Chromatin, SF-1, and CtBP
Structural and Post-Translational Modifications
Induced by ACTH/cAMP
Accelerate CYP17 Transcription Rate

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History is merely a list of surprises. It can only prepare us to be surprised yet again.

-Kurt Vonnegut
For Kate and Jeran, and Dad-
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<table>
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<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AF-1 or -2</td>
<td>transcription activation function molecular switches</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>Bt2cAMP</td>
<td>dibutryl cyclic AMP</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine 3',5' monophosphate (AMP)</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CoRNR box</td>
<td>corepressor helix for interacting with nuclear receptor</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotropin releasing hormone</td>
</tr>
<tr>
<td>CtBP</td>
<td>Adenovirus early antigen E1A C-terminal binding protein</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol, a molecule derived from membrane lipids</td>
</tr>
<tr>
<td>DHEA</td>
<td>dehydroepiandrosterone</td>
</tr>
<tr>
<td>DRB</td>
<td>5,6-dichloro-1-β-D-ribofuranosylbenzimidazole an inhibitor of CDKs</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>Ftz-F1</td>
<td>fushi tarazu-related F1 helix</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>GSK3β</td>
<td>glycogen synthase kinase 3β</td>
</tr>
<tr>
<td>HAT</td>
<td>histone acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamic-pituitary-adrenal (signaling axis)</td>
</tr>
<tr>
<td>IP</td>
<td>immunoprecipitation</td>
</tr>
<tr>
<td>LBD</td>
<td>ligand binding domain</td>
</tr>
<tr>
<td>Mc2</td>
<td>melanocortin 2</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide, reduced form</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate, reduced form</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance (imaging and structure determination)</td>
</tr>
<tr>
<td>NR</td>
<td>nuclear receptor</td>
</tr>
<tr>
<td>NR box</td>
<td>LxxLL or related helical coactivator motif</td>
</tr>
<tr>
<td>P450</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>p54</td>
<td>54 kDa nuclear RNA binding protein</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent kinase/protein kinase A</td>
</tr>
<tr>
<td>PSF</td>
<td>polypyrimidine tract binding protein associated splicing factor</td>
</tr>
<tr>
<td>PTM</td>
<td>post-translational (protein) modification</td>
</tr>
<tr>
<td>SF-1</td>
<td>steroidogenic factor-1</td>
</tr>
<tr>
<td>SRC</td>
<td>steroid receptor coactivator</td>
</tr>
<tr>
<td>StAR</td>
<td>steroidogenic acute regulatory protein</td>
</tr>
<tr>
<td>SUMO</td>
<td>small ubiquitin-like modifier protein</td>
</tr>
<tr>
<td>SWI/SNF</td>
<td>mating type switch/sucrose non-fermenting protein complex involved in chromatin remodeling</td>
</tr>
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SUMMARY

CYP17 is an ACTH/cAMP inducible gene in the human adrenal cortex encoding a cytochrome P450 enzyme with sterol 17α-hydroxylase activity and 17,20 lyase activity essential for biosynthesis of cortisol and androgens. Studies carried out during the past decade have shown that accelerated transcription of inducible eukaryotic genes involves sequential chromatin modifications by cooperative promoter-specific transcription factors and the class of proteins called transcriptional coregulators. In the present work, we aimed to first identify important chromatin modifications and chromatin modifying complexes at the CYP17 transcription start site and nearby steroidogenic factor-1 (SF-1) binding site. Then, we asked what modifications to SF-1 occur during the interaction of this nuclear receptor with the CYP17 promoter, and what their function may be. Finally, we asked how ACTH/cAMP signaling affects SF-1-containing chromatin-modifying complexes during the early phase of transcriptional induction of CYP17. Results from chromatin immunoprecipitation (ChIP) and mammalian two hybrid experiments identified complexes including one comprised of SF-1, steroid receptor coactivator-1 (SRC-1), and the histone acetyltransferase general control nonderepressed 5 (GCN5) as cAMP-inducible, but sensitive to the SF-1 antagonist sphingosine, and able to act in stimulating CYP17 transcription. Moreover, ATPases on the promoter coincided with manipulation of nucleosome histone H2 dimer content. Next, we found that SF-1 phosphorylation by glycogen synthase kinase 3β (GSK3β), reciprocal dephosphorylation by phosphatase(s), and acetylation by GCN5 at nearby sites at the ligand binding pocket opening were required for efficient CYP17 transcription. This leads us to propose that ligand binding to SF-1 is controlled by these post-translational modifications. Finally, we determined that the corepressors E1A C-terminal binding proteins (CtBP) 1 and 2 are protein kinase A (PKA) targets and are sensitive to PKA-dependent NADH accumulation. These effects of PKA activation by ACTH/cAMP in adrenal cortex cells enforce CYP17 transcription concomitant with dimerization of CtBP1 and CtBP2.
Chapter 1. Introduction

1.1 Causes and Effects of Induction of Adrenal Cortisol Synthesis

In humans, stress initiates a cascade of physiological responses, including the synthesis of the steroid hormone cortisol in the adrenal cortex. This stress response is conserved, with some minor differences, across most vertebrates. Production of steroids, called steroidogenesis, in the adrenal cortex occurs following a cascade of signaling through the hypothalamus, the anterior pituitary gland, and the adrenal gland (the HPA axis).

Neural activity initiated by the perception of fear or stress, or the early phase of the wake-sleep cycle, causes the hypothalamus to secrete the short-lived peptide hormone corticotropin releasing hormone (CRH) into local circulation between the hypothalamus and pituitary. CRH receptors in the anterior pituitary gland couple to $G_{\alpha} \text{s}$, adenylate cyclase, and its product, cyclic adenosine 3’,5’ monophosphate (cAMP). Cleavage of adrenocorticotropic hormone/melanocortin 2 (ACTH/Mc2) from a larger pro-opiomelanocortin peptide occurs as the hormone is packaged into secretory vesicles (1). Following cAMP production, ACTH is released from these vesicles into systemic circulation. The primary target of ACTH is the Mc2 receptor, which is also coupled to cAMP production in cells of the innermost layers or zones of the mature adrenal cortex, namely zona fasciculata and zona reticularis. Cells of these respective zones specialize in the ACTH- and cAMP-inducible synthesis of cortisol and dehydroepiandosterone (DHEA, an androgen).

Cortisol is a specific high-affinity agonist for the glucocorticoid receptor (GR), a transcription factor of the nuclear receptor superfamily of proteins (NRs). GR is
ubiquitously expressed, with particularly high levels in many cells of the immune system (2). The immune system and inflammatory response is strongly repressed by GR bound to cortisol in a mechanism of transcription repression, in which agonist-bound GR becomes modified, binds and stabilizes corepressor complexes on repressed gene promoters (3). In addition, through the GR, glucocorticoids activate a transcription program on other genes which are essential in altering whole body metabolism and signaling to mobilize diverse energy sources to elevate blood glucose. In particular, active GR induces genes required for liver gluconeogenesis (4), muscle proteasome-mediated catabolism of protein (5) and inhibition of protein synthesis, and adipose tissue lipolysis (4), although these effects may depend on the recent food intake of an individual.

1.1.1 Steroid Precursor Mobilization and Induced Transcription of Steroidogenic Enzymes Are Hallmarks of Acute and Chronic Steroidogenesis

Steroidogenic cells are typically have an ability to take cholesterol as a starting substrate; cholesterol then feeds into all steroid hormone-generating pathways. Such cells make up the adrenal cortex, select cells of the gonads, placenta, and brain, although the latter two tissues lack an ability to undergo rapidly induced steroid production (6). This is because they lack cAMP-inducible transcription of a labile factor that enables the high rate of cholesterol conversion to steroid inherent to acute adrenal and gonadal steroidogenesis (6, 7). In steroidogenic cells of the gonads and adrenal cortex, cholesterol esters in cytoplasmic lipid droplets are the readily available and locally stored form of cholesterol. The acute response to induction of steroidogenesis by elevated cAMP in the adrenal requires hormone sensitive lipase activity (8), whereby these fatty-
acid esters of cholesterol are hydrolyzed to release free substrate, forming cytoplasmic lipid droplets if subsequent steps do not occur. In the rate-limiting step of steroidogenesis, cholesterol is translocated to the inner leaflet of the inner mitochondrial membrane through the outer mitochondrial membrane, giving it accelerated access to inner leaflet-associated enzymes in a process requiring steroidogenic acute regulatory protein (StAR). During this process, a complex of StAR-associated proteins including cAMP-dependent kinase/protein kinase A (PKA) assembles on the outer mitochondrial membrane in response to cAMP induced activation of PKA, probably via phosphorylation-aided protein-protein interactions with StAR (9, 10). StAR is the labile factor that is rapidly transcribed, then phosphorylated by PKA at the outer mitochondrial membrane following cAMP mediated liberation of free cholesterol in steroidogenic cells of the gonads and adrenal cortex. Phosphorylation of StAR at a single residue is required for about fifty percent of its mitochondrial cholesterol import capability (9). It has also been shown that StAR is not enriched in mitochondria of cAMP-stimulated steroidogenic cells treated with a cholesterol esterase inhibitor (11); this suggests coupling of the lipase mediated release of free cholesterol and StAR membrane integration during the transfer of that cholesterol into mitochondria.

1.1.2 Cytochromes P450 Are Key Steroidogenic Enzymes

Contacting the inner leaflet of the inner mitochondrial membrane (12), a member of the heme-containing monooxygenase superfamily of cytochromes P450 begins the enzymatic conversion of cholesterol to steroid (summarized in Figure 1.1). Specifically, P450 side chain cleavage enzyme performs successive oxidations of
Figure 1.1 Steroidogenic reactions and enzymes of the three zones of the adrenal cortex. Adapted from (13). P450scc, cholesterol side chain cleavage P450; 3βHSD, 3β-hydroxy steroid dehydrogenase, the only non P450 adrenal steroidogenic enzyme; P450c17, steroid 17α-hydroxylase/17,20 lyase; P450c21, steroid 21α-hydroxylase; P450c11, steroid 11β-hydroxylase; P450c18, aldosterone synthase; Ac-CoA, acetyl-coenzyme A; HDL, high density lipoprotein particle; SR-B1, HDL receptor scavenger receptor-B1; LDL, low density lipoprotein particle; LDLR, LDL receptor. Drawing of human adrenal cortex zonation reproduced from 20th US ed. of Grey’s Anatomy of the Human Body (1918).
cholesterol at C20 and C22, generating pregnenolone, which is then directed along a cell type-specific pathway. Steroidogenic pathways specific to each of the three adrenal cortex zones are summarized with respect to cell type and intracellular location of steroidogenic enzymes in Figure 1.1.

P450s are a family of enzymes that act on diverse endogenous and xenobiotic substrates, typically increasing water solubility. Activated oxygen in a steroidogenic P450 attacks bound sterol at a site specified by the overall structure and substrate binding properties of that enzyme. Possible reactions by adrenal steroidogenic P450s are the oxidation of a C-H bond to a C-OH bond (hydroxylase activity), a C-OH moiety to a carbonyl group (C=O), or carbon-carbon bond scission between a vicinal O=C-C-OH pair due to oxidation of the remaining hydroxide and rearrangement of a hydrogen atom and electrons. Outside the adrenal, in estrogen (estradiol) producing cells, P450 aromatase (encoded by the CYP19 gene) catalyzes successive oxidations of the first sterol ring (A ring), resulting in loss of the C19 methyl group while making the A ring aromatic.

Each oxidation reaction catalyzed by a P450 requires two electrons, one each typically from the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), which does not directly interact with the heme oxygen of eukaryotic P450s but rather relays electrons individually via P450 oxidoreductase in the endoplasmic reticulum, or adrenodoxin and adrenodoxin reductase in mitochondria. In some cases, cytochrome B₅ may relay an electron from NADH to P450s such as to P450c17 for the catalysis of 17/20 bond scission (14). Stimulation of acute steroidogenesis also may
increase the availability of electrons for this process, or at least requires that the NADPH-generating pentose phosphate pathway be fully functional (15).

1.1.3 How Is Cortisol Output Chronically Upregulated in Zona Fasciculata?

Acute ACTH-induced cortisol production by the adrenal cortex is complemented by a chronic increased potential for steroidogenic capacity primarily through increased transcription of the genes encoding the enzymes required for cortisol production. Obligate roles for ACTH receptor, cAMP, and PKA in stimulating steroidogenic gene transcription in response to ACTH have been determined in cell lines of mouse and human adrenal cortex cells (reviewed in (16)).

The battery of ACTH-inducible genes in the human adrenal cell nucleus includes the genes for StAR, P450scc (CYP11A1), 3β hydroxysteroid dehydrogenase (3βHSD), P450c17 (CYP17A1), P450c21 (CYP21A1), 11β-hydroxylase (CYP11B1) (Figure 1.1). Unlike active StAR, with a half life of 3-4 hours (17), P450s are longer lived. For example, P450scc in a bovine adrenal cells has a half life in range of 38-42 hours (18). Moreover, transcription of P450s such as CYP17A1 (CYP17) is significantly elevated in response to the cAMP-coupled gonadotropin, lutenizing hormone, only after two hours in ovine theca cells (19), or following activation of cAMP signaling for two hours in a human adrenal cortex cell line (20). On the other hand, StAR mRNA expression increases in the rat adrenal within 30 minutes of ACTH stimulation that is insufficient to stimulate significant elevation of steroidogenic P450 mRNAs (21).

The CYP17 gene and the P450c17 enzyme it encodes are unique among the steroidogenic P450s in the adrenal because P450c17 expression is restricted to zones which respond positively to ACTH/cAMP and its transcription responds negatively to
signals which stimulate aldosterone production (22). Aldosterone synthesis from cholesterol in the zona glomerulosa does not require the presence of P450c17 enzyme. Thus, zona glomerulosa lacks melanocortin 2 (ACTH) receptors and rather responds acutely and chronically to angiotensin II (reviewed in (23)).

Where expressed, the propensity of P450c17 to catalyze 17α-hydroxylation (for cortisol), or 17/20 bond scission (for DHEA) is also zone specifically regulated by cell-type specific protein-protein interactions (24) and kinase activity (25). Therefore, along with 3β-hydroxysteroid dehydrogenase expression and activity (which is absent where DHEA is produced in the zona reticularis—Figure 1.1 and (26)), CYP17 expression and choice of enzyme activity determines functional zonation of the two inner zones of the adult adrenal cortex. Thus, CYP17 is an ideally suited model for the study of transcriptional regulation of an ACTH/cAMP-induced gene required for chronic cortisol production. Inducible CYP17 transcription is also determined in gonadal steroidogenic cells downstream of cAMP produced in response to other G\textsubscript{ia}-coupled receptors which are activated by gonadotropins (27), making these studies relevant for CYP17 expression in gonads as well.

### 1.1.4 Steroidogenic Factor-1 Directs Chronic Steroidogenesis in Adrenal Glands and Gonads, Enables Their Development, and Functions in the Hypothalamus and Pituitary

A major determinant of the transcription of steroidogenic genes including CYP17 in adrenal cortex ((13), review) and gonads is steroidogenic factor-1 (SF-1; NR5A1;Ad4BP). SF-1 is a primarily nuclear transcription factor which has the ability to bind to specific DNA response elements in cooperation with other transcription factors on most steroidogenic genes (13). Although SF-1 expression itself is highly tissue specific
and may be highest in adrenal and gonad cells, tissue specific knockouts have uncovered essential roles elsewhere for SF-1 (see below references and others reviewed in (28)). For example, SF-1 is found at low levels relative to abundant proteins in cells of the ventromedial hypothalamus and pituitary gonadotropes, but nowhere else in the central nervous system (29). In the hypothalamus, SF-1 appears to have a role in controlling anxiety, because a central nervous system knockout mouse displays increased anxiety-like behavior (30). Pituitary gonadotropes express the identical alpha subunit of gonadotropin, lutenizing hormone, follicle stimulating hormone, and thyroid stimulating hormone in response to cAMP following transcription mediated by SF-1 (31).

In development, SF-1 has essential roles in formation of the testes in males, and subsequent development of androgen-dependent structures, as well as formation of the adrenal gland; in SF-1 global knockout mice, neither the adrenal gland nor testes form and both male and female mice die in utero (32). Thus, SF-1 has important developmental and signaling roles in both the HPA and hypothalamic-pituitary-gonad signaling axes, as well as a requisite role in the synthesis of steroids.

1.2 Common Characteristics and Differences among Nuclear Receptors: 

NR Classes and Modules

Like GR, SF-1 is a nuclear receptor (NR), a member of a superfamily of proteins that each has as its primary function the regulation of transcription of specific target genes (33, 34). Two major subdivisions of the nuclear receptor superfamily include 1) NRs like GR, which homodimerize, translocate from cytoplasm to nucleus, and bind to short inverted or direct repeat DNA sequences (hormone response elements) on target promoters and 2) those that heterodimerize with the NR retinoid X receptor (RXR) and
are predominantly nuclear, binding to direct repeats (33, 34). Nuclear translocation, dimerization and stabilization of DNA binding may be positively affected by agonist ligands which bind to a C-terminal ligand binding domain (LBD) of individual NR peptide molecules (35, 36). A small fraction of some nuclear receptor populations may also be anchored to the plasma membrane via palmitoylation of a conserved cysteine in the LBD (37), where they can rapidly activate kinase cascades in response to ligand (38). This action is termed nongenomic, in contrast to mediation of transcription, a genomic effect of NRs.

There are, however, two major exceptions to the above general rules for genomic NR action. First, some NRs have sealed or absent ligand binding domains and thus have no ligand binding capability, while some NRs that may have the capacity to bind ligand have no identified endogenous ligand. These receptors are referred to as orphan NRs. Second, a small number of NRs do not dimerize, and bind DNA as monomers, and this is the case for the subfamily of NRs into which SF-1 is classified.

NRs bind DNA through a well-conserved zinc-finger containing DNA binding domain N-terminal relative to the LBD. The DNA binding domain and LBD are connected by a hinge. Finally, activation functions are located at amino- (N) and carboxy- (C) termini of most nuclear receptors, although the SF-1-containing subfamily does not have an N-terminal activation function, which is suited to its monomeric nature, since this activation function may play a role in receptor dimerization via interaction with the C-terminal LBD (36). In general, these activation functions are amino acid stretches of medium length in the case of ones at the N-terminus or short at the C-terminus which
enable ligand dependent (39-41) and ligand-independent (42) modulation of transcription by the NR.

### 1.2.1 SF-1 Functional Architecture from Amino to Carboxy Terminus

SF-1 shares the common modular domain architecture with other members of the nuclear receptor superfamily described above, with some variations. The isolated modules of SF-1, from N terminus to C terminus, are described as follows and are pictured as primary and tertiary structures in Figure 1.2. The N terminus begins with a well conserved 75 residue double zinc-finger DNA binding domain, followed by a 35 residue helical extension, Ftz-F1, found in a small subset of NRs and named for its sequence conservation from the *Drosophila* fushi tarazu nuclear receptor (29). The zinc-finger domain has a central role in recognizing the canonical NR-recognized DNA sequence AGGTCA, while the Ftz-F1 box improves the affinity and stability of the DNA binding

![Figure 1.2](image-url) Functional domains and solved structures for SF-1.

Domains are described in the text. Structures were imaged using Accelrys DS Visualizer v2.0 and are from protein data bank files 2FF0 (43) and 1YP0 (44).
receptor/DNA complex by extending and refining the DNA sequences that SF-1 recognizes via minor groove binding and intramolecular interaction with the core DNA binding domain ((43) and see Figure 1.6). Although the Ftz-F1 box of a SF-1 homolog, liver receptor homolog 1, does not contact DNA (45), this C-terminal extension of the SF-1 DNA binding domain may stabilize its binding to DNA as a monomer rather than a NR dimer (43), the common mode of binding for most classes of NR. The Ftz-F1 box is subject to lysine acetylation, which may affect DNA binding capability (46). In addition, there are two DNA-interacting arginine residues in this region at positions 69 and 114 that are found in motifs that are readily arginine methylated (47).

Next, a hinge region of about 100 residues connects the N and C terminal domains of SF-1. A small stretch within this region sometimes referred to as the synergy control motif is subject to phosphorylation at position S203 (48-50), lysine ε-amino SUMOylation at K194 (51-53), and modification-sensitive binding to other factors and/or intramolecular interactions which enable cooperative transcription regulation (54, 55). Finally, a large 240 residue globular ligand binding domain (LBD) is composed primarily of helices which form a three-layered sandwich typical of NRs, although SF-1 contains a 1040-1640 Å³ ligand binding pocket that is relatively large compared to the analogous pocket found in most other nuclear receptors, and even the closest relative of SF-1 (cf. (44, 56)).

The twelfth and final helix of the LBD at the C-terminus of SF-1 is a well-conserved activation function (AF-2) switch, found in all NRs that have the ability to enhance transcription (39-41). The position of this switch and the ability of SF-1 to bind transcriptional activation- or repression-associated proteins (coactivators and
corepressors, respectively—generally termed coregulators) is respectively determined by occupancy of agonistic or antagonistic ligands in those NRs that have a pocket that is ligand accessible (57). In SF-1, AF-2 is essential for the ability of this factor to induce transcription, because deletion of this amphipathic helix or mutations of hydrophobic residues reduce transcription by as much as 75 percent (58). AF-2 point mutation likewise decreased CYP17 reporter transcription 79 percent (59). The AF-2 helix has ligand-dependent effects on transcription independent of, i.e. downstream of, DNA binding, which is not directly affected by AF-2 mutation or deletion (58, 59). However, it is noteworthy that ligand binding to other NRs has been recently shown to induce post-translational modification and structural changes in the LBD and hinge regions which can affect the ability of NRs to interact with other active transcription regulators (60) and/or to target specific novel gene promoters for transcription (61). In such cases, it is probable that an NR is functioning as a coactivator for another unrelated inducible transcription factor which is being targeted to its own specific repertoire of promoters (62), and the inverse may also be true (63). Whether an NR directly or indirectly binds to DNA, AF-2 and its ligand dependent orientation, described in the next subsection, are key in enabling transcription. The roles of post-translational modifications in NR-mediated transcription are discussed further in section 1.4.

1.2.2 NR Ligands Induce Protein-Protein Interactions by Favoring Specific Modes of Coregulator Binding

Crystal structures show that the SF-1 LBD is accessible to phospholipids that may be endogenous ligands, but are also common components of plasma membranes. These include phosphatidylinositol phosphate (64), phosphatidylethanolamine (44, 65), and
possibly phosphatidic acid and phosphatidylcholine with aliphatic fatty acyl chains of 12-18 carbon length. In these crystal structures, binding of phospholipids causes AF-2 to be closely packed near the LBD, defining one boundary of a nearby hydrophobic cleft. In this configuration, the cleft is just long enough to specifically bind a complimentary LxxLL or related helical motif (NR box, (66)) specific to coactivators (44, 64, 65) and some competitive corepressors (67). This agonist bound state of the NR will be referred to as its “coactivator binding mode.”

In general, when a NR LBD is unliganded or occupied by antagonist, AF-2 is displaced from the top of the hydrophobic cleft, which is extended and more favorably binds another complimentary motif, the CoRNR box (a helical motif related to the consensus sequence Lxx(H/I)Ixxx(I/L), (68, 69)). CoRNR boxes are typically found on nuclear receptor corepressors associated with dedicated transcription repression activity—they do not contain NR boxes or coactivator enzymatic functions (E. Dammer, unpublished observations). This state of the nuclear receptor and its AF-2 will be called the “corepressor binding mode.” Both modes of LBD binding, shown for NRs with otherwise substantially different LBDs, are pictured in Figure 1.3.

NR coregulators are discussed in more detail in following sections of this introduction. At this point, it is sufficient to note that many of these coregulators are also important for regulated transcription. In addition to their NR-specific interaction modes, some, if not most, bind structurally diverse transcription factors not related to NRs (70-72). The ultimate function of coregulators may best be described as a multiprotein assembly gatekeeper that allows or disallows the chromatin-restricted formation of general transcription factor complexes (lacking gene specificity) which ultimately
recruit and license RNA polymerase II to initiate transcription of any gene. The interaction of NR with some NR box-containing coactivators is necessary and sufficient to stimulate transcription in response to nuclear receptor ligand. This was elegantly demonstrated in one study, in which swapping of a CoRNR box for a NR box in such a NR coactivator conferred the ability of an NR antagonist (tamoxifen, an estrogen receptor antagonist) to stimulate transcription and cellular proliferation in breast cancer cells (73). Transcription was presumably initiated by endogenous, unaltered estrogen receptor (ER, an NR). Chromatin-associated changes following assumption of the coactivator binding mode and binding of such coactivators are also required for transcription initiation from NR-regulated gene promoters and are considered in below sections 1.3.
1.2.3 **SF-1 Ligands Enable Corepressor and Coactivator Binding Modes**

Endogenous SF-1 ligands have only recently been identified by *in vivo* studies. In a study by Urs *et al*, a catabolic product of complex sphingolipid breakdown (and an intermediate in the formation of complex sphingolipids), sphingosine, was identified as an SF-1 ligand. Sphingosine bound purified SF-1 with an apparent $K_d$ of 0.325 µM in labeled ligand displacement studies (74). This study also confirmed by tandem mass spectrometry that sphingosine copurified with receptor and that its binding decreased in response to treatment of human adrenocortical H295R cells with membrane permeable cAMP analog, dibutyl cyclic AMP (Bt$_2$cAMP). Wild type (WT) SF-1 and LBD mutants found to alter sphingosine binding of the NR were characterized in an assay of CYP17 reporter activity. In this assay, sphingosine treatment of steroidogenic placenta cells expressing only exogenous SF-1 demonstrated that sphingosine specifically reduced the ability of SF-1 to initiate CYP17 transcription, but only if the LBD was capable of binding sphingosine at levels similar to WT receptor (74). Finally, this study showed that sphingosine treatment potentiated the repression of CYP17 reporter by a NR corepressor and abrogated activation by a NR coactivator (74). In total, this study strongly suggests that sphingosine is an antagonist of SF-1 transcription factor function on the CYP17 gene promoter.

A recent study failed to verify the role of sphingosine as a *bona fide* SF-1 antagonist (75). Either sphingosine solvation in DMSO (75) rather than ethanol (74) prior to treatment of cells, or its intracellular conversion to sphingosine-1-phosphate during treatment may explain this discrepancy. Sphingosine-1-phosphate is an autocrine/paracrine activator of CYP17 transcription in H295R cells (76) and possibly of
other SF-1 regulated genes that have roles in sterol metabolism. Thus, there is mounting evidence to indicate that sphingolipid metabolism is interconnected with the cell- and gene-specific regulation of SF-1 targets and steroidogenic potential.

Recently, phospholipid agonists of SF-1 which stimulate the coactivator binding mode (Figure 1.3B) were identified in vivo. Using H295R cells, 26 carbon phosphatidic acid specifically copurified with SF-1 from H295R cells (77). Moreover, phosphatidic acid was rapidly produced by nuclear diacylglycerol (DAG) kinase(s) in these cells in response to Bt2cAMP. Knockdown of SF-1-interacting DAG kinase theta reduced Bt2cAMP-dependent CYP17 transcription, and in opposition, its overexpression stimulated CYP17 transcription initiated by SF-1, but not when NR box motifs in the kinase were disrupted by mutation (77). This study allows one to conclude the following with some confidence. 1) One or more forms of DAG kinase is capable of interacting with SF-1. 2) This mode of interaction precedes loading of agonist, phosphatidic acid, which allows SF-1 to adopt a more stable coactivator interaction mode. 3) Agonist is synthesized in response to ACTH/cAMP signaling. The fact that a NR box is used for SF-1 interaction coincides with a feed forward mechanism where colocalization of SF-1 and its DAG kinase partner increases following agonist production (77).

SF-1 ligand binding function is positioned in the nucleus of adrenal cortex cells to enable transcriptional responses to signaling downstream of ACTH/cAMP. Mounting evidence suggests that the nuclear environment of this tissue integrates lipid metabolism and dynamics that occur under basal conditions to antagonize SF-1 (sphingosine production as an intermediate in anabolism and catabolism of sphingolipids).
Alternately, lipid metabolism which leads to activation of SF-1 targets is rapidly induced by cAMP (sphingosine kinase and DAG kinase activation).

1.3 Induced Transcription Overcomes Multiple Barriers through an Ordered Sequence of Events

Most of the SF-1 structure can be assembled from crystallographic (44, 64, 65) and NMR (43) data in which DNA recognition and binding and, separately, ligand binding, are independently understood. This raises the question of how DNA binding and ligand binding functions interrelate during transcriptional activation, particularly in the context of signaling and transcription of genes which are enmeshed in chromatin.

While SF-1 is nuclear in H295R cells, its subnuclear localization in adrenal cortex does vary in the presence and absence of steroidogenic stimuli (78). In particular, cAMP appears to increase dynamic colocalization of SF-1 with the dedicated NR coactivator steroid receptor coactivator-1 (SRC-1) (78), and recently, the Sewer lab identified cAMP-stimulated colocalization of SF-1 and DAG kinase theta (77). This raises many questions. For example, which coregulators does SF-1 require for transcription? What SF-1 complexes are cAMP/PKA responsive and dependent? Which of these complexes occur on the CYP17 promoter chromatin prior to transcription of this gene, and what is the order of events occurring there? Finally, which of the possible post-translational modifications to SF-1 (described in Section 1.4) coincide with and enforce this order of events? An ordered sequence of coregulator exchange is consistent with post-translational modification and remodeling of chromatin, transcription factor, and/or chromatin-associated proteins, and this topic and its implications are given special attention here and in the three respective results sections of this dissertation.
Considerable progress has been made in the last decade to extend our understanding of the mechanisms by which transcription factors including nuclear receptors enable a characteristic accelerated rate of transcription induced by diverse signals. In this section, both general functions specific to inducible transcription, and specifics of cAMP-induced transcription requiring SF-1/target gene interaction are reviewed. The overlap, differences, and questions raised about SF-1 specific mechanisms that arise from comparison between these two sets of results are the context for SF-1-specific studies described in the following chapters of this dissertation. In particular, the role of chromatin (the packaging of eukaryotic DNA) in gene-specific mechanisms of transcription is considered in as much detail as possible. Chromatin structure and the central role of nucleosomes are reviewed in Figure 1.4.

Transcription factors for inducible genes require specific promoter targeting function. For promoter targeting to occur, barriers are overcome as a result of mechanisms conferred by the inducing signal. Compartmentalization of the transcription factor away from its target promoters is overcome by signal-induced disassembly of sequestered, inactive complexes and/or assembly of active complexes which have access to DNA targets. For example, GR agonists cause the receptor to dissociate from chaperone in the cytoplasm (35), enabling GR homodimerization, nuclear entry, and transcriptional activation. Then, the transcription factor must bind its target promoter with high specificity at recognized binding site(s), and somehow increase the accessibility of this site. Both GR and ER overcome precise positioning and arrangement of histones within nucleosomes which obstruct the most stable NR-DNA interaction at
Figure 1.4 Chromatin organization and the role of nucleosomes.  A. DNA double helix (2 nm diameter fiber) is wrapped around a cylinder-shaped histone octamer comprised of 2 H2A/H2B dimers and an H3/H4 tetramer. The 147 base pairs (bp) of DNA plus the histone octamer make up a nucleosome. Amino-terminal tails of histones H3 and H4 are visible extending beyond the compact nucleosome cylinder. The X-ray crystallographic structure is from protein data bank 1AOI and (79). B. “Beads on a string” have been visualized by electron microscopy and are made up of nucleosomes connected by short stretches (~10 bp) of “naked” DNA. Chromatin in this configuration is called euchromatin. C. A 30 nm solenoid fiber is made up of a two-start helix of nucleosomes linked by the histone linker protein H1 (80); alternate geometries may also form and interchange with this configuration (81). D. Higher order loops of solenoid are thought to form, particularly in regions of the genome which are not transcriptionally active, aided by additional scaffolding protein(s). E. The metaphase chromosome is the most highly packed form of chromatin, entirely heterochromatin.

gene-specific promoter binding sites (nicely reviewed in (82)). Recently, positioning of nucleosomes was determined to have a strong sequence-dependent thermodynamic component (83), although there is also subtle alteration in nucleosome positioning due to the tissue context and possibly the frequency of past transcription initiation from a gene promoter (84).

As mentioned, although SF-1 is predominantly localized in the nucleus, its subnuclear localization is sensitive to cAMP, which increases interaction with a dedicated coactivator (78). Sewer et al found that in vitro translated SF-1 binds to the human CYP17 promoter in the presence or absence of cAMP at the -57/-38 segment (20) which includes the element TCAAGGTGA. This element is similar to the consensus high-
affinity SF-1 binding site confirmed by Morohashi et al, (C/T)CAAGG(T/C)CA, occurring with some variation on the promoters of all steroidogenic P450 genes with the capability of binding SF-1 in vitro (85). However, Sewer et al found that additional nuclear RNA binding proteins, 54 kDa nuclear RNA binding protein (p54nrb) and polypyrimidine tract binding protein associated splicing factor (PSF), formed a higher weight complex with SF-1 and perhaps other proteins on the CYP17 DNA element only within 120 minutes of Bt2cAMP treatment and only if protein translation was not inhibited (20). It can be concluded that cAMP is a sufficient signal to redeploy SF-1 in unique protein complexes with roles in targeting transcription function to CYP17 via a SF-1 recognized element.

1.3.1 The Central Role of Chromatin in Cyclic Transcription of Inducible Genes: An overview of progressive interactions of promoter chromatin, transcription factor(s), and coregulators

The role of chromatin in transcription has been considered in a number of other studies. In a seminal study, Shang et al identified cyclic patterns of mRNA accumulation of three ER-responsive genes in nuclei of ER-containing cells treated for specific times with estradiol, the endogenous ER agonist (73). This nuclear run-on data was considered as evidence for cycles of transcription activity, and indeed the timing following estradiol treatment at which mRNA accumulated coincided with times when ER was most stably associated with the promoter of each gene. The latter data was obtained using chromatin immunoprecipitation (ChIP). Shang et al concluded that “the regular cycling of the ERα transcription complex may represent a mechanism that favors continuous sampling of the external milieu.” That is, transcription cycles ensure that inducible transcription remains responsive to the current environment, particularly the loss and gain of inducing signals.
Since that study, cyclic transcription has been observed for multiple inducible genes with central roles for cycling of ER (86, 87), androgen receptor (88), thyroid hormone receptor (89), vitamin D receptor (90), or other inducible transcription factors outside of the NR superfamily, including p53 (91) and NFkB (92). Invariably in this phenomenon, the binding to a promoter by coactivators with different classes of chromatin-modifying capability (described in the following section), is followed by the binding of general transcription factors required for transcription of any gene (such as TATA binding protein and the mediator complex) and RNA polymerase II. The coactivators and general transcription factors do this specifically as and after an inducible sequence-specific transcription factor associates with maximum stability at a site that includes a sequence recognized by the inducible transcription factor, often also at the proximal promoter of the gene being studied.

Finally, the protein degradation function of the proteasome (86, 88) was found to be important in enabling repeatability of this process during subsequent cycles. When the proteasome was disabled (e.g. with MG-132 (73)), when the reversal of post-translational chromatin modification was inhibited (e.g. with trichostatin A (86)), or when phospho-RNA polymerase-mediated elongation was prevented by a kinase inhibitor (5,6-dichloro-1-ß-D-ribofuranosylbenzimidazole (DRB) (73)), cycles of induced stable transcription factor binding and loss stalled, either with high or low binding profiles, depending on the specific treatment. For example, enforcement of static, rather than dynamic, hyperacetylation of histones (discussed in the following section) by trichostatin A led to the loss of stable binding of polymerase and transcription factor to the promoter after 14
hours, but stalling of these factors on the chromatin could be induced in the presence of both trichostatin A and proteasome inhibitor MG-132 (73).

In total, these ChIP studies and other ground breaking studies (including UV laser crosslinking and fluorescence resonance energy transfer experiments reviewed in (82)) greatly enhanced our knowledge of the mechanistic basis by which transcription rate is determined for inducible genes. Gene promoters that were inducible in one context (‘inactive but poised’ or ‘regulated with late accessibility’) may be ‘constitutive and immediately accessible’ or ‘active’ in another context, while in other situations, such as in cell lines or tissues in which a required inducible transcription factor is absent, the gene may be fully repressed (82, 92). A distinction for inactive but poised genes was also made clearly in a study that showed that ER isoforms maintain promoter surveillance for an inducing signal on a gene ‘poised’ for transcription even in the absence of agonist by cycling on and off of a target promoter, bringing along coactivators and corepressors, albeit transiently (93). This activity keeps chromatin assembly, structure, and modifications dynamic, even to the point that general transcription factors can increase their association with the transcription start site, albeit nonproductively, i.e., not resulting in mRNA transcription (93).

Métivier and colleagues concluded from a compendium of kinetic ChIP studies of inducible gene promoter interaction with transcription factors that there are three phases of cyclic transcription on such a promoter (94). First, there is achievement of transcription competence. Then, productive rounds of transcription occur. Progress during this stage is marked by an increase in histone tail acetylation and other successive dynamic covalent modifications to chromatin, which modify nucleosome structure, and
decrease DNA-histone affinity. Finally, a clearance phase occurs, perhaps to avoid
stalled or non-specific transcription such as that seen during long term enforced
acetylation of nucleosome tails; this reverses permissive chromatin modifications, general
transcription factor accessibility, and possibly results in compaction of chromatin
structure to the least accessible poised form (e.g. a solenoid configuration, as shown in
Figure 1.4). While the first and last phases of the transcription cycle are likely to
determine the overall rate of transcription, these steps are less understood at a
mechanistic level than transcription itself. Even the most complete studies find multiple
alternate inducible complexes of inducible transcription factor and coactivators (87).
However, the functions of those coactivators with respect to chromatin post-translational
modification and structural disruption or ordering are conserved, and are considered in
detail in subsections 1.3.3-1.3.4.

1.3.2 Transient Behavior of NR-Coregulator Complexes

NR interactions with coactivators are generally activated by agonist ligand, but
these occur in an environment of competition among coregulators with similar binding
criteria, which may be as simple as display by a coactivator of a small amphipathic helix,
the NR box described in section 1.2.2. This helix could even form from an otherwise
unstructured primary sequence as a result of induced fit (94) following NR achievement
of the coactivator binding mode (95). Each transcription cycle can last 40 or more
minutes (87). However, even RNA polymerase II, which must remain bound to DNA as
it reads the length of that gene at 15 to 85 percent of the rate at which naked DNA is
transcribed (96)—which is likely to be between 300 to 1200 or more base pairs per minute
(97-99)—this transcription requires just a fraction of the time during which the gene’s
chromatin is competent for transcription. So, multiple rounds of transcription are very likely occurring during a single phase of transcription competence in what this dissertation considers a transcription cycle.

Likewise, studies using methods which do not rely on crosslinking of DNA to protein (reviewed along with seminal kinetic ChIP studies in (82)) suggest that no one NR molecule maintains its hold on a recognized DNA element for more than a small fraction of the transcription cycle, perhaps only seconds (100). So many coactivator-chromatin interactions can occur with a signature rate of stochastic progress in modifying chromatin post-translationally and structurally reordering nucleosomes (100). It follows that, although ligands can increase the DNA binding stability of a NR or the likelihood that a NR will recruit coactivators which remodel chromatin for a sufficient period of time, the act of modifying chromatin, particularly structural reordering of nucleosomes, may necessitate loss from chromatin of the very NR-coactivator complex which started that process (100), but this process enables another unique complex to possibly bind with greater affinity and act at the same promoter site.

The above logic and integration of data from study of transcription in vitro, along with chromatin kinetics in living cells on a much shorter time scale than ChIP experiments permit (2-5 minutes or greater), provide important information about the physical nature of the process by which competence for transcription is reached at an induced gene promoter. High-affinity, low specificity interactions with agonist-liganded NRs are likely to occur between NR and coactivators near NR binding sites on chromatinized DNA. In this way, NR agonist increases the local concentration of multiple coactivator-NR complexes which modify that chromatin stochastically before
competence for assembly of a stable general transcription factor complex occurs, i.e. the classical preinitiation complex. Thus, in the first stage of a transcription cycle (achieving competence for transcription), chromatin achieves competence for RNA polymerase II activity through the actions of multiple coactivator-NR complexes.

Now we have the background necessary to fully evaluate the most complete studies of transcription cycling by ChIP. One such study identified at least six unique complexes of ER with coactivators on a model ER-responsive gene promoter (87). This was accomplished by performing serial ChIP of protein pairs at times suggested in single-factor IP kinetic ChIP experiments. While the authors concluded that these complexes are alternately required in order to achieve transcription competence in a transcription cycle, given the above logic that these complexes rapidly exchange, it is just as likely that each complex has a requisite role in each transcription cycle.

1.3.3 Coregulators Manipulate the Histone Code and Nucleosome-DNA Interaction

What are the functions of NR-coactivator complexes, almost entirely encoded in the structure and sequence of the coactivators? In the review of their own work, the Gannon laboratory identified four functions for coregulators, each of which involves overcoming chromatin-imposed regulatory constraints on transcription.

First, coactivators have the ability to write, “read,” and erase the histone code, and to interact with and bridge a NR and multiple chromatin modifying enzymes into a functional complex that marks chromatin with a specific set of post translational modifications (PTMs). Histone tails which protrude from nucleosomes and their wrapped DNA (Figure 1.4A), and even the core of the nucleosome, are readily modified
post-translationally with molecular marks including lysine acetylation, lysine and arginine methylation, serine/threonine phosphorylation, and lysine ubiquitination. These PTMs, and in particular, compatible combinations of modifications (101, 102), change affinity of the local chromatin for chromatin-associating factors, including coregulators of transcription. Therefore, following combinatorial modification of chromatin initiated by early NR-coactivator complexes, chromatin-modifying coactivators which act later do not necessarily need to interact with an induced transcription factor directly, but can interact with histone tail(s) modified earlier. The particular pattern of modification of a nucleosome or a set of nucleosomes in a region of DNA defines whether that DNA is silenced or actively transcribed (if the region encodes a gene (103)), or if that region of DNA is prepared to undergo mitotic DNA replication, or in need of damage repair. Important PTM sites and covalent modifications of histones related to signaling one of these particular states are given in Table 1. In addition, a second class of modifications is listed there. These modifications are naturally occurring or engineered nucleosome structural modifications, some that helped to identify solenoid chromatin geometry, and a role for nucleosome repositioning in transcription.

The second class of histone modifications in table 1.1 primarily relates PTMs to changes in nucleosome structure or histone/DNA binding energy and stability. Indeed, the second identified role of a subset of coactivators is to alter nucleosome structural integrity, positioning on DNA, and histone/DNA interactions. This chromatin reorganization is ATP dependent and may involve disruption or restitution of intermolecular histone interactions, based on in vitro studies (104, 105). A founding class of proteins with such a function was first identified in yeast. These proteins form
Table 1.1 A list of histone modifications. See this review (106) by Cosgrove and Wolberger for more information on most of the above modifications. *Formation of solenoid chromatin with internucleosome contacts was tested by engineered cystine disulfide crosslinking of histones H4 and H2A mutated to cysteine at these positions (80). Ac, acetyl; P, phospho; Me, methyl; Ub, ubiquitinated.

<table>
<thead>
<tr>
<th>Modification</th>
<th>Associated with</th>
</tr>
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<tbody>
<tr>
<td>H3K9-Me3</td>
<td>HP1 binding, increased heterochromatin properties</td>
</tr>
<tr>
<td>H4K20-Me3</td>
<td>increased heterochromatin properties</td>
</tr>
<tr>
<td>H3K27-Me</td>
<td>increased heterochromatin properties; placed by polycomb complex</td>
</tr>
<tr>
<td>H3S10-P</td>
<td>inhibits or counters H3K9 methylation; stimulates H3K14-Ac</td>
</tr>
<tr>
<td>H2B K123-Ub</td>
<td>stimulates H3K79-Me; removed or lost during transcription</td>
</tr>
<tr>
<td>H2A K119-Ub</td>
<td>prevents efficient RNA polymerase elongation on some genes; gene silencing; prevents H2A/H2B dimer removal by chaperone(s)</td>
</tr>
<tr>
<td>H2A.X S139-P</td>
<td>DNA double strand breaks, DNA replication</td>
</tr>
<tr>
<td>H3K4-Me2 or 3</td>
<td>transcription; placed by Set1, stimulated by H2BK123-Ub; turnover of H3K9-Ac, H3K27; recruitment of nucleosome remodelers, coactivators</td>
</tr>
<tr>
<td>H3K36-Me3</td>
<td>co-transcriptional histone deacetylation; placed by Setd2</td>
</tr>
<tr>
<td>H3K79-Me</td>
<td>DNA double strand breaks and checkpoint halt; placed by Dot1L; transcription; stimulated by H2B K123-Ub</td>
</tr>
<tr>
<td>H4K20-Me</td>
<td>DNA double strand breaks and checkpoint halt</td>
</tr>
<tr>
<td>H3K9,14,18, 23, 27 -Ac</td>
<td>hyperacetylation of N-terminal tail lysines; decreased DNA binding; transcription &amp; conditions favoring nucleosome sliding, H1 dissociation</td>
</tr>
<tr>
<td>H4K5, 8, 12, 16, 20 -Ac</td>
<td>DNA CpG island methylation boundaries; H4K20-Me2</td>
</tr>
<tr>
<td>H4R3-Me2</td>
<td>counters H3K4Me2 or 3</td>
</tr>
<tr>
<td>H3R2-Me2</td>
<td>ER-mediated transcription activation</td>
</tr>
<tr>
<td>H3R17-Me</td>
<td></td>
</tr>
</tbody>
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**Structural Modifications**

<table>
<thead>
<tr>
<th>Modification</th>
<th>Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>H4K91-Ac</td>
<td>Decreased H2B E63 interaction with H4K91</td>
</tr>
<tr>
<td>H3K56-Ac</td>
<td>near DNA entry/exit site on nucleosome; transcription; H3 exchange</td>
</tr>
<tr>
<td>H4R45</td>
<td>DNA minor groove insert, sensitive to H3T118-P</td>
</tr>
<tr>
<td>H3T118-P</td>
<td>increased DNA mobility</td>
</tr>
<tr>
<td>H4 V21, N-terminal tail near globular core</td>
<td>required for solenoid formation, interaction with the H2A core of an adjacent nucleosome near H2A E64*</td>
</tr>
<tr>
<td>Lateral surface of globular domain of nucleosome</td>
<td>some mutants show SWI/SNF independent transcription in yeast</td>
</tr>
</tbody>
</table>
large complexes and when components of these complexes were functionally inactivated by mutations, the corresponding yeast strains were incapable of activating transcription of genes required for sucrose fermentation or mating type switching—thus, this set of proteins became known as mating type switch/sucrose non-fermenting proteins (SWI/SNF) (reviewed with a focus on broader transcription regulation in (107)).

SWI/SNF complexes invariably include a member with ATPase catalytic function (Brahma or Brahma related gene 1 (BRG1)), and a ubiquitously expressed large structural scaffold (BRG1 associated factor (BAF250)/p270/ARID1), as well as other structural components which may be required for function. At least in higher eukaryotes, the largest protein in SWI/SNF complexes, BAF250, is rich in NR boxes, which may explain a strong positive effect of overexpression of this protein on NR-dependent transcription (108, 109). Other subunits have also been shown to mediate SWI/SNF complex interaction with NRs (107). However, SWI/SNF ATPase overexpression or manipulation can positively or negatively regulate transcription of various genes (cf. above references, with SWI/SNF roles in NR-mediated repression found in (110, 111)). Therefore, ATP-dependent chromatin remodeling has likely roles in both activation and loss of transcription competence.

Perhaps the perfect verification of this was recently published regarding the specificity of unique SWI/SNF complexes in GR-mediated effects on gene transcription (112). Recall from section 1 that GR both activates and represses transcription of different sets of genes. The particular direction an ATPase complex acts, i.e. toward or away from achievement of transcription competence, is specified in a gene-specific manner by the membership of that complex, because while 40 percent of glucocorticoid-
activated genes required BRG1 function, only 11 percent of glucocorticoid-repressed
genes were rescued by dominant negative BRG1 (112). Therefore, chromatin remodeling
toward transcription competence is downstream of a gene-specific induction signal,
which stimulates specific ATP-dependent chromatin remodeling complex(es) with this
function. Later in a transcription cycle, and on genes which are repressed by an agonist-
bound NR, recruitment of a functionally distinct repression-specific ATP-dependent
remodeling complex that revokes transcription competence by remodeling chromatin to a
compact state must occur. Either event is likely to be based on the ability of specific
members of that complex to read a PTM pattern on histones established, respectively,
following induction or after the last round of transcription in a transcription cycle. In
support of the above, conserved domains capable of binding to either acetylated histones
or methylated histones are prevalent features of subunits of distinct classes of ATP-
dependent remodeling complexes (e.g. the SWI/SNF complex member BAF180 has
bromodomains capable of binding acetyl-lysine, while Mi-2, a member of a remodeler
complex which is typically repressive, as well as BAF155/BAF170/SMARCC2 have a
chromodomain capable of reading methyl-lysine) (113). At this point, it is highly
relevant to note that in addition to SWI/SNF complexes, four other classes of ATP-
dependent chromatin remodeling complexes with ATPase subunits related to SWI/SNF,
but with various differences or similarities in membership by other factors (and some
with previous species-specific designation) are cross-referenced in this useful review
(107).

The *in vitro* data indicating that disrupted nucleosomes support accelerated
transcription (104, 105) in conjunction with the established function of ATP-dependent
nucleosome remodeling complexes suggests that these are gatekeepers for gene promoter and downstream gene accessibility. They are functionally positioned to not only determine the rate at which a gene promoter becomes transcriptionally competent, but also should be able to determine the rate of transcription elongation by RNA polymerases through their activity at genes downstream of promoters, and recent work is starting to bear out this hypothesis (114).

A third class of chromatin-associated modification with a role in cyclic transcription that has recently been identified by the Gannon laboratory is the reversible strand-specific methylation of isolated CpG dinucleotides, found to occur on the region of 400 proximal bases of an ER-responsive model promoter (115). Apparently, this modification can occur on a cycling promoter with the recruitment of DNA methyltransferases, and this study is also the first to demonstrate eukaryotic methyl-cytosine deaminase activity of one such methyltransferase, generating thymine at the former site of cytosine methylation. As a transcription cycle proceeds, not only are these enzymes sequentially recruited in coactivator complexes, but base-excision repair machinery is also required to reverse mismatched base pairs occurring after methyl-cytosine deamination (115). The implications for methyl CpG-marked progress through transcription cycles are only beginning to be realized.

The fourth and final class of coactivator-associated functions that the Gannon lab listed (94) is histone H1 dissociation and association. It is evident that the density of nucleosomes distributed on a genomic region may underlie the likelihood of histone H1 and other linker proteins to associate with that region and promote a solenoid conformation of chromatin. H1 binding to DNA, but not necessarily H1 linkage of
nucleosomes into higher order structure, is also affected by an increased affinity for methylated linker DNA between nucleosomes, because mobility of H1 increases in cells with defective DNA methylation (116). Other internucleosome-linking proteins like HP1 are capable of reading methyl-lysine (113), and are enriched on chromatin with histone H3K9 methylated (117). Thus, there is crosstalk between DNA methylation, the histone code—particularly histone lysine methylation—and heterochromatin packing. Given the requirement for nucleosome occupancy of solenoid DNA, then nucleosome occupancy at promoters and transcription start sites (perhaps with intrinsically lower density at poised inducible genes, and definitely at housekeeping genes (118)), underlies the decreased propensity for regulatory elements of these genes to exist in solenoid conformation.

However, it is conceivable that a solenoid conformation is adopted at some point during transcription cycles. Methods which could verify whether a specific region of genomic DNA has adopted a solenoid conformation may remain to be developed. However, globally, chromatin conformations can be differentiated and quantified, and surprisingly, there is some evidence that histone H1 can regulate expression positively in a gene-specific manner (119), perhaps by helping fix the positions of specific nucleosomes ideally for transcription factor binding.

In addition to the above four functional classes of coactivator function, it is easy to envision three additional coactivator functions which coordinate transcription. For example, it has been found that certain coactivators, and early inducible viral molecular interaction hubs are capable of bridging coactivator complexes associated with inducible transcription factors to general transcription factors (120, 121).
Next, roles for topoisomerases in relieving transcription-associated supercoiling have been clearly implicated with requisite roles in promoting efficient transcription (122, 123). Therefore, it is conceivable that a class of coregulators function during achievement of competence for transcription in enhancing topoisomerase access to chromatin, perhaps in conjunction with altered H1 linker occupancy (122).

Finally, a novel function for a small set of corepressor-complex associated factors as coregulator exchange factors has been identified as necessary for disassembly of these corepressor complexes preceding functional coactivator/NR complex formation on specific promoters (124). Examples of coactivators from all seven classes with roles in achieving competence for transcription in cyclic chromatin-mediated transcription are listed in table 1.2.

1.3.4 The “Clearance Phase” of Cyclic Transcription: Corepressors enable a return to repressive chromatin structure

What causes the transition to repressive chromatin from active transcription of competent chromatin after possibly multiple rounds of transcription, and in the continued presence of signal capable of inducing transcription? As mentioned previously, Shang et al found that DRB, an inhibitor of casein kinase II and cyclin dependent kinases (CDKs) 7 and 9 (the latter target RNA polymerase II), stalled ER on promoters indefinitely, while low dose α-amanitin (blocking RNA synthesis by polymerase II directly) did not do this (73). A consistent hypothesis is that polymerase II in some configurations (i.e., the last round of transcription during a cycle, which, like all RNA elongation events, is inhibited by DRB) couples that event to chromatin remodeling that reduces accessibility. Such a process has been found to occur in yeast (125, 126) and mouse (127), where histone
Table 1.2  Representatives of seven classes of induction-specific coregulators associated with cycles of transcription. Homologs refer to mammalian homologs of yeast proteins. This list is not exhaustive.

<table>
<thead>
<tr>
<th>Class</th>
<th>Full Name</th>
<th>Associated with</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Histone PTM Enzymes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRC-1 / NCoA1</td>
<td>nuclear receptor coactivator-1</td>
<td>(lysine) acetylation; glucocorticoid response; NR boxes</td>
</tr>
<tr>
<td>SRC-2 / GRIP-1 / NCoA2</td>
<td>nuclear receptor coactivator-2</td>
<td>acetylation-associated; female reproduction; NR boxes</td>
</tr>
<tr>
<td>SRC-3 / ACTR/AIB1/NCoA3</td>
<td>general control nondepressed 5</td>
<td>acetylation; amplified in some breast cancers; NR boxes</td>
</tr>
<tr>
<td>GCN5, human homologs</td>
<td>CREB binding protein</td>
<td>acetylation; Ac-lysine binding; neural tube closure</td>
</tr>
<tr>
<td>P/CAF</td>
<td>p300</td>
<td>acetylation; Ac-lysine binding; memory and stress</td>
</tr>
<tr>
<td>CBP</td>
<td>E1A binding protein p300</td>
<td>acetylation; Ac-lysine binding; hypoxia induced txn.</td>
</tr>
<tr>
<td>TIP60 / HTATIP / ESA1</td>
<td>HIV Tat-interacting protein, 60 kDa</td>
<td>acetylation; Me-lysine and/or RNA/DNA binding</td>
</tr>
<tr>
<td>CARM1 / PRMT4</td>
<td>coactivator arginine methyltransferase 1</td>
<td>arginine methylation at H4R3; 1 of 7 PRMTs; repression</td>
</tr>
<tr>
<td>LSD1</td>
<td>lysine specific demethylase 1</td>
<td>arginine methylation at H3R17; ER-mediated transcription</td>
</tr>
<tr>
<td>Set1, human homologs</td>
<td>e.g. set-domain containing protein 1A</td>
<td>mono- and di-Me lysine demethylation; activation complex</td>
</tr>
<tr>
<td>Dot1, human homolog</td>
<td>Dot1-like</td>
<td>Histone H3K4 mono-, di-, and tri-methylation, activation</td>
</tr>
<tr>
<td>Jmj-c domain family</td>
<td>Jumonji-c domain containing proteins</td>
<td>Histone H3K79 methylation; G1 (cell cycle); transcription</td>
</tr>
<tr>
<td>II. ATP-Dependent Chromatin Remodeling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SWI2 / SNF2 homologs</td>
<td>brahma, brahma related gene 1</td>
<td>ATPases associated with SWI/SNF, RSC complexes</td>
</tr>
<tr>
<td>SNF2H, SNF2L1</td>
<td>imitation SWI complex</td>
<td>ISWI also repositions/reorders nucleosomes; txn/repression</td>
</tr>
<tr>
<td>BAF250 / p270 / ARID1</td>
<td>large structural subunit of SWI/SNF</td>
<td>a subunit of above complexes that binds DNA and NRs</td>
</tr>
<tr>
<td>BAF180</td>
<td>structural subunit of SWI/SNF</td>
<td>Ac-lysine binding subunit of above complexes</td>
</tr>
<tr>
<td>III. DNA Methylation-Associated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNMT3a, b</td>
<td>DNA methyltransferases 3a, b</td>
<td>deamination of 5'-Me cytosine</td>
</tr>
<tr>
<td>TDG</td>
<td>thymine DNA glycosylase</td>
<td>cleaves T:C mismatches; base excision; SRC interaction</td>
</tr>
<tr>
<td>p68</td>
<td>p68 RNA helicase</td>
<td>demethylation complex member; coactivator function</td>
</tr>
<tr>
<td>IV. Histone H1-Associated Solenoid Formation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMGB 1/2</td>
<td>high mobility group B 1/2</td>
<td>acidic tract; may displace linker histones; Wdhd1/AND-1 is</td>
</tr>
<tr>
<td>AND-1 / Wdhd1</td>
<td>acidic nucleoplasmic DNA binding prot.</td>
<td>an HMG factor in a SRC-1 NR coactivator complex</td>
</tr>
<tr>
<td>H1, H5</td>
<td>linker histones of the histone H1 family</td>
<td>binds methylated DNA; may help activate select genes</td>
</tr>
<tr>
<td>V. General Transcription Factor (GTF) or Structural Bridging</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MFB1</td>
<td>multiprotein bridging factor</td>
<td>links SF-1 to general transcription factors</td>
</tr>
<tr>
<td>some TFII complexes</td>
<td>general transcription factor</td>
<td>recruit RNA polymerase II, bridge to TATA binding protein</td>
</tr>
<tr>
<td>mediator complex</td>
<td>general transcription factor</td>
<td>sometimes considered as a coactivator</td>
</tr>
<tr>
<td>Med1 / TRAP220 / DRIP205</td>
<td>member of MLL1/MLL2 complex</td>
<td>bridge NRs to Mediator, TFIID; has NR boxes like NCoAs</td>
</tr>
<tr>
<td>menin</td>
<td>activating signal coactivator-2</td>
<td>H3K4 methylation complex targeting by liganded ER</td>
</tr>
<tr>
<td>ASC-2/AIB3/HoxA7/NCoA6</td>
<td>RNA recognition motif domain proteins</td>
<td>MLL3/MLL4 H3K4 Me-transferase; stimulates DNA-PK</td>
</tr>
<tr>
<td>PGC-1, p54, PSF</td>
<td>adenosine early expressed antigen 1A</td>
<td>bridge preinitiation/transcription to splicing machinery, RNA</td>
</tr>
<tr>
<td>E1A</td>
<td></td>
<td>non-mammalian, viral interaction hub; (anti)oncogenic;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>inhibits acetylation of non-histone substrates</td>
</tr>
<tr>
<td>VI. Topoisomerase Access</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA-PK (Ku70, Ku86)</td>
<td>Topoisomerase IIβ</td>
<td>causes double strand break at site of promoter induction</td>
</tr>
<tr>
<td>PARP-1</td>
<td>DNA-dep. protein kinase (associated)</td>
<td>DNA break associated DNA binding and kinase activities</td>
</tr>
<tr>
<td></td>
<td>poly(ADP-ribose) polymerase 1</td>
<td>DNA-damage associated ADP-ribose polymerization on</td>
</tr>
<tr>
<td></td>
<td></td>
<td>protein substrates, including histones; may deplete NAD'</td>
</tr>
<tr>
<td>VII. Coregulator Exchange</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBL1</td>
<td>transducin-ß-like 1</td>
<td>Rqd. for signal / ligand-dependent coregulator exchange</td>
</tr>
<tr>
<td>TBLR1</td>
<td>TBL-related 1</td>
<td>… on NR &amp; NR corepressor (NCoR)-regulated promoters</td>
</tr>
</tbody>
</table>
H3K36 methylation occurs cotranscriptionally. In yeast, this modification enables recruitment of a histone deacetylase (HDAC)-containing complex and thereby, a return to more compact chromatin, as evidenced by the function this has in preventing inappropriate transcription initiation downstream from gene promoters (within genes) (126). The SWI/SNF-related ATPase Mi-2 has been found on promoter chromatin during the clearance phase of transcription by ER (87) in a 5’-methyl CpG dependent manner (115). This fits with Mi-2 association in the nucleosome remodeling and deacetylase (NuRD) complex, which directs HDAC activity at histone tails, slides nucleosomes, and includes methyl-DNA binding proteins (reviewed in (128)). It is possible that an increase in DNA supercoiling or secondary structure occur cotranscriptionally, and this could also lead to an altered orientation of post-transcribed DNA and nucleosomes (129).

Regardless of the cause, the effects of promoter clearance are consistent with temporary desensitization, then resensitization of chromatin to transcription-inducing signal, co-transcriptional PTM of histones, restitution of more compact chromatin structure, and masking DNA to prevent spurious transcription factor binding and transcription initiation downstream of the appropriate transcription start site(s). None of these functions need be mutually exclusive. Coregulators and complexes which are confirmed or likely participants in the clearance phase of transcription, (as well as having roles in repression of genes in other contexts) are divided into the same seven functional classes described in the above section and are listed in Table 1.3. Major complexes, and readers and writers of the histone code which enable processing during transcription cycling are shown in Figure 1.5.
Figure 1.5 Major complexes involved in chromatin modification associated with cyclic NR-chromatin interaction. The formation of homogeneously packed solenoid (heterochromatin) by NR may co-occur with or follow occupancy by NR-corepressor complexes; alternatively, solenoid formation may be more heterogeneous, with short tracts of NR-accessible DNA between more highly packed regions. Refer to tables 1.2 and 1.3 for nomenclature and functions associated with the above coregulators.
Table 1.3  Transcription corepressors associated with static and signal-induced repression, and in some cases, the clearance phase of cyclic transcription. For membership of complexes containing HDACs and species cross-referenced protein nomenclature, see this review (130).

<table>
<thead>
<tr>
<th>Acronym(s)</th>
<th>Full Name</th>
<th>Associated with</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDAC1, 2, 3, 8 (class I)</td>
<td>Histone deacetylases related to Rpd3</td>
<td>Loss of Ac-lysine; CK2 (1, 2, 3) or PKA (8) phosphorylation</td>
</tr>
<tr>
<td>HDAC4, 5, 7, 9 (class IIa)</td>
<td>Histone deacetylases related to Hda1</td>
<td>14-3-3 binding; signaling; low deacetylase activity</td>
</tr>
<tr>
<td>HDAC 6, 10 (class IIb)</td>
<td>Tandem domain histone deacetylase (6)</td>
<td>Ubiquitin binding zinc finger (6); often cytoplasmic (6)</td>
</tr>
<tr>
<td>HDAC 11 (class IV)</td>
<td>Sirtuin 1, mammalian homolog of Sir2</td>
<td>Loss of Ac-lysine; calorie restricted transcription repression</td>
</tr>
<tr>
<td>SirT1 (class III HDAC)</td>
<td>Peptidylarginine demethylaminase 4</td>
<td>Arginine demethylaminase; conversion to citrulline</td>
</tr>
<tr>
<td>PADO1</td>
<td>Jumonji domain containing 6</td>
<td>Arginine demethylaminase; conversion to citrulline</td>
</tr>
<tr>
<td>LSD1</td>
<td>Jumonji AT-rich interactive domain 1C</td>
<td>H3K4-Me3, Me2, or Me demethylase activity</td>
</tr>
<tr>
<td>EHMT2 / G9a</td>
<td>Lysine specific demethylase 1</td>
<td>Mono- and di-Me lysine demethylation; CoREST complex</td>
</tr>
<tr>
<td>Pcg complexes 2 and 3</td>
<td>Euchromatin histone N-Methyltransferase</td>
<td>Lysine methylation of histone H3K9, H3K27, repression</td>
</tr>
<tr>
<td>NCoR1, 2 (SMRT)</td>
<td>Polycomb group methyltransferases</td>
<td>H3K27 methylation; differentiation</td>
</tr>
<tr>
<td>CoREST</td>
<td>NSR1</td>
<td>Nucleates HDAC/demethylase complexes; binds NR corepressor mode; SANT domain stimulates HDAC activity</td>
</tr>
<tr>
<td>RIP140</td>
<td>Receptor interacting protein, 140 kDa</td>
<td>RNA &amp; DNA binding; associated with Sin3A, CoREST</td>
</tr>
<tr>
<td>LCoR</td>
<td>Ligand-dependent corepressor</td>
<td>Nucleates LSD1, HDAC, CtbP; SANT domain-containing</td>
</tr>
<tr>
<td>MeCP2</td>
<td>Methyl-CpG binding protein 2</td>
<td>NR corepressor binding mode; HDAC/CtBP assoc.</td>
</tr>
<tr>
<td>TBL1, TBLR1</td>
<td>Transducin-β-like (related) 1</td>
<td>NSR1 binds H3K9-Me; blocks general tran activation</td>
</tr>
</tbody>
</table>

**I. Histone PTM Enzymes**

<table>
<thead>
<tr>
<th>Acronym(s)</th>
<th>Full Name</th>
<th>Associated with</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNMT1</td>
<td>DNA methyltransferase 1</td>
<td>Maintenance methylation following DNA replication</td>
</tr>
<tr>
<td>DNMT3a, b</td>
<td>DNA methyltransferases 3a, b</td>
<td>De novo DNA 5'-cytosine methylation capacity</td>
</tr>
<tr>
<td>DNMT3L</td>
<td>Inactive paralog of DNMT3</td>
<td>Binds specifically to non-H3K4-Me</td>
</tr>
<tr>
<td>MeCP2</td>
<td>Methyl-CpG binding protein 2</td>
<td>Contains a methyl-cytosine binding domain; associates with SWI/SNF</td>
</tr>
<tr>
<td>MBD2, 3</td>
<td>Methyl binding domain proteins 2, 3</td>
<td>Nucleases HDAC/demethylase complexes; binds NR corepressor mode; SANT domain stimulates HDAC activity</td>
</tr>
</tbody>
</table>

**II. ATP-Dependent Chromatin Remodeling**

<table>
<thead>
<tr>
<th>Acronym(s)</th>
<th>Full Name</th>
<th>Associated with</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mi-2</td>
<td>NuRD complex ATPase</td>
<td>Nucleates HDAC/demethylase complexes; binds NR corepressor mode; SANT domain stimulates HDAC activity</td>
</tr>
<tr>
<td>Ino80</td>
<td>INO80 complex ATPase</td>
<td>Nucleates HDAC/demethylase complexes; binds NR corepressor mode; SANT domain stimulates HDAC activity</td>
</tr>
<tr>
<td>BAF170 or 155 / SMARCC2</td>
<td>Structural subunit of SWI/SNF</td>
<td>NSR1 binds H3K9-Me; blocks general tran activation</td>
</tr>
</tbody>
</table>

**III. DNA Methylation-Associated**

<table>
<thead>
<tr>
<th>Acronym(s)</th>
<th>Full Name</th>
<th>Associated with</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNMT1</td>
<td>DNA methyltransferase 1</td>
<td>Maintenance methylation following DNA replication</td>
</tr>
<tr>
<td>DNMT3a, b</td>
<td>DNA methyltransferases 3a, b</td>
<td>De novo DNA 5'-cytosine methylation capacity</td>
</tr>
<tr>
<td>DNMT3L</td>
<td>Inactive paralog of DNMT3</td>
<td>Binds specifically to non-H3K4-Me</td>
</tr>
<tr>
<td>MeCP2</td>
<td>Methyl-CpG binding protein 2</td>
<td>Contains a methyl-cytosine binding domain; associates with SWI/SNF</td>
</tr>
<tr>
<td>MBD2, 3</td>
<td>Methyl binding domain proteins 2, 3</td>
<td>Nucleases HDAC/demethylase complexes; binds NR corepressor mode; SANT domain stimulates HDAC activity</td>
</tr>
</tbody>
</table>

**IV. Histone H1-Associated Solenoid Formation**

<table>
<thead>
<tr>
<th>Acronym(s)</th>
<th>Full Name</th>
<th>Associated with</th>
</tr>
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<tbody>
<tr>
<td>H1, H5</td>
<td>Linker histones of the histone H1 family</td>
<td>Nucleases HDAC/demethylase complexes; binds NR corepressor mode; SANT domain stimulates HDAC activity</td>
</tr>
<tr>
<td>HP1</td>
<td>Heterochromatin protein 1</td>
<td>Nucleases HDAC/demethylase complexes; binds NR corepressor mode; SANT domain stimulates HDAC activity</td>
</tr>
</tbody>
</table>

**V. General Repression Machinery / Bridging**

<table>
<thead>
<tr>
<th>Acronym(s)</th>
<th>Full Name</th>
<th>Associated with</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1A</td>
<td>Adenovirus early expressed antigen 1A</td>
<td>Non-mammalian; viral interaction hub; (anti)oncogenic</td>
</tr>
<tr>
<td>CtBP1 and 2</td>
<td>E1A C-terminal binding proteins 1 &amp; 2</td>
<td>Bridges DNA binding factors to repression machinery; sequesters acetyl-lysine binding and other (co)activators?</td>
</tr>
<tr>
<td>NCoR1, 2 (SMRT)</td>
<td>Nuclear receptor corepressors 1, 2</td>
<td>Nucleates HDAC/demethylase complexes; binds NR corepressor mode; SANT domain stimulates HDAC activity</td>
</tr>
<tr>
<td>Sin3A, B complexes</td>
<td>Homologs A and B of yeast Sin3</td>
<td>Well conserved; HDAC-containing; chromosome integrity</td>
</tr>
<tr>
<td>PSF, p54</td>
<td>PTB-associated splicing factor</td>
<td>RNA &amp; DNA binding; associated with Sin3A, CoREST</td>
</tr>
<tr>
<td>CoREST</td>
<td>Corepressor of RE1-silencing TF</td>
<td>Nucleates LSD1, HDAC, CtbP; SANT domain-containing</td>
</tr>
<tr>
<td>RIP140</td>
<td>Receptor interacting protein, 140 kDa</td>
<td>Binds NR coactivator binding mode; HDAC/CtBP assoc.</td>
</tr>
<tr>
<td>LCoR</td>
<td>Ligand-dependent corepressor</td>
<td>NSR1 binds H3K9-Me; blocks general tran activation</td>
</tr>
</tbody>
</table>

**VI. Topoisomerase Access**

<table>
<thead>
<tr>
<th>Acronym(s)</th>
<th>Full Name</th>
<th>Associated with</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown</td>
<td>Topoisomerase</td>
<td>Unknown; may only occur during transcription activation</td>
</tr>
</tbody>
</table>

**VII. Coregulator Exchange**

<table>
<thead>
<tr>
<th>Acronym(s)</th>
<th>Full Name</th>
<th>Associated with</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBL1, TBLR1</td>
<td>Transducin-β-like (related) 1</td>
<td>Part of NCoR and possibly other corepressor complexes; required for stimulated disassembly of these complexes</td>
</tr>
</tbody>
</table>
1.4 Crosstalk between PTMs, Signaling and NR Transcriptional Mechanisms

PTMs of SF-1 were briefly discussed in relation to the list of functional modules of this NR in section 1.2. The PTM most associated with signaling pathways is phosphorylation, however this section will give particular attention to SUMOylation, acetylation, and phosphorylation of NRs and coregulators. Acetylation and SUMOylation of SF-1 are also PTMs with important functional consequences, only their context in signaling pathways was not well established until recently. Nonetheless, the established downstream effects of these modifications on transcription and their potential for crosstalk, i.e. regulation of one modification by another, are profound.

1.4.1 SUMOylation / SUMOrylation

Small ubiquitin like modifier (SUMO) conjugation to lysine residues (SUMOylation, SUMOrylation) is emerging as a major PTM for functional modification of nuclear proteins. SUMOylation of the SF-1 hinge affects subnuclear compartmentalization, promoting relocalization to regions not involved in active transcription following association with a complex (53). This complex includes a distinct RNA-binding protein partner that may bring SF-1 into contact with SUMO-conjugating machinery (53), but represses a SUMO-deficient SF-1 mutant nonetheless (131). The transcription repressive role of SF-1 hinge SUMOylation has been confirmed by three studies (51-53) and is in distinction to the effect of estradiol-dependent ER hinge SUMOylation, which activates ER-mediated transcription (132). Transcription activation effects of SUMOylation are associated with SUMO competition for ubiquitination sites and proteasome targeting (e.g., in a cascade which stabilizes p53 and increases its activity as a transcription factor (133)), and uncoupling of DNA methylation
and heterochromatin-associated H3K9 methylation (134). However, direct SUMOrylation of transcription factors is more immediately associated with repression in most cases other than ER.

1.4.2 Acetylation

Acetylation on lysine residues of nuclear receptors may occur in a motif which mimics histone targets of histone acetyltransferases (HATs), and this motif is conserved phylogenetically across most nuclear receptors, the exceptions being NURR1, NGFIB, and RXRs (135, 136). Acetylation of SF-1 by the GCN5 HAT was first reported in 2001, and this report suggested that DNA binding domain residues now known to interact with DNA were potential acetylation targets (137). Therefore, mutation of these residues had strong effects on transcription (137), probably independent of acetylation. Residues at the end of the Ftz-F1 DNA binding helix were next found to be acetylated by p300 with cAMP dependence (46). These lysine residues are conserved at positions 106, 109 and 110, and are proximal to an acidic tract at the N terminus of SF-1 (Figure 1.6). However, mutating these and other lysines in the DNA binding domain did not ablate all acetylation of SF-1, suggesting additional sites of acetylation outside the DNA binding regions (46). Despite the fact that these residues do not interact directly with DNA in the below structure (Figure 1.6 and (43)), there was a significant decrease in SF-1 DNA binding in vitro and chromatin binding in vivo when these acetylation targets were mutated (46). Confocal microscopy enabled the conclusion that the Ftz-F1 box conserved acetylation
site could affect coactivator interaction(s), for example with p300 or other HATs, and affected endogenous SF-1 recruitment to p300-enriched foci, which could be sites of active transcription (46). Crosstalk of acetylation with other modifications of SF-1 has not been examined.

1.4.3 Phosphorylation of Factors with a Serine/Threonine-Proline Motif

In addition to AF-2, a region of the SF-1 hinge proximal to the SF-1 LBD was found to be necessary for transcription by SF-1 co-activated by SRC-1 (58). In this region, SF-1 can be phosphorylated at S203, and in vitro, ERK2 does so (49) (However, ERK1 does not (MB Sewer, unpublished observations)). Mutation of this site decreased SRC-2 coactivator function on a SF-1 responsive promoter (49) and S203 phosphorylation downstream of estradiol signaling enabled packing (interaction) of the
C-terminal region of the SF-1 hinge with the LBD proper (50). ERKs and the wider class of kinases to which they belong, MAPKs, are proline directed kinases.

Just recently, a novel approach using phospho-motif specific antibodies identified S203 as a proline-directed cyclin dependent kinase (CDK) target without testing ACTH/cAMP involvement, and this study called into question the specificity of the MAPK pathway inhibitor (48) used in the earlier studies which suggested MAPK pathway involvement in phosphorylation of S203 \textit{in vivo} (49). CDK7 in particular was shown to target SF-1 \textit{in vivo}, and this kinase is a member of the multifunctional general transcription factor complex TFIIH, which directs CDK7 activity toward RNA polymerase II at YSPT\textsubscript{S}PS motifs (the underlined residue is targeted by CDK7, ERK\textsubscript{1} and ERK\textsubscript{2} \textit{in vitro} (138)). This event promotes the switch from preinitiation to initiation of transcription, and mRNA 5'-end capping is also stimulated by CDK7 kinase activity (reviewed in (139)).

Disengagement of SF-1 from regulated promoters demarcates the beginning of the clearance phase of efficient transcription cycling. Therefore, evidence for a stalling of this process and a loss of cycling in the presence of the CDK inhibitor DRB presented above (73) is extremely relevant to the coupling of NR loss from promoters to events following transcription initiation. These events necessarily involve TFIIH, which contains CDK7, with specificity not only for RNA polymerase, but also SF-1. Building evidence for a recently proposed model applicable to multiple NRs (140) suggests that CDK7 in TFIIH, or MAPK, phosphorylates a Ser-Pro motif in AF-1 (141, 142) or elsewhere in NRs (143, 144) or NR-coactivator complexes (see below). This somehow allows transcription to proceed more efficiently than in the absence of phosphorylation.
Recall that AF-1 is a region N-terminal to the DNA binding domain of ER and many other NRs that is not present in SF-1; however, the region of SF-1 hinge phosphorylation (PEPYASP) is increasingly being referred to as AF-1.

There are a number of opportunities for crosstalk between S203 phosphorylation of SF-1 and other functional PTMs. Like the above motif in RNA polymerase, the phospho-Ser203-Pro motif in AF-1 is a likely substrate for the SRC-3 associated NR coactivator proline isomerase 1 (145), because this enzyme alters the conformation of the peptide backbone at any proline following a phosphorylated serine or threonine residue (146). This is consistent with a reorientation of the SF-1 hinge relative to the LBD in a phosphorylation-specific manner (50). Another effector of proximal phosphorylation may be SUMOylation at K194, as described above for SUMOylation sites proximal to phosphorylation sites. In this case, phospho-S203 antagonism of K194 SUMOylation and its transcription repressive effect described above would be consistent with the loss of function seen in unphosphorylatable S203A SF-1 (see below).

Unlike the SUMOylation site, which is well conserved across the fushi tarazu family of NRs, the phosphorylation motif of human SF-1 is unique to SF-1 of higher vertebrates, with no alignment of this sequence being possible with any other nuclear receptor sequence, including the ancestor of SF-1, fushi tarazu (unpublished observations). Coincidentally, the SF-1 paralog and closest relative liver receptor homolog-1 is also phosphorylated on the hinge at two serine-proline (Ser-Pro) motifs which are further from the conserved SUMOylation site and from the start of the LBD helical bundle, with a similar modest activating effect on transcription (147), arguing against SUMOyl-phosphoryl crosstalk on the hinge. Authors of this study took note of
the general trend that activating phosphorylation in many NRs is much more modest than agonist-mediated activation of the same receptors. This would appear to be true for SF-1, because a phospho-site mimetic or site ablation in respective mutant receptors S203E and S203A did not affect CYP17 reporter transcription more than 1.5 fold (48), generally consistent with an earlier report which found no effect of S203A on SF-1 mediated transcription of a short CYP17 promoter-fused reporter (148). Importantly, studies which do find a significant effect of S203A mutation on SF-1 reporter expression (31) used other genes with promoters that are long enough (>500bp in this example) to enable packaging of the promoter in a manner similar to native chromatin with nucleosomes. This would be a requirement to differentiate any effects of S203 phosphorylation on cyclic reporter transcription mediated through chromatin modification. However, endogenous mRNA of SF-1 target genes has not been adequately measured in response to S203 mutation in any study to date. This leaves open the possibility that S203 effects are gene-specific.

Metabolic incorporation of labeled phosphate into SF-1 decreases in response to cAMP because cAMP activates rapid transcription of phosphatase(s) in the adrenal cortex (148, 149). This contrasts the reasonable expectation that the trans activating role of elevated phosphorylation at the major phosphorylation site just described, S203, would correlate with both ACTH/cAMP treatment and elevated phosphorylation of SF-1. In a landmark study, Winnay et al identified that this is indeed the case for S203—but at particular time points following ACTH stimulation (150). This study also established that a gene promoter—that of the receptor for ACTH—is regulated by phases of cyclic SF-1 binding, coregulator recruitment, and chromatin modification in cultured,
synchronized cells, as described in previous sections. In addition, ChIP of adrenal cortex cells taken from mice treated with ACTH for specific acute periods provided the first evidence for synchronized populations of cells undergoing induced target gene transcription \textit{in vivo}. Finally, and most importantly, this study elegantly correlated transcription cycle peaks of SF-1 binding to a specific target promoter with the onset of increased cell-wide S203 phosphorylation of SF-1 (150). This supports the model that TFI IH (or ERK2) mediates events that precede disengagement of SF-1-coactivator complexes from DNA on a large population of induced, cyclic promoters. Kinetics of S203 phosphorylation, ERK2 recruitment, and/or TFI IH-associated specifically with promoter chromatin of a given target gene were not determined. Kinase integration into NR transcription cycle-associated complexes is pictured in Figure 1.7.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.7}
\caption{Kinases regulate NR-mediated transcription and other PTMs at multiple levels. Dashed lines indicate MAP kinase pathways. Drawing is based on Rochette-Egly (151).}
\end{figure}
1.4.4 Other Modifications

In addition to CDK7 and MAPKs, other kinases and PTM-writing enzymes target key participants in NR-mediated transcription cycles at different, unique, points during phases of those cycles. One of the most striking examples is NR ligand-dependent phosphorylation of corepressor complex members and subsequent dismissal of corepressors from NRs and other transcription factors (152). Specifically following ligand stimulation, casein kinase 1, glycogen synthase kinase 3, and protein kinase C target coregulator exchange factors (namely, TBLR and TBL) that respectively reside in NCoR and CtBP corepressor complexes ((152) and see tables 1.3 and 1.4 for more information)). This checkpoint preceding transcription by NRs necessarily insures that transcription cycles occur in the context of activation of these specific kinases. Therefore, it is exciting that there is accumulating evidence for the participation of these kinases in proceeding events of transcription cycles: e.g., a requirement for protein kinase C phosphorylation of a NR required for trans activation (153), and glycogen synthase kinase 3-dependent ubiquitination and temporary activation of SRC-3 coactivator function (154).

As reviewed above, NRs and coregulators are targets for the same PTMs written by histone code writing coregulators, e.g., they are targets for acetylation by HATs. So, kinases also integrate signals into a transcriptional response by targeting histone modification machinery (transcription coregulators), which in turn modifies not only histones but also trans activator modifications. Termination of SRC-3 coactivator function can be caused by coactivator arginine methyltransferase 1 (CARM1)-mediated methylation (47), which itself is downstream of kinase inactivation (155). In a second
example, the HAT p300 is phosphorylated on a conserved residue during metabolic stress or localized ATP depletion by AMP-activated protein kinase, specifically decreasing p300 interaction with NRs or NR-containing complexes (156).

The overall goal of studies detailed in the next three chapters of this dissertation was to better define the molecular events important for ACTH and cAMP-stimulated transcription of the CYP17 gene in a human adrenal cortex cell line. Because the above work demonstrates that chromatin modifications, nuclear receptor modifications, and transcription factor coregulator modifications each play a role in transcription of human (indeed, many eukaryotic) inducible genes, a chapter is dedicated to broad or specific examples of each of these types of modification as it relates to CYP17 transcription. While these studies are narrowly focused on CYP17 transcription, it may be useful to consider CYP17 as a model inducible steroidogenic gene, and more generally, as a model nuclear receptor target gene, particularly with regards to the effects of SF-1 and CtBP modification.
Chapter 2. Materials and Methods

2.1 Reagents.

Dibutyryl cAMP (Bt2cAMP) was obtained from Sigma (St. Louis, MO). D-erythro Sphingosine was obtained from Avanti Polar Lipids (Alabaster, AL). α-amanitin, H-89, tetrabromobenzotriazole (TBB), recombinant CK1, CK2, GSK3β, ERK2, and the catalytic subunit of PKA were obtained from EMD Biosciences, Inc. (La Jolla, CA). Antibodies utilized in chromatin IP and most experiments are in Table 2.1.

Table 2.1 Antibodies for ChIP experiments and nomenclature cross-reference.

<table>
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<th>Source</th>
<th>Antibody</th>
<th>Definition</th>
<th>Alternate Names</th>
<th>Cat No.</th>
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<td>ACTR</td>
<td>activator of thyroid and retinoic acid receptors</td>
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<td></td>
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<td>coactivator arginine methyltransferase-1</td>
<td>SRC-2/NCoA2/TIF2</td>
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<td>CBP</td>
<td>cAMP-responsive element binding protein</td>
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<td></td>
<td>CtBP1</td>
<td>E1A C-terminal binding protein 1</td>
<td></td>
<td>07-316</td>
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<td></td>
<td>GRIP-1</td>
<td>glucocorticoid receptor interacting protein</td>
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<td>06-270</td>
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<td>BD Biosciences (San Diego, CA)</td>
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<td>E1A C-terminal binding proteins 1 and 2 nuclear RNA and DNA binding protein</td>
<td>NonO</td>
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<td>Brahma related gene-1, SWI/SNF ATPase</td>
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<td>Dr. J. Patton</td>
<td>PSF</td>
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<td>(Vanderbilt Univ., Nashville, TN)</td>
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<td>associated splicing factor</td>
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Experiments described in chapter four or five used antibodies including anti-SF-1 from Millipore (Temecula, CA), anti-FLAG M2 from Stratagene (La Jolla, CA), and anti-ß catenin phospho-Ser33 and phospho-Ser33 and 37 (GSK3 phospho-substrate) from Sigma (St. Louis, MO).

Some experiments described in chapter five utilized 5,6-carboxy-2’,7’-dichlorodihydrofluoroscein diacetate purchased from Invitrogen (Eugene, OR). Active PAK6 was obtained from Millipore.

2.2 Cell Culture.

H295R adrenocortical cells (157, 158) were generously donated by Dr. William E. Rainey (Medical College of Georgia, Augusta, GA) and cultured in Dulbecco's modified Eagle's/F12 (DME/F12) medium (Invitrogen, Carlsbad, CA) supplemented with 10% Nu-Serum I (BD Biosciences, Palo Alto, CA), 0.5% ITS Plus (BD Biosciences), and antibiotics. Jeg3 human choriocarcinoma cells were donated by Dr. Michael R. Waterman (Vanderbilt University School of Medicine, Nashville, TN) and cultured in the same media as the H295R cells. CV-1 cells were obtained from ATCC (Manassas, VA) and cultured in MEM with 10% fetal bovine serum and antibiotics.

2.3 Plasmids and Mutagenesis.

The CYP17 57-pGL3 plasmid was constructed by ligating double stranded oligonucleotides corresponding to the region -57/-2 of the CYP17 5' flank upstream of the Firefly luciferase gene in the pGL3 vector (Promega, Madison, WI) as previously described (20). Expression plasmids were generously given by the labs of B.W.
O’Malley (Baylor College of Medicine, Houston TX)-pBKCMV.SRC-1e; M.R. Stallcup (University of Southern California, Los Angeles, CA)-pSG5.HA.GRIP-1 full length and partial constructs pSG5.HA.GRIP-1(5-1121), pSG5.HA.GRIP-1(1124-1462), and pSG5.HA.SRC-1a(977-1441) “SRC-1ΔN”; R.M. Evans (Salk Institute, La Jolla, CA)-pCMX.ACTR and pCMX.mSMRTaFL; Y. Nakatani (Dana Farber Cancer Institute, Harvard Medical School, Boston, MA)-pOZ-N.hGCN5 and pCI.P/CAF; R.H. Goodman (Vollum Institute, Oregon Health & Sciences University, Portland, OR)-pRC-RSV.mCBP; G. Chinnadurai (Institute for Molecular Virology, Saint Louis University School of Medicine, St. Louis, MO)-pRC-CMV.CtBP1; K.B. Horwitz (University of Colorado Health Sciences Center, Aurora, CO)-pCMX.mNCoR; and P.W. Tucker (University of Texas at Austin, Austin, TX)-pCR3.1.NonO and pCDNA3.1.PSF. Site-directed mutagenesis to disrupt the human GCN5 (hGCN5) HAT active site residue E214 homologous to yeast GCN5 E173 (159) was performed using the primer 5’-CCC ACC CAG GGC TTC ACG CAG ATT GTC TTC TGT GCT GTC-3’ and the reverse complement, generating hGCN5 E214Q. NADH-binding defective pAct.CtBP1 G183V (160) was generated with the primer 5’-TTG GGC ATC ATC GGA CTT GTT CGC GTG GGG CAG GCA GTG-3’ and the reverse complement. WT SF-1 was generously provided by Dr. K. Morohashi (National Institute for Basic Biology, Okazaki, Japan) and cloned into the pCMV Tag1 vector (Stratagene, La Jolla, CA). SF-1 mutants and phosphorylation sites and dimerization interface site mutants of CtBP1 and 2 were prepared using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and were confirmed by sequencing. pRC-CMV.CtBP1, pFH.CtBP2, and pFH.CtBP1.NLS were kindly donated by Dr. G. Chinnadurai (Institute for Molecular
CtBP1 and 2 were subcloned into pET42 vector (Novagen, La Jolla, CA).

2.4 **Bacterial Expression of SF-1.**

Conditions used for cloning, expression, and purification of His-tagged SF-1 have been previously described (161).

2.5 **In vitro Kinase Assays.**

Purified wild type (WT) or mutant immobilized receptor was incubated with recombinant, active CK1, CK2, GSK3β, ERK2, or the catalytic subunit of PKA and 1 μCi \( [\gamma ^{32}P]ATP \) (MP Biomedicals, Solon, OH) in an assay buffer optimal for the kinase being tested for 1 hour at 30°C. Likewise, CtBP1 or 2 bound to GST beads were incubated with ERK2, PAK1, PAK6, or the catalytic subunit of PKA. CK1 and CK2 were assayed in 20 mM Tris-Cl, pH 7.5, 10 mM MgCl₂, and 1 mM dithiothreitol (DTT). The reaction buffer for analysis of GSK3β kinase activity contained 20 mM MOPS, 25 mM glycerol phosphate, 1 mM DTT, 5 mM EGTA, 25 mM MgCl₂, and 1 mM sodium orthovanadate. ERK was assayed in 25 mM Tris-Cl, pH 7.5, 20 mM EGTA, 10 mM HEPES, 10 mM MgCl₂, and 1 mM DTT, while PKA reaction buffer contained 20 mM Tris-Cl, pH 7.5, 100 mM NaCl, 12 mM MgCl₂, and 1 mM DTT. Assay buffer for reactions containing PAK6 contained 45 mM HEPES, pH 7.9, 10 mM MgCl₂, 2 mM MnCl₂, and 0.2 mM DTT. The substrate-bound beads were washed twice with RIPA [containing PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail I (Calbiochem)] and three times with PBS, resuspended in SDS-PAGE gel loading buffer and proteins resolved on 10% acrylamide gels. Dried Coomassie-
stained gels were exposed to a phosphorimager screen and phosphorylated SF-1 imaged on a Fluor/Phospho-Imager (Fuji Film, Japan). Positive reactions were obtained for each kinase/buffer system in reactions with myelin basic protein (Sigma) or CREB PKA target fragment, CREBtide (EMD).

2.6 Chromatin Immunoprecipitation (ChIP).

For ChIP assays (162, 163), H295R cells (subcultured into 100 or 150 mm dishes) were pre-treated with 2.5 µM α-amanitin for 2 h, washed twice with PBS, then treated with 1 mM Bt2cAMP and/or Sph (5 µM) for time periods ranging from 15 minutes to 4 hours. Cross-linking was performed by the addition of formaldehyde (final concentration of 1%) for 10 minutes with gentle shaking. The reaction was stopped by the addition of glycine (0.125 M final concentration) for 5 min, the cells were washed twice in PBS and harvested into RIPA buffer. Lysates were then sonicated to obtain optimal DNA fragment lengths of 100 to 1000 base pairs followed by centrifugation for 15 minutes at 4°C. Fifty µl supernatant was retained as input. The purified chromatin solutions were cleared with 1 µg rabbit or mouse IgG and immunoprecipitated overnight at 4°C on a tube rotator using 5 µg of primary antibody (see Table 1), and protein A/G plus (Santa Cruz Biotechnology). The immobilized protein/DNA complexes were subjected to a series of 5 minute washes: three times in RIPA buffer, three times in RIPA buffer plus 500 mM NaCl, three times in washing buffer (10 mM Tris-Cl, pH 8, 0.25 M LiCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, and protease inhibitors), and three times in TE buffer, pH 8.0. The cross-links were reversed and protein digested using proteinase K (100 µg/ml). DNA was purified by phenol:chloroform extraction and ethanol precipitation. Real-time PCR was carried out using 4 µl of output, 1 µl of input
(diluted 1:4), the iTaq SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA), and the following primer pairs: forward 5′-GGC TGG GCT CCA GGA GAA TCT TTC TTC CAC-3′, reverse 5′-CGG CAG GCA AGA TAG ACA GCA GTG GAG TAG-3′, which amplify the region of the CYP17 promoter from position -104 to +43. For negative controls, primers for actin (forward 5′-TGC ACT GTG CGG CGA AGC-3′ and reverse 5′-TCG AGC CAT AAA AGG CAA-3′) or CYP17 coding regions (forward 5′-GAC AAG GGC ACA GAA GTT ATC ATC-3′ and reverse 5′-CAG GGA GGG CAG CTG CCC ATC ATC-3′) were used. PCR reactions were as follows: 1) 1 X 94°C, 5 minutes, 2) 35 X 95°C, 1 minutes, 55°C, 1 minute, 72°C, 2 minutes, 3) 1 X 72°C, 10 minutes, 4) cool to 4°C. Graphical data was normalized to input values. PCR reactions were also resolved by 2% agarose gel electrophoresis.

In some experiments, cells were treated with 1 mM Bt2cAMP and/or 20 mM LiCl for 1 hour. In studies examining the functional significance of S342 phosphorylation, cells (150 mm dishes) were first transfected with 25 μg WT or mutant pCMVTag1-SF-1 for 48 hours and then treated with α-amanitin for synchronization, followed by treatment with 1 mM Bt2cAMP for 30 minutes to 4 hours. In these experiments, PCR reactions were as follows: 1) 1 X 94°C, 5 min, 2) 45 X 95°C, 30 sec, 55°C, 30 sec, 72°C, 1 min, 3) 1 X 72°C, 10 minutes.

2.7 RNA Isolation, Real Time RT-PCR, and RNA Interference.

Cells were cultured onto 12-well plates, treated for the indicated times with 1 mM Bt2cAMP in the presence and absence of Sph and total RNA was prepared using TRIzol (Invitrogen, Carlsbad, CA). Real time RT-PCR reactions were performed in the iCycler (Bio-Rad), using 100 ng of total RNA, 100nM forward and reverse primers and the One-
Step RT-PCR SYBR Green Kit (Eurogentec, San Diego, CA). The following primers were used: CYP17 (forward 5’-CCG CAC ACC AAC TAT CAG-3’ and reverse 5’-GTC CAC AGC AAA CTC ACC-3’) and actin (forward 5’-ACG GCT CCG GCA TGT GCA AG-3’ and reverse 5’-TGA CGA TGC CGT GCT GCA TG-3’). CYP17 expression is normalized to α-actin content and calculated using the delta cycle threshold (ΔC_T) method.

RNA interference detailed in chapter 4 was performed as follows. Cells were sub-cultured into 12-well plates and 24 hours later transfected with 50 nM of small interfering RNAs (siRNAs; obtained from Dharmacon) directed against CK2 or GSK3β using HiPerfect Trasfection Reagent (Qiagen, Valencia, CA). Twenty-four hours after transfection, cells were transfected again (50 nM siRNA) and incubated for an additional 48 hours. Some cells were treated with 1 mM Bt2cAMP for 16 hours (66 hours after first transfection). Then RNA was harvested and analyzed as described above.

2.8 Transient Transfection.

In experiments performed in chapter three, cells were subcultured onto 12-well plates and 24 hours later transfected with 250 ng CYP17 57-pGL3 (the first 57-base pairs of the CYP17 promoter upstream of the start site fused to the Firefly luciferase gene) (20) using GeneJuice (Novagen, Madison, WI). Ten to 200 ng of coregulator plasmids were cotransfected as indicated. Cells were co-transfected with 5 ng of the Renilla luciferase plasmid (pRL.TK, Promega, Madison, WI) for normalization. Approximately 24 hours later, cells were treated with 1 mM Bt2cAMP and/or 1-5 µM Sph for 16-24 hours and harvested for dual luciferase assays (Promega, Madison, WI).

In chapter 4 experiments, Jeg3 cells were sub-cultured onto 24-well plates
and transfected with 100 ng of pGL3-CYP17-2x57 reporter plasmid (164) and 25 ng of WT or mutant (T334A, T335A, T338A, S342A, and S346A) pCMVTag1-SF-1 (Invitrogen, Carlsbad, CA) using GeneJuice (Novagen, Madison, WI). Cells were co-transfected with 1 ng of a Renilla luciferase plasmid (pRL-TK, Promega, Madison, WI) for normalization. Twenty-four hours after transfection, the cells were treated with 1 mM Bt₂cAMP for 16 hours and the transcriptional activity of the CYP17 reporter gene determined using a dual luciferase assay (Promega, Madison, WI).

In chapter five, reporter assays were performed in 24 welled plates and transfections relied on per well combinations of the following plasmids, unless otherwise indicated: pGL3.CYP17 2x57 or -300 (150 ng), pCR3.1.SF-1 (30 ng), pRC-CMV.CtBP1 (30 ng).

2.9 Mammalian Two-Hybrid.

Coactivators and nuclear receptor genes were cloned into the Mlu I and Xba I sites of pBIND and/or pACT vectors (Promega). Cells were transfected with pG5 firefly luciferase reporter in combination with pBIND and pACT vectors expressing fusions of Gal4 DBD and VP16 AD, respectively, with SF-1, LRH-1 (liver receptor homolog-1, NR5A2) or coregulators. The ratio of pG5 to pBIND to pACT in transient transfections was 50ng:50ng:15ng. Twenty-four hours later, cells were treated with Bt₂cAMP and/or other reagents as indicated for 16-24 hours before harvesting and assaying for dual luciferase activity.
2.10 Metabolic Labeling.

H295R cells were subcultured onto 100 mm dishes and transfected with 15 μg WT or mutant pCMVTag1-SF1. Forty-eight hours later, media was replaced with phosphate-free DMEM containing 10 μCi 32P-phosphorus/ml for 4 hours. Cells were washed twice with PBS and lysates isolated in RIPA for immunoprecipitation with 5 μg anti-FLAG and 30 μl of a 50% slurry of protein A/G plus overnight at 4°C with rotation. Immunoprecipitants were washed twice with RIPA and twice with PBS and then subjected to SDS-PAGE. Gels were stained overnight with coomassie, destained, and exposed to a phosphorimger screen. Blots were imaged by scanning screens on a Fluor/Phospho-Imager (Fuji Film, Japan).

2.11 In vitro Acetylation Assay.

WT or mutant pCMVTag1-SF1 was transiently transfected into H295R cells and then treated for 1 hours with 1 mM Bt2cAMP. Cell lysates were immunoprecipitated with an anti-FLAG antibody (Stratagene) and protein A/G agarose (Santa Cruz). The immunoprecipitants were washed three times with RIPA buffer and incubated with E. coli expressed GCN5 and 14C-acetyl coenzyme A (MP Biomedicals). The immobilized receptor was washed and subjected to scintillation counting and SDS-PAGE and fluorography.

2.12 Confocal Microscopy and Time Lapse Imaging.

A Carl Zeiss LSM 510 (Germany) was used to image live and fixed cells. Three μm crosssections were imaged via excitation with 351 nm (UV) or 543 nm laser, and emission at 385-470 nm and 560 nm recorded, respectively, for
endogenous autofluorescent pyridine nucleotide or fixed secondary antibody conjugated to DyLight 549 (Pierce Biotechnology, Rockford, IL). Regions of interest for nuclear and cytoplasmic CtBP1 and 2 were defined in the Zeiss image analysis software and background-subtracted absolute and relative intensities for time points were then calculated.

2.13 Coimmunoprecipitation and Western Blotting.

H295R cells were cultured in 100 mm dishes and cytoplasmic and nuclear fractions obtained using the NE-PER kit (Pierce), or whole cell extracts were prepared in RIPA. For exogenous CtBP expression, 5 µg of pFH.CtBP1 or 2 (WT or mutant), and/or 5µg of pACT.CtBP1 were transfected 60 hours before harvest. For kinetics by coimmunoprecipitation (coIP), one hundred µg nuclear extracts, or for other experiments an equal fraction or total sample of whole cell lysates, were precleared for 45 minutes at 4° C and then incubated overnight with protein A/G beads (Santa Cruz Biotechnology, Santa Cruz, CA) and 4 µg anti-CtBP1 (BD Biosciences), 3 µg anti-VP16 or 2 µg GCN5 (Santa Cruz), 2 µg anti-FLAG (Sigma), or 2 µg anti-SF-1 (Millipore). Beads were washed two times in RIPA with protease inhibitors (EMD) and twice in PBS, then boiled in SDS-PAGE buffer and separated in 10 % gels before transfer to PVDF membrane (Millipore) and western blotting with anti-CtBP2. Blots were developed with ECF (Amersham Biosciences, Piscataway, NJ) and imaged with a FLA-3000 (Fuji Film, Japan), then quantified using Fuji Image Gauge v3.0 software.
2.14 Biomolecular Simulation.

Re-creation of the CtBP1 homodimer (165) was performed manually to generate the symmetry of the homodimer as displayed in the publication by Kumar, et al, and the structures were merged in Swiss PDB Viewer 3.7 (166) and energy minimized. CtBP2 was threaded through one of the CtBP1 monomers in this structure and the resulting CtBP2 monomer refined using the SWISS-MODEL service (166). Following energy minimization of the resulting heterodimer, peptides comprising CtBP1 residues 133-145 and/or CtBP2 residues 139-151 were saved as an isolated structure, maintaining dimeric contacts. *In silico* mutagenesis was carried out in Swiss PDB Viewer followed by 30 cycles of energy minimization *in vacuo*, during which the total system free energy was determined to plateau within 0.1% of the energy of the previous calculation for at least 5 cycles of minimization.

2.15 Statistics.

One-way analysis of variance and Tukey’s multiple comparison tests were performed for selected data in chapter three using GraphPad Prism 4.03 (GraphPad Software, Inc., San Diego, CA). In chapter five, one sample t-tests were performed in GraphPad Prism 5.00 (GraphPad Software, Inc., San Diego, CA). Significant difference from a compared value was defined as p < 0.05.
Chapter 3. Results—Part I: Coregulator Exchange and Sphingosine-Sensitive Cooperativity of Steroidogenic Factor-1, GCN5, p54, and p160 Coactivators Regulate cAMP-Dependent CYP17 Transcription Rate

3.1 $\alpha$-Amanitin, a Valuable RNA Polymerase II (Pol II) Inhibitor

In sections 1.3, seminal ChIP studies were reviewed which revealed that inducible genes undergo cycles of chromatin modification and interaction with transcription factors such as nuclear receptors, along with coregulators. We hypothesized that combinatorial and sequential recruitment of SF-1 along with coregulators would also occur on the CYP17 promoter in adrenal cortex cells in which chronic steroidogenesis is induced by ACTH/cAMP. Some attention must be given to the means by which many of these kinetic ChIP studies achieved synchronized populations of cells. Synchronization implies a uniform promoter chromatin structure and a nucleosome modification state that is not conducive to transcription competence being achieved. Then, such conditions are reversed, transcription is induced, and ChIP is used to monitor the population-wide trends of subsequent chromatin modification and protein factor-DNA binding stabilization. $\alpha$-Amanitin is a cyclic peptide toxin from *Amanita* species of fungi that blocks dynamic movement of RNA polymerase II (Pol II) along the DNA that it is poised to continue reading after a phosphodiester bond in a nascent mRNA has been formed (167). Thus, it is not surprising that Pol II does not immediately disengage from DNA at the concentrations and treatment times used (~2 $\mu$M, 2 hours) in most of these studies—in fact, one study which examined the early kinetics of Pol II on histone promoters
undergoing timed treatment with 2 µM α-amanitin found that the toxin at this concentration doubled stable occupancy of 3’ regions of the H2AB and H3B genes within 60 minutes, decreasing towards no net change after two hours of treatment, and little change in Pol II occupancy on promoters of these genes was noted (123). Indeed, cyclic kinetics of polymerase recruitment and loss in response to estradiol on an ER responsive promoter were identical with or without low dose α-amanitin cotreatment (73). However, TATA binding protein near the transcription start sites for H2AB and H3B genes decreased in occupancy while histone solenoid linker histone H1 increased slightly in occupancy at the beginning of these histone genes (123), suggesting that dynamic Pol II competes with histone H1 binding to chromatin and maintains an open chromatin conformation at the transcription start site. Thus, a default or return to partial or full solenoid structure may very well occur in the absence of polymerase access to a genomic segment. Of note, histone genes like those studied above may be considered either constitutive and immediately accessible, or poised for further induction, e.g. during the cell cycle, and in some tissues, with circadian rhythmicity (168).

At the concentration of 2 or 2.5 µM, α-amanitin inhibition of transcription is fully reversible, simply by washing cultured cells (87). However, at higher concentrations (5-20µM), used in studies which predated the finding of synchronized, cyclic, transcription, Pol II half-life is affected in a DNA dependent manner (169), suggesting the induction of polyubiquitination and degradation of a Pol II complex which can no longer undergo any dynamic interaction with DNA (170). Strong repression of cyclic transcription with stalling of polymerase is clearly visible upon proteasome inhibition (86). In addition to ameliorating a proclivity of actively engaged transcription complexes to stall, the
proteasome may have other coactivator-like roles that guarantee an accelerated rate of transcription during gene induction (88, 171).

3.2 cAMP Induces Cycles of SF-1 Binding to the CYP17 Proximal Promoter

We have previously shown that cAMP stimulates SF-1 dependent transcription by promoting the binding of a complex containing SF-1 and the splicing factors p54<sup>nb</sup> and PSF to the cAMP-responsive sequence of the CYP17 promoter (20). Moreover, we have also demonstrated that both ligand binding (172) and phosphorylation (148) modulate the activity of SF-1. Various coregulators of transcriptional activation are known to interact with SF-1 and modulate its ability to transactivate target genes, especially in response to cAMP or PKA (46, 58, 78, 137, 173-175). In order to examine the kinetics of recruitment and combinatorial effects of a panel of coregulators on SF-1 responsive gene promoters, we treated α-amanitin synchronized H295R adrenocortical cells with 1 mM Bt<sub>2</sub>cAMP for time periods ranging from 15 to 240 minutes and carried out ChIP for SF-1. Primers for ChIP of the CYP17 promoter and transcription start site were designed to most likely capture the state of a single nucleosome, with 147 bp of wrapped DNA in a canonical model. In addition, real time PCR requires an amplicon length of more than 100 bp for maximum sensitivity. Therefore, the region selected, -104/+43, was chosen to match these criteria, with the -57/-37 SF-1 binding site centrally located.

Both ACTH and Bt<sub>2</sub>cAMP increased the binding of SF-1 to the CYP17 promoter; however, the relative enrichment of SF-1 bound CYP17 promoters mediated by Bt<sub>2</sub>cAMP was greater than that seen for ACTH (Figure 3.1A). The ACTH-/Bt<sub>2</sub>cAMP-stimulated SF-1 binding initially occurs in 120-minute cycles (Figure 3.1A), though elevated SF-1 occupancy of the promoter occurs for less than 90 minutes. Peaks indicate a stochastic
time for maximal recruitment to the promoter within the population of synchronized cells (176). To determine if the periodic binding of SF-1 results in cyclical changes in transcription, we quantified the changes in CYP17 reporter gene expression in synchronized cells over the same 240-minute time frame. As shown in Figure 3.1C, both ACTH and Bt2cAMP treatment resulted in cyclical increases in CYP17 luciferase activity. However, the periodicity of reporter expression did not overlap with SF-1 binding to endogenous promoter at early time points. CYP17 reporter gene activity peaked at the 30-, 90-, and 210-minute time points, suggesting that rhythms of ACTH/cAMP-stimulated SF-1 binding to the CYP17 promoter correspond with an overall increase in P450c17 transcription rate, and these transcription rhythms can be brought forward into corresponding rhythmic protein accumulation.

3.3 Trans Activation of CYP17 Follows SF-1 Binding

Basal transcription machinery as well as nuclear receptors are lost (86) and promoter histone modifications are uniformly reset to patterns associated with low levels of transcription (87) on the promoter when cells are exposed to α-amanitin in the absence of signals that induce transcription, so we extended our ChIP assay to examine the dynamics of Pol II recruitment to the proximal CYP17 promoter during SF-1 transactivation, particularly following peaks of SF-1 recruitment at 60 (cycle I) and 180 (cycle II) minutes of ACTH or Bt2cAMP stimulation. The antibody used recognizes the phosphorylated C-terminal domain heptad repeat. Sixty minutes of both ACTH and Bt2cAMP increase Pol II recruitment which decreases more slowly than Bt2cAMP-stimulated SF-1 binding; we reference the beginning of SF-1 recruitment until the loss of Pol II as transcription cycle I (Figures 3.2A, B). In cycle II, Bt2cAMP promotes Pol II
Figure 3.1  cAMP induces cyclic binding of SF-1 to the CYP17 promoter.

H295R cells were synchronized for 2 hours with 2.5 µM α-amanitin, then treated with 1 mM Bt2cAMP for the indicated times and subjected to ChIP using a polyclonal SF-1 antibody.  (A) Real time PCR analysis of ChIP DNA.  Output data are normalized to values obtained for 1% input controls, and results are presented as percent of baseline value obtained for untreated cells at each time point.  Data graphed represents the mean ± SEM from 11 experiments performed in duplicate.  (B) Purified DNA was amplified by PCR with primers described in chapter 2 and the samples resolved on a 2% agarose gel.  Results from a representative experiment are shown.  (C) H295R cells subcultured onto 24-well plates and transfected with 125 ng pGL3-CYP17 2x57 and 2 ng pRL-TK for 48 hours, and then treated with 2.5 µM α-amanitin for 2 hours.  Synchronized cells were washed twice with PBC, and then treated with either 50 nM ACTH or 1 mM Bt2cAMP for 30-240 minutes.  Lysates were isolated and luciferase activity quantified by luminometry.  Graphed data are expressed as fold of the control group mean for each time point and represents mean +/- SEM from two experiments performed in quadruplicate.
recruitment with more complex dynamics culminating 30 minutes after peak binding of SF-1 on the CYP17 promoter at 180 minutes. In contrast to the cycle of Pol II enrichment seen in response to Bt2cAMP, peak ACTH-stimulated Pol II binding in cycle I occurred at the 90-minute time point. Further, the comparative effect of ACTH on Pol II recruitment in cycle II was an earlier increase that was sustained longer as compared to Bt2cAMP-stimulated Pol II binding (Figure 3.2A). SF-1 and Pol II binding after acute 5 and 15 minute exposure to ACTH or Bt2cAMP revealed no additional early peaks in binding prior to the first transcription cycle (data not shown). Because kinetics of SF-1 binding to CYP17 promoter is indistinguishable between Bt2cAMP- and ACTH-stimulation (Figure 3.1A), there is a better incremental burst of protein expression with Bt2cAMP (Figure 3.1C), and timed separation of transcription cycles is greater with Bt2cAMP than with ACTH (Figure 3.2A), the cAMP analog was used as a surrogate for ACTH stimulation in subsequent experiments. SF-1 binding to the CYP17 promoter in response to Bt2cAMP is shown in all subsequent ChIP figures for comparison.

3.4 Histone H3 and H4 Acetylation Precedes Pol II Recruitment

We postulated that histone acetylation is altered during induction of SF-1 dependent transactivation, and that these changes must precede changes in Pol II occupancy of the proximal CYP17 promoter as has been demonstrated for SF-1 responsive promoters (150, 177), as well as many other genes in their native context of chromatin (178). ChIP with an antibody for acetylated histone H4 correlates with subsequent Pol II recruitment in both cycles of SF-1 mediated transcription in response to both ACTH and Bt2cAMP, while histone H3 acetylation at Lys 9 and 14 coincides with
Figure 3.2 SF-1 transcription cycles correlate with histone acetylation and HAT and Pol II recruitment.

(A) Graphical analysis of relative promoter binding of Pol II. Assays were performed as described in chapter 2 and DNA amplified by quantitative PCR using primers targeted at region -104/+43 of the CYP17 promoter. Two transcription cycles (I and II) are indicated. Data graphed represents the mean ± SEM from 3 experiments performed in duplicate. (B) Representative ethidium bromide-stained agarose gel of temporal ChIP for Pol II binding to the CYP17 promoter. (C) Temporal ChIP analysis of Bt2cAMP-stimulated acetylation of histone H3 and histone H4. Sheared chromatin was immunoprecipitated with anti-SF1, anti-acetyl histone H3, or anti-acetyl histone H4 antibodies. Output data are normalized to delta Ct values obtained for 1% input controls, and results are presented as percent of baseline value obtained for untreated cells at each time point. (D) Graphical analysis of temporal ChIP for HAT recruitment to the CYP17 promoter during Bt2cAMP stimulation. α-Amanitin synchronized H295R cells were treated for time periods ranging from 30 minutes to 4 hours with 1 mM Bt2cAMP, exposed to 1% formaldehyde and purified lysates immunoprecipitated using antibodies directed against SF-1, GCN5, p300, CBP, and P/CAF. Output data are normalized to values obtained for 1% input controls, and results are presented as percent of baseline value obtained for untreated cells at each time point. Data graphed represents the mean from 2 experiments performed in duplicate. (E) Time course of Bt2cAMP-stimulated recruitment of p160 coactivators to the CYP17 promoter. ChIP was performed on lysates purified from Bt2cAMP-treated synchronized cells using antibodies for SF-1, SRC-1, GRIP-1, ACTR. Outputs are normalized to delta Ct values obtained for 1% input controls, and results are presented as percent of delta Ct values for untreated cells at the corresponding time point. Data graphed represents the mean from 2 experiments performed in duplicate.
the presence of Pol II in cycle II (Figure 3.2C). Histone H4 acetylation is decreased just before or as Pol II moves from the -104/+43 region of the promoter, while acetylation of H3 remains after cycle II until at least the 240 minute time point. Thus, histone H4 acetylation occurs by the time that Pol II binds the proximal promoter, whereas H3 acetylation is not strictly required for Pol II recruitment but does occur at high levels throughout transcription cycle II.

3.5 Histone Acetyltransferase Recruitment Correlates with Histone Acetylation

We next performed temporal ChIP experiments in order to determine the kinetics of binding of coactivators with histone acetyltransferase (HAT) activity (Figure 3.2D). GCN5 binding peaks at 30 minutes, and again at 180 minutes of stimulation. GCN5 recruitment coincides with histone H4 acetylation in both transcription cycles (Figure 3.2B). Early in cycle II, p300 rapidly binds between 120-150 minutes, and this binding coincides with histone H3 acetylation specific to the second transcription cycle (Figure 3.2C).

HAT binding events that occur after peak SF-1 binding vary between the first and second transcription cycles. CBP binds preferentially in cycle I, in phase with SF-1 (Figure 3.2D), and this binding does not correlate with H3 or H4 acetylation. The GCN5 paralog P/CAF binds in phase with GCN5 but with lower abundance in both cycles; yet, during cycle II at 210 minutes of stimulation, there is pronounced P/CAF binding independent of GCN5 recruitment 30 minutes after peak SF-1 binding, and this corresponds with a further increase in H3 acetylation after SF-1 begins to vacate the promoter during the second transcription cycle (Figure 3.2C).
3.6 Each Transcription Cycle Has a Unique Profile of p160 Binding

Only two of the three characterized members of the p160 family of coactivators [SRC-1, GRIP-1, ACTR (see Table 2.1 for alternate names)] have confirmed intrinsic HAT activity (179, 180). All three p160s serve as scaffolding for recruitment of other HATs to nuclear receptors (179-182) thus they are good candidates for bridging HATs with weak or no ligand-dependent interaction motifs to the SF-1 activation function-2 (AF-2) motif. In temporal ChIP, p160s show binding that mirrors increases in SF-1 on the promoter in both cycles, but in the first transcription cycle, SRC-1 rapidly binds within the first 30 minutes, while SF-1 is recruited for an additional 30 minutes (Figure 3.2E). Notably, only SRC-1 is enriched on the promoter in the first transcription cycle. On the other hand, multiple overlapping peaks of lower intensity in the second transcription cycle indicate that p160s may be interchangeable in the second SF-1 transcription cycle.

3.7 GCN5 Interaction with SF-1 is Strengthened by p160 Coactivators

Initial GCN5 binding coincides with SRC-1 in intensity and timing (cf. Figures 3.2D and 3.2E), and GCN5 stimulates expression of a SF-1 responsive reporter only in the presence of SRC-1 (Figure 3.3, group 2 vs. group 6) and only with SF-1 that has an intact ligand binding pocket (group 6 vs. group 8). We have previously found that Sph inhibits the ability of SRC-1 to coactivate SF-1-dependent transcription (172). These data suggest the possibility of a concomitant GCN5, SRC-1 and SF-1 interaction on the promoter. Such a complex has been shown to form in yeast cells expressing human thyroid hormone receptor, GCN5, and SRC-1 or GRIP-1 (183). The ability of SRC-1 to mediate the interaction between GCN5 and SF-1 was tested in mammalian two hybrid
Figure 3.3 GCN5 coactivation of SF-1-mediated CYP17 expression requires SRC-1. Cells were transfected as indicated with pCDNA3.1.SF-1 or pCDNA3.1.SF-1ΔLBD with pOZ-N.hGCN5, pBKCMV.SRC-1e, and/or pSG5.HA.GRIP-1. Striped bars: cells were treated 24 hours after transfection with 1 mM Bt2cAMP. Data are from a single experiment performed in triplicate.

experiments in H295R cells. SRC-1 dose-dependently promotes interaction of GCN5 with SF-1, and this interaction is potentiated by Bt2cAMP (Figure 3.4A). Bt2cAMP-stimulated interaction is decreased by cotreatment with the SF-1 antagonist Sph (Figure 3.4B), suggesting antagonist dissociation is required for cAMP-dependent SRC-1 recruitment of GCN5. GRIP-1 was also able to potentiate interaction of SF-1 with GCN5 (data not shown), however GRIP-1 coexpression with SRC-1 modestly increased CYP17 reporter expression only in the absence of Bt2cAMP compared to SRC-1 alone, and GRIP-1 alone did not potentiate GCN5 coactivation of this reporter (Figure 3.3, group 7), consistent with a different interaction mechanism.

To determine the specificity of the GCN5/SRC-1 interaction with nuclear receptors of the NR5A subfamily, we asked whether a complex forms between GCN5,
Figure 3.4  p160 dose-dependent interaction of GCN5 with NR5A receptors is mediated by SRC-1 and sensitive to SF-1 antagonist.

(A) Mammalian two hybrid experiments were performed by transfecting H295R cells with 50 ng pG5 luciferase reporter, 25 ng pBind-SF-1, 15 ng pAct vector or pAct-GCN5, and SRC-1 using Gene Juice. Twenty-four hours after transfection, cells were treated with 1 mM Bt2cAMP and lysates harvested 16 hours later for dual luciferase assays. (B) Mammalian two hybrid experiments were carried out as described in Materials & Methods. Transfected cells were treated for 16 hours with 1 mM Bt2cAMP in the presence and absence of 1 µM Sph and then lysed for dual luciferase assays. (C) Cells were transfected for 30 hours with the two-hybrid plasmids pG5, pAct-GCN5, and pBind-LRH-1, and expression plasmids for SRC-1, GRIP-1, or ACTR and harvested for dual luciferase assays. (D) H295R cells were transfected with pG5, pBind-SF-1, pAct-GCN5, pAct-GCN5 E214Q, and pBKCMV-SRC-1, then incubated for 16 hours with 1 mM Bt2cAMP. Lysates were isolated and subjected to dual luciferase assays. Data presented in all panels are normalized to Renilla activity (pAct) and represent the mean +/- SEM from 2 experiments performed in triplicate. Statistically significant difference between transfection with the pAct empty vector versus transfection with pAct-GCN5 are denoted as follows: †, p<0.05; ††††, p<0.001. Asterisk denotes statistically significant effect of SRC-1 overexpression, where *, p<0.05 and ***, p<0.001. #, p<0.05 or ## # p<0.001 denote statistically significant difference compared to pAct-GCN5/pBind-SF-1 transfected cells with control treatment. Ampersand denotes statistically significant difference between untreated and Bt2cAMP-treated cells, where &, p<0.01 and &&, p<0.01. Caret (^, p<0.05) denotes statistically significant difference between cells transfected with wild type GCN5 versus cells transfected with the GCN5 E214Q mutant. Dollar sign, ($$, p<0.01 and $$$, p<0.001) denotes statistically significant effect of Sph.
SRC-1, and the SF-1 ortholog liver receptor homologue-1 (LRH-1). We tested for p160-mediated interaction of GCN5 with LRH-1 in the mammalian two hybrid system in Jeg3 cells. This complex does indeed form and SRC-1, but neither GRIP-1 nor ACTR potentiates the LRH-1/GCN5 interaction (Figure 3.4C). The SF-1 specific antagonist Sph has no effect on the LRH-1/GCN5 interaction (data not shown).

3.8 GCN5 Acetyltransferase Activity Limits Interaction with SF-1

We hypothesized that acetylation of SF-1 by GCN5 may be a mechanism that ensures the SF-1/p160/GCN5 complex is transient, as observed in the ChIP time course (Figures 3.2D, E). To test this hypothesis, we constructed a GCN5 acetyltransferase catalytic site defective mutant E214Q (159) and repeated two hybrid experiments in H295R cells. Interestingly, the formation of the SRC-1/SF-1/GCN5 complex was strengthened in the mutant (Figure 3.4D). These data indicate that GCN5 acetyltransferase activity may destabilize the transient GCN5/SF-1/SRC-1 interaction, and suggest that acetylation of SF-1 may be an underlying mechanism. Collectively, the two hybrid data suggest that acetylation of an unknown target by GCN5 promotes dissociation of the GCN5/SRC-1/SF-1 complex.

3.9 Class I HDACs Have Two Roles in Transcription Cycles Mediated by SF-1

There are two major classes of HDACs: class I HDACs (human HDACs 1-3, 8), which share sequence homology with the yeast transcriptional repressor Rpd3, and class II HDACs (human HDACs 4-7, 9 and 10), which are generally larger and more tissue-specifically expressed, and were identified by their homology to yeast Hda1 (184). In order to determine which class I HDACs are responsible for the loss of histone
acetylation on the CYP17 promoter (Figure 3.2B), we performed temporal ChIP. HDAC8 recruitment occurs within 30 minutes of Bt2cAMP stimulation, while an increase in HDAC1 recruitment peaks at 90 minutes (Figure 3.5A). The former peak coincides with early coactivator cooperativity while the latter peak coincides with decreases in Pol II occupancy (Figure 3.2B) and a loss of histone H4 acetylation at this time (Figure 3.2C). At 120 minutes, when SF-1 binding to the promoter is at a minimum, HDAC2 is maximally recruited, and while levels of this HDAC decline during SF-1 recruitment in the second transcription cycle, they again wax with loss of SF-1 from the promoter at 210 minutes (Figure 3.4A). HDAC3 and HDAC8 modestly increase after 120 minutes until 210 minutes in the second transcription cycle.

3.10 Corepressor Recruitment Reciprocates SF-1 Loss from the CYP17 Promoter

HDACs are often associated with corepressor complexes brought to the promoter by scaffold proteins with repression domains such as NCoR or SMRT, which can bind nuclear receptors directly in absence of agonist (185, 186), with dependence on partial agonist (187) or antagonist (172), or in the case of RIP140, with agonist dependence (188). Notably, temporal ChIP of these nuclear receptor corepressors shows recruitment that reciprocates SF-1 loss from the promoter. NCoR has modest recruitment at 90 minutes of stimulation coinciding with HDAC1 recruitment (Figure 3.5B). RIP140 and Sin3A may be members of a repression complex that is recruited to the promoter in the relative absence of SF-1 at the 120 minute time point, coinciding with initial recruitment of HDAC2. Taken together, these data indicate that HDAC1 and 2 are recruited within NCoR and RIP140/Sin3A corepressor complexes, respectively, and these putative complexes bind at the CYP17 promoter during cAMP stimulation without dependence on
Figure 3.5 Histone deacetylases (HDACs) and corepressors show promoter binding reciprocal to that of SF-1 but compatible with an increase in p54<sup>prom</sup> and PSF splicing factors.

(A) Temporal ChIP for coregulator protein binding to the CYP17 promoter was carried out as described.

(Legend continues next page)
in Materials and Methods (Chapter 2). Lysates were immunoprecipitated using antibodies against SF-1, HDAC1, HDAC2, HDAC3, and HDAC8. (B) Time course of corepressor recruitment to the CYP17 promoter in response to Bt2cAMP stimulation. α-Amanitin synchronized H295R cells were treated for 30 minutes to 4 hours with 1 mM Bt2cAMP and crosslinked using 1% formaldehyde. Purified lysates containing sheared chromatin were immunoprecipitated using anti-SF-1, anti-NCoR, anti-Sin3A, anti-RIP140, and anti-SMRT antibodies. (C) Temporal ChIP of cAMP-dependent binding of p54\textsuperscript{nrb} and PSF splicing factors, SF-1 and Pol II to the CYP17 promoter. Temporal ChIP for coregulator protein binding to the CYP17 promoter was carried out as described in Materials and Methods. Lysates were immunoprecipitated with antibodies against SF-1, p54\textsuperscript{nrb}, PSF and Pol II. Data graphed in all panels represent the mean from at least 2 experiments, each performed in duplicate. Outputs are normalized to delta Ct values obtained for 1% input controls, and results are presented as percent of delta Ct values for untreated cells at each time point.

SF-1. Moreover, these data confirm our previous findings demonstrating that Sin3A mediates CYP17 repression (20).

### 3.11 Corepressor Clearance Coincides with p54\textsuperscript{nrb}/PSF Recruitment and Precedes SF-1 Recruitment

We have shown that the interaction of p54\textsuperscript{nrb} and PSF splicing factors with SF-1 on the CYP17 promoter is stimulated by cAMP (20). Moreover, PSF has been shown to mediate nuclear receptor interactions with Sin3A and associated HDACs (189). Thus, we postulated that these splicing factors may be involved in the dynamics of corepressor-containing complexes and SF-1. Unexpectedly, PSF and p54\textsuperscript{nrb} binding increase in a SF-1 independent manner at 150 minutes (Figure 3.5C). Initial recruitment of splicing factors by 150 minutes reciprocates a loss of corepressors and precedes SF-1 binding in cycle II, and also parallels an initial increase in Pol II promoter occupancy at this time (Figures 3.2A, 3.5C). PSF and p54\textsuperscript{nrb} remain associated with the promoter as SF-1 and Pol II vacate the promoter for the second time by 240 minutes of Bt2cAMP stimulation. These findings in combination with our earlier study (20) establish a time course of cAMP-dependent assembly of the p54\textsuperscript{nrb}/PSF/SF-1 complex on the -57/-38 region of the CYP17 promoter, and suggest that PSF and p54\textsuperscript{nrb} promote or at minimum are retained during clearance of RIP140, Sin3A, and HDAC2 while SF-1 is recruited. Independent
dynamics of promoter occupancy by \( p54^{nrb} \), PSF, and SF-1 indicate unexpected plasticity in their interactions with each other and with promoter DNA and/or other coregulatory proteins bound to the CYP17 promoter.

3.12 \( p54^{nrb} \) Also Enables Assembly of a HAT/p160 Coactivator Complex

GCN5 and p300 occupancy of the promoter increase before that of SF-1 does in the second transcription cycle (Figure 3.2D). Therefore, we considered the possibility that these HATs may be recruited to the cAMP-responsive \( p54^{nrb}/PSF \) transcription complex and together act to initiate the second cycle of SF-1 mediated transcription. We tested for \( Bt2cAMP \)-dependent interaction of GCN5 with \( p54^{nrb} \) in the mammalian two hybrid system in H295R cells. This interaction does occur, and is potentiated by SRC-1 but decreased by PSF (Figure 3.6A).

![Figure 3.6](image)

**Figure 3.6** GCN5 interaction with SF-1 can also occur via PSF-sensitive complexes containing \( p54^{nrb} \) and p160 coactivators.

(A) H295R cells were transfected with pG5, pBind-GCN5, pAct-\( p54^{nrb} \), pBKCMV-SRC-1, and pCR3.1-PSF using Gene Juice. After Twenty-four h, transfected cells were treated with 1 mM \( Bt2cAMP \) and then harvested for quantification of reporter gene activity. (B) Cells were transfected with pG5, pBind-GCN5, and pAct-GRIP-1 in the presence and absence of pCR3.1-\( p54^{nrb} \), pCR3.1-PSF and/or pDNA3.1-SF-1, treated with \( Bt2cAMP \), and then harvested for dual luciferase assays. Data graphed in both panels are from 2 experiments performed in triplicate and normalized to \( Renilla \) expression from the pAct vector +/- SEM. *(Legend continues next page)*

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We next asked whether the interaction between a p160 coactivator specific to cycle II and GCN5 is fostered by p54nrβ using the two hybrid system to detect p54nrβ-dependent assembly of a p160/GCN5 complex. A higher order complex among these factors and SF-1 would coincide with simultaneous occupancy on the promoter in the second SF-1 dependent transcription cycle in ChIP time courses, when p54nrβ, GCN5, and all three p160 coactivators are enriched (see Figures 3.2D, E, 3.5C). Modest Bt2cAMP-dependent interaction between GCN5 and GRIP-1 is strengthened only when both SF-1 and p54nrβ are coexpressed (Figure 3.6B). Like the GCN5/p54nrβ interaction, this complex is sensitive to PSF. The kinetics of GCN5 binding coincides additively with both p54nrβ and SF-1 binding from 120 to 210 minute time points (Figures 3.2D and 3.5C). Together, these data implicate the assembly of a GCN5/p54nrβ complex on the CYP17 promoter in H295R cells during cycle II before recruitment of p160s and SF-1. SF-1 recruitment between 150 and 180 minutes destabilizes PSF interaction with the promoter (Figure 3.5C), and further stabilizes GCN5 and p160 binding in the complex.

3.13 CtBP Recruitment Corresponds with Exchange of Transcription Activators for Repressors on the CYP17 Promoter

CtBP corepressors were initially identified and characterized due to their ability to suppress transformation by the E1A viral oncoprotein (190), and may also have a role in cases of myeloid leukemia that involve the AML1/MDS1/EVI1 (AME) fusion oncoprotein, both to promote oncogenic cellular replication (191) and to enable
oncogenic transformation (192) via repression of AME-dysregulated genes. A recent report by Zhang et al shows that CtBP1 can repress E cadherin via promoter binding, inducing cancer cell migration in response to increases in the nuclear NADH:NAD$^+$ ratio, which occurs during acute hypoxia or with chemical manipulation of cellular NADH levels (193). This mechanism requires a DNA targeting factor with CtBP interaction motifs, and CtBP is thought to bridge such factors to HDACs (194); thus, this repression mechanism is sensitive to deacetylase inhibition.

It is not established if or how intrinsic CtBP activity is involved in mechanisms of trans repression by CtBPs. CtBP1 and 2 bind NADH and/or NAD$^+$ (165, 195-197). Upon binding NADH, CtBPs self-associate (160, 196) and/or associate with E1A (165, 196). In the absence of NADH, CtBP1 has intrinsic slow dehydrogenase activity (165, 196) and can promote or inhibit histone targeting of the HAT p300 (160). There have also been reports of NADH-dependent inhibition of CBP HAT activity (198, 199). It is thought that binding of CtBPs to the acetyl-lysine binding bromodomains of these and other HATs, resulting in loss of chromatin targeting of HAT activity in an HDAC-independent manner, is a second mechanism of CtBP trans repression (160).

In the monomeric form, CtBP1 binds a signature PxD(L/I)(S/K) motif within the p300 bromodomain (BrD) (160, 200). GCN5 and two of the three p160s have potential CtBP binding motifs proximal to a conserved NR box (Figure 3.7A). Other HATs and the CBP-related transcription factor CREB show one or more homologous motifs, while in p54$^{nbr}$ and PSF, these motifs are near a conserved aromatic residue (underlined in Figure 3.7A) that is required for snRNP binding and targeting of splicing function in the closely related factor TAT-SF1 (201). Although CtBP1 lacks a nuclear localization
**A**

<table>
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<th>Transcription Regulator</th>
<th>Domain Included</th>
<th>Demonstrated Interaction with CtBPs</th>
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<td>mGRIP-1</td>
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<td>This study</td>
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<td>hSRC-1</td>
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<td>(NR4)</td>
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<td>(199), This study</td>
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<td>(199)</td>
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**Figure 3.7** CtBP dehydrogenases disrupt cycles of cAMP-dependent SF-1 mediated transcription of the CYP17 gene via multiple interactions with coregulators. (Legend continues next page)
sequence, CtBP1-mediated repression is modulated by factors that facilitate nuclear localization of the corepressor (202) in concurrence with that of SR family splicing factors, of which PSF and p54\textsuperscript{nrb} are members (203).

In order to determine whether CtBPs interact with coregulators involved in cAMP responsiveness and CYP17 transcription, we performed mammalian two hybrid experiments. CtBP1 interacts with GRIP-1 (Figure 3.7B) and GCN5 (Figure 3.7C). Similar positive interactions were detected in both Jeg3 and H295R cells (data not shown). We next determined the effect of mutating the NADH binding site, as done by Kim et al (160), on the ability of CtBP1 to interact with GCN5. These experiments showed a greater degree of interaction of GCN5 with the CtBP1 dimerization deficient, NADH-binding site mutant when compared to wild type CtBP1 (Figure 3.7D). This finding suggests a role for GCN5 binding to CtBP1 in the disruption of CtBP oligomerization.

Because CtBP1 interacts with a number of factors involved in regulation of CYP17 transcription, we next asked whether CtBPs interact with SF-1. We found that

(A) Alignment of binding motifs in coregulatory proteins. Interactions that have been experimentally confirmed are cited. Underlined residues: NR4, the fourth nuclear receptor box interaction motif in these p160 coregulators; BrD, conserved acetyl-lysine binding bromodomain residues; SplFn, a conserved aromatic residue that is important for the splicing activity of Tat-SF-1, a homologous SR family splicing factor (201). (B-E) Cells were transfected with pG5 and (B) pBind-CtBP1 and pAct-GRIP-1, (C) pBind-GCN5 and pAct-CtBP1, (D) pBind-GCN5 and pAct-CtBP1 or pAct-CtBP1 G183V, (E) pBind-SF-1 and pAct-CtBP1 or pAct-CtBP1 G183V and then treated with 1 mM Bt2cAMP. Reporter gene activity was quantified from cell lysates using dual luciferase assays. Open bars are untreated controls and striped bars are treated with 1 mM Bt2cAMP. Graphed data (panels B-E) represent the mean +/- SEM and are from 2 experiments performed in triplicate. Statistically significant difference between cells transfected with pAct empty vector versus cells transfected with pAct-GRIP-1 (B) or pAct-CtBP1 (C and E) are denoted by ‡, p<0.05 or ‡ ‡ ‡, p<0.001. Ampersand denotes statistically significant difference between untreated and Bt2cAMP-treated cells, where &&, p<0.01 and &&&, p<0.001. Carets denote statistically significant difference between cells expressing wild type CtBP1 versus cells expressing the CtBP1 G183V mutant; ^^^, p<0.01; ^^^^, p<0.001. (F) H295R cells were synchronized for 2 hours with 2.5 M α-amanitin, then treated with 1 mM Bt2cAMP for the indicated times and subjected to ChIP using antibodies against SF-1, CtBP1, or CtBP2. Outputs are normalized to delta Ct values obtained for 1% input controls, and results are presented as percent of delta Ct values for untreated cells at the corresponding time point. Data graphed represents the mean from 2 experiments performed in duplicate.
SF-1 interacts with CtBP1, and the interaction signal was weaker in the G183V NADH-binding mutant (Figure 3.7E). Bt2cAMP weakened the interaction of SF-1 with G183V CtBP but not wild type CtBP1. This data suggests that both the NADH-sensitive oligomerization of CtBP1 and Bt2cAMP stimulation can affect recruitment of the repressor to SF-1-containing complexes.

To clarify the role of CtBPs in CYP17 transcriptional activation in the context of other coregulator interactions on this promoter, we repeated temporal ChIP on the CYP17 promoter for CtBP1 and 2. Acute CtBP2 binding between 30 and 60 minutes (Figure 3.7F) coincides with loss of GCN5 and SRC-1 (cf. Figures 3.2D and E), while loss by 90 minutes is concurrent with loss of SF-1 promoter occupancy. CtBP1 also binds to the promoter rapidly between the 180 and 210 minute time points (Figure 3.7F), corresponding with dissociation of the GCN5/p54nrb/SF-1/p160 complex from the promoter (cf. Figures 3.2D, E, 3.5C, and 3.6).

3.14 CtBP1 Alters cAMP Dependent Activation of CYP17 and Basal Interactions among SF-1, GCN5, and SRC-1

To assess the ability of CtBPs to regulate CYP17 transcription, we performed cotransfections of CtBP1 and other functional coregulators with SF-1 or LRH-1 and a luciferase reporter with a promoter containing two copies of the CYP17 SF-1 recognition motif (-57/-37 element) (Figure 3.8A). Wild type CtBP1 expression in H295R cells lowered basal (compare sets 1 and 2) and SF-1-dependent CYP17 expression (sets 3 and 4), but not LRH-1 dependent expression (sets 6 and 7) with or without Bt2cAMP treatment and this repression requires the SF-1 AF-2 hexamer (sets 4 and 5). CtBP1 therefore has coregulator function in CYP17
transcription independent of CtBP2, corresponding with increases in promoter occupancy by CtBP1 after 90 minutes of Bt2cAMP stimulation (Figure 3.7F). In light of the above results that GCN5 and GRIP-1 interact with CtBP1, and the findings of others demonstrating that GCN5 coimmunoprecipitates with CtBP1 (199), we asked whether SRC-1 coactivator function is also altered by CtBPs. Therefore, we tested whether wild type CtBP1 disrupts the GCN5/SRC-1/SF-1 interaction in the two hybrid system in H295R cells, but found that CtBP1 overexpression had a stabilizing effect on the trimer (Figure 3.8B). On the other hand, CtBP1 decreased the stability of the acetyltransferase deficient

![Figure 3.8](image)

**Figure 3.8** cAMP- and NADH-dependent CtBP1 modulation of GCN5/SRC-1 cooperativity. (A) Cells were transfected with 250 ng pGL3-CYP17 2x57, pcDNA3.1-SF-1, pcDNA3.1-SF1DAF2 mutant, pCI-LRH1, and/or pRC-CMV-CtBP1, stimulated with Bt2cAMP, and then harvested for dual luciferase assays. (B) Mammalian two-hybrid assays were performed as described in Materials and Methods by transfecting cells with pBind-SF-1, pAct-GCN5, pAct-GCN5 E214Q, pBKCMV-SRC-1, and/or pRC-CMV-CtBP1, then incubated with 1 mM Bt2cAMP for 16 hours. Interaction was quantified using dual luciferase assays. In both panels A and B, open bars represent untreated control and striped bars Bt2cAMP treatment. Graphed data represent the mean +/- SEM and are from 2 experiments performed in triplicate. In panel A, daggers denote statistically significant effect of overexpressed vectors; p<0.01. ‡ ‡ ‡, p<0.001. Statistically significant effects of CtBP1 overexpression are denoted by $$; p<0.01. Ampersand (&&, &<0.001) indicates statistically significant effect of SF-1 AF-2 deletion. Asterisks denote statistically significant differences between cells transfected with only pBind-SF-1 and pAct-GCN5 compared to groups also overexpressing SRC-1 and CtBP1; *, p<0.05 or ***, p<0.001.
3.15 Chromatin Remodeling Occurs before and after Pol II Interaction with the CYP17 Promoter

Studies characterizing cyclical ER-α binding to the pS2 promoter have linked ATP dependent chromatin remodeling to 1) time points immediately following receptor binding and 2) temporary repressive chromatin structure between cycles of ligand-bound ER-α interaction with the promoter (87). To determine if ATP dependent chromatin remodelers act as “gatekeepers” of promoter accessibility for SF-1-mediated Pol II recruitment, we examined a panel of three chromatin remodeling complex ATPase subunits in assays of temporal CYP17 promoter binding. We found that the Brahma-related gene 1 (BRG1) ATPase, a member of some SWI/SNF remodeling complexes, is associated with the promoter 30 minutes before peak SF-1 binding in cycle I, and the sucrose non-fermenting 2 (SNF2) component of the imitation SWI or ISWI complex binds early in cycle II (Figure 3.9A). As SF-1 binding peaks during this cycle, Brahma1 (Brm) ATPase exchanges for the BRG1 ATPase. This Brm binding coincides with increased H4 acetylation (Figure 3.2C). These data suggest that proximal CYP17 promoter chromatin structure is made permissive to Pol II binding and possibly transcription early in transcription cycle I by a SWI/SNF remodeling complex containing BRG1. Additional remodeling by ISWI occurs early in cycle II, reestablishing permissive conditions for PSF and p54 binding before chromatin is further remodeled by SWI/SNF complexes, concomitant with SF-1 and Pol II binding.

Each nucleosome is composed of a core tetramer and two histone H2A/H2B
Figure 3.9 Chromatin remodeling ATPase recruitment is part of SF-1-dependent CYP17 transcription cycles and coincides with rapid loss and gain of histone H2 from promoter nucleosomes.

(A) Temporal ChIP of chromatin remodeling ATPases on the CYP17 promoter and transcription start site was performed. Lysates were immunoprecipitated with antibodies against SF-1, BRG1, Brm1, and SNF2 and purified DNA subjected to real time PCR using primers that amplified the -104/+43 region of the CYP17 promoter. (B) Temporal ChIP analysis of Bt$_2$cAMP-stimulated SF-1 and histone H2B binding to the CYP17 promoter. Sheared chromatin isolated from cells treated for 30 minutes to 4 hours was immunoprecipitated with antibodies against SF-1 or histone H2B. Output data are normalized to delta Ct values obtained for 1% input controls, and results are presented as percent of baseline value obtained for untreated cells at each time point.
dimers, which may have a role in stabilizing DNA-nucleosome interaction in inactive chromatin. ATP dependent chromatin remodeling complexes have been shown to exchange histone H2 dimers between nucleosomes on different short chromatin templates in vitro, and it is thought that removal of these dimers disfavors higher order chromatin packing of heterochromatin (104). We asked whether changes in the composition of histone H2 dimers in nucleosomes coincide with remodeling by DNA-interacting ATPases in vivo. Performing temporal ChIP for histone H2B occupancy of the same promoter region, we discovered that BRG1 SWI/SNF ATPase recruitment correlates with strong depletion of H2B within 30 minutes of Bt2cAMP stimulation (compare Figures 3.9A and B), which is evidence that H2A/H2B dimers are disrupted during cAMP-dependent CYP17 activation. On the other hand, SNF2H recruitment corresponds with strong enrichment of H2B at the beginning of cycle II, followed within 30 minutes by Brm SWI/SNF ATPase binding and a subsequent return to histone H2 depletion. Thus, we postulate that SWI/SNF ATPases transfer H2B in H2A/H2B dimers to chaperones, or are otherwise exchanged from the nucleosome(s) at the proximal promoter of CYP17 during cAMP-dependent transcription.

3.16 Activating Histone Lysine Methyltransferases are Targeted to the CYP17 Promoter in Response to Bt2cAMP

Another class of histone modifications that regulate transcription is the methylation of histone H3 and histone H4 lysine or arginine residues [for a review, see (205)]. Histone monomethylation at K20 on the N terminal tail of histone H4 correlates with hyperacetylation of this tail in transcriptionally competent chromatin; trimethylation...
Histone lysine methyltransferases are recruited to the CYP17 promoter during SF-1-dependent cycles of transcription.

\[\text{α-Amanitin synchronized H295R cells were treated for time periods ranging from 30 minutes to 4 hours with 1 mM Bt2cAMP, exposed to 1% formaldehyde and the purified lysates immunoprecipitated using anti-SF-1, anti-trimethylated (K4) histone H3, anti-monomethylated (K20) histone H4, or anti-trimethylated (K20) histone. Output data are normalized to values obtained for 1% input controls, and results are presented as percent of baseline value obtained for untreated cells at each time point. Data graphed represents the mean from 2 experiments performed in duplicate.}\]

at this site represses transcription (206). On the other hand, H3 K4 trimethylation in yeast is required for ATP-dependent chromatin remodeling by Isw1p ATPase and correlates with recruitment of a factor involved in mRNA maturation (207).

Using temporal ChIP, we assayed for these Bt2cAMP stimulated changes in histone lysine methylation at the CYP17 promoter. Histone H4 K20 monomethylation is upregulated as SF-1 occupancy peaks at 60 and 180 minutes, as well as at 120 minutes (Figure 3.10); all three peaks correlate with histone H4 hyperacetylation (cf. Figure 3.2C). Repression-associated trimethylation at this site is not upregulated during the first four hours of Bt2cAMP stimulation. Trimethylation at histone H3 K4 occurs transiently during cycle I and also appears to be upregulated between 210 and 240 minutes of treatment (Figure 3.10); the initial event does in fact precede recruitment of splicing
factors by at least 60 minutes (cf. Figure 3.5C). Of the panel of ATPases examined in temporal ChIP assays (Figure 3.9A), only SNF2H, a mammalian homolog of yeast Isw1p, interacts with the promoter during the interval between histone H3 K4 trimethylation at 60 minutes and upregulation of p54nrb and PSF recruitment between 120 and 180 minutes (cf. Figures 3.5C, 3.9A, and 3.10). Thus, our data are consistent with roles for at least two histone lysine methylation events already established to correlate with (1) promoter histone H4 acetylation and transcriptional competence, i.e. monomethylated histone H4 K20, and (2) histone H3 K4 trimethylation, associated with subsequent ISWI chromatin remodeling, and then recruitment of factors with roles in RNA maturation, a pattern seen in both yeast (207) and in human adrenocortical cells.

3.17 Sph Modulates SF-1-Mediated Transcription Cycles

The SF-1 antagonist Sph decreases β2cAMP-dependent transcription of CYP17 in H295R cells (Figure 3.11A), in line with Sph-mediated disruption of SF-1/SRC-1/GCN5 cooperativity (Figure 3.4C). In order to examine the effect of Sph on SF-1-mediated transcriptional cycling, we repeated synchronized temporal ChIP experiments with 5μM Sph treatment. In the absence of β2cAMP, SF-1 in Sph-treated cells associated with the CYP17 promoter within 30 minutes of treatment and again increased after three hours with a longer delay between increases in binding compared to β2cAMP-stimulated cycling, and with muted amplitude or binding rate (Figure 3.11B). Sph strongly attenuated the ability of β2cAMP to initiate the binding of SF-1 to the CYP17 promoter in cycle I (Figure 3.11B). SF-1 recruitment during cycle II was relatively unaffected by the antagonist, indicating significant cAMP-dependent release of
Figure 3.11 cAMP-stimulated CYP17 mRNA expression and cycles of SF-1-mediated promoter binding to the CYP17 promoter are antagonized by Sph.

(A) H295R cells treated with 1 mM Bt2cAMP and/or 1 µM Sph for time periods ranging from 1 hour to 12 hours. Total RNA was isolated and subjected to quantitative RT-PCR. Data graphed are expressed as fold change compared to untreated controls and represent the mean +/- SEM of CYP17 mRNA expression normalized to the cellular α-actin mRNA content from four experiments performed in triplicate. (B) α-Amanitin synchronized H295R cells were treated for time periods ranging from 30 minutes to 4 hours with 1 mM Bt2cAMP and/or 1 µM Sph, exposed to 1% formaldehyde and the purified lysates immunoprecipitated using a polyclonal antibody against SF-1. Data graphed in all panels represent the mean from 3 experiments, each performed in duplicate. Outputs are normalized to delta Ct values obtained for 1% input controls, and results are presented as percent of untreated control delta Ct values at each time point.
Sph from SF-1 within three hours, consistent with previous results (172).

In this chapter, we have defined the sequence and cooperativity of various classes of chromatin modifying coregulators of CYP17 transcription, including HATs, HDACs, ATP-dependent chromatin remodelers, multifunctional RNA and DNA binding splicing factors, and finally, bridging-type coregulators, which appear to include the multifunctional coregulators of the p160 steroid receptor coactivator family, CtBP corepressors, and p54. Since the transcription of CYP17 in the adrenal cortex cell line examined is absolutely dependent upon cAMP stimulation and PKA (references reviewed in (16)), many of these coregulators are candidate effectors of cAMP and PKA in this context, as are the dynamic chromatin marks they leave in the wake of their productive interactions on DNA and nucleosome(s). Finally, the presence of SF-1 agonist, Sph, is overcome by cAMP after three or more hours, enabling transcription (Figure 3.11). This is consistent with opposing abilities of cAMP and Sph to strengthen or weaken interaction among members of an early cAMP-stimulated, dynamic SF-1 coactivator complex comprised of SF-1, SRC-1, and GCN5 (Figure 3.4).
Chapter 4. Results—Part II: Post-Translational Modification of Steroidogenic Factor-1 Regulates Periodic Cycles of CYP17 Transcription

4.1 CKII and GSK3β Phosphorylate SF-1 in Vitro

We hypothesized that there may be kinase targets near the entryway to the ligand binding pocket of SF-1 and that phosphorylation here regulates receptor-mediated induction of transcription. In order to identify kinases capable of phosphorylating the receptor, we carried out in vitro kinase assays using purified SF-1 and either recombinant PKA catalytic subunit, CKI, CK2, GSK3β, or ERK. As previously found (208), incubating the immobilized receptor with the catalytic subunit of PKA had no effect on the amount of radiolabeled phosphate associated with SF-1 (Figure 4.1A). In contrast to previous studies by Hammer et al. demonstrating that SF-1 is an ERK target (209), we were unable to detect phosphorylation of the receptor by ERK2 in this assay. However, both CK2 and GSK3β phosphorylated the receptor (Figure 4.1A). CK2 targets serine or threonine residues in a S/TxxE motif whereas GSK3β targets SxxxS motifs. Moreover, the ability of GSK3β to phosphorylate these S/TxxxS/T motifs is significantly enhanced by the presence of “priming” phosphorylation in the +4 position (210-213). In silico analysis of SF-1 revealed T334, T338, S342, and S346 as potential GSK3β targets (bold letters, Figure 4.1B). Within this region, T335 is a putative CK2 phosphorylation site (boxed). Although the ability of GSK3β to phosphorylate SF-1 was unaffected by the T335A mutation (data not shown), this mutation rendered CK2 unable to phosphorylate the receptor (Figure 4.1C). Mutation of S342 decreased activity of GSK3β towards SF-1 (Figure 4.1C), indicating that GSK3β targets this residue.
Figure 4.1 CK2 and GSK3β phosphorylate SF-1.

A. His-tagged SF-1 was incubated with $^{32}$P-γATP, the catalytic subunit of PKA, recombinant CK1, CK2, GSK3β, or ERK2 for 30 minutes at 30°C. The reactions were terminated and subjected to SDS-PAGE and radiolabeled SF-1 was detected by phosphorimager scanning. B. Amino acid sequence of region 334-346 in human SF-1. Putative CK2 phosphorylation site at T-335 is boxed and bold lettering indicates putative GSK3β phosphorylation sites. C. In vitro kinase assays were carried out using WT or mutant receptor, $^{32}$P-γATP, and CK2 or GSK3β. Jeg3 cells were transfected with pGL3-CYP17-2x57, pRL-TK, and WT or mutant pCMVTag1-SF1 for 24 hours and then treated for 16 hours with 1 mM Bt2cAMP. Data graphed represents the fold change in Firefly luciferase (pGL3-CYP17-2x57) activity over Renilla luciferase (pRL-TK) activity and represents the mean ± SEM of 3 experiments, each performed in triplicate. *, significantly different from untreated WT, #, significantly different from Bt2cAMP-treated WT, p < 0.05. E. Representative gel of samples obtained from H295R cells that were transfected with WT or mutant FLAG-tagged receptor for 48 hours and then incubated in phosphate-free DMEM containing $^{32}$P-phosphorus for 4 hours. Lysates were immunoprecipitated with anti-FLAG antibody and the immobilized receptors subjected to SDS-PAGE, coomassie staining, and phosphorimager scanning. Parallel transfections were carried out and nonradiolabeled lysates separated by SDS-PAGE. Proteins were transferred to PVDF membranes and western blots incubated with a rabbit polyclonal SF-1 antibody. (Legend continues next page)
We next mutated phosphorylation sites in the GSK3β-motif-rich region of SF-1 (Figure 4.1D), generating alanine mutants of T334, T335, T338, S342, and S346 and determined the effects of mutation on the transactivation potential of the receptor. A reporter gene plasmid containing two copies of the SF-1 binding site of the human CYP17 promoter fused to luciferase (pGL3-CYP17-2x57) was transfected into Jeg3 cells because they lack endogenous SF-1 and then treated for 16 hours with 1 mM Bt2cAMP. Mutation of S342 increased both basal and Bt2cAMP-stimulated luciferase activity (Figure 4.1D), suggesting that phosphorylation of S342 regulates SF-1 binding of the CYP17 promoter, and/or interaction with coregulator proteins. Interestingly, mutation of S346A also exhibited higher transactivation activity in unstimulated cells. Moreover, while T335A had no significant effect on luciferase activity in untreated cells, Bt2cAMP-dependent activity of these mutant receptors was significantly higher than WT.

To determine the effect of the mutations on the phosphorylation status of the endogenous receptor, we carried out metabolic labeling on H295R cells that were transiently transfected with WT or mutant SF-1 and then incubated in media containing 32P-phosphorus. As shown in Figure 4.1E (graphed in Figure 4.1F), mutation of T335, T338, and S342 resulted in significant decreases in the amount of radiolabeled phosphate.
incorporated into the receptor, with S342A exhibiting the greatest decrease in phosphorylation when compared to WT. We also carried out metabolic labeling studies to examine the effect of Bt2cAMP and the GSK3ß inhibitor LiCl (214, 215) on the levels of phosphorylated endogenous SF-1. Stimulation with Bt2cAMP for 1 hour increased the cellular content of radiolabeled SF-1 and incubation with LiCl significantly reduced this increase (Figure 4.1G). The presence of radiolabeled receptor in untreated cells indicates that the receptor is phosphorylated and suggests that the endogenous receptor is phosphorylated at multiple sites, possibly by multiple kinases, such as CDK7 (216).

Finally, because the region between T334 and T346 (Figure 4.1B) contains multiple putative GSK3ß phosphorylation sites, we used antibodies raised against the established GSK3ß target ß-catenin phosphorylated at S33 (pS33) or against ß-catenin phosphorylated at both S33 and S37 (pS33pS37) to assess the effect of mutations on the level of phosphorylated receptor. First, we determined if the phospho-specific ß-catenin antibodies could detect phosphorylated SF-1 by treating H295R cells with Bt2cAMP and LiCl for 1 hour. Western blotting found that the p33 ß-catenin antibody detected phosphorylated forms of both endogenous ß-catenin and SF-1 (Figure 4.1H). Although Bt2cAMP had no significant effect on the amount of pS33 ß-catenin (top arrow) in the H295R cells, it increased the levels of phosphorylated SF-1 (bottom arrow). These findings are consistent with metabolic labeling studies shown in Figure 4.1G. As predicted, incubation with LiCl prevented detection of phosphorylated ß-catenin (top arrow) and attenuated the Bt2cAMP-stimulated increase in phosphorylated SF-1 (bottom arrow). Similar findings were obtained when using an antibody raised against ß-catenin phosphorylated at both S33 and S37 (data not shown). To further examine the effect of
mutations on the phosphorylation status of SF-1, we transfected CV-1 cells with WT or mutant FLAG-tagged SF-1 and analyzed the cell lysates by western blotting using anti-pS33pS37 β-catenin or anti-FLAG antibodies. Mutation of T338 and S342 rendered the antibody unable to detect phosphorylated SF-1 (Figure 4.1I), further supporting a role for GSK3β in phosphorylating the receptor. Probing western blots with the p33 β-catenin antibody showed a similar decrease in the detection of the S342A mutant (data not shown).

4.2 SF-1 interacts with CK2 and GSK3β

To further characterize the role of CK2 and GSK3β in modulating SF-1 function, we next assessed the subcellular localization of the two kinases in nuclear and cytoplasmic extracts isolated from H295R cells treated for 5 to 30 minutes with

![Figure 4.2](image)

**Figure 4.2** Nuclear translocation of GSK3β and interaction of SF-1 with CK2 and GSK3β.

A. H295R cells were treated with 0.4 mM Bt2cAMP for 5-, 15-, or 30 minutes and nuclear and cytoplasmic fractions isolated for SDS-PAGE and western blotting. Blots were hybridized to anti-CK2 or anti-GSK3β antibodies. B. H295R cells (150 mm dishes) were treated for 1 hour with 0.4 mM Bt2cAMP and lysates subjected to immunoprecipitation with anti-SF-1 antibody and protein A/G. The immobilized proteins were separated by SDS-PAGE and transferred to PVDF membranes for western blotting. Blots were probed with antibodies directed against CK2 (top) and GSK3β (middle). A fraction (5%) of input lysates was subjected to SDS-PAGE and western blotting for SF-1 (bottom).
Bt₂cAMP. Intriguingly, cAMP stimulation promoted the nuclear translocation of GSK3β (Figure 4.2A). We also detected interactions between SF-1 and both kinases in H295R cells (Figure 4.2B).

4.3 Effect of LiCl and TBB on Complex Formation and CYP17 mRNA Expression

We next determined the effects of inhibiting the catalytic activity of GSK3β on cAMP-stimulated recruitment of GCN5, SF-1, SRC-1, and Pol II to the CYP17 promoter by carrying out ChIP assays. Synchronized populations of cells were treated for 1 hour with 1 mM Bt₂cAMP and/or 20 mM LiCl and the isolated chromatin immunoprecipitated as previously described (217). As previously shown (217), Bt₂cAMP increased the binding of SF-1, GCN5, SRC-1, and Pol II to the promoter (Figure 4.3A). Although LiCl treatment alone increased the binding of GCN5 and SF-1 to the CYP17 promoter, it prevented Bt₂cAMP-stimulated association of these proteins with the CYP17 gene, suggesting that activated signaling through the ACTH/cAMP pathway requires GSK3β activity to promote increased transcription. Indeed, inhibiting GSK3β completely inhibited the ability of Bt₂cAMP to induce the expression of CYP17 mRNA (Figure 4.3B). Inhibition of CK2 activity using TBB (218, 219) also dose-dependently attenuated Bt₂cAMP-stimulated CYP17 mRNA expression (Figure 4.3C), indicating that phosphorylation of the receptor by these two kinases is critical for attaining maximal CYP17 transcription in response to ACTH/cAMP signaling. The inhibitory effect of chemical CK2 and GSK3β inhibitors on cAMP-dependent CYP17 mRNA expression was mirrored by siRNA oligonucleotides directed against the kinases (Figure 4.3D), which
Figure 4.3 Effect of LiCl and TBB on CYP17 transcription.

A. Cells were synchronized by treatment with 2.5 μM α-amanitin for 2 hours and then treated with 1 mM Bt2cAMP and/or 10 mM LiCl for 1 hour. Cross-linking was carried out by exposing cells to 1% formaldehyde and chromatin lysates isolated for immunoprecipitation using antibodies to GCN-5, SF-1, Pol II, and SRC-1. Cross-links were reversed and DNA purified for analysis by real-time PCR and gel electrophoresis. Lane 1, control; lane 2, Bt2cAMP; lane 3, Bt2cAMP + LiCl; lane 4, LiCl. B. Total RNA was extracted from cells that were treated for 16 hours with Bt2cAMP and LiCl (panel B) or TBB (panel C). CYP17 mRNA expression was quantified by real-time RT-PCR and normalized to the mRNA content of the β-actin gene. Data is graphed as fold change in the expression of CYP17 in treated cells when compared to the mRNA expression in untreated control cells and represent the mean ± SEM of 4 experiments, each performed in triplicate. D. H295R cells were transfected with 50 nM CK2 or GSK3β siRNA oligonucleotides and 72 hours later harvested for analysis by SDS-PAGE and western blotting. Blots were incubated with anti-CK2 (left) or anti-GSK3β (right) antibodies. E. CYP17 mRNA expression was quantified in RNA isolated from cells that were transfected with siRNA oligonucleotides directed against CK2 or GSK3β. Some cells were treated with 0.4 mM Bt2cAMP 16 hours prior to RNA isolation (72 hours total incubation time). Data graphed represent the mean ± SEM of 3 experiments, each performed in either triplicate or quadruplicate and is expressed as fold change in CYP17 mRNA expression over control group mean, normalized to actin mRNA content.
also attenuated the stimulatory effect of Bt$_2$cAMP on CYP17 mRNA transcription (Figure 4.3E).

### 4.4 Phosphorylation of S342 Modulates the Association of SF-1 with the CYP17 Promoter

The function of GSK3β-catalyzed phosphorylation of SF-1 in DNA binding was further probed by performing ChIP assays on FLAG-tagged WT or S342A mutant receptor from the chromatin lysates of transiently transfected H295R cells. Primers for ChIP of the CYP17 promoter were designed so that the SF-1 binding site (-57/-37) would be centrally located within approximately a single nucleosome (220), with regions near the 5’ and 3’ ends coinciding with the predicted cis-encoded nucleosome positioning code described by Segal, et al (221). We found that Bt$_2$cAMP increased the binding of WT receptor to the CYP17 promoter by 4.9-fold (Figure 4A). Interestingly, the S342A mutant exhibited a 5.3-fold increase in receptor bound to the CYP17 promoter in unstimulated cells, consistent with transcriptional stalling and supporting the hypothesis that S342 phosphorylation promotes dissociation of SF-1 from the promoter and accelerated transcription by potentiating subsequent cycles of transcription. Both WT and S342A mutant FLAG-tagged receptor were expressed at comparable levels (data not shown).

We next asked if the kinetics of the interaction between the CYP17 promoter and S342 mutant SF-1 in response to Bt$_2$cAMP is altered by carrying out temporal ChIP assays of the same CYP17 promoter segment. Our previous studies demonstrated that SF-1 cycles on and off the CYP17 promoter in ~60 minute cycles, with GCN5 recruitment occurring early in each cycle (217). As previously shown (217), 60 minutes...
Figure 4.4 Phosphorylation modulates SF-1 interactions with the CYP17 promoter.

A. Cells were transfected with WT or S342A mutant SF-1 and treated for 1 hour with 1 mM Bt2cAMP. Proteins were cross-linked to DNA by incubation in 1% formaldehyde and the purified, sheared chromatin lysates sonicated immunoprecipitated with an anti-FLAG antibody. Cross-links were reversed and DNA purified for analysis by real time PCR and gel electrophoresis. B. Cells transfected with WT or mutant (S342A or S342D) pCMVTag1-SF-1. Forty-eight hours after transfection, cells were synchronized by incubation for 2 hours with α-amanitin and then treated for time periods ranging from 30 minutes to 4 hours with 1 mM Bt2cAMP. Cells were exposed to 1% formaldehyde and the purified, sheared chromatin solutions immunoprecipitated using an anti-FLAG antibody. Output data are normalized to Δ-Ct values obtained for input samples and the results are graphed as fold change in Bt2cAMP-stimulated binding of (Legend continues next page)
WT or S342D mutant receptor to the CYP17 promoter, where 100% represents promoter occupancy in untreated control cells at the zero time point. Data graphed represents the mean from 2 experiments performed in duplicate. 

C. α-Amanitin-synchronized cells were incubated with 1 mM Bt2cAMP and 25 nM OA for 30- to 240-min and then incubated in 1% formaldehyde. Cell lysates were isolated, sonicated and immunoprecipitated with an anti-SF-1 antibody. Cross-links were reversed and the purified DNA subjected to real time PCR. Output data are normalized to values obtained for 1% input controls, and results are presented as percent of baseline value obtained for untreated cells at each time point. Data graphed represent the mean of 2 experiments that were performed in duplicate.

Bt2cAMP increased the binding of wild type SF-1 to the CYP17 promoter, however, the amplitude of Bt2cAMP-stimulated S342A or S342D SF-1 cycling on and off of the promoter was severely blunted compared to the WT receptor (Figure 4.4B), consistent with a departure from the kinetics of promoter binding observed with WT SF-1, and providing further evidence that dynamic phosphorylation of S342 enables endogenous receptor cycling on and off of the CYP17 promoter.

4.5 Phosphatase Activity Is Required for SF-1 Cycling on and off the CYP17 Promoter

If phosphorylation enables cycling of SF-1 during iterative rounds of transcription, then phosphatase activity may be equally as important in promoting CYP17 transcription by enabling receptor recycling. We have previously established that PP2A or another okadaic acid (OA)-sensitive Ser/Thr phosphatase is essential for Bt2cAMP-dependent transcription of steroidogenic genes in the human adrenal cortex, including CYP17, CYP11A1, and CYP21 (164, 222). Moreover, Winnay et al., have shown that OA prevents ACTH-stimulated binding of SF-1 to the melanocortin 2 receptor and subsequent polymerase recruitment and transcription (223). Temporal ChIP of α-amanitin synchronized cells treated with Bt2cAMP in the presence and absence of OA showed that periodic binding of SF-1 to CYP17 was inhibited by OA (Figure 4.4C).
4.6 **K253 and R255 Modulate the Transactivation of a CYP17 Reporter Gene**

Further inspection of crystallographic data (56, 224-226), revealed the presence of two positively charged residues (K253 and R255) in a highly mobile loop near the entryway to the ligand binding pocket (Figure 4.5A) that is conserved across numerous species (Figure 4.5B). We refer to this loop, minimally encompassing residues 250-260, as the SF-1 “ligand binding gate”, which we hypothesized may interact with phospho-S342 and control receptor activity, potentially by enabling further allosteric rearrangement. To test this hypothesis, we mutated K253 and R255 to either alanine or aspartic acid and determined the effect of these mutations on SF-1 transactivation of CYP17 reporter in transiently transfected Jeg3 cells. K253D significantly increased basal and Bt2cAMP-stimulated luciferase activity (Figure 4.5C). Both K253A and R255A mutants decreased basal reporter gene activity when compared to WT. The K253D/R255D double mutant increased reporter gene activity to a level similar to that of the K253D mutant, indicating that the charge of this lysine residue plays a key role in modulating the transactivation potential of the receptor.

4.7 **Acetylation of SF-1 Modulates the Transactivation Potential of the Receptor**

We postulated that K253 is also a target for post-translational modification. Several nuclear receptors (227, 228), including SF-1 (229-231), and coactivator proteins (232) are acetylated. Moreover, we have demonstrated that CYP17 transcription depends on the SF-1/SRC-1/GCN5 AT complex (217). Based on the location of K253 and the effect of mutating this amino acid on receptor function, we speculated that this residue might be targeted by GCN5 or another AT, so we determined the acetylation status of SF-1, by immunoprecipitating it from H295R cells and probing Western blots with anti-
Figure 4.5  Mutation of SF-1 at the entryway to the ligand binding pocket alters transactivation potential of the receptor.

A. Ligand binding gate of SF-1 adopted from the crystal structure of Li et al. Receptor shown with PA docked into the ligand binding domain. Post-translationally modified amino acid residues (S342, T335, and K253) are denoted. B. Alignment of ligand binding gate across multiple species and human LRH-1 (liver receptor homologue-1). C. Jeg3 cells were transfected with pGL3-CYP17-2x57, pRL-TK, and WT or mutant pCMVTaq1-SF-1 for 24 hours and then treated with 1 mM Bt2cAMP for 16 hours. Luciferase activities in isolated lysates were quantified by luminometry. Data is graphed as the fold change in Firefly luciferase (pGL3-CYP17-2x57) activity over Renilla luciferase (pRL-TK) activity and represent the mean ± SEM of 3 experiments, each performed in triplicate. *, Significantly different from untreated WT, #, significantly different from Bt2cAMP-treated WT, p < 0.05.
acetyl lysine antibody. Acetylated lysine residues on SF-1 are present in control and Bt2cAMP-treated cells, and acetylation increased after 1 hour Bt2cAMP treatment (Figure 4.6A). In vitro acetylation of WT and K253A mutant receptor immunoprecipitated from control and Bt2cAMP-treated H295R cells indicated an approximate 55% decrease in acetylation of the K253A mutant compared to the WT receptor in untreated cells (Figures 4.6B and 4.6C). Stimulation with Bt2cAMP had no significant effect on the ability of the WT receptor to be acetylated in vitro by GCN5, however the acetylation of the K253A mutant was higher when the receptor was isolated from Bt2cAMP-treated cells when

![Diagram A](image1.png)  

**Figure 4.6** GCN5 acetylates SF-1 at K253.

A. H295R cells were treated for 1 hour with 1 mM Bt2cAMP. Cell lysates were immunoprecipitated using an anti-SF-1 antibody and the immobilized receptor subjected to SDS-PAGE and Western blotting. Upper panel is western blot hybridized to an anti-acetyl lysine antibody and lower blot is 5% of input lysate probed using an anti-SF-1 antibody. Blots were imaged using an ECF Western blotting kit. B. Representative gel showing 14C-labeled SF-1. FLAG-tagged WT or K253A mutant SF-1 was immunoprecipitated from transiently transfected H295R cells that were treated with 1 mM Bt2cAMP for 1 hour. The purified receptor was incubated with GCN5 and 14C-acetyl CoA, washed, and subjected to SDS-PAGE and fluorimetry (top) or western blotting with an anti-FLAG antibody (bottom). C. Graphical representation of acetylation assay carried out on WT or K253A mutant SF-1 isolated from H295R cells and incubated with GCN5 and 14C-acetyl CoA. Data represents the mean ± STD of four experiments, each performed in at least duplicate. D. Cells were transfected with pGL3-CYP17-2x57, pRL-TK, and WT or mutant pCMVTag1-SF-1 then treated with Bt2cAMP. Lysates were isolated and luciferase activity quantified by luminometry. Data graphed represent the mean ± SEM of 3 experiments, each performed in triplicate.
compared to isolates from control cells (Figure 4.6C). These findings identify K253 as one of several amino acids targeted by ATs. If K253 is an AT target, the loss of positive charge, rather than the post-translationally modified structure of acetyl-lysine, is expected to be integral in modulating receptor activity. Reporter gene assays using the K253Q acetyl-mimic showed a significant decrease in basal transactivation potential of the receptor and a complete loss of Bt2cAMP-stimulated CYP17 reporter activity (Figure 4.6D).

In this chapter, we have confirmed that post translational modification of the SF-1 ligand binding domain is carried out by GSK3β (Figure 4.3B) and CK2 (Figure 4.3C) kinases, and potentially the GCN5 AT (Figure 4.6C). SF-1 has evolved ligand binding domain residues with complementary charge (Figure 4.5B), the context of which (Figure 4.5A) suggests that they may interact conditionally before acetylation at K253 and after phosphorylation at T335 and S342, and thereby affect ligand binding to SF-1 allosterically. It is a notable possibility, remaining untested, as to whether these interactions are enhanced or enabled when the relevant receptor surface is buried by protein-protein interaction and not water accessible, which would presumably shield charged residues from each other.
5.1 ACTH and cAMP Induce Rapid Accumulation of Reduced Pyridine Nucleotides via PKA Activation

The array of pleiotropic effects of PKA induction by ACTH/cAMP in the adrenal cortex includes pyridine nucleotide accumulation and a multifaceted, complex mechanism of transcriptional induction. We sought to identify a mechanism of PKA-induced adrenal cortex transcription which corresponds with the induced formation of SF-1:coactivator complexes (233).

It is established that ACTH induces accumulation of reduced NADPH in adrenal cortex (234, 235), and that NADPH is the major provider of reducing equivalents to steroidogenic (15) and other pathways relying on cytochrome P450 enzymes (236, 237). In the adrenal cortex, PKA specifically induces metabolic flux through the pentose phosphate pathway (234, 238), reducing NADP$. Based on these previous studies, we first verified that ACTH and dibutyryl cAMP (Bt2cAMP) stimulate accumulation of reduced pyridine nucleotide in cultured H295R human adrenocortical cells by measuring the accumulation of NAD(P)H autofluorescence using confocal microscopy (Figure 5.1A). Increases in NAD(P)H autofluorescence typically peaked at approximately 16-fold of the levels in cells prior to stimulation, while unstimulated cells did not show an increase in cytoplasmic NAD(P)H autofluorescence for up to 60 minutes (data not shown). The overall ratio of oxidative:reductive reactions in treated cells as measured by
Figure 5.1 NAD(P)H levels respond to ACTH/cAMP.

(A) NAD(P)H confocal autofluorescence during ACTH/cAMP treatment of H295R cells. Reduced pyridine nucleotide autofluorescence (blue) with UV laser stimulation was recorded at times following addition of 100 nM ACTH or 1 mM Bt2cAMP. Reactive oxygen indicator, dichlorofluorescein diacetate, was imaged separately (green or yellow). (B) Static nuclear or cytoplasmic regions were defined as regions of interest and the signal ratio of nuclear/cytoplasmic autofluorescence was calculated and averaged for 5-9 cells under each experimental condition. Pretreatment with H-89 (10 µM) was carried out for 20 hours before ACTH stimulation and measurement.
dichlorofluorescein, a reactive oxygen species indicator, indicated that cells favored the loss of oxidative radicals during acute ACTH/cAMP stimulation (Figure 5.1A).

We next asked whether ACTH/cAMP stimulates differential pyridine nucleotide reduction in distinct subcellular compartments by defining nuclear and cytoplasmic regions of each cell in the above time courses and calculating the average ratio of nuclear to cytoplasmic NAD(P)H in the timecourses shown in Figure 5.1B. Nuclear levels of NAD(P)H, which are initially one third to one half of cytoplasmic levels, increase at a faster rate than cytoplasmic levels, but ultimately reach similarly elevated levels. PKA inhibition with 10µM H-89 severely blunted increased NAD(P)H, and prevented nucleocytoplasmic redistribution (Figure 5.1B). We conclude that ACTH/cAMP activates NAD(P)^+ reduction and redistribution in H295R cells primarily, though not exclusively, via PKA-stimulated metabolism.

5.2 Pyridine Nucleotides Activate Nuclear-Cytoplasmic CtBP1/CtBP2 Shuttling

NADH, the established cofactor for the CtBP1 dehydrogenase (165, 195) involved in its homodimerization (160), could be influenced by ACTH/cAMP along with NADPH. By serving as a substrate for lactate dehydrogenase, pyruvate depletes NADH, thereby maintaining a high NAD^+/NADH ratio (193, 239). In order to validate the use of pyruvate for the purpose of manipulating NAD(P)H, we confirmed that exogenous lactate rapidly (within 15-30 minutes) stimulates NAD(P)H autofluorescence in H295R cells, and that application of a slightly higher concentration of pyruvate more rapidly (within 1 minute) quenches this signal below initial levels (Figure 5.2A).

The ability of CtBP1 to interact with other cellular proteins is affected by NADH binding (160, 195). In addition, CtBP1 partner switching is known to affect its subcellular
Figure 5.2 Pyridine nucleotide metabolism affects CtBP1 and 2 localization in H295R cells.

(A) Confocal measurement of NAD(P)H autofluorescence during a treatment time course following addition of 3.3 mM sodium lactate, then immediately following 3.8 mM sodium pyruvate addition is shown. Some cells were detached during addition of pyruvate. (B) and (C) CtBP1 and 2 in nuclear and cytoplasmic extracts from stimulated H295R cells. Following indicated treatment with 1 mM Bt2cAMP and/or 5 mM pyruvate supplemented media and fractionation, 25 µg protein from nuclear or cytoplasmic extracts was analyzed by SDS-PAGE and western blotting of CtBP1 (B) or CtBP2 (C). Western blots of 25 µg nuclear or cytoplasmic H295R cellular extracts were analyzed with paired controls and the ratio of nuclear to cytoplasmic CtBP1 from western blot densitometry normalized to this ratio in paired controls was calculated and plotted as a function of time. The experiment was performed twice in triplicate. Pyruvate treatments in these panels and other figures are expressed as amount of sodium pyruvate supplementing the 2 mM standard media concentration.
localization (202, 203). Thus, we asked whether nuclear/cytoplasmic shuttling of CtBP1 and 2 occurs in response to ACTH/cAMP-evoked NAD(P)H changes. We also tested the effect of NAD(P)H depletion by incubating cells with excess pyruvate. H295R cells were treated with Bt2cAMP or pyruvate for times ranging from 5 to 120 minutes and then fractionated into nuclear and cytoplasmic extracts for analysis by western blotting.

Intriguingly, both CtBP1 and 2 were found to oscillate between nucleus and cytoplasm (Figures 5.2B, C). CtBP2 oscillation in response to Bt2cAMP matched that of CtBP1 during this time period. Pyruvate also stimulates CtBP1 and 2 shuttling, indicating a role for ACTH-induced modulation of pyridine nucleotide metabolism in the oscillatory fluxes of CtBP proteins across the nuclear envelope.

5.3 Pyruvate Modulates SF-1 and CtBP1 Binding to the CYP17 Promoter

As discussed earlier, CYP17 activation by Bt2cAMP involves both SF-1 and CtBP (233). In order to determine whether pyruvate can affect SF-1 binding to the CYP17 promoter, we performed ChIP of the promoter using SF-1 antibody (233) after 12 hours of exposure to 30 mM pyruvate. Excess pyruvate increased SF-1 binding to the promoter (Figure 5.3A), consistent with either stalled binding or enhanced transcription. We asked whether oscillation in the kinetics of the CtBP proteins and SF-1 on the CYP17 promoter (233) corresponds with nuclear-cytoplasmic oscillation of CtBP. Endogenous SF-1, CtBP1, and CtBP2 binding to this segment of the CYP17 promoter, -104/+43, was examined at half-hour intervals following synchronization with α-amanitin, then treatment with 5 mM pyruvate above standard concentration (Figure 5.3B). CtBP2 oscillation on the promoter corresponds with bulk nuclear/cytoplasmic flux of this protein seen in cells treated with 30 mM pyruvate, waning with nuclear export at 30 minutes.
Pyruvate affects CtBP and SF-1 interaction on the CYP17 gene. (A) ChIP measuring SF-1 binding to CYP17 -104/+43 following 12 hour treatment with 30 mM pyruvate. (B) Temporal ChIP of SF-1, CtBP1, and 2 measuring SF-1 binding to CYP17 -104/+43 following treatments with 5 mM pyruvate for indicated times. Output is normalized to paired controls (actin). (C) Quantitative RT-PCR of CYP17 normalized to actin and expressed as fold of controls was calculated for mRNA from H295R cells treated with media supplemented with 0, 2, 5, 15, or 30 mM pyruvate, as indicated for 18 hours. Experiment was repeated 4 times in quadruplicate, except for cotreatment data, from one experiment. * indicates significant difference from control.

Pyruvate stimulation and returning by 60 minutes, consistent with a return of CtBP2 to the nucleus (Figures 5.2C, 5.3B). However, CtBP1 binding to the CYP17 promoter at 120 minutes does not correspond with nuclear enrichment of this protein.

Stalling of SF-1 on the CYP17 promoter is expected to repress CYP17 mRNA accumulation, while cycling of SF-1 and coregulators is consistent with transcription.

We determined that pyruvate stimulates endogenous adrenal cortex CYP17 transcription in the absence or presence of ACTH/cAMP signaling (Figure 5.3C), consistent with pyruvate promoting cyclic binding of SF-1 and CtBPs to the CYP17 promoter (Figure 5.3A).

5.4 PKA Phosphorylates CtBP1 Dehydrogenase Domain Distal from the NADH Binding Site

Having identified CtBP1 as key for basal repression of CYP17 transcription (233), we postulated that activation of the ACTH pathway may play a role in attenuating the repressor function of the CtBP proteins. CtBP1 is phosphorylated by PAK1 at S158,
a modification that may decrease CtBP1 dehydrogenase activity in the homodimer (240). We predicted that post-translational modification by PKA (or PAK) could alter CtBP corepressor function. Using bacterially expressed GST-CtBP1 and 2 nuclear isoforms, we found that PKA phosphorylates both CtBP1 and CtBP2 \textit{in vitro} (Figure 5.4). While PAK1 has been found to phosphorylate CtBP1 (240), we found that PAK6 phosphorylates both CtBP1 and CtBP2 (Figure 5.4). Consistent with the findings of Barnes et al (240), mutation of S158 to alanine substantially reduced the amount of radiolabeled phosphate incorporated into CtBP1 (Figure 5.4), though the same mutation in a parallel assay did not decrease PKA phosphorylation (data not shown). Therefore, we conclude that PKA and PAK6 target unique sites in CtBP1.

T144, a consensus PKA (RxT motif) and possible minor PAK (RxxT motif) phosphorylation site, is conserved in both CtBP1 and 2. This site is on the NADH-dependent CtBP1 homodimerization interface (165), on a surface of the dehydrogenase domain opposite to the NADH binding site and is distal to a hydrophobic cleft on the substrate binding domain that enables binding of some CtBP partner proteins (241).

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**Figure 5.4** PAK6 and PKA phosphorylation of CtBP1 and 2.

An \textit{in vitro} kinase assay was performed with GST-CtBP1 (WT or S158A), GST-CtBP2 (WT) expressed in \textit{E. coli}, bound to glutathione beads, and incubated with active PAK6 or the catalytic subunit of PKA, and \(^{32}\text{P}\)-ATP. Coomassie staining (loading) and autoradiogram (\(^{32}\text{P}\) labeling) of the GST-CtBP1 or 2 are shown as labeled. The same assay was also performed with S100A and T144A CtBP1 mutants bound to nickel-affinity beads.
S100 in CtBP1 is in a PAK motif that is exposed to the NADH binding site on the substrate binding domain and is in direct van der Waals contact with NADH in the crystal structure (165). In vitro kinase assays using a T144A mutant exhibited a decrease in the amount of radiolabeled $^{32}$P incorporated into CtBP1 when compared to WT or S100A when PKA was the kinase. Conversely, PAK6 was still able to phosphorylate the T144A mutant, while exhibiting a decreased phosphorylation of the S100A mutant (Figure 5.4). We conclude that CtBP1 is differentially targeted by PKA and PAK6 and that T144 is a PKA target while S158 and S100 are PAK6 targets.

5.5 The CtBP Helical Bend is a Phosphorylation-Dependent Heterodimerization Motif

T144 in CtBP1 and T150 in CtBP2 are located at the dimerization interface so we further examined dimerization regions of both proteins. Manually docking and energy minimizing a CtBP1 homodimer from the available monomeric structure (165), CtBP2 was modeled using one of the CtBP1 monomers as a threading template, and the regions of interest from contacting monomers were excised as shown in Figure 5.5A. We then mutated select residues in silico to the lowest scoring rotamer using Swiss-PDB viewer (166) and energy minimized resulting structures 30 times (Figure 5.5B). We found that T144D (CtBP1) or T150D (CtBP2), stabilized dimerization at the helical bend via hydrogen bonding with the backbone at N138. We also noted that a N138G mutant or a C134A/N138G double mutant drastically increased the predicted free energy of dimerization, consistent with a loss of dimerization potential. These calculations suggest that the helical bend of CtBP1 and 2 is a heterodimerization motif, and that the specific conformation required for dimerization is favored by phosphorylation of T144, and
Figure 5.5 The dimerization motifs of CtBP1 and 2.

(A) Peptides of CtBP1 and CtBP2 which form the core of the interface between the two proteins oriented in a heterodimer, created as described in methods. (B) Minimized energy of the two peptide system with CtBP1 or CtBP2-specific residues or mutations interfering with packing of the peptides, or improving their interaction (T144D, mimicking phosphorylation). (C) CoIP of WT or C134A/N138G double mutant FLAG-CtBP1 with endogenous CtBP2 from H295R whole cell lysates. Cells treated with Bt2cAMP for 90 minutes show a 28 % decrease in levels of endogenous CtBP2.
disfavored by mutation of the helical bend motif, at C134 and N138. For reference, this motif is conserved as CxxxNxyRRxT, and occurs at a bend in an otherwise continuous helix between the two arginines in dimeric crystal structures of CtBP1 (165) and CtBP2 (Pilka et al., unpublished). To test this model experimentally, we performed coIP of WT FLAG-tagged CtBP1 and C134A/N138G (double mutant) with endogenous CtBP2 and found that the double mutant exhibits decreased binding to CtBP2, both in the presence and absence of Bt2cAMP (Figure 5.5C).

5.6 PKA-Catalyzed Phosphorylation of T144 Regulates CtBP Partnering

We have previously found that the coactivator GCN5 preferentially interacts with the monomeric, NADH-binding deficient CtBP1 G183V mutant (233). This G183V mutant of CtBP1 has also been shown to exhibit increased affinity for the coactivator p300 when compared to WT, supporting a role for NADH-sensitive CtBP1 sequestration of bromodomain containing acetyltransferases (160). We hypothesized that phosphorylation of CtBP1 by PKA at T144 disfavors GCN5 interaction with CtBP1 by inducing dimerization of CtBP1 and CtBP2 (160, 233). The hinge created in the N138G mutant could mimic the helical bend induced by phosphorylation (Figure 5.5B). Additional mutants were also made to test the role of confirmed PAK6 targets S100 and S158. To determine the effect of these CtBP1 mutants on the ability of the corepressor to interact with GCN5, we carried out mammalian two hybrid assays using GAL4:GCN5 and WT or mutant VP16:CtBP1. GAL4:GCN5 interacts strongly with VP16:CtBP1 and, loses a modest albeit significant 8 percent of interaction strength upon stimulation with Bt2cAMP (Figure 5.6A). Mutation of the PKA phosphorylation site increases interaction whereas the N138G mutant significantly disfavored interaction with GCN5. Effects of
mutation of helical bend arginines on the interaction are shown in Figure 5.6B. Alanine substitution of the PAK6-targeted site S100 severely compromises GCN5 interaction, perhaps because a decrease in side chain volume encourages more stable binding of pyridine nucleotide cofactor to CtBP1, which we have established is likely to disfavor interaction with GCN5 (233). The other PAK target, S158, had no significant effect on GCN5 interaction in the S158A mutant.

![Figure 5.6](image_url)

**Figure 5.6** CtBP1 dimerization motif mutations affect GCN5:CtBP interaction.

(A) and (B) Mammalian two hybrid interaction of GAL4:GCN5 and VP16:CtBP1 (WT or mutants) in H295R cells in the presence or absence of Bt_{2}cAMP is shown +/- SEM. pG5 reporter, GAL4:GCN5, and VP16:CtBP1 plasmids were transfected in the ratio 50 ng : 50 ng : 50 ng; n=3; N=2. Symbols indicate significant difference from control (*) or Bt_{2}cAMP stimulated (†) interaction with WT CtBP1.
5.7 SF-1:CtBP1 Interaction and CYP17 Induction Respond to CtBP1 and CtBP2 Dimerization Interface Mutations

Since CtBP1 also interacts with SF-1 (233), we next determined if phosphorylation of CtBP1 at T144 modulates interaction with the receptor. As shown in Figure 5.7A, the S158A mutant strengthened the SF-1:CtBP1 interaction compared to WT. However T144A did not affect interaction, suggesting that the absence of T144 phosphorylation does not affect the interaction with SF-1. The S100A mutation weakened interaction signal to background levels, similar to the effect on GAL4:GCN5. These data suggest that phosphorylation of CtBP1 at distinct sites differentially affects the interaction between SF-1 and CtBP1 versus GCN5 and CtBP1. It is likely that arginines define the PKA phosphorylation site at T144. CtBP1 helical bend arginine charge neutralization had no significant effect on SF-1 interaction, while the R141E charge reversal did significantly increase SF-1:CtBP1 interaction (Figure 5.7B).

We have previously shown that CtBP1 represses CYP17 reporter gene activity in H295R cells (233). To test whether CtBP interaction mutants affect CYP17 transcription, we examined the effect of the mutants on reporter gene activation. N138G and C134A/N138G significantly inhibited Bt2cAMP-mediated activation (Figure 5.7C), consistent with a requirement for CtBP1 dimerization in the loss of CtBP1-mediated repression of CYP17. S158A CtBP1 repressed CYP17 reporter more effectively than WT, while S100A lost the ability to repress the reporter.

CtBP2 partnering with CtBP1 is predicted to be energetically favorable to CtBP1 homodimerization (Figure 5.5B). Therefore, we used the reporter gene assay to determine effects of mutating residues in the dimerization domain of CtBP2 on CYP17.
Figure 5.7 Interaction of SF-1 with CtBP1 helical bend mutants.

(A) and (B) mammalian two hybrid SF-1:CtBP interaction with WT CtBP1 or mutants near the helical bend were measured. Plasmids were transfected as in Figure 5.6, and experiments were performed in triplicate and results are displayed with SEM. *, differs significantly from basal SF-1: WT CtBP1 signal.

(C) and (D) CYP17 -300 reporter (125 ng) activity was measured in the presence of SF-1 (25 ng), and WT or mutant CtBP1 or homologous site mutants of CtBP2 (25 ng). Symbols indicate significant difference from basal (^) or Bt2cAMP-stimulated (‡) SF-1 induced reporter activity in the absence of overexpressed CtBP.
transcription. CtBP2 overexpression significantly represses CYP17. Mutation of CtBP2 T150 (CtBP1 T144) which we predicted facilitates CtBP1:CtBP2 heterodimerization (Figure 5.5B), strongly attenuates CYP17 transcription. Mutation of the CtBP2 residue S164 (CtBP1 S158) reverses repression. Our findings indicate that CtBP1:CtBP2 heterodimerization may be required in efficient CYP17 transcription.

The effects of CtBP1 and 2 arginine mutations of the dimerization interfaces are shown in Figure 5.7D. The R142A mutation, which significantly strengthened GCN5:CtBP1 interaction (Figure 5.6B), also significantly enhances repression of CYP17 during Bt2cAMP stimulation.

5.8 Endogenous Interactions of CtBP1 and 2 with SF-1 and GCN5 are Sensitive to Bt2cAMP and Pyruvate

Our data suggest that ACTH/cAMP-stimulated phosphorylation promotes CtBP1 partner switching and the nuclear export of CtBP1:CtBP2 heterodimers. To further examine this phosphorylation-induced partner shuffling, we carried out coIP assays for endogenous CtBP1, 2, and SF-1 interactions in H295R cells and found that CtBP1, but not CtBP2, strongly interacts with SF-1, while CtBP2 prefers to interact with CtBP1 rather than SF-1 (Figure 5.8A). Bt2cAMP and pyruvate decreased interaction of CtBP1 with SF-1, while only Bt2cAMP decreased the GCN5:CtBP1 interaction (Figure 5.8B). Consistent with previous findings implicating NADH binding in CtBP1 function (160, 233), Bt2cAMP and pyruvate additively impacted the SF-1:CtBP1 interaction.

5.9 Kinetics of Endogenous Nuclear CtBP Heterodimerization in Response to Bt2cAMP
To further explore the roles of ACTH/cAMP-induced pyridine nucleotide accumulation and PKA-catalyzed phosphorylation in CtBP1:CtBP2 heterodimerization, we quantified Bt2cAMP-stimulated interaction kinetics between CtBP proteins in coIP experiments using the nuclear fraction of H295R cells. As shown in Figure 5.8C, CtBP1 and 2 interaction increases in nuclei within 30 minutes, peaks at 90-120 minutes, and proceeds to decay after 120 minutes, concomitant with flux of both proteins into the nucleus by 30 minutes (Figures 5.2B, C). Conditions favoring CtBP heterodimerization remain for up to 120 minutes, though this does not maintain positive flux of CtBP1 or 2 into the nucleus throughout this period (Figures 5.2B, C). We next determined the effect of excess pyruvate on CtBP1:CtBP2 interaction, repeating coIP experiments using lysates isolated from cells treated with 30 mM pyruvate. Pyruvate decreased the CtBP1:CtBP2 interaction by 25 and 19 percent in the absence and presence of Bt2cAMP, respectively (Figure 5.8D).

SF-1 coIP with a tagged CtBP1 T144A mutant also revealed a 77 percent decrease in binding compared to WT, while Bt2cAMP increased interaction only with the phosphodeficient mutant by 3-fold (Figures 5.8E and F). In contrast, while basal CtBP2 interaction was much weaker, T150A mutation increased SF-1 binding 48 percent compared to WT CtBP2, and Bt2cAMP still decreased interaction with this mutant by more than two-thirds.

5.10 A Model of CtBP-Mediated CYP17 Repression and Relief by Kinase Signaling

Bt2cAMP induces CtBP1 and 2 heterodimerization in the nucleus (Figure 5.8C). We asked what the combined effect of CtBP1 T144 and CtBP2 T150 mutation is on their
Figure 5.8 CtBP1 interaction with CtBP2 and SF-1 in H295R extracts.

A, coIP was performed on H295R whole cell lysates. B, coIP of GCN5 or SF-1 with CtBP1 was performed on whole cell lysates following the indicated treatments for 90 min: control, 1 mM Bt2cAMP and/or 5 mM pyruvate. C, excess protein A/G beads and CtBP1 antibody were incubated with 70 µg of H295R nuclear extracts taken following the indicated Bt2cAMP treatments. Western blots of washed beads were

(legend continues next page)
quantified via densitometry for CtBP2 signal and are expressed as coIP output. D, CtBP1 from cells treated as indicated for 4 hours was immunoprecipitated from nuclear extracts and interaction with CtBP2 was quantified by western blot of washed outputs. E, western blots of SF-1 following coIP of VP16-CtBP1 or FLAG-CtBP2 from H295R whole cell lysates. F, quantification of coIP results, including coIP of SF-1 with FLAG-CtBP1 not shown in E. G, western blots of tagged CtBP2 following coIP of VP16-CtBP1 in whole cell lysates of transfected cells.

heterodimerization by performing coIP of tagged WT or T144 CtBP1 and found that the T144A mutation weakened CtBP1:CtBP2 heterodimerization (Figure 5.8G). Further mutation of the corresponding PKA phosphorylation site in CtBP2 (T150) also resulted in decreased interaction, supporting our hypothesis that phosphorylation of CtBP proteins in response to ACTH signaling promotes partner switching and CYP17 induction.

Our results indicate that CtBP switches partners and subcellular localization concomitantly, and that CtBP heterodimers form in response to Bt2cAMP. Therefore, we propose a model of allosteric changes in CtBP1 which control the nuclear concentration of monomer available for regulating exchange of CYP17 coactivators from their active complexes containing SF-1 and GCN5. Nuclear export of CtBP heterodimers prevails in response to ACTH/cAMP and allows induction of CYP17 transcription (Figure 5.9).

In this chapter, we have shown that reduced pyridine nucleotide can accumulate rapidly in the nucleus in response to PKA activation (Figure 5.1), and pyridine nucleotide manipulation causes shuttling of CtBP1 and 2 across the nuclear envelope (Figure 5.2B and C), and dynamic CYP17 promoter binding (Figure 5.3B). Significantly, PKA directly phosphorylates CtBP1 and 2 (Figure 5.4), and phosphorylation of the target site at T144 in CtBP1 has the capacity to improve the stability of the CtBP dimer (Figure 5.5B). GCN5 and SF-1 interactions with CtBP1 are sensitive to T144 mutation, as well as mutation of other CtBP dimerization motif residues. We conclude from these results that PKA mediated phosphorylation of T144, and the increase in nuclear pyridine
nucleotides, probably NADH, stimulates CYP17 transcription promoting dissociation of CtBP from SF-1 coactivator complex members, thereby enabling formation of the early cAMP-induced SF-1/SRC-1/GCN5 complex which is involved in CYP17 transcription cycles in response to cAMP.

**Figure 5.9** Working model of CYP17 induction by PKA. PAK6 and NADH differentially affect the ability of SF-1 and GCN5 to interact with CtBP1.

PKA phosphorylates CtBP1 (and possibly 2), causing oligomerization of CtBP proteins in the nucleus. Reversal of PKA phosphorylation at T144 or T150 and/or oxidative conditions may prevail as cytochrome P450-mediated steroidogenesis proceeds, may cause the system to revert to its initial state.
Chapter 6. Discussion

6.1 Finding Determinants of Induced, Accelerated, Transcription Rate

Transcription of eukaryotic genes proceeds through an ordered process of chromatin modification orchestrated by transcription factors which obligatorily interact with transcriptional coregulators on promoter chromatin (Sections 1.3). Genes that are not constitutively expressed must be induced during and following developmental differentiation. This is of particular interest because there is an implication for a mechanism or mechanisms which monitor conditions (indeed, any inducing signal) and transduce that signal into a transcriptional response. The machinations of this process have ostensibly co-evolved over the course of more than a billion years along with chromatin (242). In the case of the CYP17 gene in tissues capable of acute steroidogenesis (adrenal cortex and gonads) (Sections 1.1), the process of transcriptional induction is concomitant with the cAMP-dependent induction of SF-1 transcription factor function. SF-1 is a member of the nuclear receptor superfamily of transcription factors (Sections 1.2) that are found in sponges and most, if not all, animalia descended from a common ancestor; even yeast has proteins that (via convergent or divergent evolution) structurally and functionally resemble the ligand-binding control apparatus of nuclear receptors (243). Nuclear receptors are subject not only to ligand-mediated regulation (Section 1.2.2), but also respond with unique transcription programs affecting specific sets of target genes when they are post-translationally modified (Section 1.4).

In the studies detailed in chapters three, four, and five, we respectively asked the following questions. 1) What is the order of events which occur on CYP17 promoter chromatin (nucleosome(s)) near the transcription start site, and what are some proteins...
and conditions that cooperate in stimulating these ordered events? 2) What role(s) does post translational modification of the SF-1 LBD have in coordinating these events, and how essential is it? 3) How does an incompletely characterized SF-1 coregulator protein (CtBP) help integrate the monitoring of signals which regulate and enforce ordered events during induced transcription of CYP17?

In chapter three, a fascinating team of cooperative protein players with broad roles in chromatin modification were described and related to one another through their kinetics of interaction with the CYP17 promoter and/or transcription start site. A specific coregulator complex involving SF-1, SRC-1, and GCN5 was identified as a candidate dynamic complex which has an early role in cAMP-induced chromatin modification on the CYP17 promoter. In chapter four, diverse molecular biological methods were used to identify the essential role for phosphorylation and acetylation of the SF-1 LBD in regulating the above kinetics of cooperation. In chapter five, kinase cascades and dehydrogenase metabolism involving pyridine nucleotides were demonstrated to also play a central role in CYP17 transcription. These parameters are monitored by a set of two conditionally monomeric general transcriptional corepressors, CtBP1 and CtBP2, which differentially interact with each other, or a number of essential CYP17 activators, including SF-1 and the acetyltransferase GCN5.

6.2 Key Events during CYP17 Transcription Cycles Are Reflected by Changes in Protein/DNA Interactions

Nucleosome structure, modification, position, and time-dependent coregulator complex assembly are integrated during ACTH/cAMP-induced transcription of the CYP17 gene in the adrenal cortex. Select coactivator complexes assemble or are
stabilized in the nucleus in response to ACTH/cAMP. One such complex which forms and binds CYP17 promoter within thirty minutes of stimulation is the transcription factor/HAT complex comprised of SF-1, SRC-1, and GCN5. Such complexes in turn direct a cascade of chromatin remodeling at the CYP17 promoter (Figure 6.1), including reorganization of histone H2A/H2B by ATP-dependent chromatin remodeling complexes. Recall that histone H2 dimers can interact with histones on neighboring nucleosomes (80), and with the linker histone H1 (244). Evidence for remodeling of H2 dimers on the CYP17 promoter is given by the loss of H2B occupancy of the promoter upon cAMP stimulation, followed by a 600 percent gain at the 150 minute time point (Figure 6.9B). This may reflect not only an ISWI-dependent return of H2 dimer, but also formation of higher-order chromatin structure at the promoter.

p54, PSF, and SF-1 begin to co-locate on the promoter during the second transcription cycle in what has been defined as a hallmark of cAMP-dependent CYP17 transcription (20). Other cAMP-dependent promoters rely on p54 as a bridging factor to RNA polymerase II (245), and in the case of CYP17, kinetics of this factor relative to other coregulators suggest it has a role during the switch from repression to activation (Figure 6.1).

Our findings in chapter three suggest opposing activities of SWI/SNF and ISWI-containing chromatin remodeling complexes (246). Our data (Figure 3.9) is consistent with the former ATPases causing disruption and the latter ATPase, reformation, of H2A/H2B dimers within the nucleosome at the CYP17 transcription start site, respectively. Based on the crystal structure of Luger et al. (79), it would be expected that reorganization of H2A/H2B dimers within the DNA-nucleosome complex could alter
cAMP initiates formation of a SF-1/SRC-1/GCN5 trimer that associates with the CYP17 promoter (-57/-37) followed by acetylation of histone H4 and another component of the complex. CtBP (not shown) mediates complex disruption, thereby facilitating recruitment of transcription activating factors (TAFs) and Pol II (transition 1). Corepressors bind in the absence of SF-1 during cAMP-mediated cycling (transitions 2-4), followed by exchange of the corepressor complex for a complex containing p54nrb and p300 and/or GCN5 (transition 5). This is followed by acetylation and monomethylation of histone H4, which promotes SWI/SNF-dependent remodeling and enables the cooperative binding of SF-1, p160s, and GCN5 (transition 6). Additional remodeling by BRG1 re-enables binding of Pol II and TAFs (transition 7) followed by dismissal of SF-1 and coactivators and the initiation of p54nrb/PSF mediated splicing (transition 8). **, cAMP-dependent interactions confirmed by this study; Ac, acetylation.
DNA contact with remaining histones in the core nucleosome. Since H2 dimers have the ability to contact histones of neighboring nucleosomes (See Table 1.1), it is also likely that their loss or gain reflects the propensity for chromatin reorganization at the level of the transition from euchromatin to heterochromatin.

In concert with the possibility of nucleosome repositioning, histone H2 dimer disruption alters accessibility of *cis* elements, directing the sequential recruitment of other *trans* factors to DNA, culminating in a transcription-competent complex including RNA polymerase II (Pol II). Transcription rate is further tuned by corepressor binding to and post-translational modification of the same region. This includes GCN5 acetyltransferase activity, which aids in disassembly of the SF-1/SRC-1/GCN5 complex (Figure 3.4D).

### 6.2.1 A CYP17 Transcriptional Clock Encoded in *cis* Determines Energetic Transitions in Nucleosome Organization

Post translational modification of *trans* factors, including histones in chromatin, transcription factors, and coregulators is a straightforward means by which to alter the cooperativity of *trans* factors during transcription preinitiation. However, results in chapter three also lend credence to the idea that chromatin organization intrinsic to the proximal promoter sequence is responsible for the timing and sequential nature of coregulator processing of the CYP17 proximal promoter chromatin during transcription cycles. Recently, an exhaustive study of nucleosome positioning presented a statistical model by which a simple 140 bp pattern within any DNA sequence can encode steady state nucleosome position or absence of association with nucleosomes. In this model, a dyad of two juxtaposed A:T pairs enables tight DNA wrapping around a nucleosome if repeated with periodicity in a double stranded DNA sequence (83).
This model (Figure 6.2) was used to make a template and applied to the region of the CYP17 gene used in ChIP experiments, which suggested that nucleosome positioning is strongly favored over the region -140/-90 of the CYP17 gene. However, alignment of the template 3’ of the region suggests alternate modes of chromatin organization around the remainder of the DNA binding tract of this nucleosome (Figure 6.3C, D, E). I propose three modes of nucleosome-DNA interaction, and three transitions between them during a complete transcription cycle. The first is a low energy conformation favored thermodynamically by the promoter sequence (Figure 6.3C). A region of the proximal promoter is excluded from nucleosome wrapping and would be accessible to SF-1 and SF-1/coactivator complexes detected on the CYP17 promoter at the beginning of the transcription cycle. Transition to a higher energy state would be encouraged by histone H2 dimer extrusion from the nucleosome (Figure 6.3D), consistent with 80 percent loss of H2B occupancy early in the transcription cycle (Figure 3.9B). Nucleosome-DNA contacts along the promoter even 3’ to the original loop would be lost, including at the transcription start site. This event would likely be aided by nucleosome disorganization.

![Figure 6.2](image)

**Figure 6.2** A 140 bp nucleosome positioning template.

The template for nucleosome positioning on the CYP17 proximal promoter was derived from statistical data of global nucleosome positioning relative to AA, TT, and TA dyads described in Segal et al (83) and shown in this figure. These dyads, when positioned correctly, kink the DNA helix and stabilize steady state nucleosome wrapping. A:T dyads in the CYP17 sequence were manually aligned to match peaks and avoid strong troughs in the template from -140 to -42 and from +22 to +64. Thermodynamically stable (“tight”) nucleosome wrapping of CYP17 -42 to +23 appears to be disfavored.

*Adapted by permission from Macmillan Publishers Ltd: Nature (Segal et al (83)), copyright 2007.*
Figure 6.3 Possible alternate nucleosome wrapping states of the CYP17 promoter proximal to the transcription start site.

A. Crystal structure 1AOI of a nucleosome wrapped with 146 bp palindromic human DNA. B. Representation of CYP17 proximal promoter with nucleosome wrapping and 5' positioning based on strong adherence to statistical/thermodynamic sequence requirements for optimal nucleosome positioning/organization in whole genome chromatin of chicken (83); ‘0’ denotes the center of this DNA segment, corresponding to position -71 on the CYP17 promoter. Low adherence to favored A:T dyad positioning at the 3' end (Figure 6.1) disfavors this model. C. Stable nucleosome wrapping predicted by the best alignment of the CYP17 proximal promoter and coding start region bend-capable sites to nucleosome positions which best accommodate these bends. D. Reduced nucleosome wrapping (shown without H2 dimers) enables read-through of transcription machinery. E. Tightest winding of CYP17 3' DNA, locking in the second H2 dimer (H2A, magenta; H2B, red) with bend sites (TT) favoring 2bp slack 3' to CYP17 -57 bp. Over time, possibly a consistent, predictable number of minutes, molecular “breathing” of this structure would be expected to favor motions which return the conformation to the one shown in C.
catalyzed by the SWI/SNF remodeling complex which includes BRG1 early in the first transcription cycle (Figure 3.9A). Occupancy of RNA polymerase II would then increase. Later, intrusion of H2 dimers (Figure 3.9B) occurs following corepressor enrichment on the CYP17 promoter at 120 minutes Bt2cAMP treatment. Modification of the histone code further encourages the recruitment of SNF2H ATP-dependent chromatin remodeling activity at the 150 minute time point (Figure 3.9A). This would effectively end the first transcription cycle as a fully wrapped nucleosome/DNA assembly is formed (Figure 6.3E). The third and final transition would occur spontaneously, as kinetic breathing motions of the DNA-nucleosome complex return it to the thermodynamically favored conformation pictured in Figure 6.3A. During this time, p54nrb would be one of the first factors to gain access to its recognized binding element on the promoter DNA, thus starting the second transcription cycle with a unique pattern of trans activator recruitment differing from the first cycle. Temporal ChIP data in chapter three, particularly variation over time of the chromatin occupancy of Pol II and H2B, as well as the procession of p54nrb/PSF binding leading to SF-1 recruitment in cycle II, corresponds very well with this sequence of chromatin structural rearrangements.

6.2.2 The Cooperativity Principle of Transcription and Sequential versus Concurrent Promoter Recruitment of Cooperative Transcription Factors

Extension of temporal ChIP methodology to other factors which have been confirmed to interact with SF-1 (below references) may yet reveal that some of these trans factors interact with the -104/+43 CYP17 gene segment only in cooperation of SF-1. Such factors may include other transcription factors which have been shown to synergize with SF-1, including β-catenin (247), Sp1, NF1, and CCAAT enhancer binding
protein(s) (248, 249). For example, cooperativity between SF-1 and GATA-6 is required for the most efficient transcription of CYP17 (250). Mechanisms for transition of nucleosome/promoter interaction likely underlie sequential recruitment of factors. The above reasoning and evidence for SF-1/GATA synergy pigeonholes an expected time of possible GATA recruitment to the CYP17 promoter closely following that of transcription-permissive remodeling ATPase recruitment, but preceding peak RNA polymerase II recruitment. Other genes originally thought to be strictly under SF-1-mediated control, such as anti-mullerian hormone, also may consistently involve GATA as an equal partner in transcription activation (251, 252).

These studies (251-253) in combination with the data presented in chapter three provide examples that help define a central principle of SF-1 function: cooperativity with coactivators or other transcription factors regulates transcription. Some of this cooperativity is gene-specifically encoded in cis, as is the case for GATA and SF-1 on CYP17. However, simultaneous accessibility of multiple elements is brought about through nucleosome remodeling, while protein-protein interactions among cooperating factors must be also be enabled by ligand-mediated switching of nuclear receptor coregulator binding modes, by bridging factors, and by post-translational modifications (discussed in the following section).

6.3 A Phospho-Acetyl Switch and Ligand Retention / Exchange Mechanism is Controlled by ACTH/cAMP

Based on our data on SF-1 phosphorylation presented in chapter four (Figure 4.4B), we propose that positively charged K253 acts to promote closure of the SF-1 ligand binding pocket in response to phosphorylation of T335 and S342. Further,
reversible acetylation and deacetylation of this residue is key in modulating receptor activation (Figure 4.6D), possibly by controlling ligand binding, ligand exchange, and/or modulating coregulator binding modes.

Consistent with rapid increased colocalization of SF-1 and acetyltransferases, particularly GCN5, in response to stimulation of the ACTH pathway (78, 233), our data suggests that target gene activation by agonist liganded SF-1 is enabled by K253 acetylation, then later in the transcription cycle is reversed by K253 deacetylation. Deacetylation of SF-1 is consistent with the prevalence of deacetylases acting on the CYP17 promoter later in the transcription cycle, when SF-1 cannot maintain stable occupancy upon the promoter (233).

The requirement of histone deacetylase (HDAC) activity in transcription cycles is consistent with the findings of Winnay and Hammer, in which trichostatin A prevented ACTH-stimulated phospho-RNA polymerase II binding to the ACTH receptor gene (223), possibly due to stalling of SF-1 on the promoter. In that study, HDAC-1 and -7 bound the promoter in Y1 mouse adrenocortical cells in response to ACTH, while we found that HDAC-1, -2, and -8 bind to the CYP17 promoter during Bt2cAMP-mediated transcription cycles in H295R cells (217). Despite these species- or gene-specific differences, these data and current findings indicate that while SF-1 K253 is not the sole target of acetyltransferase activity, it is a key acetylation target that modulates the ability of the receptor to induce target gene transcription.

Since we previously found that SF-1 binds to several sphingolipids and phospholipids (74, 77), we envision that the ability of SF-1 to adopt an active conformation upon ACTH/cAMP-stimulated SPH dissociation and PA binding is
controlled by the phosphorylation and acetylation status of the receptor. By opening and closing the ligand binding gate, acetylation and phosphorylation, respectively, should enforce ligand exchange and retention. Such a mechanism is consistent with our findings in chapter four, where transcription cycles mediated by SF-1 are potentiated by dynamic acetylation of the SF-1 ligand binding gate (Figure 4.6). This mechanism is also consistent with the late onset of cAMP-stimulated transcription cycling in the presence of excess sphingosine (Figure 3.11B), because sphingosine discourages acetyltransferase interaction with SF-1 (Figure 3.4B), probably by causing SF-1 to adopt a corepressor, rather than coactivator binding mode (74). Finally, the activity of OA sensitive phosphatases are required for optimal cycling (Figure 4.4C).

Thus, we propose that the changes in the strength of the electrostatic attraction between modified and unmodified K253 and S342 create a “phospho-acetyl switch” (254) that ensures PTM-coordinated monitoring of ligand occupancy by regulating opening and closing of the ligand binding gate (Figure 6.4). The nature of the mechanistic consequences for interplay of this switch operating on the ligand binding gate with agonist/antagonist balance, such as suggested at the beginning of this section, remains an open question.

However, it is clear that this phospho-acetyl switch is key in selecting which coregulator binding mode SF-1 adopts for a given time, and that this switching is mandatory during efficient induction of CYP17 transcription cycles. Specifically, S342 and T335 phosphorylation by GSK3ß and CK2, respectively, are required for cyclic, transient interaction with the CYP17 promoter. Ser/Thr phosphatase inhibition alone is sufficient to disallow cyclic association of SF-1 with the CYP17 promoter (Figure 4.4C.
and (223)), supporting a role for the reciprocal actions of kinase and phosphatase activities in controlling the transactivation potential of the receptor. A SF-1 crystal structure with bound phospholipid is in the coactivator binding mode when the ligand binding gate is open (44). So, it is reasonable to conclude that one or more of the three open gate configurations in our model (Figure 6.4) predominate during the phase of the transcription cycle involved with achieving transcription competence. We conclude that these post-translational modifications underlie SF-1 receptor cycling on the human CYP17 promoter and ensure optimal levels of gene transcription in response to ACTH/cAMP signaling, while also monitoring the presence of acetyl-coA, the mandatory cofactor for acetyltransferases acting on SF-1.

6.3.1 The Complex Pattern of Basal and cAMP-Responsive SF-1 Phosphorylation

Of note, the inability of mutations to completely inhibit phosphorylation of SF-1 in metabolic labeling studies (Figures 4.1E and 4.1G) indicates that the receptor is phosphorylated at multiple sites. The recent findings by Lewis et al., provide compelling evidence for CDK7 in modulating both the transactivation of SF-1 and the ability of the receptor to bind ligand (216). It is possible that CDK7 regulates the phosphorylation of SF-1 at S203 in the absence of ACTH signaling, while GSK3β triggers phosphorylation of additional sites in response to increased intracellular cAMP. Notably, our metabolic labeling studies demonstrate that Bt2cAMP increases phosphorylation of SF-1 (Figure 4.1G), and this correlates with phospho-specific western blotting of SF-1 (Figure 4.1H). These studies are in contrast to our metabolic labeling studies carried out in cells treated with Bt2cAMP for 12 hours (164). The one hour stimulation time used in chapter four is consistent with the rapid nuclear translocation of GSK3β (Figure 4.2A) and the cycling of
Figure 6.4 Model for regulation of SF-1 function by post-translational modifications.

ACTH/cAMP promotes modification of SF-1 by stimulating phosphorylation of the receptor (T335 and S342) and dynamic interactions between charged amino acid residues (K253 and R255) at the entryway to the LB pocket. Recruitment of GCN5 modulates access to the LB pocket by acetylating K253. Dynamic transcriptional cycling is achieved by protein phosphatase (PPase) and deacetylase (DAC) activities.
SF-1 on the CYP17 promoter (Figure 4.4 and (217)). However, it is likely that the receptor undergoes several temporally distinct changes in its phosphorylation status during ACTH signaling and CYP17 transcription.

Mass spectrometric studies aimed at comprehensive identification of phosphorylated residues on SF-1 are ongoing. Interestingly, these mass spectrometric experiments have detected several tyrosine residues that are phosphorylated on the receptor (Dammer and Sewer, unpublished observations), however, further study is required to determine the functional significance of these phosphorylation events and to identify the kinase(s) that targets these residues.

6.4 Global and Gene Specific Chromatin Modification Changes and a Coregulator Cooperativity Switch Are Integrated Outputs of CtBP Involved in Transcription Regulation

CtBP1 and 2 repress transcription of numerous genes via participation in master regulatory complexes of transcription corepressors (194, 255). These corepressors promote chromatin compaction via association with histone deacetylase (256) and methyltransferase enzymes (194). It is equally likely that the CtBPs serve as general repressors of transcription by sequestering a wide array of histone acetyltransferases that are required for promotion of heterochromatin to euchromatin, via a conserved interaction motif in bromodomains (160). By sequestering acetyl-lysine binding proteins, CtBP can regulate and inhibit a likely mechanism for histone acetyltransferase feed forward that would otherwise enable histone hyperacetylation following a single acetylation event.

The gene specificity of CtBP repression, however, is conferred by specific protein-protein interactions with trans factors and depends on the trophic status of a cell.
and NAD⁺/NADH ratio. CtBP1 interacts with a growing list of gene-specific DNA binding factors (193, 257-259), to which we add the nuclear receptor SF-1. The divergent effects of mutagenesis of the CtBP dimerization motif on the interaction with SF-1 (Figures 5.6A, 5.7E) versus the interaction with GCN5 (Figure 5.5), as well as of mutating the Rossman fold (233) suggest that conditions favoring SF-1 interaction are unique from the ones favoring GCN5 (Figure 5.9). The exception, however, is that both this SF-1 interaction and the GCN5 interaction with CtBP proteins are reduced by Bt2cAMP treatment (Figure 5.8B), in a mechanism which requires T144 (Figures 5.6, 5.8E, F). Bt2cAMP treatment also effectively, though temporarily, increases CtBP heterodimerization (Figure 5.8C), while allowing stronger GCN5/SRC-1/SF-1 interaction (Figure 3.4). Therefore, in addition to cAMP-dependent switching of SF-1 coregulator binding mode, the cAMP-dependent switch in CtBP-mediated interactions which occurs with the switch from monomeric to dimeric CtBP, particularly heterodimerization, provides for an expanded nuclear population of SF-1 and free coactivators available for participation in the coactivator binding mode.

In other words, PKA-mediated phosphorylation (Figure 5.4) and NAD(P)H modulation (Figure 5.1) are responsible for enforcing alternate protein-protein interaction networks involving CtBP, which depend on the homo- and hetero-dimerization of CtBP proteins. We term this the “CtBP coregulator cooperativity switch.” The complete list of differential bridging functions of which CtBP1 and 2 are capable in monomeric, homodimeric and heterodimeric forms will greatly aid in the prediction of how CtBP proteins affect both global and gene specific transcription programs.
6.4.1 Kinase Cascades, Overall NAD(P)H Reductive Capacity of a Cell, and Pyridine Nucleotide Metabolism Are Inputs Determining CtBP Transcription Regulation

CtBP proteins are unique not only in their switchable bridging function, but also in the large number of factors, or inputs, that are integrated into the physically determined decision of whether to dimerize. PAK phosphorylation site residues (CtBP1 S158 or CtBP2 S164), are at extreme ends of the CtBP heterodimer interaction interface, and affect the ability of Bt$_2$cAMP to induce CYP17 transcription (Figure 5.7C). The location of both S100, juxtaposed to NADH in the catalytic domains of the homodimeric crystal structure (165), and S158, at the ends of the dimerization interface closest to the NADH binding sites (240) support a role for PAK-mediated exclusion of NADH from monomers assembling into oligomeric CtBP during the shift to activation of CYP17 transcription. It is not clear if PAK affects only NADH binding to CtBP or also affects dimer stability, and hence, dimerization propensity.

CtBP proteins are 2-hydroxy acid dehydrogenases, and as such, their oligomeric state is linked to dehydrogenase activity, which is therefore also considered an input linked to CtBP ability to repress transcription. S158 phosphorylation of CtBP1 has been shown to inactivate dehydrogenase activity (240) consistent with a direct role of CtBP dehydrogenase activity in modulating target gene repression or activation. Thus, dehydrogenase activity of CtBP1 likely affects tighter binding to SF-1 in the absence of PAK signaling, such that the CtBP1 S158A mutation prevents Bt$_2$cAMP derepression of CYP17, while, in contrast, mutation of the cognate residue in CtBP2, S164A, causes CtBP2 to lose basal repressive capacity (Figure 5.7C). Therefore, the two homologs of
CtBP in the nucleus of H295R cells are likely not equivalent in how their dehydrogenase activity affects transcription of specific genes.

Consistent with the role of PAKs in modulating CtBP dehydrogenase activity, CtBP1 S100 is a novel PAK6 target (Figure 5.4), and its phosphorylation is predicted to sterically interfere with NADH binding to CtBP1. It is particularly interesting that mutation of this site decreases two hybrid interactions with both GCN5 and SF-1 (Figures 5.6, 5.7A). Our data showing PAK site effects on Bt2cAMP induction of CYP17 suggest that activation of PAK isoform(s) is linked to PKA in the adrenal cortex. PAK signaling, classically downstream of serum growth factors, and upstream of ERK signaling, could thus link the activation of ACTH/PKA signaling and ERK pathways during steroidogenic gene derepression. Progressive phosphorylation or dephosphorylation of PAK sites may induce multiple modes of preferential CtBP partner binding (Figure 5.9). Interestingly, we have found that PAK6 is acutely phosphorylated and active in response to ACTH/cAMP signaling (S. Jagarlapudi, unpublished observations).

Extrinsic dehydrogenase activity, i.e. of NAD(H)-dependent dehydrogenases other than CtBP, also affects CtBP activity by altering NAD+/NADH ratio, as would pyridine nucleotide metabolism. For these reasons, we have become interested in pathways which affect the overall reductive capacity of NAD(P)⁺/NAD(P)H pools in a cell, including transhydrogenase, the pentose phosphate pathway, NAD⁺ kinase, NAD⁺ synthesis and salvage, metabolism of glutamine, and malic enzyme. The first two in this list in particular appear to be important in NAD(P)H sensitive transcription of H295R adrenal cortex cells, because inhibition of the pentose phosphate pathway by 5 µM 6-aminonicotinamide reduces CYP17 transcription by more than forty percent in both the
presence and absence of Bt2cAMP (E. Dammer, unpublished observations). On the other hand, acute cortisol output in response to 1 mM Bt2cAMP measured by EIA is inhibited only 33 percent by concentrations of this inhibitor which are five times higher (E. Dammer, unpublished observations). Therefore, the pentose phosphate pathway appears to have an independent (if not a more central) role in providing reducing equivalents to pyridine nucleotides for potentiating chronic steroidogenesis, more so than it does in providing NAD(P)H for acute steroidogenesis by cytochromes P450. It is an open question as to whether steroidogenic P450 utilization of NAD(P)H pools imparts a rhythm to pyridine nucleotide reduction, but this is another potential factor affecting CtBP dehydrogenase activities and their ability to repress specific genes.

The net level of dehydrogenase in the nucleus is expected to affect nuclear NAD(P)+/NAD(P)H, and thus the dehydrogenase activity of CtBP1 and 2 in the nucleus. Therefore, it is extremely interesting that spikes in NADH oxidation (e.g., via acute pyruvate treatment; Figure 5.2) or NAD+ reduction (e.g. via PKA activation in H295R cells; Figure 5.1) cause rhythmic loss of CtBP1 and 2 from the nucleus and an apparent transient gain in the cytoplasm (Figure 5.2B, C). Only Bt2cAMP stimulation promotes the apparent rhythmic co-transport of both CtBP1 and 2 (cf. Figure 5.2B, C), consistent with its promotion of heterodimerization.

It is therefore of interest what the relative levels of CtBP1 and 2 are in the nuclei of cells of a given tissue, because the coregulator cooperativity switch may not function if their levels are not well matched. In fact, in H295R cells, if CtBP1 level is adjusted independent of CtBP2 by transient overexpression, then the ACTH-dependent acute rise in nuclear NAD(P)H as measured by autofluorescence takes fifty minutes with CtBP1
overexpression versus less than ten minutes without (Figure 5.1B and E. Dammer, unpublished observations). In the case of CtBP2 overexpression, the rise is not delayed, but its amplitude is blunted by about 18 percent (E. Dammer, unpublished observations).

The above evidence serves to illustrate the complexity of factors which CtBP dehydrogenase activity, and trans repression function, integrates. We have shown that CtBP is a PKA target at a unique motif which is important for CtBP dimerization. Mutation of the dimerization motif as well as PAK6 and PKA phosphorylation targets, as well as modulation of the availability of reduced pyridine nucleotide, each affect the ability of CtBP proteins to repress CYP17 in human adrenal cortex cells. These conditions affect a switch in binding partners for CtBP1 and 2, in particular facilitating assembly of CtBP1:CtBP2 heteromeric complexes, which appear to be required for efficient CYP17 transcription.

6.5 Concluding Remarks and Some Implications for These Findings

Nuclear receptors are well-studied representatives of the wider class of proteins that function as positive and negative transcription regulators. In this work, we have specifically shown that even ligand-regulated transcription factor function of the SF-1 nuclear receptor [which coordinates sequential chromatin modifications; (Chapter 3)] further requires dynamic protein complex formation involving interactions with ternary or higher-order complex members (Chapter 3), loss of interactions among CtBP corepressor and (co)activators (Chapter 5), and post-translational modification of SF-1 (Chapter 4) and of the CtBP corepressors downstream of the ACTH/cAMP signal cascade (Chapter 5). Metabolism and metabolites also play a gatekeeper role in determining the confluence of the above events (Chapter 5).
An application for the above findings may be found in the treatment of cancer in general, where transcriptional networks suffer from rewiring of metabolic (260) and signaling networks, and also loss of chromatin remodeling capacity of ATPases (261) and dysregulation of histone post-translational modification machinery (262, 263). For example, treatments which temporarily restore one or more of these networks in the correct order could enable transcription of intact, but epigenetically silenced, genes with essential roles in apoptosis. Some specific cancers are propagated through dysregulation of nuclear receptors including SF-1 and a close relative, liver receptor homolog-1 (75, 264-267). Treatments for these cases may be designed to target the kinases and acetyltransferases that may be at work in a temporally coordinated fashion underlying the phospho-acetyl switch on the LBD of these nuclear receptors, as suggested by data in chapter four. The same cases may also be responsive to metabolic manipulation of the functional interaction of NAD(P)H, SF-1, and CtBP, as suggested by the data in chapter five. A final note to be kept in mind is that since the above work was performed in a human adrenal cortex cell line, it likely provides insight into the etiology of certain previously unexplained cases of adrenal insufficiency.
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