potential sites for N-linked glycosylation are in bold.

```
GAGCGACTGCGGGCCCAGGTTGTCGCGCTGCGCTCGGTGAGTACAGGATCCAGCTTCCAG 60
GATGTGGTGGTCCTTGATCCCTCTCTCTTGTCTGCTGGCACTGACCAGTGCCCATGACAA 120
  M  W  W  S  L  I  P  L  S  C  L  L  A  L  T  S  A  H  D  K
GCCTTCCTCACACCCACTGTCGGACGACATGATTAACTATATCAACAAACAGAATACAAC 180
  P  S  S  H  P  L  S  D  D  M  I  N  Y  I  N  K  Q  N  T  T
ATGGCAGGCTGGACGCAACTTCTACAATGTTGACATAAGCTATCTGAAGAAGCTGTGTGG 240
  W  Q  A  G  R  N  F  Y  N  V  D  I  S  Y  L  K  K  L  C  G
AACTGTCCTGGGTGGACCCAACCTGCCGGAAAGGGTTGGGTTCAGCGAGGACATAAATCT 300
  T  V  L  G  G  P  N  L  P  E  R  V  G  F  S  E  D  I  N  L
ACCTGAATCCTTTGATGCACGGGAACAGTGGTCCAATTGCCCGACCATCGCACAGATCAG 360
  P  E  S  F  D  A  R  E  Q  W  S  N  C  P  T  I  A  Q  I  R
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  D  Q  G  S  C  G  S  C  W  A  F  G  A  V  E  A  M  S  D  R
AATCTGCATTCACACCAATGGCCGAGTCAATGTGGAGGTGTCTGCTGAGGACCTGCTTAC 480
  I  C  I  H  T  N  G  R  V  N  V  E  V  S  A  E  D  L  L  T
CTGCTGTGGTATCCAGTGTGGGGATGGCTGTAATGGTGGCTATCCCTCTGGAGCATGGAA 540
  C  C  G  I  Q  C  G  D  G  C  N  G  G  Y  P  S  G  A  W  N
CTTCTGGACTAGAAAAGGCCTGGTTTCTGGTGGAGTATACAATTCTCATATAGGCTGCTT 600
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ACCCTACACCATCCCTCCCTGTGAACACCATGTCAATGGCTCCCGTCCCCCATGCACTGG 660
  P  Y  T  I  P  P  C  E  H  H  V  N  G  S  R  P  P  C  T  G
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GGAAGATAAGCACTATGGGTACACTTCCTACAGTGTGTCTGACAGCGAGAAGGAGATCAT 780
  E  D  K  H  Y  G  Y  T  S  Y  S  V  S  D  S  E  K  E  I  M
GGCGGAAATCTACAAAAATGGCCCAGTGGAGGGTGCTTTTACTGTGTTTTCTGACTTCTT 840
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GACTTACAAATCAGGCGTATACAAGCATGAAGCCGGTGATGTGATGGGAGGCCATGCCAT 900
  T  Y  K  S  G  V  Y  K  H  E  A  G  D  V  M  G  G  H  A  I
CCGCATTCTGGGCTGGGGAATAGAGAATGGAGTACCCTACTGGCTGGTAGCAAACTCCTG 960
  R  I  L  G  W  G  I  E  N  G  V  P  Y  W  L  V  A  N  S  W
GAACGTTGACTGGGGTGATAATGGTTTCTTTAAAATCCTCAGAGGAGAGAACCACTGTGG 1020
  N  V  D  W  G  D  N  G  F  F  K  I  L  R  G  E  N  H  C  G
AATTGAATCAGAAATCGTGGCTGGAATCCCACGCACTCAGCAGTACTGGGGAAGATTCTA 1080
  I  E  S  E  I  V  A  G  I  P  R  T  Q  Q  Y  W  G  R  F  -
ATCTGCTGGGACTTGATTGTATAGTCCTTAGGAGACTTTTTCCAAAATTTAGCAATGCAG 1140
GCAGGGAGTGAGGCAGATAGGAGTCTTTGATTCTTTGAGTTCACCTAACATGCATGAAGC 1200
TTTCAGCAAGGTTTGGGTGACTGGACCAGAGCTGCCCCCCATCAGAGCCACCCTACCTGG 1260
CTCCCTTCTAGCCTATCCCATGACAGTGATCACAGCCTACATCCTAGCCTCTCCCTAGAC 1320
TAGTGCTGTGTGTAGCGCCTGCTGCTGTGGCTCTGTCTGCCATCCCTCCCACCCCCACCT 1380
CAGCATCCCCAGACCAAGGGCTTTGGCTGCAGGATTTCCAAAGGAATGAACACCAACTAC 1440
ATGATGAGAGGACAGATGCCACCCGCCAGCTGCACCTTGAAGCTAGTCACTTCTAGGACT 1500
GTGGCAGGCTTTGATGGCCCAATGCAGTTCTCTGGAGAAAACTACCTTTCCCCAAGGCAT 1560
CTGCACCCATTGACAATGGTAATGTGCCCATCTCTCCTTGGTCCTGCCCTCAACCGATGC 1620
TTTTCCAGTCAGGGTTTTGTTTTTTGTTTTGTTTTGTGTACCTCAACTGAGTTATGAAGA 1680
TTTGTACTGGTTTTACAGATCATCTCATCGTATGGAAAAGAACAAGCTTCGTGGTCAGTT 1740
TACTGGGTGACCGGCAGACACCACAATCAAACTAGTCTGGGGAAAACCTGCTTTTTTGTT 1800
GTAGGTGCCACGTAACCCTGTCAGTTTGACAAGGAATGACCGTGCCAATAAACCAATTCT 1860
CCCTCTGCTTGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                    1904
```

Figure 5-3: Comparison of RSG-2 Protein Sequence with Murine and Human cathepsin B

The predicted protein sequence of RSG-2 and murine and human cathepsin B were aligned using the PC-Gene software package and the CLUSTAL program. The predicted protein sequence of RSG-2 is highly conserved between species with 92.3% and 79.9% identity with the murine and human cathepsin B sequences respectively. The * indicates a residue that is perfectly conserved, the + indicates a residue which is well conserved.

```
RSG2          MWWSLIPLSCLLALTSAHDKPSSHPLSDDMINYINKQNTTWQAGRNFYNV   50
CATB_MOUSE    MWWSLILLSCLLALTSAHDKPSFHPLSDDLINYINKQNTTWQAGRNFYNV   50
CATB_HUMAN    MWQLWASLCCLLVLANARSRPSFHPVSDELVNYVNKRNTTWQAGHNFYNV   50
              **     *+****+*++*+++** **+**+++*+**+*+*******+*****

RSG2          DISYLKKLCGTVLGGPNLPERVGFSEDINLPESFDAREQWSNCPTIAQIR   100
CATB_MOUSE    DISYLKKLCGTVLGGPKLPGRVAFGEDIDLPETFDAREQWSNCPTIGQIR   100
CATB_HUMAN    DMSYLKRLCGTFLGGPKPPQRVMFTEDLKLPASFDAREQWPQCPTIKEIR   100
              *+*****+**** ****+ * ** *+**++**++********++**** +**

RSG2          DQGSCGSCWAFGAVEAMSDRICIHTNGRVNVEVSAEDLLTCCGIQCGDGC   150
CATB_MOUSE    DQGSCGSCWAFGAVEAISDRTCIHTNGRVNVEVSAEDLLTCCGIQCGDGC   150
CATB_HUMAN    DQGSCGSCWAFGAVEAISDRICIHTNAHVSVEVSAEDLLTCCGSMCGDGC   150
              ****************+***+******++*+************  *****

RSG2          NGGYPSGAWNFWTRKGLVSGGVYNSHIGCLPYTIPPCEHHVNGSRPPCTG   200
CATB_MOUSE    NGGYPSGAWSFWTKKGLVSGGVYNSHVGCLPYTIPPCEHHVNGSRPPCTG   200
CATB_HUMAN    NGGYPAEAWNFWTRKGLVSGGLYESHVGCRPYSIPPCEHHVNGSRPPCTG   200
              *****++**+***+*******+*+***+** **+*****************

RSG2          EGDTPKCNKMCEAGYSTSYKEDKHYGYTSYSVSDSEKEIMAEIYKNGPVE   250
CATB_MOUSE    EGDTPRCNKSCEAGYSPSYKEDKHFGYTSYSVSNSVKEIMAEIYKNGPVE   250
CATB_HUMAN    EGDTPKCSKICEPGYSPTYKQDKHYGYNSYSVSNSEKDIMAEIYKNGPVE   250
              ******+*+* **+****+***+***+**+******+* *+***********

RSG2          GAFTVFSDFLTYKSGVYKHEAGDVMGGHAIRILGWGIENGVPYWLVANSW   300
CATB_MOUSE    GAFTVFSDFLTYKSGVYKHEAGDMMGGHAIRILGWGVENGVPYWLAANSW   300
CATB_HUMAN    GAFSVYSDFLLYKSGVYQHVTGEMMGGHAIRILGWGVENGTPYWLVANSW   300
              ***+*+**** ***+***+* +*++*************+***+****+****

RSG2          NVDWGDNGFFKILRGENHCGIESEIVAGIPRTQQYWGRF   339
CATB_MOUSE    NLDWGDNGFFKILRGENHCGIESEIVAGIPRTDQYWGRF   339
CATB_HUMAN    NTDWGDNGFFKILRGQDHCGIESEVVAGIPRTDQYWEKI   339
              * ************+*+*******+*******+***+++
```

5.200 - Expression of cathepsin B mRNA

The cDNA insert from the RSG-2 clone was amplified by PCR and hybridized to Northern blots of poly(A)+ RNA extracted from the ventral prostate at different times after castration and from the mammary gland at different times after weaning. The cDNA insert hybridizes to an mRNA of approximately 2 kb that is expressed at low levels in the normal prostate and lactating mammary glands, but shows a dramatic induction in these tissues after ablation of the appropriate trophic hormone (Fig. 5-4). Expression of cathepsin B mRNA reaches a maximal level in the prostate 4 days after castration, at which time the level of epithelial cell death in the gland is maximum (DeKlerk and Coffey, 1978). In the mammary gland the expression of cathepsin B mRNA is also increased during regression, reaching a maximum between 2 and 4 days after weaning, before diminishing to background levels by day 8. Control hybridizations using α-tubulin cDNA on duplicate blots demonstrated that the increase in the steady state levels of cathepsin B mRNA is specific and not due to loading artifacts or to changes in the rRNA content of the tissues (results not shown).

5.300 - Localization of cathepsin B mRNA

To localize the expression of cathepsin B mRNA, *in situ* hybridizations were performed on random sections from normal and regressing prostate (Fig. 5-5) and lactating and regressing mammary gland (Fig. 5-6). The cathepsin B mRNA localizes to the epithelial cells of both tissues. In intact male animals cathepsin B mRNA showed hybridization in the prostate that is barely above background (Fig. 5-5, panel B) which correlates with the expression observed in normal prostate measured by Northern analysis. The increased expression of cathepsin B mRNA is detectable in the luminal epithelial cells of the prostate two days after castration (Fig. 5-5, panel D) and reaches maximal levels on day 4 (Fig. 5-5, panel F). The density of grains over the stroma remains relatively constant throughout the time course with no significant increase above background during regression. The expression of cathepsin B mRNA in the regressing mammary gland shows a similar pattern of expression to that seen in the prostate. The mRNA is present in the lactating mammary gland at a low level, and the expression appears to be confined to the

Figure 5-4: Northern Analysis of cathepsin B Steady-State mRNA Levels in the Regressing Prostate and Mammary Gland

Poly(A)+ RNA was extracted from the rat ventral prostate at different times (0, 1, 2, 3, and 4 days) after castration (panel A) or from the rat mammary gland at different times (0, 2, 4 , 6 and 8 days) after weaning (panel B). 2 μg per lane were electrophoresed on formaldehyde gels, transferred to nylon membranes, and hybridized to a ^{32}P-labeled cDNA specific for cathepsin B ($1x10^8$ cpm/μg). The membranes were exposed for 18 h. The position of the 1,900 nucleotide cathepsin B mRNA was established by comparison to the migration of 18S and 28S ribosomal RNA.

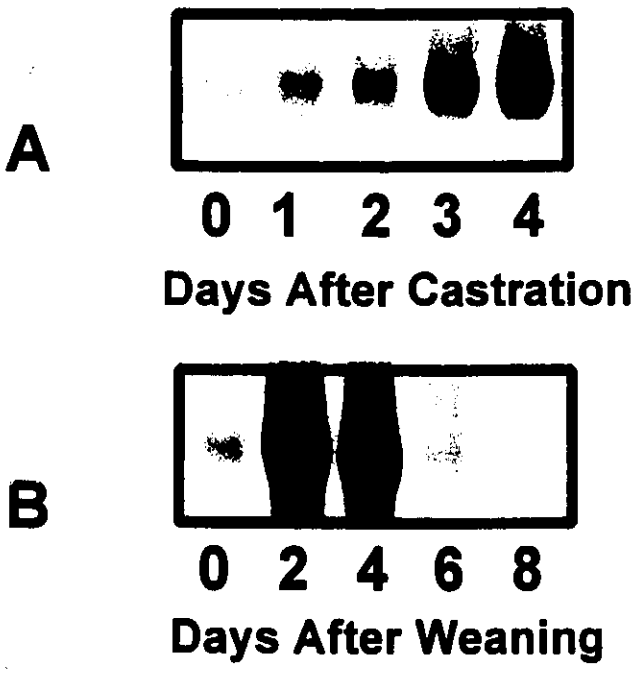

A
0 1 2 3 4
Days After Castration
B
0 2 4 6 8
Days After Weaning

Figure 5-5: *In Situ* Hybridization of cathepsin B mRNA in Regressing Rat Ventral Prostate

Random sections from the rat ventral prostate, excised at different times after castration, were hybridized to a ^{35}S-labeled cDNA insert specific for cathepsin B ($1x10^6$ cpm). The sections were exposed for 4 days. Magnification X20.

Panels A, C, E:	phase contrast showing morphology of sections
Panels B, D, F:	dark field illumination showing *in situ* hybridization
Panels A and B:	prostate from control animals
Panels C and D:	prostate 2 days after castration
Panels E and F:	prostate 4 days after castration

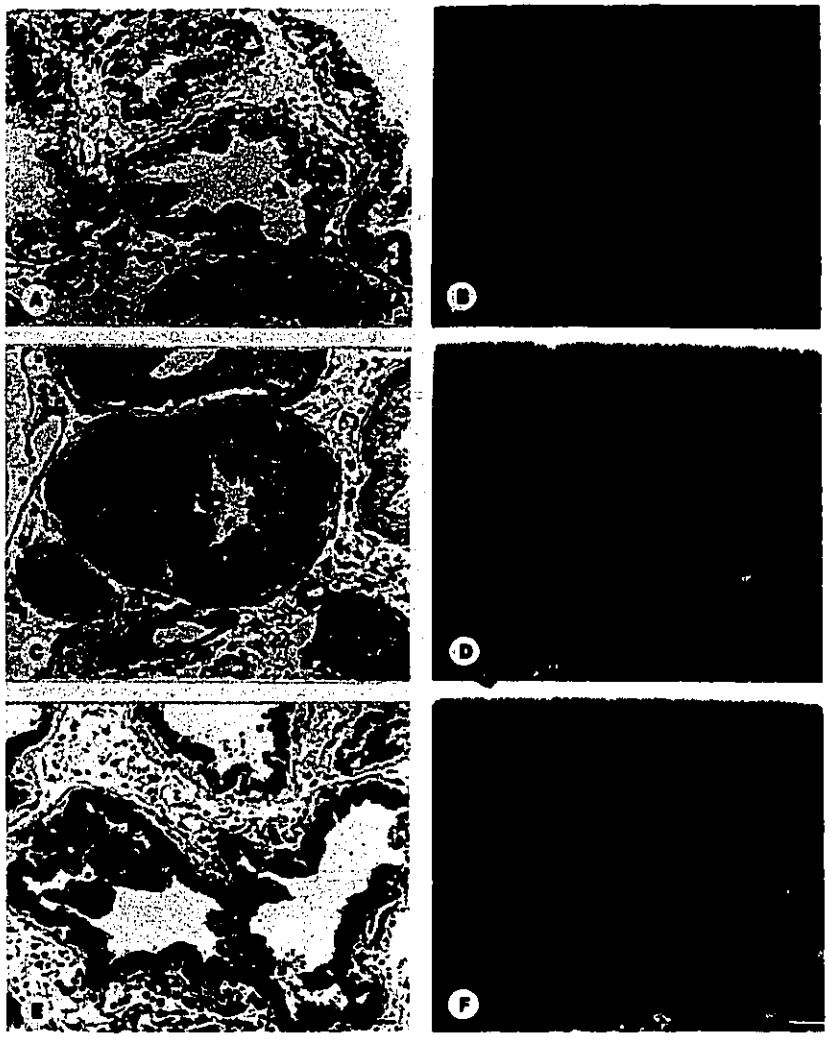
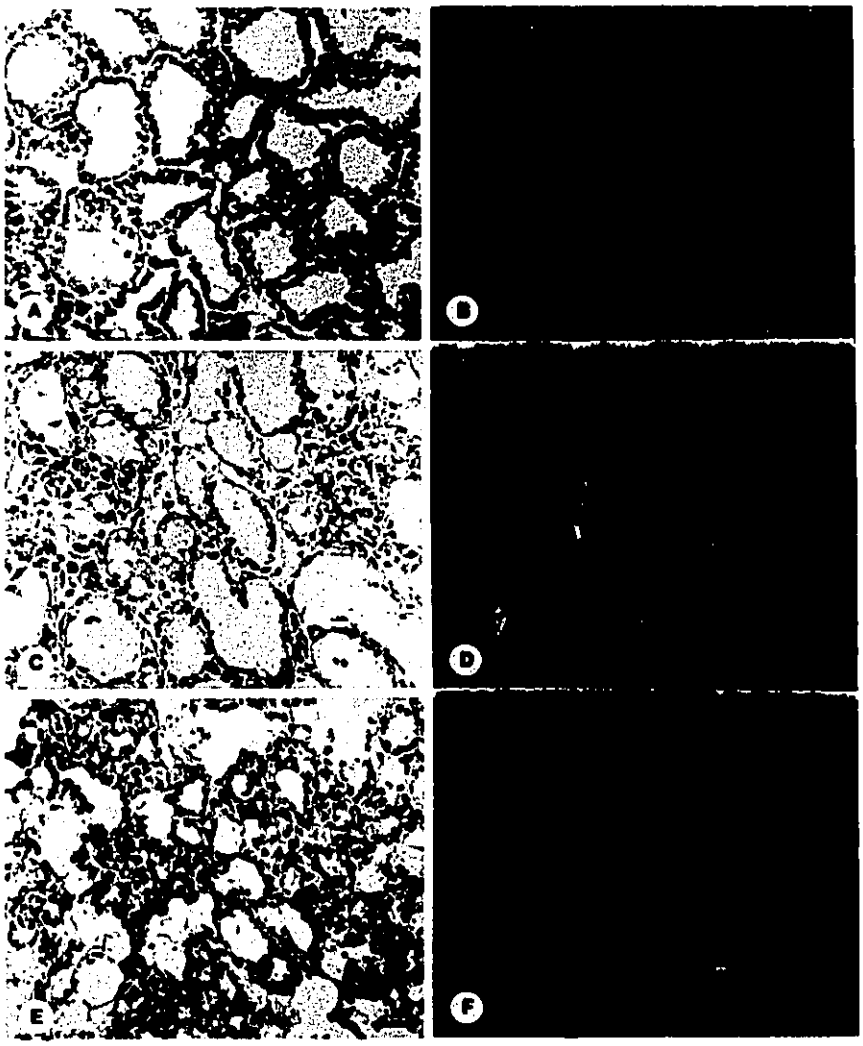

A
B
C
D
E
F

Figure 5-6: *In Situ* Hybridization of cathepsin B mRNA in Regressing Rat Mammary Gland

Random sections from the rat mammary gland, excised at different times after weaning, were hybridized to a ^{35}S-labeled cDNA insert specific for cathepsin B ($1x10^6$ cpm). The sections were exposed for 4 days. Magnification X20.

Panels A, C, E:	phase contrast showing morphology of sections
Panels B, D, F:	dark field illumination showing *in situ* hybridization
Panels A and B:	mammary glands from lactating dams
Panels C and D:	mammary gland 2 days after weaning
Panels E and F:	mammary gland 4 days after weaning;


A
B
C
D
E
F

luminal epithelium (Fig. 5-6, panel B). After weaning, the expression of cathepsin B mRNA increases significantly in the luminal epithelium, reaching a peak on day 4 (Fig. 5-6, panel D). The expression subsequently decreases to baseline levels by day 6 after weaning (Fig. 5-6, panel F), paralleling the changes in the steady state level observed on Northern analysis. The enhanced expression of the mRNA appears to be restricted to the luminal epithelium, although it is possible that there is a less dramatic increase in cathepsin B expression in the stroma and myo-epithelium that is masked by the increase in the epithelial expression.

5.400 - Immuno-Localization of cathepsin B

After identifiying that the RSG-2 cDNA coded for cathepsin B, a polyclonal anti-cathepsin B antibody was obtained from Dr. J. S. Mort and used to examine the protein expression in regressing prostate and mammary gland. In the intact prostate cathepsin B is detected as distinct punctate staining over the lysosomal compartment of the luminal epithelial cells (Fig. 5-7, panel B). The level of cathepsin B protein increases significantly after castration, and is localized predominantly in the apoptotic cells of the gland on days 2 and 4 after castration, the majority of which are immunoreactive (Fig. 5-7, panel D and F). In the lactating mammary gland cathepsin B is also detected as distinct punctate staining over the lysosomal compartment of the luminal epithelial cells (Fig. 5-8, panel B). This staining is increased 2 days after weaning, but remains confined to the lysosomes (Fig. 5-8, panel D). Four days after weaning, cathepsin B is localized in the apoptotic cells of the gland, however a significant proportion of the apoptotic cells are not immunoreactive for cathepsin B (Fig. 5-8, panel F), in contrast to the localized staining in the apoptotic cells observed in the regressing prostate. It is not clear whether this distinction represents a fundamental difference in the expression of cathepsin B between the prostate and mammary gland or whether it represents a difference in the time course of ACD in the two glands.

Figure 5-7: Immuno-Localization of cathepsin B in the Regressing Rat Ventral Prostate

Random sections from the rat ventral prostate were excised at different times after castration. Cathepsin B was immunolocalized using a rabbit polyclonal antibody raised against the human protein, and visualized with FITC conjugated goat anti-rabbit gamma globulin. Controls slides were incubated with anti-cathepsin B antibody only or with FITC conjugated goat anti-rabbit gamma globulin only to determine the levels of background immunofluoresence (data not shown). Controls slides were incubated with anti-DNP (di-nitro-phenol) antibody followed by FITC conjugated goat anti-rabbit gamma globulin to determine levels of non-specific binding. Immunofluorescent detection of cathepsin B demonstrates that the protein is expressed at a low level, in a distinctly punctate fashion in the control prostate (B). The level of the protein increases, and is localized predominantly in the apoptotic cells in the gland on day 2 and 4 after castration (as indicated by white arrows) (D and F). Magnification X20.

Panels A, C, E:	phase contrast showing morphology of sections
Panels B, D, F:	immunofluorescent detection of cathepsin B
Panels A and B:	prostate from control animals
Panels C and D:	prostate 2 days after castration
Panels E and F:	prostate 4 days after castration

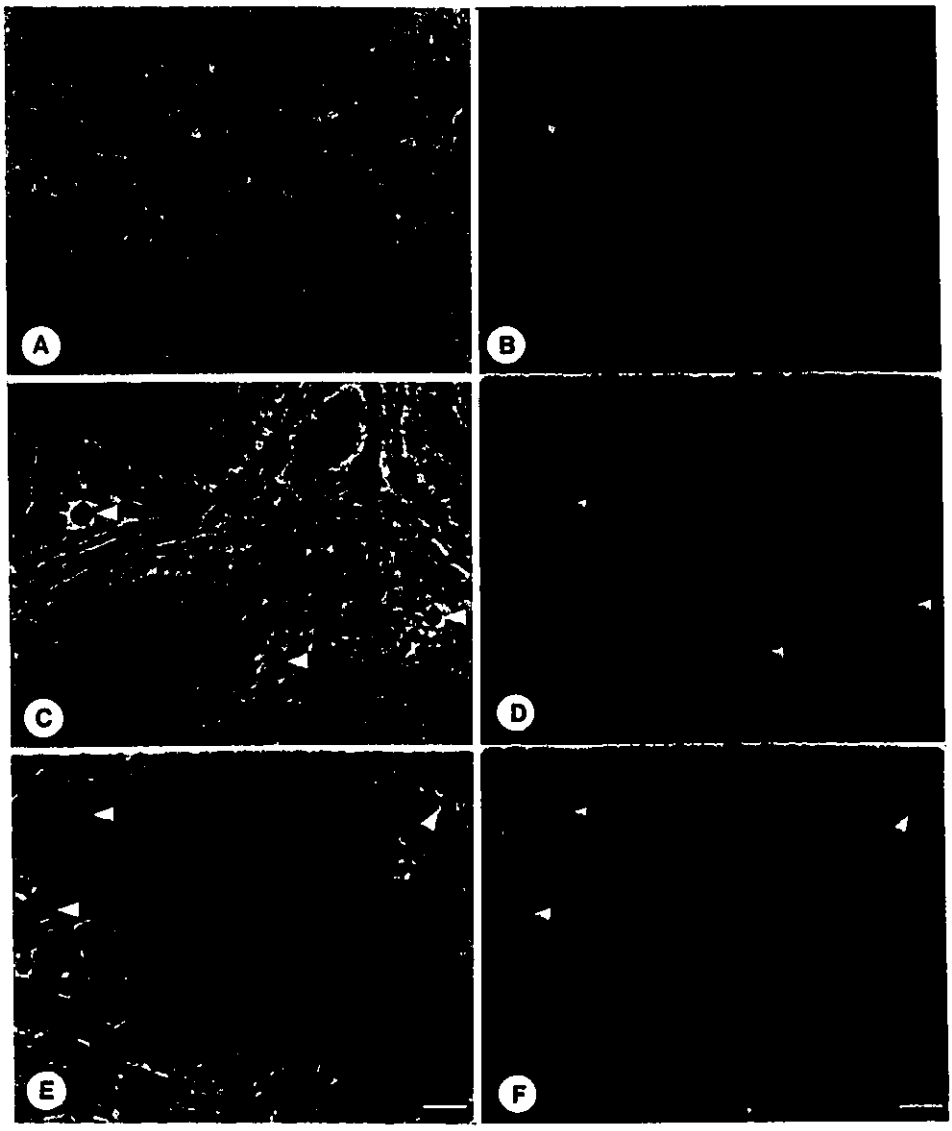
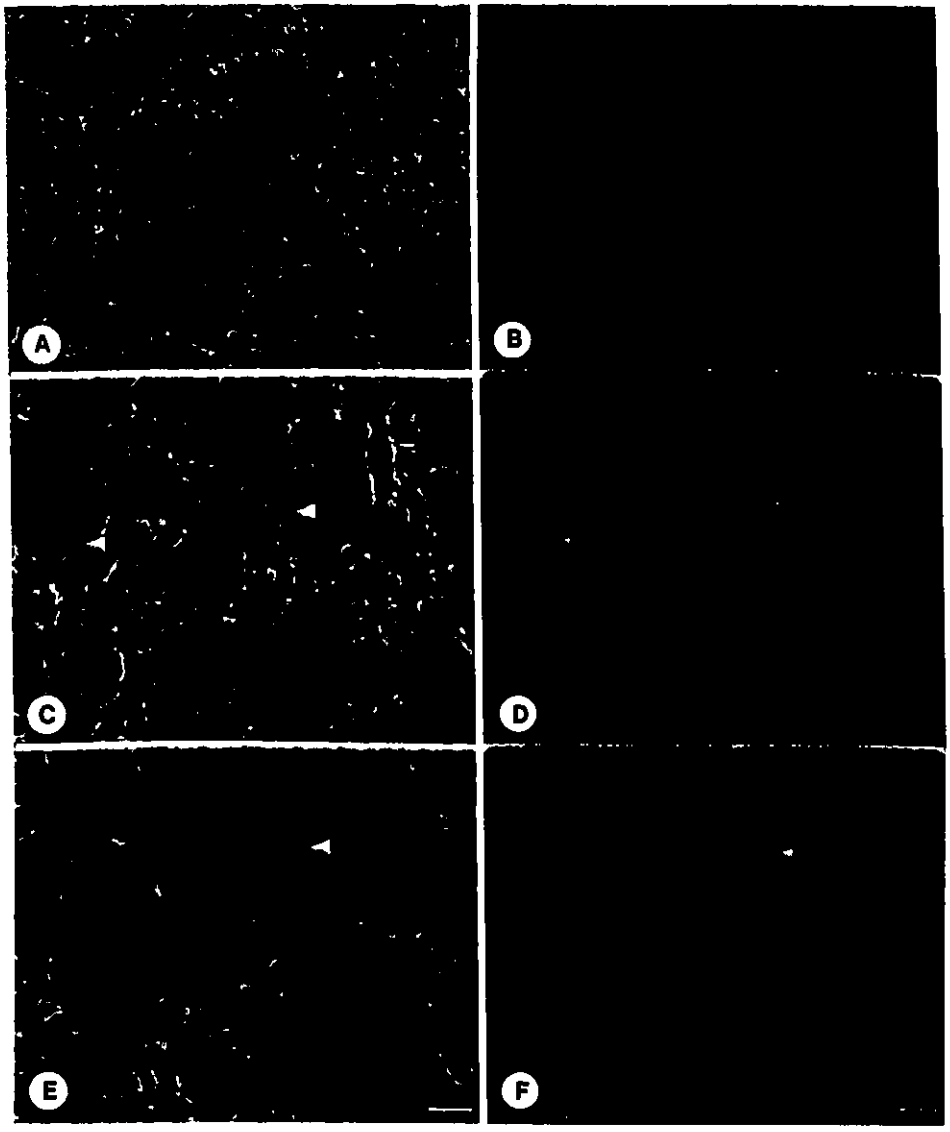

A
B
C
D
E
F

Figure 5-8: Immuno-Localization of cathepsin B in the Regressing Rat Mammary Gland

Random sections from the rat mammary gland were excised at different times after weaning. Cathepsin B was immunolocalized using a rabbit polyclonal antibody raised against the human protein, and visualized with FITC conjugated goat anti-rabbit gamma globulin. Controls slides were incubated with anti-cathepsin B antibody only or with FITC conjugated goat anti-rabbit gamma globulin only to determine the levels of background immunofluoresence (data not shown). Controls slides were incubated with anti-DNP (di-nitro-phenol) antibody followed by FITC conjugated goat anti-rabbit gamma globulin to determine levels of non-specific binding. Immunofluorescent detection of cathepsin B demonstrates that the protein is expressed at a low level, in a distinctly punctate fashion in the lactating glands (B). The level of the protein increases, and is localized predominantly in the apoptotic cells in the gland on day 2 and 4 after weaning (as indicated by white arrows) (D and F). Magnification X20.

Panels A, C, E:	phase contrast showing morphology of sections
Panels B, D, F:	immunofluorescent detection of cathepsin B
Panels A and B:	mammary glands from lactating dams
Panels C and D:	mammary gland 2 days after weaning
Panels E and F:	mammary gland 4 days after weaning;


A
B
C
D
E
F

5.500 - DISCUSSION

Sequence analysis of one of the cDNA clones, RSG-2, demonstrated that the mRNA coded for cathepsin B, a thiol protease that degrades components of the basement membrane, including collagen, fibronectin and proteoglycans (Sloane and Honn, 1984). *In situ* hybridization and immunofluorescence demonstrates that the mRNA encoding cathepsin B is induced in the dying luminal epithelial cells of the rat ventral prostate after castration and the mammary gland after weaning. Cathepsin B is expressed in the apoptotic cells and is subsequently localized in the apoptotic bodies. In both tissues this is the first demonstration of a thanatogen that is induced in the luminal epithelium and is associated with apoptotic bodies after hormone deprivation. The localization of cathepsin B to the lysosomes of the dying cells further suggests that lysosomes activated within the dying cells play an active role in the degradation of the apoptotic bodies. This is in marked contrast to the degradation of apoptotic lymphocytes, which are degraded following lysosomal activation in the engulfing macrophages (Walker et al., 1988).

Although the physiological role of cathepsin B is not known, it is present in the lysosomes of many tissues, including breast, kidney, liver, lung and spleen (Recklies and Mort, 1985; Kominami et al., 1985). In addition to the results presented here, two other reports link cathepsin B with ACD. The level of cathepsin B in the liver is significantly elevated during hepatic atrophy induced by starvation, and returns to normal levels on refeeding (Ohshita et al., 1986). Enzymatically active cathepsin B has also been detected in neuronal perikarya at various stages of disintegration within the neuritic plaques associated with Alzheimer's disease (Cataldo and Nixon, 1990; Nakamura et al., 1991). In these plaques the enzyme is associated both with lysosomes and with extracellular lipofuscin granules (Cataldo and Nixon, 1990), demonstrating that the protein is secreted into the extracellular matrix. It is of interest that another secondary thanatogen, TRPM-2/clusterin, is also expressed during ACD in the liver (Bursch et al., 1995), and in Alzheimer's tissue (Michel et al., 1992), suggesting that ACD is important in the etiology of these diseases. Over-expression of the protein has been implicated in the pathology of a

number of other diseases including nephritis, emphysema and rheumatoid arthritis (Howie et al., 1985).

The co-ordination of thanatogen expression during ACD must, of necessity, be complex since the genes are induced in a temporally and anatomically restricted manner in the dying cells. Computer aided analysis of the 5' promoter regions of the murine cathepsin B (Qian et al., 1991) and human and rat TRPM-2/clusterin (Wong et al., 1993a; Wong et al., 1994) genes does not demonstrate any obvious common DNA binding motifs. Unlike the TRPM-2/clusterin gene, which is repressed in the normal cell (Léger et al., 1987), the cathepsin B gene lacks the TATA and CAAT motifs and appears to be a housekeeping gene that is constitutively expressed at low levels and which is further induced and recruited during ACD, presumably as a result of additional DNA-protein interactions (Qian et al., 1991). While the promoter regions of the two genes are very different, it is of interest that both genes are localized to the same region of chromosome 8- 8p22 in the case of cathepsin B (Fong et al., 1991) and 8p21 in the case of TRPM-2/clusterin (Wong et al., 1993a) - raising the possibility that thanatogen induction in the dying cell may be co-ordinated at higher levels of chromatin structure.

The expression of cathepsin B during ACD is co-incident with the expression of several other extracellular proteases including collagenase, tissue type and urokinase type plasminogen activators, and cathepsin D (Muntzing, 1981; Sensibar et al., 1990; Freeman et al., 1990). This latter serine protease actively degrades cystatin C, the major inhibitor of cathepsin B (Lenarcic et al., 1991), resulting in the enhancement of the enzymatic activity of the thiol protease. These proteases form a proteolytic cascade that results in the active degradation of the basement membrane, a necessary prerequisite of cellular condensation, the first visible stage of ACD (Bursch et al., 1990a).

The level of cathepsin B expression is elevated in human breast and ovarian cancers (Woynarowska et al., 1989; Gabrijelcic et al., 1992; Lah et al., 1992;), and in several metastatic cell lines, including ras-transformed NIH 3T3 cells and Lewis lung

carcinoma cells, in which the level of expression has been correlated with the increased malignancy (Brodt et al., 1992; Chambers et al., 1992). In many malignant cell lines the enzyme is localized to the plasma membrane and is clearly secreted in most instances (Sloane et al., 1986). These data re-enforce the observation that many of the features of ACD are recapitulated in metastasis. The major distinction in the formation of an apoptotic cell and a metastatic cell lies in the destruction of the DNA as a result of the activation of one or more endogenous endonucleases (Tenniswood et al., 1992). Since the apoptotic cell induces the enzymes necessary for the degradation of the ECM, these cells have the potential to metastasize if the endonucleases are not activated appropriately. It is of interest that we have previously demonstrated that TRPM-2/clusterin, which is believed to block complement mediated lysis, is also expressed during ACD, potentially providing the metastatic cell protection from complement fixation and immune surveillance (Tenniswood et al., 1992). Identification of additional secondary thanatogens, and elucidation of their role in ACD will provide further information that can be used to test this hypothesis that the formation of the metastatic phenotype may therefore be due to "arrested" ACD.

CHAPTER SIX

CHARACTERIZATION OF RSG-8

6.000 - INTRODUCTION

PCR amplification had demonstrated that the RSG-8 clone had a 0.6 kb insert, which was probably a partial cDNA of the 3' untranslated region of the gene based on the large size of the message (6.0 kb) as determined by a preliminary Northern analysis. The first step in the characterization of RSG-8 was a complete cloning and sequence analysis of the entire cDNA.

6.100 - Identification and Sequence Analysis of RSG-8

We tried to isolate longer cDNAs specific for RSG-8 from a number of libraries including the VPR2-11, VPR2-10, VPR3-11, VPR3-10, and MGR2-10 libraries. After an exhaustive search two clones R8B and R8C were isolated that contained inserts of 1.0 and 1.1 kb respectively. These inserts were also used to screen a random primed λgt10 library (VPR3RP-10) made from regressing day 3 castrated prostate poly(A)+ RNA. Several clones were isolated from this library but none contained any further cDNA sequence. Other methods such as 5' RACE, PCR cloning and mRNA enrichment were attempted with no success probably because of the large size of the message. The cDNA inserts of the R8A, R8B, and R8C clones were subcloned into the EcoRI site of the pGEM7Zf- vector, and nested overlapping exonuclease III deletion clones were generated using the Erase-A-Base™ kit (Promega, Madison, WI). The overlapping clones were sequenced (see Fig. 6-1 for the strategy) and a 1.1 kb overlap of sequence for the RSG-8 cDNAs was determined (Fig. 6-2). The sequence was compared to the updated EMBL and Genbank databases and showed >90 % homology at the nucleotide level to the 3' untranslated region of the murine insulin growth factor binding protein five (mIGFBP-5) gene (Fig. 6-3). Hereafter the RSG-8 clones were considered to be the cDNAs for the 3' untranslated region of rat IGFBP-5. Once the identity of the RSG-8 gene was confirmed by sequence, the coding region for the RSG-8 gene was isolated using gene specific primers designed using the coding sequence of rat IGFBP-5, which was entered into the database without the 3' untranslated region. These primers were used for reverse transcriptase PCR cloning of the complete coding region of rat IGFBP-5 (Fig. 6-4, and section 2.620). The complete sequence of the IGFBP-5 RT-PCR clone was determined,

Figure 6-1: Sequencing Strategy for RSG-8 cDNA Clones

The cDNA inserts of the RSG-8A, 8B, 8C clones were subcloned into the EcoRI site of the pGEM7Zf- vector. A set of overlapping Exonuclease III deletion clones were created from the original RSG-8 clones using the Erase A Base™ kit (Promega, Madison, WI). The overlapping clones were sequenced and 1,165 bases of sequence for the RSG-8 cDNAs was determined.

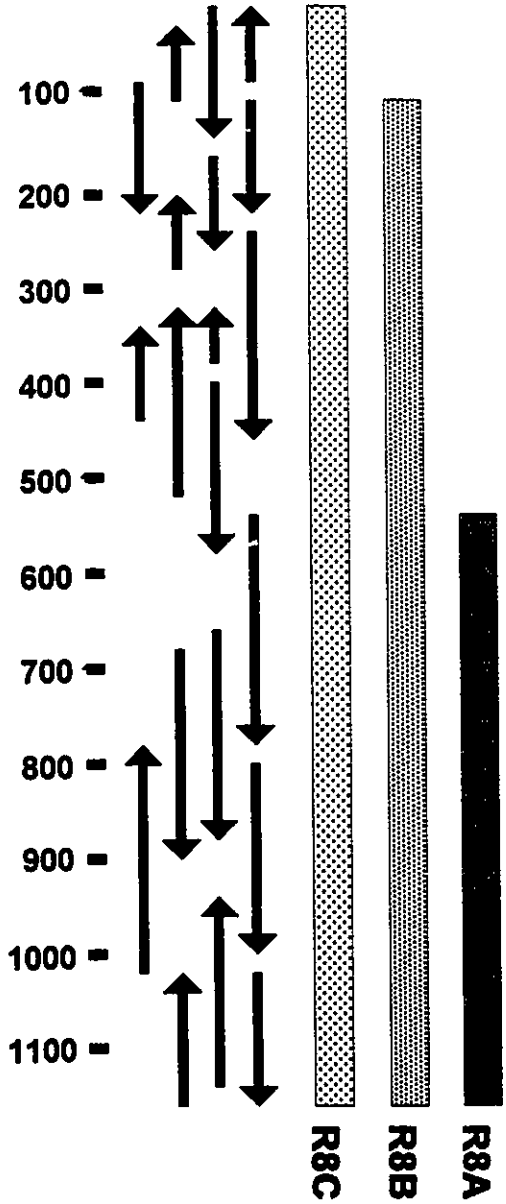

100
200
300
400
500
600
700
800
900
1000
1100
R8C
R8B
R8A

Figure 6-2: Partial Sequence of RSG-8 3' untranslated Region

The partial sequence (1165 nucleotides) of the RSG-8 cDNA including part of the 3' untranslated region. The potential poly adenylation signals are underlined. The sequence was compared to the EMBL and Genbank databases and showed 93 % homology at the nucleotide level to the murine IGFBP-5 gene.

AGGATCCGGGTACCATGGTCTGTGCTCACTCCTCTTCTCC 40

TTCCAAGACCCTCTTCCTACCATGAGTTATCACCATGGTA 80

ACATCCTCACCATCCTTCTCCCACCACCAAGGGTTGCCAC 121

AGCCTACCAAACCACCTGCCCATCCCAGGCAGGCAGCAGT 160

CCTTCTGCTCAGAACCCTTGAGGACTCTAGGTGAAAAATT 200

ACAGCCAGAGAGAGGAGTGAGCCAAGGAGAAAGGAGAAGA 240

NCCCTGTAGCCCTCTGGACTTTCAAGAGAATGGAGGTTAC 280

NNTTTAAAGCCACCAGGTAAGGAAGGGGAAAGGAAGGGCT 320

TTTCCTAATTCCAAGGCCCTTCCTTAACCTAAGGAGCCTA 360

GGGGTTGTACTGTCTTTATTTTTAAACCACTAAAGTGCAG 400

TGTTTCCTGCACTCTTGTTACTCCACCCTCTCTTCCCCGT 440

TCTTATTTCTTTGAGCAGACTTTCTTGGTGTTTTTTTAAT 480

GGAGTATAGACTTTCACCACTTCACAGGCTCTGACCTCCT 520

CTCTAAGTCTTTCTGGATGGGGAAAAGGAAGGTAGAGGGT 560

CAGAGGGGAGGGGGGGATCCTCGTCACCCCGCATCCATTC 600

ACCCCACTTCTCTGGTCCCTAGTCACCAGCCTCAGTCCCC 640

ATCTCCGACCGTCATTGTCACACAGATAGCCTGTCACACG 680

GATAGCACAGTTCAGACAAGACTCCTTCAGATTCCGAGAC 720

GCCTACCGGTTGTTTTTGGCTTTTGTTTTGTTTGTTTGTT 760

TTTTTACGACAGCAATATTCACAGCACATTATTACTATTA 800

GCTCTTTGTAGTGTTGCGTTCAGGCACCATAGCTCTGTTC 840

TCTCTTCTTTTTTGTTTTCGACTTTAAAAAAAAAAAATGC 880

TTATACTTTTTGCTGGTGAAACGGATTGAAAAAAAAATTC 920

AATAGCAAACCAGTTTGTGGGAAAAAAACAAAAAACGAAA 960

TGTGGGAAAAAAATCACCCCAATGTGGAGGGCTCAGCTCC 1000

TCTTTAGCATTTTGTACTTAAGGAAATAAAAAAGAAAAAC 1040

CTGCAAGATCTCACATTTTATTACAAGGTGAAGATTGCTG 1080

TATTCTATTTATTCAACTCATAATTTATGTTACTCCCTGA 1120

TCTTTGTCTTTCGTCGTGACAAAGCATTTATTTAATAAAA 1160

TTCCA 1165

Figure 6-3: cDNA Sequence Comparison between RSG-8 and murine IGFBP-5

The sequences of RSG-8 and murine IGFBP-5 were aligned using the PC-Gene software package and the NALIGN program. The murine IGFBP-5 (MBP5) sequence is on top with the RSG-8 sequence aligned below it. The RSG-8 sequence is 93% identical to nucleotides 4840 - 5556 of the murine 3' untranslated region. Nucleotides which are identical are indicated by the | symbol.

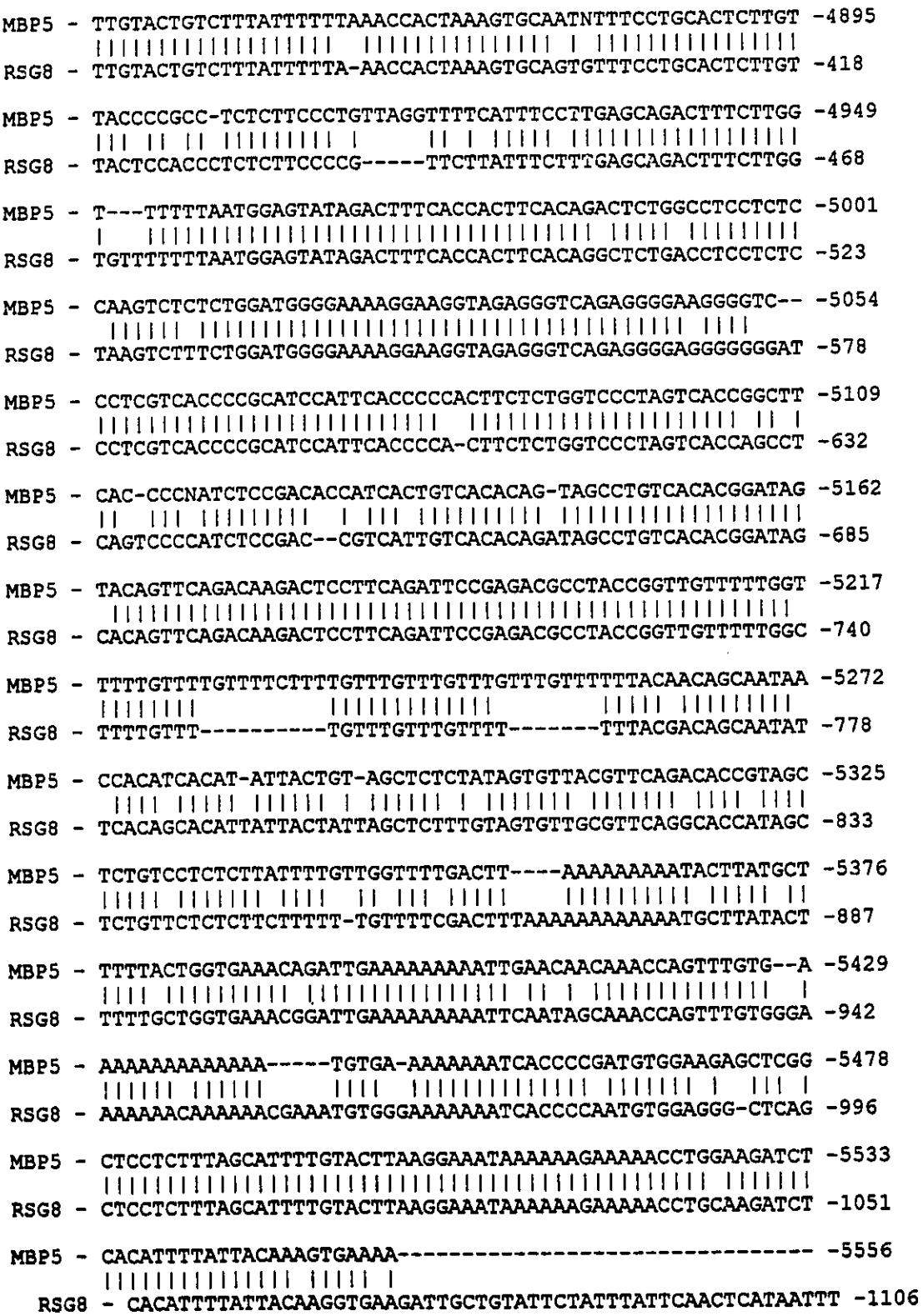

MBP5 - TTGTACTGTCTTTATTTTTTAAACCACTAAAGTGCAATNTTTCCTGCACTCTTGT -4895
RSG8 - TTGTACTGTCTTTATTTTTA-AACCACTAAAGTGCAGTGTTTCCTGCACTCTTGT -418
MBP5 - TACCCCGCC-TCTCTTCCCTGTTAGGTTTTCATTTCCTTGAGCAGACTTTCTTGG -4949
RSG8 - TACTCCACCCTCTCTTCCCCG-----TTCTTATTTCTTTGAGCAGACTTTCTTGG -468
MBP5 - T---TTTTTAATGGAGTATAGACTTTCACCACTTCACAGACTCTGGCCTCCTCTC -5001
RSG8 - TGTTTTTTTAATGGAGTATAGACTTTCACCACTTCACAGGCTCTGACCTCCTCTC -523
MBP5 - CAAGTCTCTCTGGATGGGGAAAAGGAAGGTAGAGGGTCAGAGGGGAAGGGGTC-- -5054
RSG8 - TAAGTCTTTCTGGATGGGGAAAAGGAAGGTAGAGGGTCAGAGGGGAGGGGGGGAT -578
MBP5 - CCTCGTCACCCCGCATCCATTCACCCCCACTTCTCTGGTCCCTAGTCACCGGCTT -5109
RSG8 - CCTCGTCACCCCGCATCCATTCACCCCA-CTTCTCTGGTCCCTAGTCACCAGCCT -632
MBP5 - CAC-CCCNATCTCCGACACCATCACTGTCACACAG-TAGCCTGTCACACGGATAG -5162
RSG8 - CAGTCCCCATCTCCGAC--CGTCATTGTCACACAGATAGCCTGTCACACGGATAG -685
MBP5 - TACAGTTCAGACAAGACTCCTTCAGATTCCGAGACGCCTACCGGTTGTTTTTGGT -5217
RSG8 - CACAGTTCAGACAAGACTCCTTCAGATTCCGAGACGCCTACCGGTTGTTTTTGGC -740
MBP5 - TTTTGTTTTGTTTTCTTTTGTTTGTTTGTTTGTTTGTTTTTTACAACAGCAATAA -5272
RSG8 - TTTTGTTT----------TGTTTGTTTGTTTT-------TTTACGACAGCAATAT -778
MBP5 - CCACATCACAT-ATTACTGT-AGCTCTCTATAGTGTTACGTTCAGACACCGTAGC -5325
RSG8 - TCACAGCACATTATTACTATTAGCTCTTTGTAGTGTTGCGTTCAGGCACCATAGC -833
MBP5 - TCTGTCCTCTCTTATTTTGTTGGTTTTGACTT----AAAAAAAAATACTTATGCT -5376
RSG8 - TCTGTTCTCTCTTCTTTTT-TGTTTTCGACTTTAAAAAAAAAAAATGCTTATACT -887
MBP5 - TTTTACTGGTGAAACAGATTGAAAAAAAAATTGAACAACAAACCAGTTTGTG--A -5429
RSG8 - TTTTGCTGGTGAAACGGATTGAAAAAAAAATTCAATAGCAAACCAGTTTGTGGGA -942
MBP5 - AAAAAAAAAAAAA-----TGTGA-AAAAAAATCACCCCGATGTGGAAGAGCTCGG -5478
RSG8 - AAAAAACAAAAAACGAAATGTGGGAAAAAAATCACCCCAATGTGGAGGG-CTCAG -996
MBP5 - CTCCTCTTTAGCATTTTGTACTTAAGGAAATAAAAAAGAAAAACCTGGAAGATCT -5533
RSG8 - CTCCTCTTTAGCATTTTGTACTTAAGGAAATAAAAAAGAAAAACCTGCAAGATCT -1051
MBP5 - CACATTTTATTACAAAGTGAAAA---------- -5556
RSG8 - CACATTTTATTACAAGGTGAAGATTGCTGTATTCTATTTATTCAACTCATAATTT -1106

Figure 6-4: RT-PCR Cloning Strategy For IGFBP-5

Upstream and downstream primers complementary to IGFBP-5 containing EcoRI and NotI 5' overhangs (shown as EN and NE respectively) were designed which would amplify the entire coding region of each gene. The downstream primer was used to prime first strand cDNA synthesis as described in section 2.620. The cDNA product from this reaction was used as a template in a PCR amplification reaction using both IGFBP-5 specific upstream and downstream primers. The PCR products were digested with EcoRI and sub-cloned into the pGEM7Zf- plasmid vector.

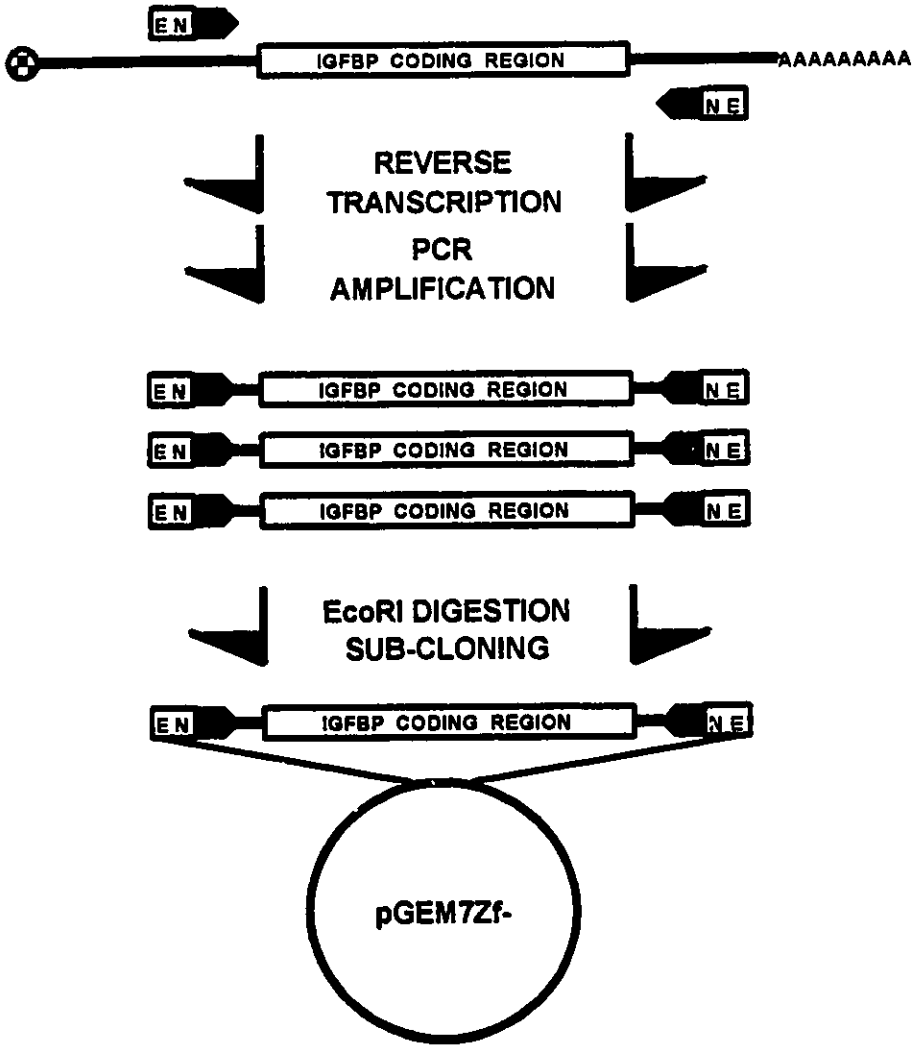
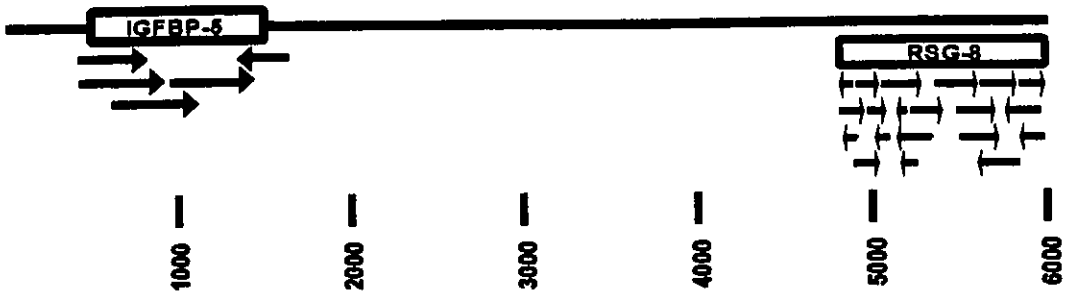

EN
IGFBP CODING REGION
AAAAAAAAA
NE
REVERSE
TRANSCRIPTION
PCR
AMPLIFICATION
EN
IGFBP CODING REGION
NE
EN
IGFBP CODING REGION
NE
EN
IGFBP CODING REGION
NE
EcoRI DIGESTION
SUB-CLONING
EN
IGFBP CODING REGION
NE
pGEM7Zf-

Figure 6-5: Sequencing Strategy and Sequence of IGFBP-5 RT-PCR Clone

Panel A: The IGFBP-5 RT-PCR product was subcloned into the EcoRI site of the pGEM7Zf- vector. A set of overlapping sequences were obtained using sequence specific primers in standard sequencing reactions. The overlapping sequences covered the complete 964 bases of the IGFBP-5 clone. The complete sequence of the IGFBP-5 mRNA has not yet been determined. The portion of the IGFBP-5 mRNAs 3' untranslated region covered by the RSG-8 cDNAs is indicated.

Panel B: The complete sequence (964 nucleotides) of the IGFBP-5 clone, including part of the 5' and 3' untranslated regions and the complete coding region. Comparison to the EMBL and Genbank databases showed 100 % homology at the nucleotide level to the rat IGFBP-5 gene. The coding region predicts a protein of 271 amino acids. The conserved cysteine residues which are found in all members of the IGFBP family are in bold.



A

```
TCCACACACTCTCGCTCTCCTGCCCCGCCCCGAGGTAAAGCCAGACTCGGAAAAAATGGT      60
                                                          M  V
GATCAGCGTGGTCCTCCTGCTGCTGGCCGCCTGTGCCGTGCCGGCTCAAGGCCTGGGCTC     120
  I  S  V  V  L  L  L  L  A  A  C  A  V  P  A  Q  G  L  G  S
TTTCGTGCATTGTGAACCCTGCGACGAGAAAGCTCTGTCCATGTGTCCCCCCAGCCCTCT     180
  F  V  H  C  E  P  C  D  E  K  A  L  S  M  C  P  P  S  P  L
GGGCTGTGAGCTGGTCAAAGAGCCCGGCTGTGGCTGCTGCATGACTTGCGCCCTGGCGGA     240
  G  C  E  L  V  K  E  P  G  C  G  C  C  M  T  C  A  L  A  E
GGGACAGTCGTGTGGCGTCTACACTGAGCGCTGCGCCCAGGGTTTGCGCTGTCTCCCCCG     300
  G  Q  S  C  G  V  Y  T  E  R  C  A  Q  G  L  R  C  L  P  R
GCAGGATGAGGAGAAGCCGCTGCACGCCCTGCTGCACGGCCGCGGGGTTTGCCTCAACGA     360
  Q  D  E  E  K  P  L  H  A  L  L  H  G  R  G  V  C  L  N  E
AAAGAGCTACGGCGAGCAAACCAAGATAGAGAGAGACTCTCGGGAGCATGAGGAACCCAC     420
  K  S  Y  G  E  Q  T  K  I  E  R  D  S  R  E  H  E  E  P  T
CACCTCCGAGATGGCTGAGGAGACCTACTCCCCGAAGGTCTTCCGGCCCAAGCACACTCG     480
  T  S  E  M  A  E  E  T  Y  S  P  K  V  F  R  P  K  H  T  R
CATTTCCGAGCTGAAGGCCGAGGCTGTGAAGAAGGATCGCAGAAAGAAGCTGACCCAGTC     540
  I  S  E  L  K  A  E  A  V  K  K  D  R  R  K  K  L  T  Q  S
TAAGTTTGTGGGGGGCGCGGAGAACACTGCCCACCCCCGAGTCATCCCTGCACCTGAGAT     600
  K  F  V  G  G  A  E  N  T  A  H  P  R  V  I  P  A  P  E  M
GAGACAGGAATCTGACCAAGGCCCCTGCCGCAGACACATGGAAGCTTCCCTCCAGGAGTT     660
  R  Q  E  S  D  Q  G  P  C  R  R  H  M  E  A  S  L  Q  E  F
CAAAGCCAGCCCACGCATGGTGCCCCGTGCCGTGTACCTGCCCAACTGTGACCGCAAAGG     720
  K  A  S  P  R  M  V  P  R  A  V  Y  L  P  N  C  D  R  K  G
ATTCTACAAGAGAAAGCAGTGCAAGCCTTCTCGTGGCCGCAAACGTGGCATCTGCTGGTG     780
  F  Y  K  R  K  Q  C  K  P  S  R  G  R  K  R  G  I  C  W  C
TGTGGACAAGTATGGGATGAAGCTGCCGGGCATGGAGTACGTCGATGGGGACTTTCAGTG     840
  V  D  K  Y  G  M  K  L  P  G  M  E  Y  V  D  G  D  F  Q  C
CCACGCCTTCGACAGCAGTAACGTTGAGTGACGCGTCCCCTCCCTTCCTCCCCCTTTCCT     900
  H  A  F  D  S  S  N  V  E  -
ACCCCCCAGCCCCAACTCCAGCCAGCGCCTCCCTCCACCCCAGGACGTCACTCATTTCAT     960
CTCA
```

B

and shows complete homology to the sequence published by Shimasaki (Shimasaki et al., 1991) (Fig. 6-5).

6.200 - Expression of IGFBP-5 mRNA

The cDNA insert from the R8A clone was amplified by PCR and hybridized to Northern blots of poly(A)+ RNA extracted from the ventral prostate at different times after castration and from the mammary gland at different times after weaning. The cDNA insert for R8A, hybridizes to an mRNA of approximately 6.0 kb which is expressed at very low levels in the normal prostate and lactating mammary glands, but shows a dramatic induction in these tissues after ablation of the appropriate trophic hormone (Fig. 6-6). Expression of IGFBP-5 mRNA reaches a maximal level in the prostate 4 days after castration, at which time the level of epithelial cell death in the gland is at a maximum. In the mammary gland the expression of IGFBP-5 mRNA is also increased during regression, reaching a maximum between 2 and 4 days after weaning, before diminishing to background levels by day 8. The smearing observed in the mammary samples is not due to general RNA degradation, as the same mammary RNA samples when probed with β-casein produce a distinct band (data not shown). Control hybridizations using α-tubulin cDNA on duplicate blots demonstrated that the increase in the steady state levels of IGFBP-5 mRNA is specific and not due to loading artifacts or to changes in the rRNA content of the tissues (results not shown).

6.300 - Localization of IGFBP-5 mRNA

To localize the expression of IGFBP-5 mRNA, *in situ* hybridization was performed on random sections from normal and regressing prostate (Fig. 6-7) and lactating and regressing mammary gland (Fig. 6-8). The IGFBP-5 mRNA localizes to the epithelial cells of both tissues. In intact male animals IGFBP-5 mRNA showed hybridization in the prostate that is barely above background (Fig. 6-7, panel B) which correlates with the expression observed in normal prostate on Northern analysis. The increased expression of IGFBP-5 mRNA is detectable in the luminal epithelial cells of the

Figure 6-6: Northern Analysis of IGFBP-5 Steady-State mRNA Levels in the Regressing Prostate and Mammary Gland

Poly(A)+ RNA was extracted from the rat ventral prostate at different times (0, 1, 2, 3, and 4 days) after castration (panel A) or from the rat mammary gland at different times (0, 2, 4 , 6 and 8 days) after weaning (panel B). 2 μg per lane were electrophoresed on formaldehyde gels, transferred to nylon membranes, and hybridized to a ^{32}P-labeled cDNA specific for IGFBP-5 ($1x10^8$ cpm/μg). The membranes were exposed for 18 h. The position of the 6,000 nucleotide IGFBP-5 mRNA was established by comparison to the migration of 18S and 28S ribosomal RNA.

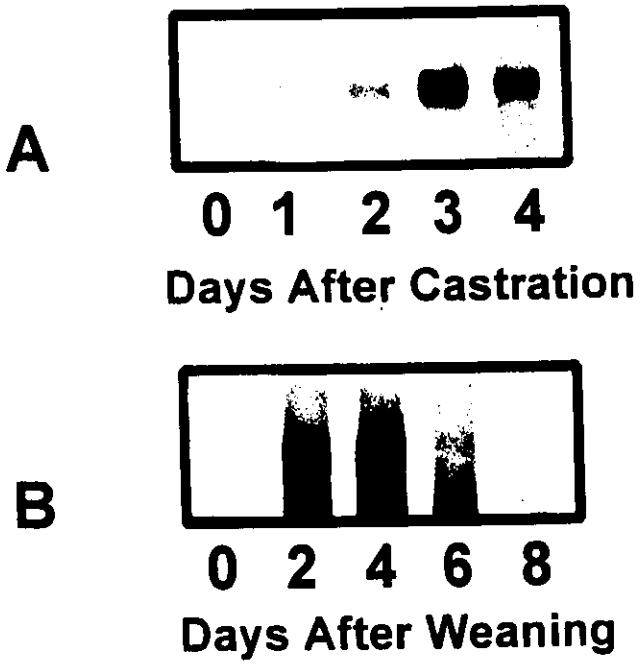

A
0 1 2 3 4
Days After Castration
B
0 2 4 6 8
Days After Weaning

Figure 6-7: *In Situ* Hybridization of IGFBP-5 mRNA in Regressing Rat Ventral Prostate

Random sections from the rat ventral prostate, excised at different times after castration, were hybridized to a ^{35}S-labeled cDNA insert specific for IGFBP-5 ($1x10^6$ cpm). The sections were exposed for 4 days. Magnification X20.

Panels A, C, E:	phase contrast showing morphology of sections
Panels B, D, F:	dark field illumination showing *in situ* hybridization
Panels A and B:	prostate from control animals
Panels C and D:	prostate 2 days after castration
Panels E and F:	prostate 4 days after castration

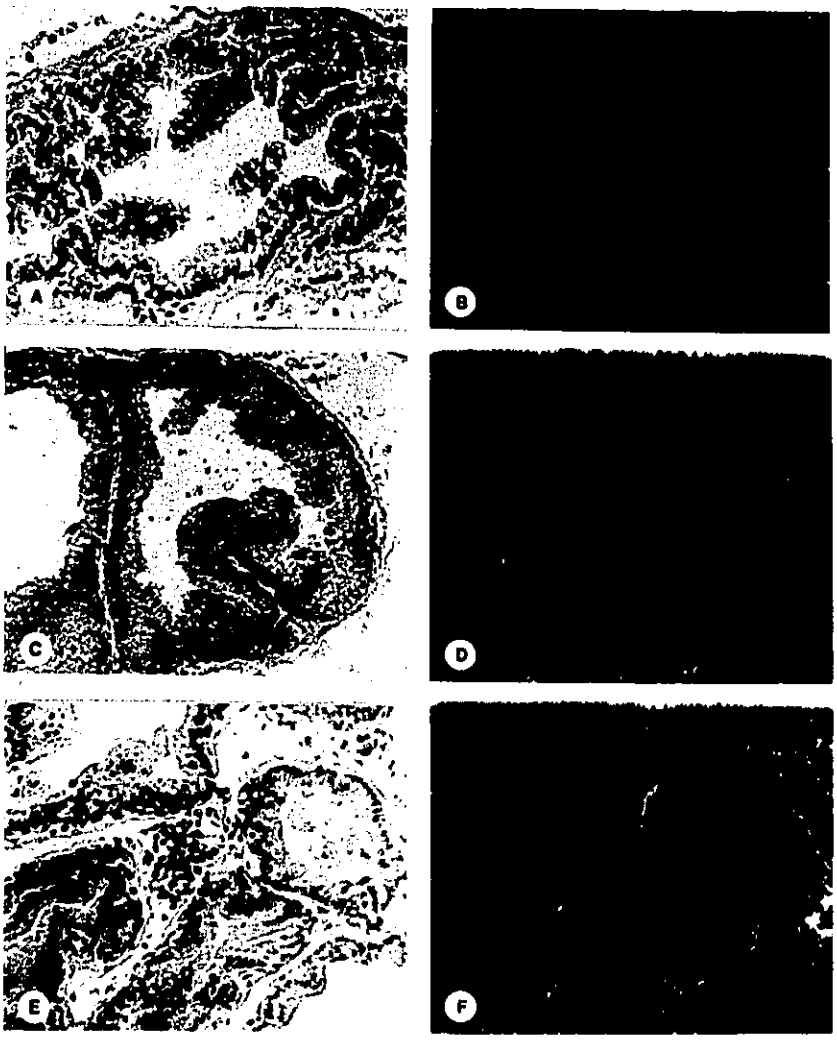
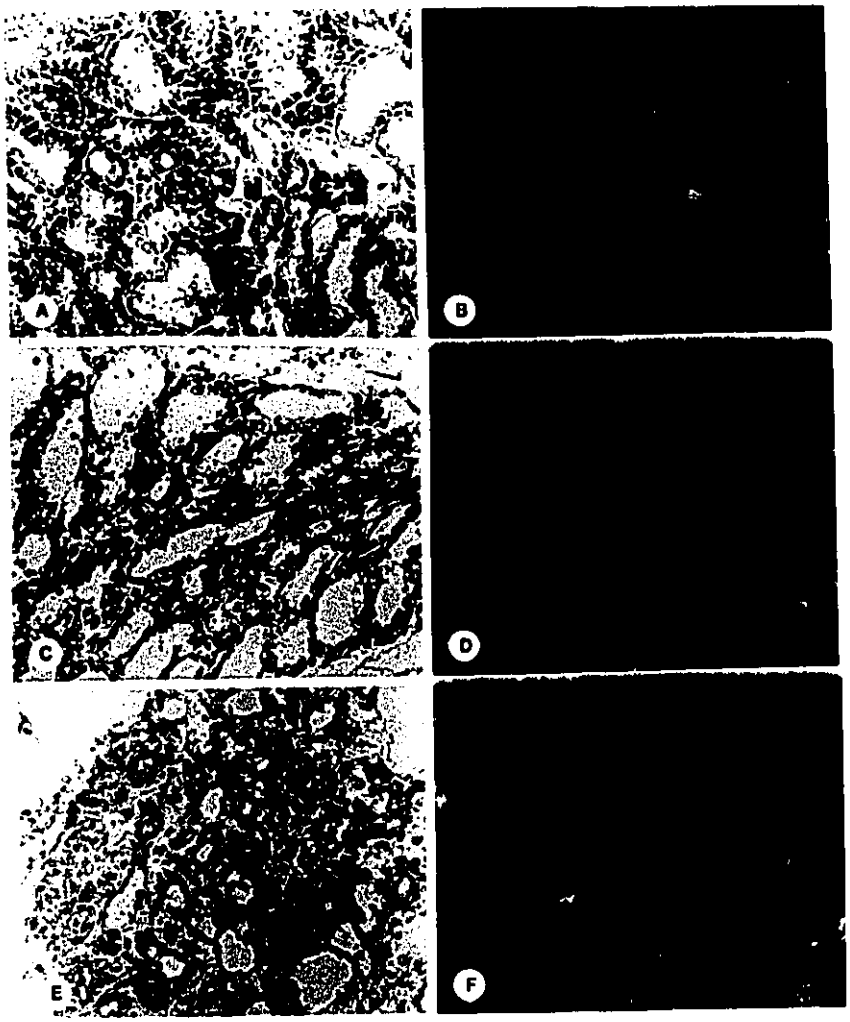

A
B
C
D
E
F

Figure 6-8: *In Situ* Hybridization of IGFBP-5 mRNA in Regressing Rat Mammary Gland

Random sections from the rat mammary gland, excised at different times after weaning, were hybridized to a ^{35}S-labeled cDNA insert specific for IGFBP-5 ($1x10^6$ cpm). The sections were exposed for 4 days. Magnification X20.

Panels A, C, E:	phase contrast showing morphology of sections
Panels B, D, F:	dark field illumination showing *in situ* hybridization
Panels A and B:	mammary glands from lactating dams
Panels C and D:	mammary gland 2 days after weaning
Panels E and F:	mammary gland 4 days after weaning


A
B
C
D
E
F

prostate two days after castration (Fig. 6-7, panel D) and reaches maximal levels on day 4 (Fig. 6-7, panel F). The density of grains over the stroma remains relatively constant throughout the time course with no significant increase above background during regression. The expression of IGFBP-5 mRNA in the regressing mammary gland shows a similar pattern of expression to that seen in the prostate. The mRNA is present at very low levels in the lactating mammary gland, and the expression appears to be confined to the luminal epithelium (Fig. 6-8, panel B). After weaning, the expression of IGFBP-5 mRNA increases significantly in the luminal epithelium, reaching a peak 4 days after weaning (Fig. 6-8, panel D). The expression subsequently decreases to baseline levels by 6 days after weaning (Fig. 6-8, panel F), paralleling the changes in the steady state level of the mRNA observed on Northern analysis. The enhanced expression of the mRNA appears to be restricted to the luminal epithelium, although it is possible that there is a small increase in IGFBP-5 expression in the stroma and myo-epithelium that is masked by the increase in the epithelial expression.

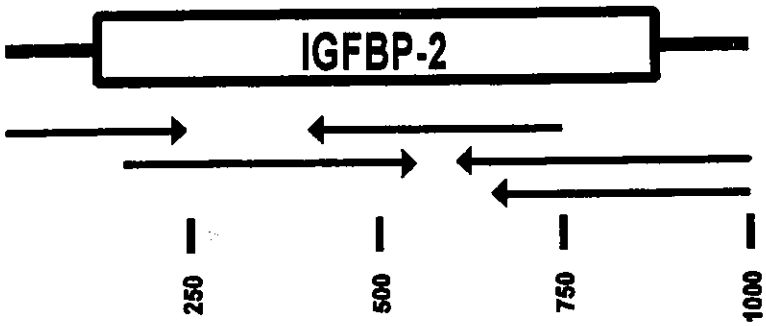
6.400 - Analysis of IGFBP-2

In order to characterize the involvement of other IGFBPs in the ACD process we cloned the cDNA for IGFBP-2 using an identical strategy to that described for IGFBP-5 (see section 6.100 this chapter, section 2.620 and Fig. 6-4). The complete sequence of the IGFBP-2 RT-PCR clone was determined, and shows complete homology to the sequence published by Margot (Margot et al., 1989) (Fig. 6-9). The literature describes a pattern of expression for IGFBP-2 and IGFBP-5 during development in which the mRNAs for these two genes often co-localize to similar cell compartments (Schuller et al., 1993; Green et al., 1994). Following development IGFBP-2 expression remains, consistent with its proposed constitutive function and the expression of IGFBP-5 is down regulated (Schuller et al., 1993; Green et al., 1994). We predicted based on this information that IGFBP-2 would not show as dramatic an induction during ACD and would be constitutively expressed in normal prostate and mammary gland.

Figure 6-9: Sequencing Strategy and Sequence of IGFBP-2 RT-PCR Clone

Panel A: The IGFBP-2 RT-PCR product was subcloned into the EcoRI site of the pGEM7Zf- vector. A set of overlapping sequences were obtained using sequence specific primers in standard sequencing reactions. The overlapping sequences covered the complete 974 bases of the IGFBP-2 clone.

Panel B: The complete sequence (974 nucleotides) of the IGFBP-2 clone, including part of the 5' and 3' untranslated regions and the complete coding region. Comparison to the EMBL and Genbank databases showed 100 % homology at the nucleotide level to the rat IGFBP-2 gene. The coding region predicts a protein of 304 amino acids. The conserved cysteine residues which are found in all members of the IGFBP family are in bold.



A

```
TAGCTCGCCGCGCTACGGTTTCCCACTAGCCAACATGCTGCCGAGATTGGGCGGCCCCGC      60
                                        M  L  P  R  L  G  G  P  A
GCTGCCGCTGCTCCTGCCGTCGCTGCTCTTGCTGCTGCTCTTGGGCGCGGGCGGTTGCGG     120
  L  P  L  L  L  P  S  L  L  L  L  L  L  G  A  G  G  C  G
TCCTGGGGTGCGCGCCGAGGTGCTGTTCCGCTGCCCACCCTGCACGCCCGAGCGTCTGGC     180
  P  G  V  R  A  E  V  L  F  R  C  P  P  C  T  P  E  R  L  A
CGCCTGCGGACCCCCACCCGACGCGCCCTGCGCCGAGCTGGTGCGAGAGCCCGGCTGCGG     240
  A  C  G  P  P  P  D  A  P  C  A  E  L  V  R  E  P  G  C  G
TTGCTGCTCCGTGTGCGCACGACAGGAGGGCGAAGCTTGCGGCGTCTACATCCCGCGCTG     300
  C  C  S  V  C  A  R  Q  E  G  E  A  C  G  V  Y  I  P  R  C
CGCCCAGACGTTACGCTGTTACCCCAACCCGGGCTCCGAGCTGCCCCTGAAGGCACTGGT     360
  A  Q  T  L  R  C  Y  P  N  P  G  S  E  L  P  L  K  A  L  V
CACCGGCGCGGGTACCTGTGAAAAGAGACGCGTGGGCGCCACCCCACAGCAGGTTGCAGA     420
  T  G  A  G  T  C  E  K  R  R  V  G  A  T  P  Q  Q  V  A  D
CAGTGAGGATGACCACTCGGAGGGAGGCCTGGTGGAGAACCATGTGGACGGAACCATGAA     480
  S  E  D  D  H  S  E  G  G  L  V  E  N  H  V  D  G  T  M  N
CATGTTGGGAGGCAGCAGTGCTGGCCGGAAGCCCCCTAAGTCAGGCATGAAGGAACTGGC     540
  M  L  G  G  S  S  A  G  R  K  P  P  K  S  G  M  K  E  L  A
TGTGTTCCGGGAGAAGGTCAACGAGCAGCACCGGCAGATGGGCAAAGGTGCCAAACACCT     600
  V  F  R  E  K  V  N  E  Q  H  R  Q  M  G  K  G  A  K  H  L
CAGCCTGGAGGAGCCCAAGAAGCTGCGCCCACCTCCTGCCAGGACCCCTTGCCAGCAGGA     660
  S  L  E  E  P  K  K  L  R  P  P  P  A  R  T  P  C  Q  Q  E
GCTGGACCAGGTCCTGGAGCGCATCTCCACCATGCGCCTTCCGGATGATCGGGGTCCTCT     720
  L  D  Q  V  L  E  R  I  S  T  M  R  L  P  D  D  R  G  P  L
GGAACATCTCTACTCCCTGCATATCCCCAACTGTGACAAGCATGGCCTGTACAACCTCAA     780
  E  H  L  Y  S  L  H  I  P  N  C  D  K  H  G  L  Y  N  L  K
ACAGTGCAAGATGTCTCTGAATGGACAGCGTGGGGAGTGCTGGTGTGTGAACCCCAATAC     840
  Q  C  K  M  S  L  N  G  Q  R  G  E  C  W  C  V  N  P  N  T
TGGGAAGCCAATCCAGGGAGCTCCCACCATCCGGGGAGACCCCGAGTGCCATCTCTTCTA     900
  G  K  P  I  Q  G  A  P  T  I  R  G  D  P  E  C  H  L  F  Y
CAACGAGCAGCAGGAGAATGATGGGGCTCACGCCCAAAGGGTGCAGTAAACCACAGCCAG     960
  N  E  Q  Q  E  N  D  G  A  H  A  Q  R  V  Q  -
TCGGTGCCTGGCTT
```

B

6.410 - Expression of IGFBP-2 mRNA

The cDNA insert from the IGFBP-2 clone was amplified by PCR and hybridized to Northern blots of poly(A)+ RNA extracted from the ventral prostate at different times after castration and from the mammary gland at different times after weaning. The cDNA insert for IGFBP-2, hybridizes to an mRNA of approximately 2.0 kb which is expressed constituitvely at significant levels in the normal prostate and lactating mammary glands (Fig. 6-10). Although the changes are not dramatic, expression of IGFBP-2 mRNA increases slightly in the prostate 4 days after castration. In the mammary gland the expression of IGFBP-2 mRNA is highly induced during regression, between 1 and 4 days after weaning, before diminishing to control levels by day 8.

6.420 - Localization of IGFBP-2 mRNA:

To localize the expression of IGFBP-2 mRNA, *in situ* hybridization was performed on random sections from normal and regressing prostate (Fig. 6-11) and lactating and regressing mammary gland (Fig. 6-12). The IGFBP-2 mRNA localizes to the epithelial cells of both tissues. In intact male animals IGFBP-2 mRNA showed hybridization in the normal prostate that is significantly above background (Fig. 6-11, panel B) and which correlates with the expression observed in normal prostate on Northern analysis. The slight increase in the expression of IGFBP-2 mRNA is detectable in the luminal epithelial cells of the prostate by day 2 (Fig. 6-11, panel D) and day 4 (Fig. 6-11, panel F) after castration. The density of grains over the stroma remains relatively constant throughout the time course with no significant increase above background during regression. The expression of IGFBP-2 mRNA in the regressing mammary gland shows a similar pattern of expression to that seen in the prostate. The mRNA is present in the lactating mammary gland, and the expression appears to be confined to the luminal epithelium (Fig. 6-12, panel B). After weaning, the expression of IGFBP-2 mRNA increases slightly in the luminal epithelium, reaching a peak 4 days after weaning (Fig. 6-12, panel D). The expression subsequently decreases to normal levels by 6 days after weaning (Fig. 6-12, panel F), paralleling the changes in the steady state level of the mRNA observed on Northern analysis. The enhanced expression of the mRNA appears to

Figure 6-10: Northern Analysis of IGFBP-2 Steady-State mRNA Levels in the Regressing Prostate and Mammary Gland

Poly(A)+ RNA was extracted from the rat ventral prostate at different times (0, 1, 2, 3, and 4 days) after castration (panel A) or from the rat mammary gland at different times (0, 1, 4 , 6 and 8 days) after weaning (panel B). 2 μg per lane were electrophoresed on formaldehyde gels, transferred to nylon membranes, and hybridized to a ^{32}P-labeled cDNA specific for IGFBP-2 ($1x10^8$ cpm/μg). The membranes were exposed for 48 h. The position of the 2,000 nucleotide IGFBP-2 mRNA was established by comparison to the migration of 18S and 28S ribosomal RNA.

A

0 1 2 3 4

Days After Castration

B

0 1 4 6 8

Days After Weaning

Figure 6-11: ***In Situ*** **Hybridization of IGFBP-2 mRNA in Regressing Rat Ventral Prostate**

Random sections from the rat ventral prostate, excised at different times after castration, were hybridized to a ^{35}S-labeled cDNA insert specific for IGFBP-2 ($1x10^6$ cpm). The sections were exposed for 4 days. Magnification X20.

Panels A, C, E:	phase contrast showing morphology of sections
Panels B, D, F:	dark field illumination showing *in situ* hybridization
Panels A and B:	prostate from control animals, RNase treated
Panels C and D:	prostate 2 days after castration
Panels E and F:	prostate 4 days after castration

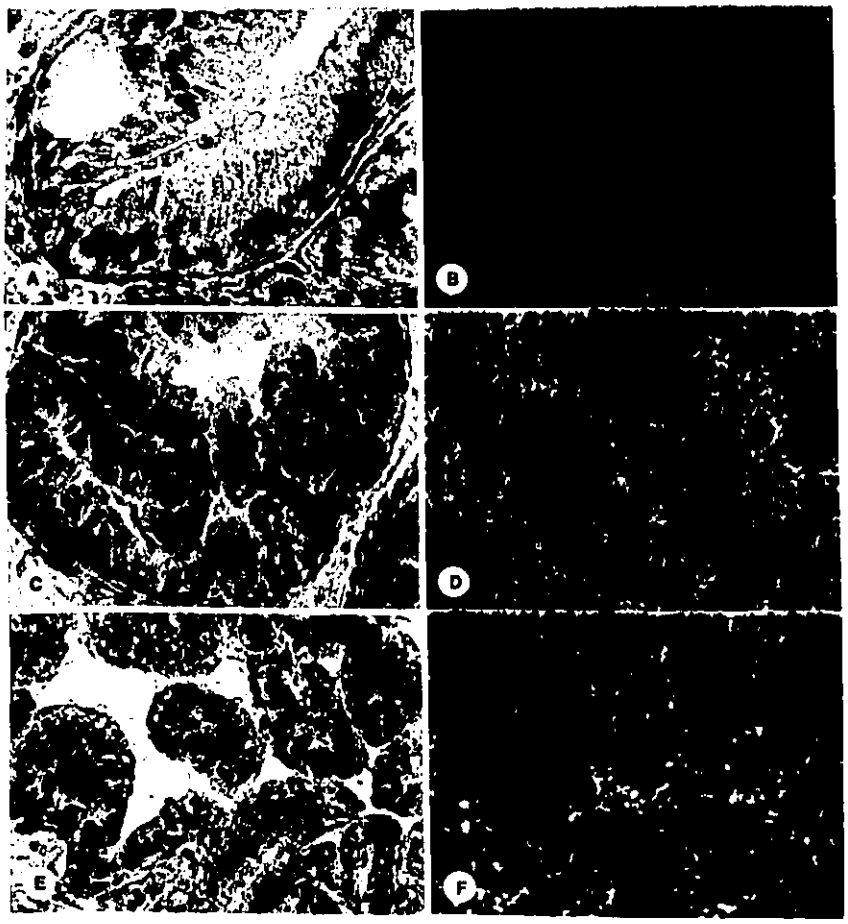
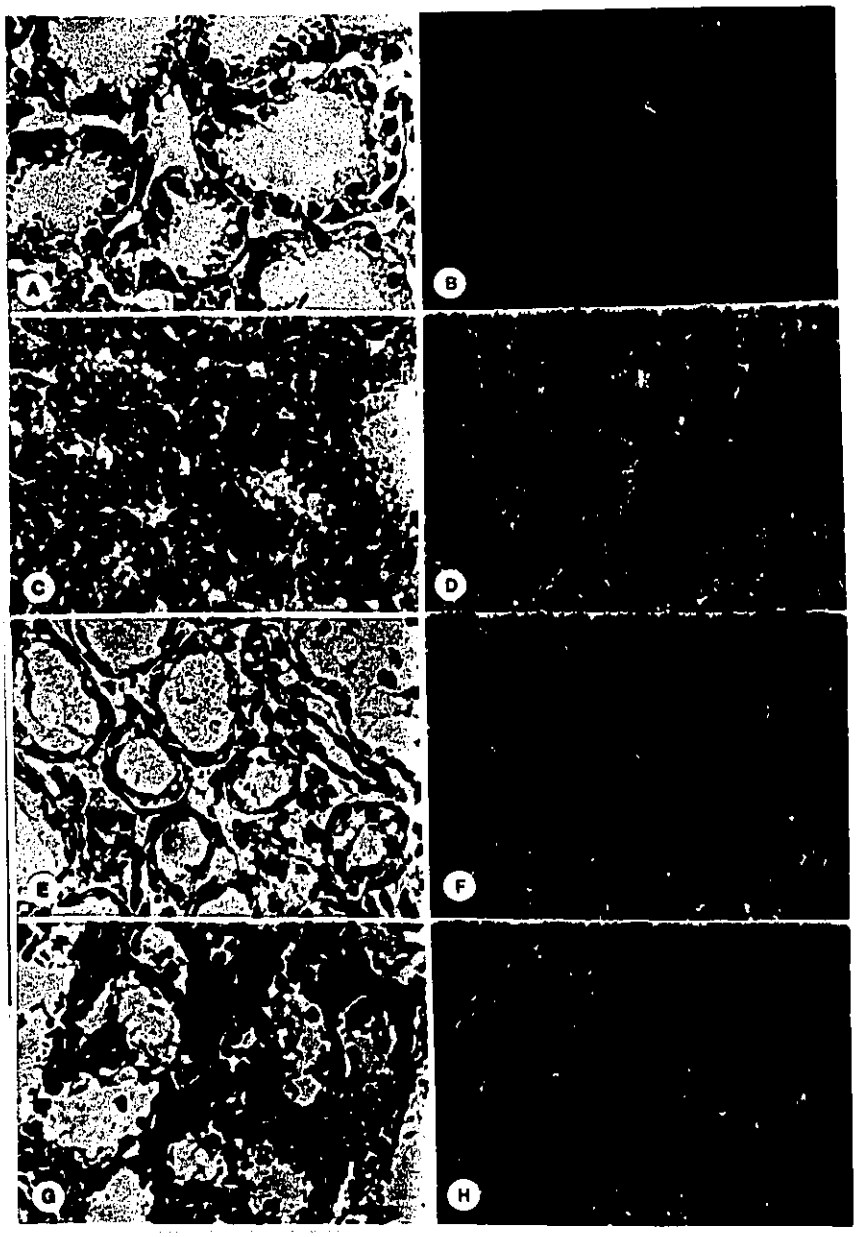

A
B
C
D
E
F

Figure 6-12: *In Situ* Hybridization of IGFBP-2 mRNA in Regressing Rat Mammary Gland

Random sections from the rat mammary gland, excised at different times after weaning, were hybridized to a ^{35}S-labeled cDNA insert specific for IGFBP-2 ($1x10^6$ cpm). The sections were exposed for 4 days. Magnification X20.

Panels A, C, E, H:	dark field illumination showing *in situ* hybridization
Panels B, D, F, G:	phase contrast showing morphology of sections
Panels A and B:	mammary glands from lactating dams, RNase treated
Panels C and D:	mammary gland from lactating dams
Panels E and F:	mammary gland 2 days after weaning
Panels G and H:	mammary gland 4 days after weaning


A
B
C
D
E
F
G
H

be restricted to the luminal epithelium, although it is possible that there is a small increase in IGFBP-2 expression in the stroma and myo-epithelium that is masked by the increase in the epithelial expression.

6.500 - DISCUSSION

Characterization of RSG-8 provided further evidence for its potential role in ACD. Sequence analysis of RSG-8 demonstrated that the cDNA is specific for IGFBP-5, a member of a small family of proteins which bind to IGF-I to modulate its actions. Northern analysis revealed that the mRNA for IGFBP-5 was induced de-novo in both regressing prostate and mammary tissue. The observed induction of IGFBP-5 mRNA was localized to the luminal epithelial cells of both glands, which are deleted during ACD, and further correlates the expression of IGFBP-5 with this process. In contrast to IGFBP-5, the mRNA for IGFBP-2 shows constitutive expression in both prostate and mammary gland with only a slight induction in the prostate during ACD. The identification of IGFBP-5 as an mRNA induced during ACD, suggests that the modulation of cellular response to the IGF growth factors, by altering expression of the various members of the IGFBP family, may play a role in the control and initiation of the ACD pathway.

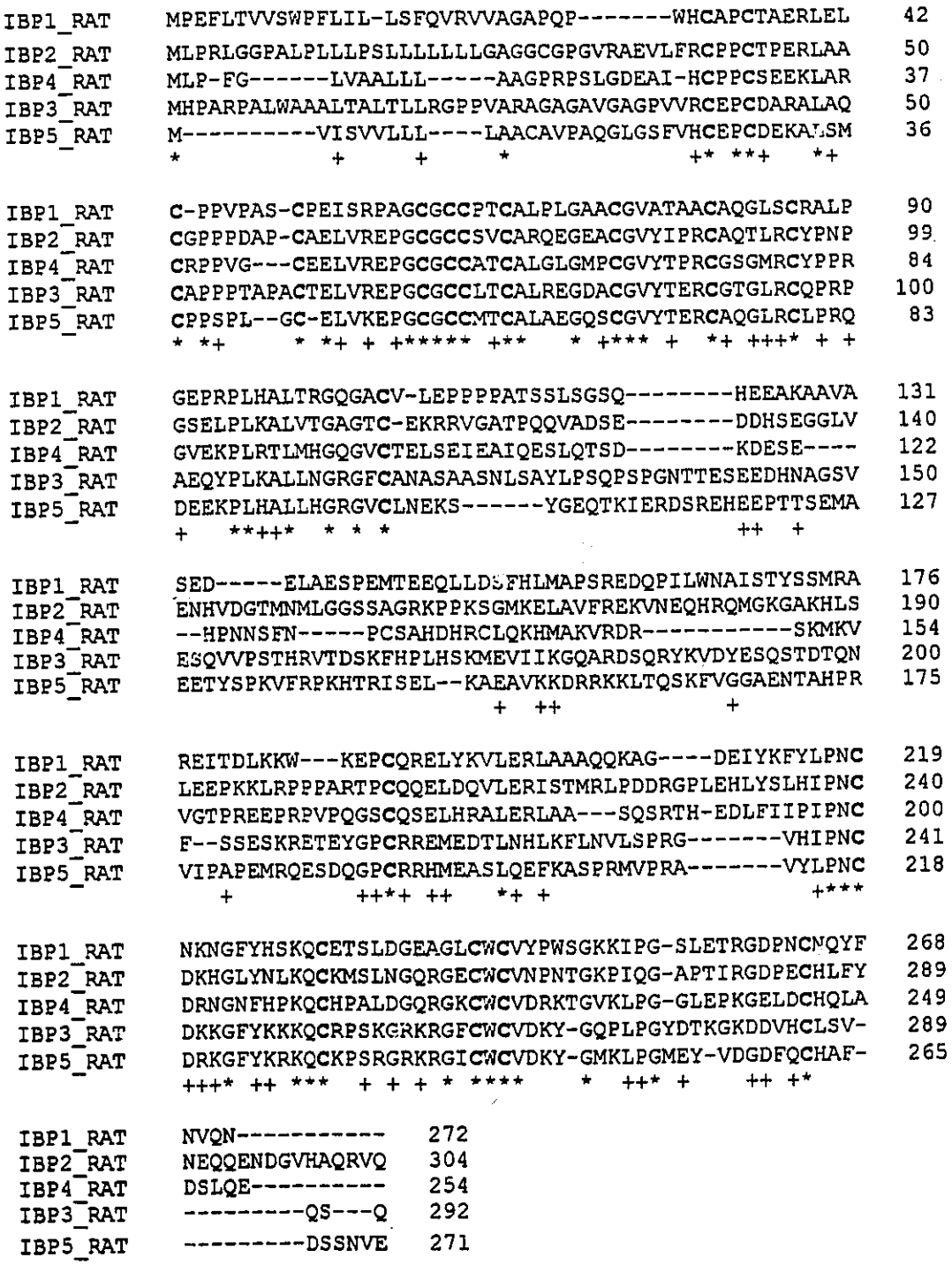
Involution of the prostate or lactating mammary gland results in the elimination of most of the secretory epithelial cells of the glands, and requires RNA and protein synthesis (Lee, 1981; Tenniswood et al., 1992). R_0t analysis has suggested that between 20-30 mRNA species are up-regulated in the regressing prostate (Montpetit et al., 1986). Some of these genes, including TRPM-2/clusterin, tissue transglutaminase and Hsp27, are induced *de novo* (Léger et al., 1987; Rouleau et al., 1990; Guenette et al., 1994a and 1994b). A few of the genes induced after hormone ablation are primary thanatogens that trigger ACD, while most others represent secondary thanatogens that ensure that the process is completed appropriately, without leakage of the intracellular components (Tenniswood et al., 1994). To date no primary thanatogens have been unequivocally identified, although it has been suggested that c-myc may trigger ACD in a variety of cell types (Evan et al., 1992). However, with the exception of the early induction of c-myc in

the prostate after castration (Buttyan et al., 1988), there is no functional evidence implicating the gene in the induction of ACD in the prostate or mammary gland. Several lines of evidence suggest that the normal function of both prostate and mammary epithelial cells is dependent on a complex interplay between epithelial cells, the extracellular matrix, and the underlying mesenchyme or stroma. Cunha and co-workers have demonstrated in elegant reconstruction experiments using the embryonic mesenchyme from normal and testicular feminized rats (Tfm) combined with normal and Tfm epithelium, that the main determinant influencing the production of a normal functional epithelium is the influence of the mesenchme it interacts with (Cuhna et al., 1987).

One possible mechanism for mesenchymal influence on normal epithelial function is via the release of growth factors. There is considerable evidence indicating that the growth and proliferation of epithelial cells in both the prostate and mammary gland are influenced by factors such as epidermal growth factor, (EGF), transforming growth factor (TGF) and insulin like growth factors (IGF). In this thesis I report the first evidence that the attenuation of the epithelial response to one particular family of growth factors, namely IGFs may be responsible for ACD in the prostate and mammary gland. A cross-screening technique designed to clone messages induced during ACD in the regressing prostate and mammary gland identified the mRNA for insulin-like growth factor binding protein 5 (IGFBP-5). The IGFBPs are a family of 6 proteins which bind to and modulate the effects of both IGF-I and IGF-II. Both human and rat IGFBPs share a highly conserved amino acid sequence particularily in the amino and carboxyl terminus, including 18 conserved cysteines, with the exception of IGFBP-6 which has 16 in human and 14 in rat (Fig. 6-13). IGFBP-1 and IGFBP-2 both have an RGD sequence located in their carboxyl terminus, and therefore may associate with integrins. The different members of the IGFBP family vary with respect to their post-translational modifications such as glycosylation, phosphorylation, and proteolytic cleavage. The expression of the various IGFBPs differs widely from tissue to tissue.

Figure 6-13: Alignment and Comparison of the Protein Sequences of the Rat IGFBPs

The sequences of 5 rat IGFBPs were aligned using the PC-Gene software package and the PALIGN program. Conserved cysteines characteristic of the IGFBPs are in bold. The * indicates a residue that is perfectly conserved, the + indicates a residue which is well conserved. The individual IGFBPs show the most similarity in the internal portions of the molecules. Sequences at the amino and carboxyl terminal are more divergent, and are used to identify the various members.


IBP1_RAT MPEFLTVVSWPFLIL-LSFQVRVVAGAPQP-------WHCAPCTAERLEL 42
IBP2_RAT MLPRLGGPALPLLLPSLLLLLLLGAGGCGPGVRAEVLFRCPPCTPERLAA 50
IBP4_RAT MLP-FG------LVAALLL-----AAGPRPSLGDEAI-HCPPCSEEKLAR 37
IBP3_RAT MHPARPALWAAALTALTLLRGPPVARAGAGAVGAGPVVRCEPCDARALAQ 50
IBP5_RAT M----------VISVVLLL----LAACAVPAQGLGSFVHCEPCDEKALSM 36
IBP1_RAT C-PPVPAS-CPEISRPAGCGCCPTCALPLGAACGVATAACAQGLSCRALP 90
IBP2_RAT CGPPPDAP-CAELVREPGCGCCSVCARQEGEACGVYIPRCAQTLRCYPNP 99
IBP4_RAT CRPPVG---CEELVREPGCGCCATCALGLGMPCGVYTPRCGSGMRCYPPR 84
IBP3_RAT CAPPPTAPACTELVREPGCGCCLTCALREGDACGVYTERCGTGLRCQPRP 100
IBP5_RAT CPPSPL--GC-ELVKEPGCGCCMTCALAEGQSCGVYTERCAQGLRCLPRQ 83
IBP1_RAT GEPRPLHALTRGQGACV-LEPPPPATSSLSGSQ--------HEEAKAAVA 131
IBP2_RAT GSELPLKALVTGAGTC-EKRRVGATPQQVADSE--------DDHSEGGLV 140
IBP4_RAT GVEKPLRTLMHGQGVCTELSEIEAIQESLQTSD--------KDESE---- 122
IBP3_RAT AEQYPLKALLNGRGFCANASAASNLSAYLPSQPSPGNTTESEEDHNAGSV 150
IBP5_RAT DEEKPLHALLHGRGVCLNEKS------YGEQTKIERDSREHEEPTTSEMA 127
IBP1_RAT SED-----ELAESPEMTEEQLLDSFHLMAPSREDQPILWNAISTYSSMRA 176
IBP2_RAT ENHVDGTMNMLGGSSAGRKPPKSGMKELAVFREKVNEQHRQMGKGAKHLS 190
IBP4_RAT --HPNNSFN-----PCSAHDHRCLQKHMAKVRDR----------SKMKV 154
IBP3_RAT ESQVVPSTHRVTDSKFHPLHSKMEVIIKGQARDSQRYKVDYESQSTDTQN 200
IBP5_RAT EETYSPKVFRPKHTRISEL--KAEAVKKDRRKKLTQSKFVGGAENTAHPR 175
IBP1_RAT REITDLKKW---KEPCQRELYKVLERLAAAQQKAG----DEIYKFYLPNC 219
IBP2_RAT LEEPKKLRPPPARTPCQQELDQVLERISTMRLPDDRGPLEHLYSLHIPNC 240
IBP4_RAT VGTPREEPRPVPQGSCQSELHRALERLAA---SQSRTH-EDLFIIPIPNC 200
IBP3_RAT F--SSESKRETEYGPCRREMEDTLNHLKFLNVLSPRG-------VHIPNC 241
IBP5_RAT VIPAPEMRQESDQGPCRRHMEASLQEFKASPRMVPRA-------VYLPNC 218
IBP1_RAT NKNGFYHSKQCETSLDGEAGLCWCVYPWSGKKIPG-SLETRGDPNCNQYF 268
IBP2_RAT DKHGLYNLKQCKMSLNGQRGECWCVNPNTGKPIQG-APTIRGDPECHLFY 289
IBP4_RAT DRNGNFHPKQCHPALDGQRGKCWCVDRKTGVKLPG-GLEPKGELDCHQLA 249
IBP3_RAT DKKGFYKKKQCRPSKGRKRGFCWCVDKY-GQPLPGYDTKGKDDVHCLSV- 289
IBP5_RAT DRKGFYKRKQCKPSRGRKRGICWCVDKY-GMKLPGMEY-VDGDFQCHAF- 265
IBP1_RAT NVQN---------- 272
IBP2_RAT NEQQENDGVHAQRVQ 304
IBP4_RAT DSLQE---------- 254
IBP3_RAT ---------QS---Q 292
IBP5_RAT ---------DSSNVE 271

Schuller and co-workers have studied IGFBP expression during development in the mouse embryo using *in situ* hybridization (Schuller et al., 1993). One interesting observation derived from their studies was that IGFBP-2 and IGFBP-5 are often co-expressed during development in tissues of ectodermal origin. Our results in the prostate and mammary gland suggest that IGFBP-2 is constituitively expressed during normal gland function, while IGFBP-5 expression is down-regulated. IGFBP-2 expression is also observed during pregnancy, but decreases dramatically during lactation in the ewe (Klempt et al., 1993).

A wealth of data has been accumulated characterizing the expression of IGFBPs in a variety of prostate and mammary cancer cell lines (Clemmons et al.; Manni et al., 1992; Pekonen et al. 1992; Perkel et al., 1990). While it is difficult to draw any definite conclusions concerning the role of any specific IGFBP members in the growth and malignancy of prostate or mammary cancer cell lines, it is clear that all levels of IGF regulation, including IGF production, binding by IGFBP proteins, and presence or absence of IGF receptors are involved. Several factors that modulate growth of breast and prostate cancer cell lines *in vitro* such as estradiol, progesterone and their antagonists also affect the expression and secretion of IGFBPs (Clemmons et al., 1991; Owens et al., 1993; Sheikh et al ., 1993). The observation that IGFBPs may modulate ACD suggests that cancer therapies that involve altering or manipulating response to IGF growth factors may function in altering a tumor's rate of cell death. A significant portion of prostate cancers metastasize to the bone. Considering this fact it is interesting to note that IGFBP-5 has been demonstrated to have a mitogenic effect on osteoclast cells in culture.

Previously we have advanced the hypothesis that metastasis in tumors may be co-related with an abortive cell death process. Theoretically some tumor cells may initiate ACD, including degradation of the extracellular matrix and removal of cell-cell contacts, but are unable to degrade the cellular DNA and complete the process (presumably having accumulated mutations in genes responsible for the final destruction of the cell) (Tenniswood et al., 1994). The observation that IGFBP-5 is induced during prostate and

mammary ACD, and that IGFBP-5 has a mitogenic effect for osteocytes (ie bone) a tissue to which a large percentage of prostate cancer metastasise, adds further evidence to support this hypothesis. Currently the serum levels of prostate specific antigen (PSA) are used to indicate the prognosis and presence of prostate cancer. PSA is a member of the kallikrein protease family and has been shown to specifically degrade IGFBP-5 and IGFBP-2 (Donna Peehl, personal communication). High levels of PSA would result in cleavage of IGFBP-5 removing its IGF binding capacity, and as our data suggests lead to lowered levels of ACD, and an un-coupled response to IGF, thereby promoting the growth of the tumor.

6.510 - Insulin like Growth Factors (IGFs) and Active Cell Death: An Hypothesis

The prostatic stroma is known to secrete Insulin Like Growth Factor-I (IGF-I), and the epithelial cells respond to IGF-I (and to a lesser extent IGF-II and insulin) through the high affinity interaction of these growth factors with the Type 1 IGF receptor (Cohen et al., 1991; Iwamura et al., 1993). This interaction is probably modulated by IGFBP-2 which presents IGF-I to the receptor and which appears to be constitutively expressed in the normal rat ventral prostate (Cohen et al., 1991). During early development IGFBP-2 and IGFBP-5 are co-expressed in tissues of ectodermal origin, however in the adult prostate IGFBP-2 is expressed while the IGFBP-5 expression is repressed (Schuller et al., 1993; Green et al., 1994). During ACD, IGFBP-5 mRNA expression is induced. IGFBP-2 has an RGD sequence located in the carboxy terminus, and is thought to associate with integrins after secretion from the basal surface of the epithelial cells (Shimasaki and Ling 1991). In this way IGFBP-2 may serve as a storage pool for IGF-I, potentiating the effect of IGF-I by facilitating interactions with the type 1 IGF receptor, in a manner that is similar to the proposed role of IGFBP-2 in small cell lung carcinoma (Reeve et al., 1993) (Fig. 6-14). During ACD the induction of IGFBP-5 expression, coupled with loss of IGFBP-2 expression, may attenuate the cellular response to IGF-I. High affinity binding of IGF-I to IGFBP-5, which associates with several components of the extracellular matrix including types III and IV collagen, laminin, and fibronectin, may sequester IGF-I away from the receptor, to interfere with the normal homeostatic

Figure 6-14: Hypothetical Role of IGF, IGFBP-2 and IGFBP-5 in Homeostasis

Normal prostate epithelial cells connected to the underlying stroma via the ECM express and secrete IGFBP-2 which binds to IGF-I growth factor being produced by the stroma. The IGFBP-2 facilitates interaction of IGF-I with the IGF-I receptor. Secondary thanatogens responsible for completion of ACD such as cathepsin B (degradation of the ECM) and TRPM-2 (membrane remodelling) are negatively regulated by IGF-I and are not expressed. The promoter for IGFBP-5 is under negative regulation by androgens and is normally repressed and not expressed. Genes such as PSBP and IGFBP-2 are constitutively expressed and are not regulated by androgens.

ECM	Extracellular matrix
IGF-I	Insulin like growth factor I
IGFBP-2	Insulin like growth factor binding protein 2
IGFBP-5	Insulin like growth factor binding protein 5
PSBP	Prostate steroid binding protein
TRPM-2	Testosterone repressed prostate message 2

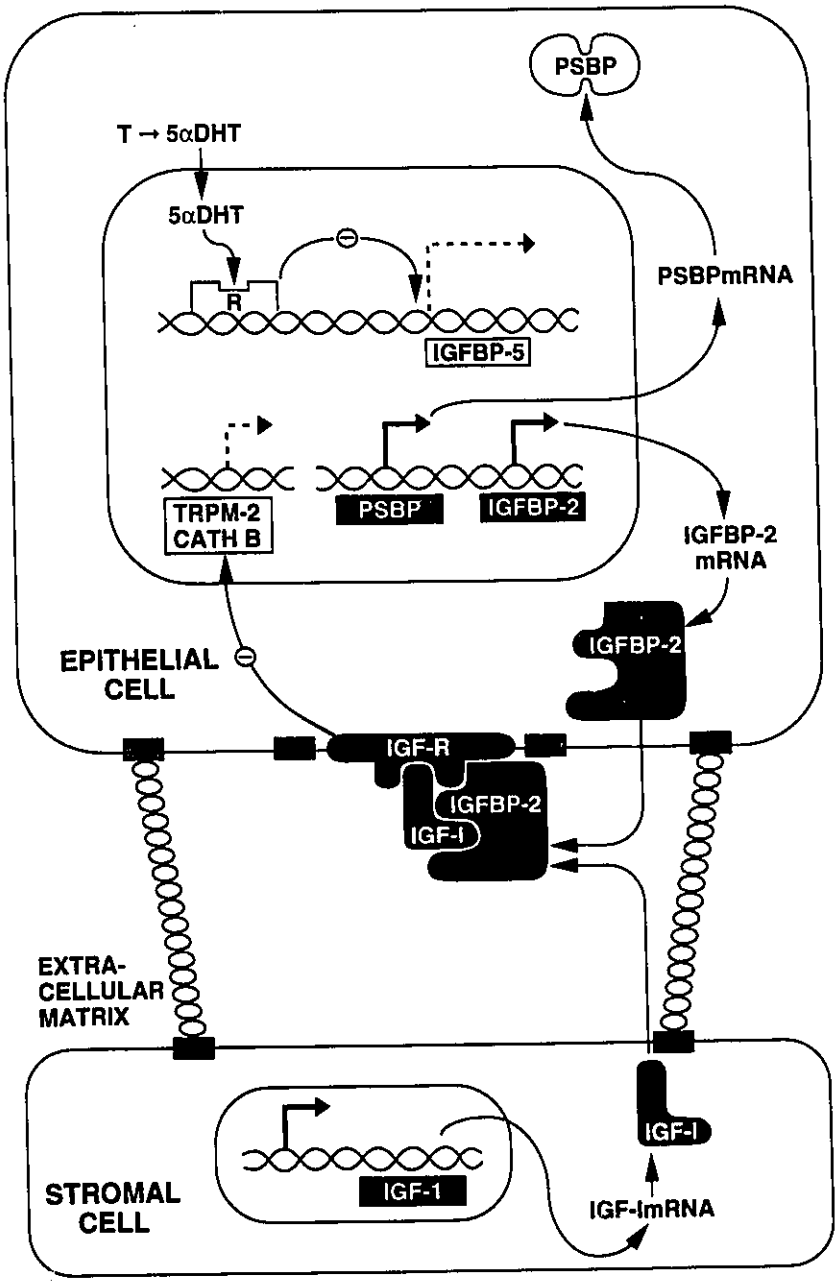
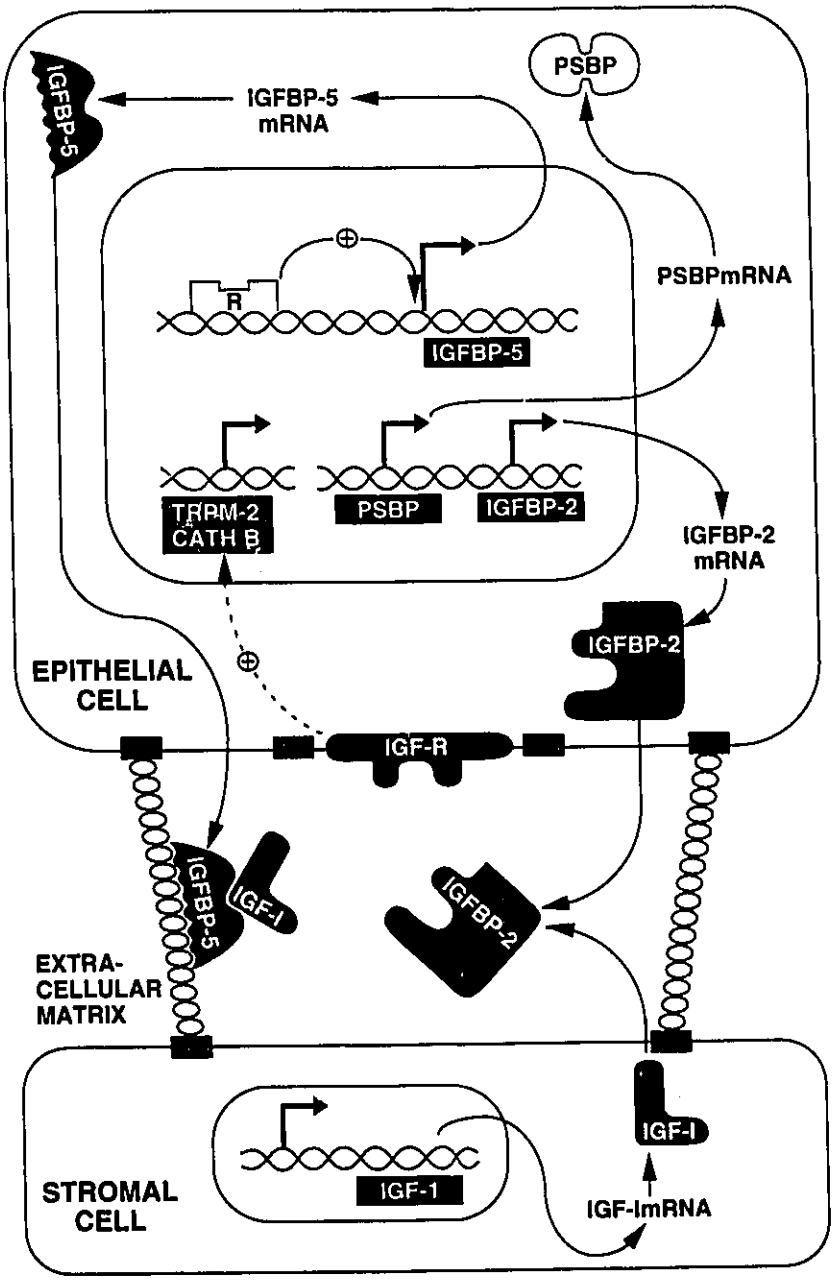

PSBP
T → 5αDHT
5αDHT
R
IGFBP-5
PSBPmRNA
TRPM-2
CATH B
PSBP
IGFBP-2
IGFBP-2
mRNA
IGFBP-2
EPITHELIAL
CELL
IGF-R
IGFBP-2
IGF-I
EXTRA-
CELLULAR
MATRIX
IGF-I
IGF-1
STROMAL
CELL
IGF-ImRNA

Figure 6-15: Hypothetical Role of IGF, IGFBP-2, and IGFBP-5 During ACD

ACD is induced following hormonal withdrawal, and the IGFBP-5 gene is induced and IGFBP-5 protein secreted following removal of repressors from its promoter which are androgen dependent. IGFBP-5 binds the IGF-I with greater affinity than IGFBP-2 and sequesters the IGF-I from the cell via binding the ECM. Genes such as TRPM-2 and cathepsin B which are required for cell death are induced in the absence of IGF-I, and the cell completes the apoptotic process.

ECM	Extracellular matrix
IGF-I	Insulin like growth factor I
IGFBP-2	Insulin like growth factor binding protein 2
IGFBP-5	Insulin like growth factor binding protein 5
PSBP	Prostate steroid binding protein
TRPM-2	Testosterone repressed prostate message 2


IGFBP-5
IGFBP-5 mRNA
PSBP
PSBPmRNA
R
IGFBP-5
TRPM-2
CATH B
PSBP
IGFBP-2
IGFBP-2 mRNA
IGFBP-2
EPITHELIAL CELL
IGF-R
IGFBP-5
IGF-I
IGFBP-2
EXTRA-CELLULAR MATRIX
IGF-1
IGF-I
STROMAL CELL
IGF-ImRNA

intracellular signaling downstream of the receptor (Clemmons 1993; Jones et al., 1993). Close examination of the relationship between IGFBP-5, IGF-I and ACD suggest that the expression of IGFBP-5 is probably an early event in ACD, and may serve to trigger the process in prostate secretory epithelial cells (Fig. 6-15). The accumulated data suggest that in the normal prostate the expression of IGFBP-5 is directly repressed by androgens, and that IGFBP-2 serves to modulate the interaction of IGF-I and the IGF-I receptor. After secretion into the extracellular matrix, IGFBP-5 could compete with IGFBP-2 for IGF-I binding and through its interaction with the extracellular matrix (rather than the integrins) essentially sequester IGF-I away from the receptor (Jones et al., 1993). This would effectively attenuate the cellular response to IGF-I which appears to be necessary for survival. The changes in second messenger signaling downstream of the receptor induces a cascade of other genes, including cathepsin B and TRPM-2, that are required for ACD. It is of interest that IGFBP-5 can be proteolytically degraded by several of the extracellular proteases that are induced during ACD, including cathepsin D. This effectively limits the duration of IGFBP-5 action and ensures that stromal-epithelial interactions between neighboring cells are not interrupted by slow diffusion of the binding protein.

More detailed studies concerning the function of IGF and IGFBPs in ACD and normal growth in both the prostate and mammary gland should lead to a greater understanding of their role in prostate and breast cancer.

CONCLUSION

CONCLUSION

The work presented in this thesis has focused on the identification and characterization of two genes involved in ACD during hormonally induced regression of the rat ventral prostate and rat mammary gland. These genes refered to as RSG-2 and RSG-8 were identified using a unique differential cross-hybridization screening method.The isolation of these genes using the whole library cross-screening strategy first described by Wong et al., 1989, provides the first validation of this method as a general technique for differential screening. RSG-2, the first gene identified in the cross-screen, codes for the cysteine protease cathepsin B. Northern analysis indicated that the message for cathepsin B was highly induced in both regressing prostate and mammary gland. *In situ* hybridization experiments localized this increased expression to the epithelial cells of each gland, which are deleted during ACD. Furthermore, immunohistochemical studies on regressing prostate and mammary tissue, clearly localizes the cathepsin B protein to the apoptotic bodies formed during ACD. During ACD the dying cell loses its cell-cell contacts and degrades the basement membrane to which it is associated. The neccessity for expression of proteases responsible for performing the degradation suggests that cathepsin B may be involved in this process. Alternatively the localization of cathepsin B to apoptotic bodies may represent a potential role in the degradation of cellular fragments occuring during ACD.

RSG-8, a second gene identified in the screening codes for insulin like growth factor binding protein five (IGFBP-5) a member of the 6 homologous proteins involved in modulating cellular responses to the IGF growth factors. Northern analysis showed a *de novo* induction of IGFBP-5 mRNA during ACD in regressing prostate and mammary gland. The induction of IGFBP-5 was localized to the epithelial component of both tissues by *in situ* hybridization. Because of its function as a modulator of growth factor action, it is tempting to speculate that IGFBP-5 may be playing a role as a primary thanatogen, and directly initiates or modulates the apoptotic process. cathepsin B and IGFBPs have been implicated in metastasis and oncogenesis respectively. Conclusive proof of their role in ACD would provide convincing evidence that cancer and metastasis are closely linked to

the process of ACD. Further characterization of both cathepsin B and IGFBP-5 and their role in ACD should provide a greater understanding of the etiology of both prostate and mammary cancer.

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APPENDIX ONE

ABBREVIATIONS

ACD	active cell death
AMV	avian myeloblastosis virus
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
CL4B	Sephadex CL4B
cpm	counts per minute
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
dNTP	deoxynucleotide 5'-triphosphate
dTTP	deoxythymidine 5'-triphosphate
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ds-cDNA	double stranded cDNA
DTT	dithiothrietol
EDTA	ethylenediaminetetra-acetic acid
G-25	Sephadex G-25
G-50	Sephadex G-50
gm	gram
h	hour(s)
HMW	high molecular weight

hnRNA	heteronuclear RNA
Hsp27	heat shock protein 27
IGF-I	insulin like growth factor I
IGF-II	insulin like growth factor II
IGFBP	insulin like growth factor binding protein
kb	kilobase
kbp	kilobase pair
min	minute
mRNA	messenger RNA
O.D.	optical density
oligo-(dT)	oligodeoxythimidylate
PAGE	polyacrylamide gel electrophoresis
PARP	poly (ADP-ribose) polymerase
PCR	polymerase chain reaction
PEG	poly ethylene glycol
pfu	plaque forming unit
pH	$-\log_{10}[H+]$
poly(A) +	RNA retained on oligo(dT)-cellulose column
poly(A)-	RNA not retained on oligo(dT)-cellulose column
PSBP	Prostate Steroid Binding Protein
RNA	ribonucleic acid
RNase	ribonuclease

rpm	revolutions per minute
rRNA	ribosomal RNA
RSG	regression selected gene
RT	reverse transcription
S	Svedberg unit
SDS	sodium dodecyl sulfate
ss-cDNA	single-stranded cDNA
Taq	*Thermus aquaticus*
TCA	trichloroacetic acid
tTGase	tissue transglutaminase
Tris	Tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
TRPM-2	testosterone repressed prostate message 2
U	unit of enzyme activity
UV	ultra violet
V	volts

APPENDIX TWO

SUPPLIES AND GENERAL SOLUTIONS

Amersham (Oakville, Ontario): [^{35}S]-dATP (3000 Ci/mmole), [^{32}P]-dCTP (3000Ci/mmole), [^{32}P]-dATP (3000 Ci/mmole)., multiprime kits, λgt10 and λgt11 cloning kit, Hybond™ nylon membranes, restriction endonucleases.

BDH Canada (Toronto, Ontario): Ficoll, polyvinylpyrrolidone.

Beckman Canada (Mississauga, Ontario): Ready-Solv ET Scintillation fluid.

Bethesda Research Laboratories (Burlington, Ontario): restriction endonucleases, Hind III digested λ DNA, Hind III / EcoRI digested λ DNA, Superscript™ reverse transcriptase, Taq polymerase.

Bill's Camera Craft (Ottawa, Ontario) and Henry's (Toronto, Ontario): Cronex X-rayfilm, Illford films, Ilford and Kodak paper and other dark room chemicals.

Bio-Can Scientific Inc (Mississauga, Ontario): Gene clean™ kits.

Bio-Rad Laboratories (Canada) Ltd (Mississauga, Ontario): Acrylamide, N'N'methylene-bisacrylamide, N,N,N,N,-tetramethylenediamine, Coomassie blue, protein molecular weight standards, Bovine serum albumin, AG 501-X8D mixed bed resin, penicillin, streptomycin.

Boehringer-Mannheim Canada (Dorval, Quebec): Ampicillin, T4 ligase, DNA polymerase I, RNAse A, yeast tRNA, calf thymus DNA, Tris, oligo(dT-cellulose),polynucleotide kinase, RNAse H, restriction endonucleases.

Dupont (Lachine, Quebec): [^{35}S]-dATP (3000 Ci/mmole), [^{32}P]-dCTP (3000 Ci/mmole), [^{32}P]-dATP (3000 Ci/mmole), multiprime labeling kits.

International Biotechnologies Incorporated (Toronto, Ontario): Polyacrylamide, SDS, N',N'-methylene-bisacrylamide, urea, agarose, N,N,N',N'-tetra-methylendiamine, ammonium persulfate, TEMED.

Life Sciences (St. Petersburg, FL): AMV reverse transcriptase

Oxoid Canada (Nepean, Ontario): Agar, tryptone, bacto-yeast extract.

Perkin-Elmer Cetus (Nepean, Ontario): Gene amplification-DNA amplification kit,Taq polymerase.

Pharmacia (Dorval, Quebec): random-sequence hexadeoxyribonucleotides, oligo dT_{12-18}, Sephadex G-50, Sepharose 4B, Sepharose CL4B, nucleotides (dNTPs).

Promega Biotec (Madison, WI): pGEM7Zf- vector, Erase A Base™ (exo III deletion kit), T7, SP6 primers.

Qiagen (Chatsworth, CA): Qiaprep™ plasmid mini and maxi prep kits. Qiaex™ DNA purification kits.

Queens University Biotechnology Research Institute (Kingston, Ontario): Synthetic oligonucleotides, λgt10, λgt11, T7, SP6 primers.

University of Ottawa Biotechnology Research Institute (Ottawa, Ontario): Synthetic oligonucleotides, λgt10, λgt11, T7, SP6 primers.

US Biochemicals Corp.(Cleveland, Ohio): Sequenase™ DNA sequencing kits.

Whatman BioSystems Inc.(Clifton, New Jersey): DE52-preswollen DEAE cellulose.

All other chemicals, of reagent grade, were purchased from Fisher Scientific Co. Ltd(Ottawa, Ontario), Sigma (St. Louis, Mo) or BDH (Toronto, Ontario).

BACTERIAL CELL LINES

NM514: selective bacterial host for the vector λgtl0 (Amersham, Oakville, Ontario).

L87: non-selective bacterial host for the vector λgt10 (Amersham, Oakville, Ontario).

Y1090: selective bacterial host for the vector λgtl0 (Amersham, Oakville, Ontario).

XL1-Blue: general bacterial host for plasmid vectors (Stratagene, Palo Alto, CA)

DH5αf' general bacterial host for plasmid vectors (Promega Biotec, Madison, WI).

GENERAL SOLUTIONS

2YT broth	1.6% bactotryptone, 1% bacto-yeast extract, 0.5% NaCl
Denhardt's	0.02% BSA, 0.02% polyvinyl pyrrolidone, 0.02% Ficoll
High TE	100 mM Tris-HCl pH 8.0, 1 mM EDTA
L-agar	1% bactotryptone, 0.5% bacto-yeast extract, 1% NaCl, 1.5%bacto-agar, pH 7.0
L-broth	1% bactotryptone, 0.5% bacto-yeast extract, 1% NaCl, pH 7.0
MOPS	20 mM morpholonopropanesulfonic acid, 5 mM NaOAc, 1 mM EDTA, pH 7.0
SM buffer	50 mM Tris, 100 mM NaCl, 10 mM $MgSO_4$, 0.1% gelatin, pH 7.5
SSC	0.15 M NaCl, 0.015 M sodium citrate, pH 7.0
SSPE	0.15 M NaCl, 0.010 M sodium phosphate, 1 mM EDTA, pH 7.4
STE	100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.0
TAE loading buffer	10X TAE buffer, 50% glycerol, 0.5 mg Xylene Cyanol/ml, 0.25 mg Bromophenol Blue
TAE buffer	40 mM Tris, 20 mM EDTA, 0.23 % glacial acetic acid, pH 7.7
TBE buffer	44 mM boric acid, 44 mM Tris, 20 mM EDTA, pH 8.3
TBE loading buffer	20 mM EDTA, 96% deionized formamide, 0.5 mg Xylene Cyanol/ml, 0.25 mg Bromophenol Blue/ml
TE	10 mM Tris-HCl pH 7.5 or 8.0, 1 mM EDTA
Top agar	1% bactotryptone, 0.5% bacto-yeast extract, 0.5% NaCl, 0.25% $MgSO_4$, 0.8% bacto-agar, pH 7.0

APPENDIX THREE

INTERPRETATION OF *IN SITU* HYBRIDIZATION METHOD

Interpretation of *In Situ* Hybridization Method

The *in situ* hybridization experiments were performed as described in section 2.780. For each experiment all photographs were taken from a set of slides that were hybridized with the same probe for the same time, and therefore can be directly compared. In all cases the final picture presented in the thesis represents the best result of at least three individual experiments. Localization of the grains to either stroma or epithelial cells was delineated by marking with white ink the outline of the stroma on a clear cellophane cover that had been attached over the bright field photograph, followed by overlaying this cellophane on the darkfield photograph. The number of grains which were stromal or epithelial in origin were counted and in all cases the expression was clearly localized predominantly to the epithelium. Figure A-1 shows the typical result obtained after delineating the stroma. Figure A-2 shows a representative bar graph of manual grain counting for the *in situ* presented in Figure A-1. One possible source of error in interpreting the *in situ* data results from the random nature of the tissue orientation during sectioning, and preparation of the slides used for the experiments. Sections which cut through areas of the gland at an oblique angle may lead to some of the grains from epithelium appearing to localize over the stroma as shown in Figure A-3.

Figure A-1: *In Situ* Hybridization of PARP mRNA in the Ventral Prostate After Castration

Random sections from the rat ventral prostate excised at different times after castration were hybridized to ^{35}S-labeled cDNA insert specific for PARP ($1x10^6$ cpm). The sections were exposed for 4 days. Stromal regions are delineated in white ink. Magnification X20.

Panels A, C, E:	phase contrast showing morphology of sections
Panels B, D, F:	dark field illumination showing *in situ* hybridization
Panels A and B:	prostate from control animals
Panels C and D:	prostate 2 days after castration
Panels E and F:	prostate 4 days after castration

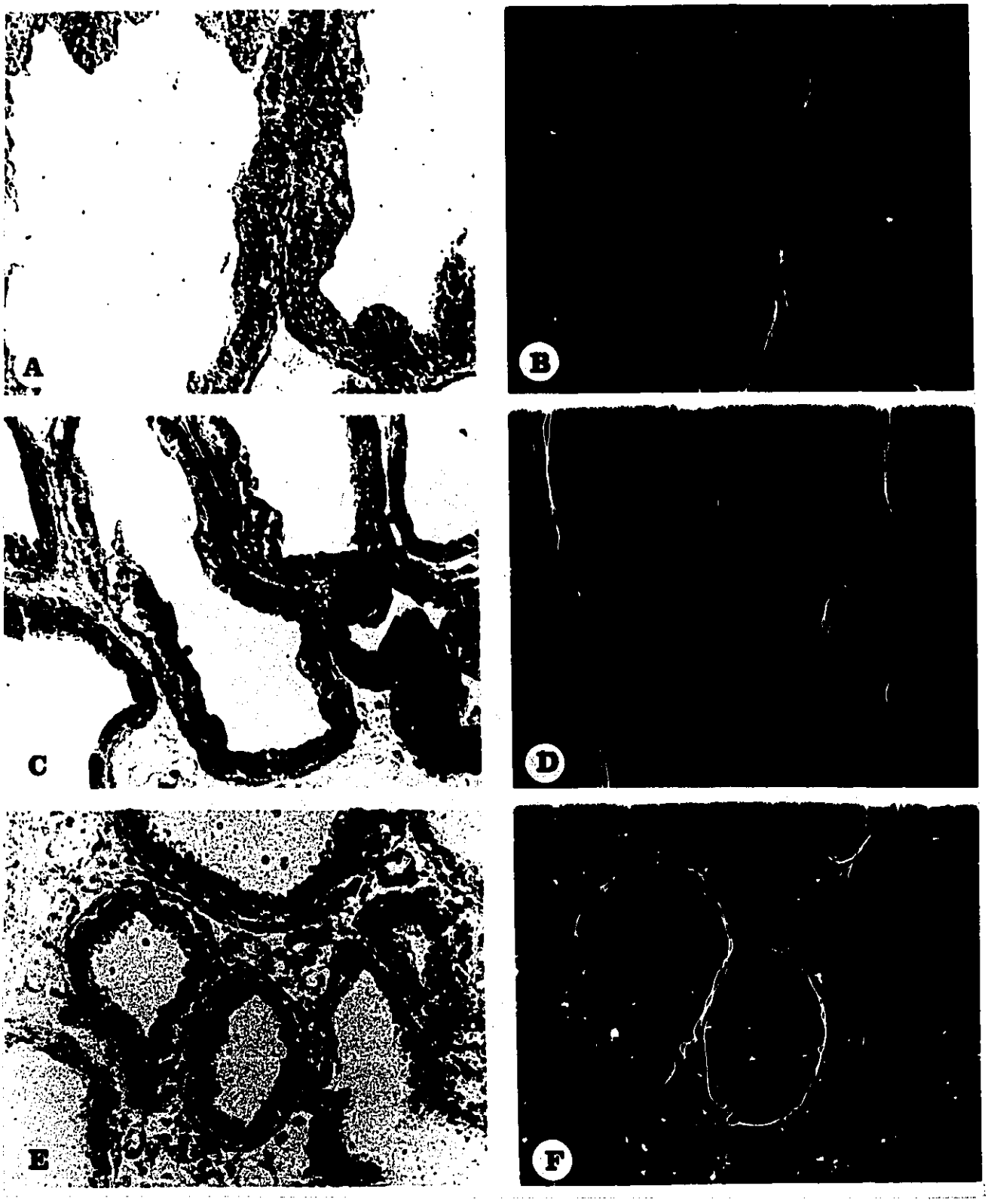

A
B
C
D
E
F

Figure A-2: Representative Results of Manual Counting For Localization of *In Situ* Hybridization Grains

A 3 cm x 3 cm square was placed on each figure in 3 different locations, and the number of grains in this area were counted and assigned as either epithelial or stromal in location. The bar graph shows the average number of grains for epithelial (E) or stromal (S) cell types +/- S.D. for each time point.

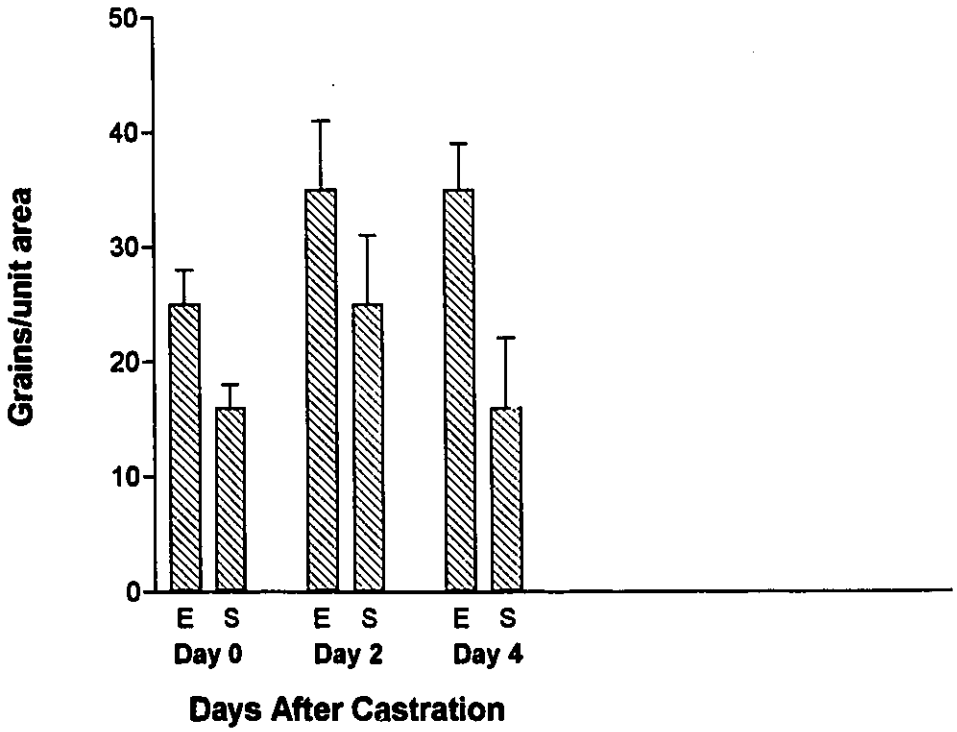

Grains/unit area
50
40
30
20
10
0
E S
Day 0
E S
Day 2
E S
Day 4
Days After Castration

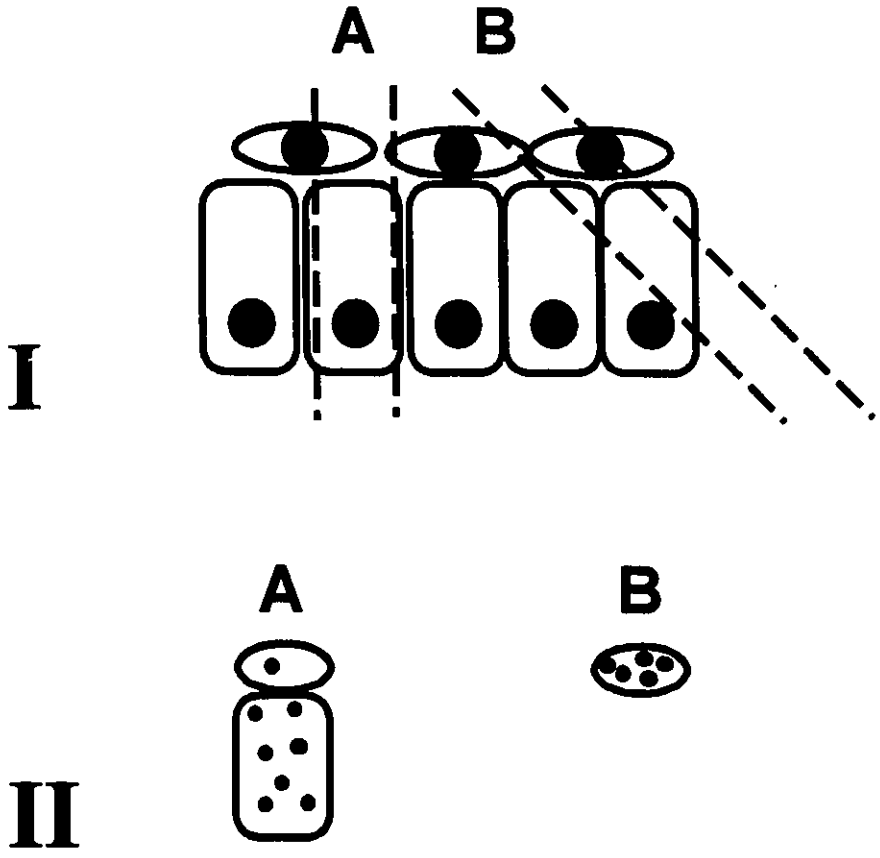
Figure A-3: Effect of Random Sectioning On *In Situ* Localization

I. Representative Structure of Prostate/Mammary Gland.

The figure shows the typical organization of a gland with the tall columnar secretory epithelial cells in a pallisade overlying the stromal cells. During sectioning random cuts are made through the tissue. A section cut at 90° (A) and a more oblique cut (B) through stroma and epithelium are illustrated.

II. *In situ* Appearance For Sections A and B

In section A, because the angle of the cut was 90° the grains localise clearly to the proper stromal or epithelial location. In section B the angle of the cut, coupled with limitations of the microscopy and thickness of the sections, causes the grains from the overlying epithelial cell to appear to localize to the stromal component of the gland.


A
B
I
A
B
II

APPENDIX FOUR

CURRICULUM VITAE

CURRICULUM VITAE

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UNIVERSITY	:	University of Ottawa
DEPARTMENT	:	Department of Biochemistry
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EDUCATION

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SCHOLARSHIPS AND ACADEMIC AWARDS

McMaster University (Faculty of Science)	**Dean's Honour List**	1988-1989
University of Ottawa Graduate School	**Entrance Scholarship**	1989-1990
University of Ottawa Graduate School	**Travel Award**	1990
Ontario Graduate Scholarship	**Competitive Studentship**	1990-1991 1991-1992 1992-1993 1993-1994

RELATED WORK EXPERIENCES

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Appointment	:	Teaching Assistant
Supervisor	:	Dr. P. Rodriguez
Course	:	Biochemistry 3946, Department of Biochemistry, University of Ottawa.

Date	:	Jan 1990 - May 1990
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MANUSCRIPTS IN REFERRED JOURNALS

1. Tenniswood M, **Guenette RS**, Lakins J, Mooibroek M, Wong P, and Welsh J. (1992) Active cell death in hormone-dependent tissues. Cancer and Metastasis Reviews 11:197-220.

2. Tenniswood MP, Taillefer D, Lakins JL, **Guenette RS**, Mooibroek M, Daehlin L., and Welsh JE (1994) Control of Gene Expression during Apoptosis in Hormone Dependent Tissues. In: Apoptosis II. The molecular basis of cell death. (Eds: Tomei L.D., and Cope F.O.) Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY. 283-311.

3. **Guenette RS**, Corbeil HB, Leger J, Wong K, Mezl V, Mooibroek M, and Tenniswood M. (1994) Induction of gene expression during involution of the lactating mammary gland of the rat. Journal of Molecular Endocrinology 12:47-60.

4. **Guenette RS**, Daehlin L, Mooibroek M, Wong K, and Tenniswood M. (1994) Thanatogen expression during involution of the rat ventral prostate after castration. Journal of Andrology 15: 200-211.

5. **Guenette RS**, Mooibroek M, Wong K, Wong P, and Tenniswood M. (1994) Cathepsin B, a cysteine protease implicated in metastatic progression, is also expressed during regression of the rat prostate and mammary glands. European Journal of Biochemistry 226:311-321

6. **Guenette RS**, and Tenniswood MP. (1994) The role of growth factors in the suppression of cell death in the prostate. Biochemistry and Cell Biology in press.

7. Tenniswood MP, **Guenette RS**, Taillefer D, and Mooibroek M. (1995) The role of growth factors and extracellular matrix proteases in active cell death in the prostate. Schering Monographs in press.

MANUSCRIPTS IN PREPARATION

1. **Guenette RS**, Mooibroek M, Wong K, and Tenniswood, M. Co-ordinate regulation of insulin like growth factor binding protein expression during active cell death in hormone dependent tissues.

2. **Guenette RS**, Mooibroek M, Sridhar K, Wilson MR, Wong K, and Tenniswood M. (1994) Characterization of Rat Embigin, a member of the immunoglobulin superfamily.

ABSTRACTS

1. **Guenette RS**, and Tenniswood, M. The Role of Insulin Like Growth Factor Binding Proteins (IGFBPS) in Regulating Active Cell Death in Regressing Rat Prostate and Mammary Gland.(Abstract of Poster Presentation at Keystone Symposium on Apoptosis, Tamarron, Coloroado, March 1995).

2. Mooibroek M, **Guenette RS**, Taillefer D, and Tenniswood, M. Attenuation of active cell death in androgen receptor positive epithelial cells of the rat ventral prostate. (Abstract of Poster Presentation at AACR Special Conference on the Mechanism of Action of Retinoids, Vitamin D and Steroid Hormones, Banff, Alberta, March 1993).

3. **Guenette RS**, Mooibroek M, Wong K, and Tenniswood, M. Cathepsin B, a cysteine protease implicated in metastatic progression, is also expressed during regression of hormone dependent tissues. (Gordon Conference on Hormonal Carcinogenesis, Salve Regina University, Newport, Rhode Island, July 1993).

4. Tenniswood M, Wong P, Daehlin L, and **Guenette RS.** Epithelial-Stromal Interactions and Androgen Sensitivity in the Prostate. (Abstract of Poster Presentation at Gordon Conference on Molecular Carcinogenesis, Colby-Sawyer College, New Hampshire, July 1991)

5. Tenniswood M, Wong P, and **Guenette RS.** Co-ordinate regulation of gene expression during apoptosis (programmed cell death) in the rat ventral prostate. (Abstract of a presentation at the annual meeting of the Society of Basic Urological Research, Toronto, Ontario, June 1991).

6. **Guenette RS**, Daehlin L, and Tenniswood MP. Characterization of regression selected genes (RSG) in the rat ventral prostate and mammary gland. (Abstract of Poster Presentation at the 34th Annual Meeting of the Canadian Federation of Biological Societies, Kingston, Ontario, June 1991).

7. **Guenette RS,** and Tenniswood M. Regression selected genes (RSG): the cloning of regression expressed sequences using a PCR based cross-screening of λgt10 and λgt11 cDNA libraries. (Abstract of Poster Presentation at the 20th Annual Meeting of the Federation of European Biological Societies, Budapest, Hungary, August 1990).

8. **Guenette RS,** and Tenniswood MP. Regression associated gene expression in the rat ventral prostate. (Abstract of Poster Presentation at the 33rd Annual Meeting of the Canadian Federation of Biological Societies, Halifax, Nova Scotia, June 1990).