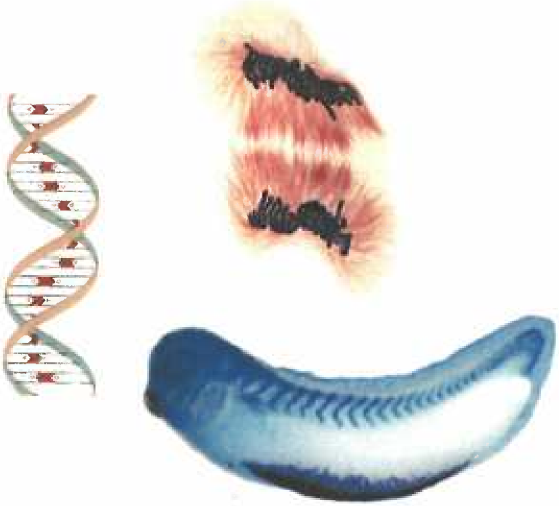

Cell and Developmental Biology

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Regulation of Prolactin Transcription – Studies of Chromatin Structure and Analysis of cAMP Action

Paul Kievit

Department of Cell & Developmental Biology
Oregon Health & Science University

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Paul Kievit

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CERTIFICATE OF APPROVAL

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Paul Kievit

has been approved

[redacted]

Professor in charge of thesis

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Member

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ABSTRACT

The studies described in this thesis describe two different aspects of PRL gene expression. One part will examine the mechanisms by which cAMP can stimulate transcription of the prolactin gene. Treatments that elevate intracellular cAMP concentrations were found to stimulate the mitogen activated protein kinase (MAPK) in GH_3 cells. Elevated cAMP was also found to stimulate activation of the GTP-binding protein, Rap1. Rap1GAP1 reduced cAMP-induced phosphorylation of MAPK, offering evidence that Rap1 may play a role in mediating activation of MAPK. Treatment of GH_3 cells with PD98059, an inhibitor of the MAPK pathway, reduced the ability of forskolin to activate a PRL reporter gene providing evidence that MAPK contributes to cAMP mediated effects on the PRL promoter. As previous studies have implicated Ets factor binding sites within the PRL promoter in mediating responses to MAPK, we expected that the Ets sites would also play a role in cAMP-responsiveness. Surprisingly, mutation of all of the consensus Ets factor binding sites in the proximal PRL promoter greatly reduced responsiveness to EGF and TRH, but did not reduce cAMP-responsiveness. Experiments using an expression vector for adenovirus 12S E1a provided evidence that the co-activators, CREB binding protein and/or p300, probably play a role in cAMP-responsiveness of the PRL promoter. Interestingly, the ability of a GAL4-p300 fusion protein to enhance reporter gene activity was stimulated by cAMP in a MAPK-dependent manner. These findings provide evidence for a model for cAMP-induced PRL transcription involving Rap1 induced MAPK activity leading to stimulation of the transcriptional co-activators, CBP and p300.

The other segment investigates the role of chromatin structure in PRL gene expression. The chromatin structure of a promoter element is an important determinant of its transcriptional activity. Many promoters are assembled into repressive polynucleosomal arrays that are subsequently remodeled to allow for the activation of gene expression. This study addresses the contribution of a single transcription factor, Pit-1, in orchestrating the chromatin structure of the prolactin gene. Utilizing an in vivo chromatin reconstitution system, we found that Pit-1 could translationally position several nucleosomes on the prolactin promoter. Interestingly, the locations of these nucleosomes are within the same regions as the Pit-1 binding sites, and cannot be positioned by Pit-1 mutants that fail to bind DNA. Further, the positioning of the nucleosomes appears to be dependent on the level of Pit-1 expression. In this reconstitution assay, Pit-1 was able to modestly active a chromatin template, and this activation was increased greatly in the presence of a deacetylase inhibitor. Thus, we propose that Pit-1 likely plays a role in setting the chromatin into an open structure that could facilitate the recruitment and subsequent activation by additional transcription factors. Together, these studies underscore the importance of Pit-1 in integrating processes ranging from chromatin remodeling to the transcriptional activation of the PRL gene.

CHAPTER I

INTRODUCTION

This thesis will investigate the molecular mechanism underlying the regulated, tissue-specific expression of the prolactin (PRL) gene. This first chapter will provide an overall background for the studies in this thesis. The second chapter reports studies on the role of cAMP mediated gene expression in a PRL secreting cell line, focusing on the signal transduction pathways and DNA elements in the 5' flanking region of the PRL gene. The third chapter describes studies that investigate the contribution of an individual transcription factor, Pit-1, to the chromatin structure of the PRL gene. The studies in the third chapter employ the use of a chromatin reconstitution system in *Xenopus laevis* oocytes.

The Mammalian Pituitary

The pituitary gland plays a central role in body function (1). This endocrine gland is located at the base of the brain, just below the hypothalamus to which it is connected via the stalk. The pituitary is comprised of two parts: the posterior pituitary (neurohypophysis) and the anterior pituitary (adenohypophysis). The posterior pituitary is derived from neuroectoderm and is considered an outgrowth of the hypothalamus. It contains the nerve endings of two populations of neurons whose cell bodies are located within the hypothalamus that secrete the two primary posterior pituitary hormones, oxytocin and vasopressin. Among others, these hormones play an important role in regulation of water metabolism and mammary gland function. In contrast to the posterior pituitary, the anterior pituitary is not directly connected to the hypothalamus. Instead, communication between the anterior pituitary and the hypothalamus is established through a humoral pathway.

The anterior pituitary is derived from the most anterior portion of the neural ridge and arises as an invagination of oral ectoderm, known as Rathke's pouch. The mature pituitary gland contains six hormone-producing cell types, including the corticotrophs secreting adrenocorticotropin; melanotrophs secreting melanocyte-stimulating hormone; thyrotrophs secreting thyroid-stimulating hormone; gonadotrophs secreting luteinizing hormone and follicle-stimulating hormone; somatotrophs secreting growth hormone; and lactotrophs secreting prolactin. Secretion of the anterior pituitary hormones is largely regulated by hormones produced in the hypothalamus. Neurons from various regions of the hypothalamus release these hormones near capillaries at the base of the hypothalamus (median eminence). From there they can be conveyed to the cells of the anterior pituitary via the hypothalamo-pituitary portal vessels and act as either stimulatory hormones (like growth hormone releasing hormone) or inhibitory hormones (like prolactin inhibitory factor (later identified as dopamine)).

The well-defined nature of the pituitary cell-types, their secreted hormones and hypothalamic regulation have proven to provide an excellent model system in which to investigate the molecular mechanisms that underlie cell specific gene expression and its regulation by extracellular signals. Relevant to this thesis is the regulated expression of the PRL gene by intracellular signaling pathways and prolactin gene promoter elements. Several factors have contributed to the usefulness of this system for the analysis of regulated gene expression. The availability of the GH3 clonal cell line (2) that expresses the PRL gene in a regulated manner has greatly facilitated the studies of PRL secretion and gene expression. In addition, the relatively early isolation of cloned cDNAs for PRL (3, 4) and the subsequent cloning and characterization of the PRL chromosomal gene (5) led to studies of PRL expression at the molecular level.

Prolactin

Prolactin was identified as the functional component of bovine pituitary gland extract that induces lactation in rabbits and crop sac growth in pigeons (6). It is a simple, polypeptide hormone composed of 199 amino acids. The sequence homology between different species can range from a striking 97% among primates to 56% when comparing primate and rodent sequences. Although prolactin is most appreciated for its effect on the mammary gland, including gland development and lactation, various other biological actions have been attributed to prolactin. For instance, it can play a role in the maintenance of the corpus luteum and certain aspects of reproductive behavior. Prolactin is also implicated in non-reproductive effects such as regulation of the immune system, osmotic balance, and angiogenesis (reviewed in (7)). Because PRL is involved in this many processes in the human body, it is not surprising that the levels of PRL in serum are tightly regulated by various mechanisms.

Prolactin release from the pituitary is influenced by many exogenous and endogenous stimuli that converge in the hypothalamus. From here, neurons project into the median eminence and release both PRL releasing and inhibitory factors. It is believed that the majority of PRL regulation is mediated through inhibition by dopamine (DA). DA is secreted by the tubero-infundibular neurons into the median eminence from where it can reach the lactotrophs. The lactotroph contains DA receptors that belong to the D2 receptor subclass of the dopamine receptor family, which in turn are members of the superfamily of G-protein coupled receptors (8). The physiological importance of these receptors in PRL regulation is underscored by the observation that mice lacking these receptors have lactotroph hyperplasia and elevated PRL serum levels (9). DA can inhibit both the secretion as well as the synthesis of PRL. A large body of work has explored the regulation of PRL secretion and has recently been reviewed and will not be further discussed (7). Rather, this introduction will examine the mechanisms of PRL gene expression.

Regulation of Prolactin Gene Expression by cAMP

The primary mechanism by which dopamine represses PRL gene expression is by inhibiting the activity of adenylate cyclases (10). Indeed, treatment of pituitary cells with the dopamine agonist ergocryptine reduced the level of PRL gene transcription. Conversely, addition of a cell-permeable form of cAMP, thus increasing intracellular levels of cAMP, to ergocryptine-pretreated cells resulted in a rapid stimulation of PRL gene expression (11). An increase in cAMP levels results in the dissociation of the cAMP dependent protein kinase holoenzyme (PKA), which consists of two catalytic and two regulatory subunits. Upon binding of cAMP to the regulatory subunits, the tetramer dissociates into two active catalytic subunits and a regulatory subunit dimer. The importance of PKA was underscored by the observation that forced expression of the catalytic subunit in the established rat lactotroph GH3 cell line could increase the level of PRL gene expression (12). Further, transfection of GH3 cells with the heat-stable inhibitor of PKA, PKI, resulted in a decrease in both basal and cAMP mediated PRL gene expression (13). Although these studies established early on that PKA was sufficient for PRL gene expression, a definite analysis of the mechanism by which cAMP acts remains elusive.

An important discovery in cAMP mediated signaling was achieved with the cloning of a cAMP response element binding protein (CREB) (14, 15). Stimulus dependent activation of CREB is mediated by phosphorylation. Exposure of cells to forskolin, an activator of adenylyl cyclase resulting in increased levels of cAMP, leads to phosphorylation of a serine residue at position 133. This serine is required for transcriptional activation since mutation of the serine residue to an alanine residue results in an inactive form of CREB (16). Interestingly, a DNA element located in the proximal region of the PRL gene promoter contains a similarity to a consensus cAMP response element. However, extensive analysis concluded that CREB was not able to bind to the PRL promoter via this DNA element. The observation that CREB might not be involved was further corroborated when it was shown that a dominant-negative form of CREB was unable to interfere with cAMP mediated PRL gene expression (17). These studies suggested that an alternate target for cAMP and/or PKA could be involved. Interestingly, mutational analysis of the 5' flanking sequence of the PRL gene identified DNA regions that were important for both basal and cAMP stimulated transcription (18). Earlier studies had shown that these regions were involved in the binding of a pituitary specific protein named Pit-1 and that the presence of Pit-1 was important for PRL gene expression (19, 20).

The Pituitary-Specific Transcription Factor Pit-1

Deletion analysis of the 5' flanking sequence of the PRL gene identified two regions that are important in cell-specific promoter activity. A distal enhancer is located approximately 1500 bp upstream of the transcription initiation site (-1581 to –1718) and a proximal promoter located 200 bp upstream of the initiation site (-200 to –38) (21, 22). The pituitary specific transcription factor Pit-1 was cloned due to its ability to bind these PRL gene sequences and related sequences in the 5' flanking sequence of the growth hormone gene (20, 23). The evolutionary relationship between growth hormone and prolactin (24) and their simultaneous expression during development (25) suggested that similar processes could be responsible for the expression of both genes. Indeed, a common cell-specific factor, designated Pit-1, was identified (21) and cloned (20, 23). Pit-1 binds to four sites within the proximal region, designated 1P to 4P, and 4 sites within the distal enhancer, named 1D to 4D (Figure 1) of the PRL gene.

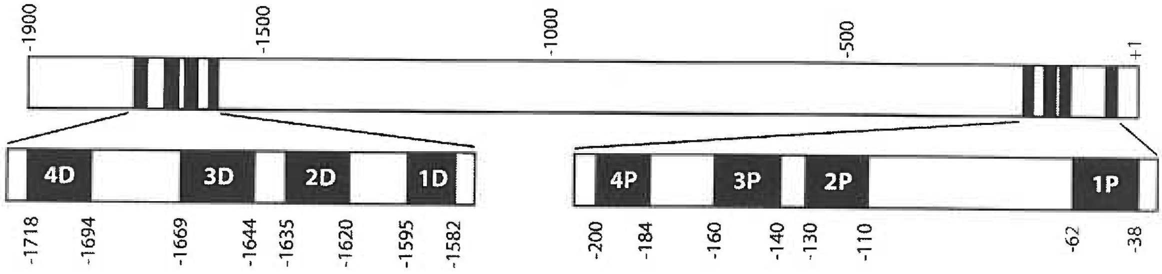


Figure 1. Overview of the Pit-1 binding sites in the PRL gene.

The expression of Pit-1 is restricted to lactotroph, somatotroph and a subset of thyrotroph cells in the anterior pituitary (26). The necessity of Pit-1 for the proper development of these cell lines is emphasized by the phenotype of mice harboring a mutation in the Pit-1 coding sequence (26). Analysis of these dwarf mice revealed that a mutation in the DNA binding domain of Pit-1 was present which resulted in the loss of the three cell lineages and a low level of Pit-1 mRNA and protein. The observation that levels of Pit-1 itself were also reduced in comparison to wild-type mice can be explained by the presence of Pit-1 binding sites within the 5' flanking sequence of the Pit-1 gene, suggesting that Pit-1 can regulate its own expression (27). Actually, similar Pit-1 binding sites have been described in other pituitary-expressed genes such as the TSHβ gene (28, 29), the GHRH receptor gene (30) and the somatostatin receptor 1 gene regulatory regions (31) among others.

DNA binding properties of Pit-1

The Pit-1 protein is a 33-kDa polypeptide that is composed of a 80 amino-acid N-terminal transactivation domain and a C-terminally located POU domain that is responsible for DNA binding, homodimerization and other protein-protein interactions (32, 33)(see figure 2). The POU domain family of transcription factors was defined following the observation that a region of homology was shared between the mammalian genes encoding Pit-1, Oct-1 and –2 and the *Caenorhabditis elegans* gene unc-86 (34). The POU-domain is a bipartite DNA binding domain consisting of a highly conserved, 75 amino-acid POU-specific domain, tethered by a variable linker to a 60 amino-acid homeodomain (Figure 2). High affinity binding by POU domain transcription factors requires both domains, which can bind DNA cooperatively even when they are not connected by the linker (35).

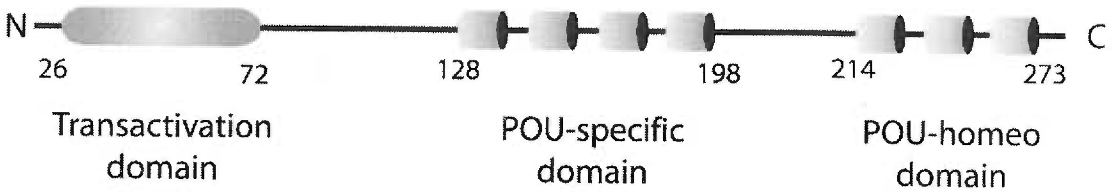
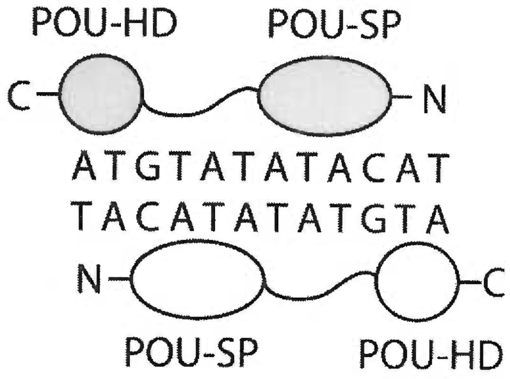


Figure 2. Structural properties of Pit-1

Recent studies have resolved the structure of the POU domain of Pit-1 bound to its cognate DNA recognition site by high resolution X-ray analysis (36). The POU-specific domain of both Oct-1 and Pit-1 consists of four α-helices, with the second and third helices forming a structure reminiscent of a helix-turn-helix motif of the λ and 434 repressors (36, 37). The POU-homeodomain consists of three α-helices, but the binding properties differ between Pit-1 and Oct-1. Whereas the POU-specific domain and the POU-homeodomain of Oct-1 contact separate sites on different sides of the helix, the Pit-1 POU domain contacts the same face of the DNA. Therefore, the binding of a Pit-1 dimer to its DNA binding site essentially surrounds its DNA binding site (Figure 3). Obviously, this conformation of the POU domain could differ dependent on the specific DNA binding site and whether Pit-1 is bound as a monomer, homodimer or a heterodimer with another DNA binding protein.

Figure 3. Binding of a Pit-1 homodimer to a DNA element. Figure is adapted from Jacobson et al (36).



Pit-1 and Hormonal Stimulation

The observation that both Pit-1 and the Pit-1 binding sites are important for cAMP mediated activation of PRL gene expression led to studies investigating the nature of the contribution by Pit-1. Indeed, several studies (38, 39) demonstrated that Pit-1 could be phosphorylated at serine residue 115 and threonine residue 220 in response to an increase in cAMP levels. Further, this phosphorylation allowed Pit-1 to bind with a higher affinity to the PRL proximal promoter (38, 40), strongly implicating that Pit-1 was a critical nuclear target of cAMP regulated signaling pathways. Moreover, *in vitro* phosphorylation of bacterially expressed Pit-1 by PKA generated a similar pattern as was observed *in vivo* (39). Interestingly, mutation of the serine residues in Pit-1 did not alter its ability to drive cAMP mediated PRL gene expression (40, 41). What then is the role of Pit-1 in regulated gene expression of PRL? A hypothesis that is consistent with the previous data is that Pit-1 could recruit another factor (42). Indeed, Pit-1 has been shown to cooperate with various transcription factors and co-activators. For instance, Pit-1 can cooperate with Lhx-3 (43), Ptx-1 (44, 45) and Ets-1 (46). It would be very tempting to suggest that a putative co-factor could actually be the target of cAMP mediated signaling instead of Pit-1. An interesting observation consistent with this postulated mechanism of cAMP activated PRL gene expression in GH3 cells is the finding that Elk-1, a member of the Ets superfamily of transcription factors, was capable of increasing transcriptional activity in response to increases in intracellular cAMP levels (47). Since Ets family members have been reported to cooperate with Pit-1 in PRL gene transcription, activation of Ets family members by cAMP might provide a parsimonious mechanism by which cAMP regulates PRL gene expression.

Regulation of PRL gene expression by ETS domain factors

The Ets family of transcription factors is comprised of DNA-binding proteins that contain a transcriptional activation domain that is regulated by extracellular signals (48). Ets-1 is the founding member of this family and its name is derived from the name of the avian erythroblastosis virus, E26, which carried the *v-ets* oncogene (49). Based on homology with the Ets-1 DNA-binding domain (ETS-domain), additional members of the family, such as TCF (50), were identified. Structural studies on this domain showed that the ETS domain is a variant of the winged helix-turn-helix motif, in which three α-helices and 4 β-sheets are highly conserved between family members (49). All ETS domain proteins bind to sequences that contain a central GGA motif, although additional residues centered around this motif can increase the binding affinity. The interaction of ETS domain proteins with other co-factors, which independently are either DNA or non-DNA binding, can selectively increase the affinity of binding to any given DNA element. Besides directing protein-DNA interactions, the ETS domain is also involved in protein-protein interactions. Such an interaction has been described between Ets-1 and the pituitary specific transcription factor Pit-1. The involvement of Ets factors in PRL gene expression was initially described in a study by Conrad et al (51) in which a dominant-negative form of Ets-1 was capable of reducing Ras-mediated activation of a transfected PRL reporter construct. Further analysis demonstrated that the proximal promoter of the PRL gene contains 5 putative Ets binding motifs within the same region of the Pit-1 binding sites. In heterologous cells, the interaction between Pit-1 and ETS factors can increase both the basal (52) and Ras-induced level of PRL promoter activity (47). These findings suggested that Ets-1 or another ETS domain factor could be required for proper PRL promoter activity and might play a role in the responsiveness of the PRL promoter to extra-cellular stimuli.

A series of signaling pathways can be activated by extra-cellular stimuli. For instance, exposure of cells to epidermal growth factor results in the activation of mitogen-activated-protein-kinases (MAPKs) ERK-1 and ERK-2. A member of the ETS domain family for which the mechanism of activation has been extensively studied is the TCF family member Elk-1. This ETS domain factor is the target of all three MAPK cascades (ERK1/2, JNK1/2 and p38) and can activate the c-fos promoter that contains an ETS domain factor binding site (reviewed in (53)). The activation of these MAPKs results in the phosphorylation of multiple residues in the C-terminal domain of Elk-1, which enhances (54) transcriptional activity. The finding that ETS domain factors can bind to the PRL promoter and the observation that increased levels of cAMP in GH3 cells can result in an increased transcriptional activity of an Elk-1 construct, resulted in the hypothesis that cAMP could activate PRL gene expression through an alternate pathway (Figure 4).

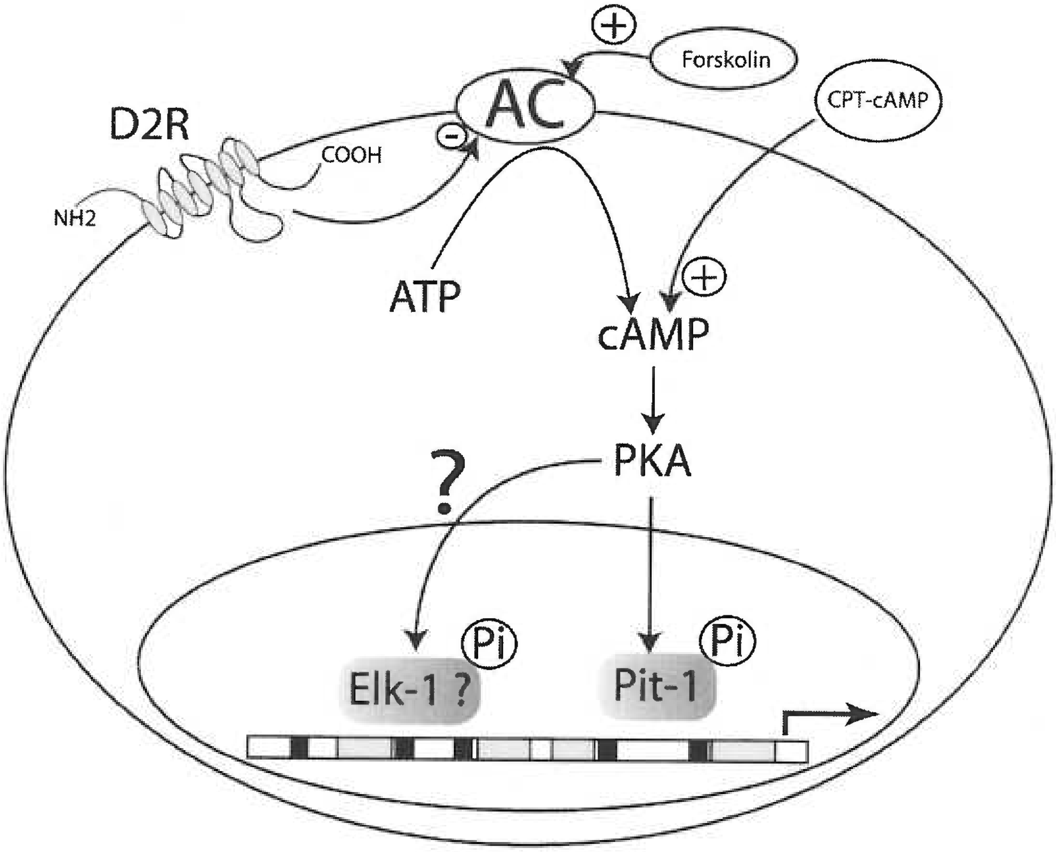


Figure 4. Model of cAMP-mediated activation of the PRL promoter.

Chapter two of this thesis will seek to understand the mechanism by which cAMP can alter PRL gene expression. It will examine the mechanism by which cAMP can modulate the activity of the MAPK pathways and the subsequent role of MAPK activation in PRL gene expression. Further, it will assess the contribution of the ETS domain factor binding sites in the proximal promoter of the PRL gene to cAMP-mediated activation of the PRL gene.

An introduction to Chromatin Structure

DNA is organized and packaged within the eukaryotic nucleus into chromatin (55). A striking property of the chromosome is the sheer quantity of DNA molecules that is incorporated and condensed within this structure. The human genome is comprised of $3x10^9$ base pairs and would extend over 3 feet when completely stretched. However, through several orders of compaction, the DNA fits in the nucleus of a mammalian cell with a diameter of approximately 10 μm. The actual process of DNA compaction is still a poorly understood phenomenon, but the primary proteins, histones, that mediate the folding of DNA into chromatin have been a topic of extensive research for many years.

The histones are universal components of eukaryotic chromosomes and are found in the nucleus at a mass level roughly equivalent to that of the DNA (56). Extraction and isolation of these proteins from nuclei resulted in the identification of five histone types, designated H1, H2A, H2B, H3, H4 (57). The H2A, H2B, H3 and H4 histones, also referred to as the core histones, are conserved in both amino acid sequence and length through evolution, with H3 and H4 showing the highest sequence conservation. For instance, the difference between the amino acid sequence of calf and pea histone H4 is two residues out of 102. The histones H2A and H2B are conserved to a lesser extent. All core histones are small basic proteins (11-16 kDa) that contain a large amount of lysine and arginine. These four histones also contain N-terminal extensions that contain a high level of lysines. Interestingly, post-translational modification of these amino acid extensions plays an important role in various cellular processes. Histone H1 is also a basic protein, but differs from the 4 core histones in other properties. H1 is slightly larger (20 kDa) and contains highly charged amino acid extensions both on the N-terminal and C-terminal end of the protein. Further, where the structure of the core histones is made up of three α-helices, the central domain of H1 contains 3 α-helices attached to a three-stranded β-sheet.

All five of the histones described here play an integral role in the formation of chromatin. The following sections will expand on the role of the histones in formation of a nucleosome and chromatin and the role that nucleosomes play in regulating transcriptional activity of a gene.

The Nucleosome

The nucleosome is the fundamental repeating unit of chromatin (Figure 5). It is comprised of a nucleosome core particle, which consists of 146 base pairs of DNA wrapped around an octamer containing two molecules of each of the core histones H2A, H2B, H3 and H4, a single linker histone (H1) and linker DNA (56). The first indication that DNA existed in the nucleus as part of these organizing structures came from nuclease experiments (55, 58) in which a series of fragments was discovered as multiples of 180-200 base pairs. In addition, electron microscopy analysis of chromatin revealed that a structure was incorporated in chromatin that gave chromatin the appearance of 'beads on a string'. These observations, along with the finding that histones existed in a precise stoichiometry (59), led to the hypothesis of a nucleosome (60). The initial studies on the nucleosome utilized the nuclease specificity of micrococcal nuclease cleavage. Micrococcal nuclease cleaves chromatin in the most accessible region first, the linker DNA, resulting in the formation of nucleosomes (Figure 5). Further digestion with this enzyme will result in trimming of the ends of the linker DNA, leaving the nucleosome core particle intact. Extensive digestion will eventually result in digestion of the DNA within the nucleosome core particle. Therefore, partial micrococcal nuclease digestions provided a convenient method to analyze the structure of chromatin.

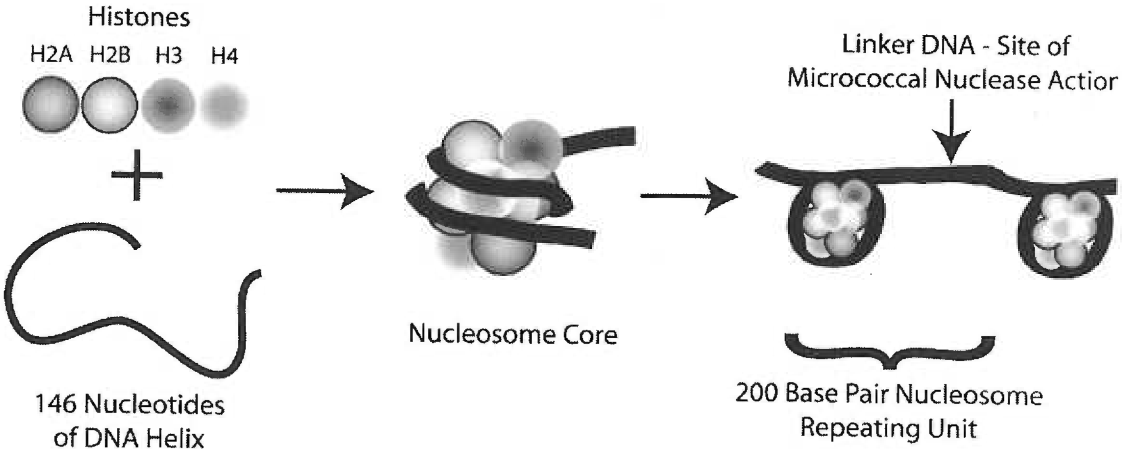


Figure 5. The components of a nucleosome.

The arrangement of DNA into nucleosomal arrays provides one level of compaction. The current knowledge of the processes involved in organizing nucleosomal arrays into higher order structures however is less developed than the understanding of the nucleosome. A key protein that plays a role in this process is histone H1. This protein contains a central, conserved globular region, which is flanked by amino-, and carboxy terminal ends that are less conserved. It is thought that the globular region of histone H1 can interact with the nucleosome core where the DNA enters and exits the structure (61). Subsequent studies have expanded this vision, postulating that the binding of H1 to nucleosomes is assymetrical ((62) and reviewed in (63)). Currently, the exact position and function of histone H1 is not fully known. However, it is of interest to postulate that asymmetric binding of histone H1 allows for a directional component in chromatin folding.

While the packaging of DNA provides the cell with a means to compact the vast amount of DNA in the nucleus, it also poses a physical barrier for non-histone DNA binding proteins to reach their DNA binding sites. Indeed, the mere packaging of promoters into polynucleosomal arrays prevents the initiation of transcription by RNA polymerases in vitro (64). To counterbalance the repressive nature of the chromatin template, a variety of complexes are present in the nucleus that can change the chromatin state, a process widely described as chromatin remodeling. This regulated alteration of chromatin structure can be accomplished by events such as the post-translational modification of the amino-terminal amino acid tails of histones and/or the recruitment of ATP-dependent chromatin remodeling complexes.

Modifications of Histones in Chromatin Remodeling

The amino-terminal amino acid tails of the core histones are central to processes that modulate nucleosome structure (55, 65, 66). The modification of these tails can alter the interaction of the histones with the DNA, thus leading to a different conformation of the nucleosome. The most described modification of the histones is their acetylation, although other processes such as phosphorylation, methylation and ubiquitination have also been documented (67-69). The acetylation of lysines in the histone H4 tail can neutralize the positive charge whereas phosphorylation of a serine residue in the histone H3 tail can introduce a negative charge. Although the exact role of these modifications is still unknown, it is suggested that the change of charges can alter the protein-protein interactions between nucleosomes and acetylation could interfere with the local condensation of nucleosomes in chromatin fibers (56).

Acetylation is most likely the best characterized post-translational modification of histones (65). Since the initial observation of this alteration, a strong correlation between the state of histone acetylation and gene transcription has developed (70). For instance, regions of the chromatin that are known to be transcriptionally silent in yeast such as the mating cassette contain hypoacetylated histone H4 proteins (71). In contrast, hyperacetylation of histone H4 appears to correlate with higher levels of transcriptional activity (72).

The physical link between the regulation of transcription and the state of acetylation was corroborated with the finding that the yeast protein Gcn5 could act as a histone acetyltransferase (HAT)(73). Gcn5 was initially identified as a co-activator of the transcription factor Gcn4 (74) and therefore provided the first example of a HAT protein that was recruited to chromatin by a transcription factor. This discovery was quickly followed by the identification of other HAT containing proteins, such as p300/CBP (75), P/CAF (76) and $TAF_{II}250$ (77). These HAT containing proteins are generally found in large complexes containing many subunits. One example is the SAGA complex (78), which was identified in yeast as a 1.8 MDa protein complex that contained the Gcn5 HAT as the catalytic subunit. Additional studies also identified the human counterpart of this complex, with the p300/CBP associated factor (P/CAF) acting as the HAT (79).

If acetylation were to play an important role in the regulation of gene expression by tethering DNA accessibility, a complex needs to be in the nucleus that can reverse the effects of HAT action. Further, it is to be expected that a protein that can reverse acetylation, could act as a repressor. Indeed, many of the proteins that have been cloned as histone deacetylase proteins were originally identified as corepressors (80). A direct link between histone deacetylases and transcription resulted from the identification of the two mammalian histone deacetylases HDAC-1 and HDAC-2, proteins homologous to the yeast corepressor RPD3 (80, 81). Similar to the HAT containing cofactors, the HDAC are found in multiprotein structures like the Sin3/RPD3 repressor complex (82).

ATP-dependent Chromatin Remodeling Complexes

The modification of histone tails by acetylation is not the only mechanism by which chromatin is modified. Genetic studies in yeast revealed a set of genes, termed SWI and SNF, whose products are found within a general activating complex required for the transcriptional induction of many yeast genes (83, 84). It was shown that this SWI/SNF complex exists as one large 2 MDa complex that is characterized by its ability to alter or disrupt the association of histones with DNA in an ATP-dependent manner (85). The SWI/SNF group of chromatin-remodeling complexes contains 11 components, of which the ATPase subunit is termed Swi2/Snf2. Similar complexes have subsequently been described in yeast (RSC complex), *Drosophila* (Brahma) and human (hBRM and hBRG1) that all contain an ATPase subunit related to the Swi2/Snf2 protein (86). A second group of ATP-dependent remodeling complexes were purified from Drosophila extracts. The most extensively studied members of this group are ACF (ATP-utilizing chromatin assembly and remodeling factor), NURF (nucleosome-remodeling factor) and CHRAC (chromatin accessibility complex) (87-89). The ATPase subunit ISWI, which is a part of ACF, NURF and CHRAC, is homologous to Swi2/Snf2 exclusively over the ATPase domain.

The mechanisms by which the ATP-dependent nucleosome remodeling factors can alter nucleosome and chromatin structure are not yet elucidated. However, studies investigating both the similarities and differences between the related ATPases ISWI and Swi2/Snf2 have provided an insight into several events. In order for chromatin to be remodeled, the remodeling complex needs to be able to recognize and bind to their substrate. For the SWI/SNF and related RSC complex, DNA and nucleosome binding occurs with a high affinity (90, 91). The binding properties of these complexes are relatively similar to the non-histone DNA binding HMG box proteins that can bind DNA nonspecifically. So far, no DNA sequence specificity has been described for the SWI/SNF family of complexes. Obviously, a certain degree of targeting is required given the observation that mutants of SWI/SNF only disrupt gene expression regulation from a select set of genes.

Once the SWI/SNF complex is bound to DNA, it can disrupt the nucleosome in an ATP-dependent manner. The mechanisms by which ATP-dependent nucleosome remodeling complexes can alter the nucleosome structure remains unclear. Very likely, many differences exist between the different families of ATP-dependent remodeling complexes. For instance, the SWI/SNF complex can destabilize the nucleosome by disrupting the rotational phasing of the DNA on the surface of the histone octamer. Therefore, the contacts between the DNA and the histones are altered and the DNA becomes sensitive to endonucleases such as DNaseI (92, 93). On the other hand, ISWI complexes do not seem to cause this perturbation of the nucleosome, instead leaving the pattern of DNaseI digestion unaltered. Mechanistic studies of the ISWI family members NURF and CHRAC complexes suggested that nucleosomes are not disrupted, but change location along the DNA by nucleosome sliding (94). Apart from the different mechanisms by which the ATP-dependent remodeling complexes function, the result of their action culminates in the increased accessibility of the chromatin to transcription factors. It is therefore not hard to appreciate the role chromatin remodeling can play in transcriptional activation of a particular gene. In this thesis, the regulation of the prolactin gene chromatin structure will be assessed. The next section will describe the current knowledge of the prolactin gene chromatin structure and introduce the hypothesis that a pituitary specific transcription factor could be responsible for chromatin remodeling of the prolactin gene.

The Chromatin Structure of the Prolactin Gene

The presence of DNaseI hypersensitive sites within a promoter often correlates with transcriptionally active genes (95). In pituitary cells that express high levels of prolactin protein, the rat PRL gene contains DNaseI hypersensitive sites located within the distal enhancer and the proximal promoter (96). These hypersensitive sites are present as early as three days after birth (97) and are specific for PRL-producing cells (96, 98). The observation that DNaseI hypersensitive sites were only present in cells from a lactotroph lineage provided the first indication that a pituitary specific protein could be responsible for the difference in DNaseI hypersensitivity.

Further studies of the hypersensitive sites in the PRL gene involved the use of minichromosomes. These episomes, each containing 1.9 kb of 5' PRL flanking sequence, were established in GH3 lactotroph cells with approximately 50-100 copies of the episome per cell. Analysis of the DNaseI hypersensitive sites suggested that estrogen treatment increased sensitivity to DNaseI in both the proximal and distal region, suggesting that chromatin structure can be altered by exogenous stimuli (99). In addition, micrococcal nuclease digestion demonstrated that the PRL minichromosomes contain phased nucleosomes in the distal enhancer of the PRL gene. Interestingly, the location of the nucleosomes was not modulated by the presence of estrogen (100). This could argue that although estrogen could increase DNaseI hypersensitivity in the PRL gene (99), the nucleosome structure in PRL producing cells is preset. Therefore, activation of PRL gene expression does not require further nucleosome rearrangement in the distal enhancer. When the minichromosomes were introduced into estrogen receptor containing Rat-1 cells, the promoter was not responsive to estrogen and the nucleosome distribution in the distal enhancer was not phased (100). Thus, within the context of a heterologous non-prolactin producing cell, it appears the estrogen receptor is not sufficient to generate a phased nucleosomal region in the distal enhancer. These studies combined proposed that pituitary specific proteins direct the establishment of nucleosomes on the prolactin gene, and that this is required for hormone induced activation of the PRL gene.

The pituitary-specific transcription factor Pit-1 could be such a factor. Pit-1 can bind to the proximal promoter and distal enhancer of the 5' flanking region of the prolactin gene, in approximately the same region where the two tissue-specific DNaseI hypersensitive sites are observed. Therefore, it seems possible that Pit-1 contributes to the development of a specific chromatin structure. Chapter three of this thesis will evaluate the role that Pit-1 might play in orchestrating the chromatin structure of the PRL gene.

CHAPTER II

Analysis of the Role of the Mitogen Activated Protein Kinase in Mediating cAMP effects on Prolactin Promoter Activity

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Introduction

Although it has been known for some time that cAMP can stimulate PRL gene expression (11, 101), it has been difficult to determine the mechanisms mediating this response. Studies utilizing expression vectors for the catalytic subunit of the cAMP-dependent protein kinase (PKA) or the heat stable inhibitor of PKA (PKI) have provided evidence that PKA is sufficient and necessary for cAMP-induced activation of the PRL gene (12, 13). However, the events downstream of PKA have been elusive. Unlike many PKA-regulated genes, stimulation of PRL gene expression by cAMP probably does not involve CREB (17, 102). Several studies have provided evidence that the pituitary-specific POU transcription factor, Pit-1, may play a role in mediating the effects of cAMP (18, 102, 103) as well as other signaling pathways (104, 105) on PRL transcription. The finding that Pit-1 is phosphorylated in response to elevated cAMP levels in GH_3 cells (38, 39) rather strongly supported the view that Pit-1 may function as a cAMP-regulated transcription factor. However, studies using Pit-1 mutants in which the phosphorylation sites are removed (40, 41) have provided evidence that cAMP-induced activation of the PRL promoter probably does not require phosphorylation of Pit-1. Although this does not exclude Pit-1 from contributing to cAMP mediated effects on PRL transcription, it is by no means clear that phosphorylation of Pit-1 mediates cAMP effects and other mechanisms need to be considered. We have obtained evidence that cAMP can activate the MAPK-responsive transcription factor, Elk1, providing indirect evidence that cAMP may stimulate MAPK in GH_3 cells (39) raising the possibility that the MAPK pathway may be involved in mediating effects of cAMP on transcription of the PRL gene. We have also recently obtained evidence that thyrotropin releasing hormone (TRH) effects on PRL transcription may be mediated at least in part through activation of MAPK (106). Thus it is possible that both the TRH and cAMP signaling pathways may utilize the MAPK cascade to stimulate PRL gene expression.

In the present study we have examined the ability of cAMP to stimulate MAPK activity and tested the role that cAMP-induced MAPK activation plays in modulating PRL transcription. We have found that cAMP can activate MAPK in GH_3 cells and that the increase in MAPK is required for full effects of cAMP on PRL transcription. Analysis of DNA sequences of the PRL gene which are required for cAMP-responsiveness reveals both similarities and differences in the role of specific Ets factor binding sites for TRH- and cAMP-responsiveness. We have also assessed the possible role that the transcriptional co-activators, CREB binding protein and p300, play in mediating cAMP-induced activation of the PRL promoter.

Materials and Methods

Materials

Tissue culture reagents and media were purchased from Life Technologies (Gaithersburg, MD). Antibodies to Rap1, ERK1, ERK2, phosphorylated ERK, anti-mouse and rabbit horseradish-peroxidase conjugated IgG and immunoprecipitation reagents were acquired from Santa Cruz Biotechnology, INC (Santa Cruz, CA). A polyclonal antibody to phosphorylated ERK was purchased from New England BioLabs (Beverly, MA). Antibodies to the FLAG epitope and anti-FLAG M2 affinity resin were purchased from Sigma (St. Louis, MO). The chemical inhibitor PD98059 was obtained from Alexis Corporation (San Diego, CA) and H-89 and genistein were purchased from Calbiochem (La Jolla, CA). Forskolin and chlorophenylthio-cAMP (CPT-cAMP) were purchased from Sigma (St. Louis, MO). Epidermal growth factor (EGF) was from Roche Molecular Biochemicals (Indianapolis, IN). Thyroid releasing hormone (TRH) was purchased from Peninsula Laboratories, Inc. (San Carlos, CA). Radioisotopes and chemiluminescence reagents were obtained from Dupont, NEN (Boston, MA).

Reporter Genes and Expression Vectors

A PRL reporter construct containing sequences representing the -255 to +34 region of the rat PRL gene (107) was obtained by polymerase chain reaction and inserted upstream of the firefly luciferase coding sequence in the pLuc-Link vector (108). Individual consensus Ets factor binding sites in the PRL 5' flanking region were disrupted by oligonucleotide-directed mutagenesis using the polymerase chain reaction. The specific mutations for each site were as follows: the Ets sites at –211 to –208 and -183 to –180 were mutated from TTCC to TGAA, the site at –162 to –159 from TTCC to GGCC, the site at –95 to –92 from GGAA to GTTC and the site at –75 to –66 from GGAAGAGGAT to GGCCGATTAT.

A GAL4-p300 construct was generated by in-frame subcloning of the full coding sequence of p300 into a vector containing the GAL4 (1-147) DNA binding domain downstream of the cytomegalovirus promoter (109). The RSV-PKI vector (13), RSV-Pit-1 vector (18), GAL4-Elk1 (110) construct and the luciferase reporter containing 5 GAL4 binding sites (109) have been described previously. Dr Philip Stork kindly provided GST-RalGDS protein and the FLAG-Rap1GAP1 and FLAG-ERK2 expression vectors. The E1a deletion mutant, Δ2-35 (111) was obtained from Dr. James Lundblad.

Cell Culture and Transfection

GH_3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2.5% fetal bovine serum and 15% equine serum. For transfection, GH_3 cells were seeded into 6-well dishes 1 day prior to transfection at an approximate density of 500,000 cells per well. DNA (1-3 μg total) was transfected using the Lipofectamine reagent (Life Technologies) according to the manufacturer's protocol. After overnight incubation, the cells were treated with agonists for 6 h after which the cells were washed, lysed, and analyzed for luciferase activity. All experiments with the PD98059 compound were performed in DMEM supplemented with BSA (6.6 mg/ml) to prevent precipitation of PD98059. Cells were transfected with CMV-β-Galactosidase expression vector as an internal standard (112).

Immuno-Complex Kinase Assay

MAPK immunoprecipitation assays were performed as previously described (110). Briefly, GH_3 cells were treated with CPT-cAMP for the indicated time intervals, washed twice with PBS and resuspended in lysis buffer (20 mM Hepes-KOH pH7.4, 2 mM EGTA, 50 mM β-Glycerophosphate, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 2 mM NaVanadate). ERK1, ERK2 and JNK1 were precipitated using the appropriate antibody linked to protein A/G agarose and MAPK activity was determined by incubating the precipitates with a bacterially expressed glutathione-S-transferase-Elk1 fusion protein in the presence of 1 μCi [γ-^{32}P]-ATP. The reactions were stopped with loading buffer, resolved on a 10% polyacrylamide, denaturing gel, and phosphorylated proteins were detected by autoradiography.

Immunoblot analysis

For immunoblot analysis, GH_3 cells were grown to ~80% confluency, cultured in serum-free medium for 24 h, and treated with agonist for specific times. Cells were lysed in BOS buffer (50 mM Tris-HCl, pH8.0, 10% Glycerol, 1% Nonidet-P40, 200 mM NaCl, 2.5 mM $MgCl_2$, 2 mM sodium vanadate) supplemented with a mixture of protease inhibitors (Complete Proteinase Inhibitor, Roche Molecular Biochemicals, Indianapolis, IN). Equal amounts of protein (50 μg) were loaded on a 10% polyacrylamide, denaturing gel. Proteins were transferred to a polyvinylidene difluoride (Millipore Co, Bedford, MA) membrane before incubation with antibodies to detect specific proteins. In many cases, membranes were treated to remove bound antibody to permit immunoblot analysis of a second protein. The membrane was incubated for 30 min in stripping buffer (100 mM β-mercaptoethanol, 1% sodium dodecyl sulfate, 62.5 mM Tris-HCl pH6.8), washed in TBS-T (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween 20), and re-probed with the appropriate antibody. All antibodies were used at a concentration of 1:1,000 in TBS-T, 5% non-fat milk, except the FLAG antibody was used at a dilution of 1:10,000. The proteins were visualized on radiography film using a secondary antibody conjugated to horseradish peroxidase and a chemiluminescence detection kit (Dupont NEN, Boston, MA). Quantitation of immunoblot analysis was performed using the Lumi-Imager F-1 system (Roche Molecular Biochemicals, Indianapolis, IN).

Affinity assay for Rap1 Activation

Rap1 activation assays were performed according to Franke et al (113). GH_3 cells were treated with forskolin for the indicated times and harvested in BOS buffer supplemented with Complete protease inhibitor. Cell lysates were cleared by brief centrifugation and adjusted with lysis buffer to a final concentration of 1 mg/ml. Equal amounts of protein were incubated for 1 h in the presence of GST-RalGDS Rap1 binding domain pre-coupled to glutathione beads. Precipitates were washed 3 times with lysis buffer before separation on a denaturing polyacrylamide gel and immunoblot analysis as described.

Results

Elevation of cAMP induces MAPK activity in GH_3 cells.

Previous studies from this laboratory used an indirect assay involving transcriptional activation of a GAL4-Elk1 fusion protein to test for cAMP effects on MAPK activation (47). To directly assess MAPK activation, we used an immunocomplex assay (Fig. 6A, 6B and 6C). For this assay, cell lysates were immunoprecipitated with an antibody to a specific MAPK family member and then the immunoprecipitated proteins were incubated with [γ-^{32}P]ATP and GST-Elk1 as a substrate. This assay demonstrated that treatment with chlorophenylthio-cAMP stimulated the activity of MAPK family members, ERK1 and ERK2 (Fig. 6A and 6B) with only a minor effect on the activity of the stress activated MAPK, JNK1 (Fig. 6C). ERK1 and ERK2 are activated by phosphorylation of specific tyrosine and threonine residues (114) and an antibody which can detect this phosphorylation was also used to assess ERK activation (Fig. 6D). Treatment of cells with forskolin, an agent that increases intracellular cAMP levels, resulted in the rapid stimulation of the phosphorylation of ERK1 and ERK2 (Fig 6D). There was little or no change in the level of total immunoreactive ERK1 and ERK2 suggesting that the increased phosphorylation is not due to a change in the total amount of these kinases (Fig. 6E). Experiments were then performed to compare the time course of ERK activation by forskolin and EGF. Cells were treated with either forskolin or EGF for varying times and ERK activation was determined using a phospho-specific antibody and the results quantitated using a Lumi-Imager system (Fig 6F and G). EGF effects on ERK phosphorylation appeared to reach maximal levels more quickly and also appeared to decline somewhat more rapidly. Several different experiments supported the view that EGF effects on ERK activity reach a maximum more quickly than cAMP effects, but decay more rapidly (data not shown). Overall, the analysis of cAMP effects on MAPK confirm our earlier results (47) and studies from Jacob et al. (115) which demonstrated cAMP-induced activation of MAPK in the GH_4 cell line. The present studies also provide information about the time course and specificity of MAPK activation.

The major signaling pathway that mediates responses to cAMP involves activation of the cAMP dependent protein kinase (PKA). An expression vector for PKI, the heat stable inhibitor of PKA (13), was used to determine if PKA is required for cAMP-induced activation of MAPK. For this study, MAPK induction was assessed indirectly through analysis of the activation of a GAL4-Elk1 fusion protein (116). GH_3 cells were transfected with a GAL4-Elk1 expression vector and a GAL4-dependent luciferase reporter gene either in the presence or absence of a PKI expression vector (Fig. 7A). PKI strongly reduced the ability of forskolin to stimulate GAL4-Elk1 activity as measured by the reporter gene assay. Although PKI reduced EGF-stimulated GAL4-Elk1 activation, PKI also had an effect on basal activity in the absence of hormonal treatment and the fold activation remained similar. The pharmacological inhibitor H-89 was also used to determine if inhibition of PKA led to a decrease in MAPK activation (Fig. 7B and 7C). GH_3 cells were treated with H-89 and then forskolin was added for 5 min and the cells were collected. Activation of MAPK was determined by immunological detection of phosphorylated ERK1 and ERK2 as described above. Addition of H-89 substantially blocked the ability of forskolin to activate ERK1 and ERK2, whereas EGF induced phosphorylation was essentially unchanged. In addition, the tyrosine kinase inhibitor genistein had relatively little effect on the induction of ERK phosphorylation by forskolin, but greatly reduced EGF-induced ERK phosphorylation. The ability of PKA inhibitors to attenuate both cAMP-induced activation of GAL4-Elk1 and phosphorylation of ERK1 and ERK2 suggests that PKA is required for cAMP-induced stimulation of MAPK activity.

Analysis of a possible role of the GTP binding protein, Rap1, in mediating responses to cAMP

Recent studies have provided new insights into cAMP signal transduction and demonstrated that in specific cell types, Rap1 can play a crucial role in mediating cAMP-induced MAPK activation (117). These studies have shown that cAMP can stimulate Rap1 activation leading to activation of B-Raf, MEK1 and MAPK. To determine if this pathway might be present in GH_3 cells, cAMP-induced activation of Rap1 was investigated (Fig 8). This assay is based on the ability of RalGDS to interact with activated, GTP-bound Rap1, but not with inactive, GDP-bound Rap1 (113). Lysates from forskolin-treated GH_3 cells were incubated with the Rap1-binding domain of RalGDS and then bound Rap1 was detected immunologically (Fig. 8A). Activation of Rap1 was detected after 1 minute, the earliest tested time point, and maximum activation was achieved in 5-10 min. In the same experiment, the time course of MAPK activation was also examined using antibodies for phospho-ERK (Fig. 8C). There appear to be some differences in the time course of Rap1 and ERK activation. At the 2-minute time point, Rap1 was only modestly activated, whereas ERK phosphorylation appears to be near maximal. This finding suggests that mechanisms other than Rap1 activation may contribute to the ability of cAMP to stimulate MAPK activity. Alternatively, the activation of Rap1 in the early time-points may be sufficient for maximal ERK activation. In any case, the ability of cAMP to stimulate Rap1 activation suggests a possible role in at least partially mediating effects on MAPK.

To test whether the activation of Rap1 is necessary for MAPK activation, an expression vector for the Rap1 GTPase activating protein (Rap1GAP1) was used (118). Forced expression of Rap1GAP1 should maintain Rap1 in the inactive, GDP-bound state, and thus act as an antagonist to this signaling pathway. Therefore, a Rap1GAP1 expression vector was transfected with an expression vector for FLAG-tagged ERK2 (Fig. 9). Tagged ERK2 was immunoprecipitated and activated ERK2 was detected immunologically using a phospho-specific antibody (Fig. 9A). Rap1GAP1 reduced both basal and forskolin-induced levels of phosphorylated ERK2 at both time points tested. The ability of Rap1GAP1 to reduce ERK2 phosphorylation offers evidence that Rap1 may contribute to cAMP-induced MAPK activation in GH_3 cells. To determine whether Rap1 contributes to cAMP mediated gene expression, GH_3 cells were transfected with the Rap1GAP1 expression vector and a PRL reporter gene (Fig. 9D). At the higher tested concentrations, Rap1GAP1 was capable of reducing both cAMP-induced and basal PRL reporter gene activity. As was observed with Rap1GAP1 effects on cAMP-induced ERK2 phosphorylation, the effects of cAMP were not completely blocked. It is not clear if the remaining cAMP response is a reflection of limited efficacy of the transfected Rap1GAP1 expression vector or if it indicates that a component of the cAMP response involves a Rap1-independent pathway.

We also tested the ability of a different reagent to interfere with the Rap1 signaling pathway. Vossler et al. (117) found that Rap1-N17 can function as a dominant negative mutant to block cAMP-induced ERK and GAL4-Elk1 activation in PC12 cells. Therefore, we tested the effects of Rap1-N17 on ERK activation in GH_3 cells. Surprisingly, we found that Rap1-N17 had no detectable effect on cAMP-induced ERK activation or GAL4-Elk1 activation (data not shown). Another group has also failed to detect an effect of Rap1-N17 on cAMP-induced ERK activation in a different cell type (119). It is possible that the differing ability of Rap1GAP1 and Rap1-N17 to block cAMP-induced ERK activation reflects a different efficacy of these reagents in blocking endogenous Rap1 signaling. Alternatively, this observation may indicate that the Rap1GAP1 and Rap1-N17 interfere with different signaling pathways. We cannot exclude the possibility that Rap1GAP1 may block a signaling pathway other than Rap1, perhaps a related small GTPase.

MAPK activation is required for cAMP-stimulated transcriptional activity

To determine if cAMP-stimulated MAPK activity is required for specific gene expression, we used the MEK1 inhibitor, PD98059. PD98059 has a relatively high affinity for MEK1 and can block the ability of MEK1 to activate ERK1 and ERK2 (120). GH_3 cells were pretreated with 100 μM PD98059 for 30 min and then treated with forskolin for 5 min and assayed for ERK phosphorylation (Fig. 10A). PD98059 treatment greatly reduced cAMP-stimulated phosphorylation of ERK1 and ERK2.

The ability to PD98059 to block cAMP induced transcriptional activation was then tested (Fig. 10B and 10C). PD98059 strongly reduced the ability of forskolin to stimulate GAL4-Elk1 activity as assessed by a GAL4-dependent reporter gene (Fig. 10B). This finding is consistent with our observation that cAMP induces MAPK activity and the known ability of MAPK to induce Elk1 activity (116). PD98059 reduced, but did not completely block forskolin effects to induce a PRL reporter gene (Fig. 10C). Similar results were obtained when a kinase-defective MEK1 was used to inhibit activation of the MAPK pathway (data not shown).

These findings provide evidence that cAMP-induced activation of MAPK plays a key role in stimulating Elk1 transcriptional activation and also contributes to cAMP-induced enhancement of PRL transcription.

Analysis of the role of Ets binding sites in mediating cAMP-induced activation of the PRL promoter

The proximal region of the PRL gene contains a number of consensus sites for members of the Ets family of transcription factors (Fig. 11A). As MAPK has been shown to stimulate the transcriptional activity of several members of the Ets family, (116, 121), the Ets sites appear to be excellent candidates for mediating the transcriptional response to cAMP-induced MAPK activation. Indeed, previous studies have shown that disruption of some of the Ets sites of the PRL gene can reduce transcriptional responses to Ras (46) or TRH (106). To determine if any of the Ets sites play a role in cAMP-dependent PRL gene expression, PRL reporter constructs were tested for cAMP, TRH and EGF responsiveness (Fig. 11B). Some of the Ets site mutants were previously described (106) and several Ets site mutants were newly prepared for this study. The only mutation which reduced cAMP, TRH and EGF responsiveness to less than 50% of wild type was mutation of the Ets site located at position –211, a site which previously has been shown to be important for MAPK responsiveness (46, 106). Disruption of other Ets sites either only modestly reduced responsiveness, or in some cases stimulated reporter gene activation in response to cAMP, TRH or EGF.

As there are multiple Ets sites in the proximal region of the PRL gene, it seemed possible that the modest effects of mutating single Ets sites reflected some redundancy in their ability to confer MAPK responsiveness. To assess the overall contribution of Ets sites within the proximal region of the PRL gene, a reporter construct was created in which all the consensus Ets sites were mutated. This construct was tested for its ability to be activated by the activators forskolin, EGF and TRH (Fig. 11C). Surprisingly, the construct in which all of the Ets sites were mutated was more responsive to forskolin treatment than the wild type PRL promoter. In contrast, and as expected, reporter gene responses to TRH and EGF were reduced in the Ets mutant. Thus although cAMP, TRH and EGF all lead to activation of MAPK, the requirements for downstream transcription factor targets of these signaling pathways appear to differ.

Elevation of cAMP enhances p300 transcriptional activity in a MAPK-dependent manner

As the preceding studies suggest that Ets factors are probably not required for transcriptional responses to cAMP, we considered a possible role for other factors. Recent studies have provided evidence that the ability of cAMP to stimulate PRL promoter activity in heterologous cells probably involves the closely related co-activator proteins, CBP and p300 (122, 123). To test the role of endogenous CBP and/or p300 in mediating responsiveness of the PRL promoter to cAMP, we used an expression vector for the adenovirus 12S E1a protein, which blocks CBP and p300 activity (124, 125). We found that increasing amounts of the E1a expression vector substantially reduced the ability of forskolin to stimulate PRL promoter activity in GH_3 cells (Fig. 12A). To test for possible non-specific effects of E1a, we compared the wild-type E1a to an E1a mutant (Δ2-35) which is defective for interaction with CBP/p300 (126). The mutant E1a had much smaller effects on cAMP- and EGF-stimulated prolactin gene expression (Fig. 12B). These findings offer support for the view that CBP/p300 plays a role in transcriptional regulation of the PRL gene. To further explore this topic, we elected to test the ability of cAMP to stimulate the transcriptional activity of p300. We prepared an expression vector for a GAL4-p300 fusion which was co-transfected with GAL4-dependent reporter gene. The results demonstrate that GAL4-p300 activity is substantially increased by treatment with forskolin (Fig. 12C). Interestingly, treatment with the MEK1 inhibitor, PD98059, almost completely blocked the ability of forskolin to enhance GAL4-p300 activity. These findings offer evidence that in GH_3 cells, cAMP stimulates p300 activity in a MAPK-dependent manner.

Discussion

These studies provide evidence that cAMP can activate the MAPK signaling pathway in GH_3 cells and that MAPK activation contributes to the ability of cAMP to stimulate PRL promoter activity. Analysis of ERK phosphorylation as well as immunocomplex assay of ERK activity have provided substantial evidence that elevated cAMP can stimulate MAPK activity. Inhibition of the MAPK pathway was found to reduce the ability of cAMP to activate the PRL promoter and a

GAL4-Elk1 fusion protein. These findings as well as previous studies (47, 106, 115, 127) suggest that the cAMP, EGF and TRH signaling pathways all converge in GH_3 cells to stimulate MAPK activity which then plays a role in stimulating PRL gene expression.

The signaling pathway that mediates cAMP effects on MAPK in GH_3 cells may involve the small GTP-binding protein, Rap1, although other pathways may be involved. Rap1 has been shown to play an important role in mediating cAMP-induced MAPK activation in PC12 cells (117). Rap1 can respond to cAMP through two different mechanisms. One mechanism involves phosphorylation of Rap1 by PKA leading to changes in activity (128). The other mechanism involves direct binding of cAMP to the Rap1 GDP exchange protein, Epac (129). We found that elevation of cAMP in GH_3 cells leads to rapid activation of Rap1. An expression vector for Rap1GAP1, which should inactivate Rap1, reduced cAMP-induced MAPK activation and PRL promoter activity. On the other hand, Rap1-N17, which should also block the Rap1 pathway (117), did not reduce cAMP effects on MAPK or the PRL promoter. It is possible that Rap1-N17 is simply not as effective in blocking signaling through the endogenous Rap1 pathway as Rap1GAP1. However, we cannot rule out the possibility that Rap1GAP1 may have effects on signaling pathways other than Rap1, perhaps blocking the activity of a related, small GTPase. In some cells, cAMP can modulate MAPK activity through activation of Ras (130). However, cAMP has little if any ability to activate Ras in GH_3 cells and a dominant negative form of Ras did not block cAMP induction of MAPK in GH_3 cells (P. Kievit, unpublished observations). Another mechanism which might mediate cAMP effects on MAPK would involve modulation of a protein phosphatase. It has been shown that PKA can phosphorylate the protein tyrosine phosphatase, PTP-SL, leading to decreased interaction between the phosphatase and ERK1 and ERK2 thus allowing for an increase in ERK phosphorylation and activation (131). We have not tested a possible role for PTP-SL in mediating PKA effects in GH_3 cells.

Concerning the events downstream of MAPK, it seemed likely that members of the Ets family of transcription factors would be involved in mediating transcriptional regulation of the PRL gene. Previous studies have led to a model in which Ras, TRH or EGF can stimulate MAPK activity leading to Ets factor phosphorylation and transcriptional activation involving several specific DNA elements in the PRL gene (46, 47, 51, 106, 132). As the present studies demonstrate that cAMP can stimulate MAPK activity, it seemed probable that Ets sites would also play a role in mediating transcriptional responses to cAMP. To test this possibility, Ets binding sites within the proximal region of the PRL promoter were mutated, including several Ets sites which have not previously been studied. Similar to previous studies examining Ras- or TRH-responsiveness (106, 132), mutation of the Ets site at position –211 decreased cAMP-responsiveness to less than 50% of wild type activity. Interestingly, this region of the PRL gene has been shown to interact with the LIM homeodomain transcription factor, Lhx3 (133), and Lhx3 has been shown to enhance Ras-responsiveness of the PRL promoter (134). Mutation of other single Ets sites either had smaller effects, or actually stimulated cAMP-responsiveness. The increases in responsiveness which were observed at some sites may be due to disruption of the binding of a repressor. It has been reported that some Ets factors can function as repressors and one has been shown to inhibit PRL promoter activity (135, 136). To further explore the overall role of Ets sites, a PRL promoter reporter gene construct in which all of the consensus Ets sites were disrupted was prepared. Surprisingly, the promoter construct with all Ets sites disrupted was even more responsive to cAMP than the wild type reporter gene. In contrast, disruption of all of the consensus Ets sites substantially reduced EGF-responsiveness and partially reduced TRH-responsiveness. Thus, it appears that there is a differential requirement for Ets sites in mediating regulation of the PRL promoter. The consensus Ets sites are not required for cAMP-responsiveness, but are necessary for full responses to EGF or TRH. This observation is somewhat surprising in view of the fact that all of these signaling pathways appear to converge to activate MAPK. One explanation may involve differing kinetics of MAPK activation. We found that cAMP- and EGF-induced activation of ERKs have somewhat different time courses. The effects of cAMP were observed to have a slower onset and appeared to persist somewhat longer than EGF effects on MAPK activation. Studies in other systems have provided evidence that the kinetics of MAPK induction may have a profound effect on the induced response. For instance, in PC12 cells, transient MAPK activation results in proliferation while sustained MAPK activation is associated with differentiation (137). Alternatively, the differential requirement for Ets sites may result from differences in the combined action of several signaling pathways.

The ability of cAMP to stimulate both PKA and MAPK may lead to activation of different transcription factors than occurs after activation of MAPK alone or MAPK combined with other signaling pathways.

The ability of the PRL promoter to respond to cAMP appears to involve the transcriptional co-activators, CBP and p300. Previous studies have used antibody blocking experiments to provide evidence that CBP and p300 play a role in mediating the ability of PRL promoter to respond to cAMP (122, 123). We have used an expression vector for adenovirus E1a as a CBP/p300 antagonist and confirmed that these co-activators appear to be required for full responsiveness to cAMP. As CBP and p300 can bind to either Pit-1 or Ets-1 in a constitutive manner (122, 123, 138, 139), both transcription factors may constitutively recruit CBP/p300 to the PRL promoter. Our studies with a GAL4-p300 fusion provide evidence that the transcriptional activity of p300 can be stimulated by elevated cAMP in a MAPK-dependent manner. Thus, it is possible that CBP/p300 is constitutively present at the PRL promoter and that the transcriptional activity of CBP/p300 is modulated by several signaling pathways including cAMP which converge on the MAPK pathway. Alternatively, Xu et al. (122) have suggested that activation of growth factor or cAMP pathways leads to recruitment of CBP/p300 to the PRL promoter. Additional studies assessing the recruitment of CBP/p300 to the PRL promoter in GH_3 cells are required to distinguish between these two models. The recent development of chromatin immunoprecipitation assays should provide an appropriate technology to address this question.

Figure 6. Time course of cAMP-induced MAPK activation

Immunocomplex kinase assays of MAPK activation (A, B, and C). GH_3 cells were treated with 0.5 mM CPT-cAMP for various periods of time. ERK1 (A), ERK2 (B), or JNK1 (C) were immunoprecipitated from 500 μg of cell lysates using antibodies conjugated to protein A/G agarose. MAPK activity was assessed by incubating the immunoprecipitate with $[^{32}P]$-γ-ATP and a GST-Elk1 fusion protein as substrate. The phosphorylated proteins were resolved on a denaturing polyacrylamide gel and detected by autoradiography. EGF (10 nM) and anisomycin (10 ng/ml) were used as a positive control for activity of ERK and JNK respectively. Immunoblot analysis of ERK activation (D, E, F and G). GH_3 cells were cultured in serum-free medium for 24 h and treated with 10 μM forskolin or 10 nM EGF for the indicated times. Cell lysates (50 μg) were resolved on a denaturing polyacrylamide gel, transferred to a membrane, and phosphorylated ERK1 and ERK2 were detected with a phosphorylation-specific antibody (D). To determine the total amount of ERK present, the membrane was stripped and reprobed with an antibody directed against ERK-1 protein (E). This antibody also detects ERK-2, although to a lesser extent. In a separate experiment GH_3 cells were treated with 10 μM forskolin or 10 nM EGF for varying times and ERK activation was detected by a phosphorylation-specific antibody and then quantitated by Lumi-Imager analysis (F and G).

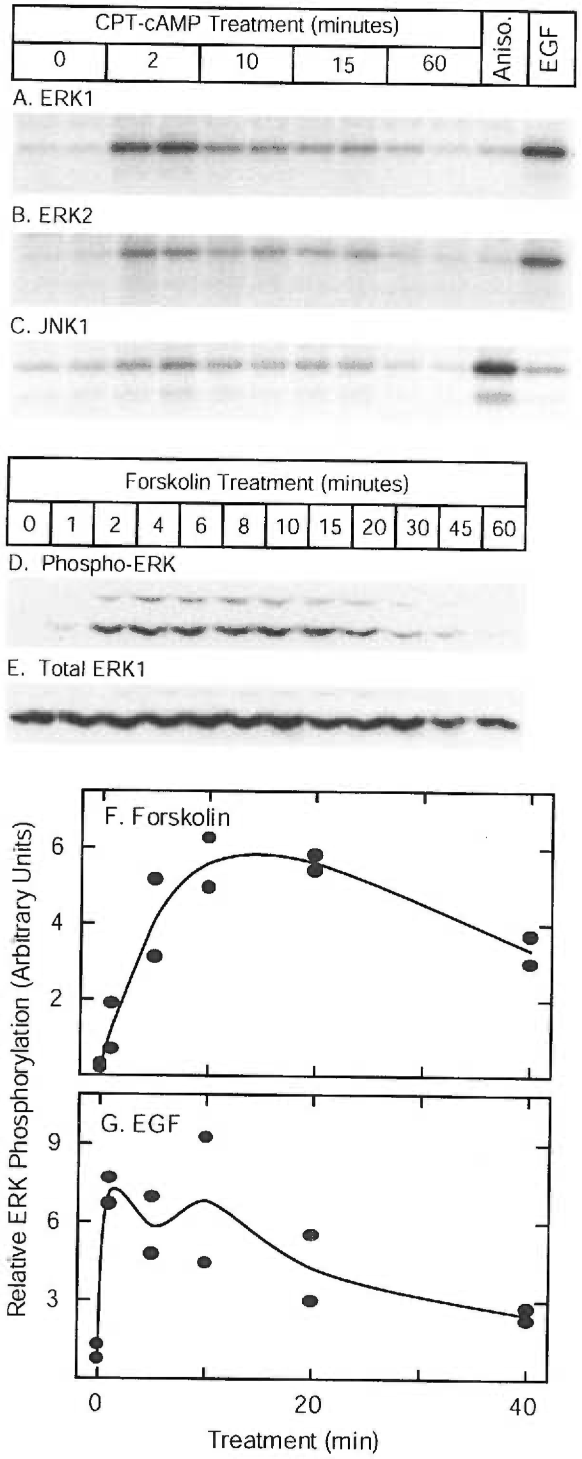

CPT-cAMP Treatment (minutes)
0
2
10
15
60
Aniso.
EGF
A. ERK1
B. ERK2
C. JNK1
Forskolin Treatment (minutes)
0
1
2
4
6
8
10
15
20
30
45
60
D. Phospho-ERK
E. Total ERK1
F. Forskolin
G. EGF
Relative ERK Phosphorylation (Arbitrary Units)
2
4
6
3
6
9
0
20
40
Treatment (min)

Figure 7. The cAMP-dependent protein kinase is required for full cAMP-induced ERK1 and ERK2 activation

Effects of inhibition of PKA by PKI on GAL4-Elk1 activity (A). GH_3 cells were transfected with a GAL4-dependent luciferase reporter gene and an expression vector encoding a GAL4-Elk1 fusion protein. The cells were also transfected with either an empty vector (control), or a vector encoding PKI. The cells were treated with EGF (10 nM) or forskolin (10 μM) for 6 h and collected for analysis of luciferase activity. Reporter gene activity is reported as luciferase activity from three independent transfections ± S.E.M. normalized to β-galactosidase activity. Effects of inhibition of PKA or tyrosine kinases on MAPK activation (B and C). GH_3 cells were treated with 20 μM of the PKA inhibitor H-89 or 40 μM of the tyrosine kinase inhibitor, genistein for 30 min. Cells were stimulated for 5 min with forskolin or EGF and harvested. Cell lysates (50 μg) were separated on a denaturing polyacrylamide gel and transferred to a membrane. Activated ERK was detected with the antibody directed against the phosphorylated form of ERK1 and ERK2 (B). To test for equal loading, the membrane was stripped and probed for total amount of immunoreactive ERK1 and ERK2 (C).

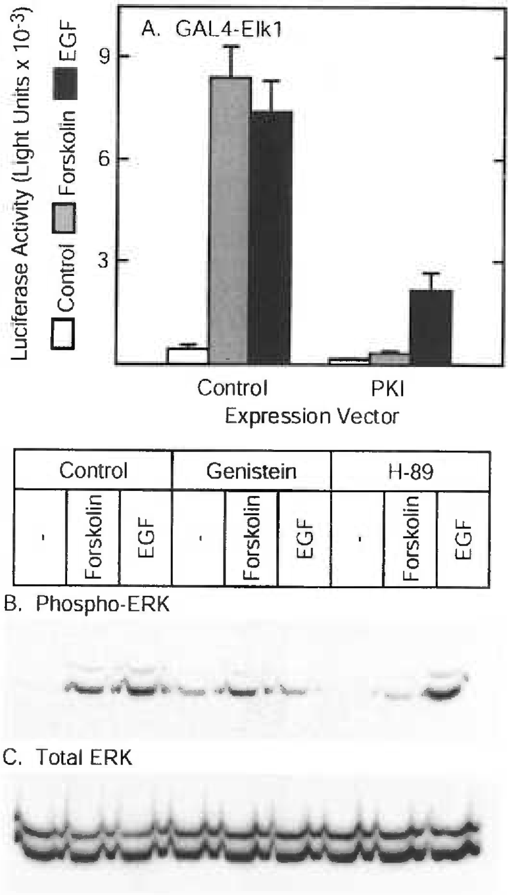
Luciferase Activity (Light Units x 10-3)
Control
Forskolin
EGF
A. GAL4-Elk1
9
6
3
Control
PKI
Expression Vector
Control
Genistein
H-89
-
Forskolin
EGF
-
Forskolin
EGF
-
Forskolin
EGF
B. Phospho-ERK
C. Total ERK

Figure 8. cAMP activates Rap1 in GH_3 cells

GH_3 cells were cultured in serum free medium for 24 h prior to treatment with 10 μM forskolin. Cells were lysed and activated Rap1 was isolated with a GST-RalGDS fusion protein bound to beads. The Rap1-RalGDS complex was washed and immobilized proteins resolved on a denaturing polyacrylamide gel. The proteins were transferred to a membrane and bound Rap1 was visualized with a polyclonal antibody (A). To verify that equal amounts of Rap1 were present in the lysates, 2% of the input was separated on a denaturing polyacrylamide gel and transferred to a membrane (B). Rap1 was detected using the polyclonal antibody. For the ERK phosphorylation time course, 50 μg of the GH_3 lysate was resolved on a denaturing polyacrylamide gel and phosphorylated ERK1 and ERK2 was detected using a phosphorylation-specific antibody (C). Equal loading was determined by stripping the blot and re-probing with an ERK1 antibody (D).

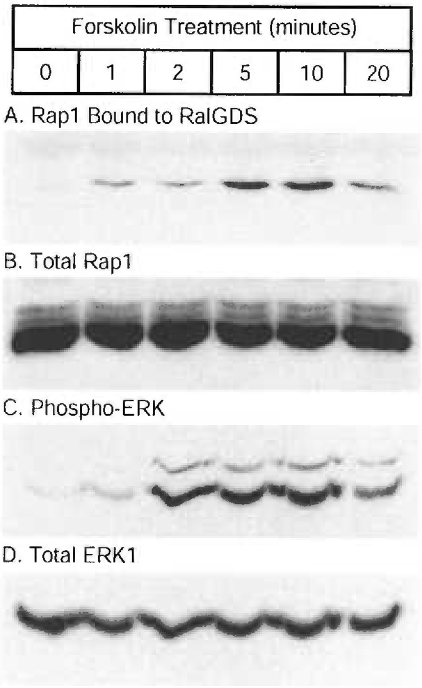

Forskolin Treatment (minutes)
0
1
2
5
10
20
A. Rap1 Bound to RalGDS
B. Total Rap1
C. Phospho-ERK
D. Total ERK1

Figure 9. Rap1 is required for full effects of cAMP to activate MAPK and gene expression

Analysis of the effects of Rap1GAP1 on ERK activation in GH_3 cells (A, B and C). GH_3 cells were transfected with FLAG-tagged ERK2 (4 μg) and either FLAG tagged Rap1GAP1 (6 μg) or empty vector (6 μg) as indicated. After serum-deprivation, 10 μM forskolin was added for the indicated times. FLAG-tagged proteins were precipitated from the cell lysates (1 mg) and phospho-ERK2 was detected with a phosphorylation-specific polyclonal antibody (A). Expression levels of Rap1GAP1 (B) and ERK2 (C) were verified by immunoblot analysis of cell lysates (100 μg) using the FLAG monoclonal antibody. Analysis of the effects of Rap1GAP1 expression on PRL reporter gene activity (D). GH_3 cells were transfected with increasing amounts of the FLAG-Rap1GAP1 construct and a reporter construct containing the –255 to +34 region of the PRL promoter (D). The total amount of transfected DNA was normalized with empty expression vector (pcDNA3). At 24 h after transfection, the cells were treated with 10 μM forskolin for 6 h and collected for analysis of luciferase activity. Reporter gene activity is reported as luciferase activity from three independent transfections ± S.E.M. normalized to β-galactosidase activity.

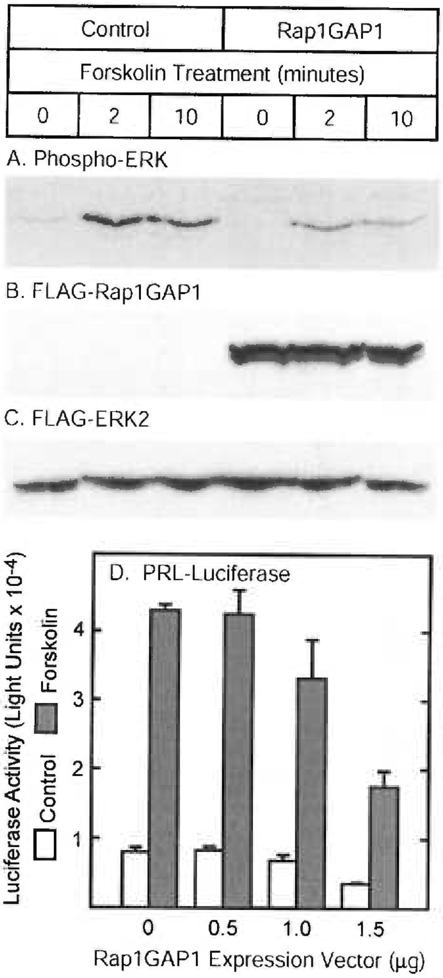
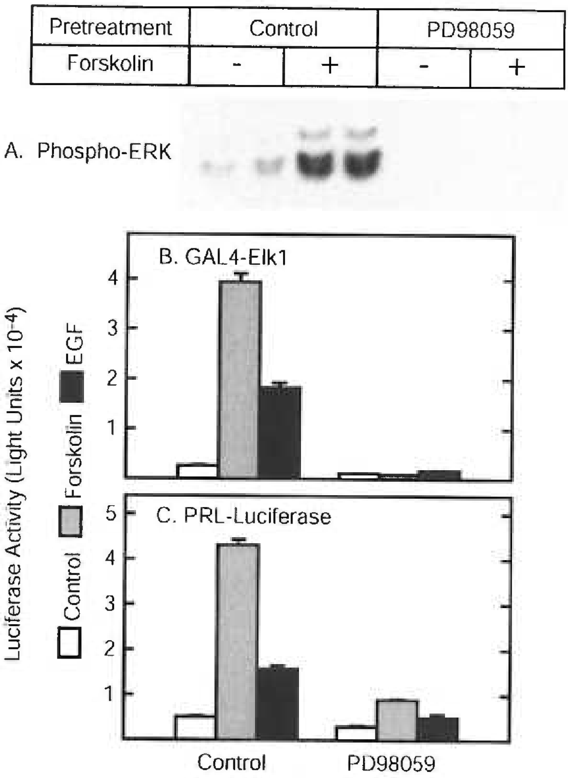

Control
Rap1GAP1
Forskolin Treatment (minutes)
0
2
10
0
2
10
A. Phospho-ERK
B. FLAG-Rap1GAP1
C. FLAG-ERK2
D. PRL-Luciferase
Luciferase Activity (Light Units x 10^{-4})
☐ Control
▨ Forskolin
1
2
3
4
0
0.5
1.0
1.5
Rap1GAP1 Expression Vector (μg)

Figure 10. MAPK Activation is required for full effects of cAMP on PRL gene expression

Analysis of the effects of the MAPK inhibitor, PD98059, on forskolin-induced ERK activity in GH_3 cells (A). GH_3 cells were cultured in serum free medium for 24 h, pretreated for 30 min with the vehicle, DMSO (Control), or 100 μM PD98059 followed by 10 μM forskolin treatment for 5 min. Cell lysates were resolved on a denaturing polyacrylamide gel, transferred to a membrane, and probed with an antibody specific to the phosphorylated form of ERK (A). Analysis of the effects of the MAPK inhibitor, PD98059 on forskolin-induced reporter gene activity (B and C). GH_3 cells were transfected with either a GAL4-dependent luciferase reporter gene and an expression vector encoding a GAL4-Elk1 fusion protein (B) or a PRL promoter containing the proximal 255 base pairs fused to the luciferase reporter gene (C). At 24 h after transfection, GH_3 cells were pretreated for 30 min with either the vehicle, DMSO (Control), or 100 μM PD98059. The cells were then treated with either 10 μM forskolin or 10 nM EGF for 6 h after which time the cells were lysed and analyzed for luciferase activity. Reporter gene activity is reported as light units from three independent transfections ± S.E.M. normalized to β-galactosidase activity.



Pretreatment
Control
PD98059
Forskolin
-
+
-
+
A. Phospho-ERK
B. GAL4-Elk1
C. PRL-Luciferase
Luciferase Activity (Light Units x 10^{-4})
Control
Forskolin
EGF
1
2
3
4
5
Control
PD98059

Figure 11. Mutational analysis of putative Ets factor binding sites in the proximal PRL promoter

Specific Ets factor binding sites within the proximal region of the PRL gene were disrupted as indicated by the schematic diagram for the PRL promoter (A) where Ets sites are indicated by black-filled rectangles and Pit-1 binding sites are indicated by gray-filled rectangles. GH_3 cells were transfected with PRL promoter reporter constructs in which individual Ets factor binding sites were disrupted (B) or a reporter gene in which all of the consensus Ets sites were disrupted (C). After serum deprivation, the cells were treated with 10 μM forskolin, 100 nM TRH or 10 nM EGF for 6 h. Cells were collected and assayed for luciferase activity. The basal levels for the various promoters were as follows (in arbitrary light units): wild type 446±93.1; mut1 617±66.1; mut2 2096±253; mut3 444±36.7; mut4 798±78.9; mut5 293±20.6. The fold induction of the wild type promoter by forskolin, TRH and EGF was 13, 3.2 and 8.7 respectively. Reporter gene activity is reported as the light units from three independent transfections ± S.E.M. normalized to β-galactosidase activity.

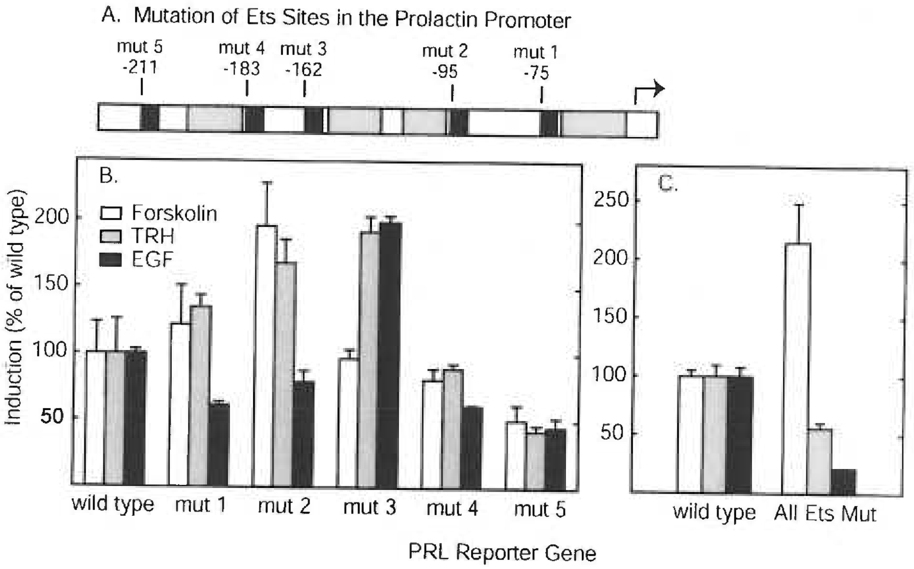
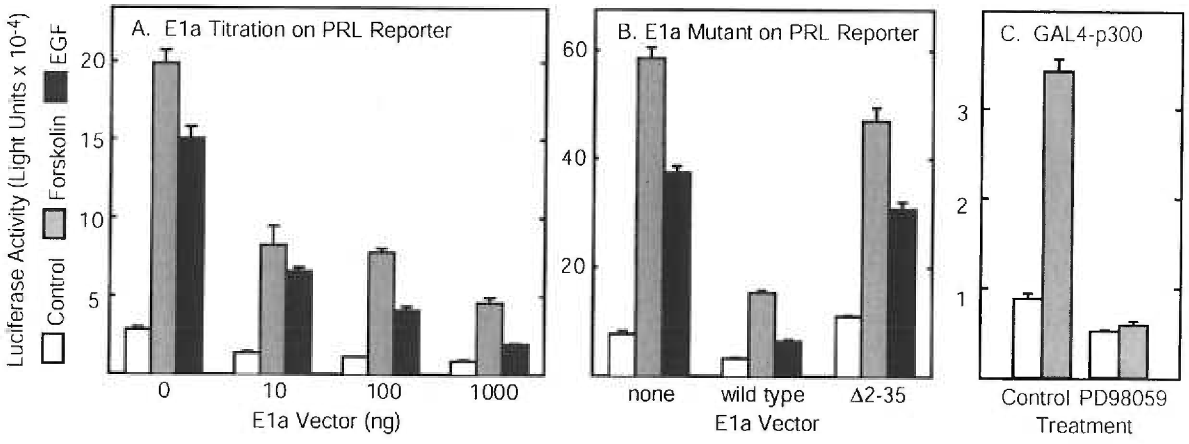

A. Mutation of Ets Sites in the Prolactin Promoter
mut 5 -211
mut 4 -183
mut 3 -162
mut 2 -95
mut 1 -75
B.
☐ Forskolin
▨ TRH
■ EGF
Induction (% of wild type)
200
150
100
50
wild type
mut 1
mut 2
mut 3
mut 4
mut 5
C.
250
200
150
100
50
wild type
All Ets Mut
PRL Reporter Gene

Figure 12. Analysis of a possible role for the co-activator CBP/p300 in cAMP-stimulated activation of the PRL promoter.

Analysis of the effects of increasing amounts of an adenovirus E1a expression vector on forskolin- and EGF-induced PRL reporter gene activity (A). GH_3 cells were transfected with a PRL reporter construct and either an empty vector (pcDNA3) or increasing amounts of E1a expression vector as indicated. The cells were treated with 10 μM forskolin or 10 nM EGF for 6 h and the cells then collected and assayed for luciferase activity. Comparison of wild type and mutant E1a on activation of the PRL promoter (B). GH_3 cells were transfected with a PRL reporter construct and 1 μg of an expression vector for either the wild type E1a or mutant E1a in which residues 2-35 have been deleted (Δ2-35). The cells were treated with 10 μM forskolin or 10 nM EGF for 6 h and the cells then collected and assayed for luciferase activity. Analysis of PD98059 effects on forskolin-induced activation of a GAL4-p300 fusion protein (C). GH_3 cells were transfected with an expression vector encoding a GAL4-p300 fusion protein and a reporter construct containing 5 GAL4 binding sites. The cells were incubated in serum-free medium for 24 h and then treated with 100 μM PD98059 for 30 min. Forskolin (10 μM) was added and cells were collected and assayed 6 h later. Reporter gene activity is reported as light units from three independent transfections ± S.E.M. normalized to β-galactosidase activity.


Luciferase Activity (Light Units x 10-4)
☐ Control
▨ Forskolin
■ EGF
A. E1a Titration on PRL Reporter
20
15
10
5
0
10
100
1000
E1a Vector (ng)
B. E1a Mutant on PRL Reporter
60
40
20
none
wild type
Δ2-35
E1a Vector
C. GAL4-p300
3
2
1
Control
PD98059
Treatment

CHAPTER III

The Pituitary-Specific Transcription Factor, Pit-1, Can Direct Chromatin Remodeling of the Prolactin Promoter

Introduction

An important aspect of transcriptional regulation in eukaryotes involves packaging the DNA of inactive genes into repressive polynucleosome arrays (140). The process of transcriptional activation for a particular gene then involves the actions of sequence-specific DNA binding factors leading to the recruitment of chromatin modifying enyzmes (141). These enzymes include factors that covalently modify histones and other chromatin proteins (142) and ATP-dependent chromatin remodeling complexes (86, 143). The actions of these enzymes lead to modifications in chromatin structure that contribute to transcriptional activation. Changes in chromatin structure may involve a loss or redistribution of nucleosomes or a positioning of nucleosomes in a way that structurally facilitates interactions of transcription factors.

There are still many unanswered questions concerning the role of individual components in modulating chromatin structure. One question concerns the ability of specific, DNA-binding transcription factors to direct remodeling of chromatin. Although eukaryotic promoters generally contain binding sites for multiple factors, there is evidence that a single factor can direct changes in chromatin structure. For instance, GAGA factor appears to play a crucial role in positioning nucleosomes on the Drosophila hsp26 promoter (144) and reconstitution experiments have shown that GAGA factor can direct chromatin remodeling in vitro (145). In vitro chromatin reconstitution studies have shown that the yeast Pho4 transcription factor can remodel the PHO5 promoter (146). Similarly it has been demonstrated that in vitro, the winged helix factor, HNF3, can direct positioning of a nucleosome on an enhancer from the albumin gene (147). An in vivo chromatin reconstitution system using Xenopus oocytes has been used to demonstrate that the thyroid hormone receptor can direct chromatin remodeling (148, 149).

In the present study we have examined the ability of the tissue-specific, POU transcription factor, Pit-1 (20, 23) to direct remodeling of the chromatin structure of the prolactin promoter. Prolactin is a simple polypeptide hormone that is synthesized and secreted by the anterior pituitary. The proximal promoter region and distal enhancer of the prolactin gene contain multiple Pit-1 binding sites and Pit-1 has been shown to be sufficient to activate the prolactin promoter in heterologous cells and in vitro (19). As cells that synthesize prolactin demonstrate changes in the chromatin structure of the prolactin gene (96, 97), it seems possible that Pit-1 contributes to the development of a specific chromatin structure. In addition, Pit-1 has been shown to interact with the co-activator, CREB binding protein (CBP) and the co-repressor N-CoR (122). As CBP has been shown to have intrinsic and associated histone acetyltransferase activity (75) (76) (150) and N-CoR associates with a histone deacetylase (151, 152), it is possible that recruitment of CBP or N-CoR by Pit-1 leads to localized changes in histone acetylation. Transfection experiments have provided evidence that Pit-1 can enhance acetylation of histone H4 on the prolactin promoter (153). Thus, it is possible that Pit-1 can modulate the status of histone acetylation on the prolactin promoter perhaps leading to changes in chromatin structure.

We have used Xenopus oocytes as an in vivo chromatin reconstitution system (154) to study the ability of Pit-1 to direct remodeling of chromatin structure. The results of these studies offer evidence that Pit-1 can lead to positioning of nucleosomes in both the distal enhancer and proximal promoter region of the prolactin gene.

Materials and Methods

DNA constructs

Luciferase reporter constructs containing approximately 0.6 or 1.9 kilobase pairs of the 5' flanking sequence and the promoter from the rat prolactin gene have been described previously (18, 155). Expression vectors encoding Pit-1 fused to the FLAG epitope were generated by subcloning the appropriate coding sequence into pCS2+ (156). Pit-1 mutants were obtained by either PCR-based site-directed mutagenesis of the FLAG-Pit-1 encoding vector (A158P) or were previously described (V153E) (157).

Preparation and microinjection of oocytes

The preparation and microinjection of *Xenopus laevis* oocytes was performed using a modification of methods previously described (154). Mature female

Xenopus laevis were anesthetized in 0.2% Tricaine (Sigma, St. Louis, MO) and the ovaries removed. Fragments of ovary were incubated with 0.2 Wünsch units/ml Liberase Blendzyme 3 (Roche Molecular Biochemicals, Indianapolis, IN) for 3 hours at 18°C in 5 mM Hepes pH7.9, 82.5 mM NaCl, 2.5 mM KCl, 1mM $MgCl_2$. The oocytes were then washed several times in ND-96 (5 mM Hepes pH 7.9, 2 mM KCl, 1.8 mM $CaCl_2$, 1mM $MgCl_2$, 50 μg/ml gentamycin). Stage VI oocytes were injected with 9.2 nl of an aqueous solution containing 100 ng/μl of supercoiled plasmid DNA using a Nanoject II (Drummond Scientific Company, Broomall, PA). After 24 to 48 h incubation at 18°C on a rotating platform, healthy oocytes were selected for analysis.

RNA preparation and primer extension analysis.

RNA was isolated from 20 oocytes using methods similar to those described previously (158). Oocytes were washed once in ND-96 and then homogenized by repeated pipetting in 0.1 ml 10 mM Tris pH 8.0, 1 mM EDTA. Then 0.5 ml of Trizol reagent (Invitrogen, Carlsbad, CA) and 0.1 ml chloroform were added and the sample vigorously mixed. After 5 minutes on ice the nuclei and membranes were pelleted by centrifugation for 15 minutes at 10,000 x g for 15 minutes. Then 0.35 ml of supernatant was removed and RNA was precipitated by adding 1 volume of isopropanol. The RNA was collected by centrifugation, the supernatant removed and the pellet rinsed with 1 ml of 70% ethanol. RNA pellets were resuspended in 40 μl of water. Primer extension analysis was performed with a primer specific for the luciferase RNA product (primer I: 5'-

GCAGTTGCTCTCCAGCGGTTCCATCCTC-3') and a primer recognizing the endogenous histone H4 mRNA (primer II: 5'-GGCTTGGTGATGCCCTGGATGTTATCC-3'), which acted as a recovery/loading control. RNA (equivalent of 2 oocytes) was incubated in a final volume of 10 μl with 0.2 pmol of primer I and 0.04 pmol of primer II for 10 minutes at 65°C, 30 minutes at 55°C and 10 minutes at 42°C in 1X First Strand Buffer (50 mM Tris pH8.3, 75 mM KCl, 3 mM Mg_2Cl_2; Invitrogen, Carlsbad, CA). Primer extension was performed in a reaction volume of 40 μl in the same buffer with the addition of 0.25 mM dNTPs, 1 mM DTT and 100 units of Molony leukemia virus RNase H^- reverse transcriptase (Superscript II, Invitrogen, Carlsbad, CA). After a 60 min incubation at 42°C, the reaction was stopped by adding 200 μl of 1% SDS, 50 mM Tris pH 8.0, 10 mM EDTA and 0.5 mg/ml Proteinase K (Roche Molecular Biochemicals, Indianapolis, IN). The samples were incubated at 55°C for 1 hour, followed by addition of an equal volume of phenol: chloroform: iso-amyl alcohol (25:24:1) and vigorous mixing. After centrifugation, the upper aqueous phase was removed and nucleic acids precipitated by addition of two volumes of ethanol. Extension products were collected by centrifugation and analyzed on a 7% polyacrylamide gel containing 8 M urea containing gel. Products were visualized by autoradiography.

Protein expression assay

Oocytes were injected with expression vectors encoding FLAG-Pit-1 and incubated for the indicated times in the presence of [^{35}S]methionine (Dupont

NEN, Boston, MA). The oocytes (n=10) were washed three times in ND96 and homogenized by repeated pipetting in 0.3 ml of 50 mM Tris-HCl, pH 8.0, 10% Glycerol, 1% Nonidet-P40, 200 mM NaCl, 2.5 mM $MgCl_2$. Homogenates were combined with an equal volume of Freon (1,1,2-trichloro-trifluoroethane; Sigma, St. Louis, MO), vortexed for 1 minute and microfuged for 5 min at 4°C. The supernatant was transferred to a new tube and incubated with a resin containing an immobilized anti-FLAG antibody (Sigma, St. Louis, MO) for 90 minutes at 4°C. The resin was washed with 50 mM Tris-HCl, pH 8.0, 10% Glycerol, 1% Nonidet-P40, 200 mM NaCl, 2.5 mM $MgCl_2$ buffer three times. Radiolabeled proteins were separated on a 12%, denaturing polyacrylamide gel and visualized by autoradiography.

Micrococcal Nuclease Assay

For analysis of chromatin structure, oocyte homogenates were treated with micrococcal nuclease after which DNA was isolated. For these assays, 30 oocytes for each group were washed twice with ND96 and homogenized in 210 μl of nuclease buffer (10 mM Hepes pH 8.0, 50 mM KCl, 5 mM MgCl2, 3 mM CaCl2, 1 mM DTT, 0.1% NP-40, 8% glycerol). Then 60 μl aliquots of the homogenate were incubated with 0, 0.83, 2.5 or 7.5 units/ml of micrococcal nuclease (Sigma, St. Louis, MO) for 20 min at room temperature. The reaction was stopped by adding 200 μl of 50 mM Tris pH8.0, 20 mM EDTA, 1% SDS. Samples were adjusted to contain 100 μg/ml RNase A and incubated for 1 hour at 37°C. Proteinase K (Roche Molecular Biochemicals, Indianapolis, IN) was then added to 0.3 mg/ml and the samples incubated overnight at 55° C. An equal volume of phenol: chloroform: iso-amyl alcohol (25:24:1) was added and mixed vigorously and the phases separated by centrifugation. DNA was precipitated from the supernatant by addition of two volumes of ethanol. After collection by centrifugation, the DNA was dissolved in 20 μl H_20 and the equivalent of DNA from 15 oocytes was used for indirect end-labeling analysis. For analysis of the proximal region of the prolactin gene, DNA was digested to completion with restriction endonucleases KpnI and EcoRI or AflIII and separated on a 1.5% agarose gel. The DNA was transferred to a nylon filter by blotting, and fragments containing prolactin gene sequences were visualized by hybridization with a radiolabeled DNA probe corresponding to the 3' end of the gene fragment.

Results

Regulation of Prolactin Gene Expression by Pit-1 in Xenopus Oocytes

We have used *Xenopus laevis* oocytes as an in vivo chromatin reconstitution system to examine a role for Pit-1 in modulating the chromatin structure of the prolactin gene. As oocytes can assemble exogenous DNA into a chromatin template with physiologically spaced nucleosomes, this system provides a very useful in vivo model for studying chromatin structure and transcription (154). We microinjected oocytes with a reporter gene containing approximately 1,900 base pairs of the 5' flanking region and promoter of the rat prolactin gene linked to luciferase (1.9PRL-Luc). We have used the oocyte system to examine a possible role for the pituitary-specific transcription factor Pit-1 in remodeling the chromatin structure of the prolactin gene. Previous studies have demonstrated the presence of multiple Pit-1 binding sites (19) in both the proximal region (designated 1P, 2P, 3P and 4P) and distal enhancer (designated 1D, 2D, 3D and 4D) of the prolactin gene (Fig. 13A). In an initial experiment we used digestion with micrococcal nuclease to assess assembly of the prolactin reporter gene into a polynucleosome array (Fig. 13B). The prolactin reporter gene was microinjected into oocytes and following incubation for various time intervals, the oocytes were homogenized and incubated with increasing amounts of micrococcal nuclease. When microinjected oocytes were homogenized immediately with no time to assemble chromatin, micrococcal nuclease yielded only a smear (compare lane 1 & 2). At 4 and 18 hours after microinjection, a regularly spaced ladder of micrococcal nuclease digestion products could be detected suggesting the assembly of a polynucleosome array. Even as early as an hour after microinjection, it appeared that mono- and di-nucleosomes had assembled on the injected DNA.

We next assessed whether Pit-1 could activate transcription from a chromatinized prolactin reporter gene in Xenopus oocytes. Oocytes were injected with prolactin reporter constructs containing both the proximal promoter and distal enhancer regions (1.9PRL-Luc) or a shorter construct containing only the proximal region and promoter (0.6PRL-Luc). Some of the oocytes were also injected with an expression vector for Pit-1. The Pit-1 expression vector modestly activated transcription in Xenopus oocytes with both prolactin gene constructs (Fig. 14A). These findings provide evidence that Pit-1 is probably capable of stimulating transcription from a chromatin-assembled template. However, we can not rule out the possibility that some of transcripts obtained with the microinjected DNA are produced from a fraction of DNA that is not assembled into chromatin. In this experiment we also tested the ability of the deacetylase inhibitor, trichostatin A (TSA), to enhance transcription from the injected DNA. Previous studies have provided evidence that in oocytes, transcription from injected DNA templates can be inhibited by recruitment of deacetylase complexes (159). Interestingly, the ability of Pit-1 to stimulate transcription was considerably enhanced by TSA, particularly for the 1.9-PRL-Luc construct. As Pit-1 in this experiment was produced following microinjection of a plasmid expression vector, it was possible that the TSA altered the synthesis of Pit-1. To test this possibility, oocytes were injected with an expression vector encoding FLAG-Pit-1, incubated overnight in the presence of [^{35}S]methionine and radiolabled Pit-1 isolated by immunoprecipitation (Fig. 14B). The TSA treatment did not appear to substantially alter the synthesis of Pit-1. Thus, the stimulatory effect of TSA on transcription is consistent with the possible recruitment of a deacetylase repressor complex to the prolactin gene in oocytes. Inhibition of a recruited deacetylase by TSA would then stimulate transcription from the injected template. The stimulatory effect of TSA also offers some support for the view that transcripts are produced from chromatin-assembled templates.

Pit-1 expression alters micrococcal nuclease digestion of prolactin 5' flanking DNA sequences when assembled into chromatin.

To examine the chromatin structure in the proximal region of the prolactin promoter, we digested homogenates of microinjected oocytes with micrococcal nuclease. To map sites within the prolactin reporter gene that were cleaved by micrococcal nuclease, isolated DNA was digested to completion with KpnI, the DNA fragments resolved by gel electrophoresis, transferred to a membrane and then specific fragments identified by hybridization to probe representing the 3' end of the region of interest (Fig. 15A). In the absence of Pit-1, multiple micrococcal nuclease sensitive sites were observed spanning most of the proximal flanking 5' flanking region. At approximately –380 relative to the transcription start site, there appeared to be a site particularly sensitive to micrococcal nuclease. These relatively distinct cleavage products could represent either a modest preference for nucleosome positioning on the DNA or some sequence specificity for micrococcal nuclease digestion. To examine this issue further, this experiment was repeated using oocytes that were homogenized immediately after microinjection so that the injected DNA is not assembled into chromatin (Fig. 15B). DNA that was not assembled into chromatin demonstrated a similar pattern of micrococcal nuclease digestion as the control, chromatinized DNA. It seems likely that in the absence of Pit-1, the pattern of selective micrococcal nuclease digestion reflects an intrinsic sequence specificity of the nuclease and there for is not evidence for an organized nucleosomal structure in the proximal region of the prolactin gene.

Expression of Pit-1 results in several distinct changes in the micrococcal nuclease digestion products obtained with a chromatinized template (Fig 15A, lanes 6-8). Enhanced cleavage was detected at positions +40, –80, -280, and –850. Cleavage was reduced at several sites, including a site at position –140. Interestingly, the enhanced cuts at –80 and -280 and the reduced cleavage at –140 suggest the possible presence of a translationally positioned nucleosome interacting with a DNA region containing several Pit-1 binding sites (Fig. 15C). In the presence of Pit-1, there are other regions which also appear to be protected from micrococcal nuclease (for instance the –400 to approximately –700 region). However, for these regions, at least one of the boundaries is not clearly defined by a strong micrococcal cleavage site. Therefore, it is not clear that the protection in these regions is due to a unique, translationally positioned nucleosome. The enhanced cleavage site at –850, occurs in region that has not been shown to play a role in modulating prolactin promoter activity (22). Thus, any possible functional significance of this change is not clear.

It seemed possible that Pit-1 stimulated changes in micrococcal nuclease observed after chromatin assembly might be a direct consequence of Pit-1 expression and binding and not reflect changes in chromatin structure. Therefore, we tested the effects of Pit-1 expression on micrococcal nuclease cleavage of DNA that was not assembled into chromatin (Fig. 15B). For this experiment, oocytes were injected with the Pit-1 expression vector and then incubated as in Fig. 14A and 15A, conditions that produced Pit-1 in a manner sufficient to activate transcription and remodel chromatin. The oocytes were then disrupted and the naked PRL reporter construct was added and incubated for 30 minutes prior to micrococcal nuclease digestion. Under these conditions, in the absence of chromatin assembly, Pit-1 expression had little or no detectable effect on micrococcal nuclease cleavage. Therefore it seems likely that the ability of Pit-1 to stimulate changes in micrococcal nuclease cleavage of chromatin assembled DNA likely reflects a remodeling of chromatin structure including translational positioning of a nucleosome in the proximal region of the prolactin gene.

In the preceding experiments we noticed that Pit-1 expression appeared to stimulate micrococcal nuclease digestion at upstream site relatively near the KpnI restriction site used for analysis. It seemed possible that this micrococcal nuclease might be in the distal enhancer of the prolactin gene. To explore this possibility further, we used different restriction enzymes to prepare the samples for indirect end labeling analysis. We found that Pit-1clearly altered micrococcal nuclease digestion of sites in the distal enhancer with enhanced cleavage at positions –1410, -1550 and –1720 (Fig. 16A). Pit-1 also appeared to reduce cleavage at approximately positions –1480 and –1620. As with the proximal region of the prolactin gene, Pit-1 expression did not alter micrococcal nuclease cleavage of DNA that was not assembled into chromatin (Fig. 16B). Based on these observations it seems likely that Pit-1 expression leads to appearance of a translationally positioned nucleosome in the –1550 to –1720 region that contains several Pit-1 binding sites (Fig. 16C). We have also tentatively indicated a possible positioned nucleosome at approximate positions –1410 to –1550. While this region appears to be too small to accommodate a nucleosome, there are limitations to accurately estimating the site of cleavage. Thus, it is possible that this region is somewhat larger than indicated and perhaps may accommodate a nucleosome. Perhaps the –1410 cleavage represents the upstream limit of downstream nucleosome. Alternatively, the enhanced cleavage of the –1410 site may reflect altered tortional forces perhaps due to changes in supercoiling of the template.

The time course of changes in chromatin structure correspond to the time course of Pit-1 expression

To further examine the ability of Pit-1 to alter chromatin structure, we examined the time course of Pit-1 stimulated changes in chromatin structure. For this experiment, the prolactin reporter gene was injected into oocytes followed by a 20 hour incubation to permit assembly of chromatin on the injected template (Fig. 17A). Then an expression vector for Pit-1 was injected and the oocytes incubate for varying times, prior to analysis. Some of the oocytes were incubated in [^{35}S]methionine to permit analysis of the time course of Pit-1 accumulation (Fig. 17B). After homogenization, other oocytes were incubated with micrococcal nuclease for indirect end labeling analysis of the chromatin structure of the proximal region of the prolactin gene (Fig. 17C). The time course of Pit-1 accumulation very closely corresponded to the time course of changes in chromatin structure as assessed by enhanced cleavage at positions –280 and –80 and decreased cleavage of the –140 site. As for the studies shown in Fig. 15, it seems likely that the changes in micrococcal nuclease cleavage indicate that expression of Pit-1 results in the presence of translationally positioned nucleosome over this region of the prolactin promoter. The excellent correlation between Pit-1 expression and these chromatin changes is consistent with a role for Pit-1 in directing chromatin remodeling.

A Pit-1 mutant that cannot bind DNA does not alter chromatin structure

The DNA binding region of Pit-1 consists of two separate domains that can individually interact with DNA, the POU-specific domain and the homeo domain (32). Structural studies have confirmed that both domains of Pit-1 make specific base contacts and that Pit-1 homodimer involves an interaction of the POU specific with the POU homeodomain domain (36). To further examine the role of Pit-1 in directing chromatin remodeling, the ability of two different Pit-1 mutants to modulate chromatin structure was examined (Fig. 18). Replacing valine at residue 153 with a glutamate residue (V153E) has previously been shown to essentially abrogate DNA binding (157). Replacement of alanine 158 with proline (A158P) has been reported to not alter DNA binding but this mutant has greatly reduced ability to activate transcription as assessed in a transfection assay (160). Both Pit-1 mutants were expressed in Xenopus oocytes, although the V153E mutant accumulated to significantly lower levels than the wild type Pit-1 (Fig. 18C). As expected, the ability of both mutants to activate transcription was reduced, particularly in the presence of TSA (Fig. 18B). Interestingly, these mutations displayed some differences in their ability to remodel chromatin. The

A158P Pit-1 mutant appeared to be capable of stimulating chromatin remodeling as assessed by enhancement of micrococcal digestion at –280 and reduction of cleavage at –140. However, in several experiments it appeared to be somewhat less efficient than wild type Pit-1. The V153E mutant did not effectively induce changes in micrococcal nuclease digestion of the proximal prolactin promoter suggesting that DNA binding is essential for directing chromatin remodeling.

Discussion

These studies have used an in vivo chromatin reconstitution system in Xenopus oocytes to examine the ability of Pit-1 to stimulate transcription and to alter chromatin structure. Although it has been known for some time that Pit-1 plays a key role in stimulating prolactin gene expression (20, 23, 26), the possible involvement of Pit-1 in establishing the chromatin structure of the prolactin promoter has not been previously explored. The present findings provide evidence that Pit-1 may play at least two roles in modulating the chromatin structure of the prolactin gene. Pit-1 expression was associated with the presence of translationally positioned nucleosomes in both the proximal promoter region and distal enhancer of the prolactin gene. Positioning of nucleosomes was not observed with a Pit-1 mutant that is unable to bind DNA. The other role for Pit-1 in modifying chromatin structure involves the apparent recruitment of a deacetylase activity. The recruitment of a deacetylase was indicated by the ability of TSA to substantially enhance transcription from chromatin-assembled templates. It seems likely that both nucleosome positioning and deacetylase recruitment are important aspects of Pit-1 function in regulating prolactin gene expression.

The ability of Pit-1 to direct nucleosome positioning could play a role in transcriptional regulation of the prolactin gene. Previous studies have used micrococcal nuclease digestion and indirect end labeling experiments to provide evidence for cell-specific differences in chromatin structure of the prolactin gene (100). It was concluded that in GH_3 pituitary tumor cells that express the prolactin gene, nucleosomes are translationally positioned in the distal enhancer region of the prolactin gene. Interestingly, the Pit-1-dependent positioned nucleosomes that we have observed in the present study probably correspond to positioned nucleosomes detected in GH_3 cells. Therefore, the present findings suggest that Pit-1-dependent changes in nucleosome positioning likely contribute to the mechanisms that establish the cell-specific chromatin structure of the prolactin gene. The ability of Pit-1 to modulate chromatin structure may enhance recruitment of other transcription factors and facilitate functional synergism between factors. This would be similar to findings that the glucocorticoid receptor can alter chromatin structure leading to enhanced recruitment of other transcription factors (161) (162). Interestingly, the synergism observed with the glucocorticoid receptor does not occur on naked DNA or with mononucleosomes, suggesting that the chromatin template is required for maximal gene activation (163).

The ability of Pit-1 to stimulate nucleosome positioning probably involves the action of an ATP-dependent remodeling complex. At the present time there is no information concerning the possible direct interaction of Pit-1 with components of an ATP-dependent remodeling complex. So it remains possible that Pit-1 directly recruits a remodeling complex. There are studies that raise the possibility of an indirect recruitment. The CCAAT-enhancer binding protein (C/EBP) has been shown to stimulate activity of the prolactin promoter (164) and there is a functional interaction between C/EBP and Pit-1 to stimulate the growth hormone promoter (165). As C/EBP has been shown to interact with the SWI/SNF complex (166), it is possible that interactions between Pit-1 and C/EBP lead to recruitment of the SWI/SNF complex or another complex leading to chromatin remodeling. This model for the role of Pit-1 in modifying chromatin structure has some similarity to the role of GAGA factor in organizing chromatin on the hsp70 promoter. GAGA factor binds to $[GA]_n$ repeats in the hsp70 promoter (167) (145) resulting in the recruitment of a chromatin remodeling complex (88). The complex contains several subunits, including the ISWI ATP-dependent chromatin remodeling factor. ISWI appears to mediate GAGA-directed nucleosome sliding resulting in the formation of an inducible heat shock promoter (168). Additional studies will be required to examine the role of ATP-dependent remodeling complexes in mediating Pit-1 effects on chromatin structure and transcriptional activation.

As TSA treatment substantially enhanced Pit-1-dependent transcriptional activation, it seems likely that in oocytes Pit-1 recruits an inhibitory, deacetylase activity. Previous studies have demonstrated that Pit-1 can bind to the co-repressor N-CoR (122), leading to the recruitment of a repressor complex containing Sin3 (169). As N-CoR and Sin3 are present and functional in oocytes (82) (170), it is reasonable to suggest that these factors may play a role in mediating the response to TSA. Similar to the present findings, studies in oocytes of the repressive effects of thyroid hormone receptors have demonstrated that TSA can decrease repression and enhance transcription (159). In oocytes it has been shown that, the unliganded receptor can recruit endogenous, N-CoR containing deacetylase complexes (171) (82). Although changes in histone acetylation are an obvious possible consequence of deacetylase recruitment, there are other possibilities. A recent study has shown that Pit-1 can be acetylated by CBP in cooperation with the co-activator p/CIP (172). To date, no functional consequences have been reported for this post-translational modification.

In summary, this work provides new insights into the ability of Pit-1 to modulate chromatin structure and transcription. We have used a Xenopus oocyte, in vivo system to assemble the prolactin gene into chromatin. The studies provide evidence that Pit-1 can direct chromatin remodeling on the prolactin gene. Pit-1 expression was found to lead to the presence of translationally positioned nucleosomes in both the proximal promoter and distal enhancer regions of the prolactin gene. Pit-1 appears to recruit an endogenous repressor complex in oocytes that can be alleviated by treatment with the deacetylase inhibitor, TSA. The Xenopus oocyte system used in the present study provides a powerful tool to further explore the mechanisms mediating these responses and to examine the role other transcription factors might play in modulating chromatin structure and prolactin transcription.

Figure 13. Time course of nucleosome formation of the prolactin promoter in oocytes.

(A) Schematic diagram indicating the relative location of Pit-1 binding sites in the prolactin gene. Pit-1 sites in the proximal region are designated 1P through 4P and sites in the distal enhancer region are designated 1D to 4D. The 1.9 PRL-Luc reporter construct used in these studies contains approximately 1.9 kilobase pairs of 5' flanking and promoter sequence of the prolactin gene including regulatory regions linked to luciferase (1.9 PRL-Luc). (B) Injection of the 1.9 PRL-Luc reporter gene into oocytes results in chromatin formation. The 1.9 PRL-Luc reporter was injected into oocytes and incubated for 1, 4 or 18 h. Homogenates were treated with 0.8, 2.5 and 7.5 U/ml of micrococcal nuclease. The digested DNA was resolved on an agarose gel, transferred to a nylon membrane and visualized by hybridization to radiolabeled DNA probe representing the prolactin gene sequences.

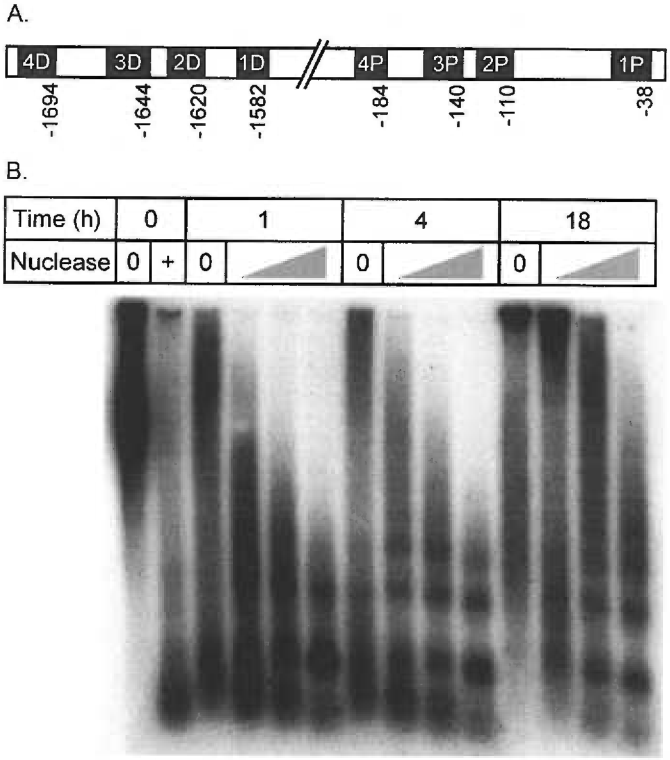

A.
4D
3D
2D
1D
4P
3P
2P
1P
-1694
-1644
-1620
-1582
-184
-140
-110
-38
B.
Time (h)
0
1
4
18
Nuclease
0
+
0
0
0

Figure 14. Pit-1 stimulates transcription from prolactin reporter genes in oocytes.

(A) Xenopus oocytes were injected with the 1.9 PRL-Luc reporter construct and a Xenopus expression vector containing Pit-1 coding sequence where indicated. After injection, groups were divided into a control group receiving DMSO and a group receiving trichostatin A (TSA; 5ng/ml). After an overnight incubation, oocytes were homogenized and RNA prepared. Primer extension analysis with a luciferase specific, radiolabeled primer was used to detect the prolactin transcript (appropriate sized product indicated with an arrow). (B) Analysis of Pit-1 expression in control and TSA treated oocytes. Xenopus oocytes were injected with an expression vector encoding FLAG-Pit-1. Some of the oocytes were treated with TSA as indicated. Ooctyes were incubated overnight in the presence of ^{35}S[methionine]. FLAG-Pit-1 was isolated by immunoprecipitation and resolved on a denaturing polyacrylamide gel.

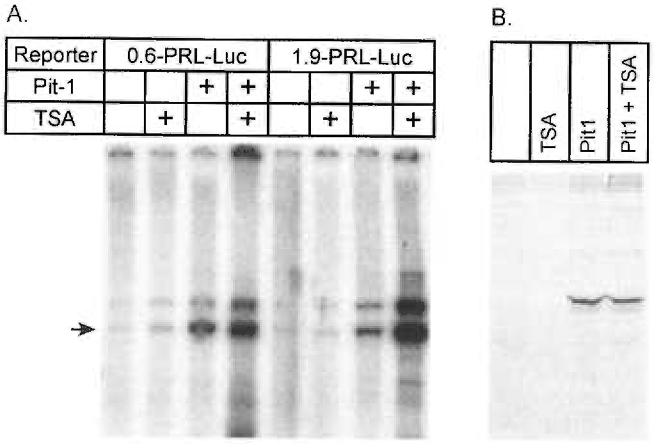

A.
Reporter
0.6-PRL-Luc
1.9-PRL-Luc
Pit-1
TSA
+
B.
TSA
Pit1
Pit1 + TSA

Figure 15. Pit-1 expression results in the presence of a positioned nucleosome in the proximal promoter region of the prolactin gene.

(A) Analysis of micrococcal cleavage of a prolactin reporter gene that has been assembled into chromatin. Oocytes were injected with the 1.9 PRL-Luc reporter construct and the FLAG-Pit-1 expression vector (Pit-1) as indicated. After overnight incubation, oocytes were homogenized and incubated with increasing amounts of micrococcal nuclease (0, 0.8, 2.5 and 7.5 U nuclease/ml). After digestion with KpnI and EcoRI, DNA was resolved on an agarose gel and transferred to a membrane. The locations of nuclease digestion were identified by hybridization to a radiolabeled EcoRI-XbaI fragment from the luciferase coding sequence. Solid arrows indicate sites where Pit-1 expression increased nuclease cleavage. An open arrow indicates sites that were either unchanged or demonstrated decreased cleavage in the presence of Pit-1. (B) Analysis of micrococcal nuclease digestion of the prolactin reporter gene that was not assembled into chromatin. Oocytes were injected with either a control empty vector or a FLAG-Pit-1 expression vector. After an overnight incubation to permit expression of Pit-1, oocytes were homogenized and prolactin reporter construct DNA was added to the homogenate. Following a 30 minute incubation, micrococcal nuclease was added to the sample (0.001, 0.005, 0.01 and 0.05 U nuclease/ml) and indirect end labeling analysis was performed as above. The locations of two sites in the proximal promoter are indicated that were sensitive to micrococcal nuclease. (C) Schematic indicating proposed position of a translationally positioned nucleosome in the prolactin proximal region.

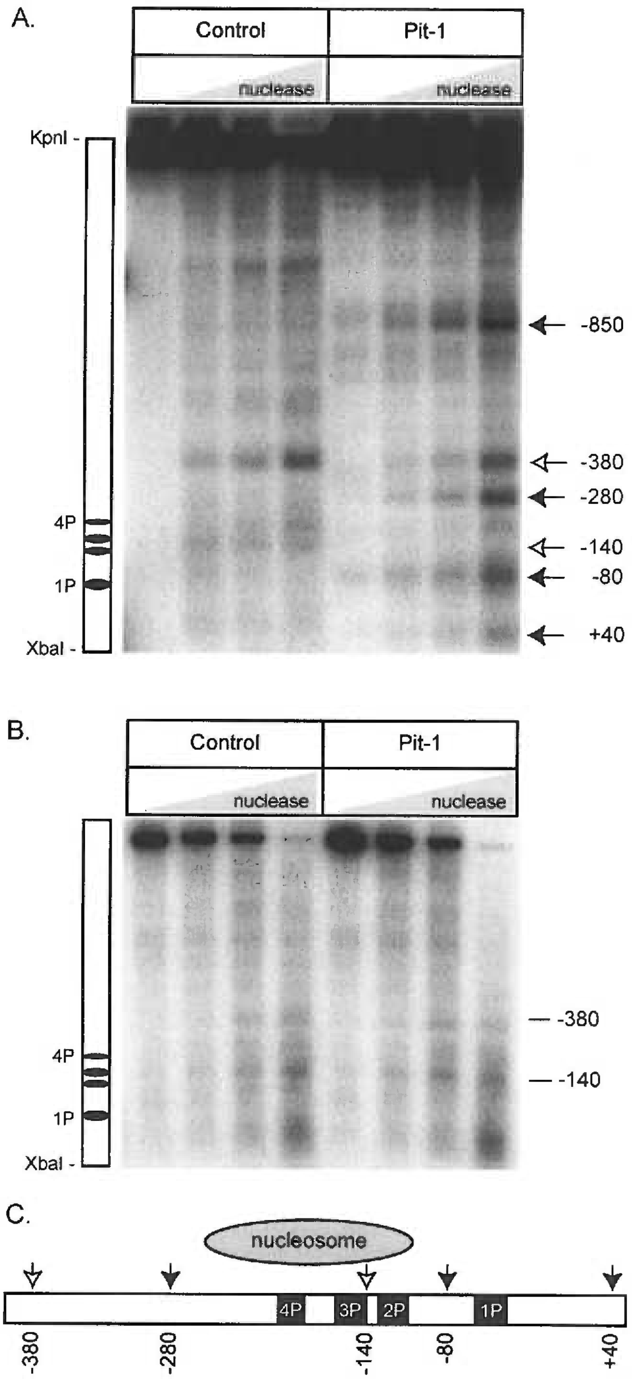

A.
Control
Pit-1
nuclease
nuclease
KpnI -
4P
1P
XbaI -
-850
-380
-280
-140
-80
+40
B.
Control
Pit-1
nuclease
nuclease
4P
1P
XbaI -
— -380
— -140
C.
nucleosome
4P
3P
2P
1P
-380
-280
-140
-80
+40

Figure 16. Pit-1 expression in oocytes results in the presence of a positioned nucleosome in the distal enhancer region of the prolactin gene.

(A) Oocytes were injected with 1.9 PRL-Luc reporter construct and a FLAG-Pit-1 expression vector (Pit-1) as indicated. After a 40 hour incubation, oocytes were homogenized and incubated with increasing amounts of micrococcal nuclease (0, 0.8, 2.5 and 7.5 U nuclease/ml). Isolated DNA was digested with AflIII. The DNA was resolved by agarose gel electrophoresis and transferred to a membrane. The locations of nuclease digestion sites were determined by hybridization to a radiolabeled AflIII-BstXI fragment that hybridizes to the 3' end of the region of interest. Solid arrows indicate sites where Pit-1 expression increased nuclease cleavage. (B) Analysis of micrococcal nuclease digestion of prolactin reporter gene that has not been assembled into chromatin. Oocytes were injected with either a control empty vector or a FLAG-Pit-1 expression vector. After an overnight incubation, oocytes were injected with the PRL reporter construct, incubated for 30 minutes and then homogenized, digested with increasing amounts of micrococcal nuclease (0.05 and 0.1 U/ml).) and then subjected to indirect endlabeling analysis performed as above. Two sites are indicated that were also present in chromatinized DNA. (C) Schematic indicating proposed nucleosome positioning in the distal enhancer. The oval with the dotted line represents the location of a tentatively assigned nucleosome.

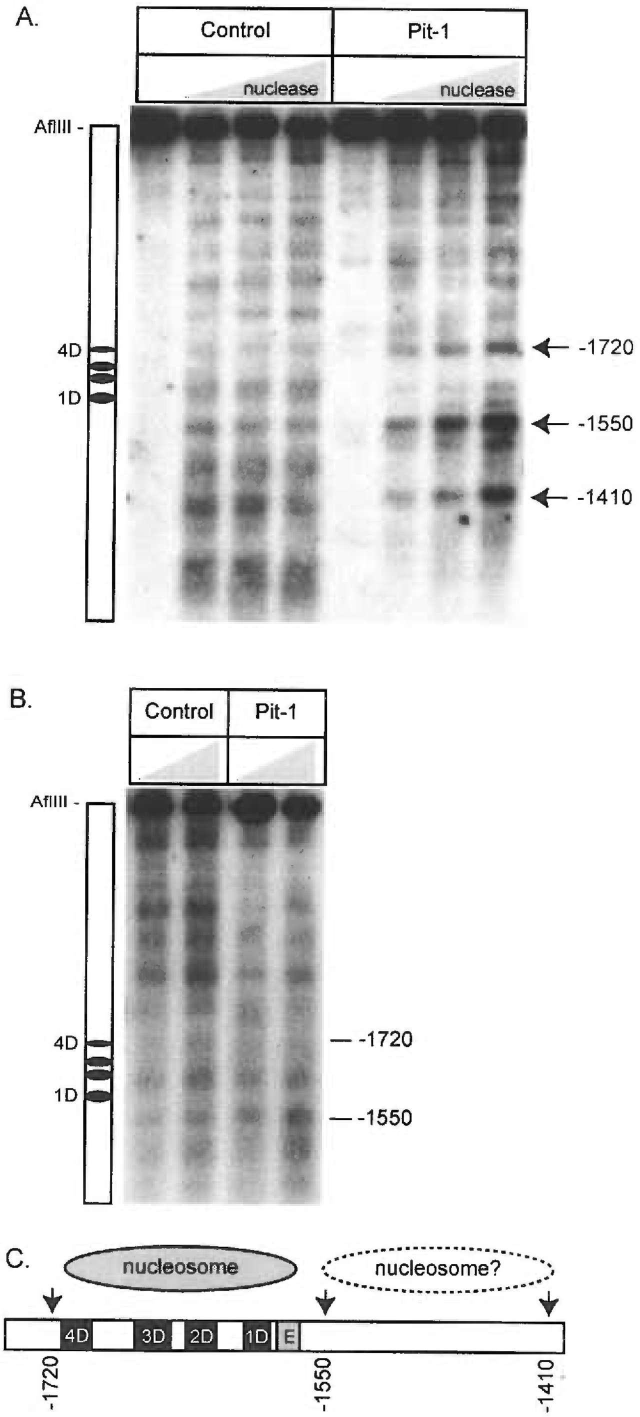

A.
Control
Pit-1
nuclease
nuclease
AflIII -
4D
1D
-1720
-1550
-1410
B.
Control
Pit-1
AflIII -
4D
1D
-1720
-1550
C.
nucleosome
nucleosome?
4D
3D
2D
1D
E
-1720
-1550
-1410

Figure 17. Time course of Pit-1 expression and chromatin remodeling in oocytes.

(A) Oocytes were injected with the PRL reporter construct and incubated overnight. A FLAG-Pit-1 expression construct was injected 72, 48, 20 or 8 hours prior to micrococcal nuclease digestion. After injection, groups of ten oocytes were incubated in [35S]methionine for analysis of Pit-1 expression. (B) Expression of Pit-1. Radiolabeled FLAG-Pit-1 was immunoprecipitated from homogenates and resolved on a denaturing polyacrylamide gel. (C) Indirect end-labeling analysis of the proximal promoter during the time-course. After the indicated incubation times, 30 oocytes were homogenized, digested with micrococcal nuclease and subjected to indirect end-labeling as in Fig. 3. Selected sites are indicated with solid arrows (Pit-1 enhanced cleavage) or open arrows (no change or decrease in cleavage).

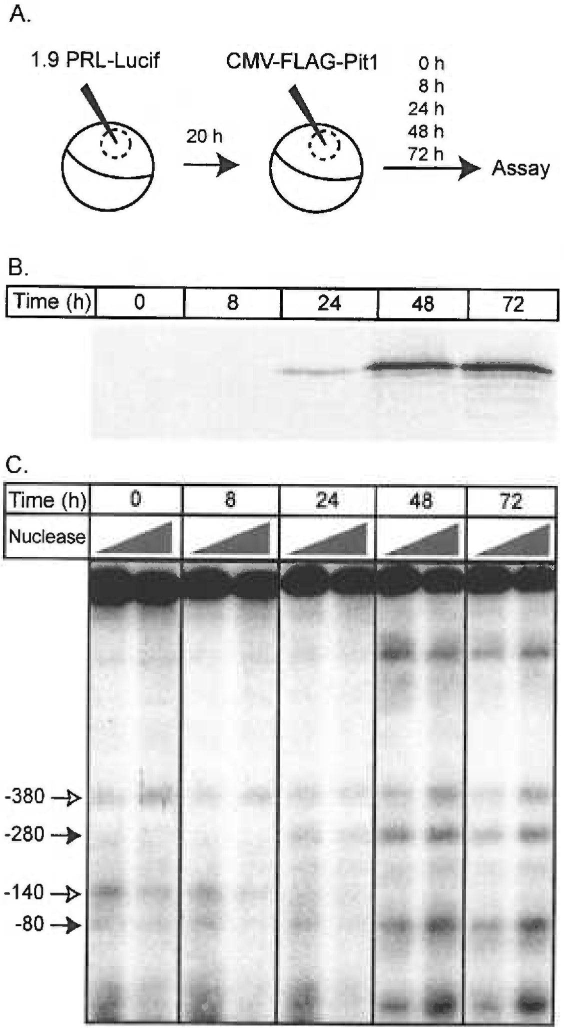
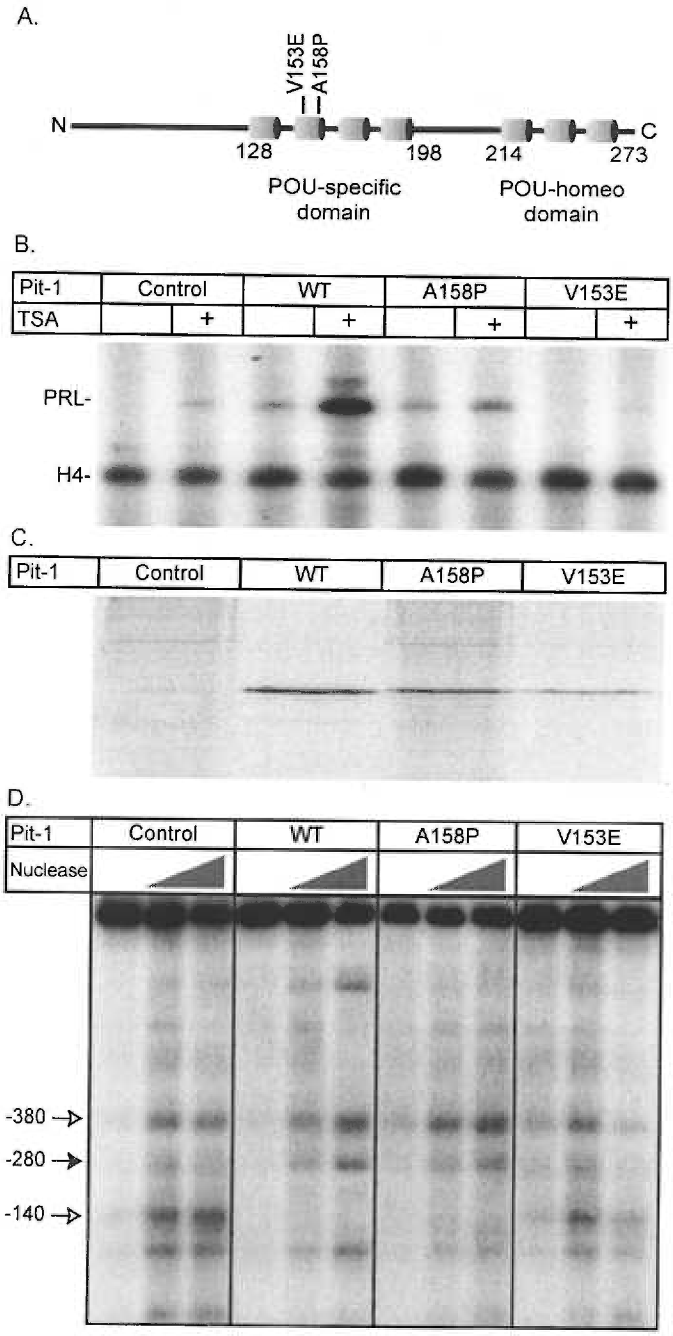

A.
1.9 PRL-Lucif
CMV-FLAG-Pit1
0 h
8 h
24 h
48 h
72 h
20 h
Assay
B.
Time (h)
0
8
24
48
72
C.
Time (h)
0
8
24
48
72
Nuclease
-380
-280
-140
-80

Figure 18. Analysis of the effects of mutations in the Pit-1 coding sequence on transcription and chromatin remodeling.

(A) Schematic diagram indicating major known helical regions of Pit-1 (rectangles) and identifying the relative locations of the mutations. (B) Transcriptional activation of the Pit-1 mutants. 1.9 PRL-Luc reporter constructs were injected into oocytes together with expression vectors encoding wild type Pit-1 (WT) or mutant Pit-1 with replacement of alanine at residue 158 with proline (A158P) or replacement of valine at residue 153 with glutamic acid (V153E). RNA was isolated and luciferase transcript was identified by primer extension analysis using a luciferase specific primer (PRL). A transcript for the endogenous Xenopus histone H4 mRNA was also identified by primer extension and used as a recovery/loading control (H4). (C) Protein expression levels were determined for each of the Pit-1 mutants. Microinjected oocytes were incubated in the presence of [35S]methionine. FLAG-Pit-1 protein was immunoprecipitated using FLAG-resin and resolved on a denaturing polyacrylamide gel. (D) Chromatin remodeling by wild type and mutant Pit-1. Oocytes were injected with 1.9 PRL-Luc reporter constructs and the indicated Pit-1 expression vectors and incubated for 40 hours. The oocytes were homogenized, digested with micrococcal nuclease and subjected to indirect end-labeling as in Fig. 3. Selected sites are indicated with solid arrows (Pit-1 enhanced cleavage) or open arrows (no change or decrease in cleavage).


A.
V153E
A158P
N
C
128
198
214
273
POU-specific domain
POU-homeo domain
B.
Pit-1
Control
WT
A158P
V153E
TSA
+
+
+
+
PRL-
H4-
C.
Pit-1
Control
WT
A158P
V153E
D.
Pit-1
Control
WT
A158P
V153E
Nuclease
-380
-280
-140

CHAPTER IV

DISCUSSION AND CONCLUSION

This thesis has explored two separate mechanisms by which the regulation of PRL gene expression can occur. In chapter II, the mechanism by which cAMP can regulate PRL gene expression was investigated. These studies demonstrated that cAMP could regulate PRL gene expression through the activation of the MAPK pathway via the GTP-binding protein Rap1. The experiments further argued that ETS domain transcription factors do not necessarily play a role in cAMP mediated activation of PRL gene expression. Instead, cAMP appeared to utilize the MAPK pathway to increase the transcriptional activity of the coactivators CBP/p300 (Figure 19).

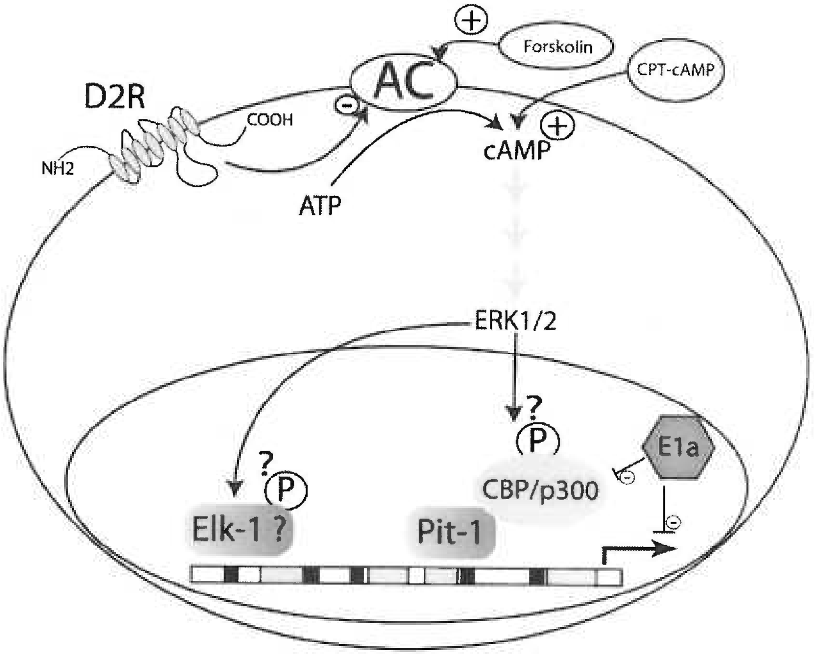


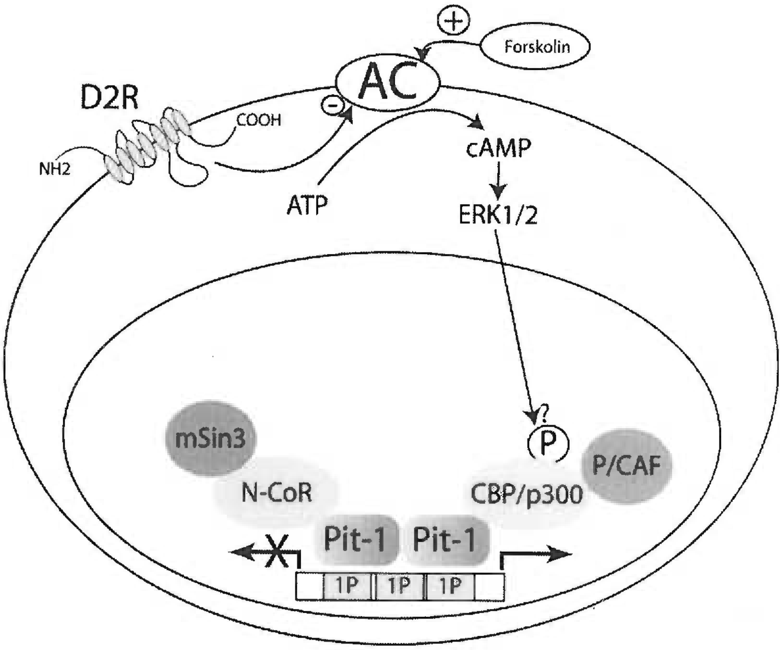
Figure 19. Possible mechanisms involved in the regulation of PRL gene expression by cAMP.

The most described mechanism by which cAMP can alter the transcriptional activity of a promoter is through the action of the cAMP response element binding protein (CREB). CREB can bind to an 8 base pair long specific sequence in DNA, termed the cAMP-response element (CRE)(173). The activation of CREB is achieved through the phosphorylation of serine 133 by PKA and other signaling pathways which results in the recruitment of the coactivator CBP/p300 (174). It is suggested that CBP/p300 could function as a molecular bridge between the CREB transcription factor and components of the general transcription machinery (175). Obviously, cAMP regulation through this pathway requires the presence of a CRE in the promoter region. Interestingly, promoters that do not contain a CRE can also be regulated by changes in cAMP levels and have been shown to require CBP/p300 (176, 177). An example of such a promoter is the prolactin gene.

The prolactin gene is regulated by cAMP, but the exact mechanisms are still unclear. The transcription factor Pit-1, an integral part of PRL gene expression, can be phosphorylated by PKA. However, this phosphorylation is not necessary for Pit-1 to mediate PKA-dependent transcriptional activation (40). The studies in this thesis suggest a role for CBP/p300 in the cAMP-mediated activation of the prolactin gene. Activation of the cAMP pathway resulted in increases in the transcriptional activity of p300 recruited to an artificial promoter. An interesting finding was the ability of a MAPK pathway inhibitor to interfere with cAMP mediated activation of p300, suggesting that p300 activity was regulated by MAPK. The ability of CBP/p300 to function as an integrator for MAPK mediated signaling was first described by Janknecht and Nordheim (178). Activation of the MAPK pathway demonstrated that a C-terminal region of CBP was increased in transcriptional activity. This observation was expanded upon when this region of CBP was shown to be sensitive to other stimuli, such as nerve growth factor (179) and phenylephrine (180). A recent study demonstrated that this region of CBP is required for PKA-mediated activation (122, 123). Also, expression of the C-terminal region of CBP/p300 enhanced Pit-1 mediated PRL gene expression (123). Thus, it is plausible that cAMP-mediated activation of the PRL promoter in GH3 cells requires the C-terminal region of CBP. The studies presented in this thesis provide not only a mechanism by which cAMP can activate gene expression through CBP/p300, but also present an example of CBP/p300 mediated gene expression of a CRE-less promoter.

The central role of Pit-1 in PRL gene expression is underscored by the observation that Pit-1 can recruit both activator and repressor complexes to the PRL promoter. In heterologous cells the binding of Pit-1 to multimers of the 1P binding site only weakly activated gene expression. However, the inhibition of various components of the Sin3 repressor complex resulted in relieve of repression. The co-repressor N-CoR, a component often associated with the Sin3 repressor complex (169), was shown to interact with Pit-1 in vivo and in vitro (122). Using a microinjection technique, several components of the Sin3 repressor complex were shown to be involved in Pit-1 mediated gene expression. Injection of antibodies to N-CoR, mSin3 A/B, HDAC2 and SAP 30 were all capable of reversing the repressive nature of Pit-1 bound to a Pit-1 response element. Thus, it appears that Pit-1 can recruit both an activator complex and a repressor complex to a Pit-1 dependent promoter. In heterologous cells, Pit-1 was capable of recruiting members of the mSin3 repressor complex, resulting in low levels of promoter activity. Stimulation of the cells with forskolin, an adenylate cyclase inducing agent, resulted in the activation of a Pit-1 binding site driven promoter. Analysis of CBP identified that the C-terminal region is imported for cAMP-mediated activation (Figure 20).

Figure 20. A model of cAMP mediated gene expression of the Pit-1 transcription factor



The recruitment of both the mSin3 repressor complex and the CBP/p300 protein suggests that Pit-1 might be capable of regulating the level of acetylation at the promoter. The regulation of acetylation at a promoter correlates strongly with the transcriptional status of a gene. Hence, targeting the co-activator

CBP/p300, which has intrinsic HAT activity (75), could provide a mechanism for Pit-1 to increase local levels of histone H4 acetylation. Indeed, overexpression of Pit-1 in PRL producing cells can induce the level of acetylated histone H4 at the PRL promoter (153). Conversely, expression of Pit-1 in non-PRL producing cells resulted in the recruitment of the repressor complex mSin3 (122), which contains a protein with histone deacetylase activity (HDAC1/2)(181). Therefore, the level of acetylation at the prolactin promoter can be regulated by the recruitment of either CBP or N-CoR. The recruitment of an inhibitory deacetylase activity by Pit-1 was also reported in this thesis.

In chapter III, we describe the role that Pit-1 plays in regulating the chromatin structure of the prolactin promoter. These studies utilized an in vivo chromatin remodeling system to assess the contribution of Pit-1 to PRL gene chromatin structure. We demonstrate that Pit-1 can translationally position nucleosomes on the proximal promoter and distal enhancer of the PRL gene. The results also suggest that Pit-1 could recruit a repressor complex and limit PRL gene transcription.

The observation that Pit-1 might recruit an inhibitory, deacetylase activity in xenopus oocytes underscores the ability of Pit-1 to repress PRL gene expression in non-PRL producing cells (122). As oocytes contain functional N-CoR and Sin3 (82, 170), it is likely that Pit-1 is capable of recruiting the Sin3 repressor complex in oocytes. The Sin3 complex in Xenopus oocytes is comprised of N-CoR, Sin3, RbAp48 and HDAC1 (82). As mentioned previously, N-CoR could function as the Pit-1 binding factor, HDAC can deacetylate histones within the nucleosomes, Sin3 likely acts as the scaffold and RbAp48 can interact with histone H4 (170). This repressor complex can interact in vivo with an unliganded thyroid hormone receptor to repress transcriptional activity in the absence of ligand (82). Since this repression can also be relieved by the addition of TSA (159), it is reasonable to suggest that these factors may play a role in Pit-1 mediated repression of the PRL gene in Xenopus oocytes.

However, the Sin3 repressor complex in Xenopus oocytes has not yet been shown to contain any ATP-dependent chromatin remodeling factors. If Pit-1 truly recruits the Sin3 repressor complex, how does the translational positioning of the nucleosomes occur on the PRL promoter? An answer might be that simply the ATP-dependent chromatin remodeling factor of the Sin3 complex has not been identified in Xenopus oocytes. Recently, a study by Sif et al (182). demonstrated that the human mSin3 complex contained Brg1 and hBrm, two ATP-dependent chromatin remodeling factors. However, this study does not report the presence of N-CoR within this complex. An alternate explanation could be that the N-CoR/Sin3/HDAC complex in Xenopus oocytes does not mediate the Pit-1 mediated repression of PRL gene expression, but another complex is involved.

The studies described in chapter III relate to the nucleosome structure in the presence of only Pit-1. However, over the years many other transcription factors have been identified that can play a role in PRL gene expression. It will be of great interest to examine the contribution to PRL chromatin structure of these factors, which includes ETS factors, estrogen receptor and Oct-1. Further, the involvement of CBP/p300 in chromatin remodeling will be worth investigating. In these studies, the majority of the nucleosome positioning by Pit-1 was directed under repressive conditions. It will prove fruitful to investigate whether the nucleosomes reside in a similar location when Pit-1 is associated with a co-activator complex.

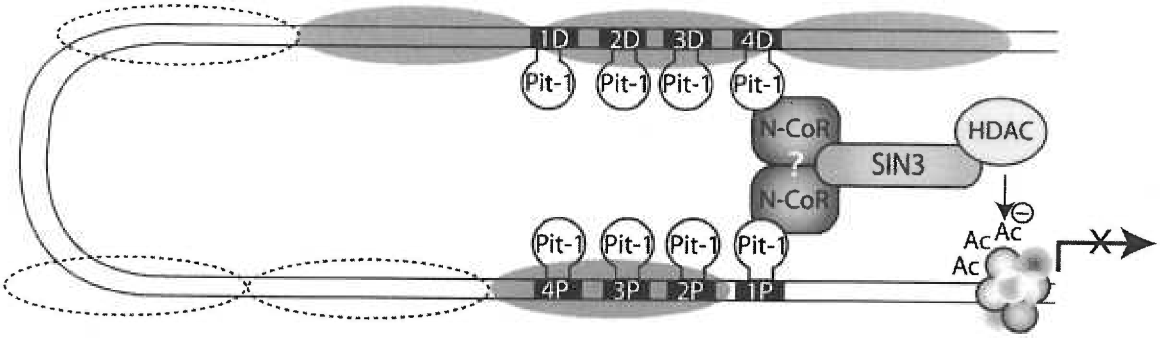


Figure 21. Overview of the positioned nucleosomes in the PRL promoter.

The data in this thesis yielded an important insight in the mechanism by which cAMP can regulate expression of the PRL gene. It also described the central role of the pituitary-specific transcription factor Pit-1 plays in PRL gene expression. Pit-1 acts as a molecular switch, recruiting both co-activator and co-repressor complexes, through which it can regulate processes ranging from orchestrating the nucleosomal structure of the PRL promoter to the cAMP-mediated activation of PRL gene expression.

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