STRUCTURE FUNCTION STUDIES OF AKR, A HOMEODOMAIN REPRESSOR OF APOVLDLII GENE EXPRESSION

by

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ABSTRACT

The apoVLDLII gene encodes a small phospholipid binding protein that comprises part of the low-density fraction of the egg-yolk. Expression of this gene is completely estrogen dependent and exclusive to the livers of the laying hens. In addition, the competence to express the apoVLDLII gene in response to estrogen is also developmentally regulated. Such tight regulation of expression is vital as misexpression of the gene can result in hyperlipidemia and hyperlipoproteinemia and lead to the development of atherosclerosis. Essentially all of the elements required for the tissue-specific and estrogen-inducible expression of the apoVLDLII gene are clustered within a region of 300 nucleotides of the proximal promoter. Within this region, an element designated F' plays a crucial role in mediating both the positive effects of estrogen and the negative effects of the Avian Knotted-Related (AKR) homeodomain protein on apoVLDLII gene expression. *In vitro,* AKR and ER individually form complexes with F' and the canonical and imperfect estrogen response elements E1 and E2 respectively, of the apoVLDLII promoter. Additionally, AKR binds to a region of the promoter designated G that is flanked by the E1 and E2 elements. While it appears that site G is not important in the AKR-mediated downregulation of apoVLDLII gene expression, mutation of the F' site negatively affects the ability of AKR to bind to both F' and E2. Similarly, mutation of F' disrupts ER contacts to the E2 element. In addition, a mutation that alters the spacing between that F' and E2 elements affects the ability of the promoter to respond to estrogen suggesting that these two elements mediate cooperative interactions by ER.

AKR is distinguished by the presence of a three amino acid insertion between helix 1 and 2 as well as the highly unusual combination of amino acids in helix 3. In addition, the hexanucleotide core sequence 5'-TGACAT-3' of F' represents an unusual homeodomain recognition element. AKR is grouped together with the Meis1 and Prep1 homeodomain proteins on the basis of their structural similarities and the sequences of their respective binding elements. However, AKR differs in the manner with which it binds to its recognition element. A combination of molecular modelling and

mutational analysis performed on the AKR homeodomain indicates that within helix 3 Asn47, Ile50 and Arg54 specify the identities of the nucleotides at positions 1 to 5 of the hexanucleotide core while and Arg3 to 5 in the NH_2-terminal arm of AKR reinforce these contacts and help determine the identity of the nucleotide at the 6^{th} position. AKR makes very discriminate contacts to a region of DNA of approximately 13 nucleotides that is much larger than the more common 6 base pair homeodomain elements.

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In memory of my beloved mother Emiliana Tejada. For all of the ways in which she enriched my life, for the love that she shared with her family and the world and for the sense of purpose that she instilled in all of her children. *I love you mami. Te estraño mucho*.

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PROLOGUE

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Chapter 1

GENERAL INTRODUCTION

Regulating gene expression is essential to all organisms. They must control the expression of thousands of genes in both a temporally and spatially regulated manner in response to intra- and extracellular stimuli. It is generally accepted that transcription is the principal stage at which gene expression is controlled and that the packaging of genomic material into chromatin is the focal point through the interplay among chromatin modifying and remodelling complexes, the DNA-binding transcriptional activators or repressors, and the components of the basal transcription machinery that recruit these complexes to their target genes.

The expression of eukaryotic genes transcribed by RNA Polymerase II (RNA Pol II) is controlled by the general transcription factors (GTFs), DNA binding activators or repressors, and non-DNA binding transcriptional coactivators and corepressors. GTFs and RNA Pol II bind to promoter DNA and, together, specify the transcriptional initiation site. The choice of initiation site determines the frequency of initiation that is described as promoter strength. The same GTFs function at most promoters. Activator and repressor proteins contribute significantly to promoter strength. These factors bind to promoter proximal and distal elements in subsets of genes, thus regulating the developmental program of gene expression and the cellular response to extracellular signals. Coactivators are proteins that bridge the interactions between the gene-specific activator proteins and the GTFs [1; 2]. Coactivators can also be associated with multiprotein complexes that acetylate lysine residues in the NH_2-termini of histones, thus increasing the accessibility of the promoter regulatory elements to GTFs [3]. In contrast, corepressors are associated with histone deacetylase (HDAC) activities [4; 5]. For the sake of clarity, this review will focus initially on basal

transcription. Subsequent sections will discuss activated transcription, the role of chromatin in the regulation of transcription and the functions of coactivator and corepressor complexes in mediating gene expression. As an example, activation or repression mediated by nuclear hormone receptors through their effects on chromatin is also discussed with special emphasis on the activation potential of the ligand-bound and unliganded estrogen receptor.

1.0 THE CORE PROMOTER AND BASAL TRANSCRIPTION

The core promoter elements are defined as the minimal DNA elements necessary for the accurate transcriptional initiation by RNA pol II *in vitro.* Several types of core promoters exist and they are classified according to their sequence motifs. The TATA-box, TATA(T/A)A(T/A), resides 20 to 30 bases upstream of the transcriptional start site of many genes and can direct the accurate initiation of transcription [6]. A G-rich element adjacent to the TATA-box that binds the general transcription factor IIB (TFIIB) has also been identified [7; 8]. In the absence of the TATA box, an initiator element (Inr), a pyrimidine-rich sequence, is used as the start site of transcription and is often found upstream of housekeeping genes. Functional studies have suggested that PyPyAN(T/A)PyPy is the optimal initiator sequence, whereas sequence comparisons of *Drosophila* genes have indicated a TCA(A/G)TPyPy consensus [9; 10]. A third motif, the downstream promoter element (DPE) with a consensus sequence of PuG(A/T)CGTG, was identified in *Drosophila* TATA-less promoters and is located approximately 30 nucleotides downstream of the transcriptional start site [11].

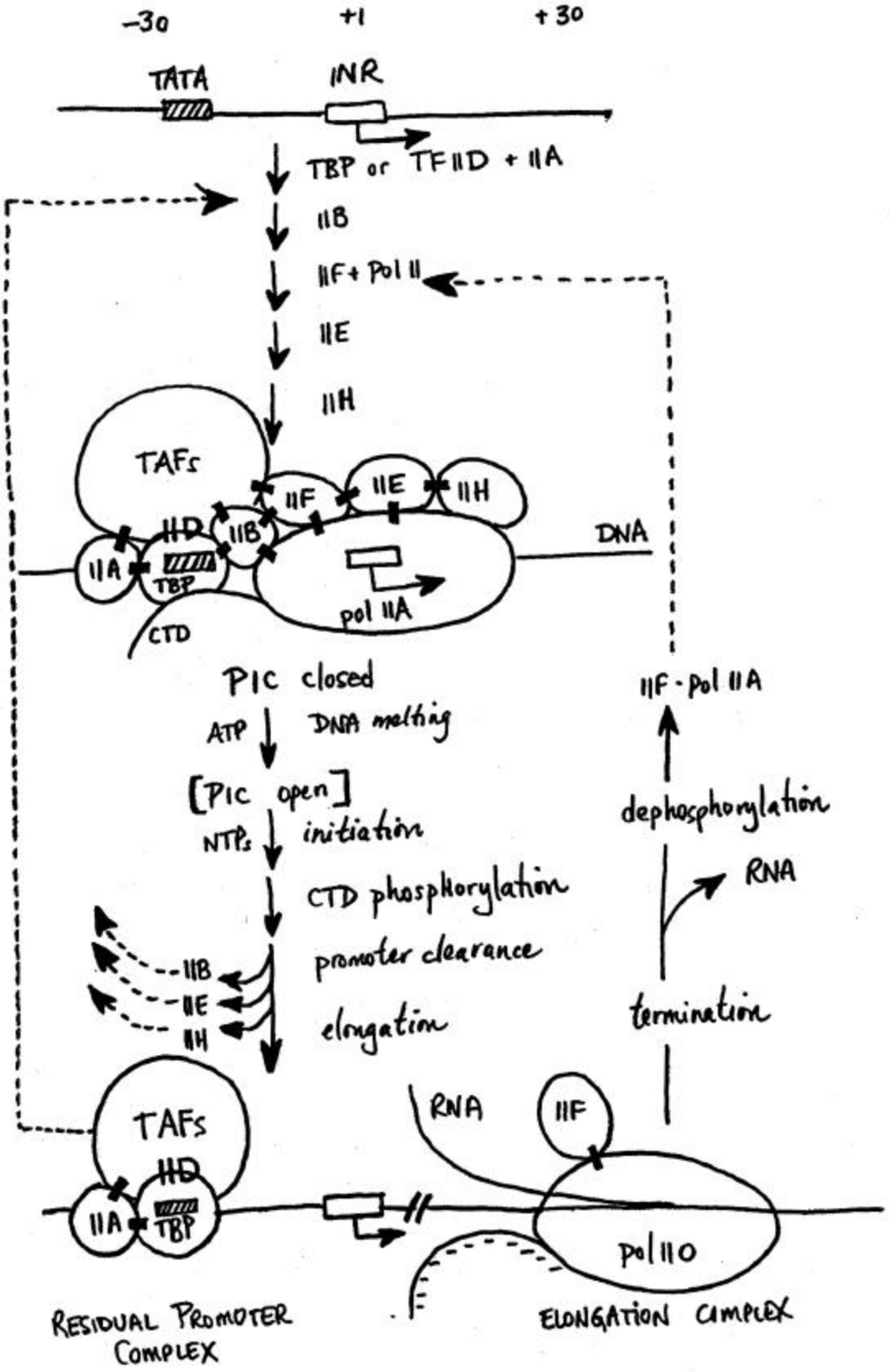
Typically, the GTFs, TFIIA, -B, -D, -E, -F and –H, comprise the minimal complement of proteins that interact with RNA pol II to reconstitute accurate basal-level transcription from a minimal promoter such as a TATA element or Inr sequence. The core promoter element nucleates the assembly of a pre-initiation complex (RNA pol II and the GTFs). *In vitro,* these factors assemble in a stepwise ordered fashion through an extensive series of protein-protein interactions [12; 13]. The conventional model of transcription initiation by RNA pol II can be described in terms of pre-

initiation complex assembly and activation, initiation of transcription, promoter clearance, and transcriptional elongation and termination (Figure 1.1). The first step in transcription initiation is TFIID binding. TFIID is a multi-component (~10 subunits) transcription factor that recognizes and binds to the promoter DNA [14]. The DNA binding subunit of TFIID recognizes the TATA element and is therefore designated the TATA-binding protein or TBP. The remaining subunits are designated either as the TBP-associated factors (TAFs) or non-TAF coactivators [1]. The association of TFIID and promoter DNA is the only step in GTF assembly that is driven entirely by protein-DNA interactions. TFIID acts to nucleate the transcription complex, recruiting the rest of the GTFs through direct interactions with TFIIB.

TFIIA can join the complex at any stage after TFIID binding and stabilizes the initiation complex [14]. TFIIA consists of two subunits in yeast and three in humans and *Drosophila,* all of which contain high concentrations of acidic amino acids. TFIIA binds directly to the TBP subunit of TFIID and stabilizes the binding to DNA through direct contacts of its own with DNA. TFIIA enhances the ability of TBP to compete with non-specific DNA-binding proteins by increasing the affinity of TBP for a promoter. In addition, TFIIA binding to TBP is mutually exclusive with the binding of some classes of negative regulatory proteins. Thus, TFIIA stabilizes TFIID binding by blocking transcriptional repressors and inhibits the binding of factors that compete with TBP for DNA.

The next factor to be recruited to the pre-initiation complex is TFIIB. It exists as a single polypeptide of 35 to 40 kDa that contains a zinc-finger domain at the NH_2-terminus and two imperfect direct repeats in the COOH-terminal domain. The primary role of TFIIB is to physically link TFIID at the promoter with the RNA pol II/TFIIF complex. Consistent with this function, TFIIB contains binding sites for the TFIID/DNA complex, TFIIF and for RNA pol II [15-18].

Figure 1.1 **Stepwise assembly of the pre-initiation complex.** TFIID recognition of the TATA element is the initial step in pre-initiation complex assembly. This is followed by the coordinated recruitment to the complex of TFIIB, the complex comprised of TFIIF and the non-phosphorylated form of the RNA pol II COOH-terminal domain, TFIIE and TFIIH. In the presence of ATP and NTPs promoter melting occurs followed by initiation of transcription. Promoter clearance follows phosphorylation of the COOH-terminal domain of RNA pol II by TFIIK. TFIIB, -E and -H disengage from RNA pol II during promoter clearance, creating an elongation complex comprised of the phosphorylated form of RNA pol II and TFIIF. Following termination, a phosphatase dephosphorylates the COOH-terminal domain allowing RNA pol II to rejoin the activated pre-initiation complex and reinitiate transcription. Adapted from reference [19].


-30
+1
+30
TATA
INR
TBP or TFIID + IIA
IIB
IIF + Pol II
IIE
IIH
TAFs
IIF
IIE
IIH
IID
IIB
IIA
TBP
DNA
pol IIA
CTD
PIC closed
ATP
DNA melting
[PIC open]
NTPs
initiation
CTD phosphorylation
promoter clearance
IIB
IIE
IIH
elongation
TAFs
IID
IIA
TBP
RESIDUAL PROMOTER COMPLEX
RNA
IIF
pol IIO
ELONGATION COMPLEX
termination
dephosphorylation
RNA
IIF - pol IIA

Once TFIID and TFIIB have assembled at the promoter, RNA pol II can enter the pre-initiation complex. However, this association is unstable and requires recruitment of RNA Pol II by TFIIF and TFIIB. TFIIF binds directly to RNA pol II and was originally isolated as an RNA Pol II-associated protein (RAP). Additionally, binding of RNA pol II by TFIIF indirectly inhibits RNA pol II binding to non-specific sites on DNA, reducing the spurious initiation of transcription. TFIIF consists of two subunits, RAP30 and RAP74 [20; 21]. The smallest subunit, RAP30, appears to be sufficient for interactions with TFIIB and recruitment of RNA Pol II. However, studies suggest that both subunits are involved in the stabilization of pre-initiation complex interactions through TFIIB and in transcriptional initiation. TFIIF also stimulates the phosphatase activity that removes the phosphate moieties from the COOH-terminal domain of the largest subunit of RNA Pol II [20; 21]. The dephosphorylated form of RNA pol II is the form of the protein that is recruited to the pre-initiation complex.

Even though RNA Pol II is stably incorporated into the pre-initiation complex, it is unable to initiate RNA synthesis and requires the presence of two additional GTFs, TFIIE and TFIIH [22]. TFIIE is a tetramer that consists of dimers of 35 or 56 kDa subunits. TFIIE binds RNA pol II, recruits TFIIH to the pre-initiation complex and modulates TFIIH kinase, helicase and ATPase activities. TFIIE appears to be required for promoter clearance of RNA polymerase II and its transition into elongation mode [23]. The addition of TFIIE and TFIIH to the pre-initiation complex completes the assembly process rendering the polymerase competent to initiate transcription.

Mammalian and yeast TFIIHs are composed of at least nine subunits [22]. The two largest subunits are ATP-dependent helicases of opposite polarity that are essential for promoter melting. This involves the separation of the two DNA strands that is required to allow the RNA pol II complex access to the template strand and the formation of the first phosphodiester bond of the RNA transcript [24]. Extension of the RNA transcript results in disruption of the RNA pol II contacts with the initiation complex, promoter clearance, and entry into the elongation phase of transcription

in which the nascent RNA strand is extended as the polymerase moves along the DNA template [25].

TFIIH is also associated with a complex, TFIIK, which consists of a CDC-like kinase and cyclin-like subunit. The COOH-terminal domain of RNA pol II consists of a heptapeptide with a consensus sequence, YSPTSPS that is identically conserved between yeast and human. This heptapeptide is repeated 26 times in the yeast enzyme and 52 times in human [26; 27]. During transcription initiation, the COOH-terminal domain becomes extensively phosphorylated on serine and threonine residues by the kinase activity associated with TFIIH, designated TFIIK [28]. This modification of RNA pol II is essential for its dissociation from the pre-initiation complex and marks the transition into the elongation phase of transcription. TFIIH is also essential for nucleotide excision repair of damaged DNA. Mutations that affect TFIIH function result in *Xeroderma pigmentosum*, a rare human hereditary syndrome characterized by hyperpigmentation of the skin under sun exposure, cutaneous abnormalities and predisposition to skin cancer, which are the result of nucleotide excision repair dysfunction [29].

The above assembly of RNA Pol II and the GTFs on the promoter DNA *in vitro* suggests a stepwise assembly of the pre-initiation complex. There is also evidence for the existence of a holoenzyme complex wherein some GTFs are observed to associate with RNA Pol II in the absence of DNA. Evidence for the existence of such a complex first arose from the characterization of the SRB (supressors of RNA pol B) group of proteins, products of genes that were identified in yeast during a genetic screen for suppressors of growth defects associated with truncations in the COOH-terminal domain of RNA Pol II [26; 30]. All nine of the genetically identified SRB proteins exist in a complex with RNA Pol II, yet this complex lacks a subset of the GTFs. However, when supplemented with TBP and TFIIE, the complex was capable of both accurate initiation and transcription in response to a transcriptional activator [31-33]. Another yeast RNA Pol II complex, termed the mediator, was identified in the laboratories of Kornberg and Young [2; 34]. This

complex included a subset of the SRB proteins, a novel set of proteins designated MEDs and an additional subcomplex composed of 4 proteins [34; 35]. Thus, the SRB and mediator complexes are structurally similar but not identical. The differences may be accounted for simply on the basis of the differences in the purification strategies used to isolate them. Similarly, this could also account for the presence of the SWI/SNF chromatin-remodelling complex in the SRB complex but not in the mediator [34; 36]. Another yeast holoenzyme complex has been isolated which is devoid of SRB and other mediator subunits, but contains a subset of the GTFs including TFIIB and TFIIF [37]. This complex was shown to be involved in regulating the expression of a unique set of genes and is therefore functionally distinct from the SRB- or mediator-containing holoenzymes. All complexes isolated to date contain common elements that are essential for transcription. For example, recent studies carried out using a temperature sensitive SRB protein, Srb4, and analysis by DNA microarray technology indicated that virtually all yeast genes require Srb4 for their transcription. The fact that all of the Srb4 in yeast extracts is associated with an RNA pol II holoenzyme complex strongly suggests that a holoenzyme is required for transcription [38; 39].

Several different mammalian RNA Pol II complexes have been isolated [26; 40]. One complex contained SWI/SNF and the histone acetyltransferases CBP and PCAF, but was devoid of GTFs. Another complex that mediated transcriptional activation did not include chromatin-modifying factors but did contain a subset of the SRB/mediator subunits and GTFs [41]. Still other RNA Pol II complexes exist that contain a variety of subunits, including the SMCC, TRAP and DRIP complexes [42; 43]. As in the case of the various yeast complexes an emerging theme in the mammalian complexes is the presence of common or shared subunits in the distinct complexes that have been characterized. This suggests that the specificity of these holoenzyme complexes is mediated by unique subunits that interact with distinct activators.

2.0 ACTIVATED TRANSCRIPTION

Basal transcription *in vitro* is not dependent upon the presence of activating factors. The minimal protein apparatus required for the accurate initiation of transcription consists of the GTFs and the subunits of RNA pol II. However, *in vivo*, all eukaryotic chromosomal DNA is coiled around an octameric core of histone, the nucleosome. This DNA packaging forms a physical barrier preventing the general transcription factors and RNA pol II from accessing the promoter. Under these circumstances, transcription must be triggered by activator proteins through their interactions with specific regulatory elements in the promoters of their target genes. Transcriptional activators function to target nucleosome-remodelling factors (*i.e.* histone acetyltransferases) and ATP-dependent chromatin remodelling complexes (*i.e.* SWI/SNF) to the core promoter.

Regulatory elements for RNA pol II transcription are located both upstream and downstream of the RNA transcription start site [27]. The regulatory elements include both the proximal promoter elements that are traditionally thought to be located from 50 to 500 base pairs upstream of the transcriptional start site, and the distal enhancer elements which may be located up to tens of thousands of base pairs from the transcriptional start site and are able to exert their effects when positioned in either direction or orientation. The regulatory regions of genes transcribed by RNA Pol II contain various combinations of these different types of promoter elements, which function independently or synergistically to modulate transcription by recruiting different combinations of DNA-binding transcriptional activators. In several cases, the binding of multiple transcription factors to a specific promoter/enhancer region is cooperative and requires a unique composition and spatial arrangement of transcription factor binding sites [44]. The assembly of these enhancer complexes, termed enhanceosomes, is facilitated by protein/protein interactions between DNA-bound factors and protein-induced DNA bending.

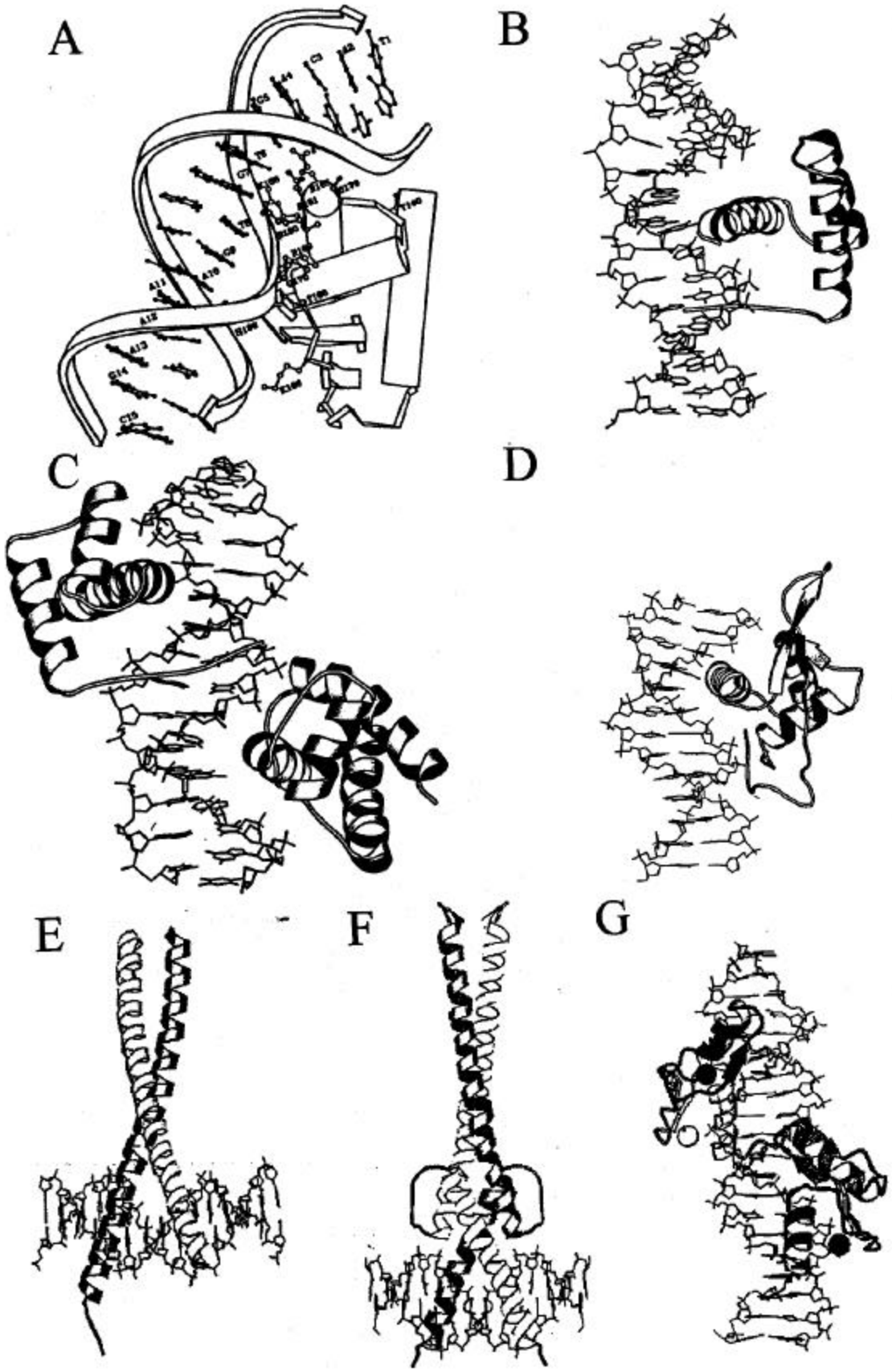
2.1 DNA binding motifs

As stated previously, the upregulation of transcription is contingent upon the recruitment of transcription activating factors to regulatory elements in the target genes. To this end, transcription activating factors are modular in nature, comprising distinct DNA-binding domains that are characteristic of specific transcription factor families and one or more transcription-activating domains [45]. These come in several varieties (*i.e.* helix-turn-helix, homeodomain, zinc-finger and leucine zipper motifs) that are a characteristic of specific classes of transcription factors (Figure 1.2). A recurring theme displayed by the structures of these DNA-binding domains is that the specificity of the interactions observed between protein and DNA is determined in most, but not all cases, by specific residues on the surface of the protein's á-helix and the major groove of DNA [46]. Sections 4 and 5 focus on two examples of well-characterized and distinct DNA-binding domains, the homeodomain and the zinc-finger domain found in nuclear hormone receptors.

2.2 Transactivation domains

Activation domains are not generally as structurally well defined as the DNA-binding domains and do not correlate with the particular type of DNA-binding domain present. Typically, the activation domains have been classified on the basis of their amino acid composition, with different groups comprised of activators rich in acidic amino acids, glutamine or proline [47-49]. Some activation domains have also been described that are rich in isoleucine, or basic amino acids [50; 51]. The first activation domains identified were those of the yeast acidic activators GAL4 and GCN4 [52; 53]. Other activation domains were subsequently identified including those of RelA [54], several steroid hormone receptors [55], c-Fos and c-Jun [56] and the herpesvirus activator VP16 [54-58]. Glutamine-rich or proline-rich activation domains were initially identified in the transcription factors Sp1 and CTF/NF1, respectively [59; 60].

Figure 1.2 **DNA-binding structural motifs complexed with DNA.** These include: A, the helix-turn-helix domain of the *Escherichia coli* catabolite activator protein; B, the *Drosophila* engrailed homeodomain; C, the Oct1 POU domain protein motif; D, the winged helix motif of HNF3 ã; E, the basic region leucine zipper structure of GCN4; F, the basic region helix-loop-helix/leucine zipper motif of the transcription factor Max; G, the zinc-finger motif of YY1 (zinc ions are depicted as spheres).


A
B
C
D
E
F
G

An interesting factor, the B-cell transcription factor Oct-2 contains both a glutamine-rich and a proline-rich activation domain that are positioned on either side of the DNA-binding POU domain and have been shown to act synergistically [61].

The TBP-associated factors, TAFs, can also be involved in activated transcription and promoter selectivity. As stated previously, the general transcription factor TFIID is a complex consisting of the TATA-binding TBP and approximately 10 TAFs [62]. Early studies suggested both that TBP alone was sufficient for TATA element recognition and basal transcription and that the TAFs were essential for transcriptional activation [1; 63]. However, there is increasing evidence suggesting that although TAFs are required for the transcriptional activation of some genes, they are not required for activation of others. For example, depletion or inactivation of seven different TAFs did not significantly affect activation by four acidic activators GCN4, ACE1, GAL4 and HSF1 [64-66]. This is in contrast to numerous results that indicate that TAFs are crucial for transcriptional activation *in vitro* [63; 67; 68]. In some situations, TAFs are required for activator-dependent recruitment of TFIIA and TFIID or of RNA pol II holoenzyme components [1]. Similarly, reconstitution studies with recombinant proteins indicate that stimulation of transcription by Sp1 or NTF-1 is dependent upon TAFs that recognize and recruit these to the TFIID complex [69]. The requirement for specific TAFs has also been observed for activators such as Bicoid, p53, Hunchback and ER [68; 70-73]. These results suggest that some activators act by contacting different TAFs, thus allowing TFIID to integrate multiple signals for different enhancer-bound regulators.

The current view is that, while TAFs may not be generally required for transcriptional activation, they selectively affect the transcription of specific genes *in vivo* [64; 74]. Curiously, the genes affected contain suboptimal, non-consensus TATA elements suggesting that TAFs may play important roles at such promoters by interacting either with components of the basic transcriptional machinery or with promoter DNA. Thus, the TAF functions emerging from these studies can be

summarized as follows: First, certain TAFs may be essential for transcription from a subclass of promoters sharing a common feature such as weak or non-consensus TATA elements. Secondly, although TAFs are not generally required for activation, an individual TAF may be required to interact with a subset of activators that affect one or more essential genes; for example, an activator involved in cell cycle progression. In this regard, the failure of glutamine-rich activation domains to stimulate transcription in yeast may reflect the absence of a yeast homologue for *Drosophila* TAF110, a target for glutamine-rich activation domains *in vivo* [74-76]. Thirdly, absence of a TAF could subtly affect transcription of many genes such that the cumulative effects lead to cell inviability.

Transcription activation also requires accessory factors, termed coactivators, adaptors or mediators, in addition to the GTFs needed for basal transcription. These coactivators typically function to either modify or remodel chromatin. Other coactivators posses scaffolding properties. These are discussed in depth in the following sections.

3.0 CHROMATIN STRUCTURE AND ITS ROLE IN TRANSCRIPTION

The basic structural unit of chromatin is the nucleosome, which consists of 146 base pairs of DNA wrapped in approximately 1.75 superhelical turns around an octamer histone core. The histone core contains 2 molecules each of histones H2A, H2B, H3 and H4. This unit is repeated once every 200 ± 40 base pairs as a nucleosomal array in chromosomal DNA. A fifth histone, the linker histone H1, binds to the nucleosome and promotes their organization into a higher order structure, the 30 nm filament. Since DNA is supercoiled around the histone octamer in the nucleosome, and these can be organized into higher order structures, it was widely accepted that the primary function of histones was to package the genome. However, chromatin in most cells exists in a form that is far less condensed than, for example, in sperm nuclei. This suggests that the main function of histones

is not to produce the most compact form of the genome but rather to provide for the proper regulation of many inducible genes [77; 78].

Over the past decade, evidence has been increasing that chromatin structure is dynamic [78; 79]. The discovery of nucleosome mobility highlighted the importance of nucleosome positioning and/or disruption, depending on the biological context [78; 80]. On the other hand, nucleosomes positioned at promoters were shown to block transcription [81; 82]. In order to allow access to the transcriptional apparatus these nucleosomes must be disrupted. In some cases, this disruption requires specific transcription factors and ATP [83; 84]. The SWI/SNF complex, a large multiprotein complex that is required for the induction of transcription at many promoters, is involved in the remodelling of chromatin during transcription [85; 86]. In other cases, histones are modified by complexes containing acetyltransferase activity that acetylates the lysine residues in the NH_2-terminal tails of the histones. This modification neutralizes their charged interactions with the phosphate backbone of DNA making the DNA available for transcription. On the other hand, the supercoiling of DNA by the nucleosomes can bring two regulatory elements into close proximity thus allowing transcription to take place. This has been observed for the heat shock elements found in the Hsp26 promoter in *Drosophila* melanogaster and in the vitellogenin B1 gene of *Xenopus laevis* [87; 88]. Also in the mouse mammary tumor virus promoter, an intact nucleosomal structure was found to be essential for the synergistic activity of the glucocorticoid receptor and nuclear factor 1 [89; 90].

3.1 Chromatin remodelling

The yeast Swi/Snf genes (Switching mating type/Sucrose non-fermenting) were first isolated as regulators of mating-type and carbohydrate pathway switches [91]. SWI/SNF is a 2 MDa multisubunit complex composed of at least 11 proteins and is an integral component of the yeast RNA pol II holoenzyme [36]. The SWI2/SNF2 subunit of the SWI/SNF complex contains

sequence motifs closely related to those found in DNA-stimulated ATPases/DNA helicases [92]. However, bacterially-expressed SWI2/SNF2 protein was found to have DNA-dependent ATPase activity but no helicase activity. Mutational studies have shown that the conserved ATPase domain is necessary for function *in vivo* [93]. *In vitro*, the SWI2/SNF2 subunit destabilizes histone-DNA interactions in reconstituted nucleosomes in an ATP-dependent manner. This destabilization increases the binding of transcription factors, such as GAL4 derivatives and TBP to histone-associated DNA [93]. SWI2/SNF2 is the prototype of an emerging class of chromatin remodelling proteins whose activities are dependent upon or associated with ATP hydrolysis. These proteins are also contained in large multisubunit complexes that contain components related to subunits of yeast SWI/SNF, specifically SWI2/SNF2 [92; 93].

Nucleosome remodelling factor (NURF) was purified from yeast as a cofactor that facilitated the interaction of the GAGA transcription factor with chromatin by perturbing a nucleosomal array in the presence of ATP. Studies suggest that NURF affects an early stage in the transcription process. One subunit of NURF, p55, has recently been identified as a WD repeat protein. Interestingly, WD repeat proteins are components of multiprotein complexes involved in a wide spectrum of cellular activities, such as cell cycle progression, signal transduction, apoptosis, and gene regulation [94; 95]. Homologues of p55 have been found associated with histone acetyltransferases and deacetylases. Thus, p55-like proteins may be involved in targeting the catalytic subunits of various chromatin-altering complexes to the histones [96].

Chromatin accessibility complex (CHRAC), a 670 kDa complex consisting of 5 subunits was identified on the basis of its ability to mobilize nucleosomes in a manner that allows enhanced access to a DNA restriction enzyme [97]. CHRAC is also able to introduce regularity into an irregular nucleosomal array by acting as a nucleosome spacing factor [97]. Both of these processes are ATP-dependent. Thus, CHRAC may function in a manner similar to histone chaperones by accepting or donating histones during chromatin assembly or disassembly. Topoisomerase II is a

subunit of the CHRAC complex but its activity can be inhibited without abrogating the remodelling activity. It is possible that the topoisomerase II subunit may function to direct CHRAC to particular chromosomal sites so that CHRAC participates in topoisomerase II-dependent processes such as chromosome condensation/decondensation, kinetochore assembly and other mitotic events [97].

ATP-utilizing chromatin assembly and remodelling factor (ACF) was purified as an ATP-dependent chromatin assembly factor. However, ACF is also involved in transcription factor-mediated chromatin disruption, similar to NURF, and can remove nucleosomes from densely packaged chromatin [98; 99].

A SWI/SNF-like complex, termed remodel structure of chromatin (RSC) was purified from *Saccharomyces cerevisiae* during a search for homologues of the SWI/SNF subunits [100]. RSC is a complex of approximately 1 MDa that contains 15 subunit proteins of which three are related to the components of the SWI/SNF complex. This is consistent with the related biochemical activities of the SWI/SNF and RSC complexes [100; 101]. However, unlike the constituents of the SWI/SNF complex, two subunits of RSC are encoded by genes that are essential for mitotic growth in yeast [100; 102]. A third subunit of RSC that is functionally equivalent to a SWI/SNF subunit is specifically phosphorylated in the G1 phase of the cell cycle. A temperature sensitive allele of this gene arrests the cell in the G2/M phase of the cell cycle at the non-permissive temperature [103; 104]. Thus, the RSC chromatin remodelling activity probably plays a role in the progression of the cell cycle.

A novel complex has been identified recently using a purified *in vitro* transcription system [105; 106]. This complex named FACT for Facilitates Chromatin Transcription is capable of alleviating a nucleosomal block to transcriptional elongation in a manner unlike other chromatin remodelling complexes, as it does not appear to require ATP hydrolysis to function. Although promoter-proximal chromatin remodelling is one critical step in gene activation, the evidence suggests that it

may be insufficient for transcription unless coupled with activities such as FACT, which permit elongation through the nucleosome [105; 106].

3.2 Histone acetylation

As mentioned above, chromatin remodelling can be accomplished by chemical modification. A relationship between acetylation of histones and transcriptional activation was proposed about 30 years ago [107; 108]. However, the major breakthrough in demonstrating such a connection was the cloning of the gene encoding p55 from *Tetrahymena thermophila*. The p55 protein, which had histone acetyltransferase activity displayed remarkable similarity to the yeast protein GCN5, already known to be a transcriptional adaptor in yeast [109]. The significance of this similarity was reinforced by the discovery that GCN5 has histone acetyltransferase (HAT) activity that is essential for its function [110]. A number of complexes with HAT activity have now been isolated and generally they can be grouped into two types designated A and B [111]. Type A HATs are localized in nuclei and most likely acetylate nucleosomal histones in reactions closely tied to transcriptional activation. To date, several transcriptional regulators have been found to possess intrinsic HAT activity: GCN5 and homologues, PCAF, p300/CBP, $TAF_{II}250$ and homologues and SRC-1/NCoA-1 and pCIP/ACTR/AIB1 [112-115]. Type B HATs can be purified from cytoplasmic fractions, and it is believed that these activities are responsible for acetylating newly synthesized histones before chromatin assembly during DNA replication [116]

GCN5 was isolated and characterized in a screen in yeast to identify mutations that impaired the GCN4-dependent general control response to amino acid starvation [117; 118]. Bacterially expressed GCN5 acetylates free histone H3 strongly at lysine 14 and histone H4 weakly at lysine 8 and 16 [119]. Biochemical and genetic studies showed that GCN5 is the catalytic subunit in at least two distinct multisubunit complexes possessing HAT activity; a 1.8 MDa complex termed SAGA (for SPT-ADA-GCN5-acetyltransferase) and a complex encoded by the Ada genes (for altered

defective activation) [120; 121]. Subunits present in both the SAGA and ADA complexes are known to play a role in activated transcription. Additionally, the SAGA complex contains the SPT proteins, which are believed to modulate TBP function [121]. The presence of these activities suggests a link between acetylation and transcription.

In addition to its HAT activity, GCN5 also contains a 110 amino acid domain termed the bromodomain [122]. This domain, which is involved in protein/protein interactions, is present in every HAT protein identified to date [122; 123]. Studies with the human PCAF bromodomain showed that it interacts specifically with acetyl lysine in short histone H3 and H4 peptides [124]. Since acetyl lysine in the NH_2-terminal tails of histones is associated with actively transcribed or poised chromatin, the results provide the first evidence that bromodomains play a role in anchoring HATs and other coactivators onto active chromatin. In addition, two subunits of the RSC complex described above each contain 2 bromodomains. Deletions that remove only the first bromodomain cause little or no phenotypic anomalies while mutations that remove only the second bromodomain cause phenotypes that are the equivalent of mutations that remove both. This suggests that multiple bromodomains in a complex have different functions.

p300 and CBP (CREB-binding protein) are two closely related proteins that share many biological functions [125]. The ligand-dependent transcription of many nuclear hormone receptors, such as RXR, RAR, TR and ER requires p300/CBP [126; 127]. p300/CBP also fills essential coactivator roles for many other classes of regulatory transcription factors including p53 [128; 129]. Recent studies have also demonstrated the recruitment of CBP by the Smad2/3 proteins in the TGF-β signalling pathway [130; 131]. The p300/CBP complex functions as an acetyltransferase and as a platform for a large number of essential associated proteins. For example, the C/H3 domain is capable of binding the adenoviral E1A oncoprotein, a protein kinase pp90rSK1 postulated to antagonize CREB function, and PCAF [132-134]. Distinct regions of CBP and associated factors

may be required for interactions with different classes of transcription factors. For example, E1A inhibition of STAT-1 or nuclear hormone receptor mediated transcriptional activation appears to require the C/H3 and p160 interaction domains, respectively [135].

A role for p300 in cell proliferation has been clearly demonstrated in the p300 -/- mouse [136]. Mice lacking p300 are embryonic lethal and show defects in neuralation and heart development. In addition, Rubenstein-Taybi syndrome, a disease characterized by mental retardation and specific skeletal abnormalities results from mutation of one CBP allele [137]. The disease is mimicked to some extent in CBP +/- mice, which exhibit skeletal abnormalities [138]. These biological effects are not observed with p300+/- mice which show reduced viability but no other complications. These results suggest that the absolute levels of the combination of p300 and CBP are critical for aspects of development. This is supported by observations that a compound heterozygous mouse (p300+/- and CBP +/-) is embryonic lethal [136].

The p300/CBP Associating Factor (PCAF) was identified through its ability to interact with p300/CBP [134]. The association between p300/CBP and PCAF is required for transcriptional activation of many genes. The COOH-terminal half of PCAF is homologous to GCN5 and, as expected, this domain contains essentially all of the intrinsic HAT activity. Like GCN5, PCAF can acetylate free histones H3 and H4 but, unlike GCN5, can also acetylate nucleosomal H3. The NH_2-terminal half of PCAF has been shown to directly contact SRC-1/NCoA-1 and CBP as well as several nuclear hormone receptors [114; 139]. PCAF associates both with p300/CBP and with a number of the nuclear receptor coactivator complexes thereby forming complexes with multiple histone acetylases [113; 114].

$TAF_{II}250$, the largest subunit of the TBP-associated factors, is unique among the various TAFs in that individually, or as part of the TFIID complex, it exhibits both a serine kinase activity and a histone acetyltransferase activity [140; 141]. The serine kinase activity $TAF_{II}250$ is specific for the

RAP74 subunit of the general transcription factor TFIIF and to a lesser extent TFIIA and TFIIE [140]. TFIIF is an interesting target for phosphorylation since it is intimately associated with RNA polymerase and is involved both in recruiting RNA pol II to the pre-initiation complex and in modulating its elongation properties. $TAF_{II}250$ also contains an intrinsic histone acetyltransferase activity that has been conserved in yeast, *Drosophila* and humans. $TAF_{II}250$ functions through the acetylation of lysine residues in histones that weakens histone-DNA interactions. This implicates TFIID as an important player in controlling access of nucleosome-bound promoter sequences to the basal machinery *in vivo*. Similarly to GCN5, $TAF_{II}250$ preferentially acetylated free histones H3 over H4 and displays little or no activity for nucleosomal histones [141]. The domain of $TAF_{II}250$ responsible for the histone acetyltransferase activity has been localized to a central region that is well conserved but shows no sequence similarity to other histone acetyltransferases. A temperature sensitive cell line, which arrests in G1 at a non-permissive temperature, has been found to encode a $TAF_{II}250$ mutant with a point mutation within the histone acetyltransferase domain, indicating that this TAF may play a specific role in regulation of the cell cycle [141].

3.3 Non-histone acetylation

The substrate specificities of histone acetyltransferases could be determined, in part, by the primary sequence surrounding the target lysine residues. The consensus recognition sequence derived from the target sites of GCN5 is K-X-X-G-G/A-K^*-X, while that of Hat1 is proposed to be G-X-G-K^*-X-G, where K^* is the acetylation site. Therefore, it is possible that proteins bearing similar sequences may also be natural substrates for acetylation.

Indeed the acetylation of several transcription factors by several known acetyltransferases has been demonstrated *in vitro* [142-144]. TFIIF can be acetylated by PCAF and p300/CBP [144]. The â-subunit of TFIIE can be acetylated by PCAF, p300/CBP and $TAF_{II}250$ [144]. Interestingly, a point mutation introduced at lysine 52 of TFIIE â was shown to dramatically reduce the efficiency with

which TFIIE â was acetylated, suggesting that lysine 52 is a primary target for acetylation. This residue is conserved among human, *Xenopus* and yeast TFIIE â homologues and appears in a sequence context similar to lysine 14 of histone H3, the most preferred site for yeast GCN5 action *in vitro* [119].

p300 is capable of acetylating p53 efficiently *in vitro* on two highly conserved lysine residues [145]. p53 is also acetylated *in vivo*, for example, in response to DNA damage [146]. Interestingly, the acetylated form of p53 is capable of binding its target DNA with a higher affinity. Thus, the acetylation of activator proteins may function to signal or enhance the binding of bromodomain-containing coactivator complexes that are important for transcriptional control, such as SAGA, RSC and SWI/SNF.

3.4 Histone deacetylation in repression of transcription

As histone acetylation is correlated with actively transcribed genes histone deacetylation has become associated with transcriptional inactivity. Like GCN5, RPD3 was first identified during genetic screening for positive and negative regulators of a subset of yeast genes [147]. Two groups independently discovered that the RPD3 gene encodes the catalytic subunit of histone deacetylase complexes. Rpd3 contains the deacetylase activity in a large complex of approximately 600 kDa called HDB (histone deacetylase B), while an RPD3-like protein in a second complex of approximately 350 kDa is encoded by the HDA1 gene (histone deacetylase complex A) [147]. The Rpd3 and Hda1 genes share approximately 49% similarity over a region of 498 amino acids. In yeast, deletion of Hda1 and Rpd3 leads to hyperacetylation of histones H3 and H4 [147].

The high level of similarity of the first histone deacetylase isolated (HDAC1) from human cells to RPD3 provided a clue as to its function *in vivo* [147]. Other human deacetylases have now been identified [148; 149]. As is the case for the acetylases, the deacetylases exist as part of larger

complexes in both mammalian cells and in yeast. Insights into the mechanism of repression in mammalian cells have come from the identification of a corepressor complex comprised of Sin3, the nuclear receptor corepressor (NCoR) and the silencing mediator for RXR and TR (SMRT). The gene encoding NCoR was cloned using TR as bait in a yeast two-hybrid screen [150; 151]. Similar approaches were also used to identify the related factors, SMRT and the TR-associated factors (TRAC) [152; 153]. The corepressor complexes also contain various stably associated protein components including several histone deacetylases, thus establishing a link between repression and histone deacetylation [4; 154]. The data suggest that transcription factors that function as transcriptional repressors do so by a common mechanism involving recruitment of a multicomponent complex containing HDAC activity and the re-establishment of a repressive chromatin state.

3.5 DNA methylation in gene expression

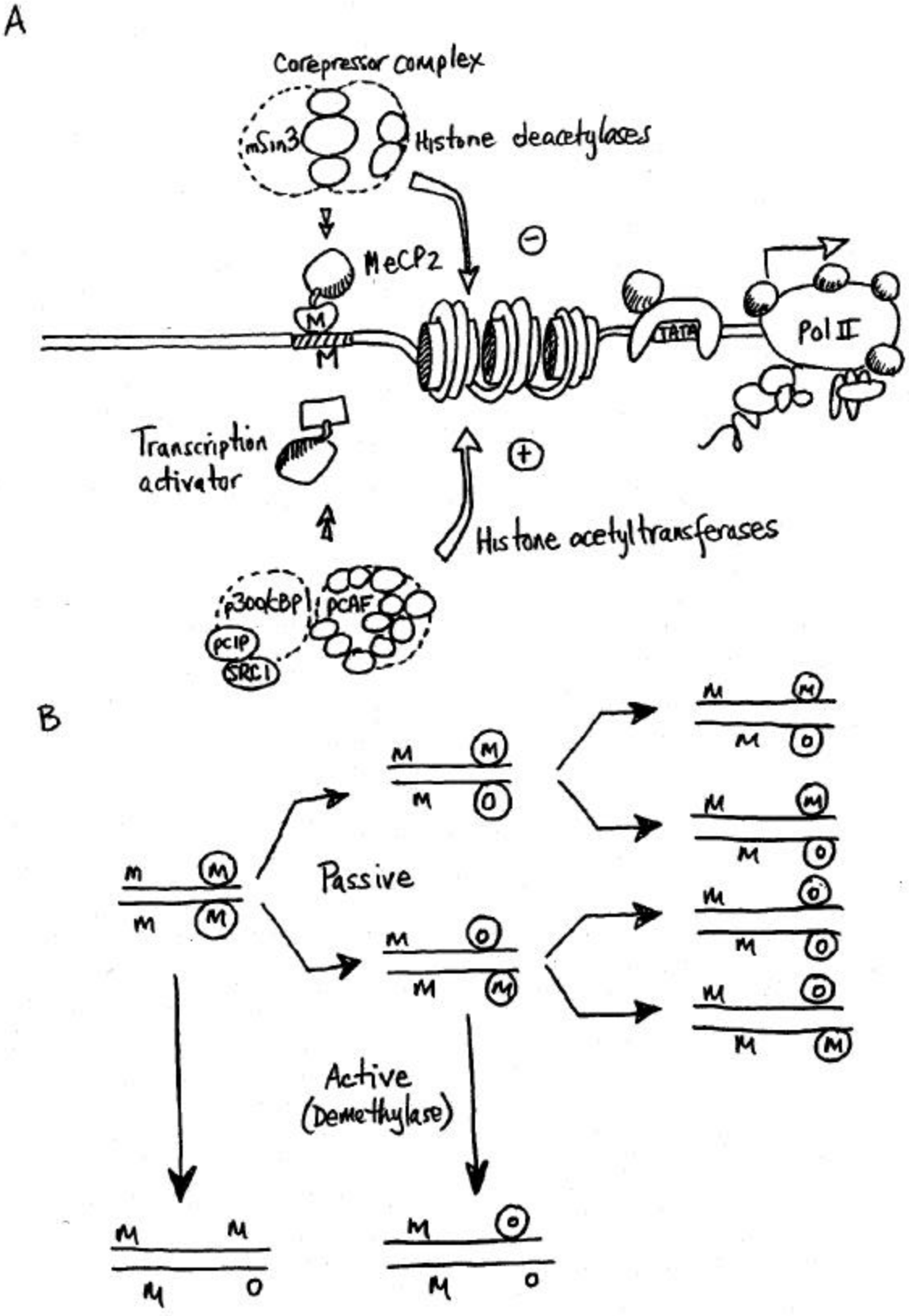
Methylation of cytosine-guanine (CpG) sites is a characteristic feature of many eukaryotic genomes. In vertebrates, approximately 60-90% of all CpGs are methylated. Many of the non-methylated sites (approximately 15% of CpGs in human DNA) are found in CpG islands, which usually encompass functional promoters. Reviewed in [155]. Microinjection and transfection experiments using *in vitro* methylated gene sequences demonstrated that DNA methylation results in the formation of inactive chromatin. The silencing effect exerted by CpG methylation is only observed following incorporation of the methylated DNA into chromatin [156; 157].

Studies in mammalian systems, where methylation clearly plays a role in gene silencing, indicate that methylation mediates the formation of a multiprotein repression complex, which induces changes in histone acetylation. Specific ally, under conditions where methylation of DNA exceeds a threshold level, methylated CpG sites have been shown to recruit MeCP2, an abundant mammalian protein that functions as a transcriptional repressor [158-160]. When bound to methylated

chromosomal DNA *in vitro* MeCP2 has been shown to displace histone H1 [158]. MeCP2 has been shown to contain both a methyl-binding domain and a transcriptional repressor domain (TRD) [158]. The TRD lies within a region that interacts directly with the corepressor mSin3A. Coimmunoprecipitation experiments show that antibodies raised against MeCp2 immunoprecipitate mSin3A as well as histone deacetylase 1 and 2 (HDAC1 and 2, respectively) [158; 161]. Other experiments used cell lines that had been stably transfected with *in vitro* methylated or unmethylated constructs driven by the *thymidine kinase* (*tk*) promoter. Immunoprecipitation of chromatin with an antibody against acetylated histone H4 revealed that in contrast to the non-methylated *tk* gene the methylated *tk* gene was not enriched for acetylated histones. Treatment with the potent histone deacetylase inhibitor trichostatin A (TSA) resulted in an increased expression of the methylated *tk* gene. Furthermore, TSA treatment increased the accessibility of the methylated *tk* gene locus to nucleases suggesting that acetylation of histones restablishes features of transcriptionally active chromatin despite the presence of DNA methylation [162]. Such studies suggest that the methylation of gene sequences induces transcriptional repression through their capacity to recruit MeCP2 [158; 161; 162]. This methyl binding protein tethers a multiprotein complex that includes the corepressor protein mSin3A and the histone deacetylases HDAC1 and HDAC2. The deacetylase activity recruited by MeCP2-bound mSin3A renders the promoter of the gene inaccessible to transcription factors by deacetylating histone H3 and H4 [85].

Presently there are several models that address the transcriptional activation of genes silenced by methylation and these are depicted in figure 1.3. In the first model, activation results from deacetylation of the gene while in the second model, demethylation alone is sufficient for reactivation, The first model is exemplified by the progesterone receptor (PR) gene. This gene is estrogen responsive and is inactivated by DNA methylation in certain types of breast cancer.

Figure 1.3 **Two models for the transcriptional activation of genes silenced by methylation.** In the first model, A, methylated (M) CpG sites in the regulatory region of gene X are occupied by a repressor complex comprised of MeCP2/mSin3A/HDAC1 and 2. Under conditions were this particular gene is induced, a transcriptional activator displaces the MeCP2 repressor complex and recruits a coactivator(s) with histone acetyltransferase activity leading to nucleosome disruption and culminating in the activation of gene X. In the second model; B, transcription factor occupation of methylated CpG sites in the regulatory region of gene Y prevents the maintenance methylase from remethylating these hemimethylated DNA sites following one round of DNA replication. At this point demethylase enzymes act upon the resulting hemimethylated regions and fully demethylate the DNA. Alternatively, these helimethylated sites could be fully unmethylated at any given point during or following DNA replication.


A
Corepressor complex
mSin3
Histone deacetylases
MeCP2
M
M
TATA
Pol II
Transcription activator
Histone acetyltransferases
p300/CBP
PCAF
PCIP
SRC1
B
Passive
Active (Demethylase)

Treatment with the DNA demethylating agent 5-aza-deoxycytidine can reactivate PR expression demonstrating that the gene is hypermethylated. Ectopic expression of ligand-bound ER can reactivate the methylated PR gene but induction by ER is dependent upon the presence of SRC-1, which has intrinsic histone acetyltransferase activity. In the second model, activation of methylated gene expression may occur through DNA demethylation. Evidence for both passive and active mechanisms of DNA demethylation is demonstrated in the case of the EBNA-1 interactions with the Epstein Barr virus latent replication origin, oriP or Sp1 interactions with variants of the â-globin gene promoter [163-165]. Demethylation could be carried out by a passive (replication-dependent) mechanism whereby the binding of a site-specific transcription factor (EBNA-1, Sp1, NFêB) and subsequent association of the general transcription factors would prevent the maintenance methylase activity from methylating post-replicative, hemimethylated DNA. After a second round of replication, fully unmethylated DNA would be generated. Alternatively, following the first round of replication the hemimethylated DNA could be further modified by a demethylase enzyme activity to generate fully unmethylated DNA.

4.0 NUCLEAR HORMONE RECEPTORS

Nuclear hormone receptors represent an evolutionarily well-conserved class of transcription factors being present from flies to mammals (Reviewed in [166-169]). They bind to small lipophilic hormones, or presently undetermined ligands in the case of the orphan receptors, and function in the cell nucleus as ligand-activated transcription factors. Nuclear hormone receptors can be classified according to the type of hormone they bind. In this manner, nuclear hormone receptors are divided into four different classes: i) receptors that bind steroids such as glucocorticoids (GR), mineralcorticoids (MR), progestins (PR), androgens (AR) and estrogens (ER); ii) those which bind steroid derivatives such as vitamin D3 (VDR); iii) those which bind non-steroids such as thyroid hormone (TR), the all-*trans* and 9-*cis* forms of retinoic acid (RAR, RXR) and the insect developmental hormone ecdysone (EcDR). Additionally, fatty acids, bile acids, oxysterols, farnesol

metabolites, leukotriene B4 and prostaglandins may act by binding to nuclear hormone receptors such as peroxisome proliferator-activated receptors (PPAR) or the receptors designated FXR and LXR; and iv) receptors for which no physiological ligand has yet been determined. These are designated the orphan receptors.

Alternatively, the nuclear hormone receptors can be divided into two broad types, the type I and type II receptors. The type I class, comprise the receptors for steroids such as glucocorticoids, progestins, aldosterone, estrogens, and androgens are associated with heat shock proteins. Hormone binding leads to dissociation of hsp and binding in dimeric form to specific palindromic DNA sequences as elements that include specific palindromic sequences. The type II class is represented by receptors such as for the thyroid hormone, retinoids, prostaglandins, vitamin D3 and ecdysone. In contrast to the class I receptors, members of this class are bound to DNA in the absence of hormone. Binding of ligand induces a conformational change that leads to transcriptional activation. Receptors of this class are predominantly bound as heterodimers to DNA response elements that include direct repeats, everted or inverted palindromes. The dimerization partner for many members of this class of receptors appears to be the 9-*cis* retinoic acid receptor RXR. Orphan receptors can bind as monomers or as dimers preferentially to direct repeat DNA elements.

Based upon this type of classification ER can be functionally classified as a type I/II hybrid by having a type II DNA binding domain, but forming only homodimers on a palindromic arrangement of two 5'-AGGTGA-3' half sites.

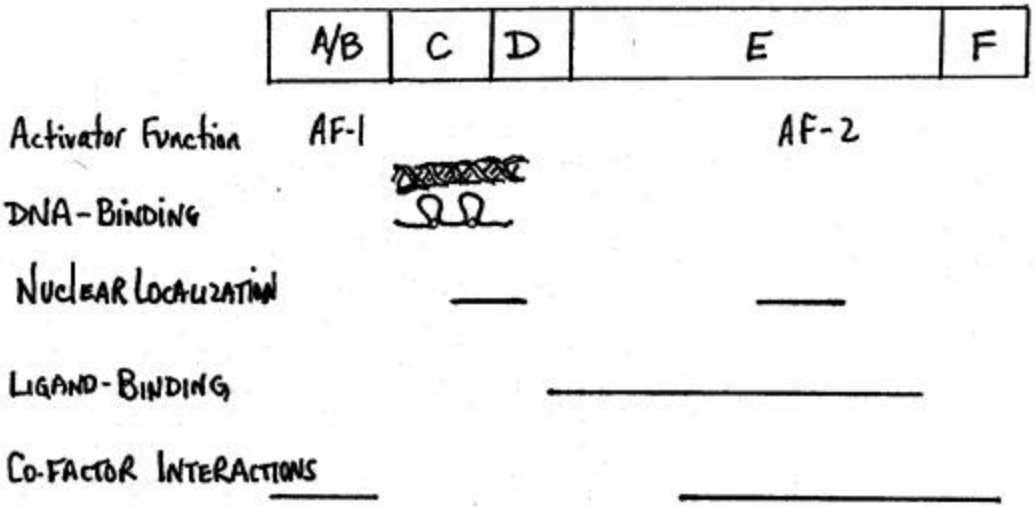
4.1. The general structure of nuclear hormone receptors

Homology studies of a number of nuclear hormone receptors indicate that their amino acid sequences can be divided into five different domains A/B, C, D, E and F (Figure 1.4A) [166; 170]. The NH_2-terminal A/B region is highly variable in length and sequence between different NHR

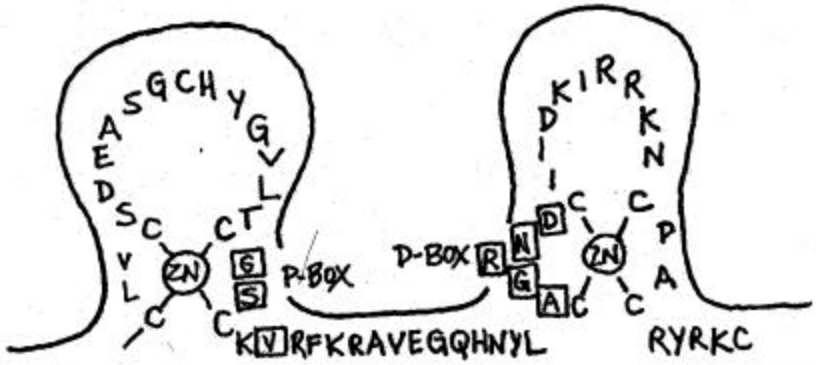
proteins but in many receptors, such as the ER and GR, a ligand-independent transactivation domain (AF-1) has been identified in the A/B region. The most conserved region among the different family members is the centrally located DNA-binding domain designated the C region, which contains two zinc-finger domains of the C_2-C_2 type. This means that the structure of the DNA-binding domain is stabilized by the tight binding of two Zn^{2+} ions that are coordinated by four cysteine residues (Figure 1.4B). The first zinc-finger located towards the NH_2-terminus is involved in specific binding to DNA whereas residues in the second, more COOH-terminal, zinc-finger are important for dimerization. The amino acids that determine DNA-binding specificity are located in a more COOH-terminal portion of the first zinc-finger designated the P-box. The DNA motifs that are recognized by the P-box are usually derivatives of the sequences 5'-AGGTCA-3' (for ER and many other nuclear hormone receptors) or 5'-AGAACA-3' (for all steroid hormone receptors except for ER) (Figure 1.4B). Members of the type II subfamily appear to heterodimerize with the retinoid X receptor (RXR) and bind to direct repeats of the consensus motif 5'-AGGTCA-3' with different spacings. In contrast, the steroid hormone receptors are predominantly known to form homodimers, which bind to two palindromically arranged copies of the sequence motifs mentioned above usually spaced by three nucleotides. Other arrangements of the DNA half-sites are also known, and many of the natural response elements to which nuclear hormone receptors can bind can diverge significantly from the consensus sequences. Some receptors such as the ER, have been shown to bind to complex arrangements of half-site elements that may be separated by long intervening DNA regions [171]. In some of the receptors a nuclear localization signal has been identified at the junction of the C and D domains. The short D domain located COOH-terminally to domain C is primarily regarded as a flexible hinge between the DBD and the E domain. It contains sequences at its NH_2- and COOH-terminal ends that contribute to the function of the adjacent domains. For example the NH_2-terminal portion is important for DNA binding and heterodimerization. This region contains the T and A boxes, which are well conserved.

Figure 1.4 **The general structure of steroid hormone receptors.** A, The functional domains of steroid hormone receptors are shown. The structure of steroid receptors can be divided into six domains designated A/B, C, D, E and F. B, the amino acid sequence of the DNA-binding domain (domain C) of the glucocorticoid receptor is shown. The P-box and D-box, which are essential for DNA-recognition and dimerization, respectively, are highlighted. Also included are the P-box sequences for a number of nuclear hormone receptors and the recognition elements they specify.

A



B



RECEPTORS	P-BOX	HALF-SITE
RAR, VDR, RxR, PPAR EAR1, rREVERBα	cEGckG	GGTCA
HNF-4	cDGckG	GGTCA
EAR-2	cEGcKS	GGTCA
SF-1	cEScKG	GGTCA
ERR-1	cEAckA	GGTCA
ER	cEGckA	GGTCA
GR, MR, PR, AR	cGSckV	TGTTCT

The A box is implicated in base contacts with the 5' extension of the half-site recognized by monomer binding receptors. The T box of RXR is implicated in the dimerization process.

The E domain is relatively large and contains many activities that include a ligand binding domain and a ligand-dependent transcriptional activation function (AF-2) domain that is recognized by coactivators or corepressors, which serve to bridge the receptor to the basal transcription machinery. In addition, a dimerization interface, an hsp-interaction region and a nuclear localization domain are present.

Recently, the three-dimensional structures of the ligand binding domains of three different nuclear hormone receptors: RXR á in the absence of ligand, and RAR ã and TR á in their ligand-bound forms have been solved [172-174]. The overall structures consist of three layers of á-helices, a structure referred to as an á-helical sandwich consisting of 12 á-helical regions, two anti-parallel â-strands (S1 and S2) and an Ù loop between helices 2 and 3. The ligand-binding pocket exists in a centrally located cavity formed by protein regions that are distally separated in the primary structure. A comparison of the ligand-bound to the unliganded structures suggests that ligand binding induces a rearrangement of helices 10 and 11 that results in the movement of the COOH-terminal á-helix (helix 12), which in the unbound state extends out from the ligand-binding domain. In the presence of ligand, helix 12 is translocated so that it effectively covers the ligand-binding pocket. It is believed that helix 12 in this position together with the adjacent helices 3 and/or 4 forms a surface by which the nuclear hormone receptors interact with the basal transcription machinery or intermediary factors.

A COOH-terminally located F domain varies greatly in length and is not present in all nuclear hormone receptors. A functional role for this domain remains unclear; nevertheless, a role for this domain has been documented in several reports. For example, the F domain of the ER has been shown to play an important role in regulating the extent of transactivation by estrogens [175].

Additionally, two RAR mutants that differ only in the presence or absence of the F' domain, exhibit significant differences in transactivation potential [176].

4.2 Transactivation by steroid hormone receptors

The steroid hormone receptor subfamily within the nuclear hormone receptor family contains two distinct transactivation domains: a ligand-dependent activation function AF-2 present in the COOH-terminal ligand binding domain and a ligand-independent function in the NH_2-terminal A/B region [177]. Experiments performed principally with ER and PR have shown that the activity of the two AFs is cell- and promoter-specific [178]. For example, it was found that a mutant ER containing only AF-1 activated transcription from chicken embryo fibroblasts but was inactive in HeLa cells, whereas an AF-2 containing receptor was active in both cell types [177]. It was also shown that AF-2 only functioned on certain promoters whereas AF-1 activity was less sensitive to variations in promoter context [177; 179].

According to the classical model, unliganded steroid hormone receptors exist in a monomeric state associated with a number of proteins belonging to the heat shock family of proteins. These proteins are believed to maintain their steroid hormone receptor partners in a conformation that exhibits high-affinity ligand binding and low DNA-binding affinity [180]. Binding of the cognate hormone induces a cascade of events including hsp-dissociation, conformational changes, phosphorylation and dimerization, ultimately resulting in the binding of steroid hormone receptor dimers to their respective hormone response elements (HREs).

Several mechanism(s) have been proposed to explain the manner by which ligand-bound receptors activate gene transcription *in vivo*. One mechanism is based upon evidence that at least four different components of the basal transcription machinery are targets of steroid hormone receptors. These direct protein/protein interactions are believed to result in the stabilization of pre-initiation

complex assembly and increased rates of transcriptional initiation. It has been demonstrated that three of these proteins are subunits of the TFIID complex including TBP and the TAFs [72]. Using *in vitro* binding assays, TBP has been shown to interact with the ER in a ligand-independent manner. This interaction was also independent of the presence of functional AF-1 or -2 elements [72; 181]. Two TAFs, hTAFII30 and dTAFII110, have also been reported to interact with ER, PR and TR, respectively [182; 183]. The $TAF_{II}110$ subunit of the *Drosophila* TFIID complex has been reported to interact with the DNA-binding domain of PR *in vitro* [183]. Results from yeast two-hybrid assays indicate that $dTAF_{II}110$ interacts with RXR and TR through AF-2 [184]. TFIIB has also been demonstrated to interact with both ER and TR [185; 186].

A second mechanism is based upon mounting evidence indicating that ligand-activated steroid hormone receptors associate with a number of different factors, termed coactivators, intermediary factors, or receptor interacting proteins, which may play decisive roles in mediating the effects of steroid hormone receptors on transcription initiation [187].

A third mechanism implicates chromatin remodelling in steroid hormone receptor transactivation [188; 189]. Nucleosome disruption is therefore an essential element in the ligand-dependent transactivation of many steroid hormone receptor-inducible genes. Nucleosome displacement by histone acetylation can be reversed by the actions of histone deacetylases that have been shown to act with steroid hormone receptors in the absence of hormone causing repression of transcription.

4.3 The receptor interacting proteins

The most basic function performed by an activated nuclear hormone receptor is to recruit and maintain a stable pre-initiation complex at the promoter of the target gene. This is accomplished through a series of direct or indirect association of the liganded receptor with the GTFs. Overexpression of the AF domains of various steroid hormone receptors were noted to interfere

specifically with the transcription of reporter genes activated by other steroid hormone receptors family members, while control promoters were relatively unaffected. These transcriptional interference or squelching experiments suggested that the steroid hormone receptors AFs are able to form specific complexes with distinct set of cofactors that existed in limiting concentrations and which were different than the basal transcription factors [190]. Evidence exists that liganded receptors are capable of directly contacting the GTFs as well as the TBP-associated factors (TAFs) [182; 191]. However, several elegant studies suggested that non-TAF proteins were important targets of liganded receptors. The first of these was a 160-kDa ER-associated protein (ERAP-160) that co-purified with ER in the presence of ligand, suggesting that ER underwent interactions with specific protein complexes prior to transcriptional initiation [192]. Others groups published similar findings with respect to several other receptor interacting proteins [193]. These endogenous factors are functionally limiting. Additionally, hormone antagonists can uncouple the interaction of receptor with these proteins (collectively termed the p160 proteins), suggesting that transcriptional activation is dependent upon the interaction of the receptor with these proteins. Several other receptor interacting proteins have now been identified and some of these are discussed below.

4.3.1 *The nuclear receptor coactivators (NCoA)*

The nuclear receptor coactivators are a group of related 160 kDa proteins that interact with the conserved activator domain, AF-2, of the retinoic acid receptor (RAR) and ER in a ligand-dependent manner [151; 192; 193]. These p160 proteins are also capable of interacting with p300/CBP through a separate domain [126; 194]. Several distinct but related members have been identified, with each family member having a number of splice variants. These include SRC-1/NCoA-1, TIF2/GRIP1/NCoA-2, pCIP/ACTR/AIB1/RAC3/TRAM-1 [114; 115; 195-197]. A weak intrinsic HAT activity has been reported for SRC-1/NCoA-1 and pCIP/ACTR/AIB1, suggesting that chromatin remodelling may also be a function of these NCoA factors although they do not appear to contain regions homologous to the HAT domain of p300/CBP or PCAF [113;

114]. SRC-1/NCoA-1 and ACTR acetylate nucleosomal histones H3 and H4 *in vitro* [113]. All three of the NCoA proteins contain two major transactivation domains that operate through distinct mechanisms: a weaker transactivation domain located in the COOH-terminus, and a stronger transactivation domain that overlaps the region that mediates interactions with p300/CBP. The catalytic domain of SRC-1/NCoA-1 has been mapped to the COOH-terminal region that bears no significant homology to the catalytic domains of the GCN5 family members. All three related NCoA proteins share a number of structural features, one of the most interesting being the presence of a well-conserved NH_2-terminal domain found in the PAS/basic helix-loop-helix (bHLH) family of transcription factors. Members of this class are involved in regulation of cell type differentiation and proliferation and are characterized by the formation of homo- or heterodimeric complexes with bHLH partners for their function (for review see [198]). Like other PAS-bHLH proteins SRC-1 and TIF2 appear to be capable of forming multimeric complexes *in vivo*, but the role of the PAS domain in this interaction remains to be clarified [199].

Yeast two-hybrid screening of a Hela cell cDNA expression library using the ligand binding domain of PR resulted in the identification of the receptor interacting protein designated steroid receptor coactivator (SRC-1) [200; 201]. Interaction of SRC-1 with PR is dependent upon ligand. SRC-1 also enhances the activity of a number of steroid hormone receptors including PR, GR, and ER as well as the non-steroid receptors TR and RXR. However, it fails to enhance E2F- or CREB-driven activity indicating that SRC-1 is not a general cofactor of transcriptional activation [126; 194]. More significantly, SRC-1 relieves the inhibition of a PR-regulated reporter that is observed when ER is coexpressed. This indicates that SRC-1 is one of the limiting cofactors for which ligand-activated steroid hormone receptors compete, as demonstrated in the original squelching experiments. SRC-1 has also been shown to enhance the functional cooperation between two separately expressed ER deletion constructs encoding AF-1/DNA-binding domain and estrogen-activated AF-2 [202]. This implies that SRC-1 functions as an adaptor protein to stabilize

cooperative interactions between AF-1 and AF-2. In this regard, it is worth noting that SRC-1 contains two interaction domains, as RAR contacts a site on mSRC-1 that is distinct from the PR interaction domain [194]. SRC-1 has also been shown to interact with p300/CBP and PCAF, both components with intrinsic histone acetyltransferase activity [113; 200]. This interaction involves part of a glutamine-rich domain located in the COOH-terminal part of CBP and a more central region of SRC-1 that is distinct from its PR- or RAR-interacting domain implying that steroid hormone receptors as well as other nuclear hormone receptors may be capable of forming ternary complexes with SRC-1 and p300/CBP at target gene promoters [113; 200]. CBP has also been shown to interact with TFIIB suggesting that this factor may function to transmit the signals of AF-1 or AF-2 of the steroid hormone receptors to the basal transcription machinery. Finally, p300/CBP interacts with PCAF implying that the two factors, in concert, may facilitate steroid hormone receptor-mediated promoter activity by disrupting chromatin structure around the target gene.

ACTR is homologous to the SRC-1/NCoA-1 and TIF2/GRIP1/NCoA-2 proteins in several motifs including the HAT catalytic domain of SRC-1 [114]. ACTR preferentially acetylates the nucleosomal histones H3 and H4. ACTR interacts directly with several ligand-bound nuclear hormone receptors and recruits PCAF and CBP to form a multicomponent complex, which is essential for ligand-dependent transactivation [114].

AIB1, a human splice variant of pCIP was identified by a technique involving chromosome microdissection of genes whose expression and copy number are elevated in human breast cancers [203]. AIB1 is overexpressed in several ER-positive breast and ovarian cancer cell lines and primary breast cancer specimens, suggesting that altered expression of pCIP/AIB1 may contribute to the development of steroid-dependent cancers [197; 203].

TIF2/GRIP1/NCoA-2 is a 160 kDa protein cloned from a human placenta cDNA expression library by screening with labelled ER ligand binding domain [204]. GRIP1, the murine orthologue of

TIF2, has been shown to interact with GR, ER and AR [196; 205]. TIF2 interacts with the ligand-binding domain of all the steroid hormone receptors and other nuclear hormone receptors tested including ER, PR, AR, TR, RAR, RXR and VDR [196; 205]. This interaction occurs through AF-2 in a ligand-dependent manner. Similarly to SRC-1, TIF2 appears to represent one of the limiting cofactors that are squelched by overexpression of ligand-activated AF-2 since inhibition resulting from coexpression of ER can be partially reversed by introducing TIF2 [204].

4.3.2 *Other classes of receptor interacting proteins*

One of the receptor interacting factors initially described was a 140-kDa nuclear protein designated RIP140 [206]. RIP140 was shown to interact with the AF-2 domain of ER in a ligand-dependent manner both *in vitro* and *in vivo*. RIP140 has subsequently been shown to be involved in transactivation by RXR, RAR and TR [207; 208]. RIP140 contains two binding sites that interact independently with the AF-2 of ER but differentially with the RXR, suggesting that one RIP140 molecule contacts both of the receptors present in nuclear receptor homo- and heterodimers. RIP140 is able to act as a potent transcriptional activator when tethered to the DNA-binding domain of GAL4 indicating that it may act as a bridging factor mediating recruitment of individual basal transcription factors or the RNA pol II holoenzyme complex [209].

Another receptor interacting protein named TIF1á was cloned from a mouse cDNA library by screening for factors that enhance the activity of a Gal4-RXR ã (AF-2) fusion protein in yeast. mSUG1, the mouse orthologue of TIF1á, was subsequently shown to interact with the ligand binding domains of ER, PR, VDR, TR, and RAR in a ligand-dependent fashion [210; 211]. TIF1á contains a RING finger domain and a bromodomain, which are present in a number of transcriptional regulators [212]. A yeast two-hybrid screen was used to identify two TIF1á-binding factors, mHP1á and mMOD1 {}. mMOD1 was subsequently demonstrated to interact with the

murine orthologue of hSNF2â, a component of the SWI/SNF complex suggesting that TIF1á participate in chromatin remodelling [213].

TRIP1/SUG1 is another receptor interacting protein isolated via yeast two-hybrid screening using the LBDs of the TR and RXR as bait [214]. The murine orthologue of TRIP1, designated mSUG1, has been shown to interact with ER, TR, RXR, RAR, and VDR [211]. Furthermore, this interaction is dependent upon an intact AF-2 domain. *In vitro* binding studies have shown that mSUG1 is capable of binding TBP, TFIIB, $TAF_{II}30$ suggesting that it may function as a molecular adapter that helps recruit the basal transcription machinery following binding of the various nuclear hormone receptors through AF-2.

4.3.3 *The LXXLL motifs*

The nuclear receptor interaction domains, termed the NR boxes, of pCIP, SRC-1 and TIF2 contain three highly conserved motifs that share a consensus amino acid sequence LXXLL (single letter amino acid code, where X is any amino acid) with a conserved spacing that is required for their interaction with nuclear hormone receptors as well as p300/CBP [115; 215]. Extensive mutational analysis has shown that different LXXLL motifs within SRC-1 are required to interact with different nuclear hormone receptors [115]. This functional specificity corresponds to the difference in affinity between each LXXLL motif and the different nuclear receptors. Such specificity appears to be influenced by the amino acid residues located adjacent to the LXXLL motifs [216]. The cocrystal structures of peptides encompassing one LXXLL motif of SCR-1 and thyroid hormone receptor (TR) have indicated that the mechanism of interactions between SRC-1 and the ligand-binding domains of nuclear receptors is fairly well conserved [217; 218].

Receptors exhibit preferential binding to different NCoA family members, for example AR preferentially binds to GRIP1 over SRC-1. In addition LXXLL motifs within a given coregulator

exhibit binding specificity. For example, the central domain of SRC-1 (NR boxes I-III) preferentially binds the ligand binding domains of ER, PR, VDR and TR, while the more COOH-terminal NR-box IV strongly binds AR and GR [219].

4.4 Nuclear receptor corepressors

Several members of the nuclear hormone receptor family including RAR and TR can act as transcriptional repressors in the absence of hormone. This repressor activity is contained in a distinct region of the ligand-binding domain that is functionally separable from the AF-2 domain. In these cases transcriptional repression is mediated by interactions with NCoR and SMRT [214; 220]. Other nuclear hormone receptors including COUP-TF, PPARá as well as steroid receptors such as the ER and PR have also been demonstrated to interact with NCoR/SMRT [221-224].

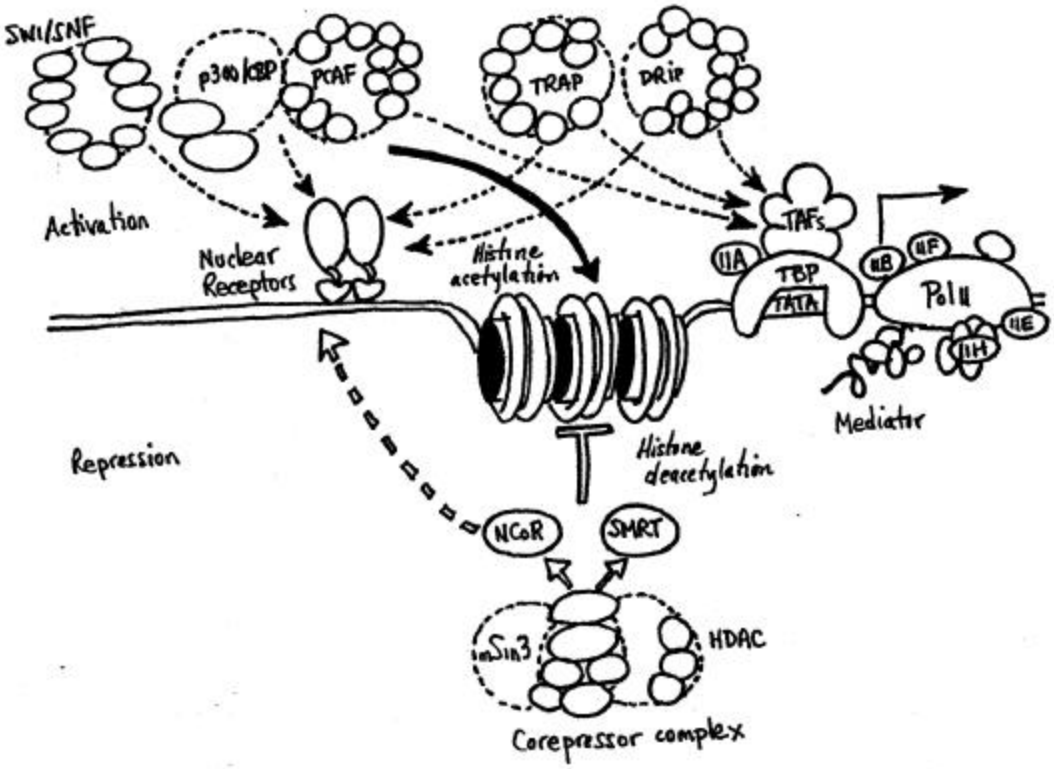
Additionally, NCoR and SMRT participate in a general mechanism of active repression by other classes of transcriptional repressors, such as Mad/Mxi1, RB, YY1 and the yeast Ume6 protein [225-228]. These factors have been shown to recruit the corepressor complexes to the promoter by either binding to the corepressor complex directly, as in the case of YY1 and RB or by contacting a component of the complex such as Sin3, NCoR and SMRT. For example, the bHLH zipper protein Max is able to dimerize with members of the Myc/Mad family to elicit opposite transcriptional responses. Whereas Myc/Max heterodimers activate transcription, Mad/Max heterodimers repress transcription. This repression is mediated through interaction with mSin3A and mSin3B [154; 229]. Based upon these and other studies, the current model suggests that unliganded nuclear hormone receptors, or Max/Mad-Mxi1 heterodimers, bind DNA and mediate repression by recruiting the NCoR/Sin3/RPD3 complex, resulting in histone deacetylation and repression of transcription. Ligand-binding to nuclear hormone receptors, or replacement of Max/Mad-Mxi1 with the Max/Myc heterodimers, results in the replacement of the corepressor complex by activator

complexes that consist of multiple acetyltransferases which then catalyze histone acetylation and result in transcriptional activation (Figure 1.5).

4.5 The Estrogen Receptor (ER)

In tissues that are estrogen-responsive, the action of the hormone is mediated through the estrogen receptor (ER). Estrogen stimulates transactivation by ER. Estrogen enters the cell by passive diffusion across the cellular membrane where it can bind to the ER present in the nucleus. In the absence of hormone, ER exists as a complex together with several heat shock proteins (hsp) hsp90, hsp70 and hsp56 [230]. The hsps are believed to act as chaperones that keep the ER in a transcriptionally inactive form that is capable of binding its ligand with high affinity. Evidence thus far indicates that among the nuclear hormone receptors only the steroid hormone receptors are thought to interact with heat shock proteins. There is evidence that binding of hormone by some steroid hormone receptors permits translocation into the nucleus *i.e.* inactive GR exists as an hsp complex in the cytoplasm and that GR translocates to the nucleus subsequent to ligand binding. Binding of ligand by ER is followed by release of the hsp complex. Subsequently, two ER molecules dimerize and in the nucleus bind to specific DNA sequences designated the estrogen response elements (EREs) that are present in the promoter regions or enhancers of specific target genes. The canonical ERE consists of a palindromic 5'-AGGTCA-3' element separated by three nucleotides. However, many functional EREs deviate substantially from this consensus sequence. Once bound to the ERE in the promoter of a target gene, the ER homodimer interacts directly or indirectly with components of the basal transcription machinery to modulate transcription.

Figure 1.5 **The current view of mechanisms underlying transcriptional activation.** Illustrated is an example of the current view of transcriptional repression effected by members of the nuclear hormone receptor family in the absence of hormone. In these cases transcriptional repression has been shown to be mediated by interactions with NCoR and SMRT. The current model suggests that DNA-bound factors such as unliganded nuclear hormone receptors, steroid hormone receptors, and various transcriptional repressors have been shown to recruit the corepressor complexes to the promoter by either binding to the corepressor complex directly or by contacting a component of the complex such as Sin3, NCoR and SMRT which exist in a complex with various histone deacetylases. This results in deacetylation of histones and repression of transcription. Ligand-binding to nuclear hormone receptors, or replacement of transcriptional repressors with transcriptional activators results in the replacement of the corepressor complex by activator complexes that consist of multiple acetyltransferases which then catalyze histone acetylation and result in transcriptional activation. Published with permission [231].


SWI/SNF
p300/CBP
PCAF
TRAP
DRIP
Activation
Nuclear Receptors
Histone acetylation
TAFs
IIA
TBP
TATA
IIB
IIF
Pol II
IIE
IIH
Mediator
Repression
Histone deacetylation
NCoR
SMRT
mSin3
HDAC
Corepressor complex

The classical estrogen receptor, ER á, was cloned from uterus in 1986 [232] and for many years it was believed that ERá was essential for life. However, a study published in 1994 described a man lacking functional ER á, who suffered from severe osteoporosis and reduced fertility, dispelling the notion that deletion of the ER gene would be lethal [233]. The persistence of estrogen binding in some tissues of the ER á knock-out mouse strain indicated the existence of a second receptor for estrogen [234]. This second receptor for estrogen, designated ER â, was cloned in 1996 [235]. ER á and â bear 95% amino acid identity through the DNA binding domain and both are capable of binding to a consensus estrogen response element. The receptors bear 55% identity through the ligand-binding domain and exhibit similar but not identical ligand binding properties [236]. ER á and â have the potential to function as heterodimers but given that their relative tissue distributions are rather different, the two receptors are more likely to function as homodimers in the majority of target cells [237; 238].

4.5.1 *Results of aberrant estrogen receptor expression*

Studies of ER- [234] or aromatase-knockout [239] mice have provided insight into the actions of estrogen highlighting the importance of estrogen and ER for many different physiological functions. The enzyme aromatase, encoded by the *CYP19*, gene converts testosterone into estradiol. The aromatase knock-out (ArKO) mice are characterized by females that are infertile as a result of a defect in ovulation that is accompanied by underdeveloped external genitalia, uteri and mammary glands. The ArKO males are fertile with increased levels of testosterone. In the ER knock-out (ERKO) mice where ER á has been inactivated, both sexes are infertile. Females have cystic haemorrhagic follicles and no corpora lutea, while males have testicular atrophy, decreased spermatogenesis and inactive sperm. The ERKO mouse exhibits abnormalities in its reproductive behaviour and breast development [240]. In addition, both sexes display a marked reduction in

bone density. The importance of functional ER for normal bone development is also evident from the single reported case of the man lacking functional ER described earlier [233]. Studies on the ER â knock-out (BERKO) mouse indicate that the males are fertile. The female mice exhibit reduced fertiliy as a result of a block in the last step of follicle development prior to ovulation and consequently no corpora lutea are present [241]. In contrast to the abnormal reproductive behaviour of the ERKO mice, BERKO mice exhibit normal sexual behaviour highlighting the importance of ER á for the normal expression of natural reproductive behaviours in both sexes [242].

5.0 HOMEODOMAIN PROTEINS

Homeotic genes are master control genes that specify the body plan and regulated development in higher organisms. They share a common sequence element of about 180 base pairs, the homeobox, that encodes a well conserved DNA binding motif termed the homeodomain [243; 244]. The first genes found to encode homeodomain proteins were the fruitfly Drosophila melanogaster developmental control genes, in particular the homeotic genes from which the name "homeo" box was derived [245]. Presently, there are several hundred homeobox gene sequences published from different species [244-247]. In vertebrates, the homeobox family of genes can be divided into two subfamilies: i) the clustered homeobox genes known as the Hox genes and ii) the non-clustered or divergent homeobox genes, the latter are scattered throughout the genome and fall into a number of groups based upon the sequence similarities [245; 248]. The popular view of homeotic gene function is largely based upon the dramatic mutant phenotypes that result from altering their patterns of expression. For review see references [249-251]. The classic example is the choice between formation of an antenna or a leg in *Drosophila*. When the Hox gene *Antennapedia* is expressed inappropriately in the antenna premordium it gives rise to a leg, and when its expression is removed it becomes an antenna [252; 253]. Observations such as these have led to the idea that

the function of the Hox genes is to generate morphological diversity during animal developmental and evolution [254-256].

A major area of focus in the field has been to examine the promiscuous nature of the DNA-binding specificity described for many homeodomain proteins. Since the amino acid sequence of the homeodomain is highly conserved among those of highly divergent organisms it is not surprising that homeodomain-containing proteins bind to their regulatory elements in a highly analogous manner. Indeed, homeodomain proteins that specify entirely different biological functions are capable of recognizing very similar, if not identical, DNA sequences *in vitro*. Therefore, it remains difficult to reconcile the biological diversity displayed by these proteins *in vivo* with the apparent redundancy and lack of specificity suggested by *in vitro* studies [257; 258]. In addition, the typical DNA-binding motifs recognized by homeodomain proteins are only 5-6 base pairs in length and as such are encountered too frequently in the genome to serve any highly specific regulatory function.

In order to explain the molecular basis of homeodomain/DNA recognition it is necessary to integrate three types of information: the structure of the homeodomain of interest, the identification of the amino acid-base pair contacts that influence the specificity of DNA/protein interactions and the mode of binding to DNA (monomeric binding, homo/heterodimerization).

5.1 The specificity of homeodomain/DNA interactions

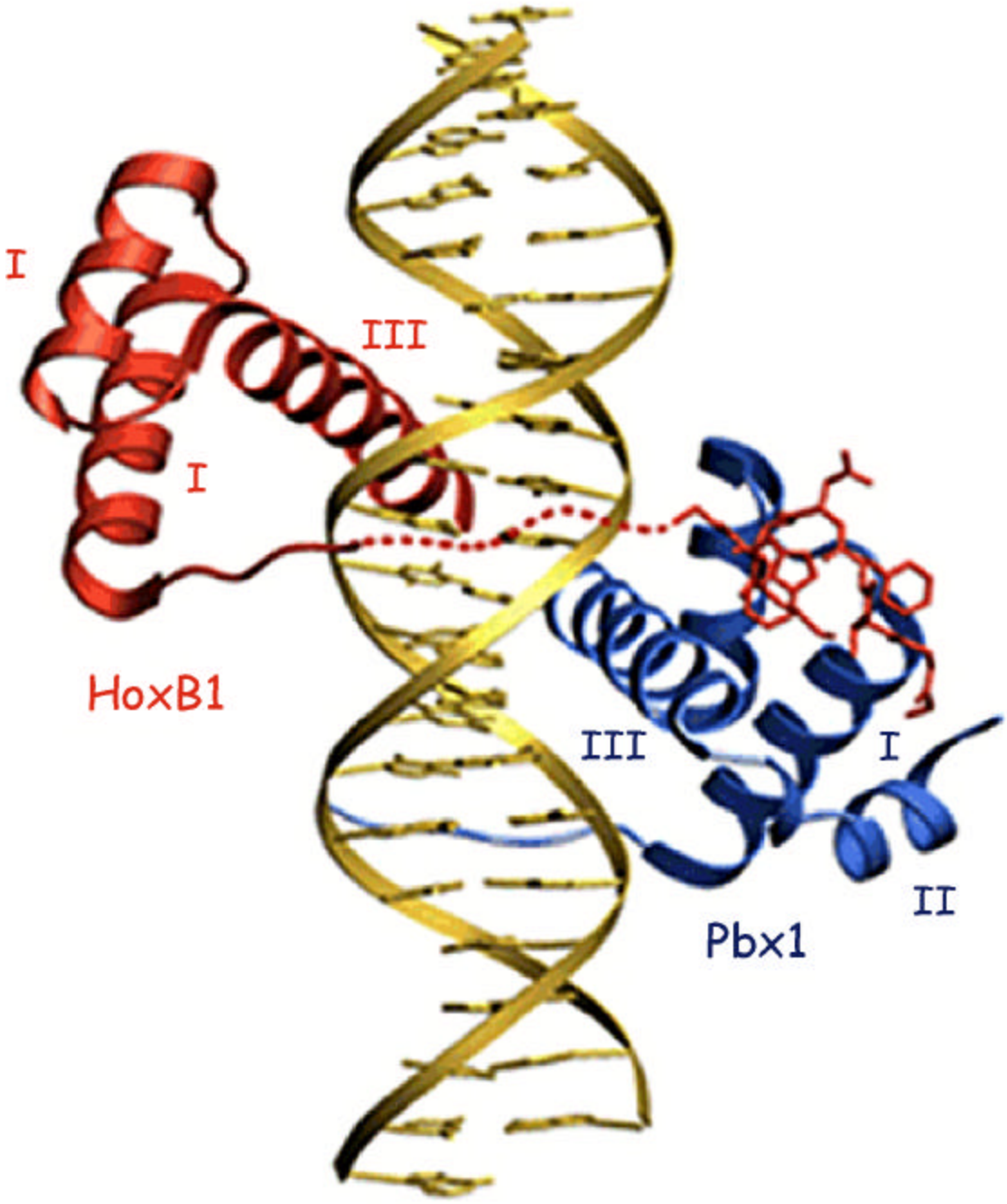
The amino acids that dictate the secondary structure of the homeodomain are highly conserved across species [259-262]. As depicted in figure 1.6, the recognition helix, helix 3 of the homeodomain is inserted into the major groove of the DNA where it establishes the majority of base-specific contacts. Within this helix, varying levels of importance have been ascribed to amino acids at positions 47, 50, 51 and 54. Of these, the residues at positions 50 and 54 have been shown

to be the most critical determinants of binding specificity [259; 263-265]. Although the amino acids that determine the DNA binding specificity of the homeodomain are predominantly located within helix 3, it has been proposed that different combinations of amino acids at positions 3 to 7 in the NH_2-terminal arm also contribute to the preference of a specific homeodomain protein for particular nucleotides within its binding site core [260; 266-268]. In these cases, it has been suggested that differences in the amino acid composition of the NH_2-terminal arm of these proteins specifically, the number of basic residues present, can result in small changes in the orientation of the amino acids side chains in the NH_2-terminus, which are then transmitted through the protein resulting in minor adjustments of side chains in helix 3. This significantly influences the stability, and consequently, the specificity with which the proteins bind to their recognition elements. Subtle repositioning of the protein has been used to explain why the Ubx and Dfd homeodomains with nearly identical recognition helices select binding sites that differ in the region presumably contacted by helix 3 [269].

Many of the individual homeodomain classes have additional conserved protein domains outside of the homeodomain. Several of these large domains have DNA-binding properties. The binding specificity of these families of homeodomain proteins is influenced by the presence of this extra domain, as in the case of the POU domain proteins, the LIM homeodomain proteins, paired domain groups, that augments the stringency or changes the specificity with which these homeodomain proteins recognize their response elements [270-275].

Atypical homeodomain proteins are those that do not have an extra DNA binding domain but where the homeodomain contains fewer or greater residues than the standard 60 amino acids. Essentially all atypical homeodomains have extra amino acids between helix 1 and helix 2 of the homeodomain or helix 2 and helix 3.

Figure 1.6 **A model of a ternary complex made up of two homeodomain-containing factors and DNA.** This model was generated from crystal structure data of the HoxB1/Pbx1/DNA complex solved to 2.35Å. The structure shows that the homeodomain of each protein binds to adjacent recognition sequences on opposite sides of the DNA through their recognition helices, helix 3. Heterodimerization occurs through contacts formed between a six amino acid hexapeptide NH_2-terminal to the homeodomain of HoxB1 and a hydrophobic pocket in Pbx1 formed between helix 3 and helices 1 and 2. A COOH-terminal extension of the Pbx1 homeodomain forms an á-helix that packs against helix 1 to form a larger four helix homeodomain. Reproduced with permission [277].


I
III
I
HoxB1
III
I
II
Pbx1

The structural analysis of yeast MATá2, which has a homeodomain containing 3 extra amino acids in the loop between helix 1 and 2, showed that such atypical homeodomains have the same structure as typical homeodomains [276]. A series of genes encoding such homeodomains have been isolated from animals, plants and fungi. They are classified as TALE homeodomain proteins on the basis of this three amino acid loop extension and are discussed below.

As discussed previously, in many instances, the high affinity binding specificity of the homeodomain can be directly attributed to the amino acid composition of its helix 3 and NH_2-terminal regions. However, an increasing number of examples highlight the involvement of a homeodomain protein partner that can also raise the binding specificity and affinity of homeodomain/DNA interactions. This is exemplified by the interactions of the yeast mating type homeodomain proteins MATa1/MATá2. In the diploid a/á cell type, the two homeodomain proteins form a heterodimers that binds to sites upstream of haploid-specific genes (hsg) [278]. In the absence of á2, the a1 protein exhibits no detectable specific binding to DNA. In contrast, the presence of a1 in solution dramatically raises the affinity of á2 for hsg operators [278; 279]. The cooperative binding of á2 with a1 depends on the 21-residue COOH-terminal tail of á2, which is located immediately COOH- terminal to its homeodomain. This tail interacts with a hydrophobic patch of a1 that is comprised of Phe15, Val19, Lys23, Leu26, Glu30, and Val34 [279]. In a manner analogous to the interactions of MATa1 and MATá2, the members of the PBC family of proteins including the mammalian oncoproteins Pbx1-3 and their various isoforms, the *Caenorhabditis elegans* ceh-20 and their *Drosophila* orthologue extradenticle (Exd), have been demonstrated to act as cofactors for a number of Hox proteins [280-285]. These interactions require a highly conserved hexapeptide motif F/IYPWMK or alternatively a motif of the form ANW, which is joined to the NH_2-terminal arm of the homeodomain by a linker that varies in length and sequence, and a hydrophobic pocket on Pbx1 that bears limited resemblance to the hydrophobic patch of a1. The

Drosophila homeodomain protein engrailed makes use of a WPAW sequence in its EH2 motif for its interactions with Pbx1 [286]. The residues that mediate the interactions of Pbx1 with the Hox proteins are very well conserved [287]. PBC/Hox dimers bind to elements that are composites of PBC and Hox recognition elements. Furthermore, the choice of Hox protein partner depends upon subtle differences within the DNA binding site. The interaction of the PBC proteins with their Hox partners is believed to influence the nature or energetic contributions of contacts formed by the NH_2-terminal arms of the Hox proteins in the minor groove resulting in the higher binding specificity of the dimer.

Recently, Pbx1 has also been shown to interact with the Ile50-containing TALE proteins, which include Meis1, the Meis-related proteins Mrg1 (Meis2) and Mrg2 (Meis3) and Prep1/pKnox1 [263; 288-290]. These proteins share a high degree of identity throughout the DNA recognition helix and as a result bind to a common recognition motif, 5'-TGACAG-3', designated the MPRE or Meis1/Prep1 Recognition Element. The sequence of the MPRE is quite divergent from the canonical 5'-TAAT-3' motifs. Meis1 and Prep1 are incapable of binding to their recognition elements with high affinity *in vitro*. Rather, they have been shown to bind to DNA as heterodimers with members of the PBC class of proteins [263; 288-290]. The Meis1/Prep1-Pbx dimers are capable of binding to a variety of regulatory elements whose sequence deviates from that of the MPRE. *In vivo* Meis1 and Prep1 have been shown to exist as heterodimers with Pbx1 in the absence of DNA suggesting that dimerization is essential for high affinity binding of their target sites [263; 288; 289]. More recently, homothorax (Hth), the *Drosophila* orthologue of Meis1, has been demonstrated to be essential to the nuclear localization of Exd [291]. This implies a higher degree of regulatory complexity for the Meis1/Pbx1 interactions. Meis1 has also been shown to dimerize with members of the AbdB-like family of homeodomain proteins in manner that increases the stability of Meis1 binding to its recognition element [292]. The members of this family lack any

of the Trp-containing motifs used for the interactions with Pbx1 and consequently they do not dimerize with any of the members of the PBC group. Recently, Jacobs *et al.* (1999) have demonstrated that trimers comprised of Pbx/Meis and Hoxb1 regulate the transcriptional activity of the *Hoxb2* enhancer [293]. These results indicate the existence of a higher order combinatorial code, which is mediated by the interdependent DNA-binding activities of the different partners and the spatial orientation of the various response elements.

6.0 THE AVIAN VERY LOW DENSITY APOLIPOPROTEIN II GENE

The avian very low-density apolipoprotein II (apoVLDLII) gene encodes a small phospholipid binding protein that comprises part of the low-density fraction of the egg-yolk [294]. ApoVLDLII is synthesized exclusively in the liver of the laying hen and transported through the bloodstream to be deposited in the developing oocytes [295; 296].

ApoVLDLII gene expression represents an excellent model demonstrating the various levels of transcriptional regulation. The acquisition of competence to express the apoVLDLII gene in response to estrogen is developmentally regulated [297]. Expression of this gene is also liver-specific and completely dependent on the presence of estrogen [298]. The levels of apoVLDLII gene expression are also influenced by estrogen-induced factors that modulate the stability of its mRNA [298-301]. The tight regulation of apoVLDLII expression is essential, since misexpression of the gene, exemplified by the estrogenized rooster model, results in accumulation in the serum of lipoproteins that would normally be shunted to the oocyte. The resulting hyperlipidemia and hyperlipoproteinemia can result in the development of atherosclerosis in these birds [302; 303].

The apoVLDLII gene is approximately 3 kb in length and consists of 4 exons encoding a 750-base pair mRNA species [295; 296; 304]. It contains three transcriptional start sites located at nucleotides -10, +1, and +17, which are used in a 10:87:3 ratio, respectively [305].

6.1 Regulatory regions in the distal promoter region of apoVLDLII

Previous members of this laboratory examined the distal promoter regions of the chicken apoVLDLII gene for changes associated with its activation or occurring at stages in development where the liver becomes competent to express this gene in response to estrogen [297; 306]. Analysis of the methylation pattern of the 5' and 3' flanking regions of the gene identified two sites that become demethylated in the liver between day 7 and 9 of embryogenesis. One of these sites, which contains an *Msp*I site, is located at -2.6 kb and the other, an *Xho*I site is located 1.6 kb downstream of the 3' end of the gene. The timing of the demethylation of these sites coincides with the acquisition of competence by the gene to respond to hormone suggesting that these two events could be associated.

Filter binding assays performed with liver nuclear extracts demonstrated the existence of factors that recognized an 850-base pair fragment spanning the region -2.8 to -1.96 kb of the apoVLDLII gene. Exonuclease III deletion analyses of this fragment identified boundaries for 3 binding sites that were designated Site 1, 2 and 3 [305; 307]. The boundaries of Site 1 (-2.61 to -2.57 kb) span the MspI site that is demethylated during embryogenesis, coinciding with the time at which the liver becomes competent to express the apoVLDLII gene in response to estrogen. The Site 1-binding activity was found to be liver enriched and developmentally regulated, and deletion of this site reduced the activity of the apoVLDLII promoter by approximately 60% [297; 308]. Although, the sequence of Site 1 is very similar to sites recognized by C/EBP á, it was shown that the proteins that

recognized Site 1 were quite distinct C/EBP á, DBP and LAP, particularly with respect to their binding specificities [307].

A day 9 embryonic chicken liver cDNA expression library was screened using site 1 concatamers. Three factors were subsequently cloned on the basis of their abilities to bind to site 1. The first proved to be the avian orthologue of the Y-box binding protein, YB-1 and was designated chkYB-1 accordingly [309]. Another Site 1 binding factor proved to be a novel member of the IRF family of transcription factors, designated chicken interferon regulatory factor 3 (cIRF3) [310]. The expression patterns chkYB-1 and cIRF-3 resemble the binding characteristics of the complex detected with site 1 [307; 309]. The third factor cloned proved to be a C_2H_2-zinc-finger encoded by an 8.5 kb mRNA that was ubiquitously expressed. Thus far, this factor remains uncharacterized.

Changes were also detected to the pattern of DNase1 hypersensitivity at 0, 0.2 and 1.5 kb upstream of the major transcriptional start site in adult tissue. In addition, two hormone-inducible DNase1 hypersensitive sites were identified at -1.8 and -3.1 kb [306]. The hormone-inducible hypersensitive site at -3.1 is proximal to a putative silencer element present within a CR1 element characterized by another member of the laboratory [311]. The close association of this silencer element with the hormone-inducible hypersensitive site at -3.1 kb suggests that changes in chromatin structure may be involved in the regulation of transcription from the apoVLDLII promoter.

6.2 Regulatory elements in the proximal promoter region of apoVLDLII

To examine regions that were critical to the hormone-responsiveness of the gene, exonuclease III deletion constructs of the apoVLDLII promoter were transfected into primary hepatocyte cultures. These studies highlighted the fact that a construct ending at -230 that included both canonical and

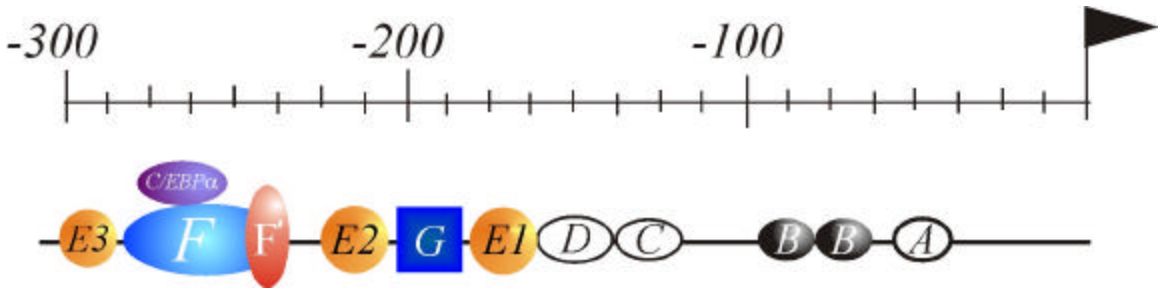
imperfect EREs displayed estrogen-inducible levels of expression that were only 2-3% of those observed with the entire 5'-flanking region. Only the most upstream protein binding site identified in vivo is missing in this construct. This footprint, designated F, is relatively large spanning a region from -237 to -284 [312]. Including an additional 3' 11 nucleotides of the F footprint in construct -247/+38 increased estrogen-dependent activity approximately 5-fold [313]. The location of this site, designated F' maps to the hypersensitive site at -0.2 kb that is present in day 7 embryos, in rooster liver and to lesser extents in hen liver and oviduct [306]. Including the remainder of the F element in the -307/+38 construct increased promoter activity a further 2-fold while extending this region to -427 did not affect any further increases in the activity of the promoter.

6.2.1 *The F' site in the apoVLDLII proximal promoter*

Functional analyses of the regulatory regions upstream and in the first intron of the apoVLDLII gene confirmed that essentially all of the regulatory elements required for the efficient hormone-dependent expression of apoVLDLII are clustered within a region of approximately 300 nucleotides of the proximal promoter that includes both canonical and imperfect EREs (Figure 1.7) [314]. This region contains binding elements for C/EBP á, DBP, COUP-TF, LAP, LF-A1 all of which lie within 160 nucleotides of the major transcriptional start site [305; 306; 312; 315; 316].

Since the sequence of the F' site did not match the sequences of any known factor binding elements, experiments carried out by my predecessor were aimed at examining developmental changes in the abundance of the F' binding activity, characterizing the protein complexes that could form on the F' site as well as cloning and identifying the factor(s) responsible for this activity [317]. EMSA performed using a synthetic binding site corresponding to the F' site sequence using liver nuclear extracts revealed the presence of two major retardation complexes.

Figure 1.7. **Diagram of the proximal promoter region of the apoVLDLII gene. The locations of eight protein binding sites in the apoVLDLII proximal promoter are depicted.** These sites were identified by *in vivo* and *in vitro* footprinting [312]. Footprint A contains a recognition element for the liver-enriched factor LF-A1/HNF4 and the ubiquitous factor COUP-TF. A second LF-A1/HNF4 binding site is present in footprint C. Footprints B and D contain binding sites for the liver-enriched factors C/EBP á and DBP. E1 and E2 contain canonical and imperfect estrogen response elements, respectively. Footprint F contains a binding element recognized by chicken VBP as well as the element designated F'. The sequence of F' is presented with the recognition sequence of AKR shown in bold [314]. Sites of methylation interference with AKR binding are indicated by ●. Additionally, an element situated at -259/-252 was designated E3, solely on the basis of its sequence similarity to a half ERE. Finally, the location and the sequence of site G, discussed in this chapter, are included.


-300
-200
-100
C/EBPα
E3
F
F'
E2
G
E1
D
C
B
B
A

A fast migrating complex (Complex 1) that is liver-enriched but present at low yet detectable levels in kidney, spleen, brain and heart. This complex is not detectable prior to day 7 of embryogenesis, becomes the predominant complex by day 9 of embryogenesis prior to declining approximately two-fold between days 9 and 20 and a further 3-5-fold in adult liver. A second, slower migrating complex (Complex 2), was also observed. This complex is relatively abundant in all tissues examined except for blood. The levels of this complex decreased two-fold between days 7 and 9 remaining relatively constant afterwards.

Methylation interference analysis carried out with partially purified extracts revealed that the two complexes had overlapping points of contact (Figure 1.7). Complex 1 contacts two G residues on each strand of DNA of the downstream boundary of the F footprint. Complex 2 contacts only the most upstream of these G residues on each strand. This information was used to introduce mutations into -305/+38 reporter that would dsrupt binding by either the liver-enriched (FM2 mutant binding site) or ubiquitous factors (FM1 binding site). Both classes of mutations decreased the ability of these reporter constructs to respond to estrogen [313]. Additional mutations made to either the canonical or imperfect EREs in these studies resulted in reporter constructs that maintained 80-90% and 10-20% of the estrogen-inducible activity of the wild-type promoter. This indicated that the EREs function additively rather than synergistically to activate expression of apoVLDLII in response to hormone [313]. The data also indicated that much of the influence on the activity of the promoter attributed to E2 was the result of protein interactions with F'. To identify the factor responsible for the F' binding activity, end-labelled concatamers of the F' binding site were used to screen a day 9 chicken embryonic liver cDNA library.

Positive clones were screened further using concatamers of the FM1 or FM2 binding sites in order to distinguish between the liver-enriched or ubiquitous complex. Subsequent sequence analysis of the liver-enriched factor identified it as the avian homologue of C/EBPα [317].

Similar analyses of the ubiquitous factor identified it as a novel and highly unusual homeodomain protein. At that time the ubiquitous factor resembled the maize homeodomain protein knotted-1 most closely, hence it was designated Avian Knotted- Related (AKR) [314]. Subsequently, the murine and human orthologues of AKR, designated TGIF for TG-interacting factor, were isolated [318; 319]. Studies carried out by a present member of the laboratory demonstrated that AKR is capable of antagonizing the estrogen-dependent activity from reporter constructs under the control of the -305/+48 region of the apoVLDLII promoter [314]. This repressive activity was mediated by the F' site as the activity of a reporter construct containing the FM1 mutation failed to be downregulated by AKR.

6.3 AKR, an atypical homeodomain protein

AKR was the first vertebrate homeobox protein to be identified with an Ile at position 50 of helix 3. It recognizes an atypical binding element within the F' site of the form 5'-TGACAT-3'[314]. Results of target site selection indicated that AKR selected an optimal binding site with a sequence 5'-TGACAG-3' [320]. Presently, AKR is classified as a member of the Ile50-containing TALE homeodomain proteins that include Meis1, the Meis-related proteins Mrg1 (Meis2) and Mrg2 (Meis3) and Prep1/pKnox1 [289; 321-323]. These proteins share a high degree of identity throughout the DNA recognition helix and as a result bind to a common recognition motif, 5'-TGACAG-3', designated the MPRE or Meis1/Prep1 Recognition Element whose sequence matches the optimal binding site determined for AKR. The sequence of these elements is quite divergent

from the canonical 5'-TAAT-3' motifs recognized by the majority of homeodomain-containing proteins.

Despite their similarities, a number of characteristics distinguish AKR from the other members of the TALE group. Structurally the homeodomain of AKR is situated near the NH_2-terminus of the protein, in contrast to the more traditional COOH-terminal location. The composition of the NH_2-terminal AKR differs from that of the other Ile50-containing TALE proteins. The TALE region, which has been shown to participate in the interactions between Exd/Ubx, MATá2/MATa1, and Pbx1/HoxB1 is well conserved across species within a given group of proteins [277; 279; 287]. In contrast, this loop exhibits a high degree of variability between different groups of TALE proteins. These three residues are LSN for members of the PBC group, LTH for Meis1, Mrg1 and Mrg2, IGH for Prep1 and RYN for AKR.

Functionally, AKR and TGIF both exhibit high affinity binding to their recognition elements *in vitro*. AKR has been demonstrated to be capable of binding with high affinity to sites that share a high degree of identity to the MPRE (F' and Opt-1) and to a site completely diverged from the MPRE [314; 320]. In contrast, the high affinity binding of the other members of this particular group of TALE homeodomain proteins is dependent upon interactions with the members of the PBC class or with other Hox proteins [263; 288-290; 292]. Studies thus far, have indicated that complexes containing Meis1 or Prep1 activate expression of the genes they regulate, whereas both AKR and TGIF have been shown to act as potent repressors of genes that are responsive to the actions of nuclear hormone receptors [314; 319; 320]. An additional role has been defined recently for TGIF in the smad2-mediated downregulation of several TGF â-responsive genes [324]. In this system, the TGIF-mediated recruitment of histone deacetylase 1 (HDAC1) occurs in the absence of

the protein binding to its recognition element. This indicates that TGIF, and by analogy AKR, may employ a different set of co-actors than those recruited by the PBC or Meis proteins.

7.0 RESEARCH OBJECTIVES

To determine the factors responsible for the differences in DNA-binding exhibited by AKR and the remainder of the Ile50-containing TALE homeodomain proteins a major focus of my research has been to define the determinants of the unique binding specificity of the AKR. Studies were performed in which specific mutations were introduced in the helix 3 and NH_2-terminal arm regions in order to examine their effects upon the DNA recognition of AKR. Subsequently, the changes effected by the various mutations made to the binding specificity of AKR were determined using PCR-mediated target site selection. The results of these studies were used to confirm a molecular model of the AKR homeodomain complexed to its optimal binding site. Analysis of the model has provided useful insights into the manner with which AKR binds to DNA.

Recent studies have indicated that TGIF, the mammalian orthologue of AKR, is capable of interactions with a histone deacetylase. Furthermore, these interactions occurred independent of TGIF binding to DNA and required regions NH_2- and COOH-terminal of the homeodomain. To determine the manner with which AKR downregulated the estrogen-dependent activity of the apoVLDLII promoter, the other major focus of my research has been to determine AKR and ER interactions within the proximal promoter of apoVLDLII. Subsequent studies used site-directed mutation to examine the relative importance of these sites to AKR or ER activity. Finally, the possible involvement of an avian histone deacetylase in the AKR-mediated repression of apoVLDLII gene expression was examined.

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