CHARACTERIZATION OF GENE EXPRESSION DURING ADENOSINE 3’:5’-CYCLIC MONOPHOSPHATE INDUCED NEUROENDOCRINE DIFFERENTIATION IN HUMAN PROSTATIC ADENOCARCINOMA

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

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ABSTRACT

The LNCaP cell line is a versatile and useful model that is suitable for the study of human prostate cancer in vitro. The elevation of LNCaP intracellular cAMP levels through the addition of membrane permeable cAMP analogues, phosphodiesterase inhibitors, adenylate cyclase activators, or components of the cAMP signal transduction pathway can induce reversible neuroendocrine differentiation. Elucidation of those genes that are differentially expressed between undifferentiated prostate cancer cells and prostate cancer cells that have been induced to differentiate may present new insights for the molecular mechanisms governing neuroendocrine differentiation, early detection of prostate cancer, and/or potential targets for gene therapy. In this study, differential display PCR was used to identify 226 differentially expressed PCR products. Twelve of the differential display PCR products were confirmed by Northern blot analysis and cloned. DNA sequencing and
database comparisons were performed. Among the differentially expressed genes, the human ribosomal \( S3a \) gene was identified as down regulated in response to LNCaP differentiation. In order to better ascertain the mechanism by which \( HRS3a \) gene expression is decreased during differentiation, the promoter region for this gene was analyzed. Electrophoretic mobility shift assay, antibody supershift assays, site-directed mutagenesis, and luciferase reporter gene analysis were employed to authenticate the roles of several transcription factors in the regulation of the \( HRS3a \) gene. Two cyclic AMP response elements, a Sp1 element and a GA-binding protein element, were involved in the regulation of \( HRS3a \) gene expression. In order to ascertain the effect of \( HRS3a \) down regulation in LNCaP cells, antisense phosphorothioate oligonucleotides were designed to inhibit \( HRS3a \) gene expression. Treatment of LNCaP cells with antisense \( HRS3a \) oligonucleotides did not influence cAMP induced neuroendocrine differentiation but antisense treatment did result in a decrease in LNCaP cell growth. In addition, it was determined that morphological changes associated with cAMP induced differentiation of LNCaP cells from the epithelial to the neuroendocrine state may not require alterations in gene expression nor the expression of novel proteins.
An education isn’t how much you have committed to memory, or even how much you know. It’s being able to differentiate between what you know and what you don’t.

Anatole France Thibault
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CHAPTER 1

Introduction and Survey of the Literature.
CHAPTER 1. INTRODUCTION AND SURVEY OF THE LITERATURE

1.1. Introduction.

The differentiation, proliferation, and survival of individual cells in a multicellular organism are carefully regulated to promote the survival of the organism as a whole. Occasionally, the regulatory mechanisms that govern normal cell growth and behavior can malfunction. This break down in individual cell regulation sometimes results in cells that grow and divide in an uncontrolled manner, invade normal tissues and organs, and eventually spread throughout the affected individual. Cancer can result from the abnormal proliferation of any of the different cell types found in the body. Even though there are over one hundred distinct cancer types, the prostate, breast, lung, and colon cancers alone account for over 60% of new cancer cases each year [1]. Prostate cancer is the most commonly diagnosed cancer in men and the second leading cause of cancer related death in men in the Western world [2].

Understanding the molecular genetic changes associated with the progression from non-cancerous to malignant states is essential in order to stop the process. The overall emphasis of this research is to understand the influence of cyclic AMP, and
factors that modulate intracellular cAMP levels, on the expression of specific genes in prostate cancer cells with emphasis on the possible functional significance of these genetic changes.

1.2. **Structure and Function of the Prostate Gland.**

The prostate gland, a single doughnut-shaped tubuloalveolar gland about the size of a chestnut, serves as an accessory sex gland of the male reproductive system [3]. The gland is located inferior to the urinary bladder with muscular fibers that surround the upper portion of the urethra. The gland consists of a median lobe and two lateral lobes. The ducts from the prostate glandular matter empty into the prostatic portion of the urethra. Prostatic secretions give semen its characteristic milky appearance and are slightly alkaline. Resultant seminal fluid alkalinity helps to protect sperm from the acidic female vagina. Prostatic secretions contain citric acid and enzymes such as acid phosphatase, clotting enzymes, and fibrinolysin. The prostate gland gradually increases in size from birth until puberty, at which time the gland grows rapidly.

The prostate is composed of three distinct zones: the peripheral (PZ), central (CZ), and transition zones (TZ) [4]. The PZ composes about 70% of prostate volume and is the most common site for prostatic intraepithelial neoplasia (PIN) and
adenocarcinomas [5]. The CZ encircles the ejaculatory ducts and composes about 25% of the prostate. The TZ comprises the remaining 5% of prostate mass. The PZ and CZ together are also referred to as the cortical or outer prostate and the TZ is sometimes referred to as the inner or periurethral prostate.

The acini and ducts of the prostate are primarily composed of secretory and basal epithelial cells with interspersed neuroendocrine cells. Prostatic secretory or luminal cells, as the name suggests, are secretory in nature and express keratins 8 and 18, prostatic acid phosphatase, prostate specific antigen, and the androgen receptor [6-8]. Secretory cells require androgens to maintain their functions [9]. Differentiation of adenocarcinomas is considered to be towards secretory cells as adenocarcinomas express phenotypic markers that are typical of secretory cells.

The prostatic basal cells are considered to be multi-potential with the capacity to give rise to all of the epithelial lineages in normal, hyperplastic, and neoplastic prostates [10]. Basal cells separate the secretory cells from the basement membrane and basal cells are known to express high molecular weight cytokeratins that are reactive with the monoclonal anti-keratin antibody 34betaE12 that is specific for keratins 1 (68 kDa), 5 (58 kDa), 10 (56.5 kDa), and 14 (50 kDa) [11]. These keratins are known to be associated with complex epitheliums. Prostate specific antigen (PSA) and prostatic acid phosphatase are not expressed by normal prostatic basal cells [12-13], but basal cells have been demonstrated to express the androgen receptor...
Neuroendocrine cells (NE) are present in each zone of the prostate, but they appear to be most abundant in the periurethral and ductal regions [16]. NE cells are found among the basal and secretory cells in the glandular component of the prostate [17]. Chromogranin A and B, secretogranin II, neurone specific enolase and serotonin are secreted by most NE cells [18]. Chromogranin A is the best overall tissue and serum marker of neuroendocrine differentiation and chromogranin A serum levels have been found to be useful in the assessment of the progression of hormone refractory prostate cancer [19]. Studies with markers such as chromogranin A and neurone-specific enolase suggest that neuroendocrine differentiation, as reflected by increased tissue expression or blood concentrations of chromogranin A and neurone-specific enolase, is associated with a poor prognosis, tumor advancement, and androgen independence [20]. Peptide hormones such as somatostatin, calcitonin and bombesin are secreted by some NE cells [21]. NE cells express PSA, that suggests a common origin with secretory cells [18], but NE cells do not express androgen receptor [22].
1.3. LNCaP Human Prostatic Adenocarcinoma Cell Line.

The most widely used cell lines, cultured in vitro, for studies of human prostate cancer are PC-3, DU-145 and LNCaP [23]. During the past few years, the human prostatic cancer cell line LNCaP has gained much interest. Needle aspiration biopsy of a 50 year old prostate cancer patient’s left supraclavicular lymph node was used to obtain small fragments of tissue from which the LNCaP line was originally established [24]. The capacity for maintaining and studying human prostatic cancers in an in vitro environment has allowed for the development of essential tools for studying many aspects of prostate cancer biology. The studies conducted with cancer cell lines over the last 20 years constitute much of the basis for major progress in the understanding of prostate cancer disease.

The LNCaP cell line exhibits an epithelial morphology in the non-cyclic AMP induced state and tends to grow in clusters with an acinar appearance [25]. Certain cell line characteristics can be examined to facilitate the acceptance of a cell line as having prostatic epithelial origin. For example, the expression of specific cytokeratins but the absence of desmin and factor VIII can be used to determine epithelial origin [26]. Responsiveness to androgens, with the expression of androgen receptors and prostate-specific antigens, can also be examined to establish prostatic epithelial origin.
LNCaP cell growth is considered to be slow with an average population doubling time of approximately 72 hours [24]. The slow growth rate that is observed in LNCaP cells appears to correlate well with the *in vivo* multiplication of malignant prostate cancer cells [28]. Despite the absence of receptors for progesterone and estradiol, the growth rate of the androgen responsive LNCaP-FGC cells has been shown to increase when cultured in the presence of either estrogens or progesterones [29].

LNCaP is an aneuploid cell line with a modal chromosome number of 84 occurring in 22% of cells. Cells with chromosome counts of 86 (20%) and 87 (18%) also occur at high frequency [30].

The LNCaP cell line produces four prostatic biomarkers: prostate specific antigen (PSA), prostatic acid phosphatase (PAP), prostate-specific membrane antigen (PSM), and prostatic inhibitory peptide (PIP) [31-32]. The continuous production of human prostatic acid phosphatase (PAP) has been found to accumulate to levels in excess of 300 µU/ml/10⁶ cells when measured by spectrophotometric methods [33]. In addition, LNCaP cells express a high-affinity mutated (codon 868, Thr->Ala) androgen receptor that can be blocked by bicalutamide, but stimulated by cyproterone acetate and flutamide [34-35].

Bang *et al.* [25] reported that elevation of intracellular cAMP through the
addition of cell permeable cAMP analogues or phosphodiesterase inhibitors induces permanent conversion from an epithelial to a neuronal morphology and that the induced cells express markers of the neuroendocrine phenotype. Subsequent analysis demonstrated that the cAMP induced neuroendocrine differentiation of LNCaP cells is a reversible phenomenon [36-37]. Three examples of neuroendocrine markers that are expressed in cAMP differentiated LNCaP cells include neuron-specific enolase (NSE), chromogranin A, and pp60c-src. NSE is a homodimeric protein composed of monomers with a molecular mass of about 46 kDa [38]. The receptor-linked protein tyrosine kinase pp60c-src has also been reported to be a marker of neuroendocrine differentiation [39]. Chromogranin A (CgA) is an acidic glycoprotein that was first identified in chromaffin granules of the adrenal medulla [40]. CgA is the major member of the chromogranin/secretogranin family of proteins, that are present in virtually all endocrine and neuroendocrine cells [41-42].
1.4 Prostate Cancer Development, Progression, and Metastasis.

An increased understanding of prostate cancer biology, progression, and metastasis are essential for improving the ability to successfully prevent, diagnose, and treat human prostate cancer. To help determine the basic biological characteristics of prostate cancer, many current research efforts are directed toward the analysis of developmental, biological, and genetic aspects of prostate disease [43]. A great deal of emphasis has been placed on the identification of molecular mechanisms that can predict the risk of developing prostate cancer.

Over the past decade, the perception of normal and neoplastic prostate pathophysiology has improved dramatically [44]. Several important details regarding the developmental, tissue, and cellular biology of the prostate gland have been characterized. For example, it is known that proliferation and development of prostate epithelium is initiated and subsequently maintained by angiogenic and surrounding mesenchymal stimuli through both autocrine and paracrine signals [45]. Furthermore, several therapeutic modalities have been created as a result of an improved understanding of hormonal influences on prostatic tissue. The recognition that androgen receptors and metabolites are important in the regulation of prostate growth, differentiation, and neoplastic progression resulted in the development of the
antiandrogens (e.g., flutamide, bicalutamide) and 5-alpha reductase inhibitors (finasteride) that are used clinically to treat prostate cancer [46].

Prostate cancer originates as a localized lesion. It subsequently progresses to acquire increased invasive, migratory, and metastatic potentials. Eventually, androgen independence develops and the tumor becomes refractory to conventional therapies [47]. The progression of prostate disease from a localized tumor to a dispersed disease state probably occurs through alterations in proliferation, cell-cell adhesion, invasion potential, and critical interactions between prostate cancer cells and surrounding host cells at both primary and metastatic tumor sites [48]. Contemporary methods for predicting how rapidly a given prostate cancer will acquire metastatic attributes and progress from an androgen-dependent to an androgen-independent state are unreliable [49]. The evolution of prostate cancer from a disease contained within the prostatic parenchyma to a systemic disease involving the seminal vesicles, lymph nodes, skeleton, and other distant organs involves fundamental changes in the cancer that are dictated by genetic as well as epigenetic (environmental, behavioral, and dietary) influences. Supporting this concept are clinical and laboratory studies that demonstrate putative roles for hereditary factors, tumor characteristics (including DNA repair, cell cycle progression, and apoptotic potential) [50], and host factors (comprising stromal-epithelial interactions, angiogenic influences, and host immune surveillance) in prostate cancer progression.
and metastasis [51].

Contemporary knowledge concerning the basic mechanisms of prostate development, carcinogenesis, tumor progression, and metastatic dispersion continue to advance. The biochemical and molecular mechanisms that control cellular interaction during prostate organogenesis, morphogenesis, and functional differentiation are the subjects of a great deal of investigation [52,10]. The inter-cellular and intra-cellular signaling pathways that regulate androgen receptor-mediated gene transcription also continue to be characterized [53]. Moreover, the specific target genes and gene products that are directly controlled by signaling molecules during the various stages of prostate cancer development and progression are under investigation [49]. Cellular signaling, cell surface receptor activity, and the interactions of prostate cancer cells with soluble and matrix-associated molecules that regulate prostate cancer growth, progression, and dissemination continue to be areas of important investigation in prostate cancer research.

Recently, new technologies such as tissue microdissection, DNA microarray, and comparative genomic hybridization have been utilized to evaluate clinical specimens, enabling the identification, cloning, and characterization of prostate cancer progression-associated molecular markers that might someday be used to define and characterize the malignant potential of prostate cancer in individual
patients [54]. Tissue microdissection is a laboratory method that is used to procure specific cells or cell populations from a histology slide under direct microscopic visualization [55]. The recovered cells can be studied with a variety of DNA, messenger RNA, and protein analysis methods, including new high-throughput gene expression and proteomics technologies.

Despite the identification of novel prostate cancer progression-associated biomarkers, validation of the findings, as well as discoveries of the molecular basis of cell communication and intra-cellular and intercellular signaling, have only begun to be performed with respect to metastatic clinical prostate cancer specimens [56]. New genetic and molecular pathways that will allow for stratification and classification of disease status based on molecular and biochemical parameters should facilitate improvements in the prevention, diagnosis, and treatment of prostate cancer.
1.5. Molecular Genetics of Prostate Cancer Initiation, Progression, and Metastasis.

1.5.1. Cytogenetics.

Karyotype analysis of many primary prostate cancer samples has typically revealed an atypical male diploid karyotype [57]. Cytogenetic changes are broadly categorized as gains, losses, rearrangements, and transversions. Loss of chromosomes 1, 2, 5, 7, 14, and Y and gains of chromosomes 7, 14, 20, and 22 have been reported [58]. Fluorescence in situ hybridization analysis has revealed trisomy of chromosome 7 and a subsequent correlation with tumor grade, pathological state, metastasis, and prostate cancer related death [59].

Loss of heterozygosity (LOH) studies resulted in the observation of LOH for at least one chromosome in 61% of primary prostate cancer tumors that were examined [60]. Approximately 20% of the examined tumors exhibited loss of heterozygosity on either chromosome 13q, 17p, 18q or a combination of these [60]. Interestingly, these regions contain the Rb (retinoblastoma), p53, and DCC (deleted in colorectal cancer) genes. In a different analysis, Bova et al. reported loss of 8p22 in approximately 70% of observed samples [86]. A study by Vocke and coworkers on
the short arm of chromosome 8 found LOH on 8p in 86% of observed prostate cancers [87]. A group of independent investigations indicated that chromosomes 2, 7, 8, 10, and 16 appear to have the greatest incidence of involvement in the progression of prostate cancer [61-63, 60].

1.5.2. Oncogenes: Introduction and Definition.

In the broadest definition, oncogenes are any genetic entities that promote tumor development [64]. Over 100 oncogenes have been identified to date. Proto-oncogenes participate in normal growth and development and they encode a wide variety of proteins that function as growth factors, growth factor receptors, regulators of DNA synthesis, regulators of gene transcription, and proteins involved in post-translational modification of targets [65].

There are a number of mechanisms by which a proto-oncogene can be converted to an oncogene. Base substitution mutations, such as observed for the ras gene, may be sufficient for conversion. Another mechanism for conversion involves the presence of extra copies of normal genes. This mechanism is called gene amplification with the N-myc and erbB-2 genes representing specific examples. Translocations, or the movement of a gene from one position on a chromosome to a different position on the same or different chromosomes, can also produce the
conversion from proto-oncogene to oncogene. The influence of novel promoters and transcriptional regulators sometimes produces an abnormal overexpression of genes that leads to oncogenesis. Translocation events may also produce fusion proteins with oncogenic potential. Such is the case with the c-Abl proto-oncogene in leukemia [66].

1.5.2.1. Ras and p21.

The ras family of cellular oncogenes encode small GTP-binding proteins and have been found to be mutated in a wide variety of human tumors. The Ras protein is a central component of intracellular signaling pathways involved in the transduction of stimuli that induce growth and differentiation. The Ras protein is activated in mammalian cells by guanine nucleotide releasing factors and, in the active state, the protein binds and activates the serine/threonine protein kinase encoded by the raf proto-oncogene. Mutant Ras proteins found in tumor cells have reduced GTPase activity and remain activated, resulting in increased levels of growth stimulatory signals.

The closely related C-H-ras, c-Ki-ras, and c-N-ras proto-oncogenes encode a 21 kDa protein (p21). Ras genes are activated after a single base mutation or with the loss of regulated expression [67]. Ras p21 proteins initiate events that lead to DNA
synthesis in response to mitogenic stimuli [68]. A great deal of evidence demonstrates that mutation in the ras genes are a frequent occurrence in prostate cancer [69-75]. LNCaP cells, transfected with non-mutated c-ras, expressed high levels of p21 protein but did not exhibit altered growth properties. However, LNCaP cells transfected with the mutated v-K-ras became androgen independent and exhibited increased anchorage-independent colony formation [76]. The frequency of H-ras point mutations in prostate cancer patients in the United States is estimated to be less than 5%. However, the occurrence of H-ras point mutations in Japanese male prostate cancer patients was found to be about 25%. Interestingly, the incidence of prostate cancer in Japanese males is about 5-fold less than that observed in the North American population, suggesting racial differences [73]. Currently, there appears to be no correlation between p21 protein expression and prostate tumor stage or grade [77]; but, experiments by Fan et al. demonstrated increases in c-H-ras mRNA expression in tumors that evolve from low grade androgen-dependent into high grade androgen-independent cancer [78-79].
1.5.2.2. **C-myc Oncogene.**

The *c-myc* oncogene is the cellular equivalent of the oncogene *v-myc* that is carried by an acutely-transforming strain of the retrovirus avian leukosis virus. Myc proteins are members of the helix-loop-helix-leucine zipper family of transcription factors. Myc proteins contain distinct transcriptional activation domains that modulate the expression of genes to which the transcription factor binds [80]. A number of genetic anomalies, such as amplification and chromosomal translocations, have been associated with *c-myc* in various human tumors. *C-myc* has been demonstrated to be involved in the chromosome translocation t(8;13)(q24;q32) found in Burkitt’s lymphoma where it is translocated into the immunoglobulin heavy chain gene.

Conflicting information exists in the literature concerning the role of *c-myc* in prostate cancer. A number of studies have demonstrated an increase in *c-myc* mRNA expression in prostate cancers compared to benign hyperplasias and normal tissue [81-85]. Additional studies did not demonstrate an association between *c-myc* and prostate cancer [88-89]. In mouse prostate models *c-myc/ras* overexpression was found to induce transformation and prostate cancer [90].
1.5.2.3. c-erbB-2 Oncogene.

The c-erbB-2 gene is located on chromosome 17q21-q22 and it encodes a transmembrane phosphoprotein that is very similar to the epidermal growth factor receptor. Over-expression by gene amplification seems to be the most common mechanism by which c-erbB-2 is activated in cancer, but activation has been observed in many tumors by over-expression of mRNA or protein without DNA amplification [91]. In breast cancer, c-erbB-2 amplification and overexpression has been correlated with poor prognoses [92-94]. But, like the c-myc gene, there is no clear consensus as to the role of c-erbB-2 in prostate cancer [95-101]. It is possible that a subset of prostatic adenocarcinomas express c-erbB-2 protein and that protein may contribute to the oncogenic state.

1.5.2.4. c-sis, c-fos, and Bcl-2.

The c-sis proto-oncogene product is identical to the beta chain of platelet derived growth factor and this gene has been studied in prostate cancer. Evidence exists to support the hypothesis that c-sis may play a role in the growth of prostatic tissue by androgens and suppression of growth by corticosteroids [82, 102]. RNA
analysis demonstrated an increase in *c-sis* expression in poorly differentiated prostatic adenocarcinomas [82].

The *fos* proto-oncogene is an "immediate early gene" in mammalian cells and is one of the first genes expressed in response to mitogenic stimuli. The *fos* gene encodes a nuclear transcription factor that forms heterodimers with Jun protein. The Fos/Jun heterodimers bind at AP-1 DNA binding sites and the Fos/Jun protein can function as both a positive or a negative regulator of gene expression. Some evidence exists to suggest a correlation between *c-fos* expression and prostate androgen receptor content [103-104] and androgen deprivation has been demonstrated to reduce *c-fos* expression by as much as 90% in some cell lines [103].

*Bcl-2* is an anti-apoptotic gene that has been associated with a translocation event correlated with follicular lymphoma. The gene is located on chromosome 18q21 and the protein is normally undetectable in the majority of hormone dependent prostate cancers. In contrast, androgen independent tumors have been shown to exhibit high levels of Bcl-2 protein [105]. Thus, the expression of *Bcl-2* may be associated with the transition to androgen independence [106].
1.5.3. Tumor Suppressor Genes.

Tumor suppressor genes are a class of genes that are thought to be involved in the regulation of normal cellular growth and development. A common characteristic of tumor suppressor genes is that their inactivation tends to favor tumor development. While oncogenes tend to favor tumor development by overexpression of the oncogenic gene product, that can be accomplished by incorrect modulation of a single gene, tumor suppressor genes must typically be inactivated at both alleles to manifest oncogenesis. Inactivation of two well known tumor suppressor genes, \( RB1 \) [107] and \( p53 \) [108] have been associated with several neoplasms [109-110].

1.5.3.1. \( RB1 \) (retinoblastoma gene).

Retinoblastoma is a malignant cancer that arises from the cells of the retina. There are two patterns of occurrence. In one group there is an onset in early childhood, with tumors in both eyes, and a propensity for development of other cancers. The other pattern of occurrence involves individuals that develop the eye cancer later in life and do not seem to be at a greater risk for development of secondary malignancies.
The first group appears to have inherited a gene that makes them more likely to develop retinoblastoma while the second group is thought to have acquired mutations of the \textit{Rbl} gene later on in life. The \textit{Rbl} gene is located on chromosome 13(13q14). Hereditary retinoblastoma is thought to involve a germ-line mutation that inactivates the \textit{RB} locus, on one of the pair of alleles, in chromosome 13. The lack of the gene in all somatic and germ cells results in enhanced capacity for tumor formation. Because the loss-of-function mutation favors the development of cancer, the \textit{Rb} gene is termed a tumor suppressor gene. \textit{Rbl} was the first identified tumor suppressor gene [107].

The Rbl protein product is a nuclear phosphoprotein termed p105\textit{Rbl} and it binds to DNA in a non-sequence dependent manner. P105\textit{Rbl} is known to interact with a large number of protein transcription factors including cell cycle related proteins. Associations between E2F and Rbl protein prevents the activation of a variety of genes, the products of which are central to the onset of DNA synthesis during S phase of the cell cycle [111]. This produces a cell cycle block in G1. Mutations of \textit{RB1} have been described in a number of human tumors [112-115]. Investigations have found the loss of a single \textit{Rbl} allele in 27\% of prostate cancers, suggesting that inactivation of this gene may play a role in prostate cancer development [116].
1.5.3.2 p53 Tumor Suppressor Gene.

The p53 tumor suppressor gene is a DNA-binding transcription factor that contains an acidic activation domain and has been identified as a tumor suppressor protein. In addition to activating transcription from promoters with p53 binding sites, it has been reported to suppress transcription from various promoters lacking p53 binding sites and also to inhibit helicase activity and DNA replication. Increased levels of p53 arrest cells in the G1 phase of the cell cycle and increased levels of p53 have been found to induce apoptosis after DNA damage, to inhibit tumor cell growth, and to preserve genetic stability [117]. P53 knockout mice demonstrate that the protein is not essential for development, but these animals do have an increased incidence of cancer [118].

1.5.4. Significance of the Androgen Receptor in Prostate Cancer.

The androgen receptor is a member of the ligand activated steroid thyroid hormone transcription factors [119]. The gene itself is located on the X chromosome and is greater than 90 kb in size with at least eight exons [120]. An 11 kb and an 8.5 kb androgen receptor mRNA have been demonstrated in prostate tissue and the
different transcripts are generated by alternative splicing of the 3’ untranslated region [121]. The cDNA sequence has a 2730 bp open reading frame that encodes a 98.5 kDa protein.

The androgen receptor contains four functional domains: (1) a 550 amino acid N-terminal portion that is highly polymorphic and regulates the amplitude [122] and specificity of the receptors target gene effects [123]; (2) a 67 amino acid DNA binding domain; (3) an 8 amino acid nuclear localization signal [123]; and (4) A C-terminal 250 amino acid hormone binding domain.

The androgen receptor N-terminal domain contains a high number of acidic residues and the DNA binding domain has a "D-box" on its C-terminal zinc finger for dimerization [124]. When bound, the hormone binding domain exhibits transcriptional regulatory properties and contributes to dimerization and nuclear localization [123,125].

Androgen receptors are required not only for the development of prostate cancer but also for normal prostate development. Androgen ablation typically produces an initial favorable response in 70-80% of prostate cancer patients, but most tumors progress to an androgen independent state within two years. The mechanism for progression to androgen independent prostate cancer involves loss of androgen receptor expression [126], androgen receptor gene amplification [127], and structural changes in the androgen receptor protein [128].
Androgen receptors have been found in both nuclear and cytosolic fractions of malignant and benign prostate cancer cells [129-130]. Nuclear androgen receptors were found to be 4-fold less common in metastases than in primary tumors [131]. In androgen independent prostate cancer, a significant decrease in androgen receptor positive cells was observed when compared to control prostate cancer of the same Gleason grade [132].

Evidence seems to indicate that mutations associated with the androgen receptor may influence the onset of androgen independence by allowing for selective outgrowth of cells with existing androgen receptor mutations during hormonal therapy rather than the acquisition of novel mutations after the beginning of treatment [133]. Many mutations have been shown in the androgen receptor ligand binding domain in both refractory prostate cancer and metastatic prostate cancer [134]. Gene amplification of the androgen receptor was observed in 30% of hormone refractory prostate cancers and is hypothesized that the increase in androgen receptor protein may facilitate tumor growth in the presence of low serum androgen levels such as those remaining after conventional hormonal deprivation therapy [127].

The transcriptional activation domain for the androgen receptor is located in the N-terminal region and this domain contains two polymorphic trinucleotide repeats of CAG [135] and GGC [136]. The CAG repeat codes for polyglutamine and GGC repeat codes for polyglycine. The length of the CAG repeat is 21±2 and removal of
the CAG repeat resulted in elevated transcriptional activation while increases in CAG correlated with a linear decrease in transcriptional activation [137]. Interestingly, Irvine et al. [138] showed that short CAG repeats were highest (75%) in African American males and lowest (49%) in Asians. African American males are considered to be one of the highest risk groups for prostate cancer while Asian men are considered to be a low risk group for prostate cancer.

1.6. **Prostate Cancer Statistics and Racial Ethnic Patterns.**

Prostate cancer incidences began to accelerate dramatically in the mid-1980s, to a peak of 190.9 per 100,000 in 1992, and then began decreasing. The corresponding rate in 1994 was 147.8 and was down to 137.2 in 1995. Mortality from prostate cancer had also been increasing for some time, reaching its peak in 1991 of 26.7 per 100,000 and subsequently decreasing to 25.9 in 1994 and to 24.9 in 1995 [139]. According to the “American Cancer Society's Cancer Facts and Figures - 1999" and as shown in the reports of the National Cancer Institute's “Surveillance, Epidemiology and End Results Program,” in 1999 alone, an estimated 179,300 men were diagnosed with prostate cancer, and some 37,000 men died from this disease. Incidence rates are substantially higher among African Americans in the U.S. and mortality rates in African American men are more than twice as high as rates in white
Asian and native American men have the lowest rates of prostate cancer. There is a remarkably sharp increase in prostate cancer incidences with age. Sixty percent of all newly diagnosed prostate cancer cases and almost 80% of all deaths occur in men 70 years of age and older. Mortality rates for prostate cancer are much lower than the incidence rates, because survival for men with this cancer is generally high [140]. Some research has suggested that diets high in fat and red meats increase risk, while a high intake of fruits and vegetables may offer some protection [141]. There is current interest in the possibility that the low risk of prostate cancer in certain Asian populations may be a result of their high intake of soy products [142].
1.7. Eukaryotic Gene Structure, Regulation of Gene Expression, and Transcription Factors in General.

Eukaryotic genes are generally considered to be those genes that are found in the nuclear genome of eukaryotic cells. Mitochondrial and chloroplast genes, though present in eukaryotes, are usually considered separately. The basic anatomy of a eukaryotic gene consists of a coding region, that encodes a polypeptide, tRNA, or rRNA molecule, and associated regulatory regions that flank the coding sequence and control the initiation of gene transcription. Eukaryotic genes exhibit a great deal of structural diversity due in part to the presence of introns that interrupt the coding sequence of many protein-coding genes and some tRNA genes. Introns are removed from primary RNA transcripts to produce functional mRNA. Protein coding genes are extremely variable in size, intron number, and regulatory flanking sequence (promoter) organization.

Eukaryotic organisms contain three different types of RNA polymerases that each transcribe different groups of genes. Ribosomal 18S, 5.8S, and 28S rRNAs are transcribed by RNA polymerase I [143]. Ribosomal 5S rRNA, tRNAs, and small nuclear and small cytoplasmic RNAs are transcribed by RNA polymerase III [144]. RNA polymerase II is responsible for transcription of all of the protein encoding
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genes as well as genes encoding U RNAs.

Multicellular organisms gain an advantageous synergy through cellular specialization and division of labor. The vast arrays of different cells within multicellular organisms synthesize proteins specific for the cell type in question and some proteins are specific to only one cell type. For example, insulin is only produced by pancreatic B cells, immunoglobulins are only produced by B lymphocytes, and hemoglobin is only produced in red cell precursors. However, the complete set of genes for the production of an entire organism is present in every cell type with few exceptions. Thus, the regulation of eukaryotic gene expression is an essential component of the multicellular condition.

In principle, any of the specific stages by which the information in DNA is ultimately encoded into a protein could be regulated to facilitate the correct gene expression for a given cell type. The processes of pre-mRNA transcription from DNA, mRNA post-transcriptional processing, transport of mRNA to the cytoplasm, and mRNA to protein translation each could be targeted for regulation. Evidence exists to confirm that gene regulation does occur at each of the before mentioned stages [145].

An abundance of evidence indicates that the majority of gene regulation occurs at the initial stage of gene transcription, by regulating the genes that are transcribed into a primary RNA transcript [145]. This type of regulation is referred to
as transcriptional control. In order for a gene to be transcribed, it is necessary for specific protein transcription factors to bind to sites in the regulatory regions of the gene to induce the gene’s transcription by RNA polymerase.

Transcription factor binding sites, in most eukaryotic genes, are organized into four distinct regions: the promoter, upstream promoter elements, regulatory elements, and enhancers. The promoter is considered to be immediately adjacent to the start site of transcription and, in many genes, contains the TATA box. The TATA box is a binding site for the TATA box binding factor TFIID \[146\]. Binding of TFIID to the TATA box is critical for the assembly of the basal transcriptional complex containing other transcription factors such as TFIIA, TFIIB, TFIIE, TFIIF and RNA polymerase II itself \[147\]. Binding of the basal transcriptional complex allows for a certain level of transcription that can be greatly enhanced by the binding of other constitutively expressed factors to upstream promoter elements (UPEs). UPEs are typically located immediately upstream of the promoter and the classical examples of UPEs include the CCAAT box, that binds a number of different transcription factors, and a GC-rich sequence that binds the constitutively expressed Sp1 transcription factor \[148\].

In addition to the before mentioned transcription factor recognition sequences, that bind constitutively expressed transcription factors, many genes contain other regulatory DNA sequences that bind transcription factors that only are active in specific cell types or under particular conditions. These regulatory sequences can
confer specific expression patterns on individual genes. The cyclic AMP response element (CRE) is a classic example of a regulatory DNA sequence that binds a transcription factor named CREB (cyclic AMP response element binding protein) that modulates the expression of specific genes under conditions of elevated intracellular cAMP [149].

Unlike the promoter, UPEs, and regulatory elements that are located proximal to the start site of transcription, the so-called “enhancer elements” exert an influence on gene expression from a distance. Enhancers can contain the same binding sites as those present in proximal promoter elements, but enhancers often contain multiple binding sites for the same transcription factor or multiple binding sites for several different transcription factors. The presence of multiple binding sites seems to allow enhancers to influence gene expression without regard to distance from the transcriptional start site, orientation of the transcription factor binding sites, or relative upstream or downstream location of the enhancer relative to the target gene promoter. Enhancers themselves are typically not capable of driving transcription, but the presence of these elements can increase the activity of target promoters by several orders of magnitude.

The binding of constitutively expressed transcription factors, such as the CCAAT-box binding factors and Sp1, alone allows for significantly high rates of transcription in some genes. This results in the constitutive expression of the genes
that are regulated by these constitutively expressed transcription factors in all tissue types. Other genes require the binding of specific regulatory transcription factors to binding sites proximal to the promoter, or in distal enhancer regions, in order for high levels of transcription to occur.

Certain regulatory transcription factors are active only in certain cell types and under certain conditions. Thus, these genes are only expressed in a limited number of cell types or after exposure to certain stimulatory signals. The control of eukaryotic gene expression is dependent upon the proper regulation of specific transcription factor activity. In some cases, the transcription factors are synthesized only in one cell type. The transcription factor Oct-2 (octamer binding transcription factor) is synthesized only in B cells and neuronal cells and the subsequent Oct-2 associated regulation ensures that immunoglobin genes are only active in B cells [150]. The transcription factor MyoD is only present in muscle and it has been demonstrated that expression of MyoD in non-muscle fibroblasts is sufficient to convert them to muscle cells [151].

In addition to the regulation of transcription factor activity through the control of transcription factor synthesis, existing transcription factors can be regulated by a variety of mechanisms in response to certain stimuli. The activity of some transcription factors is restricted by the presence of inhibitory proteins. The inhibitory proteins can dissociate under appropriate conditions in order for gene
expression to occur [152]. Transcription factors can also be activated by various post-translational modifications such as phosphorylation. This is observed in the case of the CREB transcription factor that mediates gene expression in response to cAMP. In this case, modulation of gene expression is dependent upon the ability of cAMP to stimulate the activity of the enzyme protein kinase A. Protein kinase A phosphorylates CREB and induces a conformational change in the transcription factor that renders CREB capable of stimulating the transcription of cAMP-dependent genes.

Most constitutively expressed or regulated transcription factors activate the transcription of specific genes; however, it is also possible for transcription to be inhibited by the action of transcription factors. In a similar fashion to activating transcription factors, inhibitory transcription factors can be regulated both at the level of synthesis and in response to stimuli. A number of cases exist where the same transcription factor can activate the expression of certain genes while inhibiting the transcription of other genes [153]. Protein-protein interactions between activating and repressing transcription factors can inhibit the capacity for activating transcription factors to bind DNA or activate transcription after binding to DNA.

An additional level of gene regulation is afforded to eukaryotic genes by the particular package in which they are arranged. Eukaryotic DNA is condensed into chromosomes in the form of DNA-protein complexes called chromatin. Research
suggests that genes that are not expressed in given cell types may be inaccessible to transcription factors due to the physical state of chromatin in differentiated cells [154-155]. The role of chromatin remodeling in the regulation of gene expression in various differentiated cell types continues to be an important emerging field of scientific investigation.

1.7.1 The Cellular Response to Adenosine 3′,5′ – Cyclic Monophosphate.

1.7.1.1. Adenosine 3′,5’- Cyclic Monophosphate Signal Transduction.

Cyclic 3′,5′- adenosine monophosphate (cAMP) was first identified as a mediator, or second messenger, of hormone action in 1959 by Sutherland and coworkers. Currently, cAMP has been found to be an intracellular signaling molecule in all prokaryotic and animal cells that have been studied, and cAMP has been demonstrated to regulate a variety of cellular activities [145]. In eukaryotic cells, except for higher plants, cAMP serves as a second messenger that is produced as a result of the activation of cell surface receptors coupled to GTP binding proteins [156]. The increased intracellular cAMP levels activate cyclic AMP dependent protein kinase A that is capable of phosphorylating target proteins and modulating the activity of the target proteins. In bacteria, cAMP levels are regulated by the available
nutrient levels, and cAMP controls gene expression through interaction with the cyclic AMP receptor protein [157]. In the cellular slime mold *Dictyostelium discoideum*, cAMP acts as a chemoattractant and signaling molecule during development [158].

The typical intracellular concentration of cAMP is $1 \times 10^{-7}$ M. In order for the molecule to function efficiently as an intracellular mediator, the concentration of cAMP must be capable of rapidly changing in response to extracellular signals such as hormones. Cyclic AMP is synthesized from a membrane bound enzyme called adenylyl cyclase and is continuously degraded by cyclic AMP phosphodiesterases. Phosphodiesterases hydrolyze cAMP to adenosine 5’ monophosphate (5’ AMP).

Many extracellular signaling events, such as peptide hormones, growth factors, and neuronal activity [159], influence cellular activity by altering intracellular cAMP levels. Extracellular signals alter cAMP levels by influencing the activity of adenylyl cyclase. The various extracellular receptors that recognize signaling molecules are coupled to adenylyl cyclase enzymes by trimeric G proteins [160]. The beta-adrenergic receptors, that mediate the action of adrenaline and noradrenaline, are classic examples of receptors that are coupled to the activation of adenylyl cyclase.

Trimeric G proteins are composed of alpha, beta, and gamma polypeptides. The alpha chain binds and hydrolyzes GTP and modulates adenylyl cyclase activity. G proteins are termed "stimulatory" ($G_s$) or "inhibitory" ($G_i$) depending upon the
regulatory effect of the G protein relative to adenylate cyclase. The beta and gamma G protein chains are tightly bound to one another and anchor the G protein to the cytoplasmic face of the cell membrane. When inactive, stimulatory G proteins (G\(_s\)) exist as a trimer with GDP bound to the alpha polypeptide. When stimulated by receptor binding, the alpha subunit exchanges the GDP for GTP. It is thought that the guanine nucleotide exchange facilitates dissociation of the alpha subunit from the trimeric G protein [161]. The alpha subunit is then capable of associating with and activating adenylyl cyclase to produce cAMP.

The activation of adenylyl cyclase by G-alpha is rapidly reversible. A GTPase activity of G-alpha is activated when G-alpha binds to adenylyl cyclase. The bound GTP is hydrolyzed to GDP and that inactivates the stimulatory effect of G-alpha. G-alpha is then free to re-associate with the beta and gamma G protein subunits to reform the inactive trimeric G protein.

It is interesting to note that the same extracellular messenger, such as adrenaline, can either increase or decrease cAMP levels depending on the type of receptor to which the messenger binds. In the case of \(\beta\)-adrenergic receptors, binding of adrenaline activates adenylate cyclase and increases intracellular cAMP levels [162]. Binding of adrenaline to \(\alpha_2\)-receptors inhibits adenylate cyclase. The inhibition of adenylate cyclase is accomplished by inhibitor G proteins (G\(_i\)). The alpha subunit of inhibitor G proteins differs from the G-alpha of stimulatory G
proteins, but the beta and gamma polypeptides may be identical between the two trimeric G proteins.

Nine isoforms of mammalian adenylyl cyclase have been cloned [163-167]. The primary structure of adenylyl cyclase consists of two transmembrane regions (M1 and M2) and two cytoplasmic regions (C1 and C2) [168]. Each of the transmembrane regions contains six predicted membrane-spanning helices. The cytoplasmic regions are further subdivided into C1a and C1b; and C2a and C2b. The C1a and C2a regions are homologous to each other and compose the enzyme active site [169].

Formation of cyclic AMP by adenylyl cyclase requires the deprotonation and activation of the ATP 3’-hydroxyl for nucleophilic attack; stabilization of the transition state at the α-phosphate; and stabilization of increased negative charge on the pyrophosphate leaving group [170]. There is a great deal of evidence that the formation of cAMP from ATP by adenylyl cyclase involves a two-ion mechanism [171-172]. One Mg\(^{2+}\) is thought to act as free Mg\(^{2+}\) (metal ion "A") while the other (metal ion "B") binds in a complex with all three nucleotide phosphates in ATP [173]. Metal ion "A" activates the 3’-hydroxyl and both metal ions share in stabilization of the transition state.

Elevation of intracellular cAMP levels can be enhanced through the use of reagents that stimulate adenylyl cyclase activity, inhibit phosphodiesterases, or
directly increase intracellular cAMP, such as membrane permeable cAMP analogs. Forskolin is a hydrophobic activator of mammalian adenylyl cyclases that increases activity by up to $10^3$ [170]. Forskolin is known to bind to the catalytic core of adenylyl cyclase and activate the enzyme by bringing together the C1a and C2a active site domains. Forskolin brings the active site domains together through a combination of hydrophobic and hydrogen bonding interactions [174].

The intracellular effects of cAMP are mainly exerted by cAMP-induced activation of the enzyme cyclic-AMP-dependent protein kinase (PKA). PKA catalyzes the transfer of the terminal phosphate group from ATP to specific serines or threonines of target proteins. Covalent phosphorylation of the appropriate amino acids on target proteins regulates the target protein’s activity. Protein kinase A is found in all animal cells and PKA is thought to mediate almost all of the effects of cAMP in animal cells. Different cell types have different substrates for PKA and this explains why the effects of cAMP vary in different cell types. PKA, in the inactive state, is composed of two catalytic subunits and two regulatory subunits. Association of cAMP with the regulatory subunits alters the regulatory subunit conformation and releases the catalytic subunits [175]. The catalytic subunits are then activated to phosphorylate target substrate proteins.
1.7.1.2. Regulation of Gene Expression by Adenosine 3’5’-Cyclic Monophosphate.

Increases in intracellular cAMP are known to modulate gene expression in some animal cells. Interestingly, the elevation of intracellular cAMP levels can result in either stimulation or repression of individual gene expression [156,176-177]. A short palindromic DNA sequence (5’-TGACGTCA-3’) called the cyclic AMP response element (CRE) can be recognized by a variety of different transcription factors. Variations in the CRE consensus sequence are common.

The first transcription factor capable of binding the CRE that was cloned was termed CRE-binding (CREB) protein [156]. The CREB protein was followed by the discovery of several other proteins that were capable of modulating gene expression through CRE elements. The CREM/CREB subfamily includes CREB, ATF1, CREMI and CREM II. The CRE-BP1 subfamily includes CRE-BP1 and ATF\(_{\alpha}\). In addition, ATF3, ATF4, ATF6, TREB5, and E4BP4 constitute CRE-binding proteins [156, 149]. CRE-binding proteins have been found to play an important role in the physiology of the pituitary gland, in regulating spermatogenesis, in the response to circadian rhythms, and in the molecular basis of memory [178].

CRE-binding proteins all belong to the basic region/leucine zipper (bZip)
transcription factor class [149]. The DNA binding domain of CRE-binding proteins is a conserved 30 amino acid region that is rich in basic residues. To the immediate C-terminal of the basic region is a region with a heptad leucine repeat. This region can form an amphipathic alpha-helix with the leucine residues aligned along one ridge and two of these helices can associate in a coiled-coil conformation (leucine zipper) that allows for protein dimerization [179]. The alpha helical basic region is capable of wrapping around the DNA helix and contacting specific bases in the major groove. The spacing between the basic region and the leucine zipper is highly conserved and appears to be a critical feature of the transcription factor [149].

Certain CRE-binding factors are capable of forming heterodimers with a limited number of other CRE-binding factors [180-181]. Additional complexity is granted to the CRE-binding factors by the capacity for alternative splicing. Alternative RNA splicing has been demonstrated for CREB [182], CREBP1 [183], and CREM [184]. CREM is of particular interest because the CREM transcript can encode both activators and repressors of CRE-mediated gene transcription.

CRE-binding proteins can be divided into activators and repressors of gene transcription. Examples of activator CRE-binding proteins include CREB, CREMτ, and ATF-1 [185]. Repressor CRE-binding proteins include three isoforms of CREM (α, β, γ), ICER, [186-187] E4BP2 [188], and CREB-2 [189].

The transcriptional activation domains of activating CRE-binding proteins,
such as CREB, contain two independent regions. The kinase-inducible domain, also known as the phosphorylation box (P-box), contains a number of phosphorylation sites for different kinases. Glutamine-rich domains constitute the second important region and flank the P-box on both sides [190]. The glutamine-rich domains function as transcriptional activation domains similar to the domains that are present in other activators such as AP-2 and Sp1 [191-192]. When CREB is phosphorylated at serine 133 by PKA, mitogen-activated protein kinases (MAPKs), or Ca\textsuperscript{2+}/calmodulin dependent protein kinases (CaMKs) [159], CREB is activated and can activate gene expression. However, phosphorylation of serine 133 in CREB is not sufficient for full transcriptional activation. An acidic region downstream from the P-box and another region called $\alpha_2$ contain a number of sites that can be recognized by casein kinase II [193-194].

The current understanding of the mechanism of transcriptional activation involves both phosphorylation of the P-box and the presence of the glutamine rich domains. Phosphorylation is thought to induce a change in CREB protein conformation and the conformational change is believed to expose the glutamine-rich domains. The glutamine-rich domains are thought to interact with components of the basal transcriptional complex. Phosphorylation of the P box may also be involved in the regulation of transcriptional activation domains present in other transcription factors bound nearby on the promoter [195]. CREB-binding protein (CBP) and p300
are coactivators that can bind with the phosphorylated CREB P-box [196]. The coactivators are believed to position histone acetyltransferases (HATs) near specific nucleosomes in gene promoters, interact with many additional transcriptional activators, and interact with basal transcription factors such as TFIID, TFIIB, and the RNA polymerase II holoenzyme [197]. In addition, active, Ser133-phosphorylated CREB affects transcription of CRE-dependent genes via interaction with the 265-kDa co-activator protein CREB-binding-protein, CBP, which bridges the CRE/CREB complex to components of the basal transcription complex [198]. The CREB-binding protein contains three zinc finger domains, a glutamine-rich domain, and a PKA phosphorylation site adjacent to the third zinc finger domain [199].

The DNA binding properties of CREB may or may not be affected by phosphorylation at serine 133. Some investigators have found a 2-3-fold increase in DNA binding by CREB after phosphorylation by PKA [200] while others did not find a correlation between PKA phosphorylation, protein dimerization, and DNA binding [201].

The action of protein phosphatases on phosphorylated activators provides the basis for the mechanism leading to repression of genes induced through the PKA pathway. Four groups of serine/threonine phosphoprotein phosphatases (protein phosphatases I, IIA, IIB, and IIC) work to inactivate proteins that are activated by phosphorylation. The activity of any protein that is regulated by phosphorylation
depends on the balance of activities between the kinases that phosphorylate the protein and the phosphatases that dephosphorylate the protein. The protein phosphatase-I is responsible for dephosphorylating many of the PKA targets. Protein phosphatase-I dephosphorylates CREB to counter cAMP induced activation of gene expression.

Two specific groups of repressors for CRE-induced transcription exist. The CRE element binding repressors include 1) those that are constitutively present and whose activity can be altered by phosphorylation and 2) those repressors that can be stimulated by cAMP. CREMα, CREMβ, and CREMγ are constitutively present. They have a similar structure to activator CRE-binding proteins but they lack the glutamine rich domains. Thus, repressor CREMs can bind to CRE elements and be phosphorylated by PKA but they do not stimulate transcription [184].

The discovery of an additional family of CREM isoforms, the inducible cAMP early repressors (ICER), provides for an important contribution to the understanding of the molecular mechanisms governing cAMP-dependent repression of gene expression [187]. ICER expression is cyclic AMP inducible and these small (12-13.5 kDa) CRE-binding proteins are produced from alternate transcription of the CREM gene via an intronic promoter. ICER proteins contain the DNA-binding domain but lack the P-box and glutamine rich domains; thus, these CRE-binding proteins serve as efficient repressors of CRE-mediated transcription [187]. ICER transcription factors
can repress CRE-mediated transcription either by binding to CRE sites as inactive homodimers or by forming heterodimers with activators.

1.7.2. Sp1 Family of Transcription Factors.

The general transcription factor Sp1 was named according to the original purification scheme that utilized Sephacryl and phosphocellulose columns [202]. Sp1 is one of the most common GC box binding transcription factors. GC box and GT/CACC boxes are important cis-acting elements that have been found in promoters, enhancers, and locus control regions [203]. G-rich element sequences, such as GC (GGGGCGGGG) and GT/CACC boxes (GGTGTTGGGG) have been found to be involved in the regulation of genes that are under cell cycle, hormonal, and developmental regulation [203].

Currently, 16 different mammalian proteins have been found to contain a highly conserved DNA binding domain consisting of three C2H2-type zinc fingers that are typical of the Sp1 family of transcription factors. The identified transcription factors are Sp1, Sp2, Sp3, Sp4, BTEB1, TIEG1, TIEG2, EKLF, GKLF, LKLF, ZNF741, AP-2rep, BKLF, IKLF, BTEB2, ZF9, and UKLF [203]. The amino acid sequences that are most likely to make contact with DNA from each of the three zinc fingers include KHA from zinc finger 1, RER from zinc finger 2, and RHK from zinc
finger three. These amino acids are conserved in Sp3, Sp4, BTEB1, TIEG1, and TIEG2 and these transcription factors recognize the classical Sp1 binding sites [204-206]. A leucine residue replaces the histidine residue in the first zinc finger of Sp2 and this substitution removes Sp2’s binding affinity for the classical Sp1 GC box and replaces it with a GT-rich element affinity in the T-cell receptor Va promoter [207]. EKLF, UKLF, and BKLF have substituted the lysine in the third finger with leucine and each of these transcription factors appear to have a preference for GT boxes over the classical GC box [208-211].

The majority of Sp/XKLF transcription factors have been demonstrated to act in a positive manner. However, the Sp3 factor was originally found to repress Sp1-mediated activation by binding to the Sp1 recognition site and preventing subsequent binding and activation by Sp1 [212]. In a similar circumstance, BTEB1 was found to activate the AP2 promoter while AP-2rep was found to repress the promoter by competing for the same site [213]. Examples such as these suggest a potential mechanism for gene regulation based upon competition between repressors and activators for binding sites. In addition, the location and organization of transcription factor binding sites within a particular promoter appear to determine the effect of the particular transcription factor on gene transcription. In the case of Sp3, promoters containing a single binding site exhibit activation of gene transcription while promoters with multiple binding sites may not respond to Sp3 [214]. Both
activation and repression domains have been identified in Sp1 [215], Sp3 [214], EKLF [216] and GKLF/EZF [217].

Considering that Sp1 is ubiquitously expressed and Sp1 has been demonstrated to be involved in the activation of many genes, including genes involved in cell cycle regulation, chromatin remodeling, and the propagation of methylation-free islands [218-225], it is predicted that a cell lacking Sp1 would not be able to survive. Surprisingly, Sp1 null embryonic stem (ES) cells were found to exhibit normal growth characteristics, maintained methylation-free islands, and could differentiate in vitro with similar efficiency to control ES cells [226]. However, all Sp1 null embryos in mice exhibited severely slowed growth and died after day 10 of development.

One of the most intriguing questions surrounding the Sp1 family of transcription factors concerns the capacity for specificity between the individual family members. A certain degree of specificity has been demonstrated to be provided by DNA binding site preference. All Sp1 family transcription factors bind to similar DNA targets; however, the binding affinities for specific sequences are different. For example, Sp factors are known to bind more tightly to GC boxes than to GT boxes [227] while XKLF bind more efficiently to GT boxes [228].

Sp1 family transcription factor expression patterns also play a role in specificity. Transcription factors like Sp1 and Sp3 are ubiquitously expressed but
others, such as EKLF and BTEB1 are restricted to certain cell types [229]. For many of the Sp1 family transcription factors, mRNA expression patterns alone have been analyzed without regard for protein expression patterns. Thus, it is conceivable that protein expression patterns may not correlate with observed mRNA expression as was the case for BTEB1. Competition for available DNA binding sites in tissues in which more than one Sp1 family member is expressed also may contribute to specificity. The abundance of individual transcription factor proteins is known to vary in different cell types under different conditions. In primary keratinocytes, Sp3 levels exceed Sp1 levels and the Sp3:Sp1 ratio becomes inverted if the cells are allowed to differentiate [230]. In NIH 3T3 cells, GKLF mRNA levels are high in quiescent cells, but become almost undetectable in proliferating cells [231]. Also, TIEG1 is induced by TGFβ, EGF, and estrogens in a number of different cell types [232-233].

A further level of specificity may be obtained by interaction of Sp1 transcription factors with other transcription factors, co-activators, or co-repressors. A protein named mCtPB2 (murine C-terminal binding protein 2) binds to the repression domain of BKLF and may act as a co-repressor [234]. In addition, the co-activator CRSP (cofactor required for Sp1 activation) was found to be required for Sp1 activation in vitro [235].

Post-translational modification of the Sp1 family proteins may also play a role
in providing specificity. Sp1 is known to be phosphorylated [236] and glycosylated [237]. EKLF has been demonstrated to be modified by both phosphorylation and acetylation and mutation of the phosphorylation target site abolished activity from the 40 amino acid EKLF transactivation domain [238]. Certainly, the molecular mechanisms that govern Sp1 family transcription factor specificity are complex and will remain a challenging topic for continued investigation.

1.7.3. GA-Binding Protein Transcription Factors.

The transcription factor E4TF1 was originally purified from HeLa cells based on its ability to bind to and activate transcription from the adenovirus early 4 (E4) promoter [239]. Characterization of the transcription factor revealed two subunits, an ets-related DNA binding protein designated E4TF1-60, and a non-DNA binding factor termed E4TF1-53. Each of the subunits were found to interact with one another to form the E4TF1 heterotetramer (E4TF1-60)$_2$(E4TF1-53)$_2$ on the DNA recognition sequence 5’-CGGAAGTG-3’ and binding of the transcription factor was shown to activate transcription in vitro [240]. E4TF1-47, another E4TF1 subunit, was found to be identical to E4TF1-53 in the N-terminal region but E4TF1-47 contained a different C-terminal sequence. Complexes of E4TF1-47 with E4TF1-60 did not stimulate in vitro transcription as efficiently as E4TF1-60 in complex with
Complementary DNA library sequence analysis of the E4TF1 subunits found them to be highly homologous to the GABPα (E4TF1-60), GABPβ-1 (E4TF1-53), and GABPβ-2 (E4TF1-47) GA binding protein (GABP) from rat [242-244]. Thus, the E4TF1 subunits have been renamed using the human GABP nomenclature (hGABP). hGABPα, hGABPβ1, and hGABPγ1 correspond to E4TF1-60, E4TF1-53, and E4TF1-47 respectively.

The hGABP transcription factors have been found to be involved in the regulation of a number of genes including the human heparanase 1 (HPR1) gene [245], BRCA1 [246], Fas [247], Utrophin [248], and several ribosomal protein genes [153, 249-250]. Of particular interest, Genuario et al. [153] demonstrated that GABP can serve as either an activator or a repressor of ribosomal protein gene transcription depending upon the location of the consensus sequence for the transcription factor within the promoter. Ribosomal gene promoters with GABP sites 40-80 bp upstream from the transcriptional start site were shown to act as activators of gene transcription while ribosomal promoters with the GABP consensus sequence in the immediate vicinity of the transcriptional start site were found to act as repressors of gene transcription.
1.8. **The Ribosome, Individual Ribosomal Proteins, and Multi-functionality.**

Ribosomes are the sites of protein synthesis in both prokaryotic and eukaryotic cells. Ribosomes were first characterized by ultracentrifugation and were designated based on their rates of sedimentation. Bacterial ribosomes were designated 70S and eukaryotic ribosomes were designated 80S. Ribosomes are composed of both proteins and rRNA and rapidly growing mammalian cells may contain as many as 10 million ribosomes [251].

The basic structure of both prokaryotic and eukaryotic ribosomes is similar but they differ from one another in some details. Both prokaryotic and eukaryotic ribosomes have a small and a large subunit. The prokaryotic small subunit (termed 30S) is composed of 16S rRNA and 21 protein subunits. Proteins from the small subunit are given the prefix "S" and a corresponding protein number (e.g., S6). The 50S, or large subunit of prokaryotic ribosomes, is composed of 23S and 5S rRNA and 34 protein subunits (L1-L34). Eukaryotic ribosomal subunits are larger and contain more proteins than the prokaryotic subunits. The eukaryotic small subunit (40S) consists of 18S rRNA and about 33 protein subunits (S1-S33). The large subunit (60S) is composed of 28S, 5.8S, and 5S rRNA in addition to about 50 individual
proteins (L1-L50).

The ribosomal RNA composes about two-thirds of the ribosome’s mass and the rRNA is essential for ribosomal function. Ribosomal RNA forms complex three-dimensional conformations as a result of complementary base pairing within the individual rRNAs. Ribosomal proteins bind to the rRNA, and other ribosomal proteins, to form the functional ribosome and each ribosomal subunit has been demonstrated to be able to self-assemble \textit{in vitro} from its constituent rRNAs and proteins. Crystal structures of individual ribosomal proteins and the entire ribosome itself have been determined [252]. A number of the ribosomal proteins that form a complex with rRNA have been demonstrated to share a common motif, called a \textit{Rossman} fold, with an alpha-helix and a sheet of three beta-strands, that is also seen in the structure of the RNA-binding domain of the small nuclear RNA-binding protein U1 from ribonucleoprotein A [253].

The individual ribosomal proteins are low molecular weight (M_r <35,000), mostly basic proteins. Many of the ribosomal proteins bind directly to the rRNA itself at specific primary and secondary structures. A number of the ribosomal proteins are targets for protein kinases and phosphatases (\textit{i.e.} eukaryotic ribosomal protein S6) and the associated phosphorylation events seem to play a role in the regulation of protein synthesis [254]. A small number of ribosomal proteins are acidic and have the capacity to be exchanged on and off of the ribosome [255].
Ribosomal protein genes are found in only one or two copies per haploid genome. The expression of individual ribosomal proteins is thought to be coordinately regulated to produce equimolar concentrations of protein that are stoichiometric with rRNA concentrations. However, a growing body of evidence seems to indicate that the expression of individual ribosomal proteins may not be stoichiometric with rRNA concentrations [256-257]. These observations support the concept that individual ribosomal proteins may be involved in multiple functions within the cell.

Gene recruitment is a mechanism of molecular evolution whereby a gene product can function in more than one capacity. The “one gene-dual function” phenomenon is exemplified by crystallins, structural proteins that play both specialized roles in the eye lens and also “housekeeping” enzyme roles [258]. Ribosomal proteins are integral components of the basal cellular machinery involved in protein synthesis, whose roles have been regarded collectively as important, but individually somewhat mundane. Various individual ribosomal proteins, and also translation initiation and elongation factors, have been found to play roles in regulating cell growth, transformation, and death, giving rise to increasing speculation that components of the translational apparatus can act as multifunctional proteins. Naora et al. [257] demonstrated that ribosomal protein S3a (RPS3a) plays important roles in cell transformation and death, whereby constitutively or transiently
enhanced \textit{RPS3a} expression can be regarded as “priming” a cell for apoptosis and suppression of such enhanced expression as “execution.” Ribosomal proteins P_0 and S3 possess apurinic/apyrimidinic endonuclease activity and these proteins have been suggested to participate in DNA repair [259-260]. Mutations in the ribosomal protein S6 seem to be associated with melanotic tumor formation, lymph gland hyperplasia, and abnormal blood cell differentiation [261].

Increasing evidence supports the hypothesis that some of the proteins involved in transcription and translation are shared between the two processes [262]. For some proteins, such as ribosomal proteins S10 and L4, there is strong evidence of their participation in both processes, and much is known about their role in both activities [263-264-265]. There are not, at present, many examples of such shared proteins, but the importance of understanding their behavior and intimate involvement with two major cellular machines represents a significant topic for continued investigation.
1.9. Research Objectives and Significance of the Current Study to the Investigation of Prostate Cancer.

Identifying novel molecular and genetic markers associated with prostate differentiation has the potential to facilitate our understanding of prostate development, carcinogenesis, tumor progression, and metastatic dissemination as well as contributing to subsequent advancements in prostate cancer management. The identification of the underlying mechanisms of international and racial differences in prostate cancer development and progression; the separation of passive and virulent forms of prostate disease; improvements in diagnosing and monitoring patients; and the potential to predict the ultimate malignant potential of a prostate tumor while still at a localized state may all be advanced through studies of prostate cell differentiation.

Prostate cancer is a diverse and multifocal disease. To understand the clinical characteristics of prostate cancer, we must first investigate the cellular and molecular basis of disease initiation and progression. The ultimate goals of prostate cancer research are to better understand the fundamental concepts of cancer biology, and to both prevent the development of prostate cancer and cure those men already afflicted.

One of the primary goals of the investigation presented here is to identify
genes, and characterize novel genetic biomarkers, that are differentially expressed or suppressed in the development of LNCaP prostate cancer cells during cyclic AMP induced neuroendocrine differentiation. The novel gene expression patterns recorded in this work should provide excellent targets for further evaluation and validation in clinical specimens.

A second goal of the investigation involves the analysis of cAMP related intercellular signal transduction pathways that govern and determine gene transcription and the differentiation of cancer cells to neuroendocrine cells. It is recognized that characterization of the molecular pathways that control prostate development, differentiation, and senescence, may provide exciting insights into the determinants of prostate cancer growth, proliferation, and apoptosis in response to physiological, pathological, and pharmacological perturbations.

A substantial gap remains in the comprehension of the basic cellular and molecular biology of the normal prostate, especially with regard to the events that govern the early stages of neoplastic cell transformation and the subsequent progression and acquisition of a malignant phenotype and metastatic potential [266]. Several factors have contributed to the absence of information regarding the continuum of normal prostate development, the multiple steps of carcinogenesis, and the ultimate acquisition of a malignant and metastatic phenotypes. These include, but are not limited to, a lack of relevant animal models that closely mimic the
development, transformation, and acquisition of androgen-independent and metastatic human prostate disease [267], as well as a deficiency in the understanding of basic prostate cancer developmental processes, including the regulatory mechanisms of gene transcription and cell proliferation, differentiation, and senescence.

Because of the lack of understanding of the basic processes that underlie prostate cancer development, progression, and metastasis, the ability to prevent, diagnose, and assign prostate cancer patients to appropriate treatment modalities is significantly impeded. In order to improve therapeutic and management options for men with prostate disease, it is necessary to characterize relevant genes or gene products that may serve as reliable biomarkers of prostate cancer progression.

The development of new human prostate cancer cell lines and xenografts that closely mimic initial androgen dependency, but are capable of progressing to an androgen-independent and metastatic phenotype, should provide templates for novel research [268]. New strategies to screen large quantities of cells in different physiological and pathological states for differences in gene expression affords us an opportunity to identify marker genes associated with each stage of prostate cancer progression. As a result, the causative genetic switches for each of the multiple steps from initial neoplastic transformation to disseminated, hormone-resistant disease can be identified and applied in the treatment of prostate cancer.
1.9.1. Specific Research Objectives of the Current Investigation.

A. Induction of neuroendocrine differentiation using cyclic AMP, IBMX, and forskolin in LNCaP cells.

B. Examination of neuroendocrine differentiation reversibility following withdrawal of differentiation agents in LNCaP cells.

C. Isolation and purification of total cellular RNA from both differentiated and non-differentiated LNCaP cells.

D. Differential display analysis of differentiated vs. non-differentiated LNCaP mRNA.

E. Confirmation of differential gene expression by northern blot analysis.

F. Cloning of those differential display PCR products that appear to be differentially expressed.
G. DNA sequencing of differentially expressed PCR products and computer database analysis of DNA sequences.

H. Isolation of complete cDNA sequences from computer databases for differentially expressed genes.

I. Isolation of genomic DNA sequences by genomic PCR for differentially expressed genes.

J. Promoter analysis using luciferase and/or GFP reporter genes assay.

H. Analysis of putative promoter protein binding sites using electrophoretic mobility shift assays.
1.9.2. Significance of the Current Research to the Advancement of Prostate Cancer Investigation.

The current investigation has the potential to facilitate the advancement of a number of different aspects of prostate cancer research including:

A. Advancement of the understanding of the molecular mechanisms underlying the transformation of normal cells to cancer cells.

B. The discovery of potential diagnostic markers for the early detection of prostate cancer.

C. Discovery of valid targets for gene therapy and other molecular-based treatment modalities.

D. Introduction of new molecular mechanisms of cyclic AMP action.

E. Expedition of human genome-based studies concerning the identification, cloning, and characterization of proteins relevant to normal and cancerous
prostate cells.

F. The potential to define metastasis-associated genes and their expression patterns in human cell lines. These may suggest new genes for analysis in both primary and metastatic clinical prostate specimens.

G. The capacity to provide new insights into prostate development and general developmental processes in biology to facilitate the understanding of multicellular interactions in the prostate gland and their underlying molecular basis.

H. Identification of critical housekeeping and regulatory genes that may be associated with human prostate cancer development and progression.

I. The discovery of novel genes that are expressed during prostate cancer development and the progression of prostate cancer to androgen independence.

J. Suggestion of alternative signaling pathways that regulate prostate growth, development and differentiation.
K. Suggestion of novel target genes, if any, that may explain international and racial differences in prostate cancer development, progression, and metastatic potential.

L. The potential to provide new alternatives for genes that can be considered hereditary prostate cancer genes.
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Chapter 1

Introduction and Survey of the Literature


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CHAPTER 2

Identification of differentially expressed genes during adenosine 3’:5’-cyclic monophosphate induced neuroendocrine differentiation in human prostatic adenocarcinoma cell line LNCaP.

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2.2 ABBREVIATIONS

NE : neuroendocrine

cAMP : cyclic adenosine monophosphate
db-cAMP : dibutyryl-cAMP
dd-PCR : mRNA differential display PCR

Fsk : forskolin

IBMX : isobutylmethylxanthine

Epi : epinephrine

Isop : isoproterenol

GPC3 : glypican 3

HRS3a : human ribosomal protein S3a

AP : arbitrary primer

Abbreviated Title: Differentially Expressed Genes in LNCaP

Key Words: prostate cancer; differential display-polymerase chain reaction; signal transduction.
2.3 ABSTRACT

The LNCaP cell line is a versatile and useful model that is suitable for the study of human prostate cancer \textit{in vitro}. It has been determined that the elevation of LNCaP intracellular cAMP levels through the addition of membrane permeable cAMP analogues, phosphodiesterase inhibitors, adenylate cyclase activators, or components of the cAMP signal transduction pathway can induce reversible neuroendocrine differentiation. Elucidation of those genes that are differentially expressed between undifferentiated prostate cancer cells and prostate cancer cells that have been induced to differentiate may present new insights for the molecular mechanisms governing NE differentiation, early detection of prostate cancer, and/or potential targets for gene therapy. In this study, differential display PCR was used to identify 226 differentially expressed PCR products. Twelve of the dd-PCR products were confirmed by Northern blot analysis and cloned. DNA sequencing and database comparisons were performed. This is the first report, to my knowledge, of the use of differential gene expression techniques to analyze gene expression during cAMP induced NE differentiation in LNCaP cells. Confirmation of NE differentiation reversibility was also accomplished.
2.4 INTRODUCTION

Even though prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer-related death in men in the western world, there have been no significant developments in treatment of the disease since hormonal down regulation was introduced in the 1940s [1]. Unfortunately, patients that are treated by androgen ablation inevitably develop the androgen-independent form of the disease [2]. An urgent need exists for the development of new therapeutic approaches for the treatment of advanced prostate cancer based on the specific characteristics of prostate cancer physiology.

Much of the advancement in the understanding of prostate cancer has been made possible by the study of human prostate cancer cell lines such as LNCaP. The LNCaP cell line was originally established from metastatic human prostatic adenocarcinoma cells that were isolated from the left supraclavicular lymph node of a Caucasian male [3-4].

It has been demonstrated that the LNCaP cell line is capable of both epithelial and neuroendocrine differentiation [5]. LNCaP cells grown in vitro develop features of neuronal morphology when stimulated by treatments that increase intracellular
levels of cAMP. NE differentiation is marked by the appearance of dense core granules in the cytoplasm, the extension of neuritic processes, loss of mitogenic activity, and expression of the NE markers neuron-specific enolase (NSE), parathyroid hormone-related peptide, neurotensin, serotonin, and chromogranin A [6].

Exposure of LNCaP cells to membrane permeable cAMP analogs such as db-cAMP, adenylate cyclase activators such as forskolin (Fsk), and/or phosphodiesterase inhibitors such as IBMX increase intracellular levels of cAMP and result in the induction of NE-like differentiation. Factors that operate both upstream, such as the B-adrenergic receptor agonists epinephrine (Epi) and isoproterenol (Isop) (6), and downstream, such as activated cAMP dependent protein kinase (PKA), are sufficient to induce NE characteristics [7]. In addition, LNCaP cells have been shown to develop NE characteristics in response to long term androgen ablation [8], and stimulation with the cytokines interleukin-1B and interleukin-6 [9-10].

Given that cyclic AMP is a classic second messenger in signal transduction pathways that lead to alterations in gene expression, it is reasonable to postulate that the onset of NE differentiation in LNCaP cells is facilitated by accompanying alterations in gene expression. To our knowledge, no study to date has examined the effect of the cyclic AMP induced NE differentiation process on gene expression.

The relationship between prostatic NE cells and prostate cancer progression
has generated significant interest in NE cells as potential targets for new therapeutic strategies. NE cells are typically present in the peripheral, central, and transition zones of the normal prostate but are most common in the periurethral and ductal regions [11]. NE cells are thought to play a paracrine role during prostate gland development and it is believed that NE cells participate in the regulation of secretory processes in the mature prostate. There appears to be a direct relationship between the density of NE cells and enhancement of prostate cancer characteristics such as raised Gleason grade, loss of androgen sensitivity, and autocrine/paracrine activity [12-13]. It has been suggested that NE cells may provide paracrine stimuli for the propagation of local carcinoma cells and that NE differentiation is associated with the progression of prostate cancer toward an androgen-independent state [14].

Elucidation of the molecular events that coordinate NE differentiation has the potential to contribute substantially to the resolution of the potential roles of NE cells in the development of androgen independence and to the development of new approaches for carcinoma treatment.

In this report, I initiated a project using the differential display technique in an attempt to better characterize the molecular events associated with cAMP induced NE differentiation. The differential display technique is based on the reverse transcription and PCR amplification of expressed mRNA populations from specific
cells types or cells under different conditions [15]. Reverse transcription of the 3' terminal ends of mRNAs is accomplished using one base anchored oligo dT primers which bind to the 5' boundary of the poly-A tail [16]. The reverse transcription is followed by cDNA PCR amplification using a number of 13 bp primers which are arbitrary in sequence. The resulting cDNA PCR products are subjected to electrophoretic separation that allows for side by side analysis and rapid identification of differentially expressed cDNAs that can be recovered, reamplified, cloned and used as probes for Northern blot analysis to confirm differential expression. Differential display has been successfully used to identify potential oncogenes and tumor suppressor genes involved in a variety of malignancies [17-21].

I also examine the discrepancy between Bang et al.'s [5] original description of cAMP induction of differentiation as a terminal phenomenon and the more recent report by Cox et al [6] pertaining to the reversibility of cAMP induced NE differentiation. In agreement with Cox et al, we find acquisition of cAMP induced NE characteristics to be a reversible phenomenon. In addition, I am interested in the identification of genes that are differentially expressed as a result of cAMP induced neuroendocrine differentiation. These genes may play a role in cancer promotion and advancement, may facilitate the early detection of prostate cancer, and/or provide potential targets for gene therapy.
2.5 MATERIALS AND METHODS

2.5.1 Cell culture. The human prostatic carcinoma cell line LNCaP was obtained from the American Type Culture Collection (Manassas, VA). LNCaP cells were maintained at 37°C in a humidified incubator containing 5% CO$_2$ / 95% air in RPMI 1640 medium supplemented with 50 µg/ml gentamycin, 2.5 µg/ml fungizone (Gibco. Rockville, Maryland), and 10% heat inactivated fetal bovine serum (Atlanta Biologicals. Norcross, GA). Fluid renewal was performed twice weekly and cultures were passaged upon confluency (6-8 x 10$^5$ cells/cm$^2$) by trypsinization.

2.5.2 Induction of NE differentiation and growth studies. Cells were counted using a hemacytometer and cell viability was determined by counts of trypan blue-excluding cells. Neuroendocrine differentiation was induced in the LNCaP cell line by the addition of either 1 mM dibutyryl-cAMP, 500 µM IBMX, 60 µM forskolin, or the combination of 1 mM dibutyryl-cAMP and 500 µM IBMX together. Induction of differentiation was started 24 hours after subculturing cells and repeated after 48 hours. NE Induction was carried out over a four-day period.
2.5.3 RNA isolation and preparation. Total RNA was isolated from confluent cultures of both differentiated and non-differentiated LNCaP cells by lysis in RINApure (Genehunter Corporation, Nashville, TN) guanidium thiocyanate solution as per the manufacturer’s suggested protocol. Total RNA for differential display was then purified by DNase I digestion, phenol/chloroform (3:1) extraction, and ethanol precipitation.

2.5.4 Reverse transcription of mRNA and differential display PCR. Differential display PCR reactions were performed in duplicate using RNAimage kits containing differential display primers and reagents from Genehunter. Messenger RNA was reverse transcribed using 200 units Moloney-murine leukemia virus reverse transcriptase in the presence of 2 μM one-base anchored oligo-dT primer, 20 μM deoxynucleotide triphosphate, and 10 μM DTT. PCR was performed by amplifying 10% of the cDNA mixture with 0.4 μM one base anchored oligo-dT primer, 0.4 μM 13 bp arbitrary primer, 20 μM deoxynucleotide triphosphate, 1 μCi [α-33P]dATP, and 0.1 unit Taq DNA polymerase (Qiagen, Valencia, CA). PCR reactions were performed in a Perkin Elmer 2400 thermocycler. Forty cycles of denaturing at 94°C
for 30 sec, annealing at 40°C for two minutes, and extension at 72°C for 30 sec were performed followed by an elongation step of 5 min at 72°C. The PCR products were separated on a 6% denaturing polyacrylamide gel in the presence of 8 M urea. The gel was dried and exposed to X-ray film at -80°C for 16 hrs.

2.5.5 **PCR re-amplification of differential bands.** Differentially expressed bands between the differentiated and non-differentiated LNCaP lanes were excised from the gel, soaked in 100 µl mQH2O for 10 min, precipitated with ethanol, and reamplified using the same primer set and PCR conditions for differential display except that the dNTP concentration was increased to 20 µM and no isotope was added.

2.5.6 **Verification of differential expression by Northern hybridizations.** Northern blot analysis was performed to confirm differential gene expression. Electrophoresis of 15 µg total RNA per lane was performed using 1.5% denaturing formaldehyde agarose gels. RNA was blotted onto Hybond (Amersham, Arlington Heights, IL) positively charged nylon membranes by capillary action and UV crosslinked. The probes were labeled with \([\alpha-^{32}\text{P}]d\text{ATP}\) by random-priming as
described (22). Hybridization was performed overnight at 42°C. The blots were washed with 2 X SSC, 0.1 % SDS at room temperature for 15 min. This was followed by two washes at 55°C with 0.2 X SSC, 0.1 X SDS for 15 min each wash. The blots were exposed to X-ray film at -80°C for at least 16 hrs.

2.5.7 Cloning of differentially expressed PCR products. The differentially expressed PCR products were cloned using the PCR-TRAP cloning vector (Genehunter Corporation, Nashville TN) and transformed into competent Escherichia coli cells using standard techniques [23]. Ligation reactions using reamplified PCR products from differential display and PCR-TRAP cloning vector were carried out overnight at 16°C.

2.5.8 DNA sequencing and analysis. Clones of interest were sequenced using an ABI Prism 310 automated fluorescent sequencer and the Virginia Tech DNA sequencing facility. DNA sequence data was compared to known sequences in public databases such as Genbank for homology to known sequences.
2.6 RESULTS

2.6.1 Increased levels of intracellular cAMP induce neuroendocrine morphology and continued elevation of cAMP is required to maintain neuroendocrine morphology in LNCaP cells. LNCaP cells were treated with cAMP elevating agents db-cAMP, IBMX, db-cAMP/IBMX, or Fsk for 5 days and the morphology of the cells was examined by phase-contrast microscopy (Fig. 2.1). Cells under all treatments developed features of neuronal morphology featuring a condensed cell body, elongation of narrow processes in a multipolar or bipolar fashion (Fig. 2.1), and loss of mitogenic activity (Fig. 2.2). Untreated LNCaP cells exhibited an epithelial morphology. The cells in each of the four treatments developed identical structures regardless of the stimulus agent that was used.

To better determine the level of stimulus agent required for differentiation, a range of stimulating agent concentrations was used to induce differentiation and subsequent cell counts were preformed to assess mitogenic activity (Fig. 2.3). Differentiation was assessed by microscopic observation and total cell number. Concentrations of db-cAMP of 0.5 mM and higher were sufficient to induce differentiation and inhibit cell growth while concentrations less that 0.1 mM did not
Chapter 2

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induce NE differentiation. The phosphodiesterase inhibitor IBMX suspended cell division when used in concentrations of 1 µM and higher. Concentrations of Fsk greater than 1 µM were sufficient to induce differentiation, but concentrations of 60 µM were most efficient for rapid induction.

Loss of mitogenic activity is a typical characteristic of prostatic NE cells. In order to assess the reversibility of LNCaP cell cycle arrest by cAMP induced NE differentiation, LNCaP cells were subjected to withdrawal of stimulus agents after five days of induction. Cell number was measured for LNCaP cells after five days of treatment and again five days after withdrawal from db-cAMP, IBMX, db-cAMP/IBMX, or Fsk (Fig. 2.4). Removal of cAMP-elevating agents resulted in the loss of NE characteristics and a return to the epithelial morphology typical of untreated cells. Upon removal of the differentiation agent, LNCaP cells rapidly relinquished neuritic features and regained epithelial characteristics as evident by phenotypic examination and a return to typical cell division. These results support the observation that cAMP induced NE differentiation is not a stable phenotypic alteration, but is a reversible phenomenon. Cells that were maintained in the presence of differentiating agents continued to exhibit growth inhibition (Fig. 2.4).
2.6.2 Cyclic AMP-induced NE differentiation of LNCaP cells results in alterations in gene expression when compared with undifferentiated LNCaP cells as indicated by differential display PCR. A total of 144 different PCR primer combinations were used for differential display PCR producing 226 potentially differentially expressed cDNA fragments. Differential display reactions performed in duplicate allowed for the rapid identification of uncommon banding patterns between the two cell conditions (Fig. 2.5). Twelve cDNA fragments demonstrated differential expression by Northern blot analysis (Fig. 2.6) and were subsequently cloned and sequenced (Fig. 2.7). Nine of the cDNA fragments exhibited decreased expression levels following NE differentiation while three fragments were overexpressed in differentiated cells (Table 2.1). Each primer combination produced between 50-100 bands as expected [17]. Of the 214 potentially differentially expressed cDNA fragments for which differential expression was not supported by Northern blot, about 75% failed to produce a band while the remaining 25% were false positives.

Comparison of the sequences obtained by differential display PCR to published sequences in Genebank using BLAST indicated that the isolated cDNAs represented genes encoding F1F0 H\(^+\) transporting ATP synthase subunit \(g\), L1 juxtaposed to glypican 3 (\(GPC3\)), human ribosomal \(s3a\) (\(HRS3a\)), human protein
translation initiation factor 2C1 (\textit{EIF2C1}), and a putative heme binding protein.

LINE, MER, and \textit{Alu} repetitive elements were found to be differentially expressed upon cAMP induction. In addition, two sequences with unknown function and no significant homology to published sequences were isolated. The MER and LINE (L1) repetitive elements each appeared twice over the course of differential display reflecting the significant presence of these sequences in the human genome.
2.7 DISCUSSION

The results presented in this study serve to illustrate the wide array of genes expressed differentially between two phenotypically different forms of the LNCaP human prostate cancer cell line. We hypothesized that isolation of genes expressed differentially between untreated LNCaP cells and LNCaP cells that have been induced to a quiescent NE-like state would result in the identification of genes encoding important regulatory proteins as well as proteins that can serve as diagnostic, prognostic, and therapeutic targets. In addition, we have provided additional support for the reversibility of LNCaP NE differentiation; a finding that may provide important insights for the progress of prostate cancer physiology.

The ability of cAMP elevating agents to induce NE differentiation in LNCaP cells suggests that an increased accumulation of intracellular cAMP may underlie the conversion of prostatic tumor cells to NE cells and contribute to NE cell increases during disease progression. It has been speculated that the ability of prostatic NE cells to produce possible mitogenic hormones may influence the growth of local carcinoma cells that lie in proximity to the NE cells [47]. If NE cells are able to contribute to the durability and growth of the surrounding tumor cell population and
are themselves able to reenter the cell cycle under designated physiological conditions, then tumors possessing NE cells may gain a strong selective advantage [6].

A cDNA was identified by differential display as being down-regulated during cAMP-induced NE differentiation. This cDNA was subsequently cloned, sequenced, and identified as a human gene known collectively as fte-1, v/fos transformation effector gene, and HRS3α. Enhanced expression of translation factor mRNAs such as S3α has been found in tumors and cancer cell lines [24, 25]. These genes have been alluded to as possible facilitators of colorectal cancer and liver carcinogenesis [26, 27]. S3α has also been demonstrated to be down regulated following neuronal differentiation of NTERA cells [28]. In addition, S3α has also been demonstrated to promote apoptosis and enhance malignant phenotypes [24].

Protein synthesis by mRNA translation is an essential process in living cells. Thus, every eukaryotic cell type can be expected to exhibit a certain degree of ribosomal protein expression. Upon superficial inspection, it is easy to dismiss the increased expression levels of ribosomal proteins in carcinomas as simply a by-product of the augmented metabolic activity that accompanies tumor physiology. However, an increasing body of evidence seems to suggest that individual constituents of the translational mechanism may play an important role in cancer
progression by acting as regulatory mediators of growth, proliferation, and oncogenesis.

Another cDNA identified as being down regulated was *EIF2C1*. This protein codes for a gene that is involved in the initiation of protein translation. Translation initiation is considered to be the most complex process in the myriad of consecutive steps that are required for the initiation of protein synthesis [29]. The human eukaryotic protein translation initiation factor *EIF2C1* gene is located on the short arm of chromosome 1 in the region 1p34-p35 which is frequently lost in human cancers such as Wilms tumors, neuroblastoma, and carcinomas of the breast, liver, and colon [30]. The *EIF2C1* gene is ubiquitously expressed at low to medium levels in healthy tissues, but *EIF2C1* appears to be expressed at high levels in undifferentiated LNCaP cells and down regulated following the addition of cAMP. There is a significant deficiency of investigation relative to the potential for dual functionality by this novel gene. The differential expression pattern observed for *EIF2C1* following LNCaP NE differentiation suggests that this gene may play an important role in prostate cancer physiology.

It is unlikely that increased levels of proteins such as S3a and EIF3C1 are due to increased rates of cell division because the expression of individual translational proteins are coordinately regulated by various means to produce equimolar amounts
of protein that are roughly stoichiometric with rRNA concentrations. In the course of evolution, it is thought that some of the constituents of multi-protein complexes, such as the ribosome, may have been recruited from prior cellular activities to enhance alternative cellular functions. $S3a$ in particular provides support for this hypothesis in that it has already been identified as a bifunctional ribosomal protein that interacts with the transcription factor CHOPS acting as a negative modulator of erythroid differentiation during erythropoiesis [31]. Additional functional roles for both $S3a$ and $EIF2C1$ may exist and these roles could prove to be very significant for the progression of prostate cancer disease.

The glypican 3 (GPC3) integral membrane protein is a newly discovered gene that has gained considerable attention for its role in the Simpson-Golabi-Behmel overgrowth syndrome [32]. The Simpson-Golabi-Behmel syndrome is characterized by pre- and postnatal overgrowth as well as increased risk of embryonal tumors such as neuroblastoma, medulloblastoma, and Wilms’ tumor [33]. Interestingly, $GPC3$ has recently been identified as a differentially expressed gene in several different cancer types using differential display technology. $GPC3$ was found to be transcribed in several neuroblastomas and all Wilms’ tumors but not present in medulloblastoma and malignant mesotheliomas [33-34]. Lin et al [35] found that $GPC3$ expression was lost in some ovarian cancer cell lines due to hypermethylation
Lin et al also showed that ectopic expression of GPC3 inhibited growth of ovarian cancer cell lines. An L1 element in juxtaposition to GPC3 was found to be up-regulated following NE differentiation. This observation enhances the possibility that GPC3 itself is up regulated as a result of cAMP treatment in LNCaP cells. If accurate, this finding would be in agreement with the observations of Lin et al [35] and Murthy et al. [34], indicating that GPC3 is not highly expressed in actively growing LNCaP cells but is up-regulated after cAMP induction. These results provide for the intriguing possibility that GPC3 functions as a negative regulator of LNCaP cell growth.

Cloning and sequencing of a cDNA that was down regulated during cAMP-induced NE differentiation of LNCaP identified it as being the “g” subunit of F1F0 H⁺ transporting ATP Synthase. This is particularly interesting because the ATP Synthase enzyme has been demonstrated to be involved in interactions with anti-cancer drugs such as angiostatin and endostatin. The surprising find by Moser et al [36] that angiostatin binds to ATP synthase began to shed some light on a possible mechanism by which angiostatin inhibits angiogenesis resulting in attenuated tumor growth. Loss of ATP from ATP synthase by binding of drugs such as angiostatin may prevent blood vessel growth, that is necessary for continued tumor growth, by removing an essential enzyme involved in the production of cellular energy. Specific
targeting of individual components of the ATP synthase complex may provide alternative means of blocking the ATP producing function of this enzyme and provide for novel therapeutic approaches for prostate cancer treatment.

The causal versus effectual nature of down regulation of a putative heme binding protein during cAMP induced NE differentiation in LNCaP cells is a subject for conjecture. It is known that the presence of heme binding protein seems to be linked to the abundance of uncommitted heme in the cytosol. Heme is not very soluble in aqueous solutions at standard physiological pH and ionic conditions and tends to form large aggregates at concentrations as low as $10^{-7}$M [37]. The prosthetic group heme is associated with a number of different proteins including globins, cytochromes, and enzymes. In addition, heme has been shown to regulate protein synthesis in both transcriptional and translational modalities [38-39]. These observations indicate that increased levels of heme may be present in actively growing cancer cells. Since heme is known to be intimately involved with gene expression, it is worth investigating whether or not heme and/or heme binding protein may be promoting the expression of genes that facilitate the cancer process. Studies on functions of heme binding proteins and the influence of heme on gene expression are few; thus, their participation in cellular regulation has remained poorly understood.
cDNA sequences corresponding to L1 repetitive elements were demonstrated to be up-regulated following cAMP induced NE differentiation. Differential display PCR products corresponding to L1 elements were cloned on two independent occasions in the study. LINE is an acronym for long interspersed DNA sequence elements. LINE’s are a class of interspersed repetitive retrotransposon-like sequences found in mammalian genomes. L1 is an example of a LINE that has been replicating and evolving in mammals for at least the past 100 million years and now composes at least 20% or more of some mammalian genomes [40]. L1 elements have likely had a prominent effect on the evolution, structure, and function of mammalian genomes. Because L1 elements are known to be a major source of insertional mutagenesis in humans, it is reasonable to postulate that L1 mediated gene knockout may play a significant role in the initiation of carcinogenesis and other genetic disorders. In human hepatocarcinomas, hypomethylation of LINEs has been listed as a potential contributor to increased retrotransposition and resultant genomic instability [41]. It has been shown that in addition to gene knockout, LINE sequences can also modify the transcribed coding sequence of additional genes [42].

LINE retrotransposons, which transpose through the reverse transcription of their own transcript, can also mobilize transcribed DNA not associated with the LINE sequence [43]. Because LNCaP differentiation is induced by increased levels of
intracellular cAMP, it is our hypothesis that up regulation of L1 elements is likely due to the effects of cyclic AMP response elements that juxtapose L1 elements. On the surface, this hypothesis may seem to diminish the fundamental importance of LINE elements in carcinogenesis. However, this notion suggests a potential mechanism by which mundane physiological processes that increase cAMP may inadvertently increase the retrotranspositional potential of L1 elements and contribute to genomic instability. The potential roles of LINE elements in pathogenesis make these sequences and their associated proteins interesting targets for further study.

Alu elements are short interspersed repetitive elements (SINEs) found exclusively in primates. These elements are around 300 base pairs long, are found in excess of one million copies per diploid genome, and are found throughout the human genome. MER elements are medium reiteration frequency sequences that are also primate specific [44]. Like LINE elements, Alu elements and MER elements are replicated by retrotransposition. [45]. This suggests that repetitive elements such as these have the potential to produce a multitude of effects on the genome, mostly by inactivating genes or altering gene function. Alu elements are known to assume a variety of functions depending on the influences of the surrounding genetic material into which they insert [46]. Four of the cDNAs that were isolated from differential display and found to be down regulated following induction of NE differentiation
corresponded to either SINE or MER elements. This poses an interesting question. How does the mechanism of SINE and MER retroposition differ from LINE retroposition under the influence of cAMP and/or NE physiology? Possible roles of SINE and MER elements in the onset of human disease make these elements intriguing candidates for continued investigation.

In summary, dd-PCR allowed for the examination of 226 potentially differentially expressed products between undifferentiated LNCaP cells and LNCaP cells that had been induced to NE differentiation by increases in intracellular cAMP. Twelve of the potentially differentially expressed products were confirmed by Northern analysis. Exhaustive analysis of each of the differentially expressed genes identified in this report is beyond the scope of this preliminary study. The results of this investigation provide for several fascinating candidates for continuing research in the field of LNCaP NE differentiation and prostate cancer physiology. In addition to potential regulatory roles during the process of LNCaP NE differentiation, the genes identified here allude to novel molecular mechanisms for prostate cancer disease progression and these findings have the capacity to generate new avenues for carcinoma therapy.
2.8 REFERENCES


9. **Spiotto MT, Chung TD.** STAT3 mediates IL-6-induced neuroendocrine differentiation in prostate cancer cells. Prostate 2000;42(3):186-95.

10. **Qiu Y, Robinson D, Pretlow TG, Kung HJ.** Etk/Bmx, a tyrosine kinase with a pleckstrin-homology domain, is an effector of phosphatidylinositol 3'-kinase and is involved in interleukin 6-induced neuroendocrine differentiation of prostate cancer cells. Proc Natl Acad Sci USA. 1998;95:3644-3649.


Table 2.1. Summary of Properties of Differentially Expressed cDNA Transcripts

<table>
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<th>Fragment</th>
<th>Primers</th>
<th>bp</th>
<th>Identified Sequence or Gene</th>
<th>Score</th>
<th>Expect</th>
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<tr>
<td>10C(15)</td>
<td>HT5/G/AP15</td>
<td>91</td>
<td>Unknown - no significant homology</td>
<td>N/A</td>
<td>N/A</td>
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<td>186</td>
<td>F1E+ H+ transporting ATP synthase subunit</td>
<td>262</td>
<td>5e-68</td>
</tr>
<tr>
<td>6C(15)</td>
<td>HT5/G/AP15</td>
<td>407</td>
<td>HREEs</td>
<td>668</td>
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</tr>
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<tr>
<td>probe #11</td>
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<td>LINE1 on chromosome #5</td>
<td>264</td>
<td>3e-68</td>
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<tr>
<td>B418</td>
<td>HT5/G/AP17</td>
<td>233</td>
<td>Human protein translation initiation factor 2CI (eIF-2CI)</td>
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<td>3e-14</td>
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<tr>
<td>B218</td>
<td>HT5/G/AP18</td>
<td>226</td>
<td>MER type repeat on PAC clone RP5.44.7E14 from 7</td>
<td>448</td>
<td>e-124</td>
</tr>
<tr>
<td>B1620</td>
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<td>285</td>
<td>LTR repeat on chromosome 21 region 581.05</td>
<td>597</td>
<td>e-142</td>
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Table 1. Summary of Properties of Differentially Expressed cDNA Transcripts.

Genes expressed differentially between uninduced and cAMP induced NE differentiated cells in human prostate cancer cell line LNCaP. AP: arbitrary primer; HTi/C, HTi/C, one base anchored primer composed of a HindIII recognition site, 1 thymidine residues (T), and either a guanine (G) or cytosine (C) that acts as the one base anchor specific for the 3' end of cDNA’s immediately 5' the poly A tail. Score and Expect are from Genebank homology searches.
Agents that elevate intracellular cAMP levels induce growth arrest and neuroendocrine morphology. (A). Untreated LNCaP cells. (B). LNCaP cells treated with 1 mM db-cAMP and 500 µM IBMX. (C). LNCaP cells treated with 500 µM IBMX alone. (D). LNCaP cells treated with 30 µM Forskolin. Photographs were taken 72 hours after initiation of differentiation. Changes in morphology among LNCaP cells treated with different cAMP elevating agents were identical regardless of the stimulating agent used.
Figure 2.2.  Effects of agents that increase intracellular cAMP on cell number.

$1 \times 10^5$ LNCaP cells per well in six well plates were stimulated with 1 mM db-cAMP, 500 µM IBMX, or 60 µM Fsk for 5 days. Cell counts were performed using a hemocytometer and are the average of three individual counts per stimulating agent.
Chapter 2  
Differentially Expressed Genes in LNCaP

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)
Figure 2.3. Effects of different concentrations of differentiation agents on LNCaP cell number.

1 x 10^5 LNCaP per well were exposed to different concentrations of (A) db-cAMP, (B) IBMX, and (C) Fsk. Changes in cell morphology were observed using phase-contrast microscopy and cell counts were taken after 5 days.
Chapter 2

Differentially Expressed Genes in LNCaP

A

B

Graph A and B showing cell number data for different treatments.

Graph A title: Cell Number \( \times 10^4 \)

Graph B title: Cell Number \( \times 10^4 \)

Legend: cAMP, cAMP/IBMX, IBMX, Forskolin, Untreated.
Figure 2.4. Effects of withdrawal of cAMP-elevating agents on LNCaP cell growth.

(A). $1 \times 10^5$ LNCaP cells were treated with levels of cAMP-elevating agents as indicated in Fig. 2 for 5 days and subjected to cell counting (open bars). A second group of LNCaP cells was treated with standard concentrations of cAMP-elevating agents for 10 days and then counted (filled bars). (B). $1 \times 10^5$ LNCaP cells were treated with cAMP-elevating agents for 5 days. The medium was then withdrawn and supplemented with complete medium alone. Following withdrawal of the stimulating agent, LNCaP cells were maintained for an additional five days in complete medium and then counted using a hemocytometer. The results are representative of three separate counts.
Figure 2.5. Differential Display of undifferentiated and cAMP induced NE-differentiated LNCaP mRNA.

Total RNA was isolated from uninduced (U) and cAMP induced (D) LNCaP, reverse transcribed, and amplified by PCR using 13 bp arbitrary primers and one-base anchored oligo dT primers in the presence of $[^{33}\text{P}]d\text{ATP}$. The PCR fragments were separated using 6% polyacrylamide DNA sequencing gels and subjected to autoradiography as detailed in “Materials and Methods.” The size of each fragment is listed in Table 1. Arrows indicate the bands selected for further analysis. When possible, reference bands are included to show equivalent intensity of nondifferentially expressed bands in proximity to differentially expressed bands. Scratches around differentially expressed bands are a result of band excision using the autoradiograph as a guide for the dried gel.
Figure 2.6. Confirmation of Differential Display PCR by Northern Blot.

(A) Fifteen µg of total RNA from uninduced (U) and cAMP induced (D) LNCaP cells was subjected to Northern blot analysis as detailed in “Materials and Methods.” Subclones of fragments shown in Fig. 5 were used as individual probes for Northern hybridization. (B) Representative ethidium bromide stained gels. Ribosomal RNA bands from uninduced (U) and cAMP induced (D) LNCaP cells demonstrate equal total RNA loading in the two lanes.
Figure 2.7. Homology of a dd-PCR DNA sequence with known gene sequence.

The 6C(15) dd-PCR product sequence (query) was compared to known DNA sequences using the Genbank nr database. The 6C(15) DNA sequence exhibited strong homology to the HRS3a mRNA sequence (Sbjct). Database comparisons were performed for all isolated dd-PCR sequences resulting in similar homologies with additional published DNA sequences (Table 1).
CHAPTER 3

Characterization of human ribosomal S3a gene expression during adenosine 3’:5’ cyclic - monophosphate induced neuroendocrine differentiation of LNCaP cells

Accepted for Publication: Molecular Biology Reports. April 2002.
3.1 ABSTRACT

Elevation of intracellular cAMP levels in the human prostatic adenocarcinoma cell line LNCaP results in the induction of reversible neuroendocrine differentiation and cell growth arrest. We have used the differential display technique to identify genes that are differentially expressed during cAMP induced neuroendocrine differentiation in LNCaP cells. We identified the human ribosomal S3a gene as being down regulated in response to LNCaP differentiation. The S3a gene is known to be expressed at high levels in both tumors and cancer cell lines. It has also been shown that down regulation of S3a is associated with a loss of the transformed phenotype. In order to better ascertain the mechanism by which S3a gene expression is decreased during differentiation, the promoter region for this gene was analyzed. Electrophoretic mobility shift assay, antibody supershift assays, site-directed mutagenesis, and luciferase reporter gene analysis were employed to authenticate the roles of several transcription factors in the regulation of the S3a gene. We found that two cyclic AMP response elements, an Sp1 element, and a GA-binding protein were involved in the regulation of S3a gene expression. The CRE elements were found to be necessary for high level expression of the S3a gene in undifferentiated LNCaP cells. Mutations in the CRE elements abolished CREB-1 binding and resulted in a 50% decrease in S3a gene expression. The addition of cAMP elevating agents to LNCaP cells in sufficient quantity to induce differentiation also generated a 50%
decrease in S3a gene expression. Therefore, the results indicate that the presence of the CRE elements is necessary for cAMP-induced down regulation of gene expression. Furthermore, occupation of the GABP binding site results in a significant decrease in S3a promoter activity.
## 3.2 ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3':5'-cyclic monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>DNA complementary to mRNA</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>db-cAMP</td>
<td>dibutyryl-cAMP</td>
</tr>
<tr>
<td>dd-PCR</td>
<td>mRNA differential display PCR</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
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<td>GABP</td>
<td>GA binding protein</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxy-ethyl)-piperazine-1-ethane-sulfonic acid</td>
</tr>
<tr>
<td>hRPS3a or S3a</td>
<td>human ribosomal S3a</td>
</tr>
<tr>
<td>IBMX</td>
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</tr>
<tr>
<td>MSP</td>
<td>β-microseminoprotein</td>
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<td>NE</td>
<td>neuroendocrine</td>
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<td>phenylmethylsulfonyl fluoride</td>
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3.3 INTRODUCTION

An estimated 200,000 men will be diagnosed with prostate cancer in 2001 [1]. For decades, radical prostatectomy has been the preferred treatment for men with localized prostate cancer [2]. While chemical or surgical castration does produce tumor regression, long term survival is not secured. Patients afflicted with metastatic cancer eventually relapse as the disease progresses to hormonal independence [3]. An increased understanding of the molecular mechanisms that regulate prostate cancer physiology is crucial for the development of novel treatment modalities.

The capacity for prostate cancer cells to undergo reversible neuroendocrine (NE) differentiation as a result of increased intracellular cAMP levels has generated significant interest in NE cells as potential targets for new therapeutic strategies [4-8]. In order to better ascertain the regulatory mechanism of LNCaP NE differentiation, we have begun an investigation for identification and characterization of those genes that are differentially expressed in response to cAMP.

We have previously used the differential display technique with the aim of identifying genes that are differentially expressed in response to cAMP induced NE differentiation. A PCR product was identified using the differential display technique [9] as being down-regulated during cAMP-induced NE differentiation. This cDNA
was subsequently cloned, sequenced, and identified as a human gene known collectively as *fte-1*, *v/fos* transformation effector gene, and *S3a* [10-11]. The enhanced expression of translation factor mRNAs, such as *S3a*, has been recognized in both cancer cell lines and tumors [12, 13]. Translational apparatus proteins have also been recognized as possible facilitators of both colorectal cancer and liver carcinogenesis [14,15]. *S3a* has been demonstrated to be down regulated following neuronal differentiation of NTERA cells [16]. In addition, *S3a* has been shown to modulate apoptosis and enhance malignant phenotypes [12].

It is generally accepted that the expression of individual translational proteins are coordinately regulated by various means to produce equimolar amounts of protein that are roughly stoichiometric with rRNA concentrations. Thus, it is unlikely that increased levels of proteins such as *S3a* are due to increased rates of cell division. In the course of evolution, it is thought that some of the constituents of multi-protein complexes, such as the ribosome, may have been recruited from prior cellular activities to enhance alternative cellular functions. *S3a* in particular provides support for this hypothesis in that it has already been identified as a bifunctional ribosomal protein that interacts with the transcription factor CHOP acting as a negative modulator of erythroid differentiation during erythropoiesis [17]. Additional functional roles for *S3a* may exist and these roles could prove to be very significant for the progression of prostate cancer disease.
In order to better characterize the role of cAMP in the regulation of \( S3a \) gene expression, we describe here the capacity for two potential cAMP response element (CRE) sequences to be recognized by cAMP response element binding protein (CREB). Analysis of the effects of the CRE/CREB interactions on gene regulation during NE induction and in actively growing carcinomas were performed using a luciferase reporter vector and site-directed mutagenesis of the CRE elements. A dominant negative CREB expression vector was used to verify further the functional importance of the CRE elements in regulation of \( S3a \). In addition, we demonstrate that binding of the GABP consensus sequence, that is located in the transcriptional initiation region, significantly down-regulates \( S3a \) promoter activity.
3.4 MATERIALS AND METHODS

3.4.1 Cell culture. The human prostatic carcinoma cell line LNCaP was obtained from the American Type Culture Collection (Manassas, VA). LNCaP cells were maintained at 37°C in a humidified incubator containing 5% CO\textsubscript{2} / 95% air in RPMI 1640 medium supplemented with 50 µg/ml gentamycin, and 10% heat inactivated fetal bovine serum (Atlanta Biologicals. Norcross, GA). Cells were grown to 80% confluency in 75 cm\textsuperscript{2} culture flasks and passaged into 6 well plates the day before transfection experiments. Fluid renewal was performed twice weekly and cultures were passaged upon confluency (6-8 x 10\textsuperscript{5} cells/cm\textsuperscript{2}) by trypsinization.

3.4.2 Induction of NE differentiation and growth studies. Cell counts were conducted using a hemacytometer and cell viability was determined by counts of trypan blue-excluding cells. Neuroendocrine differentiation was induced in the LNCaP cell line by addition of the combination of 1 mM dibutyryl-cAMP and 500 uM IBMX. For isolation of RNA from NE-differentiated cells, induction of differentiation was started 24 hours after subculturing the cells and repeated after 48 hours. NE induction was carried out over a four-day period. For most luciferase
activity studies, NE induction was initiated 16 hours after plasmid transfection. A limited number of luciferase activity studies were performed on cells that were exposed to cAMP/IBMX 3 days prior to plasmid transfection in order to evaluate the efficiency of NE cell transfection.

3.4.3 Isolation of S3a 5' flanking sequences by genomic PCR. Two oligonucleotides were synthesized according to the previously published hRPS3a genomic sequence (Fig 3.1A) [11]. The S3a+ATG-F primer sequence was 5'-GAGACTCGAGAGGTTTGGGGTTAGACGGTGAG-3'. This sequence includes a XhoI restriction site and an additional four nucleotides at the 5' end. The primer S3a+ATG-R sequence was 5'GAGAAAGCTTCTCCCTTTTTGCCGCCTTTCTTGTA-3' and this sequence includes a HindIII restriction site at the 5' end. The resulting 438 bp PCR product of S3a+ATG-F and S3a+ATG-R corresponds to the S3a nucleotides -344 through +73 (Fig 1B). A second downstream primer called S3a5'-R was synthesized corresponding to the sequence 5'-GAGAAAGCTTCAAAAGGGCGGAAGTGG-3'. The resulting 373 bp PCR product of S3a+ATG-F and S3a5'-R corresponds to -344 through +8 of the S3a promoter sequence (Fig 3.1B). The +8 nucleotide position is 17 bp 5' to the S3a ATG start codon. PCR was performed on 100 ng of LNCaP genomic DNA using the
following conditions: initial denaturing at 94°C for 3 min, followed by 30 cycles of denaturing at 94°C for 1 min, annealing at 58°C for 40 sec, and extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The resulting PCR products were cloned into the blunt end PCR-TRAP vector (Genehunter. Nashville, TN). The sequences of the LNCaP PCR products were confirmed by comparison to the published \textit{hRPS3a} sequence in Genbank (accession number: X87373).

3.4.4 Plasmid Reporter Constructs. All \textit{S3a} promoter segments were subcloned into the pGL3-Enhancer luciferase reporter plasmid (Promega. Madison, WI) after initial cloning of corresponding PCR products into the PCR-TRAP blunt end cloning vector. Cloned PCR products corresponding to the \textit{S3a} 5' flanking sequence were digested from the PCR-TRAP vector using \textit{XhoI} and \textit{HindIII} and subcloned into the \textit{XhoI} and \textit{HindIII} sites of pGL3-Enhancer to ensure directional cloning relative to the luciferase gene. The S3a+ATG PCR products were designed to be in frame with the pGL3 luciferase ATG start codon. Subcloned \textit{S3a} promoter sequences were confirmed by DNA sequencing using the pGL3-Enh vector sequencing primer RVprimer3 5'-CTAGCAAAATAGGCTGTCC-3'.

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3.4.5 **Point mutations in the S3a promoter elements.** Site-directed mutagenesis of the putative S3a promoter transcription factor binding sites was performed using the QuikChange site-directed mutagenesis kit (Stratagene. Cedar Creek, TX). Mutagenic primers were designed to be between 25-45 bp in size, have a T_m greater than 78°C, have at least 10-15 nucleotides flanking each side of the mutagenic nucleotides, and have a minimum 40% GC content as recommended by the manufacturer. All mutagenic oligonucleotides were purified by polyacrylamide gel electrophoresis. The mutagenesis reactions were performed in a Perkin Elmer (Wellesley, MA) 2400 thermocycler using 2.5 units of PfuTurbo DNA polymerase (Stratagene), 20 ng of plasmid template, and the following reaction conditions: denaturing at 95°C for 30 sec, followed by 16 cycles of denaturing at 95°C for 30 sec, annealing at 55°C for 1 min, and extension at 68°C for 10 min. A final hold at 37°C was used to prepare the site directed products for Dpn I digestion of parental plasmid using 10 U of enzyme as recommended. The mutagenic oligonucleotide sequences for the S3a transcription factor binding sites are listed in Table 3.1. Mutations in the S3a CRE were confirmed by DNA sequencing.
3.4.6 Nuclear Extracts and Gel Mobility Shift Assays. LNCaP nuclear protein extracts were prepared from combination of two 80% confluent monolayer cultures in 75 cm² culture flasks. Cells were either untreated (U) with differentiation agents or treated (D) with 1 mM db-cAMP/500 uM IBMX for 4 days prior to protein isolation. Cells were washed and collected using phosphate buffered saline pH 7.4. Pelleted cells were resuspended in 10 mM HEPES (pH 7.9) containing 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 1.0 µg/ml leupeptin. Resuspended cells were maintained on ice for 10 min prior to homogenization using a cold Dounce homogenizer. Nuclear pellets were obtained by centrifugation at 3300 x g for 15 min. The pellets were then suspended in 20 mM HEPES (pH 7.9) containing 25% glycerol, 1.5 mM MgCl₂, 1.2 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, and 1.0 µg/ml leupeptin. The nuclear extract was dialyzed for 30 min against 100 volumes of 20 mM HEPES (pH 7.9) containing 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, and 1.0 µg/ml leupeptin. The nuclear extract was then stored at -80°C.

The double stranded oligonucleotides corresponding to the S3a transcription factor binding sites and immediate 5' and 3' flanking sequences that were used as probes for EMSA are listed in Table 3.1. The nucleotide sequences of oligonucleotides that were used as nonspecific competitors (C, TAG, B1 and B2) are also listed in Table 1. Fifty picomoles of each forward oligonucleotide was 5' end
labeled using gamma $^{32}\text{P}$-ATP and T4 polynucleotide kinase. Following the 1 h labeling reaction incubation, T4 polynucleotide kinase activity was terminated by a 10 min incubation at 75°C. Two-fold molar excess of the appropriate reverse oligonucleotide was then added to the reaction and allowed to anneal for 10 min at room temperature. The resulting double stranded DNA probes were purified from the reaction mixture using Bio-Spin 6 columns (BioRad. Hercules, CA). The capacity for nuclear protein factors to specifically bind to the double stranded DNA probes was assessed using gel mobility shift analysis. The binding reaction buffer consisted of 10 mM HEPES (pH 7.9) containing 1 mM EDTA, 5 mM MgCl$_2$, 100 mM KCl, 6% glycerol, 0.05% Nonidet P-40, 2 µg BSA, and 0.04 µg/µl poly(dA)-poly(dT). Ten µg of LNCaP nuclear protein extract, from either undifferentiated or cAMP differentiated cells, was combined with 10,000 cpm of labeled oligonucleotide and allowed to incubate at room temperature for 30 min. Electrophoretic separation was performed using a 4% polyacrylamide gel at 185 V for 2 h 15 min in 0.5X TBE. The dried gels were exposed to X-ray film at -80°C for at least 16 h.
3.4.7 Northern Blot - Northern blot analysis was performed to confirm the expression levels of S3a as determined by differential display and to ensure that the putative S3a-luciferase fusion protein (Fig 3.1B) was not affecting observed luciferase activity levels. Electrophoresis of 15 µg total RNA per lane was performed using a 1.5% denaturing formaldehyde agarose gel. The RNA was blotted onto a Hybond (Amersham. Arlington Heights, IL) positively charged nylon membrane by capillary action and UV crosslinked. The probe was labeled with [α-32P]dATP using the random-priming method. Hybridization was performed overnight at 42°C. The blot was washed with 2 X SSC, 0.1 % SDS at room temperature for 15 min followed by two 15 min washes at 55°C with 0.2 X SSC, 0.1 X SDS. The blot was exposed to X-ray film at -80°C for at least 16 h.

3.4.8 Transfection and Luciferase Assays. Plasmid DNA for transient transfection was isolated using the plasmid maxi kit (Qiagen. Valencia, CA). LNCaP cells were plated at a density of 5 x 10^5 cells per well in 6 well plates and grown in RPMI-1640 supplemented with 10% FBS overnight prior to transfection. Transfections were performed using 8 µl Lipofectin reagent per well (Life Technologies. Carlsbad, CA) and serum free RPMI-1640 as per the manufacturer’s suggestions. A total of 10 µg of each pGL3-Enh vector and 1 µg of pRL-SV40
*Renilla* luciferase vector (Promega) were used for each transfection. The pRL-SV40 vector was used as a transfection efficiency control. The dominant negative KCREB expression vector was generously provided by the laboratory of Richard Goodman [29]. Ten µg of KCREB vector was used for each transfection. Each transfection was performed in triplicate and repeated at least twice. Firefly luciferase and *Renilla* luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega). Approximately 72 h after transfection, cells were washed with 1x PBS pH 7.4 then harvested with 400 µl passive lysis buffer (Promega). Following two rounds of freeze thawing, 10 µl of cell lysate were added to 100 µl firefly luciferase substrate and light units were measured in a Berthold luminometer. Renilla luciferase activities were measured in the same tube after the addition of 100 µl Stop and Glo reaction and 5 seconds of vigorous vortexing.

### 3.4.9 Western Blot Analysis

Twenty µg of LNCaP nuclear protein extract from undifferentiated and cAMP differentiated cells was separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes by electroblotting. The membranes were blocked with 5% nonfat milk and 1% casein enzymatic hydrolysate in 10 mM Tris-HCl, pH 7.5 containing 150 mM NaCl and 0.05% Tween-20 for 2 h at room temperature. The membranes were probed for 2 h
at room temperature with TBS containing 0.05% Tween 20 and 1:1000 dilution of anti-CREB-1 antibody (Cell Signaling Technology. Beverly, MA). The membranes were then washed twice for 10 min, then incubated with secondary antibody (goat anti-rabbit Ig(H+L)-AP human adsorbed (Southern Biotechnology, AL)) diluted 1:1000 in TBS containing 0.05 % Tween 20 for 2 h at room temperature. For detection, the membranes were first equilibrated for 10 min in alkaline phosphatase reaction buffer (100 mM Tris-HCl, pH 9.5 containing 100 mM NaCl and 5 mM MgCl₂). Alkaline phosphatase reaction buffer (15 ml) containing BCIP (110 µg/ml) and NBT (140 µg/ml) was added to each membrane then each membrane was incubated in the dark until a suitable band intensity was achieved.
3.5 RESULTS

3.5.1 The $S3a$ gene is down regulated following cAMP induced NE differentiation of LNCaP cells. To analyze the steady state levels of $S3a$ mRNA in undifferentiated and cAMP treated LNCaP cells, Northern blot analysis was performed (Fig 3.2). A significant decrease in $S3a$ mRNA levels was consistently observed when comparing undifferentiated and cAMP differentiated LNCaP cells (Fig 3.2; A,B). Considering that LNCaP NE differentiation is a result of increased intracellular levels of cAMP, we anticipated the presence of cAMP responsive elements in the promoter region of those genes that exhibit differential expression in response to cAMP induced NE differentiation. A potential CRE element had been previously identified in the $S3a$ 5' flanking sequence by computer analysis of published sequence, but the authenticity of the site had not been confirmed [11].

3.5.2 LNCaP nuclear extract and double stranded $S3a$ promoter CRE oligos form a specific DNA-protein complex. The potential CRE within the promoter region of a human lymphoblast $S3a$ identified by Nolte et al. [11] contained the sequence 5'-CGACGTCA-3'. This differs from the CRE consensus sequence by one nucleotide (5'C). We cloned the LNCaP $S3a$ promoter region and identified the
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Characterization of HRS3a Gene

S3a CRE as having the sequence 5’-AGACGTCA-3’. Thus, the sequence from LNCaP differed from the published human lymphoblast S3a sequence and the CRE consensus (5’-TGACGTCA-3’) sequence by one nucleotide at the 5' end of the element. Benbrook and Jones [18] demonstrated that base substitutions at the 5' end of the CRE consensus sequence is a common occurrence. In addition, the transactivational capacity of CRE sequence elements is strongly affected by: 1) the specific consensus sequence, 2) the 5' and 3' flanking DNA sequences, and 3) the strength of transcription factor binding. EMSA was performed in order to evaluate the capacity of the potential S3a CRE element to form a complex with LNCaP nuclear proteins (Fig. 3.3A).

A CRE element with a similar consensus sequence as the LNCaP S3a was identified in LNCaP β-microseminoprotein (MSP) [19]. To compare the electrophoretic mobility of the potential S3a CRE to the electrophoretic mobility of a known CRE from a LNCaP gene promoter, the MSP CRE element was evaluated along with the potential S3a CRE element (Fig. 3.3A). The S3a CRE oligonucleotide was designed to contain the same number of nucleotides as the MSP oligonucleotide in order to facilitate the comparison. EMSA using LNCaP nuclear protein extract and the MSP and S3a CRE probes resulted in the observation of two shifted bands. Ueyama et al., using the MSP CRE probe, showed that the band with lower mobility corresponded to the interaction between CREB and the CRE element [19]. The faster
mobility band was a non-specific DNA protein interaction that is typical of EMSA using LNCaP nuclear protein extracts. Fig 3.3A shows that the DNA/protein complex migration patterns were identical for both the MSP probe and the S3a probe, thus providing a preliminary indication that the S3a site is a true CRE.

The specificity of the S3a CRE DNA-protein interaction was determined by competition assays using an excess of non-radioactive oligonucleotides (Fig 3.3B). Again, both S3aCRE probe and MSP probe produced identically shifted bands (lanes 1 and 2). A 100-fold molar excess of unlabeled S3aCRE resulted in complete elimination of shifted bands (lane 3). A 100-fold molar excess of unlabeled MSP-CRE in combination with labeled S3aCRE also resulted in abolishment of shifted complexes (lane 4). In addition, a 100-fold molar excess of unlabeled S3aCRE in combination with labeled MSP resulted in loss of shifted bands (lane 5).

A variety of non-specific competitor probes were used to verify the specificity of the DNA-protein interaction. Nonspecific competitors C, TAG, B1, and B2 were unable to significantly compete with S3aCRE binding as indicated by the inability to remove the reduced mobility CRE/CREB bands (lanes 6-11). Neither 10-fold molar excess nor 100-fold molar excess of the non-specific competitors B1 or B2 significantly competed for binding with the labeled S3aCRE. Interestingly, a 100-fold molar excess of either B1 (lane 9) or B2 (lane 11) resulted in significant reduction of the lower band. This result supports the conclusion of Ueyama et al. that
the faster mobility band is a result of non-specific DNA protein interaction.

Figure 3.3C demonstrates that the S3aCRE/protein interaction can be abolished by mutations within the CRE consensus sequence (compare lanes 1 and 2) and that the S3aCRE/protein complex can be specifically supershifted by incubation with anti-CREB-1 antibody (lanes 4, 6, and 7). The combination of wild type and mutated S3aCRE oligonucleotides (lane 3) did not inhibit the capacity for wild type S3aCRE to produce a specifically shifted band. The degree of anti-CREB-1 supershift band intensity appears to increase in a concentration dependent manner as the quantity of anti-CREB-1 added to the DNA protein binding reactions increased (lanes 4, 6, and 7). Neither the specific CRE/CREB band nor the anti-CREB-1 supershifted band were present when mutated CRE oligonucleotide was used as a labeled probe (lane 5).

The presence of a nonspecific band at about the same migration distance as the anti-CREB-1 antibody shifted band was observed when rabbit preimmune serum was included in the DNA protein binding reactions to serve as a control (Fig 3.3C, lanes 9-12). The low mobility band seen in the presence of preimmune serum is due to binding of the antiserum itself to the labeled probe. In lane 12, rabbit preimmune serum was incubated with labeled S3aCRE oligonucleotide in the absence of LNCaP nuclear extract. Thus, the nonspecific rabbit preimmune band was present even in the absence of LNCaP nuclear proteins. Lane 8 shows that the combination of labeled
S3a CRE oligonucleotide and anti-CREB-1 antibody, unlike preimmune serum, does not result in a band shift. Lane 13 demonstrates that a combination of preimmune serum and anti-CREB-1 antibody, incubated with LNCaP nuclear extract and labeled S3a CRE oligonucleotide, does not inhibit the ability for anti-CREB-1 to produce a specifically supershifted band.

3.5.3 A second potential S3a CRE element forms specific DNA protein complexes with LNCaP nuclear proteins. Analysis of the S3a 5' flanking region using the TRANSFAC database [26] revealed the presence of a second potential CRE element at -228 to -221 bp. The second potential CRE element consensus sequence is 5' GCACGTCA 3'. This sequence differs from the consensus CRE recognition site by two bases at the 5' end of the consensus sequence. TRANSFAC analysis also identified the presence of a strong consensus sequence for the mouse MZF-1 transcription factor with the S3a gene promoter. In order to evaluate the capacity for the second potential S3a CRE element (2CRE) to bind with LNCaP nuclear proteins, EMSA was performed (Fig 3.4). Oligonucleotides corresponding to the potential MZF-1 transcription factor binding site were also synthesized and evaluated to serve as a negative control.

EMSA of the 2CRE element in the presence of LNCaP nuclear proteins resulted in a similar banding pattern to that observed for the upstream S3a CRE
element (Fig 3.4; lanes 1-2). One hundred-fold excess of unlabeled 2CRE oligonucleotide resulted in almost complete elimination of shifted bands (lane 3). One hundred fold molar excess of B1 nonspecific competitor was unable to compete with S3aCRE binding as indicated by the inability to remove the reduced mobility CRE/CREB band (lanes 4). A 100-fold molar excess of B2 nonspecific competitor did inhibit the reduced mobility CRE/CREB band (lane 5). A specifically supershifted S3aCRE/protein complex band was observed when EMSA was performed in the presence of anti-CREB-1 antibodies (lane 6). The specifically shifted S3aCRE band was not observed when EMSA was performed in the presence of rabbit pre-immune serum (lane 7). Lanes 8-12 of figure 4.4 contains EMSA reactions using the mouse MZF-1 transcription factor as a negative control. It is not expected that the mouse transcription factor should be present in human LNCaP nuclear proteins. As expected, only the single faster mobility non-specific DNA protein complex band was observed (lanes 8-9). Addition of 100-fold molar excess of unlabeled MZF-1 significantly reduced the non-specific band (lane 10). Neither the addition of anti-CREB-1 antibodies nor rabbit pre-immune serum, in the presence of labeled MZF-1 oligonucleotides, resulted in additional reduced mobility bands (lanes 11-12).

3.5.4 Potential Sp1 and GABP (E4TH) consensus sequences in the S3a
gene promoter form specific DNA protein complexes with LNCaP nuclear proteins. The GA-binding protein (GABP), also known as E4 transcription factor (E4TF1), is a heterodimeric DNA binding protein [28]. The α subunit of GABP binds weakly to DNA and the β subunit can only bind to DNA in the presence of the α subunit. Sp1 is a member of a family of transcription factors consisting of Sp1, Sp2, Sp3, and Sp4 [32]. These proteins bind to GC-rich sequences (e.g. GGGCGG) that are found in the promoters of many genes. Sp1 can activate transcription through a variety of mechanisms and can function as both a basal promoter element and as an upstream activator. In many genes lacking TATA boxes, such as S3a, a proximally positioned Sp1 site serves as a critical determinant of promoter activity. Sp1 can also enhance transcription from a distance through DNA looping mediated by protein-protein interactions [33]. In order to evaluate the capacity for potential Sp1 and GABP transcription factor consensus sequences to be recognized by LNCaP nuclear proteins, EMSA was performed (Fig 3.5).

EMSA using LNCaP nuclear protein extract and S3aE4TF1 probe resulted in the observation of multiple shifted bands (Fig 3.5A; lanes 1-2). The fastest mobility band corresponds to the typical LNCaP non-specific protein interaction. Reduced mobility bands corresponding to monomeric (α), dimeric (αβ), and tetrameric (αβ)_2 complexes appear to be present in both lanes. Disruption of the EMSA consensus sequence by site-directed mutagenesis resulted in removal of the dimeric and
tetrameric complex bands, but interestingly did not remove the monomeric band (lanes 3-4).

Reduced mobility bands were observed using labeled S3aSp1 oligonucleotides (Fig 3.5B). The S3aSp1 DNA/protein complex was specifically supershifted using anti-Sp1 antibodies (Fig 3.5B; lane 2). The supershifted band was not observed when S3aSp1 oligonucleotides were incubated with preimmune serum and LNCaP nuclear protein extract (Fig 3.5B; lane 3). Mutation of the S3aSp1 consensus sequence resulted in removal of the S3aSp1 DNA/protein complexes (Fig 3.5B; lanes 4-6).

Sawada et al. [27] demonstrated that the hGABP complex mediated efficient activation of transcription from E4 promoter synergistically with activating transcription factor (ATF) 1 or cAMP response element-binding protein (CREB). In addition, they demonstrated that physical interactions do occur between hGABP and CREB [27]. In order to test the possibility of physical interactions between CREB and GABP in LNCaP cells, gel supershift analysis was performed using anti-CREB-1 antibodies and labeled S3aE4TF1 probe (Fig 3.5C). Lanes 1-3 of Fig 3.5C demonstrate the reduced mobility bands that are typical of S3aCRE oligonucleotide incubation with LNCaP nuclear proteins (lane 2) and LNCaP nuclear proteins plus anti-CREB-1 antibody (lane 3). Lanes 4-6 include the E4TF1 consensus sequence without additional 5' and 3' S3a flanking DNA sequence. The lack of reduced mobility bands in lanes 4-6 demonstrates the importance of flanking sequences for
interactions between protein transcription factors and DNA. In the absence of LNCaP nuclear proteins, neither S3aCRE nor E4TF1 oligonucleotide probes produced a reduced mobility band (lanes 1, 4, and 7). In the presence of LNCaP nuclear proteins, the S3aE4TF1 probe exhibited multiple reduced mobility bands corresponding to the monomeric (α), dimeric (αβ), and tetrameric (αβ)_2 GABP:DNA complexes (lane 8). Interestingly, incubation of S3aE4TF1 probe with LNCaP nuclear proteins in the presence of anti-CREB-1 antibodies resulted in a dramatic decrease in the intensity of the reduced mobility bands (lane 9). These results support the potential for a physical interaction between E4TF1 and CREB that is described by Sawada et al. [27].

3.5.5 Multiple transcription factors facilitate regulation of the S3a gene promoter. In order to characterize the effects of the S3a promoter transcription factors on transcriptional activity in vivo, specific regions of the S3a gene 5' flanking sequence were subcloned into the pGL3-enhancer luciferase reporter vector. The “S3a+ATG” 5' flanking sequence contained the S3a ATG start codon and an additional 45 bp of coding sequence (Fig. 3.6A). The “S3a-ATG” sequence corresponds to -390 to -18 and did not include the S3a ATG start codon. In addition, the S3a-ATG construct lacks sequence that is necessary for binding of GABP [28]. Luciferase reporter vectors were transiently transfected into LNCaP cells and
luciferase activity was measured after 72 h. All luciferase activities were normalized using activity measurements from cotransfected pRL Renilla luciferase vector. The effect of cAMP induced NE differentiation on S3a gene expression was also evaluated.

The normalized luciferase activities for each promoter construct and CRE alteration are summarized in Figure 3.6A. The S3a-pGL3 promoter constructs produced a 130-2000-fold increase in luciferase activity over the pGL3 vector alone (pGL3-Enh) demonstrating that the S3a 5' flanking sequences act as functional promoter elements. The results indicate that the S3a-ATG promoter constructs exhibited a 6-fold increase in luciferase activity over the S3a+ATG promoter constructs. This increase occurs in both the presence and absence of 1 mM cAMP/500 µM IBMX.

Because the S3a+ATG promoter constructs include the S3a ATG and coding sequence for 15 additional amino acids from S3a (Fig 3.1B), we examined the levels of luciferase mRNA in LNCaP cells that were transfected with S3a+ATG and S3a-ATG pGL3 constructs (Fig 3.6B). Northern blot analysis showed no luciferase mRNA in non-transfected LNCaP cells (lane 1), low luciferase mRNA in S3a+ATG transfected LNCaP cells (lane 2) and high luciferase mRNA expression in LNCaP cells that had been transfected with S3a-ATG constructs (lane 3). The Northern analysis indicates that the differences in luciferase activity between the plus and
minus ATG constructs as shown in Fig 3.6A correlate well with the differences in actual luciferase mRNA present in transfected LNCaP cells (Fig 3.6B). These results also demonstrate that the differences observed in luciferase activity are due to transcriptional regulation, rather than an effect on luciferase enzyme activity due to the potential S3a-luciferase fusion protein from S3a+ATG.

In the S3a+ATG reporter gene analysis, an approximate 50% decrease was observed when comparing the luciferase activity between undifferentiated LNCaP cells and LNCaP cells that had been induced to NE differentiation by the addition of cAMP elevating agents (Fig 3.6A, filled and open bars respectively). This result agrees with the Northern analysis of S3a mRNA showing down-regulation of S3a expression in response to cAMP as shown earlier in Fig 3.2. In order to evaluate the efficiency of transfecting LNCaP cells that exhibit the epithelial versus the NE phenotype, transfection was performed on cells both 16 hrs prior to (open bars) and 3 days after (striped bars) cAMP induced NE differentiation (Fig 3.6A). Similar results were observed in the normalized levels of luciferase activity 72 hrs after transfection regardless of whether or not the LNCaP cells were NE differentiated during the transfection procedure.

In order to ascertain the putative functions of each of the S3a transcription factors, site-directed mutagenesis was performed for each of the transcription factor binding sites in the S3a+ATG and S3a-ATG luciferase plasmid constructs (Fig 3.7).
Following mutagenesis in S3a+ATG, the luciferase reporter gene was linked to S3a promoter constructs that contained individual mutations in CRE, 2CRE, Sp1 and GABP (E4TF1), double mutations of 2CRE, Sp1, and GABP in combination with CRE, mutations in CRE, 2CRE, and Sp1 together, and simultaneous mutations in each of the four identified S3a transcription factor binding sites (Fig 3.7A). All luciferase activities were normalized against the unmutated S3a+ATG pGL3-enhancer luciferase construct. Each mutant promoter construct was also evaluated in the presence and absence of 1 mM db-cAMP/500 µM IBMX.

Mutagenesis of the S3aCRE element resulted in a decrease in average luciferase activity, as compared to the non-mutated element, in the S3a+ATG construct. Interestingly, the levels of luciferase activity produced by the mutated CRE promoter were very similar to the luciferase activity of NE differentiated cells. Addition of cAMP/IBMX to the CRE mutated reporter did not result in a significant decrease in promoter activity. This suggests that an authentic CRE element is required for high level expression of S3a in undifferentiated cells. Mutation of the 2CRE element did not result in a significant decrease in luciferase activity compared to the non-mutated promoter. Mutation of the Sp1 element alone did not result in a significant decrease in average luciferase activity. Mutation of the GABP consensus sequence alone resulted in a significant increase in luciferase activity (50% higher than the non-mutated promoter). The increased expression levels observed in the
GABP mutant did not exhibit the 50% decrease in expression that was observed in the non-mutated promoter after treatment with cAMP.

Double mutations of the transcription factor binding sites for CRE in combination with either 2CRE or Sp1 abolished the high level of S3a expression observed in the non-cAMP treated LNCaP cells. However, the decreased luciferase activity for the double mutations was not significantly different than that for mutation of the CRE element alone. The mutation of CRE and GABP together resulted in high level expression of luciferase, regardless of whether or not the cells were stimulated to NE differentiation by increased intracellular cAMP, similar to the GABP mutation alone. Mutation of CRE, 2CRE, and Sp1 did not yield a significantly different level of luciferase activity from mutation of CRE alone while simultaneous mutation of CRE, 2CRE, Sp1, and GABP resulted in a 4-fold increase in luciferase expression relative to the non-mutated S3A+ATG promoter construct. Thus, analysis of the S3a+ATG promoter by site-directed mutagenesis suggests that GABP acts as a strong repressor of S3a gene expression and the CRE element is necessary for high level expression of the S3a gene in the presence of the GABP. In addition, our results suggest that the presence of the CRE element is necessary for cAMP induced down regulation of the S3a gene.

Previous EMSA analysis indicated that mutation of the GABP consensus sequence successfully inhibited the formation of dimeric and tetrameric GABP
complexes but did not remove the faster mobility monomeric GABPα band (Fig 3.5C). In order to ascertain the functional significance of GABP as a repressor of S3a gene expression, a 3' deletion product was produced that lacked necessary GABP binding sequences (Fig 3.7B). The 3' deletion product (S3a-ATG), was cloned into the pGL3-enhancer luciferase vector and site-directed mutagenesis was performed on each of the three remaining S3a transcription factors: CRE, 2CRE, and Sp1.

Average luciferase activity levels for LNCaP cells transfected with the S3a-ATG promoter construct were higher than the observed luciferase activity levels for LNCaP cells transfected with the S3a+ATG construct (Figs 3.6A and 7B). Cyclic AMP induced NE differentiation of cells transfected with the S3a-ATG promoter construct resulted in a reproducible but non-significant decrease in average luciferase activity. Mutation of the CRE element resulted in an apparent 50% decrease in luciferase activity and removal of the previously observed decrease in luciferase activity following treatment with cAMP elevating agents. Mutation of the 2CRE element did not result in a significant change in luciferase activity relative to the non-mutated S3a-ATG. Alteration of the CRE consensus sequence in combination with Sp1 or 2CRE in the S3a-ATG reporter did not result in a significant change in luciferase activity compared to mutation of the CRE element alone. Mutation of CRE, 2CRE, and Sp1 resulted in a return of luciferase activity levels to levels similar to that of non-mutated S3a-ATG. However, the reduced luciferase activity associated
with cAMP induced NE differentiation was abolished. These results further support the repressive capacity of GABP in $S3a$ gene regulation.

3.5.6 Cyclic AMP induced NE differentiation of LNCaP cells influences the binding affinity of CREB for the upstream $S3a$ CRE element. In order to assay for potential differences in transcription factor binding as a result of cAMP induced NE differentiation in LNCaP cells, EMSA was performed using nuclear protein extracts from undifferentiated and differentiated (cAMP induced) LNCaP cells (Fig 3.8). EMSA for the $S3a$ CRE element in the presence of differentiated LNCaP nuclear proteins resulted in an increased band intensity of CREB for the CRE probe as compared to EMSA using undifferentiated nuclear proteins (Fig 3.8A). An increased intensity of CREB for 2CRE was not observed in the presence of differentiated nuclear proteins. Western blot analysis using anti-CREB-1 antibodies did not indicate a difference in the relative protein level of CREB between undifferentiated and differentiated LNCaP cells (Fig 3.8D). The binding affinities for Sp1 (Fig 3.8B) and GABP (Fig 3.8C) did not appear to be affected by cAMP induced NE differentiation of LNCaP cells.

3.5.7 Quenching of CREB protein by dominant negative CREB (KCREB) results in decreased expression by the $S3a$ promoter. To better establish the contribution of the CREB transcription factor to regulation of the $S3a$
gene promoter, we used a dominant-negative cDNA construct of CREB, KCREB, that contains a mutation in the DNA binding domain [29]. Expression of the KCREB vector is expected to form heterodimers with CRE-binding proteins; however, the mutant’s poor affinity for DNA does not allow for the same degree of transcriptional activity as associated with the wild-type CREB. The addition of KCREB vector to cells that were transfected with S3a+ATG resulted in a dramatic decrease in observed luciferase activity levels (Fig 3.9). The KCREB associated decrease in luciferase activity was greater than the decrease in luciferase activity associated with mutation of both S3a promoter CRE elements. Furthermore, I suggest that the interaction between CREB and GABP is significant for repression of S3a gene expression.
3.6 DISCUSSION

The transcription factor CREB is known to regulate cyclic AMP-dependent gene expression by binding to and activating transcription from cAMP response elements (CREs) in the promoters of target genes. The transcriptional activation functions of CREB are stimulated by its phosphorylation by cAMP-dependent protein kinase A (PKA). Cyclic AMP is a quintessential second messenger produced in cells in response to a variety of hormonal and nutrient signals [20]. A consequential function of cAMP is activation of the phosphorylating enzyme, PKA. Paradoxically, we report that the S3a gene promoter is repressed by the addition of cAMP and the presence of an intact CRE is necessary for decreased S3a expression during NE differentiation in LNCaP cells.

Evidence exists to suggest potential repressive roles for CRE/CREB [21-24]. It is possible that additional transcription factors with the capacity to modulate the S3aCRE/CREB interaction are involved in this process. Nevertheless, the presence of the CRE element is necessary for attenuation of S3a gene expression during cAMP induced NE differentiation as indicated by the results shown in Fig 3.7 using the luciferase reporter gene.

The observation of decreased gene expression in response to increased...
intracellular cAMP levels for genes with known CRE elements has been observed previously. For example, the Synapsin I gene promoter is known to contain a CRE in the promoter region. Treatment of neuroblastoma cell lines with cAMP elevating agents such as IBMX and Forskolin resulted in a 50% decrease in Synapsin mRNA levels [25]. In addition, mutagenesis of the CRE consensus sequence resulted in a 50% decrease in constitutive expression of Synapsin I. These observations are virtually identical to our findings for the S3a gene promoter in LNCaP cells suggesting the potential for a similar regulatory mechanism.

The dramatic increase in luciferase activity observed for the S3a-ATG reporter construct as compared to the lower luciferase activity associated with the S3a+ATG construct suggests the presence of a negatively acting element contained within the region from -18 to +48 bp. Computer analysis of this region using the TRANSFAC database [26] indicates the presence of potential GABP (E4TF1) binding loci immediately flanking both sides of the S3a transcriptional start point. GABP has been shown to interact with members of the activation transcription factor/CREB protein family [27]. Interestingly, GABP-proteins serve as both activators and repressors of ribosomal protein gene transcription depending on the location of the GABP-binding sites within the gene promoter. GABP-binding sites 40-80 bp upstream of the transcriptional start point seem to act as activators of gene transcription while GABP-binding sites in the immediate vicinity of the
transcriptional start point have been demonstrated to facilitate down-regulation of gene expression as was demonstrated for rps16 [28]. Our results strongly suggest that the GABP binding site serves as a negative regulator of S3a gene expression.

The combination of EMSA, site-directed mutagenesis, and luciferase activity data suggest a possible mechanism by which the addition of cAMP may function to decrease S3a gene expression. Luciferase activity assays in the presence and absence of cAMP demonstrate that the distal S3a CRE element (-268 to -261) is more important than the proximal CRE element (-228 to -221) relative to cAMP induced down regulation of S3a gene expression (Fig 7). Furthermore, the results of EMSA showed that the addition of cAMP enhanced the binding of CREB to the distal CRE (Fig 8). Elevation of intracellular cAMP ultimately results in phosphorylation of CREB by the catalytic subunit of cAMP-activated protein kinase A [30]. It is thought that phosphorylation events alter the conformation of the transactivation domain of CREB, enhancing its interaction with transcriptional machinery [31]. We propose that phosphorylation of CREB enhances the ability of the transcription factor to interact with the GABP protein complexes. Occupation of the GABP binding site by dimeric or tetrameric GABP alone is sufficient to cause a significant reduction in promoter activity by interfering with the formation of the transcriptional initiation complex. Interactions between phosphorylated CREB and GABP further inhibit the formation of a stable and active initiation complex and the efficient recruitment of
RNA polymerase. Depending on the location of GABP within the promoter regions for different genes, interactions between CREB and GABP could potentially enhance or impede the assembly of the transcriptional initiation complex [28].

In summary, we demonstrate in this paper that the DNA sequence element 5'-AGACGTCA-3' acts as a functional CRE element for the $S3a$ gene promoter and this CRE sequence can be recognized by CREB as indicated by gel supershift analysis. The CRE element varies slightly from the consensus CRE, but the variation does not appear to inhibit the affinity of CREB binding within the local DNA sequence environment. We also demonstrate that the $S3a$ 2CRE DNA sequence element 5'-GCACGTCA-3' can also be recognized by CREB. Sp1 and GABP consensus sequences, that are found in the $S3a$ gene promoter, can be recognized by specific DNA binding proteins that are present in LNCaP nuclear protein extracts. The intact $S3a$ distal CRE element is required for an increased expression level of $S3a$ as indicated by site-directed mutagenesis of the CRE element, transfection of LNCaP cells with a dominant-negative CREB (KCREB) expression vector, and the observation of a subsequent decrease in luciferase activity. In contrast to typical observations, the addition of cAMP elevating agents appears to decrease $S3a$ expression levels using a mechanism that is intimately associated with the presence of the functional $S3a$ CRE. Binding of GABP complexes to the GABP consensus sequence, that is proximal to the transcriptional initiation site for $S3a$, results in
significant repression of gene expression. The GABP transcription factor is specifically important for down regulation of the $S3a$ gene and removal of DNA sequence that is necessary for GABP complex formation results in a dramatic increase in promoter activity regardless of whether or not the accessory $S3a$ transcription factors are capable of binding to the promoter.

3.7 ACKNOWLEDGMENTS

This work was supported by grants and awards from the Pfeiffer Research Foundation, the Horsley Cancer Research Fund, the Virginia Academy of Science, and the Virginia Tech Graduate Research and Development Program (GRDP).
3.8 REFERENCES


18. **Benbrook DM and Jones NC.** (1994) Different binding specificities and


and type IV involves phosphorylation of a site that negatively regulates activity. Genes Dev. 8: 2527-39.


<table>
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<tr>
<td>53amCRE-F</td>
<td>ACTTACCTCCATGTATCACAGCC</td>
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<tr>
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<tr>
<td>E4TF1-F</td>
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<td>GATCTAAATAATAATATAATATA</td>
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All sequences are written in the 5' to 3' direction. Mutated sequences are indicated by underlining and boldface type.
**Figure 3.1. S3a genomic organization and 5' flanking region.**

(A). The S3a gene spans 5013 bp and contains 6 exons. The upstream regulatory region for the gene contains several potential recognition sites for known transcription factors including cAMP response elements, Sp1, and E4TF1 (GABP) consensus sequences. (B). PCR primers were synthesized to facilitate the amplification of the S3a 5' flanking region. S3a+ATG primers were designed to amplify the upstream regulatory region and a small portion of the S3a exon 1. S3a-ATG primers were designed for production of a 3' deletion of the S3a in which necessary sequences for GABP binding were excluded.
Chapter 3

Characterization of HRS3α Gene

A

U D

B

U D

C

U D
Figure 3.2. *S3a* is down regulated during cAMP induced NE differentiation.

(A). Fifteen ug of total RNA from uninduced (undifferentiated “U”) and cAMP induced (differentiated “D”) LNCaP cells was subjected to Northern blot analysis as detailed in “Materials and Methods.” A differential display PCR product corresponding to the LNCaP *S3a* gene was $^{32}$P labeled by random priming and used as a probe for Northern hybridization. (B). The differential display PCR product was cloned into the PCR-TRAP vector and a PCR product corresponding to the cloned sequence was labeled and used as a probe for a second Northern blot (C). Ethidium bromide stained gel. Ribosomal RNA bands from uninduced (U) and cAMP induced (D) LNCaP cells demonstrate equal total RNA loading in the two lanes.
Figure 3.3. Electrophoretic mobility shift assay of the S3a gene promoter -268 to -261 CRE element.

(A). The potential S3a CRE elements were evaluated along with a known LNCaP gene CRE element (MSP CRE) to allow for side-by-side comparison of the potential and confirmed CRE elements. Each CRE probe was end-labeled with $^{32}$P and incubated with 10 µg of LNCaP nuclear protein extract. Lane 1, 10,000 cpm MSP; lane 2, 20,000 cpm MSP, lane 3, 10,000 cpm S3aCRE; lane 4, 20,000 cpm S3aCRE; lane 5, MSP probe without protein, lane 6, S3aCRE probe without protein. The arrow alone indicates the nonspecific band and the “arrow-*” denotes the specifically shifted DNA/protein band. (B) S3aCRE element EMSA competition assays. The specificity of the S3aCRE/protein interaction was evaluated using both specific and non-specific competitor sequences. Each DNA protein binding reaction contained 10 µg of LNCaP nuclear protein extract and 10,000 cpm of labeled probe. Lane 1, S3aCRE probe; lane 2, MSPCRE probe; lanes 3-5, specific competitors, lanes 6-11, nonspecific competitors. Lane 3 contains labeled S3aCRE oligo with 100 fold excess unlabeled S3aCRE oligo, lane 4 contains labeled S3aCRE oligo with 100 fold excess unlabeled MSP oligo, and lane 4 contains labeled MSP oligo with 100 fold excess unlabeled S3aCRE oligo. Lanes 6-11 contain labeled S3aCRE oligo with the
following nonspecific competitors: 6, 10 fold excess C; 7, 10 fold excess TAG; 8, 10 fold excess B1; 9, 100 fold excess B1; 10, 10 fold excess B2; 11, 100 fold excess B2. The arrow alone indicates the nonspecific band and the “arrow-#” denotes the specifically shifted DNA/protein band. (C). The potential S3CRE/nuclear protein complex is supershifted by anti-CREB-1 antibody and site directed mutagenesis of the CRE element abolishes CREB protein binding. Each CRE probe was end labeled with $^{32}$P and incubated with 10 µg of LNCaP nuclear protein extract. The conditions for each lane are as follows: lane 1: S3CRE, lane 2: mutated S3CRE (mS3CRE), lane 3: S3CRE + mS3CRE, lanes 4, 6, and 7: S3CRE + antiCREB1 antibody, lane 5: mS3CRE + antiCREB1 antibody, lane 8: S3CRE + antiCREB1 antibody without nuclear protein extract, lanes 9-11: S3CRE + preimmune serum, lane 12: S3CRE + preimmune serum without nuclear proteins, lane 13: S3CRE + antiCREB1 antibody + preimmune serum. The arrow alone indicates the nonspecific band. The “arrow-#” denotes the specifically shifted DNA/protein band and the “arrow-S” indicates the supershifted band.
<table>
<thead>
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* S, * denote specific bands.
Figure 3.4. Electrophoretic mobility of S3a gene promoter 2CRE element -228 to -221 and a potential mouse MZF1 consensus sequence following incubation with LNCaP nuclear proteins.

A second potential CRE element (2CRE) at -228 through -221, 40 bp downstream from the upstream S3a CRE element, was analyzed by EMSA. The consensus sequence for mouse MZF1 transcription factor is located within the S3a gene promoter, starting at -155 bp, and this consensus sequence was also evaluated. Each probe was end labeled with $^{32}$P and 10,000 cpm of probe was incubated with 10 ug LNCaP nuclear protein extract with the following additions: lanes 1-2: no competition, lane 3: 100 fold excess unlabeled 2CRE; lanes 4-5: 100 fold excess nonspecific competitors B1 and B2; lane 6: antiCREB1 antibody, lane 7: preimmune serum; lanes 8-9: no competition, lane 10, 100 fold excess unlabeled MZF1, lanes 11-12: nonspecific competitors. The “arrow-*$^*$” denotes the specifically shifted S3a2CRE DNA/protein band and the “arrow-S” indicates the antiCREB1 supershifted band.
Chapter 3  Characterization of *HRS*3a* Gene

A

B

Supershift:

8p1

C

Oligos:

S3a CRE  
E4TF1  
S3aE4TF1

Nuc ext:

-  +  +  
-  +  +  
-  -  +  

Anti-CREB:

1  2  3  4  5  6  7  8  9

S

R

*
Figure 3.5. EMSA of \textit{S3a} gene promoter E4TF1 (GABP) and Sp1 transcription factor binding sites.

(A). LNCaP nuclear proteins were incubated with $^{32}\text{P}$-labeled oligonucleotides corresponding to the $S3a$-E4TF1 (GABP) consensus sequence or a mutated E4TF1 consensus sequence (mutE4TF1). The positions of monomeric ($\alpha$), dimeric ($\alpha \beta$), and tetrameric ($\alpha \beta_2$) complexes are indicated. (B). EMSA for wild type $S3a$Sp1 consensus sequence and mutated $S3a$Sp1 (mut$S3a$Sp1) consensus sequence in the presence or absence of Sp1 antibodies or preimmune serum. The locations of specifically shifted Sp1 bands and supershifted bands are indicated. (C). The $S3a$CRE, E4TF1 consensus sequence without 5' and 3' flanking DNA, and the $S3a$E4TF1 were evaluated by EMSA with or without LNCaP nuclear proteins or with LNCaP nuclear proteins in the presence of antiCREB1 antibody. The “arrow-*” denotes the specifically shifted $S3a$CRE DNA/protein band and the “arrow-S” indicates the antiCREB1 supershifted band. The “arrow-R” indicates a reduction in E4TF1 bands when E4TF1 probe was incubated with anti-CREB1 antibody.
Chapter 3

Characterization of \textit{HRS3a} Gene

A

B

1 2 3
Figure 3.6. *S3a* promoter constructs and luciferase activity.

(A). The 5’ flanking region of the *S3a* gene was amplified by PCR and cloned into the pGL3-Enhancer luciferase plasmid. The S3a+ATG PCR product was designed to include the *S3a* ATG start codon. The S3a-ATG product corresponds to -390 to -18 5’ flanking sequence of *S3a* and does not contain the *S3a* start codon. pGL3-Enhancer represents the promoterless vector. Firefly luciferase activities were normalized to pRL-SV40 *Renilla* luciferase activity. Filled bars represent luciferase activity from transfected LNCaP cells without cAMP treatment (undifferentiated cells). Open bars represent activity from cells that were induced to NE differentiation by the addition of 1 mM db-cAMP/500 µM IBMX 16 hrs after transfection. Striped bars represent luciferase activity from LNCaP cells that were treated with 1 mM db-cAMP/500 µM IBMX 3 days prior to transfection with luciferase plasmids. (B). Northern blot analysis of steady state luciferase mRNA levels. Fifteen µg of total RNA from non-pGL3 transfected LNCaP cells (lane 1), LNCaP cells transfected with pGL3+ATG (lane 2), and cells transfected with pGL3-ATG (lane 3) was subjected to Northern blot analysis as detailed in “Materials and Methods.” A cDNA corresponding to the pGL3 luciferase gene was generated by PCR and labeled for use as a probe in Northern hybridization.
Chapter 3

Characterization of HRS3a Gene

A

B
Figure 3.7. Luciferase activity and site directed mutagenesis of S3a transcription factor binding sites in S3a+ATG and S3a-ATG promoter sequences.

Site-directed mutagenesis was performed on the S3a+ATG and S3a-ATG pGL3-Enhancer plasmids as described in “Materials and Methods.” LNCaP cells were transfected with 10 μg of each of the indicated site-directed plasmids. The relative positions of the transcription factor binding sites are indicated. Plus (+) signs indicate intact transcription factor binding sites and minus (-) signs indicate site-directed mutagenesis of the response element consensus sequence. Filled bars indicate luciferase activity for LNCaP cells that were not treated with cAMP. Open bars indicate luciferase activity for LNCaP cells that were treated with 1 mM-db-AMP/500 μM IBMX for three days prior to luciferase activity assays. Firefly luciferase activities were normalized by pRL-SV40 Renilla luciferase activity. (A). Luciferase activity for various S3a+ATG pGL3-Enhancer site-directed mutagenesis plasmids. Luciferase activity is normalized against the relative luciferase activity for non-mutated S3a+ATG (100%). (B). Luciferase activity for the 3’ deletion product S3a-ATG pGL3-Enhancer site-directed mutagenesis plasmids. Activity is normalized against the relative luciferase activity of non-mutated S3a-ATG (100%).
Chapter 3

Characterization of *HRS3a* Gene

![Image of gel electrophoresis and autoradiogram](image)

A. Gel electrophoresis showing bands under different conditions:

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B. Autoradiogram showing band migration under different conditions:

- U: Undifferentiated
- D: Differentiated

C. Close-up of a specific band in autoradiogram B.

D. A densitometry scan of autoradiogram B, showing intensity variations under U and D conditions.
**Figure 3.8.** Comparison of electrophoretic mobility for S3a transcription factor oligonucleotide probes when incubated with LNCaP nuclear proteins isolated from undifferentiated (Und) or cAMP differentiated (Dif) LNCaP cells.

(A). EMSA for S3a CRE and 2CRE oligonucleotide probes incubated with untreated (Und) LNCaP nuclear protein extracts or LNCaP nuclear protein extracts that were treated for 3 days with 1 mM db-cAMP/500 µM IBMX to induce NE differentiation (Dif). EMSA was also performed for the S3a Sp1 (B) and GABP (C) consensus sequences using undifferentiated (U) and differentiated (D) LNCaP nuclear protein extracts. (D). Western blot analysis of CREB protein levels in undifferentiated (U) and differentiated (D) LNCaP cells using antiCREB1 antibody.
Figure 3.9. Expression of a dominant negative CREB decreases S3a promoter activity.

Luciferase activity assays were performed for LNCaP cells that were transfected with 10 μg S3a+ATG pGL3-Enhancer and various amounts of KCREB expression vector. A construct containing site-directed mutations for both of the S3a CRE elements (ΔCRE) was also analyzed.
CHAPTER 4

Suppression of \textit{HRS3a} gene translation by antisense phosphorothioate oligonucleotides results in growth inhibition in human prostate cancer cells.
4.1 ABSTRACT

An increasing body of evidence seems to suggest that individual constituents of the translational mechanism may play an important role in cancer progression by acting as regulatory mediators of growth, proliferation, and oncogenesis. I previously identified the HRS3a gene by differential display as being down-regulated during cAMP-induced NE differentiation in LNCaP prostate cancer cells. In order to evaluate the effect of HRS3a down regulation in LNCaP cells, antisense phosphorothioate oligonucleotides were designed to inhibit HRS3a gene expression. Treatment of LNCaP cells with antisense HRS3a nucleotides did not influence cAMP induced neuroendocrine differentiation but antisense treatment did result in a significant decrease in LNCaP cell growth. The results of this investigation suggest that HRS3a plays an important role in prostate cancer cell proliferation.
4.2 ABBREVIATIONS

bp : base pair(s)
cAMP : adenosine 3':5'-cyclic monophosphate
cDNA : DNA complementary to mRNA
db-cAMP : dibutyryl-cAMP
dd-PCR : mRNA differential display PCR
HRS3a or S3a: human ribosomal S3a
IBMX : isobutylmethylxanthine
NE : neuroendocrine
ODN : oligodeoxynucleotide
4.3 INTRODUCTION

An increased understanding of prostate cancer biology and progression are essential for improving the ability to successfully prevent, diagnose, and treat human prostate cancer. The LNCaP human prostate cancer cell line exhibits an epithelial morphology in the non-cyclic AMP induced state. Bang et al. reported that elevation of intracellular cAMP through the addition of cell permeable cAMP analogues or phosphodiesterase inhibitors induces a permanent conversion from an epithelial to a neuronal morphology and that the induced cells express markers of the neuroendocrine phenotype [1]. Subsequent analysis demonstrated that the cAMP induced neuroendocrine differentiation of LNCaP cells is a reversible phenomenon [2-3].

The ability of cAMP elevating agents to induce NE differentiation in LNCaP cells suggests that an increased accumulation of intracellular cAMP may underlie the conversion of prostatic tumor cells to NE cells. It has been speculated that the ability of prostatic NE cells to produce possible mitogenic hormones may influence the growth of local carcinoma cells that lie in proximity to the NE cells [4]. If NE cells are able to contribute to the durability and growth of the surrounding tumor cell population and are themselves able to reenter the cell cycle under designated
physiological conditions, then tumors possessing NE cells may gain a strong selective advantage [2].

A cDNA was identified using differential display technology as being down-regulated during cAMP-induced NE differentiation. This cDNA was subsequently cloned, sequenced, and identified as a human gene known collectively as \textit{fte-1}, \textit{v/fos} transformation effector gene, and \textit{HRS3a} [3]. Enhanced expression of translation factor mRNAs such as \textit{S3a} has been found in tumors and cancer cell lines [5-6]. These genes have been suggested as possible facilitators of colorectal cancer and liver carcinogenesis [7-8]. \textit{S3a} has also been demonstrated to be down regulated following neuronal differentiation of NTERA cells [9]. In addition, \textit{S3a} has also been demonstrated to favor apoptosis and enhance malignant phenotypes [5].

Protein synthesis by mRNA translation is an essential process in living cells. Thus, every eukaryotic cell type can be expected to exhibit a certain degree of ribosomal protein expression. Upon superficial inspection, it is easy to dismiss the increased expression levels of ribosomal proteins in carcinomas as simply a by-product of the augmented metabolic activity that accompanies tumor physiology. However, an increasing body of evidence suggests that individual constituents of the translational mechanism may play an important role in cancer progression by acting as regulatory mediators of growth, proliferation, and oncogenesis.

It is unlikely that increased levels of proteins such as \textit{S3a} are due to increased
rates of cell division because the expression of individual translational proteins are coordinately regulated by various means to produce equimolar amounts of protein that are roughly stoichiometric with rRNA concentrations. In the course of evolution, it is thought that some of the constituents of multi-protein complexes, such as the ribosome, may have been recruited from prior cellular activities to enhance alternative cellular functions. S3a in particular provides support for these hypotheses in that S3a has already been identified as a bifunctional ribosomal protein that interacts with the transcription factor CHOP, acting as a negative modulator of erythroid differentiation during erythropoiesis [10]. Additional functional roles for S3a may exist and these roles could prove to be very significant for the progression of prostate cancer disease.

In order to better ascertain the role of S3a down regulation in LNCaP cells, antisense phosphorothioate oligonucleotides were designed to allow for simulated down regulation of S3a gene expression through antisense inhibition (Fig 4.1). While treatment of LNCaP cells with antisense S3a oligonucleotides (ODNs) does not result in NE differentiation, a decrease in LNCaP cell division was observed compared to cells that were treated with either lipofectin alone or S3a-sense ODNs, indicating that the S3a gene is significant for prostate cancer proliferation.
4.4 MATERIALS AND METHODS

4.4.1 Cell culture. The human prostatic carcinoma cell line LNCaP was obtained from the American Type Culture Collection (Manassas, VA). LNCaP cells were maintained at 37°C in a humidified incubator containing 5% CO₂ / 95% air in RPMI 1640 medium supplemented with 50 µg/ml gentamycin, and 10% heat inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA). Cells were grown to 80% confluency in 75 cm² culture flasks and passaged into 6 well plates the day before transfection experiments. Fluid renewal was performed twice weekly and cultures were passaged upon confluency (6-8 x 10⁵ cells/cm²) by trypsinization.

4.4.2 HRS3a Antisense ODN Treatments. Phosphorothioate ODNs used in this study were obtained from Integrated DNA Technologies (Coralville, Iowa). The sequences of the ODNs are listed in Fig 4.1. LNCaP cells were plated at a density of 1-2 x 10⁴ cells per well in 6 well plates the day before transfection. Before transfection, cells were washed once with 1x PBS, pH 7.4. Varying concentrations of antisense ODNs were combined with 4 µl lipofectin in a final volume of 1.2 ml serum free RPMI-1640 and added to cells. ODN uptake was allowed to proceed overnight before transfection medium was replaced with complete medium. Transfection was
repeated after 48 hours. Forty-eight hours after the second exposure to ODNs, cells were visually inspected using an inverted microscope and counted using a hemacytometer.

4.4.3 Northern hybridizations. Northern blot analysis was performed to evaluate steady state S3α mRNA levels in the presence of phosphorothioate ODNs. Electrophoresis of 15 µg total RNA per lane was performed using 1.5% denaturing formaldehyde agarose gels. RNA was blotted onto Hybond (Amersham, Arlington Heights, IL) positively charged nylon membranes by capillary action and UV crosslinked. The probes were labeled with $[\alpha^{32}\text{P}]{\text{dATP}}$ by random-priming [11]. Hybridization was performed overnight at 42°C. The blots were washed with 2 X SSC, containing 0.1 % SDS at room temperature for 15 min. This was followed by two 15 min washes at 55°C with 0.2 X SSC, containing 0.1 X SDS. The blots were exposed to X-ray film at -80°C for at least 16 h.
4.5 RESULTS AND DISCUSSION

4.5.1 Effect of Antisense S3a ODNs on LNCaP cells. Antisense ODNs were designed to be complementary to the S3a mRNA start codon and immediate flanking sequence. In order to evaluate the capacity of S3a-antisense ODNs to generate specific effects on LNCaP cell growth, different concentrations of both S3a-antisense and S3a-sense ODNs were used in LNCaP transfection experiments (Fig 4.2). Treatment of LNCaP cells with lipofectin alone (Fig 4.2, 0 nM) was sufficient to produce a decrease in viable cell number after four days of exposure compared to cells that were not treated with transfection reagent (Fig 4.2, no addition). Transfection of LNCaP cells with 100 nM and 250 nM concentrations of antisense or sense ODNs did not produce a difference in viable cell number compared to transfection with lipofectin alone. However, transfection of LNCaP cells with 500 nM and greater concentrations of S3a-antisense ODNs produced a decrease in cell number when compared to equal concentrations of S3a-sense ODNs after four days of exposure (Fig 4.2).

In order to evaluate the effect of higher concentrations of antisense and sense ODNs, 1500 nM concentrations of S3a-antisense and S3a-sense ODNs were transfected with LNCaP cells and cell numbers were determined after four days (Fig
4.3). As observed previously, treatment of LNCaP cells with lipofectin alone was sufficient to produce a significant decrease in viable cell number when compared to non-treated cells after four days (compare Fig 4.2 and Fig 4.3). Treatment of LNCaP cells with 1500 nM S3a-sense ODNs did not produce a significant difference in cell number when compared to lipofectin treated cells alone. Treatment of LNCaP cells with 1500 nM S3a-antisense ODN produced a significant decrease in cell number when compared to treatment of cells with S3a-sense ODNs or treatment with lipofectin alone. Interestingly, the cell density for cells treated with antisense ODNs was $2 \times 10^4$ after four days and this is the initial concentration of cells in the experiment. The observation of $2 \times 10^4$ cells after four days of exposure to S3a-antisense ODNs suggests that the LNCaP cell population that was exposed to S3a-antisense ODNs did not increase over the four day observation period.

**4.5.2 Treatment of LNCaP cells with S3a antisense ODNs does not result in a decrease in steady state S3a mRNA levels.** Chimeric phosphorothioate antisense ODNs have been used as specific inhibitors of target gene expression in a variety of cell types, including prostate cancer cells [12-15]. Antisense inhibition of protein expression is thought to occur by either degradation of RNA by the action of RNase H, which is known to cleave RNA from DNA-RNA hybrids [12], or by the inhibition of mRNA translation by blocking of the S3a translation initiation site in the
Northern blot analysis of S3a mRNA levels in the presence of different concentrations of S3a-antisense ODNs and S3a-sense ODNs did not indicate a decrease in the steady state levels of S3a mRNA transcript for either treatment (Fig 4.4, A and B). Similar observations in which relative mRNA levels did not decrease in the presence of antisense ODNs have been made using TGFβ1 antisense ODNs [13]. This suggests that the phenotypic effects associated with antisense mediated inhibition of protein expression may not correlate with decreases in respective mRNA levels for some genes.

The results of these experiments suggest that down regulation of HRS3a expression by antisense ODN technology may result in attenuation of cell division in LNCaP prostate cancer cells. The growth attenuation of LNCaP cells that are transfected with S3a-antisense ODNs does not correlate with a decrease in steady state S3a mRNA levels suggesting that down regulation of HRS3a may be occurring at the level of protein synthesis. HRS3a represents a novel candidate for gene therapy approaches in prostate cancer treatment and cancer treatment in general.
4.6 REFERENCES


S3a-antisense
5’C*T*TGTTCCTTGGCAACCAGCC*A*T-3’

S3a-sense
5’A*T*GGCGGTGGGCAAGAACA*A*G-3’
Figure 4.1. Phosphorothioate nucleotides and chimeric phosphorothioate oligonucleotides specific for S3a.

Specialized nuclease-resistant modifications are necessary for oligonucleotides that are used in antisense studies. Phosphorothioate nucleotides are nuclease resistant but have a lower $T_m$ than their phosphodiester counterparts. The phosphorothioate modifications were used to “cap” the 5’ and 3’ ends of the S3a-antisense and S3a-sense oligonucleotides resulting in chimeric oligonucleotides. The asterisks “*” represent phosphorothioate modifications.
Figure 4.2. Antisense inhibition of S3a gene expression: cell growth effects.

Different concentrations of S3a-sense phosphorothioate ODNs and S3a-antisense phosphorothioate ODNs were combined with $1 \times 10^4$ LNCaP cells per well in six well plates as described in the “Materials and Methods” section. Bars are a single cell count. The vertical axis scale is $N \times 10^2$ cells per well.
Chapter 4

HRS3a Antisense

![Graph showing initial cell density x10^4 for different treatments: No Addition, Lipofectin, Sense, Antisense. The graph indicates a significant decrease in cell density for the Antisense treatment compared to the other conditions.](image)
Figure 4.3. Antisense inhibition of S3a gene expression: cell growth effects.

Fifteen hundred nM concentrations of S3a-sense phosphorothioate ODNs and S3a-antisense phosphorothioate ODNs were combined with an initial concentration of 2 x 10⁴ LNCaP cells per well in six well plates as described in the “Materials and Methods” section. “Lipofectin” represents a mock transfection without oligonucleotides. Bars represent the average of cell counts from three separate wells. The vertical axis scale is N x 10⁴ cells per well.
Figure 4.4. Evaluation of steady state S3a mRNA levels in the presence of S3a-antisense and S3a-sense phosphorothioate ODNs.

Fifteen µg of total RNA from LNCaP cells that were transfected with different amount of antisense and sense phosphorothioate ODNs was subjected to northern blot analysis as detailed in “Materials and Methods.”  (A). S3a-antisense ribosomal bands from ethidium bromide stained agarose formaldehyde RNA gel and northern blot using HRS3a probe. Total RNA for northern analysis was extracted from LNCaP cells that had been transfected with the following concentrations of S3a-antisense ODNs for 4 days: Lane 1, lipofectin alone; lane 2, 100 nM; lane 3, 250 nM; lane 4, 1000 nM; lane 5, no addition.  (B). S3a-sense ribosomal bands from ethidium bromide stained agarose formaldehyde RNA gel and northern blot using HRS3a probe. Total RNA for northern analysis was extracted from LNCaP cells that had been transfected with the following concentrations of S3a-sense ODNs for 4 days: Lane 1, lipofectin alone; lane 2, 100 nM; lane 3, 250 nM; lane 4, 500 nM; lane 5, 1000 nM; lane 6, no addition.
CHAPTER 5

Treatment of LNCaP human prostate cancer cells with transcriptional and translational inhibitors does not inhibit neurite outgrowth during cAMP induced neuroendocrine differentiation.
5.1 ABSTRACT

Elevation of intracellular cAMP levels in the human prostatic adenocarcinoma cell line LNCaP results in the induction of reversible neuroendocrine differentiation and cell growth arrest. Cyclic AMP induced NE differentiation of LNCaP cells is characterized by the appearance of dense core granules in the cytoplasm, loss of mitogenic activity, and expression of the NE markers chromogranin A, neuron-specific enolase, parathyroid hormone-related peptide, neurotensin, and serotonin. In addition, the acquisition of the NE phenotype is associated with the extension of neuron-like processes following exposure to cAMP elevating agents. Here I show that morphological changes that are associated with cAMP induced differentiation of LNCaP cells from the epithelial to the neuroendocrine state do not require alterations in gene expression nor the expression of novel proteins. My experiments suggest that precursor molecules that are necessary for neurite outgrowth may be present in cells at sufficient quantity to allow for NE process development without the need for additional gene transcription and protein translation during cAMP induced NE differentiation.
5.2 ABBREVIATIONS

bp : base pair(s)
cAMP : adenosine 3'5'-cyclic monophosphate
cDNA : DNA complementary to mRNA
CgA : chromogranin A
db-cAMP : dibutyryl-cAMP
FBS : fetal bovine serum
HRS3a or S3a: human ribosomal S3a
IBMX : isobutylmethylxanthine
NGF : nerve growth factor
NE : neuroendocrine
PKA : protein kinase A
5.3 INTRODUCTION

Cyclic 3’5’ adenosine monophosphate (cAMP) is known to be a quintessential intracellular signaling molecule in animal cells and cAMP has been demonstrated to regulate a variety of cellular activities [1]. Many extracellular signaling events, such as peptide hormones, growth factors, and neuronal activities influence cellular activity by altering intracellular cAMP levels [2]. The human prostatic adenocarcinoma cell line LNCaP is known to acquire neuroendocrine characteristics following treatments that increase intracellular cAMP [3].

Cyclic AMP induced NE differentiation of LNCaP cells is characterized by the appearance of dense core granules in the cytoplasm, loss of mitogenic activity, and expression of the NE markers chromogranin A (CgA), neuron-specific enolase, parathyroid hormone-related peptide, neurotensin, and serotonin. In addition, the acquisition of the NE phenotype is associated with the extension of neuron-like processes following exposure to cAMP elevating agents.

It has been suggested that neuroendocrine cells provide paracrine stimuli to proximal cells and the NE cells may facilitate carcinoma cell growth. Considering the well characterized role of cAMP in signal transduction and gene regulation [4], it is logical to assume that the acquisition of NE characteristics is accompanied by
changes in gene expression. Indeed, the chromogranin A gene is a well characterized neuroendocrine marker and this gene is known to be up-regulated in response to cAMP in neuroendocrine cells [5].

Unlike alterations in NE marker gene expression, we found that changes in cell morphology involving the extension of NE-like processes occurred in the presence of cycloheximide and actinomycin D suggesting that cAMP induced changes in morphology do not require do novo protein synthesis or changes in mRNA stability.
5.4 MATERIALS AND METHODS

5.4.1 Cell culture. The human prostatic carcinoma cell line LNCaP was obtained from the American Type Culture Collection (Manassas, VA). LNCaP cells were maintained at 37°C in a humidified incubator containing 5% CO₂ / 95% air in RPMI 1640 medium supplemented with 50 µg/ml gentamycin, and 10% heat inactivated fetal bovine serum (Atlanta Biologicals. Norcross, GA). Cells were grown to 80% confluency in 75 cm² culture flasks and passaged into 6 well plates the day before transfection experiments. Fluid renewal was performed twice weekly and cultures were passaged upon confluency (6-8 x 10⁵ cells/cm²) by trypsinization.

5.4.2 Induction of NE differentiation, measurement of longest process length, and growth studies. Cell counts were conducted using a hemacytometer and cell viability was determined by counts of trypan blue-excluding cells. Neuroendocrine differentiation was induced in the LNCaP cell line by addition of the combination of 1 mM dibutyryl-cAMP and 500 µM IBMX. NE induction was carried out over a four-day period. Protein synthesis and RNA transcription was inhibited in LNCaP cells by the addition of 10 ng/ml cycloheximide and 3 µg/ml actinomycin D. Cycloheximide and actinomycin D were added either concurrently
with the addition of cAMP elevating agents or 2 h prior to the addition of cAMP/IBMX. Observation and measurement of NE process extension was performed using an inverted phase contrast microscope. The length of the longest cell process was determined for random cells by measuring the distance from the cell body to the end of the longest process. For luciferase activity studies, NE induction was initiated 16 hours after plasmid transfection.

5.4.3 Plasmid Constructs. Two oligonucleotides were synthesized according to the previously published $HRS3a$ genomic sequence [6]. The S3a+ATG-F primer sequence is 5'-GAGACTCGAGAGGTTTGGGGTACGGTGAG-3'. This sequence includes a XhoI restriction site and an additional four nucleotides at the 5' end. A downstream primer called S3a5'-R was synthesized corresponding to the sequence 5'-GAGAAAGCTTCAAAAGGGCGGAAGTGG-3'. The resulting 373 bp PCR product of S3a+ATG-F and S3a5'-R corresponds to -344 through +8 of the $S3a$ promoter sequence and includes a HindIII recognition site at the downstream end. The +8 nucleotide position is 17 bp 5' to the $S3a$ ATG start codon. PCR was performed on 100 ng of LNCaP genomic DNA using the following conditions: initial denaturing at 94°C for 3 min, followed by 30 cycles of denaturing at 94°C for 1 min, annealing at 58°C for 40 sec, and extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The resulting PCR product was cloned into the blunt end PCR-
TRAP vector (Genehunter. Nashville, TN). The sequence of the LNCaP PCR product was confirmed by comparison to the published *HRS3a* sequence in Genbank (accession number: X87373).

### 5.4.4 Plasmid Reporter Constructs

The *S3a* promoter segment was subcloned into the pGL3-Enhancer luciferase reporter plasmid (Promega. Madison, WI) after initial cloning of the *S3a* PCR product into the PCR-TRAP blunt end cloning vector. The cloned PCR product corresponding to the *S3a* 5' flanking sequence was digested from the PCR-TRAP vector using *XhoI* and *HindIII* and subcloned into the *XhoI* and *HindIII* sites of pGL3-Enhancer to ensure directional cloning relative to the luciferase gene. The subcloned *S3a* promoter sequence was confirmed by DNA sequencing using the pGL3-Enh vector sequencing primer RVprimer3 5'-CTAGCAAAAATAGGCTGTC -3'.

### 5.4.5 Transfection and Luciferase Assays

Plasmid DNA for transient transfection was isolated using the plasmid maxi kit (Qiagen. Valencia, CA). LNCaP cells were plated at a density of 5 x 10⁵ cells per well in 6 well plates and grown in RPMI-1640 supplemented with 10% FBS overnight prior to transfection. Transfections were performed using 8 µl Lipofectin reagent per well (Life Technologies. Carlsbad, CA) and serum free RPMI-1640 as per the manufacturer’s
suggestions. A total of 10 µg of S3a-ATG pGL3-Enh vector and 1 µg of pRL-SV40 Renilla luciferase vector (Promega) were used for each transfection. The pRL-SV40 vector was used as a transfection efficiency control. Each transfection was performed in triplicate and repeated at least twice. Firefly luciferase and Renilla luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega). Approximately 72 h after transfection, cells were washed with 1x PBS pH 7.4 then harvested with 400 µl passive lysis buffer (Promega). Following two rounds of freeze thawing, 10 µl of cell lysate was added to 100 µl firefly luciferase substrate and light units were measured in a Berthold luminometer. Renilla luciferase activities were measured in the same tube after the addition of 100 µl Stop and Glo reaction and 5 seconds of vigorous vortexing.
5.5 RESULTS AND DISCUSSION

In eukaryotic cells, except for higher plants, cAMP serves as a second messenger that is produced as a result of the activation of cell surface receptors coupled to GTP binding proteins [7]. Increased cAMP levels activate cyclic AMP dependent protein kinase A (PKA) that is capable of phosphorylating and modulating the activity of the target proteins. PKA catalyzes the transfer of the terminal phosphate group from ATP to specific serines or threonines of target proteins. Covalent phosphorylation of the appropriate amino acids on target proteins is known to regulate the target protein’s activity.

Protein kinase A is found in all animal cells and PKA is thought to mediate almost all of the effects of cAMP in animal cells. Different cell types have different substrates for PKA and this explains why the effects of cAMP vary in different cell types. PKA, in the inactive state, is composed of two catalytic subunits and two regulatory subunits. Association of cAMP with the regulatory subunits alters the regulatory subunit conformation and releases the catalytic subunits [8]. The catalytic subunits are then activated to phosphorylate target substrate proteins. The activated catalytic subunits may then influence gene expression or modulate the activity of target proteins that are not involved with gene expression.
In order to evaluate the effects of transcriptional and translational inhibitors on the ability of cAMP treated LNCaP cells to acquire NE morphological characteristics, LNCaP cells were treated with cycloheximide and actinomycin D during cAMP induced NE differentiation (Fig 5.1). The acquisition of extended cell processes is considered to be a characteristic of NE differentiation in LNCaP cells [9]. As expected, treatment of LNCaP cells with cAMP elevating agents produced a significant increase in longest process length (Fig 5.1, bars 1-2). Interestingly, when the transcriptional and translation inhibitors were added concurrently with the cAMP elevating agents, the LNCaP cells developed significantly extended NE processes in a similar fashion to cells that were not treated with cycloheximide and actinomycin D (Fig 5.1, bars 2-8).

During the initial cycloheximide and actinomycin D experiments, the inhibitors were added in parallel with cAMP elevating agents. In order to evaluate the effects of pre-treatment of LNCaP cells with transcriptional and translational inhibitors on the ability of LNCaP cells to acquire extended NE processes, LNCaP cells were pre-incubated with cycloheximide and actinomycin D for 2 h prior to the addition of cAMP elevating agents (Fig 5.2). Again, LNCaP cells acquired extended processes in the presence of cAMP elevating agents regardless of whether or not cycloheximide or actinomycin D was present.

Luciferase activity assays were performed to provide evidence that treatment
of LNCaP cells with cycloheximide and actinomycin D ultimately results in inhibited protein synthesis (Fig 5.3). Luciferase activity was relatively high in LNCaP cells using the pGL3-Enhancer S3a(-)Sp1(-)ATG vector regardless of whether or not cAMP elevating agents were present (Fig 5.3, lanes 1-2). When treated with cycloheximide and actinomycin D, relative luciferase activity decreased ten to twenty fold (Fig 5.3, lanes 3-4). The results of this experiment suggest that protein synthesis is attenuated in the presence of the transcriptional and translational inhibitors. Firefly luciferase is known to have a short half life in mammalian cells [10]. The observed luciferase activity in the presence of transcription and translational inhibitors could be due to either low level expression of luciferase or residual luciferase protein from previous protein expression.

In this chapter I show that morphological changes that are associated with cAMP induced differentiation of LNCaP cells from the epithelial to the neuroendocrine state do not require alterations in gene expression nor the expression of novel proteins. Wilson et al [11] have shown that the association of neuroendocrine differentiation factor with IGF-binding protein related protein 1 can induce neuroendocrine cell differentiation in prostate cancer cells. Interestingly, it is known that under some circumstances, neurons can be made to regenerate injured neuritic processes independent of new gene expression [12].

Neurite cell outgrowth is believed to be directed by concentrations of neurotrophic
factors, such as nerve growth factor (NGF), and concentration gradients of neurotrophic factors guide the direction of neurite cell outgrowth [13]. Our experiments suggest that precursor molecules that are necessary for neurite outgrowth may be present in cells at sufficient quantity to allow for NE process development without the need for additional gene transcription and protein translation.
5.6 REFERENCES


6. Nolte D, Taimor G, Kalff-Suske M, and Seifart KH. The human S3a ribosomal protein: sequence, location and cell-free transcription of the


cAMP/IBMX: - + - + - + - + +
Cycloheximide: - - + + - - + +
Actinomycin D: - - - - + + + +
Figure 5.1. Effect of cycloheximide and actinomycin D treatment on longest process length in cAMP induced LNCaP cells.

Protein synthesis and RNA transcription was inhibited in LNCaP cells by the addition of 10 ng/ml cycloheximide and 3 µg/ml actinomycin D. Cycloheximide and actinomycin D were added concurrently with the addition of 1 mM db-cAMP and 500 µM IBMX. Observation and measurement of NE process extension was performed using an inverted phase contrast microscope. The length of the longest cell process was determined for random cells by measuring the distance from the cell body to the end of the longest process. Process lengths were determined for 50 individual cells per well in three separate wells per treatment.
cAMP/IBMX: - + - + + +
Cycloheximide: - - - + + +
Actinomycin D: - - + - + +
Figure 5.2. Effect of cycloheximide and actinomycin D pretreatment on longest process length in cAMP induced LNCaP cells.

Protein synthesis and RNA transcription was inhibited in LNCaP cells by the addition of 10 ng/ml cycloheximide and 3 µg/ml actinomycin D. Cycloheximide and actinomycin D were added 2 h prior to the addition of 1 mM db-cAMP and 500 µM IBMX. Observation and measurement of NE process extension was performed using an inverted phase contrast microscope. The length of the longest cell process was determined for random cells by measuring the distance from the cell body to the end of the longest process. Process lengths were determined for 50 individual cells per well in three separate wells per treatment.
cAMP/IBMX: - + - +
Cycloheximide: - - + +
Actinomycin D: - - + +
Figure 5.3. Effect of cycloheximide and actinomycin D on pGL3-ATG-Sp1 reporter vector luciferase vector activity in LNCaP cells.

LNCaP cells were transfected with 10 µg pGL3-ATG-Sp1 Enhancer vector. Firefly luciferase activities were normalized to pRL-SV40 Renilla luciferase activity. Plus cAMP/IBMX bars represent activity from cells that were induced to NE differentiation by the addition of 1 mM db-cAMP/500 µM IBMX 16 hrs after transfection. Protein synthesis and RNA transcription was inhibited in LNCaP cells by the addition of 10 ng/ml cycloheximide and 3 µg/ml actinomycin D. Cycloheximide and actinomycin D were added 2 h prior to the addition of 1 mM db-cAMP and 500 µM IBMX. Each experiment was performed in triplicate.
Prostate cancer is a diverse and multifocal disease. To understand the clinical characteristics of prostate cancer, we must first investigate the cellular and molecular basis of disease initiation and progression. The ultimate goals of prostate cancer research are to better understand the fundamental concepts of cancer biology, and to both prevent the development of prostate cancer and cure those men already afflicted.

The results presented in this study serve to illustrate the wide array of genes expressed differentially between two phenotypically different forms of the LNCaP human prostate cancer cell line. I hypothesized that isolation of genes expressed differentially between untreated LNCaP cells and LNCaP cells that have been induced to a quiescent NE-like state would result in the identification of genes encoding important regulatory proteins as well as proteins that can serve as diagnostic, prognostic, and therapeutic targets. In addition, I provided additional support for the reversibility of LNCaP NE differentiation; a finding that may provide important insights for the progress of prostate cancer physiology.

Differential Display PCR technology allowed for the examination of 226 potentially differentially expressed products between undifferentiated LNCaP cells and LNCaP cells that had been induced to NE differentiation by increases in intracellular cAMP. Twelve of the potentially differentially expressed products were confirmed by Northern analysis. Exhaustive analysis of each of the differentially expressed genes identified is beyond the scope of this preliminary study. The ddPCR results provided for several fascinating candidates for continued research in the
field of LNCaP NE differentiation and prostate cancer physiology. In addition to potential regulatory roles during the process of LNCaP NE differentiation, the identified genes allude to novel molecular mechanisms for prostate cancer disease progression and these findings have the capacity to generate new avenues for carcinoma therapy.

A PCR product was identified using the differential display technique as being down-regulated during cAMP-induced NE differentiation. This cDNA was subsequently cloned, sequenced, and identified as a human gene known collectively as *gte-1, v/fos* transformation effector gene, and *S3a*. In order to better ascertain the mechanism by which *S3a* gene expression is decreased during differentiation, the promoter region for this gene was analyzed. I found that the DNA sequence element 5'-AGACGTCA-3' acts as a functional CRE element for the *S3a* gene promoter and this CRE sequence can be recognized by CREB as indicated by gel supershift analysis. The CRE element varies slightly from the consensus CRE, but the variation does not appear to inhibit the affinity of CREB binding within the local DNA sequence environment. I also demonstrated that the *S3a* 2CRE DNA sequence element 5'-GCACGTCA-3' can also be recognized by CREB. Sp1 and GABP consensus sequences, which are found in the S3a gene promoter, can be recognized by specific DNA binding proteins that are present in LNCaP nuclear protein extracts. The intact *S3a* distal CRE element is required for the increased expression level of *S3a* as indicated by site-directed mutagenesis of the CRE element, transfection of LNCaP cells with a dominant-negative CREB (KCREB) expression vector, and the
observation of a subsequent decrease in luciferase activity. In contrast to typical observations, the addition of cAMP elevating agents appears to decrease $S3a$ expression levels using a mechanism that is intimately associated with the presence of the functional $S3a$ CRE. Binding of GABP complexes to the GABP consensus sequence, that is proximal to the transcriptional initiation site for $S3a$, results in significant repression of gene expression. The GABP transcription factor is specifically important for down regulation of the $S3a$ gene and removal of the DNA sequence that is necessary for GABP complex formation results in a dramatic increase in promoter activity regardless of whether or not the accessory $S3a$ transcription factors are capable of binding to the promoter.

In order to evaluate the effect of $HRS3a$ down regulation in LNCaP cells, antisense phosphorothioate oligonucleotides were designed to inhibit $HRS3a$ gene expression. These experiments suggest that down regulation of $HRS3a$ gene expression by antisense ODN technology may result in attenuation of cell division in LNCaP prostate cancer cells. Furthermore, the growth attenuation of LNCaP cells that are transfected with S3a-antisense ODNs does not correlate with a decrease in steady state $S3a$ mRNA levels suggesting that down regulation of $HRS3a$ may be occurring at the level of protein synthesis. The $HRS3a$ gene represents a novel candidate for gene therapy approaches in prostate cancer treatment and cancer treatment in general.

I also show that the extension of neuritic processes, that is associated with cAMP induced differentiation of LNCaP cells from the epithelial to the
neuroendocrine state, does not require alterations in gene expression nor the expression of novel proteins. Interestingly, it is known that under some circumstances neurons can be made to regenerate injured neuritic processes independent of new gene expression. Neurite cell outgrowth is thought to be directed by concentrations of neurotrophic factors, such as nerve growth factor (NGF), and concentration gradients of neurotrophic factors direct the direction of neurite cell outgrowth. The results of these experiments suggest that precursor molecules that are necessary for neurite outgrowth may be present in cells at sufficient quantity to allow for NE process development without the need for additional gene transcription and protein translation.
APPENDIX A. Cloning and Reporter Vector Maps

Figure A-1. PCR-TRAP positive selection cloning vector

Figure taken from Genehunter Corporation. Nashville, TN.
Figure A-2. pGL3-Enhancer and pRL SV40 luciferase reporter vectors

Figure taken from Promega. Madison, WI.
Figure A-3. pRc/RSV vector with KCREB insert.

Figure taken from Invitrogen. Carlsbad, CA.
KCREB vector was a gift from the laboratory of Richard Goodman
Figure B-1. Human ribosomal S3a genomic sequence

Fig. 1. Nucleotide sequence of the 18S S3a gene and coding DNA sequence. The DNA sequence is shown in capital letters, introns and 3' and 5' flanking sequences are depicted in lower case letters. The major intron is indicated by a bold arrow and the polyadenylation signal is underlined. Lower case letters indicate regions of repeat elements. The sequence has been submitted to EMBL, GenBank and DDBJ Nucleotide Sequence Database accession Nos. X61205.

Figure taken from Nolte et al 1996.
APPENDIX C. DNA alignments between *HRS3a* genomic sequence and site directed mutagenesis products

Figure C-1. Alignment of *HRS3a* genomic sequence (top) with 6C(15) [(+ATG)] dd-PCR product (bottom).
Figure C-2. Alignment of *HRS3a* genomic DNA (bottom) with (-)ATG PCR product (top).
Figure C-3. Alignment of (-)CRE site directed mutagenesis product (top) with HRS3a genomic DNA (bottom).
Figure C-4. Alignment of (-)CRE(-)ATG site directed mutagenesis product (top) with \textit{HRS3a} genomic DNA (bottom).
Figure C-5. Alignment of (-)Sp1 site directed mutagenesis product (bottom) with *HRS3a* genomic DNA (top).
Figure C-6. Alignment of (-)GABP site directed mutagenesis product (bottom) with *HRS3a* genomic DNA (top).
Figure C-7. Alignment of (-)CRE(-)Sp1 site directed mutagenesis product (bottom) with HRS3a genomic DNA (top).
Figure C-8. Alignment of (-)2CRE site directed mutagenesis product (top) with \textit{HRS3a} genomic DNA (bottom).
Figure C-9. Alignment of (-)2CRE(-)ATG site directed mutagenesis product (bottom) with $HRS3a$ genomic DNA (top).
Figure C-10 Alignment of (-)CRE(-)2CRE(-)ATG site directed mutagenesis product (bottom) with the *HRS3a* genomic DNA (top).
Figure C-11. Alignment of (-)CRE(-)2CRE(-)Sp1(-)ATG site directed mutagenesis product (bottom) with *HRS3a* genomic DNA (top).
Figure C-12. Alignment of (-)CRE(-)GABP site directed mutagenesis product (bottom) with *HRS3a* genomic DNA (top).
Figure C-13. Alignment of (-)CRE(-)Sp1(-)ATG site directed mutagenesis product (bottom) with *HRS3a* genomic DNA (top).
Figure C-14. Alignment of (-)CRE(-)Sp1(-)GABP site directed mutagenesis product (bottom) with *HRS3a* genomic DNA (top).
Figure C-15. Alignment of (-)Sp1(-)ATG site directed mutagenesis product (bottom) with \textit{HRS3a} genomic DNA (top).
Molecular Cell Biology and Biotechnology

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PUBLICATIONS & PRESENTATIONS

Goodin, J.L. & C.L. Rutherford. 2002 Isolation of differentially expressed genes
during cAMP Induced NE differentiation in Human Prostate Cancer using mRNA

expression during cAMP induced neuroendocrine differentiation of LNCaP cells.
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Differentiation in Human Postatic Adenocarcinoma Cell Line LNCaP Using mRNA
EXPERIENCE

1/1999 – Present  Virginia Tech University  Blacksburg VA  
**Molecular Biology Lab Instructor**
This position provided a great deal of experience in supervision and trouble shooting of various molecular biology techniques while instructing both undergraduate and graduate students. Most of the fundamental molecular biology techniques were covered in the course. A few of the techniques used included: restriction enzymes and restriction mapping, various forms of gel electrophoresis, cDNA library manipulation, plasmid cloning and subcloning, transformation of bacteria, PCR, DNA sequencing and computer analysis of DNA sequences, Southern blotting, chromatography, western blotting, and use of various reporter genes to study gene expression.

08/22/01 – 12/22/01  Virginia Tech University  Blacksburg VA  
**Honors Biological Principles Instructor**
Developed an application based introduction to basic biological concepts with emphasis on presentation of fundamental molecular biology techniques. Course was designed to be a challenging introduction to laboratory science for honors students. Some methods covered included basic gene cloning experiments, plasmid vectors, DNA and protein sequence analysis, DNA fingerprinting (RFLP), and forensic science.

07/31/00 – 08/04/00  University of Dundee  Dundee, Scotland  
**International Dictyostelium Conference. Regulation of 5’ Nucleotidase (5NT) in Dictyostelium discoideum.**

06/01/00 – 07/31/00  Virginia Tech University  Blacksburg, VA  
**Intern Science Advisor** in the Minority Academic Opportunities Program – 2000 Summer Research Internship Session.

**Biological Principles Lab Instructor**
Taught basic laboratory methods to undergraduate science majors. A few of the basic principles covered included: use of microscopes, forensics, pH and buffers, manipulation of enzymes, dialysis, photosynthesis, and fermentation.

**General Biology Lab Instructor**
Taught freshman non-science majors basic scientific principles and application of the scientific method as a useful strategy for solving problems.

**Mentally Handicapped Special Education Instructor**
Worked with trainable mentally handicapped and severe and profoundly handicapped high school students.

8/1996 – 8/1997  Radford University  Radford, VA  
**Undergraduate Research Project. “Analysis of G6PD (Zw) gene with Respect to Species Formation in Drosophila maritiana.”** Performed DNA sequencing and analysis.
of DNA sequence polymorphism within the *Drosophila* G6PD gene. Analysis was conducted within different lines of the *D. maritiana* species and between closely related *Drosophila* species in an effort to better ascertain the evolutionary relationships between closely related *Drosophila* species.

**General and Advanced Biology Instructor**
High school biology, chemistry, and physics instructor.

08/1993 – 08/1994  Southwest Virginia Community College  Richlands VA
**Project Achieve Peer Tutor.** Primarily involved with instruction in subject areas of English, Mathematics, Chemistry, and Human Anatomy.

**AFFILIATIONS**
02/06/2002  Commissioned 1LT US Army AMEDD
05/10/2002  Promoted to CPT US Army AMEDD
1/1996 – Present  Virginia Academy of Science Member

**ADDITIONAL INFORMATION**
**Current Research Interests**
We are interested in the molecular events that regulate the cAMP induced differentiation of prostate cancer cells from the epithelial state to the neuroendocrine state. To elucidate the genes involved in this process, we have employed the differential display PCR technique. This technique has allowed for the identification of many interesting genes. We are using a variety of molecular biology techniques to better characterize the roles of each of these differentially expressed genes with the goal of discovering novel targets for cancer treatment through gene therapy. Some of the molecular methods we use include: luciferase/GFP reporter gene analysis, gel mobility shift assay, oligomer antisense gene knockout strategy, site-directed mutagenesis, and inducible protein overexpression systems.

**Awards and Membership Activities**
GRDP Grant. - $500 + $500 Biology Dept Matching funds.
Virginia Academy of Science Grant. - $1250 + $500 Biology Dept Matching Funds. 2000.
College of Arts and Sciences Millennium Grant - $5000 1999.
Dean’s Scholar. Radford University. 1996.
Virginia Academy of Science. 1996 - present